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Contents

Chapter 1

Introduction and thesis outline

1.1 Tradition of fermentation

Fermentation is one of the oldest methods of preserving food together with salting and drying. Food fermentation as a technology dates back at least 6 000 years and probably has enabled our ancestors to survive through different seasons of the year. Traditional fermentation processes are based on substrates, which were periodically available in excess (fruits, grains and dairy) and had to be preserved before spoilage. Beer brewing and wine making are one of the oldest ways to ferment sugars to ethanol that humankind has harnessed for several uses (medical, recreational, chemical and fuel).

The use of citric acid as a natural preservative has made citric acid probably the most well-known example of a commodity chemical produced by fermentation. Citric acid was originally obtained as a side product of lemon juice manufacturing, but already in 1919 the first industrial process using a filamentous fungus, *Aspergillus niger*, was established (Papagianni, 2007). The production of citric acid has reached over 1.4 million tons and the market demand is expected to grow 3.5 - 4 % annually (Anastassiadis et al., 2008). Since the invention of citric acid fermentation process by *A. niger*, also many other fermentation processes with applications in mainly Pharma, food and feed industry were developed throughout the 20th century.

1.2 Microbial production of chemicals

In the past decade, the fermentative production of chemicals that can be used as monomers for the synthesis of polymers has attracted much attention because of the depletion of fossil energy resources and environmental concerns. Biomass is a sustainable, widely abundant potential source of chemicals, most of which are currently derived from

petrochemicals with few exceptions (such as ethanol and lactic acid). In 2004, the US Department of Energy reported a list of top value-added chemicals from biomass that support the production of fuels and power in an integrated biorefinery (Werpy and Petersen, 2004). A decade later, production of 10 out the 12 top chemicals (excluding aspartic acid and 3-hydroxybutyrolactone) have been realized at commercial scale (Choi et al., 2015), Table 1.1.

Table 1.1. Overview of commercial building block chemicals[1]

Platform chemical	Structure	Company
Ethanol	C_2H_6O	ADM, Poet, Copersucar and many others
3-hydroxypropionic acid (3-HP)	$C_3H_6O_3$	Novozymes, Cargill, Perstorp
Glycerol	$C_3H_8O_3$	Solvay, Dow Chemical, Huntsman
Succinic acid	$C_4H_6O_4$	Succinicity, Bioamber, Myriant, Reverdia
Lactic acid	$C_3H_6O_3$	PURAC, Cargill and Henan Jindan Lactic Acid Technology Co and many others
Fumaric acid	$C_4H_4O_4$	Myriant
Malic acid	$C_4H_4O_4$	Novozymes
Itaconic acid	$C_5H_6O_4$	Itaconix, Qingdao Kehai biochemistry Co
Glutamic acid	$C_5H_9NO_4$	Many companies, mainly in Asia
Levulinic acid	$C_5H_8O_3$	Segetis, Biorefine Technology LLC
Glucaric acid	$C_6H_{10}O_8$	Johnson Matthey and Rennovia, Rivertop Renewables
2,5-Furan dicarboxylic acid	$C_6H_4O_5$	Avantium
Sorbitol	$C_6H_{14}O_6$	Roquette, ADM
Xylitol	$C_5H_{12}O_5$	Danisco

Derivatives of building block chemicals

1,4-Butanediol (BDO)	$C_4H_{10}O_2$	Genomatica, Bioamber
1,3-Propanediol (PDO)	$C_3H_8O_2$	DuPont, Tate and Lyle Bio

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[1] Partially adapted from (Choi et al., 2015) and information available at companies websites

In addition, production of 1,4-Butanediol and 1,3-Propanediol by direct fermentation process without succinic acid and 3-hydroxypropionic acid as precursor molecules have also been commercialized. The top list of sugar-derived building blocks has especially put more attention to the fermentative production of diols, dicarboxylic and amino acids. Among them, itaconic acid was selected as one of the promising building blocks for polymer industry.

1.3 Itaconic acid

Itaconic acid, also known as 2-methylenesuccinic acid, is an unsaturated C5-dicarboxylic acid with the stoichiometric formula $C_5H_6O_4$ and a molar weight of 130.1 g/mol, Figure 1.1. The current global production is estimated at 150 000 tons annually (Ma et al., 2013). Itaconic acid has a market potential in polymer industry as a component of resins, plastics, adhesives, elastomers and coatings (Willke and Vorlop, 2004) and it is also a prominent platform chemical for biofuel production (Geilen et al., 2010). After being listed as a top 12 value-added biobased chemical compound (Werpy and Petersen, 2004), microbial production of itaconic acid has been of special interest for a decade. Itaconic acid is already produced on a commercial scale by its native producer *Aspergillus terreus* but the costs of biobased production are relatively high (Liao and Chang, 2010). The process at an industrial scale is performed aerobically by using sugar molasses or glucose as a substrate (Okabe et al., 2009, Yahiro et al., 1995). The filamentous fungus, *A. terreus*, has limitations as a production host, such as slow growth, high viscosity and sensitivity of the filament pellets to hydro-mechanical stress, which inhibits mass transfer and consequently oxygen supply to the cells (Klement and Büchs, 2013). For this reason, more resistant and non-filamentous producer organisms are being developed which might be able to replace *A. terreus* in the future.
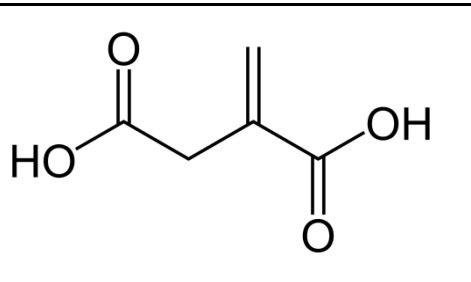
Formula	$C_5H_6O_4$
Molecular weight	130.1
Melting point	167-168 ºC
Boiling point	268 ºC
Water solubility	83.103 g/L
Density	1.632 g/L (20 ºC)
pKa	3.84 and 5.55

**Figure 1.1** Structure and properties of itaconic acid (adapted from (Willke and Vorlop, 2001).

1.4 Metabolic engineering

Over the years, strategies to evolve wild type strains together with rational metabolic engineering techniques have been developed to increase the titre and productivity of the fermentation process to its maximum. Strain breeding was traditionally achieved mainly by laboratory evolution (random mutagenesis and selection), which is still a useful tool in metabolic engineering (Portnoy et al., 2011). Since the invention of gene deletion protocols like phage lambda-derived Red recombination system (Datsenko and Wanner, 2000) and gene targeting by homologous recombination (Capecchi, 1989) together with highly efficient plasmid expression systems for heterologous gene expression (Miroux and Walker, 1996), more systematic and rational metabolic engineering has become a standard procedure in process development and optimization. One of the latest and most prominent technologies are RNA guided, Cas9-based, genome engineering tools from the microbial adaptive immune system CRISPR (clustered regularly interspaced short palindromic repeats) that have facilitated genome editing in large scale (Hsu et al., 2014, Mali et al., 2013). This has also expanded the variety of products that can be made by microorganisms.

Rapid developments in the field of molecular biology have also allowed the production of itaconic acid in heterologous hosts. The key enzyme for itaconic acid production, *cis*-aconitate decarboxylase (CadA) from *Aspergillus terreus* was described and characterized by (Kanamasa et al., 2008). For example, itaconic acid has been produced in several non-natural producers such as *Aspergillus niger* (van der Straat et al., 2014), *Yarrowia lipolytica* (Wang et al., 2011), *Saccharomyces cerevisiae* (Blazeck et al., 2014) and *Corynebacterium glutamicum* (Otten et al., 2015) through the heterologous expression of *cadA*. Although the itaconate titres obtained with heterologous hosts are still modest, these developments towards alternative production processes are necessary to expand its market potential.

1.5 Theoretical maximum yield

Maximizing yield is of importance for cost-competitive production and a way to expand market potential for itaconic acid. For each substrate/product combination, there is a maximum amount of product that can be formed from the substrate. This maximum yield (Y^E) is based on elemental balancing and is calculated from the ratio of the degree of reduction (γ) of substrate and product (Dugar and Stephanopoulos, 2011). The Y^E for itaconate (itaconate=18) from glucose (glucose=24) is 1.33 mol/mol glucose. The overall equation becomes:

Glucose + $^2/_3$ CO_2 = $1^1/_3$ Itaconate + 2 H_2O
$\Delta G_0' = -197.3 \pm 6.2$ kJ/mol glucose   equation 1.1
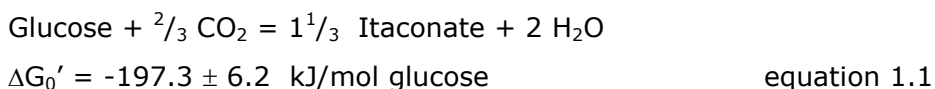
The $\Delta G_0'$ indicates that the conversion can occur spontaneously and allows synthesis of ATP. However, the maximum pathway yield (Y^P) depends on the pathway involved and the way redox cofactor

regeneration takes place. Current fermentation processes for the production of itaconic acid from sugar are executed aerobically using oxygen as the terminal electron acceptor, which has a negative influence on Y^P. In *Aspergillus terreus*, a maximum pathway yield of 0.80 mol itaconic acid / mol of glucose was found, with a maximal productivity of 1.15 g/l/h (Hevekerl et al., 2014), which is only 60 % of the Y^E.

1.6 Anaerobic fermentations

Equation 1.1 shows that efficient production of citrate does not require the input of oxygen. The same is true for the production of many other chemicals. Up till now, most industrial processes are performed aerobically. Examples are the production of citrate, L-glutamate and L-lysine. Aerobic bioconversions have a low productivity because they are rate limited by oxygen transfer. The availability of oxygen also results in a lower yield, since substrate can be completely oxidized to CO_2 and efficiently converted into microbial biomass due to a high energetic efficiency of aerobic respiration. Anaerobic product synthesis would alleviate most of the problems associated with aerobic production (Cueto-Rojas et al., 2015, Weusthuis et al., 2011), Figure 1.2. Anaerobic fermentation alleviates the oxygen transfer limitation resulting in higher productivities. Lower capital cost is required because smaller fermenters can be used, and fermenters can be less expensive because no compressors for air addition are necessary and because less cooling capacity is demanded. In addition, anaerobic fermentations have a higher yield (lower operational expenditure) due to reduced biomass formation and because full oxidation of substrate to CO_2 is not possible (Weusthuis et al., 2011).

Consequently, current pathway yields are far below their theoretical maximum yields, as in the case of itaconic acid production. Anaerobic product synthesis can avoid most of the problems associated with aerobic production and thus, allow production of itaconate closer to its Y^E.

The conversion of glucose to itaconate is an oxidation reaction, resulting in the net reduction of the NAD-cofactor. The role of oxygen is to regenerate NAD under aerobic conditions. Under anaerobic conditions, alternative methods have to be employed for cofactor regeneration. For instance, the mixed acid fermentation pathway of *Escherichia coli* offers two options: the conversion of glucose and CO_2 into succinate and the conversion of glucose into ethanol and formate (or hydrogen gas and CO_2).
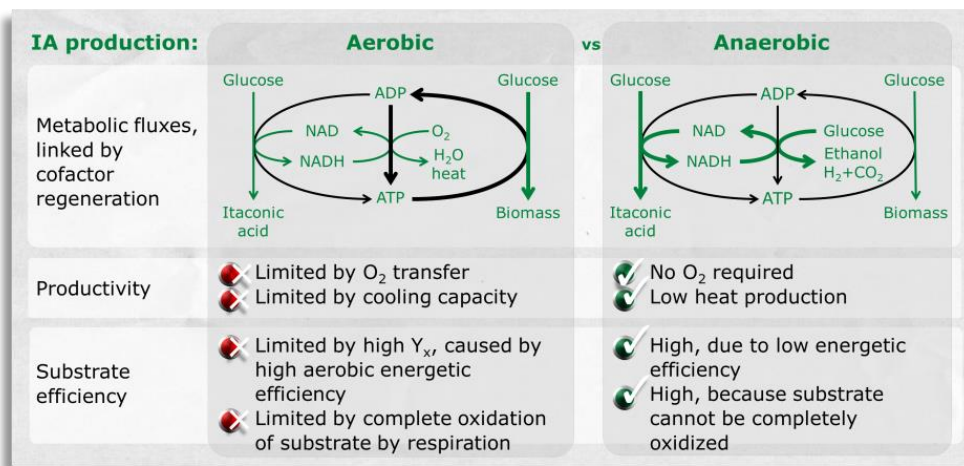

Figure 1.2 Comparison of productivity and substrate efficiency of itaconate (IA) production under aerobic and anaerobic conditions. Strength of metabolic flux is indicated by thickness of an arrow. Biomass yield Y_x.

1.7 *Escherichia coli* as a production host

The Gram-negative rod-shaped bacterium, *Escherichia coli* is a model organism that has been extensively studied to understand basic mechanisms of molecular genetics. *E. coli* has long been the favoured organism in fundamental studies and heterologous protein production due to its relative simplicity and the ease to culture it in the laboratory. These characteristics together with well-developed tools for cloning have made *E. coli* a true workhorse for molecular biology. *E. coli* is a production host for many value-added chemicals with potential applications in the food, pharmaceutical and chemical industry (Chen et al., 2013). Moreover, *E. coli* is able to grow under anaerobic conditions making it also a suitable production host for anaerobic product synthesis. During anaerobic growth, glucose is initially metabolized to pyruvate via glycolysis. These reactions generate 2 molecules of NADH and 4 molecules of ATP. In total net 2 ATP's are produced as two ATP's are consumed in early steps of the pathway. Pyruvate is further metabolized to one or more end products: lactate, acetate, ethanol, succinate, formate, CO_2 and H_2 (Clark, 1989). Production of ethanol, succinate or lactate is used to regenerate the two molecules of NADH formed in glycolysis back to NAD^+, Figure 1.3.

In addition, *E. coli* has several interesting features for anaerobic production of itaconate. It is one of the few industrial microorganisms that are able to grow under anaerobic conditions and produces a precursor of itaconate, acetyl-CoA, as a central metabolite in dissimilation processes. Under anaerobic conditions, *E. coli* converts pyruvate into acetyl-CoA and formate by pyruvate-formate lyase. Formate can subsequently be split into valuable H_2 and CO_2. Other industrial strains that are able to grow under anaerobic conditions, like *S. cerevisiae* and lactic acid bacteria, use NAD-dependent pyruvate

dehydrogenase to synthesize acetyl-CoA (Kandler, 1983, Pronk et al., 1996), which generates extra NADH and thus requires additional cofactor regeneration at the cost of substrate. Moreover, these microorganisms have a limited flux over acetyl-CoA under anaerobic conditions. In addition, *E. coli* does not have expensive growth media requirements like lactic acid bacteria (Hayek and Ibrahim, 2013) and is genetically more accessible than Clostridium species (Cho et al., 2015, Lütke-Eversloh and Bahl, 2011).

Besides other heterologous production systems, also *E. coli* has been shown to produce itaconate in earlier studies. However, only trace amounts of itaconate were obtained through expression of *cadA* (Li et al., 2011). In other words, *E. coli* possess the right characteristics to produce itaconate, but its full potential as a production host can only be revealed by implementing novel strain and process design.
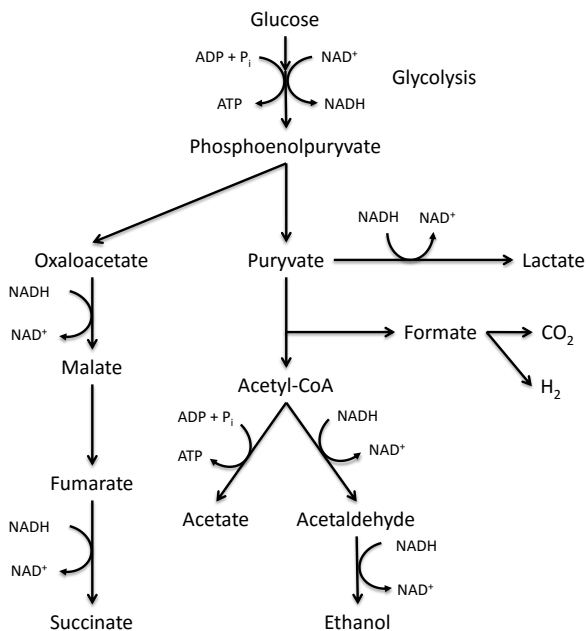

Figure 1.3 Mixed acid fermentation pathway in *E. coli*, according to (Clark, 1989).

1.8 Aim of research

The aim of this thesis was to explore options to approximate maximum yield for production of itaconic acid, which is a biobased building block monomer for polymer industry. The study was focused on itaconic acid production mechanism and in increasing the efficiency of the pathway in heterologous production host, *Escherichia coli*.

1.9 Outline of the thesis

This thesis presents different ways to enhance itaconate production in *E. coli* by metabolic engineering. Itaconic acid is not a natural product of *E. coli* metabolism and the work started by construction of an itaconate biosynthesis pathway in *E. coli*, which is described in **chapter 2**. The key enzyme of microbial itaconate production is *cis*-Aconitate decarboxylase (CadA) that converts the citric acid cycle intermediate *cis*-aconitate into itaconate. In this chapter, we focused on optimizing heterologous expression of *cadA* from *Aspergillus terreus.* Culturing conditions such as temperature and growth medium composition played an important role in the optimization. To have a well-controlled production scheme, a batch culturing process in bioreactors was introduced, Figure 1.4. In addition, availability of itaconate precursor molecules, citrate and cis-aconitate, was increased by introducing citrate synthase and aconitase from *Corynebacterium glutamicum.* To reduce by-product formation, pathways to lactate and acetate formation were deleted. The maximum itaconate yield from glucose obtained in this study was only 0.09 mol/mol, due to high flux of carbon to by-products such as acetate and pyruvate.

Although *E. coli* is a widely used cell-factory for the production of proteins and chemicals, problems such as inclusion body formation and

low enzyme activity are often associated with heterologous production of proteins, which was observed in chapter 2. As sufficient *cis*-aconitate decarboxylase activity is crucial for itaconate production, in **chapter 3** we looked at ways to increase the activity of this step. We expressed a recently characterized *cis*-aconitate decarboxylase of mammalian origin in *E. coli*. The novel cis-aconitate decarboxylase from *Mus musculus* encoded by immunoresponsive gene 1 (*irg1*) produced comparable amounts of itaconate as CadA from *A. terreus*, although they share only 24 % amino acid sequence identity. In addition, the effect of codon optimization and harmonization on enzymatic activities of heterologously expressed cadA and irg1 was studied.

E. coli is also one of the few industrial microorganisms that are able to grow under anaerobic conditions. Production of itaconate results in NADH formation and when oxygen is not present as a terminal electron acceptor, alternative ways to regenerate cofactors have to be employed. The mixed acid fermentation pathway of *E. coli* offers two options to regenerate NAD: the conversion of glucose and CO_2 into succinate and the conversion of glucose into ethanol and formate (or hydrogen and CO_2). In **chapter 4** we established a proof of principle for an anaerobic fermentation process for the production of itaconic acid with co-production of ethanol and hydrogen gas to maintain redox balance. Unexpectedly, strains started to produce significant amounts of glutamate when the itaconate pathway was introduced. Glutamate production could be suppressed by introducing nitrogen-limited growth medium. This is the first time that anaerobic production of itaconate and glutamate from glucose was reported for *E. coli*. The observed itaconate yields and productivities were still modest. We concluded that eliminating the pathways to major by-products like glutamate, succinate, and acetate, and enhancing the pathway between pyruvate

and itaconate is therefore crucial to obtain a cost-competitive anaerobic production process for itaconic acid.

To investigate how itaconate production can be improved, the insights from the previous chapters together with existing scientific literature were combined with suggestions for future pathway design in **chapter 5.** We took a closer look at the metabolic engineering of the tricarboxylic acid (TCA) cycle for the efficient production of chemicals like itaconic acid. We focused on strategies to reach theoretical maximum yield production that could, in theory, exceed the current pathway yields of many bulk chemicals. To allow high production yield of biochemicals, which can reach their theoretical maximum, carbon fluxes from both branches of the TCA cycle must be directed to product formation. The theoretical maximum might become achievable by turning around fluxes of some main fermentation pathways such as reversing the TCA or glyoxylate cycle, allowing high yield, close to the theoretical maximum production of chemicals from both branches of the TCA cycle without loss of CO_2 in the process.

Chapter 6 summarizes the main results obtained in this thesis together with concluding remarks and future perspectives.
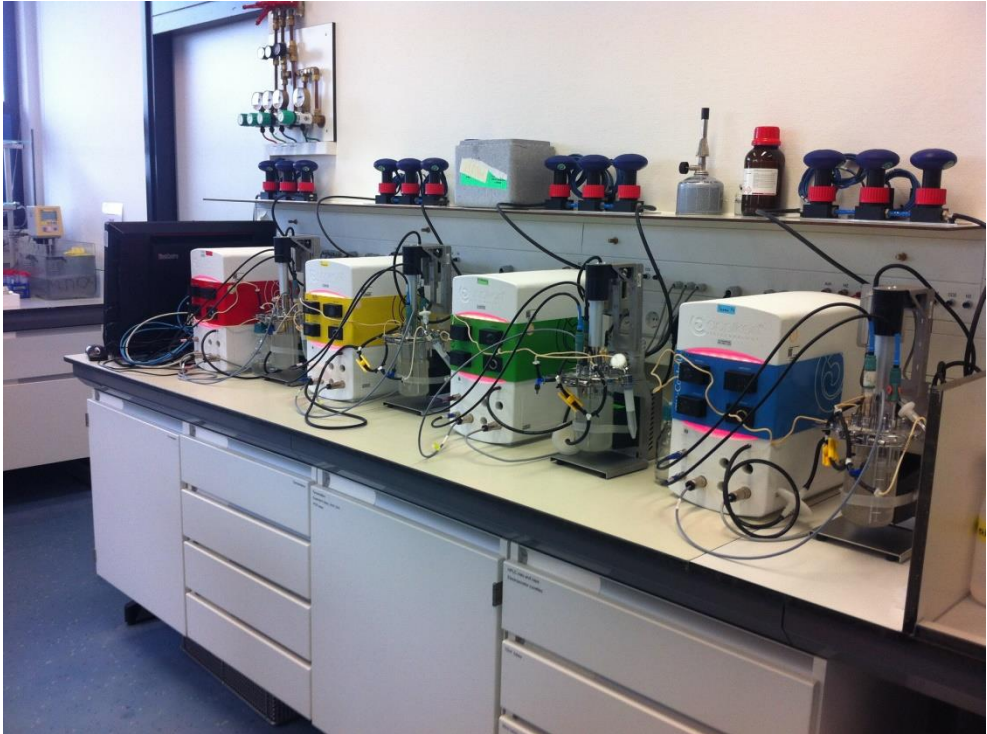
Figure 1.3 A typical fermentation setup (Applikon Mini-Bioreactors connected to myControl controlling units) of *E. coli* producing itaconate in a batch culture.

Chapter 2

Metabolic engineering of itaconate production in *Escherichia coli*

Abstract

Interest in sustainable development has led to efforts to replace petrochemical-based monomers with biomass-based ones. Itaconic acid, a C5-dicarboxylic acid, is a potential monomer for the chemical industry with many prospective applications. *cis*-Aconitate decarboxylase (CadA) is the key enzyme of itaconate production, converting the citric acid cycle intermediate *cis*-aconitate into itaconate. Heterologous expression of *cadA* from *Aspergillus terreus* in *Escherichia coli* resulted in low CadA activities and production of trace amounts of itaconate on LB medium (< 10 mg/L). CadA was primarily present as inclusion bodies, explaining the low activity. The activity was significantly improved by using lower cultivation temperatures and mineral medium, and this resulted in enhanced itaconate titres (240 mg/L). The itaconate titre was further increased by introducing citrate synthase and aconitase from *Corynebacterium glutamicum* and by deleting the genes encoding phosphate acetyltransferase and lactate dehydrogenase. These deletions in *E. coli*'s central metabolism resulted in the accumulation of pyruvate, which is a precursor for itaconate biosynthesis. As a result, itaconate production in aerobic bioreactor cultures was increased up to 690 mg/L. The maximum yield obtained was 0.09 mol itaconate / mol glucose. Strategies for a further improvement of itaconate production are discussed.

