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Biotechnological production of 1,3-propanediol from crude glycerol

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Abstract

1,3-Propanediol (1,3-PD) is one of the important products used in chemical industry, in particular for polyesters production (e.g. polyethers and polyurethanes). Using crude glycerol for producing 1,3-PD is a good solution from the economical as well as ecological point of view. Glycerol produced by cleavage of natural fats can be microbially converted to 1.3-propanediol by, among others, *Citrobacter*, *Klebsiella, Lactobacillus, Enterobacter,* and *Clostridium* strains. Biotechnological production of 1,3-PD from waste biomass is a promising and attractive alternative to the traditional chemical synthesis. The production of 1,3-PD by glycerol fermentation was already reported in 1881. The microbiological bioconversion pathway of glycerol to 1,3-PD has been known for long but the microorganisms taking part in this fermentation are not efficient. In addition, they are pathogenic. Consequently, natural producers of 1,3-PD are still being sought. In this review we present a historical outline of 1,3-PD production, as well as the microorganisms and their metabolic pathways that are involved in glycerol fermentation to 1,3-PD.

Key words: bioconversion, glycerol, HPLC, 1,3-propanediol

Introduction

The shortage of resources of crude oil has induced an increase in biofuels production. Biofuels are a wide range of fuels which are in some way derived from biomass. The most common biofuel in Europe is biodiesel. Biodiesel is made from vegetable oils, animal fats or recycled greases. An increase in biodiesel production generates a huge amount of waste glycerol – one part of glycerol is produced for every 10 parts of biodiesel. This fact has a negative influence on the price of biodiesel. In 2009, the biodiesel production rose up to 10 million tons per year in UE, and 1.5 million tons in Poland (Papanikolaou et al., 2000; Kośmider et al., 2009). In the biodiesel production process, the glycerin phase is obtained as a by-product. The glycerin phase includes glycerol, methanol, mono- and diaceloglycerols, fatty acids, and soap. Approximately 200-300 tones of the glycerin phase are generated every year, causing environmental problems regarding the management of this by-product. One solution to this threat is an application of a glycerin phase or raw glycerol as a carbon source in microbial growth media used in the production of a metabolite such as 1,3-PD (Kośmider et al., 2009; Kośmider et al., 2010). Moreover, crude glycerol may also be used as a raw material in citric acid, succinic acid, propionic acid, and dihydroxyacetone production (Rywińska, 2010; Kośmider et al., 2009).

1,3-PD is also known as a trimethylene glycol, 1.3-dihydroxypropane, propane-1,3-diol. Molecular formula of the compound is $C_3H_8O_2$ (Fig. 1), molecular mass 76.09 g $\times$ mol^{-1}, the boiling point is 210-212 °C and melting point is −28 °C (Igari et al., 2000). 1,3-PD is a typical product of glycerol fermentation (Katrlik et al., 2007). It is a valuable chemical intermediate potentially used in the manufacture of polymers (among others, polyesters, polyethers, polyurethanes), cosmetics, lubricants, medicines, and as an intermediate in the synthesis of heterocyclic compounds (Menzel et al., 1997; Biebl et al., 1999). Recently, 1,3-PD is also used as a monomer to synthesize a new type of polyester – polytrimethylene terephthalate (Biebl et al., 1999; Zeng et al., 2002; Liu et al., 2007; Zhang et al., 2007).

In the past, 1,3-PD was produced only chemically by two methods: the hydration of acrolein or the hydroformylation of ethylene. The chemical synthesis, however, has many disadvantages – it requires high pressure, high temperature and catalysts. Consequently, the costs of 1,3-PD production are very high (Igari et al., 2000). An

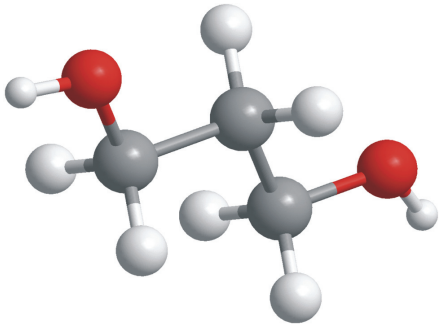

Fig. 1. A ball and stick model of the 1,3-propanediol molecule. (Werle et al., 2006)

attractive alternative for chemical synthesis is a microbial conversion of raw materials to 1,3-PD. This method is easy and does not generate toxic by-products. Nevertheless, the major limitation for industrial microbial production of 1,3-PD is the relatively high cost of the typical substrate such as glucose. The economically attractive solution to this problem might be the use of crude glycerol (without prior purification) as a fermentative substrate, (Nakumara et al., 2003; Mu et al., 2006).

