J Ind Microbiol Biotechnol (2012) 39:217–226
DOI 10.1007/s10295-011-1038-0

REVIEW

Microbial utilization of crude glycerol for the production of value-added products

Rosemary Dobson · Vincent Gray · Karl Rumbold

Received: 23 May 2011 / Accepted: 2 September 2011 / Published online: 25 September 2011

Abstract Energy fuels for transportation and electricity generation are mainly derived from finite and declining reserves of fossil hydrocarbons. Fossil hydrocarbons are also used to produce a wide range of organic carbon-based chemical products. The current global dependency on fossil hydrocarbons will not be environmentally or economically sustainable in the long term. Given the future pessimistic prospects regarding the complete dependency on fossil fuels, political and economic incentives to develop carbon neutral and sustainable alternatives to fossil fuels have been increasing throughout the world. For example, interest in biodiesel has undergone a revival in recent times. However, the disposal of crude glycerol contaminated with methanol, salts, and free fatty acids as a by-product of biodiesel production presents an environmental and economic challenge. Although pure glycerol can be utilized in the cosmetics, tobacco, pharmaceutical, and food industries (among others), the industrial purification of crude glycerol is not economically viable. However, crude glycerol could be used as an organic carbon substrate for the production of high-value chemicals such as 1,3-propanediol, organic acids, or polyols. Microorganisms have been employed to produce such high-value chemicals and the objective of this article is to provide an overview of studies on the utilization of crude glycerol by microorganisms for the production of economically valuable products. Glycerol as a by-product of biodiesel production could be used a feedstock for the manufacture of many products that are currently produced by the petroleum-based chemical industry.

Keywords Industrial biotechnology · Bioconversion · Crude glycerol · Biodiesel · Value-added products · Metabolic engineering

R. Dobson · V. Gray · K. Rumbold (✉)
Wits Industrial Biotechnology Consortium,
School of Molecular and Cell Biology, Faculty of Science,
University of the Witwatersrand, Johannesburg,
Johannesburg 2050, South Africa
e-mail: karl.rumbold@wits.co.za

Introduction

To date an effective use for the glycerol derived from biodiesel production does not exist. Similar to the petroleum industry, the biodiesel industry produces unwanted by-products. From every 10 l of biodiesel produced, 1 l of crude glycerol is obtained. Biodiesel producers have little incentive to purify this crude glycerol because the price of refined glycerol has decreased over the last 15 years from US $1/lb to US $0.34/lb [15]. In addition, the price of crude glycerol is continuously decreasing; for example, in the USA it has decreased from US $0.20/lb in 2001 to US $0.01/lb in 2006 [4]. Thus, a negative value will be attributed to crude glycerol in the future, which will increase the interest to use glycerol as a biological feedstock for the production of economically value-added products. As a result, utilization of glycerol in other commercial applications will enhance the economic viability and sustainability of the biodiesel industry.

Therefore, if the industry were to consider crude glycerol as a biological feedstock, the only costs relate to its transportation and not its market value. However, it is not economical to transport crude glycerol over large distances. Thus, it is important that biorefineries are set up close to biodiesel plants, as this will provide crude glycerol for bioprocessing at minimal transportation costs. Crude glycerol is available in numerous countries throughout the world,

since biodiesel is produced as a response to governments' 'green initiatives'. In 2005 (after a 295% production increase since 2000), global production of biodiesel resulted in 390 million l of crude glycerol and the biggest producers of biodiesel include Germany, France, Italy, and the USA [11].

At present, the most widely used feedstock in bioprocessing is glucose. Although the current price of glucose is comparable to that of crude glycerol (US $0.21–0.23/lb), strong price fluctuation has been observed in the last 15 years, with prices reaching US $0.40/lb at the beginning of 2010. Therefore, in comparison to crude glycerol, it can be noted that glucose prices are less stable and affected by a number of economical factors [32]. In addition, glucose is inherently connected to the ongoing 'food versus fuel' debate, which has socioeconomic implications that do not apply to crude glycerol. In a growing bio-based economy, glycerol can thus be seen as a complementary rather than a competing feedstock.

With this in mind, numerous studies have investigated the innovative use of crude glycerol in microbiologically based processes for the production of high-value products. Refinement of crude glycerol is expensive and thus it is not profitable, not only for biodiesel companies, but also for traditional glycerol-utilizing industries, such as fast moving consumer goods industries (e.g., the cosmetics industry).

Furthermore, the composition as well as the variety and concentration of impurities found in crude glycerol varies, depending on the parent feedstock used in the biodiesel production process, e.g., rapeseed, soybean, waste cooking oil, or even animal fats. These factors play an important role in what happens to the glycerol by-product. A recent study by Moon and co-workers [16] demonstrated the effect that crude glycerol, from different manufactures, has on 1,3-propanediol (1,3-PD) production in comparison to pure glycerol (Table 1).

Currently, the main three approaches that make up the focus of innovative glycerol utilization incorporate aqueous-phase reforming (APR), chemical conversion, and bioconversion [13]. Different chemical conversions of glycerol have been explored and analyzed; however, these have several disadvantages [33]. With regards to the use of chemical catalysts, e.g., for the production of 1,3-PD, disadvantages include the usual factors associated with chemical processing such as the use of high temperature and high pressures, the addition of toxic organic solvents, the production of unwanted by-products, and resulting low yields [12]. Industrial biotechnology, on the other hand, has all the advantages of the chemical processes but represent a much more sustainable option. This is because it offers an environmentally friendly and alternative approach for the development of existing and new products. Industrial biotechnology will enable energy consumption to be decreased, greenhouse gas emissions to be reduced, and higher product yields to be obtained with a resulting reduction in waste product. However, although in the past decade functional genomics tools have become available for application in the biotechnology field, their importance for industrial biotechnology has only recently become apparent.

