
CATÓLICA
UNIVERSIDADE CATÓLICA PORTUGUESA
ESCOLA SUPERIOR DE BIOTECNOLOGIA

# PRODUCTION OF GLYCEROL AND 1,3-PROPANEDIOL FROM RENEWABLE RESOURCES: STUDY OF NEW BIOCATALYSTS AND PROCESS STRATEGIES

Thesis submitted to the *Universidade Católica Portuguesa* to attain the degree of PhD in Biotechnology with specialization in Microbiology

by

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Under the supervision of Isabel M. Vasconcelos, Professor

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*“The whole is more than the sum of its parts.”*

Aristóteles

# Abstract

1,3-Propanediol can be biologically produced from glycerol by several microorganisms, namely clostridia. However, no natural microorganism is able to synthesize 1,3-propanediol from glucose. Different strategies have been tried to accomplish this conversion and set up a flexible process operating either on glycerol or glucose. In this work the production of 1,3 - propanediol from glucose and sugar cane molasses was investigated in a two-step process using two recombinant microorganisms. The first stage of the process was the conversion of glucose or other sugar into glycerol by a *Saccharomyces cerevisiae* strain. To increase glycerol production, different *tpi1*Δ mutant strains of *S. cerevisiae*, resulting from genetic engineering strategies, focused on the redirection of metabolic fluxes and overexpression of the key enzymes of the glycerol formation pathway, were tested. Best results were obtained with the mutant strain *S. cerevisiae* HC42 which exhibits a glycerol yield of 0.46 $g.g^{-1}$ glucose and a productivity of 3.1 mmol $glycerol.h^{-1}.g^{-1}$ dry mass on 20 $g.l^{-1}$ of glucose. Genomic analysis of this strain revealed that 384 genes had significantly changed in response to the genetic modifications introduced; also, the genetic strategy followed led to an intracellular glycerol concentration 10-fold higher than in the parent strain. To solve this issue, the overexpression of *FPS1* was exploited in *S. cerevisiae* HC42. A novel mutant strain FM62 was obtained; however no significant differences on glycerol intracellular accumulation and production were reported. The strain HC42 was not able to grow on high glucose concentrations.

In order to increase the glycerol production, the strain was adapted to high glucose concentrations (> 200 $g.l^{-1}$). The adapted strains FH100 and FH200 were able to grow on 100 and 200 $g.l^{-1}$ glucose leading to final glycerol concentrations of 49.2 $g.l^{-1}$ and 60.0 $g.l^{-1}$ respectively. FH100 was also cultivated on sugar cane molasses media; the production of glycerol increased with the initial total sugars concentration and 47.1 $g.l^{-1}$ of glycerol were produced when the strain was grown on 101.3 $g.l^{-1}$ total sugars.

The second stage of the process was carried out by the engineered strain *Clostridium acetobutylicum* DG1 (pSPD5) able to convert glycerol to 1,3-propanediol. This two-step strategy led to a flexible process, resulting in a 1,3-propanediol production and yield that depended on the initial sugar concentration. Below 56.2 $g.l^{-1}$ of sugar concentration, cultivation on molasses or glucose showed no significant differences. However, at higher molasses concentrations glycerol initially produced by yeast could not be totally converted into 1,3-propanediol by *C. acetobutylicum* and a lower 1,3-propanediol overall yield was observed. Best results were obtained with an initial glucose concentration of 103 $g.l^{-1}$, leading to a final 1,3-propanediol concentration of 25.5 $g.l^{-1}$, a productivity of 0.16 $g.l^{-1}.h^{-1}$ and 1,3-propanediol yields of 0.56 $g.g^{-1}$ glycerol and 0.24 $g.g^{-1}$ sugar, which is, to our knowledge, the highest value reported for a two-step process. For an initial sugar concentration (from molasses) of 56.2 $g.l^{-1}$, 27.4 $g.l^{-1}$ of glycerol were produced, leading to 14.6 $g.l^{-1}$ of 1.3-propanediol and similar values of productivity, 0.15 $g.l^{-1}.h^{-1}$, and overall yield, 0.26 $g.g^{-1}$ sugar.

# Resumo

O composto 1,3-propanodiol pode ser biologicamente produzido a partir de glicerol por vários microrganismos, nomeadamente clostridia. No entanto, nenhum microrganismo indígena é capaz de sintetizar 1,3-propanodiol a partir de glicose. Têm sido desenvolvidas diferentes estratégias tendo em vista esta conversão e a implementação de um processo flexível, operando quer a partir de glicerol quer a partir de glicose. Neste trabalho, foi investigada a produção de 1,3-pronanodiol a partir de glicose ou melaços de cana-de-açúcar, através de um processo em duas etapas, usando dois microrganismos geneticamente modificados. Na primeira etapa do processo a glicose, ou outro açúcar, foi convertido em glicerol por uma estirpe de *Saccharomyces cerevisiae*. Para aumentar a produção de glicerol, foram desenvolvidas e avaliadas diferentes estirpes mutantes *tpi1*Δ de *S. cerevisiae*, resultantes de estratégias de engenharia genética tendo em vista o redireccionamento dos fluxos metabólicos e a sobre-expressão de enzimas chave da via metabólica de produção de glicerol. Os melhores resultados foram obtidos com a estirpe mutante *S. cerevisiae* HC42, que apresentou um rendimento em glicerol de 0.46 $g.g^{-1}$ glicose e uma produtividade de 3.1 mmol $glicerol.h^{-1}.g^{-1}$ peso seco, em 20 $g.l^{-1}$ de glicose. A análise genómica desta estirpe revelou alterações na expressão de 384 genes em resposta às modificações genéticas introduzidas; para além disso, a estratégia genética seguida resultou numa concentração intracelular de glicerol 10 vezes maior do que na estirpe selvagem. Com o objectivo de resolver esta questão, foi investigada a

sobre-expressão de *FPS1* em *S. cerevisiae* HC42, tendo-se obtido uma nova estirpe mutante, FM62; no entanto, não foram observadas diferenças significativas na produção e na acumulação intracelular de glicerol. A estirpe HC42 não era capaz de crescer em concentrações elevadas de glicose. Para aumentar a produção de glicerol, esta estirpe foi adaptada a elevadas concentrações de glicose (> 200 $g.l^{-1}$). As estirpes adaptadas FH100 e FH200 foram capazes de crescer em 100 e 200 $g.l^{-1}$ glicose, conduzindo a concentrações finais de glicerol de 49.2 $g.l^{-1}$ e 60.0 $g.l^{-1}$ respectivamente. A estirpe FH100 também foi cultivada em meio de melaços de cana-de-açúcar. A produção de glicerol aumentou com a concentração inicial de açúcares totais e foram produzidos 47.1 $g.l^{-1}$ de glicerol quando a estirpe foi cultivada em 101.3 $g.l^{-1}$ açúcar total.

A segunda etapa do processo foi realizada pela estirpe geneticamente modificada *Clostridium acetobutylicum* DG1 (pSPD5), capaz de converter glicerol em 1,3-propanediol. Esta estratégia em dois passos conduziu a um processo flexível, resultando numa produção e rendimento de 1,3-propanodiol dependentes da concentração inicial de açúcar no meio. Para concentrações de açúcar inferiores a 56.2 $g.l^{-1}$ não foram encontradas diferenças entre os processos realizados com glicose ou com melaços. No entanto, para concentrações mais elevadas de melaços, o glicerol inicialmente produzido pela levedura não foi totalmente convertido em 1,3-propanodiol por *C. acetobutylicum* e observou-se um rendimento global em 1,3-propanodiol mais baixo. Os melhores resultados foram obtidos com uma concentração inicial de glicose de 103 $g.l^{-1}$, que conduziu a uma concentração final de 1,3-propanodiol de 25.5 $g.l^{-1}$, uma produtividade de

0.16 $g.l^{-1}.h^{-1}$ e rendimentos de 1,3-propanediol de 0.56 $g.g^{-1}$ de glicerol e 0.24 $g.g^{-1}$ de açúcar, sendo este último o valor mais elevado obtido num processo em duas etapas. Para um teor inicial de açúcar (de melaços) de 56.2 $g.l^{-1}$, foram produzidos 27.4 $g.l^{-1}$ de glicerol, que conduziram a uma concentração de 14.6 $g.l^{-1}$ de 1,3-propanodiol, a valores similares de produtividade, 0.15 $g.l^{-1}.h^{-1}$, e a um rendimento global de 0.26 $g.g^{-1}$ de açúcar.

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# Contents

# Introduction

The state-of-the-art of white biotechnology with the possibility of using renewable feedstocks for the production of many interesting compounds and the present situation of crude oil market and environmental problems toughens industrial biotechnology as an alternative to the chemical industrial production of chemicals based on petrol and derivatives. One of these interesting compounds is 1,3-propanediol (1,3-PD). Due to its simple chemical structure, it can be used in a wide range of applications, and one of the most relevant is the production of polyesters, such as PTT (polytrimetylene terephthalate) used nowadays in the textile industry.

Traditional production of 1,3-PD is based on chemical synthesis, which requires expensive production processes, the use of petroleum derivatives and produces toxic intermediates. The microbial production of 1,3-PD is a very interesting process as low cost renewable resources can be used as fermentation substrates, which provides solutions to environmental pollution and petroleum depletion. According to some studies, biological 1,3-PD has properties equivalent or superior to chemically produced 1,3-PD. Polyesters based on biological 1,3PD are also most promising in terms of biodegradability.

One of the big challenges of white biotechnology is to find cheap production sources to reduce the cost of final products. A natural pathway for the production of 1,3-PD is from glycerol and occurs in a few species of bacteria such as *Clostridium butyricum*, *Clostridium pasteurianum*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Bacillus welchii*, and during the last years various studies have been developed with these

microorganisms. With the development of biofuels production, glycerol has become a cheap source for the production of 1,3-PD. However, after the initial optimistic opinions on the use of biofuels, especially bioethanol, recent studies show new trends and some constraints concerning the benefits of biofuels. Facing the instability of the market, the study of other alternative cheap sources for the biological production of 1,3-PD should be considered.

Sugars are a desirable renewable carbon source, but till now no natural microorganism has been found to directly convert sugars to 1,3-PD. Different approaches have been suggested as possible solutions: (1) fermentation in a two-stage process where one microorganism ferments sugar to glycerol and another one converts glycerol to 1,3-PD, (2) a one stage process by mixed cultures (with genetically modified microorganisms to suppress the repression of 1,3-PD formation by glucose), (3) engineering the pathway for the conversion of sugars to 1,3-PD in a single organism. Based on these approaches, many attempts to convert sugar to 1,3-PD have been carried out in the last years with the development of metabolic and genetic engineering tools and new fermentation processes.

The aim of this work was to investigate the production of 1,3-PD from glucose and other sugar feedstocks, like sugar cane molasses, in a two-step process using two new biocatalysts. Also new strategies to obtain a *Saccharomyces cerevisiae* strain able to overproduce glycerol were evaluated.

For this purpose, the current work was divided in two parts (schematic outline of the thesis presented in Figure 1). In the first part, the study focused on

glycerol overproduction by *Saccharomyces cerevisiae* from glucose or sugar cane molasses. Several genetically engineered mutants of *Saccharomyces cerevisiae* were first tested in order to obtain the best glycerol overproducer (Chapter 3). The strain *Saccharomyces cerevisiae* HC42 was the most promising glycerol producer mutant and was chosen for further research. A metabolic and physiological study of this strain was undertaken during cultivation in bioreactors, in order to better evaluate its performance. A genomic study of this mutant, through the use of microarrays technique, is described in Chapter 4.

Some constraints to enhance the production of glycerol by the novel genetic engineered strain *Saccharomyces cerevisiae* HC42 were found. In order to avoid intracellular accumulation of glycerol, *FPS1* was overexpressed in HC42 (Chapter 5). The result was the mutant strain *Saccharomyces cerevisiae* FM62. The physiological and metabolic characterisation of this strain was also carried out during cultivations on glucose or molasses media.

The second part of this work was focused on the production of 1,3-propanediol in a two-step process by *S. cerevisiae* HC42 mutant strain and by the genetically engineered strain *Clostridium acetobutylicum* DG1 (pSPD5). Different cultivation parameters and strategies were tested in order to improve glycerol yield and titer by the strain *Saccharomyces cerevisiae* HC42 (Chapter 6). In an attempt to reduce the final cost of the biologic process, a cheaper feedstock, such as sugar cane molasses, was also evaluated. The results of *Clostridium acetobutylicum* DG1 (pSPD5) cultivations on yeast broth from glucose and sugar cane molasses media are presented in Chapter 7.

To finalise this thesis work, general conclusions and future work perspectives are presented in Chapter 8.

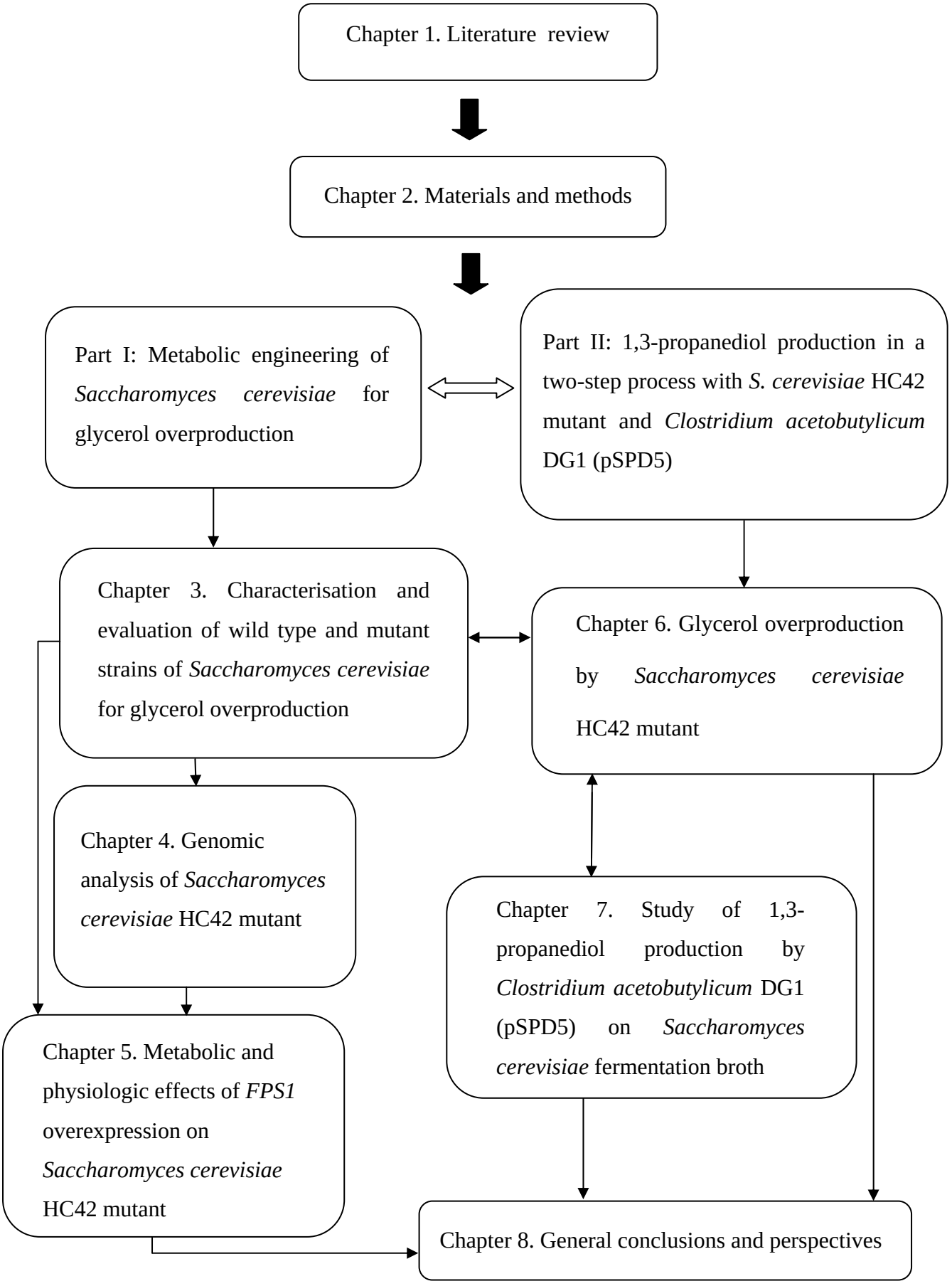



Figure 1. Diagram representing the outline of the thesis.

*Chapter 1*

# Literature review

## 1.1. Microorganisms and industry

For several thousand years microorganisms have been exploited to people's benefit. Since the accidental discovery of the usefulness of fermentation processes, microorganisms have been used in the production of a wide range of products, such as foods and beverages (e.g. bread, yoghurt, cheese, beer, wine, etc), chemicals (acetone, butanol, ethanol, organic acids, L-glutamic acid, polyhydroxyalkanoates, etc) and pharmaceutical compounds (antibiotics, insulin, etc). Studies demonstrated that the yeast *Saccharomyces cerevisiae* was used by the Egyptians for wine fermentations by at least 3150 B.C. However, millennia passed before the discovery and identification of microorganims. It was Antonie van Leeuwenhoek, who for the first time, in 1675, observed microorganisms. But only in the nineteenth century Louis Pasteur, carrying out extensive physiological studies of fermentation, proved that microbial activity was the cause of fermentation.

For over 100 years microorganisms have been successfully used for industrial proposes and their economic potential is still enormous. Microorganisms can use cheap raw materials and convert them into valuable products. Various commercial products of economic value are the result of microbial activities, such as organic acids, enzymes, alcohols, proteins, antibiotics, vitamins, etc. It is estimated that their annual added value to the chemical industry alone is about €10 billion and, in the next 10 years, 60% of synthetic fine chemicals will rely on microbial conversion (Beloqui *et al*., 2008).

### 1.1.1. Industrial (white) biotechnology

In the beginning of the twentieth century in response to a demand of the World War I, scientists developed the novel industrial-scale fermentation process, the production of acetone-butanol using the bacterium *Clostridium acetobutylicum*. After this, further innovations in fermentation technology were applied to industry, especially in the 1940s with the production of penicillin, and biotechnology began to emerge. For years biotechnology could not economically compete with the petrochemical industry in the synthesis of many products that could be produced from oil. However, growing concerns about petroleum depletion and environmental awareness are increasing the relevance of using industrial or white biotechnology. Industrial biotechnology uses living cells and enzymes, the so-called biocatalysts, to synthesise bio-based chemicals, materials and fuels. Besides the fact that most of the time these products are easily degradable, industrial biotechnology processes are considered more environmentally sustainable because generally they are low energy consuming and create less waste than the traditional chemical processes they replace. The use of biomass like starch, molasses, cellulose, vegetable oils and agricultural waste by industrial biotechnology is another advantage of those processes (Fig. 1.1).

Nevertheless, industrial biotechnology still is a relatively young technology and many of the processes have not yet proven that they are economically sustainable (Frazzetto, 2003). Some of the problems to be solved are the low product concentration, low productivity and also the high recovery cost. Although molasses and other renewable resources are cheap, the cost of downstream

processing for these cases should not be neglected and can account for more then 50% of the total production cost. Research areas such as genetic and metabolic engineering have been developed and can contribute to the renewed interest in the production of chemicals from renewable resources.

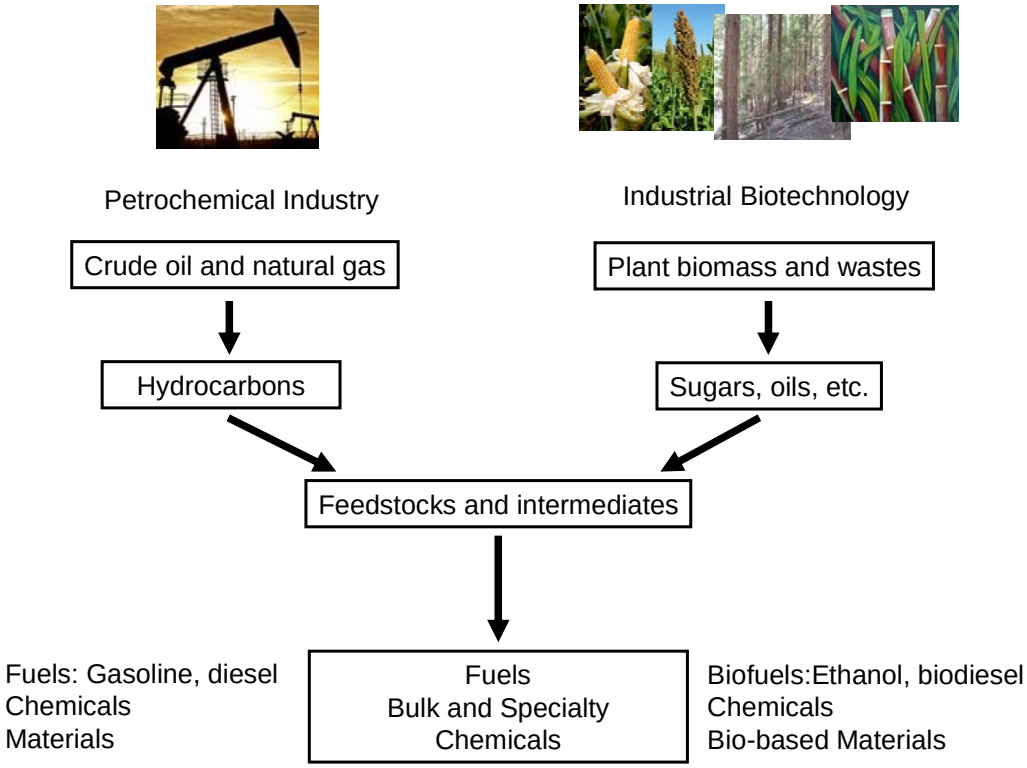



Figure 1.1. Petrochemical and biotechnological processes.

## 1.1.2. Genetic, metabolic and evolutionary engineering

Fermentations used to be carried out spontaneously, and microorganisms were unknowingly transferred from fermentation to fermentation. The first pure yeast culture used for beverage production was obtained in 1883 by Emil Christian

Hansen from the Carlsberg Brewery and in 1890 a pure culture of a wine yeast was obtained by Muller-Thurgau from Geisenheim (Germany) (Dequin, 2001). Traditional techniques to improve the performance of microorganisms consisted of selecting the best producers for the desired phenotype. The evolution of microbial physiology knowledge, genetic manipulation, biochemistry advances and the development of fermentation technologies have contributed to obtain microorganisms or its derivatives capable of producing a final desired product with high efficiency, yields and titers. With genetic engineering, new tools were available for microorganisms genetic manipulation and transformation. It became possible to develop, improve, modify or eliminate metabolic pathways in order to increase the range of microbial native products as well as exogenous products commercially more viable. The new age of "omics", including genomics, transcriptomics, proteomics and metabolomics, has brought many advances and advantages to the area of study called metabolic engineering and introduced by Bailey in 1991 (Chotani, 2000; Ostergaard, 2000; Schuller and Casal, 2005; Nevoigt 2008). Since its expansion there are many examples of emerging applications of biotechnology using metabolic engineering in the design of systems wide experiments for the production of chemicals, materials and medicines. A relevant example is the genetically engineered human insulin production, in the yeast *Saccharomyces cerevisiae*, in 1987 (Kjeldsen, 2000). However, genetic engineering is not always applicable or the sole solution. Sometimes, following the nature's "engineering" process results in a complementary strategy. Evolutionary engineering offers compelling scientific

and applied advantages for strain development and process optimization and is able to reach the desired phenotype (Sauer, 2001).

## 1.2. Glycerol: Production and applications

Glycerol, also known as glycerine, glycerin or 1,2,3-propanetriol is a simple alcohol. At normal room conditions, glycerol is a high viscous, colourless, odourless, sweet warm tasting and hygroscopic liquid. Glycerol is soluble in water and alcohol, slightly soluble in diethyl ether, ethyl acetate and dioxane, and insoluble in hydrocarbons. It can form esters, ethers, halides, amines, aldehydes and also such unsaturated compounds as acrolein. It is low-boiling, non-toxic, emollient, a good solvent and easily biodegradable. Due to all these physical properties glycerol has a wide range of applications.

It was K.W. Scheele who first discovered glycerol, in 1779 (Rahmat *et al*., 2010). This compound can be produced either by chemical synthesis, as a by-product from fats and oils use, or by microbial fermentation. Glycerol plays essential roles in different vital physiological processes, in prokaryotes and eukaryotes (including mammals). It is an important carbon source for energy and biomass production by several species. The glycerol precursor glycerol 3-phosphate (G3P) is not just involved in acylglycerol synthesis but is also involved in an energy shuttle system in cells. This shuttle is a complex mechanism used to transfer NADH electrons from cytosol to mitochondrial electrons transport chain (Brisson *et al*., 2001).

### 1.2.1. Chemical synthesis

Different methods are available for glycerol synthesis by chemical route (using petrochemical feedstocks) all of them using propylene: from chlorination of propylene to allyl chloride, from acrolein formed from the oxidation of propylene and from propylene oxide (Huang, 2002). In 2001, around 25% of the world glycerol produced by chemical industry was via the oxidation or chlorination of propylene. However, since 1970 these routes are declining, not only because of the environmental problems caused, but also due to the increase of the price of propylene (Wang *et al*., 2001). Moreover, in the last years, the search for novel renewable energy sources promoted an enormous increase of biofuels production, causing an increase of crude glycerol in the market, and the chemical synthesis had to compete with it.

### 1.2.2. Production from fats and oils

Usually glycerol is found in plants and animals in the form of fatty acid esters, the triacylglycerols (triglycerides), or bonded to fatty acid and phosphate (phospholipids). Traditionally, glycerol was a by-product of soap and detergents made from oils and fats from plants and animals. To obtain glycerol through this production process, oils and fats must be split and separated by saponification (Figure 1.2.). In recent years, glycerol is also obtained as a co-product of triglyceride trans-esterification of oils and fats from plants and animals, during the production of biodiesel. The yield of glycerol is about 10 wt% of total biodiesel production (Rahmat *et al*., 2010).

### 1.2.3. Production by fermentation

Glycerol can also be produced by fermentation. Since the investigation of Pasteur in the 19th century, glycerol production by yeast fermentation is known. Glycerol is a by-product of yeast glucose fermentation (Gancedo *et al*., 1968). In addition to yeast, there are several microorganisms able to synthesise glycerol, such as bacteria, mold, protozoa and algae (Taherzadeh *et al*., 2002).

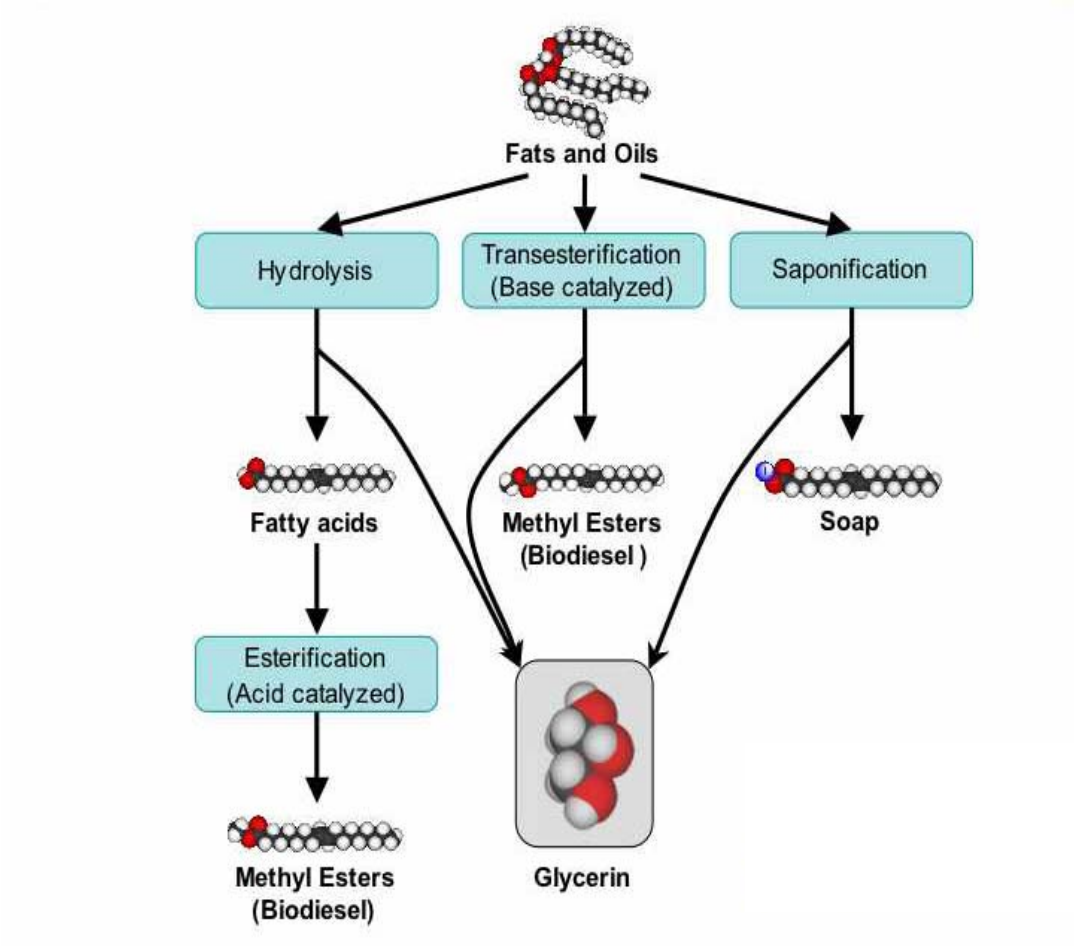



Figure 1.2. Production of glycerol from fats and oils (Yazdani and Gonzalez, 2007).

Glycerol was produced for the first time by fermentation in a large scale process during the World War I, using the so-called sulphite-steered yeast process, to supply the production of explosives. After the World War II, with the development of petrochemical synthesis of glycerol, the fermentation technology started to decline, since it was unable to compete with the new process, specially due to the inefficient down-stream technology, with a low yield and titer of glycerol from the fermentation broth (Wang *et al*., 2001).

### 1.2.3.1. Glycerol production by yeast

Among the microorganisms able to produce glycerol, yeasts, and especially *Saccharomyces cerevisiae*, are the most studied. Other osmophilic yeasts such as *Candida*, *Debaryomyces*, *Hansenula*, *Pichia*, *Schizosaccharomyces*, *Torulaspora* and *Zysaccharomyces* were also investigated for glycerol production (Ciani and Ferraro, 1996; Petrovska *et al.,* 1999; Zhuge *et al*., 2001; Djelal *et al*., 2006; Liu *et al*. 2006).

Glycerol is one of the most important by-products of sugar conversion during alcoholic fermentation by yeasts. The accumulation of this metabolite in *S. cerevisiae* can reach 10 $g.l^{-1}$, under anaerobic conditions (Radler and Schütz, 1982; Ciani and Ferraro, 1996). This is mainly a consequence of maintaining the cellular redox balance by regenerating NADH excess associated to biomass production during fermentation (Overkamp *et al.,* 2002; Remize *et al.,* 1999). Glycerol is also involved in osmoregulation (Hohmann, 2002) and can act as a cryoprotectant like trehalose (Izawa *et al.,* 2004). The osmotolerant yeast *Candida*

*glycerinogenes*, selected from an environment of high osmotic pressure, was able to convert up to 64.5% (w/w) of the glucose present in the growth medium into glycerol, with a glycerol production of 137 $g.l^{-1}$.

### 1.2.3.2. Glycerol production by bacteria

Different bacteria, such as *Crithidia fasciculate*, *Bacillus subtilis*, *Bacillus coli*, *Bacterium orleanense*, *Bacterium pasteurianum*, *Lactobacillus lycopersici*, among others, have been studied for the production of glycerol. However, the yields obtained were too low and fermentation rates too slow. *B. subtilis*, for instance, yielded 29.5% glycerol in a glucose-yeast extract medium within 8 days. According to these perspectives the production of glycerol by bacteria has not been seriously considered (Wang *et al*., 2001). However, in the last years, with the possibility of using glycerol as a feedstock for the production of new products economically advantageous, and with the help of genetic engineering, new generations of bacteria have been developed. Meynial-Salles *et al.* (2007) described a genetic engineered *Escherichia coli* that, without any fermentation process optimization, was able to produce glycerol from glucose with a yield of 1 $mol.mol^{-1}$, titer of 130 $g.l^{-1}$ and productivity up to 0.67 $g.l^{-1}h^{-1}$, in a fed-batch system.

### 1.2.3.3. Glycerol production by algae

The production of glycerol by algae has also been investigated. The use of $CO_2$ and light as carbon and energy sources to produce valuable chemical

compounds is an interesting challenge. The most studied species belong to *Dunaliella*. In these algae, glycerol is accumulated in the cells as a response to high salinity in the growth media. Depending on the salt concentration in the growth medium, more than 50% of the dry weight of *Dunaliella* may be glycerol (Kaçka and Dönmez, 2008). In a medium with 4 $mol.l^{-1}$ NaCl *D. tertiolecta*, immobilised in calcium alginate, produced up to 5 $g.l^{-1}$ of glycerol (Grizeau and Navarro, 1986). Kaçka and Dönmez (2008) also reported *Dunaliella* species isolated from a hypersaline lake in Turkey that showed high glycerol production, i.e. 0.45 $g.l^{-1}$ of culture broth at 20% NaCl concentration. Although the production of glycerol from *Dunaliella* species was shown to be technically possible, economic feasibility is still too low.

### 1.2.4. Applications and market situation

Glycerol has many different applications in several industries. It can be used as a basic material or as a chemical building block. Due to its molecular structure, glycerol is considered as a good starting material for the synthesis of many other chemicals and intermediates. The major applications of glycerol include food (when used as food additive, it is labelled as E number E422), pharmaceuticals, cosmetics, personal care and soaps, tobacco, polymers and explosives. Nevertheless, in the last years, the rise in world biodiesel production has created a glut of glycerol (Figure 1.3.).

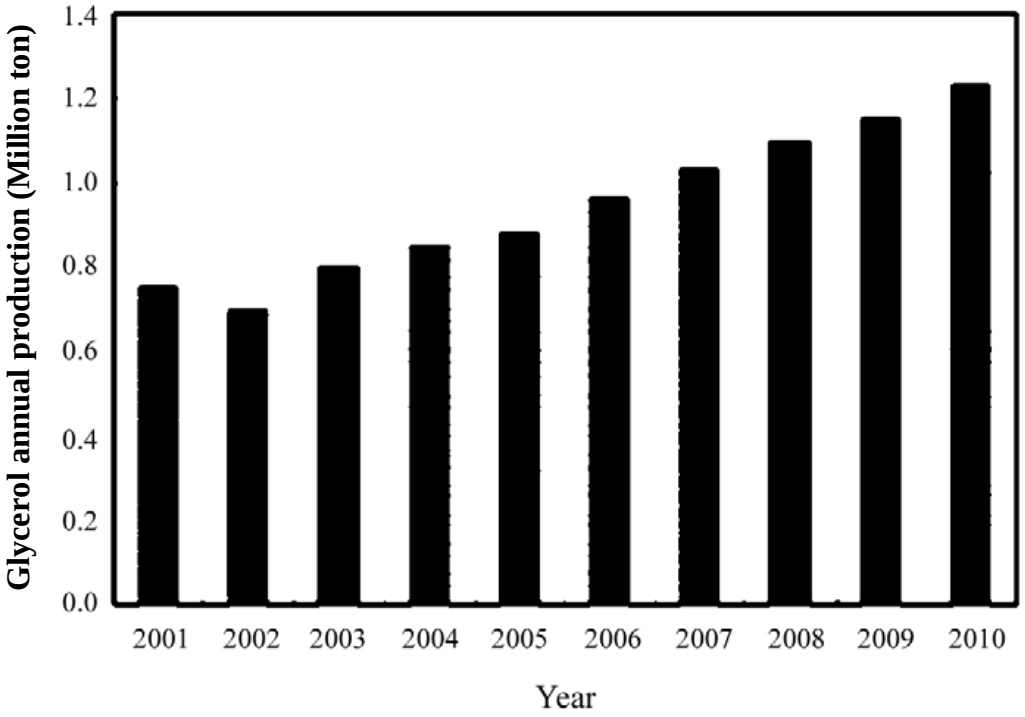



Figure 1.3. Global glycerol production (in Rahmat *et al*., 2010).

The direct effect of glycerol surplus is the decline of glycerol prices. As a consequence, new investments and efforts are now put up in research to develop new strategies and approaches for glycerol alternative applications. One of those ideas, based in the concept of "bio-refinery", is the glycerol bioconversion into new products such as 1,3-propanediol, succinic acid, lactic acid, propionic acid, ethanol, dihydroxiacetone, citric acid, pigments, polyhydroxyalconate and biosurfactants (da Silva *et al*., 2009). The use of glycerol in chemical industry is also showing particular interest when glycerol is used into fuel additives. Glycerol ethers, acetyl glycerol and glycerol acetal have been identified as valuable replacements of fuel additives that are petroleum derivatives. These glycerol-based fuel additives have demonstrated a large potential for diesel and biodiesel

formulation that assist to a decreasing in particles, hydrocarbons, carbon monoxide and unregulated aldehydes emissions (Rahmat *et al*., 2010).

## 1.3. Glycerol production by *Saccharomyces cerevisiae*

*S. cerevisiae* is not the best glycerol producer. However, this yeast has been intensively studied for several years, which resulted in a valuable knowledge about its physiology and metabolism. It is an excellent model system of the eukaryotic cell.

### 1.3.1. *Saccharomyces cerevisiae* characterization

*S. cerevisiae* is by far the most studied eukaryotic microorganism. It is classified as facultative fermentative yeast, which means that can grow aerobically but in the presence of limited oxygen concentrations exhibits alcoholic fermentation (van Dijken and Scheffers, 1986). It is also a Crabtree-positive yeast, i.e., a mixed respiro-fermentative metabolism can occur under fully aerobic conditions, specifically when glucose concentration is higher than a certain threshold concentration or when the specific growth rate is above a specific value, usually higher than two-thirds of the maximum specific growth rate on glucose (Overkamp *et al.,* 2000).

As in its natural environment of fruit and nectar the most abundant sugars are glucose and fructose, it is not surprising that *S. cerevisiae* prefers to consume these sugars. Other fermentable sugars such as galactose, sucrose and maltose, can only be used after glucose. Sucrose is extracellularly hydrolysed to glucose and

fructose by periplasmic invertases. The invertase enzymes are also responsible for degrading raffinose to melibiose and fructose. Pentose sugars such as xylose and arabinose cannot be consumed.

*S. cerevisiae* has been used in several industrial processes such as baking, wine making and brewing (Nevoigt, 2008). Due to its long history of application in everyday life, it has been classified as a GRAS (Generally Regarded as Safe) organism. The nucleotide sequence of *S. cerevisiae* is available since 1996, when the completed sequence was published and diverse and efficient genetic tools are available to genetic engineering of this yeast (Ostergaard *et al*., 2000). *S. cerevisiae* is relatively tolerant to low pH values and high sugar and ethanol concentrations, characteristics that lower the risk of contamination in industrial environments. For all these reasons *S. cerevisiae* has been widely used in industrial biotechnology, for the production of valuable fermented products such as bioethanol, glycerol, propanediol, organic acids, sugar alcohols, steroids, etc. (Nevoit, 2008).

### 1.3.2. Biochemistry of *Saccharomyces cerevisiae* fermentation

After uptake, glucose is oxidized into two pyruvate molecules, through the glycolytic pathway, resulting in the net formation of two mole of ATP per mole of glucose. Depending on whether growth is respiratory or fermentative, the reducing and oxidative reactions occur via different metabolic pathways and this has large consequences on energy generation in the cell. During respiratory metabolism, pyruvate can be further oxidized in the tricarboxylic acid cycle. In the

fermentative metabolism, pyruvate is converted into ethanol by the activity of two enzymes: pyruvate decarboxylase and alcohol dehydrogenase. During this conversion, NADH is formed in glycolysis by glyceraldehyde-3-phosphate dehydrogenase. The redox balance is assured by formation of glycerol. Glycerol is the main by-product synthesised during fermentation (Fig. 1.4.). There are other possible products of sugars fermentation involved in yeast redox balance, like succinate, acetate, acetaldehyde, acetoin and 2,3-butanodiol. Acetaldehyde is formed by the decarboxilation of pyruvate catalysed by pyruvate dacarboxilase (PDC) and can be reduced into ethanol by the enzyme ethanol dehydrogenase, or oxidized into acetate by the enzyme acetaldehyde dehydrogenase, involving the reduction of $NAD(P)^+$ to NAD(P)H. Acetaldehyde can also be converted into acetoin and 2,3-butanediol. Acetoin can be produced through 3 pathways (Romano and Suzzi, 1996). In a first pathway pyruvate is decarboxylated and originates an active acetaldehyde complex. Active acetaldehyde and pyruvate are then transformed into α-acetolactate by means of acetohydroxy acid synthetase. This compound can easily be converted to diacetyl or to acetoin, particularly in the presence of oxygen. Acetoin may also be synthesised in yeasts by condensation of active acetaldehyde with acetyl coenzyme A to form diacetyl, which is successively reduced to acetoin. The third route to form acetoin by yeast is by condensation of the active acetaldehyde (acetaldehyde-thiamine diphosphate complex) with free acetaldehyde formed from pyruvate, without the intermediate formation of α-acetolactate. 2,3-Butanodiol is formed by the reduction of acetoin

with the respective oxidation of NADH. This reaction is catalysed by the enzyme acetoin reductase.

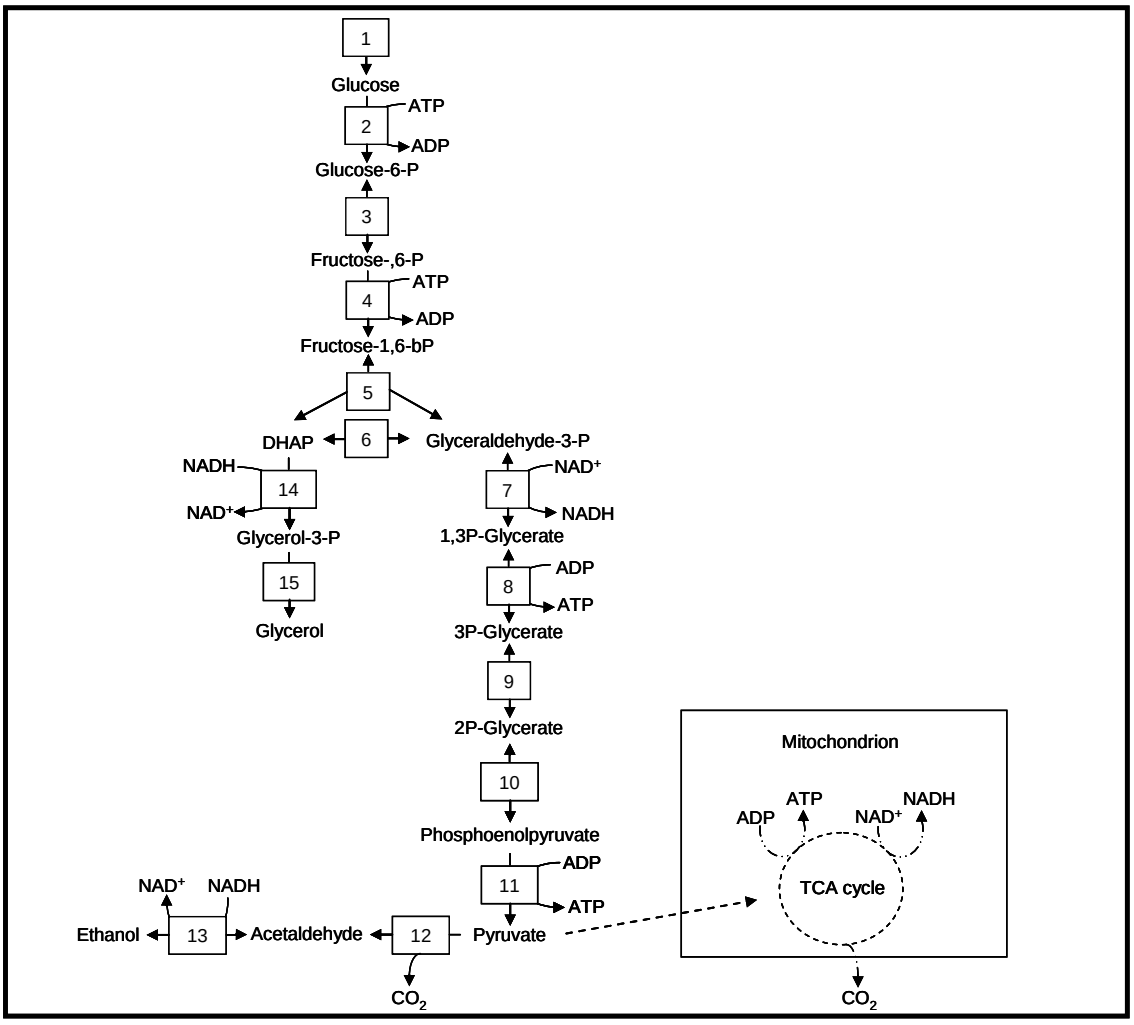



Figure 1.4. *S. cerevisiae* glucose fermentative pathway. 1 - hexose, 2 -hexokinase, 3 - glucose-6-phosphate isomerase, 4 - fructose-6-phosphate kinase, 5 – fructose-biphosphate aldolase, 6 - triose phosphate isomerise, 7 - glyceraldehyde-3-phosphate dehydrogenase, 8 – phosphoglycerate kinase, 9 - phosphoglycerate mutase, 10 - enolase, 11 - pyruvate kinase, 12 - pyruvate decarboxylase, 13 – alcohol dehydrogenase, 14 - glycerol-3-phosphate dehydrogenase, 15 - glycerol-3-phosphatase, TCA - Tricarboxylic Acid Cycle.

