GEORGE AGGELIS
EDITOR

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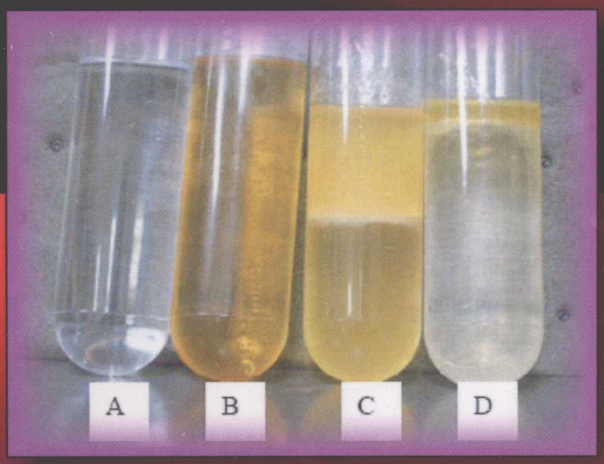
MICROBIAL CONVERSIONS OF RAW GLYCEROL



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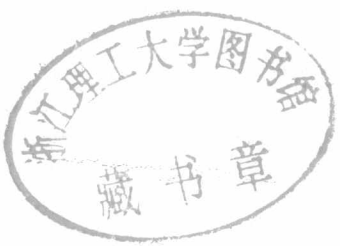

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MICROBIAL CONVERSIONS OF RAW GLYCEROL

Preface

Glycerol is a simple carbon source than can be easy assimilated by numerous eukaryotic and prokaryotic microorganisms. However, this compound had been neglected as substrate for microbial fermentations for many years due mainly to its high cost. Nevertheless, recent developments in the fuel market, led to the production in large scale of biodiesel derived from vegetable oil, reversed this situation. Currently glycerol is available in large quantities in the biodiesel production units. This industry produces enormous quantities of glycerol as a by-product, during transesterification process of the oil with methanol or ethanol. Therefore, glycerol turns into an attractive feedstock.

Biotechnology provides a broad range of methods for the valorisation of raw glycerol. Important commodities and high-added value products of industrial interest, such as organic acids, bioplastics, polyunsaturated fatty acids, carotenoids etc could be produced from raw glycerol. Glycerol can also be recycled after its conversion into microbial triacylglycerols and then to biodiesel.

In this book, the biochemical pathways of glycerol metabolism in prokaryotic and eukaryotic cells cultivated under various conditions are discussed. The various methods, proposed so far in the international literature for the valorisation of glycerol by biotechnological means, are shown and discussed by using scientific and technological criteria. Evaluation of the economic viability of the 1,3-propanediol production processes is also presented. All chapters (research articles and reviews) were reviewed by experts.

Although the examples presented are, doubtless, the more important, there are also a lot of other applications based on raw glycerol.

Chapter I - Crude glycerol represents the major byproduct of the biodiesel industry. Currently, biodiesel production in the United States is experiencing a rapid expansion; as a result, the market is being flooded with excess crude glycerol. Due to various impurities contained in the crude glycerol, it is not cost-effective to purify this waste stream for use in the food, pharmaceutical, or cosmetics industries. Various alternative methods are being developed for utilizing this crude glycerol. If this waste stream can be utilized economically, the biodiesel production process will become more profitable and more prevalent.

Chapter II - The catabolic glycerol pathways have long been elucidated, and the regulatory properties of the enzymes involved in the major pathways have been studied in some detail. The advent of molecular biology allowed for the identification and

characterization of the genes coding for the enzymes of the major catabolic pathways. Characterization of the glycerol genes, still in its infancy, produced rather confusing results and thus many of these findings are subject to revision.

