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Enhancing the Microbial Conversion of Glycerol to 1,3-Propanediol Using Metabolic Engineering

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Abstract:

1,3-Propanediol (PDO) is the starting point of a new-generation polymer with superior properties which is used in the textile and carpet industry. This product is mainly produced chemically, but high pressure, high temperature and expensive catalysts are required and toxic intermediates are released. Therefore, the biological production route has been studied intensively. DuPont and Genencor International, Inc. have modified *Escherichia coli* genetically so that this organism could produce PDO from glucose. However, due to the tremendous growth of the biofuel industry, a glycerol surplus has been created, so more and more researchers started to investigate the natural producing strains for the production of PDO from glycerol. Several metabolic engineering techniques have been used to enhance the production of PDO. This contribution gives an overview of the different strategies to increase the final titer, yield, and productivity of 1,3-propanediol in non-natural and natural producing strains.

1. Introduction: Glycerol and Its Applications

Glycerol (1,2,3-propanetriol or glycerin) is a (bio)chemical byproduct with a broad range of applications. It is produced during the manufacturing of soaps, emulsifiers, and fatty acids, but the bulk is created during biofuel production. For every 100 kg of biodiesel or bioethanol, approximately 10 or 4 kg of glycerol is produced, respectively.[1,2] Due to the tremendous growth of the biofuel industry a glycerol surplus has been created, resulting in a decrease in glycerol prices. This resulted for instance in the closure of the glycerol production plants of Dow Chemical and Procter and Gamble Chemicals.[3] Therefore, there is a need for new technologies dealing with the (bio)conversion of glycerol into value-added chemicals.

Nowadays, glycerol is mainly used in the food, pharmaceutical, and cosmetic industries as solvent, sweetener, and thickener. In addition, glycerol is used as a feedstock for several interesting compounds, produced chemically or biologically (Table 1).

As noted in Table 1, glycerol is a starting molecule for several chemical conversions. For example, glycerol can be converted in acrolein by a method based on glycerol dehydration by acidic solid catalysts. This end-product is then employed for the production of acrylic acid esters, superabsorber polymers, and detergents.[4] Furthermore, glycerol can be used as a base material for the methanol production which can be blended directly into gasoline and serve as a substitute for methyl-*tert*-butyl ether. In this way, it will be directed to addressing the European Union's requirement for a 5.75% biofuel component by 2010.[5]

In addition to chemical conversion, glycerol is used as a feedstock for bioconversion and fermentative processes. Using glycerol as a carbon and energy source instead of using the more traditional carbohydrates is advantageous because glycerol has a higher reduced state than that of sugars, such as glucose or xylose. Converting glycerol to phosphoenolpyruvate or pyruvate generates twice the amount of reducing equivalents produced from glucose or xylose. This leads to a higher yield of reduced products such as the industrially important compounds succinic acid and ethanol,[6] as proven by Lee and co-workers.[7,8] They have produced succinic acid from glucose and glycerol with *Anaerobiospirillum succiniproducens*. Using glucose as the carbon source results in a yield of 86%, while starting from glycerol produces a yield of 133%. Moreover,

Table 1. **Compounds chemically (C) or biologically (B) produced from glycerol**

chemical compound	method of production
acrolein[b]	C
dihydroxyacetone[a,b]	C/B
epichlorohydrin[b]	C
glyceric acid[b]	C
glycerol carbonate[b]	C
glycerol tertiary butyl ether[b]	C
lactic acid[g]	B
methanol[c]	C
propionic acid[a,e]	B
polyhydroxyalcanoate[a,f,g]	B
polyurethanes[g]	B
rhamnolipids[a]	B
succinic acid[a,d,e,f,h]	B
Syngas[b]	C
1,2-propanediol[b]	C
1,3-propanediol	C/B

[a] Reference 2. [b] Reference 4. [c] Reference 5. [d] Reference 7. [e] Reference 9. [f] Reference 12. [g] Reference 13. [h] Reference 20.

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the formation of the byproduct acetic acid is avoided, reducing the purification cost. The purification process for succinic acid involves desalting electrodialysis, water-splitting electrodialysis, and crystallization. When desalting and water-splitting electrodialysis are performed for the broth containing succinate and acetate salts, power consumption increases due to the presence of high acetate salts. Therefore, the reduced formation of acetic acid makes recovery of succinic acid easier and more economical.[8]

Another molecule synthesized from glycerol by bacteria such as *Propionibacterium* sp. is propionic acid, which is used as an antifungal agent in food and feed and as a basic chemical to produce cellulose-based plastics, herbicides, solvents, and perfumes.[9]

Some compounds can be produced chemically as well as biologically. For instance, dihydroxyacetone (DHA), used in sunless tanning products, is one of them. Chemically, DHA is formed by anodic oxidation of the secondary hydroxyl group. Although the yields of this process are comparable to the yields obtained with fermentative processes, the main route to produce DHA is by fermentation of glycerol with *Gluconobacter oxydans*.[4] This production process, however, also has drawbacks: both substrate and product have an inhibitory effect on bacterial growth.[2,10] Nevertheless, 220 g/L can be produced after fermentation for 30 h.[10]

Other applications of glycerol are extensively revised in Pagliaro et al. (2007), da Silva et al. (2009), Liu et al. (2010) and Wolf (2005) and the references herein. This review focuses on one conversion product of glycerol, namely 1,3-propanediol (trimethylene glycol, propylene glycol, PDO). First, a general introduction about the product 1,3-propanediol is given that describes the way of chemical production and its applications. Then follows a detailed description of the biological production pathway. Thereafter, the improvement of the final titer, yield, and productivity of 1,3-propanediol in non-natural and natural production strains using metabolic engineering is discussed. Only prokaryotic hosts are discussed, since the production of PDO in eukaryotic strains is reviewed by Celinska (2010).[11] Finally, some perspectives and economical aspects are given.

2. The Product 1,3-Propanediol

2.1. Applications of 1,3-Propanediol. PDO has been known for more than 100 years and has been applied widely in many industrial applications, such as composites, adhesives, laminates, coatings, moldings, aliphatic polyester, and antifreeze.[12] PDO is colorless, tastes slightly sweet, and is a viscous liquid which is easily miscible with water and ethanol.

As bifunctional molecule PDO can be used in the same applications as ethylene glycol, propylene glycol, 1,3-butanediol, and 1,4-butanediol. Moreover, PDO has unique properties due to its odd number of methylene units.[13,14] It is best known as a building block in poly(trimethylene terephthalic acid) (PTT) production, commercially known as Sorona, CDP Natureworks (DuPont) or Corterra (Shell Chemical). This polyester is based on terephthalic acid and PDO and is synthesized by the transesterification of dimethylterephthalic acid with PDO or by the esterification of terephthalic acid with PDO. For both processes, process economy and PTT-polymer quality are strongly dependent on the purity of PDO. Since the steps for poly(ethylene therephthalic acid) (PET) production and PTT are analogous, it is possible to convert existing PET plants into PTT.[13,15] The cost for converting a PET facility to a PTT producing facility is between 10% to 20% of the cost of building a new plant.[13] PTT can then be spinned and used in the carpet, textile, film and packaging industry. The fibers are soft, elastic, stain resistant, easy to manipulate and fast drying. Moreover, PTT combines the physical properties of PET (strength, stiffness, toughness, and heat resistance) with the processing properties of polybutylene terephthalate (low melt and mould temperatures, rapid crystallization).[13]

PDO can not only be used as a component of PTT, but also in thermoplastic poly urethane elastomers (TPUs) as replacement of 1,4-butanediol. These TPUs have the same mechanical properties as those based on 1,4-butanediol, but they are more heat-resistant.[16] In addition, PDO can be used as a monomer for cyclic compounds, as a polyglycol-type lubricant, as a solvent, in cosmetics, and in the medical sector.[2,17]

2.2. Chemical Synthesis of 1,3-Propanediol. As mentioned earlier, PDO can be produced both chemically and biologically. Several chemical conversion processes are known, but only two have industrial applications. The Degussa/DuPont process starts from acrolein and consists of two steps: the hydratation of acrolein to 3-hydroxypropionaldehyde (3-HPA) and the catalytic hydrogenation of 3-HPA to PDO. The first step is the key step, since various byproducts are generated, and the quality of PDO is dependent on the hydroxylation of acrolein.[12] The second industrial process starts from ethylene oxide and is still used by Shell. In this two-step reaction ethylene oxide is first carbonylated with carbon monoxide and hydrogen into 3-HPA, and this metabolite is then further hydrogenated to PDO.[12] These

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two processes give a yield of 40% and 80%, respectively.[18] A third route to produce PDO chemically starts from glycerol and consists of three steps. One problem with this process is the byproduct, 4-hydroxymethy-2-phenyl-1,3-dioxolane, generated in the first step, which lowers the yield of the desired compound.[12]

The main disadvantage of these chemical processes is the high pressure and high temperature requirement. Furthermore, expensive catalysts are required, and toxic intermediates are released.[19] For these reasons, the chemical PDO synthesis is too expensive to compete with diols such as 1,2-ethanediol, 1,2-propanediol, and 1,4-butanediol, opening development potential for biobased PDO.[20]

