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Chapter 9

Value-added utilization of crude glycerol from biodiesel production

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Background information

In recent years, there has been a rapid increase in demand for fuels and chemicals, and at the same time there are concerns over global warming and limited availability of fossil resources. These have put scientists in tremendous pressure to explore alternative sources for energy and chemicals. Biofuels such as biodiesel are promising and environmentally safe alternative to fossil fuels, which attracted increasing interest from academia and industry.

Biodiesel has become one of the vital renewable fuels with potential to replace fossil fuels. It is produced from animal fats and vegetable oils by reacting with a primary alcohol in the presence of a catalyst [1]. During the last few years, the production of biodiesel has increased dramatically, and the European Union (EU) is the biggest biodiesel producer in the world. In 2011, EU countries produced 178.15 thousand barrels/day of biodiesel, followed by the North America which produced 65.91 thousand barrels/day of biodiesel [2]. A typical biodiesel production process, however, produces a significant amount of crude glycerol as a core by-product, approximately 10% of the biodiesel produced. Due to its low price, glycerol is now considered as a waste instead of a useful product. The high volume of glycerol generated from biodiesel industry has become an environmental problem since it cannot be safely disposed in the environment. Crude glycerol contains methanol, salts, soaps, nonglycerol organic matter and catalysts as the main impurities which can negatively influence the bioconversion processes. Thus, the crude glycerol is considered as an organic waste that might create eutrophication problems to the environment. Due to oversupply of crude glycerol from biodiesel refinery and high cost of purification (where as pure glycerol being very low in cost), negative influence is being impacted on purification of crude glycerol. If all biodiesel producers were to invest in making pharma-grade, the present high price of pharma-grade glycerin would collapse. Therefore, conversion (chemical or biological) of crude glycerol into value-added products would lead to both environmental and economic dividends. Presently, increasing efforts around the globe have been focused on the development of novel and efficient bioconversion processes of glycerol into value-added products. Glycerol can be used as carbon and energy sources for microbial growth to produce valuable chemicals. This chapter attempts to summarize the current state of the research on various aspects of glycerol metabolism.

Glycerol chemistry & uses

Glycerol, aka, glycerine or glycerin, is a three-carbon sugar alcohol with a molecular formula of $C_3H_8O_3$ and an IUPAC name of propane-1,2,3-triol. It is an odorless, colorless, nontoxic and sweet tasting hygroscopic liquid readily soluble in water. Its molar mass is 92.09 g mol^{-1}, relative density 1.261 g/cm^3, melting point 17.8°C (64°F), boiling point 290°C (554°F), surface tension 63.4 mN/m, autoflammability 393°C, flash point 177°C (open cup) and viscosity 1410 mpas at 20°C [3]. Animal and vegetable fats and vegetable oils are the natural sources of glycerol esters (glycerides). Pure glycerol is widely used in medical, pharmaceuticals, cosmetics, paint, pulp and paper, leather, textile, food, tobacco and automotive industries. It is also used as personal healthcare product for improving smoothness, providing lubrication and moisturizing the skin. Glycerol is an important ingredient of cough syrups, expectorants, toothpaste, mouthwashes, shaving cream and soaps. One of the important uses of glycerol is suppository into the rectum as a laxative. Glycerol can cause side effects including headaches, dizziness, bloating, nausea, vomiting, thirst and diarrhea.

Biological methods for conversion

Bioconversion of glycerol into biofuel or other value-added bioproducts can play a significant role for the development of biodiesel biorefinery industry. Glycerol can be used as a promising and abundant carbon source by the biotechnological production process. Anaerobically many microorganisms are able to utilize glycerol as a sole carbon and energy source, and the use of these microorganisms has increased attention for the bioconversion of glycerol [1]. Although anaerobic condition lacks external electron acceptor hampering microbial growth, some engineered strains of bacteria and fungi can utilize glycerol to produce 1,2-propanediol (1,2-PDO), 1,3-propanediol (1,3-PDO), H_2 and other higher value molecules without external electron acceptor [4,5]. In 2005, Gonzale-Pajuelo *et al.* [6] explored that some biotechnologically important organisms could not naturally ferment glycerol. However, these engineered strains constructed by importing genes from natural glycerol fermenting organisms could ferment, for example, the 1,3-PDO producing gene from the pathway of *Clostridium butyricum* was introduced into *Clostridium acetobutylicum* resulting in efficient glycerol fermentation and 1,3-PDO synthesis [6]. To increase productivity of high value chemicals by the microbial conversion of glycerol, extensive genetic manipulation and metabolic engineering should be imposed on these biotechnologically important organisms.

