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FERMENTATIVE BIOFUELS PRODUCTION

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Abstract

The limited reserves and increasing prices of fossil carbohydrates, as well as the global warming due to their utilization, impose the finding of renewable energy sources. Because of this, since decades an increasing interest in production of alcohols, which can be used as a fuel additives or fuels for direct replacement in gasoline engines, is observed.

Alcohols can be obtained chemically or as products of microbial metabolism of different species in fermentation of sugars or starchy materials. In the present review are summarized different fermentative pathways for production of all alcohols, which are or could be used as biofuels. The focus of the paper is on production limitations, strains development and economical perspectives.

Key words: *fermentation, biofuel, alcohols*

1. INTRODUCTION

The increasing energy demand and running low stocks of fossil fuels in last decades shift the attention to alcohols production in respect of their fuel properties. Alcohols can be produced from renewable sources and reply both to energy crisis and environmental problems. A number of alcohols are candidates to replace the existed fuels, but to date only ethanol and methanol fuels are on race. Having in mind that methanol fuel is produced mainly from coal or natural gas, it remains that only ethanol fuel is received by fermentation. Nevertheless, many other alcohols have fuel properties, very similar to those of gasoline and could be used as a fuel, much better than ethanol (Table 1). The higher alcohols as butanol and branched-chain alcohols (2-methyl-1-butanol (2MB) and 3-methyl-1-butanol (3MB)) have higher energy density and lower hygroscopicity than the ethanol (Atsumi et al. 2008a). Moreover, one of the main problems of ethanol fuel - the insufficient vaporization, is partially solved, using butanol. The heat of vaporization of butanol is less than half of those of ethanol, which makes butanol preferable fuel in cold weather. Other advantage of higher alcohols is that they can be transported in already existing pipelines (Ladisch 1991, Dürre 2007).

Table 1. Properties of alcohol fuels

	Gasoline	Methanol	Ethanol	Isopropanol	Butanol
Research octane number	95	109	109	118	96
Motor octane number	85	89	90	98	78
Anti knock index	90	99	99.5	108	87
Energy density (MJ/L)	32.6	15.8	19.6	23.9	29.2
Heat of combustion (kJ/mol)	4817	725	1378	2030	2683
Heat of vaporization (MJ/kg)	0.36	1.20	0.92		0.43
Air-fuel ratio	14.6	6.4	9.0		11.1
Boiling point (˚C)		65	78	82.5	118

However, certain disadvantage of these alcohols is that to date they cannot be synthesized economically. Except ethanol, the yield of all other alcohols, obtained by fermentation is insufficient for its production in industrial scale. In the present paper are listed all known metabolic pathways of their fermentative production. The recent efforts of process optimization and strain development in respect of their industrial application are also discussed.

2. ETHANOL

Ethanol (ethyl alcohol) can be produced in industrial scale both chemically and biologically. In 2003 about 95% of the ethanol was produced by fermentation of sugar cane and corn, and 5% was a petroleum product (by catalytic hydration of ethane (ethylene) with sulfuric acid as a catalyst) (Berg 2004). On the other hand, 73% of the produced ethanol is for fuel ethanol, 17% for beverages and 10% for the industry (Sánchez and Cardona 2008). In 2010 the total world production of ethanol for fuel was 86.9 billion liters, as 88% of it was produced in United States and Brazil (Lichts 2010).

Usually ethanol is used in different mixtures with gasoline. Thus, in USA most common blends are of 10% ethanol and 90% gasoline (E10), and in Brazil since 2007 the legal blend is 25% ethanol and 75% gasoline (E25) fuel blends. High ethanol blends cannot be in use during cold weather because of their low vapor pressure. Engines working with high ethanol mixtures are built with additional heater or secondary gasoline reservoir for a cold start with pure gasoline injection. Nevertheless, last decade many cars were designed for ethanol blends up to 85% ethanol (North America) and even up to 100% (E100) in Brazil. Ethanol can be blended also with diesel fuel (Hansen et al. 2005) and even in ethanol-biodiesel-diesel fuel blend (Pidol et al. 2012).