Bioconversion

Bioconversion offers a safer and more viable alternative with the opportunity to produce a wider range of chemicals, under milder conditions [13]. For example, the biological conversion for the production of 1,3-PD from glycerol is environmentally friendly and from an economical perspective is more advantageous; this is because milder conditions are used, less energy is required, and greater yields are attainable for specific products [12].

Biocatalysts, substrates, intermediates, and the resulting product and by-products that are produced during bioconversions are biodegradable; and in most cases, water is used as a solvent. This differs greatly from synthetic chemical processes that have a high energy demand, requiring toxic chemicals and nonbiodegradable catalysts, which also result in the production of harmful waste products at the end of the process [33]. Thus, it is clearly apparent that bioconversions have several advantages over chemical conversions.

There are, however, several problems associated with biotechnological processes, when viewed from a broader perspective, most of which regard the end product; although many of these difficulties have possible solutions. Product yield and recovery are the two main aspects of bioconversion processes which are of particular interest, because they directly affect the economic viability of the product. If they can be increased and enhanced, respectively, then biotechnological processes will have increased viability and, therefore, will be industrially more appealing.

Biotechnological processes have been studied with a number of different microorganisms which have the ability to use glycerol as a carbon source for microbial bioconversions. The main chemicals that can be produced by these conversions include 1,3-PD, succinic acid, citric acid, and polyhydroxyalkanoates (PHAs) [13], while the production of other chemicals is still being explored. The chemicals produced can either be used as end products or they can be used as precursors for further processing. A main advantage is that these products are biodegradable [7]; thus bioconversion offers a way in which a nonbiodegradable feedstock can be used to create an environmentally friendly solution for the fate of crude glycerol.

Over several centuries, a wide variety of different and desirable products have been produced from the use of

Table 1 Comparison of the production parameters for the microbial utilization of glycerol

Microorganism	Product	Pure glycerol			Crude glycerol			References
		x (g dm^{-3})	P (g dm^{-3} h^{-1})	Y (g g^{-1})	x (g dm^{-3})	P (g dm^{-3} h^{-1})	Y (g g^{-1})	
Klebsiella pneumoniae ATCC 70072111	1,3-Propanediol				13.8			[18]
K. pneumoniae DSM 2026		61.90	2.00	0.49[a]	51.30[b] 53.00[b]	1.70	0.46[b] 0.47[c]	[17]
K. pneumoniae DSM 4799		51.86	0.84[e] 0.92[f]	0.50[a] 0.64	80.00[d]	1.51[e] 1.16[f]	0.67[e] 0.55[f]	[12]
Clostridium butyricum					63.40		0.69[a]	[29]
C. butyricum DSM 15410		9.70			4.10 5.80			[16]
C. butyricum DSM 2477		7.90[g] 7.90[h]			1.90[g] 2.90[h] 5.80[i] 6.20[j]	0.04[g] 0.06[h]		[16]
C. butyricum VPI 3266		29.70	2.98	0.62	30.00 31.5	3.02 3.15	0.60 0.61	[7]
C. beijerinckii NRRL B-593							0.23–0.79	[9]
Yarrowia lipolytica Wratislavia K1	Citric acid	110.00	0.66	0.44	86.00	0.05	0.43	[25]
Y. lipolytica Wratislavia AWG7		139.00	1.16	0.69	131.50 154.00*	1.05 1.05*	0.66 0.78*	[26, 27]
Y. lipolytica LGAM					35.00		0.44	[20]
Y. lipolytica NCIM 3589					77.40			[6]
Y. lipolytica 1.31		124.50	0.88	0.62	124.5	0.05	0.62	[24]
K. pneumoniae GEM 167	Ethanol	21.50 25.00[k]	0.93 0.78[k]		24.60 20.50 19.90	0.89 0.87		[19]
Kluyvera cryocrescens S26					27.00	0.61		[2]
Escherichia coli AC-521	Lactic acid	85.50	0.97	0.90[a]				[10]
C. butyricum VPI 3266				0.03[a]			0.23[l] 0.01[m]	[7]
Basfia succiniciproducens DD1	Succinic acid				5.21	0.09	1.02	[28]

P productivity, *Y* yield, *x* biomass

* 2010 study

[a] Units are mol/mol

[b] Alkali catalyzed

[c] Lipase catalyzed

[d] Highest reported

[e] 47 h fed batch

[f] 69 h fed batch

[g] *S-RWO* raw glycerol obtained from waste vegetable oil (without pretreatment)

[h] *N-RSO* raw glycerol obtained from soybean oil (without pretreatment)

[i] *S-TWO* acid pretreated S-RWO

[j] *N-TSO* acid pretreated N-RSO

[k] Gene overexpression

[l] 92% glycerol (w/v)

[m] 65% glycerol (w/v)

glycerol as a feedstock, e.g., cosmetics, paint, food, tobacco, pharmaceuticals, pulp and paper, leather, textiles, and chemicals [3]. However, in the past, there has been limited research documenting microbial conversions using glycerol as a feedstock. Therefore, the focus of this review is to explore and summarize recent studies that have used