Chapter III - The effects of different kinds of raw glycerol on yeast growth and citric acid biosynthesis in fed-batch cultures were studied. The following types of raw glycerol were used: purified and un-purified raw glycerol from rape seed methyl ester production and un-purified raw glycerol from ethyl ester production. All of them were a very good carbon and energy source for citric acid production by the acetate mutant strain of *Y. lipolytica* Wratislavia 1.31. Salt and other impurities in raw glycerol slightly influenced the production of citric acid. The highest citric acid concentration (146 g/L) was obtained with purified glycerol from methyl ester production during a fed-batch culture lasting 148 h. In this process, the yield of citric acid (0.73 g/g) was also the highest.

Chapter IV - The aim of the studies was to evaluate the dynamics and yield of oxalic acid production from biodiesel by-products, such as pure glycerol, fatty acids and glycerin waste (a mixture of glycerol and fatty acids) by *Aspergillus niger* XP in submerged cultivations. The comparative studies included: product yields, volumetric productivity and concentration of citric acid (an unwanted by-product). The maximum concentration of oxalic acid (55.7 gdm^{-3}) was obtained in the medium containing 50 gdm^{-3} of fatty acids, which also resulted in the highest oxalate yield and volumetric productivity (1.25 gg^{-1} and 0.29 $gdm^{-3}h^{-1}$, respectively). In contrast, the concentration of citric acid was very low (<1 gdm^{-3}). When the medium contained 65 gdm^{-3} of glycerin waste, *A. niger* XP produced only 42.0 gdm^{-3} of oxalic acid (without undesired by-products) at oxalate yield of 0.8 gg^{-1}. The lowest oxalic acid concentrations were obtained in the medium containing pure glycerol.

Chapter V - Crude glycerol represents the major byproduct of the biodiesel industry. As biodiesel production skyrockets, the market is being flooded with excess crude glycerol. Producing the omega-3 polyunsaturated fatty acids docosahexaenoic acid (DHA, C22:6, ω-3) and eicosapentaenoic acid (EPA, C20:5, ω-3) provides an opportunity to utilize this under-valued material. With many therapeutic benefits, DHA and EPA have been used as supplements in various human foods or animal feeds. Fish oil as the main source of DHA/EPA has several limitations such as undesirable taste and odor, heavy metal contamination, and potential shortage due to overfishing; thus, it is necessary to seek alternative sources to produce these two fatty acids. Our laboratory has been developing a microbial fermentation process to produce DHA and EPA from crude glycerol. The microalga *Schizochytrium limacinum* was used as DHA producer; the fungus *Pythium irregulare* as EPA producer. It was found that the major impurities contained in crude glycerol, methanol and soap, were inhibitory to algal/fungal culture, but they can be easily removed from the crude glycerol medium. The culture conditions, including medium composition and culture temperature, for *S. limacinum* and *P. irregulare* were optimized in flask cultures, with a high DHA yield of 4.91 g/L from S. limacinum and an EPA yield of 182 mg/L from P. irregulare. Overall, the works presented in this chapter show that biodiesel-derived crude glycerol can serve as a good carbon source for microbial production of omega-3 fatty acids. Future research should focus on (1) elucidating the mechanisms of the inhibitory effects of soap on algal/fungal growth, (2) controlling fungal morphology to create

the desired pellet form, (3) developing optimal fermenter cultures of the two species, and (4) implementing the process on a large scale.

Chapter VI - A wide variety of bacteria are able to synthesize polyhydroxyalkanoates (PHA) as an intracellular storage compound. The best-known and most common representative *one* is poly (3-hydroxybutyrate) (PHB). The properties of these isolated biodegradable polymers are very similar to polypropylene. As a rule their synthesis takes place, if the multiplication is limited by nutrients other than the carbon source. Depending on the metabolism of the involved bacteria, quite different carbon substrates can be used; the most common ones are sugars. In contrast to petrochemically produced plastics PHA can be produced from renewable carbon resources, which become more and more important in respect to the conservation of finite fossil resources like mineral oil and coal and their largely neutrality with regard to the emission of CO2.