2.1 Introduction

Itaconic acid is a C5-dicarboxylic acid that can be used as a building block for the production of a diverse set of isomeric lactones, diols, cyclic ethers (Geilen et al., 2010) and polymers (Hughes and Swift, 1993). The polymers are potential substituents for many acrylic-based materials such as resins or synthetic fibres (Okabe et al., 2009, Willke and Vorlop, 2001).

Since the 1960s, itaconic acid has been commercially produced using natural mutants of the filamentous fungus *Aspergillus terreus* (Klement and Büchs, 2013, Willke et al., 2001). Kuenz et al (2012) recently reported one of the best performing itaconic acid producing *A. terreus* cultivations, which had a productivity of 0.51 g/l/h, a maximum titre of 86.2 g/l, and a yield of 86 mol%. Better achievements seem possible because the productivity is relatively low and is caused by the low oxygen transfer rates that can be achieved with the filamentous growth form (Klement et al., 2012), the yield is only 65% of the maximum theoretical yield of 1.33 mol itaconic acid and titres over 200 g/l citric acid – a precursor of itaconic acid – have been achieved by closely related *A. niger*. Other disadvantages of *A. terreus* are sensitivity of the filament pellets to hydro-mechanical stress, laborious handling of spores (Klement et al., 2012), low reproducibility of fermentations (Kuenz et al., 2012) and the fact that an interruption of oxygen supply strongly decreases itaconic acid production (Klement et al., 2012).

Several groups have searched for other microorganisms able to produce itaconic acid. *Ustilago* sp. and *Candida* sp. (Tabuchi et al., 1981) have also been found to produce itaconic acid, but with titres below 55 g/L. Recombinant production hosts such as *Escherichia coli* have been proposed for cheaper production of itaconic acid (Yu et al., 2011). As a

facultative anaerobic bacterium, *E. coli* has many advantages as a production host, like rapid growth under both aerobic and anaerobic conditions, simple medium requirements, and well-established protocols for genetic modification.

Wild-type *E. coli* does not produce itaconate, because it misses *cis*-aconitate decarboxylase (CadA), which catalyses the conversion of *cis*-aconitate to itaconate (Yahiro et al., 1995) and is the key enzyme for itaconate biosynthesis in *A. terreus.* It has been successfully expressed in *E. coli* (Li et al., 2011), but product titres remained low.

In this paper, we investigate the potential of *E. coli* to produce itaconate by introducing *cis*-aconitate decarboxylase (CadA) from *Aspergillus terreus* and the effects of the introduction of the heterologous enzymes citrate synthase and aconitase from *C. glutamicum*, and the elimination of the native phosphate acetyltransferase and lactate dehydrogenase activities, which could stimulate itaconate production by enhancing the availability of precursors. The proposed itaconate pathway in *E. coli* is shown in Figure 2.1.
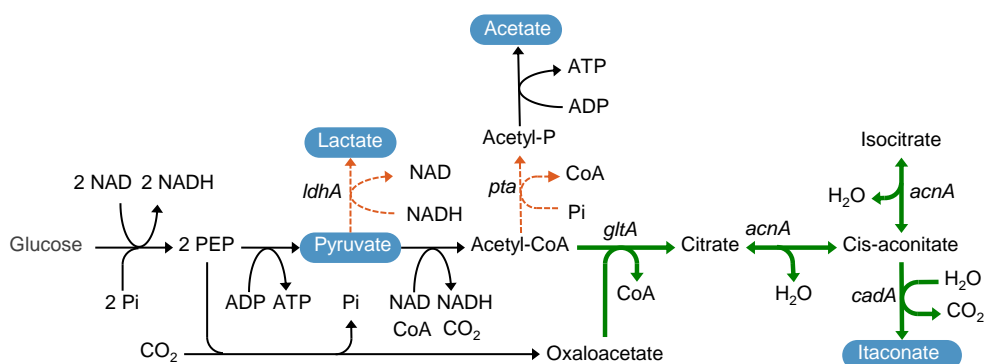

Figure 2.1 Itaconate production pathway in *E. coli*. The bold arrows indicate the introduced pathway consisting of genes encoding citrate synthase (*gltA*) and aconitase (*acnA*) from *C. glutamicum* and *cis*-aconitate decarboxylase (*cadA*) from *A. terreus*. The dotted lines indicate that phosphate acetyltransferase (*pta*) and lactate dehydrogenase (*ldhA*) were deleted.

2.2 Materials and methods

2.2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 2.1.

Table 2.1   *E. coli* strains and plasmids used in this study.

Strains and plasmids	Characteristics	References
Strains		
BW25113	lacIq rrnBT14 Δ*lac*ZWJ16 hsdR514 Δ*ara*BADAH33 ΔrhaBADLD78	CGSC[1]
BW25113 (DE3)	BW25113 DE3 T7 RNA polymerase	This study
BW25113 Δ*pta*	BW25113 Δ*pta*-779::kan	CGSC
BW25113 (DE3) Δ*pta*Δ*ldhA*	BW25113 Δ*pta* Δ*ldhA* DE3 T7 RNA polymerase	This study
Plasmids		
pET101/D/*lacZ*	pBR322 ori lacI T7 AmpR	Invitrogen
pKD46	AmpR plasmid with temperature-sensitive replication and arabinose induction of λ-red recombinase	CGSC
pKD4	KanR plasmid with R6K-γ replicon	CGSC
pCP20	AmpR and CmR plasmid with temperature-sensitive replication and thermal induction of FLP synthesis	CGSC
pEV	pACYCDuet-1 overexpression vector using T7 promoter, capable to carry two multiple cloning sites, CmR	Novagen
pKV-C	pACYCDuet-1 derivative, synthetic *cadA* gene	This study
pKV-CGA	pACYCDuet-1 derivative, synthetic *cadA*, *acnA*, and *gltA* genes	This study

[1] The Coli Genetic Stock Center at Yale University

2.2.2 Deletion of phosphate acetyltransferase (*pta*) and lactate dehydrogenase (*ldhA*) genes

The gene encoding lactate dehydrogenase (*ldhA*) was inactivated in *E. coli* BW25113 Δ*pta* by using the Lambda red-mediated gene

replacement method described by (Datsenko and Wanner, 2000). Shortly, *E. coli* BW25113 Δ*pta* was transformed with pKD46 and cultured in the presence of L-arabinose to induce λ-red recombinase expression, which is an inducer for recombination. The target gene *ldhA* was replaced by a kanamycin resistance gene flanked by flippase recognition target (*FRT*) sites. For this, a deletion cassette containing a kanamycin resistance gene with *FRT* sites was amplified from pKD4 by using Phusion High Fidelity DNA Polymerase (Thermo Scientific) and primers that contain 50 bp targeting flanks to the *ldhA* region in the genome (Table 2.2) and transformed into *E. coli* BW25113 Δ*pta* (pKD46). Transformants were screened for their proper genotype by selecting for kanamycin resistance and colony PCR (GoTaq Green polymerase, Promega) using primers that flank the target gene. The phenotype was verified in liquid cultures. The kanamycin resistance gene was subsequently eliminated by using the temperature-sensitive helper plasmid pCP20 encoding the flippase (*FLP*), followed by curing of the temperature sensitive plasmids by culturing strains at 42 °C for 16 hours.

Table 2.2 List of primers used in this study

(50bp flanking regions underlined)

Name	Sequence
For *ldhA* deletion	
ldhA flank F	AAATATTTTTAGTAGCTTAAATGTGATTCAACATCAC
	TGGAGAAAGTCTTGTGTAGGCTGGAGCTGCTTC
ldhA flank R	ATTGGGGGATTATCTGAATCAGCTCCCCCTGGAATGCAG
	GGGAGCGGCAAGAATGGGAATTAGCCATGGTCC
For PCR verifications	
ldhA check F	AAATATTTTTAGTAGCTTAAATGTG
ldhA check R	ATTGGGGGATTATCTGAATCAGCTCC

2.2.3 Site-specific integration of the λDE3 prophage

Site-specific integration of the λDE3 prophage into *E. coli* BW25113 and into its derivative *E. coli* BW25113 Δ*pta* Δ*ldhA* was done using the λDE3 Lysogenization Kit (Novagen). The integration of the λDE3 prophage and expression of T7 polymerase in strains were verified according to the protocol in the kit. Besides, the functional expression of T7 polymerase was confirmed by transforming the strains with pET101/D/lacZ. The transformants were able to cleave 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) after induction with isopropyl-beta-D-thiogalactopyranoside (IPTG).

2.2.4 Construction of pACYC expression vectors

The expression vector pACYCDuet-1 (Novagen) was used to express the genes *cadA* (NCBI Reference Sequence ID: BAG49047.1), *acnA* (ID: NP_600755.1), and *gltA* (ID: NP_600058.1) under the transcriptional control of the T7 promoter. All genes were codon-optimized according to the algorithm of OptimumGene™ (GenScript) and synthesized by GenScript, USA. The sequences of the codon-optimized *cadA*, *acnA*, and *gltA* can be retrieved from GenBank (ID: KM464677, ID: KM464678, ID: KM464679, respectively. *cadA* was ligated between the *Nco*I-*Hin*dIII restriction sites in MCS1 of pACYCDuet-1, yielding pKV-C. *acnA* and *gltA* were respectively ligated between the *Nde*I-*Xho*I and *Xho*I-*Pac*I sites in MCS2 of pKV-C, yielding pKV-CGA, Table 2.1. An additional ribosomal binding site (rbs), identical to those in pACYCDuet-1, was introduced upstream of *gltA* gene.

2.2.5 Cultivation conditions

1) Culture media

For plasmid construction and gene expression analysis, *E. coli* strains were cultured on Luria-Bertani (LB) agar plates or in LB liquid medium at either 30°C or 37°C. Recombinants harbouring temperature-sensitive plasmids were cultured at either 30°C for cultivation or 42°C to cure the selection markers. Expression of *lacZ* was detected by blue/white screening in agar plates on top of which 40 µl of 20 mg/ml X-gal in dimethyl sulfoxide and 40 µl of 1 M IPTG were spread on top of the plates. When needed, medium and plates were supplemented with ampicillin (50 µg/mL) or chloramphenicol (35 µg/mL). Induction of gene expression in liquid cultures was started by the addition of 1 mM of IPTG when the optical density at 600 nm (OD_{600}) of the culture reached approximately 0.4.

The other cultivations were done in M9 Minimal medium (MM), which contained per 1 liter: 200 mL 5×M9 Minimal Salts (BD Difco) supplemented with 50 mmoles of glucose, 2 mmoles of MgSO4, 0.1 mmoles of $CaCl_2$, 15 mg of thiamine, and 0.30 mg of selenite. Medium was buffered with 0.1 M 3-(N-morpholino) propanesulfonic acid (MOPS) and the pH was adjusted to 6.9 with NaOH.

2) Cultivation in bioreactors

E. coli BW25113 (DE3) and *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* containing either pEV, pKV-C or pKV-CGA were cultivated at 30°C in 0.5 L Mini Bioreactors, connected to myControl controller units (Applikon, The Netherlands) with a working volume of 400 ml. The pH was maintained at 6.9 by the automated addition of 2 M NaOH. Cultures were continuously stirred at 1200 rpm and sparged with medical air at

400 mL/min. Bioreactors were inoculated with 5 % (v/v) of a pre-culture that was grown in a 250 mL Erlenmeyer flasks with 50 mL of MM at 250 rpm and 30 °C for 24 hours. Samples of 2 mL were regularly taken to determine the OD_{600} of the cultures and the concentrations of substrate and products.

2.2.6 Enzymatic assays

For enzymatic assays, 50 mL of bioreactor culture was harvested by centrifugation (5 min, 7745 × *g*) after 17 hours of cultivation in the presence of IPTG and washed with MM. Cell free extracts (CFE) were made according to the Y-PER Yeast Protein Extraction Reagent kit instructions (Thermo Scientific). Protein concentrations were determined by using the Total Protein Kit, Micro Lowry, Peterson's Modification (Sigma Aldrich).

The activity of *cis*-aconitate decarboxylase (CadA) was measured by using a method adapted from (Dwiarti et al., 2002) and (Li et al., 2011): CFE's were incubated with 17 mM of *cis*-aconitate in 200 mM sodium phosphate buffer (pH 6.2) for 10 min at 30°C. Reactions were terminated by adding 1 M HCl. Supernatants were analyzed for itaconate formation by HPLC.

The activity of aconitase was measured by monitoring the formation of *cis*-aconitate at 240 nm in a UV-Vis Spectrophotometer (UV-1650PC SHIMADZU) using an extinction coefficient for *cis*-aconitate of 3.5 $mM^{-1}cm^{-1}$ (Baumgart and Bott, 2011). The assays were performed at 30°C in 100 mM Tris–HCl buffer (pH 8.0) and 20 mM trisodium citrate as a substrate.

Citrate synthase activity was determined by monitoring the hydrolysis of the thioester of acetyl coenzyme A (acetyl-CoA), which results in the formation of CoA. The thiol group of CoA reacts with 5,5'-dithiobis-(2-nitrobenzoic acid (DTNB) in the reaction mixture to form 5-thio-2-nitrobenzoicacid (TNB). The formation of TNB was measured at 412 nm by using 13.6 mM^{-1} cm^{-1} as extinction coefficient at 30°C according to (Morgunov and Srere, 1998) with minor adjustments. The reaction mixture contained 0.31 mM acetyl-CoA, 0.5 mM oxaloacetate, 0.1 mM DTNB, and ca. 0.25 % Triton X-100 in 100 mM Tris-HCl (pH 8.0).

2.2.7 Analytical methods

The cell density was determined by measuring the OD_{600} by using a spectrophotometer (Dr. Lange XION 500). The concentrations of glucose and organic acids were determined by using HPLC by using a Dionex Ultimate 3000 (Thermo Fisher) equipped with an RI detector (Shodex, RI-101) and a UV detector (Dionex, 3400 RS at 210 nm). The samples were separated on a Micro Guard Cation H pre-column (30x4.6mm, Biorad) and an Aminex HPX-87H column (300x7.8 mm, Biorad) at 35°C, using 0.6 mL/min of 5 mM H_2SO_4 as an eluent.

2.3 Results

2.3.1 Heterologous expression of *cadA, acnA*, and *gltA*

Gene *cadA* from *A. terreus* was codon optimized and expressed in *E. coli* to enable itaconate production. Small amounts of itaconate were produced (below 10 mg/L) when *E. coli* BW25113 (DE3) (pKV-C) was cultivated in LB in shake flask cultures at 37°C, but no detectable CadA activity was found in CFE of these cultures. SDS-PAGE analysis showed

that almost all CadA was present in the form of inclusion bodies (data not shown). As inclusion bodies are often associated with fast and high-level expression of heterologous proteins (Jurgen et al., 2010), two measures were taken to reduce these rates: cultivation in MM instead of LB and cultivation at lower temperatures. When *E. coli* BW25113 (DE3) (pKV-C) was grown in MM in pH-controlled bioreactors at 37°, CadA could be detected in CFE with a specific activity of 0.03 U/mg. The activity was further increased to 0.38 U/mg when the cultivation temperature was lowered to 30°C (Figure 2.2). SDS-PAGE analysis showed that the amount of soluble protein had increased by these measures (data not shown).

To channel more acetyl-CoA to itaconate, the codon optimized genes encoding citrate synthase (*gltA*) and aconitase (*acnA*) from *C. glutamicum* were overexpressed in *E. coli* together with *cadA*, yielding *E. coli* BW25113 (DE3) (pKV-CGA). The expression levels of the heterologous genes were determined by measuring the activities of the corresponding enzymes in CFE of *E. coli* BW25113 (DE3) strains containing either pEV, pKV-C or pKV-CGA. The activities of citrate synthase and aconitase in CFE's of *E. coli* BW25113 (DE3) (pKV-CGA) increased 4 and 40 times, respectively, compared to the native activities measured in *E. coli* BW25113 (DE3) (pKV-C) (Figure 2.3). It appeared that expression of *cadA* increased the native citrate synthase and aconitase activities in *E. coli* BW25113 (DE3) (pKV-C), as the activities of these enzymes were lower in *E. coli* BW25113 (DE3) (pKV-EV), which might be due to an activating effect of itaconate. Simultaneous expression of *gltA* and *acnA* together with *cadA* resulted in a lower specific CadA activity compared to the *E. coli* BW25113 (DE3) (pKV-C), which is probably due to a dilution effect caused by the overexpression of the two additional genes.
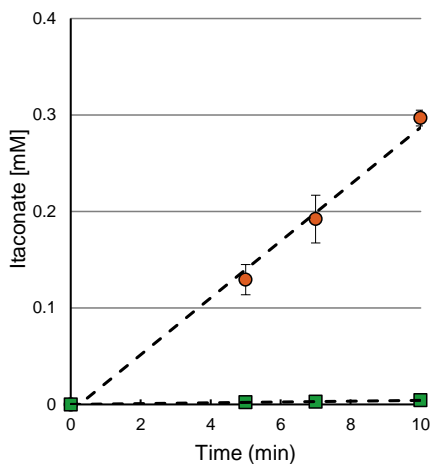
Figure 2.2 Conversion of *cis*-aconitate to itaconate in CFE of *E. coli* BW25113 (DE3) (pKV-C) that was cultured in bioreactors at either 30°C (circles) or 37°C (squares). Protein concentrations in the assay mixtures were 5.8 and 4.3 mg/mL, respectively. The activities of CadA were derived from the slopes of the lines.
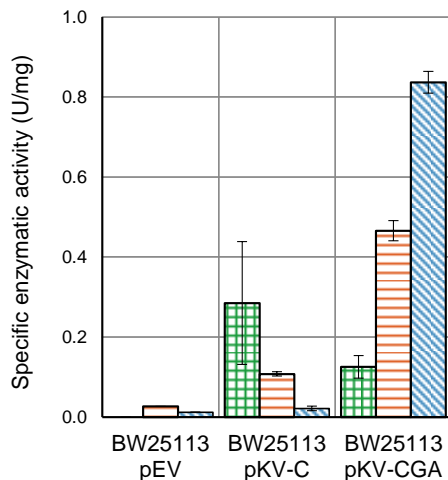

Figure 2.3 Specific enzymatic activities (U/mg) of *cis*-aconitate decarboxylase (hatched lines), citrate synthase (horizontal lines) and aconitase (diagonal lines) in CFE of *E. coli* BW25113 (DE3) containing either pEV, pKV-C, or pKV-CGA. Strains were cultured in bioreactors at 30°C on MM medium. The average values and standard deviations (SD) of duplicate parallel studies are given.

2.3.2 Itaconate production in *E. coli* BW25113 (DE3)

Itaconate production by *E. coli* BW25113 (DE3) containing either pEV, pKV-C, or pKV-CGA was monitored in pH-controlled bioreactors in MM at 30°C for 72 hours. Itaconate was produced up to 1.9 mM with both *E. coli* BW25113 (DE3) (pKV-C) and BW25113 (DE3) (pKV-CGA), but not in the control strain (Figure 2.4). Overexpression of *gltA* and *acnA* together with *cadA* had no significant impact on itaconate production under these conditions as the production profiles were similar with both pKV-C and pKV-CGA plasmids. This suggests that the availability of precursors is limiting itaconate production.

During growth on glucose, acetate was observed in all cultures after 1 day of cultivation, which accumulated up to 55 mM. When glucose was depleted from the medium, the cells started consuming acetate. In addition, low concentrations (below 5 mM) of ethanol, citrate, pyruvate, lactate, succinate and formate were detected in the medium during cultivation of all strains, and some *cis*-aconitate (< 5 mM) was formed by *E. coli* BW25113 (DE3) (pKV-C) and BW25113 (DE3) (pKV-CGA) (data not shown). Most of these compounds were only intermediary products and disappeared over time.

Figure 2.4 Batch cultivation of *E. coli* BW25113 (DE3) containing pEV (left panel), pKV-C (middle panel) and pKV-CGA (right panel) in pH-controlled bioreactors on MM at 30°C. The OD_{600} (diamond) and the concentrations of glucose (squares), acetate (triangles) and itaconate (circles) are indicated.

2.3.3 Itaconate production in *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA*

To increase the availability of precursors, *E. coli* BW25113 (DE3) was made deficient in acetate and lactate production. Deletion of *pta*, encoding phosphate acetyltransferase, is known to result in accumulation of pyruvate in the cells, which may be redirected to

itaconate. As Δ*pta* strains have been reported to convert pyruvate to lactate (Castano-Cerezo et al., 2009), this conversion was eliminated as well by deleting *ldhA*. To test the effect of these eliminations, the resulting strain *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA*, containing either pEV, pKV-C, or pKV-CGA, was cultivated in pH-controlled bioreactors in MM at 30°C.

E. coli BW25113 (DE3) Δ*pta*-Δ*ldhA* (pKV-CGA) produced three times more itaconate than its wild type equivalent. Overexpression of *gltA* and *acnA* was essential to improve itaconate production, as production was not enhanced in *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* (pKV-C) (Figure 2.5). In all *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* cultivations, pyruvate accumulated up to 30 mM, after which it was consumed. Acetate was still produced in the double knockout strain, but with a significant delay. Citrate and/or *cis*-aconitate were also observed, but at trace levels without clear correlations with strain and growth conditions (results not shown).

The results show that the simultaneous elimination of *pta* and *ldhA* and the heterologous expression of *gltA* and *acnA* increased the flux through CadA, resulting in higher itaconate titers of up to 690 mg/L, which corresponds to an itaconate yield from glucose of 0.09 mol/mol.
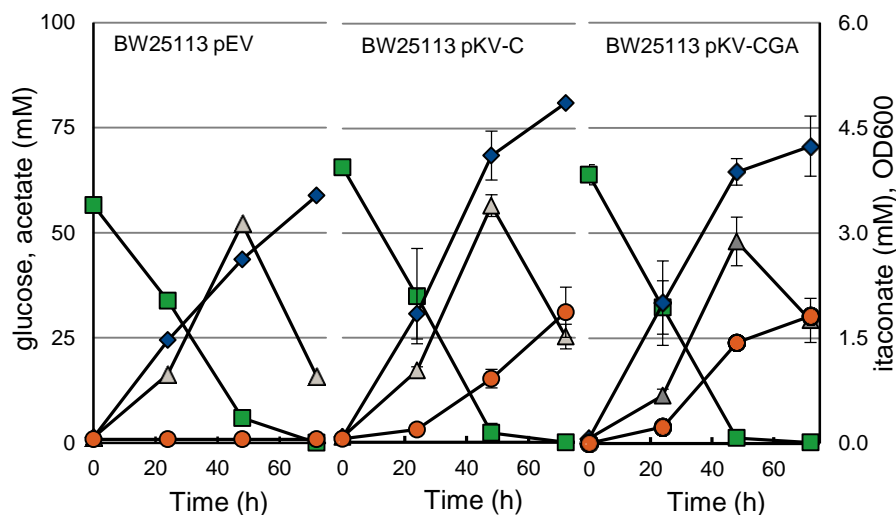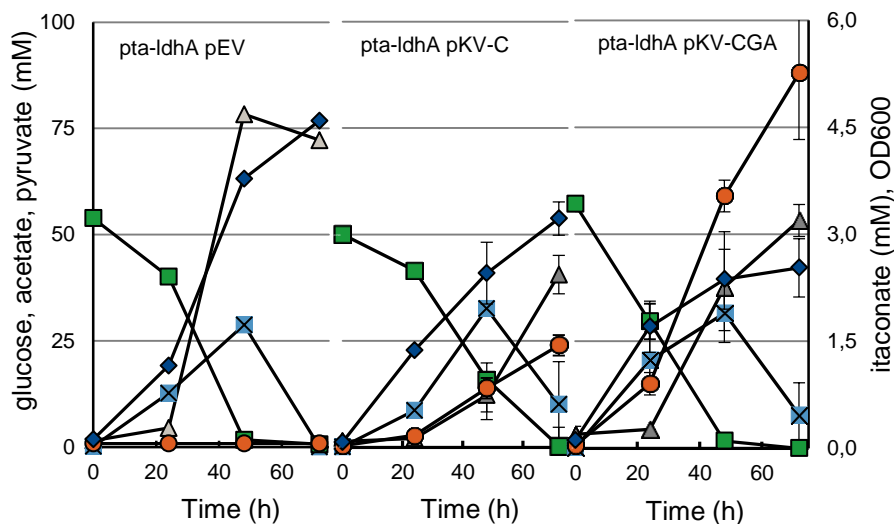
Figure 2.5 Batch cultivation of *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* containing pEV (left panel), pKV-C (middle panel) and pKV-CGA (right panel) in pH-controlled bioreactors on MM at 30°C. The OD_{600} (diamond) and the concentrations of glucose (squares), acetate (triangles), pyruvate (crosses) and itaconate (circles) are indicated.

