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REVIEW ARTICLE

Biorefinery for Glycerol Rich Biodiesel Industry Waste

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Abstract The biodiesel industry has the potential to meet the fuel requirements in the future. A few inherent lacunae of this bioprocess are the effluent, which is 10 % of the actual product, and the fact that it is 85 % glycerol along with a few impurities. Biological treatments of wastes have been known as a dependable and economical direction of overseeing them and bring some value added products as well. A novel eco-biotechnological strategy employs metabolically diverse bacteria, which ensures higher reproducibility and economics. In this article, we have opined, which organisms and what bioproducts should be the focus, while exploiting glycerol as feed.

Keywords Biorefinery · Biowastes · Biofuels · Biopolymers · Biohydrogen · Methane

Introduction

Generation of wastes is a necessary evil in the lives of all human beings. However, Nature has provided all organisms with unique metabolic abilities such that all biomaterials: raw, bioproducts, or "wastes", get transformed and prove beneficial in a cyclic manner. Natural cycles are regulated in such a manner that there is no material which can be classified as waste. The rate of production substrates and their biotransformation are very well regulated. The actual problem has cropped up because of rapid and large scale exploitation of natural resources. The waste generation, its disposal and management are completely disorganized. The magnitude is accelerating at an alarming pace [1, 2]. There is an absolute dearth of abilities and resources to dispose waste. This is leading to rapid deterioration of the environment. Another equally alarming issue which is a major cause of worry around the world is the high rates at which fossil fuels are getting consumed and associated emission of obnoxious gases. We are thus in a scenario, where rapid progress is forcing us into an unprecedented environmental pollution level and an equally fatal energy crisis. Efforts to circumvent these man-made crises have made it imperative to look for mechanisms to reduce waste generation and fuel consumption on one hand and look for alternative and cleaner sources of energy, on the other hand. Among the diverse methods to handle waste, biological mechanisms have the potential to treat wastes and produce energy as well [2, 3].

In natural ecosystems, microorganisms operate as a dynamic population. They are known to preserve the rivers and streams as pollution free and fresh source of drinkable water. These natural systems and the microbes are now being exploited to clean man made polluting sources. Wastewater treatment through aerobic routes is energy intensive and causes high energy requirements. Anaerobic digestion process, though relatively slow, is economical and extremely effective. The best option is to operate the two types of processes in a sequential manner, depending upon the bioproduct under consideration. The concept has moved into a new direction, where pure microbial culture driven processes are being replaced with the use of well defined microbes with high activity are mixed together to ensure two things: (1) at least one of

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the organisms can act under a given set of conditions, and (2) the risk of getting contaminated is reduced because the diversity of inoculum can out beat the inherent or contaminating organism(s) [4].

Biotransformation of the organic matter present in a waste takes place through a few steps [5]. The foremost in the degradation process is the hydrolytic step involving enzymes primarily responsible for depolymerization of carbohydrates, proteins and fatty substances. These solubilized intermediates can be further metabolized into materials into biofuels and biopolymers and other bioproducts—biofertilizers. Here, in this article we will focus on the effluent from the biodiesel industry.

Biodiesel Industry Waste

Generation of crude glycerol (CG) during the biodiesel production process has been witnessing a steady increase to 6.9 million ton [6]. This has lead to a drastic decline in CG prices to such an extent that its disposal has become a burden for the Environmental and Health Departments. Research efforts are being made to derive value added products from CG as a feed: propanediols, ethanol, hydrogen, methane, methanol, single cell oil, biosurfactants, and organic acids [7–13].

Propanediols

CG could be transformed via anaerobic fermentation by *Klebsiella pneumoniae* 1,3-propanediol, which can be an excellent raw material for producing polyesters and polyurethanes [14]. Large scale production of 1,3-propanediol (1,3-PDO) by *K. pneumoniae* M5al was done in step-wise manner, in reactors ranging from 5 to 5000 L capacity. The best results achieved were the production of 58.8 g/L 1,3-PDO with a yield of 0.53 mol/mol glycerol and productivity of 0.92 g/L/h [15]. Methanolysis of soybean oil by chemical treatment and enzymatic (lipase) catalysis were quite similar at 51.3–53.0 g/L. Further optimization of the bioprocess has lead to 1,3-PDO yield of 56 g/L [16]. *K. pneumoniae* co-expressing *ald4* and *rpoE* produced 9.8 g 1,3-PDO/L over a period of 24 h, which was an enhancement of 0.85-fold over strain expressing only *ald4* [17]. *Klebsiella oxytoca* M1 could convert CG more effectively than pure glycerol for producing 2,3-butanediol (2,3-BDO) (73.8 g/L). Using a double mutant of *K. oxytoca* M3 (deleting genes—*pduC,* which encodes for the large subunit of glycerol dehydratase and *Doha,* which encodes for lactate dehydrogenase) enhanced 2,3-BDO yield by 1.9-fold, under batch culture conditions. With CG, the double mutant resulted in the production of 1,3-PDO free 2,3-BDO—131.5 g/L concentration and a yield of 0.44 g/g feed [18].

