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Review

Biotechnological synthesis of 1,3-propanediol using *Clostridium* ssp.

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1,3-Propanediol (PD) is an important chemical product which can be used for synthesis reactions, in particular, as a monomer for polycondensations to produce polyesters, polyethers and polyurethanes. It is produced by two methods, chemical synthesis and microbial conversion. Recently, the increasing interest in microbial conversion was observed. Glycerol is used as a substrate in this process and it may be fermented to 1,3-PD by, among others, *Citrobacter* ssp., *Klebsiella* ssp., *Lactobacillus* ssp., *Enterobacter* ssp. and *Clostridium* ssp. strains. The process of microbiological bioconversion pathway of glycerol to 1,3-PD is well known for a long time but microorganisms taking part in this fermentation are pathogenic. Thus, natural producers of 1,3-PD that are non-pathogenic and efficient enough, are still sought. This review deals with the case of 1,3-PD production and microbial formation of 1,3-PD, especially by *Clostridium* ssp. Moreover, it presents genetic engineering methods used in increasing microorganisms' efficiency in the glycerol to 1,3 PD fermentation.

Key words: 1,3-Propanediol, Clostridium ssp., fermentation, glycerol.

INTRODUCTION

Bacteria which belong to the genus *Clostridium* are relatively large, Gram-positive, heterotrophic, endospore-forming and motile rods. They are typical anaerobic microorganisms. Most of them are mesophilic, though some are psychotropic or thermopholic. The natural environment of clostridia is anaerobic habitats with organic nutrients such as soils, feeding stuffs, aquatic sediments and the intestinal tract of humans and animals. *Clostridium* genus consists of circa 100 species that include common free-living bacteria as well as important pathogens. There are four main species responsible for diseases in humans and animals: *Clostridium botulinum* (which causes botulism), *Clostridium perfringens* (which causes surgical infection– gas gangrene), *Clostridium tetani* (responsible for deadly tetanus infections) and *Clostridium difficiele* (which causes intestine inflam-mation, especially in children and hospital patients). Some species of *Clostridium* are responsible for food poisoning, particularly in the case of canned food (*C. botulinum, C. perfringens*, *Clostridium putrefaciens*, *Clostridium butyricum*, *Clostridium tyrobutyricum* and *Clostridium sporogenes*) (Gerding, 2009; Siegrist, 2010; Songer, 2010). A considerable biochemical activity of *Clostridium* spp. is connected with an extended range of extracellular enzymes which they produce. This bacteria can cause fermentation of organic compounds such as sugars and produce large amounts of CO_2, H_2, as well as organic compounds like organic acids (especially butyric and acetic acids), butanol and acetone. Metabolism of amino acids and fatty acids by clostridia results in the formation of foul-smelling degradation products (Buckel, 2005). The non-pathogenic clostridia have a large potential industrial application. They are used for production of butyric acid (Zhang et al., 2009; Nicolaou et al., 2010), and some solvents such as butanol, acetone, isopropanol (so-called solventogenic clostridia) (Dürre, 1998, Ezeji et al., 2005; Ezeji et al., 2007) and hydrogen (Skonieczny and Yargeau, 2009; Kothari et al., 2010).

It is well known for almost 60 years that glycerol is fermented by facultative anaerobic bacteria, among others, by *Clostridium* ssp. to 1,3-propanediol (1,3-PD), 2,3-butanediol, ethanol and acetic acid. Among these

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Abbreviation: PD, 1,3-Propanediol

substances, 1,3-PD is of industrial interest as a monomer for light-insensitive plastics, and some strains indeed form this diol as the main product. The production organisms belong mainly to the *Enterobacteriaceae* family (*Citrobacter* ssp., *Entorobacter* ssp., *Citrobacter* ssp., and *Klebsiella* ssp.). Moreover, some species of *Clostridium* are also able to convert glycerol to 1,3-PD (Luers et al., 1997). In this process, *Clostridium pasteurianum, C. butyricum, Clostridium diolis* and *Clostridium acetobutylicum* are mostly used (Biebl, 2001).

