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Full Length Research Paper

The use of microorganisms in 1,3-Propanediol production

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Recently, an increasing interest in "green" processes for the production of chemicals was observed. An attractive solution is microbial fermentation processes which use renewable feedstocks such as glycerol. However, this kind of synthesis has several limitations, among others a small range of products, low productivities, as well as difficulty in recovery and purification of products. One way to overcome these limitations is the application of metabolic engineering. This paper is a review of 1,3-Propanediol (1,3-PD) characteristic and applications, of microorganisms which ferment glycerol to 1,3-PD and their metabolic pathways, and finally of the metabolic engineering methods for the microbial conversion of raw glycerol to 1,3-PD.

Key words: Fermentation, glycerol, metabolic engineering, 1,3-PD.

INTRODUCTION

1,3-Propanediol (1,3-PD) $CH_2(CH_2OH)_2$, a typical product of glycerol fermentation, is one of the most interesting raw materials for chemical industries due to its wide use in the different fields, e.g. it is a valuable chemical intermediate used in organic synthesis. It is also used as a monomer for the production of biodegradable polymers (polyesters, polyethers, polyurethanes, etc.), cosmetics, lubricants, medicines, and as an intermediate for the synthesis of heterocyclic compounds (Zeng et al., 1996; Menzel et al., 1997; Biebl et al., 1999; Malinowski, 1999; Igari et al., 2000; Zeng and Biebl, 2002; Nakamura and Whited, 2003; Liu et al., 2007; Zhang et al., 2007; Katrlik et al., 2007; Ma et al., 2009). 1,3-PD can also be used as a chain extender for the synthesis of lubricants, solvents, and precursors in the chemical and pharmaceutical industries. Moreover, biodegradability of natural plastics containing 1,3-propanediol is higher compared to those of fully synthetic polymers (Deckwer, 1995; Witt et al., 1994; Barbirato et al., 1998).

1,3-PD is produced in two ways, chemically and microbiologically. Annually over one million ton of 1,3-PD is produced by a chemical method, which needs high cost and is non-friendly to the environment (Biebl et al., 1999; Ma et al., 2009). Recently, we observe an increased interest in the production of 1, 3-PD from the renewable resources through economic and "green" processes such as microbial fermentation (Biebl et al., 1999; Huang et al., 2002; Ma et al., 2009). Glycerol becomes a good renewable resource because it is formed as a byproduct during biodiesel production with a large volume. It causes the necessary development of a new technology to convert glycerol into other products of high value, among others 1,3-PD (Kośmider and Czaczyk, 2009; Ma et al., 2009). In a chemical way, the 1,3-PD production starts from acrolein (Dupont Company) or from ethylene oxide (Shell Company). In the first case, acrolein is hydrated at moderate temperature and pressured to 3-hydroxypropionaldehyde which, in a second reaction, is hydrogenated to 1,3-PD over a rubidium catalyst under high pressure (90 bar). In the second case, ethylene oxide is transformed with synthesis gas in a hydroformylation process to 3-hydroxypropanal, but very high pressure (150 bar) is required for this reaction. The aldehyde is extracted from the organic phase with water and subjected to hydrogenation using nickel as a catalyst, again under high pressure. In the first process the yield does not exceed 65%, but in the second a yield of 80% is obtained.

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MICROORGANISMS IN 1,3-PD PRODUCTION

For almost 120 years a bacterial fermentation has been known, in which glycerol is converted to 1,3-PD (Zeng and Biebl, 2002; Nakas et al., 1983; Biebl, 1999). In 1881, August Freund identified 1,3-PD as a product of glycerol fermentation by *Clostridium pasterianum*. It is of the oldest known fermentation products. Next, in 1914 production of this diol by *Bacillus* sp. was described by Voisenet. However, no comparable strain was isolated. In 1928 at the Microbiology School of Delft, the fermentation of different *Enterobacteriaceae* producing 1,3-PD was analyzed, and it was continued at Ames, Iowa, in the U.S. Finally, as late as in 1990, a biotechnological way of 1,3-PD was recognized (Zeng and Biebl, 2002; Katrlik et al., 2007; Drożdżyńska et al., 2011; Leja et al., 2011).