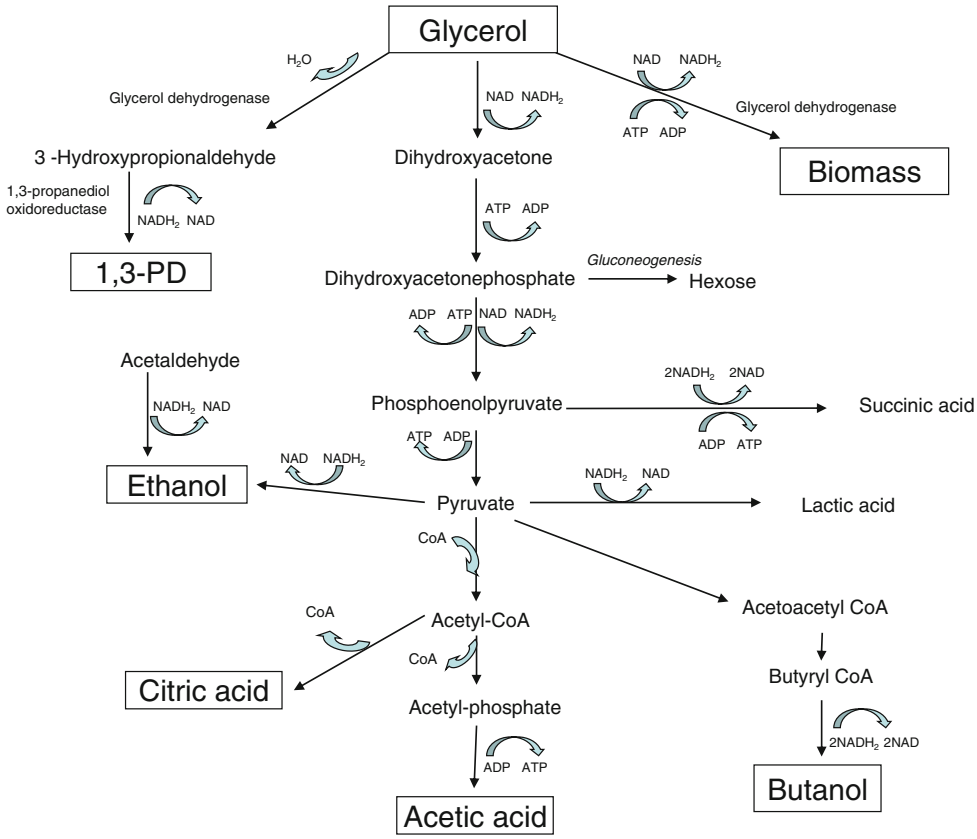

Fig. 1 Glycerol metabolism for the production of value-added products

glycerol as a feedstock in microbial conversion. With the increase in crude glycerol as a by-product of biodiesel production, the prospect to investigate and research its use in bioprocessing is likely to expand.

Crude glycerol contains several impurities, the main being alcohols, salts, heavy metals, and soaps. The composition of crude glycerol varies depending on the parent feedstock and the transesterification process. For example, depending on whether methanol or ethanol was used and the type of catalyst (sodium or potassium hydroxide), the levels of impurities will vary. The salt content can be as high as 5% (wt/wt) and the residual methanol content could be as high as 32% (wt/wt) [21]. The impact these impurities have on the bioconversion process needs to be investigated. One or more of these substances could be a problem as they may cause an inhibitory effect on microbial cells [23]. In addition, methanol is considered a hazardous waste [30]. Since it is nonbiodegradable, it is imperative that a methanol removal pretreatment system be employed to prevent crude glycerol from becoming an environmental threat. A methanol-tolerant crude glycerol bioconversion process would be advantageous.

For a bioconversion process to be successful, microorganisms which are able to tolerate these impurities are needed. Furthermore, it is an advantage if the microorganisms in any fermentation processes that uses a waste product as the main feedstock show little sensitivity to the impurities present.

Fermentative metabolism of glycerol

Glycerol is a cheap, abundant, and simple molecule, which can be taken up into the microbial cell by facilitated diffusion, and a number of microorganisms have metabolic pathways that can convert glycerol into different metabolic intermediates. This is because glycerol is found abundantly in nature in the form of triglycerides, the chemical combinations of glycerol and fatty acids.

In addition, glycerol is a highly reduced carbon source [36], which means that it can be used as a platform for the anaerobic production of chemicals of a reduced nature. A subsequent result could theoretically be higher product yields than those obtained when glucose is used in the fermentation process. Dissimilation of glycerol in microorganisms, in fermentative metabolism, is strictly linked to their ability to produce the highly reduced product 1,3-PD [36]. This process involves two pathways which are responsible for the metabolism of the carbon substrate. These pathways are widely known but not fully understood and this is a limiting factor in fermentative studies (Fig. 1).

The one way in which glycerol can be metabolized is through the oxidative pathway in which it is dehydrogenated by NAD-linked glycerol dehydrogenase to dihydroxyacetone (DHA). DHA is then phosphorylated to DHA phosphate, which can then be converted to pyruvate (glycolysis). The alternative pathway, which can occur in

parallel to the oxidative one, is a reductive route in which glycerol is dehydrated to form 3-hydroxypropionaldehyde (3-HPA). 3-HPA is then reduced to 1,3-PD in a reaction that regenerates NAD^+ [36].

The reductive pathway, on the other hand, is a necessity because of the highly reduced nature of glycerol. This pathway allows for redox balance to be maintained in the absence of electron acceptors, because the conversion of glycerol into 1,3-PD results in the net consumption of reducing equivalents [36]. Species of the genera *Propionibacterium* and *Anaerobiospirillum* have been shown to ferment glycerol independent of 1,3-PD. However, the mechanisms and pathways responsible for dissimilation of glycerol in these particular organisms have not yet been studied.

