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Microbial production of propanol

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Abstract

Both, n-propanol and isopropanol are industrially attractive value-added molecules that can be produced by microbes from renewable resources. The development of cost-effective fermentation processes may allow using these alcohols as a biofuel component, or as a precursor for the chemical synthesis of propylene. This review reports and discusses the recent progress which has been made in the biochemical production of propanol. Several synthetic propanol-producing pathways were developed that vary with respect to stoichiometry and metabolic entry point. These pathways were expressed in different host organisms and enabled propanol production from various renewable feedstocks. Furthermore, it was shown that the optimization of fermentation conditions greatly improved process performance, in particular, when continuous product removal prevented accumulation of toxic propanol levels. Although these advanced metabolic engineering and fermentation strategies have facilitated significant progress in the biochemical production of propanol, the currently achieved propanol yields and productivities appear to be insufficient to compete with chemical propanol synthesis. The development of biosynthetic pathways with improved propanol yields, the breeding or identification of microorganisms with higher propanol tolerance, and the engineering of propanol producer strains that efficiently utilize low-cost feedstocks are the major challenges on the way to industrially relevant microbial propanol production processes.

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1 Introduction

Propanol exists in the form of two isomers, 1-propanol (also called n-propanol), and 2-propanol (also called isopropanol or isopropyl alcohol). Both propanols are mainly used as solvents, but they also serve as chemical intermediates in the production of various esters and amines. Furthermore, isopropanol is often applied as a disinfectant in pharmaceutical products, or as an antifreezing agent. n-propanol is industrially produced via hydroformylation of ethylene. Isopropanol can be chemically produced by reduction of acetone in the presence of excess hydrogen, or from propylene by hydration over an acid catalyst (Papa, 2011).

Growing shortage of fossil resources has increased the interest in microbial synthesis of propanol from renewable raw materials. The development of cost-effective propanol fermentation processes may not only satisfy the demand of 2 Mt/year of isopropanol and 0.2 Mt/year of n-propanol. In fact much larger markets could be addressed if the production costs of biosourced propanol would allow using this alcohol as a biofuel (Choi et al., 2014; Dusséaux et al., 2013; Huo et al., 2011; Lee et al., 2012; Shen and Liao, 2008), or as a precursor for propylene which can be chemically produced from either propanol by dehydration (Kibby and Hall, 1972).

Whether propanol is a good target molecule to develop biofuels is still matter of debate. This alcohol provides high octane numbers and is less corrosive than ethanol (Fernando et al., 2007). On the other hand, its energy density is not much higher than that of ethanol, and significantly smaller than of alternative biofuels such as butanol or biodiesel ("Biofuels - Types of Biofuels - Bioalcohols," 2010). Hence, pure propanol may not be the ideal biofuel, but it could account for a significant fraction of biofuel alcohol mixtures that are obtained in mixed fermentation processes such as the isopropanol-butanol-ethanol (IBE) fermentation (Dusséaux et al., 2013; George et al., 1983; Lee et al., 2012), or from the fermentation of innovative feedstocks such as algae proteins (Choi et al., 2014; Huo et al., 2011).

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The development of fermentation processes for the production of pure propanol is mainly motivated by its potential use as a precursor for propylene, which is one of the most important building blocks in the chemical industry. Propylene is used for the production of plastics and is a precursor for the chemical synthesis of propylene oxide**,** acrylonitrile**,** cumene**,** butyraldehyde**,** and acrylic acid. Currently, it is exclusively produced from petrol, and its annual production volume amounted to 85 Mt in 2013 ("Propylene – Study: Market, Analysis, Trends," 2016). Thus, the development of competitive microbial propanol production processes which enable an economically viable propylene production from renewable resources represents a significant market opportunity.

It is therefore not surprising that much research efforts have been invested in recent years to improve microbial propanol production. Production organisms and process conditions have been optimized to increase the propanol yield and the final product titers; and the engineering of different metabolic pathways and production organisms has enabled the use of new feedstocks, such as protein waste, cellobiose, lignocellulose, or carbon dioxide. We herein review all of these aspects but pay particular attention to the description of the nine natural and synthetic propanol-producing pathways (and pathway variants) that have been recently identified and constructed. This growing number of pathways reflects our ever increasing capacity to conceive and implement new metabolic routes by making use of synthetic biology principles, and to use renewable carbon feedstocks more and more efficiently. However, given the current oil market prices, the replacement of petrol-based propanol and propylene production by sugar-based biochemical processes appears to be a long way ahead.

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2 Natural propanol-producing organisms

2.1 Biosynthesis of isopropanol in the mixed Isopropanol-Butanol-Ethanol (IBE) fermentation

The mixed acetone-butanol-ethanol (ABE) fermentation, which occurs during anaerobic growth of solvent-producing *Clostridia* species, was one of the most important industrial fermentation processes in the early 20th century. During World War One, the ABE fermentation was the major source for acetone which was required in the production of ammunition. In the period between the two world wars butanol became a highly used solvent in the car industry, and its supply was mainly guaranteed by ABE fermentation plants (see (Jones and Woods, 1986) for an excellent review). With the rise of the petrochemical industry, the contribution of ABE fermentation processes to the production of solvents became gradually smaller and eventually marginal. Due to the expected shortage of oil supply, and because butanol is considered as one of the most promising drop-in biofuels, this fermentation process recently regained considerable interest. Most anaerobically growing *Clostridia* species naturally produce mixtures of acetone, butanol, and ethanol (ABE) at an approximate ratio of 3:6:1, respectively (Chen and Hiu, 1986; George et al., 1983). The production of solvents is considered a defense mechanism against medium acidification that is caused by the secretion of acetic and butyric acid, which are alternative fermentation end-products (Hüsemann and Papoutsakis, 1988). At decreasing pH, these organic acids are re-assimilated and converted into alcohols. Upon adjustment of a slightly acidic pH of the cultivation medium, organic acid production can be almost completely prevented and solvents become the major fermentation products.

The acetone-butanol pathway of *Clostridia* is shown in Figure 1. Two molecules of acetyl-CoA are condensed to one molecule of acetoacetyl-CoA by acetyl-CoA: acetyl-CoA C-acetyltransferase (also named acetoacetyl-CoA thiolase, or acetoacetyl-CoA synthase). In the next step, acetoacetyl-CoA:acetate CoA-transferase (also named coenzyme A transferase, or acetoacetyl-CoA transferase)

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catalyzes the transfer of CoASH to acetate, yielding one molecule acetoacetate and regenerating one molecule acetyl-CoA. Acetoacetate is either decarboxylated by acetoacetate decarboxylase yielding acetone, or it is reduced and dehydrated via a sequence of 5 reactions to yield butanol (Figure 1). For some *Clostridia* species, reduced acetone production and concomitant accumulation of isopropanol was observed (George et al., 1983). Formation of isopropanol in these strains was shown to be a consequence of the increased specificity of the strictly NADPH-dependent primary/secondary alcohol dehydrogenase for acetone (Hiu et al., 1987). However, isopropanol production in natural *Clostridia* strains remained small compared to the production of butanol, and did not exceed 25 mol% of the total alcohol fraction (George et al., 1983) (Table 1). The metabolic pathway which produces isopropanol via the characteristic intermediate acetone is in the following sections termed the acetone-dependent isopropanol pathway (Figure 1). It has been optimized in *Clostridia* strains for the simultaneous production of isopropanol, butanol, and ethanol; and it was heterologously expressed in different bacteria and yeast to facilitate the exclusive production of isopropanol (see below).

2.2 Biosynthesis of n-propanol through the Wood-Werkman pathway

Propionibacteria and various other anaerobic bacteria, such as *Clostridium propionicum, and Clostridium neopropionicum* produce propionic acid as the major fermentative end-product (Wood, 1981). Under some cultivation conditions these bacteria also form significant amounts of n-propanol as a co-product (Table 1). The biosynthesis of propionic acid proceeds in most of these cases through the Wood-Werkman cycle (also termed dicarboxylic pathway, or methylmalonyl-CoA pathway)(Allen et al., 1964; Wood, 1981). Alternatively, propionic acid is produced via the acrylate pathway which has been less frequently observed in microorganisms (Cardon and Barker, 1946; Tholozan et al., 1992). The operation of both pathways can be distinguished by the characteristic ^{13}C-labelling patterns of the C-atoms of propionic acid which is produced from differently labelled substrates (e.g. lactate). Since the Wood-Werkman cycle proceeds via the symmetric molecules fumarate and

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succinate (Figure 2), the asymmetric ^{13}C label of the substrate becomes randomized in the product. In contrast, the acrylate is a linear pathway which conserves the initial labelling pattern of the substrate (see below). The Wood-Werkman cycle starts from pyruvate, which is converted to oxaloacetate by receiving a carboxyl group from methylmalonyl-CoA. The reaction is catalyzed by methylmalonyl-CoA carboxytransferase and releases propionyl-CoA as the second product. Oxaloacetate is then converted to succinate via several reaction steps in the reductive branch of the TCA cycle. Propionyl-CoA:succinate CoA-transferase transfers the CoA-group of the initially formed propionyl-CoA to succinate and produces succinyl-CoA and propionic acid, which is the final product of the pathway. The cycle is closed by the conversion of succinyl-CoA into methylmalonyl-CoA, which is catalyzed by methylmalonyl-CoA mutase (Figure 2)(Allen et al., 1964; Wood, 1981).

When the propionic acid bacteria *Propionibacterium acidipropionici* and *Propionibacterium freudenreichii* were grown on glucose under anaerobic conditions, they produced very little n-propanol with the molar yield being ≤0.06. When the more reduced carbon source glycerol was used instead, the propanol yields increased to 0.13, but propionic acid remained the major fermentation end-product. (Seeliger et al., 2002) studied several ethanol and lactate-fermenting anaerobic bacteria and found that *P. freudenreichii* produced n-propanol from ethanol (molar yield 0.14) when the medium was sparged with hydrogen. The actual pathway for propanol production in these bacteria has not been identified, but it is likely that propionyl-CoA is synthesized through the Wood-Werkman cycle and then reduced in two steps by a yet unknown acylating propionaldehyde dehydrogenase and a propanol dehydrogenase (Figure 2). Thus, when propanol is produced, succinyl-CoA cannot be regenerated via the propionyl-CoA:succinate CoA transferase but needs to be replenished by a different reaction. Since *Propionibacteria* do not have a functional succinyl-CoA synthetase (Falentin et al., 2010), succinyl-CoA synthesis under propanol-producing conditions appears to rely on the promiscuity of the propionyl-CoA:succinate CoA-transferase, which also accepts acetyl-CoA as an alternative CoA-group donor (Allen et al., 1964). Therefore, the

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complementation of the Wood-Werkman pathway by a succinyl-CoA synthetase could be an interesting metabolic engineering strategy to improve propanol production in these organisms.

2.3 Biosynthesis of n-propanol though the acrylate pathway

Clostridium propionicum has the capability of using the amino acids alanine and serine, as well as lactate and ethanol as growth substrates in the absence of oxygen (Cardon and Barker, 1946; Tholozan et al., 1992). The major fermentative end-products are propionic acid, acetate, and carbon dioxide. In these organisms propionic acid formation depends on the acrylate pathway which is initiated by the formation of pyruvate and proceeds via the intermediates lactate, lactyl-CoA, acryloyl-CoA, and propionyl-CoA (Figure 3). When *C. neopropionicum* and *C. propionicum* were cultivated on 30 mM ethanol, they mainly produced propionate and acetate, but also small amounts of n-propanol (Table1)(Tholozan et al., 1992). Based on enzymatic assays of potential pathway reactions, it was proposed that propanol was formed through the two-step reduction of propionyl-CoA (Figure 3). This required, however, that part of the lactyl-CoA was not produced by propionyl-CoA:lactate CoA transferase (Figure 3). The unknown lactyl-CoA-producing activity (e.g. another CoA-transferase, or lactyl-CoA synthetase) was not identified (Tholozan et al., 1992). Thus, essential information required for the improvement of this pathway through rational metabolic engineering is still missing.

