Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry

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Although biofuels such as biodiesel and bioethanol represent a secure, renewable and environmentally safe alternative to fossil fuels, their economic viability is a major concern. The implementation of biorefineries that co-produce higher value products along with biofuels has been proposed as a solution to this problem. The biorefinery model would be especially advantageous if the conversion of byproducts or waste streams generated during biofuel production were considered. Glycerol-rich streams generated in large amounts by the biofuels industry, especially during the production of biodiesel, present an excellent opportunity to establish biorefineries. Once considered a valuable 'co-product', crude glycerol is rapidly becoming a 'waste product' with a disposal cost attributed to it. Given the highly reduced nature of carbon in glycerol and the cost advantage of anaerobic processes, fermentative metabolism of glycerol is of special interest. This review covers the anaerobic fermentation of glycerol in microbes and the harnessing of this metabolic process to convert abundant and low-priced glycerol streams into higher value products, thus creating a path to viability for the biofuels industry. Special attention is given to products whose synthesis from glycerol would be advantageous when compared with their production from common sugars.

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Introduction

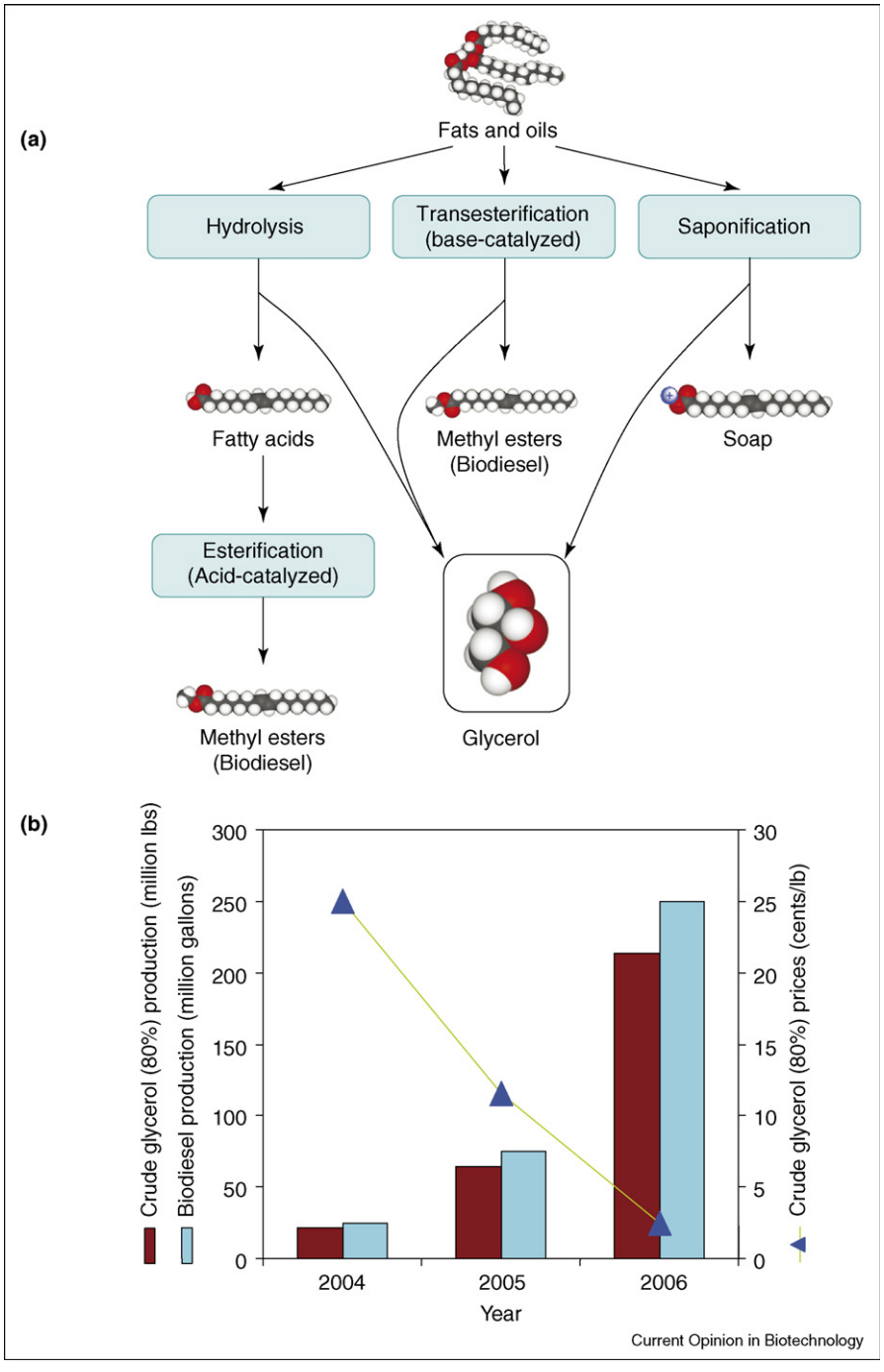
Our economy and lifestyle rely on the use of fossil resources for the generation of transportation fuels and materials; however, there has been rising concern over their cost, sustained availability, and impact on global warming and pollution [1]. This has led to a search for technologies that generate fuels and materials from renewable carbon sources, such as plant biomass. Depending on the component of the biomass used as feedstock (e.g. sugars, oils/fats) and the technology employed to transform this component into the desired product, at least three general platforms have been envisioned: the sugar [2], syngas (synthesis gas) [3], and oil [4] platforms. The sugar and oil platforms are the most well-established today, with bioethanol and biodiesel being examples of their commercial products, respectively. Bioethanol is produced through the microbial fermentation of sugars derived from corn, sugarcane or sugar beet [5]. Biodiesel is produced by the transesterification of vegetable oils or animal fats with an alcohol to produce esters [4].

