Metabolic Engineering Toward 1-Butanol Derivatives in Solvent Producing Clostridia

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Table of content

Preface and outline

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This thesis describes the results of metabolic engineering toward 1-butanol-derivatives in solvent producing clostridia. The overall aim of the project involves the chemical stress on solventogenic clostridia. Important stress factors that generally lead to this sub-optimal fermentation performance are: (i) concentration of substrates, and (ii) concentration of products (*viz*. certain products and/or by-products may lead to severe inhibition of the fermentation process). Engineering solventogenic clostridia in an attempt to reduce the stress, resulting in higher efficiency of the industrial ABE process, was thought to result in higher yields of the products 1-butanol and/or derivatives thereof. 1-Butanol and derivatives were chosen, since these become more and more attractive as biofuel or biofuel additive.

Here, we focus on the identification of alternative less toxic 1-butanol derivatives. The most attractive compounds (*iso*-butanol, 2-butanol, and 2,3-butanediol) were selected based on their toxicity, but also on the feasibility to produce them using existing enzymatic steps. Metabolic adaptations to the existing pathways, involving these enzymes, were designed and subsequently engineered into *C. acetobutylicum*.

Chapter 1 – Introduction

The first chapter gives a general overview about the history of the acetone, butanol and ethanol (ABE) fermentation. The responsible solventogenic clostridia with their central metabolism are briefly discussed. Also the toxicity of 1-butanol, together with attempts to reach higher yields of 1-butanol, is described.

Chapter 2 – The Central Catabolic pathways – *C. acetobutylicum* ATCC 824 versus *C. beijerinckii* NCIMB 8052

The second chapter gives a comparison between the central catabolic pathways of *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052. With the genome sequence of *C. beijerinckii NCIMB 8052* also available, likely candidates for the 34 involved enzymatic conversions within the central catabolic pathway of *C. beijerinckii*, could be predicted. The enzymatic conversions, involved in glucose uptake, glycolysis, gluconeogenesis, pyruvate conversion and acetyl-CoA conversion towards the different end products, are being discussed.

Chapter 3 – Toxicity of 1-butanol and derivatives thereof

The third chapter describes a novel approach in solving the problem of the 1-butanol toxicity towards *C. acetobutylicum*. Increasing of the tolerance of 1-butanol has been tried more often in other research groups. In our approach, we chose for the introduction of new biosynthesis pathways that enable the production of less toxic 1-butanol derivatives.

Chapter 4 – Characterization and Overexpression of Acetolactate Decarboxylase in *Clostridium acetobutylicum*

The fourth chapter describes the identification, heterologous production, purification and biochemical characterization of a acetolactate decarboxylase from *C. acetobutylicum ATCC 824*. Ca-ALD encoded by CAC2967 was proven to exhibit acetolactate decarboxylase activity. A shuttle vector was constructed to express *Ca-ald* under control of the strong *adc* promoter in *C. acetobutylicum ATCC 824*.

Chapter 5 – Molecular characterization of an acetoin reductase from *Clostridium beijerinckii*

Chapter 5 describes the identification, heterologous production, purification and biochemical characterization of an acetoin reductase from *C. beijerinckii*. Out of six successfully cloned genes, one (*CBEI_1464*) showed substantial acetoin reductase activity after heterologous expression in *E. coli*. To gain insight into the reaction mechanism, but also into the substrate- and cofactor-specificity, a structural model was constructed with a ketose reductase (sorbitol dehydrogenase) from *Bemisia argentifolii* (silverleaf whitefly) as template.

Chapter 6 – Heterologous expression of an acetoin reductase leads to D-2,3-butanediol production in *C. acetobutylicum*

The sixth chapter describes the production of D-2,3-butanediol production in *C. acetobutylicum ATCC 824* by heterologous expression of Cb-ACR. A gene encoding ACR from *Clostridium beijerinckii* NCIMB 8052 has been functionally expressed in *C. acetobutylicum* under control of two strong promoters, *i.e.* the constitutive *thl* promoter and the late exponential *adc* promoter.

Chapter 7 – Summary and general discussion

This final chapter is a brief summary of the findings described in this thesis. Discussed are potential novel developments and future perspectives in ABE fermentation. Therefore, three new potential biosynthetic pathways in solventogenic clostridia are described intensively. Also some concluding remarks are given.

1

Introduction

This chapter has been adapted from:

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Chapter 1: Introduction

1.1 The history of the Acetone - Butanol - Ethanol (ABE) fermentation

Nowadays, production of 1-butanol is a well known type of fermentation, and observed among several bacterial strains, mainly from the genus *Clostridium*. The first description of the microbial production of this solvent, however, was already in 1861 by Louis Pasteur. In 1912, Chaim Weizmann isolated a bacterial strain called *Clostridia acetobutylicum*, which was able to ferment sugars to a mixture of acetone, 1-butanol and ethanol (ABE fermentation). Some of the butanol-producing microorganisms produced *iso*-propanol as co-product of the fermentation instead of acetone (the IBE fermentation process) [1]. In Word War I and II, the ABE fermentation gained a lot of interest, since acetone was used for the manufacturing of cordite [2, 3]. The ABE fermentation process was the first industrial process for the production of butanol, and started to be replaced by the emerging petrochemical industry from the 1960s due to economical considerations. However, the energy crisis of the 1970s lead towards higher energy costs, which resulted in a revived interest in the competitive ABE fermentation. Since the 1980s, ABE fermentation is researched extensively, especially since new molecular techniques were developed and more genetic information of ABE fermenting strains became available [3]. Large-scale ABE plants continued to be in operation in Russia and China until the mid 1990s [4, 5]. Presently, the process is being re-introduced in China [6] and other countries. Detailed reviews covering the history from different viewpoints have been published [7-13].

1.2 Solventogenic Clostridia

Over the years, a large number of clostridial strains, able to produce neutral solvents (acetone, butanol, ethanol, *iso*-propanol) from different carbohydrates has been isolated and described in patent applications [14]. Initially, solvent-producing strains were classified mainly as *C. acetobutylicum* or *C. beijerinckii*, but important physiological and genetic differences observed between strains belonging to the same group made it necessary to make a clear classification of the existing strains. Detailed DNA similarity studies and 16S rDNA sequence comparisons between strains belonging to different culture collections showed that the existing strains can be classified into four distinct groups [15-18] that are all members of the cluster I of the clostridia: *C. acetobutylicum* (type strain ATCC 824); *C. beijerinckii* (type strain NCIMB 9362); *C. saccharoacetobutylicum* (type strain NCP 262), and *C. saccharoperbutylacetonicum* (type strain N1-4). All known solventogenic clostridia are mesophilic and contain DNA with a low GC-content.

The biochemical pathways used by solvent-producing clostridial species for the conversion of carbohydrates into hydrogen, carbon dioxide, volatile fatty acids, acetone, butanol, ethanol or *iso*-propanol have been extensively studied and characterized [1]. Hexose sugars are metabolized via the Embden-Meyerhof pathway (Figure 1). One mole of hexose is converted to 2 moles of pyruvate, with the net production of 2 moles of ATP and 2 moles of NADH. The utilization of pentoses takes place via de pentose phosphate pathway (Warburg-Dickens pathway), yielding 5 moles of ATP and 5 moles of NADH and 2 moles of fructose-6-phosphate and 1 mole of glyceraldehyde-3-phosphate (which both enter the glycolytic pathway) per 3 moles of pentoses [1]. The pyruvate resulting from the glycolysis is converted by pyruvate:ferredoxin oxidoreductase in the presence of coenzyme A to yield CO_2, acetyl-CoA and reduced ferredoxin. Pyruvate and acetyl-CoA are the

central intermediates in the branched fermentation pathways leading to both acid and solvent production (Figure 1).

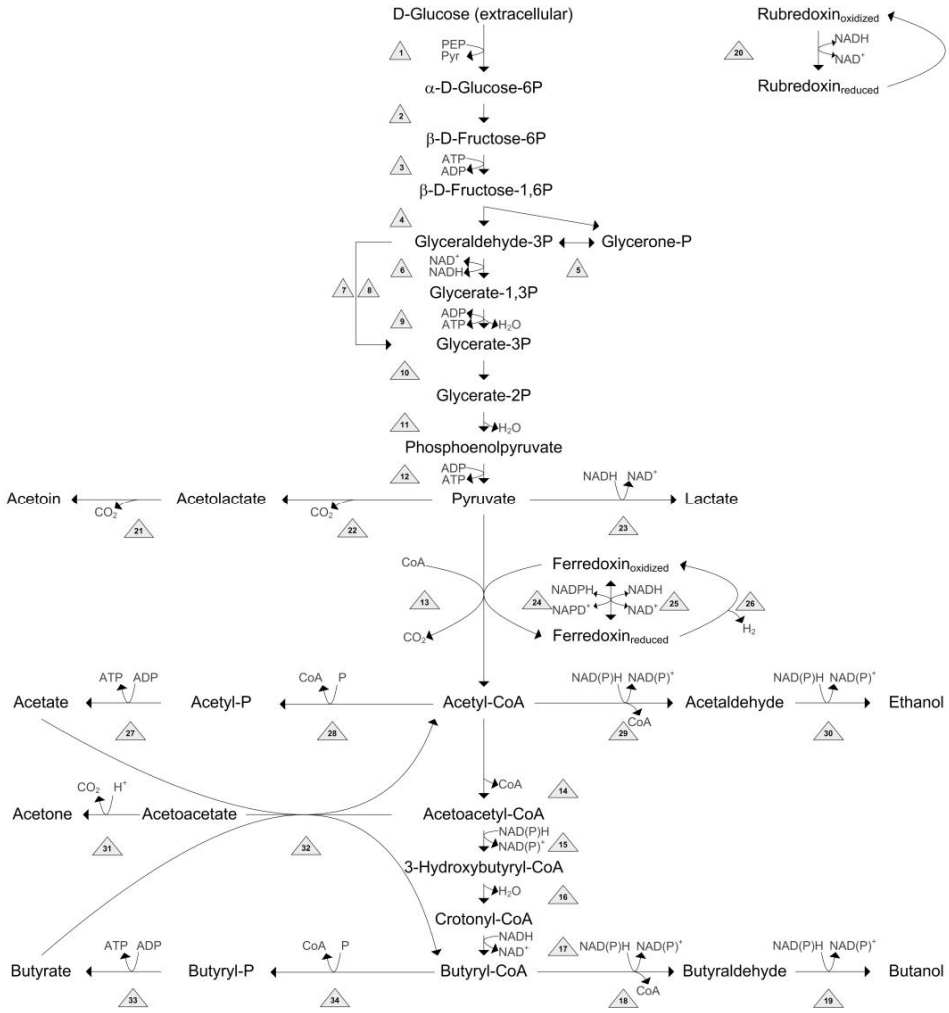

Figure 1: Central glycolytic metabolism of two solventogenic Clostridia: C. *acetobutylicum* ATCC 824 and C. *beijerinckii* NCIMB 8052, modified from Gheshlaghi [19]. Enzymes involved in this metabolism are shown with numbers. 1: PTS enzyme II, ABC components, 2: Glucose-6-phosphate isomerase (PGI), 3: 6-Phosphofructokinase (PFK), 4: Fructose-1,6-bisphosphate aldolase (FBA), 5: Triosephosphate isomerase (TPI), 6: GAP dehydrogenase (GAPC), 7: Aldehyde:ferredoxin oxidoreductase, 8: NADP-dep. GAP dehydrogenase (GAPN), 9: Phosphoglycerate kinase (PGK), 10: Phosphoglycerate mutase (PGM), 11: Enolase (ENO), 12: Pyruvate kinase (PYK), 13: Pyruvate: ferredoxin oxidoreductase (PFOR), 14: Thiolase (THL), 15: 3-Hydroxybutyryl-CoA dehydrogenase (HBD), 16: Crotonase (CRT), 17: Butyryl-CoA dehydrogenase (BCD), 18: Alcohol-aldehyde dehydrogenase (AADH), 19: Butanol dehydrogenase (BDH I,II), 20: NADH:rubredoxin-oxidoreductase, 21: Acetolactate decarboxylase, 22: Acetolactate synthase, 23: L-lactate dehydrogenase (LDH), 24: Ferredoxin:NADP oxidoreductase, 25: Ferredoxin:NAD oxidoreductase, 26: Hydrogenase (HYD) - (iron only, and nickel-iron), 27: Acetate kinase (ACK), 28: Phosphotransacetylase (PTA), 29: Alcohol-aldehyde dehydrogenase (AADH), 30: Acetaldehyde dehydrogenase (ACDH), Alcohol-aldehyde dehydrogenase (AADH), 31: Acetoacetate decarboxylase (AADC), 32: CoA transferase AB (CoAT AB), 33: Butyrate kinase (BK), 34: Phosphotransbutyrylase (PTB). Other abbreviations: ADP, Adenosine diphosphate;; ATP, Adenosine triphosphate; CoA, coenzyme A; NAD(P)H, reduced nicotinamide adenine dinucleotide (phosphate); PEP, Phosphoenolpyruvate; P, phosphate.

Despite numerous physiological studies, it is still not completely understood how solvent production is regulated at the molecular level [2]. During the exponential growth phase mainly acids are produced (acidogenic phase) by most strains, including species of *C. acetobutylicum*. By reaching the early stationary phase the production of solvents starts, a phenomenon known as "metabolic switch". Initiation of solvent formation requires low pH, threshold concentrations of acetate and butyrate, and a suitable growth-limiting factor such as phosphate or sulfate [20, 21]. Solvent formation appears to be associated with the availability of ATP and NAD(P)H [22] and can be controlled, in continuous culture, by varying the glucose concentration [23]. Increasing the reducing power in the cell by inhibiting hydrogenase can also enhance solvent formation. The reported role of a DNA-binding protein, Spo0A, on the expression of genes that are jointly involved in solvent production and sporulation in *C. beijerinckii* [24], suggests that these two phenomena may be connected.

Most of the genes that encode enzymes involved in primary metabolism have been characterized, and more and more knowledge becomes available on the regulation of the expression of genes involved in acid and solvent production and their function in the metabolic pathways [25, 26]. The best-studied solventogenic strain at the genetic level is *C. acetobutylicum ATCC 824*. In this strain, several genes involved in solvent production are located on a megaplasmid of 210 kb (pSOL1). The loss of this megaplasmid results in strains unable to make solvents [27]. The whole genome of *C. acetobutylicum ATCC 824* has been sequenced and is publicly available (http://www.ncbi.nlm.nih.gov) [28]. The presence of a similar megaplasmid in other strains has not been reported. The genome of another 1-butanol producer, strain *C. beijerinckii NCIMB 8052* has also been sequenced (http://genome.jgi-psf.org/finished_microbes/clobe/clobe.home.html),

although a scientific paper describing the outcome of the sequencing has not been published yet.

1.3 Toxicity

One of the factors which affects the feasibility of the ABE fermentation is the toxic effect of 1-butanol. Due to its hydrophobic chain and polar group, 1-butanol has membrane distorting properties, which cause severe cell damage [29, 30]. A good indicator for the strength of membrane perturbing effects of certain compounds is the log K_{ow} value (octanol:water coefficient) [31]. Generally, the lower the log K_{ow} value, the less toxic the compound is with regard to the membrane. Ethanol (log K_{ow} of -0.32), for example, has a lower log K_{ow} value than 1-butanol (log K_{ow} of 0.88). Biological ethanol production is also a known industrial process, which reaches solvent levels that are approximately 5-10 fold higher (10%-12% (v/v)) [32].

In the past, attempts have been made to increase the 1-butanol tolerance of the clostridial strains. Both, a chemical mutagenesis approach [33, 34], as well as adaptation strategy approaches [35], have been used to obtain mutants with an increased resistance towards 1-butanol. In some cases, this resulted in slight, to moderate increases in butanol production levels, while in other cases solvent production was lost [36].

In another approach, the overexpression of two heat-shock related proteins, GroES and GroEL [37], or overexpression of an endogenous putative transcriptional regulator (CAC1869) [38], resulted in *C. acetobutylicum* mutants with increased butanol tolerance and moderate butanol production levels.

1.4 Biofuels

It is estimated that the world energy consumption between 2001 and 2025 increases by about 54%, To meet these future energy needs, sustainable and carbon neutral energy sources are required. Therefore, by 2020, the European Union intends to replace 10% (energy value) of normal automotive fuels with biofuels. Liquid biofuels are attractive candidates, since little or no adaptations are required to the current petroleum-based fuel technologies [39, 40].

Nevertheless, with designing new liquid biofuel candidates, several other important factors like, energy content, combustion quality (octane or cetane number), volatility, freezing point, odor, and toxicity, need to be considered (Table 1) [41].

Different types of liquid fuels			
Fuel type	**Major components**	**Important property**	**Biosynthetic alternatives**
Gasoline	C_4-C_{12} hydrocarbons Linear, branched, cyclic, aromatic Anti-knock additives	Octane number[a] Energy content[b] Transportability	Ethanol, n-butanol and *iso*-butanol Short chain alcohols Short chain alkanes
Diesel	C_9-C_{23} (average C_{16}) Linear, branched, cyclic, aromatic Anti-freeze additives	Cetane number[c] Low freezing temperature Low vapor pressure	Biodiesel (FAMEs) Fatty alcohols, alkanes Linear or cylic isoprenoids
Jet fuel	C_8-C_{16} hydrocarbons Linear, branched, cyclic, aromatic Anti-freeze additives	Very low freezing temperature Net heat of combustion Density	Alkanes Biodiesel Linear or cylic isoprenoids

Table 1: Different types of liquid fuels with their major components, important properties and biosynthetic alternatives; [a] Measurement of knocking resistance; [b] Amount of energy produced during combustion; [c] Measurement of combustion quality of diesel fuel during compression ignition [41].

Biofuels are, by definition, the fuel products obtained from agricultural and forest waste, together with the biodegradable part of industrial and domestic waste [42]. The development of biofuels is nowadays divided into three types of generations: the first, second and third generation biofuels.

i) First generation biofuels are biofuels produced from directly available crops, containing sugar, starch or oil and are obtained using conventional technologies (i.e. bioethanol, biodiesel and biobutanol.

ii) Second generation biofuels are biofuels produced from raw waste, which is not easily hydrolysed (i.e. lignocellulosic materials). Glucose residues used for fermentation are obtained via pre-treatment of the raw feedstock (e.g. steam-explosion and/or acid treatment, followed by addition of exogenously produced cellulolytic enzymes to hydrolyse cellulose).

iii) Third generation biofuels refers to an alternative processing strategy, named consolidated bioprocessing (CBP). A single process step by microorganisms that express cellulolytic (and hemicellulolytic) enzymes, results in cellulase production, substrate hydrolysis and fermentation [40, 43].

To date, there are four main biofuels which are manufactured from biomass: i) ethanol, ii) methanol, iii) biodiesel, and hydrogen. However, other fuels like biobutanol and dimethyl ether are also made from biomass. So far, the biofuels, in a commercial perspective have been ethanol, manufactured from corn starch and sugarcane in the United States and Brazil, respectively, next to biodiesel which is produced mostly from rapeseed oil in Germany and France [44].

1.5 Production hosts

The desired host for production of liquid biofuels produces a single fermentation product and possesses high substrate utilization and processing capacities, preferably from lignocellulosic materials. It has also high metabolic

fluxes from sugar transport till product secretion and owns a good tolerance to substrate concentration, inhibitors and the fermentation product itself. The chosen starting point for engineering purposes depends on the priority one is giving to newly isolated strains with its potentials in comparison with improved recombinant, model organism with its potentials [45].

A lot of micro-organisms are capable to produce natural compounds which can be used as chemicals, pharmaceuticals or as biofuels. However, improvement of these capacities is often limited by lack of physiological knowledge or genetic tools for a particular micro-organism. Nowadays, more and more metabolic engineering is performed, to optimize these processes. *E. coli* is often chosen as the most promising host organism for microbial production of biofuels. It has, next to the above mentioned advantages, also multiple industrial advantages, like an efficient growth at industrially relevant conditions, the ability to grow in mineral salts medium with inexpensive components and in the absence of oxygen. But it also utilizes a wide variety of substrates including carbohydrates, polyols, and fatty acids [46].

Examples of known fermentative and non-fermentative pathways for the production of alcohols in *E. coli* are ethanol, 1-propanol, *iso*-propanol, 1,2-propanediol, 1,3-propanediol, 1-butanol, *iso*-butanol, 2,3-butanediol, 2-methyl-1-butanol, and 3-methyl-1-butanol [46-61].

One of the above mentioned alcohols with potential as biofuel is 2,3-butanediol [62]. Since the biosynthetic pathway to 2,3-butanediol is key subject of this thesis, the next sub-chapter is focusing on this particular diol.

1.6 2,3-butanediol

2,3-butanediol, a 1-butanol derivative (2,3 butylene glycol, CAS nr [513-85-9]) can be produced by a number of microorganisms in the genera *Bacillus*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, and *Serratia*. Three different stereomers exist, viz. D(-) or (2R,3R)-2,3-butanediol, L(+) or (2S,3S)-2,3-butanediol and *meso* or (2R, 3S)-2,3-butanediol. In literature, the biological production of all three different stereomers of 2,3-butanediol has been reported [62, 63]. The 2,3-butanediol fermentation process at pilot scale using strains of *Klebsiella oxytoca* and *Bacillus polymyxa* has been described during the years of World War II for the production of 2,3-butanediol as precursor of 1,3-butadiene used for synthetic rubber. However, the process was not commercially implemented due to competition with the petrochemical process for the production of 2,3-butanediol [62-64]. Nowadays, 2,3-butanediol is still manufactured by the petrochemical industry. The commercial product is usually either the *meso*-2,3-butanediol or D(-)-2,3-butanediol. The *meso*-form is derived from trans-2,3-epoxybutane and the D/L(-)-form is derived from cis-2,3-epoxybutane [65].

2,3-Butanediol can be used as component in anti-freeze agents, printing inks, perfumes, fumigants, moistening and softening agents, explosives and plasticizers, or carriers for pharmaceuticals [66]. Furthermore, dehydration of 2,3-butanediol results in methylethyl ketone, also usable as fuel additive, or as organic solvent for resins and lacquers. The 2,3-butanediol analogues, acetoin and diacetyl, obtained after dehydrogenation, can be used as flavoring agents in dairy products, margarines or in cosmetics [67].

Metabolic pathways

The synthesis route towards 2,3-butanediol proceeds via the central intermediate acetolactate, involved in the branched-chain amino acid (*iso*-

leucine, leucine and valine) biosynthesis pathway. Acetolactate is formed by coupling two pyruvate molecules with the concomitant formation of one carbon dioxide molecule, catalyzed by acetolactate synthase. Subsequently, acetolactate is decarboxylated by acetolactate decarboxylase to yield acetoin [68, 69]. Alternatively, acetoin can also be produced from pyruvate and acetaldehyde by pyruvate decarboxylase [70]. Reduction of acetoin by acetoin reductase results in 2,3-butanediol production (Figure 2).

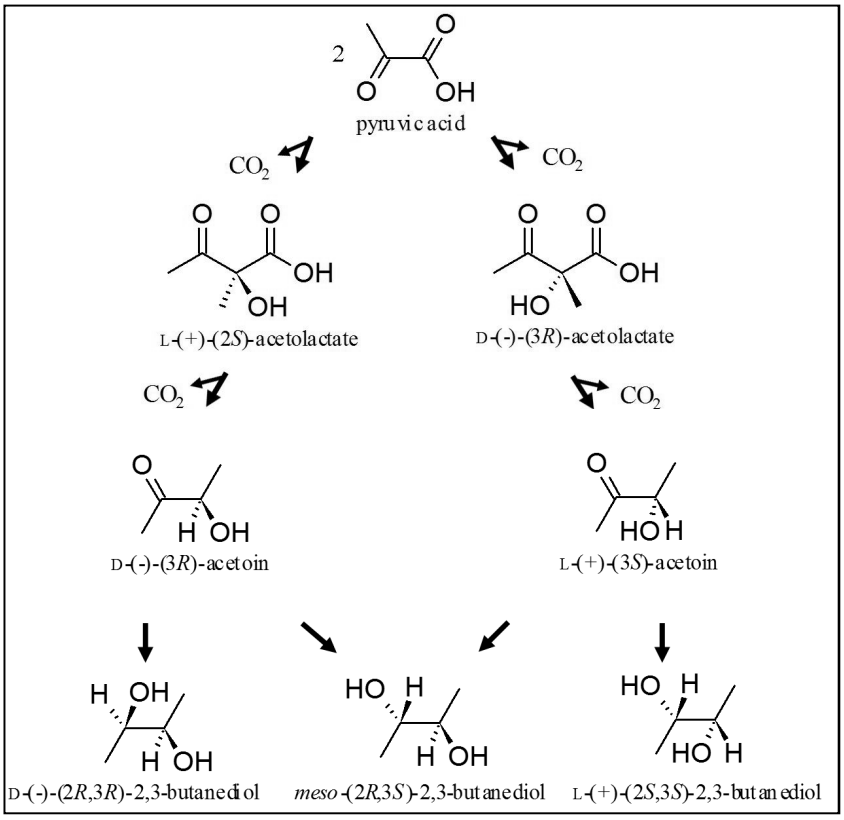

Figure 2: Different metabolic pathways for the production of stereoisomers of 2,3-butanediol from pyruvic acid.

Improvement of 2,3-butanediol producing strains

Currently, two bacterial species, *Klebsiella pneumoniae* and *Bacillus polymyxa*, have demonstrated their potential for 2,3-butanediol production on a commercial scale. *K. pneumoniae* is known to produce mainly *meso*-2,3-butanediol, while *B. polymyxa* produces mainly D(-)-2,3-butanediol [67, 71].

K. pneumoniae has several advantages over *B. polymyxa* as 2,3-butanediol producer. It grows rapidly, metabolizes a wider range of simple sugars and produces higher amounts of 2,3-butanediol. Furthermore, all of the major sugars present in hemicellulose and cellulose hydrolysates are well suited as substrates for this bacterial species [67]. The highest 2,3-butanediol levels reported so far were obtained with *K. pneumoniae* and reached values as high as 150 g 2,3-butanediol /L and a productivity of 4.21 g/L.h (see Table 2) [62, 72].

Process technology: Fermentation parameters and product separation

Several parameters have been reported to have an effect on the production of 2,3-butanediol. Culturing factors like pH, temperature, aeration, agitation, and inoculum size are important parameters influencing the 2,3-butanediol production process, as well as factors like initial substrate concentration, product concentration, medium supplements and the water activity of the medium.

In general, the pH optimum for 2,3-butanediol production lies around pH 6. The temperature optimum for growth and 2,3-butanediol production is reported to be between 30-35 °C. Furthermore, aeration of the system results in higher 2,3-butanediol yields [67]. The oxygen transfer rate is noted as the most important operating factor for 2,3-butanediol production. Decreasing the oxygen supply rate increases the 2,3-butanediol yield, although it also decreases the cell density. Therefore, an optimal balance is needed to create

optimal conditions for 2,3-butanediol production [62]. Agitation is also beneficial, since it permits, besides improved oxygen-transfer rates, a better availability of fresh substrates and prevents high local concentrations of products. Increasing the size of inoculum of *K. pneumoniae* and *B. polymyxa* did not improve 2,3-butanediol production [73, 74]. In contrast, the acclimatisation of pre-cultures to new environmental conditions improved the production tremendously specially when concentrated wood hydrolysates and agricultural residues were used as substrates [75, 76].

Carbohydrate concentrations used in most studies, using particularly wood hydrolysates or various types of molasses as substrates, vary between 5-10% (w/v). More concentrated substrates (with higher sugar concentrations) contained inhibiting concentrations of toxic substances like furfurals and phenolic compounds. Significant inhibition of 2,3-butanediol formation and sugar utilisation was observed when substrate levels of only xylose, glucose, arabinose, galactose, mannose and cellobiose exceeded 50 g/L.

It has been reported that 2,3-butanediol is less toxic than ethanol, acetone or butanol to several microbial species [75]. As an example, growth of *K. oxytoca* is sustained at 2,3-butanediol concentrations op to 105 g/L, and 2,3-butanediol production continues up to concentrations of this product up to 130 g/L. Therefore it was concluded that 2,3-butanediol at high concentrations may inhibit bacterial growth, but has little effect on its biosynthesis [77].

Another important factor is the composition of the culture medium. It must contain all necessary components for optimal growth and 2,3-butanediol production. A cheap and adequate nitrogen source in 2,3-butanediol production is urea, instead of yeast-extract. Urea has been added to substrates like hydrolysed wheat mashes and wood hydrolysates. Generally, *K. pneumoniae* has low nutritional requirements and produces already satisfactory amounts of 2,3-butanediol in media containing inorganic salts and

sugar. Addition of extra peptone/beef-, wheat-, malt- or yeast extract are proven to enhance the production of 2,3-butanediol. Next to these extracts, extra phosphate and the trace metals Fe^{2+}, and Mn^{2+} were found to significantly improve 2,3-butanediol yields.

The water activity of the medium is another important factor, related to the osmotic pressure experimented by the microorganism. Increasing the concentration of certain solutes, such as starch or sugars, in the medium results in a decrease of the water activity. *K. pneumoniae* is known to possess a relatively weak osmotolerance and therefore, water activity may have a great influence in its industrial process environment. This effect may partially explain why the butanediol process is more difficult with natural, complex sources of carbohydrates (e.g. starch-containg substrates), than with simple sugars as substrate [67].

An important aspect in the 2,3-butanediol fermentation is the recovery from the fermentation broth. Major difficulties in this recovery are due to the fact that 2,3-butanediol has a high boiling point and high affinity for water, but also the presence of dissolved and solid components of fermentation mashes interferes with the recovery [67].

In microbial production systems, the costs of the separation of 2,3-butanediol from the fermentation broth have been estimated to account for more than 50% of the total expenses. Most studies regarding downstream processing of 2,3-butanediol are focused on steam stripping, reverse osmosis, pervaporation, and solvent extraction [66]. In Table 2, a summary of recent results described for 2,3-butanediol processes at laboratory scale using innovative separation techniques is shown.

Table 2: Comparison of improved fermentation systems reported for the production of 2,3-butanediol.

Organism	*Fermentation type*	*Substrate*	*Fermentation time (h)*	*Concentration g 2,3-BD /L*	*Yield g 2,3-BD/g substrate*	*Productivity (g/L/h)*	*Recovery technique*	*Comments*	*Ref.*
Klebsiella pneumoniae CICC 10011	Fed-batch SSF	Jerusalem artichoke powder	40	84.0	0.28*	2.10	-	SSF	[78]
Klebsiella pneumoniae CICC 10011	Fed-batch	Glucose	60	63.8	~0.44*	1.06*	Aqueous two-phase extraction	Based on 2-propanol/$(NH_4)_2SO_4$ system	[79]
Klebsiella pneumoniae G31	Fed-batch	Glycerol	300	49.2	0.36*	0.16*	-		[80]
Klebsiella pneumoniae SDM	Fed-batch	Glucose	38	150	0.44*	4.21	-	Residual glucose concentration 20-30 g/l	[72]
Klebsiella oxytoca ME-UD-3	Batch	Glucose	56	95.5	0.48	1.71	-	Two-stage agitation	[81]
Klebsiella pneumoniae PTCC 1290	Batch	Glucose	35	23.01	NR	0.66	Liquid-liquid extraction	Extraction with oleyl alcohol	[82]
Klebsiella pneumoniae CICC 10011	Fed-batch	Glucose	50	92.4	0.43*	1.84*	-		[83]
Klebsiella oxyroca ATCC 8724	Fed-batch	Glucose	NR	NR	0.47	1.40	Vacuum membrane distillation	Final concentration 2,3-BD is 430 g/L	[84]

* calculated from reported data ; NR = not reported; SSF, simoultaneous saccharification and fermentation; 2,3-BD, 2,3-butanediol

Solvent extraction may also become an effective method for 2,3-butanediol recovery. Many solvents, like ethyl acetate, diethyl ether, and n-butanol are suitable for this extraction system. Countercurrent steam stripping and reversed osmosis are also regarded as feasible techniques [85]. However, these methods are still difficult and deficient, and more research is needed to improve their yield, purity and energy consumption [66].

2

The Central Catabolic pathways – *C. acetobutylicum ATCC 824* versus *C. beijerinckii NCIMB 8052*

This chapter has been adapted from:

Siemerink, M. A. J., Kuit, W., Kengen, S. W. M. (2010), "Comparative genomic analysis of the solventogenic species *Clostridium acetobutylicum* ATCC 824 and *Clostridium beijerinckii* NCIMB 8052" in *System biology of Clostridia,* ed. P. Dürre, Ulm, Germany: *Publisher*.

In preparation

Chapter 2: The Central Catabolic pathways – *C. acetobutylicum ATCC 824* versus *C. beijerinckii NCIMB 8052*

The availability of the genomes of *C. acetobutylicum* ATCC 824 [28] and *C. beijerinckii* NCIMB 8052 (http://genome.ornl.gov/microbial/cbei) now offers the opportunity to compare both solventogenic species at the gene level. This Chapter aims to provide such a comparative genomic analysis regarding the central catabolic pathway. It may reveal general features of solvent production and lead to a better understanding of both solventogenic species.

In more detail, the next paragraphs focus on all the enzymes involved in the glycolysis (Embden-Meyerhof pathway) and subsequent pathways leading to the main end products of the acidogenic and solventogenic metabolism, *viz.* acetate, butyrate, lactate, acetone, butanol, ethanol, acetoin, carbon dioxide and hydrogen (Figure 3 and Table 3).

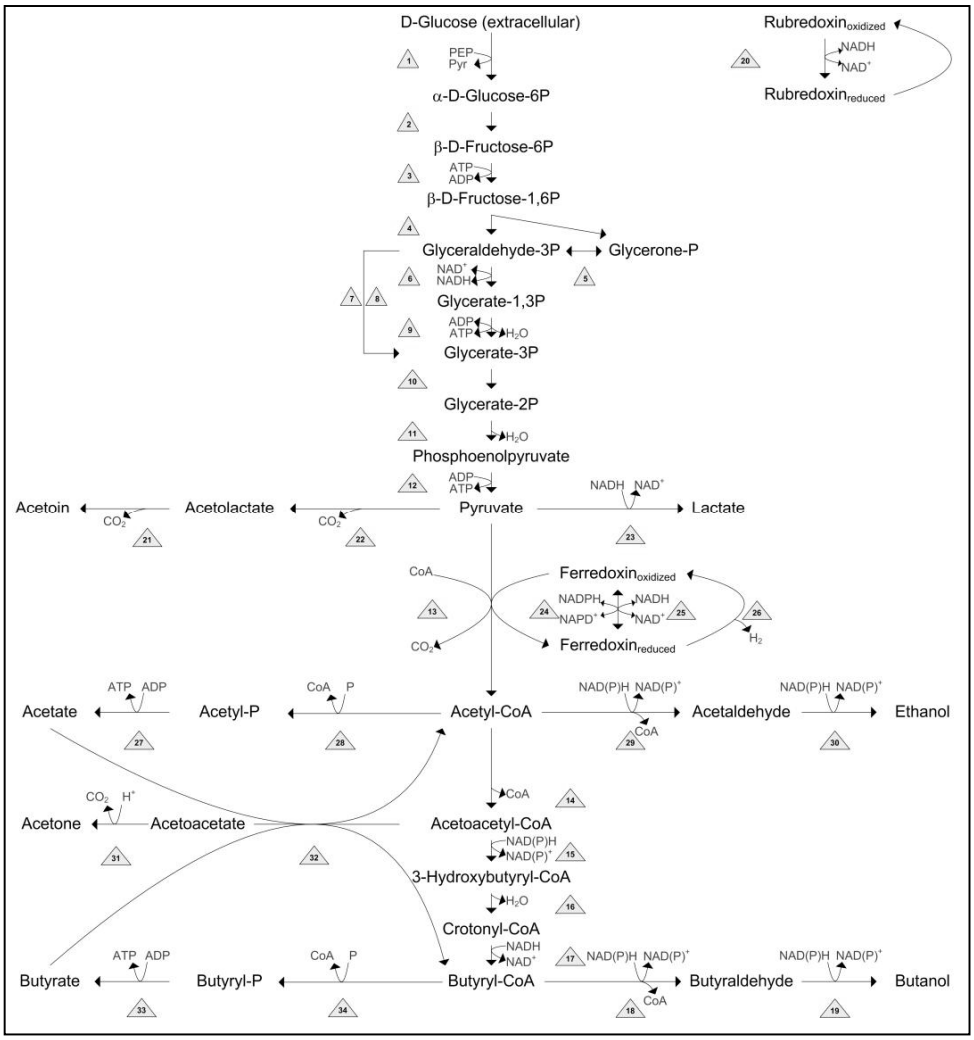

Figure 3: Central glycolytic metabolism of two solventogenic clostridia: *C. acetobutylicum ATCC 824* and *C. beijerinckii NCIMB 8052*. Numbers in triangles refer to the reaction numbering in Table 3.

Table 3: Overview of enzymes and corresponding genes involved in the central metabolism of *C. acetobutylicum* ATCC824 and *C. beijerinckii NCIMB 8052*. The table shows all candidate enzymes of *C. acetobutylicum* indicated in figure 3, their paralogs and the respective orthologs in *C. beijerinckii* on amino acid (AA) level. The cut-off value is 40% identity (id). Numbers in superscript indicate gene positions within gene clusters extracted from this table. COG numbering as indicated in IMG. The most likely enzyme candidates within *C. acetobutylicum* and *C. beijerinckii*, based on gene neighbourhood, homology, microarray data (differential expression) and literature information are shown in bold. Nd, not determined.

#	EC-number	COG	Name	Locus-tag	AA	Locus-tag	AA	Id (%)	Ref.
				C. acetobutylicum ATCC 824		*C. beijerinckii* NCIMB 8052			
1	[2.7.1.69]	1263; 1264; 2190	PTS enzyme II, ABC components	**CAC0570 *(glcG)***	665	*nd*			86, 87
			BC components	CAC3425[1] *(glvC)*	518	CBEI_4977[2]	513	80	
			A component	CAC3427[2]	165	CBEI_4982[3]	158	41	86
			BC components	CAC1353[1]	488	CBEI_4532[1]	478	41	86, 88
			A component	CAC1354[2]	159	CBEI_4533[2]	165	53	86
						CBEI_4706	158	46	
			A component	CAC2995	157	CBEI_3273	634	41	86
2	[5.3.1.9]	0166	Glucose-6-phosphate isomerase (PGI)	**CAC2680 *(pgi)***	450	**CBEI_0341**	449	72	86, 89-92
3	[2.7.1.11]	0205	6-Phosphofructokinase (PFK)	**CAC0517[1] *(pfkA)***	319	**CBEI_4852[2]**	318	76	86, 89-91, 93
4	[4.1.2.13]	3588	Fructose-1,6-bisphosphate aldolase (FBA)	CAP0064 *(alf)*	295	CBEI_3039	295	79	86
				CAC0827*(fba)*	287	**CBEI_1903[2]**	288	81	86, 88-92
5	[5.3.1.1]	0149	Triosephosphate isomerase (TPI)	**CAC0711[3] *(tpiA)***	248	**CBEI_0599[3]**	248	73	37, 86, 89-94
6	[1.2.1.12]	0057	GAP dehydrogenase (GAPC)	**CAC0709[1] *(gapC)***	334	**CBEI_0597[1]**	333	75	86, 89-93
7	[1.2.7.5]	2414	Aldehyde:ferredoxin oxidoreductase	CAC2018	684	nd			88, 90
8	[1.2.1.9]	1012	NADP-dep. GAP dehydrogenase (GAPN)	**CAC3657[2] *(gapN)***	482	**CBEI_2572**	486	65	90-92, 95
						CBEI_2282	488	63	

9	[2.7.2.3]	0126	Phosphoglycerate kinase (PGK)	**CAC0710[2] *(pgk)***	397	**CBEI_0598[2]**	391	79	86, 89, 93
10	[5.4.2.1]	0588	Phosphoglycerate mutase (PGM)	**CAC0712[4] *(pgm)***	510	**CBEI_0600[4]**	512	71	86, 89-93
				CAC2741 *(gpmA)*	243	CBEI_1759	232	70	
				CAC0167	228	CBEI_3922	237	58	37
				CAC3021	219	CBEI_1719	203	50	
11	[4.2.1.11]	0148	Enolase (ENO)	**CAC0713[5] *(eno)***	431	**CBEI_0602[4]**	430	76	37, 86, 89, 90, 92
12	[2.7.1.40]	0469	Pyruvate kinase (PYK)	**CAC0518[2] *(pykA)***	473	**CBEI_4851[1]**	476	64	86, 89-93
						CBEI_0485	472	55	
				CAC1036 *(pykA)*	472	CBEI_0485	472	60	86, 89, 92
						CBEI_4851[1]	473	57	
13	[1.2.7.1]	0674; 1013;	Pyruvate: ferredoxin oxidoreductase (PFOR)	**CAC2229 *(pfor)***	1171	**CBEI_4318**	1169	72	90-92, 96
						CBEI_1853	1171	66	
						CBEI_1458	1172	64	
				CAC2499	1173	CBEI_1458	1172	73	91
						CBEI_4318	1169	63	
						CBEI_1853	1171	62	
14	[2.3.1.9]	0183	Thiolase (THL)	**CAC2873 *(thlA)***	392	**CBEI_3630**	392	90	86, 88-93
						CBEI_0411	393	74	
				CAP0078 *(thiL)*	392	CBEI_3630	392	78	86, 90, 93
						CBEI_0411	393	76	
15	[1.1.1.157]	1250	3-Hydroxybutyryl-CoA dehydrogenase (HBD)	**CAC2708[1] *(hbd)***	282	**CBEI_0325[3]**	282	79	86, 89-93, 97

16	[4.2.1.55]	1024	Crotonase (CRT)	**CAC2712**[3] ***(crt)***	261	**CBEI_0321**[1]	261	69	37, 86, 89-93, 97
						CBEI_2034[1]	261	69	
						CBEI_4544[2]	258	57	
17	[1.3.99.2]	1960	Butyryl-CoA dehydrogenase (BCD)	**CAC2711**[2] ***(bcd)***	379	**CBEI_0322**[2]	379	79	86, 89-93, 95, 97
						CBEI_2035[2]	379	79	
						CBEI_2883	379	65	
						CBEI_4542[1]	379	60	
18	[1.2.1.10]	1012; 1454	Alcohol-aldehyde dehydrogenase (AADH)	**CAP0035** ***(adhE2)***	858	**CBEI_0305**	864	66	86, 88, 90, 92, 93, 95, 97
				CAP0162[1] ***(aad)***	862	**CBEI_0305**	862	66	86, 88-93, 95, 97
19	[1.1.1.-]	1063;	Butanol dehydrogenase (BDH I,II)	**CAC3299**[2] ***(bdhA)***	389	**CBEI_2421**	387	64	37, 86, 88-93, 95, 97
				CAC3298[1] ***(bdhB)***	390	**CBEI_2421**	387	60	37, 86, 88-93, 97
20	[1.18.1.1]	1251	NADH:rubredoxin-oxidoreductase	CAC2448	379	nd			98-101
21	[4.1.1.5]	3527	Acetolactate decarboxylase	**CAC2967**	238	nd			
22	[2.2.1.6]	0028; 0440	Acetolactate synthase AB	CAC3169[2] *(ilvB)*	554	CBEI_0217[2]	557	54	37, 86, 90
						CBEI_2646	560	40	
				CAC3176[2] *(ilvN)*	165	CBEI_0212[1]	169	48	86, 89
				CAC3652[1] *(alsS)*	563	CBEI_2868	556	63	
23	[1.1.1.27]	0039	L-lactate dehydrogenase (LDH)	**CAC0267** ***(ldh)***	313	**CBEI_1014**	316	58	37, 90, 92, 95
				CAC3552	320	CBEI_4126	553	69	86, 90, 92
						CBEI_4972[1]	535	50	
24	[1.18.1.2]	nd	Ferredoxin:NADP oxidoreductase	nd		nd			
25	[1.18.1.3]	nd	Ferredoxin:NAD oxidoreductase	nd		nd			

26	[1.12.7.2]	4624	Hydrogenase (HYD) - (iron only)	**CAC0028 *(hydA)***	582	**CBEI_3796**	461	40	37, 86, 88, 89, 93, 102
		1740	Hydrogenase (HYD) - (nickel-iron)	**CAP0141[1] *(mbhS)***	291	**CBEI_3013[2]**	291	70	86
		0374		**CAP0142[2] *(mbhL)***	471	**CBEI_3012[1]**	463	62	86
27	[2.7.2.1]	0282	Acetate kinase (ACK)	**CAC1743[2] *(askA))***	401	**CBEI_1165[2]**	400	59	86, 89, 90, 92, 93, 97
	[3.6.1.7]	1254	Acylphosphatase (ACYP)	CAC2830	91	CBEI_4233	90	52	
28	[2.3.1.8]	0280	Phosphotransacetylase (PTA)	**CAC1742[1] *(pta)***	333	**CBEI_1164[1]**	333	68	86, 89-93, 97
29	[1.2.1.10]	1012; 1454	Alcohol-aldehyde dehydrogenase (AADH)	**CAP0035 *(adhE2)***	858	**CBEI_0305**	864	66	86, 88, 90, 92, 93, 95, 97
				CAP0162[1] *(aad)*	862	**CBEI_0305**	864	66	86, 88-93, 95, 97
30	[1.1.1.1]	1063	Acetaldehyde dehydrogenase (ACDH)	CAC3375	377	nd			
30	[1.2.1.10]	1012; 1454	Alcohol-aldehyde dehydrogenase (AADH)	**CAP0035 *(adhE2)***	858	**CBEI_0305**	864	66	86, 88, 90, 92, 93, 95, 97
				CAP0162[1] *(aad)*	862	**CBEI_0305**	864	66	86, 88-93, 95, 97
31	[4.1.1.4]	4689	Acetoacetate decarboxylase (AADC)	**CAP0165[4] *(adc)***	244	**CBEI_3835[3]**	246	75	37, 86, 88-95, 97
32	[2.8.3.9]	1788; 2057	CoA transferase AB (CoAT AB)	**CAP0163[2] *(ctfA)***	218	**CBEI_3833[1]**	217	73	86, 88-93, 95, 97
						CBEI_2654[2]	238	42	
				CAP0164[3] *(ctfB)*	221	**CBEI_3834[2]**	221	69	37, 86, 88-93, 95, 97
						CBEI_2653[1]	217	51	
33	[2.7.2.7]	3426	Butyrate kinase (BK)	**CAC3075[1] *(buk)***	355	**CBEI_0204[2]**	355	64	86, 89-91, 93, 95
						CBEI_4006	356	65	
						CBEI_4609	356	64	

				CAC1660	356	CBEI_0204[2]	355	62	[103]
						CBEI_4006	356	74	
						CBEI_4609	356	71	
34	[2.3.1.19]	0280	Phosphotransbutyrylase (PTB)	**CAC3076[2] *(ptb)***	301	**CBEI_0203[1]**	302	71	[86, 89-93, 95, 97]

The metabolic network as depicted in figure 3 and table 3 was based on the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/).