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3 Engineered microorganisms expressing the acetone-dependent isopropanol pathway

3.1 Engineering of *Clostridium acetobutylicum* for increased isopropanol production

Alcohol production using *Clostridia* species is mainly motivated by the potential application of butanol as a drop in bio-fuel (Lee et al., 2008). Acetone is an unwanted by-product of the ABE fermentation since it cannot be used as a biofuel and because it is highly corrosive to car engines. Therefore, the primary/secondary alcohol dehydrogenase from *C. beijerinckii* NRRL B593 which has increased affinity of for acetone was used to reduce this compound to isopropanol, thus, shifting the spectrum of fermentation products towards alcohols that are more suitable for biofuel applications. In three very similar studies (Collas et al., 2012; Dusséaux et al., 2013; Lee et al., 2012) isopropanol production by *C. acetobutylicum* was enhanced by overexpressing the genes encoding the acetone-accepting primary/secondary alcohol dehydrogenase (*adh*B-593 from *C. beijerinckii* NRRL B593), and the endogenous acetoacetyl-CoA:acetate CoA-transferase (*ctfAB*), and acetoacetate decarboxylase (*adc*) enzymes. The resulting strain produced 8.8 g/L isopropanol, 13.7 g/L butanol, and 1.5 g/L ethanol from 90 g/L glucose in anaerobic shake flask experiments (Collas et al., 2012). When butyrate kinase (*buk*) was additionally deleted, the strains produced on average ~4.5 g/L isopropanol, ~14.4 g/L butanol, and 1.5 g/L ethanol (and no acetone) from 80 g/L glucose under very similar cultivation conditions (Table 1)(Dusséaux et al., 2013; Lee et al., 2012). These results suggest that the deletion of butyrate kinase shifts the spectrum of produced alcohols from isopropanol towards butanol.

Above the observed product titers, the alcohols became toxic for the cells (Dusséaux et al., 2013; Lee et al., 2012). Gas-stripping maintained alcohol concentrations in the cultivation vessel below inhibitory concentrations and facilitated accumulation of 35 g/L total alcohol (calculated from

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cumulated alcohol content in condensate and fermentation broth, ~6 g/L isopropanol)(Lee et al., 2012). The alcohol productivity with and without gas-stripping was ~0.6 g/(L h).

Due to the comparatively high tolerance of *Clostridia* species against butanol, research on alcohol production with this microorganism is mainly directed to increase butanol for bio-fuel applications. This is probably one reason why no study is available wherein a *Clostridium* strain was engineered to preferentially produce isopropanol. Another reason is the still considerable lack of physiological knowledge and the rather few available genetic tools for *Clostridia* which render the rational engineering of these microorganisms complicated. The heterologous expression of the *Clostridial* isopropanol pathway in genetically amenable host organisms appears to be a much more promising alternative (see below).

3.2 Engineered *Escherichia coli* for isopropanol production

Bermejo et al. first demonstrated acetone production by an *E. coli* strain that heterologously expressed the acetone pathway from *C. acetobutylicum* (Bermejo et al., 1998). This work mainly aimed at detoxifying acetate which accumulated in cultures of *E. coli* strains that expressed heterologous proteins. Acetone production was achieved by expressing the *C. acetobutylicum* genes which encode for acetoacetyl-CoA thiolase (*thl*), acetoacetyl-CoA:acetate CoA-transferase (*ctfAB*), and acetoacetate decarboxylase (*adc*) in several *E. coli* strains that had different acetate production capacities. The best acetone-producing strain accumulated 154 mM of acetone after 120 h of cultivation (acetone evaporation was not quantified) in a controlled bioreactor when glucose was used as the carbon source. Aerobic cultivation conditions and slightly acid pH 5.5 were found to be beneficial for acetone production (Bermejo et al., 1998).

Two studies independently demonstrated the production of isopropanol in *E. coli* by extending the *Clostridial* acetone pathway by one reduction step (Hanai et al., 2007; Jojima et al., 2008) (Figure 4A). The performance of this pathway was optimized by overexpressing the endogenous acetoacetyl-

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CoA transferase, encoded by *atoAD*, instead of the acetoacetyl-CoA transferase from *C. acetobutylicum* ATCC824*,* encoded by *ctfAB*. Furthermore, isopropanol production was found to be highest when the primary/secondary alcohol dehydrogenase (SADH) of *C. beijerinckii* NRRL B593, encoded by $adh_{B\text{-}593}$, was used for the reduction of acetone instead of an alternative SADH from *Thermoanaerobacter brockii*. The resulting *E. coli* strain produced 4.9 g/L isopropanol at a yield of 0.43 mol/mol in shake flasks without any further modification of the strain genotype (Hanai et al., 2007). An *E. coli* strain expressing the same isopropanol pathway entirely comprised of genes isolated from *Clostridium acetobutylicum* ATCC824 produced 13.6 g/L isopropanol in a controlled fed-batch culture at a yield of 0.51 mol/mol and with a productivity of 0.6 g/(Lh)(Jojima et al., 2008).

Furthermore, the *E. coli* strain previously developed by Hanai et al. (2007) was studied under fermentation conditions that were optimized to increase isopropanol production (Inokuma et al., 2010). It was found that isopropanol becomes inhibitory for glucose uptake and cell growth at concentrations of ~40 g/L (Inokuma et al., 2010). Product removal by continuous gas stripping increased the final isopropanol titer in a fed-batch fermentation to the equivalent of 143 g/L (calculated from isopropanol concentrations in condensate and fermentation broth, yield: 0.67 mol/mol, productivity: 0.6 g/(Lh))(Inokuma et al., 2010). It was proposed that the yield of this process could still be slightly improved by avoiding acetone formation by using an improved acetone reductase.

Another study aimed at increasing isopropanol production by optimizing the flux repartitioning between TCA cycle and isopropanol pathway using a genetic toggle switch (Soma et al., 2014). Acetyl-CoA availability for the synthesis of isopropanol was improved by decreasing the expression of the acetyl-CoA consuming citrate synthase in the production phase. Indeed, the authors showed a 3-fold increased isopropanol production (0.48 mol/mol, reference strain: 0.16 mol/mol) when the transcription of the citrate synthase was controlled by their genetic device (Soma et al., 2014).

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Recently, a slightly modified acetone pathway was presented that relies on the use of an acetoacetyl-CoA thioesterase for the deacetylation of acetoacetyl-CoA instead of an acetoacetyl-CoA::acetate CoA-transferase (May et al., 2013) (Figure 4B). The use of the thioesterase alleviates the need for the presence of free acetate during the biosynthesis of acetone. This may prove useful for augmenting strain performance because acetate availability has to be tightly controlled in the natural pathway to avoid secretion of this metabolic by-product. Three candidate thioesterases of different biological origin, TEII*srf* from *Bacillus subtilis*, YbgC from *Haemophilis influenza,* and AcsA2 from *Sinorhizobium meliloti,* were tested *in vitro* for activity on acetoacelyl-CoA. It was found that TEIIsrf and YbgC were capable of hydrolyzing acetoacetyl-CoA. Furthermore, these two enzymes enabled acetone production (May et al., 2013) in *E. coli* strains that heterologously expressed acetoacetyl-CoA thiolase (*thlA*) and acetoacetate decarboxylase (*adc*) from *C. acetobutylicum*. Unexpectedly, the capacity of the thioesterase-dependent pathway to produce acetone was strain dependent. Since the tested thioesterases were not very specific for acetoacetyl-CoA but also had a significant activity on acetyl-CoA, the authors speculated that differences in acetone production arose from different acetyl-CoA concentrations in the tested strains (May et al., 2013). Engineering of the available thioesterases to increase their specificity for acetoacetyl-CoA, or more exhaustive screenings to identify better suited enzymes may thus indeed provide an isopropanol pathway that is independent from acetate, and therefore less prone to production of this metabolic by-product.

The theoretical yield of the acetone-dependent isopropanol pathway is 1 mol of isopropanol per mol of glucose. This limitation is imposed by the carbon loss during the production of acetyl-CoA from pyruvate by the decarboxylating pyruvate dehydrogenase (PDH) reaction. It has been demonstrated that the PDH reaction can be by-passed via the carbon-conserving phosphoketolase (PKT) pathway. PKTs are capable of cleaving either fructose-6P (F6P) or xylulose-5P (X5P) into erythrose-4P (E4P) or glyceraldehyde-3P (GAP), respectively, and acetyl phosphate. The latter reaction product can be readily converted to acetyl-CoA by phosphate acetyltransferase (Pta). The PKT-Pta pathway was applied in several organisms to increase the product yield of acetyl-CoA-

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derived molecules (Henard et al., 2015). In addition, (Bogorad et al., 2013) showed that carbon-conserving conversion of sugars into acetyl-CoA could be achieved by the PKT-Pta pathway when E4P and GAP were recycled into the PKT substrates X5P or F6P (by employing pentose phosphate pathway reactions, and part of the Embden-Meyrhoff-Parnass pathway). Hence, it would be interesting to learn whether the application of the PKT-Pta pathway could also increase the product yield of microorganisms that express the acetone-dependent isopropanol pathway.

3.3 Engineered yeast for isopropanol production

Yeasts are considered to have higher stress tolerance than *E. coli* and can be cultivated at lower pH. Thus, the development of yeast strains for isopropanol production may facilitate production of this alcohol under the harsh fermentation conditions that production organisms commonly encounter when plant hydrolysates are used as the feedstock. In addition, the application of yeasts may enable propanol production under non-sterile process conditions where yeast cells can be recovered at the end of the fermentation to be reused in a new batch after removal of bacteria by mild acid treatment (Brethauer and Wyman, 2010). In a recent study, the yeast *Candida utilis* was engineered for isopropanol production (Tamakawa et al., 2013). When the codon-optimized *Clostridial* genes encoding for acetoacetyl-CoA:acetate CoA-transferase (*cftAB*), acetoacetate decarboxylase (*adc*) and the acetone-accepting alcohol dehydrogenase (adh_{B593}) were expressed in an unmodified strain, high amounts of ethanol transiently accumulated and only 1.2 g/L isopropanol were produced upon re-consumption of the ethanol. Product formation was further improved by increasing cytosolic acetyl-CoA production through overexpression of endogenous acetyl-CoA synthetase, encoded by *ACS2*, and by overexpressing the endogenous acetyl-CoA transferase, encoded by *ERG10*, instead of the *Clostridial* enzyme. When this strain was cultivated in a controlled bioreactor run in fed-batch mode, 27.2 g/l isopropanol were produced in ~200 h from 200 g/l glucose

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(Table 1)(Tamakawa et al., 2013). The corresponding isopropanol yield of 0.41 mol/mol was still significantly lower than for *E. coli* strains which reached up to 0.73 mol/mol when expressing the same pathway (Table 1). It appears, however, that isopropanol productivity of this yeast could be significantly improved when further metabolic engineering increased the capacity of the heterologous pathway to accommodate carbon flux, and when mitochondrial activity could be reduced (e.g. through oxygen limitation) to channel more carbon flux through the cytosolic isopropanol pathway.

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4 Synthetic pathways for production of n-propanol

4.1 The synthetic threonine pathway for n-propanol production

The Liao lab proposed synthetic pathways that produce n-propanol and other higher alcohols from threonine. n-propanol production proceeds via the intermediates 2-ketobutyrate (2KB) and 1-propanal (Figure 5A)(Atsumi et al., 2008; Zhang et al., 2008). Higher alcohols are obtained by successively adding acetyl-CoA-derived C2 units to the precursor 2KB, before decarboxylating and reducing the resulting 2-ketoacids (Zhang et al., 2008). The theoretical n-propanol yield of this pathway is 1 mol/mol (Shen and Liao, 2013). The synthetic alcohol-producing pathway was implemented in *E. coli* by employing endogenous threonine dehydratase, TdcB, endogenous 2-isopropylmalate synthase, LeuA, natural and mutant 2-ketoacid decarboxylase, Ll-KviD, from *Lactococcus lactis*, and the alcohol dehydrogenases, Sc-Adh2 or Sc-Adh6, from *Saccharomyces cerevisiae* (Atsumi et al., 2008; Zhang et al., 2008). These genes were expressed in a threonine-overproducing *E. coli* strain that additionally over-expressed the genes encoding the threonine pathway (*thrABC*) and genes of the isoleucine pathway that were required to facilitate chain elongation of 2KB. The product spectrum was varied by expressing different mutants of Ll-KivD, and LeuA. The resulting strains produced a mixture of higher alcohols (butanol, isobutanol, pentanol, 3-methyl-1-butanol, 3-methyl-1-pentanol) among which n-propanol represented a relatively small fraction with final concentrations that did not exceed 213 mg/L (produced from 20 g/L glucose after 40 h of shake flask cultivation (Zhang et al., 2008)).