The fermentative metabolism of glycerol has been reported in a number of different species of bacteria which fall under several genera. However, since many of these microorganisms are potentially pathogenic, e.g., *Enterobacter* species [33], the potential to use them at an industrial level is restricted. Other factors which also affect their suitability for industry include the required control of anaerobic conditions, specific nutrients and supplementations, availability of genetic engineering tools, and the physiological knowledge necessary for successful metabolic engineering [14, 36].

If the total production cost were to be divided into the different components (such as energy, waste, raw material, and wages), it would be noted that the about 50% of production cost is attributed to the feedstock price [34]. Therefore, the use of crude glycerol could potentially lower the entire production cost as long as its abundance keeps the market price low.

Numerous biotechnological methods that use renewable resources (e.g., oil plants, starch plants, sugar cane, and industrial waste products such as glycerol) exist for the replacement of chemical technologies [33]. The major concerns with these methods are, however, the knowledge of the products, the costs involved, and the metabolic pathways used by the microorganism. The advantages, on the other hand, are that biotechnological advances offer the best environmentally friendly alternative to the replacement of chemical conversions for the production of value-added products.

1,3-PD

The most widely studied value-added product which can be produced from crude glycerol is 1,3-PD and as a result it has attracted much attention in the last few years. It has a great potential to be used in commercial applications for synthetic reactions [29], particularly in the plastics industry, as a monomer of polyesters, polyethers, and polyurethanes. Poly(trimethylene terephthalate) (PTT) is a new polyester, which can also be synthesized from 1,3-PD (with terephthalic acid) and has properties that facilitate its use for the manufacturing of a variety of different products, e.g., polymers, cosmetics, food, lubricants, and medicines [12]. PTT also has end uses in the fabrics industry for the manufacturing of clothing, fibers, and carpeting [9].

The plastics incorporating 1,3-PD have unique properties and a high biodegradability factor. Currently 1,3-PD is derived from acreolein, which is a harmful reagent derived from the production of petroleum fuels [7]; thus a friendlier alternative is welcomed. As a result the current production process for this monomer is not only expensive but also unsafe. These factors have resulted in several approaches aimed at developing an economically feasible process utilizing microbial production of 1,3-PD.

Bacteria which have been studied with regards to 1,3-PD production included species in the genera *Clostridium*, *Klebsiella, Citrobacter*, and *Escherichia* [26, 29]. *Clostridia* are generally regarded as safe (most are nonpathogenic) but in comparison to *Enterobacteriacaea*, they are more difficult to handle because they are obligate anaerobes. However, in terms of 1,3-PD producers, they have very similar yields and both produce acetic acid as a by-product. Another reason why *Clostridia* species are preferred is that their vitamin and nutritional supplementation are less stringent than other organisms [33]. Table 1 summarizes such studies with regards to important parameters for microbial bioconversions.

In the studies regarding 1,3-PD production from crude glycerol, *K. pneumoniae* and *C. butyricum* are the most well-studied microorganisms. The crude glycerol used has been obtained from different biodiesel manufactures (in different countries) and this gives an extra variable to consider when comparing 1,3-PD production from crude glycerol. It has been noted that depending on the material used for the transesterification process (soybean oil, rapeseed, and canola) different product yields of 1,3-PD can be obtained. The price of microbial 1,3-PD is mainly influenced by the product yield, final product concentration, and the fermentation time [34].

Over the last decade there have been several reports of the production of 1,3-PD from crude glycerol, as well as the effect that this substrate may have on the growth of microorganisms. While a number of microorganisms can use glycerol metabolically in the presence of an external electron acceptor [35], there are a limited number of species which have the ability to metabolize glycerol in the absence of an external electron acceptor, i.e., fermentative. Fermentation processes for the production of 1,3-PD usually occur under anaerobic conditions in complex media and this, therefore, limits the type of microorganism that can be

used; although, the conversion of glycerol by bacteria has also been noted under microaerobic conditions [34].

González-Pajuelo and co-workers [8] studied *Clostridium butyricum* VPI 3266 with regards to its tolerance to crude and pure glycerol. They established that *C. butyricum* demonstrates a similar tolerance to crude glycerol in comparison to pure glycerol (of similar grade); productivity and yield were also very similar and not significantly different (Table 1).

Mu and co-workers [17] studied the production of 1,3-PD from crude glycerol by *Klebsiella pneumoniae* DSM 2026, using two types of biodiesel-derived glycerol. The crude glycerol was taken from an alkali-catalyzed methanolysis of soybean oil, as well as a lipase-catalyzed process, and the resulting productivities were similar to those of pure glycerol (Table 1). This demonstrated that crude glycerol can efficiently be used as a feedstock without pretreatment.

Jun and co-workers [12] further investigated the production of 1,3-PD by *K. pneumoniae*, with the DSM 4799 strain, using crude glycerol from a biodiesel production process without pretreatment or purification. Repeated fed-batch fermentations, with immobilized cells, were used and the study concluded that this was an efficient setup for the fermentation process. 1,3-PD was produced without any inhibitory effect on the bacteria.

A unique and novel approach to the production of 1,3-PD by Gungormusler and co-workers [9] compared immobilized and suspended culture fermentation processes with *Clostridium beijerinckii* NRRL B-593. Low product yields have been reported in the literature when suspended culture systems are used and the result of this study concluded that continuous production of 1,3-PD with immobilized cells was an efficient method for the microbial conversion of crude glycerol. In order to demonstrate this, pumice stone and ceramic rings were used, in separate bioreactor systems, because their specific characteristics make them suitable for microbial colonization.

Cell immobilization has several benefits, such as increasing production reliability, and is a suitable technique that can increase productivity and shorten time for both the upstream and the downstream process [34].

