Investigation of glycerol assimilation and cofactor metabolism in Lactococcus lactis

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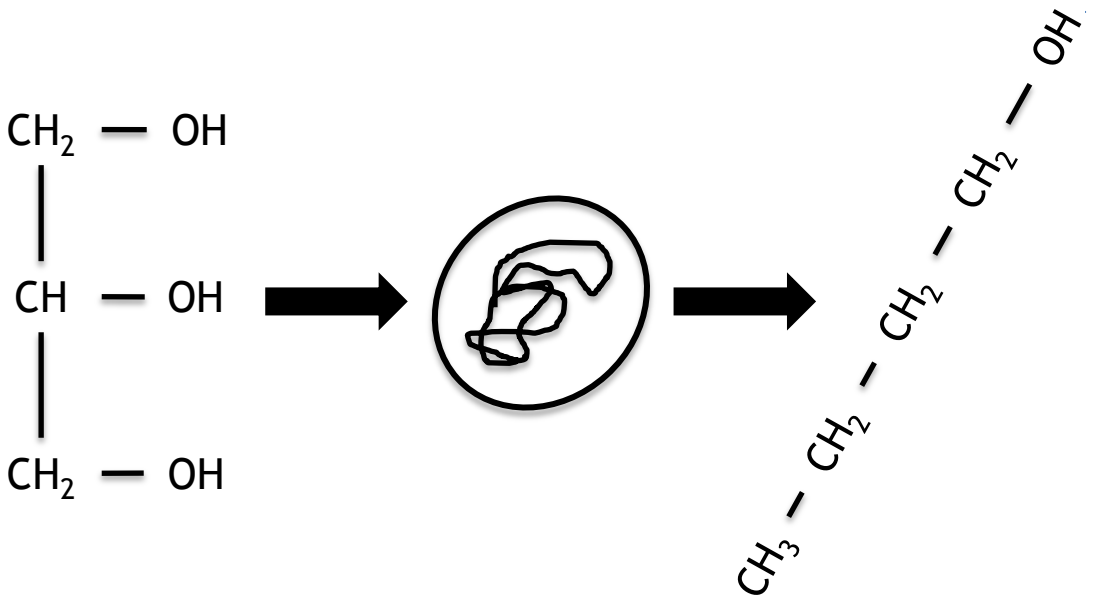

Anders Koefoed Holm

PhD. Thesis

November - 2012

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The research was carried out at the Technical University of Denmark, Department of Systems Biology, Center for Systems Microbiology.

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Investigation of glycerol assimilation and cofactor metabolism in *Lactococcus lactis*

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Summary

The production of biodiesel has been steadily increasing during the last decade, and with it crude glycerol as a byproduct. Despite being rich in glycerol, the increased supply has saturated the demand for glycerol, making purification a non-viable option. The background for this project was to investigate the suitability of lactic acid bacteria as production organisms for the production of biofuels and biochemicals. Specifically, the goal was to adapt the model organism *Lactococcus lactis* to convert crude glycerol, to value-added fuels or chemicals. Work was divided between four main areas: life cycle assessment of the GLYFINERY project, screening of *L. lactis* spp. for glycerol utilization, engineering of glycerol metabolism in *L. lactis* and finally an investigation into perturbation of energy metabolism in *L. lactis*.

The work from the life cycle assessment resulted in two reports, detailing the technological requirements for the GLYFINERY processes. These have been included in the appendix (section A).

The screening did not reveal any *L. lactis* strains capable of assimilating glycerol nor did it reveal any conditions favorable to glycerol dissimilation in *L. lactis*. The conditions evaluated were: anaerobic, aerobic and respiration permissive growth in combination with either glycerol as a sole substrate or with co-metabolization of glycerol with common sugar substrates. Although no growth on glycerol was seen, both positive and detrimental effects were observed from cultures with glycerol supplementation.

The positive effects were observed from cultivation of *L. lactis* IL1403 with trehalose as a substrate under aerated conditions. Under these conditions, the supplementation of glycerol would cause an increase in biomass production of over night cultures. The growth rate of the cultures with glycerol supplementation were determined to be 84% of the reference cultures without glycerol. The detrimental effects of glycerol were observed as reduced growth rate and decreased biomass formation. The effects were observed when cultivating plant isolates of *L. lactis* on xylose. The effect manifested itself under both anaerobic and respiration permissive conditions, but was not found to have the same profound effect on other sugar substrates such as galactose or ribose.

Supplementation of nucleosides to the growth medium or increased substrate concentration were found to counteract the inhibitory effects and improve the growth rate, though not completely to the level of the reference strain. The fact that this effect was predominantly observed while utilizing xylose implicates the involvement of the pentose phosphate pathway. A possible mechanism underlying the observed growth characteristics under anaerobic conditions could be a rise in triosephosphate levels (the entry point of

glycerol in glycolysis) regulating pyruvate formate-lyase. Under aerobic and respiration permissive conditions, the rise in the redox level from channeling glycerol into metabolism could possibly regulate both glyceraldehyde-3-phophate dehydrogenase and the pyruvate dehydrogenase complex, disrupting flow through the central metabolism and ATP production.

If this is the case, the question remains, as to why the excess redox is not simply removed by respiration. The results from this investigation have provided an initial characterization of the inhibitory effects and some possible directions for future investigations, but more work is needed to fully elucidate the mechanism and target of inhibition.

The engineering of glycerol metabolism in *L. lactis* was initiated from three different perspectives: overexpression of glycerol kinase from *L. lactis*, introduction of a heterologous glycerol assimilation pathway and construction of a library of NADH oxidase activity. Based on a preliminary analysis of transcription level data, an attempt was made to stimulate glycerol assimilation by overexpressing the glycerol kinase already present in *L. lactis*. The construction and verification of a strain with increased glycerol kinase activity was not fully completed and is still ongoing.

Similarly the construction of mutants expressing a heterologous pathway for glycerol dissimilation is also an ongoing task. An artificial glycerol assimilation operon was designed based on components from known glycerol metabolizers. Three genetic elements were placed in the operon: the glycerol facilitator *glpF* from *E. coli*, the glycerol dehydrogenase *dhaD* from *Citrobacter freundii* and the dihydroxyacetone kinase *dhaK* also from *Citrobacter freundii*. These were arranged in an operon structure where *glpF* was placed in front of *dhaD* and *dhaK*. Ribosomal binding sites from glycolytic promoters in *L. lactis* were placed in front of each gene. The operon was introduced into *L. lactis* with expression modulated by a synthetic promoter library.

Lastly, to prevent possible issues with redox accumulation during growth on glycerol, a library of mutants with NADH oxidase activity was constructed and verified by enzymatic assays. Despite the NADH oxidase activity, no growth could be detected in defined medium supplemented with glycerol as sole carbon and energy source. This could possibly be connected to the expression levels of the library, which were in the lower range.

Investigations were also made into the response of *L. lactis* mutants to perturbations in energy metabolism. The motivation was to apply, transcriptomic and metabolomic techniques that were not available at the time of the previous characterization by Koebmann et *al.*, in 2002. To minimize noise and pleiotropic effects, strains with mild perturbations were selected for transcriptomic analysis. For the purpose of investigating the changes in internal metabolite concentrations, a mutant with very high ATPase activ-

ity was included with the mildly perturbed strains. The data obtained from the metabolomic study of internal metabolites, did not provide any novel observations and did not substantiate the results from the transcriptomic investigation.

Although significance of the transcriptomic analysis was affected by technical issues, the overall impression gathered from the response to perturbation of ATP levels, was that the genes were generally downregulated. Glycolysis along with most of the anabolic pathways were downregulated in what resembled a starvation response. During hydrolysis of ATP two signals were generated, lowered energy state and increased inorganic phosphate levels (P_i). The exact contribution of each signal along with many other interesting observations will need to be confirmed by additional experiments and further investigation in future studies.

The task of making *Lactococcus lactis* grow on glycerol as a sole carbon and energy source still remains to be accomplished. It will require continued efforts in the three areas investigated in this work and others, to fulfill this task. Hopefully, future investigations can successfully bridge the integration of the complex challenges encountered, when engineering central carbon metabolism, to complete the goal.

Resume

Produktionen af biodiesel har været støt stigende gennem det sidste årti og ligeledes biproduktet råglycerin. Selvom råglycerin er rig på glycerol, har den stigende produktion mættet markedet og oprensning er ikke længere en rentabel måde at håndtere råglycerin på. Baggrunden for dette projekt var at afklare hvorvidt mælkesyrebakterier kunne anvendes som produktionsorganismer til produktion af biobrændsler og biokemikalier. Specifikt var målet at tilpasse model organismen *Lactococcus lactis* til at omdanne råglycerin til biobrændsler eller biokemikalier. Arbejdet i denne afhandling er inddelt i fire hovedområder: Livscyklusanalyse af GLYFINERY projektet, screening af *L. lactis* stammer for naturlig anvendelse af glycerol, modifikation af glycerolmetabolismen i *L. lactis* og undersøgelse af effekten ved perturbation af energimetabolismen i *L. lactis*.

Arbejdet med livscyklusanalyse af GLYFINERY projektet udmundede i to rapporter, der beskriver de tekniske krav til processerne der udvikles i GLYFINERY projektet. Disse er inkluderet i appendiks A.

Screeningen af *L. lactis* stammer for glycerol afslørede ikke nogen kandidater. Ej heller var det muligt at finde betingelser der kunne stimulere vækst på glycerol. De betingelser der blev undersøgt var: anaerob, aerob og respiratorisk vækst kombineret med enten glycerol eller kombinationer af glycerol og sukkersubstrater. Selvom det ikke var muligt at stimulere vækst på glycerol alene, blev både gavnlige og skadelige effekter observeret.

En positiv effekt kunne observeres ved øget biomasse udbytte i overnatskulturer med trehalose som substrat. Væksthastigheden af kulturer med glyceroltilsætning blev målt til 84% af værdien for kulturer uden glyceroltilsætning. De skadelige virkninger af glyceroltilsætning blev konstateret som værende sænket vækshastighed og et fald i biomasseudbytte. Indledende blev fænomenet opdaget ved vækst af planteisolater på xylose.

Glycerol inhibering gør sig gældende under både anaerobe og respiratoriske forhold, men har ikke samme hæmmende effekt med andre substrater, såsom galaktose eller ribose. Tilførsel af nukleosider til vækstmediet eller en forøgelse af xylose koncentrationen, viste sig at modvirke glycerol inhiberingen øgede vækshastigheden, dog ikke helt på niveau med referencen. Siden dette fænomen først og fremmest kunne observeres med xylose som substrat perger på pentose phosphat vejen. En mulig virkningsmekanisme kunne under anaerobe forhold være en stigning i triosephophatniveauet (indgangen for glycerol til glykolysen), hvilket regulerer pyruvate formate-lyase. Under aerobe or respiratoriske betingelser, kunne en mulig effekt være en stigning i redox niveauet, fra glycerols vej ind i glykolysen, hvilket regulerer både

glyceraldehyd-3-phosphat dehydrogenase og pyruvate dehydrogenase komplekset. Dette ville forstyrre flowet gennem den centrale metabolisme, samt ATP produktionen.

Skulle dette være tilfældet er spørgsmålet så, hvorfor den overskydende redox energi ikke blot fjernes gennem respiration. Denne undersøgelse har givet en basal undersøgelse af glycerolinhibering og leveret et udgangspunkt for fremtidige undersøgelser, selvom en større indsats er påkrævet for til fulde at karakterisere dette fænomen.

Tilpasningen af glycerol metabolismen i *L. lactis* foregik ud fra tre indgangsvinkler: overudtryk af glycerol kinase fra *L. lactis*, introduktionen af det komplette enzymatiske maskineri påkrævet til vækst på glycerol og konstruktionen af et bibliotek af NADH oxidase aktivitet. Baseret på den indledende analyse af transkriptionsdata, blev et forsøg på at stimulere vækst på glycerol ved glycerol kinase overudtryk igangsat. Konstruktion of verifikation af en sådan stamme er stadig undervejs og endnu ikke afsluttet.

Stammer med en komplet pathway til vækst på glycerol er ligeledes heller ikke færdige. Til at indsætte en komplet pathway, blev der designet en syntetisk operon. Komponenterne blev baseret på stammer der allerede var kendt for at vokse på glycerol. Sammenlagt er der tre genetiske elementer i operonstrukturen: glycerol facilitatoren *glpF* fra *E. coli*, glycerol dehydrogenasen *dhaD* fra *Citrobacter freundii* og dihydroxyacetone kinasen *dhaK* ligeledes fra *Citrobacter freundii*. Generne blev arrangeret i en operonstruktur med *glpF* placeret foran *dhaD* og *dhaK*. Ribosomale bindingsteder fra glykolystiske promotorer fra *L. lactis*, blev indsat foran hvert gen. Denne operon blev introduceret i *L. lactis* med et syntetisk promoterbibliotek indsat foran.

Til at forhindre problemer med redox niveauet under vækst på glycerol, blev der fremstillet et bibliotek af stammer med NADH oxidase aktivitet. Disse blev verificeret med et enzymatisk assay og testet for vækst på glycerol. Ingen vækst kunne konstateres i defineret medium med glycerol som eneste kulstof- og energikilde. Dette skyldes muligvis at NADH oxidase enzymaktiviteterne ikke var høje nok.

Det blev yderligere undersøgt, hvorledes *L. lactis* reagerede på pertubationer i energimetabolismen. Motivationen bag dette studie var at genoptage analysen af stammerne fra tidligere, men denne gang anvende transkriptionelle og metabolske analyseværktøjer der ikke var tilgængelige ved den tidligere analyse fra Koebmann et *al.* i 2002. For at minimere støjen og pleiotropiske effekter, blev stammer med milde perturberinger udvalgt til transkriptionsanalysen. Til analyse af interne metabolitniveauer inkluderes en stamme med kraftig pertubering, sammen med stammerne med de milde perturberinger. Data fra metabolitstudiet gav ikke anledning til nogle nye opdagelser. Transkriptionsanalysen viste en generel tendens til nedreguler-

ing af glykolysen og anabolske reaktionsveje, hvilket kunne minde om en sultrespons. Når man hydrolyserer ATP påvirkes både cellens energiniveau, samt niveauet af fri phosphat P_i. Den præcise virking og kontrol af hver af disse signaler er ukendt og skal afklares i fremtidige undersøgelser.

Målet med at få *Lactococcus lactis* til at vokse på glycerol som eneste kulstof- og energikilde, er endnu ikke nået. Det vil kræve betydelig indsats indefor områderne beskrevet her, samt muligvis andre, at opfylde dette. Forhåbentligt vil fremtidige undersøgelser kunne løfte denne opgave og fuldføre målet.

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Preface

When this project started some years ago the outset seemed simple. But as things progressed a certain feeling began to rise. Were I to describe the sensation it was not unlike trying to master the art of flying:

> There is an art, it says, or rather, a knack to flying. The knack lies in learning how to throw yourself at the ground and miss. Pick a nice day it suggests, and try it.
> The first part is easy. All it requires is simply the ability to throw yourself forward with all your weight, and the willingness not to mind that it's going to hurt.
> That is, it's going to hurt if you fail to miss the ground. Most people fail to miss the ground, and if they are really trying properly, the likelihood is that they will fail to miss it fairly hard.
> Clearly, it is the second part, the missing, which presents the difficulties.
> *-The Hitchhiker's Guide to the Galaxy, Douglas Adams*

I find that this description of putting theory to practice fairly accurately describes my own progression. What seems straightforward (like a freshly started PhD. project) usually turns out to be a more complex affair upon contact with reality. Especially if it seems like you spend more time hitting the ground than you do flying. But even though failing to miss the ground does invoke a certain feeling of discomfort, especially during those particularly determined attempts, it is the innate possibility of missing that propels us forward time and again. When reflecting on the course of the project it has overall been a great and extremely educational experience. I have been fortunate to get the chance to expand my abilities on both a scientific and a personal level. Looking back there are of course things that today I would do differently. Not just because hindsight reveals that the result did not materialize as expected, but also because my entire point of reference has changed.

Bagsværd, November 2012

Anders Koefoed Holm

Part I

General introduction

Chapter 1

Outline of the thesis

As stated in the frontmatter, the project was financed by the European Community's 7th Framework Research Programme under Grant Agreement Number 213506 (Project GLYFINERY).

Project GLYFINERY deals with the production of biofuels and biochemicals from waste glycerol derived form biodiesel production, so called crude glycerol. To that aim we set out to investigate the hypothesis that a well established model organism at the center, *Lactococcus lactis*, could in fact be used as a production organism. Although *Lactococcus lactis* had at the time mostly been studied as a dairy isolate of starter cultures for cheese production, isolates can be found in most ecological niches. Even though the model organisms isolated from dairy sources did not appear to possess the ability to grow on glycerol as sole carbon and energy source, it was our hypothesis that a plant isolate, generally being more prototrophic, could have retained the ability to utilize glycerol as sole carbon and energy source. The metabolic flexibility of *Lactococcus lactis* i.e. the ability to grow very well under anaerobic conditions while still being aerotolerant to a large extent and even being able to respire in the presence of hemin, held the promise that perhaps it was possible to coax *Lactococcus* into utilizing glycerol.

1.1 Organization of the thesis

Although there is a single matter at heart of the investigation, the thesis has been divided into 4 parts:

I A general introduction

II Screening of *Lactococcus* spp. for glycerol utilization

III Engineering glycerol metabolism and cofactor levels in *Lactococcus lactis* MG1363

IV Investigation of *Lactococcus lactis* strains with perturbed ATP levels

The division reflects mostly my assessment and interpretation of which issues were key in relation to the project. It is more a framework to convey the results of the project rather than a strict chronological account. Work in all parts have mostly been ongoing in parallel throughout the project. Each section is treated as a self contained unit while still being a part of the whole.

1.1.1 Thesis layout

The layout of the thesis was chosen to represent the format of peer reviewed articles as closely as possible. Currently additional experimental support is underway for part IV and in the future that may be the case for the other parts as well hence this layout might be beneficial for future manuscript preparations. Thus in addition to the general introduction each part has been given a separate introduction which, although short, is intended to be combined with material from the general introduction in any future work.

1.1.2 Work package 7 - GLYFINERY integrated assessment

In addition to the work outlined in the four parts described above there was an additional aspect to my work in the GLYFINERY project. I was also involved in work package 7 (WP7) which was an integrated assessment of the processes defined by the GLYFINERY consortium. It contained multiple work tasks: WT 7.1 - Technological assessment, WT 7.2 - Environmental assessment, WT 7.3 - Economical assessment, WT 7.4 - Optimization of product chains and WT 7.5 - Integrated assessment. The goal for the work package was to integrate performance from both environmental and economical aspects to evaluate each process.

My involvement in WP7

My role in WP7 was the work task (WT) 7.1 - Technological assessment. More specifically I was involved in the preparation of deliverables 7.1 and 7.4 - both are reports dealing with the technological aspects of the processes being developed in the GLYFINERY project. The initial report - D7.1 contains the preliminary introduction to the GLYFINERY projects processes and technological definitions. As such it is an excellent introduction to the entire GLYFINERY project itself. The second report - D7.4 is an updated version of the initial report to reflect the experimental results obtained in each process. Since the preparation of these reports did not directly tie in with the other parts, the reports have not been included in the thesis, but both reports can be found in appendix A.

Chapter 2

The origins of the glycerol refinery (GLYFINERY)

2.1 7th Framework programme

The project name "GLYFINERY" is an acronym playing on the words glycerol and refinery. As mentioned previously the project is funded by the 7 th Framework Programme for Research and Technological Development (FP7 for short), and aims at developing processes to handle the increased availability of crude glycerol as a byproduct of biodiesel production. The FP7 programme is scheduled to run for seven years from 2007–2013. The total budget of FP7 is over 50 billion euro. This money will mostly be spent on grants to players within the field of research all over Europe and beyond, in order to co-finance research, technological development and demonstration projects [15]. The GLYFINERY project proposal was approved in November 2007 under theme 5 - Energy. The timeline for the project was four years running from March 2008 until March 2012. The focus of the GLYFINERY project was to develop novel processes for the production of next generation biofuels and biochemicals from glycerol. The target products were the biofuels ethanol and butanol, the biochemical 1,3-propanediol and biomethane as an energy carrier.

2.1.1 Organization of the GLYFINERY consortium

The project is divided into several work packages each with one or more partners responsible for various stages. The entire project was designed to run from initial strain discovery through strain development and process optimization to a final implementation of pilot scale testing for the most promising processes. The GLYFINERY consortium consists of six partners:

- Technical University of Denmark (DTU), Denmark
- BioGasol ApS., Denmark

- A&A Biotechnology s.c. - Poland
- The Institute for Energy and Environmental Research (IFEU), Germany
- MEROCO A.S., Slovakia
- Prochimia Surfaces Sp. z.o.o., Poland

Initially MEROCO supplied the crude glycerol to be used as a substrate for the bioconversion processes, DTU together with BioGasol and A&A Biotechnology were responsible for strain isolation and development, Prochimia was responsible for product recovery and IFEU performed a life cycle and integrated assessment for the processes.

2.1.2 Life cycle assessment

As described above, part of the project contained a life cycle assessment. This work was delegated to work package 7 (WP7). Part of the work package was to perform a technological assessment of the GLYFINERY processes. I participated together with IFEU in the preparation of these reports which can be found in appendix A.

2.2 Biodiesel production

Biodiesel is a fuel produced from oleaginous materials. It is yellowish in color and has similar properties to petroleum derived diesel. Emissions from biodiesel compared to petrol diesel display lowered amounts of particulate matter, CO and soot but increased levels of NO_x (15-20%). The idea of using vegetable oils as fuel is not novel. Rudolf Diesel ran his diesel engine on peanut oil, but vegetable oils as fuels do have performance issues and were eventually replaced by the easy access to inexpensive petroleum derived fuels [8]. Realizing that petroleum reserves are finite, attention has once again been directed to the renewable oil based fuels as an alternative to fossil diesel.

There are several ways to adapt the density of oils to better suit their use as a fuel in combustion engines. These include: dilution with solvents such as ethanol to lower viscosity, thermal based approaches such as pyrolysis or cracking and transesterification [16]. Currently the most common method of producing biodiesel is by transesterification. Transesterification is a fairly simple chemical process where an alcohol (typically methanol) is used to cleave off the fatty acid sidechains of triglycerides in presence of a catalyst, leaving the glycerol backbone and fatty acid esters. As a result of this process the byproduct glycerol represents approx. 10% [wt.] of the production [33]. Many types of oleaginous materials can be used to produce biodiesel: Virgin

oils (non-used), waste oils such as waste cooking oil or animal fats, non-edible oils such as palm oil (jatropha) or even oil from algae. There is also the matter of which alcohol and catalyst to use. The most prevalent process in europe is using mainly virgin oils such as rape seed or soy bean oil with an alkali based catalyst and methanol as the alcohol. This process is efficient, cheap, and robust [70, 62, 61]. The choice of alcohol is mainly determined by price, since the type of alcohol does not severely affect the quality of biodiesel. The choice of feedstock on the other hand has a greater influence on the quality of biodiesel [8, 30]. The production of biodiesel has been increasing at an incredible rate since 1992 [16]. An overview of the biodiesel production in Europe can be seen in figure 2.1.

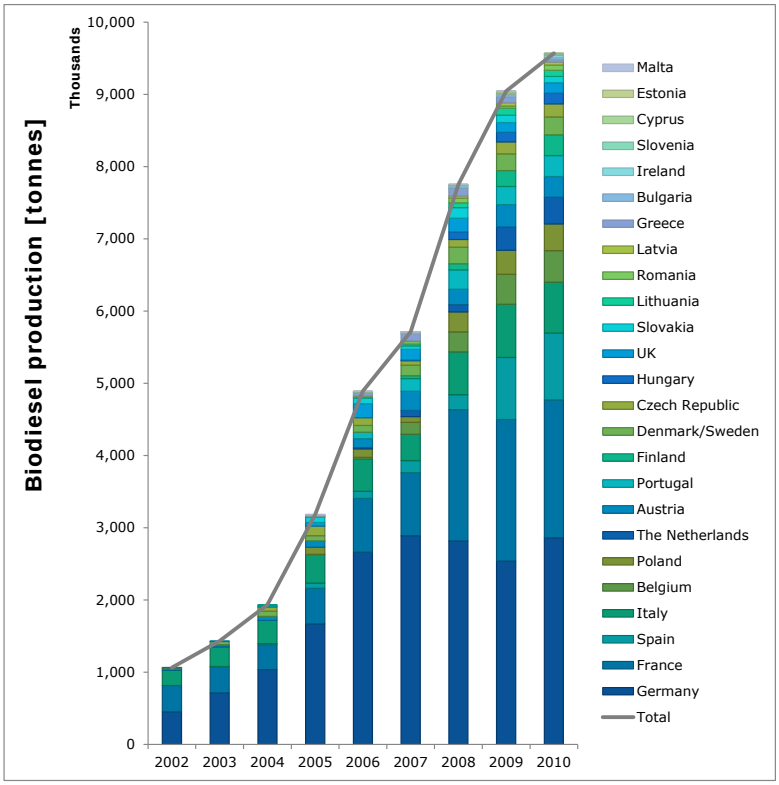

Figure 2.1: Biodiesel production in Europe from 2002–2010. Data from the European Biodiesel Board [4].

2.2.1 Waste glycerol

As seen in figure 2.1 the production of biodiesel has been steadily increasing since the beginning of the century. When the project began in 2008, production of biodiesel in Europe was 7,800,000 tonnes and expected to increase. This represents a supply of 780,000 tonnes of crude glycerol. The increasing production of biodiesel has been saturating the existing markets

for glycerol [52]. The cost of purification is relatively expensive and with prices for refined and crude glycerol decreasing, it is not an obvious choice for handling the increased amounts of crude glycerol [19]. Although the expected increase in biodiesel production had leveled off, there was still an increase in production of biodiesel in 2010 to 2,861,000 tonnes. With the traditional glycerol markets saturated, alternative solutions are needed to handle the increased production of biodiesel and with it crude glycerol. This is where the GLYFINERY project comes in.

Chapter 3

Selection of a suitable production organism

When investigating the biological production of biofuels and biochemicals, an important parameter is obviously the choice of production organism. Although there is a huge variety in microbial species, one is often faced with the needle in a haystack scenario when screening for novel production strains. This is further complicated by the fact, that the act of cultivating strains from a sample already represents a selection based on the environmental conditions that can be simulated in an efficient manner in the laboratory. Since this is a time consuming and laborious process, often the tactic is to fall back to the well established laboratory strains of microbial workhorses, since these are well described, easily amendable and well known to researchers [24].

The optimal production host is a combination of certain key traits such as flexible substrate utilization, high product yield and titer, high productivity, high product tolerance, the ability to grow in extreme temperature or pH and preferably anaerobic growth [24, 72]. Since no known microorganism exists at the moment which possesses all these traits, the selection of production organism becomes a choice of selecting an appropriate compromise. The advent of modern recombinant DNA technologies allows for the improvement or adaptation of missing traits, but there are limits to the problems which can be solved within the scope of a limited number of manipulations. Introduction of a new product pathway is achievable within a fairly small number of genetic manipulations, while engineering of temperature or osmotolerance involves complex systems operating throughout a given cell. These challenges provide a basis for the selection of traits more or less amendable to improvement in a given host.

3.1 Lactic acid bacteria

Lactic acid bacteria (LAB) are a diverse group which have had industrial relevance for many years. They have been a topic of scientific study and interest at the Center for Systems Microbiology (formerly the Bacterial Systems Biology group) for several decades. They comprise a range of genera such as *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Oenococcus*, *Pediococcus* and *Streptococcus*. They have traditionally been associated with food and feed fermentation, and have been named from a common defining trait: the production of lactic acid as a principal end product of sugar fermentation.

Lactic acid bacteria in general are typically found in habitats which are fairly rich in nutrients. This includes milk, meat and beverages, but also on plant material and leaves in nature as well as the mouth, intestine, and vagina of mammals [2, 71]. As a group LAB exhibit very broad substrate utilization and a high degree of tolerance towards environmental stress. They readily metabolize both hexoses such as glucose, mannose, galactose and fructose, pentoses such as arabinose, ribose and xylose and various disaccharides such as maltose, lactose and sucrose. Their tolerance ranges from low pH (pH 3.0, *L. suebicus*, *L. acetotolerans*, *L. acidophilus*) to high salt concentrations (above 20%, *Tetragenococcus muriaticus*, *Carnobacterium viridans*) and high ethanol tolerance (13%–15%, *L. fructivorans*, *Oenococcus oeni*) [69, 2]. Recently LAB have even been adapted to grow in 3–4% butanol, an ability which is top ranked among microbial species [44, 37].

3.1.1 From lab to industry

When looking at industrial production there are several barriers to introducing improvements from commonly used research strains into organisms with an industrial background. Often the laboratory strains are cured of plasmids and phages, leaving them unable to utilize certain substrates such as milk and vulnerable to infection by bacteriophages. This often requires that advances pioneered in common laboratory strains, have to be recreated in industrial strains, which can present further problems, since the introduction of DNA into industrial strains can be challenging [32]. Furthermore, there are often regulatory issues associated with production at an industrial scale, often not encountered in the research laboratories at the university.

This is especially important when considering the use of genetically modified microorganisms in food grade systems. This has been a challenge facing the research with model organisms such as *Lactococcus lactis* IL1403 or MG1363, which are commonly used in the production of dairy starter cultures. To answer that problem, several methods have been developed that facilitate the food-grade manipulation of genetic material [32, 53].

3.1.2 *Lactococcus lactis* - a model lactic acid bacterium

Not only is the general class of lactic acid bacteria a broad and diverse group of microorganisms - this also holds true for the individual species. The species *Lactococcus lactis* can be be divided into three subspecies: *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *hordniae*. Furthermore, to add to the complexity of this picture, *Lactococcus lactis* subsp. *lactis* includes species formerly designated: *Streptococcus lactis* subsp. *lactis*, *Streptococcus lactis* subsp. *diacetylactis* and *Lactobacillus xylosus* [59].

Traditional investigations into the metabolism of *Lactococcus lactis* have typically been associated with the dairy industry and their use in starter cultures for fermented dairy products [21, 71]. As mentioned earlier LAB can be found in many other habitats. Two intensely studied model organisms: *Lactococcus lactis* MG1363 (subsp. *cremoris*) and *Lactococcus lactis* IL1403 (subsp. lactis) both have a dairy background and are typically referred to as dairy strains. But *Lactococcus lactis* strains have been isolated from many habitats and the strains *Lactococcus lactis* NCDO2118 and KF147 (both subsp. *lactis*) were isolated from vegetables (frozen peas and mung bean sprouts respectively) and are considered to be representatives of the so called plant isolates [23, 36]. The plant isolates show evidence of being adapted to grow on substrates from plant materials (eg. arabinose and xylose) [63] and are generally more prototrophic than their dairy counterparts [13].

The choice of organism for the project

At the onset of the project a choice had to be made with respect to an interesting production organism. As mentioned, the aim of the GLYFINERY project was to produce ethanol, butanol and 1,3-PDO from glycerol. The list of natural candidates with these qualifications is rather short. The field was further narrowed by looking only at anaerobic organisms. The list of microbial producers of the target products are limited to mainly *Clostridium*, *Citrobacter*, *Enterobacter* and *Klebsiella* species. These produce butanol and 1,3-PDO from glycerol naturally, but they are somewhat difficult to work with, since there is a lack of tools and a requirements for strict anaerobic conditions [73]. All except *Citrobacter* (although it is still considered an opportunistic pathogen) are considered class 2 organisms which leads to pathogenicity issues. For *Clostridium*, the main product formation is a mix of the products, butanol, ethanol and acetone (ABE-fermentation) which further complicates product optimization [27, 76, 35].

Production of ethanol has traditionally been the domain of yeasts, normally baker's yeast, also known as *Saccharomyces cerevisiae*. This is carried out as a standard aerobic batch or fed-batch fermentation, exploiting the innate capability of the Crabtree effect in *S. cerevisiae*. When looking at an-

aerobic growth, neither *S. cerevisiae* nor *E. coli* [12] grow on glycerol without an external electron acceptor and even when grown aerobically *S. cerevisiae* grows very slowly [45, 50]. Reports of anaerobic fermentation of glycerol has been reported for *E. coli* both naturally and through engineering [48, 60].

As a part of the GLYFINERY project, other PhD. students are working on characterizing non-saccharomyces yeasts such as *Pachisolen tannophilus* or *Yarrowia lipolytica* and their use in bioconversion of glycerol. Also the two other partners engaged in strain isolation and development have both chosen biocatalysts from *Clostridium* spp. as their choice for production of butanol and 1,3-PDO respectively.

Limited availability of candidates both fermenting glycerol and producing target products imposes a choice

In this setting, with the list of potential candidates severely limited, the option of using a species which could both ferment glycerol and produce the target products seemed unattainable. Therefore the choice came down to either engineering product formation or engineering substrate utilization. In this process an alternative idea came up - to investigate the use of lactic acid bacteria as cell factories for the microbial conversion of glycerol. This approach obviously has two major flaws: the model organisms *Lactococcus lactis* MG1363, IL1403 and NCDO2118 do not natively utilize glycerol as sole carbon and energy source and they do not produce butanol or 1,3-PDO. Despite their shortcomings, they do possess several other traits that would make them very suited for use as industrial production organisms.

Lactococci do have the capability to produce many other interesting compounds including lactic acid, ethanol, acetate, acetoin and 2,3-butanediol. Furthermore they are aerotolerant and grow exceedingly well under anaerobic conditions with very high glycolytic flux. They are generally regarded as safe (GRAS) and have a high level of tolerance against alcohols and organic acids in particular. Finally the Center For Systems Microbiology have been investigating *Lactococcus lactis* for many years, producing an array of tools including several patented technologies and a huge catalog of interesting mutants to assist the development of novel production strains. So we set out to investigate the use of *Lactococcus lactis* as a potential production organism for the production of biofuels and biochemicals.

Chapter 4

Glycerol and metabolism

Glycerol (1,2,3-propanetriol) also known as glycerin, is a simple molecule which can be found in all living organisms (figure 4.1). It was discovered in 1779 from saponification of olive oil with lead oxide but was first named in 1813 by French chemist Michel Eugène Chevreul after the Greek word for sweet: $\gamma\lambda\upsilon\kappa\epsilon\rho o\sigma$ (glukeros). The most famous use of glycerol, and probably also the first industrial use, was in 1866 when Alfred Nobel produced dynamite from the trinitrate of glycerol (nitroglycerin). Today glycerol has several thousand applications ranging from pharmaceutical applications to use in food and and cosmetics [11]. Today the primary source of glycerol is the increasing production of biodiesel as outlined in section 2.2.

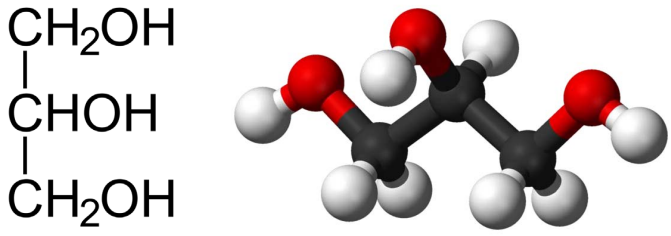

Figure 4.1: An illustration of the molecular structure of glycerol

4.1 Glycerol assimilation

In the world of bacteria, there exist only two different ways of metabolizing glycerol [43]. One starts with a dehydrogenation followed by a phosphorylation, the other proceeds in the reverse order. A schematic overview can be seen in figure 4.2 below:

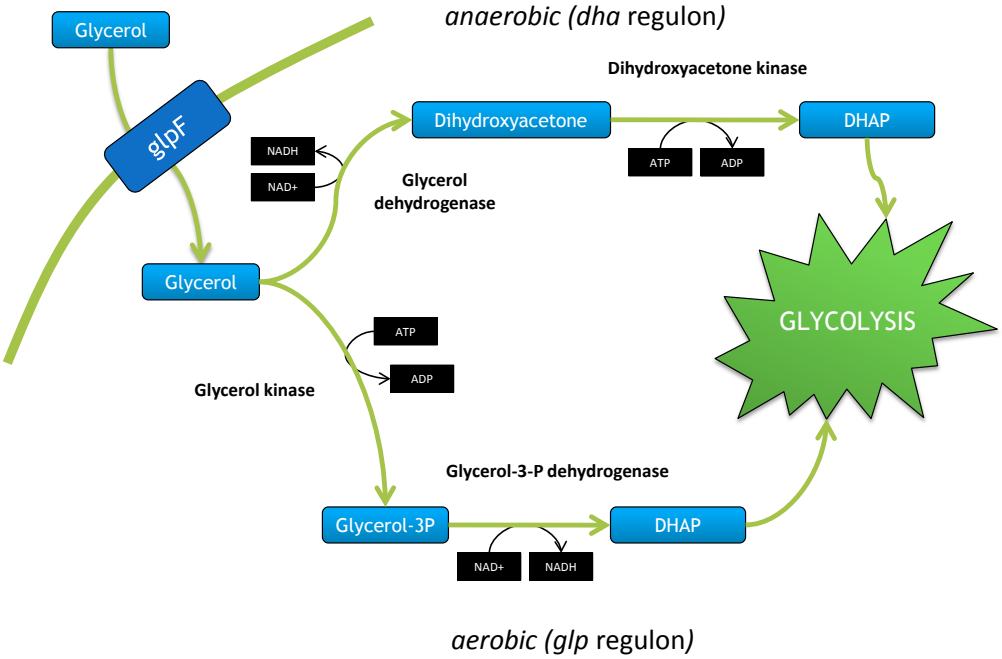

Figure 4.2: Overview of the two pathways for glycerol assimilation. The aerobic pathway (*glp* regulon) is activated by glycerol kinase trapping the glycerol as glycerol-3-phosphate followed by by the action of glycerol-3-P dehydrogenase. The anaerobic pathway (*dha* regulon) proceeds in the reverse order (glycerol dehydrogenase followed by DHA kinase). Both pathways convert glycerol to dihydroxyacetone phosphate (DHAP) a key intermediary in glycolysis.

As is often the case in biology, this seemingly simple system of course has a rich variation in the actual enzymatic machinery that performs the task. The ability and extent of glycerol assimilation is highly species and strain dependent. Among the Enterobacteriaceae only 8 species of 126 tested have been shown to grow fermentatively on glycerol. This ability was intimately tied to the existence of both glycerol dehydrogenase and 1,3-PDO dehydrogenase [5, 6].

4.2 Fermentative glycerol metabolism

Although not a lactic acid bacterium, one of the most studied organisms with respect to fermentative glycerol assimilation is the species *Klebsiella*. The reason for this interest has been partly the ability to grow fermentatively on glycerol and partly because while doing so, one of the main end products is 1,3-PDO, a chemical that has seen renewed interest recently. Several strains retain the ability to ferment glycerol among others *Klebsiella aerogenes*, *Klebsiella pneumoniae* and *Klebsiella oxytoca* [6].

4.2.1 Genetic framework of glycerol assimilation

The *Klebsiella* species mentioned are capable of metabolizing glycerol both aerobically and anaerobically. Even if molecular oxygen is not available species that are unable to metabolize glycerol fermentatively (eg. *Klebsiella planticola*) can usually do so if an alternative electronacceptor is present such as fumarate or nitrate.

When oxygen is present glycerol is metabolized via the *glp* regulon. This regulon is similar to the one present in *E. coli*. Glycerol is phosphorylated to glycerol-3-phosphate via glycerol kinase and further oxidized to dihydroxyacetonephosphate through the action of a flavin-linked glycerol-3-phosphate dehydrogenase. When no oxygen or other electron acceptors are present the glycerol is metabolized via the so called *dha* regulon, which allows for the use of glycerol itself as electron acceptor. The *dha* regulon consists of four enzymatic steps typically divided into an oxidative pathway and a reductive pathway. In the oxidative pathway a NAD-linked dehydrogenase converts glycerol to dihydroxyacetone (DHA). Then an ATP dependent kinase phosphorylates DHA into DHAP which connects to glycolysis. In parallel the reductive pathway serves as a redox sink for the redox equivalents produced in the oxidative branch. First a B_{12}-dependent dehydratase converts glycerol into 3-hydroxypropionaldehyde which is further reduced to 1,3-PDO by an NADH-linked oxidoreductase [5, 57, 34]. An overview of the process can be seen in figure 4.3

4.2.2 Regulation of glycerol metabolism

Dihydroxyacetone has been identified as the inducer of the dha regulon [34]. The oxidative branch of glycerol assimilation is regulated at a metabolic level while the reductive branch is governed by synthesis of the enzymes [1]. Overexpression of glycerol dehydratase and 1,3-pd oxidoreductase did not improve 1,3-PDO production [74]. Glycerol kinase is inhibited allosterically by 1,6-FBP (like *E. coli*). Dihydroxyacetone kinase is highly specific for DHA while glycerol kinase is non specific and will phosphorylate both glycerol and dihydroxyacetone.

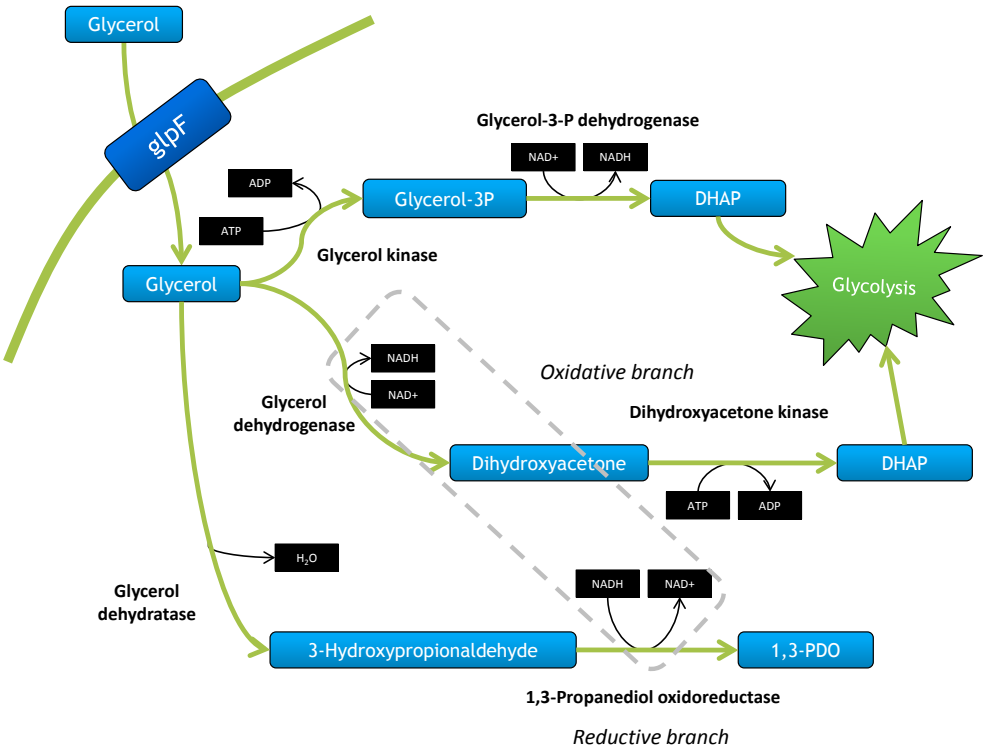

Figure 4.3: Overview of the pathways for glycerol dissimilation in *Klebsiella* spp. When oxygen is present glycerol is metabolized via the glp regulon encoding glycerol kinase and glycerol-3-P dehydrogenase. If oxygen is not present glycerol is metabolized vi the dha regulon, encoding the enzymatic activity for a parallel system consisting of an oxidative branch (glycerol dehydrogenase, DHA kinase) and a reductive branch (glycerol dehydratase, 1,3-PDO oxidoreductase). The end result for both the aerobic glp regulon and the oxidative branch of the dha regulon is the conversion of glycerol to dihydroxyacetone phosphate (DHAP) a key intermediate in glycolysis.

Chapter 5

Central metabolism of *Lactococus lactis*

5.1 Anaerobic metabolism of *Lactococcus lactis*

Lactococcus lactis is traditionally described as a facultative anaerobe growing fermentatively with lactic acid as a major fermentation end product. The lack of a complete citric acid cycle and complete respiratory chain means that *L. lactis* relies on substrate level phosphorylation for energy production. When grown in the presence of a preferred carbon source such as glucose, more than 90% of the sugar ends up in the main fermentation product, lactic acid. When a less favorable carbon source such as maltose is available, the fermentation pattern shifts and becomes a mix of lactate, formate, acetate and ethanol. These two modes of growth are generally referred to as homolactic and mixed acid fermentation [22, 14]. An overview of the central carbon metabolism can be seen in figure 5.1 and a detailed overview can be seen in figure 5.4.

Sugars are can be taken up by the phosphoenolpyruvate-dependent phosphotransferase system (PTS) or via permease systems. Glucose is mainly transported by the mannose/glucose specific PTS (PTS^{Man}) but can also be transported by the cellobiose specific PTS (PTS^{Cel}) or a glucose permease ($glcU$) requiring subsequent phosphorylation by glucokinase [10]. PTS transport have been described for the transport of galactose, lactose, trehalose, sucrose and fructose. Additional permease systems are present for transport of galactose and lactose. Permease systems are also used to transport sugars such as ribose, xylose and maltose [49, 14]. An overview of the metabolic connections between various sugar substrates and central metabolism can be found in figure 5.1.

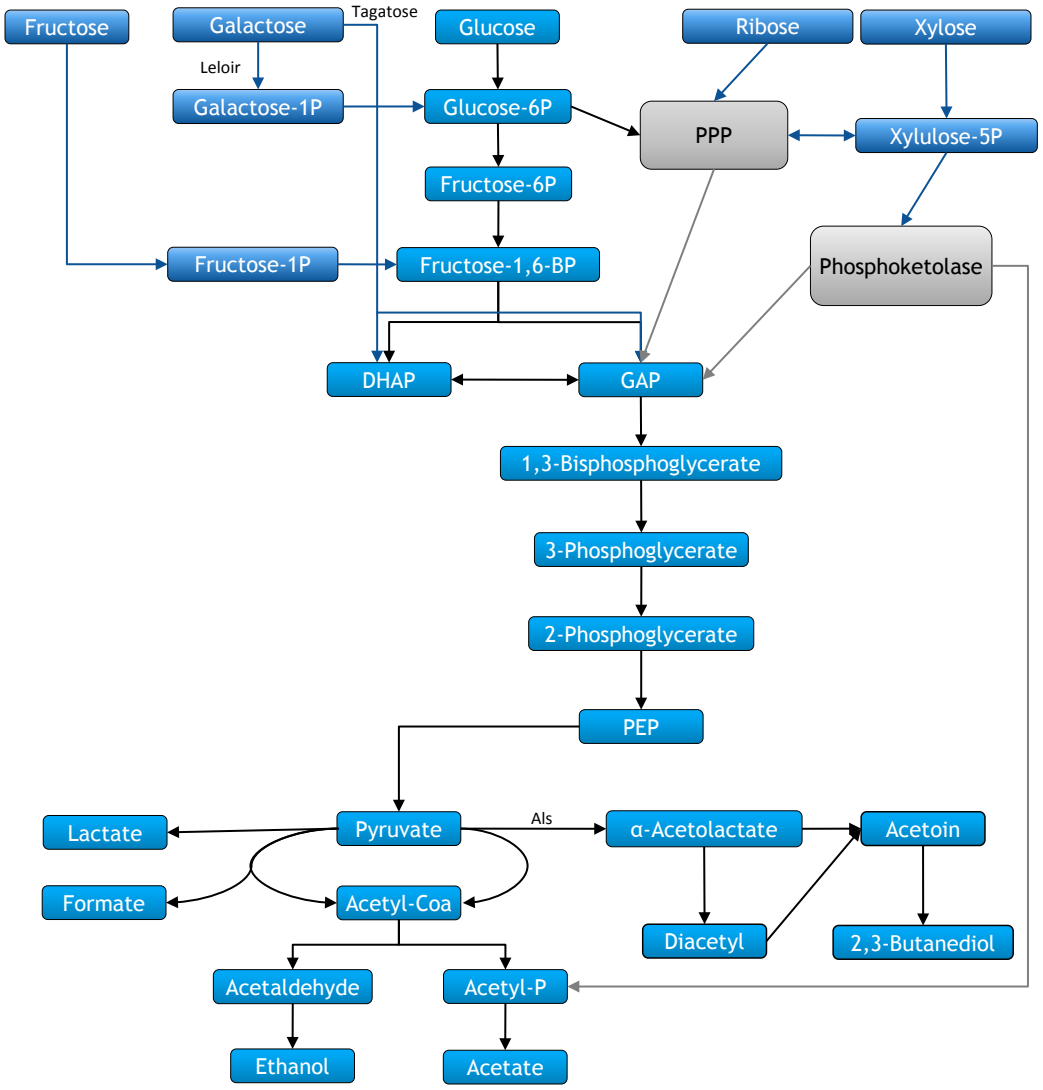

Figure 5.1: Overview of the central carbon metabolism of *Lactoccus lactis*. This is a simplified overview of the metabolites in central metabolism, without enzymes and cofactors. This illustrates the connection between different (mono) sugar substrates and central carbon metabolism. PPP - Pentose phosphate pathway, Phosphoketolase - The phosphoketolase pathway, DHAP - dihydroxyacetone phosphate, GAP - glyceraldehyde-3-phoshate, PEP - phosphoenolpyruvate. The ability to metabolize certain sugars is strain dependent ie. xylose can be metabolized by *Lactococcus lactis* NCDO2118 but not by *L. lactis* MG1363 or IL1403.

5.2 Central carbon metabolism

Several factors regulate the utilization of sugars and the shift from homolactic to mixed acid fermentation in *Lactococcus lactis*. A simplified schematic overview of central metabolism with metabolic regulation can be seen in figure 5.4.

5.2.1 Regulation of carbon metabolism by HPr and CcpA

The major regulator of carbon metabolism in *L. lactis* (and other gram positive organisms such as *Bacillus subtilis*, *Staphylococcus xylosus* and *Lactobacillus reuteri*) is the protein HPr (*ptsH*). HPr is involved in both the PTS system as a phosporyl donor and in transcriptional regulation while interacting with catabolite control protein A (CcpA) as either a transcriptional repressor (carbon catabolite repression - CCR) or a transcriptional activator (carbon catabolite activation - CCA). The active role of the protein is determined by the phosphorylation state. HPr has two different sites where it can be phosphorylated: At His-15 (utilizing PEP) and at Ser-46 (utilizing ATP). This provides four different states in which HPr can exist; HPr (unphosphorylated), HPr-His-15-P (phosphorylated on histidine 15), HPr-Ser-46-P (phosphorylated on serine 46) and HPr-His-Ser-P (doubly phosphorylated on both His-15 and on Ser-46) [47, 18].

The role of HPr is determined by the phosphorylation state

The transport and phosphorylation of sugars through the PTS system consists of a cascade involving several components. The process was initially described as comprising of two steps catalyzed by enzyme I (EI) and enzyme II (EII) with HPr as an intermediary phosphoryl donor. All three components can both accept and transfer a phosphoryl group thus causing them to cycle through a state of either being phosphorylated or not [18].

Phosphorylation of HPr at His-15 is mediated by EI (*ptsI*) utilizing PEP and in this state HPr-His-P interacts with the EIIA and EIIB components of the PTS allowing the transfer of a phosphoryl group to the incoming sugar. HPr-His-P can also interact with histidyl residues in other proteins such as glycerol kinase and non PTS transporters to exert regulatory effects.

Phosphorylation of HPr at Ser-46 is carried out by the HPr kinase/phosphorylase (HPrK/P, *hprK* or *ptsK*) utilizing either ATP or pyrophosphate (PP_i). This enzyme also catalyzes the opposite reaction, i.e. dephosphorylation of HPr-Ser-P to HPr by generating PP_i. The two opposing reactions are regulated by the metabolites: FBP, ATP and P_i, but the effects may vary depending on the species [68]. A high level of FBP together with a

low level of P_i stimulates the kinase reaction with the opposite effect on the phosphorylase reaction. When growing on a rapidly metabolizable carbon source, the levels of these metabolites result in HPr kinase activity rather than HPr-Ser-P phosphorylase activity.

Initial findings showed that phosphorylation of HPr on Ser-46 almost completely eliminated phosphorylation on His-15 in *Enterococcus faecalis*, *Bacillus subtilis* and *Streptococcus salivarius* [9, 17, 54]. Further study indicates that this may not be the case for all species as large amounts of doubly phosphorylated HPr was observed for *Lactococcus lactis* with the majority of HPr present as HPr-Ser-P and HPr-His-Ser-P. In *Lactococcus lactis* MG1363 almost 75% of HPr was detected as being doubly phosphorylated (HPr-His-Ser-P) during growth on glucose. There was also evidence that the doubly phosphorylated form could still transfer a phosphoryl group to proteins of the PTS and thus participate in sugar transport [56].

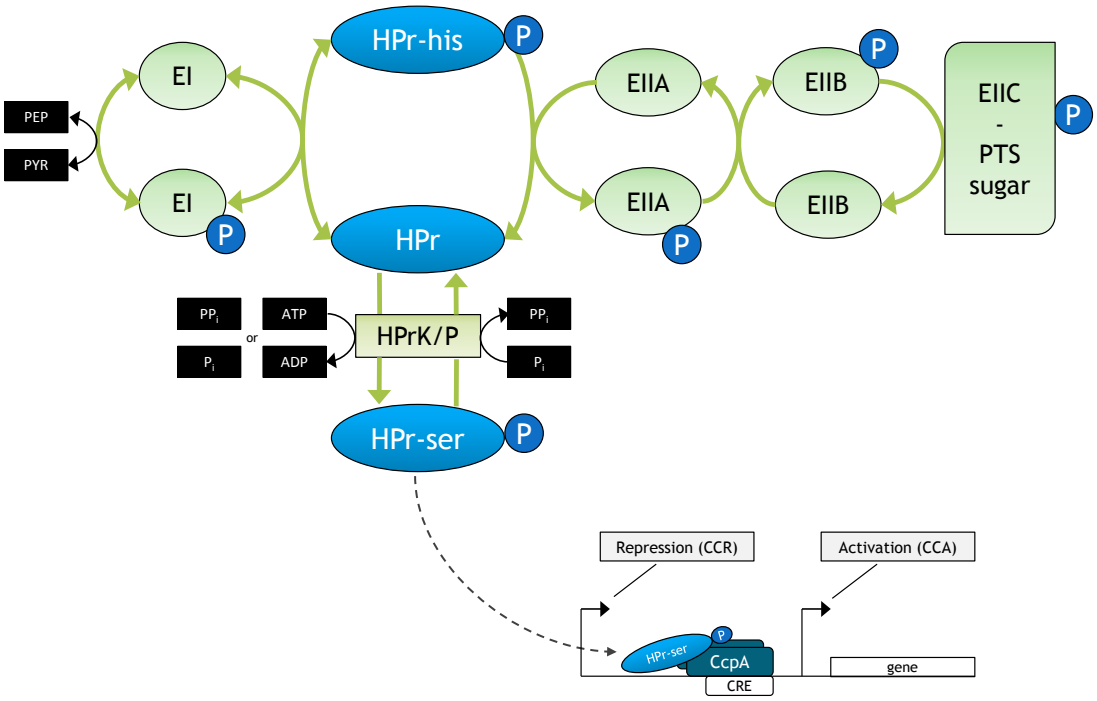

Figure 5.2: Overview of the regulation of central carbon metabolism in *Lactococcus lactis* by HPr and CcpA. HPr: HPr protein, CcpA: Catabolite control protein A, HPrK/P: HPr kinase/phosphatase, CRE: catabolite responsive element, CCR: carbon catabolite repression, CCA: carbon catabolite activation. Based on [18, 68]

HPr-Ser-P is involved in carbon catabolite repression and activation HPr-Ser-P is involved in CCR/CCA through the interaction with CcpA. Together the two proteins can bind so called catabolite responsive elements (*cre*) which results in either repression or activation of af-

fected genes depending on if the *cre* site is upstream or downstream of the promoter[47, 14, 18]. The binding of CcpA is strongly stimulated by interaction with HPr-Ser-P, although there are indications that CcpA might exhibit some degree of unspecific binding in *L. lactis* which is not observed in *Bacillus* [40]. A schematic overview of the regulatory system can be seen in figure 5.2.

CcpA targets *cre* sites located adjacent to genes involved in regulation and central metabolism which are often glycolytic genes but there are many *cre* sites throughout the chromosome of *Lactococcus lactis*. Known targets are the *gal* operon (galactose utilization), *fru* operon (fructose utilization), *las* operon (encoding phosphofructokinase, pyruvate kinase and lactate dehydrogenase) and NoxE (*noxE*) encoding the primary NADH oxidase in *Lactococcus lactis*. The *las* operon is activated by CcpA and is an example of CCA. This involvement places CcpA in direct contact key parts of both central carbon metabolism and redox metabolism via control of the *las* operon and NoxE [26, 75]. There are also indications of a link between CcpA and the proteolytic system through activation of *pepQ* which affects the pleitropic regulator CodY (*codY*).

5.3 Aerobic metabolism of *Lactococcus lactis*

5.3.1 Oxygen tolerance in *Lactococcus lactis*

When growing under aerobic conditions a cell is subjected to oxidative stress. This does not arise from oxygen itself, but rather as a side product of cellular processes in which partially reduced oxygen can generate reactive oxygen species such as O_2^- (superoxide anion), $OH^\bullet$ (hydroxyl radical) and H_2O_2 (hydrogen peroxide) [46]. *Lactococcus lactis* has several systems to combat oxidative stress [46, 31]:

- Superoxide dismutase (*sodA*), $2\,O_2^- + 2\,H^+ \longrightarrow H_2O_2 + O_2$
- Water forming NADH oxidase (*noxE*), $2\,NADH + 2\,H^+ + O_2 \longrightarrow 2\,NAD^+ + 2\,H_2O$
- Coupled NADH oxidase/NADH peroxidase (*ahpR* - *ahpF* + *ahpC*), $NADH + O_2 + H^+ \longrightarrow NAD^+ + H_2O_2$ and $H_2O_2 + NADH + H^+ \longrightarrow NAD^+ + 2\,H_2O$ (overall reaction is the same as in the water forming NADH oxidase above)

The water forming NADH oxidase (*noxE*) is believed to be the main provider of NADH oxidase activity (NOX) in *Lactococcus lactis* (*noxE* provided 95% of the NOX activity in *Lactococcus lactis* TIL46). Though very important for oxygen consumption and NOX activity, *noxE* is not essential for protection from oxidative stress nor for growth or NAD^+ regeneration under aerobic conditions and natural strains have been isolated without any NADH oxidase activity [67].

5.3.2 Respiration in *Lactococcus lactis*

The observation that lactic acid bacteria, and *Lactococcus lactis* (*Streptococcus lactis*) in particular, can respire when hemin is added to the growth medium was made more that 40 years ago [64] (heme or hemin reflects the redox state of iron atom). This added another capability to the already diverse capabilities of lactic acid bacteria. When looking into this phenomenon many years later, it was discovered that not only the two previously tested species but many lactic acid bacteria exhibit increased growth-efficiency in the presence of heme and aeration: *Lactococcus lactis*, *Enterococcus faecalis*, *Leuconostoc mesenteriodes* and *Streptococcus* spp. [7]. To enable respiration a minimal respiratory chain must exist. In LAB this consists of an electron donor, an electron shuttle and a heme-dependant terminal electron acceptor. None of the LAB synthesize heme naturally and therefore scavenge it from the surrounding environment. The degree to which the various components in the electron transport chain are synthesized varies in different species but is never completely present [41]. A small schematic overview can be seen in figure 5.3.

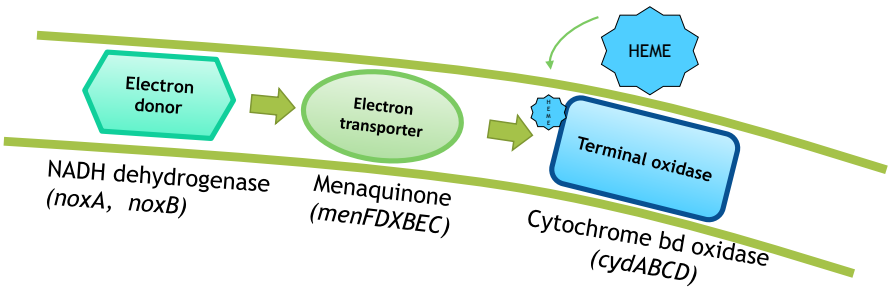

Figure 5.3: Overview of respiration in lactic acid bacteria. Genes present in *Lactoccus lactis* are indicated below each component. Adapted from [41]

Components and regulation of respiratory metabolism in *Lactococcus lactis*

The best studied model of respiration in a lactic acid bacterium is *Lactococcus lactis*. Since *L. lactis* was among the first, in which the latent ability to respire was discovered, it has received the attention of several studies over the years to elucidate the effect of respiration on the metabolism. The main benefit to respiring cells are improved long term survival and improved biomass formation [20, 25, 55] both of which has proven useful in the production of starter cultures for the dairy industry.

Initial investigations indicated that when a heme source is added to an aerated culture the growth is biphasic. First the culture grows under normal fermentative conditions only to begin respiration in late exponential

phase [20, 25]. This was later questioned by Koebmann et *al.* by their finding that ATPase deficient mutants required respiration permissive conditions for exponential growth [38].

The mechanism behind the beneficial effects on biomass formation is believed to be a combination of acetate production (yielding extra ATP compared to lactate production) and the fact that respiration-coupled expulsion of protons, spares the energy required for the ATPase complex to perform a similar task, amounting to a saving of energy rather than a production of energy through oxidative phosphorylation. Similarly any effects from amino acid catabolism or lower maintenance were also ruled out [38].

The reasons behind the increased survival rates of cultures grown under respiratory conditions stems from a lower oxygen concentration inside the cells leading to reduced oxidative stress in respiring cultures, with lower DNA damage, lower protein damage and a reduced mutational frequency, when compared to aerated cultures without heme. Another factor is reduced acid production under respiratory conditions, which in turn leads to an increase in pH, positively impacting the survival of respiring cultures [25, 55].

Regulatory mechanisms of respiration in *Lactococcus lactis*

As with many other aspects of metabolism, the control of respiration in *Lactococcus lactis* has been linked to CcpA. The regulation by CcpA on heme uptake is believed to be negative regulation [26].

Intracellular heme homeostasis is managed by HrtR, an intracellular heme sensor that regulates transcription of *hrtBA* which constitutes a heme efflux pump. HrtR acts as a transcriptional repressor of *hrtBA* which is alleviated upon binding of heme [42].

Anaerobic respiration and alternative electron acceptors

The use of alternative electron acceptors has not been extensively studied. A literature review has only revealed a single paper on the use of alternative electron acceptors in *Lactococcus lactis*. The conclusion is that even though *Lactococcus lactis* C10 (*Streptococcus lactis* C10) posseses a fumarate reductase it is not able to allow fermentative growth on glycerol [28]. This is contrary to the finding of Tachon et *al.* [66] and a genome search which only reveals the presence of a single gene *frdC*, encoding a FRD flavoprotein subunit in *Lactococcus lactis* MG1363, KF147 and IL1403 and no other components of a fumarate reductase.

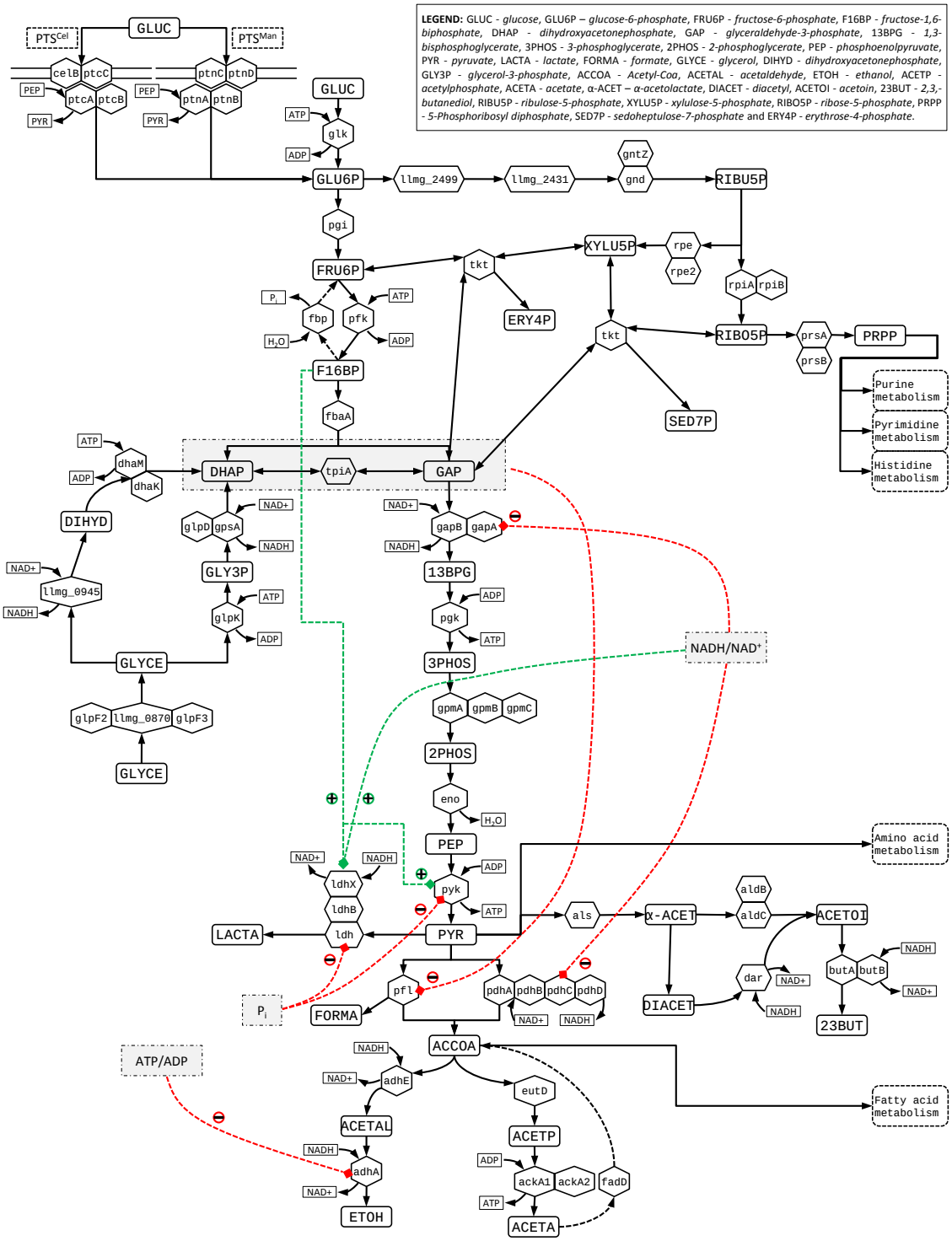

Figure 5.4: A simplified schematic overview of central metabolism in *Lactococcus lactis* MG1363. Coloured dashed lines indicates regulation (based on [49]).

5.4 Redox and energy metabolism is highly connected to central metabolism

Traditional metabolic engineering has often focused on the engineering of enzyme levels as well as inserting or deleting entire pathways to improve production of a given compound [3]. Initial attempts at investigating the control of flux through glycolysis by overexpressing various enzymes in the pathway have not been successful [39]. This implies that flux control must lie somewhere else. At the core of any metabolic network are only 12 metabolic precursors and the cofactors ATP, NADH and NADPH [65, 58]. The energy and redox cofactors are some of the most frequently used metabolites in the central metabolism of *E. coli* [51] and as such they have a large potential as regulators of metabolism. The effect of redox metabolism on the central metabolism of *Lactococcus lactis* has been well studied.

5.5 Integration of information from the transcriptomic and the metabolic level

The characterization of strains with increased expression of ATPase activity, presented later in this thesis, was intended to combine the information from several levels. The idea of combining several layers of information is similar to the work previously done for *Escherichia coli* where both transcriptional and metabolomic analysis were combined [29]. For that investigation, strains which overexpressed NADH oxidase and the soluble F_1 part of ATPase in *Escherichia coli* were constructed to lower the level of NADH and ATP, respectively. A global interaction network, comprising of protein interactions, transcriptional regulation, and metabolic networks, was used to integrate data from transcription profiles, metabolic fluxes, and the metabolite levels. High-scoring networks for the two strains were identified.

The results revealed a smaller, but denser network for perturbations of ATP level, compared with that of NADH level. The action of many global transcription factors such as ArcA, Fnr, CRP, and IHF commonly involved both NADH and ATP, whereas others responded to either ATP or NADH. Overexpressing NADH oxidase invokes response in widespread aspects of metabolism involving the redox cofactors (NADH and NADPH), whereas ATPase has a more focused response to restore ATP level by enhancing proton translocation mechanisms and repressing biosynthesis. Interestingly, NADPH played a key role in restoring redox homeostasis through the concerted activity of isocitrate dehydrogenase and UdhA transhydrogenase. A figure showing the regulatory effects of these perturbations can be seen in figure 5.5.

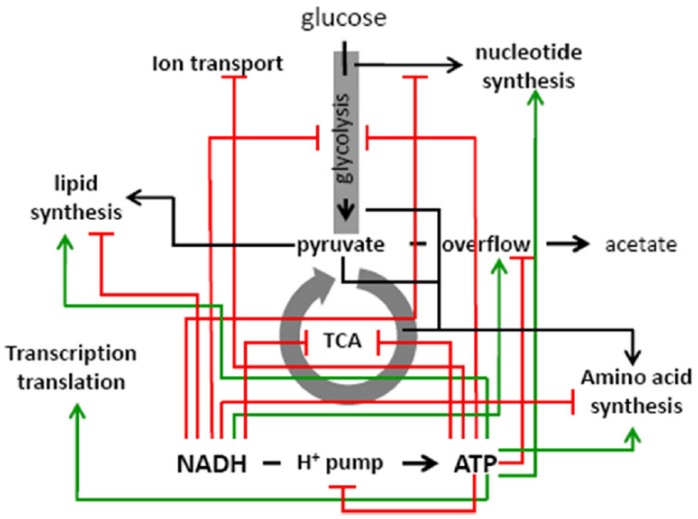

Figure 5.5: A figure showing the regulatory roles of ATP and NADH on the metabolism of *Escherichia coli*. Green lines - activation, red lines - repression. Reproduced from [29].

Manipulation of redox and energy metabolism as a potential tool to redirect cellular metabolism into producing desirable products

The redox and energy cofactors have a great influence on the metabolism in *E. coli*. This control could potentially be applied to the investigation into glycerol metabolism in *Lactotoccus lactis*. Cells overexpressing ATPase activity have a lowered energy state which might simulate growth on a poor substrate. This could lead to alleviation of catabolite repression and stimulate a metabolic response to allow *L. lactis* to use glycerol either as sole carbon and energy source or in co-metabolization. Furthermore the application of glycerol as a substrate in itself presents a challenge with respect to maintaining redox balance in the cell.

The further investigation into the response of *L. lactis* to perturbations in energy metabolism as presented in part IV, not only has implications for the utilization of glycerol as carbon and energy source but also for designing efficient cell factories for the production of essential chemical building blocks in the future. Any insights into the complex interplay between central carbon metabolism and control imposed by the redox and energy cofactors could prove valuable for engineering competitive bioconversion platforms, not only for glycerol conversion but also for other substrates such as lignocellulose or marine biomass.

Chapter 6

Potential challenges to glycerol assimilation by *Lactococcus lactis*

An initial analysis of the challenges facing *Lactococcus lactis* when utilizing glycerol as a substrate, revealed three areas that were deemed likely to present difficulties. These were: 1) when growing on glycerol the gluconeogenic flux needs to be high enough to sustain growth, 2) the biochemical pathway for glycerol dissimilation may not be entirely functional, and 3) the growth on glycerol generates a greater amount of redox energy per pyruvate than equivalent growth on glucose, which the cell needs to regenerate. An overview of the analysis and the three areas can be seen in figure 6.1.

The challenge facing gluconeogenic flux is evident in figure 6.1 when looking at the connection between glycerol and central metabolism. Besides generating energy for growth there is also a need for certain building blocks such as glucose-6-phosphate and fructose-6-phosphate in growing cells. When growing on glucose these would be generated in the normal course of glycolysis but growth on glycerol requires a flux to be channeled back up from DHAP via gluconeogenesis. A complete biochemical pathway obviously needs to be present for glycerol to be channeled into glycolysis. Even though many genetic components appear to be present there could still be structural or regulatory issues that prevents the functionality of the pathway. Unbalanced growth with a surplus of redox energy will be problematic for the cell since this would deplete the supply of cofactors. If this happens faster than new cofactors can be synthesized, it will lead to growth arrest.

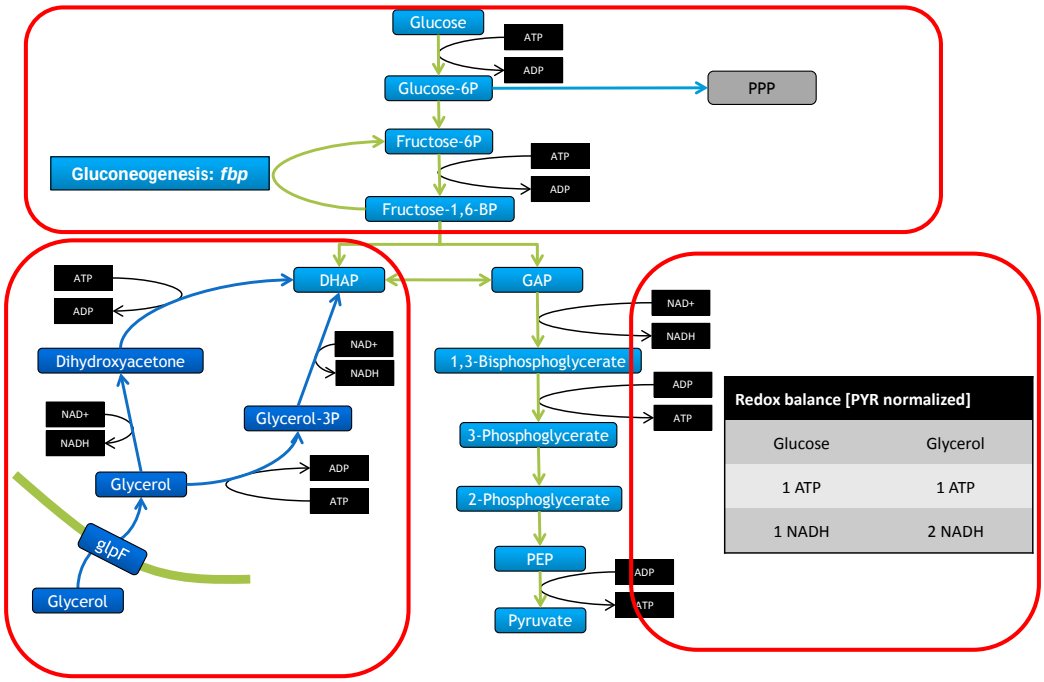

Figure 6.1: Overview of the possible problems associated with glycerol dissimilation in *Lactococcus lactis*. TOP - gluconeogenic flux, substrates and conditions, LEFT - functional pathway for glycerol uptake and dissimilation and RIGHT - redox levels needs to be balanced in growing cells.

6.0.1 Genetic components

As previously outlined (figure 4.2, page 14) the pathway from glycerol to glycolysis is made up of two routes each with their own set of enzymes.

- Aerobic: Glycerol kinase/Glycerol-3P dehydrogenase
- Anaerobic: Glycerol dehydrogenase/Dihydroxyacetone kinase

A quick scan of the genomes from the sequenced *Lactococcus lactis* strains reveals the presence of most of the components. An overview can be seen in table 6.1. Even though the presence of most of the components required for glycerol assimilation can be found in the genomes of various strains of *Lactococcus lactis*, many of them are uncharacterized or putative annotations. This opens up questions to whether they are active and functional.

A very rough idea of the activity was gained from a simple look into intensity data from microarray analysis of *Lactococcus lactis* MG1363 cultivated on glucose, maltose and galactose (manuscript in preparation) courtesy of Associate professor Christian Solem (Center for Systems Microbiology, Department of Systems Biology, Technical University of Denmark). The results can be seen in figure 6.2 and figure 6.3. This indicated that

Component	MG1363	IL1403	SK11	KF147
Transport	*glpF* (llmg_0870) *glpF2* (llmg_1097) *glpF3* (llmg_2327)	*glpF1* (L47650) *glpF2* (L0015)	(LACR_0255) (LACR_1487) (LACR_1732)	*glpF* (LLKF_0248) *glpF* (LLKF_1309)
Glycerol kinase	*glpK* (llmg_1099)	*glpK* (L014)	*glpK* (LACR_1485)	*glpK* (LLKF_1311)
G3P dehydrogenase	*glpD* (llmg_1098) *gpsA* (llmg_1114)	*glpD* (L0013) *gpsA* (L0016)	(LACR_1486) *gpsA* (LACR_1461)	*gpsA* (LLKF_1429)
Glycerol dehydrogenase	Putative (llmg_0945)	-	(LACR_1650)	*gldA* (LLKF_1680)
DHA kinase	*dhaK* (*dhaQ*, llmg_0255) *dhaL* (llmg_pseudo_05) *dhaM* (llmg_0257)	*dhaK* (*dhaQ*, L44063) *dhaL* (L45677) *dhaM* (L46694)	*dhaK* (*dhaQ*, LACR_0250)	*dhaK* (LLKF_0245) *dhaL* (LLKF_0246) *dhaM* (LLKF_0247) *dhaQ* (LLKF_0243)

Table 6.1: An overview of the genetic components required for glycerol assimilation and their presence on the genomes of sequenced *Lactococcus lactis* strains. G3P dehydrogenase - Glycerol-3-phosphate dehydrogenase, DHA kinase - Dihydroxyacetone kinase.

at least for *Lactococcus lactis* MG1363 most of the components seem to be transcribed to some extent and some almost on par with glycolytic enzymes, even on a PTS sugar such as glucose.

Glykolytic genes:		Glucose	Maltose	Galactose		
glk	llmg_2299	11.05	10.80	10.52	Glucokinase	
pgi	llmg_2448	13.41	13.17	13.27	Glucose-6-P isomerase	
fbp	llmg_0264	7.38	9.06	8.19	Fructosebisphosphatase	Gluconeogenesis
pfk	llmg_1118	16.36	15.34	15.66	6-Phosphofructokinase	
fbaA	llmg_2167	14.51	13.78	14.26	Fructosebisphosphat aldolase	
tpiA	llmg_1424	10.63	9.89	10.07	Triosephophat isomerase	
gapA	llmg_0530	8.67	9.13	9.50	Glyceraldehyd-3-P dehydrogenase	
gapB	llmg_2539	14.52	14.05	14.41	Glyceraldehyd-3-P dehydrogenase	
pgk	llmg_0253	15.14	14.75	14.96	Phosphoglycerate kinase	
pmg	llmg_0355	14.21	13.51	13.80	Phosphoglycerate mutase	
gpmB	llmg_1579	14.77	15.07	14.90	Phosphoglycerate mutase	
gpmC	llmg_1894	5.79	5.76	5.63	Phosphoglycerate mutase	
eno	llmg_0617	12.91	13.07	12.50	Phosphopyruvate hydratase (Enolase)	
pyk	llmg_1119	15.16	14.29	14.88	Pyruvate kinase	
Glycerol genes:						
glpF	llmg_0870	12.00	12.04	11.76	Glycerol uptake facilitator	
glpF2	llmg_1097	6.70	8.12	6.93	Glycerol uptake facilitator	
glpF3	llmg_2327	11.88	10.42	10.34	Glycerol uptake facilitator	
glpK	llmg_1099	6.94	8.14	6.93	Glycerol kinase	Route 1
dhaK	llmg_0255	7.68	7.73	7.91	Dihydroxyacetone kinase	Route 2
dhaM	llmg_0257	6.02	6.28	6.39	Dihydroxyacetone kinase	Route 2
glpT	llmg_0523	4.91	5.15	6.00	Glycerol-3-P transporter	
Putative	llmg_0945	12.84	12.34	11.70	Putative glycerol dehydrogenase	Route 2
gpsA	llmg_1114	13.82	13.20	13.27	Glycerol-3-P dehydrogenase	Route 1
glpD	llmg_1098	6.99	8.17	7.00	Glycerol-3-P-dehydrogenase	

Figure 6.2: Overview of transcriptomic data from *Lactococcus lactis* MG1363 cultivated on the sugars glucose, maltose and galactose. The intensity data has been averaged for each of the five different probes representing each gene. The expression unit is arbitrary intensity.

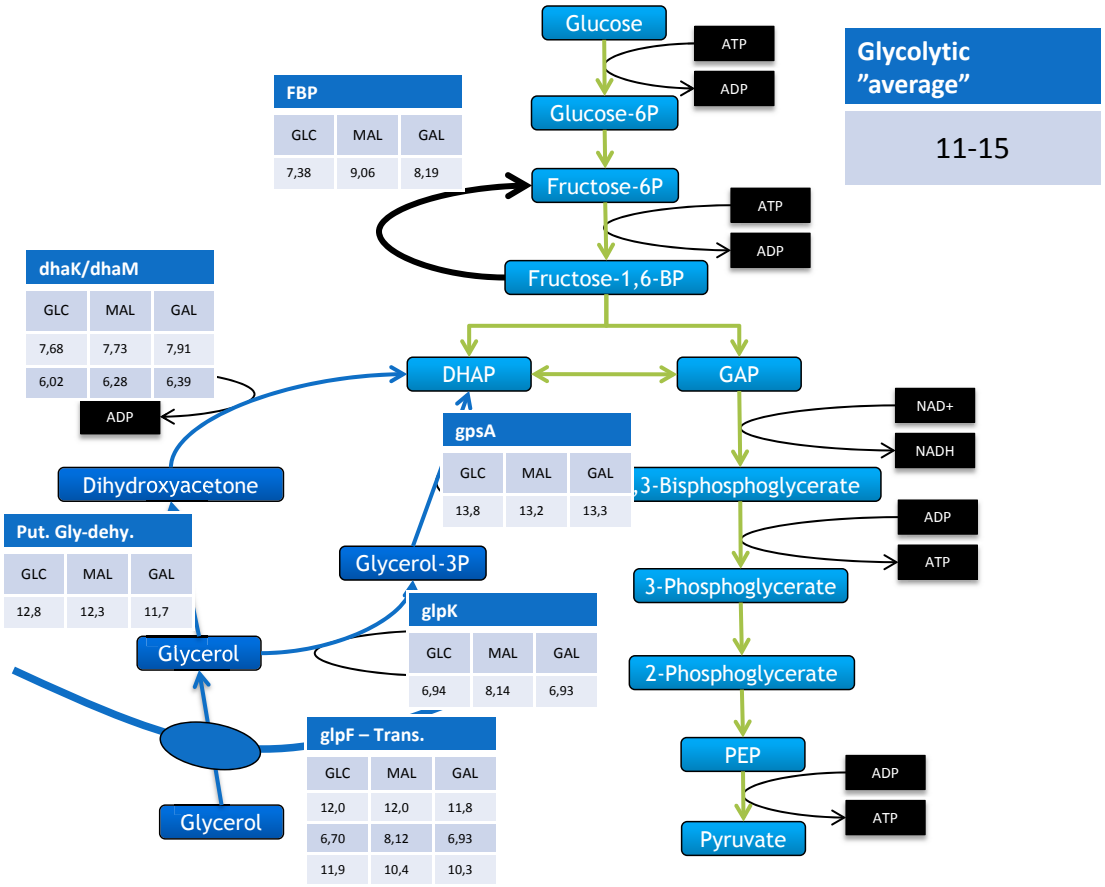

Figure 6.3: Overview of the simple averages of transcriptional data for glycolytic and glycerol assimilation genes for *Lactococcus lactis* MG1363 cultivated on glucose, maltose and galactose.

6.1 Approaches to overcome the problems faced by *Lactococcus lactis*

To investigate these possible problems a strategy was devised for each area. For the investigation into the extent of gluconeogenic flux and presence of the required enzymatic machinery, screening of growth with different substrates was used to ascertain whether gluconeogenic flux is sufficiently active and whether all the components for glycerol assimilation are functional. If this were not to be the case, the solution to the biochemical problem is a complete pathway with all necessary genes introduced into *Lactococcus lactis*. For that purpose a novel artificial operon was designed which contained all the necessary components to assimilate glycerol, these being a glycerol fascilitator from *Escherichia coli*, a glycerol dehydrogenase from *Citrobacter*

freundii and a dihydroxyacetone kinase also from *Citrobacter freundii*. To solve a potential redox problem, a library of NADH oxidase activity will be introduced into *Lactococcus lactis* to efficiently tune the level of oxidation required for cell growth. An overview of these approaches can be seen in figure 6.4.

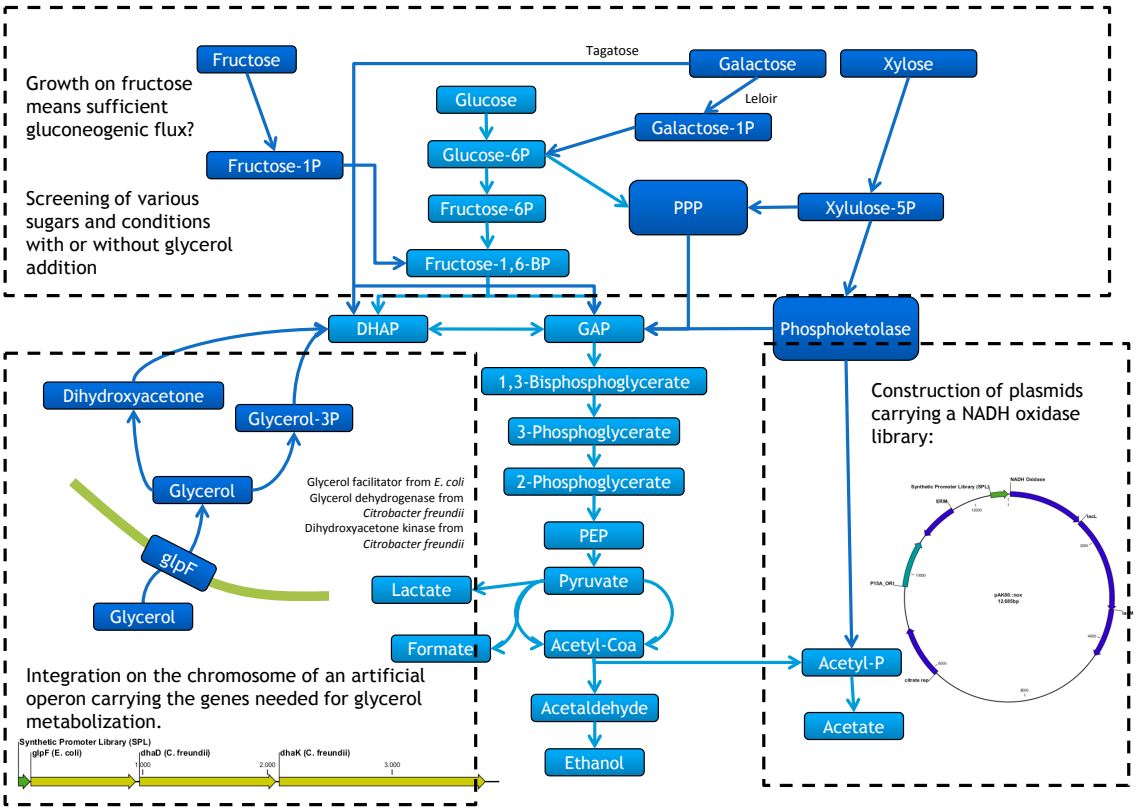

Figure 6.4: Approaches to investigate and overcome the problems associated with glycerol dissimilation in *Lactococcus lactis*. TOP - screening of growth under various conditions, LEFT - construction of an artificial operon containing all necessary genes for glycerol dissimilation and RIGHT - construction of synthetic promoter libraries with varied levels of NADH oxidsase activity.

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Part II

Screening of *Lactococcus* spp. for glycerol utilization

Chapter 7

Introduction

7.1 Glycerol has become an abundant molecule suitable as an industrial substrate

The potential use of glycerol as a substrate for industrial fermentation has emerged through increased production of biodiesel. Biodiesel production results in a byproduct of waste (or crude) glycerol which contains salts, methanol, free fatty acids and glycerol. Refinement of crude glycerol is expensive and bioconversion of crude glycerol could present a better way of handling the increased amounts of waste [19, 124, 60, 73, 48].

While many microorganisms can metabolize glycerol with an external electron acceptor such as oxygen, few are able to do so anaerobically. Microorganisms that can metabolize glycerol anaerobically have been reported for the species *Citrobacter*, *Clostridium*, *Klebsiella*, *Enterobacter*, *Lactobacillus*, *Bacillus*, *Propionibacterium* and *Anaerobiospirillum* [73, 48]. It has even been reported that by manipulating the right fermentation conditions it is possible for *E. coli* to grow fermentatively on glycerol [118, 48].

There are many advantages to anaerobic bioconversion for production of fuels and chemicals such as less biomass formation resulting in higher yields and easier production in large scale since oxygen limitation no longer presents a problem [72]. Since *Lactococcus lactis* is adept at growing anaerobically and already produces several interesting biochemicals it seemed a promising place to start. Furthermore strains that are easily amendable and well characterized are good starting points for developing a production organism [24].

7.1.1 Prototrophic plant isolates of *Lactococcus lactis* might have retained the ability to grow on glycerol

Glycerol is metabolized through glycolysis as shown in figure 7.1. There are two main pathways of glycerol assimilation either via *glycerol dehydrogenase*

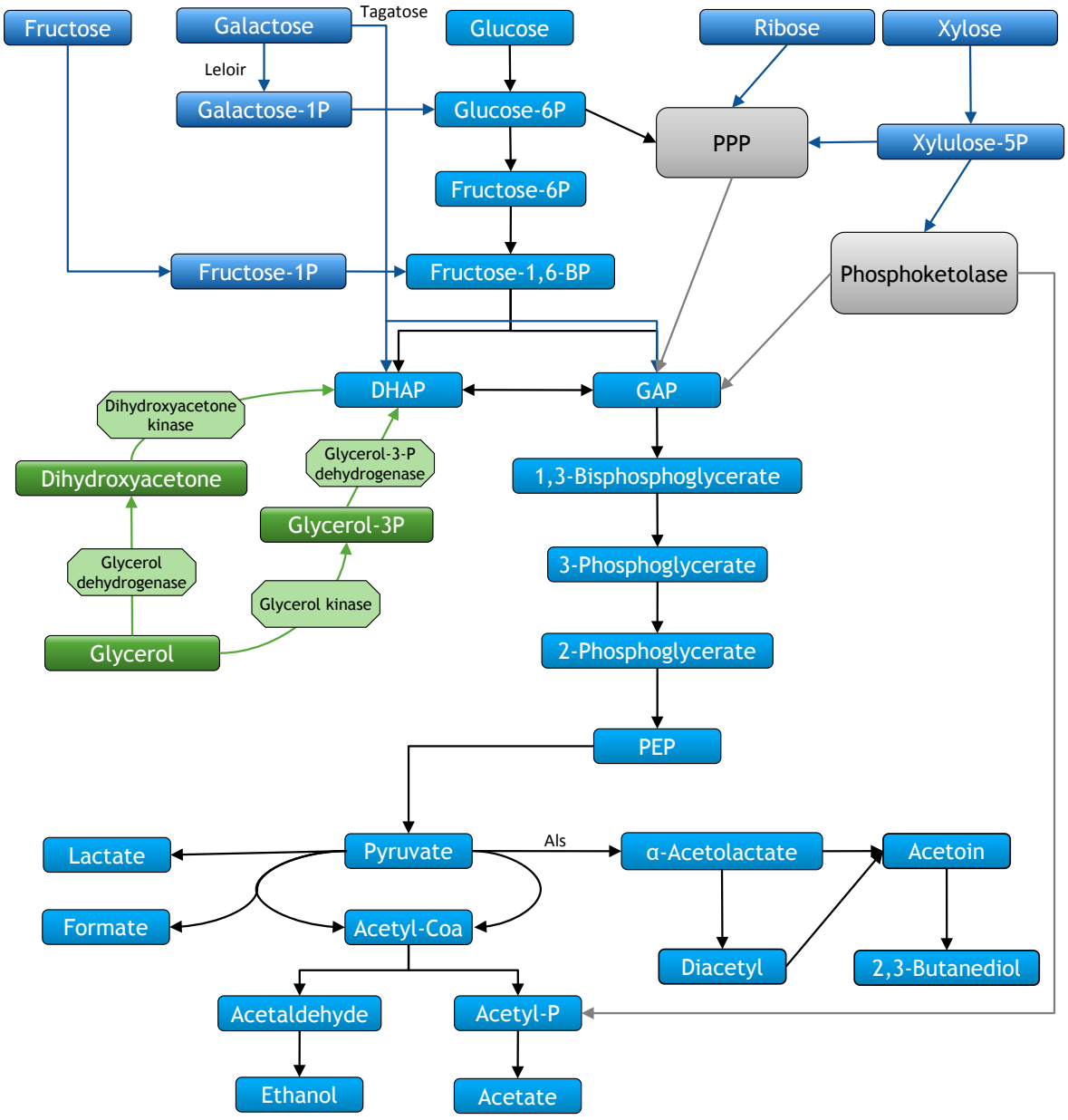

Figure 7.1: Overview of the central carbon metabolism of *Lactococcus lactis*. This is a simplified overview of the metabolites in central metabolism, without enzymes and cofactors. This illustrates the connection between glycerol and glycolysis.

and *dihydroxyacetone kinase* or via *glycerol kinase* and *glycerol-3-phosphate dehydrogenase*. The aim of this investigation was to determine whether any of the commonly used *L. lactis* strains have retained the ability to grow on glycerol as a sole energy and carbon source. The main sources of *L. lactis* strains which have been isolated to date have either a dairy or a plant origin. As presented in the general introduction, many of the required components for glycerol metabolization has been annotated for the sequenced strains of *L. lactis*. These are so far mostly of dairy origin. An overview of these genes can be seen in table 7.1.

Component	MG1363	IL1403	SK11	KF147
Transport	*glpF* (llmg_0870) *glpF2* (llmg_1097) *glpF3* (llmg_2327)	*glpF1* (L47650) *glpF2* (L0015)	(LACR_0255) (LACR_1487) (LACR_1732)	*glpF* (LLKF_0248) *glpF* (LLKF_1309)
Glycerol kinase	*glpK* (llmg_1099)	*glpK* (L014)	*glpK* (LACR_1485)	*glpK* (LLKF_1311)
G3P dehydrogenase	*glpD* (llmg_1098) *gpsA* (llmg_1114)	*glpD* (L0013) *gpsA* (L0016)	(LACR_1486) *gpsA* (LACR_1461)	*gpsA* (LLKF_1429)
Glycerol dehydrogenase	Putative (llmg_0945)	-	(LACR_1650)	*gldA* (LLKF_1680)
DHA kinase	*dhaK* (*dhaQ*, llmg_0255) *dhaL* (llmg_pseudo_05) *dhaM* (llmg_0257)	*dhaK* (*dhaQ*, L44063) *dhaL* (L45677) *dhaM* (L46694)	*dhaK* (*dhaQ*, LACR_0250)	*dhaK* (LLKF_0245) *dhaL* (LLKF_0246) *dhaM* (LLKF_0247) *dhaQ* (LLKF_0243)

Table 7.1: An overview of the genetic components required for glycerol assimilation and their presence on the genomes of sequenced *Lactococcus lactis* strains. G3P dehydrogenase - Glycerol-3-phosphate dehydrogenase, DHA kinase - Dihydroxyacetone kinase.

Many components of glycerol assimilation can be found in the genomes of sequenced *L. lactis* strains As shown in table 7.1 most components are present on the chromosomes of the sequenced *L. lactis* strains. In particular *L. lactis* MG1363 and *L. lactis* KF147 contain components for both pathways. In *L. lactis* MG1363 glycerol dehydrogenase has only a putative status, but as can be viewed in the transcriptome analysis in part IV still undergoes regulation in strains overexpressing ATPase activity.

To ascertain whether any of the strains have retained the ability to use glycerol various conditions under which glycerol assimilation might be stimulated were tested. These included different carbon sources (for co-metabolization) and environmental conditions such as aeration in combination with hemin for respiration permissive settings. Ideally a combination of these parameters will alleviate any potential problems such as regulatory issues or problems with the redox balance. The goal was to find a suitable candidate which grows on glycerol for the production of value-added fuels and chemicals such as ethanol or butanol from waste glycerol.

7.2 Xylose metabolism in *Lactococcus lactis*

The utilization of xylose as a substrate has been reported for several bacterial species such as *E. coli*, *Lactobacillus* and *Bacillus* spp. After transport of xylose into the cell by either low-affinity symport systems or other (high-affinity) systems, xylose isomerase (*xylA*) converts xylose to xylulose which is further phosphorylated by xylulokinase (*xylB*) to xylulose-5-phosphate (xylu5P) [83, 96]. Further assimilation can then proceed via two different routes depending on the capabilities of the given strain. If assimilation proceeds along the phosphoketolase pathway xylu5P is converted (cleaved) to equimolar amounts of glyceraldehyde-3-phosphate (GAP) and acetylphosphate (acetylP). These are further metabolized to lactic acid and acetate, providing a theoretical yield of 1 mol of lactic acid (and acetate) per mole of xylose. If xylose on the other hand is shuttled into the pentose phosphate pathway the final result is 5 mol of lactate from 3 mol of xylose equal to a theoretical yield of 1.67 mol lactic acid per mol of xylose [103, 89, 100].

7.2.1 The ability to utilize xylose among *Lactococcus* spp. depends on the origin

The ability to utilize xylose among *Lactococcus* spp. is strain dependant. Among the two major groups of Lactococcus dairy isolates (subsp. cremoris) such as *L. lactis* MG1363 and IL1403 are unable to utilize xylose while some plant isolates (subsp. lactis) such as *L. lactis* IO-1, KF147 and NCDO2118 have retained the ability [83]. Known manipulations of xylose metabolism include transferring the genes required for xylose assimilation (*xylRAB*) from *L. lactis* IO-1 to *L. lactis* IL1403 allowing growth on xylose as sole carbon and energy source [100] and the production of xylitol from xylose by expressing a xylose reductase (*Pichia stipitis*) in *L. lactis* NZ9800 [95]. An overview of the proposed pathways for xylose metabolism can be seen in figure 7.2.

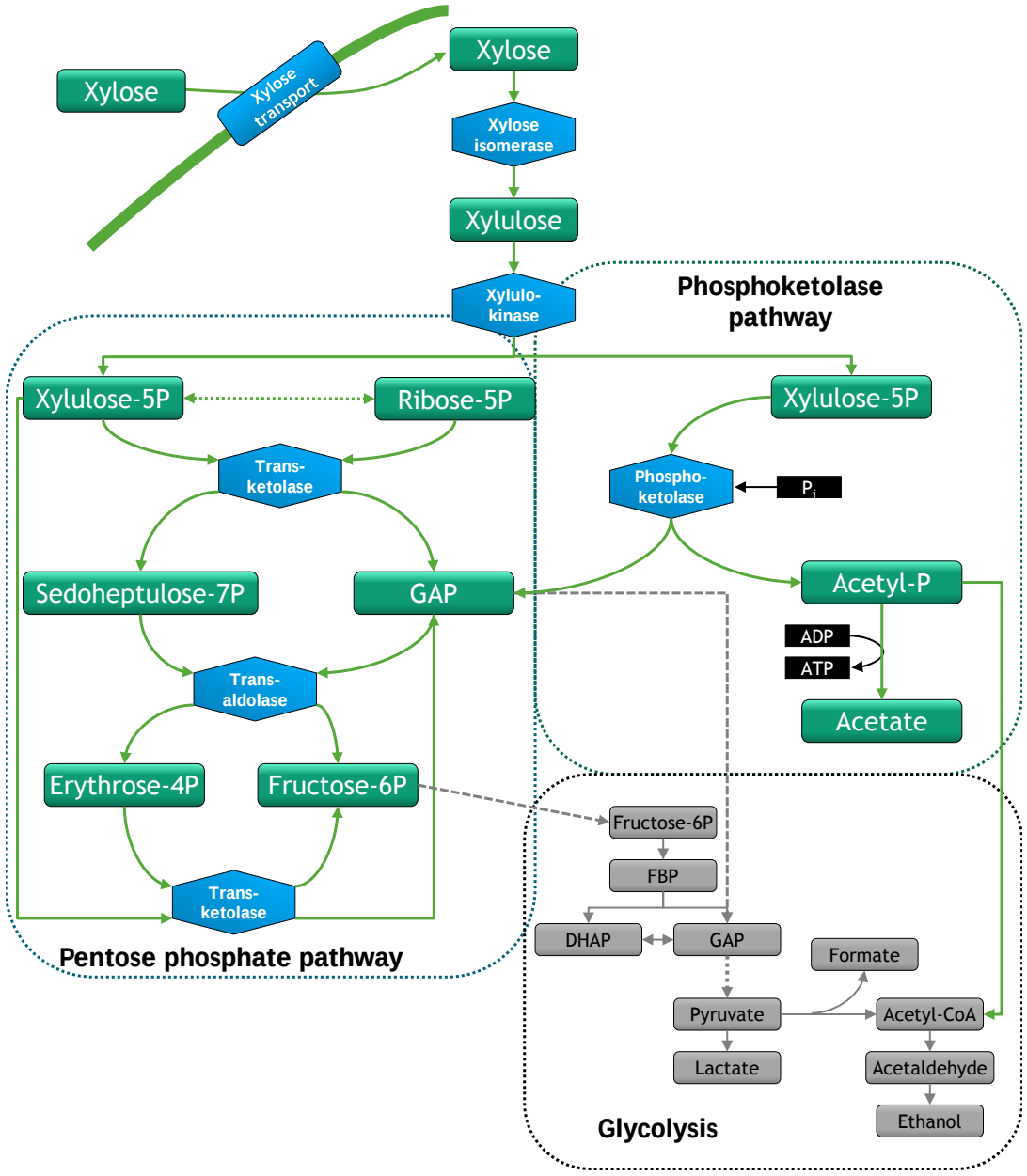

Figure 7.2: Overview of the proposed xylose metabolism in *Lactoccus lactis* IO-1 [103]. This is a simplified schematic overview of the metabolic routes for xylose assimilation in *L. lactis* strains capable of growing on xylose. Xylose is either metabolized via the phosphoketolase pathway, the pentose phosphate pathway or a combination of these. GAP - glyceraldehyde-3-phosphate, DHAP - dihydroxy-acetone phosphate, FBP - 1,6-fructosebisphosphate.

Chapter 8

Materials and Methods

8.1 Bacterial strains and growth conditions

The bacterial strains used in the project are listed in table 8.1.

Strain	Alias	Description	Source
AH64	MG1363	*Lactococcus lactis* subsp. *cremoris*, plasmid-free derivative of *L. lactis* NCDO712, dairy isolate (cheese starter culture)	[123]
AH186	IL1403	*Lactococcus lactis* subsp. *cremoris*, plasmid-free derivative of the strain IL594, dairy isolate (cheese starter culture)	[78, 77]
AH63	NCDO2118	*Lactococcus lactis* subsp. *lactis*, plant isolate (frozen peas)	[23]
AH83	KF147	*Lactococcus lactis* subsp. *lactis*, plant isolate (mung bean)	[36, 63]
AH66	NCDO1867	*Lactococcus lactis* subsp. *lactis*, plant isolate (frozen peas 1966)	NCIMB 701867, [23]
AH67	NCDO2091	*Lactococcus lactis* subsp. *lactis*, plant isolate (fermenting radishes)	NCIMB 702091, [23]
AH68	NCDO2108	*Lactococcus lactis* subsp. *lactis*, plant isolate (frozen beans, lac-)	NCIMB 702108, [23]
AH69	NCDO2110	*Lactococcus lactis* subsp. *lactis*, plant isolate (frozen peas)	NCIMB 702110, [23]
AH70	NCDO2111	*Lactococcus lactis* subsp. *lactis*, plant isolate (frozen peas)	NCIMB 702111, [23]
AH71	NCDO2125	*Lactococcus lactis* subsp. *lactis*, isolated from termite gut	NCIMB 702125, [23]
AH72	NCDO2146	*Lactococcus lactis* subsp. *lactis*, isolated from mastitis	NCIMB 702146, [23]
AH73	NCDO2633	*Lactococcus lactis* subsp. *lactis*, isolated from the rectum of cow 330	NCIMB 702633
AH74	NCDO2727	*Lactococcus lactis* subsp. *lactis*, plant isolate (mung bean)	NCIMB 702727
AH75	NCDO2738	*Lactococcus lactis* subsp. *lactis*, plant isolate ("Anchu" mash)	NCIMB 702738
AH76	NCDO2181	*Lactococcus lactis* subsp. *hordniae*, isolated from leafhopper	NCIMB 702181, [93]
AH77	NCDO2112	*Lactococcus raffinolactis*, isolated from garden carrots	NCIMB 702112
	BK1010	*L. lactis* MG1363 transformed with pAK80, Erm^r	[164]
	BK1503	*L. lactis* MG1363 transformed with pCPC4::*atpAGD*, Erm^r	[164]
	BK1506	*L. lactis* MG1363 transformed with pCPC7::*atpAGD*, Erm^r	[164]
	BK1502	*L. lactis* MG1363 transformed with pCPC3::*atpAGD*, Erm^r	[164]

Table 8.1: An overview of the bacterial strains used in the screening part of the project. All strains are wild type strains. NCIMB - NCIMB Ltd, Aberdeen, Scotland, UK.

8.1.1 Growth conditions

Strains were routinely cultivated under three different conditions: 1) anaerobically, 2) aerobically and 3) respiratory (aerobic incubation with addition of hemin). Temperature for strain cultivation was maintained at 30°C in

either rich or defined media with either no antibiotic or containing 5 μg/mL Erythromycin or 5 μg/mL Tetracycline, depending on the selection requirements. Other temperatures such as at 28°C or room temperature (22°C) were used when appropriate. Rich medium consisted of GM17 broth [149] and defined medium was either BL or SA [143]. The defined media were modified as follows: 1) SAL/BLL: SA or BL medium without sodium acetate but supplemented with 2 μg/mL lipoic acid, 2) SALN: SA medium without sodium acetate but supplemented with 2 μg/mL lipoic acid and 20 μg/mL of adenosine, guanosine, cytidine, thymidine, inosine and uridine. Growth was monitored by optical density and was measured at 600 nm (OD_{600}) on a Shimadzu UV mini 1240 spectrophotometer.

8.2 Sampling for metabolite analysis

Sampling for metabolite analysis was carried out as follows: 5 mL of culture was quenched in 5 mL of hot phenol (80°C) and subsequently frozen at -20°C for further processing. After completing the entire set of measurements, all samples were treated with 2 times chloroform extraction and the finished samples were shipped to professor Marco Oldiges, Forschungszentrum Jülich, Biotechnologie 2, Germany, for further analysis.

8.3 Correlation of cell density to cell mass

To convert OD into dry weight a correlation from Jensen et *al.* [88] is utilized. They determined that the correlation between dry weight and optical density was (on average) 0.19 $\frac{\text{g DW}}{\text{L OD450 1}}$. This was based on measurements done at 450 nm. This can be converted to 600 nm:

$$0.19\frac{\text{g DW}}{\text{L OD450 of 1}} \cdot 1.8 = 0.342\frac{\text{g DW}}{\text{L OD600 of 1}}$$

8.4 Calculations

Growth rates were calculated from plots of OD vs. time on a logarithmic scale. The growth rates were then estimated from the slope of a logarithmic regression on the data points under exponential growth. Each experiment gave rise to a set of growth parameters for each strain, and from these three separate values a mean and standard deviation was calculated.

The specific glycolytic fluxes were calculated from slopes of glucose consumption, lactate production, formate production and acetate production, which, were plotted against OD, and multiplied by the specific growth rates. Biomass yield was calculated as the inverse of the slope generated by linear regression on the glucose plot.

Chapter 9

Results

This section describes the work conducted to screen various *Lactococcus* spp. candidates for the ability to utilize glycerol as a carbon and energy source. The idea behind these experiments is that perhaps some of the problems associated with growth on glycerol can be mediated by changing the substrate and environmental conditions i.e. a redox imbalance could be remedied by the addition of heme and aeration or perhaps glycerol could be utilized in co-metabolization with another substrate. The general approach was:

- Utilization of glycerol as sole carbon and energy source
- Co-metabolization of glycerol in combination with other substrates

The results represented in the following section are representative data sets, often from different strains. Not all the data, from all the strains has been presented here. Additional information can be seen in the appendix.

9.1 Preliminary serial cultivation does not induce glycerol utilization

As an early attempt at activating glycerol utilization, the strain *Lactococcus lactis* NCDO2118 was subjected to a serial transfer regiment in order to stimulate this activation by directed evolution. The medium and conditions chosen were SALN + 1% glycerol incubated anaerobically at 30°C to represent the ideal production conditions. A total of 15 transfers (of one mL into 10mL of fresh medium) were completed without any sign of additional biomass formation above $OD_{600} \approx 0.100$. Since SALN medium contains amino acids and nucleosides, limited growth can be observed (around $OD_{600} \approx 0.100$) in this medium without any carbon and energy source added. After this period the experiment was discontinued.

9.2 Dairy isolates of *Lactococcus lactis* show improved adaptation to growth under nutrient rich conditions compared to plant isolates

When screening strains for substrate utilization in liquid cultures, it is important to ensure that the culture is only limited by the carbon and energy source. In any other case the effect of adding a supplemental carbon source such as glycerol to the growth medium might not be detected. Therefore an initial screening was performed to establish growth patterns on standard sugar substrates (glucose, fructose, galactose, maltose and for the plant isolates also xylose) for three selected model organisms. Growth characteristics of these strains when grown in SAL medium supplemented with glucose can be seen in figure 9.1 (*L. lactis* MG1363), figure 9.2 (*L. lactis* NCDO2118) and figure 9.3 (*L. lactis* KF147).

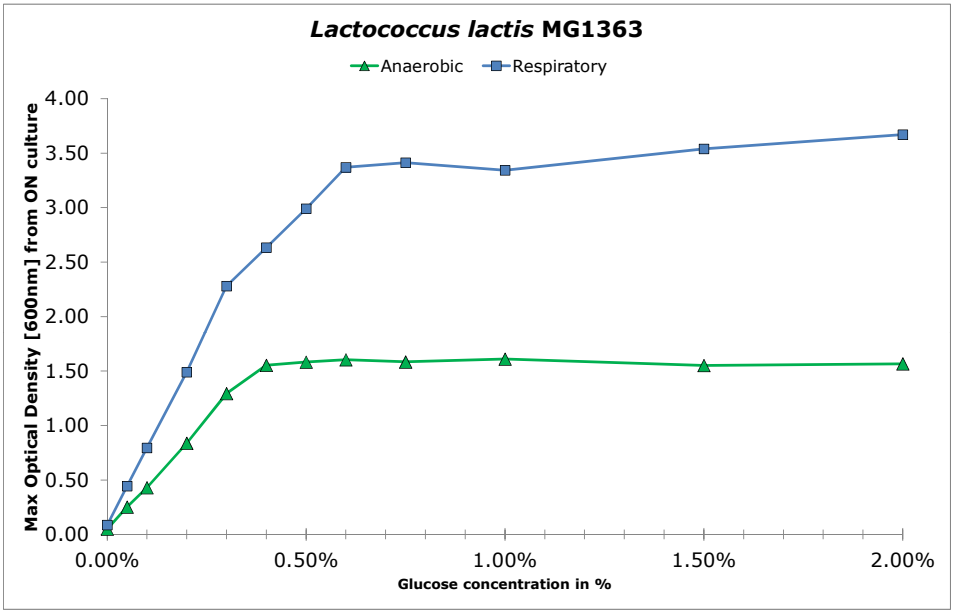

Figure 9.1: Overview of growth by *Lactococcus lactis* MG1363 in SAL medium supplemented with increasing amounts of glucose. Blue (square) - respiratory growth, Green (triangle) - anaerobic growth.

Comparing the three plots, the dairy isolate (MG1363) seems to grow 50% better under anaerobic conditions and almost twice as good under respiratory conditions as the plant isolates (NCDO2118 and KF147). This could be indicative of the specialization by the dairy strains to better utilize the nutrients found in the (fairly) rich environment with plenty of available amino acids. The full range of data for all tested sugars can be seen in appendix B. On the basis of these results sugar concentrations of 0.1%–0.2% in the

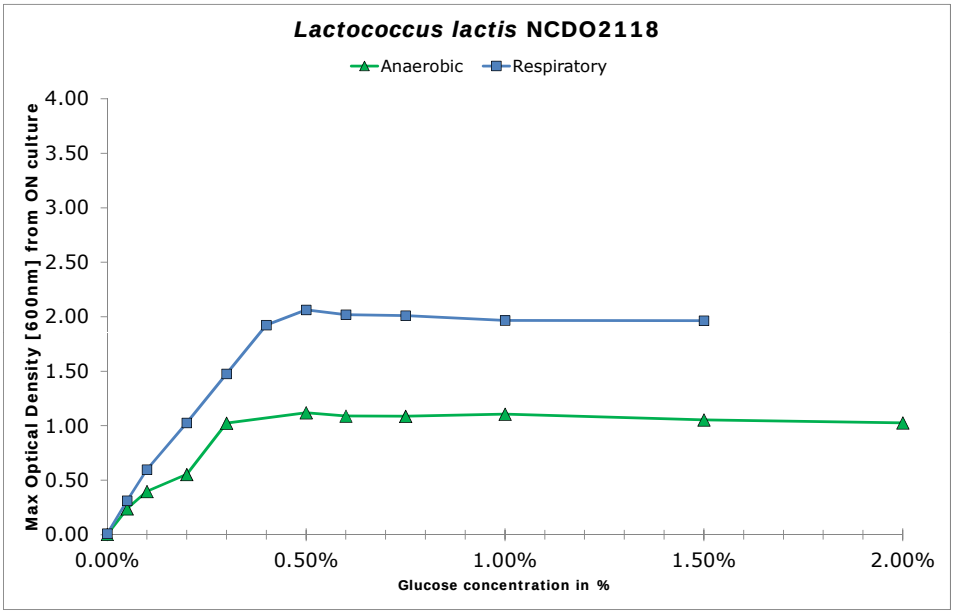

Figure 9.2: Overview of growth by *Lactococcus lactis* NCDO2118 in SAL medium supplemented with increasing amounts of glucose. Blue (square) - respiratory growth, Green (triangle) - anaerobic growth.

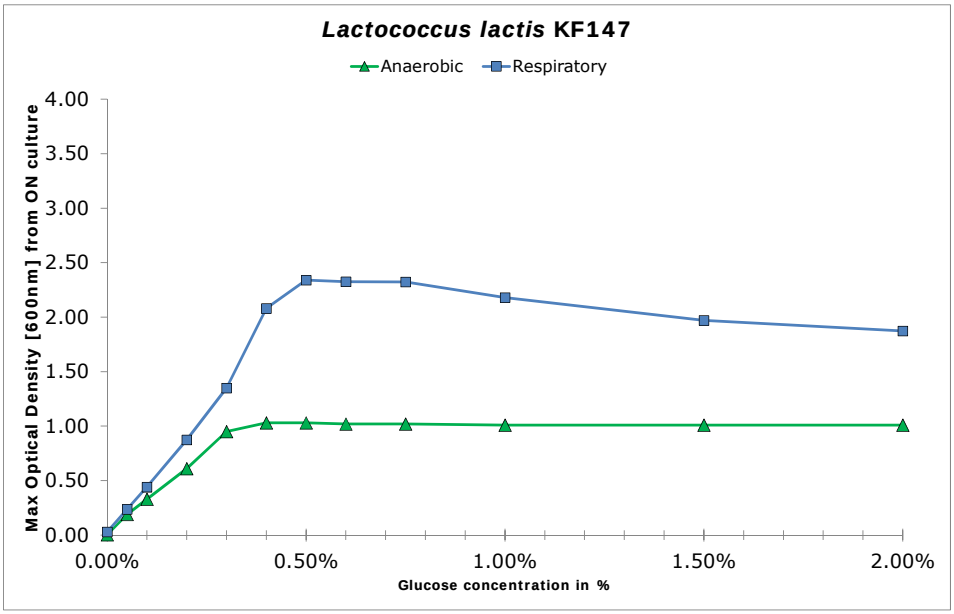

Figure 9.3: Overview of growth by *Lactococcus lactis* KF147 in SAL medium supplemented with increasing amounts of glucose. Blue (square) - respiratory growth, Green (triangle) - anaerobic growth.

linear range of growth in SAL medium, were selected as a basis for screening co-metabolism of glycerol utilization.

9.3 The selected *Lactococcus lactis* spp. were unable to utilize glycerol under all of the conditions tested

As detailed earlier to test for glycerol utilization, comparisons are made between cultures with and without glycerol addition for indications of an increase in OD_{600}. This has been done in medium containing only glycerol or in medium with a sugar substrate supplemented with glycerol. An overview of the core experimental conditions tested for the most common model organisms can be seen in table 9.1.

9.3.1 Variation of carbon source is not sufficient to stimulate glycerol utilization

One of the screening variables was to investigate the effect of cultivating *Lactococcus lactis* on different carbon sources. A change in carbon source might alleviate the effects of CCR induced by glucose and perhaps allow utilization or co-metabolization of glycerol.