2.3. Bacterial Production of 1,3-Propanediol from Glycerol. *2.3.1. Isolation of 1,3-Propanediol-Producing Organisms.* PDO was first observed as a fermentation product in 1881 by Freund. It occurred as a specific gravity product in spontaneously fermented soap lyes. Such fermentations posed a disadvantage because they reduced the quality of the glycerol prepared from these lyes.[21] The first researcher who could isolate a PDO fermenting bacteria was Braak (1928). He isolated the microorganism *Bacterium freundii* from canal water, which produced considerable quantities of trimethylene glycol from glycerol.[21] Later, researchers showed that microorganisms isolated from horse, sheep, mouse and cow manures, and various soils could form trimethylene glycol from glycerol. These microorganisms were methyl-red positive, Voges-Prokauer negative and failed to utilize uric acid as nitrogen source.[21] Citrate was one of the preferred carbon sources, hence the genus was named *Citrobacter* and the type species *Citrobacter freundii* (Braak) *comb. nov.*[21]

Several other sources were screened for microorganisms producing PDO. Enrichment cultures with glycerol were blended with mud and soil samples from various places.[22−25] Most of the isolated microorganisms were anaerobic bacteria belonging to the genera *Enterobacter*, *Lactobacillus* and *Clostridium*. More precisely, it encompasses the species *Lactobacillus brevis*, *L. buchnerii*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Enterobacter agglomerans*, *E. aerogenes*, *Clostridium welchii*, *C. pasteurianum*, *C. butyricum*, *C. acetobutylicum*, *C. beijerinckii*, and *C. kainantoi*.[2,22−24,26−29]

2.3.2. The Glycerol Metabolizing Pathway. The natural producing microorganisms apply two mechanisms for glycerol uptake. Glycerol either passes the membrane by diffusion or with the help of the glycerol transport facilitator GlpF (TC 1.A.8.1.1[30,31]). This transmembrane protein is a member of the major intrinsic protein (MIP) family (TC 1.A.8) and is involved in forming aqueous pores that selectively allow passive transport of compounds like glycerol, dihydroxyacetone, urea, and other small neutral linear molecules. Since 1,3-propanediol has a more hydrophobic character than glycerol, it is not preferred as substrate for GlpF.[32]

When glycerol has entered the cell, it can be processed by two parallel pathways (Scheme 1): an oxidative pathway and a reductive one (for more details about the genes and their topology, see Celinska (2010)). In the oxidative pathway glycerol is first oxidized by the glycerol dehydrogenase (DhaD, E.C. 1.1.1.6[33]) to dihydroxyacetone (DHA) with formation of NADH. Dihydroxyacetone is then further phosphorylated by the ATP-dependent dihydroxyacetone kinase (DhaK, E.C. 2.7.1.29) into dihydroxyacetone phosphate which enters glycolysis. This molecule can then be converted in organic acids, ethanol, 2,3-butanediol, CO_2 and H_2. *Klebsiella* sp. also have an alternative oxidative pathway, which is normally used under aerobic conditions. This branch contains a glycerol kinase (GlpK, E.C. 2.7.1.30) which catalyses the reaction of glycerol to *sn*-glycerol-3-phosphate. This compound is then further metabolized to dihydroxyacetone phosphate which is then channeled into the glycolysis.[34] *Lactobacillus* sp. however, only have the reductive pathway and need an additional carbon source for growth and generation of reducing equivalents.[26]

The second, reductive pathway consists of two enzymatic reactions. First, glycerol is dehydrated by a glycerol or diol dehydratase (GDHt or DhaB) (E.C. 4.2.1.30) in 3-hydroxypropionaldehyde (3-HPA). Then the 3-HPA is turned into PDO

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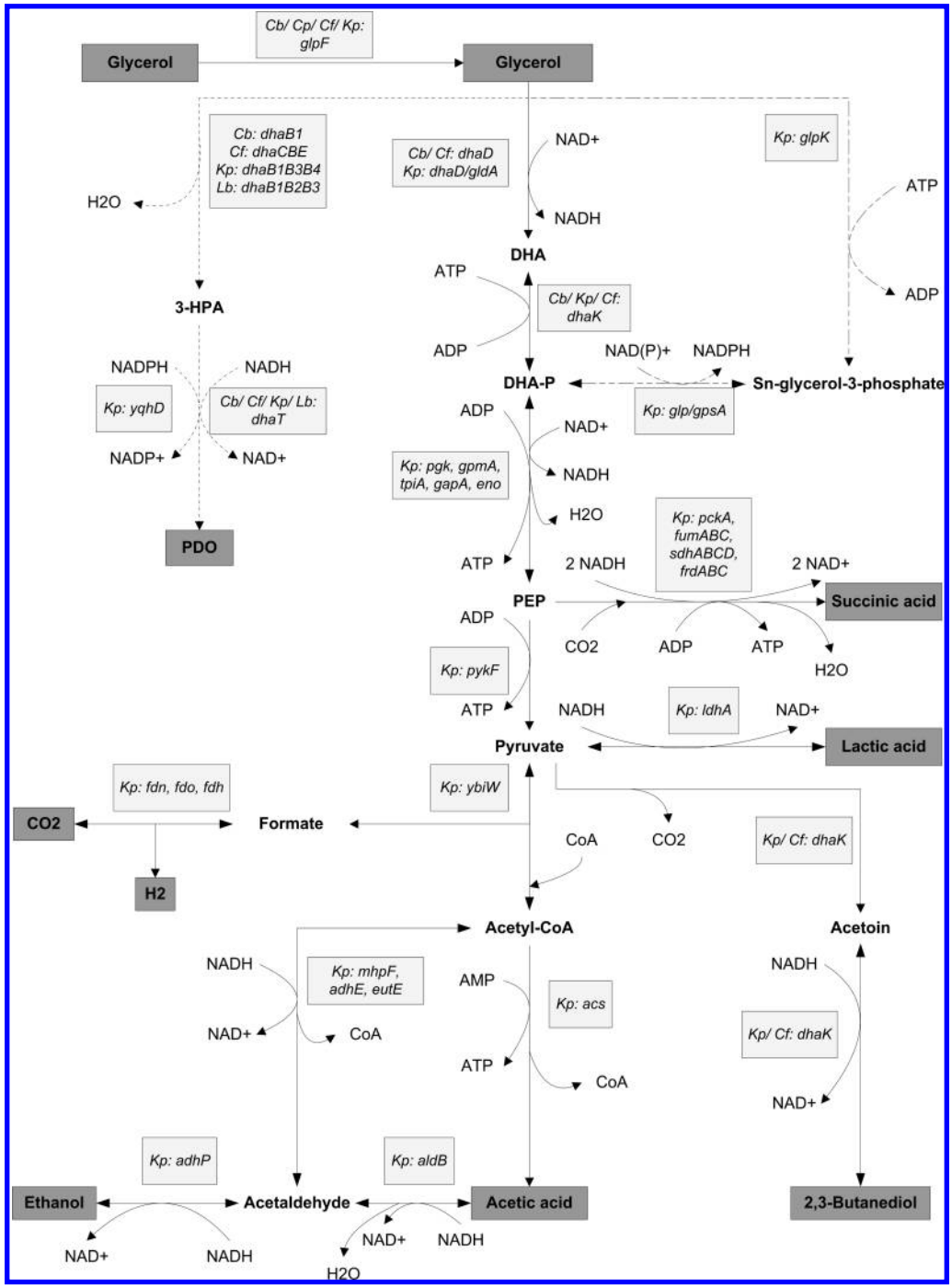
Scheme 1. **Glycerol metabolizing pathways in 1,3-propanediol-producing microorganisms: Kp = *Klebsiella pneumoniae*; Cb = *Clostridium butyricum*; Cf = *Citrobacter freundii*; Cp = *Clostridium pasteurianum*; Lb = *Lactobacillus brevis*[a]**



[a] The dotted lines are reactions of the reductive pathway, the solid lines represent the oxidative pathway and the dashed lines symbolize the alternative oxidative pathway of *K. pneumoniae*.

by the 1,3-propanediol dehydrogenase (PDODH or DhaT) or the 1,3-propanediol oxidoreductase (PDOR or YqhD) (E.C. 1.1.1.202) with formation of NAD(P).[35]

The overall reaction rate of the reductive pathway is limited by the first reaction because this reaction is mediated by cobalamine (vitamin B_{12}). In general, the reaction mechanism consists of 6 steps. First, cobalamine binds to the apoenzyme via the imidazole of a histidine residue. When the substrate is available, it will cause a homolytic cleavage of the Co−C bond of the coenzyme leading to the formation of cob(II)alamine and an adenosyl radical. In the third step, the adenosyl radical will abstract a hydrogen atom from the substrate. This results in the formation of a substrate-derived radical and 5′-deoxyadenosine. The substrate-derived radical then rearranges to a product radical by a hydroxyl group transfer from C2 to C1. In the next-to-last step the product radical abstracts a hydrogen atom back from 5′-deoxyadenosine resulting again in an adenosyl radical. Finally, the coenzyme is regenerated by binding of the adenosyl