The authors could show that crude glycerol, a by-product of the biodiesel production from rape, is a promising feedstock for the production of PHB. By co-feeding of appropriate precursors we also could produce copolymers of poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) with co-monomer contents of 12-25 mol% resulting in improved polymer properties. For the isolation of the PHA we used an enzymatic method. The polymer composition was not changed during this procedure. Applying an additional mechanical disruption of the bacteria by French press, the use of the expensive enzyme lysozyme could be minimized or omitted. The molecular weight of the isolated polymer (700.000 – 900.000 g/mol) is sufficiently high, to be processed by common methods of polymer industry.

Chapter VII - The oleaginous Zygomycete *Thamnidium elegans* was grown on raw glycerol producing high amounts of single cell oil (SCO) rich in gamma-linolenic acid (GLA). Produced biomass contained more than 40% w/w lipid, which contained 7.3% (w/w) GLA, giving a GLA yield of 664.3 mg/l GLA. This yield indicates that raw glycerol is an efficient substrate for SCO production. Lipid analysis showed that at the beginning of growth the mycelium was rich in phospholipids and glycolipids plus sphingolipids, while neutral lipids accumulated as growth proceeded. Fatty acid analysis of the major lipid fractions revealed that phospholipids were rich in linoleic acid and GLA, while the other two fractions had similar fatty acid composition.

Chapter VIII - Importance and fermentation technologies for the production of food-grade carotenoids by fungi are presented with focus on raw glycerol employment. Information about fungal growth, substrate assimilation and carotenoid production parameters such as yield, selectivity, productivity and process economics are detailed. Toxicological aspects of the raw glycerol are also discussed.

Chapter IX - The conversion of glycerin waste from ethyl ester biodiesel production into a protein source for animal feed was examined. In the study, six yeast strains were used for biomass production: *Yarrowia lipolytica* ATCC 8661, *Y. lipolytica* ATCC 8661 UV'1, *Y. lipolytica* A-101, *Y. lipolytica* Z, *Candida robusta* ATCC 60 559, and *C. utilis* ATCC 60 558. The glycerin waste, a main by-product of biodiesel industry, contained (w/w) 45% of raw glycerol, 44% of fatty acids, 3–4% of ethyl esters, and large quantities of potassium soaps. Since the pH of the substrate was 9.6, glycerin waste was added batchwise during yeast growth, in order to prevent the pH value from exceeding 4.0. All experiments were conducted in a stirred tank reactor, on media containing 30 g/L of glycerin waste as a substrate.

The results of the experiments have shown that the strain *Y. lipolytica* 8661 UV'1 is the most suitable for biomass production from this substrate. During batch cultivation, the strain simultaneously utilized glycerol and fatty acids. The biomass yield and biomass production rate obtained with this strain were the highest at 0.89 g/g and 2.7 g/Lh, respectively.

Protein concentration in the biomass varied from 26.5 to 36.5% (w/w), depending on the yeast strain used. The content of essential amino acids was in compliance with the FAO/WHO standards for fodder yeast, valine and isoleucine occurring in the yeast biomass in higher quantities. The nutritional value of the yeast obtained in a submerged culture on glycerin waste ranged between 64 and 70% according to Oser's Essential Amino Acid Index (EAAI). Analysis of technological parameters (biomass yields, volumetric biomass production rate and protein content has also revealed that the strain *Y. lipolytica* ATCC 8661 UV'1 is the most efficient biomass producer from glycerin waste. The results are very promising, as these findings may lead to a low-cost process of fodder yeast biosynthesis from a waste generated during biodiesel production.