Bioethanol can be obtained from many different agricultural feedstocks – sugar cane, grain, sweet potatoes, cassava, molasses, fruit, cotton and many types of cellulose waste. Generally the feedstocks can be divided in three main groups – sugars, starch and lignocellulosic biomass. The production process consists of: pretreatment of the raw feedstock, hydrolysis or saccharification (when starch or cellulose is used as a feedstock), microbial fermentation of sugars, distillation and dehydration.

2.1. Fermentation process

The most preferable organism used to convert sugars into ethanol is *Saccharomyces cerevisiae*. These species can ferment mono- and disaccharides to ethanol, but is unable to convert pentoses and it is not useful for lignocellulose utilization (Wyman and Hinmam 1990). The main advantage of *S. cerevisiae* is the high ethanol tolerance – up to 150 g/l. It has ability to grow under strictly anaerobic conditions (Visser 1995), but higher ethanol yield is received in presence of oxygen. In fed-batch process, Alfenore et al. (2004) yielded 147 g/l ethanol from glucose (productivity 3.3 g/l per h) in condition without oxygen limitation (vvm = 0.2) whereas in micro-aerobic conditions - 131 g/l (2.6 g/l per h). Yeast like *Pichia stipitis*, *Candida shehatae* and *Pachysolen tannophilus* are capable to convert pentoses (Claassen et al. 1999), but have 2-4 times lower ethanol tolerance (Hinamn et al. 1989) and 5 times lower ethanol production (Hahn-Hägerdal et al. 1994). Other yeast such as *S. bayanus* (Castellar et al. 1998), *Schizosaccharomyces pombe* (Bullock 2002) and *Kluyveromyces marxianus* (Oliva et al. 2003) also have been used for ethanol production, as they metabolize glucose.

In high amounts ethanol can be produced also by the bacterium *Zymomonas mobilis*. This organism has high ethanol tolerance – up to 100 g/l and produced ethanol up to 97% of the theoretical maximum (Lynd et al. 1996). However, *Z. mobilis* can metabolize only glucose, fructose and sucrose to ethanol.

Some saccharolytic *Clostridium* strains could be applied for direct ethanol production from lignocellulosic materials because of their ability to convert both hexoses and pentoses (McMillan

1997). However, the very low tolerance to ethanol (less than 30 g/l) makes clostridia strains unfeasible.

2.2. Production problems and process development

Although bioethanol is a renewable and environmentally clean source of energy, till now its production cost is higher than production cost of gasoline. To date, bioethanol can be obtained economically competitively only from sugar cane and corn starch. The economic analysis shows that 40 - 70% of the final ethanol prices, produced from these substrates are the feedstock costs (Dale and Linden 1984, Maiorella et al. 1984). By that reason, the main attempts are focused on searching for alternative, less expensive feedstocks. The most promising option is the utilization of lignocellulosic materials. Theirs low cost and great availability makes them attractive for competitive production of ethanol. However, conversion of lignocellulose requires additional steps prior to fermentation – pretreatment of raw material and cellulose hydrolysis by acids or specific enzymes. Resulting cellulose hydrolysate contains fermentable sugars which already can be easily converted to ethanol by *S. cerevisiae*. These technologies are not completely developed and need additional improvement for its industrial implementation (Cardona and Sánchez 2007). One effective solution is the combination of the enzymatic hydrolysis with glucose fermentation in a single process – simultaneous saccharification and fermentation (SSF) (Takagi et al. 1977). In this way sugars are not accumulated in the broth and their diminishing inhibitory effect allows higher yields and productivities of ethanol (Wyman 1994). Other advantage of SSF is the easier operation – no hydrolysis reactors are needed. However, the optimal conditions for hydrolysis and fermentation are different and the process is difficult for control.

