



Biological production of 2-butanol

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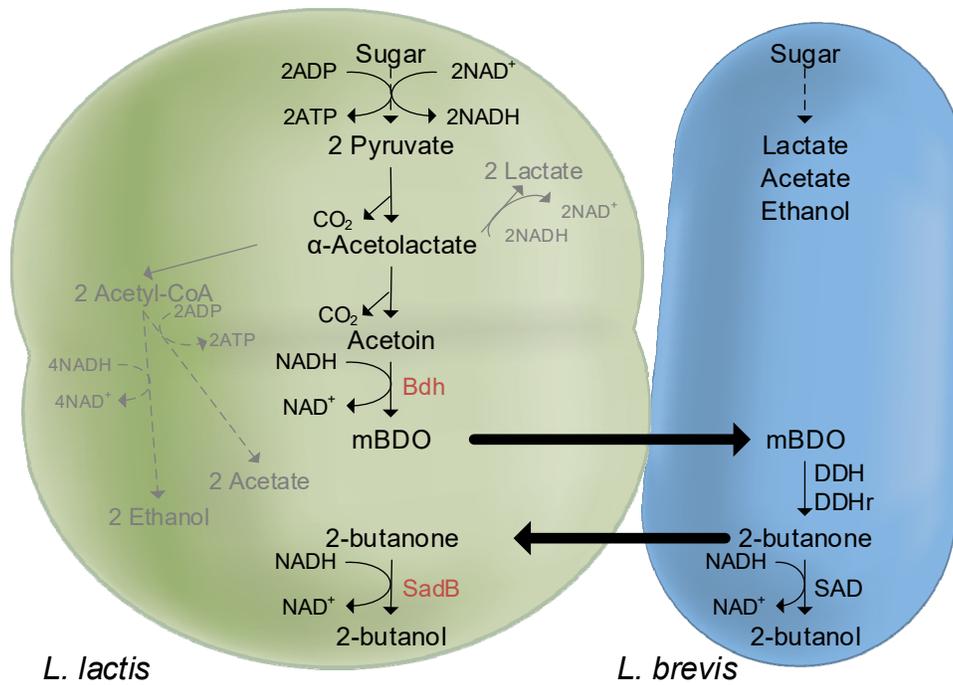
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Biological production of 2-butanol

Mette Jurlander Mar

PhD thesis

February 2020

Technical University of Denmark

National Food Institute

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Supervisors:

Prof. Peter Ruhdal Jensen

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Preface

This dissertation is submitted for a PhD degree at the Technical University of Denmark. The work was carried out in the Research Group for Microbial Biotechnology and Biorefining, National Food Institute, under the supervision of Prof. Peter Ruhdal Jensen, and Assoc. Prof. Christian Solem.

The project is part of the Flabbergast project funded by the Innovation Fund Denmark that strive to develop novel and sustainable aviation fuels from biologically derived alcohols.

Acknowledgements

My journey towards becoming a PhD has not always been easy and there are many people to whom owe a great thank for helping me on my way. I would first like to thank my supervisors Peter and Christian for providing me with this opportunity and for their guidance throughout my time at DTU.

I am thankful for the company of many the fellow PhD student, colleagues, and students I have meet over the years. Special thanks goes to Zhihao, Mathilde, Kia, Joakim, Robin, Xiaobo, Mikkel, Claus, Anders, and Tine for having made the journey much more fun. Thanks to Jianming, Viji, and Joakim for collaboration in the lab during our many failed attempts at producing butanol isomers.

I am grateful for José Arnau and Novozymes for hosting me for three months. I thank all the staff at the department for including me in their laboratory and for making me feel at home from day one. Thanks to Ana Ley and Michael Lynge Nielsen for teaching me about yeast, and for Gitte and Marianne for assisting in the laboratory. Additional thanks to José for making room for a very tired pregnant woman, and for proofreading my thesis.

Last, but certainly not least, I would like to thank my family and friends for supporting me; I could not have done this without you. A special thanks to my parents for their everlasting support and help with babysitting. To Ane and Daniel, thank you for putting up with me, especially during the final stages of my PhD. I love you, and I look forward to spending much more fun time with the both of you in the near future.

Mette Jurlander Mar

Lyngby, February 2020

Abstract

Harnessing the machinery of microorganisms for producing useful chemicals has great potential. In this project, we explore if lactic acid bacteria (LAB) are suitable for producing 2-butanol, an alcohol that is currently manufactured by chemical synthesis from compounds derived from crude oil. LAB are best known for their application in dairy fermentations, but demonstrate great potential for use in other biotechnological processes. Microbial production of 2-butanol have so far met little success with titers far below those needed for industrial production. The usual precursor of biologically produced 2-butanol is *meso*-2,3-butanediol (mBDO), a compound derived from pyruvate. mBDO is dehydrated to 2-butanone by coenzyme B12 dependent dehydratases, and subsequently reduced to 2-butanol by alcohol dehydrogenases.

In this project, I start out by evaluating if the LAB model organism *Lactococcus lactis*, a natural producer of mBDO is a suitable production platform for 2-butanol. I do this by introducing a coenzyme B12 dependent diol dehydratase from *Klebsiella oxytoca* and a 2-butanol dehydrogenase from *Achromobacter xylosoxidans*. Despite successful heterologous expression of the two enzyme activities, 2-butanol was not formed, and this I attributed to lack of an uptake system for coenzyme B12 in *L. lactis*.

Secondly, the potential of transforming *Lactobacillus brevis* SE20 into a cell factory for production of 2-butanol is assessed. The strain was found to exhibit several traits favorable to 2-butanol production e.g. it is quite robust, is capable of producing 2-butanol from α -acetolactate, and contain the coenzyme B12 salvage pathway. Thus, expression of a single enzyme, an α -acetolactate synthase should allow 2-butanol production from sugar.

Next, I investigated whether the essential mBDO dehydratase activity could be provided to *L. lactis* in-trans; a more simple strategy than introducing the B12 uptake system in *L. lactis*, or engineer the 2-butanol pathway in *L. brevis*. Using a co-cultivation strategy, where the diol dehydratase of *L. brevis* SE20 complemented an incomplete 2-butanol biosynthetic pathway in an engineered *L. lactis* strain, I demonstrated that 2-butanol formation was possible. A titer of 5.9 g/L and a yield of 0.58 mol/mol was achieved using a simple small-scale setup (50 mL), which are the highest reported from glucose in a one-step fermentation process. Initial attempts for scaling-up the production identified issues regarding process stability. The potential for scaling-up the process is therefore still being assessed with testing of different approaches for increasing the 2-butanol titer.

Resumé

Der ligger et stort potentiale i at udnytte mikroorganismer til produktion af brugbare kemikalier. I dette projekt undersøger vi om mælkesyrebakterier er egnede til produktion af 2-butanol, som i dag produceres fra råolie ved brug af kemisk syntese. Mælkesyrebakterier er mest kendt for deres anvendelse i mejeriindustrien, men har vist stort potentiale for anvendelse i andre bioteknologiske processer. Tidligere forsøg på at producere 2-butanol mikrobielt har haft begrænset succes med koncentrationer langt under det nødvendige for en rentabel industriel produktion. 2-butanol dannes normalt fra *meso*-2,3-butandiol (mBDO), et produkt der stammer fra pyruvat. mBDO omdannes til 2-butanon ved hjælp af coenzym B12-afhængige dehydrataser og reduceres derefter til 2-butanol af alkohol dehydrogenaser.

I dette projekt lægger jeg ud med at evaluere om model-mælkesyrebakterien *Lactococcus lactis*, der naturligt producerer mBDO, er egnet som cellefabrik til produktion af 2-butanol. Dette gør jeg ved at introducere en coenzym B12 afhængig diol dehydratase fra *Klebsiella oxytoca* og en 2-butanol dehydrogenase fra *Achromobacter xylosoxidans*. 2-butanol blev dog ikke dannet, på trods af succesfuld ekspression af de to enzym aktiviteter. Manglen på 2-butanol produktion synes at være et resultat af at *L. lactis* ikke indeholder et system for optag af coenzym B12.

Dernæst blev potentialet i at omdanne *Lactobacillus brevis* SE20 til en 2-butanol producerende cellefabrik vurderet. Denne stamme viste flere favorable karaktertræk for 2-butanol produktion. Den er f.eks. robust, er i stand til at producere 2-butanol fra α -acetolaktat og indeholder coenzym B12 salvage pathwayen. Dvs. introduktion af et enkelt enzym til denne bakterie, en α -acetolaktat syntase, teoretisk skulle tillade 2-butanol produktion fra sukker.

Til sidst undersøgte jeg om den essentielle mBDO dehydratase aktivitet kunne blive tilført til *L. lactis in-trans*; En mere simpel strategi end at introducere et optage system for coenzym B12 til *L. lactis*, eller at udvikle 2-butanol pathwayen i *L. brevis*. 2-butanol produktion blev opnået ved brug af en co-kultiverings strategi hvor diol dehydratase aktiviteten fra *L. brevis* SE20 komplimenterer en ikke-komplet 2-butanol pathway i *L. lactis*. En koncentration på 5.9 g/L og et udbytte på 0.58 mol/mol blev opnået i en simpelt 50 mL kultivering, hvilket er det højeste opnået fra glukose i en et-trins fermentering proces. De første forsøg på at opskalere produktionen viste problemer med stabilitet, hvorfor potentialet for opskallering af processen stadig er under vurdering, med test af forskellige opsætninger for at øge 2-butanol koncentrationen.

Outline of the thesis

Chapter I Background: An introduction to biological production of 2-butanol and other butanol isomers. The 2-butanol biosynthetic pathway, the related enzyme activities, and previous records of its microbial production is described. Afterwards follows an introduction to lactic acid bacteria, the model organism *L. lactis* and an description to why lactic acid bacteria are good candidates for production of 2-butanol.

Chapter II Production of 2-butanol using *L. lactis*: Attempt to engineer *L. lactis* for production of 2-butanol by introducing enzymes responsible for converting *meso*-2,3-butanediol into 2-butanol.

Chapter III Assessing the potential of *L. brevis* for 2-butanol production: Assessment of the potential for turning *L. brevis* into a 2-butanol producing strain by evaluating its inherent abilities to produce 2-butanol from mBDO and other intermediates, identification of genes related to 2-butanol synthesis, and confirming if it can readily be engineered.

Chapter IV Synergy at work – Linking the metabolism of two lactic acid bacteria to achieve superior production of 2-butanol: Production of 2-butanol using co-cultivation where the diol dehydratase of *L. brevis* complements an incomplete 2-butanol biosynthetic pathway in an engineered *L. lactis* strain. The manuscript has been submitted and is under second round of revision at Biotechnology for Biofuels.

Chapter V Scaling up a 2-butanol co-cultivation: Production of 2-butanol in a 1 L bioreactor using the co-cultivation strategy presented in Chapter IV.

Chapter VI Conclusion and outlook

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Chapter I Background

1. Introduction

The growing concern with global warming and depletion of fossil resources, have spurred the development of sustainable solutions for the future production of chemicals and fuels. The first-generation biofuels, ethanol and biodiesel, are the most widely used in the replacement of liquid transportation fuels [1].

In Europe, the main biofuel is biodiesel. Biodiesel constitute monoalkyl esters of long-chain fatty acids produced from non-edible, edible-, or waste-oils. Currently, most biodiesel is produced via transesterification using methanol and a catalyst, resulting in by-production of glycerol. Other production methods included pyrolysis and supercritical fluid method [2]. In addition, microorganisms have been engineered for production of biodiesel, albeit the production cannot compete with current industrial processes [3,4]

Bioethanol is currently produced via yeast fermentation by *Saccharomyces cerevisiae* as a commodity renewable energy source. In United States, the main substrate for fermentation is corn flour, whereas sugarcane juice or sugar molasses is used in Brazil [1]. Albeit the production is profitable, bioethanol is not a good candidate in the replacement of the current petroleum-based transport infrastructure. E.g. ethanol has only 70% of the energy content of gasoline, it has a higher vapor pressure, and higher hygroscopicity which leads to corrosion [1]. In order for it to be used directly in cars, specially designed engines are needed [1,5]. There is therefore a need for production of other sustainable fuels.

When compared to ethanol, higher alcohols such as butanol show greater compatibility with existing transportation infrastructure. E.g. butanol isomers can readily be used as neat or blends with conventional diesel [6] where they lead to reduced NO_x and soot emission [7]. There are four structural isomers of butanol, *n*-butanol (1-butanol), iso-butanol (2-methyl-1-propanol), 2-butanol (*sec*-butanol,) and *tert*-butanol (2-methyl-2-propanol), whereof all but the last are found naturally as products of fermentation. Compared to the other isomers, 2-butanol has the highest octane number as well as the lowest boiling point, which makes it a preferred biofuel candidate [8]. The process for preparation of 2-butanol which has achieved industrial importance is hydration of 1-butane, in an acid-catalyzed reaction [8].

2. Microbial production of butanols

Biological production of butanol has focused mainly on *n*-butanol, using ABE (acetone, butanol, ethanol) fermentation which was an industrial process in the 1900s [8]. However, the inhibitory effects of *n*-butanol on the producing strains have been a major obstacle in improving production. *n*-butanol exhibits inhibitory effects on most microbes at concentrations above 1.5 to 2 % [9–12]. Of the butanol isomers, *n*-butanol shows the highest toxic effect on the cells, whereas 2-butanol the least [11,13,14]. The difference in toxicity has been attributed to the hydrophobicity of the alcohols [13].

2.1. 2-butanol

Albeit 2-butanol show preferred characteristics as a biofuel candidate as well as lowest toxicity, its biological production has not been studied in detail and more efficient microbial strains are needed for achieving feasible production of 2-butanol [15]. Natural occurrence of 2-butanol has been reported in many alcoholic beverages such as beers, fruit wines, rice wines, and spirits [16–18] where it is produced from *meso*-2,3-butanediol (mBDO) (Figure I-1).

In section 3, Biological production of 2-butanol, additional focus is put on this isomer including the biosynthetic pathway leading to its production, the related enzyme activities, and previous records of its microbial production.

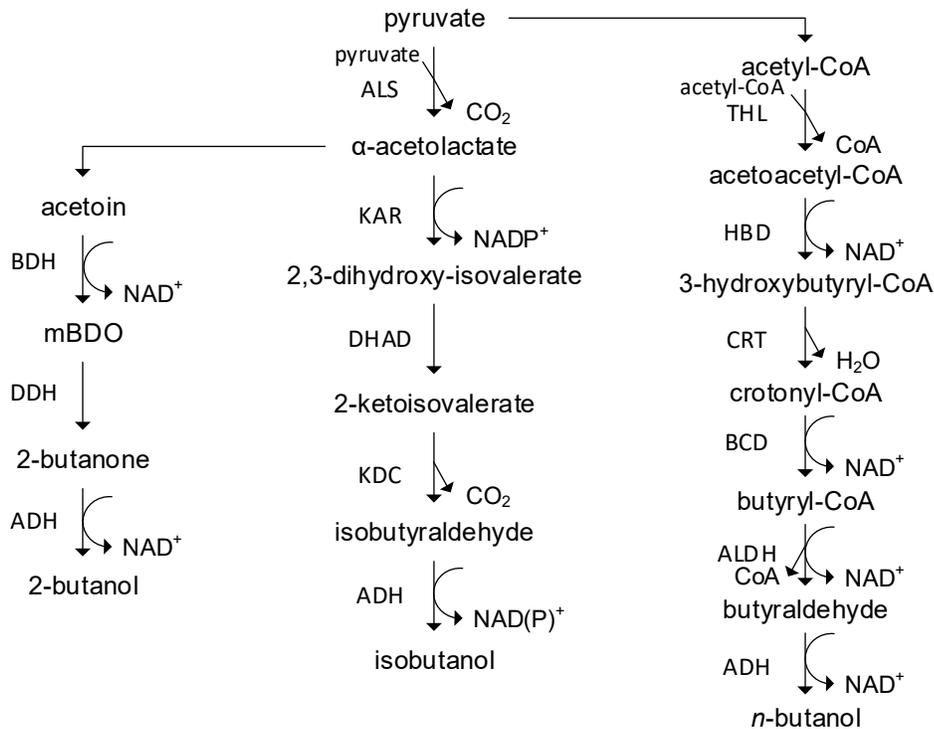


Figure I-1 Schematics of selected biosynthetic pathways for 2-butanol, iso-butanol, and *n*-butanol. BDH, butanediol dehydrogenase; DDH, diol dehydratase; ADH, alcohol dehydrogenase; ALS, acetolactate synthase; KAR, ketoacid isomeroreductase; DHAD, dihydroxy-acid dehydratase; KDC, keto-acid decarboxylase; THL, thiolase; HBD, 3-hydroxybutyryl-CoA dehydrogenase; CRT, crotonase; BCD, butyryl-CoA dehydrogenase; ALDH, aldehyde dehydrogenase.

2.2. *n*-butanol

With the recent interest in the production of biologically derived fuels, *n*-butanol has received renewed attention. Typically, *Clostridium acetobutylicum* a natural producer of *n*-butanol have been used for the production via ABE fermentation. ABE fermentation is biphasic, the first phase during exponential growth is the acidogenic phase where acetate, butyrate, hydrogen, and carbon dioxide is produced. The second phase is the solventogenic phase during which the produced acids are re-assimilated into acetone, butanol, and ethanol [19].

The natural biosynthetic pathway from *Clostridium* includes the condensation of two acetyl-CoA, followed by reduction and dehydration into *n*-butanol, and is known as the *Clostridium* CoA pathway (Figure I-1)[19]. Improvements of *n*-butanol production using ABE fermentation is limited by the complex physiological features of *Clostridium* e.g. the need of a biphasic fermentation and high oxygen sensitivity [20]. Albeit the need for oxygen removal in the culture medium has been eliminated by co-cultivation of *C. acetobutylicum* and *Nesterenkonia* sp. [21], obtaining strict control of a cultivation consisting of several strains can be difficult in an industrial production [22].

In addition, optimization using metabolic engineering of clostridia has been limited by only few available tools [19].

The main issues regarding production of *n*-butanol via ABE fermentation is issues related to the cost of substrate and product toxicity [23]. Especially product toxicity has made fed-batch or continuous fermentations economically unfeasible unless coupled with continuous product recovery [24]. Impressive titers have been reached by coupling a continuous fermentation of *Clostridium beijerinckii* with gas stripping, demonstrating a total of 460 g/L ABE with a productivity of 0.91 g/L/h [24].

In addition to production using Clostridia, the *n*-butanol synthetic pathway of *C. acetobutylicum* has been introduced to a variety of production strains such as *E. coli*, *Pseudomonas putida*, *Bacillus subtilis* [25], *S. cerevisiae* [26], and *Lactobacillus brevis* [27]. Other pathways than the *Clostridium* CoA pathway have been introduced for production of *n*-butanol; E.g. in *E. coli* by reversing the β -oxidation pathway [28] or by co-cultivation of two engineered strains of *E. coli*, where one is capable of producing butyrate when supplied with acetate, while the other converts butyrate to *n*-butanol with concurrent production of acetate [20].

Production of *n*-butanol and other higher alcohols has additionally been achieved via keto-acid intermediates of the branched chain amino acid synthesis pathways [29]. The precursor of *n*-butanol is 2-ketobutyrate from the L-threonine pathway [26].

2.3. Isobutanol

As for *n*-butanol, isobutanol can be produced from keto-acids by sequential decarboxylation and reduction [29]. The precursor for isobutanol is 2-ketoisocaproate, an intermediate in the valine biosynthetic pathway. Using this pathway, a large variety of strains such as *E. coli* and *S. cerevisiae* can inherently convert valine to isobutanol [30].

The best isobutanol producing strain was constructed by Atsumi et al. (2008) and is an engineered *E. coli* (strain JCL260) where genes encoding proteins involved with by-product formation were deleted [29]. Cultivation in shake flasks resulted in production of 22 g/L of isobutanol in 112h with a yield of 86 % of the theoretical maximum [29]. Later, the same strain was tested a 1-L bioreactor with in situ isobutanol removal from the reactor using gas stripping. Using this setup, 50 g/L isobutanol with a yield of 68 % of the theoretical maximum was achieved in 72 h.

Albeit most studies have focused on the engineering of *E. coli* for production of isobutanol [31,32], its production has also been achieved from glucose using *S. cerevisiae* [33], *B. subtilis* [34], and *Corynebacterium glutamicum* [26] and from glycerol using *Klebsiella pneumoniae* [35]. In addition,

isobutanol has been produced from cellulose using a consortium of *Trichoderma reesei* and *E. coli* [36]. In the consortium, *T. reesei* produced cellulases that hydrolyse cellulose to soluble oligosaccharides, which serve as substrates for the microbes. *E. coli* utilize glucose for production of isobutanol.

3. Biological production of 2-butanol

3.1. The 2-butanol pathway

Naturally occurring 2-butanol is derived from mBDO which is dehydrated to 2-butanone, and subsequently hydrogenated to 2-butanol by secondary alcohol dehydrogenases. So far, the reduction of pyruvate to mBDO and the conversion of mBDO to 2-butanol has not been described to occur within the same natural organism. It is, however, likely that such organism does exist as e.g. *Klebsiella pneumoniae* and *Klebsiella oxytoca* have been described as inherently containing both parts of the pathway.

Natural producers of mBDO are versatile and include members of *Bacillus* [37], *Saccharomyces* [38], *Klebsiella* [39], and *Lactococci* [40]. The pathway for conversion of mBDO to 2-butanol is typically found in organisms capable of glycerol fermentation where it is needed for restoration of the redox balance [41]. Among these organisms are species of *Citrobacter*, *Klebsiella* [42], *Lactobacillus* [43], and *Salmonella* [44]. In the pathway, coenzyme B12 (coB12) dependent diol- or glycerol dehydratases are responsible for the conversion of mBDO to 2-butanone.

3.2. Diol and glycerol dehydratases

CoB12 dependent diol and glycerol dehydratases are iso-functional enzymes catalyzing the conversion of glycerol and 1,2-propanediol (PDO) to the corresponding aldehydes. Albeit capable of catalyzing the same enzymatic reactions, they are distinguished by their substrate induction and specificity, PDO for diol dehydratases or glycerol for glycerol dehydratases [45].

These enzymatic reactions are chemically challenging, thus the enzymes catalyzing the reaction have evolved to use radical chemistry [46]. Two different radical enzymes catalyze the reactions, coenzyme B12 (coB12)-dependent and coB12-independent dehydratases. CoB12-dependent dehydratases have received much attention, whereas only one coB12 independent glycerol dehydratase from *Clostridium butyricum*, has been well characterized [47–50] and is suggested to require S-adenosylmethionine instead of coB12 for functionality [51].

3.2.1. Coenzyme B12

Vitamin B12, also known as cobalamin, is regarded as the largest and most complex “small molecule” [52] (Figure I-2). The molecule consists of a corrin ring chelating a cobalt atom. At the β -axial position is a ligand that can vary among different cobalamin derivatives. The ligand forms a covalent bond which offers unique catalytic properties to enzymes which thus can perform radical reactions which could otherwise not occur. In adenosylcobalamin, a coenzyme form of vitamin B12, this ligand has a 5'-deoxyadenosyl group [53]. Adenosylcobalamin participates in about ten enzymatic reactions of which dehydration of mBDO by the action of diol or glycerol dehydratases is one [46].

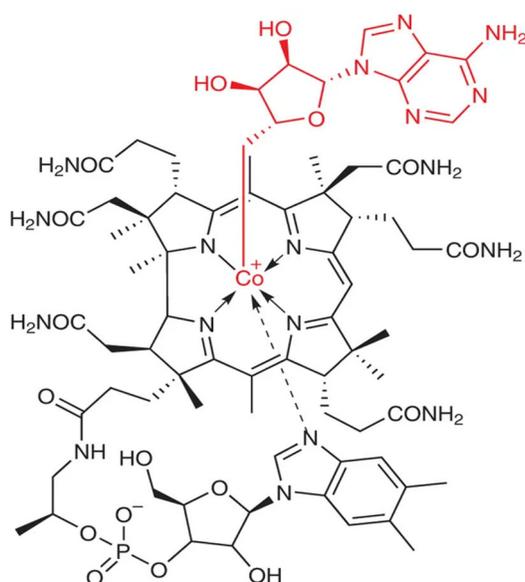


Figure I-2 Structure of adenosyl cobalamin. The cobalt atom and adenosyl group are in red. Reprinted with permission from [54].

The coB12 biosynthetic pathway involves at least 25 enzymes [53,55], and can be divided into two distinct synthetic pathways, the oxygen-dependent and -independent pathway. The oxygen-dependent pathway is found in species such as *Pseudomonas denitrificans* and *Citrobacter freundii* [56], whereas the oxygen-independent is found in *Salmonella typhimurium*, *Bacillus megaterium*, *Klebsiella pneumoniae*, *Propionibacterium shermanii* [56,57], and Lactobacilli such as *L. reuteri* [55,58], *L. coryniformis* [59], *L. plantarum* [60], and *L. rossiae* [59].

3.2.2. Reaction mechanism of dehydratases

The extensive studies of coB12 dependent diol and glycerol dehydratases form the basis for understanding the general mechanism of coB12-assisted enzymatic catalysis [46,61–64]. In this

regards, highlight should be put on the early work by Toraya and colleagues who published a whole series of manuscripts in the 1970's dealing with coenzyme B12 dependent diol dehydratases [45,65,66]

CoB12-dependent diol and glycerol dehydratases are susceptible to irreversible inactivation during catalysis and have their own chaperones to maintain catalytic activity (Figure I-3). Inactivation occurs due to undesirable side reactions that leave a damaged coenzyme bound in the active side of the enzyme. By the use of a reactivase, the damaged cofactor is substituted with intact coB12 at the expense of ATP [67–70]. Subsequently, the damaged coenzyme is reactivated by a cobalamin reductase and a cobalamin adenosyltransferase by the use of ATP [46].

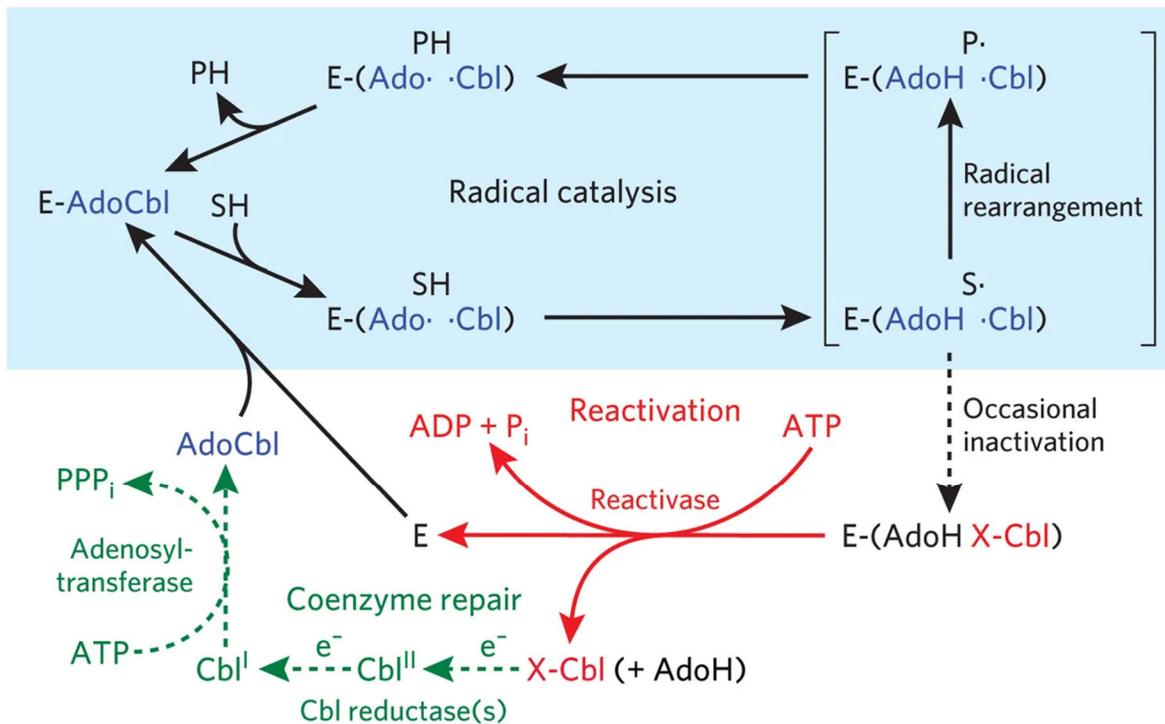


Figure I-3 Minimal mechanism and coenzyme recycling of diol and glycerol dehydratases. The addition of substrate (SH) to the apoenzyme (E) - adenosyl cobalamin (Ado) complex leads to radical catalysis and product formation (PH). Occasional inactivation leads to a loss of the adenine moiety of the coenzyme (AdoH X-Cbl). In the presence of ATP, reactivases release the adenine lacking cobalamin (X-Cbl). The apoenzyme and AdoCbl forms the holoenzyme again. X-Cbl is converted back into AdoCbl by the action of cobalamin reductase and adenosyltransferase. Reprinted with permission from [54].

The inactivation can occur by some substrates e.g. glycerol [62,66,71], by O₂ in the absence of substrate [72] and by coB12 analogues [73,74]. The substrate PDO does not cause inactivation

[62]. In regards to 2,3-butanediols, mBDO acts both as a substrate and an inhibitor, whereas the other isomers acts solely as competitive inhibitors [62,72]. As for the inactivation caused by glycerol, co-expression of the reactivase leads to improved conversion of mBDO [71].

3.2.3. Engineering of dehydratases

Few studies have attempted to make the dehydratases less susceptible to inactivation and to increase activity. The similarity between the thermodynamics in conversion of PDO and mBDO as well as their structural binding to the enzyme complex suggests that higher activity towards mBDO can be achieved using protein engineering approaches [71].

By using rational engineering, Yamanishi et al. (2012) reduced glycerol inactivation and substrate specificity of the diol dehydratase of *K. oxytoca* ATCC8724 by changing two amino acid residues in the active site at positions 301 and 336 (Ser and Gln, respectively) in the α -subunit [75]. Alanine replacement at these positions resulted in 2.7 and 2.5-fold more resistant mutants, respectively. Alanine replacement at both positions significantly increased substrate activity towards the longer 1,2-diols of 1,2-butanediol and 1,2-hexanediol. This work set the stage for several other studies to change the substrate specificity of diol and glycerol dehydratases by mutation of the corresponding residues [11,43,76].

Qi et al. (2009) applied saturation mutagenesis to two positions distant from the active site of a glycerol dehydratase from *K. pneumoniae* and achieved higher thermo- and pH stability, as well as more efficient enzymes. The mutations affected the binding of substrate and coenzyme by distorting the structure near the active site. The most active mutants showed increased catalytic power (kcat) of 8.3-fold for glycerol and 3.1-fold for PDO. Another study of saturation mutagenesis of 28 residues identified a single point mutation that increased the catalytic efficiency of mBDO dehydration by four-fold [78].

3.3. Current production strategies

2-butanol production from glucose was demonstrated by Chen et al. (2015) in *Klebsiella pneumoniae* by extension of the native mBDO synthesis pathway [11]. By elimination of a lactate dehydrogenase (encoded by the gene *ldhA*) and introducing the diol dehydratase from *L. brevis*, they initially constructed a 2-butanone producing strain [42]. Further introduction of a secondary alcohol dehydrogenase from *Clostridium autoethanogenum* extended the pathway to 2-butanol [11]. The resulting strain produced 0.72 g/L of 2-butanol after 36 h of cultivation. The diol dehydratase was identified as the limiting step due to a mBDO buildup of 19 g/L. Introduction of a point mutation in the dehydratase previously demonstrated to reduce inactivation [75], increased the production to 1.03 g/L.

Klebsiella spp. for 2-butanol production has some advantages, as they are natural producers of mBDO and some inherently produce diol and glycerol dehydratases as well as coB12. However, their pathogenic status can be a hindrance to industrial-scale production, thus efforts have been made on the removal of known virulence factors [79–81].

To this day, the work by Chen et al. (2015) [11] is the only study demonstrating a microorganism capable of producing 2-butanol from sugar. There are, however, several other studies worth mentioning.

3.3.1. Production of mBDO

Biological production of the 2-butanol precursor mBDO has received attention due to its wide variety of applications. Scientific focus has been on metabolic engineering of producing strains and extraction from fermentation broth [82–84].

Using metabolic engineering, production has been achieved in the non-native producer *E. coli* [71,85] and enhanced in native producers such as *Bacillus* spp. [37,86], *K. oxytoca* [39], *Serratia marcescens* [87], *S. cerevisiae* [38,88], and *Lactococcus lactis* [89,90]. Alternatively, synthesis from CO₂ has been achieved in cyanobacterium *Synechococcus elongatus* [91].