Chapter X - The ongoing energy crisis has resulted in increasing demands for renewable fuels in the market, and this has as an inevitable effect bio-diesel production. This situation will soon lead to the accumulation of tremendous quantities of crude – impure glycerol in every country utilizing bio-diesel. Therefore, glycerol valorization should have much to offer in the cost reduction of the overall bio-diesel production process. The most important studies that are related with the conversion of (crude) glycerol into higher added-value chemical compounds are referred to its conversion into 1,3-propanediol, a substance of noticeable importance for the chemical and the textile industry. This conversion is carried out with the aid of various prokaryotic microorganisms (principally strains belonging to the family Enterobacteriaceae, to the lactic acid group and to the genus Clostridium sp.) principally under anaerobic conditions. This chapter presents a brief survey of studies that have been carried out by various research teams (including our team – Department of Food Science and Technology of the Agricultural University of Athens and Department of Biology, Division of Genetics, Cell and Development Biology of the University of Patras) in relation with the assimilation of glycerol by bacterial strains and its conversion into 1,3-propanediol. To this end, physiological approaches related with the anaerobic assimilation of glycerol, biochemical aspects related with the biosynthesis of 1,3-propanediol and biotechnological aspects concerning the feasibility of the process in various fermentation configurations will be considered and discussed.

Chapter XI - The aim of this chapter is to present the calculations of the capital cost and the total manufacturing cost of a bioprocess that is used to convert raw glycerol to 1,3-propanediol. To this end a representative process flow diagram is developed based on well known heuristics. Data from the literature are used to solve the material and energy balances. The process equipment is then designed and the fixed capital cost estimated. Finally, the total manufacturing cost is estimated. The results of the economic analysis are particularly helpful in identifying research direction that will improve process economics and can contribute in transforming the results of fundamental research into successful industrial projects.

Contents

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Chapter I

Glycerol Waste from Biodiesel Manufacturing

Zhiyou Wen[1,2], *Denver J. Pyle*[1] *and Sneha K. Athalye*[1]
[1] Department of Biological Systems Engineering,
[2] Institute for Critical Technology and Applied Science,
Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

Abstract

Crude glycerol represents the major byproduct of the biodiesel industry. Currently, biodiesel production in the United States is experiencing a rapid expansion; as a result, the market is being flooded with excess crude glycerol. Due to various impurities contained in the crude glycerol, it is not cost-effective to purify this waste stream for use in the food, pharmaceutical, or cosmetics industries. Various alternative methods are being developed for utilizing this crude glycerol. If this waste stream can be utilized economically, the biodiesel production process will become more profitable and more prevalent.

1. Introduction

As oil prices reach historical highs and public support for alternative fuels broadens, biodiesel as a renewable energy source has attracted increasing attention. In general, for every 100 pounds of biodiesel produced, approximately 10 pounds of crude glycerol are created. Because this glycerol is expensive to purify for use in the food, pharmaceutical, or cosmetics industries, biodiesel producers must seek alternative methods for its disposal. Various methods for disposal and utilization of this crude glycerol have been attempted, including combustion, composting, anaerobic digestion, animal feeds, and thermochemical/biological conversions to value-added products. The objective of this chapter

is to provide a general background in terms of biodiesel production and waste glycerol utilization.

2. Current Status of Biodiesel Production

Biodiesel production has increased tremendously in last few years. Taking the United States as an example, the National Biodiesel Board has projected the annual U.S. biodiesel production in 2007 as 450 million gallons, a sharp increase from less than 100 million gallons prior to 2005 (NBB, 2008). On December 19, 2007, the Energy Independence and Security Act was signed into law in the U.S. Under this legislation, annual biodiesel production is supposed to increase to 1 billion gallons in 2012 (RFA 2008). Although this increment is relatively small compared to that of cellulosic ethanol, the absolute amount of biodiesel production will be increased significantly. As biodiesel production skyrockets, the market is being flooded with crude glycerol. Crude glycerol prices have dropped from 25 cents/lb in 2004 to 2.5-5 cents/lb in 2006 (Johnson and Taconi, 2007; Yazdani and Gonzalez, 2007) because the current U.S. demand for glycerol is not large enough for all of this crude glycerol. It is clear that new uses for this byproduct are needed.