2.4 Discussion

In recent years, several microorganisms have been investigated for their ability to produce itaconic acid, besides the well-known itaconic acid producer *A. terreus*. Some studies have focused on non-conventional natural producers of itaconic acid, such as *Pseudozyma antarctica* (Levinson et al., 2006) and *Ustilago maydis* (Klement et al., 2012). Besides, several heterologous production hosts, like *A. niger* (van der Straat et al., 2014)*, Yarrowia lipolytica* (Wang et al., 2011), and potato (Koops et al., 2011) were studied since *cadA* was identified as the gene responsible for itaconic acid biosynthesis in *A. terreus* (Kanamasa et al., 2008).

E. coli has been widely studied for the production of chemicals, such as lactic acid (Zhou et al., 2003), succinic acid (Lee et al., 2005), 1,3-

propanediol (Tong et al., 1991) and 1,4-butanediol (Yim et al., 2011, Yu et al., 2011). So far, *E. coli* has not been studied extensively for itaconic acid production. Only one study is published (Li et al, 2011), in which *E. coli* was used as a control strain for the identification of enzymes from *A. terreus* that are relevant for itaconic acid production. Overexpression of *cadA* in *E. coli* resulted in itaconate production, but at low titres (56 mg/L).

We focused on improving *E. coli*'s potential for itaconate production. Also in our work, the introduction of *cadA* resulted in low levels of itaconate (< 10 mg/L). Expression of heterologous genes in *E. coli* often causes problems that lead to the synthesis of inactive enzymes, such as protein misfolding and inclusion body formation (Baneyx, 1999). This was also observed in our study, but we could significantly enhance the heterologous production of active CadA by optimizing expression conditions (temperature and culture medium), which resulted in higher itaconate titres up to 240 mg/L.

The metabolic flux to itaconate does not only depend on CadA activity, but also on the availability of precursors. This availability is the resultant of the flux to itaconate and the fluxes to by-products. *E. coli* excretes acetate under aerobic conditions as an overflow metabolite when glucose is in excess (Castano-Cerezo et al., 2009). This indicates that the capacity of the glycolytic pathway is higher than the capacity of the TCA cycle. Citrate synthase is known to exert a strong control on the TCA flux, due to inhibition by high NADH concentrations (Holms, 1996), which is at least one of the reasons for acetate overflow, even at aerobic conditions (Vemuri et al., 2006). So a feasible approach to enhance itaconate production is to reduce acetate production and to diminish the control on the TCA flux.

As the citrate synthase of *C. glutamicum* (GltA) is not inhibited by high NADH concentrations (Eikmanns et al. 1994), we overexpressed *gltA* and simultaneously introduced the aconitase gene (*acnA*) from the same organism in *E. coli*. The corresponding enzymatic activities increased significantly in *E. coli*, but did not result as such in higher itaconate titres.

Acetate is produced from acetyl-CoA by phosphate acetyltransferase (Pta), which is constitutively expressed under both aerobic and anaerobic conditions (Chang et al., 1999b). Deletion of *pta* reduces the formation of acetate, and results in the accumulation of pyruvate (Chang et al., 1999b, Diazricci et al., 1991); (Tarmy and Kaplan, 1968). Acetate formation is still possible in *E. coli* Δ*pta* strains, due to the direct oxidation of pyruvate to acetate that is catalysed by pyruvate oxidase (*poxB*) (Abdel-Hamid et al., 2001).

Castano-Cerezo et al. (2009) reported a significant transient accumulation of lactate during cultivation of a Δ*pta* strain on glucose. We therefore decided to knock out both *pta* and *ldhA*, which resulted in a strain that accumulated pyruvate but not lactate, and showed a delayed acetate production. Overexpression of *cadA* in this Δ*pta*-Δ*ldhA* strain did not result in enhanced itaconate production, but itaconate production was significantly improved up to 690 mg/L when a combination of *cadA*, *gltA* and *acnA* was overexpressed in the Δ*pta*-Δ*ldhA* strain.

The production of acetate was delayed in our Δ*pta*-Δ*ldhA* strain, which is in line with studies that showed that expression of *poxB* is repressed in the early exponential phase (Castano-Cerezo et al. 2009) and is mainly activated at low growth rates (Abdel-Hamid et al. 2001). Still, acetate

remained a dominant by-product in our process. Elimination of *poxB* is an obvious strategy to decrease acetate formation, although (Phue et al., 2010) showed that acetate still accumulated when both *pta* and *poxB* were deleted in *E. coli*, indicating that more metabolic pathways are involved in acetate formation. An alternative approach is to overexpress the acetyl-CoA synthetase gene (*acs),* which is known to reduce acetate production and increase the intracellular acetyl-CoA concentration during aerobic growth on glucose (Lin et al., 2006).

A. terreus produces itaconic acid at pH values around 3, below the low pKa value of 3.85. Therefore itaconic acid is largely in its fully protonated form, which is also the required final product. *E. coli* grows is generally applied at pH values higher than 5.5, above the high pKa value of 5.45. Under these conditions itaconic acid will be largely in the fully deprotonated form. This has consequences for both fermentation and downstream process. Itaconic acid production by *E. coli* will require titration with a base to maintain a constant pH. During down-stream processing the pH will have to be decreased by adding acid to form the final fully protonated product, however with concomitant production of salts. Disposal or recycling of the salts contributes significantly to the overall process costs. On the other hand, organic acids are generally more toxic to microorganisms in their fully protonated form. Product inhibition will therefore be stronger at low-pH itaconic acid production by *A. terreus*. A similar situation occurs with the production of lactic acid, which has been more extensively studied. Down-stream processes at both low and high pH are being developed and improved (Abdel-Rahman et al., 2013), also in combination with in situ product removal to prevent product inhibition (Dafoe and Daugulis, 2014). At the moment it seems unclear if low or neutral pH processes will be the best option for production of organic acids.

To our best knowledge, 690 mg/L is the highest itaconate titre produced by metabolically engineered *E. coli* strains published in peer-review journals. This was realized without maximizing sugar concentrations and without employing growth limiting conditions. We obtained this titre by increasing the synthesis of soluble CadA and the availablity of precursors for itaconate by eliminating *pta* and *ldhA* and by overexpressing the genes that are responsible for the conversion of acetyl-CoA to the direct precursor *cis*-aconitate. The maximum itaconate yield from glucose was 0.09 mol/mol, which is only 7 % of the theoretical maximum yield of 1.33 mol/mol. A significant improvement is therefore required before the production of itaconate with *E. coli* can become economically feasible. Further optimization of *cadA* expression, and reduction of acetate formation are obvious strategies to achieve this.

2.5 Acknowledgements

Chapter 3

Heterologous expression of *Mus musculus* immunoresponsive gene 1 (*irg1*) in *Escherichia coli* results in itaconate production

Abstract

Itaconic acid, a C5-dicarboxylic acid, is a potential biobased building block for the polymer industry. It is obtained from the citric acid cycle by decarboxylation of *cis*-aconitic acid. This reaction is catalysed by CadA in the native itaconic acid producer *Aspergillus terreus*. Recently, another enzyme encoded by the mammalian immunoresponsive gene 1 (*irg1*), was found to decarboxylate *cis*-aconitate to itaconate *in vitro*. We show that heterologous expression of *irg1* enabled itaconate production in *E. coli* with production titres up to 560 mg/L.

3.1 Introduction

Itaconic acid is a biotechnologically produced monomer, which can be used as a precursor for many industrially important chemicals such as acrylic plastics, acrylate latices and absorbents. After being listed as a top 12 value-added biobased chemical compound (Werpy and Petersen, 2004), microbial production of itaconic acid has been of special interest for a decade.

Studies on the biotechnological production of itaconic acid have either focused on strain breeding of natural itaconic acid producers (mainly *Aspergillus terreus*), or on the heterologous expression of *cadA*, the key gene encoding *cis*-aconitate decarboxylase in *A. terreus* in other host organisms (Klement and Büchs, 2013). The economics of itaconic acid production with *A. terreus* are negatively influenced by the slow growth of the organism and the sensitivity of the filamentous pellets to hydro-mechanical stress, which inhibits mass transfer and consequently oxygen supply to the cells. Consequently, the production costs are still too high for industrial application of itaconic acid as a starting material (Steiger et al., 2013). Development of alternative hosts for itaconate production could overcome these problems. *E. coli* may be a suitable candidate host as it grows rapidly under both aerobic and anaerobic conditions and has well-established protocols for genetic modification.

The mammalian immunoresponsive gene 1 (*irg1*) was recently found to have *in vitro cis*-aconitate decarboxylase activity, although it has only 24 % amino acid sequence identity with CadA from *A. terreus* (Michelucci et al., 2013). However, both Irg1 and CadA share high identity with MmgE/PrpD family of proteins (Kanamasa et al., 2008, Lohkamp et al., 2006). Irg1 from *Mus musculus* was highly active in

mammalian macrophages during inflammation and was linked to having a role in immune defence by catalysing itaconic acid production (Michelucci et al., 2013), making the enzyme also an interesting candidate for biotechnological itaconic acid production. *E. coli* has been successfully used as a host for itaconic acid production by expressing *cadA* (Li et al., 2011, Okamoto et al., 2014, Vuoristo et al., 2015) and is therefore an interesting candidate to test whether *irg1* can be used for microbial itaconate production.

In earlier studies inclusion body formation was observed in *E. coli* that was overexpressing *cadA* from *A. terreus* (Vuoristo et al., 2015). Expression of heterologous genes in *E. coli* often causes problems such as protein miss-folding and inclusion body formation, which lead to synthesis of inactive enzymes. In recent years many tools, such as codon harmonization, have been developed to optimize a codon usage to its best. Compared to codon optimization, which substitutes codons by the most frequently used codons of the expression host (Makrides, 1996), codon harmonization selects the codons with usage frequencies in the expression host that most closely match the usage frequencies in the native host (Angov et al., 2008). This harmonization leads to better control of the translation speed and may prevent miss-folding of the nascent polypeptide (Angov et al., 2011).

In this study, we heterologously expressed *irg1* in *E. coli.* The proposed itaconate pathway in *E. coli* is shown in Figure 3.1. The effect of codon-harmonization on itaconate production and enzyme activity was determined. We showed that expression of mammalian immunoresponsive gene 1 (*irg1*) results in itaconate production in *E. coli*.
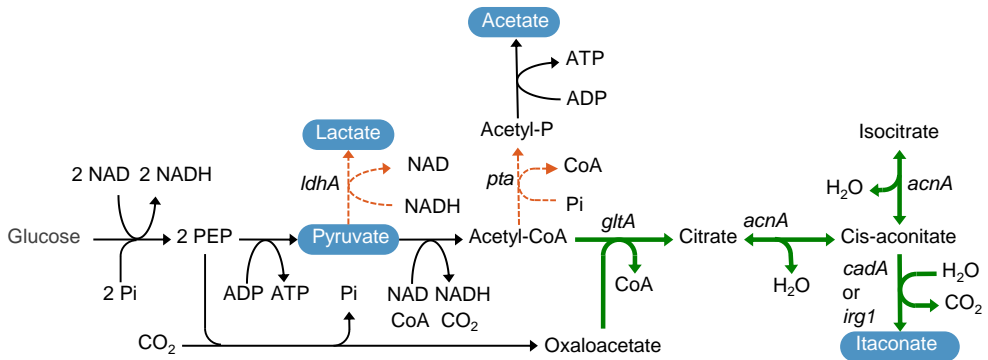
Figure 3.1 Itaconate production pathway in *E. coli*. The bold arrows indicate the introduced pathway consisting of genes encoding citrate synthase (*gltA*) and aconitase (*acnA*) from *C. glutamicum* and *cis*-aconitate decarboxylase (*cadA)* from *A. terreus* or immunoresponsive gene 1 (*irg1*) from *M. musculus*. The dotted lines indicate that phosphate acetyltransferase (*pta*) and lactate dehydrogenase (*ldhA*) were deleted.

3.2 Materials and methods

3.2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 3.1.

3.2.2 Codon-optimization and codon-harmonization

Codon-optimization was done according to the algorithm of OptimumGene^{TM} (GenScript). The codon usage of *cadA* from *A. terreus* and *irg1* from *M. musculus* was harmonized to that of *E. coli.* For this, the codon usage frequency per codon was determined for the native organism and for the host by using the graphical codon usage analyser tool (Fuhrmann et al., 2004). The codons used in the harmonized genes are the *E. coli* codons that mimicked the codon usage frequencies in the native organism the best. Genes were synthesized by GenScript and nucleotide sequences were deposited to NCBI BankIt (accession numbers: *cadA* optimized KM464677, *cadA* harmonized KT273316, *irg1* optimized KT273318 and *irg1* harmonized KT273317).

Table 3.1 *E. coli* strains and plasmids used in this study.

Strains and plasmids	Characteristics	References
Strains		
BW25113 (DE3)	**BW25113 DE3 T7 RNA polymerase**	**(Vuoristo et al., 2015)**
BW25113 (DE3) Δ*pta*Δ*ldhA*	**BW25113 Δ*pta* Δ*ldhA* DE3 T7 RNA polymerase**	**(Vuoristo et al., 2015)**
Plasmids		
pKV-GAC^{opt}	**pACYCDuet-1 derivative (Novagen), codon-optimized *gltA* and *acnA* from *Corynebacterium glutamicum*, codon-optimized *cadA* from *Aspergillus terreus***	**(Vuoristo et al., 2015)**
pKV-GA	**pACYCDuet-1 derivative, codon-optimized *gltA* and *acnA* from *C. glutamicum***	**This study**
pKV-GAC^{har}	**pACYCDuet-1 derivative (Novagen), *gltA* and *acnA* from *C. glutamicum*, codon-harmonized *cadA* from *A. terreus***	**This study**
pKV-GAI^{opt}	**pACYCDuet-1 derivative (Novagen), codon-optimized *irg1* from *M. Musculus*, *acnA*, and *gltA* from *C. glutamicum***	**This study**
pKV-GAI^{har}	**pACYCDuet-1 derivative (Novagen), codon-harmonized *irg1* from *C. glutamicum*, codon- optimized *acnA*, and *gltA* from *C. glutamicum***	**This study**

3.2.3 Construction of pACYCDuet-1 expression vectors

All strains and plasmids used in this work are given in Table 1. All optimized and harmonized *cadA* and *irg1* sequences were expressed by using the T7 promotor in MCS1 of pACYCDuet-1. The control vector pKV-GA was derived from pKV-GAC^{opt} by cloning the *acnA* and *gltA*-containing part of pKV-GAC^{opt} in MCS2 of pACYCDuet-1. In addition, harmonized *cadA*, optimized *irg1* or harmonized *irg1* sequences were cloned in MCS1 of pKV-GA resulting in pKV-GAC^{har}, pKV-GAI^{opt} and pKV-GAI^{har}, respectively. Constructed plasmids were verified by sequencing.

3.2.4 Culture media

For plasmid construction, *E. coli* strains were cultured at 37°C on lysogeny broth (LB) agar plates or in LB liquid medium and agitation rates of 200 rpm. Medium and plates were supplemented with chloramphenicol (35 µg/mL).

The other cultivations were done in M9 minimal medium (MM), which contained per 1 liter: 200 mL 5×M9 Minimal Salts (BD Difco) supplemented with 50 mM of glucose, 2 mM of MgSO4, 0.1 mM of $CaCl_2$, 15 mg of thiamine, and 0.30 mg of selenite and US* trace elements (Panke et al., 1999). Medium was buffered with 0.1 M 3-(N-morpholino) propanesulfonic acid (MOPS) and the pH was adjusted to 6.9 with NaOH. Induction of gene expression was started by the addition of 0.5 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) when the optical density at 600 nm (OD_{600}) of the culture reached approximately 0.4.

3.2.5 SDS-PAGE

For SDS-PAGE, *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* containing either pKV-GA, pKV-GAC^{opt}, pKV-GAC^{har}, pKV-GAI^{opt} or pKV-GAI^{har} was cultivated for 16 hours at 37˚C and 200 rpm in 2 mL MM. Subsequently, the overnight cultures were transferred into 20 mL of fresh MM and incubated at 25 ˚C and 200 rpm. When the cultures reached an OD_{600} of approximately 0.4, they were induced with 0.5 mM IPTG and incubated overnight at 25 ˚C and 200 rpm.

16 mL of the cultures were used to make cell free extracts (CFE) according to the Y-PER Yeast Protein Extraction Reagent kit instructions (Thermo Scientific). 4 mL of the cultures were harvested by centrifugation (2 min, 14000 rpm), resuspended in a small volume of water, and loaded on pre-casted Criterion XT SDS-PAGE gels (Bio-Rad) together with the CFE's according to manufacturer's instructions (Criterion™ Cell, Bio-Rad). The proteins were stained by Bio-Safe Coomassie staining (Bio-Rad). Protein sizes were determined by using Precision Plus Protein™ All Blue Standards ladder (Bio-Rad).

3.2.6 Bioreactor cultures

E. coli BW25113 (DE3) Δ*pta*-Δ*ldhA* containing either pKV-GA, pKV-GAC^{opt}, pKV-GAC^{har}, pKV-GAI^{opt} or pKV-GAI^{har} was cultivated at 25°C in 0.5 L Mini Bioreactors, connected to myControl controller units (Applikon, The Netherlands) with a working volume of 400 ml. The pH was maintained at 6.9 by the automated addition of 2 M NaOH. Bioreactors were inoculated with 5 % (v/v) of a pre-culture that was grown at 37 °C in a 250 mL Erlenmeyer flasks with 50 mL of MM at 250 rpm for 24 hours. The bioreactor cultures were stirred at 500 rpm and

sparged with air at 200 mL/min for 8 hours, after which the stirring speed was increased to 1200 rpm and the sparging rate was increased to at 400 mL/min. Samples of 2 mL were regularly taken to determine the OD_{600} of the cultures and the concentrations of substrate and products.

3.2.7 *cis*-Aconitate decarboxylase assay

For enzymatic assays, 30 mL of bioreactor culture was harvested by centrifugation (5 min, 7745 × *g*) after 17 hours of cultivation in the presence of IPTG. Cell free extracts (CFE) were made according to the Y-PER Yeast Protein Extraction Reagent kit instructions (Thermo Scientific). Protein concentrations were determined by using the Total Protein Kit, Micro Lowry, Onishi & Barr Modification (Sigma Aldrich).

The activity of CadA and Irg1 was measured with *cis*- and *trans*-aconitate according to Vuoristo *et al.*, 2015. Besides, the Irg1 activity was measured by using a method adapted from Michellucci *et al.*, 2013 with the following modifications: CFE's were incubated with 200 mM of *cis*-aconitate in 25 mM HEPES buffer (pH 7.1) supplied with proteinase inhibitor (cOmplete Protease Inhibitor Cocktail Tablets, Roche) for 50 min at 30°C.

For whole cell assays, 20 mL of bioreactor culture was harvested by centrifugation (5 min, 7745 × *g*) after 17 hours of cultivation in the presence of IPTG. Cells were washed with M9 salts in 100 mM MOPS (pH 7.1) and resuspended in 10 ml of the same medium. 20 mM of substrate (*cis*- or *trans*-aconitate) was added to 2 mL of the cell suspension and incubated at 30 °C and 100 rpm. 1 M HCl was added to terminate the

reaction, after which the supernatants were analysed for itaconate formation by HPLC.

3.2.8 Analytical methods

The cell density was determined by measuring the OD_{600} by using a DR6000 spectrophotometer (Hach Lange). The concentrations of glucose and organic acids were determined by using a Perkin Elmer (200 series) HPLC equipped with an RI detector (PerkinElmer, Flexar) for measuring glucose and an UV detector (PerkinElmer, Flexar UV/V, at 210 nm) for citric acid, itaconic acid, *cis-* and *trans*-aconitic acid. The samples were separated on a Micro Guard Cation H pre-column (30x4.6mm, Biorad) and an Aminex HPX-87H column (300x7.8 mm, Biorad) at 35°C, using 0.5 mL/min of 5 mM H_2SO_4 as eluent.

3.3 Results

3.3.1 Heterologous expression of *cadA* and *irg1*

Optimized and harmonized gene sequences of *cadA* from *A. terreus* and *irg1* from *M. musculus* were expressed in *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* together with *gltA and acnA* from *C. glutamicum*. The strains were cultivated in shake flasks on MM at 25 °C and induced with IPTG. Samples of culture pellets and CFE of the cultures were analysed by SDS-PAGE. A protein band of around 53 kDa appeared on the SDS-PAGE gels, which is the expected size of the proteins encoded by *cadA* and *irg1* (figure provided in the supplementary material). The samples of culture pellets gave very thick protein bands at the expected sizes, while much thinner bands were obtained with CFE's, indicating that CadA and Irg1 were mostly in the form of inclusion bodies. No large differences

were observed between the amounts of CadA or Irg1 on the gels after expression of the optimized and harmonized genes. The proteins encoded by *gltA* and *acnA* have sizes of 49 kDA and 114 kDA.

3.3.2 Enzymatic assays

cis-Aconitate decarboxylase activity was measured in CFE's of bioreactor cultures after 17 hours of cultivation at 25 ºC in the presence of IPTG. The specific conversion rate of *cis*-aconitate to itaconate in CFE of *E. coli* BW25113 (DE3) *Δpta-ΔldhA* with pKV-GAC^har was 1.39 μmol itaconate/min/mg protein, which was 1.7 times higher than with pKV-GAC^opt. CadA activities were higher than those previously obtained with cells cultivated at 30 and 37 ºC (Vuoristo et al., 2015), indicating that inclusion body formation is more suppressed at 25 ºC. Itaconate formation was not detected with CFE's of cultures with pKV-GA, pKV-GAI^opt or pKV-GAI^har. Instead, *cis*-aconitate was converted to citrate, which is likely caused by the activity of aconitase in the CFE. Itaconate formation was also absent in cell lysates of pKV-GAI^opt and pKV-GAI^har that were not centrifuged, indicating that Irg1 activity is not present in the membrane fraction of the cells. *Trans*-aconitate was not converted by any of the CFE's.

Irg1 activities were also determined according to Michellucci *et al.,* 2013. Again, no itaconate formation was detected under these conditions (results not shown). The absence of conversion of *cis*-aconitate to itaconate in CFE of *E. coli* BW25113 (DE3) *Δpta-ΔldhA* expressing *irg1* suggests that Irg1 was inactivated in the period between the harvest of the cells and the measurements.

3.3.3 Whole cells assays

Conversion of *cis*-aconitate to itaconate by cells of *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* containing pKV-GA, pKV-GAC^{opt}, pKV-GAC^{har}, pKV-GAI^{opt} and pKV-GAI^{har} was measured by incubating cells in the presence of 20 mM of *cis*- or *trans*-aconitate and measuring the formation of itaconate in the supernatant. Whole cells containing pKV-GAC^{opt}, pKV-GAC^{har}, pKV-GAI^{opt} and pKV-GAI^{har} converted *cis*-aconitate to itaconate. *Trans*-aconitate was not converted (data not shown). The highest conversion rate was observed with pKV-GAI^{har} (1.10 µmol itaconate/min/mg cells, Figure 3.2), which was much higher than with pKV-GAI^{opt} (0.19 µmol itaconate/min/mg cells). The conversion rates of pKV-GAC^{opt} and pKV-GAC^{har} were (0.41 and 0.31 µmol itaconate/min/mg cells).
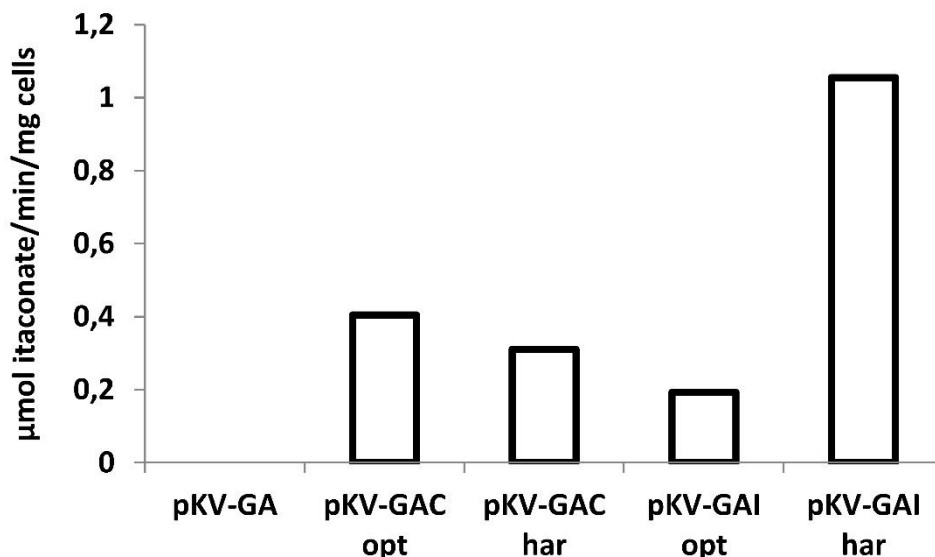

Figure 3.2 Conversion of *cis*-aconitate to itaconate (µmol itaconate/min/mg cells) by whole cells of *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* containing pKV-GA, pKV-GAC^{opt}, pKV-GAC^{har}, pKV-GAI^{opt} and pKV-GAI^{har}. The average values of duplicate measurements are given.