3.3.2. 2-butanol production in *S. cerevisiae*

2-butanol production from mBDO has been achieved in *S. cerevisiae* by expression of a diol dehydratase from *Lactobacillus reuteri* and a secondary alcohol dehydrogenase from *Gordonia* sp. [92]. A titer of 4 mg/L 2-butanol and 2 mg/L 2-butanone was achieved after 66 h of cultivation, corresponding to a conversion efficiency of approx. 13%. CoB12 was supplied to the medium and the limiting factor was speculated to be the availability of coB12 inside the cells. Albeit *S. cerevisiae* is known to produce mBDO in low amounts, additional engineering is needed to ensure feasible production from sugar [38,88].

3.3.3. 2-butanol production using a two-step fermentation strategy

Instead of introducing the mBDO pathway to *Lactobacillus diolivorans* which contains a native diol dehydratase, Russmayer et al (2019) combined the strain with an effective mBDO producer, *S. marcescens* [93]. In a two-step process, *S. marcescens* produces mBDO from glucose, followed by heat inactivation for 60 °C for 30 min. After that, fresh medium and *L. diolivorans* is added for conversion of mBDO to 2-butanol. By applying an engineered *L. diolivorans* overexpressing the endogeneous alcohol dehydrogenase, pduQ, 13.4 g/L 2-butanol and a yield of 0.24 mol/mol glucose was achieved. Besides 2-butanol, high amounts of lactate, acetate, and ethanol were produced.

Albeit the high 2-butanol titer here demonstrated seem impressive compared to previous records, there are clear limitation to this approach, e.g. the low yield and large amount of by-products produced. In addition, the use of *S. marcescens*, an opportunistic pathogen [94], and need for a 30 min heat treatment to inactivate *S. marcescens* seem not compatible with industrial application.

3.3.4. 2-butanone production in *E. coli*

Yoneda et al. (2014) demonstrated production of 151 mg/L 2-butanone from glucose in *E. coli* [71]. The strain was constructed by introducing the glycerol dehydratase and reactivase from *K. pneumoniae* into a previously engineered *E. coli* producing mBDO from glucose. Besides 2-butanone the strain produced acetoin as major by-product (12 g/L). Further extending the introduced pathway to 2-butanol, would thus require introduction of a single enzyme, a secondary alcohol dehydrogenase.

3.3.5. Other strategies

Using a different pathway than through mBDO, Srirangan et al. (2016) demonstrated production of 1.3 g/L 2-butanone, 2.9 g/L acetone, and 2.9 g/L acetate from glycerol using an engineered strain of *E. coli* [95]. The strain was constructed by introducing a set of keto-thiolases from *Cupriavidus necator* to form 3-ketovaleryl-CoA, which was subsequently converted to 2-butanone by introduction of the clostridial acetone formation pathway for thioester hydrolysis followed by decarboxylation to 2-butanone.

Using a cell free system, Zhang et al. (2018) converted ethanol into acetoin, mBDO, and 2-butanol by an artificial synthetic pathway [96]. The system was composed of ethanol dehydrogenase, formolase, 2,3-butanediol dehydrogenase, diol dehydratase, and NADH oxidase. The rate limiting step was identified as the dehydratase with up to 33mM mBDO and only 14mM 2-butanol produced. In a different cell free system, levulinic acid was used for production of 2-butanone [97].

Alternatively, 2-butanone can be synthesized by hybrid biochemical/chemical approaches where mBDO is biologically produced, followed by a catalytic process for production of 2-butanone [82,98].

4. Lactic acid bacteria as cell factories

4.1. Microorganisms in a biorefinery process

When setting up a biorefinery process, there are several considerations to take into account regarding the choice of product, substrate, and production host. When producing fuels and other

low-value added product such as 2-butanol, production costs must be kept at a minimum [99]. Consequently, the chosen substrate should be cheap and the production host effective.

The current production of bioethanol is mainly based on readily available sugars such as starch; however, there is a need to change to non-edible resources as to not compete with the production of food [10]. Second-generation biorefineries are therefore based on utilization of lignocellulose such as wood and corn stalks. In order to release carbohydrates for utilization of microorganisms, the biomass is pretreated (e.g. by dilute-acid) and processed using simultaneous saccharification and fermentation. In this process, hydrolytic enzymes degrade cellulose and hemicellulose into monomers that can be fermented by microorganisms in the same reactor [100].

The released sugars consist of both hexoses and pentoses, and albeit many organisms utilize both, achieving simultaneously utilization is challenging due to carbon catabolite repression [101,102]. In order to achieve high productivity, the producing strain should thus utilize glucose and xylose simultaneously. In addition to this, the microbe must tolerate process stresses such as high temperatures, low pH, end-product toxicity and tolerance to fermentation inhibitors released during biomass pretreatment (e.g. organic acids, phenols, and furan derivatives) [10].

Currently, the most frequent used production hosts include *E. coli* and *S. cerevisiae*, which is mainly because they are well studied and have efficient genetic tools available. They are, however, promising examples of lactic acid bacteria (LAB) as future production hosts [101,103,104].

4.2. Selected traits of LAB

Lactic acid bacteria (LAB) are lactic acid producing Gram-positive bacteria within the third class of the phylum *Firmicutes*, the *Bacilli*. The main characteristic of LAB is the production of D- or L-lactic acid. Through the ages, LAB have played an important role in the preservation of food and beverages by fermentation, where rapid acid production reduces the pH and thus prevent spoilage [105]. This long use has granted LAB involved with food fermentation a generally regarded as safe (GRAS) status. LAB are especially known for their use within the dairy industry, where they are used for acidification, texture, and formation of flavor compounds such as diacetyl and acetaldehyde. In addition, they are associated with the human gut microbiota [106] and have thus found use as probiotics [107,108].

In the production of bioethanol, bacterial contamination is a common phenomenon that can lead to profit loss as the bacteria compete with yeast for nutrients [109]. This contamination is likely because of the general robust nature of LAB. They have a broad optimal growth temperature and

are tolerant to low pH and high concentrations of salt and alcohols such as ethanol and *n*-butanol [104,110,111]. Compared to *E. coli*, LAB are able to tolerate higher concentrations of common inhibitors from pretreatment of lignocellulose [112].

The substrate utilization range of LAB is broad and highly species dependent [112,113]. Most LAB are able to utilize both hexoses and pentoses derived from lignocellulose, albeit they exhibit carbon catabolite repression and thus favor glucose. However, some LAB such as *L. brevis* [114,115] and *Lactobacillus buchneri* [116] simultaneously utilize glucose and xylose, a trait favorable in the utilization of lignocellulose.

Albeit LAB are mainly associated with the production of lactic acid, they produce other fermentation products as well, mainly acetate, ethanol, and carbon dioxide. To achieve high production yields of the desired product, the metabolic flux towards unfavorable by-products should be eliminated. Identification of targets for gene knockout depends on the type of carbohydrate metabolism of the chosen LAB.

4.3. The carbohydrate metabolism of LAB

Lactic acid bacteria are divided into homolactic- and heterolactic fermentative species. For homolactic and facultatively heterofermentative species, glucose is fermented via the Embden-Meyerhof-Parnas pathway (EMP), and lactate is the main product. Facultatively heterofermentative species ferment pentoses via the phosphoketolase pathway (PKP), whereas most homolactic fermentative species are incapable of pentose utilization. Obligate heterofermentative LAB ferment both hexoses and pentoses via the PKP, thus, produces lactate, CO₂, acetate and ethanol. The use of PKP for fermentation of one molecule of glucose result in the production of one molecule of each of lactic acid, CO₂, and ethanol, whereas xylose yields one molecule of each lactic acid and acetic acid. [117]

The actual production yield often differs greatly from independent studies using LAB strains of the same species. For heterofermentative LAB, the distribution of acetyl-phosphate towards acetate or ethanol depends on the reductive/oxidative condition of the surroundings [118]. Under reductive conditions, a shortage of NAD⁺ pushes acetyl-P towards ethanol. However, even slight oxidative conditions e.g. in the presence of a hydrogen acceptor like oxygen, acetyl-P is converted via the more favorable reaction into acetic acid and ATP. Additionally, pyruvate may be reduced to lactate, but can be converted by alternative mechanisms into several other products. This conversion depends on the growth conditions and properties of the particular organism [104].

Homofermentative LAB, such as the model LAB *Lactococcus lactis*, can switch between two different fermentation modes: homolactic fermentation and mixed-acid fermentation in which pyruvate is converted into acetate and ethanol, via acetyl-CoA [119]. The shift between the fermentation modes occur for example during carbon limitation. The mechanism determining the shift has been identified by ratios of NADH/NAD, ATP/ADP, or levels of PFL or LDH, among other, albeit the mechanism has not been fully understood [120].

A fundamental difference between heterofermentative and homofermentative LAB is typically the presence or activity of phosphofructokinase (PFK) and fructose-1,6-biphosphate aldolase (FBA). In obligate heterofermentative strains, the *pfk* gene is absent, and for many, the *fba* gene is absent as well [121]. In general, only little is known at the fundamental level about the presence of genes, regulation, and flux distribution of PKP, pentose phosphate, and EMP pathways in LAB [104]. Strains of *L. reuteri* simultaneously utilize PKP and EMP when cultivated on glucose, however, the flux distribution among the two pathways changes through growth phases and show large variation among different strains [122].

In heterofermentative LAB, the use of PKP for fermentation of sugars can be an obstacle for achieving high yields of a single product. Redirection of the metabolic flux from PKP to EMP is thus needed, in addition to elimination of competing pathways [123–125]. For homofermentative LAB achieving high yields is relatively simple as it requires only the elimination of competing pathways and expression of the desired metabolic pathway [126].

4.4. *L. lactis*, the LAB model organism

The model organism of LAB is the homofermentative *L. lactis*, which has been used for a number of metabolic engineering studies related to the production of alcohols, acids, and chemicals. The use of *L. lactis* has several advantages. *L. lactis* has simple central metabolism e.g., does not have a complete functional TCA cycle. They produces only a limited number of compounds, thus, achieving a high metabolic flux towards the desired product is relatively simple, as only few competing pathways have to be eliminated. In addition, the glycolytic flux of *L. lactis* is much higher during anaerobic cultivation than e.g. *E. coli* [127,128], which is a preferred trait in order to achieve high yields.

4.4.1. Tools for metabolic engineering

There are a wide selection of tools for metabolic engineering of *L. lactis*. One of these it the NIsin Controlled gene Expression system (NICE) that has become one of the most used systems for regulating gene expression in Gram-positive bacteria [129]. The NICE system is based on the auto-regulation mechanism of the bacteriocin nisin where the a gene of interest is placed behind

a nisin inducible promoter, P_{nisA} . Depending on the presence or absence of targeting signals, the system can be used for protein expression into the cytoplasm, the membrane, or secreted into the medium [129]. The examples where the NICE system has been used to introduce protein expression are plenty [130–135].

Metabolic engineering for modulated gene expression can be achieved using synthetic promoter libraries [136]. In the promoters, consensus sequences are separated by randomized spacer sequences, thus providing a library of promoters with different strength. Unlabeled deletion, replacement or insertion of genes into the chromosome can be achieved using a simple plasmid based system [137]. In addition, sequence specific integration can be achieved into a bacteriophage attachment site by the use of plasmid pCS1966 [138]

4.4.2. Production of pyruvate derived products

As previously demonstrated by this research group, *L. lactis* can easily be manipulated for high yield production of products from the pyruvate node. The metabolism of *L. lactis* subsp. *cremoris* MG1363 was directed towards pyruvate by eliminating native lactate dehydrogenases, a phosphotransacetylase, and an alcohol dehydrogenase (encoded by the genes *ldh*, *ldhB*, *ldhX*, *adhE*, and *pta*) [126]. The sole fermentation product from the resulting strain (CS4363) is acetoin [90], and it has been used for production of several metabolites, by extending the pathway from pyruvate.

Production of ethanol from glucose using CS4363 was achieved by introduction of a pyruvate decarboxylase (PDC) and an alcohol dehydrogenase both from *Zymomonas mobilis* (ADHB) [126]. Introduction of a plasmid encoding lactose catabolism allowed production from residual whey permeate, a waste product from the dairy industry, of 41 g/L ethanol and a yield of 70 % of the theoretical maximum [139].

By further eliminating the native butanediol dehydrogenase (encoded by the genes *butBA*) in strain CS4363 and introducing different alcohol dehydrogenases, allowed production of (2R,3R)-butanediol (rBDO) and mBDO [90]. Introduction of an alcohol dehydrogenase from *Achromobacter xylosoxidans* (encoded by *sadB*) allowed production of rBDO, whereas introduction of SadB from *Enterobacter cloacae* allowed production of mBDO (strain mBD001).

The constructed strain mBD001 produced 16 g/L mBDO from glucose in 13 h with a yield of 80 % of the theoretical maximum under aerobic conditions [90]. Subsequently introduction of a lactose plasmid allowed production of 51 g/L mBDO from residual whey permeate with a yield of 89 % of theoretical maximum [90]. Prior to this work, Gaspar et al (2011) achieved production of

mBDO at the theoretical maximum (67 %) under anaerobic conditions, with formate and ethanol as major by-products [140]. These previous records thus demonstrate that *L. lactis* is an efficient producer of the 2-butanol precursor mBDO.

4.5. LAB as cell factories for production of 2-butanol

In this study, we chose *L. lactis* subsp. *cremoris* MG1363 as a model LAB for production of 2-butanol because it is the most studied of the LAB and because it is relatively simple to manipulate. In addition, metabolic engineering of MG1363 has demonstrated production of mBDO, a 2-butanol precursor, as sole fermentation product, at levels close to the theoretical maximum from both glucose and lactose. Industrially, MG1363 might not be the preferred candidate, as it requires a complex and more expensive growth medium compared to other LAB. It might, however, be useable in the production of 2-butanol from lactose waste streams such as residual whey permeate.

In addition, we chose to evaluate the potential of turning the heterofermentative *L. brevis* SE20, an isolate from a bioethanol plant into a 2-butanol producing cell-factory. When compared to MG1363, only few genetic tools have been developed for *L. brevis* and achieving high yield production would require a shift from hetero- to homo-lactic fermentation. However, the strain does have other favorable traits; it is inherently capable of producing 2-butanol from mBDO and it can simultaneously utilize glucose and xylose. In addition, as it was isolated from a bioethanol plant, it is likely that it will outperform *L. lactis* in a lignocellulose biorefinery.

Chapter II Production of 2-butanol using *L. lactis*

1. Introduction

2-butanol is naturally occurring in wine and other fermented beverages [16] where it is produced from *meso*-2,3-butanediol (mBDO) e.g. by members of the Lactobacilli [42,43]. In the biosynthetic pathway, mBDO is converted to 2-butanone by the action of coenzyme B12 (coB12) dependent diol or glycerol dehydratases, and subsequently conversion to 2-butanol by secondary alcohol dehydrogenases. The pathway is typically found in organisms capable of glycerol fermentation, where it is needed for restoration of the redox balance [41].

Most *L. lactis* strains, including the model strain, *L. lactis* subsp. *cremoris* MG1363, typically convert more than 90% of the glucose consumed into lactate [139]. Lactate formation efficiently regenerates the NAD⁺ formed in glycolysis. Previously this research group has demonstrated high yields of pyruvate-derived products by modification of the metabolism of MG1363. By deletion of lactate dehydrogenase, phosphotransacetylase, and alcohol dehydrogenase activities, strain CS4363 was constructed which can grow only under aerated conditions where NADH oxidase regenerates NAD⁺ and its sole fermentation product is acetoin [126]. The genome of MG1363 encodes a butanediol dehydrogenase, which should allow production of mBDO from acetoin; however, a weak promoter drives its expression. Instead, overexpression of a heterologous butanediol dehydrogenase allowed aerobic production of mBDO at a yield of 0.47 g/g glucose, corresponding to 89% of theoretical maximum [90]. Anaerobic production of mBDO has additionally been demonstrated in a different *L. lactis* with formate and ethanol as major by-products [140].

These previous records show that *L. lactis* can be engineered to efficiently produce the 2-butanol precursor mBDO, thus show a potential in achieving high yields of 2-butanol. We therefore decided to explore whether *L. lactis* could serve as a chassis for producing 2-butanol.

In this chapter, the potential of using *L. lactis* for production of 2-butanol is assessed by evaluation of the tolerance of MG1363 to 2-butanol. Following this, synthesis of 2-butanol from mBDO is attempted by introducing enzymes previously described as efficient, a diol dehydratase and its reactivase from *Klebsiella oxytoca* and a secondary alcohol dehydrogenase from *Achromobacter xylosooxidans*.

2. Materials and methods

2.1. Strains and plasmids

L. lactis subsp. *cremoris* MG1363, a plasmid-free derivative of strain NCD0712 [141] and derived strains were used for engineering and expression in *L. lactis*. *Lactobacillus brevis* SE20 [142], kindly provided by Christer Larsson (Chalmers University of Technology, Sweden), was used as a positive control in the assessment of diol dehydratase activity. *Escherichia coli* strain Top10 {F-*mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80/*lacZ* Δ M15 Δ *lacX74* *recA1* *araD139* Δ (*ara-leu*) 7697 *galU* *galK* *rpsL* (StrR) *endA1* *nupG* λ -} (Invitrogen) was used for cloning purposes. Plasmids pTD6, pCI372, and derived plasmids were used for expression of heterologous enzyme activities in *L. lactis*. MG1363 derived strains and plasmids used in this study are described in Table II-1.

Table II-1 *L. lactis* strains and plasmids used in this study.

Designation	Genotype or description	Reference
Strains		
CS4363	MG1363 Δ <i>ldhX</i> Δ <i>ldhB</i> Δ <i>ldh</i> Δ <i>pta</i> Δ <i>adhE</i>	[126]
MM01	CS4363 pButop pDdrAB	This work
MM10	CS4363 pButop	This work
MM22-32	MG1363 pMM22	This work
Plasmids		
pTD6	<i>E. coli</i> / <i>L. lactis</i> shuttle vector containing <i>gusA</i> reporter, Tet	[126]
pJM001	pTD6:: <i>bdh</i> , Tet	[143]
pCI372	<i>E. coli</i> / <i>L. lactis</i> shuttle vector, Cam	[144]
pCS4518	pCI372 with <i>gusA</i> reporter	[90]
pButop	pCI372:: <i>pddABC-sadB</i>	This work
pDdrAB	pTD6:: <i>ddrAB</i>	This work
pMM12	pCI372:: <i>pddABC-ddrAB</i>	This work
pMM22	pCS4518:: <i>SPL-pdd-ddrAB</i>	This work

2.2. Growth conditions

Cultivation of MG1363 was done at 30°C in M17 medium (Oxoid, England) supplemented with 1 % glucose (GM17). For growth experiments, *L. lactis* was grown as batch cultures (flasks). Strains unable to grow anaerobically were grown as batch cultures with agitation at 140 rpm. For test of activity of the expressed diol dehydratase and alcohol dehydrogenase, cultivations were executed in GM17 medium with 7.5 μ M coenzyme B12 and 20 mM mBDO or 2-butanone. Cultivation prior

to enzyme activity assay of diol dehydratase was executed in GM17 medium containing 0, 10 μ M, or 1 mM coenzyme B12.

L. brevis was grown as batch cultures (flasks) at 30°C in modified MRS medium [145] containing: 10 g/L peptone, 10 g/L meat extract, 5 g/L yeast extract, 1 ml/L Tween 80, 2 g/L K₂HPO₄, 5 g/L sodium acetate · 3H₂O, 2 g/L triammonium citrate, 0.2 g/L MgSO₄·7H₂O, 0.05 g/L MnSO₄ · 4H₂O, 20 g/L glucose, 10 μ M coB12, and 20 mM mBDO.

E. coli strains were grown aerobically at 37 °C in Luria-Bertani broth [146].

When required, antibiotics were added in the following concentrations; tetracycline: 8 μ g/ml for *E. coli* and 5 μ g/ml for *L. lactis*; chloramphenicol: 20 μ g/mL for *E. coli* and 5 μ g/mL for *L. lactis*.

2.3. Tolerance study

The tolerance of *L. lactis* to 2-butanol was evaluated by cultivation of MG1363 in MRS medium at 0 to 3 % of 2-butanol. Cultivations were carried out using a Bioscreen-C automated growth curve analysis system (OyGrowth Curves Ab Ltd.) at 30°C under an air atmosphere. Cell growth was monitored by measuring optical density at 600 nm. Shaking was performed prior to each measurement to keep cells in suspension.

The decrease in growth rate was calculated by taking the specific growth rate of *L. lactis* without 2-butanol as 100% using the formula:

$$\% \text{ decrease in specific growth rate} = \left(1 - \left(\frac{\mu \text{ in the presence of 2butanol}}{\mu \text{ in the absence of 2butanol}} \right) \right) \cdot 100\%$$

2.4. DNA techniques

All manipulations were performed according to Sambrook and Russell (2001) [146]. *E. coli* was transformed using electroporation. *L. lactis* was made electrocompetent by growing in GM17 medium containing 1% glycine and transformed by electroporation as previously described by [147]. Chromosomal DNA from *L. lactis* was isolated using the method described for *E. coli* [146] with the modification that cells were treated with 20 μ g of lysozyme per ml for 2 h prior to lysis.

2.5. Construction of strains

For construction of the 2-butanol pathway in *L. lactis*, codon-optimized versions of the *pddABC* genes from *K. oxytoca* ATCC 8724 [148] and the *sadB* gene from *A. xylosoxidans* [149] were obtained from Genscript combined in one operon *pddABC-sadB* with the GAP promoter from MG1363 inserted in front of the genes (see Appendix A for sequences). The operon was amplified with primers VP19 and VP20 (Table II-2) and the PCR product was digested with Sall and PstI

restriction enzymes. The PCR fragment was inserted into the XbaI/KpnI and PstI/SalI sites of pCI372 and transformed into *E. coli* Top10, resulting in plasmid pButop. The plasmid was subsequently transformed into CS4363 (MG1363 $\Delta^3ldh \Delta pta \Delta adhE$), resulting in strain MM10.

To enable expression of the diol dehydratase reactivase in *L. lactis* containing the 2-butanol pathway, codon-optimized versions of the *ddrAB* genes from *K. oxytoca* ATCC 8724 [148] were obtained from Genscript with the GAP promoter from MG1363 in front (see Appendix A for sequences). The operon was amplified using primers P001 and P002 and cloned at SalI/PstI of pTD6. The plasmid was subsequently transformed into MM10, resulting in strain MM01.

Construction of an *L. lactis* with expression of *pddABC* and *ddrAB* from *Klebsiella oxytoca* ATCC 8724 [148] under various promoter strengths was done to see if this could eliminate difficulties with obtaining in vivo activity of the diol dehydratase. The vector was constructed in two steps, first by combining genes *pddABC* and *ddrAB* in one operon, secondly by inserting the *pddABC-ddrAB* operon under the expression of a synthetic promoter library (SPL). Step 1; *ddrAB* was amplified from plasmid pDdrAB using primers P059/P060 and *pddABC* together with the vector backbone using primers P057/P058 from plasmid pButop. The PCR products were digested with EcoRI/BamHI and ligated together, resulting in plasmid pMM12. Step 2; the *pddABC-ddrAB* operon was amplified using primers P062 containing the SPL and P002. The PCR products and pCS4518 were digested using XbaI/SalI, ligated, and transformed into *E. coli* Top10. The libraries were tested using colony PCR. The libraries were subsequently transformed into *L. lactis* MG1363 and plated on X-gluc containing GM17 agar plates to confirm promoter library integrity.

Table II-2 Primers used in this study

Primer name	Primer use	Primer sequence (5'->3')
VP20	GapB promoter, PstI	ATCACTGCAGGAATAAAAATTACTGACAGC
VP19	GapB promoter, SalI	TATCAGTCGACTAGTAGTTTCCTCCTTATAG
P001	<i>ddrAB</i> + gapB, ups., PstI	ACGCCTGCAGGAATAAAAATTACTGACAGCC
P002	<i>ddrAB</i> , dwn., SalI	TGCGGTGCGACTTATTCATCTTGTTGTTCCACC
P057	<i>pddABC</i> , ups., BamHI	TTAATCATCTCCTTTCAAGGATCC
P058	pCI372, down	TCTTAGTAATGCAGGAGC
P059	<i>ddrAB</i> ups., BamHI	TGAGGGATCCTCTAATCCCTATAAGGAGGAAACTAC TAATGAG
P060	<i>ddrAB</i> , dwn., EcoRI	GAATTCTTATTCATCTTGTTGTTCCACCCTAG
P062	<i>Pdd</i> , ups., XbaI	CTAGTCTAGANNNNNAGTTTATTCTTGACANNNNNNN NNNNNNNTGRTATAATNNNNAAGTAATAAAATATTCCG GAGGAAT

2.6. Quantification of cellular growth and metabolites

Cell growth was regularly monitored by measuring optical density at 600 nm (OD₆₀₀) and the quantification of glucose, xylose, lactate, acetate, acetoin, ethanol, mBDO, 2-butanone, and 2-butanol was carried out using an Ultimate 3000 high-pressure liquid chromatography system (Dionex, Sunnyvale, USA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, USA) and a Shodex RI-101 detector (Showa Denko K.K., Tokyo, Japan). The column oven temperature was set at 60 °C and the mobile phase consisted of 5 mM H₂SO₄, at a flow rate of 0.5 ml/min.

2.7. Diol dehydratase assay

Diol dehydratase activity of MM10 towards 1,2-propanediol (PDO) was determined in cellular extracts using the 3-methyl-2-benzothiazolinone hydrazone (MBTH) method [65]. MBTH reacts with the produced propionaldehyde to form an azine derivate which can be determined by spectrophotometer [150]. Cells from a 100 mL culture were harvested, washed twice, and re-suspended in 10 mM potassium phosphate and 1 mM dithiothreitol buffer, pH 7.2. The cells were then disrupted by glass beads (106 µm, Sigma, Prod. No. G4649) using a FastPrep (MP Biomedicals, Santa Ana, USA).

The enzymatic reaction was carried out in 50 mM potassium chloride, 35 mM potassium phosphate buffer pH 7, 0.015 mM coenzyme B12, 50 mM PDO, and appropriate amount of cellular extract, in a total volume of 0.5 mL. After incubation at 30 °C for 10 min, the reaction was terminated by addition of 0.5 mL potassium citrate buffer (0.1 M, pH 3.6). 0.25 mL 0.5 % MBTH hydrochloride was added and left to react at 37 °C. After 15 min 0.5 mL water was added prior measurement at 305 nm using the Infinite M1000 PRO microplate reader. Absorbance values were converted to µmol propionaldehyde using a standard curve. Protein concentration of cellular extracts was determined using the Bradford method, and bovine serum albumin served as the standard.

2.8. β-glucuronidase assay

Strains encoding *gusA* were streaked on GM17 plates containing 200 µM X-gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid) for direct estimation of reporter gene expression level. SPL screening based on *gus A* reporter gene was performed on exponentially growing cultures grown in GM17 as previously described [136].

3. Results

3.1. Tolerance of *L. lactis* to 2-butanol

In construction of a cell factory for production of chemicals, it is relevant to determine its tolerance towards the produced compound. When compared to other microorganisms, lactic acid bacteria are in general quite tolerant to low pH, high concentrations of salt, and, though with great strain dependent variations [104,110,111]. Previous studies have additionally shown that *L. lactis* is highly adaptable to various stress conditions [151,152][153], thus improved tolerance is possible via simple adaptive laboratory evolution.

In this study, I found an effect on aerobic growth from having 0.5 % 2-butanol present (Table II-3). As expected, the specific growth rates obtained under 2-butanol stress was inverse correlated to the 2-butanol concentration. At 4.0 %, growth of *L. lactis* almost ceased.

Table II-3 Effect of 2-butanol on the growth of *L. lactis* MG1363 in M17 medium.

Concentration (% v/v)	Specific growth rate (h ⁻¹)	Decrease in specific growth rate (%)
0	0.84	0
0.5	0.61	28
1.0	0.49	42
1.5	0.46	46
2.0	0.37	56
2.5	0.35	58
3.0	0.33	60
4.0	ND	ND

Cultivations were executed using a Bioscreen. Decrease in specific growth rate was calculated as described in Methods section 2.3. ND, not detected.

3.2. Construction of a 2-butanol pathway in *L. lactis*

For introduction of the 2-butanol pathway in *L. lactis*, I decided to express the enzyme activities necessary for conversion of mBDO to 2-butanol in an MG1363 derived strain, CS4363 [126]. Overall this pathway should allow redox-balanced anaerobic production of 2-butanol from mBDO, as the NAD⁺ consumed in glycolysis would be regenerated by the butanediol dehydrogenase and the alcohol dehydrogenase acting on 2-butanone (Figure II-1).

For conversion of mBDO to 2-butanone, a diol dehydratase from *K. oxytoca* (encoded by the *pddABC* genes) which has previously been characterized for this reaction [42], was introduced. Dehydration of mBDO by diol dehydratases leads to inactivation of the enzyme. In order to

address this inactivation, the diol dehydratase reactivase (ddrAB) from *K. oxytoca* was additionally introduced. The reduction of 2-butanone to 2-butanol, relied on a secondary alcohol dehydrogenase from *A. xylosooxidans* (encoded by the *sadB* gene) which has previously demonstrated efficient [149].

Besides trying to express the co B12 dependent diol dehydratase, a strategy for cloning of a coenzyme B12 independent glycerol dehydratase and its reactivase, DhaB12 from *Clostridium butyricum* was additionally attempted. Cloning was attempted in many different vectors and using different cloning strategies with both restriction enzymes and using Gibson assembly, but all attempts were unsuccessful. The enzyme has previously been introduced to *E. coli* where it was used for production of 1,3-propanediol [154].

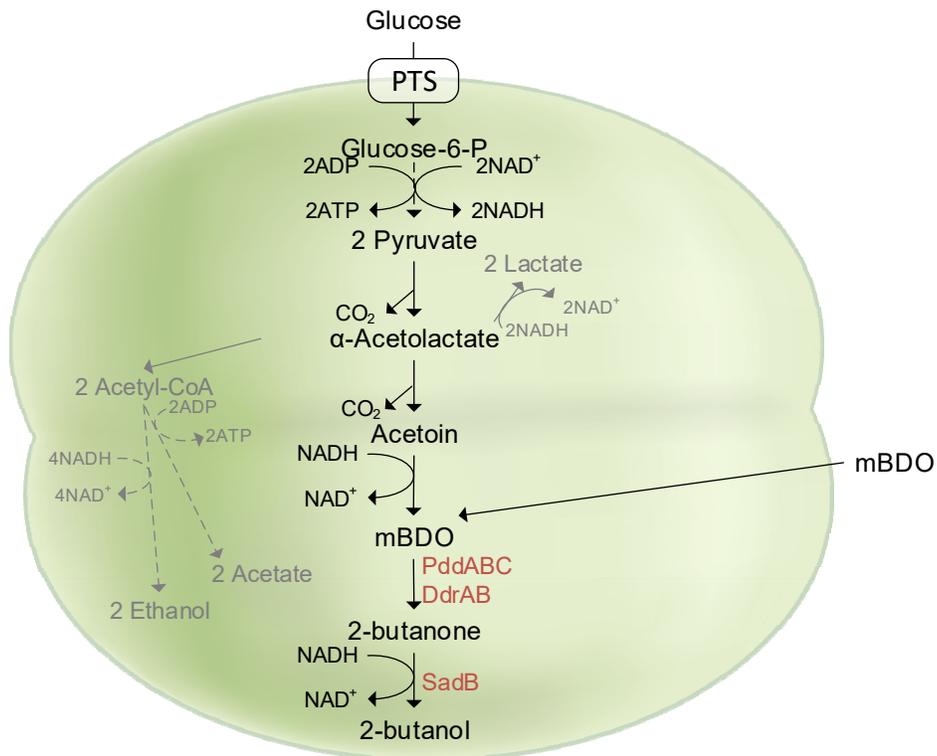


Figure II-1 Metabolic pathway based on *L. lactis* CS4363 (mBDO was added to medium). The constructed strain encodes: PddABC, diol dehydratase, and DdrAB, reactivase from *K. oxytoca*; SadB, secondary alcohol dehydrogenase from *A. xylosooxidans*. Pathways in gray indicate activities that have been eliminated. Dashed lines indicate multiple enzymatic steps.

3.3. Expression of the 2-butanol pathway

First, we verified that the enzyme activities, introduced in *L. lactis* strain MM01 were present. Strain MM01 contains both the plasmid containing the diol dehydratase and alcohol dehydrogenase, pButop, as well as the plasmid encoding the diol dehydratase reactivase (pDdrAB), showed reduced growth rate compared to the parent CS4363. Because of this, strain MM10 containing only plasmid pButop was additionally included for verification of expression.

The activity of the introduced alcohol dehydrogenase towards 2-butanone was confirmed in crude enzyme extracts of MM10, and where the control (crude extract of MG1363) did not display any activity, the extract of MM10 rapidly oxidized 2-butanone in the presence of NADH (data not shown). Restoration of anaerobic growth and 2-butanol production in 2-butanone containing medium further verified the activity.

