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REVIEW PAPER

Metabolically Engineered *Escherichia coli* as a Tool for the Production of Bioenergy and Biochemicals from Glycerol

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Abstract Currently, a variety of feedstock is utilized by metabolically engineered bacteria for the production of bioenergy and biochemicals. Recent studies have shown that glycerol can be used as an alternative feedstock for glucose, considering its higher availability, lower price, and high degree of reduction. Hence, this review focuses on recent developments in the bioconversion of glycerol to bioenergy (ethanol and hydrogen) and biochemicals (1,3-propanediol, 1,2-propanediol, 3-hydroxypropionic acid, succinic acid, lactic acid, polyhydroxyalkanoates and L-phenyl alanine) using metabolically engineered *Escherichia coli*.

Keywords: glycerol, *Escherichia coli*, bioenergy, biochemicals, metabolic engineering

1. Introduction

Glycerol (1,2,3-propanetriol, or glycerin) is a structural component of many lipids, and has been widely used in various industries including dental, detergent, pharmaceutical, paint and food [1]. Glycerol is obtained as a byproduct during the trans-esterification of vegetable oils and animal fats [2-4]. It has become a cheap and abundant carbon feedstock, since the tremendous growth of biodiesel industries has resulted in the production of enormous quantities of crude glycerol [5]. By 2016, the overall biodiesel market is estimated to reach 37 billion gallons with an average annual growth of 42% (4 billion gallons of crude glycerol) [6]. Thus, the use of glycerol as a feedstock in fermentation processes could be highly advantageous in regards to excessive availability and low cost [5,7].

Several strategies are being used to convert glycerol into more valuable products either biologically or chemically. Bio-based processes are considered to be more attractive processes than chemical processes, since the use of high temperatures/pressures and the generation of high levels of contaminants can be avoided. Fermentative metabolism of glycerol results in various reduced biochemical, such as 1,3-propanediol (1,3-PD), 1,2-propanediol (1,2-PDO), 3-hydroxypropionic acid (3-HP), D- or L-lactate or lactic acid, succinate or succinic acid, polyhydroxyalkanoates (PHAs), and L-phenylalanine at high yield. In addition, biofuels, such as ethanol and hydrogen, can be produced from glycerol in a higher yield.

These biochemicals and biofuels have many industrial applications and have drawn a great deal of attention. The global market for bioenergy and biochemicals from biomass is estimated to reach 150 billion dollars by 2050 [8]. Similarly the biorefinery market is expanding rapidly. For example, 3-HP has been ranked 3rd by the U. S. DOE among the top 12 value-added products, which can be produced from renewable biomass and the global market has been estimated to be 3.63 million tons per year [9]. Succinic acid has also been identified as one of the top 12 building block chemicals by the U. S. DOE, [10] since it can be converted to a wide variety of products [11,12].

In recent years, the increase in petroleum prices has generated a substantial interest in metabolically engineered *Escherichia coli* for the production of biofuels. Using glycerol as a feedstock, engineered *E. coli* may be capable of growing and generating a high yield of bioenergy and biorefinery-like ethanol, hydrogen, and 3-HP [13,14]. This

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review focuses on metabolically engineered *E. coli* production of bioenergy and biochemicals using glycerol as a carbon source. The available genetic tools and the large genomic, metabolic, and physiological knowledge of *E. coli* have facilitated the engineering of this bacterium [15,16]. Various strategies that have been employed to engineer *E. coli* to efficiently utilize glycerol for the production of bioenergy and biochemicals are described. This review showed that glycerol is an abundant and viable source that could be efficiently employed as a potent raw material in the bioenergy and biochemical industries.

2. Bioenergy Production from Glycerol

Bioenergy is a desirable alternative energy considering that they are compatible with storage, distribution, and engine compressibility of the current fuel infrastructure [17]. *E. coli* is considered to be one of the most promising bioenergy producers, because it can be grown efficiently under industrially relevant conditions and to its tolerance to high concentrations of substrate and products. Advances in metabolic engineering approaches have resulted in the ability to engineer *E. coli* to produce bioenergy materials far beyond the scope of what any single organism could naturally make [16,18]. The following section explains the use of glycerol as a carbon source and the engineering strategies that have been employed to modify *E. coli* metabolism (Fig. 1).