Given the increasing demand for biofuels [6], there is an urgent need to investigate new and more efficient alternatives for their production. For example, the conversion of lignocellulosic biomass to ethanol and the use of oil-accumulating algae in the production of biodiesel are being investigated [7,8]. These approaches are very promising and will provide abundant nonfood feedstocks for the production of biofuels with environmental benefits and large net energy gains. However, an outstanding issue in both current and future biofuel production platforms is economic viability. For example, the feedstock and operating costs for the production of biodiesel from soybean amount to $2.94 per gallon of biodiesel, which almost matches the market price of $3.02 per gallon of biodiesel (04/11/2007: www.thejacobsen.com). The implementation of biorefineries has been proposed as a means to increase the economic viability of the biofuels industry [9]. In its 'conventional' form, a biorefinery would make use of a fraction of the feedstock (e.g. a portion of sugars or oils) to co-produce a higher value, small-market chemical along with the biofuel(s). The higher revenue from the co-product, which benefits itself from the economies of scale available in a large biofuels plant, would improve the economics of biofuel production. A more economically viable model for a biorefinery, however, should consider the use of byproducts or waste streams generated during the production of the biofuel. Glycerol-rich streams generated by the biofuels industry (Figure 1a) have the potential to be used in this context. This review focuses on the anaerobic conversion of crude glycerol into higher value products as a means to improve the economic viability of the biofuels industry.

Conversion of glycerol into higher value products: a need and an opportunity

Glycerol is generated in large amounts during the production of both bioethanol [10] and biodiesel [11]. With every 100 lbs of biodiesel produced by the transesterification of vegetable oils or animal fats, 10 lbs of crude glycerol