The activity of the diol dehydratase towards propanediol (PDO) and mBDO is typically detected by the MBTH assay [65]. Following the enzymatic reaction, MBTH is added where it reacts with the produced aldehydes or ketones to form azine derivatives which can be determined by spectrophotometer [150]. In our hands, however, this method was found non-optimal for detection of 2-butanone. At standard conditions (15 min of reaction with MBTH, and absorbance measured at 305 nm), standard solutions of 1 mM propionaldehyde (the product from PDO) resulted in an absorbance of 3.062, whereas 1 mM 2-butanone gave rise to only 0.0228.). The wavelength giving rise to the highest absorption (λ_{max}) and the optimal reaction time depends on the formed azine derivative [150]. Thus, the MBTH reaction time was extended beyond the usual 15 min, and the absorption spectra was read from 300 to 350 nm (Figure II-2). However, changing the wavelength or extending the reaction time beyond 15 min did not significantly improve the detection limit of 2-butanone and no proper standard curve could be achieved.

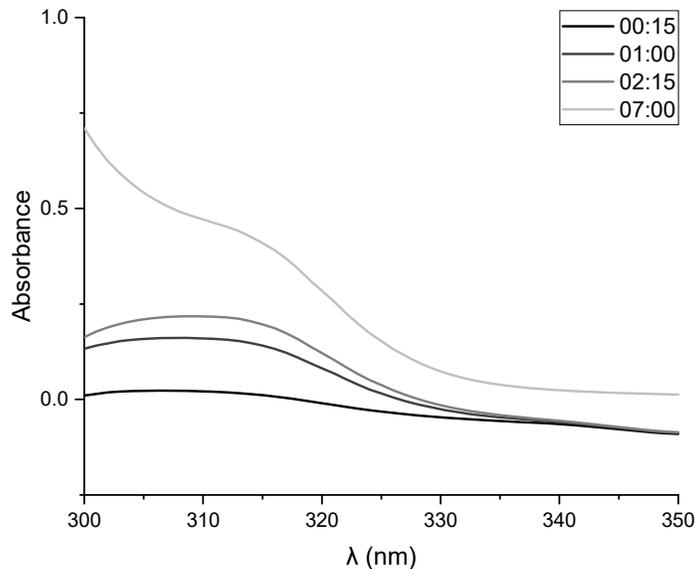


Figure II-2 Absorption spectra of the MBTH derivative of 2-butanone following different reaction time from 15 min to 7 h.

Because of the issues with detection of 2-butanone using the MBTH assay, I instead assessed the activity of the diol dehydratase towards PDO. Activity of the coB12 dependent diol dehydratase was confirmed in crude cell extracts of MM10 to be $0.32 \pm 0.01 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$. Hydrolysis of PDO does not cause inactivation of the dehydratase [62]; hence, the applied method did not allow confirmation of the activity of the diol dehydratase reactivase. Expression of the reactivase operon was confirmed by β -glucuronidase activity from the reporter gene *gusA*.

After verifying diol dehydratase activity in crude cell extracts, we attempted to produce 2-butanol from mBDO. Since the diol dehydratase relies on coenzyme B12 to function, we included this compound in the medium. Various cultivation setups were tested using both growing and non-growing cells of MM01 and MM10 in different media including arginine and maltose to supply additional ATP for the reactivation mechanism. However, no restoration of anaerobic growth was observed nor the production of 2-butanol from mBDO. This, despite of the presence of the necessary enzyme activities in crude extracts. Issues regarding detection limits of the HPLC analysis were left out by analysis using headspace GC-MS with a detection limit of approx. 1ppm. 2-butanol.

3.4. Expression of the diol dehydratase under different promotor strengths

The lack of in vivo activity of the diol dehydratase together with reduced growth rate of the constructed strain could be a result of a high level of gene expression and enzyme activity

resulting in a burden on the metabolism of the strain. We therefore expressed the diol dehydratase and reactivase with upstream synthetic promoter libraries that encodes promoters with different strengths.

Reporter gene expression level of the plasmid introduced to MG1363 was estimated by plating on X-gluc containing agar plates. A total of 10 colonies (strains MM22-MM32) with a wide distribution of expression levels were chosen for further evaluation. Strain MM22 to MM32 yielded β -glucuronidase expression levels between 7 and 310 Miller units. The strains were tested for in vivo conversion of mBDO to 2-butanone in coB12 containing medium, but none showed diol dehydratase activity, suggesting that the issues lies elsewhere. *L. lactis* is unable to generate coenzyme B12, and our results indicate that in addition, coB12 is not taken up by the intact cells.

3.5. *L. lactis* appears to be unable to take up coB12 from the medium

The observation above indicates that *L. lactis* is unable to take up coB12 from the medium. To confirm this, an experiment was conducted; *L. lactis* was cultivated in medium containing different levels of coB12, crude cell extracts were prepared of each culture, and the extracts was subsequently tested in an enzyme assay for activity towards PDO. The enzymatic assay was executed with and without addition of coB12. I hypothesized that, if coB12 is capable of entering the cells during cultivation, then some degree of activity would be seen even without further addition of coB12. This was indeed the case for the control strain *L. brevis* SE20 (Table II-4) which has an inherent dehydratase activity and is capable of taking up coB12 [142]. Albeit enzyme extracts of MM10 exhibited dehydratase activity when supplied with coB12, no activity was detected without the supplement.

Table II-4 Specific activity in crude enzyme extracts of *L. lactis* MM10 and *L. brevis* SE20 diol dehydratase. Activity towards PDO measured with or without coenzyme B12 addition. Cells were grown in medium containing 0, 10 μ M, or 1 mM coenzyme B12.

Strain	Medium addition	Specific activity (μ mol min ⁻¹ protein ⁻¹)	
		+ coB12	No coB12
<i>L. lactis</i>	No B12	0.32 \pm 0.01	0.01 \pm 0.01
	10 μ M B12	0.34 \pm 0.02	0.001 \pm 0.003
	1mM B12	0.26 \pm 0.01	0.000 \pm 0.001
<i>L. brevis</i>	10 μ M B12	0.045 \pm 0.001	0.040 \pm 0.001

4. Discussion

The results above clearly show that the lack of in vivo activity is a result of coB12 not being taken up by the cells. CoB12 and vitamin B12 biosynthesis and their role as co-factors are well understood, whereas knowledge about uptake of cobalamin by prokaryotic auxotrophs is limited. The only well described transporter of cobalamin is the ABC-type (BtuFCD) as found in *Escherichia coli* [155]. The transporter comprises the substrate-binding protein, BtuF, two copies of the membrane embedded protein, BtuC, and two ATPases, BtuD located on the cytoplasmic side of the membrane, which powers the transport by hydrolysis of ATP. The BtuFCD-operon can be identified only in two species of Lactococcus, *L. garviae* and *L. raffinolactis* (personal communication with Rodionov). Homologues of the BtuFCD are found widely in prokaryotes, albeit not represented in all bacteria which require uptake of vitamin B12 [156].

In an in silico study, Rodionov et al. (2009) predicted that the energy coupling factor (ECF)-type ABC transporter, ECF-CbrT, might be a transporter of cobalamin [157]. Later, the involvement of ECF-CbrT in the uptake of B12 has been confirmed in *Lactobacillus delbrueckii* (ECF-CbrT) [52]. Additionally, a transporter of this type was putatively identified in *L. lactis* subsp. *lactis* [157]. More recently, a different type of transporter, BtuM, believed to be of a previously un-characterized, was characterized from *Thiobacillus denitrificans* [158].

Sequence analysis of the genome of *L. lactis* subsp. *cremoris* MG1363 (using BLAST), searching for known transporters of coenzyme B12 showed no indications of the presence of similar genes related to the uptake of coenzyme B12. To the best of our knowledge, there is only one record of a vitamin B12 producing *L. lactis* (IB-2), isolated from an fermented food, *idli* batter [159].

5. Conclusion and outlook

Despite successful expression of heterologous enzymes which should allow 2-butanol production from mBDO in *L. lactis*, no 2-butanol formation was observed in vivo. The results strongly indicate that the underlying reason for the lack of production is due to *L. lactis* being unable to take up coB12 from the medium, resulting in low or absent diol dehydratase activity. In principle, one could pursue heterologous introduction of genes involved in B12 synthesis from organisms possessing these, e.g. *Lactobacillus reuteri* [160], which would require introduction of the 29 genes involved in the complete biosynthetic pathway [55]. A similar strategy has been applied to achieve biosynthesis in *E. coli* [161].

To limit the number of heterologous genes required, continued work could be limited to introduction of a vitamin B12 transporter and a coenzyme B12 activity maintaining system comprising of an adenosyl-transferase, and a cobalamin reductase. This would allow vitamin B12 uptake and conversion into coenzyme, as well as repair of damaged coenzyme following dehydratase inactivation. Using this strategy, vitamin B12 would have to be supplied to the medium.

The other cloning strategy with introduction of a coB12 independent glycerol dehydratase from *C. butyricum* was cast aside due to problems regarding construction of the expression plasmid. This strategy might, however, be worth pursuing again as production of the needed coenzyme S-adenosyl-L-methionine, has recently been demonstrated in *L. lactis* [162].

Our efforts here demonstrate, that even though *L. lactis* show good potential for 2-butanol production due to efficient production of mBDO, significant metabolic engineering is needed in order to turn it in to an efficient 2-butanol producer. Because of this, we decided to explore the use of *L. brevis* which is inherently capable of 2-butanol synthesis from mBDO when supplied with vitamin B12. In Chapter 3 follows the characterization and evaluation of the potential of *L. brevis* in 2-butanol synthesis.

Chapter III Assessing the potential of *L. brevis* for 2-butanol production

1. Introduction

Certain types of lactic acid bacteria (LAB) have been implicated in beer and wine spoilage, and the presence of these LAB often coincides with an increase in the amount of 2-butanol [163]. The 2-butanol is formed from *meso*-2,3-butanediol (mBDO), a product of the pyruvate – α -acetolactate - acetoin pathway which is present in many *Saccharomyces* spp. [38,88,92]. Among LAB capable of forming 2-butanol are strains of *L. brevis* [163,164].

Two steps are involved in the transformation of mBDO into 2-butanol. First mBDO is dehydrated to 2-butanone by the action of coenzyme B12 (coB12) dependent diol dehydratase and its reactivase (encoded by the genes *pduCDEGH*), and where after 2-butanone is reduced to 2-butanol by the action of a secondary alcohol dehydrogenase [42] (Figure III-1). It has been found that the diol dehydratase of *L. brevis* is superior to those of *Klebsiella oxytoca* and *Salmonella enterica* [42].

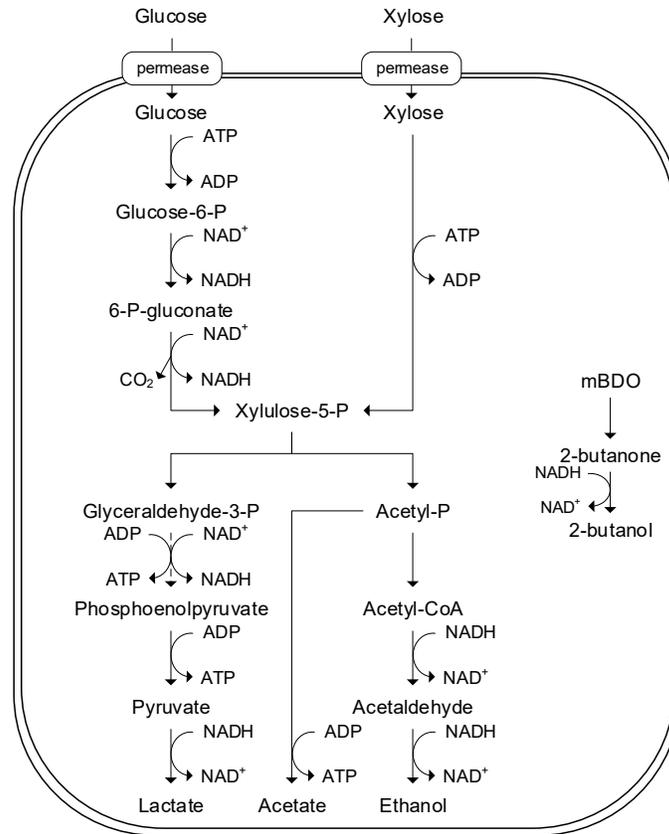


Figure III-1 Overview of glucose, xylose, and mBDO metabolism in *L. brevis*.

Lactic acid bacteria (LAB), best known for their application in dairy fermentations, have been demonstrated to have great potential for use in biotechnological applications [103]. In second-generation biorefineries, simultaneous saccharification and fermentation is often used for processing of lignocellulosic material. In this process, hydrolytic enzymes are added to lignocellulose to degrade cellulose and hemicellulose into monomers that can be fermented by microorganisms in the same reactor [100]. Albeit the microbes can utilize the released pentoses and hexoses, achieving simultaneously utilization of the sugars is challenging [101,102]. Instead, most microbes exhibit carbon catabolite repression and thus prefer glucose, which result in reduced yields. To address these issue, strains of *Escherichia coli* [165], *Lactobacillus plantarum* [166], and *Saccharomyces cerevisiae* [167] have been engineered into being able to simultaneously utilization of glucose and xylose. Other strains such as *L. brevis* [114,115] and *Lactobacillus buchneri* [116] do not exhibit carbon catabolite repression towards these sugars and are thus preferred candidates for utilization of lignocellulose. *L. brevis* have previously been used

for production of lactate upon utilization of lignocellulose hydrolysate [168] and expression of a xylanase in *L. brevis* allowed directly production of lactic acid and ethanol from xylan [169].

Only few genetic tools have been developed for engineering *L. brevis* compared to e.g. *Lactococcus lactis*. In general, focus has been on expression of heterologous enzyme activities from plasmids [27,125,170,171]. Among these efforts is the production of *n*-butanol which was achieved in *L. brevis* by introducing the *n*-butanol pathway from *Clostridium acetobutylicum* [27].

Due to its inherent ability to produce 2-butanol from mBDO and to simultaneously utilize pentoses and hexoses, *L. brevis* exhibit great potential for production of 2-butanol. In the following chapter, this potential will be further evaluated. This will be done by confirming its inherent 2-butanol pathway, identification of related genes, and evaluation of the dehydratase activity. As achieving 2-butanol production from sugar as well as eliminate the production of by-products calls for metabolic engineering of the strain, the potential for engineering and identification of gene targets will additionally be evaluated.

2. Materials and methods

2.1. Strains and plasmids

L. brevis SE20 [142], isolated from an ethanol pilot plant facility in Örnköldsvik, Sweden, was kindly provided by Christer Larsson (Chalmers University of Technology, Sweden). For evaluation of the potential for enzyme expression from plasmids in *L. brevis*, plasmids pTD6, an *L. lactis* expression vector [126], and two broad host range plasmids pAT28 [172] and pREG696 [173], were used for transformation.

2.2. Media and growth conditions

L. brevis was routinely grown as anaerobic batch cultures (flasks) at 30°C in modified MRS medium [145] containing: 10 g/L peptone, 10 g/L meat extract, 5 g/L yeast extract, 1 ml/L Tween 80, 2 g/L K₂HPO₄, 5 g/L sodium acetate ·3H₂O, 2 g/L triammonium citrate, 0.2 g/L MgSO₄·7H₂O, 0.05 g/L MnSO₄·4H₂O, and 20 g/L glucose.

Substrate utilization *L. brevis* was tested on MRS agar plates with 2% of the following carbohydrates; fructose, galactose, glucose, lactose, maltose, ribose, sucrose, and xylose. Plates were incubated for 2 days at 30 °C prior to evaluation of growth.

Carbohydrate consumption and product formation was evaluated using modified MRS medium with 20 g/L of glucose or xylose, or 10 g/L of each glucose and xylose. The production of 2-butanol using *L. brevis* was evaluated in modified MRS medium containing 7.5 µM vitamin B12 and 50 mM of pyruvate, α-acetolactate, acetoin, or mBDO.

2.3. Tolerance study

The tolerance of *L. brevis* to 2-butanol was evaluated by cultivation of *L. brevis* SE20 in MRS medium at 0 to 5 % (v/v) of 2-butanol. The decrease in growth rate was calculated by taking the specific growth rate of *L. brevis* without 2-butanol as 100% using the formula.

$$\text{Eq. 1. \% decrease in specific growth rate} = \left(1 - \left(\frac{\mu \text{ in the presence of 2butanol}}{\mu \text{ in the absence of 2butanol}} \right) \right) \cdot 100\%$$

2.4. Quantification of growth and metabolites

Cell growth was regularly monitored by measuring optical density at 600 nm (OD_{600}). Quantification of glucose, xylose, lactate, acetate, acetoin, ethanol, mBDO, 2-butanone, and 2-butanol was carried out using an Ultimate 3000 high-pressure liquid chromatography system (Dionex, Sunnyvale, USA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, USA) and a Shodex RI-101 detector (Showa Denko K.K., Tokyo, Japan). The column oven temperature was set at 60 °C and the mobile phase consisted of 5 mM H_2SO_4 , at a flow rate of 0.5 ml/min.

2.5. Activity of the dehydratase in permeabilized cells

The diol dehydratase activity of *L. brevis* SE20 was determined in permeabilized cell using the 3-methyl-2-benzothiazolinone hydrazone (MBTH) method [65]. MBTH reacts with the produced propionaldehyde to form an azine derivate which can be determined by spectrophotometer [150].

Cell cultures were quenched on ice for 15 min, harvested, washed once and re-suspended in 10 mM potassium phosphate and 1 mM dithiothreitol buffer, pH 7.0 to a final OD_{600} of 10. Permeabilized cells were prepared by treatment of 0.4 mL resuspended cells with 10 µL 0.1 % SDS and 20 µL chloroform on a vortex mixer for 10 s.

The enzymatic reaction was carried out in 50 mM potassium chloride, 35 mM potassium phosphate buffer pH 8, 0.015 mM coB12, 50mM 1,2-propanediol (PDO), and appropriate amount of permeabilized cells, in a total volume of 0.6 mL. After incubation at 30 °C for 10 min, the reaction was terminated by addition of 0.6 mL potassium citrate buffer (0.1 M pH 3.6). After centrifugation 1 mL supernatant was transferred to a 2 mL tube preheated to 37 °C, and added 0.25 mL 0.5 % MBTH hydrochloride. After 15 min, 0.5 mL water was added prior measurement at 305 nm using

a spectrophotometer. Absorbance values were converted to μmol propionaldehyde using a standard curve.

2.6. Activity of the *in vivo* dehydratase activity

The diol dehydratase activity of *L. brevis* SE20 was determined towards mBDO using an *in-vivo* assay. Cell cultures were harvested, washed once in 0.9 % NaCl, and re-suspended in defined synthetic amino-acid (SA) medium [174] with the following modification: 40mM MOPS was replaced with 100 mM potassium phosphate buffer. Reactions were executed in 2 mL tubes with a total volume of 2 mL at OD_{600} of 2.5. Incubation was done in SA medium, 20 mM mBDO, 7.5 μM vitamin B12, and 1 % glucose. After 3 h of reactions, cultures were harvested by centrifugation, filtrated, and formation of 2-butanone and 2-butanol was evaluated using HPLC.

2.7. Genome sequencing

Chromosomal DNA of *L. brevis* was isolated using DNeasy UltraClean Microbial kit (Qiagen) according to the manufacturers' instruction, with the exception of cell disruption by 3 times 1 min using a FastPrep system (MP biomedical).

The genomes were sequenced using a HiSeq 4000, Illumina system by BGI (Hong Kong). Data analysis was executed using Geneious Prime® (version 2019.1.3). Adapter trimming was done using BBDuk followed by base quality trimming with a quality threshold above a Phred score of 30 (10^{-3} likelihood of false base calling) allowing one ambiguous base by single read. Thereafter reads with a length below 50 bases were discarded for further analysis.

Phylogenetic grouping of strain SE20 was done by multi-locus sequence typing (MLST) of seven housekeeping genes (*rpoA*, *rpoB*, *recA*, *pheS*, *gyrB*, *groEL*, and *dnaK*) and compared to *L. brevis* complete and draft genomes available through NCBI at the time of writing. The genes were chosen on the basis of their use in previously published *Lactobacillus* MLST schemes [175]. The seven genes were extracted from *L. brevis* ATCC 367 (NC_008497) and used as reference sequence to map the SE20 reads to each gene, using Bowtie2 (at default setting) [176] and extracting the SE20 respective consensus sequences. The MLST analysis in Geneious was performed by extracting the seven MLST genes from each available genome, and the SE20 consensus sequences, ordering the gene sequences in alphabetical order relatively within each genomes, and concatenating the gene sequences into one sequence per genomes. The resulting sequence files were aligned using the Geneious DNA sequence aligner tool and visualized by a phylogenetic tree representation in Geneious to see what or which *L. brevis* genomes SE20 appeared closest related to by MLST.

2.8. DNA techniques

All manipulations were performed according to Sambrook and Russell (2001)[146]. *L. brevis* was made electrocompetent and transformed by electroporation as previously described by Aukrust et al. (1995), procedure 1 [177]. The method was modified so that the cells were grown in MRS medium with 1 % glycine and treated with 20 µg/mL ampicillin at OD₆₀₀ 0.4 for 50 minutes prior harvest.

3. Results and discussion

3.1. The *L. brevis* candidate and its tolerance to 2-butanol

For investigation of *L. brevis* for synthesis of 2-butanol, we chose strain SE20 previously characterized for 2-butanol synthesis from mBDO when cultivated in SM2 medium [142]. Genomic DNA of *L. brevis* SE20 was isolated and sequenced. Using multi-locus sequence typing (MLST), an effective typing approach to study the relationship among species using a specific set of genes within each species [178], *L. brevis* SE20 was compared to the 39 available *L. brevis* reference and draft genomes available through NCBI. A phylogenetic tree was constructed (depicted in Appendix B). Of the references with complete genome sequence, SE20 was found to have 99 % sequence identity *L. brevis* ATCC367 [179].

When selecting a candidate organism for bio-production, it is relevant to determine its robustness in a biorefinery environment as well as its tolerance towards the compound produced. Strain SE20 was isolated from an ethanol pilot plant facility in Örnköldsvik, Sweden. LAB are often involved with bacterial contamination of bioethanol productions where they contribute to profit loss [109]. As it originates from such an environment, it is likely that it will be robust thus more likely to handle a biorefinery environment, when compared to isolates from other sources.

To address the potential inhibitory effect of high 2-butanol concentrations, a growth experiment was performed to investigate the effect of different concentrations on growth rate. Only a slight effect (12% decrease) in specific growth rate was observed having 0.1 %(v/v) 2-butanol present. As expected, there was a direct correlation between the 2-butanol concentration and the decrease in specific growth rate. A sharp change was seen between 1.3, 2.5, and 5.0 %(v/v). At 5.0 %(v/v), the highest concentration tested, growth almost ceased.

Table III-1 Effect of 2-butanol on the growth of *L. brevis* SE20 in MRS medium. The data here shown are average of two independent experiments with standard deviation. Decrease in specific growth rate was calculated using equation (Eq.1).

2-butanol %(v/v)	Growth rate (h ⁻¹)	Decrease in specific growth rate (%)
0	0.59 ± 0.03	0
0.1	0.54 ± 0.01	8
0.3	0.54 ± 0.01	8
0.6	0.52 ± 0.02	12
1.3	0.46 ± 0.04	22
2.5	0.30 ± 0.01	49
5.0	0.02 ± 0.01	ND

3.2. Carbohydrate metabolism of *L. brevis*

For evaluation of the application of SE20 to biorefineries using various substrates, substrate utilization was assessed by cultivation on MRS agar plates containing different carbon sources. SE20 can utilize monosaccharides of xylose, glucose, and galactose, as well as disaccharides maltose and sucrose. No growth was observed on ribose and lactose plates.

L. brevis SE20 simultaneously utilized glucose and xylose (Table III-2), a common trait for *L. brevis* [114]. Cultivation on mixed sugars resulted in the highest total consumption of sugar, as well as highest biomass formation.

Glucose poorly supported growth of *L. brevis*, as compared to xylose. This is probably due to a low ATP yield on glucose of only one, whereas on xylose, the yield is two (Figure III-1) [180]. As a heterofermentative bacterium, *L. brevis* ferments glucose through the phosphoketolase pathway which, in theory, results in a net production of one mole of each lactate, ethanol, and carbon dioxide per mole of glucose [181]. Product formation by SE20 per mole of glucose was 1, 0.5, and 0.7 mole of lactate, ethanol, and acetate, respectively. The discrepancy from theory is common and the actual yields often differ from theory with great variation from independent studies of strains within the same species (Table III-3).

Table III-2 Sugar consumption and product formation by *L. brevis* SE20 following 26h of cultivation in modified MRS medium containing different carbon sources.

Sugar	Consumed sugar (mM)		Product distribution (mol/mol)			
	Glucose	Xylose	Lactate	Acetate	Ethanol	Biomass (OD600)
Glucose	20.4±1.0	ND	0.44±0.04	0.33±0.01	0.23±0.04	2.4±0.05
Xylose	ND	69.4±4.3	0.58±0.06	0.42±0.06	ND	3.5±0.11
Glucose + Xylose	53.9±0.6	62.8±0.2	0.60±0.11	0.28±0.07	0.12±0.04	9.6±0.33

Product distribution in mol pr total mol of lactate, acetate, and ethanol. The data here shown are average of two independent cultivations with standard deviation.

Table III-3. Distribution of fermentation products from glucose by different strains of *L. brevis*.

Strain no	Lactate ^a (mol/mol)	Acetate	Ethanol	
ATCC 367	0.46	0.28	0.26	[180]
s3f4	0.33	0.06	0.60	[125]
ATCC 14869	0.48	0.08	0.43	[114]
IFO 3960	0.43	0.04	0.53	[111]
IFO 12005	0.58	0.12	0.30	
IFO 12520	0.58	0.12	0.30	
IFO 13109	0.49	0.04	0.48	
IFO 13110	0.44	0.06	0.50	
IFO 27305	0.48	0.15	0.37	

^a Product distribution in mol pr total mol of lactate, acetate, and ethanol.

3.3. The 2-butanol pathway in *L. brevis*

Previously, 2-butanol production in *L. brevis* has been described from mBDO through 2-butanone [142]. Interestingly, addition of mBDO to the media exhibited different influence on cultivations when cultivated on glucose, xylose, or the mixed sugar (Table III-4). Utilization of mBDO on all three carbon sources resulted in production of 2-butanol in vitamin B12 (vB12) containing medium. No 2-butanol was detected in cultivations without mBDO or a coenzyme B12 precursor (data not shown).

The highest titer of 28.3 mM 2-butanol was achieved upon mixed sugar cultivation, corresponding to a yield of 0.24±0.02 mol/mol sugar. Cultivation on glucose resulted in the highest yield of 0.46±0.02 mol/mol sugar. Additionally, the addition of mBDO to glucose medium significantly

increased glucose uptake and biomass formation, as well as eliminated ethanol production in favor of lactate (Table III-4). The response caused by the availability of mBDO was similar to that observed during aerobic cultivation (data not shown). It is therefore likely that the changed fermentation characteristics is caused by the availability of an additional hydrogen acceptor 2-butanone, in the case of fermentation with mBDO, and O₂ during aerobic fermentation by the action of NADH oxidase activity [182]). Upon cultivation on glucose, the additional hydrogen acceptor restores the redox balance by formation of NAD⁺.

Table III-4 Sugar consumption and product formation by *L. brevis* SE20 following 26h of cultivation in modified MRS medium containing different carbon sources and 50 mM mBDO. 7.5 μM vB12

Sugar	Consumed sugar (mM)		Production (mM) 2-butanol	Product distribution (mol/mol)			Biomass (OD ₆₀₀)
	Glucose	Xylose		Lactate	Acetate	Ethanol	
Glucose	30.5±3.4	ND	14.1±1.0	0.70±0.02	0.30±0.02	ND	3.7±0.35
Xylose	ND	76.4±0.3	0.84±0.1	0.58±0.05	0.42±0.05	ND	3.8±0.02
Glucose + Xylose	55.9±0.2	63.4±0.5	28.3±2.2	0.57±0.08	0.32±0.05	0.11±0.03	9.6±0.04

Product distribution in mol pr total mol of lactate, acetate, and ethanol. The data here shown are average of two independent cultivations with standard deviation.

The results thus demonstrate that SE20 is indeed capable of producing 2-butanol from mBDO in vB12 containing medium. I then turned to determine if SE20 is additionally able to produce 2-butanol from one of the pre-cursors prior to mBDO. Production of mBDO typically involves the conversion of pyruvate into α-acetolactate, a reaction catalyzed by the α-acetolactate synthase. The α-acetolactate then undergoes decarboxylation into acetoin, followed by reduction into mBDO. Incubation of SE20 in MRS medium added vitamin B12 and α-acetolactate or acetoin, resulted in production of 2-butanol (data not shown). Formation of butanediol from acetoin has previously been described in *L. brevis* [183]. Additionally, a strain of *L. brevis* was found to produce 2-butanol from acetoin and diacetyl [164]. *L. brevis* SE20 appears to lack acetolactate synthase activity under the tested conditions as no 2-butanol was detected upon cultivation with pyruvate.

3.4. Activity of the diol dehydratase in *L. brevis* SE20

The dehydration of mBDO to 2-butanone has previously been identified as the rate limiting step for 2-butanol synthesis [11], thus use of a microbial strain possessing high activity is important for high yield production of 2-butanol. The intracellular activity of the dehydratase is known to be

influenced by several factors such as type of carbon source, the growth phase, and the availability of inducer molecules [74]. Initially the diol dehydratase activity of *L. brevis* was assessed using crude cell extracts. Using this method, however, no enzyme activity was detected. The lack of activity under these conditions might result from a low solubility [74,184] or be caused by inactivation of the enzyme by oxygen. Even the use of un-capped aerobic cuvettes can significantly influence the measured activity [185].

Because of the issues regarding the use of crude cell extracts, two other means of testing were applied. The first test was the assessment of activity towards 1,2-propanediol (PDO) in permeabilized cells of *L. brevis* SE20. As opposed to mBDO, PDO does not cause inactivation of the enzyme and is thus routinely used for assessment of diol dehydratase activity [65,142,186]. Using this method, diol dehydratase activity was confirmed towards PDO in SE20 when cultivated on glucose and mBDO (Figure III-2). The activity was influenced by the point of harvest, with a significant decreasing activity during the stationary phase.

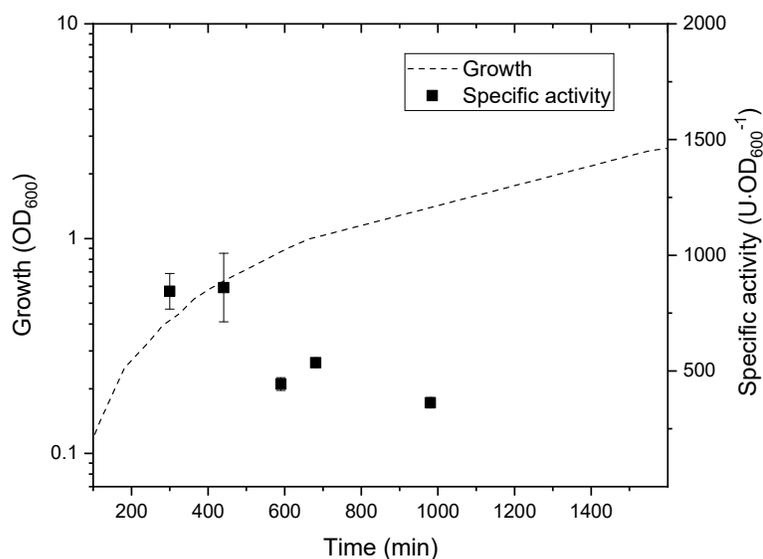


Figure III-2 Growth and PDO dehydratase activity in permeabilized cells of *L. brevis* on MRS medium containing glucose, mBDO, and vitamin B12. Values are average of three independent experiments with standard deviation.

The second test for confirming the dehydratase activity was using an in vivo assay for activity towards mBDO. In this assay, conversion of mBDO to 2-butanone and 2-butanol was assessed using cells of SE20 re-suspended in SA medium which does not support growth. The dehydratase activity was significantly higher for SE20 cultivated on glucose as compared to xylose (Figure

III-3). Thus, confirming that this pathway is used when glucose is carbon source. It has been described that cultivation using pentoses can prevent or significantly reduce the synthesis of dehydratase [183,187]. Moreover, addition of mBDO to the cultivation medium seemed to have a positive effect on the protein expression or activity. Co-utilization of glucose and mBDO resulted in the highest measured activity of 10.39 ± 0.86 U/min. As demonstrated using permeabilized cells (Figure III-2), the activity seemed to be increasing during the exponential phase and decreasing under the stationary.

