Metabolic Engineering of Acid Formation in *Clostridium acetobutylicum*

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For my family

Contents

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Chapter 1

General Introduction

1.1 Introduction

The work described in this thesis focuses on improving a known renewable process for the production of *n*-butanol through fermentation of sugars by the bacterium *Clostridium acetobutylicum*. Butanol has applications as a feedstock for the chemical industry and as a transport biofuel. This chapter will first provide a background on fossil resources, on renewable alternatives, and the on reasons for developing these. This is then followed by an introduction to the fermentative production of butanol, an inventory of genetic methods that can be applied to improve the process, and an overview is given of mutants described in the scientific literature. The chapter concludes with the proposed research plan and listing of the thesis outline.

1.2 Fossil Resources

In this day and age virtually everyone is affected, directly and indirectly, by the use of fossil resources for almost every aspect of their life. For example, we use natural gas to cook our food and to heat our houses, coal for electricity generation in power plants, and we use oil as a source for transport fuels and a chemical feedstock for various industries. These industries in turn produce very diverse products, ranging from toothpaste to plastic shopping bags.

1.3 Resource Limitations

1.3.1 Finite Resources

A resource is a source or supply from which benefit is produced. Whenever a resource is consumed at a rate higher than at which it is created, the possibility of

future shortages arises and the resource is rendered non-renewable. The fact that a resource is finite if the consumption rate exceeds the formation rate, regardless if it is coal, whale oil,[1] or wood, means that a society must keep its consumption rate below the formation rate, if it wants to use that resource on a renewable basis. Prominent resources that are used in a non-renewable way include (tropical) forests, fishing grounds, and fossil fuels, *e.g.* coal, natural gas, and petroleum.

1.3.2 Petroleum Supply

According to the biogenic oil formation theory, a petroleum deposit is created over a period of millions of years.[2,3] The current rate at which mankind is consuming these deposits, years to decades, renders petroleum a non-renewable resource.[4] Already in 1956, M. K. Hubbert, an American geologist, proposed a model for the extrapolation of a production curve from a finite resource into the future.[5] The model assumes that production levels start at zero, and return to zero when the resource has been exhausted. In between these, the production curve passes through one or several maxima. Although the shape of each production curve may vary, its integral is limited by the maximum recoverable amount of that particular resource.[6] The maximum amount that can be recovered is influenced by several factors, including technological and economical ones, and in most cases will be less than the total amount that is available. When Hubbert applied the model to oil production, a graph as plotted in Figure 1.1 was obtained.

Initially there was very little production, but as oil became accepted as a useful energy carrier, and a resource for the production of chemicals, demand increased and so did production. This was expected to increase further until a maximum production rate is reached, a point referred to as peak oil. At this point the production rate cannot increase any further as it is limited by the discovery rate of new oil reserves, technology, economics, and other factors.[4] After this peak, or plateau, production rate levels will decrease until all reserves are depleted, at least from an economical perspective. The most recent predictions of the International Energy Agency (IEA) assert that peak oil has already occurred at presently discovered fields.[7] Peak oil can of course be delayed by more quickly taking recently discovered fields into production and utilising unconventional oil sources (such as tar sand and heavy oils), but this only postpones the inevitable shortage of this non-renewable resource.

1.3.3 Petroleum Demand

The increasing demand for fossil fuels is exacerbating the situation. The increase stems from a growing world population living at increasingly higher levels of economic welfare, which results in increased petroleum usage per capita. The major share of this increase is taken up by the upcoming economies China and India, although the United States of America (U.S.A.) are still expected to be the second largest consumer of oil by 2035.[7]

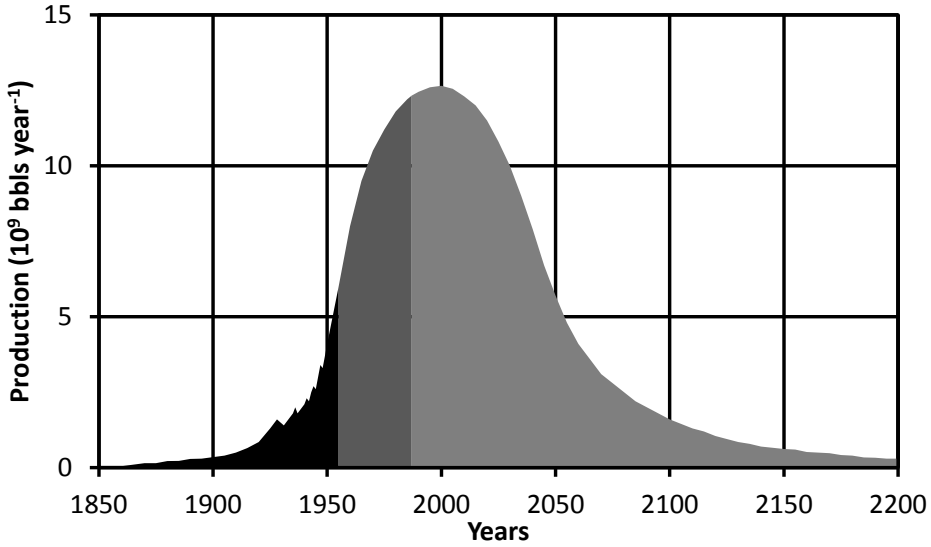


Figure 1.1: Peak oil graph (redrafted from Hubbert[5]) Black, cumulative production; dark grey, proven reserves; light grey, future discoveries; bbls, oil barrels.

The inherently finite nature of petroleum, the decrease in supply, and increase in demand has resulted in a search for alternatives for our current petroleum based society. Besides these reasons to endeavour on a search for alternatives, also other arguments exist that call for replacement of petroleum, as discussed in the next section.

1.4 Additional Drivers for Fossil Fuel Replacement

1.4.1 Global Warming

Although there is still some controversy if anthropogenic heating, *i.e.* global warming caused by human activity, actually occurs,[8–11] governmental policies have been changed in response to the reports of the Intergovernmental Panel on Climate Change (IPCC) which has been reporting on the issue since its establishment in 1988. It has been put forward that the release of several gasses, amongst which are CO_2, CH_4, and N_2O, in the atmosphere results in the so-called greenhouse effect *i.e.* part of the infra-red radiation that would normally escape into space is absorbed by the greenhouse gasses (GHG) and results in an increase in the temperature in the upper layers of the atmosphere. It is of interest to note that water, as a gas, is one of the largest contributors to the greenhouse effect,

especially due to its abundance. Its very nature however precludes its taxation as a GHG despite its large share towards global warming (36–66 %) compared to CO_2 (9–26 %).[12] It has been argued that the greenhouse impact of water is less than that of carbon dioxide, because its residence time in the upper atmosphere is much shorter than that of the latter, rendering it a feedback, rather than a forcing, component.[13] However, the influence of clouds on the earth's annual global mean energy budget is however still not fully understood.

In response to the perceived dangers of global warming, most governments have signed and ratified the Kyoto Protocol. Signees of the protocol strive to cut back on the emission of greenhouse gasses to below the levels of 1990.[14] One way of reducing net CO_2 emissions is by using carbon based energy sources that have only recently taken up CO_2 from the atmosphere, such as plant biomass.

1.4.2 Energy Security

Energy security is divined to exist "if the energy sector does not cause (major) welfare-reducing frictions in the economy at national and global levels".[15] The majority of all oil barrels produced at the moment originates from countries which are considered to be economically and politically unstable regions, like Iraq, Venezuela, and Nigeria.[16] The increasing demand for oil and gas further increases the dependence of America, Europe, and Asia on imports from the Middle East and other unstable regions. It has been recognised by the EU,[17] the U.S.A.,[16] and others that this dependency should be reduced by identifying and exploiting alternative sources of energy.

1.5 Traditional Transport Fuels

According to OECD[‡]-International Energy Agency data, a large part of today's total energy consumption of the U.S.A. and Europe, met by the use of fossil fuels, is used for transportation purposes. In 2005, transport accounted for 29 % of EU energy consumption while this figure was with 41 % even higher for the U.S.A.[18]

Petrol and diesel are the two main fuel types for cars, trucks, and other transportation vehicles at the moment, and their characteristics are shown in Table 1.1. The current resource from which these fuels are derived is oil. The reasons to develop alternatives for fossil fuels listed in the previous section, combined with the expected increase in the use of oil-derived products by the transport sector, have resulted in a world-wide search for alternative energy sources for the transport sector. Important parameters to compare these various alternatives by are their energy-content-to-weight and energy-content-to-volume ratios (see Figure 1.2). These parameters give an indication for the transportability of the energy source. For example, hydrogen gas at standard pressure and temperature,

[‡]Organisation for Economic Co-operation and Development

although very light, is not a very useful alternative as you would require a vast volume of it to be able to travel any reasonable distance. By compressing the gas, the volume is reduced and the energy-content-to-volume ratio is increased (at the expense of the energy needed to compress it).

Table 1.1: Comparison between the composition of carbohydrate-rich biomass and petroleum-based fuels[a]

Property	Petrol	Gasoil/ Diesel	Jet fuel	Carbohydrate
Carbon chain	5–10	12–20	8–16	$[5–6]_n$
O/C molar ratio	0	0	0	1
H/C molar ratio	1–2	∼2	1.9	2
Phase behaviour [at ambient T]	liquid	liquid	liquid	solid
Polarity	non polar	non polar	non polar	polar
Preferred structure	branched/ aromatic/ cyclic/ unsaturated	linear/ saturated	linear/ branched/ aromatic/ cyclic	linear/cyclic

[a] Data taken from Petrus and Noordermeer,[19] Lee *et al.*,[20] and Edwards.[21]

The high energy-content-to-weight ratio of liquid fuels compared to other alternatives such as batteries for use with electric cars, or the use of compressed hydrogen gas, to drive transport vehicles, makes liquid fuels still the most desirable energy carrier at the moment[22] although this has been contested by others based on the fact that electric cars are more efficient in conversion of stored energy to kinetic energy.[23,24] However, considering the fact that our current infrastructure is built around these liquid fuels it is likely that alternatives will have to be compatible with the existing approach, or at least in a transition phase. Renewable (liquid) fuels, commonly referred to as biofuels, and their production, will be discussed in the next sections.

1.6 Biofuels

Biofuels are energy carriers whose energy has been stored by means of biological carbon fixation over a — from a geological perspective — short period of time (up to a few decades), or that have been derived from such material. This definition excludes petroleum which builds up over much longer periods, but includes for example hydrogen gas produced by fermentation of sugars. Biofuels can be very diverse and range from gasses (*e.g.* hydrogen and methane), to liquids (*e.g.* ethanol, butanol, or fatty acid methyl esters [FAMEs]), to solids

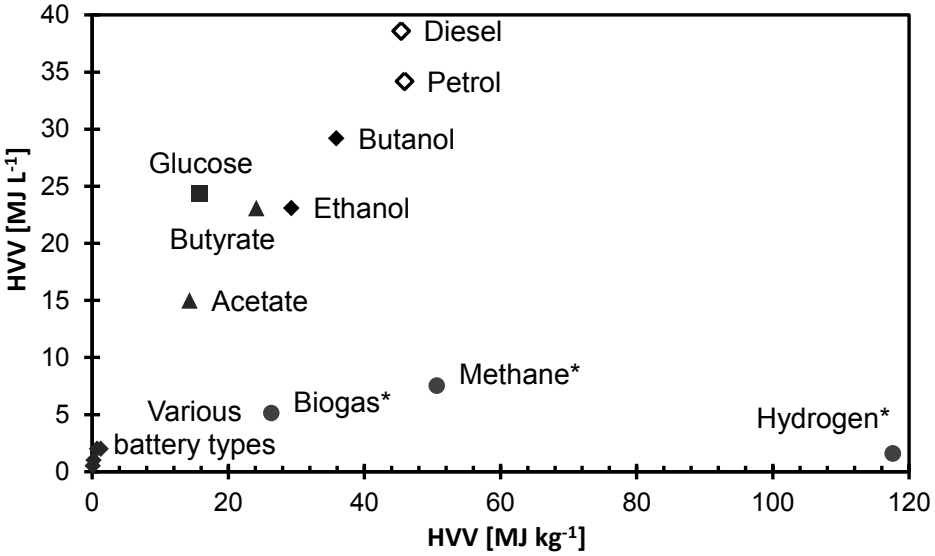


Figure 1.2: Volumetric *versus* mass energy density of glucose, various fermentation products, liquid fuels and gasses. HHV, higher heating value; * indicates value for gas compressed at 200 bar. Adapted from K. J. J. Steinbusch,[25] with permission. Additional data from Fischer *et al.*[23]

(*e.g.* wood). The most prominent biofuels are bio-ethanol and bio-diesel, both of which are currently produced at large scale.

Subsidies and regulatory requirements have created a market for the production and use of these compounds as fuel additives. In 2009 the EU adopted a directive that 10 % of all transport fuel should be renewable by 2020.[26] Also the U.S.A., in 2007, put regulation in place requiring an aggregate of 36 billion gallons ethanol equivalents (136×10^9 L), made from renewables, to be used in transport fuels by 2022.[27] Many other countries implemented similar measures.[28] These measures have resulted in a large increase in biofuel production. In 2008, the U.S.A. was the world's largest producer of bio-ethanol (33.7×10^9 L) followed by Brazil (24.3×10^9 L), whereas bio-diesel production is concentrated in Europe (9.2×10^9 L), with Germany as the main producer, the U.S.A. is second (3.1×10^9 L).[28,29] Worldwide, bio-ethanol production almost quadrupled between 2000 and 2008 from 16 940 to 65 690 million litres, while bio-diesel production increased by more than 18 times from 797 to 14 574 million litres.[28]

1.6.1 First Generation Biofuels

Currently the majority of these biofuels are so-called 'first generation' biofuels, meaning that they are derived from easily accessible and digestible sugar sources

such as starch (from corn and wheat) and sucrose (from sugar cane and sugar beet) in the case of bio-ethanol, and from oils from pressed plant seeds in the case of bio-diesel.[30] There is increasing awareness that the large-scale cultivation of crops for the production of these first generation biofuels is associated with sustainability and environmental issues, something they were intended to remedy.[31] In 2006, 20 % of the total U.S.A. corn supply was allocated to fuel ethanol production.[32] Biofuels have therefore, in part, been blamed for the increasing food prices between 2003 and 2008,[33–37] although this has also been disputed[29] based on the observation that between 2007 and 2009 many basic food commodity prices have significantly declined without there being any reduction in biofuel production.

Besides criticisms for the increased linkages between the food and energy sectors, life-cycle analysis (LCA) studies of first generation biofuels have highlighted a negative net contribution in GHG emission reduction for specific types of feedstock crops and processing techniques. Results of LCA studies show quite some divergence, however, corn (starch) based ethanol consistently showed the most harmful LCA profile.[28]

1.6.2 Second Generation Biofuels

Certain drawbacks of first generation biofuels that are based on starch, sucrose or vegetable oils, can be countered by using lignocellulosic biomass as a feedstock for the production of so called 'second generation' biofuels.[29] The advantage of using lignocellulose is that it cannot be used for human consumption and therefore does not directly compete with food production. Lignocellulosic biomass can be derived from agricultural by-products such as corn stover, wheat straw, and rice straw, residuals that now have hardly any economic value. By using these resources, synergy is created between food production (starch component) and the remaining plant parts (lignocellulose). But also dedicated, so-called 'energy crops' can serve as a source, provided that their production does not affect food crop land use. Energy crops include for example perennial grasses, such as *Miscanthus giganteus*[38] and *Panicum virgatum* (switchgrass),[39] which have low subsistence requirements and can be grown on marginal lands that are unsuitable for food crop cultivation. In addition, wood and related materials from sustainably managed forests can be used, as well as organic waste streams for municipal or industrial sources.[40] Use of these second generation substrates does require extensive pre-treatment by mechanical, chemical, and/or enzymatic methods, in order to release the sugars from the lignocellulosic matrix so that they are available for fermentation.

The nomenclature is sometimes extended to third (or even fourth[41]) generation biofuels, but this is less well-established. 'Third generation' is sometimes used to refer to examples of biofuels produced using a consolidated bioprocessing approach (*i.e.* the biomass pre-treatment step is combined with the fermentation step resulting in combined sugar release and fermentation),[42] or to refer to the

use of algae that produce oils that can be harvested for direct use as a biofuel without the need for a fermentation process.[43]

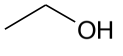
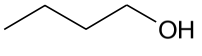
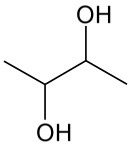
1.7 Butanol as Biofuel

Biofuel is a term to describe various, often very different, compounds that are used to store energy. Ethanol is currently the most widely produced biofuel, but this popularity is mostly a reflection of the ease with which it can be produced rather than its properties as a fuel replacement. The linear, four carbon long alcohol *n*-butanol can also be produced by fermentation. The different physical properties of butanol compared to ethanol (see Table 1.2 and Table 1.3), make it a superior fuel replacement. Its advantages over ethanol include: (i) a higher energy density (see Figure 1.2); hence more kilometres per unit of mass of fuel, (ii) a lower vapour pressure; resulting in lower tail pipe emissions in the lower blending range (0–25 %),[44] (iii) a less corrosive nature; consequently transport is possible using existing infrastructure, as opposed to ethanol, (iv) a lower water solubility; therefore reduced hygroscopicity of the resulting fuel blend and better miscibility with the fuel, and (v) the fact that cars do not require modification of their engines to use mixed butanol petrol or even pure butanol as a fuel,[45–47] where this is not the case for fuel containing more than 10 % ethanol.[48,49] The possibility to use pure butanol as a motor fuel has been shown by David Ramey from ButylFuel LLC (now part of Green Biologics Ltd.), during two long car trips in the U.S.A. in 2005 and 2007 (www.butanol.com, accessed 13 January 2009), thus building the case for the use of higher alcohols as a 'drop in' fuel replacement. The research in this thesis is therefore focused on renewable butanol production, or derivatives thereof such as 2,3-butanediol.

1.7.1 Butanol Production from Petroleum

Currently, the majority of *n*-butanol is produced by petrochemical synthesis for use in the chemical industry. The three most important processes are oxo-synthesis, Reppe synthesis, and crotonaldehyde hydrogenation.[50] In the oxo-synthesis (hydroformylation) process, carbon monoxide and hydrogen are added to the carbon–carbon double bond of propene using catalysts such as cobalt-, rhodium-, or ruthenium-substituted hydrocarbonyls. The resulting aldehyde mixtures obtained in the first reaction step are then hydrogenated to production butanol. The isomeric ratios in which the resulting butanol is produced can be controlled by reaction conditions such as pressure and temperature, as well as by the choice of catalyst. The Reppe process also starts from propane and carbon monoxide but uses water instead of hydrogen. The Reppe process directly produces butanol at low temperature and pressure. The third route for butanol synthesis shows similarities with the biological production route (subsection 1.8.2, page 12) and starts from acetaldehyde (an activated C2 compound just

Table 1.2: Physical and chemical properties of acetone and some alcohols discussed in this thesis.[a]

Property	Ethanol	*n*-Butanol	Acetone	*iso*-Propanol	2,3-Butanediol
Chemical structure					
Molecular weight	46.07	74.12	58.08	60.1	90.12
Melting point at 101.3 kPa [°C]	−114.1	−89.8	−94.7	−87.9	7.6
Boiling point at 101.3 kPa [°C]	78.29	117.7	56.1	82.3	182.5
Density[b] [g/cm^3]	0.789^{20}	0.809^{20}	0.780^{25}	0.781^{25}	1.003^{20}
Vapour pressure at 25 °C [kPa]	7.87	0.86	30.8	6.02	0.02

[a] Data from Lide.[51]
[b] Temperature as indicated in superscript.

like acetyl-CoA), followed by an aldol condensation, a dehydration, and finally a hydrogenation to yield butanol.

Butanol is used in a wide range of industrial applications, including the production of butyl acrylate, used in the manufacturing of, *e.g.* polymeric coatings, adhesives, elastomers and plastics; and butyl methacrylate, used in resins, dental products, as an oil additive and in the leather and paper industry. Other products derived from butanol are butyl glycol, used mainly as an industrial solvent, butyl acetate, used in paints and a flavouring agent in the food industry, butylamine,*e.g.* used in the production of thiocarbazides and butylbenzenesulfonamide, used as plasticizer in nylon.[45] Its annual production has been estimated to be between $3.1 - 5.4 \times 10^9$ kg[52,53] and since its market expansion is projected at 3 % per year,[50] bio-butanol could also be interesting for these applications, provided that the process is economically competitive.

Table 1.3: Properties of alcohols as fuels compared to petrol.[a]

Compound	Heat of combustion ($\Delta_c H°$) [kJ/mol]	Heat of vaporisation ($\Delta_{vap} H_m°$) [kJ/mol]	Energy density [MJ/kg]	RON[b]	MON[b]
Petrol	4817[c]	36	43.5	95	85
Methanol	726	37	22.7	133	99
Ethanol	1368	42	29.7	130	96
iso-Propanol	2006	45	33.4	121	96
n-Butanol	2670	51	36.0	113	94
2-Butanol	2661	42	35.9		
2,3-Butanediol	2461	67	27.3		

[a] Data from NIST[54] and others.[46,55,56]

[b] Abbreviations: RON, Research octane number; MON, Motor octane number; Both are octane rating numbers related to the performance of a liquid in a combustion engine.

[c] Data for heptane.

1.7.2 History of Biological Butanol Production

Although now a petrochemical product, during the first part of the 20th century, butanol was produced by bacterial species of the genus *Clostridium* at an industrial scale by fermentation of sugars (molasses) or starch, via the acetone-butanol-ethanol (ABE) process.[57,58] Butanol is one of the longest linear alcohols, together with 2,3-butanediol, that is a natural, major end-product of microbial fermentation. Microbial production of butanol was reported for the first time by Louis Pasteur in 1862, long before the production of acetone in 1905.[59] Initially, the ABE process was mainly operated for the production of acetone, rather than

butanol, as there was a huge demand for it during World War I for the production of cordite, a family of smokeless propellants used in weapons to replace gun powder.[57]

The fermentative production of acetone was pioneered by Chaim Weizmann, a Jewish Russian organic chemist working in England. He isolated a bacterial strain from the species *Clostridium acetobutylicum*, now termed the Weizmann-strain, which could grow on starch and produce acetone with high yield. During the First World War, access to acetone became of great military importance and Weizmann assisted the war effort by overseeing the implementation of his process to produce it.[§] Despite the success of the process, the increasing price of the substrates (starch or molasses) made it increasingly difficult to compete with the upcoming petrochemical processes, and from 1960 onwards fermentative production of acetone and butanol had essentially stopped in the U.S.A. and Great Britain. Several countries continued to operate the process for various reasons, including South Africa (up to 1981),[57] the U.S.S.R.,[60] and China (until the mid-1990s).[61] The increasing substrate price combined with cheaply available oil ultimately led to the downfall of the process, but increases of the oil price have rekindled interest in the process after the oil crises of 1973 and 1979. From 2007 onwards, fermentative butanol production has once again commenced in China[58] and Brazil,[62] and pilot-scale production is undertaken in, amongst others, the U.S.A. and Great Britain.

1.8 The ABE-Fermentation

1.8.1 Solvent Producing Bacteria

The best butanol-producing (also referred to as solventogenic) microorganisms known to date are all bacterial strains belonging to the *Clostridium* genus and have been classifieds into four species: (i) *Clostridium acetobutylicum* (type strain ATCC 824); (ii) *C. beijerinckii* (type strain NCIMB 9362); (iii) *C. saccharobutylicum* (type strain NCP 262); and (iv) *C. saccharoperbutylacetonicum* (type strain ATCC 27021).[63–66] Clostridia are anaerobic, endospore forming, gram positive, rod shaped bacteria. All known solventogenic clostridia contain DNA with a low GC-ratio and are mesophilic. The toxicity of butanol increases with temperature and it is likely that the fact that they are mesophilic is related to that. Fermentations by these organisms result in either production of acetone, butanol, and ethanol (ABE fermentations) or in some cases acetone is further reduced to *iso*-propanol resulting in an IBE fermentation (by *e.g. C. beijerinckii* NRRL B-593 and LMD 84.48). The *Clostridium acetobutylicum* strain ATCC 824 is the most extensively studied solventogenic bacterium. The strain was isolated from Connecticut garden soil in 1924[64] and was selected to be the *C. acetobutylicum* type strain. In this strain, most of the genes involved in solvent

[§]He later became the first president of the state of Israel in 1949.

production are located on a megaplasmid of 210 kb (pSOL1). The loss of this megaplasmid results in asporogenous strains unable to make solvents[67] which are normally produced in a 3:6:1 ratio (A:B:E). The whole genome of *C. acetobutylicum* ATCC 824 has been sequenced and is publicly available,[68] as is the genome of *C. beijerinckii* NCIMB 8052.[69] The genomes of two other *C. acetobutylicum* strains are also publically available: DSM 1731[70] and EA 2018.[71] In addition to the genome sequences, microarrays are available for *C. acetobutylicum*[71–73] and *C. beijerinckii*,[74] and both stoichiometric and kinetic metabolic models have been published for *C. acetobutylicum*,[75–80] *C. saccharoperbutylacetonicum*,[81,82] and *C. beijerinckii*.[83]

A characteristic of most, if not all, of these strains which negatively affects the stability of the cultures is their gradual loss of solvent production when they are kept in a vegetative stage for long periods of time (*i.e.* during repeated transfers or long periods of continuous cultivation), a phenomenon known as 'degeneration'.[57] In addition to their lack of solvent production, degenerated strains show different morphological and physiological characteristics compared to the parent strain; larger and translucent colonies with irregular shapes,[84] a longer or thinner cell shape and a characteristic infrared spectrum.[85] The loss of solvent production seems to be linked to the loss of the ability to sporulate, since several asporogenous degenerated mutants have been isolated during prolonged continuous cultures.[84,86] However, a solvent-producing asporogenous mutant has been described, and used in the development of a continuous ABE-process.[87] As earlier mentioned, the loss of the pSOL1 plasmid in *C. acetobutylicum* ATCC 824 results in degenerated strains,[67] but in other solvent-producing strains, a different mechanism for degeneration is expected, since they do not contain such a plasmid and the genes involved in solvent production are still present in the degenerated mutant.[88,89] Recently, a cell density-dependent regulatory mechanism (quorum sensing) has been proposed to be involved in the degeneration of strain *C. saccharoperbutylacetonicum* N1-4, since solvent production in a degenerated mutant could be restored after the addition to the cultivation medium of concentrated broth extract from a wild-type fermentation.[89]

In addition to the four solventogenic species mentioned previously, several less well-known butanol producers are also described. *C. pasteurianum*, a classic acid fermenter, is capable of ABE-solvent production under certain conditions,[90,91] and so is *C. aurantibutyricum*.[92] Also *C. tetanomorphum*[93] has been reported to produce butanol and ethanol, but no acetone or *iso*-propanol.[94] In addition, species such as *C. sporogenes* and *C. cadaveris* produce minor amounts of *n*-butanol, but acetone and *iso*-propanol are not detectable.[92]

1.8.2 Metabolism

A typical ABE batch fermentation by *C. acetobutylicum* goes through two phases: (i) a growth-associated phase during which the acids acetate and butyrate are formed, called the acidogenic phase; and (ii) a stationary phase during

which part of the acids are taken up and the solvents butanol, acetone, and ethanol are formed, termed the solventogenic phase. Common to both phases are the biochemical pathways leading from carbohydrates to acetyl-CoA (Figure 1.3). Hexose sugars are metabolised via the Embden-Meyerhof-Parnas pathway resulting in 2 moles of pyruvate per 1 mole of hexose, with the net production of 2 moles of ATP and 2 moles of NADH. The organism also seems to have the possibility to produce NADPH instead of NADH during conversion of glyceraldehyde-3-phosphate at the expense of 1 ATP when using the EMP pathway.[95,96] The utilisation of pentoses takes place via the pentose phosphate pathway (Warburg-Dickens pathway) of which only the non-oxidative part is functional.[97,98] The pyruvate resulting from glycolysis is cleaved by pyruvate ferredoxin oxidoreductase in the presence of coenzyme A to yield CO_2, acetyl-CoA and reduced ferredoxin. In the proposed mechanism of PFOR, pyruvate is decarboxylated to first form the hydroxyethyl-thiamine pyrophosphate intermediate (TPP) which then reacts with coenzyme A (CoA) to produce acetyl-CoA. Recent metabolic tracer analysis suggest that the carboxylic group in pyruvate interchanges rapidly with CO_2, although the overall PFOR reaction is essentially irreversible.[98] Reduced ferredoxin can be oxidised by the reduction of protons to molecular hydrogen by the hydrogenase enzyme. Acetyl-CoA is the central intermediate in the branched fermentation pathways leading to both acid and solvent production.

Acidogenic Phase

During the acidogenic phase, sugars are typically metabolised to acetate, butyrate, carbon dioxide and molecular hydrogen. The enzymes involved in this stage in the acetate and butyrate producing pathways are phosphate acetyltransferase (Pta), acetate kinase (Ack), butyryltransferase (Ptb), and butyrate kinase (Buk1), respectively. In both pathways additional ATP is generated in the last substrate level phosphorylation reaction. Butyryl-CoA is produced by coupling two acetyl-CoA molecules catalysed by thiolase (ThlA), resulting in acetoacetyl-CoA. This is subsequently reduced to β-hydroxybutryl-CoA using NADH by the enzyme 3-hydroxybutyryl-CoA dehydrogenase (Hbd), followed by a dehydration by crotonase (Crt) to crotonyl-CoA. The double bond of crotonyl-CoA can be reduced by butyryl-CoA dehydrogenase (Bcd) using NADH via two electron transfer flavoproteins, EtfA and EtfB. Recently it has been shown in *C. kluyveri* that this very exergonic reaction actually uses 2 NADH equivalents.[99] The reducing power that is not used for reduction of crotonyl-CoA is used to generate reduced ferredoxin, resulting in NADH mediated hydrogen production.

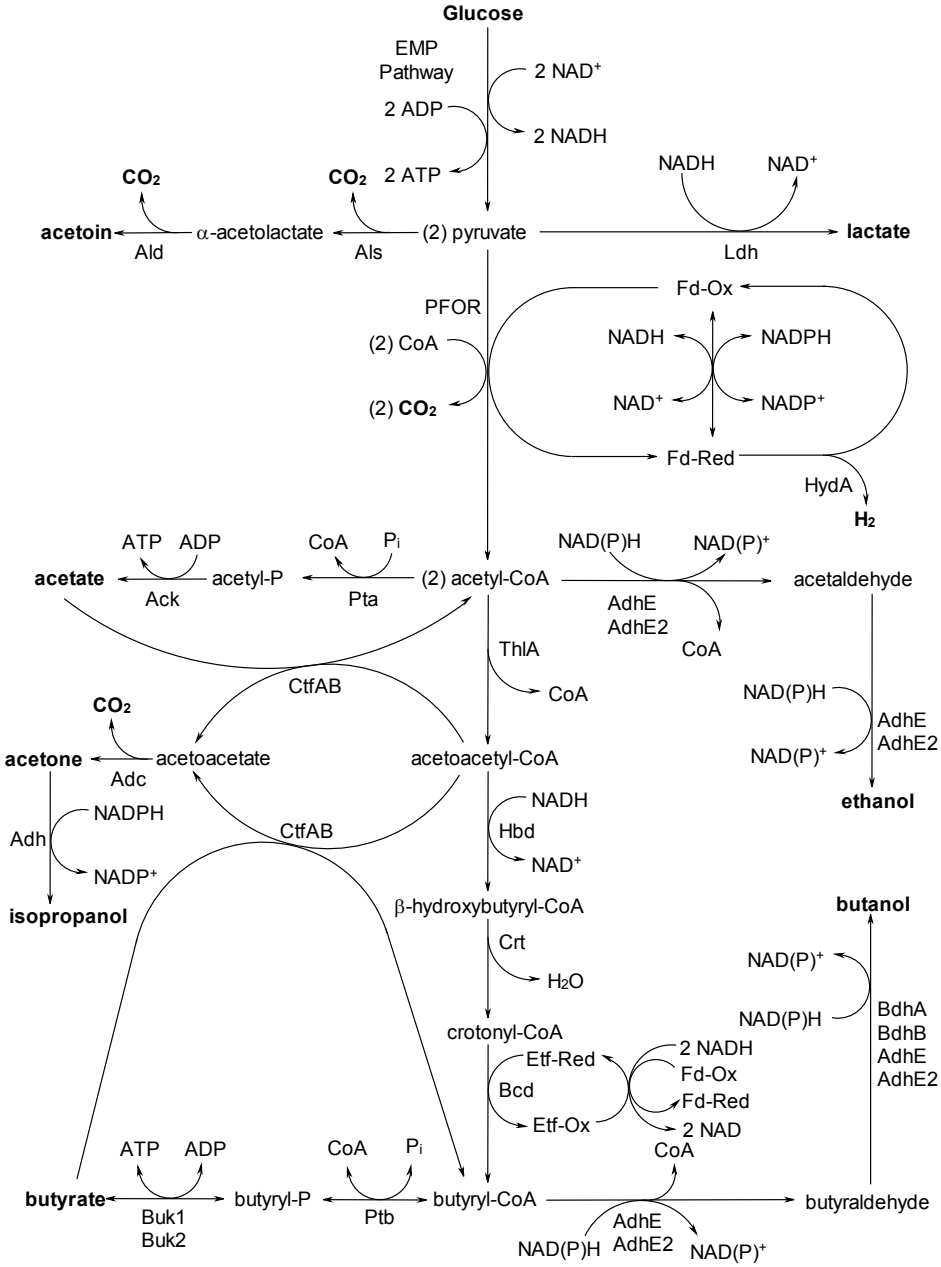
Solventogenic Phase

During this first phase, the short chain fatty acids acetate and butyrate accumulate in the extracellular medium, thereby lowering the pH. After some time, what has been called the ‘solventogenic switch’ or ‘metabolic switch’ occurs,

which triggers the organisms in taking up the previously produced acetate and butyrate, and converting them to solvents, combined with continued glucose utilisation and carbondioxide and hydrogen release. Several solvent pathways genes are now expressed and the enzyme complex acetoacetyl-CoA:acetate/butyrate:CoA transferase (CtfAB) and the enzyme acetoacetate decarboxylase (Adc) now enable the activation of excreted acids to their corresponding CoA intermediates with concomitant acetone production. In addition to this pathway, the butyrate pathway is inverted and used to take up butyrate from the medium by expression of the butyrate kinase 2 (Buk2) enzyme. Although the acetate concentration in the medium drops, there is evidence that the acetate pathway remains active to generate ATP, but the acetate production rate is lower than the acetone mediated uptake route.[76] An interesting observation is that metabolite measurements indicate that there is rapid interchange between extracellular and intracellular acetate, and that the aceate formation pathway is highly reversible.[101] Next to acetone formation, aldehyde and alcohol dehydrogenases such as AdhE, BdhA, and BdhB (sometimes referred to as Aad, Bdh I, and Bdh II respectively) are expressed and reduce acetyl-CoA and butyryl-CoA to ethanol and butanol, respectively. Under certain so-called alcohologenic conditions, AdhE2 is expressed, resulting in solventogenesis without acetone formation.

Initiation of solvent formation requires a low pH, threshold concentrations of acetate and butyrate, and a suitable growth limiting factor such as phosphate or sulphate,[102,103] Solvent formation appears to be associated with the availability of ATP and NADP(H)[104] and can be controlled, in continuous culture, by varying the pH in the bioreactor.[105] If the transition does not occur and the culture continues to produce acids, then the culture is said to undergo an 'acid crash'.[106] During the transition of an acid-forming culture to a solvent-forming one, a number of observations can be made. The pH stabilises due to termination of net acid

Figure 1.3 ***(following page)*****:** Biochemical pathways in *C. acetobutylicum* and *C. beijerinckii*.[57,99,100] Substrate (glucose) and fermentation products are shown in boldface. Enzymes catalysing the different reactions are indicated by abbreviation of their name: Ldh, lactate dehydrogenase; Als, acetolactate synthase; Ald, acetolactate decarboxylase; PFOR, pyruvate-ferredoxin oxidoreductase; HydA, hydrogenase; Pta, phosphate acetyltransferase (phosphotransacetylase); Ack, acetate kinase; ThlA, thiolase; Hbd, 3-hydroxybutyryl-CoA dehydrogenase; Crt, crotonase; Bcd, butyryl-CoA dehydrogenase; Ptb, phosphate butyryltransferase (phosphotransbutyrylase); Buk1, Buk2, butyrate kinase 1 and 2, respectively; AdhE, AdhE2, aldehyde-alcohol dehydrogenase 1 and 2, respectively; BdhA, BdhB, butanol dehydrogenase A and B, respectively; CtfAB, acetoacetyl-CoA:acetate/butyrate:CoA transferase subunits A and B; Adc, acetoacetate decarboxylase; Adh, primary and secondary alcohol dehydrogenase (in *C. beijerinckii* NRRL B593). Other abbreviations: CoA, coenzyme A; EMP, Embden-Meyerhof-Parnas pathway; Etf, electron transfer flavoprotein; Fd, ferredoxin; $NAD(P)^+$ Nicotinamide adenine dinucleotide (phosphate), oxidised; NAD(P)H Nicotinamide adenine dinucleotide (phosphate), reduced; P_i, phosphate.


Glucose
EMP Pathway
2 NAD+
2 NADH
2 ADP
2 ATP
CO2
CO2
NADH
NAD+
acetoin
α-acetolactate
(2) pyruvate
lactate
Ald
Als
Ldh
PFOR
Fd-Ox
(2) CoA
(2) CO2
NADH
NADPH
NAD+
NADP+
Fd-Red
HydA
H2
ATP
ADP
CoA
Pi
NAD(P)H
NAD(P)+
acetate
acetyl-P
(2) acetyl-CoA
acetaldehyde
Ack
Pta
AdhE
AdhE2
CoA
ThlA
CoA
CtfAB
NAD(P)H
AdhE
AdhE2
NAD(P)+
CO2
acetone
acetoacetate
acetoacetyl-CoA
ethanol
Adc
NADPH
Adh
NADP+
CtfAB
NADH
Hbd
NAD+
isopropanol
β-hydroxybutyryl-CoA
butanol
Crt
H2O
NAD(P)+
NAD(P)H
BdhA
BdhB
AdhE
AdhE2
crotonyl-CoA
Etf-Red
Bcd
Etf-Ox
2 NADH
Fd-Ox
Fd-Red
2 NAD
CoA
ATP
ADP
CoA
Pi
butyrate
butyryl-P
butyryl-CoA
butyraldehyde
Buk1
Buk2
Ptb
AdhE
AdhE2
NAD(P)H
NAD(P)+

production, cells no longer divide and undergo major physiological changes. The vegetative cells are highly motile, thin, and rod-shaped but subsequently change into swollen, phase-bright, cigar-shaped cells when the transition to solventogenesis is initiated.[107,108] The cells accumulate granulose, which is an amylopectin-like storage polymer, possibly supplying the cells with carbon and energy during sporulation.[109] The cells lose their motility and produce an extracellular capsule. This stage is normally followed by the initiation of endospore formation, which involves the production of a fore-spore septum at one or both poles of the cell, followed by endospore production and finally cell lysis, resulting in the release of the mature spore. Spores provide the organism with a possibility to withstand unfavourable conditions such as oxygen exposure, heat, desiccation, and toxic chemicals, but make it metabolically inactive.

The solventogenic phase can be seen as a response of the organism to the decreasing pH to a level where it would no longer be able to grow. By taking up the produced acids from the medium and converting them to neutral solvents, the organism can continue to generate ATP by fermenting glucose. This allows it time to form an endospore that can survive the adverse conditions by the accumulated acids and solvents. Hence solventogenesis and sporulation are expected to the intimately linked,[110] with the former ultimately resulting in the latter and thus termination of growth. It should be noted, however, that actively growing solventogenic cultures do exist, for example in continuous culture fermentations, which has an actively dividing solvent forming culture.[111,112] Recently, Tracy *et al.*, based on fluorescence-assisted cell sorting (FACS) data, suggested that not the cells with the typical swollen shape are the solvent-forming cells but rather that solvent formation is carried out by normally dividing cells. These vegetative cells are predominately responsible for butanol production, and are likely to be a precursor to clostridial-form cells.[113]

1.8.3 Drawbacks and Possible Improvements

There is significant industrial interest in the ABE process as a green process for the production of butanol to be used as chemical or as fuel, being the BP-Dupont joint venture Butamax (www.butamax.com) one of the most developed initiatives. As it occurs with other fermentation processes for the production of alcohols and chemicals from biomass resources, improvements are necessary in order to make these economically viable, including substrate costs and availability, diluted product streams and the presence of multiple fermentation products which makes the separation very costly (see Table 1.4). One of the most important bottlenecks to be solved in the ABE process is the separation of the solvent products, which are present at low concentration, from the fermentation broth in an energetically and economically efficient manner.[114]

The substrate costs of a conventional ABE plant using corn starch would make up to 79 % of the overall operating costs of the process.[115] It is therefore essential that alternative, cheap and renewable substrates are found. As put for-

Table 1.4: Key issues associated with the ABE-process[57,115–118]

Area	Issue	Possible solution
Substrate	Substrate cost (starch, molasses)	Use of alternative (ligno-cellulosic) feedstocks
	Competition with food production	
Process	Product inhibition levels around 2 % butanol	Develop more resistant microbes or use other production hosts
		Apply *in situ* product removal
	Mixed fermentation products (acids, hydrogen)	Develop microbes which produce a reduced number of by-products
	Batch process operation	Strains selected for use in a continuous fermentation process Improved process conditions
	Low substrate to product conversion efficiency	Improved microbes with higher butanol yields and/or higher butanol to other solvents ratios
	Phage infections	Isolate phage resistant strains
Product recovery	Product isolation from dilute water streams	Low energy methods for solvent recovery and purification
	The fermentation process produced large volumes of effluent, which required the development of specific processes for handling, treatment, and processing	Recycling of process water back through the fermentation

ward in subsection 1.6.2, lignocellulose is a prime target for this. Lignocellulose is composed of cellulose, hemicellulose, and lignin. Both cellulose and hemicellulose are sugar polymers, whereas lignin consists of hydrophobic, aromatic groups. Plants use lignocellulose for their structural integrity and have therefore evolved to resist degradation from outside sources. In turn, organisms that can grow on lignocellulosic plant material have found ways to degrade it. Anaerobic cellulolytic microorganisms have developed dedicated large enzyme complexes called cellulosomes,[119] while some aerobic living microorganisms secrete synergistically acting individual endoglucanases, exoglucanases, and ancillary enzymes to attack plant cell walls.[120] Unfortunately, *C. acetobutylicum* ATCC 824 cannot degrade crystalline cellulose, even though the genome encodes eleven proteins identified as cellulosomal components, ten of which are organised in an operon like cluster (ca_c0910 to ca_c0919) with a gene order similar to that of *C. cellulolyticum* and *C. cellulovorans*.[116,121] Although solventogenic strains are not cellulolytic, they are able to utilise all sugars (C5, C6) present is cellulosic biomass, which represents an important advantage compared to other organisms, such as current industrial yeasts for ethanol production. In addition, clostridial species are able to degrade and utilise poly- and oligosaccharides from (hemi) cellulose,[122,123] and

this may result in the need of less stringent methods for biomass pre-treatment and hydrolysis compared to those for other processes. An alternative strategy would be the change the butanol production host, use co-cultivation, or through heterologous expression of a functional cellulosome, enable the organism to use lignocellulosic substrates.

The main challenge for the ABE process is the low butanol concentration in the fermented broth (1.2-1.6 %), which causes problems with product recovery and large volume waste streams. The ABE fermentation results in several end-products, which has as a consequence that the yields of butanol obtained are less than optimal. Various methods to improve the efficiency (higher yields of butanol per gram of feedstock) of wild-type strains have been applied: (i) to inactivate or reduce expression of genes encoding enzymes involved in pathways leading to by-products;[124–126] (ii) by creating regulatory mutants;[127] or (iii) by overexpression of certain beneficial genes.[128,129] These mutations attempt to direct metabolic intermediates (acetyl-CoA, acetoacetyl-CoA, butyryl-CoA) towards butanol formation, thereby increasing the production selectivity (*i.e.* the amount of substrate that is used to produce butanol *versus* other products). An in-detail discussion of the various mutants is provided in section 1.11 of this chapter.

Another reason for the low efficiency of the process is the fact that butanol is toxic to the producing organism at low levels (1–2 %) and thus ultimately limits production to around 21 g/L.[130] Butanol has membrane-distorting properties due to its hydrophobic chain and polar group, which cause severe cell damage.[131,132] Several factors could be involved in the stress that microorganisms experience due to the presence of solvents, which include disruption of: (i) nutrient transport; (ii) ion transport (sodium-potassium pump); (iii) phospholipid composition of cell membranes; and (iv) cell metabolism.[130] Many efforts have been made in the past to obtain clostridial strains with increased 1-butanol tolerance, but with limited success.[133–138] An important observation is that increased resistance to 1-butanol does not necessarily impart greater butanol production by the organism: it can even result in strains producing less butanol.