Clostridium butyricum was shown to produce 1,3-PDO from synthetic medium supplemented with CG [19–22]. *C. butyricum* strain F2b could convert CG into 1,3-PDO under batch and continuous culture conditions, with yields of 44–47 g/dry matter. The yield of 1,3-PDO was negatively influenced at higher glycerol concentrations. This concentration dependent influence was because of the alternative and competitive pathways leading to butyric and acetic acid production [23]. CG generated by biodiesel production from rapeseed oil is reported to be a good feed for microbially converting it to 1,3-PDO. *C. butyricum* strain DSP1 isolated from a rumen of a cow could produce 0.65 mol 1,3-PDO/mol glycerol on bioreactor scale [24]. Metabolically active bacterial biomass acclimatized during fermentation were used to inoculate subsequent bioreactors having CG (80 and 100 g/L). Here, the maximum 1,3-PD concentrations achieved were 43.2 and 54.2 g/L, respectively [25]. Genetically modified *Escherichia coli* strain carrying *dhaB1* and *dhaB2* (encoding for vitamin B12 independent glycerol dehydratase and its activator, respectively) from *C. butyricum* along with *E. coli yqhD* gene (encoding for 1,3-PDO oxidoreductase isoenzyme, YqhD) gave a 1,3-PDO yield of 104.4 g/L and a productivity of 2.61 g/L/h from glycerol as C source [26].

Another factor which affects the biotransformation of CG is the associated chemical contaminants. Pretreatment of CG with organic solvents helps to remove residual fatty acids. Here, *Citrobacter freundii* produced 1,3-PD in quantities similar to those obtained with pure glycerol [27]. *C. freundii* strain FMCC-B 294 (VK-19) has been tested under diverse physiological conditions for exploiting CG generated as a consequence of waste-cooking oil trans-esterification. It has a high tolerance to glycerol up to 170 g/L unlike most other bacteria, which cannot withstand such high concentrations. However, maximum 1,3-PDO production of 45.9 g/L was realized at lower CG concentrations of 10 % v/v. Under non-sterilize fed-batch conditions, 176 g/L of CG were transformed to 66.3 g/L of 1,3-PDO [28]. To reduce the cost of biotransformation of glycerol to various products including 1,3-PDO, nitrogen (N_2) sparging into the bioreactors was executed. It enhanced the yield of 1,3-PDO from 0.5 to 3.0 g/L. This change was attributed to shifting from reductive to oxidative metabolism of glycerol with the co-culture of *C. butyricum* and *Enterobacter aerogenes* [29]. Naturally occurring microbes are always expected to perform the bioprocess in an economical manner. *Lactobacillus brevis* N1E9.3.3, facultative anaerobic bacteria, which was isolated through on-site enrichment technique resulted in a yield of 0.89 g 1,3-PDO/g glycerol, with a productivity value of 0.78 g 1,3-PDO/L/h. Here, Co^{+2} and vitamin B_{12}

were found to enhance the biotransformation process [30]. In a fed-batch mode, use of mixed microbial cultures could ferment CG (10 g/L) into 1,3-PDO at the rate of 3.67–3.99 g/L [31].

Production of 1,2-PDO is associated with different by-products e.g., formate, acetate, succinate, and ethanol [32]. Efforts to eliminate by-products by knocking out genes encoding for acetate kinase, phosphate acetyltransferase, and lactate dehydrogenase improved the yield from 0.11 to 0.21 g/g, but was negatively linked to enhanced production of ethanol, formate, and pyruvate. *E. coli* could transform CG to 1,2-PDO, with a yield of 0.24 g/g [33]. *K. pneumoniae* G31 was observed to transform glycerol to 2,3-BDO with a yield of 49.2 g/L. The process was greatly favoured by strong aeration and alkaline pH [34]. *Saccharomyces cerevisiae* is not a very suitable organism for using glycerol as a carbon source primarily because of its low utilization rates. Genetic modification in *S. cerevisiae* by introducing *mgsA* gene encoding for the enzyme methylglyoxal synthase and the *gldA* gene encoding for glycerol dehydrogenase of *E. coli* allowed increased production of 1,2-PDO up to 2.19 g/L [35]. Similar attempts to use engineered *E. coli* carrying genes—*mgsA, gldA*, along with *yqhD* encoding aldehyde oxidoreductase, overall responsible for the functioning of the 1,2-PDO pathway were able to produce 5.6 g 1,2-PDO/L, at a final yield of 21.3 % (w/w) [36].