3. How Biodiesel is Made

Biodiesel is made through a catalyzed transesterification between oils or fats (triglycerides) and an alcohol (usually methanol) (Figure 1). Common feedstocks are pure vegetable oil (e.g., soybean, canola, sunflower), rendered animal fats, or waste vegetable oils. The theoretical ratio of methanol to triglyceride is 3:1; which corresponds to having one methanol molecule for each of the three hydrocarbon chains present in the triglyceride molecule, and is equivalent to approximately 12% methanol by volume. In practice, this ratio needs to be higher in order to drive the reaction towards a maximum biodiesel yield; 25% methanol by volume is recommended.

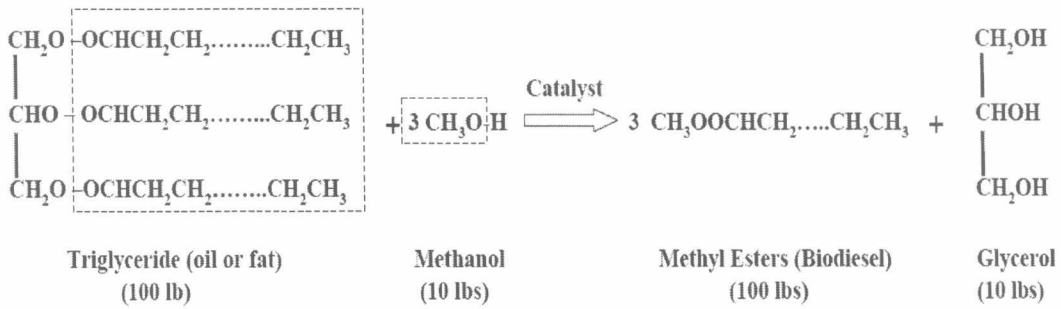


Figure 1. Transesterification reaction to produce biodiesel.

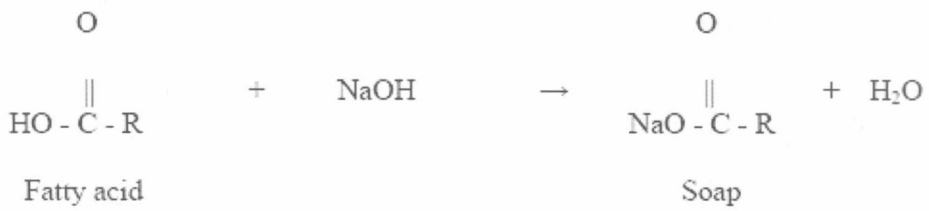
The catalyst can be alkalis, acids, or enzymes (e.g., lipase). The majority of biodiesel produced today is made using an alkali (such as NaOH or KOH) catalyzed reaction because

this reaction (1) requires only low temperature and pressure, (2) has a high conversion yield (98%) with minimal side reactions and a short reaction time, (3) is a direct conversion to biodiesel with no intermediate compounds, and (4) does not require specific construction materials. The glycerol backbone of the triglyceride remains as a waste product after the reaction is completed.

4. Characterizations of Glycerol Waste

Crude glycerol generated from biodiesel production is impure and of little economic value. In general, glycerol makes up 60% to 85% (w/w) of the crude stream (Gonzalez-Pajuelo et. al., 2005; Mu et. al., 2006, Pyle et. al., 2008). The wide range of the purity values can be attributed to different glycerol purification methods or different feedstocks used by biodiesel producers. For example, Thompson and He (2006) have characterized the glycerol produced from various biodiesel feedstocks. The authors found that mustard seed generated a lower level (62%) of glycerol, while soy oil had 67.8 % glycerol, and waste vegetable oil had the highest level (76.6 %) of glycerol.