The main fermentation problems are in consequence of substrate inhibition, product inhibition and high osmotic pressure. For decreasing the levels of substrate concentration fed-batch strategy was applied (Echegaray et al. 2000, Alfenore et al. 2002). This type of cultivation is the most employed technology because of the possibility to achieve higher final ethanol concentration and productivity. To eliminate inhibitory effect of the high ethanol concentration there is used continuous cultivation (Hojo et al. 1999, Kosaric and Velikonja 1995, Monte Alegre et al. 2003) or a system of reactors arranged in cascade (Coombs 1996). Additionaly, during the process ethanol can be removed from the broth by vacuum (Cysewski and Wilke 1977), membrane pervaporation (Groot et al. 1993), gas stripping (Taylor et al. 1998) or liquid extraction (Gyamerah and Glover 1996).

2.3. Strains development

S. cerevisiae, the most employed species for ethanol production, has no cellulolytic nor saccharolytic abilities. Still more, it is unable to convert the C5-sugars. To overcome this serious disadvantage, genes encoding xylose reductase and xylitol dehydrogenase were cloned into *S. cerevisiae*. The recombinant strain was able to produce ethanol from xylose, but with low productivity (Hahn-Hägerdal et al. 1994). Ingram et al. (1999) used for ethanol production lignocellulosic biomass where genetically strains of *Escherichia coli* and *Klebsiella oxytoca* are engineered. After acid pre-treatment, the pentoses consisting the liquid fraction was fermented to ethanol by recombinant *E. coli* strain supplemented with *Zymomonas mobili*s genes, and the solid fraction - by recombinant *K. oxytoca* strain, also supplemented with genes for ethanol production.

3. BUTANOL

Butanol (1-butanol or *n*-butyl alcohol), a four-carbon alcohol with molecular formula C_4H_9OH is an important bulk material for many chemical synthesis with wide application as a solvent in chemical and textile industry (Lee et al. 2008). Moreover, now it is considered that butanol could be used as an efficient biofuel, superior even than the ethanol. Butanol combustion is:

$$C_4H_9OH + 6O_2 \rightarrow 4CO_2 + 5H_2O + heat$$

Butanol can be obtained in acetone-butanol-ethanol (ABE) fermentation of sugars or starch by clostridia species. High amounts of butanol can be produced by four species of genus *Clostridium* – *Clostridium acetobutylicum*, *C. beijerinkii*, *C. saccharobutylicum* and *C. saccharoperbutylacetonicum* (Keis et al. 2001). The other methods for butanol production are chemical: from butyraldehyde or ethylene oxide (O'Neil et al. 2001, Yuan 2007) or by butane oxidation (Kang et al. 2007).

3.1. History

Pasteur in 1861 first reported that butanol is a fermentative product. Later Weizmann (1919) patented the production of acetone and butanol by fermentation of sugars via so called "Weizmann process" or ABE fermentation. During the World War I the ABE fermentation process was developed in industrial scale and up to the 1950s the butanol was produced mainly by fermentation of molasses and maize. However, by 1960s the increasing cost of molasses and advancement of the petrochemical industry shifted of the butanol production from fermentation to chemical synthesis (Jones and Woods 1986). After that, industrial ABE fermentation process continued to work in South Africa and Russia (Zverlov et al. 2006) until 1980s, when the process was forced to close due to transport problems and shortage of feedstock (Jones and Woods 1986). Nevertheless the increasing demand of fuels from renewable sources returns the attention back to fermentative butanol production. In 2009 British Petroleum and Dupont started ABE process in industrial scale in UK (Lopez Contreras et al. 2010). Today butanol produced by fermentation is obtained also in China from corn and in Brazil from sugar cane (Ni and Sun 2009).