An alternative to strain improvement is the continuous removal of solvents during the course of the fermentation by adsorption, gas stripping, liquid-liquid extraction, perstraction, pervaporation, reverse osmosis, or liquid demixing.[130] This results in the organism experiencing lower butanol levels. However, all these techniques are either too energy-intensive, or still require further development to improve their selectivities (pervaporation and adsorption).[114]

Phage infections were a reoccurring problem of the classical ABE-process, illustrated by the loss of solvent production as recorded for industrial process runs in South Africa in the 1980s. The infections required strain rotation and isolation of resistant strains to the particular phage causing the infection.[139]

Lastly, the batch style nature of the fermentation results in increased downtime compared to a continuous process, increasing costs. It should be noted however that semi-continuous process conditions have been employed in both Russia and China at production scale.

In summary, strain improvements towards increased butanol production, selectivity, productivity, and final production titres would contribute to once more make the ABE-fermentation process commercially viable. The application of metabolic engineering techniques and strategies could be a solution to these problems and is the goal of the work described in this thesis. In the next sections, an update of the available genetic techniques will be given and various mutants known from the scientific literature will be discussed.

1.9 Metabolic Engineering of Clostridia

Metabolic engineering (*i.e.* the purposeful modification of intermediary metabolism using recombinant DNA techniques)[140] of solventogenic clostridia is a developing field. Various basic tools have been available for some time and have recently been reviewed.[50,141] These include shuttle plasmids, like pMTL500E,[142] pIMP1,[143] pSYL2[144] three antibiotic resistance markers (*ermB* erythromycin/clarithromycin; *catP*, chloramphenicol/thiamphenicol; *tetM*, tetracycline); random chemical mutagenesis;[145] transposon mutagenesis;[146] and transformation protocols.[141] Unfortunately, the chosen type strain for the ABE fermentation, *C. acetobutylicum* ATCC 824, expresses a restriction endonuclease enzyme, *Cac*824I, that cuts any foreign, not properly methylated DNA transformed into the cell.[147] The host restriction enzyme can be circumvented by *in vivo* methylation of the DNA, using a methyltransferase gene from *Bacillus subtilis* phage ϕ3T.[147] This method allows for transformation of plasmids into *C. acetobutylicum* with increased efficiency, although it is still very low compared to organisms like *E. coli* and *B. subtilis*.

The creation of gene knock outs by single cross-over homologous recombination using suicide plasmids in *C. acetobutylicum*[148] and *C. beijerinckii*[149] was reported but these gene knock outs are not stable, because the process is reversible, resulting in revertants with a wild type genome in the absence of selective pressure. Recent developments have made it now possible to create stable knock-out and knock-in mutants. Because one of these techniques played a pivotal role in the project, the various methods will be discussed in the following section.

1.10 New Genetic Tools for Clostridia

Recently, four new technologies have emerged that make it possible to perform targeted, stable gene knock-outs in Clostridia. Additionally, these tools can also be used to integrate additional DNA sequences in the bacterial genome.

1.10.1 Mobile Group II Introns

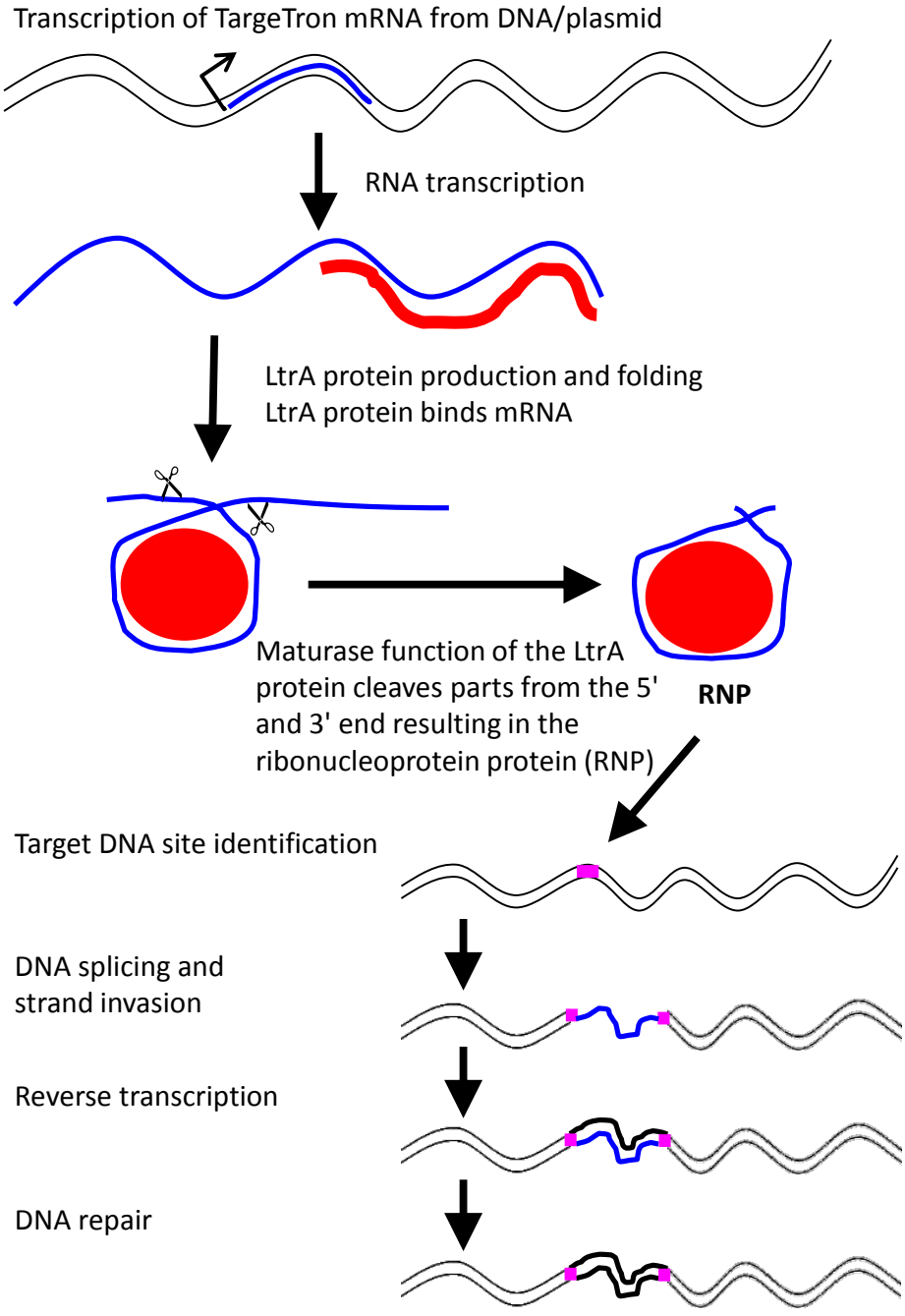
One technique is based on the use of mobile group II introns. These introns, in their active form, are catalytic RNAs, found frequently in organelles and bacteria.

The *Lactococcus lactis* Ll.LtrB intron is used as a model system for these kind of introns and the sequences in the intron RNA that determine its insertion site have been elucidated by the Lambowitz group.[150] This allows one to retarget the intron into virtually any gene in the genome, causing gene disruption. The mobility of the intron is based on a mechanism termed retrohoming, which is mediated by a ribonucleoprotein (RNP) complex (See Figure 1.4). The RNP complex, consisting of an RNA lariat and an intron encoded protein, inserts the bound RNA into one strand of a double-stranded DNA target site, resulting in an RNA/DNA hybrid strand. Reverse transcription of the RNA, removal, and replacement of it by DNA residues by the host repair machinery then results in a stable integrated intron. The system has been commercialised under the name TargeTron by Sigma Aldrich.[151]

While the intron II system is almost completely host independent, the vector and some features of the system are host dependent. The Minton group adapted the system for use with the clostridial genus.[152] The clostridial system, called ClosTron, now basically has the same features as the original *E. coli* system, and more.[153,154] The strong suit of the system is an erythromycin-based retrotransposition-activated selectable marker (RAM)[151] which is an adaptation of the kanamycin RAM present in the TargeTron system. This marker system allows one to differentiate between mutants containing a successfully integrated intron element and transformant cells were integration did not take place. The so-called second generation Clostron system also has FRT sites situated around the erythromycin gene. The *flp* recombinase system can then be used to remove the erythromycin selection marker from the genome, after successful isolation of a mutant. After only a few months, several labs had created more than 60 mutants in the clostridial species *C. difficile*, *C. sporogenese*, *C. botulinum*, and

Figure 1.4 ***(following page)*:** Schematic depiction of the way mobile group II intron insert in the genome. From top to bottom. The intron II mRNA is transcribed from the introduced plasmid using a host compatible promoter. Near the 3′-terminus of the mRNA is an open reading frame (ORF) that encodes the LtrA protein, the protein is produced, folds and binds the mRNA. The LtrA protein encodes four function and the first, the maturase, function cuts the 5′ and 3′-end of the mRNA. Included in the 3′-end is the ORF encoding the LtrA protein which is therefore lost from the final integrated intron. The matured mRNA complexed with the LtrA protein is referred to as the RNP (for ribonucleoprotein). In the second phase the RNP finds its target site in the DNA of the host using helicase activity, its second function. When the target site is found both DNA strands are cleaved (third function) and the RNA that is bound to the RNP is inserted into one of the DNA strands creating a DNA/RNA hybrid. The last function of the LtrA, reverse transcriptase activity, allows it to reverse transcribe (RT) the inserted RNA sequence. The host DNA repair systems then remove the RNA and fill in the resulting gap with the complementary sequence. Because the mobility function of the intron is encoded by the *ltrA* gene, which is removed from the host upon loss of the plasmid the mutation is a stable one.

Mechanism of mobile group II intron insertion



C. acetobutylicum, whereas up to that time in total only 10 defined, knock-out mutants were described in the literature.[155]

A specific site within the intron can be used to insert additional genetic material, *e.g.* genes, in the genome. The RNP complex will integrate the intron plus the additional inserted sequences in a site-specific manner, resulting in a stable genomic insertion. The upper size limit for this is around 2 kilo base pairs.

Other researches also reported the construction of a TargeTron-based system for use with *C. acetobutylicum*.[156] The system is similar to the Clostron but does not utilise a RAM to expedite selection of the mutant. To successfully isolate a knock-out using that system, a possibly extensive colony PCR screen is proposed to identify the correct clone. The advantage is that once isolated, the strain can immediately be used for a next round of mutagenesis.

1.10.2 Double Crossover Homologous Recombination

The three other systems that have been developed are based on homologous recombination and are aimed at stably integrating new sequences into the genome. They can also be used to delete, or mutate genes that are already present in the genome. The first system, developed by Soucaille and co-workers, uses a negative selection marker for the second cross-over event.[157] This approach, based on the same strategy in *B. subtilis*,[158] allows for selective pressure to be applied to identify the, rare, second cross-over event.

As first step to creating a knock-out using this method a mutant strain of *C. acetobutylicum* is created that lacks the uracil phosphoribosyl-transferase gene (*upp*), yielding it resistant to 5-fluorouracil. A knock-out plasmid is created containing two stretches of sequences that flank the region that is to be deleted from the genome. Between these sequence stretches, an erythromycin resistance cassette, flanked on both sides by FRT sites, is inserted. The plasmid further contains a functional copy of the *upp* gene, like the one that is removed from the genome. When the plasmid is then used to transform a *C. acetobutylicum* strain, 5-fluorouracil and erythromycin resistant colonies can be obtained. After sub-culturing and selection on erythromycin and 5-fluorouracil, clones will be obtained that underwent a double cross over event. Subsequent introduction of a plasmid that expresses a Flp recombinase, allows for removal of the erythromycin resistance cassette, and for additional knock-outs to be made. Next to the erythromycin cassette, also additional cargo can be inserted, generating stable gene integrants. This has been exemplified by Von Abendroth *et al.*, who introduced an algal hydrogenase that replaced the native clostridial gene.[159] Disadvantage of this system is that, to isolate the strain that has lost the plasmid and has undergone two homologous recombination events, extensive replica plating has to be undertaken.

A similar approach has been described by Tracy and Papoutsakis,[160], however their approach reportedly uses expression of a heterologous *B. subtilis recU* resolvase gene (involved in genetic recombination) to increase the recombination

frequency in *C. acetobutylicum*. Similar to the previously discussed approach, this method also depends on the extensive use of serial transfers and replica plating to isolate strains that have undergone the desired recombination event, and have lost the plasmid. The method has been applied successfully to generate knockout strains of various genes.[107,161,162] However, the reported use of *recU* expression for an improved recombination efficiency is unclear as all mutant reported in the peer-reviewed literature underwent only a single recombination event, not the desired two. The usefulness of the approach is therefore unclear because a similar, but better, result can be obtained by suicide plasmids reported previously as the currently reported strains have gained two antibiotic selection markers as opposed to one.

The last approach based on homologous recombination was reported by Heap *et al.*[163] and relies on an intricate combination of carefully designed plasmid components to obtain genomic integrands via homologous recombination in either a wild-type or uracil auxotroph background. The system relies on a pIM13 replicon which exhibits segregational instability in *C. acetobutylicum*, resulting in quick loss of the plasmid in the absence of selective pressure, and on asymmetrical homology arms flanking the DNA sequence that needs to be inserted into the genome. This approach allows for multi-step stable insertion of DNA into the chromosome, paving the way for expression of multiple genes or large operons, that would otherwise be too large to clone, from a chromosomal location. In addition recently also a new counter selection marker, CodA, was described for *C. difficile* that could also have applications in *C. acetobutylicum*.[164]

1.11 Metabolic Engineering Targets

Several metabolic engineering strategies have been published that resulted in the creation of recombinant strains with increased production of butanol, together or in conjunction with the other solvents. A short discussion of the most successful and interesting approaches is presented in the next paragraphs. A listing of all discussed mutants can be found in Table 1.5.

1.11.1 Catabolic Pathways

Most studies to date have focused on changing catabolic pathway fluxes directly involved in butanol or in by-product formation. Two of the first targeted knock out mutants produced were affected in their capacity to produce acids. Phosphotransacetylase (*pta*) as well as butyrate kinase (*buk1*) was inactivated by Green *et al.*[126] Both mutants were obtained by homologous, single cross-over recombination. The *pta* mutant had reduced acetate and increased butyrate levels, and the converse was true for the *buk1* inactivated mutant. However, only the solvent forming characteristics of the *buk1* mutant were affected. It turned out to be a butanol super-producer reaching up to 225 mM butanol as

end concentration in batch cultures.[128] Ethanol production by the mutant had also increased, but acetone levels were unaffected. Later studies showed that the *buk1* mutant, as expected, accumulated high levels of butyryl-phosphate. This molecule is implicated in intercellular regulation in *C. acetobutylicum* and associated with the transition into solventogenesis.[165] A recently constructed *ptb* knock-out mutant[166] confirmed the essentiality of PTB in butyrate production as the mutant did not accumulate it. Depending on the pH the mutant predominately produced ethanol with butanol becoming the second most abundant product. Butyrate feeding experiments demonstrated that the mutant was still able to take up butyrate from the medium, presumably via acetoacetyl-CoA:acyl-CoA/butyrl-CoA transferase enzyme complex.

Although Nair *et al.* demonstrated that over-expression of *adhE*, the main alcohol producing dehydrogenase, in the wild-type did not result in altered solvent production titres,[167] the over-expression of *adhE* in a *buk1* deficient background did result in increased ethanol production.[128] It was however not successful in increasing butanol production, indicating that the rate-limiting step for that compound was not the dehydrogenase, but probably lies with the production of the precursor butyryl-CoA.

In *C. acetobutylicum* ATCC 824, the only polycistronic transcript involved in solvent production is the *sol* operon. It contains the *adhE* gene and the two genes coding for the subunits CtfA and CtfB of the acetoacetyl-CoA:butyrate/acetate-CoA transferase enzyme. This complex enables the uptake of acids under the concomitant production of acetoacetate, which can then be decarboxylated to yield acetone. Over-expression of the *ctfA/B* and *adc* acetone production genes almost doubled acetone titres and reduced acid levels due to increased uptake.[168]

The sol operon is preceded by *orf5*, also referred to as *solR* for *sol* locus repressor. An initial report by Nair *et al.*[169] suggested that the gene coded for a transcriptional repressor of the *sol* operon. This was later shown to be incorrect as the gene encoded a glycosylase/deglycosylase protein, that was shown to be localised on the outer side of the cell membrane.[170] The reduction of solvent levels when over-expressing *orf5* was shown to be due to erroneously subcloning part of the regulatory region of the *sol* operon.[171] However, combined with *adhE* over-expression, the *orf5* knock-out mutant is still the top butanol producing strain to date, reaching 238 mM (17.6 g/L) in pH-controlled batch fermentations. Acetone and ethanol levels are also increased in this mutant, making up to 28 g/L of total solvents.[127]

The *C. acetobutylicum* M5 strain[172] is called degenerated because it can no longer produce solvents or sporulate due to the loss of the pSOL1 megaplasmid.[67] In several studies, it has been used as a platform to reintroduce some of these solvent genes to partly restore alcohol production without acetone production. Reintroduction of the *adhE* gene results in partly restored butanol and ethanol production.[173] Recent work by Sillers *et al.* demonstrated that the M5 strain lacks the metabolic flexibility to alter oxidation/reduction pathways to achieve electron balance.[129] Inactivation of acid production pathways in an M5

background resulted in strongly increased doubling times, and in reduced acid production of the down-regulated pathway. When attempts were made to overexpress the *adhE* gene in these acid production mutants, only the strain with an inactivated acetate kinase could produce transformants. This did however not result in increased butanol or solvent production, but rather the inverse, increased acid production and decreased solvent production.[129] This suggests that the M5 strain is limited in its ability to balance the electron flow, and mainly uses butyrate production to balance the production of reducing equivalents. The recent discovery that in *C. kluyveri*, and probably *C. acetobutylicum* too, reduction of crotonyl-CoA results in the concomitant reduction of ferredoxin, could hint at a way for the organism to regulate the flow of acetyl-CoA towards butyryl-CoA.[99] It is suggested that this inelasticity could be related to a missing gene or genes that are present on the pSOL1 megaplasmid. Alternatively, pSOL1 also encodes many transcription factors and DNA-binding proteins that could be important in facilitating the electron flow.[129]

Antisense RNA technology has been also applied to down-regulate gene expression of specific genes. By making antisense constructs against the genes *ptb*, *buk*,[174] *adc*, *ctfA*, and *ctfB*[125] the fluxes through several pathways were altered. Especially the last construct was effective in almost completely abolishing acetone production. Because the *adhE* gene, involved in butanol production, is part of the same polycistronic operon, butanol levels were also negatively affected. In a later study it was demonstrated that overexpression of *adhE* from its native promoter resulted in increased butanol and ethanol levels, however it also reintroduced limited acetone production.[124] A follow-up study demonstrated that, when using different promoters to compensate for titration effects and by constitutive expression of *adhE*, down-regulation of acetone production results in a predominantly alcohologenic fermentation.[175] The strain produced 30 gram/L of total solvents, and made ethanol, for the first time, the major product of the fermentation. In an effort to divert more acetyl-CoA units to butyryl-CoA, a butanol precursor, *thl* and *adhE* were both overexpressed. This however, resulted in slightly increased acetate production, while lowering production of all other products.[175]

Quite recently Lehmann *et al.* reported on several knock-out mutants. In one report 3-hydroxybutyryl-CoA dehydrogenase (HBD) was disrupted using the ClosTron system.[176] This is the first time that the central C2 to C4 pathway was blocked in *C. acetobutylicum*, and possibly in butyrate forming clostridia, and it resulted in a strain producing predominantly ethanol and some acetate and acetone. Lactate was reported not to be formed to any significant level, however the carbon-balances for respectively the glucose, xylose, and fed-batch glucose fermentations, was only 66 %, 91 %, and 85 % respectively (my calculations, omitting biomass formation which normally accounts for about 10 % of carbon consumption) This would suggest that in the case the glucose fermentations other products were formed besides those reported. Nonetheless their work demonstrates that *C. acetobutylicum* can survive and produce solvents without

Table 1.5: Overview of clostridial mutants and their fermentation end-point product concentrations.

Genes inactivated or repressed	Genes (over)expressed[a]	Notes	End product concentrations [mM][b]					Reference
			Acetate	Butyrate	Acetone	Butanol	Ethanol	
	C. acetobutylicum ATCC 824							
Wild type			25	44	14	44	7	Green *et al.*[148]
adhE(*aad*)			28	51	0	7	10	Green *et al.*[148]
Wild type		Fermentation pH $\geq$ 5.5	112	108	79	131	11	Green *et al.*[126]
pta		Fermentation pH $\geq$ 5.5	87	159	72	133	13	Green *et al.*[126]
buk1(*buk*)		Fermentation pH $\geq$ 5.5	149	37	39	146	16	Green *et al.*[126]
Wild type		Fermentation pH $\geq$ 5.0	60	41	85	158	16	Harris *et al.*[127,128]
buk1		Fermentation pH $\geq$ 5.0	46	16	76	225	57	Harris *et al.*[128]
buk1	*adhE*	Fermentation pH $\geq$ 5.0	51	14	66	226	98	Harris *et al.*[128]
orf5 (*solR*)		Fermentation pH $\geq$ 5.0	68	13	97	197	29	Harris *et al.*[127]
orf5	*adhE*	Fermentation pH $\geq$ 5.0	85	12	141	238	47	Harris *et al.*[127]
buk1		Not stated	n.r.	n.r.	70	200	50	Shao *et al.*[156]
orf5		Not stated	n.r.	n.r.	85	188	45	Shao *et al.*[156]
	P_{thlA}–*groES-groEL*		80	70	148	231	21	Tomas *et al.*[137]
	adhE				same as wild type			Nair *et al.*[167]
	ptb, buk1	Plasmid effect reported	60	30	80	140	10	Walter *et al.*[177,178]
	adc, ctfA, ctfB		10	0.5	149	177	31	Mermelstein *et al.*[168]
	Plasmid control		n.r.	n.r.	n.r.	110	n.r.	Borden *et al.*[135]
	ca_c1869	Flask experiment	n.r.	n.r.	n.r.	140	n.r.	Borden *et al.*[135]
ctfB (asRNA)	*adhE*		125	52	26	130	190	Tummala *et al.*[124]
ctfB (asRNA)	P_{ptb}–*adhE*		85	2	61	178	300	Sillers *et al.*[175]
ctfB (asRNA)	*adhE*		129	1	42	146	184	Sillers *et al.*[175]
ctfB (asRNA)	P_{ptb}–*adhE, thlA*		110	2	30	130	240	Sillers *et al.*[175]
Wild type			32	4	67	183	31	Lehmann *et al.*[179]
pta			41	7	52	157	28	Lehmann *et al.*[179]
adc			70	16	7	96	17	Lehmann *et al.*[179]
ctfA			74	7	—	96	20	Lehmann *et al.*[179]
pta, adc			14	67	2	26	9	Lehmann *et al.*[179]
pta, ctfA			7	62	—	29	8	Lehmann *et al.*[179]
Wild type		Fermentation pH $\geq$ 5.5	88	72	29	88	24	Lehmann *et al.*[166]
ptb		Fermentation pH $\geq$ 5.5	113	—	24	73	204	Lehmann *et al.*[166]
Wild type		Fermentation pH $\geq$ 5.0	45	5	119	161	43	Lehmann *et al.*[166]
ptb		Fermentation pH $\geq$ 5.0	32	—	91	108	263	Lehmann *et al.*[166]

Table 1.5: Overview of clostridial mutants and their fermentation end-point product concentrations (*continued*).

Genes inactivated or repressed	Genes (over)expressed[a]	Notes	End product concentrations [mM][b]					Reference
			Acetate	Butyrate	Acetone	Butanol	Ethanol	
		C. acetobutylicum ATCC 824 (*continued*)						
Wild type		FB[c] Fermentation pH $\geq$ 5.0	63	12	108	167	41	Lehmann *et al.*[166]
ptb		FB[c] Fermentation pH $\geq$ 5.0	63	—	72	105	703	Lehmann *et al.*[166]
Wild type		Flask experiment	21	1	112	170	45	Wietzke and Bahl[180]
rex		Flask experiment	20	2	44	172	178	Wietzke and Bahl[180]
		C. acetobutylicum M5 (ATCC 824 mutant deficient in pSOL1)						
M5		Fermentation pH $\geq$ 5.0	107	169	—	—	6	Nair and Papoutsakis[173]
M5	*adhE*	Fermentation pH $\geq$ 5.0	101	99	—	84	8	Nair and Papoutsakis[173]
M5		Flask experiment	23	73	—	—	6	Sillers *et al.*[129]
M5 *ack*		Flask experiment	9	75	—	—	7	Sillers *et al.*[129]
M5 *ack*	$P_{ptb-adhE}$	Fermentation pH $\geq$ 5.5	180	75	—	92	22	Sillers *et al.*[129]
M5 *ack*	$P_{ptb-adhE}$	Fermentation pH $\geq$ 6.0	179	287	—	51	21	Sillers *et al.*[129]
M5 *buk1*		Flask experiment	17	26	—	—	3	Sillers *et al.*[129]
		C. beijerinckii NCIMB 8052						
Wild type		200-L scale	n.r.	n.r.	103	171	—	Qureshi and Blaschek[181]
BA101 (chemically induced mutant)		200-L scale	n.r.	n.r.	95	240	22	Qureshi and Blaschek[181]

[a] Genes are overexpressed using their native promoter site unless otherwise noted.
[b] n.r., not reported; —, not detected.
[c] FB, fed-batch.

apparent accumulation of butyryl-phosphate.

In a second article Lehmann *et al.* report on the generation and analysis of mutants in the acetate (*pta*) and acetone (*ctfA*, *adc*) pathways and double mutants thereof.[179] Disappointingly, the mutants displayed reduced solvent production, especially in the case of the double mutants (*pta*$^-$ *adc*$^-$ and *pta*$^-$ *ctfA*$^-$) which produce only marginal amounts of solvents and their main fermentations product is butyrate. However also here the carbon-balances are incomplete (wild-type, 96 %; *pta*$^-$ *adc*$^-$, 52 %; *pta*$^-$ *ctfA*$^-$, 61 %; all not accounting for biomass). Their *pta*$^-$ knock-out mutant did not give under the tested conditions any significant differences with the wild-type, however the functional inactivation of PTA was not borne out by enzyme activity assays.

1.11.2 Increasing Butanol Resistance

The toxicity of the solvents that are produced is caused by the destabilising effect that especially butanol has on proteins and by its impact on membrane fluidity. Approaches to increase the tolerance of *C. acetobutylicum* towards butanol using metabolic engineering have been described as well. Examples include the over-expression of the GroES and GroEL heat shock proteins[137] and CAC1869,[135] a putative transcriptional regulator. The *groES* and *groEL* genes were over-expressed together as the *groESL* operon. Under stress conditions, these proteins assist in the refolding of misfolded proteins. When this operon was over-expressed, growth inhibition by butanol was reduced by 85 % and 40 %, at concentrations of 2 and 4 g/L respectively, compared to the plasmid control strain. Their increased resistance to butanol stress allowed the strain to realise a 33 % increase in final solvent titres compared to the plasmid control strain.

The *ca_c1869* gene was identified by selective enrichment of a culture that was transformed with a *C. acetobutylicum* genomic library. Serial transfers of the culture in medium with progressively higher butanol concentrations allowed for the selective enrichment of several different plasmid carrying strains. Which library fragments conferred increased resistance to butanol was detected by microarray analysis. One of the enriched fragments contained *ca_c1869*. A strain over-expressing the *ca_c1869* gene showed 80% increased resistance toward butanol, relative to the plasmid control strain.

In this context of butanol stress it is of interest to mention compatible solutes. The folding and proper conformation of macromolecules such as proteins, DNA, and RNA is in most cases essential for their stability and function. Various stressors such as high or low temperatures, hydrostatic pressure, high concentrations of inorganic salts or other compounds can destabilise these macromolecules and disrupt their functioning. Cells can accumulate various low molecular weight compounds called compatible solutes to help them withstand these stresses. The sometimes very high concentration (up to the molar range) protects the cells, while not interfering with cellular processes. For this reason they are called com-

patible solutes but also the terms counteracting solutes, compensatory solutes, and chemical chaperones have been used in the literature.[182–188] The general hypothesis is that almost all stresses, also butanol stress, are ultimately related to loss of water from the cell. The compatible solutes help to maintain water inside the cell and, to some extend, replace it, to keep proteins in solution by preventing aggregation.

Compatible solutes can be classified in four major groups based on their chemical composition. These are: a) Carbohydrates, mainly uncharged forms but diglycerol phosphate has also been described in certain archaea; b) Amino acids and derivatives, almost all are zwitterions; c) Methylamine and methylsulphonium zwitterions; d) Urea, the only class with only one member.[182] No reports have appeared in the literature as far as we could identify that describe the use application of these kind of compounds for increased butanol production.

1.11.3 *C. beijerinckii* BA101

Currently the bacterium with the highest butanol and total solvent producing characteristics belongs to the species *C. beijerinckii*. A chemically-induced (random mutagenesis) mutant of strain NCIMB 8052, strain BA101, produces significantly increased amounts of butanol[181,189–192] and constitutes one of the most interesting strains developed so far. This strain consistently produced double amounts of butanol and showed increased butanol tolerance when grown in batch cultures on glucose compared to the wild-type strain. The final butanol concentrations reached at the end of batch cultures carried out in a 200-L bioreactor were 240 mM and 171 mM by the BA101 and wild-type strains, respectively. An economic assessment of butanol production from corn using this mutant strain resulted in a price for butanol below the price of petrochemically produced butanol at that time.[193]

1.12 Alternative Butanol Production Hosts

In order to circumvent some of the intrinsic drawbacks of the solventogenic Clostridia (especially the relatively high ratio of by-products, complex life cycle, and the limited genetic tools available compared to other species), several research groups have reported attempts to produce *n*-butanol using other organisms. This approach of heterologous expression of genes from a clostridial background in well-studied, genetically easily accessible, organisms such as *E. coli* or *S. cerevisiae* comes at a price. These last mentioned organisms, amongst other drawbacks,[194] tend to be more sensitive for butanol in the growth medium than native produces and have a narrower susbtrate range. Possibly, some lactic acid bacteria, such as *Lactobacillus*, *Pediococcus*, *Enterococcus*, and *Leuconostoc* would be an interesting platform for *n*-butanol production, since they have been reported to be relatively tolerant to high concentrations of this alcohol.[195,196]

Studies have been published that are based on the production of *n*-butanol using the clostridial pathway. In *E. coli* both Atsumi *et al.*[197] and Inui *et al.*,[198] and Nielsen *et al.*[199] succeeded in detecting butanol production levels of, 550, 1200, and 520 mg/L respectively. Similar results were obtained by others using *Lactobacillus brevis* (300 mg/L),[200] *S. cerevisiae*, (2.5 mg/L)[201] *Bacillus subtilis* (25 mg/L) and *Pseudomonas putida* (122 mg/L).[199] Examples of patent applications in which the introduction of the *C. acetobutylicum* butanol pathway in alternative micro-organisms is claimed are those filed by Dupont (WO 2007/041269), DSM (WO 2008/05991) and Arbor Fuels Inc (EP 2008 0841766). In these patent applications, the results described for fermentations in batch cultures by recombinant yeast strains show butanol production at very low concentrations (< 1 mM).

The group of James Liao also explored another approach and used intermediates of amino acids biosynthesis routes present in *E. coli* as precursors for longer chain alcohols.[202] In their approach α-keto-acids were decarboxylated to the corresponding aldehyde by the keto acid decarboxylase (KDC) from *Lactococcus lactis* and then reduced to the alcohol by alcohol dehydrogenase 2 (Adh2) of *S. cerevisiae*. This resulted in various linear and branched chain alcohols (butanol, propanol, *iso*-butanol, 2-metyl-1-butanol, 3-methyl-1-butanol, 2-phenylethanol) depending on the amino acid precursor. Production of *iso*-butanol reached the highest level at 300 mM (22 g/L), whereas *n*-butanol did not surpass 9.2 mM (0.68 g/L).

The wider substrate range of clostridial butanol producers, combined with their higher butanol production levels and previous industrial application still make clostridia the organisms of choice for *n*-butanol production.

1.13 Project Approach

The goal of the research described in this thesis was to reduce the number and especially the amount of by-products formed during the ABE-process. The main focus was on reducing the amount of acetone produced as part of the fermentation, for two reasons. Firstly, acetone in the fermentation broth complicates downstream processing (DSP). The mixture of solvents — acetone, butanol, and ethanol — and water creates an azeotropic mixture which makes the butanol separation harder and more energy consuming. By preventing acetone formation that particular azeotrope is removed. Secondly, acetone formation reduces butanol yields per amount of substrate. One molecule of glucose is used for every acetone molecule produced, whereas this could also have been used to produce butanol. As expected, previous attempts to inhibit acetone production resulted in increased acid concentrations.[124,125,203] as acetone production is an important acid uptake route.

The approach taken for this thesis was therefore to impede acid production, both that of acetate and of butyrate, which is expected to lead to the desired effect

of reduced or eliminated acetone production, because there would not be acetate or butyrate to take up. Butyrate kinase was selected for inactivation of the butyrate pathways, because previous results suggested that butyryl-phosphate accumulation had a regulatory function implicated in triggering solvent formation,[165] and had resulted in increased solvent formation.[128] The analogously functioning acetate kinase was selected to inactivate acetate production, because it was shown that the enzymes of the butyrate pathway could substitute for those used in acetate pathway and possibly vice versa. It was envisioned that butanol and ethanol would become the major liquid fermentation end-products possibly combined with lactate formation. As inactivation of both acid production pathways would reduce ATP generation quite considerably, inducible expression of a functional acetate kinase gene was considered to allow establishment of sufficient biomass followed by shift to alcohols-only production by taking away the inducer. The proposed mutant could then be used in a cell recycling based process to allow semi-continues operation.

1.14 Outline of This Thesis

Chapter 2 describes the adaptation of a gene-knockout mutagenesis system based on a mobile group II intron to selectively, and permanently inactivate genes in *Clostridium acetobutylicum* and potentially other clostridia. It further also describes a method to store electro-competent *C. acetobutylicum* cells for later transformation for a period of at least two and half years.

The work discussed in **chapter 3** describes the generation and characterisation of a *C. acetobutylicum* strain with an inactivated acetate kinase gene. Batch fermentations in two different media are discussed and their results compared.

Chapter 4 then builds on the work done in chapters 2 and 3 and first describes a mutant strain with an insertionally inactivated butyrate kinase gene and how it differs in batch fermentation performance, from an earlier described mutant strain with an inactivated butyrate kinase gene. Subsequently, the acetate kinase gene in this strain is also inactivated resulting in the first ever *C. acetobutylicum* strain with targeted inactivation of genes in both acid production pathways. The effect on production formation of the fermentation pH and the addition of acetate to the medium is evaluated.

In **chapter 5** an alternative approach to circumvent the toxicity of 1-butanol while maintaining its interesting biofuels properties is explored. The goal is to produce 2-butanol and this chapter takes the first step towards that by expressing an acetoin reductes gene from *C. beijerinckii* in *C. acetobutylicum* to reduce natively produced acetoin to D–2,3–butanediol.

Lastly, **chapter 6** is a general discussion chapter, in which the results of the previous chapters are evaluated, and suggestions for future research are made. In addition it summarises the general conclusions from this thesis.

The first part of this chapter will be submitted as:
Wouter Kuit, Ana M. López-Contreras and Gerrit Eggink; "*A Method for Storing Electro-Competent* Clostridium acetobutylicum *cells*"

Chapter 2

Molecular Biology Methods for *Clostridium acetobutylicum*

2

Part 1: A Method for Storing Electro-Competent *C. acetobutylicum* Cells

Abstract

Preparation of electrocompetent cells of *C. acetobutylicum*, and related species, and their transformation is a time consuming process typically requiring more than 11 hours. Here we describe how electrocompetent cells can be stored for up to two and a half years at −80 °C, for later transformation.

Note

Clostridium *acetobutylicum* is a rod-shaped, Gram-positive, anaerobic bacterium that can ferment starch and various sugars to the acids acetate and butyrate, and the neutral solvents acetone, butanol, and ethanol (ABE).[57] The ABE-fermentation was once performed at industrial scale for the production of acetone and butanol, but was unable to compete with the more economical petrochemical process during the second half of the 20$^{\text{th}}$ century in most parts of the world.[58,60,115] Recently the process has received renewed interest for the production of butanol from various renewable feedstocks including, amongst others, molasses, starch, and lignocellulosic materials. Applications of butanol

include its use used as fuel extender and as a chemical feedstock.[45]

Although the ABE process is known for more than a hundred years, attempts to improve yield, selectivity, and productivities have had limited success.[204] Among the four clostridial species[66] know to produce butanol as their main fermentation product, *C. acetobutylicum* ATCC 824 is the most well studied strain at the molecular and genetic level. Methods and genetic tools that have been reported for this strain include transformation protocols, expression plasmids, random mutation, selective inactivation of genes, and integration of new DNA sequences in the chromosome.[141,154,157,160,163] Protocols for transformation by electroporation have been described by Oultram *et al.*,[142] Mermelstein *et al.*,[143] and others.[205]

To date, no protocols for storage of competent cells of solventogenic Clostridia have been described in literature, as it has been reported for a large range of organisms; Gram-negative bacteria (*Escherichia coli*,[206,207] *Pseudomonas aeruginosa*[208]), Gram-positive bacteria (*Lactococcus lactis*,[209] *Geobacillus stearothermophilus*,[210] *Lactobacillus casei*, *L. plantarum*,[211] and *Bacillus subtilis*[212]), and yeasts (*Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*)[213] can be stored competent at −80 °C. All reported methods for transformation of *C. acetobutylicum*, and related strains such as *C. beijerinckii*, require preparation of freshly prepared competent cells.[141,143,214] One report by Klapatch *et al.*[215] describes the storage of electrocompetent *C. thermosaccharolyticum* cells at −80 °C but gives not details as to how this was accomplished. Otherwise we have been unable to identify a publication in the literature demonstrating storage of electrocompetent obligatory anaerobic bacteria. Here we describe, to the best of our knowledge, for the first time how to store electrocompetent cells of *C. acetobutylicum* strains ATCC 824 and WUR, the later a strain closely related to ATCC 824,[216,217] for later transformation.

Competent *C. acetobutylicum* cells were essentially prepared as described by Oultram *et al.*[142] 200 mL mCGM medium[216] was inoculated with 20 mL of an overnight pre-culture from heat shocked spores (10 minutes, 80 °C) and grown up to an OD_{600} of 0.7 (corresponding to a biomass concentration of approximately 0.35 mg/mL) at 37 °C. The culture was transferred to a large centrifuge tube closed with a plug with an O-ring, and a screw cap and then wrapped with plastic paraffin film. The tube was cooled in an ice-water mixture and centrifuged for 10 minutes (5 468 × *g*, 4 °C). 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) and transferred to a smaller centrifuge tube. The tube was again closed and wrapped as before and centrifuged again under the same conditions. The supernatant was removed and the pellet was resuspended in 4 mL ice-cold electroporation buffer. The competent cell suspension can now be used in 300 µL portions for electroporation in an electroporation cuvette or stored inside 1.5 mL polypropylene cryovials closed with a rubber O-ring fitted screw cap (Greiner Bio-One, The Netherlands, Cat.-No. 717 261). Vials are then transferred to a wide bore 250 mL glass infusion

bottle and closed off with a thick (1.0–1.5 cm) butyl rubber stopper and an aluminium screw cap. The flask with cryovials was then transferred outside the anaerobic environment and stored at −80 °C.

During storage and afterwards great care must be taken not expose the glass bottle to any additional stresses as the glass might easily break due to the temperature and pressure differences. All manipulations, except for the centrifugation, were carried out anaerobically. Storing competent cells in cryovials that were not placed inside a glass bottle resulted in undetectable transformation frequencies and low numbers of viable cells (data not shown). Presumably as oxygen diffuses through the plastic walls and inactivates key enzymes resulting in non-viable cells.[218] Storing cells inside glass test tubes that are closed off with a butyl rubber stopper and a screw cap, as we do at our lab with non-sporulating strains, would facilitate storage inside −80 °C freezers due to their less bulkiness. We however did not test this. It has been reported that, at least for chemical competent cells, *E. coli* loose competence when stored in glass containers.[207]

When a transformation is performed the glass bottle was taken from the −80 °C freezer, wrapped inside a large cloth and placed inside the airlock for entry into the anaerobic chamber. After the airlock is made anaerobic the flask is opened, still wrapped in the cloth, and the desired number of cryovials is taken out. If any vials are left the flask is closed again and transferred outside for storage at −80 °C. The cells are thawed and used as fresh cells for electrotransformation. Approximately 2.4 µg of methylated plasmid DNA[147] was added to fresh or thawed cells and incubated on ice for at least 1 minute. Cells were then electroporated (0.2 cm gap with; 1.25 kV; 25 µF; 100 Ω) with 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 resuspended in 200 µL mCGM and plated with appropriate dilutions on pre-warmed mCGM plates containing erythromycin.

To compare the competency of cells stored as described above we transformed stored and freshly prepared competent cells with 2.4 µg pMTL500E plasmid.[142] Stored *C. acetobutylicum* WUR cells from batches that were 1 month (n=2), 2.5 years (n=2), and 4.5 years (n=4) old were tested and compared to fresh cells (n=2). Each transformation was plated in duplicate to determine the number of transformants. Mean values are plotted in Figure 2.1. Except for an initial increase in competence upon storage after 1 month, competence seems to remain stable for up to at least 2.5 years. After 4.5 years it is clearly reduced, although transformants were still obtained in all four independent transformations carried out with 4 ± 0.8 CFU/µg (mean ± SEM).

To determine if *C. acetobutylicum* ATCC 824 cells (obtained from H. Bahl, Rostock, Germany) behaved similar to the WUR strain, fresh competent cells were prepared and transformed with 2.4 µg pMTL500E plasmid. Similar transformation efficiencies were obtained for the fresh ATCC 824 cells compared to the WUR strain cells. Upon storage for one month at −80 °C cells remained competent to the same level compared to the fresh cells, but with lower efficiency

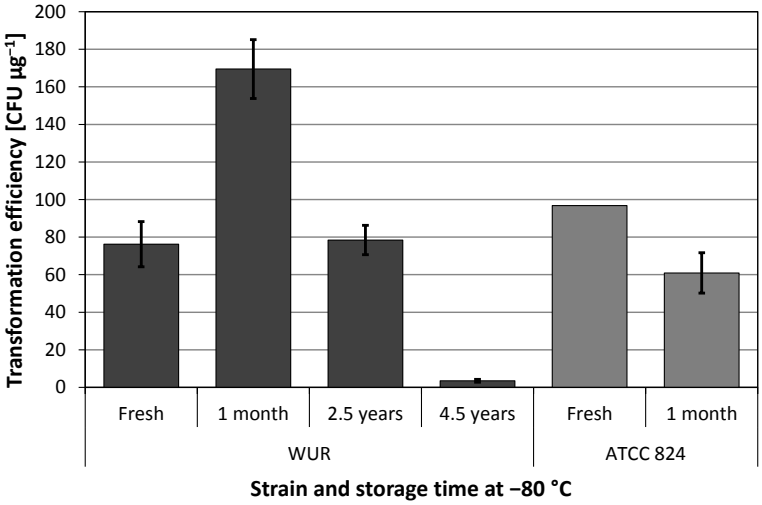


Figure 2.1: Transformation efficiency of competent *C. acetobutylicum* WUR and ATCC 824 cells. Prepared fresh or after storage at −80 °C for the indicated period of time. Error bars indicate SEM for $n = 2$ except for the 4.5 year WUR sample ($n = 4$) and the fresh ATCC 824 sample ($n = 1$).

compared to one month old WUR-strain cells.

In summary, the storage method outlined above allows electrocompetent *Clostridium acetobutylicum* cells to be stored at −80 °C and remain competent. It is essential to keep the cell suspension anaerobic by storing the plastic tubes inside a glass container with a thick rubber stopper as storage of the same tubes alone results in loss of competence and reduced viability. Use of this method will allow the storage of competent cells for later use, resulting in similar flexibility for *C. acetobutylicum* as for *E. coli* with regard to the 'ease-of-use' of competent cells. This procedure could potentially also be applied to other obligate anaerobic microorganisms.