3.3.4 Bioreactor cultures

The effect of expression of *cadA* and *irg1* on itaconate production was studied in *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA*, in which the genes encoding phosphate acetyltransferase and lactate dehydrogenase were disrupted. *gltA* and *acnA* from *C. glutamicum* were expressed together with the target genes. The strains were cultivated under aerobic conditions in pH-controlled bioreactors on MM at 25 °C. Itaconate was produced in all cultures, except in the control pKV-GA (Figure 3.3). Strains containing the harmonized genes (pKV-GAC[har] and pKV-GAI[har]) produced slightly less itaconate than those containing the optimized genes (pKV-GAC[opt] and pKV-GAI[opt]). The latter strains produced 5.5 and 4.3 mM, respectively.

3.4 Discussion

Since the identification of the gene encoding *cis*-aconitate decarboxylase (*cadA*) in *Aspergillus terreus* (Kanamasa et al., 2008), there has been a growing interest to produce itaconic acid in recombinant hosts such as *E. coli* (Li et al., 2011, Liao and Chang, 2010). Metabolic engineering strategies to increase the flux through the citric acid cycle and to reduce by-product formation have significantly increased the itaconate titres obtained with *E. coli* (Okamoto et al., 2014, Vuoristo et al., 2015), but they are still far below those obtained with *A. terreus* (Hevekerl et al., 2014). One of the possible bottlenecks in *E. coli* is the low activity of the heterologous CadA.
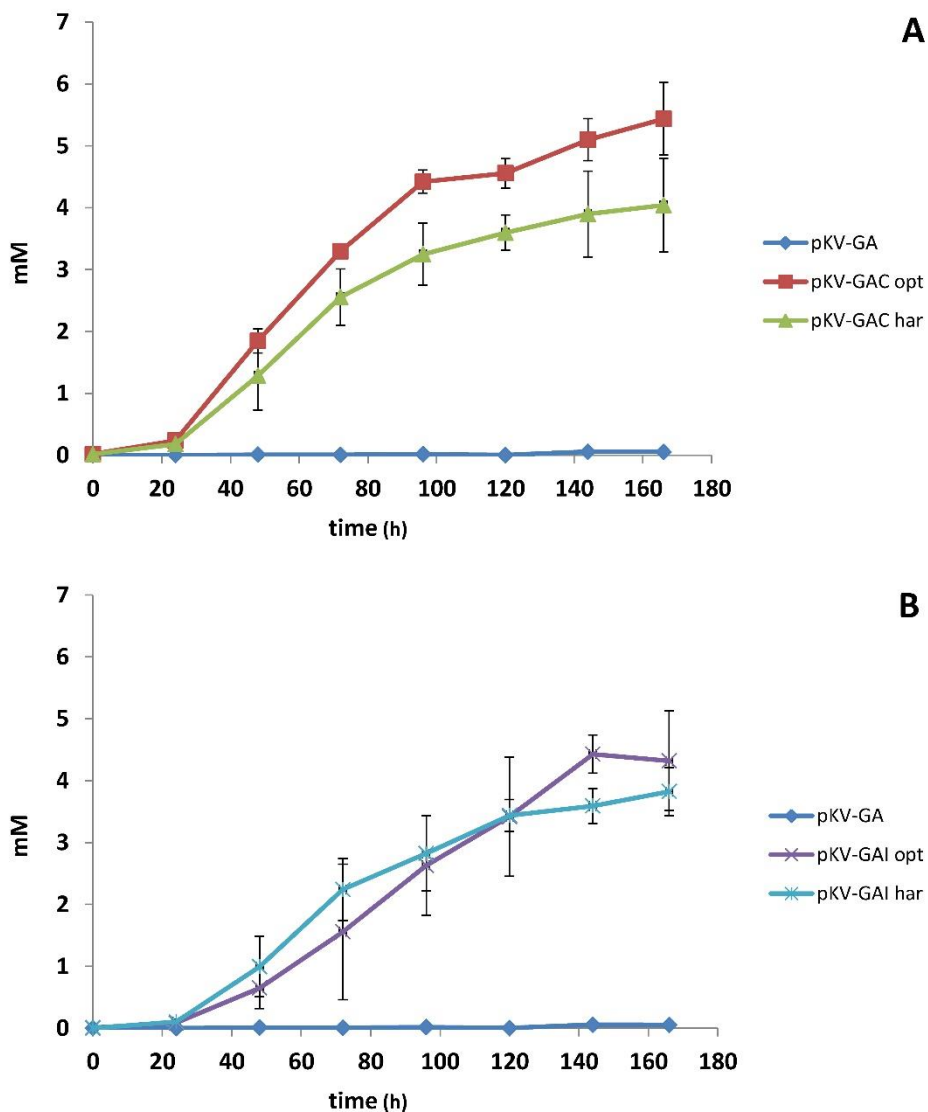
Figure 3.3 Itaconate production in bioreactor cultures containing *E. coli* BW25113 (DE3) Δ*pta-*Δ*ldhA* with pKV-GA, pKV-GAC[opt] or pKV-GAC[har] (panel A) or pKV-GA, pKV-GAI[opt] or pKV-GAI[har] (panel B). The average values and standard deviations (SD) of duplicate cultures are given.

Although *E. coli* is a widely used cell-factory for the production of proteins and chemicals, problems such as inclusion body formation and low enzyme activity are often associated with heterologous production of proteins. For instance, low enzyme activities may occur when the frequency of synonymous codons in foreign coding DNA significantly differs from that of the host (Rosano and Ceccarelli, 2014). Harmonization of the codon usage frequencies of the target gene with those of the expression host can increase their expression (Angov et al., 2008, Van Zyl et al., 2014). We compared expression of codon optimized and harmonized sequences of *cadA* and *irg1* by determining enzyme activities and performance in bioreactor cultures. The specific enzymatic activity with harmonized *cadA* was 1.7 times higher than with optimized *cadA*. In contrast, the specific conversion rate of *cis*-aconitate by whole cells containing pKV-GAC^har was only 75% of the rate observed with pKV-GAC^opt, and the titre obtained in bioreactor cultures with pKV-GAC^har was 74% of that obtained with pKV-GAC^opt. This suggests that other factors, like itaconate export, by-product formation, or levels of other enzymes involved in itaconate biosynthesis have a larger effect on itaconate production than the activity of CadA.

Okamoto *et al*., 2014 measured increased intracellular itaconate concentrations in their production strains and suggested that extracellular secretion of itaconate in *E. coli* limits the production. Itaconate transport has been studied in *Aspergillus* species and a few putative candidates have been characterized (Li et al., 2011). Expression of *A. terreus* mitochondrial transporter *mttA* and plasma membrane transporter *mfsA* were beneficial to production in *Aspergillus niger* (Li et al., 2013), (van der Straat et al., 2014). So far, itaconate transporters have not been identified in *E. coli*, and the transport

mechanism is unknown, which makes them obvious research targets for the future.

Besides CadA, alternative *cis*-aconitate decarboxylases can facilitate itaconate production. Recently, the gene product of immunoresponse gene 1 (*irg1*) from *M. musculus* was shown to catalyse the decarboxylation of *cis*-aconitate to itaconic acid *in vitro* (Michelucci et al., 2013). We were unable to detect any *cis*-aconitate decarboxylase activity in CFE's of *E. coli* cultures expressing *irg1,* although the cultures were producing itaconate. This indicates that the enzyme was inactivated during preparation of CFE's. Whole cells of pKV-GAI^har converted *cis*-aconitate nearly 5.5 times as fast as pKV-GAI^opt. Still, the titre of pKV-GAI^har was less than the titre of pKV-GAI^opt.

We showed for the first time that introduction of this mammalian enzyme also results in itaconate production in *E. coli* with titres up to 560 mg/L. The titres are similar to those obtained with *cadA*-expressing *E. coli* strains (700 mg/L), although the protein sequences are only 24 % identical.

3.5 Conclusions

We have successfully expressed a mammalian *cis*-aconitate decarboxylase encoded by *irg1* from *M. musculus* in *E. coli.* Titres up to 560 mg/L of itaconate were obtained with *irg1*, which are comparable to those obtained with *cadA* from *A. terreus* (700 mg/L). Codon harmonization increased the activity CadA in CFE, but this did not result in higher itaconate production in bioreactor cultures, suggesting that other factors are limiting. Besides *irg1* from mouse, also other immunoresponse genes are known. Many of them may possess CadA activity and it should be investigated which ones are best for itaconate production.

3.6 Supplementary material
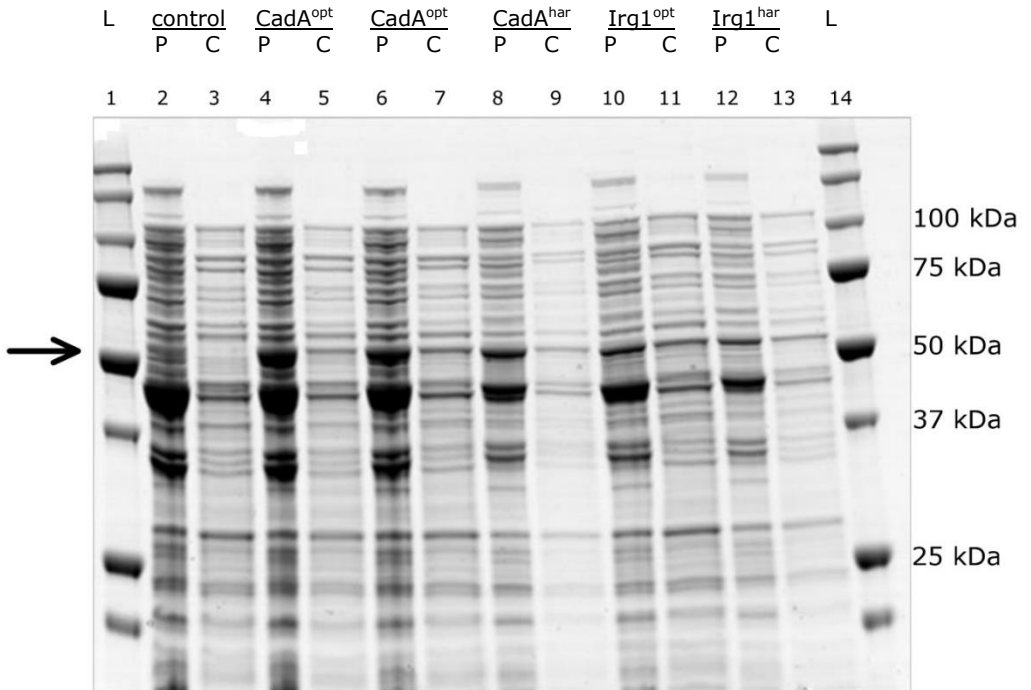
Protein profiles of *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* pKV-GA, pKV-GAC^{opt}, pKV-GAC^{har}, pKV-GAI^{opt} and pKV-GAI^{har}. Lane 1 and 14: Precision Plus Protein All Blue standards. Lane 2-3: pKV-GA, 4-7: pKV-GAC^{opt}, 8-9:pKV-GAC^{har}, 10-11:pKV-GAI^{opt}, 12-13:pKV-GAI^{har}. Even lanes contain culture pellets (P), odd lanes contain CFE's (C). The arrow indicates the position of CadA and Irg1 around 53 kDa.

Author contributions

Kiira S. Vuoristo performed the bioreactor experiments, enzymatic assays and drafted the manuscript. Astrid E. Mars participated in the design of the experiments and helped to draft the manuscript. Stijn van Loon and Enrico Orsi constructed the strains and verified the expression of heterologous genes on SDS-PAGE. Gerrit Eggink supervised the project and commented the manuscript. Johan P. M. Sanders conceived the project and commented the manuscript. Ruud A. Weusthuis conceived, designed and supervised the project, and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Chapter 4

Metabolic engineering of the mixed-acid fermentation pathway of *Escherichia coli* for anaerobic production of glutamate and itaconate

Abstract

Itaconic acid, an unsaturated C5-dicarboxylic acid, is a biobased building block for the polymer industry. The purpose of this study was to establish proof of principle for an anaerobic fermentation process for the production of itaconic acid by modification of the mixed acid fermentation pathway of *E. coli*.

E. coli BW25113 (DE3) and the phosphate acetyltransferase (*pta*) and lactate dehydrogenase (*ldhA*) deficient strain *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* were used to study anaerobic itaconate production in *E. coli*. Heterologous expression of the gene encoding *cis*-aconitate decarboxylase (*cadA)* from *A. terreus* in *E. coli* BW25113 (DE3) did not result in itaconate production under anaerobic conditions, but 0.08 mM of itaconate was formed when the genes encoding citrate synthase (*gltA*) and aconitase (*acnA*) from *Corynebacterium glutamicum* were also expressed. The same amount was produced when *cadA* was expressed in *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA*. The titre increased 8 times to 0.66 mM (1.2 % Cmol) when *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* also expressed *gltA* and *acnA*. In addition, this strain produced 8.5 mM (13 % Cmol) of glutamate. The use of a nitrogen-limited growth medium reduced the accumulation of glutamate by nearly 50 % compared to the normal medium, and also resulted in a more than 3-fold increase of the itaconate titre to 2.9 mM.

These results demonstrated that *E. coli* has potential to produce itaconate and glutamate under anaerobic conditions, closing the redox balance by co-production of succinate or ethanol with H_2 and CO_2.

4.1 Introduction

Itaconic acid, an unsaturated C5 dicarboxylic acid produced by various microorganisms such as *Aspergillus terreus*, can be used as a precursor for many relevant compounds in chemical and pharmaceutical industries. It is especially of interest for the production of polymers, because of its potential as a substitute for acrylic and methacrylic acid (Okabe et al., 2009).

Current fermentation processes for the production of itaconic acid from sugar are executed aerobically using oxygen as the terminal electron acceptor (Kuenz et al., 2012). Aerobic processes result in higher operating and capital costs compared to anaerobic processes due to the lower yields and increased demands for oxygen and heat transfer (Cuellar et al., 2013, Zeikus, 1980). Because of this, Zeikus (Zeikus, 1980) stated that anaerobic fermentations form the basis for microbial production of chemicals and fuels. It is therefore interesting to design an anaerobic process for itaconic acid production.

The conversion of glucose to itaconate is an oxidation reaction that results in the net reduction of the NAD cofactor. Respiration is used to reoxidize NADH under aerobic conditions. Under anaerobic conditions alternative methods have to be employed for cofactor regeneration (Weusthuis et al., 2011)

E. coli can produce itaconic acid under aerobic conditions when the *cis*-aconitate decarboxylase gene (*cadA*) from *Aspergillus terreus* is expressed (Li et al., 2011). We recently showed that itaconate production by *E. coli* is improved by enhancing the availability of precursors by overexpression of the first part of the citric acid cycle (citrate synthase (*gltA*) and aconitase (*acnA*) from *C. glutamicum* and

reduction of the native metabolic routes to acetate and lactate by inactivating the genes encoding phosphate acetyltransferase (*pta*) and lactate dehydrogenase (*ldhA*) (Vuoristo et al., 2015)*.

E. coli is also one of the few industrial microorganisms that is able to grow under anaerobic conditions. It is therefore a suitable candidate to test whether anaerobic production of itaconic acid is possible. The mixed acid fermentation pathway of *E. coli* offers two options to regenerate NAD: the conversion of glucose and CO_2 into succinate and the conversion of glucose into ethanol and formate (or hydrogen and CO_2). The proposed heterofermentative pathway to itaconate and succinate or ethanol and formate/H_2 is shown in Figure 4.1.
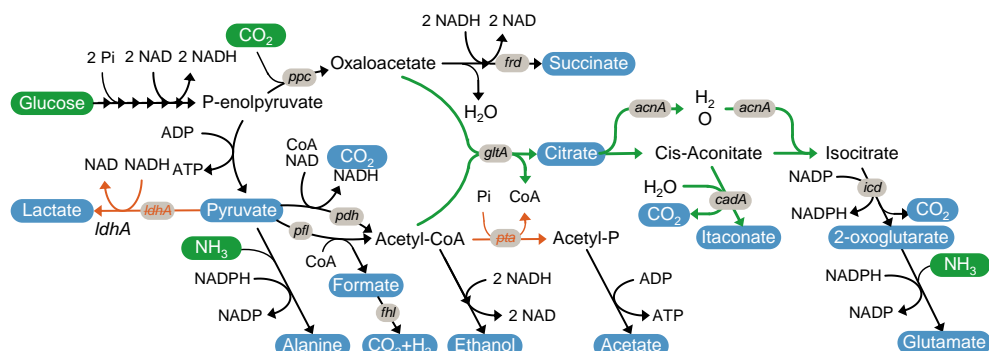
Figure 4.1 Anaerobic itaconate pathway in metabolically engineered *E. coli*. The green bold arrows indicate the introduced pathway consisting of genes encoding citrate synthase (*gltA*) and aconitase (*acnA*) from *C. glutamicum* and *cis*-aconitate decarboxylase (*cadA*) from *A. terreus*. The red lines indicate that phosphate acetyltransferase (*pta*) and lactate dehydrogenase (*ldhA*) were deleted.

In this paper, we cultivated *E. coli* strains under anaerobic conditions and analysed the effect of the introduction of *cis*-aconitate decarboxylase from *A. terreus* and citrate synthase and aconitase from *Corynebacterium glutamicum* on itaconate production, growth and formation of other fermentation products. Unexpectedly, strains started to produce significant amounts of glutamate when the itaconate

pathway was introduced. Up to 2.9 mM of itaconate was produced when nitrogen-limited growth medium was introduced.

4.2 Materials and methods

4.2.1 Construction of pACYC expression vectors

All strains and plasmids used in this work are given in Table 4.1. The expression vector pKV-GA was derived from pKV-CGA by cloning the *acnA* and *gltA*-containing part of pKV-CGA in pACYC-Duet-1.

Table 4.1 *E. coli* strains and plasmids used in this study.

Strains and plasmids	Characteristics	References
Strains		
BW25113 (DE3)	BW25113 DE3 T7 RNA polymerase	(Vuoristo et al., 2015)
BW25113 (DE3) Δ*pta*Δ*ldhA*	BW25113 Δ*pta* Δ*ldhA* DE3 T7 RNA polymerase	(Vuoristo et al., 2015)
BW25113 (DE3) Δ*pta*Δ*ldhA*Δ*icd*	BW25113 Δ*pta*Δ*ldhA*Δ*icd* DE3 T7 RNA polymerase	This study
Plasmids		
pKD46	AmpR plasmid with temperature-sensitive replication and arabinose induction of λ-red recombinase	The Coli Genetic Stock Center at Yale University (CGSC)
pKD13	KanR plasmid with R6K-γ replicon	CGSC
pCP20	AmpR and CmR plasmid with temperature-sensitive replicon and thermal induction of FLP synthesis	CGSC
pEV	pACYCDuet-1 expression vector using T7 promoter, with two multiple cloning sites, CmR	Novagen
pKV-C	pACYCDuet-1 derivative, synthetic *cadA*	(Vuoristo et al., 2015)
pKV-CGA	pACYCDuet-1 derivative, synthetic *cadA*, *acnA*, and *gltA*	(Vuoristo et al., 2015)
pKV-GA	pACYCDuet-1 derivative, synthetic *acnA*, and *gltA*	This study

4.2.2 Cultivation conditions

1) Culture media

For plasmid construction, *E. coli* strains were cultured on Luria-Bertani (LB) agar plates or in LB liquid medium at either 30°C or 37°C. Recombinants harbouring temperature-sensitive plasmids were cultured at 30°C for cultivation and at 42°C to cure the selection markers. When needed, medium and plates were supplemented with ampicillin (50 μg/mL), kanamycin (50 μg/mL) or chloramphenicol (35 μg/mL). Induction of gene expression in liquid cultures was started by the addition of 1 mM of IPTG when the optical density at 600 nm (OD_{600}) of the culture was approximately 0.4.

The other cultivations were done either in M9 Minimal medium (MM) or in nitrogen-limited minimal medium (NL-MM) with chloramphenicol (35 μg/mL). MM contained per 1 liter: 200 mL 5×M9 Minimal Salts (BD Difco) supplemented with 50 mM of glucose, 2 mM of MgSO4, 0.1 mM of $CaCl_2$, 15 mg of thiamine, and 0.30 mg of selenite. Medium was buffered with 0.1 M 3-(N-morpholino) propanesulfonic acid (MOPS) and the pH was adjusted to 6.9 with NaOH. The nitrogen limited medium (NL-MM) contained 0.5 g/L NH_4Cl, which is 50 % less than in standard M9. In some cultivations, MM and NL-MM were supplemented with US* trace elements (Panke et al., 1999), yielding MM* and NL-MM*, respectively, to reduce the lag phase.

2) Cultivation in bioreactors

E. coli BW25113 (DE3) and *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* containing either pEV, pKV-C or pKV-CGA were cultivated at 30°C in 0.5 L Mini Bioreactors, connected to myControl controller units (Applikon,

The Netherlands) with a working volume of 400 mL. The pH was maintained at 6.9 by the automated addition of 2 M NaOH. Cultures were stirred at 400 rpm and sparged with nitrogen at 16 mL/min for 17 hours, after which the stirring speed was increased to 800 rpm and the sparging rate was increased to at 35 mL/min. Bioreactor cultures that were grown in MM* or NL-MM* were stirred at 500 rpm and sparged with air at 150 mL/min for 4 hours, followed by nitrogen sparging at 35 mL/min. Bioreactors were inoculated with 5 % (v/v) of a pre-culture that was grown at 30 °C in a 250 mL Erlenmeyer flasks with 50 mL of MM at 250 rpm for 24 hours. Samples of 2 mL were regularly taken to determine the OD_{600} of the cultures and the concentrations of substrate and products. The product distribution (% Cmol) was calculated by dividing the amounts of products formed by the amount of substrate consumed after 72 hours of cultivation.

4.2.3 Deletion of isocitrate dehydrogenase (*icd*) gene

The gene encoding isocitrate dehydrogenase (*icd*) was inactivated in *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* by using the Lambda red-mediated gene replacement method described by Datsenko and Wanner (Datsenko and Wanner, 2000). Shortly, *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* was transformed with pKD46 and cultured in the presence of L-arabinose to induce λ-red recombinase expression, which is an inducer for recombination. The target gene *icd* was replaced by a kanamycin-resistance gene flanked by flippase recognition target (*FRT*) sites. For this, a deletion cassette containing a kanamycin-resistance gene with *FRT* sites was amplified from pKD13 by using Phusion High Fidelity DNA Polymerase (Thermo Scientific) and primers that contain 50 bp targeting flanks to the *icd* region in the genome (Table 2), and transformed into *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* (pKD46). Transformants were screened

for their proper genotype by selecting for kanamycin resistance and colony PCR (GoTaq Green polymerase, Promega) using primers that flank the target gene. The phenotype was verified in liquid cultures and by sequencing. The kanamycin-resistance gene was subsequently eliminated by using the temperature-sensitive helper plasmid pCP20 encoding the flippase (*FLP*), followed by curing of the temperature sensitive plasmids by culturing strains at 42 °C for 16 hours.

The presence of the 1.5 kB *cadA* region in *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA*Δ*icd* that was transformed with either pKV-C or pKV-CGA were analysed by PCR using GoTaq Green DNA polymerase (Promega) and primers pACYC MCS1 F and pACYC MCS2 R (Table 4.2).