3.2. Fermentation process

After ethanol production, ABE fermentation is the second largest fermentation process by its global industrial and historical impact (Jones and Woods 1986). In industrial scale the fermentation is carried out in a batch process prolonged 40-60 h in fermentors without mechanical agitation. As a substrate are used preliminary cooked and sterilized molasses or maize. The process is conducted at 35-37˚C under completely anaerobic conditions. ABE fermentation (Fig 1) is biphasic process. In the first phase (acidogenic phase) are formed mainly acids - butyrate and acetate, but in the second phase are activated other metabolic patways and acids are converted into solvents - acetone, butanol and ethanol (solventogenic phase). The acidogenic phase is attended with rapid decreasing of pH and occurs usually during the exponentional growth of the culture (Andersch et al. 1983). When pH reaches a critical low point, the metabolic shifts from acidogenic to solventogenic phase. The total solvent yield reaches 12-20 g/l from maize and 18-22 g/l from molasses (Jones and Woods 1986). Solvents ratio is strain dependent and for *C. acetobutylicum* (Weizmann organism) it is 6:3:1 (butanol-acetone-ethanol). In *C. beijerinckii* metabolism acetone is replaced by isopropanol, but butanol production remains almost the same.

Butanol production in solventogenic phase is complicated and the mechanism involves many enzyme reactions of different metabolic pathways. With ^{14}C-labeled acids Wood et al. (1945) showed that more than 55% of acetate and the majority (85%) of butyrate are converted to butanol by the enzyme acetoacetyl–CoA:acetate/butyrate:CoA transferase (ctfAB) (Fig 1). The other metabolic pathway is from pyruvate by pyruvate – ferredoxin oxidoreductase (pfIB), thiolase (thl), 3-xydroxybutyryl-CoA dehydrogenase (hbd), crotonase (crt), butyryl-CoA dehydrogenase (bcd), butyraldehyde dehydrogenase (adhE) and butanol dehydrogenase (bdhAB).