In *C. acetobutylicum ATCC 824*, 34 reactions are required to compose the glycolysis and subsequent pathways (Figure 3). For these 34 reactions, 53 genes (including paralogs) have been annotated in KEGG, and for most reactions it has been experimentally proven which of the paralogs is actually coding for the responsible enzyme. However, the corresponding genes of two enzymes (i.e. ferredoxin:NADP+ oxidoreductase and ferredoxin:NAD+ oxidoreductase) have not been identified, as yet, although both activities were demonstrated in cell free extract of *C. acetobutylicum* sp. [104, 105].

For the 53 genes encoding central metabolism enzymes of *C. acetobutylicum*, 59 orthologs could be retrieved from the *C. beijerinckii* genome. Despite the higher number of orthologs in *C. beijerinckii*, for seven of the *C. acetobutylicum* genes (i.e.. #1, CAC0570; #7, CAC2018; #20, CAC2448; #21, CAC2967; and #30, CAC3375), no close ortholog was detected. Obviously, these also include both previously mentioned ferredoxin:NAD(P)+ oxidoreductases, whose encoding genes are not known.
In the following paragraphs, the main enzymatic steps of the acidogenic and solventogenic pathways of both species and their encoding genes will be discussed in more detail.

Glucose uptake

Glucose is taken up via phosphoenolpyruvate (PEP)-phosphotransferases systems (PTS) . There is no indication of ABC-type transport systems or secondary transport systems for glucose in *C. acetobutylicum*. Based on the genome sequence, *C. acetobutylicum* contains

13 PTS systems. Six of these systems belong to the glucose-glucoside (Glc) family, which contains permeases that are specific for glucose, N-acetylglucosamine or different α- and β-glucosides [2]. For one system (containing all three necessary A, B and C subunits) within this family, encoded by *glcG* (CAC0570), it has been proven that it transports glucose inside the cells. However, the possibility of other active glucose specific phosphotransferases encoded by one of the other five subuit paralogs, remains [87]. Differential expression of some other PTS system subunits has even been observed [86, 88], suggesting that in glucose grown cultures, alternative uptake proteins are likely to be involved. Furthermore, it has been speculated that one of the small subunits with a IIA^{glc} domain (CAC2995) which is thought to be involved in glucose uptake, is possibly involved in maltose uptake, since the putative *mal* operon of *C. acetobutylicum* does not encode this IIA domain [2]. Remarkably, no ortholog could be found for the complete PTS system encoded by *glcG* (CAC0570) within *C. beijerinckii*, although at least two clusters coding for $IIABC^{glc}$ domains (CBEI_4982, CBEI_4983 and CBEI_4532, CBEI_4533) do exist. Additionally, CBEI_4983, part of one of the two $IIABC^{glc}$ clusters is not shown in table 3, but encodes domain IIBC (518 AA) with an identity of 33%. Since this ortholog had an identity lower than 40%, it was omitted in table 3.

Glycolysis

The PTS systems results in the formation of glucose-6-P, which subsequently follows the established glycolytic steps to fructose-6-P and fructose-bis-P. For the encoding genes of the involved enzymes, *viz*. glucose-6-P isomerase and phosphofructokinase only one candidate exists in *C. acetobutylicum* and *C. beijerinckii*. In both species, the phosphofructokinase gene (CAC0517, CBEI_4852) lies clustered with the pyruvate kinase (CAC0518, CBEI_4851), which is commonly observed in many fermentative anaerobes and

which supports their role in glycolysis. There are no indications of alternative ADP- or PPi dependent phosphofructokinases in either species, as have been reported for certain thermophilic sugar fermentors (*Pyrococcus furiosus*, *Caldicellulosiruptor saccharolyticus*, *Thermatoga tengcongensis*, *Thermatoga maritima*). For the aldolase, *C. acetobutylicum* and *C. beijerinckii* contain two candidates, a Class I and a Class II type, of which one resides on the megaplasmid for *C. acetobutylicum* (CAP0064). The chromosomally encoded Class II aldolase (CAC0827) is expressed constitutively and most likely responsible for the glycolytic activity [106].

The genes involved in the subsequent carbon-3 part of the glycolysis are clustered on the chromosome for *C. acetobutylicum* as well as *C. beijerinckii*, again supporting their concerted action in the glycolysis. These concern the genes for trisose-P-isomerase, glyceraldehyde-3-P dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, and enolase with locus tags CAC0709 - CAC0713 and CBEI_0597 - CBEI_0602 for *C. acetobutylicum* and *C. beijerinckii*, respectively. The *gap*, *pgk* and *tpi* have been shown to constitute an operon in *C. acetobutylicum* [107]. For the conversion of GAP, both species also contain a NADP-dependent *GapN* homolog (CAC3657, CBEI_2572), which directly oxidizes GAP to 3-phosphoglycerate, without formation of ATP [108]. Its role in the glycolysis is unclear, although it has been reported to be activated together with other glycolytic enzymes under conditions of oxygen stress in *C. acetobutylicum* [98]. In addition, *C. acetobutylicum* but not *C. beijerinckii*, harbors an aldehyde:ferredoxin oxidoreductase (CAC2018). It should be noted that this enzyme has not yet been characterized and proven to actually catalyze GAP oxidation, although, differential expression of CAC2018, has been observed in butanol challenged cultures of *C. acetobutylicum* [88, 90]. For the phosphoglycerate mutase, 3 paralogs were found in both *C. acetobutylicum* and *C. beijerinckii*, in addition to the

phosphoglycerate mutase gene, in the glycolytic cluster. The latter belongs to the 2,3-phosphoglycerate independent monophosphoglycerate mutases with a size of ~510 residues, whereas the paralogs are dependent on 2,3-phosphoglycerate and consist of ~ 230 residues. *C. acetobutylicum* and *C. beijerinckii* each contain two pyruvate kinase genes, of which one (CAC0518, CBEI_4851) clusters with the phosphofructokinase gene and most likely operates in the glycolysis. The role of the other pyruvate kinase (CAC1036, CBEI_0485) is not known.

Gluconeogenesis

Most enzymes of the glycolysis are reversible and can therefore also operate in the anabolic gluconeogenic direction, except for the fructose-1,6-bisphosphatase and the pyruvate kinase. Both species, *C. acetobutylicum* and *C. beijerinckii* contain two genes (CAC1088, CAC1572, CBEI_2467, CBEI_4541) annotated as fructose-1,6-bisphosphatase. The smaller protein, CAC1088 consisting of 324 amino acids has no detectable orthologs, while CAC1572 consisting of 665 amino acids has two orthologs, *viz.* CBEI_2467 (663 AA) and CBEI_4541 (653 AA) with an identity of 71% and 66%, respectively. Furthermore, CBEI_4541 is also encoded in close proximity to two other enzymes of the central metabolism, viz. 3-hydroxybutyryl-CoA dehydratase and butyryl-CoA dehydrogenase.

For the interconversion of phosphoenol pyruvate (PEP) and pyruvate, two alternatives are known next to the classical pyruvate kinase reaction, *viz.* pyruvate phosphate dikinase (PPDK) and PEP synthase (PEPS). Both can catalyze the conversion of PEP to pyruvate, albeit that both reactions are reversible and thus also catalyze the anabolic reaction from pyruvate to PEP. The difference lies in the used cofactors. PPDK requires AMP and PPi, while PEPS uses AMP and Pi to convert PEP to pyruvate and ATP. Interestingly,

C. acetobutylicum does not contain a PPDK, while *C. beijerinckii* contains three isoenzymes, encoded by CBEI_0849, CBEI_3859 and CBEI_3862. Nevertheless, both species do contain three putative PEPS enzymes, encoded by CAC0534, CAC0801, CAC0797, CBEI_2063, CBEI_2066 and CBEI_3516. Currently, there are no expression data available that would argue for a catabolic or anabolic role of any of the PPDKs (*C. beijerinckii*) and PEPS (*C. acetobutylicum* and *C. beijerinckii*).

Pyruvate conversion

As in most fermentative anaerobes pyruvate is oxidatively decarboxylated to acetyl-CoA involving a pyruvate:ferredoxin oxidoreductase (PFOR). *C. acetobutylicum* contains two paralogs (CAC2229, CAC2499), both coding for large proteins (1171 and 1173 AA) and comprising the α, β, and γ domains. Again, no transcriptional or proteomic data are available that allow us to decide which is the most important enzyme for the formation of acetyl-CoA.

Remarkably, *C. beijerinckii* has 6 PFOR paralogs, also without experimental data to strengthen their role. Alternatively, pyruvate can be reduced to lactate involving lactate dehydrogenase (CAC0267, CAC3552, CBEI_1014, CBEI_4126 and CBEI_4972) or decarboxylated to acetolactate requiring acetolactate synthase (CAC3169, CAC3176, CAC3652, CBEI_0212, CBEI_0217, CBEI_2646, CBEI_2686) and subsequently decarboxylated to acetoin involving acetolactate decarboxylase (CAC2967). Furthermore, the latter gene has no ortholog in *C. beijerinckii*.

Acetyl-CoA conversion

Acetyl-CoA is a branch point for the formation of acetate, the reduction to ethanol, and the condensation into carbon-4 compounds. Acetate formation occurs via the classical enzyme couple phosphotransacetylase (CAC1742) and acetate kinase (CAC1743), whose genes are clustered on the chromosome. For *C. beijerinckii* a similar situation exists, with both genes also clustered (CBEI_1164, CBEI_1165). However, next to this kinase, in both species, a small protein named acylphosphatase is encoded by CAC2830 and CBEI_4233. This enzyme catalysis the irreversible conversion of acetyl-P to acetate without formation of ATP. The same enzyme might also be active on the similar substrate butyryl-P. To date, no literature data are available that indicate the functional expression of these enzymes in either of both solventogenic clostridia.

Alternatively, acetyl-CoA can act as a sink for reducing equivalents by the action of NAD(P)H-dependent alcohol-acetaldehyde dehydrogenases (AADH). For the reduction to acetaldehyde and ethanol two paralogs exist in *C. acetobutylicum* and both solvent forming genes, designated as *adhE1* (or *aad*) and *adhE2*, are located on the megaplasmid (CAP0162, CAP0035, respectively). Is has been shown that ADHE1 is active during solventogenic conditions, while ADHE2 is active during alcohologenic conditions [109]. For *C. beijerinckii* only one *adhE* homolog was identified, located on the chromosome (CBEI_0305). In *C. acetobutylicum adhE1* forms an operon (*sol* operon) with *ctfA* and *ctfB*, which code for the acetoacetyl CoA:acetate/butyrate:CoA transferase [110]. The adhE homolog in *C. beijerinckii* (CBEI_0305) is not clustered with *ctfA* and *ctfB* (CBEI_3833 and CBEI_3834, respectively). The latter two genes are however, clustered with the gene coding for the acetoacetate decarboxylase (*vide infra*).

Two molecules of acetyl-CoA are converted to acetoacetyl-CoA by the action of acetyl-CoA acetyltransferase (thiolase, THL). In *C. acetobutylicum* two THL isoenzymes exist, whose genes are located on the chromosome (*thlA*; CAC2873) and on the megaplasmid *(thlB* or *thiL*, CAP0078). THLA is active during acidogenic and solventogenic conditions, whereas the role of THLB is still unclear [111]. *C. beijerinckii* also harbors two thiolase genes (CBEI_3630, CBEI_0411*),* with the former showing highest homology with *thlA*. Increased expression of a *thl* (CBEI_3630 or CBEI_0411) has been reported during the late acidogenic and solventogenic phase [112].

Acetoacetyl-CoA is reduced to 3-hydroxybutyryl-CoA by the action of 3-hydroxybutyryl-CoA dehydrogenase (HBD), and which is encoded by CAC2708 and CBEI_0325. In both species this gene lies clustered wit the genes for the subsequent steps, catalyzed by crotonase (CRT) and butyryl-CoA dehydrogenase (BRD), and identified as CAC2712 and CAC2711 in *C. acetobutylicum* and CBEI_0321 and CBEI_0322 in *C. beijerinckii.* Interestingly, in both species this gene cluster also includes two genes that code for two flavoproteins (EtfA, EtfB). For *C. kluyvveri,* these flavoproteins have recently been shown to be part of a protein complex, also comprising the BCD. This protein complex was shown to catalyze the NADH-dependent reduction of crotonyl-CoA to butyryl-CoA, and due to the exergonic nature of this reaction also enable the NADH-dependent reduction of ferredoxin [113]. Reduced ferredoxin can subsequently be used to produce hydrogen. The presence of such BCD/ETF clusters (butyryl-CoA dehydrogenase/electron transferring flavoprotein) in *C. acetobutylicum* and *C. beijerinckii* suggest that also these bacteria are capable of crotonyl-CoA-dependent ferredoxin reduction using NADH. Moreover, *C. beijerinckii* appears to contain a second BCD/ETF cluster / encoded by *bcd* (CBEI_2035), *etfA* (CBEI_2037) and *etfB* (CBEI_2036).

Butyryl-CoA can be used for the synthesis of butyrate, coupled to ATP synthesis, or it is reduced to butyraldehyde and butanol. Conversion to butyrate involves phosphotransbutyrylase (PTB) and butyrate kinase (BK), encoded by the clustered genes CAC3076, CAC3075 and CBEI_0203, CBEI_0204 for *C. acetobutylicum* and *C. beijerinckii*. In both species, paralogs exist for the butyrate kinase (CAC1660, CBEI_4006, CBEI_4609), but for *C. acetobutylicum*, it has been discussed that this butyrate kinase might be involved in the reversed direction, i.e. the phosphorylation of butyrate [103]. Reduction of butyryl-CoA to butyraldehyde is catalyzed by the same set of alcohol dehydrogenases also reported for acetyl-CoA reduction (ADHE1, ADHE2). Reduction of butyraldehyde to butanol occurs using BDHI (CAC3299) and BDHII (CAC3298). *C. beijerinckii* contains only one BDH homolog, encoded by CBEI_2421.

As mentioned above, acetoacetyl-CoA can be converted to acetoacetate involving the CoA transferases *ctfA* (CAP0163, CBEI_3833) and *ctfB* (CAP0164, CBEI_3834). In addition, *C. beijerinckii* harbors a pair of ctfA/B paralogs (CBEI_2654, CBEI_2653). Acetoacetate is finally decarboxylated to acetone by a acetoacetate decarboxylase, encoded by *adc*. In *C. acetobutylicum*, this gene is part of the sol operon (CAP0165). Similarly, its ortholog in *C. beijerinckii* (CBEI_3835) lies also clustered with the *ctfA* and *ctfB* genes.

Summary

As the overall acidogenic and solventogenic metabolism of *C. acetobutylicum* and *C. beijerinckii* is very similar, one can expect that similar pathways and enzymes are involved. Indeed, for most of the enzymes involved in de central glycolytic pathway of *C. acetobutylicum* at least one ortholog in *C. beijerinckii* exists. Moreover, genes that are clustered in *C. acetobutylicum*,

often also lie clustered in *C. beijerinckii*, *e.g.* the glycolytic carbon-3 cluster, the BDH/ETF cluster and the kinase/PTA/PTB cluster. A major difference is the presence of the megaplasmid in *C. acetobutylicum*, carrying important genes for solvent production. *C. acetobutylicum* appears to be better equipped for solvent production, as it has four ADH's (i.e. AAD, ADHE2, BDHI and BDHII), whereas *C. beijerinckii* has only two orthologs (i.e. CBEI_0305 and CBEI_2421). In contrast to the latter example, *C. beijerinkii* often contains in general more (clustered) paralogs (especially alcohol dehydrogenases) compared to *C. acetobutylicum*. The reason for this is not clear. Moreover, the identity of the genes responsible of the major fermentative steps in *C. beijerinckii* are mainly based on homology to *C. acetobutylicum* genes and gene clusters. To proof the identity of the key genes, genome wide microarrays and/or proteomic data are required. With respect to the glycolytic pathway from glucose and the central metabolism to the main end products described in this paragraph, both solventogenic clostridia show huge differences in their genetics and phenotypes [16, 114]. As yet, only limited microarray data (484 ORFs) are available for *C. beijerinckii* [112].

3

Toxicity of 1-butanol and derivatives thereof

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Chapter 3: Toxicity of 1-butanol and derivatives thereof

Introduction

1-Butanol toxicity is one of the problems hampering biobutanol production. A native producer of 1-butanol, *C. acetobutylicum,* which is also used in industry, tolerates only up to 1-2% (v/v) 1-butanol [115]. The toxic (or fluidizing) effect of 1-butanol includes membrane distorting properties, due to the hydrophobic chain and polar group of 1-butanol, which causes severe cell damage. The cell energy status is harmed by inhibition of nutrient transport, glucose transport, and membrane bound ATPase activity. Also the internal pH cannot be maintained and intracellular ATP levels decrease [29, 30, 116].

Already in 1985, it was known that the autolytic activity, lipid composition, membrane transport processes, and substrate utilization of micro-organisms was affected by contact with 1-butanol. Therefore, substantial improvements regarding these factors were thought to increase 1-butanol tolerance [117].

Ever since, many efforts have been made to obtain clostridial strains with increased 1-butanol tolerance, however with limited success. Four different approaches have been used:. i) Chemical mutagenesis [33, 34] as well as ii) adaptation strategy approaches [118] to isolate mutants with increased butanol resistance. In some cases, this resulted in slight, to moderate increases in butanol production levels, while in other cases solvent production was lost [36]. iii) The overexpression of two heat-shock related proteins, GroES and GroEL [119], and iv) overexpression of an endogenous putative transcriptional regulator (CAC1869) [38], resulted in *C. acetobutylicum* mutants with increased butanol tolerance.

Next to increasing 1- butanol tolerance in *C. acetobutylicum*, also attention is given to the usage of other suitable hosts for production of 1-butanol. An overview of 1-butanol (log K_{ow} of 0.88) tolerance of seven mesophilic, facultative anaerobic bacteria (i.e. *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Yarrowia lypolytica, Escherichia coli, Corynebacterium glutamicum, Zymomonas mobilis, Rhodococcus erythropolis)* is given by Fisher *et al*. (2008) [120]. It shows a great distribution in tolerance amongst these micro-organisms. From the seven bacteria tested, *B. subtilis* tolerates 1-butanol best, while little 1-butanol tolerance was found for *R. erythropolis*. It was stated that the ideal production host for biofuels could be created, if the desired characteristics of a production strain, were optimally combined with the interesting physiological properties of other strains [120]. For example, the production of 1-butanol in *E. coli*, indicates the potential of an, in general, more accessible strain as host. Especially, since 1-butanol is more toxic to *E. coli* than it is to *B. subtilis*, which is closely related to *C. acetobutylicum* [47, 58, 120, 121].

The toxicity of 1-butanol is expected to correlate with its lipophilicity, e.g. the tendency to accumulate in cell membranes [122]. The lipophilicity can be expressed as the logarithm of the partition coefficient with octanol and water (log K_{ow} value). In other words, it is a good indicator for the strength of membrane perturbing effects. Generally, a log K_{ow} value between 1-5 becomes toxic and the lower this value is, the less toxic a compound is with regard to the membrane [31, 123]. The log K_{ow} for a neutral compound is defined as

$$\log K_{ow} = \log\left(\frac{C_O}{C_W}\right),$$

where C_O is the concentration of a compound in the octanol phase and C_W, its concentration in the aqueous phase, when the system is in equilibrium [122].

In summary, attempts to increase the 1-butanol tolerance were successful, although 1-butanol yields were not as high as expected [34, 36, 38, 118, 119]. Also alternative strategies like concurrent butanol removal still have a lot of disadvantages. So far, it remains difficult to make significant progress in obtaining highly productive strains for ABE fermentations [124, 125].

Therefore, a different approach was chosen, which investigates the possibilities for the production of less toxic 1-butanol derivatives in *C. acetobutylicum*. These compounds should possess the same (or more attractive) valuable characteristics (energy content, combustion quality (octane or cetane number), volatility, freezing point) as 1-butanol [41], to make them suitable as biofuel or useful for other applications within the chemical or pharmaceutical industry.

In this study, eight 1-butanol derivatives with log K_{ow} values between -0.29 and 2.83 (Table 4) were tested for their toxicity effect on the fermentation profiles of *C. acetobutylicum ATCC 824*. Since the compounds differ in their value, differences regarding their toxicity effect were expected (Table 4). For comparison reasons, ethanol (log K_{ow} of -0.32) was included in the table as reference, since biological ethanol production is a known industrial process, with high ethanol yields (10%-12% (v/v)) [32]. Furthermore, for all compounds depicted below 1-butanol in table 4, with the exception of *tert*-butanol, biosynthetic pathways do exist. The final purpose is to construct new biosynthetic pathways of less toxic 1-butanol derivatives within *C. acetobutylicum*.

Table 4: 1-butanol derivatives with their corresponding K_{ow} values [126, 127, 128, 129, 130].

Name	log K_{ow}	
butyl butyrate[116]	2.70	expected to be more toxic
butyl acetate[115]	1.73	
iso-amyl alcohol[114]	1.42	
butyl lactate[117]	1.11	
1-butanol[114]	0.88	
iso-butanol[114]	0.76	expected to be less toxic
2-butanol[114]	0.61	
tert-butanol[114]	0.40	
2,3-butanediol[118]	*-0.29*	
ethanol[114]	*-0.31*	

Materials and methods

Bacterial strain

The bacterial strain used in this study is listed in table 5. Stock cultures of C. *acetobutylicum* ATCC 824 were maintained as spore suspensions in sterile 15 % (v/v) glycerol at -20 or -80 °C.

Table 5: Bacterial strain used in this study.

Bacterial strain	Relevant genotype	Remarks	Source
C. acetobutylicum ATCC 824	WT	Type strain	Laboratory stock, originally from G. Bennet (Rice University, Houston, Texas, USA)

Media and growth conditions

Prior to inoculation of clostridial pre-cultures, spore suspensions were heat shocked for 10 minutes at 75 °C. For this comparison study, modified Gapes medium (MGM) for the growth of *C. acetobutylicum ATCC 824* was used.

MGM medium was based on the semi-synthetic medium described by Nimcevic *et al*. [131], and contained per liter of water: yeast extract, 5.0 g; KH_2PO_4, 1.0 g; K_2HPO_4, 0.76 g; ammonium chloride, 2.0 g; *p*-aminobenzoic acid, 0.10 g; $MgSO_4 \cdot 7\ H_2O$, 1.0 g; $FeSO_4 \cdot 7\ H_2O$, 0.005 g; cystein-HCl, 32 mg; resazurin, 1 mg; and glucose, 60 g.

Medium for fermentation was made anaerobic by sparging with nitrogen gas. Serum flasks (125 mL), containing 50 mL MGM medium, were inoculated with 1 % (v/v) overnight pre-cultures. Clostridial experiments and

manipulations were performed anaerobically in glass serum vials with butyl rubber stoppers and aluminum crimp seals equipped with a pressure release system previously described [132]. The growth of clostridial cultures was monitored by measuring the optical density 600 nm on a Pharmacia Biotech Ultrospec 2000.

All alcohols and esters, 1-butanol (Merck), *iso*-butanol (Aldrich), 2-butanol (Janssen), *tert*-butanol (Merck), *meso*-2,3-butanediol (Acros), *iso*-amyl alcohol (Merck), butyl acetate (Aldrich), butyl butyrate (Aldrich) and butyl lactate (Aldrich) were maintained anaerobically in glass serum vials with viton rubber stoppers and aluminum crimp seals.

Toxicity tests

The cultures were started from pre-cultures in an exponentional growth phase (OD600 ≈ 0.8-1). 1-Butanol derivatives were added to the cultures (in triplicate) in the exponential growth phase (OD_{600} ≈ 0.8-1). The final concentration of 1-butanol derivatives in the growth media was 1% (v/v). During growth, samples of two mL were taken every 2-3 hours. One mL of culture was used to measure the optical density OD_{600}. The other mL was centrifuged for 1' at 13200 rpm, after which the supernatant was stored at -20 °C, until further use.

Analysis of metabolites

After thawing, an equivolume amount of the internal standard solution (20 mM crotonic acid (Sigma-Aldrich) in 0.1 M H_2SO_4) was added to the supernatant sample. The components were quantified using HPLC (SpectraSYSTEM, Thermo Electron Corporation). The order of elution was: glucose, lactic acid, acetic acid, acetoin, butyric acid, acetone, ethanol, crotonic acid and 1-butanol. The injection volume was 10 µL. An autosampler was used

and separation was achieved using a Shodex RS-pak KC811-LG column with 1.5 mM H_2SO_4 as the eluent (flow: 1.5 mL min^{-1}; runtime: 35 min; column temperature: 65 °C), equipped with a refractive index detector and a UV detector operating at 210 nm. All concentrations were determined based on the refractive index chromatogram except for butyrate for which in some cases the UV chromatogram was used. Chromquest software (Thermo Fisher Scientific) was used for machine control and data analysis.

Results

1% (v/v) 1-butanol derivatives were added to cultures in the exponential growth phase (OD_{600} = ~0.8). Table 6 indicates the concentration of the toxic compounds present in the medium at the end of the fermentations.

Table 6: Concentrations of the 1-butanol derivatives present in medium at the end of fermentations.

Name	mM
butyl acetate	76
iso-amyl alcohol	91
butyl lactate	67
butyl butyrate	60
1-butanol	115
2-butanol	95
tert-butanol	120
iso-butanol	90
meso-2,3-butanediol	160

The culture cell density and fermentation products were followed for 75h per toxicity test. The transition of late exponential to the stationary phase occurred during the night, so the exact time at which maximum OD_{600} was reached, could not be determined.

After 75 hours, the reference culture (no addition) showed no metabolic activity anymore. Figure 4 shows the optical densities of the ten cultures in time.

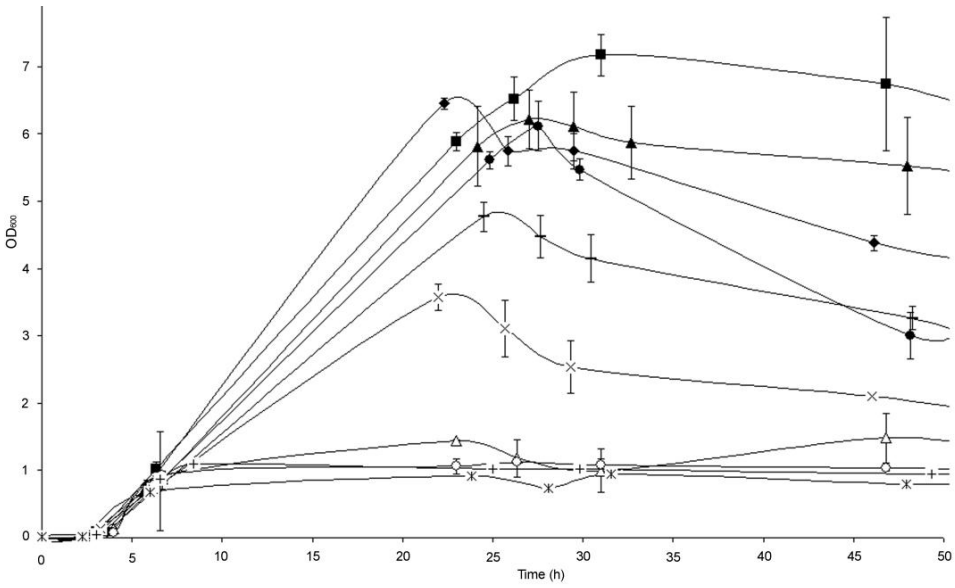

Figure 4: Growth curves of cultures of *C. acetobutylicum* ATCC 824 challenged with nine different 1-butanol derivatives. Culture codes: no challenge (closed squares); *tert*-butanol (closed diamonds); *meso*-2,3-butanediol (closed triangles); 2-butanol (closed circles); *iso*-butanol (line); 1-butanol (multiplication sign); butyl butyrate (open triangles); butyl lactate (open circles); butyl acetate (crosses); *iso*-amyl alcohol (asterisks).

Fermentation profile graphs were composed for all ten 1-butanol derivative 'stressed' cultures (Figure 5 - 10). Comparisons were made between growth profiles of these cultures (*viz*. no challenge, 1-butanol and 1-butanol derivatives).

1. No challenge

As expected, the highest optical density was reached in the unchallenged culture (Figure 5). An optimum OD_{600} of 7 was reached after 31 hours, with a consumption of 112 mM glucose. No consumption of glucose was observed after 46 hours. The total production of acetone, 1-butanol and ethanol (ABE) were 36 mM, 64 mM and 7 mM, respectively and 3 mM, 1 mM and 1 mM for lactic acid, acetic acid and butyric acid, respectively.

Furthermore, 8 mM of acetoin was produced. The solventogenic initiation of acetone, 1-butanol and ethanol occured between 6-26 hours. Butyrate re-assimilation was observed after 23 hours (Figure 5). The final concentrations of end-products were not as high as expected, however a typical ABE fermentation was performed.

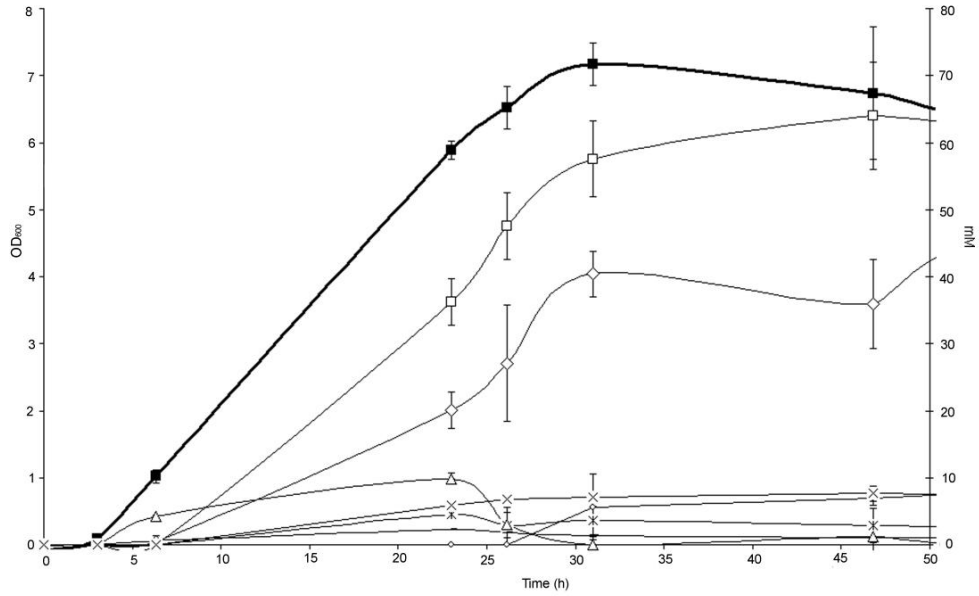

Figure 5: Fermentation profile of the unchallenged *C. acetobutylicum* ATCC 824 culture. Codes: OD_{600} (closed squares); lactic acid (asterisks); acetic acid (lines); butyric acid (open triangles); acetone (open diamonds); 1-butanol (open squares); ethanol (open circles); acetoin (multiplication sign).

2. Tert-butanol challenge

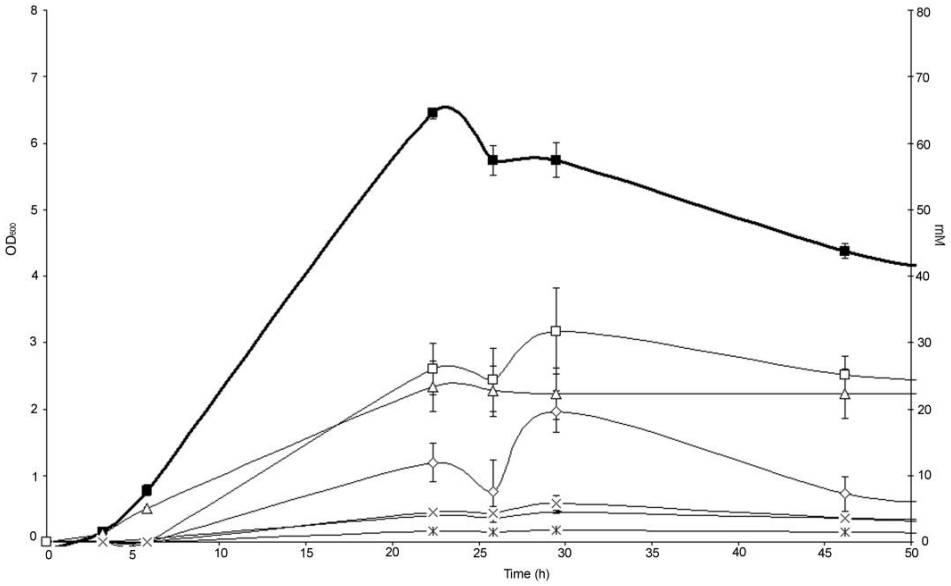

Figure 6: Fermentation profile of the *C. acetobutylicum* ATCC 824 supplemented with 120 mM *tert*-butanol. Codes: OD_{600} (closed squares); lactic acid (asterisks); acetic acid (lines); butyric acid (open triangles); acetone (open diamonds); 1-butanol (open squares); acetoin (multiplication sign).

In the 120 mM *tert*-butanol challenged culture, an optimum OD_{600} of 6.4 was reached after 22 hours, with consumption of 71 mM glucose. The total production of AB products was 7 mM, and 25 mM, respectively and 1 mM, 5 mM and 22 mM for lactic acid, acetic acid and butyric acid, respectively. Furthermore, 4 mM of acetoin was produced. Interestingly, no ethanol was produced, while there was also no re-assimilation of acetate or butyrate. The solventogenic initiation of 1-butanol and acetone occurred between 6-23 hours (Figure 6).

3. Meso-2,3-butanediol challenge

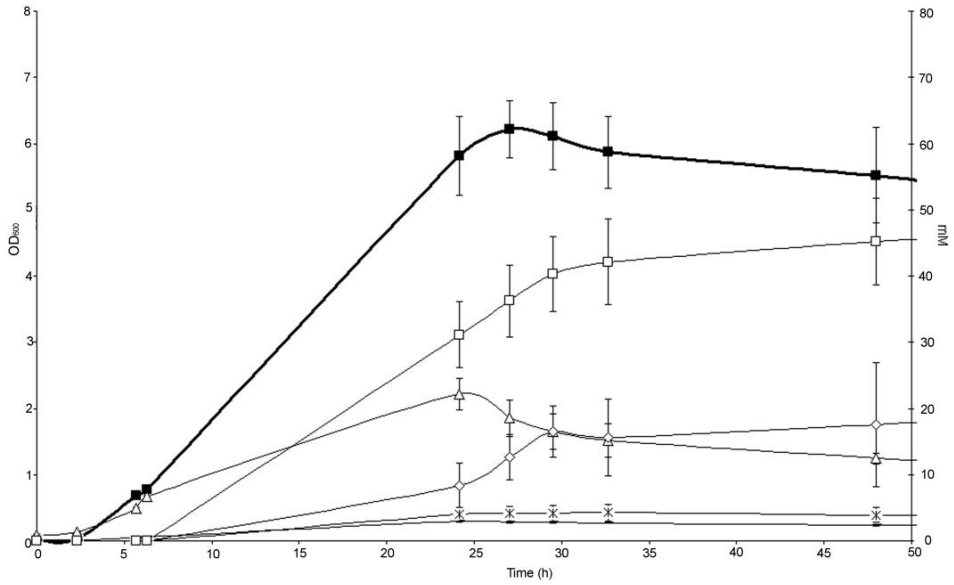

Figure 7: Fermentation profile of the *C. acetobutylicum* ATCC 824 supplemented with 160 mM *meso*-2,3-butanediol. Codes: OD_{600} (closed squares); lactic acid (asterisks); acetic acid (lines); butyric acid (open triangles); acetone (open diamonds); 1-butanol (open squares).

In the 160 mM *meso*-2,3-butanediol challenged culture, an optimum OD_{600} of 6.2 was reached after 27 hours, with consumption of 73 mM glucose. The total production of AB products was 17 mM, 45 mM, respectively and 4 mM, 2 mM and 12 mM for lactic acid, acetic acid and butyric acid, respectively. Interestingly, no ethanol was produced. The solventogenic initiation of 1-butanol and acetone occured between 6-23 hours, with butyrate partially re-assimilated (Figure 7).

4. 2-Butanol challenge

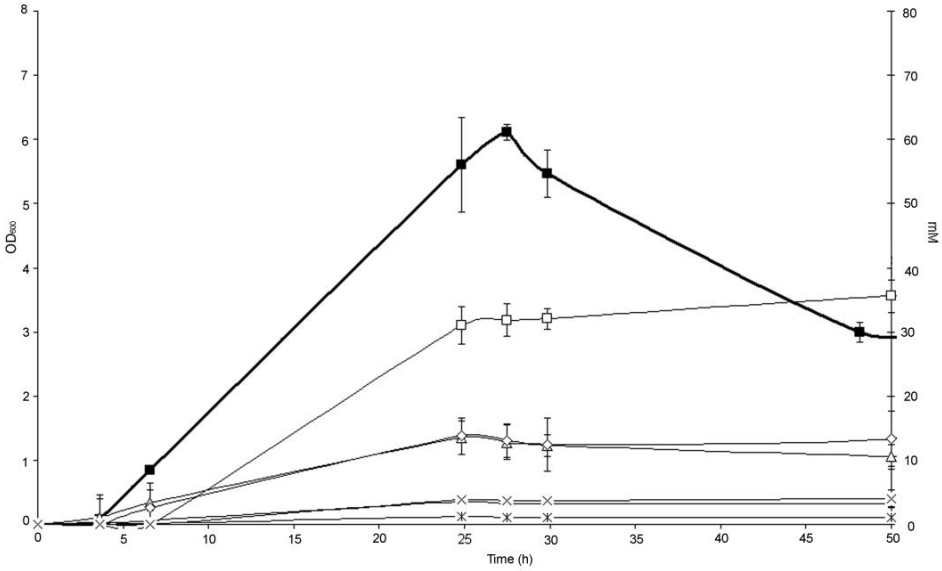

Figure 8: Fermentation profile of the *C. acetobutylicum* ATCC 824 supplemented with 95 mM 2-butanol. Codes: OD_{600} (closed squares); lactic acid (asterisks); acetic acid (lines); butyric acid (open triangles); acetone (open diamonds); 1-butanol (open squares); acetoin (multiplication sign).