The results from cultivation of *L. lactis* MG1363, IL1403, NCDO2118 and KF147 in defined medium with supplementation of different carbon sources can be seen in table 9.2. Further information can be found in appendix C. The different carbon sources were combined with either anaerobic, aerobic or respiratory conditions.

As can be seen in the table a change in carbon source to slower metabolizable substrate such as maltose did not induce glycerol utilization. Likewise neither a switch to a poorer growth medium (such as BLL) nor an increased concentration of glycerol (to 1%) appears to have any effect in stimulating the utilization of glycerol.

To investigate whether glycerol utilization actually took place but at such a slow rate that it might not immediately be evident from over night cultures a series of extended cultivations were undertaken.

9.3.2 Prolonged incubation in medium supplemented with glycerol does not stimulate utilization

Several different approaches were tested out to stimulate glycerol assimilation. These included various sugar and glycerol combinations. Since these did not indicate any glycerol utilization, experiments with prolonged incubation were performed to investigate if very slow utilization did occur.

Medium & conditions	28°C	30°C	PI	Anaerobic	Aerobic	Respiratory	MG1363	IL1403	NCDO2118	KF147
BLL + 0.2% fructose + 1.0% glycerol		•		•			•		•	•
BLL + 0.5% fructose + 1.0% glycerol		•				•	•		•	•
BLL + 0.1% xylose + 0.1% glycerol		•		•					•	
BLL + 0.2% xylose + 1.0% glycerol		•		•					•	•
BLL + 0.5% xylose + 0.5% glycerol		•		•					•	
BLL + 0.5% xylose + 0.5% glycerol		•		•					•	
BLL + 0.5% xylose + 1.0% glycerol		•				•			•	•
SAL + 0.0001% glycerol		•	•	•	•	•	•		•	•
SAL + 0.001% glycerol		•	•	•	•	•	•		•	•
SAL + 0.01% glycerol		•	•	•	•	•	•		•	•
SAL + 0.1% glycerol		•	•	•	•	•	•		•	•
SAL + 1.0% glycerol		•	•	•	•	•	•		•	•
SAL + 2.0% glycerol		•				•	•		•	•
SAL + 0.1% glucose + 0.2% glycerol		•		•	•			•		
SAL + 0.2% glucose + 0.2% glycerol	•	•	•		•	•	•		•	•
SAL + 0.2% glucose + 0.2% glycerol		•	•	•					•	•
SAL + 0.5% glucose + 1.0% glycerol		•		•		•				•
SAL + 0.1% fructose + 0.2% glycerol		•		•	•			•		
SAL + 0.1% fructose + 1.0% glycerol		•		•		•	•		•	•
SAL + 0.5% fructose + 0.5% glycerol		•		•		•			•	
SAL + 0.1% maltose + 0.2% glycerol		•		•	•			•		
SAL + 0.1% maltose + 2.0% glycerol		•				•	•		•	
SAL + 0.1% xylose + 0.1% glycerol		•		•					•	
SAL + 0.1% xylose + 1.0% glycerol		•		•		•			•	•
SAL + 0.5% xylose + 0.1% glycerol		•		•					•	
SAL + 0.5% xylose + 0.5% glycerol		•		•		•			•	

Table 9.1: Overview of the conditions tested during screening for glycerol utilization by selected *Lactococcus* spp. - *Respiratory* refers to aerobic growth with hemin added to growth medium. PI - Prolonged incubation at the given temperature, at least 4-5 days. All experiments performed in duplicates or triplicates. Dots (•) - Indication that the condition has been tested.

The results of these experiments can be found in table 9.3. Plots of additional experiments can be seen in figure 9.4 and figure 9.5. The individual strain data can be seen in appendix B.2.

Medium	Anaerobic	Aerobic	Respiratory
IL1403			
SAL + 0.1% glucose	0.25 ± 0.05	0.37 ± 0.006	0.44 ± 0.006
SAL + 0.1% glucose + 0.2% glycerol	0.27 ± 0.03	0.38 ± 0.003	0.44 ± 0.004
SAL + 0.1% maltose	0.15 ± 0.01	0.32 ± 0.03	0.36 ± 0.19
SAL + 0.1% maltose + 0.2% glycerol	0.19 ± 0.01	0.19 ± 0.01	0.16 ± 0.08
SAL + 0.1% galactose	0.18 ± 0.01	0.42 ± 0.05	
SAL + 0.1% galactose + 0.2% glycerol	0.21 ± 0.02	0.35 ± 0.03	
SAL + 0.1% cellobiose	0.25 ± 0.01	0.47 ± 0.01	0.75 ± 0.22
SAL + 0.1% cellobiose + 0.2% glycerol	0.33 ± 0.01	0.46 ± 0.01	0.82 ± 0.02
SAL + 0.1% sucrose	0.12 ± 0.01	0.14 ± 0.01	
SAL + 0.1% sucrose + 0.2% glycerol	0.12 ± 0.02	0.14 ± 0.01	
MG1363			
SAL + 0.1% fructose	0.38 ± 0.02		0.41 ± 0.08
SAL + 0.1% fructose + 1.0% glycerol	0.32 ± 0.03		0.32 ± 0.01
BLL + 0.2% fructose	0.30 ± 0.02		0.31 ± 0.06
BLL + 0.2% fructose + 1.0% glycerol	0.27 ± 0.03		0.18 ± 0.05
KF147			
SAL + 0.1% fructose	0.14 ± 0.02		0.34 ± 0.01
SAL + 0.1% fructose + 1.0% glycerol	0.16 ± 0.02		0.36 ± 0.01
BLL + 0.2% fructose	0.31 ± 0.03		0.28 ± 0.04
BLL + 0.2% fructose + 1.0% glycerol	0.30 ± 0.01		0.25 ± 0.14
NCDO2118			
BLL + 0.2% fructose	0.23 ± 0.05		0.37 ± 0.01
BLL + 0.2% fructose + 1.0% glycerol	0.24 ± 0.04		0.37 ± 0.01

Table 9.2: Results from cultivating various *Lactococcus lactis* strains in defined medium supplemented with different combinations of sugars with or without glycerol addition. OD_{600} measurements on over night cultures, averages of three biological replicates incubated at 30°C. Blank slots indicate that results from that particular set of conditions are not available. *Anaerobic* - static cultures, *Aerobic* - aerated cultures, *Repiratory* - aerated cultures with hemin supplementation.

The aim of the experiments was to investigate whether glycerol was at all metabolized, very slowly, by the strains under investigation. Neither figure 9.4 nor figure 9.5 show any signs of additional biomass formation after glucose has been consumed in the first over night culture (0.2% glucose in the medium would sustain an OD_{600} of approx. 0.8). Nor does there seem to be any effect of modulating glycerol concentration (figure 9.4).

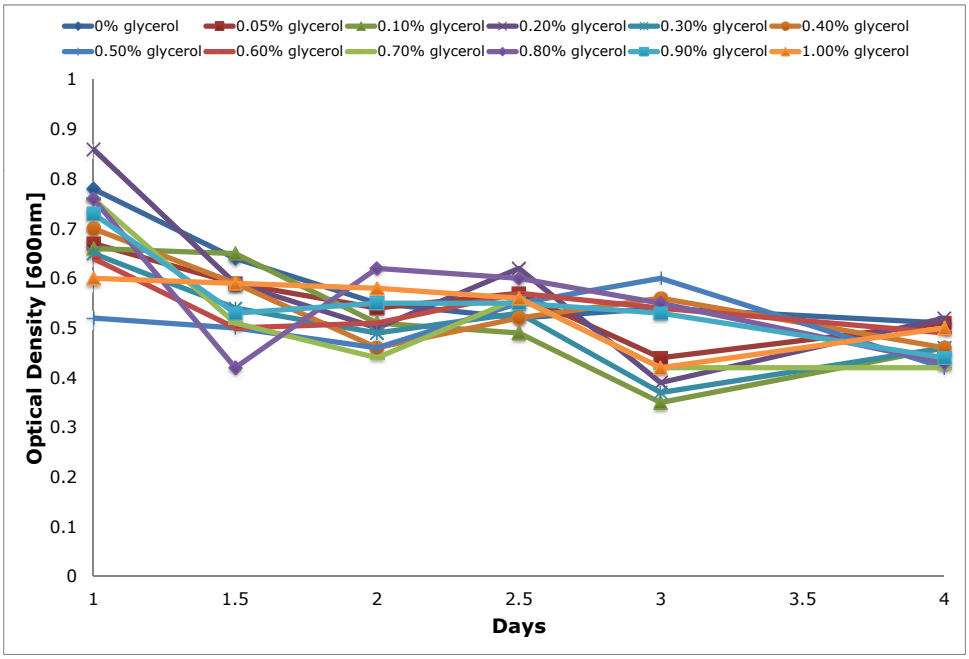

Figure 9.4: A plot of growth by *Lactococcus lactis* IL1403 in SAL medium supplemented with 0.2% glucose and increasing concentrations of glycerol. Cultivated at 30°C, under anaerobic conditions for a period of 4 days. Similar experiments were carried out for aerobic and respiratory conditions.

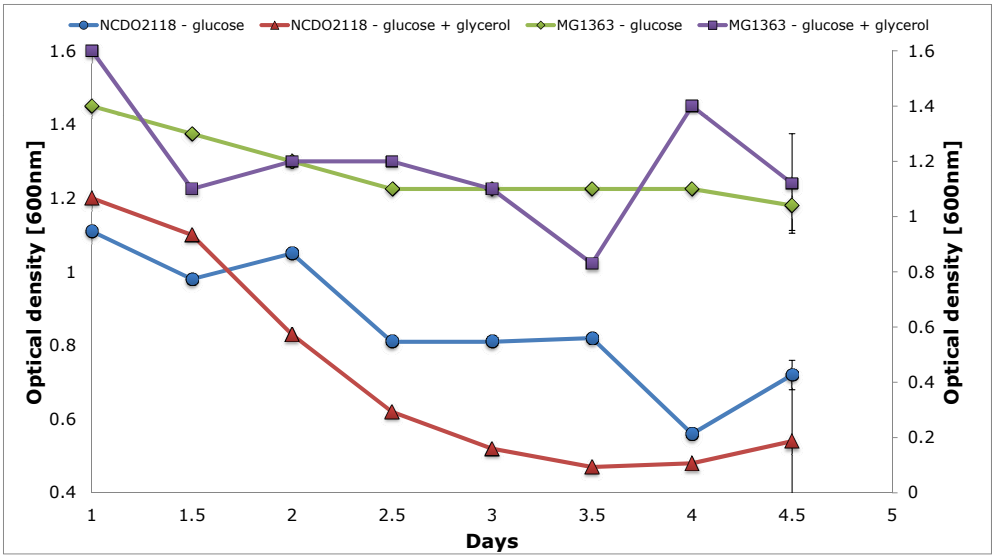

Figure 9.5: A plot of growth by *Lactococcus lactis* NCDO2118 and MG1363 in SAL medium supplemented with 0.2% glucose with or without and additional 0.2% of glycerol. Cultivated at 28°C, under respiratory conditions for a period of 4 days. Purple (square) - MG1363, glucose + glycerol, Green (diamond) - MG1363, glucose, Red (triangle) - NCDO2118, glucose + glycerol, Blue (circle), NCDO2118, glucose

Medium	Anaerobic	Aerobic	Respiratory
MG1363			
SAL	0.01 ± 0.004	0.03 ± 0.003	0.04 ± 0.02
SAL + 0.0001% glycerol	0.01 ± 0.01	0.08 ± 0.03	0.04 ± 0.05
SAL + 0.001% glycerol	0.02 ± 0.01	0.05 ± 0.01	0.04 ± 0.02
SAL + 0.01% glycerol	0.01 ± 0.01	0.06 ± 0.02	0.07 ± 0.02
SAL + 0.1% glycerol	0.01 ± 0.01	0.06 ± 0.03	0.05 ± 0.03
SAL + 1.0% glycerol	0.03 ± 0.01	0.06 ± 0.002	0.03 ± 0.01
KF147			
SAL	0.018 ± 0.01		
SAL + 0.0001% glycerol	0.02 ± 0.01		
SAL + 0.001% glycerol	0.04 ± 0.01		
SAL + 0.01% glycerol	0.03 ± 0.002		
SAL + 0.1% glycerol	0.11 ± 0.002		
SAL + 1.0% glycerol	0.08 ± 0.01		
NCDO2118			
SAL	0.01 ± 0.002		
SAL + 0.0001% glycerol	0.02 ± 0.002		
SAL + 0.001% glycerol	0.03 ± 0.001		
SAL + 0.01% glycerol	0.03 ± 0.003		
SAL + 0.1% glycerol	0.11 ± 0.01		
SAL + 1.0% glycerol	0.08 ± 0.02		

Table 9.3: Results from cultivating various *Lactococcus lactis* strains in defined medium supplemented with different concentrations of glycerol. OD_{600} measurements on cultures were monitored during the period and the values shown are the values reached from 16 days of incubation. Averages of three biological replicates incubated at 30°C.

Since glycerol is transported into the cell by facilitated diffusion, a concentration gradient screening (table 9.3) was performed to see if glycerol concentration affected uptake and metabolism. This was done in defined medium without any additional carbon and energy source. The experiment ran for 16 days during which optical density was regularly monitored. The two plant isolates NCDO2118 and KF147 showed signs of glycerol utilization, as they both reached an optical density of approximately 0.1, at a glycerol concentration of 0.1% (higher than optical density measured in pure SAL medium), after 16 days of incubation. The lack of a similar response from MG1363 (dairy strain) could indicate that the more prototrophic plant isolates might not necessarily be metabolizing glycerol, but could perhaps grow on the remaining components in the medium (19 amino acids are added to SAL, i.e. all but aspartate).

9.3.3 Overexpression of ATPase activity does not stimulate glycerol utilization

The effect of overexpressing ATPase activity was believed to simulate the growth on a poor substrate and might induce a suitable metabolic response allowing the utilization of glycerol. Indications that this was indeed the case can be seen from the transcriptomic analysis in part IV where a starvation like response is seen to expression of ATPase activity. The strains selected were BK1010 (reference), BK1506, BK1503 and BK1502 which all expressed ATPase activity (in increasing order, see table 16.1). The results of over night screening can be seen in table 9.4.

Strain	Medium	Anaerobic	Aerobic	Respiratory
BK1010	SAL + 0.1% glucose	0.36 ± 0.09	0.39 ± 0.03	0.61 ± 0.02
	SAL + 0.1% glucose + 0.1% glycerol	0.36 ± 0.02	0.39 ± 0.03	0.62 ± 0.04
BK1506	SAL + 0.1% glucose	0.32 ± 0.03	0.33 ± 0.02	0.46 ± 0.04
	SAL + 0.1% glucose + 0.1% glycerol	0.31 ± 0.02	0.32 ± 0.02	0.49 ± 0.03
BK1503	SAL + 0.1% glucose	0.32 ± 0.02	0.31 ± 0.02	0.46 ± 0.01
	SAL + 0.1% glucose + 0.1% glycerol	0.27 ± 0.03	0.30 ± 0.02	0.45 ± 0.02
BK1502	SAL + 0.1% glucose	0.31 ± 0.04	0.31 ± 0.01	0.44 ± 0.02
	SAL + 0.1% glucose + 0.1% glycerol	0.25 ± 0.02	0.31 ± 0.03	0.47 ± 0.05

Table 9.4: Results from cultivating *Lactococcus lactis* strains overexpressing ATPase activity in defined medium supplemented with 0.1% glucose and 0.1% glucose with an additional 0.1% glycerol. 5 μg/mL Erythromycin was added to over night cultures, to stabilize them. OD_{600} measurements are from over night cultures, and averages of four biological replicates incubated at 30°C are shown.

These results seem very similar to those previously shown. It is difficult to distinguish between the strains based on their growth characteristics on glycerol. When examining the values for BK1503, one might question if there is sufficient ATPase activity present in the cells. The BK1502 strain would be expected to have only half the biomass yield of the reference strains, but here it seems more in the area of 80–85% under anaerobic and aerobic conditions and around 70% under respiratory conditions. The strains with the highest expression of ATPase activity are known to be unstable and could have lost or mutated the plasmid. If one assumes the two other strains are ok, it would seem that ATPase activity in itself is not enough to stimulate glycerol assimilation.

9.3.4 Growth experiments do not reveal any glycerol utilization under respiration permissive conditions

The initial results from the screening of over night cultures, prompted a more detailed study via growth experiments. When performing growth ex-

periments further information can be obtained from the culture, namely growth rates and product formation. In this case HPLC analysis of samples taken during the experiment could clarify whether glycerol was being consumed or not. An example of the initial experiments are presented here.

Growth experiments with glucose and maltose supplemented with glycerol show no indications of glycerol utilization

Growth experiments were performed with the strain *Lactococcus lactis* MG1363 with glucose and maltose as substrates under respiratory conditions. This combination of environmental conditions and substrate was believed to be the best scenario for stimulating either growth on glycerol or co-metabolization. The choice of respiration permissive conditions meant that any excess redox produced from glycerol could be channeled to the respiratory system and thereby not cause problems. Maltose being a slower metabolizable substrate was hoped to allow induction of a starvation response which in turn could stimulate the use of the additional energy source in the medium. Growth rates, substrate flux and biomass yields can be seen in table 9.5. Product formation can be seen in figure 9.6 and figure 9.7. Further information can be viewed in appendix D.

Medium	Gen. time T_d [min.]	Growth rate μ [h^{-1}]	Average flux [mmol / g CDW · h]	Biomass yield [g CDW / mmol]
SAL + 0.1% glucose	53.53 ± 0.24	0.78 ± 0.01	18.45 ± 0.81	0.042 ± 0.0013
SAL + 0.1% glucose + 1% glycerol	54.16 ± 0.92	0.77 ± 0.01	18.35 ± 0.30	0.042 ± 0.0001
SAL + 0.1% maltose	84.80 ± 0.99	0.49 ± 0.01	20.80 ± 1.03	0.068 ± 0.004
SAL + 0.1% maltose + 1% glycerol	78.71 ± 4.05	0.53 ± 0.03	19.55 ± 0.82	0.067 ± 0.003

Table 9.5: Results from cultivating *Lactococcus lactis* MG1363 in defined medium supplemented with 0.1% glucose or maltose and 0.1% glucose or maltose with an additional 1% glycerol. Experiment performed in quadruplicates incubated at 30°C under respiratory conditions.

As with the previous investigations no significant difference, on either growth rate, biomass yield or substrate flux can be observed between cultures growing with or without glycerol. This is supported by the product formation plots in which the plot of glycerol concentration remains constant during the experiments. Thus it appears that glycerol is not consumed under these conditions.

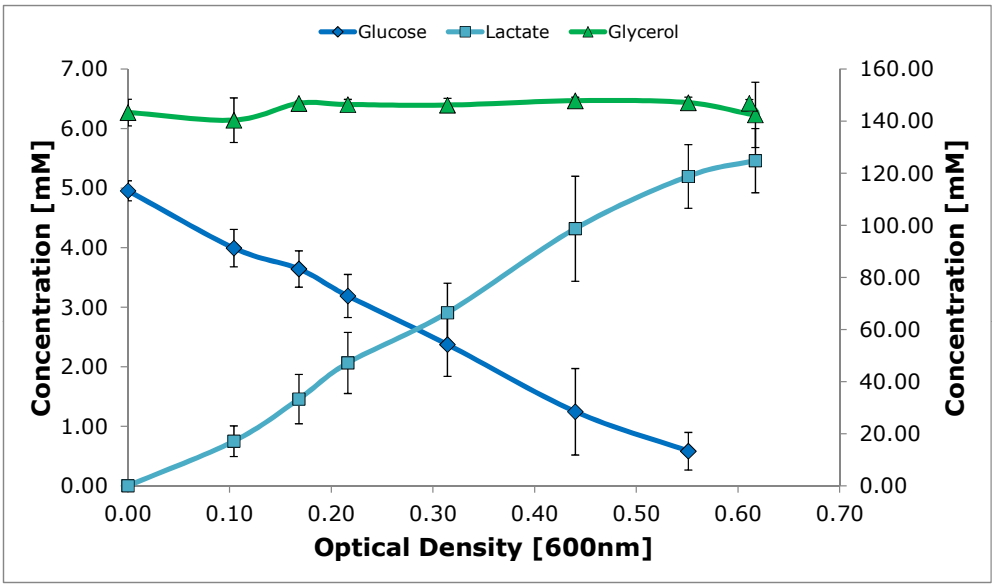

Figure 9.6: Plot of product formation and substrate consumption in a growth experiment with *Lactococcus lactis* MG1363 cultivated in SAL + 0.1% glucose + 1% glycerol at 30°C under respiratory conditions. Samples done in quadruplicates. Green (triangle) - glycerol concentration (secondary axis), Blue (diamond) - Glucose concentration, Turquoise (square) - Lactate concentration.

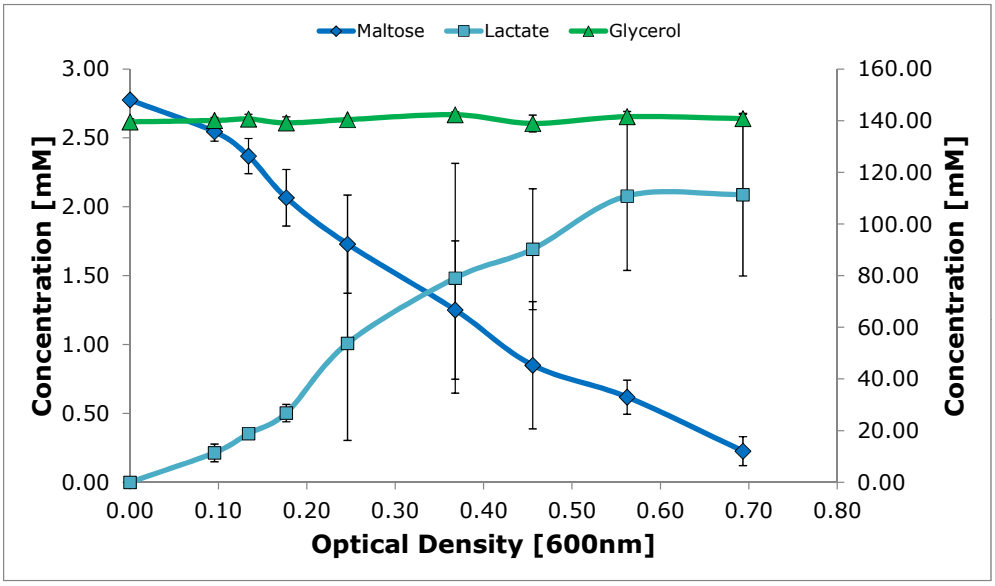

Figure 9.7: Plot of product formation and substrate consumption in a growth experiment with *Lactococcus lactis* MG1363 cultivated SAL + 0.1% maltose + 1% glycerol at 30°C under respiratory conditions. Samples done in quadruplicates. Green (triangle) - glycerol concentration (secondary axis), Blue (diamond) - Maltose concentration, Turquoise (square) - Lactate concentration.

9.4 Glycerol supplementation to the growth medium has a great effect on growth rate but only for certain substrates

Although none of the species exhibited convincing results for glycerol utilization there were a few interesting observations to come out of the screening. Firstly the results from cultivating *Lactococcus lactis* IL1403 with trehalose showed of a beneficial effect of glycerol addition to over night cultures. Secondly the addition of glycerol to cultures growing on xylose or ribose appears to affect growth rates detrimentally.

9.4.1 Supplementation of glycerol to the growth medium shows indications of increased biomass formation for strains grown under aerobic conditions with trehalose as a substrate

The only example of a beneficial effect of glycerol supplementation was observed when cultivating *L. lactis* on trehalose under aerobic conditions. The results can be seen in table 9.6.

Medium	Anaerobic	Aerobic[†]	Respiratory
SAL + 0.1% trehalose	0.26 ± 0.06	0.49 ± 0.01	0.61 ± 0.004
SAL + 0.1% trehalose + 0.2% glycerol	0.29 ± 0.01	0.61 ± 0.07	0.67 ± 0.01

Table 9.6: Results from cultivating *Lactococcus lactis* IL1403 in defined medium supplemented with 0.1% trehalose and 0.1% trehalose with an additional 0.2% glycerol. OD_{600} measurements on over night cultures, averages of three biological replicates incubated at 30°C are shown. [†]The aerobic experiments were done in six biological replicates.

It seems that under aerobic conditions, supplementation of glycerol to the growth medium leads to higher optical density. A follow up growth experiment was performed the results can be seen in table 9.7. From the growth rates it is clear that the culture with glycerol addition has a growth rate of 84% of the culture with only trehalose. Whether this is sufficient to generate the difference in optical density observed in over night cultures remains to be investigated further. The difference though obvious, is small, so it might be caused by the reduced growth rate or other effects.

Medium	Specific growth rate μ [h^{-1}]	Average generation time T_d [min.]
SAL + 0.1% trehalose	0.50 ± 0.01	83.77 ± 0.96
SAL + 0.1% trehalose + 0.2% glycerol	0.42 ± 0.01	99.53 ± 2.20

Table 9.7: Results from cultivating *Lactococcus lactis* IL1403 in defined medium supplemented with 0.1% trehalose and 0.1% trehalose with an additional 0.2% glycerol. Averages of four biological replicates incubated at 30°C with aeration.

9.4.2 Glycerol supplementation to growth medium has a detrimental effect on growth rate of cultivated strains

A second interesting observation was an apparent sign of glycerol toxicity or glycerol inhibition. This is somewhat opposite to what was hoped when adding glycerol to the medium and was initially only seen in media with xylose. In figure 9.8 and 9.9 are two illustrations of the effect. The initial observation (figure 9.8) pointed to an effect on biomass formation. The maximum optical density of cultures with glycerol added were significantly lower than those without. The second screening (figure 9.9) shows an apparent dose effect of glycerol addition in both SAL and BLL medium. The stronger effect in BLL medium points to an effect in energy metabolism as BLL compared to SAL has fever amino acids added which increases the energetic cost of growing.

9.4.3 Growth experiments show glycerol inhibition of growth rate in most types of growth media

The curious effect of apparent glycerol inhibition or toxicity, first encountered during screening of *Lactococcus lactis* NCDO2118 in medium with xylose, was investigated further by a series of well defined batch experiments with regular measurements of biomass formation (growth experiments). The aim was to characterize the effect further in an attempt to elucidate the mechanism behind the effect.

Glycerol inhibition effect in BLL medium is evident when supplemented with xylose but not when supplemented with galactose

The results from the growth experiment with xylose in BLL medium can be seen in table 9.8 and an analysis of product formation can be found in table 9.9. This initial growth experiment focused on BLL medium because the effect was higher when first encountered, compared to SAL medium (figure 9.9). Two different concentrations of xylose (high, 5% and low, 0.5%) were included in the experiment. It had previously been established that NCDO2118 grows faster at higher xylose concentrations and it would therefore be interesting to see how this affected the inhibitory effect.

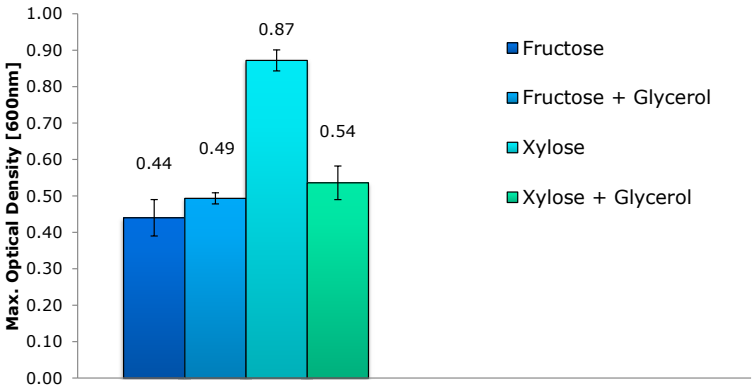

Figure 9.8: Plot of optical density measurements showing an apparent inhibitory effect of glycerol addition to defined growth medium supplemented with either fructose or xylose. The strain tested is *Lactococcus lactis* NCDO2118 cultivated in SAL + 0.5% sugar ± 0.5% glycerol at 30°C under anaerobic conditions. All samples done in triplicates.

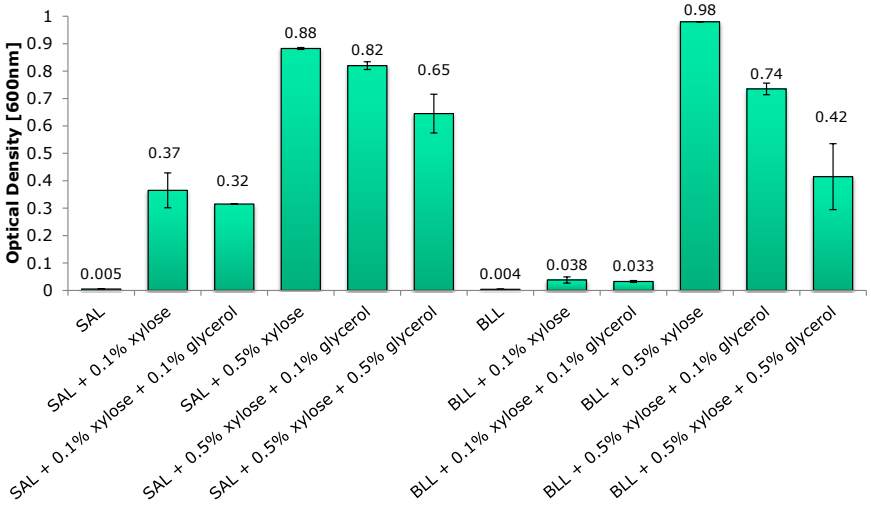

Figure 9.9: Plot of optical density measurements showing an apparent inhibitory effect of glycerol addition to two different defined growth media. BLL is essentially SAL medium with fewer amino acid supplements and greater buffering capacity but otherwise identical to SAL. The strain tested is *Lactococcus lactis* NCDO2118 cultivated in different combinations of sugar and glycerol at 30°C under anaerobic conditions. Samples done in duplicates or triplicates.

Medium	Cond.	Growth rate μ [h^{-1}]	Biomass form. [Max. OD_{600}]
BLL + 0.5% xylose	resp.	0.18	1.01
BLL + 0.5% xylose + 0.5% glycerol	resp.	0.03	0.20
BLL + 5.0% xylose	resp.	0.16	1.01
BLL + 5.0% xylose + 0.5% glycerol	resp.	0.09	0.51
BLL + 0.5% xylose	anaer.	0.096	0.75
BLL + 0.5% xylose + 0.5% glycerol	anaer.	0.018	0.069
BLL + 5.0% xylose	anaer.	0.17	0.98
BLL + 5.0% xylose + 0.5% glycerol	anaer.	0.14	0.87

Table 9.8: Results from cultivating *Lactococcus lactis* NCDO2118 in defined medium supplemented with 0.5% xylose and 0.5% xylose with an additional 0.5% glycerol. Incubated at 30°C under respiratory (resp.) and anaerobic (anaer.) conditions.

The results indicated a clear effect on both growth rate and biomass formation, for both high and low concentrations of xylose and under both conditions. The effect seems to be worse under anaerobic conditions, with low substrate concentration. The cultures with high substrate concentration seem to be less affected by the toxic effects of glycerol under anaerobic conditions. This could possibly be explained with the higher flux present in cultures with a high level of substrate generating more energy. No difference can be seen from substrate concentration on growth rate at respiratory conditions. These findings indicated the problem was associated with energy metabolism and since the substrate was xylose, it naturally also pointed to the pentose phosphate pathway.

Analysis of product formation shows formate is missing in cultures supplemented with glycerol Samples for analysis of product formation were taken during the growth experiment. The results can be seen in table 9.9. The intriguing observation from these samples is the lack of formate formation in the anaerobic cultures with glycerol addition. If glycerol inhibited pyruvate formate-lyase (PFL) via elevated levels of triosephosphates (the endpoint of glycerols assimilation into glycolysis), it could explain the growth deficiencies under anaerobic conditions, as this severely limits the capacity for generating acetyl-Coa. Ordinarily, this would not affect the cultures growing under respiratory conditions, here PDH should supply an alternative route to acetyl-CoA formation.

	Sample	Xylose mM	Glycerol mM	Lactate mM	Formate mM	Acetate mM	Ethanol mM
Anaerobic							
BLL + 0.5% xylose	1	23.48	n.a.	n.a.	n.a.	n.a.	n.a.
	2	14.22	n.a.	4.45	6.71	10.99	n.a.
	3	0.84	n.a.	10.95	14.00	22.63	n.a.
BLL + 0.5% xylose + 0.5% glycerol	1	39.77	72.12	n.a.	n.a.	n.a.	n.a.
	2	37.76	66.25	0.62	n.a.	2.89	n.a.
	3	37.26	66.41	1.19	n.a.	3.30	n.a.
	4	35.68	67.23	3.36	n.a.	3.64	7.20
BLL + 5% xylose	1	255.87	n.a.	n.a.	n.a.	n.a.	n.a.
	2	247.00	n.a.	10.03	6.18	8.77	n.a.
	3	227.28	n.a.	23.95	9.08	15.19	4.83
BLL + 5% xylose + 0.5% glycerol	1	254.09	74.67	n.a.	n.a.	n.a.	3.93
	2	245.64	76.71	10.37	n.a.	10.96	n.a.
	3	240.90	76.28	14.61	n.a.	15.11	8.40

Table 9.9: Product formation in the cultures from cultivating *Lactococcus lactis* NCDO2118 in defined medium supplemented with 0.5% xylose and 0.5% xylose with an additional 0.5% glycerol. Cultures were incubated at 30°C under anaerobic (static) conditions. Note the absence of formate in the cultures supplemented with glycerol.

Growth experiments with galactose as a substrate do not show signs of the glycerol inhibition effect To investigate whether the inhibitory effect of glycerol was specific to growth on xylose, growth experiments were carried out with galactose as main substrate. Galactose was chosen because it has a very similar profile to xylose with respect to product formation and growth rate [14]. The results from the growth experiment with galactose in BLL medium can be seen in table 9.10 and an analysis of product formation can be found in table 9.11.

The results from the growth experiment with galactose showed that the effect of glycerol on the growth rate is less pronounced, than when the cells are growing on xylose. There is perhaps evidence of a slight effect for the cultures grown anaerobically. The addition of glycerol at the mid. exponential phase did not appear to have much effect. This was done primarily to show that if an effect was present it would appear from glycerol addition.

The product formation for the anaerobic cultures still show a lack of formate indicating a possible inactivation of PFL. This observation would then seem to indicate that the disappearance of formate is not the cause of the slow down, since cultures with added glycerol grew almost as fast as those without. Then glycerol inhibition is mainly associated with xylose metabolism, which again leads to an effect in xylose utilization and the pentose phosphate pathway.

Medium	Cond.	Growth rate μ [h^{-1}]	Biomass form. [Max. OD_{600}]
BLL + 0.5% galactose	resp.	0.24	0.74
BLL + 0.5% galactose + 0.5% glycerol	resp.	0.23	0.75
BLL + 0.5% galactose + 0.5% glycerol (glycerol added mid. exp.)	resp.	0.23	0.98
BLL + 0.5% galactose	anaer.	0.19	1.02
BLL + 0.5% galactose + 0.5% glycerol	anaer.	0.16	0.88
BLL + 0.5% galactose + 0.5% glycerol (glycerol added mid. exp.)	anaer.	0.19	1.01

Table 9.10: Results from cultivating *Lactococcus lactis* NCDO2118 in defined medium supplemented with 0.5% galactose and 0.5% galactose with an additional 0.5% glycerol. One culture had glycerol added at approx. mid expontial growth to a final concentration of approx. 0.5% (mid. exp.). Incubated at 30°C under respiratory (resp.) and anaerobic (anaer.) conditions.

Medium	Glycerol mM	Lactate mM	Formate mM	Acetate mM	Ethanol mM
Respiratory					
BLL + 0.5% galactose	n.a.	7.48	n.a.	12.95	n.a.
BLL + 0.5% galactose + 0.5% glycerol	69.57	10.93	n.a.	13.14	n.a.
BLL + 0.5% galactose + 0.5% glycerol (mid exp.)	69.72	11.16	n.a.	15.77	n.a.
Anaerobic					
BLL + 0.5% galactose	n.a.	39.43	7.81	6.67	n.a.
BLL + 0.5% galactose + 0.5% glycerol	70.43	40.12	n.a.	4.92	n.a.
BLL + 0.5% galactose + 0.5% glycerol (mid exp.)	69.56	42.87	n.a.	4.49	n.a.

Table 9.11: Product formation in the cultures from cultivating *Lactococcus lactis* NCDO2118 in defined medium supplemented with 0.5% galactose and 0.5% galactose with an additional 0.5% glycerol. Incubated at 30°C under respiratory and anaerobic (static) conditions. A single sample was taken towards the end of the experiment.

The inhibitory effects of glycerol supplementation on growth rate can still be observed in richer defined media and in complex medium

The effect of glycerol supplementation was originally observed in BLL medium. This medium is a reduced version of the more commonly used SAL medium in which only 8 amino acids are added instead of the 19 added to SAL. Without these supplements growth in BLL medium is slower than in SAL, hence if the effect of glycerol was still visible in SAL medium, the growth experiments duration could be shortened significantly (compared to using BLL medium).

The inhibitory effect of glycerol is still present in cultures grown on SAL medium supplemented with xylose Growth experiments were performed in SAL medium supplemented with xylose (with or without glycerol supplementation) under anaerobic and respiratory conditions. The results can be seen in table 9.12.

Medium	Spec. growth rate μ [h^{-1}]	Biomass form. [Max. OD_{600}]
Anaerobic		
SAL + 0.5% xylose	0.37	0.892
SAL + 0.5% xylose + 0.5% glycerol	0.16	0.564
SAL + 0.5% xylose (addition of 0.5% glycerol mid. exp.)	0.35	0.676
SAL + 5% xylose	0.40	0.896
SAL + 5% xylose + 0.5% glycerol	0.34	0.796
SAL + 5% xylose (addition of 0.5% glycerol mid. exp.)	0.38	0.840
Respiratory		
SAL + 0.5% xylose	0.43	0.924
SAL + 0.5% xylose + 0.5% glycerol	0.22	0.401
SAL + 0.5% xylose (addition of 0.5% glycerol mid. exp.)	0.43	0.660
SAL + 5% xylose	0.44	1.29
SAL + 5% xylose + 0.5% glycerol	0.23	0.532
SAL + 5% xylose + (addition of 0.5% glycerol mid. exp.)	0.46	0.904

Table 9.12: Results from cultivating *Lactococcus lactis* NCDO2118 in SAL medium supplemented with xylose and glycerol. Incubation was at 30°C under respiratory or anaerobic (static) conditions.

From these results, it is clear that the same inhibitory effect initially seen in BLL medium, could also be seen for cultivations in SAL medium. In SAL medium, the growth rate difference between high and low xylose concentration is no longer present, for both conditions. The beneficial effect of increased substrate concentration is still evident but mostly for the anaerobic cultures, where the effect still seems more severe. Future investigations then focused on utilizing SAL medium instead of BLL medium.

The inhibitory effect of glycerol is still present in cultures grown on SA, SALN and M17 medium supplemented with xylose but fades when supplemented with ribose Growth experiments in a series of other media were performed to narrow down the cause of glycerol inhibition. The results can be seen in table 9.13. A summary of the inhibitory effect between cultures with and without glycerol addition can be seen in table 9.14.

From the results in table 9.13 cultivation in SALN medium appears to reduce the inhibitory effect of glycerol addition on growth rate. The same applies to cultivation in SA medium compared to SAL medium. The main difference between SA and SAL medium is the addition (to SAL) of

lipoic acid required for pyruvate dehydrogenase activity under respiratory conditions and the removal of sodium acetate.

Medium	Spec. growth rate μ [h^{-1}]	Biomass form. [Max. OD_{600}]
Lactococcus lactis NCDO2118		
Anaerobic		
†SALN + 0.5% xylose	0.35 ± 0.01	1.06 ± 0.01
†SALN + 0.5% xylose + 0.5% glycerol	0.25 ± 0.01	0.76 ± 0.01
†M17 + 0.5% xylose	0.32 ± 0.01	2.34 ± 0.03
†M17 + 0.5% xylose + 0.5% glycerol	0.18 ± 0.01	2.22 ± 0.04
‡SAL + 0.5% xylose	0.29 ± 0.01	0.95 ± 0.02
‡SAL + 0.5% xylose + 0.5% glycerol	0.14 ± 0.01	0.59 ± 0.01
‡SAL + 0.5% xylose + 0.5% glycerol + 10mM formate	0.14 ± 0.01	0.58 ± 0.02
‡SAL + 0.5% ribose	0.24 ± 0.01	0.61 ± 0.01
‡SAL + 0.5% ribose + 0.5% glycerol	0.22 ± 0.01	0.55 ± 0.01
†SA + 0.5% xylose	0.34 ± 0.01	1.02 ± 0.01
†SA + 0.5% xylose + 0.5% glycerol	0.22 ± 0.01	0.64 ± 0.01
†SA + 0.5% ribose	0.27 ± 0.01	0.58 ± 0.01
†SA + 0.5% ribose + 0.5% glycerol	0.26 ± 0.01	0.52 ± 0.01
Lactococcus lactis IL1403		
Anaerobic		
‡SAL + 0.5% ribose	0.21 ± 0.01	0.59 ± 0.01
‡SAL + 0.5% ribose + 0.5% glycerol	0.18 ± 0.01	0.51 ± 0.01

Table 9.13: Results from cultivating *Lactococcus lactis* NCDO2118 and IL1403 in defined and complex medium with various sugar substrates supplemented with or without glycerol. Incubation took place at 30°C under respiratory (heme supplementation) or anaerobic (static) conditions. †Experiments in quadruplicates. ‡Experiments in duplicates.

An experiment was conducted to which 10mM formate was added to the medium. This was to investigate the absence of formate production, seen previously (table 9.9) in anaerobic cultures with glycerol supplementation. If the inclusion of formate could counteract the effects of glycerol supplementation it would point towards C1 metabolism. Since growth in medium with both glycerol and formate is identical to growth in medium with only glycerol, it would seem the missing formate is not directly involved in the inhibitory mechanism.

Interestingly, growth in the rich medium M17 still shows signs of inhibition by glycerol. The effect is worse growth in SALN and SA medium and

only slightly better than growth in SAL.

Medium	μ [% of ref.]
Lactococcus lactis NCDO2118	
Anaerobic	
BLL + 0.5% xylose + 0.5% glycerol	18.8
SAL + 0.5% xylose + 0.5% glycerol	48.3
SA + 0.5% xylose + 0.5% glycerol	64.7
SALN + 0.5% xylose + 0.5% glycerol	71.4
M17 + 0.5% xylose + 0.5% glycerol	56.3
BLL + 0.5% galactose + 0.5% glycerol	84.2
SAL + 0.5% ribose + 0.5% glycerol	91.7
SA + 0.5% ribose + 0.5% glycerol	96.3
SAL + 5.0% xylose + 0.5% glycerol	85.0
BLL + 5.0% xylose + 0.5% glycerol	82.4
Lactococcus lactis NCDO2118	
Respiratory	
BLL + 0.5% xylose + 0.5% glycerol	16.7
SAL + 0.5% xylose + 0.5% glycerol	51.2
BLL + 0.5% galactose + 0.5% glycerol	95.8
BLL + 5.0% xylose + 0.5% glycerol	56.3
SAL + 5.0% xylose + 0.5% glycerol	52.3
Lactococcus lactis IL1403	
SAL + 0.5% ribose + 0.5% glycerol	85.7

Table 9.14: Summary of results obtained from cultivating *Lactococcus lactis* NCDO2118 and IL1403 in defined and complex medium with various sugar substrates supplemented with or without glycerol. The values are percentages of the respective glycerol cultures compared with a non glycerol culture. Incubation took place at 30°C under respiratory or anaerobic (static) conditions.

Ribose is also metabolized via xylulose-5-phosphate and PPP like xylose. Furthermore it would have been very beneficial if ribose could be used as a substrate since dairy isolates do not grow on xylose. Using ribose would open up new possibilities to access the already existing mutant collections. Unfortunately the addition of glycerol to cultivations with ribose as a substrate does not seem to have the same effect as with xylose.

To summarize the effects of glycerol addition an overview of the growth rate inhibition in the various media was compiled in table 9.14. This confirms the interesting observation about SA, SAL and SALN medium earlier stated. Interestingly cultivation in both SA medium and SALN medium improves the growth rate of strains cultivated on xylose and glycerol. It

would seem that the inhibition effect of glycerol is substrate specific and is counteracted by either supplementation of nucleosides or sodium acetate.

9.4.4 Internal metabolite measurements do not reveal significant differences between cultures grown with or without glycerol supplementation

In order to gain some insight into the internal effects of glycerol inhibition, samples were prepared from cultures grown in medium with xylose with or without glycerol supplementation. The results can be seen in table 9.15 and an illutration of the data can be found in figure 9.10.

Strain	**G6P**	**F6P**	**F16BP**	**DHAP**	**GAP**
NCDO2118 - XYL	0.79 ± 0.30	0.20 ± 0.08	14.19 ± 2.85	3.35 ± 1.40	3.90 ± 2.81
NCDO2118 - XYL/GLY	-0.21 ± 0.77	-0.05 ± 0.10	9.37 ± 2.14	2.23 ± 1.45	1.97 ± 1.34
Strain	**PEP**	**PYR**	**RIB5P**	**RI/XY5P**	**SEDO7**
NCDO2118 - XYL	2.48 ± 1.30	18.30 ± 16.34	10.41 ± 16.23	14.16 ± 9.58	5.96 ± 12.34
NCDO2118 - XYL/GLY	1.60 ± 1.25	11.32 ± 5.75	-5.62 ± 13.66	4.66 ± 2.74	-4.23 ± 6.57
Strain	**AMP**	**ADP**	**ATP**	**NAD**	**NADP**
NCDO2118 - XYL	-0.08 ± 0.57	1.18 ± 0.85	0.02 ± 0.09	1.10 ± 0.60	0.35 ± 0.36
NCDO2118 - XYL/GLY	0.47 ± 0.58	1.90 ± 0.73	0.08 ± 0.15	0.74 ± 0.54	0.41 ± 0.07

Metabolite concentrations in μM. Samples in triplicate/quadruplicate.

Table 9.15: Overview of the internal metabolite concentrations measured in the samples taken from mid. exponential growth of *Lactococcus lactis* NCDO2118. G6P - Glucose-6-phosphate, F6P - Fructose-6-phosphate, F16BP - Fructose-1,6-biphosphate, DHAP - Dihydroxyacetonephosphate, GAP - Glyceraldehyde-3-phosphate, PEP - Phosphoenolpyruvate, PYR - Pyruvate, RIB5P - Ribose-5-phosphate, RI/XY5P - Ribulose-5-phosphate and xylulose-5-phosphate, SEDO7 - Sedoheptulose-7-phosphate, AMP - Adenosine monophosphate, ADP - Adenosine diphosphate, ATP - Adenosine triphosphate, NAD - Nicotineamide adenine dinucleotide, NADP - Nicotineamide adenine dinucleotide phosphate.

From the metabolite data it seems that the only significant difference is the concentration of F16BP, which is lowered in the culture with glycerol supplementation. Whether this is an effect of glycerol or the slower growth rate remains to be elucidated. It is difficult to draw any meaningful conclusions from the internal metabolite data at this stage with regard to the levels of internal metabolites.

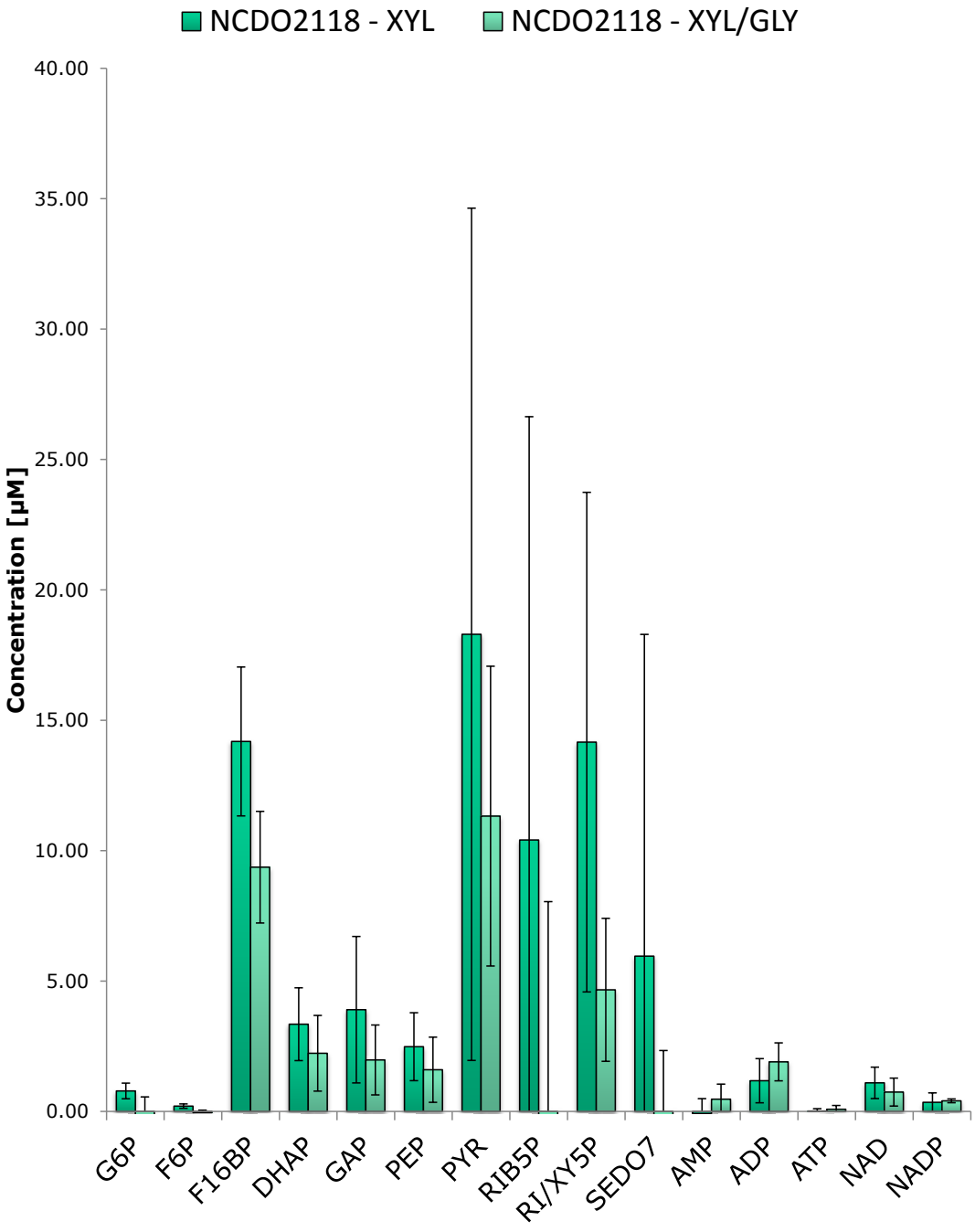

Figure 9.10: Plot of internal metabolite concentrations in samples from mid. exponential growth phase of *Lactococcus lactis* NCDO2118. GREEN - NCDO2118 cultivated on xylose, LIGHT GREEN - NCDO2118 cultivated on xylose supplemented with glycerol. G6P - Glucose-6-phosphate, F6P - Fructose-6-phosphate, F16BP - Fructose-1,6-biphosphate, DHAP - Dihydroxyacetonephosphate, GAP - Glyceraldehyde-3-phosphate, PEP - Phosphoenolpyruvate, PYR - Pyruvate, RIB5P - Ribose-5-phosphate, RI/XY5P - Ribulose-5-phosphate and xylulose-5-phosphate, SEDO7 - Sedoheptulose-7-phosphate, AMP - Adenosine monophosphate, ADP - Adenosine diphosphate, ATP - Adenosine triphosphate, NAD - Nicotineamide adenine dinucleotide, NADP - Nicotineamide adenine dinucleotide phosphate.

Chapter 10

Discussion

10.1 A serial transfer regime was not sufficient to stimulate glycerol assimilation in *Lactococcus lactis* NCDO2118

The initial attempt at stimulating glycerol usage by *Lactococcus lactis* NCDO2118 was not immediately successful. After a period of around two weeks and 15 transfers, the experiment was halted. Using SALN medium (SAL supplemented with nucleosides) allows a certain amount of growth for the slightly more prototrophic plant isolates such as NCDO2118. It was anticipated that this slight growth in a well buffered medium combined with a prolonged incubation period in the presence of glycerol, might trigger a response from latent genes or regulatory systems.

10.1.1 How can you enrich for something that does not grow?

There is of course an inherent problem with using adaptive evolution on a substrate that does not support growth. The whole foundation of the concept is to utilize naturally occurring mutations in the population as it grows and divides, to select for interesting mutants. So the main concern with the use of directed evolution is the time frame and of course growth. This approach is founded on the assumption that all genetic machinery is available, and a fairly simple set of mutational events is enough to reactivate glycerol utilization i.e. activation or inactivation of regulatory genes, sensitizing-desensitizing of regulatory effects etc. Furthermore if the mutations required are deleterious in any way the probability of success decreases further.

The dilution is approximately 10x per transfer which would allow around three generations of growth per transfer (growing from 0.010 to 0.100). If the mutational rate can be compared to that of *Escherichia coli* (even though genome size for coli is around 1.5 times that of lactis) which has been estim-

ated to $1.7 \cdot 10^{-4}$ mutations per genome per generation [91], and the density of lactococcal culture in defined SALN medium is (very) roughly $1 \cdot 10^6$ cells for an $OD_{600} \approx 0.100$, then the culture should have picked up ≈ 275 mutations per transfer. With no change after 15 transfers, it was speculated that the process of activating glycerol usage requires more drastic changes to succeed. The main difficulty of the approach is that, the lack of actual growth on glycerol as an energy and carbon source, made the utilization of glycerol by any mutant an added bonus, rather than a strict requirement. This makes enrichment of relevant mutants more difficult since the selection is based on co-metabolism, and hence exerts less selective pressure.

10.2 None of the tested conditions could stimulate the selected *Lactococcus lactis* strains to utilize glycerol

None of the strains showed signs of metabolizing glycerol from the screening done so far. A wide range of substrates and conditions have been tested, and especially respiration permissive conditions were anticipated to have allowed the use of glycerol as sole carbon and energy source.

The parameters tested were anaerobic, aerobic and respiration permissive growth, combined with several substrates such as glucose, fructose, galactose and xylose with or without glycerol supplementation. Gradients of glycerol concentration were also tested to find out if this affected usage. There were slight indications from the prolonged gradient experiment that glycerol might have been metabolized by the two plant isolates NCDO2118 and KF147. These were for the most part difficult to replicate. Both respiration permissive and prolonged incubation was tested with other substrates and conditions but did not stimulate glycerol utilization.

Only an initial screening was done on the various plant isolates from NCIMB, none of which showed immediate signs of glycerol usage. There are still many other isolates, and a repeated screening program could be interesting for further investigations, perhaps combined with isolation of strains from environmental samples. One might also consider utilizing a more sensitive analysis of product formation and substrate utilization to show whether glycerol is indeed being metabolized or not. A final approach might be to put environmental samples in a glycerol rich environment and isolate potential glycerol fermenting candidates for further study.

The results from the screening indicate that the issue of glycerol utilization may not only be a question of conditions but perhaps also of machinery as dicussed below.

10.2.1 The role of glycerol transport

A pending issue which was never fully addressed in this work is the matter of glycerol transport into the cell. Since there were no conditions which seemed to stimulate glycerol utilization, one immediate concern could be the transport of glycerol into the cell.

The initial screening was performed with an underlying assumption that glycerol can indeed enter the cell. This was based on the fact that glycerol can permeate the cell membrane simply by passive diffusion [97]. Most uptake system concerning glycerol are driven by facilitated diffusion such at the *glpF* system in *E. coli*. These simple uptake systems were annotated in several *Lactococcus lactis* species but questions remain as to whether they are functional or not. The specific model strain used in this work *L. lactis* MG1363, has a frameshift in the *glpF1* gene which might have rendered it non-functional [105]. There are two other glycerol transporting genes annotated but their capacity is yet to be verified.

The initial observations of both beneficial and detrimental effects of glycerol supplementation on growth in defined medium supplemented with xylose would seem to lend support to the fact that glycerol does indeed enter the cell. If it was only an osmotic effect by the high concentration of glycerol in the medium a similar effect should have been observed under any condition with glycerol supplementation. This was not the case as only growth in defined medium supplemented with xylose was affected by glycerol addition.

10.2.2 Measurements of internal metabolites did not provide new insights

Samples for internal metabolite analysis were prepared, as the levels of the various metabolites could have shed some light on the area of effect. The data obtained in the internal metabolite analysis was not significant enough to support any conclusions in this regard, although the level of fructose-1,6-bisphosphate (F16BP) does appear to be lower in the glycerol perturbed strain. Whether this can be attributed to glycerol or simply the lowered growth rate remains to be investigated. In general, a trend that appears (with the exception of AMP and ADP) is a lower level of most metabolites in the samples from the glycerol perturbed strain. This points in the direction of a lowered energy state in the cells exposed to glycerol but again this could also be attributed to the much lower growth rate.

Improvements in the sampling for internal metabolite measurements could increase the reliability of the data

There are several areas where improvements could be made to the current protocol, such as better extraction or more biomass present in the samples for analysis. This was a first adaptation of a protocol used successfully on *E.*

coli at the relatively same optical density of sampling. Yet the more robust *Lactococcus lactis* cells may require extra treatment, such as the use of glass beads to ensure effective extraction of internal metabolites as used successfully by Solem et *al.* [176] who sampled at slightly higher optical densities (0.5–0.8 vs. 0.3–0.6). The cells being perturbed by glycerol (and in energy metabolism in part V) generate less biomass. The level of biomass is most likely the most critical parameter to the success of generating interesting metabolic data. The turn around time of the analysis was long, and another data set could not be completed within the project time frame. It may be worthwhile repeating the experiment again in the future but with sampling at a substantially higher optical density, e.g. $OD_{600} = 1$, to improve the signal. This would ensure that enough biomass is present, and that a proper baseline from the reference samples cannot interfere with signal from the main samples.

10.2.3 Glycerol supplementation affects growth rate in medium with xylose and is counteracted by addition of nucleosides, sodium acetate and trehalose

The only indication of a beneficial effect with glycerol supplementation was with cultivation in defined medium supplemented with trehalose. When cultivated under aerobic conditions, over night cultures of *Lactococcus lactis* IL1403 supplemented with glycerol reached a higher optical density than cultures without glycerol. A single growth experiment was done under these conditions which showed that strains supplemented with glycerol grew with approximately 84% of the growth rate, of cultures without glycerol supplementation.

The beneficial effect of trehalose may be connected to the properties of the molecule itself

The positive effect in combination with glycerol has so far only been observed with trehalose. Trehalose in itself is an interesting molecule which is widespread in biology and has been associated with many roles such as energy and carbon reserve or protection from dehydration, protection from damage by oxygen radicals, protection from cold and as a sensing compound and cell wall component [82].

Since it is only during aerobic conditions the effect has materialized, it could simply be that it is not glycerol consumption which forms the basis of the difference in optical density, but rather a protective attribute of trehalose causing increased survival of the cultures with glycerol addition. It would be very interesting to study this effect further by determining if this is a strain dependant phenomenon or not. More detailed studies utilizing

transcriptome analysis or internal metabolite measurements could be used to investigate the phenomenon further.

Glycerol supplementation has a detrimental effect on growth rate in cultures grown on xylose

The inhibitory effect of glycerol was initially observed in over night cultures grown in defined medium supplemented with xylose and glycerol. To investigate this effect, further growth experiments were performed in BLL medium, initially with xylose and subsequently with galactose. The reason for choosing galactose as a substrate was that growth rate and product formation profile was very similar to that of xylose. The effect of glycerol inhibition was less pronounced on galactose.

This would indicate that the area of effect was involved with xylose metabolism, leading to the pentose phophate pathway. One other curious effect of glycerol supplementation was the apparent loss of formate production. A potential mechanism might be a high level of triosephophates regulating PFL activity (glycerol enters glycolysis as DHAP). When comparing the effect of glycerol inhibition, the effect is stronger in anaerobic cultures than in respiring ones. If the problem was shutdown of PFL, cultures respiring would have an alternative route via PDH to form acetyl-CoA. Furthermore, cultures under respiration permissive conditions also suffer from decreased growth rates indicating that there is at least one other cause to the inhibition. In this case an extraordinarily high redox level caused by the influx of glycerol to glycolysis, could be the cause. The question still remains as to why this is not simply re-oxidized through respiration.

Growth experiments with glycerol supplementation reveal its inhibitory effect in BLL, SA, SAL and M17 media Several different growth experiments have been performed with a range of different substrates and conditions. Even growth in rich medium such as M17 is affected by glycerol supplementation. When cultivated in SA or SALN medium growth rate is improved when compared to SAL. This would indicate the effect is localized to xylose metabolism and the PPP. This is further substantiated by an absence of glycerol inhibition for cultures grown on galactose and on ribose which is metabolized via the same intermediary (xylulose-5-phosphate) as xylose. Furthermore improvements in the growth rate is observed when cultivating strains in medium with a high concentration of xylose (5%).

The difference in growth rates for SA, SALN and SAL medium in connection to anaerobic cultivation on xylose is interesting, and suggests that pyruvate dehydrogenase complex (PDH) is involved (the main difference between SA and SAL is addition of lipoic acid, necessary for PDH activity and removal of sodium acetate). The absence of formate in anaerobic cul-

tivation points to inactivation of pyruvate formate-lyase (PFL) which would have a great effect under anaerobic conditions, but less so under respiratory conditions. Both PFL and PDH share a metabolite namely, acetyl-CoA, which is the product of both PFL and PDH. If xylose is metabolized via phosphoketolase the result is GAP and acetyl phosphate [103]. GAP enters glycolysis while acetyl phosphate can be converted to acetate or acetyl-CoA. If the level of triose phosphates (GAP and DHAP) is high, PFL is inhibited, which in combination with a bottleneck at acetyl-CoA would explain the more severe inhibition of growth in SAL medium compared to SA. An open question is still the lack of effect with ribose which could be caused by regulatory mechanisms governing its route through central carbon metabolism.

So far, the finding that an effect is observed under the diverse conditions of both anaerobic and respiratory growth could suggest that there may be more than one area which is affected by glycerol supplementation. One possible link is xylose uptake and metabolism via the phosphoketolase pathway. Since ribose might be metabolized via the same pathway this contradicts this model. Other factors such as redox level might also be influencing the cellular growth. Further investigations are needed to determine the exact mechanism by which glycerol interferes with xylose metabolism.

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Part III

Engineering glycerol metabolism and cofactor level in *Lactococcus lactis*

Chapter 11

Introduction

The availability of glycerol has increased dramatically. An increase in production of biodiesel has led to a concomitant rise in the byproduct waste glycerol (crude glycerol) which consists mainly of glycerol, methanol, salts and remnants of free fatty acids in varying degrees. As a result of the increased production the price of crude glycerol is low. A saturated market for pure glycerol and expensive refining costs have made crude glycerol an attractive substrate for industrial bioconversion [151, 19, 114].

A wide range of different microorganisms and value added products have been investigated as possible routes to handle excess glycerol. The main species commonly utilized to convert glycerol are *Klebsiella*, *Clostridium*, *Citrobacter*, *Pichia* and *Yarrowia*. These are natural glycerol fermenters and have been applied to the production of 1,3-propanediol, citric acid, citrate, ethanol, 2,3-butanediol and butanol [19, 130, 137, 139, 138, 121].

11.1 Metabolic engineering of glycerol utilization

Other strategies for glycerol valorization includes engineering other species that do not naturally ferment glycerol. The common work horse *Escherichia coli* has been widely used for this purpose. Initially *E. coli* was believed to only utilize glycerol in the presence of an external electron acceptor but recent studies have shown that under the right conditions anaerobic fermentation of glycerol is possible [48, 124, 118, 120]. Overexpression of the native genes *gldA* (encoding glycerol dehydrogenase) and *dhaKLM* (encoding dihydroxyacetone kinase) involved in glycerol metabolism allowed efficient production of ethanol from glycerol [60, 113].

Other strategies include the production of 1,3-propanediol by expressing the *dhaB1* (encoding a B12-independent glycerol dehydratase) and *dhaB2* (DhaB1 activating factor) genes from *Clostridium butyricum* together with the *E. coli yqhD* gene (encoding a 1,3-propanediol oxidoreductase) [148] and

the production of serinol (2-amino-1,3-propanediol) by expressing the *rtxA* gene from *Bradyrhizobium elkanii* [108] from glycerol.

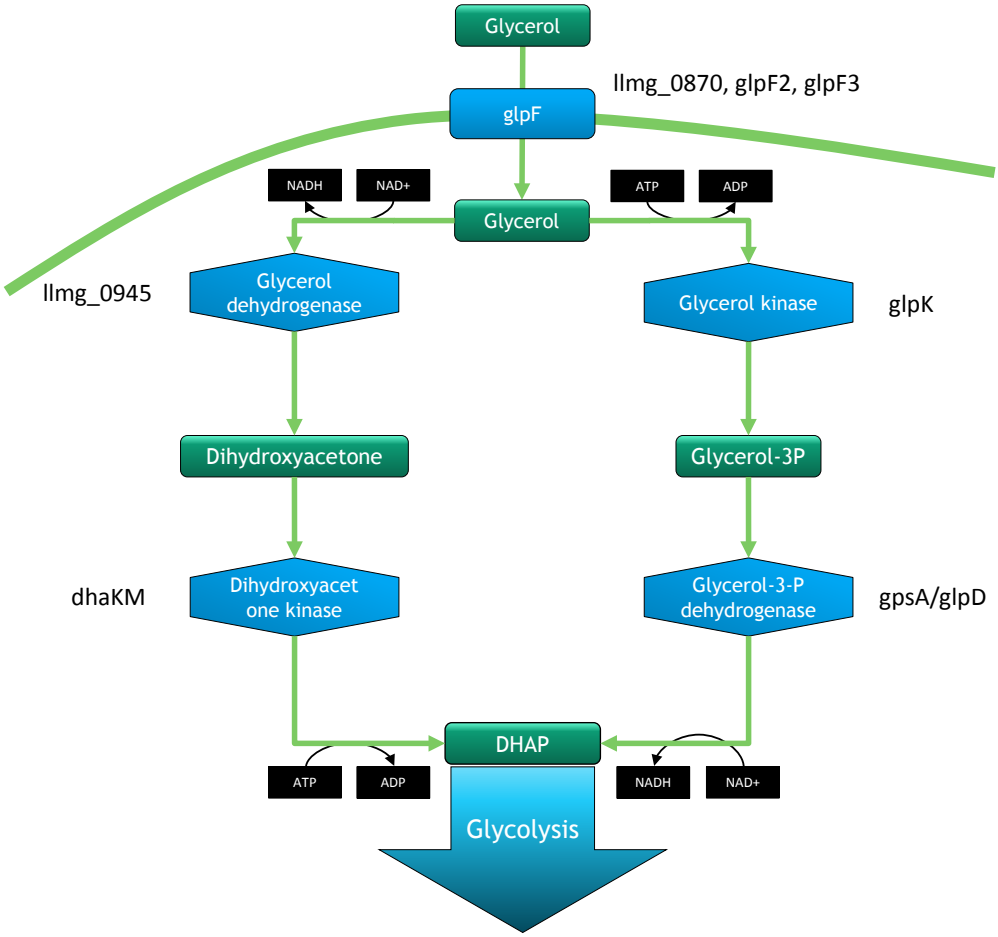

Figure 11.1: Overview of the possible routes of glycerol dissimilation. The annotated genes found in *Lactococcus lactis* are shown next to the enzymatic steps. After transport into the cell by facilitated diffusion glycerol is either oxidized to dihydroxyacetone by glycerol dehydrogenase or phosphorylated to glycerol-3-phosphate by glycerol kinase. Subsequently either dihydroxyacetone is phosphorylated by dihydroxyactone kinase or glycerol-3-phosphate is oxidized by glycerol-3-phosphate dehydrogenase to dihydroxyacetone phosphate (DHAP) which enters glycolysis.

11.2 Engineering recombinant glycerol utilization in *Lactococcus lactis* MG1363

In addition to the screening of *Lactococcus* spp. and conditions which might induce glycerol utilization a parallel course was undertaken to introduce

the required enzymatic machinery for anaerobic glycerol fermentation into *Lactococcus lactis* MG1363.

A similar approach had already been shown to work in *Escherichia coli* and *Corynebacterium glutamicum*. Rittmann *et al.* introduced genes encoding *glpF*, *glpK* and *glpD* (encoding a glycerol facilitator, a glycerol kinase and a glycerol-3-phosphate dehydrogenase respectively) from *E. coli* into *C. glutamicum* allowing production of amino acids from glycerol [141].

Daniel *et al.* introduced the entire *dha* regulon (encoding glycerol dehydrogenase - *dhaD*, dihydroxyacetone kinase - *dhaK*, glycerol dehydratase - *dhaB* and 1,3-propanediol dehydrogenase - *dhaT*) from *Citrobacter freundii* into *E. coli* which allowed anaerobic growth on glycerol [116]. Similarly the *dha* operon from *Clostridium butyricum* and *Klebsiella pneumonia* have been used to facilitate anerobic growth on glycerol in *E. coli* while producing 1,3-propanediol [115, 150].

11.2.1 Both overexpression of endogenous genes and an artificial operon containing a complete pathway for glycerol dissimilation is employed to allow anaerobic growth on glycerol by *L. lactis*

In order to allow anaerobic growth on glycerol by *L. lactis* MG1363 a parallel strategy was devised consisting of overexpression of endogenous genes involved in glycerol metabolism and the introduction of an artificial operon containing all the required genes for glycerol transport and assimilation.

An overview of the required steps and the annotated genes from *L. lactis* MG1363 can be seen in figure 11.1. The operon was based on the glycerol facilitator from *E. coli* in combination with glycerol dehydrogenase and dihydroxyacetone kinase from *Citrobacter freundii*.

Chapter 12

Materials and Methods

12.1 Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in the project are listed in table 12.1. *Escherichia coli* strains were cultivated aerobically at 28°C, 30°C or 37°C either in rich or defined medium. Rich medium consisted of Luria-Bertani broth [144] and defined medium was either MOPS medium [135] or M9 medium [122] with either no antibiotic or containing any of the following or both: 200 μg/mL Erythromycin or 100 μg/mL Ampicillin, depending on the selective requirements. When cultivating *E. coli* MC1000 in minimal media 50 μg/mL leucine was added to ensure optimal growth conditions. *Lactococcus lactis* strains were cultivated in rich medium which consisted of GM17 broth [149] (M17 + 1% glucose) or defined media which were either BL or SA [143] with the appropriate modifications as described in section 8.1.1. Strains were routinely cultivated at room temperature, 28°C or 30°C, with or without aeration.

12.2 Primers and PCR protocols

The primers used in the project can be seen in table 12.2.

Strain	Description	Source or Reference
E. coli strains		
MC1000	(F^-, *araD139*, *δ(araA-leu)7679 galU galK lac-174 rpsL thi-1*)	[111]
DH5α	(F^- *Δ(argF-lacZ)*U169 *phoA glnV44 ϕ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 endA1 endA1 thi-1 hsdR17*)	[133]
AH07	*E. coli* MG1655 transformed with pTrc99A::nox, inducible plasmid with NADH oxidase from *Streptococcus pneumoniae*	[126]
L. lactis strains		
MG1363	Prophage-cured and plasmid-free derivative of *Lactococcus lactis* subsp. cremoris NCDO 712	[123]
LB436	*Lactococcus lactis* MG1363 transformed with the plasmid pLB65 containing the phage TP901-1 integrase gene, which is necessary for site specific recombination in the chromosomal attB region of *L. lactis* MG1363, Cam^r	[110]
AH200–216	*Lactococcus lactis* MG1363 transformed with pCS574::SPL::*glpF*::*dhaD*::*dhaK*, Tet^r	This study
AH220–235	*Lactococcus lactis* MG1363 transformed with pLB85::SPL::*noxE* or pLB85::SPL::*nox*, Erm^r	This study
Plasmids		
pAK80	Promoterless vector carrying the *lacLM* genes encoding the reporter enzyme β-galactosidase, Erm^r	[160]
pLB85	Promoterless vector carrying the *gusA* gene encoding the reporter enzyme β-glucuronidase and the integrase from TP901-1 allowing for integration into the chromosome of *L. lactis* at the attB site, Erm^r,	[110]
pLB86	Promoterless vector carrying the *lacLM* genes encoding the reporter enzyme β-galactosidase, Erm^r	[110]
pTD5	Promoterless vector carrying the *lacLM* genes encoding the reporter enzyme β-galactosidase, based on pAK80 with around 3kb of sequence removed, Erm^r	[117]
pCS574	Promoterless vector carrying the *gusA* gene encoding the reporter enzyme β-glucuronidase, based on pLB85 with Tet^r instead of Erm^r	[174]
pAH184	Plasmid carrying an artificial operon with the *glpF* gene from *E. coli* and the *dhaD* and *dhaK* genes from *Citrobacter freundii* codon optimized for *Lactococcus lactis*. pUC57::*glpF*::*dhaD*::*dhaK*::*groEL2*, Amp^r	GenScript

Table 12.1: An overview of the bacterial strains and plasmids used in engineering *L. lactis* glycerol metabolism. Erm^r - Erythromycin resistance. Amp^r - Ampicillin resistance. Tet^r - Tetracycline resistance. Cam^r - Chloramphenicol resistance.

No.	Name	RES	Sequence (5'–3')
28	glpK_lactis_fwd_XbaI_SPL	XbaI	TACTGTCTAGAGGATCCATNNNNNAGTTTATTCTTGACANNNNNNNNNNNNNNNTGRTATAATNNNNAAGTAATAAAATATTCGGAGGAATTTTGAAATGTCTGAGGGATTACCA
29	glpK_lactis_rev_PstI		TACTGCTGCAGTTATTCAAAAGATCTTGTTGCC
30	glpK_lactis_seq_300_fwd		CTGGTCTCCCAATCTATC
31	glpK_lactis_seq_1200_rev		ACCTAAAATATCACGAACC
32	glpK_coli_fwd_XbaI_SPL	XbaI	TACTGTCTAGAGGATCCATNNNNNAGTTTATTCTTGACANNNNNNNNNNNNNNNTGRTATAATNNNNAAGTAATAAAATATTCGGAGGAATTTTGAAATGACTGAAAAAAAATATATCG
33	glpK_coli_rev_PstI	PstI	TACTGCTGCAGTTATTCGTCGTGTTCTTCC
36	pCS574_attL_fwd		CTACTGCTGCTTCACCAG
37	pCS574_gusA_rev_1		CGCGATCCAGACTGAATG
39	noxE_lactis_fwd_XhoI_SPL	XhoI	TACTGCTCGAGGGATCCATNNNNNAGTTTATTCTTGACANNNNNNNNNNNNNNNTGRTATAATNNNNAAGTAATAAAATATTCGGAGGAATTTTGAAATGAAAATCGTAGTTATCGGTAC
46	noxE_lactis_rev_PstI	PstI	TACTGCTGCAGTTATTTGGCATTCAAAGCTGC
47	glpF_alpha_fwd_XbaI_SPL	XbaI	TACTGTCTAGAGTATCCATNNNNNAGTTTATTCTTGACANNNNNNNNNNNNNNNTGRTATAATNNNNAGGTAAGTAATAAAATATTCGG
48	groEL2_rev_PstI	PstI	TACTGCTGCAGAAAATTCTTGATACCAGC
49	glpF_alpha_rev_PstI	PstI	TACTGCTGCAGTTACAATGATGCTTTTTGTTCAG
51	dhaD_alpha_rev_PstI	PstI	TACTGCTGCAGTTATCTAGCAAGCCATTGTT
54	dhaK_alpha_rev_SalI	SalI	TACTGGTCGACTTATCCCAATTCAGATTCAGC
58	glpF_alpha_ctrl_fwd		AGGTAAGTAATAAAATATTCGG
59	dhaD_alpha_ctrl_fwd		ATCTCAATTTCGGAGGACA
60	dhaK_alpha_ctrl_fwd		CGCCCTATAAGGAGAATTAGA
73	noxE_lactis_USER_fwd_SPL		ATTATCT**U**CATNNNNNAGTTTATTCTTGACANNNNNNNNNNNNNNNTGRTATAATNNNNAAGTAATAAAATATTCGGAGGAATTTTGAAATGAAAATCGTAGTTATCGGTAC
74	noxE_lactis_USER_rev		ACTCCTCAT**U**TTATTTGGCATTCAAAGCTGC
75	nox_strep_USER_fwd_SPL		ATTATCTUCATNNNNNAGTTTATTCTTGACANNNNNNNNNNNNNNNTGRTATAATNNNNAAGTAATAAAATATTCGGAGGAATTTTGAAATGAGTAAAATCGTTGTAG
76	nox_strep_USER_rev		ACTCCTCAT**U**TTATTTTTCAGCCGTAAG
77	nox_strep_control_fwd		ATGAGTAAAATCGTTGTAG
78	pLB85_USER_fwd_2		AATGAGGAG**U**GCCGCGGACCAGTATTATTAT

No.	Name	RES	Sequence (5'–3')
79	pLB85_USER_rev_2		AAGATAA**U**ACTGATGAACTATGGGACC
80	pLB85_USER_fwd_3		AATGAGGAG**U**AGCCGCGGACCAGTATTAT
81	pLB85_USER_rev_3		AAGATAA**U**ATGAGATAACTGATGAACTATGGGACCA
86	noxE_lactis_rev_NotI	NotI	TACTGGCGGCCGCTTATTTGGCATTCAAAGCTGC
87	nox_strep_rev_NotI	NotI	TACTGGCGGCCGCTTATTTTTCAGCCGTAAGGGCAG
88	dhaD_alpha_fwd_SPL_XhoI	XhoI	TACTGCTCGAGGTATCCATNNNNNAGTTTATTCTTGACANNNNNNNNNNNNNNNTGRTATAATNNNNATCTCAATTTCGGAGGACA
89	dhaD_alpha_rev_NotI	NotI	TACTGGCGGCCGCTTATCTAGCAAGCCATTGTT
90	dhaK_alpha_fwd_SPL_XhoI	XhoI	TACTGCTCGAGGTATCCATNNNNNAGTTTATTCTTGACANNNNNNNNNNNNNNNTGRTATAATNNNNCGCCCTATAAGGAGAATTAGA
91	dhaK_alpha_rev_NotI	NotI	TACTGGCGGCCGCTTATCCCAATTCAGATTCAGC

Table 12.2: Primers used in the construction and verification of strains. RES - restriction site if present. Restriction sites are marked by underlining and the annealing part of the longer primers is marked by italics. For primers deigned for USER cloning the uracil residue is highlighted by boldface.

12.2.1 PCR using *Taq* polymerase

The PCR program and the standard reaction mixture for PCR using Taq polymerase can be seen in table 12.3.

Reaction mixture		PCR program			
Thermopol buffer	10 μL	Initial denaturation	94°C	2 min.	1 cycle
5 mM dNTP mix	4 μL				
Primer A	2.5 μL	Denaturation	94°C	30 sec.	
Primer B	2.5 μL	Annealing	50°C	30 sec.	30 cycles
Taq polymerase	1 μL	Extension	68°C	1 min. pr. kilobase	
H_2O	80 μL	Hold	4°C	-	
Total	100 μL				

Table 12.3: Reaction mixture and program settings for colony PCR with Taq polymerase.

12.2.2 PCR using Phusion polymerase

Standard protocol

The PCR program and the standard reaction mixture for PCR with Phusion polymerase can be seen in table 12.4.

Reaction mixture		PCR program			
5x Phusion buffer	20 μL	Initial denaturation	98°C	30 sec.	1 cycle
5 mM dNTP mix	4 μL				
Primer A	5 μL	Denaturation	98°C	10 sec.	
Primer B	5 μL	Annealing	50°C	30 sec.	30 cycles
Template DNA	1 μL	Extension	72°C	30 sec. pr. kilobase	
DMSO	3 μL				
Phusion	1 μL	Final extension	72°C	10 min.	1 cycle
H_2O	61 μL	Hold	4°C	-	
Total	100 μL				

Table 12.4: Reaction mixture and program settings for standard PCR with Phusion polymerase. Reagents: 5x Phusion buffer - Finnzymes F-518, DMSO - Finnzymes F-515, Phusion polymerase - Finnzymes F-530.

Touchdown protocol

The PCR program and the standard reaction mixture for touchdown PCR using Phusion polymerase can be seen in table 12.5.

Reaction mixture		PCR program			
5x Phusion buffer	20 μL	Initial denaturation	98°C	30 sec.	1 cycle
5 mM dNTP mix	4 μL				
Primer A	5 μL	Denaturation	98°C	10 sec.	
Primer B	5 μL	Annealing	first: 65°C	30 sec.	15 cycles
Template DNA	1 μL		last: 50°C		
DMSO	3 μL	Extension	72°C	30 sec. pr. kilobase	
Phusion	1 μL				
H_2O	61 μL	Denaturation	98°C	10 sec.	
Total	100 μL	Annealing	50°C	30 sec.	20 cycles
		Extension	72°C	30 sec. pr. kilobase	
		Final extension	72°C	10 min.	1 cycle
		Hold	4°C	-	

Table 12.5: Reaction mixture and program settings for touchdown PCR with Phusion polymerase. Reagents: 5x Phusion buffer - Finnzymes F-518, DMSO - Finnzymes F-515, Phusion polymerase - Finnzymes F-530.

USER fragment amplification

The reaction conditions for amplification of fragments with USER based primers are identical to the mixtures and conditions used in Phusion PCR. The main difference is the polymerase used was PfuX7 [136] instead of Phusion.

Generating synthetic promoter libraries (SPL)

A promoter library is a collection of promoters with different strengths. The modulation of promoter strength can be done by modulating the regions flanking the bacterial promoter concensus sequences -35 (TTGACA) and -10 (TATAAT) [128, 129]. The construction of such a library has been made increasingly easy and can in fact be done in a single PCR step using oligonucleotides with randomized promoter regions [125, 146]. An example of such a primer can be seen in figure 12.1

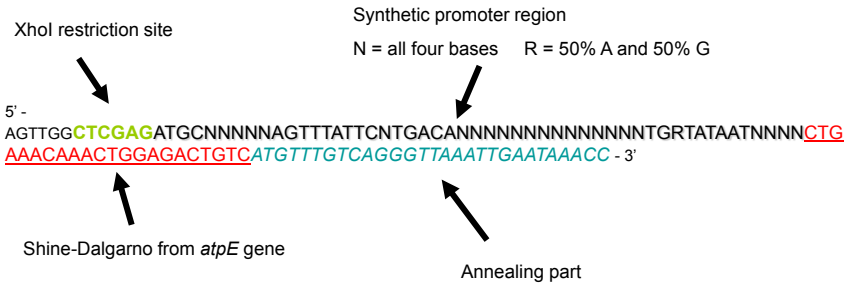

Figure 12.1: Figure depicting an oligonucleotide with a synthetic promoter region. The chosen example is an actual primer used to amplify a fragment encoding NADH kinase (*POS5*) from *Saccharomyces cerevisiae* in a previous project.

Colony PCR on *E. coli* and *L. lactis*

To perform colony PCR to verify constructs the reaction conditions for Taq PCR was used. The polymerase was either Taq (<2kb) or DreamTaq™, Fermentas (>2kb). A standard reaction of 100 μL was split into 4 seperate reactions of 25 μL. To the mixture a small amount of a single colony was added and a standard PCR program was used with one modification: An initial denaturation time of 15 min. For the *L. lactis* colony PCR it is paramount that only a very small amount of cell material is added otherwise no reaction will occur.

12.2.3 DNA techniques

Agarose gel electrophoresis

To verify DNA fragment sizes, samples were run on 0.7-1% agarose gels containing 0.3 μg/mL ethidium bromide. Samples were mixed with loading buffer and loaded next to 10 μL of Fermentas GeneRuler™DNA ladder mix (#SM0333) or Fermentas GeneRuler™1kb DNA ladder plus (#SM1333) and subsequently run for 45-90 min. at 100-150V / 200 mA. A size distribution for the ladder mix used can be seen in figure 12.2.

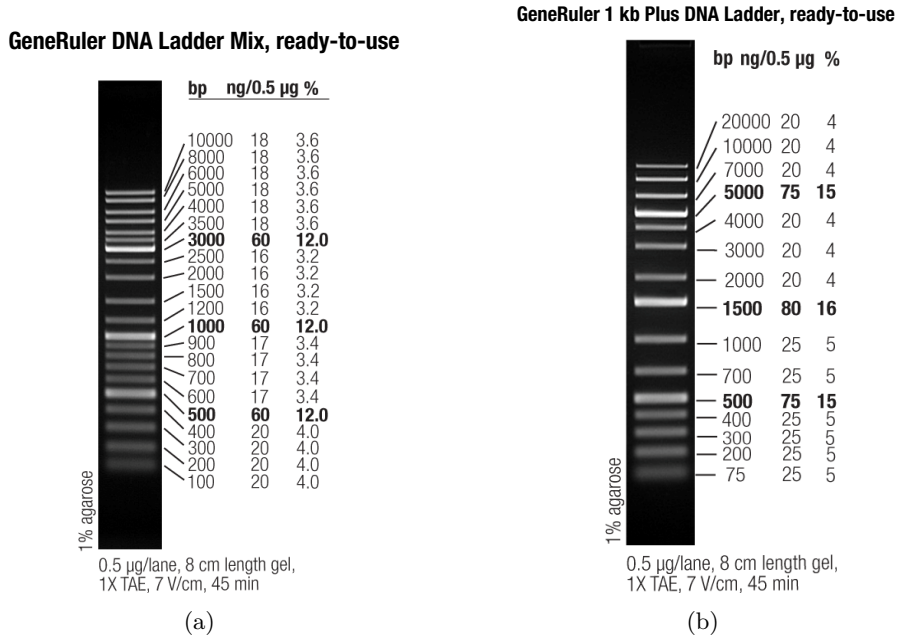

Figure 12.2: **(a)** Size distribution for Fermentas Generuler™DNA ladder mix (#SM0333) **(b)** Size distribution for Fermentas Generuler™1kb DNA ladder plus (#SM1333)

Purification of DNA

When necessary, DNA was purified from excised gel bands or from solution by using the GE Healthcare - illustra GFX™PCR, DNA and Gel Band Purification Kit (product code 28-9034-70). All purification work was done according to the manufacturers specifications.

Purification of plasmid DNA

The plasmids used in the project were prepared from either Zymo research - Zyppy™Plasmid Miniprep Kit (catalog no. D4020) or with Macherey-Nagel - NucleoBond® Xtra Midi Plus kit (ref. 740412.10). The Zyppy Miniprep kit was mainly used for smaller samples used for analytical purposes and sequencing while the NucleoBond® kit was used for large quantity plasmid preparations. The work was carried out according to the specific protocols supplied by the manufacturer.

Digestions

Two types of digestions were generally employed: Restriction analysis and preparative digestion. Digestion was performed with either traditional restriction enzymes or with the fermentas FastDigest® system. An overview can be seen in table 12.6 and 12.7. When digesting with traditional enzymes buffers were generally selected on the recommendations of Fermentas and New England Biolabs for both single and double digestions. Reaction conditions and BSA addition were based upon manufacturer's specification. When using the FastDigest® system all reactions took place in fastdigest buffer.

Standard restriction enzyme digestion		
	Restriction analysis	Peparative digest
DNA	10 μL	40–50 μL
10x reaction buffer	2 μL	10 μL
(10 x BSA)	2 μL	10 μL
Restriction enzyme	1 μL	4 μL
H_2O	5–7 μL	26–36 μL
Total	*20 μL*	*100 μL*

Table 12.6: Protocol for restriction analysis and preparative digestion of DNA with standard restriction enzymes.

For standard restriction enzymes digestions were performed for 2–4 hours at 37°C. For FastDigest® enzymes digestions were performed for 30–60 min

at 37°C. The effectiveness was gauged by running 1–5 μL samples on a 1% agarose gel after digestion as described in section 12.2.3.

FastDigest® enzyme digestion		
	Restriction analysis	Peparative digest
DNA	10 μL	40–50 μL
10x fastdigest buffer	2 μL	10 μL
Restriction enzyme	1 μL	4 μL
H_2O	7–10 μL	36–46 μL
Total	*20 μL*	*100 μL*

Table 12.7: Protocol for restriction analysis and preparative digestion of DNA with FastDigest® restriction enzymes.

Ligation

Prior to ligation, the molar ratio of vector and insert was determined by gel electrophoresis. A ratio of at least 1:3 - vector:insert is preferable. Usually a ratio of 1:5 was utilized. The ligation mix can be seen in table 12.8. Ligation was performed either at 16°C over night (O/N) or at room temperature for 1–2 hours.

Ligation mix	
Vector and insert in a ratio of (at least) 1:3	17μL
T4 ligase Buffer	2μL
T4 DNA ligase	1μL
Total	*20μL*

Table 12.8: Protocol for ligation used during the project.

Prior to use the ligation mixture was heated to 65°C for 10 min. to inactivate T4 DNA ligase.

Ligation of USER mix

For assembly of fragments with USER cloning the protocol in table 12.9 was used.

USER Ligation mix		USER program	
Vector and insert 1:1	8 μL	37°C	35 min.
10x BSA	0.5 μL	25°C	25 min.
NEBbuffer 4	0.5 μL	4°C	-
USER enzyme mix	1 μL		
Total	*10μL*		

Table 12.9: Protocol for ligation used during the project.

12.2.4 Preparation of crude enzyme extract

Culture for enzyme assays was quickly chilled on ice and cells harvested by centrifugation at 7000 rpm for 10 min. (4°C). The harvested cells were resuspended in 1mL 0.2 % (w/v) KCl, centrifuged for 2 min. in a cold microcentrifuge (4 °C) at 7000 rpm, and washed again with 1 mL 0.2% KCl. The cells were then finally resuspended in 700 μL protein extract buffer: 45 mM Tris, 15 mM tricarballylate, 20% glycerol, 4.5 mM $MgCl_2$ and 1 mM dithiothreitol. Depending on the enzymatic activity to be measured, glycerol was sometimes omitted from the extract buffer eg. in the case of glycerol kinase activity measurements. The cell suspensions were then stored at -80°C until they were needed. When samples were to be analyzed the cells suspension was disrupted with glass beads (106 microns acid washed and finer (G-4649) from Sigma). Approximately 0.5 mL glass beads were added to each tube and the samples were shaken in a Fastprep® for 45 seconds at speed rate 4. The disrupted cell suspension was subsequently centrifuged at maximum speed in a cold centrifuge for 20 min. (0°C). The supernatant was collected and used to measure relevant enzyme activity.

12.2.5 Enzyme assays

Enzymatic assays were performed in either 1 mL using a Zeiss M500 spectrophotometer [162] or in 200 μL using a Tecan infite® 200 pro microplate reader with a Corning® 96 Well Clear Flat Bottom UV-Transparent Microplate (Product 3635). Enzyme assay activity was measured either by adding 100 μL of sample to 900 μL assaymix or by addition of 20 μL sample in 180 μL assaymix.

All enzyme assays were measured from cell free crude extracts prepared as described in section 12.2.4 and performed at 30°C. Protein concentration was quantified using the Bradford assay (reagent B6916 Sigma-Aldrich) with pre-diluted BSA standards from fermentas measured in a Tecan infite® 200 pro plate reader.

NADH oxidase assay

The assay is based on measuring the decrease of NADH concentration as a function of time at a wavelength of 340 nm. Cell extract is added to assaymix containing 100 mM Triethanolamine-HCL buffer, 0.3 mM NADH and 0.3 mM EDTA at pH 7.2.

Glycerol dehydrogenase assay

The assay is a direct assay based on the linear increase in absorbance at 340nm by (NADH) [57]. Glycerol dehydrogenase activity was measured by addition of sample to assaymix containing 100mM potassium carbonate/bicarbonate buffer at pH 9.5, 30 mM ammoniumsulfate, 0.6 mM NAD^+ and 100 mM glycerol.

Dihydroxyacetone kinase assay

The assay is a coupled assay following the decrease of NADH concentration which is coupled to the reduction of dihydroxyacetonephosphate to glycerol-3-phosphate by glycerol dehydrogenase [34]. DHA kinase activity was measured by addition of sample to assaymix containing 50 mM potassium phosphate buffer at pH 7.2, 1 mM Dihydroxyacetone, 1 mM ATP, 1 mM $MgCl_2$, 0.1 mM NADH, 10U G3P dehydrogenase (rabbit) and 10 mM α,α-dipyridyl.

Glycerol kinase assay

The assay is a coupled assay based on the action of pyruvate kinase (PK) and lactate dehydrogenase (LDH) [140]. The ATP consumption of glycerol kinase is monitored by the conversion of phosphoenolpyruvate (PEP) to pyruvate (PYR) by PK which regenerates ADP to ATP. The following reduction of PYR to lactate by LDH can then be monitored by a drop in absorbance at 340nm. Glycerol kinase activity was measured by addition of sample to assaymix containing 50 mM Triethanolamine-HCL buffer at pH 7.2, 10 mM $MgCl_2$, 20 mM KCl, 2.5 U Pruvate kinase, 2.5 U Lactate dehydrogenase, 0.2 mM PEP, 0.2 mM NADH, 3 mM ATP and 10 mM glycerol.

12.2.6 Transformation

Competent *E. coli cells*

Electro competent *E. coli* cells were prepared by adding exponentially growing cells from O/N culture to 5 x 250 mL LB medium, resulting in a final OD of 0.05. When the culture reached an OD600 of 0.4 the cells were quickly cooled in a slurry of ice and water and harvested by centrifugation at 7000 rpm for 10 min. The pellet was washed with 20 mL 10% glycerol five times

(until the pellet was slightly runny) and finally resuspended in 2 mL 10% glycerol, which was aliquoted into 40 μL portions and stored at -80°C.

Competent *L. lactis cells*

Electro competent *L. lactis* cells were prepared by adding exponentially growing inoculum from O/N culture to 100 mL of solution 1, resulting in a final OD of 0.03. When the culture reached an OD600 of 0.6 the cells were quickly cooled in a slurry of ice and water and harvested by centrifugation at 5000 rpm for 10 min. The pellet was washed with 30 mL of solution 2 and finally resuspended in 1 mL solution 2, which was aliquoted into 40-200 μL portions and stored at -80°C. The composition of solution 1 and 2 can be seen in table 12.10. Glycine concentration in solution 1 is strain dependent. For MG1363 or IL1403 1.5% is used but other strains usually require lower concentrations to be added.

Solution 1		**Solution 2**	
Component	Final concentration	Component	Final concentration
15 mL of 10% stock	1.5% glycine	25 mL of 2M stock	0.5M sucrose
12.5 mL of 2M stock	0.25M sucrose	20 mL of 50% stock	10% glycerol
5 mL of 20% stock	1% glucose	55 mL dest. H_2O	
50 mL of 2x stock	1x M17	Total	100 mL
17.5 mL dest. H_2O			
Total	100 mL		

Table 12.10: Composition of solution 1 and 2 used in the preparation of competent cells from *Lactococcus lactis*.

Electroporation and transformation

Escherichia coli Transformation was done by mixing 40 μL of competent cells with 3.5 μL of plasmid preparation or ligation mix on ice. The mix was transferred to a pre-chilled Gene Pulser cuvette and electroporated on a Bio-Rad MicroPulser by a 2.5 kV pulse and resuspended in 2 mL of transformation mix (LB + 10mM $MgCl_2$ + 10mM $CaCl_2$ + 0.2 % glucose). The culture was then incubated for two hours at 37°C after which the cells were plated in dilution on suitable plates.

Lactococcus lactis Transformation was done by mixing 40 μL of competent cells with 1-5 μL of plasmid preparation or ligation mix on ice. The mix was transferred to a pre-chilled Gene Pulser cuvette and electroporated on a Bio-Rad MicroPulser by a 2.0 kV pulse (manual 2.0) and resuspended in 2 mL of transformation mix (SGM17: M17 + 0.2M sucrose + 0.5% glucose + 20mM $MgCl_2$ + 2mM $CaCl_2$). The culture was then incubated for two hours at 30°C after which the cells were plated in dilution on suitable plates.

USER transformation USER mix was transformed into chemically competent *E. coli* DH5α by first mixing competent cells and 3–5 μL of DNA gently. After a 15 min. incubation on ice, the cells were heat shocked at 45°C for 1min. after which they were incubated on ice another 10 min. Finally, 1 mL of LB was added and the cells incubated for a suitable time allowing for phenotypic expression before plating on appropriate selective plates.

Selective plates When screening a synthetic promoter library (SPL) for transformants with varying promoter strength, the following plates were used: for *E. coli* LB plates were prepared containing: Either 150–200 μg/mL Erythromycin or 100 μg/mL Ampicillin + 1 % glycerol and either 100–200 μg/mL of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) or X-gluc (5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid) depending on the reporter gene used. For *L. lactis* M17 + 1% glucose plates were prepared containing: either 5 μg/mL Erythromycin or 5 μg/mL Tetracycline + 1 % glycerol and either 100–200 μg/mL of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) or X-gluc (5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid) depending on the reporter gene used. Glycerol can be omitted if need be.

Site specific integration onto the chromosome of *Lactococcus lactis* MG1363 via the TP901-1 phage encoded integrase Site specific integration of plasmid DNA into the chromosome of *Lactococcus lactis* MG1363 can be done utilizing the attachment site (attP) and the enzyme integrase from the *L. lactis* phage TP901-1 [110]. By placing the attachment site on a vector which is unable to replicate in *L. lactis* integration onto the chromosome is the only way for the plasmid to remain. This is mediated by the phage encoded integrase between the attachment site on the vector (attP) and an attachment site on the chromosome (attB).

Integration is achieved by transforming a non-replicative plasmid such as pLB85 into a strain carrying the integrase on separate plasmid. This will allow a high frequency of recombination to occur. An example showcasing the vector pLB85 is shown in figure 12.3.

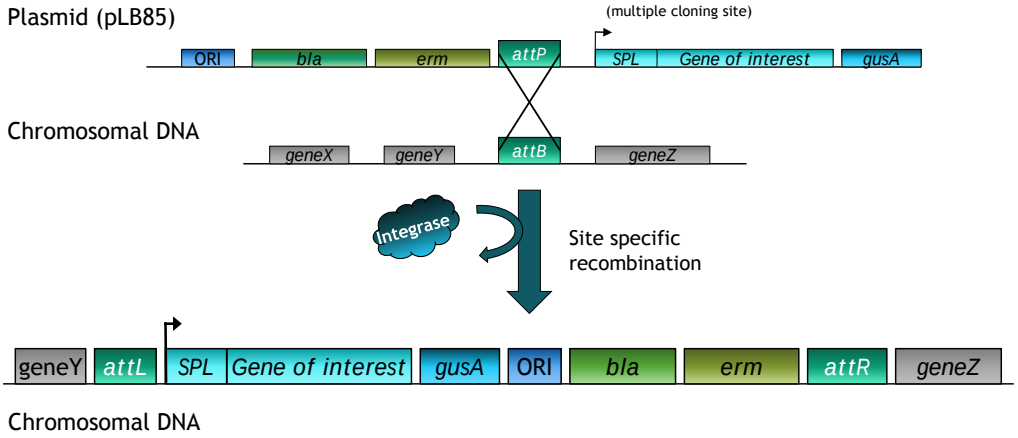

Figure 12.3: Illustration of site specific integration of plasmid DNA onto the chromosome of *Lactococcus lactis* MG1363. pLB85 (shown linearized): ORI - origin of replication for *E. coli*, *bla* - ampicillin resistance gene, *erm* erythromycin resistance gene, attP - attachment site for site-specific recombination in *L. lactis*, SPL - synthetic promoter library in front of a gene of interest inserted in the multiple cloning site, *gusA* - β-glucuronidase (reporter gene). Integration is mediated by the phage encoded integrase between the attachment site on the vector (attP) and the attachment site on the chromosome (attB).

12.3 Construction of strains overexpressing Glycerol kinase

A *glpK* (PubMed gene ID: 4798474, llmg_{1099}) fragment was amplified from chromosomal DNA of *Lactococcus lactis* MG1363. A corresponding *glpK* (PubMed gene ID: 948423, b3926) fragment was amplified from chromosomal DNA of *E. coli* MG1655.

The sequence was amplified using Phusion polymerase together with primer 28 and 29 for *Lactococcus lactis* and primer 32 and 33 for *E. coli* as described in section 12.2.2. The fragments were digested with XbaI and PstI and ligated as described in section 12.2.3 and 12.2.3 into the vector pCS574. The vector had been digested with XbaI and PstI with subsequent treatment with Shrimp Alkaline Phosphatase (SAP). The constructed plasmids were then transformed into competent *L. lactis* LB436 as described in section 12.2.6.

12.3.1 pAH184: An artificial operon with the machinery required to metabolize glycerol

As an alternative to the native enzymes present in *Lactococcus lactis* an artificial operon was constructed utilizing genes from *E. coli* and *C. freundii* [116]. The operon was designed to be comprised of three genes: *glpF*

from *E. coli* and *dhaD* plus *dhaK* from *Citrobacter freundii*. A leader sequence from a glycolytic promoter from *Lactococcus lactis* was placed in front of each gene and a terminator placed at the end. An illustration can be seen in figure 12.4.

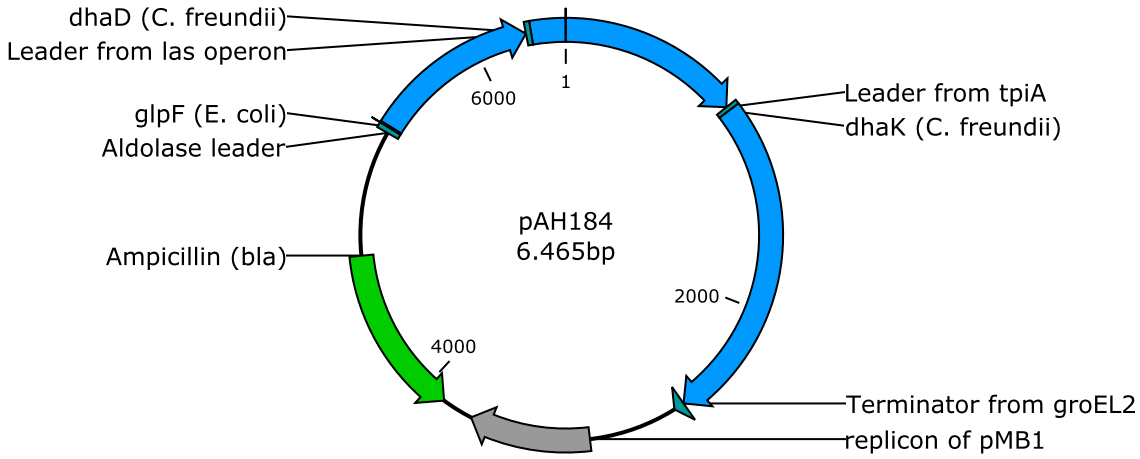

Figure 12.4: Illustration of pAH184, a plasmid carrying an artificial operon for glycerol dissimilation. The operon is constructed from a pUC57 vector with the *glpF* gene from *E. coli* (glycerol facilitator), the *dhaD* (glycerol dehydrogenase) gene from *Citrobacter freundii* and the *dhaK* (dihydroxyacetone kinase) gene also from *Citrobacter freundii*

Construction of *L. lactis* strains expressing the entire operon

Strains expressing the entire operon were constructed by amplification of a fragment containing the operon with a synthetic promoter library in front by using the primers 47 and 48 (table 12.2). The sequence was amplified using Phusion polymerase with a plasmid preparation of pAH184 as template as described in section 12.2.2. The fragment was digested with XbaI and PstI and ligated as described in section 12.2.3 and 12.2.3 into the vector pCS574. The vector had been digested with XbaI and PstI with subsequent treatment with Shrimp Alkaline Phosphatase (SAP). The constructed plasmids were then transformed into competent *L. lactis* LB436 as described in section 12.2.6.

The fragment was also ligated into the vector pLB85 and transformed into competent *L. lactis* LB436 using a similar approach as described above.

Finally a fragment without the terminator was amplified using the primers 47 and 54. The fragment was digested with XbaI and SalI and ligated as described above into the vector pLB85 with subsequent transformation into competent *L. lactis* LB436.

Construction of *L. lactis* strains expressing *dhaD* and *dhaK*

Construction of strains expressing *dhaD* and *dhaK* was done by amplifying a fragment from pAH184 with the primers 88 and 91 (table 12.2). The fragment was digested with XhoI and NotI and ligated as described in section 12.2.3 and 12.2.3 into the vector pTD5. The vector had been digested with XhoI and NotI with subsequent treatment with Shrimp Alkaline Phosphatase (SAP). The constructed plasmids were then transformed into competent *L. lactis* MG1363 as described in section 12.2.6.

Construction of *L. lactis* strains expressing *dhaD*

Construction of strains expressing *dhaD* was done by amplifying a fragment from pAH184 with the primers 88 and 89 (table 12.2) with Phusion polymerase as described in section 12.2.2. The fragment was digested with XhoI and NotI and ligated as described in section 12.2.3 and 12.2.3 into the vector pTD5. The vector had been digested with XhoI and NotI with subsequent treatment with Shrimp Alkaline Phosphatase (SAP). The constructed plasmids were then transformed into competent *L. lactis* MG1363 as described in section 12.2.6.

Construction of *L. lactis* strains expressing *dhaK*

Construction of strains expressing dhaK was done by amplifying a fragment from pAH184 with the primers 88 and 89 using Phusion polymerase as described in section 12.2.2. The fragment was digested with XhoI and NotI and ligated as described in section 12.2.3 and 12.2.3 into the vector pTD5. The vector had been digested with XhoI and NotI with subsequent treatment with Shrimp Alkaline Phosphatase (SAP). The constructed plasmids were then transformed into competent *L. lactis* MG1363 as described in section 12.2.6.

12.3.2 Construction of strains with NADH oxidase activity

Strains with NADH oxidase activity were constructed either from the *noxE* gene from *Lactococcus lactis* (Gene ID: 4796799,llmg_{0408}) or with a NADH oxidase fragment amplified from a template containing the *nox* gene from *Streptococcus pneumoniae* [109] (Genbank entry AF014458). A template with the *nox* gene was supplied by Jin Ho, working at CMB, building 223, Technical University of Denmark. This was used to construct the pTrc99A::nox plasmid which was used as a template for ampification of the *nox* fragment.

The *noxE* sequence was amplified using Phusion polymerase together with primer 39 and 46 as described in section 12.2.2. The fragment was digested with XhoI and PstI and ligated as described in section 12.2.3 and

12.2.3 into the vector pCS574. The vector had been digested with XhoI and PstI with subsequent treatment with Shrimp Alkaline Phosphatase (SAP). The constructed plasmids were then transformed into competent *L. lactis* LB436 as described in section 12.2.6.

Both the *noxE* and the *nox* sequence was amplified using Phusion polymerase together with primer 39 and 86 and with primer 17 and 87 respectively as described in section 12.2.2. The fragments were digested with XhoI and NotI and ligated as described in section 12.2.3 and 12.2.3 into the vector pTD5. The vector had been digested with XhoI and NotI with subsequent treatment with Shrimp Alkaline Phosphatase (SAP). The constructed plasmids were then transformed into competent *L. lactis* MG1363 and NCDO2118 as described in section 12.2.6.

USER cloning

When constructing strains with the USER cloning approach, both the *noxE* and the *nox* sequence was amplified using Pfu-X7 polymerase together with the primers 73 and 74 and with primers 75 and 76 respectively (table 12.2) as described in section 12.2.2. The vector pLB85 was amplified from plasmid preparations using the primers 78 and 79 or 80 and 81 (table 12.2). The amplified USER fragments were ligated together using the USER enzyme mix as described in section 12.2.3 and either transformed into competent *E. coli* DH5α as described in section 12.2.6 or directly into *Lactococcus lactis* LB436 after treatment with T4 DNA ligase. When using plasmid preparations from verified *E. coli* constructs, the preparations were transformed into *Lactococcus lactis* LB436.

Chapter 13

Results

From the initial analysis of challenges with respect to glycerol metabolism in *Lactococcus lactis*, several areas were identified as potential problem areas. Recombinant strategies were devised to investigate and handle the possible challenges associated with these. The recombinant work included:

- Construction of a strain with glycerol kinase overexpression.
- Design and construction of strains with an artificial operon containing the necessary enzymes for glycerol assimilation.
- Construction of a NADH oxidase library for tuning redox levels in connection with glycerol metabolism.

13.1 Overexpression of endogenous glycerol kinase from a synthetic promoter library as way to stimulate glycerol assimilation

Initial data indicated that glycerol kinase and dihydroxyacetone kinase were expressed at a lower level than the rest of the genes in the glycerol pathway. The solution was to construct a strain overexpressing *L. lactis*, own glycerol kinase gene, *glpK*.

Mutants made with the glycerol kinase gene were constructed with a synthetic promoter library modulating gene expression. After verification by colony PCR, the selected clones were subjected to an enzymatic assay to determine the promoter activity via the reporter gene, typically either β-galactosidase or β-glucuronidase. The results from a β-glucuronidase assay can be seen in figure 13.1.

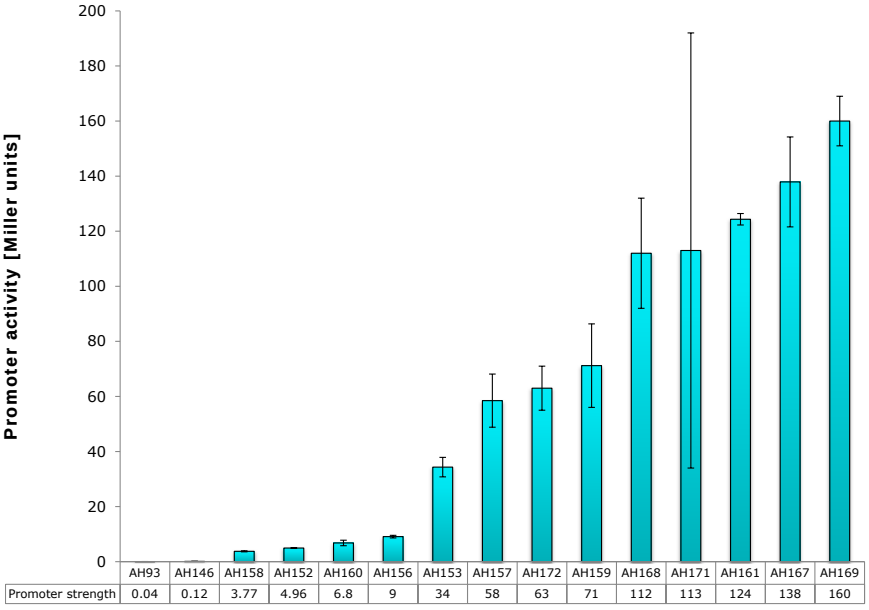

Figure 13.1: Results from promoter strength assay on strains with increased glycerol kinase expression using β-glucuronidase as a reporter gene. The strains have a single copy from an SPL library integrated on the chromosome using the vector pCS574.

From these results three candidates were selected for further analysis. The strains AH153, AH159 and AH169 were selected and samples prepared for a glycerol kinase assay. The results of the glycerol kinse assay can be found in table 13.1 and a plot of the data can be viewed in figure 13.2.

Strain	specific glycerol kinase activity [U/mg]
AH153	0.041 ± 0.001
AH159	0.079 ± 0.04
AH169	0.106 ± 0.09
MG1363	0.036 ± 0.02
MG1363 + glycerol	0.109 ± 0.06

Table 13.1: Results from glycerol kinase assay on selected strains. Strains harvested from anaerobic conditions. The strains have a single copy from an SPL library integrated on the chromosome using the vector pCS574.

None of the selected strains exhibited increased glycerol kinase activity compared to the reference. The highest activity recorded is from a reference culture which had been supplemented with glycerol during cultivation and harvest (substrate was glucose). None of the strains showed any signs of growth on plates made from defined medium supplemented with glycerol

(0.1% and 0.5%). It was later realized that the activity of glycerol kinase is regulated by the phosphotransferase system (PTS) and HPr, causing a re-evaluation of the whole strategy. After several rounds of unsuccessful cloning, no further cloning was done with glycerol kinase.

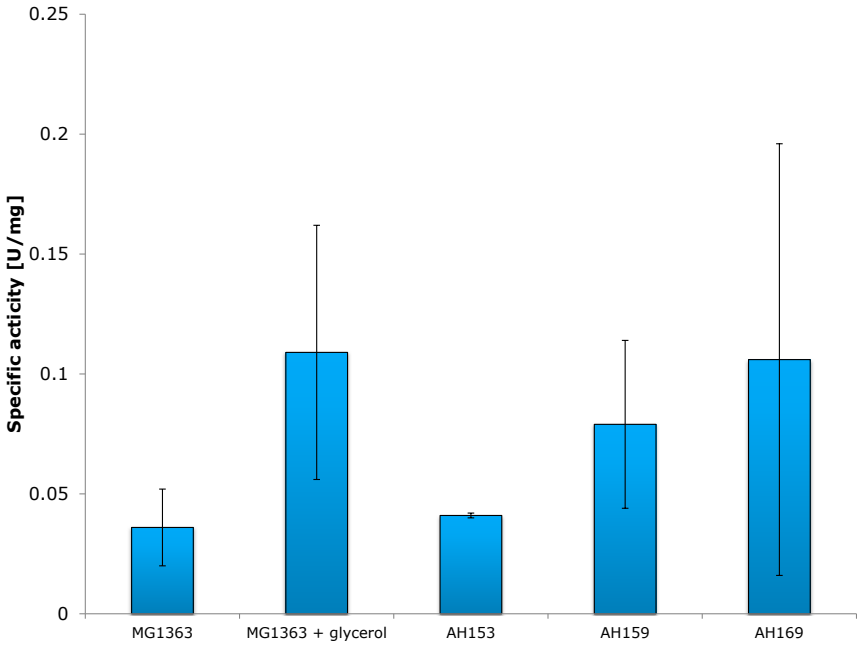

Figure 13.2: Plot of results from a glycerol kinase assay using selected strains overexpressing *glpK* from *Lactococcus lactis*. The strains have a single copy from an SPL library integrated on the chromosome using the vector pCS574. AH200 is the reference strain *Lactococcus lactis* LB436 with an empty pCS574 vector integrated on the chromosome.

13.2 Introduction of an artificial operon for glycerol dissimilation into *Lactococcus lactis*

In order to ensure a complete biochemical pathway for glycerol dissimilation an artifial operon, comprising all the necessary components, was designed. The construction was based on the work done by Daniel et *al.* [116], in which they characterized the oxidative branch of glycerol metabolism from *Citrobacter freundii*. *Citrobacter* was selected as a basis for the design because the species is known to grow fermentatively on glycerol and the genetic makeup was simple. Not having conclusively determined whether the glycerol transport system was functional in *Lactococcus lactis*, a decision was made to also include the glycerol facilitator from *E. coli*. The operon then consisted of three elements: the glycerol transport protein *glpK* from *E. coli*, the glycerol dehydrogenase *dhaD* and the dihydroxyacetone kinase *dhaK* both

from *Citrobacter freundii*. The entire operon was bought as a synthetic gene from GenScript USA Inc., codon optimized for *L. lactis*. Futher details on the operon can be found in materials and methods section 12.3.1.

Multiple rounds of cloning were performed with the artificial operon, without success. Several different combinations of cloning strategies were tested. These included integration on the chromosome of *L. lactis* and construction of plasmids carrying the operon, utilizing either traditional restriction digestion protocols or uracil based excision reagent cloning (USER). So far multiple series of clones have been generated and characterized, but so far none have been verified as containing a complete operon. An example from an initial round of cloning with the integrative vector pCS574 can be viewed in figure 13.3, figure 13.4 and figure 13.5.

Constructions made from the glycerol operon were made with a synthetic promoter library modulating gene expression of the operon. After verification by colony PCR the selected clones were subjected to an enzymatic assay to determine the promoter activity via the reporter gene, typically either β-galactosidase or β-glucuronidase. The results from such an assay can be seen in figure 13.3.

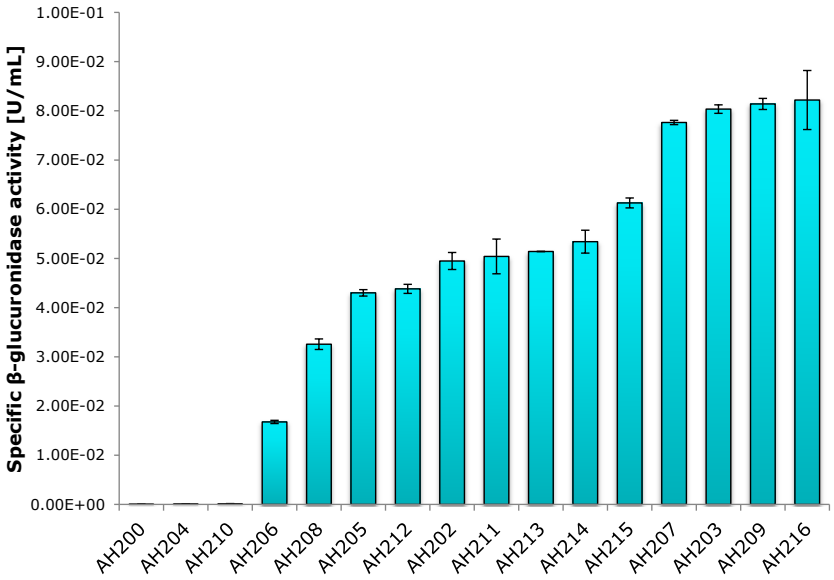

Figure 13.3: Results from promoter strength assay on strains expressing the synthetic glycerol assimilation operon using β-glucuronidase as a reporter gene. The strains have a single copy of an SPL fragment integrated on the chromosome using the vector pCS574. AH200 is the reference strain *Lactococcus lactis* LB436 with an empty pCS574 vector integrated on the chromosome.