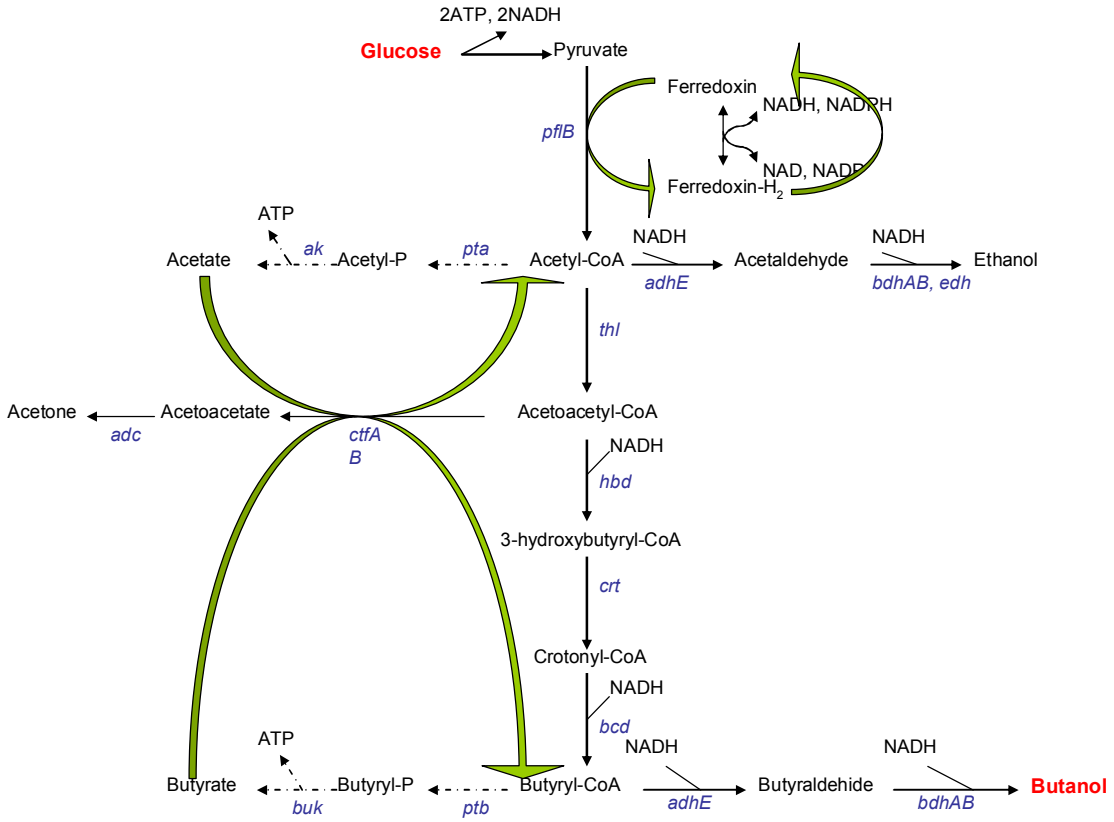


Fig. 1. Metabolic pathways in *C. acetobutylicum*. Reactions active in whole fermentation, during the acidogenic and the solventogenic phases are marked with thick, dotted and solid arrows, respectively.

3.3. Process development

Fermentative production of butanol faces with several limitations, which make its industrial application unprofitable without additional optimizations. The most critical problem is solvent toxicity. Clostridial metabolism completely ceases when the concentration of solvents reaches 20 g/l (Woods 1995). The lipophilic butanol is the most toxic of all products, leading to disruption of phospholipids envelope of the cell membrane. In batch fermentation, solvent production stops when butanol concentration reaches 13 g/l. The other solvents acetone and ethanol are also toxic, but their concentrations do not reach inhibitory levels. Thus, the low yield of fermentative butanol leads to the prime cost of production increase. Other obstacles are: (i) the formation of numerous by-products; (ii) phage contamination; (iii) high amount of energy required for butanol distillation; (iv) the use of expensive substrates like sugar and starch, which can be employed as a nutrients ("food versus fuel" debate) (Dürre 2011).

There are many attempts to improve the productivity and to reduce the prime cost of production: (i) finding new, extra-butanol-producing natural strains (Qureshi and Blaschek 2001); (ii) using less expensive substrates as a carbon source like syngas (Köpke et al. 2010), lignocellulosic hydrolysates (Schubert 2006), wheat straw hydrolysates (Qureshi et al. 2007), furfural and hydroxymethyl furfural (Ezeji et al. 2007); (iii) applying new methods for butanol recovery as pervaporation (Izàk et al. 2008),

liquid-liquid extraction (Dürre 1998) gas stripping (Ezeji et al. 2003); (iv) conducting the fermentation process in fed-batch mode (Groot et al. 1984, Ezeji et al. 2004a) or in continious mode (Qureshi and Blaschek 2000, Ezeji et al. 2004b).

3.4. Strains development

Important approach to increase the solvent production is by metabolic engineering of Clostridia strains. One direction is by amplification or inactivation of genes in recombinant Clostridia strains, another direction – by cloning and expression of Clostridium genes in other organisms.

Amplification of *adc* (encoding acetoacetate decarboxylase), *ctfAB* (encoding CoA transferase), *adhE* (encoding butyraldehyde dehydrogenase) and *groESL* operon (encoding the heat shock proteins GroES and GroEL) genes leads to increase in final butanol concentration (Mermelstein et al. 1993, Nair et al. 1994, Tomas et al. 2003b). The same effect can be observed by inactivation of *buk* (encoding butyrate kinase) or *solR* (encoding regulator of genes, involved in solventogenesis) genes (Green and Bennett 1996, Nair et al. 1999, Harris et al. 2000, 2001). Thus, the maximal butanol concentration can be increased twice, even three times, but cannot surpass 20 g/l in batch process.

Butanol production by other than Clostridia species is developed in purpose to eliminate culture degradation, by-products formation and phage infection sensitivity. Genes from Clostridia were cloned in *E. coli* (Atsumi et al. 2008b, Inui et al. 2008) and up to 16 mM butanol was achieved from glucose (Inui et al. 2008). Alternative metabolic pathways for butanol production were also tested. Shen and Liao (2008) obtained 13.5 mM butanol using *E. coli* amino acids pathway via keto-acid intermediates. In spite of all, the attempts to obtain higher amounts of butanol by recombinant *E. coli* strains are not so successful. On the contrary, the introduction of Clostridia genes in *E. coli* can lead to considerable increase in acetone (Bermejo et al. 1998) and isopropanol (Hanai et al. 2007, Jojima et al. 2008) production.