Under anaerobic conditions, succinate is mainly formed through the reductive branch of the Tricarboxylic Acid Cycle from malate via fumarate with the production of $FAD^+$.

### 1.3.3. Metabolic pathway for glycerol production

In the yeast *Saccharomyces cerevisiae* glycerol is synthesized in a two-step reaction process, involving the reduction of dihydroxyacetone phosphate (DHAP) by a NAD dependent glycerol phosphate dehydrogenase (GPD), followed by the dephosphorylation of glycerol 3-phosphate (G3P) by a glycerol phosphate phosphatase (GPP) (Albertyn *et al.,* 1994b; Ansell *et al.,* 1997; Pahlman *et al.,* 2001). Figure 1.5. shows the metabolic pathway for the production of glycerol in the yeast. Two genes *GPD1* and *GPD2* encode two isoenzymes of GPD (Larsson *et al.,* 1993) and *GPP1* and *GPP2* encode two isoforms of GPP (Pahlman *et al.,* 2001). These genes are subject to different control, *GPD1* and *GPP2* being upregulated under osmotic stress, while *GPD2* and *GPP1* are induced under anaerobic condition (Albertyn *et al.,* 1994b; Ansell *et al.,* 1997; Costenoble *et al.,* 2000; Pahlman *et al.,* 2001). Based on these considerations and on additional genetic data, it is currently admitted that the couple Gpd1p/Gpp2p is the major route for glycerol production under aerobic condition, whereas Gpd2p/Gpp1p plays a specific role in redox balance under anaerobic growth conditions (Blomberg, 1997; Hohmann, 2002).

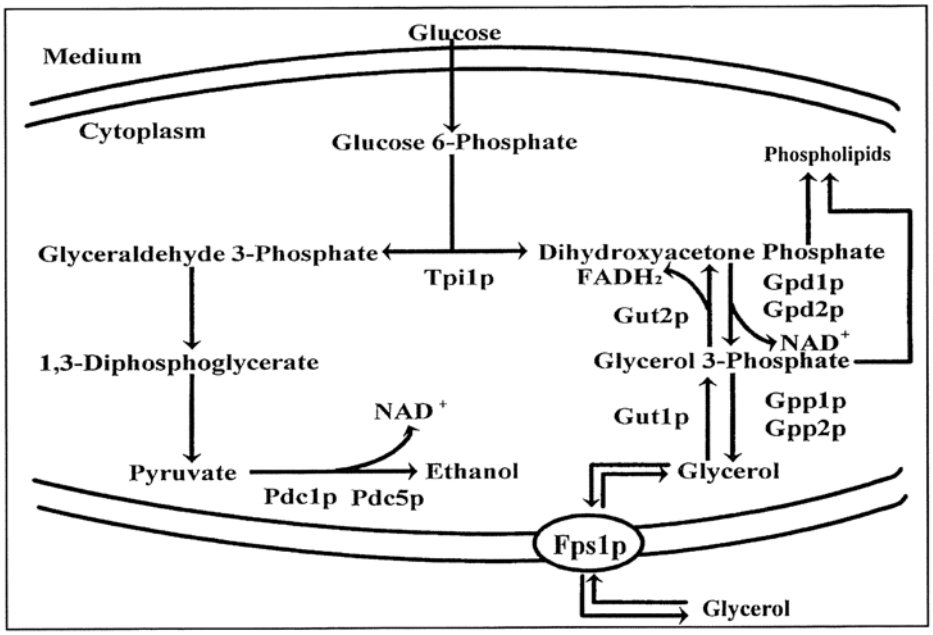



Figure 1.5. Metabolic reactions of importance in the glycerol metabolism of *S. cerevisiae* (Scanes *et al*., 1998).

Additionally, the distinct localization of Gpd1p and Gpd2p may also explain that the former cannot substitute for the latter under mitochondrial defective strains (Valadi *et al.,* 2004). Fps1p is described as the major glycerol facilitator through plasma membrane to the outside of cells (Luyten *et al.* 1995; Oliveira *et al*. 2003; Tamas *et al*. 1999).

### 1.3.3.1. Redox metabolism

Yeast growth is possible due to substrates oxidation. The transfer of electrons amongst metabolites is assured by the presence of transporters. In yeast there are two main couples of electrons transporters: $NADH/NAD^+$ and

NADPH/$NADP^+$. The first pair of cofactors participates in the catabolic and anabolic reactions whereas the pair NADPH/$NADP^+$ is related with the anabolic pathways. The maintenance of the redox balance is dependent on the equilibrium of these cofactors pools and is essential for cells survival. However, NAD(P)H/$NAD(P)^+$ are not able to cross cell membranes, inclusively the mitochondrial inner membrane, and so, once reduced, the reoxidation of the cofactors has to occur in the same cell compartment. Furthermore, *S. cerevisiae* lacks a transhydrogenase activity to convert $NAD^+$ and NADPH to NADH and $NADP^+$. Most of the anabolic reactions involving NADPH oxidation take place in the cytosol, whereas NADH production occurs in the cytosol but also in mitochondria. During aerobic condition, cells have different systems coupled to the respiratory chain to reoxidize cytosolic and mitochondrial NADH. During anaerobic conditions, as the respiratory chain is not working, alternative pathways have to be used. One of the most important pathways for the reoxidation of cytosolic NADH is that involving glycerol production. Since sugars fermentation is a redox-neutral process, *S. cerevisiae* and other yeasts found as solution to this redox problem, the production of glycerol involving the reoxidation of NADH (Heux *et al*., 2006; Bakker *et al*., 2001).

Although NADH or $NAD^+$ are not able to cross the mitochondrial inner membrane under aerobic conditions, yeasts and other eukaryotes possess redox shuttle mechanisms that allow to transfer the redox equivalents of NADH across the mitochondrial membrane (Bakker *et al*., 2001). One of these shuttles is related to the glycerol metabolic pathway. It is the glycerol 3-phosphate shuttle which

indirectly oxidizes cytosolic NADH once that does not result in the translocation of redox equivalents but does bypass the NADH dehydrogenases. This mechanism includes two components; the cytosolic $NAD^+$ dependent GPD and the mitochondrial GPD:ubiquinone oxidoreductase, also called mitochondrial glycerol-3-phosphate dehydrogenase. The latter enzyme is encoded by *GUT2*. It is FAD-linked and is located in the inner mitochondrial membrane. It transfers electrons from cytosolic GPD to ubiquinone in the respiratory chain.

### 1.3.3.2. Osmoregulation

When cells are exposed to hypertonic medium, there is a rapid diffusion of water from the cell into the surrounding medium. This process is driven by the difference between cell and environment water activities. Osmoregulation "is understood to be the cellular response directed at restoring and maintaining volume, turgor pressure and normal biological activities of the cell" ( Nevoigt and Stahl, 1997). To prevent the loss of water to the environment, yeast accumulates solutes, known as compatible solutes, to equilibrate the intra- and extracellular environment. Only some compounds are accumulated such as ions, amino acids and polyhydroxy compounds. The principal compound accumulated by *S. cerevisiae* is glycerol. It is evident that the intracellular level of glycerol is adjusted to external water activities and this is essential to growth under osmotically stressful conditions (Scanes *et al*., 1998). In a study reported by Petrovska *et al*. (1999) the glycerol yield in *S. cerevisiae* increased 2.4 times when the osmotic pressure was increased by adding 40 $g.l^{-1}$ NaCl to the medium.

Glycerol was produced as a response to the osmotic pressure caused by salt addition.

### 1.3.4. Factors affecting glycerol production

There are several factors affecting glycerol production by yeast. Strain, inoculation level, pH, temperature, water activity, oxygen level and medium composition (initial sugar concentration and carbon/nitrogen ratio) can influence the production of glycerol by yeasts. Several studies described that temperature increase resulted in higher glycerol production. The optimal temperature for glycerol production was shown to be dependent on agitation time during cultivation (Scanes *et al*., 1998). However, Remize *et al.* (2000) observed that glycerol production increased by less than 15% when temperature increased from 18 to 28 ºC and agitation had only a slight positive effect on the amount of glycerol formed (0-13%). Studies related with pH and acidity revealed that higher glycerol yields were obtained with *S. cerevisiae* under alkaline conditions. It is reported that the optimum pH range for sulphite-directed glycerol production lies between 6.7 and 7.0.

As described before, glycerol is involved in the osmotic cell balance. It was noted that in order to compensate for the osmotic stress caused by the high sugar concentration of wine must (usually 200 $g.l^{-1}$ or more), the yeast increased synthesis and accumulation of glycerol (Scanes *et al*., 1998). Nitrogen source is also reported to affect glycerol production. The glycerol yield achieved in cultures growing in ammonium was two-fold higher than the value obtained for yeast

growing in cultures with a mixture of amino acids as nitrogen source. This is related to the fact that ammonium-grown cultures require *de novo* synthesis of amino acids that generates a surplus of NADH, which must be reoxidized by glycerol synthesis. Other factor affecting glycerol production seems to be aeration. The production of glycerol is markedly affected by the degree of aeration in osmotolerant yeasts. Sahoo *et al.* (2001), in a study with a *Candida magnoliae* strain, observed that the higher oxygen availability increased the yields of cells mass and glycerol and reduced ethanol yield. When compared with this type of strains, the production of glycerol by *S. cerevisiae* seems to required oxygen-restricted condition (Wang *et al*., 2001). It is known that higher glycerol yields are obtained under anaerobic conditions compared with aerobic conditions.

### 1.3.5. Strategies to enhance glycerol production by *Saccharomyces cerevisiae*

Though *S. cerevisiae* is likely not the best glycerol producer (Wang *et al.,* 2001), is the most useful system to evaluate various engineering strategies to optimise its synthesis. Accordingly, over the past decade, there have been several attempts to manipulate yeast metabolism in order to optimise glycerol production, shifting from a bioprocess-mediated mode involving sulphite (Bisping and Rehm, 1988; Petrovska *et al.,* 1999) towards a reprogramming of the cellular metabolism by recombinant DNA technology. In spite of the relative technological advantage or difficulties encountered in implementing the various genetic strategies, the final challenge is to get the maximal glycerol yield per mol glucose consumed. This

achievement notably requires a reduction of the contribution of assimilatory glucose metabolism to anabolic and maintenance purposes, an increase in the NADH availability and eventually an increase of the average productivity rates.

Comparing various strains of *S. cerevisiae*, Radler and Schütz (1982) found a glycerol production variation between 4.2 to 10.4 $g.l^{-1}$. The authors also reported that increasing the initial amount of cells, used as inoculum, significantly increased the amount of glycerol produced. This fact is also reported by Munene *et al*. (2002). An increase in pH medium can also result in high glycerol yields. In the alkaline process, also known as Neuberg's third form of fermentation, $Na_2CO_3$ is added to the medium to maintain the pH at 7-8 and to enhance the production of acetate, leading to an increase of NADH that has to be balanced by glycerol formation, under anaerobic conditions. However, for pH values higher than 6.0 *S. cerevisiae* fermentation capacity decreased by more than 30% and so, the optimal concentration of sodium carbonate was reported to be only 5% with a glycerol yield of 0.2 $g.g^{-1}$ of sugar (Taherzadeh *et al*., 2002). In another study, medium pH was increased to 8.7 (the optimum for aldehyde dehydrogenase activity) using sodium hydroxide, reporting glycerol yields up to 40.5% of sugar consumed.

The overexpression of *GPD1* in a wine yeast strain resulted in a 4-fold increase of glycerol production at the expense of ethanol. However, this mutation also resulted in the accumulation of other by-products such as pyruvate, acetate, acetoin, 2,3-butanediol and succinate (Michnick *et al*., 1997).

When triose phosphate isomerise gene (*TPI1*) was deleted, glycerol yield reached 80-90% of the theoretical maximum yield of 1 $mol.mol^{-1}$ glucose. 0.46 g

glycerol. $g^{-1}$glucose were obtained in shake flaks with glucose as the sole carbon source. However, the mutant growth rate was too slow when compared with the wild type strain (Compagno *et al*., 1996). Likely this result was caused by the accumulation of DHAP that has been shown to suppress the production of myo-inositol by inhibiting the enzyme myo-inositol-3 phosphate synthase (Shi *et al*., 2005). The work described by Overkamp *et al.* (2002) raised the glycerol production from 0.25 g glycerol.$g^{-1}$ glucose obtained by the sulphite bioprocess to 0.42 g glycerol.$g^{-1}$ by metabolic engineering implicating the deletion of *TPI1* encoding triose phosphate isomerase and removal of the cytosolic NADH reoxidation by mitochondrial redox shuttles. The two isogenes *NDE1* and *NDE2*, encoding mitochondrial external NADH dehydrogenase, and *GUT2*, encoding the $FAD^{+}$-dependent mitochondrial GPD, were deleted. These mutations recovered the myo-inositol defect caused by the deletion of *TPI1*. An evolutionary engineering strategy described by Overkamp *et al.* (2002) allowed selecting a strain with an increased growth rate producing glycerol at a molar yield of glycerol on glucose close to unity. The strategy involved the construction of a mutant *pdc1Δ pdc5Δ pdc6Δ nde1Δ ned2Δ gut2Δ*. In another work using the same mutant strain, combined with the overexpression of *GPD2* and *FDH1*, it was possible to achieve a glycerol yield of 1.08 mol glycerol.$mol^{-1}$ glucose by co-feeding the strain with formate. In this case, formate is an external source of cytosolic NADH (Geertman *et al.*, 2006) (Table 1.1.).

Table 1.1. Glycerol final concentration, yield and productivity of different yeast fermentation processes .

| Yeast/Fermentation process | Glycerol (g/l) | $Y_{Glycerol}$ (g/g) | $r_{Glycerol}$ (g/l/h) | Reference |
|---|---|---|---|---|
| ***S. cerevisiae*** | | | | |
| Sulfite, Batch | 45 | 0.23 | 0.4 | Freeman and Donald (1957) |
| Sulfite, Fed-batch under vacuum | 82 | 0.25 | 1.35 | Kalle and Naik (1985) |
| *ADH* deletion, Shake flask | 4.6 | 0.26 | 0.1 | Drewke *et al.* (1990) |
| *pdc2Δ* mutant, Shake flask | 2.9 | 0.16 | 0.09 | Nevoit and Stahl (1996) |
| *tpiΔ* mutant, Shake flask | 36 | 0.46 | 1.5 | Compagno *et al.* (1996) |
| *tpiΔ* mutant, Shake flask | 63 | 0.44 | 1.5 | Compagno et al. (1998) |
| *GPD1* overproduction, Batch | 25 | 0.12 | 0.18 | Remize *et al*. (2001) |
| *tpiΔnde1Δ ned2Δ gut2Δ* mutant, aerated batch | 219 | 0.50 | 2.4 | Overkamp *et al.* (2002) |
| *pdc1Δ pdc5Δ pdc6Δ nde1Δ ned2Δ gut2Δ* mutant, aerated batch | 46 | 0.46 | 0.3 | Geertman *et al.* (2006) |
| *pdc1Δ pdc5Δ pdc6Δ nde1Δ ned2Δ gut2 GPD2 FDH1* overexpression mutant, aerated chemostat cultivation formate co-feeding | 8.3 | 0.55 | 0.2 | Geertman *et al.* (2006) |
| ***C. magnoliae*** | | | | |
| Batch | 80 | 0.32 | 0.65 | Sahoo and Agarwal (2001) |
| ***C. glycerinogenes*** | | | | |
| Batch | 137 | 0.64 | 1.7 | Zhuge *et al*. (2001) |

Y- Yield; r – Productivity

## 1.4. 1,3-Propanediol. Production and applications

1,3-propanediol is a three-carbon diol, colourless and odourless viscous liquid, miscible with water, alcohols, ethers and formamide.

August Freund, in 1881, identified the production of 1,3-propanediol, in a glycerol fermentation by a mixed culture containing *Clostridium pasteurianum.* It is one of the oldest known products of fermentation (Saxena *et al*., 2009). 1,3-

propanediol has several applications and among them is the use as a building block for the production of polymers.

1,3-propanediol can be produced by chemical or microbiological route. In the past, 1,3-propanediol bio-production was considerable low, due to the high production costs. However, the situation has changed. The high demand for a new polyester based on terephthalic acid and 1,3-propanediol, the polytrimethylene terephthalate (PTT), turned the production of 1,3-propanediol very profitable and interesting (Biebl et al. 1999; González-Pajuelo *et al.* 2006; Rao *et al.* 2008).

### 1.4.1. 1,3-Propanediol chemical synthesis

Nowadays, there are two main chemical processes for 1,3-propanediol synthesis. The Degussa process, now owned by DuPont, Wilmington, DE, USA, is based on acrolein obtained by the catalytic oxidation of propylene. Acrolein is hydrated to 3-hydroxypropionaldehyde, followed by hydrogenation to produce 1,3-propanediol. The yield of 1,3-propanediol obtained in this process is 40%. The other major process is owned by Shell Chemicals, USA. The process of Shell starts with the hydroformylation of ethylene oxide, followed by hydrogenation. In this process a yield up to 80% can be obtained. Both processes involve high production costs and formation of toxic intermediates (Saxena *et al*., 2009), that probably induced DuPont to develop a biological process.

### 1.4.2. 1,3-Propanediol fermentative production

1,3-Propanediol can be naturally produced by microorganisms. The carbon source used is glycerol or sugar co-fermented with glycerol, but no natural microorganism can convert only sugar into 1,3-propanediol (Cameron et al., 1998). The natural pathway for the production of 1,3-PD is from glycerol and occurs in facultative aerobic or obligate anaerobic bacteria species. The facultative aerobic group includes microorganisms belonging to genera *Klebsiella*, *Citrobacter* and *Enterobacter* (e.g. *Citrobacter freundii*, *Klebsiella pneumoniae*, *Enterobacter agglomerans*). It was also shown that some lactobacilli species such as *Lactobacillus brevis*, *Lactobacillus buchneri* and *Bacillus welchii* are able to produce 1,3-propanediol from glycerol, when cultivated with a co-substrate like glucose or fructose, under anaerobic conditions. The obligate anaerobic bacteria able to produce 1,3-propanediol are some clostridia species, like *Clostridium butyricum* and *Clostridium pasteurianum* (Schütz and Radler, 1984; Forsberg, 1987; Homann *et al*., 1990; Biebl *et al*., 1992; Dabrock *et al*., 1992; Barbirato *et al*., 1995; Daniel *et al*., 1995; Biebl *et al*., 1999). *C. acetobutylicum* was initially included among these species (Forsberg, 1987), but later on it was found that this microorganism does not carry the metabolic pathway to produce 1,3-propanediol and is not able to grow on glycerol as the sole carbon source (Vasconcelos *et al*., 1994). During the last years, various studies have been carried out with these microorganisms, aiming 1,3-PD production (Boenigk *et al*., 1993; Daniel *et al*. 1995; Papanikolaou *et al*. 2000; Zeng and Biebl 2002; González-Pajuelo *et al*. 2006).

From all microorganisms tested for 1,3-PD production, *K. pneumoniae* and *C. butyricum* have been widely studied. *K. pneumoniae* is an opportunistic pathogenic microorganism and, consequently, *C. butyricum* is preferred for potential industrial applications.

### 1.4.3 Applications and market situation

The market situation of 1,3-propanediol has changed in recent years. New applications for this chemical resulted in higher interest and demand for 1,3-propanediol. The production of a novel polyester increased the demanded of 1,3-PD and the market is growing rapidly. In the last years the development of new technologies and strategies to produce 1,3–PD resulted in a reduction of market prices. Currently the price of 1,3-PD is around $1.76 per kg and the production is over 45,400 tons per year with tendency to grow (Saxena *et al*., 2009).

Due to its simple chemical structure, 1,3-PD can be used in a wide range of applications. One of the most relevant is the production of polyesters, namely PTT (polytrimethylene terephthalate) (Biebl *et al*. 1999; González-Pajuelo *et al*. 2006; Rao *et al*. 2008). Polyester is the most widely used synthetic fiber in the world. Since its invention, the dominant polyester has been polyethylene terephthalate (PET). Comparing the properties of PTT fibers like Sorona®, the first polymer platform based on 1,3-PD commercialized by Dupont, with the conventional PET and nylon, it offers excellent and better physical and chemical properties. Among other, this fiber is highly resistant to most strains without the need for surface treatment with additives or coatings; it resists UV degradation better than other fibers, and shows both low water absorption and low electrostatic charging

(Kurian, 2005). PTT is used in the textile industry, with particular application in carpet manufacture, but it can also be used in food, cosmetics, adhesives, lubricants, laminates, solvents, antifreeze and medicines (one of its derivatives can be used to reduce rejection of transplanted organs (Németh and Sevella, 2008)). Traditional production of 1,3-PD is based on chemical synthesis from petroleum derivatives compounds, which requires expensive production processes and yields toxic intermediates, like acrolein. In contrast, 1,3-PD can be produced by fermentation of relatively low cost renewable carbon sources, and this process, though maybe more expensive than the chemical process, provides radical solutions to environmental pollution and petroleum depletion. The microbiologically produced 1,3-PD has properties superior to chemically synthesized 1,3-PD, such as a lower amount of impurities (Kurian, 2005). In addition, the polyesters based on the biological 1,3-PD are fully biodegradable.

## 1.5. Biochemistry of 1,3-propanediol production

Microbial anaerobic glycerol fermentation involves an oxidative and a reductive pathway. 1,3-Propanediol is produced through the reductive pathway in two enzymatic steps. First, glycerol is dehydrated to 3-hydroxypropionaldehyde (3-HPA) by a glycerol dehydratase, followed by the reduction of the aldehyde to 1,3-propanediol by the enzyme 1,3-propanediol dehydrogenase under the oxidation of NADH. In the species *K. pneumoniae* (Forage and Lin, 1982) and *C. freundii,* glycerol dehydratase is encoded by *dha*B gene and is $B_{12}$-dependent, and 1,3-propanediol dehydrogenase is encoded by *dha*T. For *C. butyricum* the

conversion of glycerol to 1,3-propanediol involves three genes arranged in an operon. Glycerol dehydratase is encoded by *dha*B1 and *dha*B2; *dha*T codifies for 1,3-propanediol dehydrogenase (Raynaud *et al.,* 2003). The enzyme glycerol dehydatase is very sensitive to oxygen and it was shown that for *C. butyricum* VPI 3266 the enzyme is $B_{12}$- independent (Saint-Amans *et al.,* 2001). In the second step of 1,3-propanediol biosynthesis, NADH is consumed. NADH regeneration occurs in the oxidative pathway. In the oxidative branch, glycerol is converted to dihydroxiacetone by a $NAD^+$-dependent enzyme glycerol dehydrogenase, which is phosphorylated by the enzyme dihydroxyacetone kinase and then converted into pyruvate through the glycolytic pathway. Biomass is produced and pyruvate can be converted into several end-products, depending on the microorganism (Figure 1.5.). Butyrate and *n*-butanol are produced by clostridia, while 2,3-butanediol is only formed by enterobacteria. Acetate and ethanol are produced by both bacterial groups (Biebl *et al*., 1999). The intermediate product 3-hydroxypropionaldehyde (3-HPA) is a very toxic compound produced in first enzymatic step of the reductive pathway for 1,3-PD formation, and when accumulated can cause growth inhibition.

### 1.5.1. *Enterobacteriaceae*

Glycerol fermentation by *Klebsiella* can result in the production of acetate, formate, lactate, succinate, ethanol, 2,3-butanediol, $CO_2$ and $H_2$, besides 1,3-propanediol (Streekstra *et al*., 1987; Homman *et al*., 1990; Barbirato *et al*., 1995). Acetate is the main acid formed and represents an energy source for cells (Zeng *et*

*al.*, 1993). When the substrate is in excess and the growth rate is high, small concentrations of acetoin were also observed (Solomon *et al.*, 1994).

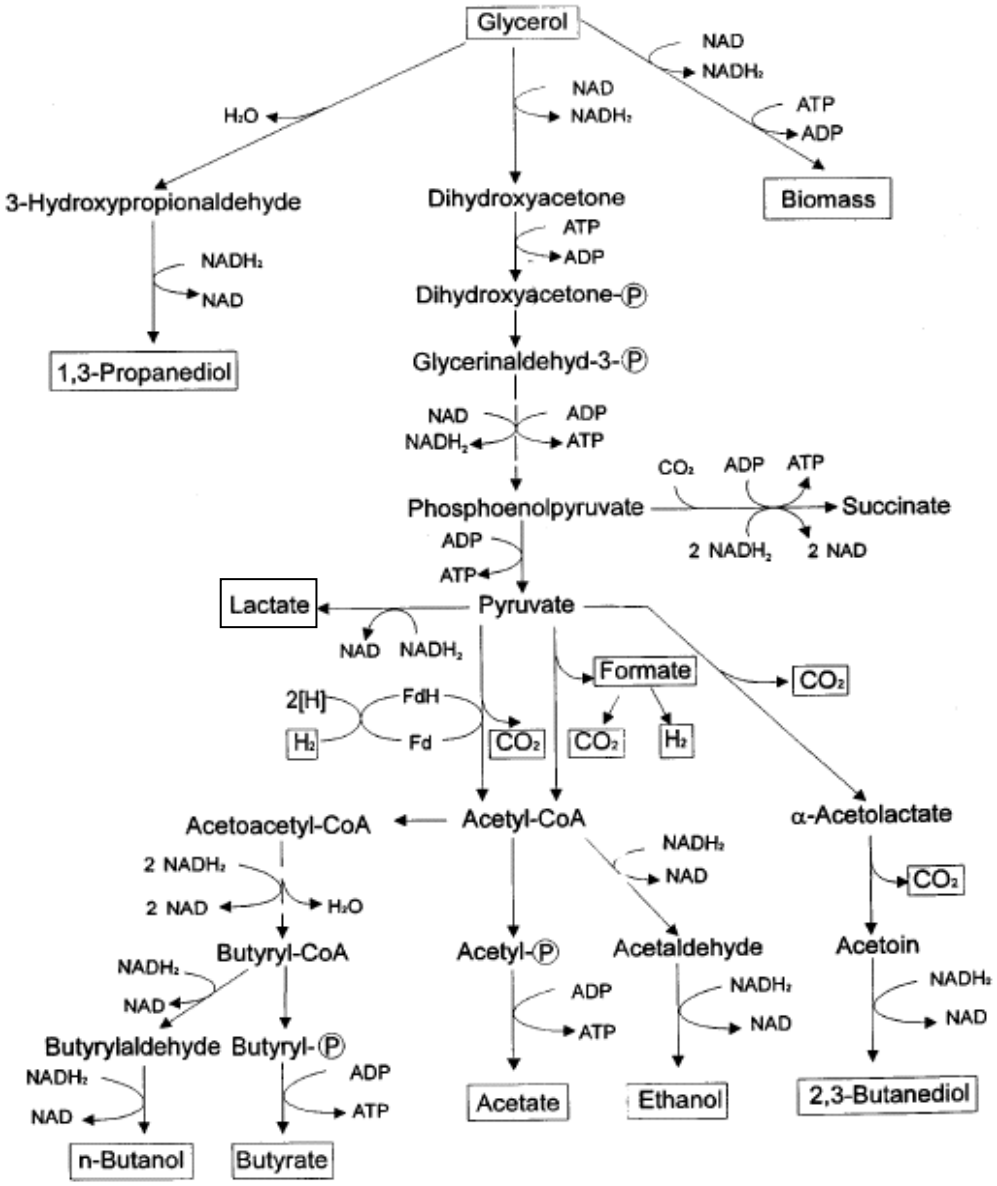



Figure 1.6. Glycerol fermentative biochemical pathways (in Biebl *et al.*, 1999). Pyruvate utilisation is indicated for different microorganisms.

*Citrobacter*, namely *C. freundii*, is able to use glycerol as the sole carbon source. The end-products of this fermentation are not only 1,3-propanediol, but also acetate, lactate, formate, pyruvate, ethanol, $CO_2$ and $H_2$ (Homann *et al*., 1990; Boenigk *et al.,* 1993). *Citrobacter* produces lower amounts of ethanol than *Klebsiella*. 1,3-Propanediol molar yield can reach 65%, and in this case the main by- product is acetate.

Barbirato *et al*. (1995) described an *Enterobacter*, *E. agglomerans*, that produces 1,3-propanediol as the major glycerol fermentation end-product. The by-products are acetate, ethanol, formate, lactate and succinate. When high glycerol concentrations were used (71 to 100 $g.l^{-1}$), 1,3-propanediol yield reached 0.61 mol/mol of glycerol. During batch cultivations after the consumption of about 40 $g.l^{-1}$ of glycerol, cell growth, glycerol uptake and 1,3-propanediol production ceased. This phenomenon was related with the lethal accumulation of 3-HPA and was also observed in *K. pneumoniae* and *C. freundii* (Barbirato *et al*., 1996). For *K. pneumoniae*, the fermentation ceased when 3-HPA concentration was over 0.79 $g.l^{-1}$ (Zheng *et al*., 2008).

A strategy using an engineered strain of *Escherichia coli*, was recently described by Tang *et al.* (2009). In this study, the first stage consisted of a significant increase of biomass using glucose and in the second stage glycerol added to the medium was converted to 1,3-PD. The overall fermentation time was only 40 h. The 1,3-PD obtained was 104.4 $g.l^{-1}$, with a productivity of 2.61 $g.l^{-1}.h^{-1}$ and a conversion of glycerol to 1.3-PD of 90.2% ($g.g^{-1}$).

### 1.5.2. *Lactobacillus*

As mentioned before, other microorganisms able to produce 1,3-PD belong to *Lactobacillus* genus, such as *L. brevis*, *L. buchneri*, *L. collinoides* and *L. reuteri* (Sauvageot *et al*., 2000; Sobolov and Smiley, 1960; Talarico *et al*., 1990; da Cunha and Foster, 1992). Some strains of lactobacilli can produce 1,3-PD from glycerol in co-fermentation with glucose or fructose. Glycerol cannot be metabolized as the sole carbon source by these species. NADH formed during the metabolism of sugars by glycolysis is reoxidized in the catabolic pathway of glycerol (Schutz and Randler, 1984). But according to da Cunha and Foster (1992), the influence of glycerol co-fermentation with sugars, appears to be more complex. They suggested not only the consumption of NADH but also its production (in order to avoid 3-HPA accumulation) via the production of acetate instead of ethanol or lactate and the reoxidation of formed lactate into acetate (via pyruvate).

### 1.5.3. *Clostridium*

#### 1.5.3.1. Bacteria characterization

Clostridia are rod-shaped, spore-forming Gram positive bacteria and typically strict anaerobes. They can naturally produce butanol, acetone, ethanol, isopropanol and 1,3-propanediol. Moreover, clostridia can produce chiral products which are difficult to obtain by chemical synthesis and degrade a number of toxic chemicals. Solventogenic clostridia, such as *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum* and *C. saccharoperbutylacetonicum*, can utilize a wide range

of substrates, from monosaccharides including many pentoses and hexoses to polysaccharides (Lee *et al*., 2008). A variety of enzymes involved in degradation of hemicellulose and starch have been identified in different strains. Cellulolytic clostridia, typified by *Clostridium thermocellum*, produce a multi-enzyme cellulase complex able to degrade cellulose, hemicellulose and starch (Mitchell, 1997). A remarkable development of clostridial toxins and spores has been their utility in the treatment of human diseases. Botulinum neurotoxin is used as a therapeutic agent for various neurological disorders, including dystonias, involuntary muscle disorders, pain, and other maladies. Spores systems of clostridia are being developed for the delivery of therapeutics to tumors (Johnson, 2009).

The nutrient requirements for the growth of clostridia are simple. Complex nitrogen sources are generally required for good growth and solvent production. For all these reasons, clostridia are a valuable source of enzymes that are used in bioprocessing and in biotransformations. Clostridia are important in fermentation processes for the production of solvents and organic acids. When growing in glucose, three different metabolic states can be found in *C. acetobutylicum* depending on pH (Girbal and Soucaille, 1998). At neutral pH *C. acetobutylicum* behaviour is acidogenic and it produces acetic and butyric acids; but when the pH is neutral and, at the same time, the availability of NAD(P)H is high, the behaviour is alcohologenic and it produces ethanol and butanol, but not acetone; at low pH the behaviour is solventogenic and it produces acetone, butanol and ethanol. Solventogenic behaviour is closely coupled to sporulation. The

transcriptional factor responsible for initiation of sporulation (Spo0A) is also responsible for the solvent production initiation in *C. acetobutylicum* (Lee *et al*., 2008) by activating transcription of acetoacetate decarboxylase, alcohol dehydrogenase and CoA transferase genes (Sullivan and Bennett, 2006). Although able to use several carbon sources, *C. acetobutylicum* cannot grow on glycerol as the sole carbon source, as it cannot re-oxidize the excess of NADH generated in glycerol catabolism (Vasconcelos *et al*., 1994). The best natural 1,3-PD producer in terms of yield and titer is *C. butyricum*. Through a genetic engineering strategy, it was possible to introduce the NADH consuming 1,3-PD pathway from *C. butyricum* into *C. acetobutylicum* and obtain the mutant strain *C. acetobutylicum* DG1 (pSPD5) able to grow on glycerol and produce 1,3-PD as the main fermentation end-product. This strain was used in the present work.

### 1.5.3.2. Fermentation end-products

Fermentation of glycerol by *Clostridium* results in production of 1,3-PD but also in several secondary products such as lactic, acetic and butyric acid, ethanol, $CO_2$ and $H_2$. The theoretical maximum yield that can be obtained from anaerobic fermentation of glycerol occurs when acetate is the only by-product produced (Saxena *et al*., 2009; Chotani *et al.,* 2000). The theoretical maximum 1,3-propanediol yield is 0.72 mol/mol glycerol, which was calculated for a culture without $H_2$ and butyric acid productions (Zeng, 1996). The production of by-products not only reduces the amount of carbon available, but can also inhibits the growth of microorganisms.

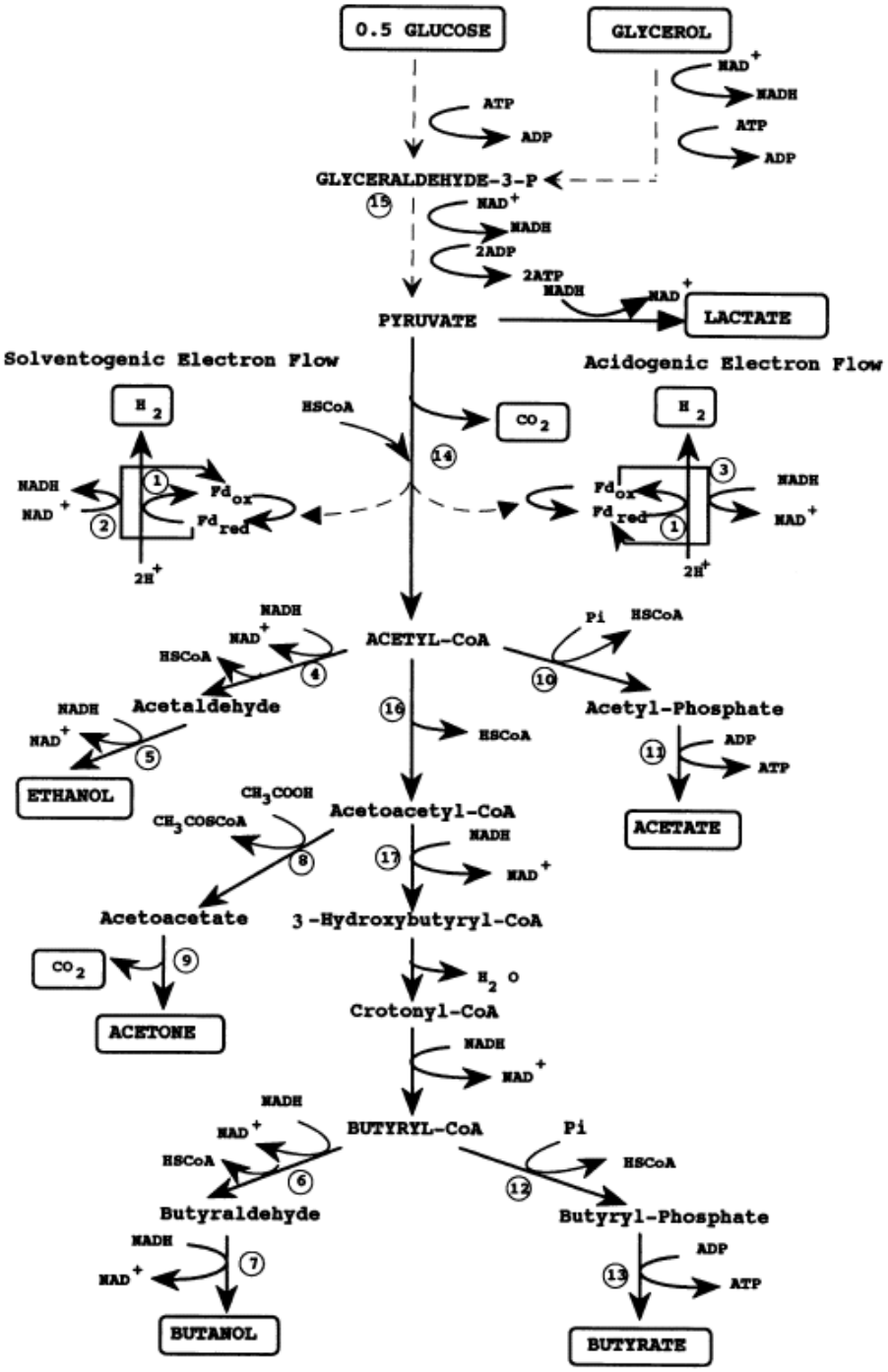



Figure 1.7. Metabolic pathways of *C. acetobutylicum* (Vasconcelos *et al.,* 1994). 1: Hydrogenase; 2: ferredoxin-NAD reductase; 3: NADH-ferredoxin reductase; 4: acetaldehyde dehydrogenase; 5: ethanol dehydrogenase; 6: butyraldehyde dehydrogenase; 7: butanol dehydrogenase; 8: CoA-transferase; 9: acetoacetate decarboxylase; 10: phosphotransacetylase; 11: acetate kinase; 12: phosphotransbutyrylase; 13: butyrate kinase; 14: pyruvate ferredoxin oxidoreductase; 15: glyceraldehyde-3-phosphate dehydrogenase; 16: thiolase; 17: 3-hydroxybutyryl-CoA dehydrogenase.

*C. pasteurianum* also synthesises butanol, sometimes the predominant product (Bieble *et al*., 1999). Other *Clostridium* able to produce butanol is *C. acetobutylicum*. Besides this product it can also produce acetone and ethanol however, it cannot produce 1,3-PD (Figure 1.6.). A characteristic reaction of *C. acetobutylicum* and others is the phosphoroclastic reaction. In this reaction pyruvate is cleaved by pyruvate ferredoxin oxireductase, in the presence of coenzyme A (CoA), to yield $CO_2$ and acetyl-CoA. The reduced ferredoxin ($Fd_{red}$) also formed is reoxidized in different reactions, and the most important one is the cleavage into hydrogen and oxidized ferredoxin ($Fd_{ox}$). Ferredoxin is an iron-sulphur-containing protein that accepts or gives electrons at a very low potential and together with NADH-ferredoxin oxiredoreductase, plays a crucial role as an electron carrier in electron distribution system in cells (Jungermann *et al*., 1973; Petitdemange *et al*., 1976). In the conversion of glycerol into 1,3-PD, the reducing equivalents ($NADH_2$) generated by the oxidative branch during the production of organic acids determines the amount of 1,3-propanediol that can be formed by the reductive branch. The genetic engineered *C. acetobutylicum* DG1 (pSPD5) contain the genes for the production of 1,3-propanediol from *C. butyricum* VPI 3266. A comparison between these two strains showed a similar physiological "global behaviour" when grown on glycerol (González-Pajuelo *et al*., 2006). In both strains the main fermentation end-product resulting from glycerol metabolism was 1,3-PD, and the hydrogen specific production rate was very slow. Most of the reduced ferredoxin produced by the decarboxylation of pyruvate was used to generate NADH, which caused the low hydrogen production. However, a major

difference was found between the two strains relatively to the oxidative glycerol pathway. While in *C. butyricum* VPI 3266 glycerol oxidation is carried out by glycerol dehydrogenase and dihydroxyacetone kinase (Saint Amans *et al.*, 2001), González-Pajuelo and co-authors (2006) demonstrated that the mutant strain *C. acetobutylicum* DG1 (pSPD5) uses only glycerol kinase and glycerol-3-phosphate dehydrogenase to oxidize glycerol. No glycerol dehydrogenase and dihydroxyacetone kinase activities were found in *C. acetobutylicum* DG1 (pSPD5). Another difference reported by the same authors, was the fact that lactate dehydrogenase activity was only detected in *C. acetobutylicum* DG1 (pSPD5). In glycerol fermentation by *C. butyricum* VPI 3266 no lactate was produced and no lactate dehydrogenase activity was found.

## 1.6. Process strategies for 1,3-pronanediol fermentation

In an attempt to enhance the production and reduce the final cost of 1,3-PD, many efforts have been focused on the development of new production processes. Fermentative production seems to be a feasible solution due to the present world situation. The use of renewable feedstocks and agricultural and industrial wastes in biological processes appears to be the desired solution to cheaper substrates and a lowered demand for diminishing crude oil supplies. Glucose, starch and glycerol appear as possible cheap carbon sources for 1,3-PD production; however, as no native microorganism can convert glucose or starch into 1,3-PD, metabolic engineering has been used to generate novel 1,3-PD producers biological systems. Whether using sugar or glycerol different

approaches have been implemented involving two-step fermentation, co-fermentation, mixed cultures and cells immobilization in batch, fed-batch or continuous cultures.

### 1.6.1. 1,3-Propanediol production from glycerol

Besides the microorganism, there are several factors influencing 1,3-PD production, such as medium composition, temperature, pH, end-products and substrate concentrations. A fed-batch process can be a solution to avoid substrate inhibition. Chemostat cultures are also interesting if high productivity can be reached. The addition of sugars as co-substrate can also improve the production of 1,3-PD from glycerol. Although sugar could not be converted into 1,3-PD, it can be used to cells growth and regeneration of reducing power (Yang *et al*., 2007). All these strategies were evaluated and the results achieved depended on the microorganism used and on the process configuration and operating conditions. Some of those results are described in Table 1.2. González-Pajuelo *et al.* (2005) described a new *C. acetobutylicum* mutant able to produce 60 $g.l^{-1}$ of 1,3-PD from glycerol with a yield of 0.64 $mol.mol^{-1}$ and a productivity of 3 $g.l^{-1}.h^{-1}$ in continuous cultures, what represented a two fold increased in 1,3-PD titer and productivity when compared to the wild type *C. butyricum* that in the same conditions produced up to 35 $g.l^{-1}$. Research on immobilized *K. pneumoniae* was carried out by Zhao *et al.* (2006) under batch, fed-batch and continuous cultures.

