Review

Recent advances in the production of value added chemicals and lipids utilizing biodiesel industry generated crude glycerol as a substrate – Metabolic aspects, challenges and possibilities: An overview

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HIGHLIGHTS

- Overview on strategies adopted for bioconversion of using crude glycerol.
- Discusses engineered strains for utilization of crude glycerol.
- Discusses improvement in process economics by utilizing crude glycerol.

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ABSTRACT

One of the major ecological concerns associated with biodiesel production is the generation of waste/crude glycerol during the trans-esterification process. Purification of this crude glycerol is not economically viable. In this context, the development of an efficient and economically viable strategy would be biotransformation reactions converting the biodiesel derived crude glycerol into value added chemicals. Hence the process ensures the sustainability and waste management in biodiesel industry, paving a path to integrated biorefineries. This review addresses a waste to wealth approach for utilization of crude glycerol in the production of value added chemicals, current trends, challenges, future perspectives, metabolic approaches and the genetic tools developed for the improved synthesis over wild type microorganisms were described.

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1. Introduction

Global energy demand is met by petrochemical sources, coal and natural gases. These sources are finite and the current high consumption rate may lead to depletion of fossil fuels which on combustion release various oxides of sulphur, oxides of nitrogen, oxides of carbon, lead and hydrocarbons that lead to global warming. The scarcity of the fossil fuels, release of hazardous chemicals will make renewable bio-based fuels from biomass attains great attraction. In the world energy consumption scenario, diesel fuels play a major role in industrial, transport and agricultural sectors in a developing country. An alternative strategy for this non renewable diesel is production of economically and environmentally feasible and acceptable fuel derived from renewable sources like plant derived oils. This fuel derived from biomass sources was termed as biodiesel (Ayoub and Abdullah, 2012; Demirbas, 2009b; Meher et al., 2006). The physical properties and chemical composition of various fossil fuels and biofuels was depicted in Table 1. Biodiesel is manufactured from vegetable, plant derived oils or animal fat feedstock consisting of long chain alkyl esters. Biodiesel is chemically synthesized by transesterification of lipids with an alcohol in the presence of catalyst resulting in a mono alkyl ester. Various methods like batch processing, supercritical, ultrasonic and microwave treatment techniques can be used for biodiesel production.

1.1. Biodiesel production and efficiency

The composition analysis of plant derived oils was found to have free fatty acids, phospholipids, sterols, water and other components, that reduce the usage of oil directly as fuel. Hence the oil was modified to produce fatty acyl esters by transesterification reaction. Monoalkyl esters of long chain fatty acids are the principle components of biodiesel derived from waste vegetable oils and plant oils. In the transesterification reaction, oils or fats on reaction with alcohol (ethanol or methanol) in the presence of alkali catalyst (potassium hydroxide or sodium hydroxide) break open to long chain fatty acid chains to form methyl/ethyl esters, separating the glycerol moiety one of long chain fatty acids as crude glycerin.

The obtained methyl or ethyl esters are termed as biodiesel, which is pale yellow in color, medium light combustion fuel (Meher et al., 2006). From the plant to yield of biodiesel comprise multiple tedious steps: crop production, harvesting, seed cleaning and drying, oil and meal production, later transesterification using extracted oil. Investment on a biodiesel production unit requires 78% in the form of price of feedstock, but 93% returns can be expected from biodiesel yield. A time consuming process in the whole production crop yield and harvesting where it takes 4–7 years for first yield based on different oil crops, for an example, *Jatropha* (4–5 yrs) and *Pongamia* (6–7 yrs) (Sheehan et al., 2000).

As the physical properties of biodiesel are more similar to petroleum derived diesel, it has profound applications as a fossil fuel alternative. It can be either used in pure form B100 or as blends with different concentrations ranging from 5% to 20%, where the remaining concentration will be petroleum derived diesel. Among these blends B20 is most preferable, as such we can use in the present motor without altering the engine make up. On combustion biodiesel effluents discharged has 41% reduction in green house gases, reduced emission of unburned hydrocarbons, carbon monoxide and particulate matter by 21%, 11% and 10% (Sheehan et al., 2000).

Till today edible vegetable oils either in fresh or used form are using as raw material for biodiesel production, but almost 400 species of non edible oil producing plant species are available, which can be cultivated to produce enough raw material. Globally waste lands, degraded forests and non-forest lands other than food and fodder cultivable lands can be used for oil based crop production, to establish a cleaner and greener environment with this clean fuel (biodiesel) reducing green house effect, environmental and ecological imbalances due to pollution cause by exhausts from vehicle tail pipes. This clean fuel is non toxic, biodegradable and suitable for sensitive environments.

1.2. Biodiesel derived glycerol and its properties

Glycerol (1,2,3-propanetriol), commonly known as glycerin is the major by-product obtained during transesterification reaction

Table 1
Comparison of the chemical characteristics and properties of fossil fuels and biofuels.

Property	Ethanol	Gasoline	No. 2 Diesel	Propane	Butanol	Isooctane
Chemical Formula	C_2H_5OH	C4–C12	C3–C25	C_3H_8	$C_4H_{10}O$	C_8H_{18}
Molecular Weight	46.07	100–105	≈ 200	44.096	74.12	114.26
Carbon	52.2	85–88	84–87	81.85	64.7	84.3
Hydrogen	13.1	12–15	33–16	18.14	13.49	15.7
Oxygen	34.7	0	0	0	21.5	0
Specific gravity, 60° F/60° F	0.796	0.72–0.78	0.81–0.89	0.5–0.51	0.81	0.691
Density, lb/gal @ 60° F	6.61	6.0–6.5	6.7–7.4	0.116	6.75	5.77
Boiling temperature, °F	172	80–437	370–650	−44	243.9	210
Reid vapor pressure, psi	2.3	8–15	0.2	9.19	0.6	1.7
Research octane no.	108	90–100	–	112	96	100
Motor octane no.	92	81–90	–	97	78	100
(R + M)/2	100	86–94	N/A			100