4. METHANOL

Although methanol has a number of drawbacks as a fuel, many authors propose it as a biofuel of the future. It has been in use for a long time as racing fuel and as a component in rocket fuel. Methanol fuel usually is used as a blend of 85% methanol and 15% unleaded gasoline (M85). The addition of methanol increases the thermal efficiency and the power output of the blended fuel due to it high octane number and high heat of vaporization.

The most of the disadvantages of methanol as a fuel are the same as these of ethanol. Both are less volatile than the gasoline and have lower combustion temperature, which make difficulties in engine starting during cold weather. Methanol like ethanol contains halide ions, which is the reason for their high corrosivity. Moreover, methanol is very hygroscopic and absorbs water from the atmosphere. Other faults of methanol are that it burns with invisible, colorless flame, and has high toxicity. Likewise, the low energy density of methanol (see Table 1) and the low air/fuel ratio of 6,42:1 means that fuel consumption will be higher than the consumption of hydrocarbon fuels. Because of this, vehicles using methanol fuel should have a larger fuel tank.

Methanol, the simplest alcohol (CH_3OH) can be produced from large variety of different carbon sources including fossil fuels, biomass, crops, forest residues, animal waste or most simply, from carbon dioxide and water. Historically, for a long time it has been produced by pyrolysis of wood chips, from where is its common name of wood alcohol (Dave 2008). Also, methanol can industrially be obtained from methane via syngas (a mixture of CO and H_2) (Fig 2), and to date the most preferred raw material for methanol production from methane is still the natural gas.

$$CH_4 + H_2O \rightarrow CO + 3\ H_2$$

$$CO + H_2O \rightarrow CO_2 + H_2$$

$$CO + 2\ H_2 \rightarrow CH_3OH$$

$$CO_2 + 3\ H_2 \rightarrow CH_3OH$$

Fig.2. Reactions involved in methanol production from methane via syngas

4.1. Fermentation process

Biologically, methanol can be produced as an intracellular intermediate by methane-utilizing bacteria (methanotrophs) under anaerobic conditions. These organisms use methane as a sole carbon source to produce CO_2 and cell carbon via methanol, formaldehyde and formic acid (Hanson and Hanson 1996, Trotsenko and Khymelenina 2005):

$$CH_4 \rightarrow CH_3OH \rightarrow HCHO \rightarrow HCOOH \rightarrow CO_2$$

Organisms capable of this conversion are strains of *Methylophilus methylotrophus*, *Methylococcus capsulatus*, *Methylomonas sp.*, *Methylosinus trichosporium*, *Mycobacterium smegmatis* (Lee et al. 2004, Weisman and Ballou 1988).

On the other hand, methanol is the major end product of pectin metabolism. Strains of *Clostridium*, *Erwinia* and *Pseudomonas* species are capable to metabolize pectin to methanol without further degradation. However, the accumulated methanol is only from 9.2 mM for strain of *Erwinia chrysanthemi* to 16.0 mM for *Clostridium butyricum* strain (Schink and Zeikus 1980).

4.2. Fermentation problems and solutions

The amounts of methanol produced by microorganisms are insufficient for its large-scale use. Nevertheless, many attempts to increase methanol production are made.

One way is to inhibit methanol dehydrogenase (MDH) – the enzyme converting methanol to formaldehyde in methanotrophic metabolism. Addition of NaCl prevents further oxidation of methanol to formaldehyde in methane utilization by *Methylosinus trichosporium* (Lee et al. 2004). Thus, authors obtained 7.7 mM methanol in 36 hours. Other chemicals like cyclopropanol (Shimoda and Okura 1991, Takeguchi et al. 1997, Furuto et al. 1999), cytochrome (Mukai et al. 1990), EDTA (Carver et al. 1984), and cyclopropane (Frank et al. 1989) also inhibit MDH.