A number of microorganisms can use glycerol as the sole carbon and energy source, such as *Klebsiella* strains (among others *Klebsiella pneumonia* (Biebl et al., 1998), *Klebsiella oxytoca* (Homann et al., 1990), *Klebsiella aerogenes* (Biebl et al., 1998)), *Lactobacillus* strains (among others *Lactobacillus reuterii*, *Lactobacillus buchnerii*, *Lactobacillus collinoides* (da Silva et al., 2009)), *Enterobacter* strains (among others *Enterobacter agglomerans* (Barbirato et al., 1998), *Enterobacter aerogenes* (da Silva et al., 2009), and some *Clostridium* strains (among others *Clostridium butyricum* (Colin et al., 2000), *Clostridium pasteurianum* (Biebl et al., 1992; Dabrock et al., 1992), *Clostridium diols*, *Clostridium acetobutylicum*, *Clostridium butylicum*, *Clostridium perfingens* (Youngleson et al., 1998; Hao et al., 2008). Also other bacteria are able to convert glycerol to 1,3-PD, such as *Citrobacter freundii* (Boenigk et al., 1993; Daniel et al., 1995a; Malinowski, 1999), *Pelobacter carbinolicus*, *Rautella planticola*, and *Bacillus welchii* (Saxena et al., 2009). However, no natural microorganisms have been found so far which can directly convert glucose to 1,3-PD (Hartlep et al., 2002).

In all microorganisms glycerol is converted to 1,3-PD by two steps, catalyzed by enzyme. The first one is when a dehydratase catalyzes the conversion of glycerol to 3-hydroxypropionaldehyde (3-HP) and water. In the second step, 3-HP is reduced to 1,3-PD by NAD^+-linked oxidoreductase. 1,3-PD is not metabolized further, it accumulates in high concentration in media (Nakamura et al., 2000). Generally, the production of 1,3-PD from glycerol is performed under anaerobic conditions and glycerol as a carbon source and energy is used. There are no other exogenous reducing equivalent acceptors. Among others in *Citrobacter*, *Klebsiella*, and *Clostridium* strains, a parallel pathway for glycerol conversion exists. In the first case, glycerol is oxidated to dihydroxyacetone (DHA) by NAD^+- (or $NADP^+$) linked glycerol dehydrogenase. The next step is phosphorylation of the DHA to dihydroxyacetone phosphate (DHAP) by a DHA kinase. The DHA in this form is available for biosynthesis and for supporting ATP generation through metabolic pathway, for example glycolysis. This is an oxidative pathway (Macis et al., 1998; Zhu et al., 2002; Nakamura et al., 2000). In the second case, glycerol is converted to 3-HP which is catalyzed by coenzyme B_{12}-dependent glycerol dehydratase and related diol dehydratases. Then, the 3-HP reduced to 1,3-PD and NAD^+ is regenerated by the $NADH^+H^+$-dependent enzyme, 1,3-propanediol dehydrogenase (1,3-propanediol-oxydoreductase). This is a reducing pathway (Ahrens et al., 1998; da Silva et al., 2009; Forage and Foster, 1982; Nakamura et al., 2000; Knietsch et al., 2003; Németh et al., 2003).