Citric acid

Citric acid is an important sought-after microbial product and because of its low toxicity it is used for a number of different applications, particularly in the food and pharmaceutical industry [27]. Fungal fermentations (with sucrose or molasses) have been used in the past, with *Aspergillus niger*, for citric acid production [6]. However, alternative fermentation processes for the production of citric acid are desirable, since the demand for this value-added product is increasing annually [27]. Yeast strains of *Yarrowia lipolytica* and several *Candida* species have been preferred, in research, for an alternative fermentation process that will produce high yields of citric acid. These fermentations for citric acid have been established using batch culture fermentation, incorporating glucose, ethanol, plant oil, paraffin, and sucrose, and have been explored using wild-type, mutant, and recombinant yeast stains. If these species and fermentations are found to be effective, they may replace the traditional processes that have used filamentous fungal species.

In order to establish whether or not fermentation by *Y. lipolytica* was possible using crude glycerol as the sole carbon source, Rywińska and co-workers [26] studied citric acid production by *Y. lipolytica* Wratislavia AWG7 and *Y. lipolytica* Wratislavia K1. The results obtained from these acetate-negative mutants of *Y. lipolytica* were similar to those obtained when pure glycerol was used (Table 1). The reason for the lower ethanol concentration produced by the Wratislavia K1 strain was a consequence of the erythritol by-product in the fermentation broth [26]. By-product production is a disadvantage to the process and in order to enhance the citric acid productivity, the percentage of by-products produced needs to decrease.

One way this can be achieved is through metabolic engineering or changing the type of fermentation setup. A repeated batch operation is a more efficient mode of fermentation that has productively been employed to produce ethanol, lactic acid, and acetic acid [27].

In a more recent study, Rywińska and Rymowicz [27] used knowledge obtained when citric acid was produced using batch and fed-batch fermentation separately to establish if product yield could be increased when a repeated batch fermentation was used. Two different stains were compared (same as in their previous study [26]) to test their suitability in long-term repeated batch systems for citric acid production from crude glycerol. The study concluded that the concentration of biomass could greatly be affected by the percentage of medium replaced during the repeated batch fermentation process. An increase in citric acid could thus be produced when the amount of replaced media decreased, as this resulted in lower biomass level (Table 1); the only disadvantage was that with an increase in product came an increase in erythritol, which is an undesirable by-product.

In addition, an important factor that Rywińska and Rymowicz [27] used as an indicator for the overproduction of citric acid was the content of intracellular protein, since the literature shows that a decrease in intracellular protein content to between 17 and 24% is a sign that there is overproduction of citric acid by yeasts. In addition to other results, they concluded that *Y. lipolytica* Wratislava AWG7

was a better strain for the production of citric acid. It had decreased protein concentration (from 28 to 22%) whereas *Y. lipolytica* Wratislavia 1.31 had an increase in intracellular protein concentration (from 27 to 31%).

Further aspects that were considered to determine the stains' ability to produce citric acid from crude glycerol were the citric acid concentration and yield. *Y. lipolytica* Wratislava AWG7 achieved the best result when 40% of the culture media was replaced (Table 1), while for *Y. lipolytica* Wratislavia 1.31 the effect of replaced media did not follow the same trend and thus no particular percentage of replaced media gave better results for both the concentration and yield of citric acid for this strain.

Additional research has produced similar results in which differences occur between the strains used in the study as well as the experimental conditions employed. However, the ultimate aim is to develop a process that can use microbial conversion of crude glycerol (without pretreatment or purification) to afford value-added products that are economically feasible.

In order to compare new processes that use crude glycerol to industrial and conventional processes that use either pure glycerol or glucose as a carbon source, it is important to employ a mathematic model with defined parameters. Although predictions may not always be accurate it does allow for a means to make comparisons and hence move forward to finding the best process that can be used by industry.

An example of such a study by Papanikolaou and Aggelis [20] used a different stain of *Y. lipolytica* to model aspects of the biotechnological valorization or crude glycerol. The kinetic behavior of the microorganism was quantified and it was established that crude glycerol could be used as an alternative substrate for citric acid production. The importance of crude glycerol as a feedstock for bioconversions was established by comparing the results obtained in their study to those reported in different literature reviews.

In addition to its quantifying ability, it was suggested that the estimated parameter values can be further used to select for other strains that may have the potential to improve bioconversions from glycerol, as well as provide data for process optimization. Process optimization is one of the major factors that researchers need to take into consideration, since industrial acceptance relies on a process that can produce the largest amount of a particular product, in the least amount of time, at the lowest cost.

Ethanol

Posada and Cardona [21] reviewed and experimented with ethanol production from glycerol with particular interest in commercial prices of glycerol, purification costs of crude glycerol, and bioconversion costs for ethanol production from crude glycerol. The authors' economic assessment showed that there is a potential to use *E. coli* for the production of fuel ethanol from crude glycerol. There are several different types of feedstocks which can be used for ethanol production (e.g., sugarcane, corn starch, and cassava); however, the low cost of crude glycerol is advantageous.

Microbial fermentations have been used for the production of ethanol, using the oxidative metabolic pathway (Fig. 1) and in most of the studies pure glycerol has been used as a carbon source. The incorporation of crude glycerol as a sole carbon source has been limited in studies producing ethanol. However, Choi and co-workers [2] have identified a nonpathogenic microorganism, *Kluyvera cryocrescens*, which is able to metabolize crude glycerol to ethanol with high yield and productivity (Table 1). *Klebsiella planticola* is another organism that has been studied for its ethanol production, with ethanol levels of 30 mol l^{-1} [6].