Figure 1



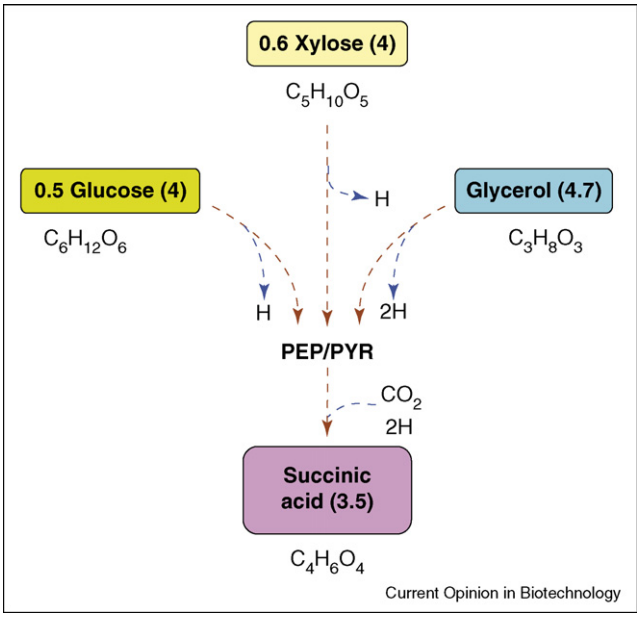
Oils, fats, biodiesel and glycerol. **(a)** Biodiesel production from vegetable oils and animal fats and its relationship with primary oleochemical platform pathways [39,40]. Glycerol is an inevitable by-product of oils/fats processing, regardless of the pathway. **(b)** US biodiesel production and its impact on crude glycerol prices (www.thejacobsen.com). Crude glycerol produced by the biodiesel industry was estimated assuming the generation of 0.853 lb of glycerol per gallon of biodiesel.

are generated. The tremendous growth of the biodiesel industry created a glycerol surplus that has resulted in a dramatic 10-fold decrease in crude glycerol prices over the past two years (Figure 1b). Glycerol-producing/refining operations by companies such as Dow Chemical and Procter and Gamble Chemicals were shut down [12] and the oleochemical industry has been negatively affected by the glycerol surplus [13]. The negative impact of low

glycerol prices is more evident in the biodiesel industry itself. Once considered a desirable co-product that could contribute to the economic viability of biodiesel production, many now regard crude glycerol as a 'waste stream' with a disposal cost associated to it. For example, an analysis of the feedstock and processing costs in the production of biodiesel from soybean oil yields a gross processing margin of about $0.079 per gallon of biodiesel (including a glycerol credit of $0.021, but excluding any interest expense, tax credits or fixed costs) (04/11/2007: www.thejacobsen.com). Essentially, if 2004 glycerol prices (Figure 1b) were still valid, the glycerol revenues by themselves would amount to about three times the current gross processing margin (i.e. crude glycerol at $0.25/lb × 0.85 lb/gal would result in a glycerin credit of ~$0.21). Clearly, the development of processes to convert crude glycerol into higher value products is both an urgent need and a 'target of opportunity' for the development of biorefineries. Such technologies could be readily integrated into existing biodiesel facilities, thus establishing true biorefineries and revolutionizing the biodiesel industry by dramatically improving its economics. Moreover, waste streams containing high levels of glycerol are generated in almost every industry that uses animal fats or vegetable oils as starting material (Figure 1a). For example, the oleochemical industry generates waste streams containing 55–90% glycerol [14]. Such glycerol surplus will not only result in a further reduction in prices, but the disposal of these streams will become a major issue [12].

Several strategies based on chemical and biological transformations are being pursued to convert glycerol into more valuable products. Biological conversion could help circumvent the disadvantages of chemical catalysis (e.g. low product specificity, use of high pressure and/or temperatures, inability to use crude glycerol with high levels of contaminants, etc.), while offering the opportunity to synthesize a large array of products and functionalities. At current prices (~2.5 cents/lb), glycerol is very competitive with sugars used in the production of chemicals and fuels via microbial fermentation. Given the highly reduced nature of carbon atoms in glycerol, additional advantages can be realized by using glycerol instead of sugars. For example, conversion of glycerol into the glycolytic intermediates phosphoenolpyruvate (PEP) or pyruvate generates twice the amount of reducing equivalents produced by the metabolism of glucose or xylose (Figure 2). Fermentative metabolism would then enable higher yield of fuels and reduced chemicals from glycerol compared with those obtained from common sugars such as glucose or xylose. The advantages of glycerol are evident when the synthesis of a reduced compound, such as succinic acid, is considered. Although production from glycerol can be achieved through a redox-balanced pathway, the use of glucose or xylose results in a shortage of reducing equivalents that clearly limits succinic acid yield (Figure 2). Anaerobic fermentation is also preferred because of its lower capital and operational costs when compared to aerobic fermentations (anaerobic fermenters are less expensive to build and operate than their aerobic counterparts and also use less energy). In the next two sections we first describe the fermentative metabolism of glycerol in microbes and then discuss the use of anaerobic fermentation processes for the conversion of glycerol into higher value products.