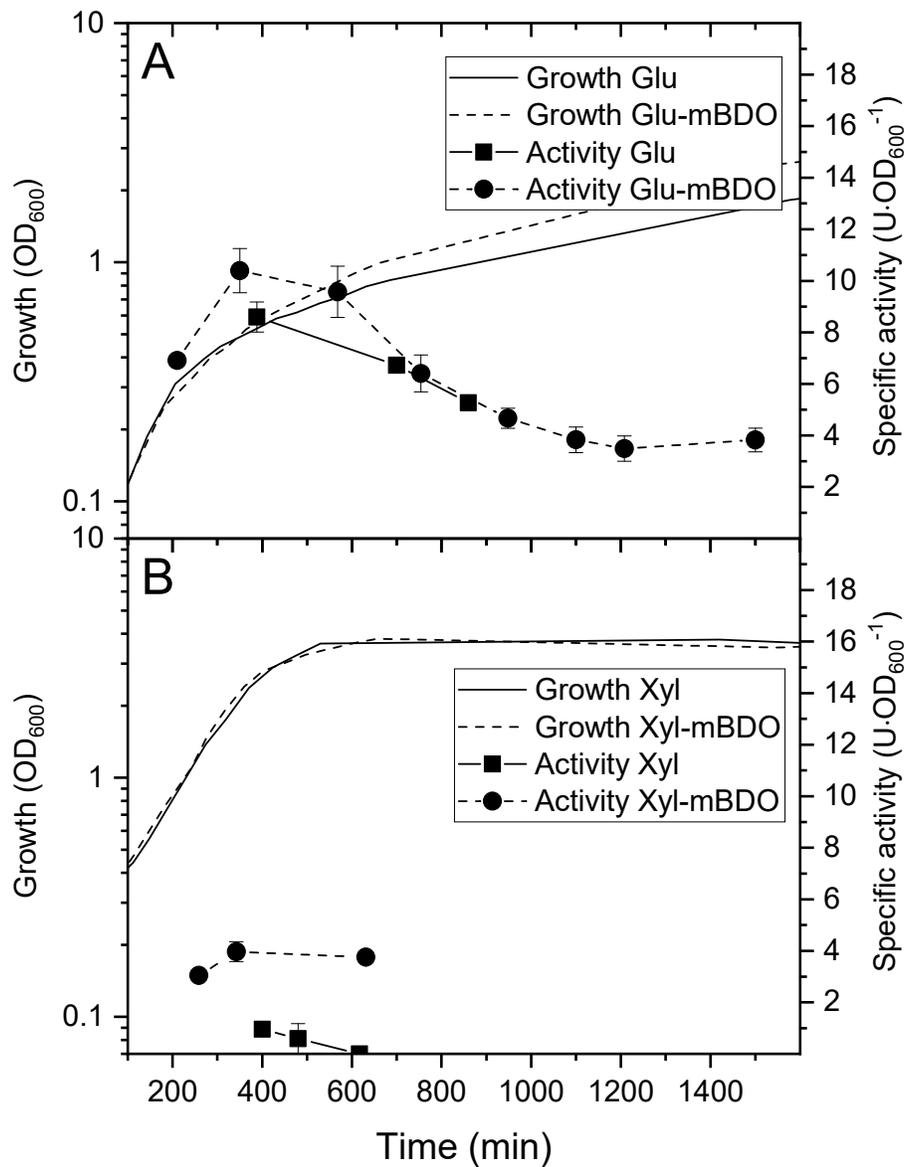


Figure III-3 Growth and in vivo mBDO dehydratase activity of *L. brevis* on MRS medium containing vitamin B12 and (A) glucose, glucose plus mBDO, or (B) xylose and xylose plus mBDO. Values are average of three independent experiments with standard deviation.

3.5. Confirmation of genes involved with dehydratase activity

In order to allow metabolic engineering of the genes involved with 2-butanol synthesis, genomic identification of genes involved in the 2-butanol pathway is of relevance. E.g. for protein engineering of the diol dehydratase. Using the genome sequencing data, and mapping the obtained sequence reads from *L. brevis* SE20 against the most similar genome reference, *L. brevis* ATCC 367, showed the dehydratase operon and surrounding genes to be conserved (Figure III-4). Low DNA complexity in library preparation was seen as gaps in the reads (Appendix C). Therefore re-sequencing or gap filling by Sanger sequencing of the operon should be done prior to genome engineering to verify sequence identity to the reference.

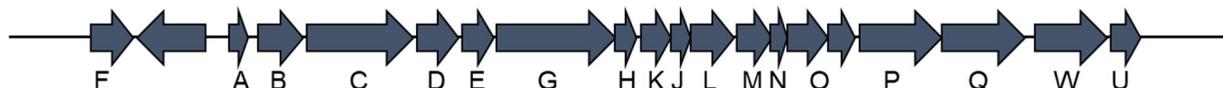


Figure III-4 The *pdu* operon of *L. brevis* SE20. Genes in the cluster are depicted by arrows indicating the transcription direction. The genes corresponds to *L. brevis* ATCC367 locus tag LVIS_1619 to LVIS_1600 (from left to right).

In vB12 producing organisms, the *pdu* operon and the cobalamin biosynthesis cluster (*cbi-cob-hem*) are located adjacent to each other and are co-regulated by the same protein (PocR), a structure which represents the close association of the two operons [160,188,189]. The *pdu* and vB12 gene clusters appears to have been acquired by lateral gene transfer and inserted independently in these genomes [160].

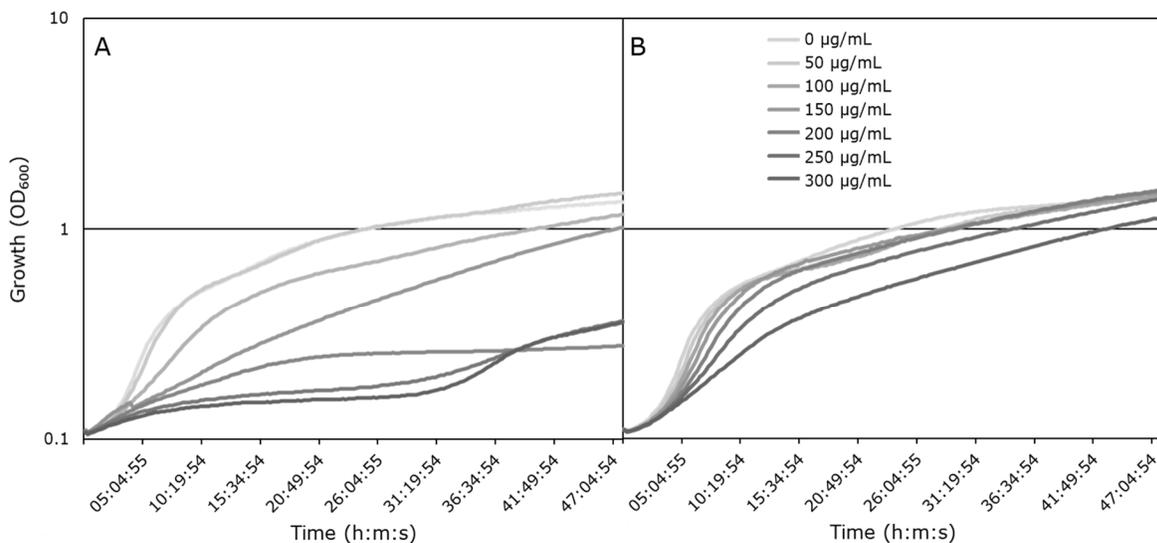
The dehydratase operon (*pdu*) displays a conserved gene order in *Lactobacillus reuteri*, *Lactobacillus collinoides*, *Lactobacillus rossiae* and *L. brevis* [59,160]. Exceptionally, among these *L. brevis* is the only strain in which the vB12 biosynthetic gene cluster has not been identified. Instead, it contain only part of the biosynthesis pathway, known as the salvage pathway also present in *E. coli*, which allow coB12 synthesis from intermediates such as cobinamide or vitamin B12 [190,191]. The presence of the salvage pathway was additionally suggested from the experimental findings that coB12 or vB12 is needed for production of 2-butanol in SE20.

3.6. Tools for engineering of *L. brevis*

For the assessment of the opportunities and bottlenecks for cell factory development of *L. brevis* SE20, an evaluation of available tools for engineering of the metabolism is essential. Transformation of *Lactobacillus* is typically done by electroporation [169,192–194], or by protoplast transformation for higher transformation efficiency of plasmids larger than 10 kb [27,195].

Due to the heterogeneity within the genus, electroporation protocols needs to be optimized for each species and strain [177]. *L. brevis* SE20 was confirmed to be transformable using the protocol described for *Lactobacillus* spp.[177] and modifications thereof. During preparation of the electrocompetent cells, addition of 1 % glycine in the medium, as well as treatment with 20 µg/mL ampicillin for 50 min after the culture had reached OD₆₀₀ of 0.4, was found to increase the transformation efficiency (data not shown). Transformation and replication of pTD6, an *L. lactis* expression vector, and two broad host range plasmids pAT28 and pREG696 were confirmed from increased antibiotics resistance of the transformants (Figure III-5 for transformation of pAT28).

Figure III-5 Growth of *L. brevis* SE20 (A) and *L. brevis* SE20 with pAT28 (B) in MRS with different concentrations of spicitinomycin.



3.7. Targets for engineering of *L. brevis*

3.7.1. Engineering of the 2-butanol pathway

By evaluation of annotations from NCBI, putative acetolactate synthase, acetolactate decarboxylase, and two butanediol dehydrogenases were identified in the genome of *L. brevis* ATCC 367 (LVIS_0491, LVIS_0492, LVIS_1802, and LVIS_0187). Thus, *L. brevis* genome appear to contain all genes necessary for production of 2-butanol from glucose, albeit the expression or activity of the acetolactate synthase appears to be insufficient for production under the tested conditions. Improving expression of the related genes may be a valid strategy to obtain 2-butanol production from sugar.

Additionally, protein engineering of the diol dehydratase has previously been applied to improve performance [78]. Introduction of an engineered enzyme to *K. pneumoniae*, resulted in improved 2-butanol production [11]. It has also been reported that over-expression of the transcription factor PocR can boost the diol dehydratase activity of *L. brevis* [189].

As *L. brevis* contains only the coB12 salvage pathway, it might be necessary to incorporate the full biosynthetic pathway for coB12 into the genome of *L. brevis*, to keep the production costs to a minimum. This has previously been done in *E. coli* by expression of the full pathway from *Salmonella typhimurium* [161]. The complete coB12 operon has been characterized in *Lactobacillus coryniformis* [59] and *Lactobacillus reuteri* [160].

3.7.2. Engineering of the central metabolism

For heterolactic bacteria, the theoretical yield of pyruvate derived products such as lactate is only 1 mol/mol sugar, whereas for homolactic bacteria the yield is 2 mol/mol glucose or 1.67 mol/mol xylose. Shifting the metabolism of *L. brevis* from heterofermentative to homofermentative, meaning fermentation via the Embden-Meyerhof-Parnas pathway, could therefore also increase the production of pyruvate derived products.

Fructose-6-phosphate kinase (PFK) and fructose-1,6-biphosphate aldolase (FBA) have been identified as an important difference between heterofermentative and homofermentative LAB. In general, in obligate heterofermentative strains, PFK is absent and many also lack FBA [121]. Although efforts to identify *pfk* and *fba* genes in the genome of *L. brevis* have been negative [121,125,175], their enzyme activities have been detected upon cultivation of fructose [196] and xylose [125]. Additionally, flux analysis suggest their metabolic involvement upon cultivation of

mixed glucose and xylose [180]. Those reports suggests that *L. brevis* do infact possess the initial enzymes of glycolysis.

Homolactic fermentation from glucose was achieved in *L. brevis* s3f4 by expression of *fba* from the homofermentative *Lactobacillus rhamnosus* [125]. Cultivation on xylose, however, showed no change in product formation from the reference. Flux analysis suggested that the activity of the phosphoketolase was higher than the transketolase, which shunted all xylulose-5-phosphate to the phosphate ketolase pathway as opposed to the desired pentose phosphate pathway. This issue was solved in *Lactobacillus plantarum* by expression of a transketolase and eliminating the inherent phosphoketolase, thus achieving homolactic fermentation from pentoses [123,124].

4. Conclusion

The work here presented demonstrate that *L. brevis* SE20 can indeed produce 2-butanol when supplied vB12 and that contains all enzyme activities needed for production from α -acetolactate. Additionally, a putative acetolactate synthase was identified in the genome albeit no enzyme activity was detected under the tested conditions.

L. brevis show no carbon catabolite repression upon glucose and xylose cultivation and is thus a good candidate for biorefineries using lignocellulosic feedstock as it can simultaneously utilize the released monosaccharides. However, the enzyme activity of the diol dehydratase catalyzing the conversion of mBDO to 2-butanone was found to be significant dependent on the available carbon source. In order to achieve high yields of 2-butanol, metabolic engineering for optimization of the 2-butanol pathway as well as achieving constantly high expression of the dehydratase is thus advisable. *L. brevis* SE20 can easily be transformed with a selection of broad host range plasmids, thus increasing the activity of the need enzymes should be possible.

Chapter IV Synergy at work – Linking the metabolism of two lactic acid bacteria to achieve superior production of 2- butanol

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1 Synergy at work – Linking the metabolism of two lactic acid bacteria to achieve superior
2 production of 2-butanol

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8
9 **Abstract**

10 Background:

11 The secondary alcohol 2-butanol has many important applications, e.g. as a solvent. Industrially, it is
12 usually made by sulfuric acid-catalyzed hydration of butenes. Microbial production of 2-butanol has
13 also been attempted, however, with little success as witnessed by the low titers and yields reported.
14 Two important reasons for this, are the growth-hampering effect of 2-butanol on microorganisms, and
15 challenges associated with one of the key enzymes involved in its production, namely diol
16 dehydratase.

17 Results:

18 We attempt to link the metabolism of an engineered *Lactococcus lactis* strain, which possesses all
19 enzyme activities required for fermentative production of 2-butanol from glucose, except for diol
20 dehydratase, which acts on *meso*-2,3-butanediol (mBDO), with that of a *Lactobacillus brevis* strain
21 which expresses a functional dehydratase natively. We demonstrate growth-coupled production of 2-

22 butanol by the engineered *L. lactis* strain, when co-cultured with *L. brevis*. After fine-tuning the co-
23 culture setup, a titer of 80 mM (5.9 g/L) 2-butanol, with a high yield of 0.58 mol/mol is achieved.

24 Conclusions:

25 Here, we demonstrate that it is possible to link the metabolism of two bacteria to achieve redox-
26 balanced production of 2-butanol. Using a simple co-cultivation setup, we achieved the highest titer
27 and yield from glucose in a single fermentation step ever reported. The data highlight the potential that
28 lies in harnessing microbial synergies for producing valuable compounds.

29

30 **Keywords**

31 2-butanol, *Lactococcus lactis*, co-cultivation, *Lactobacillus brevis*, diol dehydratase

32

33 **Background**

34 Fermentative production of bio-ethanol is a classic example of microbial solutions for bio-based fuel
35 production [1]. Ethanol, however, compared to medium length alcohols, such as butanol, has less
36 desirable fuel properties [2]. Atsumi et al. successfully demonstrated the feasibility of producing
37 different butanol isomers by coupling branched chain amino acid synthesis with the Ehrlich pathway
38 [3], however, this approach is not applicable for producing 2-butanol and despite several attempts at
39 its bio-production, so far only limited success has been reported.

40 Production of 2-butanol therefore relies on chemical synthesis, and currently 811,000 tons are being
41 produced annually [4]. Besides its potential to serve as a biofuel, 2-butanol has numerous
42 applications, e.g. as solvent or in perfume manufacturing [4].

43 Microbial production of 2-butanol from sugar has been achieved in *Klebsiella pneumonia* [5] albeit with
44 low titers. Very recently, it was reported that 13.4 g/L 2-butanol could be produced from mBDO. The
45 mBDO was generated by *Serratia marcescens* and subsequently converted into 2-butanol by
46 *Lactobacillus diolivorans* [6]. There are clear limitations to using this approach, e.g. a very low yield of
47 only 0.24 mol/mol glucose, and formation of large amounts of by-products such as acetate, ethanol,
48 and lactate (in total 815 mM, 4.5 mol per mol 2-butanol). Furthermore, the need for a 30 min. heat
49 treatment to inactivate *S. marcescens*, and the use of this opportunistic pathogen for producing
50 mBDO, appear not to be compatible with large scale production of 2-butanol. Cell-free multi-enzyme
51 catalysis has also been utilized for synthesis of 2-butanol from ethanol through continued supply of
52 coenzyme B12 and ATP [7]. Additionally, 1.3 g/L butanone was made from glycerol through 3-
53 ketovaleryl-CoA and subsequent decarboxylation [8], however, significant amounts of acetone was
54 generated as byproduct. Thus, there is room for further improvements in microbial 2-butanol
55 production.

56 Production of 2-butanol in one-step fermentation setups typically involves the conversion of pyruvate
57 into α -acetolactate, a reaction catalyzed by the α -acetolactate synthase. The α -acetolactate then
58 undergoes decarboxylation into acetoin and reduction into mBDO. mBDO is subsequently dehydrated
59 to 2-butanone followed by reduction 2-butanol.

60 Notably, the dehydration of mBDO to 2-butanone is carried out by the coenzyme B12 dependent diol
61 or glycerol dehydratases [9], which are typically found in microorganisms capable of producing 1,3-
62 propanediol [10]. B12 independent dehydratases have been described in *Clostridium butyricum*,
63 however, these require an S-Adenosyl methionine co-factor [11]. The coenzyme B12 dependent
64 dehydratase reaction is oxygen sensitive and susceptible to irreversible inactivation when substrates
65 such as glycerol and mBDO are used [12,13]. To maintain catalytic activity, the microorganisms rely
66 on dehydratase re-activation systems, consisting of reactivases, that consume ATP to restore catalytic

67 activity [14]. The intracellular activity of the dehydratase is known to be influenced by several factors
68 such as carbon source, growth phase, and the availability of inducer molecules [15].

69 Interestingly, the obligate heterofermentative *Lactobacillus brevis* was found to be capable of
70 producing 2-butanol from the mBDO produced by yeast during wine fermentation [16]. Later, the diol
71 dehydratases from *Lactobacillus brevis* were found to be superior to dehydratases from *Klebsiella*
72 *oxytoca* and *Salmonella enterica* [17]. Lactic acid bacteria (LAB), best known for their application in
73 dairy fermentations and as human probiotics, have recently been demonstrated to have great potential
74 for use in biotechnological applications [18]. The emergence of tools for genetic engineering of LAB
75 [19], combined with their high metabolic rates and fast growth [20], make them interesting candidates
76 for production of biofuels. One particular LAB, *Lactococcus lactis*, has received a lot of attention, and
77 has been metabolically engineered into producing a broad variety of useful compounds [21].

78 In our previous work, we constructed an *L. lactis* strain that could be used as a platform for producing
79 various pyruvate-derived compounds, with little byproduct formation [22]. Recently we expanded the
80 metabolic repertoire of this strain by introducing genes needed for production of mBDO [23], the
81 precursor for 2-butanol.

82 In the current study, we first investigate whether *L. lactis* is the right platform for producing 2-butanol
83 and we do this by introducing a diol dehydratase from *Klebsiella oxytoca* and a 2-butanol
84 dehydrogenase from *Achromobacter xylosoxidans*. Challenges in achieving a functional diol
85 dehydratase prompt us to try out a different strategy, namely co-cultivation, where we explore whether
86 the diol dehydratase of *L. brevis* can complement an incomplete 2-butanol biosynthetic pathway in an
87 engineered *L. lactis* strain. We show that co-cultivation is an efficient approach for producing 2-
88 butanol, and achieve the highest reported titer and yield from glucose in a one-step fermentation
89 process.

90 **Results and discussion**

91 **Assessing the potential of *L. lactis* for 2-butanol production**

92 *L. lactis* is an established industrial workhorse within the dairy industry, where it is used to ferment in
93 excess of 100 mio. tonnes of milk annually [24] . This lactic acid bacterium grows well, is easy to
94 manipulate genetically [25–27] and there are many reports on its use as an efficient cell factory for
95 producing useful compounds [21,23,28,29]. Here we explore whether *L. lactis* can be transformed into
96 a 2-butanol producing cell factory. To assess the potential of *L. lactis* to become an efficient 2-butanol
97 producer, we first introduced two genes necessary for 2-butanol formation from mBDO, namely a diol
98 dehydratase for converting BDO into 2-butanone, and an alcohol dehydrogenase for reducing 2-
99 butanone into 2-butanol. We used the *L. lactis* strain CS4363, which lacks lactate dehydrogenase,
100 phosphotransacetylase, and alcohol dehydrogenase activities, and can only grow under aerated
101 conditions where NADH oxidase regenerates NAD⁺ and its sole fermentation product is acetoin. By
102 introducing the diol dehydratase and alcohol dehydrogenase enzyme activities into CS4363, redox-
103 balanced production of 2-butanol from mBDO should in principle be possible (Figure 1A). For the diol
104 dehydratase, we decided to rely on the enzyme complex from *K. oxytoca* (PddABC), and the alcohol
105 dehydrogenase was obtained from *A. xylosoxidans* (SadB). SadB has previously been found to be
106 efficient at converting 2-butanone into 2-butanol [30] and the diol dehydratase from *K. oxytoca* has
107 previously been demonstrated to be efficient at dehydrating mBDO [17]. One concern when using diol
108 dehydratases for dehydrating mBDO, is substrate inactivation, and the enzyme needs to be re-
109 activated by a dedicated re-activase. For this reason we additionally introduced the diol dehydratase
110 reactivase from *K. oxytoca* (DdrAB), as the beneficial effect of this has been demonstrated previously
111 [31].

112 After introducing the genes, we verified the respective enzyme activities. We found that the
113 recombinant strain, in contrast to its parent lacking 2-butanol dehydrogenase activity (SadB), could
114 grow anaerobically in the presence of 2-butanone with concurrent formation of 2-butanol, which
115 confirmed the presence of SadB activity. The diol dehydratase activity was measured in crude cell
116 extracts and were shown to be $0.32 \pm 0.01 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$. We subsequently examined if the
117 engineered strain could grow and produce 2-butanol from mBDO in medium containing coenzyme
118 B12, a co-factor needed for the function of the diol dehydratase. However, we did not observe
119 restoration of anaerobic growth or formation of 2-butanol when mBDO was supplied. *L. lactis* lacks
120 genes involved in coenzyme B12 biosynthesis, and our findings that coenzyme B12 is not taken up by
121 the intact cells, is in accordance with the absence of an uptake system for vitamin B12 in *L. lactis* [32].

122 ***L. brevis* can serve as a whole-cell diol dehydratase catalyst**

123 The observation above strongly indicates that the reason why our engineered *L. lactis* strain cannot
124 produce 2-butanol is due to low or no diol dehydratase activity resulting from the lack of coenzyme
125 B12 uptake. In principle, we could pursue heterologous introduction of genes involved in B12
126 synthesis from organisms possessing these, e.g. *Lactobacillus reuteri* [33], but the B12 synthesis
127 pathway is encoded by 29 genes [34]. As an alternative, we decided to explore whether the diol
128 dehydratase activity could be supplied, in-trans, from a second strain, used as a whole cell catalyst. In
129 the subsequent experiments we chose to express the *Enterobacter cloacae* meso-2,3-butanediol
130 dehydrogenase (Bdh) and the 2-butanol dehydrogenase (SadB) in *L. lactis*, thus generating a strain
131 which in principle only lacks a diol dehydratase in order to be able to generate 2-butanol (Figure 1B).
132 As a source of the diol dehydratase we chose *L. brevis* SE20, which previously has been shown to
133 produce 2-butanol when supplied with mBDO and vitamin B12 [35].

134 Our hypothesis was that the mBDO formed in *L. lactis* would leave the cells and enter the *L. brevis*
135 cells to be dehydrated into 2-butanone. 2-butanone would subsequently leave the *L. brevis* cells,
136 reenter the *L. lactis* cells and be reduced into 2-butanol. In this way, the metabolism of *L. lactis* would
137 be redox balanced, since the two NADH generated in glycolysis would be consumed by the 2,3-
138 butanediol dehydrogenase and the 2-butanol dehydrogenase.

139 We found that glucose was a poor substrate for *L. brevis*, probably due to a low ATP yield on glucose
140 of only one [36]. On xylose, however, the ATP yield is two (See Supplementary Figure S1, Additional
141 File 1). Using xylose as a fermentation substrate could be of interest, since this sugar is abundant in
142 lignocellulose. However, since we intend to use *L. brevis* as an mBDO dehydratase cell catalyst, it is
143 relevant to investigate on which substrate the highest in vivo enzyme activity is attained. We found a
144 nine-fold higher mBDO dehydratase activity for cells grown on glucose when compared to cells grown
145 on xylose, and that mBDO acted as an inducer of activity (Table 1). For the following experiments, we
146 therefore decided to rely on *L. brevis* cells grown on glucose.

147

148 **Table 1 In vivo mBDO dehydratase activity of *L. brevis* on different carbon sources.**

Carbon source +/- inducer	Activity (U/OD ₆₀₀)
Glucose	8.6±0.6
Glucose + mBDO	10.4±0.9
Xylose	1.0±0.03
Xylose + mBDO	4.0±0.4

149 Values are average of three independent measurements with standard deviations.

150

151 The next step was to test if the diol dehydratase activity from *L. brevis* could complement the
152 metabolism of the engineered *L. lactis*, and thereby enable production of 2-butanol by *L. lactis*.
153 Indeed, 2-butanol synthesis was achieved in defined medium (SA) supplemented with 7.5 μ M vitamin
154 B12 and 5 mM 2-butanone. After 20 h, a titer of 14.2 ± 0.6 mM, with a yield of 0.5 ± 0.02 mol/mol was
155 obtained. Production of 2-butanol was not observed when the cultures were incubated in medium
156 without a small “catalytic” amount of 2-butanone added, which we speculate helped in the linking of
157 the metabolisms of the two bacteria. We also demonstrated that 2-butanol production could be
158 accomplished using a mix of lactose and xylose, although with a lower titer and yield (See
159 Supplementary Table S1, Additional File 1).

160

161 **Co-cultivation of engineered *L. lactis* and *L. brevis* in M17 broth**

162 After demonstrating proof-of-principle, we established a fermentation setup for co-culturing the two
163 strains to enable more efficient 2-butanol production. The aim was to create an environment
164 supporting a high metabolic flux in *L. lactis*, thus enabling efficient 2-butanol production, while
165 concurrently preserving a high dehydratase activity in *L. brevis*. For the latter, an active *L. brevis*
166 metabolism is needed, as re-activation of the diol dehydratase requires ATP. We decided to use rich
167 M17 medium supplemented with 2 % glucose, which supports optimal growth of *L. lactis* and to this
168 medium 7.5 μ M B12 was added. It has been shown previously that the ratio between the different
169 strains present in a co-culture has a great impact on product formation [37]. For this reason, three
170 different inoculation ratios of *L. lactis* to *L. brevis* were tested, 1:1, 1:4, and 4:1, using cell densities
171 corresponding to an OD_{600} of either 0.06 or 0.24.

172 We found that 2-butanol was formed, when using M17 medium as well (Figure 2 and Table 2). When
173 using M17 medium, it was not necessary to add 2-butanone to facilitate 2-butanol generation. The best

174 performance was observed when an excess of *L. lactis* was used (inoculation ratio 4:1), with a
175 production of 80.0 ± 1.0 mM (5.9 ± 0.1 g/L) 2-butanol and a yield of 0.58 ± 0.01 mol/mol.

176 The 4:1 culture also resulted in the lowest production of the by-products acetate, ethanol, and lactate.
177 None of the co-cultivations showed significant buildup of 2-butanol precursors, which suggests an
178 effective transfer of intermediates between the two strains. 2-butanol was not produced in any of the
179 control cultivations with *L. lactis* or *L. brevis* alone, and only modest glucose consumption was observed
180 in these cultures (data not shown). Additionally, growth of the *L. lactis* strain was dependent on the
181 catalytic activity of the *L. brevis* strain.

182 Formation of the by-products acetate, ethanol, and lactate during co-cultivation was from 79 to 136 mM,
183 as compared to *L. brevis* alone where 56 ± 1.5 mM was produced. The increase in by-product formation
184 observed in the co-cultures suggests that *L. brevis*, in addition to catalyzing the conversion of mBDO to
185 2-butanone, reduce some of the 2-butanone to 2-butanol. This issue becomes more pronounced at
186 higher initial culture ratios where the lack of 2-butanone in combination with the high acid production by
187 *L. brevis* begins to inhibit *L. lactis*, which then reaches lower CFU/mL.

188 It therefore appears to be important to restrict the amount of *L. brevis* cells present to avoid excessive
189 consumption of 2-butanone, while simultaneously ensuring that a sufficient diol dehydratase activity is
190 available. We tested other inoculation ratios as well, however, this did not lead to higher yields of 2-
191 butanol (Figure 3). Previous research into co-culture fermentations highlights division of labor and
192 functional enzyme expression to be the main burden of monocultures, whereas co-culturing is
193 constrained by the need of population control and possible limitation by transfer of intermediates [38,39].

194

195 **Table 2 Co-culture fermentation yield of products after 96 hours at different ratios of**
196 **inoculation.**

<i>L. lactis</i> :	Glucose cons. ^a	2-butanol	2-butanone	By-products ^b
<i>L. brevis</i>	mM	mol/mol glucose		
1 : 1	120±3.4	0.48±0.02	0.03±0.01	0.80±0.05
1 : 4	82.6±3.3	0.08±0.01	0.02±0.001	1.64±0.02
4 : 1	135±3.4	0.58±0.01	0.04±0.01	0.58±0.01

197 ND, not detected. ^a Glucose consumed. ^b By-products, sum of acetate, ethanol, and lactate. Yield was
 198 calculated from the fermentation experiment shown in Figure 2. Average of three independent
 199 experiments with standard deviations.

200

201 We believe that there is potential for improving the titer and yield of 2-butanol by further engineering of
 202 the strains and by optimizing the fermentation setup. In the setup used here, we relied on a wild-type
 203 *L. brevis* strain to supply the important diol dehydratase activity. Protein engineering has been used to
 204 improve the diol dehydratase performance [40], and when the improved enzyme was introduced into
 205 *K. pneumoniae*, this resulted in improved 2-butanol production [5]. It has also been reported that over-
 206 expression of the transcription factor PocR in *L. brevis* can boost the diol dehydratase activity of *L.*
 207 *brevis* [41].

208

209 **Conclusion**

210 Our work highlights the possibility of linking the metabolisms of living microorganisms for producing
 211 useful compounds. Here, we have used an engineered *L. lactis* and a wild-type *L. brevis* strain for
 212 producing 2-butanol, where the *L. lactis* strain depends on the diol dehydratase activity of the *L. brevis*
 213 strain. We achieved the highest titer (5.9 g/L) and yield (0.58 mol/mol glucose) ever reported in a one-
 214 step production setup, and we believe that our work sets the stage for future studies where
 215 metabolisms of microorganisms are linked to enable superior production of a variety of useful
 216 compounds.

217

218

219 **Methods**

220 **Strains and plasmids**

221 Strain construction in *L. lactis* was based on MG1363, a plasmid-free derivative of *L. lactis* subsp.
222 *cremoris* strain NCD0712 [42]. For optimized production of the precursor acetoin, CS4363 (MG1363
223 $\Delta^3Idh \Delta pta \Delta adhE$), was used [22]. Expression of heterogeneous genes in *L. lactis* was done using
224 plasmids pCI372 and pTD6. Derivatives of MG1363 and plasmids used in this study are described in
225 Table 3.

226 *L. brevis* SE20 [35], isolated from an ethanol pilot plant facility in Örnköldsvik Sweden was kindly
227 provided by Christer Larsson (Chalmers University of Technology, Sweden). *Escherichia coli* strain
228 Top10 {F- *mcrA* $\Delta(mrr-hsdRMS-mcrBC)$ $\phi 80/lacZ\Delta M15 \Delta lacX74$ *recA1 araD139* $\Delta(ara-leu)$ 7697 *galU*
229 *galK rpsL* (StrR) *endA1 nupG* λ -} was used for cloning purposes.

230

231 **Table 3 *L. lactis* strains and plasmids used in this study.**

Designation	Genotype or description	Reference
Strains		
CS4363	MG1363 $\Delta^3Idh \Delta pta \Delta adhE$	[22]
MM01	CS4363 pButop pDdrAB	This work
MM10	CS4363 pButop	This work
MM06	CS4363 pMM06	This work
Plasmids		
pTD6	<i>E. coli</i> / <i>L. lactis</i> shuttle vector containing <i>gusA</i> reporter, Tet	[22]
pJM001	pTD6:: <i>bdh</i> , Tet	[43]
pCI372	<i>E. coli</i> / <i>L. lactis</i> shuttle vector, Cam	[44]

pButop	pCI372:: <i>pddABC-sadB</i>	This work
pDdrAB	pTD6:: <i>ddrAB</i>	This work
pMM06	pTD6:: <i>bdh-sadB</i>	This work

232

233 **Growth conditions**

234 Cultivation of *L. lactis* and *L. brevis* were carried out in 125 mL flasks with 100 mL medium and slow
 235 magnetic stirring at 30 °C.