Butanol

Taconi and co-workers [30] used glycerol for the production of butanol, which is an important biorefinery chemical. As an alternative fuel, butanol offers better physical properties in comparison to ethanol and thus its production is of particular interest [34]. Fermentative studies have researched *C. acetobutylicum* and *C. beijerinckii* species for butanol production; however, in the case of *C. acetobutylicum*, glycerol can only be metabolized in the presence of glucose [30]. Furthermore, Taconi and co-workers [30] have experimented with *C. pasteurianum* for the production of butanol (via anaerobic fermentation) from crude glycerol and found it to produce significant concentrations of ethanol, as well as 1,3-PD and ethanol. In contrast to *C. acetobutylicum*, *C. pasteurianum* has the ability to use glycerol as its sole carbon source.

In comparison to the other two *Clostridia* strains, *C. pasteurianum* produces significantly higher yield of butanol from a glycerol feedstock [30]. Although using crude glycerol results in slightly lower yields, these can be compensated for by the economic saving that is a result of pretreatment of the crude glycerol being unnecessary. Taconi and co-workers [30] found the maximum butanol yield obtained using crude glycerol to be 0.3 g/g, which is comparable to the yields obtained when *C. acetobutylicum* metabolizes glucose.

Furthermore, when *C. pasteurianum* was used in their fermentation process, acetone was not produced as a by-product; this subsequently simplifies the purification of butanol at the end of the process. The disadvantages of by-product production have already been mentioned, and in

this example the absence of acetone can be very beneficial, from both a practical and economical perspective.

Metabolic engineering

There are, however, many microorganisms which are unable to grow in the presence of glycerol but have the metabolic ability to produce important valuable products. In order for these microorganisms to be incorporated into an industrial process for the conversion of glycerol, they need to be engineered for the uptake and utilization of this particular carbon source. Several studies have initiated the metabolic engineering of microorganism for this purpose with promising results. Currently, the majority of studies have only grown recombinant species in pure glycerol. However, the knowledge obtained will provide a platform for further studies, which can be based upon integrating the use of engineered species into fermentation processes that contain crude glycerol as the sole carbon source.

Since *Clostridia* are valuable microorganisms in bioconversions, it is not surprising that they are of interest for the metabolic engineering of pathways concerning glycerol utilization. One such study introduced the 1,3-PD pathway from *C. butyricum* into *C. acetobutylicum* (which does not naturally ferment glycerol) and the result was efficient glycerol fermentation for the production of 1,3-PD [8]. Studies using *C. butyricum* along with *K. pneumoniae* have shown promising results for the conversion of crude glycerol into value-added products especially 1,3-PD.

In addition, *Corynebacterium glutamicum* has been engineered for the production of amino acids (glutamate and lysine) from pure glycerol [22]. *C. glutamicum*, a non-pathogenic, gram-positive soil bacterium, cannot metabolize glycerol but its products are desirable. Therefore, to enable glutamate production from glycerol, *C. glutamicum* was engineered with the *E. coli* genes for glycerol uptake (*glp*F), glycerol kinase (*glp*K), and glycerol dehydrogenase (*glp*D) by Rittmann and co-workers [22]. The production of lysine by a similarly engineered *C. glutamicum* lysine-producing strain (DM1730) was also considered. Their study demonstrated that the lysine yields from glycerol were comparable to the lysine yield on glucose. It was concluded that other organisms can also be engineered to grow on glycerol [using the vector they constructed (pVWEx1)]. The production of amino acids from a glycerol feedstock is a process which is likely to be integrated into a biorefinery.

It is well known that *E. coli* can utilize glycerol [5] and this has developed into a fundamental platform for microbial bioconversions using glycerol. There have been numerous studies which have documented the engineering of *E. coli* for the production of chemicals and fuels from different sugars. *E. coli* is a very important microorganism with regards to modern biotechnology and this knowledge can thus be used to create *E. coli*-based platforms for the anaerobic production of reduced chemicals from glycerol. The result will subsequently be the production of yields higher than those obtained from common sugars (e.g., glucose or xylose) [35].

A study by Yazdani and Gonzalez [36] identified the environmental conditions that are needed for the metabolic conversion of glycerol, by *E. coli*, as well as the pathways and mechanisms responsible for this process. *E. coli* was engineered for the efficient conversion of crude glycerol into ethanol. The two different stains created for the co-production of ethanol–hydrogen and ethanol–formate were SY03 and SY04, respectively; they were able to produce ethanol–hydrogen and ethanol–formate with yields that exceeded 95% of the theoretical maximum and specific rates. These conversions were superior to those of other organisms which produce the same products and the study was able to increase the attainable yield and productivity using engineered strains. The dehydrogenase (gldA) and dihydroxyacetone kinase (dhaKLM) genes, coding for the enzymes responsible for the conversion of glycerol into the metabolic intermediate dihydroxyacetone phosphate (DHAP), were simultaneously expressed. They facilitated increased rates of glycerol utilization and synthesis of the respective products [36].

Furthermore, Tang and co-workers [31] have experimented with engineered *E. coli* strains for the conversion of glycerol to 1,3-PD. Using two genes from *C. butyricum*, namely dhaB1 and dhaB2 (encoding for the vitamin B_{12}-independent glycerol dehydrogenase and its activating factor, respectively), as well as *pdu*P and *pha*C1 (propionaldehyde dehydrogenase of *Salmonella enterica* and polyhydroxyalkanoate synthase of *Ralstonia eutropha*, respectively), they effectively increased 1,3-PD yield and productivity. *E. coli* does not naturally produce 1,3-PD; therefore, for the improved expression of genes required for the 1,3-PD pathway, an artificial operon has to be constructed. Since *E. coli* presents an abundance of genetic engineering tools and it is closely related to a natural 1,3-PD producer [29], there are benefits to using this microorganism for metabolic engineering.