Figure 2

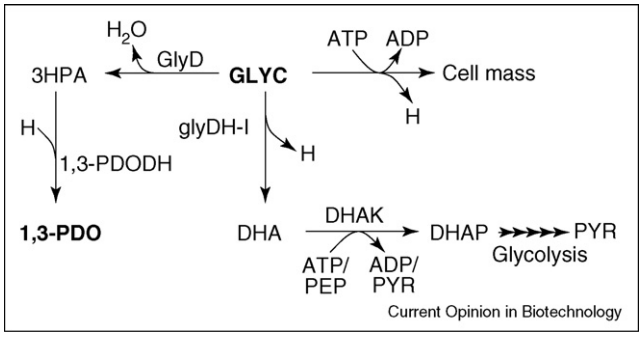


Generation of reducing equivalents during the conversion of glucose, xylose and glycerol into the glycolytic intermediates phosphoenolpyruvate (PEP) or pyruvate (PYR) in bacteria. The conversion of PEP into succinic acid is also illustrated. Broken lines represent pathways composed of several reactions. H, reducing equivalents (H = NADH/NADPH/$FADH_2$). The degree of reduction per carbon, κ, is indicated in parenthesis; κ was estimated as described elsewhere [41].

Fermentative metabolism of glycerol by microbes

Although many microorganisms are able to metabolize glycerol in the presence of external electron acceptors (respiratory metabolism) [15,16], few are able to do so fermentatively (i.e. in the absence of electron acceptors). Fermentative metabolism of glycerol has been studied in great detail in several species of the *Enterobacteriaceae* family, such as *Citrobacter freundii* and *Klebsiella pneumoniae*. Dissimilation of glycerol in these organisms is strictly linked to their capacity to synthesize the highly reduced product 1,3-propanediol (1,3-PDO) [17]. It is known that a dismutation process involving two pathways is responsible for this phenomenon (Figure 3) [15]. Through the oxidative pathway, glycerol is dehydrogenated by an NAD-linked glycerol dehydrogenase (glyDH) to dihydroxyacetone (DHA), which is then phosphorylated by PEP- and ATP-dependent DHA kinases (DHAK). Through the

Figure 3



Current model for the 1,3-PDO-dependent fermentative metabolism of glycerol in species of the *Enterobacteriaceae* family, such as for the genera *Klebsiella* and *Citrobacter*. Abbreviations: DHA, dihydroxyacetone; DHAK, DHA kinase; DHAP, DHA phosphate; GLYC, glycerol; GlyD, glycerol dehydratase; glyDH-I, glycerol dehydrogenase type I; PEP, phosphoenolpyruvate; PYR, pyruvate; 1,3-PDO, 1,3-propanediol; 1,3-PDOH, 1,3-PDO dehydrogenase; 3HPA, 3-hydroxypropionaldehyde.

parallel reductive pathway, glycerol is dehydrated by the coenzyme B_{12}-dependent glycerol dehydratase to form 3-hydroxypropionaldehyde (3-HPA). 3-HPA is then reduced to the major fermentation product 1,3-PDO by the NADH-linked 1,3-PDO dehydrogenase (1,3-PDODH), thereby regenerating NAD^+ (Figure 3). Only eight taxa of the *Enterobacteriaceae* (out of 1123 strains from 128 taxa tested) are reported to grow fermentatively on glycerol and all produce 1,3-PDO and possess both glyDH type I and 1,3-PDODH [17]. The above-described pathways provide the basis of the current model for the fermentative metabolism of glycerol in microorganisms. In addition to *Klebsiella* and *Citrobacter*, species from the genera *Enterobacter*, *Clostridium*, *Lactobacillus* and *Bacillus* have also been reported to ferment glycerol in a 1,3-PDO-dependent manner. The need for an active 1,3-PDO pathway results from the highly reduced state of carbon in glycerol, whose incorporation into cell mass generates reducing equivalents (Figure 3). As conversion of glycerol into 1,3-PDO results in the net consumption of reducing equivalents, this pathway provides a means to achieve redox balance in the absence of electron acceptors.