In the 95 mM 2-butanol challenged culture, an optimum OD_{600} of 7 was reached after 31 hours, with an consumption of 112 mM glucose. The total production of AB products was 13 mM, 36 mM, respectively and 1 mM, 3 mM and 11 mM for lactic acid, acetic acid and butyric acid, respectively. Furthermore, 4 mM of acetoin was produced. The solventogenic initiation of 1-butanol and acetone occured between 6-23 hours. Butyrate was partially re-assimilated after 25 hours (Figure 8).

5. Iso-butanol challenge

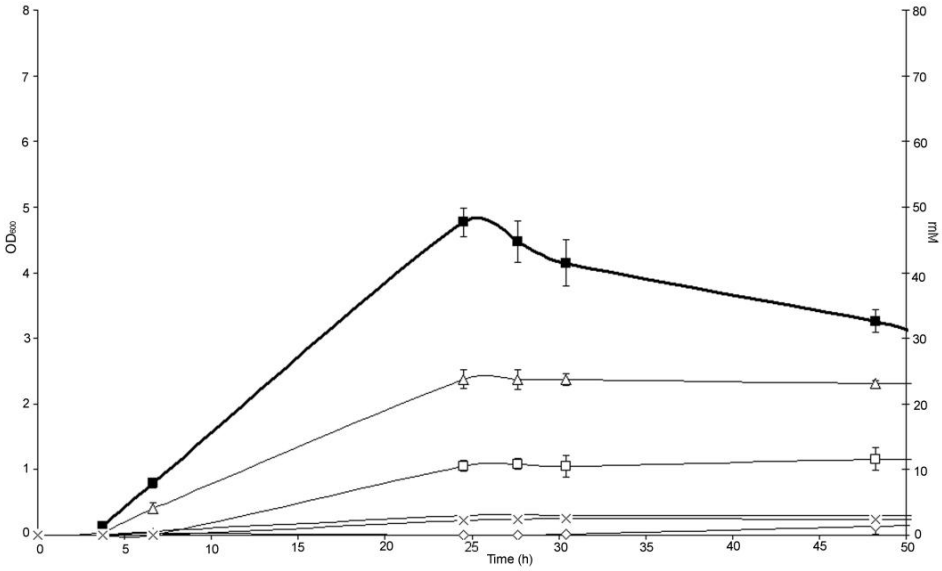

Figure 9: Fermentation profile of the *C. acetobutylicum* ATCC 824 supplemented with 90 mM *iso*-butanol. Codes: OD_{600} (closed squares); acetic acid (lines); butyric acid (open triangles); acetone (open diamonds); 1-butanol (open squares); acetoin (multiplication sign).

In the 90 mM *iso*-butanol challenged culture, an optimum OD_{600} of 4.8 was reached after 24.5 hours, with a consumption of 112 mM glucose. The total production of AB products was 1 mM, and 12 mM, respectively and 3 mM, and 23 mM for acetic acid and butyric acid, respectively. No lactic acid nor ethanol were produced. Furthermore, 2 mM of acetoin was produced. The solventogenic initiation of 1-butanol and acetone occured between 6-23 hours. Partial re-assimilation of butyrate did not occur (Figure 9).

6. 1-Butanol challenge

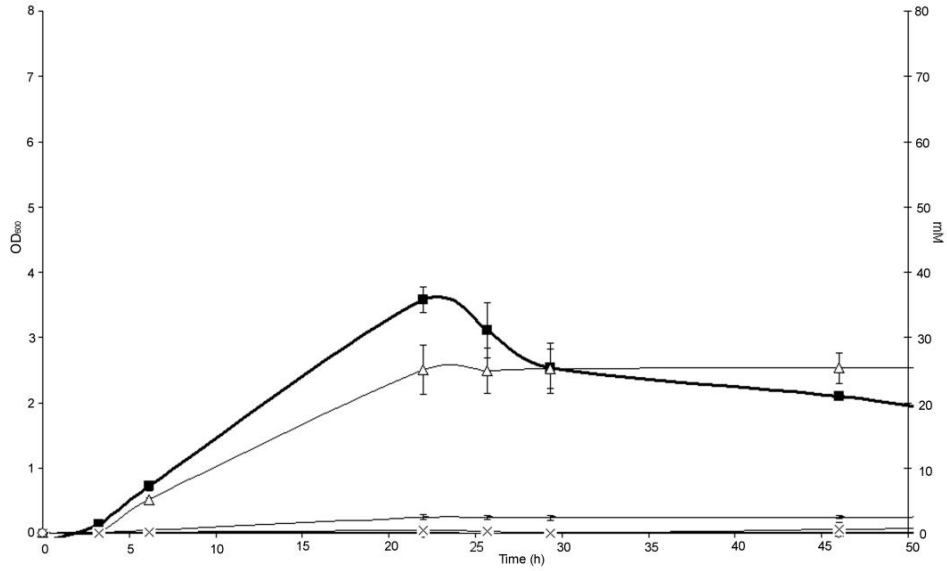

Figure 10: Fermentation profile of the *C. acetobutylicum* ATCC 824 supplemented with 115 mM 1-butanol. Codes: OD_{600} (closed squares); acetic acid (lines); butyric acid (open triangles); acetoin (multiplication sign).

In the 115 mM 1-butanol challenged culture, an optimum OD_{600} of 3.6 was reached after 22 hours, with a consumption of 112 mM glucose. The consumption of glucose ceased after 25 hours. No ABE products were produced, while only 2 mM acetic acid and 25 mM butyric acid were produced. Still, 1 mM of acetoin was produced. No partial re-assimilation was observed (Figure 10).

7. Butyl butyrate / butyl lactate / butyl acetate / iso-amyl alcohol challenge

The figures regarding the three butyl esters and *iso*-amyl alcohol challenges resulted in no growth, nor production of ABE products. Therefore, it was decided to omit these figures.

Discussion

The interpretation of the different data sets was hampered by the fact, that in addition to the toxic effect of the added solvents, also accumulation of additional solvents by the fermentation process occurred, which both affected the overall growth of the organism. Therefore, the growth inhibition was a result of the combined effect of added and produced solvents. Thus, for instance, the growth in the presence of *meso*-2,3-butanediol was not only inhibited by *meso*-2,3-butanediol itself, but also by the end products, like 1-butanol and acetone. Attempts to use a degenerated (non-solventogenic) strain to avoid this effect were not successful. Nevertheless, the potential toxicity, as indicated by the log K_{ow}, correlated nicely with the amount of glucose converted and the final OD. Remarkably, growth in the presence of *iso*-butanol gave a higher OD compared to 1-butanol, whereas the amount of converted glucose was the same. This could be explained by the observation that in the presence of 1-butanol substantially less acetate was produced, which causes a lower ATP-yield and thus a lower optical density. Whereas in the presence of *iso*-butanol small amounts of additional solvents were produced, this did not occur in the presence of 1-butanol, suggesting that the former compound was indeed less inhibitory.

The compounds, *iso*-amyl alcohol, butyl acetate, butyl lactate and butyl butyrate were shown to be far more toxic than 1-butanol. In these cultures no production of ABE by fermentation was observed. The low consumption of glucose and small increase in OD observed in the presence of butyl-butyrate could be explained by the hydrolysis of the added ester by secreted endogenous esterases, thereby diminishing its toxic effect.

Interestingly, the log K_{ow} values of the 1-butanol derivatives corresponded perfectly with the toxicity data. From more toxic to less toxic to *C. acetobutylicum ATCC 824*, the C4 alcohol compounds could be ordered as

follows: 1-butanol, *iso*-butanol, 2-butanol, *tert*-butanol, and *meso*-2,3-butanediol (Table 7).

Table 7: Overview of calculation data from all growth profiles of the challenged cultures.

Compound	log K_{ow}		Consumed glucose (mM)	OD_{600}	
butyl butyrate	2.83	expected to be more toxic	35	1,4	verified to be more toxic
butyl acetate	1.78		12	1	
iso-amyl alcohol	1.42		5	0,8	
butyl lactate	1.10		8	1	
1-butanol	0.88		55	1,9	
iso-butanol	0.76	expected to be less toxic	55	3	verified to be less toxic
2-butanol	0.61		80	3,5	
tert-butanol	0.40		75	4,1	
meso-2,3-butanediol	-0.92		95	5,3	
no challenge			135	6,5	

With respect to these results, it can be stated that the isomers of 1-butanol, e.g. *iso*-butanol, 2-butanol and *tert*-butanol, together with 2,3-butanediol are suitable as replacement for 1-butanol production in *C. acetobutylicum ATCC 824* or even in other solventogenic clostridia. For 3 out of these 4, natural biosynthetic pathways exist, namely for *iso*-butanol, 2-butanol and 2,3-butanediol. These and several other alcohol biosynthesis pathways have been described.

Additionally, *iso*-butanol, 2-butanol and 2,3-butanediol can theoretically be produced via small modifications of the central metabolism of *C. acetobutylicum ATCC 824*.

Iso-butanol can be produced from the reduction of *iso*-butyraldehyde. The aldehyde can be produced from the energy rich compound *iso*-butyryl-CoA, while this intermediate can be produced from the clostridial central

metabolite butyryl-CoA. An enzyme, *iso*-butyryl-CoA mutase is known to catalyze this latter reaction [133-138]. An additional oxidoreductase should catalyze the two subsequent reactions.

2-butanol can be produced from the reduction of 2-butanone, which can be produced by a dehydration step of 2,3-butanediol. 2,3-butanediol can be produced by the reduction of acetoin, which is already a minor fermentation product in *C. acetobutylicum ATCC 824* [1]. 2,3-butanediol production by *C. acetobutylicum* is possible as is described in chapter 5 of this thesis. 2-butanone reductase activity is needed to reduce 2-butanone to 2-butanol.

4

Characterization and overexpression of acetolactate decarboxylase in *Clostridium acetobutylicum*

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In preparation

Chapter 4: Characterization and Overexpression of Acetolactate Decarboxylase in *Clostridium acetobutylicum*

Summary

2-acetolactate decarboxylase (ALD) catalyzes the conversion of 2-acetolactate to acetoin. The natural acetoin producer *Clostridium acetobutylicum* ATCC 824, contains one gene (CAC2967) that potentially codes for 2-acetolactate decarboxylase. This gene was successfully cloned and expressed in *E.coli* and the purified recombinant protein (Ca-ALD) was proven to exhibit 2-acetolactate decarboxylase activity. Size exclusion chromatography revealed that the native enzyme mainly exists as dimer of 27 kDa subunits. Optimal activity was found around 40 °C, and at pH 5.2. The enzyme is dependent on the presence of bivalent metal ions, like Zn^{2+} or Co^{2+}. The half life is estimated as 25 hours at 37 °C. The Ca-ALD binds acetolactate cooperatively with a Hill coefficient of 1.49. Also, a $K_{1/2}$ of 16.8 mM and a V_{max} of 51.9 U/mg was determined. Furthermore, a shuttle vector was constructed to express *Ca-ald* under control of the strong *adc* promoter in *C. acetobutylicum ATCC 824*. Despite successful transformation, no significant increase in acetoin production was observed in the *Ca-ALD* overexpressing strain.

Introduction

The production of acetone, 1-butanol and ethanol by solventogenic *Clostridia*, also known as ABE fermentation, has been studied extensively in the past. The industrial application of this process has a long history, dating back to the beginning of the twentieth century. However, starting around 1960, the fermentation was no longer economically competitive with the petrochemical process [1]. Recently, the ABE-fermentation has received renewed interest for the renewable production of 1-butanol from simple and complex carbohydrates. Applications of 1-butanol include its use as a biofuel or fuel extender, or as industrial bulk chemical [124]. A well-known member of the solventogenic *Clostridia* is *C. acetobutylicum ATCC 824*. It is able to ferment several C5 and C6 sugars, like glucose, arabinose, fructose, galactose, and xylose into lactate, acetate, butyrate, acetone, 1-butanol, ethanol, hydrogen, carbon dioxide and small amounts of acetoin [18, 139, 140]. Whereas most research has focused on the metabolic shift from acids to solvents, little is known about the pathway leading to acetoin.

Acetolactate, the precursor for acetoin, is produced via the condensation of an activated aldehyde moiety derived from pyruvate with a second molecule of pyruvate. This reaction is catalyzed by a two-subunit α-acetolactate synthase (acetohydroxy acid synthase; EC 2.2.1.6) encoded by the *ilvB* and *ilvN* genes. This anabolic pathway results in 2-acetolactate biosynthesis as precursor for the branched chain amino acids (BCAA), isoleucine, leucine and valine.

A second catabolic pathway for acetolactate biosynthesis involves an one-subunit α-acetolactate synthase (EC 2.2.1.6), condensing two molecules of pyruvate to form acetolactate with the release of one carbon dioxide. Acetolactate is subsequently converted to acetoin, either by a spontaneous

decarboxylation at low pH, or enzymatically using acetolactate decarboxylase (EC 4.1.1.5) [141-143].

As part of our aim to investigate the acetoin pathway in *C. acetobutylicum ATCC 824*, we focused here on the functional production of the acetolactate decarboxylase. The gene was heterologously expressed in *E. coli*, purified to homogeneity and characterized with respect to substrate specificity, temperature and pH optima, and kinetics. To our knowledge this is the first description of a characterized acetolactate decarboxylase of clostridial origin. Attempts to overexpress to enhance the acetoin production by overexpressing acetolactate decarboxylase in *C. acetobutylicum* ATCC824 will be discussed.

Material and methods

Chemicals, primers and plasmids.

All chemicals were of analytical grade and purchased from Sigma (unless stated otherwise). Primers were obtained from Biolegio. The restriction enzymes were obtained from Invitrogen. For heterologous expression the vector pET-24d (kanamycin resistance, Novagen, Darmstadt, Germany) was used. The *C. acetobutylicum* / *E. coli* shuttle vector pMTL500E was used for expression of *Ca-ald* in *C. acetobutylicum* (Table 8).

Organisms and growth conditions.

Commercially obtained, chemically competent, *E. coli* NEB 5-alpha F' I^q cells were used for cloning and vector maintenance and *E. coli* BL21(DE3) was used as overproduction strain in this study (Table 8). *E. coli* DH10B, containing methylation plasmid pAN1, was used to methylate plasmid DNA before transformation into *C. acetobutylicum*. All *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C, 200 rpm (unless stated otherwise), and kanamycin (50 µg/ml) was added when appropriate.

Clostridial spore suspensions were made by gently resuspending *C. acetobutylicum* spores from agar plates (14 days anaerobic growth at 37 °C) in sterile 15 % (v/v) glycerol. Agar plates for sporulation contained per liter of water: yeast extract, 5.0 g; KH_2PO_4, 1.0 g; K_2HPO_4, 0.76 g; ammonium acetate, 2.9 g; *p*-aminobenzoic acid, 0.10 g; $MgSO_4 \cdot 7\ H_2O$, 1.0 g; $FeSO_4 \cdot 7\ H_2O$, 0.005 g; glucose, 20.0 g; and agar, 15 g.

Stock cultures of *C. acetobutylicum ATCC 824* were maintained as spore suspensions in sterile 15 % (v/v) glycerol at -20 or -80 °C. Prior to inoculation of clostridial pre-cultures, spore suspensions were heat shocked for 10 minutes at 80 °C. *C. acetobutylicum* were grown in Gapes medium or modified CGM (mCGM) medium as indicated.

Gapes medium was based on the semi-synthetic medium described by Nimcevic *et al*. [131], and contained per liter of water: yeast extract, 5.0 g; KH_2PO_4, 1.0 g; K_2HPO_4, 0.76 g; ammonium acetate, 3.0 g; *p*-aminobenzoic acid, 0.10 g; $MgSO_4 \cdot 7\ H_2O$, 1.0 g; and $FeSO_4 \cdot 7\ H_2O$, 0.5 g.

mCGM medium contained per liter of water: yeast extract, 5.0 g; KH_2PO_4, 0.75 g; K_2HPO_4, 0.75 g; $MgSO_4 \cdot 7\ H_2O$, 0.4 g; $MnSO_4 \cdot H_2O$, 0.01 g; $FeSO_4 \cdot 7\ H_2O$, 0.01 g; NaCl, 1.0 g; asparagine, 2.0 g; $(NH_4)_2SO_4$, 2.0 g; cysteine, 0.125 g; and glucose, 12.5 g.

Medium for fermentation was made anaerobic by sparging with nitrogen gas. Serum flasks (250 mL), containing 100 mL Gapes medium, were inoculated with 2 % (v/v) overnight pre-cultures. Clostridial experiments and manipulations were performed anaerobically in; (i) an anaerobic chamber (Sheldon Manufacturing, Oregon U.S.A.; gas mixture consisting of 15 % CO_2, 4 % H_2 and 81 % nitrogen); or (ii) glass serum vials with butyl rubber stoppers and aluminum crimp seals equipped with a pressure release system previously described [132].

Culture media were supplemented with ampicillin (100 μg mL^{-1}), chloramphenicol (30 μg mL^{-1}), erythromycin (40 μg mL^{-1} for liquid cultures and plates; 25 μg mL^{-1} for transformant isolation on plates), kanamycin (50 μg mL^{-1}), *iso*-propyl-β-D-thiogalactopyranoside (IPTG; 50 μg mL^{-1}) or 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal; 40 μg mL^{-1}), when appropriate.

The growth of clostridial cultures was monitored by measuring the optical density spectrophotometrically at 600 nm on a Pharmacia Biotech Ultrospec 2000.

DNA isolation, transformation and manipulation

Genomic DNA from *C. acetobutylicum* was isolated using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich), while plasmid DNA from *E. coli* was isolated by the GenElute Plasmid Miniprep Kit (Sigma-Aldrich). Amplification of clostridial DNA by PCR was done using the *Pfu* polymerase (Stratagene), while *E. coli* colony PCR reactions were carried out using REDTaq DNA polymerase (Sigma-Aldrich). Transformations of chemically competent *E. coli* strains were conducted using New Engeland Biolab's "High Efficiency Transformation Protocol" (http://www.neb.com/nebecomm/products/protocol119.asp). Prior to transformation into *C. acetobutylicum*, plasmids were methylated *in vivo* by electroporation into *E. coli* DH10B (pAN1) [144].

A modified procedure, based on the method of Oultram *et al.* [145], was used for transformation of *C. acetobutylicum* by electroporation. Briefly, this was carried out as follows; *C. acetobutylicum* was grown up to an OD_{600} of 0.7 (corresponding to a biomass concentration of approximately 0.35 mg mL^{-1}) in 100 mL mCGM medium at 37 °C. The culture was centrifuged for 10 minutes (5 468 × *g*, 4 °C), after which the supernatant was removed and the pellet was washed with 20 mL ice-cold anaerobic electroporation buffer (270 mM sucrose, 1 mM $MgCl_2$, 7 mM sodium phosphate buffer, pH 7.4). The pellet was again resuspended in 4 mL of fresh electroporation buffer. 300 µL of the suspension was transferred into an electroporation cuvette with a 0.2 cm gap width. After addition of approximately 1 µg of methylated plasmid DNA, the cells were electroporated (1.25 kV; 25 µF; 100 Ω) resulting in time constants between 1.2 and 1.8 ms. The electroporated cells were cooled on ice and then incubated with 3 mL pre-warmed mCGM at 37 °C for 3 hours. The cells were concentrated and plated on pre-warmed mCGM plates containing erythromycin. All manipulations were carried out anaerobically and on ice, unless noted otherwise.

Cloning of the *Ca-ald* gene and transformation of *E. coli*.

The expression plasmid pWUR499 (Table 8) was constructed as follows. The pET24d was linearized by digestion with *Nco*I, *Xho*I and dephosphorylated to prevent self-ligation. The gene *CAC2967* was amplified by PCR from *C. acetobutylicum* genomic DNA using primers 1 and 2 (Table 9). Primer 1 contained a *Bsp*HI site, an isoschizomer of *Nco*I and primer 2 a *Xho*I site. All PCR products were digested using the restriction enzymes for the aforementioned sites in the primers and ligated into their corresponding pET24d vector yielding pWUR499. The reversed primer lacks a stop codon which results in a hexahistidine-tag at the C-terminus of the expressed enzyme to facilitate purification.

Chemical competent *E. coli* NEB 5-alpha cells were transformed with the ligation mixtures according to the manufacturers instructions and selected for kanamycin resistance. Colonies were checked by colony PCR, after which, restriction analysis and sequence analysis of both DNA strands confirmed the correct constructs. Chemical competent *E. coli* BL21(DE3) cells were transformed with pWUR499, selected for kanamycin resistance and stored until further use.

Cloning of *Ca-ald* and transformation of *C. acetobutylicum*

The clostridial expression plasmid pWUR500 was constructed as follows. The pMTL500E vector was linearized by digestion with *Sph*I and *Xho*I. The *adc* promoter region was amplified by PCR from *C. acetobutylicum ATCC 824* genomic DNA using primers 3 and 4 (Table 9), respectively. The acetolactate decarboxylase (*Ca-ald*) gene was amplified by PCR from *C. acetobutylicum ATCC 824* genomic DNA using primers 1 and 2. Primer 1 contained a *BspHI* restriction site, primer 2, a *XhoI* restriction site,

primer 3 a *Sph*I restriction site, and primer 4 an *NcoI* restriction site. All PCR products were digested using the restriction enzymes for the aforementioned sites. Plasmid pWUR500 was obtained by a three way ligation reaction, which contained the linearized vector, the *Ca-ald* gene and the *adc* promoter. Competent *E. coli* NEB 5-alpha cells were transformed with the ligation mixtures and subjected to blue-white screening and selected for ampicillin resistance. White colonies were checked by colony PCR for the presence of the corresponding construct, after which, restriction analysis and sequencing confirmed the correct constructs. *E. coli* DH10B (pAN1) methylation strain was electroporated with plasmids pWUR500 and transformants were selected for ampicillin and chloroamphenicol resistance. Correct methylation was checked by restriction analysis using *Fnu4*HI [144].

Competent *C. acetobutylicum* cells were transformed with plasmid pWUR500, and the control plasmid pMTL500E, containing no insert. Each transformation resulted in multiple erythromycin resistant colonies. After re-streaking, selected colonies were used to prepare spore suspensions for further experiments.

Table 8: Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant genotype	Remarks	Source
E. coli NEB 5-alpha F' I^q	$proA^+B^+$ $lacI^q$ Δ(lacZ)M15 recA1	Cloning strain	Invitrogen / New England Biolabs
E. coli BL21(DE3)	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3)	Expression strain	Novagen
C. acetobutylicum ATCC 824	WT	Type strain	Laboratory stock, originally from G. Bennet (Rice University, Houston, Texas, USA)
pWUR499	pET24d; P_{T7}-*Ca-ald*	Expression plasmid of *CAC2967* with His-tag under control of a T7 promoter	This study
pAN1	p15A ori; Cm^r Φ3tI	Expression plasmid for phage Φ3tI methylase gene [144]	Laboratory stock
pMTL500E	ColE1 ori; pAMβ1 ori; MLS^r; Ap^r	clostridial/*E. coli* shuttle vector [145]	Laboratory stock
pWUR500	ColE1 ori; pAMβ1 ori; MLS^r; Ap^r; P_{adc}-*CAC2967*	Expression plasmid of *Ca-ald* under control of the *C. acetobutylicum* acetoacetate decarboxylase promoter	This study

Table 9: Primer sequences used in this study; Restriction sites incorporated in the primers are underlined.

No.	Primer	Sequence (5' → 3')[a]	Target DNA region
1	BG2201	GCGCGTCATG Attgaagaag tgatccctaa tc	*CAC2967*
2	BG2202	GCGCGCTCGA Gtttctcaac tttacttatt tcatc	*CAC2967*
3	BG2606	gcgcgGCATGCatgggaaagccaacattgc	Promoter *adc*; *cap0165*
4	BG2607	cgcgcCCATGGaagtcaccttcctaaatttaataatg	Promoter *adc*; *cap0165*

Production and purification of heterologous Ca-ALD from E.*coli*.

Escherichia coli BL21(DE3) harboring pWUR499 was used to inoculate a 5 mL overnight LB medium culture supplemented with 50 µg/mL kanamycin. The preculture was used to inoculate 500 mL LB medium (1 : 1000) supplemented with 50 µg/mL kanamycin in conical flasks (2L) and incubated for 8 h. The culture was put on ice for 1 h to induce chaperones [146] and subsequently induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

E. coli cells were harvested after 15 h of growth at 20 °C and resuspended in 20 mM Tris-HCl pH 7.0. After passing twice through a French Press at 110 MPa, the total cell extract was centrifuged for 20 minutes (16 500 × g, 4 °C) resulting, after passing through a 0.45 µm filter (Whatman), in clear cell free extract (CFE). The cell-free extract was applied with a flow rate of 2 mL min^{-1} to a Ni-chelating column (20 mL) equilibrated in 20 mM Tris-HCl buffer (pH 7.5) containing 300 mM NaCl. Proteins were eluted with a linear gradient of 20–500 mM imidazole and fractions (10 mL) were collected. The most active fractions were pooled and applied at a flow rate of 10 mL min^{-1} to

a HiPrep desalting column (53 mL) (Amersham Biosciences, Piscataway, NJ, USA), equilibrated in 20 mM Tris-HCl buffer (pH 7.5). Fractions of 10 mL were collected and stored at -20 °C, until further use.

Acetolactate decarboxylase activity assay

The activity of ALDC was determined colorimetrically according to a modified assay as originally described by Stormer (1975) [147]. The substrate, 2-acetolactate is unstable, so 25 mL 2-acetolactate solution was prepared freshly by hydrolyzing 50 µL ethyl 2-acetoxy-2-methylacetoacetate ester (10 mM final) with 1.5 mL 1 M NaOH for 20 minutes, yielding acetolactate, but also ethanol and acetate. After 20 minutes, the volume was set to 20 mL with 10 mM sodium citrate buffer pH 5.0. The pH was set to 6 with 0.5 M HCl, and de volume was subsequently adjusted to 25 mL with 10 mM sodium citrate buffer pH 6.0, unless stated otherwise.

The enzymatic product, acetoin, reacts with guanido groups of creatin in an alkaline environment, yielding a pink colour. This can be measured at 522 nm [148, 149].

The assay mixture containing 200 µl enzyme solution and 200 µl substrate solution (10 mM ethyl 2-acetoxy-2-methylacetoacetate, 0.06 N NaOH, 10 mM citrate buffer pH 6.0) was incubated for 20 minutes at 30 °C. Simultaneously, a blank containing only substrate solution was incubated to correct for spontaneous decarboxylation of 2-acetolactate.

After the incubation, 4.5 mL colouring solution (70 mM α-Naphtol, 7.6 mM creatine in 1N NaOH) was added to stop the enzymatic reaction and to stain the solution by the reaction with the produced acetoin. After 45 minutes at room temperature, the absorption of the sample was measured at 522 nm and corrected for the blank measurement.

Acetoin concentrations were calculated using a calibration curve (0 to 1.135 mM), made with an acetoin standard. .

Size exclusion chromatography

The native molecular mass was determined by size exclusion chromatography on a Superdex 200 high-resolution 10/30 column (24 ml; Amersham Biosciences) equilibrated in 50 mM Tris-HCl, 100 mM NaCl (pH 7.8). Two hundred fifty microliters of enzyme solution in 50 mM Tris-HCl buffer (pH 7.8) was injected on the column. Proteins used for calibration were Blue dextran 2000 (>2 000 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and RNase A (13.7 kDa).

Optimal pH, temperature and thermostability

The optimal pH of the reaction was determined using a 10 mM sodium citrate and Tris-HCl buffer system, respectively, that was adjusted in the range of pH 4 - 11 at 30°C.

The optimal temperature was determined in 10 mM sodium citrate buffer (pH 5.0) that was adjusted in the temperature range of 20 °C – 85 °C. The final pH of the solution after addition of enzyme to the substrate buffer was checked.

Thermal inactivation assays were performed by incubating Ca-ALD at 37 °C and taking aliquots at regular intervals until 70 hour, followed by a standard activity assay.

Substrate, co-factor specificity and kinetics

The Ca-ALD kinetic parameters K_m and V_{max} were calculated from multiple measurements with 2-acetolactate (0.39, 0.78, 1.56, 3.125, 6.25, 12.5,

12.5, and 25 mM). The enzyme kinetics followed a Hill-type rather than a Michaelis-Menten type of kinetics. A software programme, Tablecurve 2D, v5 (Systat Software Inc., San Jose, CA, USA) was used to directly fits the data to the Hill curve. The turnover number (*k*cat, s^{-1}) was calculated per catalytic subunit.

Salts, metal ions, and inhibitors

The effect of metal ions on acetoin reductase activity was determined using different metal salts ($CuCl_2$, $CoCl_2$, $FeSO_4$, $FeCl_3$, $NiCl_2$, $MnCl_2$ and $ZnCl_2$) at final concentrations of 0.2 mM using the standard activity assay. The enzyme was first pre-incubated in 10 mM EDTA at 37 °C for 60 minutes, after which the EDTA was removed by buffer exchange. The enzyme was pre-incubated in 100 mM sodium phosphate (pH 6.5) in the presence of the metals at 37 °C for 60 minutes. Subsequently, samples were cooled on ice and the residual activities were measured using the standard method. The activity of Ca-ALD without pre-treatment with EDTA or addition of metal ions was used as control.

Analysis of metabolites

The concentration of sugars, acids and solvents in culture supernatants was determined by HPLC. Samples taken during fermentation (approximately 1.5 mL) were centrifuged (5 min, 20 800 × *g*) and the supernatants were stored at -20 °C. After thawing, an equivolume amount of internal standard solution (30 mM 4-methyl valeric acid (Sigma-Aldrich) in 0.5 M H_2SO_4) was added to the supernatant sample. The sample with internal standard was filtered through a 0.2 µm pore size membrane filter (Whatman) and a 10 µL-sample was injected on the HPLC-column. Separation was achieved using a Shodex Ionpack KC-811(RP) column with 3 mM H_2SO_4 as the eluent (flow: 1 mL min^{-1}; column

temperature: 85 °C), equipped with a refractive index detector (Waters 2414) and a UV detector (Waters 2487) operating at 210 nm. The order of elution was: glucose, lactic acid, acetic acid, acetoin, *meso*-2,3-butanediol, D/L-2,3-butanediol, butyric acid, acetone, ethanol, valeric acid, 4-methyl valeric acid and 1-butanol. All concentrations were determined based on the refractive index chromatogram except for butyrate for which in some cases the UV chromatogram was used. Empower 2 software (Waters Corporation) was used for machine control and data analysis.

Results

In silico identification and examination of an acetoin reductase

The *C. acetobutylicum CAC2967* gene encodes a protein of 238 amino acids and has a calculated molecular mass of approximately 27 kDa with a theoretical pI of 5.31 (http://www.expasy.ch/tools/protparam.html). It is annotated in the NCBI database (http://www.ncbi.nlm.nih.gov/) as an alpha-acetolactate decarboxylase. The sequence belongs to COG 3527 (alpha-acetolactate decarboxylase) (http://www.ncbi.nlm.nih.gov/COG/). The two most significant hits of a BLAST-P analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi/) were a (putative) alpha-acetolactate decarboxylase from *Pelotomaculum thermopropionicum* SI (43% identity, *alsD*) and a (putative) acetolactate decarboxylase from *Desulfuromonas acetoxidans* DSM 684 (44% identity, *Dace_0685*).

Identification of the protein family and protein domains was performed using an Interpro scan from EMBL-EBI (http://www.ebi.ac.uk/interpro/). This analysis revealed that the protein is a member of the alpha-acetolactate decarboxylase family (IPR005128). Conserved context analysis with STRING (http://string.embl.de/) of *Ca-ald* revealed a conserved neighborhood links with the two subunits of the anabolic acetolactate synthase, as well with the catabolic acetolactate synthase from other related micro-organisms (*Bacillus* sp., and *Lactobacillus* sp.), supporting the predicted acetolactate decarboxylase activity of Ca-ALD.

Purification of recombinant Ca-ALD

Ca-ALD was successfully purified to homogeneity from cell free extract of *Escherichia coli* BL21(DE3) containing pWUR499 by using two sequential chromatographic columns, namely a Ni-chelating affinity column and a HiPrep desalting size exclusion column. Fractions containing the purified protein were

pooled. The migration of Ca-ALD with his-tag shows on SDS/PAGE, a molecular subunit mass of approximately 27 kDa (not shown). Size-exclusion chromatography suggested that the multimeric structure of Ca-ALD is, next to small amounts of tetramer, mainly dimeric.

Kinetics

Ca-ALD was proven to exhibit acetolactate decarboxylase activity, with specific activities amounting to 35 $U.mg^{-1}$. The kinetic parameters were determined for acetolactate in the presence of 0.2 mM Zn^{2+}.

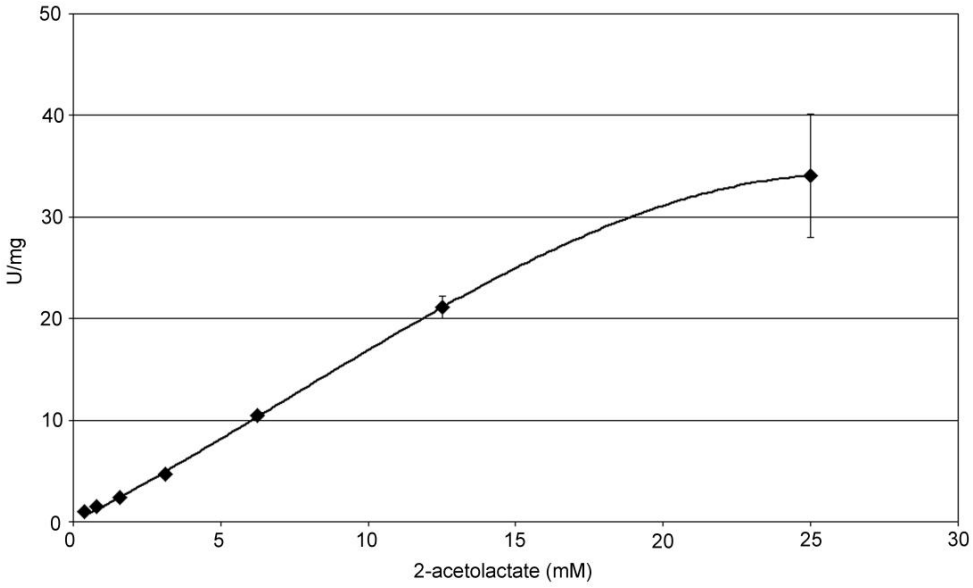
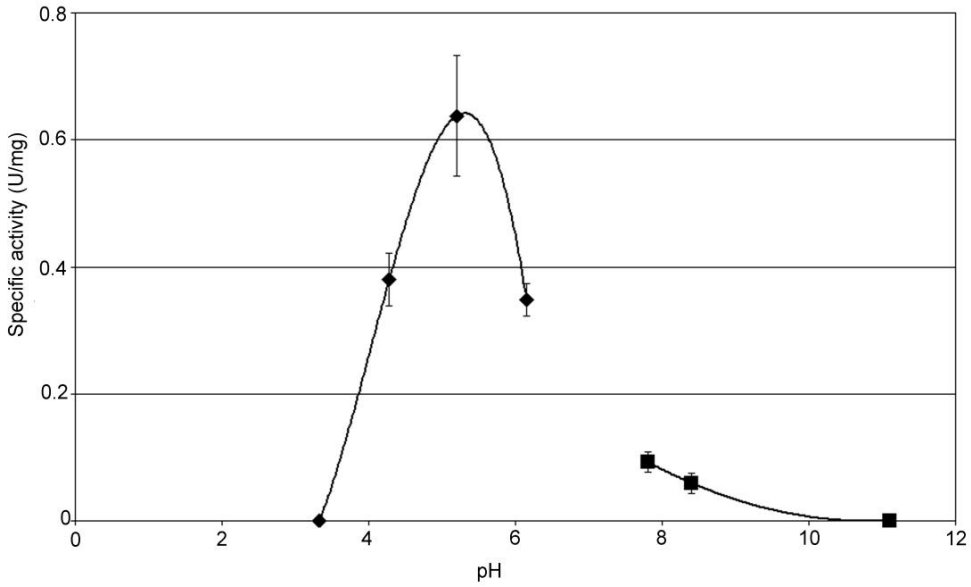


Figure 11: The specific activity as a function of the 2-acetolactate concentration (mM).

The dependence of the enzyme activity on the substrate concentration appears to follow a sigmoid curve indicative of cooperative binding. Kinetic analysis according to the Hill equation, yielded a $K_{1/2}$ of 16.8 mM, a k_{cat} of 23 s^{-1} (k_{cat}/K_m = 1.39 s^{-1} * mM^{-1}) and a Hill coefficient of 1.49. The enzyme was saturated at approximately 35 mM of acetolactate (Figure 11).

Optimal pH, temperature and thermostability

A)



B)

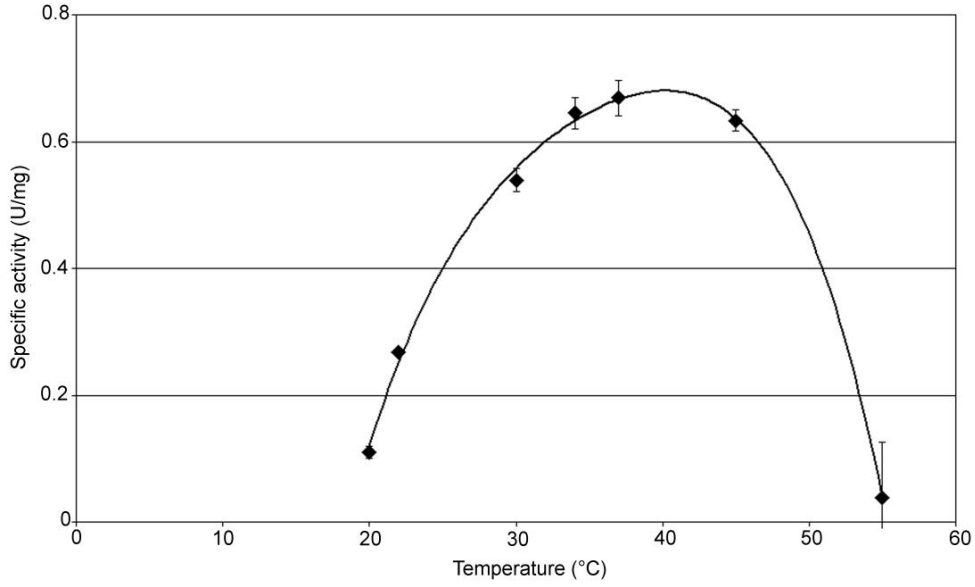


Figure 12: Effect of (A) pH and B) temperature on acetolactate decarboxylase activity.

Ca-ALD activity was measured in a pH range of 3.8 – 11.0 (Figure 12A). Ca-ALD displayed >50% of its maximal activity in the pH range of 4.3 to 6.2, with an optimal pH at approximately 5.2. For determining the optimal temperature of the decarboxylase, the temperature was increased from 20 °C upwards until 55 °C (Figure 12B), revealing an optimal activity at approximately 40 °C.

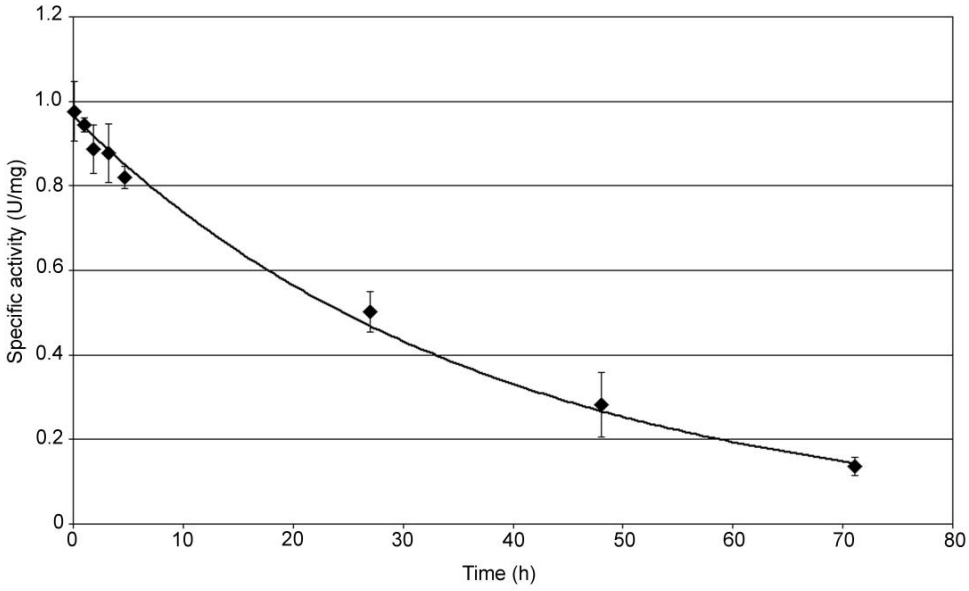


Figure 13: Thermostability of acetolactate decarboxylase.

Furthermore, Ca-ALD has a high resistance to inactivation at the mesophilic growth temperature of 37 °C, with a half-life value of approximately 25 hours (Figure 13).

Salts, metals, and inhibitors

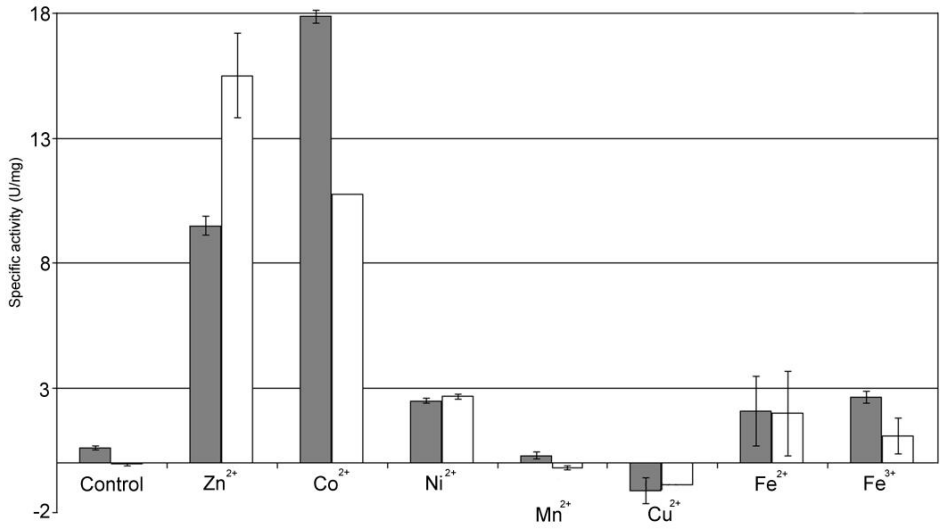


Figure 14: Effect of metal ions on Ca-ALD activity. Samples were incubated without (grey) and with (white) 10 mM EDTA before measuring the acetolactate decarboxylase activity.