Part 2:
Development of a Mobile Group II Intron-Based Gene Knock-Out Method for Clostridia

Abstract

Creating targeted gene knock-out mutants is one of the requirements to establish a metabolic engineering 'toolbox' for the modification and remodelling of metabolic pathways. Here we describe the adaptation of a mobile group II intron-based gene knock-out system for its use with *Clostridium acetobutylicum*. The systems is expected to work in a wide range of clostridia due to it pAMβ1 replicon and generally recognised thiolase promoter for the expression of the intron. Application of the system for the generation of an acetate kinase knock-out mutant is demonstrated by PCR. A pure culture could not be obtained after colony PCR screening or attempts at phenotypical selection, indicating that screening methods need to be improved. This method has been used for the generation of a second knock-out in a recombinant strain, described in chapter 4 of this thesis.

2.1 Introduction

Prior to 2007 no genetic tools were available in the literature to stably inactivate genes in a targeted manner in clostridial species. Up to that time clostridial genes could only be inactivated using non-replicating, so-called 'suicide', plasmids. Using this method, the cells are transformed with a plasmid that cannot replicate itself inside the new host. The plasmid further contains a gene that confers a selectable trait (normally an antibiotic resistance gene) and contains an internal fragment that is homologous to a targeted region on the chromosome (*i.e.* an internal part of the gene to be inactivated). The internal gene fragment allows for host mediated homologous recombination to occur (Figure 2.2). During this process the DNA strands are exchanged between the two homologous sites and this results in an interrupted targeted site, of which there are now two copies on the chromosome, interleaved by the sequence of the rest of the plasmid. If the inserted plasmid sequence is in the coding region of the gene then this normally results in an inactive gene product. The presence of the selection marker on the plasmid, for example an antibiotic resistance gene, allows for the selection of a genome integrated plasmid, because the plasmid does not contain an origin of replication. This situation is however not a stable one. The genome still has two homologous regions (indicated in grey in Figure 2.2) and so the process just described can be reversed and the plasmid will be removed from

the genome. If this happens when the selective pressure is still applied these cells will not survive.

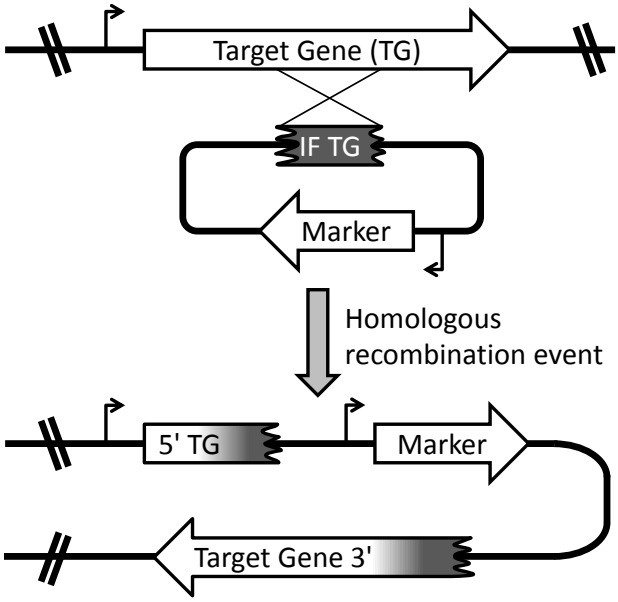


Figure 2.2: Schematic depiction of a homologous recombination event resulting in disruption of the targeted gene (abbreviation TG). The introduced plasmid does not contain an origin of replication that will function in the host, thus for the host to acquire the marker gene it must recombine the plasmid in the genome. To guide this process and increase the efficiency, the plasmid contains an internal fragment of the targeted gene (IF TG). Once recombination has occurred the marker gene is integrated in the genome of the host.

Because the recombination frequency in clostridia is very low and there was no known method to select for the second recombination event (*i.e.* a negative selection marker on the stretch of plasmid DNA that should be lost on completion of the second homologous recombination event), it proved impossible to screen for those particular mutants. Instead various single cross-over mutants have been constructed[126,148,149,169] and assessed for how quickly the integrated plasmid was lost from the culture when the selective pressure (antibiotic) was removed.[149,219] Although reportedly loss of some integrated plasmids was low, none were highly stable. In addition, a separate positive selection marker (*i.e.* antibiotic resistance marker) is needed for each separate insertion. The main differences of *C. acetobutylicum* compared to other organisms where double cross-over mutants can more easily be obtained are the low frequency of both transformation and recombination, and the absence of a conditional replication system for clostridial

vectors.

To find a way to obtain stable gene knock-out mutants we searched the literature for possible methods. We focussed on one called TargeTron which is based on the targeted insertion of mobile group II introns for two reasons; (i) the TargeTron approach works almost independent from any host factors to complete the mutagenesis process;[150] (ii) Chen *et al.*[220] had inactivated the alpha toxin gene in *C. perfringens* using this system, demonstrating that it can function in a clostridial species.

The TargeTron system is built around a mobile group II intron present in *Lactococcus lactis* called Ll.LtrB. These kind of catalytic RNA elements occur in a wide range of prokaryotic and eukaryotic organisms (in their chloroplasts and mitochondria) and consist of six domains (I through VI) that are a result of the intricate folding of the intron.[221] The tertiary structure that is a result of this folding assures that specific nucleotides are positioned in such a way that they can perform their catalytic function. Mobile group II introns have an intron-encoded-protein (IEP) that has RNA splicing, DNA endonuclease, and reverse transcriptase activities.[150] In the Ll.LtrB intron the IEP is called LtrA and is 599 amino acids long.

Mobility initiates when the IEP helps the intron RNA fold into the catalytically active RNA structure to promote splicing, resulting in ligated exons and an intron lariat-IEP ribonucleoprotein (RNP) complex. In the modified *L. lactis* intron the LtrA encoding gene is placed outside the intron lariat boundaries resulting in an RNP complex without an encoded IEP on the RNA part, as opposed to the wild-type system where the *ltrA* gene is located on domain IV of the intron lariat. This arrangement assures that the gene is not present anymore after removal of the plasmid. The RNP complex recognizes specific DNA target sites and promotes integration by reverse splicing of the intron RNA directly into one strand of the target DNA. The IEP then cleaves the opposite strand and uses it as a primer for target DNA-primed reverse transcription of the inserted intron RNA. The opposite strand of the resulting cDNA copy of the intron is generated by host DNA repair mechanisms resulting in an dsDNA intron copy inserted in the target site.

The direction in which the intron is inserted in the gene is referred to as the sense, or anti-sense orientation. If the intron is oriented in sense then the LtrA protein, expressed from the plasmid, is able to recognise the intron sequence in the mRNA of the inactivated gene and remove it from the transcript, restoring the wild-type mRNA.[222] It is therefore important in cases of sense intron insertion to ascertain if the plasmid, and therefore the LtrA encoding gene, has been lost from the mutant. If the intron is inserted anti-sense then the intron sequence cannot be removed from the mRNA transcript even if the LtrA protein is expressed.

Although the group II intron itself performs almost all required function in a host independent manner several modifications to the plasmid that carried the intron were needed. These were:

i an additional Gram-positive origin of replication compatible with clostridia;
ii another selection marker then ampicillin on the TargeTron carrying plasmid;
iii a suitable promoter to replace the T7 promoter and lacZ system used to drive and control the expression of the TargeTron as it is not expected to work in clostridia;
iv removal of the RAM[§] as *C. acetobutylicum* is not sensitive to kanamycin and larger introns have reduced insertion efficiency.

The work in this chapter describes how the *E. coli* based TargeTron system was modified to work in *C. acetobutylicum* and describes its application to generate an acetate kinase (*ack*) knock-out mutant. Three methods to isolate the generated mutant are described but were unsuccessful. Later work, described in chapter 4, was after all able to demonstrate the system's successful application to obtain the desired mutation. The approach there relied on a new colony PCR protocol to screen a large number of colonies for the mutant.

2.2 Material and Methods

2.2.1 Strains, Plasmids and Primers

Table 2.1 lists all bacterial strains and plasmids used for the study. Stock cultures of *C. acetobutylicum* were maintained as spore suspensions in sterile 15 % (v/v) glycerol at −20 °C. *E. coli* XL1-Blue was used for vector maintenance and cloning and stored as competent cells or glycerol stock at −80 °C.

2.2.2 Media and Growth Conditions

Lysogeny broth (LB) medium[223,224] was used for growth of *E. coli* strains at 37 °C, 250 RPM supplemented with the appropriate antibiotics. Clostridial spore suspensions were prepared as previously described[216] and heat shocked for 10 minutes at 70 °C, prior to using them as an inoculum for pre-cultures. *C. acetobutylicum* was plated on RCM (Oxoid) or mCGM medium containing 1.2 % agarose. Liquid cultures were grown in mCGM medium. Liquid media were made anaerobic by flushing with nitrogen gas for 10 to 30 minutes depending on the volume of the liquid. All clostridial culture experiments were performed at 37 °C, without shaking, and anaerobically in (i) an anaerobic chamber (Sheldon Manufacturing, Oregon U.S.A.; gas mixture consisting of 15 % CO_2, 4 % H_2, and

[§]Retrotransposition Activated Marker, see subsection 1.10.1.

81 % N_2); or (ii) in glass serum vials as described previously.[225] Culture media were supplemented with ampicillin (100 µg/mL), chloramphenicol (30 µg/mL), thiamphenicol (15 µg/mL), or erythromycin (40 µg/mL standard; 5–10 µg/mL for initial mutant isolation) when appropriate.

2.2.3 Mutant Selection

For mutant isolation plates appropriate amounts of a filter sterilised (0.2 µm, Sartorius) 4 M sodium fluoroacetate solution were added to not yet solidified RCM-agar after it was autoclaved. Overnight liquid cultures of wild-type and transformants were plated and incubated and checked for growth after 24, 48, 72 hours. For selection on chloroacetate the same procedure was followed except that a 1 M sodium chloroacetate solution was used for medium supplementation.

2.2.4 DNA Isolation and Modification

Standard molecular work was done according to established protocols.[224] Restriction enzymes were from New England Biolabs (Bioké, The Netherlands) and used according to their instructions. DNA from *C. acetobutylicum* was isolated using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) using the

Table 2.1: Bacterial strains an plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source
Strains		
C. acetobutylicum WUR	Wild type	Laboratory stock[216,217]
E. coli XL1-Blue	*rec*A1 *end*A1 *rel*A1 Tn*10*(Tet^r) *gyr*A96(nal^r) *lac* *lac*I^q Δ(*lac*Z)M15	Laboratory stock
E. coli DH10B(pAN1)	Δ(*mrr-hsdRMS-mcrBC*)	Laboratory stock
Plasmids		
pAN1	p15A ori; Cm^r ϕ*3tI*	Laboratory stock[147]
pMTL500E	ColE1 ori; pAMβ1 ori; MLS^r Ap^r; *lac*Z′	Laboratory stock[142]
pACD4K-C	p15A ori; Cm^r; intronII-RAM-*ltrA*-T1T2 terminator	Sigma-Aldrich
pWOK001::Cac-*ack*-84s	ColE1 ori; pAMβ1 ori; MLS^r Ap^r; P_{thl}-intronII-ΔRAM-*ltrA*-T1T2 terminator	This study
pWOK001::Cac-*ack*-1027a	ColE1 ori; pAMβ1 ori; MLS^r Ap^r; P_{thl}-intronII-ΔRAM-*ltrA*-T1T2 terminator	This study
pWOK002::Cac-*ack*-1027a	ColE1 ori; pAMβ1 ori; MLS^r Ap^r; P_{adc}-intronII-ΔRAM-*ltrA*-T1T2 terminator	This study

[a] MLS^R, confers resistance to erythromycin, amongst others; p15A ori, *E. coli* origin of replication; ϕ*3tI*, *Bacillus subtilis* phage gene encoding the ϕ3T I methyltransferase; ColE1 ori, *E. coli* origin of replication; pAMβ1 ori, clostridial origin of replication; Cm^R, chloramphenicol resistance; *ltrA*, the intron-encoded protein of the *Lactococcus lactis* Ll.LtrB group II intron

Gram-positive isolation procedure which incorporates a lysozyme (from chicken egg white; Fluka) treatment. *E. coli* plasmid DNA was isolated by the GenElute Plasmid Miniprep Kit (Sigma-Aldrich). Both kits were used according to the manufacturer's instructions. PCR products intended for cloning were amplified using PWO master mix or the High Fidelity PCR kit (both Roche) and used according to the manufacturer's instructions. Clostridial colony PCRs were done with PWO master mix and for *E. coli* colony PCRs RedTaq master mix (Sigma) was used. The retargeting and related procedures of the TargeTron system (Sigma-Aldrich) were performed according to the manufacturer's instructions. Prior to transformation into *C. acetobutylicum*, plasmids were methylated *in vivo*[147] by electroporation into *E. coli* DH10B(pAN1) cells. Electrotransformation of *C. acetobutylicum* was carried out as previously described.[142] All manipulations were carried out anaerobically and on ice. After transformation and recovery, the cells were plated on pre-warmed plates containing erythromycin.

2.2.5 Primers and DNA Sequencing

All DNA primers used in the study are listed in Table 2.2. Primers were obtained from Eurogentec (Seraing, Belgium). DNA sequencing of clones was done by BaseClear (Leiden, The Netherlands).

Table 2.2: Primers used in this study

Primer name	Sequence ($5' \rightarrow 3'$)[a]
AK1027a_ibs	aaaaaagctt ataattatcc ttaTGTTTcT GCATAgtgcg cccagatagg gtg
AK1027a_ebs1d	cagattgtac aaatgtggtg ataacagata agtcTGCATA TTtaacttac ctttctttgt
AK1027a_ebs2	tgaacgcaag tttctaattt cgGttAAACA Tcgatagagg aaagtgtct
AK84s_ibs	aaaaaagctt ataattatcc ttaGTACTcG CTAAAgtgcg cccagatagg gtg
AK84s_ebs1d	cagattgtac aaatgtggtg ataacagata agtcGCTAAA GGtaacttac ctttctttgt
AK84s_ebs2	tgaacgcaag tttctaattt cgAttAGTAC Tcgatagagg aaagtgtct
EBS_Univ	cgaaattaga aacttgcgtt cagtaaac
TT1027Apa_Fw	AAAAGGgcCC ataattatcc ttatgtttct g
TT84Apa_Fw	AAAAGGgcCC ataattatcc ttagtactcg c
TT_XhoI_Rev	CCTCGAGcgt tctgctttcc tgatgc
CA_Pthl_Fw	GGCATGCgaa tttagaatga agtttcttat gc
CA_Pthl_Rev	AAAAGGGCCC ccatagttta tccctaattt atacg
CA_Padc_Fw	GGCATGCatg ggaaagccaa cattgc
CA_Padc_Rev	AAAAGGGCCC cttcacatta taaatcgcct ct
Cac_ack_fwd	atgaaaaact tagttattaa ctgcg
Cac_ack_rev	ttattttaac ttgcctacta tatcttt

[a] Nucleotides that are not complementary to the template sequence are given in uppercase. *Hin*dIII, *Bsr*GI, *Apa*I, and *Xho*I restriction sites incorporated in the primers are underlined.

2.3 Results and Discussion

2.3.1 Clostridial TargeTron Construction

The TargeTron kit supplies the pACD4K-C vector for application in *E. coli* mutagenesis, however this is unsuitable for work in clostridia. Therefore a clostridial version of the plasmid was constructed. Because the plasmid is supplied as a linearised version with the kit, it needs to be circularised using a retargeted region. This region determines where the intron will insert in the genome and is generated using a one-step SOEing PCR with a specially prepared template supplied with the kit. As our initial target will be the acetate kinase (*ack*) gene from *C. acetobutylicum* we chose to select two insertion sites within that gene. Although the sites were insertion can take place are flexible, recognition of certain nucleotides is dependent on the protein part of the RNP complex and cannot be changed.[150] Where potential insertions sites are located within a given DNA sequence can be computed using a developed and published algorithm.[226] Sigma, the company selling the TargeTron kit, has set up a website (http://www.sigma-genosys.com/targetron/) to provide these insertion sites within a specified sequence, using an access code supplied when buying a kit. There is however also a free alternative available (http://clostron.com/clostron2.php) that is based on the aforementioned Perutka *et al.*[226] algorithm. Besides possible insertion sites the websites also provide details on the PCR primers needed to re-target the intron to the selected insertion site.

Two top scoring sites within the *ack* gene were selected from the Sigma-website results, and the corresponding PCR primers (AK1027a_ibs, AK1027a_ebs1d, AK1027a_ebs2; and AK84s_ibs, AK84s_ebs1d, AK84s_ebs2; were used, combined with the universal primer EBS_Univ (Table 2.2) supplied with the kit, to generate two re-targeted regions, one aimed at insertion after base pair 84 in the sense orientation, and one after base pair 1027 in the anti-sense orientation of the *ack* gene, respectively. The PCR products were isolated from gel, digested with *Hin*dIII and *Bsr*GI and ligated into the pACD4K-C vector. The resulting vectors, pACD4K-C::Cac-*ack*-1027a and pACD4K-C::Cac-*ack*-84s, were sequence verified and then digested with *Mlu*I, gel purified, and self-ligated to remove the RAM-cassette from the intron, creating pACD4K-C::Cac-*ack*-1027aΔRAM and pACD4K-C::Cac-*ack*-84sΔRAM. The intron and *ltrA* gene containing section of these plasmids were PCR amplified using primers TT1027Apa_Fw and TT84Apa_Fw respectively as the forward primers and TT_XhoI_Rev as the reverse primer in both reactions. Both forward primers were designed to mutate the *Hin*dIII site, that is used to insert the re-targeted region into the intron, to an *Apa*I site because the intended clostridial shuttle vector already contained a *Hin*dIII site in its backbone and it was therefore necessary to remove it from the intron sequence. This also means that future IBS primers, which will have a *Hin*dIII site incorporated near the 5′ end, will have to be adapted to so that the corresponding nucleotides are changed to an *Apa*I site.

The pACD4K-C vector relies on the T7 promoter for transcription of the intron RNA but is not expected to work in *C. acetobutylicum*. Two native *C. acetobutylicum* promoters were selected to drive transcription of the intron RNA, P_{thl} and P_{adc}. The thiolase promoter (P_{thl}) provides strong, constitutive expression while the acetoacetate decarboxylase promoter (P_{adc}) results in even stronger expression, but is expressed in the late-exponential and stationary phase.[227,228] Both promoters were amplified from *C. acetobutylicum* genomic DNA using primers CA_Pthl_Fw and CA_Pthl_Rev, and CA_Padc_Fw and CA_Padc_Rev, for P_{thl} and P_{adc} respectively.

The *E. coli*-*C. acetobutylicum* shuttle vector pMTL500E was selected to supply the *E. coli* (ColE1) and clostridial origin of replication (pAMβ1) and resistance genes (Amp^R, MLS^R¶) for our gene knock-out plasmids. The three parts, vector backbone, promoter, and intron\LtrA components were three-way ligated to obtain plasmids pWOK001::Cac-*ack*-1027a and pWOK001::Cac-*ack*-84s, containing the *thl* promoter, and pWOK002::Cac-*ack*-1027a and pWOK002::Cac-*ack*-84s, containing the *adc* promoter.

2.3.2 Generation of *C. acetobutylicum* Mutants

Plasmids pWOK001::Cac-*ack*-1027a and pWOK002::Cac-*ack*-1027a were used to transform *C. acetobutylicum* to test if the system worked. Several erythromycin resistant colonies were isolated after transformation and genomic DNA was isolated from them after liquid cultivation under erythromycin selective pressure to allow for intron expression. Using primers Cac_ack_fwd and AK1027a_ebs2 to amplify across the left gene-intron border, and primers EBS_Univ and Cac_ack_rev to amplify across the right gene-intron border (Figure 2.3a), resulted in PCR products of the correct sizes (Figure 2.3b and c) for transformants harbouring plasmid pWOK001::Cac-*ack*-1027a, which is based on the *thl* promoter. For the *adc* based pWOK002 system the efficiency seemed to be much lower as only very low levels of mutated genes were present (Figure 2.3b, lane 4).

These results suggest that *adc* driven expression of the intron II did only result in low levels of correct mutants. There could be several reasons for this observation. First of all, the *adc* promoter only becomes active during late exponential and stationary phase resulting in induction of the system is in a later stage of growth. It is possible that cells at that stage are in such a condition that the RNP complex cannot assemble, or is no longer active, because of the drop in intracellular pH that occurs during acid production.[229] However, as the intron used is derived from *Lactococcus lactis* that experiences a stronger drop in internal pH,[230] this would seem less likely.

Another possibility is that cells expressing *adc* enter cell differentiation state ultimately leading to spore formation, that could make their DNA unrecoverable

¶MLS; Macrolide-Lincosamide-Streptogramin resistance gene conferring resistance to, amongst other antibiotics, erythromycin.

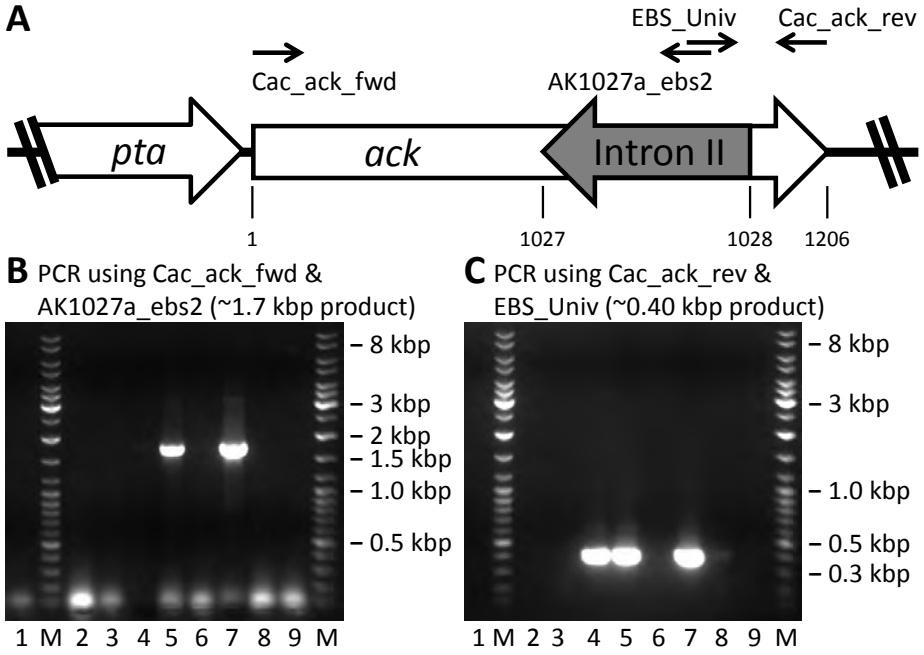


Figure 2.3: (**A**) The *ack* gene of the *C. acetobutylicum* WUR *ack*$^{-}$ strain after successful insertion of the group II intron and the location of the confirmation primers relative to the gene; (**B**) PCR products after separation on an 1.0 % agarose gel using primers spanning the 5′-end of the intron;(**C**) PCR products after separation on an 1.0 % agarose gel using primers spanning the 3′-end of the intron. Templates for PCRs in lanes for both **B** and **C**: **1**, Water; **M**, Marker; **2**, gDNA WT; **3**, gDNA isolate #32 (pMTL500E); **4**, gDNA isolate #38 (pWOK002::Cac-*ack*-1027a); **5**, gDNA isolate #45 (pWOK001::Cac-*ack*-1027a); **6**, plasmid pWOK001::Cac-*ack*-1027a; **7**, gDNA isolate #47 (pWOK001::Cac-*ack*-1027a); **8**, plasmid pMTL500E; **9**, plasmid pWOK002::Cac-*ack*-1027a.

during a normal genomic DNA isolation. The recent observation that solvent producing cells are also dividing cells seems to negate that possibility.[113]

Lastly, it is possible that not enough cells of a colony growing on mCGM medium, that only contains 12.5 g/L glucose, enter solventogenesis. Prolonged incubation and/or a growth medium that allows for solventogenesis to occur could both help to increase the number of mutants using the *adc* construct.

Sequencing of the PCR products amplifying across the gene-intron borders of the pWOK001 derived mutants, showed that insertion had taken place as predicted by the algorithm used to detect the insertion site. Amplification of the acetate kinase gene from genomic DNA using primers Cac_ack_fwd and Cac_ack_rev did however show that only a small fraction of liquid culture used to

isolate the genomic DNA from contained the insert (Figure 2.4). These results demonstrated that the system was functional and able to insert into the insertion site it was designed for. However, the (very) low levels of insertion detected by amplification of the whole *ack* gene indicated that a quick screening method was needed to isolate the mutants.

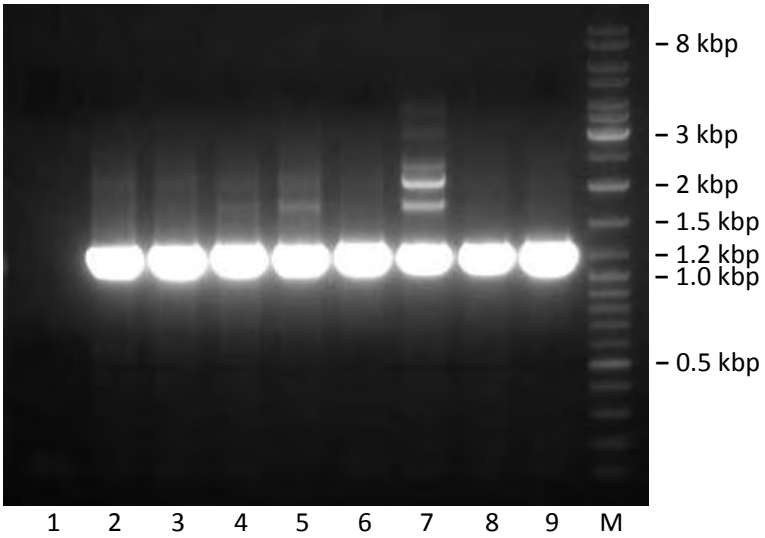


Figure 2.4: PCR amplification of the *ack* gene using primers Cac_ack_fwd and Cac_ack_rev from genomic DNA isolated from liquid cultures of transformants harbouring the pWOK001::Cac-*ack*-1027a plasmid. The wild type gene product is 1.2 kbp in size the expected mutant product is 2.3 kps. **Lane 1**, Water; **2** & **3**, gDNA *C. acetobutylicum*WUR (pMTL500E); **4**, gDNA *C. acetobutylicum* WUR (pWOK002::Cac-*ack*-1027a), clone 1; **5** & **6** & **7**, gDNA of three different clones of *C. acetobutylicum* WUR pWOK001::Cac-*ack*-1027a; **8**, gDNA *C. acetobutylicum*WUR (pMTL500E); **9**, gDNA *C. acetobutylicum* WUR (pWOK002::Cac-*ack*-1027a), clone 2; **M**, Marker.

2.3.3 Attempts at Mutant Isolation

Use of colony PCR would allow for the analysis of large numbers of colonies and immediately gives the desired information. To test if a *C. acetobutylicum* colony PCR method could be established a preliminary test was run using standard *E. coli* conditions (10 minutes at 94 °C) and primers flanking the *ack* gene. No product could be detected for colony samples, genomic DNA used as a positive control did give a product (data not shown). A literature search for a specific method for *C. acetobutylicum* was fruitless. Various approaches were tried to find a suitable method, these included the following; (i) heating the cells to 99 °C for

5 minutes, (ii) microwaving the cells for 30 or 60 seconds at 900 W, (iii) addition of up to 3 % isoamyl alcohol to the reaction, and (iv) addition of up to 3 % *n*-butanol to the reaction, however not one was successful. A *C. tetani* colony PCR protocol, employing TE-buffer and two subsequent heating steps (60 °C, 30 minutes and 95 °C for 10 minutes), from the literature[231] was tried but without success.

As we failed to get a colony PCR method working at this stage it was decided to move to a phenotypical screening method for the, presumed, acetate kinase knock-out mutant. In the literature the isolation of various acid producing mutants after random mutagenesis has been described due to their resistance to halogen analogues of these acids.[232–235] Initially fluoroacetate was used to try to discriminate between wild-type and mutants. Fluoroacetate resistance of the wild-type was tested at concentrations of 5 to 50 mM in 5 mM increments, and at 70 mM added to reinforced clostridial medium (RCM) plates. Although some growth inhibition was seen towards the higher concentrations this was not enough for a useful screening method. To increase the toxicity of fluoroacetate Brown *et al.*[232] used pyruvate as a carbon source instead of glucose.

As *C. acetobutylicum*can also grow on pyruvate as the sole carbon source this could be tested, but concerns about the highly toxic nature of fluoroacetate to mammals (oral LD_{50} 0.1–5.0 mg/kg), led to the testing of chloroacetate as an alternative (oral LD_{50} 180–350 mg/kg). Chloroacetate sensitivity of the wild-type was assessed on RCM plates containing 0.5, 1, 2, 3, 5, and 10 mM chloroacetate. Growth of the wild-type was reduced at 2 and 3 mM and completely repressed at 5 mM levels. Colonies of *C. acetobutylicum* transformants harbouring the pWOK plasmids with introns targeted to insert in the acetate kinase gene were plated on 5 mM chloroacetate containing plates. Between 3 (pWOK002) and 20 (pWOK001) resistant colonies could be seen on the plates. After restreaking on chloroacetate containing plates genomic DNA was isolated from these strains and the acetate kinase gene was amplified by PCR using gene flanking primers. All tested colonies showed wild-type length PCR fragments. Amplification with an intron specific and a gene flanking primer did show that mutants were still present but apparently were not enriched by the chloroacetate selection.

Although chloroacetate was previously successfully applied to isolate acid-pathway mutants of various clostridial species that were subjected to random mutagenesis, it failed to aid in isolation of a targeted *ack* insertional mutant.[234] Interestingly 5 mM chloroacetate containing plates did result in selected growth of a low number of colonies. These colonies were however not mutants, nor enriched in the mutant population. It is therefore unlikely that *ack* inactivation gave a growth advantage. It is possible that the presence of the intron II bearing plasmid resulted in an additional mutation, or mutations, in the genome that resulted in a growth advantage over the wild type cells in the presence of chloroacetate. These mutations would most likely be associated with other acetate production or uptake routes. The resistant cells were not further characterised but likely targets are enzymes that are part of the acetone production pathway (*ctfA* and *ctfB*) as

well as phospho-transacetylase (*pta*). To determine if additional insertions had taken place a Southern blot, using the intron sequence as a probe, would have been able to identify if this were the case. Inverse PCR could then have been used to identify the gene or genes affected.

During the execution of the work described we gained access to the pMTL007 system which was also a TargeTron based knock-out system for clostridia and it was equipped with an erythromycin-RAM allowing for selection of the integrants.[152] As this new system would facilitate rapid isolation of the mutant the current work was abandoned in favour of the pMTL007 vector system. This resulted in the successful isolation of an *ack*$^-$ mutant as described in chapter 3.

2.4 Conclusions and Recommendations

In this chapter we describe the development of a clostridial gene knock-out system based the TargeTron methodology. The systems was shown to function in *C. acetobutylicum* for the insertion of the intron into the acetate kinase gene. During the development of the system we noted that P_{thl} seemed to be a better promoter for expression of the intron II then the *adc* promoter with regard to the tested insertion sites. Work by Heap *et al.* seems to confirm this as they concluded that constitutive expression was more beneficial for mutant isolation then inducted expression.[154] The disadvantage of the original pMTL007 system is however that system still relies on a non-removable selectable marker making multiple mutations impossible when using the same system. We therefore later applied a modified version of the here described pWOK001 plasmid for the targeted inactivation of acetate kinase in an erythromycin resistant strain (see chapter 4), thereby demonstrating that our system can be used to obtain targeted mutants.

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Chapter 3

Disruption of the Acetate Kinase (*ack*) Gene of *Clostridium acetobutylicum* Results in Delayed Acetate Production

Abstract

In microorganisms, the enzyme acetate kinase (AK) catalyses the formation of ATP from ADP by dephosphorylation of acetyl phosphate into acetic acid. A mutant strain of *Clostridium acetobutylicum* lacking acetate kinase activity is expected to have reduced acetate and acetone production compared to the wild type. In this work, a *C. acetobutylicum* mutant strain with a selectively disrupted *ack* gene, encoding AK, was constructed and genetically and physiologically characterized. The *ack*$^-$ strain showed a reduction in acetate kinase activity of more than 97 % compared to the wild type. The fermentation profiles of the *ack*$^-$ and wild-type strain were compared using two different fermentation media, CGM and CM1. The latter contains acetate and has a higher iron and magnesium content than CGM. In general, fermentations by the mutant strain showed a clear shift in the timing of peak acetate production relative to butyrate and had increased acid uptake after the onset of solvent formation. Specifically, in acetate containing CM1 medium, acetate production was reduced by more than 80 % compared to the wild type under the same conditions, but both strains produced similar final amounts of solvents. Fermentations in CGM showed similar peak acetate and butyrate levels, but increased acetoin (60 %), ethanol (63 %) and

butanol (16 %) production and reduced lactate (−50 %) formation by the mutant compared to the wild type. These findings are in agreement with the proposed regulatory function of butyryl phosphate as opposed to acetyl phosphate in the metabolic switch of solventogenic clostridia.

3.1 Introduction

Expected shortage of petroleum in the near future and concerns regarding the net increase of carbon dioxide emissions by fossil fuel combustion have resulted in a search for sustainable sources for the production of transport fuels and chemicals. Butanol derived from lignocellulosic materials could provide such an alternative method to the current petrochemical production process.[45,50] Fermentative butanol production is carried out by various clostridial species as part of the acetone–butanol–ethanol (ABE) process.[50,57,115,194] Two of the most important drawbacks of the ABE process are the low butanol yields, due to the formation of by-products and the toxicity of butanol itself to the cultures, and the high costs of the separation of the different products.[236]

Several strategies to increase the production of butanol and reduce by-product formation by metabolic engineering have been described.[204,237] To date, only two gene knockouts (phosphotransacetylase, *pta*; butyrate kinase, *buk1*)[126,156] and five asRNA constructs (acetoacetyl-CoA:acetate/butyrate:CoA-transferase, *ctfA* and *ctfB*; acetoacetate decarboxylase, *adc*; phosphotransbutyrylase, *ptb*; *buk1*)[124,125,174] pertaining directly to the reduction of by-product formation in *C. acetobutylicum* ATCC 824 have been reported. Inactivation of *buk1* resulted in a 42 % increase in butanol levels[128] while inactivation of the *pta*, involved in acetate formation, did not show improved solvent production (WT A:B:E production, 72:133:13 mM vs. 79:131:11 mM for the pta^- mutant).[126]

In related strains, three genes have been knocked out: *adc* in *C. acetobutylicum* EA 2018[203] and acetate kinase (*ack*) and *buk1*, both in *C. acetobutylicum* M5.[129] Impairment of the acetone pathway results in all cases in increased acid accumulation, as expected for strains that can no longer efficiently take-up acids from the growth medium. Strain M5 is a degenerated strain, isolated after chemical mutagenesis, which no longer has the pSOL1 megaplasmid and therefore is devoid of solvent production.[172] To restore solvent productivity, Nair and Papoutsakis[173] expressed the alcohol dehydrogenase gene (*adhE*), normally located on pSOL1, in strain M5 from a replicative plasmid. Butanol and ethanol production was restored without acetone formation, but at reduced levels compared to the wild-type ATCC 824 strain, while large amounts of acetate and butyrate accumulated in the growth medium. To reduce the acid production in this M5 background, Sillers *et al.*[129] created an acetate kinase knockout strain (M5 AKKO) and a butyrate kinase knockout strain (M5 BKKO). Both strains had reduced, but not eliminated, acid accumulation corresponding to the inactivated pathway and grew more slowly. Attempts to restore solvent formation by these

acid production mutants by plasmid based *adhE* expression were unsuccessful for the M5 BKKO strain. M5 AKKO could be transformed, but it still produced large amounts of acetate and butyrate with reduced alcohol production compared to the M5 *adhE* expressing strain.

Based on the fermentation results of the *pta*$^-$ and *buk1*$^-$ mutants and a later study measuring the intracellular levels of phosphorylated acids in *C. acetobutylicum*, it was proposed that butyryl phosphate is a regulatory molecule involved in the transition from acidogenesis into solventogenesis.[165] The enzymes involved in acetate production in *C. acetobutylicum*, phosphotransacetylase and acetate kinase, are encoded by the *pta-ack* operon. A *C. acetobutylicum* strain in which the acetate kinase (*ack*) gene is inactivated is expected to accumulate acetyl phosphate, a phosphorylated intermediate with a potential regulatory function in *Escherichia coli*.[238,239] On the contrary, a *pta*$^-$ mutant is not expected to accumulate this intermediate.[239] The influence of *ack* disruption on solvent production in a wild-type background has not been studied yet. Formation of acetate, butyrate, ethanol and acetone limits the amount of metabolic precursors available for butanol production. In our current research, we therefore focus on reducing formation of these by-products by a targeted gene disruption. By inactivating the genes involved in their production, we wished to investigate the effects on the overall metabolism and butanol production. In this study, we describe a mutant of *C. acetobutylicum* with an inactivated acetate kinase (*ack*) gene, obtained using the ClosTron system for selective gene inactivation in Clostridia.[152] The resulting *ack*$^-$ mutant has been characterized, and its fermentation profile under batch conditions has been compared to that of the wild-type strain.

3.2 Materials and Methods

3.2.1 Bacterial Strains and Plasmids

All bacterial strains and plasmids used in this study are listed in Table 3.1. Stock cultures of *C. acetobutylicum* were maintained as spore suspensions in sterile 15 % (v/v) glycerol at −20 °C. Chemical competent *E. coli* TOP 10 cells (Invitrogen) were used for vector maintenance and cloning.

3.2.2 Media and Growth Conditions

E. coli strains were grown in lysogeny broth (LB) medium[223,224] at 37 °C, 200 rpm supplemented with the appropriate antibiotics. Clostridial spore suspensions were prepared as previously described[216] and heat-shocked for 10 min at 70 °C, prior to using them as an inoculum for precultures. Liquid cultures of *C. acetobutylicum* were grown in CM1 medium, based on Nimcevic *et al.*,[240] containing per litre: yeast extract, 5.00 g; KH_2PO_4, 1.00 g, K_2HPO_4, 0.76 g; ammonium acetate, 3.00 g; *para*-aminobenzoic acid (*p*ABA), 0.10 g; $MgSO_4 \bullet 7\ H_2O$, 1.00 g; $FeSO_4 \bullet 7\ H_2O$, 0.50 g and glucose, 100 g or in CGM[102] containing per litre:

Table 3.1: Strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Remarks	Source
Strains			
E. coli TOP10		Cloning strain	Invitrogen
E. coli TOP10 (pAN2)		Methylation strain	This study
C. acetobutylicum WUR		Originally obtained as ATCC 824 but shown to deviate from type strain behaviour[216]	Laboratory stock
C. acetobutylicum WUR AK	*ack*$^-$	Acetate kinase knockout mutant of WUR strain	This study
Plasmids			
pAN2	p15A ori; Tc^R ϕ*3tI*	Methylation plasmid derived from pAN1 but with tetracycline resistance selection	Laboratory stock[152]
pMTL007::Cac-*spo0A*-242a	ColE1 ori, pCB102 ori; Cam^R LtrA intron II	Supplied pMTL007 backbone and is a positive control targeted to insert in *spo0A* between bp 242 and 243 in the antisense orientation	Laboratory stock[152]
pMTL007::Cac-*ack*-1027a	ColE1 ori, pCB102 ori; Cam^R LtrA intron II	Targeted to insert in *ack* between bp 1027 and 1028 in the antisense orientation	This study
pMTL007::Cac-*ack*-84s	ColE1 ori, pCB102 ori; Cam^R LtrA intron II	Targeted to insert in *ack* between bp 84 and 85 in the sense orientation	This study

[a] *ermB*, confers resistance to erythromycin; p15A ori, *E. coli* origin of replication; Tc^R, tetracycline resistance; ϕ*3tI*, *Bacillus subtilis* phage gene encoding the ϕ3T I methyltransferase; ColE1 ori, *E. coli* origin of replication; pCB102 ori, clostridial origin of replication; Cam^R, chloramphenicol resistance; LtrA, the intron-encoded protein of the *Lactococcus lactis* Ll.LtrB group II intron.

yeast extract, 5.00 g; KH_2PO_4, 0.75 g; K_2HPO_4, 0.75 g; asparagine•H_2O, 2.27 g; $(NH_4)_2SO_4$, 2.00 g; cysteine, 0.50 g; $MgSO_4$•7 H_2O, 0.40 g; $MnSO_4$•H_2O, 0.01 g; $FeSO_4$•7 H_2O, 0.01 g and glucose, 80 g. Media were made anaerobic by flushing with nitrogen gas for 10 to 30 min depending on the volume of the liquid. All clostridial culture experiments were performed at 37 °C, without shaking, and anaerobically in (a) an anaerobic chamber (Sheldon Manufacturing, Oregon, USA; gas mixture consisting of 15 % CO_2, 4 % H_2 and 81 % N_2) or (b) in glass serum vials as described previously.[225]

Culture media were supplemented with ampicillin (100 µg/mL), chloramphenicol (30 µg/mL), thiamphenicol (15 µg/mL), erythromycin (40 µg/mL standard; 5–10 µg/mL for initial mutant isolation) or kanamycin (50 µg/mL) when appropriate. Biomass was determined spectrophotometrically (Pharmacia Biotech Ultrospec 2000) based on a experimentally determined relationship between

optical density measurements (OD_{600}) and cell dry weight [CDW = OD_{600} − 0.40/2.91].

3.2.3 DNA Isolation, Manipulation and Transformation

Standard molecular work was done according to established protocols.[224] DNA from *C. acetobutylicum* was isolated using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) using the Gram-positive isolation procedure which incorporates a lysozyme (from chicken egg white; Fluka) treatment. *E. coli* plasmid DNA was isolated by the GenElute Plasmid Miniprep Kit (Sigma-Aldrich). Both kits were used according to the manufacturers' instructions. DNA amplification by PCR on *C. acetobutylicum* DNA was done using Pwo polymerase (Roche Diagnostics), and *E. coli* colony PCR reactions were carried out using REDTaq DNA polymerase (Sigma-Aldrich).

Prior to transformation into *C. acetobutylicum*, plasmids were methylated *in vivo*[147] by electroporation into *E. coli* TOP 10 (pAN2) cells. Electrotransformation of *C. acetobutylicum* was carried out as previously described.[142] All manipulations were carried out anaerobically and on ice. After transformation and recovery, the cells were plated on pre-warmed plates containing thiamphenicol.

3.2.4 Primers and DNA Sequencing

All DNA primers used in the study are listed in Table 3.2. Primers were obtained from Eurogentec (Seraing, Belgium). DNA sequencing of clones was done by BaseClear (Leiden, The Netherlands).

Table 3.2: Primer sequences

Primer name	Sequence (5′ → 3′)[a]
AK1027a_ibs	aaaaaagctt ataattatcc ttaTGTTTcT GCATAgtgcg cccagatagg gtg
AK1027a_ebs1d	cagattgtac aaatgtggtg ataacagata agtcTGCATA TTtaacttac ctttctttgt
AK1027a_ebs2	tgaacgcaag tttctaattt cgGttAAACA Tcgatagagg aaagtgtct
AK84s_ibs	aaaaaagctt ataattatcc ttaGTACTcG CTAAAgtgcg cccagatagg gtg
AK84s_ebs1d	cagattgtac aaatgtggtg ataacagata agtcGCTAAA GGtaacttac ctttctttgt
AK84s_ebs2	tgaacgcaag tttctaattt cgAttAGTAC Tcgatagagg aaagtgtct
EBS_Univ	cgaaattaga aacttgcgtt cagtaaac
Cac_ack_fwd	atgaaaaact tagttattaa ctgcg
Cac_ack_rev	ttattttaac ttgcctacta tatcttt
5402F_fwd	ttaaggaggt gtatttcata tgaccatgat tacg
pMTL007_rev	agggtatccc cagttagtgt taagtcttgg
ErmRAM-F	acgcgttata ttgataaaaa taataatagt ggg
ErmRAM-R	acgcgtgcga ctcatagaat tatttcctcc cg
TT_Probe_f	aatctgtagg agaacctatg ggaacg
TT_Probe_r	cgcgtcgcca cgtaataaat atctgg

[a] Nucleotides that are not complementary to the template sequence are given in uppercase. *Hin*dIII and *Bsr*GI restriction sites incorporated in the primers are underlined.

3.2.5 Construction of ClosTron Plasmids

Plasmids were constructed according to the protocol by Heap *et al.*[152] using the Sigma Targetron design website (www.sigma-genosys.com/targetron). The retargeted cassettes were ligated into the pMTL007 backbone, resulting in plasmids pMTL007::Cac-*ack*-1027a and pMTL007::Cac-*ack*-84s. Correct plasmids were identified by restriction digestion and by sequencing the retargeted region using primers 5402F_fwd and pMTL007_rev.