To overcome the disadvantages of using microorganisms another approach was developed – to use purified enzymes to catalyze the desired reactions. Metyhanol can be produced by NADH depended enzymatic reactions from CO_2 (Attwood 1990, Popov and Lamzin 1994, Reid and Fewson 1994). Obert and Dave (1999) used system of three enzymes to convert carbon dioxide to methanol via format and formaldehyde (formate dehydrogenase, formaldehyde dehydrogenase and alcohol dehydrogenase):

$$CO_2 \rightarrow HCOOH \rightarrow HCHO \rightarrow CH_3OH$$

Methanol production can be additionally enhanced, when these enzymes are entrapped into sol-gel matrix (Obert and Dave 1999).

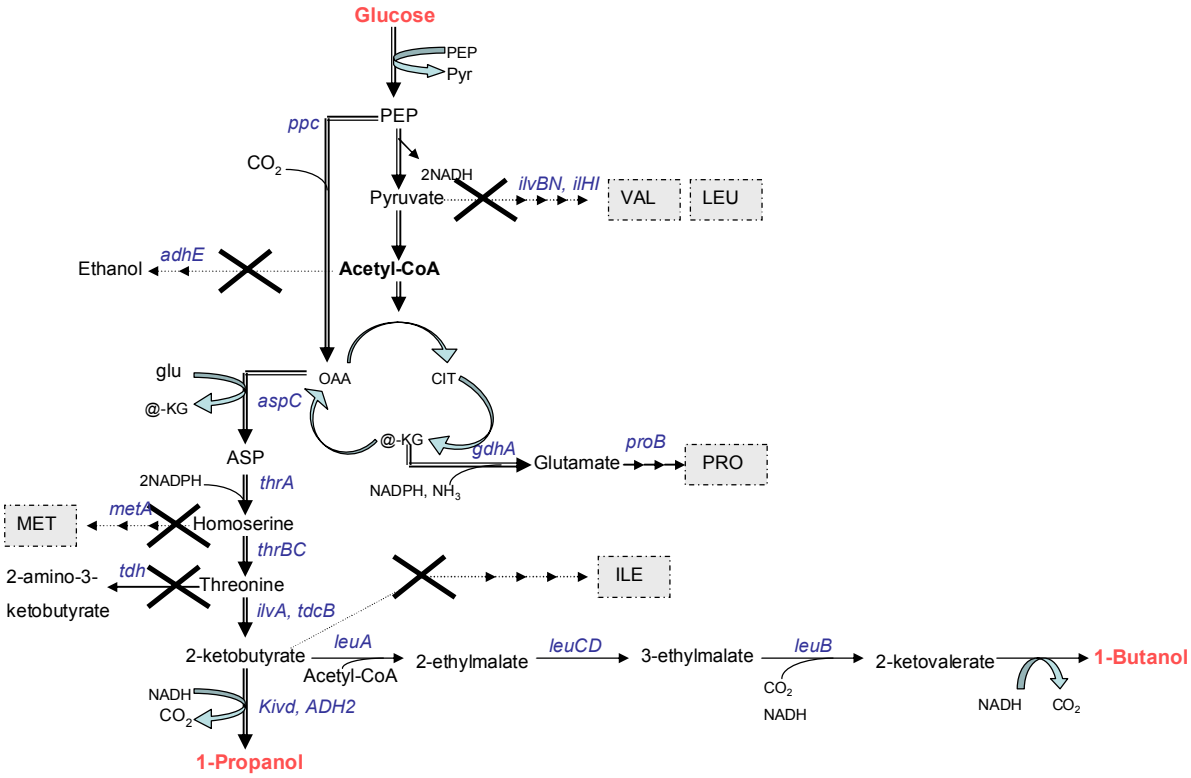


Fig.3. 1-propanol and 1-butanol production via threonine pathway in genetically engineered *E. coli* strain (Shen and Liao 2008)

5. PROPANOL

To date 1-Propanol (C_3H_7OH) is produced from ethane via propionaldehyde in petrochemical industry and has application generally in drug, cosmetic and textile industry. Just recently, it was attended as potential biofuel in respect of its good fuel characteristics close to these of butanol. Nevertheless, to date its production is not in relevant quantities for industrial application as a fuel.