for biodiesel production. The ratio of crude glycerol to biodiesel is 1:10 for every 10 kg of biodiesel produced crude glycerin is produced to tune of 1 kg. The crude glycerol phase get settled at the lower part of the production tank, which can be easily separated from biodiesel. As it is crude form the concentration of glycerol varies from (70–98%) and rest of impurities includes fatty acid methyl esters, fatty acids, methanol, water, soap and ash content. Due to various impurities viscosity of crude glycerin ranges between 15 and 1213 mpa. s, and density ($1.01–1.2$ g/cm^3) is found to be less than the pure glycerol (1.31 g/cm^3) (Tan et al., 2013; Yang et al., 2012). The unique physical and chemical properties of glycerol, non-toxic nature made it as a compatible renewable raw material with various applications. As biodiesel is globally accepted for a better alternative fossil fuel, entrepreneurs and industrial relevance increased and scale up in an industrial scale was established, which limits the question of waste glycerol management available in surplus amounts. It has become an economical issue for biodiesel industries. As glycerol is mostly used as a commodity chemical in various chemical synthesis, food and pharmaceutical industries, the crude form can be distilled to obtain the pure glycerol, but the cost of distillation and purification is comparatively higher than the market price, which made the process uneconomical (Meher et al., 2006; Demirbas, 2009a).

The structural configuration of glycerol shows three carbon back bone with three hydroxyl groups, responsible for its hygroscopic nature and water solubility. Its highly reduced nature, make glycerol a better raw material for the production of chemicals and value added products. The elemental analysis of the crude glycerol derived from a biodiesel industry is shown in Table 2. In the commercial scale, glycerol is available in three different forms, (i) crude glycerol (ii) purified/refined glycerol (iii) commercially synthesized glycerol. As biodiesel is being produced from cheaper substrates, the surplus glycerol produced cannot meet market potential in terms of purity and cost. Instead the crude glycerol can be directly used as the feedstock or substrate for the production of value added chemicals which increases the economic value of glycerol

1.3. Understanding the glycerol market

The glycerol market was into existence from the time before World War I. Nitroglycerin the leading explosive raw material is manufactured from glycerol. Invented by Sobrero in 1846, later in 1866 using mixture of kieselguhr and nitroglycerine Nobel discovered 'dynamite' for blasting purpose. In the war DuPont was the only industry manufacturing smokeless powder and dynamites. Later glycerol manufacturers were establish in other parts of the world like Europe, Japan, Russia and United states. Until 2003 the bioglycerol has come into limelight the global demand for glycerol was met by petrochemical industries and soap manufacturers.

The melt down of crude glycerol manufacturers begun in October 2005 in Japan, Dow Chemical Texas, Procter & Gamble, London, Solvay France in 2006. Though the price of glycerol slashed from 2006 to 2009, due to increased end applications in 2012 the prices were started to recover with global demand in food and pharmaceutical applications. The glycerol market size was expected to exceed USD million by 2022 with a gain of 7.9% from 2015 to 2022. In today scenario worlds 68% of glycerol was produced from biodiesel industries. By application personnel care, pharmaceutical accounts for 38%, food and beverages around 7% respectively. The world's leading glycerol manufacturers are IOI, Oleon, Kao Sipong, P&G, Wilmar and Emery supplies the consolidated 65% of glycerol and other to be mentioned manufacturers Dow Chemical, P&G, Solvay SA, BASF, Sofiproteol Group, Godrej Industries, Kuala Lumpur Kepong Berhad, Croda International, Archer Daniel Midland, United Coconut Chemicals, Vitusa Products, Cargill, Ecogreen Oleochemicals and Evonik (Global Market Insights, 2016).

Table 2
Metabolites produced by various microorganisms using glycerol as the substrate.

S. No.	Product	Microorganism	Titers(g/l)	Yield[**] (g/g)	References
1	Succinic acid	*Actinobacillus succinogenes* *Anaerobiospirillum succiniciproducens* *Basfia succiniciproducens*	4.9–35	0.6–0.87	Kongruang and Kangsadan (2015), Lee et al. (2001, 2010), Scholten et al. (2009), Blankschien et al. (2010)
2	Citric acid	*Y. lipolytica*	86.5–157.5	0.59–0.9	Rywińska et al. (2011), Rywińska et al. (2012), Rywinska et al. (2010), Rywińska and Rymowicz (2010), Rymowicz et al. (2010), Morgunov et al. (2013)
3	Propionic acid	*Propionibacterium acidipropionici* *Propionibacterium acnes* *Clostiridum propionicum* *Propionibacterium jensenii* *P. freudenreichii subsp. shermanii*	11.5–47.28	0.3–0.54	Barbirato et al. (1997), Liu et al. (2011), Liu et al. (2015), Wang et al. (2015), Zhu et al. (2010)
4	Lactic acid	*E. coli* *Lactobacillus rhamanosus* *Enterobacter faecalis*	26.53–85.8	0.5–0.9	Hong et al. (2009), Prada-Palomo et al. (2012), Murakami et al. (2016)
5	Glyceric acid	*Gluconobacter cerinus* *G. frateurii* *Acetobacter tropicalis*	57–101	0.7–0.9	Habe et al. (2009a,b,c, 2010), Hong et al. (2015)
6	1,3-Propanediol	*Clostridium butyricum* *Clostridium diolis* *Klebsiella pneumoniae* *Citrobacter freundii* *Lactobacillus reuteri* *Lactobacillus brevis* *Lactobacillus diolivorans*	20–98	0.5–0.7	Zhong et al. (2014), Guo et al. (2010) Wilkens et al. (2012) Otte et al. (2009) Pflügl et al. (2014) Vivek et al. (2016) Celińska et al. (2015) Vaidyanathan et al. (2011) Tang et al. (2009)

1.4. Traditional chemosynthetic utilization of glycerol

Functionality of glycerol resembles the reactive behavior of hydrocarbon derived from petrochemical derivatives; hence various chemicals and value added products can be synthesized from glycerol through various chemical reactions. Primarily glycerol is used in manufacturing of cosmetics, soaps, resins, food, drinks, esters, polymers and other products. Later due to high reducing nature found in glycerol by its structural and physical characterization made biorefineries to utilize crude glycerol as the raw material either in oxidation or reduction process to produce many chemicals like dihydroxyacetone, mesoxalic acid, glyceraldehydes, glyceric acid, malonic acid, hydroxypyruvic acid, lactic acid, pyruvic acid, propylene glycol, propionic acid, glycidol, acrylic acid, propanol, isopropanol, acetone, propylene oxide, propionaldehyde, allyl alcohol, acrolein, acetol, glycerol carbonate etc (Luo et al., 2016; Zheng et al., 2008; Santibáñez et al., 2011).