Table 4.2 List of primers used in this study. The 50 bp targeting flanks are underlined.

Name and description	Sequence
For *icd* deletion	
icd flank F	<u>CCCGTTAATAAATTTAACAAACTACGGCATTACA</u> <u>TGTTTTCGATGATCGC</u>TGTAGGCTGGAGCTGCTTCG
icd flank R	<u>AACGTGGTGGCAGACGAGCAAACCAGTAGCGCT</u> <u>CGAAGGAGAGGTGAATG</u>ATTCCGGGGATCCGTCGACC
For PCR verifications	
icd check F	ACGTGGTGGCAGACGAGCAAAC
icd check R	TTAATAAATTTAACAAACTACGG
pACYC MCS1 F	GGATCTCGACGCTCTCCCT
pACYC MCS1 R	GATTATGCGGCCGTGTACAA
Sequencing	
icd200seqF	GGATCACACGCGTGGGCTG
icd200seqR	GGTGTAAGGAGTGGTAATTCA

4.2.4 Analytical methods

The cell density was determined by measuring the OD_{600} by using a spectrophotometer (Dr. Lange XION 500). For CDW determination, 100 ml of fermentation medium was centrifuged (7745×g, 10 min), and the pellet was washed with 0.7 % (w/v) NaCl. The pellet was resuspended in deionized water and dried at 100°C until constant weight. The relation between OD and CDW was found to be CDW[g/l]=0.25×OD600. The molar ratio of $CH_{1.83}O_{0.5}N_{0.22}P_{0.017}$, which corresponds to a molecular weight of 24.63 g per mol C was used to determine the amount of C in biomass.

The concentrations of glucose, ethanol and organic acids were determined by using HPLC by using a Dionex Ultimate 3000 (Thermo Fisher) equipped with an RI detector (Shodex, RI-101) and a UV detector (Dionex, 3400 RS at 210 nm). The samples were separated on a Micro Guard Cation H pre-column (30x4.6mm, Biorad) and an Aminex HPX-87H column (300x7.8 mm, Biorad) at 35°C, using 0.6 mL/min of 5 mM H_2SO_4 as eluent.

The concentrations of glutamate and alanine were determined by using UPLC Dionex RSLC system with an UltiMate 3000 Rapid Separation pump as described by Meussen et al. 2014 (Meussen et al., 2014). Glutamate concentrations were also determined by using L-Glutamic Acid Assay Kit (K-GLUT07/12, Megazyme).

The concentrations of CO_2 and H_2 in the off-gas of the bioreactors were determined by using BlueSens Off-Gas Sensors (Gas Sensor, GmbH).

4.3 Results

4.3.1 Itaconate production under anaerobic conditions

E. coli BW25113 (DE3) and *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* containing pEV. pKV-C or pKV-CGA were grown on M9 Minimal medium (MM) at pH 6.9 in pH-controlled bioreactors under anaerobic conditions with glucose as carbon source. The main fermentation products of *E. coli* BW25113 (DE3) (pEV) were lactate, ethanol, formate and acetate (Figure 4.2), which accounted for 74 % of the carbon that was added to the culture (Table 4.3). As a lot of formate (16 % Cmol) was formed in *E. coli* BW25113 (DE3) (pEV), only low amounts of CO_2 (3 mmol/L, < 1 % Cmol) and H_2 (12 mmol /L) were produced. The formation of acetate was redox balanced with the co-production of ethanol and succinate.

E. coli BW25113 (DE3) Δ*pta*-Δ*ldhA* (pEV), in which *pta*, encoding phosphate acetyltransferase, and *ldhA,* encoding lactate dehydrogenase were eliminated, still produced acetate in comparable amounts as *E. coli* BW25113 (DE3) (pEV), but lactate was no longer formed (Figure 4.3; Table 4.3). *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* (pEV) did not produce formate. Instead, the production of CO_2 (30 mmol/L, 14 % Cmol) and H_2 (> 100 mmol) were both more than 10 times higher than observed with *E. coli* BW25113 (DE3) (pEV) and the amount of succinate was doubled. Also high amounts of pyruvate and some citrate accumulated in the culture.

pKV-C and pKV-CGA both express codon-optimized *cadA*, which encodes the *cis*-aconitate decarboxylase from *Aspergillus terreus* that was previously shown to enable the production of itaconate in *E. coli* (Vuoristo et al., 2015). pKV-CGA also expresses citrate synthase (*gltA*) and aconitase (*acnA*) from *Corynebacterium glutamicum*. These genes

enhanced the production of itaconate in *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* under aerobic conditions (Vuoristo et al., 2015).

Expression of *cadA* did not result in itaconate production in *E. coli* BW25113 (DE3) (pKV-C) (Figure 4.2), but 0.08 mM of itaconate was produced by *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* (pKV-C) (Figure 4.3). A similar amount of itaconate was formed by *E. coli* BW25113 (DE3) (pKV-CGA) (Figure 4.2). *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* (pKV-CGA) produced eight times more itaconate (Figure 4.3), showing that both the expression of *gltA* and *acnA* and the elimination of *pta* and *ldhA* stimulate the production of itaconate in *E. coli* under anaerobic conditions.

The fermentation products that were formed by the strains carrying either pKV-C or pKV-CGA were similar to those formed by the strains carrying pEV. However, the carbon recovery for strain *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* (pKV-CGA) was initially much lower (84 %) than for the other strains, indicating that unidentified products were formed. Amino acid analysis of the culture supernatants showed that this strain accumulated significant amounts of glutamate and alanine. Alanine was also produced by all other strains but glutamate was only produced in large amounts by strain *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* (pKV-CGA). This indicates that expression of *gltA* and *acnA* increased the flux through the citric acid cycle, resulting in more itaconate and the accumulation of glutamate (Figure 4.3). Carbon balances for all strains were satisfactory (90-110 %) when the production of glutamate and alanine was taken into account (Table 4.3).

Figure 4.2 Anaerobic cultivation of *E. coli* BW25113 (DE3) containing pEV (diamonds), pKV-C (squares) pKV-CGA (triangles) or pKV-GA (circles) in pH-controlled bioreactors on MM at 30°C. The average values of duplicate cultures are given. Standard deviations are based on replicas of two parallel cultivations.
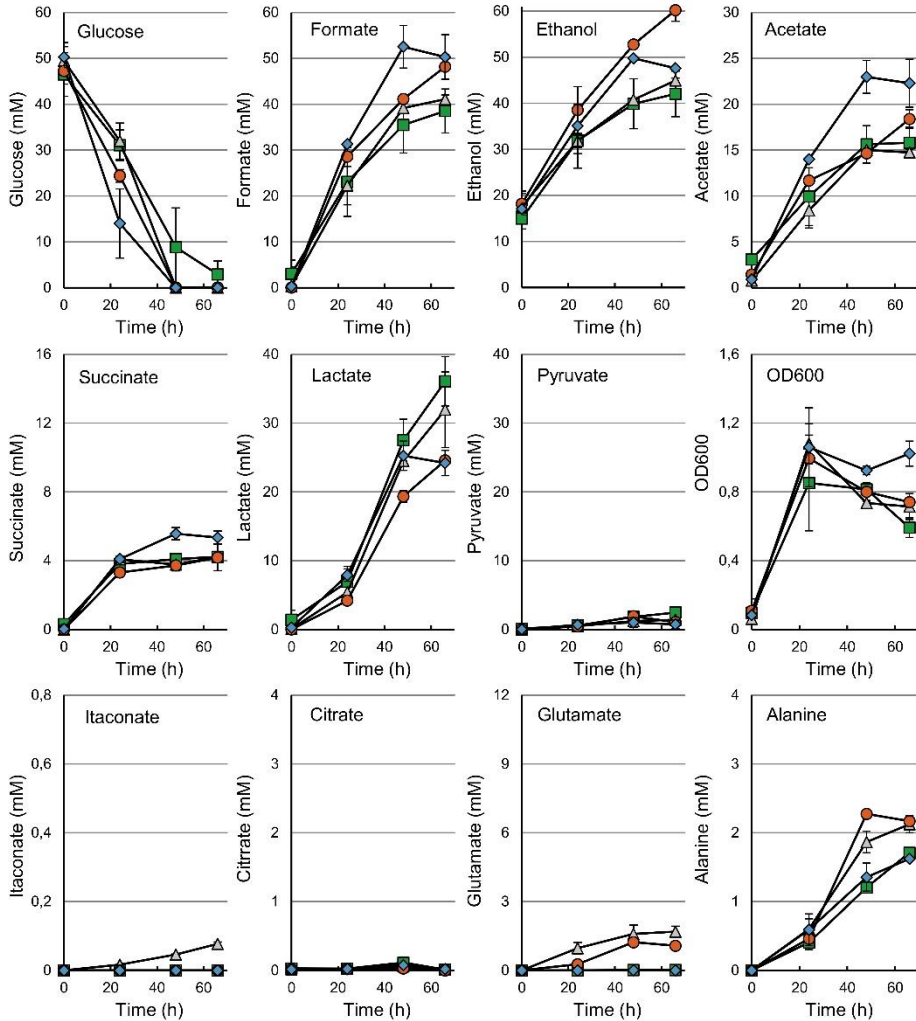
Figure 4.3 Anaerobic cultivation of *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA* containing pEV (diamonds), pKV-C (squares) pKV-CGA (triangles) or pKV-GA (circles) in pH-controlled bioreactors on MM at 30°C. The average values of duplicate cultures are given. Standard deviations are based on replicas of two parallel cultivations.
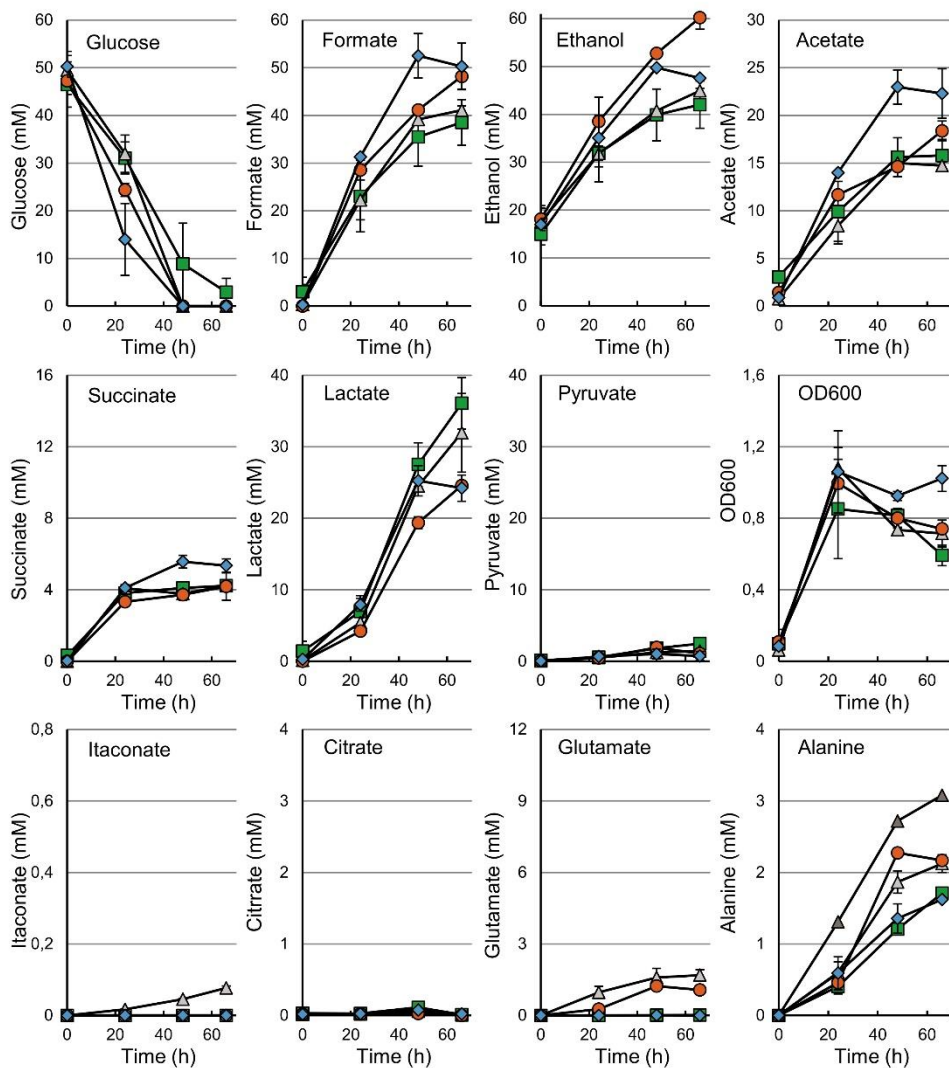
Table 4.3 Product distribution in % Cmol in culture supernatants of *E. coli* BW25113 (DE3) and *E. coli* BW25113(DE3) Δ*pta-*Δ*ldhA* containing pEV, pKV-C, pKV-CGA or pKV-GA after 66 hours in pH-controlled bioreactors on MM at 30°C. * based on theoretical CO_2 production. The average values of duplicate cultures are given.

Strains and plasmids	formate	ethanol	acetate	succinate	lactate	pyruvate	itaconate	citrate	glutamate	alanine	biomass	CO2	C-recovery %
E. coli BW25113 (DE3)													
pEV	15.8	20.4	12.8	6.8	26.7	1.4	0.0	0.0	0.0	1.7	3.0	0.9	89.5
pKV-C	14.3	26.2	11.4	5.3	33.5	2.3	0.0	0.5	0.0	1.5	2.2	1.4	98.6
pKV-CGA	13.5	18.7	9.2	5.9	37.9	1.9	0.1	0.0	2.5	2.3	2.0	1.1	95.0
pKV-GA	17.9	27.4	13.0	7.0	25.9	1.1	0.0	0.0	1.7	2.2	2.4	0.9	99.4
E. coli BW25113 (DE3) Δ*pta-*Δ*ldhA*													
pEV	0.0	23.0	10.4	18.0	0.1	20.2	0.0	2.7	0.2	3.7	1.8	14.4 *	94.4
pKV-C	0.0	29.7	5.2	17.6	0.1	36.0	0.1	1.5	0.3	3.5	3.2	13.2	110.3
pKV-CGA	0.0	29.8	3.3	12.1	0.8	17.9	1.2	4.0	13.0	3.6	2.8	16.4	104.9
pKV-GA	0.1	24.9	3.7	11.0	0.3	13.8	0.0	5.1	19.5	4.2	3.2	15.4	101.2

The average values of duplicate cultures are given

* Based on theoretical CO_2 production

4.3.2 Glutamate production under anaerobic conditions

To further investigate the production of glutamate under anaerobic conditions, pKV-GA (*gltA* and *acnA* were expressed without *cadA*) in *E. coli* BW25113 (DE3) and *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA*. The latter strain produced eight times more glutamate than the former one, resulting in the excretion of 8.6 mM of glutamate. This accounts for 19.5 % of the carbon that was added to the culture.

4.3.3 Elimination of isocitrate dehydrogenase (*icd*)

E. coli BW25113 (DE3) Δ*pta*-Δ*ldhA* strains produced significant amounts of glutamate under anaerobic conditions when *gltA* and *acnA* from *C. glutamicum* were expressed. Glutamate is produced in *E. coli* via the citric acid cycle suggesting that citrate and *cis*-aconitate are more efficiently channelled through the citric acid cycle than towards itaconate formation. To prevent isocitrate conversion to 2-oxoglutarate and further to glutamate, isocitrate dehydrogenase (*icd)* was deleted from *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA.* This resulted in the glutamate auxotrophic strain *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA*-Δ*icd*. Growth of this strain was hampered under anaerobic conditions (OD_{600} < 0.5 after 66 hours). When pKV-CGA and pKV-C were transformed into this strain, PCR analyses with cells of colonies that were grown on LB plates with chloramphenicol (35 µg/mL) for 16 h after transformation yielded fragments of 1.5 kB, indicating that *cadA* was present. However, all transformants lost a part of *cadA* during 16 h cultivation on liquid LB medium as PCR analysis with these cultures as template yielded fragments of only 0.3 kB (data not shown). The loss of a part of *cadA* was also already occurring in several colonies on the LB plates, as some

PCR reactions yielded fragments of both 1.5 kB and 0.3 kB. Different ways to improve the stability of *cadA* like altered media and growth conditions were tried without success. The results indicate that *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA*-Δ*icd* is not able to maintain a complete *cadA*.

4.3.4 Itaconate production under nitrogen limited conditions

Itaconate production in *E. coli* BW25113 (DE3) Δ*pta*-Δ*ldhA*-Δ*icd* turned out to be impossible due to the partial loss of *cadA*. Another option to prevent glutamate formation is to limit the availability of ammonium, as it is required for glutamate synthesis. This strategy was tested in bioreactors by using medium in which the amount of nitrogen was reduced to 50 %. Besides, US* trace elements were added to the medium, and a short aerobic growth phase was added to fermentation scheme to reduce the length of the lag phase. The use of nitrogen-limited medium resulted in a 50 % reduction of the production of glutamate and a 3-fold increase of   the itaconate yield (Figure 4.4). The itaconate titre of the latter culture was 2.9 mM.

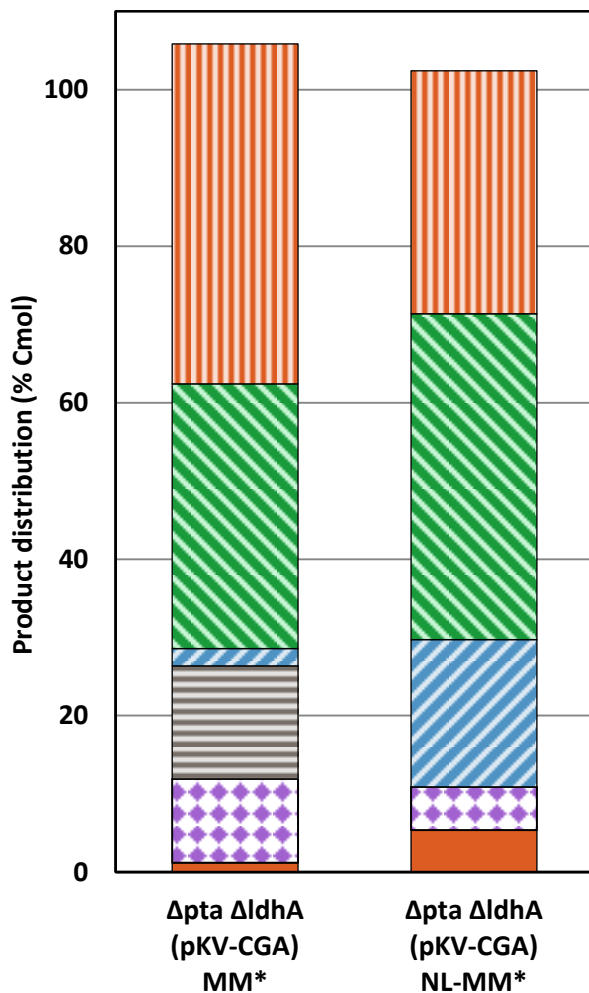**Figure 4.4** Product distribution of bioreactor cultures of *E. coli* BW25113 (DE3) Δ*pta-*ΔldhA* (pKV-CGA) cultivated in MM* and NL-MM* after 72 hours. Itaconate (solid), glutamate (diamonds), citric acid (horizontal stripes), pyruvate (upward diagonal stripes), ethanol (downward diagonal stripes), and other products (vertical stripes). The average values of duplicate cultures are given.

4.4 Discussion

We have previously constructed an *E. coli* strain that is able to produce itaconate under aerobic conditions by overexpressing *cis*-aconitate decarboxylase (*cadA*) from *A. terreus*. The flux to itaconate was enhanced by overexpressing the genes encoding citrate synthase (*gltA*) and aconitase (*acnA*) from *C. glutamicum* and eliminating the genes encoding phosphotransacetylase (*pta*) and lactate dehydrogenase (*ldhA*) (Vuoristo et al., 2015). Under anaerobic conditions, *E. coli* uses a mixed acid fermentation in which various products like acetate, succinate, ethanol, formate, lactate, hydrogen and carbon dioxide are synthesized (Clark, 1989). The fluxes to these products and to biomass in *E. coli* is combined in such a way that a redox balance is maintained. Itaconate production, as well as acetate and pyruvate production - results in cofactor reduction, which can be balanced by the co-production of succinate and/or ethanol. In this study we investigated whether it is possible to realize anaerobic production of itaconate in *E. coli*.

The *E. coli* strains that were previously developed for aerobic production of itaconate were cultivated under anaerobic conditions. Expression of *cadA* in *E. coli* BW25113 (DE3) was not sufficient to initiate itaconate production. Citrate synthase from *E. coli* is controlling the flux through the citric acid cycle because it is allosterically inhibited by the high NADH concentrations that occur under anaerobic conditions, and this may have prevented the production of itaconate. The citrate synthase from *C. glutamicum* (GltA) is not affected by such a negative feedback (Eikmanns et al., 1994). Indeed, co-expression of *acnA* and *gltA* from *C. glutamicum* in *E. coli* BW25113 (DE3) had a positive effect on itaconate production as it resulted in itaconate production, but the titres were low.

As the itaconate titre and yield were low, it is important to prevent unnecessary by-product formation. Knocking out *ldhA* completely suppressed lactate production, but the elimination of *pta* did not result in a significant reduction of acetate production. A similar observation was made under aerobic conditions (Vuoristo et al., 2015) and confirms the existence of alternative pathways for acetate production.

Expression of *cadA* in *E. coli* BW25113 (DE3) Δ*pta*Δ*ldhA* was already sufficient to evoke itaconate production, but the strain also accumulated pyruvate and citrate, indicating that the pathway to itaconate was restrained. Pyruvate accumulation is likely caused by a redox imbalance. To maintain redox balance, *E. coli* BW25113 (DE3) Δ*pta*Δ*ldhA* has to produce itaconate together with ethanol and/or succinate. When the flux to itaconate is too low compared to the fluxes to ethanol and succinate, the strains will become NADH-limited, which resulted in pyruvate accumulation. Additional expression of *gltA* and *acnA* from *C. glutamicum* strongly stimulated itaconate production and reduced the amount of pyruvate that was formed, resulting in an 8 times increased titre of itaconate.

We earlier showed that heterologous expression of *cadA* leads to inclusion body formation (Vuoristo et al., 2015). Strategies to increase the solubility of CadA in *E. coli* such as laboratory-directed protein evolution (Yuan et al., 2005) or codon harmonization (Angov et al., 2008) are likely to increase the flux from aconitate. Another option is that intracellular concentration of itaconate and the lack of transport capacity might become rate limiting, which was also proposed by (Okamoto et al., 2014). DauA was characterized as the main succinate transporter in *E. coli*, but it was shown to transport also other dicarboxylic acids at pH 7 (Karinou et al., 2013), suggesting that overexpression of *dauA* may boost itaconate export. Several putative

itaconate transporters have recently been characterized in *Aspergillus* species (Li et al., 2011, van der Straat et al., 2014), but their functionality *in E. coli* has not been tested.

Unexpectedly, glutamate was produced in *E. coli* BW25113 (DE3) Δ*pta*Δ*ldhA* cultures in which *gltA* and *acnA* of *C. glutamicum* were expressed. The UPLC method used to determine glutamate also revealed that significant amounts of alanine were produced (1.5-3 % C-mol) in all *E. coli* strains. Apparently, alanine is a standard fermentation product of this *E. coli* strain. Literature search did not reveal other studies in which alanine was found as a standard fermentation product of *E. coli*.

Both itaconate and glutamate synthesis compete for the same intermediates. To increase the flux to itaconate it is therefore necessary to repress glutamate production. Glutamate auxotrophs of *E. coli* have been realized by knocking out the genes encoding either citrate synthase (*gltA*) (Mainguet et al., 2013), aconitase (*acnA*) (Gruer et al., 1997) or isocitrate dehydrogenase (*icd*) (Lakshmi and Helling, 1976). *GltA* and *acnA* are involved in itaconate production and are therefore unsuitable candidates. Both *icd* and *acnA* knockouts are known to be instable under aerobic conditions as they lead to inactivation of *gltA*, possibly because of a toxic effect of intracellularly accumulating citrate (Gruer et al., 1997). Still, Gruer *et al.* (Gruer et al., 1997) showed that *acnA* knockouts were stable under anaerobic conditions, which suggests that less citrate accumulates under anaerobic conditions, possibly because of the regulation of the activity of GltA by NADH. Introduction of the NADH-insensitive citrate synthase of *C. glutamicum* in *E. coli* BW25113 (DE3) Δ*pta*Δ*ldhA*Δ*icd* may therefore not be feasible. Indeed, attempts to express the genes of pKV-CGA in the strain were unsuccessful. Even expression of pKV-C, which only contains *cadA*,

resulted in the loss of a part of *cadA* during growth, which suggests that the instability of *icd* knockouts may also be caused by the accumulation of high intracellular itaconate concentrations.