Figure 14 shows that no acetolactate activity was left in the control after pre-incubation in EDTA, while 0.6 U/mg was measured with residual metals bound after growth and purification. It can be concluded that Ca-ALD is metal dependent. In the EDTA treated samples, the activity with Zn^{2+} is most optimal, with cobalt being second best. Addition of Ni^{2+}, Fe^{2+} and Fe^{3+} showed also a reactivation in EDTA treated samples, although with very low activities. Copper and manganese gave no recovery of the enzymatic activity, however high background activity by chemical decarboxylation was observed in the presence of Cu^{2+}, Mn^{2+} and Fe^{3+}. In the non-EDTA treated samples, similar patterns were found, except that Ca-ALD is more active with Co^{2+} than with Zn^{2+}.

Characterization of C. *acetobutylicum* transformants expressing Ca-ALD

Wild type *C. acetobutylicum ATCC 824* is known to produce significant levels of acetoin. Increased levels of acetolactate decarboxylase in *C. acetobutylicum ATCC 824* cells may result in an elevated production of acetoin. Therefore, *C. acetobutylicum* transformants were constructed, containing the *Ca-ald* gene under control of the *adc* promoter (pWUR499), and their fermentation pattern was analyzed. As a result, transformants expressing additional Ca-ALD were found to produce the same amount of acetoin (10.8 ± 0.4) as the control (10.1 ± 0.6 mM), containing the empty vector.

Discussion

Acetoin is an important physiological metabolite, and an unusual fermentation product of certain anaerobic bacteria. Therefore, it has been used as microbial classification marker, which can be easily demonstrated with the Voges Proskauer test. Its physiological importance is to avoid acidification, to participate in the regulation of the NAD/NADH ratio and to store excess carbon [69, 143].

Acetoin is produced by the decarboxylation of its precursor, acetolactate by a decarboxylase. Only a few acetolactate decarboxylases have been described to date. Some of these proteins were found within the Gram-negative bacteria *Klebsiella aerogenes* and *Klebsiella terrigena*, and others in the Gram-positive bacteria *Bacillus brevis, Lactococcus lactis* and *Streptococcus lactis* [141, 142, 150-152].

The well-studied $Gram^+$ solventogenic bacterium, *C. acetobutylicum ATCC 824*, also produces acetoin and a putative acetolactate decarboxylase gene has been identified in the genome. Gene neighborhood analysis showed a link with mannose-1-phosphate guanylyltransferase, however, without an obvious physiological explanation. In other species, acetolactate decarboxylase is often linked to acetolactate synthase, which can have an anabolic (production of branched chain amino acids) or a catabolic (production of acetoin / 2,3-butanediol) role. This link suggests a possible role in the branched chain amino acid production or in acetoin / 2,3-butanediol production. In *L. lactis*, the acetolactate decarboxylase is even clustered with the genes for the synthesis of branched-chain amino acids (BCAA). In this bacterium, it was proven to have a dual role, as (i) key regulator of valine and leucine biosynthesis, by controlling the acetolactate flux towards catabolism and (ii) as an enzyme catalyzing the second step to acetoin of the 2,3-butanediol pathway [141, 142].

After functional expression of CAC2967 (*Ca-ALD*) in *E. coli*, acetolactate decarboxylase activity could indeed be confirmed. Size exclusion chromatography indicated mainly a dimeric quaternary structure. The ALD from *L. lactis* has been shown to obtain a hexameric structure. However, the ALD from *Lactobacillus casei* and *Brevibacterium acetylicum* showed both also a dimeric oligomerization [153, 154].

Ca-ALD displayed sigmoidal kinetics with a $K_{1/2}$ of 16.8 mM for acetolactate. Saturation occurred at approximately 35 mM acetolactate. In addition, a Hill coefficient of 1.49 was obtained, indicating a positive cooperativity between the two subunits. Similarly, a sigmoidal saturation curve was observed for ALD from *L. lactis* with a K_m for D-α-acetolactate of 74 mM and a Hill coefficient of 2.62 (saturation at 120 mM of D-α-acetolactate), which also suggests a strong positive cooperativity in substrate binding. However, after addition of 40 mM leucine, the K_m (10 mM) and Hill coefficient (1.18) were lowered, while the enzyme was saturated at 50 mM D-α-acetolactate [155]. The effect of leucine was not tested for Ca-ALD in this study.

A temperature and pH optimum for Ca-ALD was found around 40 °C and pH 5.2, indicating that *in vivo* (after transcription, translation and without any enzymatic regulation) relatively high activities might be expected, at all stages during growth of *C. acetobutylicum* (temperature of 37 °C and internal pH between 5.5 and 6.7) [156, 157]. Nevertheless, with a $K_{1/2}$ of 16.8 mM for acetolactate, the maximum activity of Ca-ALD could not be reached. Ca-ALD was stable at mesophilic temperatures, with a half-life of approximately 25 h at 37 °C. After 71 h, 14 percent of the activity was still present. Compared to the ALD from Lactobacillus casei, with a half-life of 15 minutes at 37 °C, Ca-ALD is relatively stabile [153].

Incubation of Ca-ALD with the chelating agent EDTA results in complete inactivation, indicating its dependence for bivalent metal ions.

Reactivation was observed in the presence of Zn^{2+} and Co^{2+}. The effect of the EDTA-treatment was, however, confusing. Whereas Zn^{2+} gave the highest activity in the EDTA-treated samples, Co^{2+} was best in the untreated samples. These data suggest that saturation with one type of metal ion (either Zn^{2+} or Co^{2+} in the EDTA-treated samples) is less optimal than a combination of different metal ions (Co^{2+} together with some residual metal ion) that is likely to occur in the untreated sample. Metal analysis is needed to clarify this observation. A stimulatory effect of Zn^{2+} was also observed for the ALD from *L. casei*. Also Mn^{2+} stimulated the ALD from *L. casei,* whereas Co^{2+} did not. Mn^{2+} could not restore the activity of Ca-ALD.

Clostridium acetobutylicum ATCC 824 is known to produce acetoin as a minor fermentation product [68, 139]. Our analysis of fermentations of the ATCC 824 type strain, as well as transformants harboring the empty pMTL500 confirms this. Transformants containing the expression plasmid did not show an increased acetoin production. The transformed and control strain produced 10.8 ± 0.4 mM and 10.1 ± 0.6 mM, respectively. Although, functional overexpression of CAC2967 was not confirmed, several reasons can be given to explain the same acetoin yields in the *in vivo* experiments. In *L. lactis*, ALD is regulated at transcriptional and translational level. It has been shown that it can be transcribed from three different promoters. Entrapment of the ribosome binding site of the mRNA in a stem-loop -like secondary structure results in a 100-fold decrease in the level of translation. A fivefold additional decrease is caused by an additional secondary structure element upstream of the stem-loop, resulting in a complete translation stop. Also at protein level, regulation has been observed. An allosterical activation by branched chain amino acids is found for the decarboxylase [141, 142, 155].

With respect to these regulations, it is possible that Ca-ALD is also regulated on different levels as it has been observed in *L. lactis* [142]. Even with a

strong promoter like the *adc* promoter, specific secondary structures of mRNA might cause imperfect translation or no translation at all. Nevertheless, it should be noted that correct transcription or translation of the here presented overexpression experiment has not yet been analysed. In addition, allosterical acetoin feedback inhibition of Ca-ALD could also exist. However, the most obvious reason for the similar acetoin production levels is that this enzyme is not controlling the flux from pyruvate to acetolactate, in other words there is another bottleneck. The amount of acetolactate present during fermentation, which is not needed for the branched chain amino acids anabolism, is already completely converted to acetoin in both strains, by the natively produced acetolactate decarboxylase. Increasing the flux from pyruvate to acetolactate by increasing the expression of the catabolic acetolactate synthase might result in higher acetoin / 2,3-butanediol production.

5

Molecular Characterization of a acetoin reductase from *Clostridium beijerinckii*

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In preparation

Chapter 5: Molecular characterization of an acetoin reductase from *Clostridium beijerinckii*

Summary

Acetoin reductase is an important enzyme for the fermentative production of 2,3-butanediol. *Clostridium acetobutylicum* ATCC 824 produces acetoin, but is not able to perform the subsequent conversion to 2,3-butanediol. A bioinformatic screening within the genome of *C. beijerinckii NCIMB 8052*, revealed eight potential acetoin reductases. Out of six successfully cloned genes, one (*CBEI_1464*) showed substantial acetoin reductase activity after heterologous expression in *E. coli*. The purified enzyme (Cb-ACR) was found to exist predominantly as homodimer of 37 kDa subunits. The enzyme has a preference for NADPH (K_m = 0.32 μM) as electron donor, with a specific activity amounting to 76 U. mg^{-1}. Optimal activity was found around 68 °C, for both reactions and at pH 6.5 and 9.5, for the reduction and oxidation reaction, respectively. ICP-AES analysis revealed the presence of ~2 Zn^{2+} atoms and ~1 Ca^{2+} atom per monomer. To gain insight into the reaction mechanism, but also into the substrate- and cofactor-specificity, a structural model was constructed with a ketose reductase (sorbitol dehydrogenase) from *Bemisia argentifolii* (silverleaf whitefly) as template. The catalytic zinc atom is likely coordinated by Cys37, His70, Glu71 in Cb-ACR, while the structural zinc site is probably composed of Cys100, Cys103, Cys106, and Cys114.

Introduction

The production of acetone, 1-butanol and ethanol by solventogenic clostridia, also known as ABE fermentation, has been studied extensively over the past [1]. A well-known member of the solventogenic clostridia is *C. beijerinckii* NCIMB8052 (formerly *C. acetobutylicum*). It is able to ferment several C5 sugars (xylose, arabinose) and C6 sugars (glucose, fructose, galactose) into lactate, acetate, butyrate, acetone, 1-butanol, ethanol, acetoin, hydrogen and carbon dioxide [18, 139, 140]. In addition, certain *C. beijerinckii* sp. are known to reduce acetone further to *iso*-propanol [1].

Solvent production in these organisms is the result of the activity of alcohol dehydrogenases. This group of enzymes (ADHs; EC 1.1.1.), which are found in all three domains of life, are capable of catalyzing the reversible reaction between aldehydes and ketones and their corresponding alcohols [158, 159].

The ADHs show variations within their structural organization and catalytic mechanisms and moreover between their substrate specificity. Therefore, they can be classified in three major groups, based on their cofactor specificity (a) NAD(P)-dependent alcohol dehydrogenases, (b) NAD(P)-independent alcohol dehydrogenases, which use pyrroloquinoline quinone (PQQ) or F_{420} as cofactor, and (c) FAD-dependent alcohol oxidases [159]. Furthermore, the NAD(P)-dependent alcohol dehydrogenases are subdivided into four distinct classes according to the polypeptide size; (i) type I, medium-chain dehydrogenases/reductases (MDR, ~350 amino acids per subunit, (ii) type II, short-chain dehydrogenases/reductases (SDR, ~250 amino acids per subunit), and (iii) type III, long-chain dehydrogenases/reductases (LDH, ~360-550 (up to 900) amino acids per subunit [159]. Next to the above described ADHs, a class of the NAD(P)-dependent ADHs is described, which are not closely related to type I, II or III. This superfamily is called the aldo-keto reductase

superfamily [160]. Members within this family share a common fold, and are mostly monomeric proteins that bind a nicotinamide cofactor without a Rossmann-fold motif [161-165]. Acetoin reductases (EC: 1.1.1.5) are enzymes within this group of ADHs, catalyzing reversibly the reaction from acetoin to 2,3-butanediol. Since *C. acetobutylicum* is a native producer of acetoin, the search for this reductase is a logical next step to produce 2,3-butanediol within *C. acetobutylicum*.

The work reported here describes the search for this acetoin reductase within *C. beijerinckii* NCIMB 8052. A carefully selected alcohol dehydrogenase gene was heterologously expressed in *E. coli*, purified to homogeneity and characterized with respect to substrate specificity, temperature and pH optima, and kinetics. Since CBEI_1464 (Cb-ACR) was found to exhibit acetoin reductase activity, it is an attractive candidate for 2,3-butanediol production in solventogenic Clostridia sp, like *C. acetobutylicum* ATCC 824, since acetoin is already known to be produced by this species [1].

Materials and methods

Chemicals, primers and plasmids.

All chemicals were of analytical grade and purchased from Sigma-Aldrich (unless stated otherwise). Primers were obtained from Biolegio (Nijmegen, The Netherlands). The restriction enzymes were obtained from Invitrogen (Breda, The Netherlands). For heterologous expression the vector pET-24d (kanamycin resistance, Novagen, Darmstadt, Germany) is used.

Organisms and growth conditions.

Escherichia coli DH5α was used as a host for cloning vectors and *E. coli* BL21 (DE3) was used as overproduction strain to obtain recombinant protein for purification (Table 10). All *E. coli* strains were grown at 37°C, 200 rpm in Luria-Bertani (LB) medium supplemented with $MgSO_4$ (1 mM), kanamycin (50 μg/mL) and $ZnSO_4$ (250 μM) (unless stated otherwise). Additional zinc was added to ensure full occupance of the zinc binding sites of the enzyme [166], while additional magnesium was added to increase *E. coli* biomass [167].

Data mining

A screening for possible acetoin reductases was performed within the genomes of *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052. The sequences for these reductases were identified by performing BLAST searches with the sequence from a characterized acetylacetoin reductase/2,3-butanediol dehydrogenase from the closely related $Gram^+$ *Bacillus cereus* YUF-4 (http://www.ncbi.nlm.nih.gov/blast) [168, 169]. The secondary structure was obtained using Psipred [170]. Phylogenetic analysis was performed by aligning the amino acid sequence of Cb-ACR and its homologues with the amino acid sequences of known characterized reductases using ClustalW [171]. A

bootstrapped phylogenetic tree was constructed and displayed using the neighbor-joining method with TreeView version 1.6.5 [172].

Cloning and sequencing of the potential acetoin reductase encoding genes.

The expression plasmids pWUR[1-6] (Table 10) were constructed as follows. The pET24d en pET26b vector was linearised by digestion with *Nco*I, *Xho*I and *NdeI*, *XhoI*, respectively and dephosphorylated to prevent self-ligation. The genes, *CBEI_0223, CBEI_0685, CBEI_1464, CBEI_2243, CBEI_3864, CBEI_3890* were amplified by PCR from *C. beijerinckii* genomic DNA using the indicated primers 1 - 12 (Table 11). Primers 1,3 and 5 contained a *Bsp*HI site, an isoschizomer of *Nco*I; primer 7, an *Nde*I site; primers 9 and 11 an *Ase*I site, an isoschizomer of *Nde*I and primer 2; 4, 6, 8, 10, and 12 an *Xho*I site. All PCR products were digested using the restriction enzymes for the aforementioned sites in the primers and ligated into their corresponding pET24d or pET26b vector yielding pWUR[1-6]. The reversed primers (except primer BG2408) are lacking a stopcodon resulting in a hexahistidine-tag at the C-terminus of the enzymes after expression. Chemical competent *E. coli* NEB 5-alpha cells were transformed with the ligation mixtures according to the manufacturers protocol and selected for kanamycin resistance. Colonies were checked by colony PCR, after which, restriction analysis and sequence analysis confirmed the correct constructs. Chemical competent *E. coli* BL21 (DE3) cells were transformed with pWUR[1-6], selected for kanamycin resistance and stored until further use.

Table 10: Bacterial strains and plasmids used in this study.

	Relevant genotype	Remarks	Source
Bacterial strains			
E. coli NEB 5-alpha F' I^q	$proA^+B^+$ $lacI^q$ Δ(lacZ)M15 recA1	Cloning strain	Invitrogen / New England Biolabs
E. coli BL21 (DE3)	F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3)	Expression strain	Novagen
Plasmids			
pWUR463[1]	pET24d; P_{T7}-*CBEI_0223*	Expression plasmid of *CBEI_0223* with His-tag under control of a T7 promoter	This study
pWUR461[2]	pET24d; P_{T7}-*CBEI_0685*	Idem for *CBEI_0685*	This study
pWUR450[3]	pET24d; P_{T7}-*Cb-acr*	Idem for *CBEI_1464 (Cb-acr)*	This study
pWUR464[4]	pET26b; P_{T7}-*CBEI_2243*	Idem for *CBEI_2243*	This study
pWUR462[5]	pET26b; P_{T7}-*CBEI_3864*	Idem for *CBEI_3864*	This study
pWUR452[6]	pET26b; P_{T7}-*CBEI_3890*	Idem for *CBEI_3890,* without His-tag	This study

Table 11: Primer sequences used in this study; Restriction sites incorporated in the primers are underlined.

No.	Primer name	Sequence (5' → 3')[a]	Target DNA region
1	BG2405	GCGCGTCatg aaagcattaa caaaaacaaa tccag	*CBEI_0223*
2	BG2524	GCGCGCTCGA Gagatcttat tactactttt aattc	*CBEI_0223*
3	BG2413	GCGCGTCatg aaaacaaaag tattgttaat taaagc	*CBEI_0685*
4	BG2527	GCGCGCTCGA Gattattaat atctttaaga cttac	*CBEI_0685*
5	BG2203	GCGCGTCatg aaagcagcat tatggtatgc	*CBEI_1464*
6	BG2204	GCGCGCTCGA Gagatttaga tacaagttct ttg	*CBEI_1464*
7	BG2411	GCGCGCATat gattacaaca attcaaaatg agaaag	*CBEI_2243*
8	BG2528	GCGCGCTCGA Gcacaactgt aagcacg	*CBEI_2243*
9	BG2415	GCGCGATTAa tggatgtgttt tttatgaaac taatg	*CBEI_3864*
10	BG2529	GCGCGCTCGA Gttccatagt taaaacagct c	*CBEI_3864*
11	BG2407	GCGCGATTAa tgcgtgcttt aaaatataat ggg	*CBEI_3890*
12	BG2408	GCGCGCTCGA Gttataacttttgcattcttttc	*CBEI_3890*

Production and purification of heterologous Cb-ACR from *E. coli*.

Escherichia coli BL21 (DE3) containing pWUR[1-6] was used to inoculate a 5 mL overnight LB medium culture supplemented with 1 mM $MgSO_4$, 250 μM $ZnSO_4$ and 50 μg/mL kanamycin. The preculture was used to inoculate 50 mL LB (Luria-Bertani) in conical flasks and incubated for 8 h (37 °C, 120 rpm).

A larger volume of cultures with *Escherichia coli* BL21 (DE3) containing pWUR[3] was produced by inoculating 2 litres of LB (Luria-Bertani) medium. After eight hours of growth, the cultures were placed on ice for 1 hour to induce host chaperones [146] and subsequently induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). *E. coli* cells were harvested after 15 hours of growth at 20 °C and resuspended in 20 mM Tris-HCl pH 7.5 containing 1 mM tris(2-carboxyethyl)phosphine (TCEP) (reducing agent) [173]. After three

sonication cycles (20 W for 20 seconds; with intervals of 30 seconds on ice), the total cell extract was centrifuged for 30 minutes (16 000 × g, 4 °C) resulting in clear cell free extract (CFE). The unpurified *E. coli* cell free extracts of pWUR[1-6] were used for screening purposes only.

For characterization purposes, overproduced Cb-ACR was purified from *E. coli* cell free extracts harboring pWUR[3]. After passing twice through a French Press at 110 MPa, the total cell extract was centrifuged for 20 minutes (16 500 × g, 4 °C) resulting, after passing through a 0.45 µm filter (Sartorius Stedim Biotech), in clear cell free extract (CFE). It was applied to a 20 mL Matrix Red A affinity column (Amicon) equilibrated in 20 mM Tris-HCl buffer (pH 7.5).

After removal of unbound protein by washing with two column volumes of buffer, the recombinant protein was eluted by a linear gradient of 0-2 M NaCl in the same buffer. Subsequently, the fractions containing Cb-ACR activity were pooled and applied with a flow rate of 2 mL min^{-1} to a Ni-chelating column (20 mL) equilibrated in 20 mM Tris-HCl buffer (pH 7.5) containing 300 mM NaCl. Proteins were eluted with a linear gradient of 20–500 mM imidazole and fractions (10 mL) were collected. The most active fractions were pooled and applied at a flow rate of 10 mL min^{-1} to a HiPrep desalting column (53 mL) (Amersham Biosciences, Piscataway, NJ, USA), equilibrated in 20 mM Tris-HCl buffer (pH 7.5). Fractions of 10 mL were collected. After re-addition of 1 mM TCEP, the fractions were frozen at -20 °C and kept under anaerobic conditions, until further use.

Screening for acetoin reductase activity

An initial screening for D/L-acetoin reduction was performed in crude cell free extracts of *E. coli* BL21 (DE3) harboring pWUR[1-6]. Each reduction

reaction was performed in a degassed reaction mixture containing 100 mM sodium phosphate buffer (pH 6.5), 50 mM D/L-acetoin, and 0.28 mM NAD(P)H. The following controls were included: (1) Crude CFE of *E. coli* BL21 (DE3) harbouring an empty pET24d vector (control); (2) blank with no substrate. A correction was made for the temperature dependent spontaneous degradation of NAD(P)H. Reactions in this screening were initiated by adding D/L-acetoin.

Acetoin reductase activity assays

Unless stated otherwise, all alcohol oxidations and aldehyde/ketone reductions were determined at 37 °C under standard reactions conditions as described below, by following either the reduction of $NAD(P)^+$ or the oxidation of NAD(P)H at 340 nm, using a Hitachi U2010 spectrophotometer equipped with a temperature-controlled cuvette holder.

Each oxidation reaction was performed in a degassed reaction mixture containing 100 mM glycine-NaOH (pH 9.5), 50 mM alcohol, and 0.28 mM $NAD(P)^+$. Each reduction reaction was performed in a degassed reaction mixture containing 100 mM sodium phosphate buffer (pH 6.5), 50 mM aldehyde or ketone, and 0.28 mM NAD(P)H.

In all assays, the reaction was initiated by the addition of an appropriate amount of purified enzyme. One unit of Cb-ACR was defined as the amount of enzyme that oxidized or reduced one micromole of NAD(P)H or $NAD(P)^+$ per minute, respectively. A correction was made for the (temperature-dependent) spontaneous degradation of NAD(P)H. The protein concentration was determined using Bradford reagents (Bio-Rad) with bovine serum albumin as a standard [174].

Size exclusion chromatography

The molecular mass of the native enzyme was determined by size exclusion chromatography on a Superdex 200 high-resolution 10/30 column (24 ml; Amersham Biosciences) equilibrated in 50 mM Tris-HCl containing 100 mM NaCl (pH 7.8). Two hundred fifty microliters of enzyme solution in 50 mM Tris-HCl buffer (pH 7.8) was applied to the column. Proteins used for calibration were Blue dextran 2000 (>2 000 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and RNase A (13.7 kDa).

Optimal pH and temperature

The optimal pH of the reduction and oxidation reaction was determined using a 100 mM NaP_i (pH 5.5-7.5) and a 100 mM glycine-NaOH (pH 8.0-10.0) buffer system, respectively, at 37°C for both reactions.

The optimal temperature of the oxidation and reduction reaction was determined (20 °C – 85 °C) using the optimal pH buffer, either 100 mM NaP_i (pH 6.5) or 100 mM glycine-NaOH (pH 9.5). The pH of the buffers was set at 25°C, and temperature corrections were made using their temperature coefficients (±0.0028 pH/°C for sodium phosphate buffer and ±0.025 pH/°C for glycine buffer).

Substrate, co-factor specificity and kinetics

The Cb-ACR kinetic parameters K_m and V_{max} were calculated from multiple measurements with D/L-acetoin (0.0244, 0.0488, 0.098 0.19, 0.391, 0.781, 1.563, 3.125, 12.5, and 50.0 mM) and the co-factors NADH and NADPH (0.014, 0.028, 0.035, 0.042, 0.056, 0.070, 0.14, and 0.28 mM) by performing a computer-aided direct fit to the Michaelis-Menten equation (Tablecurve 2D, v5

(Systat Software Inc., San Jose, CA, USA). All the reactions followed Michaelis-Menten-type kinetics.

Metal salts

The effect of metal ions on acetoin reductase activity was determined using different metal salts ($CaCl_2$, $CoCl_2$, $FeCl_3$, $FeSO_4$, $NiCl_2$, $MgCl_2$, $MnCl_2$ and $ZnCl_2$) at final concentrations of 1 mM using the standard activity assay. The enzyme was first pre-incubated in 1 mM EDTA at 37 °C for 60 minutes, after which the EDTA was removed. Subsequently, the residual activity of the acetoin reductase was measured. The activity of Cb-ACR without pre-treatment with EDTA or addition of metal ions was defined as 100%.

The metal content (assayed for Ca^{2+}, Cd^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}) of the purified enzyme was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) using 20 mM Tris-HCl buffer (pH 7.5) as a blank.

Results

Data mining

A number of acetoin reductase / 2,3-butanediol dehydrogenases have been characterized with respect to their substrates, and cofactors. These characterized proteins from the eukaryotic micro-organism *S. cerevisiae*, the Gram-negative bacteria *Klebsiella pneumoniae* and the Gram-positive bacteria *Bacillus cereus* YUF-4, *B. polymyxa*, *B. subtilis, B. stearothermophilus* and *L. lactis* were used to find potential acetoin reductases within *C. acetobutylicum* and *C. beijerinckii*.

A phylogenetic tree was made with characterized acetoin reductases / butanediol dehydrogenases [68, 169, 175-183] and the predicted acetoin reductases from the two clostridial origins (Figure 15). The (acetyl)acetoin reductase from the closely related *Bacillus cereus* YUF-4 has been demonstrated to reduce both D- and L-acetoin isomers and to oxidize the stereoisomers D- and *meso*-2,3-butanediol [169, 180, 183].

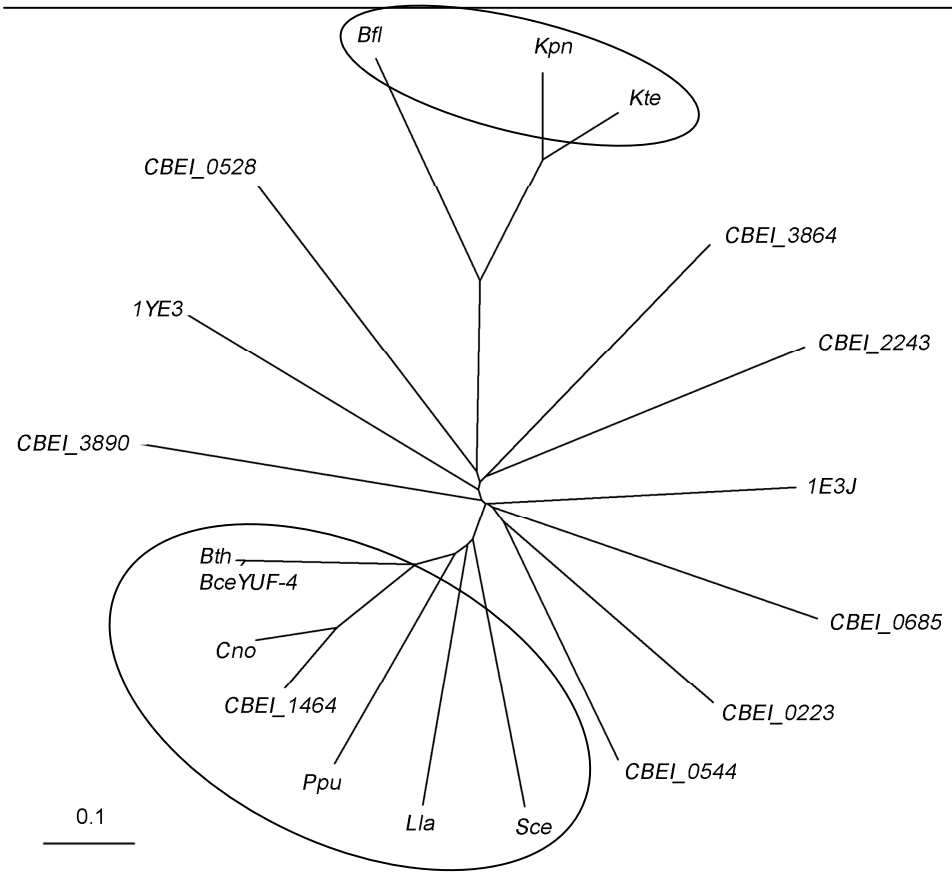


Figure 15: Phylogenetic tree of potential and described acetoin reductases / butanediol dehydrogenases in literature. Published acetoin reductases / 2,3-butanediol dehydrogenases (except for CBEI_1464 and *Cno*) are shown within circles. The following symbols are used: ***Bfl***: L-2.3-butanediol dehydrogenase from *Corynebacterium glutamicum (Brevibacterium flavum)*, ***Kpn***: D-acetoin (diacetyl) reductase from *Klebsiella pneumoniae*, ***Kte***: D-Acetoin (diacetyl) reductase from *Klebsiella terrigena*, ***Sce***: D-butanediol dehydrogenase from *Saccharomyces cerevisiae*, ***Lla***: 2,3-butanediol dehydrogenase from *Lactococcus lactis*, ***Ppu***: 2,3-butanediol dehydrogenase from *Pseudomonas putida* (strain KT2440), ***Cno***: 2,3-butanediol dehydrogenase from *Clostridium novyi* NT, ***Bth***: D-butanediol dehydrogenase from *Bacillus thuringiensis* ATCC 35646, ***BceYUF-4*** (acetyl)acetoin reductase from *Bacillus cereus* YUF-4, ***1E3J***: sorbitol dehydrogenase from white fly, *Bemisia argentifolii*, , ***1YE3***: horse liver alcohol dehydrogenase. ***CBEI_3864***, ***CBEI_0528***, ***CBEI_0685***, ***CBEI_0223***, ***CBEI_0544***, ***CBEI_1464, CBEI_2243 and CBEI_3890*** are eight potential acetoin reductases from *C. beijerinckii NCIMB 8052*. Bar indicates 10% of estimated phylogenetic divergence.

Based on homology with the acetylacetoin reductase / 2,3-butanediol dehydrogenase gene from *Bacillus cereus* YUF-4 [169], which is described to possess acetoin reductase activity, eight putative acetoin reductase-encoding genes were found within the genome of *C. beijerinckii* NCIMB 8052. The predicted acetoin reductases encoded by *CBEI_0223, CBEI_0528*, *CBEI_0544, CBEI_0685*, *CBEI_1464*, *CBEI_2243*, *CBEI_3864*, and *CBEI_3890* were all annotated in NCBI (http://www.ncbi.nlm.nih.gov/) as alcohol dehydrogenase. Only the clostridial reductases, *CBEI_1464* and an uncharacterized reductase from *C. novyi* NT cluster together with the *Bacillus cereus* YUF-4 reductase, suggesting a potential similar acetoin reductase activity. This suggest that from the selected clostridial *CBEI_1464* is a likely candidate to possess acetoin reductase activity.

In addition to the phylogenetic tree, an alignment was made with only the characterized acetylacetoin reductase from *B. cereus* YUF-4 and the selected clostridial acetoin reductases (Figure 16). The conserved residues, are most likely involved in binding the catalytic zinc atom (Cys37, His70, Glu71; shown with circumflexes in the alignment) and the structural zinc atom (Cys100, Cys103, Cys106, and Cys114; shown with asterisks in the alignment)

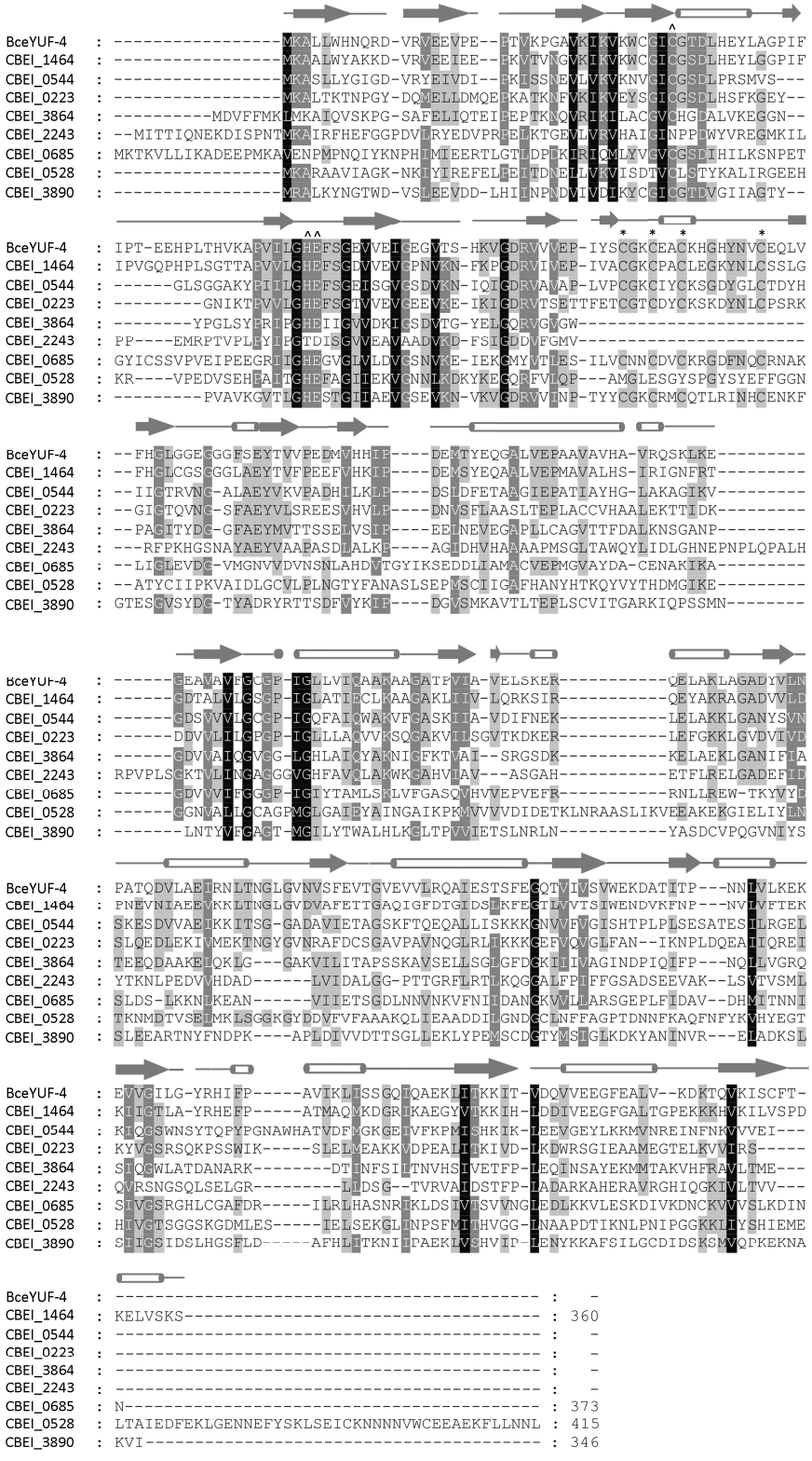


Figure 16: Amino acid sequence alignment of the characterized acetylacetoin reductase/2,3-butanediol dehydrogenase from *Bacillus cereus* YUF-4 with potential acetoin reductases within *C. beijerinckii* NCIMB 8052. The secondary structure based on Cb-ACR, obtained using the psipred program, is shown above the alignment. The sequences were aligned using the CLUSTALX program. Symbols indicate residues involved in binding of the catalytic zinc (circumflex) and structural zinc (asterisk).

Screening for acetoin reductase activity

After successful expression in *E. coli* of six potential acetoin reductases, an initial screening for this activity in crude cell free extract showed that CBEI_1464 (Cb-ACR) exhibited significant acetoin reductase activity with racemic acetoin as substrate (Figure 17). Transformation of *E. coli* with the ligation mixes of other two did not result in any transformants. Still, minor acetoin reductase activity was also found with CBEI_0685, CBEI_2243, and CBEI_3864. Remarkably, an increase in NADH or NADPH was observed, indicating oxidation of acetoin to diacetyl in case of CBEI_3890.

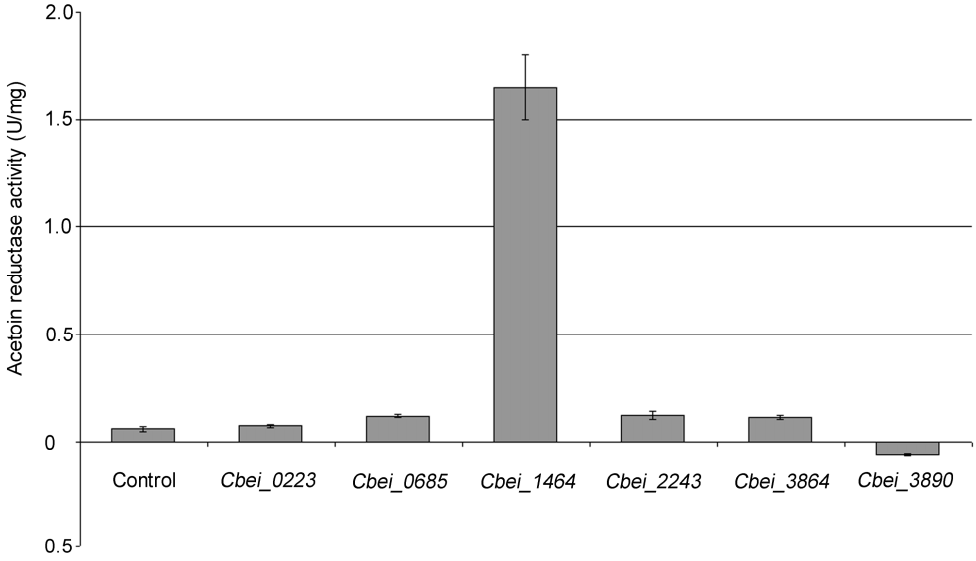


Figure 17: Acetoin reductase activity in crude cell free extracts of *E. coli* BL21 (DE3) expressing six potential acetoin reductases.

In silico identification and examination of an acetoin reductase encoded by *CBEI_1464* from *C. beijerinckii*

The *C. beijerinckii CBEI_1464* gene encodes a protein of 360 amino acids and has calculated molecular mass of approximately 39 kDa with a theoretical pI of 6.04 (http://www.expasy.ch/tools/protparam.html). It is annotated in the NCBI database (http://www.ncbi.nlm.nih.gov/) as an alcohol dehydrogenase. The encoded proteins belongs to COG1063 (cluster of orthologous groups of proteins), which represents (L-threonine dehydrogenase (TDH) and related Zn-dependent dehydrogenases) (http://www.ncbi.nlm.nih.gov/COG/). The three most significant hits with BLAST-P analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) concern a (putative) sorbitol dehydrogenase from *Clostridium butyricum* 5521 (95% identity, *CBY_2472*), an oxidoreductase that belongs to the zinc-binding dehydrogenase family from *Clostridium botulinum* E1 strain 'BoNT E Beluga' (92% identity, *CLO_3146*), and a (putative) (2*R*,3*R*)-butanediol dehydrogenase from *Clostridium botulinum* (92% identity, *CLL_A0664*).

Identification of the protein family and protein domains was performed using an Interpro scan from EMBL-EBI (http://www.ebi.ac.uk/interpro/). This scan revealed that the protein is a member of the zinc-containing alcohol dehydrogenase superfamily (IPR002085). It is composed of a GroES-like alcohol dehydrogenase domain (IPR011032, IPR013154) (AA 1-178), and a GroES-related zinc and NAD(P)-binding alcohol dehydrogenase domain (IPR011597, IPR013149 and IPR016040) (AA 147-334).

Conserved context analysis with STRING (http://string.embl.de/) of Cb-ACR revealed a functional interaction link with a predicted xylulokinase encoded by *CBEI_2384*. Manual inspection of the neightborhood of *CBEI_1464* (Figure 18) also revealed a sigma54 specific transcriptional regulator from the

Fis family (*CBEI_1463*). A Blast-P search indicated that *CBEI_1463* is highly similar to the aco operon expression regulatory protein [*Clostridium butyricum* 5521]. The aco operon is known to be involved in the catabolic pathway of acetoin as a carbon source. Similar genes like the genes encoded by CBEI_1463 and Cb-ACR are conserved within several species in the firmicutes phylum.

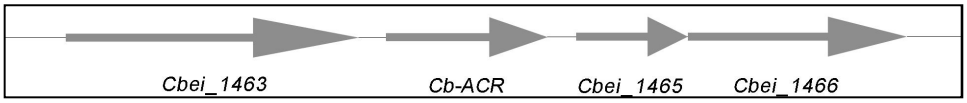


Figure 18: Neighborhood of *Cb-ACR*; *CBEI_1463* encoded an putative GAF modulated sigma54 specific transcriptional regulator from the Fis family, *Cb-ACR* encodes an putative alcohol dehydrogenase, *CBEI_1467* encodes an ferredoxin-NADP(+) reductase subunit alpha, *CBEI_1466* encodes an putative oxidoreductase.