In a subsequent study, the production strain was further optimized for the parallel production of n-propanol and 1-butanol by the deletion of competing pathways (Shen and Liao, 2008). In particular, threonine and 2-ketovalerate production was increased by overexpressing genes encoding threonine (*thrABC*) and leucine (*leuABCD*) pathway enzymes, and by deleting *metA* and *tdh* which

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encode for homoserine O-succinyltransferase and threonine dehydratase, respectively. 2KB availability was increased by inactivating the 2KB-consuming acetohydroxy acid synthases through deletion of *ilvB* and *ilvI*, acetyl-CoA availability was increased by deletion of alcohol dehydrogenase, AdhE. Ll-KviD and Sc-Adh2 were employed as ketoacid decarboxylase and propanol/butanol dehydrogenase, respectively. The optimized strain produced 1 g/L isopropanol (+ 1 g/L butanol) in a shake flask. Propanol yield and productivity were 0.11 mol/mol and 0.01 g/(Lh) (Table 1)(Shen and Liao, 2008).

4.2 The synthetic citramalate pathway for n-propanol production

Another 2KB-synthesizing pathway was identified in *Archaea* and does not require the formation of the intermediate threonine (Xu et al., 2004). The archeal 2KB pathway commences by the condensation of pyruvate and acetyl-CoA to yield citramalate. This intermediate is then further processed to citraconate and beta-methyl-malate to yield 2KB (Figure 6). The pathway was applied to produce n-propanol (Atsumi and Liao, 2008). The citramalate synthase, Mj-Cim1, of the thermophilic organism *Methanocaldococcus jannaschii* was evolved in *E. coli* to obtain improved enzymatic activities at moderate temperatures. The improved Cim1 mutant was expressed together with 2-ketoacid decarboxylase, Ll-KviD, alcohol dehydrogenase, Sc-Adh2, and the *leuABCD* operon in an *E. coli ΔilvI ΔilvB* strain. Product titers of 3.5 g/L propanol (+ 0.5 g/L butanol) were achieved in shake flask cultures (isopropanol yield: 0.5 mol/mol, productivity: 0.03 g/Lh) (Table 1)(Atsumi and Liao, 2008).

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4.3 Simultaneous expression of the threonine and citramalate pathways in *E. coli*

The n-propanol-producing citramalate and threonine pathways differ in regard to their co-factor requirement. It was shown in a subsequent study that the simultaneous operation of both pathways in a single host organism had a synergistic effect on propanol production (Shen and Liao, 2013). The theoretical n-propanol yield increases from 1 mol/mol for each individual pathway to 1.3 mol/mol when both pathways work together (Shen and Liao, 2013). It was demonstrated that this increase of the theoretical yield also translated into actually improved n-propanol production: when the threonine and citramalate pathways were working individually, the corresponding propanol yields only reached 0.27 mol/mol and 0.33 mol/mol, respectively (Shen and Liao, 2013). When both pathways were active simultaneously, 8 g/L propanol were produced in shake flask experiments at a molar yield of 0.45 mol/mol and with a productivity of 0.12 g/(L h) (Table1).

In a different study it was shown that it was possible to convert 2KB to n-propanol by making use of the 'spontaneous' decarboxylation of 2KB (this 'spontaneous' decarboxylation reaction is likely catalyzed by endogenous pyruvate oxidase, PoxB (Chang and Cronan, 2000)) and by converting the resulting propionylphosphate via the intermediates propionate, propionyl-CoA and 1-propanal into the desired alcohol (Figure 5B)(Choi et al., 2012). A previously constructed threonine-overproducing strain (Lee et al., 2007) was optimized for the overproduction of 2KB by expressing an isoleucine-insensitive threonine dehydratase mutant, encoded by $ilvA_{C1139T\ G1341T\ C1351G\ T1352C}$, by deleting the 2KB-consuming enzymes encoded by *ilvI, ilvH, ilvB* and *ilvN*, by expressing the citramalate synthase from *Methanocaldococcus jannaschii*, encoded by *cimA*, and by deleting the stationary phase sigma factor, encoded by *rpoS*. The conversion of 2KB into n-propanol was facilitated by overexpressing the bifunctional acetate/propionate kinase, the bifunctional acetyl-CoA/acetoacetyl-CoA synthase, and the bifunctional alcohol/aldehyde dehydrogenase, which are encoded by the endogenous genes *ackA, atoAD*, and *adhE*, respectively. To enable effective reduction of 1-propanal to n-propanol under aerobic conditions, an oxygen-resistant mutant of the endogenous AdhE enzyme was used. The

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engineered strain produced 10.7 g/L n-propanol with a yield and productivity of 0.16 mol/mol and 0.14 g/(Lh) in a fed-batch culture using glucose as the carbon source (Choi et al., 2012). When glycerol was used as the carbon source, the strain produced 10.4 g/L n-propanol at a yield and productivity of 0.2 mol/mol and 0.08 g/(Lh) (Table 1).

4.4 The synthetic succinate pathway for n-propanol production

A synthetic pathway which produces n-propanol from succinate in a reaction sequence that proceeds via succinyl-CoA, methylmalonyl-CoA, propionyl-CoA, and propanal (Figure 7) was developed by (Srirangan et al., 2013). In *E. coli*, the genes that encode the enzymatic activities required to convert succinyl-CoA into propionyl-CoA are located in the so called 'sleeping beauty mutase' operon (SBMO). To achieve propanol production, the SBMO genes coding for methylmalonyl-mutase (*sbm*), arginine kinase (*ygfD*), and methylmalonyl-CoA decarboxylase (*ygfG*) were overexpressed together with endogenous succinyl-CoA synthetase (*sucCD*) and bifunctional butyraldehyde/alcohol dehydrogenase from *C. acetobutylicum* (Ca-*adh2*). Using glucose as the carbon source, 150 mg/L propanol were produced in an anaerobic shake flask experiment when 0.2 µM cyanocobalamin, the cofactor of Sbm, was added to the medium (Srirangan et al., 2013). Furthermore, it was shown that overexpression of arginine kinase (YgfD) was indispensable for propanol production, and that other endogenous alcohol dehydrogenases (YqhD, oxygen tolerant $AdhE_{Mut}$, and AdhP), could partially replace Ca-Adh2 (Srirangan et al., 2013).

In a companion study (Srirangan et al., 2014), overexpression of the *sbm, ygfD*, and *ygfG* genes was achieved by replacing the natural SBMO promoter by the strong *trc* promoter (Brosius et al., 1985). Reduction of propionyl-CoA to propanol relied on spontaneously expressed endogenous activities. The production strain was further optimized by deletion of *ldhA* and produced 7 g/L isopropanol along with 31 g/L ethanol in an anoxic fed-batch process using glycerol as the carbon

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source (molar yield was 0.1 mol propanol per mol glycerol, productivity: 0.04 g/(Lh)) (Table 1) (Srirangan et al., 2014).

4.5 The synthetic 1,2-propanediol pathway for n-propanol production

Another synthetic pathway for the production of 1-propanol relies on the dehydration of 1,2-propanediol (1,2-PDO) which is followed by the reduction of the resulting 1-propanal (Jain and Yan, 2011). To facilitate 1,2-PDO production, the glycolytic intermediate dihydroxyacetonephosphate (DHAP) is first converted into methylglyoxal (MG) by methylglyoxal synthase. MG can be transformed into 1,2-PDO via the intermediates hydroxyacetone or lactaldehyde (Figure 8).

Jain and Yan (2011) characterized several candidate enzymes for the methylglyoxal synthase, secondary alcohol dehydrogenase, and 1,2-PDO dehydratase reaction steps, and found that methylglyoxal synthase from *Bacillus subtilis* (Bs-MgsA), secondary alcohol dehydrogenase from *Klebsiella pneumoniae* (Kp-BudC), and 1,2-PDO dehydrogenase from *Klebsiella oxytoca* (Ko-PpdABC), respectively, were the best suited enzymes to catalyze these reactions. A wild-type strain expressing these three synthetic pathway enzymes produced 0.25 g/L 1-propanol and 0.46 g/L 1,2-PDO in 48 h during anaerobic shake flask cultivations from 20 g/L glucose (Table 1). This result indicated that the other enzymatic activities of the pathway were provided by spontaneous expression of endogenous activities (Jain and Yan, 2011).

The propanol-producing strain was further optimized for 1,2-PDO production by inactivating the ethanol and lactate-producing fermentative pathways (*ΔadhE, Δldh*), by increasing entry of carbon flux into the 1,2-PDO pathway (*Δtpi, Δzwf, ΔgloA*), and by overexpressing methylglyoxal synthase (Bs-MgsA), glycerol dehydrogenase (Ec-GldA), and lactaldehyde reductase (Ec-FucO). Strain optimization was completed by overexpressing formate dehydrogenase, Fdh1 from *Candida boidinii,* to increase NADH-cofactor supply through oxidation of formate that was added to the culture medium (Jain et

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al., 2014). The engineered strain produced 5.1 g/L 1,2-PDO at a yield of 1.1 mol/mol from glucose and formate in an anaerobic shake flask (productivity was 0.03 g/(Lh)). It was found that the 1,2-PDO dehydratase significantly lost activity when coexpressed with the 1,2-PDO pathway. Therefore, a two-strain strategy was devised where the 1,2-PDO-producing strain was simultaneously or sequentially incubated with the n-propanol-producing strain that expressed the 1,2-PDO dehydratase from *K. oxytoca* as a fusion protein (Ko-Ppd-A-B-C). When the PDO dehydratase-expressing strain was added after 1,2-PDO production by the first strain had been ceased, ~3 g/L n-propanol were produced after 220 h incubation in an anaerobic shake flask (Jain et al., 2014). It remains somewhat obscure why the PDO dehydratase enzyme could not be stabilized in the 1,2-PDO-producing strain. However, if the enzyme stability problem could be solved, and if efficient 1,2-PDO production could be achieved by tightly controlling the accumulation of the toxic pathway intermediate methylglyoxal, this metabolic route may represent an interesting alternative or complement for the above described synthetic n-propanol pathways.

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5 Maximum propanol yield and stoichiometric evaluation of pathways

The maximum *thermodynamically* feasible yield of a microbial product synthesis can be calculated from the ratio of the degrees of reduction (γ) of the product and the carbon source (Cueto-Rojas et al., 2015). Thus, when glucose (γ = 24) is chosen as the carbon source, the maximum propanol (γ = 18) yield is 1.33 mol/mol. To evaluate whether the stoichiometries of the different propanol pathways allow to achieve this thermodynamic optimum, we have extended a previously published stoichiometric model of the central metabolism of *E. coli* (Stelling et al., 2002) by the net reactions representing these pathways and calculated the maximum *stoichiometrically* feasible yield (Table 2) using the CellNetAnalyzer software package (Klamt et al., 2007). We found that the Wood-Werkman, acrylate, succinate, and 1,2-propanediol pathways have the stoichiometric potential to achieve the maximum yield of 1.33 mol/mol. In contrast, the acetone, threonine, and citramalate pathways have a theoretical yield which is up to 25 % smaller (Table 2).

It is clear that the optimization and use of pathways with favorable stoichiometry is preferable over the application of pathways with limited stoichiometric potential. However, our stoichiometric analysis does not provide any information on whether the corresponding optimal carbon flux distributions can actually be achieved, or whether physiological constraints (e.g. a toxic intermediate) reduce the practical performance of a pathway despite an optimal stoichiometry. Therefore, the theoretical yields listed in Table 2 provide only a first and very rough estimate on the potential of the natural metabolic network of *E. coli* to support propanol production via the different pathways.