METABOLIC PATHWAY OF 1,3-PD PRODUCTION

Biochemical pathways for glycerol fermentations with 1,3-PD as the end-product is presented in Figure 1. Glycerol is dehydrogenated to dihydroxyacetone, after phosphorylation, and can be converted to pyruvate in the course of the well known sequence of glycolytic reactions involving another dehydrogenation and two ATP-forming steps. The reductive glycerol conversion involves a vitamin B12-mediated dehydration to 3-hydroxypropionaldehyde and a reduction of the aldehyde to 1,3-PD (Daniel et al., 1995b; Luers et al., 1997; Macis et al., 1998; Biebl, 1999). The fate of pyruvate is different in different bacteria strains. For example, in the enterobacteria it is cleaved to acetyl-CoA and formed in a reaction catalyzed by the enzyme pyruvate formate-lyase. From acetyl-CoA, acetic acid is formed through acetyl-phosphate, yielding extra ATP as well as ethanol, involving two NADH-oxidizing steps with acetaldehyde as the intermediate. Formate is usually cleaved to hydrogen and carbon dioxide by a formate lyase. As in sugar fermentation, pyruvate can also be condensed to acetolactate to give acetoin and 2,3-butanediol. Lactic acid (a reduction product of pyruvate) and succinic acid (originates from phosphoenolpyruvate) are also the end-products of the enterobacterial fermentation. In *Clostridium* strains two products are formed in addition to 1,3-PD. They are acetic and butyric acids. Butyric acid is formed after condensation of two molecules of acetyl-CoA in a reaction chain that involves two NADH-oxidizing steps and the generation of ATP. *Clostridium pasteurianum* produces butanol in addition, which in some situations becomes the main product (Dabrock et al., 1992; Biebl et al., 1999). In *Clostridium butyricum* glycerol enters the cell by diffusion and next it can be oxidized to dihydroxyacetone, and subsequently converted to pyruvate, which reacts to various fermentation products known from the fermentation of glucose, or it can be dehydrated to 3-hydroxypropionaldehyde. The latter compound is reduced to 1,3-propanediol with a regeneration of NAD.