In oxidation and reduction process, oxidation is easier where glycerol in the presence of potassium permanganate is converted to tartronic acid where as in the presence of nitrous acid it is converted to glyceric acid. Two important chemicals fuel oxygenate a fuel additive is produced by etherification of glycerol and isobutene and quinoline is produced from glycerol and aniline (Díaz-Álvarez and Cadierno, 2013). These chemicals have an established market with higher value than the crude glycerol which makes this conversion process more efficient than distillation of glycerol to avail pure form in the market.

Highly reduced nature of glycerol makes it a better raw material for the production of chemicals and value added products. Primarily glycerol is used in manufacturing of cosmetics, soaps, resins, food, drinks, esters, polymers and other products. The dissimilation of glycerol can produce many chemicals like dihydroxyacetone, mesoxalic acid, glyceraldehydes, glyceric acid, malonic acid, hydroxypyruvic acid, lactic acid, pyruvic acid, propylene glycol, propionic acid, glycidol, acrylic acid, propanol, isopropanol, acetone, propylene oxide, propionaldehyde, allyl alcohol, acrolein, acetol and glycerol carbonate (Luo et al., 2016; Santibáñez, 2011; Zheng et al., 2008). Various value added chemicals derived from glycerol were depicted in Table 2.

Utilization of this crude glycerol for the production of value added chemicals seems promising. This review addresses conversion of this waste glycerol to value added chemicals using green processes, current trends, challenges and future perspectives.

2. Value added products from crude glycerol

2.1. Organic acids

2.1.1. Succinic acid

Succinic acid, four carbon dicarboxylic acid is used as a substrate for various commodity and special chemicals like adipic acid, 1,4-butanediol, tetrahydrofuran, N-methyl pyrrolidinone, succinate salts, gamma-butyolactone polyurethane, food and beverages, resins, coatings and pigments, plasticizers, pharmacy, de-icer solutions, PBS/PBST, solvents and lubricants, personal care and succinic acid has its own role in production of other biodegradable polymers, currently succinate is synthesized from petrochemical derivatives like maleic anhydride via catalytic hydrogenation in reductive environment resulting in lower yields (Pinazo et al., 2015). But the global succinic acid market was expected to be $ 486.7 million by 2019, with Europe as the largest global market and Germany being the main consumer utilizing succinic acid in field of chemicals and pharmaceutical applications (Bechthold et al., 2008). As succinic acid is the intermediate metabolite in the TCA cycle, an active oxidation pathway from the renewable

and cheap substrates as the carbon sources leads to greater production levels but in anaerobic metabolism alternative pathways in different microorganisms resulted in higher yields compared to oxidative pathway. Various microorganisms are reported for succinic acid production from agro residual wastes like wood hydrolysates, cane molasses, straw hydrolysates, whey and industrial wastes like glycerol as substrates, *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, *Anaerobiospirillum succiniciproducens*, *Clostridium thermosuccinogenes*, *Klebsiella pneumoniae*, *Ruminococcus albus*, *Prevotella ruminicola*, *Bacteroides amylophilus* and *Bacteroides fragiles* as well as few fungi like *Aspergillus niger, Aspergillus fumigatus, Byssochlamys nivea, Lentinus degenee, Paecilomyces varioti, Penicillium viniferum* and yeast like *Saccharomyces cerevisiae,* production was reported in both aerobic and anaerobic conditions (de Barros et al., 2013; Vlysidis et al., 2011; Zheng et al., 2009; Vlysidis et al., 2009, 2008; Agarwal et al., 2006).

Lee et al. (2001) reported succinic acid production under anaerobic conditions using *Anaerobiospirillum succiniciproducens*. The succinic acid concentration of 19 g/L with a higher yield of 1.3 g/g and productivity of 0.155 g/L/h was observed. Fed-batch strategy with an intermittent addition of glycerol and yeast extract yielded 1.6 g/g with final titer 19 g/L of succinic acid. This was found to be five times higher than batch fermentation.

Blankschien et al. (2010) reported heterologous overexpression of pyruvate carboxylase (*pyc*) in *E. coli* from *Lactococcus lactis* resulting in an increased titer of succinic acid 5.3 g/L where as the wild strain produced 2.1 g/L of succinic acid. A two fold increase in succinate production was observed in this strategy. Further genetic modulation resulted in a 4.5 fold increase in succinic acid production. A quadruple deletion mutant with over expressed pyruvate carboxylase strain was constructed by deleting alcohol dehydrogenase (*adh E*), phosphoacettyl transferase (*pta*), pyruvate oxidase (*poxB*) lactate dehydrogenase (*ldh A*), *E. coli* Δ*pf1 B* Δ*pox B* Δ*ldh A* Δ*adh E* Δ *ack A* Δ*pta* {*pZS-pyc*} mutant strain on fermentation resulted in succinate concentration of 8.8 g/L in 48 hrs, 60% yield. Factors which favors increased succinic acid yield are immediate availability of electron donors, favorable pH conditions along with sufficient substrate concentrations.