Glycerol uptake & catabolism

An earlier report indicated that the glycerol transport facilitated its diffusion across the *Escherichia coli* cytoplasmic membrane [7]. In this pathway, glycerol facilitator and glycerol kinase are the two proteins which are principally involved in the entry of external glycerol into cellular metabolism of bacterial cell. The glycerol facilitator acts as a carrier, whereas the kinase traps the glycerol inside the cell as glycerol-3-phosphate. Therefore, the kinase activity is inspired by an interaction between the glycerol facilitator protein and glycerol kinase. In the first step, glycerol passes the biological membrane through energy independent passive diffusion process which is catalyzed by glycerol facilitator, a membrane protein [7]. After entering into a bacterial cell, intracellular glycerol is then converted to glycerol-3-phosphate by glycerol kinase enzyme. Glycerol facilitator operon is the inducer in many bacteria. Nevertheless, glycerol-3-phosphate is not a substrate for the glycerol facilitator, and it remains trapped in the cell until it is further metabolized. However, glycerol uptake in most fungi occurs by active transport which requires energy to operate. Several yeasts and other fungi can take up glycerol by proton symporters. In *saccharomyces cerevisiae*, active transport is mediated by permeases, which are highly specific membrane proteins [8].

Glycerol conversion to 1,3-PDO under anaerobic conditions by Enterobacteriaceae takes two pathways. These are oxidative pathway and reductive pathway. In the oxidative pathway glycerol is dehydrogenated by an NAD^+-dependent glycerol dehydrogenase to dihydroxyacetone (DHA), which is then phosphorylated by phosphoenolpyruvate and ATP-dependent DHA kinase. On the other hand, glycerol is dehydrated by the coenzyme B_{12}-dependent glycerol dehydratase to 3-hydroxypropionaldehyde in the parallel reductive pathway. With the help of the NADH-dependent 1,3-PDO dehydrogenase, this is then reduced to the major product 1,3-PDO, which leads to regenerating NAD^+ [9].

Value-added products from glycerol

Bioconversion of glycerol waste is initiated by microorganisms such as bacteria and fungi which are able to utilize glycerol as a carbon source. In biotechnological process, biodiesel waste could be used to produce high value chemicals to avoid waste disposal and increase the profit of biorefineries. Throughout this section, biotechnological production processes as well as the economic value of microbial metabolites from glycerol are evaluated.

Alcohols

1,3-propanediol

A simple organic chemical 1,3-propanediol (1,3-PDO) obtained from microbial fermentation of glycerol is one of the high value products that has several interesting applications. The potential uses of this chemical are in the preparation of plastic, laminates, ultraviolet cured coating, adhesives material, antifreeze and it is also used as a solvent. The 1,3-propanediol-based polymers possess some better features than that generated from 1,2-propanediol, butanediol or ethylene glycol. Now, 1,3-PDO is used to produce poly-trimethylene terephthalate, a biodegradable polyester which is widely used in carpet and textile manufacturing [10]. The precursor 1,3-PDO of poly-trimethylene terephthalate is produced through chemical synthesis and fermentatively from glucose by microbes. Nowadays, many researchers are trying to develop more competent technology for the production of 1,3-PDO by more cost effective glycerol fermentation technique. Thus, the anaerobic fermentation is the most promising option for bioconversion of glycerol by *Klebsiella, Citrobacter, Clostridium, Lactobacillus* and *Bacillus* [11]. The organisms best known to produce 1,3-PDO are *Clostridium butyricum, C. pasteurianum* and *Klebsiella pneumonia*. Some approaches have achieved 1,3-PDO levels close to 100 g/l by adopting advantage of both genetic and bioprocess strategies [12]. In the recent report on pilot scale production of 1,3-PDO, crude glycerol from Jatropha biodiesel was converted by using *K. pneumonia* ATCC 15380 to yield 56 g/l of 1,3-PDO, with a purity and recovery of 99.7 and 34%, respectively [13]. Also, several strains of *C. butyricum* and an engineered strain of *E. coli* could convert glycerol to 1,3-PDO [14].