Table 1.2. 1,3-Propanediol final concentration, yield and productivity of different glycerol (and co-substrate) fermentation processes.

| Fermentation process | Microorganism | 1,3-PD (g/l) | YPD (mol/mol) | $q_{1,3\text{-PD}}$ (g/l/h) | Reference |
|---|---|---|---|---|---|
| Batch | *C. butyricum* DSM 5431 | 56.0 | 0.62 | 2.2 | Biebl *et al.* (1992) |
| | *C. butyricum* VPI 3266 | 35.0 | 0.65 | 0.6 | Saint-Amans *et al.* (1994) |
| | *C. butyricum* CNCM1211 | 67.0 | 0.63 | - | Himmi *et al*. (1999) |
| | *K. pneumoniae* M 5al | 58.8 | 0.53 | 0.92 | Cheng *et al.* (2007) |
| Fed-batch | *C. butyricum* DSM 5431 | 58.0 | 0.68 | 2.7 | Günzel et al. (1991) |
| | *C. butyricum* VPI 3266 | 65.0 | 0.69 | 1.0 | Saint-Amans et al. (1994) |
| | *C. butyricum* DSM 5431 | 70.3 | 0.68 | 1.5 | Abbad-Andaloussi et al. (1995) |
| | *C. butyricum* E5 | 65.6 | 0.65 | 1.2 | Petitdemange *et al*. (1995) |
| | *C. butyricum* DSM 5431 | 70.4 | 0.68 | 1.4 | Reimann and Biebl (1996) |
| | *C. butyricum* mutnt 2/2 | 70.5 | 0.66 | 0.9 | Reimann and Biebl (1996) |
| | *K. pneumoniae* ATCC 25955 | 73.3 | 0.48 | 0.92 | Cameron *et al.* (1998) |
| | *C. acetobutylicum DG1(pSPD5)* | 84.0 | 0.65 | 1.8 | González-Pajuelo *et al.* (2005) |
| | *K. pneumoniae* DSM 2026 | 53.0 | 0.47 | 1.7 | Mu *et al.* (2006) |
| | *K. pneumoniae* DSM 2026 | 75.0 | 0.61 | 2.2 | Liu *et al*. (2007) |
| | *K. oxytoca* LDH 3 | 83.5 | 0.62 | 1.39 | Yang *et al*. (2007) |
| | *K. pneumoniae* AC 15 | 71.0 | 0.64 | 2.37 | Zheng *et al.* (2008) |
| | *K. pneumoniae* DSM 2026 | 61.1 | 0.51 | 2.0 | Mu *et al.* (2008) |
| Continuous | *K. pneumoniae* DSM 2026 | 48.5 | 0.61 | 4.9 | Menzel *et al.* (1997) |
| | *C. butyricum* mutant 2/2 | 34.2 | 0.70 | 5.13 | Reimann *et al.* (1998) |
| | *C. butyricum* VPI 3266 | 30.0 | 0.65 | 10.3 | González-Pajuelo *et al.* (2005) |
| | *C. acetobutylicum DG1(pSPD5)* | 60.0 | 0.64 | 3.0 | González-Pajuelo *et al.* (2005) |
| Continuous two-stage | *C. freundii* DSM 30040 | 41.5 | 0.62 | 1.38 | Boenigk *et al.* (1993) |
| | *C. butyricum* F 2b | 46.0 | 0.53 | 3.4 | Papanikolaou *et al.* (2000) |
| | *C. butyricum* F 2b | 43.5 | 0.49 | 1.33 | Papanikolaou *et al*. (2008) |
| Microaerobic | *K. pneumoniae* DSM 2026 | 59.5 | - | 1.57 | Chen *et al.* (2003) |
| Microaerobic fed-batch | *K. pneumoniae* DSM 2026 | 72.0 | 0.57 | 2.1 | Liu *et al.* (2007) |
| | *K. pneumoniae* M 5al | 83.56 | 0.62 | 1.61 | Yang *et al*. (2007) |
| Aerobic fed-batch | *K. pneumoniae* TUAC01 | 70.6 | 0.56 | 1.05 | Hao *et al.* (2008) |
| | *K. pneumoniae* XJPD-Li | 65.26 | 0.56 | 3.16 | Ma *et al.* (2009) |
| Immobilized cells | *C. freundii* DSM 30040 | 16.4 | 0.57 | 8.2 | Pflugmacher and Gottschalk (1994) |
| | *K. pneumoniae* ZJU 5205 | 4.1 | 0.30 | 16.4 | Zhao *et al*. (2006) |

Bacteria were trapped in microcapsules of sodium cellulose sulphate/poly-dimethyl-diallyl-ammonium chloride. In the batch fermentation, 63.1g.$l^{-1}$ of 1,3-PD were produced; however, in continuous cultures the production decreased to 13.6 g.$l^{-1}$ with a dilution rate of 0.33 $h^{-1}$. In a recent study (Jin *et al*., 2010), hemicellulosic hydrolysates (corn straw) were used as co-substrate to improve 1,3-PD production. The use of the co-substrate resulted in higher biomass level and higher reducing power for the production of 1,3-PD. The final 1,3-PD concentration, yield and productivity were 71.58 g.$l^{-1}$, 65 mol.$mol^{-1}$ and 1.93 g.$l^{-1}$.$h^{-1}$, respectively, in fed-batch cultivations.

1,3-PD maximum concentration was obtained in batch and fed-batch cultures with produced titers up to 84 g.$l^{-1}$. Fed-batch fermentation with a non lactic acid producer *K. oxytoca* mutant, using sucrose as co-substrate under microaerobic conditions, resulted in 83.56 g.$l^{-1}$ of 1,3-PD (Yang *et al*., 2007). However, the highest productivities were achieved in continuous cultures, 3.0 – 10.3 g.$l^{-1}$.$h^{-1}$ (González-Pajuelo *et al*., 2005). The highest value reported by Zhao *et al.* (2006) of 16.4 g.$l^{-1}$.$h^{-1}$ was obtained in a continuous fixed-bed bioreactor at a dilution rate of 4.0 $h^{-1}$.

With the expansion of biofuels production, glycerol, which represents a secondary product, may become an abundant and cheap source for the biological production of 1,3-PD. Papanikolaou and co-workers (2000) showed that equivalent growth characteristics were obtained for batch cultures of *C. butyricum* F2b on pure or raw glycerol, from the biodiesel production process. A 1,3-PD yield of around 0.55 g.$g^{-1}$ of glycerol was observed for batch and chemostat

cultures on raw glycerol; 48 $g.l^{-1}$ of 1,3-PD were produced from 90 $g.l^{-1}$ of raw glycerol, at a dilution rate of 0.02 $h^{-1}$, and a volumetric productivity of 5.5 $g.l^{-1}.h^{-1}$ was obtained for a dilution rate of 0.21 $h^{-1}$. In order to reach simultaneously high volumetric productivity and product concentration, a two-stage continuous fermentation may be used, where the first reactor operates with a high dilution rate to achieve a high productivity and a low dilution rate is applied to the second reactor to increase product concentration. This strategy led to a 1,3-PD production of 41 - 46 $g.l^{-1}$ by *C. butyricum* F2b, growing on raw glycerol, with an overall volumetric productivity of 3.4 g $l^{-1}$ $h^{-1}$ as the highest value (Papanikolaou et al. 2000, 2008). González-Pajuelo et al. (2004) showed that *C. butyricum* VPI 3266 presented the same tolerance to raw and to commercial glycerol, when both had a similar grade (above 87% w/v); however, a 39% increase of growth inhibition was observed in the presence of 100 g $l^{-1}$ of 65% (w/v) grade raw glycerol. *C. acetobutylicum* DG1 (pSPD5), an engineered strain able to produce 1,3-PD from glycerol, also exhibited similar fermentation patterns on commercial (87% w/v) and raw (65% w/v) glycerol (González-Pajuelo et al. 2005). Hirschmann et al. (2005) reported a final 1,3-PD concentration of 87 $g.l^{-1}$ and a productivity of 2.2 $g.l^{-1}.h^{-1}$ in fed-batch cultures of a wild type *Clostridium* strain IK124 on treated raw glycerol. Economic production of 1,3-PD from raw glycerol may be possible with optimized operating conditions and novel technological strategies, but the main unknown cost factor is the strongly fluctuating glycerol market (Willke and Vorlop, 2008).

### 1.6.2. 1,3-Propanediol production from glucose

Glucose is not the natural substrate to produce 1,3-propanediol. This conversion requires the combination of two natural pathways. First the conversion of glucose into glycerol and then the production of 1,3-propanediol from glycerol. This can be achieved by two different approaches: a one-step process or a two step-process. For this purpose several strategies have been investigated.

One-step process

A one-step strategy is using mixed cultures, with genetically modified microorganisms to suppress the repression of 1,3-PD formation by the glucose organism (Cameron *et al.*, 1998). Through the application of mixed cultures, it would be possible to use one microorganism to convert sugars to glycerol and another one to convert glycerol to 1,3-PD. This can be achieved, but microbial repression of 1,3-propanediol production by glucose appears not to be favourable and the use of genetically modified microorganisms to suppress the repression of 1,3-PD formation is needed (Biebl *et al.*, 1999 and Cameron *et al*., 1998). Other one-step strategy is to combine the two pathways in one microorganism. Biebl *et al*. (1999) proposed three approaches to reach this goal. One is the introduction of the genes responsible for 1,3-propanediol synthesis in one microorganism that possesses the metabolic pathway for glycerol production. The other is the introduction of the genes responsible for glycerol production in a 1,3-popanediol producer microorganism. And the third way is to express both pathways in one microorganism. Many efforts have been carried out to produce 1,3-PD from sugars

via a single microorganism. However, this strategy is not satisfactory since the production of 1,3-PD by metabolic engineered *S. cerevisiae* or *K. pneumoniae* was only 0.4 or 1.2 $g.l^{-1}$ and 0.58 $g.l^{-1}$ respectively (Ma *et al.*, 2009; Rao *et al*., 2008; Zheng *et al*., 2008). Dupont and Genencor developed a glucose-based process using a recombinant *E. coli* carrying the genes for the production of glycerol from *S. cerevisiae* and the genes for the production of 1,3-PD from *K. pneumoniae*. 1,3-PD was produced with a titer of 135 $g.l^{-1}$ (Nakamura and Whited, 2003); however, this process is dependent on the addition of vitamin B12 inflating the cost of the production process.

Tow-step process

Other strategy is the fermentation in a two stage process, where one microorganism ferments sugar to glycerol in a first step and another one converts glycerol to 1,3-PD in a second step. Studies applying the two-stage process strategy have been already tried either by a recombinant E. *coli* or by a yeast strain to produce glycerol in the first stage and *K. pneumoniae* to convert glycerol into 1,3-PD in the second stage (Cheng *et al*. 2006 and Hartlep *et al.* 2002). However, the 1,3-PD yield reached was not high (0.41 - 0.53 $g.g^{-1}$ of glycerol) and according to Hartlep *et al.* (2002) the overall 1,3-PD yield from glucose in the two-stage process was 0.17 $g.g^{-1}$. In addition, *K. pneumoniae* is known as a facultative pathogen which limits its application in industry. Based on these approaches, many attempts have been carried out in the last years with the development of metabolic and genetic engineering tools and new fermentation processes.

*Chapter 2*

# Materials and methods

# 2.1. Microorganisms

## 2.1.1. Yeast strains

*Saccharomyces cerevisiae* strains used in this work are listed in Tables 2.1 and 2.2.

Table 2.1. *S. cerevisiae* industrial and oenological model strains.

| Strain | Characteristics | Source |
|---|---|---|
| IMD | Obtained from industrial strains JT6100 and JT6101 | Lesaffre Company (France) |
| V5 | *MAT**a** ura3* – derivative from Champagne wine strain 8130 | INRA (France) |

INRA - Institut National de la Recherche Agronomique

Yeast strains were kept for short periods at 4ºC on YPD or YM medium (described in 2.3) and stored in 20% (v/v) glycerol at -80ºC.

## 2.1.2. *Clostridium* strain

*Clostridium acetobutylicum* DG1 (pSPD5), a recombinant strain of *C. acetobutylicum* DG1, was used to perform the second part of this work. It was gently provided by Professor Philippe Soucaille (INSA, Toulouse, France). This genetically engineered strain does not own the megaplasmid pSOL1, carrying the genes for solvent production and consequently does not produce acetone and butanol. The transformation with the plasmid pSPD5, carrying the 1,3-

propanediol operon genes from *C. butyricum* VPI 3266, conferred the ability to consume glycerol as the sole carbon source and produce 1,3-propanediol. This mutant strain was stored in 20% (v/v) glycerol at -80ºC.

Table 2.2. *S.cerevisiae* laboratory strains used in this study.

| Strain | Genotype | References |
|---|---|---|
| CEN.PK2 | *MAT***a**/*MAT*α *leu2/leu2, trp1/trp1, ura3-52/ura3-52, his3/his3* | Van Dijken *et al* (2000) |
| HC13 | *MAT*α *leu2::GPD1-LEU2, trp1, ura3-52, his3* | Cordier *et al* (2007) |
| HC14 | *MAT*α *leu2, trp1, ura3-52, his3, tpi1□::Kan$^R$* | Cordier *et al* (2007) |
| HC16 | *MAT*α *leu2::GPD1-LEU2, trp1, ura3-52, his3, tpi1::Kan$^R$* | Cordier *et al* (2007) |
| HC17 | *MAT*α *adh1::Kan$^R$ leu2 trp1 ura3-52 his3* | Cordier *et al* (2007) |
| HC23 | *MAT*α *leu2::GPD1-LEU2 trp1::ALD3-TRP1ura3-52 his3* | Cordier *et al* (2007) |
| HC30 | *MAT*α *leu2::GPD1-LEU2 trp1::ALD3-TRP1tpi1::Kan$^R$ ura3-52 his3* | Cordier *et al* (2007) |
| HC32 | *MAT***a** *adh1::Kan$^R$leu2::GPD1-LEU2 tpi1::Kan$^R$ trp1 ura3-52 his3* | Cordier *et al* (2007) |
| HC42 | *MAT***a** *adh1::Kan$^r$ leu2::GPD1-LEU2 tpi1::Kan$^r$ trp1::ALD3-TRP1 ura3-52 his3* | Cordier *et al* (2007) |
| FH100 | *MAT***a** *adh1::Kan$^r$ leu2::GPD1-LEU2 tpi1::Kan$^r$ trp1::ALD3-TRP1 ura3-52 his3* (adapted to high glucose concentrations) | This work |
| FH200 | *MAT***a** *adh1::Kan$^r$ leu2::GPD1-LEU2 tpi1::Kan$^r$ trp1::ALD3-TRP1 ura3-52 his3* (adapted to high glucose concentrations) | This work |
| FM62 | *MAT***a** *adh1::Kan$^r$, leu2::GPD1-LEU2, tpi1::Kan$^r$, trp1::ALD3-TRP1, ura3-52, his3, ADH1-FPS1::URA3* | This work |

## 2.2. Yeast mutant construction

In order to construct the yeast mutant FM62, the linearized plasmid pHC33 (described by Cordier *et al*., 2007) was introduced by homologous

recombination at the *URA3* locus into HC42 strain. Yeast cells were transformed following the lithium acetate method as described by Woods and Gietz (2001). From a culture grown to an $OD_{600}$ between 0.5 and 0.6, 5 ml of cells were harvested by centrifugation (5 minutes, 4800 rpm, at 4ºC) and resuspended in 10 ml sterile ultra-pure cold water. The suspension was centrifuged for 5 minutes, at 4800 rpm and 4ºC, and the pellet was resuspended in 1 ml lithium acetate (LiAc) 100 mM. After transferred to microtubes and centrifuged for 15 seconds at maximum speed, the pellet was resuspended in 50 µl LiAc 100 mM. For the transformation, ingredients were added by the following order: 240 µl PEG 4000 50% (w/v), 36 µL LiAc 1 M , 50 µl salmon sperm DNA (2 mg/ml), 5 µl of plasmid DNA, and 29 ml sterile ultra-pure water. The mixture was incubated for 30 minutes at 30ºC with agitation. Cells were heat shocked at 42ºC for 45 min, microcentrifuged at maximum speed (15 seconds) and resuspended in 200 µl sterile ultra-pure water. After complete resuspension, cells were plated on synthetic defined $ura^-$ selective medium for uracil auxotrophy (described in 2.3) and incubated at 30ºC.

## 2.2.1. Polymerase chain reaction (PCR)

The genotype of the selected clones was confirmed by PCR. Genomic DNA was isolated using Master pure Yeast DNA Purification Kit (Epicentre) according to manufacturer instructions. The presence of *FPS1* gene cassette was confirmed by diagnostic PCR using primers:

HC_FPS1HS: 5’ AAGCTTATGAGTAATCCTCAAAAAGC 3’

HC_FPS1HAS: 5' AAGCTTTCATGTTACCTTCTTAGCAT 3'

The PCR was performed using DyNazyme II PCR Master Mix kit (Finnzymes) according to the manufacturer's specification.

## 2.3. Culture Media

All culture media were sterilized by autoclaving at 121ºC for 20 minutes, except molasses media that were sterilized twice for 15 minutes.

### 2.3.1 Yeast Malt Broth

Yeast Malt Broth (YM) from DIFCO was used to stock the yeast strains at 4ºC. This medium supplemented with agar and different glucose concentrations (50, 100 and 200 g $l^{-1}$) was also used to adapt the strain CEN.PK HC42 to high sugar concentrations.

### 2.3.2. Yeast Peptone Dextrose medium

Solid Yeast Peptone Dextrose (YPD) medium was used to maintain yeast strains at 4ºC and liquid YPD (Table 2.3) was used to prepare the inocula and to assess growth efficiency of engineered yeast strains. Different amounts of glucose were added to this medium according to the strain used.

This medium was also used to select transformed strains resistant to geneticin. In this case, YPD medium contained 0.2 $g.l^{-1}$ of G418 (Sigma) (according to Wach *et al.,* 1994).

Table 2.3. YPD medium composition.

| Components | Amount (per liter of deionised water) |
|---|---|
| Yeast extract | 10 g |
| Peptone | 20 g |
| Glucose | 20 g to 200 g |
| Agar* | 20 g |

* Only used for solid medium.

## 2.3.3. Synthetic Defined medium

Synthetic Defined (SD) medium was also used to prepare the inocula and to assess growth efficiency of engineered yeast strains (Table 2.4). For these purposes different glucose concentrations were used (20, 100, and 200 $g.l^{-1}$), according to the strain tested.

The "drop-out solution" containing the amino acids and vitamins required for yeast growth was previously sterilized by filtration and added to the autoclaved medium (Table 2.5).

For strains CEN.PK2 and IMD, 71.4 $mg.l^{-1}$ of leucine were also added to alleviate their auxotrophy, and for FM62 the addition of uracil was not required.

When necessary, silicon anti-foaming agent (Merck) was used at 0.1 ml $l^{-1}$.

This medium was buffered by addition of 85 mM sodium succinate (pH 5.0) when necessary, to avoid high pH changes during cultivation in shake flasks.

Table 2.4. SD medium composition.

| **Components** | **Amount (per liter of deionised water)** |
|---|---|
| Yeast nitrogen base without amino acids and $(NH_4)_2SO_4$ (DIFCO) | 1.7 g |
| $(NH_4)_2SO_4$ | 5 g |
| Glucose | 20 g to 200 g |
| Adenine | 0.01 g |
| Uracil | 0.01 g |
| Tyrosine | 0.05 g |
| Drop-out $Leu^-$ $Ura^-$ solution | 10 ml |

Table 2.5. Drop-out $Leu^-$ $Ura^-$ solution.

| **Components** | **Amount (per liter of deionised water)** |
|---|---|
| L-Arginine | 0.02 g |
| Histidine | 0.01 g |
| Threonine | 0.05 g |
| L-Tryptophan | 0.04 g |
| L-Isoleucine | 0.06 g |
| Lysine | 0.04 g |
| Methionine | 0.01 g |
| Phenylalanine | 0.06 g |

### 2.3.4. Medium for anaerobic cultivation of *Saccharomyces cerevisiae*

For *S. cerevisiae* cultivation under anaerobic conditions, the SD medium described before was supplemented with 0.01 $g.l^{-1}$ of ergosterol and 0.42 $g.l^{-1}$ of tween 80, needed for the yeast membrane stability in the oxygen absence conditions. 0.05 $g.l^{-1}$ of cysteine were added to the medium to ensure reduced environment and resazurin was used as an anaerobic indicator. SD medium was boiled before the distribution into serum bottles and degassed with $O_2$-free nitrogen. Cysteine was added to reduce the medium after sterilization (121ºC, 20 minutes).

### 2.3.5. Molasses medium

Molasses medium had the same composition as SD medium, except that glucose was replaced by a sugar cane molasses solution at different concentrations: 5%, 10%, 15% and 20% w/v (around 25 $g.l^{-1}$, 50 $g.l^{-1}$, 75 $g.l^{-1}$ and 100 $g.l^{-1}$ of sugars respectively in the final medium). The sugar cane molasses solutions were previously autoclaved for 15 minutes.

The composition of sugars in sugar cane molasses was confirmed by HPLC analysis: sucrose 82.7%, glucose 7.7%, fructose 5.9% and raffinose 3.7%.

### 2.3.6. Basic medium for *Clostridium acetobutylicum* cultivation

The basic medium used in this work to prepare *C. acetobutylicum* inocula was adapted from the synthetic liquid medium described by González-Pajuelo *et al.* (2005) (Table 2.6).

Table 2.6. Basic medium composition for *C. acetobutylicum* growth.

| **Components** | **Amount (per liter of deionised water)** |
|---|---|
| Glycerol | 30 g |
| $KH_2PO_4$ | 0.5 g |
| $K_2HPO_4$ | 0.5 g |
| $MgSO_4.7H_2O$ | 0.2 g |
| $FeSO_4.7H_2O$ | 0.01g |
| $CoCl_2.6H_2O$ | 0.01 g |
| Acetic acid | 2 g |
| Biotin | 0.04 mg |
| *p*-Aminobenzoic acid | 8 mg |
| Yeast extract | 4 g |
| Resazurin | 1 mg |

## 2.4. Inocula preparation

### 2.4.1. Yeast

For cultivation in shake flasks or in bioreactors, a loopful of the stock culture was inoculated into Erlenmeyer flasks containing SD medium (or YPD, since no difference was detected), which were incubated in a rotary shaker at 150 rpm. After 24h cultivation at 30ºC, cells necessary to start the fermentation with 0.05 to 0.1 $OD_{600}$ units were harvested by centrifugation and the pellet was resuspended in SD medium and transferred to the shake flasks or to the bioreactor. For anaerobic cultivation, a volume of the inoculum culture (2% v/v) was inoculated into sealed bottles using sterile syringes.

### 2.4.2. *Clostridium acetobutylicum*

As this microorganism is an obligate anaerobic bacterium the culture medium (described in Table 2.6.) was boiled, distributed in 100 ml bottles and sparged with $O_2$-free nitrogen for 15 to 20 minutes. Bottles were closed with butyl rubber stoppers and sealed, before autoclaving. Sterilized bottles were inoculated with 10% (v/v) of a cells suspension stored at -80ºC, and incubated for 24h at 35ºC (Figure 2.1).

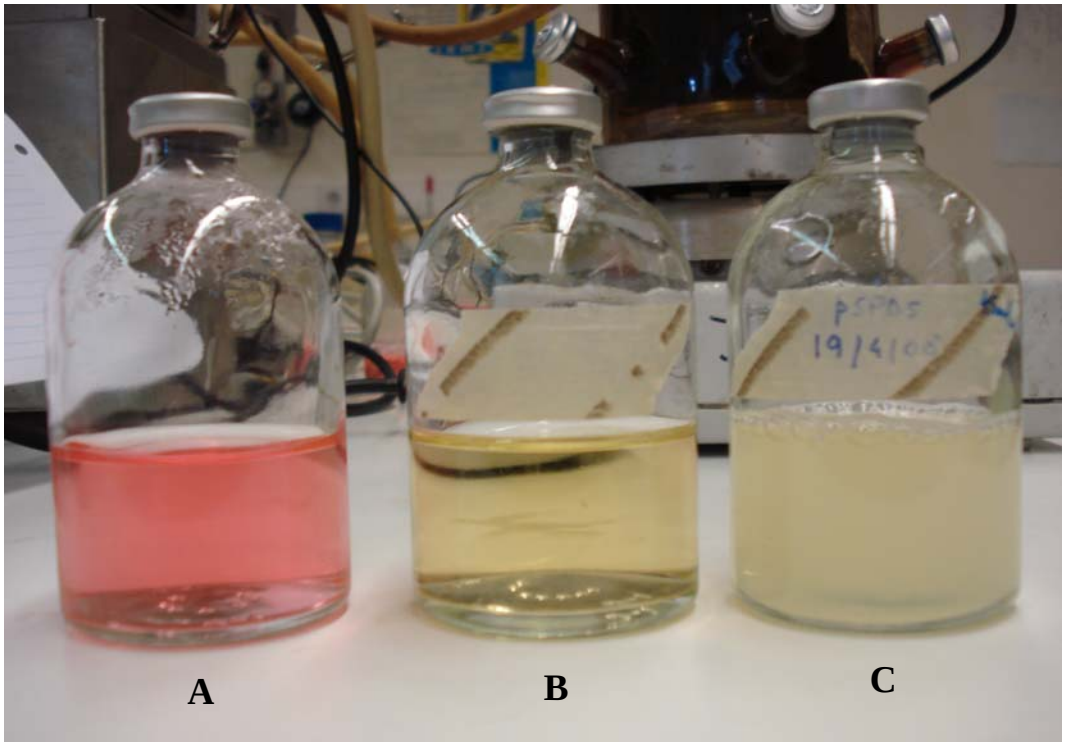



Figure 2.1. Bottles with medium for cultivation in anaerobic conditions. A- before autoclaving; B – after autoclaving; C – with the inoculum culture.

## 2.5. Batch cultures

### 2.5.1. Yeast anaerobic cultures

Some preliminary tests were performed with yeasts under anaerobic conditions. Fermentations were carried out in serum bottles of 100 ml with 50 ml of SD medium supplemented as described before, at 30ºC and 150 rpm.

### 2.5.2. Shake flasks cultivation

To assess growth efficiency, the wild type and engineered yeast strains were cultivated at 30ºC in 0.5 l Erlenmeyer flasks containing 150 ml of culture medium.

### 2.5.3. Bioreactor cultivation

The tow-step process cultivations were carried out in a 2-liter bioreactor (Biostat MD, Braun, Melsungen, Germany).

In the first stage of the process yeast cultures were carried out at 30ºC, with continuous stirring at 150 rpm and aeration rate of 0.5 vvm. The pH was maintained at 6.5 by automatic addition of NaOH 1 M.

For the second stage of the process, carried out by *Clostridium acetobutylicum*, operating conditions were changed. To assure the anaerobic environment needed for bacterium growth, yeast broth was degassed by sparging sterile $O_2$-free nitrogen for around 8 hours. The gas out-let of the bioreactor was connected to a pyrogallol arrangement (Vasconcelos *et al*., 1994), which trapped any oxygen entry.

The fermentation was carried without pH regulation and agitation until exponential phase was reached. Only than, pH was maintained at 6.5 with the automatic addition of $NH_4OH$ 6N and agitation was settled at 100 rpm. Temperature was set at 35ºC and the fermentation broth was continuously flushed with sterile $O_2$-free nitrogen, to avoid oxygen entry.

## 2.6. Fed-batch cultures

*S. cerevisiae* fed-batch cultures were carried out in a 2-liter bioreactor (Biostat MD, Braun, Melsungen, Germany) with an initial volume of 1 l. SD medium was used, with an initial glucose concentration of 50 $g.l^{-1}$. Feed medium was a 500 $g.l^{-1}$ glucose solution, sterilised by autoclaving for 15 minutes at 121ºC. Glucose concentration in the culture medium was controlled by HPLC. To obtain 50 $g.l^{-1}$ of glucose in the fermentation broth, the culture was fed each time glucose concentration in the medium reached 10 $g.l^{-1}$.

The pH was kept constant at 6.5 by automatic addition of 1M sodium hydroxide. Culture was aerated at a flow rate of 0.5 vvm.

## 2.7. DNA microarray analysis

This technique allows the analysis of global yeast genome expression. In this work, using this technique, it was possible to compare the expression of the 6200 genes from the wild strain CENPK2 and the mutant strain HC42. Figure 2.2 represents a general overview of the method used to perform the analysis of the genome-wide transcription of the two strains mentioned above. Particular steps of this method are described in the following subchapters.

The wild type strain and HC42 strain were cultivated in SD medium, and cells were harvested in early log phase ($OD_{600}$ = 1.0 unit) by centrifugation from four independent cultures and kept at -80°C until use.

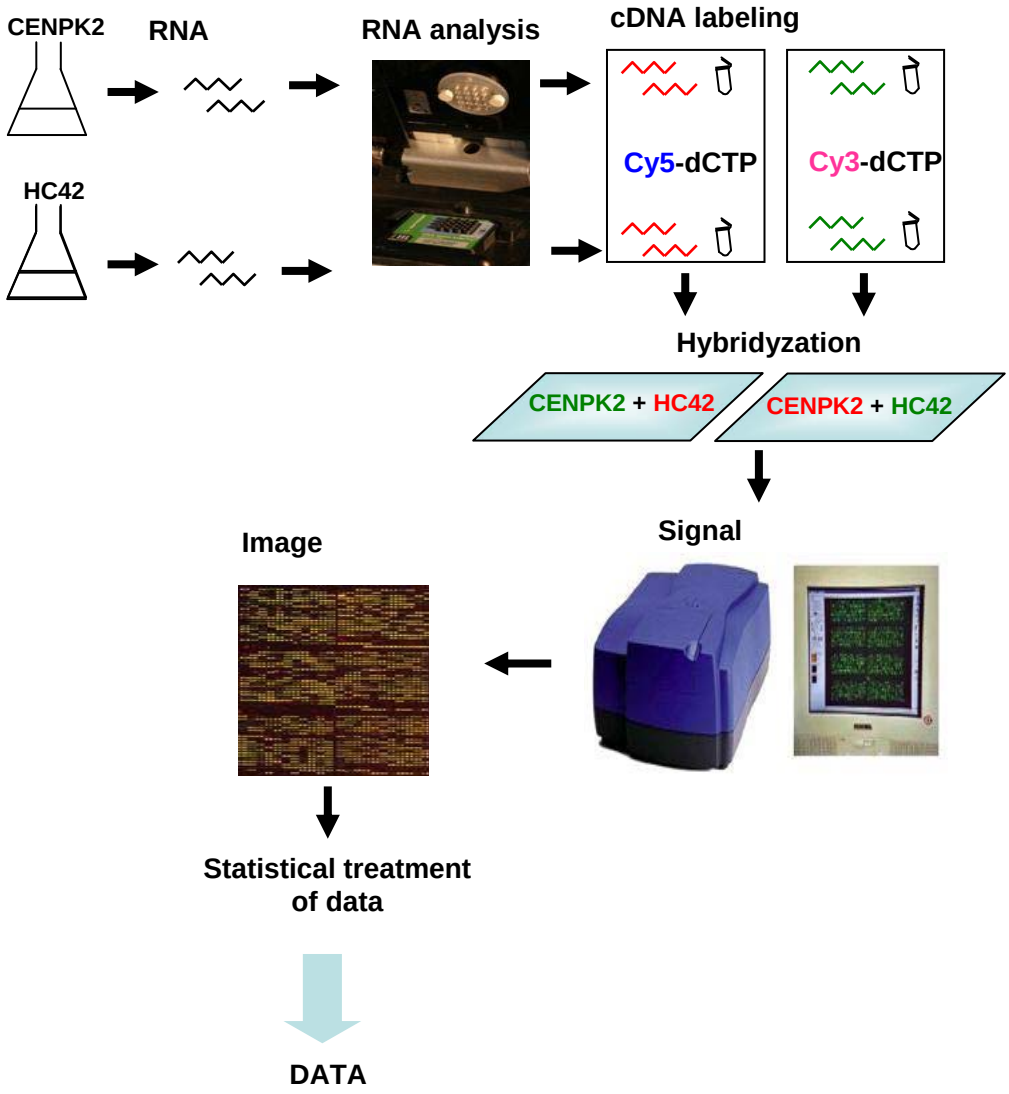



Figure 2.2. DNA microarray – a scheme of the method.

## 2.7.1. RNA isolation, cDNA synthesis and hybridization

Frozen cells (10 units of $OD_{600}$) were mechanically disrupted (MicroDismembrator Braun, Melsungen) and total RNA was isolated using RNeasy Mini kit (Qiagen) following the protocol of the manufacturer. The quantity and the quality of the extracted RNA were determined by microcapillary

electrophoresis using Bioanalyzer 2100 and the RNA 6000 Nano Assay Kit (Agilent Technologies, Wilmington, DE, USA). Incorporation of Cyanine 3- and Cyanine 5-dCTP (Amersham Bioscience) was performed during reverse transcription of total RNA using LabelStar Reverse Transcriptase (Qiagen). Labelled cDNA was purified on MinElute spin columns (Qiagen) and was hybridized on dendrimer-activated glass slides, which bears the whole yeast genome by covalently attached DNA probes (70-mer oligonucleotides) made according to LeBerre *et al.,* 2003. Hybridization was carried out in an automatic hybridization chamber (Discovery from Ventana Medical System, Inc) for 10 h at 42°C. After hybridization, the slides were washed in 2 x SSC/0.2% (v/v) SDS, immersed briefly in isopropanol and then dried under a stream of air. Biological replicates of DNA arrays experiments were made by using total RNA that were extracted from four independent cultures (four cultures for both wild type and the engineered strains). Transcripts from two independent cultures (for both wild type and engineered strains) were retro transcribed using dCTP-CY3, and the two other reciprocally with dCTP-CY5. Labelled cDNA from both strains were competitively hybridized on DNA microarrays. This resulted in four DNA arrays, with eight intensity values for each gene, since each gene was represented two times on an array. The hybridization signal was detected by scanning using GenePix 4000B laser Scanner (Axon Instruments), and the signal quantification was transformed to numerical values using the integrated GenePix software version 3.01.

### 2.7.2. Transcript data acquisition and treatment

The data were statistically treated using home-made Bioplot/Bioclust software accessible at http://biopuce.insa-toulouse.fr/ExperimentExplorer/doc/BioPLot/. Raw intensities were corrected from the background, log transformed and normalized by the mean log-intensity of all spots. Log-ratios of normalized intensities from quadruplicate samples were tested for statistical significance using Student's *t*-test with Benjamini and Hockberg test correction, with a prediction for false discovery rate to about 5% of the genes identified. The differentially expressed genes were further narrowed with fold changes in expression of at least 1.5-fold. They were classified according to functional categories following MIPS (http://mips.gsf.de/proj/funcatDB/search_main_frame.html). Other publicly available resources for data expression analysis were used including Go Term Finder (http://yeastgenome.org) and FunSpec (http://funspec.med.utoronto.ca/) for Gene ontology classification and YEASTract (http://www.yeastract.com/) to search of transcriptional factors.

## 2.8. Analytical procedures

### 2.8.1. Cell concentration determination

Yeast cells concentration was measured by three different methods: optical density (OD), cell dry weight (DW) and Colony Forming Unit (CFU). The latter was used for the fermentations with molasses.

Correlations between OD and DW and between OD and CFU were established.

#### 2.8.1.1. Optical density

Optical density of samples of fermentation broth was measured at 600 nm. Samples presenting an OD value higher than 0.6 were diluted with deionised water. OD was measured immediately after collecting the sample.

#### 2.8.1.2. Cell dry weight

Yeast dry weight was determined by filtering 40 ml of the culture using pre-weighted filters (pore size 0.45 μm). Filters were washed twice with an equal volume of deionised water and dried until the weight reached a stable value.

#### 2.8.1.3. Colony Forming Units (CFU)

The number of yeast cells, expressed as $cfu.ml^{-1}$, was determined using the classical plate count method in YM agar (Difco) or YPD agar, after incubation at 30ºC for 48h.

### 2.8.2. Analysis of substrates and fermentation end-products

Substrates and fermentation end-products concentrations were determined by High Performance Liquid Chromatography (HPLC). Glycerol concentration was also determined by an enzymatic analysis.

#### 2.8.2.1. HPLC analysis

Glucose, fructose, sucrose, raffinose, succinate, acetate, acetoin, acetaldehyde, ethanol, glycerol, 1,3-PD and butyrate were determined by HPLC.

A sample of the fermentation broth was centrifuged, and the supernatant was filtered through 0.22 μm pore size nylon filters prior to perform the separation on a Bio-Rad Aminex column HPX-87H (300 x 7.8 mm; Richmond, California, USA). The detection was achieved by using refractive index. Operating conditions were as follows: mobile phase, 1 mM sulphuric acid; flow rate, 0.5 $ml.min^{-1}$ and temperature, 30ºC.

#### 2.8.2.2. Enzymatic glycerol analysis

The Boehringer-Mannheim enzymatic kit for glycerol analysis (Cat. No. 148 270) was used to confirm results from HPLC analysis and for concentrations under the HPLC detection limit.

### 2.8.3. Determination of enzyme activity

#### 2.8.3.1. Cell extracts

Cells from yeast culture were harvested (about 25 mg dry mass) and disrupted by vortexing at 4ºC, with 1 g glass beads (0.4-0.5 mm diameter), 0.5 ml of 20 mM Hepes pH 7.1, 20 mM KCl, 1 mM EDTA, 1 mM DTT and protease inhibitor cocktail from Boehringer (1 capsule for 10 ml of buffer), for four cycles of 30 seconds. After centrifugation at 8000*g* (15 minutes at 4ºC), the supernatant was kept in ice and used for enzymatic assays.

### 2.8.3.2. Enzymatic assays

Enzymatic assays for dehydrogenases enzymes were performed on ELISA plates (final volume per well 250 µl) at 30ºC. NADH oxidation or $NAD^+$ reduction was measured at 340 nm. A calibration curve was established correlating OD values of NADH and the related concentrations values.

For the phosphatase, released inorganic phosphate (Pi) concentration was measured at 820 nm using a spectrophotometer (Pharmacia Biotech UV / Visible Ultrospec 1000). A standard curve was established for Pi.

All enzymatic assays were performed in triplicate. The results are the mean of three independent experiments.

One unit of enzyme activity is defined as the amount of enzyme necessary to catalyze the conversion of 1µmol of substrate per minute.

#### 2.8.3.2.1. Glycerol-3-phosphate dehydrogenase

Glycerol-3-phosphate dehydrogenase activity was measured following the modified assay described by Gancedo *et al.* (1968).

Reaction: DHAP + NADH ⟷ Glycerol-3-P + $NAD^+$

Assay mixture:

| | |
|---|---|
| - Hepes (pH 7.1) | 20 mM |
| - NADH | 0.15 mM |
| - DHAP | 5 mM |
| - Cell extract | 10 - 100 µg of protein |

The reaction was started by the addition of DHAP.

#### 2.8.3.2.2. Glycerol-3-phosphate phosphatase

Glycerol-3-phosphate phosphatase activity was determined as described by Gancedo et al. (1968), with some modifications in the method.

Reaction: Glycerol-3-P ⟶ Glycerol + Pi

Assay mixture:

| | |
|---|---|
| - Hepes (pH 7.1) | 20 mM |
| - $MgCl_2$ | 10 mM |
| - DTT | 0.5 mM |
| - Glycerol-3-P | 10 mM |
| - Cell extract | 100 – 200 µg of protein |

The reaction was started with the addition of glycerol-3-P. 0.1 ml were taken in 0.1 ml of hot water (in Eppendorf tube kept in a water bath at 80ºC) at 3, 6, 9, 12, 18 and 30 min after starting the reaction. Released Pi was measured by the Elon-Molybdate method.

#### 2.8.3.2.3. Glycerol dehydrogenase

The $NAD^+$ and $NADP^+$-dependent glycerol dehydrogenase were measured according to Vries *et al.* (1989).

Reaction: Glycerol + $NAD(P)^+$ ⟶ Dihydroxyacetone + NAD(P)H

Assay mixture:

- Hepes (pH 7.1) 20 mM
- KCl 5 mM
- DTT 0.5 mM
- $MgSO_4$ 2.5 mM
- $NAD^+$ or $NADP^+$ 1.2 mM
- Glycerol 40 mM
- Cell extract 150 – 300 μg of protein

The reaction was started by addition of glycerol 10% (w/v).

The enzyme activity was also measured at pH 9.0. For this propose the reaction buffer Hepes was changed to $K_2CO_3$ 100 mM.

**2.8.3.2.4. Acetaldehyde dehydrogenase**

The activities of acetaldehyde dehydrogenase $NAD^+$ and $NADP^+$-dependent were measured by following the method of Postma *et al.* (1989).

Reaction: Acetaldehyde + $NAD(P)^+$ ⟶ Dihydroxyacetone + NAD(P)H

Assay mixture:

- $KH_2PO_4$ (pH 8.0) 100 mM
- Pyrazol 15 mM
- DTT 0.4 M

- $MgCl_2$ 10 mM
- $NAD^+$ or $NADP^+$ 0.4 mM
- Acetaldehyde 100 mM
- Cell extract 50 – 100 μg of protein

The reaction was started by addition of acetaldehyde.

#### 2.8.3.2.5. Alcohol dehydrogenase

Alcohol dehydrogenase activity was assayed by following the $NAD^+$ reduction at 340 nm.

Reaction: Ethanol + $NAD^+$ ⟷ Acetaldehyde + NADH

Assay mixture:

- $KH_2PO_4$ (pH 8.0) 50 mM
- $NAD^+$ 1.0 mM
- Ethanol 100 mM
- Cell extract < 5 μg of protein

The reaction was started by the addition of ethanol.

#### 2.8.3.2.6. Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase activity was measured following the formation of NADPH.

Reaction: Glucose-6-phosphate + $NADP^+$ ⟷ 6 -P -Glucolactone + NADPH

Assay mixture:

- Hepes (pH 7.1) 20 mM
- $NADP^+$ 0.4 mM
- Glucose-6-phosphate 4.5 mM
- Cell extract 2 – 10 µg of protein

The reaction was started by the addition of glucose-6-P.

#### 2.8.3.2.7. Glucose-6-phosphate isomerase

Glucose-6-phosphate isomerase (GPI) activity was measured indirectly by following the formation of NAPH in the coupled reaction using glucose-6-phosphate dehydrogenase (G6PD) (Sigma).

Reaction: (1) Fructose-6-P $\xleftrightarrow{\text{GPI}}$ Glucose-6-P

(2) Glucose-6-P + $NADP^+$ $\xleftrightarrow{\text{G6PD}}$ 6-P-Gluconolactone + NADPH

Assay mixture:

- Hepes (pH 7.1) 20 mM
- $NADP^+$ 0.4 mM
- Glucose -6-P dehydrogenase 1 $U.ml^{-1}$
- Fructose-6-phosphate 4 mM

- Cell extract < 6 µg of protein

The reaction was started by the addition of fructose-6-phosphate.

## 2.8.4. Determination of protein content

The protein concentration in cell extracts was determined by the Bradford method (Bio-Rad Kit assay nº 500-002) adapted to ELISA. The final volume was 250 µl. 50 µl of the dye reagent from Bio-Rad was mixed with 200 µl of cell extract (diluted from 100 to 1000 times) and incubated at room temperature. The extinction was measured at 550 nm and OD values were accepted till 0.6. Bovine serum albumin (BSA) was used as standard. A calibration curve was settled with BSA concentrations between 0 and 20 $\mu g.ml^{-1}$.

## 2.8.5. Quantification of intracellular glycerol

For measuring intracellular glycerol, yeast cells were collected at the exponential growth phase. 20 ml of a cell sample were quickly filtered through a 0.45 µm nitrocellulose filter and immediately washed with the same volume of pre-cold 60% methanol solution (-20ºC) for quench metabolism. The filters with the collected cells were immersed in 2 ml of ice-cold ultra-pure water. After briefly shaking the tubes with the filters to assure that the cells were separated from the filters, the tubes were kept for 10 min in a water bath set at 90ºC. Cells debris were separated by centrifugation at 10000*g* for 5 min and glycerol was

measured in the supernatant using HPLC or a NADH-coupling enzymatic assay at 340 nm (as described in points 2.8.2.1 and 2.8.2.2).

The assay mixture for the reaction contained:

- Glycine 0.1 M
- Hydrazine 0.5 M
- ATPMg 1 mM
- NAD 2 mM
- Glycerokinase 1 $U.ml^{-1}$
- Glycerol-3-phosphate dehydrogenase 0.2 $U.ml^{-1}$

Glycerokinase was from *E. coli* (Sigma) and glycerol-3-phosphate dehydrogenase from rabbit muscle (Sigma). The increase of NADH concentration, measured by the change in absorbance, is proportional to the amount of glycerol.

## 2.8.6. Determination of intracellular nucleotide pools

### 2.8.6.1. Extraction procedure

Intracellular coenzymes $NAD^+$ and NADH were extracted from yeast cells according to Gonzalez *et al.* (1997) (Figure 2.3). In this method, 1 volume (25 ml in this work) of culture broth at an $OD_{600}$ around 1.0 was collected and added to 5 volumes of a 60% (v/v) methanol solution buffered with 10 mM-Hepes (final concentration). After centrifugation, 5 ml of a boiling 75% (v/v) ethanol solution, buffered with 10 mM-Hepes (final concentration) pH 7.5 was added to the pellet.

The extraction was fast, requiring only 3 minutes incubation of yeast cells in the ethanol-buffered mixture maintained at 80ºC.

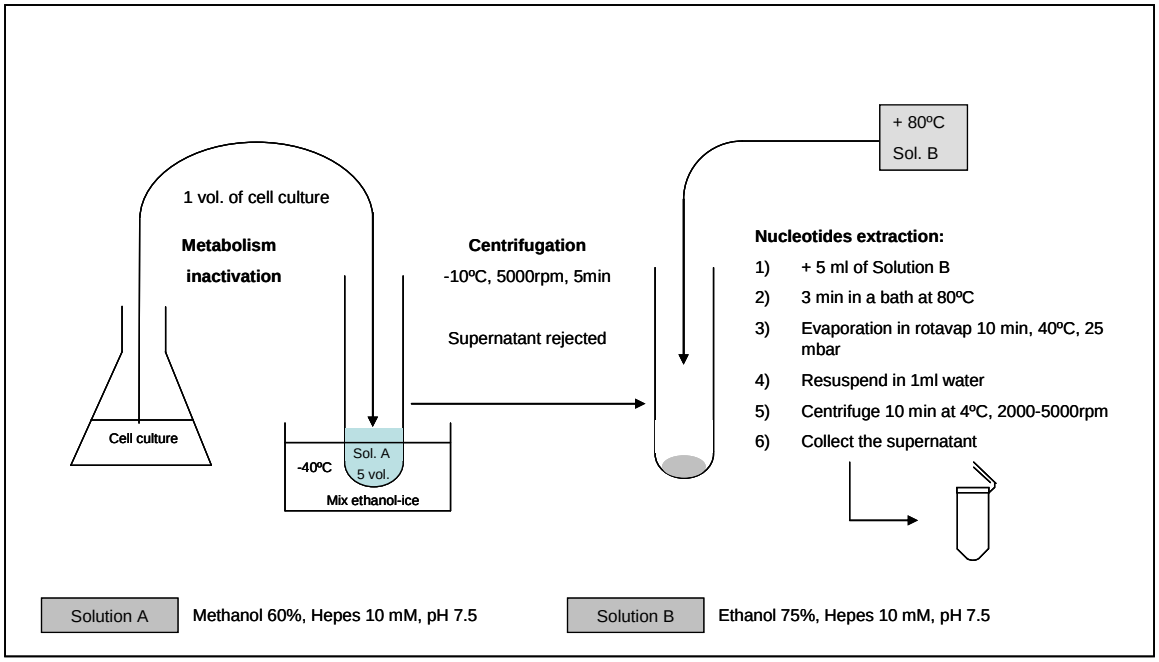



Figure 2.3. Method for nucleotides extraction (according to Gonzalez *et al.*, 1997).