2.1.2. Citric acid

Citric acid is a weak organic intermediate acid of tricarboxylic acid or Krebs cycle and is the first organic acid produced by fermentation. It is widely used as preservative in food and beverages as well as in chemical synthesis, medical, metallurgy and textile industries, chelating agent and as an additive in surfactants and detergents. Initially *Aspergillus niger* is termed as sole citric acid producer utilizing molasses as the substrate, but later significant research contribution led to isolation of fungal wild type *Yarrowia lipolytica* and its mutant strains yielding higher titers compared to *A. niger* strain. The metabolic pathway from external glycerol substrate to final product i.e., citric acid production, follows glycolytic pathway when glycerol was converted to glycerol-3-phosphate by glycerol kinase and isomerization the intermediate dihydroxyacetone converts into citric acid and storage lipids, the key enzymes responsible for the production were glycerol kinase, NAD-dependent glycerol dehydrogenase, FAD dependent glycerol dehydrogenase, enzymes involved in fatty acid metabolism in glyoxylate cycle are isocitrate lyase and malate synthase, important Krebs cycle enzymes like citrate synthase, aconitase hydratase, NAD-dependent isocitrate dehydrogenase and NADP-dependent isocitrate dehydrogenase (Morgunov and Kamzolova, 2015). The biochemical and morphological characterization of these enzymes throughout three phases like exponential phase, early stationary phase and citric acid production phase was observed, glycerol kinase is the limiting enzyme catalyzing the initial step of glycerol

utilization was induced in the start of growth phase and in higher concentration throughout the incubation process,

A classical mode of strain improvement strategy of chemical mutagenesis using NTG was carried out on wild type *Yarrowia lipolytica* A101. Fermentation was carried out in batch, batch process with cell recycling and repeated batch process resulting in citric acid production of 112, 107 and 124.2 g/L respectively. Rywińska and Rymowicz (2010) used 40% of the spent medium showed a highest production of 154 g/L.

Morgunov et al. (2013) studied the physiological and biochemical aspects of metabolic pathway for citric acid production using glycerol as the sole substrate in *Yarrowia lipolytica* NG40/UV7 a mutant strain produced from a wild type *Yarrowia lipolytica* VKM Y-2373. The study revealed that the mutant strain has metabolite profile ratio of citrate: isocitrate as 53:1 while the wild type has 1.7:1 ratio of citrate: isocitrate. The physical parameters like temperature, pH, dissolved oxygen and growth limiting inorganic supplement concentrations of nitrogen, phosphorous and sulphur were optimized. Under optimized conditions with pure and crude glycerol produced 115 g/L and 112 g/L respectively. This eukaryotic yeast mutant strain was found to be best producer of citric acid utilizing crude glycerol as the sole substrate.

2.1.3. Propionic acid

Propionic acid is a carboxylic acid with pungent odour and colorless properties which finds applications as an ingredient in thermoplastics, anti-arthritic drugs, inhibitory activity against molds, antifungal agents in food and feed, perfumes, artificial derived flavors, solvents, herbicides, cellulose plastics and in production of vitamin E (Himmi et al., 2000). The propionic acid is traditionally synthesized by hydrocarboxylation of ethylene or aerobic oxidation of propionaldehyde, in the presence of expensive catalysts like nickel carbonyl, cobalt or manganese at elevated temperatures. The chemical synthesis was replaced by microbial production using genus *Propionibacteria* from various carbon sources like hexoses, pentoses, glycerol, whey lactose and sorbitol. In the pathway of glycerol dissimilation, pyruvate produced enters into the Wood-Werkman cycle by forming oxaloacetate catalyzed by pyruvate dehydrogenase. In the presence of reducing equivalents NADH, oxaloacetate is converted to succinate, with malate and fumarate as intermediates in a three step pathway catalyzed by malate dehydrogenase, fumarate hydratase and succinate dehydrogenase. Later on addition of coenzyme A forming succinyl-CoA by CoA transferase, then isomerization results in malonyl CoA and sequential decarboxylation produce propionyl CoA which converts into propionate by leaving Coenzyme A. The metabolic reaction of propionic acid production is a cyclic reaction which depends on concentration of pyruvate and NADH equivalents (Zhang et al., 2015).

Liu et al. (2011) studied propionic acid production using different substrates like glucose, glycerol using *P. acidipropionici* CGMCC1.2225 strain were 0.475 g/g, 0.303 g/g yield, 0.108 g/L/h, 0.068 g/L/h productivity and 18.1 g/L, 11.5 g/L of propionic acid was produced when glucose and glycerol was used as individual substrates, with higher cell dry weight in glucose fermentation and higher production levels when glycerol was used as substrate. The study revealed that co-fermentation using glycerol and glucose gave better fermentation yields. Higher yield was observed in fed batch than in batch fermentation. In batch mode the cell growth and propionic acid titers were 21.9 g/L with 57.2% while in fed batch mode the cell growth and propionic acid titers were 29.2 g/L and 54.4% conversion in production media containing glycerol and glucose in a 4:1 ratio.

1 mole of glycerol directly provides 2 moles of NADH and utilizes more CO_2 and hence there is no release of CO_2 and there is no generation of acetic acid. Redox balance was maintained by shifting the metabolic flux towards the reductive pathway producing succinic acid and propionic acid. Comparing glucose and glycerol fermentation, exogenous CO_2 supply favors glycerol utilization by CO_2 fixation and initiate oxaloacetic acid production, which consumes NADH to form malate and formate which regenerate NAD^+ and maintaining $NADH/NAD^+$ ratio. Zhang et al. (2015) observed that exogenous supply of CO_2 varied propionic acid production from 1.56 g/L/day to 2.94 g/L/day, but there is no specific change in glucose fermentation. Enhanced glycerol metabolism and increase volumetric productivity was observed when CO_2 is supplied to glycerol dissimilation process.

During fermentation, to enhance the end products, substrate flux towards byproduct synthesis should be inhibited or shift the flux towards the end product. Wang and Yang (2009) knocked out *ack* gene in *P. acidipropionici* ATCC 4875 which catalyses the production of acetic acid. The constructed Tet mutant strain, produced 106 g/L of propionic acid which is higher when compared to wild type. The three key enzymes involved in the biosynthesis of propionic acid are glycerol dehydrogenase (GDH), malate dehydrogenase (MDH) and fumarate dehydratase (FUM), higher activities of these enzymes brings change in glycerol uptake and propionic acid production.

The production levels increased from 26.95 to 39.43 g/L of propionic acid. An identical observation was reported by Wang et al. (2015) carrying out homologous over expression of propionyl CoA: succinate CoA transferase in *P. freudenreichii* subspecies *shermanii* resulted in 10% and 46% increase in yield and productivity. Production of propionic acid using waste substrate like glycerol seems promising and economically viable than other sugars (Zhu et al.,).