1,3-PD, a typical product of glycerol fermentation, is a valuable chemical intermediate potentially used in the facture of polymers (among others, polyesters, polyethers and polyurethanes), cosmetics, foods, lubricants, medicines and as an intermediate for the synthesis of heterocyclic compounds (Menzel et al., 1997; Biebl et al., 1999; Katrlik et al., 2007). Recently, 1,3-PD was also used as a monomer to synthesize a new type of a polyester– polytrimethylene terephthalate (Biebl et al., 1999; Zeng and Biebl, 2002; Liu et al., 2007; Zhang et al., 2007).

In this article, recent progress in this field, including the use of *Clostridia* spp. in the production of 1,3-PD, is reviewed.

THE CASE OF 1,3-PD PRODUCTION

For a long time, the chemical industry considered bio-technology as a very expensive high-tech tool that is not appropriate for bulk chemical synthesis on a large scale. This thesis is gradually changing as reflected by several current development projects of major chemical companies wishing to produce bulk chemicals by biotechnological way. It mainly concerns 1-3 PD, lactic acid and succinic acid (Zeng and Biebl, 2002; Zhang et al., 2007).

1-3 PD has several promising properties for many synthetic reactions as a monomer for polycondensation to produce polyethers, polyesters and polyurethanes. In the past, this diol was used only as a solvent for the production of dioxanes and specialty polymers that have small market volumes. This limited interest in 1,3-PD production lasted up to 1995 to 1996 when two big chemical companies, Shell and Dupont, announced their commercialization of a new polyesters based on 1,3-PD, polythrimethylene terephthalate (Shell) and terephthalate polymer (DuPont) (Biebl, 1999; Zeng and Biebl, 2002). This copolyester is a condensation product of 1,3-PD and terephthalic acid and has such beneficial properties as good resilience, low static generation and stain resistance. Moreover, it is particularly suitable for fiber and textile applications. This diol can also improve properties of solvents (it increases flexibility in blending ester quats), laminates, adhesives, detergents (it prevents phase separation and loss of enzyme activity) and cosmetics (it gives long-lasting effects). Due to these, new applications production of 1,3-PD increased up to 70000 to 80000 t/a in 2000 (Zeng and Biebl, 2002), and up to 100000 t/a in 2009 (da Silva, 2009).

In the past, 1,3-PD was produced only chemically in two pathways: by the hydratation of acrolein or by hydroformylation of ethylene (however, both methods are very expensive). The chemical synthesis has many disadvantages: it requires high pressure, high temperature and catalysts, which increases the costs of this process (Igari et al., 2000). An attractive alternative is a microbial conversion of raw materials (such as glycerol or glycerol phase) to 1,3-PD. This way is easy and does not generate toxic by-products (Nakumura and Whited, 2003; Mu et al., 2006).

MICROBIAL FORMATION OF 1-3 PD.

Recently, microbial production of 1-3 PD, a socially beneficial route to obtain chemicals from renewable resources, is widely researched as a competitor to traditional petrochemical routes (Ma et al., 2009; Zhao et al., 2006).

The bacterial fermentation in which glycerol is converted to 1,3-PD has been known already for 120 years (Zeng and Biebl, 2002). 1,3-PD was identified in 1881 as a product of glycerol fermentation by *C. pasteurianum*. Nowadays, a number of microorganism which can grow anaerobically on glycerol are known, these are: *C. diolis*, *C. acetobutylicum*, *C. butylicum*, *C. perfingens* (Hao et al., 2008), *C. butyricum* (Colin et al., 2000), *C. pasteurianum* (Biebl, 2001), *Enterobacter aerogenes* (da Silva et al., 2009), *Enterobacter agglomerans* (Barbirato et al., 1998), *Klebsiella oxytoca* (Homann et al., 1990), *Klebsiella pneumonia* (Biebl et al., 1998), *Klebsiella aerogenes*, *Citrobacter freundii* (Malinowski, 1999), *Lactobacillus collinoides, Lactobacillus reuterii*, *Lactobacillus buchnerii*, *Pelobacter carbinolicus*, *Rautella planticola* (Saxena et al., 2009) and *Bacillus welchii* (da Silva et al., 2009).