3.2.6 Induction of the ClosTron System and Mutant Isolation and Verification

One-millilitre CGM with thiamphenicol was inoculated with a 100-µL stationary phase overnight culture and incubated for 1.5 h at 37 °C. Then IPTG was added to a final concentration of 1 mM, and incubation was continued for 3 h. Cells were centrifuged at 5 200 × *g* for 1 min, and the supernatant was removed. After addition of 0.5 mL PBS, cells were resuspended and centrifuged again at 5 200 × *g* for 2 min. The supernatant was removed and replaced with 1-mL CGM without antibiotics. The cells were resuspended and incubated for 3 h at 37 °C before plating on CGM agar plates containing 5 µg/mL erythromycin and incubated for 1 or 2 days at 37 °C. Selected colonies were restreaked on plates containing 10 µg/mL erythromycin. Isolated mutants were checked for absence of the 1.3-kb plasmid sized, intron I containing, *ermB* gene using primers ErmRAM-F and ErmRAM-R.

3.2.7 Southern Blot

Genomic DNA of the wild type, the AK mutant and the pMTL007 plasmid was restriction digested using four different mixes for each sample (*Kpn*I and *Sac*I, *Bgl*II and *Eco*RV, *Hin*dIII, *Hpa*II). Digests were then subjected to Southern blot analysis using a random-labelled DIG probe (Roche) and was performed according to the manufacturer's instructions. Primers TT_Probe_f and TT_Probe_r were used to generate the probe, with pMTL007 plasmid DNA as a template. The resulting probe hybridised to nucleotides 312 to 694 of the inserted intron II sequence. Irrespective of restriction enzyme used, no DNA fragment was released from the wild-type genomic DNA that hybridised to the probe. In contrast, restriction fragments of the expected size were derived from both the genome of the AK mutant and the ClosTron plasmid which gave a positive signal on the Southern blot.

3.2.8 Cell-free Extracts and Enzymatic Activity Assays

C. acetobutylicum cells were harvested from cultures with an OD of approximately 1.0 by centrifugation (15000 × *g*, 7 min, 4 °C) and resuspended in 50 mM

MOPS buffer (pH=7.0) containing 1 mM DTT. Crude cell extracts were prepared by French press (Thermo Scientific) homogenization (two passes at 16 000 psi). The homogenates were centrifuged (20 817 × *g*, 15 min, 4 °C) decantated and centrifuged as before. The cell-free extracts were stored at −80 °C until analysed. AK and BK were assayed at 29 °C in the acyl phosphate forming direction according to the method of Rose.[241] Units of AK or BK activity were defined as micromoles of substrate converted per minute. Acetyl phosphate was used for preparation of a calibration curve for both reactions. Phosphotransacetylase (PTA) and phosphotransbutyrylase (PTB) activities were measured in the acyl phosphate forming direction by measuring the release of coenzyme A as previously described.[242] Units of PTA or PTB activity were defined as micromoles of CoA released per minute. Homogenisation and enzyme assays were carried out aerobically. Total protein in crude extracts was determined using Quick Start Bradford Protein Assay (Bio-Rad) with bovine serum albumin as a standard.

3.2.9 Batch fermentations of *C. acetobutylicum*

Fermentations were performed in 2 L bioreactors (1 L working volume) controlled via a Bio Controller ADI 1010 by a PC running Bioexpert software (all Applikon, The Netherlands). No antibiotics were used during the fermentations. CGM batch fermentations were set up as follows: Yeast extract, phosphates, ammonium sulphate and asparagine were autoclaved with the reactor, whereas autoclaved glucose was added to the reactor after cooling. Metal sulphates and cysteine were added filter-sterilised. CM1 medium-based batch fermentations were setup by autoclaving yeast extract, phosphates and ammonium acetate with the reactor. Autoclaved glucose was added to the reactor after cooling. Metal sulphates and *p*ABA were added as a filter sterilised mix.

The medium in the reactor was sparged overnight with nitrogen. Prior to inoculation, Sigma antifoam 204 was added to the reactor (0.1 ‰). The reactor was inoculated with 50 mL of an overnight pre-culture grown on the same medium but supplemented with erythromycin in the case of the mutant. After inoculation, the starting pH was allowed to fall to 5.0, after which it was controlled by addition of 6 M ammonium hydroxide solution (CGM) or 4 M KOH (CM1). During the fermentation, the headspace was flushed with nitrogen and the outgoing gas flow was passed through a condenser at 4 °C. The agitation rate was 200 rpm.

3.2.10 Analysis of Metabolites

Metabolites present in culture supernatants (glucose, acetate, butyrate, lactate, acetoin, *meso*-2,3-butanediol, acetone, butanol, and ethanol) were determined by HPLC as described previously[216] using valeric acid as an internal standard.

3.3 Results

3.3.1 *ack* Gene Disruption

Sequencing of the acetate kinase gene (*ack* or *askA*, CA_C1743) of the *C. acetobutylicum* WUR strain showed that it is identical to the published genomic sequence of the ATCC 824 strain (data not shown). After submitting the gene sequence to the TargeTron website, ten possible insertion sites were returned. The two top scoring ones were selected for our mutagenesis work. Each site requires its own set of three unique primers and a general one (EBS_Univ) for the splicing by overlap extension (SOEing) PCR[152,243] that is used to replace the standard sequence with a sequence that will recognise the desired insertion site. The first set of unique primers, consisting of AK1027a_ibs, AK1027a_ebs1d and AK1027a_ebs2, was used to target the intron to insert in the anti-sense orientation between base pairs 1027 and 1028 of *ack*. The second set of unique primers, consisting of AK84s_ibs, AK84s_ebs1d and AK84s_ebs2, targeted the intron to insert in the sense orientation between base pairs 84 and 85 (Table 3.2).

The retargeted plasmids were constructed as described in the materials and methods section. Transformation of the plasmids, selection and induction of the intron II system resulted in erythromycin-resistant colonies. Colonies were screened for insertion in the *ack* gene by PCR on genomic DNA using primers Cac_ack_fwd and Cac_ack_rev. Of the eight colonies screened, three were positive for insertion between base pairs 1027 and 1028. Of the 16 screened colonies that contained the pMTL007::Cac-*ack*-84s construct, none showed insertion in the *ack* gene. All of the screened colonies tested positive by PCR for a correctly sized *ermB* gene of approximately 900 bps (the length of the active, genomically inserted, *ermB* gene). This shows that in the cases where the *ack* gene was not affected, the intron II must have integrated somewhere else in the genome.

The insertion efficiency at the intended site of the pMTL007::Cac-*ack*-84s system appeared to be lower than that for the pMTL007::Cac-*ack*-1027a system, and therefore, no further attempts were made to isolate a mutant with insertion at the 84/85 site. One of the successful 1027a insertants was selected and further purified by re-streaking on fresh erythromycin plates. DNA isolated from this mutant strain was analysed by PCR amplification of the full-length *ack* gene using primers Cac_ack_fwd and Cac_ack_rev. Only a 3.0-kbp PCR product was obtained, corresponding to the *ack* gene with an inserted intron. The absence of a wild type, 1.2 kbp amplicon, indicated that a pure culture was obtained (Figure 3.1). Loss of the pMTL007 plasmid by the mutant was confirmed by PCR using the *ermB* gene primers (not shown). To check if only one copy of the intron had inserted in the genome, a Southern blot was performed. The probe employed was complementary to nucleotides 312 to 694 of the inserted intron sequence. Only bands, corresponding to the expected sizes, were detected (Figure 3.2 and Figure 3.3). Also this analysis confirmed that pMTL007 plasmid DNA was not present in the sample. Insertion of the intron II at the intended site in the *ack*

gene was confirmed by sequencing (data not shown). Spore suspensions from the obtained mutant, designated *C. acetobutylicum* AK, were used for further studies.

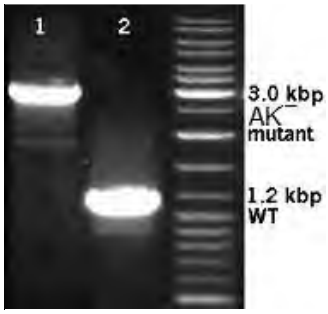

Fig. S1 PCR products obtained after amplification of the *askA* gene, using primers Cac_askA_fwd and Cac_askA_rev, from genomic DNA isolated from the AK mutant (lane 1) and wild type (lane 2)

Figure 3.1: PCR products obtained after amplification of the *ack* gene, using primers Cac_ack_fwd and Cac_ack_rev, from genomic DNA isolated from the AK mutant (lane 1) and wild type (lane 2).

3.3.2 Activities of Acetate and Butyrate Pathway Enzymes

The characterisation of the mutant was performed using two different media. CGM medium, which has been used previously for the characterisation of similar mutants[126] and does not contain acetate, was selected in order to be able to compare our results to earlier reports and to observe acid production without background levels, while CM1 medium is used routinely in our laboratory for growth experiments and contains 39 mM acetate and a higher concentration of iron and magnesium than CGM.

To confirm functional inactivation of acetate kinase, cell-free extracts of exponentially growing cultures were assayed for enzyme activities involved in acid production. The determined specific enzyme activities are shown in Table 3.3. Acetate kinase enzyme activity in cells from cultures grown in both media was reduced by more than 97 % compared to the wild type. The PTA activity, encoded by the *pta* gene which resides upstream of *ack* in the same operon, was also negatively affected by the insertion, being reduced by 41 % in CM1 medium and 29 % in CGM. Interestingly, BK activity, involved in butyrate production and encoded by the *ptb-buk1* operon, also showed reduced activity in both media. While PTB activity was still comparable (90 % and 100 % activity of the wild type in CM1 and CGM medium, respectively), BK activity was reduced to 72 % and 53 % activity of the wild type in, respectively, CM1 and CGM medium.

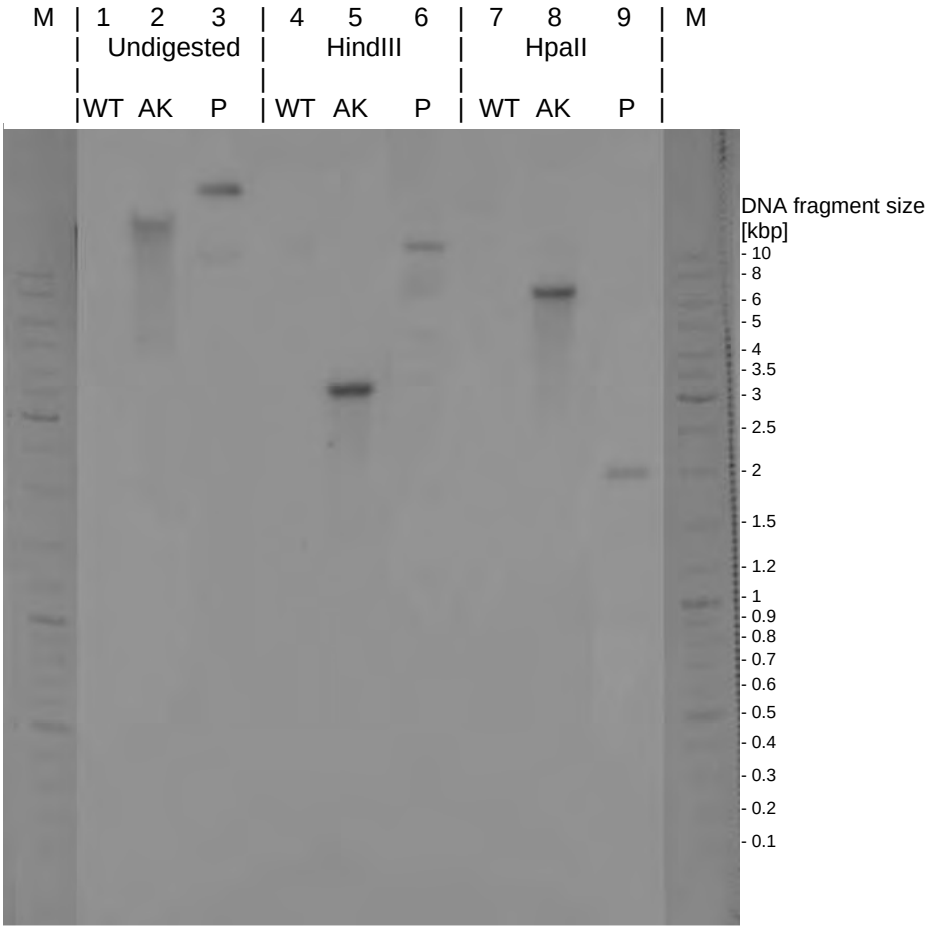

Fig. S2.2: Southern blot analysis of undigested (lanes 1 through 3) and digested (lanes 4 through 9) genomic DNA of: *C. acetobutylicum* WUR (WT), *C. acetobutylicum* WUR *ack*− mutant (AK), and plasmid pMTL007 (P) DNA. The probe hybridises to a 383 bp long part of the inserted intron II sequence and was generated as described in the materials and methods. Samples in lanes 4 through 6 were digested with *Hind*III and samples in lanes 7 through 9 were digested with *Hpa*II. Expected fragment sizes are 3391 bp (*Hind*III) and 6848 bp (*Hpa*II) for the AK strain. Marker lanes are indicated with M and sizes of the bands are shown on the right. Only AK and plasmid DNA samples gave a positive signal.

Southern blot analysis of undigested (lanes 1 through 3) and digested (lanes 4 through 9) genomic DNA of: *C. acetobutylicum* WUR (WT), *C. acetobutylicum* WUR *ack*− mutant (AK), and plasmid pMTL007 (P) DNA. The probe hybridises to a 383 bp long part of the inserted intron II sequence and was generated as described in the materials and methods. Samples in lanes 4 through 6 were digested with HindIII and samples in lanes 7 through 9 were digested with HpaII. Expected fragment sizes are 3391 bp (HindIII) and 6848 bp (HpaII) for the AK strain. Marker lanes are indicated with M and sizes of the bands are shown on the right. Only AK and plasmid DNA samples gave a positive signal.

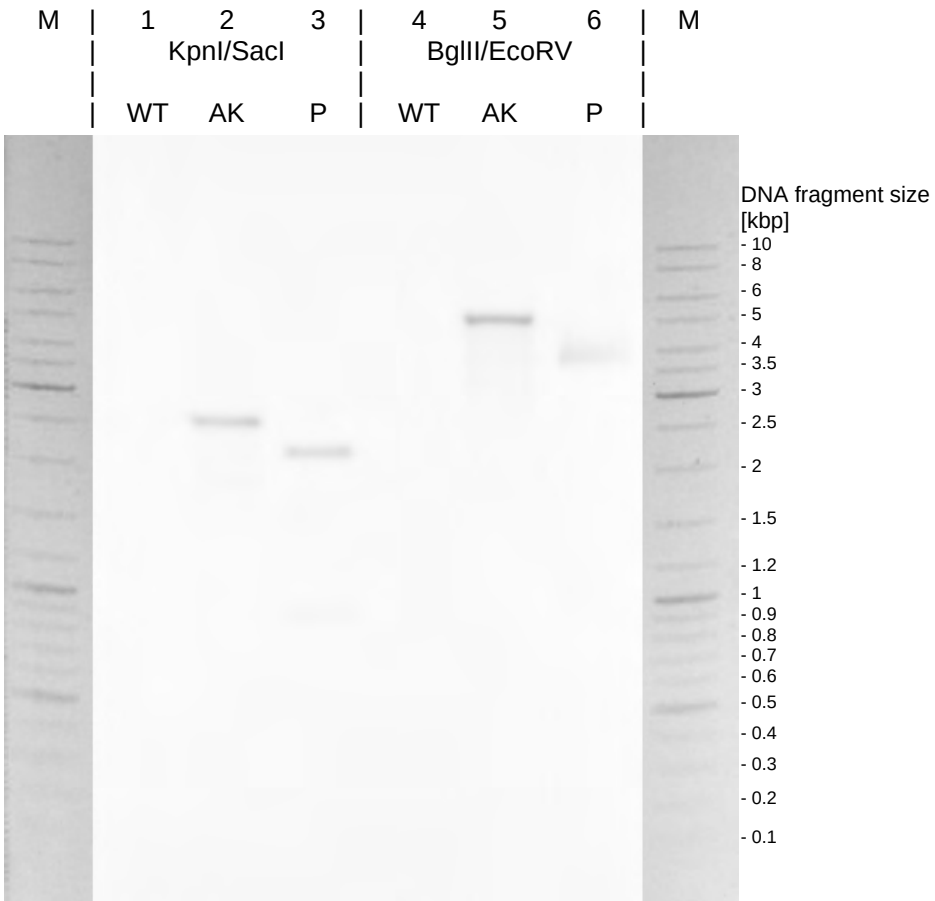

Figure 3.3: Southern blot analysis of digested genomic DNA of: *C. acetobutylicum* WUR (WT), *C. acetobutylicum* WUR *ack*$^-$ mutant (AK), and plasmid pMTL007 (P) DNA. The probe hybridises to a 383 bp long part of the inserted intron II sequence and was generated as described in the materials and methods. Samples in lanes 1 through 3 were digested with both *Kpn*I and *Sac*I, samples in lanes 4 through 6 were digested with both *Bgl*II and *Eco*RV. Expected fragment sizes are 2634 bp (*Kpn*I/*Sac*I) and 5056 bp (*Bgl*II/*Eco*RV) for the AK strain. Marker lanes are indicated with M and sizes of the bands are shown on the right. Only AK and plasmid DNA samples gave a positive signal.

Fig. S2b Southern blot analysis of digested genomic DNA of: *C. acetobutylicum* WUR (WT), *C. acetobutylicum* WUR *ack*$^-$ mutant (AK), and plasmid pMTL007 (P) DNA. The probe hybridises to a 383 bp long part of the inserted intron II sequence and was generated as described in the materials and methods. Samples in lanes 1 through 3 were digested with both KpnI and SacI, samples in lanes 4 through 6 were digested with both BglII and EcoRV. Expected fragment sizes are 2634 bp (KpnI/SacI) and 5056 bp (BglII/EcoRV) for the AK strain. Marker lanes are indicated with M and sizes of the bands are shown on the right.

Table 3.3: Specific enzyme activities involved in production of acetate and butyrate in wild-type and AK mutant homogenates

Growth medium[a]	Strain	Specific activities [U mg^{-1}][b]			
		Acetate pathway		Butyrate pathway	
		PTA	AK	PTB	BK
CM1	WT	0.40 ± 0.05	3.1 ± 0.4	9.18 ± 0.02	1.4 ± 0.1
	AK	0.24 ± 0.01 (59 %)	0.08 ± 0.02 (2.7 %)	8.3 ± 0.5 (90 %)	1.0 ± 0.3 (72 %)
CGM	WT	0.30 ± 0.03	3.4 ± 0.1	7.4 ± 1.3	1.2 ± 0.1
	AK	0.212 ± 0.007 (71 %)	0.064 ± 0.004 (1.9 %)	7.5 ± 0.1 (100 %)	0.66 ± 0.05 (53 %)

[a] Cells were harvested when $OD_{600} = 1.0$

[b] Data are reported as the mean ± SEM ($n = 2$). In the case of homogenates from the AK mutant, the relative activity compared to that of the wild type is given in parentheses.

3.3.3 Characterisation of the *C. acetobutylicum* AK Mutant in Batch Cultures on CM1 Medium

Product formation by the mutant strain was studied using pH-controlled batch cultures. The initial pH of the cultures was 6.6, and it was allowed to drop to 5.0 before it was controlled by addition of potassium hydroxide. Final product concentrations of duplicate fermentations after approximately 47 h are presented in Table 3.4. Figure 3.4 shows a typical fermentation profile in this medium. During fermentations by the wild-type strain on CM1 medium, both acetate and butyrate accumulated in the broth during the acidogenic phase of the fermentations. Butyrate production reached an average maximum of 62 mM, whereas the maximum average acetate concentration was 85 mM, a 43-mM increase relative to the starting concentration of 42 mM (Table 3.4). Both acids were, to some extent, taken up during the solventogenic phase of the fermentation. Butyrate levels dropped to around 16 mM, but acetate levels remained 27 mM above the starting concentration (Figure 3.4a). Solvent production by the wild type reached concentrations of 161, 101 and 30 mM for butanol, acetone and ethanol, respectively. The *C. acetobutylicum* AK strain consumed somewhat less glucose but had increased acetate assimilation. Final concentrations of solvents of *C. acetobutylicum* AK strain fermentations (157, 98 and 35 mM for butanol, acetone and ethanol, respectively) were similar to those in wild-type fermentations. The production of acids by the AK mutant was markedly different. Acetate levels increased with only 9 mM, a reduction of 79 % compared to the wild-type strain. The maximum butyrate levels were also lower compared to the wild-type fermentations (47 vs. 62 mM; Figure 3.4c). During the solventogenic phase of fermentations by the AK strain, butyrate was re-assimilated to reach similar levels as in fermentations by the wild-type strain. Contrary to butyrate, the acetate re-assimilation by the AK mutant resulted in net consumption (7 mM) of initially present acetate.

3.3.4 Characterisation of the AK Mutant in Batch Cultures on CGM Medium

To allow more precise monitoring of acetate levels, we characterised the mutant in CGM medium, which contrary to CM1 does not contain any acetate. In addition, CGM medium has been used in earlier fermentation studies with *C. acetobutylicum*.[126] Figure 3.5 shows typical optical density and production profiles of acids and solvents from wild-type and AK strain fermentations. The average product levels for three independent fermentations per strain after a minimum of 72 h are shown in Table 3.4 (page 65). Fermentations by the AK mutant strain consistently reached a higher biomass concentration than the wild type. Typical values of dry weight biomass concentration obtained were 4.5 ± 0.3 mg/mL for the wild-type and 5.5 ± 0.1 mg/mL for the AK mutant (Table 3.4).

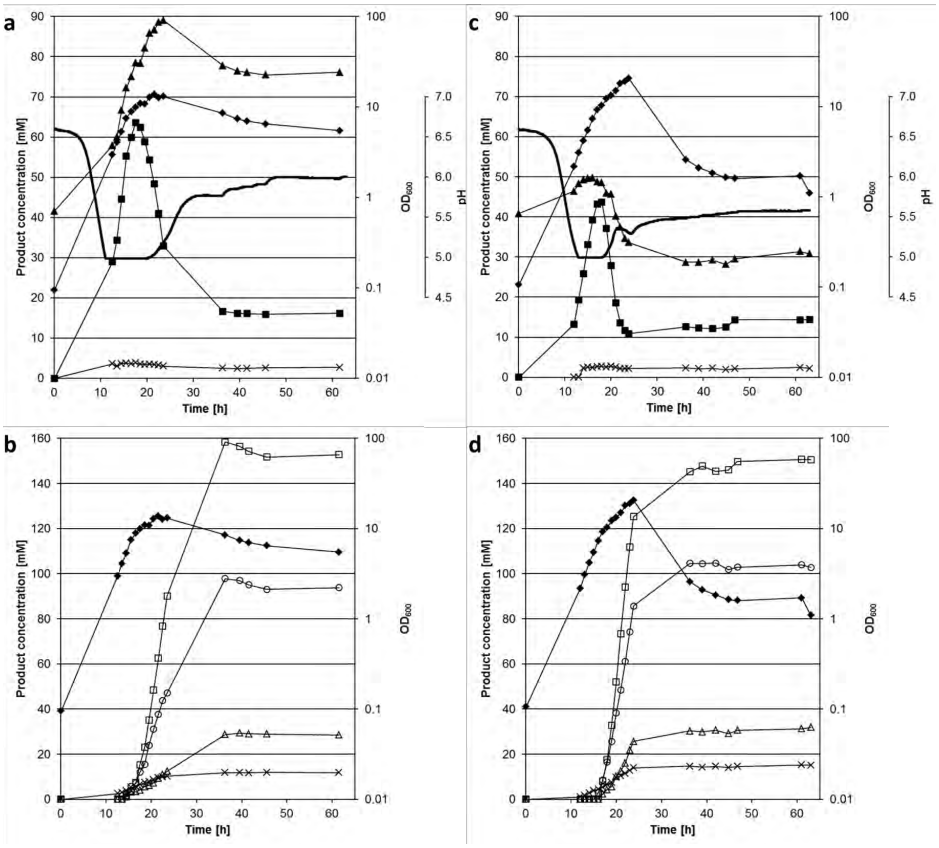

Figure 3.4: Typical optical density and production profile of a wild-type (**a** and **b**) and AK mutant strain (**c** and **d**) fermentation in pH 5.0 controlled batch reactors using CM1 medium containing 100 g/L glucose. **a**, **c** Production of acids −▲− acetate, −■− butyrate, − × − lactate, *solid line* pH, −♦− OD_{600}. **b**, **d** Production of solvents −△− ethanol, −□− butanol, −○− acetone, − × − acetoin, −♦− OD_{600}.

An interesting observation was that the fermentation broths from the AK mutant strain showed an intense yellow colour at the end of every fermentation, which was not the case for wild-type cultures. Spectrofotometrical analysis of the broth supernatant gave a local absorption maximum at 450 nm, suggesting increased build-up of riboflavin (vitamin B_2) in the medium.

During the fermentation, acetate was still produced by the AK strain but at slightly reduced maximum levels (WT 30 mM, AK 26 mM) compared to the wild type, as is shown in Figure 3.5, c. Maximum butyrate levels also did not seem to be affected to a great extent, with somewhat higher values for the mutant

Table 3.4: End-point product concentrations and fermentation parameters of 1-L batch fermentations (pH 5.0) using CM1 and CGM as cultivation media by the *C. acetobutylicum* WT strain and the AK mutant strain.[a]

		CM1			CGM		
		Wild type	AK	Change (%)[e]	Wild type	AK	Change (%)[e]
Fermentations (n)		2	2		3	3	
Consumed glucose		372 ± 5	353 ± 6		358 ± 7	345 ± 11	
Acetic acid	Final	69 ± 7	34 ± 4		30 ± 5	29 ± 4	
	Relative to start[b]	27 ± 7	−7 ± 4	−128	—[f]	—[f]	
Butyric acid		16 ± 0.4	13 ± 1		29 ± 3	4.3 ± 2	−85
Lactic acid		2 ± 0.2	3 ± 0.7		57 ± 7	28 ± 2	−51
Butanol[c]		161 ± 2	157 ± 7		131 ± 1	152 ± 2	+16
Acetone[c]		101 ± 3	98 ± 7		40 ± 6	36 ± 5	
Ethanol[c]		30 ± 1	35 ± 4		24 ± 2	39 ± 2	+59
Acetoin[d]		11 ± 1	19 ± 5		15 ± 0.7	24 ± 1	+58
Biomass [mg/mL]		4.57 ± 0.01	6.5 ± 0.4		4.5 ± 0.3[g]	5.5 ± 0.1[g]	+22
Carbon recovery (including biomass)		93 %	98 %		83 %	86 %	
(Butanol + Ethanol)/Acetone [mol/mol]		1.9	2.0		4.0	5.5	
Solvent yield based on							
mol butanol/mol glucose		0.43	0.45		0.37	0.44	
g ABE/g glucose		0.29	0.30		0.20	0.24	+20

[a] Data are concentrations in mM unless otherwise indicated and given as the mean ± SEM of the indicated number of independent fermentations.
[b] Relative acetate is calculated by the subtracting the initial acetate level at time of inoculation from the measured acetate levels later during the fermentation.
[c] Due to evaporation, ethanol and acetone levels dropped at the end of the fermentation. Peak recorded values are reported.
[d] Both the wild-type and the AK mutant strain also produced low levels (2-4 mM) of *meso*-2,3-butanediol as previously described.[216]
[e] If significant (t-test, > 95 % confidence), then the relative change in product levels is given.
[f] See row above, as no acetate is present in the medium at $t = 0$, nett acetic acid production equals the final acetic acid concentration.
[g] Data based on two independent experiments.

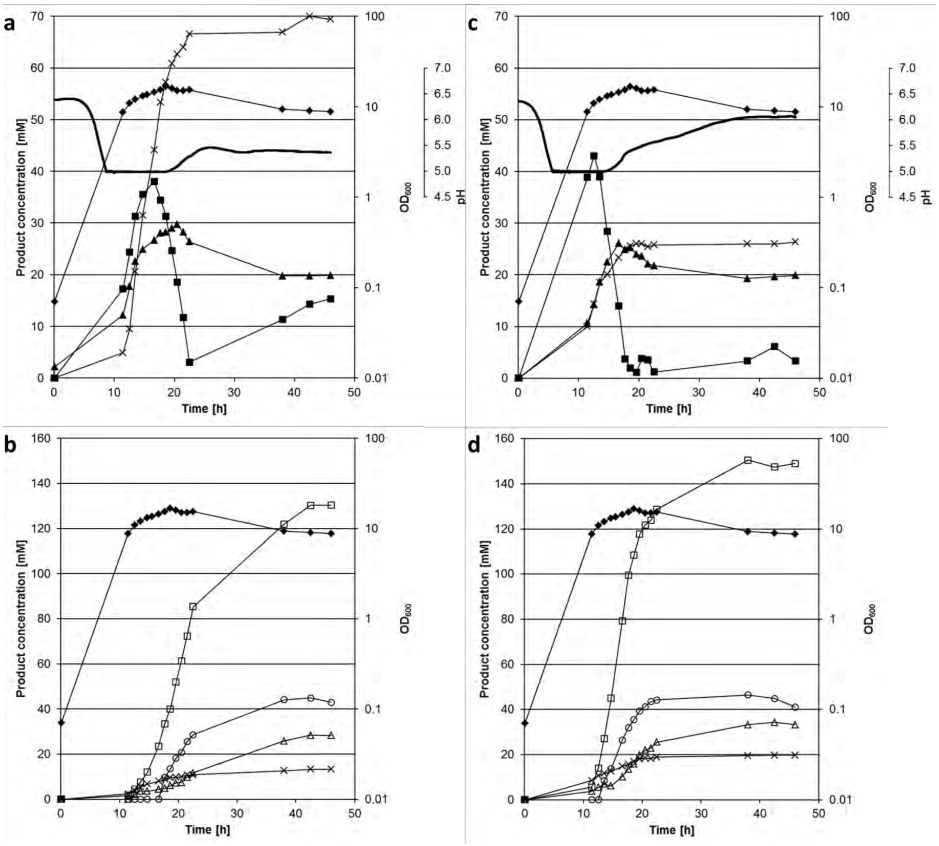

Figure 3.5: Typical optical density and production profile of a wild-type (**a** and **b**) and AK mutant strain (**c** and **d**) fermentation in pH 5.0 controlled batch reactors using CGM containing 80 g/L glucose. **a**, **c** Production of acids −▲− acetate, −■− butyrate, − × − lactate, *solid line* pH, −♦− OD_{600}. **b**, **d** Production of solvents −△− ethanol, −□− butanol, −○− acetone, − × − acetoin, −♦− OD_{600}.

(WT 38 mM, AK 43 mM). There was, however, a clear difference at what time point, compared to one another, the maximum acetate and butyrate levels were reached. The production of acetate by the AK mutant was delayed compared to that of butyrate. This difference was best observed by plotting the ratio between butyrate and acetate at various time points (Figure 3.6). From the start of the fermentation by the wild type, the ratio between butyrate and acetate rose, levelling off after 9 h at 1.5 and stayed at that ratio for 7 h. The ratio in fermentations by the AK strain continued to increase with a maximum of 4.3 after 14 h. The ratio then decreased to similar levels as those observed for the wild type, and both reached a minimum of 0.1. Analysis of the fermentations with CM1 medium gave a similar difference in acid ratio production (Figure 3.6b).

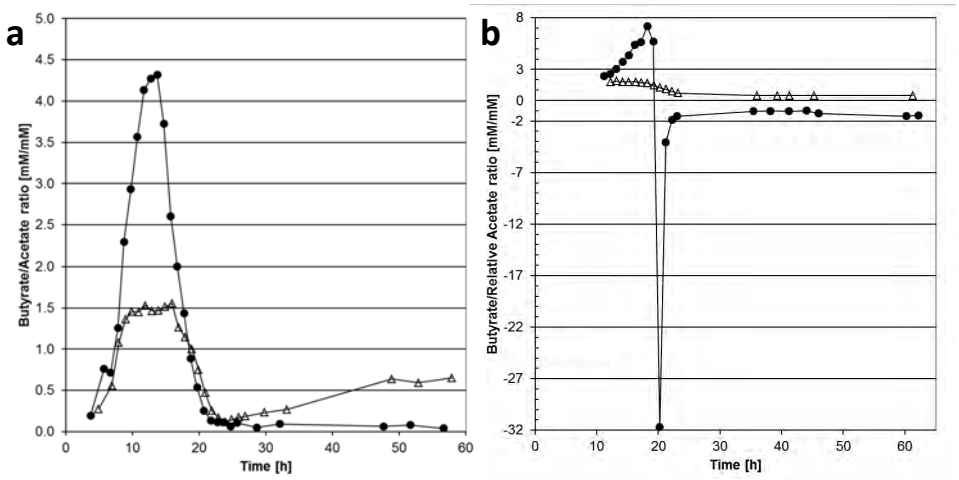

Figure 3.6: Butyrate–acetate ratios in CGM (**a**) or butyrate-relative acetate ratios CM1 (**b**) of a wild-type strain (−△−) and AK mutant strain (−●−) fermentation in pH 5.0 controlled batch reactors. On the horizontal axis, normalized time is plotted to account for variations in lag time of the cultures. In the graph, 9 h corresponds to an OD_{600} of 1.0. Relative acetate is calculated by the subtracting the initial acetate level at time of inoculation from the measured acetate levels later during the fermentation.

Lactate, normally only detected at low levels,[57,126] if at all, in *C. acetobutylicum* ATCC 824 fermentations in CGM medium, was produced in all three fermentations by *C. acetobutylicum* WUR (57 ± 7 mM). The AK mutant also produced lactate but consistently at lower levels (28 ± 2 mM). Various attempts — filter sterilisation of minerals, reduced stirring speed, extended sparging time with nitrogen gas before inoculation — were made to reduce lactate production in fermentations by the wild-type, but without success.

Butanol and ethanol production by the AK mutant were, respectively, 16 % and 59 % higher compared to the wild-type production. Acetone production levels were not significantly affected. The conversion of glucose to butanol by the mutant had increased with 19 %, and total solvent yields from glucose were up with 21 % (0.54 mol ABE/mol glucose consumed vs. 0.66 mol ABE/mol glucose consumed). The final concentration of the by-product acetoin had increased with 58 %, from 15 mM with the wild type to 24 mM for the mutant.

3.4 Discussion

The inactivation of the butyrate kinase (*buk1*) gene of *C. acetobutylicum* has previously been shown to result in reduced butyrate formation, increased peak acetate levels and increased butanol and ethanol production.[128] Acetone

is one of the major by-products of butanol production as it is formed as a consequence of the uptake of acids from the medium. It has been shown through a computational approach that even under solvent forming conditions acetate production continues, while extracellular acetate levels drop.[75] Produced acetate is continuously converted to acetyl-CoA, mediated by the enzyme acetoacetyl-CoA:acetate/butyrate:CoA transferase, ultimately resulting in acetone production. This approach allows *C. acetobutylicum* to continue acetate-mediated ATP generation during the solventogenic phase. With the aim to reduce the formation of acetate and indirectly that of acetone, by *C. acetobutylicum*, we have inactivated the acetate kinase (*ack*) gene which encodes the enzyme catalysing the last step in acetate production. In this substrate level phosphorylation reaction, the phosphate group of acetyl phosphate is transferred to ADP, generating ATP and acetate. In addition, we were interested to see if solvent formation would be similarly beneficially affected, as reported for *buk1* gene disruption.[128]

In this study, site-directed gene disruption was accomplished by integration of a group II intron using the ClosTron system.[152] This system is markedly different from the non-replicative plasmid integration-based approach used for the previously generated *buk1* and *pta* mutants of *C. acetobutylicum*.[126] Mutations based on single cross-over recombination are inherently unstable, whereas insertion of the group II intron in the anti-sense orientation, relative to the gene, results in permanent inactivation.[152] The ClosTron and a similarly adapted TargeTron system[156] have been used to generate various mutants, including *adc* and *hbd* knockout mutants.[176,203]

Successful insertion of the intron after transformation of pMTL007::Cac-*ack*-1027a would, after translation of the mutated gene, truncate the native sequence of the AK protein at amino acid 342. However, additional intron sequence encoded amino acids will be added to the native sequence. In the case of insertion after nucleotide 1027, the sequence would be extended by 86 amino acids, so intron insertion actually results in a protein that is 27 amino acids longer than the native enzyme.

Although the retrohoming system of the group II introns is expected to be site-specific, it is possible that no or only a limited number of correctly positioned insertants can be found.[152,226] Our results show efficient insertion into insertion site 1027a, but for insertion site 84s, 16 screened colonies did not yield a correct insertant. These results emphasise the need to test multiple insertion sites when using TargeTron-based systems.

AK activity in the AK mutant was less than 3 % of the wild-type enzyme activity (Table 3.3), demonstrating that while the insertion was close to the 3′-side of the *ack* gene, it still resulted in functional inactivation of the enzyme. A study by Singh-Wissmann *et al.*[244] showed that a conserved glutamate residue in sequence of the AK of *Methanosarcina thermophila* near the C-terminus is essential for enzyme activity. This glutamate is conserved in acetate kinase sequences from widely divergent organisms and is also present in the *C. acetobutylicum* AK.[244] In our AK strain, this glutamate residue is not present anymore, so no

activity from the enzyme encoded by the disrupted gene is expected, which is in line with the low enzyme activities observed (Table 3.3).

It was expected that disruption of acetate kinase would result in decreased acetate levels and increased butyrate levels to compensate for the loss in ATP production,[245] but this was not immediately observed. In both tested media, the AK mutant strain had peak butyrate levels that were lower than those for the wild type, while peak acetate levels were reduced compared to wild-type fermentations. Final acetate levels were equal (CGM), or reduced in comparison to wild-type fermentations.

The fact that inactivation of AK does not abolish acetate production has been previously observed in both *Clostridium tyrobutyricum*[246] and the degenerated *C. acetobutylicum* strain M5.[129] In the *C. tyrobutyricum ack*$^-$ strain, the fermentation time was extended and both acetate and butyrate levels surpassed those of the wild type. The M5 *ack*$^-$ strain (M5 AKKO) produced acetate at reduced levels but with similar butyrate formation. When a plasmid-based alcohol dehydrogenase (*aad*) is expressed in M5 AKKO, acetate production is again high (180 mM) while butyrate production levels are depended on the fermentation pH ranging from 75 mM for pH 5.5 to 287 mM for pH 6.0 fermentations.[129] Lee *et al.*[245] suggest that an alternative acetate-producing pathway is operational in the M5 AKKO mutant. This could also be applicable to our WUR strain and derived mutants.

Green *et al.*[126] reported on another acetate pathway mutant in *C. acetobutylicum* which the *pta* gene was inactivated. They reported reduced acetate levels and increased butyrate levels compared to the WT under conditions favouring acid formation (pH 5.5). Later data from Zhao *et al.*[165] from static flask batch cultures showed that acetate levels were reduced by approximately 50 %, while butyrate levels were unaffected under these conditions. Both culturing conditions are different from those employed in this study and complicate a direct comparison. While higher butyrate levels seem to correlate with increasing pH, the reduction in acetate is common to both. This would be in agreement with our lower butyrate levels at pH 5.0, but not with the acetate levels that we observed. Apparently an AK negative mutant is more likely to produce acetate than a mutant in which PTA is inactivated. A similar observation can be made for *C. tyrobutyricum pta*$^-$ and *ack*$^-$ mutants[246,247] and is likely due to the operon structure in both species in which *ack* is located downstream of *pta*.

Besides an alternative acetate forming pathway, another possible explanation for the continued acetate production by the *ack*$^-$ mutants could be that acetate production is catalysed by BK. BK from *C. acetobutylicum* is active on acetate with 6 % relative activity, in the acetyl phosphate-forming direction, compared to butyrate, based on *in vitro* data.[248] Although BK activity is reduced in CGM medium (Table 3.3), Desai and Papoutsakis[174] showed that even if BK activity is reduced by more the 80 % in an asRNA mutant, butyrate fluxes remain unaffected and butyrate accumulates in the growth medium. Apparently the butyrate production pathway has a far greater catalytic capacity, which could possibly be

used for acetate formation.

CM1 and CGM media differ mainly in the respect that the former contains more iron, magnesium and 39 mM acetate. The marked differences between fermentation performances by the wild-type and *C. acetobutylicum* AK strains grown on each medium are evident from the data presented in Table 3.4. In CM1 medium acetate production is strongly reduced (AK mutant 9 mM vs. WT 43 mM, both in addition to the starting concentration of 42 mM), while in CGM medium the peak acetate concentrations are quite similar (AK 26 mM, WT 30 mM). The possible effect that additional acetate in the medium has could be an indication that the intracellular metabolism involved in acetate production in the AK strain is sensitive to elevated levels of extracellular acetate. Future experiments using CM1 medium with varying, initial, concentrations of acetate can help to determine if the same maximum acetate concentration of 50 mM is reached for both lower and higher starting concentrations of acetate. This would support our current observation that acetate production is inhibited at a lower concentration (50 mM) in the AK strain than for the wild type, which accumulates more than 80 mM of acetate in CM1 medium under the same conditions. This is similar to strain ATCC 824 that accumulates up to 71 mM in CGM medium.[128]

In both media, however, an important difference that was observed between the mutant and the wild-type strain was the delay in the acetate production. In the wild-type strain fermentations, acetate and butyrate production occurs with approximately 1.5 (CGM) or 1.8 times (CM1) as much butyrate formed compared to acetate. Figure 3.6 clearly shows that the AK mutant produces considerably more butyrate [1.86 times (CGM) and 7.2 times (CM1)] than acetate in the beginning of the fermentation, in line with the expectation that acetate production would be negatively affected by an AK disruption. It appears that when the butyrate concentration has reached its maximum level, only then acetate production is allowed to reach its full potential if no extracellular acetate is present (CGM medium). If indeed BK is involved in acetate formation, then competition of acetyl-phosphate and butyryl-phosphate for the available enzyme could explain the delayed acetate production.

In CGM fermentations, lactate production was seen with both the wild type and the mutant. Interestingly enough, the AK mutant strain made considerably less lactate, but more acetoin. The increase in acetoin (9 mM), however, is not enough to account for the full reduction in lactate production (29 mM), but only for up to 62 %. Production of acetoin does not require oxidation of a reducing equivalent (NADH)[249] as is needed for lactate production. This additional reduction capacity could have possibly been used for solvent formation instead. Lactate production in CGM fermentations has been reported before (approximately 70 mM)[126] but only for mutants, not for the WT. It seems that in our hands, *C. acetobutylicum* WUR, contrary to the ATCC 824 strain, is more likely to produce lactate under these fermentation conditions in CGM, whereas it does not do so in CM1 medium. This is the second difference that we have

observed, next to *meso*-2,3-butanediol production.[216] A likely explanation for this behaviour could be that the WUR strain is sensitive to lower levels of iron and/or magnesium compared to the ATCC 824 strain. Both these metal ions are co-factors in various oxidoreductase enzymes. If the activity of these enzymes is compromised, then remodelling of the flow of reducing equivalents within the cell, ultimately leading to lactate formation, could be the result.

Inactivation of the acetate pathway in the AK mutant is expected to lead to accumulation of acetate-preceding intracellular intermediates, such as acetyl-CoA and acetyl-phosphate. These higher intracellular levels could then alter product formation of ethanol and metabolically more upstream intermediates such as lactate and acetoin.[250] The increased acetoin and ethanol levels in the AK mutant CGM fermentations follow the expected trend (Table 3.4). The reduced lactate production levels of the AK mutant, relative to those by the wild-type, are not in line with this expectation. The wild-type *C. acetobutylicum* WUR strain showed (high) lactate production levels on CGM in contrast to strain ATCC 824 which produced none under similar conditions.[128,174] This dissimilarity could be due to a different regulation of gene expression or different enzymatic properties of the iso-enzymes involved in lactate formation in both strains.

Out rationale for disrupting AK activity was that it is expected to result in intracellular accumulation of acetyl-phosphate. This accumulation could potentially be a molecular trigger[239] resulting in an observable phenotype, akin to butyryl-phosphate accumulation after *buk1* disruption.[128] In CGM, there was significant increase in acetoin, ethanol and butanol formation by the AK mutant; however, the fermentations of both the wild type and to a lesser extent the AK mutant were affected by lactate production. In CM1 medium, hardly any lactate was produced by both strains. The AK mutant produced at peak levels 34 mM (79 %) less acetate compared to the wild-type strain, but no changes in the other fermentation products were seen. This supports the earlier observations of Green *et al.*[126] and Zhao *et al.*[165] that acetate pathway disruption in *C. acetobutylicum* does not result in altered solvent production.