Butanol

Only the bacterial species *Clostridium pasteurianum* has the natural ability to use glycerol as a sole carbon and energy source for bioconversion into butanol. Butanol is a high value chemical which is widely used in industrial applications. Many valuable chemicals like acrylates, glycol ethers and butyl acetate can be obtained from the industrially converted butanol, which are utilized in the formulation of paints, lacquers and resin. As an alternative biofuel, butanol could be mixed with standard oil based fuels directly. Butanol has greater advantage over the commonly used ethanol, because it has lower enthalpy of vaporization, lower solubility in water, less corrosiveness and a much higher energy density. As a result, without making any kind of structural modification of the engine, it is possible to replace 100% fossil fuels. Likewise, using existing infrastructure, butanol can also be blended directly at the refinery for delivery [15]. *Clostridium pasteurianum* is the only microorganism that can convert glycerol directly into butanol through anaerobic fermentation process, which is the current attraction to produce high-energy and high value biofuel. Recently, it was observed that a mutant strain *C. pasteurianum* MNO6 developed by chemical mutagenesis using ethane methyl sulfonate is capable of utilizing an average of 2.49 g/l/h to a maximum of 4.08 g/]/h glycerol and a total of 111 g/l of crude glycerol and the maximum production of butanol was 12.6 g/l with productivity 1.8 g/l/h at the maximum glycerol utilization rate [16]. Due to slow growth rate of this organism and requiring long time for fermentation, immediate attention should be given to construct super strain or to look for new microorganism for increasing the production rate. It remains an interesting and challenging research topic to engineer new microooganism for the production of high value butanol from the low value and fermentable glycerol.

2,3-butanediol

The 2,3-butanediol (BDO) is widely used as an antifreeze chemical and lubricant. It can also be used for the manufacturing of printing ink, perfumes and fumigants, polymer, pharmaceutical carrier, moistening and softening agents, and reagent in different asymmetric chemical synthesis [17]. It has been observed that *K. pneumoniae* G31 strain is able to produce 2,3-BDO as a major product of glycerol fermentation under certain cultivation conditions. The maximum 2,3-BDO concentration obtained was 70 g/l, with a maximum yield and productivity of 0.39 g/g glycerol and 0.47 g/l/h, respectively, in a batch culture under aerobic condition [17].

Ethanol

Ethanol is a biofuel and widely used as a solvent and chemical intermediate. It is mainly produced from sugarcane sucrose, corn starch and lignocellulosic feedstock through the fermentation of yeast. Recently, more attention has been paid to the production of ethanol from bioconversion of glycerol. Many bacterial species of the Enterobacteriaceae, anaerobic *Clostridium* and yeasts are able to produce bioethanol by the fermentation of glycerol [5,6]. However, *Escherichia coli* can utilize glycerol both aerobically and anaerobically. It was demonstrated that *E. coli* SY4 can convert glycerol to ethanol in a batch culture, and the total yield was 85%, productivity 0.15 g/l/h and product concentration 7.8 g/l [18]. The bacterium *Enterobacter aerogenes* is also capable of bioconversion of glycerol to ethanol. It has been investigated that the strain *E. aerogenes* HU-101 exhibited high yields and high production rate of ethanol. The maximum production yield of ethanol was 0.85 mol/mol glycerol [6]. Moreover, a mutant strain *K. pneumoniae* GEM167 greatly enhanced ethanol production by the utilization of glycerol to a maximum level of 25.0 g/l [19].