2.1. Ethanol and hydrogen

Hydrogen is used as an energy carrier for fuel cells in portable electronics, power plants, and internal combustion engines [19]. It can be produced by the pyrolysis of glycerol at high temperatures and the resulting gas has a complex composition [20]. Currently, the biological production of hydrogen by microorganisms is getting attention because of its lower energy requirement than conventional thermal and electrolytic processes [21]. The process of hydrogen production by the anaerobic fermentation of glycerol has been studied in *Enterobacter aerogenes* [22,23], *E. coli* [24,25], *Klebsiella pneumonia* [26], and mixed cultures [27]; these systems were found to be attractive since they could produce both hydrogen and ethanol [22].

Identification of key pathways and environmental conditions affecting glycerol metabolism has provided an alternative way to engineer *E. coli* into an efficient ethanol producer [24,25]. In the native *E. coli* pathway, pyruvate decarboxylase (Pdc) converts pyruvate directly to acetaldehyde and CO_2. The alcohol dehydrogenase (AdhE) then converts acetaldehyde into ethanol [28]. In contrast, ethanol production in *Zymomonas mobilis* is a two-step process, in

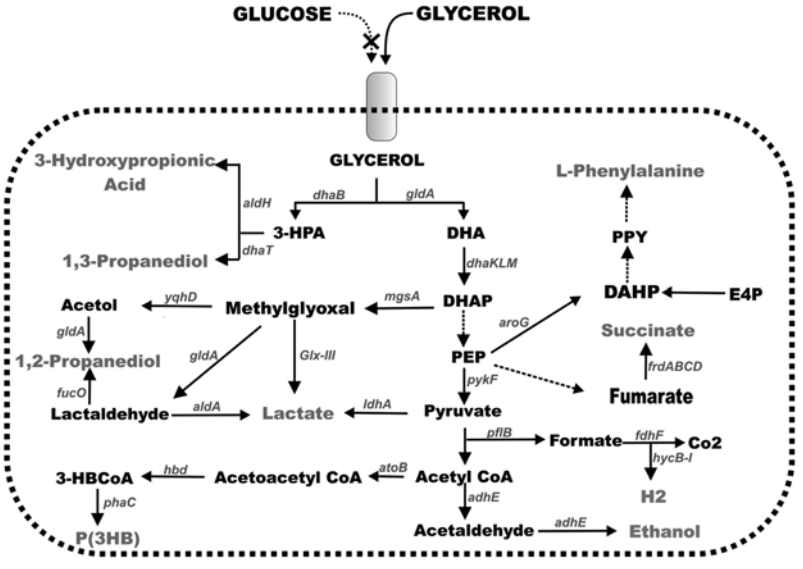

Fig. 1. The *E. coli* pathways involved in the synthesis of bioenergy and biochemical products during the fermentative metabolism of glycerol. Broken lines illustrate multiple steps. Biochemicals are violet and biofuels are red. Relevant reactions are represented by the genes that code for the enzyme of that reaction. Gene/enzyme names: *adhE*, alcohol dehydrogenase; *aldA*, lactaldehyde dehydrogenase; *aldH*, aldehyde dehydrogenase; *aroG*, DAHP synthase; *atoB*, thiolase; *dhaB*, glycerol dehydratase; *dhaKLM*, dihydroxyacetone kinase; *dhaT*, 1,3 PD-dehydrogenase; *fdhF*, formate dehydrogenase; *frdABCD*, fumarate reductase; *fucO*, 1,2-propanediol reductase; *gldA*, glycerol dehydrogenase; *Glx-III*, glyoxylase type III; *hbd*, hydroxybutyryl-CoA dehydrogenase; *hycB-I*, hydrogenase; *ldhA*, lactate dehydrogenase; *mgsA*, methylglyoxal synthase; *pflB*, pyruvate formate lyase; *phaC*, polyhydroxyalkanoic acid synthase; *pykF*, pyruvate kinase; *ydhD*, aldehyde oxidoreductase. Abbreviations: DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; PEP, phosphoenol pyruvate; DAHP, 3-deoxy-d-arobino- heptulosonate 7-phosphate; 3-HPA, 3-hydroxypropionaldehyde; 3HB-CoA, 3-hydroxybutyrl CoA; E-4P, erythrose 4-phosphate; CO_2, carbon dioxide; H_2, hydrogen; PPY, phenylpyruvate.