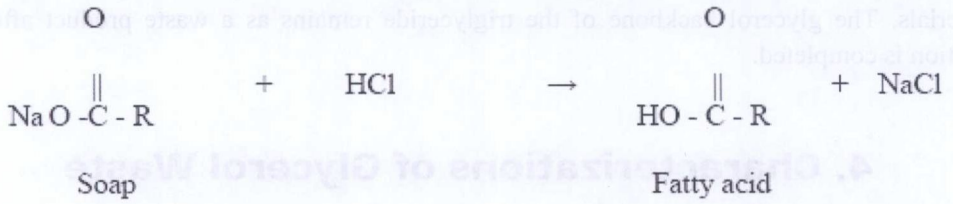
Methanol and free fatty acids (soaps) are the two major impurities contained in crude glycerol (Thompson and He, 2006). The existence of methanol is due to the fact that biodiesel producers use excess methanol to drive the chemical transesterification and do not recover all the methanol. The soaps, which are soluble in the glycerol layer, originate from a reaction between the free fatty acids present in the initial feedstock and the catalyst (base).i.e.,



In addition to methanol and soaps, crude glycerol also contains a variety of elements such as calcium, magnesium, phosphorous, or sulfur. Thompson and He (2006) reported that the elements present in the glycerol of different feedstock sources (such as canola, rapeseed, and soybean) were similar. Calcium was in the range of 3-15 ppm, magnesium was 1-2 ppm, phosphorous was 8-13 ppm, and sulfur was 22-26 ppm. However, when crambe (a perennial oilseed plant) was used as feedstock, crude glycerol contained the same elements, but at vastly different concentrations (Thompson and He, 2006). Schröder and Südekum (1999) also reported the elemental composition of crude glycerol from rapeseed oil feedstock. Phosphorous was found to be between 1.05 % and 2.36 % (w/w) of the crude glycerol. Potassium was between 2.20 % and 2.33%, while sodium was between 0.09% and 0.11%. Cadmium, mercury, and arsenic were all below detectable limits.

The crude glycerol derived from alkali-catalyzed transesterification usually has a dark brown color with a high pH (11-12). When used in microbial fermentations, crude glycerol is

dissolved in the medium solution and the pH is usually adjusted to a neutral range. Under this condition, soaps will be converted into free fatty acids, as shown in the following equation



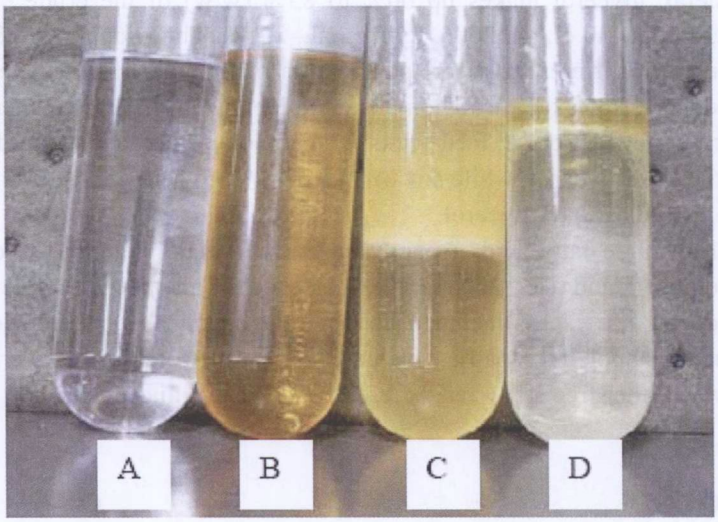


Figure 2. Appearance of different glycerol streams. A: pure glycerol; B: raw crude glycerol with pH at 12; C: crude glycerol after pH adjusted to 7.0; D: crude glycerol after pH adjusted to 7.0 and phase separation by centrifugation.

After pH adjustment, the free fatty acids in the crude glycerol stream resulted in a cloudy solution. After centrifugation, this cloudy solution will be separated into two clear phases, with the top layer being the free fatty acid phase, and bottom layer the glycerol phase. Figure 2 shows the appearance of pure glycerol, raw crude glycerol (with pH 12), crude glycerol after pH adjustment (pH 7), and crude glycerol after pH adjustment and phase separation by centrifugation.