Hydrogen

Hydrogen (H_2) is a potential next generation renewable fuel with low molecular weight. It has exceptional environmental capability. H_2 is highly flammable and the cleanest fuel that turns into water when it is burned. In chemical processes and reactions, H_2 is most commonly used and it is an essential part. Moreover, H_2 can be used to fuel cells to produce electricity and heat without emission of CO_2 [5]. Therefore, more and more attention has been paid to the production of H_2 from glycerol bioconversion [4,5]. It has been proved that many bacterial strains like *Enterobacter aerogenes* HU-101 and *Clostridium pasteurianum* DSMZ 525 are capable of biological production of H_2 from glycerol [4,5]. Recent report displayed that the strain

E. aerogenes HU-101 exhibited high yield and high production rate of H_2. The maximum rate of H_2 production was 80 mmol/l/h in a continuous culture with a packed-bed reactor using self-immobilized cells [5]. The biological production of H_2 can be one of the promising future fuel solutions.

Dihydroxyacetone

A simple carbohydrate dihydroxyacetone (DHA) produced from glycerol fermentation is primarily used as an ingredient in sunless tanning skin-care products and works as a valuable building block for the synthesis of many fine chemicals [20]. Chemical synthesis of DHA is very expensive due to laborious safety requirements. Thus, recent attention has been set for the production of DHA economically by using the microbial process. Very few studies have been done on microbial production of DHA through oxidative fermentation by *Gluconobacter oxydans* via a membrane-bound glycerol dehydrogenase [20]. However, both substrate and product have an inhibitory effect on microbial DHA synthesis. To overcome this problem, the strain *G. oxydans* has been developed by mutagenesis, and at the same time the medium was optimized for increasing production rate of DHA even at a higher concentration of glycerol. As a result, the developed *G. oxydans* ZJB09112 strain significantly enhanced glycerol conversion to DHA by optimization of medium and fermentation condition, and the maximum rate of DHA production was 165.5 g/l with a corresponding productivity of 2.3 g/l/h in a feed batch process [21].

Biogas

Biogas is a clean and renewable energy, because the renewable carbon comes from plant sources. Anaerobic digestion of animal waste is a biological process that converts organic matter into biogas like methane (CH_4) by microorganisms. Biogas production from agricultural biomass offers environmental benefits and is an additional revenue source for farmers. The utilization of large quantities of crude glycerol produced from biodiesel industries could lead as a boost for biogas production. Biogas plants have been consuming crude glycerol for increasing CH_4 production. In last few years, much attention has been attained on the improvement of digester biogas production and for improving methane yields in co-digestion system, especially in Denmark [22]. It has been proved that glycerol addition can boost biogas yields [22]. Recent report explored that the reactor treating the sewage sludge produced 1106 ± 36 ml CH_4/d before the addition of glycerol and 2353 ± 94 ml CH_4/d after the addition of glycerol 1% (v/v) in the feed [22]. They also showed that the extra glycerol added to the feed did not have a negative effect on reactor performance, but seemed to increase the active biomass (volatile solids) concentration in the system.