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radical with the cob(II)alamine radical.[36,37] However, in the case of glycerol and some other substrates, the enzymes are subject to suicide inactivation. Inactivation by glycerol involves irreversible cleavage of the Co−C bond of coenzyme B_{12}, forming 5′-deoxyadenosine and an alkylcobalamine-like species. Inactivation is then affected by tight binding of the modified alkylcobalamine to the enzyme.[36] It has been shown for the diol dehydratase of *Klebsiella oxytoca* that the glycerol-inactivated enzyme is reactivated by an exchange of the modified coenzyme for intact coenzyme B_{12} with consumption of ATP, in the presence of Mg^{2+} and a complex of the proteins DdrA and DdrB. Homologous proteins were also found in *Klebsiella pneumoniae* (GdrA and GdrB) and in *Citrobacter freundii* (DhaF and DhaG).[38,39] So in order to avoid rate limitations, the expensive cobalamine will have to be added to the fermentation broth.[40]

An alternative glycerol dehydratase was found in *Clostridium butyricum*. This enzyme is B_{12}-independent and a member of the radical S-adenosylmethionine superfamily. This superfamily utilizes S-adenosylmethionine instead of adenosylcobalamin to catalyze radical reactions at enzymatic sites. O'Brien and coworkers (2004) have elucidated the crystal structure of this glycerol dehydratase. The monomer has a β/α-barrel structure; it forms a core 10-stranded β-barrel motif which is entirely surrounded by β-helices. A disadvantage is that the vitamin B_{12}-independent dehydratase is oxygen sensitive and thus cannot be used in an aerobic production system.[40]

2.3.3. The Maximum Theoretical Yield. The maximum theoretical yield calculated for the anaerobic fermentation of PDO from glycerol is 0.875 mol/mol glycerol. In this case all acetyl-CoA formed has to enter the citrate cycle without oxidative phosphorylation to acetate, leading to a highly efficient generation of reducing equivalents. In most cases however acetate is still formed for ATP generation. If no hydrogen is released, the maximum theoretical yield with acetate as the sole byproduct is 0.72 mol PDO/mol glycerol.[20,26] In real fermentation processes, various pyruvate-derived byproducts are produced apart from acetic acid. The formation of these byproducts competes with the biosynthesis of PDO for NADH, resulting in a decrease of the PDO yield.[41]

3. Metabolic Engineering

3.1. Considerations for the Development of a Biotechnological Process. Stephanopoulos (1999) defines metabolic engineering as the directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or introduction of new ones with the use of recombinant DNA technology.[42] Thus, the aim of metabolic engineering is to optimize an important biotechnological process.

However, this optimization requires some strategic considerations. A first consideration is whether to screen for natural producing microorganisms or to apply well-known, easy to modify ones. A natural producing strain has the advantage that process optimization does not have to start from scratch; however, genetic tools are rarely available and thus need to be developed. Furthermore, a natural producing strain will have obtained resistance to the compound they produce, which is not guaranteed in a non-natural producing strain. Overproduction of certain compounds can be inhibitory or even lethal for the microorganism. Thus, resistance has to be created which is not always an easy task.[20] Moreover, transferring genes from one organism to another is not always straightforward. Eukaryotic genes, for example, are composed of introns and exons, while prokaryotes do not possess such mechanisms. In addition, codon usage between organisms can differ. Therefore a gene that is transferred between organisms has to be adapted to the appropriate codon usages of the host organism. Besides, the protein can also require post translational modifications to be functional, such as glycosylations.[20]

In the case of PDO, both strategies are used. Most attention to natural producing strains has gone to *Klebsiella* sp. and *Clostridium* sp. These microorganisms have a higher substrate tolerance, yield, and productivity than other natural producing strains.[12,18] However, DuPont and Genencor International, Inc. have created a genetically modified *Escherichia coli* strain. These companies opted for an easy-to-modify organism, widely used in industrial biotechnology, rather than for a natural producing organism.[20]

3.2. The Non-natural Producing Strain *E. coli.* *3.2.1. Production of 1,3-Propanediol from Glucose.* DuPont and Genencor International, Inc. have modified *E. coli* genetically so that this organism can produce PDO from glucose. Since wild type *E. coli* cannot efficiently produce glycerol from glucose and is not able to produce PDO from glycerol, seven genes had to be introduced in the genome. Two of these genes (*dar1* and *gpp2*) originated from *Saccharomyces cereviseae* and 5 (*dhaB1*, *dhaB2*, *dhaB3*, *dhaBX* and *orfX*) from *Klebsiella pneumoniae* (Scheme 2).

The genes encoding for the glycerol 3-phosphate dehydrogenase (*dar1*) (E.C. 1.1.1.8) and the glycerol 3-phosphate

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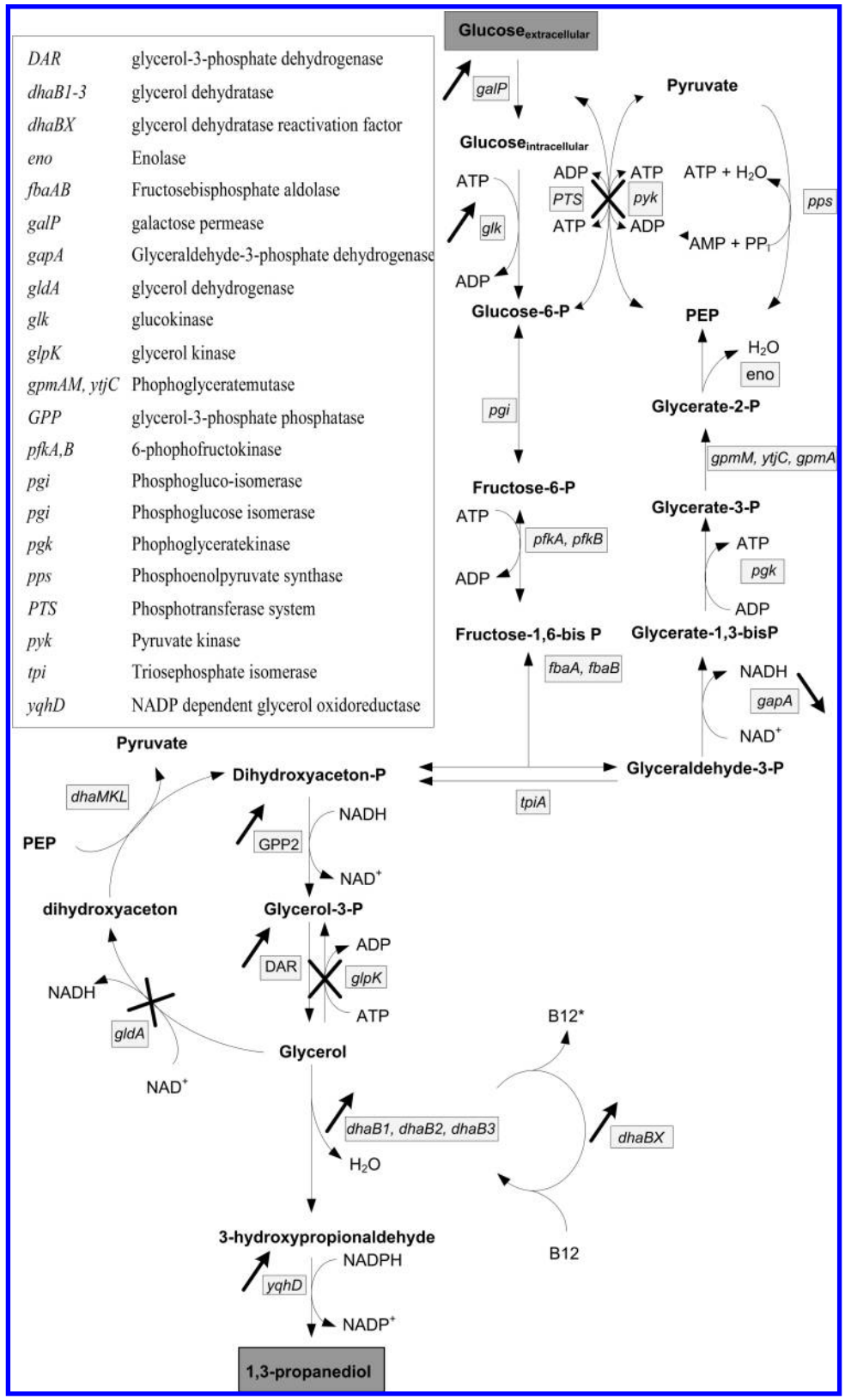
Scheme 2. **Metabolic engineering scheme for the production of 1,3-propanediol in *E. coli* with glucose as substrate[a]**



[a] The arrows slanted up, the arrows slanted down, and the times symbol represent respectively upregulation, downregulation, and elimination of the gene.