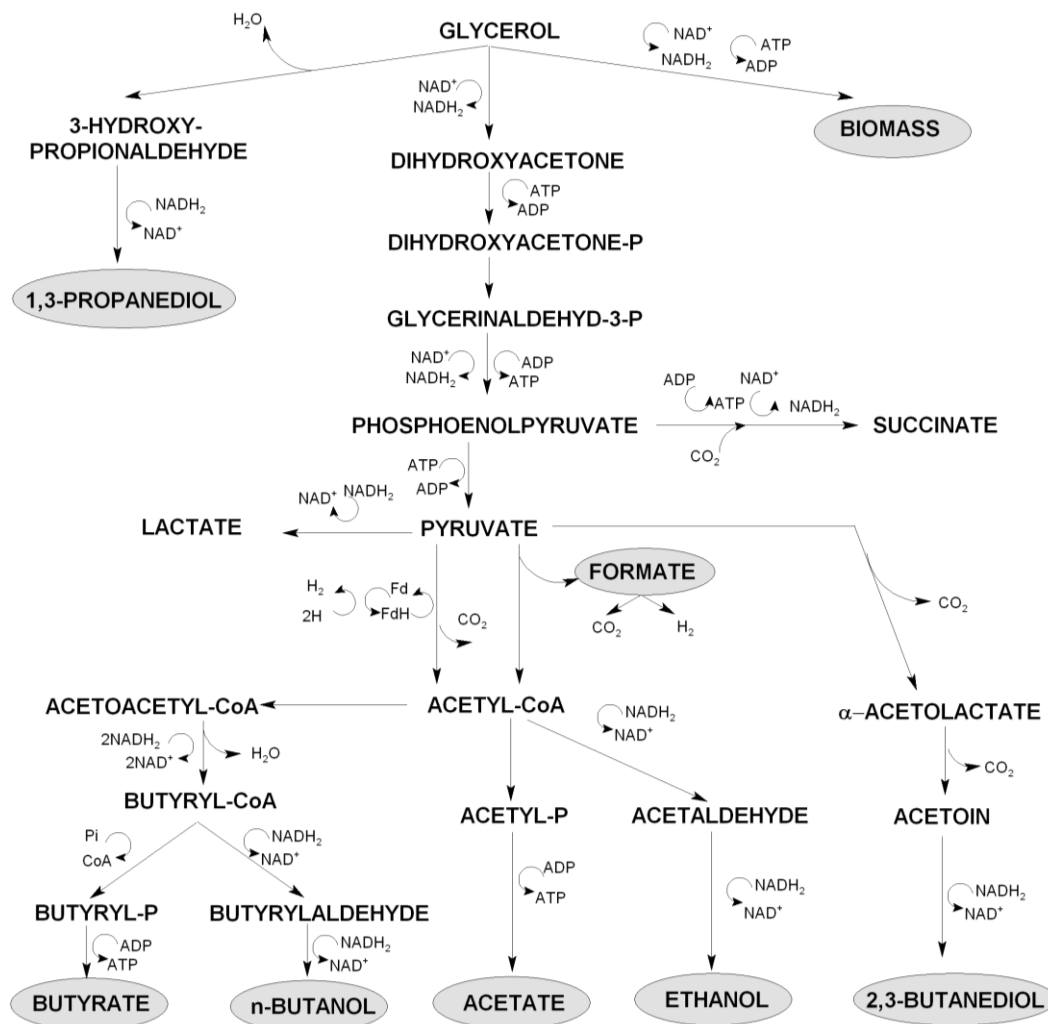

Figure 1. Biochemical pathway of glycerol fermentation.

Regeneration of the reducing equivalents is the physiological role of the 1,3-propanediol pathway. The reducing equivalents are released from the formation of dihydroxyacetone and during the oxidation of glyceraldehydephosphate to bisphosphoglycerate, as well as from biosynthesis. The cleavage of pyruvate to acetyl-CoA and CO_2 is carried out by the enzyme pyruvate, ferredoxin oxidoreductase. Under normal culture conditions acetic acid and butyric acid are the main fermentation products of pyruvate. Metabolic pathways of anaerobic glycerol fermentation by *C. butyricum* is presented in Figure 2 (Lin, 1976; Forage and Foster, 1982; Biebl, 1991; Biebl et al., 1992; Zeng et al., 1993; Zeng, 1996).

METABOLIC ENGINEERING OF MICROORGANISMS IN 1,3-PD SYNTHESIS

Metabolic engineering of the microbial 1,3-PD production concerns the native 1,3-PD producers (among others *K. pneumoniae*, *K. oxytoca*, and *C. butyricum*), as well as microorganisms which are able to form 1,3-PD thanks to genetic manipulations (among others *E. coli*, *S. cerevisiae*, and *C. acetobutylicum*) (Cameron et al., 1998; Chotani et al., 2000; Gill, 2003; Mukhopadhyay et al., 2008; Celińska, 2010).

Klebsiella is a widely recognized genus of opportunistic pathogenic bacteria. It belongs to the KES group of pathogens, including *Enterobacter* and *Serratia* species. As an opportunistic pathogen, the bacteria belonging to the genus *Klebsiella* primarily attack immune compromised individuals who are hospitalized. *K. pneumoniae* is very ubiquitous in nature, it is present in surface water, soil, plants, and also as a saprophyte over the mucuses and intestine of mammals. In spite of its pathogenic properties, *K. pneumoniae* has a complex metabolism that may lead to potential biotechnological applications such as 1,3-PD production (Podschun and Ullman, 1992; Tobimatsu et al., 1996; Podschun and