In glycerol to 1,3-PD bioconversion, *K. pneumoniae* has been widely investigated due to its high productivity (Menzel et al., 1999; Liu et al., 2007). *K. pneumoniae* is a facultative bacterium. Glycerol can be dissimilated to 1,3-PD under anaerobic or aerobic conditions (Chen et al., 2003; Liu et al., 2007) (Figure 1). In the presence of exogenous electron acceptors, the dissimilation is initiated by an ATP-dependent kinase. This enzyme is encoded by the *glp* regulon and catalyzes the phosphorylation of glycerol to glycerol-3-phosphate. The next step is to convert the phosphorylated intermediate to dihydroxyacetone phosphate by one of the flavin adenine dinucleotide-dependent dehydrogenases coupled to an electron transport chain (Lin and Magasanik, 1960; Johnson and Lin, 1987). In the absence of oxidant, glycerol is fermented by a dismutation process involving two parallel pathways encoded by the *dha* regulon. In the

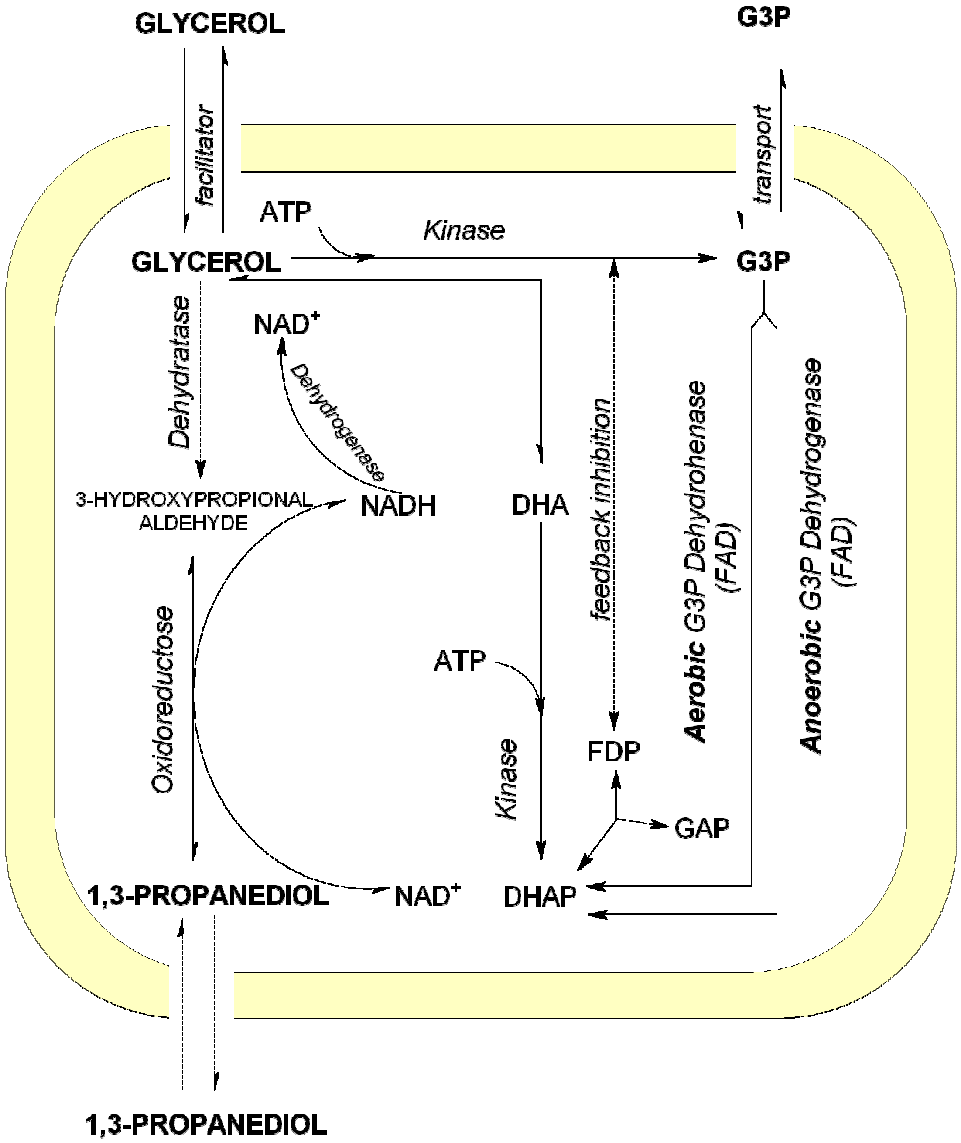

Figure 1. Pathway for the dissimilation of glycerol in *K. pneumoniae* (modified, based on Chen et al., 2003; Liu et al., 2007; Lin and Magasanik, 1960).

first pathway, glycerol is dehydrogenated by an NAD-linked enzyme to dihydroxyacetone. Then, dihydroxyacetone is phosphorylated by an ATP-dependent kinase. In the other pathway, glycerol is dehydrated by a B12-dependent enzyme to form 3-hydroxypropionaldehyde. Next, 3-hydroxypropionaldehyde is reduced to 1,3-propanediol by an NADH-linked oxidoreductase, thereby regenerating NAD^+ (Lee and Abeles, 1963; Forage and Foster, 1982; Forage and Lin, 1982; Johnson and Lin, 1987). *C. freundii* and *E. agglomerans* has the same mechanism of glycerol dissimilation as *K. pneumoniae* (Daniel et al., 1995; Hatayama and Yagishita, 2009). Some strains of *Lactobacillus brevis*, *Lactobacillus buchneri*, as well as *Lactobacillus reuteri* can grow on glycerol by cofermenting it with glucose or fructose. All these bacteria have a coenzyme B12-dependent dehydratase that converts glycerol to 3-hydroxypropionaldehyde (3-HPA) (Veiga-DA-Cunha and Foster, 1992). *L. reuteri* is unique among bacteria with its ability to produce and excrete a broad spectrum of an antimicrobial agent during anaerobic dissimilation of glycerol. This agent, reuterin, inhibits the growth of Gram-positive and negative bacteria, and lower eucaryotic organisms (Todd et al., 1990).

The yield of 1,3-PD production using diverse bacteria strains is presented in Table 1. As a carbon source, pure and crude glycerols were used.

Table 1. Production of 1,3-PD from glycerol using diverse bacteria strains.

Substrate	Culture	By-product	Product (concentration/yield)	Reference
Crude glycerol	*Clostridium butyricum* F2b	Acetate, butyrate	(48 g/l, Y= 0.66)	Papanikolaou et al., 2000
Crude glycerol	*Clostridium acetobutylicum* DG1 (pSPD5)	Acetate, butyrate	30.5 g/l, Y=0.61	Gonzales-Pajuelo, 2005
Pure glycerol	*Clostridium acetobutylicum* DG1 (pSPD5)	Acetate, butyrate	86 g/l, Y= 0.65	Sacrabal, 2007
Pure glycerol	*Clostridium butyricum* VPI3266	Butyrate	66.6 g/l, Y= 0.69	Soucaille, 2008
Crude glycerol	*Clostridium butyricum* CNCM 1211	Acetate, butyrate	63.4 g/l, Y=0.69	Barbirato et al., 1998

Yield: Mol product per mol glycerol.