phosphatase (*gpp2*) (E.C. 3.1.3.21) were transformed in *E. coli* K12 for the conversion of glucose to glycerol. Furthermore, the glycerol dehydratase (*dhaB1*, *dhaB2*, *dhaB3*) (E.C.4.2.1.30) and its reactivating factors (*dhaBX*, *orfX*), obtained from *K. pneumoniae*, enable the conversion of glycerol to 3-hydroxypropionaldehyde. An oxidoreductase (*yqhD*) (E.C. 1.1.1.202) from *E. coli* itself completes the pathway.[43,44] The production was further optimized by altering the D-glucose transport. The phosphotransferase system (PTS) (T.C. 4.A.1) was knocked out, and the galactose permease (*galP*) (T.C. 2.A.2.2.3) and glu-

cokinase (*glk*) (E.C. 2.7.1.2) were overexpressed. This decouples glucose uptake from the conversion of PEP into pyruvate. To prevent glycerol from re-entering the central carbon metabolism, glycerol kinase (*glpK*) (E.C. 2.7.1.30) and glycerol dehydrogenase (*gldA*) (E.C. 1.1.1.6) had to be knocked out. Further, glyceraldehyde 3-phosphate dehydrogenase (*gapA*) (E.C. 1.2.1.12) was down-regulated. Finally, the flux of the triosephosphate isomerase (*tpi*) (E.C. 5.3.1.1) was rearranged from an equilibrium to a reaction going in one direction, namely from glyceraldehyde 3-phosphate (GAP) to dihydroxyacetone phosphate (DHAP). The last two modifications were carried out to increase the efficiency of the conversion of DHAP into glycerol.

In this way, a metabolically engineered organism is created that provides 1,3-propanediol at a rate of 3.5 g/L/h, an end concentration of 135 g/L, and a yield of 0.62 mol/mol.[44]

3.2.2. Conversion of Glycerol to 1,3-Propanediol. Due to the upcoming need to valorize glycerol and because *E. coli* can grow on glycerol, researchers started to manipulate *E. coli* to produce 1,3-propanediol starting from glycerol instead of from glucose. Ma et al. (2009) have constructed three plasmids: one with the PDO dehydrogenase gene of *K. pneumoniae*, one with the *K. pneumoniae* glycerol dehydratase gene, and one with both genes in the same direction. When only the *dhaT* gene is expressed, no PDO is formed. However, when only the *dhaB* gene is expressed, a small quantity of PDO can be detected in the fermentation broth. This is probably due to the NADPH-linked alcohol dehydrogenase encoded by *yqhD*.[43] Expressing the two genes in the same direction led to a final 1,3-propanediol concentration of 11.3 g/L with the consumption of 40 g/L glycerol.[45]

Zhang et al. (2006) have constructed a similar recombinant *E. coli* strain, but they used the *dhaB* gene of *Citrobacter freundii* together with the *yqhD* gene of *E. coli*. In addition, the fermentation conditions were further optimized by the response surface method. The model estimated that a maximal yield of PDO of 43.86 g/L could be obtained when the fermentation time was 30 h and the concentrations of glycerol, yeast extract and vitamin B_{12} were set at 61.8 g/L, 6.2 g/L and 49 mg/L respectively. Using this optimized medium, the titer and productivity reached a value of 43.1 g/L and 1.54 g/L/h respectively.[46]

However, the highest PDO-concentration using *E. coli* as production strain starting from glycerol has been reached by Tang and co-workers (2009). The difference with the strategy of other researchers was dual. First, they created a vector comprising the *E. coli yqhD* gene together with the genes for the production of 3-HPA of *Clostridium butyricum*, *dhaB1* and *dhaB2*. The advantage of using the genes of *C. butyricum* is that *dhaB1* encodes the vitamin B_{12}-independent glycerol dehydratase, so no expensive cofactors need to be added to the medium. The second difference is that a two-stage fermentation process is used. During the first stage, aerobic fermentation is utilized to obtain a high cell density culture on glucose at 30 °C. Thereafter, a switch is made to 42 °C, and an anaerobic fermentation process is used to promote the production of PDO from glycerol in the second stage. In this way a PDO titer of 104.4 g/L was achieved with a productivity of 2.61 g/L/h and a conversion rate of glycerol to PDO of 1.09 mol/mol.[47]

3.3. Glycerol Conversion by *Klebsiella* sp. As stated before, two types of strains can be employed to optimize a biotechnological production process. Either a non-natural producer, such as *E. coli*, can be genetically modified or a natural producing strain which shows a significant yield and productivity, such as *K. pneumoniae* can be optimized. Several optimization schemes have already been employed for *K. pneumoniae* (Table 2). For instance, the oxidative pathway can be knocked out or all competitive byproduct routes can be eliminated. In the case of the glycerol oxidative pathway deletion, the genes coding for glycerol dehydrogenase (*dhaD*) or dihydroxyacetone kinase (*dhaK*) have to be knocked out.[48,49] In another case, the key competitors in *Klebsiella* sp., particularly lactate, ethanol, succinic acid, and 2,3-butanediol need to be removed because they compete for the reducing agent NADH with PDODH.[50,51]

Alternatively, PDO production can be enhanced by overexpressing the reductive pathway genes that lead to PDO formation. This can be done by overexpressing the first enzyme, GDHt,[52] or the second enzyme, PDODH,[53,54] or by overex-

(43) Emptage, M.; Haynie, S. L.; Laffend, L.; Pucci, J. P.; Whited, G. Process for the biological production of 1,3-propanediol with high titer. U.S. Pat. 7,067,300 B2, 1999.

(44) Nakamura, C. E.; Whited, G. M. Metabolic engineering for the microbial production of 1,3-propanediol. *Curr. Opin. Biotechnol.* **2003**, *14* (5), 454–459.

(45) Ma, Z.; Rao, Z. M.; Xu, L. Y.; Liao, X. R.; Fang, H. Y.; Zhuge, B.; Zhuge, J. Production of 1,3-propanediol from glycerol by engineered *Escherichia coli* using a novel co-expression vector. *Afr. J. Biotechnol.* **2009**, *8* (20), 5500–5505.

(46) Zhang, X. M.; Li, Y.; Zhuge, B.; Tang, X. M.; Shen, W.; Rao, Z. M.; Fang, H. Y.; Zhuge, J. Construction of a novel recombinant *Escherichia coli* strain capable of producing 1,3-propanediol and optimization of fermentation parameters by statistical design. *World J. Microbiol. Biotechnol.* **2006**, *22* (9), 945–952.

(47) Tang, X. M.; Tan, Y. S.; Zhu, H.; Zhao, K.; Shen, W. Microbial Conversion of Glycerol to 1,3-Propanediol by an Engineered Strain of *Escherichia coli*. *Appl. Environ. Microbiol.* **2009**, *75* (6), 1628–1634.

(48) Seo, M. Y.; Seo, J. W.; Heo, S. Y.; Baek, J. O.; Rairakhwada, D.; Oh, B. R.; Seo, P. S.; Choi, M. H.; Kim, C. H. Elimination of by-product formation during production of 1,3-propanediol in *Klebsiella pneumoniae* by inactivation of glycerol oxidative pathway. *Appl. Microbiol. Biotechnol.* **2009**, *84* (3), 527–534.

(49) Horng, Y. T.; Chang, K. C.; Chou, T. C.; Yu, C. J.; Chien, C. C.; Wei, Y. H.; Soo, P. C. Inactivation of *dhaD* and *dhaK* abolishes by-product accumulation during 1,3-propanediol production in *Klebsiella pneumoniae*. *J. Ind. Microbiol. Biotechnol.* 2010.

(50) Yang, G.; Tian, J. S.; Li, J. L. Fermentation of 1,3-propanediol by a lactate deficient mutant of *Klebsiella oxytoca* under microaerobic conditions. *Appl. Microbiol. Biotechnol.* **2007**, *73*, 1017–1024.

(51) Xu, Y. Z.; Guo, N. N.; Zheng, Z. M.; Ou, X. J.; Liu, H. J.; Liu, D. H. Metabolism in 1,3-Propanediol Fed-Batch Fermentation by a D-Lactate Deficient Mutant of *Klebsiella pneumoniae*. *Biotechnol. Bioeng.* **2009**, *104* (5), 965–972.

(52) Zheng, P.; Wereath, K.; Sun, J. B.; van den Heuvel, J.; Zeng, A. P. Overexpression of genes of the *dha* regulon and its effects on cell growth, glycerol fermentation to 1,3-propanediol and plasmid stability in *Klebsiella pneumoniae*. *Process Biochem.* **2006**, *41* (10), 2160–2169.

(53) Hao, J.; Wang, W.; Tian, J. S.; Li, J. L.; Liu, D. H. Decrease of 3-hydroxypropionaldehyde accumulation in 1,3-propanediol production by over-expressing *dhaT* gene in Klebsiella pneumoniae TUAC01. *J. Ind. Microbiol. Biotechnol.* **2008**, *35* (7), 735–741.

(54) Zhao, L.; Zheng, Y.; Ma, X. Y.; Wei, D. Z. Effects of over-expression of glycerol dehydrogenase and 1,3-propanediol oxidoreductase on bioconversion of glycerol into 1,3-propanediol by *Klebsiella pneumoniae* under micro-aerobic conditions. *Bioprocess Biosyst. Eng.* **2009**, *32* (3), 313–320.