In a recent study (Okamoto et. al 2014), CadA was successfully expressed in Δ*icd* strain when cultured on LB medium under aerobic conditions. Overexpression of aconitase *(acnB)* together with *cadA* in the Δ*icd* strain led to enhanced itaconate production (4.34 g/L). However, a complex growth medium like LB, which seemed to stabilize expression of *cadA* in Δ*icd* background, is not preferred for bulk chemical production due to its high price. In addition, the Δ*icd* strain accumulated a substantial amount of acetate without a deletion in metabolic pathways involved in acetate metabolism, such as *pta,* and the authors recommended to inactivate the acetate forming pathways. In another study, Icd activity of *C. glutamicum* was lowered by exchanging the ATG start codon to GTG or TTG, which together with a heterologous CadA expression resulted in 60 mM of itaconate (Otten et al., 2015).

As glutamate production depends on the availability of nitrogen in the medium, an alternative strategy to diminish glutamate production was tested by culturing cells in nitrogen-limited medium. This enhanced the production of itaconate to up to 5.4 % Cmol with *E. coli* BW25113 (DE3) Δ*pta*Δ*ldhA* pKV-CGA. Enhancing the flux from aconitate to itaconate would probably further reduce the amount of glutamate production.

E. coli has several interesting features for anaerobic production of itaconate: It is one of the few industrial microorganisms that is able to grow under anaerobic conditions. Acetyl-CoA – a precursor of itaconate – is a central metabolite in dissimilation processes in *E. coli*, which is not the case in eukaryotes like *Saccharomyces cerevisiae* - although several

groups are trying to change this (Kozak et al., 2014, Lian et al., 2014). *E. coli* converts pyruvate into acetyl-CoA and formate by pyruvate-formate lyase under anaerobic conditions. Formate can subsequently be split into valuable H_2 and CO_2. Other industrial strains that are able to grow under anaerobic conditions, like *S. cerevisiae* and lactic acid bacteria, use NAD-dependent pyruvate dehydrogenase to synthesize acetyl-CoA, which generates extra NADH and thus requires additional cofactor regeneration at the cost of substrate.

The microbial production of organic acids is studied by many groups (see e.g. Wendisch *et al.* (Wendisch et al., 2006) and Yu *et al.* (Yu et al., 2011)). Addition of base (lime) is necessary when organic acids are produced at neutral pH. During downstream processing the organic acid salt has to be converted into the organic acid, which is usually done by adding sulphuric acid. This results in the production of vast quantities of salts (gypsum). An alternative approach is to produce the organic acid at low pH. *E. coli* is unable to grow at low pH values and organic acid production with *E. coli* can therefore only be done at neutral pH values.

This study shows that it is possible to synthesize itaconate anaerobically by using the mixed acid pathway of *E. coli*, in which the synthesis of ethanol/H_2 and succinate regenerate NAD. Ethanol/H_2 seems to be the best set of co-products for industrial application as these products can be simply separated based on their boiling temperatures, and easily marketed as bulk chemicals. Itaconate and succinate are more difficult to separate because they are both dicarboxylic acids with a C4 backbone. Moreover they may copolymerize, which will have an impact on polymer properties. Deleting fumarate reductase (*frd*) is one of the obvious solutions to prevent succinate formation (Zhou et al., 2006).

This is the first time that anaerobic production of itaconate from glucose was reported for *E. coli*. The observed yields and productivities are still modest. Eliminating the pathways to major by-products like glutamate, succinate, and acetate, and enhancing the pathway between pyruvate and itaconate is therefore crucial to obtain a cost-competitive anaerobic production process for itaconic acid.

Competing interests

Ethical statement

No animal or human subjects were used in this work.

Acknowledgements

Chapter 5

Metabolic engineering of TCA cycle for production of chemicals

Abstract

The tricarboxylic acid (TCA) cycle has been used for decades in microbial production of chemicals like citrate, L-glutamate and succinate. Maximizing yield is key for cost-competitive production. However, for most TCA cycle products the maximum pathway yield is much lower than the theoretical maximum yield (Y^E). For succinate, this was solved by creating two pathways to the product, using both branches of the TCA cycle, connected by the glyoxylate shunt (GS). A similar solution cannot be applied directly for production of compounds from the oxidative branch of the TCA cycle because irreversible reactions are involved. Here, we describe how this can be overcome and what the impact is on the yield.

5.1 Microbial production of chemicals from the TCA cycle

The depletion of the fossil energy resources of the Earth, accompanied by their impact on the environment, has raised the demand to replace oil-based chemicals with biomass-based alternatives. Over the years, strategies to evolve wild-type strains together with rational metabolic-engineering techniques have been developed to create high-performance microorganisms that convert inexpensive substrates into value-added chemicals, fuels, and polymers (Keasling, 2010, Lee et al., 2008).

The TCA cycle is one of the major biochemical hubs of the cell. It has two main functions: generation of energy and synthesis of precursors. When a terminal electron acceptor is present it functions as a true cycle, oxidizing substrate to CO_2 and generating NADH for respiration. Intermediates of the TCA cycle are used as precursors for anabolism. The latter is the only remaining function of the TCA cycle when no terminal electron acceptor is available. In this case, the TCA cycle is not functioning as a cycle, but as two separate branches: the reductive branch from oxaloacetate to succinyl-CoA and the oxidative branch from citrate to 2-oxo-glutarate (Figure 5.1A).

The ability to synthesise building blocks has been used since decades by the fermentation industry to produce chemicals like citric acid (Papagianni, 2007) and L-glutamic acid (Hermann, 2003) (Figure 5.1A). More recently, it has been applied in the production of new chemicals like succinic acid (Song and Lee, 2006), itaconic acid (Hevekerl et al., 2014, Vuoristo et al., 2015) and 1,4-butanediol (Yim et al., 2011). These chemicals have to be cost-competitive with analogues made from fossil resources. Maximizing yield is one of the key elements to do so.

However, the maximum attainable pathway yield of some TCA cycle products is currently much lower than the theoretical maximum yield.

5.2 Maximum pathway yields are lower than theoretical maximum yields

For each substrate/product combination, there is a maximum amount of product that can be formed from the substrate. This maximum yield (Y^E) is based on elemental balancing and can be calculated from the ratio of the degree of reduction (γ) of substrate and product (Dugar and Stephanopoulos, 2011). For example, Y^E for citrate ($\gamma_{citrate}=18$) from glucose ($\gamma_{glucose}=24$) is 1.33 mol/mol glucose. The overall equation becomes:

Glucose + 2 CO_2 = $1^1/_3$ citrate + $^2/_3$ H_2O
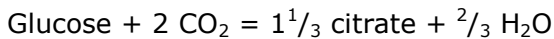
$\Delta G_0' = -191.4 \pm 12.1$ kJ/mol glucose   equation 5.1

The $\Delta G_0'$ is comparable to that of the production of ethanol from glucose ($\Delta G_0'=-230.4 \pm 12.9$ kJ/mol), indicating that the conversion can occur spontaneously and allows synthesis of ATP.

The maximum pathway yield (Y^P) depends on the pathway involved and the way redox cofactor regeneration takes place. The citric acid biosynthesis pathway in *Aspergillus niger*, in which oxygen is used to regenerate NADH, results in the overall equation (Equation 5.2)
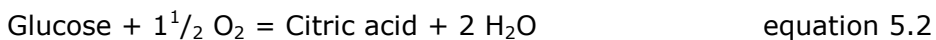
Glucose + $1^1/_2$ O_2 = Citric acid + 2 H_2O equation 5.2
$\Delta G_0' = -888.3 \pm 6.4$ kJ/mol glucose

Thus, Y^P is 1 mol/mol glucose, which is 33% lower than the Y^E.

We have done the same exercise for other products derived from the TCA cycle (Table 5.1). For many products, Y^P is significantly lower than Y^E. Fumarate and malate are exceptions. These can be made in redox balance by using the reductive branch of the TCA cycle, and do not require an additional pathway for cofactor regeneration. The practical yield is still low, because these pathways do not form ATP and therefore require the activity of respiration to generate ATP. Formation of the other products does require cofactor regeneration. For the calculation of Y^P, oxygen is reduced for cofactor regeneration, whereas reduction of CO_2 is required to reach Y^E.

5.3 Reaching Y^E by combining the reductive and oxidative branch of the TCA cycle

Many anaerobic microorganisms produce succinate via the reductive branch of the TCA cycle. This requires the input of 2 NADH units. 1 mol of glucose, when converted to formate and acetate via the glycolytic pathway, provides 2 moles of NADH, which means that only one mole of succinate can be produced per mole of glucose under anaerobic conditions (Thakker et al., 2012). Production of succinate via the GS does not require input of NADH. Therefore, the succinate yield under anaerobic conditions can be increased by preventing formation of formate and acetate, and activation of the GS. This way, Y^P can be obtained that is identical to Y^E (Box 5.1, Figure 5.1B).

Formation of products from the oxidative branch of the TCA cycle such as citric acid and L-glutamic acid results in cofactor reduction and oxygen is typically used for NAD^+ regeneration. So also here a combination of pathways using both the reductive and oxidative branches of the TCA cycle seems a valuable option, as it allows the

regeneration of cofactor while synthesizing more product. It is thermodynamically easy to channel intermediates from the oxidative branch to the reductive branch, as described above for succinate. However, the opposite action requires the reversal of either the GS or the TCA cycle, which is hampered by irreversible reactions.

Box 5.1 Product case study: succinic acid

Fermentative production of succinic acid has two advantages over petrochemical production. First, fermentative production of succinic acid can compete with petroleum based process (Pinazo et al., 2015), as the chemical conversion of maleic anhydride to succinic acid is expensive. Second, CO_2 fixation occurs during succinate formation via the reductive branch of the TCA cycle, which allows for higher product yields, and also helps to reduce CO_2 emissions.

The Y^E for succinate from glucose is 1.71 mol/mol (Table 5.1). Many microorganisms produce succinate using the reductive branch of the TCA cycle:

Glucose + 2 CO_2 + 2 NADH = 2 succinate + 2 NAD   equation 5.6
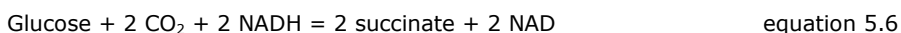
These microorganisms typically convert glucose into products like acetate and formate (Van der Werf et al., 1997) to regenerate the required NADH:

Glucose + 2 NAD = 2 acetate + 2 formate + 2 NADH   equation 5.7

The overall formula becomes:

Glucose + CO_2 = succinate + acetate + formate   equation 5.8
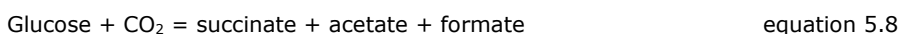
So wild-type anaerobic microorganisms produce succinate in a heterofermentative fashion, which reduces the pathway yield to 1 mol/mol.

Sánchez et al. (2005) engineered a second succinate pathway with net NADH production. They activated the GS through inactivation of *iclR*, a transcriptional repressor of GS genes in *E. coli*. The purpose of the *iclR* deletion was to direct acetyl-CoA to succinate and thus prevent acetate accumulation. In addition, the carbon flux through the GS was supported by overexpression of NADH-insensitive citrate synthase *citZ* from *Bacillus subtilis.* To force the microorganism to use this pathway the formation of other by products (ethanol, lactate and acetate) was disrupted. The strain developed by Sánchez et al. (2005) converted pyruvate to oxaloacetate and acetyl-CoA + formate by the action of heterologous pyruvate carboxylase from *Lactococcus lactis* and *E. coli*'s native pyruvate formate lyase (Pfl). The activity of these two enzymes can be replaced by a pyruvate dehydrogenase complex, which allows for an increased Y^P due to synthesis of additional NADH (Figure 1B) (Skorokhodova et al., 2011, Zhu et al., 2014).

The reaction equation of the pathway for succinate using the oxidative branch of the TCA cycle and the GS is:

3 Glucose + 8 NAD + 2 H_2O = 4 succinate + 2 CO_2 + 8 NADH   equation 5.9

Combined with the reductive branch of the TCA cycle (equation 5.6) results in:

7 glucose + 6 CO_2 = 12 succinate + 6 H_2O equation 5.10
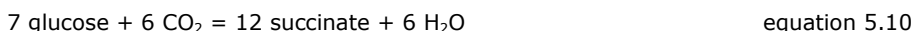
Equation 5.10 shows that the Y^P of the combined pathway is equal to Y^E.

5.4 Reversing the GS

The GS consists of isocitrate lyase (Icl) and malate synthase (Ms). Icl catalyses the conversion of isocitrate to succinate and glyoxylate. This reaction has a $\Delta G_0'$ of 8.6, which means that it is reversible. Ms catalyses the synthesis of malate from glyoxylate and acetyl-CoA, which is an exothermic reaction with a $\Delta G_0'$ of -32.7 kJ/mol. Reversing the GS is therefore thermodynamically unfavourable.

Recently, a synthetic, reverse glyoxylate shunt (rGS) that converts malate to glyoxylate and acetyl-CoA was constructed in *E. coli* (Mainguet et al., 2013) (Box 5.2). The reaction is coupled to the hydrolysis of ATP, which makes it thermodynamically feasible. By using this rGS it is possible to channel intermediates from the reductive branch to the oxidative branch of the TCA cycle, resulting in a Y^P of 1.33 mol/mol, which is equal to Y^E (Figure 5.1C, Table 5.1).

Box 5.2 Product case study: rGS by Mainguet

The GS is a bypass of part of the TCA cycle that allows plants and some microorganisms to grow on simple carbon compounds like acetate, because it avoids the decarboxylation steps of the TCA cycle. This enables conversion of acetyl-CoA to TCA cycle intermediates without loss of CO_2.
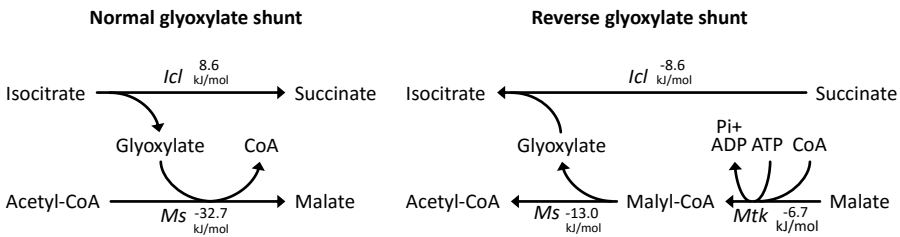
Figure 5.2 The reactions taking place in A) the GS and B) the rGS. Icl: isocitrate lyase, Ms: malate synthase, Mtk: malate thiokinase

The GS consists of isocitrate lyase (Icl) and malate synthase (Ms). The bottleneck of the GS is MS, which converts glyoxylate and acetyl-CoA to malate. The reversal of this reaction is thermodynamically unfavourable ($\Delta G_0' = 32.7$ kJ/mol) (Figure 2). To enable conversion of malate to glyoxylate and acetyl-CoA, (Mainguet et al., 2013) coupled the reaction to hydrolysis of ATP, making it thermodynamically more favourable ($\Delta G_0' = 13$ kJ/mol). For this, *E. coli*'s native Ms was replaced by an ATP-dependent malate thiokinase (Mtk) and a malyl-CoA lyase (Mcl), resulting in a synthetic rGS that converts malate and succinate to oxaloacetate and two molecules of acetyl-CoA in the thermodynamically unfavourable direction, but at the expense of ATP (Figure 1C). The reverse glyoxylate shunt (rGS) enables efficient conversion of glucose to acetyl-CoA without the loss of CO_2, which can be directed into production of industrially relevant chemicals such as citrate and amino acids that originate from intermediates of the oxidative branch of the TCA cycle. This way, the Y^P of these products becomes equal to Y^E

5.5 Reversing the TCA cycle

The second option for combining the reductive and oxidative branch of the TCA cycle for the formation of products from the oxidative branch is to reverse the TCA cycle itself. Most of the TCA cycle reactions are reversible, except for two steps: citrate synthase and 2-oxoglutarate dehydrogenase. For the production of chemicals from the oxidative part of the TCA cycle, only the reversion of the reaction catalysed by 2-oxoglutarate dehydrogenase is required. With NADH as a cofactor, formation of 2-oxoglutarate from succinyl-CoA and CO_2 has a $\Delta G_0'$ of 35.8 kJ/mol and hence, this reaction is not likely to occur. There are, however, autotrophic microorganisms that use a modified reductive branch of the TCA cycle to fix CO_2 (Saini et al., 2011). In this case, reduced ferredoxin is used as a cofactor for the conversion of succinyl-CoA to 2-oxoglutarate. This reaction, which is catalysed by a ferredoxin-dependent 2-oxoglutarate oxidoreductase (OGOR), has a $\Delta G_0'$ of 15.5 $\pm$ 12.9 kJ/mol[2], which means that it can occur under non-standard conditions. OGOR from *Hydrogenobacter thermophilus* was heterologously produced in *E. coli* (Yamamoto et al., 2003). *In vitro* carboxylation of succinyl-CoA by this OGOR occurred when hetero-logously produced ferredoxin and pyruvate:ferredoxin oxidoreductase (POR) from *Hydrogenobacter thermophiles* (Ikeda et al., 2006) were added to the assay mixture, together with pyruvate (Yamamoto et al., 2010). The latter enabled the regeneration of reduced ferredoxin by POR in the assay mixture.

Citrate production with ferredoxin-dependent OGOR and POR would have the following equation (Figure 5.1D):

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[2] Many different ferredoxins exist, with various standard electron potentials. The $\Delta G_0'$ was estimated based on a ferredoxin with an E_0' of -418 mV.

Oxidative branch of the TCA cycle:

2 Glucose + 4 Fd^{ox} + 4 NAD = 2 citrate + 4 Fd^{red} + 4 NADH

equation 5.3

Reductive branch of the TCA cycle:

Glucose + 6 CO_2 + 4 Fd^{red} + 4 NADH = 2 citrate + 4 Fd^{ox} + 4 NAD + 2 H_2O　　　　　　　　　　　　　　　　　　　equation 5.4
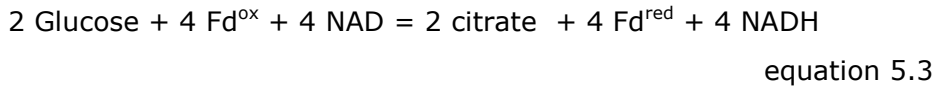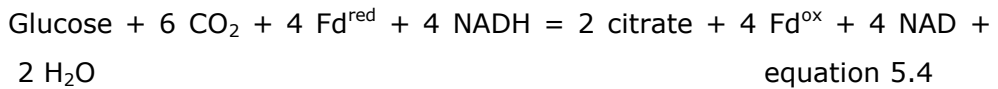
The total citrate forming reaction by using both branches of the TCA cycle would have the following equation:

Glucose + 2 CO_2 = $1\frac{1}{3}$ citrate + $\frac{2}{3}$ H_2O　　　　　　　equation 5.5
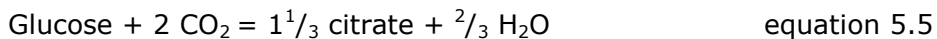
Equation 5 shows that this approach results in a Y^P of 1.33 for citrate production, which is identical to the Y^E.

5.6 ATP requirement

If product formation results in ATP formation, this can be used for cellular growth and maintenance. However, the reverse TCA cycle is ATP neutral and the rGS requires the input of ATP . We calculated the amount of ATP formed or consumed for the production of citrate, itaconate and L-glutamate using the rGS or reverse TCA cycle, ignoring ATP expenditure in transport steps. The ATP yield was respectively negative or zero (results not shown), indicating that product formation will not sustain growth and maintenance. Nevertheless, the $\Delta G_0'$ of these reactions (Table 1) is sufficiently low to allow ATP formation. Therefore, alternative ATP-generating steps such as using PEP carboxykinase

instead of PEP carboxylase (Moon et al., 2008) may be applied. Alternatively, micro-aerobic production conditions may be used to generate sufficient amounts of ATP for growth and maintenance.

5.7 Additional benefits

The pathways described above that couple the reductive and oxidative branches of the TCA cycle optimally use the reducing equivalents that are generated to bind CO_2, which results in an increased Y^P that becomes equal to the Y^E. Oxygen will only be necessary for growth and maintenance if the product pathway does not generate sufficient ATP. This is very beneficial as the presence of oxygen has multiple disadvantages. Oxygen permits the complete oxidation of substrate to CO_2. This respiration generates high amounts of ATP that can be used for the conversion of substrate to microbial biomass. Both have a negative impact on product yield, and explain why the practical yield is usually significantly lower than the Y^P under aerobic conditions (Table 1). This is less of a problem in processes in which product formation is not stoichiometrically linked to oxygen utilization or which can be operated anaerobically. In those cases, the practical yield can be up to 90-95% of the Y^P (Weusthuis et al., 2011).