Purification of recombinant Cb-ACR

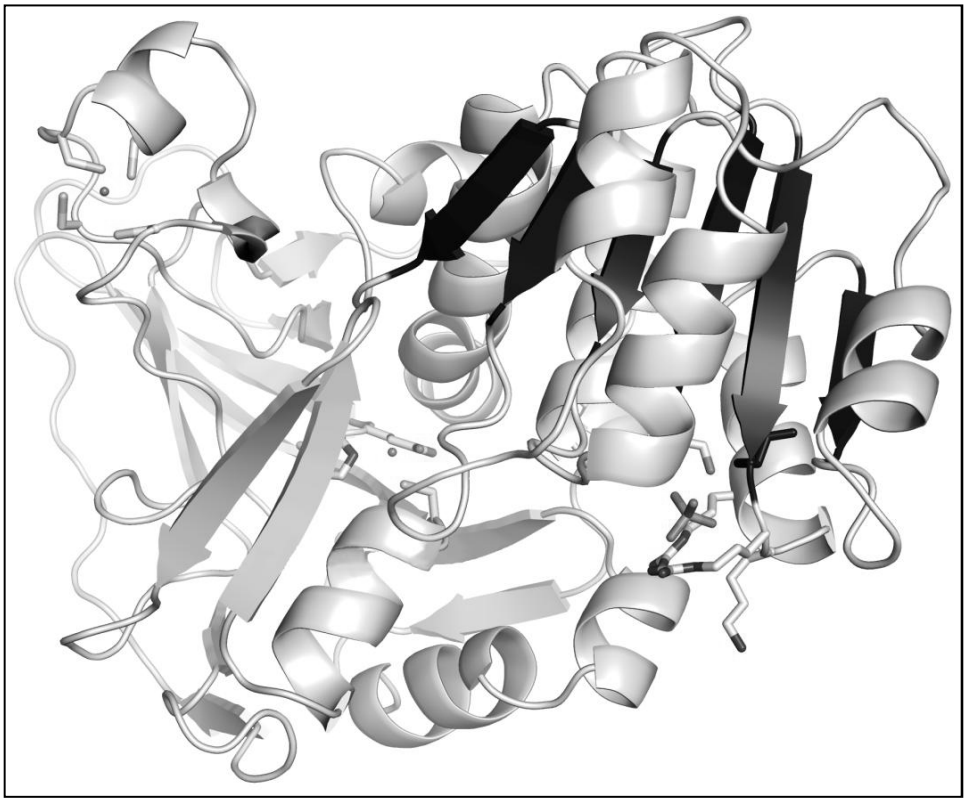
Cb-ACR was successfully purified to homogeneity from cell free extract of *Escherichia coli* BL21(DE3) containing pWUR450[3] by using three subsequent chromatographic columns, namely a matrix Red A affinity column (Amicon), a Ni-chelating affinity column and a HiPrep desalting size exclusion column. The migration of Cb-ACR with his-tag on SDS PAGE, indicated a molecular subunit of approximately 45 kDa (not shown). Size-exclusion chromatography suggested that the multimeric structure of Cb-ACR is predominantly a dimeric structure state.

Structural modeling

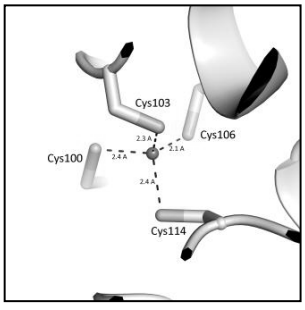
A model of Cb-ACR was made using the 3D-structural program PHYRE [184]. An algorithm is able to find similar template proteins in a database that structurally fits well to a query sequence. Several fits with structural

similarities were found. The sorbitol dehydrogenase BaKR from *Bemisia argentifolii* (silverleaf whitefly) (PDB code 1E3J) (http://www.pdb.org/pdb/home/home.do) was used to build the model of Cb-ACR (Figure 19). BaKR consist of 352 amino acids.

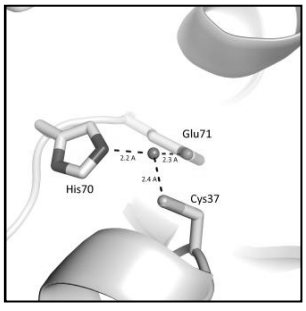
The quality of the model towards stereochemistry and geometry was analyzed using PROCHECK [185]. The Ramachandran plot (not shown) indicated that most of the residues are not present in the core and allowed regions. Bond lengths, bond angles and torsion angles were also considered not good. The three dimensional structure deposit 1E3J in the pdb databank (http://www.pdb.org/pdb/home/home.do) showed quality checks similar to the ones of Cb-ACR with PROCHECK [185].



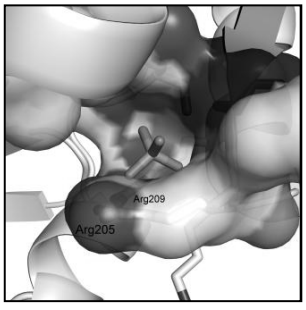
(A).



(B).



(C).



(D).

Figure 19: Three dimensional model of Cb-ACR. (A) The overall cartoon structure in light grey with the six β-sheets of the predicted Rossman fold in dark grey. The three colored regions are (B) the structural zinc with coordinating amino acids, (C) the catalytic zinc with coordinating amino acids, and (D) the cavity in which phosphate is bound.

In contrast, the root mean square deviation (RMSD) of the structural backbone alignment, a frequently-used measure of the differences between values predicted by a model and the values actually observed, with 1E3J was 0.0. Therefore, although there are major concerns about the details of the 3D structure, we considered the obtained model useful to gain insight into the three dimensional structure of Cb-ACR (Figure 19), certainly in combination with the available sequence alignment (Figure 16). Meanwhile, crystallization studies are ongoing.

According to the model, the catalytic zinc is coordinated by (at least) Cys37, His70, Glu71 in Cb-ACR as it is in the sorbitol reductase (1E3J) to Cys41, His66, Glu67 and a water molecule; in addition, the structural zinc is most likely coordinated to Cys100, Cys103, Cys106, and Cys114 as it is in the sorbitol reductase to Cys96, Cys99, Cys102, and Cys110. A phosphate ion in the structural cavity within 4 Å of Gly180, Ser181, Gly182 and Gln204, Arg205, Lys206 and Arg209 marks the likely position for the adenyl phosphate of NADP(H) as it is in the sorbitol reductase within 4Å of Gly176, Ala177, Gly178 and Ala199, Arg200, Ser201 and Arg204 (Figure 19) [186].

Substrate, co-factor specificity and kinetics

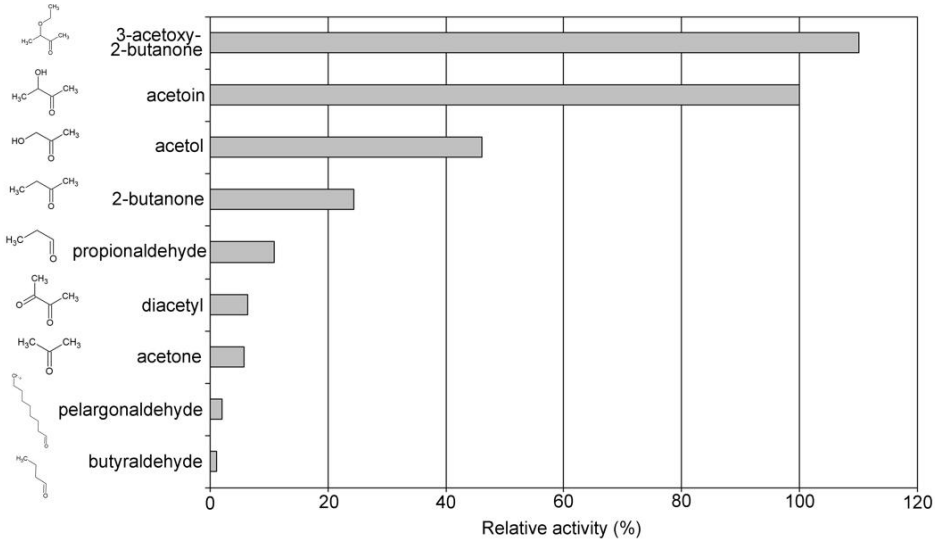


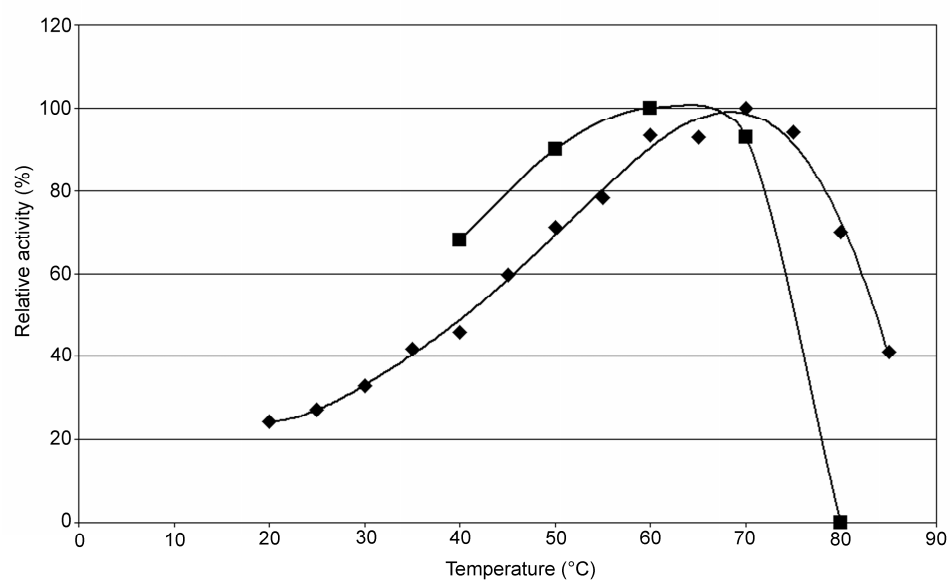
Figure 20: Substrate specificity of Cb-ACR. D/L-acetoin was set hundred percent.

The specific activity of Cb-ACR was found to be highest with 3-acetoxy-2-butanone and D/L-acetoin. Also the conversion of acetol and 2-butanone were shown to proceed with over twenty percent of the activity found with D/L-acetoin as substrate. Low activities were observed with acetone, diacetyl, and C3, C4, C9- aldehydes (Figure 20).

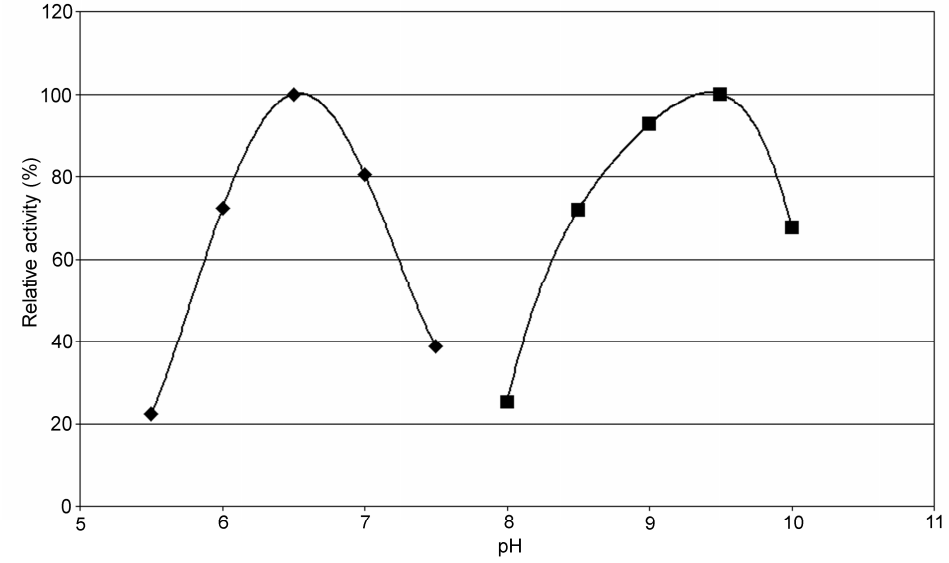
The kinetic parameters of Cb-ACR were determined for the substrate D/L-acetoin with NADH or NADPH, as cofactor. Cb-ACR has a ten-fold times higher catalytic efficiency for D/L-acetoin (K_m 0.139 mM, V_{max} 75.9 U/mg, k_{cat}/K_m 0.337 s^{-1} * mM^{-1}) and NADPH (K_m 31.9 µM, V_{max} 75.9 U/mg) than the affinity for D/L-acetoin (K_m 0.280 mM, V_{max} 15.4 U/mg, k_{cat}/K_m 0.034 s^{-1} * mM^{-1}) and NADH (K_m 314 µM, V_{max} 15.4 U/mg). In addition, the specific activity of Cb-ACR with NADPH was almost five times higher than with NADH.

Optimal pH and temperature

A)



B)



C)

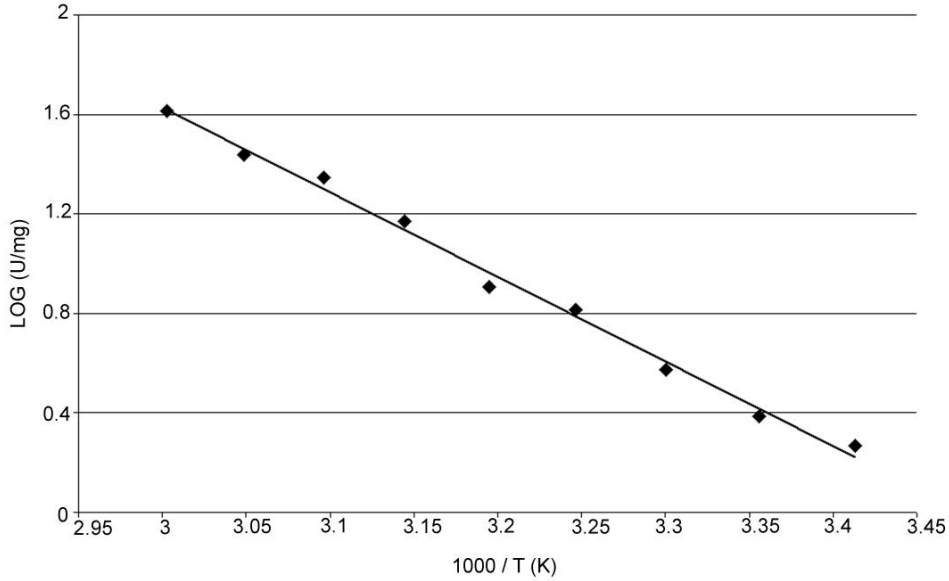


Figure 21: Effect of (A) temperature and pH (B) on acetoin reductase activity. The effect of pH (pH 5.5 -7.5 for the reduction reaction and 8.0-10.0 for the oxidation reaction) and temperature (20 °C – 85 °C for the reduction reaction and 40 °C – 80 °C for the oxidation reaction) were studied using racemic acetoin and NADH as substrate and cofactor, respectively. Graph C shows the temperature dependence from 20°C - 60°C as an Arrhenius plot.

The effect of temperature on Cb-ACR activity was studied using racemic acetoin and NADH as cofactor. The reductase activity increased from 20 °C upwards until 80 °C (Figure 21A), with an optimal temperature at approximately 70 °C for the reduction of acetoin, and an optimal temperature at approximately 65 °C for the oxidation of 2,3-butanediol. An Arrhenius analysis resulted in a linear plot in the temperature range of 20 °C to 60 °C (Figure 21C), with a calculated activation energy for the formation of the enzyme/substrate complex of 96 J/mol. To determine the optimal pH for both, the reduction of acetoin and oxidation of 2,3-butanediol, the activity of Cb-ACR was measured in a pH range of 5.5 – 10.0 (Figure 21B). Cb-ACR displayed >70% of its maximal activity in the pH range of 6 to 7, with an optimal pH at approximately 6.5 for the reduction of acetoin and >90% of its maximal activity

in the pH range of 9 to 9.5, with an optimal pH at approximately 9.5 for the oxidation of 2,3-butanediol.

Effect of metal ions and oxygen

ICP-AES showed the presence of approximately two zinc atoms and one calcium per monomer of Cb-ACR. Remarkably, the residual activity in the control after pre-incubation in EDTA remained 100%. The affinity of zinc within Cb-ACR seems to be higher than the affinity with EDTA. Addition of most salts and metals caused no significant inhibition or activation of Cb-ACR. Only Fe^{2+}, Fe^{3+} and Ca^{2+} resulted in lower residual activity. Furthermore, Cb-ACR was found to be highly oxygen sensitive, with a half-life time of approximately 6 hours (aerobic at 0 °C). This suggests that thiol groups are involved in the active site or in structural events. This is in agreement with the above described model. However, inhibition is observed when dithiothreitol (DTT) or β-mercaptoethanol is added to the reaction, therefore tris(2-carboxyethyl)phosphine (TCEP) is used as reducing agent [173].

Discussion

2,3-butanediol is a final product of fermentation of several bacteria. More specifically, in extracts of *Acetobacter suboxydans*, *Aeromonas hydrophila*, *Bacillus polymyxa*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Erwinia carotovora*, *Serratia marcescens*, and *Staphylococcus aureus*, specific activities of 2,3-butanediol dehydrogenases have been found, albeit with differences in their substrates specificities (D-2,3-butanediol, L-2,3-butanediol and *meso*-2,3-butanediol) [62, 179].

A number of acetoin reductase / 2,3-butanediol dehydrogenases are characterized with respect to their substrates, and cofactors. The amino acid sequences of characterized proteins from the eukaryotic micro-organism *S. cerevisiae*, the Gram-negative bacterium *Klebsiella pneumoniae* and the Gram-positive bacteria *B. cereus* YUF-4, *B. polymyxa*, *B. subtilis, B. stearothemophilus* and *L. lactis* [68, 169, 175-183], were used to find potential acetoin reductases within *C. acetobutylicum* and *C. beijerinckii.*

A sequence alignment showed that the acetoin reductases from the Gram-negative species (e.g. *Klebsiella*) are only five percent identical to the Gram-positive *Bacillus* species, and that the former are approximately hundred amino acids shorter in length. It might reflect different physiological roles (anabolic or catabolic) within these micro-organisms [68, 187, 188].

A comparative genomic analysis of the characterized acetoin reductases of these bacteria revealed the presence of eight potential acetoin reductases within *C. beijerinckii* genome. Clearly, one potential reductase (CBEI_1464) is clustering best with the acetoin accepting acetylacetoin reductase from *Bacillus cereus YUF-4 (identity 53%).* This gene (*CBEI_1464*) was originally annotated as an alcohol dehydrogenase. A more detailed sequence analysis revealed the presence of a GroES-like alcohol dehydrogenase domain together with a GroES-related zinc and NADP binding domain (Rossman fold)

[189]. After functional expression in *E. coli*, zinc dependent acetoin reductase (Cb-ACR) activity could indeed be demonstrated. However, also CBEI_0685, CBEI_2243 and CBEI_3864 showed small acetoin reductase activity. In contrast, CBEI_3890 seemed to oxidize acetoin to diacetyl, since NADH increased in time. Further research is needed to investigate this observation.

Next to acetoin (100%), also relatively high activities were found with 3-acetoxy-butanone (110%), acetol (46%), and 2-butanone (24%). Minor activities were found with propionaldehyde (11%), diacetyl (6%), acetone (6%), pelargonaldehyde (2%) and butyraldehyde (1%). Since a 10% higher activity was found with 3-acetoxy-butanone in stead of acetoin, it seems that the acetoxy group at the C3 position stabilizes the substrate better than the hydroxyl group (3-acetoxy-2-butanone vs acetoin). Furthermore, exchange of the additional group (hydroxyl- or acetoxy-group) at the C3 for a hydrogen, decreased the activity with at least 80% (3-acetoxy-2-butanone and acetoin vs 2-butanone). However, replacing a C4 methyl group of acetoin for a hydrogen, resulted in a 54% decrease of the activity (acetoin vs acetol). Removal of the C1 methyl group of acetoin resulted in a 66% decrease of the activity (2-butanone vs propionaldehyde). Altogether, for optimal activity, a large polar group should be positioned at the C3 of the C4 skeleton. The C1 and C4 methyl group of acetoin also seems to be necessary for stabilization of the substrate in the active site. Similarly, it has been described that the reductase from YUF-4 also shows a 23 percent higher activity with acetylacetoin (e.g. an acetyl group positioned at the C3) than with acetoin [180].

Cb-ACR has a ten times higher affinity for racemic acetoin (K_m 0.139 mM, V_{max} 75.9 U/mg, k_{cat}/K_m 0.337 s^{-1} * mM^{-1}) and NADPH (K_m 31.9 μM, V_{max} 75.9 U/mg) than for racemic acetoin (K_m 0.280 mM, V_{max} 15.4 U/mg, k_{cat}/K_m 0.034 s^{-1} * mM^{-1}) and NADH (K_m 314 μM, V_{max} 15.4 U/mg). More specifically, it converts D-acetoin in D-2,3-butanediol and L-acetoin in *meso*-2,3-butanediol

(data not shown). In addition, the specific activity of Cb-ACR with NADPH was almost five times higher than with NADH. In *B. cereus* YUF-4 two acetylacetoin reductases (namely acetylacetoin reductases I and II) were described. From acetylacetoin reductase I the sequence is known and used in all previous *in silico* analysis. For comparison, this acetylacetoin reductase I from *B. cereus* YUF-4 is also NADPH dependent, although kinetic parameters are not given. It reduces acetylacetoin optimally at pH 6.3, but the activity (acetoin as substrate) is only 35% compared to the activity on acetoin by acetylacetoin reductase II. In contrast, this acetylacetoin reductase II from *B. cereus* YUF-4 is only NADH dependent and accepts also both isomers of acetoin (K_m of 0.72 mM for D/L-acetoin), however a V_{max} value is not reported. It reduces acetoin optimally at pH 5.8. The K_m for D-2,3-butanediol and *meso*-2,3-butanediol are 6.90 and 10.35, respectively [169, 180].

The diacetyl (acetoin) reductase from *B. stearothermophilus* is also strictly NADH dependent. It reduces optimally at pH 6.5, with a similar V_{max} of 81.4 U/mg, but with a higher K_m of 2.2 mM. The K_m for L-2,3-butanediol is 1.2 mM [176]. Likewise, the L-2,3-butanediol dehydrogenase from *B. saccharolyticum* is also strictly NADH dependent and it accepts only L-acetoin (K_m of 0.44 mM). It reduces optimally at pH 6.0. The K_m for L-2,3-butanediol is 0.22 mM [190]. The D-2,3-butanediol dehydrogenase from the eukarotic *S. cerevisiae* again is NADH dependent and accepts also both isomers of acetoin (K_m of 4.5 mM for D/L-acetoin). It reduces acetoin optimally at pH 5.8. The K_m for D-2,3-butanediol and *meso*-2,3-butanediol is 14 mM and 65 mM, respectively [177, 178]. The *meso*-2,3-butanediol dehydrogenase of the Gram-negative bacterium *K. pneumoniae* IAM1063 is also only NADH dependent and it accepts only D-acetoin (K_m of 0.72 mM). It reduces optimally between pH 5-6. The K_m for *meso*-2,3-butanediol is 5.20 mM [188]. To summarize, the acetoin reductase Cb-ACR is NADPH linked, while also relatively high activity is obtained with NADH. The

other characterized acetoin reductase appear to accept only a single type of co-factor. The usage of NADPH might suggest that Cb-ACR might fulfill an anabolic rather than a catabolic role.

Cb-ACR showed a temperature optimum for the reduction and oxidation reaction around 68 °C. This is remarkable, since the origin host, *C. beijerinckii NCIMB 8052* is a mesophilic micro-organism. However, it has been observed more often that ADHs from *C. beijerinckii* were stable or became with a few directed amino acid mutation stable at moderate temperatures [191-193]. Also, closely related thermophilic *Clostridium* sp. are known to possess proteins with activity optima at higher temperatures [194]. No stability data is available for Cb-ACR, but only 14% of residual acetoin reductase was measured for the reductase from YUF-4 after an incubation for 30 minutes at 70 °C [169]. The Arrhenius plot of Cb-ACR was linear at temperatures from 20 - 60 °C, indicating that the conformation of Cb-ACR does not change throughout this temperature range. Also the pH optimum of 6.5 for the reduction reaction was slightly different for the reductase from YUF-4 (pH 5.8). The oxidation of 2,3-butanediol was confirmed, however no further data about the optima of this reaction was shown [169].

ICP-AES showed the presence of approximately two zinc atom and one calcium atom per monomer of CBEI_1464. Two zinc atom can easily be explained, by sequence and structural homology comparisons, as the structural zinc and catalytic zinc. However, further research is needed to explain the presence of the calcium atom. Addition of extra calcium did not result in differences in specific activities. Inhibition was observed when β-mercaptoethanol or DTT was added to the reaction. β-mercaptoethanol and DTT, a C2 and C4 compound, seem to mimic the substrate acetoin in the active site, resulting in inhibition.

An interesting result of the neighborhood analysis is the apparent conserved genomic connection of Cb-ACR with an xylulokinase encoded by *CBEI_2384*. However, this kind of enzymes participate in pentose interconversions, in which also dehydrogenases are present, which are highly similar in structure. As an example, a blast search with an xylitol dehydrogenase encoded by *YLR070c* from *Saccharomyces cerevisiae,* proven to be functional, shows these similar dehydrogenase domains, like the structural and catalytic zinc domains, NADPH binding domain and the TDH domain [195]. This *YLR070c* is also clustered with a xylulokinase in *Saccharomyces cerevisiae.* More interesting is the link with the transcriptional regulatory protein of the *aco* operon. This protein is described intensively for *Bacillus subtilis*, involved in catabolism of acetoin. *B. subtilis* secretes acetoin in the medium. After depletion of an external C-source, it is able to use acetoin as carbon source again by expressing its *aco* operon [187]. The expression of this *aco* operon is regulated by *acoR*, a higly similar protein with *CBEI_1463*.

With respect to the physiological role of Cb-ACR, the presence of *CBEI_1463* suggest that Cb-ACR of *C. beijerinckii* functioned as the last step for temporary storage of carbon as 2,3-butanediol, like *Bacillus subtilis* does with acetoin. In addition, *C. beijerinckii* itself is not able to produce acetoin, but *C. acetobutylicum* does have the capacity to produce acetoin [1]. In *C. magnum,* an acetoin dehydrogenase enzyme system, resembling the one encoded by the *aco* operon of *Bacillus subtilis*, is described, which has also a similar AcoR-like protein. These observations might explain the spread of the genes within these clostridia and predict the former physiological role of Cb-ACR.

6

Heterologous expression of an acetoin reductase leads to D-2,3-butanediol production in *C. acetobutylicum ATCC 824*

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Submitted

Chapter 6: Heterologous expression of an acetoin reductase leads to D-2,3-butanediol production in *C. acetobutylicum*

Summary

Acetoin reductase (ACR) catalyzes the conversion of acetoin to 2,3-butanediol. Under certain conditions *Clostridium acetobutylicum* ATCC 824 (and derived strains) generates both D- and L-stereoisomers of acetoin, but due to the lack of an ACR enzyme, does not produce 2,3-butanediol. A gene encoding ACR from *Clostridium beijerinckii* NCIMB 8052 has been functionally expressed in *C. acetobutylicum* under control of two strong promoters, i.e. the constitutive thl promoter and the late exponential adc promoter. Both ACR-overproducing strains have been grown in batch cultures, during which 89-90% of the natively produced acetoin has been converted to 20-22 mM D-2,3-butanediol. Addition of a racemic mixture of acetoin did lead to the production of both, D-2,3-butanediol and *meso*-2,3-butanediol. A metabolic network is proposed that is in agreement with the experimental data. Native 2,3-butanediol production is a first step towards a potential homo-fermentative 2-butanol producing strain of *C. acetobutylicum*.

Introduction

The world energy consumption has been estimated to increase with more than 50% between 2001 and 2025 [196]. Hence, the development of sustainable and carbon-neutral energy sources is required to meet future needs. As a result, the European Union intends to replace 10% (energy value) of normal automotive fuels with biofuels by 2020. Liquid biofuels are attractive candidates, since little or no change is needed to the current petroleum-based fuel technologies [39]. For this purpose, biological production of several alcohols is under investigation, including ethanol and butanol [197, 198].

Several clostridial species are able to ferment carbohydrates to acetone, 1-butanol and ethanol (ABE), and are therefore referred to as solventogenic. The industrial application of this process, also known as the ABE-fermentation, has a long history, dating back to the beginning of the twentieth century. However, starting around 1960, the fermentation was no longer economically competitive with the petrochemical process [8]. Recently, the ABE-fermentation has once more received interest for the renewable production of 1-butanol from simple and complex carbohydrates. Applications of 1-butanol include its use as a biofuel or fuel extender, or as industrial bulk chemical [10]. However, the inefficiency of the fermentation still hampers the commercial reintroduction of the process. Improving the yields and productivities of the solvent products are key to the successful reintroduction of the ABE process.

Reflecting the renewed interest in the ABE fermentation, the genomes of the two best known solventogenic strains have been sequenced in recent years. The first genome to be sequenced was that of *Clostridium acetobutylicum* ATCC 824 [199] and more recently, the genome sequence of *C. beijerinckii NCIMB 8052* has also become available (http://genome.ornl.gov/microbial/cbei/). Both species are able to ferment

several C5 and C6 sugars, as well as disaccharides and more complex carbohydrates such as raffinose, dextrin, and starch, into acetate, butyrate, lactate, acetone, 1-butanol and ethanol [200]. Besides these fermentation products, some strains can produce additional products. Certain *C. beijerinckii* strains are known to reduce acetone further to *iso*-propanol, and *C. acetobutylicum ATCC 824* has been reported to produce small amounts of acetoin [8].

One of the factors reducing the fermentation efficiency is the toxic effect that 1-butanol has on the culture. Butanol has membrane distorting properties, due to its hydrophobic chain and polar group, which cause severe cell damage [201, 202]. Many efforts have been made in the past to obtain clostridial strains with increased 1-butanol tolerance, however with limited success [33, 118, 119, 203-205].

As an alternative to increasing 1-butanol tolerance, we propose to replace the production of 1-butanol by the production of a compound that has similar physical and chemical properties (heat of combustion, heat of vaporization, and energy density), but which is less toxic to the cell, making higher titers possible. 2-Butanol matches these criteria and has a lower log K_{ow} value (octanol:water coefficient) than 1-butanol. The log K_{ow} value is a good indicator for the strength of membrane perturbing effects [206]. Generally, the lower the log K_{ow} value, the less toxic the compound is to the membrane. However, *C. acetobutylicum* is not known to produce 2-butanol nor its potential precursor 2,3-butanediol (2,3-BD) [2]. Nevertheless, it is known to produce acetoin as a minor fermentation product [8].

The 2,3-butanediol biosynthesis route proceeds via pyruvate, acetolactate and acetoin to 2,3-butanediol. Acetolactate is formed in vivo by coupling two molecules of pyruvate with the concomitant release of carbon dioxide, catalyzed by acetolactate synthase. Subsequently, it is decarboxylated

by acetolactate decarboxylase to yield acetoin [207]. Reduction by an acetoin reductase results in 2,3-butanediol formation.

Our aim is to construct a 2,3-butanediol producing *C. acetobutylicum* strain as a first step towards biological 2-butanol production. In this study, the cloning and functional expression is described of an acetoin reductase (ACR)-encoding gene from *C. beijerinckii* NCIMB 8052 in *C. acetobutylicum*, resulting in the production of D-2,3-butanediol.

Materials and methods

Bacterial strains and plasmids

All bacterial strains and plasmids used during this study are listed in table 12. Stock cultures of *C. acetobutylicum* strains and *C. beijerinckii NCIMB 8052* were maintained as spore suspensions in sterile 15 % (v/v) glycerol at -20 or -80 °C. Commercially obtained, chemically competent, *E. coli* NEB 5-alpha F' Iq cells were used for cloning and vector maintenance. *E. coli* DH10B, containing methylation plasmid pAN1, was used to methylate plasmid DNA before transformation into *C. acetobutylicum*. The clostridial/E. *coli* shuttle vector pMTL500E was used for expression of *Cb-acr* in *C. acetobutylicum*.

Media and growth conditions

E. coli strains were cultured in lysogeny broth (LB) medium at 37 °C, 200 RPM and *E. coli* stocks were stored in sterile 20 % (v/v) glycerol at -80 °C. Clostridial spore suspensions were made by gently resuspending *C. acetobutylicum* spores from agar plates (14 days anaerobic growth at 37 °C) in sterile 15 % (v/v) glycerol. Agar plates for sporulation contained per liter of water: yeast extract, 5.0 g; KH_2PO_4, 1.0 g; K_2HPO_4, 0.76 g; ammonium acetate, 2.9 g; *p*-aminobenzoic acid, 0.10 g; $MgSO_4 \cdot 7\ H_2O$, 1.0 g; $FeSO_4 \cdot 7\ H_2O$, 0.005 g; glucose, 20.0 g; and agar, 15 g.

Prior to inoculation of clostridial pre-cultures, spore suspensions were heat shocked for 10 minutes at 70 or 80 °C. *C. acetobutylicum* strains were grown in MG medium or modified CGM (mCGM) medium as indicated.

MG medium was based on the semi-synthetic medium described by Nimcevic *et al*. [131], and contained per liter of water: yeast extract, 2.5 g; KH_2PO_4, 1.0 g; K_2HPO_4, 0.76 g; ammonium acetate, 3.0 g; p-aminobenzoic acid, 0.10 g; $MgSO_4 \cdot 7\ H_2O$, 1.0 g; and $FeSO_4 \cdot 7\ H_2O$, 0.01 g.

mCGM medium contained per liter of water: yeast extract, 5.0 g; KH_2PO_4, 0.75 g; K_2HPO_4, 0.75 g; $MgSO_4 \cdot 7\ H_2O$, 0.4 g; $MnSO_4 \cdot H_2O$, 0.01 g; $FeSO_4 \cdot 7\ H_2O$, 0.01 g; NaCl, 1.0 g; asparagine, 2.0 g; $(NH_4)_2SO_4$, 2.0 g; cysteine, 0.125 g; and glucose, 12.5 g.

Medium for fermentation was made anaerobic by sparging with nitrogen gas. Serum flasks (250 mL), containing 100 mL MG medium, were inoculated with 2 % (v/v) overnight pre-cultures. Clostridial experiments and manipulations were performed anaerobically in; (i) an anaerobic chamber (Sheldon Manufacturing, Oregon U.S.A.; gas mixture consisting of 15 % CO2, 4 % H2 and 81 % nitrogen); or (ii) glass serum vials with butyl rubber stoppers and aluminum crimp seals equipped with a pressure release system as described previously [132].

Culture media were supplemented with ampicillin (100 μg mL^{-1}), chloramphenicol (30 μg mL^{-1}), erythromycin (40 μg mL^{-1} for liquid cultures and plates; 25 μg mL^{-1} for transformant isolation on plates), kanamycin (50 μg mL^{-1}), iso-propyl-β-d-thiogalactopyranoside (IPTG; 50 μg mL^{-1}) or 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal; 40 μg mL^{-1}), when appropriate.

The growth of clostridial cultures was monitored spectrophotometrically at 600 nm on a Pharmacia Biotech Ultrospec 2000.

DNA isolation, transformation and manipulation

Genomic DNA from *C. acetobutylicum* or from *C. beijerinckii* was isolated using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich), while plasmid DNA from *E. coli* was isolated by the GenElute Plasmid Miniprep Kit (Sigma-Aldrich). Amplification of clostridial DNA by PCR was done using the *Pfu* polymerase (Stratagene) enzyme, *E. coli* colony PCR reactions were carried out using REDTaq DNA polymerase (Sigma-Aldrich).

Transformations of chemically competent *E. coli* strains were conducted using New Engeland Biolab's "High Efficiency Transformation Protocol" (http://www.neb.com/nebecomm/products/protocol119.asp). Prior to transformation into *C. acetobutylicum*, plasmids were methylated in vivo by electroporation into *E. coli* DH10B (pAN1) [144].

A modified procedure, based on the method of Oultram *et al*. [145], was used for transformation of *C. acetobutylicum* by electroporation. Briefly, this was carried out as follows; *C. acetobutylicum* was grown up to an OD_{600} of 0.7 (corresponding to a biomass concentration of approximately 0.35 mg mL^{-1}) in 100 mL mCGM medium, at 37 °C. The culture was centrifuged for 10 minutes (5 468 × g, 4 °C), after which the supernatant was removed and the pellet was washed with 20 mL ice-cold anaerobic electroporation buffer (270 mM sucrose, 1 mM MgCl2, 7 mM sodium phosphate buffer, pH 7.4). The pellet was again resuspended in 4 mL of fresh electroporation buffer. 300 µL of the suspension was transferred into an electroporation cuvette with a 0.2 cm gap width. After addition of approximately 1 µg of methylated plasmid DNA, the cells were electroporated (1.25 kV; 25 µF; 100 Ω), resulting in time constants between 1.2 and 1.8 ms. The electroporated cells were cooled on ice and then incubated with 3 mL pre-warmed mCGM at 37 °C for 3 hours. The cells were concentrated and plated on pre-warmed mCGM plates containing erythromycin. All manipulations were carried out anaerobically and on ice, unless noted otherwise.

Construction of plasmids

The clostridial expression plasmids pWUR459 and pWUR460 were constructed as follows. The pMTL500E vector was linearized by digestion with *SphI* and *XhoI*. *adc* and *thl* promoter regions were amplified by PCR from *C. acetobutylicum* genomic DNA using primers 1 & 2, and 3 & 4 (Table 13),

respectively. The acetoin reductase (*Cb-acr*) gene was amplified by PCR from *C. beijerinckii NCIMB 8052* genomic DNA using primers 5 and 6. Primer 5 contained an artificial RBS (AGGAGG), which was separated from the ATG start codon by the 9-nucleotide sequence GGCGGCGGC (Table 13).

Primers 1 and 3 contained a *SphI* restriction site, primers 2, 4, and 5 an *ApaI* restriction site, and primer 6 a *XhoI* restriction site. All PCR products were digested using the restriction enzymes for the aforementioned sites. Plasmids pWUR459 and pWUR460 were obtained by three-way ligation reactions which contained the linearized vector, the *Cb-acr* gene and the *adc* promoter or the *thl* promoter, respectively (Figure 22). Competent *E. coli* NEB 5-alpha cells were transformed with the ligation mixtures and subjected to blue-white screening and selected for ampicillin resistance. White colonies were checked by colony PCR for the presence of the corresponding construct, after which restriction analysis and sequencing confirmed the correct constructs. *E. coli* DH10B (pAN1) methylation strain was electroporated with plasmids pWUR459 and pWUR460 and transformants were selected for ampicillin and chloroamphenicol resistance. Correct methylation was checked by restriction analysis using *Fnu4HI* [144].

Table 12: Plasmids and bacterial strains.

Bacterial strain or plasmid	Relevant genotype	Remarks	Source
C. acetobutylicum WUR	WT	Originally obtained as ATCC 824, but shown to deviate from type strain behavior	Laboratory stock
C. acetobutylicum ATCC 824	WT	Type strain	Laboratory stock, originally from G. Bennett (Rice University, Houston, Texas, USA)
C. beijerinckii NCIMB 8052	WT		Laboratory stock
E. coli DH10B (pAN1)	Δ(mrr-hsdRMS-mcrBC)	Methylation strain	Laboratory stock
pAN1	p15A ori; Cmr Φ3tI	Expression plasmid for phage Φ3tI methylase gene [208].	Laboratory stock
pMTL500E	ColE1 ori; pAMβ1 ori; MLSr; Apr	Clostridial/*E. coli* shuttle vector [209].	Laboratory stock
pWUR459	ColE1 ori; pAMβ1 ori; MLSr; Apr; P_{adc}-Cb-acr	Expression plasmid of Cb-acr under control of the *C. acetobutylicum* acetoacetate decarboxylase promoter	This study
pWUR460	ColE1 ori; pAMβ1 ori; MLSr; Apr; P_{thl}-Cb-acr	Expression plasmid of Cb-acr under control of the *C. acetobutylicum* thiolase promoter	This study

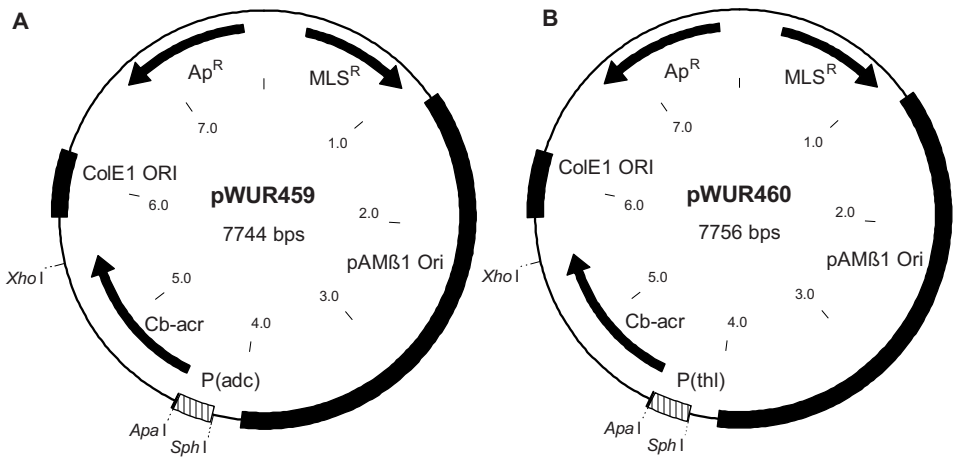

Figure 22: Plasmid map of A. pWUR459 - Expression plasmid of *Cb-acr* under control of the *C. acetobutylicum* acetoacetate decarboxylase promoter and B. pWUR460 - Expression plasmid of *Cb-acr* under control of the *C. acetobutylicum* thiolase promoter.

Table 13: Primer sequences used in this study; [a] Nucleotides that are not complementary to the genomic sequence are given in uppercase. Restriction sites are underlined. The artificial ribosome binding site is shown in bold.

No.	Primer name	Sequence (5' → 3')[a]	Target DNA region
1	005CA_Pthlf	GGCATGCgaa tttagaatga agtttcttat gc	Promoter *thl*; *cac2873*
2	017CA_Pthlr	AAAAGGGCCC ccatagttta tccctaattt atacg	Promoter *thl*; *cac2873*
3	007CA_Padcf	GGCATGCatg ggaaagccaa cattgc	Promoter *adc*; *cap0165*
4	018CA_Padcr	AAAAGGGCCC cttcacatta taaatcgcct ct	Promoter *adc*; *cap0165*
5	BG2588	CGCGCGGGCC CAGGAGGGgC GGCGGCatga aagcagcatt atg	*CBEI_1464* (*Cb-acr*)
6	BG2402	GCGCGCTCGA Gttaagattt agatacaagt tctt	*CBEI_1464* (*Cb-acr*)

Cloning of the Cb-acr gene into *C. acetobutylicum*

Competent *C. acetobutylicum* cells were transformed with plasmids pWUR459, pWUR460, and the control plasmid pMTL500E, containing no insert. Each transformation resulted in multiple erythromycin resistant colonies. After re-streaking, selected colonies were used to prepare spore suspensions for further experiments.