2.1.4. Lactic acid

Lactic acid is an industrially important organic acid produced by lactic acid bacteria. Lactic acid exists in D(+), L(−) or in racemic mixture (+/−) form, the conventional chemical synthesis is known to produce racemic mixture, where as biological fermentation was observed to yield all the isomeric forms either individually or in a mixture. Along with lactic acid bacteria few other natural microorganisms like *Enterobacteriaceae* members, yeast and genetically engineered microbes like *Escherichia coli* are reported for efficient yields and productivity. Lactic acid is known for its derivatives like acrylic acid, 2,3-pentanedione, biodegradable and biocompatible poly lactic acid production, which finds applications in food, cosmetics and pharmaceuticals. Various substrates like glucose, lactose, corn and potato starch are reported for lactic acid production. Utilization of biodiesel industry generated crude glycerol as sole carbon source will make the process economically viable.

Chen et al. (2015) observed two conventional chemical synthesis of lactic acid with higher yields using glycerol as the sole carbon source, using sodium hydroxide as catalyst under fed-batch mode with a feed inlet of 1.1 M glycerol. The reactor temperature was maintained at 300 °C yielded 82% of lactic acid with a glycerol conversion rate of 93%. Ftouni et al. (2015) used an inert catalyst Pt/ZrO_2 and reaction was carried out at 180 °C, under helium atmosphere maintained at 30 bar pressure resulted in 80% glycerol conversion after 8 hours. Since the catalyst Pt/ZrO_2 is expensive make the process economically non viable at industrial scale. Hong et al. (2009) reported lactic acid production from a soil isolate *E. coli* Ac-521 produced 85.8 g/L of lactic acid, with 0.9 mol/mol yield and 0.97 g/L/h productivity under optimized process conditions like 42 °C and pH 6.5.

Posada et al. (2012) reported a co-substrate metabolism where glycerol and acetic acid were dissimilated simultaneously to produce lactic acid. Glycolytic production of pyruvate from glycerol requires 2 moles of NADH, from which one mole of NAD^+ is regen-

erated, when pyruvate is converted to lactic acid, as the conversion prolongs the intracellular redox imbalance occurs and NADH accumulates and for preventing the imbalance, acetic acid bioconversion to ethanol takes place which requires 2 moles of NADH, regenerating 2 moles of NAD⁺ molecules.

Murakami et al. (2016) proved the concept of glycerol and acetic acid co-metabolism in *Enterobacter faecalis* QU11 strain, where acetic acid co-metabolism takes place when glycerol alone is supplied as substrate and co-metabolism does not takes place with glucose. The experiment validation was carried out using ^{13}C tracer technique; assumption of acetic acid bioconversion to ethanol was determined by final result obtaining ^{13}C ethanol and ^{13}C acetic acid, after prolonged fermentation ^{12}C ethanol and ^{12}C acetic acid was observed in gas chromatogram explaining no loss of carbon from glycerol as well as acetic acid dissimilation. In this co-metabolism 26.53 g/L vs 55.3 g/L with 0.8 vs 0.99 $g_{Lactic\ acid}/g_{Glycerol}$ yield was observed in batch and fed batch mode of fermentation, with feed inlet of 2:1 ratio of glycerol and acetic acid.

2.1.5. Glyceric acid

Glyceric acid (GA) also known as 2,3-dihydroxy propionic acid, is a functional organic acid and multifunctional monomer. The structural characterization revealed presence of one carboxyl group and two hydroxyl groups, helping the monomer to act as trifunctional monomer in polycondensation reactions to form polymers. The conventional chemical methods using AU/charcoal, AU/graphite or photocatalyst titanium disilicide (TiSi₂) has 100% selectivity to glyceric acid production under mild conditions resulting in 60–90% yield (Carrettin et al., 2002; Kondamudi et al., 2012). The biological production of the chemicals requires microorganisms as inexpensive catalysts compared to chemical methods. Two genera of acetic acid bacteria *Gluconobacter* and *Acetobacter* sp. were reported biocatalysts producing GA from glycerol. Acetic acid bacteria oxidize sugar alcohols through oxidative fermentation to produce acids. Bioconversion of glycerol to glyceric acid is a two step reaction, where alcohol dehydrogenase (adhAB) oxidize glycerol to glyceraldehyde and to glyceric acid, in excess dihydroxyacetone (DHA) is obtained as by-product, mediated by sldAB gene encoding glycerol dehydrogenase oxidizing glycerol to DHA. The crude glycerol concentration has significant effect on GA production, the activity of glycerol dehydrogenase was observed to be directly proportional to the concentration of glycerol. Crude glycerol concentration above 20% decreased the enzyme activity due to accumulated methanol. The crude glycerol has methanol as an impurity which has inhibitory effect on growth and metabolic activity of the microorganism. Crude glycerol with lower concentrations of methanol would be a promising substrate for GA production (Sato et al., 2013).

Gluconobacter cerinus IF03262, *G. frateurii* NBRC 103465 and *Acetobacter tropicalis* NBRC GA 57 g/L, 136.5 g/L and 101.8 g/L respectively (Habe et al., 2009a, 2009b, 2009c). *Gluconobacter* sp. were observed to produce both D and L forms of GA and in higher titers than the *Acetobacter* sp., but two limitations where strains can produce 71–79% of only (D)-GA and accumulation of DHA as by-product in higher concentrations. To inhibit the concentration of DHA accumulation, sldA gene encoding subunit of glycerol dehydrogenase was deleted in *G. frateurii* THD 32 and constructed a strain *G. frateurii* ΔsldA and fermentation was carried out at different glycerol concentrations, the growth of mutant strain is weaker than wild type with lag phase of 4 days, but no DHA was accumulated in the fermented broth (Habe et al., 2010). The bioenergy required for the mutant strain to reduce the lag period the experiments were carried out to use D-sorbitol as external carbon source, as *Gluconobacter* sp. have high dehydrogenase specificity to D-sorbitol by pyrroloquinine quinine dependent alcohol dehydrogenase (PQQ-ADH) and FAD dependent sorbitol dehydrogenase

(FAD-SlDH). The co-substrate fermentation of glycerol and 1% sorbitol resulted in 89.1 g/L GA with no DHA production. An enzymatic conversion of glycerol to glyceric acid using immobilized laccase from *Trametes versicolor* was resulted in 90% yield with 7% wt GA after 24 h at 25 °C using 30 mM 2,2,6,6-tetramethyl piperidine N-oxyl (TEMPO) as mediator (Hong et al., 2015).