236 For growth experiments, *L. lactis* was cultivated in M17 medium (Oxoid, England) or defined synthetic
 237 amino-acid (SA) medium [45] with the following modification: 40mM MOPS was replaced with 100 mM
 238 potassium phosphate buffer. Both media were supplemented with 1 % glucose. For test of activity of
 239 the expressed diol dehydratase and alcohol dehydrogenase, cultivations were executed in M17
 240 medium with 7.5 µM coenzyme B12 and 20 mM mBDO or 2-butanone. Strains unable to grow
 241 anaerobically were cultivated aerobically.

242 *L. brevis* was grown in modified MRS medium [46] containing per liter: peptone, 10 g; meat extract, 10
 243 g; yeast extract, 5 g; Tween 80, 1 ml; K₂HPO₄, 2 g; sodium acetate ·3H₂O, 5 g; triammonium citrate, 2
 244 g; MgSO₄·7H₂O, 0.2 g; MnSO₄ ·4H₂O, 0.05 g; glucose or xylose, 20 g, 7.5 µmol vB12. When needed,
 245 20 mM mBDO was added to stimulate expression of diol dehydratase.

246 *E. coli* strains were grown aerobically at 37 °C in Luria-Bertani broth [47].

247 When required, antibiotics were added in the following concentrations; tetracycline: 8 µg/ml for *E. coli*
 248 and 5 µg/ml for *L. lactis*; chloramphenicol: 20 µg/mL for *E. coli* and 5 µg/mL for *L. lactis*.

249 **DNA techniques**

250 All manipulations were performed according to Sambrook and Russell (2001). *E. coli* was transformed
 251 using electroporation. *L. lactis* was made electrocompetent by growing in GM17 medium containing 1

252 % glycine and transformed by electroporation as previously described by Holo and Nes, 1989 [48].
253 Chromosomal DNA from *L. lactis* was isolated using the method described for *E. coli* by Sambrook
254 and Russel (2001) with the modification that cells were treated with 20 µg of lysozyme per ml for 2 h
255 prior to lysis.

256 **Construction of strains**

257 For construction of a 2-butanol producing *L. lactis*, the diol dehydratase and reactivase from *K.*
258 *oxytoca* ATCC 8724 [49] and 2-butanol dehydrogenase from *A. xylosoxidans* [30] were codon
259 optimized for *L. lactis* and synthesized by Genscript. *pddABC* and *sadB* and GapB promoter from *L.*
260 *lactis* was amplified using the primers VP19 (Sall) and VP20 (PstI) (Table 4). The PCR products were
261 further cloned into the XbaI/KpnI and PstI/Sall sites of pCI372, resulting in plasmid pButop. The
262 plasmid was further transformed into strain CS4363 (MG1363 $\Delta^3ldh \Delta pta \Delta adhE$), a plasmid-free
263 derivative of *L. lactis* subsp. *cremoris* strain NCD0712 [42], resulting in strain MM10. *ddrAB* and GapB
264 promoter from *L. lactis* was amplified using primers P001 and P002 and cloned at Sall/PstI of pTD6.
265 The plasmid was transformed into MM10, resulting in strain MM01.

266 For application in co-cultivation, construction of a vector for high production of the precursor mBDO
267 and expression of the 2-butanol dehydrogenase, *sadB*, was based on plasmid pJM001 [43]. pJM001
268 encode a codon optimized butanediol dehydrogenase from *E. cloacae*, *bdh*. Plasmid pMM06 was
269 constructed using Gibson assembly of *sadB* amplified using primers P038 and P039 and pJM001
270 amplified using primers P041 and P036. The plasmid was further transformed into *L. lactis* strain
271 CS4363 to generate MM06.

272

273 **Table 4 Primers used in this study**

Primer name	Primer use	Primer sequence (5'→3')
VP20	GapB promoter, PstI	ATCACTGCAGGAATAAAAATTACTGACAGC
VP19	GapB promoter, Sall	TATCAGTCGACTAGTAGTTTCCTCCTTATAG
P001	<i>ddrAB</i> + gapB, ups., PstI	ACGCCTGCAGGAATAAAAATTACTGACAGCC
P002	<i>ddrAB</i> , dwn., Sall	TGCGGTTCGACTTATTCATCTTGTTGTTCCACC
P038	<i>sadB</i> + gapB, ups., gibson	CCCTATAAGGAGGAACTACTAATGAAAGCATTAGTATATCATGGAG
P039	<i>sadB</i> , dwn., gibson	AATTCTGTGTTGCGCATGCGGGTACCTTATGCTGCTCCT
P041	pJM001, gibson	TCGAGCTCCATGGCATATG
P036	pJM001, gibson	TAGTAGTTTCCTCCTTATAGGGATTAGTTAATTAATACCATAACCACCATCA

274

275

276 Analytical methods

277 Cell growth was regularly monitored by measuring optical density at 600 nm (OD₆₀₀) and the
278 quantification of glucose, xylose, lactate, acetate, acetoin, ethanol, mBDO, 2-butanone, and 2-butanol
279 was carried out using an Ultimate 3000 high- pressure liquid chromatography system (Dionex,
280 Sunnyvale, USA) equipped with a Aminex HPX-87H column (Bio-Rad, Hercules, USA) and a Shodex
281 RI-101 detector (Showa Denko K.K., Tokyo, Japan). The column oven temperature was set at 60 °C
282 and the mobile phase consisted of 5 mM H₂SO₄, at a flow rate of 0.5 ml/min.

283 Assays

284 Diol dehydratase activity of MM10 towards 1,2-propanediol (PDO) was determined in cellular extracts
285 using the 3-methyl-2-benzothiazolinone hydrazone (MBTH) method [50]. MBTH reacts with the
286 produced propionaldehyde to form an azine derivate which can be determined by spectrophotometer
287 [51]. Cells from a 100 mL culture were harvested, washed twice, and re-suspended in 10 mM
288 potassium phosphate and 1 mM dithiothreitol buffer, pH 7.2. The cells were then disrupted by glass
289 beads (106 µm, Sigma, Prod. No. G4649) using a FastPrep (MP Biomedicals, Santa Ana, USA). The
290 reaction of 0.5 mL contained 50 mM potassium chloride, 35 mM potassium phosphate buffer pH 8,
291 0.015 mM coenzyme B12, 50 mM PDO, and appropriate amount of cellular extract. After incubation at
292 37 °C for 10 min, the reaction was terminated by addition of 0.5 mL potassium citrate buffer (0.1 M pH
293 3.6). 0.25 mL 0.5 % MBTH hydrochloride was added, left to react at 37 °C after 15 min 0.5 mL water
294 was added prior measurement at 305 nm using the Infinite M1000 PRO microplate reader.
295 Absorbance values were converted to µmol propionaldehyde using standard curve. Protein
296 concentration of cellular extracts was determined using the Bradford method, and bovine serum
297 albumin served as the standard.

298 mBDO dehydratase activity was determined in vivo in cells of SE20 cultivated in modified MRS
299 medium with 2 % glucose or xylose, with or without addition of 20 mM mBDO. Cultures were
300 harvested at late exponential phase, washed with 0.9 % sodium chloride, and re-suspended to OD₆₀₀
301 of 2.5 for conversion of 20 mM mBDO. Incubations were executed at 30 °C in SA medium added 7.5
302 µM vB12 and 1 % glucose or xylose. Product formation was determined as the sum of 2-butanone
303 and 2-butanol produced after 3h of incubation.

304 **Co-cultivation in SA medium**

305 For co-cultivations, the strains were pre-cultivated separately to obtain biomass, harvested at late
306 exponential phase by centrifugation (5000 g, 10 min), and re-suspended in co-cultivation medium at

307 the desired inoculum OD₆₀₀. Pre-cultivation of *L. lactis* strains was done in SA medium with glucose,
308 tetracycline and 20 mM 2-butanone to sustain anaerobic growth. *L. brevis* cultivations were done in
309 modified MRS medium containing 2 % glucose and 20 mM mBDO.

310 Co-cultivations were done in SA medium with 2 % glucose, 7.5 µM vB12 and 5 mM 2-butanone.
311 Inoculations were done in start OD₆₀₀ of 1:1, 1:0, and 0:1, of *L. lactis* to *L. brevis*. Product formation
312 was determined after 20 h of incubation. Cultures were prepared as biological triplicates.

313 **Co-cultivation in M17 medium**

314 For co-cultivations *L. lactis* MM06 and *L. brevis* SE20 were grown separately and harvested by
315 centrifugation at late exponential phase (OD₆₀₀=0.7 and OD₆₀₀=0.4, respectively). *L. lactis* was
316 cultivated in M17 supplemented with 1 % glucose, tetracycline and 10 mM 2-butanone. *L. brevis* was
317 cultivated in modified MRS with 1 % glucose and supplemented with 20 mM mBDO.

318 Co-cultivations were executed in M17 with 2 % glucose and 7.5 µM vB12. Minimal stirring was applied
319 to the 50 mL tubes using 1 cm rod-shaped stirring magnets to keep the culture turbid while avoiding
320 aeration. Cultures were inoculated to a final OD₆₀₀ value of either 0.06 or 0.24 for each strain resulting
321 in combinations of *L. lactis*:*L. brevis* of 1:1, 1:4, 4:1, 1:0, and 0:1. Cultures were incubated for 96 hours
322 and samples taken every 24 hours for OD₆₀₀, HPLC, and CFU analysis. Cultures were prepared as
323 biological triplicates. To verify batch-to-batch replicability, an additional co-cultivation was executed
324 using inoculation ratios of *L. lactis*:*L. brevis* of 1:4, 1:2, 1:1, 2:1, and 4:1. Product formation was
325 evaluated after 72 h of cultivation.

326 Determination of colony forming units (CFU) during co-cultivation of *L. lactis* MM06 and *L. brevis*
327 SE20, were done on agar plates consisting of a semi-defined medium [52] supplemented with 1.5 %
328 agar (w/v), 1 % glucose (w/v) and 200 µM X-gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid)

329 for colorimetric detection of β -glucuronidase activity. On these plates, the *L. lactis* appear as large blue
330 colonies whereas *L. brevis* appear as small white colonies.

331

332 **List of abbreviations**

333 *L. lactis*: *Lactococcus lactis*; *L. brevis*: *Lactobacillus brevis*; *S. marcescens*: *Serratia marcescens*; *K.*
334 *oxytoca*: *Klebsiella oxytoca*; *A. xylosoxidans*: *Achromobacter xylosoxidans*; *E. cloae*: *Enterobacter*
335 *cloae*; mBDO: meso-2,3-butanediol; LAB: lactic acid bacteria; NADH or NAD⁺: reduced or oxidized
336 form of nicotinamide adenine dinucleotide; PddABC; diol dehydratase from *K. oxytoca*; SadB: alcohol
337 dehydrogenase from *A. xylosoxidans*; DdrAB: diol dehydratase reactivase from *K. oxytoca*; Bdh:
338 butanediol dehydrogenase from *E. cloae*; OD₆₀₀: optical density at wavelength 600 nm; SA: synthetic
339 amino acid medium.

340

341 **Declarations**

342 **Ethics approval and consent to participate**

343 Not applicable.

344 **Consent for publication**

345 Not applicable.

346 **Availability of data and materials**

347 All data generated and analyzed during the current study are included in this published article and its
348 supplementary information file.

349 **Competing interest**

350 The authors declare that they have no competing interests.

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354 **Authors' contributions**

355 MJM carried out all the experimental work, analyzed the data, and wrote the manuscript. JMA
356 analyzed the data and wrote the manuscript. VJ was involved in strain construction and revised the
357 manuscript. JL provided useful suggestions for experimental design and revised the manuscript
358 critically. CS and PRJ participated in the design of the study and wrote the manuscript. All authors
359 read and approved the final manuscript.

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367 **References**

368 1. Mussatto SI, Dragone G, Guimarães PMR, Silva JPA, Carneiro LM, Roberto IC, et al.
369 Technological trends, global market, and challenges of bio-ethanol production. *Biotechnol Adv.*

- 370 2010;28(6)817–30.
- 371 2. Nanda S, Golemi-Kotra D, McDermott JC, Dalai AK, Gökalp I, Kozinski JA. Fermentative
372 production of butanol: Perspectives on synthetic biology. *N Biotechnol.* 2017;37(B)210–21.
- 373 3. Atsumi S, Hanai T, Liao JC. Non-fermentative pathways for synthesis of branched-chain higher
374 alcohols as biofuels. *Nature.* 2008;45186–9.
- 375 4. Hahn H-D, Dämbkes G, Rupprich N, Bahl H, Frey GD. Butanols. In: *Ullmann's Encyclopedia of*
376 *Industrial Chemistry.* Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA; 2013.
377 doi:10.1002/14356007.a04_463.pub3.
- 378 5. Chen Z, Wu Y, Huang J, Liu D. Metabolic engineering of *Klebsiella pneumoniae* for the de novo
379 production of 2-butanol as a potential biofuel. *Bioresour Technol.* 2015;197260–5.
- 380 6. Russmayer H, Marx H, Sauer M. Microbial 2-butanol production with *Lactobacillus diolivorans*.
381 *Biotechnol Biofuels.* 2019;12(262).
- 382 7. Zhang L, Singh R, Sivakumar D, Guo Z, Li J, Chen F, et al. An artificial synthetic pathway for
383 acetoin, 2,3-butanediol, and 2-butanol production from ethanol using cell free multi-enzyme
384 catalysis. *Green Chem.* 2018;20(1)230–42.
- 385 8. Srirangan K, Liu X, Akawi L, Bruder M, Moo-Young M, Chou CP. Engineering *Escherichia coli*
386 for microbial production of butanone. *Appl Environ Microbiol.* 2016;82(9)2574–84.
- 387 9. Jiang W, Wang S, Wang Y, Fang B. Key enzymes catalyzing glycerol to 1,3-propanediol.
388 *Biotechnol Biofuels.* 2016;9(57).
- 389 10. Yang M, Yun J, Zhang H, Magocha TA, Zabed H, Xue Y, et al. Genetically engineered strains:
390 application and advances for 1,3-propanediol production from glycerol. *Food Technol*
391 *Biotechnol.* 2018;56(1)3–15.
- 392 11. Raynaud C, Sarçabal P, Meynial-Salles I, Croux C, Soucaille P. Molecular characterization of
393 the 1,3-propanediol (1,3-PD) operon of *Clostridium butyricum*. *Proc Natl Acad Sci U S A.*
394 2003;100(9)5010–5.
- 395 12. Toraya T, Shirakashi T, Kosuga T, Fukui S. Substrate specificity of coenzyme B12-dependent
396 diol dehydratase: glycerol as both a good substrate and a potent inactivator. *Biochem Biophys*
397 *Res Commun.* 1976;69(2)475–80.
- 398 13. Moore KW, Richards JH. Stereospecificity and mechanism of adenosylcobalamin-dependent
399 diol dehydratase. Catalysis and inactivation with meso- and DL-2,3-butanediols as substrates.
400 *Biochem Biophys Res Commun.* 1979;87(4)1052–7.
- 401 14. Mori K, Hosokawa Y, Yoshinaga T, Toraya T. Diol dehydratase-reactivating factor is a
402 reactivase - evidence for multiple turnovers and subunit swapping with diol dehydratase. *FEBS*

- 403 J. 2010;277(23)4931–43.
- 404 15. Sauvageot N, Muller C, Hartke A, Auffray Y, Laplace J-M. Characterisation of the diol
405 dehydratase pdu operon of *Lactobacillus collinoides*. FEMS Microbiol Lett. 2002;209(1)69–74.
- 406 16. Hieke E, Vollbrecht D. On the formation of butanol-2 by lactic acid bacteria and yeast. Arch
407 Microbiol. 1974;99(1)345–51.
- 408 17. Chen Z, Sun H, Huang J, Wu Y, Liu D. Metabolic engineering of *Klebsiella pneumoniae* for the
409 production of 2-butanone from glucose. PLoS One. 2015;10(10).
- 410 18. Mazzoli R, Bosco F, Mizrahi I, Bayer EA, Pessione E. Towards lactic acid bacteria-based
411 biorefineries. Biotechnol Adv. 2014;32(7)1216–36.
- 412 19. Börner RA, Kandasamy V, Axelsen AM, Nielsen AT, Bosma EF. Genome editing of lactic acid
413 bacteria: Opportunities for food, feed, pharma and biotech. FEMS Microbiol Lett.
414 2019;366(fny291).
- 415 20. Sauer M, Russmayer H, Grabherr R, Peterbauer CK, Marx H. The efficient clade: Lactic acid
416 bacteria for industrial chemical production. Trends Biotechnol. 2017;35(8)756–69.
- 417 21. Liu J, Wang Z, Kandasamy V, Lee SY, Solem C, Jensen PR. Harnessing the respiration
418 machinery for high-yield production of chemicals in metabolically engineered *Lactococcus*
419 *lactis*. Metab Eng. 2017;4422–9.
- 420 22. Solem C, Dehli T, Jensen PR. Rewiring *Lactococcus lactis* for ethanol production. Appl Environ
421 Microbiol. 2013;79(8)2512–8.
- 422 23. Kandasamy V, Liu J, Dantoft SH, Solem C, Jensen PR. Synthesis of (3R)-acetoin and 2,3-
423 butanediol isomers by metabolically engineered *Lactococcus lactis*. Sci Rep. 2016;6(36769).
- 424 24. Teuber M. The genus *Lactococcus*. In: Wood BJB, Holzapfel WH, editors. The genera of lactic
425 acid bacteria. Boston: Springer; 1995. 173–234.
- 426 25. Petersen KV, Martinussen J, Jensen PR, Solem C. Repetitive, marker-free, site-specific
427 integration as a novel tool for multiple chromosomal integration of DNA. Appl Environ Microbiol.
428 2013;79(12)3563–9.
- 429 26. Solem C, Jensen PR. Modulation of gene expression made easy. Appl Environ Microbiol.
430 2002;68(5)2397–403.
- 431 27. Kleerebezem M, Beerthuyzen MM, Vaughan EE, De Vos WM, Kuipers OP. Controlled gene
432 expression systems for lactic acid bacteria: Transferable nisin-inducible expression cassettes
433 for *Lactococcus*, *Leuconostoc*, and *Lactobacillus* spp. Appl Environ Microbiol.
434 1997;63(11)4581–4.
- 435 28. Gaspar P, Neves AR, Ramos A, Gasson MJ, Shearman CA, Santos H. Engineering

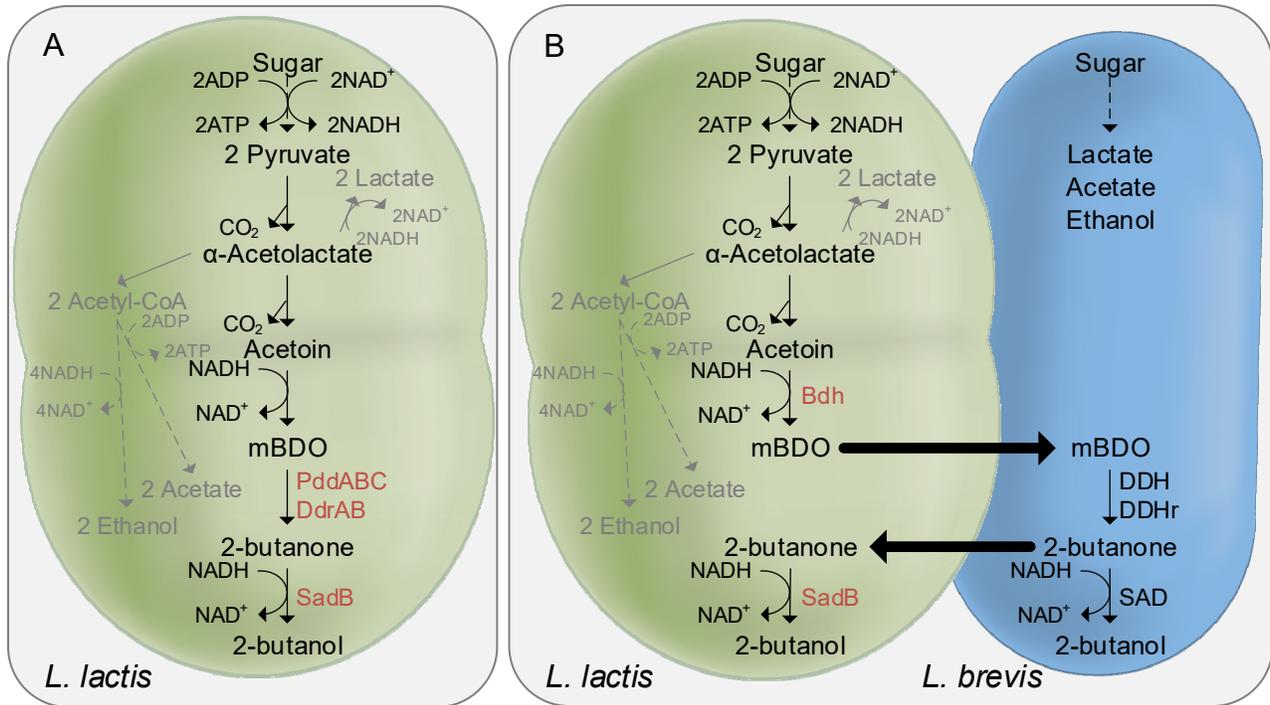
- 436 *Lactococcus lactis* for production of mannitol: high yields from food-grade strains deficient in
437 lactate dehydrogenase and the mannitol transport system. Appl Environ Microbiol.
438 2004;70(3)1466–74.
- 439 29. Liu J, Kandasamy V, Würtz A, Jensen PR, Solem C. Stimulation of acetoin production in
440 metabolically engineered *Lactococcus lactis* by increasing ATP demand. Appl Microbiol
441 Biotechnol. 2016;100(22)1–9.
- 442 30. Bramucci MG, Eliot AC, Maggio-Hall LA, Nakamura CE. Butanol dehydrogenase enzyme from
443 the bacterium *Achromobacter xylosoxidans*. United States patent; US 8,188,250 B2, 2012.
- 444 31. Yoneda H, Tantillo DJ, Atsumi S. Biological production of 2-butanone in *Escherichia coli*.
445 ChemSusChem. 2014;7(1)92–5.
- 446 32. Wegmann U, O’Connell-Motherway M, Zomer A, Buist G, Shearman C, Canchaya C, et al.
447 Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp.
448 *cremoris* MG1363. J Bacteriol. 2007;189(8)3256–70.
- 449 33. Morita H, Hidehiro T, Fukuda S, Horikawa H, Oshima K, Suzuki T, et al. Comparative genome
450 analysis of *Lactobacillus reuteri* and *Lactobacillus fermentum* reveal a genomic island for
451 reuterin and cobalamin production. DNA Res. 2008;15(3)151–61.
- 452 34. Santos F, Vera JL, van der Heijden R, Valdez G, de Vos WM, Sesma F, et al. The complete
453 coenzyme B12 biosynthesis gene cluster of *Lactobacillus reuteri* CRL 1098. Microbiology.
454 2008;154(1)81–93.
- 455 35. Ghiaci P, Lameiras F, Norbeck J, Larsson C. Production of 2-butanol through meso-2,3-
456 butanediol consumption in lactic acid bacteria. FEMS Microbiol Lett. 2014;360(1)70–5.
- 457 36. Zhang Y, Zeng F, Hohn K, Vadlani P V. Metabolic flux analysis of carbon balance in
458 *Lactobacillus* strains. Biotechnol Prog. 2016;32(6)1397–403.
- 459 37. Jones JA, Vernacchio VR, Sinkoe AL, Collins SM, Ibrahim MHA, Lachance DM, et al.
460 Experimental and computational optimization of an *Escherichia coli* co-culture for the efficient
461 production of flavonoids. Metab Eng. 2016;3555–63.
- 462 38. Zhang H, Wang X. Modular co-culture engineering, a new approach for metabolic engineering.
463 Metab Eng. 2016;37114–21.
- 464 39. Jones JA, Wang X. Use of bacterial co-cultures for the efficient production of chemicals. Curr
465 Opin Biotechnol. 2018;5333–8.
- 466 40. Maddock DJ, Gerth ML, Patrick WM. An engineered glycerol dehydratase with improved activity
467 for the conversion of meso-2,3-butanediol to butanone. Biotechnol J. 2017;1700480.
- 468 41. Santos F, Spinler JK, Saulnier DM, Molenaar D, Teusink B, de Vos WM, et al. Functional

- 469 identification in *Lactobacillus reuteri* of a PocR-like transcription factor regulating glycerol
470 utilization and vitamin B12 synthesis. *Microb Cell Fact.* 2011;10(55).
- 471 42. Gasson MJ. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic
472 streptococci after protoplast-induced curing. *J Bacteriol.* 1983;154(1)1–9.
- 473 43. Liu J, Chan SHJ, Brock-Nannestad T, Chen J, Lee SY, Solem C, et al. Combining metabolic
474 engineering and biocompatible chemistry for high-yield production of homo-diacetyl and homo-
475 (S,S)-2,3-butanediol. *Metab Eng.* 2016;3657–67.
- 476 44. Hayes F, Daly C, Fitzgerald GF. Identification of the minimal replicon of *Lactococcus lactis*
477 subsp. *lactis* UC317 plasmid pCI305. *Appl Environ Microbiol.* 1990;56(1)202–9.
- 478 45. Jensen PR, Hammer K. Minimal requirements for exponential growth of *Lactococcus lactis*.
479 *Appl Environ Microbiol.* 1993;59(12)4363–6.
- 480 46. Elferink SJWHO, Krooneman J, Jan C, Spoelstra SF, Faber F. Anaerobic conversion of lactic
481 acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri*. *Appl Environ Microbiol.*
482 2001;67(1)125–32.
- 483 47. Sambrook J, Russell DW. *Molecular cloning: A laboratory manual*. Third. New York: Cold
484 Spring Harbor Laboratory Press; 2001.
- 485 48. Holo H, Nes IF. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp.
486 *cremoris* grown with glycine in osmotically stabilized media. *Appl Environ Microbiol.*
487 1989;55(12)3119–23.
- 488 49. Tobimatsu T, Hara T, Sakaguchi M, Kishimoto Y, Wada Y, Isoda M, et al. Molecular cloning,
489 sequencing, and expression of the genes encoding adenosylcobalamin-dependent diol
490 dehydrase of *Klebsiella oxytoca*. *J Biol Chem.* 1995;270(13)7142–8.
- 491 50. Toraya T, Ushio K, Fukui S, Hogenkamp HPC. Studies on the mechanism of the
492 adenosylcobalamin-dependent diol dehydrase reaction by the use of analogs of the coenzyme.
493 *J Biol Chem.* 1977;(3)963–70.
- 494 51. Paz MA, Blumenfeld OO, Rojkind M, Henson E, Furfine C, Gallop PM. Determination of
495 carbonyl compounds with N-methyl benzothiazolone hydrazone. *Arch Biochem Biophys.*
496 1965;109(3)548–59.
- 497 52. Kimmel SA, Roberts RF. Development of a growth medium suitable for exopolysaccharide
498 production by *Lactobacillus delbrueckii* ssp. *bulgaricus* RR. *Int J Food Microbiol.* 1998;40(1–
499 2)87–92.

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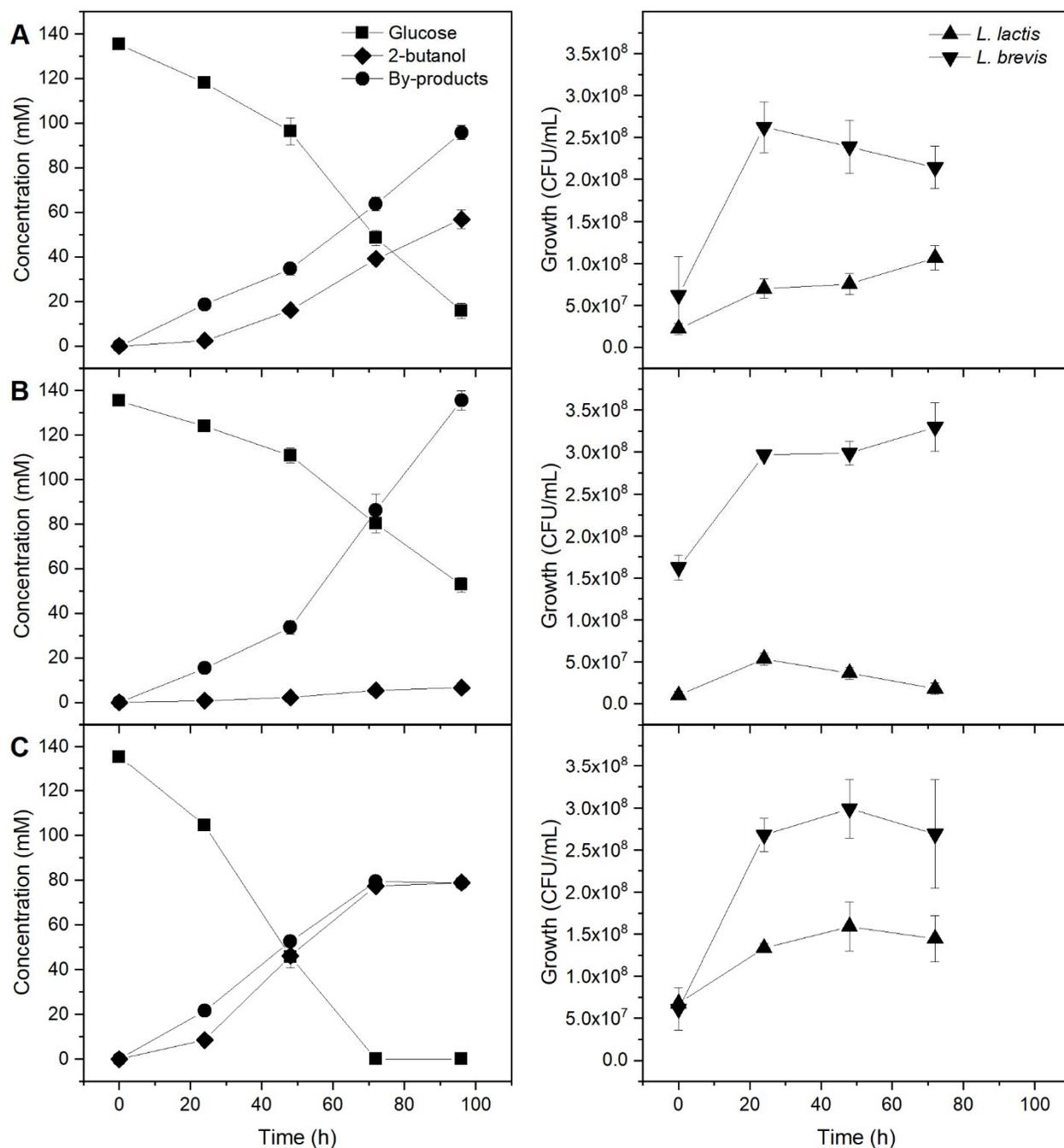
502 **List of figures**



503

504 **Figure 1 Linking the metabolism of *L. lactis* and *L. brevis* to achieve 2-butanol production. A:**
 505 **Metabolic pathway based on *L. lactis* CS4363 (mBDO added in the medium). The constructed strain**
 506 **encodes: PddABC, diol dehydratase, and DdrAB, reactivase from *K. oxytoca*; SadB, secondary**
 507 **alcohol dehydrogenase from *A. xylooxidans*. Pathways in gray indicate activities that have been**
 508 **eliminated. Dashed lines indicate multiple enzymatic steps. B: The combined metabolic pathway for *L.***
 509 ***lactis* and *L. brevis*. Bdh, butanediol dehydrogenase from *Enterobacter cloae*. DDH, diol dehydratase.**
 510 **DDHr, diol dehydratase reactivase. SAD, secondary alcohol dehydrogenase. Only heterologously**
 511 **expressed gene activities in *L. lactis* and activities related to 2-butanol synthesis in *L. brevis* are**
 512 **highlighted.**

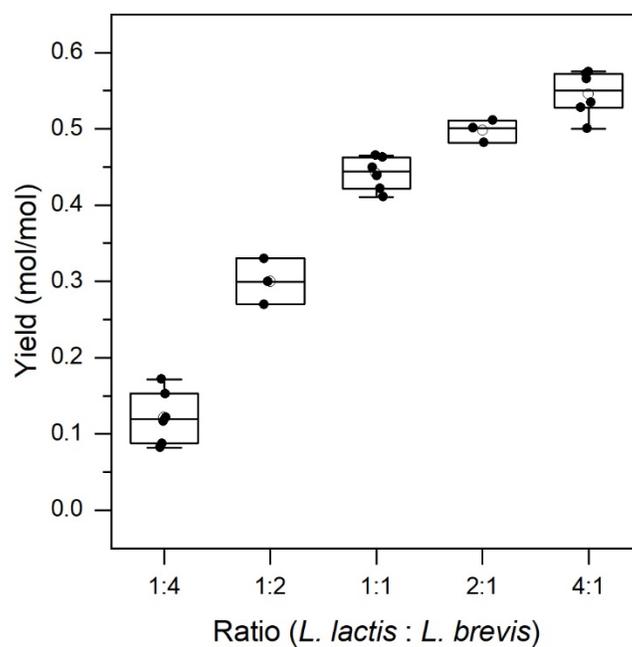
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514

515 **Figure 2 Product formation (left) and growth (right) for co-cultivation of recombinant *L. lactis***
 516 **and *L. brevis*.** Inoculation ratios *L. lactis*:*L. brevis* of 1:1, 1:4, and 4:1, panel A, B, and C, respectively.