which ethanol synthesis from pyruvate results in the consumption of one reducing equivalent, as opposed to the two consumed in the *E. coli* pathway. The expression of *pdc* and *adhE* genes of *Z. mobilis* in *E. coli* results in much higher yields of ethanol (about 95% of the total fermentation products) [28].

To improve the rate of glycerol utilization, genes that mediate glycerol dissimilation (*gldA* and *dhaKLM*) were overexpressed, which accelerated the formation of dihydroxyacetone phosphate (DHAP) from glycerol. To minimize the formation of by-products such as succinate and acetate, fumarate reductase (*frdA*), and phosphotransacetylase (*pta*) genes were simultaneously disrupted [29]. This resulted in an engineered strain that co-produced ethanol and hydrogen in nearly equimolar amounts and also reached the maximum theoretical yield of 1 mole each per mole of glycerol fermented. The ethanol production rate was 4.6 mmol/L/h.

The same strategy of overexpressing *gldA* and *dhaKLM* was used to engineer *E. coli* to simultaneously consume the carbon sources present in thin stillage. Thin stillage is a complex mixture of chemicals, which includes a low concentration of sugars such as glucose, maltose, and glycerol (up to 2%), and is produced in large quantities as a by-product of sugar based ethanol production process. The overexpression of *gldA* and *dhaKLM* in the engineered strain results in the efficient production of ethanol by simultaneously consuming glycerol and sugars in a mineral salt medium [30].

E. coli BW25113, which lacks *frdC*, underwent adaptive evolution and chemical mutagenesis to form the HW2 strain. The HW2 strain showed increased growth on glycerol and produced 0.68±0.16 mmol/L/h of hydrogen, which was 20-fold higher than the production rate by *E. coli* BW25113. Whole transcriptomic studies also revealed that the HW2 strain repressed the pathways that decreased the hydrogen yield by inducing the DHAP production pathway. In addition, the engineered strain shows higher ethanol production and grows five times faster than *E. coli* BW25113 [31].

3. Biochemical Production from Glycerol

Several metabolic engineering studies have been done for the efficient production of biorefinery products. Presently, glycerol is highly competitive with sugars for use in the production of chemicals by microbial fermentation (Fig. 1). In the following section, the conversion of glycerol to higher value chemicals is discussed in detail.

3.1. 1, 3-Propanediol

The first product obtained from glycerol fermentation is 1,3-PD, which can be used as monomer of polyester fibers, polyurethanes, and polyethers [4,32]. To avoid the drawbacks of current chemical procedures, such as toxic intermediates, high pressure, high temperature and expensive catalysts, the biological process of 1,3-PD production has been studied [33,34].

In the biological setting, glycerol is converted to 1,3-PD through a two-step enzymatic reaction. Glycerol is converted to 3-hydroxypropionaldehyde (3-HPA) by vitamin B12-dependent glycerol dehydratase (*dhaB*) and 3-HPA is further converted to 1,3-PD by NADH-dependent 1,3-PD oxidoreductase (*dhaT*) (Fig. 1) [35].Although microorganisms have been widely used to produce 1,3-PD from glycerol, the complete formation of 1,3-PD has not been achieved because of the need for additional reducing equivalents [36]. Since wild-type *E. coli* cannot ferment glycerol directly to 1,3-PD [37], recombinant *E. coli* strains have been used to produce 1,3-PD [38]. However, the excess requirement of Vitamin B12 hinders the large scale production of 1,3-PD based solely on glycerol. Vitamin B12-independent glycerol dehydratase (*dhaB1*) and its activating factor (*dhaB2*) were arrayed in tandem with the *E. coli* 1,3-PD oxidoreductase isoenzyme (*yqhD*), which can directly convert glycerol to 1,3-PD [39]. The overall yield and productivity of 104.4 g/L and 2.61 g/L/h were achieved, respectively.