A few studies have reported fermentative metabolism of glycerol in the absence of 1,3-PDO synthesis. For example, we found that *Escherichia coli* is able to ferment glycerol when appropriate conditions are maintained. These conditions include an acidic pH, avoiding accumulation of fermentation gas hydrogen, and appropriate medium composition [18••] (R Gonzalez *et al.*, unpublished). An active formate hydrogen-lyase system was identified as a requirement for glycerol fermentation to proceed [18••]. In more recent studies, we have demonstrated the fermentative nature of glycerol metabolism, the use of glycerol as carbon source for the synthesis of cell mass, along with the identification of oxidative and reductive pathways that mediate this metabolic process (R Gonzalez *et al.*, unpublished). Although the 1,3-PDO-independent fermentation of glycerol has also been reported in species of the genera *Propionibacterium* [19•] and *Anaerobiospirillum* [20], the mechanisms and pathways mediating the dissimilation of glycerol in these organisms have not been investigated.

Anaerobic fermentation of glycerol as a source of fuels and chemicals

Use of *Klebsiella*, *Citrobacter*, *Enterobacter* and *Clostridium* species

Several attempts have been made to achieve efficient production of chemicals and fuels in microorganisms that ferment glycerol in a 1,3-PDO-dependent manner. The largest body of work has focused on optimizing the conversion of glycerol into 1,3-PDO, a major product of glycerol fermentation in these species. Strain-based improvements include the inactivation of the aldehyde dehydrogenase gene [21] and overexpression of genes of the *dha* regulon [22], the latter coding for the enzymes in the 1,3-PDO pathway (Figure 3). Process-based improvements, on the other hand, include micro-encapsulation of the production strain [23], electrodialysis of crude substrate [24], media additives [25], study of growth inhibitors [26], and manipulation of culture parameters [27,28]. Some approaches have taken advantage of both genetic and bioprocess strategies, achieving 1,3-PDO levels close to 100 g/L [29•,30]. An alternative that has been explored is the engineering of organisms that do not naturally ferment glycerol by importing pathways from natural glycerol-fermenting organisms; for example, the 1,3-PDO pathway from *Clostridium butyricum* was introduced into *Clostridium acetobutylicum* resulting in efficient glycerol fermentation and 1,3-PDO synthesis [31••].

The synthesis of products other than 1,3-PDO using these glycerol-fermenting organisms has also been reported. For example, butanol was found to be the major product of glycerol fermentation by *Clostridium pasteurianum* under certain cultivation conditions [32]. The maximum butanol concentration obtained was 17 g/L, apparently due to product toxicity. In another report, ethanol and formate were shown to be the two main products of glycerol fermentation by a *Klebsiella planticola* strain isolated from the rumen of red deer [33]; they were produced in equimolar amounts at concentrations up to 2 g/L, but very long fermentation times were required to achieve significant fermentation of glycerol. The co-production of ethanol and hydrogen from glycerol-containing wastes by an *Enterobacter aerogenes* mutant has also been reported [34]. The highest yields and productivities were achieved in a packed-bed reactor that used porous ceramics as support material to immobilize the cells. Ethanol concentrations of up to 10 g/L were reported. The co-production of ethanol-hydrogen or ethanol-formate presents an excellent