Ethanol

To over-come the limited availability of fuels and use renewable resources as feed, attempts were made to utilize algal biomass. Bacterial fermentation of algal biomass to glycerol and its further use was explored. *Clostridium pasteurianum* was reported to have the capacity to convert a mixture of algal biomass (*Dunaliella bardawii*) and glycerol (4 %) into a mixture of n-butanol, 1,3-PDO, and ethanol at the rate of 16 g/L of culture [37]. Glycerol rich effluent from the biodiesel manufacturing process could be converted to ethanol by *E. aerogenes*. Glycerol metabolism resulted in ethanol yield of 0.8 mol/mol feed. The yield of ethanol by fermenting glycerol could be enhanced to 0.85 mol/mol using immobilized bacteria [38]. Upscaling from small laboratory level reagent bottles to 3.6 L continuous culture stir tank reactors resulted in utilizing 1.5 % w/v glycerol for producing 0.75 mol ethanol/mole feed [39].

Glycerol fermentation by *E. coli* was associated with the presence of CO_2, which is produced by the enzyme formate-hydrogenlyase under acidic conditions. Reduced compounds like ethanol and succinate constituted 93 % of the products. These yields were higher than those obtained from common sugars like glucose [40]. *E. coli* strains were engineered to co-produce hydrogen (H_2) and formate along with ethanol from glycerol as feed. The genetically modified *E. coli* strain SY03 yielded 1 mol ethanol/mol glycerol, with a productivity of 0.051 g/L/h and at a concentration of 5 g/L [41]. Fermentation of glycerol by *Klebsiella planticola,* isolated from the rumen of red deer (*Cervus elaphus*) resulted in ethanol and formate as major products at concentrations in the range of 30–32 mmol/L [42]. *K. pneumoniae* and *Kluyvera cryocrescens* S26 have a high capacity to produce ethanol from CG [43, 44]. The genetic modifications in *K. pneumoniae* were achieved by inactivating lactate dehydrogenase and introducing pyruvate decarboxylase and aldehyde dehydrogenase from *Zymomonas mobilis*. The modified strain of *K. pneumoniae* showed an enhanced yield of 0.89 mol ethanol/mol glycerol, productivity of 1.2 g/L/h, and a concentration of 31.0 g/L [45].

In view of the well established ability of *S. cerevisiae* to naturally produce ethanol from sugars as feed, it becomes a strong contender for this bioprocess. However, biotransformation of glycerol to ethanol by *S. cerevisiae* is not as effective as sugars. Genetic modifications for over expressing glycerol dehydrogenase (Gcy) and dihydroxyacetone kinase (Dak) in *S. cerevisiae* improved the ethanol concentration by 2.4-fold to 1.66 g/L [46]. A further 3.3-fold enhancement in ethanol production was achieved by over expression of glycerol utilizing pathway genes responsible for glycerol uptake protein GUP1 [35]. Another yeast—*Pachysolen tannophilus* strain CBS4044 was shown to be a robust organism, which could tolerate a wide range of CG concentrations as feed. The process was not influenced by the oxygen transfer rate, which greatly affects the ethanol production process. At high CG concentrations of 5 % v/v, 17.5 g/L of ethanol production was equivalent to 56 % of the theoretically achievable yield. Further optimization of the bioprocess allowed production of 28.1 g/L ethanol [47]. Bacterial susceptibility to high ethanol concentration in the medium is one of the major limiting factors of this bioprocess. *Klebsiella variicola* strain TB-83 was genetically modified to obtain strain TB-83D, which could grow well in the presence of 7 % (v/v) ethanol. *K. variicola* strain TB-83D transformed glycerol supplemented with corn steep liquor and yeast extract produced 34 g/L ethanol [48].

Hydrogen

Bacterial ability to produce H_2 from pure glycerol and CG as feed has been recently reviewed [11, 49]. Hence, the major emphasis in the present article has been laid here on works published in 2015 and 2016.

Citrobacter, *Clostridium*, *Enterobacter*, *Klebsiella*, and *Thermotoga* have been shown to use glycerol and yield H_2 ranging from 0.14 to 1.23 mol/mol [11]. In contrast to batch culture conditions, a few studies have demonstrated H_2 production from glycerol under continuous culture conditions [50, 51]. Improvements in H_2 production from glycerol have been reported by changing the nitrogen source: (1) 0.542 mol/mol glycerol with 1 % w/v ammonium chloride and (2) 0.748 mol/mol glycerol with sodium nitrate, and (3) 0.646 mol/mol glycerol with ammonium nitrate [52]. Similar enhancement in H_2 yield from glycerol was observed previously as well with ammonium sulphate [53]. Biological hydrogen production on a regular basis can be achieved where ever feasible, by continuous culture digestions, especially using immobilized bacterial cultures. Continuous culture stir tank reactors (3.6 L capacity) were found to be operating in a stable manner by converting 1.5 % w/v glycerol for producing 0.86 mol H_2/mole feed [39].