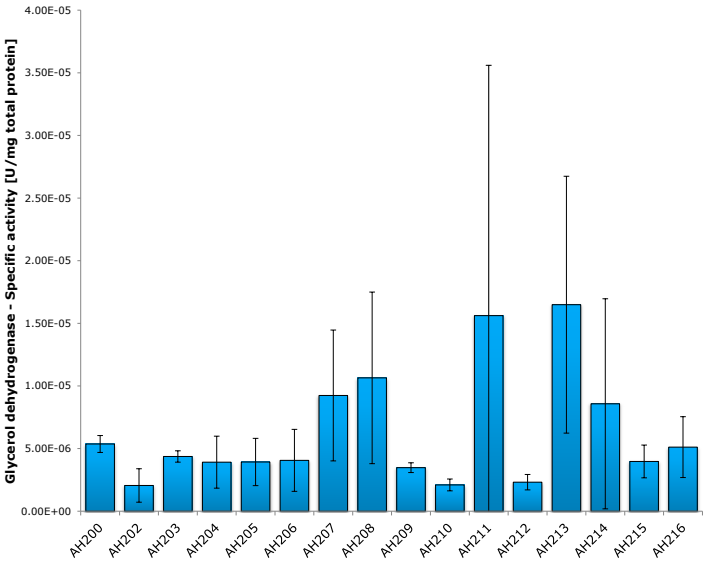

Figure 13.4: Results from glycerol dehydrogenase assay on strains expressing the synthetic glycerol assimilation operon. The strains have a single copy of an SPL fragment integrated on the chromosome using the vector pCS574. AH200 is the reference strain *Lactococcus lactis* LB436 with an empty pCS574 vector integrated on the chromosome.

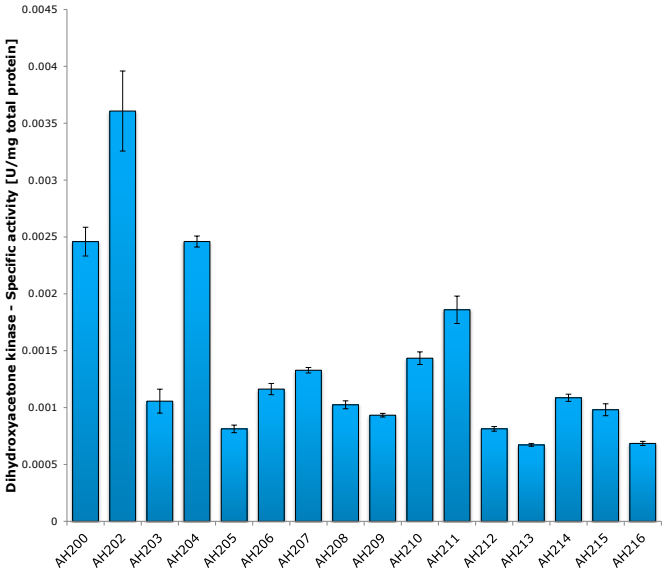

Figure 13.5: Results from dihydroxyacetone kinase assay on strains expressing the synthetic glycerol assimilation operon. The strains have a single copy of an SPL fragment integrated on the chromosome using the vector pCS574. AH200 is the reference strain *Lactococcus lactis* LB436 with an empty pCS574 vector integrated on the chromosome.

The glycerol dehydrogenase assay did not reveal significant differences between the strains and it was therefore difficult to draw conclusions based on the assay. Standard deviations for the dihydroxyacetone kinase assay, but the results indicate that the majority of the clones constructed had lower enzyme activities than the wild type/reference strain. Despite the inconclusive results the constructed strains where tested in defined medium supplemented with glycerol, for the ability to metabolize glycerol. The results can be seen in table 13.2.

Strain	Optical Density [600nm]	
	Anaerobic	Respiratory
AH200	0.012	0.020
AH202	0.010	0.020
AH203	0.016	0.043
AH204	0.010	0.038
AH205	0.020	0.039
AH206	0.012	0.041
AH207	0.010	0.038
AH208	0.012	0.040
AH209	0.005	0.038
AH210	0.008	0.043
AH211	0.014	0.057
AH212	0.002	0.037
AH213	0.007	0.042
AH214	0.015	0.038
AH215	0.012	0.045
AH216	0.024	0.058

Table 13.2: Results from screening strains with a synthetic operon for glycerol assimilation for growth in defined medium supplemented with glycerol. The strains were inoculated in SAL + 0.1% glycerol + 5 μg/mL tetracycline. Optical density was measured after 4 days at 30°C.

The strains were tested for glycerol assimilation and from the results, it is clear that none of tested strains would appear to metabolize glycerol. This is not surprising given the results from the glycerol dehydrogenase and dihydroxyacetone kinase assays.

13.3 Overexpression of NADH oxidase is not sufficient to enable growth on glycerol

When *Lactococcus lactis* grows on glycerol, more redox is formed in the form of NADH, than when growing on glucose. In order to re-oxidize the cofactors, flux must be diverted towards ethanol. This might present a problem for the cell even though *Lactococcus lactis* is fairly alcohol tolerant, as the metabolism is not geared towards producing only ethanol. Furthermore, when growing under respiration permissive conditions, additional redox is formed by the action of the pyruvate dehydrogenase complex (PDH). When the possibility to respire is there, any excess redox would in theory be re-oxidized by re-routing it to respiration. To make sure that there were no regulatory effects causing problems, an alternative route is introduced via NADH oxidase. The water forming NADH oxidase enzyme utilizes excess NADH to reduce molecular oxygen to water.

As with the previous constructions, strains with NADH oxidase activity were constructed using a synthetic promoter library to modulate gene expression. After verification by colony PCR, the selected clones were subjected to an enzymatic assay to determine the promoter activity via the reporter gene, either β-galactosidase or β-glucuronidase. The results from such an assay can be seen in figure 13.6.

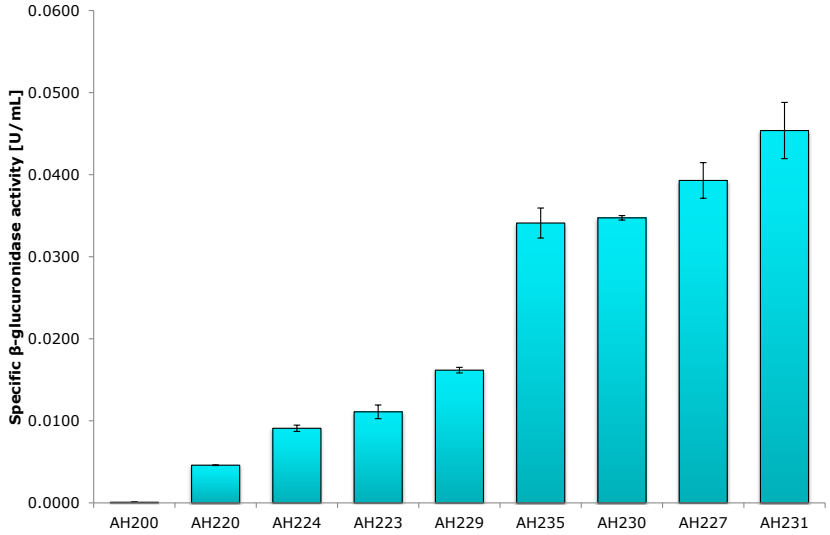

Figure 13.6: Results from promoter strength assay on strains expressing NADH oxidase activity using *beta*-glucuronidase as a reporter gene. The strains have a single copy from an SPL library integrated on the chromosome using the vector pLB85. AH200 is the reference strain *Lactococcus lactis* LB436 with an empty pCS574 vector integrated on the chromosome.

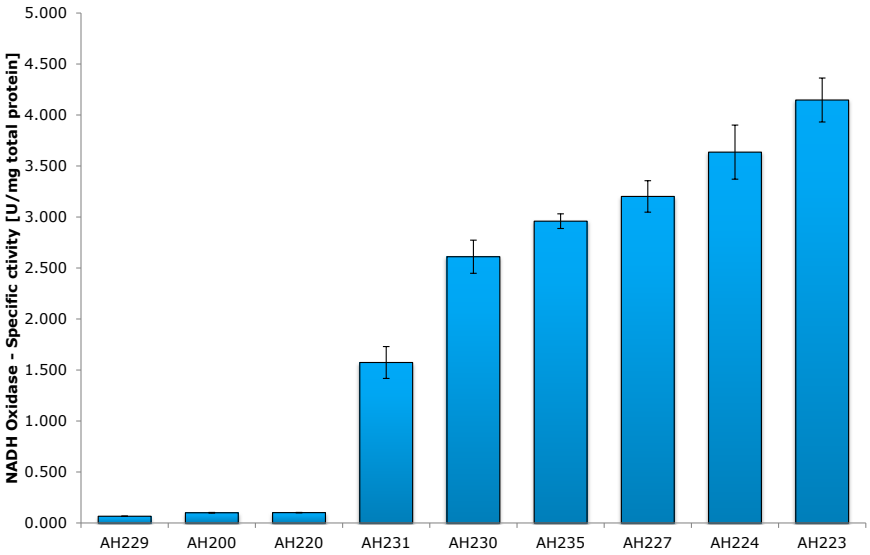

Figure 13.7: Results from NADH oxidase assay on strains expressing NADH oxidase activity. The strains have a single copy from an SPL library integrated on the chromosome using the vector pLB85. AH200 is the reference strain *Lactococcus lactis* LB436 with an empty pCS574 vector integrated on the chromosome.

To verify the activity of the constructs they were subjected to a NADH oxidase assay. The results of this can be seen in figure 13.7. The reason for lack of correlation between the promoter strength measurements and the NADH oxidase assay is most likely that two different NADH oxidase genes were used in the construction of the strains. Both *L. lactis* own gene, *noxE*, and the *nox* gene from *Streptococcus pneumoniae* were used. Following the enzymatic assay the strains were tested for glycerol metabolization. None of the strains with increased NADH oxidase activity showed signs of growth on defined medium supplemented with glycerol.

Chapter 14

Discussion

Several recombinant cloning strategies were initiated to solve the potential challenges faced by glycerol dissimilation in *Lactococcus lactis*. Initially the construction of a strain overexpressing the glycerol kinase gene *glpK* from *L. lactis* was investigated. This was done on the basis that initial transcriptomic analysis showed that glycerol kinase and dihydroxyacetone kinase were downregulated during growth on glucose, maltose and galactose.

Although several rounds of cloning and multiple clones were constructed, none were shown to possess increased glycerol kinase activity, when compared to the reference strain. It was later learned that glycerol kinase activity is regulated by HPr and the PTS system. The cells used in the glycerol kinase assay were harvested from static cultures, grown on glucose. These might not be the best conditions for a glycerol kinase activity assay, yet the sample with the highest activity was the reference strain, which had been supplemented with glycerol during cultivation and harvest.

14.1 Design and introduction of a complete recombinant pathway for glycerol assimilation into *Lactococcus lactis*

The artificial operon created for glycerol assimilation was transformed into *Lactococcus lactis* as a synthetic promoter library. Similar to the overexpression of glycerol kinase, characterization of the constructed clones revealed few with expression levels of glycerol dehydrogenase activity or dihydroxyacetone kinase activity above the level of the reference strain. No assay was done for the activity of the *glpF* gene encoding the glycerol facilitator.

From the results so far, there is still much to be done to construct and verify a fully operational operon. There have been many setbacks under the construction. It is not possible to entirely rule out that perhaps the original design of the artificial operon could be flawed in some way or that

there are other places where errors could have ocured, such as during the synthesis of the operon or in the codon optimization. An alternative could be to construct a similar operon or another construct with native genes from *Lactococcus lactis*. If this is done, great care has to be taken in order to first make sure the components are themselves functional.

Overexpression of the transcriptional regulators *dhaS* (transcriptional activator) and *dhaQ* (co-activator) might also provide a route to increased enzymatic activity for dihydroxyacetone kinase since the system in *L. lactis* functions as an activator rather than repressor [112].

14.2 Overexpression of NADH oxidase activity is not enough to allow growth of *Lactococcus lactis* on glycerol as sole carbon and energy source

The construction of strains overexpressing NADH oxidase was completed and a selection of clones were characterized by enzymatic assays. They displayed a range of different NADH oxidase activity levels, albeit to the lower side.

The strains were not able to grow in defined medium supplemented only with glycerol, indicating that the redox problem was not the only thing preventing the strains from metabolizing glycerol. Now that a selection of strains with NADH oxidase activity have been generated it would be a logical next step to combine these with the other constructs under way.

Since the additional mutants are still being constructed, initial work might focus on generating strains with higher levels of NADH oxidase activity. One might also explore the possibility of utilizing alternative electron acceptors such as fumarate to possibly develop anaerobic systems for future use.

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Part IV

Investigation of *Lactococcus lactis* strains with perturbed ATP levels

Chapter 15

Introduction

Working from the assumption that flux control does not necessarily reside within the metabolic pathways, the focus of metabolic engineering has shifted towards the role played by the redox and energy cofactors. The work of Koebmann *et al.* [165] showed that glycolysis in *E. coli* is mostly controlled by the demand for ATP but also that the degree of control can be highly specific to both organism and growth conditions [39]. Another study involving redox and energy cofactors was done by Vemuri *et al.* [179] where levels of NADH were reduced, resulting in decreased acetate production and biomass yield. Furthermore, indications of increased flux through glycolysis and the TCA cycle suggests that reducing the level of NADH has similar effects, as those observed by Koebmann *et al.* [165]. These observations lend further support to the theory that control of glycolytic flux might lie outside the pathway itself, perhaps governed by demand for global redox and energy cofactors. The interesting observation that glycolytic flux in exponentially growing *Lactoccus lactis*, an organism that relies solely on glycolysis as a source of ATP, does not increase as a consequence of ATP hydrolysis still puzzles today. This was the main reason behind reinvestigating the strains constructed by Koebmann *et al.* 2002. The idea was to utilize the advances in analytics and new high throughput methods, not available at the time of the original paper, to look a little deeper into the inner workings of the strains with increased expression of ATPase activity.

15.1 Investigations into glycolytic flux control in *Lactococcus lactis*

One way to determinine if a given enzymatic step in a pathway is flux limiting, is to overexpress that given enzymatic activity and record the effect. In order to determine the control of glycolytic flux in *L. lactis* the effect of overproducing most enzymes in glycolysis has been investigated: Phosphofructo kinase (Koebmann *et al.* 2005 [162]), Triosephosphate isomerase (Solem *et*

al. 2005 [175]), Glyceraldehyde-3-phosphate dehydrogenase (Solem *et al.* 2003 [174]), Phosophoglycerate mutase (Solem *et al.* 2010 [177]), Phosphoglycerate enolase (Koebmann *et al.* 2006 [163]), Pyruvate kinase (Koebmann *et al.* 2005 [162], Ramos *et al.* 2004 [172]) and Lactate dehydrogenase (Andersen *et al.* 2001 [152]). The conclusion was that no one enzyme showed signs of controlling flux at wild type levels of enzymatic activity and a significant reduction in enzymatic activity was required to affect the flux. The remaining enzymes showed a similar response [Christian Solem, unpublished]. Even a strain in which the activity of all the enzymes were overproduced to twice the level of the wild type did not show any increase in flux, indicating that control was not shared between all the enzymes [Christian Solem, unpublished]. This pointed in the direction of the remaining factors governing flux control such as energy demand and transport processes such as sugar uptake or lactic acid export.

15.1.1 The effect of ATP demand on glycolytic flux

In the light that no single enzyme appeared to have control over glycolytic flux Koebmann *et al.* set out to investigate the effect of energy demand. To disrupt the level of ATP in the cell, a system which is based on the ATP synthase complex, responsible for energy generation in both eukaryotic and prokaryotic cells was developed. The complex consist of two parts: A membrane bound part (F_0) and a cytoplasmic part (F_1). The cytoplasmic part of the ATP synthase complex is capable both of generating but also of hydrolyzing ATP in prokaryotic cells [182]. The soluble F_1 part of the ATP synthase complex contains the catalytic sites for ATP synthesis or hydrolysis and can remain active without the F_0 part. Expression of the genes *atpA*, *atpD* and *atpG* (*atpADG*), located in the atp operon encoding the F_1 part of the ATP synthase complex therefore represents an easy way of introducing an ATP hydrolysing process into the cell, which simply removes ATP without affecting any other parts of metabolism [165, 182].

Overexpressing F_1 in *L. lactis* did not results in any increase in glycolytic flux, as mentioned previously. When applying the same system in resting cells the flux increased and approached the level in exponential growing wild type cells but never surpassed this. Hence energy demand does not seem to control the glycolytic flux in *L. lactis*.

15.2 Experimental setup

The main idea behind the reinvestigation was to combine data from both transcriptional analysis and information on the levels of corresponding internal metabolites. This combination could hopefully uncover new clues which might point to why control of the glycolytic flux of *L. lactis* remains so elusive. Furthermore, the availability of a library expressing different

levels of ATPase activity meant that strains could be selected such that the pertubation was as minimal as possible but still allowed a significant effect to be studied. Every time a perturbation is introduced into a system there is a danger that additional pleiotropic effects are introduced if the perturbation level is too severe. By carefully selecting strains with low but defined levels of perturbation it would allow the transcriptomic studies to filter out these effects leading to more robust biological results but would of course increase the demands on the data analysis.

The experimental setup used in the following study includes four strains from the previous study by Koebmann *et al.* [164]: BK1010, BK1506, BK1503 and BK1502. The strains are listed in increasing order of ATPase activity (BK1010 being the reference strain, REF (containing the empty promotorless vector pAK80)).

The selected strains were investigated with respect to two levels of analysis: transcriptomic profilling and metabolomic profilling. The strain with the highest degree of perturbation (BK1502) was only used for metabolomic analysis, as a positive control, since this level of perturbation would most certainly have a profound effect on the metabolism of the affected cells. No transcriptional samples were taken from this strain since the goal was to utilize strains with as little perturbation (but still with a significant effect) as possible to reduce noise and pleiotropic effects.

Chapter 16

Materials and Methods

16.1 Bacterial strains, plasmids and growth conditions

The bacterial strains used in this part of the project are listed in table 16.1.

Strain	Description	Source or Reference
L. lactis strains		
BK1010	*L. lactis* MG1363 transformed with pAK80, Erm^r	[164]
BK1503	*L. lactis* MG1363 transformed with pCPC4::*atpAGD*, Erm^r	[164]
BK1506	*L. lactis* MG1363 transformed with pCPC7::*atpAGD*, Erm^r	[164]
BK1502	*L. lactis* MG1363 transformed with pCPC3::*atpAGD*, Erm^r	[164]
Plasmids		
pAK80	Promoterless vector carrying the *lacLM* genes encoding the reporter enzyme β-galactosidase, Erm^r	[160]

Table 16.1: An overview of the bacterial strains and plasmids used in part IV of the project. Erm^r - Erythromycin resistance.

Lactococcus lactis strains were cultivated in the defined medium SAL, as described in section 8.1.1. Inoculum for growth experiments were incubated over night with selective pressure (5 μg/mL Erythromycin) at 30°C. Growth experiments were carried out in 100 mL flasks filled with 100 mL of SAL medium supplemented with 0.2% glucose. No antibiotics were added to the growth experiment cultures to minimize noise in the microarray samples (antibiotics were added to over night culture to ensure strain stability). Cultivation was performed under static conditions in a Julabo SW-20C type waterbath set to 30°C without agitation. Cultures were kept in suspension by slow stirring at 300 rpm using 5mm magnets. Optical density was measured at 600 nm (OD_{600})on a Shimadzu UV mini 1240 spectrophotometer.

16.2 High-pressure liquid chromatography analysis

Sampling for high-pressure liquid chromatography (HPLC) analysis consisted of about 1.5 mL of sample withdrawn from the growing cultures at different OD600 values (approx. 0.1, 0.2, 0.3,...). The sample was immediately filtered through a 0.22 μm filter and stored at -20°C until it was analyzed. The concentrations of glucose, lactate, formate and acetate were determined by HPLC equipment from Shimadzu Corporation, Kyoto, Japan, the system was controlled by the program class VP 5.0. Separation was performed with a Bio-Rad HPX-87H heated to 30°C. A securityGuard cartridge system (Phenomenex) with a Carbo-H cartridge (Phenomenex) was used to protect the HPLC column. The mobile phase consisted of 5 mM H_2SO_4, with a flow rate of 0.5 mL/min and all products were detected on the Shodex refractive index detector, RID-10A. [153]

16.3 RNA purification

Purification of RNA was performed using the QIAGEN RNAprotect™bacterial reagent (cat. no. 76506) and the QIAGEN RNeasy Mini Kit (cat. no. 74104). Cells were treated with RNAprotect™before proceeding with purification using the RNeasy Mini Kit. Two samples of 10 ml were taken during the growth experiment at OD600≈0.5 and mixed with 20 ml RNAprotect™. Cells were harvested after 10 min incubation at room temperature by centrifugation at 2400 g for 15 min. Pellets were stored at -80°C for later purification of RNA.

RNA extraction protocol: To extract RNA the cell pellet was lysed using mechanical disruption in a modified version of protocol 3 and purified according to protocol 7 of RNAprotect™Bacteria Reagent Handbook. The pellet was resuspended in 700 μL RLT and transferred to safelock tubes containing 0.5 ml acid washed glass beads (106 microns acid washed and finer (G-4649) from Sigma). The cells were then disrupted in FastPrep™-24 by three rounds of 4.0 m/s for 45 s with 2 min of rest on ice in between. Afterwards samples were centrifuged at 15.000 g in 20 s, and the supernatant was supplemented with 70% ethanol (1:1). The purifcation on column was done according protocol 7 with final elution in RNAse free water.

RNA quantification and quality assessment: RNA was quantified and its purity estimated using Tecan infinite m200 pro with a NanoQuant plate™and Magellan 7 software. Absorbance at 260nm was utilized for quantification using extinction coefficient $\epsilon = 25\ \mu L\ \mu g^{-1}\ cm^{-1}$ and Lambert-Beers law $A = \epsilon \cdot c \cdot l$. For assessment of purity A260/A280 and A260/A230 were measured. Further assessment of RNA degradation and general quality was done with Agilent Bioanalyzer according to manufacturer's protocol. RNA Integrity Number (RIN) was inspected to evaluate the RNA quality.

16.4 DNA microarray analysis

Microarray handling: The Microarray analysis was handled by DTU Multiassay Core (DMAC), using an Agilent chip with all known and putative genes of *L. lactis* MG1363. Each gene was tested with five different probes, but occasionally less than five probes per gene existed. RNA was labeled with one color, yielding one signal from each microarray chip.
Software analysis: The software used to analyse the transcriptomic data was the statistical software package R and the Bioconductor suite of available packages [157] (in particular the packages *limma* [173], *oligo*[155] and *affy*). A complete overview of the commands used can be found in appendix F.

16.5 Carbon labeling experiments

Carbon flux analysis was performed using two different combinations of labeled sugar: 1) 100% ^{13}C-1 glucose and 2) 80% ^{13}C-6 glucose (fully labeled) combined with 20% regular glucose. Both O/N cultures and growth experiments were prepared using these combinations. Two samples of 3 ml were harvested from exponentially growing cells at OD600≈0.4. Medium was removed by centrifugation (2500 g, 10 min), the pellet was washed with 2 ml of 0.9% NaCl, centrifuged again (7000 g, 2 min) and the cell pellet stored at -80°C for later processing. When all samples were ready the pellets were resuspended in 150 μl 6M HCl and the samples were then placed at 105°C for 24 hours and subsequently dried in a heating block (placed in a fume hood) at 85°C until black/brown. After drying, the samples were shipped to Dr.-Ing. Lars M. Blank, Department of Biochemical and Chemical Engineering TU Dortmund, Germany, for further analysis.

16.6 Sampling for metabolite analysis

Sampling for metabolite analysis was carried out as previously described in section 8.2.

16.7 Correlation of cell density to cell mass

Correlation of cell density to cell mass was done as previously described in section 8.3

16.8 Calculations

Calculations were performed as previously detailed in section 8.4.

Chapter 17

Results

The following section contains the results from the two levels of analysis performed on the BK strains expressing ATPase activity. The underlying idea was to integrate data from both metabolome measurements and transcriptome level analysis into an overall framework.

17.1 Physiological characterization of strains with varying degrees of ATPase activity

To make sure that the results from these experiments correlate with the previous findings an initial comparison of the physiological data obtained in the growth experiments was done. The growth rates for each of the four strains from the experiments with sampling for transcriptome and metabolome samples respectively can be seen in table 17.1. The original values reported by Koebmann et *al.* are listed as well.

Strain	Metabolomic samples μ [h^{-1}]	Transcriptomic samples μ [h^{-1}]	Koebmann et *al.* 2002 μ [h^{-1}]	% of REF		
				Met.	Trans.	Koeb.
BK1010	0.81 ± 0.01	0.83 ± 0.01	0.74	100	100	100
BK1506	0.75 ± 0.01	0.81 ± 0.03	0.67	93	97	92
BK1503	0.68 ± 0.01	0.70 ± 0.01	0.58	83	84	79
BK1502	0.59 ± 0.01		0.51	72		69

Table 17.1: Growth rates from the experiments used to sample for transcriptomic and metabolomic analysis. Metabolomic and transcriptomic samples in quadruplicates except BK1502 which was done in triplicate, Koebmann et *al.* 2002 taken from [164].

Even though the specific growth rates differ slightly there is a good correlation with the results observed by Koebmann et *al.* previously when looking

at perturbation levels (% of REF). The slightly faster growth rates could be caused by the difference in medium composition (SA vs SAL medium). Notice also that the level of perturbation is less than 10% and 20% for BK1506 and BK1503 respectively.

17.2 Metabolomic profiling of strains expressing various degrees of ATPase activity

By looking at the concentrations of internal metabolites clues to the mechanisms that govern glycolytic flux control can possibly be uncovered. Samples were taken as described in materials and methods section 16.6 in order to analyze the levels of internal metabolites. The results from the analysis can be seen below. Quadruplicate samples were analyzed for the strains: BK1010, BK1506 and BK1503 while triplicate samples were analyzed for BK1502. For each replicate two different samples were taken: one sample and one cell free reference. A summary of the measured metabolite concentrations can be seen in table 17.2 and a plot is found in figure 17.1.

Strain	**G6P**	**F6P**	**F16BP**	**DHAP**	**GAP**	**PEP**	**PYR**	**RIB5P**
Metabolite concentrations in μM. Samples in triplicate/quadruplicate.								
BK1010	7.26 ± 0.85	0.56 ± 0.29	16.92 ± 4.31	3.66 ± 1.08	3.19 ± 3.04	1.12 ± 0.42	2.28 ± 2.16	0.42 ± 0.34
BK1506	8.04 ± 2.22	1.03 ± 0.18	19.24 ± 2.41	3.06 ± 0.15	1.86 ± 1.04	1.13 ± 0.21	2.04 ± 6.79	0.34 ± 0.15
BK1503	6.45 ± 2.93	1.26 ± 0.53	16.19 ± 3.89	2.69 ± 0.38	0.41 ± 2.25	0.97 ± 0.14	2.78 ± 7.11	0.15 ± 0.06
BK1502	7.93 ± 2.17	1.11 ± 0.10	17.59 ± 1.54	2.53 ± 0.18	0.35 ± 0.34	1.07 ± 0.15	-3.54 ± 4.71	0.32 ± 0.12

Strain	**RI/XY5P**	**SEDO7**	**AMP**	**ADP**	**ATP**	**NAD**	**NADP**
BK1010	1.04 ± 0.21	0.00 ± 0.00	0.19 ± 0.03	0.94 ± 0.06	0.01 ± 0.01	4.07 ± 0.68	0.31 ± 0.11
BK1506	1.25 ± 0.28	0.00 ± 0.00	0.24 ± 0.03	1.03 ± 0.12	0.11 ± 0.15	2.87 ± 0.38	0.24 ± 0.18
BK1503	1.37 ± 0.02	0.00 ± 0.00	0.33 ± 0.10	1.26 ± 0.10	0.06 ± 0.11	2.88 ± 0.91	0.22 ± 0.06
BK1502	1.30 ± 0.11	0.00 ± 0.00	0.47 ± 0.06	1.56 ± 0.20	0.00 ± 0.00	2.46 ± 0.44	0.00 ± 0.01

Table 17.2: Overview of selected internal metabolite concentrations measured in the samples taken from mid. exponential growth phase of BK1010, BK1506, BK1503 and BK1502. G6P - Glucose-6-phosphate, F6P - Fructose-6-phosphate, F16BP - Fructose-1,6-biphosphate, DHAP - Dihydroxyacetonephosphate, GAP - Glyceraldehyde-3-phosphate, PEP - Phosphoenolpyruvate, PYR - Pyruvate, RIB5P - Ribose-5-phosphate, RI/XY5P - Ribulose-5-phosphate and xylulose-5-phosphate, SEDO7 - Sedoheptulose-7-phosphate, AMP - Adenosine monophosphate, ADP - Adenosine diphosphate, ATP - Adenosine triphosphate, NAD - Nicotineamide adenine dinucleotide, NADP - Nicotineamide adenine dinucleotide phosphate.

As evident from table 17.2 and figure 17.1 there was a lot of noise in the data set, making any conclusions from the data difficult. One of the significant results are the levels of ADP and AMP. There is a significant difference between the levels of the reference strain and the perturbed strains.

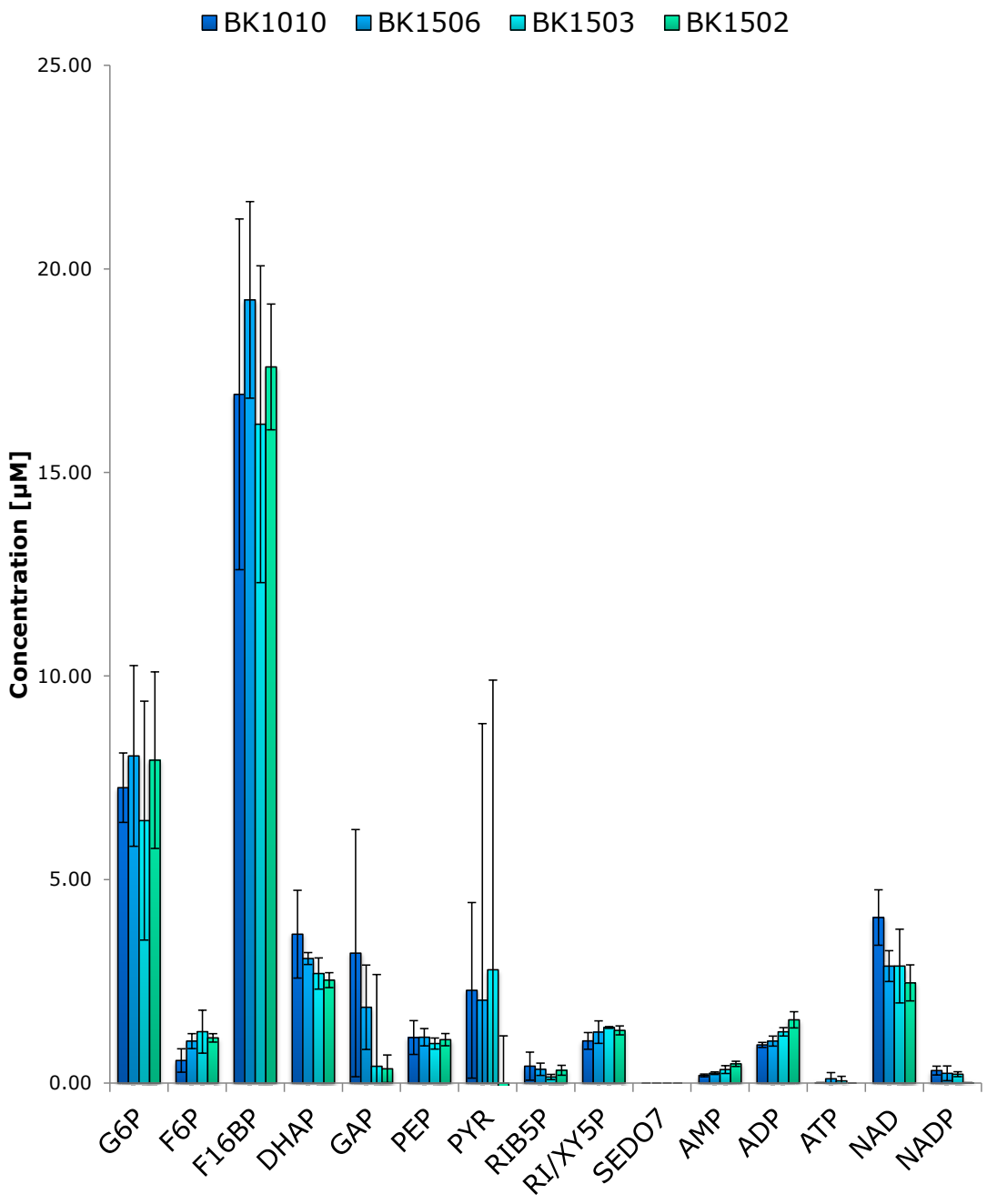

Figure 17.1: Plot of internal metabolite concentrations in samples from mid. exponential growth from ATPase overexpressing strains: BK1010 (REF, dark blue), BK1506 (LOW, blue), BK1503 (MED, turqoise) and BK1502 (HIGH, green). G6P - Glucose-6-phosphate, F6P - Fructose-6-phosphate, F16BP - Fructose-1,6-biphosphate, DHAP - Dihydroxyacetonephosphate, GAP - Glyceraldehyde-3-phosphate, PEP - Phosphoenolpyruvate, PYR - Pyruvate, RIB5P - Ribose-5-phosphate, RI/XY5P - Ribulose-5-phosphate and xylulose-5-phosphate, SEDO7 - Sedoheptulose-7-phosphate, AMP - Adenosine monophosphate, ADP - Adenosine diphosphate, ATP - Adenosine triphosphate, NAD - Nicotineamide adenine dinucleotide, NADP - Nicotineamide adenine dinucleotide phosphate.

This correlation between the ATPase activity level and the increase in concentrations of ADP and AMP indicates that the system to perturb the ATP level works. When hydrolyzing ATP the expected result would be a lowered ATP level and increased levels of AMP and ADP. This would appear to hold true for all four strains and there is a significant difference between the reference strain BK1010 and the more perturbed ATPase expressing strains BK1503 and BK1502. Unfortunately there is no information on the level of ATP. The other main observations are the level of fructose-6-phosphate (F6P) which is significantly higher in the perturbed strains and the level of NAD which is lowered in the perturbed strains.

17.3 Analysis of transcriptome levels in strains expressing ATPase activity reveals starvation like response to pertubation of ATP level

To keep the pleiotropic effects of perturbing a key energy cofactor such as ATP to a minimum, strains with low ATPase activity were chosen. For that reason only the strains BK1010 (ref), BK 1506 (low) and BK1503 (medium) were included in the growth experiments meant for transcriptome analysis. Initially twelve samples were harvested, four replicates from each strain. These were subsequently analyzed in two rounds (chip 1 and 2). Unfortunately it was not possible to compare all twelve samples together because of batch variations in the handling and processing of the chips. As a result the main analysis focused on chip 1 from which BK1010 and BK1506 were analyzed in duplicate while BK1503 was analyzed in triplicate. An overview of the entire workflow is shown in figure 17.2. The organization of the results follows the pipeline outlined in figure figure 17.2 and includes a section on each step prior to the differential gene expression analysis. A graphical summary of the results can be found in figure 17.5.

17.3.1 Data quality check and normalization

The first step in the analysis of expression data is an inspection of the obtained data from the image of the chip. One typically looks for spatial anomalies (rings or shadows) or other non-homogeneous patterns and artifacts. These issues can sometimes be corrected by normalization but may be to severe to improve. Figures from intensity plots and the raw chip images can be found in supplementary material, section S.1.

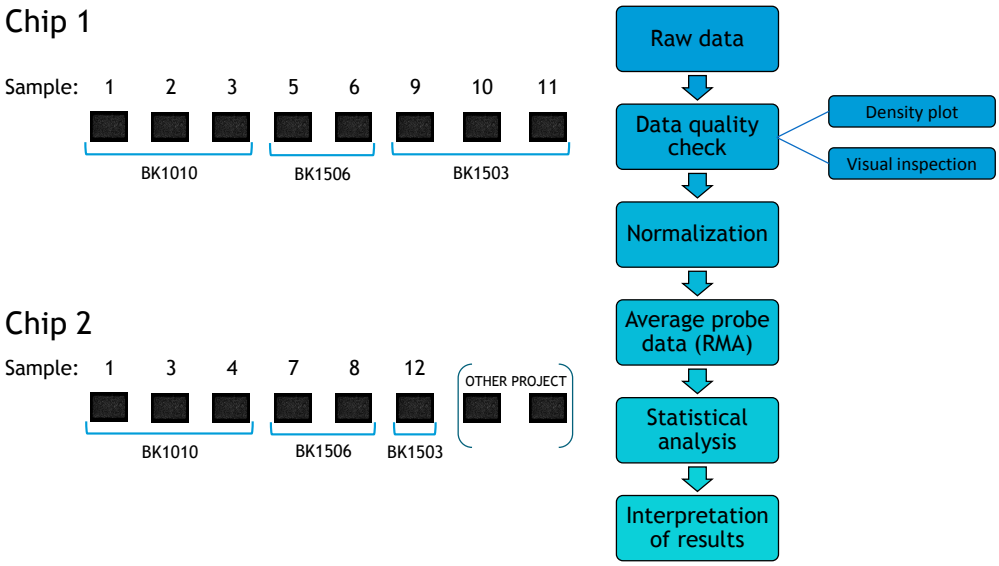

Figure 17.2: A schematic overview of the workflow in the analysis of microarray samples from the ATPase perturbed strains, BK1010, BK1506 and BK1503. To the left are the samples analyzed in each round and to the right a short flowchart describing the steps involved in the data analysis.

The visual inspection of chip 1 and 2 raw images reveals artifacts and clear differences between them

A visual inspection of the chip 1 image (supplementary material, section S.1, page 2) shows that sample 1 has what looks like a very faint ring pattern in the middle of the array. This is very faint and can hopefully be compensated for by the spatial distribution of the probes. Also sample 3 looks slightly darker than the other samples. This may affect the quality of the data but can perhaps also be compensated for by normalization if the biological information is retained.

Looking at the image of chip 2 (supplementary material, section S.1, page 3) the samples look nicely uniform. The intensity of sample 4 looks slightly below the rest but this should be compensated during normalization. What is also evident is a clear example of something affecting quality of sample 7 of the microarray (row 2 sample 3 from the left). One would not expect that gene expression levels in a sample would naturally form a ring shaped pattern (of course depending on the sample being analyzed). This was not a sample from this project but it illustrates that events during the preparation and handling of the microarray can affect the quality of the expression data quite considerably.

Singular value decomposition analysis shows clear signs of batch effects between arrays

Since the transcriptomic data has been generated by two different arrays one would not be surprised to find additional noise added by the separate runs. When including the data set from chip 2 into the analysis, robustness of the data actually went down (i.e. became less significant). More noise than signal was added by inclusion of the additional replicates. A heatmap (figure 17.3) of all the samples provided a clue to why this might be the case. As shown in the singular value decomposition (SVD) analysis, the most significant component appears to be the chip number. The samples clearly segregate into two groups based on the chip number. This makes an analysis of all samples together more complicated as the chip specific noise has to be removed. So far this has not been accomplished and no further investigations were made on the entire data set of 12 samples. Instead the analysis focused on chip 1 instead.

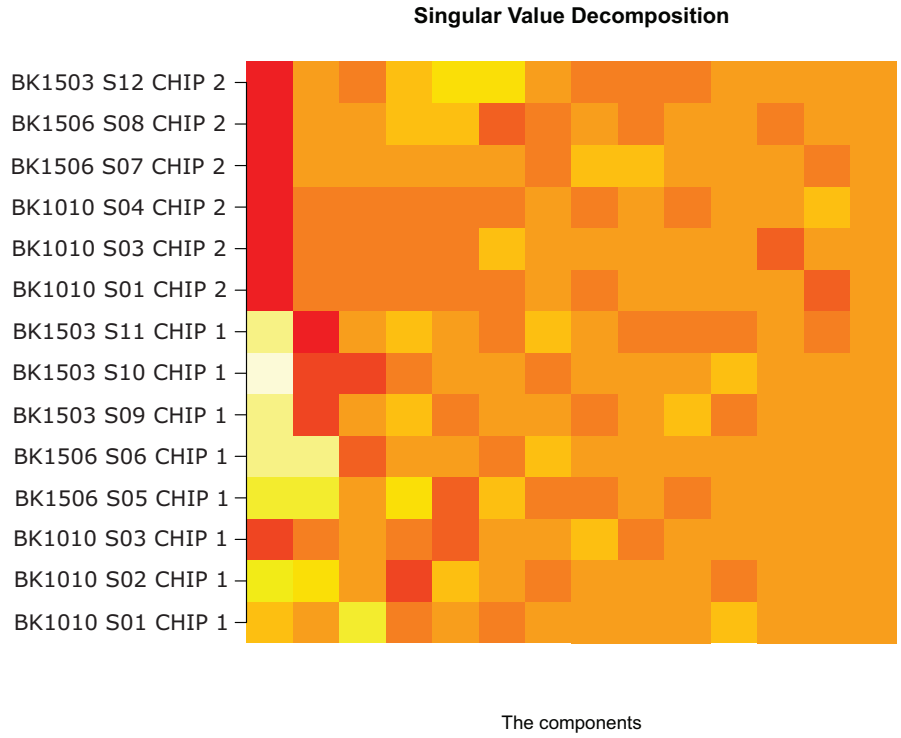

Figure 17.3: A heatmap based on SVD for all the samples. The samples clearly fall into two groups based on chip number.

Sample 3 on chip 1 is discarded based on intensity distribution

As was seen in the analysis of the raw image files, sample 3 (BK1010) on chip 1 looked darker than the other two samples from BK1010. By plotting

the intensity distribution for all the samples of chip 1 after normalization (figure 17.4) it was clear that sample 3 deviated from the remaining two.

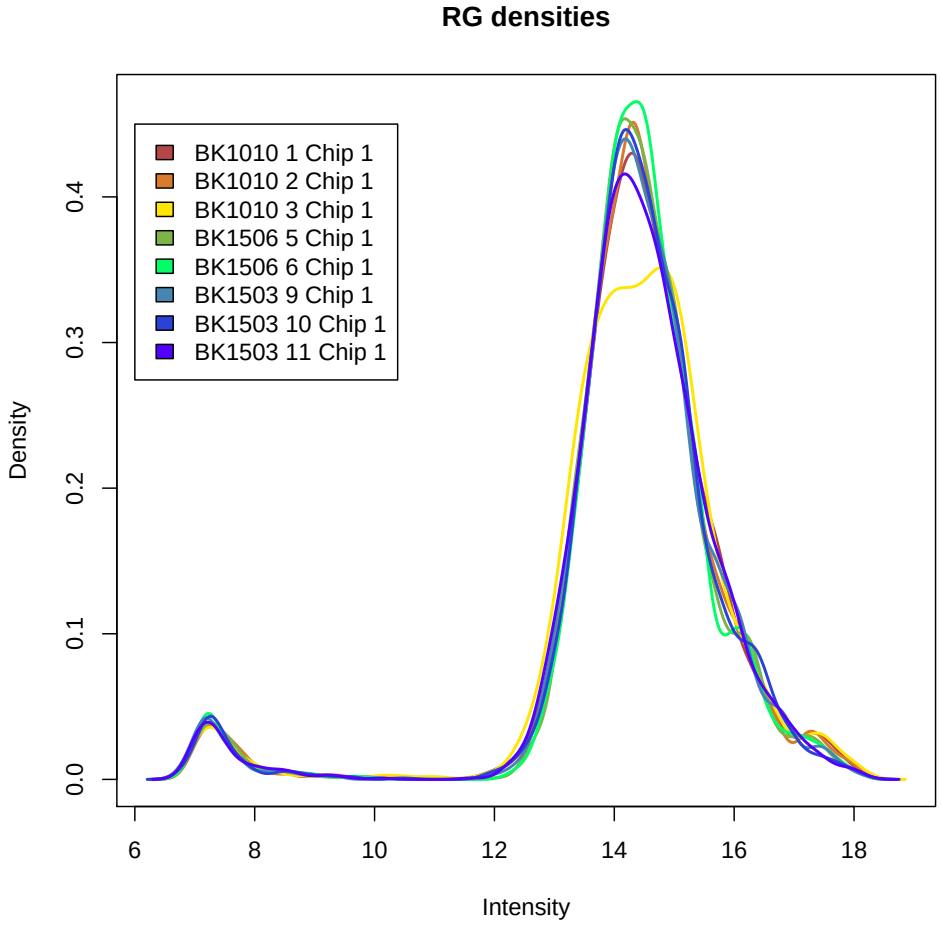

Figure 17.4: A plot of intensity data for each of the eight samples on the first chip after normalization. Sample 3 (yellow) has very different profile compared to the rest which indicates the darker appearance on the image file could not be corrected by normalization.

In order to see how different sample 3 was from the rest, a SVD analysis was made to visualize the variance captured by the different components. The analysis tries to capture the contribution of the different components to the variance in the samples. A plot of the results can be seen in the supplementary material (section S.1 page 11 and 12). Based on the intensity distribution plot, the heatmap and the plot of the SVD, sample 3 was omitted from the dataset for the analysis.

Estimation of false detection rate for BK1506 and BK1503 as an indicator for robustness of the data set

A t-test supplies a single p-value for each gene when two groups of samples are compared. The p-value signifies the probability that the difference would occur by random and the lower a p-value, the more confident is the hypothesis that the two values actually do differ in expression under the assumption that the p-values follow a normal distribution. When comparing large sets of expression data, the many tests performed will mean that some will be significant by chance. This can be avoided either by adjusting the p-values (Bonferroni) or by estimating the false detection rate (FDR).

A way to visualize the false detection rate is done by plotting a volcano plot in which samples are grouped together in random groups and the p-value is plotted against the $\log_2$ fold change (FC). This is best achieved when there is a balanced (even) set of samples from each group, but can also be done on an unbalanced set of samples such as the one in this study. For the data obtained from chip 1 the uneven number of samples in the BK1503 group (three compared to two in BK1010 and BK1506) means the random permutations will be biased by the inclusion of an additional sample from the BK1503 group.

Nevertheless one can still construct a volcano plot from these pseudo-balanced permutations. The plots can be seen in the suppplementary material (section S. 1, page 9 and 10). The volcano plots indicate that p-values above a threshold of 0.0024 (BK1506) and 0.0016 (BK1503) could be false positives. This is not a true FDR but can give a measure of the FDR to keep in mind when viewing the results of differential gene expression analysis.

17.3.2 Normalization and averaging of probe data using robust multi-array averaging

The final step before performing an analysis of differential gene expression is the averaging of probe level data into one value for each gene. Most have 4–6 probes but many such as the pseudo genes have just one. For identical probes in different locations on the array, all are included in the analysis and duplicate probes are treated as being unique. Pseudo genes and other genes with less than 4 probes have not been included in the analysis.

The data set was normalized between arrays using a quantile algorithm such that all samples follow an average density distribution, after which the probe level data was averaged into a single value for each gene using Robust Multi-array Averaging (RMA) [159].

17.3.3 Transcriptome level analysis of *Lactococcus lactis* strains displaying varying degrees of ATPase activity

There are several ways of looking at a transcriptomic data set such as the one currently under investigation. Typically a list of the most differentially expressed genes will the first thing one looks at. This would show which genes were most affected by the differential conditions tested. This experiment was designed to deliberately minimize the perturbation effect of ATPase activity on the cells to minimize any pleiotropic effects that might otherwise affect the results. This also has the effect that the difference in response between the mutants and the wild type becomes less pronounced than if we had used the BK1502 strain with a very high ATPase activity. So just looking at the lists of top regulated genes may not be the best approach. Rather a more pattern oriented approach that is looking at the response of whole pathways or families of genes instead of just looking at the top up- or down regulated genes might be more interesting. Since both approaches have their strengths results from both are presented in the following section.

The most heavilly up- and downregulated genes indicate signs of stress in BK1503 but not in BK1506

The two mutant strains BK1506 and BK1503 had different levels of ATPase activity and hence also different perturbation levels. BK1506 had the lowest perturbation ranking at less than 10% (growth rate) and BK1503 was perturbed at around 16-17% (growth rate). Lists with the 50 most up and downregulated genes can be viewed in table 17.3 and table 17.4 for BK1506 and in table 17.5 and 17.6 for BK1503.

Upregulated genes show the ATPase system to be working in both strains A convenient control that the ATPase system is working is the appearance of the *atpAGD* genes among the top ranked upregulated genes in both BK1506 and BK1503. The response by BK1503 is interestingly showing signs of stress response by the presence of *sodA* (superoxide dismutase) and *uspA* (universal stress protein A) among the top upregulated genes. These are not among the top upregulated genes in BK1506. Even though BK1503 is the more perturbed strain the level of perturbation may already be sufficient to induce pleiotropic effects at this level. Components involved in glucose transport are also present in the most upregulated genes in both strains, namely *celB* for BK1506 and *ptcA* and *ptcB* for BK1503, all part of the cellobiose PTS. Finally the presence of a global regulatory gene (*codY*) among the top regulated genes in BK1503 would suggest a global response to perturbation in ATP levels.

BK1506 - Top 50 upregulated genes

Gene/locus	LogFC	p-value	Annotation
llmg_1460	1.66	1.8E-06	putative di/TRI-peptide transport ATP-binding protein
atpG	1.36	4.8E-07	F0F1 ATP synthase subunit gamma
celB	1.34	6.3E-06	cellobiose-specific PTS system IIC component
atpD	1.27	1.5E-06	F0F1 ATP synthase subunit beta
atpA	1.24	2.0E-06	F0F1 ATP synthase subunit alpha
llmg_0258	1.22	1.2E-05	hypothetical protein
llmg_1458	1.21	4.7E-04	putative di/TRI-peptide transport system permease protein
llmg_0186	1.19	8.9E-06	hypothetical protein
llmg_1166	1.09	5.7E-03	putative endoglucanase
llmg_1457	1.06	6.3E-03	putative di/TRI-peptide transport ATP-binding protein
llmg_1165	1.00	2.1E-02	hypothetical protein
llmg_1459	0.99	2.7E-05	putative di/TRI-peptide transport system permease protein
llmg_1619	0.99	4.7E-04	hypothetical protein
llmg_1167	0.96	5.7E-03	putative endoglucanase
tagG	0.96	6.9E-04	teichoic acid ABC transporter permease protein
llmg_1824	0.95	4.6E-03	hypothetical protein
ps124	0.94	3.0E-04	hypothetical protein
dppC	0.92	3.7E-05	dipeptide transport system permease protein DppC
ps453	0.92	4.3E-04	phage tail component
uxuT	0.92	4.1E-02	Na-galactoside symporter
llmg_1164	0.91	3.3E-02	hypothetical protein
llmg_1127	0.90	8.8E-03	cell wall surface anchor family protein
llmg_1620	0.89	9.7E-05	hypothetical protein
llmg_1461	0.89	8.1E-05	putative di/TRI-peptide binding protein precursor
lplC	0.88	3.5E-02	sugar ABC transporter permease
llmg_0470	0.88	3.3E-03	hypothetical protein
maa	0.87	1.2E-03	maltose O-acetyltransferase
llmg_0921	0.86	1.6E-03	putative secreted protein
llmg_1128	0.86	1.2E-02	hypothetical protein
ulaA, sgaT	0.85	3.9E-03	PTS system ascorbate-specific transporter subunit IIC
leuC	0.84	2.5E-02	isopropylmalate isomerase large subunit
ps454	0.84	2.1E-03	hypothetical protein
llmg_0487	0.83	3.7E-04	putative trehalose/maltose hydrolase
llmg_0489	0.82	8.4E-04	sugar transport system permease protein
llmg_0922	0.81	2.4E-03	putative secreted protein
llmg_1618	0.81	1.7E-03	hypothetical protein
llmg_1393	0.81	5.3E-02	hypothetical protein
hadL	0.80	5.7E-04	cryptic haloacid dehalogenase 1
llmg_1283	0.79	7.7E-02	hypothetical protein
ps515	0.78	2.8E-03	phage protein by Glimmer/Critica
llmg_0479	0.77	6.5E-03	hypothetical protein
galP	0.76	7.1E-02	galactose permease
ps514	0.76	4.6E-04	phage protein by Glimmer/Critica
dppD	0.76	2.6E-04	dipeptide transport ATP-binding protein DppD
polC	0.76	1.1E-04	DNA polymerase III PolC
ps457	0.75	5.1E-03	serine/threonine-rich protein precursor
ps511	0.75	3.2E-04	hypothetical protein
aguA	0.74	8.3E-04	AguA protein
llmg_0963	0.73	8.7E-03	PTS system, IIC component
llmg_2513	0.73	2.8E-03	putative transport protein
ps517	0.73	5.2E-04	putative DNA primase

Table 17.3: The 50 most differentially upregulated genes in BK1506 (p=0.01).

BK1506 - Top 50 downregulated genes

Gene/locus	LogFC	p-value	Annotation
pbuO	-0.73	1.5E-04	xanthine/uracil/vitamin C permease
tig	-0.70	3.1E-04	trigger factor
llmg_1359	-0.67	2.3E-03	hypothetical protein
llmg_1230	-0.66	1.4E-03	hypothetical protein
pstA	-0.65	5.1E-03	phosphate transporter ATP-binding protein
dfrA	-0.64	1.3E-03	DfrA protein
tmk	-0.64	5.7E-03	thymidylate kinase
uvrC	-0.64	1.5E-03	excinuclease ABC subunit C
nagA	-0.64	2.9E-02	NagA protein
llmg_0760	-0.64	7.9E-03	putative transglycosylase
grpE	-0.63	7.7E-04	heat shock protein GrpE
llmg_1366	-0.63	2.1E-03	hypothetical protein
llmg_2395	-0.63	3.1E-03	hypothetical protein
murA2	-0.63	7.7E-04	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
llmg_1252	-0.62	1.1E-02	hypothetical protein
llmg_1347	-0.62	1.8E-03	hypothetical protein
llmg_0873	-0.61	1.1E-03	putative (di)nucleoside polyphosphate hydrolase
llmg_1362	-0.61	1.3E-02	hypothetical protein
llmg_1348	-0.61	5.6E-03	hypothetical protein
pyrF	-0.60	1.8E-03	orotidine 5'-phosphate decarboxylase
llmg_1645	-0.60	3.2E-03	general stress protein GSP13
llmg_1360	-0.59	5.8E-03	hypothetical protein
llmg_0218	-0.59	6.5E-03	putative glycosyl transferase
llmg_1475	-0.59	7.7E-03	hypothetical protein
mutS	-0.59	2.0E-03	DNA mismatch repair protein MutS
llmg_0599	-0.59	2.2E-03	hypothetical protein
dtpT	-0.59	6.2E-03	di-/tripeptide transporter
ps205	-0.57	1.4E-02	cI-like repressor
purQ	-0.57	3.6E-03	phosphoribosylformylglycinamidine synthase I
glyQ	-0.57	2.3E-02	glycyl-tRNA synthetase subunit alpha
llmg_1115	-0.57	6.5E-03	XpaC-like protein
llmg_1708	-0.56	3.7E-03	putative glycosyltransferase
llmg_1026	-0.56	8.5E-03	putative methyltransferase
ps303	-0.55	1.1E-03	hypothetical protein
bmpA	-0.55	7.1E-03	basic membrane protein A
llmg_1550	-0.54	1.2E-03	hypothetical protein
ftsL	-0.54	2.3E-03	cell division protein
ps203	-0.54	2.4E-02	hypothetical protein
fabH	-0.54	1.2E-02	3-oxoacyl-(acyl carrier protein) synthase III
recX	-0.53	2.8E-02	recombination regulator RecX
atpH	-0.53	1.4E-02	F0F1 ATP synthase subunit delta
nusB	-0.53	1.1E-02	transcription termination protein NusB
llmg_0315	-0.53	4.7E-03	phosphonate ABC transporter permease
llmg_2426	-0.52	5.3E-02	hypothetical protein
potD	-0.51	6.8E-03	spermidine/putrescine ABC transporter substrate-binding protein
def	-0.51	4.5E-03	peptide deformylase
deoD	-0.51	1.2E-02	purine nucleoside phosphorylase
ldh	-0.51	1.5E-02	L-lactate dehydrogenase
dnaB	-0.51	3.5E-03	chromosome replication initiation / membrane attachment protein
pgmA	-0.51	4.9E-03	alpha-phosphoglucomutase
rpmD	-0.51	2.6E-02	50S ribosomal protein L30

Table 17.4: The 50 most differentially downregulated genes in BK1506 (p=0.01).

BK1503 - Top 50 upregulated genes

Gene/locus	LogFC	p-value	Annotation
sodA	1.40	1.1E-05	SodA protein
atpG	1.38	1.9E-07	F0F1 ATP synthase subunit gamma
mtsA	1.22	6.3E-07	manganese ABC transporter substrate binding protein
atpA	1.11	2.4E-06	F0F1 ATP synthase subunit alpha
mtsC	1.09	2.3E-06	manganese transport system membrane protein
atpD	1.08	2.9E-06	F0F1 ATP synthase subunit beta
dnaN	1.05	2.5E-06	DNA polymerase III subunit beta
llmg_0165	1.04	2.7E-06	hypothetical protein
ptcA	1.00	1.1E-04	cellobiose-specific PTS system IIA component
pgmA	1.00	1.9E-05	alpha-phosphoglucomutase
llmg_0601	0.99	7.7E-05	putative secreted protein
pmi	0.98	4.0E-05	mannose-6-phosphate isomerase
yfiA	0.97	6.2E-06	putative sigma 54 modulation protein
llmg_2145	0.97	2.5E-04	hypothetical protein
msrA	0.95	6.9E-04	MsrA protein
llmg_1203	0.95	2.1E-05	ABC transporter ABC binding and permease protein
rex	0.92	7.3E-05	redox-sensing transcriptional repressor REX
llmg_0602	0.92	8.0E-05	hypothetical protein
thyA	0.92	7.2E-06	thymidylate synthase
llmg_0585	0.92	7.5E-05	hypothetical protein
llmg_1135	0.90	8.0E-05	hypothetical protein
vacB2	0.89	1.1E-04	putative exoribonuclease R
mtsB	0.87	1.7E-04	manganese ABC transporter ATP binding protein
era, bex, rbaA,	0.86	1.0E-03	GTP-binding protein Era
hslB	0.86	6.6E-05	HU-like DNA-binding protein
uspA	0.85	2.3E-04	universal stress protein A
codY	0.84	1.5E-04	transcriptional repressor CodY
llmg_0146	0.84	3.0E-05	aryl-alcohol dehydrogenase
llmg_1214	0.84	1.9E-04	hypothetical protein
llmg_2146	0.83	2.0E-04	hypothetical protein
ptcB	0.83	1.1E-03	cellobiose-specific PTS system IIB component
llmg_0600	0.83	3.2E-05	glycosyl transferase
ppiA	0.82	1.5E-05	peptidyl-prolyl cis-trans isomerase, cyclophilin-type
llmg_0152	0.80	8.9E-05	hypothetical protein
msmK	0.80	1.8E-04	multiple sugar-binding transport ATP-binding protein
llmg_2323	0.80	3.4E-05	hypothetical protein
nagD	0.80	3.4E-05	putative N-acetylglucosamine catabolic protein
llmg_0184	0.80	5.7E-05	putative lactoylglutathione lyase
llmg_0726	0.80	2.4E-03	hypothetical protein
llmg_1667	0.79	1.5E-04	glycosyltransferase
llmg_0242	0.79	8.6E-04	hypothetical protein
aroF	0.79	1.5E-04	phospho-2-dehydro-3-deoxyheptonate aldolase
llmg_2212	0.79	3.6E-05	hypothetical protein
llmg_0584	0.78	1.7E-04	hypothetical protein
llmg_1918	0.78	2.3E-04	hypothetical protein
ps302	0.78	2.9E-05	hypothetical protein
ps303	0.78	4.2E-05	hypothetical protein
rpsD	0.78	1.6E-04	30S ribosomal protein S4
pepC	0.77	1.5E-04	PepC protein
llmg_1299	0.77	1.7E-03	hypothetical protein
llmg_0599	0.77	1.6E-04	hypothetical protein

Table 17.5: The 50 most differentially upregulated genes in BK1503 (p=0.01).

BK1503 - Top 50 downregulated genes

Gene/locus	LogFC	p-value	Annotation
purH	-1.15	2.9E-05	bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
purL	-1.10	1.8E-04	phosphoribosylformylglycinamidine synthase II
atpH	-1.09	5.7E-05	F0F1 ATP synthase subunit delta
pyk	-1.09	1.3E-05	pyruvate kinase
llmg_1794	-1.08	8.8E-06	hypothetical protein
fabZ	-1.07	5.2E-05	(3R)-hydroxymyristoyl-ACP dehydratase
pyrDB	-1.07	1.1E-04	dihydroorotate dehydrogenase 1B
accB	-1.05	8.0E-05	acetyl-CoA carboxylase biotin carboxyl carrier protein subunit
fabF	-1.05	3.4E-05	3-oxoacyl-[acyl-carrier-protein] synthase II
rpsJ	-1.01	6.0E-04	30S ribosomal protein S10
atpF	-1.01	1.5E-04	F0F1 ATP synthase subunit B
rpsE	-1.01	2.6E-05	30S ribosomal protein S5
pyrP	-1.00	3.7E-05	uracil permease (uracil transporter)
llmg_1780	-1.00	1.1E-05	hypothetical protein
carB	-1.00	4.6E-05	carbamoyl phosphate synthase large subunit
llmg_0872	-0.98	3.1E-05	putative (di)nucleoside polyphosphate hydrolase
glmS	-0.98	1.4E-05	glucosamine--fructose-6-phosphate aminotransferase
llmg_1793	-0.96	3.2E-05	hypothetical protein
rplM	-0.96	5.3E-04	50S ribosomal protein L13
rpsG	-0.95	2.0E-04	30S ribosomal protein S7
rplR	-0.94	9.7E-05	50S ribosomal protein L18
atpE	-0.94	6.0E-05	F0F1 ATP synthase subunit C
glnQ	-0.94	1.3E-04	glutamine ABC transporter ATP-binding protein
atpB	-0.94	8.4E-05	F0F1 ATP synthase subunit A
rpoB	-0.92	3.6E-04	DNA-directed RNA polymerase subunit beta
fabG	-0.92	2.6E-04	3-ketoacyl-(acyl-carrier-protein) reductase
purF	-0.90	7.9E-04	amidophosphoribosyltransferase
telB	-0.90	1.0E-05	putative tellurium resistance protein
llmg_0208	-0.89	1.0E-04	hypothetical protein
glnP	-0.89	3.7E-05	glutamine ABC transporter permease and substrate binding protein
llmg_1007	-0.89	8.0E-05	hypothetical protein
rmlB	-0.88	7.4E-05	dTDP-glucose 4,6-dehydratase
nusA	-0.88	3.1E-05	transcription elongation factor NusA
rpsB	-0.88	1.4E-05	30S ribosomal protein S2
metK	-0.87	1.4E-04	S-adenosylmethionine synthetase
rpsS	-0.87	7.1E-05	30S ribosomal protein S19
rplF	-0.86	4.1E-05	50S ribosomal protein L6
priA	-0.85	1.3E-04	primosome assembly protein PriA
guaA	-0.85	1.1E-04	GMP synthase
nupC	-0.84	3.8E-04	purine/cytidine ABC transporter permease protein
rplV	-0.84	1.9E-04	50S ribosomal protein L22
purQ	-0.84	1.3E-04	phosphoribosylformylglycinamidine synthase I
pstD	-0.84	1.2E-03	phosphate transport system permease protein PstD
nupA	-0.84	7.2E-05	purine/cytidine ABC transporter ATP-binding protein
rpoC	-0.84	1.0E-03	DNA-directed RNA polymerase subunit beta'
llmg_1353	-0.83	1.3E-04	putative tellurite resistance protein
rpoA	-0.82	5.2E-05	DNA-directed RNA polymerase subunit alpha
llmg_0376	-0.82	1.9E-04	amino acid permease
nupB	-0.82	3.7E-04	purine/cytidine ABC transporter permease protein
phnC	-0.81	1.6E-04	phosphonates import ATP-binding protein PhnC
rpmD	-0.81	1.1E-03	50S ribosomal protein L30

Table 17.6: The 50 most differentially downregulated genes in BK1503 (p=0.01).

Key glycolytic genes present among the most downregulated genes Among the most downregulated genes we find genes associated with the ATPase complex such as *atpH* (both) and *atpE* (BK1503), perhaps indicative of a response to the overproduction of the remaining components. Interestingly both lactate dehydrogenase *ldh* in BK1506 and pyruvate kinase *pyk*, a key glycolytic enzyme, are among the most downregulated in both strains. A final curious observation is the gene *pgmA*, involved in connecting the leloir pathway and glycolysis [168] (conversion of glucose-1-phosphate to glucose-6-phosphate), which is upregulated in BK1503 but downregulated in BK1506.

17.3.4 Mapping the response of strains expressing varying degrees of ATPase activity to metabolic pathways reveals a global picture of the transcriptomic response

The 50 most up and down regulated genes in both BK1506 and BK1503 confirmed that the ATPase system was active. It also revealed that two key glycolytic genes were among the most heavily down regulated in both strains. To further compare and contrast the response by BK1506 and BK1503 tables were generated based on the response of particular metabolic pathways such as glycolysis.

Tables were generated such that all the genes of a particular pathway (based on information from Kyoto Encyclopedia of Genes and Genomes - KEGG) were listed and the response of BK1506 and BK1503 as compared to the reference BK1010 was listed in two adjacent columns together with the p-values. The foldchange response of the individual genes were color coded green (upregulation) and red (downregulation) with a gradient representing the severity of the response. The scale of the gradient was adjusted to the extremes of the response in the current data set so that bright red indicates the most downregulated, bright green indicates the most upregulated and white (no color) is the center indicating no change.

The values were further processed by using a p-value cutoff of 0.10 (10%) which is high. Traditionally either a p-value cutoff (1% or 5%) or a foldchange cutoff is used to assist in data analysis. Our selected p-value cutoff is higher than normal since for foldchanges in the range of 1.5 times up or downregulation, statistical significance would be harder to achieve than for greater spans of regulation (the more extreme the foldchange the higher the likelyhood of a significant p-value). Since the experimental setup was deliberately designed to minimize perturbations more extreme foldchanges were automatically less likely.

Glycolysis

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
glk	-0.25	7.0E-02	-0.57	4.9E-04	glucokinase
pgi			0.54	4.0E-03	glucose-6-phosphate isomerase
pfk	-0.26	6.1E-02	-0.56	6.9E-04	6-phosphofructokinase
pyk	-0.27	9.4E-02	-1.09	1.3E-05	pyruvate kinase
ldh	-0.51	1.5E-02	-0.45	1.8E-02	L-lactate dehydrogenase
fbp	0.60	7.9E-03	0.65	2.8E-03	fructose-bisphosphatase
fbaA	-0.36	1.2E-02	-0.46	1.8E-03	fructose-bisphosphate aldolase
tpiA			-0.68	9.7E-04	triosephosphate isomerase
gapB					glyceraldehyde 3-phosphate dehydrogenase
gapA	0.22	8.5E-02			glyceraldehyde 3-phosphate dehydrogenase
pgk	-0.35	5.5E-02			phosphoglycerate kinase
llmg_1894					phosphoglycerate mutase family protein
gpmA	-0.30	5.5E-02	-0.67	4.0E-04	phosphoglyceromutase
gpmB			-0.51	9.1E-04	phosphoglycerate mutase
gpmC	0.25	6.4E-02	-0.56	5.3E-04	phosphoglycerate mutase
eno	-0.28	8.0E-02			phosphopyruvate hydratase
pdhA			-0.51	1.1E-02	pyruvate dehydrogenase E1 component alpha subunit
pdhB			-0.36	9.6E-02	pyruvate dehydrogenase E1 component beta subunit
pdhC			-0.35	9.8E-02	pyruvate dehydrogenase complex E2 component
pdhD					dihydrolipoamide dehydrogenase
ldhX			0.45	1.6E-02	L-lactate dehydrogenase
ldhB					L-lactate dehydrogenase
adhE	0.52	6.7E-02			bifunctional acetaldehyde-CoA/alcohol dehydrogenase
adhA, adhP					alcohol dehydrogenase
llmg_0955					alcohol dehydrogenase
fadD	-0.26	7.1E-02			long-chain acyl-CoA synthetase
galM	0.57	6.2E-02			aldose 1-epimerase
bglA2					6-phospho-beta-glucosidase
arb			0.61	4.1E-04	6-phospho-beta-glucosidase
celA					6-phospho-beta-glucosidase

Table 17.7: Comparison of the expression level changes for the genes involved in glycolysis, between BK1506 and BK1503. Green colour indicates increased expression, red colour decreased. A colour gradient indicates the relative foldchange, i.e. stronger colour means bigger foldchange. Empty spots indicate original values had a p-value below the cuttoff (0.10).

A complete overview of all the data can be viewed in the supplementary material (chip 1) and in the appendix (chip 2). Tables from chip 1 are included in supplementary material section S.2 while tables from chip 2 (appendix E, section E.1) and merged tables, contrasting the results from both analysis side by side with 0.10 cutoff applied, (appendix E, section E.2) are included in the appendix. In the following section a selection of the tables generated from chip 1 will be presented.

The response of glycolytic genes and genes involved in pyruvate metabolism to expression of varying degrees of ATPase activity
The response of genes connected to glycolysis can be seen in table 17.7. The response is mostly downregulation as witnessed by pyruvate kinase (*pyk*),

fructose-bisphosphate aldolase (*fbaA*), triosephosphate isomerase (*tpiA*) and phosphoglyceromutase (*gpmA/B/C*) which is evident for both strains with the most pronounced effect in BK1503. Genes with noteworthy upregulation is fructose-bisphosphatase (*fbp*) and glucose-6-phosphate isomerase (*pgi*)

Although not a glycolytic gene lactate dehydrogenase (*ldh* and *ldhX*) has also been included in this table. Interestingly *ldh* is downlregulated while the alternative lactate dehydrogenase *ldhX* is upregulated.

Phosphotransferase system (PTS) and glucose uptake

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
ptsI			-0.37	2.1E-02	phosphoenolpyruvate-protein phosphotransferase
ptsH			0.24	7.5E-02	phosphocarrier protein HPr
ptsK	-0.25	1.0E-01	0.32	3.0E-02	HPr kinase/phosphorylase
hprT			0.30	6.4E-02	HprT protein
llmg_1426					sucrose-specific PTS system IIBC component
bglP					PTS system, beta-glucosides specific enzyme IIABC
llmg_0453					sucrose-specific PTS enzyme IIABC
llmg_0454			-0.25	6.3E-02	beta-glucoside-specific PTS system IIABC component
ptcA			1.00	1.1E-04	cellobiose-specific PTS system IIA component
ptcB			0.83	1.1E-03	cellobiose-specific PTS system IIB component
llmg_1244	0.40	7.3E-02			hypothetical protein
ptcC	0.59	1.5E-02			cellobiose-specific PTS system IIC component
celB	1.34	6.3E-06	0.33	3.8E-02	cellobiose-specific PTS system IIC component
mtlF					PTS system, mannitol-specific IIA component
mtlA					PTS system, mannitol-specific IIBC component
ptnAB			-0.78	1.6E-04	PTS system, mannose-specific IIAB components
ptnC			-0.65	6.3E-04	mannose-specific PTS system component IIC
ptnD			-0.49	1.8E-03	mannose-specific PTS system component IID
llmg_0866	0.42	1.0E-02			PTS system, unknown pentitol phosphotransferase enzyme IIB component
ulaA, sgaT	0.85	3.9E-03			PTS system ascorbate-specific transporter subunit IIC
fruA	0.37	4.7E-02			PTS system, fructose specific IIBC components
glcU					putative glucose uptake protein GlcU

Table 17.8: Comparison of the expression level changes for the genes involved in the PTS, between BK1506 and BK1503. Green colour indicates increased expression, red colour decreased. A colour gradient indicates the relative foldchange, i.e. stronger colour means bigger foldchange. Empty spots indicate original values had a p-value below the cuttoff (0.10).

The response of genes involved in transport processes to varying degrees of ATPase activity The genes associated with the PTS system and ABC transporters can be seen in table 17.8 and table 17.9. The response by the PTS system for glucose transport indicates a shift from PTS^{Man} to PTS^{Cel}. The two systems have similar capacity but differ in their affinity with PTS^{Man} being a high affinity system and PTS^{Cel} (in combination with the permease *glcU*) being a low affinity system with a preference for β-glucose [10]. For the ABC transporters two interesting observations can be made. There appears to be a high degree of upregulation in BK1506

of dipeptide transport which seem to be missing in BK1503. Secondly a very strong downregulation of phosphate transport is present in BK1503 and is also somewhat evident in BK1506. Whether the downregulation of phosphate transport is a response to ATPase activity or whther it is a general response, is an interesting question.

Regulatory responses to expression of varying degrees of ATPase activity The responses of genes under CodY regulation or CcpA mediated carbon catabolite repression (CCR) or carbon catabolite activation (CCA) can be found in table 17.10 and table 17.11. CodY (*codY*) was among the highest upregulated genes in BK1503. Being a transcriptional repressor the trend of downregulation as seen in table 17.10 seems to support this.

The response of genes under CcpA regulation seem to indicate relief of CCR as most of the genes under repression are upregulated. Also notably one of the most prominent targets of CcpA the *las* operon (LDH, PFK and PYK) which is under CCA is downregulated. The effect is again most prominent in BK1503 but also discernable in BK1506. This suggests a global response to ATPase expression in accordance with the previous observations of decrease in general metabolic capacity.

The transcription level response of strains expressing ATPase activity resembles a starvation response

The key examples of metabolic pathway maps from the transcription level analysis presented show that the response to a perturbation in ATP level is a downregulation of glycolysis. This response is counter intuitive to what might be expected from a perturbation of the energy level in the cell, since glycolysis is the main pathway for generating energy in *Lactococcus lactis*. The same trend is seen for other central metabolic pathways such as the pentose phosphate pathway, the TCA cycle and pyruvate metabolism and fatty acid metabolism (supplementary material S.2, page 18, 19, 20 and 28). There is an indication of a dose effect as the pattern is usually more pronounced in BK1503 (the strain with higher expression of ATPase activity) compared to BK1506. Also among the other anabolic pathways such as purine metabolism, pyrimidine metabolism and fatty acid metabolism there seems to be more downregulation in BK1503. This is in contrast to the response which can be seen for transporters and arginine metabolism where the effect appears to be upregulation in BK1506 which unfortunately cannot be verified in BK1503.

ABC transporters

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
potD	-0.51	6.8E-03	0.47	7.1E-03	spermidine/putrescine ABC transporter substrate-binding protein
potC					spermidine/putrescine ABC transporter permease
potB					spermidine/putrescine ABC transporter permease
potA	-0.28	4.3E-02			spermidine/putrescine ABC transporter ATP-binding protein
busAB	-0.28	9.6E-02	0.30	5.1E-02	glycine betaine-binding periplasmic protein precursor
busAA			-0.35	1.1E-02	glycine betaine/proline ABC transporter
choS			0.35	7.8E-02	choline ABC transporter permease and substrate binding protein
choQ			-0.33	1.5E-02	choline ABC transporter ATP binding protein
malE					maltose ABC transporter substrate binding protein
malF	0.72	4.2E-02			maltose transport system permease protein MalF
malG					maltose ABC transporter permease protein MalG
msmK			0.80	1.8E-04	multiple sugar-binding transport ATP-binding protein
rbsB					ribose ABC transporter substrate binding protein RbsB
rbsC					ribose transport system permease protein RbsC
rbsD					ribose ABC transporter permease protein RbsD
rbsD					D-ribose pyranase
pstE	-0.48	4.3E-03	-0.54	1.1E-03	phosphate transport substrate binding protein PstE
pstF			-0.45	4.4E-03	phosphate transport substrate binding protein PstF
pstD			-0.84	1.2E-03	phosphate transport system permease protein PstD
pstC			-0.76	6.0E-04	phosphate transport system permease protein PstC
pstA	-0.65	5.1E-03	-0.62	3.9E-03	phosphate transporter ATP-binding protein
pstB			-0.69	6.3E-04	phosphate transporter ATP-binding protein
phnD	-0.33	4.5E-02	-0.32	3.5E-02	phosphonate ABC transporter, phosphonate-binding protein PhnD
phnB			-0.79	1.1E-04	phosphonate transport system permease protein PhnB
llmg_0315	-0.53	4.7E-03	-0.78	1.7E-04	phosphonate ABC transporter permease
phnC			-0.81	1.6E-04	phosphonates import ATP-binding protein PhnC
plpA					D-methionine-binding lipoprotein PlpA precursor
plpB					D-methionine-binding lipoprotein PlpB precursor
plpC			-0.28	3.5E-02	D-methionine-binding lipoprotein PlpC precursor
plpD			-0.25	3.9E-02	D-methionine-binding lipoprotein PlpD precursor
llmg_0342	0.44	6.7E-03	-0.22	8.5E-02	amino acid ABC transporter permease protein
llmg_0341	0.44	5.9E-03	-0.24	6.1E-02	amino acid ABC transporter ATP binding protein
dppA					dipeptide-binding protein precursor
dppB	0.48	7.6E-03			dipeptide transport system permease protein DppB
dppC	0.92	3.7E-05			dipeptide transport system permease protein DppC
dppD	0.76	2.6E-04	-0.23	9.1E-02	dipeptide transport ATP-binding protein DppD
dppF	0.60	2.0E-03			dipeptide transport ATP-binding protein DppF
fhuD					ferrichrome ABC transporter substrate binding protein
fhuB					ferrichrome ABC transporter permease protein
fhuG					ferrichrome ABC transporter permease protein
llmg_1281					putative ABC transporter ATP-binding protein
fhuC					ferrichrome ABC transporter FhuC
zitS			0.48	5.2E-03	zinc ABC transporter substrate binding protein
zitP	0.61	3.1E-02			zinc ABC transporter permease protein
zitQ			0.29	7.6E-02	zinc ABC transporter ATP binding protein
cbiQ	0.50	1.7E-02			putative cobalt ABC transporter permease protein
cbiQ2			-0.57	2.4E-03	putative cobalt ABC transporter permease protein
cbiO	0.39	2.2E-02			putative cobalt ABC transporter ATP-binding protein
cbiO	-0.40	2.5E-02			cobalt transporter ATP-binding subunit
cbiO			-0.55	8.8E-04	cobalt transporter ATP-binding subunit
llmg_1552			0.38	2.3E-02	putative ABC type transport system permease protein
drrB					daunorubicin resistance transmembrane protein
llmg_0262	0.58	1.9E-03	0.25	7.2E-02	ABC transporter permease protein
llmg_1553					putative ABC transporter ATP-binding protein
drrA					daunorubicin resistance ABC transporter ATP-binding subunit
tagG	0.96	6.9E-04	0.39	5.7E-02	teichoic acid ABC transporter permease protein
tagH	0.51	1.1E-02			teichoic acid export ATP-binding protein TagH
ftsX			-0.30	4.2E-02	cell division protein FtsX-like protein
ftsE	-0.40	4.6E-02			cell division ATP-binding protein
llmg_1202	-0.24	9.2E-02	0.45	3.7E-03	ABC transporter ABC binding and permease protein
cydD					cytochrome d ABC transporter ATP binding and permease protein
cydC					cytochrome d ABC transporter ATP binding and permease protein
llmg_0989					ABC transporter ATP binding and permease protein

Table 17.9: Comparison of the expression level changes for the genes involved in the ABC transporters, between BK1506 and BK1503. Green colour indicates increased expression, red colour decreased. A colour gradient indicates the relative foldchange, i.e. stronger colour means bigger foldchange. Empty spots indicate original values had a p-value below the cuttoff (0.10).

Genes under codY regulation

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
dppA					dipeptide-binding protein precursor
dppB	0.48	8.1E-03			dipeptide transport system permease protein DppB
dppC	0.92	4.9E-05			dipeptide transport system permease protein DppC
dppD	0.76	3.2E-04	-0.23	8.8E-02	dipeptide transport ATP-binding protein DppD
dppF	0.60	2.3E-03			dipeptide transport ATP-binding protein DppF
gltD			-0.22	9.4E-02	glutamate synthase subunit beta
gltB			-0.77	3.1E-03	glutamate synthase, large subunit
lysA					LysA protein
ilvD			-0.61	8.9E-02	dihydroxy-acid dehydratase
ilvB			-0.57	8.9E-02	acetolactate synthase catalytic subunit
ilvN			-0.64	5.3E-02	acetolactate synthase 3 regulatory subunit
ilvC			-0.64	2.5E-02	ketol-acid reductoisomerase
ilvA			-0.56	6.2E-03	threonine dehydratase
aldB	-0.39	1.8E-02	-0.34	2.4E-02	AldB protein
hisC					histidinol-phosphate aminotransferase
hisZ					ATP phosphoribosyltransferase regulatory subunit
hisG					ATP phosphoribosyltransferase catalytic subunit
hisD					HisD protein
hisB					imidazoleglycerol-phosphate dehydratase
hisH					imidazole glycerol phosphate synthase subunit HisH
hisA					1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)-methylideneamino] imidazole-4-carboxamide isomerase
hisF					imidazole glycerol phosphate synthase subunit HisF
hisI					bifunctional phosphoribosyl-AMP cyclohydrolase/phos-phoribosyl-ATP pyrophosphatase protein
hisK					histidinol-phosphatase
ctrA			-0.44	3.0E-03	putative amino-acid transporter
oppD			-0.73	1.7E-04	oligopeptide transport ATP-binding protein OppD
oppF			-0.63	2.8E-04	oligopeptide transport ATP-binding protein OppF
oppB			-0.38	1.4E-02	peptide transport system permease protein OppB
oppC			-0.40	7.9E-03	oligopeptide transport system permease protein OppC
oppA					oligopeptide-binding protein OppA precursor
pepO					endopeptidase O
asnB			-0.29	3.1E-02	asparagine synthetase B
gltA					citrate synthase
citB					aconitate hydratase
icD					isocitrate dehydrogenase
serC					phosphoserine aminotransferase
serA	-0.31	4.9E-02	-0.64	6.0E-04	D-3-phosphoglycerate dehydrogenase
serB			-0.49	8.5E-03	SerB protein
ArcD1					arginine/ornithine antiporter
ArcC1					carbamate kinase
ArcC2					carbamate kinase
amtB			-0.45	6.8E-03	ammonium transporter AmtB
dapB					dihydrodipicolinate reductase
FtsW1					cell division protein ftsW1

Table 17.10: Comparison of the expression level changes for genes regulated by CodY, between BK1506 and BK1503. Green colour indicates increased expression, red colour decreased. A colour gradient indicates the relative foldchange, i.e. stronger colour means bigger foldchange. Empty spots indicate original values had a p-value below the cuttoff (0.10). Genes adapted from [158].

Genes under ccpA regulation

Gene/locus	BK1506		BK1503		Effect	Annotation
	LogFC	p. Value	logFC	P.value		
galP	0.76	8.5E-02			Rep	galactose permease
galM	0.57	7.3E-02			Rep	aldose 1-epimerase
galK					Rep	galactokinase
galT					Rep	galactose-1-phosphate uridylyltransferase
galE	-0.32	5.7E-02	0.33	3.8E-02	Rep	GalE protein
mtlA					Rep	PTS system, mannitol-specific IIBC component
mtlR					Rep	transcriptional regulator mtl operon MtlR
mtlF	0.23	9.6E-02			Rep	PTS system, mannitol-specific IIA component
mtlD					Rep	mannitol-1-phosphate 5-dehydrogenase
llmg_0453					Rep	sucrose-specific PTS enzyme IIABC
llmg_0454			-0.25	6.0E-02	Rep	beta-glucoside-specific PTS system IIABC component
trePP			-0.21	5.3E-02	Rep	putative trehalose/maltose hydrolase
pgmB			0.36	1.2E-02	Rep	beta-phosphoglucomutase
llmg_0431	0.41	2.0E-02			Rep	putative acyl carrier protein phosphodiesterase 2
llmg_0432					Rep	transcription regulator
ptcB			0.83	1.6E-03	Rep	cellobiose-specific PTS system IIB component
ptcA			1.00	1.8E-04	Rep	cellobiose-specific PTS system IIA component
llmg_0439			0.38	1.7E-02	Rep	LacI family transcription regulator
ptcC	0.59	1.8E-02			Rep	cellobiose-specific PTS system IIC component
bglA					Rep	6-phospho-beta-glucosidase
arcA	0.63	7.2E-02			Rep	arginine deiminase
arcB					Rep	ornithine carbamoyltransferase
arcD1					Rep	arginine/ornithine antiporter
arcC1					Rep	carbamate kinase
arcC2					Rep	carbamate kinase
pfk	-0.26	5.7E-02	-0.56	7.3E-04	Act	6-phosphofructokinase
pyk	-0.27	9.4E-02	-1.09	2.1E-05	Act	pyruvate kinase
ldh	-0.51	1.8E-02	-0.45	2.1E-02	Act	L-lactate dehydrogenase
pgiA			0.54	4.7E-03	Act	glucose-6-phosphate isomerase
fruA	0.37	5.0E-02			Act	PTS system, fructose specific IIBC components
fruC			-0.39	8.4E-03	Act	tagatose-6-phosphate kinase
fruR					Act	lactose transport regulator

Table 17.11: Comparison of the expression level changes for genes regulated by CcpA, between BK1506 and BK1503. Green colour indicates increased expression, red colour decreased. A colour gradient indicates the relative foldchange, i.e. stronger colour means bigger foldchange. Empty spots indicate original values had a p-value below the cuttoff (0.10). The grey sectioning indicates genes which share regulation, indicated in the effect column.

In conclusion the metabolic pattern has indications of a starvation like reponse to ATPase activity denoted by a downregulation of central metabolism (notably key glycolytic enzymes such as phosphofructokinase, phosphoglyceromutase, triosephosphate isomerase and pyruvate kinase) and lactate dehydrogenase. In addition anabolic pathways such as fatty acid metabolism and purine/pyrimidine metabolism are also downregulated in combination with a shift towards the use of alternative substrates, illustrated by upregulation of peptide transporters, upregulation of *fbp* as a gluconeogenic respons, and the relief of carbon catabolite repression regulated by ccpA. A graphical summary of the results can be seen in figure 17.5.

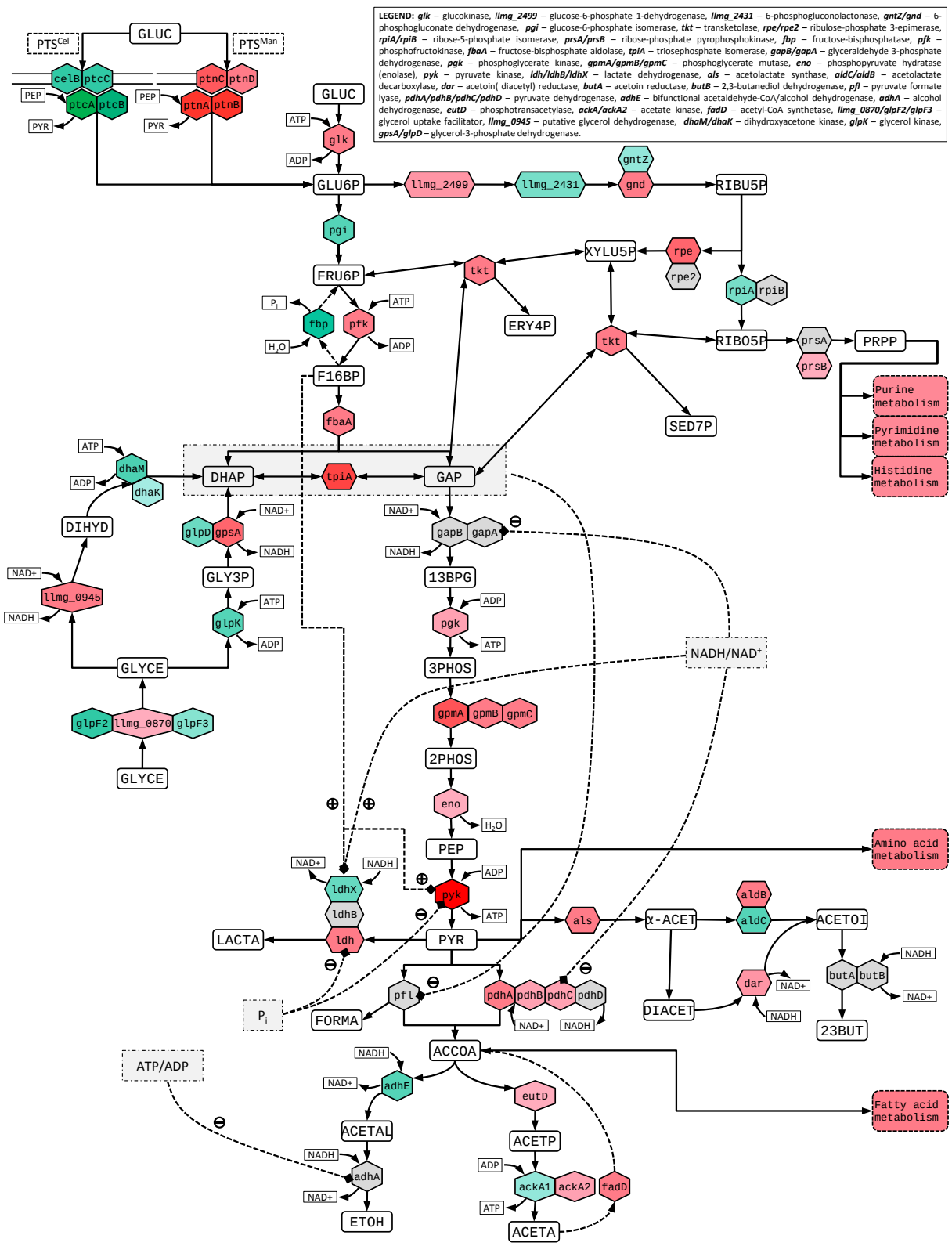

Figure 17.5: A schematic overview of the effects from ATPase expression on the central metabolism of *Lactococcus lactis*. Green - upregulation, Red - downregulation and Grey - no information. A colour gradient indicates the relative foldchange, i.e. stronger colour means bigger foldchange.

Chapter 18

Discussion

The aim of the experiments described was to get a deeper insight into the response of *Lactococcus lactis* to various degrees of ATPase activity. The approach was to combine data from a metabolomic analysis with data from a transcriptomic analysis to characterize the response of two selected strains with only slight perturbations to their metabolism.

18.1 Metabolomic profiling of strains expressing ATPase activity confirms the ATPase system as working but reveals the need for further optimization

The previous study by Koebmann *et al.* [165] measured selected metabolites and it was noted, that the concentration of glucose-6-phosphate almost doubles from reference to BK1502, being 16mM in the reference and 32mM in BK1502 while fructose-1,6-bisphosphate drops around 20%. This effect could most likely be explained by the shift in glucose transport as described in the transcriptional investigations. There is no sign of the pattern in this data set generated in this work.

From a study by Solem *et al.* [176] the concentrations of DHAP was reported to be approximately 7mM, the concentration of G6P was 15mM and the concentration of FBP (F16BP) was 50mM, in the wild type *L. lactis* IL1403. This pattern of four times the concentration of F16BP compared to G6P corresponds better with the data in the present study.

What does seem to fit nicely with the strains examined are the measurements of ADP and AMP. There seems to be a correlation of AMP and ADP concentrations, ranging from low in reference to high in BK1502 which fits the increase in ATPase expression. In contrast, the levels of NAD are lower in response to the higher ATPase expression. This could be taken as an indirect measure that NADH levels are rising, since growth rates are lowered

and biomass is more reduced than glucose (degree of reduction: biomass = 4.2, glucose = 4 [161]). It would be very interesting to repeat this analysis with the optimizations already outlined in the discussion of the results from screening of *Lactococcus lactis* for glycerol utilization.

18.2 Transcriptome profiling of strains expressing varying degrees of ATPase activity indicates a starvation like response to perturbations in energy level

When comparing the response of the strains perturbed by ATPase activity, the general trend seems to be repression of anabolic pathways such as glycolysis, fatty acid metabolism, purine metabolism and pyrimidine metabolism. This response is complemented by activation of both *fbp* (fructose-1,6-bisphosphatase) and *pgi* (glucose-6-phosphate isomerase), indicating a response in the pentose phosphate pathway and gluconeogenesis. Further there are indications of a shift in glucose transport and signs of relieved catabolite repression possibly connected to the transcription factor CodY (*codY*) and CCR/CCA via CcpA.

18.2.1 Proper handling and processing of microarrays plays a vital role in further data analysis

The original experimental design was anchored on analysis of four replicates of each strain. This number of samples were more than could be handled on a single chip which meant that the samples had to be distributed among two separate microarray chips.

Even without the added complexity of batch effects between the chips the signal from the (intentionally) slight perturbations in the experiment were already at risk of being drowned out by noise introduced in the handling preparation. The actual handling and running of the chips was done over a period of several months and one cannot exclude degradation of the samples in that time period.

Future investigations should take care to minimize the handling effects experienced during this analysis by distributing samples evenly across the purification step and the microarray chips, in order to ensure even distribution of the noise across replicates. Also it is advantageous to ensure an even amount of samples for the FDR analysis and complete the arrays within as short a period as possible.

Alternatively a more advanced bioinformatic pre-processing will be able to extract the noise introduced by the batch effect and provide an effective means of combatting these, allowing all samples to be compared. Several attempts have been made so far to combat the issue of batch variation such

as Frozen robust multiarray analysis (fRMA) [170] and an empirical Bayes approach [180]. Future application of such advances in bioinformatics might prove helpful in further analysis of such samples.

18.2.2 Implications of the observations made in the transcriptome level analysis of strains expressing varying degrees of ATPase activity

The following section contains a general discussion about the observed responses of both BK1506 and BK1503 as a whole.

Activity of global regulators such as transcription factors and CcpA suggest a systemic response to ATPase activity

The effect on transcription factors could indicate a global response similar to relief of catabolite repression. Unfortunately there is no significant information on CcpA (*ccpA*, but the genes regulated by CcpA respond in a fashion that indicates downregulation of *ccpA*. MalR (*malR*) is crucial for maltose transport (despite being named as a repressor) [154] and ArgR (*argR*) is involved in arginine metabolism but is believed to have other targets on the genome (ARC boxes) [167].

The are indications of upregulation of the transcriptional regulator CodY. CodY functions as a repressor for many genes in the chromosome. CodY is associated with starvation response, biofilm formation, sporulation and virulence in pathogenic organisms. CodY interacts with GTP (sensing of energy level) and branched-chain amino acids (BCAA) (sensing of nutrients) which is thought to increase binding affinity to DNA. A lowered level of GTP or BCAA's is connected with the transition to stationary phase and causes CodY to detach from the target sites thereby relieving repression [178].

The response of genes in glycolysis and other anabolic pathways suggest a lowered metabolic state which might present a bottleneck for glycolytic flux

Looking at the central metabolism, the downregulation of glycolysis could explain the phenomenon that glycolytic flux does not increase with increased ATPase activity as seen in *E. coli*. A downregulation of the main energy producing pathway in anaerobic/static growth of *Lactococcus lactis* as a response to a perturbation of the energy level in the cell could also indicate that the signal from the lowered ATP/ADP ratio is not directed specifically to energy homeostasis, but interpreted more as a general starvation response.

This triggers a change in metabolism towards slower growth and relieves catabolite repression which in turn affects maltose transport, dipeptide transport and causes a the shift in glucose transport, with the downregulation of the PTS^{Man} system (*ptnAB*, *ptnC* and *ptnD*) in BK1503 and upreg-

ulation of the PTS^{Cel} system (*ptcA*, *ptcB* and *ptcC*). The two systems have the same transport capacity but their affinity is very different. PTS^{Man} has much higher affinity (Km of 13μM) compared to the PTS^{Cel} system (Km of $\approx$9mM) [10]. The shift from high affinity to a lower affinity system would indicate a starvation response since the uptake of glucose is done at the expense of PEP, which acts as an energy reserve during starvation [169]. The heavy downregulation of phosphate transport is intriguing. It could be connected to the hydrolysis of ATP or be a general response similar to glycolysis other major anabolic pathways which appear to be turned down.

More anabolic pathways exhibit a response similar to glycolysis The trend observed for the genes in glycolysis is repeated in several other anabolic pathways. In amino sugar metabolism *glmS* which acts on fructose-6-phosphate (and glucosamine-6-phosphate) is heavily downregulated. In fatty acid metabolism the genes *accB*, *accC* and *accD*, which are involved in conversion of Acetyl-Coa to Malonyl-CoA (a precursor for fatty acid synthesis), are also heavily downregulated. In pyruvate metabolism *pycA*, which is involved in the conversion of pyruvate to oxaloacetate is downregulated (OAA is a precursor to aspartate, which in turn can be used in pyrimidine metabolism or as a nitrogen donor for purine metabolism) [181].

ATPase activity as a stimulus for glycerol utilization works - on a transcriptional level In glycerolipid metabolism it seems that the initial idea of using ATP hydrolysis to stimulate a metabolic response favorable to glycerol utilization, worked on a transcriptional level to some extent. The anaerobic branch had a partial upregulation, with dihydroxyacetone kinase being upregulated but had a downregulation of glycerol dehydrogenase. The aerobic branch saw upregulation of glycerol kinase and a shift of glycerol-3-phosphate dehydrogenase from *gpsA* to *glpD*. The shift might be caused by the cofactor utilization, which in the case of *gpsA* is NADPH and for *glpD* is NADH.

The downregulation of glycolysis and other anabolic pathways combined with the shift in glucose transport might provide part of the answer to why glycolytic flux does not increase as a response to ATP hydrolysis

The two main effects of hydrolyzing ATP is an increased P_i level and a lowered energy state (ATP/ADP level). Recently Levering et *al.* investigated the phosphate metabolism of *Lactococcus lactis* and *Streptococcus pyogenes*, where they found that during starvation, most phosphate resides in the phosphoenolpyruvate pool ($\approx$20mM) and as inorganic P_i (45mM). Whereas during glycolysis it shifts towards fructose-1,6-bisphosphate (50mM) and ATP (8mM) [169].

Could the increased P_i level be sufficient to elicit the observed response to ATPase activity? The strains used in these experiments were deliberately chosen to have slight perturbations. The difference in concentrations of the phosphate pool components between starvation and glycolysis are significant. Although the differences in concentration are big, the transition has been reported to be gradual with a concentration of P_i at 25-30mM at glucose depletion rising to 40-45mM over a period of time [172, 171]. This raises the question whether the effects of hydrolyzing ATP could increase the P_i level sufficiently to simulate the conditions of poorer growth?

Since even the slight perturbation of BK1506 is enough to induce a response to ATPase activity it suggests that only a limited signal is needed. The study by Levering et *al.* also found a strong connection between extracellular phosphate concentration and the intracellular level of fructose-1,6-bisphosphate (which is connected to regulation of pyruvate kinase and lactate dehydrogenase). This relationship adds a further level of complexity to the system implicating transport of phosphate in the control of flux through glycolysis and should be considered in future studies.

The indications of a profound metabolic shift as a response to ATPase activity, as presented here, might be part of the explanation for why the glycolytic flux of *Lactococcus lactis* does not increase as a result of increased energy demand.

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Conclusions

Conclusions

The background for this project was to investigate the suitability of lactic acid bacteria as production organisms for the production of biofuels and biochemicals. Specifically the goal was to adapt the model organism *Lactococcus lactis* to convert crude glycerol, generated as a byproduct from biodiesel production, to value-added fuels or chemicals. Work was divided between four main areas, life cycle assessment of the GLYFINERY project, screening of *Lactococcus lactis* spp. for glycerol utilization, engineering of glycerol metabolism in *L. lactis* and finally an investigation into perturbation of energy metabolism in *L. lactis*.

Life cycle assessment - work package 7

The overall outcome of work package 7 is the integrated assessment of the processes developed in the GLYFINERY project. The integrated assessment evaluates the processes according to environmental, economic and technological factors. This serves as guideline to future decision makers. For example one could envision a process of great economic value but with terrible environmental effects. Two reports outlining the technological aspects of the glycerol conversion processes and which further served as a basis for the economical and the environmental assessments, can be seen in appendix A. The main conclusions from these reports show several of the processes under investigation, particularly the processes investigating butanol and 1,3-PDO production, as interesting from an economic perspective. Although further work is needed to improve yields and reduce downstream purification costs for truly competitive processes.

Screening of *Lactococcus lactis* spp. for glycerol utilization

Initially an attempt was made to use directed evolution to stimulate glycerol assimilation. The experiment was discontinued after 2 weeks of serial transfers, during which time, no change in the phenotype, i.e. ability to

utilize glycerol, was observed. It was concluded that a more drastic selective environment was perhaps necessary to stimulate this phenotype in the strain. The main challenge in applying this approach is most likely that no glycerol assimilation was apparent initially. It is perhaps better suited for further adaptation of strains already metabolizing glycerol. Furthermore the successful application of directed evolution to the challenge of glycerol assimilation, would only be possible if a fairly simple set of mutational events were enough to trigger the change, which is probably not the case for this setting.

The beneficial effect on glycerol inhibition when utilizing trehalose as a substrate might be connected to the properties of trehalose itself

Many different combinations of strains and conditions were tested for the ability to stimulate glycerol assimilation, but none showed convincing signs of achieving this goal. There were however indications of a beneficial effect to glycerol supplementation, when cultivating strains in defined medium supplemented with the disaccharide trehalose. When cultivating the strain *L. lactis* IL1403 over night, cultures exhibited higher optical density under aerobic conditions than cultures without added glycerol. Growth experiments revealed that the growth rate of the two cultures were similar, with the growth rate of the glycerol supplemented culture being approx. 84% of the culture without glycerol.

Since this observation was only seen under aerobic conditions, the reason for the improvement could be related to the activation of systems for protection against oxidative stress. One of these is an NADH oxidase, *noxE*, which might have have influenced redox issues. Trehalose has been implicated in numerous cellular functions in connection with stress, among which is protection against oxidative stress, so the explanation could also simply be the combination of glycerol and trehalose simply protects the cells better in the aerobic environment. Further investigations are needed to determine if this observation is in fact connected to glycerol metabolization.

Supplementation of glycerol in cultivations with xylose as a substrate has a detrimental effect on growth rate

Besides the possible beneficial effects of glycerol supplementation in cultures growing on trehalose, a detrimental effect of glycerol supplementation was observed when cultivating *L. lactis* in defined medium with xylose as the substrate. This was initially observed in over night cultures and was later confirmed by growth experiments. The effect was mainly found in connection with xylose and cultivations with other substrates, such as galactose or

ribose, did show the same degree of inhibition when subjected to glycerol supplementation.

The detrimental effect of glycerol persists in many different types of growth media and under both anaerobic and respiratory cultivation

One can observe a significant detrimental effect on growth rate in *L. lactis* NCDO2118 cultures growing growing on xylose, when supplemented with glycerol. The level of inhibition varies with the medium composition and the initial observations in BLL medium recorded a reduction of growth rate for cultures supplemented with 0.5% glycerol, to approx. 20% of the level found in cultivations without glycerol.

Samples from cultures cultivated in defined medium supplemented with xylose and with or without the addition of glycerol, were sent for analysis of internal metabolite concentrations, but the quality of the data was not sufficient to provide additional information about the effect.

From a series of growth experiments it was determined that the effect is most likely connected to xylose metabolism and its metabolism through the pentose phosphate pathway/phosphoketolase pathway, but the actual mechanism remains unknown. The pentose phosphate pathway is implicated through xylose as a substrate and from experiments in which nucleosides were added to the cultivation medium, which improved both growth rate and biomass yield although not completely to the level of the reference cultures (growth rate of approx. 70% of reference).

Cultivation in complex medium showed a similar reduction as in SAL medium. Since many of the components for cell biomass are present in this medium, this indicates that the problem might be connected to a bottleneck in energy metabolism. This is also supported by the reduction of glycerol inhibition when switching from BLL to SAL medium, since BLL medium has fewer amino acids added thus requiring a larger energy input for growth.

The mechanism for glycerol inhibition remains elusive A suggested mechanism for the inhibitory effect of glycerol under anaerobic conditions was the inhibition of pyruvate formate-lyase by high levels of triosephosphates. Since DHAP is the metabolite connecting glycerol to glycolysis, any assimilation would naturally cause the levels to rise. A further indication of this was seen in external metabolite measurements, in which the metabolite formate was absent in samples from glycerol supplemented cultures.

Under respiration permissive conditions the redox level ($NADH/NAD^+$), affecting glycerol-3-phosphate dehydrogenase and the pyruvate dehydrogenase complex might be the cause of effect. Glycerol is more reduced than glucose leading to a higher redox level. This is contradicted by the fact that

under respiration permissive conditions the cell should be able to channel excess redox to respiration and thereby solve the issue.

The curious persistence of inhibition under both anaerobic and respiratory conditions might indicate that there are several components to the inhibitory mechanism. The improvement of growth rate in SA medium compared to SAL would point in the direction of the metabolite acetate, since sodium acetate is omitted in SAL medium. Acetate can be metabolized to acetyl-CoA which is the common product of both PFL and PDH. Furhermore xylose metabolized via the phosphoketolase pathway is converted to GAP and acetyl phosphate. Any inhibition of flux from glycolysis (GAP) to acetyl-CoA would require a redirection of flux away from acetate production thereby producing less ATP for growth. The precise components and their contributions still remain to be investigated further in subsequent studies.

Engineering of glycerol metabolism in *Lactococcus lactis*

Several different constructs were initiated to help alleviate some of the possible difficulties of glycerol metabolization. In a response to the initial analysis, strains featuring overexpression of glycerol kinase were constructed and characterized. Unfortunately, the constructs could not be verified, as glycerol kinase activity was not detected under experimental conditions. This could possibly be caused by native regulatory issues interfering with the system. More work need to be done if this strategy is to be pursued in future studies.

Introduction of a recombinant pathway for glycerol metabolization as an alternative route to the possibly non-funtional endogenous pathway

As a response to the possibility that the genetic components already present in *Lactococcus lactis* may not be fully functional, an alterntive route for glycerol dissimilation was devised.

The system consisted of three genes, glycerol dehydrogenase (*dhaD*) from *Citrobacter freundii*, dihydroxyacetone kinase (*dhaK*) also from *Citrobacter freundii* and a glycerol facilitator (*glpF*) from *Escherichia coli*. The genes were cloned into an artificial operon structure with *glpF* in front of *dhaD* and *dhaK*. Gene expression was modulated with a synthetic promoter library, to ensure a suitable level of expression could be found without knowing the exact level required, in advance.

Several different combinations of the operon were transformed into *Lactococcus lactis*. This included integrative and plasmid versions, constructed

by either traditional or USER cloning, as full operons or as combinations of either *dhaD* and *dhaK* together or separate. Despite this no strains were verified as having a functional operon, and none have been found that metabolized glycerol.

Further work is needed to determine if the operon could be used to introduce glycerol dissimilation into *L. lactis*. Since this is an artificial operon many of the problems could stem from flawed design or unforeseen issues. The ribosomal binding sites were taken from glycolytic genes, which for the glycerol facilitator (being a membrane protein) might not have been the best choice. The idea of introducing the required machinery under a known control is still valid and many other options exist for the different components.

Introduction of NADH oxidase activity alone is not enough to allow growth on glycerol by *Lactococcus lactis*

An initial analysis predicted that growth on glycerol might give rise to issues with redox balance for *L. lactis*. To provide a solution to that issue strains overexpressing NADH oxidase activity were constructed. The two water forming NADH oxidases, *noxE* (*L. lactis*) and *nox* (*S. pneumoniae*) were used to construct libraries of strains with NADH oxidase activity.

Selected strains were characterized and displayed varying degrees of NADH oxidase activity in the lower range. The strains were cultivated in defined medium supplemented with glycerol as the sole carbon and energy source, but did not grow. This indicated that a redox problem was not the only issue preventing the strains from metabolizing glycerol. The next step would be to combine these libraries with the glycerol operon or other constructs. This combined effort might provide the basis for growth on glycerol. Alternatively strains with higher levels of NADH oxidase activity might be constructed and tested, to assess whether that it is simply a higher level of NADH oxidase activity which is necessary to stimulate glycerol assimilation by *L. lactis*.

Several challenges remain for glycerol assimilation by *Lactococcus lactis*

The most pressing question is the functional characterization of the existing metabolic pathway for glycerol assimilation. In particular glycerol transport into the cell, which was assumed to be functional, may be non-functional or perhaps not have sufficient capacity to be able to sustain growth on glycerol.

Several glycerol facilitators have been annotated, but their individual contribution remains unknown. Furthermore a major intrinsic protein (MIP), a group which can be subdivided into aquaporins, glycerol facilitators and aquaglyceroporins, from *L. lactis* was shown to be permeable to glycerol in

the same manner as *glpF* from *E. coli*. A more systematic characterization of the endogenous components would therefore benefit further studies.

Transcriptomic analysis of perturbations of energy metabolism in *Lactococcus lactis* shows a starvation like response to varied degrees of ATPase activity.

The transcriptomic analysis of *Lactococcus lactis* strains with overexpression of ATPase activity revealed that the effect of hydrolyzing ATP, and thus lowering the energy state of the cell (ATP/ADP ratio), was a downregulation of glycolysis and anabolic pathways in general. This might indicate that the perturbation triggers a more general starvation response, signaling a metabolic shift to prepare the cell for slower growth on a broader range of substrates. P_i released from hydrolysis of ATP is already connected to regulation of pyruvate kinase and lactate dehydrogenase. This regulation appears to be mediated by HPr and CcpA regulation of central carbon metabolism. These results provide some interesting clues to direct further work on elucidating the control of glycolysis in *Lactococcus lactis*.

Metabolomic profiling of the four strains BK1010, BK1506, BK1503 and BK1502 did unfortunately not reveal any novel clues to the control of glycolysis. There was a lack of signal in the data prepared from analysis of the samples, which was most likely caused by a lack of sufficient biomass. It would be very interesting to repeat this analysis on samples with more biomass present, to get a better understanding of the metabolic response caused by perturbing the energy metabolism of *Lactococcus lactis*.

Outlook

The task of making *Lactococcus lactis* grow on glycerol as sole carbon and energy source still remains to be completed. It will most likely require the combined efforts on all of the three areas investigated, to accomplish this task. It was from the beginning the foundation of the project, that the results from one part of the project, would be combined with results from others.

Strains with the synthetic glycerol assimilation operon would additionally be outfitted with a library of NADH oxidase activity and would eventually be cultivated under the most stimulating of conditions. Further work would involve more rigorous investigations into each area to ascertain why the constructs did not work, and perhaps devise novel approaches to constructing these strains.

The results from the transcriptome analysis needs further experimental validation

There are several avenues which can be investigated further. The validation by experimental work and the further analysis of the results from the transcriptome level analysis by metabolite measurements or enzymatic assays of key regulated enzymes would be very interesting. The ultimate goal of determining control of glycolytic flux in *Lactococcus lactis* and ultimately increasing it, still remains to be accomplished.

Perhaps the indications gathered from the transcriptome analysis can be used as a starting point for future investigations into counter acting the downregulation of the cental metabolic pathways in response to a drain in energy level. An obvious candidate for further studies is the regulation of central metabolism by HPr and CcpA.

More light might be shed on the inhibitory effect of glycerol in combination with xylose by further metabolomic investigations

The glycerol inhibition phenomenon also merits further study. Initially just a coincidental observation, some effort has been put into characterizing the phenomenon, but the observations made in this thesis are far from exhaustive. A suitable approach for further investigations could be similar to the one from the work described here, consisting of growth experiments, transcriptome analysis and metabolome analysis, repeating the internal metabolite measurements.

Engineering of glycerol metabolism in *Lactococcus lactis* requires a more robust characterization of the existing components

Finally the goal of engineering glycerol metabolism of *Lactococcus lactis* by modulation of redox cofactor levels or the introduction of a synthetic pathway for glycerol assimilation, is still ongoing.

The initial attempts at cloning an artificial operon containing all the necessary genes for glycerol assimilation met with adversity, prompting a step back and re-evaluation of this approach. Perhaps the design principles should be modified before attempting again. This might entail a more complete characterization of the existing enzymatic machinery and introduction of genes from *E. coli* which has been shown to work previously.Especially the role of glycerol transport needs to be clarified since this step is crucial to further assimilation.

The modulation of cofactor levels, in particular the modulation of NADH level was achieved. However the activities and range of the library was limited and should be expanded upon in further work.

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Supplementary Material

S.1 – Quality assessment data, volcano plots and heat maps from chip 1 and 2.

S.2 – Results from the differential gene expression analysis of chip 1.

SUPPLEMENTARY MATERIAL

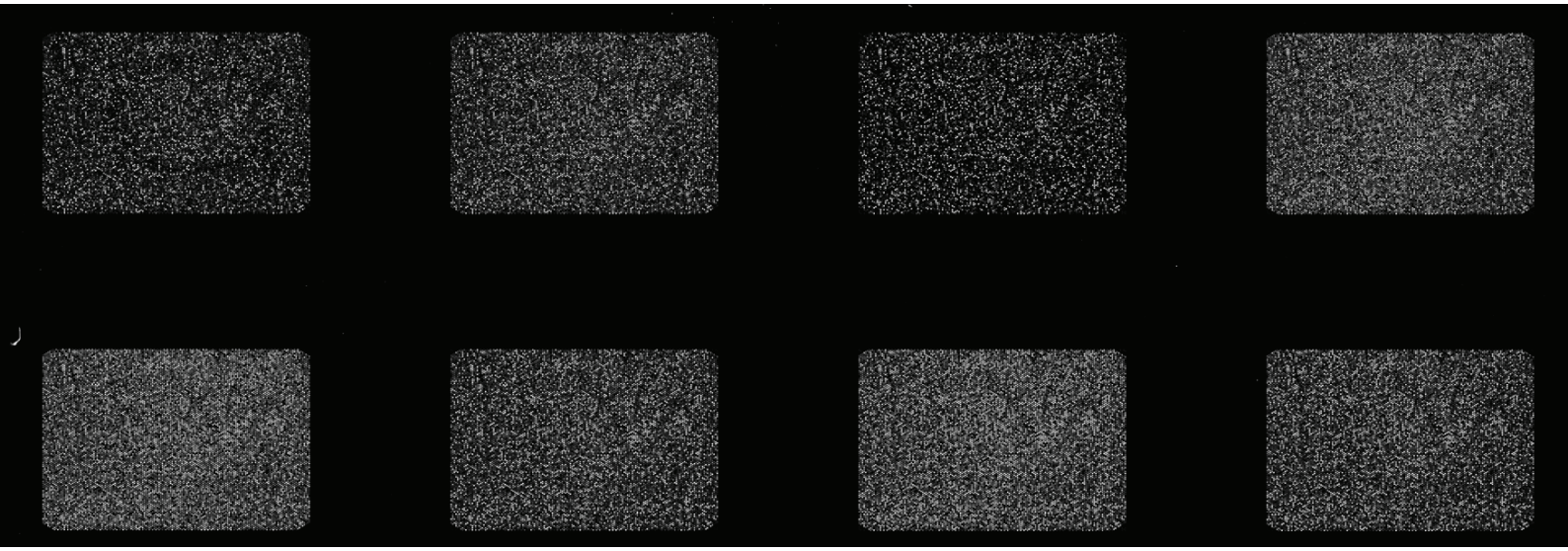
S.1 - Quality assessment data, volcano plots and heatmaps from chip 1 and 2.

This section contains the raw chip image files, intensity plots, volcano plots and heat maps generated from singular value decomposition (SVD) from chip 1 and 2.