Extracts were subsequently concentrated by evaporation under partial vacuum and the residue was resuspended in 1ml water. After centrifugation, the supernatant was collected for nucleotide determination.

### 2.8.6.2. Nucleotide pool assays

$NAD^+$ and NADH were measured by fluorescence spectrophotometry (excitation wavelength 340 nm, emission wavelength 460 nm) using enzymatic coupled reactions as described by Klingengberg (1974).

**$NAD^+$ assay**

For $NAD^+$ determination the following reaction was used, catalyzed by the enzyme alcohol dehydrogenase:

$$Ethanol + NAD^+ \longrightarrow Acetaldehyde + NADH + H^+$$

The assay mixture (3 ml) contained:

| | |
|---|---|
| - Pyrophosphate with 0.25% (v/v) semicarbazide (pH 8.8) | 50 mM |
| - Ethanol | 1% (v/v) |
| - Alcohol dehydrogenase | 10 $U.mL^{-1}$ |
| - Cell extract | 50 µL |

Fluorescence was measured before and after the addition of alcohol dehydrogenase.

**NADH assay**

For NADH assay the fluorescence decrease was measured, corresponding to NADH consumption in the following reaction:

$$NADH + H^+ + Pyruvate \longrightarrow NAD^+ + Lactate$$

The assay mixture (3 ml) contained:

| | |
|---|---|
| - Triethanolamine (pH 7.8) | 100 mM |
| - $MgSO_4$ | 3 mM |
| - EDTA | 0.8 mM |
| - Pyruvate | 5 mM |
| - Lactate dehydrogenase | 10 $U.mL^{-1}$ |
| - Cell extract | 250 µL |

Fluorescence was measured before and after the addition of lactate dehydrogenase.

## 2.9. Calculations

The maximal specific consumption and production rates in batch cultivations were calculated by fitting glucose, biomass and metabolites concentration versus time data to a polynomic function. The derivative of the equations for glucose consumption, glycerol and ethanol production was determined and divided by the corresponding biomass concentration.

The volumetric glucose uptake rate was calculated by dividing the amount of glucose consumed by the corresponding cultivation time. Products volumetric productivities were calculated dividing the maximum concentration obtained by the corresponding cultivation time.

Carbon recovery in yeast cultivation was calculated from the balance between substrate consumed and metabolites produced, including $CO_2$ and biomass. $CO_2$ amount was determined from ethanol production according to the equation:

1 mol glucose $\longrightarrow$ 2 mol ethanol + 2 mol of $CO_2$

It was assumed that 24.95 g of yeast biomass correspond to 1 mol of carbon.

# Part I: Metabolic engineering of *Saccharomyces cerevisiae* for glycerol overproduction

*Chapter 3*

# Characterisation and evaluation of wild type and mutant strains of S*accharomyces cerevisiae* for glycerol overproduction

## 3.1. Metabolic and physiologic studies of *Saccharomyces cerevisiae* mutant strains for glycerol overproduction

*S. cerevisiae* is a natural glycerol producer. Glycerol is formed as a by-product in production of ethanol and baker's yeast during fermentation of *S. cerevisiae* under anaerobic and aerobic growth conditions, respectively. It is known that one of the physiological roles of glycerol formation by yeast is to reoxidize NADH, formed in synthesis of biomass and secondary fermentation products, to $NAD^+$. Glycerol is one of the most important by-products of glucose conversion by yeast in anaerobic conditions. Nevertheless, the amounts of glycerol produced under these conditions are relatively small, reaching 0.1 $g.g^{-1}$ consumed glucose during fermentation. In *S. cerevisiae* glycerol is synthesised in two reaction steps from the glycolytic intermediate dihydroxiacetone phosphate (DHAP) (Fig. 3.1). DHAP is reduced by a $NAD^+$-dependent glycerol-3-phosphate dehydrogenase (GPD), followed by the dephosphorylation of glycerol-3-phosphate (G3P) thus formed by a glycerol-3-phosphate phosphatase (GPP) (Albertyn *et al*., 1994b; Ansell *et al*., 1997; Pahlman *et al*., 2001). The two GPD isoenzymes are encoded by *GPD1* and *GPD2* genes (Larsson *et al*., 1993) and the two GPP isoenzymes are encoded by *GPP1* and *GPP2* genes (Pahlman *et al*., 2001). *GPD1* and *GPP2* are induced under osmotic stress, whereas *GPD2* and *GPP1* are induced under anaerobic conditions (Albertyn *et al*., 1994b; Ansell *et al*., 1997; Costenoble *et al*., 2000; Pahlman *et al*., 2001).

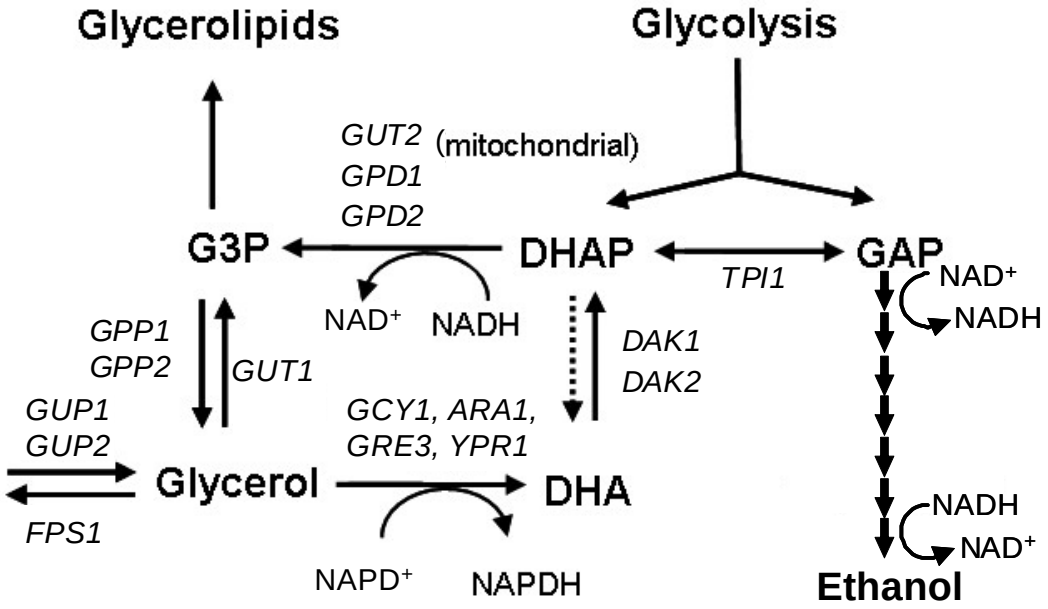



Figure 3.1. Pathway and genes involved in glycerol metabolism. GAP, glyceraldehyde 3-phosphate; G3P, glycerol 3-phosphate; DHAP, dihydroxyacetone phosphate; DHA, dihydroxyacetone; *GUT2*, mitochondrial glycerol-3-phosphate dehydrogenase 2; *GPD1*, glycerol-3-phosphate dehydrogenase 1; *GPD2*, glycerol 3-phosphate dehydrogenase 2; *DAK1*, dihydroxyacetone kinase 1; *DAK2*, dihydroxyacetone kinase 2; *GPP1*, glycerol-3-phosphatase 1; *GPP2*, glycerol-3-phosphatase 2; *GUP1*, glycerol uptake protein 1; *GUP2*, glycerol uptake protein 2; *GUT1*, glycerol kinase 1; *FPS1*, plasma membrane glycerol channel; *GCY1,* putative $NADP^{(+)}$ coupled glycerol dehydrogenase; *YPR1*, *ARA1*, *GRE3* and *YPR1*, *GCY1* homologous.

Over the past decade, there have been attempts to improve glycerol production in yeast, from the classical Neuberg bioprocess-mediated mode involving sulphite towards a strain engineered by recombinant DNA technology for a rational reprogramming of the cellular metabolism. Genetic engineering has been relatively successful since it raised the glycerol production from 0.25 $g.g^{-1}$ glucose obtained by the sulphite process (Bisping and Rehm, 1988; Petrovska *et al*., 1999) to 0.42 $g.g^{-1}$ glucose by rerouting glucose to glycerol through deletion of *TPI1* encoding triose phosphate isomerase together with removal of the cytosolic NADH reoxidation by mitochondrial redox shuttles (Overkamp *et al*., 2002). In a recent strategy developed by Geertman *et al.* (2006), *S. cerevisiae* was able to produce glycerol close to the theoretical maximum yield of 1 mol glycerol per mol of glucose that is expected from a *TPI1* deletion.

With the variety of genetic tools available for *S. cerevisiae* and the possibility of several genetic manipulations, the most important challenge is to reach the maximal glycerol yield, at the fastest rate by reducing the contribution of assimilatory glucose metabolism to anabolic and maintenance purposes, and to eventually increase the average productivity rates. Based on these considerations, the aim of this study was to evaluate another metabolic engineering strategy of the central carbon metabolism to redirect the metabolic flow towards glycerol synthesis.

## 3.1.1. Preliminary studies for glycerol overproduction

Preliminaries studies have been carried out to enhance glycerol production by the yeast *S. cerevisiae*. Several strains were tested in order to choose the best one considering growth rate, biomass production, glycerol synthesis and ability to undergo genetic modifications. Moreover, the strain must have sufficient selection markers that allow the execution of the genetic engineered strategy described in the following chapters. The strains analysed comprised the wine strain V5, expected to produce higher amounts of glycerol, the strain IMD1, obtained from crossing industrial derived strains, the CEN.PK2 strain, a laboratory strain selected within an European Cell Factory program project (van Dijken *et al.,* 2000), and CEN.PK2, with *GPD1* gene overexpressed, called HC13. In order to evaluate whether the presence or absence of oxygen would influence glycerol production and yeasts performance, anaerobic and aerobic conditions were tested.

### 3.1.1.1. Anaerobic experiments

The four strains V5, IMD1, CEN.PK2 and HC13 were cultivated in batch cultures without pH regulation, under anaerobic conditions. SD medium was used, supplemented with ergosterol and tween 80, needed for yeast membrane stability under oxygen absence conditions. Cysteine was used to ensure a reduced environment and resazurin was added to the medium as an anaerobic indicator. The major fermentation end-products from all strains were glycerol, acetate and ethanol (Table 3.1). None of the strains IMD1, V5 or CEN.PK2 showed a particularly interesting glycerol yield. Strain HC13 showed the best glycerol

production, 4 to 15 times higher than the values obtained for the other strains. The glycerol yield obtained with this mutant was 0.42 mol.mol$^{-1}$ of glucose. Residual glucose was not detected in all cultures.

Table 3.1. Anaerobic batch cultivations of *S. cerevisiae* strains (20 g.l$^{-1}$ glucose, 25ºC, initial pH 4.7).

| Strain | Initial glucose (g.l$^{-1}$) | Residual glucose (g.l$^{-1}$) | Glycerol (g.l$^{-1}$) | Acetate (g.l$^{-1}$) | Ethanol (g.l$^{-1}$) | $Y_{glycerol}$ (mol.mol$^{-1}$) |
|---|---|---|---|---|---|---|
| IMD1 | 16.08 | bd | 1.09 | 0.085 | 9.29 | 0.13 |
| V5 | 18.09 | bd | 0.84 | 0.089 | 10.0 | 0.09 |
| CEN.PK2 | 18.47 | bd | 0.26 | 0.073 | 9.88 | 0.03 |
| HC13 | 18.43 | bd | 3.94 | 0.068 | 7.34 | 0.42 |

bd- below detection

### 3.1.1.2. Aerobic experiments

Strains IMD1, V5, CEN.PK2 and HC13 were also cultivated under aerobic conditions, in batch cultures without pH regulation. Results obtained showed that glycerol, acetate and ethanol were also the major cultivation end-products, as they were in anaerobic conditions (Table 3.2). However, some differences were observed. Strain HC13 was again the highest glycerol producer, reaching almost the same yield as under anaerobic conditions. Glycerol yield from stain CEN.PK2 was the same in both conditions, 0.3 mol.mol$^{-1}$ of glucose. For the other two strains, the presence of oxygen reduced the yield of glycerol by a factor of 2. Another difference found between the two conditions was related to acetate

production. In aerobic conditions the production of acetate increased for all strains. Again, HC13 produced the highest amount, 0.84 $g.l^{-1}$, corresponding to a 12-fold increase of the value obtained in anaerobic conditions.

Table 3.2. Aerobic batch fermentations of *S. cerevisiae* strains (20 $g.l^{-1}$ glucose, 25ºC, initial pH 4.7).

| Strain | Initial glucose ($g.l^{-1}$) | Residual glucose ($g.l^{-1}$) | Glycerol ($g.l^{-1}$) | Acetate ($g.l^{-1}$) | Ethanol ($g.l^{-1}$) | $Y_{glycerol}$ ($mol.mol^{-1}$) |
|---|---|---|---|---|---|---|
| IMD1 | 18.67 | bd | 0.59 | 0.47 | 8.68 | 0.06 |
| V5 | 18.65 | bd | 0.34 | 0.14 | 8.84 | 0.04 |
| CEN.PK2 | 18.54 | bd | 0.31 | 0.28 | 8.22 | 0.03 |
| HC13 | 18.01 | bd | 3.54 | 0.84 | 6.03 | 0.39 |

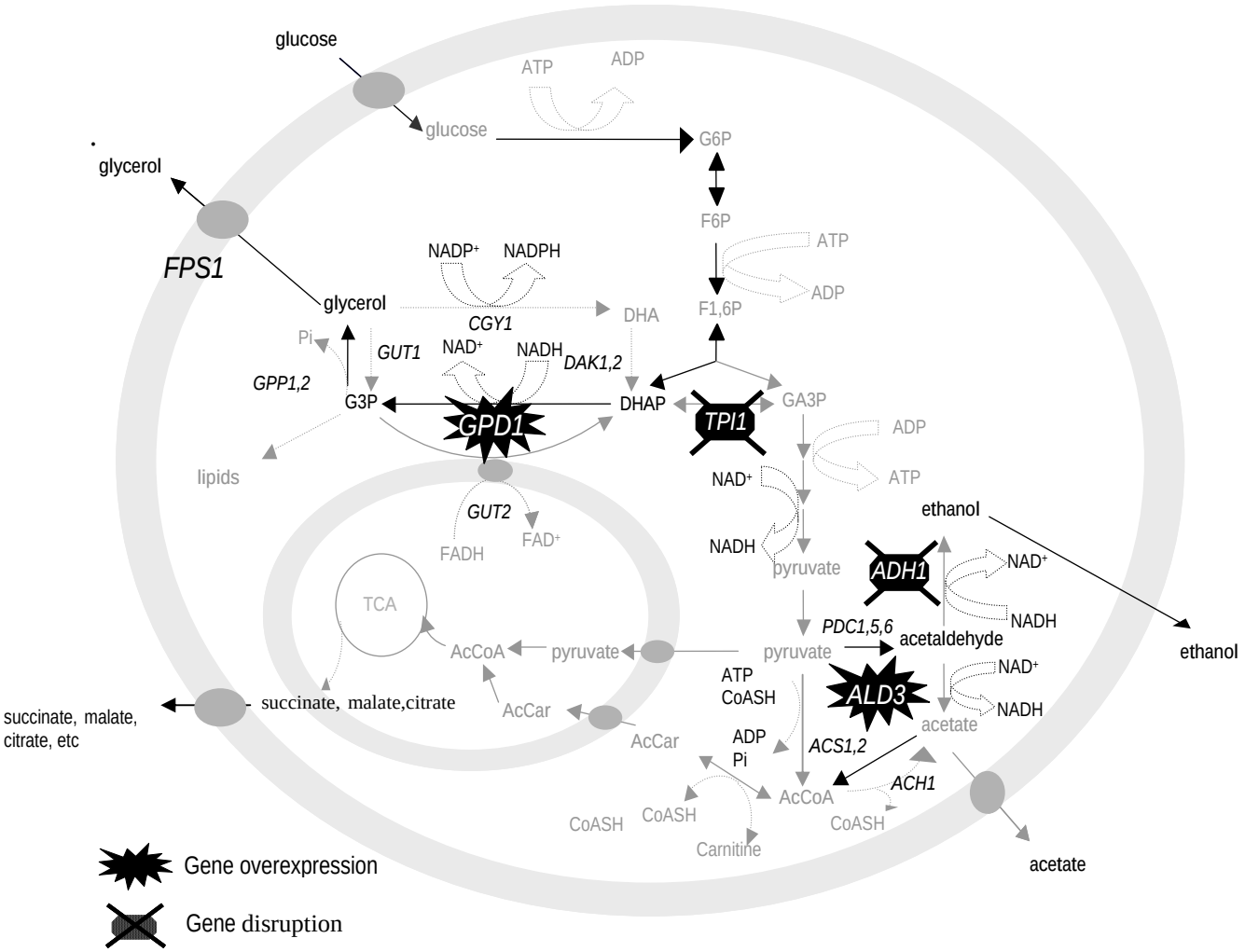
bd- below detection

As no significant differences were observed for glycerol production among the industrial strains IMD1, V5 and CEN.PK2, and the fact that the overexpression of *GPD1* in this latter strain was possible, originating the mutant strain HC13, the best glycerol producer, the strain CEN.PK2 was chosen as the wild type for further experiences.

## 3.1.2. Mutant strains description

*S. cerevisiae* mutants evaluated during this work were the result of genetic modifications carried out in order to increase glycerol production (Figure 3.2).

The metabolic engineered strategy comprised the overexpression of *GPD1* encoding glycerol phosphate dehydrogenase (the rate limiting step for glycerol production), followed by the disruption of *TPI1,* encoding triose phosphate isomerase, and of *ADH1*, encoding the major enzyme of alcohol formation from acetaldehyde. To prevent acetaldehyde accumulation, due to the disruption of *ADH1*, and to favour NADH production, *ALD3* encoding a $NAD^+$-dependent acetaldehyde dehydrogenase was overexpressed. The combination of these genetic modifications resulted in various engineered strains: HC13 with *GPD1* overexpressed; HC16 with *GPD1* overexpressed and *tpi1* disrupted; HC17 with *adh1* disrupted; HC23 with *ALD3* overexpressed and *GPD1* overexpressed; HC30 with *ALD3* overexpressed, *GPD1* overexpressed and *tpi1* disrupted; HC32 with *GPD1* overexpressed, *tpi1* disrupted and *adh1* disrupted and HC42 with *ALD3* overexpressed, *GPD1* overexpressed, *tpi1* disrupted and *adh1* disrupted. In the following points a careful biochemical analysis of the genetically modified strains is described in order to evaluate the metabolic consequences of these genetic interventions, and to identify cellular targets that can be responsible for restraining yeast cells for higher glycerol production performance.




| | | | |
|---|---|---|---|
| *GPD1* | glycerol 3 phosphate dehydrogenase | *DAK1, 2* | dihydroxyacetone kinase |
| *GPP1, 2* | glycerol 3 phosphatase | *TPI1* | triose phosphate isomerase |
| *FPS1* | glycerol channel | *ALD3* | acetaldehyde dehydrogenase |
| *GUT1* | glycerol kinase | *ADH1* | alcohol dehydrogenase |
| *GUT2* | glycerol 3 phosphate dehydrogenase (FAD) | *PDC1, 5, 6* | pyruvate decarboxylase |
| *CGY1* | glycerol dehydrogenase | *ACH1* | acetyl-CoA hydrolase |
| | | ACS1,2 | acetyl-CoA synthetase |

Figure 3.2. Genetic engineering strategy of *S. cerevisiae* for glycerol overproduction (Cordier *et a*l., 2007).

### 3.1.3. Mutant strains evaluation and selection for glycerol overproduction in shake flasks cultures

In order to evaluate engineered strains for growth efficiency and glycerol production, wild type and mutant strains were cultivated in Erlenmeyer flasks with SD medium. As showed in Table 3.3, all mutant strains presented a glycerol yield higher than the value obtained from the wild type strain CEN.PK2. Best results where achieved with HC32 and HC42 mutant strains, reaching a glycerol yield of 0.46 $g.g^{-1}$ glucose, while CEN.PK2 reached a maximum of 0.02 $g.g^{-1}$ glucose consumed. The deletion of *TPI1* should redirect half of the glucose carbon towards glycerol production. In fact, all the *TPI1Δ* mutants were those with the highest glycerol yields, 0.36 to 0.46 $g.g^{-1}$ glucose. However, as only half of the glucose can be used for cells growth, specific growth rates of the *TPI1* disrupted mutants were approximately 4-fold lower than in the wild type strain. In *GPD1* overexpressed strain HC13, glycerol yield (0.19 $g.g^{-1}$ glucose) was 10-fold higher than in the wild type strain. This result was quite similar to the one reached with the disruption of *ADH1* in the strain HC17; however the deletion of *ADH1* resulted in a 2-fold reduction of the maximal growth rate. Overexpression of *ADL3* only slightly enhanced glycerol yield to 0.42 $g.g^{-1}$ glucose in the mutant HC30, when compared to the other *TPI1* mutant HC16. Comparing glycerol yield of HC32 and of the quadruple mutant HC42 no differences were observed.

Table 3.3. Growth rate, biomass and main end-products yield obtained from shake flasks cultures of wild type and engineered strains (SD medium with 20 $g.l^{-1}$ of glucose at pH 5.0 and 30ºC)

| Strains | Genetic Modification | Growth rate ($h^{-1}$) | Biomass yield | Ethanol | Glycerol | Acetate |
|---|---|---|---|---|---|---|
| CEN.PK2 | Wild type | 0.44 ± 0.04 | 12 ± 0.5 | 41 ± 3.0 | 2.0 ± 0.4 | 4.0 ± 0.5 |
| HC13 | *GPD1* overexpression | 0.44 ± 0.04 | 10 ± 0.3 | 32 ± 3.0 | 19 ± 1.4 | 5.0 ± 0.5 |
| HC16 | *GPD1* overexpression and *TPI1* deletion | 0.09 ± 0.01 | 9.0 ± 0.4 | 19 ± 2.0 | 36 ± 3.6 | 9.0 ± 0.4 |
| HC17 | *ADH1* deletion | 0.26 ± 0.03 | 11 ± 0.5 | 29 ± 2.0 | 19 ± 2.5 | 13 ± 0.8 |
| HC23 | *GPD1* and *ALD3* overexpression | 0.39 ± 0.03 | 10 ± 1.3 | 33 ± 1.0 | 19 ± 2.0 | 5.0 ± 0.6 |
| HC30 | *GPD1* and *ALD3* overexpression, and *TPI1* deletion | 0.13 ± 0.03 | 9.0 ± 1.4 | 19 ± 2.0 | 42 ± 2.5 | 8.0 ± 0.4 |
| HC32 | *GPD1* overexpression, and *TPI1* + *ADH1* deletion | 0.10 ± 0.01 | 9.1 ± 0.5 | 8.0 ± 0.4 | 46 ± 2.5 | bd |
| HC42 | *GPD1* and *ALD3* overexpression, and *ADH1* + *TPI1* deletion | 0.13 ± 0.01 | 8.1 ± 1.3 | 8.0 ± 1.4 | 46 ± 2.7 | 6.0 ± 0.3 |

Y – yield, expressed as g product per 100 g glucose consumed. Results are the mean value ± standard deviation of four independent experiments. bd – below detection.

### 3.1.4. Enzymatic activities of engineered strains

The activities of the key enzymes encoded by the genes whose expression was changed by genetic modifications and of others enzymes were evaluated. Results are shown in Table 3.4. As expected, in all mutants strains with *GPD1* overexpressed, glycerol-3-phosphate dehydrogenase activity was higher than in the wild type strain and in mutant strain HC17. In these transformed strains the activity of GPD was up to 10-fold higher than the activity in CEN.PK2. The activity of the other enzyme involved in glycerol production, glycerol-3-phosphate phosphatase, although it was not a direct target of the genetic strategy applied, seemed to be directly affected by the down-regulation of triose phosphate isomerase, since a slightly increase of this activity in the strains tpi1*Δ*, HC16, HC30, HC32 and HC42 was observed.

The activity of the enzyme responsible for ethanol production was drastically decreased in the strains HC17, HC32 and HC42 caring *ADH1* disruption. In the strain HC30, the activity of ADH was not detected. The overexpression of *ALD3* did not result in an increase of acetaldehyde dehydrogenase activity. The activity of glycerol dehydrogenase (GDH), the enzyme that catalyses the conversion of glycerol into dihydroxyacetone, was also measured in the mutant strains HC32 and HC42. Comparing the results obtained with those from the wild type strain, there was a 2-fold increase of the activity of the $NAD^+$-dependent glycerol dehydrogenase (pH 9.1) in the two strains. The activities of glucose-6-phosphate dehydrogenase (G6PD) and glucose-6-phosphate isomerase (PGI) were measured as a control.

Table 3.4. Enzymes activities in engineered strains for glycerol overproduction

| Strains | **Enzyme activity (nmol.min$^{-1}$. mg protein$^{-1}$)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | G6PD | PGI | GPD | GPP | GDH | | | | ADH | "ALD" | |
| | | | | | pH 7.1 | | pH 9.1 | | | | |
| | | | | | $NAD^+$ | $NADP^+$ | $NAD^+$ | $NADP^+$ | | $NAD^+$ | $NADP^+$ |
| CEN.PK2 | 312 ± 3.1 | 1580 ± 214 | 23.0 ± 4.0 | 450 ± 30 | 13.6 ± 0.7 | 13.0 ± 1.7 | 21.2 | 19 | 4610 ± 10 | 57 ± 6.4 | 149 ± 6.0 |
| HC13 | 64.0 ± 0.5 | 1318 ± 69.0 | 360 ± 24 | 440 ± 30 | 2.60 ± 0.3 | bd | nd | nd | 3762 ± 50 | nd | 115 ± 3.6 |
| HC16 | 214 ± 5.4 | 1831± 109 | 387 ± 12 | 660 ± 34 | 9.0 | bd | nd | nd | 4449 ± 43 | 15 ± 3.0 | 46.0 ± 3.1 |
| HC17 | 245 ± 2.5 | 1635± 124 | 31.8 ± 2.0 | 420 ± 20 | 14.2 | bd | nd | bd | 108 ± 7.8 | 11 ± 0.5 | 157 ± 3.5 |
| HC23 | 225 ± 4.0 | 1780 ± 58.0 | 344 ± 44 | 520 ± 45 | bd | bd | nd | nd | 3690 ± 30 | 20 ± 2 | 180 ± 6.7 |
| HC30 | 302 ± 3.0 | 2620 ± 94.0 | 318 ± 23 | 840 ± 30 | 10.9 ± 0.5 | 8.50 | nd | nd | nd | 18 ± 0.4 | 103 ± 3.5 |
| HC32 | 238 ± 8.0 | 1681 ± 24.0 | 259 ± 11 | 530 ± 40 | 8.10 ± 0.4 | 12.8 | 53.6 ± 1.0 | 31 ± 2.3 | 274 ± 23 | 12 ± 2.0 | 177 ± 3.0 |
| HC42 | 267 ± 14 | 1821 ± 62.0 | 357 ± 78 | 680 ± 40 | 10.8 ± 4.1 | bd | 31.8 ± 10 | 38 ± 2. 4 | 358 ± 34 | 15 ± 3.0 | 166 ± 16.4 |

Results presented are the mean values ± standard deviation of three independent experiments. G6PD - glucose-6-P dehydrogenase; PGI -glucose-6-phosphate isomerase; GPD - glycerol-3-P dehydrogenase; GPP - glycerol-3-P phosphatase; GDH - NAD or NADP-dependent glycerol dehydrogenase; ADH - alcohol dehydrogenase; ALD - acetaldehyde dehydrogenase. nd – not determined. bd –below detection.

## 3.2. *Saccharomyces cerevisiae* HC42 mutant strain cultivation in batch bioreactor

The strain HC42 was chosen to evaluate the consequences of the genetic strategy adopted and for further experiences to enhance glycerol production, as it exhibited a weak, but reproducible, 5-10% increase in glycerol production, when compared to the strain HC32.

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### 3.2.1. Macrokinetics analysis of engineered strains for glycerol overproduction

*S. cerevisiae* HC42 was cultivated on SD medium in pH-controlled batch bioreactors. For comparison, wild type strain CEN.PK2 and mutant strain HC16 were also cultivated under the same conditions. As shown in Figure 3.3 the time necessary to consume the same amount of glucose was longer in yeast mutants HC16 and HC42 than in wild type strain. However, mutant strain HC42 was faster than HC16, result that is in accordance with a better performance showed by HC42 for glucose consumption (Table 3.5). Growth rate ($\mu_{max}$) was also higher for HC42 (0.17 $h^{-1}$) than for HC16 (0.13 $h^{-1}$), although still lower than for the wild type strain, with a $\mu_{max}$ of 0.44 $h^{-1}$. Glycerol production rate was higher in yeast mutants then in the parent strain and in the multi-engineered strain HC42 was 3.1 $mmol.h^{-1}.g^{-1}$dry mass.

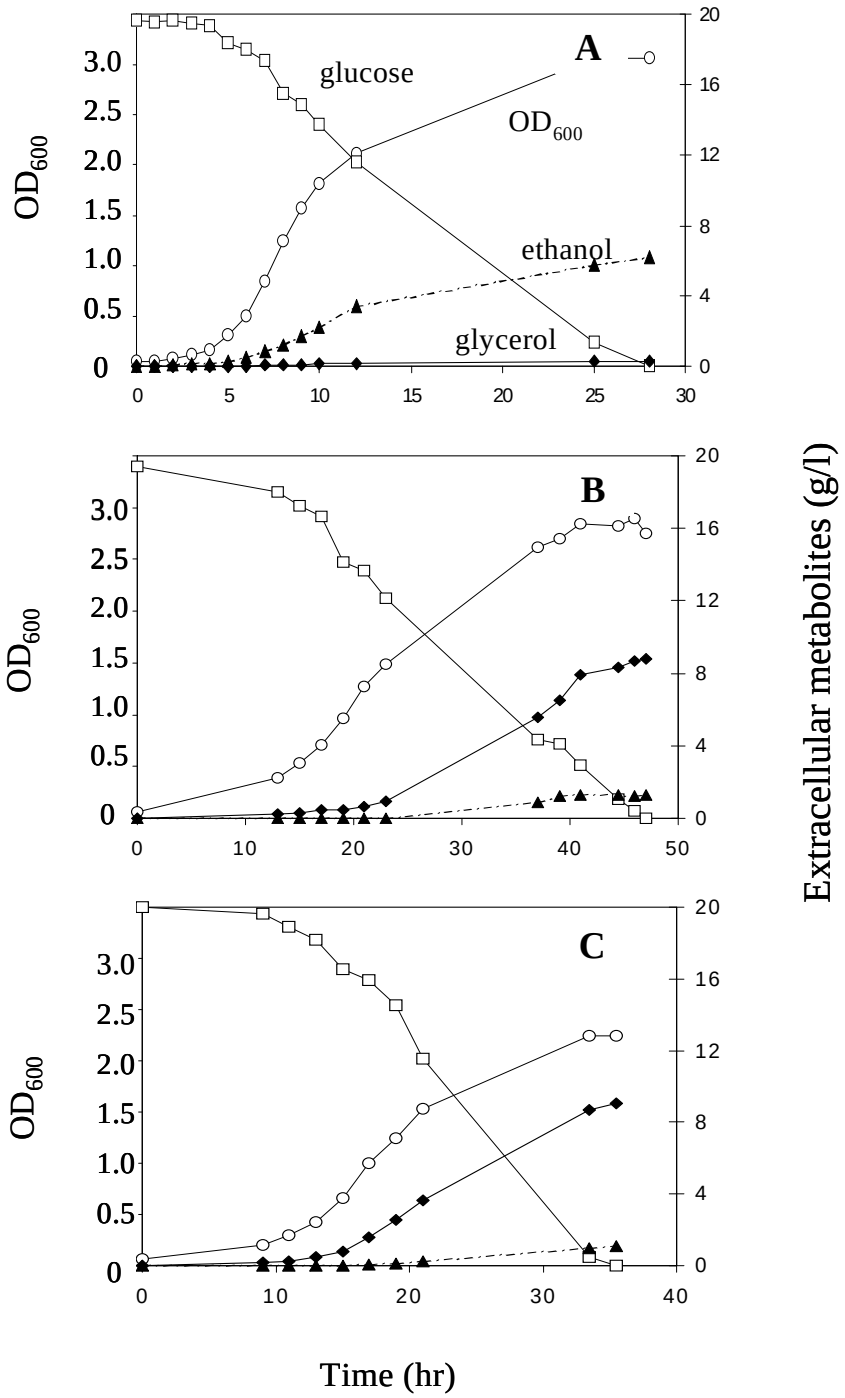



Figure 3.3. Macrokinetics behaviour of wild type and engineered strains for glycerol production during aerobic batch cultures on glucose. Growth was carried

out at 30°C in SD medium containing 20 $g.l^{-1}$ glucose and supplemented with autotrophy requirement. A – CEN.PK2; B – HC16; C – HC42.

Table 3.5. Specific rates of growth, glycerol and ethanol production and glucose consumption of wild type and engineered strains cultivated in aerobic batch cultures on 20 $g.l^{-1}$ glucose.

| **Kinetic parameters** | **Strains** | | |
|---|---|---|---|
| | Wild type | HC16 | HC42 |
| $\mu_{max}$ ($h^{-1}$) | 0.44 ± 0.15 | 0.13 ± 0.05 | 0.17 ± 0.05 |
| Glucose ($mmol.h^{-1}.g$ dry $mass^{-1}$) | 12.1 ± 0.23 | 2.9 ± 0.2 | 3.6 ± 0.24 |
| Ethanol ($mmol.h^{-1}.g$ dry $mass^{-1}$) | 17.9 ± 1.4 | 2.1 ± 0.4 | 1.5 ± 0.2 |
| Glycerol ($mmol.h^{-1}.g$ dry $mass^{-1}$) | 0.34 ± 0.06 | 2.0 ± 0.14 | 3.1 ± 0.35 |

The data are the mean values of two independent cultures ± standard deviation.

As expected, glycerol became the major cultivation product in the engineered strains (Table 3.6). As in shake flasks cultivations, HC42 mutant strain exhibited a glycerol yield of 0.46 g $g^{-1}$glucose, which was 10% higher than in HC16. In addition, it was not surprising that an enhanced production of glycerol was accompanied by a reduction of biomass yield, since glycerol synthesis is an ATP consuming process. The production of the other major end-products, ethanol and acetate, showed some differences between the engineered strains and the wild type. Ethanol production was drastically decreased in HC42 strain with a yield of 0.12 g of ethanol per g of glucose. In contrast, the acetate level in HC42 was not significantly different from HC16, and was lower than in the wild type strain,

although *ALD3* encoding a $NAD^+$-aldehyde dehydrogenase was overexpressed in HC42. Table 3.6 also showed that acetaldehyde and acetoin were significantly higher in HC42 than in HC16 and wild type strain, which was expected due to the lack of *ADH1*.

Carbon balance was estimated taking into account the cultivation products reported in Table 3.6. This balance was close to 100 % for the wild type, while it reached around 90-92% for the two engineered strains studied, HC16 and HC42. A possible explanation for this 10% carbon balance missing in the engineered strain could be an underestimation of acetaldehyde and $CO_2$ production from the respiratory activity, which was apparently more effective than in the wild type strain. 2.3-butanodiol production, resulting from the conversion of acetoin was also not determined.

## 3.2.2. Yeast mutant constraints to enhance glycerol production

The construction of engineered strains for glycerol overproduction caused several transformations on yeast's metabolism. Some of the impacts provoked did not positively contribute to glycerol overproduction. Evidence that the glycerol export could be rate limiting in glycerol production was given by the finding that HC16 and HC42 accumulated 7 to 10 times more intracellular glycerol than the wild type (Table 3.7). A second metabolic question was to evaluate effects of molecular manipulation on changes in levels of $NAD^+$ and NADH. As indicated in Table 3.7, the genetic transformations introduced in the yeast mutant HC42 led to a significant decrease in NADH/ $NAD^+$ ratio, which was essentially due to a 4-

to 5- fold reduction in NADH pools. This reduction in NADH, together with an increase of $NAD^+$, does not favour the Gpd1p activity, since $NAD^+$ is a competitive inhibitor of NADH on Gpd1p activity (Albertyn *et al.,* 1992).

Table 3.6. Cultivation products measured at the end of batch fermentations of the wild type and two engineered strains. The fermentation was carried out in controlled batch reactor at pH 5.0 in SD medium containing 20 $g.l^{-1}$ glucose.

| **Cultivation products** | **Strains** | | |
|---|---|---|---|
| | Wild type | HC16 | HC42 |
| Biomass | 12.5 ± 1.3 | 9.98 ± 1.5 | 8.70 ± 1.1 |
| Ethanol | 40.4 ± 2.7 | 17.4 ± 0.7 | 11.6 ± 0.7 |
| Glycerol | 1.50 ± 0.2 | 41.8 ± 3.3 | 45.8 ± 2.5 |
| Acetate | 4.90 ± 0.34 | 1.98 ± 0.70 | 2.80 ± 0.70 |
| Pyruvate | 0.45 ± 0.10 | 0.35 ± 0.05 | 0.66 ± 0.10 |
| Acetaldehyde | 0.40 ± 0.10 | 0.21 ± 0.05 | 2.90 ± 0.07 |
| Acetoine | 0.65 ± 0.07 | 0.27 ± 0.04 | 2.10 ± 0.20 |
| Succinate | bd | 0.25 ± 0.04 | 0.55± 0.14 |
| Citrate | bd | 0.14 | 0.68 ± 0.10 |
| Carbon balance (%) | 99.9 | 92.2 | 90.0 |

Results are expressed in g per 100 g glucose consumed and are the mean values of two independent experiments ± standard deviation. $CO_2$ was calculated from ethanol production. bd – below detection.

Table 3.7. Metabolites levels in engineered strains for glycerol production. Yeast strains were cultivated in SD medium.

| | Intracellular levels (µmol.g dry mass$^{-1}$) | | |
|---|---|---|---|
| Metabolites | Wild type | HC16 | HC42 |
| $Glycerol_{in}$ | 26.0 ± 3.8 | 115 ± 29 | 197 ± 16 |
| $NAD^+$ | 2.75 ± 0.79 | 3.52 ± 0.64 | 2.95 ± 0.59 |
| NADH | 0.167 ± 0.08 | 0.039 ± 0.01 | 0.038 ± 0.012 |
| $NAD^+$/NADH | 16.7 | 79.0 | 77.0 |

Metabolites were measured in samples taken at the exponential growth phase. Results reported are the mean value ± standard deviation of four independent experiments.

## 3.3. Discussion and conclusions

Among the different tested strains, the CEN.PK2 turned out to be the best choice to carry out the genetic engineering strategy proposed for this work. When the wild type yeast strain of the CEN.PK2 family was cultivated under well-aerated conditions in a glucose synthetic medium (20 g.l$^{-1}$ glucose), the production of glycerol reached a maximum of 0.02 g.g$^{-1}$ glucose consumed. This level of glycerol, which is around 5-fold lower than under anaerobic or oenological growth conditions (van Dijken and Scheffers, 1986; Michnick *et al.*, 1997) was not unexpected since yeast cells are accomplished with several redox readjustment mechanisms for maintenance of the intracellular $NAD^+$/NADH

balance (Bakker *et al*., 2001; Heux *et al*., 2006), and also because the $NAD^+$-dependent glycerol-3-phosphate dehydrogenase that catalyses the reduction of DHAP into glycerol-3-phosphate is rate-limiting in glycerol production (Cronwrigh *et al*., 2002; Larsson *et al*., 1993; Nevoigt and Stahl, 1996; Remize *et al*., 2003). Therefore, as a first step to boost up glycerol synthesis, the activity of GPD was increased several fold by overexpressing *GPD1* gene encoding the enzyme, in CEN.PK2 genome. Glycerol production and GPD activity were measured in the constructed mutant strain HC13. In confirmation that GPD was rate limiting in glycerol synthesis, the HC13 strain produced 10-times more glycerol than the wild type strain, and this increase in glycerol production was correlated with a 10-fold rise in the GPD activity, which showed a hierarchical flux control in the metabolic glycerol pathway (ter Kuile and Westerhoff, 2001). Results from different works suggested that the final yield of glycerol is largely determined by the amount of available NADH that could be regenerated through its pathway (Cambon *et al*, 2006; Geertman *et al*., 2006; Overkamp *et al*., 2002). To increase NADH availability, an obvious genetic intervention was to delete *TPI1* encoding triose phosphate isomerase into the *GPD1* overexpressing strain. In theory, this genetic intervention should redirect half of the glucose molecule into the glycerol pathway. However, the consequence of this intervention is the inability of *tpi1Δ* mutant to grow on glucose as the sole carbon source (Compagno *et al*., 1996; Overkamp *et al*., 2002), because of its inositol defective phenotype. This latter phenotype is a consequence of the strong inhibition of the enzyme *myo*-inositol synthase by the accumulation of DHAP in a *tpi1Δ* mutant (Shi *et al*.,

2005). Overexpression of *GPD1* restored the growth of *tpi1Δ* mutant, however the maximal growth rate of the *tpi1ΔGPD1*-overexpressing strain was still 4-fold lower than that of the wild type strain. The mutant strain resulting from this strategy, HC16 exhibited a glycerol production yield of 0.36 $g.g^{-1}$ glucose, similar to that of the quadruple *tpi1Δnde1Δnde2Δgut2Δ* mutant reported by Overkamp *et al*. (2002). Nevertheless, during cultivation in aerobic shake flasks, HC16 was growing about 2 times faster than the compared engineered strain (0.045 $h^{-1}$ versus 0.09 $h^{-1}$).

An alternative option to increase glycerol production was through removing the major alcohol dehydrogenase encoded by *ADH1*. The aim of this modification was to reduce the competition and leave more NADH available for glycerol production. A crude extract of the mutant defective in *ADH1*, HC17, still exhibited 5% of total alcohol dehydrogenase activity, likely because of the expression of other minor *ADH* genes (Drewke *et al*., 1990). Nevertheless, the deletion of *ADH1* in CEN.PK2 resulted in a mutant strain that exhibited a glycerol performance almost comparable to that of the *GPD1*-overexpressing strain HC13. However, this genetic intervention resulted in a maximal growth rate that was 2-fold lower than that of the wild type and of the *GPD1*-overexpressing strain. In addition, the *adh1Δ* mutant accumulated 3 times more acetate than the wild type. The reduction of growth rate could be due to a lower rate of reoxidation of NADH by the mutant *GPD1* and/or to an accumulation of acetaldehyde, a potential toxic molecule for cell growth, caused by the shift towards oxidized metabolites (Michnick *et al*., 1997). An additional modification that may allow increasing the

availability of NADH was to favour the conversion of pyruvate to acetate via pyruvate decarboxylase and a $NAD^+$-dependent acetaldehyde dehydrogenase. Overexpression of gene encoding ALD was expected to reduce acetaldehyde accumulation entailed by *ADH1* disruption, and increase the excess of NADH formed that could later on be used for glycerol production. In the mutants analysed in this study, *ALD3* was the gene overexpressed. This genetic modification increased by about 15% the final glycerol production when introduced in the *tpi1ΔGPD1*, HC16 mutant to obtained HC30 strain, but this overexpression had no effect in the *GPD1*-overexpressing strain HC13. As the amount of acetate was not increased the results are in accordance with those reported by Saint-Prix *et al*. (2004) and reinforced the suggestion that *ALD3* has other rules, namely being implicated in alanine biosynthesis (White *et al.*, 2003). Taken into account the results given above, the effect of *GPD1* overexpression together with the deletion of *TPI1* and *ADH1* on glycerol production was evaluated. The resulting engineered HC32 strain exhibited a glycerol yield of 0.46 $g.g^{-1}$ glucose, which was the same as the one described by Geertman *et al*. (2006) in a mutant *pdc1Δpdc2Δpdc5Δnde1Δnde2Δgut2Δ*. However, there still was a significant production of ethanol of about 0.1 $g.g^{-1}$ glucose that could result, as referred to before, from activity of alternative minor alcohol dehydrogenase. The overexpression of *ALD3* in HC32 originated HC42. In this latter mutant no metabolic consequences on either acetate or ethanol production were observed, which may confirm that this enzyme does not act on acetaldehyde *in vivo*. The activity of glycerol dehydrogenase, which converts glycerol into

dihydroxyacetone, increased by about 2-fold in the strains HC32 and HC42 which can negatively affect glycerol production. Other constraints found in the strain HC42 were the accumulation of intracellular glycerol, the reduction of NADH pools and the increase of the production of toxic compounds such as acetaldehyde. To eliminate acetaldehyde, yeast mutant used the alternative pathways acetoin/2,3-butanediol production. Higher levels of acetoin were found in engineered mutant strain HC42. Acetoin is less toxic to the cell and this pathway can contribute to alleviate acetaldehyde toxic effect (Heux *et al*., 2006; Remize *et al*., 1999). Nevertheless, the latter multi-engineered HC42 strain was used for further physiological investigation in order to increase glycerol production.