CG to H_2 by *Bacillus thruingiensis* immobilized on ligno-cellulosic materials at 0.393 mol/mol feed proved to be 2.3-fold better than free floating bacterial cultures [52]. In comparison to *Bacillus*, continuous culture H_2 yields from CG with *Clostridium* and *Thermotoga* spp., were reported to be 0.27–0.77 mol/mol [11]. An up-flow column bioreactor was operated to produce H_2 from CG in a continuous mode. With bacteria being immobilized to the biofilm reactor, it resulted in achieving an H_2 productivity level of 107 L/kg waste glycerol. Glycerol consumption was found to vary from 73 to 96 %, depending upon the operation period [54].

Recent approaches in tackling issues related to use of biowastes as feed include: (1) microbial contaminants, (2) a shift from pure single cultures to defined mixed cultures and (3) co-substrate utilization. It helps to ensure successful completion of the bioprocess [4, 55, 56]. Apple pomace and CG mixture provide nutrition suitable for microbial processes. Mixed cultures of *E. aerogenes* and *C. butyricum* could convert a mixture of pomace and CG (15 % w/v) to H_2—1.7 mmol/mol of CG [57]. Subsequent optimization by avoiding pre-treatment and use of reducing agents, this co-culture was found to yield 19.46 mmol H_2/L which was higher than that recorded with monocultures [58]. Comparisons of mono-, co- and mixed cultures revealed that maximum H_2 production of 29.8 mmol/L at 2 % w/v CG with mixed cultures. Another factor which helped to enhance H_2 production process efficiency was heat treating the feed—100 °C for 15 min, primarily to get rid of H_2 consumers [59].

Approaches to integrate dark and photosynthetic hydrogen production processes are being advocated to break the barrier of 4 mol H_2/mol hexose sugar [60, 61]. *Rhodopseudomonas palustris* grows well and can produce H_2 on glycerol as feed. However, the presence of saponified fatty acids, a contaminant in CG is a major hurdle for using *R. palustris*. Strategies like use of activated carbon, making necessary pH adjustment, solvent extraction and precipitating fatty acids helped to improve the process efficiency. H_2 production rate of untreated CG was 5 ml/g_{dw}/h, where as pretreatments improve the production rate by fourfold to fivefold, with a maximum being 27 ml/g_{dw}/h with calcium precipitation. It was almost as good as 29 ml H_2/g dry weight/h recorded with pure glycerol [62].

Biohydrogen production from CG by *Klebsiella* sp. TR17 and *R. palustris* TN1 was followed during dark and photo fermentative processes, respectively [63]. The dark fermentative process resulted in H_2 production of 64.24 mmol/L with a final yield of 5.74 mmol/g COD consumed. The effluent from the previous process resulted in an additional H_2 yield of 0.68 mmol/g COD consumed. The overall H_2 yield of 6.42 mmol/g COD consumed [63]. An analysis of process parameters influencing H_2 production from CG reveals that cost of medium, inoculum and energy input needs to be reduced to improve the overall economy and sustainability [64].

H_2 evolution by *E. coli* was detected during fermentation of glycerol [40, 65]. *E. coli* can grow well and produce H_2 at 1 % w/v of glycerol, but higher concentrations, prove inhibitory. Depending upon the pH, the H_2 production rate varied from 4.57 to 6.79 mmol/min/g dry weight. H_2 yields from co-metabolism of glycerol (0.5 %) and glucose (0.1 %) were twofold to threefold higher than those observed with individual substrates [66]. Genetic modification of *E. coli* metabolic pathway by deleting certain genes, especially, *frdC, ldhA, fdnG, ppc, narG, mgsA*, and *hycA*, allowed a fivefold enhancement in H_2 yield. Utilizing this strain under low partial pressure fermentor further proved effective in increasing H_2 production process efficiency [67].

Methane

Microbial digestion of glycerol as feed for producing biogas containing methane as an energy source has been presented in detail in a recent article [49]. Hence, after a brief summary of the previous works, the major emphasis in the present article has been laid here on works published in 2015 and 2016.

Anaerobic digestion of biowastes leads to almost complete conversion of its organic matter content. Biogas with a CH_4 content of 55–70 %, has been observed with a wide range of feed material. The overall biogas yields are in the range of 200–400 L/kg Total solids [2]. The use of CG, as a co-substrate with sewage sludge and wastes from

municipal solids and slaughterhouse could enhance hydrogen and methane production [68–70].

Continuous culture digestion of glycerol in a stirred reactor produced 74 mL CH_4/L/d, whereas 993 mL CH_4/L/d was recorded in a baffled anaerobic reactor [71]. Co-digestion of wastes is more advantageous than single feed [4, 71]. Digestion of mixed wastes along with 3–6 % glycerin lead to 570–680 L CH_4/kg VS, which was equal to a threefold enhancement over animal manure alone [72]. Digestion of mixture of animal manure and CG range from 5 to 10 %, w/w produced 0.82 L/g in continuously stirred reactors. Animal manure mixed with CG resulted in 5.9 m^3 biogas/tonne of fresh waste [73]. Under continuous culture conditions, animal manure and glycerol (6 %) combination generated 590 L CH_4/kg VS [74]. Digestion of mixture of animal manure, food waste and 2–6 % glycerol produced 0.65–2.57 L CH_4/L/d in thermophilic reactors [75]. Sewage sludge and CG (2 %, v/v) produced 1 L CH_4/L/d [70, 76]. Co-digestion of macroalgae: (1) *Gracilaria vermiculophylla* and 2 % glycerol resulted in 18 % enhancement, and (2) *Sargassum* sp. and 0.3 % glycerol lead to 1.5-fold increase in CH_4 yield and 1.38-fold enhancement in CH_4 productivity [77].