Glycerol is a renewable resource found as a by-product of ethanolic fermentation of glucose (Petitdemange et al., 1995; Kośmider et al., 2009). Glycerol can be converted to 1,3-PD by many microorganisms such as *Klebsiella pneumonia*, *Bacillus welchi, Lactobacillus* ssp., *Enterobacter* spp., *Citrobacter* spp., and *Clostridia* spp. (Liu et al., 2007). It is fermented by a dismutation process involving two parallel pathways. In one pathway, glycerol is transformed to dihydroxyacetone by a glycerol dehydrogenase and in the other one a coenzyme B_{12}-dependent glycerol dehydratase converts glycerol to 3-hydroxypropionaldehyde (Daniel et al., 1999; Zhang et al., 2007). Other metabolites can also be obtained from glycerol, e.g. dihydroxyacetone, succinic acid, citric acid, docosahexanoic acid, propionic acid, hydrogen, and ethanol (Daniel et al., 1999; Kośmider et al, 2009).

This review deals with the biotechnological production of 1,3-PD as a good alternative to the conventional chemical synthesis of this compound.

Historical outline

1,3-PD – one of the oldest known fermentation products – was identified in 1881 by August Freund, as a product of glycerol fermentation by *Clostridium pasterianum*. Next, in 1914, the *Bacillus* ssp. which produces this substance was described by Voisenet. In 1928, the Microbiology School of Delft commenced analyses of the fermentation process with different *Enterobacteriaceae* producing 1,3-PD. This research was later continued at Ames, Iowa, in the U.S. However, the first *Clostridium* ssp. producing 1,3-PD were described as late as in 1983, (Nakas et al., 1983; Biebl, 1999).

In the 1990s, three technical processes for the production of 1,3-PD were developed: the first one uses acrolein, the second ethylene oxide, and in the third one glucose is used as a raw material. Nowadays, there is an increasing interest in microbial production of 1,3-PD especially from oil industry (Katrlík et al., 2007).

Microorganisms

A number of microorganisms can grow anaerobically on glycerol as the sole carbon and energy source. This group includes: *Clostridium butyricum* (Colin et al., 2000), *Clostridium pasteurianum* (Biebl et al., 1992), *Clostridium diols*, *Clostridium acetobutylicum*, *Clostridium butylicum*, *Clostridium perfingens*, (Hao et al., 2008), *Enterobacter agglomerans* (Barbirato et al., 1998), *Enterobacter aerogenes* (da Silva et al., 2009), *Klebsiella pneumonia* (Biebl et al., 1998), *Klebsiella oxytoca* (Homann et al., 1990), *Klebsiella aerogenes*, *Citrobacter freundii* (Malinowski, 1999), *Lactobacillus reuterii*, *Lactobacillus buchnerii*, *Lactobacillus collinoides, Pelobacter carbinolicus*, *Rautella planticola* (Saxena et al., 2009), and *Bacillus welchii* (da Silva et al., 2009). Unfortunately, a huge problem is that the best 1,3-PD producers are pathogenic. In Table 1 two potential industrial producers of 1,3-PD from glycerol – *Clostridia* and *Enterobacteriaceae* are compared, and Table 2 presents production of 1,3-PD from crude and pure glycerol by *Clostridium butyricum*, *Clostridium acetobutyricum*, and *Klebsiella pneumoniae*.