1,3-PD processes that use more abundant substrates, such as starch and glucose, are being studied to decrease process cost [40]. In addition, research is being conducted to identify effective non-pathogenic microorganisms capable of producing 1,3-PD from glycerol.

3.2. 1,2-Propanediol

1,2-PDO is a major commodity chemical with global demand estimated to be around 3 billion lb/yr. It plays an important role in the production of less-toxic antifreeze, heat-transfer fluids, cosmetics, plasticizers, thermoset plastics, and chiral pharmaceutical products [41]. 1,2-PDO was produced from glycerol by *Saccharomyces cerevisiae* [42].

1,2-PDO has been reported to be a natural product of anaerobic fermentation of glycerol in *E. coli* [24]. 1,2-PDO can be produced via two different pathways; one through deoxy sugar and the other through the methyglyoxal (MG). Considering the low cost of substrate and high yield of product [5], MG pathway is considered to be the more promising one. In the MG pathway, DHAP is converted to MG by methylglyoxal synthase (*mgsA*). Then, MG is further converted to 1,2-PDO through acetol or lactaldehyde [43,44] (Fig. 1).

The native *E. coli* PEP-dependent dihydroxyacetone kinase (DhaK) was replaced with an ATP-dependent DhaK

from *C. freundii* to increase the DHAP production. Then, *mgsA*, *gldA*, and *yqhD* genes were overexpressed for the efficient conversion of DHAP to 1,2- PDO. Thus, the engineered strain produced 5.6 g/L of 1,2- PDO with a yield of 21.3%. The strain achieved the same yield even when crude glycerol was used as a substrate [45].

3.3. 3-Hydroxypropionic acid

3-HP serves as a precursor for several key compounds, such as 1,3-PD, acrylic acid, methyl acrylate, acrylamide, ethyl 3-HP, and malonic acid. In the chemical industries, it is used as a cross-linking agent for polymer coatings, metal lubricants, and antistatic agents for textiles [46].

In general, glycerol cannot be converted to 3-HP in *E. coli*, because of the lack of glycerol dehydratase (DhaB) and a low expression level of the aldehyde dehydrogenase (AldH). Hence, overexpression of DhaB and AldH is required for 3-HP production in *E. coli* [47-49]. The expression of DhaB and KGSADH (α-ketoglutaric semialdehyde dehydrogenase), along with glycerol dehydratase reactivase (GDR) [50] showed the highest level of 3-HP production of 38.7 g/L with an average yield of 35%, which is the highest reported level of 3-HP production from glycerol. However, due to the loss of enzyme activity and redox imbalance, the volumetric productivity and final titer was inadequate for commercial applications. So, intensive studies are needed to improve the stability of DhaB, the activity of KGSADH, and the appropriate NAD regeneration system.

3.4. Succinic acid

Succinic acid is a dicarboxylic acid produced as an intermediate of the tricarboxylic acid (TCA) cycle and as one of the fermentation products of anaerobic metabolism [51]. It is widely used in industries to produce food additive, pharmaceutical products, surfactants, detergents, green solvents, biodegradable plastics, and as ingredients to stimulate animal and plant growth [11]. The production of succinate from glycerol involves a redox-balanced pathway that includes CO_2 fixation onto a three-carbon intermediate and then conversion to succinate through the TCA cycle [52]. *E. coli* uses PEP carboxylase (Ppc) as the main carboxylation enzyme for succinate generation.

Under anaerobic conditions, glycerol dehydrogenase and phosphotransferase-dependent dihydroxyacetone kinase are the primary route for glycerol metabolism. But, neither of these pathways remains suitable for efficient succinate production due to the absence of net ATP production and the requirement for PEP as a phosphoryl donor for dihydroxyacetone [24,29]. Hence engineered *E. coli* produced succinate efficiently from glycerol by deleting genes essential for the phosphotransferase system. Together, combined mutations of phosphoenol pyruvate carboxykinase (*pck*), phosphotransferase system (*ptsI*), and *pflB* produced succinate with a maximum theoretical yield of 80% (0.8 mol/mol glycerol) [53].