In this context, the work of Shen and colleagues is of interest since it showed that the co-functioning of the citramalate and threonine propanol pathways had a synergistic effect on the theoretical (1.33 mol/mol combined) and experimentally achieved propanol yield (Shen and Liao,

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2013). Furthermore, the development of carbon-conserving phosphoketolase-dependent pathways for the biosynthesis of acetyl-CoA (Bogorad et al., 2013; Henard et al., 2015) appears to be a major lever to improve the practical and theoretical yield of propanol synthesis via the acetone pathway (Figure 2). This pathway outperforms all other metabolic routes when regarding the experimentally achieved propanol productivity (Table 1), but its maximum theoretical yield is limited to 1 mol/mol when implemented in the natural *E. coli* metabolic network. However, the theoretical propanol yield of this pathway increases to 1.33 mol/mol if the concomitant expression of a phosphoketolase is assumed (Table 2), which shows that the optimization of the 'surrounding' metabolic network may have a major impact on pathway performance.

Taking together these considerations it is not straightforward to rank the different propanol pathways according to their biotechnological interest. All of them have a similar stoichiometric potential to produce propanol from glucose, and it will require experimental testing to see which of them is best compatible with the host strain's physiology.

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6 Feedstocks for propanol production

In most of the above described studies glucose was used as the carbon source. However, for sustainable production of propanol it is preferable to use feedstocks that do not compete with human nutrition. One promising alternative to glucose is glycerol, which has become an abundant raw material due to the rise of the biodiesel industry (Bournay et al., 2005; Yang et al., 2012). It should be noted that all of the described propanol pathways are compatible with the use of this carbon source. It can even be expected that the use of glycerol for propanol production is more advantageous than glucose, because the additional NADH released during its assimilation can be used for the reduction of propanol precursors. In line with this notion, it was shown that propanol production via the acetone and succinate-dependent pathways had a higher carbon yield when glycerol was used as the substrate (Choi et al., 2012; Srirangan et al., 2014).

Furthermore, several production organisms have been engineered to enable the use of lignocellulosic biomass, protein waste, syngas or carbon dioxide as starting substrate for the biosynthesis of propanol. Cellulose is a major component of lignocellulosic biomass and cannot be directly utilized by frequently used propanol producers such as *E. coli*. Cellulose can be saccharified by the synergistic action of the cellulolytic enzymes beta-glucosidase (BGL), cellobiohydrolase, and endoglucanase (Soma et al., 2012). BGL catalyzes the last saccharification step by cleaving cellobiose into glucose. It could be shown that expression of BGL from *T. fusca* on the cell surface of *E. coli* cells that had been engineered with the acetone-dependent isopropanol pathway enabled propanol production from cellobiose (Soma et al., 2012). Product titers of 4.2 g/L were reached in shake flask cultures with a yield of 0.25 mol isopropanol per mol glucose equivalent (Table 1). This result can be considered a first step towards direct use of cellulose as a substrate for propanol production with *E. coli*, as it can be expected that the two other cellulolytic activities can be likewise expressed on the cell surface of this organism. In a different approach, the cellulolytic bacterium *T. fusca* was employed for n-propanol production from untreated lignocellulosic biomass (Deng and Fong, 2011).

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It was found that this bacterium naturally produced propionic acid from lignocellulosic material. Two potential pathways that start from threonine or succinate (compare to Figures 5B and 7) were proposed to be responsible for propionic acid production via the common intermediate propionyl-CoA (Deng and Fong, 2011). Even though the propionic acid/propionyl-CoA pathway in *T. fusca* could not unambiguously be identified, it was shown that heterologous expression of the bifunctional butyraldehyde/alcohol dehydrogenase, Adh2 from *C. acetobutylicum* enabled production of n-propanol from propionyl-CoA. The engineered *T. fusca* strain produced 0.48 g/L from 20 g/L of untreated switchgrass. Moreover, this bacterium was cultivated at 46 °C under aerobic conditions and tolerated propanol concentrations of up to 50 g/L (Deng and Fong, 2011). These phenotypic characteristics are highly advantageous when considering that efficient propanol production commonly requires continuous product removal by gas stripping.

A carbon source that becomes increasingly popular for the synthesis of value-added chemicals is syngas (Munasinghe and Khanal, 2010; Wilkins and Atiyeh, 2011). Syngas can be produced from natural gas by steam reforming, or from lignocellulosic material and coal by gasification. It was suggested that gasification of agricultural or municipal waste materials and direct fermentation of the resulting syngas has several advantages over processes that rely on hydrolysis of these materials and fermentation of the released sugars. In particular, it was proposed that gasification bypasses expensive biomass pretreatment and hydrolysate detoxification steps, it converts the entire biomass into a homogeneous substrate (including e.g. lignin), it provides a substrate which is free of inhibitors, and it is an inherently sterile process due to the high working temperatures (Munasinghe and Khanal, 2010). Thus, syngas can be considered an abundant low-cost feedstock which has great potential to render microbial production processes more cost efficient and sustainable. It was shown that mixed cultures of *Alkalibaculum bacchi* and *Clostridium propionicum* transiently accumulated up to 6 g/L n-propanol in continuous syngas fermentations, when a pure culture of *A. bacchi* mainly produced ethanol and no propanol (Liu et al., 2014). Although the exact physiology of propanol formation in the microbial consortium was not entirely revealed, it was proposed that propanol was

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produced by *C. propionicum* (through the acrylate pathway, Figure 3) from the ethanol which had been produced by *A. bacchi* directly from syngas (Liu et al., 2014). It remains to be seen whether the development of genetic tools for the manipulation of syngas-fermenting microorganisms, and further optimization of process conditions (mainly to overcome mass transfer limitations for the transport of the gaseous substrate into the cultivation liquid (Munasinghe and Khanal, 2010)) will allow cost-efficient propanol production from this feedstock.

In another interesting approach, proteins were used as a feedstock to produce higher alcohols (mainly butanol, and methylbutanol)(Choi et al., 2014; Huo et al., 2011). Alcohol production proceeded via the deamination of amino acids, which was followed by optional chain elongation, decarboxylation, and reduction of the resulting oxo-acids. These studies did not target propanol as a product, but it is conceivable that 1-propanol could be obtained from threonine by the pathways described above (Figure 5). Such a process would allow production of a mixture of higher alcohols from otherwise unused protein waste, or algal biomass that could be produced in large quantities from carbon dioxide and light. In addition, the ammonium released during the process could be recycled as a fertilizer (Choi et al., 2014; Huo et al., 2011). These characteristics make this process a highly interesting and sustainable alternative to sugar-based biofuel production. However, since the reaction product is a complex alcohol mixture, this process is most likely less suitable for the production of pure alcohols. It appears that the use of protein feedstocks is restricted to the production of biofuels which do not require further product separation.

Increasing research efforts are dedicated to the use of photosynthetic organisms for the production of value-added chemicals. In line with this trend, the cyanobacterium *Synechococcus elongatus* was recently engineered with the isopropanol pathway to produce the alcohol from carbon dioxide (Kusakabe et al., 2013). When the acetoacetyl-CoA thiolase (*thl*), the acetoacetate decarboxylase (*adc*) from *C. acetobutylicum*, the acetone-accepting alcohol dehydrogenase from *C. beijerinckii* (adh_{B593}), and the acetoacetyl-CoA:acetate CoA-transferase from *E. coli* (*atoAD*) were expressed in a wild-type strain, ~22 mg/L isopropanol could be accumulated after 9 days of

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cultivation in the dark under anaerobic, nitrogen-limiting conditions (Table 1)(Kusakabe et al., 2013). In a subsequent study, this propanol titer was improved to 146 mg/L by further optimizing the cultivation conditions (Table 1)(Hirokawa et al., 2015). Lack of suitable genetic engineering tools, the poorly understood physiology of cyanobacteria, and lacking experience with efficient carbon dioxide delivery strategies were discussed by the authors as the major obstacles for the optimization biochemical production processed that employ these photosynthetic organisms. These results indicate that increasing propanol production by photosynthetic organisms to industrially relevant levels still requires major research efforts.

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7 Conclusions

Both, n-propanol and isopropanol are industrially attractive value-added molecules that can be produced by microbes. The growing interest in the development of biochemical propanol production processes is mainly motivated by the potential chemical conversion of these alcohols into propylene, which is one of the most important petrol-derived molecules used in the chemical industry. However, given the rather low price of propylene (<1 $/kg), the development of competitive biochemical propanol syntheses is extremely challenging. It appears that biochemical propanol production processes have to be improved at several levels before becoming competitive with chemical synthesis.

The best reported propanol producer is an *E. coli* strain, which was engineered with the acetone-dependent isopropanol pathway from *Clostridia*. This organism accumulated 40 g/L isopropanol in a fed-batch process (Inokuma et al., 2010). The alcohol became toxic to the cells when present at higher concentrations and had to be removed by gas stripping to extend propanol production. The process costs for continuous gas-liquid extractions are determined by various physical parameters, and the product concentrations and cultivation temperatures that are tolerated by the microorganism (Oudshoorn et al., 2009; Xue et al., 2014a, 2014b). It was recently shown that higher alcohol concentrations and moderately increased temperatures of the feed solution strongly improved the efficiency of butanol recovery by gas stripping (Xue et al., 2014a). Thus, it can be expected that the breeding (or identification) of microorganisms with both higher propanol and thermo-tolerance will be highly beneficial for propanol production. The phenotypic characteristics of *T. fusca,* which can be cultivated at ~50 °C and which tolerates propanol concentrations of up to 50 g/L (Deng and Fong, 2011), show that it is indeed possible to identify such propanol-producing organisms.

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Currently, glucose-based propanol syntheses achieve the highest product titers and productivities (Table 1). The maximum thermodynamically feasible propanol yield on glucose is 1.33 mol/mol (see above). Thus, at current market prices for glucose (~0.35 $/kg, 0.063 $/mol) and propanol (1-1.5 $/kg, 0.06-0.09 $/mol), the price of the product would hardly cover the price of the raw material even if the theoretical yield could actually be achieved. However, the use of alternative feedstocks such as glycerol, or sugars that are derived from lignocellulosic material with minimized pretreatment may greatly reduce the price of the raw material. Indeed, promising results with significant propanol titers were obtained when glycerol, cellobiose, or untreated lignocellulosic material were used as the substrate (Deng and Fong, 2011; Soma et al., 2012). Thus, efficient lignocellulose or glycerol-based propanol production processes appear to be at reach. In contrast, considering the extremely low product titers and productivities which were obtained when a photosynthetic cyanobacterium was used as propanol producer (Hirokawa et al., 2015; Kusakabe et al., 2013), the direct use of carbon dioxide and light for propanol production still lies a long way ahead.

The engineering of strains with improved propanol yields, the breeding or identification of microorganisms with higher propanol tolerance, and the engineering of propanol producer strains that efficiently utilize low-cost feedstocks are the major challenges on the way to industrially relevant propanol production processes.

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9 References

Allen, S.H., Kellermeyer, R.W., Stjernholm, R.L., Wood, H.G., 1964. Purification and properties of enzymes involved in the propionic acid fermentation. J. Bacteriol. 87, 171–187.

Atsumi, S., Hanai, T., Liao, J.C., 2008. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. Nature 451, 86–89. doi:10.1038/nature06450

Atsumi, S., Liao, J.C., 2008. Directed evolution of *Methanococcus jannaschii* citramalate synthase for biosynthesis of 1-propanol and 1-butanol by *Escherichia coli*. Appl. Environ. Microbiol. 74, 7802–7808. doi:10.1128/AEM.02046-08

Bermejo, L.L., Welker, N.E., Papoutsakis, E.T., 1998. Expression of *Clostridium acetobutylicum* ATCC 824 genes in *Escherichia coli* for acetone production and acetate detoxification. Appl. Environ. Microbiol. 64, 1079–1085.

Biofuels - Types of Biofuels - Bioalcohols [WWW Document], 2010. URL http://biofuel.org.uk/bioalcohols.html (accessed 12.15.15).

Bogorad, I.W., Lin, T.-S., Liao, J.C., 2013. Synthetic non-oxidative glycolysis enables complete carbon conservation. Nature 502, 693–697. doi:10.1038/nature12575

Bournay, L., Casanave, D., Delfort, B., Hillion, G., Chodorge, J.A., 2005. New heterogeneous process for biodiesel production: A way to improve the quality and the value of the crude glycerin produced by biodiesel plants. Catal T 106, 190–192.