Analysis of metabolites

The concentration of sugars, acids and solvents in culture supernatants was determined by HPLC. Samples taken during fermentation (approximately 1.5 mL) were centrifuged (5 min, 20 800 × g) and the supernatants were stored at -20 °C. After thawing, an equivolume amount of internal standard solution (either 100 mM valeric acid (Sigma-Aldrich) in 1 M H_2SO_4, or 30 mM 4-methyl valeric acid (Sigma-Aldrich) in 0.5 M H_2SO_4) was added to the supernatant sample. The sample with internal standard was filtered through a 0.2 µm pore size membrane filter (Whatman) and components were quantified using HPLC. Separation of 10 µL sample was achieved using a Shodex Ionpack KC-811(RP) column, equipped with a refractive index detector (Waters 2414) and a UV detector (Waters 2487) operating at 210 nm, with 3 mM H2SO4 as eluent (flow: 1 mL min-1; column temperature: 85 °C). The order of elution was: glucose, lactic acid, acetic acid, acetoin, *meso*-2,3-butanediol, d/l-2,3-butanediol, butyric acid, acetone, ethanol, valeric acid, 4-methyl valeric acid and 1-butanol. All concentrations were determined based on the refractive index chromatogram except, for butyrate, for which in some cases the UV chromatogram was used. Empower 2 software (Waters Corporation) was used for machine control and data analysis.

Chiral GC-MS analysis

To determine the enantiomeric distribution of the produced 2,3-butanediol and acetoin, the fermentation samples were treated like the HPLC samples. However, after thawing, the samples were additionally saturated with sodium chloride and extracted once with an equivolume amount ethyl acetate. To prevent co-extraction of acids, 10 M sodium hydroxide was added, since these compounds interfered with chromatographic analysis. Samples used for acetoin analysis were not treated with sodium hydroxide. The extract was then analyzed on a Trace DSQ GC-MS system (Thermo) equipped with a CP-Chirasil-Dex CB (Varian) fused silica capillary column (25 m x 0.25 mm x 0.25 µm). Helium was used as the carrier gas (48 kPa). The injection port temperature was set at 250 °C, with a split ratio of 1:10. The oven temperature program was as follows: 80 °C (10 min), increased to 120 °C at 10 °C min-1 followed by a ramp of 40 °C min-1 to 200 °C (4 min). Samples (1 or 5 µL) were injected using an autosampler AS3000 (Thermo). The ion source (EI) temperature was set to 200 °C. Chromatograms were analyzed using Xcalibur 1.4 SR1 software (Thermo). These conditions allowed for separation of the enantiomers and diasteriomers of both acetoin and 2,3-butanediol. Compound identification by column retention time was confirmed by analysis of the mass spectrum. Retention times of chiral standards of D-(-)-(2R,3R) and L-(+)-(2S,3S)-2,3-butanediol (Sigma-Aldrich) were used to identify the product peak. The elution order of the acetoin enantiomers was inferred from the stereochemistry of the product D-(2R,3R)-2,3-butanediol. An analogous reasoning was applied by González *et al*. [210]. The order of elution was: (3R)-acetoin, (3S)-acetoin, L-(2S,3S)-2,3-butanediol, D-(2R,3R)-2,3-butanediol and *meso*-2,3-butanediol.

Results

Characterization of *C. acetobutylicum* transformants

Wild type *C. acetobutylicum ATCC 824* is known to produce significant levels of acetoin, but no 2,3-butanediol [8]. Introduction of an acetoin reductase (ACR)-encoding gene from *C. beijerinckii* NCIMB 8052 might enable conversion of acetoin into 2,3-butanediol. Therefore, *C. acetobutylicum* transformants were constructed, containing the Cb-acr gene under control of either the *thl* promoter (pWUR459) or the *adc* promoter (pWUR460), and their fermentation pattern was analyzed. As a result, both types of transformants were found to produce 2,3-butanediol, and more specifically, its D-stereoisomer (Table 14). No D-2,3-butanediol was produced by the control strain, containing the empty vector. Acetoin was found to accumulate transiently at the end of the exponential growth phase of both transformant strains, with levels reaching 4 ± 1.6, 8 ± 0.7 and 9 ± 0.3 mM for pWUR460 (*thl* promoter), pWUR459 (*adc* promoter) and pMTL500E (empty vector), respectively. These data suggest that the conversion of acetoin to 2,3-butanediol is limiting, especially in the case of the *adc* promoter. The final 2,3-butanediol concentrations for both Cb-ACR strains did not differ significantly from one another: 22 mM for the P_{thl}-*Cb-acr* strain, and 20 mM for the P_{adc}-*Cb-acr* strain. Acetoin levels of the control fermentation reached 19 mM. However, in fermentations of Cb-ACR expressing strains, acetoin was still detected at levels of 2 to 3 mM at the end of the fermentation (Figure 23 and Table 14). The level of conversion of acetoin into 2,3-butanediol at the end of the fermentation was similar for both strains: 90% (pWUR459) and 89% (pWUR460).

Analysis of medium samples of fermentations by the *C. acetobutylicum* control strain harboring pMTL500E (EV), showed a ratio of approximately 12:1 (D:L) in the concentrations of the two acetoin enantiomers (data not shown).

The transformants expressing the *Cb-acr* gene showed similar acid and solvent production patterns as the control strain harboring the empty vector (Table 14).

Remarkably, all three transformants produced small but nonetheless detectable amounts of *meso*-2,3-butanediol (Table 14). To confirm this finding, we also looked at fermentations by our wild type *C. acetobutylicum* strain, in the same (MG) and other media (mCGM and CGM). At the end of all fermentations, small amounts of 1 to 3 mM, *meso*-2,3-butanediol were found (Figure 23 and data not shown). In all these fermentations, no D- or L-2,3-butanediol was detected.

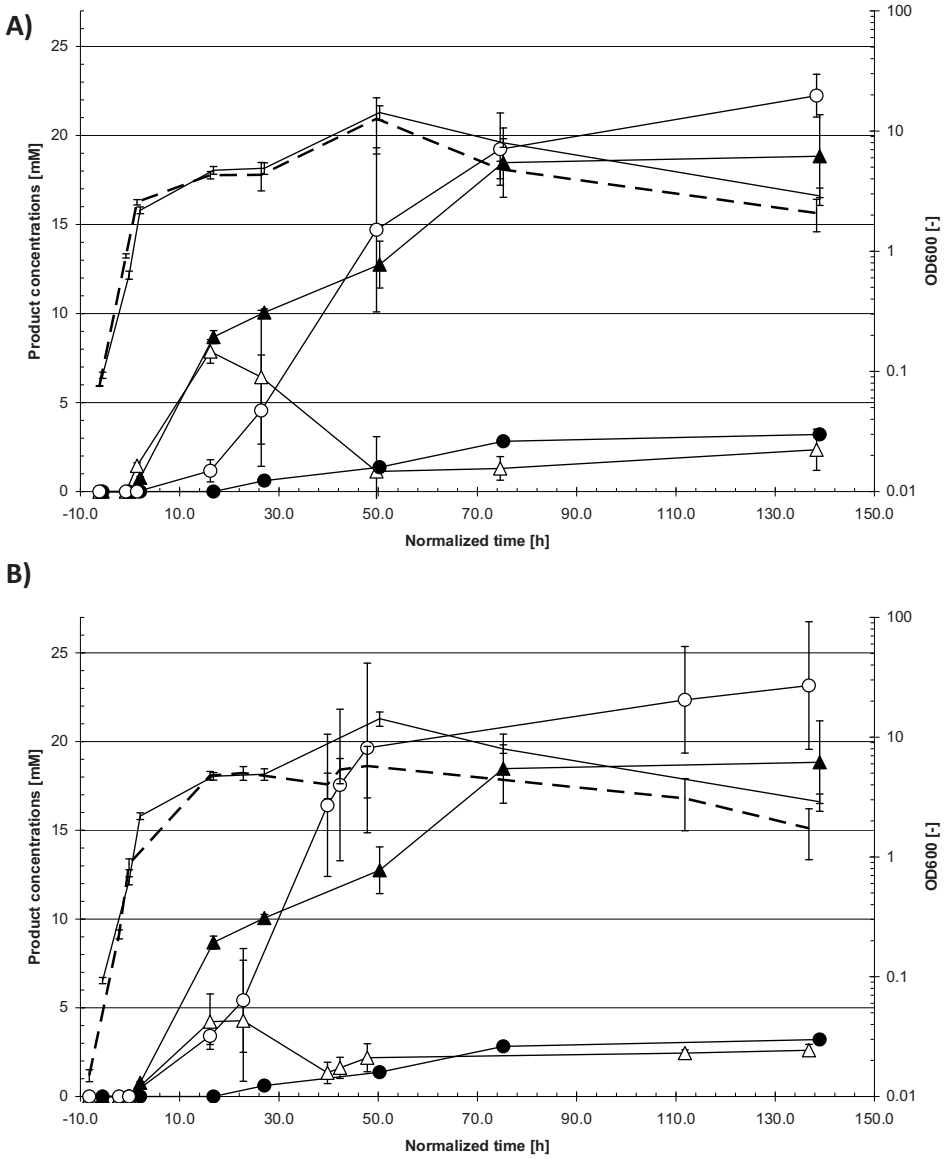

Figure 23: Production of acetoin (triangles) and 2,3-butanediol (circles) during batch fermentations of glucose by transformant strains of *C. acetobutylicum*; Strains codes: EV (panel A and B, closed symbols), *C. acetobutylicum* (pMTL500E), empty vector control strain; ADC (panel A, open symbols), *C. acetobutylicum* (pWUR459); THL (panel B, open symbols), *C. acetobutylicum* (pWUR460). Optical densities of the cultures are shown by a solid line (EV) and a dashed line (ADC and THL). Normalized time was plotted on the horizontal axis by setting and optical density of 1 at time is zero. The control strain did not produce any D-2,3-butanediol, only *meso*-2,3-butanediol. Data represent the mean of triplicate fermentations averaged for each time interval. Error bars indicate standard deviations.

Table 14: Concentrations of substrate and products of 100 mL batch fermentations of *C. acetobutylicum* harboring pMTL500E, pWUR459 or pWUR460 on MG medium after 145 hours. Data are given as the mean of triplicate fermentations ± standard deviation. [a] acetate is present at the beginning (t = 0) as a medium component and is consumed during the fermentation.

	pMTL500E Control			pWUR459 Padc-Cb-acr			pWUR460 Pthl-Cb-acr		
Consumed glucose [mM]	318	±	15	307	±	29	320	±	18
Acetoin [mM]	19	±	2	2	±	1	3	±	0.3
meso-2,3-Butanediol [mM]	3	±	0.1	2	±	0.1	2	±	0.3
D-2,3-Butanediol [mM]	0			20	±	1	22	±	3
Acetoin + 2,3-butanediol [mM]	22	±	2	25	±	2	26	±	4
Yield of (Acetoin + 2,3-BD) per glucose [mM/mM]	7	±	0.8 %	8	±	0.9 %	8	±	1 %
Initial acetic acid[mM] [a]	37	±	0.2	38	±	0.4	36	±	0.8
Final acetic acid [mM]	14	±	0.7	20	±	6	19	±	2
Butyric acid [mM]	2	±	1	5	±	3	6	±	3
Lactic acid [mM]	3	±	0.7	6	±	1	4	±	0.4
Acetone [mM]	80	±	9	78	±	12	86	±	6
Butanol [mM]	161	±	6	157	±	15	166	±	6
Ethanol [mM]	40	±	2	34	±	5	56	±	14

Product stereochemistry

There are three stereoisomeric forms of 2,3-butanediol. The main stereoisomer produced by fermentation in MG medium was identified as D-(2R,3R)-2,3-butanediol. However, also low levels of *meso*-2,3-butanediol were detected in transformant strains, as well as in wild type fermentations by both HPLC and GC-MS analysis. In all fermentations, L-(2S,3S)-2,3-butanediol was below our detection threshold. Figure 24 shows the gas chromatographic analysis of extracts of standard and medium samples of cultures of the *C. acetobutylicum ATCC 824* and WUR wild types, the plasmid control, and the P_{thl}-Cb-acr transformant strain.

The observation that both the plasmid control strain as well as the wild type strain produced *meso*-2,3-butanediol is a new observation for *C. acetobutylicum* and prompted us to investigate this further. An independently obtained *C. acetobutylicum ATCC 824* type strain sample that was grown under identical conditions, did not produce *meso*-2,3-butanediol. This suggests that our ATCC 824 lab strain has diverged from the type strain. We therefore refer to our lab strain as *C. acetobutylicum* WUR (Table 12).

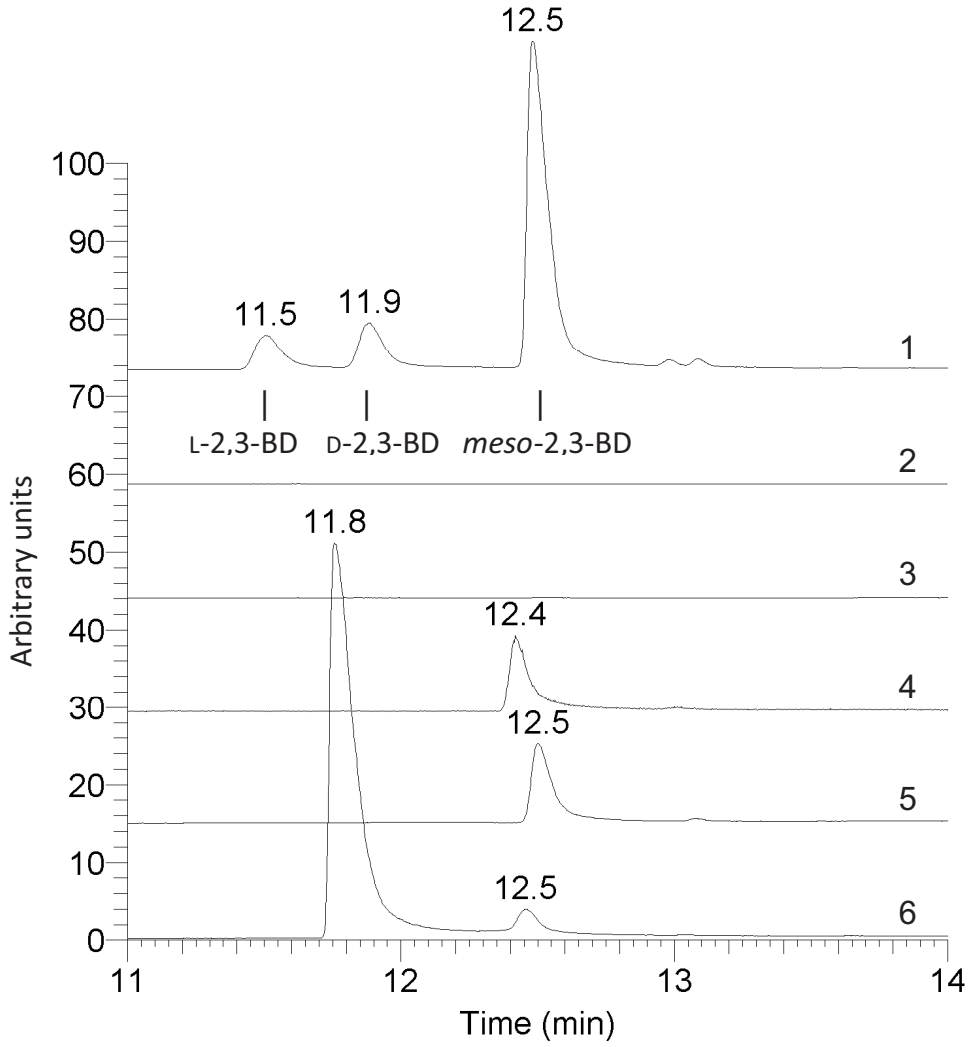

Figure 24: GC-MS chromatograms (single ion mode, m/z = 45) of extracts of, a standard, GM medium and samples taken at the end of the fermentation of wild-type and transformant cultures. Chromatogram 1, mixture of all three 2,3-butanediol stereoisomers; Chromatogram 2, GM medium blank; Chromatogram 3, fermentation of *C. acetobutylicum ATCC 824*; Chromatogram 4, fermentation of *C. acetobutylicum* WUR; Chromatogram 5, fermentation of *C. acetobutylicum* WUR harboring the empty vector (EV); Chromatogram 6, fermentation of *C. acetobutylicum* WUR harboring the pWUR460 construct containing the *acr* gene. The retention time of the D-2,3-butanediol peak in chromatogram 6 is somewhat different due to the high concentration. Spiking experiments confirmed that it is indeed the D-stereoisomer.

Acetoin and 2,3-butanediol challenged batch fermentations

In order to determine possible inhibitory effects of 2,3-butanediol on the cultures of *C. acetobutylicum* transformants expressing the *Cb-acr*-gene, fermentations in media supplemented with 20 mM D-(2R,3R) or 20 mM *meso*-2,3-butanediol were performed. The concentrations of these compounds were in the same range as those encountered at the end of the fermentations shown in table 15. Additionally, to determine if the amount of produced acetoin was limiting for the production of 2,3-butanediol, we also supplemented culture media with racemic acetoin to levels normally reached at the end of the fermentation of the control strain (20 mM). Acetoin and 2,3-butanediol were added to the medium prior to inoculation with the pre-culture.

In cultures challenged with D-2,3-butanediol, both transformants containing the *Cb-acr* gene produced additional D-2,3-butanediol in similar amounts (12 mM for strain pWUR459 and 20 mM for strain pWUR460) compared to their non-challenged controls (15 mM and 17 mM respectively). The use of *meso*-2,3-butanediol resulted in similar behavior (Table 15).

As observed in cultures without supplementation, the final concentrations of the main fermentation products (acetone, butanol and ethanol, and butyric and acetic acids) were similar in the case of the *Cb-acr* containing transformants and the control (data not shown).

In cultures supplemented with racemic acetoin, it was observed that both D- and L-enantiomers were converted by the transformant strains into D- and *meso*-2,3-butanediol (Table 15). This demonstrates that the Cb-ACR enzyme is able to convert both acetoin enantiomers and is therefore not stereoselective for the configuration at the C3 position. The total amount of both 2,3-butanediol diastereomers produced in the challenged cultures, 36 mM

(pWUR459) and 41 mM (pWUR460), corresponds with the total amount of acetoin consumed.

Interestingly, the plasmid control strain produced more *meso*-2,3-butanediol in the acetoin challenged cultures. It increased significantly from 2 ± 0.4 mM in the non challenged control to 4 ± 0.2 mM in the racemic acetoin challenged culture. However, still no D- or L-2,3-butanediol was observed.

Table 15: Nett change of extra-cellular acetoin and 2,3-butanediol concentrations in challenged batch cultures of *C. acetobutylicum* transformants harboring pMTL500E, pWUR459 or pWUR460

Strain	Challenge	Nett change in extra-cellular concentrations [mM]			
		Acetoin	d-2,3-BD [d]	*meso*-2,3-BD	Acetoin + 2,3-BD
pMTL500E (control)	No challenge	15 ± 1	0	2 ± 0.4	17 ± 1
	Acetoin [a]	13 ± 2	0	4 ± 0.2	17 ± 2
	D-2,3-BD [b]	12 ± 2	-0.7 ± 1	1 ± 0.05	13 ± 2
	meso-2,3-BD [c]	15 ± 0.2	0.3 ± 0.6	2 ± 1	18 ± 1
pWUR459 (P_{adc}-*Cb-acr*)	No challenge	0.2 ± 0.4	15 ± 1	1 ± 0.2	16 ± 1
	Acetoin	-20 ± 0.4 [e]	24 ± 1	11 ± 0.5	15 ± 1
	D-2,3-BD	0.1 ± 0.2	12 ± 1	0.9 ± 0.1	13 ± 1
	meso-2,3-BD	0.2 ± 0.4	17 ± 2	-0.5 ± 0.2	17 ± 2
pWUR460 (P_{thl}-*Cb-acr*)	No challenge	0 ± 0.3	17 ± 1	0.8 ± 0.1	18 ± 1
	Acetoin	-21 ± 0.7 [e]	29 ± 4	11 ± 0.2	20 ± 4
	D-2,3-BD	0.2 ± 0.3	20 ± 3	0.9 ± 0.1	21 ± 3
	meso-2,3-BD	0.4 ± 0.4	21 ± 0.5	0.1 ± 1	22 ± 1

Discussion

Acetoin reductase (ACR) is an enzyme that catalyses the reduction of acetoin to 2,3-butanediol. Although *Clostridium beijerinckii* NCIMB 8052 contains a homologue of a *Bacillus cereus* 2,3-butanediol dehydrogenase gene in its genome [211], we did not find any report in the scientific literature mentioning the production of either the ACR substrate acetoin or its product 2,3-butanediol by *C. beijerinckii*. This acetoin reductase encoding gene (*CBEI_1464*) was originally annotated as an alcohol dehydrogenase, but after functional expression in *E. coli*, acetoin reductase activity could indeed be confirmed (Siemerink and Kengen, unpublished results). Therefore, we decided to clone the *acr* gene into *C. acetobutylicum ATCC 824* to supplement the existing acetoin pathway and enable 2,3-butanediol biosynthesis. In this study, we showed conclusively that when the *C. beijerinckii acr* (*CBEI_1464*) gene is expressed in *C. acetobutylicum*, natively produced acetoin is indeed reduced to D-(2R,3R)-2,3-butanediol.

Clostridium acetobutylicum ATCC 824 is known to produce acetoin as a minor fermentation product, but has never been reported to produce *meso*-2,3-butanediol [212, 213]. Our analysis of fermentations of the ATCC 824 type strain confirms this. The *C. acetobutylicum* WUR strain, however, used to prepare the Cb-ACR expressing strains, did produce *meso*-2,3-butanediol, both the wild type and the plasmid control strain. It is concluded that our strain is a direct descendent of the ATCC 824 type strain, but apparently one or more random mutations have resulted in a slightly divergent phenotype. Despite this unexpected result we continued to investigate the fermentative behavior of our transformant strains, as they had otherwise interesting properties.

An earlier report describing an attempt to engineer *C. acetobutylicum* ATCC 824 to produce 2,3-butanediol from natively produced

acetoin by heterologous expression of a *Klebsiella pneumoniae* ACR was unsuccessful [213].

Our approach differed with the one reported by Wardwell *et al*. in the following aspects. In order to obtain high levels of acetoin reductase activity, we chose to express the *C. beijerinckii acr* gene, which has a comparable GC content (35%) to the GC content of the DNA of the *C. acetobutylicum* host (31%) and similar codon usage [214]. We also used a different shuttle vector (pMTL500E) with another origin of replication, in an attempt to increase the gene dosage. The pMLT500E plasmid (pAMβ1 origin) has a higher copy-number in *C. acetobutylicum* [145, 215] than the low-copy-number pSOS84 (pIM13 origin) derived plasmids used by Wardwell *et al*. [213]. Our approach, combined with a strong promoter (either P_{adc} or P_{thl}), resulted in expression levels of the *Cb-acr* gene by *C. acetobutylicum* high enough to lead to 2,3-butanediol production.

Our results agree with recent data obtained for an acetoin reductase (BdhA) of *Bacillus subtilis* [216]. The amino acid sequence of this enzyme is very similar to the proposed acetoin reductase of *C. beijerinckii* with 51% identical residues and 66% similar residues. A targeted *bdhA* knock-out mutant, with no detectable ACR activity in vitro, accumulated acetoin in the medium and lacked 2,3-butanediol production.

Two different expression constructs, pWUR459 (P_{adc}-*Cb-acr*) and pWUR460 (P_{thl}-*Cb-acr*), were transformed into *C. acetobutylicum* to test the influence of the two different expression profiles of the promoters on the fermentation. Upon expression, both transformant strains were able to convert approximately 90% of the natively produced acetoin into D-2,3-butanediol, with values reaching 22-23 mM. Compared to the industrial strain *Klebsiella pneumoniae* SDM, which reaches concentrations up to 1664 mM, this is relatively low [217]. However, for this species, 2,3-butanediol is

the main fermentation product, whereas for our strain, 2,3-butanediol is only a side product next to the solvents butanol and acetone.

Although similar final concentrations of 2,3-butanediol were produced by both transformants, a difference was observed in fermentation profiles during the exponential growth phase. During the acidogenic phase, the pWUR460 (*thl*-controlled) fermentation showed a significantly lower acetoin level compared to the pWUR459 (*adc*-controlled) culture or the empty vector control. The level of 2,3-butanediol was concomitantly higher in the *thl*-controlled culture. This difference in acetoin and 2,3-butanediol levels between the *adc* and *thl* driven *acr* expression is in good agreement with the differences in promoter activity [218, 219]. Later during the fermentation, this difference is leveled out. Therefore, there is no expected advantage, with respect to final 2,3-butanediol levels under our conditions, to express *Cb-acr* constitutively.

Despite constitutive expression of *Cb-acr* by the strain containing pWUR460, accumulation of acetoin in the medium was still observed. Apparently, the acetoin production flux under these conditions is higher than the flux from acetoin to 2,3-butanediol can accommodate.

The final levels of the main fermentation products, acetone, butanol and ethanol, were similar for all three strains. This may seem contrary to the expectation that, when a new product is formed that is more reduced than its substrate (2,3-butanediol vs. acetoin), the distribution of the other fermentation products is also affected based on the redox balance for the fermentation. Either less of the other alcohols or less hydrogen is produced, or more of the oxidized products, acetate and butyrate, is made. However, as only 7% of the glucose is converted into 2,3-butanediol, the impact on the other fermentation products is small. Even more so because the change,

caused by the increased NAD(P)H use, is probably distributed over the various fermentation products.

Acetoin and 2,3-butanediol stereochemistry

The expression of ACR from *C. beijerinckii* in *C. acetobutylicum* resulted in production of D-(2R,3R)-2,3-butanediol and not in *meso*- or L-(2S,3S)-2,3-butanediol production (Figure 24). The transformant harboring the empty vector produced acetoin enantiomers in an approximately 12:1 (d:l) ratio. Which acetoin enantiomer is the predominant one that *C. acetobutylicum* produces is not known from direct measurements, but it can be inferred from the stereochemistry of the product that it must be D-acetoin.

Figure 25 shows the stereochemistry of the reactions involved in 2,3-butanediol formation [220]. The joining of two pyruvic acid molecules creates a chiral center, which, after decarboxylation, results in either D or L-acetoin. A reduction of either enantiomer can result in formation of *meso*-2,3-butanediol, but only reduction of D-acetoin can result in formation of D-butanediol. The reason for this is, that the configuration of the third carbon atom is already defined in the acetoin molecule. Figure 25 is based on the assumption that the enzymatic mechanism does not involve racemization of the second or third carbon atom. This assumption is in agreement with the proposed reaction mechanisms for this enzyme [221] and has been applied before to assign absolute configurations of acetoin following enzymatic conversion [210].

As *C. acetobutylicum* produces D-acetoin, the inability of the transformant described by Wardwell *et al*. to produce 2,3-butanediol can not be related to the stereochemical preference of the overexpressed enzyme from *K. pneumoniae* CG21. An almost identical acetoin reductase from K. *pneumoniae* IAM 1063 converts D-acetoin to *meso*-2,3-butandiol, whereas it does not accept L-acetoin as a substrate [222]. This suggests that the

stereochemistry of acetoin is not the key problem in the earlier approach of Wardwell *et al*..

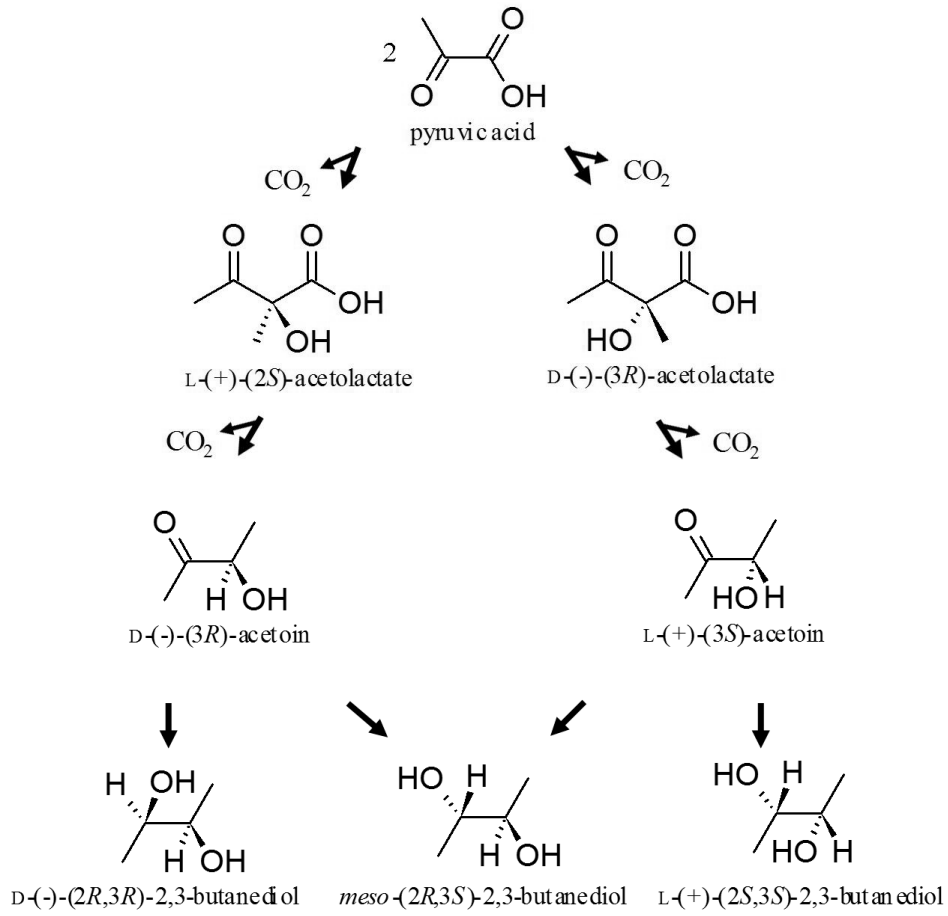

Figure 25: The influence of the chirality of the precursors on the 2,3-butanediol stereochemistry. Scheme adapted from Taylor and Juni [223] and Ui *et al.* [224].

Identification of bottlenecks for 2,3-butanediol production

In our experiments, transformant cultures were challenged by the addition of 20 mM racemic acetoin, resembling the levels observed in the final stages of normal growth. The observation that similar amounts of acetoin were produced by the control strain in both unchallenged and acetoin challenged fermentations (Table 15) indicates that acetoin production does not seem to

be regulated by its extracellular concentration, at the concentrations tested. This is in agreement with the fact that in non-challenged fermentations of *Cb-acr* expressing transformants, the combined levels of acetoin and 2,3-butanediol do not significantly exceed those of the acetoin levels of the control strain fermentation. It was observed that in the acetoin challenged cultures the *meso*-2,3-butanediol formation by the control strain (pMTL500E) doubled from 2 ± 0.4 mM to 4 ± 0.2 mM. Most likely, one or more of the several dehydrogenases that are present in *C. acetobutylicum* accept, to some extent, acetoin as a substrate.

In racemic acetoin supplemented fermentations with Cb-ACR expressing strains, all of the additional acetoin was converted into D-2,3-butanediol and *meso*-2,3-butanediol. This indicates that ACR from *C. beijerinckii* is not stereospecific for the configuration at the third carbon position of its substrate acetoin and can convert both enantiomers. This lack of selectivity for the stereochemistry of the substrate is not uncommon amongst acetoin reductases [225].

When exogenously added D-2,3-butanediol (20 mM) is present during the fermentation, the amount of converted acetoin is not affected. This shows that at the tested levels, the amount of 2,3-butanediol is not inhibitory to the reaction. Addition of the same amount of *meso*-2,3-butanediol did not affect the fermentation product profile either.

Based on these results, we conclude that the acetoin formation, for the fermentation as a whole, is the limiting factor for the production of 2,3-butanediol. Even though, initially, acetoin accumulates in the medium (Figure 23). Apparently, the catalytic conditions for, and/or the amount of, Cb-ACR at the beginning of the fermentations are not optimal. However, total acetoin production during the fermentation is limiting D-2,3-butanediol production as exogenously added acetoin increases the 2,3-butanediol yield.

Additionally, both the *thl* and *adc* promoter driven strains achieve the same final 2,3-butanediol levels. If ACR activity would be a severely limiting factor in the production of 2,3-butanediol, then the P_{thl}-*Cb-acr* strain would be expected to have a higher final concentration of 2,3-butanediol, because it expresses *Cb-acr* over a longer period of time.

Our data suggest that acetoin formation is not inhibited by the extracellular acetoin levels, because in acetoin challenge experiments of the plasmid control strain, a similar amount of acetoin was produced in addition to the acetoin already present in the medium. This suggests that another regulatory system for acetoin production is in place.

2,3-Butanediol production model

Based on the combined results of wild type, plasmid control and *Cb-acr* expressing strains, in normal and challenged cultures, we propose the model shown in figure 26 for acetoin and 2,3-butanediol production in our *C. acetobutylicum* strain.

The wild type and the plasmid control strain produced small amounts of *meso*-2,3-butanediol and no detectable levels of D- or L-2,3-butanediol, as confirmed by GC-MS analysis. Apparently acetoin is reduced by an endogenous oxido-reductase, most likely in a side reaction of the numerous alcohol dehydrogenases present in *C. acetobutylicum*. As *meso*-2,3-butanediol can be produced from both acetoin enantiomers it can not be said which enantiomer, or perhaps both, is used to produce it.

Interestingly, when performing racemic acetoin challenge experiments, the amount of *meso*-2,3-butanediol produced by the empty vector control strain doubled from 2 mM to 4 mM. This would suggest that it is D-acetoin that is the source of the endogenous *meso*-2,3-butanediol production. The rationale for this is that the levels of D-acetoin are approximately doubled,

while the L-acetoin concentration increased more then nine-fold. If L-acetoin would be the source of the endogenous 2,3-butanediol production, then also a similar, approximately nine-fold, increase of *meso*-2,3-butanediol production would be expected.

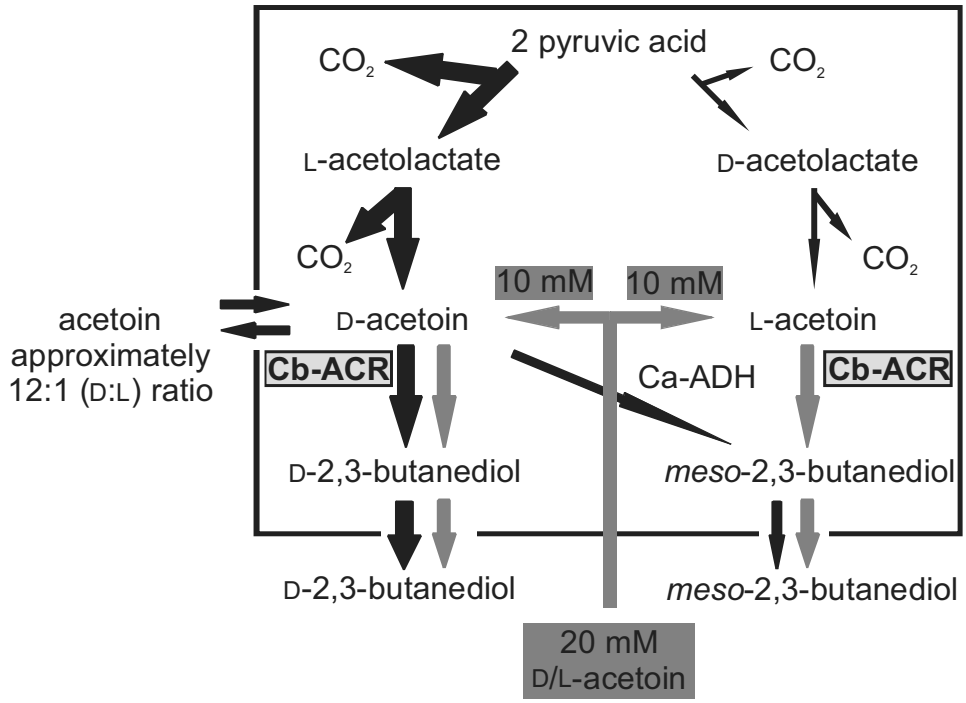

Figure 26: Proposed 2,3-butanediol biosynthesis pathway in the ACR expressing *C. acetobutylicum* transformants of this study. The boxed area indicates the intracellular space. The size of the arrows indicates the relative flux towards D- and L-acetoin from pyruvic acid. The gray arrows illustrate the impact of medium supplementation with 20 mM racemic D/L-acetoin. The *C. beijerinckii* acetoin reductase enzyme is indicated in bold on a gray background. The proposed conversion of D-acetoin to *meso*-2,3-butanediol by an endogenous alcohol dehydrogenase (Ca-ADH) is indicated. In the control strain fermentation the ratio of the two acetoin enantiomers is 12:1 (D:L).

Future prospective

If a pathway could be established in which 2,3-butanediol is dehydrated to 2-butanone and then further reduced to 2-butanol, then potentially a redox balanced fermentation of glucose to 2-butanol and carbon dioxide could be established in this organism. Because of the less toxic nature of 2-butanol compared to 1-butanol [206], this could be an alternative to circumvent the limited butanol yield of the classic ABE-fermentation. 2-Butanol has similar physical and chemical properties compared to 1-butanol and it is very likely that, just as 1-butanol, it could be used as a fuel additive or replacement, making it an interesting metabolic engineering target.

7

Summary and general discussion

Chapter 7: Summary and general discussion

Summary

Chapter 1 of this thesis gives an overview about the history of the acetone, butanol and ethanol (ABE) fermentation. The responsible solventogenic clostridia with their central metabolism are briefly discussed. Despite the fact that scientific research on the key organisms of the ABE process has continued over the past decades and even increased in recent years, still numerous aspects remain unclear.

Economically, the biggest challenge within the ABE fermentation field, remains the 1-butanol toxicity. Due to its toxicity the yield of 1-butanol does not exceed 1-2% (w/v), and only little progress has been made over the past years. Nevertheless, the ABE fermentation process became interesting again, because of the global interest in biofuels or biofuel additives.

Nowadays, in an attempt to reach higher yields, other microorganisms are also being explored as hosts for the production of solvents. *E. coli* is often chosen as a promising host organism for the microbial production of biofuels. Nevertheless, clostridial hosts remain interesting, due to several reasons, like i) availability of a genetic system, ii) natural production of solvents and iii) relatively high tolerance towards solvents. A disadvantage of the usage of these solventogenic organisms is the inability to use cellulose and hemi-cellulose as substrate.

With respect to the production of potentially interesting 1-butanol derivatives, we focussed on 2,3-butanediol. This industrially valuable compound is already produced in nature by several bacteria, but not by *C. acetobutylicum*. In this thesis, the production of 2,3-butanediol by

Clostridium acetobutylicum is investigated. In Chapter 1 the 2,3-butanediol biosynthesis pathway is extensively described.

The two industrially most important and sequenced solventogenic clostridia are *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052. A lot of biochemical information is known about the various metabolic steps of the central catabolic pathway of *C. acetobutylicum ATCC 824*. In **Chapter 2**, comparisons are made between the pathways of both species. With the genome sequence of *C. beijerinckii* NCIMB 8052 also available, likely candidates for the 34 involved enzymatic conversions within the central catabolic pathway of *C. beijerinckii*, could be predicted. The enzymatic conversions involved in glucose uptake, glycolysis, gluconeogenesis, pyruvate conversion and acetyl-CoA conversion towards the different end products are being discussed.

Chapter 3 describes and investigates a novel approach in solving the problem of the 1-butanol toxicity towards *C. acetobutylicum*. Increasing of the tolerance of 1-butanol has been tried more often in other research groups. In our approach, we chose for the introduction of new biosynthesis pathways that enable the production of less toxic 1-butanol derivatives. The toxicity of compounds is expected to correlate with its lipophilicity, e.g. the tendency to accumulate in cell membranes. It can be expressed as the logarithm of the partition coefficient with octanol and water (log K_{ow} value). Chapter 3 describes the growth experiments that were performed to access the toxicity of the various derivatives that were selected, viz. *iso*-butanol, 2-butanol, *tert*-butanol, 2,3-butanediol, *iso*-amyl alcohol, butyl acetate, butyl butyrate and butyl lactate. *Iso*-butanol, 2-butanol and 2,3-butanediol emerged as likely alternatives to 1-butanol, based on their log K_{ow} value and their behaviour in the toxicity test.

2,3-Butanediol appeared to be a potential candidate, which is less toxic to *C. acetobutylicum* than 1-butanol. The precursor of 2,3-butanediol, acetoin, which is already produced by *C. acetobutylicum* in small amounts, is formed by the decarboxylation of acetolactate. The conversion is catalyzed by acetolactate decarboxylase. In **Chapter 4** the identification, heterologous production, purification and biochemical characterization of a acetolactate decarboxylase from *C. acetobutylicum ATCC 824* is described. Ca-ALD encoded by CAC2967 was proven to exhibit acetolactate decarboxylase activity. Size exclusion chromatography revealed that the native enzyme mainly exists as dimer of 27 kDa subunits. Optimal activity was found around 40 °C, and at pH 5.2. The enzyme is dependent on the presence of bivalent metal ions, like Zn^{2+} or Co^{2+}. The half life is estimated as 25 hours at 37 °C. The Ca-ALD binds acetolactate cooperatively with a Hill coefficient of 1.49. Also, a $K_{1/2}$ of 16.8 mM and a V_{max} of 51.9 U/mg was determined. Furthermore, a shuttle vector was constructed to express *Ca-ald* under control of the strong *adc* promoter in *C. acetobutylicum ATCC 824*. However, despite successful transformation, no significant increase in acetoin production was observed in the *Ca-ALD* overexpressing strain.

Only one additional enzyme is needed to complete the 2,3-butanediol biosynthesis pathway in *C. acetobutylicum*. This enzyme, catalyzing the reduction of acetoin to 2,3-butandiol is called an acetoin reductase. **Chapter 5** describes the identification, heterologous production, purification and biochemical characterization of an acetoin reductase from *C. beijerinckii*. A bioinformatic screening within the genome of *C. beijerinckii* , revealed eight putative acetoin reductases. Out of six successfully cloned genes, one (*CBEI_1464*) showed substantial acetoin reductase activity after heterologous

expression in *E. coli*. This purified enzyme (Cb-ACR) was found to exist predominantly as homodimer of 37 kDa subunits. The enzyme has a preference for NADPH (K_m = 0.32 µM) as electron donor, with a specific activity amounting to 76 U. mg^{-1}. Optimal activity was found around 68 °C, for both reactions and at pH 6.5 and 9.5, for the reduction and oxidation reaction, respectively. ICP-AES analysis revealed the presence of ~2 Zn^{2+} atoms and ~1 Ca^{2+} atom per monomer. To gain insight into the reaction mechanism, but also into the substrate- and cofactor-specificity, a structural model was constructed with a ketose reductase (sorbitol dehydrogenase) from *Bemisia argentifolii* (silverleaf whitefly) as template. The catalytic zinc atom is likely coordinated by Cys37, His70, Glu71 in Cb-ACR, while the structural zinc site is probably composed of Cys100, Cys103, Cys106, and Cys114.