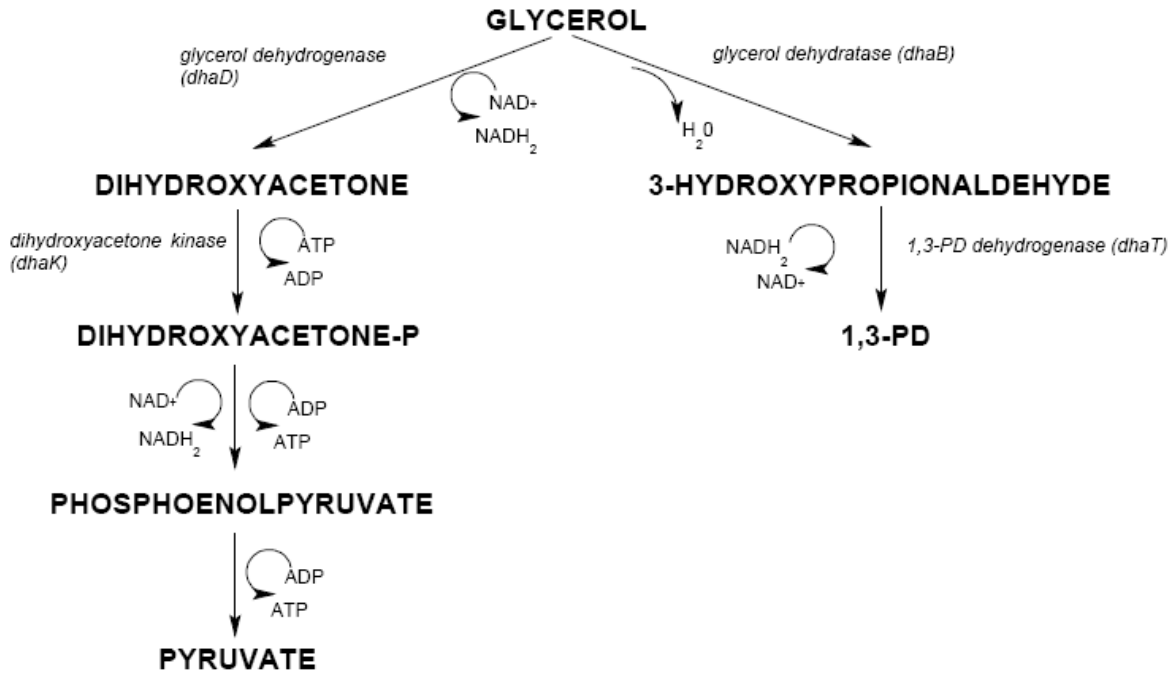

Figure 2. Biochemical pathways of glycerol fermentation (based on Biebl et al., 1999).

APPLICATION OF *CLOSTRIDIUM* STRAINS TO 1-3 PD PRODUCTION

Production of 1-3 PD by several groups of bacteria is known for a long time. However, in recent years, interest in application of *Clostridium* ssp. increased (Vasconcelos et al., 1994; Abbad-Andaloussi et al., 1995). In 1983, fermentation of *C. pasteurianum* was first described (Nakas et al., 1983). The main product in this fermentation was n-butanol, while 1,3-PD, ethanol and acetic acid were also produced (Biebl, 2001). A typical biochemical pathway of glycerol fermentation is presented in Figure 2, and the metabolic pathway in the glycerol fermentation by *C. pasteurianum* is presented in Figure 3. The difference is in NAD+ re-generation which additionally occurs in *Clostridium* ssp. via the reactions leading to butyric acid biosynthesis from acetyl-CoA, since this series of reactions involves two $NADH_2$-oxidizing steps per molecule of butyrate produced. This pathway should be considered as an antagonistic one to