517 Average of three independent experiments with standard deviations.



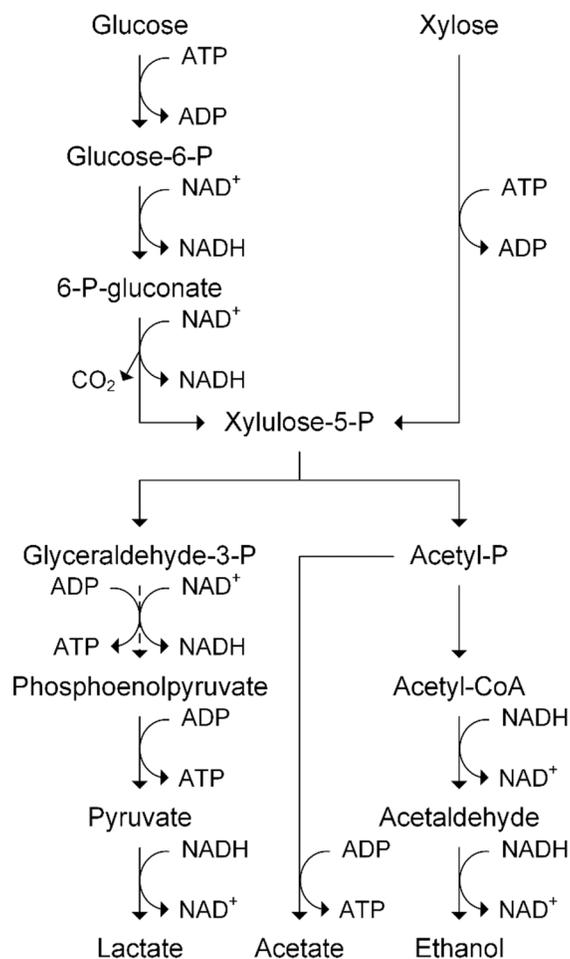
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519 **Figure 3 Co-culture yield of 2-butanol at different inoculation ratio of *L. lactis* and *L. brevis*.**

520 Production after 72 h of cultivation. Average of 3 or 6 independent experiments.

521

522



524

525 **Figure S1 Overview of glucose and xylose metabolism in *L. brevis*.** On glucose, two NADH are
 526 formed in the oxidative pentose phosphate pathway, and these have to be oxidized through ethanol
 527 formation from acetyl-CoA. Thus, the Acetyl-P cannot give rise to ATP formation through the action of
 528 acetate kinase. On xylose, however, there is no such constraint, and the acetyl-P can be used for
 529 generating ATP.

530

531 **Table S1 Production of 2-butanol from lactose and xylose in defined SA medium using resting**
532 **cells of *L. lactis* and *L. brevis*.**

Sugar	2-butanol mM	Yield ^a mol/mol
Glucose	14.2±0.6	0.5±0.02
Lactose + Xylose	13.0±0.01	0.1±0.001

533 To allow lactose utilization in our constructed *L. lactis*, we re-introduced the lactose plasmid pLP712
534 by transformation. On the mixed carbon, *L. lactis* can utilize only lactose whereas *L. brevis* can utilize
535 only xylose.

536 Strains were incubated in defined SA medium with glucose or lactose and xylose, 2-butanone, and
537 vitamin B12 for 20 h. Values are average of three independent experiments with standard deviations. ^a
538 2-butanol yield per mol sugar in glucose units, excluding the added 2-butanone.

Chapter V Scaling up a 2-butanol co-cultivation

1. Introduction

Microbial production of 2-butanol using single-cultures has faced limited success mainly due to the challenging dehydration of *meso*-2,3-butanediol (mBDO) to 2-butanone. So far, the only report of 2-butanol production from glucose using a single organism was achieved through metabolic engineering of *Klebsiella pneumonia*, where 1.03 g/L of 2-butanol was achieved [11]. The highest titer of 2-butanol 13.4 g/L reported was achieved using a two-step fermentation of *Serratia marcescens* and an engineered *Lactobacillus diolivorans* [93]. In the first step, mBDO is produced from glucose using *S. marcescens*. Then the microbe is inactivated by heat and new medium is added together with *L. diolivorans*. In the second step, *L. diolivorans* converts mBDO into 2-butanol. This strategy is a good example of taking advantage of the two microbes' strengths. However, the low yield of 0.24 mol/mol glucose and the use of a two-step fermentation including a 30 min heat inactivation might not be compatible with industrial application.

Albeit a two-step fermentation process might not be applicable with large-scale fermentation, taking advantage of different organisms strengths for production of 2-butanol could be beneficial. Some of the benefits related to use of co-cultures, as opposed to mono-cultures, is to reduce the metabolic burden of the constituent strains and the easy modulation of the strength between different parts of the pathway by simply changing the inoculation ratio [197]. There are, however, also some critical challenges to overcome when using co-cultivations. The main issues is how to maintain the co-existence of the constituent strains and achieving a stable ratio between them throughout the fermentation [22]. This is especially an issue for competitive co-cultivations, where the different microbes are utilizing the same carbon source.

The challenges with achieving stable co-cultivations might influence the scale-ability as the environment in large-scale fed-batch bioreactors is very dynamic with large spatial and temporal heterogeneities [198]. As the response to environmental oscillations is organism-specific [199], it is likely that the constituent strains of the co-cultivation will be influenced differently upon scale-up. An evaluation of the cultivation dynamics in a bioreactor is therefore needed to assess the prospects of achieving industrial scale production of 2-butanol using co-cultivation.

In order to achieve high yields of fermentation products, it is essential that the producing organism is able to maintain the redox balance. This has previously been demonstrated for production of a wide selection of chemicals in various organisms e.g. 1-butanol in *Escherichia coli* [200], acetoin, diacetyl, and butanediol isomers in *Lactococcus lactis* [143,201], as well as 2-butanol production in *Saccharomyces cerevisiae* [92]. The microbial need for maintaining the redox balance can thus be used as a driving force to direct the flux.

In Chapter 4, a strategy for 2-butanol production using two-strain co-cultivation is presented. In the process, the metabolism and growth of an engineered *Lactococcus lactis* is coupled to the butanediol dehydratase activity of a wild-type *Lactobacillus brevis* (Figure V-1). In the co-cultivation, *L. lactis* is constructed so that it will maintain its redox balance when producing 1 mol of mBDO and 1 mol of 2-butanol per mol of glucose. *L. brevis* is additionally capable of converting 2-butanone to 2-butanol. In order to achieve high yields, the metabolism of *L. brevis* should therefore be minimized while maintaining activity of the essential diol dehydratase. Using this setup, it was possible to produce 5.9 g/L 2-butanol with a yield of 0.58 mol/mol in 50 mL cultivation tubes, which is the highest reported using a single step fermentation. In the current study, we investigate 2-butanol production using the co-cultivation strategy with *L. lactis* and *L. brevis* is investigated in a 1 L bioreactor.

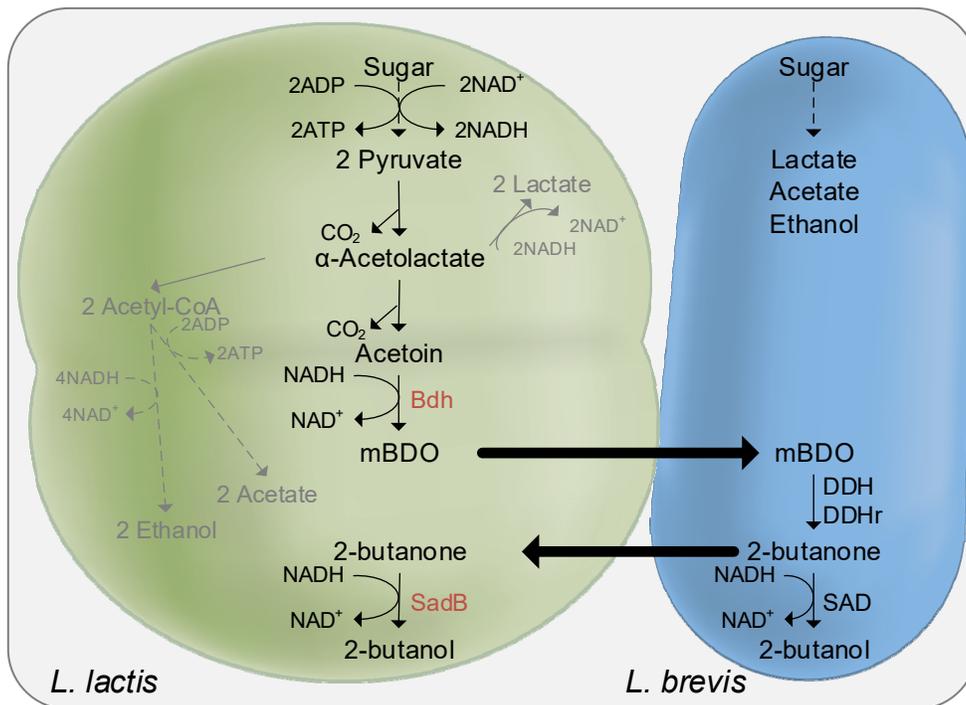


Figure V-1 Linking the metabolism of *L. lactis* and *L. brevis* to achieve 2-butanol production. The constructed *L. lactis* is based on strain MG1363 $\Delta^3ldh \Delta pta \Delta adhE$ with expression of Bdh, butanediol dehydrogenase from *E. cloae* and SadB, secondary alcohol dehydrogenase from *A. xylosoxidans*. Pathways in gray indicate activities that have been eliminated. Dashed lines indicate multiple enzymatic steps. DDH, diol dehydratase. DDHr, diol dehydratase reactivator. SAD, secondary alcohol dehydrogenase. Only heterologously expressed gene activities in *L. lactis* and activities related to 2-butanol synthesis in *L. brevis* are highlighted. (Chapter IV).

2. Methods

2.1. Strains

Co-cultivation was executed using *L. lactis* MM06 (MG1363 $\Delta^3ldh \Delta pta \Delta adhE$ pTD6::*bdh-sadB*) (Chapter IV) and *L. brevis* SE20 [142]. *L. brevis* was kindly provided by Christer Larsson (Chalmers University of Technology, Sweden).

2.2. Growth conditions

Prior to co-cultivations, *L. lactis* and *L. brevis* were grown separately as batch cultures (flasks) at 30 °C and harvested at late exponential phase ($OD_{600}=0.7$ and $OD_{600}=0.4$, respectively). *L. lactis* was cultivated in M17 medium (Oxoid, England) supplemented with 10 g/L glucose, 20 mM 2-butanone, and 5 µg/mL tetracycline. *L. brevis* was grown in modified MRS medium [145] containing: 10 g/L peptone, 10 g/L meat extract, 5 g/L yeast extract, 1 ml/L Tween 80, 2 g/L K_2HPO_4 , 5 g/L sodium acetate $\cdot 3H_2O$, 2 g/L triammonium citrate, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L $MnSO_4 \cdot 4H_2O$, 20 g/L glucose, 10 µM vitamin B12, and 20 mM mBDO was added for induction of diol dehydratase activity.

2.3. Bioreactor cultivation

Co-cultivations were performed in a 1 L Biostat® A (Sartorius) with a working volume of 500 mL in M17 medium supplemented with glucose and 7.5 µM vitamin B12. The medium was inoculated with exponentially growing pre-cultures to OD_{600} of 0.24 for *L. lactis* and 0.06 for *L. brevis*, unless stated otherwise. During fermentation, the temperature was maintained at 30 °C, agitation speed at 140 rpm, and pH controlled by automatic addition of 1 M NaOH. Samples were withdrawn every 24 h for analysis of cell growth and product formation.

Different fermentation settings were tested in three batches of fermentations, batch A, B, and C, each comprising four fermenters (for overview, see Table V-1).

2.3.1. Fermentation batch A – pH control

Prior to inoculation, the cultivation medium was flushed with CO₂ at 6000 ccm for 25 min. The cultivation medium was supplemented with 130 mM glucose and pH was maintained at 6.0, 5.5, 5.0, or 4.5. During cultivation CO₂ was sparged through the medium at 150 ccm.

2.3.2. Fermentation batch B – relieving CO₂ partial pressure

Prior to inoculation, the cultivation medium was flushed with CO₂ or air at 6000 ccm for 25 min. Cultivation medium was supplemented with 75 mM glucose, with or without addition of 5 mM 2-butanone. During cultivation, the headspace was flushed with CO₂ or air at 40 ccm and pH was maintained at 6.0.

2.3.3. Fermentation batch C – reducing the inoculation of *L. brevis*

Prior to inoculation, the cultivation medium was flushed with CO₂ at 6000 ccm for 25 min. During cultivation, the headspace was flushed with air at 40 ccm and pH was maintained at 6.0. Cultivation medium was supplemented with 100 mM glucose and 5 mM 2-butanone. The medium was inoculated with *L. lactis* to a starting OD₆₀₀ of 0.24 and of *L. brevis* to a starting OD₆₀₀ of 0.06, 0.03, 0.015, and 0.0075, corresponding to an inoculation ratio of 4:1, 4:0.5, 4:0.25, and 4:0.125, respectively.

Table V-1 Experimental settings used for bioreactor tests. For further description of the settings, see section 2.3.1 to 2.3.3.

Batch	Ferm No.	Purge	Glucose (mM)	2-butanone (mM)	Inoculum ratio	Sparging	Headspace	pH
A	F1	CO ₂	130	None	4:1	CO ₂	None	6
	F2	CO ₂	130	None	4:1	CO ₂	None	5.5
	F3	CO ₂	130	None	4:1	CO ₂	None	5
	F4	CO ₂	130	None	4:1	CO ₂	None	4.5
B	F5	CO ₂	75	5	4:1	None	CO ₂	6
	F6	CO ₂	75	5	4:1	None	Air	6
	F7	Air	75	5	4:1	None	CO ₂	6
	F8	Air	75	None	4:1	None	CO ₂	6
C	F9	CO ₂	100	5	4:1	None	Air	6
	F10	CO ₂	100	5	4:0.5	None	Air	6
	F11	CO ₂	100	5	4:0.25	None	Air	6
	F12	CO ₂	100	5	4:0.125	None	Air	6

2.4. Analysis of cellular growth

Cell growth was monitored by measuring optical density at 600 nm (OD₆₀₀). Viability and cellular ratio of *L. lactis* and *L. brevis* was assessed by determination of colony forming units (CFU) using agar plates consisting of a semi-defined medium [202] supplemented with 1.5 % agar (w/v), 1 % glucose (w/v) and 200 μM X-gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid) for colorimetric detection of β-glucuronidase activity. On these plates, the *L. lactis* appear as large

blue colonies whereas *L. brevis* appear as small white colonies. The results obtained by this method thus indicate the ratio of the viable cells rather than the ratio of these cells' overall biomass.

2.5. Metabolite quantification

Quantification of glucose, lactate, acetate, acetoin, ethanol, mBDO, 2-butanone, and 2-butanol was carried out using an Ultimate 3000 high-pressure liquid chromatography system (Dionex, Sunnyvale, USA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, USA) and a Shodex RI-101 detector (Showa Denko K.K., Tokyo, Japan). The column oven temperature was set at 60 °C and the mobile phase consisted of 5 mM H₂SO₄, at a flow rate of 0.5 ml/min.

2.6. Calculations

Calculation of carbon balance was done on the basis of Cmol. Production of CO₂ was estimated as one mole per mole acetate and ethanol, and two moles per mole of mBDO, 2-butanone, and 2-butanol. In fermentations with initial addition of 2-butanone, this was corrected for in the balances as well as the 2-butanol yield. Dry cell weight was estimated from measurement of OD₆₀₀ to be 0.35 g/L*OD₆₀₀, as for *L. lactis* MG1363 [151]. The elemental composition of the biomass was estimated to be CH_{1.8}O_{0.5}N_{0.2} [203]. Volume change resulting from sampling was included in the calculations.

3. Results and discussion

3.1. pH control influences 2-butanol production

As a starting point for the setup in the bioreactor, the best performing settings from cultivations tubes was used as reference; cultivation was executed in M17 medium at 30 °C with minimal stirring and inoculation with *L. lactis* to *L. brevis* at a 4:1 ratio. Through a series of fermentation batches, different fermentation conditions were tested and their influence on fermentation performance evaluated.

For industrial productions, the ability to run fermentations at pH below 6.0 can reduce the risk of contamination and decrease the costs [101]. Lactic acid bacteria and especially lactobacilli and pediococci are among the most acid and low-pH tolerant bacteria [104]. There are, however, large variations among species, and neither *L. lactis* or *L. brevis* are resistant to pH below 4.0 [104]. Evaluation of the effect of pH control on the production of 2-butanol was therefore tested under fixed pH of 6.0, 5.5, 5.0, and 4.5.

2-butanol production correlated with the pH and was achieved at pH 5.0 to 6.0 (Table V-2). The best performing fermentation was at pH 6.0 with a 2-butanol titer of 4.1 mM and yield of 0.6 mol/mol after 48 h of cultivation. No significant amounts of pre-cursors were identified.

In all fermenters, the 2-butanol concentration peaked at 24 or 48 h, followed by loss of product by evaporation. The highest amount of viable *L. lactis* was additionally identified after 24 h of fermentation (Figure V-2). As for the production of 2-butanol, fermentation at pH 6.0 resulted in the highest amount of viable *L. lactis*. All four fermentations resulted in a loss of viable *L. lactis* over time. Decreasing pH below pH 6.0 diminish the catabolic flux and growth rate *L. lactis* [204] but can also increase the flux towards the 2-butanol pre-cursor acetoin [205].

Growth of *L. brevis* as well as formation of by-products lactate, acetate, and ethanol continued to increase throughout the duration of the fermentation. As *L. lactis* MM06 can produce only negligible amounts of lactate, *L. brevis* is assumed to be responsible for production of the three by-products. The decreased production of by-products at lower pH indicates that the metabolism of *L. brevis* was additionally reduced.

Table V-2 Co-culture fermentation products at different pH. ^aBy-products is the sum of lactate, acetate, and ethanol. ^bYield 2-butanol in mol/mol glucose. ^cCarbon balance calculated in Cmol, as described in section 2.6.

Time (h)	pH	Glucose consumed (mM)	2-butanol	By-products ^a	Yield ^b (mol/mol)	Balance ^c
24	6.0	37.0	3.9	43.2	0.11	1.1
	5.5	32.0	3.2	41.3	0.10	1.0
	5.0	30.1	2.4	42.9	0.08	1.1
	4.5	30.0	0.0	33.6	0.00	1.0
48	6.0	64.7	4.1	104.8	0.06	1.1
	5.5	54.7	1.9	87.3	0.04	1.0
	5.0	62.5	1.6	81.2	0.03	0.9
	4.5	49.6	0.0	72.0	0.00	0.9

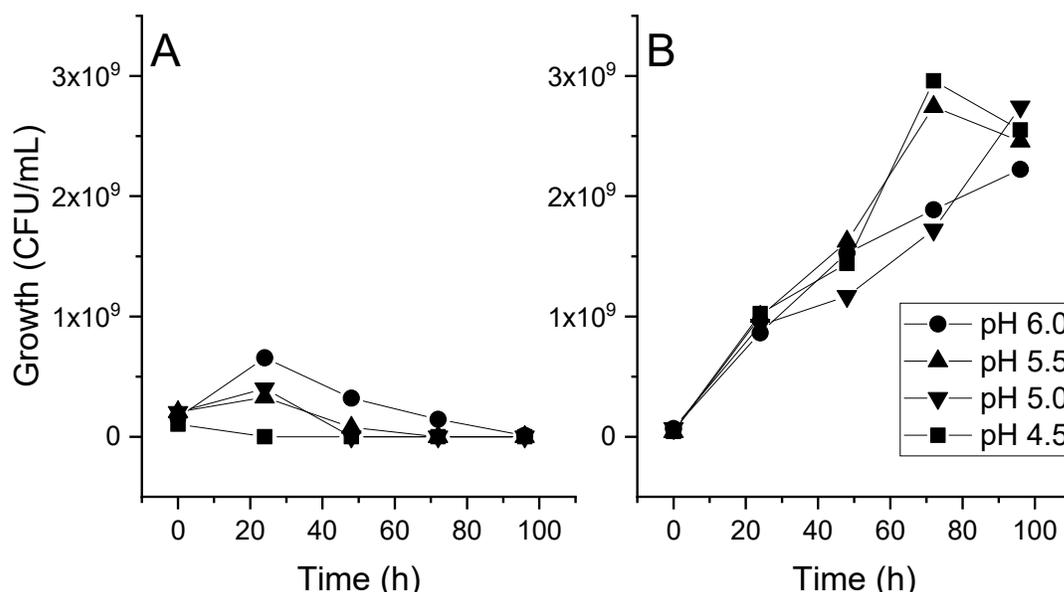


Figure V-2 Growth of *L. lactis* (A) and *L. brevis* (B) in co-cultures at different pH.

3.2. Removing CO₂ sparging leads to improved production

After demonstrating that 2-butanol production using co-cultivation of *L. lactis* and *L. brevis* was possible in 1 L bioreactor, we set out to optimize the production with pH fixed at 6.0. The obtained titer and yield in the bioreactors were, however, significant below those obtained in 50 mL cultivations, thus improvements should be possible. Additionally, the used settings seemed unable to sustain growth of *L. lactis* beyond 24 h. Increasing the initial inoculation of *L. lactis* did not significantly improve the production (data not shown). We therefore speculated if changing the operating conditions e.g. the means of which anaerobic conditions was achieved, could improve 2-butanol synthesis.

In the fermentations above, anaerobic conditions were achieved by sparging of CO₂ during the fermentation. This could result in inhibition of the metabolism as the partial pressure of CO₂ is known to inhibit metabolic reactions such as acetolactate synthase [206]. Exchanging CO₂ for nitrogen negatively influenced the production (data not shown). As a batch process, supplying CO₂ only to the headspace as opposed to sparging, should be sufficient for maintaining anaerobic conditions. Initial addition of a small amount of 2-butanone or oxygen could additionally result in a boost of the production, as was previously seen for 50 mL co-cultivations in defined SA medium (Chapter IV).

To test the hypothesis, three parameters were chosen for; purge of the fermentation broth prior to inoculation using CO₂ or air, flushing of headspace throughout the length of the fermentation using CO₂ or air, and addition of initial 5 mM 2-butanone to the cultivation medium. Four fermentations were executed, F5 to F8 (for detailed description of settings, see Methods section 2.3).

Changing the fermentation settings resulted in increased 2-butanol production (Table V-3).. After 24 h of cultivation, the highest production of 20.0 mM 2-butanol was achieved in F6 and F7. This was further increased to 38 and 21 mM after 48 h of cultivation in F6 and F7, respectively. Glucose was exhausted at 48 h (F6 and F7), 72 h (F5), or remained throughout the duration of the fermentation (F8).

Fermentation F6 was preferred over the other fermentation setups in terms of the performance at 24 and 48 h with respect to both 2-butanol titer and yields, as well as by-product formation. Issues regarding the carbon balances of the fermentation (closing between 0.6 and 1.4) interfered with proper evaluation of the results, thus no further comparison will be done.

The issues with the carbon balances is likely a result of several factors, mainly: Following sterilization by autoclave of the bioreactors containing media, the four fermenters showed variation in the resulting volume and color of the media; The HPLC instrument was not running properly at the time of analysis; And the amount of base used for pH adjustment was not measured, thus could not be accounted for in the calculations.

Table V-3 Co-culture fermentation products at different fermentation settings.

Time (h)	Ferm ^a	Setting	Glucose consumed (mM)	2-butanol	By-products ^b	2-butanol ^c (mol/mol)	Balance ^d
24	F5	MT CO ₂ ; HS CO ₂ ; B	41.1	15.7	36.8	0.24	1.2
	F6	MT CO ₂ ; HS air; B	51.8	19.9	40.5	0.27	1.2
	F7	MT air; HS CO ₂ ; B	59.2	20.0	77.7	0.24	1.4
	F8	MT air; HS CO ₂	55.5	4.1	23.6	0.07	0.6
48	F5	MT CO ₂ ; HS CO ₂ ; B	71.7	26.1	71.0	0.28	1.1
	F6	MT CO ₂ ; HS air; B	75.1	37.8	83.7	0.42	1.4
	F7	MT air; HS CO ₂ ; B	73.2	21.2	105.4	0.21	1.3
	F8	MT air; HS CO ₂	55.2	11.2	83.0	0.20	1.3

^aFermentation number and setting, see section 2.3 for details. ^bSum of by-products lactate, acetate, and ethanol. ^cYield of 2-butanol was corrected for the added 2-butanone. ^dCarbon balance calculated in Cmol, as described in section 2.6. MT, medium treatment; HS, headspace; B, 2-butanone

3.3. Reducing the inoculation of *L. brevis* influences the initial product formation

After demonstrating that improved titer and yield could be achieved by removing the sparging of CO₂, I continued evaluating the effect of changing the inoculation ratio. This was done using fermentation setup F6 from above, which resulted in the highest 2-butanol yield. From the previous 50 mL cultivations an inoculation ratio of *L. lactis* to *L. brevis* of 4:1 showed best performance. In the bioreactors, four different inoculation ratios of *L. lactis* to *L. brevis* were tested, 4:1, 8:1, 16:1, and 32:1, based on starting OD₆₀₀ at *L. lactis* of 0.24, and *L. brevis* of 0.06 to 0.0075. These ratios were chosen as previous findings from 50 mL cultivations found it beneficial to use a lower *L. brevis* inoculum as that of *L. lactis*.

2-butanol production was achieved in all four fermentations (Table V-4). The highest production was obtained after 48 h of cultivation with 2-butanol titer and yield ranging 27 to 30 mM and 0.27 to 0.28 mol/mol, respectively.

Interestingly, the majority of mBDO, 2-butanone, and 2-butanol was produced during the initial 24 h and the titers correlated with the amount of *L. brevis* inoculated. The fermentation with the lowest inoculum of *L. brevis* (ratio 4:0.125) does, however, not appear to follow the same tendency. After glucose exhaustion at 48 h the variation in yield among the fermentations was diminished from 20 % to 2 %.

Table V-4 Co-culture fermentation products at different inoculation ratios.

Time (h)	Ino. ratio	Glucose consumed (mM)	2-butanol	2-butanone	mBD O	By-products ^a	2-butanol ^b (mol/mol)	Balance ^c
24	4:1	73.5	24.1	4.0	10.2	51.8	0.30	1.1
	4:0.5	76.5	20.4	3.4	7.4	49.0	0.24	1.0
	4:0.25	78.4	15.9	3.1	6.3	51.2	0.17	0.9
	4:0.125	73.0	20.8	3.8	12.0	66.4	0.28	1.1
48	4:1	92.2	27.1	6.3	ND	68.2	0.27	1.1
	4:0.5	98.2	29.7	5.4	ND	68.3	0.28	1.0
	4:0.25	100.0	29.7	4.6	ND	84.2	0.27	0.9
	4:0.125	102.9	28.7	5.4	ND	82.4	0.28	1.0

^aSum of by-products lactate, acetate, and ethanol. ^bYield of 2-butanol was corrected for the added 5 mM 2-butanone. ^cCarbon balance calculated in Cmol, as described in section 2.6.

Fermentations with the highest inoculations sustained the growth of both bacteria the longest, as well as resulted in the highest amount of viable cells (Figure V-3). Reducing the inoculation amount of *L. brevis* below the reference OD₆₀₀ of 0.06, negatively influences the viability of *L. lactis* both *L. brevis*.

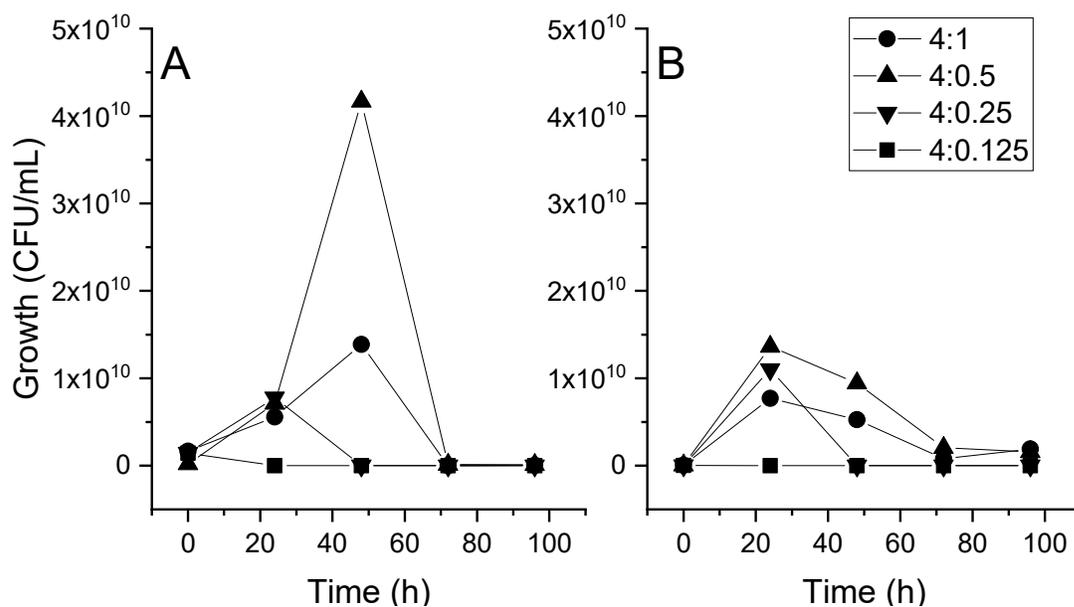


Figure V-3 Growth of *L. lactis* (A) and *L. brevis* (B) in co-cultivation with different inoculation ratios of *L. lactis*:*L. brevis*.

3.4. Summing up

Execution of the co-cultivations in the bioreactors revealed some previously unseen issues with reproducibility. For co-cultivations, batch-to-batch variability can be high as even small environmental perturbations can lead to great fluctuations in the population composition [22,207]. Additionally, fermentations in the batch bioreactors were more prone to contaminations and loss of the *L. lactis* plasmid. Biological factors which are affected by scale include mutation probability, vulnerability to contamination, plasmid instability and numbers [208], selection pressure, and the number of generations associated with the inoculum development and production phases [209].

As compared to fermentation in 50 mL cultivation tubes, fermentation in bioreactors showed increased glucose consumption and different distribution of *L. lactis* and *L. brevis*. Similar tendency has previously been demonstrated in competitive co-cultivations [210]. Especially the sparging of CO₂ reduced the viability of *L. lactis*. During cultivation, *L. lactis* and *L. brevis* compete not only on utilizing glucose and other nutrients, but also the available 2-butanone, which constitutes a growth advantage for both organisms. Continued optimization should therefore focus on achieving a stable culture by changing the operating conditions such as substrate concentration, flowrate to headspace, and availability of oxygen, as these showed great impact on the process. If substantial optimization is wanted, then it is advisable to use model prediction of conditions for optimal production [21,36,197]. Maintaining the co-existence of the individual species and stabilization of population ratio are some of the main issues related to the use of co-cultures as compared to mono-cultures [22]. Unfortunately, the experimental work had to be stopped at this point, as the PhD contract had ended, but the further optimization of the process is ongoing.

4. Conclusion

In this work, we demonstrate production of 2-butanol in a batch bioreactor using a co-cultivation of *L. lactis* and *L. brevis*. The operating condition showing the best performance was without CO₂ sparging, air supplied to the headspace, and addition of 5 mM 2-butanone to the medium. Using this setting, the highest 2-butanol titer of 38 mM and a yield of 0.42 mol/mol was achieved after 48 h of cultivation (section 3.2, fermentation no. F6). At this point glucose was exhausted. Reducing the inoculation of *L. brevis* lead to decreased 2-butanol production and a buildup of mBDO prior glucose exhaustion. The diol dehydratase activity of *L. brevis* therefore appears to be the limiting step during these fermentation settings.

Chapter VI Conclusion and outlook

In the present study, I set out to engineer a single strain of *L. lactis* for production of 2-butanol. Albeit my attempts was not successful, much can be learned from the results. I believe that an effective 2-butanol producing LAB can indeed be constructed, however, significant cloning is needed for achieving this.

As the production requires the involvement of coenzyme B12, the producing strain should preferable be able to produce this compound. One of the enzymes in the 2-butanol biosynthetic pathway is coenzyme B12 dependent diol dehydratase, which is susceptible to occasional inactivation. The re-activation of the coenzyme requires ATP and thus poses a burden on the producing strain. Thus, to achieve efficient production of 2-butanol, this enzyme should be engineered to eliminate the inactivation.