Brethauer, S., Wyman, C.E., 2010. Review: Continuous hydrolysis and fermentation for cellulosic ethanol production. Bioresour. Technol. 101, 4862–4874. doi:10.1016/j.biortech.2009.11.009

Brosius, J., Erfle, M., Storella, J., 1985. Spacing of the -10 and -35 regions in the tac promoter. Effect on its in vivo activity. J. Biol. Chem. 260, 3539–3541.

Cardon, B.P., Barker, H.A., 1946. Two New Amino-Acid-Fermenting Bacteria, *Clostridium propionicum* and *Diplococcus glycinophilus*. J. Bacteriol. 52, 629–634.

Chang, Y.Y., Cronan, J.E., 2000. Conversion of *Escherichia coli* pyruvate oxidase to an "alpha-ketobutyrate oxidase." Biochem. J. 352 Pt 3, 717–724.

Chen, J.S., Hiu, S.F., 1986. Acetone-butanol-isopropanol production by *Clostridium beijerinckii* (synonym *Clostridium butylicum*). Biotechnol Lett 8, 371–376.

Choi, K.-Y., Wernick, D.G., Tat, C.A., Liao, J.C., 2014. Consolidated conversion of protein waste into biofuels and ammonia using *Bacillus subtilis*. Metab. Eng. 23, 53–61. doi:10.1016/j.ymben.2014.02.007

Choi, Y.J., Park, J.H., Kim, T.Y., Lee, S.Y., 2012. Metabolic engineering of *Escherichia coli* for the production of 1-propanol. Metab. Eng. 14, 477–486. doi:10.1016/j.ymben.2012.07.006

Collas, F., Kuit, W., Clément, B., Marchal, R., López-Contreras, A.M., Monot, F., 2012. Simultaneous production of isopropanol, butanol, ethanol and 2,3-butanediol by *Clostridium acetobutylicum* ATCC 824 engineered strains. AMB Express 2, 45. doi:10.1186/2191-0855-2-45

Cueto-Rojas, H.F., van Maris, A.J.A., Wahl, S.A., Heijnen, J.J., 2015. Thermodynamics-based design of microbial cell factories for anaerobic product formation. Trends Biotechnol. 33, 534–546. doi:10.1016/j.tibtech.2015.06.010

Deng, Y., Fong, S.S., 2011. Metabolic engineering of *Thermobifida fusca* for direct aerobic bioconversion of untreated lignocellulosic biomass to 1-propanol. Metab. Eng. 13, 570–577. doi:10.1016/j.ymben.2011.06.007

Dusséaux, S., Croux, C., Soucaille, P., Meynial-Salles, I., 2013. Metabolic engineering of *Clostridium acetobutylicum* ATCC 824 for the high-yield production of a biofuel composed of an isopropanol/butanol/ethanol mixture. Metab. Eng. 18, 1–8. doi:10.1016/j.ymben.2013.03.003

ACCEPTED MANUSCRIPT

Falentin, H., Deutsch, S.-M., Jan, G., Loux, V., Thierry, A., Parayre, S., Maillard, M.-B., Dherbécourt, J., Cousin, F.J., Jardin, J., Siguier, P., Couloux, A., Barbe, V., Vacherie, B., Wincker, P., Gibrat, J.-F., Gaillardin, C., Lortal, S., 2010. The complete genome of *Propionibacterium freudenreichii* CIRM-BIA1, a hardy actinobacterium with food and probiotic applications. PloS One 5, e11748. doi:10.1371/journal.pone.0011748

Fernando, S., Adhikari, S., Kota, K., Bandi, R., 2007. Glycerol based automotive fuels from future biorefineries. Fuel 86, 2806–2809.

George, H.A., Johnson, J.L., Moore, W.E., Holdeman, L.V., Chen, J.S., 1983. Acetone, Isopropanol, and Butanol Production by *Clostridium beijerinckii* (syn. *Clostridium butylicum*) and *Clostridium aurantibutyricum*. Appl. Environ. Microbiol. 45, 1160–1163.

Hanai, T., Atsumi, S., Liao, J.C., 2007. Engineered synthetic pathway for isopropanol production in *Escherichia coli*. Appl. Environ. Microbiol. 73, 7814–7818. doi:10.1128/AEM.01140-07

Henard, C.A., Freed, E.F., Guarnieri, M.T., 2015. Phosphoketolase pathway engineering for carbon-efficient biocatalysis. Curr. Opin. Biotechnol. 36, 183–188. doi:10.1016/j.copbio.2015.08.018

Himmi, E.H., Bories, A., Boussaid, A., Hassani, L., 2000. Propionic acid fermentation of glycerol and glucose by *Propionibacterium acidipropionici* and *Propionibacterium freudenreichii* ssp. *shermanii*. Appl. Microbiol. Biotechnol. 53, 435–440.

Hirokawa, Y., Suzuki, I., Hanai, T., 2015. Optimization of isopropanol production by engineered cyanobacteria with a synthetic metabolic pathway. J. Biosci. Bioeng. 119, 585–590. doi:10.1016/j.jbiosc.2014.10.005

Hiu, S.F., Zhu, C.X., Yan, R.T., Chen, J.S., 1987. Butanol-Ethanol Dehydrogenase and Butanol-Ethanol-Isopropanol Dehydrogenase: Different Alcohol Dehydrogenases in Two Strains of *Clostridium beijerinckii* (*Clostridium butylicum*). Appl. Environ. Microbiol. 53, 697–703.

Huo, Y.-X., Cho, K.M., Rivera, J.G.L., Monte, E., Shen, C.R., Yan, Y., Liao, J.C., 2011. Conversion of proteins into biofuels by engineering nitrogen flux. Nat. Biotechnol. 29, 346–351. doi:10.1038/nbt.1789

Hüsemann, M.H., Papoutsakis, E.T., 1988. Solventogenesis in *Clostridium acetobutylicum* fermentations related to carboxylic acid and proton concentrations. Biotechnol. Bioeng. 32, 843–852. doi:10.1002/bit.260320702

Inokuma, K., Liao, J.C., Okamoto, M., Hanai, T., 2010. Improvement of isopropanol production by metabolically engineered *Escherichia coli* using gas stripping. J. Biosci. Bioeng. 110, 696–701. doi:10.1016/j.jbiosc.2010.07.010

Jain, R., Sun, X., Yuan, Q., Yan, Y., 2014. Systematically Engineering *Escherichia coli* for Enhanced Production of 1,2-Propanediol and 1-Propanol. ACS Synth. Biol. doi:10.1021/sb500345t

Jain, R., Yan, Y., 2011. Dehydratase mediated 1-propanol production in metabolically engineered *Escherichia coli*. Microb. Cell Factories 10, 97. doi:10.1186/1475-2859-10-97

Jojima, T., Inui, M., Yukawa, H., 2008. Production of isopropanol by metabolically engineered *Escherichia coli*. Appl. Microbiol. Biotechnol. 77, 1219–1224. doi:10.1007/s00253-007-1246-8

Jones, D.T., Woods, D.R., 1986. Acetone-butanol fermentation revisited. Microbiol. Rev. 50, 484–524.

Kibby, C.L., Hall, W.K., 1972. Studies of acid catalyzed reactions: XII. Alcohol decomposition over hydroxyapatite catalysts. J Catal 29, 144–159.

Klamt, S., Saez-Rodriguez, J., Gilles, E.D., 2007. Structural and functional analysis of cellular networks with CellNetAnalyzer. BMC Syst. Biol. 1, 2. doi:10.1186/1752-0509-1-2

Kusakabe, T., Tatsuke, T., Tsuruno, K., Hirokawa, Y., Atsumi, S., Liao, J.C., Hanai, T., 2013. Engineering a synthetic pathway in cyanobacteria for isopropanol production directly from carbon dioxide and light. Metab. Eng. 20, 101–108. doi:10.1016/j.ymben.2013.09.007

Lee, J., Jang, Y.-S., Choi, S.J., Im, J.A., Song, H., Cho, J.H., Seung, D.Y., Papoutsakis, E.T., Bennett, G.N., Lee, S.Y., 2012. Metabolic engineering of *Clostridium acetobutylicum* ATCC 824 for isopropanol-butanol-ethanol fermentation. Appl. Environ. Microbiol. 78, 1416–1423. doi:10.1128/AEM.06382-11

ACCEPTED MANUSCRIPT

Lee, K.H., Park, J.H., Kim, T.Y., Kim, H.U., Lee, S.Y., 2007. Systems metabolic engineering of Escherichia coli for L-threonine production. Mol. Syst. Biol. 3, 149. doi:10.1038/msb4100196

Lee, S.K., Chou, H., Ham, T.S., Lee, T.S., Keasling, J.D., 2008. Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels. Curr. Opin. Biotechnol. 19, 556–563. doi:10.1016/j.copbio.2008.10.014

Liu, K., Atiyeh, H.K., Stevenson, B.S., Tanner, R.S., Wilkins, M.R., Huhnke, R.L., 2014. Continuous syngas fermentation for the production of ethanol, n-propanol and n-butanol. Bioresour. Technol. 151, 69–77. doi:10.1016/j.biortech.2013.10.059

May, A., Fischer, R.-J., Maria Thum, S., Schaffer, S., Verseck, S., Dürre, P., Bahl, H., 2013. A modified pathway for the production of acetone in *Escherichia coli*. Metab. Eng. 15, 218–225. doi:10.1016/j.ymben.2012.08.001

Munasinghe, P.C., Khanal, S.K., 2010. Biomass-derived syngas fermentation into biofuels: Opportunities and challenges. Bioresour. Technol. 101, 5013–5022. doi:10.1016/j.biortech.2009.12.098

Oudshoorn, A., van der Wielen, L.A.M., Straathof, A.J., 2009. Assessment of Options for Selective 1-Butanol Recovery from Aqueous Solution. Ind Eng Chem Res 48, 7325–7336.

Papa, A.J., 2011. Propanols, in: Ullmann's Encyclopedia of Industrial Chemistry.

Propylene – Study: Market, Analysis, Trends [WWW Document], 2016. URL http://www.ceresana.com/en/market-studies/chemicals/propylene/ (accessed 12.15.15).

Seeliger, S., Janssen, P.H., Schink, B., 2002. Energetics and kinetics of lactate fermentation to acetate and propionate via methylmalonyl-CoA or acrylyl-CoA. FEMS Microbiol. Lett. 211, 65–70.

Shen, C.R., Liao, J.C., 2013. Synergy as design principle for metabolic engineering of 1-propanol production in *Escherichia coli*. Metab. Eng. 17, 12–22. doi:10.1016/j.ymben.2013.01.008

Shen, C.R., Liao, J.C., 2008. Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. Metab. Eng. 10, 312–320. doi:10.1016/j.ymben.2008.08.001

Soma, Y., Inokuma, K., Tanaka, T., Ogino, C., Kondo, A., Okamoto, M., Hanai, T., 2012. Direct isopropanol production from cellobiose by engineered *Escherichia coli* using a synthetic pathway and a cell surface display system. J. Biosci. Bioeng. 114, 80–85. doi:10.1016/j.jbiosc.2012.02.019

Soma, Y., Tsuruno, K., Wada, M., Yokota, A., Hanai, T., 2014. Metabolic flux redirection from a central metabolic pathway toward a synthetic pathway using a metabolic toggle switch. Metab. Eng. 23, 175–184. doi:10.1016/j.ymben.2014.02.008

Srirangan, K., Akawi, L., Liu, X., Westbrook, A., Blondeel, E.J., Aucoin, M.G., Moo-Young, M., Chou, C.P., 2013. Manipulating the sleeping beauty mutase operon for the production of 1-propanol in engineered *Escherichia coli*. Biotechnol. Biofuels 6, 139. doi:10.1186/1754-6834-6-139

Srirangan, K., Liu, X., Westbrook, A., Akawi, L., Pyne, M.E., Moo-Young, M., Chou, C.P., 2014. Biochemical, genetic, and metabolic engineering strategies to enhance coproduction of 1-propanol and ethanol in engineered *Escherichia coli*. Appl. Microbiol. Biotechnol. 98, 9499–9515. doi:10.1007/s00253-014-6093-9

Stelling, J., Klamt, S., Bettenbrock, K., Schuster, S., Gilles, E.D., 2002. Metabolic network structure determines key aspects of functionality and regulation. Nature 420, 190–193. doi:10.1038/nature01166

Tamakawa, H., Mita, T., Yokoyama, A., Ikushima, S., Yoshida, S., 2013. Metabolic engineering of *Candida utilis* for isopropanol production. Appl. Microbiol. Biotechnol. 97, 6231–6239. doi:10.1007/s00253-013-4964-0

Tholozan, J.L., Touzel, J.P., Samain, E., Grivet, J.P., Prensier, G., Albagnac, G., 1992. *Clostridium neopropionicum* sp. nov., a strict anaerobic bacterium fermenting ethanol to propionate through acrylate pathway. Arch. Microbiol. 157, 249–257.