Anaerobic digestion of a mixture of glycerol waste (1 % v/v) and canned seafood wastewater yielded 577 mL CH_4/g organic solids fed, equivalent to 5.8 m^3 CH4/m^3 or 207 MJ or 58 kWh of electricity. It was 8 % more than waste water digestion alone. Continuous culture digestion in up-flow anaerobic sludge blanket (UASB) reactors resulted in 2.33 L CH4/L-reactor/day [78]. For value addition to the process of biodegradation of glycerol, the simultaneous production of acetate and methane were investigated. Under thermophilic conditions, 13.0 g acetate/L was recorded under fed-batch reactors. Continuous stirred tank reactor reached a stable level within 100 days of operation. These processes allowed 0.74–0.80 mol CH_4 and 0.63–0.70 mol-acetate/mol feed, with the aid of *Thermoanaerobacter* spp. [79]. Stable biogas production with 68 % CH_4 content was reported over a period of a few months in a small scale UASB reactor of 7 L capacity [80]. It has been estimated that a 25 m^3 bioreactor has the potential to produce 4.4 MW of thermal energy, which can be transformed into heat (4.4 GW) of electricity (1.2 GW) [81]. Glycerol waste along with impurities are physiologically not very conducive for methanogens. Acclimatization of bacteria to a feed is always helpful in improving the efficiency of the process. Shock loading and gradual acclimatization of bacterial consortia to glycerol allowed a CH_4 production rate of 21 mmol/L/day [82].

Poly(hydroxyalkanoates)

Bacteria have a unique characteristic to produce Poly(hydroxyalkanoates) (PHA), which have properties similar to petrochemically produced polymers. Being biodegradable, PHAs are good substitutes for polyesters. Large scale production of PHAs is limited by the high cost of the feed and the recovery process. It is proposed that the use of cheap raw materials and biowastes are expected to reduce production cost by around 45 % [83, 84]. With availability of glycerol being easy and cheaper, *Cupriavidus necator* was found to produce up to 51 g/L, accounting for 38–65 % of dry cell mass [49]. Large-scale production of polyhydroxybutyrate (PHB) by *C. necator* DSM 545 from CG was also limited by the contaminating sodium salt [85]. *Zobellella denitrificans* MW1 could tolerate NaCl and still produces PHB [86]. Mixed microbial consortia could be used to overcome the potential threat from methanol to produce PHA CG. *Pseudomonas oleovorans* NRRL B-14682, an organism well recognized for its metabolic activities has also been shown to produce PHB from 1 to 5 %, v/v CG [87]. A biodiesel plant with a capacity of 10 million gallon per year can be expected to yield 20.9 ton PHB [88].

Molecular analysis of metabolic gene expression has provided important clues towards the limiting factors in converting glycerol to PHAs. It has been realized that glycerol uptake itself reduced during active synthesis of mcl-PHA by *Pseudomonas putida* strain LS46 WG. Subsequently, the enzymes responsible for supplying monomers were also found to express differentially on waste glycerol and waste free fatty acids. PhaJ1 gene seems to be critical in this process especially the supply of C8 monomers [89]. *C. necator,* a well known PHA producing organism was tested on a range of fatty acid rich wastes. It was realized that limiting the intracellular depolymerization by knocking out *phaZ1* gene resulted in enhanced PHA yield [90]. Using response surface methodology for optimizing feed as carbon source, glycerol waste (0.85 v/v), with mixed wastes (0.26 % w/v) leads to 1.87 g/L of co-polymer of PHA: PHB and medium chain length 3-hydroxyalkanoates by *Pannonibacter phragmitetus* [91].

Bacillus spp. are quite versatile in their metabolic activities [51, 55, 56]. Different *Bacillus* species can produce PHA from glycerol (1–5 %, v/v): *B. cereus, B. licheniformis, B. megaterium, B. sphaericus,* and *B. thuringiensis* [49]. Unlike most organisms, *Bacillus* has another unique ability to produce PHA, independent of carbon: nitrogen ratios. *B. thuringiensis,* produced 1.54–1.83 g/L of PHA on glycerol combined with high nitrogen content media. Even co-polymer containing

13.4 % 3hydroxyvalerate was produced by *B. thuringiensis* under these non-limiting N conditions [92].