In addition, the microbe should be robust, be able to utilize different carbohydrates without carbon repression, and produce 2-butanol at a high yield. I do not believe that *L. lactis* nor *L. brevis* are perfect candidates for this, but with the continuously development of new metabolic tools and models, such a strain might be within reach in a few years' time.

Instead of achieving 2-butanol production using a single strain, I develop a co-cultivation where the diol dehydratase of *L. brevis* complemented an incomplete 2-butanol biosynthetic pathway in an engineered *L. lactis*. Using this setup, I demonstrated the highest titer and yield of 2-butanol reported using a one-step fermentation. The use of engineered co-cultivations to overcome obstacles with single cultures is an emerging field that demonstrates great potential. Albeit, fermentations using co-cultivations are more difficult to control than single cultures, my achievements here demonstrate that in some cases, it is indeed the better choice.

References

1. Peralta-Yahya PP, Zhang F, del Cardayre SB, Keasling JD. Microbial engineering for the production of advanced biofuels. *Nature*. 2012;488(7411)320–8.
2. Singh D, Sharma D, Soni SL, Sharma S, Kumar Sharma P, Jhalani A. A review on feedstocks, production processes, and yield for different generations of biodiesel. *Fuel*. 2020;262.116553.
3. Steen EJ, Kang Y, Bokinsky G, Hu Z, Schirmer A, McClure A, et al. Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature*. 2010;463(7280)559–62.
4. Shafiei Alavijeh R, Karimi K, van den Berg C. An integrated and optimized process for cleaner production of ethanol and biodiesel from corn stover by *Mucor indicus*. *J Clean Prod*. 2020;249 doi.10.1016/j.jclepro.2019.119321.
5. Demirbas A. Competitive liquid biofuels from biomass. *Appl Energy*. 2011;88(1)17–28.
6. Liu H, Li S, Zheng Z, Xu J, Yao M. Effects of n-butanol, 2-butanol, and methyl octynoate addition to diesel fuel on combustion and emissions over a wide range of exhaust gas recirculation (EGR) rates. *Appl Energy*. 2013;112(x)246–56.
7. No SY. Application of biobutanol in advanced CI engines – A review. *Fuel*. 2016;183641–58.
8. Hahn H-D, Dämbkes G, Rupprich N, Bahl H, Frey GD. Butanols. In: Ullmann's Encyclopedia of Industrial Chemistry. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA; 2013. doi:10.1002/14356007.a04_463.pub3.
9. Dunlop MJ. Engineering microbes for tolerance to next-generation biofuels. *Biotechnol Biofuels*. 2011;4(Figure 1)1–9.
10. Fischer CR, Klein-Marcuschamer D, Stephanopoulos G. Selection and optimization of microbial hosts for biofuels production. *Metab Eng*. 2008;10(6)295–304.
11. Chen Z, Wu Y, Huang J, Liu D. Metabolic engineering of *Klebsiella pneumoniae* for the de novo production of 2-butanol as a potential biofuel. *Bioresour Technol*. 2015;197260–5.

12. Knoshaug EP, Zhang M. Butanol tolerance in a selection of microorganisms. *Appl Biochem Biotechnol*. 2009;153(1–3)13–20.
13. Ghiaci P, Norbeck J, Larsson C. Physiological adaptations of *Saccharomyces cerevisiae* evolved for improved butanol tolerance. *Biotechnol Biofuels*. 2013;6(1)101.
14. Kanno M, Katayama T, Tamaki H, Mitani Y, Meng XY, Hori T, et al. Isolation of butanol- and isobutanol-tolerant bacteria and physiological characterization of their butanol tolerance. *Appl Environ Microbiol*. 2013;79(22)6998–7005.
15. Pereira JPC, Lopez-Gomez G, Reyes NG, van der Wielen LAM, Straathof AJJ. Prospects and challenges for the recovery of 2-butanol produced by vacuum fermentation – a techno-economic analysis. *Biotechnol J*. 2017;12(7)1–13.
16. Jung A, Jung H, Muntean DL. Toxicological patterns of alcoholic beverages based on congeners gas-chromatographic analysis. *Rom J Leg Med*. 2012;20(4)299–302.
17. Kim HM, Yang G, Kim JY, Yoon SJ, Shin BK, Lee J, et al. Simultaneous determination of volatile organic compounds in commercial alcoholic beverages by gas chromatography with flame ionization detection. *J AOAC Int*. 2017;100(5)1492–9.
18. Spaho N, Dürr P, Grba S, Velagić-Habul E, Blesić M. Effects of distillation cut on the distribution of higher alcohols and esters in brandy produced from three plum varieties. *J Inst Brew*. 2013;119(1–2)48–56.
19. Lee SY, Park JH, Jang SH, Nielsen LK, Kim J, Jung KS. Fermentative butanol production by *Clostridia*. *Biotechnol Bioeng*. 2008;101(2)209–28.
20. Saini M, Hong Chen M, Chiang C-J, Chao Y-P. Potential production platform of n-butanol in *Escherichia coli*. *Metab Eng*. 2015;2776–82.
21. Ebrahimi E, Amiri H, Asadollahi MA, Shojaosadati SA. Efficient butanol production under aerobic conditions by co-culture of *Clostridium acetobutylicum* and *Nesterenkonia* sp. strain F. *Biotechnol Bioeng*. 2019; doi.10.1002/bit.27221.
22. Zhang H, Wang X. Modular co-culture engineering, a new approach for metabolic engineering. *Metab Eng*. 2016;37114–21.
23. Kolesinska B, Fraczyk J, Binczarski M, Modelska M, Berlowska J, Dziugan P, et al. Butanol synthesis routes for biofuel production: Trends and perspectives. *Materials* (Basel).

- 2019;12(3) doi.10.3390/ma12030350.
24. Ezeji TC, Qureshi N, Blaschek HP. Bioproduction of butanol from biomass: from genes to bioreactors. *Curr Opin Biotechnol.* 2007;18(3)220–7.
 25. Nielsen DR, Leonard E, Yoon S-H, Tseng H-C, Yuan C, Prather KLJ. Engineering alternative butanol production platforms in heterologous bacteria. *Metab Eng.* 2009;11(4–5)262–73.
 26. Lan EI, Liao JC. Microbial synthesis of n-butanol, isobutanol, and other higher alcohols from diverse resources. *Bioresour Technol.* 2013;135339–49.
 27. Berezina O V., Zakharova N V., Brandt A, Yarotsky S V., Schwarz WH, Zverlov V V. Reconstructing the clostridial n-butanol metabolic pathway in *Lactobacillus brevis*. *Appl Microbiol Biotechnol.* 2010;87(2)635–46.
 28. Dellomonaco C, Clomburg JM, Miller EN, Gonzalez R. Engineered reversal of the β -oxidation cycle for the synthesis of fuels and chemicals. *Nature.* 2011;476(7360)355–9.
 29. Atsumi S, Hanai T, Liao JC. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature.* 2008;45186–9.
 30. Dickinson JR, Harrison SJ, Dickinson JA, Hewlins MJE. An investigation of the metabolism of isoleucine to active amyl alcohol in *Saccharomyces cerevisiae*. *J Biol Chem.* 2000;275(15)10937–42.
 31. Smith KM, Liao JC. An evolutionary strategy for isobutanol production strain development in *Escherichia coli*. *Metab Eng.* 2011;13(6)674–81.
 32. Atsumi S, Wu TY, Eckl EM, Hawkins SD, Buelter T, Liao JC. Engineering the isobutanol biosynthetic pathway in *Escherichia coli* by comparison of three aldehyde reductase/alcohol dehydrogenase genes. *Appl Microbiol Biotechnol.* 2010;85(3)651–7.
 33. Chen X, Nielsen KF, Borodina I, Kielland-Brandt MC, Karhumaa K. Increased isobutanol production in *Saccharomyces cerevisiae* by overexpression of genes in valine metabolism. *Biotechnol Biofuels.* 2011;41–12.
 34. He MX, Wu B, Qin H, Ruan ZY, Tan FR, Wang JL, et al. *Zymomonas mobilis*: A novel platform for future biorefineries. *Biotechnol Biofuels.* 2014;7(1) doi.10.1186/1754-6834-7-101.

35. Oh B-RR, Heo S-YY, Lee S-MM, Hong W-KK, Park JM, Jung YR, et al. Production of 2-butanol from crude glycerol by a genetically-engineered *Klebsiella pneumoniae* strain. *Biotechnol Lett.* 2014;36(1)57–62.
36. Minty JJ, Singer ME, Scholz SA, Bae CH, Ahn JH, Foster CE, et al. Design and characterization of synthetic fungal-bacterial consortia for direct production of isobutanol from cellulosic biomass. *Proc Natl Acad Sci U S A.* 2013;110(36)14592–7.
37. Park JM, Oh BR, Kang IY, Heo SY, Seo JW, Park SM, et al. Enhancement of 2,3-butanediol production from Jerusalem artichoke tuber extract by a recombinant *Bacillus* sp. strain BRC1 with increased inulinase activity. *J Ind Microbiol Biotechnol.* 2017;44(7)1107–13.
38. Kim S-JJ, Seo S-OO, Jin Y-SS, Seo J-HH. Production of 2,3-butanediol by engineered *Saccharomyces cerevisiae*. *Bioresour Technol.* 2013;146274–81.
39. Cho S, Kim T, Woo HM, Kim Y, Lee J, Um Y. High production of 2,3-butanediol from biodiesel-derived crude glycerol by metabolically engineered *Klebsiella oxytoca* M1. *Biotechnol Biofuels.* 2015;8(1)1–12.
40. Crow VL. Properties of 2,3-butanediol dehydrogenases from *Lactococcus lactis* subsp. *lactis* in relation to citrate fermentation. *Appl Environ Microbiol.* 1990;56(6)1656–65.
41. Saint-Amans S, Girbal L, Andrade J, Ahrens K, Soucaille P. Regulation of carbon and electron flow in *Clostridium butyricum* VPI 3266 grown on glucose-glycerol mixtures. *J Bacteriol.* 2001;183(5)1748–54.
42. Chen Z, Sun H, Huang J, Wu Y, Liu D. Metabolic engineering of *Klebsiella pneumoniae* for the production of 2-butanone from glucose. *PLoS One.* 2015;10(10) doi.10.1371/journal.pone.0140508.
43. Chen L, Hatti-Kaul R. Exploring *Lactobacillus reuteri* DSM20016 as a biocatalyst for transformation of longer chain 1,2-diols: Limits with microcompartment. *PLoS One.* 2017;12(9)e0185734.
44. Bobik T a., Xu Y, Jeter RM, Otto KE, Roth JR. Propanediol utilization genes (pdu) of *Salmonella typhimurium*: Three genes for the propanediol dehydratase. *J Bacteriol.* 1997;179(21)6633–9.
45. Toraya T, Fukui S. Immunochemical evidence for the difference between coenzyme-B12-dependent diol dehydratase and glycerol dehydratase. *Eur J Biochem.* 1977;76(1)285–9.

46. Toraya T. Cobalamin-dependent dehydratases and a deaminase: Radical catalysis and reactivating chaperones. *Arch Biochem Biophys*. 2014;54440–57.
47. O'Brien JR, Raynaud C, Croux C, Girbal L, Soucaille P, Lanzilotta WN. Insight into the mechanism of the B12-independent glycerol dehydratase from *Clostridium butyricum*: preliminary biochemical and structural characterization. *Biochemistry*. 2004;43(16)4635–45.
48. Demick JM, Lanzilotta WN. Radical SAM activation of the B12-independent glycerol dehydratase results in formation of 5'-deoxy-5'-(methylthio)adenosine and not 5'-deoxyadenosine. *Biochemistry*. 2011;50(4)440–2.
49. Feliks M, Ullmann GM. Glycerol dehydration by the B12-independent enzyme may not involve the migration of a hydroxyl group: a computational study. *J Phys Chem B*. 2012;116(24)7076–87.
50. Jiang W, Wang S, Yang Z, Fang B. B 12-independent glycerol dehydratase and its reactivase from *Clostridia butyricum* : Optimizing cloning by uniform design logic. *Eng Life Sci*. 2015;15(5)519–24.
51. Raynaud C, Sarçabal P, Meynial-Salles I, Croux C, Soucaille P. Molecular characterization of the 1,3-propanediol (1,3-PD) operon of *Clostridium butyricum*. *Proc Natl Acad Sci U S A*. 2003;100(9)5010–5.
52. Santos JA, Rempel S, Mous STM, Pereira CT, Beek J Ter, de Gier JW, et al. Functional and structural characterization of an ECF-type ABC transporter for vitamin B12. *Elife*. 2018;71–16.
53. Roth JR, Lawrence JG, Bobik TA. Cobalamin (coenzyme B12): synthesis and biological significance. *Annu Rev Microbiol*. 1996;50137–81.
54. Toraya T. G-Protein signaling: A switch saves b12 radical status. *Nat Chem Biol*. 2013;9(9)530–1.
55. Santos F, Vera JL, van der Heijden R, Valdez G, de Vos WM, Sesma F, et al. The complete coenzyme B12 biosynthesis gene cluster of *Lactobacillus reuteri* CRL 1098. *Microbiology*. 2008;154(1)81–93.
56. Lawrence JG, Roth JR. Evolution of coenzyme B12 synthesis among enteric bacteria: Evidence for loss and reacquisition of a multigene complex. *Genetics*. 1996;142(1)11–24.

57. Scott AI, Roessner CA. Biosynthesis of cobalamin (vitamin B12). *Biochem Soc Trans.* 2002;30(4)613–20.
58. Taranto MP, Vera JL, Hugenholtz J, Graciela F De Valdez FS. *Lactobacillus reuteri* CRL1098 Produces Cobalamin. *J Bacteriol.* 2003;185(18)5643–7.
59. Torres AC, Vannini V, Bonacina J, Font G, Saavedra L, Taranto MP. Cobalamin production by *Lactobacillus coryniformis*: Biochemical identification of the synthesized corrinoid and genomic analysis of the biosynthetic cluster. *BMC Microbiol.* 2016;16(1)1–9.
60. Li P, Gu Q, Yang L, Yu Y, Wang Y. Characterization of extracellular vitamin B12 producing *Lactobacillus plantarum* strains and assessment of the probiotic potentials. *Food Chem.* 2017;234494–501.
61. Abeles RH, Lee HA. Purification and Properties of Dioldehydrase, an Enzyme Requiring a Cobamide Coenzyme. *J Biol Chem.* 1963;238(7)2367–73.
62. Bachovchin WW, Eagar RG, Moore KW, Richards JH. Mechanism of action of adenosylcobalamin: glycerol and other substrate analogues as substrates and inactivators for propanediol dehydratase--kinetics, stereospecificity, and mechanism. *Biochemistry.* 1977;16(6)1082–92.
63. Yamanishi M, Yunoki M, Tobimatsu T, Sato H, Matsui J, Dokiya A, et al. The crystal structure of coenzyme B12-dependent glycerol dehydratase in complex with cobalamin and propane-1,2-diol. *Eur J Biochem.* 2002;269(18)4484–94.
64. Bilić L, Barić D, Banhatti RD, Smith DM, Kovačević B. Computational Study of Glycerol Binding within the Active Site of Coenzyme B 12 -Dependent Diol Dehydratase. *J Phys Chem B.* 2019;123(29)6178–87.
65. Toraya T, Ushio K, Fukui S, Hogenkamp HPC. Studies on the mechanism of the adenosylcobalamin-dependent diol dehydrase reaction by the use of analogs of the coenzyme. *J Biol Chem.* 1977;(3)963–70.
66. Toraya T, Shirakashi T, Kosuga T, Fukui S. Substrate specificity of coenzyme B12-dependent diol dehydratase: glycerol as both a good substrate and a potent inactivator. *Biochem Biophys Res Commun.* 1976;69(2)475–80.
67. Mori K, Hosokawa Y, Yoshinaga T, Toraya T. Diol dehydratase-reactivating factor is a reactivase - evidence for multiple turnovers and subunit swapping with diol dehydratase.

- FEBS J. 2010;277(23)4931–43.
68. Kajiura H, Mori K, Tobimatsu T, Toraya T. Characterization and Mechanism of Action of a Reactivating Factor for Adenosylcobalamin-dependent Glycerol Dehydratase. *J Biol Chem.* 2001;276(39)36514–9.
 69. Honda S, Toraya T, Fukui S. In situ reactivation of glycerol-inactivated coenzyme B12-dependent enzymes, glycerol dehydratase and diol dehydratase. *J Bacteriol.* 1980;143:1458–65.
 70. Ushio K, Honda S, Toraya T, Fukui S. The mechanism of in situ reactivation of glycerol-inactivated coenzyme B12-dependent enzymes, glycerol dehydratase and diol dehydratase. *J Nutr Sci Vitaminol (Tokyo).* 1982;28(3)225–36.
 71. Yoneda H, Tantillo DJ, Atsumi S. Biological production of 2-butanone in *Escherichia coli*. *ChemSusChem.* 2014;7(1)92–5.
 72. Moore KW, Richards JH. Stereospecificity and mechanism of adenosylcobalamin-dependent diol dehydratase. Catalysis and inactivation with meso- and DL-2,3-butanediols as substrates. *Biochem Biophys Res Commun.* 1979;87(4)1052–7.
 73. Abeles R, Lee H. Properties of diol dehydratase, an enzyme requiring a cobamide coenzyme. In: *Federation Proceedings.* 1962. 253.
 74. Sauvageot N, Pichereau V, Louarme L, Hartke A, Auffray Y, Laplace JM. Purification, characterization and subunits identification of the diol dehydratase of *Lactobacillus collinoides*. *Eur J Biochem.* 2002;269(22)5731–7.
 75. Yamanishi M, Kinoshita K, Fukuoka M, Saito T, Tanokuchi A, Ikeda Y, et al. Redesign of coenzyme B(12) dependent diol dehydratase to be resistant to the mechanism-based inactivation by glycerol and act on longer chain 1,2-diols. *FEBS J.* 2012;279(5)793–804.
 76. Wang J, Jain R, Shen X, Sun X, Cheng M, Liao JC, et al. Rational engineering of diol dehydratase enables 1,4-butanediol biosynthesis from xylose. *Metab Eng.* 2017;40(February)148–56.
 77. Qi X, Chen Y, Jiang K, Zuo W, Luo Z, Wei Y, et al. Saturation-mutagenesis in two positions distant from active site of a *Klebsiella pneumoniae* glycerol dehydratase identifies some highly active mutants. *J Biotechnol.* 2009;144(1)43–50.

78. Maddock DJ, Gerth ML, Patrick WM. An engineered glycerol dehydratase with improved activity for the conversion of meso-2,3-butanediol to butanone. *Biotechnol J*. 2017;1700480 doi.10.1002/biot.201700480.
79. Shrivastav A, Lee J, Kim HY, Kim YR. Recent insights in the removal of *Klebsiella* pathogenicity factors for the industrial production of 2,3-butanediol. *J Microbiol Biotechnol*. 2013;23(7)885–96.
80. Jung SG, Jang JH, Kim AY, Lim MC, Kim B, Lee J, et al. Removal of pathogenic factors from 2,3-butanediol-producing *Klebsiella* species by inactivating virulence-related *wabG* gene. *Appl Microbiol Biotechnol*. 2013;97(5)1997–2007.
81. Paczosa MK, Meccas J. *Klebsiella pneumoniae*: Going on the Offense with a Strong Defense. *Microbiol Mol Biol Rev*. 2016;80(3)629–61.
82. Drabo P, Tiso T, Heyman B, Sarikaya E, Gaspar P, Förster J, et al. Anionic Extraction for Efficient Recovery of Biobased 2,3-Butanediol—A Platform for Bulk and Fine Chemicals. *ChemSusChem*. 2017;10(16)3252–9.
83. Anvari M, Khayati G. In situ recovery of 2,3-butanediol from fermentation by liquid–liquid extraction. *J Ind Microbiol Biotechnol*. 2009;36(2)313–7.
84. Matsumoto M, Okuno R, Kondo K. Extraction of 2,3-Butanediol with Aqueous Two-Phase Systems Formed by Water-Miscible Organic Solvents and Inorganic Salts. *Solvent Extr Res Dev Japan*. 2014;21(2)181–90.
85. Reshamwala SMS, Deb SS, Lali AM. A shortened, two-enzyme pathway for 2,3-butanediol production in *Escherichia coli*. *J Ind Microbiol Biotechnol*. 2017;44(9)1273–7.
86. Qiu Y, Zhang J, Li L, Wen Z, Nomura CT, Wu S, et al. Engineering *Bacillus licheniformis* for the production of meso-2,3-butanediol. *Biotechnol Biofuels*. 2016;9(1)117.
87. Zhang L, Sun J, Hao Y, Zhu J, Chu J, Wei D, et al. Microbial production of 2,3-butanediol by a surfactant (serrawettin)- deficient mutant of *Serratia marcescens* H30. *J Ind Microbiol Biotechnol*. 2010;37(8)857–62.
88. Ng CY, Jung M-Y, Lee J, Oh M-K. Production of 2,3-butanediol in *Saccharomyces cerevisiae* by in silico aided metabolic engineering. *Microb Cell Fact*. 2012;11(1)68.
89. Gaspar P, Neves AR, Ramos A, Gasson MJ, Shearman CA, Santos H. Engineering

- Lactococcus lactis* for production of mannitol: high yields from food-grade strains deficient in lactate dehydrogenase and the mannitol transport system. *Appl Environ Microbiol.* 2004;70(3)1466–74.
90. Kandasamy V, Liu J, Dantoft SH, Solem C, Jensen PR. Synthesis of (3R)-acetoin and 2,3-butanediol isomers by metabolically engineered *Lactococcus lactis*. *Sci Rep.* 2016;6(36769) doi.10.1038/srep36769.
 91. Oliver JWK, Machado IMP, Yoneda H, Atsumi S. Cyanobacterial conversion of carbon dioxide to 2,3-butanediol. *Proc Natl Acad Sci.* 2013;110(4)1249–54.
 92. Ghiaci P, Norbeck J, Larsson C. 2-Butanol and butanone production in *Saccharomyces cerevisiae* through combination of a B12 dependent dehydratase and a secondary alcohol dehydrogenase using a TEV-based expression system. *PLoS One.* 2014;9(7)e102774.
 93. Russmayer H, Marx H, Sauer M. Microbial 2-butanol production with *Lactobacillus diolivorans*. *Biotechnol Biofuels.* 2019;12(262) doi.10.1186/s13068-019-1594-5.
 94. DSMZ. *Serratia marcescens* DSM 14187 [Internet]. DSMZ-German Collection of Microorganisms and Cell Cultures GmbH, Leibniz Institute. [cited 2019 Nov 13]. Available from: <https://www.dsmz.de/collection/catalogue/details/culture/DSM-14187>
 95. Srirangan K, Liu X, Akawi L, Bruder M, Moo-Young M, Chou CP. Engineering *Escherichia coli* for microbial production of butanone. *Appl Environ Microbiol.* 2016;82(9)2574–84.
 96. Zhang L, Singh R, Sivakumar D, Guo Z, Li J, Chen F, et al. An artificial synthetic pathway for acetoin, 2,3-butanediol, and 2-butanol production from ethanol using cell free multi-enzyme catalysis. *Green Chem.* 2018;20(1)230–42.
 97. Min K, Kim S, Yum T, Kim Y, Sang BI, Um Y. Conversion of levulinic acid to 2-butanone by acetoacetate decarboxylase from *Clostridium acetobutylicum*. *Appl Microbiol Biotechnol.* 2013;97(12)5627–34.
 98. Multer A, McGraw N, Hohn K, Vadlani P. Production of methyl ethyl ketone from biomass using a hybrid biochemical/catalytic approach. *Ind Eng Chem Res.* 2013;52(1)56–60.
 99. Abdel-Rahman MA, Tashiro Y, Sonomoto K. Lactic acid production from lignocellulose-derived sugars using lactic acid bacteria: overview and limits. *J Biotechnol.* 2010;156(4)286–301.

100. John RP, Nampoothiri KM, Pandey A. Fermentative production of lactic acid from biomass: an overview on process developments and future perspectives. *Appl Microbiol Biotechnol.* 2007;74(3)524–34.
101. Zhao K, Qiao Q, Chu D, Gu H, Dao TH, Zhang J, et al. Simultaneous saccharification and high titer lactic acid fermentation of corn stover using a newly isolated lactic acid bacterium *Pediococcus acidilactici* DQ2. *Bioresour Technol.* 2013;135481–9.
102. Ou MS, Ingram LO, Shanmugam KT. L(+)-Lactic acid production from non-food carbohydrates by thermotolerant *Bacillus coagulans*. *J Ind Microbiol Biotechnol.* 2011;38(5)599–605.
103. Mazzoli R, Bosco F, Mizrahi I, Bayer EA, Pessione E. Towards lactic acid bacteria-based biorefineries. *Biotechnol Adv.* 2014;32(7)1216–36.
104. Bosma EF, Forster J, Nielsen AT. Lactobacilli and Pediococci as versatile cell factories – Evaluation of strain properties and genetic tools. *Biotechnol Adv.* 2017;35(4)419–42.
105. Gaspar P, Carvalho AL, Vinga S, Santos H, Neves AR. From physiology to systems metabolic engineering for the production of biochemicals by lactic acid bacteria. *Biotechnol Adv.* 2013;31(6)764–88.
106. Jonganurakkun B, Wang Q, Xu SH, Tada Y, Minamida K, Yasokawa D, et al. *Pediococcus pentosaceus* NB-17 for probiotic use. *J Biosci Bioeng.* 2008;106(1)69–73.
107. van Hylckama Vlieg JE, Rademaker JL, Bachmann H, Molenaar D, Kelly WJ, Siezen RJ. Natural diversity and adaptive responses of *Lactococcus lactis*. *Curr Opin Biotechnol.* 2006;17(2)183–90.
108. Vinderola CG, Reinheimer JA. Lactic acid starter and probiotic bacteria: A comparative “in vitro” study of probiotic characteristics and biological barrier resistance. *Food Res Int.* 2003;36(9–10)895–904.
109. Lucena BTL, dos Santos BM, Moreira JL, Moreira APB, Nunes AC, Azevedo V, et al. Diversity of lactic acid bacteria of the bioethanol process. *BMC Microbiol.* 2010;10(1)298.
110. Liu S, Qureshi N. How microbes tolerate ethanol and butanol. *N Biotechnol.* 2009;26(3–4)117–21.
111. Gold RS, Meagher MM, Hutkins R, Conway T. Ethanol tolerance and carbohydrate

- metabolism in lactobacilli. *J Ind Microbiol.* 1992;10(1)45–54.
112. Boguta A, Bringel F, Martinussen J, Jensen P. Screening of lactic acid bacteria for their potential as microbial cell factories for bioconversion of lignocellulosic feedstocks. *Microb Cell Fact.* 2014;1397.
 113. Zheng J, Ruan L, Sun M, Gänzle M. A genomic view of lactobacilli and pediococci demonstrates that phylogeny matches ecology and physiology. *Appl Environ Microbiol.* 2015;81(20)7233–43.
 114. Kim JHJ-H, Shoemaker SP, Mills DA. Relaxed control of sugar utilization in *Lactobacillus brevis*. *Microbiology.* 2009;155(4)1351–9.
 115. Jeong KH, Israr B, Shoemaker SP, Mills DA, Kim J. Impact of lactic acid and hydrogen ion on the simultaneous fermentation of glucose and xylose by the carbon catabolite derepressed *Lactobacillus brevis* ATCC 14869. *J Microbiol Biotechnol.* 2016;26(7)1182–9.
 116. Liu S, Bischoff KM, Hughes SR, Leathers TD, Price NP, Qureshi N, et al. Conversion of biomass hydrolysates and other substrates to ethanol and other chemicals by *Lactobacillus buchneri*. *Lett Appl Microbiol.* 2009;48(3)337–42.
 117. Endo A, Dicks LMT. Physiology of the LAB. In: Holzapfel WH, Wood BJB, editors. *Physiology of the LAB, lactic acid bacteria: Biodiversity and taxonomy.* 1st ed. John Wiley & Sons, Ltd; 2014. 13–30.
 118. Kandler O. Carbohydrate metabolism in lactic acid bacteria. *Antonie Van Leeuwenhoek.* 1983;49(3)209–24.
 119. Cocaign-Bousquet M, Garrigues C, Loubiere P, Lindley ND. Physiology of pyruvate metabolism in *Lactococcus lactis*. *Int J Gen Mol Microbiol.* 1996;70(2–4)253–67.
 120. Teusink B, Bachmann H, Molenaar D. Systems biology of lactic acid bacteria: A critical review. *Microb Cell Fact.* 2011;10(SUPPL. 1)1–17.
 121. Sun Z, Harris HMB, McCann A, Guo C, Argimón S, Zhang W, et al. Expanding the biotechnology potential of Lactobacilli through comparative genomics of 213 strains and associated genera. *Nat Commun.* 2015;6(83222) doi.10.1038/ncomms9322.
 122. Burgé G, Saulou-Bérion C, Moussa M, Allais F, Athes V, Spinnler HE. Relationships between the use of Embden Meyerhof pathway (EMP) or Phosphoketolase pathway (PKP)

- and lactate production capabilities of diverse *Lactobacillus reuteri* strains. J Microbiol. 2015;53(10)702–10.
123. Okano K, Yoshida S, Tanaka T, Ogino C, Fukuda H, Kondo A. Homo-D-lactic acid fermentation from arabinose by redirection of the phosphoketolase pathway to the pentose phosphate pathway in L-lactate dehydrogenase gene-deficient *Lactobacillus plantarum*. Appl Environ Microbiol. 2009;75(15)5175–8.
 124. Shinkawa S, Okano K, Yoshida S, Tanaka T, Ogino C, Fukuda H, et al. Improved homo L-lactic acid fermentation from xylose by abolishment of the phosphoketolase pathway and enhancement of the pentose phosphate pathway in genetically modified xylose-assimilating *Lactococcus lactis*. Appl Microbiol Biotechnol. 2011;91(6)1537–44.
 125. Guo W, He R, Ma L, Jia W, Li D, Chen S. Construction of a constitutively expressed homo-fermentative pathway in *Lactobacillus brevis*. Appl Microbiol Biotechnol. 2014;98(15)6641–50.
 126. Solem C, Dehli T, Jensen PR. Rewiring *Lactococcus lactis* for ethanol production. Appl Environ Microbiol. 2013;79(8)2512–8.
 127. Koebmann BJ, Solem C, Pedersen MB, Nilsson D, Jensen PR. Expression of genes encoding F1-ATPase results in uncoupling of glycolysis from biomass production in *Lactococcus lactis*. Appl Environ Microbiol. 2002;68(9) doi.10.1128/AEM.68.9.4274-4282.2002.
 128. Koebmann BJ, Westerhoff H V, Snoep JL, Nilsson D, Jensen PR. The glycolytic flux in *Escherichia coli* is controlled by the demand for ATP. J Bacteriol. 2002;184(14)3909–16.
 129. Mierau I, Kleerebezem M. 10 Years of the nisin-controlled gene expression system (NICE) in *Lactococcus lactis*. Appl Microbiol Biotechnol. 2005;68(6)705–17.
 130. Mierau I, Olieman K, Mond J, Smid EJ. Optimization of the *Lactococcus lactis* nisin-controlled gene expression system NICE for industrial applications. Microb Cell Fact. 2005;4(16) doi.10.1186/1475-2859-4-16.
 131. Kleerebezem M, Beerthuyzen MM, Vaughan EE, De Vos WM, Kuipers OP. Controlled gene expression systems for lactic acid bacteria: Transferable nisin-inducible expression cassettes for *Lactococcus*, *Leuconostoc*, and *Lactobacillus* spp. Appl Environ Microbiol. 1997;63(11)4581–4.