The production of 1,3-PD from glycerol is generally performed under anaerobic conditions using glycerol as the sole carbon source in the absence of other exogenous reducing equivalent acceptors (Nakamura et al., US Patent, 2000). However, such strains like *Klebsiella Pneumoniae*, *Klebsiella oxytoca*, *Klebsiella aerogenes*, *Enterobacter agglomerans*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Lactobacillus reuteri*, *Lactobacillus buchnerii*, *Lactobacillus collinoides*, *Pelobacter carbinolicus*, and *Rautella planticola* can produce 1,3-PD in micro-aerobic fermentation (Biebl et al., 1998; Saxena et al., 2009, da Silva et al., 2009).

Table 1. Comparison of two potential industrial producers of 1,3-PD from glycerol – *Clostridium* and *Enterobacteriaceae* (Willke and Vorlop, 2008)

Clostridium	*Enterobacteriaceae*
Risk Class 1 (GRASS)	Risk Class 2 (potentially pathogenic)
Strictly anaerobic, therefore difficult to handle	Facultative aerob, robust organisms, easy handling
Spore forming	No sporulation
Main by-products: acetic acid, butyric acid	Main by-products: ethanol, acetic acid
Yield: about 0.5 kg PDO/kg glycerol	Yield: about 0.4 kg PDO/kg glycerol
Max. theor. yield: 0.72 mol/mol	Max. theor. yield: 0.72 mol/mol

Table 2. Production of 1,3-PD from crude and pure glycerol by *Clostridium* and *Klebsiella pneumoniae* strains (modified, based on Choi, 2008)

Product (concentration, yield)	By-products	Substrate	Strain
1,3-propanediol (48 g/l, Y = 0.66)	acetate, butyrate	crude glycerol	*Clostridium butyricum* F2b
1,3-propanediol (30.5 g/l, Y = 0.61)	acetate, butyrate	crude glycerol	*Clostridium acetobutylicum* DG1 (pSPD5)
1,3-propanediol (86 g/l, Y = 0.65)	acetate, butyrate	pure glycerol	*Clostridium acetobutylicum* DG1 (pSPD5)
1,3-propanediol (66.6 g/l, Y = 0.69)	butyrate	pure glycerol	*Clostridium butyricum* VPI3266
1,3-propanediol (48.5 g/l)	butanediol, acetate	pure glycerol	*Klebsiella pneumoniae* DSM 2026
1,3-propanediol (63.4 g/l, Y = 0.69)	acetate, butyrate	crude glycerol	*Clostridium butyricum* CNCM 1211
1,3-propanediol (70.5 g/l, Y = 0.699)	acetate, ethanol, lactic acid, 2,3-butanediol	pure glycerol	*Klebsiella pneumoniae* DA-1HB
1,3-propanediol (63.2 g/l, Y = 0.6)	not determined	crude glycerol	*Klebsiella pneumoniae* ACCC10082

Factors influencing product formation

In the 1,3-PD fermentations, product formation depends mainly on the ability of the sources of carbon and energy as well as on hydrogen concentration. In the case of fermentation by *Klebsiella pneumoniae* when glycerol is a limiting factor, the formation of cell biomass is optimized and large amounts of ethanol are produced. However, if only glycerol appears in the medium (because of increasing inhibition by the products), ethanol formation ceases and the 1,3-PD yield approaches its maximum value. When the excess of glycerol increases, lactic acid, 2,3-butanediol and succinic acid can be observed, in yields respectively to about 10, 7 and below 2% of the glycerol. The formation of butyrate in *Clostridia* is comparable to ethanol formation in *Klebsiella*. However, it is more dependent on the growth rate. In the case of the absence of substrate excess, rapid decrease of butyrate is observed (Biebl et al., 1998; Biebl et al., 1999).

In batch fermentation with *Enterobacteriaceae* the accumulation of 3-hydroxypropionaldehyde is observed. It is a toxic metabolite which inhibits the growth of bacteria and the formation of 1,3-PD (Zeng et al., 1993; Zeng et al., 1994; Barbirato et al., 1996; Biebl et al., 1998; Biebl et al., 1999).

The productivity of 1,3-PD can be improved through the application of metabolic and genetic engineering

procedures. Basically, it may be done in three ways: (i) by introduction an additional gene coding for the enzyme that allows the formation of glycerol from sugars or intermediates of glycolysis, into a bacterial strain that already produces 1,3-PD from glycerol; (ii) by introduction of genes coding for enzymes which allow the conversion of glycerol into 1,3-PD into an organism which could not do it naturally; or (iii) by introduction of both types of genes into an organism which could not produce glycerol, intermediates of glycolysis and 1,3-PD (Laffend et al., 1997; Nevoigt and Stahn, 1997; Saxena et al., 2008; Celińska, 2010).