*Chapter 4*

# Genomic analysis of *Saccharomyces cerevisiae* HC42 mutant strain

## 4.1. Transcriptomic analysis of glycerol hyper-producer strain

*S. cerevisiae* HC42 was the result of genetic engineering strategies, focused on the redirection of metabolic fluxes and overexpression of the key enzymes of the glycerol formation pathway. Experiments described in Chapter 3 demonstrated the effect of genetic transformations on HC42 cultivation. The engineered strain exhibited a glycerol yield of 0.46 $g.g^{-1}$ glucose. To better understand the performance of this strain in response to the several genetic transformations, genome-wide analyses were carried out. It was possible to evaluate the effects of those modifications at the genomic scale, via transcriptomic analysis, comparing genes expression from wild type CEN.PK2 and multi-engineered HC42 strains.

The two most commonly used supports for global gene array analysis are nylon filters (known as macroarrays) and glass slides (known as microarrays). In each case the array is a reproducible pattern of probes in the form of oligonucleotides or PCR products, each probe representing a different target gene sequence, and these are spotted onto the support. In this work only microarrays technology was used. During experiment, cDNA prepared from HC42 and CEN.PK2 mRNA was labelled with fluorescent dyes (Cy5 and Cy3) for glass slides. It was then hybridized to the immobilised probes on the solid support, the glass slides. The array was after washed to remove non-specifically bound cDNA and read in an appropriated detector to determine which probes have labelled signal bound to them. Strains were cultivated on SD medium with 20 $g.l^{-1}$ glucose

in well-aerated batch cultures. Samples were taken early in log phase, at the maximal growth rate. Cultures of each strain were repeated twice to obtain 4 independent data sets that could allow statistical analysis.

## 4.1.1. Analysis of genes differentially expressed

Using two filtering criteria, the ratio > 1.5 or < -1.5, and *p* value < 0.05, the genome-wide expression analyses revealed a total of 384 differentially expressed genes between HC42 and CEN.PK2, from which 184 were down-regulated and 200 were up-regulated (Appendix).

### 4.1.1.1. Analysis by functional categories

Genes differentially expressed were first grouped according to the correspondent functional category (Figure 4.1). They were distributed into 13 functional categories according to the MIPS nomenclature (Mewes *et al*., 1997; Ruepp *et al*., 2004). In this analysis the number of up- and down-regulated transcripts was expressed in each of the categories as a percentage of the list of total differentially expressed genes and compared this functional classification with the functional catalogues of genes described in MIPS. This procedure has the advantage to easily identify the major gene expression remodelling on a genomic scale. The result of the several genetic modifications introduced in CENPK2 to reorient the carbon flux towards glycerol production was the significant enrichment in transcripts of genes that belong to carbon and energy metabolism

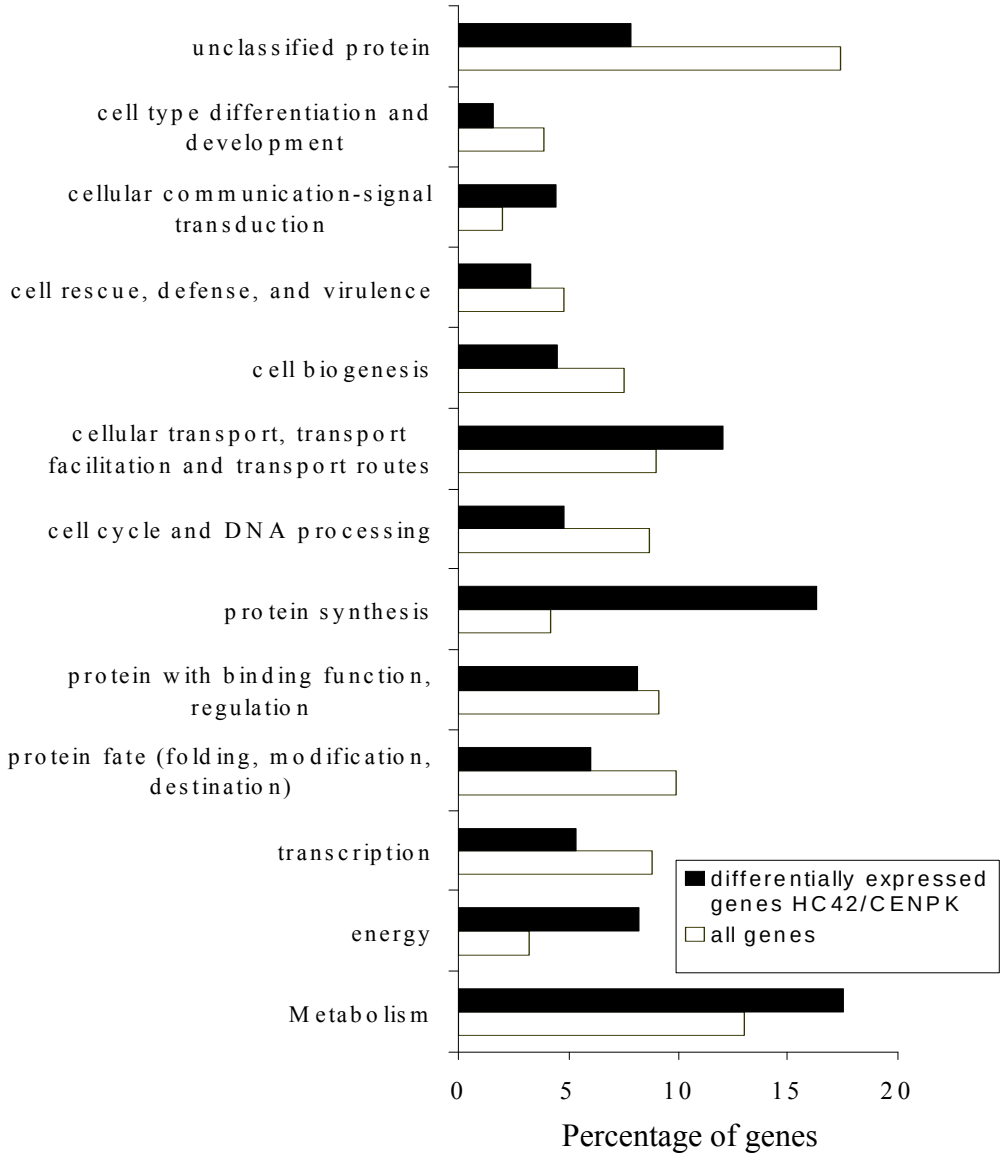
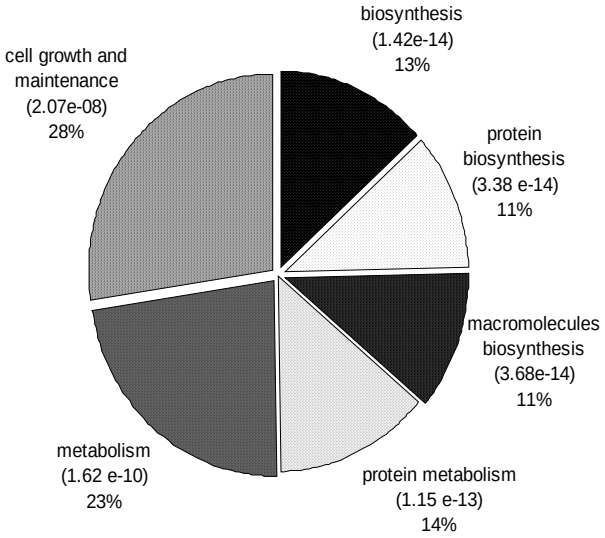



Figure 4.1. Distribution of the differentially expressed genes in the engineered strain HC42 according to the functional categories. Open histograms represent the functional catalogue of 6200 genes according to the MIPS classification. Solid histograms represent the distribution of the 384 differentially expressed genes from engineered HC42 strain versus CEN.PK2 in the functional categories of MIPS. The percentage of each category was calculated as the number of up- and down-regulated genes from each of the 13 categories divided by the total number of differentially expressed genes in the engineered strain.

and to protein synthesis, and to a less extent, to ionic homeostasis and cellular communication. In contrast, genes encoding products involved in transcription, cell biogenesis, cell cycle and DNA processing were significantly under-represented. This was further consolidated by Gene ontology (GO) analysis of the expression data, which allowed a better identification of the biological processes and cellular components. Enrichment of biological processes and cellular components in engineered strain HC42 were estimated using Term Finder from SGD (http://www.genome.org). The analysis revealed that biological processes and cellular components that were particularly affected by the several genetic interventions in the mutant strain HC42 were those implicated in cell growth, energy metabolism, ribosomal protein synthesis and ribosomal biogenesis (Figure 4.2). Closer inspections of genes belonging to the ribosomal protein synthesis and ribosome biogenesis showed that > 90 % of these genes were down-regulated.

**A**: GO biological processes




**B**: GO cellular components

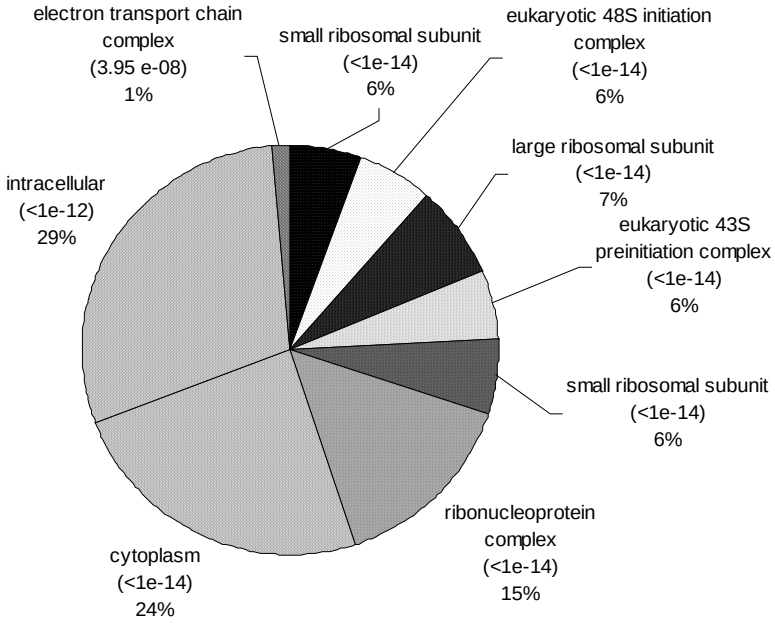



Figure 4.2. Gene ontology classification. To calculate *p* value (given in parenthesis) the number of genes representing a given process in the transcription profile was compared with the number of genes representing this process in the whole genome, without taking into account their ratio of expression.

### 4.1.1.2. Analysis of genes up and down-regulated

Other important information could be obtained analysing the list of genes whose expression was enhanced or reduced. The functional categories of genes most affected by the genetic modifications introduced on CEN.PK2 to reach HC42 were carbon metabolism, energy and cellular transport, transport facilitation and transport routes.

#### 4.1.1.2.1. Effect of genetic modifications for glycerol overproduction on genes responsible for metabolism and energy

Most of the overexpressed genes belong to metabolism and energy categories (Table 4.1). As expected, *ALD3* and *GPD1* were overexpressed (respectively 6.56 and 4.14-fold). Despite not being a direct target of the genetic modifications, *GPD2* (2.1-fold), which encodes the second GPD isoform, and *ALD2* and *ALD4* (1.57 and 1.95-fold respectively), encoding other isoforms of acetaldehyde dehydrogenase, were also found among the genes over-expressed. A 3.27 fold upregulation of *INO1* encoding *myo*-inositol-3-phosphate (MIP) synthase could be noticed. The up-regulation of 1.91-fold from the gene *ARA1*, one of the four genes encoding the $NADP^+$-dependent glycerol dehydrogenase (GDH), was also detected.

Among the genes down-regulated found in these categories are *ADH1* (-2.33-fold) and *TPI1* (-2.56-fold), both targets of the genetic modifications. Curiously, the gene *ADH2*, encoding the alcohol dehydrogenase responsible for the conversion of ethanol into acetaldehyde, was also down-regulated (-5.88-fold).

Other interesting genes found in the list were *PDC1* (-1.92-fold), encoding the major pyruvate decarboxylase, and *PYK1* (-1.82-fold), encoding pyruvate kinase. As the mutant HC42 exhibited a gross defected on redox balance, due to the lower rate of reoxidation of NADH, a particular attention was dedicated to the expression of genes involved in the redox metabolism. In the list of genes responsible for metabolism and energy several genes were found encoding proteins with $NAD^+$/$NADP^+$ binding function like: *NDI1, PDH1, ALD4, ARA1, GDH2, IDH1*, *ALD2, ETR1*, *GPD1, GPD2*, *PDC1* and *ADH1*, that were up and down-regulated.

Also, several genes responsible for energy generation were differentially expressed. Genes *ATP2*, *ATP3*, *ATP5*, *ATP14* and *ATP18* and *AAP1* encoding components of the mitochondrial F0/F1 ATP synthase were increased to the same extent in the engineered strain.

Genes belonging to Citric Acid Cycle *SDH2*, *SDH3*, *SDH4*, *FUM1*, *IDH1*, *GDH2* were over-expressed. *SDH2*, encoding a subunit of the complex succinate dehydrogenase, showed an increase of 3.58 fold.

#### 4.1.1.2.2. Genes involved in cellular transport, transport facilitation and transport routes

The "cellular transport and facilitation" category stood as another important functional category that was significantly perturbed by genetic engineering for glycerol overproduction (Table 4.2).

Table 4.1. List of differentially expressed genes in HC42 versus CEN.PK2 that belong to metabolism and energy categories.

| ORF | Gene name | Fold change | Description |
|---|---|---|---|
| *YMR169C* | *ALD3* | 6.56 | Cytoplasmic aldehyde dehydrogenase uses NAD+ as the preferred coenzyme |
| *YDL022W* | *GPD1* | 4.14 | NAD-dependent glycerol-3-phosphate dehydrogenase, key enzyme of glycerol synthesis |
| *YLL041C* | *SDH2* | 3.58 | Iron-sulfur protein subunit of succinate dehydrogenase |
| *YML120C* | *NDI1* | 3.37 | NADH:ubiquinone oxidoreductase |
| *YJL153C* | *INO1* | 3.27 | Inositol 1-phosphate synthase |
| *YFL014W* | *HSP12* | 3.1 | Plasma membrane localized protein induced by heat shock |
| *YPR002W* | *PDH1* | 2.81 | Mitochondrial protein that participates in respiration |
| *YPL058C* | *PDR12* | 2.39 | Plasma membrane weak-acid-inducible ATP-binding cassette (ABC) transporter |
| *YOL096C* | *COQ3* | 2.29 | O-methyltransferase, component of a mitochondrial ubiquinone-synthesizing complex |
| *YDR216W* | *ADR1* | 2.21 | Carbon source-responsive zinc-finger transcription factor |
| *YBR002C* | *RER2* | 2.13 | Cis-prenyltransferase involved in dolichol synthesis |
| *YBL015W* | *ACH1* | 2.1 | Acetyl-coA hydrolase |
| *YCL004W* | *PGS1* | 2.09 | Phosphatidylglycerolphosphate synthase |
| *YOL059W* | *GPD2* | 2.08 | NAD-dependent glycerol 3-phosphate dehydrogenase, homolog of Gpd1p |
| *Q0110* | *BI2* | 2.07 | mRNA maturase BI2 |
| *YDR242w* | *AMD2* | 2.06 | Amidase |
| *Q0080* | *AAP1* | 1.98 | Subunit 8 of the F0 sector of mitochondrial inner membrane F1-F0 ATP synthase |
| *YOR065W* | *CYT1* | 1.98 | Cytochrome c1, component of the mitochondrial respiratory chain |
| *YOR374W* | *ALD4* | 1.95 | Mitochondrial aldehyde dehydrogenase, required for conversion of acetaldehyde to acetate |
| *YDL215C* | *GDH2* | 1.92 | $NAD^+$-dependent glutamate dehydrogenase, degrades glutamate to ammonia and alpha-ketoglutarate |
| *YBR149W* | *ARA1* | 1.91 | Large subunit of NADP+ dependent arabinose dehydrogenase |
| *YKL093W* | *MBR1* | 1.9 | Protein involved in mitochondrial functions and stress response |

| ORF | Gene name | Fold change | Description |
|---|---|---|---|
| *YDR298C* | *ATP5* | 1.88 | Subunit 5 of the stator stalk of mitochondrial F1F0 ATP synthase, (oligomycin sensitivity-conferring protein |
| *YOR011W* | *AUS1* | 1.84 | Transporter of the ATP-bindig cassette family, involved in uptake of sterols and anaerobic growth |
| *YGL191W* | *COX13* | 1.83 | Subunit VIa of cytochrome c oxidase |
| *YJR121W* | *ATP2* | 1.81 | Beta subunit of the F1 sector of mitochondrial F1F0 ATP synthase |
| *YDR213W* | *UPC2* | 1.81 | Regulatory protein involved in sterol uptake |
| *YIL042C* | *PKP1* | 1.81 | Mitochondrial kinase, phosphorylates pyruvate dehydrogenase alpha subunit Pda1p |
| *YJR034W* | *PET191* | 1.78 | Protein required for assembly of cytochrome c oxidase |
| *YGR040W* | *KSS1* | 1.77 | Ser/thr protein of the MAP kinase family |
| *YHR001W* | *OSH7* | 1.76 | Member of an oxysterol-binding protein family with seven members in S. cerevisiae |
| *YGR161C* | *RTS3* | 1.76 | Putative component of the protein phosphatase type 2A complex |
| *YHR073W* | *OSH3* | 1.74 | Member of an oxysterol-binding protein family with seven members in S. cerevisiae |
| *YNL037C* | *IDH1* | 1.73 | Subunit of mitochondrial $NAD^+$-dependent isocitrate dehydrogenase |
| *YLR295C* | *ATP14* | 1.72 | Subunit h of the F0 sector of mitochondrial F1F0 ATP synthase |
| *YPL053C* | *KTR6* | 1.72 | Mannosylphosphate transferasse |
| *YMR217W* | *GUA1* | 1.71 | GMP synthase, enzyme that catalyzes the second step in the biosynthesis of GMP from inosine 5'-phosphate |
| *YEL024W* | *RIP1* | 1.69 | Ubiquinol-cytochrome-c reductase |
| *YBR039W* | *ATP3* | 1.67 | Gamma subunit of the F1 sector of mitochondrial F1F0 ATP synthase |
| *YGL205W* | *POX1* | 1.67 | Fatty-acyl coenzyme A oxidase, localized to the peroxisomal matrix |
| *YKL198C* | *PTK1* | 1.67 | Polyamine transport enhancing protein |
| *YKL141W* | *SDH3* | 1.67 | Cytochrome b subunit of succinate dehydrogenase |
| *YDR530C* | *APA2* | 1.66 | ATP adenylyltransferase II |
| *YBR092C* | *PHO3* | 1.66 | Constitutive acid phosphatase precursor |
| *YOR377W* | *ATF1* | 1.64 | Alcohol acetyltransferase with potential roles in lipid and sterol metabolism |
| *YPL262W* | *FUM1* | 1.63 | Fumarase, converts fumaric acid to L-malic acid in the TCA cycle |
| *YMR036C* | *MIH1* | 1.63 | M-phase inducing protein tyrosine phosphatase |
| *YDL232W* | *OST4* | 1.62 | Oligosaccharyltransferase subunit |

| ORF | Gene name | Fold change | Description |
|---|---|---|---|
| *YBR093C* | *PHO5* | 1.59 | Repressible acid phosphatase |
| *YDR074W* | *TPS2* | 1.59 | Phosphatase subunit of the trehalose-6-phosphate synthase/phosphatase complex |
| *YMR170C* | *ALD2* | 1.57 | Cytoplasmic aldehyde dehydrogenase |
| *YML081CA* | *ATP18* | 1.56 | Subunit of the mitochondrial F1F0 ATP synthase; termed subunit I or subunit j |
| *YMR256C* | *COX7* | 1.55 | Subunit VII of cytochrome c oxidase |
| *YBR026C* | *ETR1* | 1.54 | 2-enoyl thioester reductase |
| *YBR006W* | *UGA2* | 1.54 | Succinate semialdehyde dehydrogenase involved in the utilization of gamma-aminobutyrate |
| *YBL033C* | *RIB1* | 1.53 | GTP cyclohydrolase II; |
| *YDR178W* | *SDH4* | 1.53 | Membrane anchor subunit of succinate dehydrogenase |
| *YFL017C* | *GNA1* | 1.51 | Glucosamine-6-phosphate acetyltransferase |
| *YGL187C* | *COX4* | 1.5 | Subunit IV of cytochrome c oxidase |
| *YMR303C* | *ADH2* | -5.88 | Alcohol dehydrogenase II |
| YBR208C | *DUR1,2* | -3.23 | Urea amidolyase |
| YGL009C | *LEU1* | -3.03 | 3-isopropylmalate dehydratase |
| YDR050C | *TPI1* | -2.56 | Triose-phosphate isomerase |
| YLR157C | *ASP3-2* | -2.50 | L-asparaginase II |
| YOL086C | *ADH1* | -2.33 | Alcohol dehydrogenase I |
| YMR202W | *ERG2* | -2.22 | C-8 sterol isomerase |
| YPR074C | *TKL1* | -2.22 | Transketolase 1 |
| YMR116C | *ASC1* | -2.17 | 40S subunit ribosomal protein |
| YLR044C | *PDC1* | -1.92 | Pyruvate decarboxylasse, isozyme 1 |
| YBL011W | *SCT1* | -1.89 | Glycerol 3-phosphate/dihydroxyacetone phosphate dual substrate acyltransferase |
| YAL038W | *CDC19/PYK1* | -1.82 | Pyruvate kinase |
| YLR155C | *ASP3-1* | -1.79 | L-asparaginase II |
| YDL103C | *QRI1* | -1.75 | UDP-N-acetylglucosamine pyrophosphorylase |
| *YJR149W* | *YJR149W* | -1.72 | Putative 2-nitropropane dioxygenase |
| YKL055C | *OAR1* | -1.67 | Putative 3-oxoacyl-(acyl carrier protein) reductase |
| YHR128W | *FUR1* | -1.64 | Uracil phosphoribosyltransferase |
| YOR128C | *ADE2* | -1.61 | Phosphoribosylaminoimidazole carboxylase |
| YBR166C | *TYR1* | -1.61 | Prephenate dehydrogenase ($NADP^+$) |
| YAR015W | *ADE1* | -1.59 | Phosphoribosylaminoimidazole-succinocarboxamide synthase |
| YDL229W | *SSB1* | -1.59 | Heat shock protein of HSP70 family |
| YOL061W | *PRS5* | -1.56 | Ribose-phosphate pyrophosphokinases |

| ORF | Gene name | Fold change | Description |
|---|---|---|---|
| *YBR022W* | *POA1* | -1.54 | Phosphatase of ADP-ribose 1-phosphate, role in regulation of tRNA splicing |
| *YLR355C* | *ILV5* | -1.52 | Ketol-acid reducto-isomerase |

Among others, transcript levels of *JEN1*, which encodes a glucose-repressible lactate/pyruvate permease (Bojunga and Entian, 1999; Casal *et al.,* 1999), were about 2 - fold higher in HC42. An activation of *PHO84*, encoding the high affinity phosphate permease (Bun-ya *et al.,* 1991), and a 2-fold up-regulation of *SLT1*, encoding the glycerol /$H^+$ symporter (Ferreira *et al.,* 2005) can also be reported. The latter activation could be a consequence of the increased osmotic potential of the cytoplasm due to intracellular accumulation of glycerol. Several genes *COX4*, *COX7* and *COX13* encoding subunits of cytochrome *c* oxidase were over-expressed.

## 4.2. *INO1* up-regulation in HC42 mutant

During inositol synthesis, glucose 6-phosphate is converted to *myo*-inositol-3-phosphate in a reaction catalysed by the enzyme MIP synthase, followed by the dephosphorylation of MIP into *myo*-inositol by the enzyme inositol monophosphatase.

Table 4.2. List of differentially expressed genes in HC42 versus CEN.PK2 that belong to categories cellular transport, transport facilitation and transport routes category.

| ORF | Gene name | Fold change | Description |
|---|---|---|---|
| *YOR098C* | *NUP1* | 3.93 | Nuclear pore complex (NPC) subunit, involved in protein import/export and in export of RNAs |
| *YLL041C* | *SDH2* | 3.58 | Iron-sulfur protein subunit of succinate dehydrogenase |
| *YPL058C* | *PDR12* | 2.79 | Plasma membrane weak-acid-inducible ATP-binding cassette (ABC) transporter, required for weak organic acid resistance |
| *YML013W* | *SEL1* | 2.6 | UBX (ubiquitin regulatory X) domain-containing protein required for degradation of a ubiquitylated model substrate |
| *YKL217W* | *JEN1* | 2.55 | Lactate transporter, required for uptake of lactate and pyruvate |
| *Q0080* | *AAP1* | 1.98 | Subunit 8 of the F0 sector of mitochondrial inner membrane F1-F0 ATP synthase |
| *YOR065W* | *CYT1* | 1.98 | Cytochrome c1, expression is regulated by glucose-repressed Hap2p/3p/4p/5p |
| *YDR298C* | *ATP5* | 1.88 | Subunit 5 of the stator stalk of mitochondrial F1F0 ATP synthase |
| *YOR011W* | *AUS1* | 1.84 | Transporter of the ATP-binding cassette family, involved in uptake of sterols and anaerobic growth |
| *YGL191W* | *COX13* | 1.83 | Subunit VIa of cytochrome c oxidase |
| *YDR536w* | *STL1* | 1.82 | Glycerol proton symporter, transiently induced when cells are subjected to osmotic shock. |
| *YJR121W* | *ATP2* | 1.81 | β-subunit of the F1 sector of mitochondrial F1F0 ATP synthase |
| *YOL122C* | *SMF1* | 1.79 | Divalent metal ion transporter with a broad specificity for di-valent and tri-valent metals |
| *YNL214W* | *PEX17* | 1.79 | Peroxisomal membrane protein component of the peroxisomal translocation machinery |
| *YML123C* | *PHO84* | 1.73 | High-affinity inorganic phosphate (Pi) transporter and low-affinity manganese transporter |
| *YNL036W* | *NCE103* | 1.73 | Carbonic anhydrase; involved in non-classical protein export pathway |
| *YLR295C* | *ATP14* | 1.72 | Subunit h of the F0 sector of mitochondrial F1F0 ATP synthase |

| ORF | Gene name | Fold change | Description |
|---|---|---|---|
| *YLR440C* | *SEC39* | 1.7 | Protein of unknown function proposed to be involved in protein secretion |
| *YAR002W* | *NUP60* | 1.7 | Subunit of the nuclear pore complex |
| *YBR283C* | *SSH1* | 1.68 | Subunit of the Ssh1 translocon complex; involved in co-translational pathway of protein translocation |
| *YHL047C* | *TAF1* | 1.68 | Transporter, member of the ARN family of transporters that specifically recognize siderophore-iron chelates |
| *YOL123W* | *HRP1* | 1.68 | Subunit of cleavage factor I, required for the cleavage and polyadenylation of pre-mRNA 3' ends |
| *YKL141W* | *SDH3* | 1.67 | Cytochrome b subunit of succinate dehydrogenase |
| *YCR098C* | *GIT1* | 1.67 | Plasma membrane permease, mediates uptake of glycerophosphoinositol and glycerophosphocholine as sources of the nutrients inositol and phosphate |
| *YBR039W* | *ATP3* | 1.67 | Gamma subunit of the F1 sector of mitochondrial F1F0 ATP synthase |
| *YBR092C* | *PHO3* | 1.66 | Constitutively expressed acid phosphatase similar to Pho5p |
| *YML116W* | *ATR1* | 1.59 | Multidrug efflux pump of the major facilitator superfamily |
| *YML081C* | *ATP18* | 1.56 | Subunit of the mitochondrial F1F0 ATP synthase, termed subunit I or subunit j |
| *YMR256C* | *COX7* | 1.55 | Subunit VII of cytochrome c oxidase |
| *YDR178W* | *SDH4* | 1.53 | Membrane anchor subunit of succinate dehydrogenase |
| *YGL187C* | *COX4* | 1.5 | Subunit IV of cytochrome c oxidase |
| *YPR194C* | *OPT2* | -3.3 | Oligopeptide transporter; member of the OPT family |
| *YBL011W* | *SCT1* | -2.1 | Glycerol 3-phosphate/dihydroxyacetone phosphate dual substrate-specific sn-1 acyltransferase of the glycerolipid biosynthesis pathway |
| *YER009W* | *NTF2* | -2 | Nuclear envelope protein, interacts with GDP-bound Gsp1p and with proteins of the nuclear pore to transport Gsp1p into the nucleus |
| *YER119C* | *AVT6* | -1.85 | Vacuolar transporter, exports aspartate and glutamate from the vacuole |
| *YLL042C* | *APG10* | -1.8 | E2-like conjugating enzyme that mediates formation of the Atg12p-Atg5p conjugate, a critical step in autophagy |
| *YKL057C* | *NUP120* | -1.81 | Subunit of the Nup84p subcomplex of the nuclear pore complex |
| *YDL145C* | *COP1* | -1.7 | Alpha subunit of COPI vesicle coatomer complex, which surrounds transport vesicles in the early secretory pathway |

| ORF | Gene name | Fold change | Description |
|---|---|---|---|
| *YGR020C* | *VMA7* | -1.7 | Subunit F of the eight-subunit V1 peripheral membrane domain of vacuolar $H^+$-ATPase |
| *YDL084W* | *SUB2* | -1.57 | Component of the TREX complex required for nuclear mRNA export |
| *YGL255W* | *ZRT1* | -1.56 | High-affinity zinc transporter of the plasma membrane, responsible for the majority of zinc uptake |
| *YBR127C* | *VMA2* | -1.5 | Subunit B of the eight-subunit V1 peripheral membrane domain of the vacuolar $H^+$-ATPase |
| *YLR447C* | *VMA6* | -1.52 | Subunit D of the five-subunit V0 integral membrane domain of vacuolar $H^+$-ATPase |

During the genome-wide transcriptomic analysis of HC42 strain, a 3.27-fold enhancement of the expression of the gene *INO1* encoding MIP synthase was detected, as well as *GIT1* (1.7-fold) encoding a permease for uptake of glycerophosphoinositol. These results could be a consequence of *TPI1* deletion. When the activity of the enzyme TPI is abolished the intracellular levels of DHAP and G3P increase (Shi *et al*. 2005) and DHAP is a MIP synthase competitive inhibitor.

In order to test the possibility of an inositol defect in yeast mutant HC42 and the possibility of correct it, the stain was cultivated in SD medium supplemented with 100 $\mu g.ml^{-1}$ of inositol (Figure 4.3). The strain consumed all the glucose after 45 hours, reaching a glycerol yield of 0.46 $g.l^{-1}$. Growth rate was 0.14 $h^{-1}$. Ethanol and acetate were also produced during batch cultivation, as well as succinate, acetaldehyde and acetoin (data not shown).

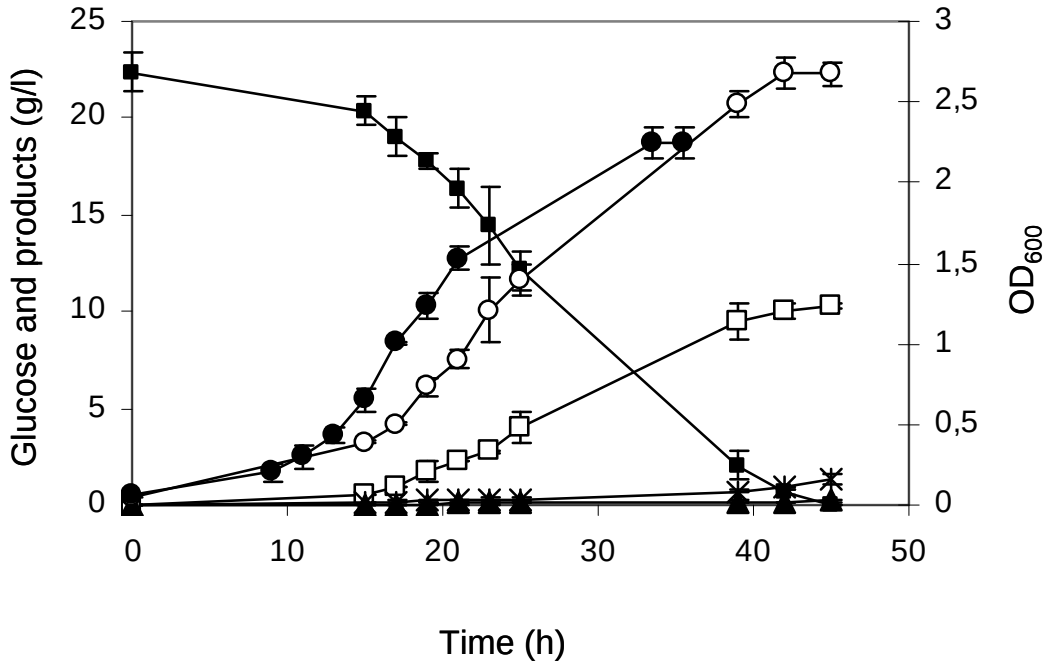



Figure 4.3. *S. cerevisiae* batch cultivation in SD medium with and without inositol addition at 30ºC, 150 rpm, 0.5 vvm and pH 5. With inositol: ■ – Glucose; □ – glycerol; ▲- acetate; ✳- ethanol; ● – $OD_{600}$. Without inositol: ○ - $OD_{600}$.

Comparing the growth curves, representing the two differential HC42 cultivation conditions, with and without inositol addition (Figure 4.3) no significant differences can be reported. Glycerol yield obtained in both conditions was also the same.

## 4.3. Discussion and conclusions

Geneome-wide transcriptomic analysis became possible a better comprehension of the new mutant strain HC42 performance. This analysis

revealed that the transcription of 384 genes had significantly changed in response to the genetic engineering modifications introduced to reach a high glycerol producer yeast strain. From those genes, 184 were down-regulated and 200 were up-regulated. Most of the genes differentially expressed belong to metabolism, energy and cellular transport, transport facilitation and transport routes.

Genetic modifications were confirmed through this analysis. Genes *TPI1* and *ADH1* were down-regulated -2.56 and -2.33 fold respectively, and *GPD1* and *ALD3* were overexpressed 4.14 and 6.56 fold respectively. However, other genes belonging to the same families of genetic engineer target genes, like *GPD2*, *ALD2* and *ALD4* and *ADH2* were also differentially expressed.

In accordance to the bottlenecks found during metabolic analyses, secondary effects of molecular manipulations were identified during transcriptomic analysis. The problems mentioned in Chapter 3, like the significant decrease in NADH/ $NAD^+$ ratio, caused by the reduction in NADH pools can be related to the several amounts of up and down-regulated genes encoding proteins with $NAD^+$/$NADP^+$ binding function (i.e., *NDI1, PDH1, ALD4, ARA1, GDH2, IDH1, ALD2, ETR1*, *GPD1, GPD2*, *PDC1*, *ADH1*, etc). Consequently the down pools of NADH could originate the reduction on ATP levels related with the low growth rate of the engineered strain HC42 (Cordier *et al*. 2007). Several genes responsible for energy generation were overexpressed, like *ATP2*, *ATP3*, *ATP5*, *ATP14* and *ATP18* and *AAP1* and also genes belonging to Citric Acid Cycle *SDH2*, *SDH3*, *SDH4*, *FUM1*, *IDH1*, *GDH2* and genes encoding subunits of cytochrome *c* oxidase. This fact can be the answer from the mutant strain in an

attempt to respond to the energetic lack, revealing a partial derepression of the glucose-repressed respiratory activity. This supports the notion that repression of these cellular functions is dependent on the rate of glucose assimilation (Blank and Sauer, 2004).

Curiously the gene *PCD1* encoding the main isoenzyme of pyruvate decarboxylase was down-regulated. Geertman *et al*. (2006), in order to enhance the production of glycerol by a *S. cerevisiae* strain, described a strategy which included the deletion of all pyruvate decarboxylases encoding genes (*PDC1, PDC5* and *PDC6*). In the present study, the reduction of *PDC1* gene expression occurred as a secondary effect of molecular modifications. Probably a spontaneous self strategy occurred in the mutant HC42 to reduce the carbon flux through the fermentative pathway, since *ADH1* was disrupted and acetaldehyde levels were higher than in the wild strain (see Chapter 3). The gene *PYK1* that encodes pyruvate kinase was also down-regulated. The reduction of the activity of this enzyme, subjected to allosteric regulation, seemed to reduce significantly yeast growth rate on glucose and the glycolytic flux was also reduced, whereas the TCA cycling was increased (Pearce *et al*., 2001).

Izawa *et al*. (2004) mentioned the gene *ARA1* as one of the four genes encoding GDH. In the present work genomic analysis revealed the over-expression of gene *ARA1* in strain HC42 when compared to CEN.PK2, what is in accordance with the increase by about 2-fold of the activity of glycerol dehydrogenase (that converts glycerol into dihydroxyacetone) in the mentioned strain and noticed in Chapter 3.

The overexpression of *INO1* was exploited by cultivating the strain HC42 in SD medium supplemented with inositol in accordance with the finding that deletion of *TPI1* caused a partial derepression of *INO1* (Shi *et al.,* 2005). The physiological behaviour of HC42 strain was the same in both media (with/ without inositol), which allowed concluding that overexpression of *GPD1* repaired the inositol defect.

*Chapter 5*

# Metabolic and physiologic effects of *FPS1* overexpression on *Saccharomyces cerevisiae* HC42 mutant strain

## 5.1. FM62 mutant strain construction

As described in Chapter 3, the strategy followed to enhance glycerol production by *Saccharomyces cerevisiae* CEN.PK2 resulted in an engineered strain with a higher glycerol yield of 0.46 $g.l^{-1}$ of glucose, close to the maximum theoretical value. However, this strategy also induced some negative effects on glycerol overproduction. One of these effects was a 10-fold accumulation of intracellular glycerol in the mutant strain HC42, when compared to the wild type strain. This result suggested that glycerol export could be a bottleneck to glycerol production in the mutant strain. As reported by Oliveira *et al.* (2003), regulation of the intracellular glycerol concentration can be linked to the regulation of the entire glycerol pathway activity and affect different vital global regulation processes of cell metabolism.

It is known that the major glycerol facilitator through plasma membrane to the outside of cells is Fps1p (Luyten *et al.* 1995; Oliveira *et al*. 2003; Tamas *et al*. 1999) (see Chapter 3, Figure 3.2). Based on these reports, a strategy to reduce the glycerol accumulation and increase glycerol export was developed. *FPS1*, enconding Fps1p, was overexpressed in strain HC42 as described in Chapter 2. From a total of 46 $Ura^+$ transformants obtained, 10 were randomly chosen and the genomic DNA was extracted and analysed by PCR. Genomic DNA isolated from HC42 strain was used as an empty control. *FPS1* overexpression cassette integration on FM62 strain was confirmed in 7 transformants, as shown in Figure 5.1 on lanes 1, 2, 4, 5, 6, 7, 9. This Figure also shows that *FPS1* cassette was not

amplified from HC42, indicating that this mutant did not carry *FPS1* overexpression cassette.

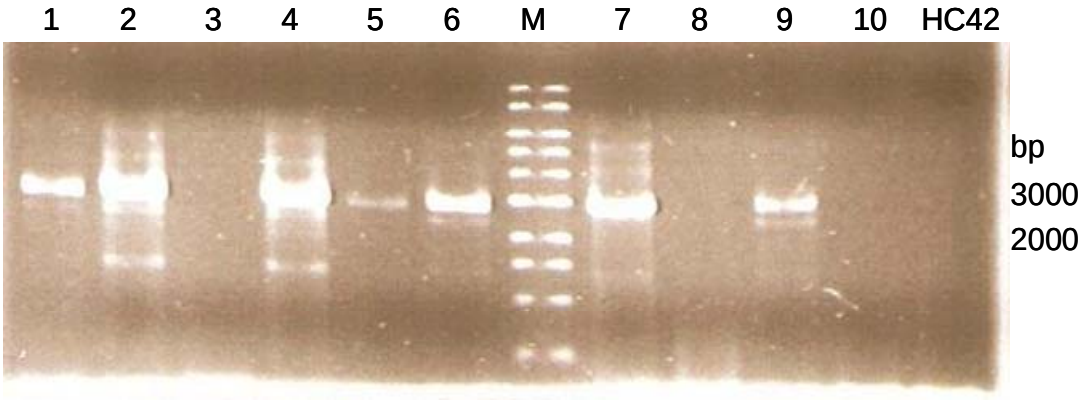



Figure 5.1. PCR amplification of *FPS1* overexpression cassette with a size of 2.7 kb from FM62 transformants on lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and from HC42.

Furthermore, positive clones were plated on SD medium and selective media to confirm the phenotype in comparison with other strains (Figure 5.2). FM62 was able to grow on synthetic medium without uracil, confirming the $Ura^+$ phenotype. The control used was a non-auxotrophic *S. cerevisiae* for the different selective media used and indicated in Figure 5.2.

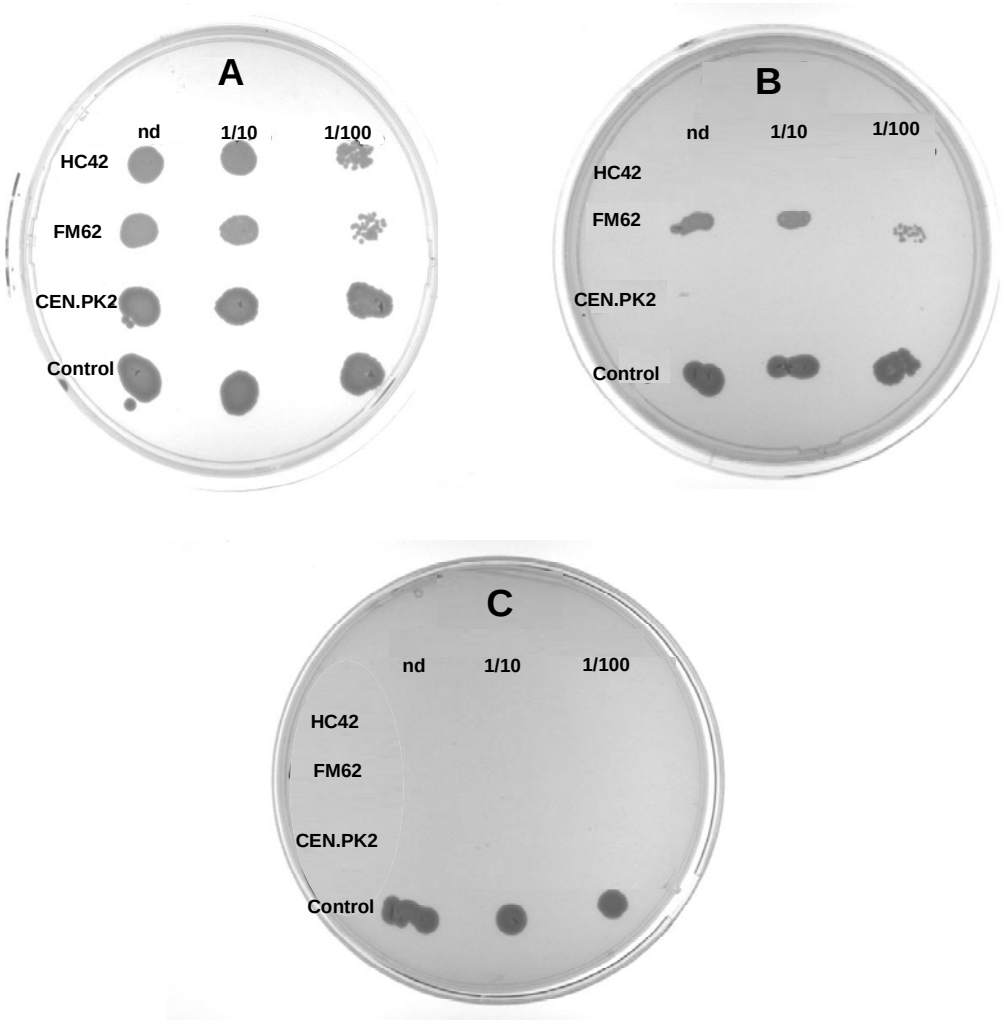



Figure 5.2. Growth of *S. cerevisiae* strains at different dilutions (nd – not diluted, dilution $10^{-1}$ and $10^{-10}$) on agar plates with: A - synthetic medium, B – synthetic medium without uracil, C - synthetic medium without histidine. Plates were incubated at 30ºC.