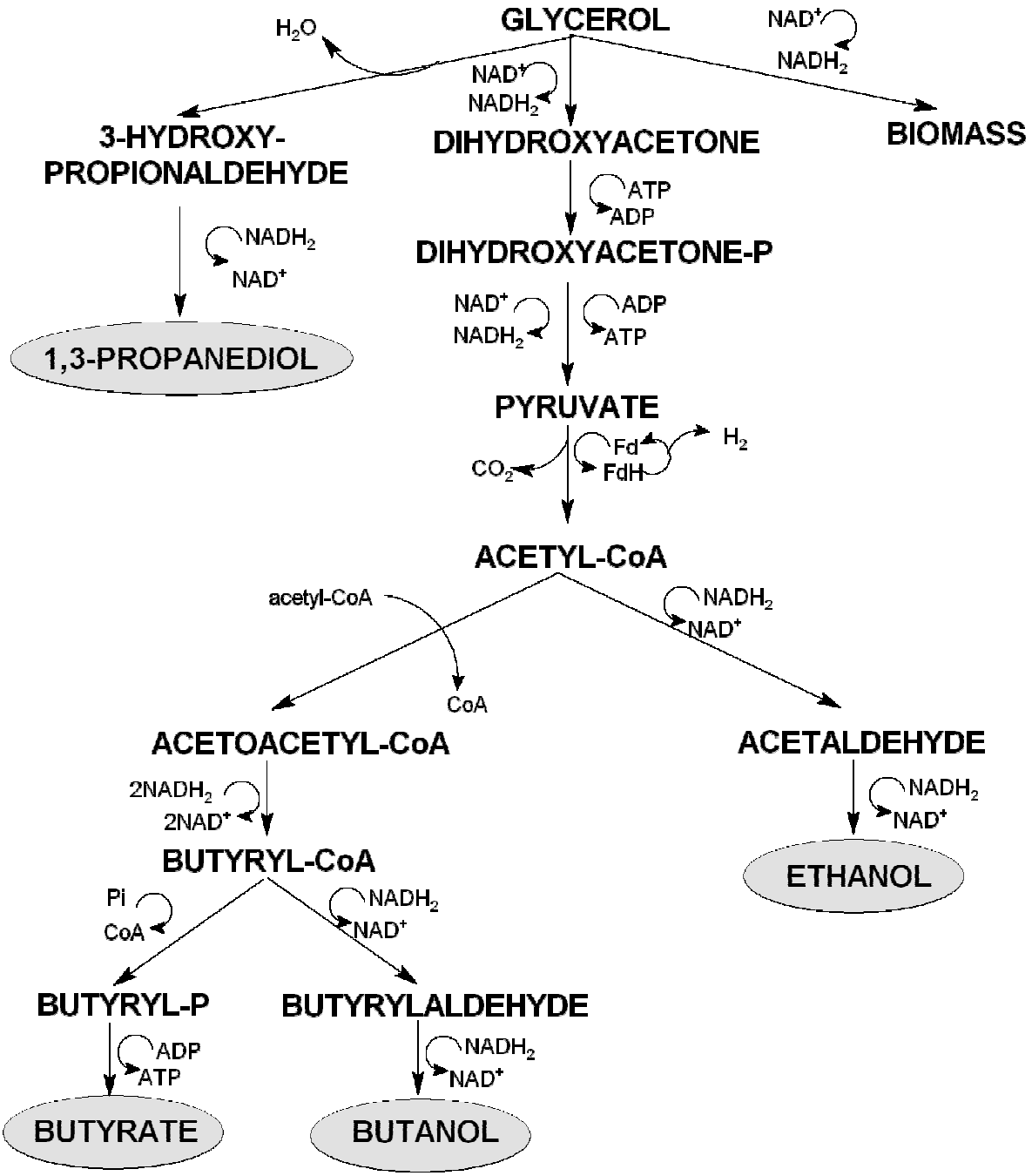

Figure 3. Metabolic pathways in the glycerol fermentation of *C. pasteurianum* (based on Biebl, 2001).

that of 1,3-propanediol production (Papanikolaou et al., 2004; Drożdżyńska, 2011).

The other *Clostridium* species, among other four strains of *C. acetobutylicum*, six strains of *C. butylicum*, two strains of *Clostridium beijerinckii*, one strain of *Clostridium kainantoi*, and three strains of *C. butyricum*, has also been reported as strains that are able to ferment glycerol with the production of many fermentation products, including acetic acid, butyric acid, ethanol, acetone, butanol, acetoin and 1,3-PD (Rorsberg, 1987; Papanikolaou et al., 2004).

C. butyricum is a very important strain here. Its high fermentation yields and relatively simple conditions of fermentation make it a microorganism of high industrial value in the production of 1,3-propanediol from glycerol (Colin et al., 2001). The production of 1,3-propanediol by this microorganism is not a vitamin B12-dependent process, which is clearly an economical advantage for

an industrial application (Gonzales-Pajuelo et al., 2006).