There are a few scientists who use these strategies today. Among them is Nakamura who together with his colleagues reported that 1,3-PD can be produced by recombinant microorganisms from various sugars; e.g. from glucose, fructose, lactose, sucrose, maltose, and mannose (Nakamura et al. 2000). The other one, Chotani, constructed a strain of *E. coli* containing the genes from *Saccharomyces* and *K. pneumoniae* for glycerol and 1,3-PD production, respectively (Chotani et al. 2000). In addition, Saxena and co-workers obtained a genetically modified *E. coli* strain which can produce 1,3-PD from glucose (Saxena et al., 2008).

In a report of DuPont and Genecor International Inc., a metabolically engineered *E. coli* could produce up to 135 g/l of 1,3-PD with a yield of 0.6 mol 1,3-PD/mol glucose (Nakamura et al., 2003; Saxena et al., 2008).

Metabolic pathways

Klebsiella spp., *Citrobacter* spp., *Clostridium* spp., *and Enterobacter* spp. metabolize glycerol both oxidatively and reductively. In the oxidative pathway the conversion of glycerol to dihydroxyacetone is catalyzed by the NAD^+-dependent glycerol dehydrogenase. The next step is phosphorylation of the latter product, which is catalyzed by the glycolytic enzyme dihydroxyacetone kinase. Then, the phosphorylated product is subjected to glycolysis (Daniel et al., 1995; Luers et al., 1997; Macis et al., 1998; Zhu et al., 2002; da Silva et al., 2009). In the reducing pathway, glycerol is converted to 3-hydroxypropionaldehyde in reactions catalyzed by coenzyme B_{12}-dependent glycerol dehydratase and related diol dehydratases. Next, 3-hydroxypropionaldehyde is reduced to 1,3-PD and the NAD^+ is regenerated by the $NADH^+H^+$-dependent enzyme 1,3-propanediol dehydrogenase (1,3-propanediol-oxydoreductase) (Ahrens et al.,1998; da Silva et al., 2009; Forage and Foster, 1982; Knietsch et al., 2003; Németh et al., 2003).

Figure 2 shows the biochemical pathways for glycerol fermentation to 1,3-PD as the end product. During this process, glycerol is dehydrogenated to dihydroxyacetone which then can be converted (after phosphorylation) to pyruvate. This step is catalyzed by the enzyme glycerol NAD^+ dehydrogenase. Glycerol dehydrogenase is a coenzyme B_{12}-dependent enzyme composed of three polypeptides. It catalyzes the free radical mediated conversion of glycerol to 3-hydroxypropionaldehyde (Nakumara et al., 2003). The reductive glycerol conversion consists in a vitamin B_{12}-mediated dehydration to 3-hydroxypropionaldehyde and a reduction of the aldehyde to 1,3-PD (Biebl et al., 1999; Katrlík et al., 2007). Unfortunately, in all of the wild 1,3-PD producers, there is a low molar yield of 1,3-PD to glycerol conversion – 0.51 to 0.65 or 0.54 to 0.64 when 20 g/l or 70 g/l of glycerol is added to the culture media respectively (Zhang et al., 2007).

Pyruvate formed during glycerol conversion may be utilized in different ways. For example, in the *Enterobacteriaceae* it is cleaved to acetyl-CoA. It can also be condensed to α-acetolactate and finally transformed to acetoin and 2,3-butanediol. As a result of glycerol fermentation by *Enterobacteriaceae*, the accumulation of two main products, 1,3-PD and acetate, is observed. Depending on the culture conditions, the secondary products, lactate, formate, succinate and ethanol, are generated in variable amounts (Dabrock et al., 1992; Biebl et al., 1999). In case of 1,3-PD production by *Lactobacillus brevis* acetic acid, ethanol, and lactic acid are also obtained as by-products (Willke and Vorlop, 2008). The 1,3-PD is the main product together with butyric acid and acetic acid as by-products when fermentation is conducted by *Clostridium butyricum*. *Cl. butyricum* is one of the best "natural producers" of 1.3-PD. Also a variety of metabolic end products, such as 1,3-PD, n-butanol, ethanol, acetic acid, butyric acid, and lactic acid, are obtained as a result of glycerol fermentation by *C. pasteurianum* (Homann, 1990; Barbirato et al., 1998; Biebl, 2001; da Silva et al., 2009) (Fig. 3). The synthesis of 1,3-PD by this microorganism is not a vitamin B12-dependent process, which is clearly an economical advantage for an industrial application (Saint-Amans et al., 1994; Gonzàlez-Pajuelo et al., 2006).