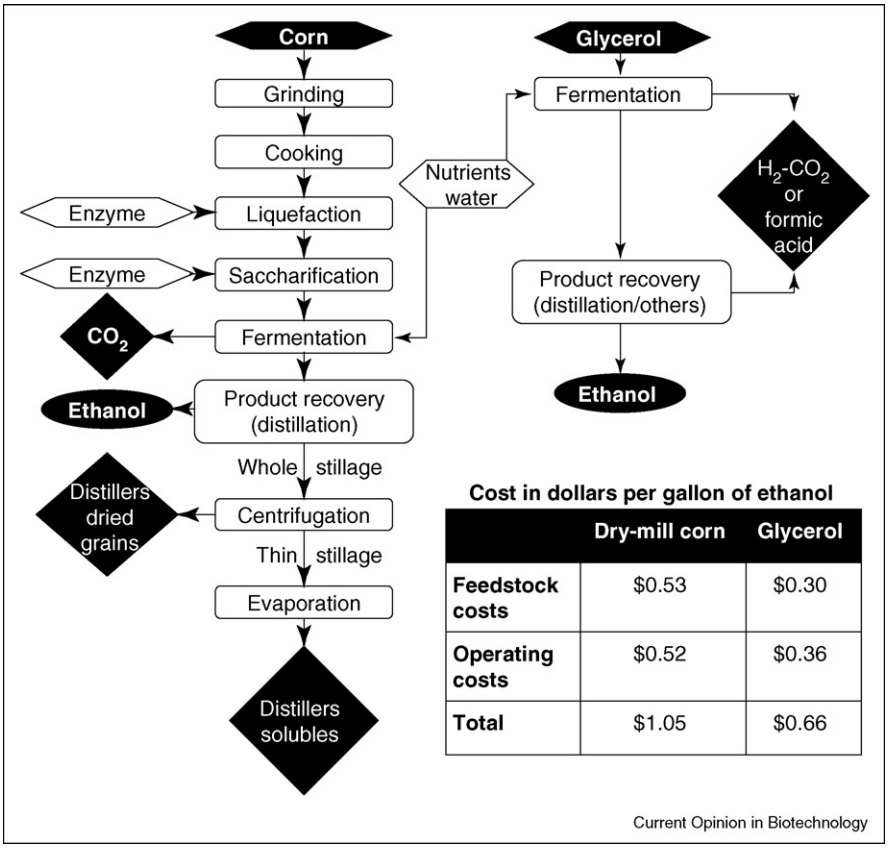
opportunity to illustrate the advantages of glycerol fermentation. Figure 4 compares ethanol production from corn-derived sugars with ethanol production from glycerol (including H_2-CO_2 or formic acid as co-products) in terms of the type of manufacturing facility required and the feedstock and operational costs associated with it. Clearly, the layout of a facility for the production of ethanol from corn is more complex (Figure 4) and thus more capital intensive. Furthermore, the operational costs are almost 40% lower in the production of ethanol from glycerol, even when in this analysis no credit was given to the formate or hydrogen co-produced along with ethanol (Figure 4). Although this comparison relates to ethanol production, the same cost advantages can be realized by the production of other fuels and reduced chemicals from glycerol.

Use of *Propionibacterium*, *Anaerobiospirillum* and *Escherichia* species

Several microorganisms for which no 1,3-PDO synthesizing activity has been reported are also being considered for the conversion of glycerol into higher value products. For example, the synthesis of propionic acid by strains *Propionibacteria acidipropionici* and *Propionibacteria freudenreichii* ssp. *shermanii* was reported [19•]. Immobilizing the cells on calcium alginate and increasing the initial concentration of glycerol allowed concentrations of propionic acid as high as 42 g/L to be achieved. Moreover, the conversion of glycerol into succinic acid using *Anaerobiospirillum succiniciproducens* has been reported [20]. This study demonstrated that succinic acid could be produced with little formation of by-product acetic acid by using glycerol as a carbon source, thus facilitating purification of succinic acid. The highest yield was obtained by intermittently feeding glycerol and yeast extract, a strategy that resulted in the production of about 19 g/L of succinic acid. It was noted, however, that unidentified nutritional components present in yeast extract were needed for glycerol fermentation to take place.

Although we have described the progress on the use of microbial species able to ferment glycerol that belong to

Figure 4



Cost in dollars per gallon of ethanol

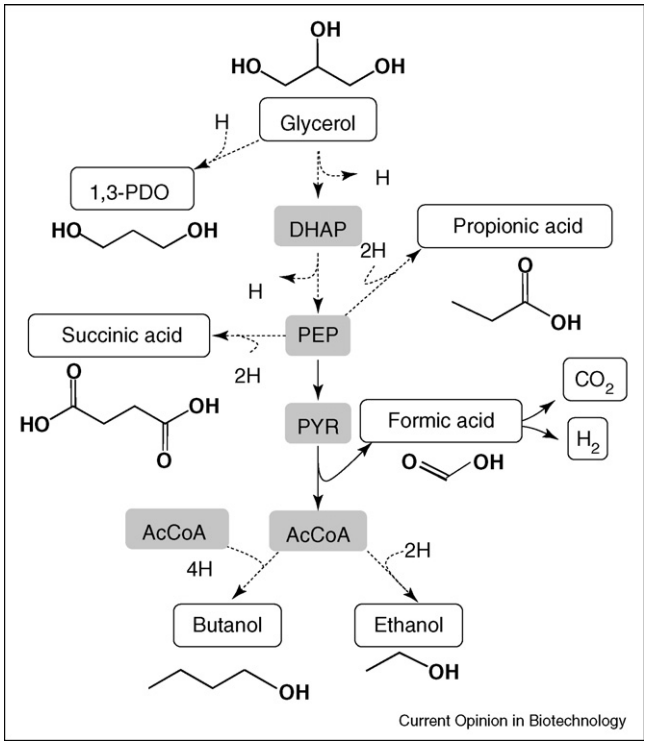
	Dry-mill corn	Glycerol
Feedstock costs	$0.53	$0.30
Operating costs	$0.52	$0.36
Total	$1.05	$0.66