Table 2. **Summary of the different strategies of metabolic engineering for *Klebsiella* sp. with the glycerol concentration used (g/L), the concentration PDO produced (g/L), the yield (mol/mol), and the productivity (g/L/h)[a]**

	B/FB	Ae/Ana	glycerol used (g/L)	concentration PDO (g/L)	yield (mol/mol)	productivity (g/L/h)
1. Knockout Strains						
K. pneumoniae Cu[c]	flask	ND	ND	7.7	0.47	ND
Δ*dhaD* Δ*dhaK*	flask	ND	ND	5.2	0.57	ND
K. oxytoca M5a1[d]	FB	Micro-Ae	139.46	58.79	0.51	0.98
Δ*ldhA*	FB		164.67	83.56	0.62	1.39
K. pneumoniae HR526[e]	FB	Ae	ND	95.39	0.48	1.98
Δ*ldhA*	FB	Ae	ND	102.06	0.52	2.13
K. pneumoniae YMU2[b]	FB	Ana	181.46	53.16	0.26	0.81
Δ*aldA*	FB	Ana	122.3	70.58	0.70	1.07
2. Overexpression of Genes from the Reductive Branch						
K. pneumoniae TUAC01[f]						
dhaT overexpressed	B	Ae	30	15	0.61	1.36
	B	Ae	30	13.75	0.56	1.06
K. pneumoniae KG1[g]	FB	Ae	236.46	98.84	0.51	3.29
dhaT overexpressed	FB	Ae	171.9	90.85	0.64	2.16
K. pneumoniae ACCC10082[l]	B/FB	Ae	30/ND	15.2/58.8	0.61/ND	0.76/ND
dhaT overexpressed	B/FB	Ae	48/ND	23.6/59.2	0.595/ND	1.18/ND
K. pneumoniae Cu with *yqhD*[h]	B	Ae	20	7.9	0.48	0.44
Δ*dhaD* Δ*dhaK* with *yqhD*	B	Ae	17.5	7.7	0.53	0.26
K. pneumoniae ME-308[m]	FB	Ae	120	ND	0.53	1.32
K. pneumoniae ME-308 with *yqhD*	FB	Ae	130	67.6	0.62	1.69
3. Cofactor Manipulations						
K. oxytoca YMU1[i]	FB	Ana	ND	ND	0.387	ND
K. oxytoca met *fdh*	FB	Ana	ND	ND	0.454	ND
4. Directed Evolution						
K. pneumoniae 10018[j]						
K. pneumoniae β-Q42F						
K. pneumoniae β-Q42L						
K. pneumoniae β-Q42S						
K. pneumoniae DSM2026[k]						

[a] B = Batch; F = Fed-Batch; Ae = aerobic; Ana = anaerobic; ND = exact values not given. [b] Reference 41. [c] Reference 48. [d] Reference 50. [e] Reference 51. [f] Reference 53. [g] Reference 54. [h] Reference 55. [i] Reference 56. [j] Reference 57. [k] Reference 58. [l] Reference 62. [m] Reference 63.

pressing both enzymes.[52] A 1,3-propanediol oxidoreductase isoenzyme, *yqhD*, can also be introduced.[55]

Third, cofactors can be manipulated in order to increase the concentration of them, favoring the reactions of the reductive pathway and more precisely the conversion of 3-HPA into PDO. One example will be given, where a formate dehydrogenase of *Candida boidinii* is introduced in *K. oxytoca*.[56]

The last option to improve the production of PDO encompasses directed evolution of the enzymes of the reductive pathway to increase their catalytic power[57] or to relax coenzyme specificity.[58]

In the next few paragraphs, these four options will be discussed in more detail for *Klebsiella* species, natural producing microorganisms.

3.3.1. Knockout Mutants. The inactivation of the oxidative pathway is extensively studied. Seo et al. (2009) constructed two mutant strains of *K. pneumoniae* Cu, a derivative of ATCC 200721. In one mutant strain the genes encoding for glycerol dehydrogenase (*dhaD*) and the dihydroxyacetone kinase (*dhaK*) were deleted (AK) (Scheme 3). In the other strain the putative transcription factor (*dhaR*) was inactivated (AR), which controls transcription of *dhaK* and *dhaD*. Both mutant strains lacked *dhaT*, encoding for PDODH. These mutations result in reduced growth rates when grown on a defined medium containing glycerol as sole carbon source. Although the PDODH was knocked out, the mutants still produce PDO, which suggests a putative oxidoreductase catalyzing the production of PDO at a lower level compared to DhaT. This activity was attributed to the recently identified *K. pneumoniae* gene *yqhD*.[48] Enzyme assays showed that this enzyme prefers NADPH over NADH as cofactor.[59]

Hong et al. (2010) investigated a *dhaD*−*dhaK* gene-deleted mutant of a clinical isolated *K. pneumoniae*. They concluded that the mutant converts glycerol into PDO more efficiently but produces less biomass and total concentration PDO. Furthermore, the mutant did not produce the byproducts lactate, 2,3-butanediol, and ethanol, whereas the wild type produces these

(55) Seo, J. W.; Seo, M. Y.; Oh, B. R.; Heo, S. Y.; Baek, J. O.; Rairakhwada, D.; Luo, L. H.; Hong, W. K.; Kim, C. H. Identification and utilization of a 1,3-propanediol oxidoreductase isoenzyme for production of 1,3-propanediol from glycerol in *Klebsiella pneumoniae*. *Appl. Microbiol. Biotechnol.* **2010**, *85* (3), 659–666.

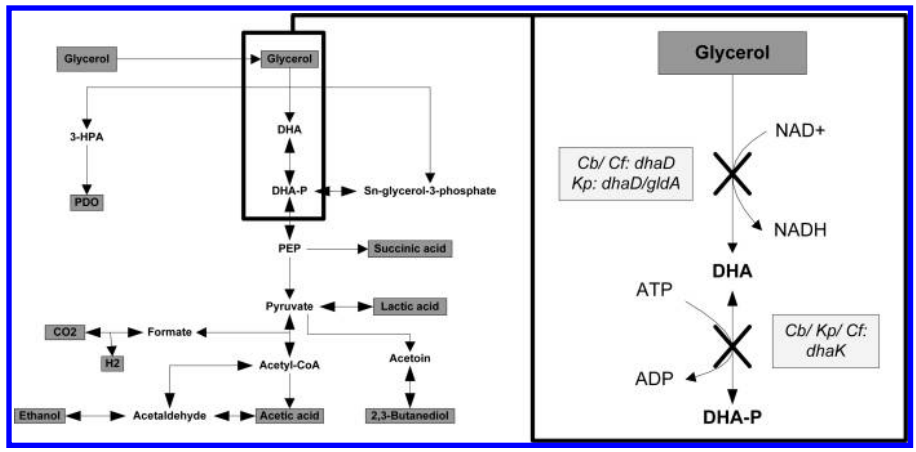
(56) Zhang, Y. P.; Huang, Z. H.; Du, C. Y.; Li, Y.; Cao, Z. A. Introduction of an NADH regeneration system into *Klebsiella oxytoca* leads to an enhanced oxidative and reductive metabolism of glycerol. *Metab. Eng.* **2009**, *11* (2), 101–106.

(57) Qi, X. H.; Chen, Y. L.; Jiang, K.; Zuo, W. P.; Luo, Z. F.; Wei, Y. T.; Du, L. Q.; Wei, H.; Huang, R. B.; Du, Q. S. Saturation-mutagenesis in two positions distant from active site of a *Klebsiella pneumoniae* glycerol dehydratase identifies some highly active mutants. *J. Biotechnol.* **2009**, *144* (1), 43–50.

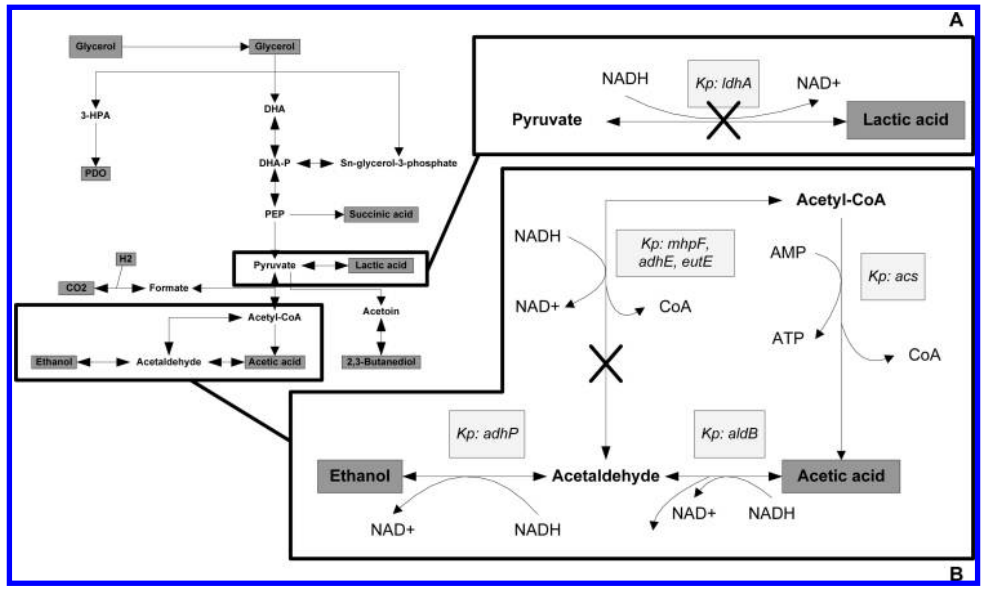
(58) Ma, C.; Zhang, L.; Dai, J.; Xiu, Z. Relaxing the coenzyme specificity of 1,3-propanediol oxidoreductase from *Klebsiella pneumoniae* by rational design. *J. Biotechnol.* **2010**, *146*, 5500–5505.