2.2. Mono and diols

2.2.1. 1,3-Propanediol

Anaerobic fermentation of glycerol to 1,3-propanediol was extensively studied. 1,3-PDO, a diol monomer is well known for numerous applications in cosmetics, solvents, adhesives, detergents, and resins. Recently, monomer has gained much attention in production of polyester polytrimethylene terephthalate, having significant application in carpet and textile industries. The conventional techniques like Degussa and Shell process were developed by DuPont and Shell chemicals using acrolein and ethylene oxide as raw materials (da Silva et al., 2009).

Various wild type strains like *Klebsiella pneumonia* (Zhong et al., 2014; Guo et al., 2010), *Clostridium butyricum* (Wilkens et al., 2012; Abbad-Andaloussi et al., 1995), *Clostridium diolis* (Otte et al., 2009), *Citrobacter freundii* (Celinska et al., 2015), *Lactobacillus diolivorans* (Pflügl et al., 2014), *Lactobacillus brevis* (Vivek et al., 2016), *Lactobacillus reuteri* (Vaidyanathan et al., 2011) and genetically modified strains of *K. pneumoniae, E. coli* (Tang et al., 2009) and *Clostridium pasteurianum* (Jensen et al., 2012) were reported for 1,3-Propanediol production. Metabolism of glycerol in these microbes is a coupled oxido reductive process, where glycerol acts as the sole carbon source for oxidative as well as reductive pathway. In oxidative pathway, NAD + dependent glycerol dehydrogenase enzyme encoded by dha D gene converts glycerol to dihydroxyacetone (DHA), which is phosphorylated to dihydroxyacetone phosphate (DHAP) by transfer of phosphate group from ATP in the presence of kinase enzyme encoded by dha K gene. This DHAP is further metabolized to phosphoenol pyruvate and pyruvate, synthesizing reducing equivalents and energy, for growth and development of microorganism. To maintain equilibrium concentrations of NAD+/NADH+H+ inside the micro compartments and cytoplasm, reductive pathway was observed which depends on reducing equivalents generated by oxidative pathway (Celinśka, 2010; Kaur et al., 2012; Nakamura and Whited, 2003).

In anaerobic fermentation with glycerol as the sole carbon source *K. pneumoniae* strains were reported to produce 1,3-PDO titers between 60 and 90 g/L (Lee et al., 2014; Zhao et al., 2009) and *Clostridium butyricum* strains were observed to produce greater than 60 g/L (Wilkens et al., 2012). Otte et al. (2009) reported genome shuffling of chemically (NTG) mutated strains of *Clostridium diolis* DSM 15410. The strain was improved such a way to tolerate a glycerol concentration of 138.15 g/L (1.5 M) and 1,3-propanediol concentration of 91 g/L (1.2 M) compared to wild type strain tolerance level of 80% was increased. As the metabolic prospective reduced environment of glycerol metabolism favors 1,3-propanediol production, hence the oxido-reduction potential (ORP) as a parameter mutations are carried out on *Klebsiella pneumoniae* M5aL strain. A combined physical and chemical mutation is carried out using UV light and LiCl. In the fermentation each strain has specific ORP values which favors the growth rate and end product synthesis, for *Klebsiella pneumoniae* M5aL wild type strain value lies between −160 and −190 mV. Hence the mutants are selected in a way that it tolerates a wide range of reduced environments, YMU1 mutant of the parent strain can tolerate −280 mV and has enhanced 1,3-propanediol production of 63.1% compared to parent strain (Du et al., 2007).

Yang et al. (2007) carried out knockout of lactate dehydrogenase in *Klebsiella oxytoca* M5aL *ldh*A. The mutants were observed

to produce 83.56 g/L, 60.11 g/L of 1,3-propanediol and 2,3-butanediol when sucrose is used as co-substrate for glycerol.

Inactivation of acetoin reductase gene and expression of formate dehydrogenase gene was observed in *Klebsiella pneumoniae* by Wu et al. (2013). This resulted in increased 1,3-propanediol production around 15.9% and 21.7% in batch and fed-batch process with a final concentration of 72.2 g/L.

Highest 1,3-PDO titers were observed in a genetically engineered strain *Escherichia coli* K-12 ER2925 by Tang et al. (2009). NADPH dependent yqhD gene from *Escherichia coli* wild type strain was tandemly arranged along with glycerol dehydratase subunits dhaB1 and dhaB2 genes instead of NADH dependent dha T gene. Pflügl et al. (2014) reported 85 g/L of 1.3-PDO by *Lactobacillus diolivorans* using crude glycerol as sole carbon source.

2.2.2. 2,3-Butanediol

Yet another chemical in competitiveness with 1,3-PDO for commercial scale production is 2,3-butanediol (2,3-BD) with various applications in food, pharmaceuticals, cosmetics and polymer industry. A wide genus of microorganisms of genera *Lactobacillus, Enterobacter, Klebsiella, Corynebacterium, Serratia* were reported for 2,3-BD production from glucose and other lignocellulosic biomass as carbon sources (Celińska and Grajek, 2009). Though higher yields and productivities of 2,3-BD was obtained using glucose as carbon source, several researches and developmental activities are going on for the utilization of biodiesel industry derived crude glycerol which makes the process economically viable (Yang et al., 2015).

The oxidative dissimilation of glycerol is initiated by glycerol dehydrogenase *DhaD* gene resulting in dihydroxyacetone, the sequential oxidations produce 2 molecules of pyruvate. Acetolactate synthase (ALS) oxidizes pyruvate to α-acetolactate, further acetolactate decarboxylase (ALDC) mediates production of acetoin and NADH dependent aceotin reductase (ACR) catalyze rate limiting acetoin degradation to produce 2,3-butanediol.