Aerobic processes are known to generate more heat than anaerobic processes. Cooling is required to maintain the temperature in the physiological range and the maximum cooling capacity will decrease the maximum productivity of the process. In general, anaerobic processes therefore show up to 5 times higher productivities (Weusthuis et al., 2011)

Table 5.1. Yields and reaction equations of selected chemicals derived from the TCA cycle

Compound	Yield type	Equation	Yield (mol/mol glucose)	$\Delta_r G'^0$ (kJ/mol glucose)[‡]	Reference
Citric acid	Y^E	Glucose + 2 CO_2(g) = $1^1/_3$ Citrate + $^2/_3$ H_2O	1.33	-191.4±12.1	
	Y^P	Glucose + $1^1/_2$ O_2(g) = Citrate + 2 H_2O	1	-851.6±9.2	
	Practical		0.88		(Jianlong et al., 2000)
Itaconic acid	Y^E	Glucose + $^2/_3$ CO_2(g) = $1^1/_3$ Itaconate + 2 H_2O	1.33	-197.3±6.2	
	Y^P	Glucose + 1 ½ O_2(g) = Itaconate + 3 H_2O + CO_2(g)	1	-868.6±11.7	
	Practical		0.80		(Hevekerl et al., 2014)
Malic acid	Y^E	Glucose + 2 CO_2(g) = 2 Malate	2	-142.9±12.3	
	Y^P	Glucose + 2 CO_2(g) = 2 Malate	2	-142.9±12.3	
	Practical		1.49		(Knuf et al., 2014)
Fumaric acid	Y^E	Glucose + 2 CO_2(g) = 2 Fumarate + 2 H_2O	2	-136.0±12.3	
	Y^P	Glucose + 2 CO_2(g) = 2 Fumarate + 2 H_2O	2	-136.0±12.3	
	Practical		0.55		(Cao et al., 1996)
Succinic acid	Y^E	Glucose + $^6/_7$ CO_2 = $^{12}/_7$ Succinate + $^6/_7$ H_2O	1.71	-256.5±8.2	
	Y^P	Glucose + $^6/_7$ CO_2 = $^{12}/_7$ Succinate + $^6/_7$ H_2O	1.71	-256.5±8.2	
	Practical		1.61		(Sánchez et al., 2005)
Glutamic acid	Y^E	Glucose + $^2/_3$ CO_2(g) + $^4/_3$ NH_3= $^4/_3$ Glutamate + 2 H_2O	1.33	-219.0±5.3	
	Y^P	Glucose + $^1/_3$ O_2 + NH_3 = Glutamate + CO_2 + 3 H_2O	1	-884.8±11.3	
	Practical		0.57		(Chinen et al., 2007)

[‡] Based on http://equilibrator.weizmann.ac.il
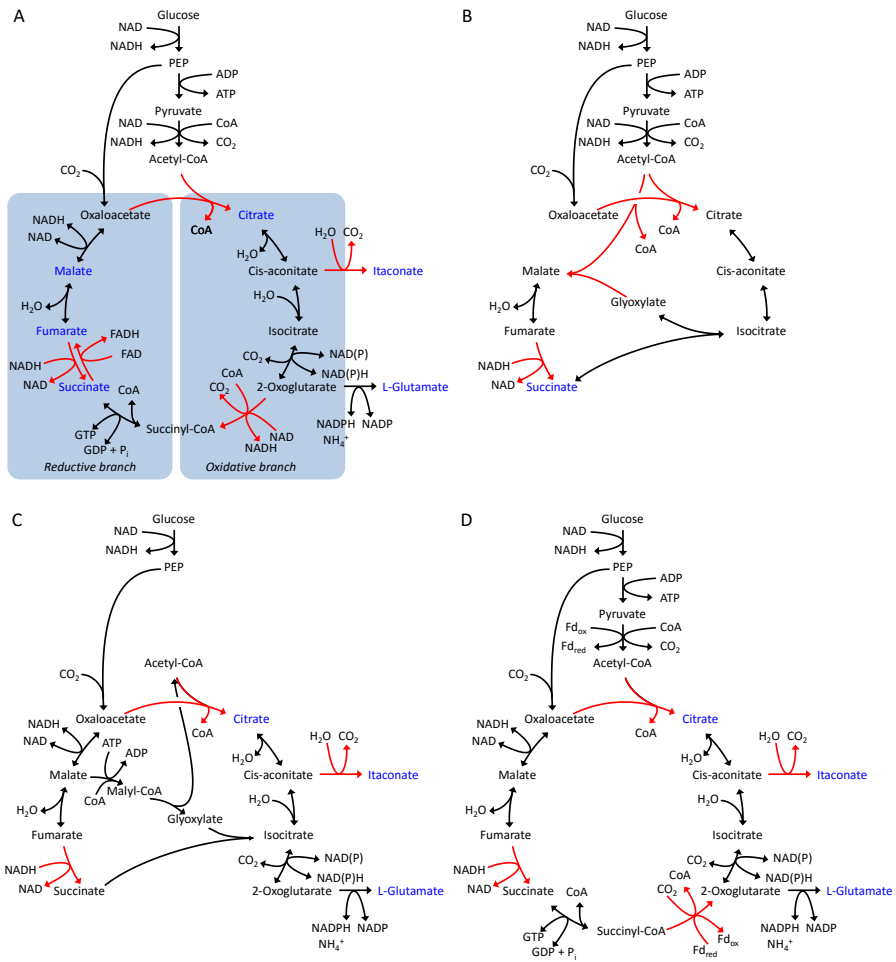
Figure 5.1 Several configurations of the TCA cycle and reactions of the GS. A. Conventional configuration of the TCA cycle for substrate oxidation and production of citrate, itaconate and L-glutamate via de oxidative branch and malate, fumarate and succinate via the reductive branch. B. Production of succinate using both oxidative and reductive branches of the TCA cycle, connected by the GS. C. Production of citrate, itaconate and L-glutamate using both reductive and oxidative branches of the TCA cycle, connected by reactions of the rGS. D. Production of citrate, itaconate and L-glutamate using both reductive and oxidative branches of the TCA cycle, connected by a ferredoxin-dependent 2-oxoglutarate oxidoreductase. Chemicals in blue: produced chemicals. Reactions in red: irreversible reactions

5.8 Concluding remarks

Maximizing Y^P is crucial to lower manufacturing costs and to make microbial production of chemicals competitive with analogues made from fossil resources. Currently, the Y^P of some TCA cycle products is much lower than the Y^E. To reach Y^E, carbon fluxes from both branches of the TCA cycle must be directed to product formation, as has already been shown for the production of succinate. Production of compounds from the oxidative branch of the TCA cycle, such as citrate and L-glutamate cannot directly use both branches as irreversible reactions are involved. In this opinion paper, we presented solutions to overcome these irreversible steps and show how the oxidative and reductive branch of the TCA can be connected. This way, the Y^P for citrate, itaconate and L-glutamate becomes identical to the Y^E, which is a 33% increase of the Y^P of the currently used pathways for these products. Similar improvements are expected for other products that emerge from the oxidative TCA branch. Processes based on these reversed pathways can be run under anaerobic conditions and will therefore require less cooling and will result in less loss of carbon to CO_2 and biomass.

Future research should focus on implementing these solutions in suitable production hosts, and increasing the ATP yield of the production pathways. This will minimize the oxygen requirement of the process, or even allow for anaerobic operation, and should lead to reduced operational costs and maximal product yields.

Conflicts of interest statement

The authors declare that they have no competing interests.

Acknowledgements

Chapter 6

General discussion

Itaconic acid, an unsaturated C5-dicarboxylic acid, is considered to be an important building block monomer in the synthesis of bio-based chemicals such as methyl methacrylate (MMA) and fuels. One of the most promising applications for itaconic acid is as a precursor for the fuel component, 3-methyltetrahydrofuran (3-MTHF) (Geilen et al., 2010) (Voll and Marquardt, 2012). At present, itaconic acid is mainly produced by fungal fermentation with *Aspergillus terreus* (Klement and Büchs, 2013), but the yield and productivity of the process must be significantly improved to reduce the production cost to expand its market potential.

One of the key factors to obtain a cost competitive production process is to maximize yield. For itaconic acid, the maximum pathway yield in the current process with *A. terreus* is much lower than the theoretical maximum yield. The maximum pathway yield (Y^P) depends on the pathway involved and the way redox cofactor regeneration takes place. Itaconic acid production in *A. terreus* requires oxygen as a terminal electron acceptor, which limits Y^P. Thus, Y^P is 1 mol/mol glucose, which is 33% lower than theoretical maximum yield (Y^E), Chapter 5. Therefore, alternative production hosts like *Escherichia coli* and metabolic engineering of production pathways are needed to optimize the production process.

The aim of this thesis was to explore options to approximate maximum yield for production of itaconic acid, which is a biobased building block monomer for polymer industry. The study was focused on the itaconic acid production mechanism and in increasing the efficiency of the pathway in a heterologous production host, the *Escherichia coli* bacterium.

In this final chapter, the obtained results are discussed and interpreted in the context of available scientific literature. Based on the findings of

this study, challenges of itaconate production in *E. coli* are briefly summarized together with suggestions to solve some of the current bottlenecks. Finally, targets for future research and conclusions of this study will be presented.

6.1 Towards efficient itaconate production in heterologous hosts

In order to expand market potential for itaconic acid, cost competitive production is of key importance. The economics of itaconic acid production with *A. terreus* are negatively influenced by the slow growth of the organism and the sensitivity of the filamentous pellets to hydro-mechanical stress, which inhibits mass transfer and consequently oxygen supply to the cells (Klement et al., 2012). The current price for itaconic acid ranges between US$ 1.2–2.5 kg (Klement and Büchs, 2013, Okabe et al., 2009) depending on the market situation. According to U.S. Department of Energy (DOE) report, the production cost of itaconic acid needs to be reduced at least to $0.5/kg to be economically competitive with the present petrochemical counterparts (Werpy and Petersen, 2004). A decade later, the production costs are still too high for industrial application of itaconic acid as a starting material (Steiger et al., 2013) and it was excluded from the DOE's report in 2010 because the market has not grown as expected (Choi et al., 2015).

Development of an alternative production host could be a solution to lower the production costs. Characterization of *cis*-aconitate decarboxylase from *A. terreus* in 2008 by (Kanamasa et al., 2008), followed by successful expression of *cadA* in *E. coli* (Li et al., 2012), enabled a good basis to study the itaconate production mechanism in *E. coli*. Although the itaconate titre remained low, *E. coli* has many characteristics that could, after implementing a proper strain design,

make it an efficient itaconate producer organism. This has already been shown over the years for a number of other products such as lactate (Chang et al., 1999a), succinate (Sánchez et al., 2005) and 1,4-butanediol (Yim et al., 2011). The common factor for these high-yield production pathways is that the products are derived from central metabolic pathways and production is possible under (micro)anaerobic conditions in a redox balanced way. This enables high flux towards product formation, which in combination with lower biomass production, improves substrate efficiency compared to aerobic fermentations (Weusthuis et al., 2011), as explained in chapter 1.

Chapter 2 focuses on the construction of the itaconate biosynthesis pathway in *E. coli*. It was realised, that the activity of CadA might limit itaconate production due to low enzymatic activity caused by improperly folded protein. Fermentation conditions such as culturing conditions and medium composition seemed to play an important role to facilitate conversion of *cis*-aconitate to itaconate. In addition, providing sufficient amounts of precursor molecules such as citrate and cis-aconitate and maintaining the flux to the TCA cycle was shown to increase itaconate concentrations. Acetate is often an unwanted side product of *E. coli* fermentations, like it was also shown in this work. Knocking out acetate and lactate forming pathways resulted in an increased itaconate titre, up to 690 mg/L, but also pyruvate accumulation was observed. Normally pyruvate would be converted to lactate, but as the conversion was prevented, this indicated that the flux to the TCA cycle is limiting itaconic acid production. The significance of itaconate transport remained unclear.

We looked at different ways to improve the solubility of heterologously expressed *cadA* in Chapter 3. Typically, a method called codon

optimization is used to optimise DNA sequence of a gene of interest when expressed in a heterologous host. Codon optimization changes the codons to match with the most prevalent tRNAs in the expression host. Compared to codon optimization, which substitutes codons by the most frequently used codons of the expression host (Makrides, 1996), codon harmonization selects the codons with usage frequencies in the expression host that most closely match the usage frequencies in the native host (Angov et al., 2008). We found out that codon harmonization increased the activity of CadA in cell free extracts, but this did not improve itaconate production in bioreactors. Besides the well-known CadA from *Aspergillus terreus*, also other enzymes have shown to exhibit *cis*-aconitate decarboxylating activity (Michelucci et al., 2013, Strelko et al., 2011). We successfully expressed the *Mus musculus* immunoresponsive gene 1 (*irg1*) in *E. coli* and as first authors described that this gene can be used for itaconic acid production. Titres up to 560 mg/L of itaconate were obtained with *irg1*, which are comparable to those obtained with *cadA* from *A. terreus* (700 mg/L). Also in this chapter 3 it was concluded, that a higher *cis*-aconitate decarboxylase activity did not result in a higher itaconate production, so the role of factors such as competing pathways leading to by-products and transporters should be carefully investigated.

In chapter 4, the first steps towards anaerobic itaconate production were realized. A heterofermentative pathway was designed for the overproduction of itaconate in *E. coli*, with the concomitant production of ethanol and formate or hydrogen gas plus carbon dioxide. Expression of *cadA* from *A. terreus* in *E. coli* did not result in itaconate production under anaerobic conditions, but 0.66 mM of itaconate was formed when the genes encoding citrate synthase (*gltA*) and aconitase (*acnA*) from *Corynebacterium glutamicum* were co-expressed. Overexpression of *C.*

glutamicum genes unexpectedly led to significant glutamate production under anaerobic conditions, up to 13 Cmol%. Both itaconate and glutamate synthesis compete for the same intermediates, so elimination of glutamate production is crucial. Production of glutamate requires nitrogen and by applying nitrogen-limited growth medium, the itaconate titre was increased to 2.9 mM. This proof of principle study showed that it is possible to synthesize itaconate anaerobically by using the mixed acid pathway of *E. coli*, in which the synthesis of ethanol/H_2 and succinate regenerate NAD. The obtained titres were still modest and eliminating the pathways to major by-products like glutamate, succinate and acetate is required so anaerobic itaconate synthesis could compete with *A. terreus* process.

To make itaconate production cost efficient, the product yield must be as high as possible. Whereas the maximum yield (Y^E) is 1.33 mol/mol, the maximum pathway yield (Y^P) of itaconate in *A. terreus* is 1 mol/mol, which is largely due to inefficient cofactor regeneration. The maximum obtainable pathway yield for itaconate could be reached by creating two pathways to the product, one using the normal oxidative TCA branch, and one using the reductive branch. Combination of these two pathways involves thermodynamically challenging steps. Solutions to overcome these challenging reactions were provided in chapter 5.

To reach high yield, which is close to theoretical maximum, carbon fluxes from both sides of TCA cycle must be directed to itaconate formation. Two theoretical alternative pathway options to realize homofermentative itaconate production were found: either by reversing the glyoxylate shunt or citric acid cycle. Recently, the envisaged synthetic, reverse glyoxylate shunt (rGS) that converts malate and succinate to acetyl-CoA and isocitrate was published in *E. coli* (Mainguet

et al., 2013). For this reason, we focused on the second option: reversing the TCA cycle. Combining both reductive and oxidative branches of the TCA cycle seems a valuable option, as it allows the regeneration of cofactor while product is formed. However, reversal of the TCA cycle requires reversion of some thermodynamically challenging reactions. Most of the TCA cycle reactions are reversible, except for two steps: citrate synthase and 2-oxoglutarate dehydrogenase. For the production of chemicals from the oxidative part of the TCA cycle like itaconate, only the reversion of the reaction catalysed by 2-oxoglutarate dehydrogenase is required. *E. coli*'s native 2-oxoglutarate dehydrogenase uses NADH as a cofactor, formation of 2-oxoglutarate from succinyl-CoA and CO_2 has a $\Delta G_0'$ of 35.8 kJ/mol, so the reaction is irreversible.

The fixation of inorganic carbon is one of the main ways of accumulating biomass and organic products. For this process a reductive TCA cycle, which does not naturally exist in *E. coli*, plays an important role. The reductive TCA cycle has been found from some anaerobic and microaerobic bacteria such as *Hydrogenobacter thermophilus* (Yamamoto et al., 2003).

H. thermophilus has a capacity to fix carbon by using two ferredoxin dependent enzymes: Pyruvate Oxidoreductase (POR) and 2-Oxoglutarate oxidoreductase (OGOR) (Yamamoto et al., 2010). In this case, reduced ferredoxin is used as a cofactor for the conversion of succinyl-CoA to 2-oxoglutarate and has a $\Delta G_0'$ of 15.5 ± 12.9 kJ/mol, which means that it can occur under non-standard conditions.

We tested reversion of the TCA cycle in *E. coli* by expressing OGOR coupled with ferredoxin (*fdx*) from *H. thermophiles* in a glutamate-

auxotroph isocitrate dehydrogenase (*icd*) deficient strain. Initially, expression of recombinant OGOR in *E. coli* lead to misfolded protein and no enzymatic activity was detected. Therefore, OGOR was also expressed in a glutamate auxotroph isocitrate dehydrogenase (*icd*) deficient *E. coli* strain. The strain did not restore its ability to grow on glutamate free medium, suggesting that the reverse TCA cycle (rTCA) was not fully functional *in vivo*. This can be due to loss of enzymatic activity (either the OGOR or ferredoxin) under mesophilic growth conditions or disparity/compatibility of ferredoxins of different species. More research using other enzymes, strains and ferredoxins is necessary to realize this conversion. In addition, overexpression of *E. coli*'s native pyruvate:ferredoxin oxidoreductase (*ybdK*) (Akhtar and Jones, 2009) might be required to provide sufficient amount of acetyl-CoA for the oxidative part of TCA cycle and reduced ferredoxin.

Besides already implemented *ldhA* and *pta* knockouts, an additional pyruvate formate lyase (*pflB*) gene deletion is required to get rid of the formate, CO_2 and H_2 production. This would result in a homofermentative itaconate production pathway, Figure 6.1. The reaction equation is then 3 glucose + 2 CO_2 → 4 itaconate + 6 H_2O, which has a $\Delta G_0'$ of -197.3 ± 6.2 kJ/mol, indicating that the conversion can occur spontaneously and allows synthesis of ATP, chapter 5. The equation shows that this approach results in a Y^P of 1.33 for itaconate production, which is identical to the Y^E.
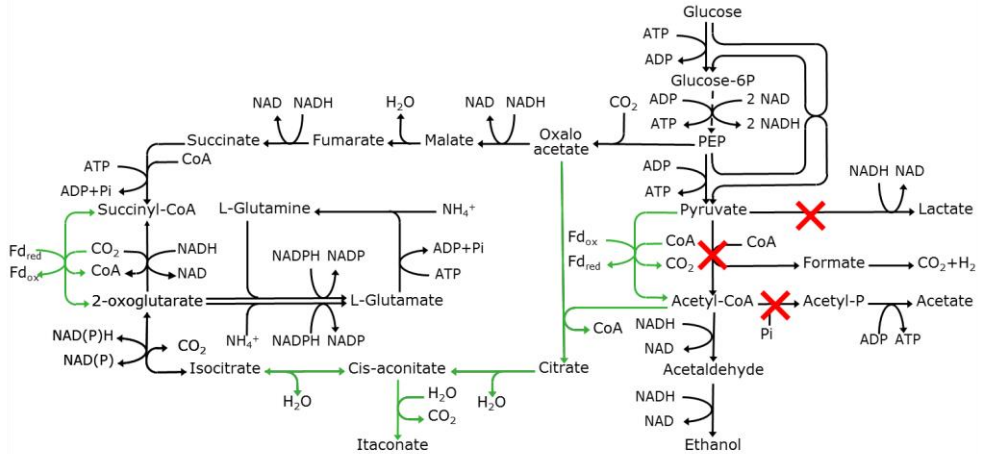
Figure 6.1 The pathway design for anaerobic homofermentative itaconate production in metabolically engineered *E. coli*. The green bold arrows indicate the introduced pathway consisting of genes encoding citrate synthase (*gltA*) and aconitase (*acnA*) from *C. glutamicum*, *cis*-aconitate decarboxylase (*cadA*) from *A. terreus,* 2-oxoglutarate:ferredoxin oxidoreductase (*korAB*) coupled with ferredoxin (*fdx*) from *H. thermophilus* and pyruvate:ferredoxin oxidoreductase (*ybdk*). The red crosses indicate gene deletions: phosphate acetyltransferase (*pta*), lactate dehydrogenase (*ldhA*) and pyruvate formate lyase (*pflB*).

6.2 Possible solutions for current bottlenecks

6.2.1 Insufficient CadA activity

E. coli is a commonly used workhorse for the production of proteins and chemicals, still problems in heterologous expression arise. Most of the expression vectors were initially designed to boost expression of genes to its maximum, which is usually not desired in pathway engineering where a balanced flux distribution is required. Overexpression of heterologous genes in *E. coli* often leads to protein instability and strong promoter takes control over the whole metabolism, lowering the expression of native genes that creates pathway bottlenecks. When a

foreign gene is expressed in *E. coli*, conditions like pH, osmolarity, redox potential, cofactors, and folding mechanisms often differ from that of the donor organism, which in the worst case scenario means complete loss of enzymatic activity (Rosano and Ceccarelli, 2014). Overall, expression of heterologous genes under the control of strong promoters is causing stress for cells and multicopy number plasmids can be considered as a metabolic burden for *E. coli* (Jones et al., 2000). Indeed, we showed that heterologous expression of *cadA* leads to inclusion body formation. An insufficient capacity of CadA to decarboxylate *cis*-aconitate to itaconate activity was proposed as an explanation to low itaconate titre and high flux to by-products such pyruvate and glutamate. Under anaerobic conditions, pyruvate accumulation is likely caused by a redox imbalance. To maintain redox balance, *E. coli* has to produce itaconate together with ethanol and/or succinate. When the flux to itaconate is too low compared to the fluxes to ethanol and succinate, the strains will become NADH-limited, which resulted in pyruvate accumulation like explained in Chapter 4. Nitrogen limited growth medium suppressed glutamate production, which simultaneously facilitates itaconate production as both itaconate and glutamate originate from oxidative part of TCA cycle. Acetate accumulation, however, is a typical phenomenon under aerobic conditions when TCA cycle is not capable of metabolizing all glucose that cells have taken up. Better control over glucose concentration in the medium is an efficient way to prevent accumulation of this overflow metabolite. In Chapter 2 and 3, a couple of strategies such as optimization of the expression conditions and harmonization of codon usage were realized to increase the solubility of CadA. Moreover, laboratory-directed protein evolution has proven to be an efficient tool to evolve proteins into desired directions, while selective pressure is applied (Yuan et al., 2005). However, the number of generations that is required to obtain the desired genotype is often difficult to predict.

Perhaps, a faster alternative to the protein evolution and more stable option to plasmid expression is gene integrations. (Koma et al., 2012) developed a Red-mediated recombination based method, which allows insertion of multiple genes into target loci on the *E. coli* chromosome. This method is of special interest to create new metabolic pathways, like reversing the TCA cycle, where a number of enzymes are required. In addition, other recently developed tools like CRISPR/Cas (Hsu et al., 2014) provides an efficient platform for genome editing in a large scale.

6.2.2 The role of itaconate transport

Even a significant increase in CadA activity did not result in substantially higher itaconate concentrations and *E. coli* produced a variety of by-products such as succinate, pyruvate and acetate. Besides CadA activity, itaconate production might be limited by inefficient transport capacity. Okamoto et al. (2014) (Okamoto et al., 2014) measured increased intracellular itaconate concentrations in their production strains and suggested that extracellular secretion of itaconate in *E. coli* is limiting the production. Since itaconic acid gene cluster of *A. terreus* has a putative mitochondrial tricarboxylate transporter (Li et al., 2011), the existence of a specific transporter is expected. Expression of *A. terreus* mitochondrial transporter *mttA* and plasma membrane transporter *mfsA* were beneficial to production of itaconate in *A. niger* (Li et al., 2013, van der Straat et al., 2014). The functionality of these fungal transporters in *E. coli* has not been confirmed. A preliminary BLAST search revealed that there is no putative itaconate transporter found in the bacterial domain. Transporters for other dicarboxylic acids are known for *E. coli,* and many of them possess a broad substrate specificity and have the ability to transport different molecules across the membrane (Janausch et al., 2002). These transporters are often present in the membranes

and are in charge of catalysing nutrient uptake, but also help mediating the effluxes. Considering the structural similarity of itaconate with some of the intermediates of the citric acid cycle like succinic acid it could be hypothesised that itaconate may be transported across the membrane by one, or more, of *E. coli*'s endogenous transporters. For instance, the main succinate transporter, DauA, was shown to transport also other dicarboxylic acids at pH 7 (Karinou et al., 2013), suggesting that overexpression of *dauA* may boost itaconate export. Other prominent candidates are DcuC and DcuB, which are members of dicarboxylate uptake involved in fumarate-succinate exchange and uptake (Zientz et al., 1999). However, it needs to be taken into account that the transporters are regulated in different ways and their level of expression is influenced by environmental conditions such as aerobic or anaerobic growth and presence or absence of substrate in the medium (Golby et al., 1999). As itaconate is being secreted to culture medium, most likely some of the endogenous transporters are already transporting it across the membrane but with low rate.

6.2.3 Synthesising additional ATP

Glucose oxidation is the most common way to produce energy and synthesize ATP in aerobic microorganisms. Oxygen allows complete oxidation of substrate to CO_2, which generates high amounts of ATP that can be used for the conversion of substrate to microbial biomass. This has a negative impact on product yield and productivity. However, oxygen is only necessary for growth and maintenance if the product pathway does not generate sufficient ATP. When product formation results in ATP generation, this can be used for cellular growth and maintenance.

Anaerobic itaconate production, with co-production of ethanol and hydrogen gas, yields 8 times less ATP compared to aerobic production (Chapter 4). The lowered ATP production will reduce biomass formation and also the anaerobic co-production of itaconate, ethanol and hydrogen gas will not be limited by oxygen transfer, which both can result in higher productivities.

The ATP yield of the two homofermentative itaconate production examples (rGS and rTCA cycle) presented in Chapter 5, is respectively negative or zero, indicating that product formation will not sustain growth and maintenance. In addition, ATP requirement for itaconate transport was not taken into account. Therefore, alternative ATP-generating steps such as using PEP carboxykinase instead of PEP carboxylase (Moon et al., 2008) may be necessary to add. Alternatively, micro-aerobic production conditions may be used to generate sufficient amounts of ATP for growth and maintenance, like (Mainguet et al., 2013) showed with rGS.

6.2.4 Production host

Due to number of aspects presented earlier in this work and by others (Cueto-Rojas et al., Weusthuis et al., 2011), it is suggested that bulk chemical production should be anaerobic. We chose to use *E. coli* in this work, as it is one of the few industrial micro-organisms able to grow under anaerobic conditions and has other desired features (such as simple media requirements, tools for genetic modifications, see Chapter 1). However, compared to filamentous fungi, yeasts and thermophilic bacterium, *E. coli* is not considered as a robust production host. For high yield organic acid production, it has a few major disadvantages: a high pH optimum, low product tolerance and susceptibility to bacteriophages.