(59) Marcal, D.; Rego, A. T.; Carrondo, M. A.; Enguita, F. J. 1,3-Propanediol Dehydrogenase from *Klebsiella pneumoniae*: Decameric Quaternary Structure and Possible Subunit Cooperativity. *J. Bacteriol.* **2009**, *191* (4), 1143–1151.

Scheme 3. **Strategy 1: inactivation of *dhaD* and *dhaK***



Scheme 4. **Strategy 2: inactivation of important byproducts; (A) inactivation of lactic acid by knockout of lactate dehydrogenase; (B) inactivation of ethanol by the deletion of the acetaldehyde dehydrogenase**



compounds. The production of acetate, however, increases as compared to that by the wild-type.[49]

A second manner to block the byproducts of no benefit is done by first determining the key competitors and then eliminating them. The key competitors for the production of PDO are those enzymes that use NADH, for example the lactate dehydrogenase (1.1.1.27) for the production of lactate and the acetaldehyde (E.C. 1.2.1.3) and ethanol dehydrogenase (E.C. 1.1.1.1) for the production of ethanol. Yang et al. (2007) and Xu et al. (2009) stopped the production of lactate by knocking out the *ldhA* gene in *K. oxytoca* M5a1 and *K. pneumoniae* HR526, respectively (Scheme 4, A). In both cases no lactate is produced, and the PDO concentration, the yield, and the productivity increase. 2,3-Butanediol becomes the main byproduct in both bacteria. This molecule has applications as a feedstock and as liquid fuel.[60] Comparing the two strains, the mutant of *K. pneumoniae* HR526 could produce the highest concentration PDO, namely 102.06 g/L, with a yield of 0.52 mol/mol and a productivity of 2.13 g/L/h. Even on crude glycerol from biodiesel, the strain leads to economically viable process parameters. Therefore, this strain has been tested on pilot scale by Hunan Rivers Bioengineering Co. Ltd., an industrial demonstration base.[51]

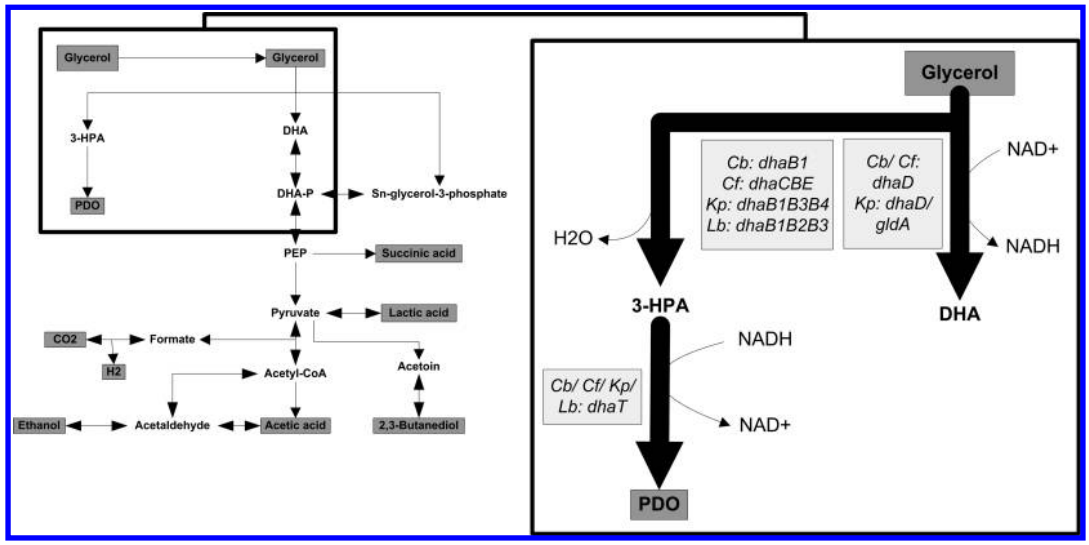
Zhang et al. (2006) reasoned that ethanol is a key competitor to the production of PDO by *K. pneumoniae* YMU2. For each ethanol molecule produced, 2 NADH molecules are oxidized: acetyl-CoA is converted into acetaldehyde with acetaldehyde dehydrogenase (ALDH) and NADH, whereafter ethanol dehydrogenase oxidizes NADH with the formation of ethanol (Scheme 4, B).

By inactivating the gene coding for ALDH, the yield, titer, and productivity could be improved up to 0.7 mol/mol, 70.58 g/L, and 1.07 g/L/h, respectively. However, apart from an improved PDO titer, acetate production is also enhanced with this mutation. Other byproducts which compete for NADH, such as succinate, lactate, or 2,3-butanediol, do not remarkably alter in concentration.[41]

3.3.2. Overexpression of the Genes from the Reductive Pathway. Apart from gene deletions, many researchers have tried to optimize production via the overexpression of the reductive pathway to reduce the intermediary metabolite 3-HPA (Scheme 5). Accumulation of this compound has been observed

(60) Syu, M. J. Biological production of 2,3-butanediol. *Appl. Microbiol. Biotechnol.* **2001**, *55* (1), 10–18.

Scheme 5. **Overexpression of *dhaT*; *dhaT* and *dhaD*; *dhaB* and *dhaT***



at the early phase of the fermentation by *K. pneumoniae*. 3-HPA, however, has a bacteriostatic effect: the reactivity of the aldehyde group of the compound causes DNA damage and inhibits DNA synthesis.[61,62] Furthermore, PDODH is very sensitive to 3-HPA. When 3-HPA increases to a certain level, this enzyme loses its activity, terminating PDO production.[53] So, a decreased accumulation of 3-HPA is necessary for the production of PDO. Hao et al. (2008) introduced the *dhaT* gene coding for 1,3-propanediol dehydrogenase, on a high-copy plasmid in *K. pneumoniae* TUAC01. In flask and batch bioreactor experiments the 1,3-propanediol production did not differ from the control. Fed-batch conditions, however, increased the yield when *dhaT* was overexpressed, but reduced growth rate in comparison to the wild-type strain. This was probably due to the existence of the multicopy plasmid pDK-*dhaT* which might affect the normal grown cell. This reduced growth rate led further to a reduction in volumetric productivity but also reduced byproduct formation. Apart from a steep decrease in 3-HPA formation, the final lactate, succinate, and ethanol concentrations were reduced 51.8, 50.6, and 47.4% respectively.[54]

Alternatively, *dhaT* can be overexpressed together with *dhaD*. *DhaD* encodes for glycerol dehydrogenase, an enzyme that converts glycerol into dihydroxyacetone while producing NADH. The activity of this enzyme will thus supply additional NADH for PDO formation. In batch conditions, this mutation increased the PDO production with 56.3% in comparison to the control, but in fed-batch mode this increase could not be reproduced. Remarkably, this mutant strain is more resistant: it does not experience any adverse effect during fermentation with high glycerol concentrations, whereas the wild-type experiences growth cessation at concentrations higher than 20 g/L.[62]

Since the first reaction, converting glycerol into 3-HPA, is the rate-limiting reaction,[36] Zheng and co-workers have studied the effect of overexpressing *dhaB*, solely or with *dhaT* in *K. pneumoniae* DSM 2026. The plasmids containing the *dhaB* gene were stable in aerobic cultures even without antibiotic pressure, but unstable in anaerobic cultures, especially when glycerol was used as sole carbon source. It was postulated that the instability of the plasmids bearing the *dhaB* gene is associated with the toxicity of 3-HPA. When glycerol is available, the overexpression of GDHt can lead to a rapid accumulation of this intermediate which seriously damages the host cells. The damage can be so severe that the plasmid-bearing cells can no longer grow or grow more slowly. The cells that lose the plasmid then grow faster, even with antibiotic pressure. Due to this plasmid instability no improvement was seen.[52]

With the annotation of *yqhD* in *E. coli*, another strategy became possible. Because YqhD prefers NADPH over NADH, the effect of these cofactors on PDO production could be tested. Overexpression of YqhD in *E. coli* showed that the cofactor NADPH is more favorable than NADH for the production of PDO.[44] Seo et al. (2009) identified a homologous gene to *yqhD* in *K. pneumoniae* and overexpressed this gene in a *K. pneumoniae* strain in which *dhaT* was knocked out. As a result, the PDO production was enhanced indicating the importance of NADPH as cofactor for PDO production (Scheme 6).[48]