Although the study by Tang and co-workers [31] reported the highest yield and productivity efficiency (at the time of publication) of 1,3-PD, it did not explore the effect that crude glycerol would have on product yield. However, the high efficiency fermentation process and engineered strain are important for further studies as they provide a platform for the development of methods suitable for 1,3-PD production from more abundant renewable feedstocks, such as biodiesel-derived crude glycerol.

Escherichia coli has also been engineered for the production of poly(3-hydroxpropionate) [poly(3HP)] using a

glycerol dehydratase gene from *Clostridium butyricum* [1]. The study compared the final poly(3H) content using both pure and crude glycerol (11.98 and 5.2% [wt/wt (cell dry weight)] respectively). This demonstrates successful engineering of the poly(3HP) pathway in bacteria.

One of the main disadvantages associated with the metabolic engineering of microorganisms for the production of value-added products is that no metabolic engineering techniques are known for many of these organisms which are good producers of specific products. For example, *Citrobacter freundii*, *Enterobacteragglomerans*, and *Clostridia* have been studied for their production of 1,3-PD but no genetic engineering tools are available to enhance the production [14]. However, the future will see more investigations into the development of genetic engineering tools, because metabolic engineering is essential for the construction of strains that are able to grow on crude glycerol (obtained from the production of biofuels).

Conclusion

Various strategies have been explored for the production of value-added products, harnessing the microbial fermentation process. A progression can be seen, starting from studies that have established wild-type strains that can metabolize glycerol, to studies that have engineered these strains to improve product yield (of substances such as 1,3-PD, ethanol, and citric acid). At the same time, the metabolic engineering of organisms that are non-natural producers of these products, such as *E. coli*, have been studied. The knowledge obtained from these investigations has led to studies exploring the use of crude glycerol for the microbial production of high-value products, which has ultimately progressed to engineering stains for the use of crude glycerol.

Historically, glycerol was a high-value chemical. Compared to using other feedstock, glycerol generates more reducing equivalents that must be oxidized and this results in higher yields of reduced compounds, such as 1,3-PD, butanol, and ethanol. However, crude glycerol is not competing with other feedstocks, especially sugars, but should be considered as a complementary feedstock. It is an important biological feedstock that when produced from biodiesel production contains a number of impurities. This makes purification expensive, but bioconversions enable crude glycerol to be used as a feedstock without any pretreatment.

This important discovery will offer a great benefit to not only the biodiesel industry, but also the expansion of biorefineries and the bio-based economy as a whole. With continued research, suitable industrial processes that can use crude glycerol for fermentations may be employed for the production of high-value platforms, as well as valued end products. This will have a tremendous impact on the economic and environmental sectors. In addition, the use of suitable microorganisms for anaerobic fermentations represents a promising solution to achieve economic viability in the biofuel industry. Currently, a process that uses crude glycerol for the production of value-added products has, to our knowledge, not yet been incorporated into an industrial process.

There are a number of microorganisms that have the ability to ferment glycerol and synthesize products, with a variety of functionalities. At present *K. pneumoniae*, *C. butyricum*, and *Y. lipolytica* appear to be three of the best suited microorganisms for the conversion of glycerol to value-added products. There are various advantages for the use of glycerol as opposed to common sugars. If the synthesis of fuels and reduced chemicals is explored and achieved, then this will render higher product yields, lower operational costs, and decreased monetary investment.

References

1. Andreeßen B, Lange AB, Robenek H, Steinbüchel A (2009) Conversion of glycerol to poly(3-hydroxypropionate) in recombinant *Escherichia coli*. Appl Environ Microbiol 76:622–626
2. Choi WJ, Hartono MR, Chan WH, Yeo SS (2011) Ethanol production from biodiesel-derived crude glycerol by newly isolated *Kluyvera cryocrescens*. App Microbiol Biotechnol 89:1255–1264
3. Çelik E, Ozbay N, Oktar N, Çalik P (2008) Use of biodiesel byproduct crude glycerol as the carbon source for fermentation processes by recombinant *Pichia pastoris*. Ind Eng Chem Res 47:2985–2990
4. Dasari M (2007) Crude glycerol potential described. Feedstuffs 79:1–3
5. Dharmadi Y, Murarka A, Gonzalez R (2006) Anaerobic fermentation of glycerol by *Escherichia coli*: a new platform for metabolic engineering. Biotechnol Bioeng 94:821–829
6. Fan X, Burton R, Zhou Y (2010) Glycerol (byproduct of biodiesel production) as a source for fuels and chemicals—mini review. Open Fuels Energy Sci J 3:17–22
7. González-Pajuelo M, Andrade JC, Vasconcelos I (2004) Production of 1,3-propanediol by *Clostridium butyricum* VPI 3266 using a synthetic medium and raw glycerol. J Ind Microbiol Biotechnol 31:442–446
8. González-Pajuelo M, Meynial-Salles I, Mendes F, Andrade JC, Vasconcelos I, Soucaille P (2005) Metabolic engineering of *Clostridium acetobutylicum* for the industrial production of 1,3-propanediol from glycerol. Metab Eng 7:329–336
9. Gungormusler M, Gonen C, Azbar N (2011) Continuous production of 1,3-propanediol using raw glycerol with immobilized *Clostridium beijerinckii* NRRL B-593 in comparison to suspended culture. Bioprocess Biosyst Eng. doi: 10.1007/s00449-011-0522-2
10. Hong A, Cheng K, Peng F, Zhou S, Sun Y, Lui C, Lui D (2009) Strain isolation and optimization of process parameters for bioconversion of glycerol to lactic acid. J Chem Technol Biotechnol 84:1576–1581
11. International Energy Agency (2007) Energy technology essentials, biofuel production. http://www.iea.org/techno/essentials.htm. Accessed 22 August 2011