Other chemicals

Most of the work on the productivity of chemicals and biofuels from glycerol fermentation by microorganisms have been focused on the production of 1,3-PDO, DHA, ethanol and butanol. There are many other valuable chemicals such as citric acid, succinic acid, lactic acid, oxalic acid, propionic acid, glyceric acid, mannitol, arabitol, biosurfactants, phytase, erythritol and 3-Hydroxypropanoic acid that can be achieved by the glycerol fermentation. The production of these chemicals through bioconversion of glycerol has also been reported briefly. Succinic acid is a dicarboxylic organic acid that is largely used in the production of various pharmaceutical products including vitamins, amino acids and antibiotics. It is an important intermediate chemical used to produce tetrahydrofuran, γ-butyrolactone, 1,4-diaminobutane, 1,4-butanediol and many other important chemicals [23]. In 2010, Yuzbashev *et al.* [23] reported that the recombinant yeast strain *Yarrowia lipolytica* Y-3314 exhibited maximum production of succinic acid at 45.5 g/l and a yield of 0.45 g per gram of glycerol. Citric acid is a weak organic acid used as an additive and preservative of foods and candies. It is commercially produced through fermentation of molasses by the fungus *Aspergillus niger*. Because of the increase of global demand for citric acid, its production from glycerol as a feed stock is also of great interest. The strain *Yarrowia lipolytica* A-101–1.22 showed high level of citric acid production (124.2 g/l) with a yield of 0.77 g/g and a productivity of 0.85 g/l/h during batch cultivation in the medium with glycerol-containing waste of biodiesel industry [24]. Lactic acid is widely used in food, cosmetic and pharmaceutical industries. It is produced from glycerol through chemical synthesis. Therefore, more attention has recently been paid to the fermentation of glycerol to lactic acid since the process is more cost effective and the yield is higher. Several microorganisms including *Klebsiella, Clostridia* and *Lactobacillus* enable to produce lactic acid by bioconversion of glycerol. Hong *et. al.* [25] reported that *E. coli* AC-521 could produce lactic acid in high amounts up to 85.8 g/l, and the total yield and productivity were 0.9 mol/mol and 0.49 g/l/h, respectively. Oxalic acid is another important organic acid used in dying process, bleaching, baking powder, paper and ceramic manufacturing. A recent report explored that *Aspergillus niger* could produce oxalic acid by using glycerol as a feed stock, and the maximum production was recorded as 48.9 g/l with yield of 0.88 g/g [26]. An industrially important chemical, 3-Hydroxypropanoic acid (3-HPA) is a natural biodegradable platform chemical used as a precursor for the preparation of many valuable chemicals such as 1,3-propanediol, acrylic acid, methyl acrylate, malonic acid and its esters, hydroxy amide and acrylamide [27]. Many bacteria are able to produce 3-HPA by anaerobic fermentation of glycerol. An engineered strain of *Klebsiella pneumoniae* has been reported for the maximum production of 3-HPA 142.63 mmol/l with

Table 9.1. **Value-added products produced from bioconversion of glycerol.**

Products	Microorganisms/strains	Product	Productivity	Ref.
1,3-propanidiol	*K. pneumonia* LDH 526	102.1 g/l	2.13 g/l/h	[32]
	C. pasteurianum MNO6	–	1.21 g/l/h	[4]
	Engineered *E. coli K12*	104.4 g/l	2.16 g/l/h	[14]
Butanol	*C. pasteurianum* MNO6	12.6 g/l	1.80 g/l/h	[16]
2,3-butanidol	*K. pneumoniae* G31	70.0 g/l	0.47 g/l/h	[17]
Ethanol H_2	*K. pneumoniae* GEM167	25.0 g/l	–	[19]
	E. coli SY4	7.8 g/l	0.15 g/l/h	[18]
	E. aerogenes HU-101	–	80 mmol/l/h	[5]
1,3-DHA	*G. oxydans* ZJB09112	165.5 g/l	2.3 g/l/h	[21]
3-HPA	*K. pneumonia*	142.63 mmol/l	–	[28]
Succinic acid	*Y. lipolytica* Y-3314	45.5 g/l	0.27 g/l/h	[23]
Citric acid	*Y. lipolytica* 101–1.22	124.2 g/l	0.85 g/l/h	[24]
Lactic acid	*E. coli* AC521	85.8 g/l	0.49 g/l/h	[25]
Oxalic acid	*A. niger* XP	48.9 g/l	0.29 g/l/h	[26]
Mannitol	*Candida magnolia*	51.0 g/l	0.53 g/l/h	[29]
Erythritol	*Y. lipolytica* Wratislavia K_1	80.0 g/l	1.00 g/l/h	[30]
Arabitol	*Debaryomyces hansenii* SBP1	15 g/l	0.13 g/l/h	[31]

yield 26.7% [28]. It was observed that mannitol, erythritol and arabitol could be produced with biodegradation of glycerol (**Table 9.1**) [29–31].