Succinate production was significantly increased in *E. coli* by expressing *Lactococcus lactis* pyruvate carboxylase (Pyc), which lacks the pathways of competing by-products (lactate, ethanol and acetate). This results in a maximum specific productivity of approximately 400 mg succinate/g cell/h with a yield of 0.69 g succinate/g glycerol [54]. As metabolic engineering strategies coupled cell growth to succinate production, it remains as the primary route for NAD^+ regeneration.

3.5. Lactic acid

Lactic acid has many applications in food, cosmetics, pharmaceuticals, polymers, and agricultural industries [55-57]. As chemical conversion of glycerol to lactic acid requires high temperature, high pressure, and expensive catalysts [58], much attention has recently been focused on the bioprocess. The use of metabolically engineered *E. coli* holds great promise for the efficient production of lactate [59-63] by improving glycerol metabolism.

Eight bacterial strains were investigated with the goal of identifying a strain suitable for producing a high concentration of lactic acid. Among those, the *E. coli* strain AC-521 was found to be the most suitable for producing lactic acid from glycerol, due to its 16S rDNA sequences and physiological characteristics. The maximum lactic acid concentration of 85.8 g/L and a yield of 0.9 mol/mol glycerol was achieved [64].

E. coli was engineered to produce D-lactic acid (D-lactate) from glycerol. The lactic acid pathway related enzymes were overexpressed, and competing pathways were blocked. Engineered recombinant strain produced 32 g/L of D-lactate from glycerol with a maximum yield of 85% [65].

Although engineered strains hold great promise for the conversion of glycerol to D-lactate, process-based modifications including fed-batch cultivations and high-density cultures are needed to further improve the volumetric rates of D-lactate production.

3.6. Polyhydroxyalkanoates

PHAs are widely used in renewable, biodegradable, and biocompatible thermoplastics for industrial applications [66]. Poly (3-hydroxybutyrate) (PHB) belongs to the group of PHAs are synthesized naturally in a wide variety of bacterial species as a reserve compound for carbon and energy [67,68].

The advantages of employing recombinant *E. coli* for the production of PHA include fast growth, high cell density, the ability to use several inexpensive carbon sources, and easy purification [69,70]. A recombinant *E. coli* which

contains the genomic DNA fragment of *Streptomyces aureofaciens* was capable to accumulate PHB as a cytoplasmic inclusion body. Interestingly the production of PHB is 25 ~ 28 fold more than that of native *S. aureofaciens* when grown under glycerol [71].

Using glycerol as the main carbon source, recombinant *E. coli* arcA2 mutant produced 3.52 g/L PHB. In fed-batch cultures, while glycerol was added to maintain concentrations above 5 g/L, the PHB concentration reached 10.81 g/L, respectively. Thus, the 2.57-fold increase in volumetric productivity was seen in microaerobic fed-batch culture when compared to batch culture and it remains as a suitable strategy for PHB synthesis [72].

3.7. L-Phenylalanine

L-phenylalanine is used as a nutritional supplement and as a precursor for various catecholamines and for aspartame (an artificial sweetener). It has also been used in infusion fluids, food additives, in synthesis of active compounds, and as a flavor enhancer. Precursors of the L-phenylalanine biosynthetic pathway include PEP and erythrose-4-phosphate (E-4P) (Fig. 1). PEP is formed during glycolysis and during the pentose phosphate pathway; it supplies E-4P. Commercially, L-phenylalanine is most often produced by fermentation involving recombinant *E. coli* [73]. When using sucrose, the yield is about 0.20 ~ 0.25 g/g and the final concentration in the fermentation broth is nearly 50 g/L.

Glycerol based L-phenylalanine production was studied by genetically modified *E. coli* BL21 (DE3). Fermentation was carried out in a stirred tank bioreactor at various impeller agitation speeds and aeration rates. The yield of L-phenylalanine on glycerol was 0.58 g/g, which is more than twice the yield (0.25 g/g) attained on sucrose. The final L-phenylalanine concentration of 5.6 g/L was comparable to those obtained in the glucose or sucrose-based batch fermentation. Glycerol usage has substantially reduced production costs relative to sucrose- and glucose-based fermentations [74].