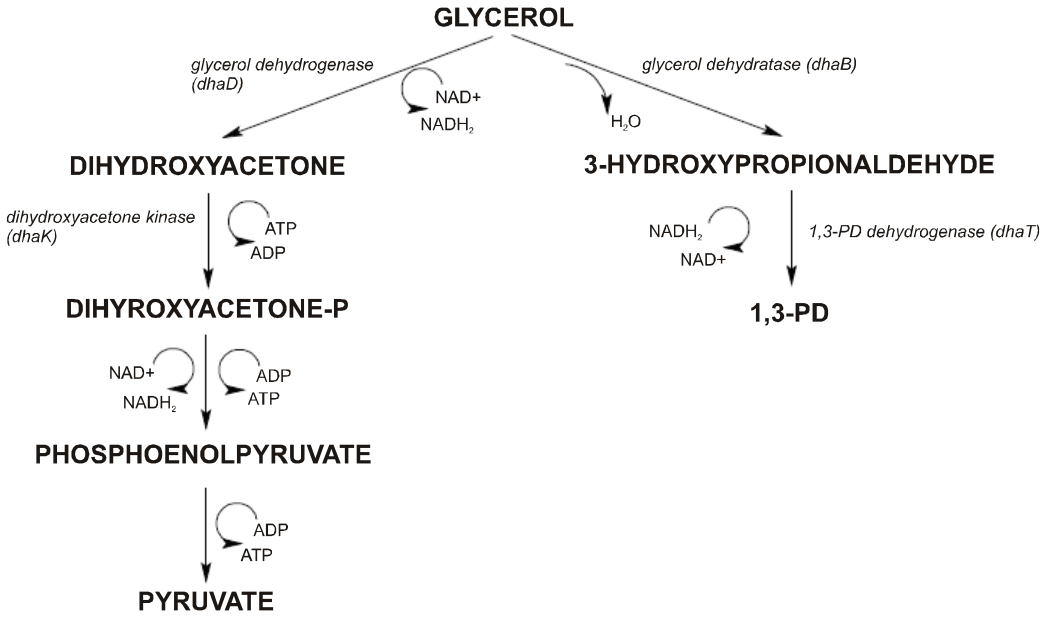

Fig. 2. Biochemical pathways of glycerol fermentation (Biebl et al., 1999)