12. Jun S, Moon C, Kang C, Kong S, Snag B, Um Y (2010) Microbial fed-batch production of 1,3-propanediol production using raw glycerol with suspended and immobilized *Klebsiella pneumoniae*. Appl Biochem Biotechnol 161:491–501
13. Koutinas AA, Wang R, Webb C (2007) The biochemurgist—bioconversion of agricultural raw materials for chemical production. Biofuels Bioprod Bioref 1:24–38. doi:10.1002/bbb.6
14. Maervoet VET, De Mey M, Beauprez J, De Maeseneire S, Soetaert WK (2011) Enhancing the microbial conversion of glycerol to 1,3-propanediol using metabolic engineering. Org Process Res Dev 15:189–202
15. Min YN, Yan F, Liu FZ, Coto C, Waldroup PW (2011) Glycerine—a new energy source for poultry. Int J Poult Sci 9:1–4
16. Moon C, Ahn J, Kim S, Sang B, Um Y (2010) Effect of biodiesel-derived raw glycerol on 1,3-propanediol production by different microorganisms. Appl Biochem Biotechnol 161:502–510
17. Mu Y, Teng H, Zhang DJ, Wang W, Xiu ZL (2006) Microbial production of 1,3-propanediol by *Klebsiella pneumoniae* using crude glycerol from biodiesel preparations. Biotechnol Lett 28:1755–1759
18. Oh B, Seo J, Choi MH, Kim CH (2008) Optimization of culture conditions for 1,3-propanediol production from crude glycerol by *Klebsiella pneumoniae* using response surface methodology. Biotechnol Bioprocess Eng 13:666–670
19. Oh B, Seo J, Heo S, Hong W, Luo L, Joe M, Park D, Kim C (2011) Efficient production of ethanol from crude glycerol by a *Klebsiella pneumoniae* mutant strain. Bioresour Technol 102:3918–3922
20. Papanikolaou S, Aggelis G (2003) Modelling aspects of the biotechnological valorization of raw glycerol: production of citric acid by *Yarrowia lipolytica* and 1,3-propanediol by *Clostridium butyricum*. J Chem Technol Biotechnol 78:542–547
21. Posada JA, Cardona CA (2010) Design and analysis of fuel ethanol production from raw glycerol. Energy 35:5286–5293
22. Rittmann D, Lindner SN, Wendisch VF (2008) Engineering of a glycerol utilization pathway for amino acid production by *Corynebacterium glutamicum*. Appl Environ Microbiol 74:6216–6222
23. Rumbold K, van Buijsen HJ, Overkamp KM, van Groenstijn JW, Punt PJ, van der Werf MJ (2009) Microbial production host selection for converting second-generation feedstocks into bioproducts. Microb Cell Fact 8:64
24. Rymowicz W, Rywińska A, Żarowska B, Juszczyk P (2006) Citric acid production from raw glycerol by acetate mutants of *Yarrowia lipolytica*. Chem Pap 60:391–394
25. Rymowicz W, Rywińska A, Goldkowski W (2008) Simultaneous production of citric acid and erythritol from crude glycerol by *Yarrowia lipolytica*. Chem Pap 62:239–246
26. Rymowicz W, Rywińska A, Żarowska B, Wojtatowicz M (2009) Biosynthesis of citric acid from glycerol by acetate mutants of *Yarrowia lipolytica* in fed-batch fermentation. Food Technol Biotechnol 47:1–6
27. Rywińska A, Rymowicz W (2010) High-yield production of citric acid by *Yarrowia lipolytica* on glycerol in repeated-batch biorectors. J Ind Microbiol Biotechnol 37:431–435
28. Scholten E, Renz T, Thomas J (2009) Continuous cultivation approach for fermentative succinic acid production from crude glycerol by *Basfia succiniciproducens* DD1. Biotechnol Lett 31:1947–1951
29. Saxena RK, Anand P, Saran S, Isar J (2009) Microbial production of 1,3-propanediol: recent developments and emerging opportunities. Biotechnol Adv 27:895–913
30. Taconi KA, Venkataramanan KP, Johnson DT (2009) Growth and solvent production by *Clostridium pasteurianum* ATCC® 6013™ utilizing biodiesel-derived crude glycerol as the sole carbon source. Environ Prog Sustain Energy 28:100–110. doi:10.1002/ep.10350
31. Tang X, Tan Y, Zhu H, Zhao K, Shen W (2009) Microbial conversion of glycerol to 1,3-propanediol by an engineered strain of *Escherichia coli*. Appl Environ Microbiol 75:1628–1634
32. United States Department of Agriculture (2010) Sugar: world markets and trade. http://usda.mannlib.cornell.edu/usda/current/sugar/sugar-05-23-2011.pdf. Accessed 22 August 2011
33. Willke T, Vorlop K (2004) Industrial bioconversion of renewable resources as an alternative to conventional chemistry. Appl Microbiol Biotechnol 66:131–142
34. Willke T, Vorlop K (2008) Biotransformation of glycerol into 1,3-propanediol. Eur J Lipid Sci Technol 110:831–840
35. Yazdani SS, Gonzalez R (2007) Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. Curr Opin Biotechnol 18:213–219
36. Yazdani SS, Gonzalez R (2008) Engineering *Escherichia coli* for the efficient conversion of glycerol to ethanol and co-products. Metab Eng 10:340–351