The acetoin reductase (Cb-ACR) found in *C. beijerinckii* is used for *in vivo* experiments in *C. acetobutylicum*. In **Chapter 6** the production of D-2,3-butanediol production in *C. acetobutylicum ATCC 824* by heterologous expression of Cb-ACR is described. Under certain conditions *Clostridium acetobutylicum* ATCC 824 (and derived strains) generates both D- and L-stereoisomers of acetoin, but due to the lack of an ACR enzyme, does not produce 2,3-butanediol. A gene encoding ACR from *Clostridium beijerinckii* NCIMB 8052 has been functionally expressed in *C. acetobutylicum* under control of two strong promoters, *i.e.* the constitutive *thl* promoter and the late exponential *adc* promoter. Both ACR-overproducing strains have been grown in batch cultures, during which 89-90% of the natively produced acetoin has been converted to 20-22 mM D-2,3-butanediol. Addition of a racemic mixture of acetoin did lead to the production of both, D-2,3-butanediol and *meso*-2,3-butanediol. A metabolic network is proposed that is in agreement with the experimental data. Native 2,3-butanediol production is a first step

towards a potential homo-fermentative 2-butanol producing strain of *C. acetobutylicum* as will be discussed below.

General discussion

In industrial ABE fermentations, solventogenic clostridia in general are exposed to relatively harsh environments. This results in unwanted stress, and therefore in a sub-optimal fermentation performance. The overall aim of the project described in this thesis involves the chemical stress on these clostridia. Important stress factors that generally lead to this sub-optimal fermentation performance are: (i) concentration of substrates, and (ii) concentration of products (*viz*. certain products and/or by-products may lead to severe inhibition of the fermentation process). Engineering solventogenic clostridia in an attempt to reduce the stress, resulting in higher efficiency of the industrial ABE process, was thought to result in higher yields of the products 1-butanol and/or derivatives thereof. 1-Butanol and its derivatives were chosen, since these become more and more attractive as biofuel or biofuel additive. 1-Butanol is a highly relevant sustainable feedstock for transport fuel, especially for gasoline.

Economically, the current 1-butanol production is not viable, since feedstock and product recovery costs are too high and only 26% of the theoretical maximum of 41% conversion of glucose to 1-butanol is reached. Apart from the subjects involved in the overall project, like (i) isolating new strains with increased 1-butanol production and increased tolerance to 1-butanol, and (ii) obtaining single route mutants towards 1-butanol of known species, this thesis concentrates on 1-butanol stress related issues. It focuses on the identification of alternative less toxic 1-butanol derivatives. The most attractive compounds (*iso*-butanol, 2-butanol, *tert*-butanol and 2,3-butanediol) were selected based on their toxicity but also on the feasibility to produce

them using existing enzymatic steps. Metabolic adaptations to the existing pathways, involving these enzymes, were designed and subsequently engineered into *C. acetobutylicum* (Figure 27). Only natural biosynthetic pathways could be defined for *iso*-butanol, 2,3-butanediol and 2-butanol, while no biosynthetic pathway could be defined for *tert*-butanol.

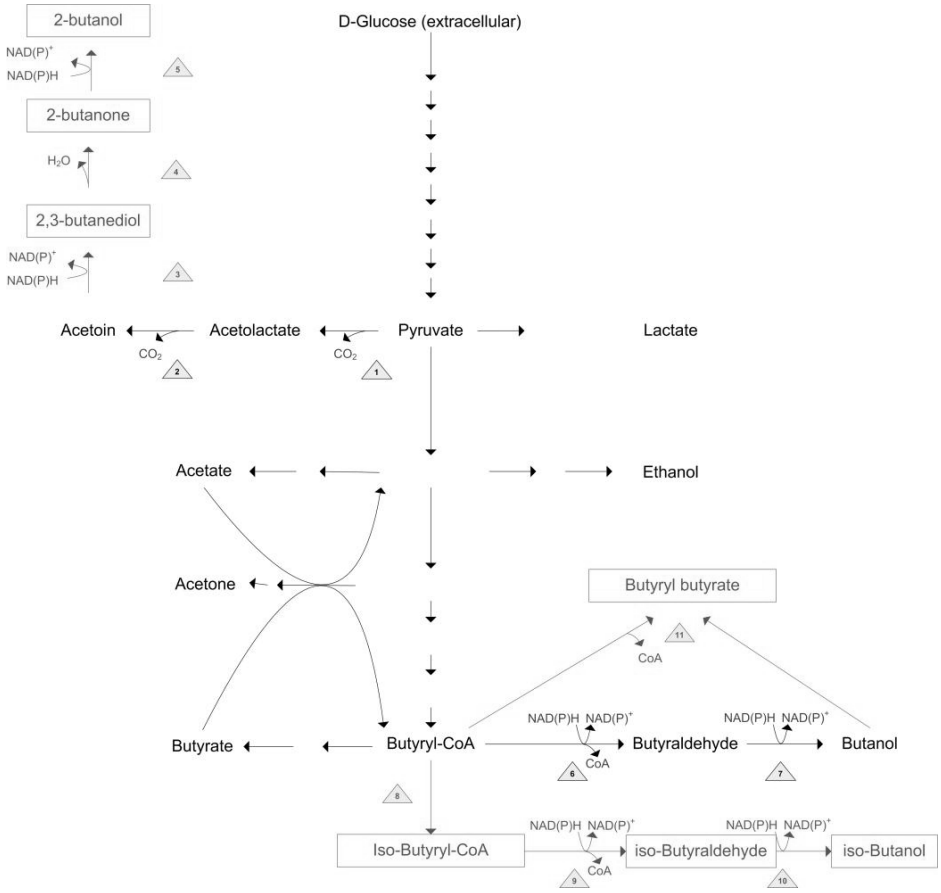

Figure 27: Simplified adapted central glycolytic metabolism of two solventogenic Clostridia: *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052, modified from Gheshlaghi [19]. Numbers in triangles show enzymes involved in the modified biosynthetic pathways. 1: Acetolactate synthase; 2: Acetolactate decarboxylase; 3: Acetoin reductase; 4: 2,3-butanediol dehydratase; 5: 2-butanone reductase; 6: Alcohol-aldehyde dehydrogenase (AADH), 7: Butanol dehydrogenase (BDH I,II), 8: *Iso*-butyryl-CoA mutase; 9: Alcohol-aldehyde dehydrogenase (AADH), 10: *Iso*-butanol dehydrogenase; 11: Alcohol acetyl transferase.

Novel developments and future perspectives in ABE fermentation

Iso-butanol

The 1-butanol isomer, *iso*-butanol is also a primary alcohol, with only a small difference in the log K_{ow} value (0.76 vs. 0.88, respectively). Nevertheless, *iso*-butanol appears to be a little less toxic than 1-butanol and is therefore an interesting 1-butanol replacer with similar chemical properties. In nature, the antibiotic monensin A producing *Streptomyces* sp., are known to promote the interconversion of n-butyrate and *iso*-butyrate [136]. These species harbour *iso*-butyryl-CoA mutase, catalyzing the interconversion of n-butyryl-CoA to *iso*butyryl-CoA. In this reaction, a CO-S-CoA group migrates to an adjacent methyl group, while a hydrogen atom is transferred in the reverse direction [138]. Since, butyryl-CoA is a central precursor for 1-butanol, this pathway could be elongated to *iso*-butyryl-CoA by introducting the enzyme *iso*-butyryl-CoA mutase in solventogenic Clostridia. Two subsequent reduction steps are needed to produce *iso*-butanol, namely the reduction steps of *iso*-butyraldehyde-CoA to *iso*-butyraldehyde and from *iso*-butyraldehyde to *iso*-butanol. The responsible enzyme consist of two subunits and is coenzyme B_{12} dependent. Interestingly, the *C. acetobutylicum* genome contains the genes for vitamin B_{12} (anaerobic) biosynthesis [93, 226]. Other related *Clostridium* sp. were found to possess coenzyme B_{12} dependent enzymes [227-232], suggesting its potential to produce coenzyme B_{12} within *C. acetobutylicum*. Nevertheless, one should keep in mind the difference in GC-content between the species *Streptomyces coelicolor* and *C. acetobutylicum* (70% vs. 30%, respectively). Thus, for expression of the *iso*butyryl-CoA mutase operon from *Streptomyces cinnamonensis* in *C. acetobutylicum* ATCC824 an optimization of the codon usage is needed.

2,3-Butanediol / 2-butanol

The two 1-butanol derivatives, 2,3-butanediol and 2-butanol, show already bigger differences in their log K_{ow} values (-0.29 and 0.61 vs. 0.88) and appear also to be less toxic than 1-butanol to *C. acetobutylicum*. 2-butanol is also a good replacer for 1-butanol with similar chemical properties, while 2,3-butanediol has a lower energy of combustion, since it is more oxidized. Nevertheless, these compounds are in a biological sense interesting, since a 2,3-butanediol biosynthesis pathway is known and described extensively [62, 66, 67, 233]. Furthermore, 2-butanol can be produced from the reduction of 2-butanone, which can be produced by a dehydration step of 2,3-butanediol catalysed by diol dehydratases.

As is described in **chapter 6**, we succeeded in the production D-2,3-butanediol in *C. acetobutylicum* by a reduction of acetoin, a minor fermentation product in *C. acetobutylicum ATCC 824* [1]. Dehydration of 2,3-butanediol, however, has only been observed with *meso*-2,3-butanediol, as substrate, while D-2,3-butanediol inhibits this reaction [234]. Since D-2,3-butanediol is the main isomer of 2,3-butanediol in the mutants of *C. acetobutylicum* expressing Cb-ACR, metabolic engineering is still necessary. Nevertheless, **chapter 5** reveals also that Cb-ACR is not stereoselective, racemic acetoin is reduced to both, D-2,3-butanediol and *meso*-2,3-butanediol by Cb-ACR. Therefore, engineering the steps before the reduction, e.g. from pyruvate to acetolactate towards L-acetoin production could lead to *meso*-2,3-butanediol production in Cb-ACR expressing *C. acetobutylicum* strains. Subsequently, 2-butanone reductase activity is needed to reduce 2-butanone to 2-butanol. This reaction might be catalyzed by the acetoin reductase (Cb-ACR) as well, because it was shown (**Chapter 5**) that the acetoin reductase accepts also 2-butanone, however to a lower extent.

Homo-fermentative production of D-2,3-butanediol production is not possible regarding the redox balance in *C. acetobutylicum*, while homo-fermentative production of 2-butanol is possible. In other words, 2,3-butanediol production is always accompanied with other reduced (unwanted) by-products. Elongating the 2,3-butanediol pathway towards 2-butanol circumvents this problem by reducing 2-butanone to 2-butanol.

Although only *meso*-2,3-butandediol can be dehydrated enzymatically, the D-2,3-butanediol isomer remains interesting for chemical dehydration. The product 2-butanone, can also be used as fuel additive [62].

A step towards improving production of 2,3-butanediol in our system would be to increase the flux towards acetoin. Doremus *et al.* observed acetoin accumulation in the ABE-fermentation under conditions of low agitation or high partial pressure of H_2 [212]. These observations could be the starting point for further pathway optimization to increase the flux towards acetoin and 2,3-butanediol in these new mutants of *C. acetobutylicum*. Preliminary micro-array data obtained with above described conditions (not shown), indicate that the genes coding for the enzymes of the two acetoin biosynthesis pathways (anabolic and catabolic) were differentially expressed (CAC3169 or *ilvB* and CAC3176 or *ilvN,* CAC3652 and CAC2967). These higher fluxes to acetoin in the conducted experiments seem to be the result of a higher expression of *ilvB* and *ilvN* encoding acetolactate synthase, and CAC2967 encoding acetolactate decarboxylase. Overexpression of these enzymes together could result in a higher stereospecific acetoin / 2,3-butanediol production.

Butyl acetate / butyl butyrate / butyl lactate

The 1-butanol derived esters, butyl acetate, butyl butyrate, and butyl lactate have log K_{ow} values higher than 1-butanol and indeed appeared to be

more toxic (chapter 3). In that respect, these compound are not interesting. On the other hand, these compounds are more attractive as biofuel additive than 1-butanol, due to their higher energies of combustion. Therefore, in ABE fermentations, the three above described esters remain interesting as 1-butanol replacements.

Esters are formed by the condensation of acids with alcohols by esters and lipases. In the presence of water, they catalyze the hydrolysis of an ester bond resulting in the formation of an alcohol and a carboxylic acid. However, in an organic solvent, they can catalyze the reverse reaction or even a trans-esterification reaction [235]. Since the secreted end products in the ABE fermentation are mainly the two fatty acids, acetate and butyrate, together with the two alcohols, ethanol and 1-butanol, the formation of esters by esterases could still be rather interesting. One of the additional challenges is that solventogenic clostridia only grow in a watery environments, which makes extracellular hydrolysis of esters very likely. As a matter of fact, the added butyl butyrate and butyl acetate in the toxicity tests, described in **chapter 3** were found to be hydrolysed, apparently by secreted esterases / lipases of *C. acetobutylicum.*

However, it might be possible to design a two phase system in which the cells are growing in the watery phase, while secreted engineered esterases/lipases with non-polar surfaces are only present in a non-polar phase, leading to esters by condensation of produced fatty acids and alcohols in the ABE fermentation. Simultaneously, 1-butanol concentrations are kept low, leading to higher efficiencies.

Another way of producing esters is via the help of alcohol acetyltransferase. This enzyme catalyzes the coupling of the acetyl moiety of acetyl-CoA to an alcohol substrate in watery environments. Of all known

alcohol acetyl transferases, ATF1 gene (YOR377w) is the most important one for the production of flavour-active acetate esters in *Saccharomyces cerevisiae*. While it has high activity with *iso*-amyl alcohol, also activity was found with C_2-C_8 acetate esters [236]. Horton *et al.* published already the heterologous expression of ATF2 from *S. cerevisiae* in *C. acetobutylicum* with *iso*-amyl alcohol as substrate. It was shown that *iso*-amyl acetate was produced in time. However, as described above, Horton *et al*. also observed degradation of the produced esters by secreted esterases/lipases from *C. acetobutylicum* [237]. In the future, metabolic engineering by deleting the responsible ester(s)/lipase(s), is possible option to produce esters via this route. Two phase systems could result in separation of the products from the cells, or even improve down stream processing events. Production of butyl butyrate in a two phase system by optimization of an alcohol acetyl transferases to couple the butyryl moiety of butyryl-CoA to 1-butanol, in combination with knock-outs of the responsible clostridial esterases / lipases, would be, in my opinion, the most ideal situation in modern ABE fermentation.

References

1. **Jones, D. T., Woods, D. R.** (1986). Acetone-butanol fermentation revisited. Microbiol Rev **50**, 484-524.
2. **Dürre, P. (ed.).** 2005. Handbook on Clostridia. Taylor & Francis Group, Boca Raton, FL.
3. **Ezeji, T. C., Qureshi, N., Blaschek, H. P.** (2004). Butanol fermentation research: upstream and downstream manipulations. Chem Rec **4**, 305-14.
4. **Chiao, J. S., Sun, Z. H.** (2007). History of the acetone-butanol-ethanol fermentation industry in China: development of continuous production technology. J Mol Microbiol Biotechnol **13**, 12-4.
5. **Zverlov, V. V., Berezina, O., Velikodvorskaya, G. A., Schwarz, W. H.** (2006). Bacterial acetone and butanol production by industrial fermentation in the Soviet Union: use of hydrolyzed agricultural waste for biorefinery. Appl Microbiol Biotechnol **71**, 587-97.
6. **Ni, Y., Sun, Z.** (2009). Recent progress on industrial fermentative production of acetone-butanol-ethanol by *Clostridium acetobutylicum* in China. Appl Microbiol Biotechnol **83**, 415-23.
7. **Rose, A. H.** 1961. Acetone-butanol fermentation, p. 160-166. *In* A. H. Rose (ed.), Industrial Microbiology. Butterworth, London.
8. **Jones, D. T., Woods, D. R.** (1986). Acetone-butanol fermentation revisited. Microbiological Reviews **50**, 484-524.
9. **Dürre, P., Bahl, H.** 1996. Microbial production of acetone/butanol/isopropanol, p. 229-268, Products of primary metabolism, second ed. VCH Publisher, Weinheim, Germany.
10. **Dürre, P.** (1998). New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation. Applied Microbiology and Biotechnology **49**, 639-648.

11. **Walton, M. T., Martin, J. L.** 1979. Production of Butanol-Acetone by Fermentation, p. 187-209. *In* H. J. Peppler,D. Perlman (ed.), Microbial Technology, 2 ed, vol. 1. Academic press, New York.

12. **Jones, D. T.** 2001. Applied acetone-butanol fermentation, p. 125-168. *In* H. Bahl,P. Dürre (ed.), Clostridia. Biotechnology and medical application. Wiley-VCH Verlag GmbH, Weinheim, Germany.

13. **Rogers, P., Chen, J.-S., Zidwick, M. J.** 2006. Organic acid and solvent production. Part III: Butanol, acetone and iso-propanol; 1,3 and 1,2 propanediol production; and 2,3 butanediol production, p. 672-755. *In* M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, E. Stackebrandt (ed.), The Prokaryotes: Symbiotic associations, biotechnology, applied microbiology, 3 ed, vol. 1. Springer.

14. **Jones, D. T., Keis, S.** (1995). Origins and relationship of industrial solvent-producing clostridial strains. FEMS Microbiology Reviews **17**, 233-240.

15. **Johnson, J. L., Chen, J.-S.** (1995). Taxonomic relationships among strains of *Clostridium acetobutylicum* and other phenotypically similar organisms. FEMS Microbiol Rev 233-240.

16. **Johnson, J. L., Toth, J., Santiwatanakul, S., Chen, J. S.** (1997). Cultures of "*Clostridium acetobutylicum*" from various collections comprise *Clostridium acetobutylicum*, *Clostridium beijerinckii*, and two other distinct types based on DNA-DNA reassociation. Int J Syst Bacteriol 47, 420-4.

17. **Jones, D. T., Keis, S.** (1995). Origins and relationship of industrial solvent-producing clostridial strains. FEMS Microbiol Rev 233-240.

18. **Keis, S., Shaheen, R., Jones, D. T.** (2001). Emended descriptions of *Clostridium acetobutylicum* and *Clostridium beijerinckii*, and descriptions

of *Clostridium saccharoperbutylacetonicum* sp. nov. and *Clostridium saccharobutylicum* sp. nov. Int J Syst Evol Microbiol **51**, 2095-103.

19. **Gheshlaghi, R., Scharer, J. M., Moo-Young, M., Chou, C. P.** (2009). Metabolic pathways of clostridia for producing butanol. Biotechnol Adv **27**, 764-81.
20. **Roos, J. W., McLaughlin, J. K., Papoutsakis, E. T.** (1985). The effect of pHon nitrogen supply, cell lysis, and solvent production in fermentations of *C. acetobutylicum*. Biotechnology and Bioengineering **27**, 681-694.
21. **Andreesen, J. R., Bahl, H., Gottschalk, G.** 1989. Introduction to the Physiology and Biochemistry of the Genus *Clostridium*. *In* N. P. Minton,D. J. Clark (ed.), Biotechnology Handbooks, vol. 3. Plenum press, New York.
22. **Meyer, C. L., Papoutsakis, E. T.** (1989). Increased levels of ATP and NADH are associated with increased solvent production in continuous cultures of *Clostridium acetobutylicum*. Applied Microbiology and Biotechnology **30**, 450-459.
23. **Green, E. M., Boynton, Z. L., Harris, L. M., Rudolph, F. B., Papoutsakis, E. T.,Bennett, G. N.** (1996). Genetic manipulation of acid formation pathways by gene inactivation in *Clostridium acetobutylicum* ATCC 824. Microbiology **142 (Pt 8)**, 2079-86.
24. **Ravagnani, A., Jennert, K. C., Steiner, E., Grunberg, R., Jefferies, J. R., Wilkinson, S. R., Young, D. I., Tidswell, E. C., Brown, D. P., Youngman, P., Morris, J. G., Young, M.** (2000). *Spo0A* directly controls the switch from acid to solvent production in solvent-forming clostridia. Mol Microbiol **37**, 1172-85.
25. **Woods, D. R.** (1995). The genetic engineering of microbial solvent production. Trends Biotechnol **13**, 259-64.
26. **Girbal, L.,Soucaille, P.** (1998). Regulation of solvent production in *Clostridium acetobutylicum*. Trends in Biotechnology **16**, 11-16.

27. **Cornillot, E., Nair, R. V., Papoutsakis, E. T., Soucaille, P.** (1997). The genes for butanol and acetone formation in *Clostridium acetobutylicum* ATCC 824 reside on a large plasmid whose loss leads to degeneration of the strain. J Bacteriol **179**, 5442-7.

28. **Nolling, J., Breton, G., Omelchenko, M. V., Makarova, K. S., Zeng, Q., Gibson, R., Lee, H. M., Dubois, J., Qiu, D., Hitti, J., Wolf, Y. I., Tatusov, R. L., Sabathe, F., Doucette-Stamm, L., Soucaille, P., Daly, M. J., Bennett, G. N., Koonin, E. V.,Smith, D. R.** (2001). Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. J Bacteriol **183**, 4823-38.

29. **Bowles, L. K., Ellefson, W. L.** (1985). Effects of butanol on *Clostridium acetobutylicum*. Appl Environ Microbiol **50**, 1165-70.

30. **Vollherbst-Schneck, K., Sands, J. A., Montenecourt, B. S.** (1984). Effect of butanol on lipid composition and fluidity of *Clostridium acetobutylicum* ATCC 824. Appl Environ Microbiol **47**, 193-4.

31. **Vermue, M., Sikkema, J., Verheul, A., Bakker, R., Tramper, J.** (1993). Toxicity of homologous series of organic solvents for the gram-positive bacteria *Arthrobacter* and *Nocardia* Sp. and the gram-negative bacteria *Acinetobacter* and *Pseudomonas* Sp. Biotechnol Bioeng **42**, 747-58.

32. **Demain, A. L., Adrio, J. L.** (2008). Contributions of microorganisms to industrial biology. Mol Biotechnol **38**, 41-55.

33. **Allcock, E. R., Reid, S. J., Jones, D. T., Woods, D. R.** (1982). *Clostridium acetobutylicum* protoplast formation and regeneration. Applied and Environmental Microbiology **43**, 719-721.

34. **Hermann, M., Fayolle, F., Marchal, R., Podvin, L., Sebald, M.,Vandecasteele, J. P.** (1985). Isolation and characterization of butanol-resistant mutants of *Clostridium acetobutylicum*. Appl Environ Microbiol **50**, 1238-43.

35. **Lin, Y. L., Blaschek, H. P.** (1983). Butanol Production by a Butanol-Tolerant Strain of *Clostridium acetobutylicum* in Extruded Corn Broth. Appl Environ Microbiol **45**, 966-973.

36. **Baer.** (1987). Effect of Butanol Challenge and Temperature on Lipid Composition and Membrane Fluidity of Butanol-Tolerant *Clostridium acetobutylicum*.

37. **Tomas, C. A., Welker, N. E., Papoutsakis, E. T.** (2003). Overexpression of groESL in *Clostridium acetobutylicum* results in increased solvent production and tolerance, prolonged metabolism, and changes in the cell's transcriptional program. Appl Environ Microbiol **69**, 4951-65.

38. **Borden, J. R., Papoutsakis, E. T.** (2007). Dynamics of genomic-library enrichment and identification of solvent-tolerance genes in *Clostridium acetobutylicum*. Appl Environ Microbiol

39. **Antoni, D., Zverlov, V. V., Schwarz, W. H.** (2007). Biofuels from microbes. Appl Microbiol Biotechnol **77**, 23-35.

40. **Carere, C. R., Sparling, R., Cicek, N., Levin, D. B.** (2008). Third Generation Biofuels via Direct Cellulose Fermentation. Int J Mol Sci **9**, 1342-60.

41. **Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K., Kim, J., Jung, K. S.** (2008). Fermentative butanol production by Clostridia. Biotechnol Bioeng **101**, 209-28.

42. **Dufey, A.** (2006). Biofuels production, trade and sustainable development: emerging issues.

43. **Barnard, D.** (2010). Extremophiles in Biofuel Synthesis.

44. **Solomon, B. D.** (2010). Biofuels and sustainability. Ann N Y Acad Sci **1185**, 119-34.

45. **Alper, H., Stephanopoulos, G.** (2009). Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential? Nat Rev Microbiol **7**, 715-23.

46. **Clomburg, J. M., Gonzalez, R.** (2010). Biofuel production in *Escherichia coli*: the role of metabolic engineering and synthetic biology. Appl Microbiol Biotechnol **86**, 419-34.

47. **Atsumi, S., Cann, A. F., Connor, M. R., Shen, C. R., Smith, K. M., Brynildsen, M. P., Chou, K. J., Hanai, T., Liao, J. C.** (2007). Metabolic engineering of *Escherichia coli* for 1-butanol production. Metab Eng

48. **Atsumi, S., Li, Z., Liao, J. C.** (2009). Acetolactate synthase from Bacillus subtilis serves as a 2-ketoisovalerate decarboxylase for isobutanol biosynthesis in *Escherichia coli*. Appl Environ Microbiol **75**, 6306-11.

49. **Cann, A. F., Liao, J. C.** (2008). Production of 2-methyl-1-butanol in engineered *Escherichia coli*. Appl Microbiol Biotechnol

50. **Cann, A. F., Liao, J. C.** (2010). Pentanol isomer synthesis in engineered microorganisms. Appl Microbiol Biotechnol **85**, 893-9.

51. **Connor, M. R., Liao, J. C.** (2008). Engineering of an *Escherichia coli* strain for the production of 3-methyl-1-butanol. Appl Environ Microbiol **74**, 5769-75.

52. **Connor, M. R., Cann, A. F., Liao, J. C.** (2010). 3-Methyl-1-butanol production in *Escherichia coli*: random mutagenesis and two-phase fermentation. Appl Microbiol Biotechnol **86**, 1155-64.

53. **Hanai, T., Atsumi, S., Liao, J. C.** (2007). Engineered synthetic pathway for isopropanol production in *Escherichia coli*. Appl Environ Microbiol

54. **Huang, K., Rudolph, F. B., Bennett, G. N.** (1999). Characterization of methylglyoxal synthase from *Clostridium acetobutylicum* ATCC 824 and its use in the formation of 1, 2-propanediol. Appl Environ Microbiol **65**, 3244-7.

55. **Inui, M., Suda, M., Kimura, S., Yasuda, K., Suzuki, H., Toda, H., Yamamoto, S., Okino, S., Suzuki, N., Yukawa, H.** (2007). Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*. Appl Microbiol Biotechnol

56. **Jojima, T., Inui, M., Yukawa, H.** (2007). Production of isopropanol by metabolically engineered *Escherichia coli*. Appl Microbiol Biotechnol

57. **Nielsen, D. R., Yoon, S. H., Yuan, C. J., Prather, K. L.** (2010). Metabolic engineering of acetoin and *meso*-2, 3-butanediol biosynthesis in E. *coli*. Biotechnol J **5**, 274-84.

58. **Shen, C. R., Liao, J. C.** (2008). Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. Metab Eng

59. **Tang, X., Tan, Y., Zhu, H., Zhao, K., Shen, W.** (2009). Microbial conversion of glycerol to 1,3-propanediol by a engineered strain of *Escherichia coli*. Appl Environ Microbiol

60. **Tong, I. T., Liao, H. H., Cameron, D. C.** (1991). 1,3-Propanediol production by *Escherichia coli* expressing genes from the *Klebsiella pneumoniae* dha regulon. Appl Environ Microbiol **57**, 3541-6.

61. **Yan, Y., Lee, C. C., Liao, J. C.** (2009). Enantioselective synthesis of pure (R,R)-2,3-butanediol in *Escherichia coli* with stereospecific secondary alcohol dehydrogenases. Org Biomol Chem **7**, 3914-7.

62. **Syu, M. J.** (2001). Biological production of 2,3-butanediol. Appl Microbiol Biotechnol **55**, 10-8.

63. **Voloch, M., Jansen N.B., Ladisch M.R. Tsao, G.T. Narayan, R. and Rodwell, V.W.** 1985. 2,3-Butanediol, Comprehensive Biotechnology: The principles , Applications and Regulations of Biotechnology in Industry, Agriculture and Medicine. Pergamon Press, Oxford.

64. **Celinska, E., Grajek, W.** (2009). Biotechnological production of 2,3-butanediol--current state and prospects. Biotechnol Adv **27**, 715-25.

65. **O'Neil, M. J., Smith, A., Heckelman, P. E. (ed.).** 2001. The Merck index. Merck & Co, INC, Whitehouse Station.

66. **Xiu, Z. L., Zeng, A. P.** (2008). Present state and perspective of downstream processing of biologically produced 1,3-propanediol and 2,3-butanediol. Appl Microbiol Biotechnol **78**, 917-926.

67. **Garg, S. K., Jain A.** (1995). Fermentative production of 2,3-butanediol: A review. Bioresour Technol **51**, 103-109.

68. **Wardwell, S. A., Yang, Y. T., Chang, H. Y., San, K. Y., Rudolph, F. B.,Bennett, G. N.** (2001). Expression of the *Klebsiella pneumoniae* CG21 acetoin reductase gene in *Clostridium acetobutylicum* ATCC 824. J Ind Microbiol Biotechnol **27**, 220-7.

69. **Xiao, Z., Xu, P.** (2007). Acetoin metabolism in bacteria. Crit Rev Microbiol **33**, 127-40.

70. **Crout, D. H. G., Littlechild, J., Morrey, S. M.** (1986). Acetoin Metabolism - Stereochemistry of the Acetoin Produced by the Pyruvate Decarboxylase of Wheat-Germ and by the Alpha-Acetolactate Decarboxylase of *Klebsiella* Aerogenes. Journal of the Chemical Society-Perkin Transactions 1 105-108.

71. **Qin, J.** (2006). Production of 2,3-butanediol by *Klebsiella pneumoniae* using glucose and ammonium phosphate.

72. **Ma, C., Wang, A., Qin, J., Li, L., Ai, X., Jiang, T., Tang, H., Xu, P.** (2009). Enhanced 2,3-butanediol production by *Klebsiella pneumoniae* SDM. Appl Microbiol Biotechnol **82**, 49-57.

73. **Yu, E. K., Saddler, J. N.** (1982). Enhanced production of 2,3-butanediol by *Klebsiella pneumoniae* grown on high sugar concentrations in the presence of acetic acid. Appl Environ Microbiol **44**, 777-784.

74. **Laube, V. M., Groleau, D., Martin, S. M.** (1984). 2,3-Butanediol production from xylose and other hemicellulosic components by *Bacillus polymyxa*. Biotechnology Letters **6**, 257-262.

75. **Yu, E. K., Saddler, J. N.** (1983). Fed-batch approach to production of 2,3-butanediol by *Klebsiella pneumoniae* grown on high substrate concentrations. Appl Environ Microbiol **46**, 630-5.

76. **Grover, B. P.** (1990). Production of 2,3-butanediol from wood hydrolysate by *Klebsiella pneumoniae*. World J Microbiol Biotechno 328-332.

77. **Fond, O., Jansen, N.B., and Tsao G.T.** (1985). A model of acetic acid and 2,3-butanediol inhibition of the growth and metabolism of *Klebsiella oxytoca*. Biotechnology Letters **7**, 727-732.

78. **Sun, L. H., Wang, X. D., Dai, J. Y., Xiu, Z. L.** (2009). Microbial production of 2,3-butanediol from Jerusalem artichoke tubers by *Klebsiella pneumoniae*. Appl Microbiol Biotechnol **82**, 847-52.

79. **Sun, L. H., Jiang, B., Xiu, Z. L.** (2009). Aqueous two-phase extraction of 2,3-butanediol from fermentation broths by isopropanol/ammonium sulfate system. Biotechnol Lett **31**, 371-6.

80. **Petrov, K., Petrova, P.** (2009). High production of 2,3-butanediol from glycerol by *Klebsiella pneumoniae* G31. Appl Microbiol Biotechnol

81. **Ji, X. J., Huang, H., Du, J., Zhu, J. G., Ren, L. J., Hu, N.,Li, S.** (2009). Enhanced 2,3-butanediol production by *Klebsiella* oxytoca using a two-stage agitation speed control strategy. Bioresour Technol

82. **Anvari, M., Khayati, G.** (2009). In situ recovery of 2,3-butanediol from fermentation by liquid-liquid extraction. Journal of Industrial Microbiology and Biotechnology **in press**,

83. **Qin, J.** (2006). Production of 2,3-butanediol by *Klebsiella pneumoniae* using glucose and ammonium phosphate. Chinese Journal of Chemical Engineering **14**, 132-136.

84. **Qureshi, N.** (1994). Recovery of 2,3-Butanediol by Vacuum Membrane Distillation. Separation Science and Technology **29**, 1733-1748.

85. **Syu, M. J.** (2001). Biological production of 2,3-butanediol. Applied microbiology and biotechnology **55**, 10-18.

86. **Jones, S. W., Paredes, C. J., Tracy, B., Cheng, N., Sillers, R., Senger, R. S., Papoutsakis, E. T.** (2008). The transcriptional program underlying the physiology of clostridial sporulation. Genome Biol **9**, R114.

87. **Tangney, M., Mitchell, W. J.** (2007). Characterisation of a glucose phosphotransferase system in *Clostridium acetobutylicum* ATCC 824. Appl Microbiol Biotechnol **74**, 398-405.

88. **Tomas, C. A., Alsaker, K. V., Bonarius, H. P., Hendriksen, W. T., Yang, H., Beamish, J. A., Paredes, C. J., Papoutsakis, E. T.** (2003). DNA array-based transcriptional analysis of asporogenous, nonsolventogenic *Clostridium acetobutylicum* strains SKO1 and M5. J Bacteriol **185**, 4539-47.

89. **Alsaker, K. V., Papoutsakis, E. T.** (2005). Transcriptional program of early sporulation and stationary-phase events in *Clostridium acetobutylicum*. J Bacteriol **187**, 7103-18.

90. **Alsaker, K. V., Spitzer, T. R., Papoutsakis, E. T.** (2004). Transcriptional analysis of spo0A overexpression in *Clostridium acetobutylicum* and its effect on the cell's response to butanol stress. J Bacteriol **186**, 1959-71.

91. **Tomas, C. A., Beamish, J., Papoutsakis, E. T.** (2004). Transcriptional analysis of butanol stress and tolerance in *Clostridium acetobutylicum*. J Bacteriol **186**, 2006-18.

92. **Tummala, S. B., Junne, S. G., Paredes, C. J., Papoutsakis, E. T.** (2003). Transcriptional analysis of product-concentration driven changes in

cellular programs of recombinant *Clostridium acetobutylicum*strains. Biotechnol Bioeng **84**, 842-54.

93. **Paredes, C. J., Rigoutsos, I., Papoutsakis, E. T.** (2004). Transcriptional organization of the *Clostridium acetobutylicum* genome. Nucleic Acids Res **32**, 1973-81.

94. **Fiedler, T., Mix, M., Meyer, U., Mikkat, S., Glocker, M. O., Bahl, H., Fischer, R. J.** (2008). The two-component system PhoPR of *Clostridium acetobutylicum* is involved in phosphate-dependent gene regulation. J Bacteriol **190**, 6559-67.

95. **Zhao, Y., Tomas, C. A., Rudolph, F. B., Papoutsakis, E. T., Bennett, G. N.** (2005). Intracellular butyryl phosphate and acetyl phosphate concentrations in *Clostridium acetobutylicum* and their implications for solvent formation. Appl Environ Microbiol **71**, 530-7.

96. **Kosaka, T., Uchiyama, T., Ishii, S., Enoki, M., Imachi, H., Kamagata, Y., Ohashi, A., Harada, H., Ikenaga, H., Watanabe, K.** (2006). Reconstruction and regulation of the central catabolic pathway in the thermophilic propionate-oxidizing syntroph Pelotomaculum thermopropionicum. J Bacteriol **188**, 202-10.

97. **Tummala, S. B., Junne, S. G., Papoutsakis, E. T.** (2003). Antisense RNA downregulation of coenzyme A transferase combined with alcohol-aldehyde dehydrogenase overexpression leads to predominantly alcohologenic *Clostridium acetobutylicum* fermentations. J Bacteriol **185**, 3644-53.

98. **Hillmann, F., Riebe, O., Fischer, R. J., Mot, A., Caranto, J. D., Kurtz, D. M., Jr., Bahl, H.** (2009). Reductive dioxygen scavenging by flavo-diiron proteins of *Clostridium acetobutylicum*. FEBS Lett **583**, 241-5.

99. **Kawasaki, S., Sakai, Y., Takahashi, T., Suzuki, I., Niimura, Y.** (2009). O2 and reactive oxygen species detoxification complex, composed of O2-

responsive NADH:rubredoxin oxidoreductase-flavoprotein A2-desulfoferrodoxin operon enzymes, rubperoxin, and rubredoxin, in *Clostridium acetobutylicum*. Appl Environ Microbiol **75**, 1021-9.

100. **Kawasaki, S., Watamura, Y., Ono, M., Watanabe, T., Takeda, K., Niimura, Y.** (2005). Adaptive Responses to Oxygen Stress in Obligatory Anaerobes *Clostridium acetobutylicum* and *Clostridium* aminovalericum. Appl Environ Microbiol **71**, 8442-50.
101. **Riebe, O., Fischer, R. J., Wampler, D. A., Kurtz, D. M., Jr., Bahl, H.** (2009). Pathway for H2O2 and O2 detoxification in *Clostridium acetobutylicum*. Microbiology **155**, 16-24.
102. **Gorwa, M. F., Croux, C., Soucaille, P.** (1996). Molecular characterization and transcriptional analysis of the putative hydrogenase gene of *Clostridium acetobutylicum* ATCC 824. J Bacteriol **178**, 2668-75.
103. **Huang, K. X., Huang, S., Rudolph, F. B.,Bennett, G. N.** (2000). Identification and characterization of a second butyrate kinase from *Clostridium acetobutylicum* ATCC 824. J Mol Microbiol Biotechnol **2**, 33-8.
104. **Petitdemange, H., Cherrier, C., Bengone, J. M., Gay, R.** (1977). [Study of the NADH and NADPH-ferredoxin oxidoreductase activities in *Clostridium acetobutylicum*]. Can J Microbiol **23**, 152-60.
105. **Vasconcelos, I., Girbal, L., Soucaille, P.** (1994). Regulation of carbon and electron flow in *Clostridium acetobutylicum* grown in chemostat culture at neutral pH on mixtures of glucose and glycerol. J Bacteriol **176**, 1443-50.
106. **Sullivan, L., Bennett, G. N.** (2005). Proteome analysis and comparison of *Clostridium acetobutylicum* ATCC 824 and Spo0A strain variants. J Ind Microbiol Biotechnol 1-11.

107. **Schreiber.** (2000). Differential Expression of Genes Within the gap Operon of *Clostridium acetobutylicum*.

108. **Iddar, A., Valverde, F., Serrano, A., Soukri, A.** (2002). Expression, purification, and characterization of recombinant nonphosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase from *Clostridium acetobutylicum*. Protein Expr Purif **25**, 519-26.

109. **Fontaine, L., Meynial-Salles, I., Girbal, L., Yang, X., Croux, C., Soucaille, P.** (2002). Molecular characterization and transcriptional analysis of adhE2, the gene encoding the NADH-dependent aldehyde/alcohol dehydrogenase responsible for butanol production in alcohologenic cultures of *Clostridium acetobutylicum* ATCC 824. J Bacteriol **184**, 821-30.

110. **Fischer, R.-J., Helms, J., Dürre, P.** (1993). Cloning, sequencing, and molecular analysis of the sol operon of *Clostridium acetobutylicum*, a chromosomal locus involved in solventogenesis. J. Bacteriol. **175**, 6959-6969.

111. **Winzer, K., Lorenz, K., Zickner, B., Durre, P.** (2000). Differential regulation of two thiolase genes from *Clostridium acetobutylicum* DSM 792. J Mol Microbiol Biotechnol **2**, 531-41.

112. **Shi, Z., Blaschek, H. P.** (2008). Transcriptional analysis of *Clostridium beijerinckii* NCIMB 8052 and the hyper-butanol-producing mutant BA101 during the shift from acidogenesis to solventogenesis. Appl Environ Microbiol **74**, 7709-14.

113. **Li, F., Hinderberger, J., Seedorf, H., Zhang, J., Buckel, W., Thauer, R. K.** (2008). Coupled ferredoxin and crotonyl coenzyme A (CoA) reduction with NADH catalyzed by the butyryl-CoA dehydrogenase/Etf complex from *Clostridium* kluyveri. J Bacteriol **190**, 843-50.

114. **Tangney, M., Mitchell, W. J.** (2000). Analysis of a catabolic operon for sucrose transport and metabolism in *Clostridium acetobutylicum* ATCC 824. J Mol Microbiol Biotechnol **2**, 71-80.

115. **Winkler, J., Rehmann, M., Kao, K. C.** (2010). Novel *Escherichia coli* hybrids with enhanced butanol tolerance. Biotechnol Lett

116. **Ezeji, T., Milne, C., Price, N. D., Blaschek, H. P.** (2010). Achievements and perspectives to overcome the poor solvent resistance in acetone and butanol-producing microorganisms. Appl Microbiol Biotechnol **85**, 1697-712.

117. **Haggstrom, L.** (1985). Acetone-butanol fermentation and its variants. Biotechnol Adv **3**, 13-28.

118. **Lin, Y.-L., Blaschek, H. P.** (1983). Butanol production by a butanol-tolerant strain of *Clostridium acetobutylicum* in extruded corn broth. Applied and Environmental Microbiology **45**, 966-973.

119. **Tomas, C. A., Welker, N. E., Papoutsakis, E. T.** (2003). Overexpression of *groESL* in *Clostridium acetobutylicum* results in increased solvent production and tolerance, prolonged metabolism, and changes in the cell's transcriptional program. Applied and Environmental Microbiology **69**, 4951-4965.