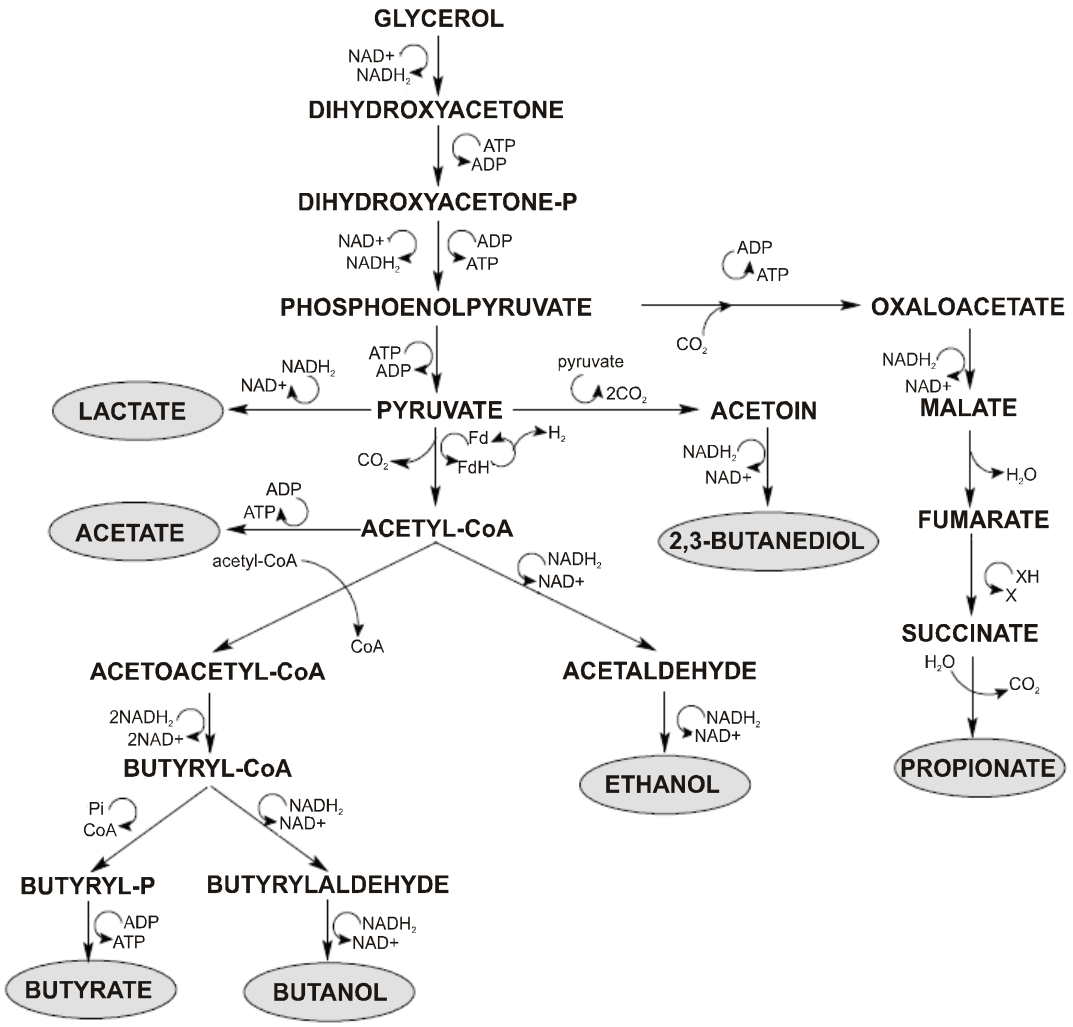

Fig. 3. The end-products of glycerol fermentation by different microorganisms (da Silva et al., 2009)

Problems in microbial production of 1,3-PD

The main reason for the low yield and productivity of glycerol fermentation to of 1,3-PD is that bioprocesses are carried out at physiological temperature, atmospheric pressure and mostly in batch operation mode (Zeng et al., 2002). A process approach can have a significant effect on volumetric production rates by increasing cell density in the bioreactor and by developing a continuous process. This is mostly achieved by the optimization of the process conditions along with the application of fed-batch and continuous operation mode with cell recycling or with immobilized cells (Saxena et al., 2009; Zeng et al., 2002). The rather low product concentration, compared with chemical processes resulting in high downstream processing costs, is the other limitation of bioprocesses. This is mainly caused by product-mediated inhibition of cell growth and biosynthesis. Physiological improvements in cell growth and product formation have only a limited impact. Chemical or directed mutagenesis may provide a better chances to overcome this problem. Unfortunately, the molecular mechanisms of product-mediated inhibition are not well understood (Zeng et al., 2002).

To make 1,3-PD production economical, by-products or waste streams generated during the production of the biofuels are used. In this context, glycerol-rich streams, which occur during biodiesel production, may have a potential use. (Saxena et al., 2009). Utilization of raw glycerol in the fermentation process offers a remarkable advantage against pure glycerol. However, it is still hard to escape the general conclusion that utilizing raw glycerol as the starting feedstock for microbial production is difficult. It is due to such reasons like high crude glycerol prices as have been the case over the past two years and very low value of crude glycerol derived as a by-product from biofuels production processes (Yazdani et al., 2007; Rehman et al., 2008; Saxena et al., 2009).