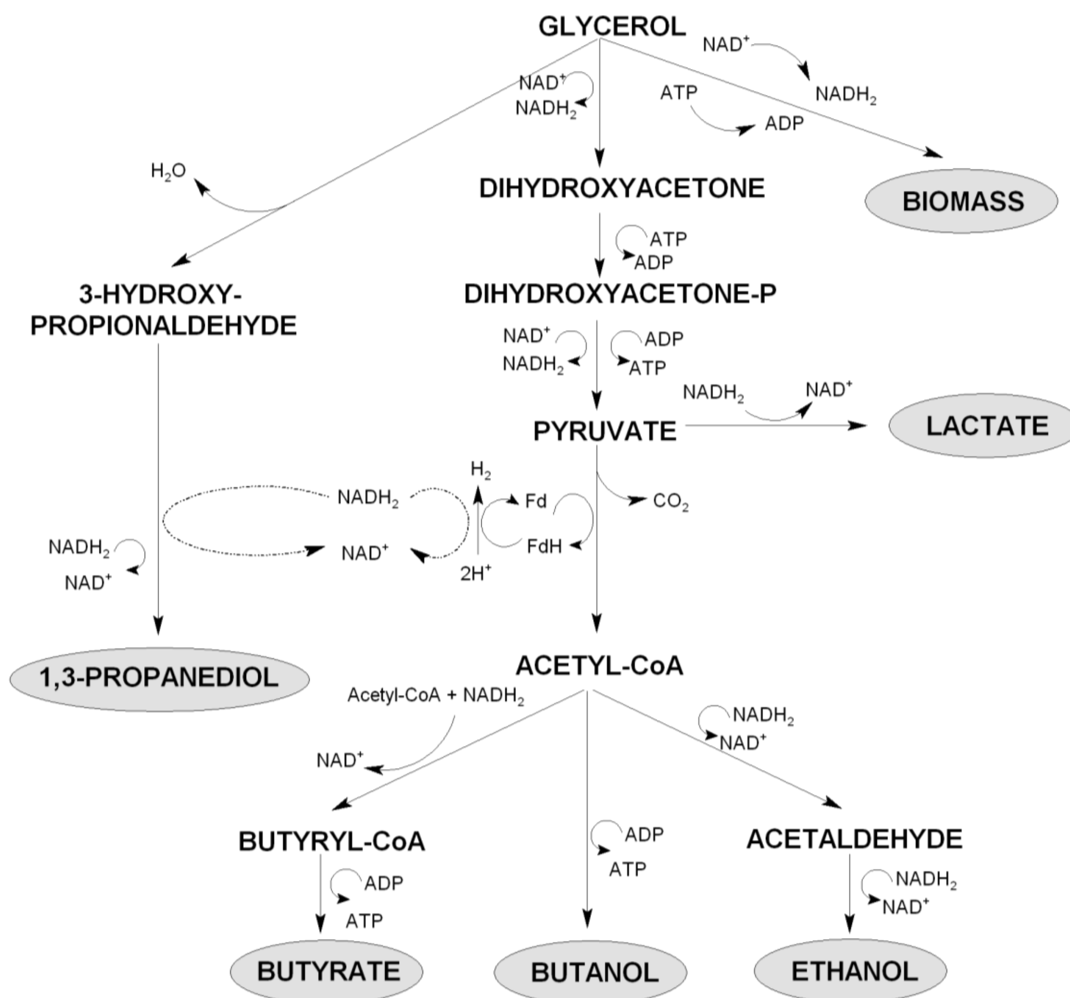

Figure 2. Metabolic pathways of anaerobic glycerol fermentation by *C. Butyricum.*