Yang et al. (2015) reported heterologous overexpression of *DhaD* and *ACR* genes in *Bacillus amyloliquefaciens* resulting 2.0 and 3.0 fold higher enzyme activities compared to parental strain respectively. Reduced biomass growth and substrate utilization and 13.6% increase in 2,3-BD production was observed. Later by increasing the copy number of plasmid carrying DhaD/ACR genes resulted in increased intracellular NADH/NAD$^+$ concentrations in mutated strain.

Chen et al. (2014) investigated the differential expression of 2,3-BD cluster genes and influence on production of different isoforms. Wild strain of *K. pneumoniae* CICC 1.6366 was observed to produce 2R, 3R-BD, which increases when glycerol was supplemented as the carbon source but glucose decreased the ratio of metabolites. Even three different mutated strains were constructed by deleting glycerol dehydrogenase dhaD (*K. pneumoniae ΔdhaD*), acetolactate decarboxylase budA (*K. pneumoniae Δ budA*) and butanediol dehydrogenase budC (*K. pneumoniae Δ budC*). The strain lacking dhaD gene was not able to oxidize glycerol so 2R, 3R -butanediol production was not observed, where as budA deletion reduced 2R,3R-butanediol production and deletion of budC increased 2R, 3R- BD concentrations.

Cho et al. (2015) observed the dependence of 1,3-PDO and 2,3-BD as the end-products by inactivating dhaB glycerol dehydratase gene by deletion of pduC the larger subunit of dhaB gene and deletion of lactate dehydrogenase ldhA the major by product of glycerol dissimilation in *K. oxytoca* M1 strain. The mutated strain was reported to produce 115 g/L, 131.5 g/L with pure and crude glycerol respectively in fed-batch fermentations. Lactate dehydrogenase can regenerate NAD$^+$ ions by shifting the flux of reducing equivalents towards lactate synthesis, this result in reduced acetoin reductase activity reducing 2,3-BD synthesis. Presence of

nitrogen sparging resulted in 1,3-PDO formation in glycerol fermentation medium, whereas same strain under aerobic conditions with oxygen supply produced 2,3-BD (Metsoviti et al., 2012; Yen et al., 2014).

Though genetic engineering of wild type isolates produced higher yields, few were opportunistic pathogens, which cannot be developed for commercial scale that requires biosafety levels. Hence a GRAS (Generally Recognized as Safe) strains are required for scale up to meet the demand and market potential. 2,3-BD gene cluster from *Enterobacter cloacae* was heterologously expressed in *E. coli* strain. Initially gene clusters of *Bacillus subtilis* 168, *B. licheniformis* 10-1-A, *K. pneumoniae* CICC 1029, *S. marcescens* ATCC 14041, *E. cloacae* subsp. *dissolvens* SDM. In *K. pneumoniae* and *E. cloacae* all the three genes budABC coding for ALDC, ALS and BDH genes were observed as one operon but in other three strains ALDC and ALS was observed as single cluster, than BDH gene was overlapped with the other two genes and all clusters were individually expressed in pET 28a vector, here after fermentation the *E. coli* strain expressing gene cluster of *E. cloacae* subsp. *dissolvens* SDM produced 12.8 g/L of 2,3-BD, optimization of selective promoters, media engineering improved 2,3-BD production to 73.8 g/L 62 hours of fermentation (Xu et al., 2014).

2.3. Production of lipids

Biodiesel consists of fatty acids methyl esters produced by the trans-esterification of triacylglycerols (TAGs). It is obtained mostly from plant sources and is considered as a major resource to face high energy prices, and the animal fats may not be sufficient to meet the crude energy requirements. Currently, there has been an increased demand on green processes for the production of biodiesel from non-edible oils including oleaginous microorganism such as microalgae, bacteria, yeast and fungi. The lipids obtained from oleaginous microorganisms can be trans-esterified into fatty acid methyl esters (FAMEs) and the composition is similar to plant-derived oils (Chatzifragkou et al., 2011; Yang et al., 2012; Papanikolaou et al., 2008). Several research and developmental activities are going on to improve the intracellular lipid accumulation. This is done either by media engineering or by metabolic engineering of microbial strains either to improve their lipid accumulation capacities or to synthesize TAGs with desired fatty acid profiles. Several genera of oleaginous yeasts have been identified and tested for the production of TAGs, like *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodosporidium*, *Cryptococcus* and *Lypomyces* (Raimondi et al., 2014).

Rakicka et al. (2015) reported effective production of TAGs from crude glycerol with a continuous culture method and resulted in 24.2 g/L of lipids with productivity of 0.43 g/L/h which is highest among the reported lipid production from yeast. Chatzifragkou et al. (2011) evaluated the capability of fifteen eukaryotic microorganisms to convert crude glycerol to value added metabolic products. The study revealed that yeasts can accumulate intracellular lipids up to 22% (w/w), while fungi produced higher amounts of lipids (18.1–42.6%, w/w) in their mycelia.

Liang et al. (2010a) observed marine microalgae, *Schizochytrium limacinum* SR21 to grow efficiently on crude glycerol derived from used cooking oils (yellow grease), and with 35 g/L crude glycerol the cellular lipid content was the highest – 73.3%. Fed batch system appears to be more advantageous over batch systems for the increased accumulation of intracellular TAGs. The main advantage in using fed batch system is less substrate inhibition compared to batch cultivation (Luo et al., 2016; Liang et al., 2010b; Chen and Walker, 2011). In batch mode, the biomass and lipid concentration of fresh water micro algae *Chlorella protothecoides* cultivated in a crude glycerol medium were 23.5 g/L and 14.6 g/L respectively. The fed batch mode of cultivation improved the biomass and lipid

Table 3
Biological conversion of crude glycerol into lipids.

Strain	Biomass (g/L)	Lipids (%cdw)	Lipid (g/L)	Lipid productivity (g/L/h)	Reference
Yarrowia lipolytica	51	31	1.6	–	Sestric at al. (2014)
Rhodotorula graminis	19	54	10.3		Xu et al. (2012)
Candida freyschussi	33	4.6	–	0.43	Raimondi et al. (2014)
Cryptococcus curvatus	50.4	45	–	0.17	Thiru et al. (2011)
Rhodotorula glutinis	8.17	52.9	4.33	–	Galafassi et al. (2012)
Chlorella protothecoides	45.2	24.6	3	–	Chen and Walker (2011)
Aspergillus niger	–	–	3.5	–	André et al. (2010)
Rhodotorula toruloides	26.5		10	0.083	Xu et al. (2016)

accumulation to 45.2 and 24.6 g/L respectively (Chen and Walker, 2011).