SUPPLEMENTARY MATERIAL

Raw image file from chip 1

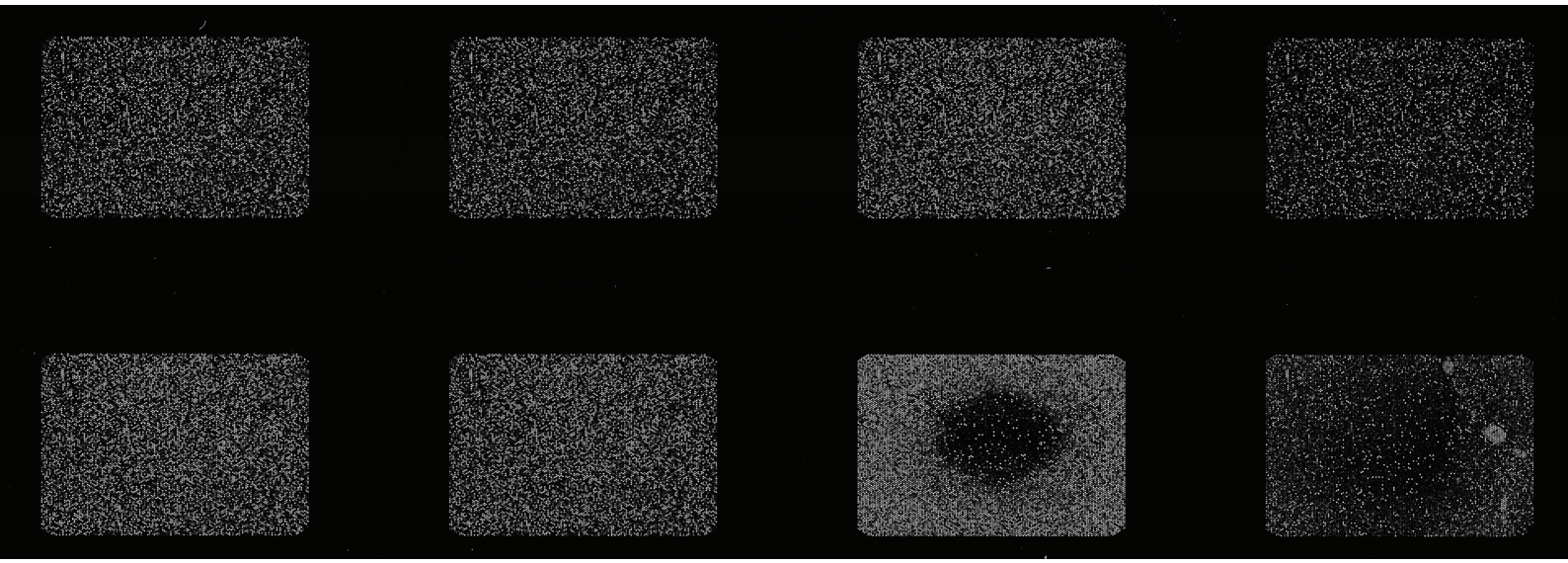
This is the raw image file of the chip. Just by a visual inspection of this file the difference in intensities can be seen between the samples



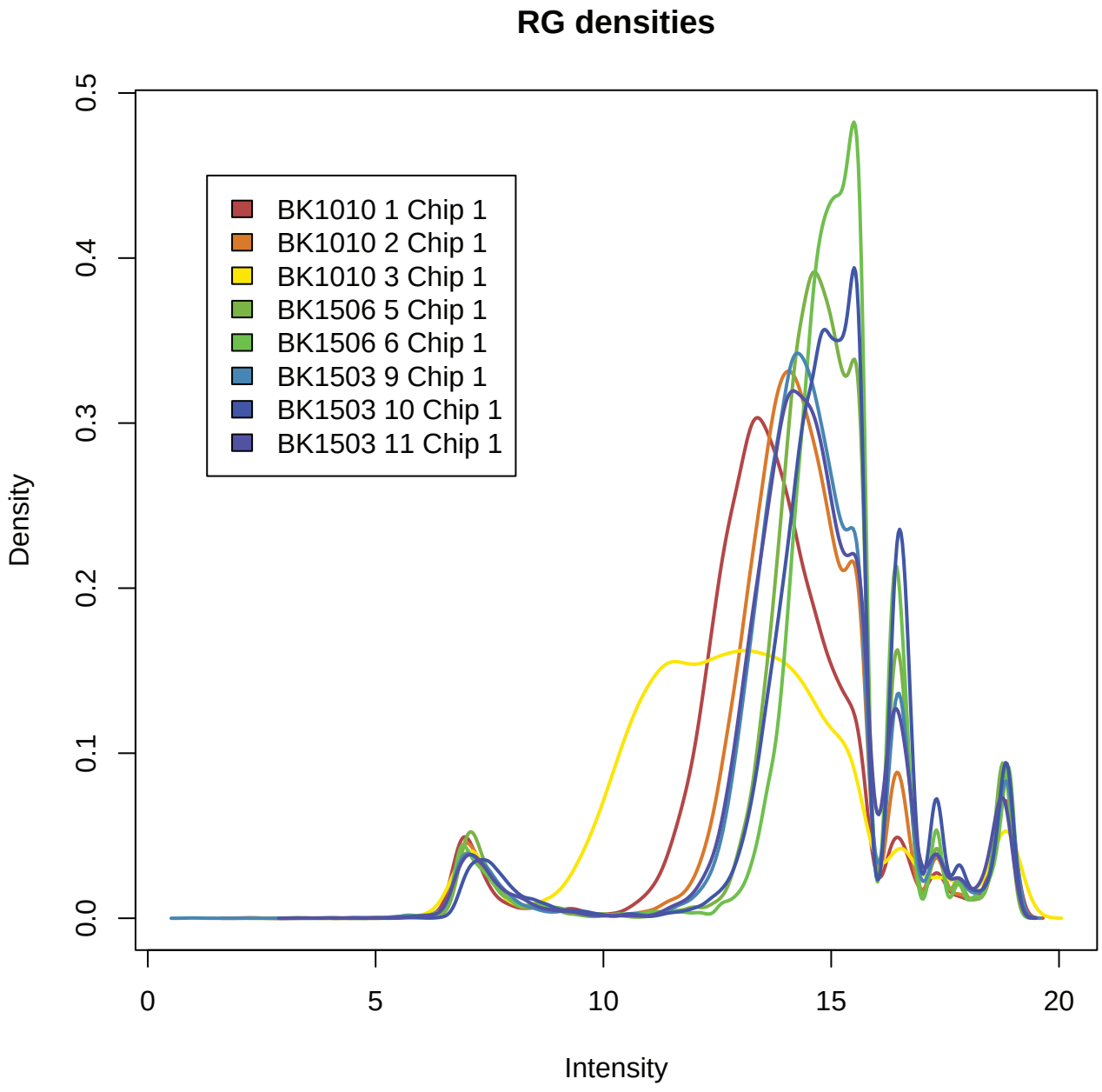
SUPPLEMENTARY MATERIAL

Raw image file from chip 2

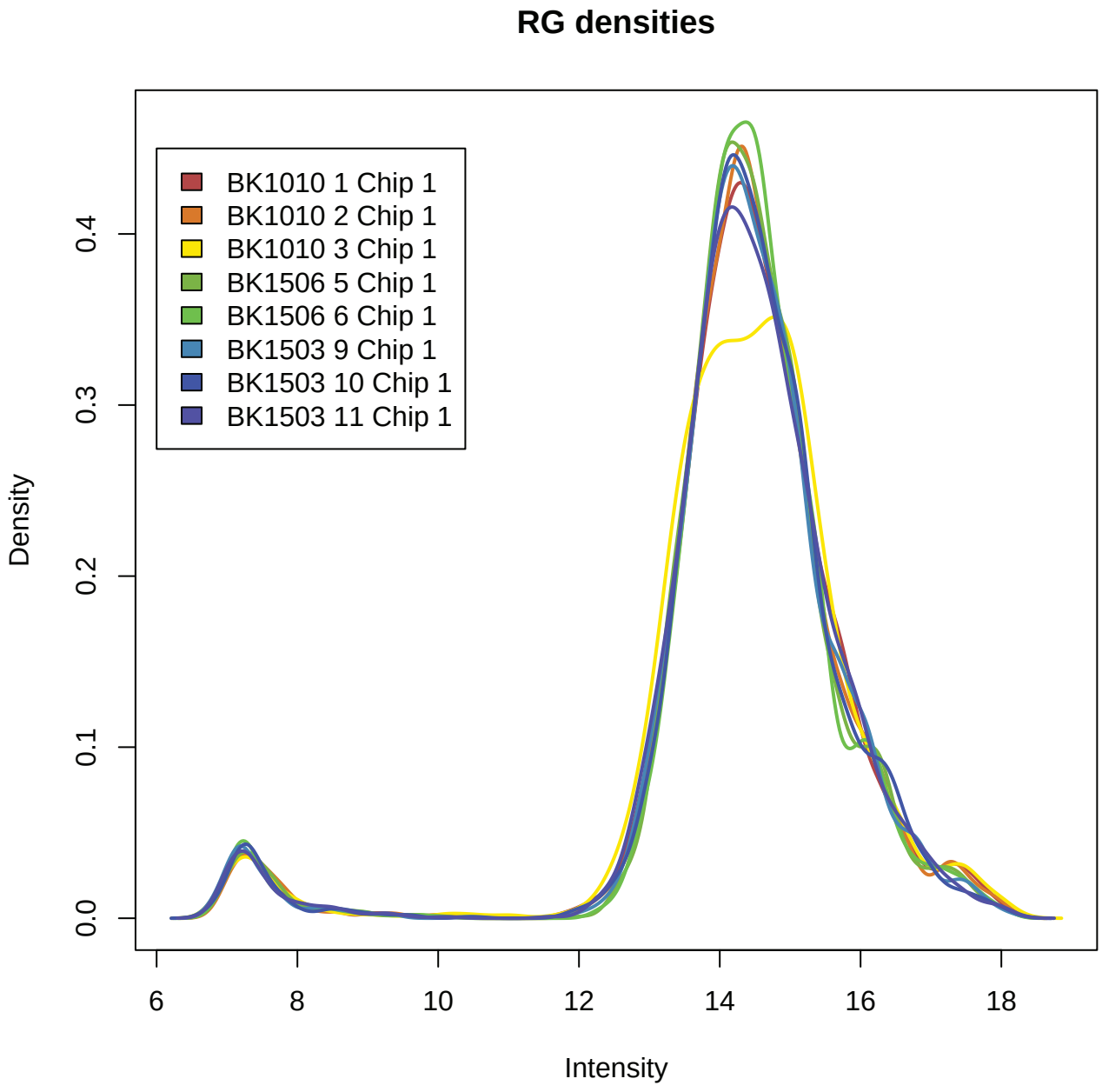
This is the raw image file of the chip. Just by a visual inspection of this file the difference in intensities can be seen between the samples



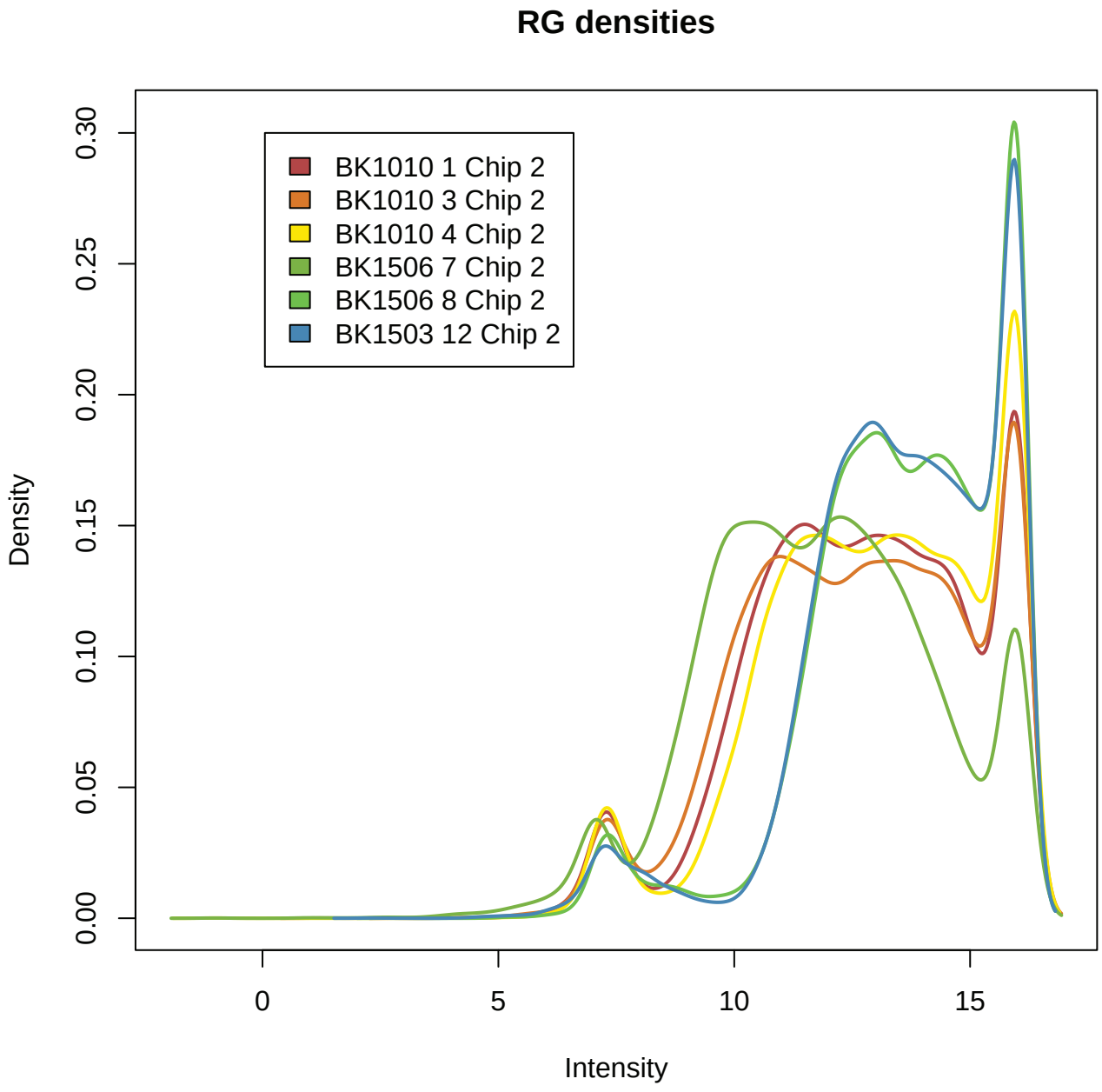
Density plot of all the samples from chip 1 before normalization.



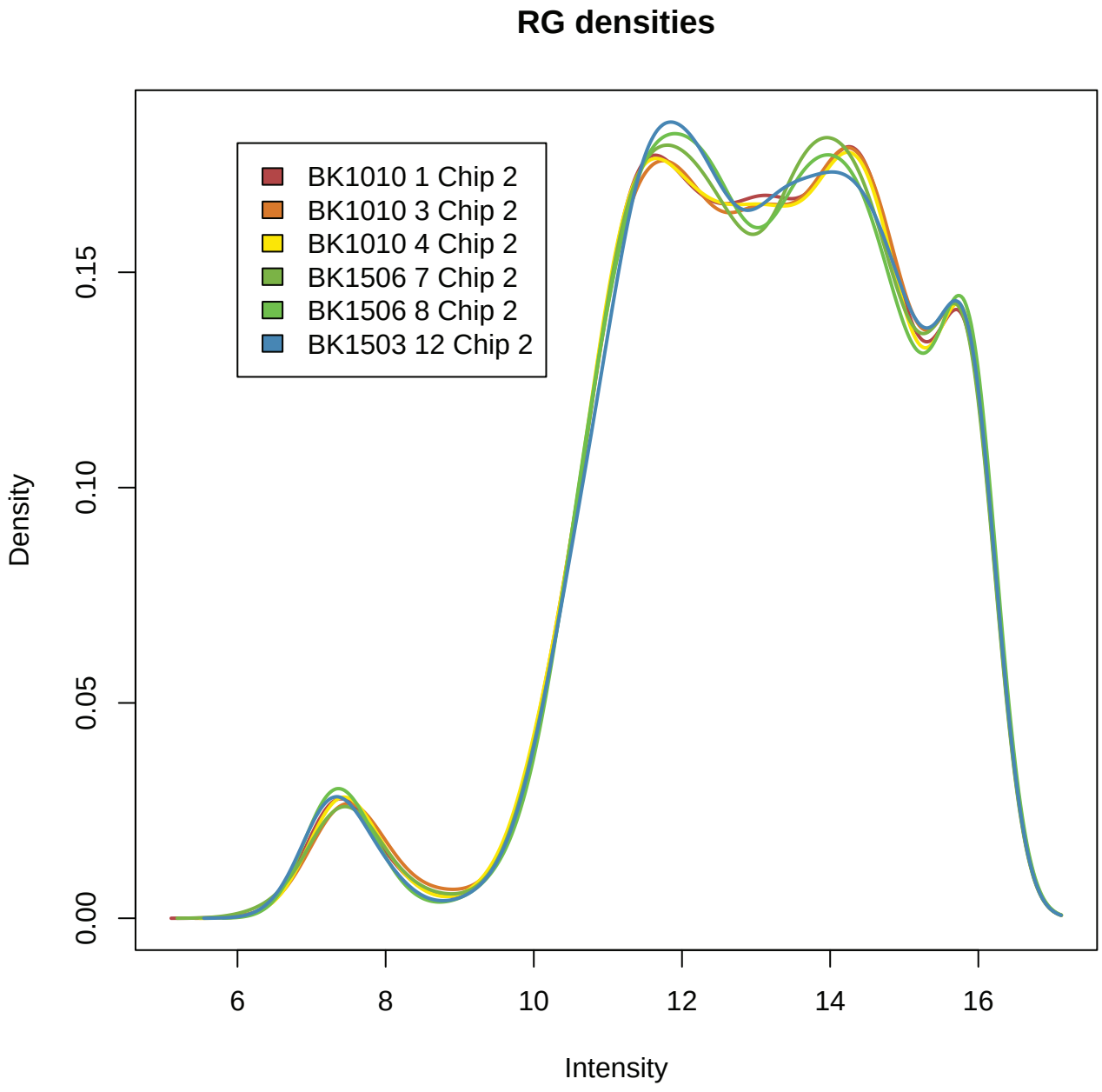
Density plot of all samples from chip 1 after nomalization (note sample 3).



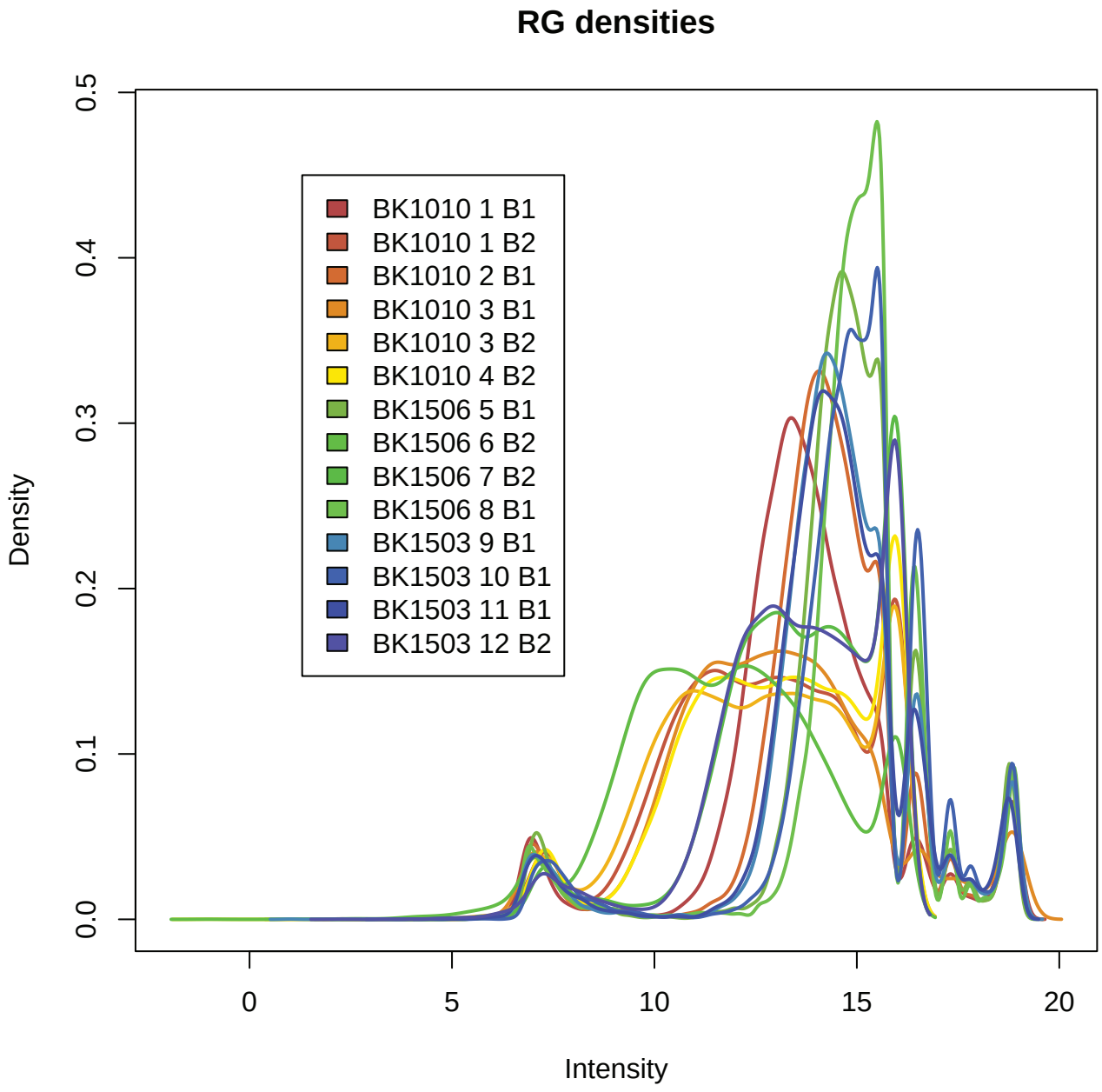
Density plot of all samples from chip 2.



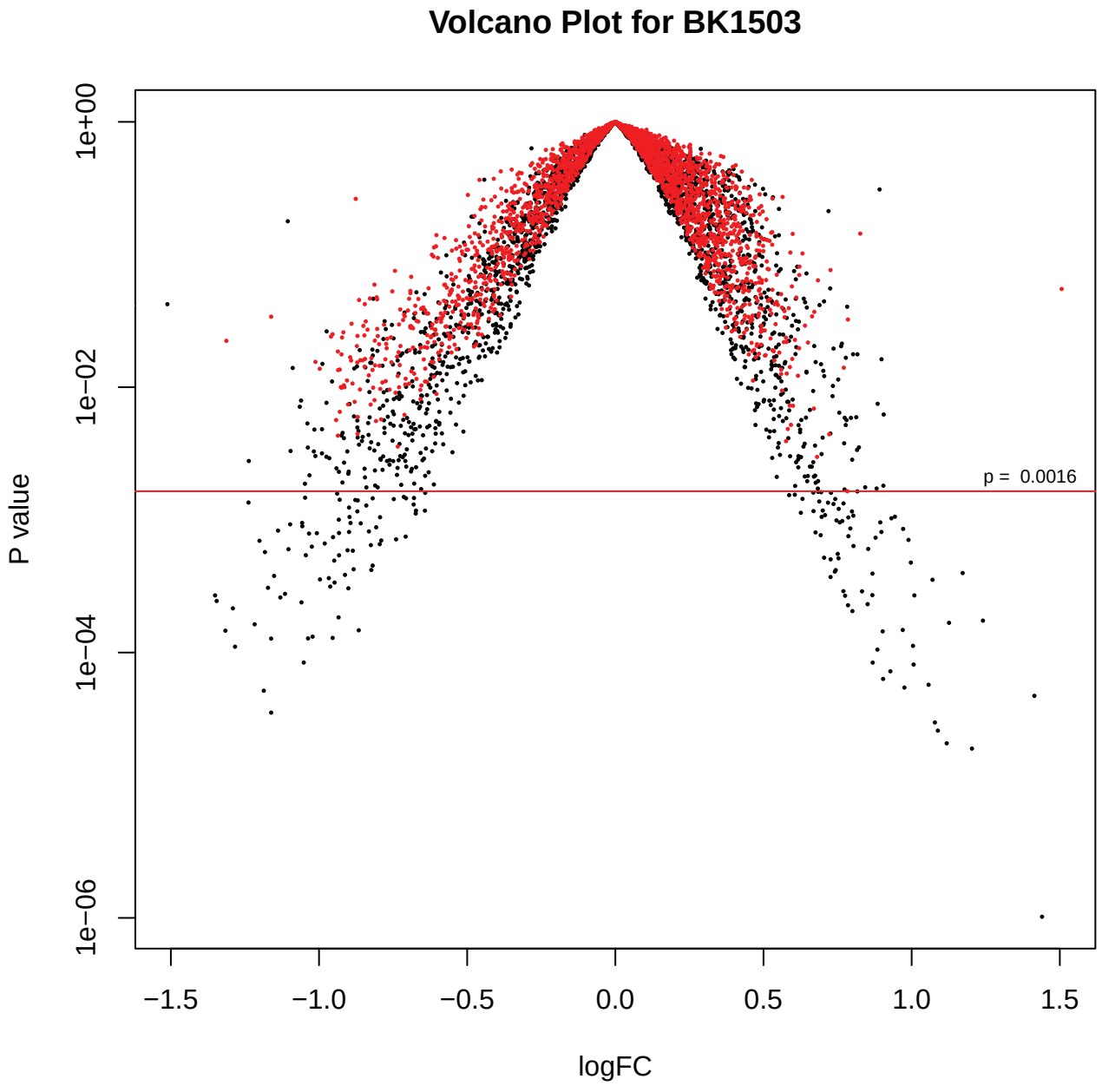
Density plot of all samples from chip 2 after normalization



Density plot of all samples (chip 1 and 2) before normalization

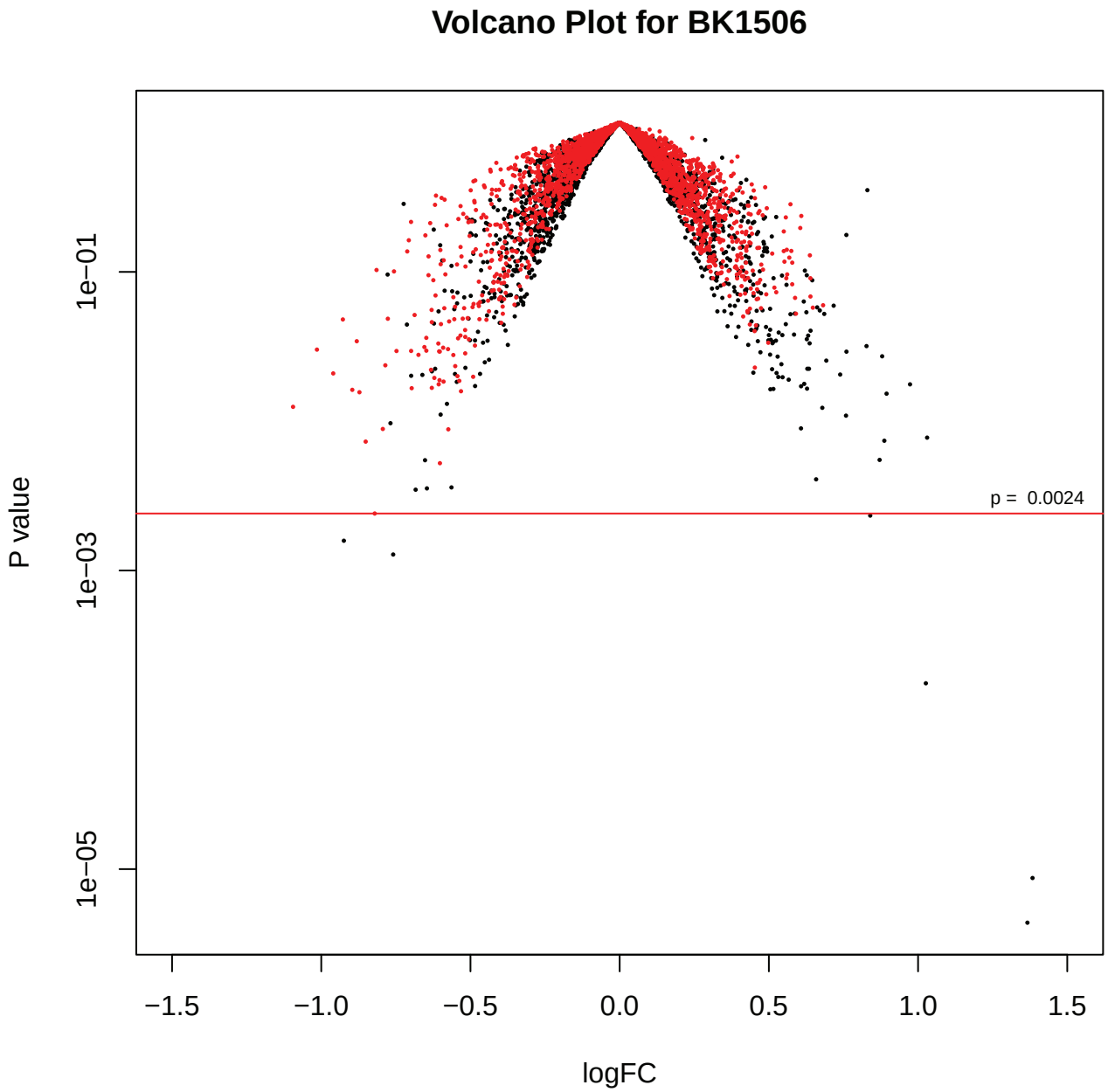


Volcano plot for chip 1, BK1503 vs. BK1010



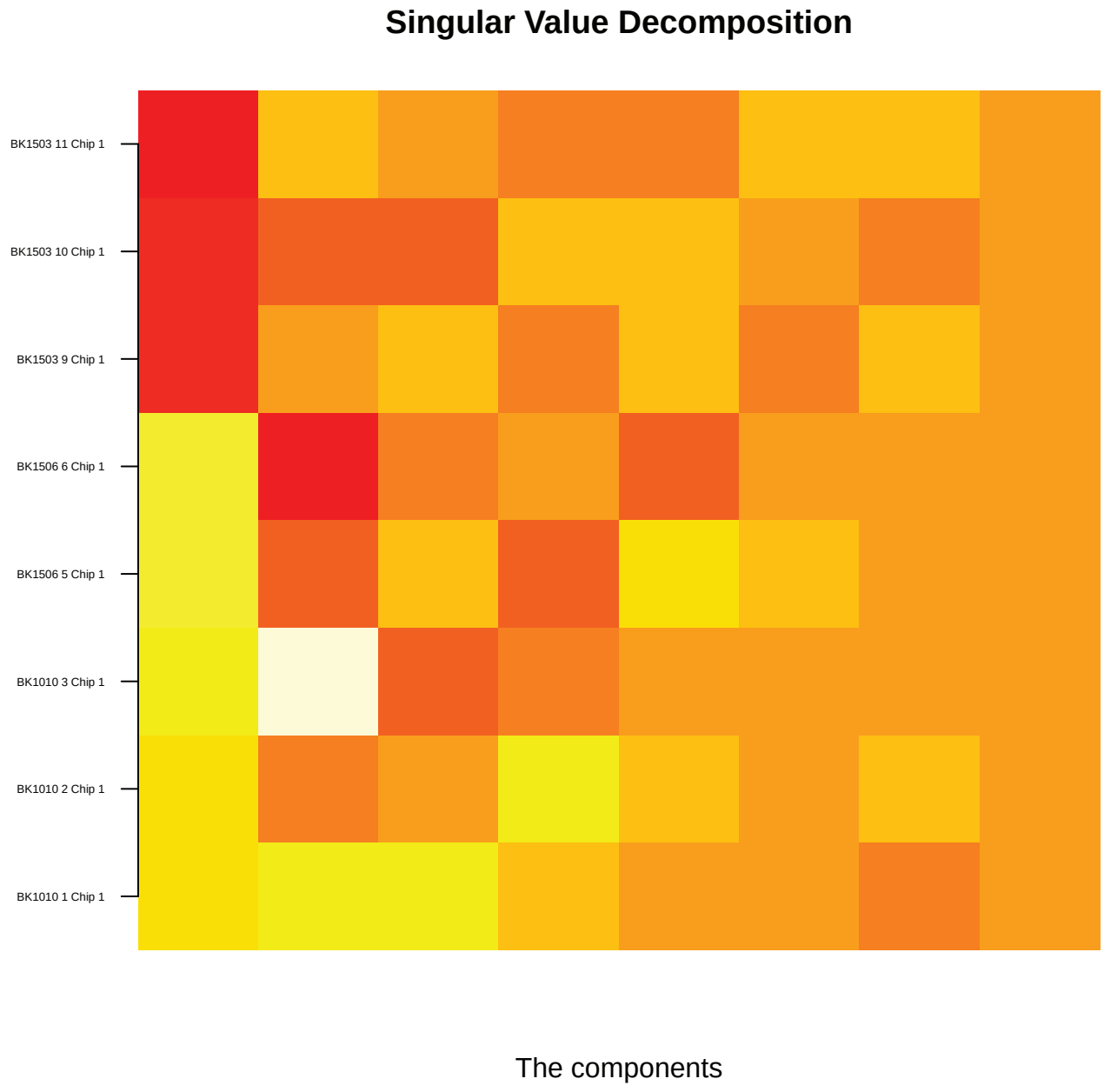
A visualization of the false detection rate (FDR) by random permutation of samples.

Volcano plot for chip 1, BK1506 vs. BK1010



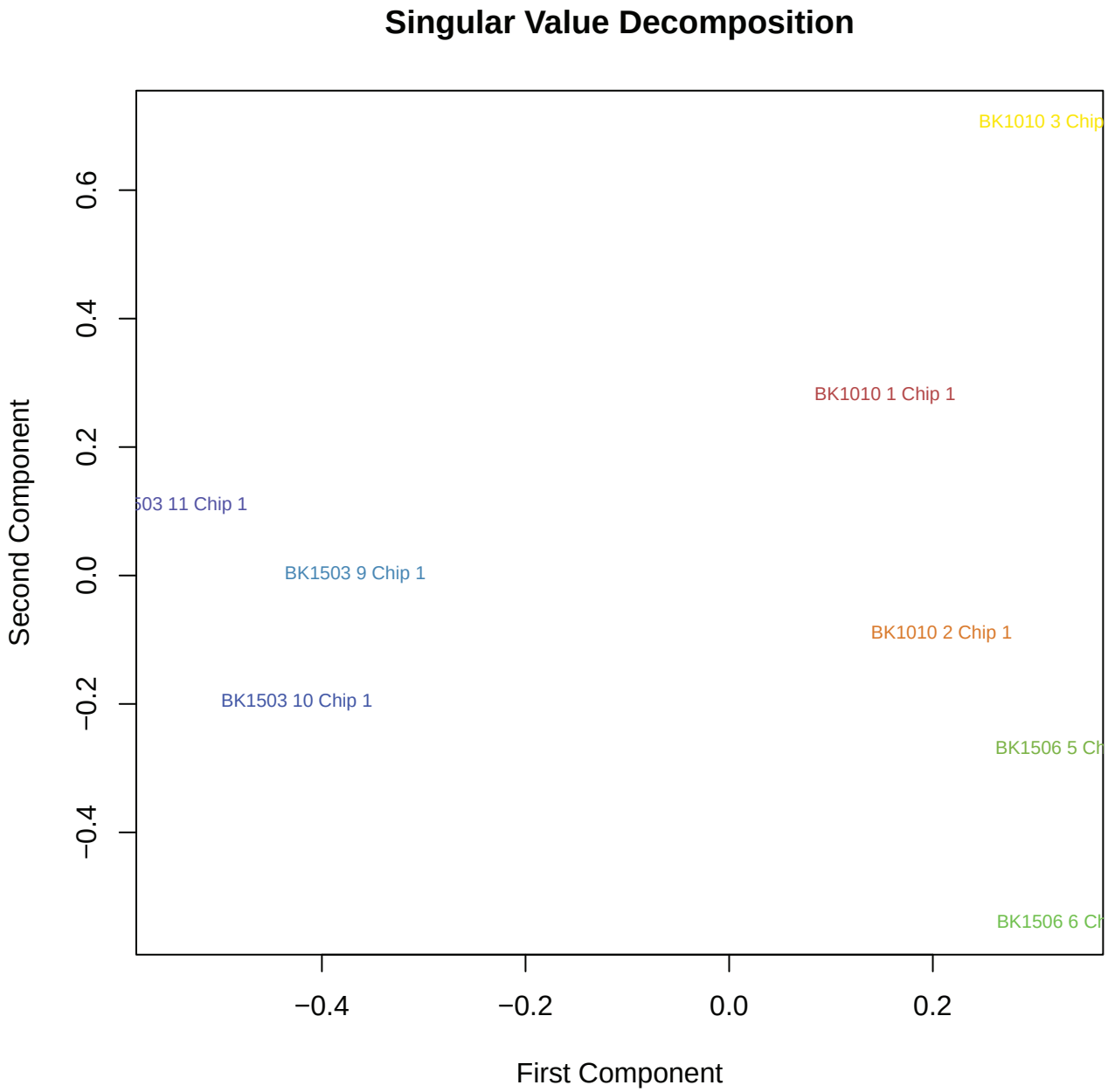
A visualization of the false detection rate (FDR) by random permutation of samples.

Heat map of singular value decomposition of the samples from chip 1.

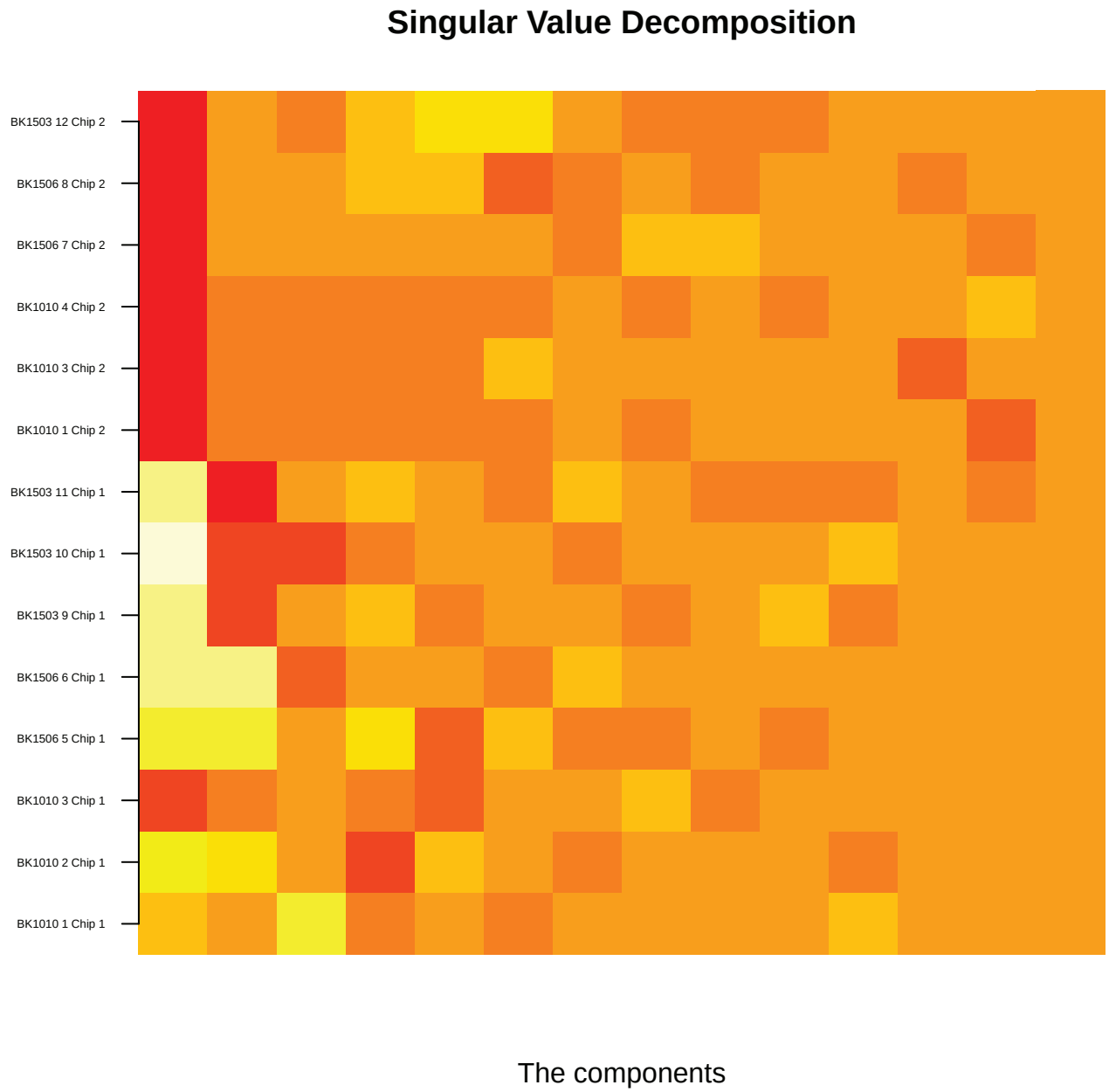


Note that the samples from each strain groups together.

Plot of the singular value decomposition of the samples from chip 1.

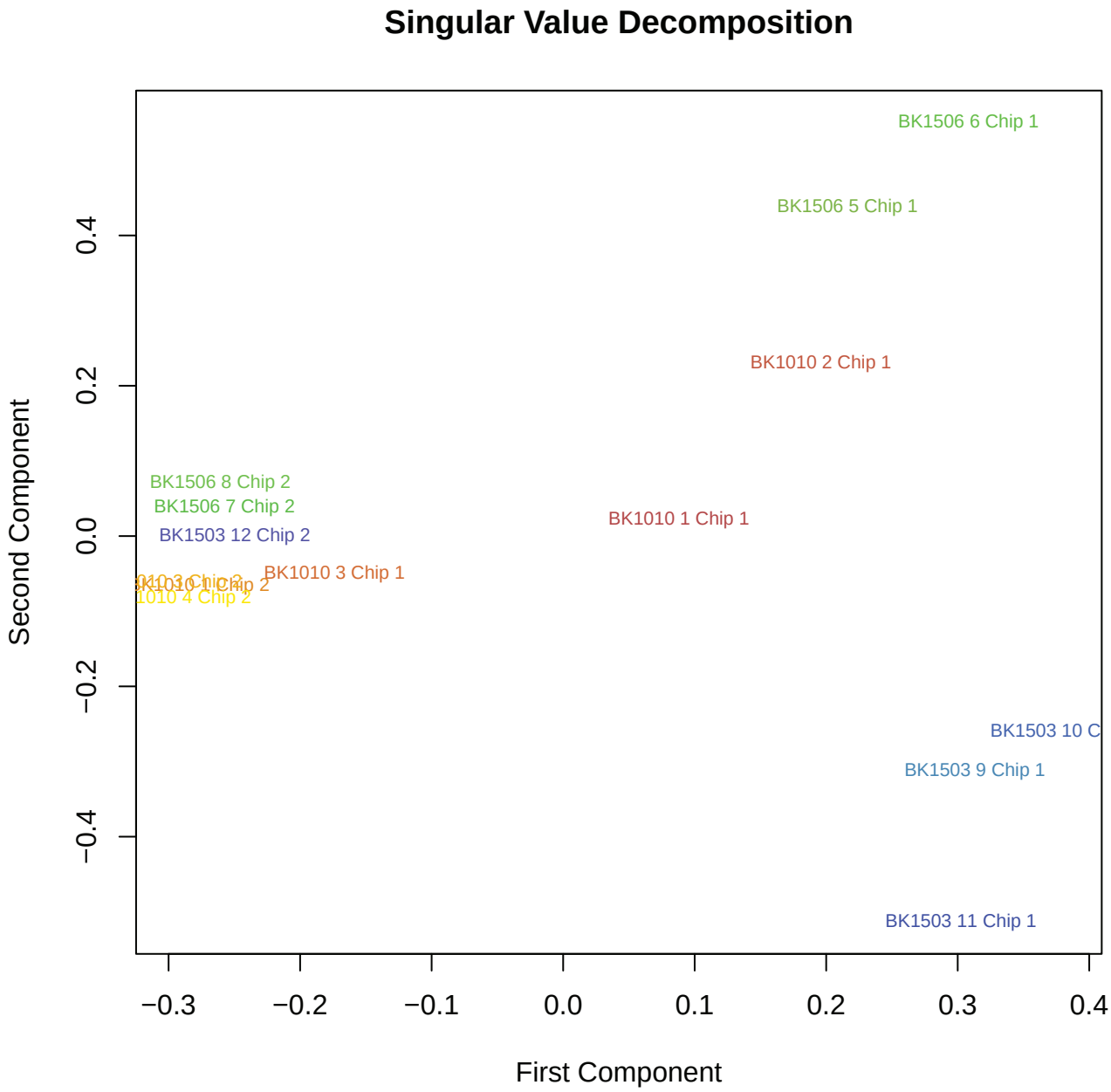


Singular value decomposition of the samples from both chip 1 and chip 2.



Note that the component which captures the most variance (first column) appears to be chip number.

Plot of singular value decomposition of all samples from both chip 1 and chip 2.



S.2 – Results from the differential gene expression analysis of chip 1.

This section contains all the results from the analysis of differential gene expression from chip 1 sorted by pathways and regulatory groups.

Trancsription factors

Gene/locus	BK1506		BK1503		Annotation
	logFC	p-value	logFC	p-value	
adaA	-0.31	3.5E-02	0.01	9.3E-01	AraC family transcriptional regulator, regulatory protein of adaptative response / methylphosphotriester-DNA alkyltransferase methyltransferase [EC:2.1.1.-]
fruR	-0.05	7.2E-01	0.10	4.7E-01	lactose transport regulator, DeoR family transcriptional regulator, fructose operon transcriptional repressor
malR	-0.16	1.8E-01	0.55	4.2E-04	maltose operon transcriptional repressor
ccpA	-0.10	5.3E-01	0.01	9.2E-01	catabolite control protein A
rbsR	-0.01	9.6E-01	-0.15	2.3E-01	ribose operon repressor
llmg_0956	-0.14	5.9E-01	0.45	7.4E-02	LacI family transcription regulator
treR	-0.31	3.5E-02	-0.47	2.5E-03	trehalose operon transcriptional repressor
rgrB	0.04	7.4E-01	0.57	6.2E-04	GntR family transcriptional regulator
rmaG	-0.46	4.4E-02	-0.47	2.8E-02	MarR family transcriptional regulator
glnR	0.11	4.6E-01	-0.27	6.4E-02	glutamine synthetase repressor
fur	-0.45	4.5E-03	0.07	5.7E-01	ferric uptake regulation protein
flpB	0.14	3.8E-01	-0.03	8.6E-01	transcriptional regulator FNR like protein B
flpA	-0.01	9.7E-01	-0.02	8.9E-01	FNR like protein A
llmg_1224	-0.08	5.5E-01	0.42	6.6E-03	transcriptional regulator
llmg_0709	-0.25	2.2E-01	0.10	5.9E-01	PadR-like family transcriptional regulator
llmg_2339	0.09	4.8E-01	0.09	4.5E-01	transcriptional regulator
arsR	0.23	1.8E-01	0.07	6.6E-01	regulator of arsenical resistance
birA1	-0.23	9.3E-02	-0.04	7.1E-01	biotin--[acetyl-CoA-carboxylase] ligase and biotin operon repressor (EC:6.3.4.15)
birA2	-0.01	9.5E-01	0.20	8.1E-02	acetyl-CoA carboxylase ligase / biotin operon repressor bifunctional protein (EC:6.3.4.
codY	0.01	9.7E-01	0.84	1.5E-04	transcriptional repressor CodY
ps602	0.31	4.4E-02	0.53	1.5E-03	hypothetical protein
mtlR	-0.03	8.6E-01	-0.10	4.7E-01	transcriptional regulator mtl operon MtlR
ahrC	-0.22	1.7E-01	-0.08	5.9E-01	arginine transcriptional regulator
argR	0.05	6.8E-01	0.34	8.0E-03	arginine repressor
cspE	-0.12	4.8E-01	0.41	1.9E-02	cold shock-like protein CspE
cspD2	0.06	6.7E-01	0.30	5.2E-02	cold shock-like protein cspD2
cspB	-0.23	3.0E-01	0.02	9.0E-01	cold shock-like protein CspB
hrcA	-0.45	1.7E-02	0.34	4.0E-02	heat-inducible transcription repressor
tenA	-0.19	2.0E-01	-0.10	4.3E-01	transcriptional activator TenA
ctsR	0.18	1.6E-01	0.17	1.4E-01	transcriptional regulator CtsR
parA	0.43	9.8E-02	-0.11	6.2E-01	chromosome partitioning protein ParA
pyrR	-0.18	2.3E-01	-0.63	7.9E-04	bifunctional pyrimidine regulatory protein PyrR uracil phosphoribosyltransferase
nrdR	-0.30	4.1E-02	-0.39	8.3E-03	transcriptional regulator NrdR
purR	-0.40	4.1E-02	0.57	4.5E-03	pur operon repressor
comX	-0.39	5.7E-02	-0.11	5.1E-01	competence regulator ComX
rpoD	-0.31	1.2E-01	0.09	6.2E-01	RNA polymerase sigma factor RpoD

Glycolysis

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
glk	-0.25	7.0E-02	-0.57	4.9E-04	glucokinase
pgi	-0.16	3.5E-01	0.54	4.0E-03	glucose-6-phosphate isomerase
pfk	-0.26	6.1E-02	-0.56	6.9E-04	6-phosphofructokinase
pyk	-0.27	9.4E-02	-1.09	1.3E-05	pyruvate kinase
ldh	-0.51	1.5E-02	-0.45	1.8E-02	L-lactate dehydrogenase
fbp	0.60	7.9E-03	0.65	2.8E-03	fructose-bisphosphatase
fbaA	-0.36	1.2E-02	-0.46	1.8E-03	fructose-bisphosphate aldolase
tpiA	-0.24	1.6E-01	-0.68	9.7E-04	triosephosphate isomerase
gapB	-0.07	5.2E-01	0.03	7.8E-01	glyceraldehyde 3-phosphate dehydrogenase
gapA	0.22	8.5E-02	-0.08	4.5E-01	glyceraldehyde 3-phosphate dehydrogenase
pgk	-0.35	5.5E-02	-0.12	4.3E-01	phosphoglycerate kinase
llmg_1894	0.06	6.6E-01	-0.01	9.1E-01	phosphoglycerate mutase family protein
gpmA	-0.30	5.5E-02	-0.67	4.0E-04	phosphoglyceromutase
gpmB	-0.04	7.5E-01	-0.51	9.1E-04	phosphoglycerate mutase
gpmC	0.25	6.4E-02	-0.56	5.3E-04	phosphoglycerate mutase
eno	-0.28	8.0E-02	-0.17	2.2E-01	phosphopyruvate hydratase
pdhA	0.01	9.7E-01	-0.51	1.1E-02	pyruvate dehydrogenase E1 component alpha subunit
pdhB	0.27	2.4E-01	-0.36	9.6E-02	pyruvate dehydrogenase E1 component beta subunit
pdhC	0.14	5.0E-01	-0.35	9.8E-02	pyruvate dehydrogenase complex E2 component
pdhD	0.02	9.2E-01	-0.20	3.4E-01	dihydrolipoamide dehydrogenase
ldhX	-0.18	3.1E-01	0.45	1.6E-02	L-lactate dehydrogenase
ldhB	0.17	2.3E-01	0.10	4.5E-01	L-lactate dehydrogenase
adhE	0.52	6.7E-02	-0.10	6.8E-01	bifunctional acetaldehyde-CoA/alcohol dehydrogenase
adhA, adhP	0.16	3.0E-01	-0.24	1.1E-01	alcohol dehydrogenase
llmg_0955	-0.12	6.2E-01	0.04	8.4E-01	alcohol dehydrogenase
fadD	-0.26	7.1E-02	-0.12	3.2E-01	long-chain acyl-CoA synthetase
galM	0.57	6.2E-02	0.24	3.4E-01	aldose 1-epimerase
bglA2	0.24	1.1E-01	0.07	5.7E-01	6-phospho-beta-glucosidase
arb	-0.07	5.9E-01	0.61	4.1E-04	6-phospho-beta-glucosidase
celA	0.25	1.1E-01	-0.02	9.1E-01	6-phospho-beta-glucosidase

TCA cycle

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
gltA	-0.02	8.6E-01	-0.13	2.8E-01	citrate synthase
citB	-0.10	4.4E-01	-0.13	2.5E-01	aconitate hydratase
icd	-0.09	4.6E-01	-0.01	9.5E-01	isocitrate dehydrogenase
pdhD	0.02	9.2E-01	-0.20	3.4E-01	dihydrolipoamide dehydrogenase
frdC	-0.29	5.8E-02	-0.28	4.2E-02	fumarate reductase flavoprotein subunit
pycA	-0.36	4.8E-02	-0.60	2.3E-03	pyruvate carboxylase
pdhA	0.01	9.7E-01	-0.51	1.1E-02	pyruvate dehydrogenase E1 component alpha subunit
pdhB	0.27	2.4E-01	-0.36	9.6E-02	pyruvate dehydrogenase E1 component beta subunit
pdhC	0.14	5.0E-01	-0.35	9.8E-02	pyruvate dehydrogenase complex E2 component

Pentose phosphate pathway

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
pgi	-0.16	3.5E-01	0.54	4.0E-03	glucose-6-phosphate isomerase
llmg_2499	0.04	8.5E-01	-0.38	4.4E-02	glucose-6-phosphate 1-dehydrogenase
llmg_2431	0.10	4.1E-01	-0.06	5.7E-01	hypothetical protein
gntZ	0.29	3.3E-02	-0.12	2.9E-01	6-phosphogluconate dehydrogenase-like protein
gnd	-0.19	1.4E-01	-0.50	1.0E-03	6-phosphogluconate dehydrogenase
rpe	-0.16	2.9E-01	-0.60	1.3E-03	ribulose-phosphate 3-epimerase
rpe2	0.08	6.3E-01	0.24	1.2E-01	ribulose-phosphate 3-epimerase
tkt	-0.02	8.9E-01	-0.54	1.2E-03	transketolase
rpiA	-0.26	9.8E-02	0.47	5.9E-03	ribose-5-phosphate isomerase A
rpiB	-0.14	3.0E-01	0.08	5.3E-01	ribose-5-phosphate isomerase B
deoC	-0.03	8.0E-01	-0.58	4.5E-04	deoxyribose-phosphate aldolase
rbsK	0.07	6.1E-01	0.08	5.1E-01	ribokinase
deoB	-0.25	7.1E-02	-0.76	6.4E-05	phosphopentomutase
prsB	-0.32	3.6E-02	-0.26	6.1E-02	ribose-phosphate pyrophosphokinase
prsA	-0.16	3.6E-01	0.06	7.0E-01	ribose-phosphate pyrophosphokinase
kdgA	0.55	2.7E-02	-0.06	7.5E-01	keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase
gntK	0.03	8.2E-01	-0.26	8.3E-02	gluconate kinase
fbaA	-0.36	1.2E-02	-0.46	1.8E-03	fructose-bisphosphate aldolase
fbp	0.60	7.9E-03	0.65	2.8E-03	fructose-bisphosphatase
pfk	-0.26	6.1E-02	-0.56	6.9E-04	6-phosphofructokinase

Pyruvate metabolism

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
fadD	-0.26	7.1E-02	-0.12	3.2E-01	long-chain acyl-CoA synthetase
pdhA	0.01	9.7E-01	-0.51	1.1E-02	pyruvate dehydrogenase E1 component alpha subunit
pdhB	0.27	2.4E-01	-0.36	9.6E-02	pyruvate dehydrogenase E1 component beta subunit
pdhC	0.14	5.0E-01	-0.35	9.8E-02	pyruvate dehydrogenase complex E2 component
pdhD	0.02	9.2E-01	-0.20	3.4E-01	dihydrolipoamide dehydrogenase
pfl	0.03	8.4E-01	0.19	1.5E-01	formate acetyltransferase
adhE	0.52	6.7E-02	-0.10	6.8E-01	bifunctional acetaldehyde-CoA/alcohol dehydrogenase
ackA2	-0.33	5.7E-02	-0.35	2.9E-02	acetate kinase
ackA1	0.30	7.5E-02	-0.04	7.8E-01	AckA1 protein
eutD	-0.25	6.9E-02	-0.23	6.4E-02	phosphotransacetylase
pyk	-0.27	9.4E-02	-1.09	1.3E-05	pyruvate kinase
accA	-0.22	1.2E-01	-0.01	9.5E-01	AccA protein
accB	-0.23	2.2E-01	-1.05	8.0E-05	acetyl-CoA carboxylase biotin carboxyl carrier protein subunit
accC	-0.26	5.7E-02	-0.71	9.2E-05	acetyl-CoA carboxylase biotin carboxylase subunit
accD	-0.14	2.4E-01	-0.28	2.6E-02	acetyl-CoA carboxylase subunit beta
llmg_0568	-0.04	8.1E-01	-0.27	9.8E-02	acylphosphatase
poxL	0.24	2.0E-01	-0.11	4.9E-01	pyruvate oxidase
ldhX	-0.18	3.1E-01	0.45	1.6E-02	L-lactate dehydrogenase
ldhB	0.17	2.3E-01	0.10	4.5E-01	L-lactate dehydrogenase
ldh	-0.51	1.5E-02	-0.45	1.8E-02	L-lactate dehydrogenase
llmg_0184	0.07	5.9E-01	0.80	5.7E-05	putative lactoylglutathione lyase
mleS	0.03	8.7E-01	-0.17	2.9E-01	malate dehydrogenase
pycA	-0.36	4.8E-02	-0.60	2.3E-03	pyruvate carboxylase
thiL	-0.19	2.2E-01	0.10	5.0E-01	ThiL protein

Amino sugar and nucleotide sugar metabolism

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
chiC	0.08	5.8E-01	-0.16	2.3E-01	acidic endochitinase precursor
nagZ	0.07	5.9E-01	-0.02	8.8E-01	putative beta-N-acetylglucosaminidase
murQ, yfeU	0.31	5.1E-02	-0.08	5.5E-01	N-acetylmuramic acid-6-phosphate etherase
glmU	-0.30	4.6E-02	-0.56	1.0E-03	bifunctional N-acetylglucosamine-1-phosphate uridyltransferase/glucosamine-1-phosphate acetyltransferase
llmg_1317	-0.38	4.8E-02	0.49	9.4E-03	N-acetylmannosamine-6-phosphate 2-epimerase
murA2	-0.63	7.7E-04	-0.35	1.6E-02	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
murA1	-0.02	9.0E-01	0.00	9.8E-01	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
murB	0.02	8.8E-01	-0.02	8.9E-01	UDP-N-acetylenolpyruvoylglucosamine reductase
glmM	-0.12	3.8E-01	0.13	3.0E-01	phosphoglucosamine mutase
nagA	-0.64	2.9E-02	-0.45	7.7E-02	NagA protein
scrK	0.53	1.1E-03	-0.22	6.6E-02	fructokinase
nagB	-0.23	1.5E-01	0.40	1.2E-02	glucosamine-6-phosphate isomerase
glmS	-0.37	2.0E-02	-0.98	1.4E-05	glucosamine--fructose-6-phosphate aminotransferase
llmg_1608	0.35	1.7E-01	-0.09	7.0E-01	putative glycosyl hydrolases
llmg_1320	-0.12	4.5E-01	-0.16	2.8E-01	putative xylan beta-1,4-xylosidase
glk	-0.25	7.0E-02	-0.57	4.9E-04	glucokinase
pgi	-0.16	3.5E-01	0.54	4.0E-03	glucose-6-phosphate isomerase
galU	-0.14	4.5E-01	0.19	2.7E-01	UDP--glucose-1-phosphate uridylyltransferase
galT	0.16	3.6E-01	0.02	9.0E-01	galactose-1-phosphate uridylyltransferase
ugd	-0.01	9.7E-01	-0.09	5.1E-01	UDP-glucose 6-dehydrogenase
galK	0.25	2.0E-01	0.06	7.1E-01	galactokinase
galE	-0.32	5.6E-02	0.33	3.7E-02	GalE protein
llmg_2003	0.09	5.7E-01	0.23	1.2E-01	UDP-glucose 4-epimerase
llmg_0247	0.65	8.5E-03	0.06	7.5E-01	putative UDP-glucose 4-epimerase
ptnAB	-0.20	2.0E-01	-0.78	1.6E-04	PTS system, mannose-specific IIAB components
ptnC	-0.03	8.6E-01	-0.65	6.3E-04	mannose-specific PTS system component IIC
ptnD	-0.18	1.9E-01	-0.49	1.8E-03	mannose-specific PTS system component IID
pmi	-0.05	7.2E-01	0.98	4.0E-05	mannose-6-phosphate isomerase
glgD	0.17	3.6E-01	0.07	6.8E-01	glucose-1-phosphate adenylyltransferase
glgC	0.31	1.6E-01	0.11	5.5E-01	glucose-1-phosphate adenylyltransferase

Phosphotransferase system (PTS) and glucose uptake

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
ptsI	-0.11	4.9E-01	-0.37	2.1E-02	phosphoenolpyruvate-protein phosphotransferase
ptsH	0.04	7.5E-01	0.24	7.5E-02	phosphocarrier protein HPr
ptsK	-0.25	1.0E-01	0.32	3.0E-02	HPr kinase/phosphorylase
hprT	-0.26	1.4E-01	0.30	6.4E-02	HprT protein
llmg_1426	0.22	1.0E-01	-0.15	2.1E-01	sucrose-specific PTS system IIBC component
bglP	0.17	3.5E-01	0.01	9.3E-01	PTS system, beta-glucosides specific enzyme IIABC
llmg_0453	0.07	5.7E-01	-0.01	9.6E-01	sucrose-specific PTS enzyme IIABC
llmg_0454	0.23	1.1E-01	-0.25	6.3E-02	beta-glucoside-specific PTS system IIABC component
ptcA	-0.10	5.7E-01	1.00	1.1E-04	cellobiose-specific PTS system IIA component
ptcB	0.15	4.7E-01	0.83	1.1E-03	cellobiose-specific PTS system IIB component
llmg_1244	0.40	7.3E-02	0.32	1.1E-01	hypothetical protein
ptcC	0.59	1.5E-02	-0.06	7.5E-01	cellobiose-specific PTS system IIC component
celB	1.34	6.3E-06	0.33	3.8E-02	cellobiose-specific PTS system IIC component
mtlF	0.23	1.0E-01	0.02	8.8E-01	PTS system, mannitol-specific IIA component
mtlA	0.23	1.4E-01	-0.15	2.9E-01	PTS system, mannitol-specific IIBC component
ptnAB	-0.20	2.0E-01	-0.78	1.6E-04	PTS system, mannose-specific IIAB components
ptnC	-0.03	8.6E-01	-0.65	6.3E-04	mannose-specific PTS system component IIC
ptnD	-0.18	1.9E-01	-0.49	1.8E-03	mannose-specific PTS system component IID
llmg_0866	0.42	1.0E-02	0.15	2.4E-01	PTS system, unknown pentitol phosphotransferase enzyme IIB component
ulaA, sgaT	0.85	3.9E-03	0.12	5.7E-01	PTS system ascorbate-specific transporter subunit IIC
fruA	0.37	4.7E-02	-0.19	2.4E-01	PTS system, fructose specific IIBC components
glcU	-0.19	2.0E-01	0.06	6.1E-01	putative glucose uptake protein GlcU

RNA degradation

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
nudH	-0.05	6.8E-01	-0.17	1.8E-01	dinucleoside polyphosphate hydrolase
eno	-0.28	8.0E-02	-0.17	2.2E-01	phosphopyruvate hydratase
pnpA	-0.12	4.8E-01	-0.56	4.1E-03	polynucleotide phosphorylase/polyadenylase
vacB1	0.10	6.6E-01	-0.49	3.0E-02	putative exoribonuclease R
vacB2	-0.21	2.0E-01	0.89	1.1E-04	putative exoribonuclease R
rheA	-0.09	7.1E-01	0.06	7.9E-01	ATP-dependent RNA helicase
recQ	-0.01	9.4E-01	-0.38	1.5E-02	ATP-dependent DNA helicase RecQ
llmg_0302	0.21	2.8E-01	-0.60	5.4E-03	putative Zn-dependent hydrolase
dnaK	-0.25	1.1E-01	-0.19	1.9E-01	molecular chaperone DnaK
groEL	-0.14	2.6E-01	-0.31	1.5E-02	chaperonin GroEL

ABC transporters

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
potD	-0.51	6.8E-03	0.47	7.1E-03	spermidine/putrescine ABC transporter substrate-binding protein
potC	-0.09	5.8E-01	-0.03	8.4E-01	spermidine/putrescine ABC transporter permease
potB	-0.01	9.5E-01	-0.11	3.9E-01	spermidine/putrescine ABC transporter permease
potA	-0.28	4.3E-02	-0.17	1.5E-01	spermidine/putrescine ABC transporter ATP-binding protein
busAB	-0.28	9.6E-02	0.30	5.1E-02	glycine betaine-binding periplasmic protein precursor
busAA	-0.10	4.1E-01	-0.35	1.1E-02	glycine betaine/proline ABC transporter
choS	-0.17	4.1E-01	0.35	7.8E-02	choline ABC transporter permease and substrate binding protein
choQ	-0.01	9.2E-01	-0.33	1.5E-02	choline ABC transporter ATP binding protein
malE	-0.09	5.0E-01	-0.16	1.8E-01	maltose ABC transporter substrate binding protein
malF	0.72	4.2E-02	0.14	6.3E-01	maltose transport system permease protein MalF
malG	0.61	1.2E-01	0.36	2.9E-01	maltose ABC transporter permease protein MalG
msmK	-0.01	9.6E-01	0.80	1.8E-04	multiple sugar-binding transport ATP-binding protein
rbsB	0.09	5.9E-01	0.00	1.0E+00	ribose ABC transporter substrate binding protein RbsB
rbsC	-0.02	9.2E-01	-0.08	6.8E-01	ribose transport system permease protein RbsC
rbsD	0.24	1.7E-01	-0.04	7.9E-01	ribose ABC transporter permease protein RbsD
rbsD	0.06	6.7E-01	-0.02	8.8E-01	D-ribose pyranase
pstE	-0.48	4.3E-03	-0.54	1.1E-03	phosphate transport substrate binding protein PstE
pstF	-0.01	9.7E-01	-0.45	4.4E-03	phosphate transport substrate binding protein PstF
pstD	0.15	4.7E-01	-0.84	1.2E-03	phosphate transport system permease protein PstD
pstC	0.17	3.4E-01	-0.76	6.0E-04	phosphate transport system permease protein PstC
pstA	-0.65	5.1E-03	-0.62	3.9E-03	phosphate transporter ATP-binding protein
pstB	-0.16	3.2E-01	-0.69	6.3E-04	phosphate transporter ATP-binding protein
phnD	-0.33	4.5E-02	-0.32	3.5E-02	phosphonate ABC transporter, phosphonate-binding protein PhnD
phnB	-0.22	1.4E-01	-0.79	1.1E-04	phosphonate transport system permease protein PhnB
llmg_0315	-0.53	4.7E-03	-0.78	1.7E-04	phosphonate ABC transporter permease
phnC	-0.10	5.1E-01	-0.81	1.6E-04	phosphonates import ATP-binding protein PhnC
plpA	-0.09	5.1E-01	-0.05	7.0E-01	D-methionine-binding lipoprotein PlpA precursor
plpB	-0.19	1.8E-01	-0.13	3.0E-01	D-methionine-binding lipoprotein PlpB precursor
plpC	0.11	4.0E-01	-0.28	3.5E-02	D-methionine-binding lipoprotein PlpC precursor
plpD	-0.03	8.1E-01	-0.25	3.9E-02	D-methionine-binding lipoprotein PlpD precursor
llmg_0342	0.44	6.7E-03	-0.22	8.5E-02	amino acid ABC transporter permease protein
llmg_0341	0.44	5.9E-03	-0.24	6.1E-02	amino acid ABC transporter ATP binding protein
dppA	0.14	2.8E-01	-0.16	1.8E-01	dipeptide-binding protein precursor
dppB	0.48	7.6E-03	-0.14	3.2E-01	dipeptide transport system permease protein DppB
dppC	0.92	3.7E-05	0.01	9.2E-01	dipeptide transport system permease protein DppC
dppD	0.76	2.6E-04	-0.23	9.1E-02	dipeptide transport ATP-binding protein DppD
dppF	0.60	2.0E-03	-0.18	1.9E-01	dipeptide transport ATP-binding protein DppF
fhuD	0.05	7.3E-01	-0.05	7.1E-01	ferrichrome ABC transporter substrate binding protein
fhuB	0.43	1.6E-01	-0.06	8.1E-01	ferrichrome ABC transporter permease protein
fhuG	0.25	2.1E-01	0.09	6.0E-01	ferrichrome ABC transporter permease protein
llmg_1281	0.24	4.5E-01	-0.14	6.3E-01	putative ABC transporter ATP-binding protein
fhuC	0.28	2.4E-01	-0.02	9.2E-01	ferrichrome ABC transporter FhuC
zitS	0.11	4.8E-01	0.48	5.2E-03	zinc ABC transporter substrate binding protein
zitP	0.61	3.1E-02	0.34	1.6E-01	zinc ABC transporter permease protein
zitQ	0.28	1.2E-01	0.29	7.6E-02	zinc ABC transporter ATP binding protein
cbiQ	0.50	1.7E-02	0.15	3.6E-01	putative cobalt ABC transporter permease protein
cbiQ2	-0.25	1.3E-01	-0.57	2.4E-03	putative cobalt ABC transporter permease protein
cbiO	0.39	2.2E-02	-0.01	9.4E-01	putative cobalt ABC transporter ATP-binding protein
cbiO	-0.40	2.5E-02	-0.19	1.9E-01	cobalt transporter ATP-binding subunit
cbiO	0.04	7.3E-01	-0.55	8.8E-04	cobalt transporter ATP-binding subunit
llmg_1552	0.13	4.0E-01	0.38	2.3E-02	putative ABC type transport system permease protein
drrB	-0.39	1.5E-01	-0.21	3.9E-01	daunorubicin resistance transmembrane protein
llmg_0262	0.58	1.9E-03	0.25	7.2E-02	ABC transporter permease protein
llmg_1553	0.25	2.3E-01	0.04	8.4E-01	putative ABC transporter ATP-binding protein
drrA	0.18	3.3E-01	0.03	8.4E-01	daunorubicin resistance ABC transporter ATP-binding subunit
tagG	0.96	6.9E-04	0.39	5.7E-02	teichoic acid ABC transporter permease protein
tagH	0.51	1.1E-02	0.11	4.7E-01	teichoic acid export ATP-binding protein TagH
ftsX	-0.24	1.2E-01	-0.30	4.2E-02	cell division protein FtsX-like protein
ftsE	-0.40	4.6E-02	-0.09	6.1E-01	cell division ATP-binding protein
llmg_1202	-0.24	9.2E-02	0.45	3.7E-03	ABC transporter ABC binding and permease protein
cydD	-0.08	6.3E-01	-0.09	5.3E-01	cytochrome d ABC transporter ATP binding and permease protein
cydC	-0.08	5.2E-01	-0.08	4.8E-01	cytochrome d ABC transporter ATP binding and permease protein
llmg_0989	0.09	4.6E-01	-0.03	7.6E-01	ABC transporter ATP binding and permease protein

Two-component system

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
apl	0.30	1.9E-01	0.24	2.4E-01	alkaline phosphatase
pstE	-0.48	4.3E-03	-0.54	1.1E-03	phosphate transport substrate binding protein PstE
pstF	-0.01	9.7E-01	-0.45	4.4E-03	phosphate transport substrate binding protein PstF
kinC	-0.15	3.0E-01	-0.13	3.3E-01	sensor histidine kinase
llrC	-0.17	2.8E-01	-0.03	8.5E-01	two-component system regulator
llmg_0458	-0.19	1.7E-01	0.15	2.3E-01	hypothetical protein
dnaA	-0.36	3.7E-02	0.63	1.2E-03	chromosomal replication initiation protein
mleS	0.03	8.7E-01	-0.17	2.9E-01	malate dehydrogenase
comX	-0.39	5.7E-02	-0.11	5.1E-01	competence regulator ComX
frdC	-0.29	5.8E-02	-0.28	4.2E-02	fumarate reductase flavoprotein subunit
llmg_0018	0.09	5.3E-01	0.30	3.5E-02	beta-lactamase A
kinD	-0.14	2.4E-01	-0.27	3.1E-02	sensor protein kinase KinD
glnB	-0.30	4.0E-02	-0.33	1.9E-02	nitrogen regulatory protein P-II
glnA	-0.16	3.0E-01	-0.61	1.3E-03	GlnA protein
thiL	-0.19	2.2E-01	0.10	5.0E-01	ThiL protein
cydA	-0.07	6.2E-01	-0.27	4.7E-02	cytochrome bd-I ubiquinol oxidase subunit I
cydB	0.03	8.1E-01	0.07	5.3E-01	cytochrome d ubiquinol oxidase, subunit II

Genes under codY regulation

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
dppA	0.14	2.7E-01	-0.16	1.7E-01	dipeptide-binding protein precursor
dppB	0.48	8.1E-03	0.00	3.1E-01	dipeptide transport system permease protein DppB
dppC	0.92	4.9E-05	0.01	9.1E-01	dipeptide transport system permease protein DppC
dppD	0.76	3.2E-04	-0.23	8.8E-02	dipeptide transport ATP-binding protein DppD
dppF	0.60	2.3E-03	-0.18	1.9E-01	dipeptide transport ATP-binding protein DppF
gltD	-0.02	8.7E-01	-0.22	9.4E-02	glutamate synthase subunit beta
gltB	-0.07	7.4E-01	-0.77	3.1E-03	glutamate synthase, large subunit
lysA	-0.13	3.1E-01	-0.19	1.3E-01	LysA protein
ilvD	-0.12	7.4E-01	-0.61	8.9E-02	dihydroxy-acid dehydratase
ilvB	-0.16	6.4E-01	-0.57	8.9E-02	acetolactate synthase catalytic subunit
ilvN	-0.36	2.8E-01	-0.64	5.3E-02	acetolactate synthase 3 regulatory subunit
ilvC	-0.17	5.3E-01	-0.64	2.5E-02	ketol-acid reductoisomerase
ilvA	-0.29	1.3E-01	-0.56	6.2E-03	threonine dehydratase
aldB	-0.39	1.8E-02	-0.34	2.4E-02	AldB protein
hisC	-0.06	7.8E-01	0.28	1.9E-01	histidinol-phosphate aminotransferase
hisZ	0.15	6.6E-01	0.07	8.1E-01	ATP phosphoribosyltransferase regulatory subunit
hisG	0.08	7.0E-01	-0.28	1.5E-01	ATP phosphoribosyltransferase catalytic subunit
hisD	0.08	5.7E-01	-0.05	7.0E-01	HisD protein
hisB	0.09	5.1E-01	0.04	7.6E-01	imidazoleglycerol-phosphate dehydratase
hisH	-0.08	5.3E-01	-0.03	8.2E-01	imidazole glycerol phosphate synthase subunit HisH
hisA	-0.01	9.7E-01	0.00	5.9E-01	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)-methylideneamino] imidazole-4-carboxamide isomerase
hisF	-0.19	1.4E-01	-0.04	7.4E-01	imidazole glycerol phosphate synthase subunit HisF
hisI	0.03	8.1E-01	0.10	4.5E-01	bifunctional phosphoribosyl-AMP cyclohydrolase/phos-phoribosyl-ATP pyrophosphatase protein
hisK	0.05	7.4E-01	0.17	1.9E-01	histidinol-phosphatase
ctrA	-0.14	2.8E-01	-0.44	3.0E-03	putative amino-acid transporter
oppD	-0.07	6.1E-01	-0.73	1.7E-04	oligopeptide transport ATP-binding protein OppD
oppF	-0.09	4.7E-01	-0.63	2.8E-04	oligopeptide transport ATP-binding protein OppF
oppB	0.11	4.5E-01	-0.38	1.4E-02	peptide transport system permease protein OppB
oppC	0.01	9.1E-01	-0.40	7.9E-03	oligopeptide transport system permease protein OppC
oppA	-0.07	5.9E-01	0.17	1.9E-01	oligopeptide-binding protein OppA precursor
pepO	0.02	8.8E-01	-0.05	6.7E-01	endopeptidase O
asnB	-0.21	1.2E-01	-0.29	3.1E-02	asparagine synthetase B
gltA	-0.02	8.5E-01	-0.13	2.7E-01	citrate synthase
citB	-0.10	4.3E-01	-0.13	2.4E-01	aconitate hydratase
icD	-0.09	4.4E-01	-0.01	9.5E-01	isocitrate dehydrogenase
serC	-0.08	5.3E-01	-0.14	2.4E-01	phosphoserine aminotransferase
serA	-0.31	4.9E-02	-0.64	6.0E-04	D-3-phosphoglycerate dehydrogenase
serB	-0.27	1.3E-01	-0.49	8.5E-03	SerB protein
ArcD1	0.50	1.5E-01	-0.12	6.8E-01	arginine/ornithine antiporter
ArcC1	0.28	3.4E-01	-0.15	5.6E-01	carbamate kinase
ArcC2	0.25	2.8E-01	-0.07	7.3E-01	carbamate kinase
amtB	-0.16	2.8E-01	-0.45	6.8E-03	ammonium transporter AmtB
dapB	-0.02	8.7E-01	-0.20	1.5E-01	dihydrodipicolinate reductase
FtsW1	-0.30	1.6E-01	-0.01	9.5E-01	cell division protein ftsW1

Genes under ccpA regulation

Gene/locus	BK1506		BK1503			Annotation
	LogFC	p. Value	logFC	P.value	Effect	
galP	0.76	8.5E-02	0.08	8.3E-01	Rep	galactose permease
galM	0.57	7.3E-02	0.24	3.6E-01	Rep	aldose 1-epimerase
galK	0.25	2.1E-01	0.06	7.1E-01	Rep	galactokinase
galT	0.16	3.7E-01	0.02	9.0E-01	Rep	galactose-1-phosphate uridylyltransferase
galE	-0.32	5.7E-02	0.33	3.8E-02	Rep	GalE protein
mtlA	0.23	1.4E-01	-0.15	2.9E-01	Rep	PTS system, mannitol-specific IIBC component
mtlR	-0.03	8.6E-01	-0.10	4.6E-01	Rep	transcriptional regulator mtl operon MtlR
mtlF	0.23	9.6E-02	0.02	8.7E-01	Rep	PTS system, mannitol-specific IIA component
mtlD	0.21	1.0E-01	0.01	9.4E-01	Rep	mannitol-1-phosphate 5-dehydrogenase
llmg_0453	0.07	5.5E-01	-0.01	9.6E-01	Rep	sucrose-specific PTS enzyme IIABC
llmg_0454	0.23	1.1E-01	-0.25	6.0E-02	Rep	beta-glucoside-specific PTS system IIABC component
trePP	-0.16	1.6E-01	-0.21	5.3E-02	Rep	putative trehalose/maltose hydrolase
pgmB	0.15	2.7E-01	0.36	1.2E-02	Rep	beta-phosphoglucomutase
llmg_0431	0.41	2.0E-02	0.02	8.7E-01	Rep	putative acyl carrier protein phosphodiesterase 2
llmg_0432	0.15	2.3E-01	0.08	4.9E-01	Rep	transcription regulator
ptcB	0.15	4.8E-01	0.83	1.6E-03	Rep	cellobiose-specific PTS system IIB component
ptcA	-0.10	5.7E-01	1.00	1.8E-04	Rep	cellobiose-specific PTS system IIA component
llmg_0439	0.22	1.5E-01	0.38	1.7E-02	Rep	LacI family transcription regulator
ptcC	0.59	1.8E-02	-0.06	7.5E-01	Rep	cellobiose-specific PTS system IIC component
bglA	0.25	1.1E-01	-0.02	9.1E-01	Rep	6-phospho-beta-glucosidase
arcA	0.63	7.2E-02	0.02	9.4E-01	Rep	arginine deiminase
arcB	0.43	1.3E-01	-0.06	8.2E-01	Rep	ornithine carbamoyltransferase
arcD1	0.50	1.5E-01	-0.12	6.8E-01	Rep	arginine/ornithine antiporter
arcC1	0.28	3.4E-01	-0.15	5.6E-01	Rep	carbamate kinase
arcC2	0.25	2.8E-01	-0.07	7.3E-01	Rep	carbamate kinase
pfk	-0.26	5.7E-02	-0.56	7.3E-04	Act	6-phosphofructokinase
pyk	-0.27	9.4E-02	-1.09	2.1E-05	Act	pyruvate kinase
ldh	-0.51	1.8E-02	-0.45	2.1E-02	Act	L-lactate dehydrogenase
pgiA	-0.16	3.5E-01	0.54	4.7E-03	Act	glucose-6-phosphate isomerase
fruA	0.37	5.0E-02	-0.19	2.4E-01	Act	PTS system, fructose specific IIBC components
fruC	-0.18	1.9E-01	-0.39	8.4E-03	Act	tagatose-6-phosphate kinase
fruR	-0.05	7.2E-01	0.10	4.7E-01	Act	lactose transport regulator

Fatty acid biosynthesis and fatty acid metabolism

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
accA	-0.22	1.2E-01	-0.01	9.5E-01	AccA protein
accB	-0.23	2.2E-01	-1.05	8.0E-05	acetyl-CoA carboxylase biotin carboxyl carrier protein subunit
accC	-0.26	5.7E-02	-0.71	9.2E-05	acetyl-CoA carboxylase biotin carboxylase subunit
accD	-0.14	2.4E-01	-0.28	2.6E-02	acetyl-CoA carboxylase subunit beta
fabD	-0.21	2.2E-01	-0.76	5.6E-04	malonyl CoA-acyl carrier protein transacylase
fabF	-0.23	1.8E-01	-1.05	3.4E-05	3-oxoacyl-[acyl-carrier-protein] synthase II
fabH	-0.54	1.2E-02	-0.51	9.9E-03	3-oxoacyl-(acyl carrier protein) synthase III
fabG	-0.33	9.6E-02	-0.92	2.6E-04	3-ketoacyl-(acyl-carrier-protein) reductase
fabG	-0.38	9.0E-02	-0.58	1.1E-02	3-ketoacyl-(acyl-carrier-protein) reductase
fabZ	-0.26	1.6E-01	-1.07	5.2E-05	(3R)-hydroxymyristoyl-ACP dehydratase
llmg_0538	-0.30	7.2E-02	0.66	8.0E-04	(3R)-hydroxymyristoyl-(acyl-carrier-protein) dehydratase
fabI	-0.24	1.2E-01	-0.22	1.1E-01	enoyl-(acyl carrier protein) reductase
llmg_1415	-0.14	2.7E-01	0.58	5.4E-04	hypothetical protein
thiL	-0.19	2.2E-01	0.10	5.0E-01	ThiL protein
llmg_1965	0.27	1.5E-01	-0.02	9.1E-01	putative AMP-binding enzyme
adhE	0.52	6.7E-02	-0.10	6.8E-01	bifunctional acetaldehyde-CoA/alcohol dehydrogenase
adhA, adhP	0.16	3.0E-01	-0.24	1.1E-01	alcohol dehydrogenase
llmg_0955	-0.12	6.2E-01	0.04	8.4E-01	alcohol dehydrogenase

Glycerolipid and glycerol metabolism

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
llmg_0870	0.07	6.5E-01	-0.30	5.3E-02	transporter
glpF2	0.67	6.6E-04	-0.10	4.6E-01	glycerol uptake facilitator
glpF3	-0.15	2.5E-01	0.45	3.5E-03	putative glycerol uptake facilitator protein
dhaK	0.35	4.4E-02	0.11	4.5E-01	DhaKLM operon coactivator DhaQ
dhaM	0.57	2.8E-03	-0.04	7.4E-01	dihydroxyacetone kinase DhaM
glpK	0.52	2.0E-02	-0.24	1.9E-01	glycerol kinase
plsX	0.16	3.0E-01	-0.21	1.5E-01	putative glycerol-3-phosphate acyltransferase PlsX
llmg_1540	-0.17	3.7E-01	0.05	7.8E-01	putative glycerol-3-phosphate acyltransferase PlsY
llmg_0119	-0.18	2.0E-01	-0.18	1.6E-01	putative acyltransferase
dgkA	-0.34	4.4E-02	0.38	1.8E-02	DgkA protein
llmg_2421	-0.01	9.2E-01	0.17	1.9E-01	hypothetical protein
glpD	0.44	2.9E-02	-0.18	2.8E-01	GlpD protein
gpsA	-0.18	3.4E-01	-0.59	4.9E-03	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase
llmg_0945	-0.12	3.2E-01	-0.49	1.0E-03	putative glycerol dehydrogenase

Purine metabolism

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
llmg_2075	-0.30	2.8E-02	-0.70	8.2E-05	ADP-ribose pyrophosphatase
deoB	-0.25	7.1E-02	-0.76	6.4E-05	phosphopentomutase
prsB	-0.32	3.6E-02	-0.26	6.1E-02	ribose-phosphate pyrophosphokinase
prsA	-0.16	3.6E-01	0.06	7.0E-01	ribose-phosphate pyrophosphokinase
purF	-0.25	2.6E-01	-0.90	7.9E-04	amidophosphoribosyltransferase
purD	-0.50	7.0E-03	-0.69	4.9E-04	phosphoribosylamine--glycine ligase
purN	-0.19	2.2E-01	0.09	5.2E-01	phosphoribosylglycinamide formyltransferase
purS	-0.21	9.8E-02	-0.12	3.0E-01	phosphoribosylformylglycinamidine synthetase PurS
purQ	-0.57	3.6E-03	-0.84	1.3E-04	phosphoribosylformylglycinamidine synthase I
purL	-0.37	1.0E-01	-1.10	1.8E-04	phosphoribosylformylglycinamidine synthase II
purM	-0.32	3.8E-02	-0.39	9.1E-03	phosphoribosylaminoimidazole synthetase
purK	-0.31	7.8E-02	-0.02	9.0E-01	phosphoribosylaminoimidazole carboxylase ATPase subunit
purE	-0.06	6.9E-01	-0.59	1.4E-03	phosphoribosylaminoimidazole carboxylase catalytic subunit
purC	-0.19	1.3E-01	0.11	3.1E-01	phosphoribosylaminoimidazole-succinocarboxamide synthase
purB	-0.27	9.0E-02	0.33	3.5E-02	adenylosuccinate lyase
purH	-0.43	3.3E-02	-1.15	2.9E-05	bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
apt	-0.11	4.7E-01	0.19	2.0E-01	adenine phosphoribosyltransferase
nucA	-0.29	2.4E-01	0.22	3.3E-01	5'-nucleotidase
llmg_0192	0.18	2.3E-01	-0.09	5.1E-01	5'-nucleotidase precursor
deoD	-0.51	1.2E-02	0.64	1.8E-03	purine nucleoside phosphorylase
hprT	-0.26	1.4E-01	0.30	6.4E-02	HprT protein
hpt	-0.02	9.1E-01	0.29	7.9E-02	hypoxanthine phosphoribosyltransferase
guaB	-0.35	7.8E-02	-0.77	8.6E-04	inosine 5'-monophosphate dehydrogenase
llmg_1188	-0.21	1.0E-01	-0.62	1.7E-04	hypothetical protein
xpt	-0.42	9.6E-03	-0.44	4.2E-03	xanthine phosphoribosyltransferase
guaA	-0.20	1.9E-01	-0.85	1.1E-04	GMP synthase
guaC	-0.36	1.9E-02	-0.12	3.4E-01	guanosine 5'-monophosphate oxidoreductase
gmk	0.03	8.5E-01	0.22	1.2E-01	guanylate kinase
pyk	-0.27	9.4E-02	-1.09	1.3E-05	pyruvate kinase
nrdE	-0.14	2.9E-01	-0.20	1.1E-01	ribonucleotide-diphosphate reductase subunit alpha
nrdF	-0.41	2.6E-02	-0.08	5.8E-01	ribonucleotide-diphosphate reductase subunit beta
llmg_0281	0.11	3.6E-01	-0.21	8.3E-02	anaerobic ribonucleoside triphosphate reductase
rpoA	-0.21	1.4E-01	-0.82	5.2E-05	DNA-directed RNA polymerase subunit alpha
rpoB	-0.05	7.8E-01	-0.92	3.6E-04	DNA-directed RNA polymerase subunit beta
rpoC	-0.14	4.9E-01	-0.84	1.0E-03	DNA-directed RNA polymerase subunit beta'
rpoE	-0.26	6.2E-02	0.14	2.4E-01	DNA-directed RNA polymerase subunit delta
rpoZ	-0.37	2.8E-02	-0.01	9.1E-01	DNA-directed RNA polymerase subunit omega
polA	0.28	6.7E-02	0.11	4.2E-01	DNA polymerase I
dnaE	0.08	5.4E-01	0.22	8.6E-02	DNA polymerase III DnaE
polC	0.76	1.1E-04	-0.05	6.4E-01	DNA polymerase III PolC
dnaN	-0.08	5.0E-01	1.05	2.5E-06	DNA polymerase III subunit beta
dnaX	0.19	1.7E-01	-0.44	4.4E-03	DNA polymerase III subunits gamma and tau
holA	-0.39	2.9E-02	-0.16	2.7E-01	DNA polymerase III subunit delta
holB	-0.24	9.0E-02	-0.29	3.2E-02	DNA polymerase III subunit delta'
dnaQ	-0.14	3.3E-01	0.21	1.2E-01	DNA polymerase III, epsilon chain
relA	-0.21	1.4E-01	0.04	7.7E-01	GTP pyrophosphokinase
llmg_0382	0.15	3.7E-01	-0.07	6.3E-01	putative GTP pyrophosphokinase
purA	-0.20	1.4E-01	-0.28	3.7E-02	adenylosuccinate synthetase
add	-0.32	3.7E-02	0.23	8.3E-02	adenosine deaminase
cpdC	-0.32	2.1E-02	-0.39	4.2E-03	2',3'-cyclic-nucleotide 2'-phosphodiesterase
adk	-0.06	6.7E-01	0.54	2.4E-03	adenylate kinase
pnpA	-0.12	4.8E-01	-0.56	4.1E-03	polynucleotide phosphorylase/polyadenylase
arcC2	0.25	2.7E-01	-0.07	7.2E-01	carbamate kinase
arcC1	0.28	3.3E-01	-0.15	5.4E-01	carbamate kinase

Pyrimidine metabolism

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
carB	-0.33	6.3E-02	-1.00	4.6E-05	carbamoyl phosphate synthase large subunit
carA	-0.12	4.7E-01	-0.71	6.7E-04	carbamoyl phosphate synthase small subunit
pyrB	-0.07	5.8E-01	-0.77	6.8E-05	aspartate carbamoyltransferase catalytic subunit
pyrC	-0.33	4.4E-02	-0.55	2.0E-03	dihydroorotase
pyrDA	-0.38	5.4E-02	0.23	1.8E-01	dihydroorotate dehydrogenase 1A
pyrDB	0.05	7.9E-01	-1.07	1.1E-04	dihydroorotate dehydrogenase 1B
pyrE	-0.33	2.0E-02	-0.48	1.3E-03	orotate phosphoribosyltransferase
pyrF	-0.60	1.8E-03	-0.70	3.4E-04	orotidine 5'-phosphate decarboxylase
cmk	-0.33	3.9E-02	0.13	3.4E-01	cytidylate kinase
pyrH	-0.16	2.0E-01	-0.19	1.1E-01	uridylate kinase
pnpA	-0.12	4.8E-01	-0.56	4.1E-03	polynucleotide phosphorylase/polyadenylase
llmg_1188	-0.21	1.0E-01	-0.62	1.7E-04	hypothetical protein
pyrG	0.05	7.1E-01	-0.31	2.8E-02	CTP synthetase
rpoA	-0.21	1.4E-01	-0.82	5.2E-05	DNA-directed RNA polymerase subunit alpha
rpoB	-0.05	7.8E-01	-0.92	3.6E-04	DNA-directed RNA polymerase subunit beta
rpoC	-0.14	4.9E-01	-0.84	1.0E-03	DNA-directed RNA polymerase subunit beta'
rpoE	-0.26	6.2E-02	0.14	2.4E-01	DNA-directed RNA polymerase subunit delta
rpoZ	-0.37	2.8E-02	-0.01	9.1E-01	DNA-directed RNA polymerase subunit omega
polA	0.28	6.7E-02	0.11	4.2E-01	DNA polymerase I
dnaE	0.08	5.4E-01	0.22	8.6E-02	DNA polymerase III DnaE
polC	0.76	1.1E-04	-0.05	6.4E-01	DNA polymerase III PolC
dnaN	-0.08	5.0E-01	1.05	2.5E-06	DNA polymerase III subunit beta
dnaX	0.19	1.7E-01	-0.44	4.4E-03	DNA polymerase III subunits gamma and tau
holA	-0.39	2.9E-02	-0.16	2.7E-01	DNA polymerase III subunit delta
holB	-0.24	9.0E-02	-0.29	3.2E-02	DNA polymerase III subunit delta'
dnaQ	-0.14	3.3E-01	0.21	1.2E-01	DNA polymerase III, epsilon chain
udk	-0.20	9.8E-02	0.59	2.1E-04	uridine kinase
nucA	-0.29	2.4E-01	0.22	3.3E-01	5'-nucleotidase
llmg_0192	0.18	2.3E-01	-0.09	5.1E-01	5'-nucleotidase precursor
udp	-0.45	3.6E-03	-0.30	1.9E-02	uridine phosphorylase
upp	-0.27	7.1E-02	0.34	2.0E-02	uracil phosphoribosyltransferase
pyrR	-0.18	2.3E-01	-0.63	7.9E-04	bifunctional pyrimidine regulatory protein PyrR uracil phosphoribosyltransferase
pdp	-0.18	1.8E-01	-0.58	4.6E-04	pyrimidine-nucleoside phosphorylase
trxB1	-0.19	1.5E-01	0.21	8.2E-02	TrxB1 protein
trxB2	-0.05	7.1E-01	0.21	1.3E-01	TrxB2 protein
llmg_0281	0.11	3.6E-01	-0.21	8.3E-02	anaerobic ribonucleoside triphosphate reductase
nrdE	-0.14	2.9E-01	-0.20	1.1E-01	ribonucleotide-diphosphate reductase subunit alpha
nrdF	-0.41	2.6E-02	-0.08	5.8E-01	ribonucleotide-diphosphate reductase subunit beta
ps428	-0.26	8.6E-02	-0.11	3.9E-01	deoxyuridine 5'-triphosphate nucleotidohydrolase
ps325	-0.06	6.9E-01	0.04	7.3E-01	deoxyuridine 5'-triphosphate nucleotidohydrolase
dut	0.23	1.5E-01	-0.04	7.8E-01	deoxyuridine 5'-triphosphate nucleotidohydrolase
thyA	0.04	7.3E-01	0.92	7.2E-06	thymidylate synthase
cdd	-0.05	7.0E-01	-0.43	4.2E-03	Cdd protein
deoD	-0.51	1.2E-02	0.64	1.8E-03	purine nucleoside phosphorylase
llmg_1416	0.30	1.5E-01	0.33	8.9E-02	hypothetical protein
ntd	0.11	4.0E-01	-0.13	3.2E-01	nucleoside deoxyribosyltransferase
tdk	-0.24	1.4E-01	0.07	6.0E-01	thymidine kinase
tmk	-0.64	5.7E-03	-0.32	8.1E-02	thymidylate kinase
cpdC	-0.32	2.1E-02	-0.39	4.2E-03	2',3'-cyclic-nucleotide 2'-phosphodiesterase

Arginine and proline metabolism

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
speG	0.18	2.6E-01	0.25	9.6E-02	spermidine acetyltransferase
llmg_0177	0.30	2.4E-01	-0.06	7.7E-01	amidase
proC	-0.39	1.7E-02	-0.37	1.5E-02	pyrroline-5-carboxylate reductase
ocd	-0.09	5.9E-01	0.01	9.6E-01	ornithine cyclodeaminase, mu-crystallin-like protein
proB	-0.17	1.6E-01	-0.47	1.1E-03	gamma-glutamyl kinase
proA	-0.11	4.1E-01	-0.04	7.2E-01	gamma-glutamyl phosphate reductase
kdgA	0.55	2.7E-02	-0.06	7.5E-01	keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase
arcB	0.43	1.2E-01	-0.06	8.1E-01	ornithine carbamoyltransferase
argF	0.16	4.8E-01	-0.27	2.0E-01	ornithine carbamoyltransferase
argG	-0.08	6.2E-01	-0.41	1.7E-02	argininosuccinate synthase
argH	-0.10	4.9E-01	-0.19	1.7E-01	argininosuccinate lyase
arcA	0.63	6.1E-02	0.02	9.4E-01	arginine deiminase
glnA	-0.16	3.0E-01	-0.61	1.3E-03	GlnA protein
arcC2	0.25	2.7E-01	-0.07	7.2E-01	carbamate kinase
arcC1	0.28	3.3E-01	-0.15	5.4E-01	carbamate kinase
argJ	0.11	5.1E-01	-0.29	8.4E-02	bifunctional ornithine acetyltransferase/N-acetylglutamate synthase protein
argB	0.29	2.7E-01	-0.25	3.0E-01	acetylglutamate kinase
argC	0.14	3.6E-01	-0.12	3.9E-01	N-acetyl-gamma-glutamyl-phosphate reductase
argD	0.59	8.6E-02	-0.21	4.7E-01	acetylornithine aminotransferase
argE	-0.23	1.4E-01	-0.45	6.9E-03	acetylornithine deacetylase

Vitamin and cofactor metabolism

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
					One carbon pool by folate
dfrA	-0.64	1.3E-03	0.28	6.2E-02	DfrA protein
fhs	-0.27	5.1E-02	0.16	1.8E-01	formate--tetrahydrofolate ligase
folD	-0.36	1.1E-02	-0.35	9.3E-03	dehydrogenase/5,10-methylene-tetrahydrofolate cyclohydrolase
glyA	-0.25	9.8E-02	-0.33	2.6E-02	serine hydroxymethyltransferase
purN	-0.19	2.2E-01	0.09	5.2E-01	phosphoribosylglycinamide formyltransferase
purH	-0.43	3.3E-02	-1.15	2.9E-05	bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
fmt	-0.20	1.2E-01	-0.52	9.0E-04	methionyl-tRNA formyltransferase
thyA	0.04	7.3E-01	0.92	7.2E-06	thymidylate synthase
metF	-0.07	5.9E-01	-0.10	3.9E-01	5,10-methylenetetrahydrofolate reductase
llmg_0181	0.23	1.0E-01	0.15	2.2E-01	5-formyltetrahydrofolate cyclo-ligase family protein
					Thiamine metabolism
thiD1	0.41	6.6E-02	-0.09	6.1E-01	phosphomethylpyrimidine kinase
nifS	-0.27	1.3E-01	-0.17	2.7E-01	putative iron-sulfur cofactor synthesis protein
nifZ	-0.50	4.9E-03	0.47	4.2E-03	pyridoxal-phosphate dependent aminotransferase
llmg_1972	0.09	6.4E-01	-0.61	6.4E-03	hypothetical protein
thiI	0.19	3.0E-01	-0.40	2.7E-02	thiamine biosynthesis protein ThiI
thiM	0.00	9.7E-01	-0.11	3.7E-01	hydroxyethylthiazole kinase
thiE	-0.24	8.2E-02	-0.08	5.1E-01	thiamine-phosphate pyrophosphorylase
thiN	-0.02	9.1E-01	0.16	2.4E-01	thiamin pyrophosphokinase
tenA	-0.19	2.0E-01	-0.10	4.3E-01	transcriptional activator TenA
					Riboflavin metabolism
ribA	0.24	1.1E-01	0.02	8.5E-01	riboflavin biosynthesis protein RibA
ribD	0.15	2.2E-01	0.02	8.4E-01	riboflavin biosynthesis protein RibD
ribH	0.08	5.8E-01	-0.02	8.6E-01	6,7-dimethyl-8-ribityllumazine synthase
ribB	0.40	8.3E-03	0.15	2.1E-01	riboflavin synthase subunit alpha
ribC, ribF	-0.12	4.1E-01	0.51	3.0E-03	bifunctional riboflavin kinase/FMN adenylyltransferase
					Vitamin B6 metabolism
thiD2	-0.09	6.2E-01	0.00	1.0E+00	phosphomethylpyrimidine kinase
serC	-0.08	5.4E-01	-0.14	2.5E-01	phosphoserine aminotransferase
thrC	-0.33	3.4E-02	-0.45	4.4E-03	threonine synthase
					Nicotinate and nicotinamide metabolism
pncB	-0.34	3.5E-02	-0.38	1.4E-02	nicotinate phosphoribosyltransferase
deoD	-0.51	1.2E-02	0.64	1.8E-03	purine nucleoside phosphorylase
nucA	-0.29	2.4E-01	0.22	3.3E-01	5'-nucleotidase
llmg_0192	0.18	2.3E-01	-0.09	5.1E-01	5'-nucleotidase precursor
nadD	-0.38	1.8E-02	-0.38	1.2E-02	nicotinic acid mononucleotide adenylyltransferase
nadD1	0.00	9.8E-01	-0.25	1.9E-01	nicotinate-nucleotide adenylyltransferase
nadE	-0.19	2.1E-01	0.14	2.8E-01	NAD synthetase
ppnK	0.08	6.9E-01	-0.22	2.4E-01	inorganic polyphosphate/ATP-NAD kinase
					Pantothenate and CoA biosynthesis
als	-0.13	5.1E-01	-0.35	6.9E-02	acetolactate synthase
ilvB	-0.16	6.2E-01	-0.57	7.6E-02	acetolactate synthase catalytic subunit
ilvH	-0.36	2.6E-01	-0.64	4.3E-02	acetolactate synthase 3 regulatory subunit
ilvC	-0.17	5.1E-01	-0.64	2.0E-02	ketol-acid reductoisomerase
ilvD	-0.12	7.3E-01	-0.61	7.5E-02	dihydroxy-acid dehydratase
panE	-0.13	4.3E-01	0.72	6.9E-04	2-dehydropantoate 2-reductase
coaA	-0.40	2.4E-02	0.37	2.3E-02	pantothenate kinase
dfpB	-0.11	4.0E-01	-0.38	7.6E-03	phosphopantothenate--cysteine ligase
dfpA	0.14	4.2E-01	-0.45	1.4E-02	phosphopantothenoylcysteine decarboxylase
coaD	-0.03	8.6E-01	0.14	3.6E-01	phosphopantetheine adenylyltransferase
coaE	0.07	5.5E-01	0.19	9.1E-02	dephospho-CoA kinase
acpS	-0.19	1.8E-01	-0.48	2.7E-03	4'-phosphopantetheinyl transferase
ilvE	-0.19	2.7E-01	0.04	8.1E-01	branched-chain amino acid aminotransferase
					Biotin metabolism
birA2	-0.01	9.5E-01	0.20	8.1E-02	acetyl-CoA carboxylase ligase / biotin operon repressor
birA1	-0.23	9.3E-02	-0.04	7.1E-01	repressor

Investigation of glycerol assimilation and cofactor metabolism in *Lactococcus lactis*

PhD. Thesis – Anders Koefoed Holm

Appendix

Appendix A

Reports from Work Package 7, work task 7.1 and 7.4 of the GLYFINERY project.

A.1 – The report from WT 7.1 of WP 7, Technological assessment

A.2 – The report from WT 7.4 of WP 7, Technological assessment (updated)

A.1 – Report from WT 7.1 – Technological assessment

This section contains the report which was made as an initial technological basis for the GLYFINERY project.

GLYFINERY WP 7: Integrated assessment

WT 7.1 – Technological assessment

Update 8 October 2010

This report contains deliverable 7.1: Technological assessment, as described in the proposal for the GLYFINERY project. This deliverable deals with both, general definitions and specifications as well as with the specific scenario settings and an overview of the pathways investigated for the conversion of glycerol into: ethanol, butanol, 1,3-propanediol and biogas. Included is written information detailing an overview of the various steps in each process and flowcharts depicting them.

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DTU

GLYFINERY WP 7: Integrated assessment

WT 7.1 – Technological assessment

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8 October 2010

Contents

1. Introduction, goal and scope

According to the EU directive 2009/28/EC of 23 April 2009, all member countries shall ensure by 2020 that the share of energy from renewable sources in transport is at least 10 % of the total fuel consumption. For achieving this goal an increased production of biodiesel from biomass plays an important role. In biodiesel production, glycerol is derived as by-product. Due to an increased biodiesel production in recent years high amounts of glycerol entered the market. This development led to a saturation of the glycerol market over the last years with prices for crude glycerol falling considerably.

The objective of the GLYFINERY project is to search for alternative uses for glycerol. Thereby, sustainable usage pathways for glycerol shall be determined and the biodiesel production as a whole shall be optimized. This is achieved by designing biorefinery production schemes for the production of biofuels, bio-energy and green chemicals from glycerol. Accompanying, the objective of WP7 is to assess the economic and ecological sustainability of biodiesel production and glycerol processing and to derive optimisation potentials.

There are two core questions for which the assessments in WP 7 will provide answers:

1. From an ecological and economic point of view, what is the best way to use glycerol resulting from biodiesel production?

2. How do the different usage pathways for glycerol from biodiesel production affect the economic and ecological performance of biodiesel production as a whole?

To address the core questions, the following issues will be assessed:

- What are the advantages and disadvantages of innovative glycerol usage pathways in comparison to the currently existing pathways?
- What is the best use of the products derived from glycerol processing?
- How does the production of green energy from glycerol compare to the material usage or the usage in chemical industries?
- What is the influence of different usage pathways for the by-products on the overall results and which usage shall be preferred?
- What is the relative importance of various life cycle steps on the overall results and which optimisation potentials can be identified?

This paper was prepared as a fulfilment of work task 7.1: *Technological assessment* as part of work package 7: *Integrated assessment*. The paper describes the technological aspects of glycerol production and processing and defines the life cycles and system boundaries. The definitions and settings will be used in the following tasks, especially for the ecological (WT 7.2) and economic (WT 7.3) assessment.

Each conversion process is dealt with in a separate section and the main focus of the report is the single process systems i. e. conversion of glycerol to a single product such as the conversion of glycerol to ethanol. No multi-product scenarios have been considered so far.

2. Background

2.1 Biodiesel: A source of crude glycerol

The focus of the GLYFINERY project is the conversion of glycerol to other useful compounds. The biggest source of glycerol is associated with the increasing production of biodiesel. In biodiesel production the main byproduct is glycerol (glycerin, glycerine, 1,2,3-Propanetriol, 1,2,3-Trihydroxypropane). When producing biodiesel approx. 10 % of the reaction volume in a given biodiesel production process ends up as glycerol. When considering that biodiesel production in Europe has increased to an annual production of around 5.7 million tonnes in 2007 the amount of glycerol which could be utilized from this production alone would be in the order of 570,000 ton (1). An overview of a typical biodiesel production process can be seen in Figure 1 below.

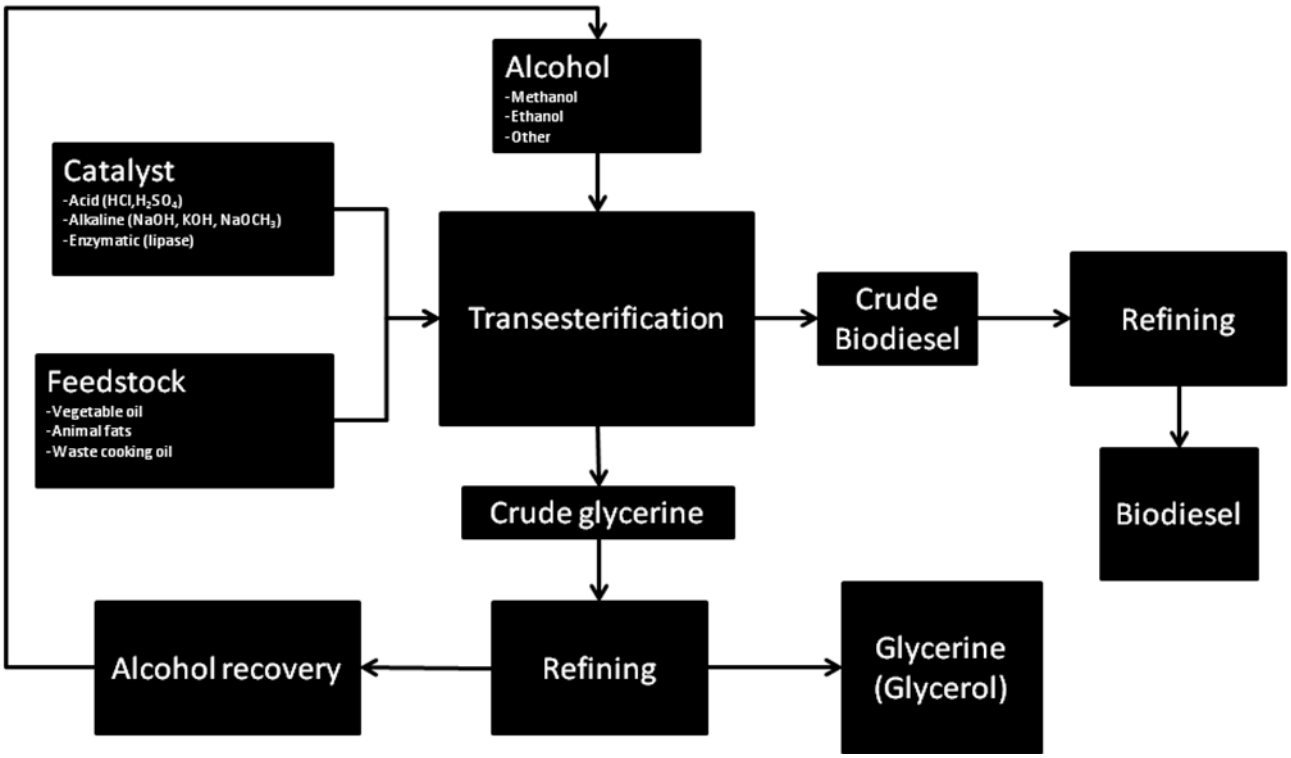

Figure 1: Schematic overview of a standard biodiesel production process. Two main products are produced in this process are biodiesel and glycerol.

2.2 Production of biodiesel

The basic process of producing biodiesel is a chemical transesterification reaction converting triglycerides into fatty acid alkyl monoesters (FAAM) in the presence of a catalyst. An overview of the reaction can be seen in Figure 2.

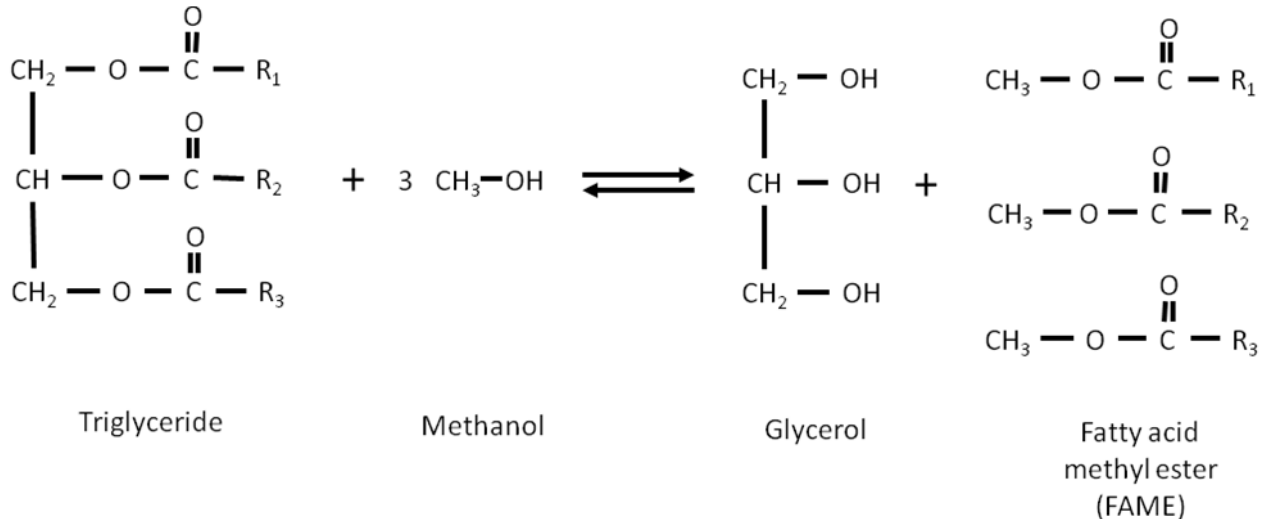

Figure 2: Stoichiometry of transesterification reaction. In this example the R_1-R_3 groups represent fatty acid radicals and methanol is used as alcohol. The reaction takes place in the presence of a catalyst (not shown) yielding fatty acid methyl ester (FAME).

As shown in Figure 2 the transesterification reaction requires three moles of alcohol to convert 1 mole of triglyceride which yields three moles of fatty acid alkyl esters and one mole of glycerol. Even though the stochiometric demand is three moles of alcohol higher ratios (6:1 or higher) are generally used in practice to ensure complete reaction (2; 3; 4; 5).

When setting up a biodiesel production process there are a several different parameters to take into account: the feedstock to use, choice of catalyst and type of alcohol. In principle any oleaginous material can be used as feedstock for a biodiesel process. Most biodiesel production comes from virgin vegetable oil of crops such as rape seed, sunflower or soy bean. Other feedstocks include nonedible oils (jatropha, pongamia), waste cooking oils or animal fats. However these feedstocks can have higher levels of free fatty acids (FFA) and water which leads to problems with saponification during biodiesel production with an alkali catalyst.

The choice of alcohol to use is mainly an economic concern. Branched chain alcohols may improve the cold flow properties of biodiesel produced from very saturated sources but generally no difference in yield can be observed from various types of alcohols. Since methanol is often the cheapest alcohol available and can be obtained in a very anhydrous formulation it is often the alcohol of choice (6; 7).

There are three major types of catalysts used for biodiesel production: acid catalysts (HCl, H_2SO_4, etc.), alkali catalysts (NaOH, KOH, etc.) and enzymatic catalysts (lipase). Other catalytic schemes have been proposed such as reaction with super critical methanol or using heterogeneous catalysts such as ZrO_2 or ZnO. The only catalysts used on an industrial scale are the acid and alkali catalysts. The predominant catalyst is the alkali catalyst which is preferably selected on the basis that is has high yield, fast reaction time and is also cheap. The main drawback of alkali catalysis is the risk of saponification in feedstocks containing high levels of FFA's and water. Acid catalyst have higher tolerances for FFA's and water but require higher alcohol to oil ratios and have slower reaction times. Enzymatic catalysts have moderate reaction conditions and do not require the same amount of excess alcohol ratios that the chemical catalysts do. Furthermore the use of enzymatic catalysts also eases downstream processing and product recovery.

The main inhibitory factor in their widespread use is cost (7; 4; 5; 2). An overview of catalytic strategies can be seen in Table 1.

Alkali catalyst	Acid catalyst	Enzymatic catalyst
+ Fast	+ One step process	+ No soap formation
+ High conversion ratios	+ Tolerates high levels of FFA's and water	+ Moderate reaction conditions
÷ Energy intensive		+ Low alcohol to oil/ratio
÷ Difficult glycerol recovery	÷ Energy intensive	+ Easy product recovery
÷ Process susceptible to interference by FFA's and water	÷ Higher alcohol/oil ratio required	÷ Very expensive
÷ Soap formation		

Table 1: Overview of the different catalytic strategies employed for biodiesel production.

On a commercial scale the most prevalent process is alkali catalyzed transesterification with methanol as alkyl receptor. To reduce the risk of saponificaion from FFA and water content in the oil feedstock a common strategy is to first employ an acid catalyzed transesterificarion step to remove FFA's and water followed by alkali catalyzed transesterification (2).

2.3 Composition of biodiesel and crude glycerol

Since biodiesel production is a simple chemical transesterification reaction the composition of biodiesel is closely related to the composition of the feedstock used. The most suitable feedstocks for biodiesel production are: almond, olive, corn, rapeseed and high oleic sunflower oils (certified according to standard UNE-EN 14214) (8). This direct relationship also means that biodiesel produced from animal fats or other saturated sources often have difficulty in achieving desired cold flow properties although this can be partly remedied by the right choice of alcohol.

When considering the nature of crude glycerol produced from different feedstocks, the composition of the crude glycerol obtained after transesterification only has an indirect relation to the choice of feedstock. It is the feedstock which dictates the most appropriate choice of catalyst which then imposes a set of characteristics on the crude glycerol obtained after transesterifcation. These characteristics together with the extent of postproduction purification of the crude glycerol, determines the final characteristics of the commercially available glycerol feedstock. Generally the composition of available glycerol will be different from different producers of biodiesel and depend on the process used (9). Therefore product limits will need to be defined for the contents of various components in the feedstock in order to be compatible with the GLYFINERY process or pre-treatment of feedstock should be considered. As an example the composition of glycerol feedstock obtained from GLYFINERY partner MEROCO can be seen in Table 2.

Parameters	Unit	Value	Limit
Glycerol	% wt.	82.4	min. 82,0
Water	% wt.	9.7	max. 10
Methanol	% wt.	0.01	max. 0.5
Ash	% wt.	6.4	max. 7.0

Table 2: Composition of crude glycerol feedstock obtained from GLYFINERY partner MEROCO. Data taken from a certificate of quality obtained directly from MEROCO.

Most glycerol feedstock falls within the range of 60-90% glycerol w/w with varying amounts of the other components (water, methanol and salts) (10; 11; 9).

3. GLYFINERY scenarios

In the following chapters, the GLYFINERY scenarios are described. The description follows the life cycle of glycerol production and processing until the use of the main products and by-products. First, general specifications and settings are defined in chapter 3.1. In chapter 3.2, the systems of biodiesel and glycerol production as well as the raw materials for biodiesel production are described. Subsequently, the scenarios for glycerol processing to innovative products are presented in chapter 3.3. The description covers the reference use of glycerol (i. e. the use of glycerol that would be realized without the innovative pathways), the use of the main products with the conventional products that are replaced, the plant design as well as the use of by-products. These scenarios are summarized in a table in chapter 3.4. The respective flow charts can be found in chapter 4.