Despite the absence of an effect on acetone and butanol formation in fermentations in CM1 medium, AK inactivation did result in increased acetate uptake in that medium and increased butyrate uptake in CGM fermentations (Table 3.4). Acetate production was only impaired when acetate was present in the medium (CM1), but in both CGM and CM1, an altered initial acetate/butyrate product ratio was observed showing a reduced acetate production rate. The absence of an impact on solvent production by the *pta*$^-$ mutant of Green *et al.*[126] and our AK mutant in CM1 medium is in agreement with the prediction by Zhao *et al.*[165] that levels of acetyl phosphate are not linked with induction of solvent formation. Potential other roles of acetyl phosphate cannot be excluded based on these experiments. It is of further interest to note that the accumulation of a yellow compound in the fermentation broth is likely to be riboflavin[251,252] which can help to facilitate the economic competitiveness of the ABE fermentation process.[253]

We now propose the construction of an alcohologenic strain, which is unable

to produce either acetate or butyrate and subsequently will not produce acetone because there are no acids to be taken up from the medium. Ideally this strain would produce only the alcohols butanol and ethanol when grown on glucose. Construction of such a strain would require the combined knockout of both acetate kinase (*ack*) and butyrate kinase (*buk1*), so that it would no longer be able to perform the, proposed, *in vivo* complementation of acetate production by butyrate kinase. Such a mutant would also allow us to test whether the remaining acetate production in our AK mutant is due to complementation of the enzyme activity by butyrate kinase or if one or more alternative acetate-forming pathways operate in *C. acetobutylicum*. We are currently investigating this option.

3.5 Acknowledgements

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Chapter 4

Fermentation Analysis of a Novel *Clostridium acetobutylicum buk1*$^-$ Mutant and *C. acetobutylicum buk1*$^-$ *ack*$^-$ Double Mutant

4

Abstract

Clostridium acetobutylicum ATCC 824 with an inactivated butyrate kinase (*buk1*) gene is under optimised conditions one of the best butanol producing strains reported to date. By comparison a *buk1* knock-out mutant of the *C. acetobutylicum* WUR strain does not sporulate and no longer has a bi-phasic growth pattern. It produces mainly butanol and acetate combined with low levels of acetone, ethanol and butyrate. At a pH of 5.5, in medium without added acetate the mutant strain produces 195 mM butanol, 127 % more than the wild type under the same conditions and 21 % more than the wild type under its optimal conditions. In order to decrease acid formation an *ack*$^-$ *buk1*$^-$ double mutant was constructed by selectively disrupting acetate kinase. This mutant however continued to produce both acetate and butyrate during fermentation at various tested pH levels. In a non-pH controlled batch fermentation final acids levels were very low (9 mM), but still 55 mM acetone was produced combined with 131 mM butanol and 98 mM ethanol. At pH 6.0 alcohol production was lower (butanol 125 mM, ethanol 23 mM), no acetone was formed, but high levels of

acetate (120 mM) and butyrate (47 mM) were produced combined with acetoin (61 mM) and lactate (21 mM). These results suggest that there are alternative acid producing pathways active in these mutants and possibly in the wild type strain.

4.1 Introduction

The current energy demand of the human population of this earth is far from sustainable with now in use methods. The expected trend for the future is that the energy demand will only increase, mainly due to increasing material wealth of an ever growing population.[254] These trends, combined with increasing energy security demands, have resulted in a search for sustainable sources for production of transport fuels and chemicals. Fermentation of lignocellulosic materials into butanol could be such an alternative method for the current petrochemical production process.[20,45] Fermentative production of butanol is carried out by various Clostridial species as part of the acetone-butanol-ethanol (ABE) process.[57,115,194] To date, two of most important drawbacks that prevent commercial reintroduction of the ABE process are the low butanol yields, due to the formation of by-products and the toxicity of butanol itself to the cultures, and the high costs of the separation of the different products.[236]

Several strategies to increase the production of butanol and reduce by-product formation by metabolic engineering have been described.[204,237] One of the most successful approaches to increase butanol production levels has been the inactivation of the enzyme butyrate kinase (BK) of *Clostridium acetobutylicum* involved in the ATP generating conversion of butyryl-phosphate to butyrate.[128] Butyryl-phosphate itself is produced from butyryl-CoA through the action of phospho-transbutyrylase (*ptb*). Single crossover homologous recombination of a suicide plasmid into the *buk1* gene resulted in a decrease of 81 % of *in vitro* BK activity[126] and 42 % more butanol compared to wild type levels.[128] Shao *et al.* disrupted *buk1* using a TargeTron based system and also observed a similar relative increase in butanol production.[156] Besides an increase in butanol levels also ethanol and, acid phase associated, acetate production is increased in *buk1*$^-$ mutant fermentations. Conversely, butyrate peak and final levels are reduced by 76 % and 61 %, respectively.

Recently Lehmann and co-workers reported on acid pathway mutants with an inactivated phospho-transacetylase *pta*[179] or *ptb* gene.[166] The *pta* knock-out mutant was unaffected in its fermentation profile under the conditions tested, contrary to the results of Green *et al.*[126] who reported reduced acetate and increased peak butyrate levels. When the acetone production pathway was also inactivated in a *pta*$^-$ background a reduction of acetate and an increase in butyrate levels were observed combined with overall severely reduced solvent levels.[179] The *ptb*$^-$ mutant no longer produced any butyrate, ethanol was increased 17-fold, but acetone and butanol levels were reduced.

In the related degenerated strain *C. acetobutylicum* M5, lacking the pSOL1 megaplasmid and therefore devoid of solvent production,[172] Sillers *et al.* created a butyrate kinase knock-out strain (M5 BKKO) and acetate kinase knock-out strain (M5 AKKO).[129] Both M5 knock-out strains had reduced, but not eliminated, acid accumulation corresponding to the inactivated pathway, and grew more slowly. Attempts to restore solvent formation by these acid production mutants using plasmid based expression of an alcohol dehydrogenase, *adhE*, was unsuccessful for the M5 BKKO strain. M5 AKKO could be transformed but it still produced large amounts of acetate and butyrate with reduced alcohol production compared to the wild type and M5 *adhE* expressing strain.[173] Sillers *et al.* attributed their inability to transform M5 BKKO with the *adhE* expression plasmid to the organism's low growth rate and the inflexibility of the M5 strain to redistribute reducing equivalents over the various metabolic end products.[129]

The wild type is expected to be endowed with this flexibility as demonstrated by the alcoholic cultures that have been reported which produce mainly butanol and ethanol.[255–259] Although this is a desirable fermentation characteristic it requires either reduced co-substrates such as glycerol or the use of redox dyes such as methyl viologen. We wanted to achieve an alcohologenic fermentation through metabolic engineering of *C. acetobutylicum* by inhibiting production of acetate and butyrate and in this way, remove acetone production. By inactivating acetate kinase (AK) in *C. acetobutylicum* we endeavoured to reduce acetate production and increase solvent formation.[217] Acetate production by this *C. acetobutylicum* AK mutant was not stopped, but only delayed, and overall butanol formation was not significantly improved.

In various publications it has been suggested that the corresponding enzyme of the other acid pathway compensates the inactivated one.[126,179] To test the hypothesis that the BK enzyme complemented AK enzyme activity in the *ack*$^-$ mutant we aimed to construct an *ack*$^-$ *buk1*$^-$ double mutant. As a first step we wanted to confirm that the *buk1* gene in our *C. acetobutylicum* WUR strain could be disrupted as reported for the ATCC 824 strain.[126,156] Others have reported that attempts to disrupt the *buk1* gene in all cases also resulted in inactivation of the upstream gene *ptb*, encoding phospho-transbutyrylase.[260] Here we demonstrate that *buk1* disruption in strain *C. acetobutylicum* WUR is possible, while retaining PTB activity, but results in a different phenotype than previously described for the ATCC 824 *buk1*$^-$ strain (PJC4BK). The resulting strain was asporogenic and produced mainly acetate and butanol without the characteristic solvent switch. Subsequently an *ack*$^-$ *buk1*$^-$ double mutant was generated resulting in wild type-like behaviour, including an initial acid phase, but with reduced final butyrate levels and strongly increased acetoin accumulation.

4.2 Materials and Methods

4.2.1 Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are listed in Table 4.1. Stock cultures of *C. acetobutylicum* were maintained as spore suspensions in sterile 15 % (v/v) glycerol at −20 °C. The non-sporulating strains BUK1KO and DAPKO were stored by adding glycerol (87 % (v/v) to an in mCGM grown overnight culture) to a final concentration of 15 % (v/v). The culture was then transferred, inside the anaerobic cabinet, to a thick-walled glass tube with a screw cap [supplied with Hach Lange (NL) test kits]. A butyl-rubber stopper [hight: 8 mm, Rubber B.V. (NL)] was inserted in the opening before screwing the cap on, to prevent oxygen entering the tube. Tubes were then stored at −80 °C. Alternatively 5 mL of grown liquid cultures were added to nitrogen flushed serum flasks(10 mL, capped with a thick butylrubber stopper and a crimp cap seal) containing glycerol, also these were stored at −80 °C. Chemical competent *E. coli* TOP 10 cells (Invitrogen) were used for vector maintenance and cloning.

4.2.2 Media and growth conditions

E. coli strains were grown in lysogeny broth (LB) medium[223,224] at 37 °C, 200 rpm supplemented with the appropriate antibiotics. Clostridial spore suspensions were prepared as previously described[216] and heat-shocked for 10 min at 70 °C, prior to using them as an inoculum for precultures. Non-sporulating strains were revived from storage at −80 °C by overnight growth in mCGM.[216] Liquid cultures of *C. acetobutylicum* strains were grown in CM1 medium[217] either with or without acetate as indicated, and 100 g/L glucose. Media were made anaerobic by flushing with nitrogen gas for 10 to 30 min depending on the volume of the liquid. All clostridial culture experiments were performed at 37 °C, without shaking, and anaerobically in (a) an anaerobic chamber (Sheldon Manufacturing, Oregon, USA; gas mixture consisting of 15 % CO_2, 4 % H_2 and 81 % N_2) or (b) in glass serum vials as described previously.[225]

Culture media were supplemented with ampicillin (100 µg/mL), chloramphenicol (12.5 µg/mL), thiamphenicol (12.5 µg/mL), erythromycin (40 µg/mL standard; 5–10 µg/mL for initial mutant isolation), or kanamycin (50 µg/mL) when appropriate. Biomass was determined spectrophotometrically (Pharmacia Biotech Ultrospec 2000) as previously reported.[217]

4.2.3 DNA isolation, manipulation and transformation

Standard molecular work was done according to established protocols.[224] DNA from *C. acetobutylicum* was isolated using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) using the Gram-positive isolation procedure which incorporates a lysozyme (from chicken egg white; Fluka) treatment. *E. coli* plasmid

Table 4.1: Strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Remarks	Source
Strains			
E. coli TOP10		Cloning strain	Invitrogen
E. coli TOP10 (pAN2)		Methylation strain	Laboratory stock
C. acetobutylicum WUR		Originally obtained as ATCC 824 but shown to deviate from type strain behaviour[216]	Laboratory stock
C. acetobutylicum WUR BUK1KO	*buk1*$^-$	Butyrate kinase knockout mutant	This study
C. acetobutylicum WUR DAPKO	*buk1*$^-$ *ack*$^-$	Butyrate kinase and acetate kinase double knockout mutant	This study
Plasmids			
pCR-BluntII	pUC ori; Kan^R	TOPO cloning vector for blunt-ended PCR products	Invitrogen
pCR-BluntII::*catP*	pUC ori; Kan^R; Cam^R	Cloning vector carrying the functional *catP* gene	Invitrogen
pAN2	p15A ori; Tc^R ϕ*3tI*	Methylation plasmid derived from pAN1 but with tetracycline resistance selection	Laboratory stock[152]
pMTL500E	ColE1 ori; pAMβ1 ori; MLS^R, Amp^R; *lacZ′*	*E. coli–C. acetobutylicum* shuttle vector	Laboratory stock[142]
pMTL500E/C	ColE1 ori; pAMβ1 ori; MLS^R, Cam^R, Amp^R	pMTL500E derived shuttle vector with *catP* inserted in the MCS	This study
pMTL500C	ColE1 ori; pAMβ1 ori; Cam^R	pMTL500E derived shuttle vector Amp^S, MLS^S, and Cam^R	This study
pMTL007::Cac-*buk1*-49s	ColE1 ori, pCB102 ori; Cam^R LtrA intron II	ClosTron vector targeted to insert in *buk1* between bp 49 and 50 in the sense orientation	This study
pWOK001::Cac-*ack*-1027a	ColE1 ori; pAMβ1 ori; MLS^R Amp^R; P_{thl}-intronII-ΔRAM-*ltrA*-T1T2 terminator	Source of the TargeTron cassette cloned into pMTL500C	Laboratory stock[261]
pWOK003::Cac-*ack*-1027a	ColE1 ori; pAMβ1 ori; Cam^R; P_{thl}-intronII-ΔRAM-*ltrA*-T1T2 terminator	Markerless TargeTron mutagenesis system for insertion into *ack* between bp 1027 and 1028 in the antisense orientation	This study

[a] pUC ori, *E. coli* origin of replication; Kan^R, kanamycin resistance; Cam^R, chloramphenicol resistance; p15A ori, *E. coli* origin of replication; Tc^R, tetracycline resistance; ϕ*3tI*, *Bacillus subtilis* phage gene encoding the ϕ3T I methyltransferase; ColE1 ori, *E. coli* origin of replication; pAMβ1 ori, clostridial origin of replication; MLS^R, confers resistance to erythromycin; Amp^R, ampicillin resistance; pCB102 ori, clostridial origin of replication; LtrA, the intron-encoded protein of the *Lactococcus lactis* Ll.LtrB group II intron; P_{thl}, thiolase promoter from *C. acetobutylicum*; intronII-ΔRAM, TargeTron system group II intron with removed RAM marker.

DNA was isolated by the GenElute Plasmid Miniprep Kit (Sigma-Aldrich). Both kits were used according to the manufacturers' instructions. Restriction enzymes were from Fermentas or NEB, T4 DNA polymerase and T4 DNA ligase were from NEB. DNA amplification by PCR on isolated *C. acetobutylicum* DNA was done using Pwo polymerase (Roche Diagnostics). Colony PCRs (cPCRs) on *E. coli* were carried out using REDTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich) and cPCRs on *C. acetobutylicum* were done using 2× DreamTaq Green PCR Master Mix (Fermentas, now Thermo Fischer Scientific).

cPCRs were carried out by using the tip of a hypodermic needle to take an absolute minimum amount of colony material and streaking this on the inside of the PCR-tube containing 5 µL water. After that the needle was used to inoculate a new plate by stabbing it into the agar. After removing the PCR-tubes from the anaerobic chamber, 15 µL reaction mix was added, containing 5 µL water and primers, and 10 µL 2× DreamTaq master mix. The cells were lysed before regular cycling, by heating at 94 °C for 10 minutes. It was essential that the PCR always resulted in a product to detect reactions that failed due to too little, or too much colony material.

Prior to transformation into *C. acetobutylicum*, plasmids were methylated *in vivo*[147] by electroporation into *E. coli* TOP 10 (pAN2) cells. Electrotransformation of *C. acetobutylicum* was carried out as previously described.[142] Erythromycin was added to all culture media for the preparation of competent BUK1KO cells. All manipulations were carried out anaerobically and on ice. After transformation and recovery, the cells were plated on pre-warmed plates containing thiamphenicol, no erythromcyin was added.

4.2.4 Primers and DNA sequencing

All DNA primers used in the study are listed in Table 4.2. Primers were obtained from Eurogentec (Seraing, Belgium). DNA sequencing of clones was done by BaseClear (Leiden, The Netherlands).

4.2.5 Construction of ClosTron plasmid pMTL007::Cac-*buk1*-49s

Plasmids were constructed according to the protocol by Heap *et al.*[152] In short, the retargeted cassette, generated in a SOEing PCR using the following primers, BK49a_ibs, BK49a_ebs1, BK49a_ebs2, and EBS_Univ, and the template from the TargeTron kit, was ligated into the pMTL007 backbone, resulting in plasmid pMTL007::Cac-*buk1*-49s. Correct plasmids were identified by restriction digestion and by sequencing the retargeted region using primers 5402F_fwd and pMTL007_rev.

Table 4.2: Primer sequences

Primer name	Sequence ($5' \rightarrow 3'$)[a]
BK49a_ibs[b]	aaaaaagctt ataattatcc ttaCAACTcA AATTGgtgcg cccagatagg gtg
BK49a_ebs1[b]	cagattgtac aaatgtggtg ataacagata agtcAAATTG GTtaacttac ctttctttgt
BK49a_ebs2[b]	tgaacgcaag tttctaattt cgGttAGTTG Tcgatagagg aaagtgtct
EBS_Univ	cgaaattaga aacttgcgtt cagtaaac
5402F_fwd	ttaaggaggt gtatttcata tgaccatgat tacg
pMTL007_rev	agggtatccc cagttagtgt taagtcttgg
CatP_fwd	AACTTAAGct aaagaggtcc ctagcgcc
CatP_rev	AACTTAAGct ctgaaaatat aaaaaccaca gattgatac
Cac_buk1_fwd	gtatagatta ctaataatca atcctggc
Cac_buk1_rev	gtattcctta gctttttctt ctcctc
Cac_ack_fwd	atgaaaaact tagttattaa ctgcg
Cac_ack_rev	ttattttaac ttgcctacta tatcttt
CA_Pthl_Fw	Ggcatgcgaa tttagaatga agtttcttat gc
TT_Probe_r	cgcgtcgcca cgtaataaat atctgg

[a] Nucleotides that are not complementary to the template sequence are given in uppercase. *Hin*dIII and *Bsr*GI restriction sites incorporated in the primers are underlined.
[b] Primer sequences for this insertion site were based on Shao *et al.*[156]

4.2.6 Construction of plasmid pWOK003::Cac-*ack*-1027a

As the previous constructed plasmid pWOK001::Cac-*ack*-1027a,[261] employed erythromycin resistance for plasmid maintenance. The plasmid needed to be changed to use another resistance marker. The *catP* gene was isolated by PCR with primers CatP_fwd and CatP_rev using a pMLT007 vector as template and TOPO cloned into pCR-BluntII (Invitrogen). After verification of function by growth on chloramphenicol containing LB plates the *catP* gene was released from the pCR-BluntII::*catP* vector using *Kpn*I and *Xba*I and inserted in the pMTL500E vector which was linearised with the same enzymes creating plasmid pMTL500E/C. To inactivate and remove as much as is possible of the erythromycin resistance gene pMTL500E/C was subsequently cut with *Eam*1105I and *Xcm*I, with a gel purification step in between. The 5.86 kbp fragment was then blunted using T4 DNA polymerase, gel purified, and self-ligated with T4 DNA ligase. Upon transformation in *E. coli* the plasmid, pMLT500C, was maintained using chloramphenicol as also a large part of the ampicillin resistance gene was deleted. Plasmid pWOK001::Cac-*ack*-1027a was then cut with *Xho*I and *Sph*I to release the TargeTron system which was then gel purified and ligated into pMTL500C which was linearised with the same enzymes resulting in a plasmid designated as pWOK003::Cac-*ack*-1027a (see Table 4.1).

4.2.7 Isolation of mutants

4.2.7.1 BUK1KO strain

After transformation of pMTL007::Cac-*buk1*-49s, colonies resistant to thiamphenicol were picked, and plated on mCGM plates containing erythromycin. After incubation for several days at 37 °C colonies were visible on plate. After inoculation of a liquid culture genomic DNA was prepared and PCR amplification of *buk1* gene using primers Cac_buk1_fwd and Cac_buk1_rev was used to confirmed successful integration.

4.2.7.2 DAPKO strain

The double mutant was made by transforming vector pMTL007::Cac-*buk1*-49s into competent BUK1KO cells. After transformant isolation a random colony was picked and re-streaked on a plate containing thiamphenicol. After growth, colonies were suspended in mCGM and a serial dilution was made. Various dilutions were plated on mCGM plates containing thiamphenicol to obtain discrete colonies. Approximately 200 individual colonies were screened by colony PCR using primers Cac_ack_fwd and Cac_ack_rev and re-streaked on library plates. Of these, three PCR reactions gave a positive signal for a pure mutant. However, only one of these could be recovered from the library plate, the other two had not

grown. This was presumably due to the extremely small amounts taken from initial colonies that were used for cPCR sampling and subsequently used for library plate streaking.

4.2.8 Southern blot

Genomic DNA of the BUK1KO mutant was checked using the same procedure as described previously.[217] Irrespective of restriction enzyme used, no DNA fragment was released from the wild-type genomic DNA that hybridised to the probe. In contrast, restriction fragments of the expected size were derived from both the genome of the BUK1KO mutant and the ClosTron plasmid which gave a positive signal on the Southern blot.

4.2.9 Cell-free extracts and enzymatic activity assays

Cell-free extracts preparation, enzymatic activity assays (PTA, AK, PTB, BUK), and protein assays were performed as described in Kuit *et al.*[217]

4.2.10 Batch fermentations of *C. acetobutylicum*

Fermentations were performed in 2-L bioreactors (1-L working volume) controlled via a Bio Controller ADI 1010 by a PC running Bioexpert software (all Applikon, The Netherlands). No antibiotics were used during the fermentations. CM1 medium-based batch fermentations were setup by autoclaving yeast extract, phosphates, and ammonium acetate or ammonium sulphate as applicable, with the reactor. Autoclaved glucose (final concentration 100 g/L unless otherwise noted) was added to the reactor after cooling. Metal sulphates and *p*ABA were added as a filter sterilised mix.

The medium in the reactor was sparged for at least 6 hours with nitrogen. Prior to inoculation, Sigma antifoam 204 was added to the reactor (0.1 ‰). The reactor was inoculated with 50 mL of an overnight pre-culture grown on the same medium but supplemented with erythromycin in the case of the mutants. After inoculation, the starting pH was allowed to fall to the pre-set pH, after which it was controlled by addition of 4 M KOH. During the fermentation, the head space was flushed with nitrogen and the outgoing gas flow was passed through a condenser at 4 °C. The agitation rate was 200 rpm.

4.2.11 Analysis of metabolites

Metabolites present in culture supernatants (glucose, acetate, butyrate, lactate, acetoin, *meso*-2,3-butanediol, acetone, butanol, and ethanol) were determined by HPLC as described previously[216] using valeric acid or 4-methyl-valeric acid as an internal standard.

4.3 Results

4.3.1 Isolation and characterisation of a *buk1*$^-$ strain

A *buk1* gene disruption was previously shown to impart a favourable phenotype regarding butanol production. We therefore wanted to recreate this strain using the ClosTron method. The *C. acetobutylicum* mutant strain BUK1KO (Table 4.1) was obtained as described in the Materials and methods section. The mutant has a group II intron inserted in the sense orientation between basepair positions 49 and 50 of the wild type gene sequence. The BUK1KO strain did not produce any spores, both on plate and in liquid culture, as determined by microscopic inspection and heat shock treatment (data not shown). To confirm gene inactivation, enzyme activity assays of all four enzymes involved in acetate and butyrate formation from acetyl-CoA and butyryl-CoA, respectively, were carried out using cell-free extracts of exponentially growing cultures of the wild type and mutant strains (Table 4.3, page 85). BK activity was essentially at background levels while also PTB, present upstream in the same operon, was somewhat reduced by approximately 30 %. Enzyme activities of the acetate pathway were not significantly affected in the BUK1KO strain under the condition tested. To ascertain that only one insertion had taken place a Southern blot was prepared using a probe that hybridizes to the intron II DNA sequence.[217] Only one band, of the expected size, was observed in all three digests of genomic DNA isolated from the BUK1KO strain (data not shown).

4.3.2 Fermentation characteristics of strain BUK1KO

The fermentation characteristics of the BUK1KO strain were initially determined for a pH controlled batch fermentation using CM1 medium in which the initial pH of 6.6 is allowed to drop to 5.0 and then stabilised by the addition of KOH. CM1 medium was selected based on our previous experiences with the *C. acetobutylicum* WUR strain[217] and pH 5.0 was chosen as it is reported to be essential for good butanol production in a similar mutant.[128]

The course of fermentations of the wild type strain showed the typical biphasic pattern of first acid accumulation followed by a switch to solvent production (Figure 4.1A and B, page 87). Both butanol and acetone were detected at the same time point, approximately three hours before butyrate levels peaked, with an average of 62 mM. Peak acetate levels were reached five hours after peak butyrate levels. Levels of both acids then decreased due to their uptake from the medium with the concomitant production of acetone. Besides acetone and butanol also ethanol was produced resulting in a final acetone-butanol-ethanol (A:B:E) ratio of 3:5:1 after 36 hours. In addition also low levels of acetoin (12 mM), lactic acid (3 mM), and *meso*-2,3-butanediol (3 mM) were produced.

Fermentations by the BUK1KO strain under the same conditions gave a very different result as shown in Figure 4.1C and D, and Table 4.4(page 90). Butyrate

Table 4.3: Specific enzyme activities involved in production of acetate and butyrate in wild type and BUK1KO and DAPKO mutant strain homogenates grown on CM1 medium

Strain[a]	Specific activities [U mg^{-1}][b]			
	Acetate pathway		Butyrate pathway	
	PTA	AK	PTB	BK
Wild type	0.79 ± 0.06	3.84 ± 0.45	3.157 ± 0.062	1.72 ± 0.16
BUK1KO	0.76 ± 0.18 (96 %)	4.109 ± 0.065 (107 %)	2.23 ± 0.19 (71 %)	0.001 ± 0.001 (0.1 %)
DAPKO	0.42 ± 0.05 (53 %)	0.055 ± 0.001 (1.4 %)	1.3 ± 0.10 (41 %)	0.003 ± 0.0002 (0.2 %)

[a] Cells were harvested when OD_{600} was approximately 2.5.
[b] Data are reported as the mean ± SEM ($n = 2$). In the case of the mutants, the relative activity compared to that of the wild type is given in parentheses.

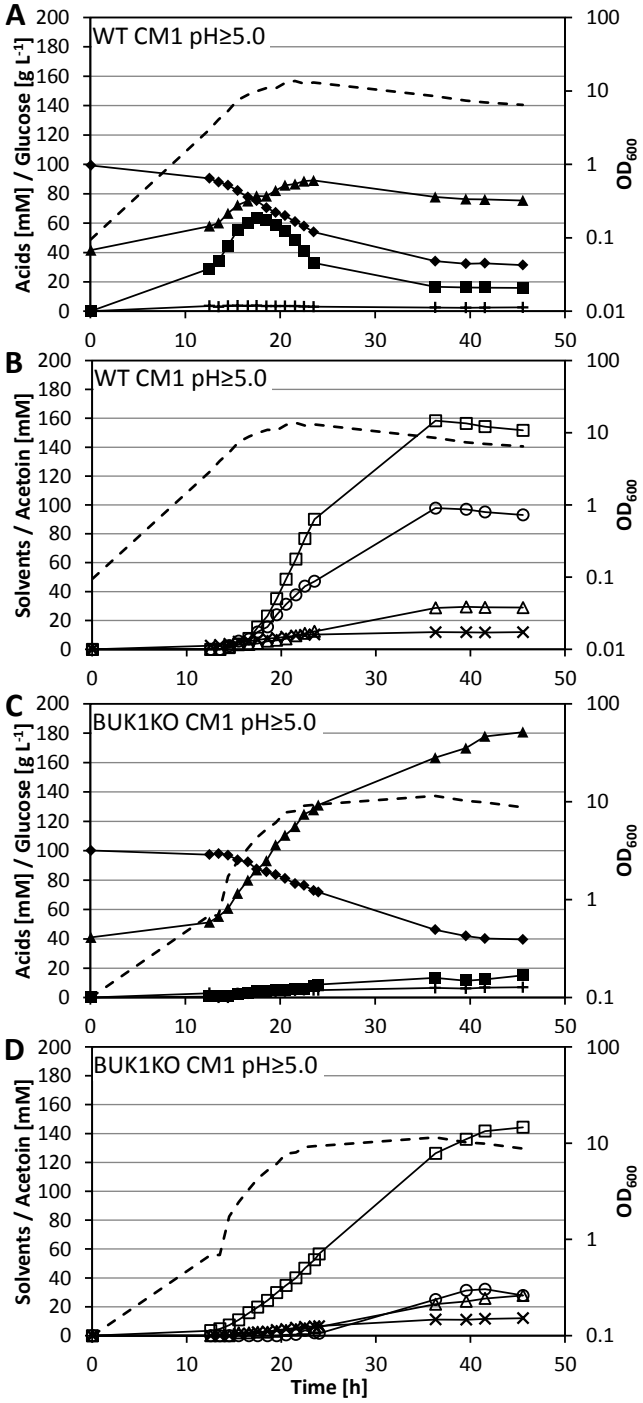
accumulates in the medium only up to 13 mM and acetone is detected for the first time after more than 23 hours. Contrary to the wild type fermentations, acetate levels increased over the entire course of the fermentation, reaching a maximum of 180 mM from a starting level of 40 mM. Butanol production commenced before butyrate was detected in the medium and continued throughout the fermentation, levelling off, after 46 hours, at approximately 140 mM, a reduction of 15 % compared to the wild type.

While the pH of the medium of wild type fermentations increased again after a period at pH 5.0, those of the BUK1KO strain remained at pH 5.0 in line with the continued acetate production under pH controlled conditions. Acetone levels in fermentations by the BUK1KO strain fermentations reached 24 mM, which was only 24 % of the wild type acetone levels. Ethanol, acetoin and lactate levels were similar to those in wild type fermentations (Table 4.4).

Butanol toxicity is generally regarded as the main cause for termination of the fermentation by *C. acetobutylicum*.[50] The butanol levels produced by the BUK1KO strain at pH 5.0 were lower than for those for the wild type strain suggesting that a different factor might have halted the fermentation progress before glucose was depleted. For any concentration of short chain fatty acids, *e.g.* acetic acid and butyric acid, the fraction of the acids that exists as undissociated fatty acids (UFAs) will increase when the pH is lowered, whereas increasing the pH will decrease the levels. UFAs can traverse the membrane and enter the cell where the higher intracellular pH allows them to dissociate again, effectively transporting protons across the membrane and thereby counteracting the transmembrane pH gradient.[229,262] In addition, it increases the level of acids inside the cell, compromising cellular functions.[263] A higher external pH is therefore expected to allow for increased acetate accumulation in the medium. We hypothesised that acetate might have accumulated at inhibiting levels in the mutant strain fermentations. To investigate how growth and product formation of the BUK1KO mutant was influenced by the external pH several fermentations under different pH-conditions were carried out. The results are shown in Table 4.4, and Figure 4.2A and B.

In fermentations in which the pH was allowed to drop below the initially tested pH setting of 5.0, growth and product formation was negatively affected. In the pH uncontrolled fermentation the lowest value reached was 4.35, and production of acetate and butanol was reduced with 58 % and 71 % respectively, compared to the fermentations at pH 5.0. Fermentations controlled at pH 4.5 gave similar

Figure 4.1 ***(following page)*:** Typical optical density and production profile of a wild-type (**A** and **B**) and a BUK1KO strain (**C** and **D**) fermentation in pH 5.0 controlled batch reactors using CM1 medium containing 100 g/L glucose. **A**, **C** Production of acids −▲− acetate, −■− butyrate, −✚− lactate, −♦− glucose, *dashed line* OD_{600}. **B**, **D** Production of solvents −△− ethanol, −□− butanol, −○− acetone, − × − acetoin, *dashed line* OD_{600}.


A
WT CM1 pH≥5.0
Acids [mM] / Glucose [g L-1]
OD600
B
WT CM1 pH≥5.0
Solvents / Acetoin [mM]
OD600
C
BUK1KO CM1 pH≥5.0
Acids [mM] / Glucose [g L-1]
OD600
D
BUK1KO CM1 pH≥5.0
Solvents / Acetoin [mM]
OD600
Time [h]

results. In fermentations with an increased pH limit, 5.5 and 6.5, acetate production did not increase, but rather decreased, compared to the fermentations at pH 5.0, with 18 % and 65 %, respectively. However in the case of fermentations by the BUK1KO strain at pH 5.5 compared to those at pH 5.0, butanol levels increased significantly from 137 mM to 169 mM and glucose consumption increased from 314 mM to 405 mM.

Although butanol levels of BUK1KO strain fermentations at pH 5.5 had increased significantly compared to fermentations at pH 5.0 in CM1 medium, these were not higher than wild type strain fermentations at pH 5.0. To ascertain if acetate levels, despite the lower concentration at a higher pH, were still influencing butanol production levels, fermentations in CM1 medium without added acetate were conducted (Table 4.4, and Figure 4.2C and D). This resulted in another increase in butanol production levels for the BUK1KO strain to 195 mM (14.5 g/L), 21 % above the wild type levels under optimal conditions (161 mM, 12.0 g/L) and 127 % above wild type levels under the same conditions (CM1 medium without acetate at pH 5.5, Table 4.4). Unfortunately the increase in butanol and ethanol production of the BUK1KO fermentations at pH 5.5 in CM1 without acetate, could not offset the reduced acetone levels resulting in a lower ABE per glucose (g/g) productivity of 23 % compared to 30 % for the wild type (Table 4.4).

Fermentations by the BUK1KO strain showed enhanced butanol selectivity (butanol per total solvents on a mole basis) in the range of 68 %–79 %, compared to the wild type levels of 55 %, due to severely reduced acetone formation under all tested pH levels. However, the amount of butanol produced per glucose consumed was in all cases lower than the 46 % recorded for the wild type. The most predominant product, next to butanol (195 mM at pH 5.5 in CM1 without acetate) of the fermentation by BUK1KO strain is acetate (143 mM). We therefore elected acetate kinase (AK) for the next targeted gene disruption.

4.3.3 Acetate kinase inactivation in BUK1KO strain

The BUK1KO strain was made using the first generation pMTL007 ClosTron,[152] therefore the erythromycin resistance gene could not be removed as it lacks the *frp* sites used in the second generation ClosTron system.[154] Using the pMTL007 vector again for disruption of the *ack* gene (encoding acetate kinase, AK) would not give a selection advantage over the parental BUK1KO strain

Figure 4.2 ***(following page)*****:** Typical optical density and production profile of a BUK1KO strain fermentation in pH 5.5 controlled batch reactors using CM1 medium (**A** and **B**) or CM1 medium without acetate (**C** and **D**), both contained 100 g/L glucose. **A**, **C** Production of acids −▲− acetate, −■− butyrate, −✚− lactate, −♦− glucose, *dashed line* OD_{600}. **B**, **D** Production of solvents −△− ethanol, −□− butanol, −○− acetone, − × − acetoin, *dashed line* OD_{600}.

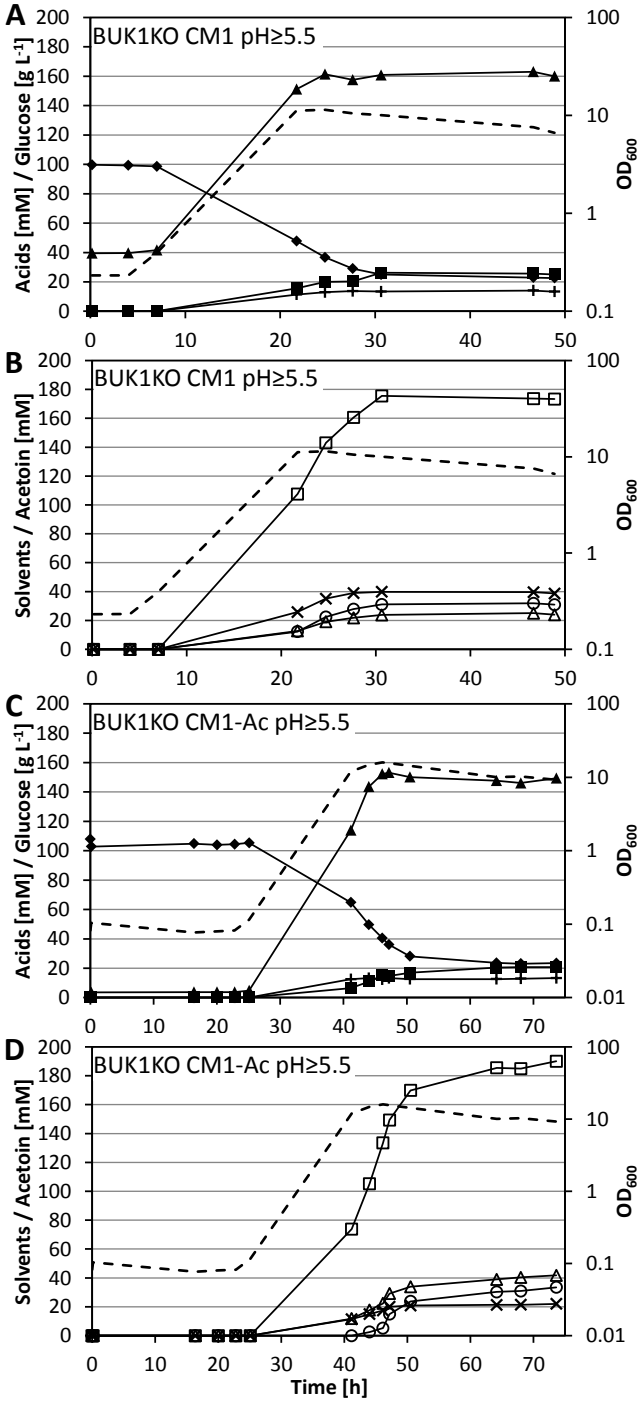

A
BUK1KO CM1 pH≥5.5
Acids [mM] / Glucose [g L-1]
OD600
B
BUK1KO CM1 pH≥5.5
Solvents / Acetoin [mM]
OD600
C
BUK1KO CM1-Ac pH≥5.5
Acids [mM] / Glucose [g L-1]
OD600
D
BUK1KO CM1-Ac pH≥5.5
Solvents / Acetoin [mM]
OD600
Time [h]

Table 4.4: Fermentation performance of the *C. acetobutylicum* wild type and BUK1KO mutant strain in CM1-based media in 1-L bioreactors at different pH conditions, as indicated[a]

Medium		CM1						CM1 without acetate	
Strain		WT[b]	BUK1KO					WT	BUK1KO
pH		5.0	Uncontrolled (4.35)	4.5	5.0	5.5	6.5	5.5	5.5
Fermentations (n)		2	1	2	2	3	1	2	2
Consumed glucose		351 ± 3	120	169 ± 34	315 ± 21	405 ± 12	371	420 ± 15	452 ± 12
Acetic acid	Final	72 ± 6	101	105 ± 5	180 ± 0.4	154 ± 11	89	92 ± 1	143 ± 7
	Relative to start	30 ± 6	59	64 ± 5	139 ± 0.3	114 ± 12	49	—[d]	—[d]
Butyric acid		16 ± 0.3	13	9 ± 4	13 ± 2	20 ± 3	14	125 ± 7	20 ± 1
Lactic acid		3 ± 0.04	3	13 ± 4	8 ± 1	11 ± 3	59	6 ± 0.3	11 ± 3
Acetoin		12 ± 0.3	9	8 ± 2.1	12 ± 0.03	30 ± 6	49	25 ± 1	21 ± 1
meso-2,3-butanediol		2.5 ± 0.2	0	0.2 ± 0.2	1.0 ± 0.002	2.1 ± 0.5	1.1	2.6 ± 0.1	1.7 ± 0.2
Acetone		101 ± 3	7	19 ± 7	24 ± 4	40 ± 6	0	36 ± 2	40 ± 6
Butanol		161 ± 2	40	65 ± 21	137 ± 7	169 ± 6	119	86 ± 3	195 ± 5
Ethanol		30 ± 1	4	13 ± 8	27 ± 0.6	34 ± 6	32	19 ± 0.2	44 ± 3
OD_{600}		14 ± 0.02	5.64	7 ± 2	11 ± 0.5	15 ± 2	8.04	5 ± 0.2	16 ± 0.3
Biomass [g/L]		4.6 ± 0.01	1.7	2.1 ± 0.5	3.7 ± 0.2	4.9 ± 1	2.6	1.6 ± 0.03	5.4 ± 0.1
Carbon recovery		101 ± 2 %	95 %	94 ± 1 %	95 ± 2 %	91 ± 1 %	73 %	82 ± 2 %	91 ± 1 %
Selectivity[c]									
B/(A+B+E) [mol/mol]		55 %	78 %	68 %	73 %	70 %	79 %	61 %	70 %
Productivity[c]									
ABE/glucose [g/g]		30 %	16 %	21 %	23 %	23 %	15 %	12 %	23 %
B/glucose [mol/mol]		46 %	33 %	38 %	44 %	42 %	32 %	21 %	43 %
B/(Ac+Bu) [mol/mol]		3.5	0.56	0.89	0.90	1.3	1.9	0.40	1.2

[a] Data is given as mean ± SEM and are concentrations in mM unless otherwise indicated.
[b] Data taken from Kuit *et al.*[217]
[c] Abbreviations under the Selectivity and Productivity headings are as follows: A = acetone, B = butanol, E = ethanol, Ac = acetate relative to the starting concentration, and Bu = butyrate.
[d] See row above, as no acetate is present in the medium at $t = 0$, nett acetic acid production equals the final acetic acid concentration.

and possibly be a disadvantage due the the large size of the group II intron, which is known to make the process less efficient.[222] We had previously constructed a TargeTron based system similar to the pMTL007 plasmid but without a retrotransposition activated marker (RAM). Mutant selection is dependent on phenotypical selection or screening, for example by colony PCR, to identify insertants.[261]

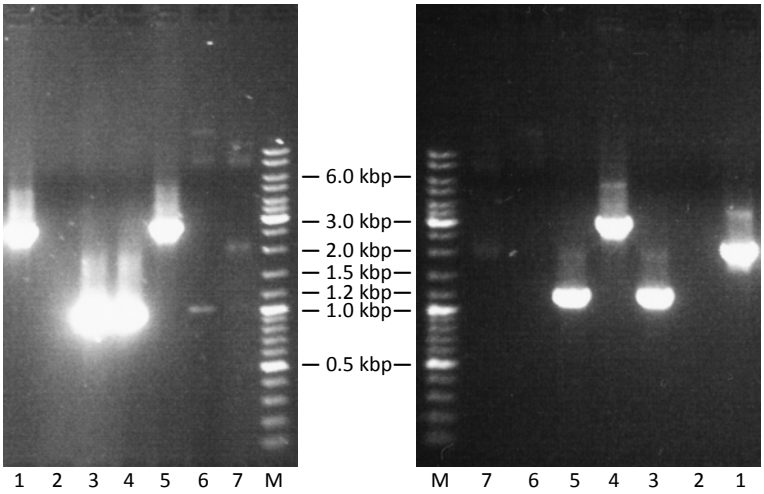


Figure 4.3: PCR products after separation on an 1.0 % agarose gel using primers amplifying *buk1* (left side) using primers Cac_buk1_fwd & Cac_buk1_rev, and *ack* (right side) using primers Cac_ack_fwd & Cac_ack_rev. Templates for PCRs in lanes for both the left and right side: **M**, Marker; **1**, gDNA DAPKO strain; **2**, Water; **3**, gDNA WUR strain (wild type); **4**, gDNA WUR *ack*$^{-}$ strain[217]; **5**, gDNA BUK1KO strain; **6**, plasmid pMTL007::Cac-*buk1*-49s; **7**, plasmid pWOK003::Cac-*ack*-1027a. Expected product sizes are: wild type *buk1* (lanes 3, 4), 1.1 kbp bp; wild type *ack* (lanes 3, 5), 1.2 kbp; inactivated *buk1* (lanes 1, 5), 2.9 kbp; inactivated *ack*, 2.1 kbp (lane 1) and 3.0 kbp (lane 4). The size of the inactivated *ack* in the DAPKO strain is smaller as it does not contain an erythromycin resistance gene.

Plasmid pWOK003::Cac-*ack*-1027a was constructed as detailed in Materials and Methods and after methylation used to transform BUK1KO competent cells. Transformants and subsequent isolation of the double mutant is described in the Materials and Methods. The identified putative double knock-out strain designated *C. acetobutylicum* DAPKO was cultured in mCGM and five times transferred to fresh medium and then plated on solid media to check for loss of the pWOK003::Cac-ack-1027a plasmid which was verified by sensitivity to chloramphenicol and PCR amplification of two different plasmid regions, the *catP* gene (primers CatP_fwd and CatP_rev) and part of the intron (primers CA_Pthl_Fw

and TT_Probe_r). Further PCR reactions confirmed both a mutant sized *buk1* and *ack* gene (Figure 4.3). Sequencing of the *pta-ack* and *ptb-buk1* operons and their surrounding regions confirmed that only the intended regions were mutated (data not shown). The mutant strain, like the BUK1KO parental strain, did not sporulate and grew slower than the wild type. The DPAKO mutant was assayed for enzyme activities involved in acid production and showed no significant acetate kinase or butyrate kinase activity, where as phospho-transacetylase and phospho-transbutyrylase activities were halved compared to the wild type activities (Table 4.3).