120. **Fischer, C. R., Klein-Marcuschamer, D., Stephanopoulos, G.** (2008). Selection and optimization of microbial hosts for biofuels production. Metab Eng **10**, 295-304.

121. **Zheng, Y. N., Li, L. Z., Xian, M., Ma, Y. J., Yang, J. M., Xu, X., He, D. Z.** (2009). Problems with the microbial production of butanol. J Ind Microbiol Biotechnol **36**, 1127-38.

122. **Poole, S. K., Poole, C. F.** (2003). Separation methods for estimating octanol-water partition coefficients. J Chromatogr B Analyt Technol Biomed Life Sci **797**, 3-19.

123. **Isken, S., de Bont, J. A.** (1998). Bacteria tolerant to organic solvents. Extremophiles **2**, 229-38.

124. **Durre, P.** (1998). New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation. Appl Microbiol Biotechnol **49**, 639-48.

125. **Knoshaug, E. P., Zhang, M.** (2008). Butanol Tolerance in a Selection of Microorganisms. Appl Biochem Biotechnol

126. **Schultz, T. W., Tichy, M.** (1993). Structure-toxicity relationships for unsaturated alcohols to Tetrahymena pyriformis: C5 and C6 analogs and primary propargylic alcohols. Bull Environ Contam Toxicol **51**, 681-8.

127. **Hansch, C.** (1968). The Linear Free-Energy Relationship between Partition Coefficients and the Aqueous Solubility of Organic Liquids.

128. **Weiner, P. H.** (1973). Prediction of Some Physical Properties of Organic Molecules by Factor Analysis of Gas Chromatographic Retention Indices.

129. **Bowmer, C. T., Hooftman, R. N., Hanstveit, A. O., Venderbosch, P. W., van der Hoeven, N.** (1998). The ecotoxicity and the biodegradability of lactic acid, alkyl lactate esters and lactate salts. Chemosphere **37**, 1317-33.

130. **Eiteman, M. A.** (1991). Predicting partition coefficients in polyethylene glycolpotassium phosphate aqueous two-phase systems.

131. **Nimcevic, D., Schuster, M.,Gapes, J. R.** (1998). Solvent production by *Clostridium beijerinckii* NRRL B592 growing on different potato media. Applied Microbiology and Biotechnology **50**, 426-428.

132. **López-Contreras, A. M., Claassen, P. A., Mooibroek, H.,De Vos, W. M.** (2000). Utilisation of saccharides in extruded domestic organic waste by *Clostridium acetobutylicum* ATCC 824 for production of acetone, butanol and ethanol. Applied Microbiology and Biotechnology **54**, 162-167.

133. **Banerjee, R.** (2003). Radical carbon skeleton rearrangements: catalysis by coenzyme B12-dependent mutases. Chem Rev **103**, 2083-94.

134. **Cracan, V., Padovani, D.,Banerjee, R.** (2010). IcmF is a fusion between the radical B12 enzyme isobutyryl-CoA mutase and its G-protein chaperone. J Biol Chem **285**, 655-66.

135. **Matthies, C., Schink, B.** (1992). Reciprocal Isomerization of Butyrate and Isobutyrate by the Strictly Anaerobic Bacterium Strain WoG13 and Methanogenic Isobutyrate Degradation by a Defined Triculture. Appl Environ Microbiol **58**, 1435-1439.

136. **Ratnatilleke, A., Vrijbloed, J. W., Robinson, J. A.** (1999). Cloning and sequencing of the coenzyme B(12)-binding domain of isobutyryl-CoA mutase from Streptomyces cinnamonensis, reconstitution of mutase activity, and characterization of the recombinant enzyme produced in *Escherichia coli*. J Biol Chem **274**, 31679-85.

137. **Vrijbloed, J. W., Zerbe-Burkhardt, K., Ratnatilleke, A., Grubelnik-Leiser, A., Robinson, J. A.** (1999). Insertional inactivation of methylmalonyl coenzyme A (CoA) mutase and isobutyryl-CoA mutase genes in Streptomyces cinnamonensis: influence on polyketide antibiotic biosynthesis. J Bacteriol **181**, 5600-5.

138. **Zerbe-Burkhardt, K., Ratnatilleke, A., Philippon, N., Birch, A., Leiser, A., Vrijbloed, J. W., Hess, D., Hunziker, P., Robinson, J. A.** (1998). Cloning, sequencing, expression, and insertional inactivation of the gene for the large subunit of the coenzyme B12-dependent isobutyryl-CoA mutase from Streptomyces cinnamonensis. J Biol Chem **273**, 6508-17.

139. **Doremus.** (1984). Agitation and Pressure Effects on Acetone-Butanol Fermentation.

140. **Mitchell, W. J.** (1998). Physiology of carbohydrate to solvent conversion by clostridia. Adv Microb Physiol **39**, 31-130.

141. **Goupil-Feuillerat, N., Cocaign-Bousquet, M., Godon, J. J., Ehrlich, S. D., Renault, P.** (1997). Dual role of alpha-acetolactate decarboxylase in Lactococcus lactis subsp. lactis. J Bacteriol **179**, 6285-93.

142. **Goupil-Feuillerat, N., Corthier, G., Godon, J. J., Ehrlich, S. D., Renault, P.** (2000). Transcriptional and translational regulation of alpha-acetolactate decarboxylase of Lactococcus lactis subsp. lactis. J Bacteriol **182**, 5399-408.

143. **Renna, M. C., Najimudin, N., Winik, L. R., Zahler, S. A.** (1993). Regulation of the Bacillus subtilis alsS, alsD, and alsR genes involved in post-exponential-phase production of acetoin. J Bacteriol **175**, 3863-75.

144. **Mermelstein, L. D., Papoutsakis, E. T., Petersen, D. J., Bennett, G. N.** (1993). Metabolic Engineering of *Clostridium acetobutylicum* ATCC 824 for Increased Solvent Production by Enhancement of Acetone Formation Enzyme-Activities Using a Synthetic Acetone Operon. Biotechnology and Bioengineering **42**, 1053-1060.

145. **Oultram, J. D., Loughlin, M., Swinfield, T. J., Brehm, J. K., Thompson, D. E., Minton, N. P.** (1988). Introduction of plasmids into whole cells of *Clostridium acetobutylicum* by electroporation. FEMS Microbiology Letters **56**, 83-88.

146. **Brouns, S. J., Smits, N., Wu, H., Snijders, A. P., Wright, P. C., de Vos, W. M., van der Oost, J.** (2006). Identification of a novel alpha-galactosidase from the hyperthermophilic archaeon Sulfolobus solfataricus. J Bacteriol **188**, 2392-9.

147. **Stormer, F. C.** (1975). 2,3-Butanediol biosynthetic system in Aerobacter aerogenes. Methods in Enzymology **41**, 518-532.

148. **Westerfeld.** (1945). A colorimetric determination of blood acetoin.

149. **Dulieu, C.** (1999). Spectrophotometric assay of x-acetolactate decarboxylase.

150. **Blomqvist, K., Suihko, M. L., Knowles, J., Penttila, M.** (1991). Chromosomal Integration and Expression of Two Bacterial alpha-Acetolactate Decarboxylase Genes in Brewer's Yeast. Appl Environ Microbiol **57**, 2796-803.

151. **Diderichsen, B., Wedsted, U., Hedegaard, L., Jensen, B. R., Sjoholm, C.** (1990). Cloning of aldB, which encodes alpha-acetolactate decarboxylase, an exoenzyme from Bacillus brevis. J Bacteriol **172**, 4315-21.

152. **Goelling, D., Stahl, U.** (1988). Cloning and expression of an alpha-acetolactate decarboxylase gene from Streptococcus lactis subsp. diacetylactis in *Escherichia coli*. Appl Environ Microbiol **54**, 1889-91.

153. **Rasmussen, A. M., Gibson, R. M., Godtfredsen, S. E., Ottesen, M.** (1985). Purification of α-acetolactate decarboxylase from Lactobacillus casei DSM 2547. Carlsberg Research Communications **50**, 73-82.

154. **Ohshiro, T., Aisaka, K., Uwajima, T.** (1989). Purification and Characterization of α-Acetolactate Decarboxylase from Brevibacterium acetylicum. Agricultural and Biological Chemistry **53**, 1913-1918.

155. **Phalip, V., Monnet, C., Schmitt, P., Renault, P., Godon, J. J., Divies, C.** (1994). Purification and properties of the alpha-acetolactate decarboxylase from Lactococcus lactis subsp. lactis NCDO 2118. FEBS Lett **351**, 95-9.

156. **Gottwald, M., Gottschalk, G.** (1985). The internal pH of *Clostridium acetobutylicum* and its effect on the shift from acid to solvent formation. Archives of Microbiology **143**, 42-46.

157. **Terracciano, J. S., Kashket, E. R.** (1986). Intracellular conditions required for initiation of solvent production by *Clostridium acetobutylicum*. Applied and environmental microbiology **52**, 86-91.

158. **Radianingtyas, H., Wright, P. C.** (2003). Alcohol dehydrogenases from thermophilic and hyperthermophilic archaea and bacteria. FEMS Microbiol Rev **27**, 593-616.

159. **Reid, M. F., Fewson, C. A.** (1994). Molecular characterization of microbial alcohol dehydrogenases. Crit Rev Microbiol **20**, 13-56.

160. **Jez, J. M., Flynn, T. G., Penning, T. M.** (1997). A nomenclature system for the aldo-keto reductase superfamily. Adv Exp Med Biol **414**, 579-600.

161. **Borhani, D. W., Harter, T. M., Petrash, J. M.** (1992). The crystal structure of the aldose reductase.NADPH binary complex. J Biol Chem **267**, 24841-7.

162. **Khurana, S., Powers, D. B., Anderson, S., Blaber, M.** (1998). Crystal structure of 2,5-diketo-D-gluconic acid reductase A complexed with NADPH at 2.1-A resolution. Proc Natl Acad Sci U S A **95**, 6768-73.

163. **Rondeau, J. M., Tete-Favier, F., Podjarny, A., Reymann, J. M., Barth, P., Biellmann, J. F., Moras, D.** (1992). Novel NADPH-binding domain revealed by the crystal structure of aldose reductase. Nature **355**, 469-72.

164. **Wierenga, R. K., Terpstra, P., Hol, W. G.** (1986). Prediction of the occurrence of the ADP-binding beta alpha beta-fold in proteins, using an amino acid sequence fingerprint. J Mol Biol **187**, 101-7.

165. **Machielsen, R., Uria, A. R., Kengen, S. W., van der Oost, J.** (2006). Production and characterization of a thermostable alcohol dehydrogenase that belongs to the aldo-keto reductase superfamily. Appl Environ Microbiol **72**, 233-8.

166. **Brouns, S. J., Turnbull, A. P., Willemen, H. L., Akerboom, J., van der Oost, J.** (2007). Crystal structure and biochemical properties of the D-arabinose dehydrogenase from Sulfolobus solfataricus. J Mol Biol **371**, 1249-60.

167. **Shiloach, J., Fass, R.** (2005). Growing E. *coli* to high cell density--a historical perspective on method development. Biotechnol Adv **23**, 345-57.

168. **Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., Lipman, D. J.** (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25**, 3389-402.

169. **Hosaka, T., Ui, S., Ohtsuki, T., Mimura, A., Ohkuma, M., Kudo, T.** (2001). Characterization of the NADH-linked acetylacetoin reductase/2,3-butanediol dehydrogenase gene from Bacillus cereus YUF-4. J Biosci Bioeng **91**, 539-44.

170. **McGuffin, L. J., Bryson, K., Jones, D. T.** (2000). The PSIPRED protein structure prediction server. Bioinformatics **16**, 404-5.

171. **Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., Higgins, D. G.** (2007). Clustal W and Clustal X version 2.0. Bioinformatics **23**, 2947-8.

172. **Page, R. D.** (1996). TreeView: an application to display phylogenetic trees on personal computers. Comput Appl Biosci **12**, 357-8.

173. **Getz, E. B., Xiao, M., Chakrabarty, T., Cooke, R., Selvin, P. R.** (1999). A comparison between the sulfhydryl reductants tris(2-carboxyethyl)phosphine and dithiothreitol for use in protein biochemistry. Anal Biochem **273**, 73-80.

174. **Bradford, M. M.** (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72**, 248-54.

175. **Crow, V. L.** (1990). Properties of 2,3-Butanediol Dehydrogenases from Lactococcus lactis subsp. lactis in Relation to Citrate Fermentation. Appl Environ Microbiol **56**, 1656-1665.

176. **Giovannini, P. P., Medici, A., Bergamini, C. M., Rippa, M.** (1996). Properties of diacetyl (acetoin) reductase from Bacillus stearothermophilus. Bioorg Med Chem **4**, 1197-201.

177. **Gonzalez, E., Fernandez, M. R., Larroy, C., Pares, X., Biosca, J. A.** (2001). Characterization and functional role of Saccharomyces cerevisiae 2,3-butanediol dehydrogenase. Chem Biol Interact **130-132**, 425-34.

178. **Gonzalez, E., Fernandez, M. R., Larroy, C., Sola, L., Pericas, M. A., Pares, X., Biosca, J. A.** (2000). Characterization of a (2R,3R)-2,3-butanediol dehydrogenase as the Saccharomyces cerevisiae YAL060W gene product. Disruption and induction of the gene. J Biol Chem **275**, 35876-85.

179. **Hohn-Bentz, H., Radler, F.** (1978). Bacterial 2,3-butanediol dehydrogenases. Arch Microbiol **116**, 197-203.

180. **Hosaka, T., Ui, S., Mimura, A.** (1999). Separation and properties of two acetylacetoin reductases from Bacillus cereus YUF-4. Biosci Biotechnol Biochem **63**, 199-201.

181. **Nicholson, W. L.** (2008). The Bacillus subtilis ydjL (bdhA) gene encodes acetoin reductase/2,3-butanediol dehydrogenase (AR/BDH): comparative genomic identification and experimental verification. Appl Environ Microbiol

182. **Ui, S.** (1986). Mechanism for the formation of 2,3-butanediol stereoisomers in *Bacillus polymyxa*.

183. **Ui, S., Hosaka, T., Mizutani, K., Mimura, A.** (1998). Purification and properties of acetylacetoin synthase from Bacillus sp. YUF-4. Biosci Biotechnol Biochem **62**, 795-7.

184. **Bates, P. A., Kelley, L. A., MacCallum, R. M., Sternberg, M. J.** (2001). Enhancement of protein modeling by human intervention in applying the automatic programs 3D-JIGSAW and 3D-PSSM. Proteins **Suppl 5**, 39-46.

185. **Laskowski, R. A., MacArthur, M.W., Moss, D.S., Thornton, J.M.** (1993). Procheck: a program to check the stereochemical quality of protein structures.

186. **Banfield, M. J., Salvucci, M. E., Baker, E. N., Smith, C. A.** (2001). Crystal structure of the NADP(H)-dependent ketose reductase from Bemisia argentifolii at 2.3 A resolution. J Mol Biol **306**, 239-50.

187. **Ali, N. O., Bignon, J., Rapoport, G., Debarbouille, M.** (2001). Regulation of the acetoin catabolic pathway is controlled by sigma L in Bacillus subtilis. J Bacteriol **183**, 2497-504.

188. **Ui, S.** (1997). Sequence Analysis of the Gene for and Characterization of D-Acetoin Forming *meso*-2,3-Butanediol Dehydrogenase of *Klebsiella pneumoniae* Expressed in *Escherichia coli*.

189. **Rossmann, M. G., Moras, D., Olsen, K. W.** (1974). Chemical and biological evolution of a nucleotide-binding protein. Nature **250**, 194-199.

190. **Takusagawa, Y., Otagiri, M., Ui, S., Ohtsuki, T., Mimura, A., Ohkuma, M., Kudo, T.** (2001). Purification and characterization of L-2,3-butanediol dehydrogenase of Brevibacterium saccharolyticum C-1012 expressed in *Escherichia coli*. Biosci Biotechnol Biochem **65**, 1876-8.

191. **Bogin, O., Peretz, M., Hacham, Y., Korkhin, Y., Frolow, F., Kalb, A. J., Burstein, Y.** (1998). Enhanced thermal stability of *Clostridium beijerinckii* alcohol dehydrogenase after strategic substitution of amino acid residues with prolines from the homologous thermophilic

Thermoanaerobacter brockii alcohol dehydrogenase. Protein Sci **7**, 1156-63.

192. **Ismaiel, A. A., Zhu, C. X., Colby, G. D., Chen, J. S.** (1993). Purification and characterization of a primary-secondary alcohol dehydrogenase from two strains of *Clostridium beijerinckii*. J Bacteriol **175**, 5097-105.

193. **Li, C., Heatwole, J., Soelaiman, S., Shoham, M.** (1999). Crystal structure of a thermophilic alcohol dehydrogenase substrate complex suggests determinants of substrate specificity and thermostability. Proteins **37**, 619-27.

194. **Demain, A. L., Newcomb, M., Wu, J. H.** (2005). Cellulase, clostridia, and ethanol. Microbiol Mol Biol Rev **69**, 124-54.

195. **Richard, P., Toivari, M. H., Penttila, M.** (1999). Evidence that the gene YLR070c of Saccharomyces cerevisiae encodes a xylitol dehydrogenase. FEBS Lett **457**, 135-8.

196. **Carere, C. R., Sparling, R., Cicek, N., Levin, D. B.** (2008). Third generation biofuels via direct cellulose fermentation. International Journal of Molecular Sciences **9**, 1342-1360.

197. **Atsumi, S., Hanai, T., Liao, J. C.** (2008). Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. Nature **451**, 86-89.

198. **Fischer, C. R., Klein-Marcuschamer, D., Stephanopoulos, G.** (2008). Selection and optimization of microbial hosts for biofuels production. Metabolic Engineering **10**, 295-304.

199. **Nölling, J., Breton, G., Omelchenko, M. V., Makarova, K. S., Zeng, Q., Gibson, R., Lee, H. M., Dubois, J., Qiu, D., Hitti, J., Wolf, Y. I., Tatusov, R. L., Sabathe, F., Doucette-Stamm, L., Soucaille, P., Daly, M. J., Bennett, G. N., Koonin, E. V., Smith, D. R.** (2001). Genome sequence and

comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. Journal of Bacteriology **183**, 4823-4838.

200. **Keis, S., Shaheen, R., Jones, D. T.** (2001). Emended descriptions of *Clostridium acetobutylicum* and *Clostridium beijerinckii*, and descriptions of *Clostridium saccharoperbutylacetonicum* sp. nov. and *Clostridium saccharobutylicum* sp. nov. International Journal of Systematic and Evolutionary Microbiology **51**, 2095-2103.
201. **Bowles, L. K., Ellefson, W. L.** (1985). Effects of butanol on *Clostridium acetobutylicum*. Applied and Environmental Microbiology **50**, 1165-1170.
202. **Vollherbst-Schneck, K., Sands, J. A., Montenecourt, B. S.** (1984). Effect of butanol on lipid composition and fluidity of *Clostridium acetobutylicum* ATCC 824. Applied and Environmental Microbiology **47**, 193-194.
203. **Baer, S. H., Blaschek, H. P., Smith, T. L.** (1987). Effect of butanol challenge and temperature on lipid-composition and membrane fluidity of butanol-tolerant *Clostridium acetobutylicum*. Applied and Environmental Microbiology **53**, 2854-2861.
204. **Borden, J. R., Papoutsakis, E. T.** (2007). Dynamics of genomic-library enrichment and identification of solvent tolerance genes for *Clostridium acetobutylicum*. Applied and Environmental Microbiology **73**, 3061-3068.
205. **Hermann, M., Fayolle, F., Marchal, R., Podvin, L., Sebald, M., Vandecasteele, J. P.** (1985). Isolation and characterization of butanol-resistant mutants of *Clostridium acetobutylicum*. Applied and Environmental Microbiology **50**, 1238-1243.
206. **Vermuë, M., Sikkema, J., Verheul, A., Bakker, R., Tramper, J.** (1993). Toxicity of homologous series of organic solvents for the Gram-positive

bacteria *Arthrobacter* and *Nocardia* sp. and the Gram-negative bacteria *Acinetobacter* and *Pseudomonas* sp. Biotechnology and Bioengineering **42**, 747-758.

207. **Andersch, W., Bahl, H., Gottschalk, G.** (1983). Level of Enzymes Involved in Acetate, Butyrate, Acetone and Butanol Formation by *Clostridium acetobutylicum*. European Journal of Applied Microbiology and Biotechnology **18**, 327-332.

208. **Hartmanis, M. G. N., Gatenbeck, S.** (1984). Intermediary Metabolism in *Clostridium acetobutylicum:* Levels of Enzymes Involved in the Formation of Acetate and Butyrate. Applied and Environmental Microbiology **47**, 1277-1283.

209. **McNeil, B., Kristiansen, B.** (1985). Effect of Temperature Upon Growth-Rate and Solvent Production in Batch Cultures of *Clostridium acetobutylicum*. Biotechnology Letters **7**, 499-502.

210. **González, E., Fernández, M. R., Larroy, C., Solà, L., Pericàs, M. A., Parés, X., Biosca, J. A.** (2000). Characterization of a (2*R*,3*R*)-2,3-butanediol dehydrogenase as the *Saccharomyces cerevisiae* YAL060W gene product. Disruption and induction of the gene. Journal of Biological Chemistry **275**, 35876-35885.

211. **Hosaka, T., Ui, S., Ohtsuki, T., Mimura, A., Ohkuma, M., Kudo, T.** (2001). Characterization of the NADH-linked acetylacetoin reductase/2,3-butanediol dehydrogenase gene from *Bacillus cereus* YUF-4. Journal of Bioscience and Bioengineering **91**, 539-544.

212. **Doremus, M. G., Linden, J. C., Moreira, A. R.** (1985). Agitation and Pressure Effects on Acetone-Butanol Fermentation. Biotechnology and Bioengineering **27**, 852-860.

213. **Wardwell, S. A., Yang, Y. T., Chang, H. Y., San, K. Y., Rudolph, F. B., Bennett, G. N.** (2001). Expression of the *Klebsiella pneumoniae* CG21

acetoin reductase gene in *Clostridium acetobutylicum* ATCC 824. Journal of Industrial Microbiology & Biotechnology **27**, 220-227.

214. **Nakamura, Y.** 2007, posting date. Codon Usage Database. [Online.]
215. **Minton, N. P., Oultram, J. D.** (1988). Host: vector systems for gene cloning in *Clostridium*. Microbiological Sciences **5**, 310-315.
216. **Nicholson, W. L.** (2008). The *Bacillus subtilis ydjL* (*bdhA*) gene encodes acetoin reductase/2,3-butanediol dehydrogenase. Applied and Environmental Microbiology **74**, 6832-6838.
217. **Ma, C., Wang, A., Qin, J., Li, L., Ai, X., Jiang, T., Tang, H.,Xu, P.** (2009). Enhanced 2,3-butanediol production by *Klebsiella pneumoniae* SDM. Applied Microbiology and Biotechnology **82**, 49-57.
218. **Alsaker, K. V., Papoutsakis, E. T.** (2005). Transcriptional program of early sporulation and stationary-phase events in *Clostridium acetobutylicum*. Journal of Bacteriology **187**, 7103-7118.
219. **Tummala, S. B., Welker, N. E., Papoutsakis, E. T.** (1999). Development and characterization of a gene expression reporter system for *Clostridium acetobutylicum* ATCC 824. Applied and Environmental Microbiology **65**, 3793-3799.
220. **Taylor, M. B., Juni, E.** (1960). Stereoisomeric specificities of 2,3-butanediol dehydrogenases. Biochimica et Biophysica Acta **39**, 448-457.
221. **Jörnvall, H., Persson, B., Krook, M., Atrian, S., Gonzàlez-Duarte, R., Jeffery, J., Ghosh, D.** (1995). Short-chain dehydrogenases/reductases (SDR). Biochemistry **34**, 6003-6013.
222. **Ui, S., Odagiri, M., Mimura, A., Kanai, H., Kobayashi, T., Kudo, T.** (1996). Preparation of a chiral acetoinic compound using transgenic *Escherichia coli* expressing the 2,3-butanediol dehydrogenase gene. Journal of Fermentation and Bioengineering **81**, 386-389.

223. **Ballongue, J., Amine, J., Masion, E., Petitdemange, H., Gay, R.** (1985). Induction of Acetoacetate Decarboxylase in *Clostridium acetobutylicum*. FEMS Microbiology Letters **29**, 273-277.

224. **Harris, L. M., Blank, L., Desai, R. P., Welker, N. E., Papoutsakis, E. T.** (2001). Fermentation characterization and flux analysis of recombinant strains of *Clostridium acetobutylicum* with an inactivated *solR* gene. Journal of Industrial Microbiology & Biotechnology **27**, 322-328.

225. **Ui, S., Masuda, T., Masuda, H., Muraki, H.** (1986). Mechanism for the formation of 2,3-butanediol stereoisomers in *Bacillus polymyxa*. Journal of Fermentation Technology **64**, 481-486.

226. **Rodionov, D. A., Vitreschak, A. G., Mironov, A. A., Gelfand, M. S.** (2003). Comparative genomics of the vitamin B12 metabolism and regulation in prokaryotes. J Biol Chem **278**, 41148-59.

227. **Michel, C., Buckel, W.** (1991). Coenzyme B12-dependent 2-methyleneglutarate mutase from *Clostridium* barkeri. Protection by the substrate from inactivation by light. FEBS Lett **281**, 108-10.

228. **Zelder, O., Buckel, W.** (1993). On the role of two different cobalt(II) species in coenzyme B12-dependent 2-methyleneglutarate mutase from *Clostridium* barkeri. Biol Chem Hoppe Seyler **374**, 85-90.

229. **Zelder, O., Beatrix, B., Buckel, W.** (1994). Cloning, sequencing and expression in *Escherichia coli* of the gene encoding component S of the coenzyme B12-dependent glutamate mutase from *Clostridium* cochlearium. FEMS Microbiol Lett **118**, 15-21.

230. **Macis, L., Daniel, R., Gottschalk, G.** (1998). Properties and sequence of the coenzyme B12-dependent glycerol dehydratase of *Clostridium* pasteurianum. FEMS Microbiol Lett **164**, 21-8.

231. **Tollinger, M., Konrat, R., Hilbert, B. H., Marsh, E. N., Krautler, B.** (1998). How a protein prepares for B12 binding: structure and dynamics of the

B12-binding subunit of glutamate mutase from *Clostridium* tetanomorphum. Structure **6**, 1021-33.

232. **Chen, H. P., Hsu, H. J., Hsu, F. C., Lai, C. C.,Hsu, C. H.** (2008). Interactions between coenzyme B12 analogs and adenosylcobalamin-dependent glutamate mutase from *Clostridium tetanomorphum*. FEBS J **275**, 5960-8.
233. **Voloch, M., Jansen N.B., Ladisch M.R. Tsao, G.T. Narayan, R. and Rodwell, V.W.** (1985). 2,3-Butanediol. Comprehensive Biotechnology: The principles , Applications and Regulations of Biotechnology in Industry, Agriculture and Medicine, Oxford, Pergamon Press
234. **Moore, K. W., Richards, J. H.** (1979). Stereospecificity and mechanism of adenosylcobalamin-dependent diol dehydratase. Catalysis and inactivation with *meso*- and dl-2,3-butanediols as substrates. Biochem Biophys Res Commun **87**, 1052-7.
235. **Levisson, M., van der Oost, J., Kengen, S. W.** (2009). Carboxylic ester hydrolases from hyperthermophiles. Extremophiles **13**, 567-81.
236. **Verstrepen, K. J., Van Laere, S. D., Vercammen, J., Derdelinckx, G., Dufour, J. P., Pretorius, I. S., Winderickx, J., Thevelein, J. M., Delvaux, F. R.** (2004). The Saccharomyces cerevisiae alcohol acetyl transferase Atf1p is localized in lipid particles. Yeast **21**, 367-77.
237. **Horton, C. E., Huang, K. X., Bennett, G. N., Rudolph, F. B.** (2003). Heterologous expression of the Saccharomyces cerevisiae alcohol acetyltransferase genes in *Clostridium acetobutylicum* and *Escherichia coli* for the production of isoamyl acetate. J Ind Microbiol Biotechnol **30**, 427-32.

Nederlandse samenvatting

Hoofdstuk 1 van dit proefschrift geeft een overzicht over de geschiedenis van de aceton, butanol en ethanol (ABE) fermentatie. Het centrale metabolisme van solventogene clostridia wordt kort besproken. Ondanks het feit dat wetenschappelijk onderzoek is toegenomen in de afgelopen jaren, zijn veel aspecten binnen het ABE fermentatie proces nog steeds onduidelijk.

De grootste uitdaging binnen de ABE fermentatie, blijft de 1-butanol toxiciteit. Vanwege deze toxiciteit is de opbrengst van 1-butanol niet meer dan 1-2% (w/v). Met maar weinig vooruitgang geboekt in de afgelopen jaren is de ABE fermentatie toch werd weer interessant geworden, vanwege de wereldwijde belangstelling voor biobrandstoffen of biobrandstoffen additieven.

Tegenwoordig, in een poging om hogere opbrengsten te bereiken, zijn ook veel micro-organismen onderzocht welke geschikt konden zijn als gastheer voor de productie van oplosmiddelen. *E. coli* wordt vaak gekozen als een veelbelovende gastheer voor de microbiële productie van biobrandstoffen. Toch blijven solventogene clostridia, als potentiële gastheer, interessant om verschillende redenen, zoals i) de beschikbaarheid van een genetisch systeem, ii) de natuurlijke productie van oplosmiddelen en iii) een relatief hoge tolerantie ten opzichte van oplosmiddelen. Een nadeel van het gebruik van deze solventogenic clostridia is het onvermogen om cellulose en hemi-cellulose te gebruiken als substraat.

Met betrekking tot de productie van potentieel interessante 1-butanol derivaten, richtten we ons op 2,3-butaandiol. Deze industrieel waardevolle verbinding wordt al geproduceerd in de natuur door verschillende bacteriën, maar nog niet door *C. acetobutylicum*. In dit proefschrift wordt de productie van 2,3-butaandiol door *C. acetobutylicum* onderzocht en beschreven. In hoofdstuk 1 wordt de 2,3-butaandiol biosynthese route uitgebreid beschreven.

De twee belangrijkste industriele en gesequencete solventogenic clostridia zijn *C. acetobutylicum* ATCC 824 en *C. beijerinckii* NCIMB 8052. Veel biochemische informatie bekend is over de verschillende metabole routes van het centrale metabolisme van *C. acetobutylicum* ATCC 824. In hoofdstuk 2 worden vergelijkingen gemaakt tussen de biosynthetische fermentatie routes van glucose naar ABE producten tussen beide soorten. Met de genoom sequentie van *C. beijerinckii* NCIMB 8052 nu ook beschikbaar, kunnen de meest waarschijnlijke kandidaten voor de 34 betrokken enzymatische omzettingen binnen de centrale afbraakproduct van *C. beijerinckii*, worden voorspeld. De enzymatische omzettingen, die betrokken zijn bij glucose-opname, glycolyse, gluconeogenese, pyruvaat conversie en acetyl-CoA conversies naar de verschillende eindproducten, worden besproken.

Hoofdstuk 3 beschrijft een nieuwe benadering bij het oplossen van het probleem van de 1-butanol toxiciteit bij *C. acetobutylicum*. Het vergroten van de tolerantie van 1-butanol is vaker geprobeerd in andere onderzoeksgroepen. In onze aanpak hebben we gekozen voor de invoering van nieuwe biosynthesewegen dat de productie van minder toxische 1-butanol derivaten mogelijk maakt. De toxiciteit van verbindingen is te correleren met de lipofiliteit (de neiging om te accumuleren in de celmembranen). Het kan worden uitgedrukt als de logaritme van de verdelingscoëfficiënt octanol en water met (log K_{ow}-waarde). Hoofdstuk 3 beschrijft de groei van *C. acetobutylicum*, die werden uitgevoerd om de toxiciteit van de verschillende derivaten in kaart te brengen. Iso-butanol, 2-butanol, tert-butanol, 2,3-butaandiol, iso-amyl alcohol, butyl acetaat, butyl butyraat en butyl lactaat warden gekozen om te testen. Iso-butanol, 2-butanol en 2,3-butaandiol kwamen, gebaseerd op hun log K_{ow} waarde en hun gedrag in de toxiciteit, naar voren als potentiële alternatieven voor 1-butanol.

2,3-Butanediol bleek de beste potentiële kandidaat, die minder toxisch is voor *C. acetobutylicum*, dan 1-butanol. De voorloper van 2,3-butaandiol, acetoin, dat reeds door *C. acetobutylicum* in kleine hoeveelheden geproduceerd wordt, wordt gevormd door de decarboxylatie van acetolactaat. Deze omzetting wordt gekatalyseerd door het enzym acetolactaat decarboxylase. In hoofdstuk 4 worden de identificatie, heterologe productie, zuivering en biochemische karakterisering van een acetolactaat decarboxylase van *C. acetobutylicum* ATCC 824 wordt beschreven. Acetolactaat decarboxylase activiteit gekatalyseerd door Ca-ALD (CAC2967) werd bewezen. 'Size exclusion' chromatografie toont aan dat het natieve enzym voornamelijk bestaat als dimeer van 27 kDa subeenheden. De optimale activiteit werd gevonden rond de 40 °C en bij een pH van 5,2. Het enzym is afhankelijk van de aanwezigheid van tweewaardige metaalionen, zoals Zn^{2+} of Co^{2+}. De halfwaardetijd ligt rond 25 uur bij 37 °C. De Ca-ALD bindt acetolactaat coöperatief met een Hill coëfficiënt van 1,49. Ook werd een $K_{1/2}$ van 16,8 mM en een V_{max} van 51,9 U/mg gevonden. Verder is er een shuttle vector geconstrueerd om, onder controle van de sterke ADC promotor, Ca-ALD in *C. acetobutylicum* ATCC 824 tot expressie te brengen. Echter, ondanks een succesvolle transformatie, werd geen significante toename in de productie acetoin waargenomen in de Ca-ALD overexpressie stam.

Slechts één extra enzym is nodig om de 2,3-butaandiol biosynthese pathway in *C. acetobutylicum* voltooien. Dit enzym, dat de reactie van acetoin naar 2,3-butanediol katalyseerd heet acetoin reductase. Hoofdstuk 5 beschrijft de identificatie, heterologe productie, zuivering en biochemische karakterisering van een acetoin reductase uit *C. beijerinckii*. Door middel van een bioinformatica screening in het genoom van *C. beijerinckii*, werden acht

vermeende acetoin reductases gevonden. Van deze acht werden er zes succesvol gekloneerd, waarvan één (CBEI_1464) een aanzienlijke acetoin reductase activiteit vertoonde na heterologe expressie in *E. coli*. Dit gezuiverde enzym (Cb-ACR) bleek voornamelijk voor te komen als homodimeer van 37 kDa subeenheden. Het enzym heeft een voorkeur voor NADPH (Km = 0,32 uM), als elektronen donor, met een specifieke activiteit van 76 $U.mg^{-1}$. De optimale activiteit werd gevonden bij ongeveer 68 °C, voor zowel de reductie als oxidatie reactie en bij een pH van 6,5 en 9,5, voor de reductie en oxidatie reactie, respectievelijk. ICP-AES analyse toonde de aanwezigheid van ~ 2 Zn^{2+} atomen en ~ 1 Ca^{2+}-atoom per monomeer. Om inzicht te krijgen in het reactie mechanisme, maar ook in het substraat- en cofactor-specificiteit, werd een structureel model gebouwd met een ketose reductase (sorbitol dehydrogenase) van de Bemisia argentifolii als template. De katalytische zink is waarschijnlijk gepositioneerd tussen Cys37, His70, Glu71 in Cb-ACR, terwijl de structurele zink site waarschijnlijk is gepositioneerd tussen Cys100, Cys103, Cys106 en Cys114.

De acetoin reductase (Cb-ACR) gevonden in *C. beijerinckii* wordt gebruikt voor in vivo experimenten in *C. acetobutylicum*. In hoofdstuk 6 wordt de productie van D-2,3-butaandiol in *C. acetobutylicum* ATCC 824, door de heterologe expressie van de CB-ACR, beschreven. Onder bepaalde omstandigheden produceerd *C. acetobutylicum* ATCC 824 (en afgeleide stammen) zowel D- en L stereo-isomeren van acetoin. Door het ontbreken van een ACR enzym wordt er geen 2,3-butaandiol geproduceerd. Een gen dat codeert voor ACR van *C. beijerinckii* NCIMB 8052 blijkt functioneel te zijn wanneer deze tot expressie komt in C. *acetobutylicum* onder controle van twee sterke promoters, de constitutieve THL promotor en de laat-exponentiële ADC promotor. Deze ACR producerende stammen zetten rond de 90% van het natieve geproduceerd e

acetoin om naar ongeveer 21 mM D-2,3-butaandiol. Het toevoegen van een racemisch mengsel van acetoin leidde tot de productie van zowel, D-2,3-butaandiol en *meso*-2,3-butaandiol. De voorspelde metabole route is in overeenstemming met de experimentele data. Native 2,3-butaandiol productie is een eerste stap naar een potentiële 2-butanol producerende *C. acetobutylicum*.

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About the author

Marco Antonius Jozef Siemerink was born on the 29th of March, 1980, in Enschede, The Netherlands. He started his education at the public primary school “St. Jozefschool” in Rietmolen. This was followed by secondary education at the “Scholengemeenschap ‘t Assink” in Haaksbergen. After graduating in 1997, he got in 2002 his Bsc. in Biology en Medical Laboratory research at the Saxion Hogeschool Enschede with the specialisation of biochemistry / biotechnology. In 2005, he obtained his MSc. in biotechnology at the Wageningen University with the specialisation of cellular / molecular. His major thesis was done at the laboratory of Microbiology in the Bacterial Genetics Group of Professor John van der Oost and the minor thesis was done partly at the same laboratory, but also partly at BioExplore, the High-Throughput Screening (HTS) Facility of the Groningen Biomolecular Sciences and Biotechnlogy Institute (GBB) of the University of Groningen. During this research he studied histidinol-phosphate aminotransferases and KDG aldolases from thermophilic *Sulfolobus* sp. After graduating, he started, in the same year, his PhD at the department of Microbiology in Wageningen, under supervision of Assistant Professor Servé Kengen and Professor John van der Oost. During this research, alternative biosynthetic pathways were explored and used to construct solventogenic clostridia producing interesting 1-butanol derivatives, which are less toxic than 1-butanol itself.

List of publications

Bongers, R. S., M. H. Hoefnagel, M. J. Starrenburg, **M. A. J. Siemerink**, J. G. Arends, J. Hugenholtz, and M. Kleerebezem. 2003. IS981-mediated adaptive evolution recovers lactate production by ldhB transcription activation in a lactate dehydrogenase-deficient strain of Lactococcus lactis. J Bacteriol. 185:4499-507.

Wolterink-van Loo, S., A. van Eerde, **M. A. J. Siemerink**, J. Akerboom, B. W. Dijkstra, and J. van der Oost. 2007. Biochemical and structural exploration of the catalytic capacity of Sulfolobus KDG aldolases. Biochem J. 403:421-30.

Wolterink-van Loo, S., **M. A. J. Siemerink**, G. Perrakis, T. Kaper, S. W. M. Kengen, and J. van der Oost. 2009. Improving low-temperature activity of Sulfolobus acidocaldarius 2-keto-3-deoxygluconate aldolase. Archaea. 2:233-239.

López-Contreras, A. M., Kuit, W., **Siemerink, M. A. J.**, Kengen, S. W. M., Springer, J., and Claassen, P. A. M. 2010. "Production of longer-chain alcohols from biomass - butanol, isopropanol and 2,3-butanediol," in Bioalcohol production, ed. K. Waldron, London: Woodhead Publishing Ltd. 415-160.

Levisson, M., Hendriks, S., **Siemerink, M. A. J.**, Deller, M. C., Kengen, S. W. M. and van der Oost, J. Characterization of a lipase from the hyperthermophilic archaeon *archaeoglobus fulgidus*. In preparation.

Siemerink, M. A. J.[*], Kuit, W[*], López Contreras, A. M, Eggink, G., van der Oost, J, Kengen, S. W. M. Heterologous expression of an acetoin reductase leads to D-2,3-butanediol production in *Clostridium acetobutylicum*. Submitted.

Siemerink, M. A. J., Levisson, M., van der Oost, J., Kengen, S. W. M. Molecular characterization of an acetoin reductase from *Clostridium beijerinckii*. In preparation.

Siemerink, M. A. J., Schwarz, K., Kuit, W., Liebl, W., Ehrenreich, A., Kengen S. W. M., "Comparative genomic analysis of the solventogenic species *Clostridium acetobutylicum* ATCC 824 and *Clostridium beijerinckii* NCIMB 8052" in *System biology of Clostridia,* ed. P. Dürre, Ulm, Germany: *Publisher*. In preparation.

Siemerink, M. A. J., Krol, J., van der Oost, J., Kengen, S. W. M., Characterization and overexpression of acetolactate decarboxylase in *Clostridium acetobutylicum*. In preparation.

[*] contributed equally

Overview of completed training activities

Discipline specific activities

Meetings:

Annual Meetings Study Group Molecular Genetics. 2006. Lunteren, NL.
Annual Meetings Study Group Protein Research. 2006. Lunteren, NL.
Annual Meetings Study Group Molecular Genetics. 2007. Lunteren, NL.
Annual Meetings Study Group Protein Research and Nucleic Acids. 2007. Lunteren, NL.
Conference: 'Non-pathogenic Clostridia'. 2008. Toulouse, France.*
Conference: '*Clostridium*X'. 2008. Wageningen, NL.*

*Oral speaker

Courses:

Applied Genetics of Industrial Fermentation. Adv. 2005. Wageningen, NL.
Systems Biology: "Principles of ~omics data analysis". 2005. Nijmegen, NL.
Workshop: 'Gene transfer techniques for Clostridia'. 2006. Nottingham, UK.

General courses
VLAG PhD week. 2006. Bilthoven, NL
Hoorcollege geven. 2006. Wageningen, NL
Scientific writing. 2008. Wageningen, NL.

Optionals
Preparation PhD research proposal.
Bacterial Genetics group meetings (weekly).
Lab of Microbiology PhD/PostDoc meetings (every two weeks).
Project meetings, Shell Amsterdam (every six months).
PhD student trip. 2006. California, USA.
Organizing PhD student trip. 2006. California, USA.

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