5. Utilization of Glycerol Waste

As a waste material, crude glycerol can be utilized through a variety of methods such as combustion (Johnson and Taconi, 2007), composting, or anaerobic digestion (Holm-Nielsen et. al., 2008). Crude glycerol has also been used as a feed additive for various animals such as pigs (Lammers et. al., 2008b), broiler chickens (Cerrate et. al., 2006), and laying hens

(Lammers et. al., 2008a). It was found that the metabolizable to digestible energy ratio of glycerol is similar to that of corn or soybean oil when fed to pigs (Lammers et. al., 2008b). Birds fed 2.5 % to 5% glycerol-diets had higher breast yield than the control group (Cerrate et. al., 2006). Crude glycerol has also been used to feed dairy cows in order to prevent ketosis, but the result was not positive (DeFrain et. al., 2004).

Converting crude glycerol into valued-added products through thermo-chemical methods or biological methods is an alternative for utilizing this waste stream. It has been reported that glycerol can be thermochemically converted into propylene glycol (Alhanash et. al., 2008; Dasari et. al., 2005), acetol (Chiu et. al., 2006), or a variety of other products (Johnson and Taconi 2007). Cortright et al. (2002) have developed an aqueous phase reforming process that transforms glycerol into hydrogen. Virent Energy Systems is currently trying to commercialize this technology and claim that sodium hydroxide, methanol, and high pH levels within crude glycerol help the process (Nilles 2005). For biological conversions of crude glycerol, the glycerol serves as a feedstock in various fermentation processes. For example, Lee et al. (2001) have used glycerol in the fermentation of *Anaerobiospirillum succiniciproducens* for the production of succinic acid. The fermentation of *E. coli* on glycerol leads to the production of a mixture of ethanol, succinate, acetate, lactate, and hydrogen (Dharmadi et. al., 2006). Glycerol can also be converted to citric acid by the yeast *Yarrowia lipolytica*. It has been reported that this organism produces the same amount of citric acid when grown on glucose or on raw glycerol (Papanikolaou et. al., 2002). Rymowicz et al. (2006) found that acetate mutant strains of *Y. lipolytica* can produce high levels of citric acid while producing very little isocitrate. Furthermore, it has been shown that *Clostridium butyricum* can utilize biodiesel-derived glycerol to produce 1,3-propanediol (an important chemical building block with many industrial uses) in both batch and continuous cultures. During the fermentation process, the organism also produces byproducts of acetic and butyric acid (Papanikolaou et. al., 2004).

6. Conclusion

Because glycerol is the major byproduct of the biodiesel manufacturing process, the disposal of crude glycerol has been a major issue faced by biodiesel producers. Research has shown that biodiesel-derived crude glycerol can be utilized to form a multitude of products. There are several thermochemical and biological methods that have been investigated as possible value-added outlets for this currently under-utilized and under-valued byproduct.

References

Alhanash, A., Kozhevnikova, E.F. and Kozhevnikov, I.V. (2008). Hydrogenolysis of glycerol to propanediol over Ru: Polyoxometalate bifunctional catalyst. *Catalysis Letters, 120,* 307-311.

Cerrate, S., Yan, F., Wang, Z., Coto, C., Sacakli, P. and Waldroupand, P.W. (2006). Evaluation of glycerine from biodiesel production as a feed ingredient for broilers. *International Journal of Poultry Science, 5,* 1001-1007.

Chiu, C.W., Dasari, M.A., Sutterlin, W.R. and Suppes, G.J. (2006). Removal of residual catalyst from simulated biodiesel's crude glycerol for glycerol hydrogenolysis to propylene glycol. *Industrial and Engineering Chemistry Research, 45,* 791-795.

Cortright, R.D., Davda, R.R. and Dumesic, J.A. (2002). Hydrogen from catalytic reforming of biomass-derived hydrocarbons in liquid water. *Nature, 418,* 964-967.

Dasari, M.A., Kiatsimkul, P.P., Sutterlin, W.R. and Suppes, G.J. (2005). Low-pressure hydrogenolysis of glycerol to propylene glycol. *Applied Catalysis a-General 281,* 225-231.