Analogously, the gene *yqhD* of *E. coli* was expressed in *K. pneumoniae*. This modification led to an increase of 125% in PDO production (67.6 g/L) in comparison to the wild type strain. In addition, the yield increased from 0.53 mol/mol glycerol to 0.62 mol PDO/mol glycerol, most likely due to a steep decrease in 3-HPA accumulation (22.4% decrease).[63]

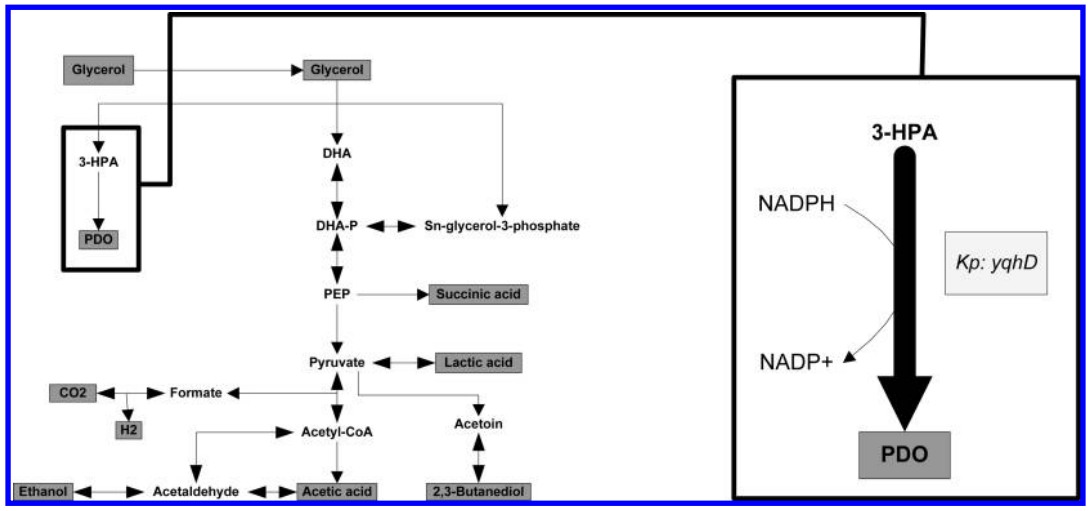
Apart from manipulating cofactor availability by eliminating competing reactions, such as lactate dehydrogenase and ethanol dehydrogenase, heterologous genes can be introduced to increase NADH availability.[41,50,51,56] For instance, formate dehydrogenase can be introduced to regenerate NADH, similar

(61) Barbirato, F.; Grivet, J. P.; Soucaille, P.; Bories, A. 3-Hydroxypropionaldehyde, an inhibitory metabolite of glycerol fermentation to 1,3-propanediol by enterobacterial species. *Appl. Environ. Microbiol.* **1996**, *62* (4), 1448–1451.

(62) Chen, Z.; Liu, H. J.; Liu, D. H. Regulation of 3-hydroxypropionaldehyde accumulation in *Klebsiella pneumoniae* by overexpression of *dhaT* and *dhaD* genes. *Enzyme Microb. Technol.* **2009**, *45* (4), 305–309.

(63) Zhu, J. G.; Li, S.; Ji, X. J.; Huang, H.; Hu, N. Enhanced 1,3-propanediol production in recombinant *Klebsiella pneumoniae* carrying the gene yqhD encoding 1,3-propanediol oxidoreductase isoenzyme. *World J. Microbiol. Biotechnol.* **2009**, *25* (7), 1217–1223.

Scheme 6. **Introduction and overexpression of *yqhD***



to biocatalysis coenzyme regeneration systems.[64] The *fdh* gene of *Candida boidinii*, encoding for formate dehydrogenase, was introduced in *K. oxytoca* YMU1 to create a regeneration system. For each formate molecule added to the fermentation broth one NADH will be formed with carbon dioxide. Introducing the gene encoding the enzyme in *K. oxytoca* did not disturb the redox balance of the organism, so that the cell growth was not significantly affected. The NADH-dependent pathways, including those from glycerol to PDO and from pyruvate to ethanol and lactate, were all improved by the introduction of the NADH regeneration system. More precisely, the molar yields of PDO, ethanol, and lactate were respectively 17.3%, 42.7%, and 18.7% higher than those of the parent strain.[56]

3.3.3. Directed Evolution for the Improvement of PDO Production. A powerful algorithm in nature to create diversity is evolution. Directed evolution uses the same algorithm to evolve enzymes in a laboratory as nature does. Via several rounds of mutation and selection new or improved enzymes are created.[65] In this way some crucial enzymes for the production of PDO were modified. As mentioned before, GDHt is the major rate-limiting enzyme concerning the production of PDO, so it is necessary to improve this critical enzyme in some way, for example by increasing its catalytic power (k_{cat}). The enzyme consists of three homodimers in the form of $\alpha_2\beta_2\gamma_2$, and it has a typical $(\beta/\alpha)_8$ barrel in its α-subunit. This barrel forms the active site of the enzyme.[57] Two positions in the α- and β-subunits were identified, which seem to be responsible for the prolonged stability of the GDHt at somewhat acidic pH. Unexpectedly, alteration of these sites resulted in enzymes with either higher enzyme activities or changed specificities. After saturation−mutagenesis of these two positions three variants were found: one with a high activity (β-Q42F) and two with very good substrate specificity (β-Q42L and β-Q42S). The catalytic activity of the mutant β-Q42F is 8.3-fold higher than the wild type, and the enzyme efficiencies of the two other mutants for the substrate glycerol were 336-fold and 80-fold higher than that for 1,2-propanediol. The effect of these mutations *in vivo* was not studied.[57]

The second enzyme of the reductive pathway has also been modified via directed evolution. Because PDODH overexpression does not result in increased PDO production but cofactor promiscuity does, relaxation of cofactor specificity should affect PDO production significantly. Directed evolution of Asp41 led to a more promiscuous enzyme. The kinetic parameters, K_m, V_{max}, and k_{cat} for NADPH changed from a nondetermining level to 0.104 g/L, 102.7 g/L/min, 187.35 s^{-1}, respectively.[58] *In vivo* studies have not been performed yet.

3.4. Metabolic Engineering in *Clostridium* sp. Although *Clostridia* achieve the highest PDO concentrations reported, there is rarely a metabolic engineering strategy behind the production processes. This is due to the fact that no genetic tools are currently available for the PDO-producing *Clostridia*. *C. acetobutylicum* is a nonproducing microorganism which cannot reoxidize the NADH generated in the glycerol catabolism. However, the species is quite resistant towards high solvent concentrations (ABE-process), which makes it an interesting strain for PDO production. Furthermore, there are genetic tools for gene deletion and overexpression available.

By cloning the *C. butyricum* genes into *C. acetobutylicum*, *C. acetobutylicum* DG1(pSPD5) was created. This mutant is able to grow on glycerol and produce PDO together with the byproducts acetate and butyrate. When fed-batch cultures were carried-out, a high concentration of 84 g/L PDO, a yield of 0.65 mol/mol, and a productivity of 1.7 g/L/h were obtained (see Table 3). The PDO concentration and productivity are higher for the mutant strain as compared to those for the wild type, but the yield is lower.[66] Comparing the natural producing strain *C. butyricum* VPI3266 and the genetically engineered *C. acetobutylicum* DG1(pSPD5), revealed two significant differences. First, there is a difference in the regulation of their electron flow, and second there is a difference in their pathway for glycerol oxidation. While *C. butyricum* only uses a glycerol dehydrogenase and dihydroxyacetone kinase, *C. acetobutylicum*

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Table 3. **Summary of the different strategies of metabolic engineering for *Clostridium* sp. with the glycerol concentration used (g/L), the concentration PDO produced (g/L), the yield (mol/mol), and the productivity (g/L/h); all the fermentations were done anaerobically in fed-batch mode**

	glycerol used (g/L)	concentration PDO (g/L)	yield (mol/mol)	productivity (g/L/h)
		Production in a Nonproducing Strain[a]		
C. butyricum VPI3266	114	64.98	0.69	1.21
C. acetobutylicum DG1(pSPD5)	165.03	84	0.65	1.70
		Directed Evolution[b]		
C. diolis DSM15410	91.08	47.48	0.586	2.147
genome shuffling mutant 2	156.09	78.52	0.642	2.821
genome shuffling mutant 4	167.14	84.76	0.632	2.745

[a] Reference 66. [b] Reference 67.

uses a glycerol kinase and a glycerol-3-phosphate dehydrogenase to oxidize glycerol. This is in agreement with the genome sequence of *C. acetobutylicum*, where only a glycerol kinase and glycerol-3-phosphate dehydrogenase gene were found.[19]