Comparison of ethanol production from corn-derived sugars (dry grind ethanol) with ethanol production from glycerol. Table shown as inset compares the feedstock and processing costs. Calculations are based on the 2003–2005 period, except for glycerol prices, which are current. Corn-based ethanol calculations are as reported [42]. Glycerol-based ethanol calculations were conducted as follows. Feedstock cost considers current glycerol prices (Figure 1b) and a 95% molar yield of ethanol from glycerol [18••]. Operating cost was assumed to be similar to that estimated for molasses, raw, and refined sugar [42]. Feedstock cost is 'net' for corn-derived ethanol (includes revenue from co-products), but not for glycerol-derived ethanol (does not include revenue from co-products H_2 or formic acid). Same cost advantages can be realized in the production of other fuels and reduced chemicals from glycerol.

the genera *Klebsiella*, *Citrobacter*, *Enterobacter*, *Clostridium*, *Propionibacterium* and *Anaerobiospirillum*, the potential for using these organisms at industrial level could be limited. Among the most relevant issues are pathogenicity, requirement of strict anaerobic conditions, the need for supplementation with rich nutrients, and a lack of appropriate genetic tools and physiological knowledge necessary for their effective manipulation. Heterologous expression of the 1,3-PDO pathway genes in organisms more amenable to industrial applications, such as *E. coli*, has been reported [35,36]. As in the case of *Clostridium* species [37], efforts have been limited to the production of 1,3-PDO.

A recent development in the microbial fermentation of glycerol is our discovery that *E. coli*, an organism considered the workhorse of modern biotechnology, can anaerobically ferment glycerol [18••] (R Gonzalez *et al.*, unpublished). The use of *E. coli* as biocatalyst could help overcome the problems associated with the use of microorganisms that are not amenable to industrial applications, as discussed above. In addition, the feasibility of engineering *E. coli* for the production of chemicals and fuels from sugars has been extensively documented [38]. Therefore, metabolic engineering strategies can be used to develop *E. coli*-based platforms for the anaerobic production of reduced chemicals from glycerol at yields higher than those obtained from common sugars, such as glucose or xylose. Examples of such products are given in Figure 5; in all cases the maximum theoretical yields are significantly higher than those obtained with the use of sugars. Through strain- and process-based strategies we have achieved several fold improvements (yield and/or productivity) in the production of ethanol-formate, ethanol-hydrogen, succinic acid, and other products (R Gonzalez *et al.*, unpublished).

Figure 5



Examples of products whose synthesis from glycerol is a redox-balanced or redox-consuming process. The maximum theoretical yield in each case is higher than that obtained from the use of common sugars, such as glucose and xylose. Broken lines represent pathways composed of several reactions. Abbreviations: AcCoA, acetyl-coenzyme A; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; PYR, pyruvate; 1,3-PDO, 1,3-propanediol.

Conclusions

The use of anaerobic fermentation to convert abundant and low-priced glycerol streams generated in the production of biodiesel into higher value products represents a promising route to achieve economic viability in the biofuels industry. Several organisms are able to ferment glycerol and synthesize products with a wide-range of functionalities. If fuels and reduced chemicals are targeted, there are many advantages for the use of glycerol over sugars, which together translate into higher yields and lower capital and operational costs. The success of these technologies greatly depends on the use of microorganisms amenable to industrial applications, especially those for which metabolic engineering strategies can be readily implemented.

Acknowledgements

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