4.3.4 Fermentation characteristics of strain DAPKO

The DAPKO strain was analysed for its fermentative properties in CM1, with and without acetate, in a pH controlled batch setup. Fermentation profiles can be found in Figure 4.4. Fermentation data for the different pH and media compositions are listed in Table 4.5 (page 95). All fermentations in CM1 showed a large reduction in final, and produced acetate levels compared to the BUK1KO strain fermentations. Final levels were reduced with 84 %, 87 %, and 66 % for respectively the uncontrolled, pH 5.0, and pH 5.5 fermentations, while the amount of acetate produced was reduced with 142 %, 112 %, and 90 %, respectively, demonstrating the impact of inactivating the *ack* gene.

Although fermentations under pH uncontrolled conditions, at pH 5.0 and at pH 5.5 by the DAPKO strain showed reduced final acetate levels compared to the BUK1KO strain, acid production was never completely abolished. Surprisingly, fermentations in CM1 without acetate at pH 5.5 and 6.0 resulted again in high acetate levels being produced by the DAPKO strain (110 mM and 120 mM, respectively). An important observation, made from the time course data of the DAPKO strain fermentations, was that the bi-phasic growth pattern of the wild type strain, absent from the BUK1KO fermentations, was restored (Figure 4.4C). Although no high temporal resolution was achieved during the DAPKO strain fermentations the data still clearly showed that acid levels first increased and later decreased with the concomitant production of acetone. Acetone production had increased for the DAPKO strain, suggesting increased uptake of acids compared to the BUK1KO strain. CM1 fermentations without added acetate produced similar amounts of acetone compared to CM1 fermentations, with the exception of pH 5.5 and 6.0 were no acetone at all could be detected (see Figure 4.5, page 97).

Also for this strain the fermentation pH is of importance, but whereas with the BUK1KO strain increasing fermentation pHs paralleled butanol production, the DAPKO strain produced more butanol at pH 5.0. The highest recorded butanol level (174 mM) was for the pH 5.0 fermentation in CM1, but CM1 without acetate gave similar levels of butanol. In general the DAPKO strain fermentations showed better solvent formation in CM1 at lower fermentations pHs (ABE/glucose yield is 29 % at pH 4.86 and 17 % at pH 6.0) whereas the BUK1KO strain again showed the inverse behaviour (pH 4.35, 16 %; pH 5.5, 23 %), except for the highest pH

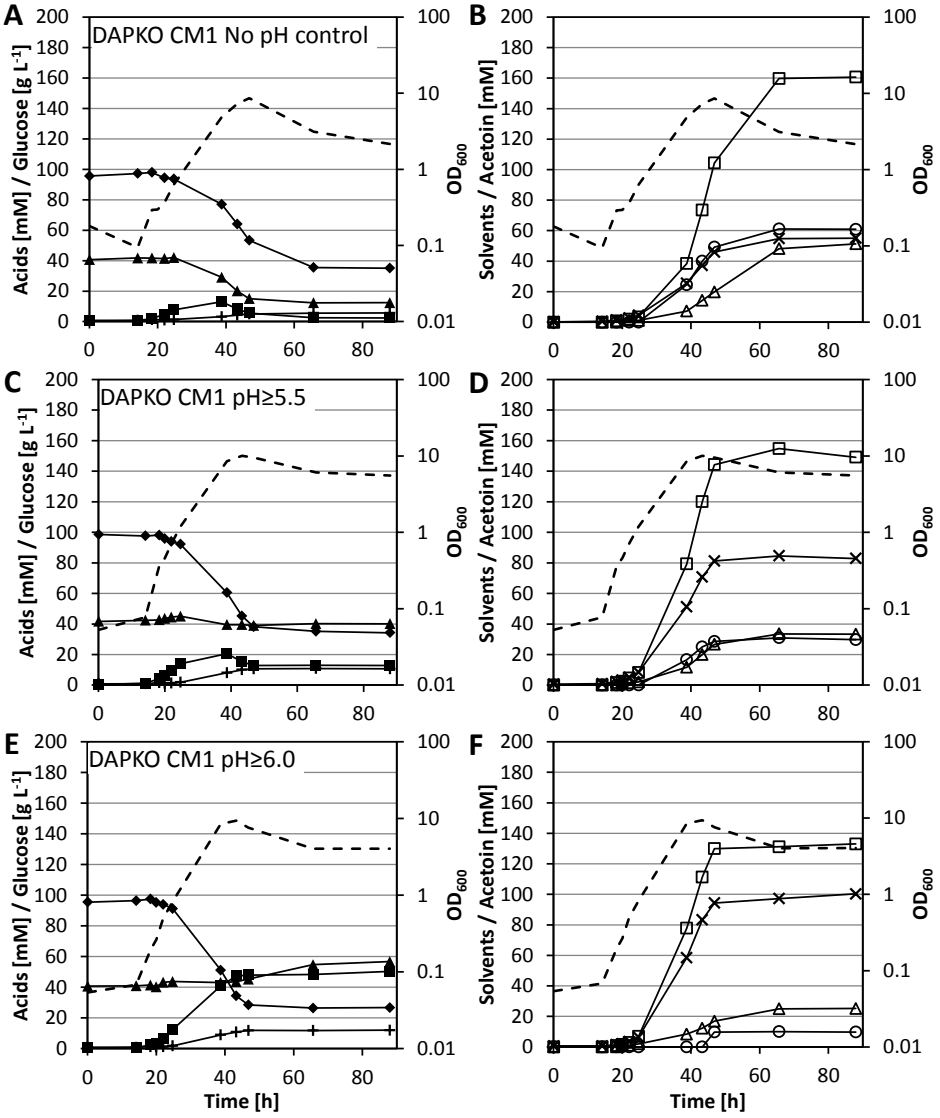


Figure 4.4: Optical density and production profiles of DAPKO strain fermentation under uncontrolled pH conditions (**A** and **B**), and controlled at pH 5.5 (**C** and **D**), and at pH 6.0 (**E** and **F**) in batch reactors using CM1 medium containing 100 g/L glucose. **A**, **C**, **E** Production of acids −▲− acetate, −■− butyrate, −✚− lactate, −♦− glucose, *dashed line* OD_{600}. **B**, **D**, **F** Production of solvents −△− ethanol, −□− butanol, −○− acetone, −×− acetoin, *dashed line* OD_{600}.

of 6.5.

Another striking effect of AK inactivation in the DAPKO strain, compared to BUK1KO fermentations, was the strong increase in acetoin levels which were more than five-fold up in fermentations at pH 5.0 in CM1 and two-fold in CM1 without acetate at pH 5.5. The highest concentration, 100 mM, was measured for the CM1 fermentation at pH 6.0, to our knowledge more than ever reported for this organism. In line with this also the *meso*–2,3–butanediol levels increased by 124 % to 5.6 mM.

4.4 Discussion

Acetate and butyrate are the main metabolites during the acidogenic phase of *C. acetobutylicum* and provide the organism with additional ATP from substrate level phosphorylation, besides the ATP generated during glycolysis. Acid pathway mutants in wild type *C. acetobutylicum* have been reported by Green *et al.*(PJC4PTA and PJC4BK with an inactivated phospho-transacetylase (PTA) and butyrate kinase (BK) enzyme respectively),[126] by Shao *et al.* (BK),[156] by Lehmann *et al.* (PTA,[179] and phospho-transbutyrylase,[166] PTB), and by Kuit *et al.* (acetate kinase, AK).[217] In all cases production of the acid corresponding to the pathway of the inactivated enzyme continued for unknown reasons with the exception of the *ptb* mutant.

It has been suggested that the enzymes of the butyrate pathway can catalyse the homologous reaction of the acetate pathway, which might explain the continued production. *In vitro* enzyme studies of acetate kinase (AK) showed that it could not accept butyryl-phosphate as a substrate,[264] whereas butyrate kinase (BK) showed 6 % cross-reactivity towards acetate in the reverse reaction.[248] Two recent publications seem to support this hypothesis for the analogous situation of PTA and PTB enzymes. A *pta*$^-$ strain continued to produce acetate at wild type levels, presumably due to compensation by PTB. Conversely, a *ptb*$^-$ strain did not produce any butyrate as PTA, *in vivo*, does not except butyryl-CoA as a substrate. Attempts by the authors to isolate a *pta*$^-$ *ptb*$^-$ double mutant were unsuccessful, which suggested to them that such a mutant is not viable under the conditions used.[166]

Previously we showed that an AK negative mutant continued to produce wild type levels of acetate, although delayed compared to butyrate production.[217] The behaviour was medium depended as CM1, containing 45 mM acetate, severely inhibited acetate production by the mutant, but not by the wild type. This behaviour might be expected if the BK enzyme would complement the AK activity *in vivo*. To investigate whether *in vivo* complementation occurred we set out to construct an acetate kinase and butyrate kinase double mutant. In addition to inhibiting acid formation, this double mutant was expected to have an increased alcohol (butanol and ethanol) versus acetone ratio due to this decreased acid production.

Table 4.5: Fermentation performance of the *C. acetobutylicum* DAPKO mutant strain in CM1-based media in 1-L bioreactors at different pH conditions, as indicated[a]

Medium	CM1					CM1 without acetate				
Strain	WT[b]	DAPKO				BUK1KO[b]	DAPKO			
pH	5.0	Uncontrolled (4.86)	5.0	5.5	6.0	5.5	Uncontrolled (?)	5.0	5.5	6.0
Consumed glucose	351 ± 3	334	372	352	382	452 ± 12	265	354	375	398
Acetic acid Final	72 ± 6	16	24	52	75	143 ± 7	9	33	110	120
Acetic acid Relative to start	30 ± 6	−25	−17	11	34	—[e]	—[e]	—[e]	—[e]	—[e]
Butyric acid	16 ± 0.3	4	5	20	63	20 ± 1	0	3	42	47
Lactic acid	3 ± 0.04	6	7	11	12	11 ± 3	1	2	11	21
Acetoin	12 ± 0.3	55	62	85	100	21 ± 1	25	23	46	61
meso-2,3-butanediol	2.5 ± 0.2	4.0	6.4	5.9	5.6	1.7 ± 0.2	2.4	3.3	3.6	3.5
Acetone	101 ± 3	61	54	31	10	40 ± 6	55	77	0	0
Butanol	161 ± 2	160	174	155	133	195 ± 5	131	166	132	125
Ethanol	30 ± 1	48	64	34	25	44 ± 3	98	83	24	23
OD_{600}[c]	14 ± 0.02	8.62	10.9	10.0	9.38	16 ± 0.3	10.5	14.4	12.3	8.84
Biomass [g/L]	4.6 ± 0.01	2.8	3.6	3.3	3.1	5.4 ± 0.1	3.5	4.8	4.1	2.9
Carbon recovery	101 ± 2 %	95 %	94 %	98 %	96 %	91 ± 1 %	109 %	102 %	86 %	85 %
Selectivity[d]										
B/(A+B+E) [mol/mol]	55 %	59 %	59 %	71 %	79 %	70 %	46 %	51 %	85 %	84 %
Productivity[d]										
ABE/glucose [g/g]	30 %	29 %	28 %	23 %	17 %	23 %	36 %	32 %	16 %	14 %
B/glucose [mol/mol]	46 %	48 %	47 %	44 %	35 %	43 %	49 %	47 %	35 %	32 %
B/(Ac+Bu) [mol/mol]	3.5	40	35	5.0	1.4	1.2	15	4.6	0.87	0.75

[a] Values are concentrations in mM unless otherwise indicated.
[b] Data from Table 4.4, shown for reasons of comparison and given as the mean ± SEM of two independent fermentations ($n = 2$).
[c] No detailed OD_{600}-data for DAPKO-fermentations were recorded so reported values might not be peak values.
[d] Abbreviations under the Selectivity and Productivity headings are as follows: A = acetone, B = butanol, E = ethanol, Ac = acetate relative to the starting concentration (set at 0 if negative), and Bu = butyric acid.
[e] See row above, as no acetate is present in the medium at $t = 0$, nett acetic acid production equals the final acetic acid concentration.

Our first target was butyrate kinase inactivation as this had previously been accomplished by two different groups, one of which also used a mobile group II intron for inactivation of the gene at the same insertion site.[156] The results presented in this work (Table 4.4) demonstrated that for our *buk1*$^-$ strain the phenotypical outcome of inactivation is different from that reported by the previous two groups.[126,156] Butyrate accumulation in the medium was severely reduced in fermentations by the BUK1KO strain, in line with the observed *in vitro* BK enzyme activities. The highest butyrate levels were observed at the end of the fermentation (13 mM for pH 5.0). Although that is not significantly different from final wild type levels, peak butyrate for the wild type (62 mM) was significantly higher and reduced by 79 %. It should be noted however that the fermentations could not be monitored during the time window of 24 to 36 hours, theoretically allowing for a peak to occur.

When comparing our results of fermentations at pH 5.0 with the results of Harris *et al.*,[128] data for butyrate levels is similar, but the other product levels are not. Although Harris *et al.* also report increased acetate production, their time course data shows a peak acetate level of 111 mM, which then drops to a final level of 46 mM, whereas our strain continues to produce acetate to apparently inhibitory levels (180 mM, Table 4.4). Furthermore, they report higher than wild type solvent levels for butanol and ethanol, where we observe a reduction of all final solvent levels.

Our efforts to increase the solvent production by optimising the pH again illustrates a difference between our BUK1KO strain and the previously reported PJC4BK strain. The higher pH of 5.5 is optimal for solvent production by the BUK1KO strain compared to the other tested pH levels (Table 4.4), contrary to the PJC4BK strain which has an optimum of 5.0.[128]

Acetate toxicity is caused by proton translocation across the membrane and increased intracellular acetate levels impeding cellular functions.[265] The likelihood that acetate was the inhibitory product for the fermentation in CM1 medium is illustrated by the improved butanol levels and a similar final acetate levels at pH 5.5 (143 mM vs. 154 mM). Contrary to the expectation that acetate levels at pH 5.0 would be lower than those at 5.5, due to increased extra-cellular levels of undissociated acids, a final level of 180 mM was recorded. This would suggest that the mechanism by which acetate is toxic varies, or that it is not the growth inhibiting compound after all.

Another difference between fermentations by our strain at pH 5.5 and those of Green *et al.* at the same pH is the much lower lactate formation (11 mM vs. 69 mM).[126] We also measured an acetoin concentration of 30 mM which was not reported by Green *et al.*. Various reasons can be put forward to account for the observed differences, *e.g.* difference in growth medium, effect of antibiotic pressure, inoculum size, and strain differences. Our WUR strain, grown under the same conditions as, and compared to the ATCC 824 strain, showed excessive lactate formation during growth on CGM.[217] Our strain also produces *meso*–2,3–butanediol, a product normally not found in *C. acetobutylicum* fermentations.[216]

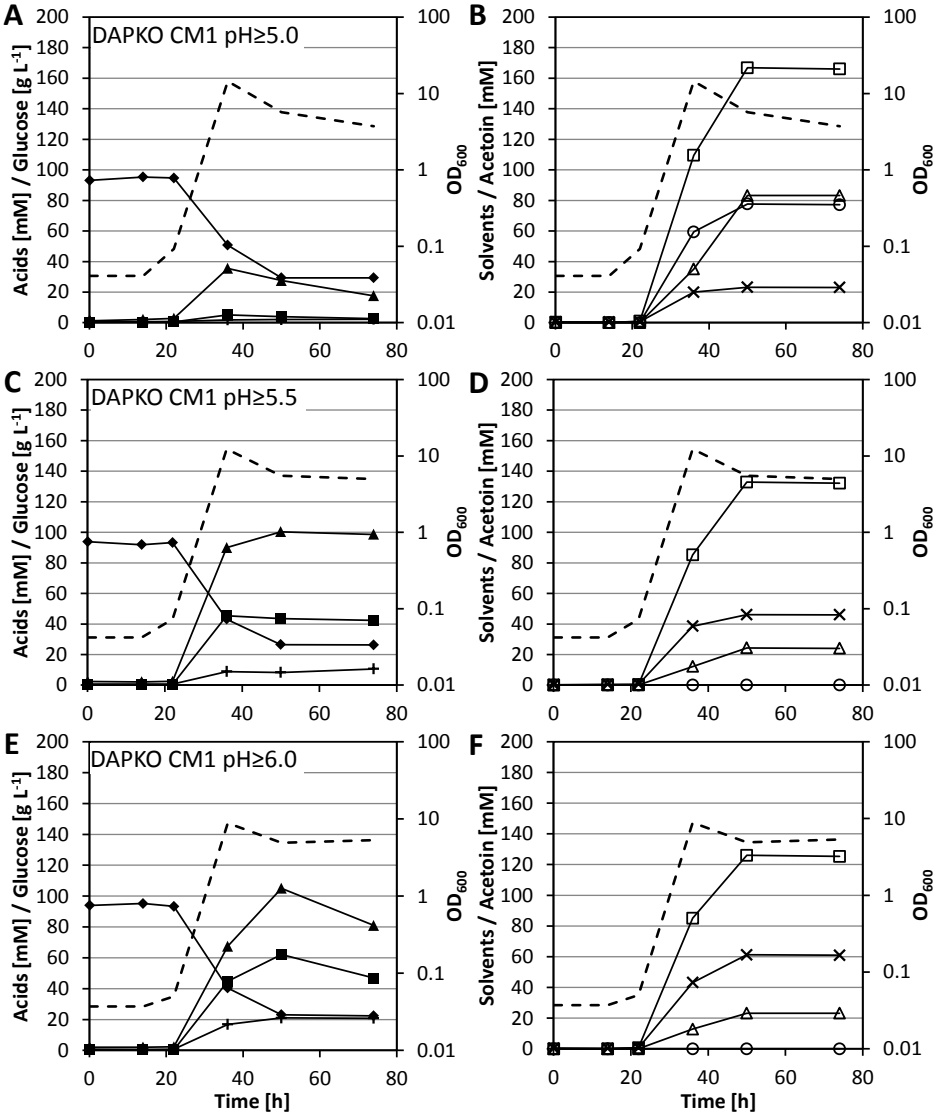


Figure 4.5: Optical density and production profiles of DAPKO strain fermentation under uncontrolled pH conditions (**A** and **B**), and controlled at pH 5.5 (**C** and **D**), and at pH 6.0 (**E** and **F**) in batch reactors using CM1 medium without acetate, containing 100 g/L glucose. **A**, **C**, **E** Production of acids −▲− acetate, −■− butyrate, −✚− lactate, −♦− glucose, *dashed line* OD_{600}. **B**, **D**, **F** Production of solvents −△− ethanol, −□− butanol, −○− acetone, − × − acetoin, *dashed line* OD_{600}.

It could be that additional strain variations result in a different phenotype upon inactivation of the same gene. It is of interest to note that also others have reported differences when attempting to create a *buk1*$^-$ mutant. When attempting to make a *buk1* deletion mutant, Soucaille and co-workers noted that in each successful case of *buk1* removal, also the preceding *ptb* gene was mutated rendering it inactive.[260] They concluded that a *buk1* deletion on its own was lethal for their strain. Although all strains in these reports are referred to as ATCC 824 it seems that laboratory stocks have diverged over time. Comparison of strains by whole genome resequencing using next generation sequencing (NGS) techniques could help to see if this is the case and to possibly identify which mutations are required to be able to obtain a *buk1*$^-$ knock-out strain.

The successful isolation of the PJC4BK and BUK1KO strains, could also be explained by selection for new secondary mutations during the mutant isolation procedure. If so, then these mutations must be present elsewhere on the genome because the strain still showed PTB activity and the fragment on the Southern blot was of the expected size. The fact that both the BUK1KO and DAPKO strain did not sporulate and did not give rise to clostridial cell shapes is another important observation. Normally loss of the pSOL1 megaplasmid is the cause of this, but our strains continued to produce acetone indicating that they did not degenerate. During frequent microscopic inspection of the culture we never observed spores or clostridial-stage cells. For the BUK1KO strain this fits with observed production profile as the cells do not seem undergo a solventogenic shift which is associated with the appearance of clostridial-stage cells ultimately resulting in spore formation. For the DAPKO strain the link with the production profile is less clear but also in that case spores were never seen.

To further improve upon the results of the BUK1KO strain, and to address the hypothesis that AK and BK enzymes can complement each others activities, we have generated, to our knowledge, the first *C. acetobutylicum* mutant with targeted inactivation of genes in both acid production pathways. The successful inactivation of both *buk1* and *ack* was demonstrated by enzyme activity assays showing less than 2 % (AK), and 0.2 % (BK) of wild type activities (Table 4.3) and by sequence analysis. Surprisingly this did not result in the expected fermentation behaviour. Although DAPKO fermentations of CM1 at various pH levels showed drastic reduction of acetic acid levels compared to the BUK1KO strain, in addition to the already low butyrate levels, but it also had increased acetone levels. The later indicating uptake, and activation, of previously produced, or already present, acetate and/or butyrate. Surprisingly, when CM1 without acetate is used for the fermentation acetate production increases suggesting that an initial concentration of 45 mM acetate inhibits additional acetate production. The reasons for this are not known, but the same effect was seen for the *C. acetobutylicum* WUR *ack*$^-$ mutant.

One of the proposed mechanisms by which the PJC4BK strain, described by Green *et al.*, could continue to produce butyrate was that the analogous enzyme in the acetate pathway, acetate kinase, converted butyryl-phosphate to butyrate.[126]

Later tests with purified acetate kinase from *C. acetobutylicum* DSM 1731 had shown that AK does not exhibit activity towards butyryl-phosphate *in vitro*.[264] Despite inactivation of both AK and BK, as demonstrated by *in vitro* enzyme activities, production of both acetate and butyrate continued. Combined with the *in vitro* enzyme characterisation data this disproves the hypothesis that expression of AK from *ack* causes butyrate formation in the PJC4BK and BUK1KO strains. Therefore an alternative explanation is needed for the continued acid production in our BUK1KO and DAPKO mutants. For example the presence of isozymes, as also suggested by Green *et al.*,[126] or alternative production pathways.

For butyrate kinase an isozyme, BKII encoded by *buk2*,[266,267] has been described, however this enzyme has a very narrow substrate specificity an can not accept acetate as a substrate. Furthermore, based on its enzymatic properties it was thought to be involved in butyrate uptake rather than its production. However it could perhaps still explain the observed low butyrate levels in the DAPKO and PJC4BK fermentations. For acetate kinase no such isozyme has been described although this does rule out the possibility that it exists. One reason why this may have gone unnoticed for so far is that acetate kinase enzyme assays are regularly performed according to the method of Rose[241,268]. This method was also employed when the acetate kinase enzyme was purified and isolated.[264,269,270] However a possible isozyme might not function in the activity test and go undetected.

Alternatively, other pathways, involving different enzymes, could also lead to the observed acetate (and butyrate) formation. Interestingly also an *E. coli* BL21 triple knock-out strain of genes involved in acetate production (*ackA*$^-$, *pta*$^-$, and *poxB*$^-$) showed acetate accumulation up to 0.4 g/L.[271] The authors conclude that additional pathways must exist that account for the observed acetate formation. Such an enzyme could for example be an acetyl-CoA hydrolase, suggested to exist in *Saccharomyces cerevisiae*, which might be expressed to deal with the, presumably, increased acetyl-CoA pool.[272,273] However, the view that such an enzyme might exist has recently been contested as the *S. cerevisiae* enzyme was shown to actually be an acetyl-CoA-transferase.[274] The suggestion that a CoA-transferase might play a role in the observed continued acid production is intriguing and deserves further investigation.

The fact that inactivation of both acid production pathways results in increased acetoin production (Table 4.5) could indicate that intracellular accumulation of pyruvate takes place and is, partially, remedied by the cell by converting it to acetoin. Due to the high levels of acetoin also production of *meso*–2,3–butanediol increased as has been observed for exogenously added acetoin.[216] Acetoin production reached a maximum of 100 mM in fermentations at pH 6.0, to our knowledge, the highest level ever reported for this organism. Lactate production by the DAPKO mutant (12 mM) also increased but reached not more than 4 times the wild type level, similar to the PJ4BK strain at pH 5.0. The incomplete carbon balance of some of the fermentations done at higher pH levels

(see Table 4.4 and Table 4.5) suggests that alternative products are formed which merits further investigation as this can also be seen in other publications.[179]

The aim of our work was to impede acid and acetone production and create an alcohologenic fermentative strain. Neither of the two created strains had these properties. The two mutants did show improved butanol selectivities of 70 % (BUK1KO, CM1 without acetate, pH 5.5) and 85 % (DAPKO, CM1 without acetate pH 5.5, compared to 50 % got the wild-type. However productivity is reduced as more glucose is used for formation of other products such as acetate (BUK1KO, DAPKO) and acetoin (DAPKO). Lehmann *et al.* observed that their *pta* knock-out mutant continued to produce acetate at wild type levels and was seemingly unaffected also in the presence of acetate in the growth medium. Unfortunately no enzymatic activity data was presented for the mutant.[179] A *pta*$^-$ mutant with in addition an inactivated acetone pathway did show reduced acetate levels.

Also in our study acid production was never totally abolished although final acid levels were very low for the DAPKO strain in CM1 without acetate under uncontrolled pH conditions. Acetone levels in that case were also reduced by 45 % compared to the wild type fermentation but were still at 55 mM. This indicated that acid production still had taken place. As with the *ack*$^-$ knock-out mutant[217] the DAPKO strain is sensitive to acetate present in the medium suggesting that the alternative acetate production pathway is sensitive for, or it expression regulated by, the initial acetate concentration.

In our effort to create an alcohologenic strain we only partially succeeded. The DAPKO strain fermentation in CM1 without acetate at pH 6.0 produced 22 % less butanol, but no acetone and similar ethanol levels. Butanol production selectivity was increased from 55 % for the wild type to 84 % for the mutant, however also acetate (120 mM) and butyrate (47 mM) were produced in addition to acetoin (61 mM) and lactate(21 mM). By comparison the approach of Nair and Papoutsakis to create an alcohologenic fermentation by expressing an alcohol dehydrogenase (*adhE*) in the degenerated M5 strain resulted in a better butanol to ethanol ratio of 84:8 (mM:mM)(91 % selectivity) but also led to massive acid accumulation.[173] Both acetate and butyrate were produced at around 100 mM each. A new approach, again using the M5 strain as a host to express *adhE* now under control of the *ptb* promoter improved butanol and ethanol levels to almost wild type concentrations but with extremely high acetate (226–256 mM) and elevated butyrate levels (73–144 mM).[129] Inactivation of AK or BK in an M5 background did not improve this.

The challenging regulation of, and the complex metabolic network underpinning the *C. acetobutylicum* fermentation makes the rational metabolic engineering approaches of this organism difficult. Our lack of knowledge on some of the very basic metabolic pathways this organism employs seem to hamper this approach. Recent reports about regulatory genes[107,161,162,180,275] and techniques[72,73,98,276] will aid in understanding the system but it will continue to be a challenging organism to work with. In an effort to better understand the

fermentative behaviour of our DAPKO mutant we are currently undertaking a whole genome re-sequencing approach using the Illumnia platform, to determine differences between the DAPKO mutant and wild type strain that could explain the phenotypical differences. The preliminary results indicate that although the DAPKO strain has approximately 190 point mutations compared to the published genome sequence, the parental WUR strain has these too. The search for further difference is ongoing. Hopefully we will be able to obtain new targets for the rational manipulation of the metabolism of our strains.

4.5 Acknowledgements

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[#] These authors contributed equally to this work.

Chapter 5

D-2,3-Butanediol Production Due to Heterologous Expression of an Acetoin Reductase in *Clostridium acetobutylicum*

Abstract

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

5.1 Introduction

To meet our future energy needs, it is necessary to develop sustainable and carbon-neutral energy sources. Liquid biofuels are attractive candidates, since little or no change is needed to the current petroleum-based fuel technologies.[277] For this purpose, biological production of several alcohols is under investigation, including ethanol and butanol.[202,278]

Several clostridial species are able to ferment carbohydrates to acetone, 1-butanol, and ethanol (ABE). Industrial application of this process, also known as ABE fermentation, has a long history, but the process economics after 1960 became unfavourable compared to the petrochemical process, and its commercial exploitation was gradually abandoned.[57] The inefficiency of the fermentation still hampers commercial reintroduction of this renewable butanol production process. Improving the yields and productivities of the solvent products is key to its successful reintroduction.

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.[131,132] Many efforts have been made in the past to obtain clostridial strains with increased 1-butanol tolerance but have had limited success.[133–137,279]

As an alternative to increasing 1-butanol tolerance, we propose replacing the production of 1-butanol with production of a compound that has similar physical and chemical properties (heat of combustion, heat of vaporization, and energy density) but that is less toxic to the cell, making higher titres possible. 2-Butanol matches these criteria and has a lower log P_{ow} value (octanol-water coefficient) than 1-butanol. The log P_{ow} value is a good indicator for the strength of membrane-perturbing effects.[280] Generally, the lower the log P_{ow} value, the less toxic the compound is to the membrane. However, *Clostridium acetobutylicum* is not known to produce 2-butanol nor its potential precursor, 2,3-butanediol (2,3-BD).[281] Nevertheless, it is known to produce acetoin as a minor fermentation product.[57]

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, catalysed by acetolactate synthase. Decarboxylation by acetolactate decarboxylase yields acetoin,[282] which can be reduced by an acetoin reductase (ACR) to 2,3-butanediol.

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

5.2 Materials and Methods

5.2.1 Bacterial strains, plasmids and primers

All bacterial strains and plasmids used during this study are listed in Table 5.1. Escherichia coli stocks were stored in 20 % (vol/vol) glycerol at −80 °C. Stock cultures of *Clostridium acetobutylicum* strains and *C. beijerinckii* NCIMB 8052 were maintained as spore suspensions in 15 % (vol/vol) glycerol at −20 or −80 °C. Chemically competent, *E. coli* NEB 5α F′ *lacI*^{q} cells were used for cloning and vector maintenance. Electro-competent *E. coli* DH10B(pAN1) cells were used to methylate plasmid DNA before transformation into *C. acetobutylicum*.[147]

5.2.2 Media and growth conditions

E. coli strains were cultured in lysogeny broth (LB) medium at 37 °C and 200 rpm. Sporulation plates were based on the media used by Nimcevic *et al.*[240] but also contained 15 g/L agar. Prior to inoculation of clostridial precultures, spore suspensions were heat shocked for 10 min at 70 or 80 °C. *C. acetobutylicum* strains were grown in MG medium or modified CGM (mCGM) medium as indicated below.

MG medium was based on the semisynthetic medium described by Nimcevic *et al.*[240] and contained the following (per litre of water): yeast extract, 2.50 g; KH_2PO_4, 1.00 g, K_2HPO_4, 0.76 g; ammonium acetate, 3.00 g; *para*-aminobenzoic acid (*p*ABA), 0.10 g; $MgSO_4 \bullet 7\ H_2O$, 1.00 g; $FeSO_4 \bullet 7\ H_2O$, 0.01 g and glucose, 60 g.

mCGM medium contained the following (per litre of water): yeast extract, 5.00 g; KH_2PO_4, 0.75 g; K_2HPO_4, 0.75 g; $MgSO_4 \bullet 7\ H_2O$, 0.4 g; $MnSO_4 \bullet H_2O$, 0.01 g; $FeSO_4 \bullet 7\ H_2O$, 0.01 g; NaCl, 1.0 g; asparagine, 2.0 g; $(NH_4)_2SO_4$, 2.00 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 % (vol/vol) overnight precultures. Clostridial culture experiments were performed at 37 °C, without shaking, and anaerobically in (i) an anaerobic chamber; or (ii) in glass serum vials as described previously.[225]

Culture media were supplemented with ampicillin (100 µg/mL), chloramphenicol (30 µg/mL), erythromycin (40 µg/mL for liquid cultures and plates; 25 µg/mL for transformant isolation), kanamycin (50 µg/mL), isopropyl-β-D-thiogalactopyranoside (IPTG) (50 µg/mL), and 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) (40 µg/mL) when appropriate. For challenge experiments, acetoin or 2,3-butanediol (2,3-BD) was added to the medium prior to inoculation with the preculture.

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

Table 5.1: Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant genotype[a]	Remark	Source
Bacterial strains			
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	H. Bahl, Rostock, Germany
C. beijerinckii NCIMB 8052	WT		Laboratory stock
E. coli NEB 5α F′ *lacI*^{q}	*lacZ*Δ*M15 endA1 recA1 hsdR17 fhuA2 lacI*^{q}	Cloning strain	Laboratory stock
E. coli DH10B(pAN1)	Δ(*mrr-hsdRMS-mcrBC*)	Methylation strain	Laboratory stock
Plasmids			
pAN1	p15A ori; Cm^{R} ϕ*3tI*	Plasmid that expresses the phage ϕ*3tI* methylase gene[147]	Laboratory stock
pMTL500E	ColE1 ori; pAMβ1 ori; MLS^{R} Ap^{R}	Clostridial/*E. coli* shuttle vector[142]	Laboratory stock
pWUR459	ColE1 ori; pAMβ1 ori; MLS^{R} Ap^{R}; P$_{adc}$-*Cb-acr*	Plasmid that expresses the *Cb-acr* gene under the control of the *C. acetobutylicum* acetoacetate decarboxylase promoter	This study
pWUR459	ColE1 ori; pAMβ1 ori; MLS^{R} Ap^{R}; P$_{thl}$-*Cb-acr*	Plasmid that expresses the *Cb-acr* gene under the control of the *C. acetobutylicum* thiolase promoter	This study

[a] Abbreviations: WT, wild type; ori, origin; Cm^{R}, chloramphenicol resistance; MLS^{R}, macrolide-lincosamide-streptogramin B resistance; Ap^{R}, ampicillin resistance; *Cb-acr*, *C. beijerinckii acr* (*Cb-acr*) gene.

5.2.3 DNA isolation, transformation, and manipulation

Standard molecular work was performed according to established protocols.[224] Genomic DNA from *C. acetobutylicum* or from *C. beijerinckii* was isolated using the GenElute bacterial genomic DNA kit (Sigma-Aldrich). Plasmid DNA from *E. coli* was isolated by the GenElute plasmid miniprep kit (Sigma-Aldrich). PCR amplification of clostridial DNA was done using *Pfu* polymerase (Stratagene). *E. coli* colony PCRs were carried out using REDTaq (Sigma-Aldrich). Methylated plasmids were electroporated into *C. acetobutylicum* by the method of Oultram *et al.*[142] Correct methylation was checked by restriction analysis using *Fnu*4HI.[147]

5.2.4 Primers and DNA sequencing

All DNA primers used in the study are listed in Table 5.2. Primers were obtained from Eurogentec (Seraing, Belgium) or Biolegio (Nijmegen, The Netherlands). DNA sequencing of clones was done by BaseClear (Leiden, The Netherlands).

Table 5.2: Primer sequences

#	Primer name	Sequence ($5' \rightarrow 3'$)[a]	Target DNA region
1	005CA_Pthlf	GGCATGCgaa tttagaatga agtttcttat gc	Promoter *thl*; *Ca_c2873*
2	017CA_Pthlr	AAAAGGGCCC ccatagttta tccctaattt atacg	Promoter *thl*; *Ca_c2873*
3	007CA_Padcf	GGCATGCatg ggaaagccaa cattgc	Promoter *adc*; *Ca_p0165*
4	018CA_Padcr	AAAAGGGCCC cttcacatta taaatcgcct ct	Promoter *adc*; *Ca_p0165*
5	BG2588	CGCGCGGGCC CAGGAGGGgC GGCGGCatga aagcagcatt atg	*Cbei_1464* (*Cb-acr*)
6	BG2402	GCGCGCTCGA Gttaagattt agatacaagt tctt	*Cbei_1464* (*Cb-acr*)

[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.

5.2.5 Cloning of the *C. beijerinckii acr* (*Cb-acr*) gene into *C. acetobutylicum*

The clostridial expression plasmids pWUR459 and pWUR460 were constructed as follows. The pMTL500E vector was linearised by digestion with *Sph*I and *Xho*I. *adc* and *thl* promoter regions were amplified by PCR from *C. acetobutylicum* genomic DNA using primers 1 & 2, and 3 & 4, 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. Primers 1 and 3 contained a *Sph*I restriction site, primers 2, 4, and 5 an *Apa*I restriction site, and primer 6 a *Xho*I 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 linearised vector, the *Cb-acr* gene and the *adc* promoter or the *thl* promoter, respectively (Figure 5.1).

Competent *E. coli* NEB 5αcells 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 *Fnu*4HI.

The clostridial expression plasmids pWUR459 and pWUR460 were constructed as detailed in the supplemental material. These plasmids and the control plasmid pMTL500E were used to transform *C. acetobutylicum*. Each transformation resulted in multiple erythromycin-resistant colonies. After restreaking, selected colonies were used to prepare spore suspensions for further experiments.

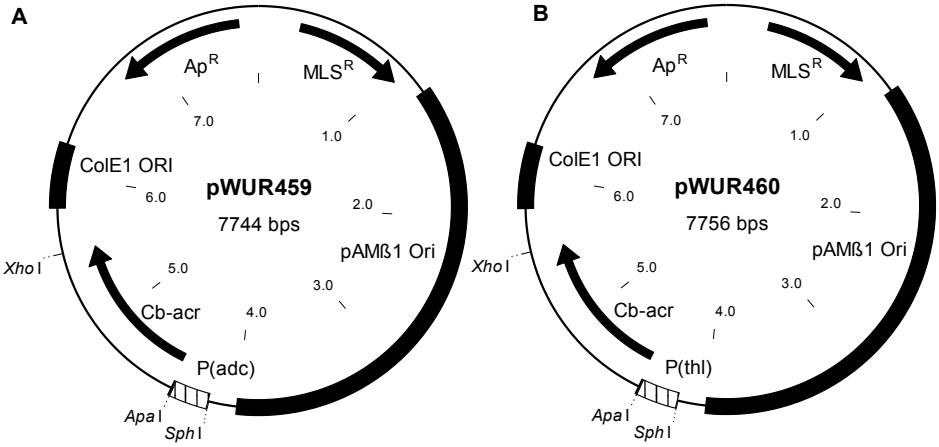

Figure 5.1: Plasmid map of A. pWUR459 - Expression plasmid of *Cb-acr* under control of the *C. acetobutylicum* acetoacetate decarboxylase promoter and B. pWUR460 - Expression plasmid of *Cb-acr* under control of the *C. acetobutylicum* thiolase promoter.

5.2.6 Acetoin reductase (ACR) enzyme assays

C. acetobutylicum cells were harvested from cultures with an optical density (OD) of 5 by centrifugation (4 816 × *g*, 15 min, 4 °C) and resuspended in 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM tris(2-carboxyethyl)phosphine (TCEP) as reducing agent, because 2-mercaptoethanol was shown to inhibit enzyme activity. Crude cell extracts were prepared by French press homogenization

(two passes at 16 000 psi) and immediately assayed for enzyme activity. Assays were carried out at 37 °C in 100 mM phosphate buffer (pH 6.5) containing 1 mM TCEP, 50 mM D/L-acetoin, and 0.28 mM NADPH. The reaction was started by the addition of acetoin. The decrease in absorbance at 340 nm due to NADPH oxidation was monitored on a Hitachi U2010 spectrophotometer with correction for background NADPH oxidation. One unit of enzyme activity was defined as the amount of enzyme required for the oxidation of 1 µmol of NADPH per minute. Total protein in crude extracts was determined using Roti-Nanoquant (Carl Roth, Karlsruhe, Germany) with bovine serum albumin (BSA) as a standard.

5.2.7 High-performance liquid chromatography (HPLC) analysis of glucose and metabolites

Fermentation samples were centrifuged (20 800 × *g*, 5 min), and the supernatants were stored at −20 °C. After the supernatants were thawed, an equal volume 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 and then filtered (0.2 µm; Whatman). Separation of a 10 µL sample was achieved using a Shodex Ionpack KC-811 column, equipped with a refractive index detector (Waters 2414) and a UV detector (Waters 2487) operating at 210 nm, with 3 mM H_2SO_4 as eluent (flow, 1 mL/min; column temperature, 85 °C). The order of elution was glucose, lactic acid, acetic acid, acetoin, *meso*-2,3-BD, D/L-2,3-BD, butyric acid, acetone, ethanol, valeric acid, 4-methyl valeric acid, and 1-butanol.

5.2.8 Chiral GC-MS analysis

To determine the enantiomeric distribution of the 2,3-BD and acetoin produced, 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 equal volume of ethyl acetate. To prevent coextraction of acids, 10 M sodium hydroxide was added, since these compounds interfered with chromatographic analysis. Samples for acetoin analysis were not treated with sodium hydroxide to prevent potential racemization. The extract was then analysed on a Finnigan Trace DSQ (dual-stage quadrupole) gas chromatography (GC)-mass spectrometry (MS) system (Thermo Electron Corporation) equipped with a CP-Chirasil-Dex CB (Varian) fused silica capillary column (25 m by 0.25 mm by 0.25 µm) with helium as the carrier gas. 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 °Cmin followed by a ramp of 40 °Cmin to 200 °C (4 min). Samples (1 or 5 µL) were injected using an AS3000 autosampler (Thermo). The ion source (electron ionization [EI]) temperature was set at 200 °C. Compound identification by column retention time was confirmed by analysis of the mass spectrum. The retention times of chiral standards

of D-(−)-(2*R*,3*R*)- and L-(+)-(2*S*,3*S*)-2,3-BD (Sigma-Aldrich) were used for peak identification. The elution order of the acetoin enantiomers was inferred from the stereochemistry of the D-(2*R*,3*R*)-2,3-BD product.[283] The order of elution was (3*R*)-acetoin, (3*S*)-acetoin, L-(2*S*,3*S*)-2,3-BD, D-(2*R*,3*R*)-2,3-BD, and *meso*-2,3-BD.

5.3 Results

5.3.1 Characterization of *C. acetobutylicum* transformants

Wildtype *C. acetobutylicum* ATCC 824 is known to produce significant levels of acetoin, but no 2,3-butanediol (2,3-BD).[57] Recently, a gene from *C. beijerinckii* NCIMB 8052, *Cbe_1464* annotated as an alcohol dehydrogenase, was functionally expressed in *E. coli* and demonstrated to possess acetoin reductase (ACR) activity (M. A. J. Siemerink and S. W. M. Kengen, unpublished results). Introduction of this acetoin reductase (ACR)-encoding gene might enable conversion of acetoin to 2,3-BD. Therefore, *C. acetobutylicum* transformants, containing the *C. beijerinckii acr* (*Cb-acr*) gene under the control of either the *thl* promoter (pWUR459) or the *adc* promoter (pWUR460), were constructed, and their fermentation pattern was analyzed. Both types of transformants were found to produce D-2,3-BD (Table 5.3, page 112). No D-2,3-BD 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, respectively (Figure 5.2). These data suggest that the conversion of acetoin to 2,3-BD is limiting, especially in the case of the *thl* promoter construct, which should result in constitutive expression. The final 2,3-BD concentrations and conversion levels for both *Cb-acr* strains did not differ significantly from one another: 22 mM and 90 % acetoin conversion for the P_{thl}-*Cb-acr* strain, respectively, and 20 mM and 89 % acetoin conversion, respectively, for the P_{adc}-*Cb-acr*. Acetoin levels of the control fermentation reached 19 mM. However, in fermentations of *C. beijerinckii* acetoin reductase (Cb-ACR)-expressing strains, acetoin was still detected at levels of 2 to 3 mM at the end of the fermentation (Figure 5.2 and Table 5.3).