Optimization of 1,3-PD production by *Klebsiella pneumoniae* bioconversion process

As it was mentioned before, in addition to the product-mediated inhibition, also by-products (i.e. ethanol, 2,3-butanediol, acetic acid) can limit the yield of 1,3-PD production. 3-hydroxypropionaldehyde seems to be the strongest inhibitor. This compound is normally an intermediate that does not accumulate within the cell. However, under conditions of high glycerol excess, it may be excreted into the medium (Zeng et al., 2002).

The main ways to optimize the microbial production of 1,3-PD from glycerol are: preventing undesired by-product formation in order to achieve high product yield; increasing the productivity of the bioreactor; and increasing the tolerance for 1,3-PD in order to maximize the final product concentration (Zeng et al., 2002).

In the case of *Klebsiella pneumoniae*, in continuous culture, under conditions of high glycerol excess and significant product inhibition, other by-products (inter alia 2,3-butanediol and lactic acid) appear in the medium diminishing the propanediol yield (Zeng et al., 1994; Menzel et al., 1997; Zeng et al., 2002). The pH lower than 6.5 is not favorable to 1,3-PD production because low pH stimulates the production of 2,3-butanediol (Biebl et al., 1998; Zeng et al., 2002; Celińska et al., 2009). It was also observed that hydrogen gas released from pyruvate cleavage to acetyl coenzyme A increases the efficacy of propanediol formation (Zeng et al., 1993; Zeng, 1996; Zeng et al., 2002). *K. pneumoniae* simultaneously use two enzymes pyruvate dehydrogenase and pyruvate formate lyase for anaerobic cleavage of pyruvate in the glycerol fermentation. Pyruvate dehydrogenase generates $NADH_2$ from pyruvate cleavage (instead of forming formate with pyruvate formate lyase) leading to an increased yield of 1,3-PD. However, if acetyl-CoA is channeled into the tricarboxylic acid cycle, the yield of 1,3-PD is much higher (Zeng et al., 2002). *Klebsiella pneumoniae* is a good 1,3-PD producer, however the use of this microorganism is limited because of its pathogenic properties.

Future prospects

The demand for energy sources is increasing together with the increasing population of the world. The overall biodiesel production in the EU increased twice during the last six years. Moreover, biodiesel production in the EU is forecast to increase to 12 Mt in 2011. By 2016, the global biodiesel market is estimated to reach 37 billion gallons (Sims, 2007; Behr et al., 2008; Saxena et al., 2009).

Bioconversion of crude glycerol obtained in biodiesel production seems to be highly recommended. First of all, it provides substrates for the production of biodegradable polymers and this way directly benefit the environ-

ment. In addition, wide application of biodegradable polymers would promote the use of biodiesel and reduce petroleum dependency (Saxena et al., 2009). An interesting example of application of the product of glycerol fermentation is a polyethylene terephthalate (PTT) production. PTT polymer is a member of a family of polymers based on fiber-grade 1,3-PD. It is a linear crystallizable polymer with a melting temperature of about 228 °C and a glass transition temperature of about 50 °C. Production of such polymers with the use of biological systems has a number of advantages over classical chemical technologies e.g. greenhouse gas emission in the manufacture of bio-1,3-PD has been demonstrated to be about 40% less than for petrochemical 1,3-PD. Moreover, recycling of PTT is made much easier owing to the absence of heavy metals in the product, compared to PET and Nylon (Kurian, 2005).

Conclusion

Today there is considerable industrial interest in microbial-based 1,3-PD production, as it appears to be competitive with traditional technologies utilized to obtain this compound. 1,3-PD is a very useful bulk chemical, with a variety of applications. It is used in the manufacture of polymers, cosmetics, food, lubricants, and medicines. 1,3-PD can be produced by chemical synthesis or by biotechnological routes from waste biomass, for example from crude glycerol obtained in the production of biodiesel. The biotechnological method seems to be an attractive alternative to the traditional chemical production. However, the main microorganisms which can be used in 1,3-PD production are pathogenic. Our task for the future is to find effective non-pathogenic microorganisms capable of producing 1,3-PD from glycerol.

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