## 5.2. Batch cultivations of *Saccharomyces cerevisiae* FM62 mutant strain

In order to evaluate the metabolic and physiologic effects of *FPS1* overexpression on the engineered strain, batch cultivations of *S. cerevisiae* FM62 were carried out with the operating conditions previously used to characterise the mutant strain *S. cerevisiae* HC42. Two carbon sources were tested, glucose and molasses. The use of cheap renewable carbon sources can reduce the production cost of microbiological products.

### 5.2.1. Cultivation in SD medium

As described in Chapter 2, SD medium contains glucose (20 $g.l^{-1}$) as carbon source. When FM62 mutant strain was cultivated in this medium at 30ºC, 150 rpm, 0.5 vvm and pH 5.0, glucose was completely consumed (Figure 5.3). As expected, the major fermentation end-product was glycerol, reaching a yield of 0.46 $g.g^{-1}$ glucose. Ethanol, acetate, succinate, acetaldehyde and acetoin were also produced but in minor concentrations. However, the concentrations of acetaldehyde and acetoin were higher than those obtained for acetate and succinate, even if acetaldehyde concentration decreased at the end of cultivation. The maximum specific growth rate of FM62 was 0.15 $h^{-1}$ (Table 5.1).

Intracellular glycerol was also measured in order to analyse *FPS1* overexpression effect when compared to HC42. The value obtained was 115 $\mu mol.g^{-1}$dry mass (Table 5.2).

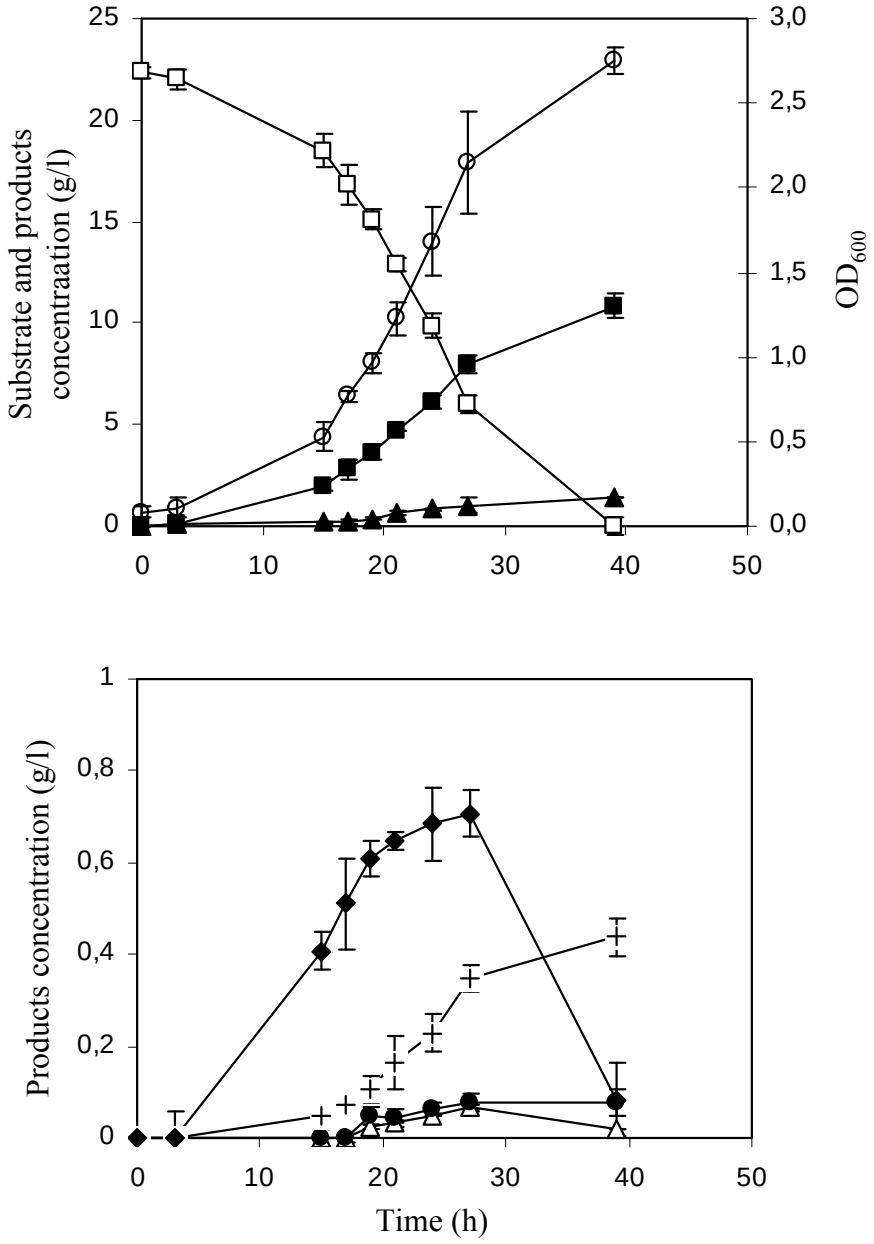



Figure 5.3. Batch culture of *S. cerevisiae* FM62 in SD medium at pH 5.0, 0.5 vvm, 150 rpm and 30ºC. ○ – OD; □ – glucose; ■ – glycerol; ▲ – ethanol; ● – acetate; Δ – succinate; ◆ – acetaldehyde; + – acetoin.

Table 5.1. Kinetic parameters of *S. cerevisiae* FM62 and HC42 grown in SD medium (20 $g.l^{-1}$ glucose, pH 5.0, 0.5 vvm, 150 rpm and 30ºC).

| **Specific rate** | ***S. cerevisiae*** | |
|---|---|---|
| | FM62 | HC42 |
| $\mu_{max}$ ($h^{-1}$) | 0.15 ± 0.05 | 0.17 ± 0.05 |
| $q_{glucose}$ ($mmol.h^{-1}.g$ dry $mass^{-1}$) | 3.4 ± 0.24 | 3.6 ± 0.24 |
| $q_{ethanol}$ ( $mmol.h^{-1}.g$ dry $mass^{-1}$) | 1.4 ± 0.20 | 1.5 ± 0.20 |
| $q_{glycerol}$ ( $mmol.h^{-1}.g$ dry $mass^{-1}$) | 3.4 ± 0.55 | 3.1 ± 0.35 |

q- specific rate (calculated as described in Chapter 2). The data are the mean values ± standard deviation of four independent experiments.

Table 5.2. Intracellular glycerol concentration of *S. cerevisiae* FM62 and HC42 grown in SD medium (20 $g.l^{-1}$ glucose, pH 5.0, 0.5 vvm, 150 rpm and 30 ºC).

| **Strains** | Intracellular glycerol concentration ($\mu mol.g$ dry $mass^{-1}$) |
|---|---|
| FM62 | 115 ± 35 |
| HC42 | 212 ± 36 |

The data are the mean values ± standard deviation of four independent experiments.

## 5.2.2. Cultivation in molasses medium

*S. cerevisiae* FM62 mutant strain was cultivated in molasses medium containing 25 $g.l^{-1}$ total sugars, at pH 5.0, 0.5 vvm, 150 rpm and 30ºC (Figure 5.4).

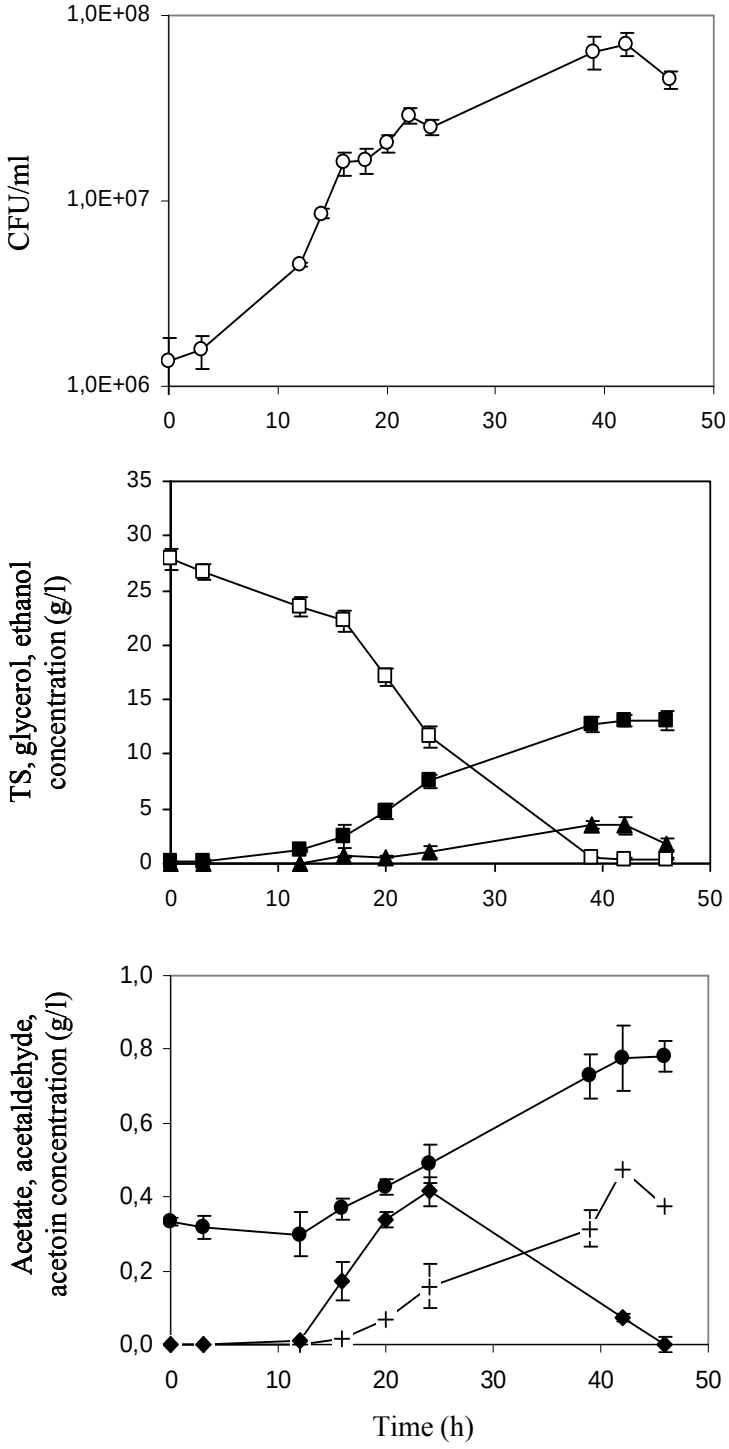



Figure 5.4. Batch fermentation of *S. cerevisiae* FM62 in molasses medium at pH 5.0, 0.5 vvm, 150 rpm and 30ºC. ○ – CFU; □ – Total sugar (TS); ■ – glycerol; ▲ – ethanol; ● – acetate; ◆ – acetaldehyde; + – acetoin.

After 46 hours of growth most of the sugar was consumed. The amount of residual sugar was not significant, 0.41 $g.l^{-1}$. The main fermentation product was glycerol, reaching a yield of 0.47 $g.g^{-1}$ of total sugar (Table 5.3). The other fermentation end-products found were ethanol, acetate, acetoin and acetaldehyde. Part of the acetaldehyde produced may have been converted into acetoin.

Table 5.3. Growth rate and product yields of *S. cerevisiae* FM62 grown in molasses and SD medium.

| Strain | FM62 | |
|---|---|---|
| Medium | Molasses | SD |
| $\mu_{max}$ ($h^{-1}$) | 0.14 ± 0.03 | 0.15 ± 0.05 |
| Glycerol yield ($g.g^{-1}$ sugar) | 0.47 ± 0.01 | 0.46 ± 0.00 |
| Ethanol yield ($g.g^{-1}$ sugar) | 0.08 ± 0.02 | 0.05 ± 0.01 |
| Acetate yield ($g.g^{-1}$ sugar) | 0.02 ± 0.01 | 0.02 ± 0.00 |

The data are the mean values ± standard deviation of four independent experiments.

## 5.3. Discussion and conclusions

In this chapter, a solution for the intracellular glycerol accumulation in the engineered strain *S. cerevisiae* HC42 was envisaged. The *FPS1* gene was overexpressed in the strain HC42, originating a novel mutant strain FM62. Batch cultivations of this strain were performed in SD and molasses media to evaluate the performance of the mutant strain and to compare it with *S. cerevisiae* HC42.

It can be conclude that the overexpression of *FPS1* resulted in a 2-fold reduction of intracellular glycerol concentration in the mutant strain FM62 (Table 5.2), when compared to the value obtained for *S. cerevisiae* HC42. However, the increase in glycerol specific formation rate caused by this reduction of intracellular glycerol was not significant (Table 5.1). An explanation for the minor effect obtained in glycerol efflux could be the fact that high intracellular content in glycerol can mediate its own closure (Karlgren *et al*., 2005). The existence of other aquaglyceroporins participating in glycerol efflux is another possibility, as referred to by Luyten *et al*. (1995) and Petterson *et al*. (2005). Accumulation of intracellular glycerol has also been reported to trigger a Hog1- dependent osmoprotective pathway (Albertyn et al., 1994; Siderius et al., 2000), which ultimately leads to a slow down of glycolysis and growth rate by a yet uncharacterized mechanism (Blomberg, 2000; Oliveira *et al*., 2003). The conjugation of these effects could also be an explanation to the fact that the overexpression of *FPS1* did not lead to a significant increase of glycerol productivity.

For both media (SD medium and sugar cane molasses medium) similar results were obtained for strain FM62. The cultivation end-products were the same. The major final product was glycerol with a yield around 0.46 - 0.47 $g.g^{-1}$ of sugar in both conditions. During cultivation in molasses, HPLC succinate results showed an initial concentration higher than the final concentration, probably, due to molasses contamination with another compound with the same retention time as succinate. For this reason, this compound was not considered in

the analysis. According to the results, the type of medium did not interfere with microorganism growth. Growth rate in SD and molasses media was similar, varying from 0.14 to 0.15 $h^{-1}$.

# Part II: 1,3-propanediol production in a two-step process by *Saccharomyces cerevisiae* HC42 and *Clostridium acetobutylicum* DG1 (pSPD5)

*Chapter 6*

# Glycerol overproduction by *Saccharomyces cerevisiae* HC42 mutant strain

## 6.1. *Saccharomyces cerevisiae* HC42 adaptation to high glucose concentrations

In order to produce glycerol from high sugar concentrations by the genetically modified yeast strain HC42, cultivation on 100 $g.l^{-1}$ of glucose was performed. However, the strain was not able to grow on a media with such a high glucose concentration. Therefore, we searched for individual clones from this engineered strain able to grow in the presence of high glucose concentrations. The strategy was quite simple since firstly HC42 was streaked onto YM agar plates containing 50 $g.l^{-1}$ of glucose. After one week, individual clones were transferred to plates with the same medium. Seven days later, the 50 $g.l^{-1}$ glucose adapted clone was streaked onto YM agar plates containing 100 $g.l^{-1}$ of glucose. After two further transfers onto the same agar plates several individual clones were picked and cultivated in liquid media in the presence of 100 $g.l^{-1}$ of glucose. One of them, showing the fastest growth in these conditions, and termed FH100, was streaked onto an YM agar plate containing 200 $g.l^{-1}$ of glucose and incubated for a week. After two more transfers into the same medium (seven days of incubation before transfer), individual clones were cultivated in liquid medium with 200 $g.l^{-1}$ of glucose. The strain obtained was called FH200. At higher glucose concentration, the selected FH200 clone could weakly grow on agar plates containing 400 $g.l^{-1}$ glucose, but not on liquid media with the same glucose concentration.

## 6.2. Parameters affecting glycerol production by *Saccharomyces cerevisiae* HC42 mutant strain

Like in others microorganisms, yeast growth and metabolism are dependent on several parameters. Environmental conditions, such as temperature, pH, water activity, oxygen, hydrostatic pressure and availability of nutrients (e.g. carbon and other energetic sources), are determinant for the biosynthesis of cellular components necessary for growth, maintenance and reproduction of microorganisms. Under natural conditions, microorganisms can be found in environments more favourable for their growth. In the laboratory, it is possible to create the most favourable conditions for the production of certain metabolites, by optimizing microbial requirements and environmental conditions.

Glycerol production by *S. cerevisiae* plays an important role on redox balance and as a response to osmotic stress. Growth conditions, such as an excess of salts in the culture medium, result in an increase of internal concentrations of glycerol by yeast. This accumulation of glycerol functions as an osmolyte to prevent loss of turgor pressure by the cells (Blomberg and Adler, 1992). The addition of sulphites and bisulphites to the growth medium (the Neuberg's method) binds acetaldehyde and shifts the metabolism towards glycerol production by the need for reoxidising NADH.

In order to set up the fermentation conditions for glycerol production, the effects of pH (4.0, 5.0, and 6.5), agitation rate (150 and 300 rpm) and aeration flow (0.5, 1.0, 1.5 vvm) on various macrokinetic parameters, including rate and

glycerol yield, were evaluated using the metabolically engineered strain HC42. The strain was cultivated in SD medium with 20 $g.l^{-1}$ of glucose at 30ºC.

## 6.2.1. pH

The effect of pH was tested at an agitation rate of 150 rpm and an aeration rate of 0.5 vvm. *S. cerevisiae* HC42 was cultivated at pH 4.0, 5.0 and 6.5.

Table 6.1. Effect of pH on *S. cerevisiae* HC42 batch cultivation (SD medium with 20 $g.l^{-1}$ of glucose, temperature of 30 ºC, aeration 0.5 vvm and agitation 150 rpm).

| pH | 4.0 | 5.0 | 6.5 |
|---|---|---|---|
| Residual glucose ($g.l^{-1}$) | 0.23 ± 0.08 | 0.02 ± 0.00 | 0.02 ± 0.01 |
| Glycerol concentration ($g.l^{-1}$) | 9.6 ± 0.2 | 9.4 ± 0.4 | 9.5 ± 0.2 |
| Biomass concentration ($g.l^{-1}$) | 1.23 ± 0.11 | 1.31 ± 0.30 | 0.96 ± 0.10 |
| Products yield ($g.g^{-1}$ glucose) | | | |
| Glycerol | 0.47 ± 0.01 | 0.46 ± 0.02 | 0.47 ± 0.00 |
| Ethanol | 0.05 ± 0.03 | 0.06 ± 0.00 | 0.04 ± 0.01 |
| Acetate | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.03 ± 0.00 |
| $\mu_{max}$ ($h^{-1}$) | 0.19 ± 0.02 | 0.18 ± 0.03 | 0.12 ± 0.02 |
| $r_{glucose}$ ($g.l^{-1}.h^{-1}$) | 0.48 ± 0.04 | 0.53 ± 0.04 | 0.48 ± 0.05 |
| Cultivation time (h) | 42.0 ± 5.7 | 38.0 ± 3.5 | 43.5 ± 4.4 |

Results are the mean values ± standard deviation from at least four determinations $\mu_{max}$ maximum specific growth rate ($h^{-1}$ ; slope of the least squares regression line of the natural logarithm of optical density vs. time data during the exponential growth phase), $r_{glucose}$ maximum volumetric glucose uptake rate ($g\ l^{-1}\ h^{-1}$)

These values were chosen because: pH 5.0 seems to be the most favourable pH for *S. cerevisiae* growth; pH 6.5 it is the optimal pH for 1,3-PD production by *Clostridium acetobutylicum*; and pH 4.0 to test the effect of a lower value. In the range of pH values tested, no differences were observed on glycerol and ethanol yields (Table 6.1). Glycerol was the major cultivation end-product and ethanol and acetate were produced in minor concentrations. However, in the case of acetate, the yield was slightly increased at pH 6.5. Slight effects were observed on biomass production and specific growth rate. At pH 6.5 the biomass and growth rate values obtained, 0.96 $g.l^{-1}$ and 0.12 $h^{-1}$ respectively, were lower than those obtained at pH 4.0 and 5.0. Although the differences in cell growth, cultivation time was more or less the same, approximately 42 h. Glucose uptake rate was not affected by changing the pH of cultivation medium.

## 6.2.2. Agitation

The effect of agitation on glycerol production by *S. cerevisiae* HC42 was evaluated at pH 5.0 and aeration rate of 0.5 vvm. In the two agitations rates tested, 150 and 300 rpm, no significant differences were observed on glycerol yield (Table 6.2). However, increasing agitation rate to 300 rpm resulted in a dramatic reduction of the glucose consumption rate, and hence increased by 3.5 fold the cultivation time for complete sugar fermentation. Ethanol was not detected at 300 rpm.

Table 6.2. Effect of agitation on S. *cerevisiae* batch cultivation (SD medium with 20 $g.l^{-1}$ of glucose, temperature of 30 ºC, aeration 0.5 vvm and pH 5.0).

| Agitation (rpm) | 150 | 300 |
|---|---|---|
| Residual glucose ($g.l^{-1}$) | 0.02 ± 0.00 | 0.02 ± 0.00 |
| Glycerol concentration ($g.l^{-1}$) | 9.4 ± 0.4 | 9.3 ± 0.1 |
| Biomass concentration ($g.l^{-1}$) | 1.31 ± 0.30 | 1.42 ± 0.41 |
| Products yield (g $g^{-1}$ glucose) | | |
| Glycerol | 0.46 ± 0.02 | 0.45 ± 0.03 |
| Ethanol | 0.06 ± 0.00 | nd |
| Acetate | 0.01 ± 0.00 | 0.01 ± 0.00 |
| $\mu_{max}$ ($h^{-1}$) | 0.18 ± 0.03 | 0.21 ± 0.02 |
| $r_{glucose}$ ($g.l^{-1}.h^{-1}$) | 0.53 ± 0.04 | 0.13 ± 0.02 |
| Cultivation time (h) | 38.0 ± 3.5 | 157.0 ± 8.6 |

Results are the mean values ± standard deviation from at least four determinations $\mu_{max}$ - maximum specific growth rate ($h^{-1}$ ; slope of the least squares regression line of the natural logarithm of optical density vs. time data during the exponential growth phase), $r_{glucose}$ - maximum volumetric glucose uptake rate ($g.l^{-1}.h^{-1}$ ), nd - not detected

## 6.2.3. Aeration

Oxygen supply was tested varying the aeration flow. Values of 0.5, 1 and 1.5 vvm were used for cultivation of *S. cerevisiae* HC42 at pH 5 and agitation rate 150 rpm. According to the results obtained (Table 6.3.), there was no effect of aeration rate on glycerol yield, which was around 0.47 $g.g^{-1}$ of glucose for the three aeration rates tested. The biomass produced increased with the aeration rate

from 1.31 $g.l^{-1}$ at an aeration rate of 0.5 vvm to 1.63 and 2.14 $g.l^{-1}$ at aeration rates of 1 and 1.5 vvm respectively. Cultivation time also increased when higher aeration rates were tested. However, for the two higher aeration rates, ethanol yield was lower than at 0.5 vvm and when the aeration rate was 1.5 vvm, acetate was not detected.

Table 6.3. Effect of aeration rate on *S. cerevisiae* HC42 batch cultivation (SD medium with 20 $g.l^{-1}$ of glucose, temperature of 30ºC, agitation 150 rpm and pH 5.0).

| Aeration rate (vvm) | 0.5 | 1.0 | 1.5 |
|---|---|---|---|
| Residual glucose ($g.l^{-1}$) | 0.02 ± 0.00 | 0.02 ± 0.01 | 0.04 ± 0.02 |
| Glycerol concentration ($g.l^{-1}$) | 9.4 ± 0.4 | 9.4 ± 0.2 | 9.7 ± 0.1 |
| Biomass concentration ($g.l^{-1}$) | 1.31 ± 0.30 | 1.63 ± 0.34 | 2.14 ± 0.15 |
| Products yield ($g.g^{-1}$ glucose) | | | |
| Glycerol | 0.46 ± 0.02 | 0.47 ± 0.02 | 0.47 ± 0.02 |
| Ethanol | 0.06 ± 0.00 | 0.02 ± 0.00 | 0.02 ± 0.01 |
| Acetate | 0.01 ± 0.00 | 0.02 ± 0.00 | nd |
| $\mu_{max}$ ($h^{-1}$) | 0.18 ± 0.03 | 0.18 ± 0.01 | 0.18 ± 0.03 |
| $r_{glucose}$ ($g.l^{-1}.h^{-1}$) | 0.53 ± 0.04 | 0.37 ± 0.03 | 0.42 ± 0.05 |
| Cultivation time (h) | 38.0 ± 3.5 | 50.0 ± 4.7 | 44.0 ± 5.1 |

Results are the mean values ± standard deviation from at least four determinations $\mu_{max}$ - maximum specific growth rate ($h^{-1}$ ; slope of the least squares regression line of the natural logarithm of optical density vs. time data during the exponential growth phase), $r_{glucose}$ - maximum volumetric glucose uptake rate ($g.l^{-1}.h^{-1}$ ), nd - not detected

According to the results, the several factors tested did not cause significant differences in the production of glycerol by *S. cerevisiae* HC42. The parameter that could impair efficient fermentation of glucose into glycerol was the agitation rate.

## 6.3. Glycerol production by adapted *Saccharomyces cerevisiae* strains to high glucose concentrations

### 6.3.1. Batch cultivation

Based on the results previously attained with the strain HC42 at low glucose concentrations, an aeration rate of 0.5 vvm, an agitation rate of 150 rpm and a pH 6.5 were chosen to test for glycerol production by FH100 and FH200 at high glucose concentrations, 100 and 200 $g.l^{-1}$ respectively (Figures 6.1 and 6.2).

As expected, glycerol was the major fermentation end-product, as it was for strain HC42, yielding 0.47 $g.g^{-1}$ of glucose for FH100 and 0.40 $g.g^{-1}$ of glucose for FH200; this latter strain was unable to exhaust the glucose in the medium. Ethanol, acetate, succinate, acetoin and acetaldehyde were also found among cultivation end-products. The biomass produced by the strain FH100 (1.1 $g.l^{-1}$) was almost the same as the value reached by the strain FH200 (1.3 $g.l^{-1}$). The growth rate was higher for strain FH100 than for FH200, reaching 0.11 $h^{-1}$ and 0.08 $h^{-1}$ respectively. To conclude, *S. cerevisiae* FH100 and FH200 could be cultivated on 100 and 200 $g.l^{-1}$ glucose, leading to a 5-fold increase of final glycerol concentration, 49.2 $g.l^{-1}$ and 60.0 $g.l^{-1}$ respectively, when compared to strain HC42 growing on 20 $g.l^{-1}$.

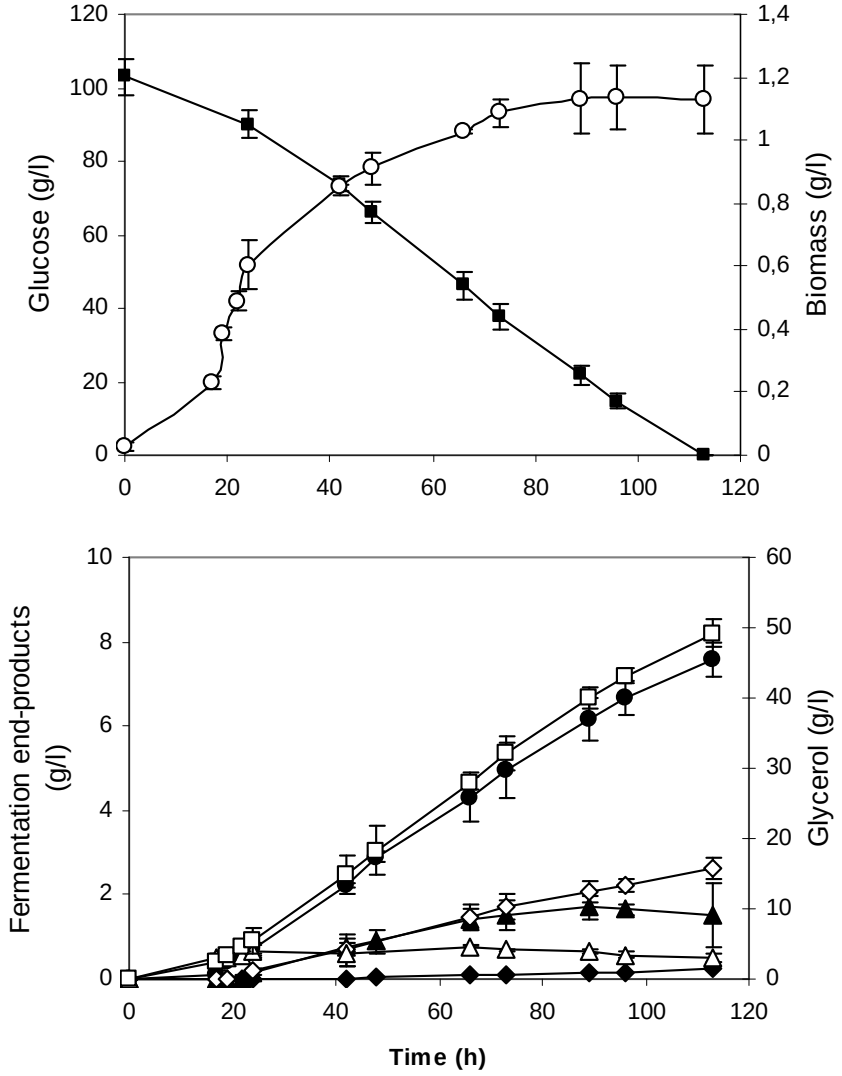



Figure 6.1. *S. cerevisiae* FH100 batch cultivation in SD medium with 100 $g.l^{-1}$ glucose, at pH 6.5, 30ºC, 150 rpm and 0.5 vvm. ■ – Glucose, ● – ethanol, ▲ – acetate, ♦ - succinate, ○ – biomass, □ - glycerol, ◊ - acetoin, Δ – acetaldehyde.

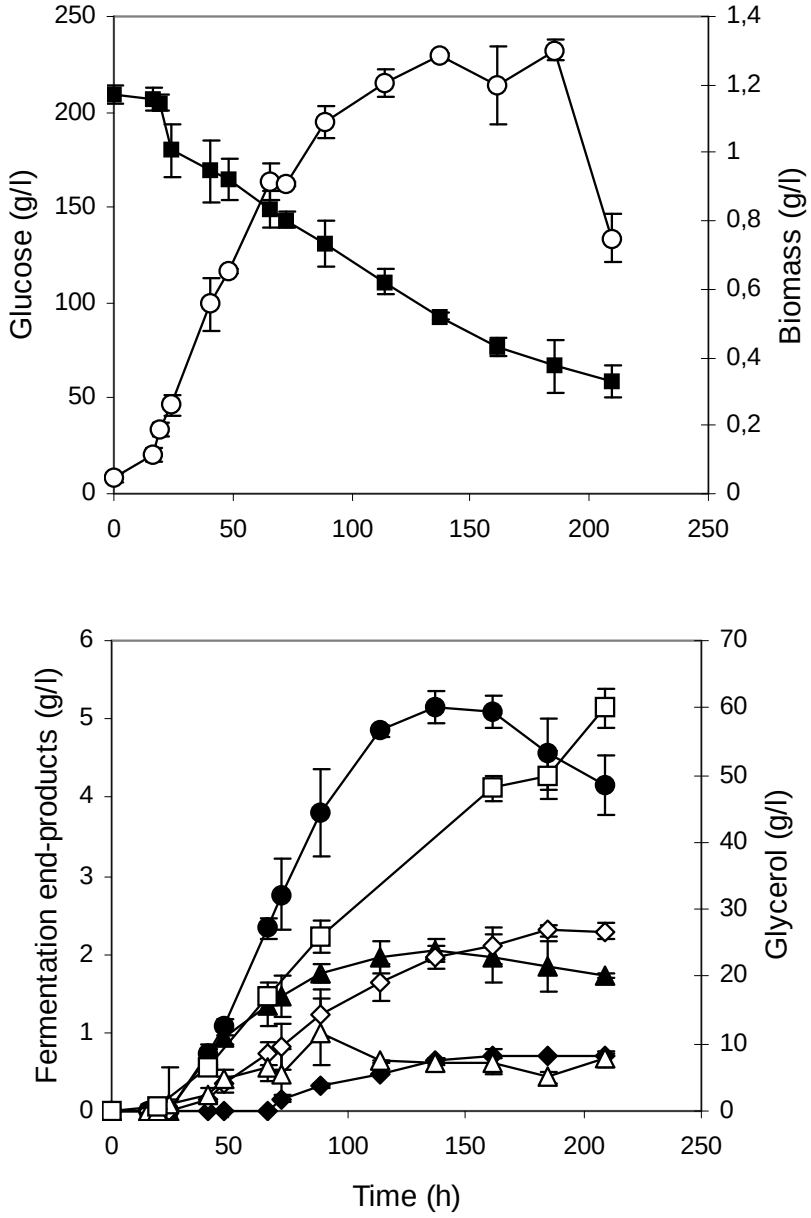



Figure 6.2. *S. cerevisiae* FH200 batch cultivation in SD medium with 200 $g.l^{-1}$ glucose, at pH 6.5, 30ºC, 150 rpm and 0.5 vvm. ■ – Glucose, ● – ethanol, ▲ – acetate, ♦ - succinate ○ – biomass, □ - glycerol, ◊ - acetoin, Δ – acetaldehyde.

## 6.3.2. Fed-batch cultivation

In an attempt to increase the final glycerol concentration, *S. cerevisiae* FH100 fed-batch cultures were carried out and glycerol production was compared with results obtained from batch cultures. The medium used was SD, with an initial glucose concentration of 50 $g.l^{-1}$ and culture was fed each time glucose concentration in the medium reached 10 $g.l^{-1}$. The volume of feed medium necessary to increase glucose to 50 $g.l^{-1}$ was manually added. The same operating conditions used in batch cultures were applied: aeration rate of 0.5 vvm, agitation rate of 150 rpm and pH 6.5.

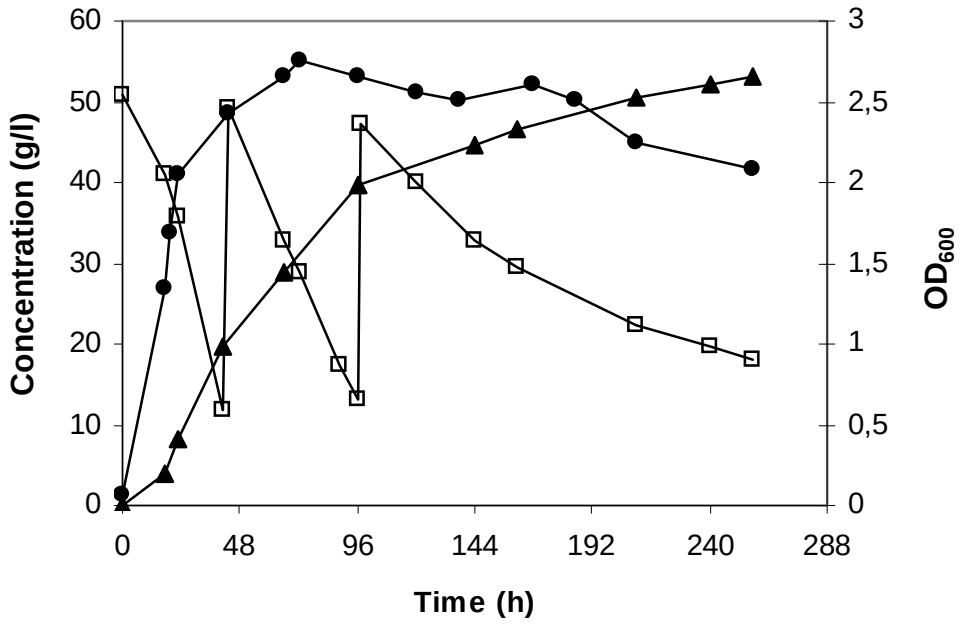



Figure 6.3. Fed-bach cultivation of *S. cerevisiae* FH100 in SD medium at pH 6.5, 30ºC, 150 rpm and 0.5 vvm. □ – glucose, ▲ – glycerol, ● – $OD_{600}$.

In fed-batch cultivation (Figure 6.3.) after the second addition of glucose, corresponding to 96 h of cultivation, the consumption rate of sugar started decreasing and glycerol production slowed down. Cells growth also started to decline. When the cultivation time reached 258 h and the medium pH increased, the experience was stopped. Glycerol yield reached 0.48 $g.g^{-1}$ of glucose, which corresponds to a glycerol production of 53 $g.l^{-1}$.

## 6.4. Cultivation of *Saccharomyces cerevisiae* HC42 and FH100 in molasses media

Two different carbon sources were evaluated in this work. Commercial glucose (SD medium) was used as substrate for yeast cultivation during the first part of the process, and has been studied in the previous subchapters. Another carbon source tested was sugar cane molasses, a by-product from sugar industry (Figure 6.4), already evaluated as a carbon source for strain FM62 growth (Chapter 5).

Strains HC42 and FH100 were also cultivated in sugar cane molasses media, using the same aeration flow, agitation rate and pH conditions as cultures on SD media.

Figure 6.4. Bioreactor with sugar cane molasses medium.

### 6.4.1. Molasses fermentation by S*accharomyces cerevisiae* HC42

*S. cerevisiae* HC42 batch experiments with 5% ($w.v^{-1}$) of sugar cane molasses, corresponding to 27.6 $g.l^{-1}$ of total sugars (TS), were performed and results were compared with cultivations on SD medium. The influence of the sugar source on glycerol production and yeast growth kinetics was evaluated. After around 50 to 60 hours, the medium pH started to increase and this was considered the end of fermentation (Figure 6.5).

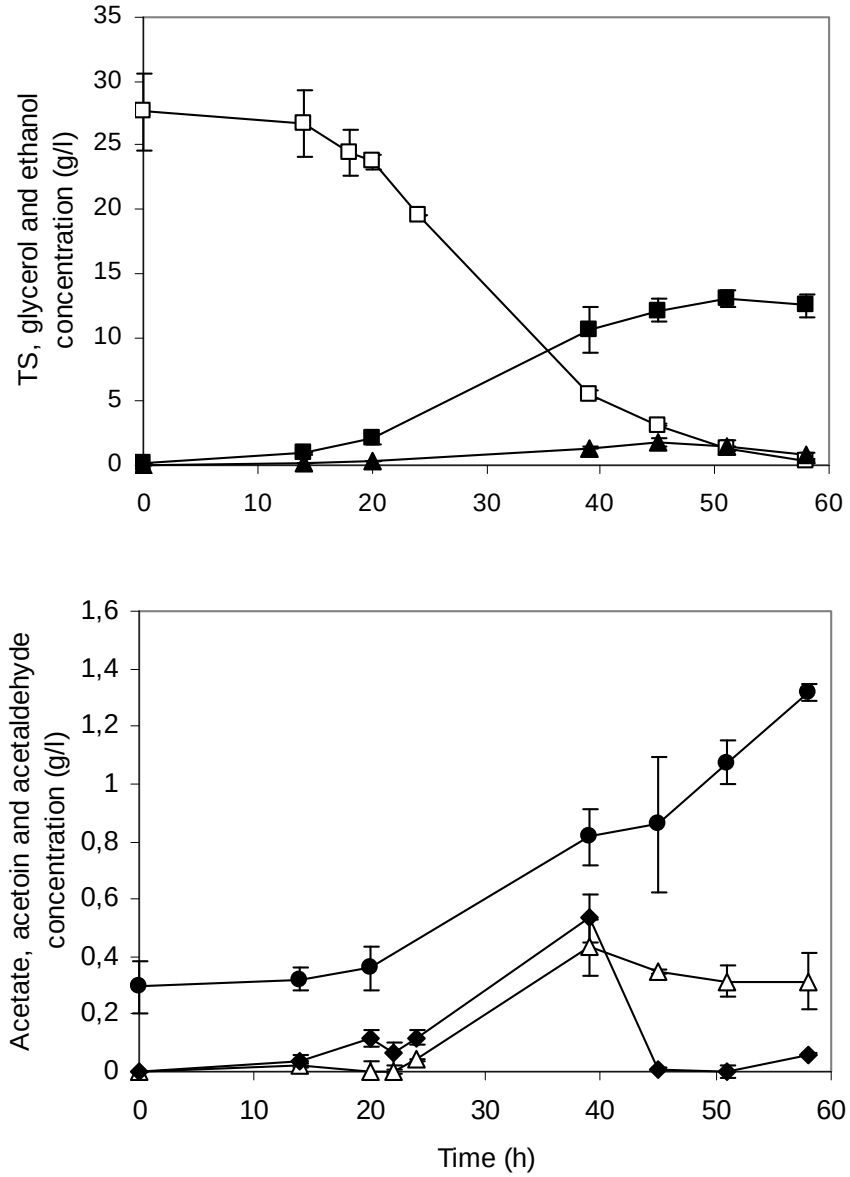



Figure 6.5. Batch fermentation of *S. cerevisiae* HC42 on molasses medium (27.6 $g.l^{-1}$ total sugars) at pH 6.5, 0.5 vvm, 150 rpm and 30ºC. ○ – CFU; □ – Total sugars; ■ – glycerol; ▲ – ethanol; ● – acetate; Δ – acetoin; ◆ – acetaldehyde.

The strain HC42 grew slightly better on molasses medium than in SD medium, with a biomass production of 2.48 $g.l^{-1}$. Glycerol was the main fermentation end-product with a production of 12.7 $g.l^{-1}$ and a yield of 0.47 $g.g^{-1}$ of TS (total sugars). Apart from biomass and ethanol, other fermentation products where also produced, such as acetate, acetaldehyde and acetoin. Acetaldehyde started to be produced during the early stage of exponential phase and the amount produced was up to 0.5 $g.l^{-1}$. Some residual sugar was detected; however, the amounts were negligible, with concentrations lower than 0.5 $g.l^{-1}$.

### 6.4.2. Molasses fermentation by *Saccharomyces cerevisiae* FH100

*S. cerevisiae* FH100 was also cultivated in molasses medium, in order to evaluate the metabolic and physiologic behaviour of this mutant strain during cultivation on a renewable carbon source. Three different concentrations of molasses were tested, 10%, 15% and 20% corresponding to 56.2, 77.8 and 101.3 $g.l^{-1}$ TS respectively. Figure 6.6. shows a representative batch cultivation on 20% $w.v^{-1}$ of molasses, the maximum concentration used to grow the strain in sugar cane molasses medium. The different sugars present in sugar cane molasses were followed, during fermentation by strain FH100: sucrose, glucose, fructose and raffinose. In the beginning of cultivation, sucrose was the main sugar in the medium at 74.0 $g.l^{-1}$.

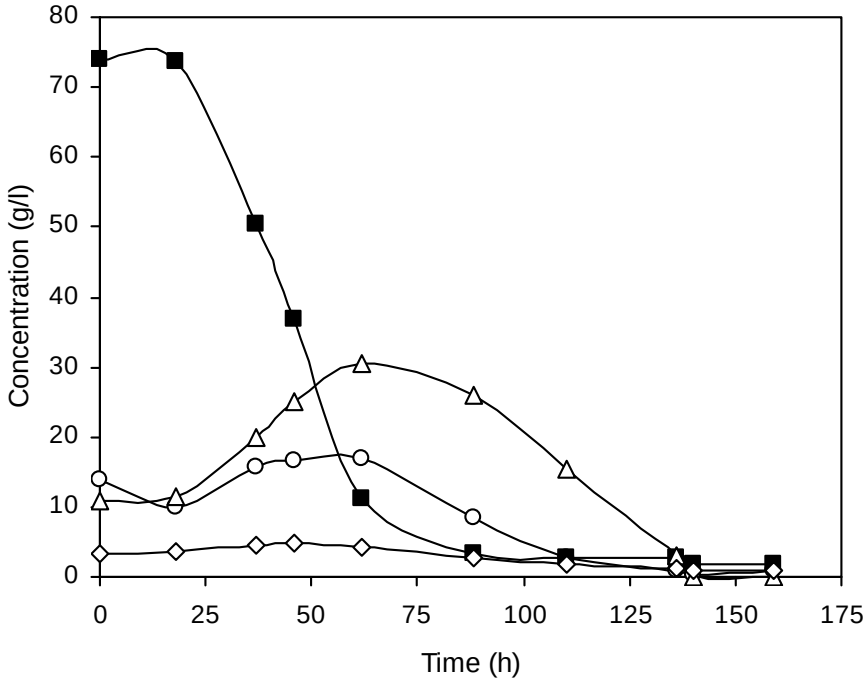



Figure 6.6. Use of the different sugars from cane molasses by the strain FH100 during representative batch fermentation on 20% w.v$^{-1}$ molasses medium. ■ – sucrose; Δ – fructose; ○ – glucose; ◊ - raffinose.

During fermentation, sucrose was hydrolysed to fructose and glucose. Due to this fact and since their production rates were faster than the consumption rates, the concentrations of the two hexoses increased after the initial 24 hours cultivation. Later on, their concentrations started decreasing, corresponding to the complete conversion of sucrose. Raffinose is the sugar present in lower percentage in sugar cane molasses medium. It was only used by the strain after around 80 hours of cultivation, when sucrose concentration in the medium was

less than 10 $g.l^{-1}$, and at the end of the fermentation it was not completely consumed.

The results from batch cultivation of FH100 in media with different sugar cane molasses concentrations are shown in Table 6.4.