In the last decade, many studies on glycerol fermentation by *Clostridium* ssp. were undertaken. In 1999, Himmi et al. (1999) worked on methods to determine essential nutrient requirements of *C. butyricum* for glycerol fermentation. The aim of their work was also to define a low-nutrient medium which allows high production yield and to test this minimal medium in microbial transformation of industrial glycerin into 1,3-PD. They found out that high 1,3-PD production from industrial glycerin and low nutrient contents of low fermentation cost medium is possible. Five years later, in another work, these scientists tested feasibility of 1,3-PD production by *C. butyricum* with the use of a synthetic medium and raw glycerol. *C. butyricum* presents the same tolerance to raw and commercial glycerol if both are of a similar grade. These authors proved that there are no significant differences between raw glycerol fermentation and commercial glycerol fermentation. The 1,3-PD yield and volumetric productivity are on the same level. It was shown from economical and environmental point of view, that raw glycerol is a valuable substrate for 1,3-PD biological production (Gonzales-Pajuelo, 2004).

METABOLIC ENGINEERING IN 1-3 PD PRODUCTION BY *CLOSTRIDIUM* SSP.

Biotechnological production of 1,3-PD and other metabolites is attractive since microorganisms usually utilize renewable feedstock and do not produce toxic by-products. However, there are many limitations of microbial synthesis, mainly, limited yields, titers and productivities, and difficulties in the product separation. These limitations can be significantly decreased through the application of metabolic engineering (Mukhopadhyay et al., 2008; Celińska, 2010).

Metabolic engineering can improve product formation or cellular properties through the directed modification of specific biochemical reactions or the introduction of new one with the use of recombinant DNA technology (Stephanopoulos et al., 1998). Most of the metabolic engineering experiments are related to modification of *C. acetobutylicum*. This is because there is currently no genetic tool available for *C. butyricum* which is the best natural 1,3-PD producer from glycerol and the only microorganism identified so far to use a coenzyme B12-independent glycerol dehydratase. Moreover, all scientists` efforts to develop them have been unsuccessful so far (Gonzales-Pajuero, 2005; Celińska, 2010).

During acid production in the biochemical pathways for the conversion of carbohydrates to acids and solvents by *C. acetobutylicum*, acetyl-CoA and butyryl-CoA function as key intermediates for acetate and butyrate. Although, both acids are produced by similar pathways, the enzymes involved are unique to each pathway. Acetyl and butyryl phosphate are first produced from their corresponding CoA derivatives in reactions catalyzed by phosphotransacetylase (PTA) and phosphortransbutyrylase (PTB). In the next step, the acyl phosphates are metabolized to acetate or butyrate. These reactions are catalyzed by acetate and butyrate kinase and during this, ATP is generated. In solvent production, acetyl-CoA and butyryl-CoA are first reduced to acetaldehyde and butyraldehyde, and then to ethanol and butanol, respectively. Acetate and butyrate are also reassimilated in a reaction coupled to the irreversible production of acetoacetate from acetoacetyl-CoA by acetoacetyl-CoA: acetate/butyrate transferase. Acetone and carbon dioxide are produced from the decarboxylation of acetoacetate by acetoacetate decarboxylase (Perego, 1993; Green et al., 1996; Boynton et al., 1996). In 1996, Green et al. used non-replicative integrational plasmids containing internal butyrate kinase (*buk*) and phosphotransacetylase (*pta*) gene fragments to inactivate *buk* and *pta* on the chromosome. Plasmid constructs, containing either clostridial *pta* or *buk* gene fragments, were integrated into homologous regions on the chromosome. It disrupted metabolic pathways leading to acetate and butyrate formation in *C. acetobutylicum.* By inactivating genes involved in acid formation, it may be possible to redirect carbon flow towards solvent production and increase solvent yields. In 2005, González-Pajuelo et al. introduced the 1,3-PD pathway from *C. butyricum* on a plasmid in several mutants of *C. acetobutylicum* altered in product formation. The recombinant acquired the ability to grow on glycerol as the sole carbon source, while the wild-type strains are unable to grow on glycerol due to lack of sufficient NADH regeneration system. The aim of this work was to obtain a better vitamin B12-free biological process. The recombinant produced 1,3-PD, butyrate and acetate. However 1,3-PD was the main product of glycerol metabolism in them.

OPTIMIZATION OF GLYCEROL BIOCONVERSION

Among the ways to optimize the microbial production of 1,3-PD from glycerol, the most common methods include the prevention of undesired by-product formation to achieve high product yield, increasing of tolerance for 1,3-PD to achieve higher final product concentration, and increasing of the productivity of the bioreactor (Zeng and Biebl, 2002; Chen et al., 2003).