Analysis of medium samples of the control strain harbouring pMTL500E, showed a ratio of approximately 12:1 (D:L) in the concentrations of the two acetoin enantiomers (data not shown). The acid and solvent production patterns of the transformants expressing the *Cb-acr* gene were similar to those of the control strain harbouring the empty vector (Table 5.3). Remarkably, all three transformants produced small but nonetheless detectable amounts of *meso*-2,3-BD (Table 5.3). To confirm this finding, we also looked at fermentations by our wild-type *C. acetobutylicum* strain, in the same (MG medium) and other media

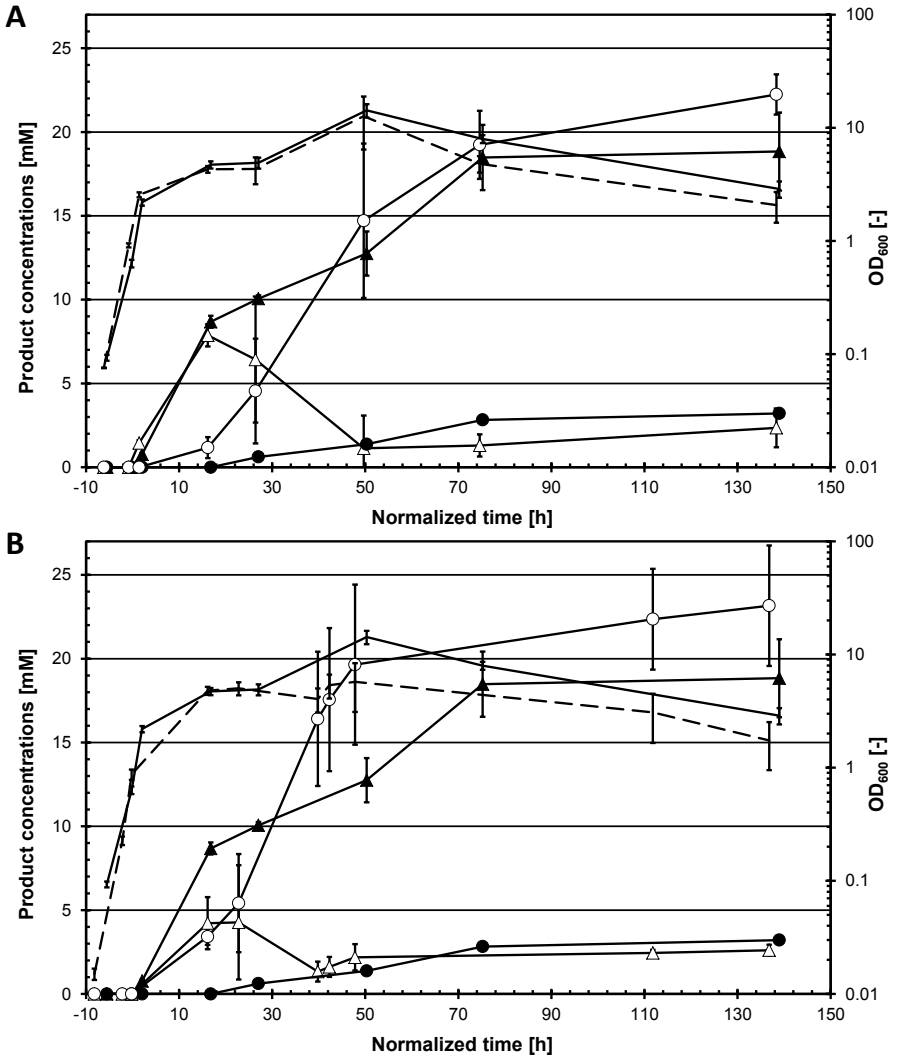

Figure 5.2: Production of acetoin (triangles) and 2,3-BD (circles) during batch fermentations of glucose by transformant strains of *C. acetobutylicum*. Strain codes: EV (closed symbols in panels **A** and **B**), *C. acetobutylicum* carrying pMTL500E, the empty vector control strain; ADC (open symbols in panel **A**), *C. acetobutylicum* carrying pWUR459; THL (open symbols in panel **B**), *C. acetobutylicum* carrying pWUR460. Optical densities of the cultures are shown by a solid line (EV) and a broken line (ADC and THL). On the horizontal axis, normalized time is plotted such that 0 h corresponds to an OD_{600} of 1.0. The control strain did not produce any D-2,3-BD, only *meso*-2,3-BD. Data represent the mean of triplicate fermentations averaged for each time interval. Error bars indicate standard deviations. OD_{600}, optical density at 600 nm.

Table 5.3: Concentrations of substrate and products of 100-mL batch fermentations of *C. acetobutylicum* harbouring pMTL500E, pWUR459, or pWUR460 on MG medium after 145 h

Substrate or product concentration or yield[a]	Value for parameter for *C. acetobutylicum* carrying the following plasmid[b]		
	pMTL500E (control)	pWUR459 (P_{adc}-*Cb-acr*)	pWUR460 (P_{thl}-*Cb-acr*)
Substrate concns [mM]			
Consumed glucose	318 ± 15	307 ± 29	320 ± 18
Initial acetic acid[c]	37 ± 0.2	38 ± 0.4	36 ± 0.8
Final acetic acid	14 ± 0.7	20 ± 6	19 ± 2
Concns of 2,3-BD pathway products [mM]			
Acetoin	19 ± 2	2 ± 1	3 ± 0.3
meso-2,3-Butanediol	3 ± 0.1	2 ± 0.1	2 ± 0.3
D-2,3-Butanediol	0	20 ± 1	22 ± 3
Acetoin + 2,3-BD	22 ± 2	25 ± 2	26 ± 4
% yield of acetoin + 2,3-BD per glucose [mM/mM]	7 ± 0.8	8 ± 0.9	8 ± 1
Concns of other fermentation products [mM]			
Butyric acid	2 ± 1	5 ± 3	6 ± 3
Lactic acid	3 ± 0.7	6 ± 1	4 ± 0.4
Acetone	80 ± 9	78 ± 12	86 ± 6
Butanol	161 ± 6	157 ± 15	166 ± 6
Ethanol	40 ± 2	34 ± 5	56 ± 14

[a] Abbreviation: 2,3-BD, 2,3-butanediol.
[b] Data are given as the means ± standard deviations of three replicate fermentations.
[c] Acetate is present at the beginning (time zero) as a medium component and is consumed during the fermentation.

(mCGM and CGM media). At the end of all fermentations, small amounts of 1 to 3 mM *meso*-2,3-BD were found (Figure 5.3 and data not shown). In all these fermentations, no D- or L-2,3-BD was detected.

Cell homogenates of wild-type and transformed *C. acetobutylicum* strains were assayed for acetoin reductase activity. A low, but significant, activity level of 0.042 ± 0.0035 U/mg and 0.042 ± 0.0044 U/mg could be detected in cell extracts for strains transformed with pWUR459 and pWUR460, respectively. The background levels were 0.018 ± 0.0015 U/mg and 0.024 ± 0.0021 U/mg for the *C. acetobutylicum* WUR strain and strain transformed with pMLT500E vector control, respectively. Detection was complicated by the rapid loss of activity also seen with purified enzyme isolated from *E. coli* extracts (Siemerink and Kengen, unpublished).

5.3.2 Product stereochemistry

There are three stereoisomeric forms of 2,3-BD. The main stereoisomer produced by fermentation in MG medium was identified as D-(2*R*,3*R*)-2,3-BD. However, low levels of *meso*-2,3-BD were also detected in transformant strains, as well as in wild-type fermentations by both HPLC and GC-MS analysis. In all fermentations, L-(2*S*,3*S*)-2,3-BD was below our detection threshold. Figure 5.3 (page 114) shows the gas chromatographic analysis of extracts of standard and medium samples of cultures of the various *C. acetobutylicum* strains.

The observation that both the plasmid vector control strain as well as our wild-type strain produced *meso*-2,3-BD 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-BD. 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 5.1).

5.3.3 Acetoin- and 2,3-BD-challenged batch fermentations

To determine possible inhibitory effects of 2,3-BD on the cultures of *C. acetobutylicum* transformants expressing the *Cb-acr* gene, fermentations in media supplemented with 20 mM D-(2*R*,3*R*) or 20 mM *meso*-2,3-BD were performed. In cultures challenged with D-2,3-BD, both transformants containing the *Cb-acr* gene produced additional D-2,3-BD in similar amounts (12 mM for strain pWUR459 and 20 mM for strain pWUR460) compared to their nonchallenged controls (15 mM and 17 mM, respectively). The use of *meso*-2,3-BD resulted in similar behaviour (Table 5.4, page 115).

We also supplemented media with racemic acetoin (20 mM) to check whether the amount of produced acetoin was limiting for the production of 2,3-BD. Supplemented cultures of ACR-expressing strains converted both D- and L-enantiomers into D- and *meso*-2,3-BD (Table 5.4). This demonstrates that the Cb-ACR enzyme is able to convert both acetoin enantiomers and is therefore not stereoselective for the configuration at the C-3 position. The total amount of both 2,3-BD 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-BD in the acetoin-challenged cultures. It increased significantly from 2 ± 0.4 mM in the nonchallenged control to 4 ± 0.2 in the racemic-acetoin-challenged culture. However, still no D- or L-2,3-BD was observed.

5.4 Discussion

Acetoin reductase (ACR) is an enzyme that catalyzes the reduction of acetoin to 2,3-butanediol (2,3-BD). Although *Clostridium beijerinckii* NCIMB 8052 con-

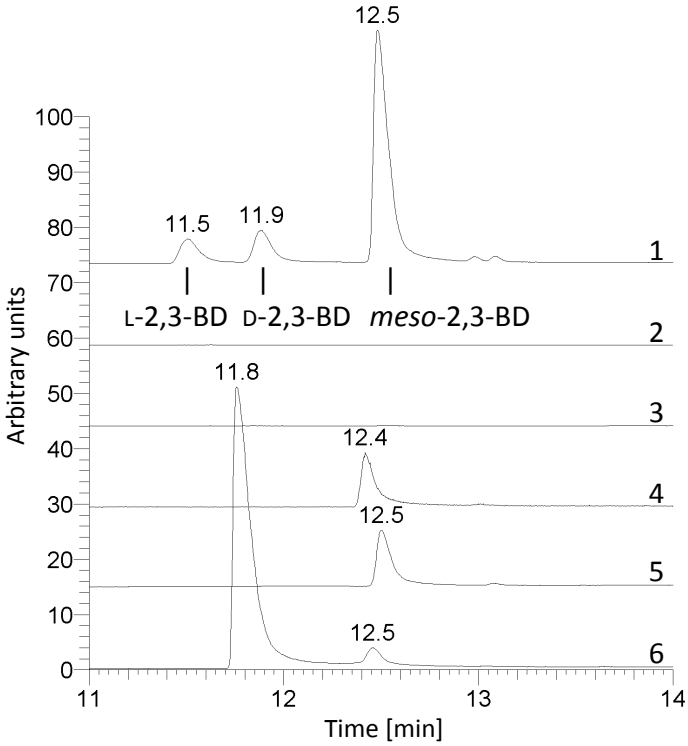

Figure 5.3: GC-MS chromatograms (single ion mode; $m/z = 45$) of extracts of a standard, GM medium, and samples taken at the end of the fermentation of wild-type and transformant cultures. Chromatogram 1, mixture of all three 2,3-BD stereoisomers; chromatogram 2, GM medium blank; chromatogram 3, fermentation of *C. acetobutylicum* ATCC 824; chromatogram 4, fermentation of *C. acetobutylicum* WUR; chromatogram 5, fermentation of *C. acetobutylicum* WUR harbouring the empty vector (pMTL500E); chromatogram 6, fermentation of *C. acetobutylicum* WUR harbouring the pWUR460 construct containing the *acr* gene. The retention time of the D-2,3-BD peak in chromatogram 6 is somewhat different due to the high concentration. Spiking experiments confirmed that it is indeed the D-stereoisomer.

tains a homologue (*Cbe_1464*) of a *Bacillus cereus* 2,3-butanediol dehydrogenase gene in its genome,[284] 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*. In this study, we showed that when the *C. beijerinckii acr* (*Cb-acr*) gene is expressed in *C. acetobutylicum*, natively produced acetoin is reduced to D-(2*R*,3*R*)-2,3-BD (Table 5.3). This conversion is in agreement with the proposed acetoin reductase function of the cloned *C. beijerinckii*

Table 5.4: Net change of extracellular acetoin and 2,3-butanediol concentrations in challenged batch cultures of *C. acetobutylicum*transformants harbouring pMTL500E, pWUR459, or pWUR460, after 72 h of fermentation, compared to their inoculation levels

Plasmid	Challenge[a]	Nett change in extracellular concn [mM] of:[b]			
		Acetoin	D-2,3-BD	*meso*-2,3-BD	Acetoin + 2,3-BD
pMTL500E (control)	No challenge	15 ± 1	0	2 ± 0.4	17 ± 1
	Acetoin[c]	13 ± 2	0	4 ± 0.2	17 ± 2
	D-2,3-BD[d]	12 ± 2	−0.7 ± 1	1 ± 0.05	13 ± 2
	meso-2,3-BD[e]	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[g]	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	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

[a] Racemic acetoin, D-2,3-BD, or *meso*-2,3-BD was added to the medium before inoculation at a concentration of 20 mM.

[b] Data are given as the means ± standard deviations for three replicate fermentations and calculated by subtracting the concentration after 72 h from the concentration at the time of inoculation. For example, for the D-2,3-BD challenge of *C. acetobutylicum* carrying pWUR460, the initial D-2,3-BD concentration of 20 mM was subtracted from the final concentration of 40 mM, resulting in a nett production of 20 mM D-2,3-BD.

[c] The medium was supplemented with racemic acetoin.

[d] The medium was supplemented with D-(2*R*,3*R*)-2,3-BD.

[e] The medium was supplemented with *meso*-2,3-BD, which also contained approximately 10 % racemic D/L-2,3-BD.

[f] Analysis was done by nonchiral HPLC, so no separation of enantiomers was possible; however, on the basis of previous results, the D-enantiomer is expected to have been formed.

[g] Negative values indicate consumption of acetoin relative to inoculation conditions.

gene based on functional expression in *E. coli* and with a recent publication on an acetoin reductase (BdhA) of *Bacillus subtilis*.[285] The amino acid sequence of this enzyme is very similar to the *C. beijerinckii* ACR with 51 % identical residues and 66 % similar residues.

Clostridium acetobutylicum ATCC 824 is known to produce acetoin as a minor fermentation product, but it has never been reported to produce *meso*-2,3-BD.[286,287] Our analysis of fermentations of an independently obtained ATCC 824 type strain confirms this. In the course of this study, however, we found that both the wild type and the plasmid control of the *C. acetobutylicum* WUR strain does produce *meso*-2,3-BD. Apparently our laboratory stock, originally acquired as the ATCC 824 type strain, evolved a divergent phenotype. Despite this, we con-

tinued to investigate the fermentative behaviour of our transformant strains, as they showed interesting properties.

An earlier report describing an attempt to engineer *C. acetobutylicum* to produce 2,3-butanediol by heterologous expression of a *Klebsiella pneumoniae* ACR was unsuccessful.[287] In contrast to the reported approach, we decided to express the *C. beijerinckii acr* gene, which has a GC content (35 %) which is comparable to that of the DNA of the *C. acetobutylicum* host (31 %), and a similar codon usage (Codon Usage Database [http://www.kazusa.or.jp/codon/]). 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*[142,288] than the plasmids derived from low-copynumber pSOS84 (pIM13 origin) used by Wardwell *et al.*[287] Combined with strong promoters (either P_{adc} or P_{thl}), our approach resulted in levels of expression of the *Cb-acr* gene by *C. acetobutylicum* high enough to lead to 2,3-BD production, despite the fact that the detected specific activity is relatively low.

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 fermentation. Both transformant strains were able to convert approximately 90 % of the natively produced acetoin into D-2,3-BD, with values reaching 22 to 23 mM. Compared to the industrial strain *Klebsiella pneumoniae* SDM, which reaches concentrations up to 1 664 mM, this is relatively low.[289] However, for this species, 2,3-BD is the main fermentation product, whereas for our strain, 2,3-BD is only a side product next to the solvents butanol and acetone whose levels remained unaltered.

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-BD was concomitantly higher in the *thl*-controlled culture. This difference in acetoin and 2,3-BD levels between the *adc*- and *thl*-driven *acr* expression is in good agreement with the differences in promoter activity.[227,228] Later during the fermentation, this difference levelled out. Despite constitutive expression of *Cb-acr* by the strain containing pWUR460, accumulation of acetoin in the medium was still observed, which suggests that the acetoin production flux under these conditions is higher than the flux from acetoin to 2,3-BD can accommodate.

5.4.1 Identification of bottlenecks for 2,3-BD 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 5.4) indicates that acetoin production is not affected by its extracellular concentration at the concentrations tested. This is in agreement with the

fact that in nonchallenged fermentations of *Cb-acr*-expressing transformants, the combined levels of acetoin and 2,3-BD do not significantly exceed those of the acetoin levels of the control strain fermentation. In racemic-acetoin-supplemented fermentations with Cb-ACR expressing strains, all of the additional acetoin was converted into D-2,3-BD and *meso*-2,3-BD. This lack of selectivity for the stereochemistry of the substrate is not uncommon among acetoin reductases.[290]

When exogenously added D-2,3-BD or *meso*-2,3-BD (20 mM) is present during fermentation, the amount of converted acetoin is not affected, resulting in final D-2,3-BD levels of 40 mM. This shows that at the levels tested, the amount of 2,3-BD is not inhibitory to the reaction or its production. On the basis of these results, we conclude that the formation of acetoin, for the fermentation as a whole, is the limiting factor for the production of 2,3-BD, even though, initially, acetoin accumulates in the medium (Figure 5.2).

5.4.2 2,3-BD production model

On the basis of the combined results of the wild-type strain, strain carrying the plasmid control, and strains expressing the *Cb-acr* gene, in normal and challenged cultures, we propose the model shown in Figure 5.4 (page 118) for acetoin and 2,3-BD production in our *C. acetobutylicum* strain. The introduced ACR enzyme can convert both D- and L-acetoin enantiomers into D-2,3-BD and *meso*-2,3-butanediol, respectively. The wild type and the plasmid control strain produced small amounts of *meso*-2,3-BD and no detectable levels of D- or L-2,3-BD, as confirmed by GC-MS analysis. In the acetoin-challenged cultures, the *meso*-2,3-BD formation by the control strain (pMTL500E) doubled from 2 mM to 4 mM. Most likely, one or more of the dehydrogenases that are present in *C. acetobutylicum* WUR do, to some extent, accept acetoin as a substrate. This would suggest that it is D-acetoin that is the source of the endogenous *meso*-2,3-BD production as the native acetoin enantiomer ratio is 12:1 (D:L). Thus, in the acetoin challenge experiment, the levels of D-acetoin are increased approximately 1.7-fold (from 14 mM to 24 mM), while the L-acetoin concentration increased more than 10-fold (from 1 mM to 11 mM). If L-acetoin were the source of the endogenous 2,3-BD production, then a more-substantial increase of *meso*-2,3-BD production would be expected.

5.4.3 Future perspective

If a pathway could be established in which 2,3-BD 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. The less-toxic nature of 2-butanol compared to 1-butanol[280] would make it an alternative approach to circumvent the limited butanol yield of the classic ABE fermentation.

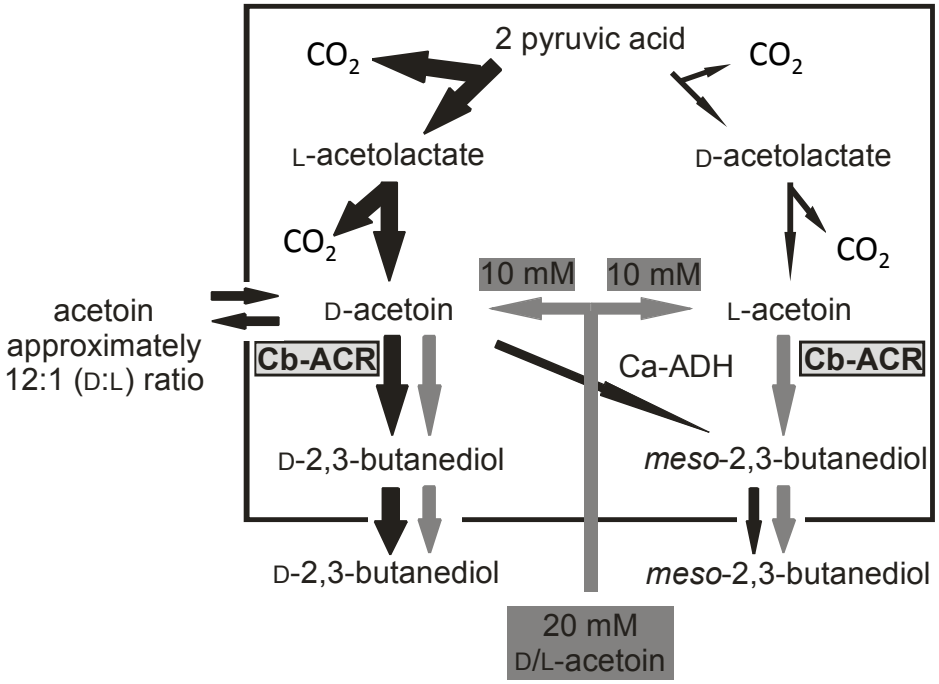

Figure 5.4: Proposed 2,3-BD biosynthesis pathway in the ACR-expressing *C. acetobutylicum* transformants of this study. The boxed area indicates the intracellular space. The size of the arrows indicates the relative flux toward D- and L-acetoin from pyruvic acid. The grey arrows illustrate the impact of supplementing the medium with 20 mM racemic D/L-acetoin. The *C. beijerinckii* acetoin reductase (Cb-ACR) enzyme is indicated in bold type on a gray background. The proposed conversion of D-acetoin to *meso*-2,3-BD by an endogenous alcohol dehydrogenase (*C. acetobutylicum* alcohol dehydrogenase [Ca-ADH]) is indicated. In the control strain fermentation, the ratio of the two acetoin enantiomers is 12:1 (D:L).

5.5 Acknowledgments

We thank P. Simon Extremera for performing the challenged culture experiments. This work was financially supported by the Netherlands Ministry of Economic Affairs and the B-Basic partner organizations (www.b-basic.nl) through B-Basic, a public-private NWO-ACTS program (ACTS stands for Advanced Chemical Technologies for Sustainability).

5

Chapter 6

General discussion and conclusions

6.1 Introduction

Since the oil crises of the 1970s more research efforts were focused on the production of chemicals from renewable resources that are normally derived from petroleum. Those events and later developments and made our increasing dependence on oil and its derived products clear. Besides the, in short term, non-renewable nature of petroleum and the insecurity of supplies, another driver for a search for alternatives is the supposed anthropogenic global warming that occurs as a result of increasing carbon dioxide concentration in the atmosphere.

Production of some of these chemicals through fermentation offers the possibility of using sugars from plant materials, which are in principal renewable, as a new resource. Butanol has applications as a replacement of transport fuel, superior to ethanol, and is used as a feedstock for the chemical industry. The biological production of butanol as part of the acetone-butanol-ethanol-process has been known for over a century, but despite many efforts to improve the butanol yield, the process is currently economically uncompetitive.

The research described in this thesis was undertaken with the aim of improving the butanol production by *Clostridium acetobutylicum* using metabolic engineering techniques. Here the work reported in the preceding chapters will be put into perspective and the initial assumptions of the work will be discussed.

6.2 Premiss

Production of butanol through fermentation is performed by various Clostridial species, most notably *C. acetobutylicum* and *C. beijerinckii*. However, in

all wild-type strains, production of other products is observed next to butanol, such as the short-chain fatty acids acetate and butyrate, the solvents acetone and ethanol, and hydrogen gas. Acids are produced by the organism to maximise ATP-production but results in a decreasing pH of the extracellular medium. After a certain time the organism starts to produce neutral solvents, presumably to prevent further acidification which would lead to inactivation of cellular processes resulting in cell death.[265] The premiss for this work was that it should theoretically be possible to modify the metabolic network in such a way that a homo-fermentative butanol strain can be obtained, as per Equation 6.1.[‖]

$$C_6H_{12}O_6 + 2\ ADP + 2\ P_i + 2\ H^+ \rightarrow 2\ ATP + 3\ H_2O + 2\ CO_2 + \ C_4H_{10}O \quad (6.1)$$

Fermentation of 1 mole of glucose could then result in 1 mole of butanol being formed in addition to 2 moles of carbon dioxide and 2 moles of ATP. This is in fact equivalent to homo-fermentative ethanol production as carried out by yeasts and, from an energy yielding perspective, also to the lactic acid fermentation which results in the same amount of ATP being generated.

Research by Maddox and Bahl *et al.* on fermentation of whey by *C. acetobutylicum* gives further support for the idea that the organism is able to grow on production of *almost* exclusively ethanol and butanol with very little acid and acetone production.[105,291] Later studies into the so-called alcoholgenic fermentation identified other conditions under which predominantly alcohols are produced.[255–259] However, no studies are published that address the question if the ATP production from glycolysis alone is enough for growth or cellular maintenance of *C. acetobutylicum*, as this would have be the case for a true butanol-only, or alcohol-only mutant.

6.3 Biological butanol production

There is no known organism that only produces butanol anaerobically as major liquid product, either naturally or by optimising the process conditions. Therefore, there are two options if we want to use biological means to convert biomass to butanol. One is to search for a novel organism, or combination of organisms, that can perform this conversion. The other is to metabolically engineer an organism to fit the requirements. The challenge with the former approach is that there is no known method to quickly select for (enhanced) butanol production, leaving screening as an alternative, which is a virtually insurmountable task. The lack of a positive selection mechanism or screening system for increased butanol

[‖]This representation should not be misconstrued to seem to indicate continous proton removal, because formed ATP will eventually be hydrolysed releasing the same amount of protons. Neither is there a charge inbalance as ADP, ATP, and P_i also carry (unwritten) negative charges in this equation.

production also hampers an approach using random mutagenesis of butanol producing strains. This leaves metabolic engineering as the only avenue to rationally develop a butanol-only strain.

One of the most important parameters in a metabolic engineering approach is the choice of host organism. The host that is to be used for butanol production should be assessed based on the following criteria:

(**i**) resistance to butanol;
(**ii**) natural butanol production;
(**iii**) amenability to genetic engineering;
(**iv**) knowledge on the metabolic pathways and regulatory network;
(**v**) ability to ferment lignocellulosic substrates and other renewable substrates;
(**vi**) robustness of the strain under industrial process conditions; and
(**vii**) resistance to extreme conditions (pH, temperature) to facilitate non-sterile process conditions.

These various criteria will be discussed in the following sections.

Butanol toxicity

Although several clostridia are native butanol producers, their resistance to this product is limited. Above a butanol concentration of 1.0 %, growth is already severely reduced and typical production titres are hardly ever above 2.0 % (w/v). These low concentrations make product recovery more costly. Ethanol concentration after yeast fermentation is for example much higher, reaching up to 12 to 16 % (w/v). A similar sensitivity for butanol has been observed for a wide range of organisms including various *E. coli* and *Zymomonas mobilis* strains, and *Pichia* and *Saccharomyces* species. Positive exceptions are *Lactobacillus delbrueckii* and *L. brevis*, that can tolerate up tot 2.5 % and 3.0 % butanol in the growth medium, respectively.[195] Also *Pediococcus pentosaceus*, *P. acidilactici*, *Enterococcus casseliflavus*, *Leuconostoc pseudomesenteroides*, and *Lactobacillus plantarum* were reported to still show growth above a concentration of 3.0 % butanol in the medium.[196]

Because resistance to general stresses, such as those induced by butanol, is difficult to engineer, as they are dependent on multiple genes and regulatory systems, resistance to the final product should be one of the most important parameters for host species selection. This phenotype is not difficult to select for, as has been shown for, amongst others, *E. coli*[292,293] and *C. acetobutylicum*.[133,134,136] The results of the selection procedures for 1-butanol resistance have been limited however, not reaching above those already obtained by the mentioned lactobacilli.

Endogenous butanol production

Before the advent of genetic engineering the organism to be used for production of a certain compound was required to already be able to produce this

compound (or a very similar one), if only suboptimal. Random mutagenesis could then be employed to try to increase production or eliminate additional unwanted reactions. Although butanol production can nowadays be engineered into an non-natural producer, there are inherent advantages to using natural butanol producing *Clostridium* species. These organisms have naturally been selected to achieve maximum growth within the constrains of a certain survival strategy. At the same time this is also a pitfall as it is important to realise that an organism was selected for growth, not for the optimised production of a certain compound. It is therefore highly beneficial in an metabolic engineering approach to couple (desired) product formation to growth as then a laboratory evolution approach could be used, to further improve production.

The fact that butanol as a (main) fermentation product was the exclusive domain of the solventogenic clostridia, made these bacteria the production host of choice for almost a century. This resulted in *C. acetobutylicum* becoming the model organism for solvent production. However, using genetic engineering techniques, it is nowadays relatively easy to endow a host with a new biochemical pathway for production of a desired compound, making this requirement far less important. But due to its 'industrial heritage', including extensive knowledge of the process, a wide substrate range including pentose sugars, and process robustness, and other factors,[115] *C. acetobutylicum* and related species, still feature prominently as the platform for butanol production.

Amenability to genetic engineering

Genetic tractability of the host is essential for a metabolic engineering approach to succeed. The possibilities of clostridia in this regard were not very good up to recently, with only limited genetic tools available. But the gene knock-out system for clostridia described in **chapter 2 part 2** and the recent plethora of options that has become available for *C. acetobutylicum* and other clostridia (see **chapter 1**) strengthens its position for continued use as a butanol production platform.

Metabolic pathways and regulatory networks

A thorough understanding of all pathways involved in production of a compound, and how they a regulated, is essential for a rational modification of the metabolism, especially when no positive selection pressure can be applied for production of the desired compound. The, only recent, discovery that the BCD/EtfAB enzyme complex, that catalyses the reduction of crotonyl-CoA to butyryl-CoA, of *C. kluyveri*, requires oxidised ferredoxin to function, as it gets co-reduced, highlights that one of the most basic pathways was not fully understood.[99] This discovery explained how in crude extracts an NADH-stimulated hydrogen production could be observed despite the theoretical limitations. It is also

still not elucidated what the actual reducing cofactor of the main aldehyde/alcohol-dehydroganese (*adhE* also known as *aad*) is.[294]

Next to these enzyme-related issues, also the global regulation mechanism of processes like sporulation and solventogenesis are very complex and still not really understood, although also in that area progress is being made.[107,161,162,180,275,295]

Fermenting renewable substrates

For producing second generation biofuels, it would be advantages if the host is able to break down complex lignocellulosic biomass into simple sugars (*i.e.* towards a consolidated bio-based process), and can utilise the various resulting sugars. In absence of these characteristics it becomes essential that these functionalities can be engineered into the host, which in all likelihood will be a complex undertaking. Alternatively other renewable substrates such as seaweed, glycerol from biodiesel and food wastes could be used which are, somewhat, less resistant to hydrolysis.

There are strong indications that the *C. acetobutylicum* genome encodes a cellulosome,[68] similar to various cellulolytic Clostridia. However they can not grow on cellulose as a substrate. They can grow on cellobiose and can convert various C5 (and C6) sugars, including xylose,[66,121] which is an advantage over various organisms regularly employed in metabolic engineering approaches such as *E. coli* and *S. cerevisiae*.

Extreme fermentation conditions

It makes the fermentation process in some regards easier when it can be operated under more or less extreme conditions. If the process can be operated at higher temperatures then huge savings can be made with regard to cooling costs and it might be possible to operate the process under non-sterile conditions. Similarly, if more extreme pH levels are tolerated by the organisms, then also this might allow for non-sterile operating conditions te be used. Unfortunately, *C. acetobutylicum* is neither thermophilic, nor tolerates pH extremes were many other bacteria can not grow. The need for sterile process conditions is evident from the known problems of contamination and spoilage of ABE-fermentations by lactic acid bacteria.[296,297]

6.4 Non-native butanol production hosts

The genetic recalcitrance and complex fermentation behaviour of *Clostridial* species, have let various researchers to heterologously express the clostridial genes of the butanol production pathway in genetically very accessible organisms such as, *E. coli*,[197,198,298] *S. cerevisiae*,[199,201] *P. putida*, *B. subtilis*,[199] and *L. brevis*.[200] In all cases, the produced amounts of butanol are below 0.1 g/L, except when a highly concentrated cell suspension is used (1.2 g/L)[198,298] or when

using rich medium with glycerol (0.55 g/L).[197] In our hands, *C. acetobutylicum* WUR by comparison routinely produces 12 g/L butanol and the *C. acetobutylicum* WUR *buk1*$^{-}$ strain produces 14.5 g/L (see **chapter 4**). All these approaches utilised the BCD/EtfAB enzyme complex of *C. acetobutylicum* for the reduction of crotonyl-CoA to butyryl-CoA, which, as was mentioned above, requires oxidised ferredoxin as a co-substrate for activity. This is most likely the cause for the very low butanol yields.

Liu *et al.* developed an approach in *Lactobacillus buchneri* dependent on the natively present enzymes for conversion of acetoacetyl-CoA to butanol.[299] Acetoacetyl-CoA was made by the bacterium by introducing the clostridial thiolase gene and this resulted in a butanol titre of 66 mg/L. The breakthrough in exogenous butanol production was made by Shen *et al.*. who recognised that the clostridial BCD/EtfAB complex was one of the main reasons for the low yields and they instead employed an NADH dependent *trans*-enoyl-CoA reductase from *Treponema denticola*.[300] In addition, they employed a strategy where deletion of all endogenous NADH-consuming pathways from the *E. coli* host, resulted in an 'NADH driving force' to utilise the butanol production pathway. By doing so, they coupled survival (growth) to butanol production, allowing them to reach a final butanol 'concentration' of 30 g/L, using gas-stripping to overcome the, apparent, toxic limit of 3.8 g/L for their strain. Although the production properties of the modified *E. coli* strain are impressive at 70 % yield of theoretical, it is still very sensitive towards butanol and the proposed pathway should ideally be transferred to a more resistant host organism to see if the results can be improved.

Another novel approach by the same lab was to use non-fermentative pathways for the production of various linear and especially branched chain alcohols.[202] The method relied on the use of the highly active amino acid biosynthetic pathways and diverts the 2-keto acid intermediates for alcohol synthesis using a 2-keto-acid decarboxylase (KDC) enzyme for aldehyde production and an aldehyde dehydrogenase from yeast for reduction to the corresponding alcohol. Also the β-oxidation pathway has been used to produce butanol by deriving butyryl-CoA from operating the pathway in reverse to the normal physiological situation.[301] The method has the advantage of generating a higher ATP-yield than the 2-keto-acid pathway for linear alcohols (see Figure 6.1). Especially the last two methods look promising, but the low butanol resistance of *E. coli* means that these methods need to be transferred to a more resistant host, possibly even *C. acetobutylicum* itself.

To take advantage of the versatile substrate utilisation properties of clostridia, its reduced number of fermentation products (only acetate and butyrate), and its less complex life-cycle *Clostridium tyrobutyricum*, a non-solventogenic species, has been modified to over-express the AdhE2 aldehyde/alcohol dehydrogenase from *C. acetobutylicum*.[302] Depending on the plasmid used, the mutants produced butanol between 0.019 and 1.1 g/L, compared to approximately 7.8 g/L butyrate and 2.8 g/L acetate. Over-expression of AdhE2 in an acetate kinase knock-out background improved butanol production 9-fold to 10.0 g/L, but still 5.8 g/L

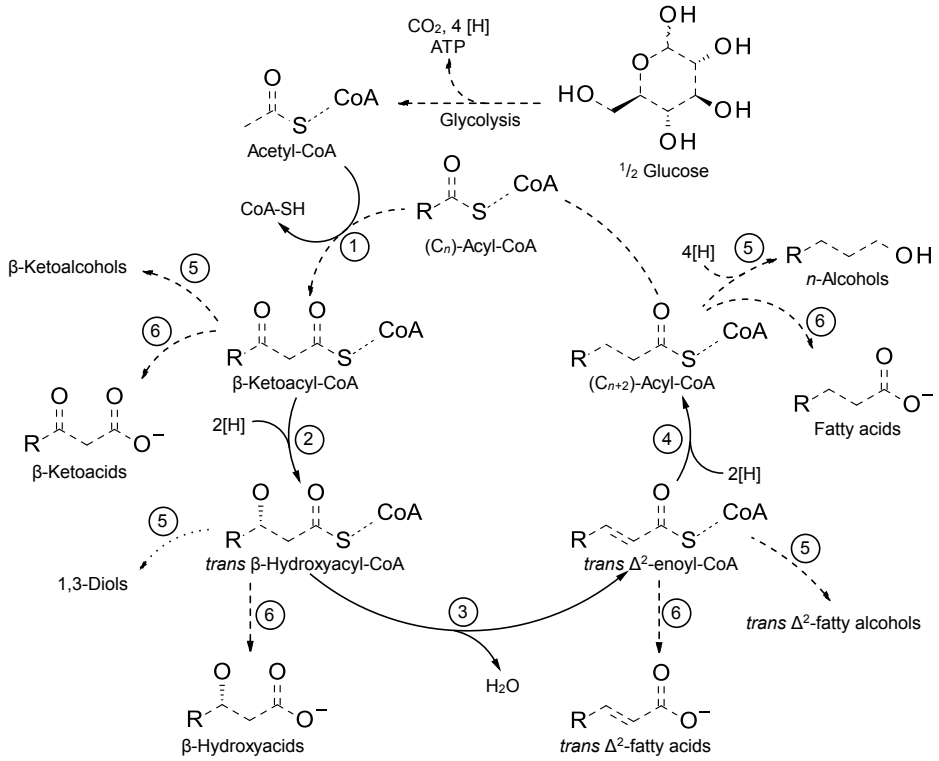

Figure 6.1: Illustration of the functional reversal of the β-oxidation cycle as demonstrated by Dellomonaco *et al.*[301]. The following enzymes (gene names in parentheses) are involved in the functional reversal of the β-oxidation cycle: (1) thiolase (*yqeF*, *fadA*); (2) hydroxyacyl-CoA dehydrogenase (*fadB*); (3) enoyl-CoA hydratase (*fadB*); (4) enoyl-CoA reductase (*ydiO*). Each turn of the cycle generates an acyl-CoA that is two carbons longer than the initial acyl-CoA thioester (indicated as C_{n+2}). Intermediates in the engineered pathway can be converted to a functionally diverse set of molecules using aldehyde-forming acyl-CoA reductases and alcohol dehydrogenases (5) and acyl-CoA thioesterases (6). R, side chain attached to the acyl-CoA group of the starter molecule (for example, R = H for acetyl-CoA and R = CH_3 for propionyl-CoA); 2[H], NADH/NADPH/ferredoxin. Dotted lines indicate multiple steps. Dashed lines without arrowheads connect identical metabolites of different chain length. Picture redrawn from Dellomonaco *et al.*[301]

butyrate and minor amounts of acetate were produced. The yield from glucose was 66 %. These initial results are interesting, especially the high butanol to ethanol ratio and high butanol yield combined with the absence of acetone is promising and demonstrate the compatibility of the pathway between the two clostridial species.

6.5 Metabolic engineering of *Clostridium acetobutylicum*

The industrial history of the ABE-fermentation, the large body of research available on solventogenic clostridia, and the (very) low levels of butanol production by heterologous hosts are all reasons for the continued research efforts into the clostridial ABE fermentation. The mix of fermentation products, with a different preferred product depending on the circumstances at the time, has always been a target for optimisation, with initially acetone as the preferred product, later butanol.

At the start of the research project described in this thesis, the first 'mature' metabolic engineering tools started to become available for the *Clostridium* genus (**chapter 1**). Targeted gene inactivation using group II introns was followed by double cross-over homologous recombination based approaches that improved upon the already available single cross-over method which had significant disadvantages.[107,163]

Electroporation for the transformation of DNA into *C. acetobutylicum* was already an established technique, although preparing the cells is laborious and time consuming. A useful addition was our discovery of storage conditions for electro-competent cells, allowing for the batch preparation of these cells for later use (**chapter 2 part 1**). It was shown that over a period of more than 30 months, cells retained their original competence and that even after 54 months cells were still transformable. Although it is of limited use to keep cells for such a long period, it does illustrate the robustness of the method. The principle on which it is based, exclusion of oxygen, suggests that it might also be applicable to the storage of other obligate anaerobes.

The focus of the research described in this thesis was to eliminate the production of especially acetone to improve butanol formation since impeding the acetone pathway results in high acetate levels.[124,175] The attempts of Tummala *et al.*and Sillers *et al.*both resulted in approximately 50 % acetone reduction and more than 15-fold increased ethanol production, but acetate production continued. I sought another method of eliminating it without the associated increased acetate accumulation. As acetone is formed by the uptake of acids, it should be possible, by eliminating their production, to stop acetone accumulation, and divert product formation exclusively to the alcohols.

6.5.1 Acetate kinase (*ack*) inactivation

In line with our strategy to eliminate acid formation and force *C. acetobutylicum* to ferment glucose exclusively to alcohols, we inactivated acetate kinase as described in **chapter 3**. The choice to inactivate the second enzyme, *ack*, of the *pta-ack* operon, was made to investigate the suggestion by Zhao *et al.*[165] that accumulation of acetyl-phosphate is not linked to initiation of solvent formation,

but that it is instead the intra-cellular butyryl-phosphate levels that might have a regulatory function, similar to acetyl-phosphate in *E. coli*.[238]

Although no intracellular concentrations for acetyl-phosphate were determined to prove that it accumulates, it is expected that it does, analogous to the *buk1*$^-$ mutant. The *ack* mutation was expected not to affect the initiation, or the level, of solvent formation, except for acetone, which is expected to be reduced.

The results described in **chapter 3** show that when the *ack*$^-$ mutant is grown in CGM, which does not contain acetate, there is no impact on acetate production. When grown on medium with acetate (CM1), a reduction of 128 % is seen in final acetate levels, while alcohol levels are not affected, confirming the expected behaviour. However, contrary to our expectations, acetone levels are unaffected.

The sensitivity to exogenously added acetate at the moment of inoculation is also observed when the *ack*$^-$ mutation is present in a *buk1*$^-$ background (**chapter 4**). These results combined suggest an alternative acetate producing pathway. However, it is either not expressed when increased acetate levels are present during inoculation, or the enzymes of the pathway might be easily inhibited.

6.5.2 Butyrate kinase (*buk1*) inactivation

We sought confirmation of the high butanol producing phenotype of a *buk1* knock-out mutant, as was published previously for the ATCC 824 strain.[128] In our WUR strain, the phenotype of this mutation turned out to different. Although butanol there too was a major fermentation product, it did not accumulate to the same level as that of the mutant of the ATCC 824 strain (**chapter 4**). Instead, we observed co-production of butanol and acetate, with fermentations at different pH-levels and medium compositions suggesting that it is acetate that was accumulating to toxic levels. Butyrate production was indeed severely inhibited, with enzyme assays confirming the inactivation of butyrate kinase.

The increased acetate production was also observed in the ATCC 824 strain mutant, but in that case, a distinct solvent switch, like for the wild-type, is observed, resulting in re-uptake of the acids and in solvent production. In our mutant strain we did not observe this switch. Butanol is detected at the same time point as acetate. To improve butanol production, acetate, normally included in CM1 medium, was not added and the pH of the fermentation was optimised. This resulted in a 21 % increase in butanol production and an improvement in butanol selectivity from 55 % to 70 % (expressed as butanol divided by the sum of acetone, butanol, and ethanol). Although this improved butanol production selectivity, it still resulted in acetone production and massive acetate accumulation in the medium. The absence of the solventogenic switch fits with the asporogenic nature of the strain. In addition, it is another example, next to solventogenic continuous cultures, of cells that can produce all solvents during vegetative growth as also observed by cell sorting experiments.[113]

Despite high acetate levels, the mutation does seem to trigger production of what could be called an 'over-flow metabolite', namely acetoin. Normally only

present at low levels (∼10 mM) but in fermentations of the BUK1KO strain at higher pH levels this was increased almost five-fold to 49 mM. Unlike lactate formation which would be redox-neutral, the formation of acetoin results in net NADH production which the organism must dispose of in some other way.

6.5.3 Dual acid pathway inactivation

To eliminate further acetate and thereby acetone production, we targeted acetate kinase for gene disruption using our own TargeTron based system (**chapter 2 part 2**). A mutant was successfully isolated and enzyme assays confirmed the inactivation of both butyrate kinase and acetate kinase. The fermentation pattern of the mutant however was unexpectedly similar to the wild-type at all tested pH levels in CM1 medium. If acetate was omitted from the CM1 medium and the pH was not controlled, then acid levels were extremely low, however there was still a large amount of acetone produced (55 mM), indicating continued acid production. The results indicate that continued acid production in the case of the *ack* mutant (**chapter 3**) and most likely also for the *pta* mutant[126,179] is, at least in part, due to an additional acetate producing pathway and not, or not only, due to catalysis by the homologous enzyme of the butyrate pathway. The existence of such a pathway might also fit with the observation that exogenously added ^{13}C labelled acetate quickly labels acetyl-phosphate pools,[101] while this is not expected on the basis of the PTA-ACK pathway. However, what the physiological function of the additional pathway, or alternative enzyme or enzymes with these characteristics would be is unclear.

Up to now it is not known which pathway could be involved, and if the *buk1*$^-$ *ack*$^-$ (DAPKO) strain had accumulated additional mutations explaining the phenotype. We therefore initiated a whole genome resequencing project using the Illumnia platform, of both the WUR strain and the WUR DAPKO mutant strain (data not shown). The preliminary results indicate that although the DAPKO strain has approximately 190 point mutations compared to the published genome sequence, the parental WUR strain has these too. It is of interest to note that also the ATCC 824 reference strain contains most of the same point mutations, suggesting errors in the reference sequence (unpublished results W. Kuit & N. P. Minton).