Docosahexaenoic Acid

Docosahexaenoic acid (DHA) has great commercial value in the cosmetic industry. DHA is used as a self-tanning agent, weight gain and reduction, antioxidant, therapeutic for recalcitrant vitiligo. Alga *Schizochytrium limacinum* has been reported to yield DHA by fermenting CG at a concentration of 75–100 g/L. The critical parameters which regulated this process were temperature and ammonium acetate concentration, which under optimum conditions resulted a DHA yield of 4.91 g/L [93]. DHA-containing algae are a potential replacement for omega-3 fatty acids generally obtained from fish oil [94]. A high oxygen transfer in the fermentation reactor inoculated with *Schizochytrium* sp. S31 was reported to effectively increase the DHA productivity and conversion yield. In fed-batch culture, the DHA concentration of 28.93 g/L was recorded at a volumetric mass transfer co-efficient of 1802/h. The resultant DHA productivity at 301 mg/L/h and conversion yield of 0.44 g/g was significantly higher than previous reports [95]. *S. limacinum* SR21 could transform CG (3 % w/v) to DHA, with a maximum productivity of 233.73 mg/g biomass [96]. *Gluconobacter* strains are extensively used to produce DHA via the incomplete oxidation of glycerol with the help of glycerol dehydrogenases. The efficiency of DHA yield in fed-batch culture by *Gluconobacter frateurii* was enhanced by high oxygen transfer coefficient of 82.14/h to 0.89 g/g [97].

Eicosapentaenoic Acid

Fungus *Pythium irregulare* could grow on CG and its biomass served as foods or feeds rich in eicosapentaenoic acid (EPA). In a medium supplemented with CG (30 g/L) had an EPA yield of 90 mg/L [94]. The growth kinetics and behaviors of the algae on CG has been effectively studied in continuous culture mode [98].

Lipids

With CG as C source, algae *S. limacinum* SR21 could grow and produce lipids, to be utilized as a sustainable feedstock for biodiesel production. These processes are limited by methanol and high concentrations of glycerol. At 35 g CG/L, the cellular lipid content reached up to 73.3 % [99]. Under fed-batch fermentative conditions, supplementation with ammonium sulfate and Tween 20 proved helpful in enhancing the accumulation of lipids—10 g/L, lipid yield—60.7 % and carotenoids—6.10 g/L. The lipid yield was 0.31 g/g or 3 g/L CG with *Chlorella protothecoides* [100, 101]. Marine diatom, *Fistulifera solaris* was engineered to over express glycerol kinase gene. With enhanced ability to metabolize glycerol, improvements in biomass and lipid productions were observed. A 12 % increase in the lipid production was recorded, which can be exploited for biodiesel fuel production [102].

Yeast, *Cryptococcus curvatus*, under fed-batch conditions could produce 44.2–52 % lipid, with a high component of monounsaturated fatty acid. It thus was regarded as a good biodiesel feedstock [103]. *Rhodotorula glutinis* TISTR 5159, an oleaginous red yeast produced lipids and carotenoids by metabolizing CG [104]. Comparative study revealed that yeast *Rhodotorula* sp. accumulated only up to 22 % (w/w), where as fungi have higher capacity to accumulate up to 42.6 % (wt/wt, of dry biomass) [105]. Accumulation of lipid by *Lipomyces lipofer* with CG as feed was reported to be 5.46 g/L, which constituted 57.64 % of the total biomass. In this study, *R. glutinis* produced TAG with 68.3 % as linoleic acid (C18:2) where as *Lipomyces starkeyi* TAG had 39.3 % as palmtic acid (C16:0) [106].

S. cerevisiae strain was engineered for overproducing triacylglycerol (TAG) from glycerol as a feed. Over expression of gene encoding for glycerol kinase, resulted in 2.4-fold enhancement accumulation of TAG, primarily due to its ability to increase utilization of glycerol. Over expression of genes responsible diacylglycerol acyltransferase and phospholipid diacylglycerol acyltransferase lead to 23.0 mg/L lipids, a 1.4 fold enhancement and 8.2 % TAG yield, equivalent to 2.3-fold increase [107].

Producing lipid by fungus, *Cunninghamella echinulata* by using biowastes such as tomato waste hydrolysate is quite lucrative as the feed is going to be dirt cheap. However, in order to maintain an imbalanced C:N ratio, which favours this process, removal of N from biowaste was attempted. Bioconversion by using glucose as C source, resulted in lipid production of 0.48 g/g of dry biomass, equivalent to 8.7 g/L of growth medium. Replacing glucose with glycerol the biomass production was effective, but lipid production was lowered to 4.5 g/L. Although, comparatively low lipid productivity is achieved, however, the major interest lies in replacing costly glucose with cheaper glycerol [108].