132. Bryan EM, Bae T, Kleerebezem M, Dunny GM. Improved vectors for nisin-controlled expression in gram-positive bacteria. *Plasmid*. 2000;44(2)183–90.
133. De Ruyter PGGA, Kuipers OP, De Vos WM. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. *Appl Environ Microbiol*. 1996;62(10)3662–7.
134. Liang X, Sun Z, Zhong J, Zhang Q, Huan L. Adverse effect of nisin resistance protein on nisin-induced expression system in *Lactococcus lactis*. *Microbiol Res*. 2010;165(6)458–65.
135. Yu L, O'Sullivan DJ. Production of galactooligosaccharides using a hyperthermophilic β -galactosidase in permeabilized whole cells of *Lactococcus lactis*. *J Dairy Sci*. 2014;97(2)694–703.
136. Solem C, Jensen PR. Modulation of gene expression made easy. *Appl Environ Microbiol*. 2002;68(5)2397–403.
137. Biswas I, Grussa, Ehrlich SD, Maguin E. High efficiency gene inactivation and replacement system for Gram-positive bacteria. *J Bacteriol*. 1993;175:(11)3628-3635.
138. Solem C, Defoor E, Jensen PR, Martinussen J. Plasmid pCS1966, a new selection/countersélection tool for lactic acid bacterium strain construction based on the *oroP* gene, encoding an orotate transporter from *Lactococcus lactis*. *Appl Environ Microbiol*. 2008;74(15)4772–5.
139. Liu J, Dantoft SH, Würtz A, Jensen PR, Solem C. A novel cell factory for efficient production of ethanol from dairy waste. *Biotechnol Biofuels*. 2016;9(1)33.
140. Gaspar P, Neves AR, Gasson MJ, Shearman C a., Santos H. High yields of 2,3-butanediol and mannitol in *Lactococcus lactis* through engineering of NAD⁺ cofactor recycling. *Appl Environ Microbiol*. 2011;77(19)6826–35.
141. Gasson MJ. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. *J Bacteriol*. 1983;154(1)1–9.
142. Ghiaci P, Lameiras F, Norbeck J, Larsson C. Production of 2-butanol through meso-2,3-butanediol consumption in lactic acid bacteria. *FEMS Microbiol Lett*. 2014;360(1)70–5.
143. Liu J, Chan SHJ, Brock-Nannestad T, Chen J, Lee SY, Solem C, et al. Combining metabolic engineering and biocompatible chemistry for high-yield production of homo-

- diacetyl and homo-(S,S)-2,3-butanediol. *Metab Eng.* 2016;3657–67.
144. Hayes F, Daly C, Fitzgerald GF. Identification of the minimal replicon of *Lactococcus lactis* subsp. *lactis* UC317 plasmid pCI305. *Appl Environ Microbiol.* 1990;56(1)202–9.
 145. Elferink SJWHO, Krooneman J, Jan C, Spoelstra SF, Faber F. Anaerobic conversion of lactic acid to acetic acid and 1,2-propanediol by *Lactobacillus buchneri*. *Appl Environ Microbiol.* 2001;67(1)125–32.
 146. Sambrook J, Russell DW. *Molecular cloning: A laboratory manual*. Third. New York: Cold Spring Harbor Laboratory Press; 2001.
 147. Holo H, Nes IF. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Appl Environ Microbiol.* 1989;55(12)3119–23.
 148. Tobimatsu T, Hara T, Sakaguchi M, Kishimoto Y, Wada Y, Isoda M, et al. Molecular cloning, sequencing, and expression of the genes encoding adenosylcobalamin-dependent diol dehydrase of *Klebsiella oxytoca*. *J Biol Chem.* 1995;270(13)7142–8.
 149. Bramucci MG, Eliot AC, Maggio-Hall LA, Nakamura CE. Butanol dehydrogenase enzyme from the bacterium *Achromobacter xylosoxidans*. United States patent; US 8,188,250 B2, 2012.
 150. Paz MA, Blumenfeld OO, Rojkind M, Henson E, Furfine C, Gallop PM. Determination of carbonyl compounds with N-methyl benzothiazolone hydrazone. *Arch Biochem Biophys.* 1965;109(3)548–59.
 151. Chen J, Shen J, Ingvar Hellgren L, Jensen PR, Solem C. Adaptation of *Lactococcus lactis* to high growth temperature leads to a dramatic increase in acidification rate. *Sci Rep.* 2015;5(14199) doi.10.1038/srep14199.
 152. Ryssel M, Meisner Hviid AM, Dawish MS, Haaber J, Hammer K, Martinussen J, et al. Multi-stress resistance in *Lactococcus lactis* is actually escape from purine-induced stress sensitivity. *Microbiol.* 2014;160(2014)2551–9.
 153. Hviid AMM. Improving second generation biorefinery processes using clues from the stress response in *Lactococcus lactis*. Technical University of Denmark; 2017.
 154. Tang X, Tan Y, Zhu H, Zhao K, Shen W. Microbial conversion of glycerol to 1,3-propanediol

- by an engineered strain of *Escherichia coli*. *Appl Environ Microbiol*. 2009;75(6)1628–34.
155. Borths EL, Poolman B, Hvorup RN, Locher KP, Rees DC. In vitro functional characterization of BtuCD-F, the *Escherichia coli* ABC transporter for vitamin B12 uptake. *Biochemistry*. 2005;44(49)16301–9.
 156. Zhang Y, Rodionov DA, Gelfand MS, Gladyshev VN. Comparative genomic analyses of nickel, cobalt and vitamin B12 utilization. *BMC Genomics*. 2009;10 doi.10.1186/1471-2164-10-78.
 157. Rodionov DA, Hebbeln P, Eudes A, Ter Beek J, Rodionova IA, Erkens GB, et al. A novel class of modular transporters for vitamins in prokaryotes. *J Bacteriol*. 2009;91(1)42–51.
 158. Rempel S, Colucci E, de Gier JW, Guskov A, Slotboom DJ. Cysteine-mediated decyanation of vitamin B12 by the predicted membrane transporter BtuM. *Nat Commun*. 2018;9(1)1–8.
 159. Iyer BK, Singhal RS, Ananthanarayan L. Characterization and in vitro probiotic evaluation of lactic acid bacteria isolated from idli batter. *J Food Sci Technol*. 2013;50(6)1114–21.
 160. Morita H, Hidehiro T, Fukuda S, Horikawa H, Oshima K, Suzuki T, et al. Comparative genome analysis of *Lactobacillus reuteri* and *Lactobacillus fermentum* reveal a genomic island for reuterin and cobalamin production. *DNA Res*. 2008;15(3)151–61.
 161. Raux E, Lanois A, Levillayer F, Warren MJ, Brody E, Rambach A, et al. *Salmonella typhimurium* cobalamin (vitamin B12) biosynthetic genes: Functional studies in *S. typhimurium* and *Escherichia coli*. *J Bacteriol*. 1996;178(3)753–67.
 162. Xu C, Shi Z, Shao J, Yu C, Xu Z. Metabolic engineering of *Lactococcus lactis* for high level accumulation of glutathione and S-adenosyl-L-methionine. *World J Microbiol Biotechnol*. 2019;35(12)1–9.
 163. Radler F, Zorg J. Characterization of the enzyme involved in formation of 2-butanol from meso-2, 3-butanediol by lactic acid bacteria. *Am J Enol Vitic*. 1986;37(3)206–10.
 164. Speranza G, Corti S, Fontana G, Manitto P, Galli A, Scarpellini M, et al. Conversion of meso-2,3-butanediol into 2-butanol by Lactobacilli. Stereochemical and enzymatic aspects. *J Agric Food Chem*. 1997;45(9)3476–80.
 165. Nichols NN, Dien BS, Bothast RJ. Use of catabolite repression mutants for fermentation of sugar mixtures to ethanol. *Appl Microbiol Biotechnol*. 2001;56(1–2)120–5.

166. Yoshida S, Okano K, Tanaka T, Ogino C, Kondo A. Homo-D-lactic acid production from mixed sugars using xylose-assimilating operon-integrated *Lactobacillus plantarum*. Appl Microbiol Biotechnol. 2011;92(1)67–76.
167. Ho NWY, Chen Z, Brainard AP. Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. Appl Environ Microbiol. 1998;64(5)1852–9.
168. Zhang Y, Vadlani P V. Lactic acid production from biomass-derived sugars via co-fermentation of *Lactobacillus brevis* and *Lactobacillus plantarum*. J Biosci Bioeng. 2015;119(6)694–9.
169. Hu C-Y, Chi D-J, Chen S-S, Chen Y-C. The direct conversion of xylan to lactic acid by *Lactobacillus brevis* transformed with a xylanase gene. Green Chem. 2011;13(7)1729.
170. Chaillou, S., Yeou-Cherng, Bor, Batt, C. A., Postma, P. W., and Pouwels PH. Molecular cloning and functional expression in *Lactobacillus plantarum* 80 of xylT, encoding the D-xylose-H⁺ symporter of *Lactobacillus brevis*. Appl Environ Microbiol. 1998;64(12)4720–8.
171. Lyu CJ, Zhao WR, Hu S, Huang J, Lu T, Jin ZH, et al. Physiology-oriented engineering strategy to improve gamma-aminobutyrate production in *Lactobacillus brevis*. J Agric Food Chem. 2017;65(4)858–66.
172. Trieu-Cout P, Carlier C, Poyart-Salmeron C, Courvalin P. A pair of mobilization shuttle vectors conferring resistance to spectinomycin for molecular cloning in *Escherichia coli* and in gram-positive bacteria. NuclAcids Res. 1998;18(14)4296.
173. Grady R, Hayes F. Axe – Txe , a broad-spectrum proteic toxin – antitoxin system specified by a multidrug-resistant , clinical isolate of *Enterococcus faecium*. 2003;471419–32.
174. Jensen PR, Hammer K. Minimal requirements for exponential growth of *Lactococcus lactis*. Appl Environ Microbiol. 1993;59(12)4363–6.
175. Salvetti E, Harris HMB, Felis GE, O’Toole PW. Comparative genomics of the genus *Lactobacillus* reveals robust phylogroups that provide the basis for reclassification. Appl Environ Microbiol. 2018;84(17)1–16.
176. Langmead B, Salzberg S. Bowtie2. Nat Methods. 2013;9(4)357–9.
177. Aukrust TW, Brurberg MB, Nes IF. Transformation of *Lactobacillus* by electroporation. In:

- Nickoloff JA, editor. *Methods in Molecular Biology*. Totowa: Humana Press; 1995. 201–8.
178. Maiden MCJ. Multilocus sequence typing of bacteria. *Annu Rev Microbiol*. 2006;60(1)561–88.
 179. Makarova K, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E, et al. Comparative genomics of the lactic acid bacteria. *Proc Natl Acad Sci U S A*. 2006;103(42)15611–6.
 180. Zhang Y, Zeng F, Hohn K, Vadlani P V. Metabolic flux analysis of carbon balance in *Lactobacillus* strains. *Biotechnol Prog*. 2016;32(6)1397–403.
 181. DeMoss RD, Bard RC, Gunsalus IC. The mechanism of the heterolactic fermentation: a new route of ethanol formation. *J Bacteriol*. 1951;62(4)499–511.
 182. Geueke B, Riebel B, Hummel W. NADH oxidase from *Lactobacillus brevis*: A new catalyst for the regeneration of NAD. *Enzyme Microb Technol*. 2003;32(2)205–11.
 183. Schütz H, Radler F. Anaerobic reduction of glycerol to propanediol-1.3 by *Lactobacillus brevis* and *Lactobacillus buchneri*. *Syst Appl Microbiol*. 1984;5(2)169–78.
 184. Tobimatsu T, Sakai T, Hashida Y, Mizoguchi N, Miyoshi S, Toraya T. Heterologous expression, purification, and properties of diol dehydratase, an adenosylcobalamin-dependent enzyme of *Klebsiella oxytoca*. *Arch Biochem Biophys*. 1997;347(1)132–40.
 185. Sankaranarayanan M, Seol E, Kim Y, Chauhan AS, Park S. Measurement of crude-cell-extract glycerol dehydratase activity in recombinant *Escherichia coli* using coupled-enzyme reactions. *J Ind Microbiol Biotechnol*. 2017;44(3)477–88.
 186. Németh Á, Balássy A, Sevelle B. Difficulties and solutions for the assays of the key enzymes of a new enzymatic glycerol bioconversion. *Period Polytech Chem Eng*. 2008;52(1)17–22.
 187. Sauvageot N, Muller C, Hartke A, Auffray Y, Laplace J-M. Characterisation of the diol dehydratase pdu operon of *Lactobacillus collinoides*. *FEMS Microbiol Lett*. 2002;209(1)69–74.
 188. Bobik TA, Ailion M, Roth JR. A single regulatory gene integrates control of vitamin B12 synthesis and propanediol degradation. *J Bacteriol*. 1992;174(7)2253–66.
 189. Santos F, Spinler JK, Saulnier DM, Molenaar D, Teusink B, de Vos WM, et al. Functional identification in *Lactobacillus reuteri* of a PoxR-like transcription factor regulating glycerol

- utilization and vitamin B12 synthesis. *Microb Cell Fact.* 2011;10(55) doi.10.1186/1475-2859-10-55.
190. Lawrence JG, Roth JR. The cobalamin (coenzyme B12) biosynthetic genes of *Escherichia coli*. *J Bacteriol.* 1995;177(22)6371–80.
 191. Capozzi V, Russo P, Dueñas MT, López P, Spano G. Lactic acid bacteria producing B-group vitamins: A great potential for functional cereals products. *Appl Microbiol Biotechnol.* 2012;96(6)1383–94.
 192. Bhowmik T, Steele JL. Development of an electroporation procedure for gene disruption in *Lactobacillus helveticus* CNRZ 32. *J Gen Microbiol.* 1993;139(7)1433–9.
 193. Wei MQ, Rush CM, Norman JM, Hafner LM, Epping RJ, Timms P. An improved method for the transformation of *Lactobacillus* strains using electroporation. *J Microbiol Methods.* 1995;21(1)97–109.
 194. Zhang Y, Cheng K, Gao Q, Gao NF. Elaboration of an Electroporation Protocol for *Lactobacillus brevis*. *Adv Mater Res.* 2011;393–395(November 2011)729–32.
 195. Morelli L, Cocconcelli PS, Bottazzi V, Damiani G, Ferretti L, Sgaramella V. *Lactobacillus* protoplast transformation. *Plasmid.* 1987;17(1)73–5.
 196. Saier MH, Ye JJ, Klinke S, Nino E. Identification of an anaerobically induced phosphoenolpyruvate-dependent fructose-specific phosphotransferase system and evidence for the Embden-Meyerhof glycolytic pathway in the heterofermentative bacterium *Lactobacillus brevis*. *J Bacteriol.* 1996;178(1)314–6.
 197. Jones JA, Vernacchio VR, Sinkoe AL, Collins SM, Ibrahim MHA, Lachance DM, et al. Experimental and computational optimization of an *Escherichia coli* co-culture for the efficient production of flavonoids. *Metab Eng.* 2016;3555–63.
 198. Hewitt CJ, Nienow AW. The scale-up of microbial batch and fed-batch fermentation processes. *Adv Appl Microbiol.* 2007;62105–35.
 199. Olughu W, Deepika G, Hewitt C, Rielly C. Insight into the large-scale upstream fermentation environment using scaled-down models. *J Chem Technol Biotechnol.* 2019;94(3)647–57.
 200. Shen CR, Lan EI, Dekishima Y, Baez A, Cho KM, Liao JC. Driving forces enable high-titer

- anaerobic 1-butanol synthesis in *Escherichia coli*. *Appl Environ Microbiol*. 2011;77(9)2905–15.
201. Liu J, Wang Z, Kandasamy V, Lee SY, Solem C, Jensen PR. Harnessing the respiration machinery for high-yield production of chemicals in metabolically engineered *Lactococcus lactis*. *Metab Eng*. 2017;44(May)22–9.
 202. Kimmel SA, Roberts RF. Development of a growth medium suitable for exopolysaccharide production by *Lactobacillus delbrueckii* ssp. *bulgaricus* RR. *Int J Food Microbiol*. 1998;40(1–2)87–92.
 203. Kumara Behera B, Varma A. Material-Balance Calculation of Fermentation Processes. In: *Microbial Biomass Process Technologies and Management*. Cham: Springer; 2017. 257–98.
 204. Even S, Lindley ND, Loubière P, Coccagn-Bousquet M. Dynamic response of catabolic pathways to autoacidification in *Lactococcus lactis*: Transcript profiling and stability in relation to metabolic and energetic constraints. *Mol Microbiol*. 2002;45(4)1143–52.
 205. Cachon R, Diviés C. Generalized model of the effect of pH on lactate fermentation and citrate bioconversion in *Lactococcus lactis* ssp. *Lactis biovar. diacetylactis*. *Appl Microbiol Biotechnol*. 1994;41(6)694–9.
 206. Kaushal A, Pabbi S, Sharma P. Characterization of 2,3-butanediol-forming and valine-sensitive α -acetolactate synthase of *Enterobacter cloacae*. *World J Microbiol Biotechnol*. 2003;19487–93.
 207. Zhou K, Qiao K, Edgar S, Stephanopoulos G. Distributing a metabolic pathway among a microbial consortium enhances production of natural products. *Nat Biotechnol*. 2015;33(4)377–83.
 208. Schmidt FR. Optimization and scale up of industrial fermentation processes. *Appl Microbiol Biotechnol*. 2005;68(4)425–35.
 209. Junker BH. Scale-Up methodologies for *Escherichia coli* and yeast fermentation processes. *J Biosci Bioeng*. 2004;97(6)347–64.
 210. Zhang H, Li Z, Pereira B, Stephanopoulos G. Engineering *E. coli*-*E. coli* cocultures for production of muconic acid from glycerol. *Microb Cell Fact*. 2015;14(1)1–10.

Appendix A

Sequence of codon-optimized genes introduced to *L. lactis*.

Sequence of codon optimized *pddA* gene:

ATGCGTAGTAAAAGATTTGAAGCACTTGCTAAACGACCTGTTAATCAAGATGGATTTGTAAA
AGAATGGATTGAAGAAGGATTTATTGCAATGGAATCACCAAATGATCCAAAACCTTCTATTA
AAATTGTTAATGGTGCTGTAAGTGAATTGGATGGAAAACCTGTTTCTGATTTTGTCTTATTG
ATCATTTTATTGCAAGATATGGAATTAATCCTAATCGAGCAGAAGAAGTTATGGCTATGGAT
TCTGTAAAACCTTGCTAATATGTTGTGTGATCCAAATGTTAAACGTAGTGAAATTGTACCATTA
ACAACCTGCAATGACACCTGCTAAAATTGTTGAAGTTGTATCACATATGAATGTTGTAGAAAT
GATGATGGCAATGCAAAAATGCGTGCTCGTAGAACTCCATCTCAACAAGCACATGTTACA
AATGTAAAAGATAATCCTGTTCAAATTGCTGCTGATGCTGCTGAAGGTGCATGGCGTGGAT
TTGATGAACAAGAAACAACCTGTTGCTGTAGCAAGATATGCTCCATTTAATGCTATTGCATTA
CTTGTTGGTTCACAAGTAGGTCGTCCTGGAGTTTTGACTCAATGTTCTTTAGAAGAAGCAAC
AGAACTTAAATTGGGAATGTTAGGACATACATGTTATGCTGAAACTATTAGTGTATTATGGTA
CAGAACCAGTATTTACTGATGGAGATGATACACCTTGGAGTAAAGGTTTTCTTGCATCATCT
TATGCTTTCACGTGGATTGAAAATGAGATTTACTAGTGGTTCAGGATCTGAAGTTCAAATGGG
TTATGCAGAAGGAAAATCAATGCTTTTATCTTGAAGCTAGATGTATTTATATTACAAAAGCTGC
TGGTGTTCAAGGATTACAAAATGGTAGTGTTCATGTATTGGAGTTCATCTGCTGTACCTA
GTGGTATTCGAGCTGTTCTTGCAGAAAATCTTATTTGTAGTTCATTGGATTTAGAATGTGCA
TCTAGTAATGATCAAACATTTACTCATTCTGATATGCGACGTAAGTCTGCTCGTTTGTAAATGCAA
TTTTTACCAGGTACAGATTTTATTTTATCTGGATATAGTGCAGTTCCTAATTATGATAATATG
TTTGCAGGATCAAATGAAGATGCTGAAGATTTTGTATGATTATAATGTTATTCAAAGAGATTTA
AAAGTAGATGGTGGACTTAGACCAGTTCGAGAAGAAGATGTAATTGCTATTCGAAATAAAG
CTGCACGTGCTTTACAAGCAGTTTTTGTGCTGGTATGGGACTTCCACCTATTACTGATGAAGAA
GTTGAAGCTGCAACATATGCACATGGAAGTAAAGATATGCCTGAACGAAATATTGTTGAAG
ATATTAATTTGCTCAAGAAATTATTAATAAAAATCGTAATGGTTTGAAGTTGTAAAAGCAT
TAGCACAAGGTGGATTTACTGATGTTGCACAAGATATGCTTAATATTCAAAAAGCTAAACTT
ACTGGAGATTATTTGCATACATCTGCAATTATTGTTGGTGTATGGACAAGTACTTAGTGCTGT
TAATGATGTAAATGATTATGCAGGTCTGCTACAGGATATAGACTTCAAGGTGAACGATGG
GAAGAAATTAATAATATTCCAGGAGCACTTGATCCAAATGAAATTGATTAA

Sequence of codon optimized *pddB* gene:

ATGGAAATTAATGAAAAATTGTTGCGACAAATTATTGAAGATGTATTGAGTGAAATGAAAGG
AAGTGATAAACCTGTTAGTTTTAATGCACCTGCTGCAAGTGTGCAACCAAGCTACACCA
CCAGCTGGTGTATGGATTTTTAACTGAAGTTGGTGAAGCTCGACAAGGAACACAACAAGATG
AAGTTATTATTGCTGTAGGTCCTGCATTTGGACTTGTCAAACCTGTTAATATTGTAGGTATTC
CACATAAATCAATTCTTCGTGAAGTAATTGCAGGTATTGAAGAAGAAGGAATTAAGCTCGT
GTTATTAGATGTTTTAAATCATCTGATGTAGCTTTTGTGTCAGTAGAAGGTAATAGACTTAGT
GGTTCAGGAATTTCTATTGGTATTCAAAGTAAAGGAACAACCTGTTATTCATCAACAAGGACT
TCCACCTTTGTCTAATTTGGAATTTTCTCAAGCACCATTACTTACATTAGAACTTATAG
ACAAATTGGAAAAATGCTGCACGATATGCAAAACGTGAATCACCACAACCTGTTCCAACAT
TGAATGATCAAATGGCTCGACCTAAATATCAAGCTAAATCTGCAATTTTACATATTAAGAAA
CTAAATATGTTGTAAGTGGTAAAAATCCTCAAGAACTTCGTGTAGCATTGTAA

Sequence of codon optimized *pddC* gene:

ATGAATACTGATGCAATTGAAAGTATGGTTCGTGATGTTTTATCTCGTATGAATAGTTTACAA
GGTGAAGCACCAGCAGCAGCTCCTGCTGCTGGTGGAGCATCACGATCTGCTCGTGTTC
GATTATCCACTTGCAAATAACATCCTGAATGGGTAAAAACAGCTACTAATAAACATTAGA
TGATTTTACTCTTGAAAATGTTTTGTCTAATAAAGTAACAGCACAAGATATGCGTATTACACC
AGAAACTTTGAGATTACAAGCTAGTATTGCAAAGATGCTGGTCGTGATAGATTGGCAATG
AATTTTGAACGAGCTGCTGAATTAACAGCTGTTCCAGATGATAGAATTTTAGAAATTTATAAT
GCACTTAGACCTTATCGAAGTACTAAAGAAGAATTACTTGCTATTGCAGATGATTTAGAATC
ACGTTATCAAGCTAAAATTTGTGCTGCATTTGTTAGAGAAGCAGCAACTTTGTATGTAGAAC
GTAAAAAATTGAAAGGAGATGATTAA

Sequence of codon optimized *ddrA* gene:

ATGAGATATATTGCTGGTATTGATATTGGTAATTCTAGTACAGAAGTTGCATTGGCTACACT
TGATGAAGCTGGTGCATTGACTATTACACATTCAGCTCTTGCAGAAACAACCTGGTATTAAG
GAACTCTTCGTAATGTTTTTGGTATTCAAGAAGCTCTTGCACCTGTTGCTCGTGGAGCAGGT
ATTGCTGTTTCAGATATTTCTTATTTCGTATTAATGAAGCAACACCAGTTATTGGAGATGTT
GCTATGGAAACAATTACTGAAACAATTACTGAATCTACAATGATTGGTCATAATCCAAAA
ACACCAGGTGGAGCTGGATTAGGTACAGGAATTACTATTACACCTCAAGAATTACTTACAC
GTCCAGCTGATGCACCTTATATTCTTGTGTTTCATCTGCTTTTTGATTTTGCAGATATTGCTT
CAGTTATTAATGCATCTTACGTGCTGGTTATCAAATTACTGGAGTTATTCTTCAACGTGATG
ATGGAGTTCTTGTTCAAATCGTTTAGAAAAACCACTTCCTATTGTTGATGAAGTTCTTTATA
TTGATCGTATTCCACTTGGTATGCTTGTGCAATTGAAGTTGCAGTTCCAGGAAAAGTTATT
GAAACTCTTTCAAATCCTTATGGTATTGCTACAGTTTTTAATCTTTCTCCAGAAGAACTAAA
AATATTGTTCTATGGCTCGTGCACCTATTGGAATCGTTCAGCTGTTGTTGTTAAAACCTCC
ATCTGGTGTATGTTAAAGCTCGTGCAATTCCAGCTGGAAATTTAGAATTACTTGCTCAAGGAC
GTTCAAGTTCGTGTTGATGTTGCTGCTGGAGCTGAAGCAATTATGAAAGCTGTTGATGGTTG
TGGACGTTTAGATAATGTTACTGGTGAATCTGGAACAAATATTGGAGGAATGCTTGAACATG
TTCGTCAAACCTATGGCTGAATTAACAAATAAACCATCATCTGAAATTTTTATTCAAGATTTAC
TTGCAGTTGATACTTCAGTTCCTGTTTCTGTTACAGGTGGATTAGCTGGTGAATTTTCATTA
GAACAAGCAGTTGGAATTGCTTCAATGGTTAAATCTGATCGTCTTCAAATGGCAATGATTGC
TCGTGAAATTGAACAAAAAATTAATATTGATGTTCAAATTGGAGGAGCTGAAGCAGAAGCTG
CAATTTTAGGTGCACTTACAACCTCCAGGAACAACCTCGTCCTCTTGGCTATTTTAGATTTAGGT
GCAGGATCAACAGATGCTTCTATTATTAATCCAAAAGGAGATATTATTGCAACACATTTAGC
TGGTGCAGGAGATATGGTACTATGATTATTGCTCGTGAATTAGGTCTTGAAGATCGTTATC
TTGCAGAAGAAATCAAAAAATATCCATTAGCTAAAGTTGAATCATTATTTTCATCTTCGTCATG
AAGATGGATCAGTTCAATTTTTCTCAACACCATTACCACCAGCTGTTTTTGTCTCGTGTGTTGT
GTTGTTAAAGCAGATGAATTAGTTCCACTTCCTGGTGAATTTAGCTCTTGA AAAAGTTTCGTGC
AATTCGTGTTTCAGCTAAAGAACGTGTTTTTGTACAAATGCATTACGTGCTCTTCGTCAAG
TTTCTCAAACCTGGAAATATTCGTGATATTCCTTTTGTGTTTTAGTTGGAGGATCATCATTAG
ATTTTGAAGTTCCACAACCTTGTACAGATGCTCTTGCACATTATCGTTTAGTTGCTGGTTCGT
GGAAATATTCGTGGTTCAGAAGGACCTCGTAATGCTGTTGCAACTGGACTTATTCTTTCTTG
GCATAAAGAATTTGCTCATGAACGTTAA

Sequence of codon optimized *ddrB* gene:

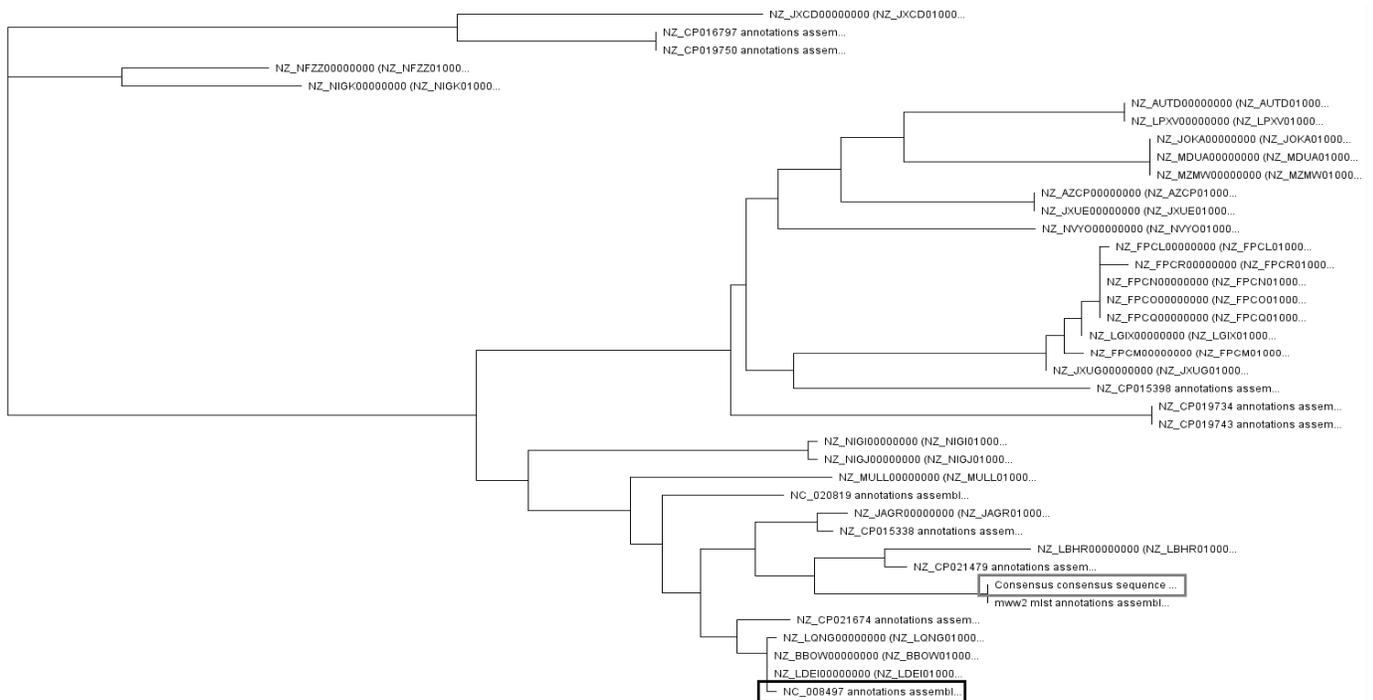
ATGAATGGTAATCATTACAGCTCCAGCAATTGCTATTGCAGTTATTGATGGTTGTGATGGACT
TTGGCGTGAAGTTTTACTTGGTATTGAAGAAGAAGGTATTCCATTTTCGTTTACAACATCATC
CAGCTGGAGAAGTTGTTGATTCTGCTTGGCAAGCTGCACGTTTCATCTCCATTACTTGTTGG
TATTGCTTGTGATCGTCATATGCTTGTGTTTCATTATAAAAATTTACCAGCTTCAGCACCTTT
ATTTACACTTATGCATCATCAAGATTCTCAAGCTCATCGTAATACTGGTAATAATGCTGCAC
GTTTAGTTAAAGGTATTCCATTTAGAGATTTGAATAGTGAAGCAACAGGTGAACAACAAGAT
GAATAA

Sequence of codon optimized *sadB* gene:

ATGAAAGCATTAGTATATCATGGAGATCATAAAATTAGTCTTGAAGATAAACCAAAACCAAC
ATTACAAAACCAACAGATGTAGTAGTTCGAGTACTTAAAACAACACTATTTGTGGTACAGATT
TGGGAATTTATAAAGGTAAAAATCCAGAAGTTGCTGATGGACGTATTTTAGGTCATGAAGGT
GTTGGAGTAATTGAAGAAGTTGGTGAATCTGTAACACAATTTAAAAAAGGAGATAAAGTTCT
TATTAGTTGTGTAAGTGTGTTTCATGTGATTATTGTAAAAACAATTGTATTCTCATTG
TCGTGATGGTGGATGGATTTTAGGTTATATGATTGATGGAGTTCAAGCTGAATATGTACGTA
TTCCACATGCAGATAATTCACCTTTATAAAATTCCTCAAACAATTGATGATGAAATTGCAGTTC
TTCTTTCTGATATTTTGCCAACTGGACATGAAATTGGTGTTCATATGGAAATGTACAACCA
GGTGATGCTGTTGCAATTGTAGGTGCTGGACCTGTTGGAATGAGTGTATTATTAACAGCAC
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TATTTGTCAAGAAATTGTTAAACCTGGTGCTCATATTGCAAATGTTGGTGTACATGGAGTTA
AAGTAGATTTTGAAATTCAAAAATTGTGGATTAATAATCTTACAATTACAACACTGGACTTGTTA
ATACTAATAACAACCTCCAATGCTTATGAAAGTTGCTTCAACAGATAAACTTCCTTTAAAGAAAA
TGATTACTCATCGTTTTGAAATTAGCTGAAATTGAACATGCATATCAAGTTTTCTTAAATGGAG
CAAAGAAAAAGCAATGAAAATTATTCTTAGTAATGCAGGAGCAGCATAA

Appendix B

Phylogenetic relationship between the genomes of *L. brevis* SE20 and other sequenced strains of *L. brevis*, inferred from MLST comparison. Highlighted in boxes; gray, SE20; black, ATCC367.



Appendix C

Mapping the obtained sequence reads from *L. brevis* SE20 *pdu*-operon against the most similar genome reference, *L. brevis* ATCC 367. Low DNA complexity in library preparation is seen as gaps in the reads.