The results thus far showed that no additional mutations occurred while isolating the DAPKO mutant strain, establishing that the phenotype observed is due only to the two introduced mutations. This supports the hypothesis that alternative acetate and/or butyrate production pathways are present in wild-type *C. acetobutylicum* and that these are responsible for the observed acetate production.

It should be mentioned that also Lehmann *et al.* attempted to isolate a mutant with both acid producing pathways inactivated. Their strategy was based on obtaining a *pta*$^-$ *ptb*$^-$ mutant, however while both the *pta*$^-$ and *ptb*$^-$ single mutant could be obtained, a double mutant could not.[166] At this point we can

only speculate why this would be the case as we were able to obtain a double mutant even without selection pressure, contrary to their approach. While their *pta*⁻ mutant, similarly to our *ack*⁻ mutant, showed continued acetate production, their *ptb*⁻ mutant produced no butyrate. It could be that this gene, or its product, becomes essential in an *pta*⁻ background. Additionally, strain differences or even growth medium, could also explain the inability to obtain the mutant.

6.6 Butanol stress

The general consensus in the ABE-fermentation field is that it is the toxicity of butanol that limits further production[57,130] due to disruption of cellular functions caused by solubilisation of the cellular membrane[132] and other mechanisms.[130,131] Many efforts have been made in the past to obtain clostridial strains with increased butanol tolerance. Both chemical mutagenesis[133] and adaptation strategies[136] have been used 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.[134] In the case of Van Der Westhuizen *et al.*,[303] the increased butanol resistance was an unexpected trait of an autolysis-deficient mutant. Soucaille *et al.*[304] reported a butanol resistant mutant displaying increased autobacteriocin activity.

In different approaches, the over-expression of two heat-shock related proteins, GroES and GroEL,[137] over-expression of an endogenous putative transcriptional regulator (CAC1869),[135] or disruption of SMB_G1518 or SMB_G1519,[305] resulted in *C. acetobutylicum* mutants with increased butanol tolerance. But since butanol resistance and production do not seem to be directly related these approaches have met with limited success. This indirectly challenges the assumption that it is butanol toxicity that inhibits the ABE-fermentation. This is supported by various engineered strains that have a higher butanol production without specifically being optimised for butanol resistance. It therefore seems that what actually stops the fermentation is more complex than generally assumed.

In our research we tried two alternative approaches to either deal with, or reduce, the stress resulting from 1-butanol accumulation in the growth medium. The first one was to help the cells withstand the stresses they are exposed to by adding various compounds, known as compatible solutes, to the medium and monitoring the butanol production level of these cultures. The second approach was a first step towards modifying the metabolism of *C. acetobutylicum* so that it would no longer produce 1-butanol, but ferment glucose to 2-butanol: a compound less toxic for *C. acetobutylicum* cells, while still retaining the same beneficial bio-fuel properties.

6.6.1 Compatible solutes

We selected a range of compatible solutes and their applied concentrations from the literature.[306–315] When these were added to CM1 growth medium,[216] we tested whether this resulted in increased butanol production. The compounds tested and at what concentration is shown in Table 6.1. Normally the 60 g/L glucose present in the medium is almost completely fermented. In order to allow the cells to produce more solvents, the glucose concentration was raised to 90 g/L. Tests showed that the increased glucose concentration did not affect the fermentation. 1 mL of an overnight pre-culture was used to inoculate flasks in duplicate. Flasks contained 50 mL CM1 medium with the compatible solute and the fermentations were monitored over a period of 6 days. Unfortunately, none of the compatible solute test flasks contained significantly more butanol or total solvents than the control flasks. We therefore had to conclude that under the tested conditions the selected solutes did not enable *C. acetobutylicum* to produce more butanol, or solvents.

Table 6.1: Potential compatible solutes and their tested concentrations

Tested concentrations of potential compatible solutes in ABE fermentations		
0.01 M and 0.1 M	0.05 M and 0.5 M	0.2 M and 1 M
trimethylamine *N*-oxide (TMAO)	L-carnitine	sucrose
putresceine	L-glutamate	D-(−)-sorbitol
ectoine	L-proline	D-(+)-trehalose
hydroxyectoine	choline	*meso*-erythritol
	dimethyl glycine	glycerol
	glycine betaine	

6.6.2 Alternative solvent products

An alternative strategy for avoinding butanol stress that we explored was to see if less toxic butanol alternatives could be produced by *C. acetobutylicum*. Data from the literature suggests[280], and experimental results[316] confirm, that several 1-butanol analogues are less toxic for *C. acetobutylicum*. Amongst these are *iso*-butanol (2-methyl-1-propanol), 2-butanol, *tert*-butanol (2-methyl-2-propanol), and *meso*-2,3-butanediol. 2-butanol has almost the same properties as 1-butanol as far as its application as a bio-fuel is concerned and biochemical production seemed possible. It was therefore selected for further experimental work.

2-Butanol can be produced by reduction of 2-butanone, which in turn can be produced by dehydration of 2,3-butanediol. Although *C. acetobutylicum*

ATCC 824 does not produce 2,3-butanediol, it does produce its precursor acetoin (3-hydroxybutanone). In **chapter 5** we demonstrated that *C. acetobutylicum* WUR, but not *C. acetobutylicum* ATCC 824, natively produces minor amounts of *meso*-2,3-butanediol and through expression of a *C. beijerinckii* acetoin reductase (ACR) from plasmid pWUR459 (P_{adc}-*acr*) or pWUR460 (P_{thl}-*acr*) can also produce D-(2*R*,3*R*)-2,3-butanediol.

For the next step, a dehydratase was needed to convert the 2,3-butanediol diasteriomer to methyl ethyl ketone (2-butanone). However, only a dehydratase with *meso*-2,3-butanediol specificity[317,318] has been described but not one for the D or L diasteriomers.[319] This therefore precludes its use in combination with the *C. beijerinckii* ACR.

The specificity of the *C. beijerinckii* ACR was not known and had to be experimentally tested. To be able to achieve 2-butanol production, it would be easiest to express a known *meso*-2,3-butanediol forming acetoin reductase such as one from *Klebsiella pneumoniae* IAM 1063[320], but optimise its expression to avoid the problems Wardwell *et al.* had,[287] instead of the *C. beijerinckii* ACR and combine this with the *meso*-2,3-butanediol dehydratase enzyme from *Lactobacillus brevis*.[317,318]

If production of 2-butanol is possible then an attempt at a homo-fermentative 2-butanol strain can be made by inactivating the pyruvate:ferredoxin oxidoreductase enzyme complex (PFOR) by gene disruption of one of its components. This enzyme complex catalyses the conversion of pyruvate to acetyl-CoA and CO_2 while reducing ferredoxin. The resulting metabolic system can then possibly bypass the regulatory network that is operating in *C. acetobutylicum* so that a solvent switch is not necessary and neither is the initiation of sporulation. Both these processes make the fermentation production-wise inefficient due to reduced productivity because of these non-productive phases. Although these finding would be of academic interest, it should also be investigated if current 2,3-butanediol producers, certain *Bacillus* and *Klebsiella* species amongst others, are sufficiently resistant to, and can be used for, production of 2-butanol.

6.7 Recommendations

6.7.1 Butanol-only production strain

In section 6.2 it was explained that it is generally assumed[194] that a homo-fermentative 1-butanol strain can be made because substrate and product are in redox balance. It has however not been proven that the organism can grow and survive on the yield of 2 ATP from that conversion, or that some of the intermediates are somehow essential for cell survival.

A straightforward way to tackle this question would be to basically turn *C. acetobutylicum* into a lactic acid fermenting organism. When a lactate dehydrogenase (*ldh*) gene would be constitutively expressed in *C. acetobutylicum* it would enable a homo-lactic acid fermentation if the new pathway would be able

to sustain the full glycolytic pyruvate flux. The major pathway competing with lactate formation would be the conversion to acetyl-CoA. If an ATP-yield of 2 ATP per glucose is enough for cell growth, then a strain expressing *ldh* should allow inactivation of the PFOR enzyme complex, similar as discussed for the proposed 2-butanol producing pathway in subsection 6.6.2.

An alternative approach could be a homo-ethanologenic strain, as this would seem synonymous from a redox and ATP-yield basis. Would seem synonymous, as no Rnf-complex has been detected in the genome of *C. acetobutylicum*.[166] It could however be that, *via* an undetected Rnf-complex, or some other mechanism, *C. acetobutylicum* is able to generate energy from the low redox-potential electrons from reduced ferredoxin like many other Clostridia. A recently constructed strain, defective in C4 product formation, by disruption of the 3-hydroxybutyryl-CoA dehydrogenase (*hbd*) gene, can apparently exist. If further inactivation of the acetate pathway can be accomplished, then only ethanol production should remain. However, as we have shown in **chapter 4**, it seems that there are multiple acetate/acid producing pathways in *C. acetobutylicum* WUR, and most likely *C. acetobutylicum* ATCC 824, which hampers this approach.

In my opinion it should first be investigated if a homo-fermantative 1-butanol strain can in theory exist using for example the above mentioned strategies, before attempting further metabolic engineering strategies aimed at improving butanol production.

6.7.2 Alternative production hosts: Lactic acid bacteria

The analysis of suitable alternative production hosts (see **section 6.3**) and the necessity to ascertain that the cell can support growth, or at the very least cellular maintenance, from the 2 ATP-yield from fermenting glucose to butanol, suggests that alternative production hosts should be more closely looked at. Notably some of the lactic acid bacteria (LAB) seem ideal production hosts due to their high 1-butanol resistance and ability to require only 2 ATP per fermented glucose.

Some researchers already looked at the possibilities of using LABs for butanol production.[200,299] But Berezina *et al.*[200] probably did not realise the associated problems with using the BCD enzyme. This in all likelihood negatively impacted their production which was only 300 mg/L. Expression of thiolase alone in *Lactobacillus buchneri* by Liu *et al.* already resulted in butanol production by endogenous enzymes but at low levels (66 mg/L). Although interesting in its own right, the authors realised that it will require further metabolic engineering to optimise butanol production in this strain. However, so far no follow-up reports were published.

The provocative approach taken by Dellomonaco *et al.*[301] to reverse the β-oxidation cycle in *E. coli* to couple two acetyl-CoA units, and more, in a cyclic manner, which could then be reduced by an bi-functional alcohol/aldehyde dehydrogenase to give butanol, or even higher linear alcohols, is a versatile method. It could potentially be used in various organisms, and lactic acid bacteria should

be a prime target to test this approach as a more suitable 1-butanol production organism. The main disadvantage of using lactic acid bacteria that should be mentioned is the extensive nutritional requirements these organisms have, which could be difficult to sustain for a large scale process such as envisioned for biobutanol production.

6.8 Concluding remarks

After more than a century, the biological production of butanol seems to be approaching an historic moment with the convergence of new genetic techniques in *C. acetobutylicum* and more knowledge on the enzymes and regulatory pathways involved in solvent formation. The insertional inactivation of genes involved in the two major acid-pathways as reported in this thesis seemed a logical approach to eliminate acetone production. However, the reported *C. acetobutylicum* strain with a double-knock-out of homologous enzymes in both the acetate and butyrate pathway, a first, did not result in the desired phenotype. Under certain conditions the mutant showed an improved butanol production selectivity compared to the wild-type, but it did clearly not perform an alcohologenic fermentation. The results further suggest that an alternative pathway, or pathways, is active in *C. acetobutylicum* which further research should identify, if this approach is to be successful in eliminating acid and acetone production. Future research should also focus on the conversion of pyruvate to acetyl-CoA to see if the organism is able to conserve energy stored in the reduced ferredoxins when it is not generating hydrogen gas, and related to that how its is able to reduce other cofactors involved in solvent formation.

The rapid strides made by researchers using heterologous hosts for the production of butanol have not gone unnoticed and alternative pathways for the production of higher linear and branched chain alcohols have been developed. Future projects should carefully consider for each process what the desirable characteristics of a production host are, and make a balanced decision with those requirements in mind. Each one will have its own pro's and con's but it should be selected based on those arguments and not merely because it has always been so, as we have now entered the era of synthetic biology.

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Summary

During the last few decades, there has been an increasing search for alternative resources for the production of products traditionally derived from oil, such as plastics and transport fuels. This has been prompted by the finite nature of our oil reserves, the desire for energy security, and by concerns about anthropogenic global warming. Petrol and diesel are the two main fuel types for land based transportation and are currently derived from oil. Butanol, a four-carbon alcohol that can be produced by certain bacteria in a renewable way, can be used as a direct petrol replacement. It also has multiple applications as chemical intermediate and as a solvent. Although it is similar to ethanol it has superior properties with regard to energy density, vapour pressure, and water solubility when applied a biofuel.

The acetone-butanol-ethanol (ABE) fermentation of sugars as carried out by various bacteria of the genus *Clostridium* has been widely applied in the first part of the 1900s as a commercial method to produce butanol and acetone. The two most used species have been *Clostridium acetobutylicum* and *C. beijerinckii*. Both produce not only solvents but also the unwanted acids acetate and butyrate. In the second part of the 20th century, the ABE-process became no longer economically competitive with the petrochemical process for the production of these solvents. But today's high oil prices make the fermentation process interesting again, although there are still challenges that have to be tackled before the process can be re-commercialised. These include finding ways to make it possible to use cheap biomass feedstocks (such as lignocelluloses) as substrate rather than using traditional feedstocks such as starch and molasses, which are relatively expensive. In addition this replacement would avoid the food-versus-fuel dilemma. Another challenge is to improve butanol production, yield, and titre. The work described in this thesis focuses on the enhancing of butanol production and diminishing acid formation by *C. acetobutylicum*.

A metabolic engineering approach was taken to reduce the number and amount of by-products in *C. acetobutylicum* fermentations. Production pathways of the acids acetate and butyrate were targeted, as we hypothesised that inhibiting acid formation would also prevent acetone production by *C. acetobutylicum*, resulting in only alcohols as the liquid fermentation products. To carry out our metabolic engineering work, we first developed an essential tool for gene disruption.

During this work we studied storage conditions for electro-competent *C. acetobutylicum* cells, allowing for the batch preparation of these cells for later use for up to 54 months (**chapter 2 part 1**). The principle on which it is based, exclusion of oxygen, suggests that it might also be applicable to the storage of other obligate anaerobes.

The **second part** of **chapter 2** describes the adaptation of the TargeTron gene knock-out system for use in *C. acetobutylicum*. The TargeTron system uses a mobile group II intron that can be 'retargeted', *i.e.* reprogrammed, to insert into a specific site in the genome in a process called retrohoming. We targeted the acetate kinase (*ack*) gene and successful insertion of the intron was demonstrated using a PCR test. But only after the development of a colony PCR protocol for *C. acetobutylicum* as described in **chapter 4**, we were able to apply our system and quickly detect pure mutants amongst the parental strain.

Another research group also developed a clostridial version of the TargeTron system and called it ClosTron. The advantage of this system over the one we developed is that inserted intron copies carry an activated erythromycin resistance gene and can therefore easily be selected. In **chapter 3** we used this system to obtain an acetate kinase gene knockout, which was extensively characterised in pH-controlled batch fermentations on two media; CGM and Clostridial Medium 1 (CM1). Enzyme assays showed a 98 % reduction in *in vitro* acetate kinase activity, however the mutant strain continued to produce wild-type levels of acetate in CGM which does not contain any added acetate. In CM1 that does contain acetate, acetate production could still be seen, but was severely reduced. These results suggest that alternative ways of acetate production may be active in *C. acetobutylicum*. The solvent production of the *ack*$^-$ strain was not significantly affected in CM1. When grown on CGM our wild-type strain produced large amounts of lactate and was therefore not suitable as a production medium. Interestingly our *ack*$^-$ mutant strain performed better.

Subsequently we created a strain with an inactivated butyrate kinase gene termed BUK1KO, as described in **chapter 4**. The phenotype of this strain was essentially that of an acetate-butanol producer. Analysis of the fermentation behaviour indicated that the strain never seemed to switch from an acidogenic to an solventogenic state, as the wild-type did. Furthermore, the growth on CM1 in batch culture demonstrated a strong influence of the pH on the fermentation behaviour. There was a good correlation between increasing fermentation pH and higher acetate levels within the pH range from 4.5 to 5.5, suggesting that the produced acetate levels might actually be the growth inhibiting compound. In addition, the mutant cells never produced the clostridial cell-types associated with spore formation. This is in line with the absence of a solventogenic switch. Also in parallel with the increasing fermentation pH was an increased acetoin accumulation with a maximum of 49 mM at pH 6.5 compared to 12 mM for the wild type under control conditions. Growth on CM1 without acetate at a pH of 5.5 resulted in a 21 % increase in butanol levels to 195 mM (14.5 g/L) compared to the wild type under its optimal conditions and 127 % under the same conditions.

There was also a 60 % reduction in acetone levels and slightly increased ethanol levels.

A subsequent inactivation of the acetate kinase gene in the *buk1*$^-$ negative background using our own TargeTron system (see **chapter 2**) resulted in isolation of an *ack*$^-$ *buk1*$^-$ double mutant. Despite abolishment of both acetate kinase and butyrate kinase enzyme activity *in vitro*, the mutant continued to produce both acids. In CM1, acetate levels were severely reduced compared to the parenteral *buk1*$^-$ strain, but when acetate was removed from the medium, large amounts of acetate were produced again. This behaviour is reminiscent of the *ack*$^-$ mutant and supports the hypothesis that unknown alternative acid producing pathways or enzymes exist in *C. acetobutylicum*. Alcohol production was negatively affected as compared to the parental strain and acetone production was not eliminated. Also at certain pH-levels acetoin production was even further increased to 100 mM, the highest reported value for this organism.

In an alternative take on improving butanol production titre, we envisioned a homo-fermentative 2-butanol strain. 2-butanol is less toxic to the cell and should, in the proposed pathway, be produced redox-neutral from glucose. In addition it retains all the beneficial biofuel properties. As a first step towards this goal, we demonstrated in **chapter 5** that an alcohol dehydrogenase from *Clostridium beijerinckii*, over-expressed in *C. acetobutylicum*, can accept natively produced D- and L-acetoin as its substrate and reduce it to D- and *meso*-2,3-butanediol. In addition we showed that our *C. acetobutylicum* WUR strain already produces small amounts (approximately 3 mM) of *meso*-2,3-butanediol through an unknown pathway, most likely from D-acetoin. No production of *meso*-2,3-butanediol was observed for the ATCC 824 strain. Completion of the pathway requires a dehydratase and a secondary-alcoholdehydrogenase to produce methyl-ethyl ketone and 2-butanol respectively.

In the general discussion (**chapter 6**) the results described in this thesis were put into perspective, and the existence of an alternative acid pathway in *C. acetobutylicum* is suggested. Furthermore the disadvantages and advantages of *C. acetobutylicum* as a butanol production platform are discussed together with developments of butanol production in heterologous hosts.

Samenvatting

De afgelopen decennia is er steeds meer belangstelling voor alternatieve manieren om producten te maken zoals plastics en brandstoffen, die traditioneel van olie gemaakt worden. Dit vanwege de realisatie dat aardoliereserves eindig zijn, dat landen die olie exporteren vaak politiek instabiel zijn en vanwege de vrees dat de aarde opwarmt door de uitstoot van CO_2. Benzine en diesel zijn de twee belangrijkste brandstoffen voor transport over land en worden momenteel vervaardigd uit olie.

Ethanol is een bekende biobrandstof die kan worden gemaakt uit maïs en suikerriet, maar heeft als nadeel dat deze voor onze huidige auto's slechts tot maximaal 15 % kan worden bijgemengd in benzine. Butanol is net als ethanol een alcohol, maar bestaat uit moleculen met een langere koolstofketen. Het voordeel van butanol is dat het hierdoor als volwaardige vervanger van benzine kan worden gebruikt in de huidige auto's. Het heeft daarnaast nog een aantal andere eigenschappen die het een betere benzinevervanger maken dan ethanol, zoals een hogere energiedichtheid, lagere dampspanning en een lagere wateroplosbaarheid. Naast de mogelijkheid om butanol als biobrandstof te gebruiken, is het ook een grondstof voor de chemische industrie en een oplosmiddel.

Net zoals ethanol kan butanol via biologische weg worden geproduceerd door micro-organismen. Waar ethanol echter door veel verschillende organismen gemaakt kan worden, is biologische butanolvorming beperkt tot het bacteriegeslacht *Clostridium*. Dit geslacht bestaat uit anaeroob groeiende, Gram-positieve, staafvormige, endospoorvormende bacteriesoorten. In het eerste deel van de twintigste eeuw, voor de opkomst van de petroleumindustrie, werd al op commerciële wijze butanol via fermentatie geproduceerd. Hiervoor werden hoofdzakelijk twee soorten gebruikt; *Clostridium acetobutylicum* en *C. beijerinckii*. Doordat alle butanolproducerende bacteriën naast butanol ook nog andere producten maken, zoals aceton en ethanol en de organische zuren acetaat (azijnzuur) en butyraat (boterzuur), is het biologische proces niet erg efficiënt. Door het commerciële succes van butanolproductie op basis van petroleum raakte het biologische proces in onbruik. De huidige hoge olieprijzen maken het fermentatieproces echter weer interessant, al zijn er nog wel hindernissen die overwonnen moeten worden voordat het kan worden geher-commercialiseerd. Het is onder andere essentieel om goedkopere biomassa te kunnen gebruiken als uitgangsmateriaal (zoals lignocellulose)

in plaats van de traditionele substraten zetmeel en molasses, die relatief duur zijn. Ook voorkomt deze vervanging het omstreden "voedsel-versus-brandstof" (*food-versus-fuel*) dilemma. Een andere uitdaging is om de butanolproductie te verbeteren, zowel de opbrengst per kilogram uitgangsmateriaal, als ook de uiteindelijke concentratie. In dit proefschrift ligt de nadruk op het verbeteren van butanolproductie en het verminderen van de productie van de zuren acetaat en butyraat door *C. acetobutylicum*.

Er is gekozen voor een aanpak waarbij de stofwisseling in de cel wordt geherprogrammeerd (*metabolic engineering*; het door middel van genetische veranderingen aanpassen welke stoffen, in welke mate worden geproduceerd door een cel) om zo het aantal en de hoeveelheid van de bijproducten in *C. acetobutylicum* fermentaties te verminderen. Hiervoor werden de metabole productieroutes van acetaat en butyraat aangepakt, omdat de hypothese was dat door zuurproductie te voorkomen ook acetonproductie zou worden gestopt, met als resultaat een fermentatie door *C. acetobutylicum* waarbij alleen alcoholen gevormd zouden worden als vloeibare eindproducten. Om het *metabolic engineering* werk te kunnen verrichten, hebben we eerst een essentieel hulpmiddel voor het maken van gen-uitschakeling in *C. acetobutylicum* ontwikkeld.

We hebben tijdens deze studie ook de opslagcondities voor electro-competente *C. acetobutylicum* cellen onderzocht, wat ons in staat stelde bulkhoeveelheden hiervan te maken en voor ten minste 54 maanden op te slaan voor later gebruik (**hoofdstuk 2 deel 1**). Het principe voor succesvolle opslag is het uitsluiten van zuurstof en dit zou de methode ook geschikt kunnen maken voor andere obligaat anaeroben.

Het **tweede deel** van **hoofdstuk 2** beschrijft de aanpassing van het TargeTron gen-uitschakelssysteem voor gebruik met *C. acetobutylicum*. Het TargeTron systeem gebruikt een mobiel groep II intron dat kan worden aangepast zodat het selectief op een andere locatie in het genoom integreert middels een proces dat *retrohoming* genoemd wordt. Ons eerste doelwit was het *ack* gen dat codeert voor het enzym acetaat kinase (AK) en betrokken is bij acetaatproductie. Met behulp van PCR is succesvolle insertie in het gen aangetoond, maar pas na de ontwikkeling van een kolonie-PCR protocol voor *C. acetobutylicum* zoals beschreven in **hoofdstuk 4**, konden we ons systeem toepassen en snel de mutanten herkennen temidden van de wildtypestam.

Een andere onderzoeksgroep had ook een clostridiale variant van het TargeTron systeem ontwikkeld, genaamd ClosTron. Het voordeel van dat systeem boven degene die wij ontwikkeld hadden is dat in gevallen waar het intron zich in het genoom heeft geïnserteerd het organisme erythromycineresistent is geworden en de mutanten daarom makkelijk te onderscheiden zijn van de wildtypestam. In **hoofdstuk 3** hebben we dit systeem gebruikt om een acetaat kinase (ack^-) mutant te isoleren. Deze mutant is gekarakteriseerd in pH-gecontroleerde batch-fermentaties op twee verschillende media; CGM en Clostridial Medium 1 (CM1). Enzymactiviteitsmetingen lieten een reductie van 98 % zien in *in vitro* acetaatkinase-activiteit, echter de gemuteerde stam bleef wildtype hoeveelheden

acetaat produceren in CGM, een medium dat zelf geen acetaat bevat. In CM1 dat wel acetaat bevat, was productie van acetaat meetbaar, maar sterk gereduceerd. Deze resultaten suggereren dat er alternatieve manieren zijn voor *C. acetobutylicum* om acetaat te produceren. De oplosmiddelproductie van de *ack*$^-$ stam was niet significant anders in CM1. Wanneer het wildtype echter CGM fermenteerde, werden grote hoeveelheden lactaat geproduceerd waardoor het geen geschikt productiemedium is. Interessant genoeg deed de *ack*$^-$ stam het hierop veel beter.

Daarna hebben we een stam gemaakt met een geïnactiveerd butyraat kinase (*buk1*) gen genaamd BUK1KO, beschreven in **hoofdstuk 4**. Het fenotype van deze stam was in essentie dat van een acetaat-butanol producent. Analyse van het fermentatiegedrag liet zien dat de stam schijnbaar nooit wisselde van de acidogene naar de solventogene staat, zoals het wildtype wel doet. Verder toonde groei op CM1 in batch-cultures aan dat de fermentatie-pH van grote invloed was op de gevormde producten. Er was een goede correlatie tussen toenemende fermentatie-pH en hogere acetaatniveaus in het pH-bereik van 4.5 tot 5.5, hetgeen suggereert dat het geproduceerde acetaat feitelijk de groei-inhiberende component was. Daarnaast werden er bij de mutant nooit clostridiale cel-typen aangetroffen, die betrokken zijn bij sporeformatie of de aanzet daartoe. Dit past binnen het beeld van de afwezigheid van een wisseling naar de solventogene staat. Parallel met de toenemende fermentatie-pH trad ook een toenemende accumulatie op van acetoïne in het medium met een maximum van 49 mM bij pH 6.5, terwijl het wildtype onder dezelfde condities slechts 12 mM vormt. Kweek in CM1 zonder toegevoegd acetaat bij een pH van 5.5 resulteerde in een toename van 21 % in butanolconcentraties tot 195 mM (14.5 g/L) in vergelijking tot wat het wildtype onder optimale condities kan produceren, en een toename met 127 % onder dezelfde condities. Verder werd er ook een reductie van acetonproductie gerealiseerd van 60 % samen met licht gestegen ethanolniveaus.

De daaropvolgende inactivatie van het acetaatkinase-gen in de *buk1*$^-$ stam met ons eigen aangepaste TargeTron systeem (zie **hoofdstuk 2**) resulteerde in de isolatie van een *ack*$^-$ *buk1*$^-$ dubbelmutant. Ondanks de eliminatie van zowel acetaatkinase- en butyraatkinase-enzymactiviteit *in vitro* bleef de mutant beide zuren produceren. In CM1 waren acetaatniveaus sterk gereduceerd ten opzichte van de *buk1*$^-$ stam, maar wanneer acetaat uit het groeimedium werd weggelaten werden weer grote hoeveelheden acetaat geproduceerd. Dit gedrag lijkt sterk op dat van de *ack*$^-$ mutant en sterkt de hypothese dat er een onbekende alternatieve zuurproducerende metabole route of enzymen bestaat in *C. acetobutylicum*. Alcoholproductie was negatief beïnvloed in vergelijking tot de wildtype stam en ook aceton productie was niet gestopt. Bij bepaalde fermentatie-pH's was de acetoineproductie zelfs nog sterker toegenomen ten opzichte van de *buk1*$^-$ stam, tot 100 mM, de hoogst gerapporteerde waarde voor dit organisme.

In **hoofdstuk 5** wordt de eerste stap gezet om op een andere manier een hogere butanolconcentratie in het medium te bewerkstelligen. 2-butanol is minder toxisch voor de cellen, waardoor een 2-butanol producerende stam zeer interessant zou zijn, mede omdat het net als 1-butanol redox-neutraal geproduceerd kan

worden ten opzichte van glucose. Daarnaast heeft 2-butanol dezelfde voordelen bij toepassing als biobrandstof als 1-butanol ten opzichte van ethanol. Door over-expressie van een alcoholdehydrogenase (acetoinereductase) van *Clostridium beijerinckii* in *C. acetobutylicum* kan natuurlijk geproduceerd D- and L-acetoine worden gereduceerd tot D- and *meso*-2,3-butaandiol. Verder toonden we aan dat onze *C. acetobutylicum* WUR stam al kleine hoeveelheden (ongeveer 3 mM) *meso*-2,3-butaandiol produceert door middel van een onbekende metabole route, zeer waarschijnlijk door reductie van D-acetoine. Voor de ATCC 824 stam kon geen productie van *meso*-2,3-butanediol worden waargenomen. Voor de voltooiing van de metabole route naar 2-butanol is naast een acetoinereductase verder nog een dehydratase en een secundair-alcoholdehydrogenase nodig om respectievelijk methyl-ethyl keton (MEK) en 2-butanol to maken.

In de algemene discussie (**hoofdstuk 6**) worden de resultaten die staan beschreven in de voorgaande hoofdstukken in perspectief geplaatst, en het bestaan van een alternatieve zuurproducerende metabole route in *C. acetobutylicum* wordt gesuggereerd. Verder worden voor- en nadelen van *C. acetobutylicum* als een butanolproductieplatform bediscussieerd, samen met ontwikkelingen op het gebied van butanolproductie in niet-natuurlijke productieorganismen.

Acknowledgements

The race is long and, in the end,
it's only with yourself.

—Mary Schmich[†]

Although the writing of this thesis was as Mary Schmich wrote, a race with myself, I would have been destined to fail if not for the support and understanding of many people. Here I would like to thank some of them.

Firstly, I'm indebted to my co-supervisor, Ana López-Contreras, for guiding me into the world of the solventogenic clostridia. Ana, my direct communication style and "stubborn" attitude did not always agree with you, and I'm aware that as your first PhD-student I was not an easy one to deal with. But I have learned to better appreciate your knowledge and experience and incorporate them into my work. I can only hope that my journey has also been of value to you. Secondly, my thanks go to my thesis supervisor Gerrit Eggink, without whom I would not be defending my thesis here. Thank you for the leeway you gave me, showing me how to appreciate my own results, and forcing me to better articulate my own thoughts.

As a PhD-student, you have many more mentors than appear on the second page of a thesis. A lot of people have added to the combined knowledge and practical experience that have resulted in this thesis. Hetty, Bwee, Miriam, Marc, and Emile: thank you all for your company, practical advice and help, and for keeping the labs running the way they do, it is truly invaluable. Also Ruud, Truus, Pieternel, Hans, Matthé, Alniek, Elinor, Jan, Twan, Patrick, Frits, and Lolke: thank you for the discussions and different views on many issues during lunch breaks or at other times.

The best way to reflect on much that goes on during your thesis work is to discuss it with your office mates. Astrid, thank you, amongst a lot of other things for being my first office mate and providing comprehensive background information on A&F politics and bureaucracy, I could not have coped without

[†]Published in the Chicago Tribune on 1 June 1997 as a column with the title "*Advice, like youth, probably just wasted on the young*", but received wider circulation via the music single "*Everybody's Free (To Wear Sunscreen)*", released in 1999, by Baz Luhrmann.

it. After the move to the TurboTron I shared an office with Anaïs, Roelof, and Tijs, ... and frustration, disappointment, laughter, and joy; thank you for these great times! The other A&F PhD-students; Helena, Catarina, David, Floor, and Maarten thank you for the good times, your company on PhD-trips, and the nice lab-atmosphere.

To the first and only student I supervised during my PhD, Pilar, thank you for teaching me a lot about myself and my assumptions. We had a lot of ground to cover and you had the bad luck that the compatible solutes study did not yield any leads despite your efforts. Fortunately your work on the 2,3-butanediol producing strains was more fruitful and was used in the fifth chapter.

Marco, we go back a long time: having made the same journey from secondary school to PhD-defence, we arrived at the same point, here in the Aula. Without you, pointing out the vacancy for a PhD-position on metabolic engineering in acetobutylicum here in Wageningen, my life would probably be very different. I would not be in Nottingham now, we would not have co-authored a paper together, worked on two book chapters, done some truly collaborative research, and had fun with it at the same time. I hope that we continue to meet each other on our future journeys.

There weren't many PhD-students in Wageningen working on solventogenic clostridia, but just as Marco, Florent also shared in that experience. Florent, your humour and personality were a great addition to the A&F crowd and it was very nice to work with you, also knowing that there was someone else to share the aceto frustrations with.

Paul and Ernest: thank you for being my paranymphs, I know you both will be a great support just as you have been all these years. Paul, when I found out that you would also be starting at A&F, around the same time as I did, I was very glad. At least one familiar face around there to the share the PhD-experience with, to laugh with and to discuss and contemplate life's challenges. Thank you for you support, council, and humour. One of the people I have known the longest since starting my studies in Wageningen is Ernest. There are few people that I feel so comfortable to be around with as you. Fencing provides the backdrop of some of my happiest memories of my student times and you are one of the lead actors. Thank you for your insight, your almost encyclopaedic knowledge, shared appreciation for whisky, and friendship!

One of the ways to unwind for me was to go fencing on Thursdays which, besides the physical exercise, also provided relaxation through *bitterballen*, beer, and talking with friends. Thanks for those great times Marieke, Michiel, Rolf, Camille, Fedor, Jouke, Bas, Hans, Ioanna[2], Yorgos, and many others.

During my "post-doc" at the laboratory of Microbiology in 2010 and 2011 I experienced the welcoming and friendly atmosphere that characterises the BacGen-group and Microbiology in general. I have learned now to even better appreciate the group-meetings with the BacGen-group. Thank you all, but especially, Bram, Edze, Elleke, John, Mark, Matthijs, Peer, Philippe, Sjon, and Tom. I also want to mention specifically John van der Oost and Servé. I really appreciate your

support in finishing some of the work that was left after my time at A&F was over and your involvement with the manuscript dealing with the ACR expressing strains. I hope that besides that one chapter, we will soon have another article together based on the BUKKO and DAPKO strains. Ana Paulo, Marjet and Rozelin: thanks for the nice office atmosphere and understanding the stress of a young father! Anja and Wim thank you for all the support.

Although I was stationed at A&F, there was always the link with the (Bio)Process Engineering group. Thanks to many for the friendly faces and especially to Koen, Dorinde and Anja for the experience of organising a PhD-trip to a country as challenging as Japan.

Pa en ma, I want to thank you both for the love and support that you have given me throughout the years, from a secondary school student with language issues, to a WUR alumnus. For believing in me, and knowing when not to ask about the thesis writing progress, for supporting my family, even if that means coming over to England to babysit your grandchildren. I can't thank you enough. Koen, your support is probably the most visible: everybody reading this has looked at your work, thank you for the great cover design. Pieter, thanks for your interest in what I was doing and making sure that I realised that not everyone finds chemistry interesting. ;-) My family-in-law: thank you for your interest in how I was progressing through these many years.

It is wonderful to join this new scientific home that I was welcomed into by so many in Nottingham, including Alex, Anne, Ann-Kathrin, Eric, Gareth, Jacqueline, Kati, Katrin, Klaus, Lili, Muhammad, Nigel, Sara, Sarah, Sheryl, Steve, Tullia, Ying and many others, a huge thank you.

Vera and Aron: you are both too young to realise what has been going on these past three years, but know that I love you with all my heart. I hope you can forgive me for wasting so many weekends behind the computer.

As the song goes, what is a man without a woman; I would be nothing without you Eveline. You have made more sacrifices than can be asked from you. Your help in making sure I would get this thesis finished, checking my manuscripts, your confidence that I could do it, they make you undeniably the greatest supporter of all. And I will be for always in your debt for letting me succeed.

Curriculum Vitae

Wouter Kuit was born in Enschede, the Netherlands, on the 2nd of July 1980. He obtained his H.A.V.O. diploma in 1997 at Het Assink in Haaksbergen. Following this, he completed BSc courses in both Organic Chemistry and Molecular Biology/Biotechnology in 2001 at Saxion Hogeschool Enschede. For his BSc thesis he went to the lab of Romano Orrū at VU University, Amsterdam, were he worked under the guidance of Günther Scheid and synthesised acyloin acetates and used lipases to perform kinetic resolution of their enantiomers.

After a break of a year he continued his studies by doing a Molecular Sciences MSc course at Wageningen University which was completed in 2005. For his minor thesis he went to New Zealand, to the lab of Max Scott (Massey University, Palmerston North) where he worked on establishing protein interactions of MSL2 with other proteins that are part of the dosage compensation complex in *Drosophila melanogaster*. Back in the Netherlands his major thesis work was entitled "Synthesis of Fumagillin-Related Spiro Epoxides as Natural Substrate Analogues for Yeast Epoxide Hydrolase", focusing once more on organic chemistry.

He started the work for his PhD-thesis in January 2006 at Food and Biobased Research of Wageningen University and Research Centre. It focused on metabolic engineering of the solvent-forming bacterium *Clostridium acetobutylicum* to improve butanol production, the results of which are described in this thesis.

From 2010, he worked at the Laboratory of Microbiology of Wageningen University for over a year, focusing on quenching and extraction techniques for metabolome sampling of *C. acetobutylicum*. In January 2012 he moved to the Clostridial Research Group of Nigel Minton at the University of Nottingham (United Kingdom) where he is currently employed as a post-doctoral researcher.

List of Publications

Ludger A. Wessjohann, Günther Scheid, Uwe Bornscheuer, Erik Henke, **Wouter Kuit** and Romano V. A. Orrū; "*Epothilone Synthesis Building Blocks III and IV: Asymmetrically Substituted Acyloins and Acyloin Derivatives, Methods for Their Production and Methods for the Production of Epothilones B, D and Epothilone Derivatives*", WO Patent 2,002,032,844.

Günther Scheid, **Wouter Kuit**, Eelco Ruijter, Romano V. A. Orrū, Erik Henke, Uwe Bornscheuer and Ludger A. Wessjohann; "*A New Route to Protected Acyloins and Their Enzymatic Resolution with Lipases*", European Journal of Organic Chemistry, 2004, **2004**:5, 1063–1074, DOI: 10.1002/ejoc.200300338.

Ludger A. Wessjohann, Günther Scheid, Uwe Bornscheuer, Erik Henke, **Wouter Kuit** and Romano V. A. Orrū; "*Epothilone Synthesis Components I: Asymmetrically Substituted Acyloins and Acyloin Derivatives, Method for the Production Thereof and Method for the Production of Epithilone and Epothilone Derivatives*", EP Patent 1,358,144.

Ana M. López-Contreras, **Wouter Kuit**, Marco A. J. Siemerink, Servé W. M. Kengen, Jan Springer and Pieternel A. M. Claassen; "*Production of longer-chain alcohols from biomass — butanol,* iso*-propanol and 2,3-butanediol*", *In*: Bioalcohol production: Biochemical conversion of lignocellulosic biomass; Editor Keith Waldron, Woodhead Publishing, 2010, ISBN 978–1–84569–510–1, DOI: 10.1533/9781845699611.

Marco A. J. Siemerink,[‡] **Wouter Kuit**,[‡] Ana M. López-Contreras, Gerrit Eggink, John van der Oost and Servé W. M. Kengen; "D*–2,3–Butanediol Production Due to Heterologous Expression of an Acetoin Reductase in* Clostridium acetobutylicum", Applied and Environmental Microbiology, 2011, **77**:8, 2582–2588, DOI: 10.1128/AEM.01616–10.

Wouter Kuit, Nigel P. Minton, Ana M. López-Contreras and Gerrit Eggink; "*Disruption of the acetate kinase (*ack*) gene of* Clostridium acetobutylicum *results in delayed acetate production*", Applied Microbiology and Biotechnology, 2012, **94**:3, 729–741, DOI: 10.1007/s00253–011–3848–4.

[‡]These authors contributed equally to this work.

Ana M. López-Contreras, **Wouter Kuit**, Jan Springer and Pieternel A. M. Claassen; "*Novel Strategies for Production of Medium and High Chain Length Alcohols*", *In*: Microbial Technologies in Advanced Biofuels Production; Editor Patrick C. Hallenbeck, Springer US, 2012, ISBN: 978–1–4614–1208–3, DOI: 10.1007/978–1–4614–1208–3_11.

Katrin M. Schwarz, **Wouter Kuit**, Christina Grimmler, Armin Ehrenreich and Servé W. M. Kengen; "*A Transcriptional Study of Acidogenic Chemostat Cells of* Clostridium acetobutylicum-*Cellular Behavior in Adaptation to* n-*Butanol*", Journal of Biotechnology, 2012, **161**:3, 366–377, DOI: 10.1016/j.jbiotec.2012.03.018.

Florent Collas, **Wouter Kuit**, Benjamin Clement, Remy Marchal, Ana M. López-Contreras and Frédéric Monot; "*Simultaneous production of isopropanol, butanol, ethanol and 2,3-butanediol by* Clostridium acetobutylicum *ATCC 824 engineered strains*", Applied Microbiology and Biotechnology (AMB) Express, 2012, **2**:45, DOI: 10.1186/2191–0855–2–45.

Marco A. J. Siemerink, Katrin Schwarz, Christina Grimmler, **Wouter Kuit**, Armin Ehrenreich, and Servé W. M. Kengen; "*Comparative Genomic Analysis of the Central Metabolism of the Solventogenic Species* Clostridium acetobutylicum *ATCC 824 and* Clostridium beijerinckii *NCIMB 8052*", *In*: Systems Biology of Clostridium; Editor Peter Dürre, Imperial College Press.
In press.

Wouter Kuit, Ana M. López-Contreras and Gerrit Eggink; "*A Method for Storing Electro-Competent* Clostridium acetobutylicum *cells*".
To be submitted.

Wouter Kuit, John van der Oost, Servé W. M. Kengen, Ana M. López-Contreras and Gerrit Eggink; "*Fermentation Analysis of a Novel* Clostridium acetobutylicum buk1$^-$ *Mutant and* C. acetobutylicum buk1$^-$ ack$^-$ *Double Mutant*".
To be submitted.

Overview of Completed Training Activities

Discipline specific activities

Courses

- Workshop Gene Transfer Techniques for Clostridia (Nottingham, UK, 2006)
- Advanced Course Microbial Physiology and Fermentation Technology (Delft, the Netherlands 2009)

Meetings

- B-Basic symposia (The Netherlands, 2006, 2007, 2008, 2009)
  Oral presentation (2009) and posters (2006, 2007, 2008)
- The Netherlands Biotechnology Congress – 11 (Ede, 2006)
- 3rd DRC-LST/BSDL Symposium (Delft, the Netherlands; 2007)
- Non-Pathogenic Bacteria — A conference (Toulouse, France, 2008)
- Workshops on the Genetics and Physiology of Acid- and Solvent-producing Clostridia (Wageningen, the Netherlands, 2008; San Diego, USA, 2010; Nottingham, UK, 2012)
  Oral presentation (2008), posters (2010, 2012)

General courses

- VLAG PhD week (Ermelo, the Netherlands, 2006)
- Teaching and Supervising Thesis Students (Wageningen, the Netherlands, 2007)
- Scientific Writing (Wageningen, the Netherlands, 2008)
- Career Perspectives (Wageningen, the Netherlands, 2009)
- Presentation Skills (Wageningen, the Netherlands, 2009)

Optionals

- Preparation PhD research proposal (2006)
- Project meetings with the industrial partner (2006–2009)
- Bioconversion group meetings (every 2 weeks, 2006–2009)
- Recycling group meetings (every 2 weeks, 2006–2007
- Process Engineering PhD study tour to Denmark and Sweden (2006)
- Bacterial Genetics group meetings (every week, 2008–2009)
- Organising Process Engineering study tour to Japan (2008)
- Process Engineering PhD study tour to Japan (2008)
- Brainstorm day Process Engineering (2009)

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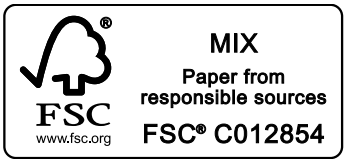

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