Lipid accumulation from crude glycerol can be increased further by using two stages fed batch cultivation. Liang et al. (2010b) reported the use of two stage fed batch cultivation in *Cryptococcus curvatus* with crude glycerol derived from yellow grease. This strategy resulted in higher biomass accumulation (32.9 g/L) and lipid accumulation (1.5 g/L/day) than that obtained by the one-stage fed-batch cultivation. *Rhodosporidium toruloides* AS 2.1389, as a result of two stage fed batch cultivation, 26.5 g/L biomass and 10 g/L lipid accumulation was reported by Xu et al. (2016). Table 3 gives an overview of biological conversion of crude glycerol into lipids.

2.3.1. Metabolic engineering strategies for improving lipid productivity

Metabolic engineering strategies involve either improvement their lipid storage capacities or engineering the steps in synthesis of lipids with specific fatty acid content. Several metabolic engineering studies have been focused on increasing TAG production through metabolic engineering of TAG biosynthetic / degradation pathways (Qiao et al., 2015; Ledesma-Amaro et al., 2016). To produce fatty acids, the introduction of acyl carrier protein and thioesterase is essential to release fatty acid from generated in fatty acid synthetic cycle. Other interaction strategies include the enhanced expression of malonyl-CoA, overexpression of acetyl-CoA carboxylase, deletion of acyl-CoA synthetase in order to repress the utilization of fatty acids, regeneration of cofactors (NADPH), enhancement of fatty acid elongation cycle. The overexpression of a thioesterase from *Cinnamomum camphora* in *E. coli* accumulated the free fatty acid up to 2.5 g/L from glycerol (Lu et al., 2008). Later Lennen et al. (2010) reported that high copy number of thioesterase decreases the accumulation of fatty acids and low copy thioesterase was found to be more beneficial for FFAs production. Enhanced NADPH generation was attempted to enhance fatty acid accumulation by overexpressing *NAD kinase* (NADK) dehydrogenase (*pntAB*) and resulted in the enhanced production (4.82 g/L and 0.3 g/g) (Wu et al., 2014). Glycerol transport is another barrier to glycerol utilization in a most of the microbial cells including *Saccharomyces cerevisiae*. *P. tannophilus* has been shown to be capable of utilize and transport crude glycerol efficiently. The genes involved in glycerol transport in *P. tannophilus* were expressed in a *S. cerevisiae* STL1 knockout strain to evaluate their function and to address the possibility of their use in *S. cerevisiae* to improve their growth on glycerol (Liu et al., 2013a, 2013b). Oleaginous marine diatom, *Fistulifera solaris* JPCC DA0580 has been metabolically engineered by Muto et al. (2015) for the over expression of glycerol kinase gene which accelerates glycerol metabolism and resulted in improved lipid accumulation and biomass productivities.

The enzymes *DGA1* (diacylglycerol acyltransferase type 2) and *DGA2* (diacylglycerol acyltransferase type 1) have been identified as crucial components of the lipid biosynthetic pathway. Metabol-

ically engineered *Y. lipolytica* strain JMY3886 proved to be an efficient cell factory for the development of biodiesel production processes. Several metabolic engineering strategies have been established in *Yarrowia lipolytica* to improve lipid accumulation. Overexpression of the *Y. lipolytica DGA1* and *DGA2* genes have efficiently increased lipid yield (Blazeck et al., 2014; Gajdoš et al., 2015) and it has been found that the overexpression of diacylglycerol acyltransferase an important target gene for high levels lipid accumulation in oleaginous organisms t (Courchesne et al., 2009; Tai and Stephanopoulos, 2013)). It has also been proved that *DGA1* and *DGA2* overexpression is beneficial for lipid accumulation in *R. toruloides* (Zhang et al., 2016a). Effect of promoter replacement in *Yarrowia lipolytica* was reported by Runguphan and Keasling, 2014. They replaced all the promoters of fatty acid biosynthesis genes with highly active constitutive promoter TEF1. They demonstrated the overexpression of key fatty acid and TAG biosynthesis enzyme resulted in enhanced TAG accumulation. Another study by Xie et al. (2015) developed a *Yarrowia lipolytica* strain by deleting the *POX1–6* genes (*POX1–POX6*) that encode acyl-coenzyme A oxidases and the *TGL4* gene, which encodes an intracellular triglyceride lipase. The deletion leads to blocking the β-oxidation pathway and blocks TAG mobilization. The genes *YlDGA2* and *YlGPD1*, were constitutively over expressed which codes for the acyl-CoA, diacyl glycerol acyl transferase and glycerol-3-phosphate dehydrogenase.

Lipid synthesis is a highly complex biosynthetic process due to the repeated elongation cycles of the long carbon chain and subsequent multiple assembly step for TAG. Thus advanced metabolic engineering tools are essential to facilitate the metabolic engineering of lipid biosynthetic pathway. The rapid development of computational tools like metabolic flux analysis and computational modeling tools and metabolic pathway reconstruction tools has extended possibilities for the simulation of complex metabolic networks. A large number of studies have applied genome-scale modeling of lipid biosynthetic pathway for conducting rational metabolic engineering (Zhang et al., 2016b; Ranganathan et al., 2012).

3. Conclusions

Crude glycerol generated as a biodiesel industry byproduct is creating several environmental problems and can be used as a substrate for the production of value added chemicals One of main limitations of crude glycerol utilization is that most microorganisms exhibits substrate mediated inhibition. To overcome this, several research and developmental activities are going on throughout the world for effective utilization of crude glycerol as a sole carbon source by metabolic engineering as well as by utilizing tolerant strains. Fine tuning of process variables will make it feasible on industrial scale. This addresses dual benefits to society by converting a waste stream to value added product.

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