Bacterium, *Rhodococcus opacus* MITXM-61 produced intracellular TAGs at high concentrations of glucose and xylose. However, with CG as feed, *R. opacus* MITXM-61 did not grow well and no TAGs were detected. A mutant strain MITGM-173 could utilize glycerol (1.6 % w/v) to produce 0.1444 g TAG/g of glycerol consumed. In a medium containing glycerol, glucose and xylose, *R. opacus* MITGM-173 produced 14.3 g TAGs/L [109]. It thus raises

the possibility of producing precursor for lipid-based biofuels from biodiesel industry waste.

The usage of single-cell-oils as alternative biodiesel feedstock depends on their fatty acid composition. The focus is on isolating yeast strains with high lipid producing ability and better oil quality, i.e. the higher proportion of medium-chain fatty acids. TFA accumulation by *Yarrowia lipolytica*/pYLEX-CpFatB2 reached a maximum after 5 days of growth, where it was 0.168 g g^{-1} dry weight basis, having 17 % lipid i.e. 0.82 g/L. In *Y. lipolytica*/pYLEX strain, the absence of *fatB2* gene, lead to lowering of TFA content down to 10–12 %. It thus elucidated that thioesterase CpFatB2 of *Cuphea palustris* triggered oleic acid accumulation in *Y. lipolytica* [110].

Other Bioproducts

Biotransformation of CG to citric acid by *Y. lipolytica* was as effective as that reportedly produced on sugar-based medium [19]. It also resulted in the production of citric acid and single-cell oil in a single reaction [21, 111]. Acetate-negative mutant strain of *Y. lipolytica* Wratislavia AWG7 fed on CG lead to citric acid yield of 131.5 g/L, which was as effective as that produced on pure glycerol (139 g/L). *Y. lipolytica* strain Wratislavia K1 co-produced citric acid (87–89 g/L) and erythritol (47 g/L) [112]. An interesting feature of strain K1 was its ability to selectively produce erythritol from CG [113]. Higher (71 g/L) citric acid yields from CG have also been reported [114]. Glycerol (30 g/L) mixed with acetic acid (10 g/L) as feed for *Enterococcus faecalis* produced pure L-lactic acid at the rate of 55.3 g/L, a yield of 0.991 mol/mol glycerol. This process was linked to the ethanol producing capacity of this strain [115].

Fermentation of CG to 3-hydroxypropionic acid (3-HP) was obtained by recombinant *Klebsiella pneumoniae* carrying gene for aldehyde dehydrogenase of *Saccharomyces cerevisiae* [116, 117]. Here, it was realized that preferential expression of *ald4* from *S. cerevisiae* is the most instrumental gene regulating 3-HP yield. Further enhancements were achieved through ligation of *puuc* gene from *K. pneumoniae* [117]. *Lactobacillus reuteri* metabolizes glycerol through CoA-dependent propanediol utilization route leading to the production of 3-HP. Genes *pduP*, *pduL* and *pduW* of *L. reuteri* DSM 20016 were expressed in *E. coli*. The recombinant strain could produce 1 mol 3-HP/mol via 3-HPA. These growing cells were also observed to co-produce 1,3-propanediol [118].

Cellulases are important enzymes for hydrolytic digestions. High biomass production of *Trichoderma harzianum* was achieved using glycerol as feed. Cellulase production was achieved by induction with sugarcane bagasse. The resulting enzymes: cellulase, xylanase, and β-glucosidase were observed to have 2.27 FPU/mL, 106 IU/mL, and 9 IU/mL, respectively. These enzymatic units were twofold higher in comparison to control where glucose instead of glycerol was used [119].

Continuous cultivation process for producing succinic acid by bacterium *Basfia succiniciproducens* DD1 was reported to be stable, economical [120]. CG, as the C source, was good enough to produce phytase in industrial scale with recombinant *Pichia pastoris* possessing a pGAP-based constitutive expression vector [121] and producing butanol 0.30 g/g, with *C. pasteurianum* [122]. Canola oil-derived CG seems to have a great potential to produce high value products such as the recombinant human erythropoietin [123].

A new avenue for value added products is the production of 0.1 g bacterial cellulose (BC)/L from CG [124]. *Gluconobacter* sp. NBRC3259 could produce glyceric acid (49.5 g/L) and 28.2 g/L dihydroxyacetone from 174 g/L of CG [125]. Solvent tolerant lipase from CG was found to be produced by *Staphylococcus caseolyticus* EX17 [126]. *Ustilago maydis* was able to convert CG to glycolipid-type biosurfactants [127]. *Rhizopus microsporus* var. oligosporus produced protein rich in threonine biomass from CG [128]. Enzymatic hydrolysis of lignocellulosic biomass was enhanced by using CG as a high boiling point solvent [129]. Bioremediation efficiency of the wastewater denitrification process, especially for removing nitrate was enhanced by adding 2.0–5.0 mg NO_3/L CG [130]. Microbial fuel cells can use CG as fuel for generating electricity [131].