1-Propanol is difficult for biological production. There are no microorganisms, identified to produce it as a major product or in significant amounts. This alcohol is only by-product in fermentation of potatoes or grains in commercial ethanol production. Similarly, 1-propanol has been detected as a metabolic product of threonine fermentation by some Clostridia species (Janssen 2004).

5.1. Metabolic engineering

Because of insufficient amounts of 1- propanol yielded by natural microorganisms, recent attempts are connected with the possibility to design new metabolic engineered pathways receiving 1-propanol as end product.

Shen and Liao (2008) constructed metabolically engineered *Escherichia coli* strain, capable to produce 1-propanol and 1-butanol from glucose. Authors used threonine pathway to 2-ketobutyrate with deletion of the competing pathways. 2-ketobutyrate is a precursor of 1-propanol, but is also a key intermediate in biosynthesis of 1-butanol via 2-ketovalerate (Fig 3). Total alcohol production was 2 g/l with ratio 1:1 of butanol and propanol.

Some organisms like *Methanococcus jannaschii* converted pyruvate and acetyl-CoA to 2-ketobutyrate via citramalate (citramalate pathway) (Howell et al. 1999). Atsumi and Liao (2008) used this way to produce 1-propanol and 1-butanol from glucose, bypassing threonine biosynthesis. The gene encoding citramalate synthase (CimA) from *M. jannaschii* was expressed in *E. coli* strain. After additional evolving of CimA, the final production of 1-propanol and 1-butanol was 9 and 22 fold higher, respectively.

Another novel metabolic pathway for 1-proppanol production was designed by Jain and Yan (2011). They extended the known pathway to 1,2-propanediol with two additional steps. First, expressed in *E.coli mgsA* gene encoded methylglyoxal synthase (from *Bacillus subtilis*), and *budC* - encoded alcohol dehydrogenasegene (from *Klebsiella pneumoniae*), authors achieved higher 1,2-propanediol production. Next, by additional expression of operon *ppdABC* (from *Klebsiella oxytoca*) that encoded 1,2-propanediol dehydratase – 0.25 g/l 1-propanol was achieved (Fig 4).

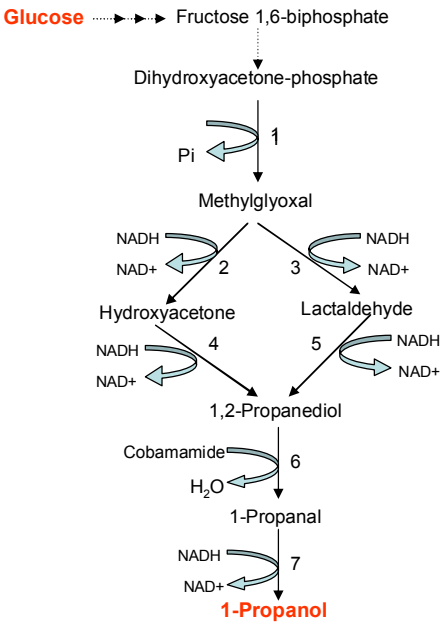


Fig.4. Designed metabolic pathway for 1-propanol production via 1,2-propanediol (Jain and Yan 2011). Enzymes and genes 1: methylglyoxal synthase (*mgsA*); 2: methylglyoxal reductase (*ydjG*); 3, 4: secondary alcohol dehydrogenase (*gldA/budC*); 5: primary alcohol dehydrogenase (*fucO*); 6: diol dehydratase (*ppdABC*); 7: primary alcohol dehydrogenase (*yqhD*).

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