3.1 General specifications, definitions and settings

For the ecological and economic analysis of the GLYFINERY scenarios, general definitions and settings are necessary. They are used in both analyses and guarantee their consistency. The general definitions and settings are described and explained below. Definitions and settings specific for either the economic or the ecological assessment will be described and explained within the reports of these tasks (reports for WT 7.2 and WT 7.3, respectively).

- **Technical reference (pilot or mature):** The project aims at comparing glycerol processing technologies, i. e. future innovative technologies with existing (conventional) pathways. Therefore, mature technologies are used as basis for the ecological and economic assessment.
- **Time frame:** The analysed technologies are currently in development and not yet existent. First pilot plants might be available in 2015. As the objective of this project is to compare mature technologies (see 'Technical reference'), 2020 is set as reference year.
  In addition, by 2020 10 % renewable fuels in transport will be mandatory within the European Union.
- **Functional unit:** The questions to be answered result in different functional units. As the main objective of the GLYFINERY project is to optimize glycerol processing, the benefit from using 1 tonne of crude glycerol is set as functional unit.
  With regard to the second question – the optimization of biodiesel production as a whole with an optimal use of glycerol playing an important role – the benefit from using the output of the biodiesel production is used as functional unit, i.e. all results are related to 1 tonne biodiesel.
- **Geographical coverage:** Europe is the main producer of biodiesel in the world and therewith of glycerol. As glycerol is traded world wide and as critical amounts of glycerol are needed for a successful implementation of innovative technologies, Europe as a whole (and not a specific country within Europe) is set as geographical reference. This implies the use of EU27 average values for prices, yields, power mixes, etc.
  Sensitivity analyses will show the dependency of single productions steps from certain factors. These factors might differ between European countries, like wages, power mix etc. Therefore, if

it becomes evident that the country specific conditions have significant influence on the results, for the geographical coverage single countries may be chosen to show the dependencies. In the ecological assessment, this might be the case for the power mix in energy production from biogas. Here, the power mix will be varied based on different countries, e. g. France (with a great share of nuclear power), Sweden (with a great share of renewable energy carriers) and Poland (with a great share of fossil energy carriers). In the economic analysis, labour costs might play an important role. Here, a Western European country with high labour costs (e. g. Germany) and an Eastern European country with low labour costs (Romania) could be assessed.

3.2 Glycerol production

Glycerol is derived as by-product in biodiesel production. In order to answer the core questions described in chapter 1, two different systems need to be assessed. They are presented below, followed by the description of raw materials which are used in biodiesel production.

3.2.1. Systems to be studied

In chapter 1, two core objectives have been defined: the optimization of glycerol processing and use in innovative pathways as well as the optimization of the biodiesel production as a whole. To analyse both issues, two different systems are regarded:

1. Glycerol processing

   The analysis starts with crude glycerol as it leaves the biodiesel plant (80 % purity). Transports (for centralized systems), processing as well as the use of main and by-products are examined. The aim is to deliver a detailed analysis of glycerol processing and usage as well as to identify best possible use options. Based on these analyses, optimization potentials are derived.
   A schematic overview on the glycerol processing is given in Figure 3.

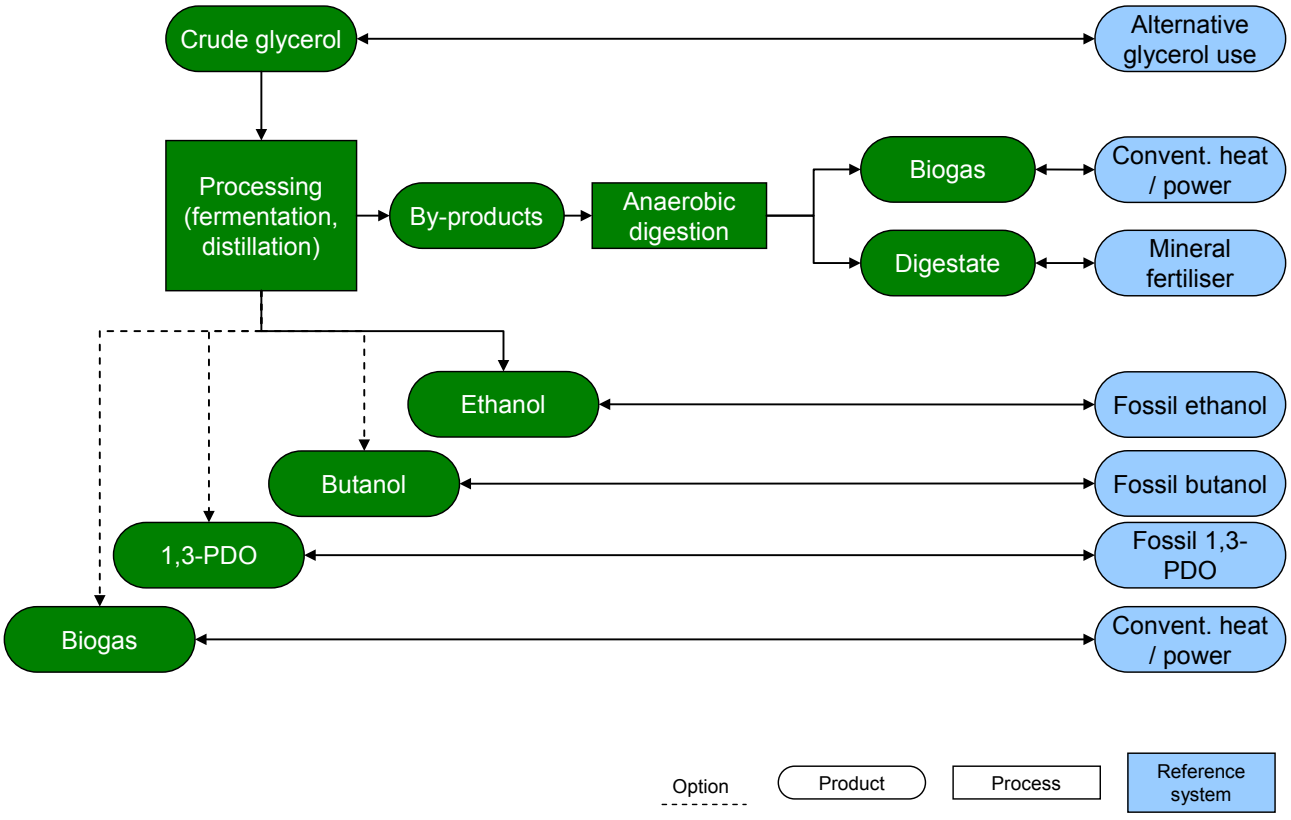

Figure 3: Life cycle of glycerol processing.

2. Biodiesel production as a whole

The whole life cycle of biodiesel production is examined, i.e. the production of oil crops, their processing to biodiesel as well as the processing and usage of all by-products including glycerol. Besides different pathways for glycerol usage other parameters are varied as well, e. g. the raw materials. This analysis aims at depicting the impact of different glycerol usage pathways on the whole biodiesel production and at optimizing the biodiesel production from an economic and ecological point of view.

A schematic overview on the whole biodiesel production system is shown in Figure 4.

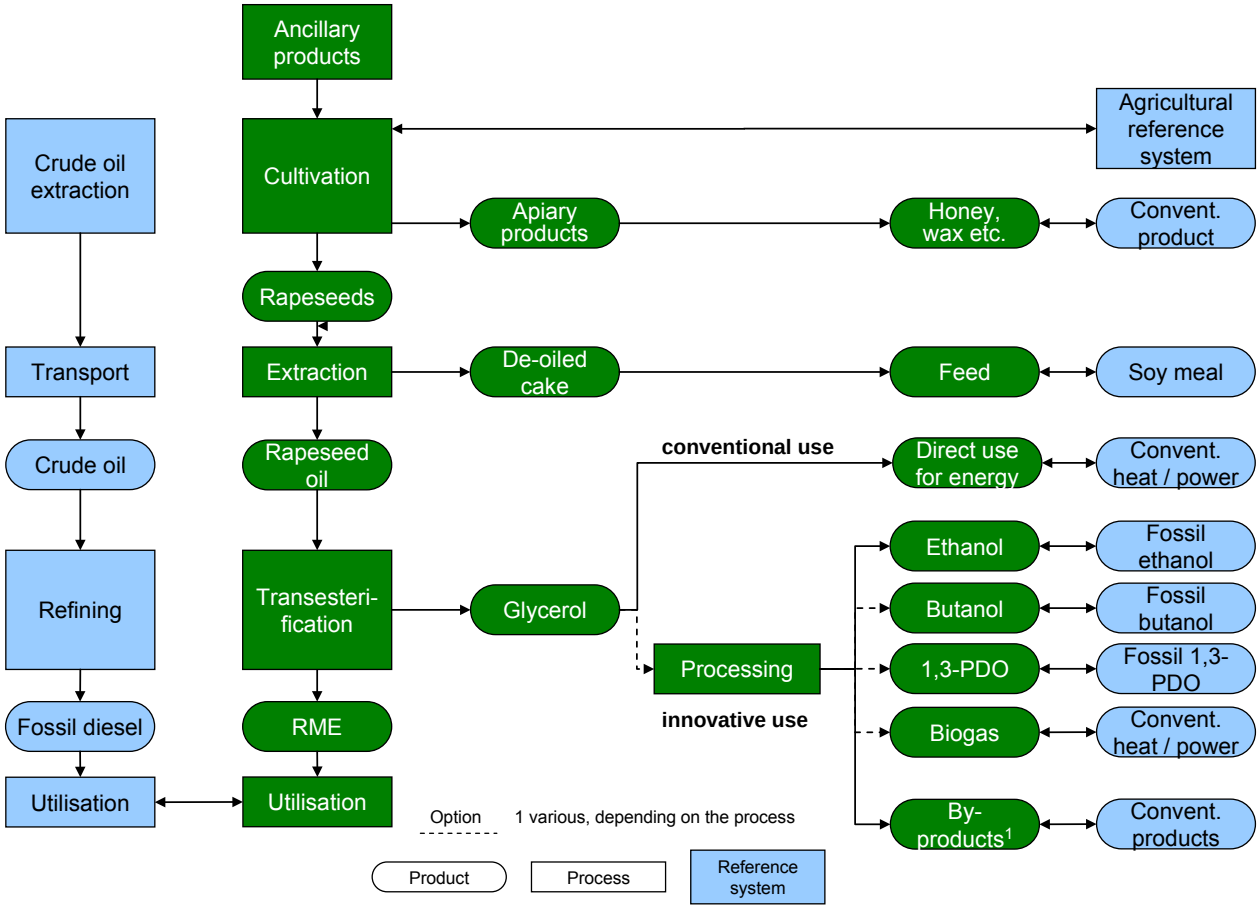

Figure 4: Life cycle of biodiesel production.

3.2.2. Raw materials

Biodiesel can be produced from different oil crops as well as from waste cooking oil and waste animal fats. The feedstock used for biodiesel production influences different factors that are relevant for the economic and ecological assessments, such as the yields of the main product and the by-products as well as the type and amount of ancillary products needed. In contrast, the feedstock has no relevant impact on the glycerol properties and its processing. Therefore, the variation of feedstock is made only within the analysis of the whole biodiesel system (biodiesel production combined with glycerol processing) and not for the analysis of glycerol processing and usage options.

For analysing the influence of the feedstock on the whole biodiesel system, the following feedstocks are regarded:

- Rape seed oil from Europe
- Soybean oil imported from Brazil
- Palm oil imported from South East Asia

The baseline scenario is the production of biodiesel from rape seed oil. Regarding one single oil crop serves the purpose to point out the impacts of different processing steps as well as of different glycerol usage pathways.

In reality, however, biodiesel producers use a mixture of different vegetable oils, mostly rape seed oil, soybean oil and palm oil. Therefore these mixtures are assessed as well. To fulfill the European biodiesel standard DIN EN 14214, the proportions need to be as follows:

1. 70 % rapeseed oil and 30 % soybean oil or
2. 70 % rapeseed, 20 % soybean oil, 10 % palm oil

It has to be noted that the cultivation of soybeans and oil palm may cause land use changes that might massively affect the results of the ecological assessment.

Besides the usage of oil crops, the processing of waste cooking oil and waste animal fats is an option increasingly put into praxis. However, the potential of these feedstocks in Europe is low. The amount of waste oils available for biodiesel production within Europe is estimated as 85 000 tonnes. Since the amount of glycerol produced is only 10 % of the produced biodiesel, the amount of glycerol from waste oils would be too small to justify innovative processing as described within this project. Therefore, waste oils are not included in the GLYFINERY scenarios.

3.3 Glycerol processing

The GLYFINERY project has several products under investigation to determine the potential for adding value to the glycerol by-product. The products under investigation are:

- Ethanol
- Butanol
- 1,3-Propanediol
- Biogas

In the following chapters (chapter 3.3.2 to 3.3.5), these four pathways are described in detail. In chapter 3.3.6 the use of the by-products derived in ethanol, butanol and 1,3-PDO production is described, followed by a summary of all scenarios in chapter 3.4.

However, first a reference system needs to be defined for the glycerol use, i. e. the use that would be realized without the innovative pathways defined in this project. This reference use is presented in chapter 3.3.1.

3.3.1. Reference system for glycerol use

The reference system describes in which way glycerol from biodiesel production would be used if the innovative usage pathways described within the GLYFINERY project were not realized, i. e. the conventional usage pathway for the reference year 2020. The innovative usage pathways are compared to the conventional uses in order to identify respective advantages and disadvantages.

The glycerol market is divided into two sections: a market for crude glycerol with about 80 % purity (crude glycerol as accrued in the biodiesel plants) and a market for refined glycerol with above 99.5 % purity. During biodiesel production, first crude glycerol is produced which has to be purified and refined at relatively high efforts. Due to an increased production of biodiesel, the quantity of crude glycerol worldwide rose remarkably in recent years. In 2008, 12.24 million tonnes of biodiesel have been produced which is equivalent to 1.224 million tonnes of crude glycerol. Compared to that, the world market for refined glycerol was estimated at only 900 000 tonnes in 2005 (16).

Although the capacities for glycerol refining are rising due to the construction of new plants, this increase is much slower than the increase in crude glycerol production. Even a further extension in refining facilities would not be able to absorb all crude glycerol entering the market. One of the main reasons for the slow growth is the high variation in amounts and prices of crude glycerol due to political uncertainties with regard to biodiesel subsidies. These uncertainties lead to a great reluctance towards investments. One consequence of the glycerol glut is a massive decline in prices. In September 2009, the price for crude glycerol was only 100 € / tonne, while the price for refined glycerol was about 300 € / tonne (17). While large biodiesel producers increasingly refine the glycerol themselves, small producers give it away for free

or even have to pay for its disposal. Regarding the current decrease in prices, the price for the energetic use is commonly seen as the minimum value to which the price might decline.

To cover the whole range of future possible glycerol usage pathways, two different reference systems are analyzed:

1. The direct use for energy defines the minimum value for the use of excess crude glycerol. Glycerol is used at 80 % purity without further refining. The following scenarios are analysed:

    a. Provision of energy to biodiesel plant replacing the average electricity and heat mix

    b. External use of energy replacing the average electricity and heat mix

    c. External use of energy replacing the average electricity mix

    d. External use of energy replacing the average heat mix

    In the environmental assessment, replaced power and heat mixes are known to have a significant influence on the results. Therefore, these mixes will be varied based on different countries, e.g. France (with a large share of nuclear power), Sweden (with a large share of renewable energy carriers) and Poland (with a large share of fossil energy carriers).

2. However, part of the crude glycerol will enter the markets due to an increase in refining capacities. To integrate these usage pathways and to define an upper limit for the glycerol value range, its material use is analyzed as second reference system. Precisely, the direct usage as a component of chemicals, pharmaceutical and cosmetic products is analyzed. The analysis includes the expenditures for crude glycerol refining to above 99.5 % purity.

The two reference uses for glycerol are shown in Figure 5.

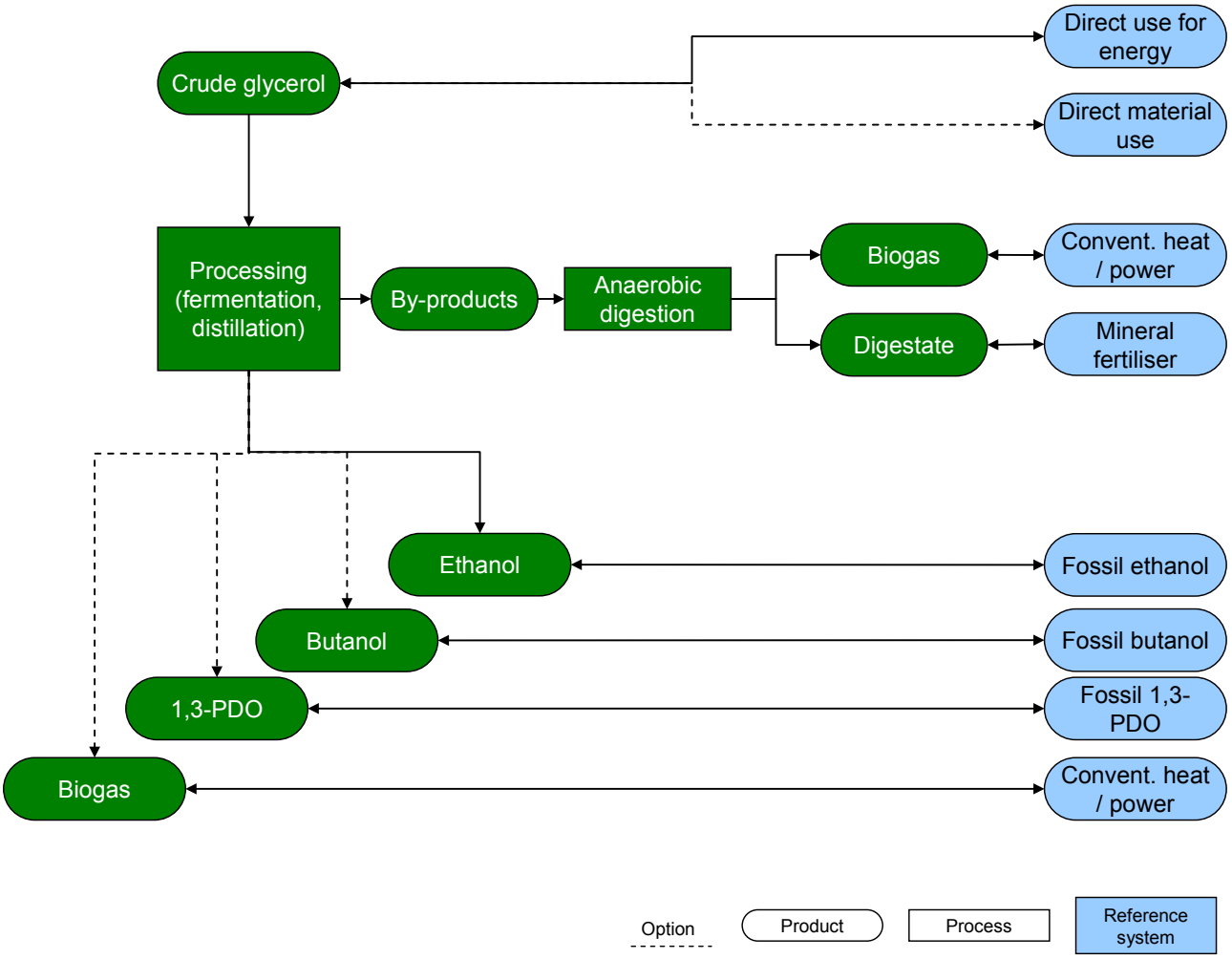

Figure 5: Life cycle of glycerol processing including the reference use of glycerol.

3.3.2. Glycerol to ethanol

Current situation

Ethanol is currently produced in two different pathways: synthetic ethanol from ethylene (which is produced from crude oil; see Figure 6) and ethanol from the fermentation of renewable resources (cereals, sugar crops, lignocellulose). 95 % of the currently produced ethanol is derived from fermentation and only 5 % synthetically. Ethanol is used for beverages, in the chemical industry and as fuel. In 2005, the world wide uses were distributed as follows: 12 % of the whole ethanol production were used for beverages, 16 % in the chemical industry, and 72 % as fuel. Most of the synthetic ethanol is used in the industrial pathway where it is mostly used as solvent (18).

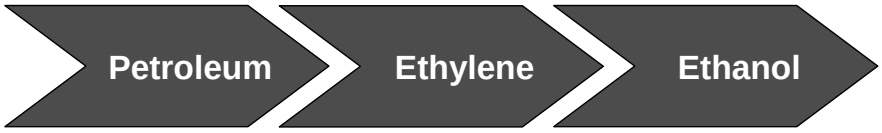

Figure 6: Flowchart depicting the synthesis of ethanol from fossil crude oil.

Use and replaced conventional products (reference products)

Within the GLYFINERY project, the following pathways for the ethanol from glycerol fermentation are analysed:

- Usage in the chemical industry as a substitute for ethanol from fossil sources (crude oil ➔ naphtha ➔ ethylene ➔ ethanol)
- Usage as fuel as a substitute for fossil gasoline

Even though only a small amount of ethanol is used in the chemical industry, this ethanol is of high importance as a solvent. Moreover, the biggest producers of synthetic ethanol are located in Europe (19). Ethanol from glycerol substitutes ethanol from fossil resources, i. e. synthetic ethanol produced with ethylene or rather naphtha as raw material.

Most of the ethanol produced today is used as fuel. As is the case for all ethanol from biomass, ethanol from glycerol substitutes fossil gasoline. Already with today's amounts of glycerol, a considerable amount of bioethanol could be produced. If all glycerol from the European wide biodiesel production (around 8 million tonnes; 19) would be used for ethanol production, 720 000 tonnes ethanol could be produced, which equals about one third of the current production of 2.2 million tonnes in Europe (20).

In the medium or long term, the biofuels produced in Europe will stabilize on a certain level (due to politically determined conditions). The production of ethanol from glycerol is independent from this development. Therefore, ethanol from glycerol is not in direct competition with bio-ethanol from other biomass sources; i. e. glycerol most likely will not replace other biomass feedstocks for fuel ethanol production. These other sources thus are not analyzed as reference products within this project.

Ethanol from glycerol fermentation could also be used to replace methanol in MTBE in order to produce ETBE. In Europe, ethanol in ETBE usually is produced from biomass. Here, the same line of argument can be used as above: since glycerol production is independent from the development of the biofuel market, ethanol from glycerol production does not compete with bio-ethanol from other biomass sources. Therefore, it is not regarded as reference product.

Another option is practiced in the USA. Here, the use of MTBE is forbidden and thus ETBE is used as additive by default. In this case, bio-ethanol from glycerol fermentation would replace conventionally produced ethanol from fossil sources. This corresponds to the first scenario and thus is included.

Use of by-products

During ethanol production from glycerol, different by-products are derived. These are – depending on the environmental conditions – hydrogen and carbon dioxide or formic acid. While carbon dioxide is emitted to the atmosphere, hydrogen or formic acid remains in the fermentation broth. The fermentation broth can at least be used for biogas production without by-product extraction (see chapter 3.3.6 By-product use for biogas production).

Alternatively, use options with a higher value might be realisable. This has to be clarified in the course of this project. If such alternative use options turn out to be realisable, the use of the processed by-product will be compared to a respective conventional equivalent product:

- Formation of hydrogen and carbon dioxide: hydrogen from glycerol processing substitutes hydrogen from fossil natural gas whilst carbon dioxide is released to the atmosphere
- Formation of formic acid: formic acid from glycerol processing replaces formic acid from fossil natural gas (via methanol)

Plant design

Ethanol from glycerol fermentation can only be produced in large centralized plants. An average biodiesel plant produces about 10 000 tonnes of glycerol, yielding in about 3 500 tonnes of ethanol. Existing ethanol plants in Europe have capacities between 50 000 tonnes and 2 000 000 tonnes. Even to meet the lower limit, glycerol from at least 14 biodiesel plants is needed. The glycerol with 80 % purity is transported by lorry to the ethanol processing plant. This procedure is shown in Figure 7. Considering the biodiesel and glycerol fermentation plant sizes and the average distribution of biodiesel plants in Europe, the average transportation distance for glycerol is around 300 km.

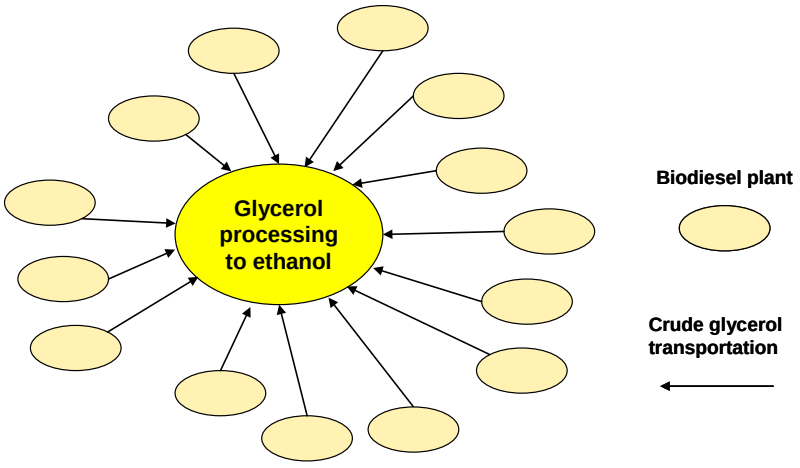

Figure 7: Scheme of the centralized ethanol production from locally produced glycerol

3.3.3. Glycerol to butanol

Current situation

Butanol mostly is used in chemical industries. It is an important platform chemical and can be used as a solvent for surface coatings such as varnishes or in the plastics and textile industry.

Almost all butanol used today is synthesized from fossil sources. This can be done in a variety of ways but the most prevalent is propylene (propene) hydroformylation (oxo synthesis) from fossil propylene (12). A schematic overview of the process can be seen in Figure 8.

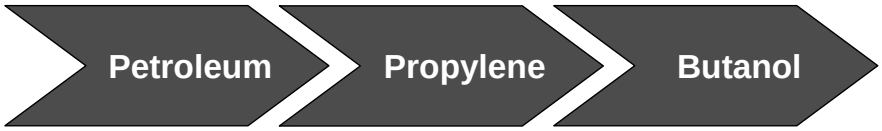

Figure 8: Flowchart depicting the synthesis of butanol from fossil crude oil.

Use and replaced conventional products (reference products)

Theoretically, the production of bio-butanol has the potential to substitute conventional butanol in all aspects of its use. Furthermore, if the production costs can be kept low enough this would open up new venues for the use of butanol. Within the GLYFINERY project the following pathway for butanol from glycerol fermentation is analyzed:

- Usage of butanol in chemical industries as substitute for butanol from fossil sources (crude oil ➔ naphtha ➔ propylene➔ butanol)

The usage of butanol from glycerol processing as transport fuel is theoretically possible but not realistic. The amount of butanol that can be produced in Europe by glycerol processing is very small. Even if all glycerol from European biodiesel production was used for butanol production, only 160 000 tonnes of butanol could be produced at the best. Compared to that, the total ethanol production in Europe is about 2.2 million tonnes, which again is only a small part of the total biofuel production. The infrastructure adaptations in the petroleum industries or, alternatively, technical adaptations in the vehicle motors are not viable from an economic point of view for these small amounts of butanol.

Use of by-products

During butanol production from glycerol, different by-products are derived. Depending on the conditions, carbon dioxide, water, hydrogen, acetone, ethanol and 1,3-PDO can be obtained. Carbon dioxide is released to the atmosphere while the other by-products remain part of the fermentation broth which at least can be used for biogas production (see chapter 3.3.6 By-product use for biogas production).

If it turns out in the course of the project that other by-products are more likely and that other value-adding uses for these by-products are possible, the use of the processed by-product will be compared to a respective conventional equivalent product:

- hydrogen from glycerol processing substitutes hydrogen from natural gas
- ethanol from glycerol processing substitutes ethanol from crude oil processing (via ethylene)
- acetone from glycerol processing substitutes acetone from crude oil (via benzene and propylene)
- 1.3-PDO from glycerol processing substitutes PDO from crude oil (via ethylene)

However, these options still need to be examined in the course of the project.

Plant design

The butanol recovery in glycerol fermentation is only 20 % on average. An average biodiesel plant with an annual production of 100 000 tonnes of biodiesel produces 2 000 tonnes of butanol at the best. For this reason, only larger centralized butanol plants are economically viable. As for ethanol production, crude glycerol with 80 % purity is transported to a central facility for fermentation and separation of butanol. In Europe, a realistic size for a butanol processing facility may be around 10 000 tonnes / yr (21). Thus, around five biodiesel plants are needed to supply one central butanol processing unit, resulting in an average transport distance of 200 km (see Figure 9).

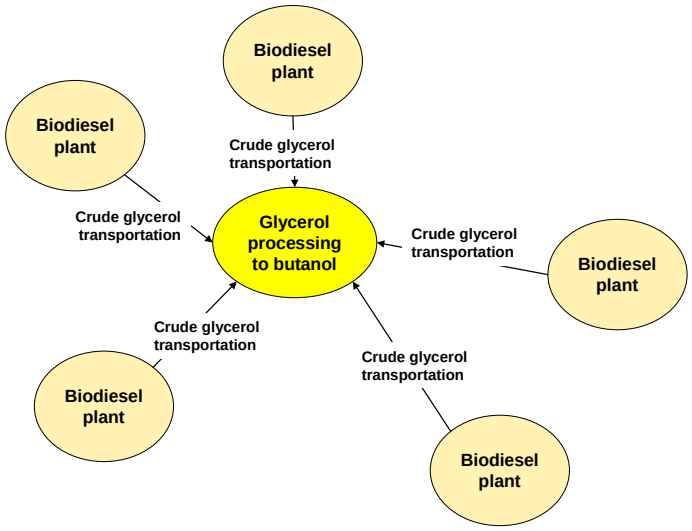

Figure 9: Scheme of the centralized butanol production from locally produced glycerol

3.3.4. Glycerol to 1,3-propanediol (PDO)

Current situation

1,3-propanediol or trimethylene glycol (1,3-PDO) is a chemical mostly used in polymeric applications for the production of textiles or biodegradable plastics. Nowadays, one of the main field of applications for 1,3-PDO is the production of polytrimethyleneterephthalate (PTT). This is a relatively new fiber which, in certain fields of applications, has superior characteristics compared to nylon and PET. For the PTT market – and thus for 1,3-PDO – a strong growth is predicted.

The production of 1,3-PDO stems mostly from petrochemical sources although some biological production has been implemented. The latter is applied since 2006 by DuPont that produces PDO from corn starch fermentation (capacity: 45 000 tonnes /yr). The chemical production of PDO starts with acrolein or ethylene but only has a yield of around 45 %. A schematic overview can be seen in Figure 10. This complex synthesis resulting in relatively high costs process has limited the use of PDO (13; 14). Out of both processes, currently only the production with ethylene as raw material is realized. The main producer is Shell with a production capacity of 73 000 tonnes / yr.

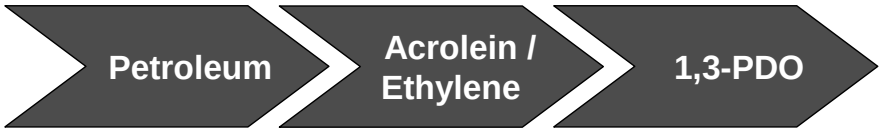

Figure 10: Flowchart depicting the synthesis of 1,3-propanediol from fossil crude oil.

Use and replaced conventional products (reference products)

The GLYFINERY project covers the following usages of 1,3-PDO:

- Usage in chemical industries as substitute for PDO from fossil sources (crude oil ➔ naphtha ➔ ethylene ➔ PDO)
- Usage in chemical industries as substitute for PDO from corn starch fermentation

Synthetic PDO is still the main input for chemical industries. However, the use of bio-based 1,3-PDO has the potential to increase the uses of the chemical if the production cost can be kept low enough. In view of low glycerol prices and rising crude oil prices the usage of glycerol for PDO production and as a substitute for ethylene is of rising attractiveness. With growing PTT markets, there are enough market capacities to absorb the bio-PDO from glycerol processing.
Furthermore, 1,3-propanediol produced by the GLYFINERY process has the potential to also replace the recently developed process of producing 1,3-propanediol from sugar using first generation technology. Corn starch as a renewable resource is currently used only by one company (see above) which comes up with a considerable part of the world's PDO production. If PDO production from glycerol is realized on

industrial scale, this would probably lead to the displacement of corn starch. For this reason, the substitution of corn starch by glycerol is analyzed as a second scenario.

Use of by-products

Also in PDO production from glycerol organic compounds are obtained as by-products. Depending on the bacteria used for fermentation and on the conditions, this can be only oxygen or additionally butyric acid, acetate and ethanol. Organic compounds formed during PDO synthesis can at least be used in a biogas plant for biogas production (see chapter 3.3.6 By-product use for biogas production), but alternative and more valuable usages may be identified in course of this project. In case such alternative pathways are found to be realisable, the organic compounds from glycerol processing are compared to respective equivalent products from fossil sources:

- butyric acid from glycerol processing substituting butyric acid from petroleum processing (via butanol and propylene)
- acetate from glycerol processing substituting acetate from natural gas (via methanol)
- ethanol from glycerol processing substituting ethanol from crude oil processing (via ethylene)

Plant design

The PDO yield from glycerol processing is about 60 %. Thus, an average biodiesel plant with an annual production of 100 000 tonnes produces 6 000 tonnes PDO at the best. Even for the smaller currently running PDO plant (with a capacity of about 45 000 tonnes / yr), the glycerol of about 8 biodiesel plants is needed to supply one PDO plant. Therefore, PDO production – just as ethanol and butanol production – can only be realized in centralized facilities. The crude glycerol (with 80 % purity) produced in biodiesel plants is transported to PDO plants for further processing (see Figure 11). The average transport distance is about 220 km.

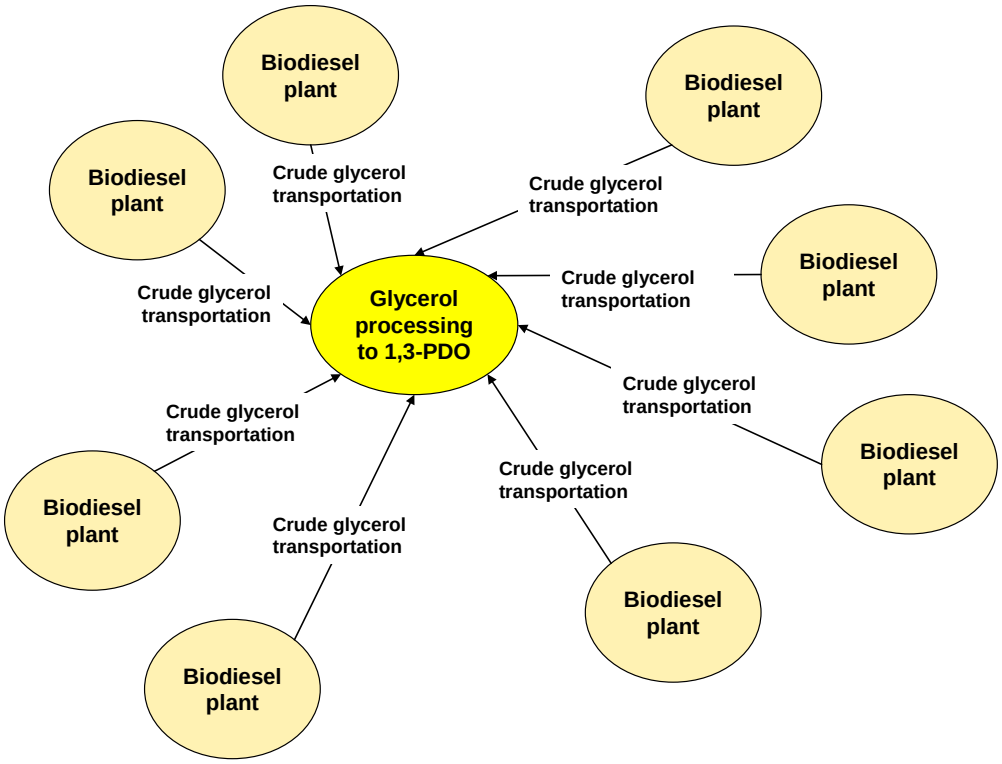

Figure 11: Scheme of the centralized PDO production from locally produced glycerol

3.3.5. Glycerol to biogas

Biogas production in the GLYFINERY system

Besides the above described use pathways of glycerol, it also can be directly fermented in a biogas plant. For this purpose, no refining of the crude glycerol is necessary, i.e. it can be directly used at 80% purity.

Two options are regarded for the biogas production:

- Mono-fermentation of glycerol
- Use of glycerol as co-substrate

The first option has been discussed controversially at the progress meeting in Bratislava (8 / 9 April 2010). Experiments on glycerol mono-fermentation have shown that this is difficult to realize. However, research on this subject is ongoing. Therefore, this option will be kept for the time being and further information will be collected in the course of the project.

The use of glycerol as co-substrate is the more common use option. The addition of glycerol can increase the biogas yields of the different substrates considerably. In order to cover the whole bandwidth of glycerol co-fermentation, two options are assessed:

- Co-fermentation with manure (from cattle and pig)
- Co-fermentation with corn

To both substrates, 6 % glycerol (by weight) is added, an amount being still favourable to the anaerobic digestion (22; 23; 24; 25; 26). The addition of glycerol changes the performance of biogas plants. In particular compared to corn silage, much higher biogas yields per tonne of feedstock are achieved. In fact, a share of 6 wt-% of glycerol leads to a share of 23 % in energy output for corn silage and of 69 % for manure co-fermentation. Therefore, it is most likely that on the long term glycerol will be co-fermented in newly established or restructured biogas plants which have been designed for a feedstock mix with higher energy yield. As a result, no conventional feedstock is going to be replaced by glycerol.

Use and replaced conventional products (reference products)

The biogas obtained from glycerol fermentation can be used in different ways. The following options are assessed:

1. The biogas is used for process energy generation in the biodiesel plant and replaces conventional energy sources.
2. The biogas is used to produce power and heat which is exported and replaces conventional power and heat.
3. The biogas is further processed into biomethane and
    a) used as chemical replacing natural gas
    b) used as transport fuel replacing conventional gasoline and natural gas, respectively,
    c) used for heat and power production which replaces conventional heat and power.

In option 1, glycerol is processed with mono-fermentation within the biodiesel plant. In options 2 and 3, both co- and mono-fermentation are assessed.

In environmental assessments, replaced power and heat mixes are known to have a significant influence on the results. Therefore, these mixes will be varied in all three use options based on different countries, e. g. France (with a large share of nuclear power), Sweden (with a large share of renewable energy carriers) and Poland (with a large share of fossil energy carriers).

Use of by-products

In biogas production, digestate is obtained as a by-product. The digestate can be applied on fields replacing mineral fertiliser. However, since glycerol digestate has low nutrient contents, the application to

agricultural land might not be feasible from an economic point of view. In this case its treatment in a sewage plant might be an alternative option. This still needs to be clarified in the course of the project.

Plant design for mono-fermentation of glycerol

The amount of glycerol produced in an average biodiesel plant is enough to feed a 2 MW_{el} biogas plant. Therefore, glycerol is fermented directly at the biodiesel plant (referred to as 'local' use) and no transportation is necessary. No processing (i.e. refining) of the glycerol is needed for its fermentation.

The processing of biogas into biomethane[1] is economically viable only for larger plants. For example, in Germany, the plants need a capacity of at least about 2-2.5 MW (equivalent electrical power). Since in an average biodiesel plant enough glycerol is derived for feeding such big plants, biogas can be processed locally into biomethane (i.e. directly adjacent to the biodiesel plant).

The principle of local biogas / biomethane production is shown in Figure 12.

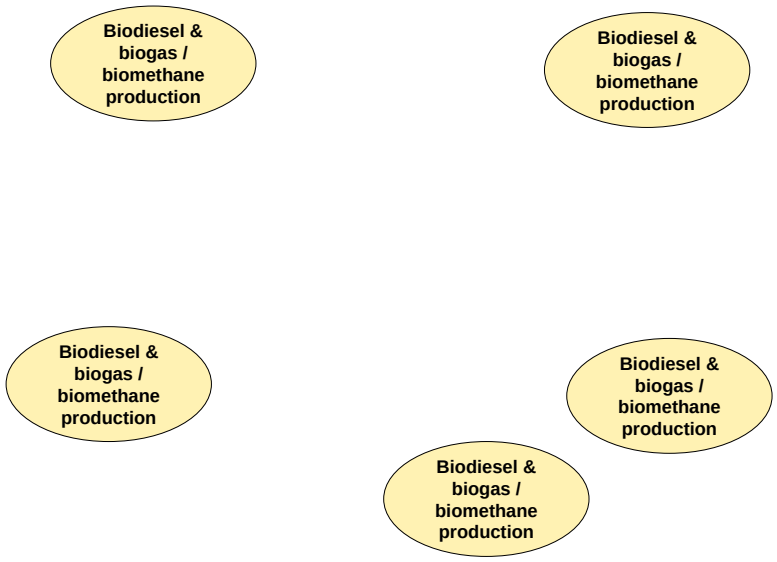

Figure 12: Local biogas / biomethan production in biodiesel plants (mono-fermentation).

As crude glycerol contains hardly no mineral nutrients (except for some potassium), nutrient supplementation is needed for mono-fermentation which has to be taken into account for the environmental and the economic analysis.

Plant design for co-fermentation of glycerol

In co-fermentation, only 6% of glycerol (by weight) is added as co-substrate. Since in an average biodiesel plant, high amounts of glycerol are obtained, the glycerol has to be distributed among several biogas plants (here referred to as 'decentralized' option). Based on an average biogas plant capacity of 0.5 MW, about 2-5 plants (depending on plant design) are needed to co-ferment 6% glycerol with manure, and about 15 plants to co-ferment 6% glycerol with corn silage. Based on the current distribution of biogas plants in Europe, the average transport distance is about 5-70 km (depending on co-ferment type and region).

[1] Biogas which is concentrated via a biogas upgrader to the same standards as fossil natural gas

The processing of biogas into biomethane is economically viable only for big plants of at least about 2-2.5 MW (equivalent electrical power; see above). Three of such big biogas plants are needed to co-ferment the glycerol production of one biodiesel plant. Assuming that every 12th biogas plant is a 2.5 MW plant, the average transport distance is about 15-50 km.

The principle of a decentralized biogas / biomethane production is shown in Figure 13.

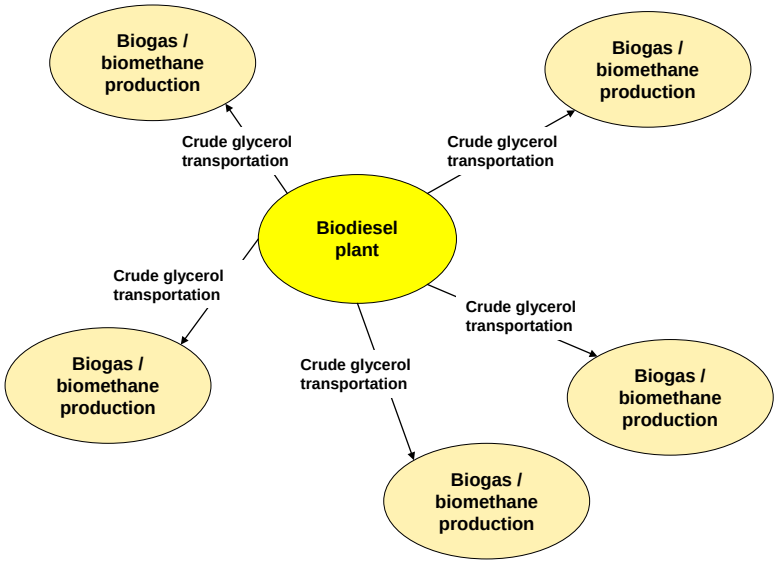

Figure 13: Decentralized biogas / biomethane production (co-fermentation)

3.3.6. By-product use for biogas production

Biomass

During the processing of glycerol into ethanol, butanol and 1,3-PDO, different by-products are derived. They are contained in the fermentation broth as (solid) distillation residue (see chapters 3.3.2, 3.3.3, 3.3.4). Furthermore, liquid residues from glycerol filters are obtained in the production of each of the three products. Both types of residues can be used as a feedstock for biogas production providing part of the process energy required for glycerol processing. The biogas use option is assessed as the basic scenario as long as the extraction of by-products from the fermentation broth is not proved to be feasible.

This means that the fermentation broth as well as filter residues obtained during the production of ethanol, butanol and 1,3-PDO are used in biogas plants which are directly attached to the glycerol processing facilities. The biogas is used for the production of process energy which is used for glycerol processing and replaces fossil energy carriers. In case surplus power is produced it is exported to the grid replacing conventionally produced electricity.

The feasibility of biogas production among others depends on the amount of biogas that can be produced, i.e. on the amount of by-products that is obtained during glycerol processing. This is subject to further research in WP 4. Depending on the outcomes of this work-package, other options of residual treatment might be taken into consideration, e. g. the disposal in a sewage plant.

For the solid use fraction the use as animal feed has been suggested as an alternative use option beside the fermentation in a biogas plant. However, the amount of residue is expected to be too small to justify its collection, processing and use as animal feed. If there is no livestock farming adjacent to the glycerol plant, the residues would have to be concentrated (i.e. dried) to make them transportable and storable, which would result in extra costs.

Biogas digestate

If the residues are fermented, digestate is obtained as a by-product. For the time being, its use as fertilizer replacing mineral fertilizer is assessed. It has to be noted that in case GMO are used for glycerol processing, the use of the digestate as fertilizer needs to be clarified.

However, it might turn out that the digestate has low nutrient contents meaning that an application to agricultural land would not be feasible from an economic point of view. In this case its treatment in a sewage plant might be an alternative option. This needs to be clarified in the course of the project.

3.4 Summary: GLYFINERY scenarios

Table 3 summarizes the main GLYFINERY scenarios including the use of the main product, the plant design (local / decentralized) as well as the reference products. The last column depicts the number allocated to each scenario. For a better orientation, these numbers are also displayed in the flow charts in chapter 4.

Table 3: Summary of main GLYFINERY scenarios

Main product	Centralized / decentralized	Use	Reference product	Scenario N°
Ethanol	Centralized	Chemical	Fossil ethanol via ethylene (crude oil)	1 a
		Transport fuel	Gasoline	1 b
Butanol	Centralized	Chemical	Fossil butanol via propylene (crude oil)	2
1,3-PDO	Centralized	Chemical	Fossil 1,3-PDO via ethylene (crude oil)	3 a
			1,3-PDO from glucose (corn starch)	3 b
Biogas – Mono-fermentation	Local[2]	Process energy for biodiesel production	Conventional heat / electricity	4 a I
		Export of heat/electricity	Conventional heat / electricity	4 a II
	Local[3]	Biomethane for heat/electricity production	Conventional heat / electricity	4 b I
		Biomethane as transport fuel	Gasoline	4 b II
		Biomethane as transport fuel / chemical	Natural gas	4 b III
Biogas – Co-fermentation	Decentralized[3]	Export of heat/electricity	Conventional heat / electricity	4 c I
	Decentralized[4]	Biomethane for heat/electricity production	Conventional heat / electricity	4 d I
		Biomethane as transport fuel	Gasoline	4 d II
		Biomethane as transport fuel / chemical	Natural gas	4 d III

[2] i.e. biogas / biomethane production directly at biodiesel plant
[3] i.e. transportation of crude glycerol from biodiesel plant to decentralised plants for biogas / biomethane production

4. GLYFINERY scenarios: Qualitative flowcharts

This section contains the qualitative flowcharts for each of the pathways under investigation which has been described in the previous chapters. For each main product (ethanol, butanol, 1,3-PDO, biogas) two flowcharts are shown: to the first one illustrates the various process steps within that particular pathway including all inputs and outputs. The second one deals with the glycerol processing as black box and focuses on the outputs and the respective conventional products they replace (reference products).

4.1 Glycerol to ethanol

The flowchart for the production of ethanol can be seen in Figure 14. The purification method is simple distillation. The purification of ethanol by liquid/liquid extraction or solid phase extraction (as specified in WT 5.2 and WT 5.6) was not investigated. Hence the purification is done by simple distillation.

All other by-products are left in the residual fermentation broth for use in the downstream anaerobic degradation step.

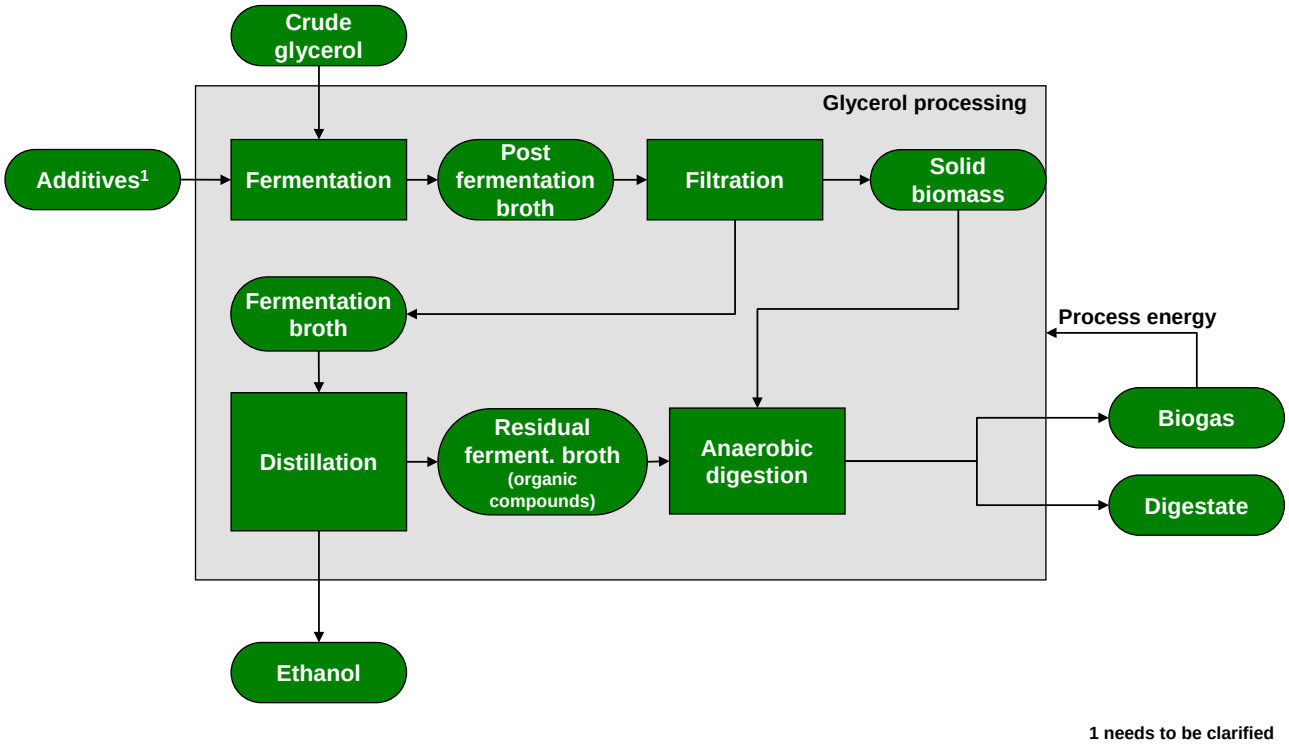

Figure 14: Detailed flowchart depicting the glycerol processing to ethanol.

Figure 15 shows all products derived in ethanol production from glycerol fermentation and the respective reference products. The numbers in the flow charts indicate the scenario. An overview on all scenarios can be found in chapter 3.4.

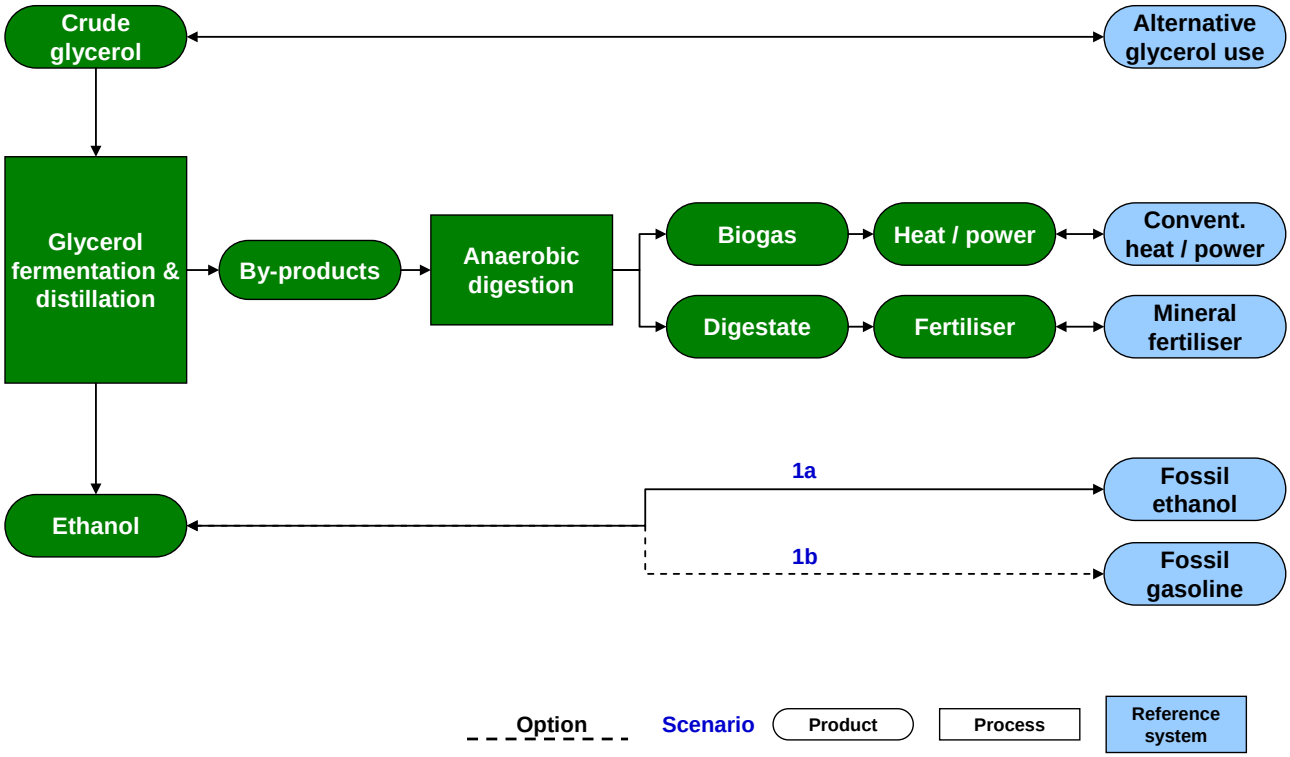

Figure 15: Flowchart depicting all products from ethanol production including all reference products.

4.2 Glycerol to butanol

The flowchart for the production of butanol can be seen in Figure 16. The process has two main products: butanol and 1,3-propanediol. Since there is no main product, the purification will depend on the cost and profitability of each component in the mix. Any residual products are left in the residual fermentation broth for use in the downstream anaerobic degradation.

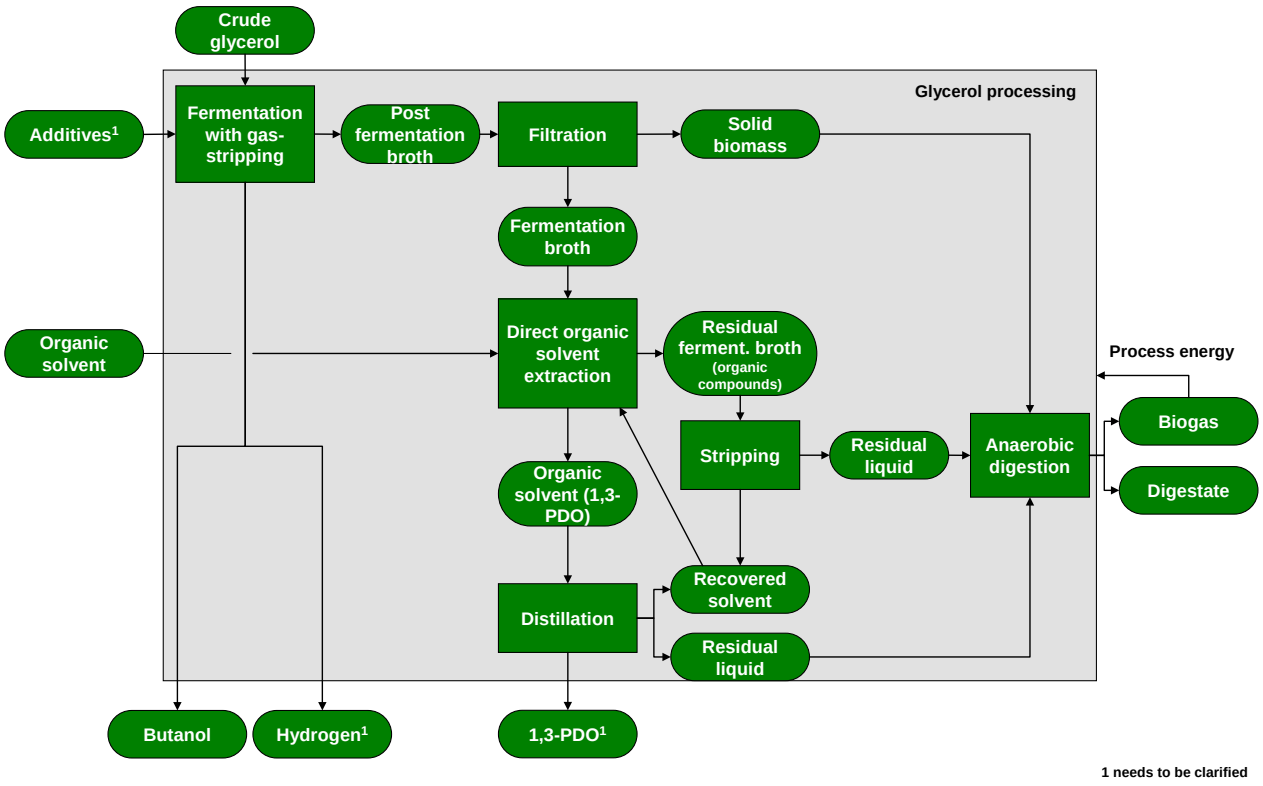

Figure 16: Detailed flowchart depicting the glycerol processing to butanol.

Figure 17 shows all products derived in butanol production from glycerol fermentation and the respective reference products. The numbers depicted in the flow charts indicate the scenario. An overview on all scenarios can be found in chapter 3.4.

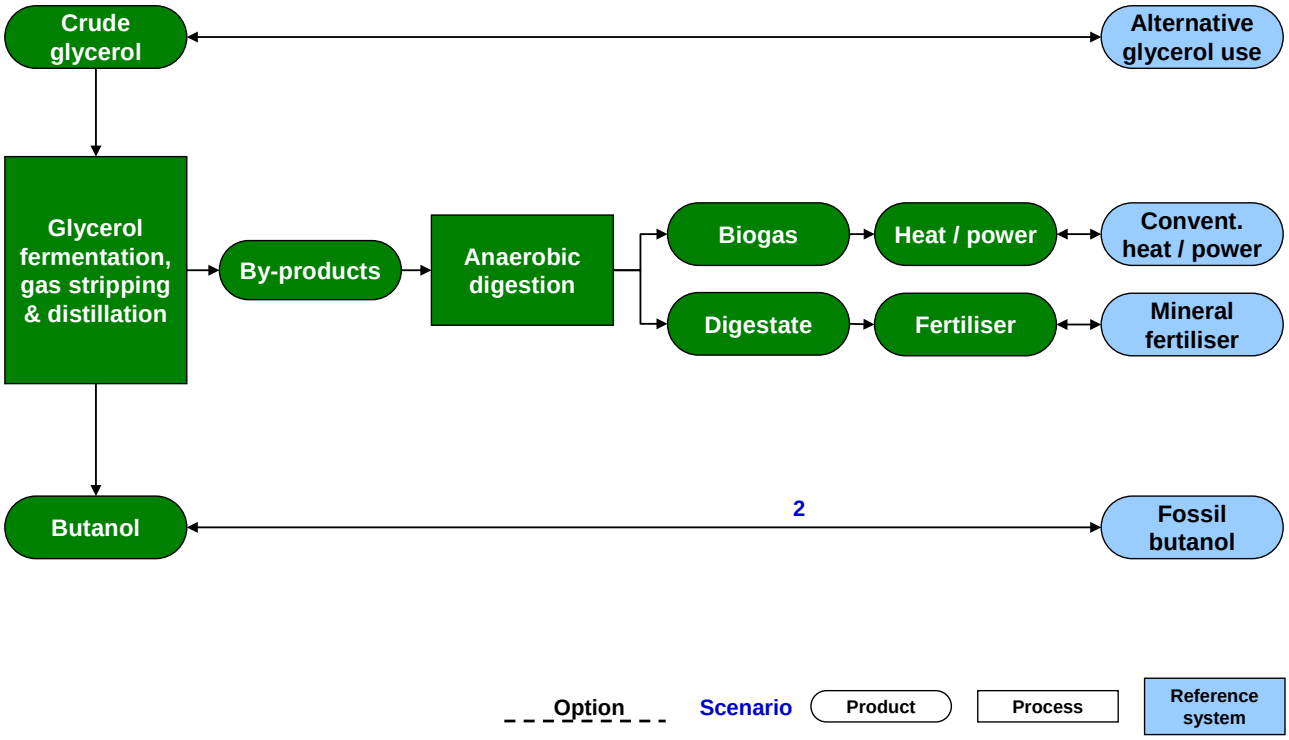

Figure 17: Flowchart depicting all products from butanol production including all reference products.

4.3 Glycerol to 1,3-propanediol

The flowchart for the production of 1,3-propanediol can be seen in Figure 18. As was determined in WP 5 the most promising purification method for 1,3-propanediol is continuous direct liquid/liquid extraction. As in the other processes the remaining products and residual glycerol and nutrients are left in the residual fermentation broth for use in anaerobic degradation.

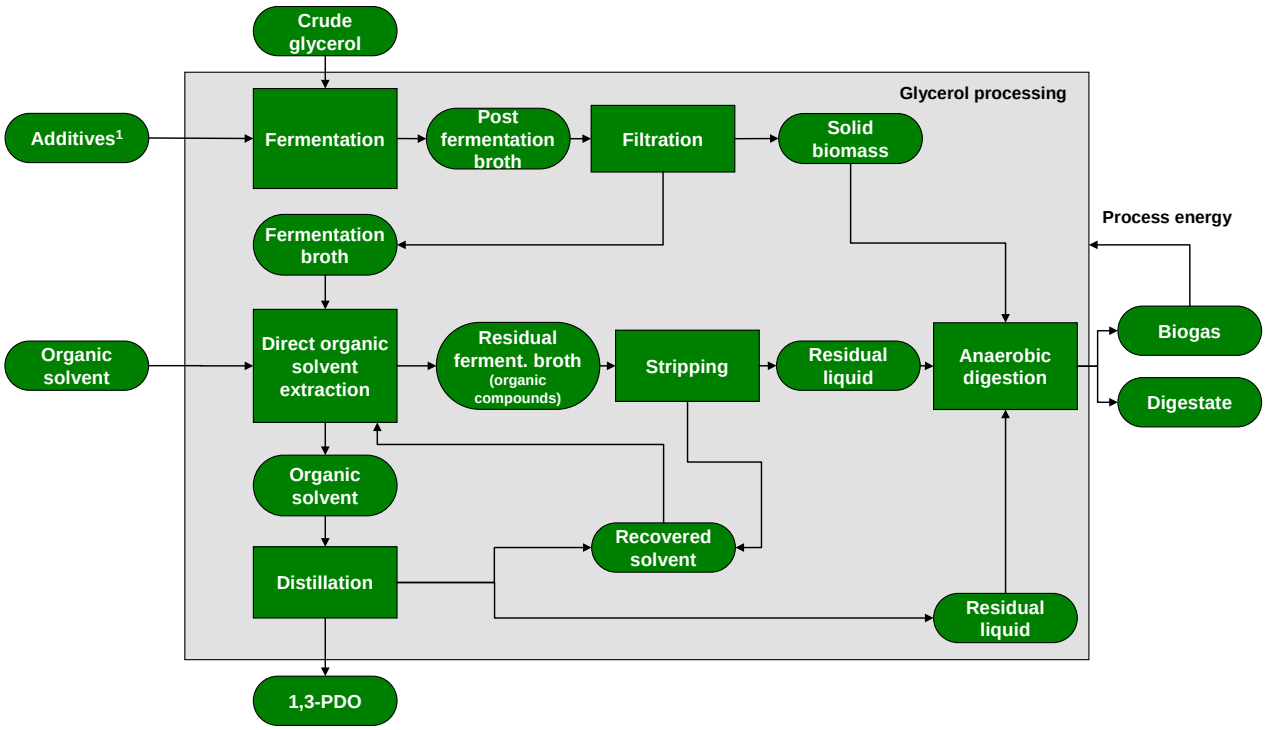

Figure 18: Detailed flowchart depicting the glycerol processing to 1,3-PDO.

Figure 19 shows all products derived in PDO production from glycerol fermentation and the respective reference products. The numbers depicted in the flow charts indicate the scenario. An overview on all scenarios can be found in chapter 3.4.

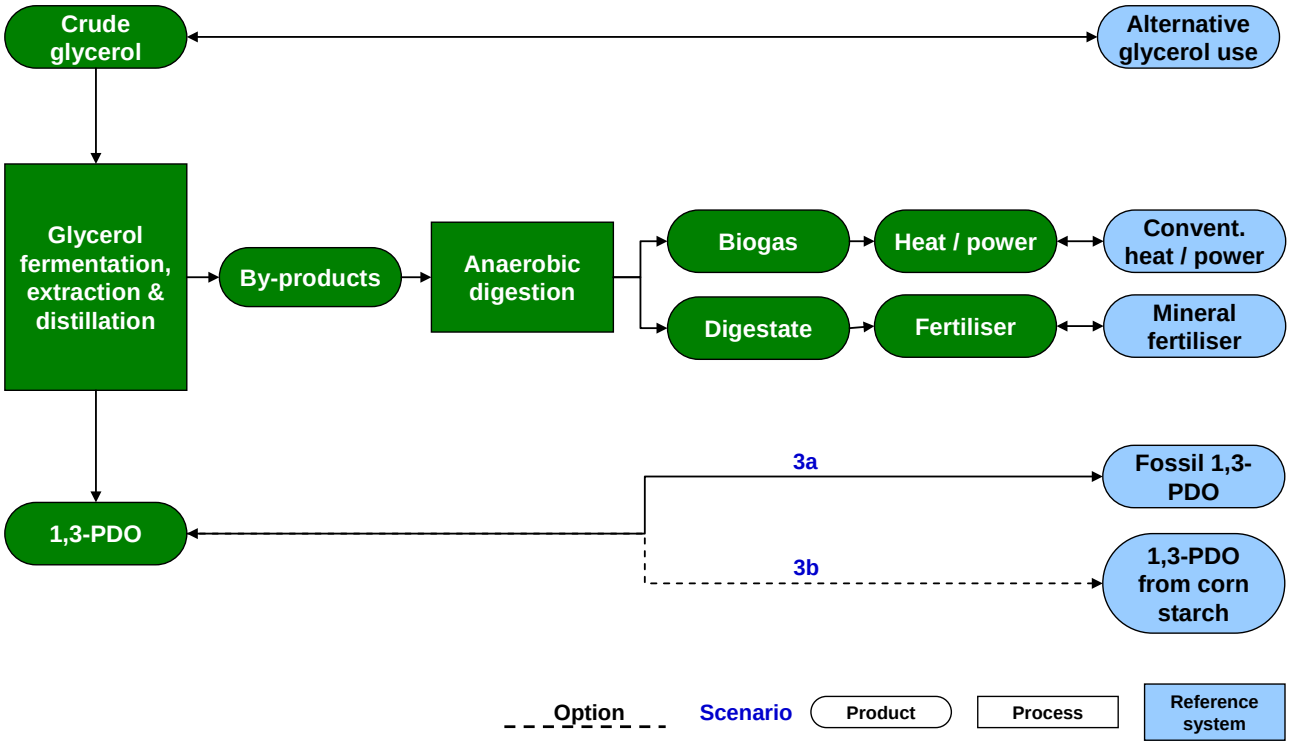

Figure 19: Flowchart depicting all products from 1,3-PDO production including all reference products.

4.4 Glycerol to biogas

The flowchart for the direct production of biogas from glycerol is shown in Figure 20. The biogas is either used directly for energy production or it is further processed into biomethane.

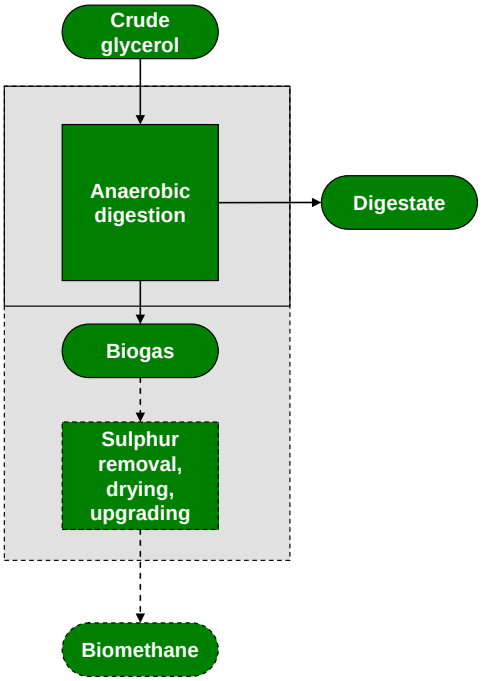

Figure 20: Detailed flowchart depicting the glycerol processing to biogas and biomethane.

Figure 21 shows all products derived in biogas production from glycerol fermentation and the respective reference products. The numbers depicted in the flow charts indicate the scenario. An overview on all scenarios can be found in chapter 3.4.

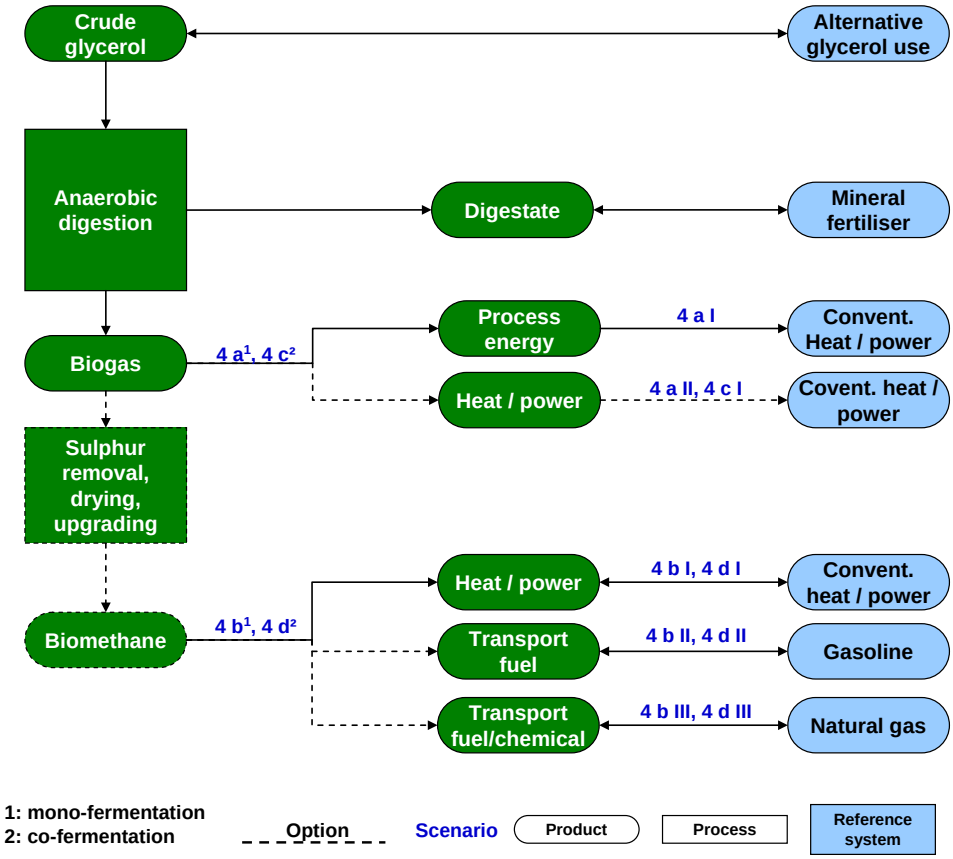

Figure 21: Flowchart depicting all products from biogas and biomethane production including all reference products

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Report from WT 7.4 – Technological assessment (updated)

This section contains an updated version of the initial technological assessment updated with experimental results.

GLYFINERY WP 7: Integrated assessment

WT 7.4 – Technological assessment

26th March 2012

This report contains deliverable 7.4: The previous technological assessment (7.1) updated with respect to experimentally derived results. This deliverable presents the latest versions of the flowcharts and current state of the art for the respective processes: Production of ethanol, butanol, 1,3-PDO and biogas.

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GLYFINERY WP 7: Integrated assessment

D 7.4 – Technological assessment

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Contents

1 Introduction

According to the EU directive 2009/28/EC of 23 April 2009, all member countries shall ensure by 2020 that the share of energy from renewable sources in transport is at least 10 % of the total fuel consumption. For achieving this goal an increased production of biodiesel from biomass plays an important role. In biodiesel production, glycerol is derived as by-product. Due to an increased biodiesel production in recent years high amounts of glycerol entered the market. This development led to a saturation of the glycerol market over the last years with prices for crude glycerol falling considerably.

The objective of the GLYFINERY project is to search for alternative uses for glycerol. Thereby, sustainable usage pathways for glycerol shall be determined and the biodiesel production as a whole shall be optimized. This is achieved by designing biorefinery production schemes for the production of biofuels, bio-energy and green chemicals from glycerol. Accompanying, the objective of WP7 is to assess the economic and ecological sustainability of biodiesel production and glycerol processing and to derive optimisation potentials.

There are two core questions for which the assessments in WP 7 will provide answers:

1. From an ecological and economic point of view, what is the best way to use glycerol resulting from biodiesel production?

2. How do the different usage pathways for glycerol from biodiesel production affect the economic and ecological performance of biodiesel production as a whole?

To address the core questions, the following issues will be assessed:

- What are the advantages and disadvantages of innovative glycerol usage pathways in comparison to the currently existing pathways?
- What is the best use of the products derived from glycerol processing?
- How does the production of green energy from glycerol compare to the material usage or the usage in chemical industries?
- What is the influence of different usage pathways for the by-products on the overall results and which usage shall be preferred?
- What is the relative importance of various life cycle steps on the overall results and which optimisation potentials can be identified?

This paper was prepared as a fulfilment of work task 7.4: *Technological assessment* as part of work package 7: *Integrated assessment*. The paper contains an update of the previous technological assessment (WT 7.1) and describes the most recent flowcharts together with current state of the art concerning the technological aspects of the GLYFINERY processes.

The following areas are addressed in this updated version:

- Latest versions of the flowcharts describing the individual processes
- Latest findings on the composition of crude glycerol
- Current state of the art concerning the production of the fuels and chemicals as proposed by the GLYFINERY processes

2 Crude glycerol composition

The primary feedstock for the GLYFINERY processes is crude glycerol derived as a waste stream from biodiesel production. In the previous report the composition of glycerol was reported to vary between producers [ref del.7.1?]. Furthermore the production biodiesel by a single producer can also be subject to variation. In the GLYFINERY project we have in total received three different batches of crude glycerol from Meroco:

1. (B1) Based on 100% rape seed oil feedstock

2. (B2) Based on a mix of 90% rape seed oil with a blend of 10% waste cooking oil

3. (B3) Based on 100% rape seed oil feedstock

The characteristics of each batch vary since they are derived from different production runs. A picture of the three batches can be seen below:

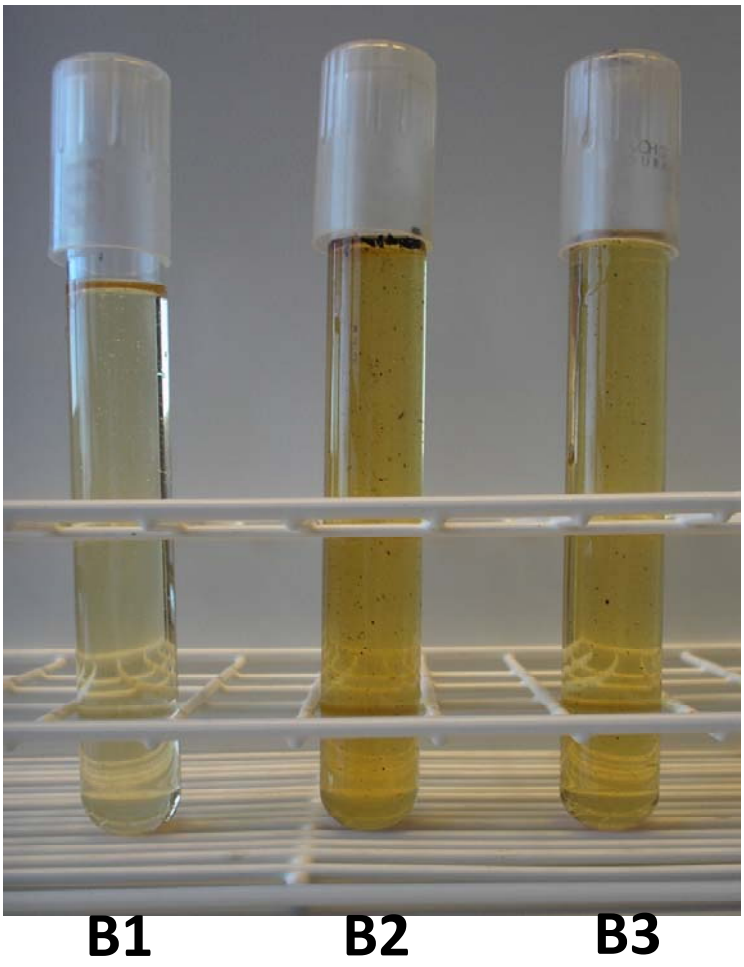

Figure 1: Test tubes with the three different batches of crude glycerol received from Meroco. B1 - Batch 1 (100% rape seed), B2 - batch 2 (90% rape seed with 10% waste cooking oil) and B3 - batch 3 (100% rape seed).

As evident by visual inspection the three batches have different appearance. This prompted a further investigation into the composition of the batches.

The reason for the interest in analyzing the batches further is that some microbial species namely *Clostridium spp.* are sensitive to inhibitory compound present in the crude glycerol. This means that certain batches from a manufacturer or certain manufacturers supply crude glycerol which is unsuitable for growth with the particular microorganisms. Since batch variations were detected in the GLYFINERY processes a decision was made to look into the composition.

It should be mentioned that the selection of microorganisms and processes for the GLYFINERY project have been done with regards to the tolerance towards inhibitors present in the crude glycerol provided by Meroco.

2.1 Further analysis

The three batches were subjected to the following analytical procedures:

- Ion chromatography
- Gas chromatography – mass spectrometry
- High Pressure Liquid Chromatography – mass spectrometry

The method of analysis is comparative. Since we have three different batches of varied composition different degrees of recalcitrance is assumed. The initial batch looked very clean even on visual inspection (as shown above) while the following appear more turbid. Hence the batches were categorized as:

B1 – Best, least amount of impurities

B2 – Worst, highest amount of impurities

B3 – Medium, more impurities than B1 but less than B2

The following analyses were focused more on finding the differences between the three batches than on quantifying the exact composition of each and every compound found within the three batches.

2.1.1 Ion chromatography

The initial analysis with ion chromatography revealed some differences when comparing chromatograms from the three samples. An example is shown in figure 2 below.

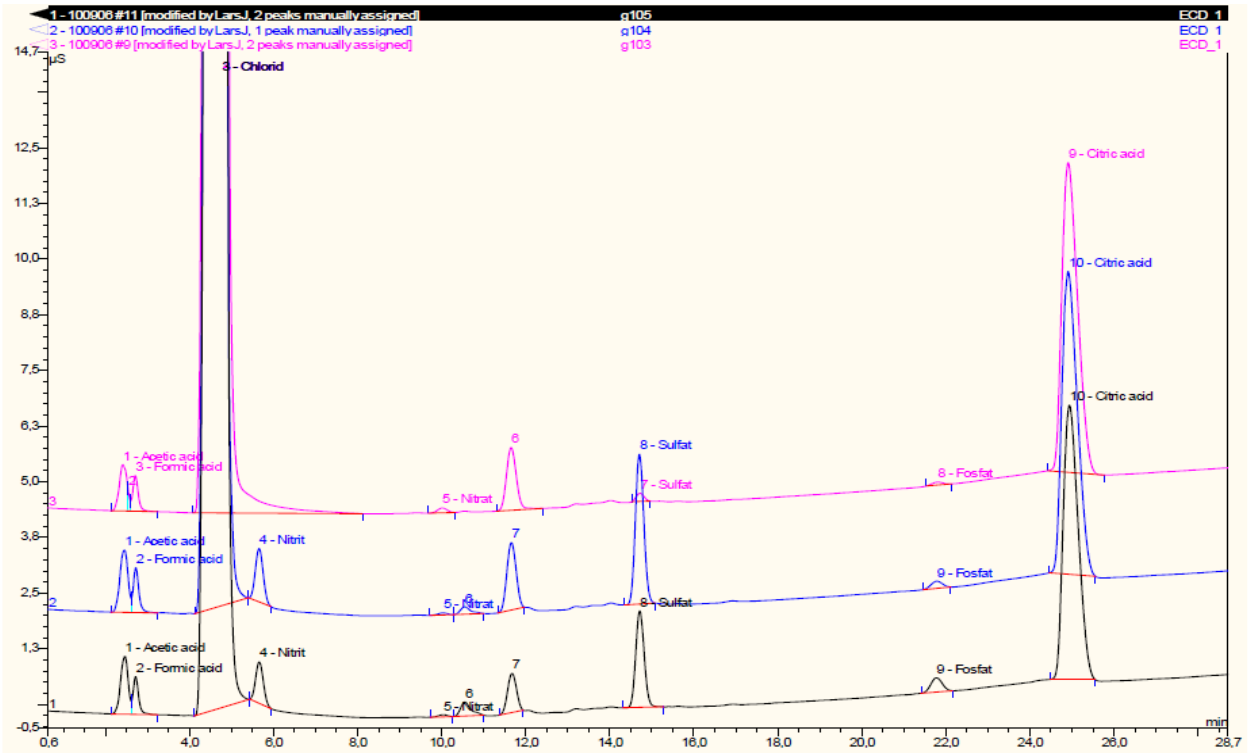

Figure 2: A chromatogram showing the results of ion chromatography. BLACK: B3 – batch three, BLUE: B2 – batch 2 and RED: B1 – batch 1. The various peaks indicate the presence of compounds in the three samples. The area of the peak indicates the quantity of the given compounds. A quick comparison indicates that the initial batch crude glycerol has a cleaner profile than the two other batches.

The advantage of ion chromatography is that it is also possible to quantify the amount of compound present. The results can be seen in table 1 below:

Compound	B1 [g/kg]	B2 [g/kg]	B3 [g/kg]
Acetic acid	0.34	0.47	0.41
Formic acid	0.073	0.10	0.083
Chloride	36.95	31.12	32.35
Nitrite	0	0.20	0.15
Sulphate	0.01	0.57	0.36
Phosphate	0.014	0.063	0.15
Citrate	6.81	6.57	5.73

Table 1: Results from ion chromatography analysis of the three batches of crude glycerol: B1, B2 and B3

The main components appear to be chloride and citrate. In the amounts present: 3.1 – 3.6% it could be a source of inhibition for less halotolerant species.

2.1.2 Gas Chromatography / Mass spectrometry

Another analytical method is gas chromatography combined with mass spectrometry. Here volatile molecules are charged and accelerated through a magnetic field. The resulting fragments are measured as fragments of a given mass, yielding a spectrogram of varying masses. An example is shown in figure 2 below:

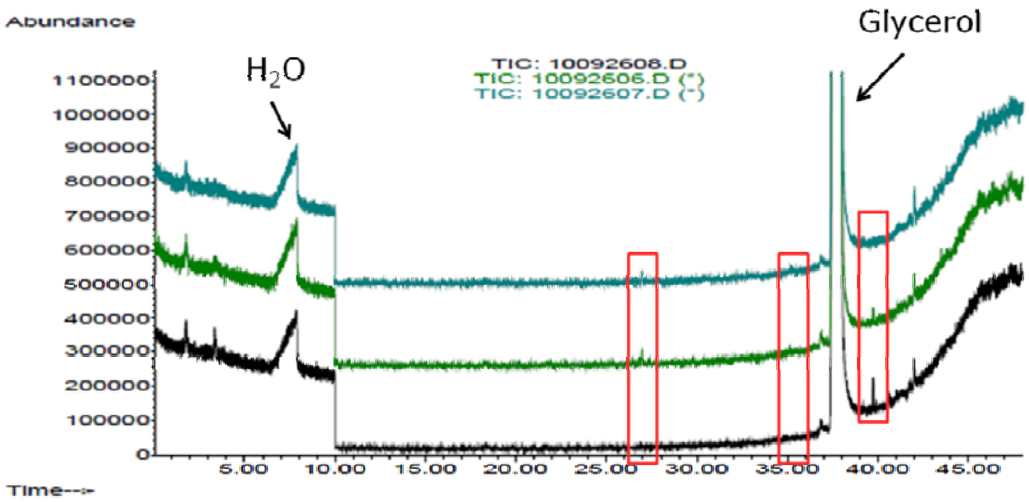

Figure 3: A mass spectrogram comparing 3 batches of glycerol: BLACK (bottom) – B1, GREEN (middle) – B2 and TEAL (top) - B3. Again the peaks indicate the presence or absence of compounds. From the spectra it is possible to identify the compounds present. Again the interesting areas are the ones that differ between the samples, here highlighted in red. As shown in the figure there appears to be areas in the B2 and B3 batches which are not present in B1.

As evident in figure 3 there are several peaks present in samples from B2 and B3 that do not appear in B1. This might give an indication to further investigation into the identity of the inhibitory compounds. From the masses alone a crude identification is possible. These are only indicative and the results can be seen in table 2 below:

Retention time (min.)	Component
26,4	1,3-propanediol
26,9	1,2-propanediol, 3-methoxy
29,7	5-O-Methyl-d-gluconic acid dimethylamide*
36,8	1,2,3-propanetriol, 1 acetate
39,7	16-octadecanoic acid, methyl ester
42,0	3-tert-butyl-4-hydroxyanisole*

Table 2: This table contains an overview of the compounds detected in figure 2. The identification is tentative and serves as an indication only.

Comparing the results from table 2 with the peaks in figure 2 the peaks found in the three red brackets are from the left: 26,9 min. – 1,2-propanediol, 3-methoxy, 36,8 min. – 1,2,3-propanetriol, 1 acetate and 42,0 min. – 3-tert-butyl-4-hydroxyanisole.

2.1.3 High pressure liquid chromatography / Mass spectromertry

As with the previous analysis the same comparative approach can be taken with a liquid chromatography system. An example is shown in figure 4:

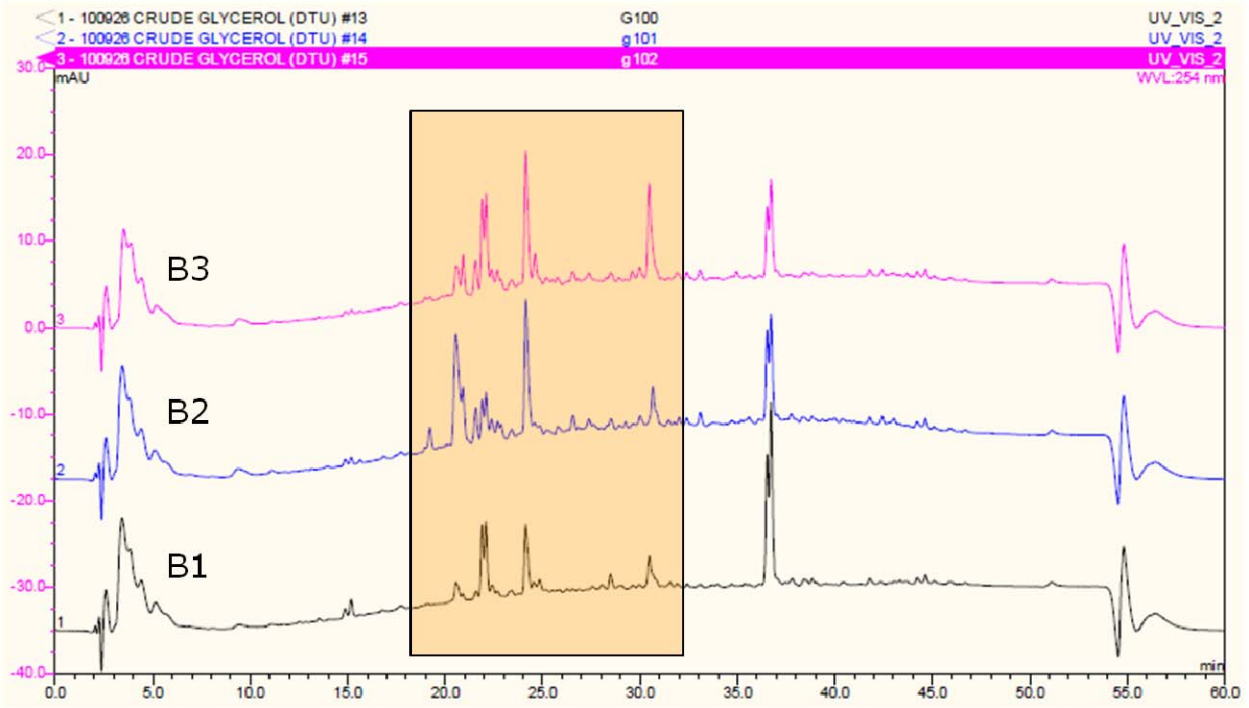

Figure 4: HPLC chromatogram showing data from UV at 254 nm. BLACK (bottom) – B1, BLUE (middle) – B2 and PINK (top) – B3. Again peaks are evident in samples B2 and B3 that are not evident in B1. These are

Again there is some more complexity in the samples B2 and B3 when compared to B1. More analysis is needed to determine the identity of the compounds in question.

2.1.4 Effects of the crude glycerol on the microorganisms

The wild type strain of *C. pasteurianum* was highly affected by the different glycerol. In order to determine the inhibition of the different batches of crude glycerol, the amount of cell mass was measured after 20 hours of incubation at different initial concentration of crude glycerol (Figure 5). B1 glycerol appeared as the least toxic of the different of glycerol batches. This corresponds very well with the amounts of impurities observed by visual inspection. B3 glycerol has lower amounts of impurities than B2 but it was almost impossible to detect growth by wild type *C. pasteurianum* within 20 hours of incubation. On B2 glycerol little growth was detected at low initial glycerol concentration. This proves that glycerol made from different feedstock at the same plant differs significantly in toxicity. To address this challenge, simple steps for detoxifying the crude glycerol was tested.

Among the tested pretreatments for the crude glycerol was Carbonation, Electrodialysis, Purification by activated stone carbon (charcoal), and supplementation of activated stone carbon. Furthermore, it was observed that storage of the glycerol affected the toxicity.

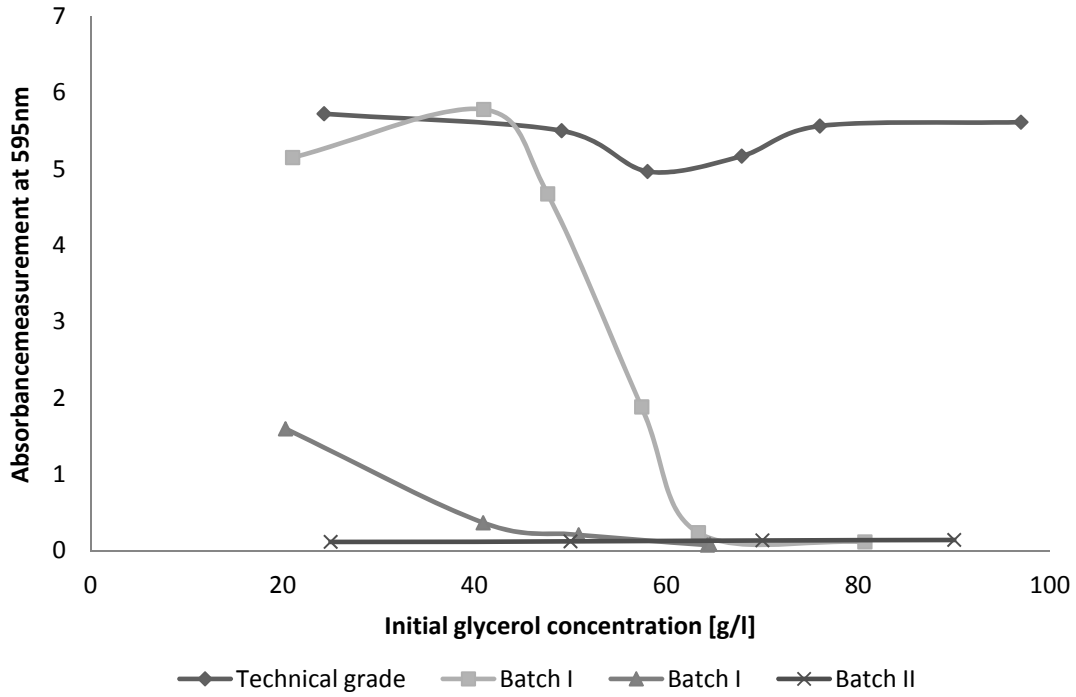

Figure 5: Growth (optical density measurements) on the three batches of glycerol and the technical glycerol. Measurements were carried out after 20 h incubation.

The purification method of Carbonation was adapted from the sugar industry, where it is used for purifying sugar juice. Milk of lime was added to the glycerol then CO_2 was bobbled through the solution. Excess calcium will react with CO_2 to form calcium carbonate which precipitates together with impurities. The purification was tested on B2 glycerol, and growth was significantly stimulated. At initial glycerol concentration of 35 g/l growth was stimulated with 152% compared to non-treated crude glycerol. The method was not proven sufficient as a single detoxifying process, because the effect at high initial glycerol concentrations was limited.

Electrodialysis was proven to be a cheap pretreatment method. It has previously been described that a considerable concentration of NaCl is present in the crude glycerol (see Table 1). A NaCl concentration of approximately 6g/L has been shown to inhibit the related organism *C. butyricum*, therefore, it was desirable to remove the NaCl. By electrodialysis it was possible to remove 98% of the NaCl. Unfortunately, the treatment affected the growth rate negatively. This treatments were therefore not of further interest.

Several investigations have pointed out that activated stone carbon added to substrates containing inhibitory compounds can act as an adsorbent of toxic compounds. Therefore, was this method tested on the crude glycerol. It was shown that activated stone carbon significantly released the inhibition, but was insufficient as a stand-alone process.

As the glycerol was stored, changes did occur releasing the inhibition. The stored crude glycerol supplemented with activated stone carbon was found to stimulate growth in a very high degree. At initial glycerol concentration of 70 g/l growth was 154% compared to the wild type strain grown technical grade glycerol. Even at initial glycerol concentration of 98 g/l growth was constituted 68% compared to the wild

type on technical grade glycerol. By supplementing with activated stone carbon to the media considerable growth by the wild type strain was possible on the very toxic glycerol.

Another approach to address the challenges with the toxicity of the crude glycerol is to identify/develop strains with increased abilities to grow on the crude glycerol.

2.1.5 Summary

All though there is still more work to be done characterizing the contents of the crude glycerol a summary of the initial observations are:

- Chloride and citric acid were present in fairly large amounts
- 1 peak identified in sample B2 (cooking oil) which was not present in the other samples: Molecular mass of 262. It was present only under negative ionization only (not pos.) indicative of it containing an acid group (-COOH)?
- Samples B2 and B3 are more ”complex” in the area of 20-24 min. of HPLC. Further analysis is needed to determine the identity of compounds eluting in this region.
- There seems to be a fair amount of variance within the batches of glycerol received from Meroco although later batches (second and third batch) are more similar than the initial batch received.
- Supplementation of activated stone carbon was found to release the toxicity of the crude glycerol significantly. Enabling the wild type strain of *C. pasteurianum* to utilize this crude glycerol.

3 The GLYFINERY scenarios

Since the completion of the initial technological assessment outlined in deliverabe report 7.1 there have been slight modifications to the flowcharts. These modifications have been updated in the present section and are included with the rest of the original analysis. Specifically the changes concern:

- Figure 16: Detailed flowchart depicting the glycerol processing to butanol
- Figure 18: Detailed flowchart depicting the glycerol processing to 1,3-PDO

In the following chapters, the GLYFINERY scenarios are described. The description follows the life cycle of glycerol production and processing until the use of the main products and by-products. First, general specifications and settings are defined in chapter 3.1. In chapter 0, the systems of biodiesel and glycerol production as well as the raw materials for biodiesel production are described. Subsequently, the scenarios for glycerol processing to innovative products are presented in chapter 3.4. The description covers the reference use of glycerol (i. e. the use of glycerol that would be realized without the innovative pathways), the use of the main products with the conventional products that are replaced, the plant design as well as the use of by-products. These scenarios are summarized in a table in chapter 3.11. The respective flow charts can be found in chapter 1.

3.1 General specifications, definitions and settings

For the ecological and economic analysis of the GLYFINERY scenarios, general definitions and settings are necessary. They are used in both analyses and guarantee their consistency. The general definitions and settings are described and explained below. Definitions and settings specific for either the economic or the ecological assessment will be described and explained within the reports of these tasks (reports for WT 7.2 and WT 7.3, respectively).

- **Technical reference (pilot or mature):** The project aims at comparing glycerol processing technologies, i. e. future innovative technologies with existing (conventional) pathways. Therefore, mature technologies are used as basis for the ecological and economic assessment.
- **Time frame:** The analysed technologies are currently in development and not yet existent. First pilot plants might be available in 2015. As the objective of this project is to compare mature technologies (see 'Technical reference'), 2020 is set as reference year.
  Functional unit: The questions to be answered result in different functional units. As the main objective of the GLYFINERY project is to optimize glycerol processing, the benefit from using 1 tonne of crude glycerol is set as functional unit.
  With regard to the second question – the optimization of biodiesel production as a whole with an optimal use of glycerol playing an important role – the benefit from using the output of the biodiesel production is used as functional unit, i.e. all results are related to 1 tonne biodiesel.
- **Geographical coverage:** Europe is the main producer of biodiesel in the world and therewith of glycerol. As glycerol is traded worldwide and as critical amounts of glycerol are needed for a successful implementation of innovative technologies, Europe as a whole (and not a specific country within Europe) is set as geographical reference. This implies the use of EU27 average values for prices, yields, power mixes, etc.

Glycerol production

Glycerol is derived as by-product in biodiesel production. In order to answer the core questions described in chapter 1, two different systems need to be assessed. They are presented below, followed by the description of raw materials which are used in biodiesel production.

3.2 Systems to be studied

In chapter 1, two core objectives have been defined: the optimization of glycerol processing and use in innovative pathways as well as the optimization of the biodiesel production as a whole. To analyse both issues, two different systems are regarded:

1. Glycerol processing

   The analysis starts with crude glycerol as it leaves the biodiesel plant (80 % purity). Transports (for centralized systems), processing as well as the use of main and by-products are examined. The aim is to deliver a detailed analysis of glycerol processing and usage as well as to identify best possible use options. Based on these analyses, optimization potentials are derived.
   A schematic overview on the glycerol processing is given in Figure 6.

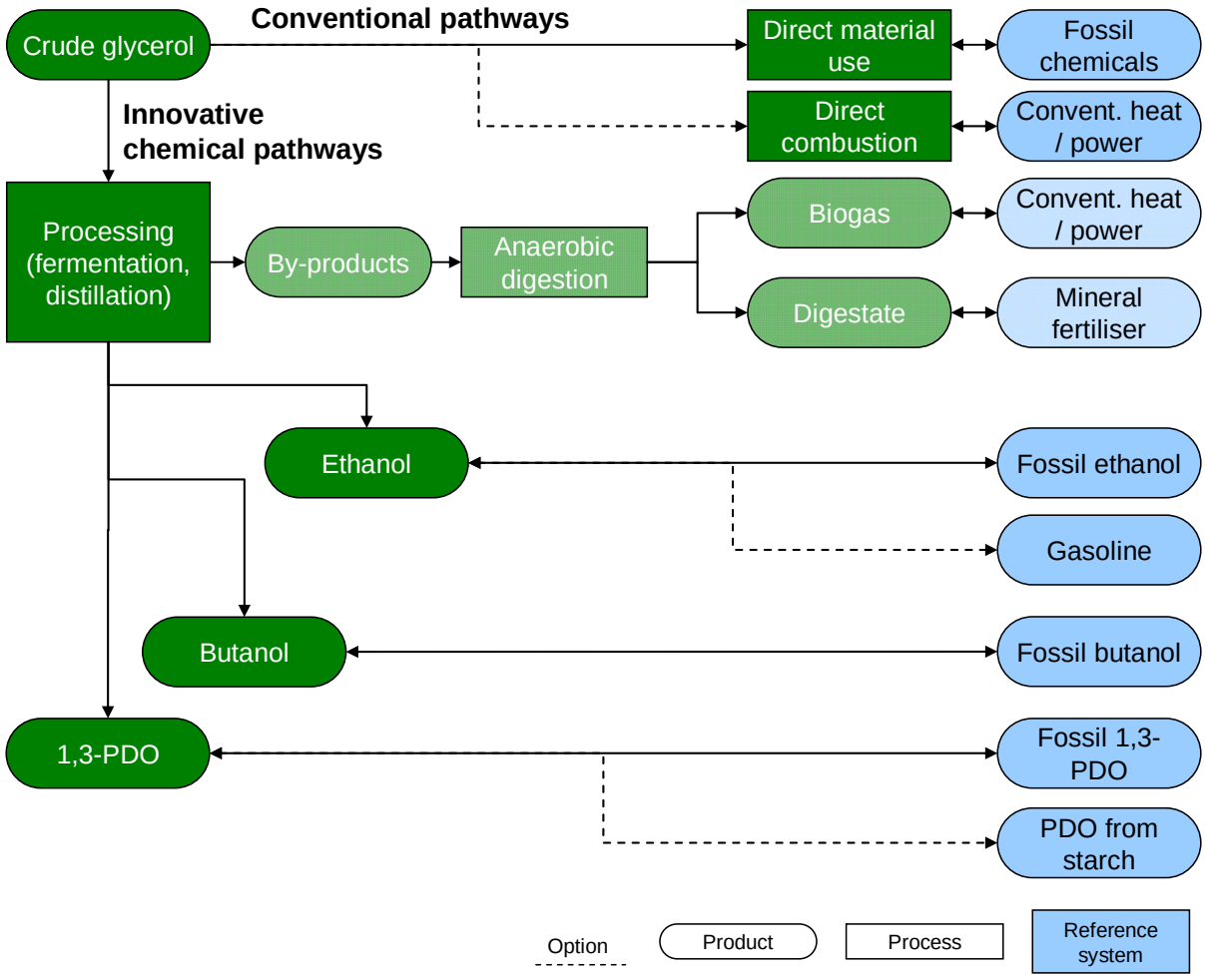

Figure 6: Life cycle of glycerol processing (updated).

2. Biodiesel production as a whole

The whole life cycle of biodiesel production is examined, i.e. the production of oil crops, their processing to biodiesel as well as the processing and usage of all by-products including glycerol. Besides different pathways for glycerol usage other parameters are varied as well, e. g. the raw materials. This analysis aims at depicting the impact of different glycerol usage pathways on the whole biodiesel production and at optimizing the biodiesel production from an economic and ecological point of view.
A schematic overview on the whole biodiesel production system is shown in Figure 7.

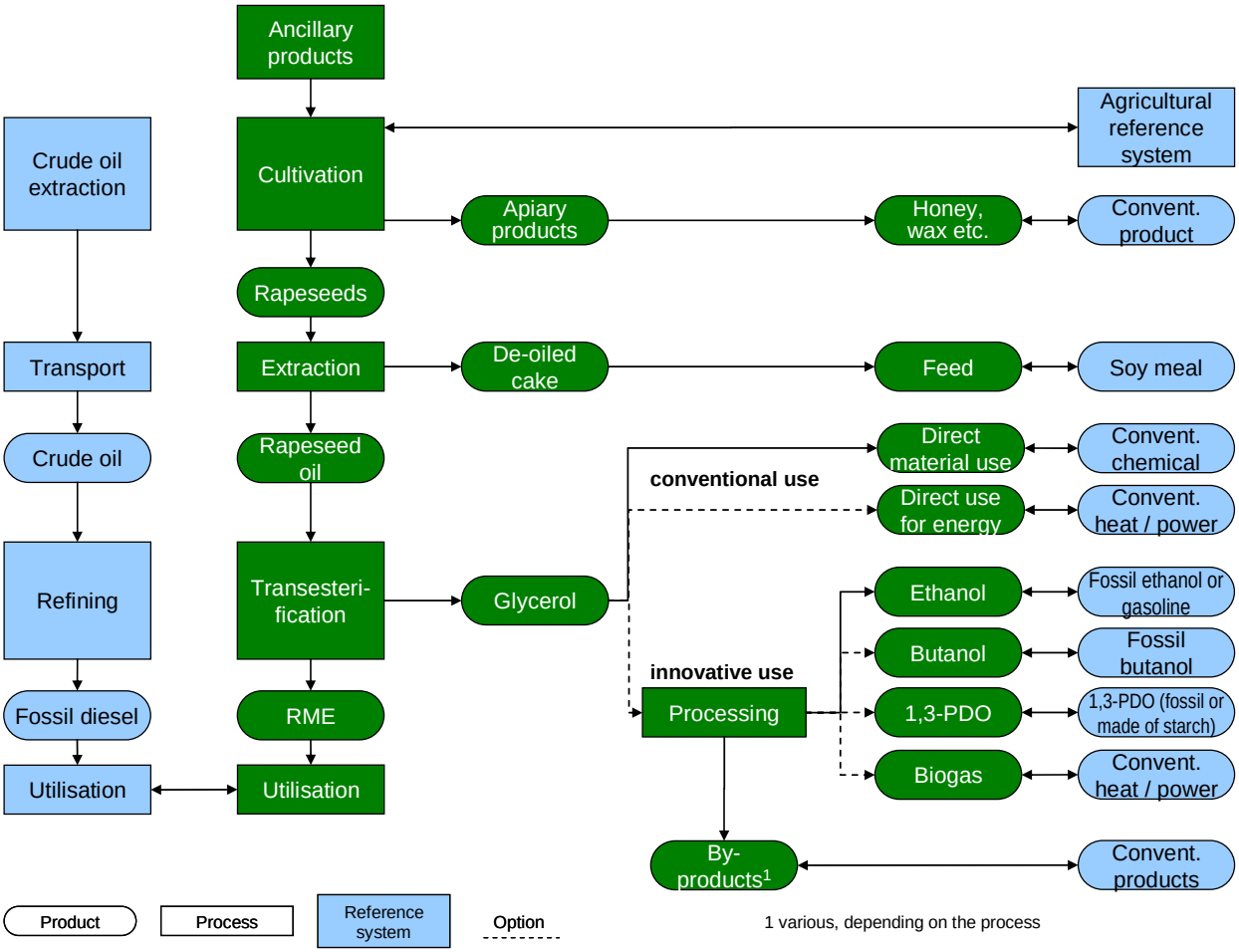

Figure 7: Life cycle of biodiesel production (updated).

3.3 Raw materials

Biodiesel can be produced from different oil crops as well as from waste cooking oil and waste animal fats. The feedstock used for biodiesel production influences different factors that are relevant for the economic and ecological assessments, such as the yields of the main product and the by-products as well as the type and amount of ancillary products needed. In contrast, the feedstock has no relevant impact on the glycerol properties and its processing. Therefore, the variation of feedstock is made only within the analysis of the

whole biodiesel system (biodiesel production combined with glycerol processing) and not for the analysis of glycerol processing and usage options.

For analysing the influence of the feedstock on the whole biodiesel system, the following feedstocks are regarded:

- Rape seed oil from Europe
- Soybean oil imported from Brazil
- Palm oil imported from South East Asia

The baseline scenario is the production of biodiesel from rape seed oil. Regarding one single oil crop serves the purpose to point out the impacts of different processing steps as well as of different glycerol usage pathways.

In reality, however, biodiesel producers use a mixture of different vegetable oils, mostly rape seed oil, soybean oil and palm oil. Therefore these mixtures are assessed as well. To fulfil the European biodiesel standard DIN EN 14214, the proportions need to be as follows:

1. 70 % rapeseed oil and 30 % soybean oil or
2. 70 % rapeseed, 20 % soybean oil, 10 % palm oil

It has to be noted that the cultivation of soybeans and oil palm may cause land use changes that might massively affect the results of the ecological assessment.

Besides the usage of oil crops, the processing of waste cooking oil and waste animal fats is an option increasingly put into praxis. However, the potential of these feedstocks in Europe is low. The amount of waste oils available for biodiesel production within Europe is estimated as 85 000 tonnes. Since the amount of glycerol produced is only 10 % of the produced biodiesel, the amount of glycerol from waste oils would be too small to justify innovative processing as described within this project. Therefore, waste oils are not included in the GLYFINERY scenarios.

3.4 Glycerol processing

The GLYFINERY project has several products under investigation to determine the potential for adding value to the glycerol by-product. The products under investigation are:

- Ethanol
- Butanol
- 1,3-Propanediol
- Biogas

In the following chapters (chapter 3.6 to 3.9), these four pathways are described in detail. In chapter 3.10 the use of the by-products derived in ethanol, butanol and 1,3-PDO production is described, followed by a summary of all scenarios in chapter 3.11.

However, first a reference system needs to be defined for the glycerol use, i. e. the use that would be realized without the innovative pathways defined in this project. This reference use is presented in chapter 3.5.

3.5 Reference system for glycerol use

The reference system describes in which way glycerol from biodiesel production would be used if the innovative usage pathways described within the GLYFINERY project were not realized, i. e. the conventional usage pathway for the reference year 2020. The innovative usage pathways are compared to the conventional uses in order to identify respective advantages and disadvantages.

The glycerol market is divided into two sections: a market for crude glycerol with about 80 % purity (crude glycerol as accrued in the biodiesel plants) and a market for refined glycerol with above 99.5 % purity. During biodiesel production, first crude glycerol is produced which has to be purified and refined at relatively high efforts. Due to an increased production of biodiesel, the quantity of crude glycerol worldwide rose remarkably in recent years. In 2008, 12.24 million tonnes of biodiesel have been produced which is equivalent to 1.224 million tonnes of crude glycerol. Compared to that, the world market for refined glycerol was estimated at only 900 000 tonnes in 2005 (16).

Although the capacities for glycerol refining are rising due to the construction of new plants, this increase is much slower than the increase in crude glycerol production. Even a further extension in refining facilities would not be able to absorb all crude glycerol entering the market. One of the main reasons for the slow growth is the high variation in amounts and prices of crude glycerol due to political uncertainties with regard to biodiesel subsidies. These uncertainties lead to a great reluctance towards investments. One consequence of the glycerol glut is a massive decline in prices. In September 2009, the price for crude glycerol was only 100 € / tonne, while the price for refined glycerol was about 300 € / tonne (17). While large biodiesel producers increasingly refine the glycerol themselves, small producers give it away for free or even have to pay for its disposal. Regarding the current decrease in prices, the price for the energetic use is commonly seen as the minimum value to which the price might decline.

To cover the whole range of future possible glycerol usage pathways, two different reference systems are analyzed:

1. The direct use for energy defines the minimum value for the use of excess crude glycerol. Glycerol is used at 80 % purity without further refining. The following scenarios are analysed:

   a. Provision of energy to biodiesel plant replacing the average electricity and heat mix

   b. External use of energy replacing the average electricity and heat mix

   c. External use of energy replacing the average electricity mix

   d. External use of energy replacing the average heat mix

2. However, part of the crude glycerol will enter the markets due to an increase in refining capacities. To integrate these usage pathways and to define an upper limit for the glycerol value range, its material use is analyzed as second reference system. Precisely, the direct usage as a component of chemicals, pharmaceutical and cosmetic products is analyzed. The analysis includes the expenditures for crude glycerol refining to above 99.5 % purity.

The two reference uses for glycerol are shown in Figure 8.

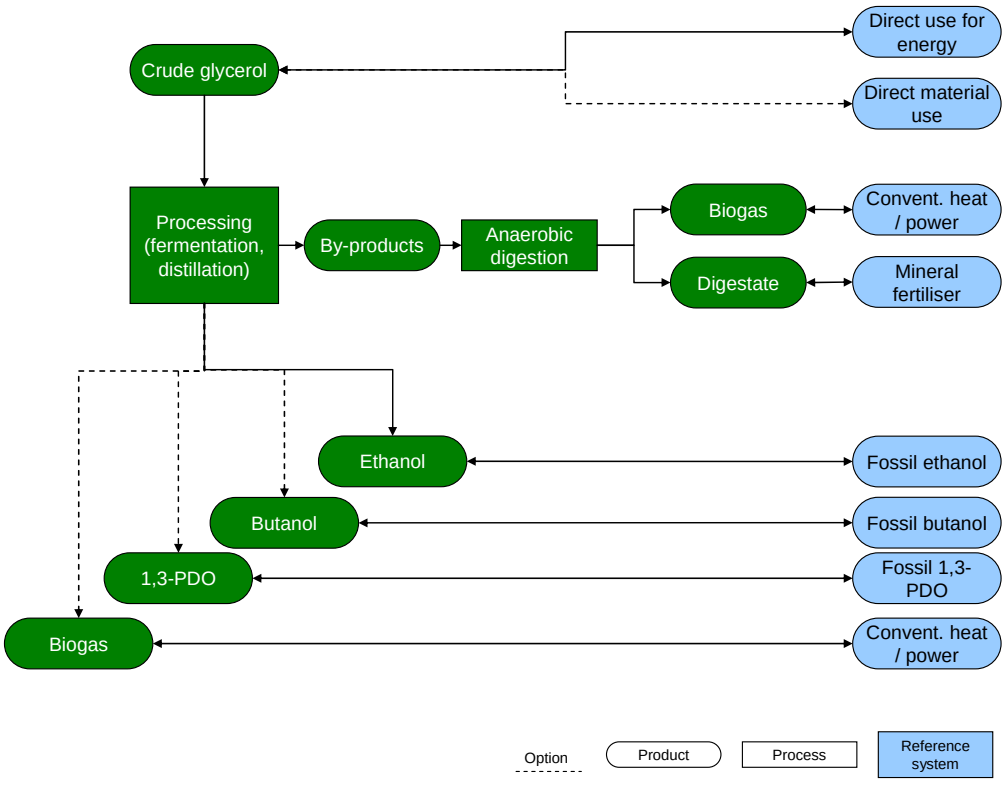

Figure 8: Life cycle of glycerol processing including the reference use of glycerol.

3.6 Glycerol to ethanol

Ethanol is currently produced in two different pathways: synthetic ethanol from ethylene (which is produced from crude oil; see Figure 9) and ethanol from the fermentation of renewable resources (cereals, sugar crops, lignocellulose). 95 % of the currently produced ethanol is derived from fermentation and only

5 % synthetically. Ethanol is used for beverages, in the chemical industry and as fuel. In 2005, the world wide uses were distributed as follows: 12 % of the whole ethanol production were used for beverages, 16 % in the chemical industry, and 72 % as fuel. Most of the synthetic ethanol is used in the industrial pathway where it is mostly used as solvent (18).

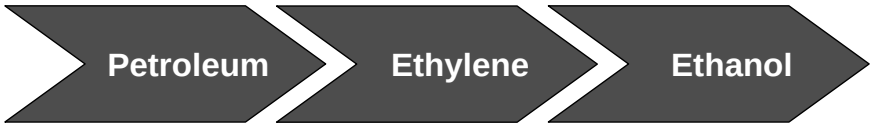

Figure 9: Flowchart depicting the synthesis of ethanol from fossil crude oil.

3.6.1 Use and replaced conventional products (reference products)

Within the GLYFINERY project, the following pathways for the ethanol from glycerol fermentation are analysed:

- Usage in the chemical industry as a substitute for ethanol from fossil sources (crude oil ➔ naphtha ➔ ethylene ➔ ethanol)
- Usage as fuel as a substitute for fossil gasoline

Even though only a small amount of ethanol is used in the chemical industry, this ethanol is of high importance as a solvent. Moreover, the biggest producers of synthetic ethanol are located in Europe (19). Ethanol from glycerol substitutes ethanol from fossil resources, i. e. synthetic ethanol produced with ethylene or rather naphtha as raw material.

Most of the ethanol produced today is used as fuel. As is the case for all ethanol from biomass, ethanol from glycerol substitutes fossil gasoline. Already with today's amounts of glycerol, a considerable amount of bioethanol could be produced. If all glycerol from the European wide biodiesel production (around 8 million tonnes; 19) would be used for ethanol production, 720 000 tonnes ethanol could be produced, which equals about one third of the current production of 2.2 million tonnes in Europe (20).

In the medium or long term, the biofuels produced from primary biomass in Europe will stabilize on a certain level (due to politically determined conditions). The production of ethanol from glycerol is independent from this development. Therefore, ethanol from glycerol is not in direct competition with bio-ethanol from other biomass sources, i. e. glycerol most likely will not replace other biomass feedstocks for fuel ethanol production. These other sources thus are not analyzed as reference products within this project.