Non-biological Products

Animal Foodstuff

As an animal feed for broilers, hens and swine, CG is easily absorbed in the digestive system. It has digestible energy values of around 14.9–15.3 MJ/kg, of which more than 90 % is metabolizable energy [132]. It can supplement feed up to 9 % [133], which can enhance its performance by 10 % [134]. In ruminants diets, lambs, goats, cows, CG can make up 5–15 % dry matter [135, 136]. These additions help animals to gain weight through efficient feed consumption [137]. It can thus be used to replace corn in animal diet [138]. The other advantages associated with the use of CG are weighed down by presence of impurities, especially methanol [139–141].

Fuel Additives

Glycerine acetates, which are regarded as valuable transportation fuel additives can be produced by esterification of glycerol with acetic acid. An etherification process to synthesize oxygenated compounds, like glycerol ethers,

Table 1 Potential biotechnological applications of biodiesel industry waste (glycerol)

Organism	Bioproduct from glycerol						References
	PDOs	EtOH	H_2	PHA	DHA	Lipids	
Bacterium							
Citrobacter freundii	+[a]	−[b]	+	−	−	−	[11, 27, 28]
Clostridium butyricum	+	−	+	−	−	−	[11, 19–25, 29, 57, 58]
Clostridium pasteurianum	−	+	−	−	−	−	[37]
Recombinant *C. butyricum*	+	−	−	−	−	−	[26]
Enterobacter aerogenes	−	+	+	−	−	−	[38, 39, 57, 58]
Klebsiella oxytoca	+	−	−	−	−	−	[18]
Klebsiella planticola	−	+	−	−	−	−	[42]
Klebsiella pneumoniae	+	+	+	−	−	−	[11, 14–17, 34, 44, 45]
Klebsiella variicola	−	+	−	−	−	−	[48]
Klebsiella sp.	−	−	+	−	−	−	[63]
Kluyvera cryocrescens	−	+	−	−	−	−	[43]
Lactobacillus brevis	+	−	−	−	−	−	[30]
Recombinant *Escherichia coli*	+	+	+	−	−	−	[36, 40, 41, 67]
Escherichia coli	−	−	+	−	−	−	[40, 65, 66]
Thermotoga	−	−	+	−	−	−	[11]
Bacillus thruingiensis	−	−	+	+	−	−	[49, 52, 92]
Bacillus cereus	−	−	+	+	−	−	[49, 52]
Bacillus licheniformis	−	−	+	+	−	−	[49, 52]
Bacillus megaterium	−	−	+	+	−	−	[49, 52]
Bacillus sphaericus	−	−	+	+	−	−	[49, 52]
Rhodopseudomonas palustris	−	−	+	−	−	−	[62, 63]
Cupriavidus necator	−	−	−	+	−	−	[49, 85, 90]
Pseudomonas oleovorans	−	−	−	+	−	−	[87]
Pseudomonas putida	−	−	−	+	−	−	[89]
Pannonibacter phragmitetus	−	−	−	+	−	−	[91]
Zobellella denitrificans	−	−	−	+	−	−	[86]
Gluconobacter frateurii	−	−	−	−	+	−	[97]
Rhodococcus opacus	−	−	−	−	−	+	[109]
Yeast							
Pachysolen tannophilus	−	+	−	−	−	−	[47]
Saccharomyces cerevisiae	+	+	−	−	−	+	[35, 46, 107]
Cryptococcus curvatus	−	−	−	−	−	+	[103]
Rhodotorula glutinis	−	−	−	−	−	+	[104, 106]
Lipomyces lipofer	−	−	−	−	−	+	[106]
Yarrowia lipolytica	−	−	−	−	−	+	[110]
Algae							
Schizochytrium limacinum	−	−	−	−	+	+	[93, 95, 96, 99]
Chlorella protothecoides	−	−	−	−	−	+	[100, 101]
Fistulifera solaris	−	−	−	−	−	+	[102]
Fungus							
Cunninghamella echinulata	−	−	−	−	−	+	[108]

The information presented here on bioproducts is based on the works cited in this article

PDOs propanediols, *EtOH* ethanol, *PHA* polyhydroxyalkanoates, *DHA* docosahexaenoic acid

[a] Yes

[b] No

from glycerol can help in reducing emissions and also improve thermal efficiency of the fuel [142].

Opinion

A perusal of the works being carried out with glycerol as feed, provide an insight into the organisms and the bio-products which are being pursued (Table 1). On the basis of this information, Can we predict the fuel or chemical which is likely to be making significant contributions in alleviating the problem of managing this biodiesel industry waste. The answer is that *Bacillus, Clostridium*, *Enterobacter*, *Klebsiella*, *Pseudomonas* and *Saccharomyces* can be exploited for generating mixed cultures for converting glycerol to H_2, PHA, ethanol and PDOs, whereas *Cupriavidus*, *Zobellella, Schizochytrium, Gluconobacter Chlorella, Rhodotorula,* and *Yarrowia* are good options for DHA, TAGs, Lipids, and acids. It will be better to integrate different processes in a sequential manner to make best use of the feed material.

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