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Wilkins, M.R., Atiyeh, H.K., 2011. Microbial production of ethanol from carbon monoxide. Curr. Opin. Biotechnol. 22, 326–330. doi:10.1016/j.copbio.2011.03.005

Wood, H.G., 1981. Metabolic cycles in the fermentation by propionic acid bacteria. Curr. Top. Cell. Regul. 18, 255–287.

Xue, C., Du, G.Q., Sun, J.-X., Chen, L.-J., Gao, S.S., Yu, M.L., Yang, S.T., Bai, F.-W., 2014a. Characterization of gas stripping and its integration with acetone–butanol–ethanol fermentation for high-efficient butanol production and recovery. Biochem Eng J 83.

Xue, C., Zhao, J.-B., Chen, L.-J., Bai, F.-W., Yang, S.-T., Sun, J.-X., 2014b. Integrated butanol recovery for an advanced biofuel: current state and prospects. Appl. Microbiol. Biotechnol. 98, 3463–3474. doi:10.1007/s00253-014-5561-6

Xu, H., Zhang, Y., Guo, X., Ren, S., Staempfli, A.A., Chiao, J., Jiang, W., Zhao, G., 2004. Isoleucine biosynthesis in *Leptospira interrogans* serotype lai strain 56601 proceeds via a threonine-independent pathway. J. Bacteriol. 186, 5400–5409. doi:10.1128/JB.186.16.5400-5409.2004

Yang, F., Hanna, M.A., Sun, R., 2012. Value-added uses for crude glycerol--a byproduct of biodiesel production. Biotechnol. Biofuels 5, 13. doi:10.1186/1754-6834-5-13

Zhang, K., Sawaya, M.R., Eisenberg, D.S., Liao, J.C., 2008. Expanding metabolism for biosynthesis of nonnatural alcohols. Proc. Natl. Acad. Sci. U. S. A. 105, 20653–20658. doi:10.1073/pnas.0807157106

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10 Figure and table legends

Figure 1: Natural isopropanol and butanol pathways in *Clostridia* species. (I) acetyl-CoA: acetyl-CoA C-acetyltransferase (acetoacetyl-CoA thiolase, or acetoacetyl-CoA synthase), (II) acetoacetyl-CoA:acetate CoA-transferase (coenzyme A transferase, acetoacetyl-CoA transferase), (III) acetoacetate decarboxylase, (IV) isopropanol dehydrogenase, (V) 3-hydroxybutyryl-CoA dehydrogenase, (VI) (S)-3-hydroxybutanoyl-CoA hydro-lyase, (VII) crotonyl-CoA reductase, (VIII) butyryl-CoA reductase, (IX) butyraldehyde reductase (butanol dehydrogenase).

Figure 2: Natural propionate and n-propanol pathway in propionic acid bacteria (Wood-Werkman cycle). (I) methylmalonyl-CoA carboxytransferase, (II) malate dhydrogenase, (III) fumarase, (IV) fumarate reductase, (V) propionyl-CoA:succinate CoA-transferase , (VI) methylmalonyl-CoA mutase, (VII) putative acylating propionaldehyde dehydrogenase, (VIII) putative propanol dehydrogenase.

Figure 3: Natural acrylate pathway for biosynthesis of propionic acid and n-propanol. (I) lactate dehydrogenase, (II) propionyl-CoA:lactate CoA-transferase, (III) lactyl-CoA dehydratase, (IV) acryloyl-CoA reductase, (V) propylaldehyde dehydrogenase, (VI) propanol dehydrogenase, (VII) phosphate acetyltransferase, (VIII) propionate kinase, (IX) unknown lactyl-CoA-forming CoA transferase.

Figure 4: Isopropanol pathway from *Clostridia* sp. heterologously expressed in engineered bacteria or yeast. (A) Natural pathway. (B) Modified pathway. (I) acetyl-CoA:acetyl-CoA C-acetyltransferase, (IIa) acetoacetyl-CoA:acetate CoA-transferase, (IIb) acetoacetyl-CoA thiolase (thioesterase), (III) acetoacetate decarboxylase, (IV) alcohol dehydrogenase.

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Figure 5: Synthetic pathway for isopropanol production from threonine. (A) Pathway implemented by (Zhang et al., 2008) (B) Pathway implemented by (Choi et al., 2012) (I) aspartate transaminase, (II) homoserine pathway, (III) threonine pathway, (IV) threonine deaminase, (V) 2-ketobutyrate decarboxylase, (VI) 1-propanol dehydrogenase, (VII) "spontaneous" decarboxylation of 2-ketobutyrate as proposed by (Choi et al., 2012), (VIII) propionyl-phosphate reductase, (IX) propionyl-CoA synthetase, (X) acylating propionaldehyde dehydrogenase, (XI) putative decarboxylation reaction for 2-ketobuturate (catalyzed by endogenous PoxB, ubi_{ox} – ubiquinone, ubi_{red} - ubiquinol).

Figure 6: Synthetic pathway for production of isopropanol from citramalate. (I) citramalate synthase, (II/III) isopropylmalate isomerase, (IV) β-isopropylmalate dehydrogenase, (V) 2-ketobutyrate decarboxylase, (VI) 1-propanol dehydrogenase.

Figure 7: Synthetic pathway for production of isopropanol from succinate. (I) succinyl-CoA synthetase, (II) methylmalonyl-CoA mutase, (III) methyl-malonyl decarboxylase, (IV) propionyl-CoA reductase, (V) 1-propanol dehydrogenase.

Figure 8: Synthetic pathway or isopropanol production from 1,2-propanediol. (I) fructose-1,6-bisphosphate aldolase, (II) triosephosphate isomerase, (III) methylglyoxal synthase, (IV) methylglyoxal reductase, (V, VI) secondary alcohol dehydrogenase, (VII) primary alcohol dehydrogenase, (VIII) 1,2-propanediol dehydratase, (IX) isopropanol dehydrogenase.

Table 1: Overview of the yields, productivities, and product titers for different propanol-producing organisms and processes

Table 2: Maximum theoretical propanol yields of different pathways on glucose in the metabolic network of *E. coli*.

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11 Tables

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Table 1: Overview of the yields, productivities, and product titers for different propanol-producing organisms and processes

Microorganism	Culture conditions	Final conc. [g/L]	Yield [mol/mol]	Productivity [g/(L h)]	Ref.
Natural propanol-producing pathways and microorganisms					
Acetone pathway to isopropanol					
C. beijerinckii	Screening of wild-type strains in anaerobic bottles on complex medium containing 5 g/l glucose	5 total alc. 0.48 isopropanol 4.5 butanol	nr	nr	(George et al., 1983)
C. beijerinckii	Wild-type strain Anaerobic batch cultivation in bioreactor on mineral medium with 80 g/L glucose, complemented with yeast extract and asparagine, no active pH control	13.0 total alc. 4.5 isopropanol 8.4 butanol 0.1 ethanol	 0.36 isopropanol 0.55 butanol 0.01 ethanol	0.37 for total alcohol	(Collas et al., 2012)
Wood-Werkman (dicarboxylate, methylmalonyl-CoA) pathway to n-propanol					
P. acidipropionici	Wild-type strain Anaerobic batch cultivation, shake flask, rich medium with 5 g/L tryptone and 10 g/L yeast extract, 20 g/L glucose (glu) or 20 g/L glycerol (gly) as carbon source	1.75 (on gly) 0.6 (on glu)	0.13 (on gly) 0.04 (on glu)	nr	(Himmi et al., 2000)
P. freudenreichii	Wild-type strain Anaerobic batch cultivation, shake flask, rich medium with 5 g/L tryptone and 10 g/L yeast extract, 20 g/L glucose (glu) or 20 g/L glycerol (gly) as carbon source	1.2 (on gly) 0.9 (on glu)	0.09 (on gly) 0.06 (on glu)	nr	(Himmi et al., 2000)
P. freudenreichii	Wild-type strain Anaerobic batch cultivation, shake flask, mineral medium, sparging with CO2/N/H, 20 mM ethanol as carbon source	0.19	0.14	nr	(Seeliger et al., 2002)
Acrylate pathway to n-propanol					
C. neopropionicum	Wild-type strain Anaerobic batch cultivation, 30 mM ethanol, major fermentation products were propionate and acetate	0.06	0.03	nr	(Tholozan et al., 1992)
A bacchii + *C. neopropionicum*	Mixed culture of wild-type strains Anaerobic cultivation in continuous bioreactor, syngas as carbon source, 20 g/L corn-steep liquor, transient accumulation of n-propanol, propanol production depends on presence of *C. neopropionicum*	6	nr	nr	

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Engineered propanol-producing pathways and microorganisms

Acetone pathway (for isopropanol)

C. acetobutylicum	Solvent-producing strain overexpressing primary/secondary alcohol dehydrogenase (*adh*B-593) from *C. beijerinckii* NRRL B593, and endogenous *ctfAB*, and *adc*. Deletion of butyrate kinase (*buk*). Anaerobic batch cultivation in bioreactor on mineral medium with 80 g/L glucose, complemented with yeast extract and asparagine	20.4 total alc. 4.4 isopropanol 14.1 butanol 1.9 ethanol	0.2 isopropanol 0.5 butanol 0.1 ethanol	0.7 for total alcohol	(Lee et al., 2012)
C. acetobutylicum	Solvent-producing strain overexpressing primary/secondary alcohol dehydrogenase (*adh*B-593) from *C. beijerinckii* NRRL B593, and endogenous *ctfAB*, and *adc*. Deletion of butyrate kinase (*buk*). Anaerobic fed-batch cultivation on mineral medium complemented with yeast extract and asparagine Gas stripping	35.6 total alc. ~6 isopropanol 25.1 butanol ~3 ethanol	0.2 isopropanol 0.5 butanol 0.1 ethanol	0.8 for total alcohol	(Lee et al., 2012)
C. acetobutylicum	Solvent-producing strain overexpressing primary/secondary alcohol dehydrogenase (*adh*B-593) from *C. beijerinckii* NRRL B593, and endogenous *ctfAB*, and *adc*. Deletion of butyrate kinase (*buk*). Anaerobic batch cultivation in bioreactor on mineral medium with 90 g/L glucose, complemented with yeast extract and asparagine	24.4 8.8 isopropanol 13.3 butanol 1.5 ethanol	0.34 isopropanol 0.47 butanol 0.08 ethanol	0.8 for total alcohol	(Collas et al., 2012)
C. acetobutylicum	Solvent-producing strain overexpressing primary/secondary alcohol dehydrogenase (*adh*B-593) from *C. beijerinckii* NRRL B593, and endogenous *ctfAB*, and *adc*. Deletion of butyrate kinase (*buk*). Anaerobic batch cultivation in bioreactor on mineral medium with 80 g/L glucose, complemented with yeast extract and asparagine	21 4.7 isopropanol 14.6 butanol 1 ethanol	0.2 isopropanol 0.5 butanol 0.1 ethanol	0.8 for total alcohol	(Dusséaux et al., 2013)
E. coli	Overexpression of *thl* and *adc* from *C. acetobutylicum*, endogenous acetoacetyl-CoA transferase (*atoAD)*, and primary/secondary alcohol dehydrogenase *adh*B-593 from *C. beijerinckii* NRRL B593. Shake flask culture, mineral medium with 10 g/L yeast extract, repeated glucose addition. Acetone was major by-product	4.8	0.43	0.41	(Hanai et al., 2007)
E. coli	Overexpression of *Clostridial* genes *thl, cftAB, acd, adhB-593* Shake flask culture, mineral medium with 10 g/L yeast extract, repeated glucose addition	13.6	0.51	0.6	(Jojima et al., 2008)
E. coli	Overexpression of *thl* and *adc* from *C. acetobutylicum*, endogenous acetoacetyl-CoA transferase (*atoAD)*, and primary/secondary alcohol dehydrogenase *adh*B-593 from *C. beijerinckii* NRRL B593. Controlled fed-batch culture in bioreactor, mineral medium with 10 g/L yeast extract, repeated glucose addition	40	0.73	0.66	(Inokuma et al., 2010)
E. coli	Overexpression of *thl* and *adc* from *C. acetobutylicum*, endogenous acetoacetyl-CoA transferase (*atoAD)*, and primary/secondary alcohol dehydrogenase *adh*B-593 from *C. beijerinckii* NRRL B593. Controlled fed-batch culture in bioreactor, mineral medium with 10 g/L yeast extract, repeated glucose addition Gas stripping to avoid growth inhibition by isopropanol	143*	0.67	0.6	(Inokuma et al., 2010)
E. coli	Overexpression of *thl* and *adc* from *C. acetobutylicum*, endogenous acetoacetyl-CoA transferase (*atoAD)*, and primary/secondary alcohol dehydrogenase *adh*B-593 from *C. beijerinckii* NRRL B593. Cell	4.2	0.25 (glucose equivalents)	0.17	(Soma et al., 2012)