One report has been published so far about metabolic engineering of a natural producing *Clostridium* sp.[67] Herein, a classical strain improvement strategy was combined with genome shuffling.[68,69] The first method is a slow process, and the mutations are predominantly neutral or detrimental.[70] Genome shuffling on the other hand offers the advantages of accumulated beneficial mutations and the removal of unnecessary mutations due to simultaneous changes at different positions throughout the genome and, therefore, yields microbes of superior fitness.[68] First, the wild-type strain *C. diolis* DSM15410 was mutated with the chemical *N*-methyl-*N*′′-nitro-*N*-nitrosoguanidine (NTG) to obtain mutants that were more resistant to glycerol. After five rounds of mutagenesis and selection, five mutants were isolated with enhanced abilities to grow on glycerol concentrations (up to 138.14 g/L). In a similar way a PDO tolerant and a superior PDO producer were obtained. Four mutants are able to grow on PDO at concentrations up to 91.31 g/L, and one mutant produces up to 18.41 g/L PDO, which is 62% more than the wild-type strain. All mutants were used for genome shuffling to get organisms which are able to grow on high PDO and high organic acid concentrations. Two strains were selected and tested in fed-batch fermentations with pharmaceutical and crude glycerol. When pharmaceutical glycerol was used as the carbon source, the mutants produced more PDO than the parent strain; the mutants produced respectively 78.5 g/L and 85 g/L PDO as compared to the wild type, which produced 47 g/L (see Table 3). However, the strains produced more acids and biomass than the wild-type. The enhanced growth rate may reflect mutations that allow cells to use nutrients in the medium more effectively or to export inhibitory molecules more rapidly, leading to increased acid excretion.[67,70] When crude glycerol was used as carbon source, the mutants and the wild-type strain produce less PDO and display a longer lag phase.[67] This indicates inhibitory compounds using crude glycerol.

4. Perspectives

4.1. Multiple Knockout Mutants. Until now, the best PDO-producing organism is a modified *E. coli*, but this strain starts the conversion from glucose. A lot of effort has been put in to increase the production in natural producing strains like *Klebsiella* sp. and *Clostridium* sp., but none of them reached an as high final titer, yield or productivity as the *E. coli* production strain. However, only single knockout mutants are made in *Klebsiella* sp. or single genes are up-regulated, while in the *E. coli* strain several genes are knocked out or up-regulated. Similar to *E. coli*, the methods used for *Klebsiella* sp. to create knockout mutants are based on homologous recombination. A gene is replaced by an antibiotic-resistant gene lying on a plasmid and flanked by up to 500 bp[48] homology. One big disadvantage is that an antibiotic-resistant gene is introduced in the genome of the bacterium, which is not favorable for industrially applied strains or when multiple knockout mutants need to be created. Furthermore, plasmid construction is very laborious. A far more efficient method is the method of Datsenko and Wanner using linear DNA created by a PCR with 35-nucleotide homology extended primers. More of this technique can be read in several papers and reviews.[71−73] This system is already applied for several microorganisms, such as *E. coli*,[72,74] *Pseudomonas aeruginosa*,[75] *Salmonella enterica*,[76] *Pantoea ananatis*,[77] *Shigella* sp.[78] and *Yersinia* sp.[79] Moreover, this technique is used to make a mutant of *E. coli* where eight genes are knocked out.[74] The advantage of this method is that more than one gene can be disrupted so the productivity, yield, and final concentration can be enhanced compared to a single knockout. In the case of the production of PDO lactate, ethanol, and other byproducts can be eliminated so that acetate is the only byproduct. This is favorable for the downstream processing. By allowing acetate as the only byproduct, a maximum theoretical yield of 0.72 mol/mol glycerol can be obtained.[20,80]

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Therefore, broadening the applicability of this technique to *Klebsiella* and/or other natural producing strains might be interesting to study.

4.2. Fine-Tuning Genes. The overexpression of genes of suboptimal fluxes can have dramatic consequences on the overall metabolism if the resulting flux is not optimal.[81−83] Thus, it is essential to tune the expression of genes, to tune the flux through a certain reaction.[84] A first method for fine-tuning gene expression is with the aid of an inducible expression system. These systems need an inducer for the activation of the promoter. Most systems, however, show an all-or-nothing phenomenon: although the mean expression is linear to the concentration of the inducer, only a fraction of the cells is induced.[85,86] Using the technique for metabolic engineering, it is desirable to induce all cells.

An alternative method is the insertion of a constitutive promoter with an optimal strength. Several methods are described in literature to develop artificial promoter libraries for a certain microorganism or for a group of organisms.[84,87−89] The library covers a wide range of promoter activities, in small steps of increasing activity. In this case the promoter can be chosen which enhances the productivity and retains an optimal flux.

In the case of PDO it is favorable to up-regulate the genes coding for the GDHt and the PDODH/PDOR. Therefore, a suitable stronger promoter can replace the natural one. However, the *dhaD* and *dhaK* genes should be down-regulated to enhance the flux to the reductive pathway, but still produce some reducing equivalents needed for the production of PDO. A (strain specific) promoter library can be used to tune expression of these genes.

4.3. Other Natural Producing Strains. Although *K. pneumoniae* and *C. butyricum* are the most well-studied microorganisms regarding PDO production, there are several other strains that produce 1,3-propanediol from glycerol for which techniques for genetic engineering are known. *Lactobacillus* sp., for example, cannot grow on glycerol alone but can convert glycerol into PDO during growth on available carbohydrates. In this way, they can produce PDO anaerobically, growing on a medium with a glycerol/glucose mixture. Tobajas et al. (2008) have produced 12.99 g/L PDO starting from 20 g/L glucose and 18.4 g/L glycerol.[90] Although several *Lactobacillus* sp. have been engineered,[91−93] these strains have not been engineered for PDO production.

Other naturally producing strains such as *Enterobacter agglomerans* and *Citrobacter freundii* have been studied for their conversion of glycerol to PDO, but there are no metabolic engineering techniques known yet. Thus, these organisms are less likely to be used, since the tools need to be developed first.

4.4. Economical Perspectives. An important aspect concerning research is the economical viability of the developed process. To this end, the product concentration, yield, and productivity should minimally be 100 g/L PDO, 0.41 mol/mol glucose, and 1.67 g/L/h, respectively.[94] However, to be competitive to the DuPont/Degussa process, the parameters should respectively be in the range of 135 g/L PDO, 0.62 mol/mol glucose, and 3.5 g/L/h. A comprehensive study has been performed in 2007 by the University of Utrecht wherein techno-economic prospects of production routes for biobased bulk

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chemicals are evaluated.[94,95] The first conclusion is that the production cost is about twice as high using glycerol as carbon source compared to the cost of sugar-based fermentation. This is due to the higher cost of glycerol as compared to glucose (700 €/t as compared to 200−400 €/t). However, important industrial players speculate that the price of glycerol will drop to the price of glucose or further, meanwhile positively influencing production costs starting from glycerol. Although the production cost plus profits (PCPP) (which is a proxy for the market price) for the petrochemical route of PDO production is 1120 €/t, the market price for PDO is 2410 €/t. This is due to the production in relatively small facilities nowadays, the use of PDO for higher-value applications such as PTT, and the imperfect market conditions.[95] Therefore, it is more reliable to choose the PCPP of petrochemically produced PDO as a benchmark instead of the market price. The study concluded that biobased PDO (produced according to the glucose-based process[43]) is competitive with petrochemically produced PDO for a sugar price up to 200 €/t and crude oil prices of 0.126 €/L. Increasing the crude oil price to 0.252 €/L results in an increase of the PCPPs of the petrochemically produced PDO by 23% on average as a result of higher feedstock and energy costs. However, the PCPPs of the biobased PDO increase only by 4−6% due to higher utility costs.[94] On the basis of this assumption, the study considered the biobased route competitive to the petrochemical route for a sugar price of up to 400 €/t. Taking this into consideration, together with the fact that the current crude oil price already reaches 0.378 €/L (ref 2010), production routes starting from glycerol are likely to become economically viable and even beneficial.

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5. Conclusion

Although a lot of research has already been done, there is only one strain available for the industrial production of PDO from glycerol. A lot of progress has been made by creating single knockout mutants of *Klebsiella* sp., but the final titer still stays under 100 g/L for most of them. The use of multiple knockout mutants and the fine-tuning of genes in natural PDO producing microorganisms would probably create the opportunity to reach end concentrations comparable to the glucose-derived pathway in *E. coli*. Moreover, taking all currently playing economical factors into reflection, PDO production starting from glycerol might become economically favorable.

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Abbreviations

3-HPA = 3-hydroxypropionaldehyde
DHA = dihydroxyacetone
FDH = formate dehydrogenase
GDHt = glycerol dehydratase
MIP = major intrinsic protein
NTG = *N*-methyl-*N*′′-nitro-*N*-nitrosoguanidine
PCPP = production cost plus profits
PDO = 1,3-propanediol
PDODH = 1,3-propanediol dehydrogenase
PDOR = 1,3-propanediol oxidoreductase
PET = poly(ethylene terephthalic acid)
PTS = phosphotransferase system
PTT = poly(trimethylene terephthalic acid)
TPUs = thermoplastic polyurethane elastomers

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