As for cultivation on SD medium, glycerol was the major end-product in all fermentations. The strain *S. cerevisiae* FH100 seemed to grow better on 10% molasses (56.2 $g.l^{-1}$ TS). The growth rate (0.13 $h^{-1}$) and the biomass concentration (2.2 $g.l^{-1}$) were higher at this molasses concentration, and decreased when higher sugar concentrations were used (growth rate 0.10 $h^{-1}$ and biomass concentration of 1.3 $g.l^{-1}$). The production of glycerol increased with the initial total sugars (TS) concentration, from 25.8 $g.l^{-1}$, when the strain was cultivated on 10% molasses (56.2 $g.l^{-1}$ TS), to 47.1 $g.l^{-1}$, when the strain was grown on an initial molasses concentration of 20% (101.3 $g.l^{-1}$ TS). However, glycerol yield was the same for all fermentations, 0.47 - 0.48 $g.g^{-1}$ of sugar. Other fermentation end-products were acetate and ethanol; acetoin (0.02 $g.g^{-1}$ TS) and acetaldehyde (0.01 $g.g^{-1}$ TS) were also detected in the final broth. Succinate was also detected, but because of other compound with the same HPLC retention time, it was not possible to determine its concentration.

Table 6.4. *S. cerevisiae* FH100 batch cultivations on molasses medium with different initial sugar concentration at pH 6.5, 30 ºC, aeration 0.5 vvm and 150 rpm.

| Strain | FH100 | | |
|---|---|---|---|
| Sugar cane molasses ( $w.v^{-1}$) | 10% | 15% | 20% |
| Initial TS concentration ($g.l^{-1}$) | 56.2 ± 4.1 | 77.8 ± 2.6 | 101.3 ± 4.4 |
| Glycerol concentration ($g.l^{-1}$) | 25.8 ± 3.0 | 36.8 ± 3.0 | 47.6 ± 3.0 |
| Biomass concentration ($g.l^{-1}$) | 2.22 ± 0.27 | 1.27 ± 0.04 | 1.25 ± 0.16 |
| Products yield ($g.g^{-1}$ of sugar) | | | |
| Glycerol | 0.48 ± 0.01 | 0.48 ± 0.00 | 0.47 ± 0.02 |
| Ethanol | 0.07 ± 0.01 | 0.08 ± 0.01 | 0.07 ± 0.00 |
| Acetate | 0.03 ± 0.01 | 0.04 ± 0.00 | 0.05 ± 0.00 |
| Acetoin | 0.02 ± 0.00 | 0.02 ± 0.00 | 0.02 ± 0.00 |
| Acetaldehyde | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.01 ± 0.00 |
| $\mu_{max}$ ($h^{-1}$) | 0.13 ± 0.01 | 0.10 ± 0.01 | 0.10 ± 0.02 |
| Glycerol productivity ($g.l^{-1}.h^{-1}$) | 0.34 ± 0.04 | 0.25 ± 0.03 | 0.30 ± 0.04 |

TS - total sugars, $\mu_{max}$ - maximum specific growth rate ($h^{-1}$ ; slope of the least squares regression line of the natural logarithm of colony forming units per milliliter vs. time data during the exponential growth phase)

## 6.5. Discussion and conclusions

In an attempt to improve glycerol production performance by the yeast, different growth condition had been tested. The effects of agitation rate, pH and aeration flow on glycerol production were investigated. According to the results obtained, the parameter that could impair efficient fermentation of glucose into glycerol was the agitation rate. Agitation rate favours oxygen supply to the cells, and in this study this can be the cause of the low glucose uptake rate and the 3.5 fold increase of cultivation time, when the agitation rate was 300 rpm. Increasing aeration rate resulted in a slight enhancement of biomass production that was accompanied by a reduction of the glucose uptake rate and of ethanol and acetate yields. This indicates that oxygen was more available to cells and to stimulate the aerobic metabolism resulting in higher ATP availability for biomass production. These results are reminiscent to the so-called Pasteur Effect (aerobic inhibition of glycolysis) (Lloyd *et al.,* 1983). In *S. cerevisiae* the Pasteur Effect is especially effective under slow growth condition, as upon nitrogen limitation (Lagunas *et al*., 1982). The strain used in this study has a growth rate twofold lower than the wild type and its global physiology may be more sensitive to the presence of oxygen than the isogenic wild type.

The use of high sugar concentration in the cultivation medium was an obvious choice to enhance the production of glycerol. However, glycerol-engineered *S. cerevisiae* HC42 was found unable to grow in the presence of more than 20 g $l^{-1}$ of glucose. By selecting spontaneous clones able to grow on 100 and 200 g.$l^{-1}$ glucose, we isolated adapted strains that were able to grow in sugar

medium containing up to 200 $g.l^{-1}$ glucose, strains FH100 and FH200 growing on 100 and 200 $g.l^{-1}$ respectively. However, FH200 did not consume all the glucose and cultivation stopped with 60.8 $g.l^{-1}$ of residual sugar. Fed-batch cultivation did not result in a higher glycerol yield for the strain FH100, that took a longer time to consume the same amount of glucose than in batch cultivation.

Yeast strains HC42 and FH100 were able to grow in sugar cane molasses, but only FH100 was able to grow on 20% molasses $w.v^{-1}$, which contained up to 100 $g.l^{-1}$ TS. At high sugar concentrations the growth rate of the adapted strain decreased. This could be caused either because of the high osmotic pressure of the media or because of an inhibitory effect due to some by-products, like acetaldehyde, since the amount of acetaldehyde in these culture conditions could reach up to 0.6 – 1.0 $g.l^{-1}$. A previous work found that acetaldehyde has a toxic effect and can impair cells growth (Heux *et al*., 2006; Remize *et al*., 1999; Remize *et al*., 2001). It is also described in literature that, increasing molasses concentration, *S. cerevisiae* growth rate, biomass yield and ethanol productivity significantly decreased, although with a higher range of concentrations tested (from 96.7 $g.l^{-1}$ till 323.5 $g.l^{-1}$) (Atiyeh and Duvnjak, 2003).

*Chapter 7*

# 1,3-Propanediol production by *Clostridium acetobutylicum* DG1 (pSPD5) on *Saccharomyces cerevisiae* fermentation broth

## 7.1. The use of sugars for 1,3-propanediol production

1,3-Propanediol is a commercially interesting compound. Due to its chemical and physical characteristics it can be used in a large variety of applications (as previously described in Chapter I). The development of a new polyester called polytrimethylene terephthalate (PTT) has increased the demand for 1,3-propanediol and several studies have been carried out in order to optimize its production. 1,3-PD is naturally produced from glycerol. Glycerol is a by-product of biodiesel industry; with the exponential increase of biodiesel production, glycerol is also generated in large amounts, with the consequent reduction of its market price. Nowadays, the production of glycerol from glucose is economically not viable. However, the production of 1,3-PD from glucose or other sugars continues to be an attractive topic, since the process must be flexible and adjustable to market fluctuations. As no natural microorganisms are known to produce 1,3-propanediol from sugar, genetic engineered strains were developed to carry out the process in one or two steps. Probably, the best 1,3-propanediol natural producer is *Clostridium butyricum*; however, no genetic tools are currently available for this microorganism (González-Pajuelo *et al.*, 2005). *C. acetobutylicum*, a related microorganism, appears as an alternative as genetic tools to be used in this microorganism are well known. When growing in glucose, three different metabolic states can be found in *C. acetobutylicum* depending on medium pH (Girbal and Soucaille, 1998). At neutral pH *C. acetobutylicum* shows an acidogenic behaviour and produces acetic and butyric acids; if pH is neutral and the availability of NAD(P)H is high, the behaviour is alcohologenic and it

produces ethanol and butanol, but not acetone. At low pH the behaviour is solventogenic and it produces acetone, butanol and ethanol. It is known that the genes responsible for the production of solvents are located in the megaplasmid pSOL1 (Cornillot and Soucaille, 1996; Cornillot *et al*., 1997). *C. acetobutylicum* cannot grow on glycerol alone (Vasconcelos et al., 1994) and does not produce 1,3-propanediol.

The strain used in this work is the recombinant strain *C. acetobutylicum* DG1 (pSPD5) (Fig. 7.1). It was obtained by introducing the pSPD5 plasmid, carrying the 1,3-propanediol operon genes (*dhaB1-dhaB2-dhaT*) from *C. butyricum* in the mutant strain *C. acetobutylicum* DG1 (Gonzalez-Pajuelo *et al*., 2005). The strain DG1 does not possess the megaplasmid pSOL1 and then is unable to produce solvents and sporulate (Cornillot *et al.*, 1997; Nair, 1995). As a result, the strain *C. acetobutylicum* DG1 (pSPD5) can grow on glycerol as the sole carbon source and the main product of this fermentation is 1,3-PD. In continuous culture, *C. acetobutylicum* can produce 60 $g.l^{-1}$ of 1,3-PD with a yield of 0.64 $mol.mol^{-1}$ of glycerol and a productivity of 3 $g.l^{-1}.h^{-1}$, which represents an increase of almost 2 fold when compared to the natural producer *C. butyricum* under the same culture conditions (González-Pajuelo *et al*., 2005). According to the same authors, the final concentration of 1,3-PD was increased up to 84 $g.l^{-1}$ when growing *C. acetobutylicum* DG1 (pSPD5) in fed-batch cultures for 47.5 h. The production of acids decreased, and acetic and lactic acids were the most affected. No butanol, acetone or ethanol where detected in the fermentation broth.

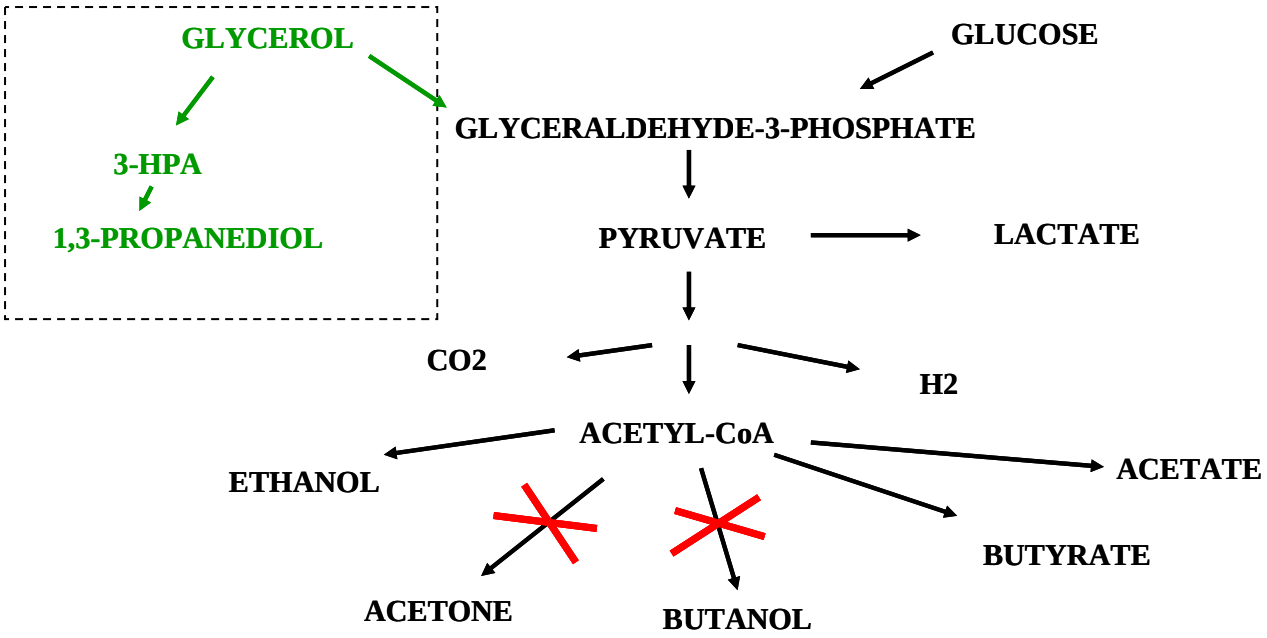



Figure 7.1. Schematic representation of *C. acetobutylicum* DG1 (pSPD5) metabolic pathways. Inside the dashed square the metabolic pathway inserted.

## 7.2. 1,3-Propanediol production on yeast broths

In this work, growth of *C. acetobutylicum* DG1 (pSPD5) in yeast fermentation broths was evaluated. Broths with different glycerol concentrations, resulting either from glucose or sugar cane molasses fermentations, were used without any previous treatment (Fig.7.2.). Several batch fermentations, were carried out in order to evaluate the possibility of using those broths to produce 1,3-propanediol by *C. acetobutylicum* DG1 (pSPD5).

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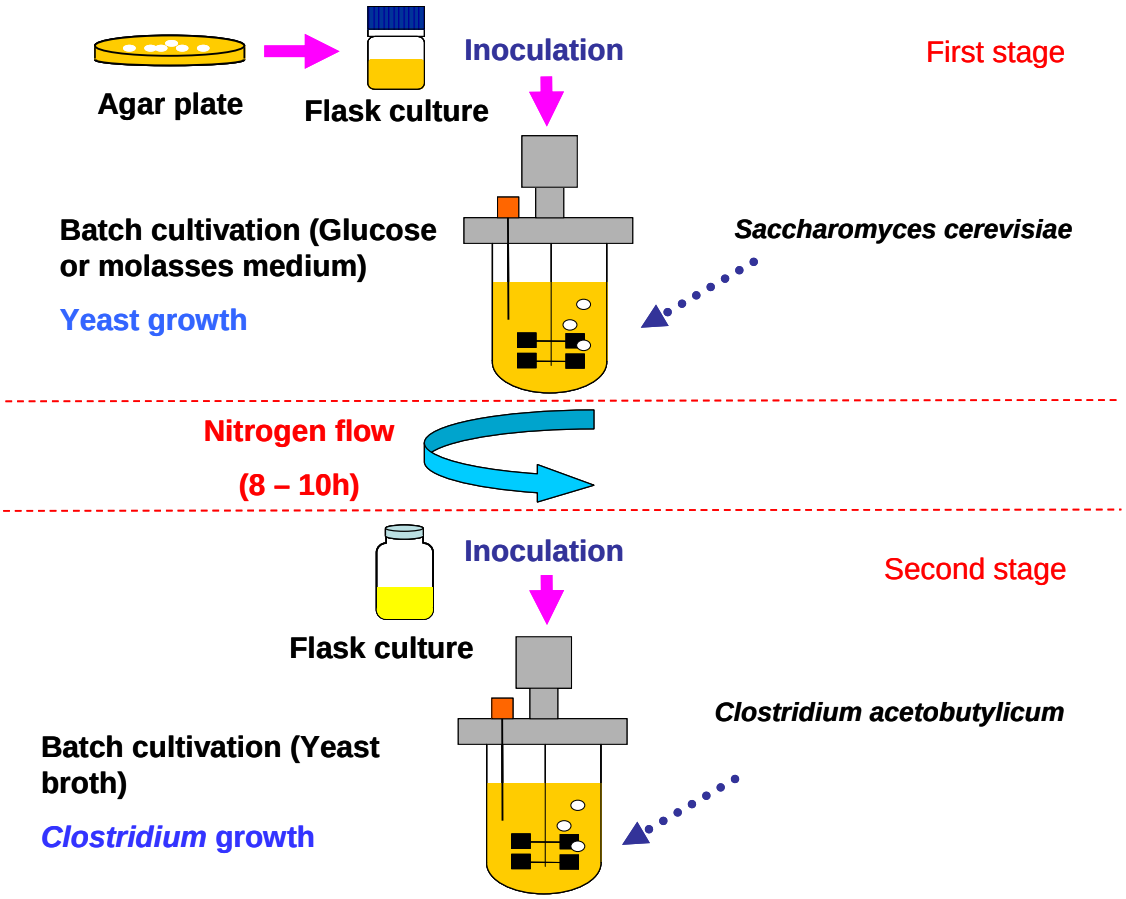


Figure 7.2. Scheme of a two-step strategy for 1,3-propanediol production.

### 7.2.1. 1,3-Propanediol production on yeast broth from glucose fermentation

Production of 1,3-propanediol by *C. acetobutylicum* DG1 (pSPD5) was tested on *S. cerevisiae* HC42 and FH100 broths with glycerol obtained from glucose fermentation. Two different initial glucose concentrations, resulting in broths with different glycerol concentrations, were tested (Table 7.1.). For low initial glucose concentrations, 22 $g.l^{-1}$, i.e. 13.4 $g.l^{-1}$ of glycerol, the 1,3-PD yield

was 0.57 $g.g^{-1}$ of glycerol, and 0.25 $g.g^{-1}$ of glucose, corresponding to 7.16 $g.l^{-1}$ of 1,3-PD, and 95% of the initial glycerol present in the broth was consumed.

Table 7.1. Batch cultures of *C. acetobutylicum* DG1 (pSPD5) on yeast broth from SD medium, at pH 6.5, 35ºC and 100 rpm.

| Yeast strain used in first fermentation stage | HC42 | FH100 |
|---|---|---|
| Sugar concentration ($g.l^{-1}$) | 22.0 | 103.0 |
| Initial glycerol concentration ($g.l^{-1}$) | 13.4 | 51.8 |
| Residual glycerol ($g.l^{-1}$) | 0.77 | 5.95 |
| 1,3-PD concentration ($g.l^{-1}$) | 7.16 | 25.5 |
| 1,3-PD yield ($g.g^{-1}$ of glycerol) | 0.57 | 0.56 |
| 1,3-PD overall yield ($g.g^{-1}$ of sugar) | 0.25 | 0.24 |
| 1,3-PD productivity ($g.l^{-1}.h^{-1}$) | 0.12 | 0.16 |

In glucose broth, it was possible to cultivate *C. acetobutylicum* on 103 $g.l^{-1}$ of initial glucose concentration, reaching 25.5 $g.l^{-1}$ of 1,3-PD which represents a yield of 0.56 $g.g^{-1}$ of glycerol. The residual glycerol was 5.95 $g.l^{-1}$ which means that 88% of the glycerol present in the broth was consumed. The 1,3-PD productivity obtained was 0.16 $g.l^{-1}.h^{-1}$. The fermentation took 141 hours to reach the highest 1,3-PD concentration (Fig. 7.3.). The increase in the acetate amount in the medium was insignificant and the second main product of fermentation was butyrate, reaching a final concentration of 6.7 $g.l^{-1}$.

The 1,3-PD overall yield was the same for both sugar concentrations, reaching 0.24 - 0.25 $g.g^{-1}$ of glucose.

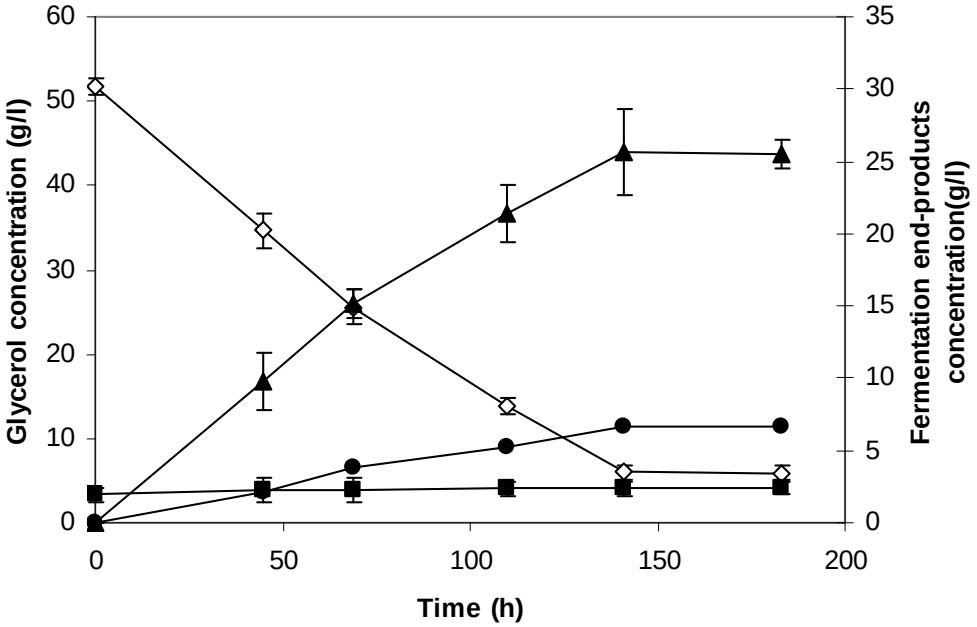


Figure 7.3. 1,3-Propanediol production on yeast broth from glucose (103 $g.l^{-1}$) fermentation. Second stage with *C. acetobutylicum* under anaerobic conditions. ◊ - glycerol, ▲- 1,3-propanediol, ■ – acetate, ● – butyrate.

## 7.2.2. 1,3-Propanediol production on yeast broth from sugar cane molasses fermentation

The possibility of using sugar cane molasses to produce 1,3-PD was also evaluated in this work. Various sugars initial concentrations were tested, leading to culture broths with different glycerol concentrations (Table 7.2.).

Table 7.2. Batch cultures of *C. acetobutylicum* DG1 (pSPD5) on yeast broths from sugar cane molasses, at pH 6.5, 35ºC and 100 rpm.

| Yeast strain used in first fermentation stage | HC42 | FH100 | | |
|---|---|---|---|---|
| Molasses Total Sugars ($g.l^{-1}$) | 27.6 | 56.2 | 77.8 | 101.3 |
| Initial glycerol concentration ($g.l^{-1}$) | 14.0 | 27.4 | 37.8 | 48.1 |
| Residual glycerol ($g.l^{-1}$) | 0.73 | 2.10 | 14.9 | 40.3 |
| 1,3-PD concentration ($g.l^{-1}$) | 7.63 | 14.6 | 12.7 | 4.30 |
| 1,3-PD yield ($g.g^{-1}$ of glycerol) | 0.57 | 0.58 | 0.55 | 0.55 |
| 1,3-PD overall yield ($g.g^{-1}$ of sugar) | 0.26 | 0.26 | 0.18 | 0.04 |
| 1,3-PD productivity ($g.l^{-1}.h^{-1}$) | 0.12 | 0.15 | 0.05 | 0.08 |

*C. acetobutylicum* DG1 (pSPD5) was able to grow on culture broths from sugar cane molasses; however, significant differences were observed in the range of sugar concentrations tested. For low initial sugar concentration, 27.6 $g.l^{-1}$ of molasses TS, i.e. 14.0 $g.l^{-1}$ of glycerol, results were similar to those achieved in yeast broth from SD medium. 1,3-PD yield was 0.57 $g.g^{-1}$ of glycerol, corresponding to 7.63 $g.l^{-1}$ of 1,3-PD, and around 95% of the initial glycerol was consumed. The maximum 1,3-PD production from yeast fermentation molasses broth was obtained with an initial TS concentration of 56.2 $g.l^{-1}$. In these conditions 14.6 $g.l^{-1}$ of 1,3-PD were reached, with a productivity of 0.15 $g.l^{-1}.h^{-1}$ and an overall yield of 0.26 $g.g^{-1}$ sugar. This fermentation was carried out for 95 hours; 3.4 $g.l^{-1}$ of butyrate and 1.22 $g.l^{-1}$ of acetate were also produced (Fig. 7.4.).

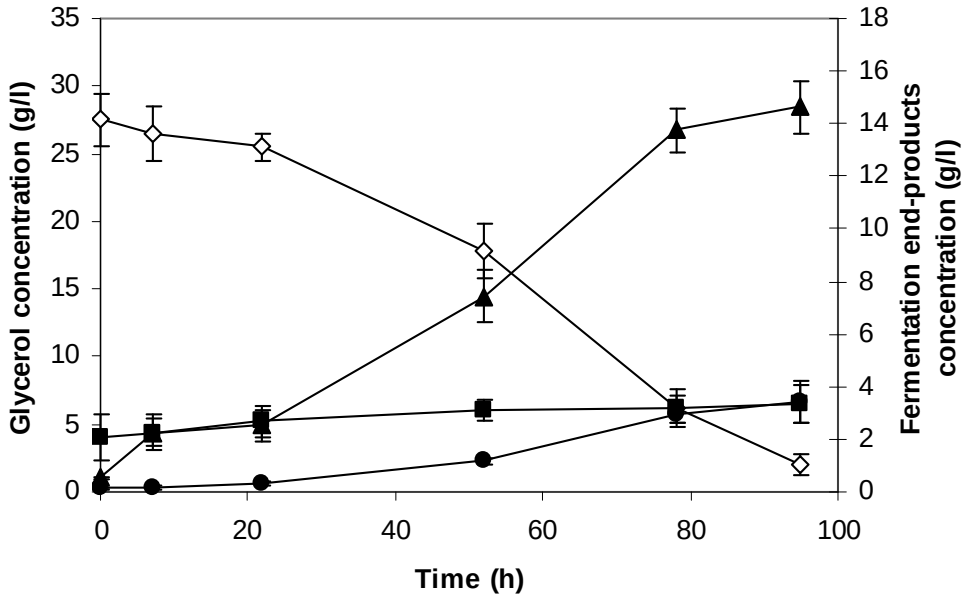


Figure 7.4. 1,3-Propanediol production on yeast broth from sugar cane molasses (56.2 $g.l^{-1}$) fermentation. Second stage with *C. acetobutylicum* under anaerobic conditions. ◊ - glycerol, ▲- 1,3-propanediol, ■ – acetate, ● – butyrate.

However, when higher sugar concentrations were used in the first stage, an inhibitory effect on bacteria's growth was found and a considerable residual glycerol concentration was observed in the medium. For initial TS concentrations of 77.8 and 101.3 $g.l^{-1}$, corresponding to 37.8 and 48.1 $g.l^{-1}$ glycerol in the broth, only 60.5% and 16% of glycerol were respectively consumed.

## 7.3. Discussion and conclusions

Production of 1,3-propanediol was carried out by inoculating the engineered strain *C. acetobutylicum* DG1 (pSPD5) on yeast fermentation broth

that contained glycerol obtained from glucose or sugar cane molasses conversion. Various initial concentrations of carbon sources, originating broths with different glycerol concentrations, were tested in batch fermentation. It was found that direct inoculation of *C. acetobutylicum* DG1 (pSPD5) into the yeast broth successfully converted glycerol previously produced to 1,3-PD. The procedure only required degassing of the medium by sparging sterile $O_2$-free nitrogen for 8-10 hours to assure the strict anaerobic conditions needed for *C. acetobutylicum* growth. However, the process only operated efficiently when using a synthetic growth medium with up to 100 g glucose per litre, or a molasses medium with up to 56.2 $g.l^{-1}$ of sugar, but not on medium with higher concentrations of molasses. In this latter situation, the rate of glycerol conversion to 1,3-PD was strongly inhibited, suggesting the presence of inhibitors of *C. acetobutylicum* growth in sugar molasses. This can be due to the high levels of salt, as it was shown in a previous work using blackstrap molasses for the production of acetone and butanol by *C. acetobutylicum*; the presence of a high level of salts in the molasses affected the microorganism's growth and the final products concentration (Fouad *et al*., 1982). Jiang *et al*. (2009) also described that molasses contain considerable amounts of metal ions like calcium, zinc, sodium, iron, magnesium, manganese, copper, etc, and suspended colloids that may cause critical problems during fermentation as they can impair microorganism's growth, influence substrate pH, and are involved in the inactivation of enzymes associated to product biosynthesis. Furthermore, melanoidins and other pigments present in molasses, which are toxic and hardly

decomposed compounds (Chandra *et al.* 2008), may also affect glycerol conversion to 1,3-PD by *C. acetobutylicum*.

*C. butyricum* was able to ferment glycerol from different industrial sources with high efficiency; a 1,3-PD yield of 0.49 $g.g^{-1}$ was obtained on glycerol coming from concentrated wine stillage and no significant inhibitory effect on cells growth was noticed; moreover, 63.4 $g.l^{-1}$ of 1,3-PD were achieved on glycerol issued from industrial ester production (Barbirato *et al.* 1998).

In the present work, best results were obtained with an initial glucose concentration of 103 $g.l^{-1}$, leading to a final 1,3-propanediol concentration of 25.5 $g.l^{-1}$ and yields of 0.56 $g.g^{-1}$ glycerol (0.67 $mol.mol^{-1}$) and 0.24 $g.g^{-1}$ glucose. Similar results were reported by González-Pajuelo *et al*. (2005) with the same strain, in continuous cultures with commercial (87%) and raw glycerol (65%). The 1,3-PD yield obtained was 0.64 $mol.mol^{-1}$ and 0.61 $mol.mol^{-1}$ in commercial and in raw glycerol respectively. Papanikolaou *et al.* (2008) obtained a 47.1 $g.l^{-1}$ final concentration of 1,3-PD, with a yield of 0.53 $g.g^{-1}$, in a batch fermentation of 90 $g.l^{-1}$ of raw glycerol by *C. butyricum* F2b. In chemostat cultures the same strain was able to produce 48 $g.l^{-1}$ of 1,3-PD from raw glycerol, with similar yield and a volumetric productivity of 0.96 $g.l^{-1}.h^{-1}$; 1,3-PD global productivity was improved to 3.4 $g.l^{-1}.h^{-1}$ in a two-stage steady-state continuous fermentation of raw glycerol and the 1,3-PD concentration remained at 41- 46 $g.l^{-1}$ (Papanikolaou *et al*. 2000). The two-stage continuous culture strategy was also applied by Boenigk *et al.* (1993) to the conversion of commercial glycerol to 1,3-PD by *Citrobacter freundii* DSM 30040, reaching a final concentration of 41 $g.l^{-1}$ and a global

productivity of 1.38 $g.l^{-1}.h^{-1}$. These productivity values are higher than those obtained with the novel two-step process used in this work.

In a previous work, where *Klebsiella pneumoniae* was directly inoculated into yeast broth in batch cultivation, Cheng *et al*. (2006) reported a 1,3-PD productivity of 0.89 $g.h^{-1}.l^{-1}$ and a yield of 0.36 $g.g^{-1}$ glycerol which represented a final 1,3-PD concentration of 21.6 $g.l^{-1}$. In the same work, using a fed-batch process with electrodialytically pre-treated glycerol broth as substrate, 53 $g.l^{-1}$ of 1,3-PD were achieved with a yield of 0.41 $g.g^{-1}$ glycerol and a productivity of 0.94 $g.l^{-1}.h^{-1}$. Hartlep *et al*. (2002) also described a two-step batch process for 1,3-PD production from glucose. In this case, *K. pneumoniae* was directly inoculated into *E. coli* glycerol broth to obtain 14.1 $g.l^{-1}$ 1,3-PD with a yield of 0.53 $g.g^{-1}$ of glycerol and a productivity of 2.0 $g.l^{-1}.h^{-1}$. The overall 1,3-PD yield from glucose in the two-step process was 0.17 $g.g^{-1}$. The overall yield obtained in our work was higher than those previously published using a two step process, either on glucose or molasses broth, reaching up to 0.24 $g.g^{-1}$ - 0.26 $g.g^{-1}$ respectively, but the productivity was lower. The bacteria strain used in this work may be too sensitive to inhibitors present in molasses broth. A treatment like sulphuric acid treatment method could be tried to remove heavy metals from molasses.

*Chapter 8*

# General conclusions and future work

## 8.1. *Saccharomyces cerevisiae* as a glycerol overproducer

One of the purposes of this work was to explore an alternative metabolic engineering strategy for glycerol overproduction by *S. cerevisiae* to those previously reported (Campagno *et al.*, 1996; Nevoigt and Stahl, 1996; Overkamp *et al.*, 2002; Remize *et al.*, 1999) and to evaluate the consequences of this strategy at physiologic, metabolic and genomic levels. The strategy followed involved the overexpression of *GPD1* and *ALD3* combined with the deletion of *TPI1* and *ADH1*. It resulted in an engineered strain with a high glycerol performance in terms of final yield, 0.46 $g.g^{-1}$ glucose, and maximal productivity rate, 3.1 $g.g.h^{-1}$. This mutant strain was significantly better than the previous one reported by Overkamp *et al.* (2002) using the same strain background. In that work, the adopted strategy promoted glycerol production by preventing regeneration of NADH by mitochondrial NADH dehydrogenase through deletion of *NDI1*, *NDI2* and *GUT2* in a *tpi1* mutant. Since in both strategies *TPI1* was deleted, the higher glycerol performance in the strategy applied in the present study can be reasonably explained by faster $NADH/NAD^+$ recycling due to the 10-fold increase in GPD activity. Also, in both cases, the growth defect of a *tpi1Δ* strain was recovered. A previous report showed that the growth defect of this mutant on glucose was due to its inositol-defective phenotype. This deficiency is due to the potent inhibition of MIP synthase that catalyzes the synthesis of inositol-6-P from glucose-6-P, by DHAP and glycerol-3-P, which accumulate to very high levels in a *tpi1Δ* mutant (Shi *et al.*, 2005). In the present work, it was found that the inositol auxotrophy of the mutant *tpi1Δ* was fully alleviated by overexpression of

*GPD1* which subsequently reduced DHAP and G3P levels. However, the levels of DHAP and glycerol-3-P did not return to those of a wild type strain, suggesting either a problem of NADH recycling or that the rate-limiting step of glycerol production was no longer at the level of GPD enzyme. The fact that a large accumulation of intracellular glycerol was observed in the engineered strain indicated that the control of glycerol synthesis was transferred from GPD to glycerol efflux. In yeast, the major glycerol facilitator is encoded by *FPS1* (Luyten *et al.,* 1995; Tamas *et al*., 1999). By overexpressing *FPS1* in HC42 strain, intracellular glycerol accumulation could be reduced twofold. However, this reduction of intracellular glycerol had a minor effect on glycerol productivity. This may be explained by the fact that high internal levels of glycerol can mediate the closure of Fps1p (Karlgren *et al.,* 2005). Alternatively, it is possible that other aquaglyceroporins are also required for glycerol efflux ( Luyten *et al.,* 1995; Petterson *et al.,* 2005). Accumulation of intracellular glycerol has been also reported to trigger a Hog1-dependent osmoprotective pathway (Albertyn *et al.*, 1994a; Siderius *et al.,* 2000), which ultimately leads to a slow down of glycolysis and growth rate by a yet uncharacterized feedback mechanism (Blomberg, 2000). As a consequence, the removal of glycerol along its own production should be considered as a pragmatic method to enhance its productivity.

Other constraints found in the strain HC42 were the reduction of NADH pools and the increase of the production of toxic compounds such as acetaldehyde. To eliminate acetaldehyde, yeast mutant used the alternative

pathways acetoin/2,3-butanediol production. Higher levels of acetoin were found in the engineered mutant strain HC42.

Besides these metabolic bottlenecks, other characteristics of the new strain, that may hamper glycerol production, were revealed by transcriptomic analysis. A major trait identified in this global genome analysis was a significant upregulation of genes encoding $NAD^+$/$NADP^+$ binding proteins and aldehyde dehydrogenases (*i.e. ARA1*, *NDI1*, *GDH2*, *ALD4*, *PDH1*, *ALD2*, etc). The $NAD^+$-dependent glycerol dehydrogenase activity, that converts glycerol into dihydroxyacetone (DHA), was also increased. Altogether, these metabolic and transcriptomic perturbations may have at least two negative effects on glycerol production, namely a possible withdrawing of NADH from glycerol 3-P dehydrogenase enzyme and a wasteful glycerol cycle (Molin *et al.,* 2003). A partial derepression of the glucose-repressive genes belonging to the TCA cycle and respiratory activity was also detected.

To summarize, in this part of the work, a high glycerol producer strain was generated, exhibiting a high glycerol yield and productivity; however, as expected, this genetic engineering strategy incidentally caused several secondary effects that could not be predicted by metabolic reconstruction modelling (Blank *et al.,* 2005). Furthermore, future work with the new mutant strain can be suggested:

1. Expression of other *ALD* gene to reduce the accumulation of acetaldehyde. The overexpression of *ALD3* in the strain HC42 did not contribute to increase the acetate production and, consequently, to reduce the acetaldehyde concentration. Besides *ALD3*, there are four other aldehyde dehydrogenases (*ALD2*, *ALD3*, *ALD5* and *ALD6*) in *S. cerevisiae*. The microarray results showed the upregulation of *ALD2* and *ALD4* in strain HC42. The direct overexpression of these genes by genetic engineering may increase the conversion of acetaldehyde into acetate.

2. Elimination of interfering $NAD^+$ regenerating reaction that can compete with the glycerol pathway, since NADH is required to the conversion of dihydroxyacetone phosphate into glycerol-phosphate.

3. Co-feed engineered strain with another substrate that acts as a direct source of cytosolic NADH. To compensate the reduction of NADH pools found in HC42 strain, the use of an external source of NADH, like formic acid, should be tested.

## 8.2. 1,3-Propanediol production in a two-stage process

In order to implement a two-stage process for 1,3-propanediol production from glucose, using at the first stage a metabolic engineered yeast strain for glycerol production, followed by an engineered *Clostridium* strain to convert it to

1,3-PD at the second stage, two major issues had to be solved. A first one was to optimise the conditions for maximal glycerol production from high sugar concentration and the second one was to verify whether conversion of glycerol to 1,3-PD was possible by direct inoculation of the bacterial strain in the fermentation broth. It was found that the major parameter that could impair efficient fermentation of glucose into glycerol was the agitation rate. It is known that agitation rate favours oxygen supply to the cells, and in this study this can be the cause of the low glucose uptake rate and the 3.5 fold increase of cultivation time, when the agitation rate was 300 rpm. Increasing aeration rate resulted in a slight enhancement of biomass production that was accompanied by a reduction of the glucose uptake rate and of ethanol and acetate yields. This indicates that oxygen was more available to cells to stimulate the aerobic metabolism resulting in higher ATP availability for biomass production. Interestingly, this glycerol engineered *S. cerevisiae* mutant strain exhibits a growth rate twofold lower than the wild type and hence its global physiology may be more sensitive to oxygen availability than the isogenic wild type.

The use of high sugar concentrations in the cultivation medium was an obvious choice to enhance the production of glycerol. However, glycerol-engineered *S. cerevisiae* HC42 was found unable to grow in the presence of more than 20 $g.l^{-1}$ of glucose. In this study, adapted strains that were able to grow in sugar medium containing up to 200 $g.l^{-1}$ glucose were isolated by selecting spontaneous clones able to grow on 100 and 200 $g.l^{-1}$ glucose. Yeast strains HC42

and FH100 were able to grow on sugar cane molasses, but only FH100 was able to grow on 20% ($w.v^{-1}$) molasses, which contained up to 100 $g.l^{-1}$ total sugars. The production of glycerol reached was 47.1 $g.l^{-1}$ with a yield of 0.47 - 0.48 $g.g^{-1}$ of sugar. This adapted strain showed reduced growth rate only at high sugar concentration, either because of the high osmotic pressure of these media or because of an inhibitory effect due to some by-products, like acetaldehyde, since the amount of acetaldehyde in these culture conditions could reach up to 0.6 – 1.0 $g.l^{-1}$. This concentration is within the range showed to exert inhibition (Heux *et al*., 2006; Remize *et al*., 1999; Remize *et al*., 2001). Also, in a previous work with *S. cerevisiae* the increase in molasses concentration resulted in a significant decrease of growth rate, biomass yield and ethanol productivity, although with a higher range of concentrations tested (from 96.7 $g.l^{-1}$ till 323.5 $g.l^{-1}$) (Atiyeh and Duvnjak, 2003).

Concerning the second issue, we found that direct inoculation of the engineered strain *C. acetobutylicum* DG1 (pSPD5) into the yeast culture broth successfully converted glycerol previously produced by yeast into 1,3-PD. This only required the degassing of the medium by sparging sterile $O_2$-free nitrogen for several hours to assure the strict anaerobic conditions needed for *C. acetobutylicum* growth. However, the process only operated efficiently using a synthetic growth medium with up to 100 $g.l^{-1}$ glucose, or a molasses medium with up to 56.2 $g.l^{-1}$ of sugar, but not on media with higher concentrations of sugar molasses. In this latter situation, the rate of conversion of glycerol into 1,3-PD

was strongly inhibited suggesting the presence of inhibitors of *C. acetobutylicum* fermentation in sugar molasses. This can be due to the high levels of salts as it was shown in a previous work using blackstrap molasses for the production of acetone and butanol by *C. acetobutylicum* (Fouad *et al*., 1982). It is also described by Jiang *et al*. (2009) that molasses contain considerable amounts of metal ions and also suspended colloids that can cause critical problems during fermentation as they can impair microorganism's growth, influence substrate pH, and are involved in the inactivation of enzymes associated to product biosynthesis. Furthermore, melanoidins and other pigments present in molasses, which are toxic and hardly decomposed compounds (Chandra *et al*., 2008), may also affect glycerol conversion to 1,3-PD by *C. acetobutylicum*. In the present work, best results were obtained with an initial glucose concentration of 103 $g.l^{-1}$, leading to a final 1,3-propanediol concentration of 25.5 $g.l^{-1}$ and yields of 0.56 $g.g^{-1}$ of glycerol (0.67 $mol.mol^{-1}$) and 0.24 $g.g^{-1}$ glucose. To our knowledge the 1,3-PD overall yield obtained in this work was higher than those previously reported using a two step process, either on glucose or molasses broth, reaching up to 0.24 $g.g^{-1}$ - 0.26 $g.g^{-1}$ respectively, but the productivity was lower. The bacterial strain may be too sensitive to inhibitors present in molasses broth.

The strategy followed in this work showed the possibility of using renewable feedstocks as carbon sources for the production of 1,3-PD. The fact that two stages were developed, using genetically modified strains of *Saccharomyces cerevisiae* for the conversion of sugar into glycerol and of

*Clostridium acetobutylicum* for the production of 1,3-PD from glycerol, shows that this process is flexible, although it still requires optimisation. It is possible to choose operating either on sugar or glycerol feedstocks, which is a very suitable characteristic in our days, due to fluctuations of the market prices of substrates.

Based on these conclusions future work can be suggested:

1. Use of other evolutionary engineering strategy for strain development to obtain a more performant *S. cerevisiae* strain adapted to higher sugar concentrations. The strain obtained in this work was not able to recover the growth rate of the original wild type strain. Strategies like Sequencing Batch Reactor using either gradual or fast increases of glucose concentration in the medium could result in a better performing strain able to grow faster on higher sugar concentrations.

2. Microarrays analysis of FH100 compared to HC42 to better understand the differences between the two strains. This study can contribute to investigate the phenotype changes found in strain FH100 when compared to its wild type HC42. This analysis can also provide guidelines to possible metabolic engineering strategies for improving strain FH100 performance.

3. Study of the growth inhibition of *C. acetobutylicum* in molasses medium.

4. It is possible that the inhibition of *C. acetobutylicum* growth on molasses media is caused by the presence of heavy metals. A treatment like the sulphuric acid treatment method could be implemented to remove those heavy metals from molasses.