At low production pH, itaconic acid could directly be crystallized after filtration (Klement and Büchs, 2013), but *E. coli* process can only operate at near neutral conditions. *E. coli* has a growth pH optimum of seven and addition of base (lime) is necessary when organic acids are produced at neutral pH. During downstream processing the organic acid salt has to be converted into the organic acid, which is usually done by adding sulphuric acid. This results in the production of vast quantities of salts (gypsum). From an economic point of view, adding extra downstream processing steps are obviously not desired. In addition, an increased itaconate concentration might become toxic for the bacterium. To avoid toxicity, an *in-situ* product removal might be necessary. For instance, ethanol fermentation at low pressure (Cysewski and Wilke, 1977) and lactic acid production in continuous membrane reactor (Tejayadi and Cheryan, 1995) are successful examples of already existing *in-situ* processes. A continuous itaconic acid production with integrated cell retention and product recovery has already been realized using *Ustilago myadis* in a membrane bioreactor (Carstensen et al., 2013). In addition, production of itaconic acid by immobilizing *A. terreus* biomass has been tested by using porous disk reactor (Ju and Wang, 1986) and in agar gel cubes (Kautola et al., 1985) among other techniques.

To overcome these issues, a strain improvement by laboratory evolution could help to make strains more resistant to organic acid production. In general, both pH optimum and product tolerance are difficult to engineer, so perhaps a better alternative is to screen for a more robust production organism and engineer that to produce itaconate. As such, thermophilic bacterium could offer an interesting production platform, but the lack of genetic tools for thermophiles has up till now limited their use. Recently, a facultative anaerobic thermophilic *Bacillus smithii* was

isolated from compost (Bosma et al., 2015b). *B. smithii* is able to produce organic acids, can utilize C5 and C6 sugars, has a relatively broad pH range and grows at elevated temperature (55°C), which are all desired characteristics for an industrial production host. In addition, a markerless gene deletion protocol was developed for this bacilli (Bosma et al., 2015a), which represents the first steps towards rational metabolic engineering of thermophilic bacilli.

6.2.5 Strain and process design

Aerobic itaconate production in *E. coli* is unlikely to be able to compete with the current *A. terreus* process, so in this paragraph strain and process design focuses on anaerobic pathway and process design. As presented in Chapter 1, anaerobic production of itaconate and other bulk chemicals would offer substantial benefits over aerobic product synthesis.

When the process is fully optimized, anaerobic itaconate production could result in a similar volumetric productivity (10-15 $kg/m^3/h$) than other classical fermentations like ethanol and lactic acid. That is a significant improvement compared to the best performing aerobic processes such as glutamic acid and lysine production with a volumetric productivity of only around 5 $kg/m^3/h$. The higher yield, titer and productivity of the anaerobic process can be obtained, because oxygen transfer does not limit production and substrate cannot be fully oxidised resulting in lower biomass formation and higher substrate efficiency. The proof of concept for anaerobic itaconate production was realized in Chapter 4, but the product concentrations remained low mainly due to a high flux towards by-products. Itaconate transport as a potential

bottleneck was already discussed earlier in this chapter, so here we focus on strategies to reduce flux to other organic acids.

Heterofermentative itaconate production requires coproduction of ethanol or succinate in combination with hydrogen gas to maintain redox balance. Ethanol and hydrogen gas seem to be the best set of co-products for industrial application as these products can be simply separated based on their boiling temperatures, and easily marketed as bulk chemicals. Separation of itaconate and succinate is more difficult due to structural similarity and possibility to co-polymerase. Succinate production can easily be reduced by deleting fumarate reductase (*frd*) (Zhou et al., 2006).

Knocking out *ldhA* completely suppressed lactate production, but the elimination of *pta* did not result in a significant reduction of acetate production. Deletion of additional acetate forming pathways such as *ackA* and *poxB* is proposed. However, literature shows that *E. coli* is capable of producing acetate after deletion of all three known acetate forming pathways (Phue et al., 2010). It is known that there is a correlation between redox balance and acetate production (Vemuri et al., 2006). Glucose is not completely oxidized under anaerobic conditions, which causes accumulation of mixed acid fermentation products to maintain redox balance. If the glucose consumption rate is faster under aerobic conditions than cells' capacity to reoxidize reduced cofactors, a similar kind of response, known as acetate overflow metabolism, is observed. A feedback controlled glucose feed is an effective way to prevent overflow metabolism and thus reduce acetate formation under aerobic conditions (Akesson et al., 2001). Complete elimination of acetate production was achieved even at a very high glucose consumption rate by deletion of aerobic respiration control

protein encoded by *arcA* together with expression of NADH oxidase (Vemuri et al., 2006).

In addition, glutamate accumulation was observed when *gltA* and *acnA* from *C. glutamicum* were overexpressed to boost carbon flux through TCA cycle. A nitrogen-limited growth medium suppressed glutamate production, which resulted in an increase in itaconate production. However, the decrease in glutamate production was not completely directed to itaconate production, but also towards pyruvate formation. Pyruvate accumulation is a typical sign of redox imbalance and in this case is expected to be related to insufficient itaconate export. A strategy to delete isocitrate dehydrogenase (*icd*), that leads to glutamate auxotrophic strains was also studied. The triple mutant strains (*pta*$^-$, *ldhA*$^-$ and *icd*$^-$) were no longer able to stably express CadA under anaerobic conditions on minimal medium. Instead of completely deactivating *icd*, a lowered enzymatic activity might be sufficient to suppress glutamate production. This was achieved in *C. glutamicum* by exchanging the ATG start codon to GTG or TTG, which resulted in 60 mM itaconate production together with nitrogen limitation and CadA expression (Otten et al., 2015).

Most importantly, the two homofermentative itaconate production pathways that were presented in Chapter 5, have potential to overcome these issues with by-product formation as itaconate biosynthesis is coupled to co-factor regeneration. Thus, the major by-product forming pathways (*pfl*, *ldhA* and *pta*) can be deleted, allowing the maximal theoretical yield.

Glucose is too expensive substrate for bulk itaconic acid production (Huang et al., 2014) and utilization of low cost substrates like starch,

molasses, hydrolysates of syrups or lignocellulosic biomass are needed for cost-efficient microbial production. Conversion of lignocellulose to itaconate requires a microorganism that can effectively ferment both hexose and pentose sugars. An *E. coli* strain, which is able to ferment glucose and xylose to ethanol with a high yield was isolated after subjecting to mutagenesis with ethyl methane sulfonate (Kim et al., 2007). An essential mutation was found at *pdh* operon (*pdhR aceEF lpd*), which encodes components of the pyruvate dehydrogenase complex. They proposed that the ethanol production is a result of a novel pathway combining fermentative alcohol dehydrogenase with pyruvate dehydrogenase, which is usually linked to aerobic metabolism. To expand substrate range of the itaconate producing *E. coli* strains, perhaps the same principle of directed laboratory evolution could also be applied. However, new feedstocks are a potential cause of undesired side effects because additional salts and inhibiting compounds could enter the process. To avoid inhibitory effects, extra pre-treatment steps of the substrates might be required. Thus, a proper characterisation of novel substrates together with techno-economic analysis must be carried out before conclusive remarks on different substrates can be made.

6.3 Future perspectives and conclusions

According to the latest report by Transparency Market Research (TMR, 2015), the global itaconic acid market was valued at US$ 126.4 million in 2014 and is expected to reach US$ 204.6 million by 2023. Currently, the high costs of production has limited the use of itaconic acid as a substituent in several applications including synthetic latex, unsaturated polyester resins, detergents, superabsorbent polymers and as a component of biofuels. For instance, itaconic acid is being developed as

an alternative way for production of methyl metacrylates (MMA), which have a global consumption of more 3.2 million metric tons per year. Lucite International has patented production of bio-MMA from itaconic acid through a fermentation process (Johnson et al., 2013). Ongoing developments in fermentation process, scale-up and synthetic biology are expected to lower the production costs, which is of key importance to expand itaconic acid market potential.

This work represents the first steps towards heterologous itaconate production in *E. coli*. Although the obtained itaconate product titres were still modest, the study created a basis of understanding the production mechanism in *E. coli*. We realized a proof of principle for anaerobic heterofermentative production of itaconate, in combination of ethanol and hydrogen gas. In addition, ways to reach theoretical maximum yield of itaconate and other industrially important chemicals from TCA cycle were presented in an opinion paper. This concludes that the maximum theoretical yield of itaconate can be reached, but only future will show can it be realized in *E. coli* as a production host.

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Summary

Summary

Interest in sustainable development together with limited amounts of fossil resources have increased the demand for production of chemicals and fuels from renewable resources. The market potential for bio-based products is growing and a transition from petrochemicals to biomass-based chemicals is ongoing. Itaconic acid is a C5-dicarboxylic acid which can be produced by microbial conversion processes. It can be easily polymerized and is an appealing building block for the chemical industry with many potential applications. However, biobased chemicals have to compete with their petrochemical counterparts, and yield and productivity of the microbial processes are therefore of the utmost importance. Traditionally itaconic acid is produced using the ascomycete *Aspergillus terreus.* This process is not competitive with petrochemical processes due to high production costs caused by low yields, and difficult and expensive product recovery. Maximizing product yield is important to lower production costs. This thesis looked at ways to reach theoretical maximum yield in a recombinant production host, *Escherichia coli*.

Chapter 2 describes the construction of an itaconate biosynthesis pathway in *E. coli*. The key enzyme of microbial itaconate production is *cis*-Aconitate decarboxylase (CadA) that converts the citric acid cycle intermediate *cis*-aconitate into itaconate. We focused on optimizing heterologous expression of *cadA* from *Aspergillus terreus* in *E. coli.* Initially this resulted in low CadA activities and production of trace amounts of itaconate. CadA was primarily present as inclusion bodies, explaining the low activity. The activity was significantly improved by using lower cultivation temperatures and mineral medium and this resulted in enhanced itaconate titres. The itaconate titre was further increased in aerobic bioreactor cultures by introducing citrate synthase and aconitase from *Corynebacterium glutamicum* and by deleting genes

encoding phosphate acetyltransferase and lactate dehydrogenase. The maximum itaconate yield from glucose obtained in this study was only 0.09 mol/mol, due to high flux of carbon to by-products such as acetate and pyruvate. Pyruvate is a precursor molecule for itaconate biosynthesis and its accumulation suggested that the activity of CadA might be one of the rate limiting steps. It was concluded that further optimization of *cadA* expression, and reduction of acetate formation should be achieved to obtain higher itaconate yield.

As sufficient *cis*-aconitate decarboxylase activity is crucial for itaconate production, in **chapter 3** ways to increase the activity of CadA were investigated. A recently characterized *cis*-aconitate decarboxylase of mammalian origin was therefore expressed in *E.coli*. The novel *cis*-aconitate decarboxylase from *Mus musculus* encoded by immunoresponsive gene 1 (*irg1*) produced comparable amounts of itaconate as CadA from *A. terreus.* In addition, the effects of codon optimization and harmonization on enzymatic activities of heterologously expressed *cadA* and *irg1* were studied. Codon harmonization increased the activity of CadA in cell free extracts, but this did not result in higher itaconate production in bioreactor cultures. This suggests that other factors such as itaconate transport may limit the production.

In **chapter 4**, proof of principle for an anaerobic fermentation process for the production of itaconic acid was obtained by using the mixed acid fermentation pathway of *E. coli*. Itaconic acid production was redox balanced by co-producing succinate or ethanol with H_2 and CO_2. Expression of *cadA* together with citrate synthase (*gltA*) and aconitase (*acnA*) from *Corynebacterium glutamicum* resulted in 0.66 mM (1.2 % Cmol) itaconate under anaerobic conditions. Unexpectedly, strains started to produce significant amounts of glutamate when the itaconate

pathway was introduced. As glutamate production depends on the availability of nitrogen in the medium, a nitrogen-limited medium was tested to diminish glutamate production. This enhanced the production of itaconate to up to 2.9 mM (5.4 % C mol %). Here, anaerobic production of itaconate from glucose was reported for the first time. The observed itaconate yields and productivities were still modest. Eliminating the pathways to major by-products like glutamate, succinate, and acetate, and enhancing the pathway between pyruvate and itaconate is crucial to obtain a cost-competitive anaerobic itaconic acid process production.

To investigate how itaconate production can be improved, the insights from the previous chapters together with existing scientific literature were combined with our pathway design proposals in **chapter 5.** The tricarboxylic acid (TCA) cycle is an important source of precursors for biobased chemicals. The opinion article takes a closer look at the metabolic engineering of TCA cycle for the production of chemicals high yield. For most TCA cycle products the maximum pathway yield is much lower than the theoretical maximum yield. For succinate, this was solved by creating two pathways to the product, using both branches of the TCA cycle, connected by the glyoxylate shunt. A similar solution cannot be applied directly for production of compounds from the oxidative branch of the TCA cycle because irreversible reactions are involved: the conversion of acetyl-CoA and glyoxylate to malate in the glyoxylate shunt and the conversion of 2-oxoglutarate into succinyl-CoA in the TCA cycle. This way, the pathway yield for products originating from the oxidative TCA cycle branch such as citrate, itaconate and L-glutamate becomes identical to the theoretical maximum. Future research should focus on implementing these solutions in suitable production hosts, and increasing the ATP yield of the production pathways. This will minimize

the oxygen requirement of the process, or even allow for anaerobic operation, and should lead to reduced operational costs and maximal product yields.

In **chapter 6** the implications of the overall results of this thesis for the current research status of itaconate production are presented. Solutions to optimize itaconate production strains and production process were proposed.

Samenvatting

De vraag naar chemicaliën en brandstoffen uit hernieuwbare grondstoffen neemt toe omdat de fossiele grondstofvoorraden afnemen en door een groeiende interesse in duurzame ontwikkeling. Het marktpotentieel voor bio-based producten groeit en een overgang van petrochemie naar biomassa gebaseerde chemicaliën vindt plaats. Itaconzuur is een C5-dicarbonzuur, dat geproduceerd kan worden met behulp van microbiele conversie processen. Het kan worden gepolymeriseerd en is daarom een aantrekkelijke bouwsteen voor de chemische industrie met vele toepassingsmogelijkheden. Biobased chemicaliën moeten concurreren met hun petrochemische tegenhangers, en de opbrengst en productiviteit van de microbiële processen zijn daarom van groot belang. Momenteel wordt itaconzuur voornamelijk gemaakt met de ascomycete *Aspergillus terreus*. Dit proces is niet competitief met petrochemische processen omdat een lage opbrengst en de moeilijke productopwerking het proces duur maken. Het maximaliseren van de productopbrengst is belangrijk om de productiekosten te verlagen. In dit proefschrift is onderzocht hoe de theoretische maximale opbrengst in een recombinante productie gastheer, *Escherichia coli*, bereikt kan worden.

Hoofdstuk 2 beschrijft de constructie van een itaconaat biosynthese *pathway* in *E. coli*. Het sleutelenzym van microbiële itaconaat productie is cis-aconitaat decarboxylase (CADA). Het zet het citroenzuurcyclus tussenproduct cis-aconitaat om in itaconaat. De heterologe expressie van Cada uit *Aspergillus terreus* in *E. coli* werd geoptimaliseerd. Aanvankelijk was de CadA activiteit laag en warden er slechts kleine hoeveelheden itaconaat geproduceerd. CadA was vooral aanwezig in de vorm van *inclusion bodies*, hetgeen de lage activiteit verklaard. De activiteit werd significant verbeterd door te kweken bij lagere temperaturen en door een anorganisch medium te gebruiken. Dit

resulteerde in verhoogde titers itaconaat. De itaconaat titer werd verder verhoogd in aërobe bioreactor cultures door het introduceren van citrate synthase en aconitase uit *Corynebacterium glutamicum*, en door genen die coderen voor fosfaat acetyltransferase en lactaat dehydrogenase te schrappen. De maximale itaconaat opbrengst uit glucose verkregen in deze studie was slechts 0,09 mol / mol. Dit komt met name door de vorming van bijproducten zoals acetaat en pyruvaat. Pyruvaat is een precursor molecule voor itaconaat biosynthese en ophoping ervan suggereert dat de activiteit van CadA snelheidsbeperkende is. Geconcludeerd werd dat verdere optimalisering van CadA expressie en vermindering van acetaat formatie nodig was voor een hogere itaconaat opbrengst.

Voldoende cis-aconitaat decarboxylase activiteit is cruciaal voor itaconaat productie. In **hoofdstuk 3** werd onderzocht hoe de CadA activiteit kon worden verhoogd. Een onlangs gekarakteriseerde cis-aconitaat decarboxylase van zoogdieren werd daarom tot expressie gebracht in *E. coli.* Expressie van de nieuwe cis-aconitaat decarboxylase van *Mus musculus* "immunoresponsive gene 1" (irg1) resulteerde in vergelijkbare hoeveelheden itaconaat als CadA van *A. terreus*. Ook werd het effect van codon optimalisatie en harmonisatie op de enzymatische activiteiten van cadA en irg1 bestudeerd. Codon harmonisatie verhoogde de activiteit van CadA in celvrije extracten, maar dit resulteerde niet in een hogere itaconaat productie in bioreactor cultures. Dit suggereert dat andere factoren, zoals itaconaat transport, de productie kunnen beperken

In **hoofdstuk 4** werd proof of principle verkregen voor een anaëroob fermentatieproces voor de productie van itaconzuur met behulp van de gemengd zure fermentatie *pathway* van *E. coli*. De redox balans werd

gehandhaafd door itaconzuur productie te combineren met de coproductie van succinaat of ethanol met H_2 en CO_2. Expressie van cadA met citraat synthase (gltA) en aconitase (acnA) van *Corynebacterium glutamicum* resulteerde in 0,66 mM (1,2% Cmol) itaconaat onder anaërobe omstandigheden. De stammen begonnen onverwachts aanzienlijke hoeveelheden glutamaat te produceren. Glutamaat produktie is afhankelijk van de beschikbaarheid van stikstof in het medium. Daarom werd getest of de glutamate productie verlaagd kon worden door een stikstof-gelimiteerd medium te gebruiken. Hierdoor nam de productie van itaconaat toe tot maximaal 2,9 mM (5,4% C mol%). Dit is de eerste keer dat anaërobe productie van itaconaat van glucose werd gerapporteerd. De waargenomen itaconaat opbrengsten en productiviteiten waren nog bescheiden. Het elimineren van de *pathways* naar belangrijke bijproducten zoals glutamaat, succinaat en acetaat, en verbetering van de route tussen pyruvaat en itaconaat is cruciaal voor het verkrijgen van een efficiënter anaëroob itaconzuur productieproces.

Om te onderzoeken hoe itaconaat productie kan worden verbeterd, werden in **hoofdstuk 5** de inzichten uit de voorgaande hoofdstukken samen met bestaande wetenschappelijke literatuur gecombineerd met onze *pathway* ontwerpen. De tricarboxylzuur (TCA) cyclus is een belangrijke bron van *precursors* voor biobased chemicaliën. Voor de meeste TCA cyclus producten is de maximale *pathway* opbrengst veel lager dan de theoretisch maximale opbrengst. Voor succinaat werd dit opgelost door het creëren van twee *pathways* naar het product, door gebruik te maken van beide takken van de TCA cyclus, verbonden door de glyoxylaat shunt. Een soortgelijke oplossing kan niet direct worden toegepast voor de productie van verbindingen van de oxidatieve tak van de TCA cyclus omdat hier onmkeerbare reacties bij betrokken zijn: de omzetting van acetyl-CoA en glyoxylaat in de glyoxylaat shunt en de

omzetting van 2-oxoglutaraat in succinyl-CoA in de TCA cyclus. Hierdoor wordt de *pathway* opbrengst van producten uit de oxidatieve TCA cyclus tak zoals citraat, itaconaat en L-glutamaat gelijk aan het theoretische maximum. Toekomstig onderzoek moet zich richten op de implementatie van deze oplossingen in geschikte productie gastheren, en het verhogen van de ATP-opbrengst van de productie routes. Dit minimaliseert de zuurstofbehoefte van het proces, of maakt het zelfs mogelijk deze processen anaeroob uit te voeren.

De implicaties van de resultaten van dit proefschrift voor de stand van zaken rond onderzoek naar itaconzuurproductie worden gepresenteerd in **hoofdstuk 6.** Hierin worden oplossingen voorgesteld voor het optimaliseren van stammen en processen voor itaconzuur productie.

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About the author, publications and training activities

About the author

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During her BSc studies Kiira did an internship at Valio ltd. in Helsinki, Finland, where she characterized probiotic bacteria from cheese microbial cultures. During her second internship she was an assistant teacher in gene technology laboratory course at the University of Helsinki. After completion of her BSc, she continued with her MSc at the same university.

She completed her MSc thesis titled 'Expression of heterologous genes in the filamentous fungus *Aspergillus niger*' in the Centre of Excellence project 'White Biotechnology-Green Chemistry' at Technical Research Center of Finland VTT in Espoo, Finland. After completion of her thesis she continued to work at VTT's Cell factory in a Future Biorefinery (Fubio) project, which focused on developing transformation methods for filamentous fungi and novel modification tools for wood derived terpenoids.

In 2011, Kiira moved to Netherlands and started her PhD study at Wageningen University under supervision of Ruud Weusthuis, Gerrit Eggink and Johan Sanders. During this PhD thesis, she worked on metabolic engineering of *Escherichia coli* for the production of itaconic acid. Since September 2015, she has been working as a post-doctoral researcher at Protein Engineering and Proteomics group at Norwegian University of Life Sciences (NMBU) in Ås, Norway.

List of publications

Kiira S. Vuoristo, Astrid E. Mars, Jose Vidal Sangra, Jan Springer,
Gerrit Eggink, Johan P. M. Sanders, and Ruud A. Weusthuis (2015),
Metabolic engineering of itaconate production in Escherichia coli,
Appl Microbiol Biotechnol. 99(1):221-8

Kiira S. Vuoristo, Astrid E. Mars, Stijn van Loon, Enrico Orsi,
Gerrit Eggink, Johan P. M. Sanders, and Ruud A. Weusthuis (2015),
*Heterologous expression of Mus musculus immunoresponsive gene 1
(irg1) in Escherichia coli results in itaconate production,*
Frontiers in Microbiology 2015.00849

Kiira S. Vuoristo, Astrid E. Mars, Jose Vidal Sangra, Jan Springer,
Gerrit Eggink, Johan P. M. Sanders, and Ruud A. Weusthuis (2015),
*Metabolic engineering of the mixed-acid fermentation pathway of
Escherichia coli for anaerobic production of glutamate and itaconate,*
AMB Expr (2015) 5:61

Kiira S. Vuoristo, Astrid E. Mars, Johan P. M. Sanders,
Gerrit Eggink, and Ruud A. Weusthuis (2015),
Metabolic engineering of TCA cycle for production of chemicals
Trends in Biotechnology 1316, No. of pages 7, article in press

Overview of completed training activities

Discipline specific activities

Meetings and conferences

- BE-BASIC annual conferences, Noordwijkerhout (NL) 2012-2015
- Renewable Resources & Biorefineries RRB8, Toulouse (FR), 2012
- Metabolic engineering X, Vancouver (CA), 2014
- Netherlands Biotechnology Congress NBC15, Ede (NL) 2014
- KNVM Spring meeting, Arnhem (NL), 2015
- 23rd European Biomass Conference and Exhibition, Vienna (AT), 2015

Courses

- Advanced Course Microbial Physiology and Fermentation Technology, Delft (NL), 2014
- 2nd Biorefining summer school, Wageningen (NL), 2012
- Bioreactor design, Wageningen (NL), 2014
- Biorefinery for Biomolecules Wageningen (NL), 2012
- Symposium Microbes for sustainability Wageningen (NL), 2012
- Business Awareness Workshop , Utrech (NL), 2014

General courses

- VLAG PhD week, Baarlo (NL), 2012
- Project and time management, Wageningen (NL), 2012
- Philosophy and Ethics of Food Sc./Tech. , Wageningen (NL), 2012
- Career perspectives, Wageningen (NL), 2015
- Scientific writing, Wageningen (NL), 2014
- Techniques for writing and presenting scientific papers, Wageningen (NL), 2013

Optional

- Preparation of PhD research proposal
- PhD study trips to Spain (2012) and Brazil (2013)
- Member of organizing committee to PhD trip to Brazil
- Monthly group meetings (BCH and BPE)