In continuous cultures by *K. pneumonia*, the production of ethanol is restricted to conditions of limitation by glycerol. But in the case of high glycerol excess and severe product inhibition such by-products such as lactic acid and 2,3-butanediol are present in the medium. Thus, the 1,3-propanediol yield diminishes. A similar situation is observed in *C. bytyricum*. The formation of butyricum by this bacteria decreases under excess substrate; however, it seems to depend also on the growth rate (Menzel et al.,

1997; Altaras and Cameron, 2000; Zeng and Biebl, 2002).

The hydrogen gas released from pyruvate cleavage to acetyl-CoA added to liquid products has a significant influence on the 1,3-PD production, too. In *C. butyricum,* the reducing equivalents from this reaction are transferred to ferredoxin. Next, ferredoxin can be transferred to NAD by the NAD:ferredoxin oxidoreductase instead of being released as molecular hydrogen, thus contributing to additional 1,3-PD formation. The NAD:ferredoxin oxidoreductase enzyme is active under excess substrate. The same situation is observed in *K. pneumoniae*. These bacteria simultaneously use two enzymes, pyruvate dehydrogenase and pyruvate:formate lyase, for anaerobic cleavage of pyruvate in the glycerol fermentation, the former particularly, is under substrate-sufficient conditions. The enzyme pyruvate dehydrogenase generates $NADH_2$ from pyruvate cleavage instead of forming formate with pyruvate formate lyase. It leads to an increased yield of 1,3-PD (0.72 mol/mol glycerol). 1,3-PD yield can be increased up to 0.88 mol/mol if acetyl-CoA from pyruvate cleavage is channeled into the tricarboxylic acid cycle for reducing power and adenosine triphosphate generation (Zeng and Biebl, 2002).

The strongest inhibitor properties in the glycerol fermentation are shown by 3-hydroxypropionaldehyde, which is normally an intracellular intermediate that does not accumulate, but in the case of high glycerol excess, it may be excreted into the medium. *K. pneumoniae* reduces accumulated 3-hydroxypropanal to 1,3-PD, *Enterobacter agglomerans* is killed by aldehyde when the concentration of glycerol is 2.2 g/l., and *C. butyricum* excretes only very small amounts of 3-hydroxypropanal. High glycerol concentration of 60 to 70 g/l is achieved with wild-type strains (Colin et al., 2001; Zeng and Biebl, 2002).

OUTLOOK AND CONCLUSIONS

It is generally recognized that bulk chemicals originating from biotechnology are promising in the important task of relieving both the modern industry and society from growing dependence on diminishing and fragile supplies of fossil feedstocks. We also hope that they can help optimize the impact of international industry on the global climate and our environment (Zeng and Biebl, 2002).

Today there is a considerable industrial interest in microbial 1,3-PD production, as it could compete with 1,3-PD made by petrochemistry. The biotechnological way of 1,3-PD production from waste biomass (e.g., crude glycerol) is an attractive alternative to traditional chemical production. For instance, in Germany glycerol has been used as an industrial waste since 2007 (Papanikolaou and Aggelis, 2009; Anand et al., 2010).

The crude glycerol contains contamination such as methanol, soaps, free fatty acids and biodiesel which makes it unfit for any useful applications in chemistry and pharmacy without purification. Nevertheless, the high cost of purification can make this application completely unprofitable. Accumulation of the glycerol phase from biodiesel production induces increase of prices of this fuel. Thus, the use of crude glycerol as a raw material in 1,3-PD production may help limit this problem (Anand et al., 2010). However, there are still several technical barriers and economical challenges preventing the growth of biobased production of bulk chemicals, reflected in high raw material and downstream processing costs, low reaction rates and limited substrate spectrum (Zeng and Biebl, 2002). Moreover, the major microorganisms which can be used in 1,3-PD production are pathogens. Nowadays, one notices a tendency to isolate *Clostridium* ssp. strains as a non-pathogenic microorganism which is able to give 1,3-PD from glycerol production effectively.

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