Table 1. Engineered *E. coli* strains yielding maximum production of biofuels and biochemicals

Compound	Strategy	Yield	Ref
Bioenergy			
Ethanol	Over expression of genes *gldA* and *dhaKLM* that mediate glycerol dissimilation; accelerated the formation of DHAP; deleting genes *frdA* and *pta* to minimize by-product formation.	4.6 mmol/L/h	[29]
Hydrogen	*E. coli* underwent adaptive evolution and chemical mutagenesis to grow efficiently in glycerol; lacking *frdC*.	0.68 mmol/L/h	[31]
Biochemicals			
1,3-propanediol	Expression of *dhaB1* and *dhaB2* genes of *Clostridium butyricum* were arranged in tandem with the *yqhD* gene of *E. coli;* direct conversion of glycerol to 1,3-PDO.	104.4 g/L	[39]
1,2-propanediol	Overexpression of genes for *mgsA*, *gldA*, *yqhD;* manipulation by replacing the native *E. coli* PEP-dependent dihydroxyacetone kinase (DHAK) with an ATP-dependent DHAK from *C. freundii.*	5.6 g/L	[45]
3-Hydroxypropionic acid	Expression of DhaB and AldH, along with glycerol dehydratase reactivase (GDR), and an alternate aldehyde dehydrogenase (KGSADH) in *E. coli;* aerobic fed batch process.	38.7 g/L	[50]
Succinic acid	By deletion of genes essential for the phosphotranferase system *pck*, *ptsI*, and *pflB;* anaerobic fermentation.	0.8 mol/mol glycerol	[53]
	Expression of *Lactococcus lactis* pyruvate carboxylase (*pyc*) in *E. coli;* by obstructing the synthesis pathways of lactate, ethanol, and acetate.	0.69 g/g glycerol	[54]
Lactic acid	Overexpression of the pathways involved in the conversion of glycerol to D-lactate; Synthesis of competing by-products blocked; aerobic utilization pathway of D-Lactate was mutated.	32 g/L	[65]
	E. coli AC-521 was selected for lactic acid overproduction; due to 16S rDNA sequence and physiological characteristics; by fed batch fermentation process.	85.8 g/L	[64]
Polyhydroxyalkanoates	*E. coli arcA* mutant, poly (3-hydroxybutyrate) (PHB) synthesis was analyzed under microaerobic condition, by fed-batch cultures.	10.81 g/L	[72]
L-phenylalanine	By using the genetically modified *E. coli*; in a stirred tank bioreactor with various impeller agitation speeds and aeration rates.	0.58 g/g glycerol	[74]

All strains utilized glycerol as a substrate for biofuel and biochemical production.

4. Conclusion

Glycerol availability has increased enormously as a byproduct of the biodiesel industries. Besides using glycerol in the chemical industry, it can serve as a feedstock for the production of high value chemicals and fuels by *E. coli* (Table 1). Innovations in metabolic engineering strategies have increased the ability of using engineered *E. coli* for the production of a broad range of novel biofuels and biochemicals through the exploitation of various metabolic pathways. Developments in gene expression tools have reduced the time required to make genetic constructs, while increasing their predictability and reliability. This greatly improved the metabolic engineering techniques for the effective production of a wide variety of fuels [75] and chemicals [76].

Processes that convert inexpensive glycerol into higher-value bioenergy products, such as ethanol and hydrogen, are expected to make biodiesel production more economical and will help in the establishment of more biorefineries [77]. Glycerol conversion to 1,3-PD, 1,2-PDO, 3-HP, and PHAs create a new platform in polyester chemistry. Similarly, the bioconversion of glycerol to organic acids, such as lactic acid and succinic acid, demonstrates the potential of using glycerol in the food and pharmaceutical industries. The conversion of glycerol to the essential amino acid phenylalanine has broadened its application in the medical and nutritional fields. All of these recent applications have created interest in producing more industrially applicable amino acids from glycerol.

Future research should focus on using glycerol as a carbon source to obtain other valuable microbial products, such as recombinant proteins and enzymes, medicinal drugs, antibiotics, and fine chemicals.

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