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	surface display of beta-glucosidase Tfu0937 from *T. fusca*. Shake flask culture, rich medium, **cellobiose** as carbon source				
E. coli	**Genetic toggle switch** to control acetyl-CoA repartitioning between TCA cycle and isopropanol pathway by shutting down transcription of *gltA.* Overexpression of *thl* and *adc* from *C. acetobutylicum*, endogenous acetoacetyl-CoA transferase (*atoAD)*, and primary/secondary alcohol dehydrogenase *adh*B-593 from *C. beijerinckii* NRRL B593. Shake flask culture, M9 mineral medium, 20 g/L glucose. Acetate was major by-product	3.0	0.48	0.04	(Soma et al., 2014)
C. utilis	Heterologously expressed *Clostridial* genes *cftAB, adc,* adh_{B593}. Overexpressed endogenous *ACS2*, and *ERG10*. $CaCO_3$-buffered medium with yeast extract, peptone, glucose addition in fed-batch mode (cumulated 200 g/l)	27	0.41	0.13	(Tamakawa et al., 2013)
S. elongatus	Heterologously expressed *thl, adc, adhB593* from *Clostridia*, and *atoAD* from *E. coli* CO_2 as carbon source. Cultivation in dark, anaerobic, nitrogen-limited conditions	0.026	nr	nr	(Kusakabe et al., 2013)
S. elongatus	Heterologously expressed *thl, adc, adhB593* from *Clostridia*, and *atoAD* from *E. coli* CO_2 as carbon source. Aerobic cultivation in light in production phase. Optimized medium.	0.146	nr	nr	(Hirokawa et al., 2015)
Synthetic pathways (for 1-propanol)					
E. coli	Engineered with **threonine pathway**. Overexpression of *Ll-kviD, Sc-ADH2*, and endogenous threonine dehydratase, *tdcB*, and threonine (*thrABC)* and leucine (*leuABCD*) biosynthetic pathways. Deletion of competing reactions encoded by *ilvB, ilvE*, *adhE*, *tdh, metA* M9 mineral medium with 72 g/L glucose and 5 g/L yeast extract. Micro-aerobic shake flask cultures	1 (+ 1 g/L 1-butanol)	0.11	0.01	(Shen and Liao, 2008)
E. coli	Engineered with **citramalate pathway**. Overexpression of *Ll-kviD, Sc-ADH2,* and the endogenous *leuABCD* operon. Deletion of competing pathways encoded by *ilvI* and *ilvB*. M9 mineral medium with 72 g/L glucose and 5 g/L yeast extract. Micro-aerobic shake flask cultures	3.5 (+0.5 g/L 1-butanol)	0.54	0.03	(Atsumi and Liao, 2008)
E. coli	Co-expressed **threonine and citramalate pathways.** Overexpression of *Ll-kviD,* evolved *Mj-cimA2* (Atsumi and Liao, 2008), endogenous *yqhD* and *leuBCD*. Deletion of *ilvI*, *ilvB* in combination with other genes chosen among *avtA, tdcE, poxB, thrB, gltA, frdA* M9 mineral medium with 72 g/L glucose and 5 g/L yeast extract. Micro-aerobic shake flask cultures	8	0.45	0.12	(Shen and Liao, 2013)
E. coli	Co-expressed **threonine (propionyl-CoA version) and citramalate pathways**. Threonine-overproducing parental strain (Lee et al., 2007) with additional deletions *rpoS*, *ilvI, ilvH, ilvB* and *ilvN*. Expresses feedback resistant threonine dehydratase $ilvA_{C1139T\ G1341T\ C1351G\ T1352C}$, overexpression of *ackA, atoDA, cimA,* $adhE_{mut}$ Yeast extract, peptone, fed-batch culture with glucose (Glu) or glycerol(Gly) as carbon sources	10.7_{Glu} 10.4_{Gly}	0.16_{Glu} 0.2_{Gly}	0.14_{Glu} 0.08_{Gly}	(Choi et al., 2012)
E. coli	Engineered with **succinate pathway**. Plasmid-born overexpression of endogenous methylmalonyl-mutase (*sbm*), arginine kinase (*ygfD*), and methylmalonyl-CoA decarboxylase (*ygfG*), succinyl-CoA	0.15	nr	nr	(Srirangan et al., 2013)

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	synthetase (*sucCD*) and bifunctional butyraldehyde/alcohol dehydrogenase from *C. acetobutylicum* (*Ca-adh2*). Anaerobic shake flask, 0.2 µM cyanocobalmine, 5 g/L yeast extract, glucose as carbon source				
E. coli	Engineered with **succinate pathway**. Genomic overexpression of endogenous methylmalonyl-mutase (*sbm*), arginine kinase (*ygfD*), and methylmalonyl-CoA decarboxylase (*ygfG*). Deletion of *ldhA*. Anoxic fed-batch bioreactor cultivation, 0.2 µM cyanocobalmine, 10 g/L yeast extract, glycerol as carbon source	7 + 31 g/L ethanol	0.1	0.04	(Srirangan et al., 2014)
E. coli	Engineered with **1,2-propanediol pathway**. Overexpressed methylglyoxal synthase (Bs-mgsA), secondary alcohol dehydrogenase (Kp-budC), 1,2-propanedil dehydratase (*Ko-ppdABC*), otherwise wild-type. Anaerobic shake flask culture, 20 g/L glucose, 5 g/L yeast extract, 13 g/L NaHCO3, low phosphate medium	0.25	nr	0.005	(Jain and Yan, 2011)
E. coli	Two-strain co-cultivation strategy with one strain being optimized for **1,2-propanediol** production, and the other expressing the 1,2-PDO dehydratase. The 1,2-PDO-optimized strain overexpressed *Bs-mgsA, Ec-gldA, Ec-fucO*, *Cb-FDH1*, carried deletions in *zwf1, tpi, ldh, gloA, adhE*. The other strain expressed the PDO-dehydratase fusion protein PpdA-B-C (genes extracted from *K. oxytoca*). Test of sequential or simultaneous cultivation of both strains in anaerobic shake flask culture, 40 g/L glucose, 5 g/L yeast extract, 13.3 g/L NaHCO3, 50 mM sodium formate, 10 µM coenzyme B12, low phosphate medium	3	nr	nr	(Jain et al., 2014)
T. fusca	Pathway for 1-propanon production not unambiguously identified. **Propionyl-CoA-dependent propanol** production upon expression of bifunctional butyraldehyde/alcohol dehydrogenase from *C. acetobutylicum* (*Ca-adh2*). Different substrates: glucose, **cellobiose, untreated switchgrass, or corn stover**. Cultivation at 46 °C.	0.45	nr	nr	(Deng and Fong, 2011)

nr- not reported, (*) cumulated from propanol amount in culture and recovery vessel, alc – alcohol, glu – glucose, gly – glycerol

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Table 2: Maximum theoretical propanol yields of different pathways on glucose in the metabolic network of *E. coli*.

Pathways	Product	Shown in Figure	Max. yield** [mol/mol]	Remarks/Observation
Wood-Werkman[1]	n-propanol	2	1.33	assuming functional succinyl-CoA synthetase
Acrylate[2]	n-propanol	3	1.33	assuming functional lactyl-CoA synthase, maximum yield depends on cyclization of pentose phosphate pathway
Acetone[3]	isopropanol	4	(A, B)* 1.0	
			(A, B)* 1.33	assuming functional phosphoketolase
Threonine[4]	n-propanol	5	(A)* 1.2	maximum yield depends on cyclization of pentose phosphate pathway
			(B)* 1.1	
Citramalate[5]	n-propanol	6	1.00	
Succinate[6]	n-propanol	7	1.33	
1,2-PDO[7]	n-propanol	8	1.33	

(*) Pathway variant in corresponding figure. (**) In the absence of growth. Net stoichiometric reactions representing the propanol pathways in the stoichiometric metabolic model of *E. coli*: (1) succinyl-CoA + pyruvate + 2 NADPH -> propanol + CoASH + oxaloacetate + 2 NADP; (2) lactate + ATP + 3 NADPH -> propanol + ADP + 3 NADP; (3) (A) acetyl-CoA + acetate + NADPH -> propanol + CoASH + CO_2 + NADP, (B) 2 acetyl-CoA + NADPH -> propanol + 2 CoASH + CO_2 + NADP; (4) (A) oxaloacetate + 2 ATP + 3 NADPH + glutamate -> propanol + 2 ADP + 3 NADP + α-ketoglutarate + NH_3 + CO_2 , (B) oxaloacetate + 3 ATP + 4 NADPH + Glutamate + Qui_{ox} -> propanol 3 ATP + 4 NADP + α-ketoglutarate + NH_3 + CO_2 + Qui_{red}; (5) pyruvate + acetyl-CoA + NADPH + NAD -> propanol + 2 CO_2 + NADP + NADH; (6) succinate + ATP + 2 NADPH -> propanol + CO_2 + ADP + 2 NADP; (7) DHAP + 2 NADH + NADPH -> propanol + 2 NAD + NADP

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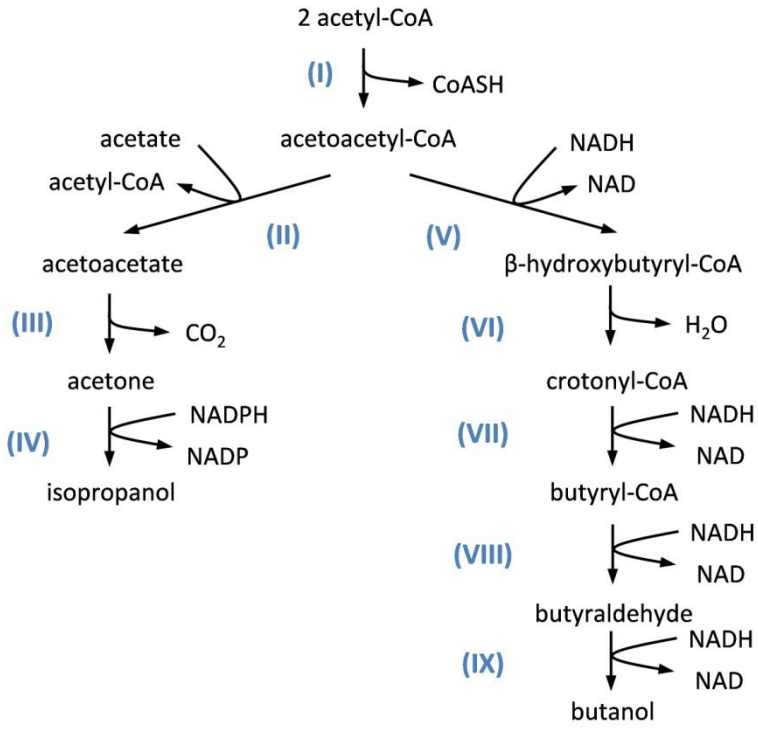