# List of figures

# List of tables

# Symbols and abbreviations

ADH - alcohol dehydrogenase

ADP - adenosine diphosphate

ALD - acetaldehyde dehydrogenase

ATP - adenosine triphosphate

BSA - Bovine serum albumine

CFU - Colony Forming Unit

$CO_2$ - carbon dioxide

CoA - coenzyme A

$CoCl_2.6H_2O$ - cobalt (II) chloride hexahydrate

DAK - dihydroxyacetone kinase

DNA - deoxyribonucleic acid

DHAP - dihydroxyacetone phosphate

DTT - dithiotreitol

DW - dry weight

EDTA - ethylene diamine tetra-acetate

$FAD^+$ - Flavin adenine dinucleotide

Fd - ferredoxin

$Fd_{ox}$ - oxidised ferredoxin

$Fd_{red}$ - reduced ferredoxin

$FeSO_4.7H_2O$ - ferrous sulfate hepta-hydrate

FPS - plasma membrane glycerol channel

GDH - glycerol dehydrogenase

GO - Gene Ontology

G3P - glycerol 3-phosphate

G6PD - glucose-6-phosphate dehydrogenase

GPD - glycerol phosphate dehydrogenase

GPI - Glucose-6-phosphate isomerase

GPP - glycerol phosphate phosphatase

GUP - glycerol uptake protein

GUT - glycerol kinase

GCY - putative $NADP^{(+)}$ coupled glycerol dehydrogenase

$H_2$ - hydrogen

$H_2O$ - water

3-HPA - 3-hydroxypropionaldehyde

HPLC - High-performance liquid chromatography

INSA - Institut National des Sciences Appliquées

KCl - potassium chloride

$K_2HPO_4$ - Dipotassium phosphate

$KH_2PO_4$ - Potassium dihydrogen phosphate

Leu - Leucine

LiAc - lithium acetate

MIP - *myo*-inositol-3-phosphate

MIPS - Munich Information Center for Protein Sequences

$MgCl_2$ - magnesium chloride

$MgSO_4.7H_2O$ - magnesium sulfate heptahydrate

NaCl - sodium chloride

$Na_2CO_3$ - sodium carbonate

$NAD^+$ - nicotinamide adenine nucleotide

$NADP^+$ - nicotinamide adenine nucleotide phosphate

NADH - reduced nicotinamide adenine nucleotide

NADHP - reduced nicotinamide adenine nucleotide phosphate

NaOH - sodium hydroxide

NDE - mitochondrial external NADH dehydrogenase

$(NH_4)_2SO_4$ - Ammonium Sulphate

$O_2$ - oxygen

OD - Optical density

P - inorganic phosphate

PCR - Polymerase Chain Reaction

1,3-PD - 1,3-propanediol

PDC - pyruvate dacarboxilase

PEG - polyethylene glycol

PET - polyethylene terephthalate

PGI - glucose-6-phosphate isomerase

PYK - pyruvate kinase

PTT - polytrimethylene terephthalate

RNA - Ribonucleic acid

Q - specific rate

SD - Synthetic Defined Medium

TPI - triose phosphate isomerase

TS - total sugars

Ura - uracile

UV - ultraviolet light

$\mu_{max}$ - maximum growth rate

Y - yield

YPD - Yeast peptone dextrose medium

YM -Yeast Malt Broth

# Identification of abbreviations of genera names

*B. - Bacillus*

*C. - Clostridium*

*D. - Dunaliella*

*E. - Escherichia*

*K. - Klebsiella*

*L. - Lactobacillus*

*S.- Saccharomyces*

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# List of differentially expressed genes between HC42 and CEN.PK2

Table 1- List of 384 differentially expressed genes between HC42 and CEN.PK2, from which 184 were down-regulated and 200 were up-regulated. Sorting criterion: *Functional category* in descending order

| Gene id | Ratio |
|---|---|
| ***UNCLASSIFIED PROTEINS** | |
| YBR191WA | 0.44 |
| YER135C | 0.49 |
| YLR122C | 0.51 |
| YDL196W | 0.54 |
| YER121W | 0.56 |
| YDL152W | 0.57 |
| YLR262CA | 0.58 |
| YDR051C | 0.58 |
| YMR157C | 0.61 |
| YLR446W | 0.61 |
| YCR097WA | 0.62 |
| YPL184C | 0.62 |
| YKL107W | 0.62 |
| YPL208W | 0.63 |
| YLR201C | 0.65 |
| YBR134W | 0.65 |
| YCR082W | 0.65 |
| YLR199C | 1.5 |
| YIL102C | 1.51 |
| YBR232C | 1.51 |
| YPR195C | 1.51 |
| YPL068C | 1.53 |
| YIR020C | 1.54 |
| YOL118C | 1.54 |
| YGR025W | 1.55 |
| YHR218W | 1.56 |
| YML050W | 1.56 |
| SYP1 | 1.58 |
| YNR077C | 1.58 |
| YGL138C | 1.61 |
| YGL024W | 1.62 |

| Gene id | Ratio |
|---|---|
| YJR023C | 1.64 |
| YJL052CA | 1.65 |
| YFL063W | 1.65 |
| YGR051C | 1.66 |
| YOL159C | 1.71 |
| YPL199C | 1.71 |
| YER182W | 1.71 |
| YAL069W | 1.71 |
| YLR402W | 1.74 |
| YMR103C | 1.75 |
| YOR215C | 1.76 |
| YKL147C | 1.79 |
| YDR274C | 1.8 |
| YHR217C | 1.8 |
| YNL195C | 1.81 |
| YPL185W | 1.89 |
| YDR438W | 1.94 |
| YAL037W | 1.94 |
| YDR314C | 1.95 |
| YLR041W | 1.98 |
| YGL258WA | 1.99 |
| YPR044C | 2 |
| YJR162C | 2.08 |
| YNL337W | 2.09 |
| YIL054W | 2.13 |
| BOP2 | 2.18 |
| YKR018C | 2.43 |
| YDR034WB | 2.43 |
| YBR116C | 2.46 |
| Q0297 | 2.95 |
| YDL026W | 3.05 |
| ***CELL TYPE DIFFERENTIATION** | |
| **+fungal/microorganismic cell type differentiation** | |
| **-fungal and other eukaryotic cell type differentiation** | |
| **development of asco- basidio- or zygospore** | |
| IRA1 | 0.55 |
| ECM33 | 0.66 |

| Gene id | Ratio |
|---|---|
| EMI5 | 1.56 |
| YLR054C | 1.58 |
| SPT3 | 1.64 |
| SMA1 | 1.72 |
| NEM1 | 1.92 |
| **hyphae formation** | |
| ACH1 | 2.1 |
| **budding, cell polarity and filament formation** | |
| CLB2 | 1.53 |
| SPT3 | 1.64 |
| KSS1 | 1.77 |
| HSP150 | 1.81 |
| ***BIOGENESIS OF CELLULAR COMPONENTS** | |
| **+vacuole or lysosome** | |
| VAM10 | 0.55 |
| VTC4 | 0.58 |
| PBI2 | 1.92 |
| **+peroxisome** | |
| PEX17 | 1.79 |
| PEX28 | 1.93 |
| ADR1 | 2.21 |
| **+mitochondrion** | |
| FUM1 | 1.63 |
| MRPL13 | 1.75 |
| MBR1 | 1.9 |
| ALD4 | 1.95 |
| PGS1 | 2.09 |
| VAR1 | 2.09 |
| RPM2 | 2.17 |
| NDI1 | 3.37 |
| **+nucleus** | |
| **-nuclear membrane** | |
| NUP120 | 0.6 |
| NUP1 | 3.93 |
| **-organization of chromosome structure** | |
| SPT3 | 1.64 |
| HPA3 | 2.38 |

**List of differentially expressed genes between HC42 and CEN.PK2**

| Gene id | Ratio |
|---|---|
| **+nucleus** | |
| NEM1 | 1.92 |
| **+cytoskeleton** | |
| **-actin cytoskeleton** | |
| VIP1 | 1.51 |
| **+cell wall** | |
| IRS4 | 0.46 |
| QRI1 | 0.57 |
| ECM30 | 0.59 |
| ECM25 | 0.66 |
| ECM33 | 0.66 |
| RPC34 | 1.51 |
| YLR054C | 1.58 |
| ECM7 | 1.62 |
| KTR6 | 1.72 |
| YBR005W | 1.72 |
| HSP150 | 1.81 |
| PGS1 | 2.09 |
| PIR3 | 2.1 |
| RER2 | 2.13 |
| ***DEVELOPMENT (Systemic)** | |
| **+fungal/microorganismic development** | |
| **-mating (fertilization)** | |
| SPT3 | 1.64 |
| A1 | 2.08 |
| ***CELL FATE** | |
| **+cell aging** | |
| ATP2 | 1.81 |
| **+cell growth / morphogenesis** | |
| CPR7 | 1.6 |
| ***TRANSPOSABLE ELEMENTS, VIRAL AND PLASMID PROTEINS** | |
| YLR256WA | 1.53 |
| YOR343WA | 1.56 |
| YOL103WA | 1.99 |
| YDR170WA | 2.06 |
| YML045WA | 2.09 |
| YNL284CA | 2.11 |

| Gene id | Ratio |
|---|---|
| YDR210CC | 2.3 |
| ***INTERACTION WITH THE CELLULAR ENVIRONMENT** | |
| **+cellular sensing and response** | |
| **-temperature perception and response** | |
| HSP12 | 3.1 |
| **-chemoperception and response** | |
| **osmosensing** | |
| HSP12 | 3.1 |
| GPD1 | 4.14 |
| **pheromone response, mating-type determination, sex-specific proteins** | |
| MF(ALPHA)1 | 0.12 |
| RFA1 | 0.42 |
| RPS1A | 0.47 |
| ORC4 | 1.56 |
| SAN1 | 1.6 |
| SPT3 | 1.64 |
| KSS1 | 1.77 |
| A1 | 2.08 |
| **perception of nutrients and nutritional adaptation** | |
| IRA1 | 0.55 |
| PTK1 | 1.67 |
| **+cell adhesion** | |
| HSP12 | 3.1 |
| **+ionic homeostasis** | |
| **-homeostasis of anions** | |
| **homeostasis of phosphate** | |
| PHO84 | 1.73 |
| **-homeostasis of cations** | |
| **homeostasis of protons** | |
| VMA7 | 0.61 |
| VMA2 | 0.66 |
| VMA6 | 0.66 |
| ATP3 | 1.67 |
| ATP14 | 1.72 |
| PHO84 | 1.73 |
| ATP2 | 1.81 |

**List of differentially expressed genes between HC42 and CEN.PK2**

| Gene id | Ratio |
|---|---|
| ATP5 | 1.88 |
| AAP1 | 1.98 |
| **homeostasis of metal ions (Na, K, Ca etc.)** | |
| ZRT1 | 0.64 |
| TAF1 | 1.68 |
| SMF1 | 1.79 |
| ***CELL RESCUE, DEFENSE AND VIRULENCE** | |
| **+detoxification** | |
| **-oxygen and radical detoxification** | |
| PRX1 | 1.51 |
| YNR064C | 1.8 |
| **+detoxification** | |
| ATR1 | 1.59 |
| TAF1 | 1.68 |
| LAP3 | 1.8 |
| **+disease, virulence and defense** | |
| **-resistance proteins** | |
| **antibiotic resistance** | |
| LAP3 | 1.8 |
| **+stress response** | |
| **-nutrient starvation response** | |
| ASP3-2 | 0.4 |
| ASP3-1 | 0.56 |
| PHO5 | 1.59 |
| **-DNA damage response** | |
| RFA1 | 0.42 |
| **-unfolded protein response (ER quality control)** | |
| SSB1 | 0.63 |
| CPR7 | 1.6 |
| PDR13 | 2.11 |
| **-heat shock response** | |
| HSP12 | 3.1 |
| **-osmotic and salt stress response** | |
| HSP12 | 3.1 |
| GPD1 | 4.14 |
| **-oxydative stress response** | |
| PRX1 | 1.51 |

| Gene id | Ratio |
|---|---|
| UGA2 | 1.54 |
| HSP12 | 3.1 |
| **+stress response** | |
| PAU4 | 0.64 |
| TPS2 | 1.59 |
| SNO2 | 1.61 |
| HSP150 | 1.81 |
| PIR3 | 2.1 |
| ALD3 | 6.56 |
| ***CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM** | |
| **+transmembrane signal transduction** | |
| MF(ALPHA)1 | 0.12 |
| **+intracellular signalling** | |
| **-enzyme mediated signal transduction** | |
| **G-protein mediated signal transduction** | |
| **small GTPase mediated signal transduction** | |
| IRA1 | 0.55 |
| RIN1 | 1.55 |
| **protein kinase cascades** | |
| **MAPKKK cascade** | |
| KSS1 | 1.77 |
| **protein kinase cascades** | |
| MIH1 | 1.63 |
| **+intracellular signalling** | |
| IRS4 | 0.46 |
| ***CELLULAR TRANSPORT, TRANSPORT FACILITATION AND TRANSPORT ROUTES** | |
| **+Unknown function** | |
| **-transport routes** | |
| **cellular import** | |
| PHO84 | 1.73 |
| SMF1 | 1.79 |
| **cellular export and secretion** | |
| ATR1 | 1.59 |

| Gene id | Ratio |
|---|---|
| YLR440C | 1.7 |
| NCE103 | 1.73 |
| SEL1 | 2.12 |
| **vacuolar transport** | |
| AVT6 | 0.58 |
| APG10 | 0.59 |
| VMA7 | 0.61 |
| VMA2 | 0.66 |
| VMA6 | 0.66 |
| VPS71 | 1.8 |
| **peroxisomal transport** | |
| PEX17 | 1.79 |
| **vesicular transport (Golgi network, etc.)** | |
| **ER to Golgi transport** | |
| COP1 | 0.62 |
| **non-vesicular ER transport** | |
| SSH1 | 1.68 |
| **mitochondrial transport** | |
| ATP3 | 1.67 |
| ATP14 | 1.72 |
| ATP2 | 1.81 |
| ATP5 | 1.88 |
| AAP1 | 1.98 |
| **nuclear transport** | |
| RPS15 | 0.44 |
| NTF2 | 0.55 |
| NUP120 | 0.6 |
| NUP60 | 1.7 |
| NUP1 | 3.93 |
| **-transport routes** | |
| PTK1 | 1.67 |
| **-transport facilitation** | |
| **ABC transporters** | |

| Gene id | Ratio |
|---|---|
| ATR1 | 1.59 |
| AUS1 | 1.84 |
| PDR12 | 2.39 |
| **transport ATPases** | |
| VMA7 | 0.61 |
| VMA2 | 0.66 |
| VMA6 | 0.66 |
| ATP3 | 1.67 |
| ATP14 | 1.72 |
| ATP2 | 1.81 |
| ATP5 | 1.88 |
| AAP1 | 1.98 |
| PDR12 | 2.39 |
| **channel / pore class transport** | |
| **nuclear pore forming protein** | |
| NUP60 | 1.7 |
| **-transport facilitation** | |
| OPT2 | 0.31 |
| SCT1 | 0.53 |
| **-transported compounds (substrates)** | |
| **General** | |
| **drug transport** | |
| ATR1 | 1.59 |
| TAF1 | 1.68 |
| PDR12 | 2.39 |
| **vitamin/cofactor transport** | |
| PHO3 | 1.66 |
| **RNA transport** | |
| NUP120 | 0.6 |
| SUB2 | 0.63 |
| HRP1 | 1.68 |
| NUP1 | 3.93 |
| **electron / hydrogen transport** | |
| VMA7 | 0.61 |
| VMA2 | 0.66 |

| Gene id | | Ratio |
|---|---|---|
| VMA6 | | 0.66 |
| COX4 | | 1.5 |
| SDH4 | | 1.53 |
| COX7 | | 1.55 |
| ATP18 | | 1.56 |
| ATP3 | | 1.67 |
| SDH3 | | 1.67 |
| ATP14 | | 1.72 |
| ATP2 | | 1.81 |
| COX13 | | 1.83 |
| ATP5 | | 1.88 |
| CYT1 | | 1.98 |
| AAP1 | | 1.98 |
| SDH2 | | 3.58 |
| **lipid transport** | | |
| GIT1 | | 1.67 |
| AUS1 | | 1.84 |
| **amine / polyamine transport** | | |
| PTK1 | | 1.67 |
| **protein transport** | | |
| RPS15 | 0.44 | |
| NUP120 | 0.6 | |
| SSH1 | 1.68 | |
| SEL1 | 2.12 | |
| NUP1 | 3.93 | |
| **peptide transport** | | |
| OPT2 | | 0.31 |
| **amino acid transport** | | |
| AVT6 | | 0.58 |
| **C-compound and carbohydrate transport** | | |
| **C4-dicarboxylate transport (e.g. malate, succinate, fumarate)** | | |
| JEN1 | | 2.05 |
| **C-compound and carbohydrate transport** | | |

| Gene id | Ratio |
|---|---|
| PHO84 | 1.73 |
| PDR12 | 2.39 |
| **ion transport** | |
| **anion transport (Cl, SO4, PO4, etc.)** | |
| **phosphate transport** | |
| PHO84 | 1.73 |
| **cation transport (Na, K, Ca , NH4, etc.)** | |
| **heavy metal ion transport (Cu, Fe, etc.)** | |
| **siderophore-iron transport** | |
| TAF1 | 1.68 |
| **heavy metal ion transport (Cu, Fe, etc.)** | |
| ZRT1 | 0.64 |
| PHO84 | 1.73 |
| SMF1 | 1.79 |
| **cation transport (Na, K, Ca , NH4, etc.)** | |
| VMA7 | 0.61 |
| VMA2 | 0.66 |
| VMA6 | 0.66 |
| ATP18 | 1.56 |
| ATP3 | 1.67 |
| ATP14 | 1.72 |
| ATP2 | 1.81 |
| ATP5 | 1.88 |
| AAP1 | 1.98 |
| **+Unknown function** | |
| YNL187W | 1.5 |
| ***PROTEIN ACTIVITY REGULATION** | |
| **+Transcription** | |
| **-target of regulation** | |
| **RNA processing** | |
| **regulator of transcription factor** | |
| SPT3 | 1.64 |
| A1 | 2.08 |

| Gene id | Ratio |
|---|---|
| **regulator of G-protein signalling** | |
| IRA1 | 0.55 |
| **enzymatic activity regulation / enzyme regulator** | |
| **enzyme inhibitor** | |
| **protease inhibitor** | |
| PBI2 | 1.92 |
| **GTPase inhibitor (GIP)** | |
| RIN1 | 1.55 |
| **enzyme inhibitor** | |
| IRS4 | 0.46 |
| **enzyme activator** | |
| **GTPase activator (GAP)** | |
| IRA1 | 0.55 |
| **enzyme activator** | |
| UBA2 | 1.53 |
| **enzymatic activity regulation / enzyme regulator** | |
| CLB2 | 1.53 |
| MIH1 | 1.63 |
| COX13 | 1.83 |
| CIS1 | 1.87 |
| **-mechanism of regulation** | |
| **Other** | |
| **binding / dissociation** | |
| IRS4 | 0.46 |
| PBI2 | 1.92 |
| **modification** | |
| MIH1 | 1.63 |

***conserved hypotheticals with an orthologue in M. bovis**

| Gene id | Ratio |
|---|---|
| **+PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic)** | |
| **-Regulatory functions** | |
| **complex cofactor/cosubstrate binding** | |
| **biotin binding** | |
| DUR1,2 | 0.31 |
| **FE/S binding** | |
| SDH4 | 1.53 |
| SDH3 | 1.67 |
| SDH2 | 3.58 |
| **NAD/NADP binding** | |
| IDH1 | 1.73 |
| ARA1 | 1.91 |
| GPD2 | 2.08 |
| INO1 | 3.27 |
| GPD1 | 4.14 |
| **nucleotide binding** | |
| **ATP binding** | |
| DUR1,2 | 0.31 |
| PRP43 | 0.49 |
| SNU114 | 0.58 |
| SUB2 | 0.63 |
| SSB1 | 0.63 |
| SAN1 | 1.6 |
| AUS1 | 1.84 |
| DED81 | 1.95 |
| PDR12 | 2.39 |
| **nucleotide binding** | |
| FUR1 | 0.61 |
| LAP3 | 1.8 |
| **lipid binding** | |
| OSH7 | 1.76 |
| **structural protein** | |

| Gene id | Ratio |
|---|---|
| NUP120 | 0.6 |
| ATP5 | 1.88 |
| NUP1 | 3.93 |
| **nucleic acid binding** | |
| **Protein interactions** | |
| **RNA binding** | |
| RPL36B | 0.43 |
| RPL28 | 0.52 |
| RPL26B | 0.57 |
| RPL15B | 0.58 |
| RPL15A | 0.64 |
| RPL26A | 0.64 |
| PRP39 | 0.64 |
| RPL36A | 0.65 |
| RPL6A | 0.65 |
| HRP1 | 1.68 |
| **DNA binding** | |
| RFA1 | 0.42 |
| SMP1 | 0.62 |
| HTB2 | 0.63 |
| HHF2 | 0.65 |
| HMO1 | 0.65 |
| RPC34 | 1.51 |
| CDC45 | 1.78 |
| **protein binding** | |
| **Other** | |
| IRS4 | 0.46 |
| NTF2 | 0.55 |
| TRM82 | 0.55 |
| SUB2 | 0.63 |
| SSB1 | 0.63 |
| CPR7 | 1.6 |

| Gene id | Ratio |
|---|---|
| SAN1 | 1.6 |
| OST4 | 1.62 |
| PEX17 | 1.79 |
| PDR13 | 2.11 |
| **Gene id** | **Ratio** |
| NUP1 | 3.93 |
| ***PROTEIN FATE (folding, modification, destination)** | |
| **+Protein synthesis** | |
| **-protein degradation** | |
| **cytoplasmic and nuclear protein degradation** | |
| **proteasomal degradation (ubiquitin/proteasomal pathway)** | |
| RPL40B | 0.46 |
| RPL40A | 0.58 |
| APG10 | 0.59 |
| SAN1 | 1.6 |
| SEL1 | 2.12 |
| **cytoplasmic and nuclear protein degradation** | |
| RPS31 | 0.44 |
| MAP1 | 0.61 |
| UBA2 | 1.53 |
| LAP3 | 1.8 |
| PBI2 | 1.92 |
| **-protein degradation** | |
| YPR151C | 2.44 |
| **-assembly of protein complexes** | |
| RPL10 | 0.64 |
| VMA6 | 0.66 |
| COX7 | 1.55 |
| SAN1 | 1.6 |
| PET191 | 1.78 |
| YLR327C | 2.3 |

| Gene id | Ratio |
|---|---|
| **-protein modification** | |
| **protein processing (proteolytic)** | |
| **autoproteolytic processing** | |
| APG10 | 0.59 |
| **protein processing (proteolytic)** | |
| MAP1 | 0.61 |
| **modification by ubiquitin-related proteins** | |
| APG10 | 0.59 |
| UBA2 | 1.53 |
| **modification by ubiquitination, deubiquitination** | |
| SAN1 | 1.6 |
| **modification by acetylation, deacetylation** | |
| VIP1 | 1.51 |
| SPT3 | 1.64 |
| HPA3 | 2.38 |
| **modification by phosphorylation, dephosphorylation, autophosphorylation** | |
| MIH1 | 1.63 |
| PTK1 | 1.67 |
| SCC2 | 1.69 |
| RTS3 | 1.76 |
| KSS1 | 1.77 |
| **modification with sugar residues (e.g. glycosylation)** | |
| **N-directed glycosylation** | |
| OST4 | 1.62 |
| KTR6 | 1.72 |
| **modification with sugar residues (e.g. glycosylation)** | |
| RER2 | 2.13 |
| **modification with fatty acids (e.g. myristylation, palmitylation, farnesylation)** | |
| ERF2 | 2.71 |

| Gene id | Ratio |
|---|---|
| **-protein targeting, sorting and translocation** | |
| **tRNA and rRNA base modification** | |
| RPS15 | 0.44 |
| NTF2 | 0.55 |
| APG10 | 0.59 |
| NUP120 | 0.6 |
| SSH1 | 1.68 |
| NUP60 | 1.7 |
| SMF1 | 1.79 |
| VPS71 | 1.8 |
| SEL1 | 2.12 |
| ERF2 | 2.71 |
| NUP1 | 3.93 |
| **-protein folding and stabilization** | |
| **Other** | |
| CPR7 | 1.6 |
| **+Protein synthesis** | |
| QRI7 | 1.73 |
| ***PROTEIN SYNTHESIS** | |
| **+Mobile and extrachromosomal element functions** | |
| **-aminoacyl-tRNA-synthetases** | |
| GRS1 | 0.58 |
| DED81 | 1.95 |
| **-translational control** | |
| RPS9A | 0.42 |
| CAM1 | 0.61 |
| RPS2 | 0.63 |
| RPS9B | 0.64 |
| RPL30 | 0.66 |
| **-translation** | |
| **translation elongation** | |
| TEF4 | 0.56 |
| CAM1 | 0.61 |
| RPP1A | 0.65 |

**List of differentially expressed genes between HC42 and CEN.PK2**

| Gene id | Ratio |
|---|---|
| EFB1 | 0.65 |
| **translation initiation** | |
| SUI1 | 0.51 |
| **-translation** | |
| ASC1 | 0.46 |
| SNU114 | 0.58 |
| **-ribosome biogenesis** | |
| **Plasmid functions** | |
| **ribosomal proteins** | |
| RPS21A | 0.37 |
| RPS22A | 0.4 |
| RPL20B | 0.4 |
| RPS7A | 0.41 |
| RPS18A | 0.42 |
| RPS9A | 0.42 |
| RPL12B | 0.43 |
| RPL42A | 0.43 |
| RPL36B | 0.43 |
| RPS29A | 0.44 |
| RPS31 | 0.44 |
| RPS15 | 0.44 |
| RPL27B | 0.44 |
| RPL21A | 0.45 |
| RPS26A | 0.45 |
| RPS8B | 0.46 |
| RPL40B | 0.46 |
| RPS1A | 0.47 |
| RPL37A | 0.47 |
| RPS29B | 0.47 |
| RPL4A | 0.47 |
| RPS19A | 0.47 |
| RPS28A | 0.47 |
| RPS8A | 0.48 |

| Gene id | Ratio |
|---|---|
| RPL13A | 0.48 |
| RPL37B | 0.48 |
| RPS17A | 0.48 |
| RPS28B | 0.49 |
| RPL3 | 0.49 |
| RPL39 | 0.49 |
| RPS26B | 0.5 |
| RPS10A | 0.5 |
| RPL42B | 0.5 |
| RPS17B | 0.5 |
| RPS27A | 0.51 |
| RPS22B | 0.51 |
| RPL7B | 0.51 |
| RPL28 | 0.52 |
| RPL32 | 0.52 |
| RPL9B | 0.52 |
| RPL9A | 0.54 |
| RPS1B | 0.54 |
| RPL31B | 0.54 |
| RPL19A | 0.54 |
| RPL33B | 0.54 |
| RPL33A | 0.54 |
| RPL32 | 0.55 |
| RPL32 | 0.55 |
| RPL32 | 0.55 |
| RPL4B | 0.56 |
| RPL32 | 0.56 |
| RPL32 | 0.56 |
| RPL32 | 0.56 |
| RPS6B | 0.57 |
| RPS30B | 0.57 |
| RPL20A | 0.57 |
| RPL26B | 0.57 |
| RPS16A | 0.58 |
| RPL32 | 0.58 |
| RPL40A | 0.58 |

| Gene id | Ratio |
|---|---|
| RPL15B | 0.58 |
| RPS24B | 0.59 |
| RPL32 | 0.59 |
| RPL13B | 0.6 |
| RPL32 | 0.6 |
| RPL32 | 0.6 |
| RPL17A | 0.6 |
| RPL35B | 0.6 |
| RPS24A | 0.61 |
| RPS6B | 0.61 |
| RPL19B | 0.61 |
| RPS16B | 0.61 |
| RPL41A | 0.61 |
| RPL32 | 0.62 |
| RPL41B | 0.62 |
| RPL34B | 0.63 |
| RPL32 | 0.63 |
| RPS4A | 0.63 |
| RPS4B | 0.63 |
| RPS7B | 0.63 |
| RPS2 | 0.63 |
| RPL15A | 0.64 |
| RPL26A | 0.64 |
| RPL10 | 0.64 |
| RPS6B | 0.64 |
| RPL32 | 0.64 |
| RPS10B | 0.64 |
| RPS9B | 0.64 |
| RPL21B | 0.64 |
| RPP1A | 0.65 |
| RPS6B | 0.65 |
| RPL32 | 0.65 |
| RPL32 | 0.65 |
| RPS30A | 0.65 |
| RPL36A | 0.65 |
| RPL6A | 0.65 |

| Gene id | Ratio |
|---|---|
| RPL22A | 0.65 |
| RPS6B | 0.66 |
| RPS6B | 0.66 |
| RPS6B | 0.66 |
| RPS6B | 0.66 |
| RPL30 | 0.66 |
| RPL43B | 0.66 |
| RPL8B | 0.66 |
| MRPL13 | 1.75 |
| VAR1 | 2.09 |
| **Plasmid functions** | |
| ASC1 | 0.46 |
| YKL056C | 0.56 |
| **+Mobile and extrachromosomal element functions** | |
| SSB1 | 0.63 |
| PDR13 | 2.11 |
| RPM2 | 2.17 |
| ***TRANSCRIPTION** | |
| **-RNA modification** | |
| **tRNA modification** | |
| TRM82 | 0.55 |
| CCA1 | 1.52 |
| **rRNA modification** | |
| DIM1 | 4.15 |
| **-RNA processing** | |
| **mRNA processing (splicing, 5'-, 3'-end processing)** | |
| **3'-end processing** | |
| HRP1 | 1.68 |
| **splicing** | |
| **regulation of splicing** | |
| RPL30 | 0.66 |
| **splicing** | |
| PRP43 | 0.49 |
| SNU114 | 0.58 |
| SUB2 | 0.63 |

| Gene id | Ratio |
|---|---|
| PRP39 | 0.64 |
| HRP1 | 1.68 |
| ISY1 | 1.72 |
| BI2 | 2.07 |
| **mRNA processing (splicing, 5'-, 3'-end processing)** | |
| UBA2 | 1.53 |
| **tRNA processing** | |
| YBR022W | 0.65 |
| RPM2 | 2.17 |
| **rRNA processing** | |
| RPS4A | 0.63 |
| RPL30 | 0.66 |
| DIM1 | 4.15 |
| **-RNA synthesis** | |
| **mRNA synthesis** | |
| **transcriptional control** | |
| SMP1 | 0.62 |
| HTB2 | 0.63 |
| HHF2 | 0.65 |
| ORC4 | 1.56 |
| SRB2 | 1.59 |
| CPR7 | 1.6 |
| SPT3 | 1.64 |
| LAP3 | 1.8 |
| UPC2 | 1.81 |
| MBR1 | 1.9 |
| A1 | 2.08 |
| ADR1 | 2.21 |
| **general transcription activities** | |
| **transcription termination** | |
| GRS1 | 0.58 |

| Gene id | Ratio |
|---|---|
| **general transcription activities** | |
| HMO1 | 0.65 |
| SRB2 | 1.59 |
| SPT3 | 1.64 |
| UPC2 | 1.81 |
| A1 | 2.08 |
| **mRNA synthesis** | |
| HRP1 | 1.68 |
| **tRNA synthesis** | |
| RPC34 | 1.51 |
| **rRNA synthesis** | |
| IRS4 | 0.46 |
| HMO1 | 0.65 |
| RPC34 | 1.51 |
| IDH1 | 1.73 |
| ***CELL CYCLE AND DNA PROCESSING** | |
| **+conserved hypotheticals** | |
| **-Hypothetical proteins** | |
| **cell cycle** | |
| **nuclear and chromosomal cycle** | |
| **chromosome segregation/division** | |
| MTW1 | 1.51 |
| **chromosome condensation** | |
| BRN1 | 1.58 |
| SCC2 | 1.69 |
| **meiosis** | |
| ECM33 | 0.66 |
| SAE2 | 1.78 |
| NEM1 | 1.92 |

| Gene id | Ratio |
|---|---|
| **mitotic cell cycle and cell cycle control** | |
| **cell cycle checkpoints (checkpoints of morphogenesis, DNA-damage,-replication, mitotic phase and spindle)** | |
| RFA1 | 0.42 |
| NIS1 | 1.59 |
| **mitotic cell cycle** | |
| **mitosis** | |
| BRN1 | 1.58 |
| **G2/M transition of mitotic cell cycle** | |
| CLB2 | 1.53 |
| MIH1 | 1.63 |
| SCC2 | 1.69 |
| **mitotic cell cycle** | |
| NIS1 | 1.59 |
| **mitotic cell cycle and cell cycle control** | |
| RPL10 | 0.64 |
| MTW1 | 1.51 |
| GNA1 | 1.51 |
| CDC45 | 1.78 |
| CIS1 | 1.87 |
| PGS1 | 2.09 |
| **DNA processing** | |
| **Domain** | |
| **DNA restriction or modification** | |
| **DNA conformation modification (e.g. chromatin)** | |
| IRS4 | 0.46 |
| HTB2 | 0.63 |
| HHF2 | 0.65 |
| ORC4 | 1.56 |
| SAN1 | 1.6 |
| SPT3 | 1.64 |
| VPS71 | 1.8 |
| HPA3 | 2.38 |

| Gene id | Ratio |
|---|---|
| **DNA recombination and DNA repair** | |
| **DNA recombination** | |
| **meiotic recombination** | |
| SAE2 | 1.78 |
| **DNA recombination** | |
| RFA1 | 0.42 |
| **DNA repair** | |
| RFA1 | 0.42 |
| TDP1 | 1.5 |
| BI2 | 2.07 |
| HSP12 | 3.1 |
| **DNA synthesis and replication** | |
| **extension/ polymerization activity** | |
| RFA1 | 0.42 |
| CDC45 | 1.78 |
| **ori recognition and priming complex formation** | |
| ORC4 | 1.56 |
| CDC45 | 1.78 |
| **DNA topology** | |
| RFA1 | 0.42 |
| **-Hypothetical proteins** | |
| HDR1 | 1.62 |
| ***ENERGY** | |
| **+information pathways** | |
| **-Biosynthesis of cofactors, prosthetic groups, and carriers** | |
| **energy conversion and regeneration** | |
| **energy generation (e.g. ATP synthase)** | |
| ATP18 | 1.56 |
| ATP3 | 1.67 |

| Gene id | Ratio |
|---|---|
| ATP14 | 1.72 |
| ATP2 | 1.81 |
| ATP5 | 1.88 |
| CYT1 | 1.98 |
| AAP1 | 1.98 |
| **energy conversion and regeneration** | |
| ALD2 | 1.57 |
| **oxidation of fatty acids** | |
| POX1 | 1.67 |
| **metabolism of energy reserves (e.g. glycogen, trehalose)** | |
| TPS2 | 1.59 |
| **fermentation** | |
| **alcohol fermentation** | |
| ADH2 | 0.17 |
| ADH1 | 0.43 |
| PDC1 | 0.52 |
| ALD4 | 1.95 |
| **fermentation** | |
| ASC1 | 0.46 |
| ALD2 | 1.57 |
| ATF1 | 1.64 |
| ALD3 | 6.56 |
| **respiration** | |
| **aerobic respiration** | |
| OAR1 | 0.6 |
| COX4 | 1.5 |
| SDH4 | 1.53 |
| MRF1' | 1.54 |
| COX7 | 1.55 |
| SDH3 | 1.67 |
| RIP1 | 1.69 |
| COX13 | 1.83 |
| MBR1 | 1.9 |
| CYT1 | 1.98 |
| NDI1 | 3.37 |
| SDH2 | 3.58 |

| Gene id | Ratio |
|---|---|
| **respiration** | |
| PDC1 | 0.52 |
| ATP18 | 1.56 |
| ATP3 | 1.67 |
| ATP14 | 1.72 |
| PET191 | 1.78 |
| ATP2 | 1.81 |
| ATP5 | 1.88 |
| ALD4 | 1.95 |
| AAP1 | 1.98 |
| **electron transport and membrane-associated energy conservation** | |
| **accessory proteins of electron transport and membrane-associated energy conservation** | |
| CYT1 | 1.98 |
| **electron transport and membrane-associated energy conservation** | |
| COX4 | 1.5 |
| SDH4 | 1.53 |
| COX7 | 1.55 |
| ATP18 | 1.56 |
| ATP3 | 1.67 |
| SDH3 | 1.67 |
| RIP1 | 1.69 |
| ATP14 | 1.72 |
| ATP2 | 1.81 |
| COX13 | 1.83 |
| ATP5 | 1.88 |
| AAP1 | 1.98 |
| NDI1 | 3.37 |
| SDH2 | 3.58 |
| **tricarboxylic-acid pathway (citrate cycle, Krebs cycle, TCA cycle)** | |
| **Heme, porphyrin, and cobalamin** | |
| SDH4 | 1.53 |
| FUM1 | 1.63 |
| SDH3 | 1.67 |
| IDH1 | 1.73 |

| Gene id | Ratio |
|---|---|
| GDH2 | 1.92 |
| SDH2 | 3.58 |
| **pentose-phosphate pathway** | |
| **Riboflavin, FMN, and FAD** | |
| TKL1 | 0.45 |
| YPR118W | 0.6 |
| PRS5 | 0.64 |
| **glycolysis and gluconeogenesis** | |
| **Other** | |
| TPI1 | 0.39 |
| CDC19 | 0.55 |
| ***METABOLISM** | |
| **+lipid metabolism** | |
| **-Amino acid biosynthesis** | |
| **secondary metabolism** | |
| **biosynthesis of secondary products derived from primary amino acids** | |
| **biosynthesis of nonprotein amino acids** | |
| ALD2 | 1.57 |
| ALD3 | 6.56 |
| **biosynthesis of derivatives of dehydroquinic acid, shikimic acid and chorismic acid** | |
| **biosynthesis of ubiquinone** | |
| COQ3 | 2.29 |
| **biosynthesis of acetic acid derivatives** | |
| ATF1 | 1.64 |
| **metabolism of primary metabolic sugars derivatives** | |
| **biosynthesis of secondary monosaccharides** | |
| INO1 | 3.27 |

| Gene id | Ratio |
|---|---|
| **utilization of vitamins, cofactors, and prosthetic groups** | |
| PHO5 | 1.59 |
| PHO3 | 1.66 |
| **biosynthesis of vitamins, cofactors, and prosthetic groups** | |
| RIB1 | 1.53 |
| SNO2 | 1.61 |
| COQ3 | 2.29 |
| **metabolism of vitamins, cofactors, and prosthetic groups** | |
| TAF1 | 1.68 |
| ACH1 | 2.1 |
| **lipid, fatty acid and isoprenoid metabolism** | |
| **regulation of lipid, fatty acid and isoprenoid metabolism** | |
| ACH1 | 2.1 |
| **lipid, fatty acid and isoprenoid utilization** | |
| HSP12 | 3.1 |
| **degradation of lipids, fatty acids and isoprenoids** | |
| **fatty acid degradation (alpha- and beta-oxidation)** | |
| POX1 | 1.67 |
| **degradation of lipids, fatty acids and isoprenoids** | |
| YJL068C | 1.58 |
| **lipid, fatty acid and isoprenoid biosynthesis** | |
| **isoprenoid biosynthesis** | |
| **tetracyclic and pentacyclic triterpenes (cholesterin, steroids and hopanoids) biosynthesis** | |
| ERG2 | 0.45 |
| OSH3 | 1.74 |
| OSH7 | 1.76 |
| **isoprenoid biosynthesis** | |
| RER2 | 2.13 |

| Gene id | Ratio |
|---|---|
| **fatty acid biosynthesis** | |
| OAR1 | 0.6 |
| MRF1' | 1.54 |
| **phospholipid biosynthesis** | |
| SCT1 | 0.53 |
| PGS1 | 2.09 |
| **lipid, fatty acid and isoprenoid biosynthesis** | |
| UPC2 | 1.81 |
| **lipid, fatty acid and isoprenoid metabolism** | |
| ATF1 | 1.64 |
| GPD2 | 2.08 |
| GPD1 | 4.14 |
| **C-compound and carbohydrate metabolism** | |
| **Aromatic amino acid family** | |
| **regulation of C-compound and carbohydrate utilization** | |
| ADR1 | 2.21 |
| **C-compound and carbohydrate utilization** | |
| **C-compound, carbohydrate anabolism** | |
| **aminosaccharide biosynthesis** | |
| QRI1 | 0.57 |
| GNA1 | 1.51 |
| **C-2 compound and organic acid anabolism** | |
| ADH2 | 0.17 |
| ALD4 | 1.95 |
| **polysaccharide biosynthesis** | |
| **peptidoglycan biosynthesis** | |
| OST4 | 1.62 |
| **sugar, glucoside, polyol and carboxylate anabolism** | |
| TKL1 | 0.45 |

| Gene id | Ratio |
|---|---|
| YPR118W | 0.6 |
| INO1 | 3.27 |
| **C-compound, carbohydrate catabolism** | |
| **C-1 compound catabolism** | |
| YJL068C | 1.58 |
| **aminosaccharide catabolism** | |
| QRI1 | 0.57 |
| GNA1 | 1.51 |
| **C-2 compound and organic acid catabolism** | |
| ADH2 | 0.17 |
| ALD4 | 1.95 |
| **sugar, glucoside, polyol and carboxylate catabolism** | |
| TPI1 | 0.39 |
| TKL1 | 0.45 |
| CDC19 | 0.55 |
| YPR118W | 0.6 |
| SDH4 | 1.53 |
| FUM1 | 1.63 |
| SDH3 | 1.67 |
| IDH1 | 1.73 |
| ARA1 | 1.91 |
| SDH2 | 3.58 |
| **C-compound and carbohydrate utilization** | |
| ADH1 | 0.43 |
| PDC1 | 0.52 |
| ALD2 | 1.57 |
| TPS2 | 1.59 |
| PHO5 | 1.59 |
| ATF1 | 1.64 |
| PHO3 | 1.66 |
| KTR6 | 1.72 |
| YNR071C | 1.83 |
| GPD2 | 2.08 |

| Gene id | Ratio |
|---|---|
| ACH1 | 2.1 |
| HSP12 | 3.1 |
| GPD1 | 4.14 |
| ALD3 | 6.56 |
| **Aromatic amino acid family** | |
| PDR12 | 2.39 |
| PDH1 | 2.81 |
| **phosphate metabolism** | |
| **Aspartate family** | |
| **phosphate utilization** | |
| CDC19 | 0.55 |
| QRI1 | 0.57 |
| DUT1 | 0.58 |
| SSB1 | 0.63 |
| PRS5 | 0.64 |
| YBR022W | 0.65 |
| TPS2 | 1.59 |
| PHO5 | 1.59 |
| MIH1 | 1.63 |
| PHO3 | 1.66 |
| APA2 | 1.66 |
| PTK1 | 1.67 |
| SCC2 | 1.69 |
| RTS3 | 1.76 |
| KSS1 | 1.77 |
| YIL042C | 1.81 |
| AUS1 | 1.84 |
| **nucleotide metabolism** | |
| **Glutamate family** | |
| **polynucleotide degradation** | |
| BI2 | 2.07 |
| **metabolism of cyclic and unusual nucleotides** | |
| RIB1 | 1.53 |

| Gene id | Ratio |
|---|---|
| APA2 | 1.66 |
| **pyrimidine nucleotide metabolism** | |
| **pyrimidine nucleotide anabolism** | |
| FUR1 | 0.61 |
| PRS5 | 0.64 |
| **pyrimidine nucleotide catabolism** | |
| DUT1 | 0.58 |
| **pyrimidine nucleotide metabolism** | |
| QRI1 | 0.57 |
| GNA1 | 1.51 |
| APA2 | 1.66 |
| **purine nucleotide metabolism** | |
| **purine nucleotide anabolism** | |
| ADE2 | 0.62 |
| ADE1 | 0.63 |
| PRS5 | 0.64 |
| **purine nucleotide catabolism** | |
| APA2 | 1.66 |
| **purine nucleotide metabolism** | |
| DUR1,2 | 0.31 |
| YDR020C | 0.53 |
| GUA1 | 1.71 |
| **Glutamate family** | |
| CDC19 | 0.55 |
| TDP1 | 1.5 |
| **nitrogen and sulfur metabolism** | |
| **Pyruvate family** | |
| **nitrogen and sulfur utilization** | |
| **catabolism of nitrogenous compounds** | |
| **urea catabolism (not urea cycle)** | |

| Gene id | Ratio |
|---|---|
| DUR1,2 | 0.31 |
| **nitrogen and sulfur utilization** | |
| ASP3-2 | 0.4 |
| ASP3-1 | 0.56 |
| YJR149W | 0.58 |
| YNL335W | 1.6 |
| YFL061W | 1.79 |
| GDH2 | 1.92 |
| AMD2 | 2.06 |
| **amino acid metabolism** | |
| **Serine family** | |
| **metabolism of the pyruvate family (alanine, isoleucine, leucine, valine) and D-alanine** | |
| **metabolism of leucine** | |
| **biosynthesis of leucine** | |
| LEU1 | 0.33 |
| ILV5 | 0.66 |
| **metabolism of valine** | |
| **biosynthesis of valine** | |
| ILV5 | 0.66 |
| **metabolism of isoleucine** | |
| **biosynthesis of isoleucine** | |
| ILV5 | 0.66 |
| **metabolism of the cysteine - aromatic group** | |
| **metabolism of histidine** | |
| **biosynthesis of histidine** | |

| Gene id | Ratio |
|---|---|
| PRS5 | 0.64 |
| **metabolism of tryptophan** | |
| **biosynthesis of tryptophan** | |
| TYR1 | 0.62 |
| PRS5 | 0.64 |
| **metabolism of tyrosine** | |
| **biosynthesis of tyrosine** | |
| TYR1 | 0.62 |
| **metabolism of tyrosine** | |
| UGA2 | 1.54 |
| **metabolism of phenylalanine** | |
| **biosynthesis of phenylalanine** | |
| TYR1 | 0.62 |
| **metabolism of the aspartate family** | |
| **metabolism of methionine** | |
| **biosynthesis of methionine** | |
| YPR118W | 0.6 |
| **metabolism of asparagine** | |
| **degradation of asparagine** | |
| ASP3-2 | 0.4 |
| ASP3-1 | 0.56 |
| **metabolism of aspartate** | |
| ASP3-2 | 0.4 |
| ASP3-1 | 0.56 |

| Gene id | Ratio |
|---|---|
| **metabolism of urea cycle, creatine and polyamines** | |
| **metabolism of urea (urea cycle)** | |
| DUR1,2 | 0.31 |
| **metabolism of polyamines** | |
| **degradation of polyamines** | |
| ALD2 | 1.57 |
| ALD3 | 6.56 |
| **assimilation of ammonia, metabolism of the glutamate group** | |
| **metabolism of glutamate** | |
| **degradation of glutamate** | |
| UGA2 | 1.54 |
| **biosynthesis of glutamate** | |
| IDH1 | 1.73 |
| **metabolism of glutamate** | |
| GDH2 | 1.92 |
| **Serine family** | |
| TKL1 | 0.45 |
| YIL042C | 1.81 |
| **Not revised genes** | |
| Randomly_generated_negative_control | 1.56 |
| Randomly_generated_negative_control | 1.57 |
| Randomly_generated_negative_control | 1.59 |
| Randomly_generated_negative_control | 1.62 |
| YJL012CA | 1.63 |
| YDR210WC | 1.89 |
| AF149267 | 3.97 |
| AF149267 | 4.14 |
| AF149267 | 4.29 |
| AF149267 | 4.4 |

| Gene id | Ratio |
|---|---|
| AF149267 | 4.96 |
| AF149267 | 5.16 |
| AF149267 | 5.42 |
| AF149267 | 5.79 |
| AF149267 | 6.23 |
| AF149267 | 6.32 |
| AF149267 | 6.41 |
| AF149267 | 6.43 |
| AF149267 | 6.47 |
| AF149267 | 6.59 |
| AF149267 | 6.72 |
| AF149267 | 7.9 |