Ethanol from glycerol fermentation could also be used to replace methanol in MTBE in order to produce ETBE. In Europe, ethanol in ETBE usually is produced from biomass. Here, the same line of argument can be used as above: since glycerol production is independent from the development of the biofuel market, ethanol from glycerol production does not compete with bio-ethanol from other biomass sources. Therefore, it is not regarded as reference product.

Another option is practiced in the USA. Here, the use of MTBE is forbidden and thus ETBE is used as additive by default. In this case, bio-ethanol from glycerol fermentation would replace conventionally produced ethanol from fossil sources. This corresponds to the first scenario and thus is included.

3.6.2 Use of by-products

During ethanol production from glycerol, different by-products are derived. These are – depending on the environmental conditions – hydrogen and carbon dioxide or formic acid. While carbon dioxide is emitted to the atmosphere, hydrogen or formic acid remain in the fermentation broth. The fermentation broth can at least be used for biogas production without by-product extraction (see chapter 3.10 By-product use for biogas production).

Alternatively, use options with a higher value might be realisable. If such alternative use options turn out to be realisable, the use of the processed by-product will be compared to a respective conventional equivalent product.

3.6.3 Plant design

Ethanol from glycerol fermentation can only be produced in large centralized plants. An average biodiesel plant produces about 10 000 tonnes of glycerol, yielding in about 3 500 tonnes of ethanol. Existing ethanol plants in Europe have capacities between 50 000 tonnes and 2 000 000 tonnes. Even to meet the lower limit, glycerol from at least 14 biodiesel plants is needed. The glycerol with 80 % purity is transported by lorry to the ethanol processing plant. This procedure is shown in Figure 10. Considering the biodiesel and glycerol fermentation plant sizes and the average distribution of biodiesel plants in Europe, the average transportation distance for glycerol is around 300 km.

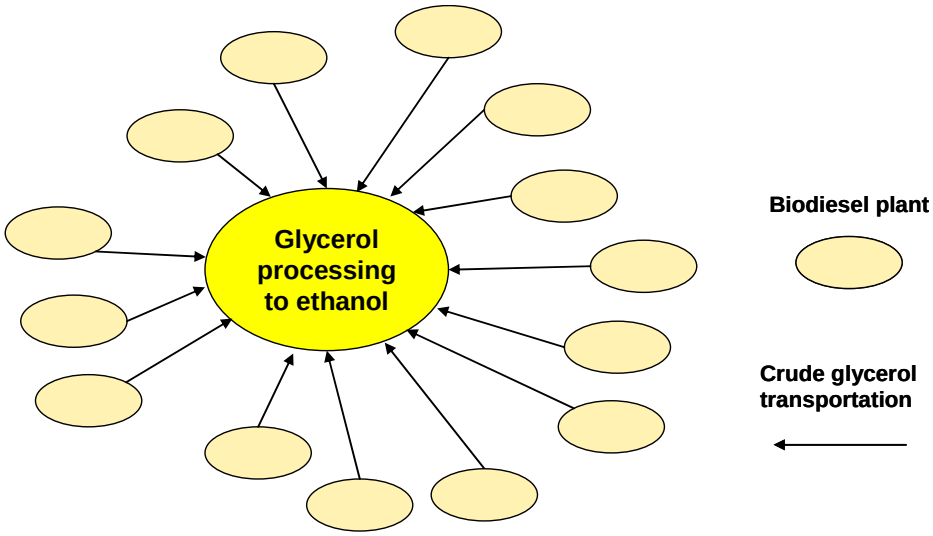

Figure 10: Scheme of the centralized ethanol production from locally produced glycerol

3.7 Glycerol to butanol

Butanol mostly is used in chemical industries. It is an important platform chemical and can be used as a solvent for surface coatings such as varnishes or in the plastics and textile industry.

Almost all butanol used today is synthesized from fossil sources. This can be done in a variety of ways but the most prevalent is propylene (propene) hydroformylation (oxo synthesis) from petrochemical sources (12). A schematic overview of the process can be seen in Figure 11.

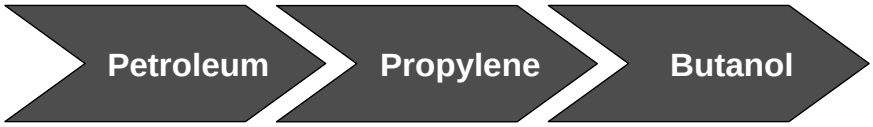

Figure 11: Flowchart depicting the synthesis of butanol from fossil crude oil.

3.7.1 Use and replaced conventional products (reference products)

Theoretically, the production of bio-butanol has the potential to substitute conventional butanol in all aspects of its use. Furthermore, if the production costs can be kept low enough this would open up new venues for the use of butanol. Within the GLYFINERY project the following pathway for butanol from glycerol fermentation is analyzed:

- Usage of butanol in chemical industries as substitute for butanol from fossil sources (crude oil ➔ naphtha ➔ propylene➔ butanol)

The usage of butanol from glycerol processing as transport fuel is theoretically possible but not realistic. The amount of butanol that can be produced in Europe by glycerol processing is very small. Even if all glycerol from European biodiesel production was used for butanol production, only 160 000 tonnes of butanol could be produced at the best. Compared to that, the total production in Europe is about 2.2 million tonnes, which again is only a small part of the total biofuel production. The infrastructure adaptations in the petroleum industries or, alternatively, technical adaptations in the vehicle motors are not viable from an economic point of view for these small amounts of butanol.

3.7.2 Use of by-products

During butanol production from glycerol, different by-products are derived. Depending on the conditions, carbon dioxide, water, hydrogen, acetone, ethanol and 1,3-PDO can be obtained. Carbon dioxide is released to the atmosphere while the other by-products remain part of the fermentation broth which at least can be used for biogas production (see chapter 3.10 By-product use for biogas production).

3.7.3 Plant design

The butanol recovery in glycerol fermentation is only 20 % on average. An average biodiesel plant with an annual production of 100 000 tonnes of biodiesel produces 2 000 tonnes of butanol at the best. For this reason, only larger centralized butanol plants are economically viable. As for ethanol production, crude glycerol with 80 % purity is transported to a central facility for fermentation and separation of butanol. In Europe, a realistic size for a butanol processing facility may be around 10 000 tonnes / yr (21). Thus, around five biodiesel plants are needed to supply one central butanol processing unit, resulting in an average transport distance of 200 km (see Figure 12).

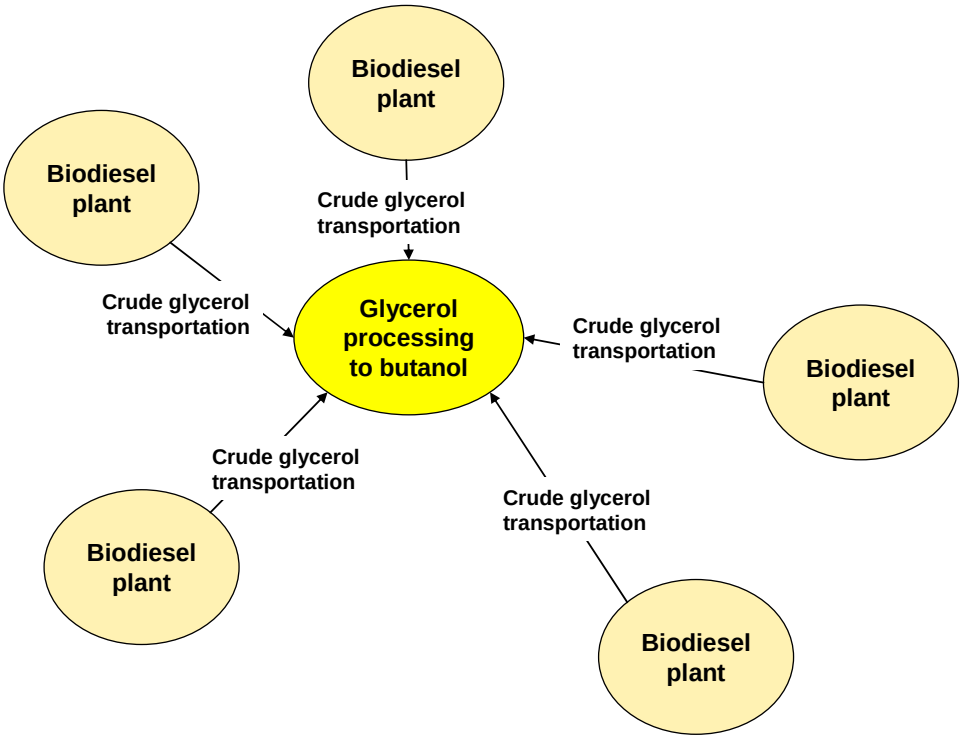

Figure 12: Scheme of the centralized butanol production from locally produced glycerol

3.8 Glycerol to 1,3-propanediol (1,3-PDO)

1,3-propanediol or trimethylene glycol (1,3-PDO) is a chemical mostly used in polymeric applications for the production of textiles or biodegradable plastics. Nowadays, one of the main field of applications for 1,3-PDO is the production of polytrimethyleneterephthalate (PTT). This is a relatively new fiber which, in certain fields of applications, has superior characteristics compared to nylon and PET. For the PTT market – and thus for 1,3-PDO – a strong growth is predicted.

The production of 1,3-PDO stems mostly from petrochemical sources although some biological production has been implemented. The latter is applied since 2006 by DuPont that produces 1,3-PDO from corn starch fermentation (capacity: 45 000 tonnes /yr). The chemical production of 1,3-PDO starts with acrolein or ethylene but only has a yield of around 45 %. A schematic overview can be seen in Figure 13. This complex synthesis resulting in relatively high costs process has limited the use of 1,3-PDO (13; 14). Out of both processes, currently only the production with ethylene as raw material is realized. The main producer is Shell with a production capacity of 73 000 tonnes / yr.

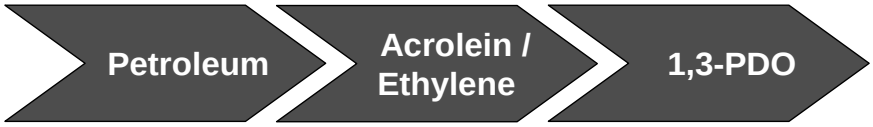

Figure 13: Flowchart depicting the synthesis of 1,3-propanediol from fossil crude oil.

3.8.1 Use and replaced conventional products (reference products)

The GLYFINERY project covers the following usages of 1,3-PDO:

- Usage in chemical industries as substitute for 1,3-PDO from fossil sources (crude oil ➔ naphtha ➔ ethylene ➔ 1,3-PDO)
- Usage in chemical industries as substitute for 1,3-PDO from corn starch fermentation

Synthetic 1,3-PDO is still the main input for chemical industries. However, the use of bio-based 1,3-PDO has the potential to increase the uses of the chemical if the production cost can be kept low enough. In view of low glycerol prices and rising crude oil prices the usage of glycerol for 1,3-PDO production and as a substitute for ethylene is of rising attractiveness. With growing PTT markets, there are enough market capacities to absorb the bio-1,3-PDO from glycerol processing.
Furthermore, 1,3-propanediol produced by the GLYFINERY process has the potential to also replace the recently developed process of producing 1,3-propanediol from sugar using first generation technology. Corn starch as a renewable resource is currently used only by one company (see above) which comes up with a considerable part of the world's 1,3-PDO production. If 1,3-PDO production from glycerol is realized on industrial scale, this would probably lead to the displacement of corn starch. For this reason, the substitution of corn starch by glycerol is analyzed as a second scenario.

3.8.2 Use of by-products

Also in 1,3-PDO production from glycerol organic compounds are obtained as by-products. Depending on the bacteria used for fermentation and on the conditions, this can be only oxygen or additionally butyric acid, acetate and ethanol. Organic compounds formed during 1,3-PDO synthesis can at least be used in a biogas plant for biogas production (see chapter 3.10 By-product use for biogas production. In case such alternative pathways are found to be realisable, the organic compounds from glycerol processing are compared to respective equivalent products from fossil sources:

- butyric acid from glycerol processing substituting butyric acid from petroleum processing (via butanol and propylene)
- acetate from glycerol processing substituting acetate from natural gas (via methanol)
- ethanol from glycerol processing substituting ethanol from crude oil processing (via ethylene)

3.8.3 Plant design

The 1,3-PDO yield from glycerol processing is about 60 %. Thus, an average biodiesel plant with an annual production of 100 000 tonnes produces 6 000 tonnes 1,3-PDO at the best. Even for the smaller currently running 1,3-PDO plant (with a capacity of about 45 000 tonnes / yr), the glycerol of about 8 biodiesel plants is needed to supply one 1,3-PDO plant. Therefore, 1,3-PDO production – just as ethanol and butanol production – can only be realized in centralized facilities. The crude glycerol (with 80 % purity) produced in biodiesel plants is transported to 1,3-PDO plants for further processing (see Figure 14). The average transport distance is about 220 km.

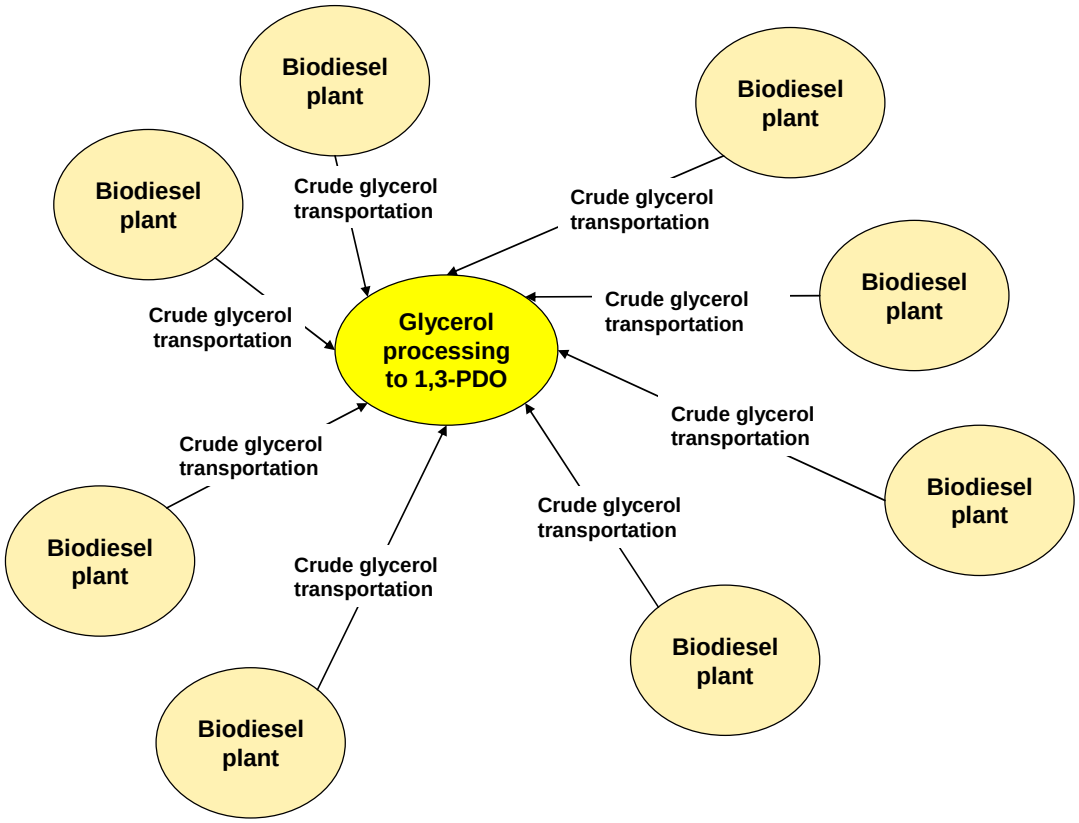

Figure 14: Scheme of the centralized 1,3-PDO production from locally produced glycerol

3.9 Glycerol to biogas

Besides the above described use pathways of glycerol, it also can be directly fermented in a biogas plant. For this purpose, no refining of the crude glycerol is necessary, i.e. it can be directly used at 80% purity.

Two options are regarded for the biogas production:

- Mono-fermentation of glycerol
- Use of glycerol as co-substrate

The first option has been discussed controversially at the progress meeting in Bratislava (8 / 9 April 2010). Experiments on glycerol mono-fermentation have shown that this is difficult to realize. However, co-fermentation was later agreed to be a relevant option.

The use of glycerol as co-substrate is the more common use option. The addition of glycerol can increase the biogas yields of the different substrates considerably. In order to cover the whole bandwidth of glycerol co-fermentation, two options are assessed:

- Co-fermentation with manure (from cattle and pig)
- Co-fermentation with corn

To both substrates, 6 % glycerol (by weight) is added, an amount being still favourable to the anaerobic digestion (22; 23; 24; 25; 26). The addition of glycerol changes the performance of biogas plants. In particular compared to corn silage, much higher biogas yields per tonne of feedstock are achieved. In fact, a share of 6 wt-% of glycerol leads to a share of 23 % in energy output for corn silage and of 69 % for manure co-fermentation. Therefore, it is most likely that on the long term glycerol will be co-fermented in newly established or restructured biogas plants which have been designed for a feedstock mix with higher energy yield. As a result, no conventional feedstock is going to be replaced by glycerol.

3.9.1 Use and replaced conventional products (reference products)

The biogas obtained from glycerol fermentation can be used in different ways. The following options are assessed:

1. The biogas is used for process energy generation in the biodiesel plant and replaces conventional energy sources.
2. The biogas is used to produce power and heat which is exported and replaces conventional power and heat.
3. The biogas is further processed into biomethane and
   a) used as chemical replacing natural gas
   b) used as transport fuel replacing conventional gasoline and natural gas, respectively,
   c) used for heat and power production which replaces conventional heat and power.

In option 1, glycerol is processed with mono-fermentation within the biodiesel plant. In options 2 and 3, both co- and mono-fermentation are assessed.

3.9.2 Use of by-products

In biogas production, digestate is obtained as a by-product. The digestate can be applied on fields replacing mineral fertiliser. However, since glycerol digestate has low nutrient contents, the application to agricultural land might not be feasible from an economic point of view. In this case its treatment in a sewage plant might be an alternative option.

3.9.3 Plant design for mono-fermentation of glycerol

The amount of glycerol produced in an average biodiesel plant is enough to feed a 2 MW_{el} biogas plant. Therefore, glycerol is fermented directly at the biodiesel plant (referred to as 'local' use) and no transportation is necessary. No processing (i.e. refining) of the glycerol is needed for its fermentation.

The processing of biogas into biomethane[1] is economically viable only for larger plants. For example, in Germany, the plants need a capacity of at least about 2-2.5 MW (equivalent electrical power). Since in an average biodiesel plant enough glycerol is derived for feeding such big plants, biogas can be processed locally into biomethane (i.e. directly adjacent to the biodiesel plant).

[1] Biogas which is concentrated via a biogas upgrader to the same standards as fossil natural gas

The principle of local biogas / biomethane production is shown in Figure 15.

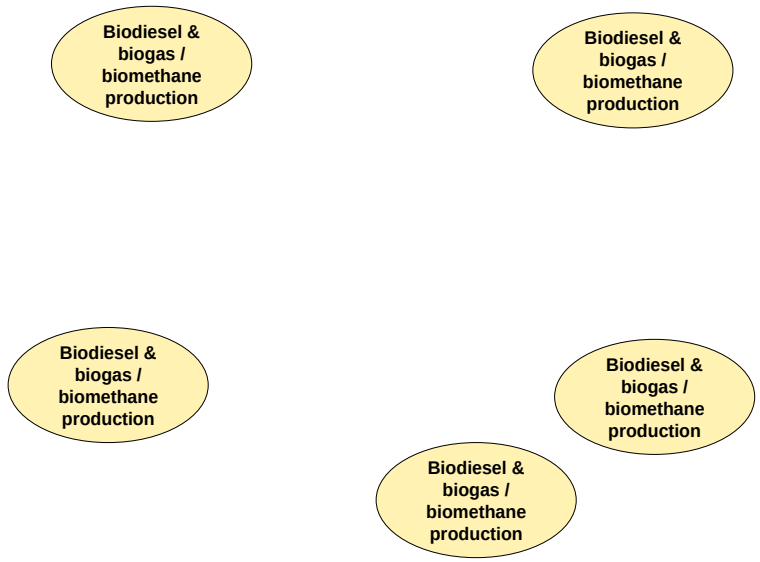

Figure 15: Local biogas / biomethan production in biodiesel plants (mono-fermentation).

As crude glycerol contains hardly no mineral nutrients (except for some potassium), nutrient supplementation is needed for mono-fermentation which has to be taken into account for the environmental and the economic analysis.

3.9.4 Plant design for co-fermentation of glycerol

In co-fermentation, only 6% of glycerol (by weight) is added as co-substrate. Since in an average biodiesel plant, high amounts of glycerol are obtained, the glycerol has to be distributed among several biogas plants (here referred to as 'decentralized' option). Based on an average biogas plant capacity of 0.5 MW, about 2-5 plants (depending on plant design) are needed to co-ferment 6% glycerol with manure, and about 15 plants to co-ferment 6% glycerol with corn silage. Based on the current distribution of biogas plants in Europe, the average transport distance is about 5-70 km (depending on co-ferment type and region).

The processing of biogas into biomethane is economically viable only for big plants of at least about 2-2.5 MW (equivalent electrical power; see above). Three of such big biogas plants are needed to co-ferment the glycerol production of one biodiesel plant. Assuming that every 12^{th} biogas plant is a 2.5 MW plant, the average transport distance is about 15-50 km.

The principle of a decentralized biogas / biomethane production is shown in Figure 16.

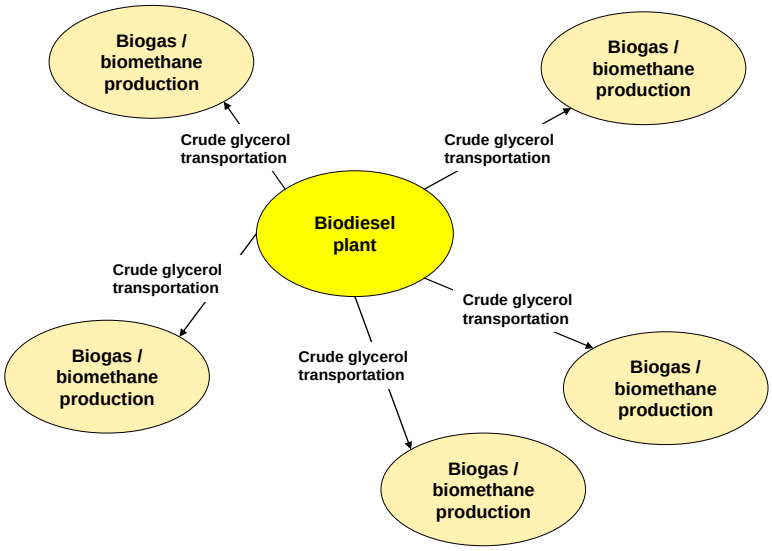

Figure 16: Decentralized biogas / biomethane production (co-fermentation)

3.10 By-product use for biogas production

3.10.1 Biomass

During the processing of glycerol into ethanol, butanol and 1,3-PDO, different by-products are derived. They are contained in the fermentation broth as (solid) distillation residue (see chapters 3.6, 3.7, 3.8). Furthermore, liquid residues from glycerol filters are obtained in the production of each of the three products. Both types of residues can be used as a feedstock for biogas production providing part of the process energy required for glycerol processing. The biogas use option is assessed as the basic scenario as long as the extraction of by-products from the fermentation broth is not proved to be feasible.

This means that the fermentation broth as well as filter residues obtained during the production of ethanol, butanol and 1,3-PDO are used in biogas plants which are directly attached to the glycerol processing facilities. The biogas is used for the production of process energy which is used for glycerol processing and replaces fossil energy carriers. In case surplus power is produced it is exported to the grid replacing conventionally produced electricity.

The feasibility of biogas production among others depends on the amount of biogas that can be produced, i.e. on the amount of by-products that is obtained during glycerol processing. For the solid use fraction the use as animal feed has been suggested as an alternative use option beside the fermentation in a biogas plant. However, the amount of residue is expected to be too small to justify its collection, processing and use as animal feed. If there is no livestock farming adjacent to the glycerol plant, the residues would have to be concentrated (i.e. dried) to make them transportable and storable, which would result in extra costs.

3.10.2 Biogas digestate

If the residues are fermented, digestate is obtained as a by-product. For the time being, its use as fertilizer replacing mineral fertilizer is assessed. It has to be noted that in case GMO are used for glycerol processing, the use of the digestate as fertilizer needs to be clarified.

However, it might turn out that the digestate has low nutrient contents meaning that an application to agricultural land would not be feasible from an economic point of view. In this case its treatment in a sewage plant might be an alternative option.

3.11 Summary: GLYFINERY scenarios

Table 3 summarizes the main GLYFINERY scenarios including the use of the main product, the plant design (local / decentralized) as well as the reference products. The last column depicts the number allocated to each scenario. For a better orientation, these numbers are also displayed in the flow charts in chapter 1.

Table 3: Summary of main GLYFINERY scenarios

Main product	**Centralized / decentralized**	**Use**	**Reference product**	**Scenario N°**
Ethanol	Centralized	Chemical	Fossil ethanol via ethylene (crude oil)	1 a
		Transport fuel	Gasoline	1 b
Butanol	Centralized	Chemical	Fossil butanol via propylene (crude oil)	2
1,3-PDO	Centralized	Chemical	Fossil 1,3-PDO via ethylene (crude oil)	3 a
			1,3-PDO from glucose (corn starch)	3 b
Biogas – Mono-fermentation	Local[2]	Process energy for biodiesel production	Conventional heat / electricity	4 a I
		Export of heat/electricity	Conventional heat / electricity	4 a II
	Local[3]	Biomethane for heat/electricity production	Conventional heat / electricity	4 b I
		Biomethane as transport fuel	Gasoline	4 b II
		Biomethane as transport fuel / chemical	Natural gas	4 b III
Biogas – Co-fermentation	Decentralized[3]	Export of heat/electricity	Conventional heat / electricity	4 c I
	Decentralized[4]	Biomethane for heat/electricity production	Conventional heat / electricity	4 d I
		Biomethane as transport fuel	Gasoline	4 d II
		Biomethane as transport fuel / chemical	Natural gas	4 d III

[2] i.e. biogas / biomethane production directly at biodiesel plant

[3] i.e. transportation of crude glycerol from biodiesel plant to decentralised plants for biogas / biomethane production

4 GLYFINERY scenarios: Qualitative flowcharts

This section contains the qualitative flowcharts for each of the pathways under investigation which has been described in the previous chapters. For each main product (ethanol, butanol, 1,3-PDO, biogas) two flowcharts are shown: to the first one illustrates the various process steps within that particular pathway including all inputs and outputs. The second one deals with the glycerol processing as black box and focuses on the outputs and the respective conventional products they replace (reference products).

4.1 Glycerol to ethanol

The flowchart for the production of ethanol can be seen in Figure 17. The purification method is simple distillation. The purification of ethanol by liquid/liquid extraction or solid phase extraction (as specified in WT 5.2 and WT 5.6) was not investigated. Hence the purification is done by simple distillation.

All other by-products are left in the residual fermentation broth for use in the downstream anaerobic degradation step.

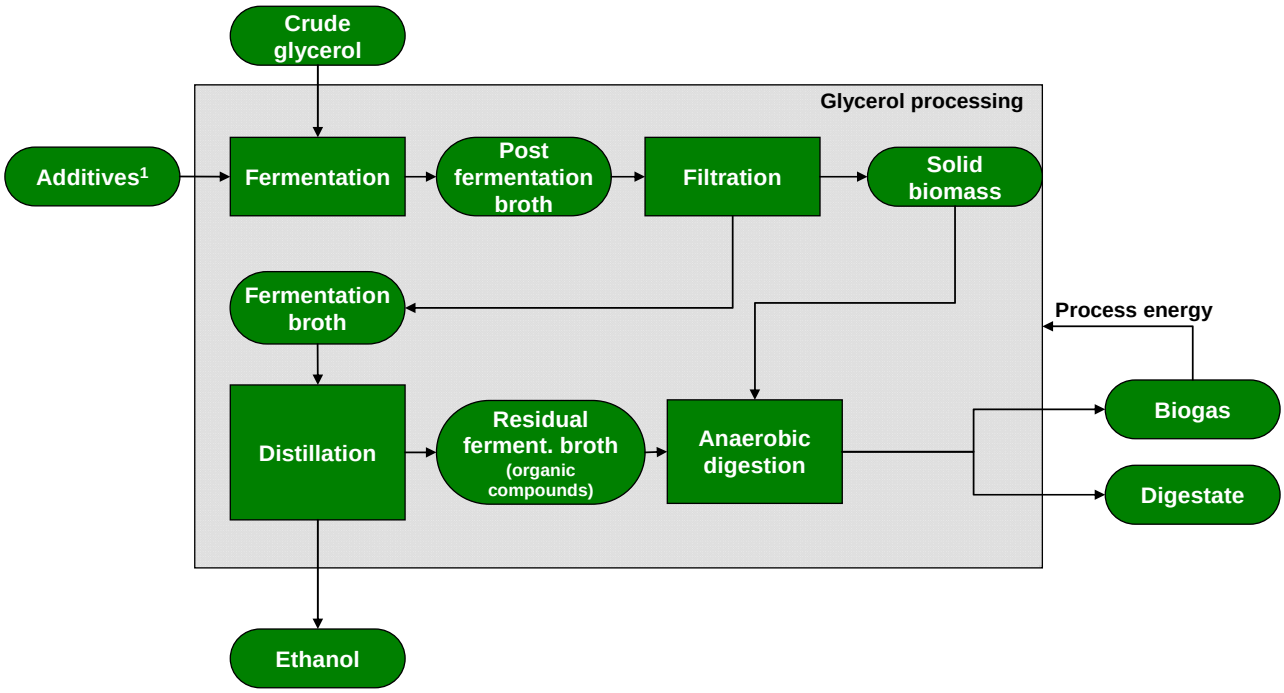

Figure 17: Detailed flowchart depicting the glycerol processing to ethanol.

Figure 18 shows all products derived in ethanol production from glycerol fermentation and the respective reference products. The numbers in the flow charts indicate the scenario. An overview on all scenarios can be found in chapter 3.11.

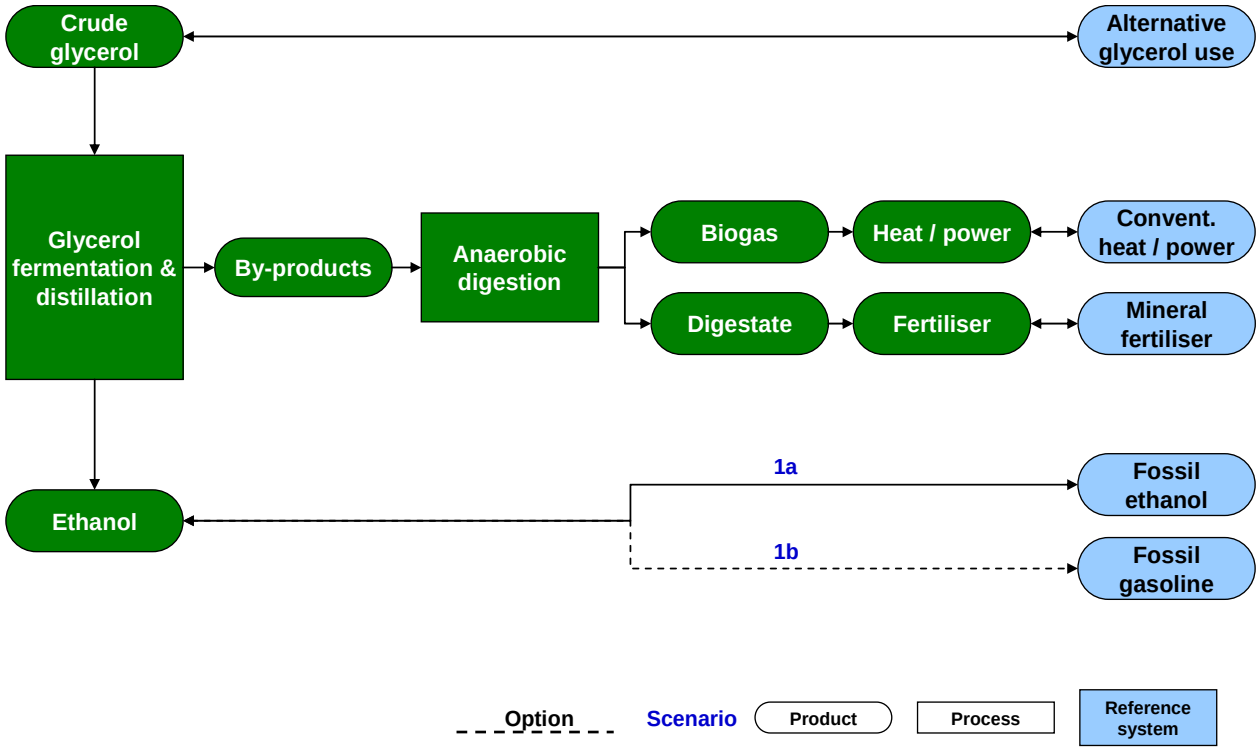

Figure 18: Flowchart depicting all products from ethanol production including all reference products.

4.2 Glycerol to butanol

The flowchart for the production of butanol can be seen in Figure 19. The process has two main products: butanol and 1,3-propanediol. Since there is no main product, the purification will depend on the cost and profitability of each component in the mix. Any residual products are left in the residual fermentation broth for use in the downstream anaerobic degradation.

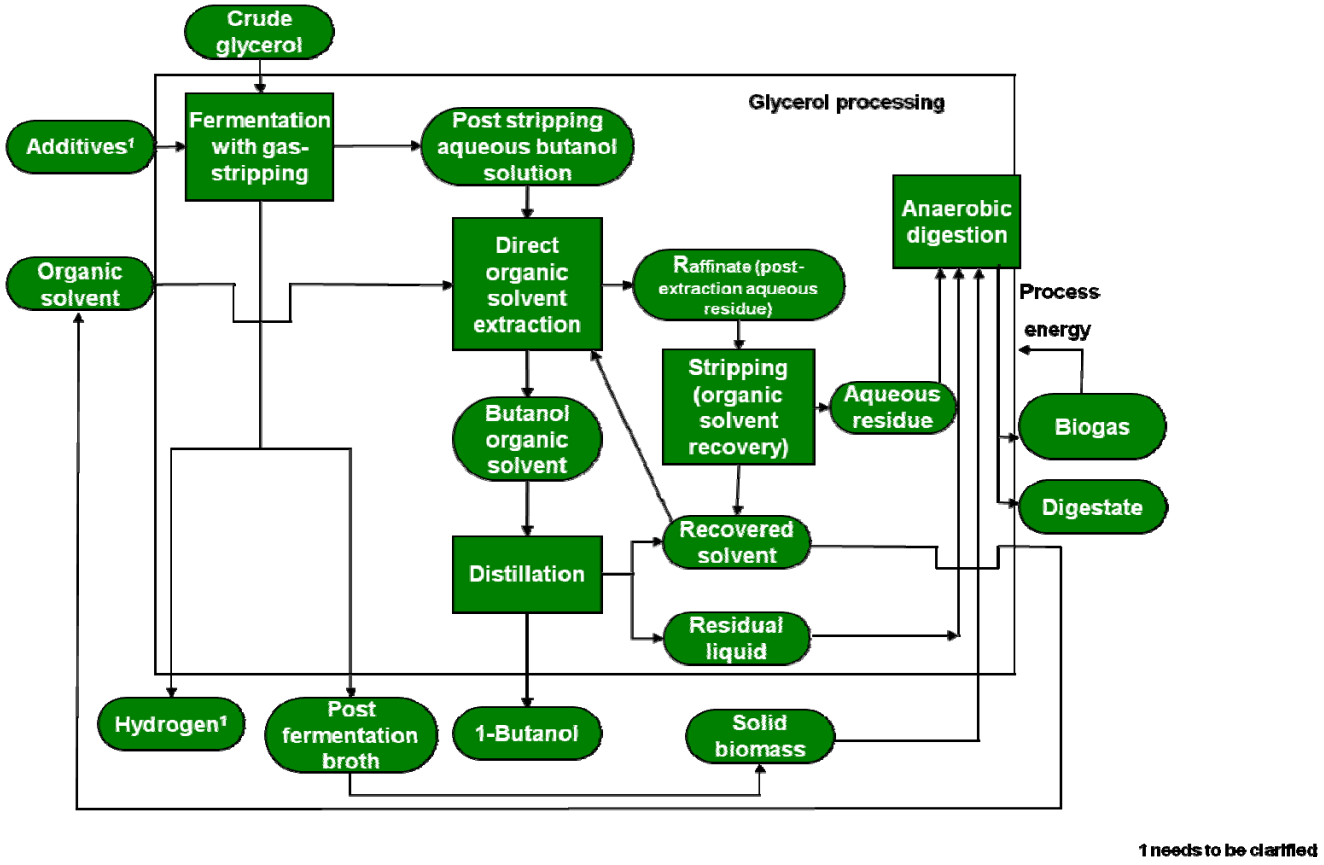

Figure 19: Detailed flowchart depicting the glycerol processing to butanol.

Figure 20 shows all products derived in butanol production from glycerol fermentation and the respective reference products. The numbers depicted in the flow charts indicate the scenario. An overview on all scenarios can be found in chapter 3.11.

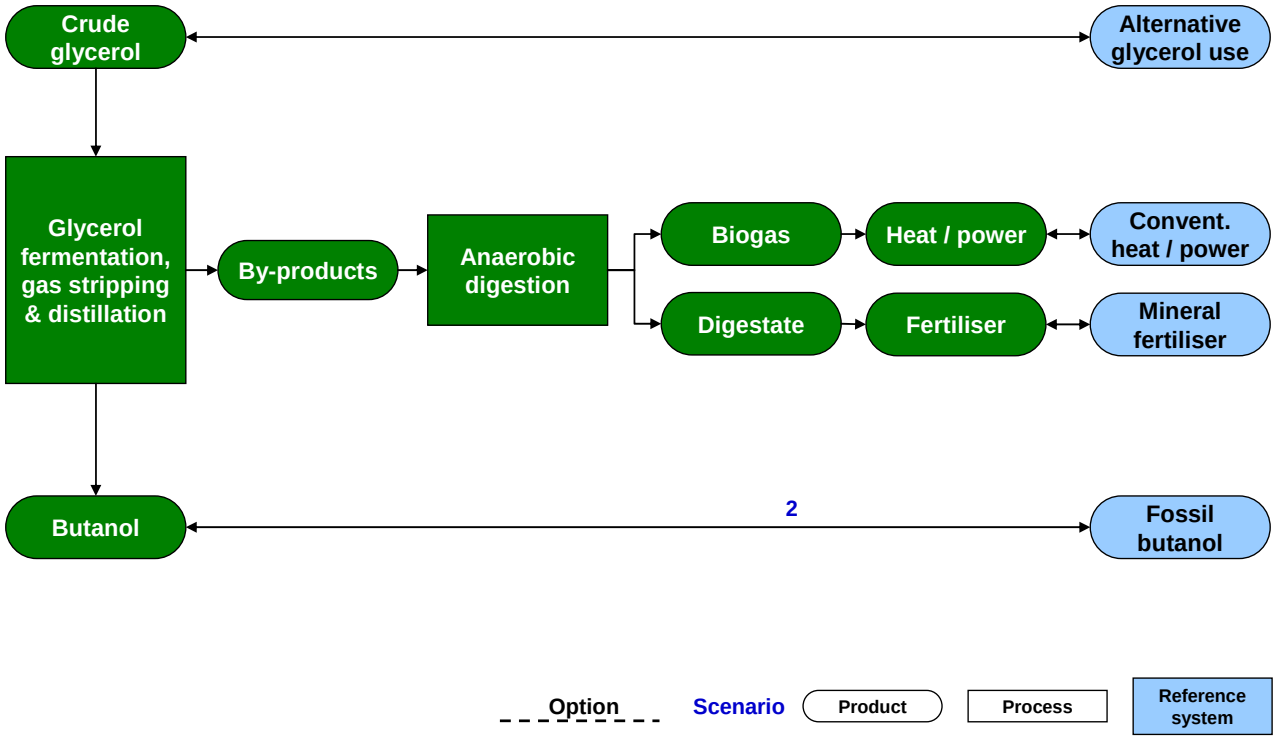

Figure 20: Flowchart depicting all products from butanol production including all reference products.

4.3 Glycerol to 1,3-propanediol

The flowchart for the production of 1,3-propanediol can be seen in Figure 21. As was determined in WP 5 the most promising purification method for 1,3-propanediol is continuous direct liquid/liquid extraction. As in the other processes the remaining products and residual glycerol and nutrients are left in the residual fermentation broth for use in anaerobic degradation.

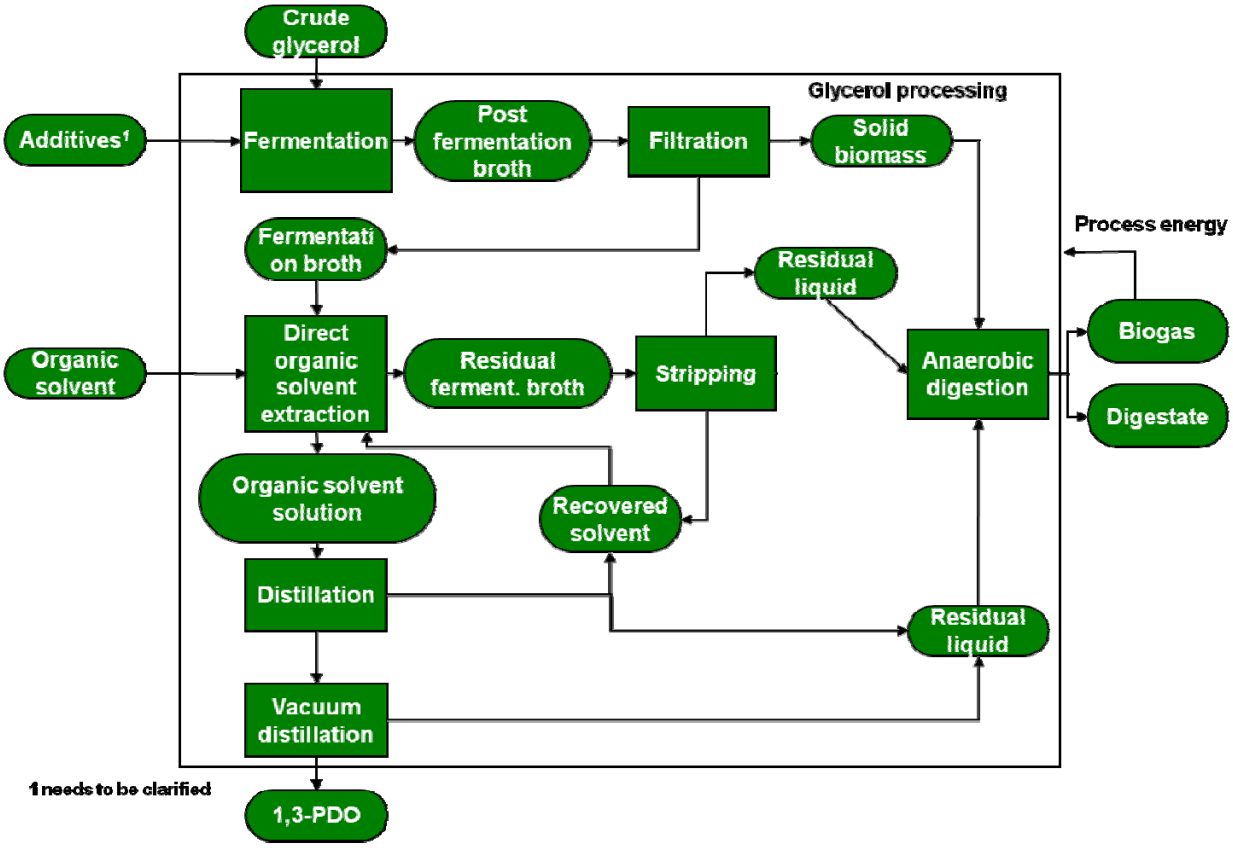

Figure 21: Detailed flowchart depicting the glycerol processing to 1,3-PDO.

Figure 22 shows all products derived in 1,3-PDO production from glycerol fermentation and the respective reference products. The numbers depicted in the flow charts indicate the scenario. An overview on all scenarios can be found in chapter 3.11.

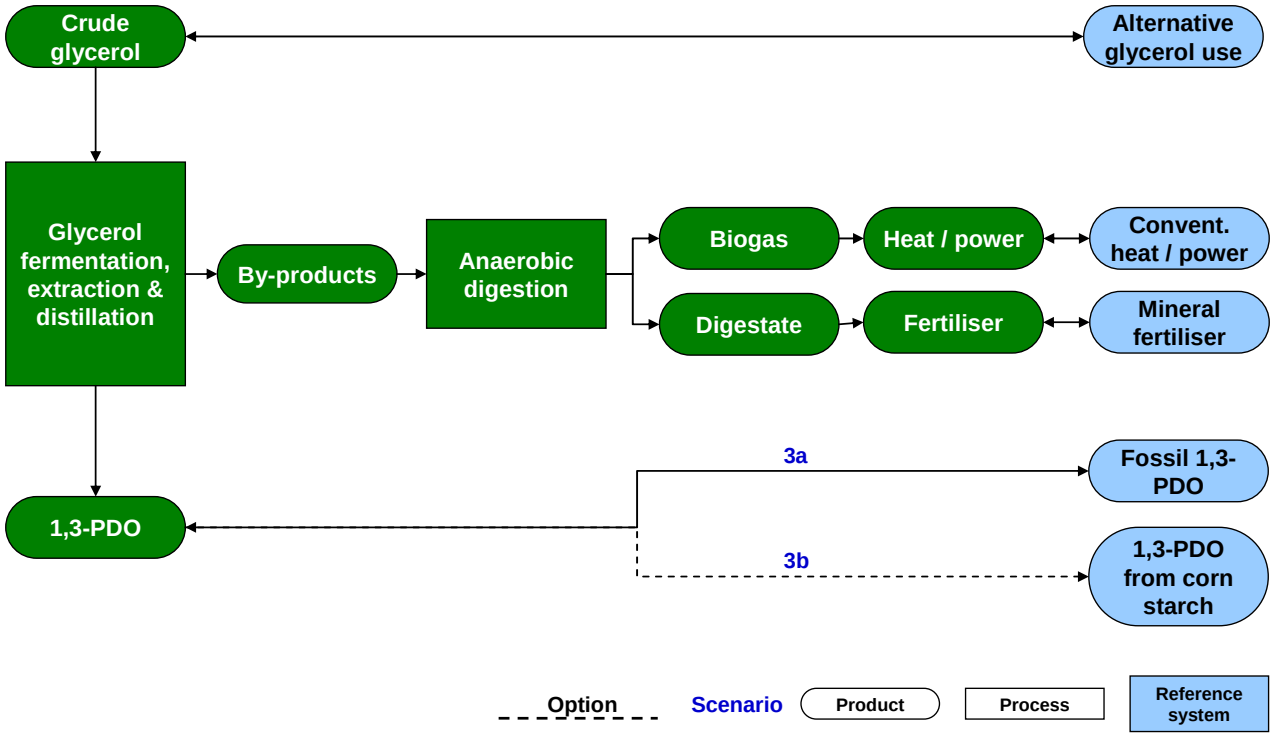

Figure 22: Flowchart depicting all products from 1,3-PDO production including all reference products.

4.4 Glycerol to biogas

The flowchart for the direct production of biogas from glycerol is shown in Figure 23. The biogas is either used directly for energy production or it is further processed into biomethane.

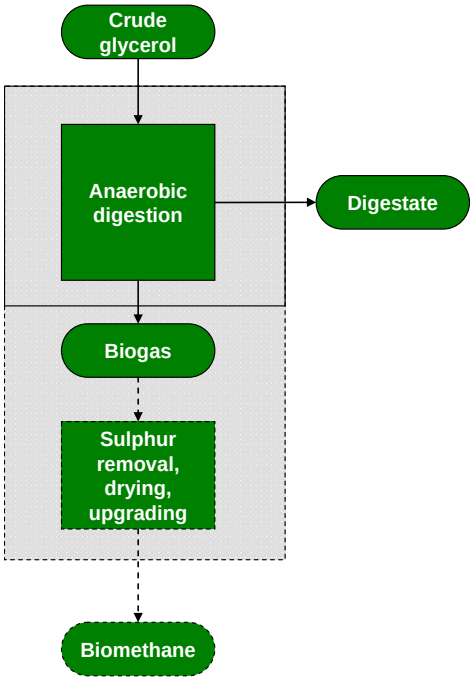

Figure 23: Detailed flowchart depicting the glycerol processing to biogas and biomethane.

Figure 24 shows all products derived in biogas production from glycerol fermentation and the respective reference products. The numbers depicted in the flow charts indicate the scenario. An overview on all scenarios can be found in chapter 3.11.

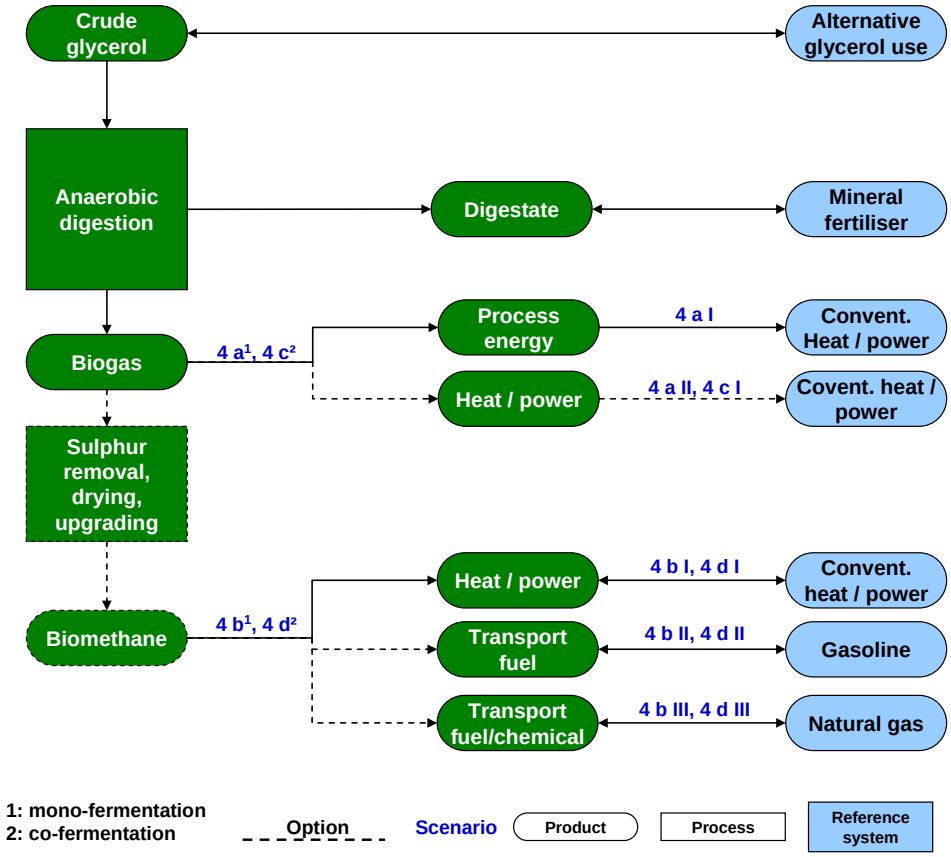

Figure 24: Flowchart depicting all products from biogas and biomethane production including all reference products

5 Current state of the art

This chapter deals with the current state of the art concerning the production of the target fuels and chemicals from the GLYFINERY processes.

5.1 Production of ethanol

5.1.1 Introduction

An ethanol production process has been developed and optimized at DTU based on the non-conventional yeast *Pachysolen tannophilus*. This organism is capable of growing on glycerol, and has been shown to produce ethanol on this substrate in previous studies (ethanol production levels of 4g/L). However, until now, this process has not been optimized to allow for ethanol production levels which could be considered relevant for larger scale production. An ethanol producing process with *P. tannophilus* has been optimized based on knowledge we have gained on the physiology of this organism during the GLYFINERY project. The current process produces 28g/L ethanol (56% of the theoretical yield). Further improvements in production levels would be possible through evolutionary engineering to produce strains which are more ethanol tolerant.

5.1.2 Benchmarking ethanol production from glycerol

It has been shown that a number of (typically anaerobic) bacteria are capable of growing on glycerol as the sole carbon and energy source. Glycerol can be converted to a wide range of biochemicals and biofuels such as ethanol, butanol, 1, 3-propanediol, succinate, dihydroxyacetone, propionic acid and pigments (da Silva et al., 2009). The newly isolated bacterium, *Kluyvera cryocrescens* can produce up to 27g/L ethanol from crude glycerol under microaerobic batch fermentation (Choi et al., 2011). *Eschericia coli* has been investigated to be an ethanol production platform on glycerol, with up to 10g/L achievable by engineered *E.coli* growing on 22g/L crude glycerol and with hydrogen and formate as byproducts under anaerobic condition (Shams Yazdani and Gonzalez, 2008). An engineered *Klebsiella pneumonia* strain has been shown to achieve 25g/L ethanol on crude glycerol (Oh et al., 2011). However, these processes require a controlled anaerobic environment, maintained through sparing with nitrogen.

For ethanol production from glycerol, only two genetically engineered yeasts have been reported which can convert glycerol into ethanol. The industrial work horse *Saccharomyces cerevisiae* has been genetically engineered to produce ethanol from glycerol and the several rounds of genetic engineering, the production level achieved was only 3.1g/L highest production level in the modified strain reached 4.4g/L (Yu et al., 2010). The methylotrophic yeast *Hansenula polymorpha* was engineered to improve ethanol production by expression of varied genes from bacteria, however after (Hong et al., 2010). Results of previous studies are summarized in Table 4.

Table 4: Comparison of ethanol production from glycerol by different bacteria and yeasts

Organism	Fermentation method	Ethanol production (g/L)	Vol. Ethanol productivity (g/L/h)	Reference
Escherichia coli EH05	Batch	20.7	0.22	Durnin et al., 2009
Klebsiella pneumoniae GEM167/pBR-pdc-adh	Fed-batch	25.0	0.78	Oh et al., 2011
Kluyvera cryocrescens S26	Batch	27.0	0.61	Choi et al., 2011
Hansenula polymorpha HpDL1-L/pYH-pdc-adhB- dhaDKLM	Batch	3.1	0.02	Hong et al., 2010
Saccharomyces cerevisiae YPH499fps1Δgpd2	Batch	4.4	0.04	(Yu et al., 2010)
Pachysolen tannophilus CBS4044	Staged-Batch			Present study
	Phase I	18.7	0.16	
	Phase II	27.5	0.18	
	Phase III	28.1	0.06	

Pachysolen tannophilus was the first yeast shown to be capable of fermenting xylose sugars to ethanol (Kurtzman, 1983) and the xylose utilisation pathway has been extensively studied in this organism (Sathesh-Prabu and Murugesan, 2011; Slininger et al., 1987; Zhao et al., 2010). In a previous study, it was reported that *P. tannophilus* could accumulate 4g/L ethanol on glycerol under aerobic growth (Maleszka et al., 1982), however, the conditions for ethanol production were not precisely defined or controlled and the physiology during growth on glycerol has not been extensively studied in this organism. The possibility for studying the physiology of glycerol conversion to ethanol in this organism provides an interesting prospect for the future production of biofuels.

This studies performed in the Glyfinery project show that crude glycerol can be utilized as a potential low cost substrate for producing fuel ethanol for transportation by *P.tannophilus* (CBS4044). After a series of batch experiments for fermentation optimization, the highest yield obtained was 0.28±0.03 g ethanol g^{-1} glycerol which corresponds to 56% of the theoretical yield. The maximum production achieved was 28.1 g/L ethanol in a staged-batch process. This is the highest value for glycerol conversion to ethanol reported to date. The process could be further optimized through fed-batch design and employment of a more ethanol tolerant strain. This strain could then be cultivated in a fed-batch process which could further optimize productivity and yields.

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5.2 Production of butanol

5.2.1 Introduction

Microbial-production of butanol has been studied very intensively for many years. Louis Pasteur was the first (in 1862) to describe the production of butanol by microbes [4]. Around the 1900, research was conducted in isolating and describing solvent producing bacteria. At the same time considerable interest in synthetic rubber started (butanol was used as a precursor for butadiene, the starting material for synthetic rubber production). Around 1912, Chaim Weizmann isolated an acetone-butanol producing strain. This strain was later named *C. acetobutylicum* and has been one of the most widespread acetone-butanol-ethanol producers (ABE-producers). The process evolved (also boosted by the World Wars demand for acetone) until the 1950's where the price of substrate (molasses) increased and the cheap crude oil was available, consequent closure of many plant. Production only continued in countries that were cut off international supplies for political or monetary reasons, such as South Africa where ABE fermentation persisted until 1982).

As focus on sustainable energy is increasing interest in the microbial production of butanol is rising. New plants are planned and built. The table below (5) lists some of the companies operating with butanol production in US and Europe. None of the companies are using glycerol as substrate, but are focused on a sugar platform. In the table it is pronounced that in situ removal of butanol is applied in all processes. However, different strategies may be used.

Table 5: A list of companies in US and in EU working on butanol production.

Company	Organism	Fermentation process	Separation strategy	Development status	Additional notes
Butamax (DuPont/BP)	1. *Clostridium* 2. *E. coli*	Semi batch	Continuous *in situ* removal followed by distillation trains	2013 Commercial Additional Feedstocks 2013+	Formed in 2009
Green Biologics (UK)	*Clostridium*. Mixed populations	Continuous fermentation	*In situ* removal Unknown	Building demo in India. Consulting with Chinese firms	
Metex (FR)	"Well known bacteria"	Unknown	*In situ* removal Unknown	Unknown	Produces also 1,3-PDO 1,3-PDO in pilot scale
Butalco (Switzerland)	Yeast	Unknown	*In situ* removal Unknown	Unknown	Developing an integrated lignocellulose-based bioethanol/ biobutanol production process.
Gevo (Isobutanol)	Yeast	Semi batch	Vacuum flash *in situ* removal followed by distillation trains	2010 Operating pilot in St. Johns, MO. 2011 Commercial	Technology designed to retrofit existing ethanol plants
Cobalt Biofuels	*Clostridium*	Continuous	Vapor compression distillation	2010 Pilot 2011 demo 2012 commercial	Plan to launch cellulosic plant in April 2012
Tetra Vitae	*Clostridium beijerinckii*	Semi batch	Carbondioxide - stripping continuous *in situ* followed by distillation trains	2009 300 l bench 2010 10,000 l pilot	Focused on butanol and acetone production.
ButylFuel	*Clostridium sp.*	Continuous two stage dual path anaerobic fermentation	Gas-stripping	Unknown	

5.2.2 Benchmarking butanol production from glycerol

The process for producing butanol from glycerol is based on a mutant strain of *C. pasteurianum*. The mutant strain was developed with respect to better crude glycerol tolerance and increase conversion rates. In order facilitate growth for an extended period of time, removal of butanol is necessary. This was done gas-stripping. A medium composition with very low cost was chosen/developed, thus, increasing the feasibility of the process.

The process of pilot scale butanol fermentation was performed in a 30 liters fermentor with the *C.pasteurianum* mutant strain. The process of the butanol fermentation is inhibited by the presence of butanol when its conc. exceeds 10 g/L. Therefore, during the fermentation process the butanol was removed by the stripping method with nitrogen (Figure 25). The fermentation data and results are provided in Table 6.

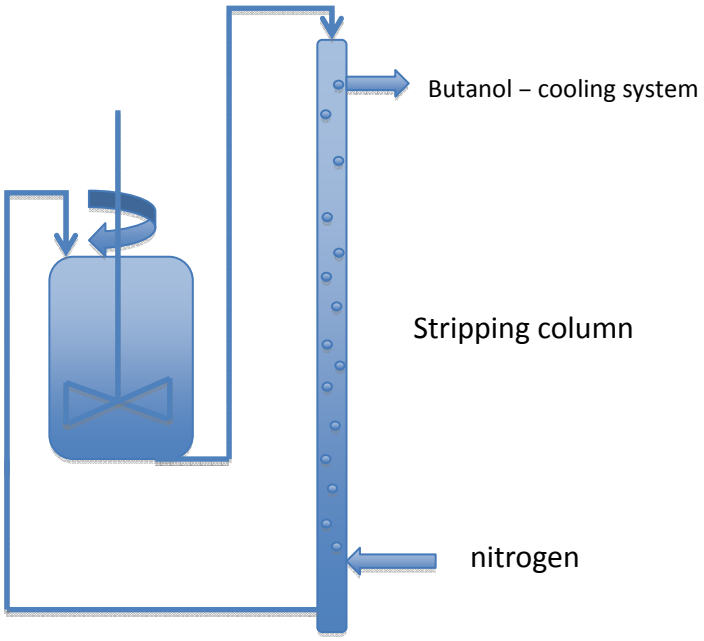

Figure 25: A schematic overview of the butanol fermentation system

Table 6: Fermentation parameters for the butanol process.

Parameter	Value
Crude glycerol initial conc.	50 g/l
Headspace overpressure	0.2 Bar
pH control level	6.0
Fermentation volume of fermenter A	30 liters
Medium	Biogasol medium
Butanol production efficiency	0.23 g 1,3-PDO / 1g Glycerol
The best observed butanol productivity	0.7 g/l/h
Glycerol uptake	3.0 g/l/h
Final butanol conc.	12.5 g/l
Final glycerol conc.	5.0 g/l
Final biomass content.	2.83 g/l

During the fermentation, the fermentation process system was controlled by pH control, temperature control and headspace overpressure control. The product and substrate content was monitored by HPLC analysis.

There are a limited number of publications dealing with utilization of glycerol as substrate for production of butanol [1,2,5,6]. The widespread ABE producer *C. acetobutylicum*, can metabolize glycerol, but only in the presence of glucose [6] therefore, another strain has been used. *C. pasteurianum* can, however, utilize glycerol as sole carbon source and produce butanol. Both Biebl [1] and Dabrock *et al.* [2] has described fermentation processes for utilization of glycerol by *C. pasteurianum*. Taconi *et al.* [5] has dealt with the challenges of utilizing crude glycerol as substrate, also with *C. pasteurianum* as production organism.

Taconi *et al.* [5] was the first to publish fermentation of crude glycerol to butanol. Even though, the yields achieved were comparable to yields from fermentation on technical grade glycerol, the rates were highly affected by the crude glycerol. Fermentation of 22g/l crude glycerol persisted for 24 days with an inhibited period of 10 days. The use of this process for immediate industrial application is evaluated as limited by Dellomonaco et al. [3] due to the very low production rates.

In order to produce high amounts of butanol, a high amount of glycerol needs to be converted. Biebl [1] showed that 63.6 g/l technical grade glycerol could be utilized. The process developed during this project almost doubled the glycerol utilization, even on crude glycerol. In addition, the utilization rates were significantly increased. The maximum utilization rate in batch fermentation reported by Biebl was 2.62g/l/h, the Glyfinery butanol process was able to increase this rate by more than 2.5 times, still utilizing crude glycerol. This high rate was not achieved by reduced butanol production; the butanol productivity was more than 1.5g/l/h.

The strain developed within the project, tolerates high concentrations of crude glycerol. Never before has initial crude glycerol concentration of 120g/l been reported, emphasizing the robustness of the strain.

By applying gas stripping, circulating the gas-phase of the fermentation, butanol was removed from the fermentation broth continuously assuring non-toxic conditions. As can be seen in table 5 *in situ* removal and especially gas stripping is applied by different industrial research companies (ABE) but it has never been utilized as part of glycerol fermentation. The reason could be that the toxicity of the crude glycerol caused the fermentation to cease before reaching butanol titers critical for the microorganisms. By the development of the butanol producing strain, the butanol toxicity issue became pronounced. Gas-stripping was applied with success assuring non product inhibition.

There are challenges illustrated by Dellomonaco *et al.* [3] with the conversion of glycerol to butanol. The strain/process developed in this project unambiguously copes with these challenges, bringing the process closer to industrial application.

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5.3 Production of 1,3-propanediol (1,3-PDO)

5.3.1 Introduction

The global biodiesel production was over 15 billion liters in 2009 and it is still increasing. The forecast for the worldwide production is over 45 billion liters in 2020 [1]. Glycerol is produced as a by-product at a level of 5-10 % [2]. The conversion of glycerol to higher-value products might be the way to decrease the costs of biofuels production. 1,3-propanediol (1,3-PDO) is one of the products that could be produced from the crude glycerol. The main application of 1,3-PDO is a substrate in the polymerization of polytrimethylene terephthalate (PTT), a type of polyester used in the engineering thermoplastics area and in the production of carpets and textile fibers [3]. Biological production of 1,3-propanediol would be a sustainable alternative to the chemical methods. There are several microorganisms which are able to ferment glycerol with the 1,3-PDO as final product. Moreover, the genetically modified *E. coli* strains might be also used.

Table 7: Biological methods of 1,3-PDO production.

Organism	Carbon source	yield *	remarks	References
Lactobacillus hilgardii	glycerol+glucose or fructose	?		[5]
Citrobacter freundii	Glycerol	0,62 mol/mol		[6]
Clostridium saccharobutylicum	glycerol	0,36 mol/mol	high substrate utilization	[7]
Clostridium butyryicum	crude glycerol	68 g/l 0,55 g/g	non-sterile fermentation	[8]
Clostridium diolis	glycerol	85 g/l	chemical mutagenesis and genome shuffling	[9]
Klebsiella HR526	glycerol	42 g/l	D-lactate dehydrogenase inactivation/deletion	[10]
Klebsiella pneumoniae	crude glycerol	53 g/l		[11]
Klebsiella pneumoniae	crude glycerol + glucose	63 g/l 0,6 mol/mol		[12]
E. coli	sucrose	3 g/l	genes for the sucrose utilization of another *E. coli* strain	[13]
E. coli	glucose	129 g/l 0,34 g/g	genes of dha regulon of *K. pneumoniae*	[14]

*molar and mass yields were calculated in relation to the consumed carbon source

Glycerol fermentation by the glycerol-fermenting microorganism is a two-branched pathway. The 1,3-PDO produced in a reductive branch is catalyzed by two enzymes, (i) glycerol dehydratase and (ii)1,3-PDO

oxidoreductase, with a 3-hydroxypropionealdehyde as an intermediate. On the other hand, in the oxidative branch, glycerol is dehydrogenated by glycerol dehydrogenase to dihydroxyacetone (DHA). DHA is then phosphorylated by ATP or phosphoenolopyruvate to the phosphohihydroxyacetone which is an intermediate to the pyruvate synthesis [4].The main microorganisms and methods of the biological 1,3-PDO production were summarized in the table above.

5.3.2 Glyfinery 1,3-PDO process

During the project A&A Biotehcnology developed the process of crude glycerol fermentation and 1,3-PDO production based on the non-GMO mutant strain of *C. butyricum*. The process is continuously performed in two fermenters A and B (Fig. 25).

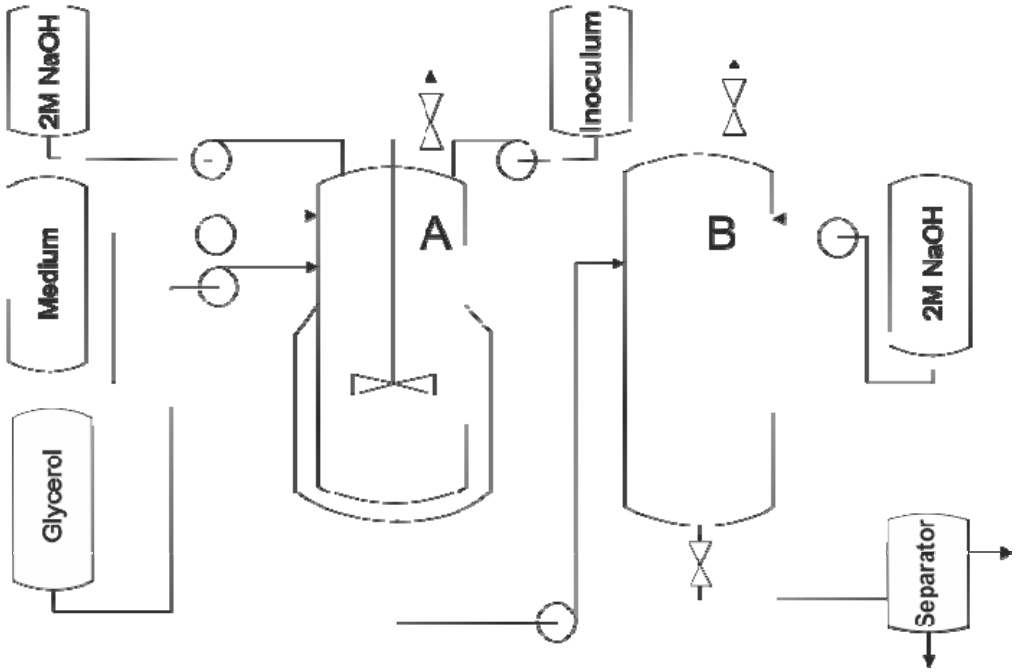

Figure 26: Schema of crude glycerol continuously fermentation system.

The fermenter A is highly controlled system where the main fermentation is carried out. The fermenter has the following controlling systems: pH control, level control, temperature control, headspace overpressure control. The first fermentation stage is performed in the steady glycerol concentration and the 1,3-PDO high production efficiency is observed (Table 7). The fermenter B is a storage tank with pH control. The second stage of fermentation allows for complete removal of residual glycerol, so the whole used for fermentation glycerol is consumed. The low content of glycerol in the final fermenter is necessary to obtain efficient recovery of 1,3-PDO by extraction.

Table 8: Fermentation process parameters for the 1,3-PDO process

Parameter	Value
Crude glycerol initial conc.	60 g/l
Headspace overpressure	0.2 Bar

pH control level	6.5
Fermentation volume of fermenter A	30 liters
Medium	YNB reduced
Glycerol Feeding	0.05 l/h
1,3-PDO production efficiency	0.56 g 1,3-PDO / 1g Glycerol (0.63 g/g theoretical yield)
The best observed 1,3 PDO productivity	0.85 g/l/h
Glycerol uptake	1.31 g/l/h
Final 1,3 –PDO conc.	30.2 g/l
Final glycerol conc.	0.2 g/l
Final biomass	2.13 g/l

Based on the pilot experiment data, the total time and fermentation volume was estimated for 1 ton of glycerol (Table 9).

Table 9: Fermentation parameters pr. ton of glycerol, based on experimental data.

Parameter	Value
Glycerol	1000 kg
Final 1,3-PDO production	560 kg
Total volume of fermentation media	10 000 liters
Fermenter A	500 liters
Fermenter B	10 000 liters
The total time of fermentation	14-20 days

After the second fermentation in fermenter B, the biomass was separated by pilot scale continuous flow centrifugation (14.000 rpm) with a feed rate of 300 ml/h. Clear supernatant was used for the 1,3-PDO recovery experiments in the pilot scale.

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5.4 Production of biogas

The interest in biogas is bigger than ever in Europe. The number of biogas plants has increased greatly during the last years. In 2010 the highest number of new installed biogas plants was observed in Germany, Hungary and Czech Republic [1]. Different substrates are used and also the field of application differs between countries in Europe. The biogas production in Germany, Denmark and Austria takes place mainly on farm based plants, while in for example Sweden and Poland the biogas is for the most part produced at sewage treatment plants [1, 2]. The biogas produced in Europe is mainly used for the production of electricity. Less than 10% of total biogas output was in 2010 upgraded to biomethane quality and injected into the gas grid or used as vehicle fuel [1]. There are only eight countries: Germany, Sweden, Netherlands, Switzerland, Austira, UK, France and Finland, that upgrades the quality of the biogas to a higher standard. In Europe, Sweden was the first county to use biogas as vehicle fuel on larger scale and has today the highest ratio of biogas in the vehicle fuel (51%) [3]. Except for electricity production and vehicle fuel, biogas is used for production of heat, steam and cooling, production of chemicals and in fuel cells [4].

However, the driving forces for the development of biogas in the European counties are different. In Denmark the main purpose of producing biogas from agricultural byproducts is to avoid nitrogen leakage. There is also an economical driving force behind the production of biogas. It can be tax relief on biogas as vehicle fuel which is common in Sweden and Switzerland or governmental support for the produced electricity which is found in Germany, Austria and France.

5.4.1 Future of biogas in Europe

The European Commission has set up a goal where 20% of the European energy demands will come from renewable energy in 2020. Two Danish researchers predict that biogas produced from energy crops, animal manure and industrial organic waste can supply nearly half of the European natural gas consumption in the coming decades and it will represents at least 25% of all bioenergy.

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5.5 Recovery processes

5.5.1 Introduction

Due to increasing price of petrochemical feedstocks and extensive oil consumption, a considerable effort has been made t oadvance the production of biofuels. Among these, butan-1-ol and propane-1,3-diol (1,3-PDO) were targeted as very promising. In case of butanol besides pervaporation and traditional distillation, other solvent recovery techniques have been developed, i.eg. gas-stripping. The separation techniques studied for 1,3-PDO include ion-exchange chromatography, evaporation, distillation, pervaporation, solvent and reactive extraction.

5.5.2 State-of-art butanol recovery process

Recent publications concern mainly ABE (acetone-butanol-ethanol) fermentation performed by *Clostridia* strains. In the ABE fermentations where butanol is usually the main product, the maximum achievable butanol concentration in the fermentation broth is ~20g/L. The final ABE composition depends on product inhibition and butanol toxicity. [1-4] With regards to above mentioned facts all synthesis approaches have focused on in situ separation of butanol from fermentation broth.

Distillation is the traditional technique of product recovery for the ABE fermentation process. Due to high boiling point of water, most of energy requirement during distillation originates from the water evaporation in the fermentation broth. Distillation efficiency is related to the energy integration applied, as the energy requirement determines the operational costs [5].

Pervaporation is a well-described method of butanol recovery. It is a combination of membrane filtration and solvent evaporation from fermentation broth [6-8]. The process is based on volatiles diffusion through a solid membrane and remaining the nutrients, macromolecules and microbial cells in the feed. Selectivity of product recovery and velocity of membrane penetration depends on the membrane properties, its thickness, composition of liquid and gas-phase, process temperature and pressure[9-13.]

Gas-stripping has been described as the most important industrial technique of butanol recovery in fermentation-integrated systems. The method allows for selective separation of volatile products from the feed with no membrane usage. The process is based on product concentration difference in liquid and gas-phase. The gas-phase is sparged into the fermentor and butanol is condensed and recovered from the condenser. After product removal gas is recycled to continue gas-stripping. During gas-stripping it is possible to maintain the anaerobic conditions by using oxygen-free gas (nitrogen, carbon dioxide, hydrogen).

Application of gas-stripping in butanol fermentation using *C. acetobutylicum* was first described by Ennis et. al. [14] Butanol recovery method has many advantages over other removal processes, for example, it is simple and inexpensive to perform. Integrated system of gas-stripping and fermentation leads to decreased toxicity and increased butanol production [15]. The list of butanol separation techniques and companies operating with butanol in Europe and US are shown in the section 5.2 „Production of butanol" (Table 5).

5.5.3 Glyfinery 1-butanol recovery process

Based on WP 3,4,5 interactions the final WP6 system proposed for recovery of 1-butanol is a three stages integrated process which combines following steps: gas-stripping, liquid-liquid extraction, distillation and solvent recovery.

Gas stripping is the most important technique for removal of 1-butanol from fermentation broth. The 1-butanol volatile properties allows for selective in situ product removal from fermentation broth without using any membranes. The gas stripping process has many advantages, e.g. it is simple and inexpensive to operate. Moreover, integrated fermentation process involving gas stripping allows to avoid the inhibitory effect of 1-butanol on the culture during fermentation and obtain high concentration of target product. The 1-butanol toxicity can be kept below the inhibitory levels by feeding the reactor at a slow and controlled rate, while the product-removal technique is applied simultaneously to remove the 1-butanol being produced. It is widely known method as described in the state-of-art section.

The post-stripping aqueous solution of 1-butanol is then subjected to liquid-liquid extraction (LLE) performed by means of the most efficient organic solvent. Main advantage of the process is high efficiency (99.5%) and low energy requirement (0.5MJ/kg of product).

Subsequent operation step is distillation of post-extraction solution of 1-butanol organic solution at yield reaching 95%. The target final product is finally obtained at very high purity (99.90%).

Solvent recovery is the side step in proposed separation process of 1-butanol from fermentation broth. Due to economical and environmental reasons stripping is the most viable technique. Recycled solvent can be successfully reused for 1-butanol extraction from fermentation broth. Regarding to low toxicity of selected solvents even some traces of solvent remaining in the raffinate would be environmentally acceptable as it is commonly utillized in biological treatment systems.

Table 10: Summary of results of the integrated 1-butanol recovery system

Recovery process efficiency [%]	99
Total energy requirement [MJ/kg of product]	57.4
Product purity [%]	99.90

According to available data and publications the proposed system has never been utillized before. It offers an obvious advantage of lower energy requirement due to liquid-liquid extraction stage and resulting reduced volume of 1-butanol containing stream subjected to distillation process.

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5.5.4 State-of-art 1,3-PDO recovery process

Several methods for the separation and purification o f 1,3-propanediol (1,3-PDO) from fermentation broth or similar processes have been reported in many previous studies and patents.

One of the 1,3-PDO recovery techniques was based on the reactive extraction (Malinowski 2000). Malinowski (2000) proposed the formation of 2-methyl -1,3-dioxane (2-MD), a product of reaction of acetic aldehydes with 1,3-propanediol catalyzed by Dowex or Amberlite ion-exchange resin with simultaneous extraction of the product (2-MD) by organic solvents. In another method, propionaldehyde, butyraldehyde, and isobutyraldehyde were used as reactants as well as extractants to form substituted 1,3-dioxane (Hao et al . 2005, 2006). Fang and Zhou (2006) proposed the kinetic study of formation of 2 –MD by 1,3-

propanediol and acetaldehyde catalyzed by cation exchange resin HD-8. All these processes are complicated, and besides the additional need to regenerate 1,3-propanediol from its dioxolane derivative, the complexity, and the cost of the chemicals used make the extraction process quite prohibitive. Moreover, if this process is used for real fermentation broth, then acetaldehyde can react with other by-products and proteins, making this process inefficient.

Malinowski (1999) proposed liquid – liquid extraction where the distribution of 1,3-propanediol into extraction solvents appeared to be not good enough to make simple extraction efficient. Another attempt to separate 1,3-propanediol from a dilute solution by normal physical or complex extraction was also not successful (Xiang et al. 2001). Although many solvent extractants are given in a patent, the hydrophilic 1,3-propanediol in diluted broth fails to enter into hydrophobic solvents, except when adding a large amount of solvents into a concentrated broth (Baniel et al. 2004). Similarly, ethyl acetate was used in phase separation of 1,3-propanediol where the ethyl acetate phase which contained 1,3-propanediol and 1,2-propanediol was subsequently used for chromatographic purification . In addition, the partition coefficient of the target product was below 1.9 (Cho et al. 2006). However, this process has low separation efficiency and also requires the handling of large quantities of solvents.

The pervaporation method based on the ZSM -5 zeolite membrane had drawbacks such as a low flux and selectivity (Li et al. 2001).

Vacuum distillation is preferred over traditional distillation as it saves energy due to the decline of boiling point. Ames (2002) and Kelsey (1996) in their patents and Sanz et al. (2001) evaluated the vacuum distillation- based separation process. However, desalination and deproteinization are required before evaporation which makes the entire process complicated and non-profitable. Gong et al. (2004) and Hao and Liu (2005) evaluated the potential of electrodialysis before evaporation, but low product yield and membrane pollution make this process undesirable.

The available methods for separation of 1,3-PDO from fermentation broths are summarized in the table 10.

Table 11: Comparison of different separation techniques for 1,3-propanediol.

Separation methods or unit operation	Application / investigation	Drawbacks or problems	References
Evaporation / distillation or vacuum distillation	Evaporation was applied for the removal of water from the fermentation broth. Distillation was applied for the final purification of 1,3-PDO	Evaporation and distillation suffer from a large amount of energy consumption. Moreover, desalination and deproteinization are required before evaporation which makes the entire process complicated and non-	Kelsey 1996; Sanz et al. 2001; Ames 2002;

		profitable.	
Pervaporation	Na-ZSM-5 and X-type zeolite membranes were used to separate 1,3-PDO from an aqueous mixture by pervaporation. The high 1,3-PDO /glycerol selectivity was due to referential adsorption of 1,3-PDO Zeolites combined with a cross-flow filtration module were applied to separate the biomass and enrch 13-PDO in fermentation broth, respectively.	The performance of pervaporation needs to be verified by using real fermentative broth in the presence of impurities, e.g., proteins and salts	Li et al.2001a, b, c, 2002; Corbin and Norton 2005
Electrodialysis	Electrodialysis has been used for desalination before evaporation	Low product yield due to loss of 1,3-PDO during electrodialysis. Membrane pollution can be very serious. High energy input for further removal of water.	Gong et al. 2004; Hao and Liu 2005
Chromatography	Combined strongly acidic cationic and weakly basic anionic resins were used to desalinate in the fermentation broth. A cationic exchange resin was used for recovery of 1,3-PDO. Adsorption of 1,3-PD on hydrophobic zeolites or active charcoal was investigated for separation of 1,3-PDO. A preparative silica gel liquid chromatography was	Although high overall purity and yield of 1,3-PDO could be obtained, the 1,3-PDO solution was not concentrated but diluted because of the low selectivity and capacity of resin or adsorbent. This method consumed more energy than the simple evaporation and distillation. In addition, the chromatographic matrix had to be regenerated frequently if the feed was not desalinated or	Roturier et al. 2002; Hilaly and Binder2002; Corbin and Norton 2003; Wilkins and Lowe2004; Adkesson et al.2005; Cho et al. 2006 Roturier et al. 2007;

	used to separate 1,3-PDO after phase separation or concentration of protein-free broth.	deproteinized. This situation also occurred for ion-exchange resins used to desalinate due to high salt concentrations.	Anand et al. 2011
Solvent extraction / liquid –liquid extraction	Many extractants have been investigated for the recovery of 1,3-PDO from dilute broth. It is partly partitioned into the solvent phase only when adding a large amount of solvent into a concentrated broth	No effective extractant has been so far found for liquid –liquid extraction of 1,3-PDO. Major problem is because 1,3-PDO is hydrophilic	Malinowski 1999; Xiang et al. 2001; Baniel et al. 2004; Cho et al. 2006
Reactive extraction	Reactive extraction includes three key steps: reaction, extraction, and hydrolysis. A reversible reaction between 1,3-PDO and aldehyde was used to form a dioxolane derivative (e.g., 2-MD). 2-MD is then extracted into an organic solvent and finally hydrolyzed into 1,3-PDO	This process is quite complicated. The removal of proteins and ethanol as well as salts is necessary before reaction. Additionally, the trace amount of aldehyde in 1,3-PDO is prohibitive for polymerization of PTT	Broekhuis et al. 1994, 1996; Malinowski 2000; Hao et al. 2005, 2006 Fang and Zhou 2006

So far, no economically feasible strategy for recovery of 1,3-PDO from fermentation broth based on the glycerol has been developed and published.

5.5.5 Glyfinery 1,3-PDO recovery process

The optimal procedure of isolation of 1,3-PDO from fermentation broth, developed in WP6, is based on the following steps:
-extraction of fermentation broth
-recovery of solvent (from extract) by distillation
-vacuum distillation
-recovery of solvent (from raffinate) by stripping

Liquid-liquid extraction is complex and always requires some type of pilot plant experiments to generate the necessary data for process design. This is especially true in the case of biotechnological applications. The fermentation broth can often vary in composition and contain trace quantities of other materials that affect the phase separation or efficiency of the process. Any pilot plant testing should be performed with actual

fermentation broth, as synthetic blends will not reveal any problems. There are many types of devices available to accomplish the liquid-liquid extraction process, including mixer-settlers, packed columns, sieve tray columns, agitated columns, and centrifugal units. Two types of agitated column were tested. Liquid-liquid extraction efficiency is 96%.

The solvent recovery step is the critical aspect of any liquid-liquid extraction process design. Efficient solvent recycling greatly affects the economics of the process. In the proposed process solvent recycling is being recovered by distillation at 90% efficiency. The recovered solvent can be returned directly to extraction step without any further purification. Vacuum distillation is a final purification stage of 1,3-PDO recovery. The yield of distillation is 99% with 99.99% purity of target product. This process requires a low energy input due to extremely low volumes being processed.

Recovery of solvent from raffinate can be performed by stripping. The recovered solvent can be successfully reused in liquid-liquid extraction of target product.

The 1,3-PDO integrated recovery system is summarized in the table 11.

Table 12: Summary of 1,3-PDO integrated recovery system.

Recovery process efficiency [%]	90.3
Total energy requirement [MJ/kg of product]	158*
Product purity [%]	99.99

70% of energy can be recovered as heat energy that can be utillized in heat demanding processes (i.e. fermentation)

The proposed 1,3-PDO recovery system integrated with bioconversion of glycerol represents a unique process that can be easily adopted by industry. Clearly there are no existing counterparts to the proposed process that have been applied in industrial scale.

References

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6 Summary

This section summarizes the key indicators from each technology described in the preceding chapters of this technological assessment.

Table 12: Key technological indicators for the three main processes proposed for the GLYFINERY.

Indicator	**1,3-propanediol**	**Butanol**	**Ethanol**
Type of process	Two stage process: Continuous and batch	Fed Batch with gas stripping	Batch/fed-batch
Current yield (crude glycerol)	537 kg/ton	>225 kg/ton	260 kg/ton
Productivity	0.85 g/l/h	> 1.5g/l/h	Phase I 0.16 g/l/h Phase II 0.18 g/l/h Phase III 0.06 g/l/h
Highest achievable titre	30.2 g/L		28.1 g/L
Yield on substrate basis	0.56 g/g	>0.280 mol/mol	0.56 mol/mol
Percentage of theoretical maximum	-	>70%	56%
% Energy recovered from substrate	92%	72%	54%
GLYFINERY Development stage	Large-scale	Large-scale	Large-scale
World Market development stage	Commercial production based on plant sugars	Butanol from sugar has been commercialised	Ethanol from plant sugars commercialised at industrial scale
Production of effluents	Recycle water, biomass to biogas	Recycle water, biomass to biogas	Recycle water, biomass to biogas
Risk associated with chemicals involved	Solvents	Solvents	None
GMO technology	no	No	no
Odor emissions	no	No	no
Technological challenges/bottlenecks		*In situ* removal of butanol	Improve ethanol tolerance

6.1 Biosafety issues

The term GMO has many definitions. One definition is:

The term genetically modified organism (GMO) means an organism in which the genetic material has been altered in a way that does not occur naturally through fertilisation and/or natural recombination. GMOs may be plants, animals or micro-organisms, such as bacteria, parasites and fungi

-efsa - European Food Safety authority (http://www.efsa.europa.eu/en/topics/topic/gmo.htm)

The operative word here is natural alteration. The strains used in the GLYFINERY project have all been isolated from the environment and are as such in their natural state. The improvements that have followed have all been by classical mutagenesis a process common in nature especially on a sunny day. The mutations have been caused by a naturally occurring process and strains with improved properties have been isolated and used for further development. In this iterative process no non natural manipulation of the genetic material has been applied. Hence none of the microorganisms are GMO.

The EC DIRECTIVE 2000/54/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 18 September 2000, on the protection of workers from risks related to exposure to biological agents at work defines the risk associated with biological agents. Directive 2000/54/EC and Directive 90/679/EEC (adopted 20 November, 1990; revised 18 September 2000) provides the classification of biological agents into four infection risk groups on the basis of the following criteria:

Group 1: biological agent means one that is unlikely to cause human disease.

Group 2: biological agent means one that can cause human disease and might be a hazard to workers; it is unlikely to spread to the community; there is usually effective prophylaxis or treatment available.

Group 3: biological agent means one that can cause severe human disease and present a serious hazard to workers; it may present a risk of spreading to the community, but there is usually effective prophylaxis or treatment available.

Group 4: biological agent means one that causes severe human disease and is a serious hazard to workers; it may present a high risk of spreading to the community; there is usually no effective prophylaxis or treatment available.

Many species from *Clostridium* are class 2 but the inclusion of the general *Clostridium* spp. does not indicate that all are dangerous. It is under the assumption that those organisms that are generally non pathogenic are excluded from the list.

The organisms used in this project are *C. pasteurianum* (butanol) and *C. butyricum* (1,3-PDO)

The EC directive does not mention *C. pasteurianum* or *C. butyricum*. Bundesanstalt für Arbeitsschutz und Arbeitsmedizin (BAuA), in Germany classifies *C. pasteurianum* as class 1 and *C. butyricum* as class 2 in their TRBA 466: Technical Rules for Biological Agents - Classification of Prokaryotes (Bacteria and Archaea) into Risk Groups (http://www.baua.de/en/Topics-from-A-to-Z/Biological-Agents/TRBA/TRBA-466_content.html).

Further certain strains of *C. butyricum* are used as a probiotics in Asia (Seki, H., Shiohara, M., Matsumura, T., Miyagawa, N., Tanaka, M., Komiyama, A. & Kurata, S. (2003). Prevention of antibiotic-associated diarrhea in children by *Clostridium butyricum* MIYAIRI. Pediatr Int 45, 86–90). The American Type Culture Collection (ATCC) classifies *C. butyricum* as biosafety level 1 in accordance with the recommended guidelines of Centers for Disease Control and Prevention (http://www.lgcstandards-atcc.org/BiosafetyLevels/tabid/1157/Default.aspx and http://www.cdc.gov/od/eaipp/). With this in mind the risks from the microorganisms used in the GLYFINERY project would be considered low.

7 Executive summary

Glycerol is an attractive substrate for current and future bioconversion due to the increasing volumes available on the market concomitant with rising biodiesel production, particularly in Europe. Crude glycerol obtained from biodiesel producers varies in composition dependent on the oil feedstock used. Several microorganisms and the respective submerged cultivation processes, particularly bacterial, have been shown to be inhibited by components found in the crude biodiesel. One success of the GLYFINERY project has been to develop robust bioprocesses based on organisms which are not sensitive (either naturally or through random mutagenesis) to variations in crude glycerol available from a typical biodiesel producer.

Three main product streams have been investigated as being part of the proposed glycerol biorefinery: two anaerobic processes based on *Clostridium* species producing 1,3-PDO and butanol respectively, and a micro-aerobic process based on the yeast *P. tannophilus* producing ethanol. There are currently no commercial processes based on conversion of glycerol to these products.

The envisaged GLYFINERY scenario includes all the described processes, in a typical (bio)refinery concept with conversion of the feed substrate to several (bio)products. The spent biomass from the processes as well as some of the recovered liquid would be fed into biogas production on-site to generate energy for the biorefinery. Further water recycling to the bioprocesses is also envisaged. Based on the results obtained in the GLYFINERY project and summarized in Table 12, it is clear that on the basis of energy recovered from substrate, that 1,3-PDO is the most technologically favorable product. However, large amounts of solvent are required for recovery which are likely to cause problems concerning chemical recycling and waste effluent treatment. The butanol production process could also be technologically favorable if the challenge of in situ removal of butanol at pilot scale could be overcome. Further improvements in yield for the ethanol process would be required to ensure the technological viability. This process has a yield of ethanol at the required level for making distillation technically feasible; this should be improved upon for optimized recovery.

Appendix B

Results from the initial screening for glycerol utilization.

B.1 – Baseline screening of growth on various substrates for selected Lactococcus lactis strains

B.2 – Results from glycerol utilization screening

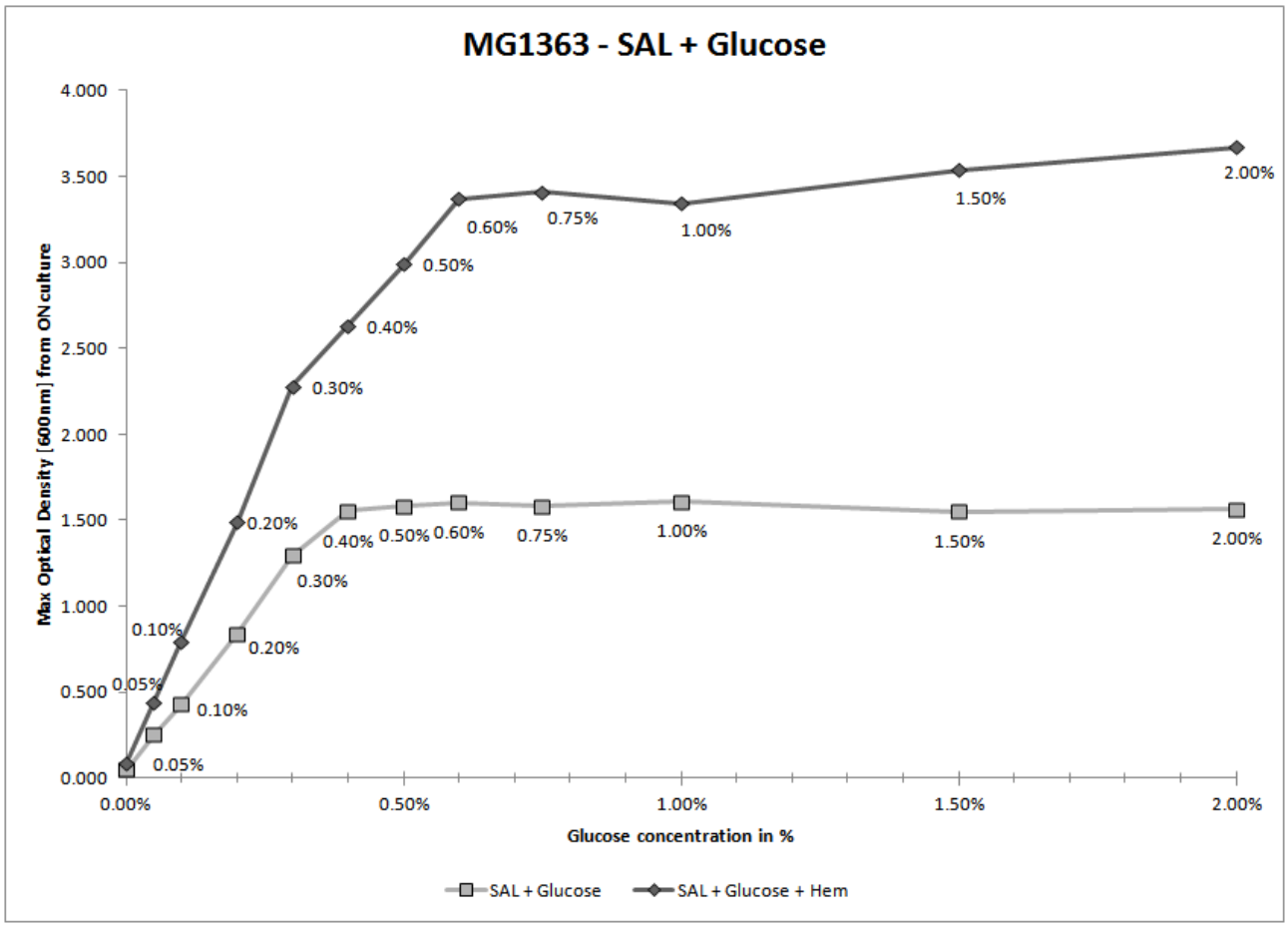
APPENDIX B

B.1 - Baseline screening of growth on various substrates for selected *Lactococcus lactis* strains

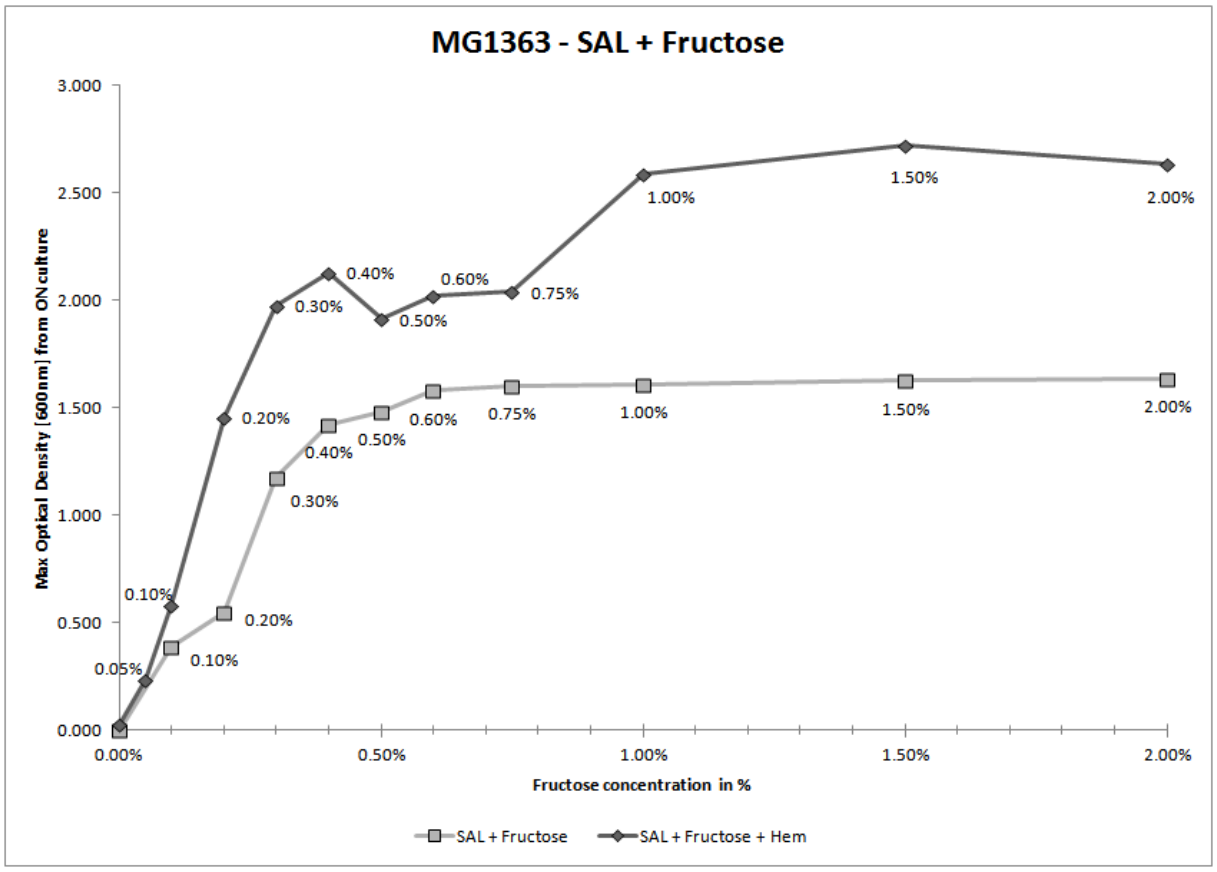
This section contains the results from the baseline screening of growth on various sugars by *Lactococcus lactis* strains MG1363, NCDO2118 and KF147.

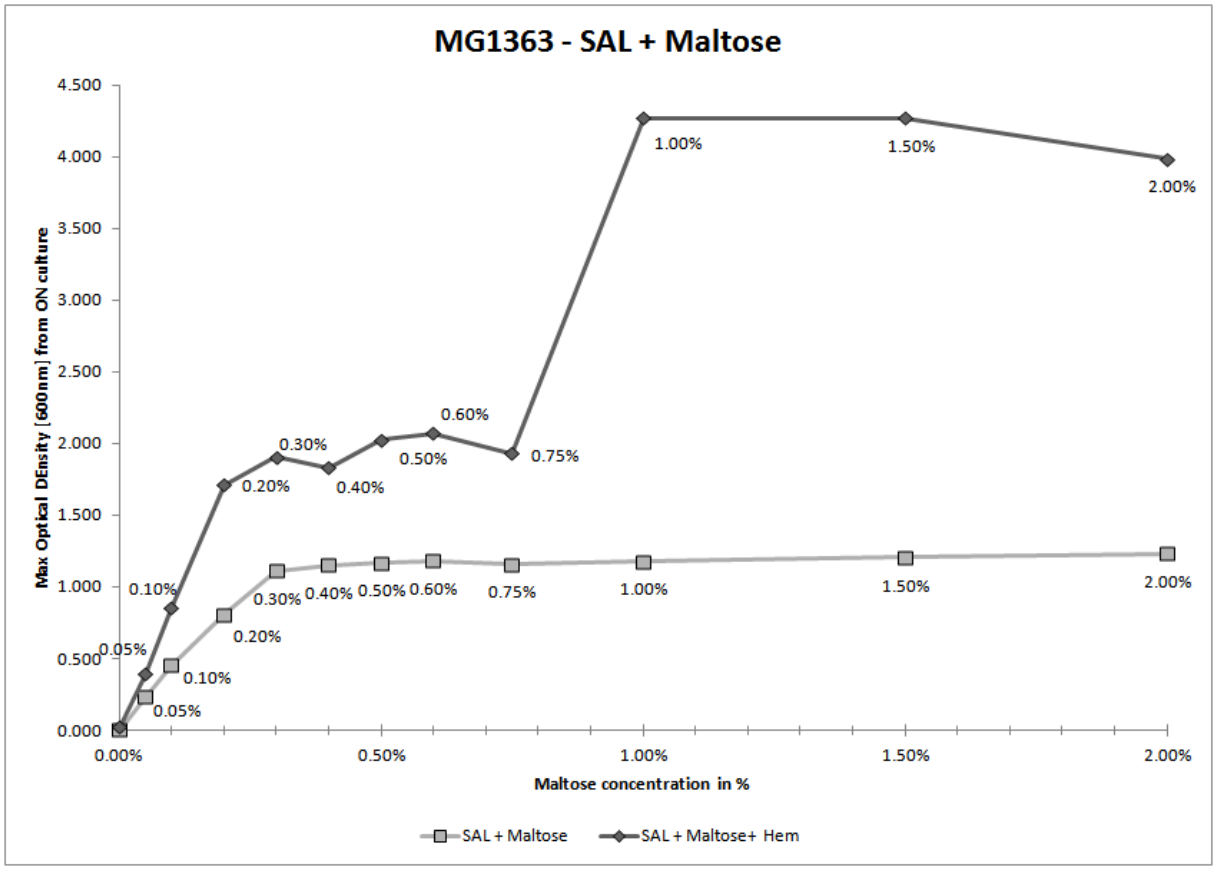
APPENDIX B

Lactococcus lactis MG1363

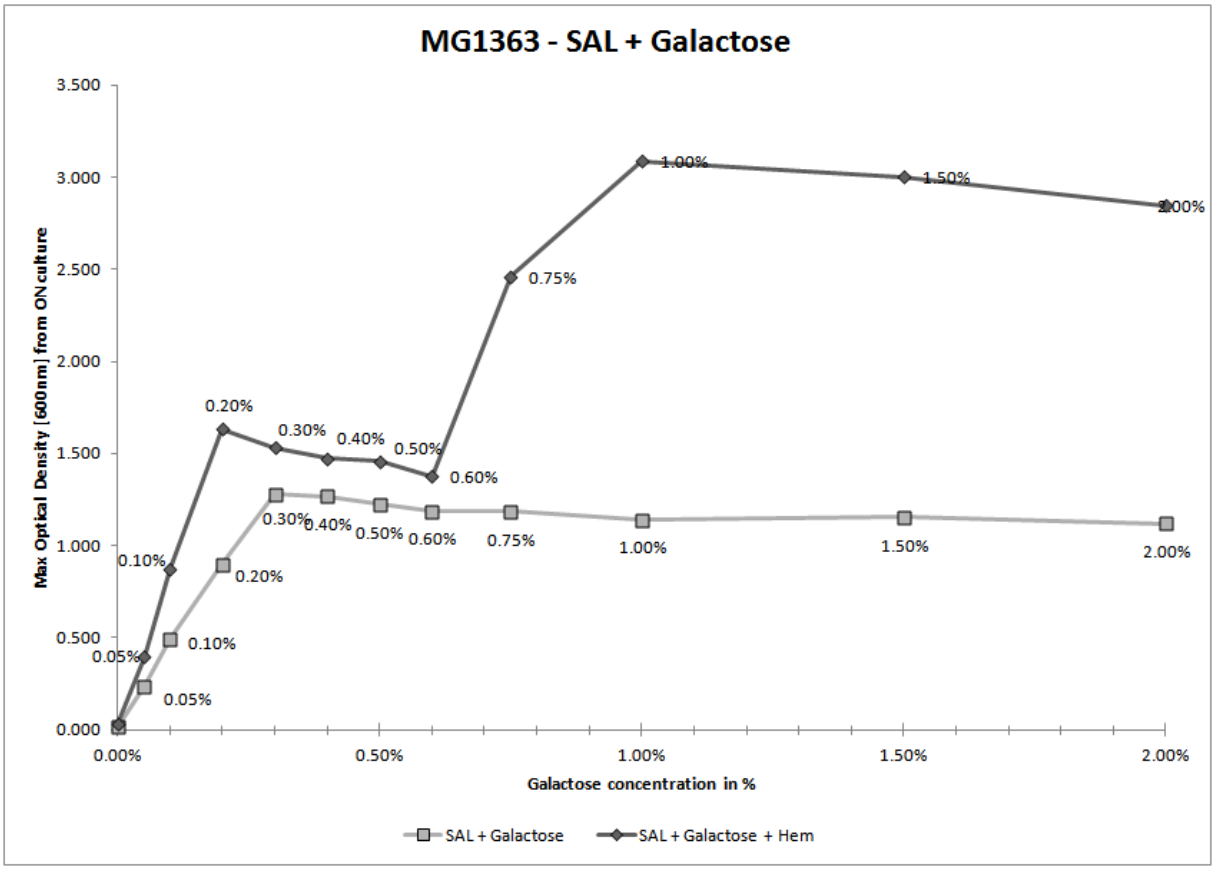


APPENDIX B


MG1363 - SAL + Fructose
Max Optical Density [600nm] from ON culture
3.000
2.500
2.000
1.500
1.000
0.500
0.000
0.00%
0.50%
1.00%
1.50%
2.00%
Fructose concentration in %
0.05%
0.10%
0.20%
0.30%
0.40%
0.50%
0.60%
0.75%
1.00%
1.50%
2.00%
SAL + Fructose
SAL + Fructose + Hem


MG1363 - SAL + Maltose
Max Optical DEnsity [600nm] from ON culture
4.500
4.000
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
0.00%
0.50%
1.00%
1.50%
2.00%
Maltose concentration in %
0.05%
0.10%
0.20%
0.30%
0.40%
0.50%
0.60%
0.75%
1.00%
1.50%
2.00%
SAL + Maltose
SAL + Maltose+ Hem

APPENDIX B

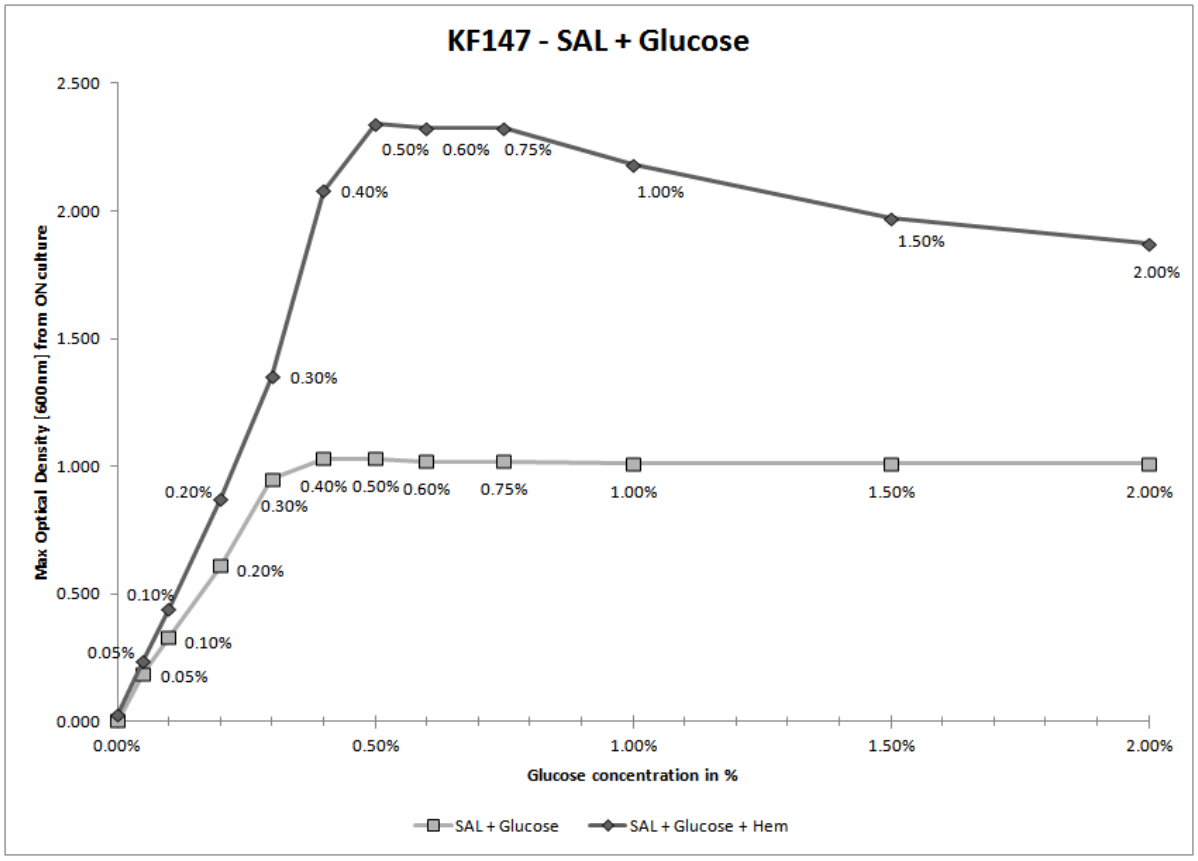


MG1363 (max ON OD600, duplicates+triplicates)												
Medium	0%	std. Dev.	0.05%	Std. Dev.	0.10%	Std. Dev.	0.20%	Std. Dev.	0.30%	Std. Dev.	0.40%	Std. Dev.
SAL + Glucose	0.050	0.009	0.252	0.004	0.430	0.031	0.838	0.057	1.29	0.10	1.55	0.07
SAL + Glucose + Hem	0.088	0.012	0.445	0.000	0.794	0.006	1.490	0.020	2.28	0.28	2.63	0.12
SAL + Fructose	0.003	0.001	#N/A	#N/A	0.390	0.021	0.547	0.066	1.17	0.03	1.42	0.02
SAL + Fructose + Hem	0.028	0.001	0.232	0.010	0.579	0.012	1.457	0.098	1.98	0.17	2.13	0.06
SAL + Galactose	0.014	0.000	0.236	0.001	0.495	0.008	0.899	0.023	1.28	0.03	1.27	0.06
SAL + Galactose + Hem	0.029	0.003	0.395	0.016	0.875	0.035	1.633	0.023	1.53	0.06	1.48	0.01
SAL + Maltose	0.000	0.000	0.231	0.000	0.448	0.017	0.805	0.016	1.11	0.01	1.15	0.00
SAL + Maltose+ Hem	0.023	0.001	0.391	0.014	0.853	0.015	1.710	0.044	1.91	0.09	1.83	0.00

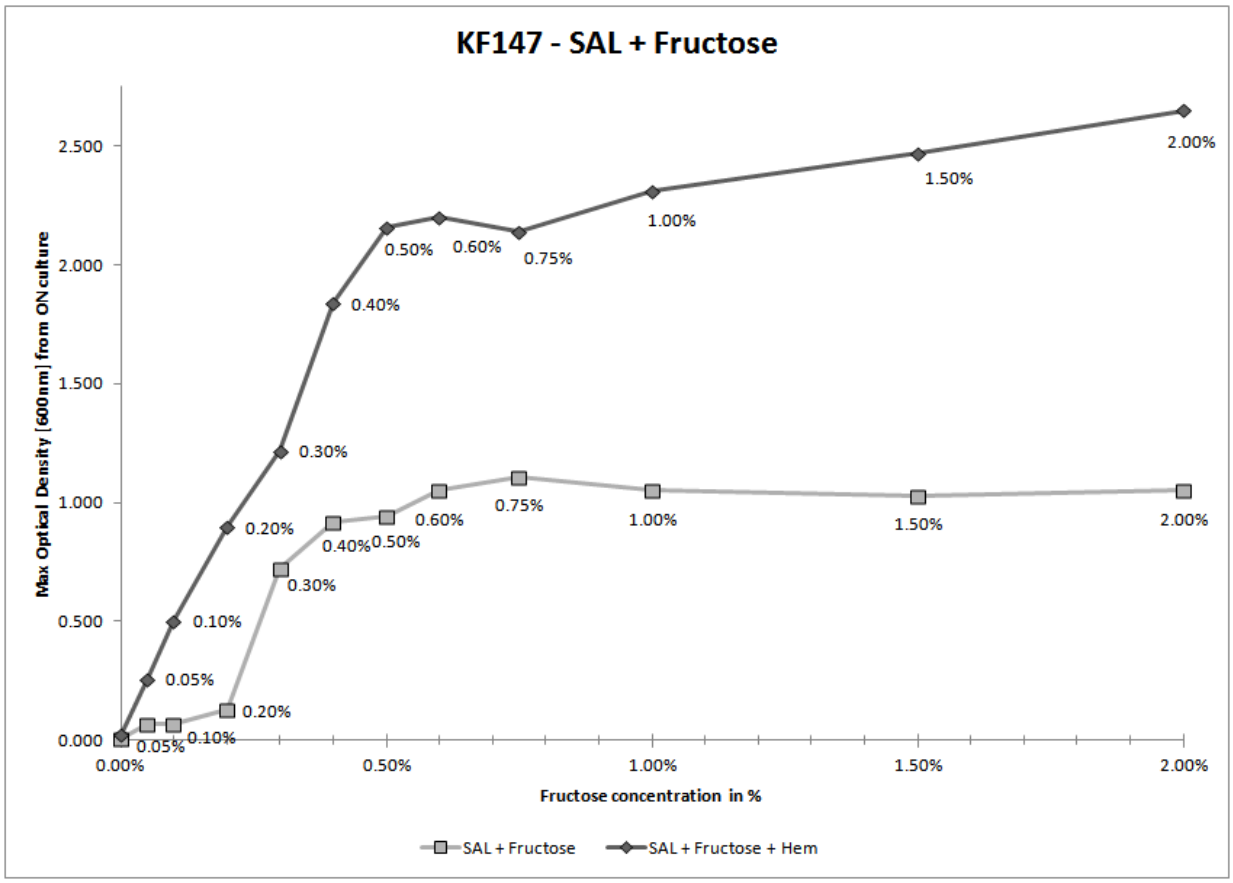
0.50%	Std. Dev.	0.60%	Std. Dev.	0.75%	Std. Dev.	1.00%	Std. Dev.	1.50%	Std. Dev.	2.00%	Std. Dev.
1.58	0.03	1.60	0.00	1.58	0.02	1.61	0.00	1.55	0.02	1.57	0.02
2.99	0.10	3.37	0.39	3.41	0.42	3.34	0.40	3.54	0.14	3.67	0.08
1.48	0.04	1.58	0.02	1.60	0.02	1.61	0.05	1.63	0.01	1.63	0.01
1.91	0.10	2.02	0.07	2.04	0.07	2.59	0.22	2.72	0.14	2.63	0.12
1.22	0.01	1.19	0.00	1.18	0.01	1.14	0.00	1.15	0.00	1.12	0.01
1.46	0.11	1.38	0.06	2.46	0.18	3.09	0.09	3.00	0.22	2.85	0.04
1.17	0.02	1.18	0.02	1.16	0.01	1.18	0.02	1.20	0.00	1.23	0.01
2.02	0.12	2.07	0.33	1.93	0.04	4.27	0.07	4.27	0.27	3.98	0.49