Problems of glycerol bioconversion

Although a lot of work has been done at different routes to establish a glycerol fermentation process by using wild type natural microbes and engineered strains, there are many obstacles to increase the productivity. Impurities present in glycerol by-product obtained from biofuel refineries inhibit microbial growth and result in decrease in production yield and production rate. Another problem for bioconversion is the concentration of impurities varies in crude glycerol which is dependent on the biodiesel production process and nature of feed stock. Purification and partial purification of the crude glycerol is an alternative solution to reduce inhibition problem of microbial growth. The wild type microbial strains are employed to improve productivity of a desired high value product by the optimization of fermentation conditions. But end product toxicity, need of anaerobic conditions or lack of genetic tools hindered their industrial application. However, due to absence of external electron accepter in anaerobic fermentation process the redox potential is dropped and microbial growth is inhibited. During anaerobic growth, drop of redox potential might be a result of redox active metabolites produced by bacteria into the culture medium that leads to decrease of external pH, or might be connected with the process on the bacterial membranes. The other problem is that the high concentration of glycerol as well as fermentation products inhibits cell growth during fermentation. To overcome these problems, a better understanding of metabolic pathways and development of new technologies are needed, such as optimizing reaction parameters and fermentation conditions, developing mutant and engineered strains. Moreover, metabolic engineering approaches have to be used to optimize metabolite synthesis through homologous and heterologous pathways. If the metabolic pathways and enzymes are identified, they can be used for the development of recombinant strains.

Conclusion

Renewable products from biodiesel co-product waste glycerol could add great advantage to the reduction of waste treatment cost and increase the economy value of by-products. In addition to the economy value, bioconversion of a large amount of biodiesel glycerol waste would directly benefit the environment by obtaining renewable products, encouraging the use of biodiesel and reducing fossil fuel use. Nowadays, effective utilization of glycerol by-product obtained from biodiesel production is globally important for the commercialization and improvement of biodiesel production. The renewable value-added products derived from glycerol will not only shrink our dependence on nonrenewable products, but also will enforce the development of biorefinery. Thus, bioconversion processes of crude glycerol waste by microorganisms have been imposed on a new dimension for biorefinery. The conversion of abundant and low priced glycerol co-product of biodiesel biorefinery into higher priced products denotes a promising way to attain economic sustainability in biofuel industry. Except the production of small alcohols from glycerol, more attention should be taken for the production of other economically important pharmaceutical and industrial products like enzymes, proteins, antibiotics, vitamins, drugs and other fine chemicals by using glycerol as a sole carbon source through microbial fermentation.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Summary

- Glycerol waste produced from biorefineries is a global oversupply crisis due to lack of refining capacity.
- Microorganisms are able to utilize glycerol as a sole carbon and energy source.
- Engineered and mutated strains of bacteria and fungi have utilized glycerol to produce biofuels and other higher value molecules.
- Different strategies employed to produce biofuels and industrially important chemicals by microbial fermentation of glycerol.
- Bioconversion processes of crude glycerol waste by microorganisms represent a remarkable alternative to add value to the biodiesel production helping biorefineries development.
- Bioconversion of a large amount of biodiesel glycerol waste would directly benefit the environment by obtaining renewable products, encouraging the use of biodiesel and reducing fossil fuel use.
- The bright future of biofuels (alcohols) and many other valuable chemicals that can be achieved by the glycerol biofermentation.

Key terms

Biodiesel:	a biodegradable, nontoxic and clean combustible fuel which is derived from transesterification of fat and vegetable oils in the presence of a catalyst leading to fatty acid methyl ester.
A bioconversion of glycerol:	the conversion of glycerol into valuable products or energy sources by biological process using microorganisms.
Engineered strain:	a microorganism constructed by introducing a gene or genes from other organisms.
Anaerobic fermentation:	a process in which microorganisms convert organic compounds into alcohol or acids under oxygen free conditions.

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