Ullman, 1998; Podschun et al., 2000). Moreover, *K. pneumoniae* is the most widely studied microorganism as far as 1,3-PD production goes. Scientists work on the improvement of 1,3-PD production by wild-type strains and on how to make this process economically more feasible (Zeng et al., 1996; Zhang et al., 2006; Zhang and Xiu, 2009; Celińska, 2010). Cameron et al. (1998) cloned the genes for glycerol dehydratase (*dhaB*) and 1,3-PD oxidoreductase (*dhaT*) from *K. pneumoniae* to *E. coli*. *DhaB* is a coenzyme B_{12}- dependent enzyme composed of three polypeptides that catalyzes the free radical mediated conversion of glycerol to 3-HPA. Both genes consist of several open reading frames (ORFs) - dhaB3, dhaB3a, dhaB4, and dhaB4a. Then the scientists constructed a series of synthetic 1,3-PD operons with *dhaT* and all of the *dhaB* genes transcribed in the same direction and under the control of a single promoter. This operon contains a variety of restriction sites which make it of broad utility for metabolic engineering of 1,3-PD production in bacteria. Figure 3 shows an example of operon (pTC49). Finally, other scientists proved that the ORF called by Cameron et al. (1998) as *dhaB3* is not necessary for glycerol dehydratase activity. Currently, the role of *dhaB3* is still not known (Tobimatsu et al., 1996; Tong et al., 1997; Cameron et al., 1998; Skaly and Cameron, 1998; Nakamura and Whited, 2003). Clostridia are strictly anaerobic, endospore forming prokaryotes of major importance to cellulose degradation, human and animal health and physiology, anaerobic degradation of simple and complex carbohydrates (Petitdemange, 1995; Paredes, 2005; Papoutsakis, 2008). *C. butyricum* requires the absolute absence of oxygen to grow. This bacteria is the best natural 1,3-propanediol producer from glycerol and the only microorganism identified so far to use a coenzyme B12-independent glycerol dehydratase. The production of 1,3-PD by *C. butyricum* is not economical and it should be improved by metabolic engineering. However, no genetic tools are currently available for *C. butyricum*. Thus, scientists used a metabolic engineering to obtain a better "vitamin B12-

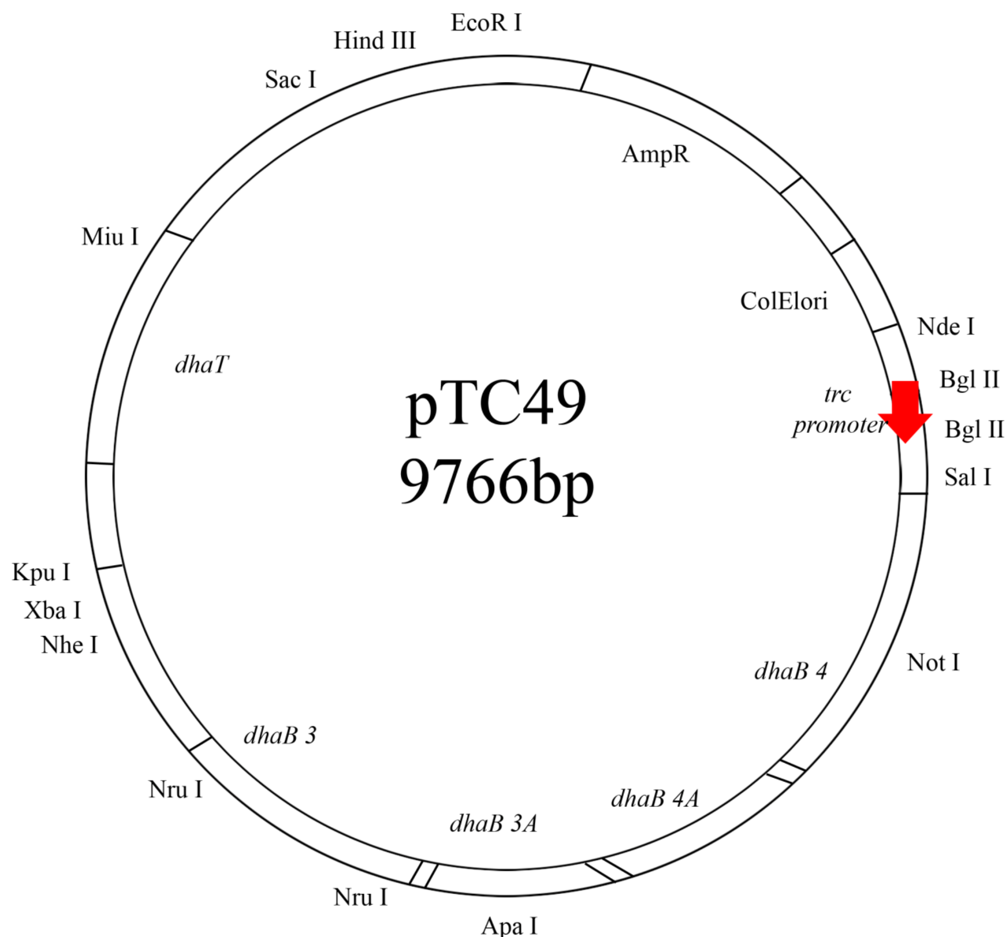

Figure 3. Plasmid pTC49.