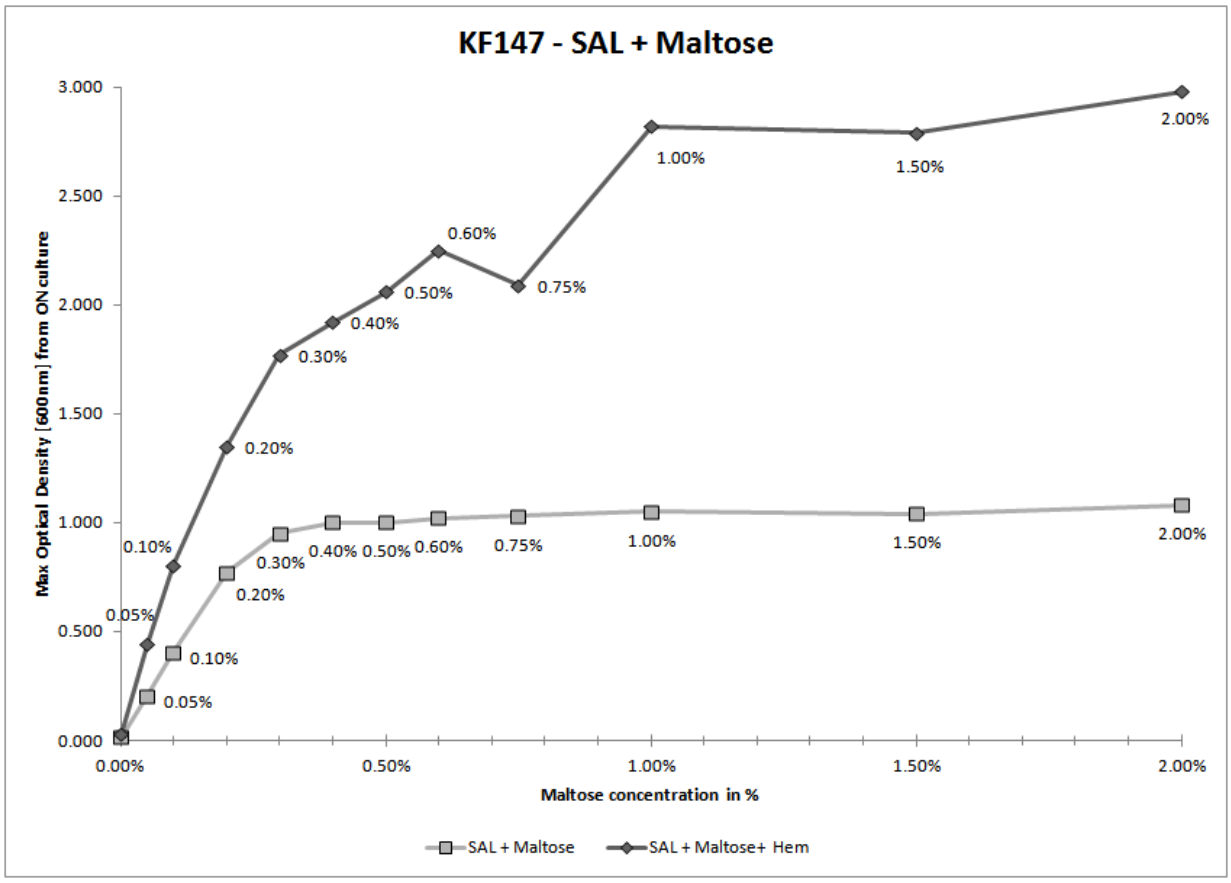
APPENDIX B

Lactococcus lactis KF147

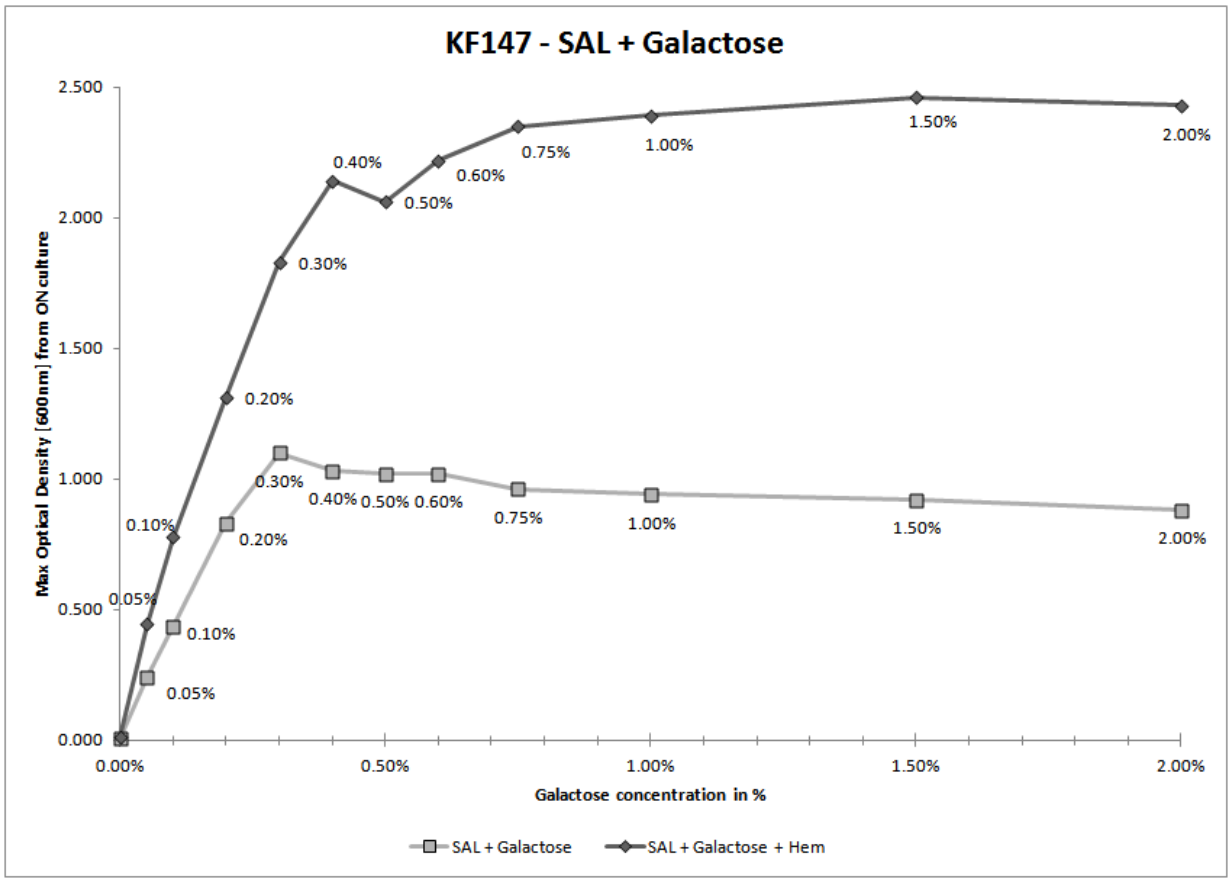


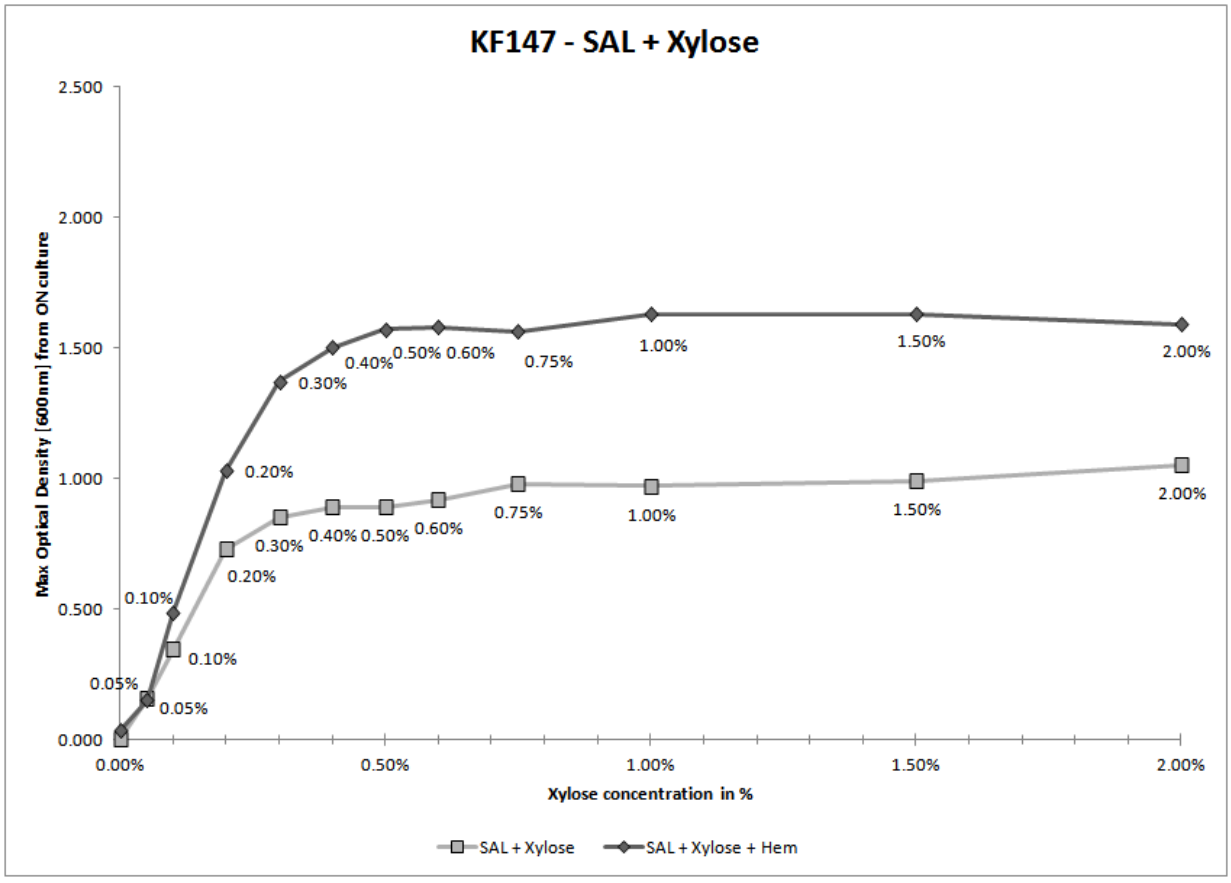
APPENDIX B


KF147 - SAL + Fructose
Max Optical Density [600nm] from ON culture
2.500
2.000
1.500
1.000
0.500
0.000
0.00%
0.50%
1.00%
1.50%
2.00%
Fructose concentration in %
SAL + Fructose
SAL + Fructose + Hem


KF147 - SAL + Maltose
Max Optical Density [600nm] from ON culture
3.000
2.500
2.000
1.500
1.000
0.500
0.000
0.00%
0.50%
1.00%
1.50%
2.00%
Maltose concentration in %
SAL + Maltose
SAL + Maltose+ Hem

APPENDIX B


KF147 - SAL + Galactose
Max Optical Density [600nm] from ONculture
2.500
2.000
1.500
1.000
0.500
0.000
0.00%
0.50%
1.00%
1.50%
2.00%
Galactose concentration in %
SAL + Galactose
SAL + Galactose + Hem


KF147 - SAL + Xylose
Max Optical Density [600nm] from ONculture
2.500
2.000
1.500
1.000
0.500
0.000
0.00%
0.50%
1.00%
1.50%
2.00%
Xylose concentration in %
SAL + Xylose
SAL + Xylose + Hem

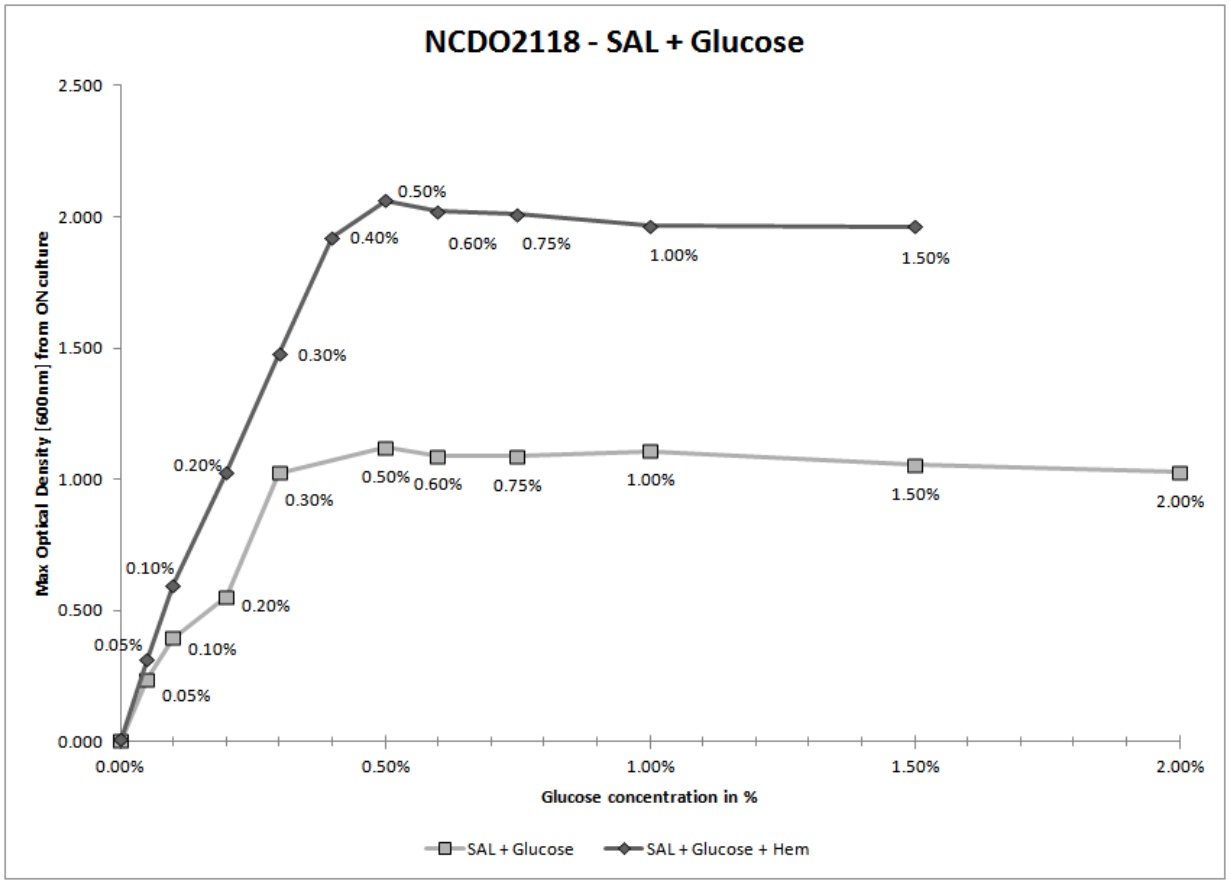
APPENDIX B

Medium	0%	std. Dev.	0.05%	Std. Dev.	0.10%	Std. Dev.	0.20%	Std. Dev.	0.30%	Std. Dev.	0.40%	Std. Dev.
SAL + Glucose	0.003	0.002	0.190	0.002	0.330	0.002	0.610	0.008	0.95	0.02	1.03	0.01
SAL + Glucose + Hem	0.030	0.001	0.237	0.003	0.440	0.000	0.873	0.006	1.35	0.04	2.08	0.30
SAL + Fructose	0.010	0.002	0.070	0.020	0.070	0.003	0.130	0.020	0.72	0.06	0.92	0.01
SAL + Fructose + Hem	0.026	0.002	0.258	0.006	0.505	0.016	0.897	0.051	1.22	0.10	1.84	0.14
SAL + Galactose	0.006	0.001	0.241	0.001	0.435	0.003	0.829	0.064	1.10	0.11	1.03	0.02
SAL + Galactose + Hem	0.012	0.001	0.443	0.005	0.776	0.017	1.310	0.040	1.83	0.15	2.14	0.11
SAL + Maltose	0.012	0.001	0.204	0.005	0.403	0.005	0.770	0.030	0.95	0.01	1.00	0.03
SAL + Maltose+ Hem	0.026	0.003	0.441	0.019	0.804	0.014	1.350	0.110	1.77	0.17	1.92	0.09
SAL + Xylose	0.002	0.001	0.158	0.000	0.346	0.000	0.727	0.009	0.85	0.02	0.89	0.01
SAL + Xylose + Hem	0.032	0.004	0.151	0.003	0.487	0.035	1.030	0.030	1.37	0.08	1.50	0.00

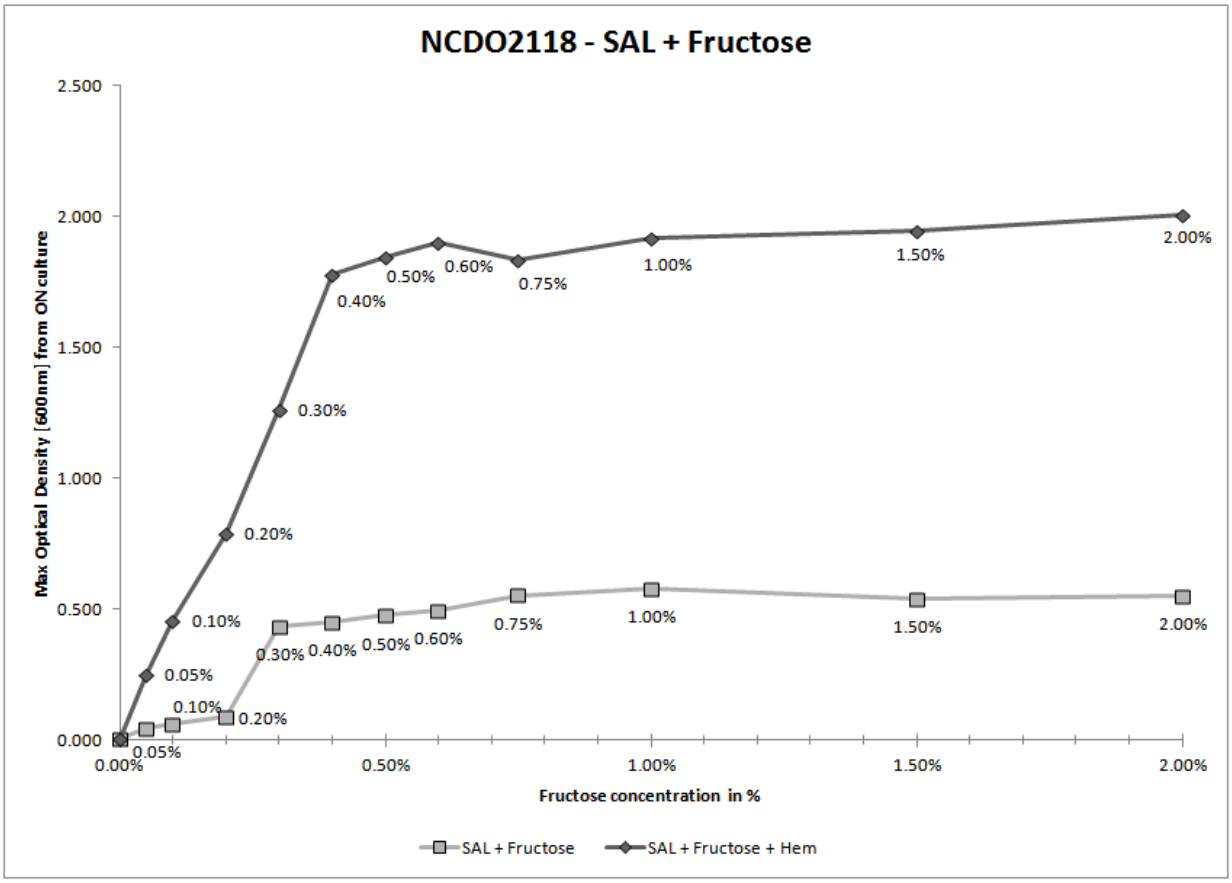
0.50%	Std. Dev.	0.60%	Std. Dev.	0.75%	Std. Dev.	1.00%	Std. Dev.	1.50%	Std. Dev.	2.00%	Std. Dev.
1.03	0.01	1.02	0.01	1.02	0.01	1.01	0.00	1.01	0.02	1.01	0.00
2.34	0.03	2.33	0.18	2.32	0.39	2.18	0.00	1.97	0.28	1.87	0.03
0.94	0.04	1.05	0.07	1.11	0.01	1.05	0.01	1.03	0.01	1.05	0.00
2.16	0.00	2.20	0.05	2.14	0.00	2.31	0.00	2.47	0.08	2.65	0.10
1.02	0.01	1.02	0.01	0.96	0.03	0.94	0.06	0.92	0.02	0.88	0.01
2.06	0.08	2.22	0.05	2.35	0.10	2.39	0.15	2.46	0.10	2.43	0.12
1.00	0.00	1.02	0.02	1.03	0.01	1.05	0.02	1.04	0.01	1.08	0.06
2.06	0.30	2.25	0.59	2.09	0.18	2.82	0.01	2.79	0.06	2.98	0.28
0.89	0.00	0.92	0.01	0.98	0.02	0.97	0.06	0.99	0.00	1.05	0.03
1.57	0.02	1.58	0.09	1.56	0.04	1.63	0.03	1.63	0.05	1.59	0.06

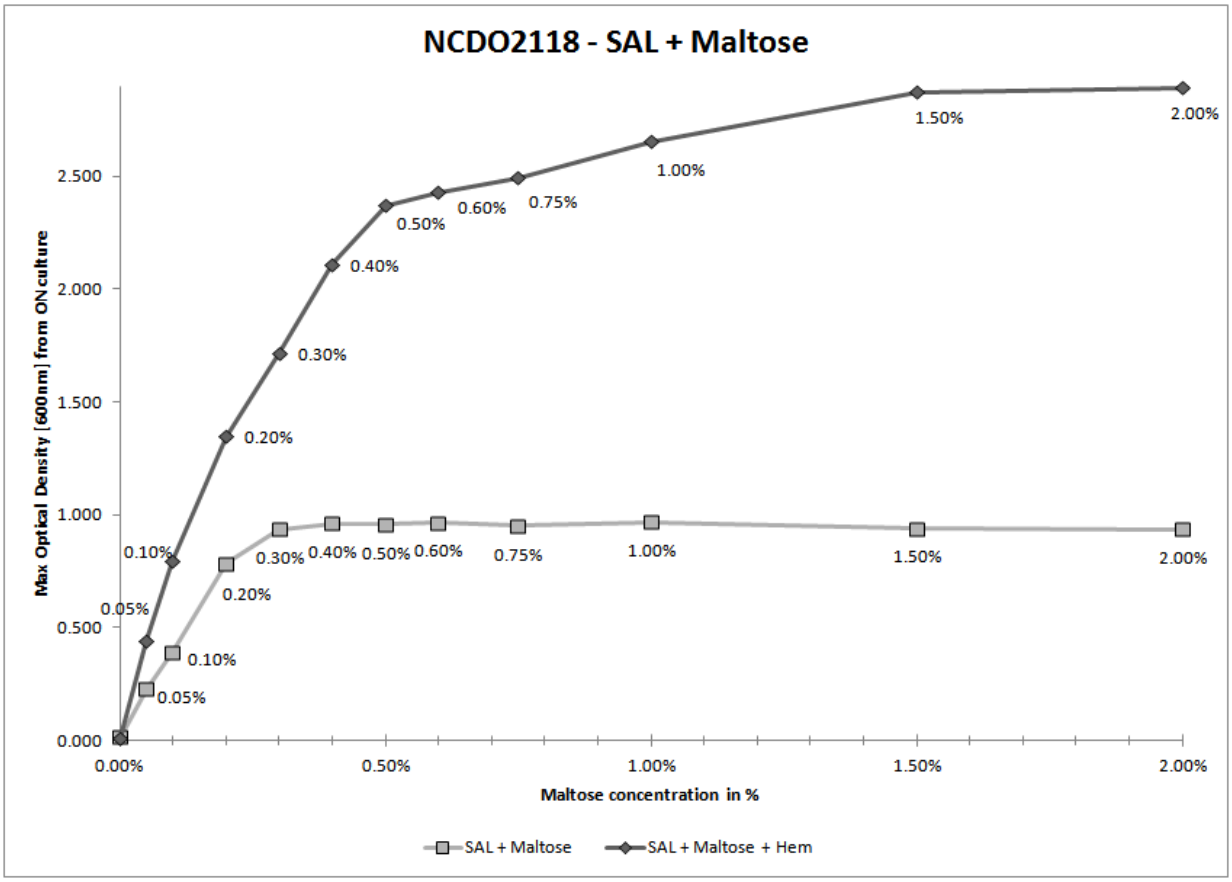
APPENDIX B

Lactococcus lactis NCDO2118

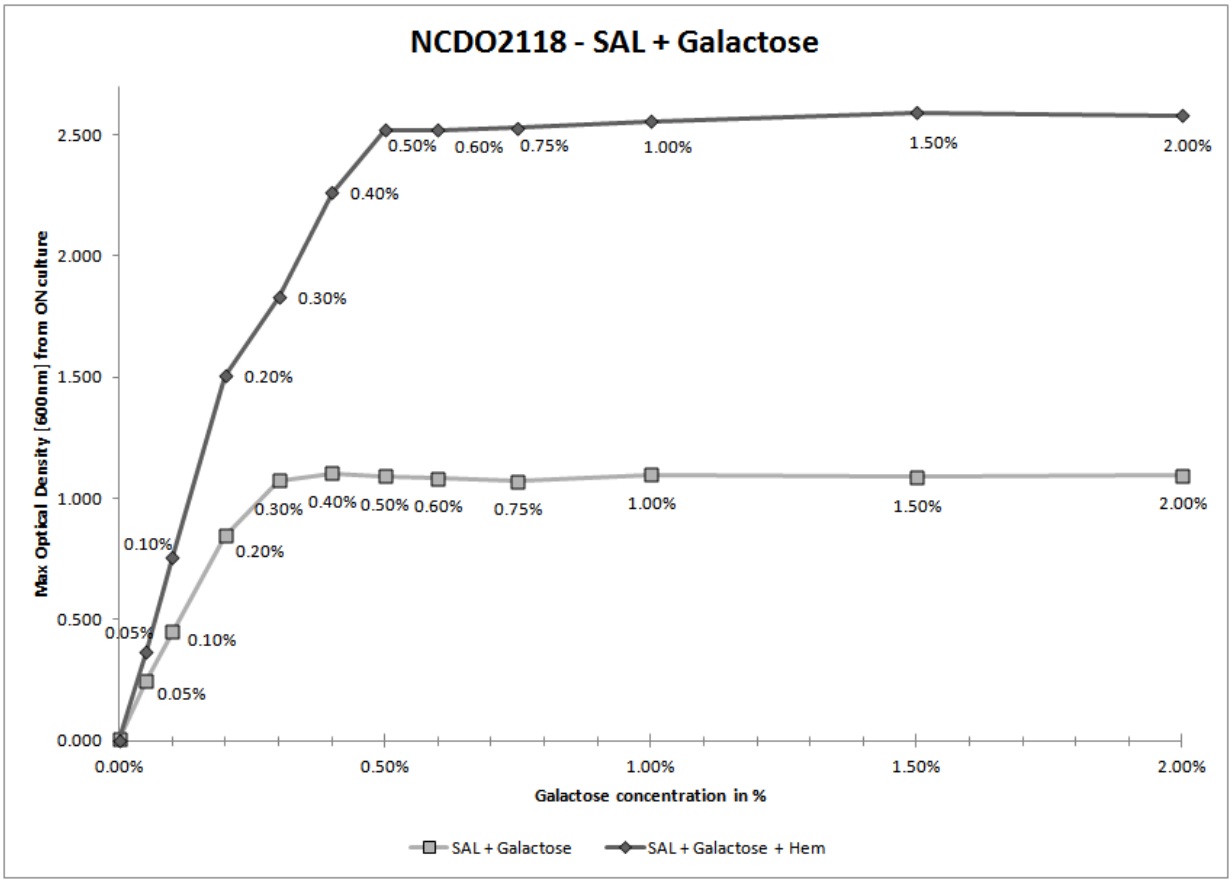


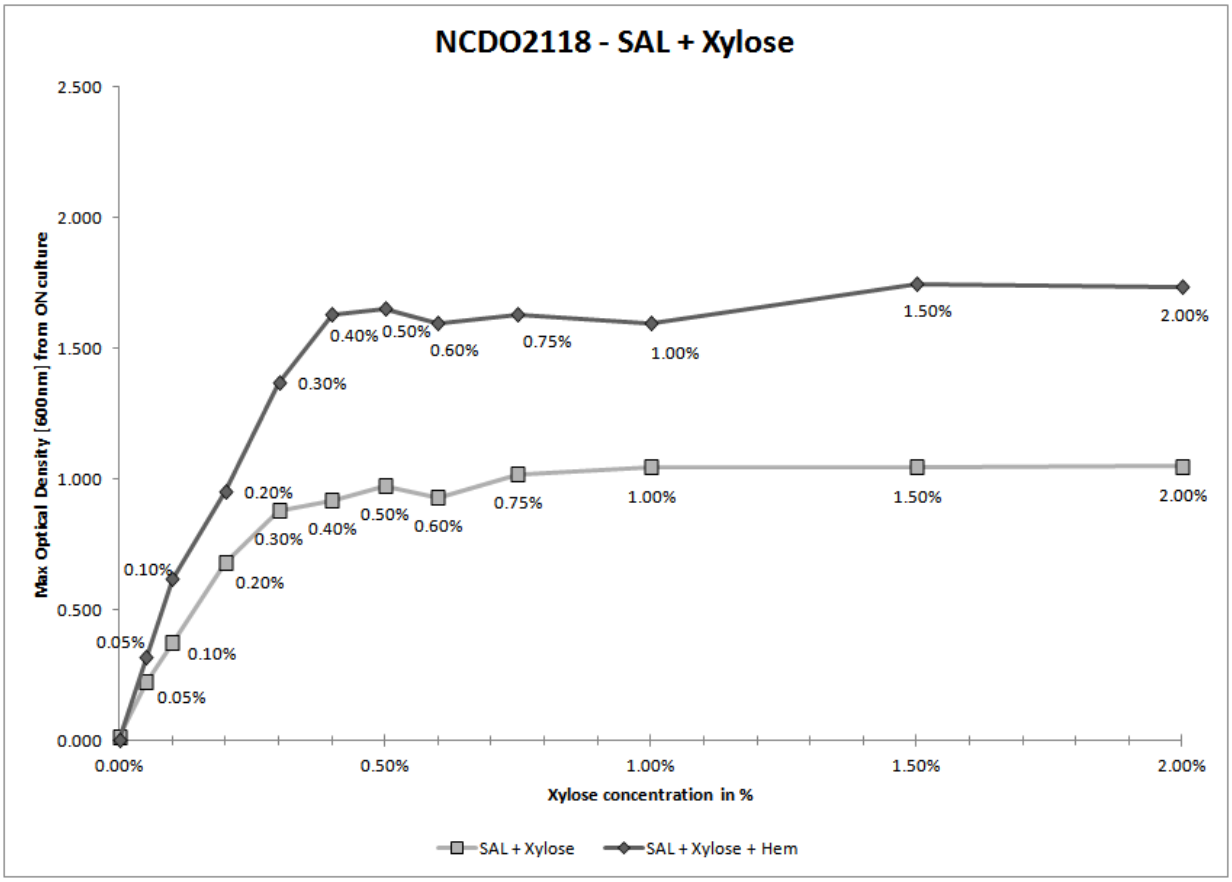
APPENDIX B


NCDO2118 - SAL + Fructose
Max Optical Density [600nm] from ONculture
2.500
2.000
1.500
1.000
0.500
0.000
0.00%
0.50%
1.00%
1.50%
2.00%
Fructose concentration in %
SAL + Fructose
SAL + Fructose + Hem


NCDO2118 - SAL + Maltose
Max Optical Density [600nm] from ONculture
2.500
2.000
1.500
1.000
0.500
0.000
0.00%
0.50%
1.00%
1.50%
2.00%
Maltose concentration in %
SAL + Maltose
SAL + Maltose + Hem

APPENDIX B


NCDO2118 - SAL + Galactose
Max Optical Density [600nm] from ONculture
2.500
2.000
1.500
1.000
0.500
0.000
0.00%
0.50%
1.00%
1.50%
2.00%
Galactose concentration in %
0.05%
0.10%
0.20%
0.30%
0.40%
0.50%
0.60%
0.75%
1.00%
1.50%
2.00%
SAL + Galactose
SAL + Galactose + Hem


NCDO2118 - SAL + Xylose
Max Optical Density [600nm] from ONculture
2.500
2.000
1.500
1.000
0.500
0.000
0.00%
0.50%
1.00%
1.50%
2.00%
Xylose concentration in %
0.05%
0.10%
0.20%
0.30%
0.40%
0.50%
0.60%
0.75%
1.00%
1.50%
2.00%
SAL + Xylose
SAL + Xylose + Hem

APPENDIX B

Medium	0%	std. Dev.	0.05%	Std. Dev.	0.10%	Std. Dev.	0.20%	Std. Dev.	0.30%	Std. Dev.	0.40%	Std. Dev.
SAL + Glucose	0.003	0.001	0.237	0.003	0.396	0.011	0.552	0.000	1.02	0.02	#N/A	#N/A
SAL + Glucose + Hem	0.010	0.001	0.312	0.005	0.597	0.012	1.027	0.012	1.48	0.02	1.92	0.03
SAL + Fructose	0.006	0.000	0.048	0.013	0.064	0.030	0.088	0.032	0.43	0.02	0.45	0.01
SAL + Fructose + Hem	0.009	0.002	0.252	0.000	0.459	0.005	0.790	0.044	1.26	0.11	1.78	0.08
SAL + Galactose	0.005	0.001	0.250	0.005	0.449	0.002	0.845	0.027	1.07	0.03	1.10	0.01
SAL + Galactose + Hem	0.002	0.001	0.369	0.016	0.760	0.010	1.507	0.032	1.83	0.01	2.26	0.02
SAL + Maltose	0.014	0.006	0.225	0.004	0.390	0.000	0.782	0.048	0.94	0.04	0.96	0.02
SAL + Maltose + Hem	0.009	0.000	0.443	0.013	0.797	0.025	1.345	0.078	1.72	0.08	2.11	0.08
SAL + Xylose	0.013	0.001	0.225	0.009	0.371	0.002	0.680	0.087	0.88	0.03	0.92	0.05
SAL + Xylose + Hem	0.003	0.000	0.320	0.004	0.619	0.016	0.950	0.017	1.37	0.05	1.63	0.08

0.50%	Std. Dev.	0.60%	Std. Dev.	0.75%	Std. Dev.	1.00%	Std. Dev.	1.50%	Std. Dev.	2.00%	Std. Dev.
1.12	0.06	1.09	0.02	1.09	0.02	1.11	0.07	1.05	0.02	1.03	0.01
2.06	0.17	2.02	0.07	2.01	0.04	1.97	0.02	1.96	0.02	#N/A	#N/A
0.48	0.01	0.50	0.01	0.55	0.00	0.58	0.04	0.54	0.03	0.55	0.03
1.84	0.07	1.90	0.04	1.84	0.02	1.92	0.06	1.95	0.05	2.01	0.04
1.09	0.01	1.08	0.01	1.07	0.01	1.10	0.03	1.09	0.02	1.09	0.01
2.52	0.08	2.52	0.01	2.53	0.07	2.56	0.02	2.59	0.01	2.58	0.04
0.96	0.03	0.96	0.03	0.95	0.01	0.97	0.00	0.94	0.03	0.94	0.02
2.37	0.01	2.43	0.00	2.49	0.08	2.65	0.14	2.87	0.07	2.89	0.20
0.97	0.02	0.93	0.02	1.02	0.07	1.04	0.04	1.04	0.03	1.05	0.04
1.65	0.05	1.59	0.01	1.63	0.03	1.60	0.06	1.75	0.16	1.74	0.11

APPENDIX B

B.2 – Results from glycerol utilization screening with increasing concentrations of glycerol

This section contains the results from the screening of selected *Lactococcus lactis* spp. for utilization of glycerol in medium supplemented with 0.2% glucose and 0-1% glycerol.

APPENDIX B

Medium 1:	SAL + 0.2% glucose + 0-1% glycerol						
Medium 2:	SAL + 0.2% glucose + 0-1% glycerol						
Medium 3:	SAL + 0.2% glucose + 0-1% glycerol + Hemin						
		270312		280312		290312	300312
	% glycerol	Optical density [600nm]					
1.1A	0.00	0.78	0.64	0.55	0.52	0.52	0.51
1.2A	0.05	0.67	0.59	0.54	0.57	0.44	0.51
1.3A	0.10	0.66	0.65	0.51	0.49	0.35	0.46
1.4A	0.20	0.86	0.59	0.50	0.62	0.39	0.52
1.5A	0.30	0.65	0.54	0.49	0.53	0.37	0.46
1.6A	0.40	0.70	0.59	0.46	0.52	0.56	0.46
1.7A	0.50	0.52	0.50	0.46	0.55	0.60	0.42
1.8A	0.60	0.64	0.50	0.51	0.57	0.54	0.49
1.9A	0.70	0.76	0.51	0.44	0.56	0.42	0.42
1.10A	0.80	0.76	0.42	0.62	0.60	0.55	0.43
1.11A	0.90	0.73	0.53	0.55	0.55	0.53	0.44
1.12A	1.00	0.60	0.59	0.58	0.56	0.42	0.50
	300312 catalase test : ok						
		270312		280312		290312	300312
	% glycerol	Optical density [600nm]					
2.1A	0.00	0.48	0.32	0,19	0.28	0.22	0.22
2.2A	0.05	0.43	0.35	0,23	0.24	0.21	0.25
2.3A	0.10	0.44	0.33	0,24	0.34	0.23	0.00
2.4A	0.20	0.37	0.31	0,23	0.39	0.27	0.31
2.5A	0.30	0.37	0.31	0,33	0.45	0.31	0.38

APPENDIX B

2.6A	0.40	0.54	0.31	0.29	0.46	0.30	0.26
2.7A	0.50	0.54	0.35	0.32	0.48	0.33	0.24
2.8A	0.60	0.41	0.30	0.31	0.51	0.38	0.26
2.9A	0.70	0.56	0.28	0.28	0.43	0.40	0.31
2.10A	0.80	0.63	0.44	0.27	0.50	0.46	0.29
2.11A	0.90	0.47	0.38	0.23	0.38	0.39	0.27
2.12A	1.00	0.58	0.42	0.27	0.49	0.38	0.33
	300312 catalase test : ok						
		270312		280312		290312	300312
	% glycerol	Optical density [600nm]					
3.1A	0.00	1.00	0.92	0.82	1.27	0.73	0.54
3.2A	0.05	0.00	0.24	0.42	x	0.56	0.44
3.3A	0.10	1.03	0.71	0.87	1.46	0.76	0.68
3.4A	0.20	0.82	0.49	0.48	1.20	0.72	0.52
3.5A	0.30	1.01	0.66	0.65	1.19	0.87	0.55
3.6A	0.40	0.92	0.72	0.51	0.93	0.83	0.50
3.7A	0.50	0.20	0.71	0.51	1.26	0.64	0.66
3.8A	0.60	0.95	0.73	0.51	1.01	0.95	0.60
3.9A	0.70	0.95	0.81	0.40	1.13	0.82	0.63
3.10A	0.80	0.90	0.64	0.51	1.25	0.79	0.62
3.11A	0.90	0.76	0.65	0.48	1.03	0.71	0.59
3.12A	1.00	0.85	0.71	0.47	1.07	0.83	0.63
	300312 catalase test : ok						

APPENDIX B

28°C, Respiratory
Medium 1 - SAL + 0.2% glucose
Medium 2 - SAL + 0.2% glucose + 0.2% glycerol

Strain		Day 1		Day 2		Day 3		Day 4		Average
NCDO2118	1A	1.11	0.98	1.05	0.81	0.81	0.82	0.56	0.75	0.72 ± 0.04
NCDO2118	1B								0.67	
NCDO2118	1C								0.73	
NCDO2118	2A	1.2	1.1	0.83	0.62	0.52	0.47	0.48	0.47	0.54 ± 0.14
NCDO2118	2B								0.44	
NCDO2118	2C								0.70	
MG1363	1A	1.4	1.3	1.2	1.1	1.1	1.1	1.1	1.13	1.04 ± 0.09
MG1363	1B								0.96	
MG1363	1C								1.04	
MG1363	2A	1.6	1.1	1.2	1.2	1.1	0.86	1.4	1.12	1.19 ± 0.18
MG1363	2B								1.4	
MG1363	2C								1.1	

Data from cultivation of Lactococcus lactis NCDO2118 and MG1363 in SAL medium supplemented with 0.2% glucose and with or without 0.2% glycerol. Strains were cultivated at 28°C under respiration permissive conditions (aeration and hemine added to growth medium).

Appendix C

Results from the screening of *Lactococcus lactis* strains for glycerol utilization.

This section contains the results from screening of selected Lactococcus lactis strains MG1363, NCDO2118 and KF147 for growth on glycerol.

APPENDIX C

Screen 15

NCDO2118, 30°C, ON, anaerob

SAL + 0.5% sugar

SAL + 0.5% sugar + 0.5% glycerol

Tube	Medium	Dilut.		OD600 meas.	OD600 calc.	Average OD600		SD
19	SAL + Fructose	10	x	0.039	0.390	0.44		0.050
20	SAL + Fructose	10	x	0.044	0.440			
21	SAL + Fructose	10	x	0.049	0.490			
22	SAL + Fructose + Glycerol	10	x	0.049	0.490	0.49		0.015
23	SAL + Fructose + Glycerol	10	x	0.051	0.510			
24	SAL + Fructose + Glycerol	10	x	0.048	0.480			
25	SAL + Xylose	10	x	0.084	0.840	0.87		0.029
26	SAL + Xylose	4	x	0.224	0.896			
27	SAL + Xylose	4	x	0.220	0.880			
28	SAL + Xylose + Glycerol	4	x	0.147	0.588	0.54		0.046
29	SAL + Xylose + Glycerol	4	x	0.130	0.520			
30	SAL + Xylose + Glycerol	4	x	0.125	0.500			

APPENDIX C

Screen 16

MG1363, 30°C, ON, respiratory

SAL + 1% sugar

Tube	Medium	dilut.		OD600 meas.	OD600 calc.	Average OD600	SD	pH
1	SAL + HEM(5γ)	1	x	0.003	0.003	0.002	0.001	7
2	SAL + HEM(5γ)	1	x	0.001	0.001			7
3	SAL + HEM(5γ)	1	x	0.001	0.001			7
4	SAL + HEM(5γ) + Glucose (1%)	13	x	0.152	1.976	2.12	0.22	6
5	SAL + HEM(5γ) + Glucose (1%)	13	x	0.155	2.015			6
6	SAL + HEM(5γ) + Glucose (1%)	13	x	0.183	2.379			6
7	SAL + HEM(5γ) + Fructose (1%)	13	x	0.192	2.496	2.53	0.03	5
8	SAL + HEM(5γ) + Fructose (1%)	13	x	0.194	2.522			5
9	SAL + HEM(5γ) + Fructose (1%)	13	x	0.197	2.561			5
10	SAL + HEM(5γ) + Xylose (1%)	1	x	0.028	0.028	0.021	0.007	7
11	SAL + HEM(5γ) + Xylose (1%)	1	x	0.022	0.022			7
12	SAL + HEM(5γ) + Xylose (1%)	1	x	0.014	0.014			7
13	SAL + HEM(5γ) + Galactose (1%)	13	x	0.146	1.898	1.89	0.01	5
14	SAL + HEM(5γ) + Galactose (1%)	13	x	0.145	1.885			5
15	SAL + HEM(5γ) + Galactose (1%)	13	x	0.146	1.898			5

APPENDIX C

KF147, 30°C, ON, respiratory

SAL + 1% sugar

Tube	Medium	dilut.		OD600 meas.	OD600 calc.	Average OD600	SD	pH
1	SAL + HEM(5γ)	1	x	0.015	0.015	0.018	0.004	7
2	SAL + HEM(5γ)	1	x	0.017	0.017			7
3	SAL + HEM(5γ)	1	x	0.022	0.022			7
4	SAL + HEM(5γ) + Glucose (1%)	10	x	0.146	1.460	1.44	0.03	4
5	SAL + HEM(5γ) + Glucose (1%)	10	x	0.145	1.450			4
6	SAL + HEM(5γ) + Glucose (1%)	10	x	0.140	1.400			4
7	SAL + HEM(5γ) + Fructose (1%)	10	x	0.155	1.550	1.55	0.02	4
8	SAL + HEM(5γ) + Fructose (1%)	10	x	0.154	1.540			4
9	SAL + HEM(5γ) + Fructose (1%)	10	x	0.157	1.570			4
10	SAL + HEM(5γ) + Xylose (1%)	10	x	0.112	1.120	1.11	0.09	6
11	SAL + HEM(5γ) + Xylose (1%)	10	x	0.120	1.200			6
12	SAL + HEM(5γ) + Xylose (1%)	10	x	0.102	1.020			6
13	SAL + HEM(5γ) + Galactose (1%)	10	x	0.216	2.160	2.30	0.27	5
14	SAL + HEM(5γ) + Galactose (1%)	10	x	0.261	2.610			5
15	SAL + HEM(5γ) + Galactose (1%)	10	x	0.212	2.120			5

APPENDIX C

NCDO2118, 30°C, ON, respiratory

SAL + 1% sugar

Tube	Medium	dilut.		OD600 meas.	OD600 calc.	Average OD600	SD	pH
1	SAL + HEM(10γ)	1	x	0.002	0.002	0.006	0.003	7
2	SAL + HEM(10γ)	1	x	0.007	0.007			7
3	SAL + HEM(10γ)	1	x	0.008	0.008			7
4	SAL + HEM(10γ) + Glucose (1%)	10	x	0.182	1.820	1.82	0.05	4
5	SAL + HEM(10γ) + Glucose (1%)	10	x	0.187	1.870			4
6	SAL + HEM(10γ) + Glucose (1%)	10	x	0.177	1.770			4
7	SAL + HEM(10γ) + Fructose (1%)	10	x	0.106	1.060	1.10	0.04	5
8	SAL + HEM(10γ) + Fructose (1%)	10	x	0.111	1.110			4
9	SAL + HEM(10γ) + Fructose (1%)	10	x	0.113	1.130			4
10	SAL + HEM(10γ) + Xylose (1%)	10	x	0.022	0.220	0.293	0.067	7
11	SAL + HEM(10γ) + Xylose (1%)	10	x	0.035	0.350			7
12	SAL + HEM(10γ) + Xylose (1%)	10	x	0.031	0.310			7
13	SAL + HEM(10γ) + Galactose (1%)	10	x	0.045	0.450	1.37	0.54	7
14	SAL + HEM(10γ) + Galactose (1%)	10	x	0.125	1.250			6
15	SAL + HEM(10γ) + Galactose (1%)	10	x	0.148	1.480			5

APPENDIX C

Screen 17

MG1363, KF147, NCDO2118, 30°C, ON, anaerobic

SAL + 0.1% sugar

SAL + 0.1% sugar + 1% glycerol

Strain	Tube	Medium	Dilut.		OD600 meas.	OD600 calc.	Average OD600	SD
MG1363	1	SAL + Fructose	5	x	0.075	0.38	0.38	0.02
	1		1	x	0.344	0.34	0.35	0.01
MG1363	2	SAL + Fructose	5	x	0.074	0.37	0.37	0.02
	2		1	x	0.345	0.35		
MG1363	3	SAL + Fructose	5	x	0.080	0.40		
	3		1	x	0.369	0.37		
MG1363	4	SAL + Fructose + Glycerol	5	x	0.063	0.32	0.32	0.03
	4		1	x	0.296	0.30	0.30	0.02
MG1363	5	SAL + Fructose + Glycerol	5	x	0.069	0.35	0.31	0.02
	5		1	x	0.326	0.33		
MG1363	6	SAL + Fructose + Glycerol	5	x	0.058	0.29		
	6		1	x	0.279	0.28		
KF147	7	SAL + Fructose	5	x	0.031	0.16	0.14	0.02
	7		1	x	0.143	0.14	0.13	0.02
KF147	8	SAL + Fructose	5	x	0.025	0.13	0.13	0.02
	8		1	x	0.109	0.11		
KF147	9	SAL + Fructose	5	x	0.029	0.15		
	9		1	x	0.125	0.13		
KF147	10	SAL + Fructose + Glycerol	5	x	0.031	0.16	0.16	0.02
	10		1	x	0.134	0.13	0.14	0.02
KF147	11	SAL + Fructose + Glycerol	5	x	0.030	0.15	0.15	0.02
	11		1	x	0.128	0.13		
KF147	12	SAL + Fructose + Glycerol	5	x	0.037	0.19		
	12		1	x	0.167	0.17		
KF147	13	SAL + Xylose	5	x	0.032	0.16	0.15	0.01
	13		1	x	0.158	0.16	0.15	0.01
KF147	14	SAL + Xylose	5	x	0.029	0.15	0.15	0.01

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	14		1	x	0.137	0.14		
KF147	15	SAL + Xylose	5	x	0.028	0.14		
	15		1	x	0.140	0.14		
KF147	16	SAL + Xylose + Glycerol	5	x	0.016	0.08	0.09	0.01
	16		1	x	0.067	0.07	0.07	0.01
KF147	17	SAL + Xylose + Glycerol	5	x	0.017	0.09	0.08	0.01
	17		1	x	0.067	0.07		
KF147	18	SAL + Xylose + Glycerol	5	x	0.019	0.10		
	18		1	x	0.082	0.08		
NCDO2118	19	SAL + Fructose	5	x	0.014	0.070	0.07	0.00
	19		1	x	0.046	0.046	0.04	0.01
NCDO2118	20	SAL + Fructose	5	x	0.013	0.065	0.05	0.02
	20		1	x	0.036	0.036		
NCDO2118	21	SAL + Fructose	5	x	0.014	0.070		
	21		1	x	0.037	0.037		
NCDO2118	22	SAL + Fructose + Glycerol	5	x	0.016	0.080	0.09	0.02
	22		1	x	0.062	0.062	0.06	0.01
NCDO2118	23	SAL + Fructose + Glycerol	5	x	0.022	0.110	0.08	0.02
	23		1	x	0.072	0.072		
NCDO2118	24	SAL + Fructose + Glycerol	5	x	0.015	0.075		
	24		1	x	0.053	0.053		
NCDO2118	25	SAL + Xylose	5	x	0.020	0.100	0.15	0.05
	25		1	x	0.092	0.092	0.15	0.06
NCDO2118	26	SAL + Xylose	5	x	0.030	0.150	0.15	0.05
	26		1	x	0.154	0.154		
NCDO2118	27	SAL + Xylose	5	x	0.041	0.205		
	27		1	x	0.208	0.208		
NCDO2118	28	SAL + Xylose + Glycerol	5	x	0.020	0.100	0.11	0.02
	28		1	x	0.084	0.084	0.09	0.01
NCDO2118	29	SAL + Xylose + Glycerol	5	x	0.019	0.095	0.10	0.01
	29		1	x	0.085	0.085		
NCDO2118	30	SAL + Xylose + Glycerol	5	x	0.025	0.125		
	30		1	x	0.094	0.094		

APPENDIX C

Screen 18

MG1363, KF147, NCDO2118, 30°C, ON, Respiratory

BLL + 0.5% sugar

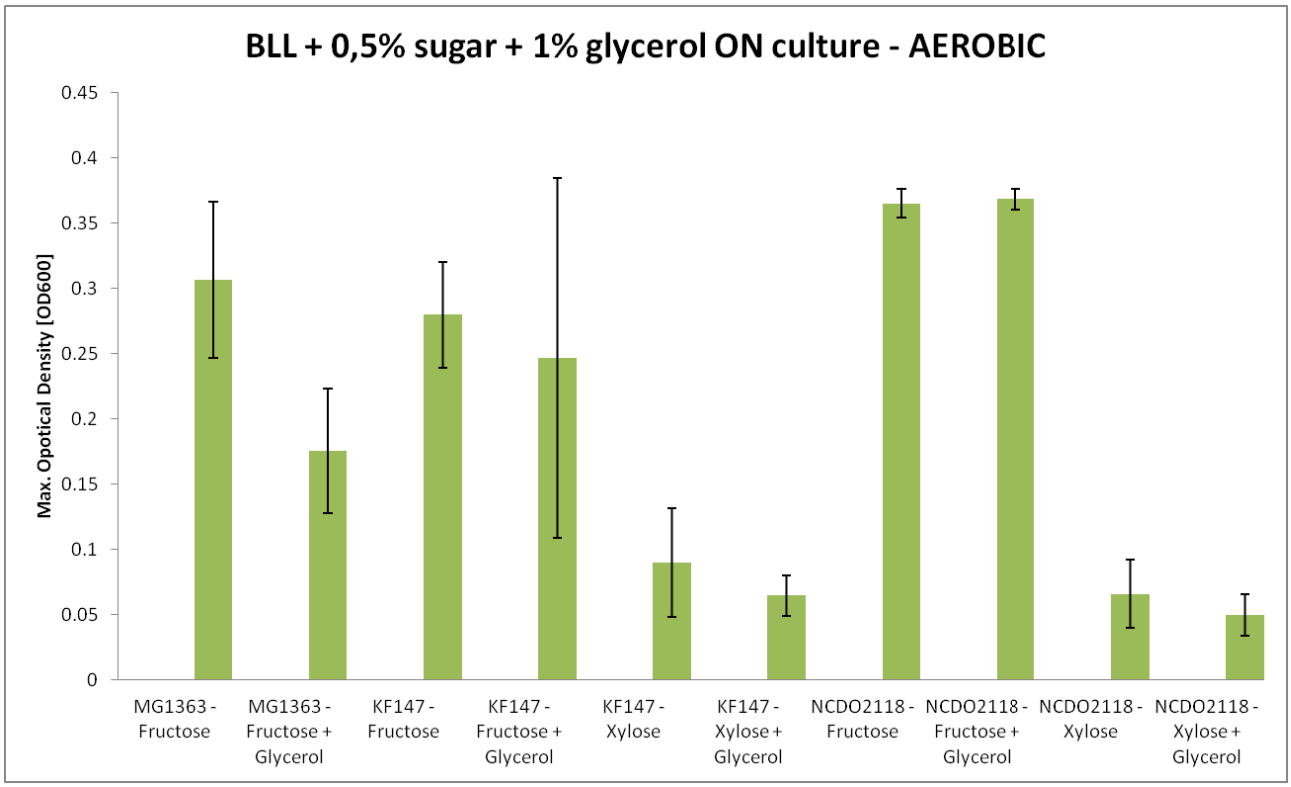
BLL + 0.5% sugar + 1% glycerol

Strain	Rør	Medium	dilut.		OD600 meas.	OD600 calc.	Average OD600	SD
MG1363	31	BLL + HEM + Fructose	5	x	0.075	0.38	0.31	0.06
MG1363	32	BLL + HEM + Fructose	5	x	0.053	0.27		
MG1363	33	BLL + HEM + Fructose	5	x	0.056	0.28		
MG1363	34	BLL + HEM + Fructose + Glycerol	2	x	0.115	0.23	0.18	0.05
MG1363	35	BLL + HEM + Fructose + Glycerol	1	x	0.155	0.16		
MG1363	36	BLL + HEM + Fructose + Glycerol	1	x	0.141	0.14		
KF147	37	BLL + HEM + Fructose	2	x	0.144	0.29	0.28	0.04
KF147	38	BLL + HEM + Fructose	2	x	0.158	0.32		
KF147	39	BLL + HEM + Fructose	2	x	0.118	0.24		
KF147	40	BLL + HEM + Fructose + Glycerol	2	x	0.107	0.21	0.25	0.14
KF147	41	BLL + HEM + Fructose + Glycerol	2	x	0.199	0.40		
KF147	42	BLL + HEM + Fructose + Glycerol	2	x	0.064	0.13		
KF147	43	BLL + HEM + Xylose	1	x	0.138	0.14	0.09	0.04
KF147	44	BLL + HEM + Xylose	1	x	0.062	0.06		
KF147	45	BLL + HEM + Xylose	1	x	0.070	0.07		
KF147	46	BLL + HEM + Xylose + Glycerol	1	x	0.067	0.07	0.06	0.02
KF147	47	BLL + HEM + Xylose + Glycerol	1	x	0.048	0.05		
KF147	48	BLL + HEM + Xylose + Glycerol	1	x	0.079	0.08		
NCDO2118	49	BLL + HEM + Fructose	2	x	0.180	0.360	0.37	0.01
NCDO2118	50	BLL + HEM + Fructose	2	x	0.179	0.358		
NCDO2118	51	BLL + HEM + Fructose	2	x	0.189	0.378		
NCDO2118	52	BLL + HEM + Fructose + Glycerol	2	x	0.182	0.364	0.37	0.01
NCDO2118	53	BLL + HEM + Fructose + Glycerol	2	x	0.182	0.364		
NCDO2118	54	BLL + HEM + Fructose + Glycerol	2	x	0.189	0.378		

APPENDIX C

NCDO2118	55	BLL + HEM + Xylose	2	x	0.024	0.048	0.07	0.03
NCDO2118	56	BLL + HEM + Xylose	2	x	0.027	0.054		
NCDO2118	57	BLL + HEM + Xylose	2	x	0.048	0.096		
NCDO2118	58	BLL + HEM + Xylose + Glycerol	1	x	0.031	0.031	0.05	0.02
NCDO2118	59	BLL + HEM + Xylose + Glycerol	1	x	0.059	0.059		
NCDO2118	60	BLL + HEM + Xylose + Glycerol	1	x	0.059	0.059		

Series	Max OD	SD
MG1363 - Fructose	0.31	0.06
MG1363 - Fructose + Glycerol	0.18	0.05
KF147 - Fructose	0.28	0.04
KF147 - Fructose + Glycerol	0.25	0.14
KF147 - Xylose	0.09	0.04
KF147 - Xylose + Glycerol	0.06	0.02
NCDO2118 - Fructose	0.37	0.01
NCDO2118 - Fructose + Glycerol	0.37	0.01
NCDO2118 - Xylose	0.07	0.03
NCDO2118 - Xylose + Glycerol	0.05	0.02



Appendix D

Results from growth experiments with the sugars glucose and maltose with or without glycerol supplementation.

This section contains the results from growth experiments with glucose and maltose with or without glycerol supplementation.

Growth 02: Glucose + glycerol

Growth 03: Glucose

Growth 04: Maltose

Maltose + glycerol

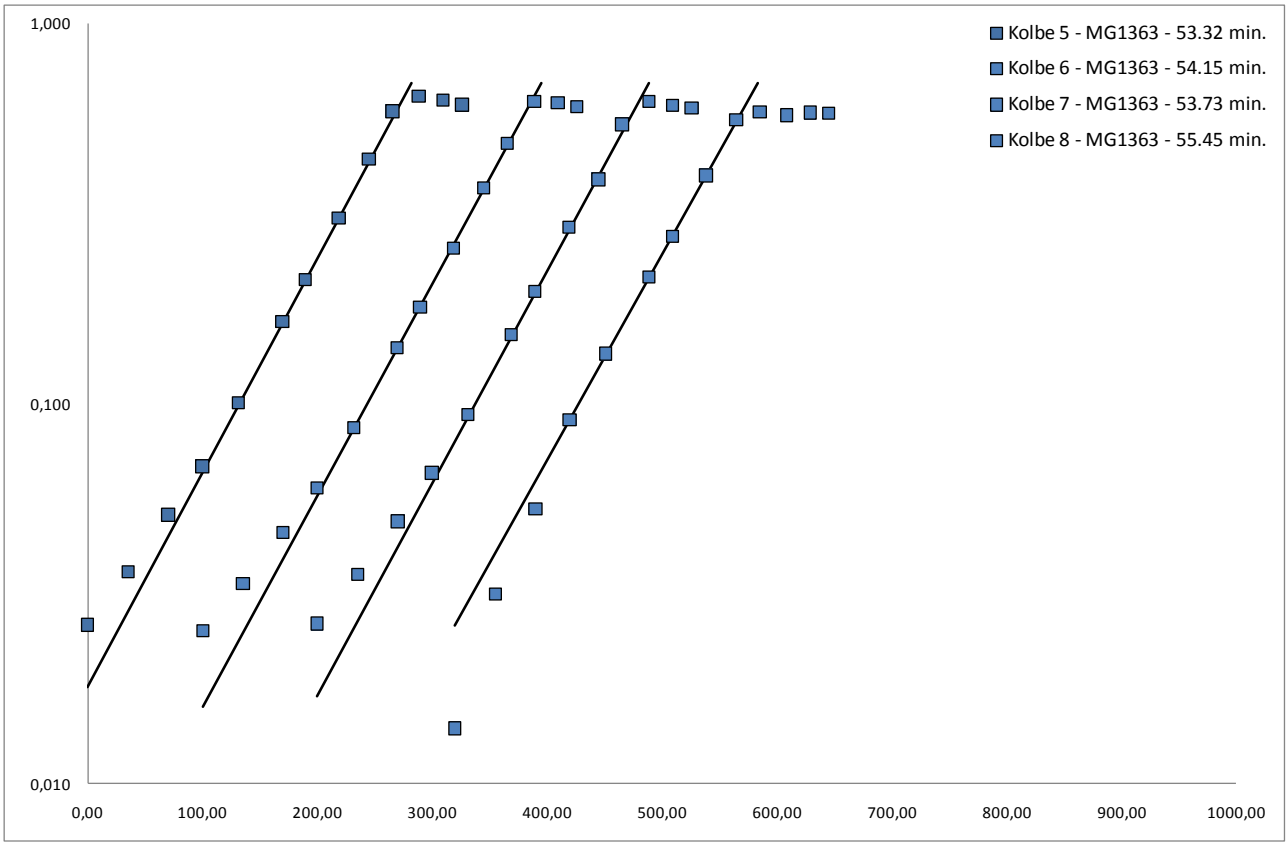
APPENDIX D

Experiment: Growth 02 – glucose and glycerol.

Contain.	**Strain**	**Alias**	**Medium**
Kolbe 5:	AH 64	MG1363	SAL + 0,1% glucose + 1% glycerol + 5 µg/mL Hemin
Kolbe 6:	AH 64	MG1363	SAL + 0,1% glucose + 1% glycerol + 5 µg/mL Hemin
Kolbe 7:	AH 64	MG1363	SAL + 0,1% glucose + 1% glycerol + 5 µg/mL Hemin
Kolbe 8:	AH 64	MG1363	SAL + 0,1% glucose + 1% glycerol + 5 µg/mL Hemin

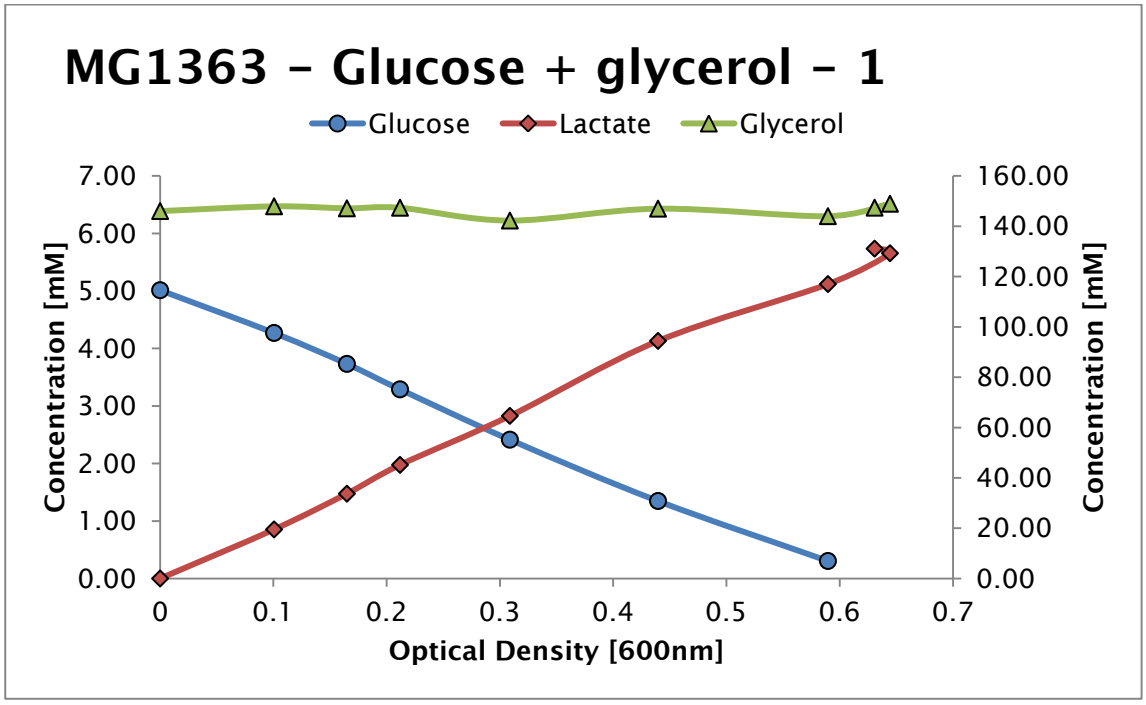
Kolbe	Laktat [mM]
Kolbe 5	5,73
Kolbe 6	5,75
Kolbe 7	5,63
Kolbe 8	5,79

Aver. T_d	SD T_d	Aver. µ	SD µ
54,16	0,92	0,77	0,01



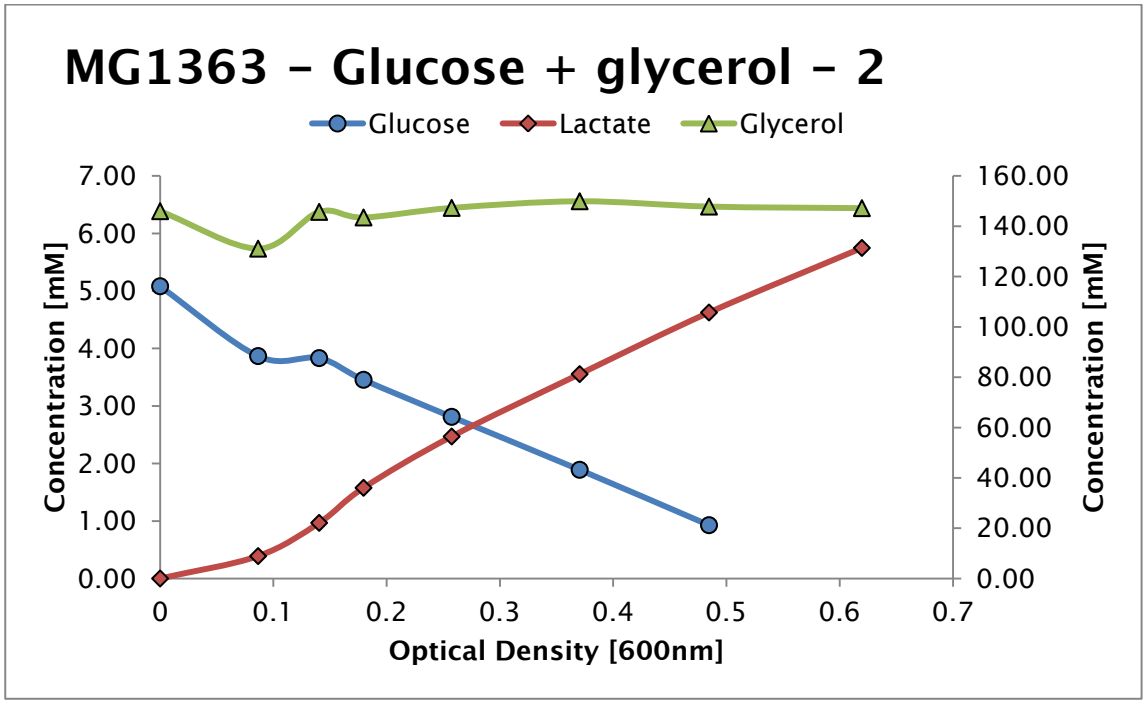
APPENDIX D

		mM	mM	mM	mM	mM	mM
Sample	OD600	Glucose	Lactate	Glycerol	Formate	Acetate	Ethanol
G02 K5P0	0	5.01	n.a.	146.01	n.a.	n.a.	n.a.
G02 K5P1	0.101	4.27	0.85	147.90	n.a.	n.a.	n.a.
G02 K5P2	0.165	3.73	1.47	147.10	n.a.	n.a.	n.a.
G02 K5P3	0.212	3.29	1.98	147.33	n.a.	n.a.	n.a.
G02 K5P4	0.309	2.41	2.83	142.24	n.a.	n.a.	n.a.
G02 K5P5	0.440	1.35	4.13	146.96	n.a.	n.a.	n.a.
G02 K5P6	0.590	0.30	5.12	143.98	n.a.	n.a.	n.a.
G02 K5P7	0.644	n.a.	5.65	148.92	n.a.	n.a.	n.a.
G02 K5P8	0.631	n.a.	5.73	147.35	n.a.	n.a.	n.a.



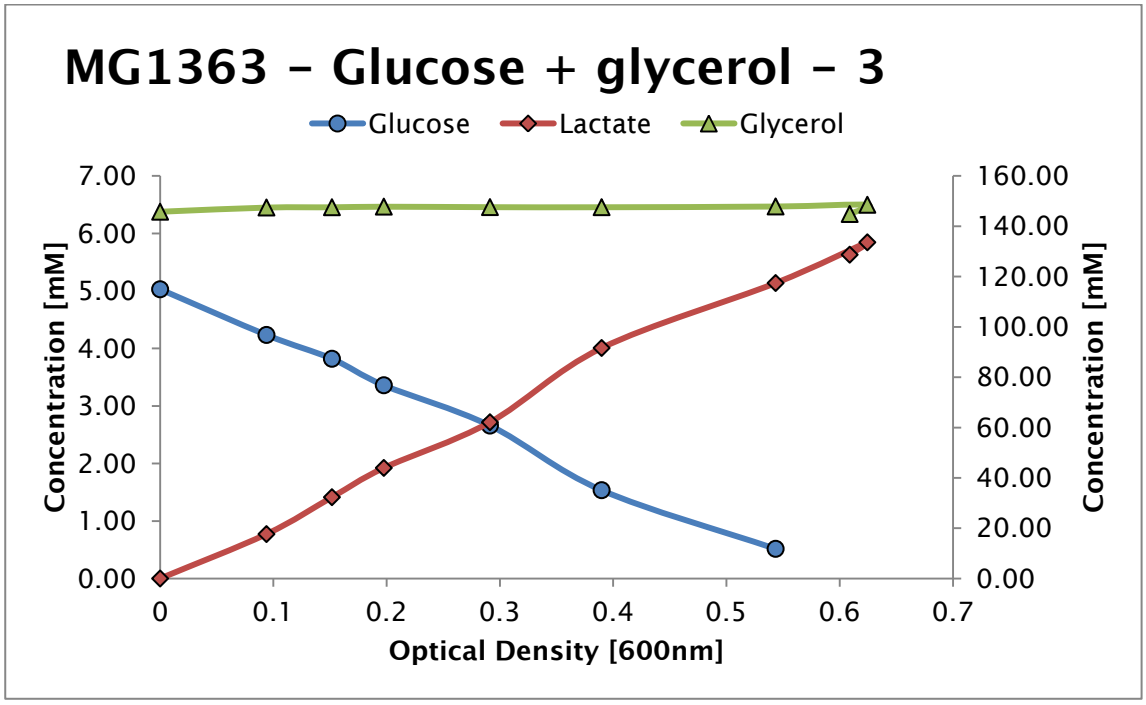
APPENDIX D

		mM	mM	mM	mM	mM	mM
Sample	OD600	Glucose	Lactate	Glycerol	Formate	Acetate	Ethanol
G02 K6P0	0	5.08	n.a.	145.95	n.a.	n.a.	n.a.
G02 K6P1	0.087	3.87	0.39	131.07	n.a.	n.a.	n.a.
G02 K6P2	0.140	3.83	0.97	145.67	n.a.	n.a.	n.a.
G02 K6P3	0.180	3.45	1.58	143.45	n.a.	n.a.	n.a.
G02 K6P4	0.257	2.81	2.47	147.25	n.a.	n.a.	n.a.
G02 K6P5	0.370	1.89	3.55	149.89	n.a.	n.a.	n.a.
G02 K6P6	0.485	0.93	4.62	147.78	n.a.	n.a.	n.a.
G02 K6P7	0.624	n.a.	4.66	123.66	n.a.	n.a.	n.a.
G02 K6P8	0.620	n.a.	5.75	147.16	n.a.	1.47	n.a.



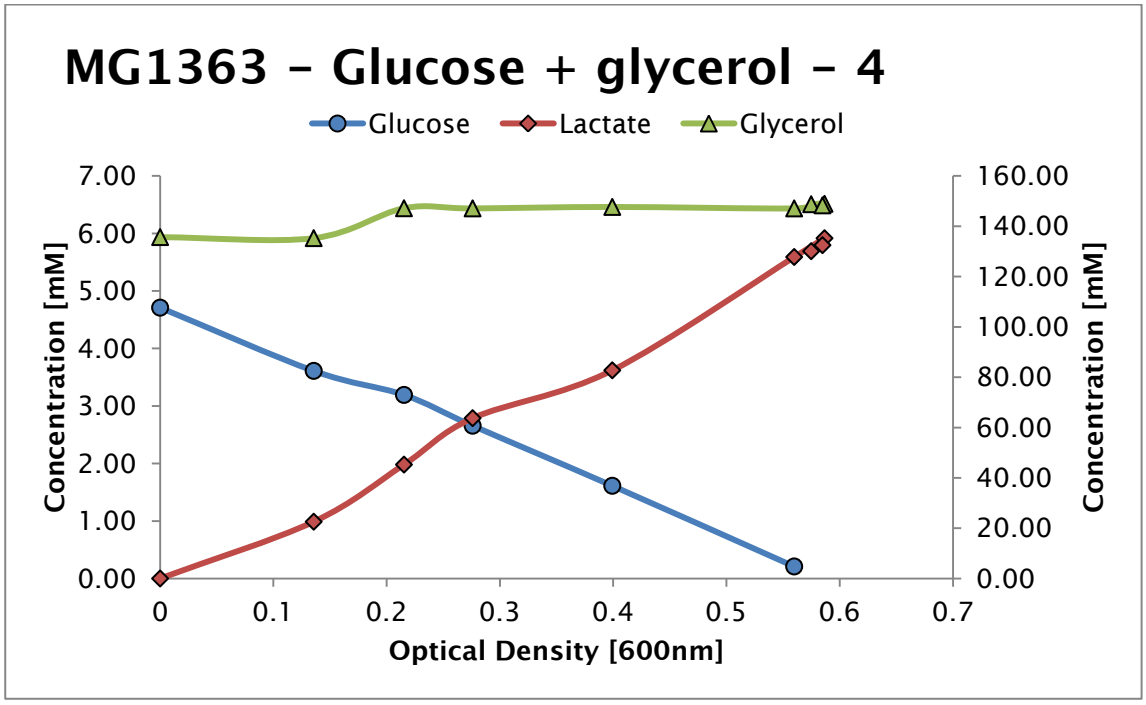
APPENDIX D

		mM	mM	mM	mM	mM	mM
Sample	OD600	Glucose	Lactate	Glycerol	Formate	Acetate	Ethanol
G02 K7P0	0	5.03	n.a.	145.75	n.a.	n.a.	n.a.
G02 K7P1	0.094	4.23	0.77	147.42	n.a.	n.a.	n.a.
G02 K7P2	0.152	3.82	1.41	147.53	n.a.	n.a.	n.a.
G02 K7P3	0.198	3.36	1.92	147.77	n.a.	n.a.	n.a.
G02 K7P4	0.291	2.66	2.72	147.58	n.a.	n.a.	n.a.
G02 K7P5	0.390	1.54	4.01	147.55	n.a.	n.a.	n.a.
G02 K7P6	0.543	0.52	5.14	147.84	n.a.	n.a.	n.a.
G02 K7P7	0.624	n.a.	5.84	148.51	n.a.	n.a.	n.a.
G02 K7P8	0.609	n.a.	5.63	144.84	n.a.	1.69	n.a.



APPENDIX D

		mM	mM	mM	mM	mM	mM
Sample	OD600	Glucose	Lactate	Glycerol	Formate	Acetate	Ethanol
G02 K8P0	0	4.71	n.a.	135.66	n.a.	n.a.	n.a.
G02 K8P1	0.136	3.61	0.99	135.25	n.a.	n.a.	n.a.
G02 K8P2	0.215	3.19	1.98	147.12	n.a.	n.a.	n.a.
G02 K8P3	0.276	2.66	2.79	147.07	n.a.	n.a.	n.a.
G02 K8P4	0.399	1.61	3.62	147.65	n.a.	n.a.	n.a.
G02 K8P5	0.560	0.21	5.59	147.07	n.a.	1.26	n.a.
G02 K8P6	0.587	n.a.	5.92	148.89	n.a.	1.07	n.a.
G02 K8P7	0.575	n.a.	5.69	148.72	n.a.	0.96	n.a.
G02 K8P8	0.585	n.a.	5.79	148.27	n.a.	1.30	n.a.



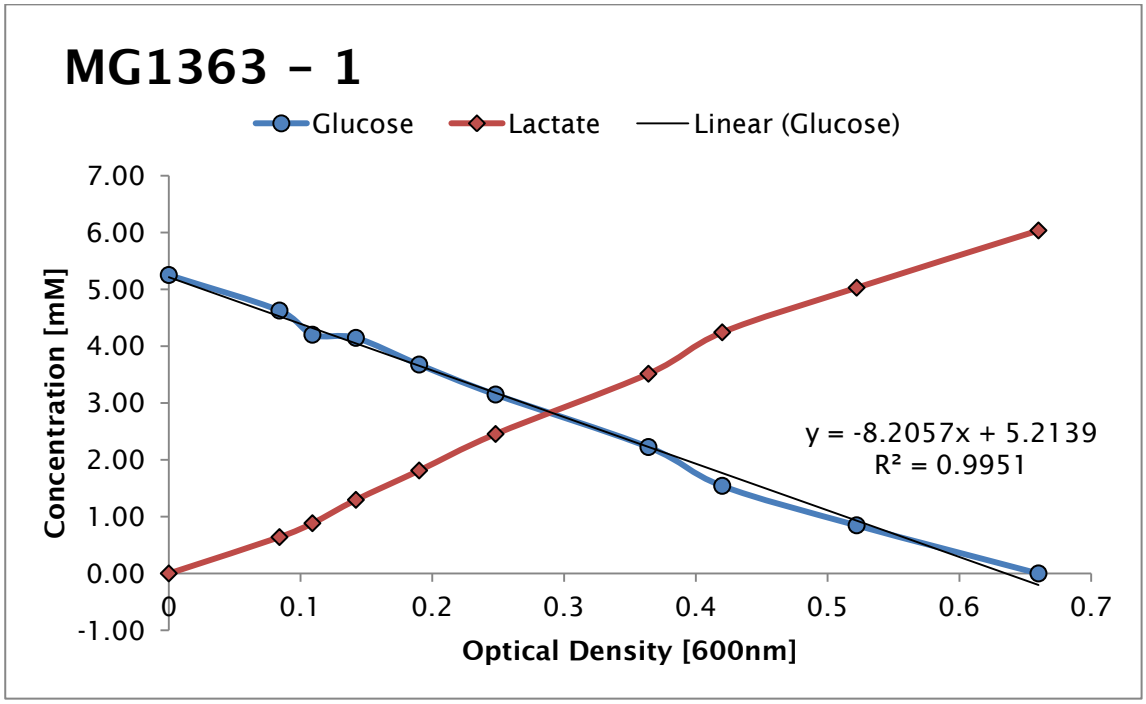
Experiment: Growth 03 – glucose without glycerol.

Contain.	**Strain**	**Alias**	**Medium**
Kolbe 1:	AH 64	MG1363	SAL + 0,1% glucose + 5 µg/mL Hemin
Kolbe 2:	AH 64	MG1363	SAL + 0,1% glucose + 5 µg/mL Hemin
Kolbe 3:	AH 64	MG1363	SAL + 0,1% glucose + 5 µg/mL Hemin
Kolbe 4:	AH 64	MG1363	SAL + 0,1% glucose + 5 µg/mL Hemin

Aver. T_d	SD T_d	Aver. µ	SD µ
53,53	0,24	0,78	0,01

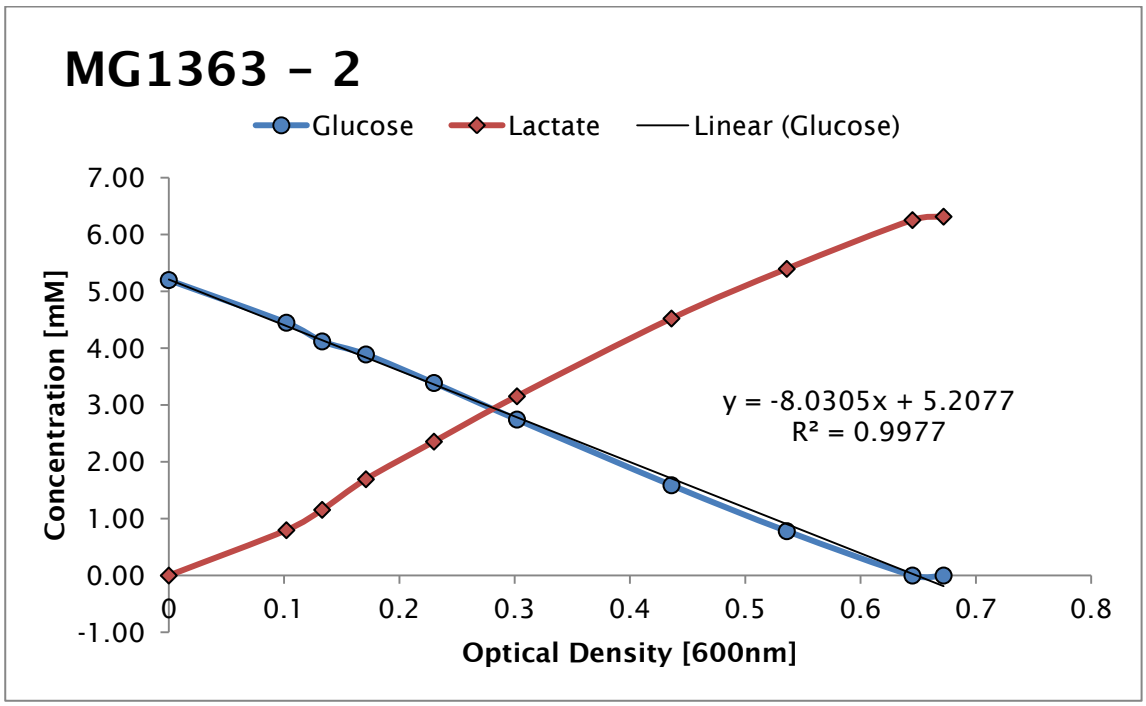
APPENDIX D

		mM	mM	mM	mM	mM	mM
Sample	OD600	Glucose	Lactate	Glycerol	Formate	Acetate	Ethanol
G03 K1P0	0	5.25	n.a.	n.a.	n.a.	n.a.	n.a.
G03 K1P1	0.084	4.63	0.64	n.a.	n.a.	n.a.	n.a.
G03 K1P2	0.109	4.20	0.88	n.a.	n.a.	n.a.	n.a.
G03 K1P3	0.142	4.15	1.30	n.a.	n.a.	n.a.	n.a.
G03 K1P4	0.190	3.68	1.81	n.a.	n.a.	n.a.	n.a.
G03 K1P5	0.248	3.15	2.45	n.a.	n.a.	n.a.	n.a.
G03 K1P6	0.364	2.22	3.51	n.a.	n.a.	n.a.	n.a.
G03 K1P7	0.420	1.54	4.24	n.a.	n.a.	n.a.	n.a.
G03 K1P8	0.522	0.85	5.03	n.a.	n.a.	n.a.	n.a.
G03 K1P9	0.660	n.a.	6.04	n.a.	n.a.	n.a.	n.a.



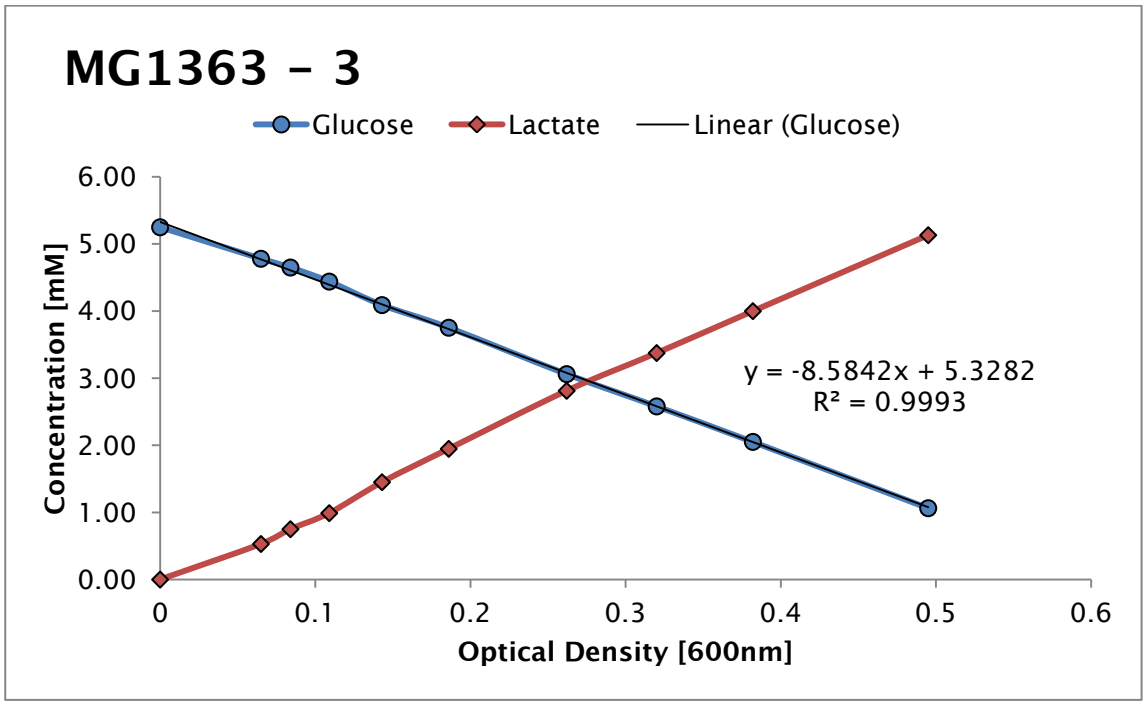
APPENDIX D

		mM	mM	mM	mM	mM	mM
Sample	OD600	Glucose	Lactate	Glycerol	Formate	Acetate	Ethanol
G03 K2P0	0	5.20	n.a.	145.95	n.a.	n.a.	n.a.
G03 K2P1	0.102	4.45	0.80	131.07	n.a.	n.a.	n.a.
G03 K2P2	0.133	4.12	1.16	145.67	n.a.	n.a.	n.a.
G03 K2P3	0.171	3.89	1.69	143.45	n.a.	n.a.	n.a.
G03 K2P4	0.230	3.39	2.36	147.25	n.a.	0.2213	n.a.
G03 K2P5	0.302	2.75	3.15	149.89	n.a.	0.6903	n.a.
G03 K2P6	0.436	1.59	4.52	147.78	n.a.	1.0496	n.a.
G03 K2P7	0.536	0.78	5.40	123.66	n.a.	1.242	n.a.
G03 K2P8	0.645	n.a.	6.25	147.16	n.a.	n.a.	n.a.
G03 K2P9	0.672	n.a.	6.32		n.a.	n.a.	n.a.



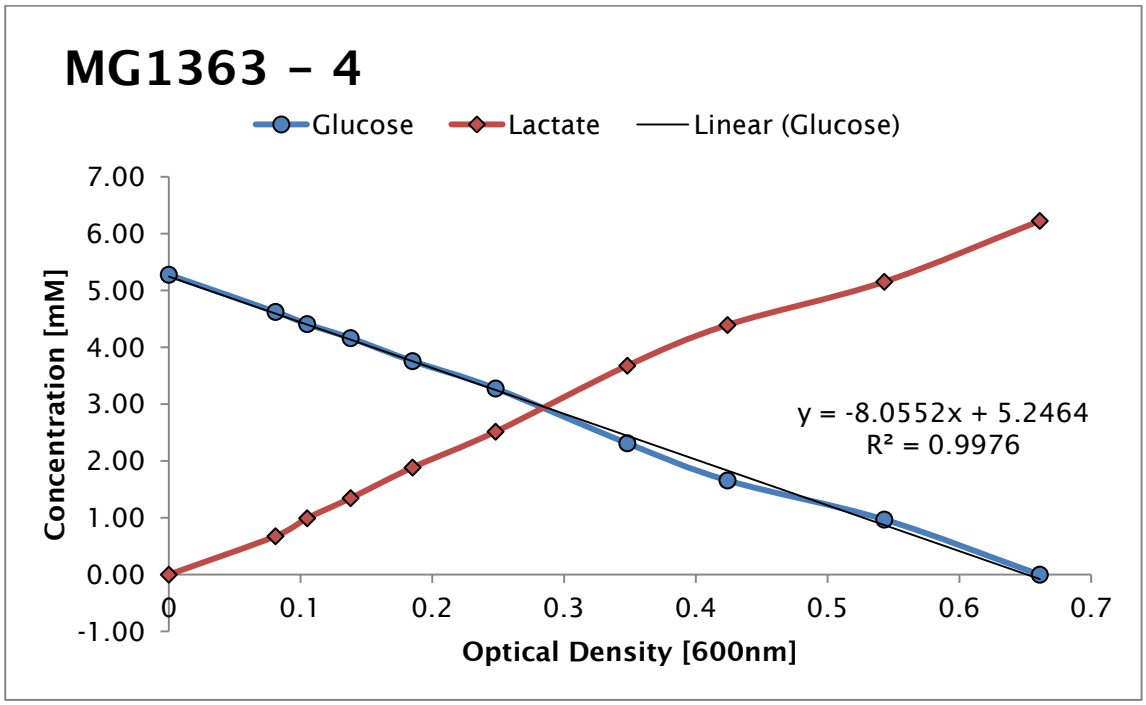
APPENDIX D

		mM	mM	mM	mM	mM	mM
Sample	OD600	Glucose	Lactate	Glycerol	Formate	Acetate	Ethanol
G03 K3P0	0	5.25	n.a.	n.a.	n.a.	n.a.	n.a.
G03 K3P1	0.065	4.78	0.53	n.a.	n.a.	n.a.	n.a.
G03 K3P2	0.084	4.65	0.75	n.a.	n.a.	n.a.	n.a.
G03 K3P3	0.109	4.44	0.99	n.a.	n.a.	n.a.	n.a.
G03 K3P4	0.143	4.09	1.45	n.a.	n.a.	n.a.	n.a.
G03 K3P5	0.186	3.75	1.95	n.a.	n.a.	n.a.	n.a.
G03 K3P6	0.262	3.06	2.81	n.a.	n.a.	0.294	n.a.
G03 K3P7	0.320	2.58	3.38	n.a.	n.a.	0.7929	n.a.
G03 K3P8	0.382	2.05	4.00	n.a.	n.a.	0.9502	n.a.
G03 K3P9	0.495	1.06	5.13	n.a.	n.a.	1.3559	n.a.



APPENDIX D

		mM	mM	mM	mM	mM	mM
Sample	OD600	Glucose	Lactate	Glycerol	Formate	Acetate	Ethanol
G03 K4P0	0	5.28	n.a.	n.a.	n.a.	n.a.	n.a.
G03 K4P1	0.081	4.62	0.68	n.a.	n.a.	n.a.	n.a.
G03 K4P2	0.105	4.41	0.99	n.a.	n.a.	n.a.	n.a.
G03 K4P3	0.138	4.16	1.35	n.a.	n.a.	n.a.	n.a.
G03 K4P4	0.185	3.76	1.89	n.a.	n.a.	n.a.	n.a.
G03 K4P5	0.248	3.28	2.52	n.a.	n.a.	0.1929	n.a.
G03 K4P6	0.348	2.31	3.68	n.a.	n.a.	0.7652	n.a.
G03 K4P7	0.424	1.66	4.39	n.a.	n.a.	1.1512	n.a.
G03 K4P8	0.543	0.97	5.15	n.a.	n.a.	1.1444	n.a.
G03 K4P9	0.661	n.a.	6.23	n.a.	n.a.	1.8259	n.a.



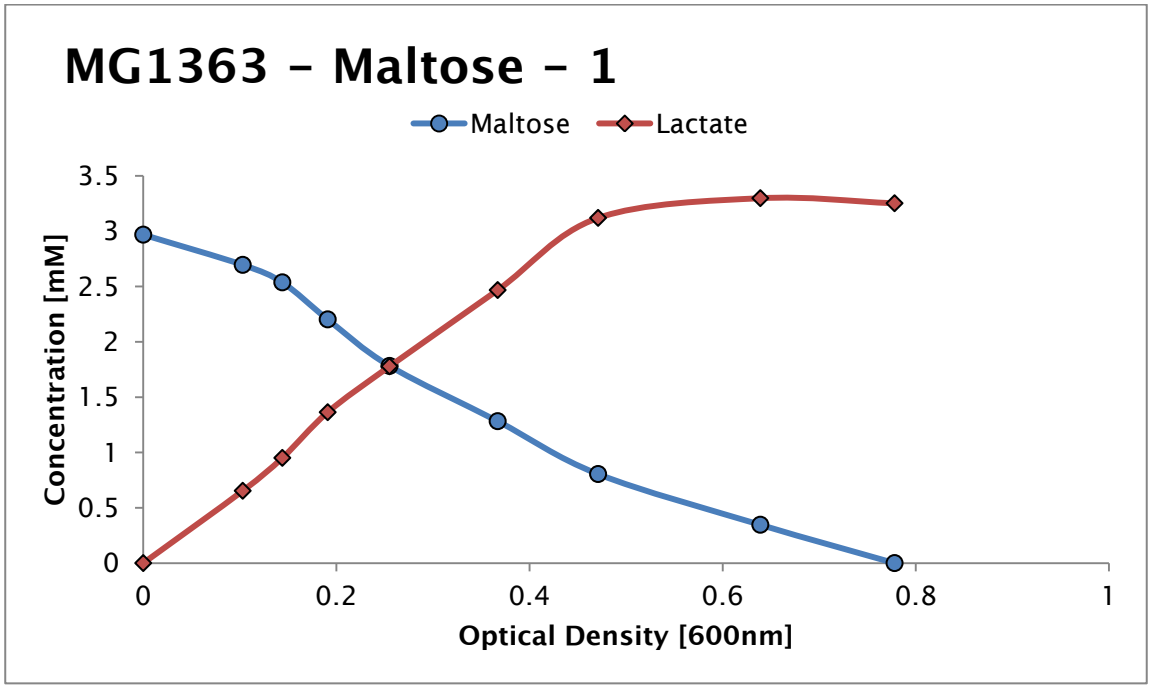
APPENDIX D

Experiment: Growth 04 – maltose with and without glycerol.

Contain.	Strain	Alias	Genetics	Medium
Kolbe 1:	AH 64	MG 1363	WT	SAL + 0,1% maltose + 5 µg/mL hemin
Kolbe 2:	AH 64	MG 1363	WT	SAL + 0,1% maltose + 5 µg/mL hemin
Kolbe 3:	AH 64	MG 1363	WT	SAL + 0,1% maltose + 5 µg/mL hemin
Kolbe 4:	AH 64	MG 1363	WT	SAL + 0,1% maltose + 5 µg/mL hemin
Kolbe 5:	AH 64	MG 1363	WT	SAL + 0,1% maltose + 1% glycerol + 5 µg/mL hemin
Kolbe 6:	AH 64	MG 1363	WT	SAL + 0,1% maltose + 1% glycerol + 5 µg/mL hemin
Kolbe 7:	AH 64	MG 1363	WT	SAL + 0,1% maltose + 1% glycerol + 5 µg/mL hemin
Kolbe 8:	AH 64	MG 1363	WT	SAL + 0,1% maltose + 1% glycerol + 5 µg/mL hemin

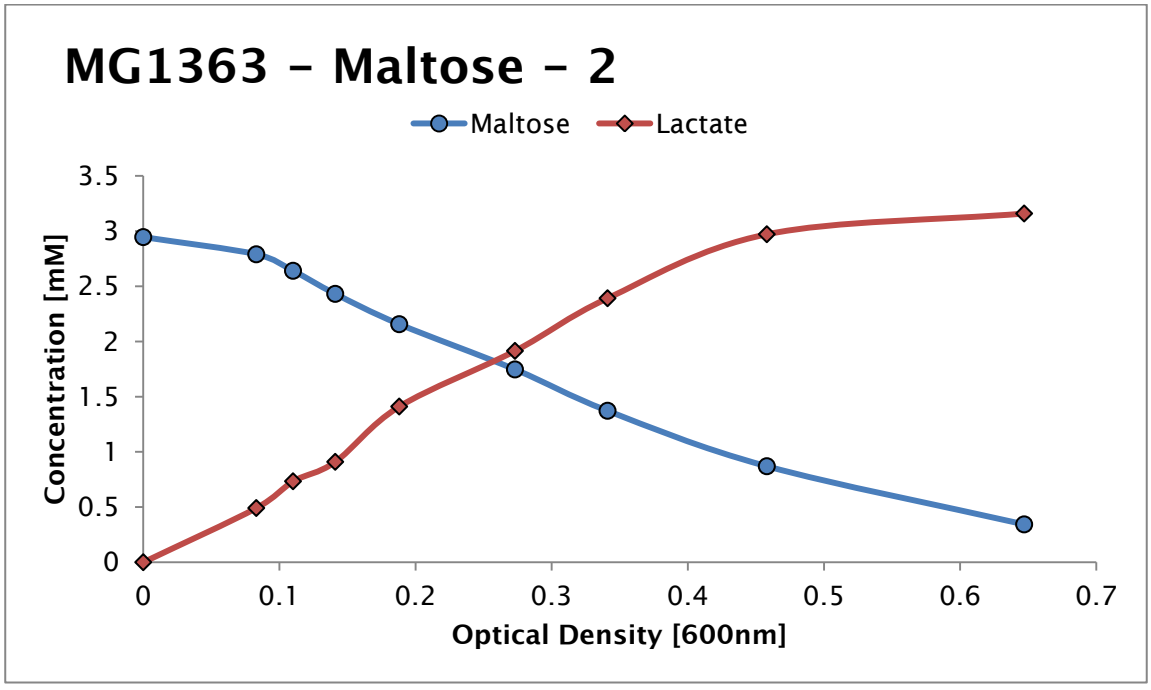
APPENDIX D

		mM	mM	mM	mM	mM	mM
Sample	OD600	Maltose	Lactate	Glycerol	Formate	Acetate	Ethanol
G04 K1P0	0	2.9685	n.a.	n.a.	n.a.	n.a.	n.a.
G04 K1P1	0.103	2.6954	0.6535	n.a.	n.a.	0.6723	n.a.
G04 K1P2	0.144	2.5373	0.9509	n.a.	n.a.	1.1425	n.a.
G04 K1P3	0.191	2.2027	1.3636	n.a.	n.a.	1.5563	n.a.
G04 K1P4	0.255	1.7819	1.7783	n.a.	n.a.	2.3001	n.a.
G04 K1P5	0.367	1.2826	2.4677	n.a.	n.a.	3.3815	n.a.
G04 K1P6	0.471	0.8049	3.1198	n.a.	n.a.	4.4085	n.a.
G04 K1P7	0.639	0.3465	3.2984	n.a.	n.a.	5.664	n.a.
G04 K1P8	0.778	n.a.	3.2523	n.a.	n.a.	6.6182	n.a.



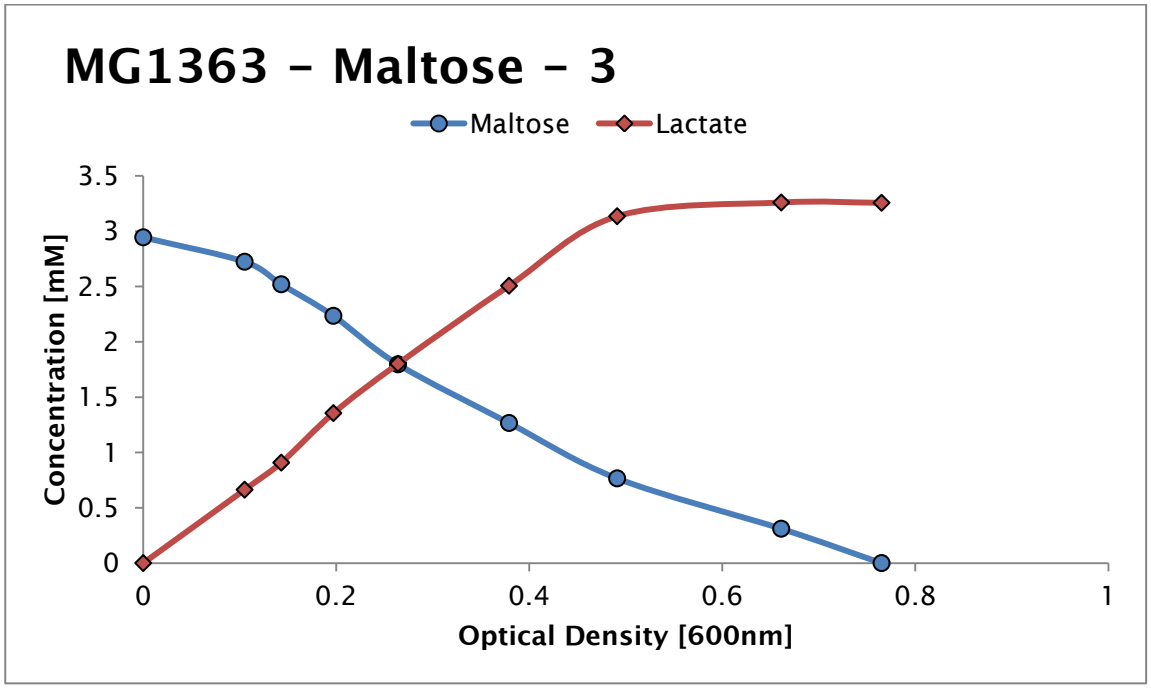
APPENDIX D

		mM	mM	mM	mM	mM	mM
Sample	OD600	Maltose	Lactate	Glycerol	Formate	Acetate	Ethanol
G04 K2P0	0	2.945	n.a.	n.a.	n.a.	n.a.	n.a.
G04 K2P1	0.083	2.7892	0.4908	n.a.	n.a.	0.5876	n.a.
G04 K2P2	0.110	2.6398	0.7332	n.a.	n.a.	0.846	n.a.
G04 K2P3	0.141	2.4308	0.9093	n.a.	n.a.	1.0819	n.a.
G04 K2P4	0.188	2.1561	1.4104	n.a.	n.a.	1.8593	n.a.
G04 K2P5	0.273	1.7463	1.9143	n.a.	n.a.	2.5423	n.a.
G04 K2P6	0.341	1.3718	2.3912	n.a.	n.a.	3.3137	n.a.
G04 K2P7	0.458	0.8694	2.971	n.a.	n.a.	4.2283	n.a.
G04 K2P8	0.647	0.3427	3.159	n.a.	n.a.	5.4664	n.a.



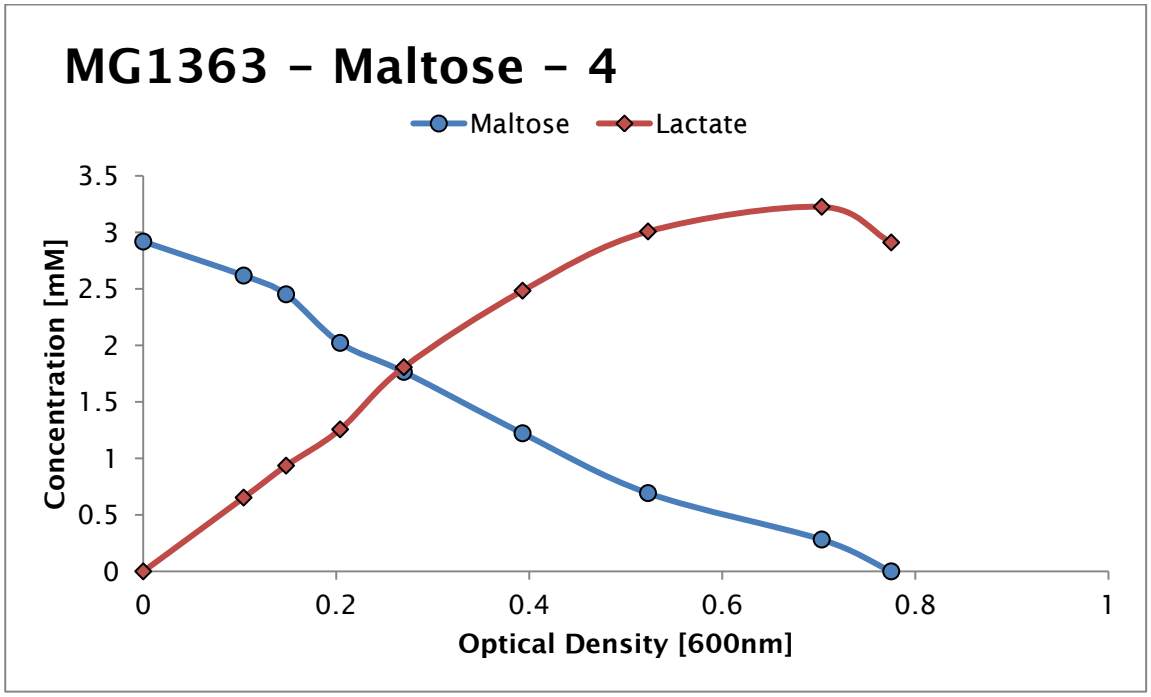
APPENDIX D

		mM	mM	mM	mM	mM	mM
Sample	OD600	Maltose	Lactate	Glycerol	Formate	Acetate	Ethanol
G04 K3P0	0	2.9443	n.a.	n.a.	n.a.	n.a.	n.a.
G04 K3P1	0.105	2.7236	0.6631	n.a.	n.a.	0.5929	n.a.
G04 K3P2	0.143	2.5198	0.908	n.a.	n.a.	1.1385	n.a.
G04 K3P3	0.197	2.2348	1.3555	n.a.	n.a.	1.7684	n.a.
G04 K3P4	0.264	1.7963	1.8018	n.a.	n.a.	2.466	n.a.
G04 K3P5	0.379	1.2661	2.5066	n.a.	n.a.	3.5127	n.a.
G04 K3P6	0.491	0.7657	3.1351	n.a.	n.a.	4.5463	n.a.
G04 K3P7	0.661	0.3107	3.2592	n.a.	n.a.	5.7534	n.a.
G04 K3P8	0.765	n.a.	3.2563	n.a.	n.a.	6.751	n.a.



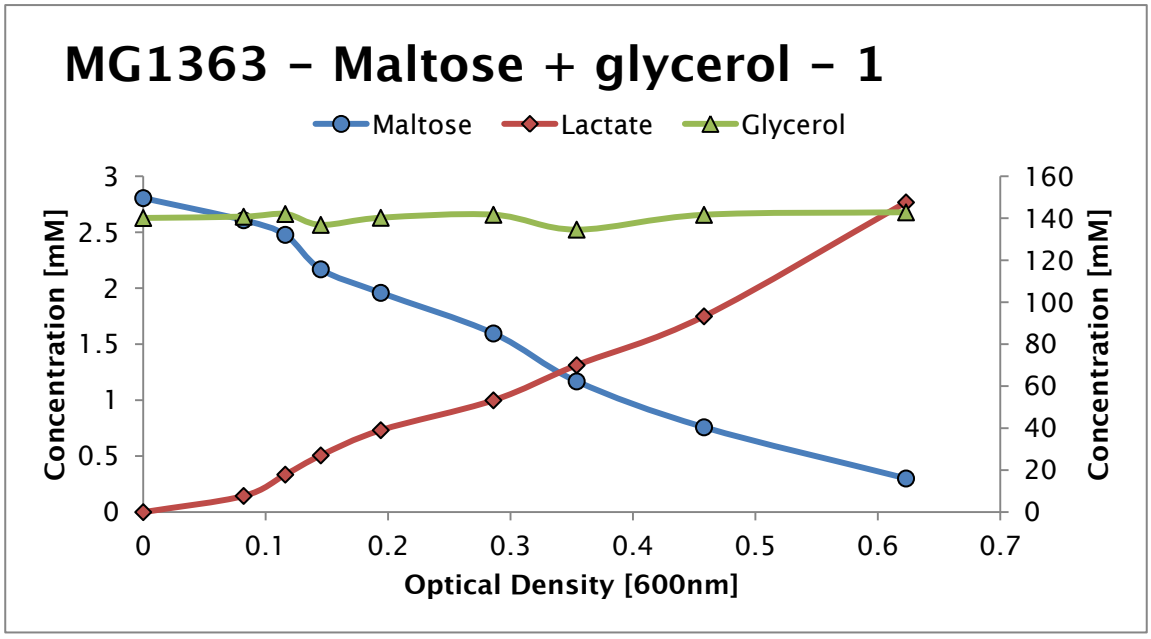
APPENDIX D

		mM	mM	mM	mM	mM	mM
Sample	OD600	Maltose	Lactate	Glycerol	Formate	Acetate	Ethanol
G04 K4P0	0	2.9184	n.a.	n.a.	n.a.	n.a.	n.a.
G04 K4P1	0.104	2.6174	0.653	n.a.	n.a.	0.7601	n.a.
G04 K4P2	0.148	2.4514	0.9355	n.a.	n.a.	1.2065	n.a.
G04 K4P3	0.204	2.0218	1.2563	n.a.	n.a.	1.6115	n.a.
G04 K4P4	0.270	1.7654	1.8069	n.a.	n.a.	2.4548	n.a.
G04 K4P5	0.393	1.2219	2.4837	n.a.	n.a.	3.5321	n.a.
G04 K4P6	0.523	0.6926	3.0079	n.a.	n.a.	4.4451	n.a.
G04 K4P7	0.703	0.2818	3.2262	n.a.	n.a.	5.8639	n.a.
G04 K4P8	0.775	n.a.	2.9105	n.a.	n.a.	6.0905	n.a.



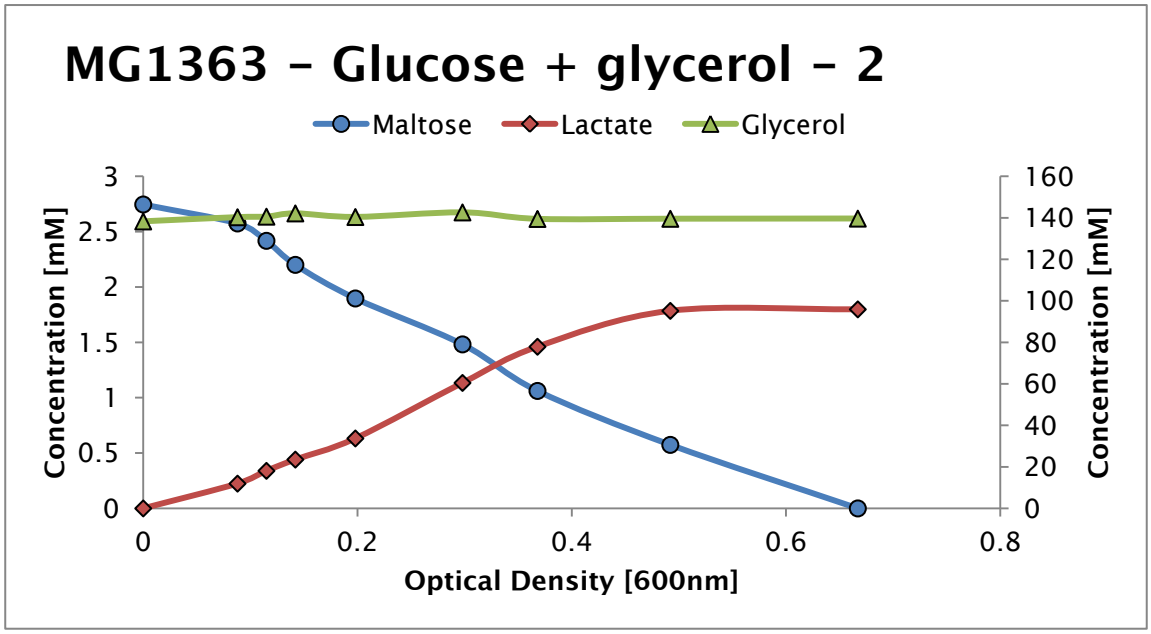
APPENDIX D

		mM	mM	mM	mM	mM	mM
Sample	OD600	Maltose	Lactate	Glycerol	Formate	Acetate	Ethanol
G04 K5P0	0	2.8054	n.a.	140.1603	n.a.	n.a.	n.a.
G04 K5P1	0.082	2.6081	0.1447	140.7857	n.a.	n.a.	n.a.
G04 K5P2	0.116	2.4774	0.3351	142.0676	n.a.	0.5208	n.a.
G04 K5P3	0.145	2.1695	0.5069	136.8945	n.a.	1.1843	n.a.
G04 K5P4	0.194	1.9591	0.7321	140.264	n.a.	1.7064	n.a.
G04 K5P5	0.286	1.5963	0.999	141.6949	n.a.	2.4831	n.a.
G04 K5P6	0.354	1.1682	1.3124	134.6202	n.a.	3.3886	n.a.
G04 K5P7	0.458	0.758	1.7493	141.6658	n.a.	4.5161	n.a.
G04 K5P8	0.623	0.3006	2.7673	142.8575	n.a.	5.8613	n.a.



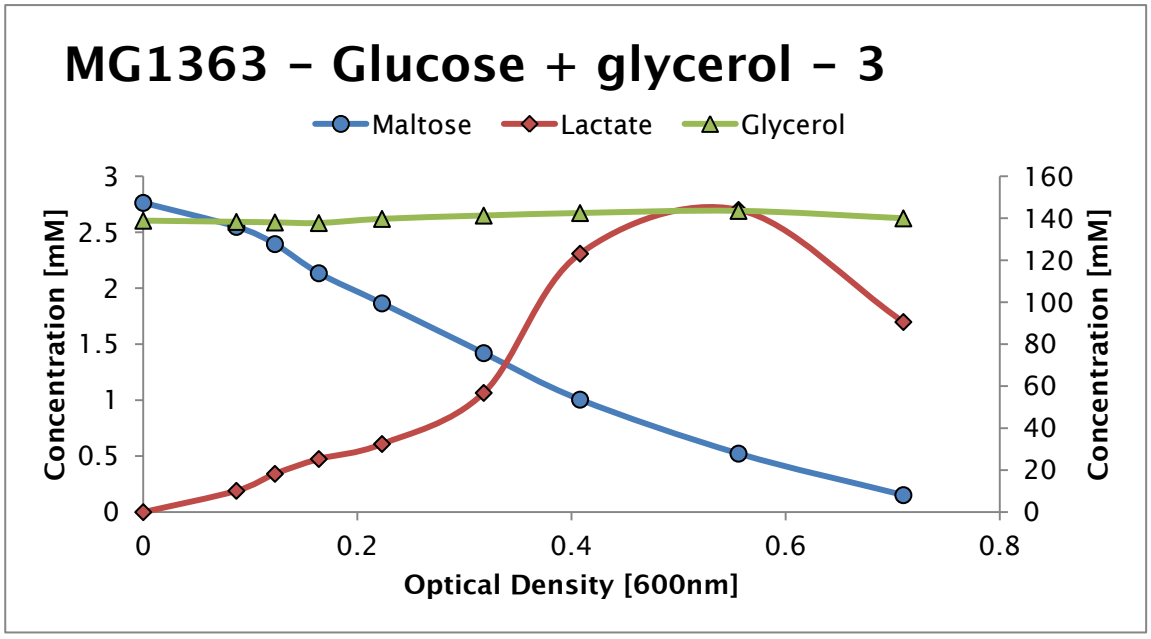
APPENDIX D

		mM	mM	mM	mM	mM	mM
Sample	OD600	Maltose	Lactate	Glycerol	Formate	Acetate	Ethanol
G04 K6P0	0	2.7457	n.a.	138.2757	n.a.	n.a.	n.a.
G04 K6P1	0.088	2.5745	0.2238	140.314	n.a.	n.a.	n.a.
G04 K6P2	0.115	2.4176	0.3393	140.5768	n.a.	0.6361	n.a.
G04 K6P3	0.142	2.2003	0.4411	142.1424	n.a.	1.3765	n.a.
G04 K6P4	0.198	1.8967	0.6322	140.4135	n.a.	1.9242	n.a.
G04 K6P5	0.298	1.4819	1.1338	142.6165	n.a.	2.9611	n.a.
G04 K6P6	0.368	1.0614	1.4604	139.4777	n.a.	3.7193	n.a.
G04 K6P7	0.492	0.5741	1.7847	139.5443	n.a.	4.7446	n.a.
G04 K6P8	0.667	n.a.	1.7981	139.6447	n.a.	5.9573	n.a.



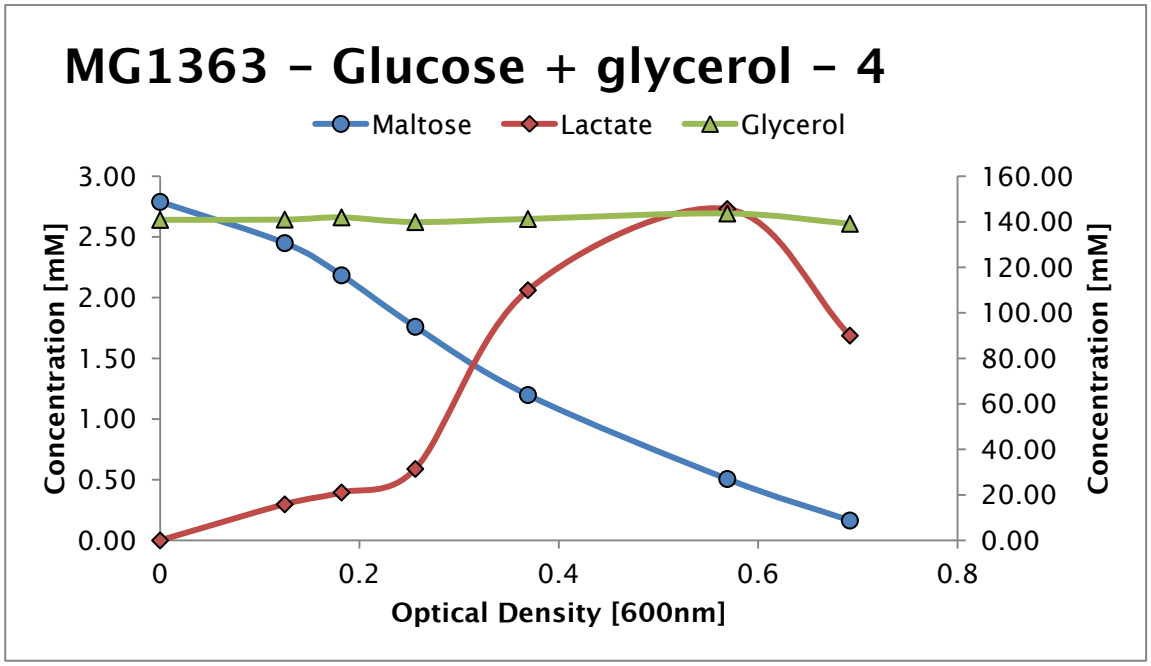
APPENDIX D

		mM	mM	mM	mM	mM	mM
Sample	OD600	Maltose	Lactate	Glycerol	Formate	Acetate	Ethanol
G04 K7P0	0	2.7629	n.a.	138.8586	n.a.	n.a.	n.a.
G04 K7P1	0.087	2.5484	0.1905	138.2967	n.a.	n.a.	n.a.
G04 K7P2	0.123	2.3947	0.342	138.0249	n.a.	1.0095	n.a.
G04 K7P3	0.164	2.1342	0.4746	137.7373	n.a.	1.3369	n.a.
G04 K7P4	0.223	1.8651	0.6088	139.721	n.a.	2.1488	n.a.
G04 K7P5	0.318	1.4203	1.0653	141.2958	n.a.	3.0866	n.a.
G04 K7P6	0.408	1.005	2.309	142.5051	n.a.	4.054	n.a.
G04 K7P7	0.556	0.5227	2.7002	143.5436	n.a.	5.1805	n.a.
G04 K7P8	0.710	0.152	1.6988	139.9812	n.a.	6.1769	n.a.