Fig. 1

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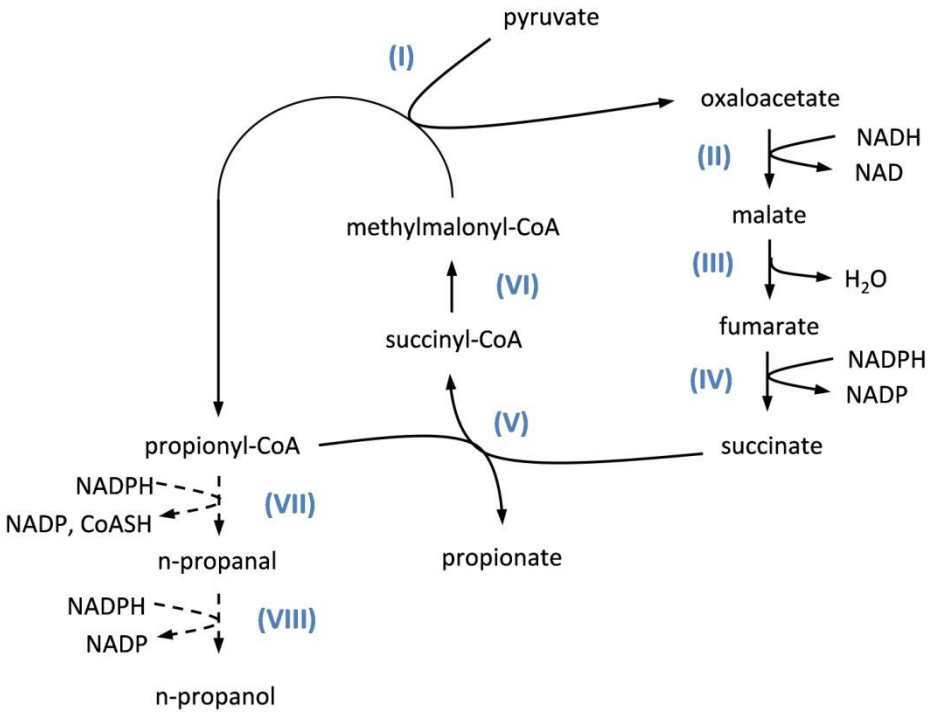

Fig. 2

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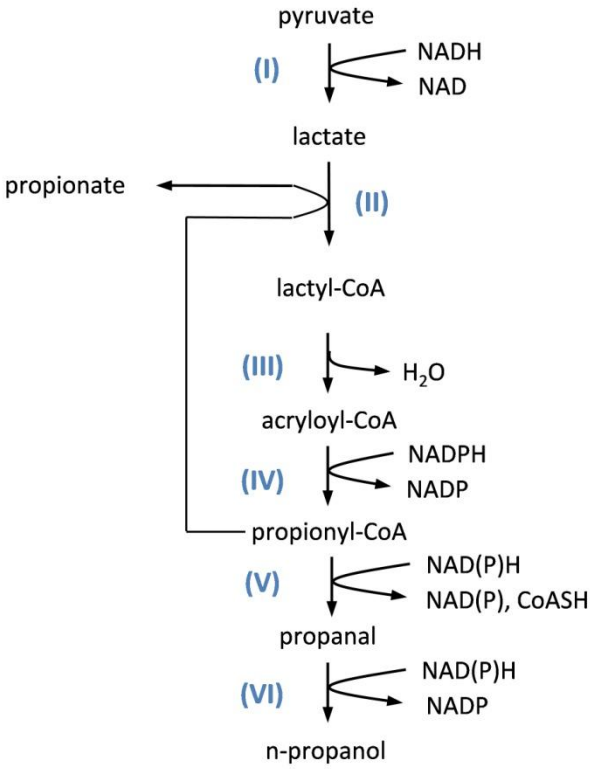

Fig. 3

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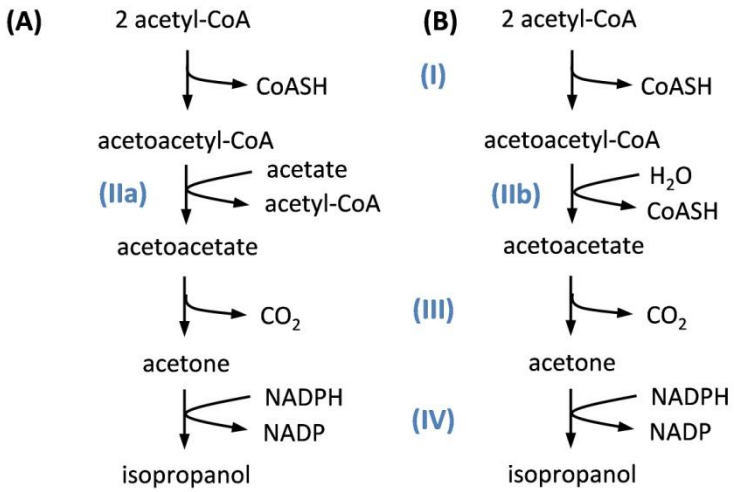

Fig. 4

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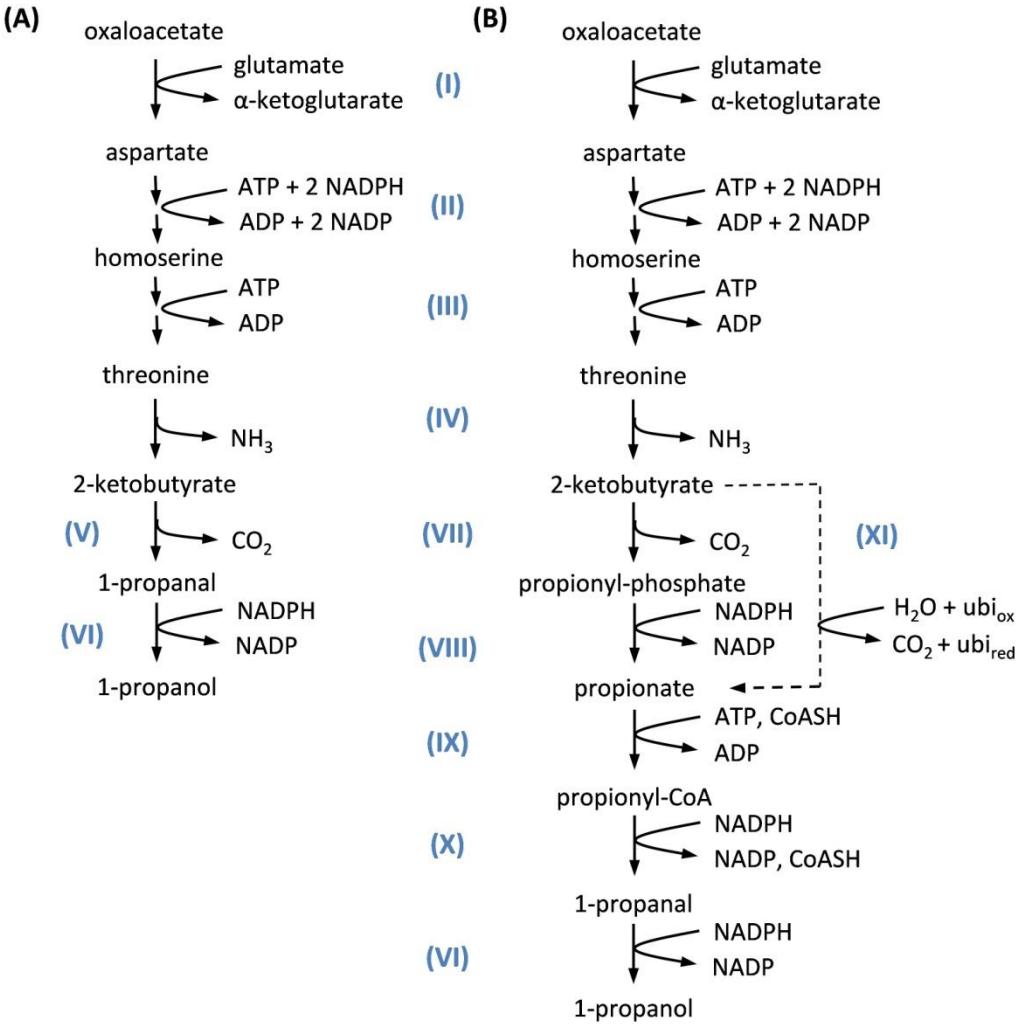

Fig. 5

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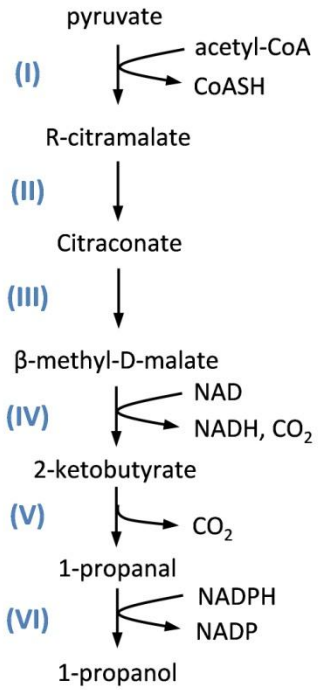

Fig. 6

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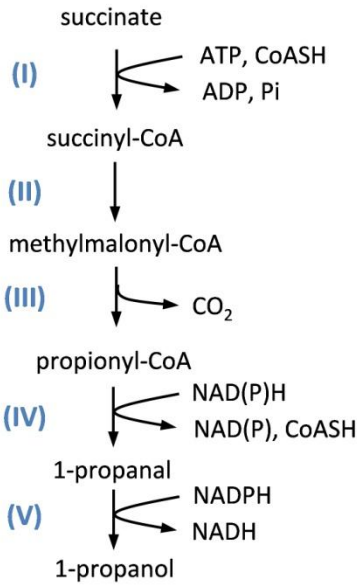

Fig. 7

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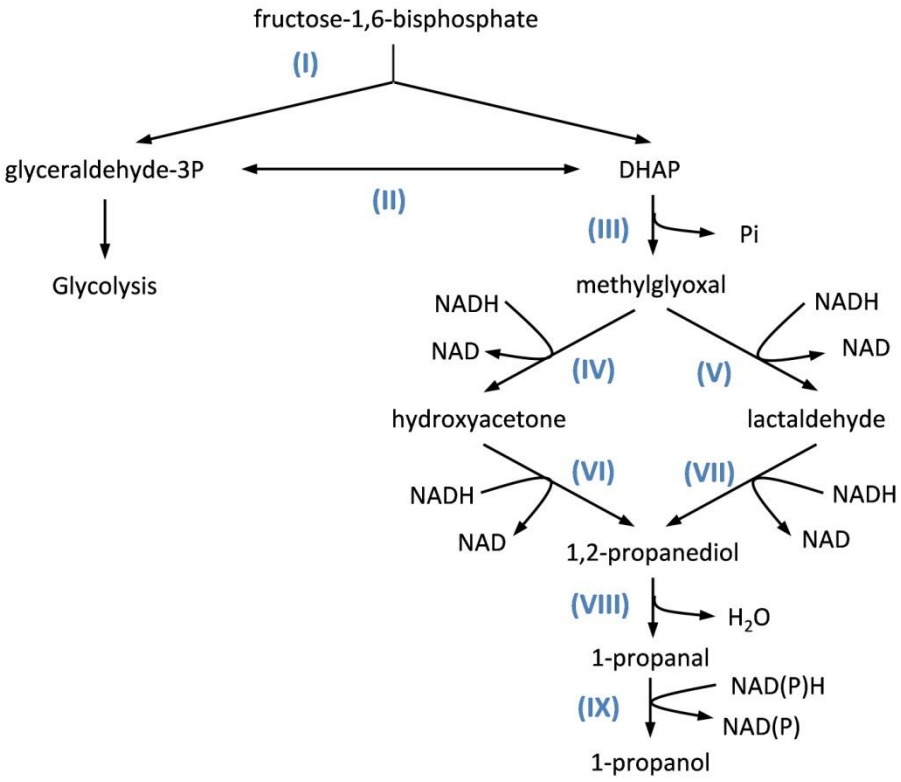

Fig. 8

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