free'' biological process in other *Clostridium* strain, *C. acetobutyricum*. The 1,3-PD pathway from *C. butyricum* was introduced on a plasmid in several mutants of *C. acetobutylicum* altered in product formation (Forsberg, 1987; Gonzales-Pajuelo et al., 2005). This recombinant acquired the ability to grow on glycerol as the sole carbon source and was the most efficient strain in continuous cultures, 788 mM of 1,3-PD was obtained at the yield of 0.64 mol mol^{-1} and the volumetric productivity of 3 gL^{-1} h^{-1} (Gonzales-Pajuelo et al., 2005; Celińska, 2010).

Tong et al. (1991) constructed a genomic library of *K. pneumoniae* enriched in clones that are able to grow anaerobically with glycerol and DHA as a carbon source. In a selected clone, which is able to produce 1,3-PD, activities of the four enzymes of the dha regulon (GDHt, 1,3-PD DH, GDH, and DHAK) were detected. The yield of 1,3-PD production was 0.47 mol mol^{-1} of glycerol (>0.5 gL^{-1}) under anaerobic conditions and in complex medium. The same yield was obtained when the *K. pneumoniae* strain was used as a donor of the genes to produce 1,3-PD. The dominant by-products were acetate, formate, and lactate. In further study the yield of 1,3-PD from glycerol was improved to 0.63 mol mol^{-1} (Tong and Cameron, 1992). The theoretical yield of 1,3-PD from glycerol (as the exclusive carbon source) is of 0.75 mol mol^{-1}, when acetate is the sole by-product, or 0.667 mol mol^{-1}, when formate is also produced. The difference between obtained and theoretical yields may be due to the presence of GDH and DHAK activities which divert glycerol from the 1,3-PD pathway to production of cell biomass and by-products. Deletion of DHAK rather than GDH would alleviate the yield, since DHA is an inducer of the *dha* regulon, and its elimination is not desirable (Cameron et al., 1998; Celińska, 2010).

Saccharomyces cerevisiae is a safe microorganism used in fermentation industry. It is a well-known glycerol producer and the genetic engineering of this bacteria is one of the most advanced processes (Nevoigt, 2008). Wild-type *S. cerevisiae* can only produce glycerol, but has no capacity to produce 1,3-propanediol (Laffend et al., 1996; Rao et al., 2008). Rao et al. (2008) integrated genes *dhaB* and *yqhD* (an alcohol dehydrogenase in *E. coli*) into the chromosome of *S. cerevisiae* by *Agrobacterium tumefaciens* mediated transformation, and

constructed a new strain W303-1A-ZR. The engineered *S. cerevisiae* W303-1A-ZR can directly produce 1,3-propanediol from low-cost feedstock d-glucose. However, obtained 1,3-PD concentration was relatively low (0.4 gL^{-1} of 1,3-PD from 2% glucose in a complex medium). The authors suggest that the obtained yield of 1,3-PD was due to low availability of glycerol.

CONCLUSIONS

Nowadays, biology offers alternate catalysts for the production of chemicals using starting materials not available through traditional chemistry. There is an increasing interest in 1,3-PD production by this way. This diol is a very useful bulk chemical, with a variety of applications (among others, it is used in the manufacture of polymers, cosmetics, food, lubricants, and medicines). The direct fermentation makes possible use of renewable feedstock in 1,3-PD production, for example from crude glycerol obtained from biodiesel production. However, the yield of 1,3-PD from the microbiological production is low. One solution to this problem is the use of genetic engineering tools to improve the role of the bacteria strains in this diol production.

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