APPENDIX D

		mM	mM	mM	mM	mM	mM
Sample	OD600	Maltose	Lactate	Glycerol	Formate	Acetate	Ethanol
G04 K8P0	0	2.79	n.a.	140.90	n.a.	n.a.	n.a.
G04 K8P1	0.125	2.45	0.30	140.99	n.a.	0.84	n.a.
G04 K8P2	0.182	2.18	0.40	141.98	n.a.	1.47	n.a.
G04 K8P3	0.256	1.76	0.59	139.90	n.a.	2.12	n.a.
G04 K8P4	0.369	1.20	2.06	141.28	n.a.	3.49	n.a.
G04 K8P5	0.569	0.51	2.73	143.70	n.a.	5.07	n.a.
G04 K8P6	0.692	0.16	1.69	139.17	n.a.	5.98	n.a.
G04 K8P7	0.743	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
G04 K8P8	0.773	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.



Appendix E

Quality control data and results from microarray analysis of chip 1 and 2

E.1 – Results from the differential gene expression analysis of chip 2.

E.2 – Merged tables of the differential gene expression analysis from chip 1 and chip 2.

APPENDIX E

E.1 – Results from the differential gene expression analysis of chip 2.

This section contains all the results from the analysis of differential gene expression from chip 2 (together with the results from chip 1) sorted by pathways and regulatory groups.

APPENDIX E

Trancsription factors

Gene/locus	BK1506		BK1503		Annotation
	logFC	p-value	logFC	p-value	
adaA	-0.24	1.7E-02	-0.11	2.9E-01	AraC family transcriptional regulator, regulatory protein of adaptative response / methylphosphotriester-DNA alkyltransferase methyltransferase [EC:2.1.1.-]
fruR	0.04	7.2E-01	0.34	3.6E-02	lactose transport regulator, DeoR family transcriptional regulator, fructose operon transcriptional repressor
malR	-0.17	5.4E-02	-0.09	3.6E-01	maltose operon transcriptional repressor
ccpA	-0.10	3.8E-01	-0.23	1.3E-01	catabolite control protein A
rbsR	0.07	5.2E-01	-0.19	1.9E-01	ribose operon repressor
llmg_0956	-0.05	4.7E-01	0.15	8.4E-02	LacI family transcription regulator
treR	0.15	7.1E-02	0.16	1.1E-01	trehalose operon transcriptional repressor
rgrB	-0.12	1.1E-01	0.22	3.0E-02	GntR family transcriptional regulator
rmaG	-0.20	1.8E-02	-0.28	1.2E-02	MarR family transcriptional regulator
glnR	0.02	7.7E-01	-0.04	6.1E-01	glutamine synthetase repressor
fur	-0.35	1.9E-02	-0.02	9.0E-01	ferric uptake regulation protein
flpB	0.07	3.7E-01	0.05	5.4E-01	transcriptional regulator FNR like protein B
flpA	-0.44	2.5E-03	-0.54	2.8E-03	FNR like protein A
llmg_1224	-0.43	1.6E-03	-0.61	9.0E-04	transcriptional regulator
llmg_0709	-0.44	4.8E-04	-0.42	2.3E-03	PadR-like family transcriptional regulator
llmg_2339	-0.20	1.2E-01	-0.35	5.1E-02	transcriptional regulator
arsR	-0.06	5.1E-01	-0.28	3.7E-02	regulator of arsenical resistance
birA1	-0.32	5.6E-03	-0.33	1.4E-02	biotin--[acetyl-CoA-carboxylase] ligase and biotin operon repressor (EC:6.3.4.15)
birA2	0.04	6.0E-01	0.18	7.9E-02	acetyl-CoA carboxylase ligase / biotin operon repressor bifunctional protein (EC:6.3.4.
codY	-0.07	2.1E-01	-0.01	8.4E-01	transcriptional repressor CodY
ps602	-0.02	8.0E-01	-0.01	8.8E-01	hypothetical protein
mtlR	0.36	1.5E-03	0.15	1.2E-01	transcriptional regulator mtl operon MtlR
ahrC	0.12	1.4E-01	0.19	7.6E-02	arginine transcriptional regulator
argR	0.35	4.5E-03	0.75	2.6E-04	arginine repressor
cspE	0.04	6.7E-01	0.12	3.4E-01	cold shock-like protein CspE
cspD2	0.13	4.5E-01	0.15	5.0E-01	cold shock-like protein cspD2
cspB	-0.15	5.9E-01	-0.39	2.8E-01	cold shock-like protein CspB
hrcA	-0.09	5.7E-01	0.09	6.4E-01	heat-inducible transcription repressor
tenA	-0.23	7.1E-03	-0.27	1.1E-02	transcriptional activator TenA
ctsR	0.09	1.4E-01	0.17	4.5E-02	transcriptional regulator CtsR
parA	-0.06	4.1E-01	-0.17	7.9E-02	chromosome partitioning protein ParA
pyrR	-0.03	6.8E-01	0.03	6.7E-01	bifunctional pyrimidine regulatory protein PyrR uracil phosphoribosyltransferase
nrdR	0.10	1.3E-01	0.14	1.2E-01	transcriptional regulator NrdR
purR	-0.17	2.1E-02	-0.22	2.3E-02	pur operon repressor
comX	-0.05	6.5E-01	-0.10	4.8E-01	competence regulator ComX
rpoD	-0.15	5.2E-02	-0.26	1.6E-02	RNA polymerase sigma factor RpoD
ptsH	0.01	8.74E-01	-0.10	2.19E-01	phosphocarrier protein HPr

APPENDIX E

Glycolysis

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
glk	0.03	6.8E-01	0.02	8.5E-01	glucokinase
pgi	-0.02	8.0E-01	-0.10	2.3E-01	glucose-6-phosphate isomerase
pfk	0.01	9.1E-01	-0.11	1.7E-01	6-phosphofructokinase
pyk	-0.04	5.6E-01	-0.10	2.8E-01	pyruvate kinase
ldh	-0.05	4.2E-01	-0.62	1.1E-04	L-lactate dehydrogenase
fbp	0.19	7.1E-02	0.39	1.3E-02	fructose-bisphosphatase
fbaA	0.04	4.8E-01	0.04	5.7E-01	fructose-bisphosphate aldolase
tpiA	-0.33	9.0E-03	-0.64	9.8E-04	triosephosphate isomerase
gapB	-0.04	5.6E-01	-0.10	2.8E-01	glyceraldehyde 3-phosphate dehydrogenase
gapA	-0.28	1.0E-02	-0.21	7.7E-02	glyceraldehyde 3-phosphate dehydrogenase
pgk	-0.06	3.2E-01	-0.07	3.2E-01	phosphoglycerate kinase
llmg_1894	0.19	7.0E-02	0.07	5.6E-01	phosphoglycerate mutase family protein
gpmA	-0.09	2.1E-01	-0.05	5.5E-01	phosphoglyceromutase
gpmB	0.22	3.3E-02	0.28	3.3E-02	phosphoglycerate mutase
gpmC	0.26	1.5E-02	0.13	2.4E-01	phosphoglycerate mutase
eno	0.05	4.1E-01	-0.07	4.2E-01	phosphopyruvate hydratase
pdhA	0.07	2.6E-01	0.08	2.9E-01	pyruvate dehydrogenase E1 component alpha subunit
pdhB	0.03	6.7E-01	-0.08	3.2E-01	pyruvate dehydrogenase E1 component beta subunit
pdhC	-0.02	7.1E-01	0.07	4.1E-01	pyruvate dehydrogenase complex E2 component
pdhD	0.12	1.7E-01	0.12	2.6E-01	dihydrolipoamide dehydrogenase
ldhX	-0.17	2.4E-02	-0.34	3.2E-03	L-lactate dehydrogenase
ldhB	0.08	3.1E-01	0.15	1.7E-01	L-lactate dehydrogenase
adhE	0.49	6.0E-02	0.64	5.5E-02	bifunctional acetaldehyde-CoA/alcohol dehydrogenase
adhA, adhP	0.20	1.5E-01	0.30	1.0E-01	alcohol dehydrogenase
llmg_0955	0.11	1.7E-01	0.06	5.6E-01	alcohol dehydrogenase
fadD	-0.10	4.1E-01	-0.06	6.9E-01	long-chain acyl-CoA synthetase
galM	0.13	1.7E-01	0.28	3.7E-02	aldose 1-epimerase
bglA2	0.17	2.2E-02	0.23	1.6E-02	6-phospho-beta-glucosidase
arb	0.12	4.6E-01	0.35	1.1E-01	6-phospho-beta-glucosidase
celA	0.28	2.3E-03	0.34	3.1E-03	6-phospho-beta-glucosidase

APPENDIX E

TCA cycle

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
gltA	0.26	2.2E-02	0.33	2.1E-02	citrate synthase
citB	0.15	1.5E-01	0.40	1.2E-02	aconitate hydratase
icd	0.25	3.6E-03	0.41	1.0E-03	isocitrate dehydrogenase
pdhD	0.12	1.7E-01	0.12	2.6E-01	dihydrolipoamide dehydrogenase
frdC	-0.09	4.4E-01	-0.07	6.1E-01	fumarate reductase flavoprotein subunit
pycA	-0.06	3.6E-01	-0.25	1.3E-02	pyruvate carboxylase
pdhA	0.07	2.6E-01	0.08	2.9E-01	pyruvate dehydrogenase E1 component alpha subunit
pdhB	0.03	6.7E-01	-0.08	3.2E-01	pyruvate dehydrogenase E1 component beta subunit
pdhC	-0.02	7.1E-01	0.07	4.1E-01	pyruvate dehydrogenase complex E2 component

APPENDIX E

Pentose phosphate pathway

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
pgi	-0.02	8.0E-01	-0.10	2.3E-01	glucose-6-phosphate isomerase
llmg_2499	0.07	3.1E-01	0.11	2.4E-01	glucose-6-phosphate 1-dehydrogenase
llmg_2431	0.30	9.5E-03	0.53	1.7E-03	hypothetical protein
gntZ	-0.02	7.9E-01	0.03	7.2E-01	6-phosphogluconate dehydrogenase-like protein
gnd	-0.02	8.3E-01	-0.21	5.1E-02	6-phosphogluconate dehydrogenase
rpe	0.15	9.4E-02	0.22	7.0E-02	ribulose-phosphate 3-epimerase
rpe2	-0.17	3.1E-02	-0.20	4.3E-02	ribulose-phosphate 3-epimerase
tkt	0.11	2.3E-01	0.11	3.2E-01	transketolase
rpiA	-0.25	3.9E-02	-0.38	2.1E-02	ribose-5-phosphate isomerase A
rpiB	-0.11	3.1E-01	-0.13	3.3E-01	ribose-5-phosphate isomerase B
deoC	0.05	4.7E-01	-0.02	7.9E-01	deoxyribose-phosphate aldolase
rbsK	0.17	6.8E-02	0.03	7.9E-01	ribokinase
deoB	0.01	9.0E-01	0.01	8.6E-01	phosphopentomutase
prsB	-0.22	8.1E-03	-0.30	5.5E-03	ribose-phosphate pyrophosphokinase
prsA	-0.03	6.3E-01	0.05	5.5E-01	ribose-phosphate pyrophosphokinase
kdgA	0.22	8.4E-03	0.40	1.3E-03	keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase
gntK	0.13	7.5E-02	0.05	5.6E-01	gluconate kinase
fbaA	0.04	4.8E-01	0.04	5.7E-01	fructose-bisphosphate aldolase
fbp	0.19	7.1E-02	0.39	1.3E-02	fructose-bisphosphatase
pfk	0.01	9.1E-01	-0.11	1.7E-01	6-phosphofructokinase

APPENDIX E

Pyruvate metabolism

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
fadD	-0.10	4.1E-01	-0.06	6.9E-01	long-chain acyl-CoA synthetase
pdhA	0.07	2.6E-01	0.08	2.9E-01	pyruvate dehydrogenase E1 component alpha subunit
pdhB	0.03	6.7E-01	-0.08	3.2E-01	pyruvate dehydrogenase E1 component beta subunit
pdhC	-0.02	7.1E-01	0.07	4.1E-01	pyruvate dehydrogenase complex E2 component
pdhD	0.12	1.7E-01	0.12	2.6E-01	dihydrolipoamide dehydrogenase
pfl	0.05	4.4E-01	0.04	6.2E-01	formate acetyltransferase
adhE	0.49	6.0E-02	0.64	5.5E-02	bifunctional acetaldehyde-CoA/alcohol dehydrogenase
ackA2	0.15	2.8E-01	0.23	2.0E-01	acetate kinase
ackA1	-0.09	4.4E-01	0.03	8.5E-01	AckA1 protein
eutD	-0.47	1.4E-02	-0.61	1.2E-02	phosphotransacetylase
pyk	-0.04	5.6E-01	-0.10	2.8E-01	pyruvate kinase
accA	-0.05	5.5E-01	-0.10	3.2E-01	AccA protein
accB	0.02	7.2E-01	-0.10	2.1E-01	acetyl-CoA carboxylase biotin carboxyl carrier protein subunit
accC	0.05	4.8E-01	0.05	5.1E-01	acetyl-CoA carboxylase biotin carboxylase subunit
accD	0.03	6.1E-01	0.06	4.2E-01	acetyl-CoA carboxylase subunit beta
llmg_0568	0.39	5.5E-04	0.45	9.3E-04	acylphosphatase
poxL	0.19	6.2E-02	0.12	2.8E-01	pyruvate oxidase
ldhX	-0.17	2.4E-02	-0.34	3.2E-03	L-lactate dehydrogenase
ldhB	0.08	3.1E-01	0.15	1.7E-01	L-lactate dehydrogenase
ldh	-0.05	4.2E-01	-0.62	1.1E-04	L-lactate dehydrogenase
llmg_0184	-0.21	1.6E-01	-0.14	4.3E-01	putative lactoylglutathione lyase
mleS	-0.13	1.2E-01	-0.26	2.9E-02	malate dehydrogenase
pycA	-0.06	3.6E-01	-0.25	1.3E-02	pyruvate carboxylase
thiL	-0.34	2.2E-03	-0.28	1.6E-02	ThiL protein

APPENDIX E

Amino sugar and nucleotide sugar metabolism

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
chiC	0.43	2.5E-03	0.50	3.8E-03	acidic endochitinase precursor
nagZ	0.04	6.7E-01	-0.01	9.6E-01	putative beta-N-acetylglucosaminidase
murQ, yfeU	0.15	5.8E-02	-0.10	2.6E-01	N-acetylmuramic acid-6-phosphate etherase
glmU	0.20	7.6E-02	0.27	6.1E-02	bifunctional N-acetylglucosamine-1-phosphate uridyltransferase/glucosamine-1-phosphate acetyltransferase
llmg_1317	-0.04	8.3E-01	0.17	4.5E-01	N-acetylmannosamine-6-phosphate 2-epimerase
murA2	-0.14	2.2E-01	-0.12	3.8E-01	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
murA1	-0.22	8.0E-03	-0.27	1.0E-02	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
murB	0.01	8.8E-01	0.02	8.0E-01	UDP-N-acetylenolpyruvoylglucosamine reductase
glmM	-0.30	1.2E-03	-0.32	3.1E-03	phosphoglucosamine mutase
nagA	-0.25	1.9E-02	-0.21	8.1E-02	NagA protein
scrK	0.40	1.1E-03	0.68	2.1E-04	fructokinase
nagB	-0.33	7.9E-04	-0.44	5.8E-04	glucosamine-6-phosphate isomerase
glmS	-0.07	3.0E-01	-0.23	1.9E-02	glucosamine--fructose-6-phosphate aminotransferase
llmg_1608	0.20	4.5E-02	0.50	2.4E-03	putative glycosyl hydrolases
llmg_1320	-0.14	7.7E-02	-0.26	2.0E-02	putative xylan beta-1,4-xylosidase
glk	0.03	6.8E-01	0.02	8.5E-01	glucokinase
pgi	-0.02	8.0E-01	-0.10	2.3E-01	glucose-6-phosphate isomerase
galU	-0.01	8.8E-01	0.04	5.8E-01	UDP--glucose-1-phosphate uridylyltransferase
galT	0.23	4.7E-02	0.56	2.8E-03	galactose-1-phosphate uridylyltransferase
ugd	0.00	9.5E-01	-0.13	2.0E-01	UDP-glucose 6-dehydrogenase
galK	0.06	3.3E-01	0.32	3.4E-03	galactokinase
galE	-0.17	5.1E-02	-0.36	5.1E-03	GalE protein
llmg_2003	-0.11	2.6E-01	-0.39	1.1E-02	UDP-glucose 4-epimerase
llmg_0247	0.15	2.2E-01	0.10	4.8E-01	putative UDP-glucose 4-epimerase
ptnAB	0.02	7.7E-01	-0.10	1.9E-01	PTS system, mannose-specific IIAB components
ptnC	0.02	7.2E-01	-0.10	2.1E-01	mannose-specific PTS system component IIC
ptnD	-0.02	7.1E-01	0.06	4.5E-01	mannose-specific PTS system component IID
pmi	0.02	7.8E-01	0.40	1.8E-03	mannose-6-phosphate isomerase
glgD	0.01	9.3E-01	0.29	1.2E-02	glucose-1-phosphate adenylyltransferase
glgC	0.24	8.1E-03	0.28	1.3E-02	glucose-1-phosphate adenylyltransferase

APPENDIX E

Phosphotransferase system (PTS) and glucose uptake

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
ptsI	0.02	7.3E-01	0.02	7.8E-01	phosphoenolpyruvate-protein phosphotransferase
ptsH	0.01	8.7E-01	-0.10	2.2E-01	phosphocarrier protein HPr
ptsK	-0.02	8.2E-01	-0.03	7.7E-01	HPr kinase/phosphorylase
hprT	-0.08	2.2E-01	0.08	3.5E-01	HprT protein
llmg_1426	0.07	5.4E-01	0.09	5.3E-01	sucrose-specific PTS system IIBC component
bglP	0.46	7.0E-03	0.42	3.1E-02	PTS system, beta-glucosides specific enzyme IIABC
llmg_0453	0.03	6.2E-01	0.03	7.5E-01	sucrose-specific PTS enzyme IIABC
llmg_0454	0.02	7.4E-01	0.05	5.5E-01	beta-glucoside-specific PTS system IIABC component
ptcA	0.35	7.9E-02	0.55	3.6E-02	cellobiose-specific PTS system IIA component
ptcB	0.31	5.9E-02	0.43	4.2E-02	cellobiose-specific PTS system IIB component
llmg_1244	0.16	3.4E-02	0.35	3.0E-03	hypothetical protein
ptcC	0.45	9.8E-04	0.47	2.8E-03	cellobiose-specific PTS system IIC component
celB	0.33	1.0E-02	0.44	7.6E-03	cellobiose-specific PTS system IIC component
mtlF	0.25	1.4E-02	0.04	6.8E-01	PTS system, mannitol-specific IIA component
mtlA	0.53	2.6E-03	0.73	1.7E-03	PTS system, mannitol-specific IIBC component
ptnAB	0.02	7.7E-01	-0.10	1.9E-01	PTS system, mannose-specific IIAB components
ptnC	0.02	7.2E-01	-0.10	2.1E-01	mannose-specific PTS system component IIC
ptnD	-0.02	7.1E-01	0.06	4.5E-01	mannose-specific PTS system component IID
llmg_0866	0.02	9.0E-01	-0.01	9.4E-01	PTS system, unknown pentitol phosphotransferase enzyme IIB component
ulaA, sgaT	0.39	4.3E-04	0.63	9.5E-05	PTS system ascorbate-specific transporter subunit IIC
fruA	0.23	7.8E-02	0.41	2.4E-02	PTS system, fructose specific IIBC components
glcU	0.05	4.5E-01	-0.15	1.1E-01	putative glucose uptake protein GlcU

APPENDIX E

RNA degradation

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
nudH	-0.08	4.4E-01	-0.18	1.9E-01	dinucleoside polyphosphate hydrolase
eno	0.05	4.1E-01	-0.07	4.2E-01	phosphopyruvate hydratase
pnpA	-0.01	9.4E-01	-0.10	4.4E-01	polynucleotide phosphorylase/polyadenylase
vacB1	-0.08	3.3E-01	-0.15	1.5E-01	putative exoribonuclease R
vacB2	-0.40	4.4E-03	-0.53	3.7E-03	putative exoribonuclease R
rheA	-0.09	2.3E-01	-0.26	2.3E-02	ATP-dependent RNA helicase
recQ	0.02	7.4E-01	0.04	6.7E-01	ATP-dependent DNA helicase RecQ
llmg_0302	0.05	4.7E-01	0.12	2.0E-01	putative Zn-dependent hydrolase
dnaK	0.02	7.5E-01	-0.08	3.2E-01	molecular chaperone DnaK
groEL	0.08	6.4E-01	0.08	7.2E-01	chaperonin GroEL

APPENDIX E

ABC transporters

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
potD	-0.01	8.7E-01	-0.18	5.3E-02	spermidine/putrescine ABC transporter substrate-binding protein
potC	0.07	3.0E-01	0.16	8.5E-02	spermidine/putrescine ABC transporter permease
potB	0.17	3.4E-02	-0.02	7.6E-01	spermidine/putrescine ABC transporter permease
potA	0.21	3.3E-02	0.29	2.4E-02	spermidine/putrescine ABC transporter ATP-binding protein
busAB	0.03	7.2E-01	0.02	8.5E-01	glycine betaine-binding periplasmic protein precursor
busAA	0.09	3.4E-01	0.04	7.3E-01	glycine betaine/proline ABC transporter
choS	0.19	4.8E-02	0.55	1.1E-03	choline ABC transporter permease and substrate binding protein
choQ	0.13	5.7E-02	0.38	1.7E-03	choline ABC transporter ATP binding protein
malE	-0.03	8.7E-01	-0.07	7.3E-01	maltose ABC transporter substrate binding protein
malF	0.22	3.1E-02	0.23	5.8E-02	maltose transport system permease protein MalF
malG	0.15	1.6E-01	0.48	5.8E-03	maltose ABC transporter permease protein MalG
msmK	0.31	8.7E-02	0.67	1.3E-02	multiple sugar-binding transport ATP-binding protein
rbsB	0.12	9.9E-02	-0.12	1.9E-01	ribose ABC transporter substrate binding protein RbsB
rbsC	0.13	1.2E-01	0.15	1.5E-01	ribose transport system permease protein RbsC
rbsD	0.23	2.3E-02	0.11	3.1E-01	ribose ABC transporter permease protein RbsD
rbsD	0.16	5.7E-02	0.07	4.2E-01	D-ribose pyranase
pstE	0.07	4.4E-01	-0.07	5.3E-01	phosphate transport substrate binding protein PstE
pstF	0.31	1.6E-02	0.57	2.9E-03	phosphate transport substrate binding protein PstF
pstD	0.24	1.7E-02	0.16	1.6E-01	phosphate transport system permease protein PstD
pstC	0.27	5.6E-03	0.20	4.9E-02	phosphate transport system permease protein PstC
pstA	-0.04	5.1E-01	-0.20	3.9E-02	phosphate transporter ATP-binding protein
pstB	0.15	3.2E-02	0.21	2.4E-02	phosphate transporter ATP-binding protein
phnD	-0.14	6.8E-02	-0.09	3.1E-01	phosphonate ABC transporter, phosphonate-binding protein PhnD
phnB	0.01	8.8E-01	-0.18	1.3E-01	phosphonate transport system permease protein PhnB
llmg_0315	0.08	5.4E-01	-0.03	8.4E-01	phosphonate ABC transporter permease
phnC	-0.06	4.0E-01	-0.28	1.2E-02	phosphonates import ATP-binding protein PhnC
plpA	0.10	3.0E-01	0.16	2.0E-01	D-methionine-binding lipoprotein PlpA precursor
plpB	0.03	7.3E-01	0.10	3.5E-01	D-methionine-binding lipoprotein PlpB precursor
plpC	0.11	1.6E-01	0.14	1.5E-01	D-methionine-binding lipoprotein PlpC precursor
plpD	-0.01	8.7E-01	-0.01	9.0E-01	D-methionine-binding lipoprotein PlpD precursor
llmg_0342	0.17	3.0E-02	0.14	1.3E-01	amino acid ABC transporter permease protein
llmg_0341	0.20	3.4E-02	0.21	6.2E-02	amino acid ABC transporter ATP binding protein
dppA	0.04	6.1E-01	-0.06	5.0E-01	dipeptide-binding protein precursor
dppB	0.08	2.8E-01	0.06	5.3E-01	dipeptide transport system permease protein DppB
dppC	0.08	1.9E-01	0.13	1.3E-01	dipeptide transport system permease protein DppC
dppD	0.16	4.1E-02	0.10	2.4E-01	dipeptide transport ATP-binding protein DppD
dppF	-0.02	7.9E-01	-0.10	2.8E-01	dipeptide transport ATP-binding protein DppF
fhuD	-0.21	7.4E-02	-0.38	2.1E-02	ferrichrome ABC transporter substrate binding protein
fhuB	0.16	4.6E-02	0.15	1.1E-01	ferrichrome ABC transporter permease protein
fhuG	0.02	8.3E-01	-0.07	6.3E-01	ferrichrome ABC transporter permease protein
llmg_1281	0.05	5.8E-01	-0.27	6.3E-02	putative ABC transporter ATP-binding protein
fhuC	-0.02	7.6E-01	0.08	3.8E-01	ferrichrome ABC transporter FhuC
zitS	0.30	2.3E-03	-0.14	1.3E-01	zinc ABC transporter substrate binding protein
zitP	0.42	4.9E-03	0.07	6.0E-01	zinc ABC transporter permease protein
zitQ	0.47	2.0E-03	0.15	2.6E-01	zinc ABC transporter ATP binding protein
cbiQ	0.05	5.9E-01	0.01	9.3E-01	putative cobalt ABC transporter permease protein
cbiQ2	0.13	1.4E-01	0.07	4.7E-01	putative cobalt ABC transporter permease protein
cbiO	0.05	5.2E-01	0.12	2.5E-01	putative cobalt ABC transporter ATP-binding protein
cbiO	-0.04	5.9E-01	0.01	9.5E-01	cobalt transporter ATP-binding subunit
cbiO	0.22	1.7E-02	0.23	3.6E-02	cobalt transporter ATP-binding subunit
llmg_1552	-0.03	8.3E-01	0.10	5.8E-01	putative ABC type transport system permease protein
drrB	0.47	4.7E-04	0.58	5.6E-04	daunorubicin resistance transmembrane protein
llmg_0262	0.09	3.8E-01	0.36	2.7E-02	ABC transporter permease protein
llmg_1553	0.10	1.7E-01	0.31	7.8E-03	putative ABC transporter ATP-binding protein
drrA	0.32	1.4E-02	0.39	1.7E-02	daunorubicin resistance ABC transporter ATP-binding subunit
tagG	0.14	1.4E-01	-0.02	8.5E-01	teichoic acid ABC transporter permease protein
tagH	0.16	8.0E-02	-0.14	2.0E-01	teichoic acid export ATP-binding protein TagH
ftsX	-0.11	1.8E-01	-0.25	3.4E-02	cell division protein FtsX-like protein
ftsE	-0.06	3.3E-01	-0.06	4.8E-01	cell division ATP-binding protein
llmg_1202	0.02	7.6E-01	0.13	1.3E-01	ABC transporter ABC binding and permease protein
cydD	0.10	2.1E-01	0.06	5.4E-01	cytochrome d ABC transporter ATP binding and permease protein
cydC	0.21	2.6E-02	0.11	2.7E-01	cytochrome d ABC transporter ATP binding and permease protein
llmg_0989	0.06	5.3E-01	0.10	3.6E-01	ABC transporter ATP binding and permease protein

APPENDIX E

Two-component system

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
apl	-0.16	1.5E-01	-0.25	1.0E-01	alkaline phosphatase
pstE	0.07	4.4E-01	-0.07	5.3E-01	phosphate transport substrate binding protein PstE
pstF	0.31	1.6E-02	0.57	2.9E-03	phosphate transport substrate binding protein PstF
kinC	0.00	9.6E-01	0.04	6.4E-01	sensor histidine kinase
llrC	-0.08	2.1E-01	0.02	7.9E-01	two-component system regulator
llmg_0458	-0.17	2.7E-01	-0.17	3.7E-01	hypothetical protein
dnaA	0.05	5.3E-01	0.08	3.7E-01	chromosomal replication initiation protein
mleS	-0.13	1.2E-01	-0.26	2.9E-02	malate dehydrogenase
comX	-0.05	6.5E-01	-0.10	4.8E-01	competence regulator ComX
frdC	-0.09	4.4E-01	-0.07	6.1E-01	fumarate reductase flavoprotein subunit
llmg_0018	0.11	2.1E-01	0.13	2.2E-01	beta-lactamase A
kinD	-0.02	7.1E-01	0.14	1.3E-01	sensor protein kinase KinD
glnB	-0.53	1.7E-03	-0.79	6.9E-04	nitrogen regulatory protein P-II
glnA	0.01	9.2E-01	-0.10	2.1E-01	GlnA protein
thiL	-0.34	2.2E-03	-0.28	1.6E-02	ThiL protein
cydA	0.04	5.6E-01	0.14	9.7E-02	cytochrome bd-I ubiquinol oxidase subunit I
cydB	0.10	2.7E-01	0.01	9.2E-01	cytochrome d ubiquinol oxidase, subunit II

APPENDIX E

Genes under codY regulation

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
dppA	0.04	6.1E-01	-0.06	5.0E-01	dipeptide-binding protein precursor
dppB	0.08	2.8E-01	0.06	5.3E-01	dipeptide transport system permease protein DppB
dppC	0.08	1.9E-01	0.13	1.3E-01	dipeptide transport system permease protein DppC
dppD	0.16	4.1E-02	0.10	2.4E-01	dipeptide transport ATP-binding protein DppD
dppF	-0.02	7.9E-01	-0.10	2.8E-01	dipeptide transport ATP-binding protein DppF
gltD	0.11	1.2E-01	0.06	4.2E-01	glutamate synthase subunit beta
gltB	0.15	4.9E-02	0.12	1.7E-01	glutamate synthase, large subunit
lysA	-0.08	2.4E-01	-0.22	3.2E-02	LysA protein
ilvD	0.19	6.8E-02	0.14	2.5E-01	dihydroxy-acid dehydratase
ilvB	0.24	3.1E-02	0.22	8.6E-02	acetolactate synthase catalytic subunit
ilvH	0.29	3.4E-02	0.17	2.7E-01	acetolactate synthase 3 regulatory subunit
ilvC	0.31	1.8E-02	0.21	1.4E-01	ketol-acid reductoisomerase
ilvA	0.26	3.9E-02	-0.04	7.5E-01	threonine dehydratase
aldB	0.01	8.9E-01	-0.11	2.4E-01	AldB protein
hisC	-0.02	7.9E-01	0.18	5.7E-02	histidinol-phosphate aminotransferase
hisZ	0.27	3.2E-03	0.52	3.0E-04	ATP phosphoribosyltransferase regulatory subunit
hisG	0.26	2.9E-02	0.47	5.9E-03	ATP phosphoribosyltransferase catalytic subunit
hisD	0.22	3.3E-02	0.58	1.1E-03	HisD protein
hisB	0.14	1.1E-01	0.45	2.4E-03	imidazoleglycerol-phosphate dehydratase
hisH	0.17	1.0E-01	0.27	4.9E-02	imidazole glycerol phosphate synthase subunit HisH
hisA	0.19	6.4E-02	0.42	7.3E-03	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase
hisF	0.25	7.7E-02	0.47	1.7E-02	imidazole glycerol phosphate synthase subunit HisF
hisI	0.36	2.4E-02	0.72	3.0E-03	bifunctional phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphatase protein
hisK	0.25	3.8E-02	0.60	2.0E-03	histidinol-phosphatase
ctrA	0.02	6.6E-01	0.06	4.4E-01	putative amino-acid transporter
oppD	-0.01	8.7E-01	-0.04	6.0E-01	oligopeptide transport ATP-binding protein OppD
oppF	0.02	7.3E-01	-0.10	2.2E-01	oligopeptide transport ATP-binding protein OppF
oppB	-0.01	9.0E-01	0.06	5.4E-01	peptide transport system permease protein OppB
oppC	-0.04	5.2E-01	0.02	8.2E-01	oligopeptide transport system permease protein OppC
oppA	-0.04	5.2E-01	0.02	8.2E-01	oligopeptide-binding protein OppA precursor
pepO	-0.01	8.4E-01	-0.05	5.2E-01	endopeptidase O
asnB	-0.08	2.8E-01	-0.02	8.3E-01	asparagine synthetase B
gltA	0.26	2.2E-02	0.33	2.1E-02	citrate synthase
citB	0.15	1.5E-01	0.40	1.2E-02	aconitate hydratase
icd	0.25	3.6E-03	0.41	1.0E-03	isocitrate dehydrogenase
serC	0.09	2.2E-01	0.18	8.3E-02	phosphoserine aminotransferase
serA	0.18	3.1E-02	0.24	2.5E-02	D-3-phosphoglycerate dehydrogenase
serB	0.12	1.8E-01	0.04	6.8E-01	SerB protein
arcD1	0.34	5.4E-03	0.21	9.5E-02	arginine/ornithine antiporter
arcC1	0.31	4.3E-03	0.29	1.7E-02	carbamate kinase
arcC2	0.07	4.0E-01	-0.10	3.6E-01	carbamate kinase
amtB	-0.35	2.9E-02	-0.55	1.2E-02	ammonium transporter AmtB
dapB	0.25	7.0E-03	0.30	8.7E-03	dihydrodipicolinate reductase
ftsW1	-0.01	8.9E-01	-0.01	9.2E-01	cell division protein ftsW1

APPENDIX E

Genes under ccpA regulation

Gene/locus	BK1506		BK1503			Annotation
	LogFC	p. Value	logFC	P.value	Effect	
galP	0.34	9.5E-03	0.49	4.8E-03	Rep	galactose permease
galM	0.13	1.7E-01	0.28	3.7E-02	Rep	aldose 1-epimerase
galK	0.06	3.3E-01	0.32	3.4E-03	Rep	galactokinase
galT	0.23	4.7E-02	0.56	2.8E-03	Rep	galactose-1-phosphate uridylyltransferase
galE	-0.17	5.1E-02	-0.36	5.1E-03	Rep	GalE protein
mtlA	0.53	2.6E-03	0.73	1.7E-03	Rep	PTS system, mannitol-specific IIBC component
mtlR	0.36	1.5E-03	0.15	1.2E-01	Rep	transcriptional regulator mtl operon MtlR
mtlF	0.25	1.4E-02	0.04	6.8E-01	Rep	PTS system, mannitol-specific IIA component
mtlD	0.22	9.9E-02	0.27	1.1E-01	Rep	mannitol-1-phosphate 5-dehydrogenase
llmg_0453	0.03	6.2E-01	0.03	7.5E-01	Rep	sucrose-specific PTS enzyme IIABC
llmg_0454	0.02	7.4E-01	0.05	5.5E-01	Rep	beta-glucoside-specific PTS system IIABC component
trePP	-0.03	6.2E-01	-0.01	8.7E-01	Rep	putative trehalose/maltose hydrolase
pgmB	0.07	3.6E-01	0.13	2.0E-01	Rep	beta-phosphoglucomutase
llmg_0431	0.30	3.3E-03	0.16	1.0E-01	Rep	putative acyl carrier protein phosphodiesterase 2
llmg_0432	0.04	5.3E-01	0.02	8.1E-01	Rep	transcription regulator
ptcB	0.31	5.9E-02	0.43	4.2E-02	Rep	cellobiose-specific PTS system IIB component
ptcA	0.35	7.9E-02	0.55	3.6E-02	Rep	cellobiose-specific PTS system IIA component
llmg_0439	0.26	1.5E-01	0.34	1.4E-01	Rep	LacI family transcription regulator
ptcC	0.45	9.8E-04	0.47	2.8E-03	Rep	cellobiose-specific PTS system IIC component
bglA2	0.17	2.2E-02	0.23	1.6E-02	Rep	6-phospho-beta-glucosidase
arcA	0.18	7.5E-02	0.27	4.3E-02	Rep	arginine deiminase
arcB	0.28	2.0E-02	0.10	4.2E-01	Rep	ornithine carbamoyltransferase
arcD1	0.34	5.4E-03	0.21	9.5E-02	Rep	arginine/ornithine antiporter
arcC1	0.31	4.3E-03	0.29	1.7E-02	Rep	carbamate kinase
arcC2	0.07	4.0E-01	-0.10	3.6E-01	Rep	carbamate kinase
pfk	0.01	9.1E-01	-0.11	1.7E-01	Act	6-phosphofructokinase
pyk	-0.04	5.6E-01	-0.10	2.8E-01	Act	pyruvate kinase
ldh	-0.05	4.2E-01	-0.62	1.1E-04	Act	L-lactate dehydrogenase
pgi	-0.02	8.0E-01	-0.10	2.3E-01	Act	glucose-6-phosphate isomerase
fruA	0.23	7.8E-02	0.41	2.4E-02	Act	PTS system, fructose specific IIBC components
fruC	0.20	7.9E-02	0.44	1.0E-02	Act	tagatose-6-phosphate kinase
fruR	0.04	7.2E-01	0.34	3.6E-02	Act	lactose transport regulator

APPENDIX E

Fatty acid biosynthesis and fatty acid metabolism

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
accA	-0.05	5.5E-01	-0.10	3.2E-01	AccA protein
accB	0.02	7.2E-01	-0.10	2.1E-01	acetyl-CoA carboxylase biotin carboxyl carrier protein subunit
accC	0.05	4.8E-01	0.05	5.1E-01	acetyl-CoA carboxylase biotin carboxylase subunit
accD	0.03	6.1E-01	0.06	4.2E-01	acetyl-CoA carboxylase subunit beta
fabD	-0.03	6.7E-01	-0.06	4.8E-01	malonyl CoA-acyl carrier protein transacylase
fabF	-0.04	5.5E-01	0.05	5.2E-01	3-oxoacyl-[acyl-carrier-protein] synthase II
fabH	0.00	9.9E-01	-0.07	4.1E-01	3-oxoacyl-(acyl carrier protein) synthase III
fabG	0.03	6.6E-01	-0.09	2.6E-01	3-ketoacyl-(acyl-carrier-protein) reductase
fabG	-0.08	4.2E-01	-0.02	8.5E-01	3-ketoacyl-(acyl-carrier-protein) reductase
fabZ	-0.02	7.9E-01	-0.10	2.3E-01	(3R)-hydroxymyristoyl-ACP dehydratase
llmg_0538	-0.12	7.6E-02	-0.07	3.6E-01	(3R)-hydroxymyristoyl-(acyl-carrier-protein) dehydratase
fabI	-0.16	3.5E-02	-0.10	2.5E-01	enoyl-(acyl carrier protein) reductase
llmg_1415	-0.16	6.2E-02	0.05	5.7E-01	hypothetical protein
thiL	-0.34	2.2E-03	-0.28	1.6E-02	ThiL protein
llmg_1965	0.06	5.9E-01	-0.09	5.1E-01	putative AMP-binding enzyme
adhE	0.49	6.0E-02	0.64	5.5E-02	bifunctional acetaldehyde-CoA/alcohol dehydrogenase
adhA, adhP	0.20	1.5E-01	0.30	1.0E-01	alcohol dehydrogenase
llmg_0955	0.11	1.7E-01	0.06	5.6E-01	alcohol dehydrogenase

APPENDIX E

Glycerolipid and glycerol metabolism

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
llmg_0870	-0.26	2.8E-02	-0.48	5.5E-03	transporter
glpF2	0.54	7.6E-05	0.60	1.6E-04	glycerol uptake facilitator
glpF3	-0.22	1.8E-02	-0.60	4.1E-04	putative glycerol uptake facilitator protein
dhaK	-0.25	1.4E-02	-0.25	3.3E-02	DhaKLM operon coactivator DhaQ
dhaM	0.07	2.8E-01	0.20	2.7E-02	dihydroxyacetone kinase DhaM
glpK	0.20	9.8E-03	0.15	6.4E-02	glycerol kinase
plsX	-0.17	2.3E-02	-0.24	1.7E-02	putative glycerol-3-phosphate acyltransferase PlsX
llmg_1540	-0.17	1.2E-01	-0.40	1.5E-02	putative glycerol-3-phosphate acyltransferase PlsY
llmg_0119	-0.40	5.2E-04	-0.64	1.2E-04	putative acyltransferase
dgkA	0.03	5.7E-01	0.19	3.5E-02	DgkA protein
llmg_2421	-0.19	1.8E-02	-0.21	3.2E-02	hypothetical protein
glpD	0.25	6.3E-03	-0.02	8.4E-01	GlpD protein
gpsA	-0.03	6.1E-01	-0.11	2.2E-01	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase
llmg_0945	0.05	4.8E-01	0.08	3.9E-01	putative glycerol dehydrogenase

APPENDIX E

Purine metabolism

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
llmg_2075	0.08	3.8E-01	-0.18	1.3E-01	ADP-ribose pyrophosphatase
deoB	0.01	9.0E-01	0.01	8.6E-01	phosphopentomutase
prsB	-0.22	8.1E-03	-0.30	5.5E-03	ribose-phosphate pyrophosphokinase
prsA	-0.03	6.3E-01	0.05	5.5E-01	ribose-phosphate pyrophosphokinase
purF	-0.01	9.0E-01	0.06	5.4E-01	amidophosphoribosyltransferase
purD	-0.03	6.9E-01	0.05	5.6E-01	phosphoribosylamine--glycine ligase
purN	-0.40	6.1E-03	-0.58	3.1E-03	phosphoribosylglycinamide formyltransferase
purS	0.02	7.2E-01	-0.10	2.1E-01	phosphoribosylformylglycinamidine synthetase PurS
purQ	-0.04	5.6E-01	0.06	5.1E-01	phosphoribosylformylglycinamidine synthase I
purL	0.00	1.0E+00	-0.05	4.7E-01	phosphoribosylformylglycinamidine synthase II
purM	-0.04	5.8E-01	-0.17	7.5E-02	phosphoribosylaminoimidazole synthetase
purK	-0.01	8.3E-01	0.04	6.2E-01	phosphoribosylaminoimidazole carboxylase ATPase subunit
purE	-0.02	7.6E-01	-0.10	2.4E-01	phosphoribosylaminoimidazole carboxylase catalytic subunit
purC	-0.05	4.2E-01	0.03	6.7E-01	phosphoribosylaminoimidazole-succinocarboxamide synthase
purB	-0.09	1.6E-01	-0.08	2.7E-01	adenylosuccinate lyase
purH	-0.07	2.9E-01	-0.08	3.2E-01	bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
apt	-0.12	8.3E-02	-0.36	2.5E-03	adenine phosphoribosyltransferase
nucA	-0.28	1.5E-02	-0.12	3.2E-01	5'-nucleotidase
llmg_0192	0.03	6.1E-01	0.28	8.9E-03	5'-nucleotidase precursor
deoD	-0.10	3.3E-01	-0.15	2.5E-01	purine nucleoside phosphorylase
hprT	-0.08	2.2E-01	0.08	3.5E-01	HprT protein
hpt	0.09	2.3E-01	0.02	8.4E-01	hypoxanthine phosphoribosyltransferase
guaB	-0.19	2.7E-02	-0.32	7.5E-03	inosine 5'-monophosphate dehydrogenase
llmg_1188	0.13	2.0E-01	0.03	7.8E-01	hypothetical protein
xpt	-0.08	2.5E-01	-0.25	2.2E-02	xanthine phosphoribosyltransferase
guaA	-0.23	1.1E-01	-0.32	8.6E-02	GMP synthase
guaC	-0.47	1.9E-03	-0.61	1.7E-03	guanosine 5'-monophosphate oxidoreductase
gmk	-0.03	6.1E-01	0.08	2.7E-01	guanylate kinase
pyk	-0.04	5.6E-01	-0.10	2.8E-01	pyruvate kinase
nrdE	-0.03	6.1E-01	-0.08	3.7E-01	ribonucleotide-diphosphate reductase subunit alpha
nrdF	0.12	4.6E-01	0.10	6.0E-01	ribonucleotide-diphosphate reductase subunit beta
llmg_0281	-0.24	5.5E-03	-0.32	4.0E-03	anaerobic ribonucleoside triphosphate reductase
rpoA	0.06	3.9E-01	0.06	4.8E-01	DNA-directed RNA polymerase subunit alpha
rpoB	0.01	9.1E-01	-0.01	9.2E-01	DNA-directed RNA polymerase subunit beta
rpoC	-0.03	6.2E-01	0.06	5.2E-01	DNA-directed RNA polymerase subunit beta'
rpoE	-0.15	2.0E-01	-0.16	3.0E-01	DNA-directed RNA polymerase subunit delta
rpoZ	0.13	1.6E-01	-0.08	4.6E-01	DNA-directed RNA polymerase subunit omega
polA	-0.04	5.7E-01	-0.09	3.1E-01	DNA polymerase I
dnaE	-0.11	7.7E-02	-0.04	5.6E-01	DNA polymerase III DnaE
polC	-0.04	7.0E-01	-0.13	3.2E-01	DNA polymerase III PolC
dnaN	0.06	4.8E-01	0.09	4.6E-01	DNA polymerase III subunit beta
dnaX	0.09	2.5E-01	-0.03	7.8E-01	DNA polymerase III subunits gamma and tau
holA	-0.06	4.2E-01	-0.09	3.2E-01	DNA polymerase III subunit delta
holB	-0.06	5.4E-01	0.09	4.5E-01	DNA polymerase III subunit delta'
dnaQ	-0.25	4.9E-03	-0.21	3.2E-02	DNA polymerase III, epsilon chain
relA	0.00	9.6E-01	-0.06	4.3E-01	GTP pyrophosphokinase
llmg_0382	0.13	1.4E-01	0.19	9.3E-02	putative GTP pyrophosphokinase
purA	-0.01	8.6E-01	-0.10	2.2E-01	adenylosuccinate synthetase
add	-0.27	1.7E-02	-0.60	1.0E-03	adenosine deaminase
cpdC	-0.26	4.5E-03	-0.50	4.7E-04	2',3'-cyclic-nucleotide 2'-phosphodiesterase
adk	-0.09	3.5E-01	0.33	2.3E-02	adenylate kinase
pnpA	-0.01	9.4E-01	-0.10	4.4E-01	polynucleotide phosphorylase/polyadenylase
arcC2	0.07	4.0E-01	-0.10	3.6E-01	carbamate kinase
arcC1	0.31	4.3E-03	0.29	1.7E-02	carbamate kinase

APPENDIX E

Pyrimidine metabolism

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
carB	-0.01	8.5E-01	-0.07	3.7E-01	carbamoyl phosphate synthase large subunit
carA	0.00	9.9E-01	0.00	9.9E-01	carbamoyl phosphate synthase small subunit
pyrB	0.06	3.7E-01	-0.06	4.3E-01	aspartate carbamoyltransferase catalytic subunit
pyrC	-0.03	6.7E-01	-0.07	3.5E-01	dihydroorotase
pyrDA	-0.39	1.1E-02	-0.53	8.5E-03	dihydroorotate dehydrogenase 1A
pyrDB	0.01	8.5E-01	-0.10	2.0E-01	dihydroorotate dehydrogenase 1B
pyrE	0.01	9.1E-01	0.09	2.7E-01	orotate phosphoribosyltransferase
pyrF	-0.27	1.2E-02	-0.53	1.3E-03	orotidine 5'-phosphate decarboxylase
cmk	-0.13	1.1E-01	0.14	1.5E-01	cytidylate kinase
pyrH	-0.01	8.3E-01	0.07	4.0E-01	uridylate kinase
pnpA	-0.01	9.4E-01	-0.10	4.4E-01	polynucleotide phosphorylase/polyadenylase
llmg_1188	0.13	2.0E-01	0.03	7.8E-01	hypothetical protein
pyrG	-0.03	6.0E-01	0.00	9.9E-01	CTP synthetase
rpoA	0.06	3.9E-01	0.06	4.8E-01	DNA-directed RNA polymerase subunit alpha
rpoB	0.01	9.1E-01	-0.01	9.2E-01	DNA-directed RNA polymerase subunit beta
rpoC	-0.03	6.2E-01	0.06	5.2E-01	DNA-directed RNA polymerase subunit beta'
rpoE	-0.15	2.0E-01	-0.16	3.0E-01	DNA-directed RNA polymerase subunit delta
rpoZ	0.13	1.6E-01	-0.08	4.6E-01	DNA-directed RNA polymerase subunit omega
polA	-0.04	5.7E-01	-0.09	3.1E-01	DNA polymerase I
dnaE	-0.11	7.7E-02	-0.04	5.6E-01	DNA polymerase III DnaE
polC	-0.04	7.0E-01	-0.13	3.2E-01	DNA polymerase III PolC
dnaN	0.06	4.8E-01	0.09	4.6E-01	DNA polymerase III subunit beta
dnaX	0.09	2.5E-01	-0.03	7.8E-01	DNA polymerase III subunits gamma and tau
holA	-0.06	4.2E-01	-0.09	3.2E-01	DNA polymerase III subunit delta
holB	-0.06	5.4E-01	0.09	4.5E-01	DNA polymerase III subunit delta'
dnaQ	-0.25	4.9E-03	-0.21	3.2E-02	DNA polymerase III, epsilon chain
udk	-0.52	4.9E-04	-0.37	1.0E-02	uridine kinase
nucA	-0.28	1.5E-02	-0.12	3.2E-01	5'-nucleotidase
llmg_0192	0.03	6.1E-01	0.28	8.9E-03	5'-nucleotidase precursor
udp	0.05	5.3E-01	0.15	1.7E-01	uridine phosphorylase
upp	-0.30	7.2E-03	-0.49	2.2E-03	uracil phosphoribosyltransferase
pyrR	-0.03	6.8E-01	0.03	6.7E-01	bifunctional pyrimidine regulatory protein PyrR uracil phosphoribosyltransferase
pdp	0.16	4.7E-02	0.26	1.6E-02	pyrimidine-nucleoside phosphorylase
trxB1	-0.21	4.0E-02	-0.07	5.4E-01	TrxB1 protein
trxB2	-0.27	7.9E-03	-0.31	1.2E-02	TrxB2 protein
llmg_0281	-0.24	5.5E-03	-0.32	4.0E-03	anaerobic ribonucleoside triphosphate reductase
nrdE	-0.03	6.1E-01	-0.08	3.7E-01	ribonucleotide-diphosphate reductase subunit alpha
nrdF	0.12	4.6E-01	0.10	6.0E-01	ribonucleotide-diphosphate reductase subunit beta
ps428	0.25	3.0E-02	0.27	5.8E-02	deoxyuridine 5'-triphosphate nucleotidohydrolase
ps325	0.14	5.4E-02	0.20	4.1E-02	deoxyuridine 5'-triphosphate nucleotidohydrolase
dut	-0.07	4.1E-01	-0.10	3.6E-01	deoxyuridine 5'-triphosphate nucleotidohydrolase
thyA	-0.47	5.0E-04	-0.38	5.4E-03	thymidylate synthase
cdd	-0.03	6.9E-01	0.00	9.9E-01	Cdd protein
deoD	-0.10	3.3E-01	-0.15	2.5E-01	purine nucleoside phosphorylase
llmg_1416	-0.27	3.2E-02	-0.24	1.0E-01	hypothetical protein
ntd	0.17	3.2E-02	0.07	4.1E-01	nucleoside deoxyribosyltransferase
tdk	0.03	6.4E-01	-0.05	5.7E-01	thymidine kinase
tmk	-0.13	1.6E-01	-0.22	7.1E-02	thymidylate kinase
cpdC	-0.26	4.5E-03	-0.50	4.7E-04	2',3'-cyclic-nucleotide 2'-phosphodiesterase

APPENDIX E

Arginine and proline metabolism

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
speG	-0.13	6.6E-02	0.07	3.5E-01	spermidine acetyltransferase
llmg_0177	0.28	5.7E-03	0.40	2.9E-03	amidase
proC	-0.18	5.3E-02	-0.23	5.0E-02	pyrroline-5-carboxylate reductase
ocd	-0.34	1.5E-03	-0.46	1.0E-03	ornithine cyclodeaminase, mu-crystallin-like protein
proB	-0.13	9.5E-02	-0.28	1.3E-02	gamma-glutamyl kinase
proA	-0.15	7.4E-02	-0.26	2.3E-02	gamma-glutamyl phosphate reductase
kdgA	0.22	8.4E-03	0.40	1.3E-03	keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase
arcB	0.28	2.0E-02	0.10	4.2E-01	ornithine carbamoyltransferase
argF	-0.53	7.8E-05	-0.67	7.5E-05	ornithine carbamoyltransferase
argG	0.17	6.2E-02	0.30	1.8E-02	argininosuccinate synthase
argH	0.00	9.7E-01	-0.07	3.4E-01	argininosuccinate lyase
arcA	0.18	7.5E-02	0.27	4.3E-02	arginine deiminase
glnA	0.01	9.2E-01	-0.10	2.1E-01	GlnA protein
arcC2	0.07	4.0E-01	-0.10	3.6E-01	carbamate kinase
arcC1	0.31	4.3E-03	0.29	1.7E-02	carbamate kinase
argJ	-0.17	1.1E-01	-0.24	7.0E-02	bifunctional ornithine acetyltransferase/N-acetylglutamate synthase protein
argB	-0.34	2.4E-03	-0.23	4.0E-02	acetylglutamate kinase
argC	-0.39	8.9E-04	-0.47	1.2E-03	N-acetyl-gamma-glutamyl-phosphate reductase
argD	-0.10	2.7E-01	0.05	6.4E-01	acetylornithine aminotransferase
argE	-0.10	2.5E-01	-0.18	1.0E-01	acetylornithine deacetylase

APPENDIX E

Vitamin and cofactor metabolism

Gene/locus	BK1506		BK1503		Annotation
	LogFC	p. Value	logFC	P.value	
					One carbon pool by folate
dfrA	-0.14	4.7E-02	-0.02	8.4E-01	DfrA protein
fhs	-0.12	1.8E-01	-0.04	6.7E-01	formate--tetrahydrofolate ligase
folD	-0.08	3.1E-01	0.03	7.5E-01	bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase/5,10-methylene-tetrahydrofolate cyclohydrolase
glyA	-0.13	6.6E-02	-0.25	1.5E-02	serine hydroxymethyltransferase
purN	-0.40	6.1E-03	-0.58	3.1E-03	phosphoribosylglycinamide formyltransferase
purH	-0.07	2.9E-01	-0.08	3.2E-01	bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
fmt	0.12	1.5E-01	0.00	9.7E-01	methionyl-tRNA formyltransferase
thyA	-0.47	5.0E-04	-0.38	5.4E-03	thymidylate synthase
metF	-0.24	6.4E-02	-0.34	4.3E-02	5,10-methylenetetrahydrofolate reductase
llmg_0181	0.00	9.8E-01	-0.06	5.6E-01	5-formyltetrahydrofolate cyclo-ligase family protein
					Thiamine metabolism
thiD1	0.11	1.9E-01	0.05	6.3E-01	phosphomethylpyrimidine kinase
nifS	-0.02	7.6E-01	0.19	4.6E-02	putative iron-sulfur cofactor synthesis protein
nifZ	-0.33	1.8E-03	-0.48	9.5E-04	pyridoxal-phosphate dependent aminotransferase
llmg_1972	0.12	9.4E-02	0.04	5.9E-01	hypothetical protein
thiI	-0.46	6.3E-04	-0.42	3.7E-03	thiamine biosynthesis protein ThiI
thiM	-0.10	1.9E-01	-0.03	7.0E-01	hydroxyethylthiazole kinase
thiE	-0.04	4.8E-01	-0.02	8.3E-01	thiamine-phosphate pyrophosphorylase
thiN	-0.07	3.5E-01	0.10	3.2E-01	thiamin pyrophosphokinase
tenA	-0.23	7.1E-03	-0.27	1.1E-02	transcriptional activator TenA
					Riboflavin metabolism
ribA	-0.03	6.5E-01	0.00	9.6E-01	riboflavin biosynthesis protein RibA
ribD	-0.10	1.6E-01	-0.12	1.9E-01	riboflavin biosynthesis protein RibD
ribH	0.12	1.5E-01	0.27	2.0E-02	6,7-dimethyl-8-ribityllumazine synthase
ribB	-0.01	8.2E-01	0.00	9.7E-01	riboflavin synthase subunit alpha
ribC, ribF	-0.11	2.3E-01	-0.01	9.6E-01	bifunctional riboflavin kinase/FMN adenylyltransferase
					Vitamin B6 metabolism
thiD2	0.03	7.8E-01	-0.10	4.9E-01	phosphomethylpyrimidine kinase
serC	0.09	2.2E-01	0.18	8.3E-02	phosphoserine aminotransferase
thrC	0.06	3.7E-01	-0.01	8.6E-01	threonine synthase
					Nicotinate and nicotinamide metabolism
pncB	0.06	3.5E-01	0.00	9.9E-01	nicotinate phosphoribosyltransferase
deoD	-0.10	3.3E-01	-0.15	2.5E-01	purine nucleoside phosphorylase
nucA	-0.28	1.5E-02	-0.12	3.2E-01	5'-nucleotidase
llmg_0192	0.03	6.1E-01	0.28	8.9E-03	5'-nucleotidase precursor
nadD	-0.16	1.8E-01	-0.12	3.8E-01	nicotinic acid mononucleotide adenylyltransferase
nadD1	0.22	2.1E-02	0.28	2.0E-02	nicotinate-nucleotide adenylyltransferase
nadE	0.10	1.2E-01	0.11	1.8E-01	NAD synthetase
ppnK	-0.09	1.3E-01	-0.04	5.5E-01	inorganic polyphosphate/ATP-NAD kinase
					Pantothenate and CoA biosynthesis
als	-0.25	1.5E-02	-0.43	3.7E-03	acetolactate synthase
ilvB	0.24	3.1E-02	0.22	8.6E-02	acetolactate synthase catalytic subunit
ilvH	0.29	3.4E-02	0.17	2.7E-01	acetolactate synthase 3 regulatory subunit
ilvC	0.31	1.8E-02	0.21	1.4E-01	ketol-acid reductoisomerase
ilvD	0.19	6.8E-02	0.14	2.5E-01	dihydroxy-acid dehydratase
panE	-0.42	1.2E-03	-0.14	1.8E-01	2-dehydropantoate 2-reductase
coaA	-0.19	4.5E-02	-0.13	2.2E-01	pantothenate kinase
dfpB	-0.05	4.5E-01	0.06	5.3E-01	phosphopantothenate--cysteine ligase
dfpA	0.03	6.2E-01	0.12	2.1E-01	phosphopantothenoylcysteine decarboxylase
coaD	-0.07	4.8E-01	-0.09	4.3E-01	phosphopantetheine adenylyltransferase
coaE	-0.09	2.9E-01	-0.10	3.3E-01	dephospho-CoA kinase
acpS	-0.03	6.5E-01	-0.06	4.9E-01	4'-phosphopantetheinyl transferase
ilvE	-0.36	3.7E-03	-0.55	1.4E-03	branched-chain amino acid aminotransferase
					Biotin metabolism
birA2	0.04	6.0E-01	0.18	7.9E-02	acetyl-CoA carboxylase ligase / biotin operon repressor bifunctional protein
birA1	-0.32	5.6E-03	-0.33	1.4E-02	biotin--[acetyl-CoA-carboxylase] ligase and biotin operon repressor

E.2 – Merged tables of the differential gene expression analysis from chip 1 and chip 2.

This section contains the complete collection of results from the analysis of differential gene expression from both chip 1 and chip 2 (sorted by pathways, filtered with p-value 0.10).

APPENDIX E

Transcription factors

Gene/locus	Chip 1 BK1506 logFC	Chip 1 BK1503 logFC	Chip 2 BK1506 logFC	Chip 2 BK1503 logFC	Annotation
adaA	-0.31		-0.24		AraC family transcriptional regulator, regulatory protein
fruR				0.34	lactose transport regulator, fructose operon transcriptional repressor
malR		0.55	-0.17		maltose operon transcriptional repressor
ccpA					catabolite control protein A
rbsR					ribose operon repressor
llmg_0956		0.45		0.15	LacI family transcription regulator
treR	-0.31	-0.47	0.15		trehalose operon transcriptional repressor
rgrB		0.57		0.22	GntR family transcriptional regulator
rmaG	-0.46	-0.47	-0.20	-0.28	MarR family transcriptional regulator
glnR		-0.27			glutamine synthetase repressor
fur	-0.45		-0.35		ferric uptake regulation protein
flpB					transcriptional regulator FNR like protein B
flpA			-0.44	-0.54	FNR like protein A
llmg_1224		0.42	-0.43	-0.61	transcriptional regulator
llmg_0709			-0.44	-0.42	PadR-like family transcriptional regulator
llmg_2339				-0.35	transcriptional regulator
arsR				-0.28	regulator of arsenical resistance
birA1	-0.23		-0.32	-0.33	biotin--[acetyl-CoA-carboxylase] ligase and biotin operon repressor
birA2		0.20		0.18	acetyl-CoA carboxylase ligase / biotin operon repressor bifunctional protein
codY		0.84			transcriptional repressor CodY
ps602	0.31	0.53			hypothetical protein
mtlR			0.36		transcriptional regulator mtl operon MtlR
ahrC				0.19	arginine transcriptional regulator
argR		0.34	0.35	0.75	arginine repressor
cspE		0.41			cold shock-like protein CspE
cspD2		0.30			cold shock-like protein cspD2
cspB					cold shock-like protein CspB
hrcA	-0.45	0.34			heat-inducible transcription repressor
tenA			-0.23	-0.27	transcriptional activator TenA
ctsR				0.17	transcriptional regulator CtsR
parA	0.43			-0.17	chromosome partitioning protein ParA
pyrR		-0.63			bifunctional pyrimidine regulatory protein PyrR
nrdR	-0.30	-0.39			transcriptional regulator NrdR
purR	-0.40	0.57	-0.17	-0.22	pur operon repressor
comX	-0.39				competence regulator ComX
rpoD			-0.15	-0.26	RNA polymerase sigma factor RpoD

APPENDIX E

Glycolysis

Gene/locus	Chip 1 BK1506 logFC	Chip 1 BK1503 logFC	Chip 2 BK1506 logFC	Chip 2 BK1503 logFC	Annotation
glk	-0.25	-0.57			glucokinase
pgi		0.54			glucose-6-phosphate isomerase
pfk	-0.26	-0.56			6-phosphofructokinase
pyk	-0.27	-1.09			pyruvate kinase
ldh	-0.51	-0.45		-0.62	L-lactate dehydrogenase
fbp	0.60	0.65	0.19	0.39	fructose-bisphosphatase
fbaA	-0.36	-0.46			fructose-bisphosphate aldolase
tpiA		-0.68	-0.33	-0.64	triosephosphate isomerase
gapB					glyceraldehyde 3-phosphate dehydrogenase
gapA	0.22		-0.28	-0.21	glyceraldehyde 3-phosphate dehydrogenase
pgk	-0.35			-0.07	phosphoglycerate kinase
llmg_1894			0.19		phosphoglycerate mutase family protein
gpmA	-0.30	-0.67			phosphoglyceromutase
gpmB		-0.51	0.22	0.28	phosphoglycerate mutase
gpmC	0.25	-0.56	0.26		phosphoglycerate mutase
eno	-0.28				phosphopyruvate hydratase
pdhA		-0.51			pyruvate dehydrogenase E1 component alpha subunit
pdhB		-0.36			pyruvate dehydrogenase E1 component beta subunit
pdhC		-0.35			pyruvate dehydrogenase complex E2 component
pdhD					dihydrolipoamide dehydrogenase
ldhX		0.45	-0.17	-0.34	L-lactate dehydrogenase
ldhB					L-lactate dehydrogenase
adhE	0.52		0.49	0.64	bifunctional acetaldehyde-CoA/alcohol dehydrogenase
adhA, adhP				0.30	alcohol dehydrogenase
llmg_0955					alcohol dehydrogenase
fadD	-0.26				long-chain acyl-CoA synthetase
galM	0.57			0.28	aldose 1-epimerase
bglA2			0.17	0.23	6-phospho-beta-glucosidase
arb		0.61			6-phospho-beta-glucosidase
celA			0.28	0.34	6-phospho-beta-glucosidase

APPENDIX E

TCA cycle

Gene/locus	Chip 1 BK1506 logFC	Chip 1 BK1503 logFC	Chip 2 BK1506 logFC	Chip 2 BK1503 logFC	Annotation
gltA			0.26	0.33	citrate synthase
citB				0.40	aconitate hydratase
icd			0.25	0.41	isocitrate dehydrogenase
pdhD					dihydrolipoamide dehydrogenase
frdC	-0.29	-0.28			fumarate reductase flavoprotein subunit
pycA	-0.36	-0.60		-0.25	pyruvate carboxylase
pdhA		-0.51			pyruvate dehydrogenase E1 component alpha subunit
pdhB		-0.36			pyruvate dehydrogenase E1 component beta subunit
pdhC		-0.35			pyruvate dehydrogenase complex E2 component

APPENDIX E

Pentose phosphate pathway

Gene/locus	Chip 1		Chip 2		Annotation
	BK1506 logFC	BK1503 logFC	BK1506 logFC	BK1503 logFC	
pgi		0.54			glucose-6-phosphate isomerase
llmg_2499		-0.38			glucose-6-phosphate 1-dehydrogenase
llmg_2431			0.30	0.53	hypothetical protein
gntZ	0.29				6-phosphogluconate dehydrogenase-like protein
gnd		-0.50		-0.21	6-phosphogluconate dehydrogenase
rpe		-0.60	0.15		ribulose-phosphate 3-epimerase
rpe2			-0.17	-0.20	ribulose-phosphate 3-epimerase
tkt		-0.54			transketolase
rpiA	-0.26	0.47	-0.25	-0.38	ribose-5-phosphate isomerase A
rpiB					ribose-5-phosphate isomerase B
deoC		-0.58			deoxyribose-phosphate aldolase
rbsK			0.17		ribokinase
deoB	-0.25	-0.76			phosphopentomutase
prsB	-0.32	-0.26	-0.22	-0.30	ribose-phosphate pyrophosphokinase
prsA					ribose-phosphate pyrophosphokinase
kdgA	0.55		0.22	0.40	keto-hydroxyglutarate-aldolase
gntK		-0.26	0.13		gluconate kinase
fbaA	-0.36	-0.46			fructose-bisphosphate aldolase
fbp	0.60	0.65	0.19	0.39	fructose-bisphosphatase
pfk	-0.26	-0.56			6-phosphofructokinase

APPENDIX E

Pyruvate metabolism

Gene/locus	Chip 1 BK1506 logFC	Chip 1 BK1503 logFC	Chip 2 BK1506 logFC	Chip 2 BK1503 logFC	Annotation
fadD	-0.26				long-chain acyl-CoA synthetase
pdhA		-0.51			pyruvate dehydrogenase E1 component alpha subunit
pdhB		-0.36			pyruvate dehydrogenase E1 component beta subunit
pdhC		-0.35			pyruvate dehydrogenase complex E2 component
pdhD					dihydrolipoamide dehydrogenase
pfl					formate acetyltransferase
adhE	0.52		0.49	0.64	bifunctional acetaldehyde-CoA/alcohol dehydrogenase
ackA2	-0.33	-0.35			acetate kinase
ackA1	0.30				AckA1 protein
eutD	-0.25	-0.23	-0.47	-0.61	phosphotransacetylase
pyk	-0.27	-1.09			pyruvate kinase
accA					AccA protein
accB		-1.05			acetyl-CoA carboxylase biotin carboxyl carrier protein subunit
accC	-0.26	-0.71			acetyl-CoA carboxylase biotin carboxylase subunit
accD		-0.28			acetyl-CoA carboxylase subunit beta
llmg_0568		-0.27	0.39	0.45	acylphosphatase
poxL			0.19		pyruvate oxidase
ldhX		0.45	-0.17	-0.34	L-lactate dehydrogenase
ldhB					L-lactate dehydrogenase
ldh	-0.51	-0.45		-0.62	L-lactate dehydrogenase
llmg_0184		0.80		-0.14	putative lactoylglutathione lyase
mleS				-0.26	malate dehydrogenase
pycA	-0.36	-0.60		-0.25	pyruvate carboxylase
thiL			-0.34	-0.28	ThiL protein

APPENDIX E

Amino sugar and nucleotide sugar metabolism

Gene/locus	Chip 1 BK1506 logFC	Chip 1 BK1503 logFC	Chip 2 BK1506 logFC	Chip 2 BK1503 logFC	Annotation
chiC			0.43	0.50	acidic endochitinase precursor
nagZ					putative beta-N-acetylglucosaminidase
murQ, yfeU	0.31		0.15		N-acetylmuramic acid-6-phosphate etherase
glmU	-0.30	-0.56	0.20	0.27	bifunctional N-acetylglucosamine-1-phosphate
llmg_1317	-0.38	0.49			N-acetylmannosamine-6-phosphate 2-epimerase
murA2	-0.63	-0.35			UDP-N-acetylglucosamine 1-carboxyvinyltransferase
murA1			-0.22	-0.27	UDP-N-acetylglucosamine 1-carboxyvinyltransferase
murB					UDP-N-acetylenolpyruvoylglucosamine reductase
glmM			-0.30	-0.32	phosphoglucosamine mutase
nagA	-0.64	-0.45	-0.25	-0.21	NagA protein
scrK	0.53	-0.22	0.40	0.68	fructokinase
nagB		0.40	-0.33	-0.44	glucosamine-6-phosphate isomerase
glmS	-0.37	-0.98		-0.23	glucosamine--fructose-6-phosphate aminotransferase
llmg_1608			0.20	0.50	putative glycosyl hydrolases
llmg_1320			-0.14	-0.26	putative xylan beta-1,4-xylosidase
glk	-0.25	-0.57			glucokinase
pgi		0.54			glucose-6-phosphate isomerase
galU					UDP--glucose-1-phosphate uridylyltransferase
galT			0.23	0.56	galactose-1-phosphate uridylyltransferase
ugd					UDP-glucose 6-dehydrogenase
galK				0.32	galactokinase
galE	-0.32	0.33	-0.17	-0.36	GalE protein
llmg_2003				-0.39	UDP-glucose 4-epimerase
llmg_0247	0.65				putative UDP-glucose 4-epimerase
ptnAB		-0.78			PTS system, mannose-specific IIAB components
ptnC		-0.65			mannose-specific PTS system component IIC
ptnD		-0.49			mannose-specific PTS system component IID
pmi		0.98		0.40	mannose-6-phosphate isomerase
glgD				0.29	glucose-1-phosphate adenylyltransferase
glgC			0.24	0.28	glucose-1-phosphate adenylyltransferase

APPENDIX E

Phosphotransferase system (PTS) and glucose uptake

Gene/locus	Chip 1		Chip 2		Annotation
	BK1506 logFC	BK1503 logFC	BK1506 logFC	BK1503 logFC	
ptsI		-0.37			phosphoenolpyruvate-protein phosphotransferase
ptsH		0.24			phosphocarrier protein HPr
ptsK	-0.25	0.32			HPr kinase/phosphorylase
hprT		0.30			HprT protein
llmg_1426					sucrose-specific PTS system IIBC component
bglP			0.46	0.42	PTS system, beta-glucosides specific enzyme IIABC
llmg_0453					sucrose-specific PTS enzyme IIABC
llmg_0454		-0.25			beta-glucoside-specific PTS system IIABC component
ptcA		1.00	0.35	0.55	cellobiose-specific PTS system IIA component
ptcB		0.83	0.31	0.43	cellobiose-specific PTS system IIB component
llmg_1244	0.40		0.16	0.35	hypothetical protein
ptcC	0.59		0.45	0.47	cellobiose-specific PTS system IIC component
celB	1.34	0.33	0.33	0.44	cellobiose-specific PTS system IIC component
mtlF			0.25		PTS system, mannitol-specific IIA component
mtlA			0.53	0.73	PTS system, mannitol-specific IIBC component
ptnAB		-0.78			PTS system, mannose-specific IIAB components
ptnC		-0.65			mannose-specific PTS system component IIC
ptnD		-0.49			mannose-specific PTS system component IID
llmg_0866	0.42				PTS system, enzyme IIB component
ulaA, sgaT	0.85		0.39	0.63	PTS system ascorbate-specific transporter subunit IIC
fruA	0.37		0.23	0.41	PTS system, fructose specific IIBC components
glcU					putative glucose uptake protein GlcU

APPENDIX E

RNA degradation

Gene/locus	Chip 1 BK1506 logFC	Chip 1 BK1503 logFC	Chip 2 BK1506 logFC	Chip 2 BK1503 logFC	Annotation
nudH					dinucleoside polyphosphate hydrolase
eno	-0.28				phosphopyruvate hydratase
pnpA		-0.56			polynucleotide phosphorylase/polyadenylase
vacB1		-0.49			putative exoribonuclease R
vacB2		0.89	-0.40	-0.53	putative exoribonuclease R
rheA				-0.26	ATP-dependent RNA helicase
recQ		-0.38			ATP-dependent DNA helicase RecQ
llmg_0302		-0.60			putative Zn-dependent hydrolase
dnaK					molecular chaperone DnaK
groEL		-0.31			chaperonin GroEL

APPENDIX E

ABC transporters

Gene/locus	Chip 1 BK1506 logFC	Chip 1 BK1503 logFC	Chip 2 BK1506 logFC	Chip 2 BK1503 logFC	Annotation
potD	-0.51	0.47		-0.18	spermidine/putrescine ABC transporter substrate-binding protein
potC				0.16	spermidine/putrescine ABC transporter permease
potB			0.17		spermidine/putrescine ABC transporter permease
potA	-0.28		0.21	0.29	spermidine/putrescine ABC transporter ATP-binding protein
busAB	-0.28	0.30			glycine betaine-binding periplasmic protein precursor
busAA		-0.35			glycine betaine/proline ABC transporter
choS		0.35	0.19	0.55	choline ABC transporter permease and substrate binding protein
choQ		-0.33	0.13	0.38	choline ABC transporter ATP binding protein
malE					maltose ABC transporter substrate binding protein
malF	0.72		0.22	0.23	maltose transport system permease protein MalF
malG				0.48	maltose ABC transporter permease protein MalG
msmK		0.80	0.31	0.67	multiple sugar-binding transport ATP-binding protein
rbsB			0.12		ribose ABC transporter substrate binding protein RbsB
rbsC					ribose transport system permease protein RbsC
rbsD			0.23		ribose ABC transporter permease protein RbsD
rbsD			0.16		D-ribose pyranase
pstE	-0.48	-0.54			phosphate transport substrate binding protein PstE
pstF		-0.45	0.31	0.57	phosphate transport substrate binding protein PstF
pstD		-0.84	0.24		phosphate transport system permease protein PstD
pstC		-0.76	0.27	0.20	phosphate transport system permease protein PstC
pstA	-0.65	-0.62		-0.20	phosphate transporter ATP-binding protein
pstB		-0.69	0.15	0.21	phosphate transporter ATP-binding protein
phnD	-0.33	-0.32	-0.14		phosphonate ABC transporter, phosphonate-binding protein PhnD
phnB		-0.79			phosphonate transport system permease protein PhnB
llmg_0315	-0.53	-0.78			phosphonate ABC transporter permease
phnC		-0.81		-0.28	phosphonates import ATP-binding protein PhnC
plpA					D-methionine-binding lipoprotein PlpA precursor
plpB					D-methionine-binding lipoprotein PlpB precursor
plpC		-0.28			D-methionine-binding lipoprotein PlpC precursor
plpD		-0.25			D-methionine-binding lipoprotein PlpD precursor
llmg_0342	0.44	-0.22	0.17		amino acid ABC transporter permease protein
llmg_0341	0.44	-0.24	0.20	0.21	amino acid ABC transporter ATP binding protein
dppA					dipeptide-binding protein precursor
dppB	0.48				dipeptide transport system permease protein DppB
dppC	0.92				dipeptide transport system permease protein DppC
dppD	0.76	-0.23	0.16		dipeptide transport ATP-binding protein DppD
dppF	0.60				dipeptide transport ATP-binding protein DppF
fhuD			-0.21	-0.38	ferrichrome ABC transporter substrate binding protein
fhuB			0.16		ferrichrome ABC transporter permease protein
fhuG					ferrichrome ABC transporter permease protein
llmg_1281				-0.27	putative ABC transporter ATP-binding protein
fhuC					ferrichrome ABC transporter FhuC
zitS		0.48	0.30		zinc ABC transporter substrate binding protein
zitP	0.61		0.42		zinc ABC transporter permease protein
zitQ		0.29	0.47		zinc ABC transporter ATP binding protein
cbiQ	0.50				putative cobalt ABC transporter permease protein
cbiQ2		-0.57			putative cobalt ABC transporter permease protein
cbiO	0.39				putative cobalt ABC transporter ATP-binding protein
cbiO	-0.40				cobalt transporter ATP-binding subunit
cbiO		-0.55	0.22	0.23	cobalt transporter ATP-binding subunit
llmg_1552		0.38			putative ABC type transport system permease protein
drrB			0.47	0.58	daunorubicin resistance transmembrane protein
llmg_0262	0.58	0.25		0.36	ABC transporter permease protein
llmg_1553				0.31	putative ABC transporter ATP-binding protein
drrA			0.32	0.39	daunorubicin resistance ABC transporter ATP-binding subunit
tagG	0.96	0.39			teichoic acid ABC transporter permease protein
tagH	0.51		0.16		teichoic acid export ATP-binding protein TagH
ftsX		-0.30		-0.25	cell division protein FtsX-like protein
ftsE	-0.40				cell division ATP-binding protein
llmg_1202	-0.24	0.45			ABC transporter ABC binding and permease protein
cydD					cytochrome d ABC transporter ATP binding and permease protein
cydC			0.21		cytochrome d ABC transporter ATP binding and permease protein
llmg_0989					ABC transporter ATP binding and permease protein

APPENDIX E

Two-component system

Gene/locus	Chip 1 BK1506 logFC	Chip 1 BK1503 logFC	Chip 2 BK1506 logFC	Chip 2 BK1503 logFC	Annotation
apl				-0.25	alkaline phosphatase
pstE	-0.48	-0.54			phosphate transport substrate binding protein PstE
pstF		-0.45	0.31	0.57	phosphate transport substrate binding protein PstF
kinC					sensor histidine kinase
llrC					two-component system regulator
llmg_0458					hypothetical protein
dnaA	-0.36	0.63			chromosomal replication initiation protein
mleS				-0.26	malate dehydrogenase
comX	-0.39				competence regulator ComX
frdC	-0.29	-0.28			fumarate reductase flavoprotein subunit
llmg_0018		0.30			beta-lactamase A
kinD		-0.27			sensor protein kinase KinD
glnB	-0.30	-0.33	-0.53	-0.79	nitrogen regulatory protein P-II
glnA		-0.61			GlnA protein
thiL			-0.34	-0.28	ThiL protein
cydA		-0.27		0.14	cytochrome bd-I ubiquinol oxidase subunit I
cydB					cytochrome d ubiquinol oxidase, subunit II

APPENDIX E

Genes under codY regulation

Gene/locus	Chip 1 BK1506 logFC	Chip 1 BK1503 logFC	Chip 2 BK1506 logFC	Chip 2 BK1503 logFC	Annotation
dppA					dipeptide-binding protein precursor
dppB	0.48				dipeptide transport system permease protein DppB
dppC	0.92				dipeptide transport system permease protein DppC
dppD	0.76	-0.23	0.16		dipeptide transport ATP-binding protein DppD
dppF	0.60				dipeptide transport ATP-binding protein DppF
gltD		-0.22			glutamate synthase subunit beta
gltB		-0.77	0.15		glutamate synthase, large subunit
lysA				-0.22	LysA protein
ilvD		-0.61	0.19		dihydroxy-acid dehydratase
ilvB		-0.57	0.24	0.22	acetolactate synthase catalytic subunit
ilvN		-0.64	0.29		acetolactate synthase 3 regulatory subunit
ilvC		-0.64	0.31		ketol-acid reductoisomerase
ilvA		-0.56	0.26		threonine dehydratase
aldB	-0.39	-0.34			AldB protein
hisC				0.18	histidinol-phosphate aminotransferase
hisZ			0.27	0.52	ATP phosphoribosyltransferase regulatory subunit
hisG			0.26	0.47	ATP phosphoribosyltransferase catalytic subunit
hisD			0.22	0.58	HisD protein
hisB				0.45	imidazoleglycerol-phosphate dehydratase
hisH				0.27	imidazole glycerol phosphate synthase subunit HisH
hisA			0.19	0.42	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)
hisF			0.25	0.47	imidazole glycerol phosphate synthase subunit HisF
hisI			0.36	0.72	bifunctional phosphoribosyl-AMP cyclohydrolase/phosphoribosyl
hisK			0.25	0.60	histidinol-phosphatase
ctrA		-0.44			putative amino-acid transporter
oppD		-0.73			oligopeptide transport ATP-binding protein OppD
oppF		-0.63			oligopeptide transport ATP-binding protein OppF
oppB		-0.38			peptide transport system permease protein OppB
oppC		-0.40			oligopeptide transport system permease protein OppC
oppA					oligopeptide-binding protein OppA precursor
pepO					endopeptidase O
asnB		-0.29			asparagine synthetase B
gltA			0.26	0.33	citrate synthase
citB				0.40	aconitate hydratase
icD			0.25	0.41	isocitrate dehydrogenase
serC				0.18	phosphoserine aminotransferase
serA	-0.31	-0.64	0.18	0.24	D-3-phosphoglycerate dehydrogenase
serB		-0.49			SerB protein
ArcD1			0.34	0.21	arginine/ornithine antiporter
ArcC1			0.31	0.29	carbamate kinase
ArcC2					carbamate kinase
amtB		-0.45	-0.35	-0.55	ammonium transporter AmtB
dapB			0.25	0.30	dihydrodipicolinate reductase
FtsW1					cell division protein ftsW1

APPENDIX E

Genes under ccpA regulation

Gene/locus	Chip 1 BK1506 logFC	Chip 1 BK1503 logFC	Chip 2 BK1506 logFC	Chip 2 BK1503 logFC	Effect	Annotation
galP	0.76		0.34	0.49	Rep	galactose permease
galM	0.57			0.28	Rep	aldose 1-epimerase
galK				0.32	Rep	galactokinase
galT			0.23	0.56	Rep	galactose-1-phosphate uridylyltransferase
galE	-0.32	0.33	-0.17	-0.36	Rep	GalE protein
mtlA			0.53	0.73	Rep	PTS system, mannitol-specific IIBC component
mtlR			0.36		Rep	transcriptional regulator mtl operon MtlR
mtlF	0.23		0.25		Rep	PTS system, mannitol-specific IIA component
mtlD			0.22		Rep	mannitol-1-phosphate 5-dehydrogenase
llmg_0453					Rep	sucrose-specific PTS enzyme IIABC
llmg_0454		-0.25			Rep	beta-glucoside-specific PTS system IIABC component
trePP		-0.21			Rep	putative trehalose/maltose hydrolase
pgmB		0.36			Rep	beta-phosphoglucomutase
llmg_0431	0.41		0.30		Rep	putative acyl carrier protein phosphodiesterase 2
llmg_0432					Rep	transcription regulator
ptcB		0.83	0.31	0.43	Rep	cellobiose-specific PTS system IIB component
ptcA		1.00	0.35	0.55	Rep	cellobiose-specific PTS system IIA component
llmg_0439		0.38			Rep	LacI family transcription regulator
ptcC	0.59		0.45	0.47	Rep	cellobiose-specific PTS system IIC component
bglA			0.17	0.23	Rep	6-phospho-beta-glucosidase
arcA	0.63		0.18	0.27	Rep	arginine deiminase
arcB			0.28		Rep	ornithine carbamoyltransferase
arcD1			0.34	0.21	Rep	arginine/ornithine antiporter
arcC1			0.31	0.29	Rep	carbamate kinase
arcC2					Rep	carbamate kinase
pfk	-0.26	-0.56			Act	6-phosphofructokinase
pyk	-0.27	-1.09			Act	pyruvate kinase
ldh	-0.51	-0.45		-0.62	Act	L-lactate dehydrogenase
pgiA		0.54			Act	glucose-6-phosphate isomerase
fruA	0.37		0.23	0.41	Act	PTS system, fructose specific IIBC components
fruC		-0.39	0.20	0.44	Act	tagatose-6-phosphate kinase
fruR				0.34	Act	lactose transport regulator

APPENDIX E

Fatty acid biosynthesis and fatty acid metabolism

Gene/locus	Chip 1		Chip 2		Annotation
	BK1506 logFC	BK1503 logFC	BK1506 logFC	BK1503 logFC	
accA					AccA protein
accB		-1.05			acetyl-CoA carboxylase biotin carboxyl carrier protein subunit
accC	-0.26	-0.71			acetyl-CoA carboxylase biotin carboxylase subunit
accD		-0.28			acetyl-CoA carboxylase subunit beta
fabD		-0.76			malonyl CoA-acyl carrier protein transacylase
fabF		-1.05			3-oxoacyl-[acyl-carrier-protein] synthase II
fabH	-0.54	-0.51			3-oxoacyl-(acyl carrier protein) synthase III
fabG	-0.33	-0.92			3-ketoacyl-(acyl-carrier-protein) reductase
fabG	-0.38	-0.58			3-ketoacyl-(acyl-carrier-protein) reductase
fabZ		-1.07			(3R)-hydroxymyristoyl-ACP dehydratase
llmg_0538	-0.30	0.66	-0.12		(3R)-hydroxymyristoyl-(acyl-carrier-protein) dehydratase
fabI			-0.16		enoyl-(acyl carrier protein) reductase
llmg_1415		0.58	-0.16		hypothetical protein
thiL			-0.34	-0.28	ThiL protein
llmg_1965					putative AMP-binding enzyme
adhE	0.52		0.49	0.64	bifunctional acetaldehyde-CoA/alcohol dehydrogenase
adhA, adhP				0.30	alcohol dehydrogenase
llmg_0955					alcohol dehydrogenase

APPENDIX E

Glycerolipid and glycerol metabolism

Gene/locus	Chip 1 BK1506 logFC	BK1503 logFC	Chip 2 BK1506 logFC	BK1503 logFC	Annotation
llmg_0870		-0.30	-0.26	-0.48	transporter
glpF2	0.67		0.54	0.60	glycerol uptake facilitator
glpF3		0.45	-0.22	-0.60	putative glycerol uptake facilitator protein
dhaK	0.35		-0.25	-0.25	DhaKLM operon coactivator DhaQ
dhaM	0.57			0.20	dihydroxyacetone kinase DhaM
glpK	0.52		0.20	0.15	glycerol kinase
plsX			-0.17	-0.24	putative glycerol-3-phosphate acyltransferase PlsX
llmg_1540				-0.40	putative glycerol-3-phosphate acyltransferase PlsY
llmg_0119			-0.40	-0.64	putative acyltransferase
dgkA	-0.34	0.38		0.19	DgkA protein
llmg_2421			-0.19	-0.21	hypothetical protein
glpD	0.44		0.25		GlpD protein
gpsA		-0.59			NAD(P)H-dependent glycerol-3-phosphate dehydrogenase
llmg_0945		-0.49			putative glycerol dehydrogenase

APPENDIX E

Purine metabolism

Gene/locus	Chip 1 BK1506 logFC	Chip 1 BK1503 logFC	Chip 2 BK1506 logFC	Chip 2 BK1503 logFC	Annotation
llmg_2075	-0.30	-0.70			ADP-ribose pyrophosphatase
deoB	-0.25	-0.76			phosphopentomutase
prsB	-0.32	-0.26	-0.22	-0.30	ribose-phosphate pyrophosphokinase
prsA					ribose-phosphate pyrophosphokinase
purF		-0.90			amidophosphoribosyltransferase
purD	-0.50	-0.69			phosphoribosylamine--glycine ligase
purN			-0.40	-0.58	phosphoribosylglycinamide formyltransferase
purS	-0.21				phosphoribosylformylglycinamidine synthetase PurS
purQ	-0.57	-0.84			phosphoribosylformylglycinamidine synthase I
purL		-1.10			phosphoribosylformylglycinamidine synthase II
purM	-0.32	-0.39		-0.17	phosphoribosylaminoimidazole synthetase
purK	-0.31				phosphoribosylaminoimidazole carboxylase ATPase subunit
purE		-0.59			phosphoribosylaminoimidazole carboxylase catalytic subunit
purC					phosphoribosylaminoimidazole-succinocarboxamide synthase
purB	-0.27	0.33			adenylosuccinate lyase
purH	-0.43	-1.15			bifunctional phosphoribosylaminoimidazolecarboxamide….
apt			-0.12	-0.36	adenine phosphoribosyltransferase
nucA			-0.28		5'-nucleotidase
llmg_0192				0.28	5'-nucleotidase precursor
deoD	-0.51	0.64			purine nucleoside phosphorylase
hprT		0.30			HprT protein
hpt		0.29			hypoxanthine phosphoribosyltransferase
guaB	-0.35	-0.77	-0.19	-0.32	inosine 5'-monophosphate dehydrogenase
llmg_1188		-0.62			hypothetical protein
xpt	-0.42	-0.44		-0.25	xanthine phosphoribosyltransferase
guaA		-0.85		-0.32	GMP synthase
guaC	-0.36		-0.47	-0.61	guanosine 5'-monophosphate oxidoreductase
gmk					guanylate kinase
pyk	-0.27	-1.09			pyruvate kinase
nrdE					ribonucleotide-diphosphate reductase subunit alpha
nrdF	-0.41				ribonucleotide-diphosphate reductase subunit beta
llmg_0281		-0.21	-0.24	-0.32	anaerobic ribonucleoside triphosphate reductase
rpoA		-0.82			DNA-directed RNA polymerase subunit alpha
rpoB		-0.92			DNA-directed RNA polymerase subunit beta
rpoC		-0.84			DNA-directed RNA polymerase subunit beta'
rpoE	-0.26				DNA-directed RNA polymerase subunit delta
rpoZ	-0.37				DNA-directed RNA polymerase subunit omega
polA	0.28				DNA polymerase I
dnaE		0.22	-0.11		DNA polymerase III DnaE
polC	0.76				DNA polymerase III PolC
dnaN		1.05			DNA polymerase III subunit beta
dnaX		-0.44			DNA polymerase III subunits gamma and tau
holA	-0.39				DNA polymerase III subunit delta
holB	-0.24	-0.29			DNA polymerase III subunit delta'
dnaQ			-0.25	-0.21	DNA polymerase III, epsilon chain
relA					GTP pyrophosphokinase
llmg_0382				0.19	putative GTP pyrophosphokinase
purA		-0.28			adenylosuccinate synthetase
add	-0.32	0.23	-0.27	-0.60	adenosine deaminase
cpdC	-0.32	-0.39	-0.26	-0.50	2',3'-cyclic-nucleotide 2'-phosphodiesterase
adk		0.54		0.33	adenylate kinase
pnpA		-0.56			polynucleotide phosphorylase/polyadenylase
arcC2					carbamate kinase
arcC1			0.31	0.29	carbamate kinase

APPENDIX E

Pyrimidine metabolism

Gene/locus	Chip 1 BK1506 logFC	Chip 1 BK1503 logFC	Chip 2 BK1506 logFC	Chip 2 BK1503 logFC	Annotation
carB	-0.33	-1.00			carbamoyl phosphate synthase large subunit
carA		-0.71			carbamoyl phosphate synthase small subunit
pyrB		-0.77			aspartate carbamoyltransferase catalytic subunit
pyrC	-0.33	-0.55			dihydroorotase
pyrDA	-0.38		-0.39	-0.53	dihydroorotate dehydrogenase 1A
pyrDB		-1.07			dihydroorotate dehydrogenase 1B
pyrE	-0.33	-0.48			orotate phosphoribosyltransferase
pyrF	-0.60	-0.70	-0.27	-0.53	orotidine 5'-phosphate decarboxylase
cmk	-0.33		-0.13		cytidylate kinase
pyrH					uridylate kinase
pnpA		-0.56			polynucleotide phosphorylase/polyadenylase
llmg_1188		-0.62			hypothetical protein
pyrG		-0.31			CTP synthetase
rpoA		-0.82			DNA-directed RNA polymerase subunit alpha
rpoB		-0.92			DNA-directed RNA polymerase subunit beta
rpoC		-0.84			DNA-directed RNA polymerase subunit beta'
rpoE	-0.26				DNA-directed RNA polymerase subunit delta
rpoZ	-0.37				DNA-directed RNA polymerase subunit omega
polA	0.28				DNA polymerase I
dnaE		0.22	-0.11		DNA polymerase III DnaE
polC	0.76				DNA polymerase III PolC
dnaN		1.05			DNA polymerase III subunit beta
dnaX		-0.44			DNA polymerase III subunits gamma and tau
holA	-0.39				DNA polymerase III subunit delta
holB	-0.24	-0.29			DNA polymerase III subunit delta'
dnaQ			-0.25	-0.21	DNA polymerase III, epsilon chain
udk	-0.20	0.59	-0.52	-0.37	uridine kinase
nucA			-0.28		5'-nucleotidase
llmg_0192				0.28	5'-nucleotidase precursor
udp	-0.45	-0.30			uridine phosphorylase
upp	-0.27	0.34	-0.30	-0.49	uracil phosphoribosyltransferase
pyrR		-0.63			bifunctional pyrimidine regulatory protein PyrR …
pdp		-0.58	0.16	0.26	pyrimidine-nucleoside phosphorylase
trxB1		0.21	-0.21		TrxB1 protein
trxB2			-0.27	-0.31	TrxB2 protein
llmg_0281		-0.21	-0.24	-0.32	anaerobic ribonucleoside triphosphate reductase
nrdE					ribonucleotide-diphosphate reductase subunit alpha
nrdF	-0.41				ribonucleotide-diphosphate reductase subunit beta
ps428	-0.26		0.25	0.27	deoxyuridine 5'-triphosphate nucleotidohydrolase
ps325			0.14	0.20	deoxyuridine 5'-triphosphate nucleotidohydrolase
dut					deoxyuridine 5'-triphosphate nucleotidohydrolase
thyA		0.92	-0.47	-0.38	thymidylate synthase
cdd		-0.43			Cdd protein
deoD	-0.51	0.64			purine nucleoside phosphorylase
llmg_1416		0.33	-0.27		hypothetical protein
ntd			0.17		nucleoside deoxyribosyltransferase
tdk					thymidine kinase
tmk	-0.64	-0.32		-0.22	thymidylate kinase
cpdC	-0.32	-0.39	-0.26	-0.50	2',3'-cyclic-nucleotide 2'-phosphodiesterase

APPENDIX E

Arginine and proline metabolism

Gene/locus	Chip 1 BK1506 logFC	Chip 1 BK1503 logFC	Chip 2 BK1506 logFC	Chip 2 BK1503 logFC	Annotation
speG		0.25	-0.13		spermidine acetyltransferase
llmg_0177			0.28	0.40	amidase
proC	-0.39	-0.37	-0.18	-0.23	pyrroline-5-carboxylate reductase
ocd			-0.34	-0.46	ornithine cyclodeaminase, mu-crystallin-like protein
proB		-0.47	-0.13	-0.28	gamma-glutamyl kinase
proA			-0.15	-0.26	gamma-glutamyl phosphate reductase
kdgA	0.55		0.22	0.40	keto-hydroxyglutarate-aldolase…
arcB			0.28		ornithine carbamoyltransferase
argF			-0.53	-0.67	ornithine carbamoyltransferase
argG		-0.41	0.17	0.30	argininosuccinate synthase
argH					argininosuccinate lyase
arcA	0.63		0.18	0.27	arginine deiminase
glnA		-0.61			GlnA protein
arcC2					carbamate kinase
arcC1			0.31	0.29	carbamate kinase
argJ		-0.29		-0.24	bifunctional ornithine acetyltransferase…
argB			-0.34	-0.23	acetylglutamate kinase
argC			-0.39	-0.47	N-acetyl-gamma-glutamyl-phosphate reductase
argD	0.59				acetylornithine aminotransferase
argE		-0.45			acetylornithine deacetylase

APPENDIX E

Vitamin and cofactor metabolism

Gene/locus	Chip 1 BK1506 logFC	BK1503 logFC	Chip 2 BK1506 logFC	BK1503 logFC	Annotation
			One carbon pool by folate		
dfrA	-0.64	0.28	-0.14		DfrA protein
fhs	-0.27				formate--tetrahydrofolate ligase
folD	-0.36	-0.35			bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase…
glyA	-0.25	-0.33	-0.13	-0.25	serine hydroxymethyltransferase
purN			-0.40	-0.58	phosphoribosylglycinamide formyltransferase
purH	-0.43	-1.15			bifunctional phosphoribosylaminoimidazolecarboxamide…
fmt		-0.52			methionyl-tRNA formyltransferase
thyA		0.92	-0.47	-0.38	thymidylate synthase
metF			-0.24	-0.34	5,10-methylenetetrahydrofolate reductase
llmg_0181	0.23				5-formyltetrahydrofolate cyclo-ligase family protein
			Thiamine metabolism		
thiD1	0.41				phosphomethylpyrimidine kinase
nifS				0.19	putative iron-sulfur cofactor synthesis protein
nifZ	-0.50	0.47	-0.33	-0.48	pyridoxal-phosphate dependent aminotransferase
llmg_1972		-0.61	0.12		hypothetical protein
thiI		-0.40	-0.46	-0.42	thiamine biosynthesis protein ThiI
thiM					hydroxyethylthiazole kinase
thiE	-0.24				thiamine-phosphate pyrophosphorylase
thiN					thiamin pyrophosphokinase
tenA			-0.23	-0.27	transcriptional activator TenA
			Riboflavin metabolism		
ribA					riboflavin biosynthesis protein RibA
ribD					riboflavin biosynthesis protein RibD
ribH				0.27	6,7-dimethyl-8-ribityllumazine synthase
ribB	0.40				riboflavin synthase subunit alpha
ribC, ribF		0.51			bifunctional riboflavin kinase/FMN adenylyltransferase
			Vitamin B6 metabolism		
thiD2					phosphomethylpyrimidine kinase
serC				0.18	phosphoserine aminotransferase
thrC	-0.33	-0.45			threonine synthase
			Nicotinate and nicotinamide metabolism		
pncB	-0.34	-0.38			nicotinate phosphoribosyltransferase
deoD	-0.51	0.64			purine nucleoside phosphorylase
nucA			-0.28		5'-nucleotidase
llmg_0192				0.28	5'-nucleotidase precursor
nadD	-0.38	-0.38			nicotinic acid mononucleotide adenylyltransferase
nadD1			0.22	0.28	nicotinate-nucleotide adenylyltransferase
nadE					NAD synthetase
ppnK					inorganic polyphosphate/ATP-NAD kinase
			Pantothenate and CoA biosynthesis		
als		-0.35	-0.25	-0.43	acetolactate synthase
ilvB		-0.57	0.24	0.22	acetolactate synthase catalytic subunit
ilvH		-0.64	0.29		acetolactate synthase 3 regulatory subunit
ilvC		-0.64	0.31		ketol-acid reductoisomerase
ilvD		-0.61	0.19		dihydroxy-acid dehydratase
panE		0.72	-0.42		2-dehydropantoate 2-reductase
coaA	-0.40	0.37	-0.19		pantothenate kinase
dfpB		-0.38			phosphopantothenate--cysteine ligase
dfpA		-0.45			phosphopantothenoylcysteine decarboxylase
coaD					phosphopantetheine adenylyltransferase
coaE		0.19			dephospho-CoA kinase
acpS		-0.48			4'-phosphopantetheinyl transferase
ilvE			-0.36	-0.55	branched-chain amino acid aminotransferase
			Biotin metabolism		
birA2		0.20		0.18	acetyl-CoA carboxylase ligase / biotin operon repressor…
birA1	-0.23		-0.32	-0.33	biotin--[acetyl-CoA-carboxylase] ligase and biotin operon…

Appendix F

The script used to perform the analysis of microarray data using R and Bioconductor.

This section contains the R-script used to perform the analysis in the statistical software package R. The analysis was performed by running a subset of the commands found within the entire script.

APPENDIX F

```
# Source:
http://matticklab.com/index.php?title=Single_channel_analysis_of_Agilent_microarray_dat
a_with_Limma

# work in progress...

# basicRMA() comes from the oligo package

library(limma)

library(oligo)

library(affy)

# specify the a folder in relative of full path where plots are saved

path <- 'Plots chip 1'

#Set working directory, tell R where to find the samples

setwd('C:/Users/achh/Documents/Projekter/02 - PhD projekt - GLYFINERY
(open)/Microarray/02 - ATPase stammer - August-November 2011/Data analyse')

# Read the targets file (filenames and conditions)

targets <- readTargets("Samples.txt")

# Array of the slide index numbers used internally in the program

nArrays = length(targets$FileName)

slideIndex = c(1:nArrays)

# Initialization of sample names and colors for plotting of Densities. The color array
should be tweaked

sampleNames = paste(targets$Condition, targets$Sample, sep=" ")

if(length(targets$Chip) == length(sampleNames)) sampleNames <- paste(sampleNames, "
Chip ", targets$Chip, sep="")

#sampleNames without "chip"

sampleNamesNoChip = paste(targets$Condition, targets$Sample, sep=" ")

b10 <- targets$Condition == "BK1010"

b06 <- targets$Condition == "BK1506"

b03 <- targets$Condition == "BK1503"

colors <- c()

colors[b10] =  hsv(seq(0, 0.15,    length.out = sum(b10)), seq(0.6, 1, length.out =
sum(b10)),seq(0.7, 1, length.out = sum(b10)) ) # red hues to BK1010

colors[b06] =  hsv(seq(0.25, 0.4,  length.out = sum(b06)), seq(0.6, 1, length.out =
sum(b06)),seq(0.7, 1, length.out = sum(b06)) ) # green hues to BK1506

colors[b03] =  hsv(seq(0.57, 0.72, length.out = sum(b03)), seq(0.6, 1, length.out =
sum(b03)),seq(0.7, 1, length.out = sum(b03)) ) # blue hues to BK1506
```

APPENDIX F

```
# Read raw datafiles. Only G and Gb is used later. The red channels need to be filled
with data of the same size for commands to work.

RG <- read.maimages(targets, columns = list(G = "gMedianSignal", Gb =
"gBGMedianSignal", R = "gProcessedSignal", Rb = "gIsPosAndSignif"), annotation =
c("Row", "Col","FeatureNum", "ControlType","ProbeName"))

# Subtract background - Background subtraction often introduce noise - Here only used
in some plots

RGBgCor <- limma::backgroundCorrect(RG, method="normexp", offset=1)

# Paranoid me put zeroes into the background intensities

RG$Gb = RG$Gb * 0

RGBgCor$Gb = RG$Gb

# Normalize data

RG.norm <- RG

RG.norm$G <- normalizeBetweenArrays(RG$G, method="quantile")

RG.spline <- RG

RG.spline$G <- normalize.qspline(RG.spline$G)

# Extract loci from ProbeNames. Normal probes: "llmg_geneNumber_probeIndex", get their
probeIndex stripped. In this way genes from the same locus can be identified.

SpecialCases <- c("rRNA", "tRNA", "pseudo", "r60") # probenames which must be taken
care of seperately. OBS! there is only one probe for each of "rRNA", "tRNA", "pseudo"
genes.

RG.norm$genes$locus <-sapply(strsplit(RG.norm$genes$ProbeName, "_"), function(V){

            if(length(V)==1){return(V)}

            if(V[1]=="llmg" && sum(V[2] == SpecialCases)) {return(paste(V,
collapse="_"))}

            if(V[2] == "phage") {return(paste(V[1:length(V)-1], collapse= "_"))}

            if(V[1] == "llmg") {return(paste(V[1],"_",V[2], sep=""))}

            paste(V, collapse="_")}

)

# Find all non-control probes

# Pseudo genes pilles fra da de kun har en enkelt probe...

BV.nonC <- sapply(RG$genes$ProbeName, function(e){sum(RG$genes$ProbeName == e) <= 2})

BV.highCopy <- sapply(RG.norm$genes$locus, function(e){sum(RG.norm$genes$locus == e) >
3})
```

APPENDIX F

```
# Make RMA (output in log2)

Green = basicRMA(RG.norm$G[BV.nonC & BV.highCopy,], RG.norm$genes$locus[BV.nonC &
BV.highCopy], normalize=FALSE, background = FALSE)

G.norm <- normalize.qspline(Green)

# G.norm <-  basicRMA(RG$G[BV.nonC & BV.highCopy,], RG.norm$genes$locus[BV.nonC &
BV.highCopy], normalize=TRUE, background = FALSE)



# Make MAList object for further processing

reducedLoci = as.data.frame(rownames(Green))

colnames(reducedLoci) = "Locus"

E.norm <- new("MAList", list(targets=RG.norm$targets, genes=reducedLoci,
source=RG.norm$source, M=Green*0, A=G.norm))

E  <- new("MAList", list(targets=RG.norm$targets, genes=reducedLoci,
source=RG.norm$source, M=Green*0, A=Green))

# Plot densities. First comes the red channels then the green. We have gProcessedSignal
in the red channels

plotDensities(E, singlechannels= c((nArrays + 1):(2 * nArrays)), groups = c(
1:nArrays), col=colors )

legend(x = 6.0, y = 0.35, legend = sampleNamesNoChip, fill = colors, col = "black")

dev.print( device = pdf, file = paste(path, "/densities.after.RMA.pdf", sep=""))



plotDensities(RG, singlechannels= c((nArrays + 1):(2 * nArrays)), groups =
c(1:nArrays), col=colors )

legend(x = 1.3, y = 0.45, legend = sampleNames, fill = colors, col = "black")

dev.print( device = pdf, file = paste(path, "/densities.pdf", sep=""))



plotDensities(RGBgCor, singlechannels= c((nArrays + 1):(2 * nArrays)), groups =
c(1:nArrays), col=colors )

legend(x = 1.3, y = 0.45, legend = sampleNames, fill = colors, col = "black")

dev.print( device = pdf, file = paste(path, "/densitiesAfterBackgroundCorrection.pdf",
sep=""))



plotDensities(RG, singlechannels= c(1:nArrays), groups = c(1:nArrays), col=colors )

legend(x = 1.3, y = 0.45, legend = sampleNames, fill = colors, col = "black")

dev.print( device = pdf, file = paste(path, "/densitiesProcessedSig.pdf", sep=""))
```

APPENDIX F

```
plotDensities(RG.norm, singlechannels= c((nArrays + 1):(2 * nArrays)), groups = c(
1:nArrays), col=colors )

legend(x = 15.7, y = 0.33, legend = sampleNames, fill = colors, col = "black")

dev.print( device = pdf, file = paste(path, "/densities.after.norm.quantile.pdf",
sep=""))


plotDensities(RG.spline, singlechannels= c((nArrays + 1):(2 * nArrays)), groups = c(
1:nArrays), col=colors )

legend(x = 15.7, y = 0.33, legend = sampleNames, fill = colors, col = "black")

dev.print( device = pdf, file = paste(path, "/densities.after.norm.qspline.pdf",
sep=""))


plotDensity(E.norm, singlechannels= c((nArrays + 1):(2 * nArrays)), groups = c(
1:nArrays), col=colors  )

legend(x = 6, y = 0.33, legend = sampleNames, fill = colors, col = "black")

dev.print( device = pdf, file = paste(path,
"/densities.after.norm.RMA.norm.qspline.pdf", sep=""))


# Make SVD

SVD <- svd(G.norm - rowMeans(G.norm))

S <-  matrix(0 , ncol(G.norm), ncol(G.norm))

diag(S)<-SVD$d


image( S%*%t(SVD$v), main="Singular Value Decomposition",  xlab = "The components",
axes = FALSE)

axis(side = 2, at = seq(0, 1, length.out = nArrays), labels = sampleNames, las =1,
cex.axis = 0.4)

lines(c(0,1),c((sum(b10)-0.5)/(nArrays-1),(sum(b10)-0.5)/(nArrays-1)))

lines(c(0,1),c((sum(b10+b06)-0.5)/(nArrays-1),(sum(b10+b06)-0.5)/(nArrays-1)))

dev.print( device = pdf, file = paste(path,"/SVD heatmap.pdf", sep = ""))


#PCA lignende clustering

plot(SVD$v[,1], SVD$v[,2], col = "white", main = "Singular Value Decomposition",

            xlab = "First Component", ylab = "Second Component")
```

```
text(SVD$v[,1], SVD$v[,2], sampleNames, col = colors, cex = 0.7)

dev.print( device = pdf, file = paste(path,"/SVD1-2.pdf", sep = ""))

# Plot intensity vs. intensity

# After RMA

# plot(E$A[,1], E$A[,2], pch = 20, cex = 0.3, xlab = paste("Microarray ", 1), ylab =
paste("Microarray ", 2))

# dev.print( device = pdf, file = paste(path,"/Slide ", 2, " Vs slide ", 1, ".pdf"))

#

# plot(E$A[,3], E$A[,4], pch = 20, cex = 0.3, xlab = "Microarray 4"   , ylab =
"Microarray 5")

# dev.print( device = pdf, file = paste(path",/Slide ", 5, " Vs slide ", 4, ".pdf"))

#

# for (i in 1:11) {

#           for (j in (i+1):12) {

#                       plot(E$A[,j], E$A[,i], pch = 20, cex = 0.3, xlab =
paste("Microarray ", j), ylab = paste("Microarray ", i))

#                       dev.print( device = pdf, file = paste(path,"/sample ", i , " Vs
sample ", j , " after RMA.pdf"))

#           }

# }


#plot(log(RG$G[,4],2), log(RG$G[,5],2), pch = 20, cex = 0.3)

# Setup analysis

f <- factor(targets$Condition, levels = unique(targets$Condition))

design <- model.matrix(~0 + f)

colnames(design) <- levels(f)

fit <- lmFit(E$A, design)

contrast.matrix <- makeContrasts("BK1506-BK1010", "BK1503-BK1010", levels=design)

fit2 <- contrasts.fit(fit, contrast.matrix)

fit2 <- eBayes(fit2)


# export data
```

APPENDIX F

```
output <- topTable(fit2, adjust="BH", coef="BK1506-BK1010", genelist=E$genes,
number=40000)

write.table(output, file= paste(path, "/BK1506-BK1010.csv", sep = ""),
sep="\t",row.name=FALSE, quote=FALSE)

output <- topTable(fit2, adjust="BH", coef="BK1503-BK1010", genelist=E$genes,
number=40000)

write.table(output, file=paste(path, "/BK1503-BK1010.csv", sep = ""), sep="\t",
row.name=FALSE, quote=FALSE)



output <- topTable(fit2, adjust="BH", genelist=E$genes, number=40000)

write.table(output, file=paste(path, "/all.csv", sep=""), sep="\t", row.name=FALSE,
quote=FALSE)

# Make volcanoplots

# Volcano BK1506 vs BK1010

# Permuted conditions

#f <- factor(targets$Condition[c(1,4, 2,3, 5:7) ], levels = unique(targets$Condition))

permP <- c()

iter <- 30

for(i in 1:iter) {

f <- factor(sample(targets$Condition), levels = unique(targets$Condition))

design <- model.matrix(~0 + f)

colnames(design) <- levels(f)

fitPerm <- lmFit(E$A, design)

contrast.matrix <- makeContrasts("BK1506-BK1010", "BK1503-BK1010", levels=design)

fitPerm2 <- contrasts.fit(fitPerm, contrast.matrix)

fitPerm2 <- eBayes(fitPerm2)

permP <- rbind(permP, fitPerm2$p.value)

}

pO <- sort(fit2$p.value[,1])

pP <- sort(permP[,1])

nO <- 1

nP <- 1

FDR <- vector(mode = "numeric", length = length(pO))
```

```
while (nO <= length(pO)) {

            while (pO[nO] > pP[nP] & nP <= length(pP)) {nP <- nP+1}

            FDR[nO] <- (nP-1)/nO/iter

            nO <- nO + 1

}

plot(FDR, main = "FDR BK1506", xlab = "significant genes", ylab = "False discovery
rate", type = "p", col= "#00000030")

dev.print( device = pdf, file = paste(path,"/FDR_BK1506.pdf", sep = ""))

# p1 <- permP[order(permP[,1]),1]

#

# count <- c(1:length(fit2$p.value[,1]))

#

# porder1 <- fit2$p.value[order(fit2$p.value[,1]),1]

#

# y1 <- sapply(count,  function(n) {sum(p1 < porder1[n])/n})/iter

#

# plot(y1, main = "FDR BK1506", xlab = "significant genes", ylab = "False discovery
rate", type = "l")

# dev.print( device = pdf, file = paste(path,"/FDR_BK1506.pdf", sep = ""))

# # y1 <- apply(count, function(n) {sum(p1 <
fit2$p.value[order(fit2$p.value[,1]),1][n])/n})

pO <- sort(fit2$p.value[,2])

pP <- sort(fitPerm2$p.value[,2])

nO <- 1

nP <- 1

FDR <- vector(mode = "numeric", length = length(pO))

while (nO <= length(pO)) {

            while (pO[nO] > pP[nP] & nP <= length(pP)) {nP <- nP+1}

            FDR[nO] <- (nP-1)/nO/ncol(fmat)

            nO <- nO + 1

}

plot(FDR, main = "FDR BK1503", xlab = "significant genes", ylab = "False discovery
rate", type = "l")
```

```
dev.print( device = pdf, file = paste(path,"/FDR_BK1503.pdf", sep = ""))

# p2 <- permP[order(permP[,2]),2]

#

# count <- c(1:length(fit2$p.value[,2]))

#

# porder2 <- fit2$p.value[order(fit2$p.value[,2]),2]

#

# y2 <- sapply(count,  function(n) {sum(p2 < porder2[n])/n})/iter

#

# plot(y2, main = "FDR BK1503", xlab = "significant genes", ylab = "False discovery
rate", type = "l")

# dev.print( device = pdf, file = paste(path,"/FDR_BK1503.pdf", sep = ""))

################################################################################

# The volcanoplot - OBS! c(..) er datasæt specifikt!

f <- factor(targets$Condition[c(1,4,6, 2,7, 3,5,8) ], levels =
unique(targets$Condition))

design <- model.matrix(~0 + f)

colnames(design) <- levels(f)

fit <- lmFit(E$A, design)

contrast.matrix <- makeContrasts("BK1506-BK1010", "BK1503-BK1010", levels=design)

fitPerm2 <- contrasts.fit(fit, contrast.matrix)

fitPerm2 <- eBayes(fitPerm2)

plot(fit2$coefficients[,1],fit2$p.value[,1], log = "y", pch = 20, cex = 0.3, main =
"Volcano Plot for BK1506", xlab = "logFC", ylab = "P value", xlim = c(-1.5, 1.5))

points(fitPerm2$coefficients[,1],fitPerm2$p.value[,1], pch = 20, cex = 0.3, col =
"red")

lowP = min(fitPerm2$p.value[,1])

lines(c(-2, 2), c(rep(lowP,2)), col = "red" )

text(1.4, lowP*1.25, paste("p = ", round(lowP,4)), cex = 0.7)

dev.print( device = pdf, file = paste(path, "/volcanoBK1506.pdf", sep=""))


################################################################################
```

APPENDIX F

```
# Volcano BK1503 vs BK1010

# Permuted conditions

# The volcanoplot

plot(fit2$coefficients[,2],fit2$p.value[,2], log = "y", pch = 20, cex = 0.3, main =
"Volcano Plot for BK1503", xlab = "logFC", ylab = "P value", xlim = c(-1.5, 1.5))

points(fitPerm2$coefficients[,2],fitPerm2$p.value[,2], pch = 20, cex = 0.3, col =
"red")

lowP = min(fitPerm2$p.value[,2])

lines(c(-2, 2), c(rep(lowP,2)), col = "red" )

text(1.4, lowP*1.25, paste("p = ", round(lowP,4)), cex = 0.7)

dev.print( device = pdf, file = paste(path, "/volcanoBK1503.pdf", sep=""))

################################################################################################
```