DeFrain, J.M., Hippen, A.R., Kalscheur, K.F. and Jardon, P.W. (2004). Feeding glycerol to transition dairy cows: Effects on blood metabolites and lactation performance. *Journal of Dairy Science, 87,* 4195-4206.

Dharmadi, Y., Murarka, A. and Gonzalez, R. (2006). Anaerobic fermentation of glycerol by Escherichia coli: A new platform for metabolic engineering. *Biotechnology and Bioengineering, 94,* 821-829.

Gonzalez-Pajuelo, M., Meynial-Salles, I., Mendes, F., Andrade, J.C., Vasconcelos, I. and Soucaille, P. (2005). Metabolic engineering of *Clostridium acetobutylicum* for the industrial production of 1,3-propanediol from glycerol. *Metabolic Engineering, 7,* 329-336.

Holm-Nielsen, J.B., Lomborg, J., Oleskowicz-Popiel, P. and Esbensen, K.H. (2008). On-line near infrared monitoring of glycerol-boosted anaerobic digestion processes: Evaluation of process analytical technologies. *Biotechnology and Bioengineering, 99,* 302-313.

Johnson, D.T. and Taconi, K.A. (2007). The glycerin glut: Options for the value-added conversion of crude glycerol resulting from biodiesel production. *Environmental Progress, 26,* 338-348.

Lammers, P.J., Kerr, B.J., Honeyman, M.S., Stalder, K., Dozier, W.A., Weber, T.E., Kidd, M.T. and Bregendahl, K. (2008a). Nitrogen-corrected apparent metabolizable energy value of crude glycerol for laying hens. *Poultry Science, 87,* 104-107.

Lammers, P.J., Kerr, B.J., Weber, T.E., Dozier, W.A., Kidd, M.T., Bregendahl, K. and Honeyman, M.S. (2008b). Digestible and metabolizable energy of crude glycerol for growing pigs. *Journal of Animal Science, 86,* 602-608.

Lee, P.C., Lee, W.G., Lee, S.Y. and Chang, H.N. (2001). Succinic acid production with reduced by-product formation in the fermentation of Anaerobiospirillum succiniciproducens using glycerol as a carbon source. *Biotechnology and Bioengineering, 72,* 41-48.

Mu, Y., Teng, H., Zhang, D.J., Wang, W. and Xiu, Z.L. (2006). Microbial production of 1,3-propanediol by Klebsiella pneumoniae using crude glycerol from biodiesel preparations. *Biotechnology Letters, 28,* 1755-1759.

NBB. (2008). National Biodiesel Board. Available from: http://www.biodiesel.org/pdf_files/fuelfactsheets/Production_Graph_Slide.pdf. (Accessed on Feburary 11, 2009)

Nilles, D. (2005). A glycerin factor. Biodiesel Magazine August/September. Grand Forks, ND: BBI International.

Papanikolaou, S., Muniglia, L., Chevalot, I., Aggelis, G. and Marc I. (2002) *Yarrowai lipolytica* as a potential producer of citric acid from raw glycerol. *Journal of Applied Microbiology*, 92, 737-744.

Papanikolaou, S., Fick M. and Aggelis., G. (2004). The effect of raw glycerol concentration on the production of 1,3-propanediol by *Clostridium butyricum*. *Journal of Chemical Technology and Biotechnology*, *79*, 1189-1196.

Pyle, D. Garcia, R. and Wen, Z. (2008). Producing docosahexaenoic acid (DHA)-rich algae from biodiesel-derived crude glycerol: effects of impurities on DHA production and algal biomass composition. *Journal of Agricultural and Food Chemcistry, 56,* 3933-3939.

RFA. (2008). Renewable Fuels Association. Available from http://www.ethanolrfa.org/resource/standard/ (Accessed on Feburary 11, 2009)

Rymowicz, W., Rywińska, A., Żarowska B. and Juszczyk P. (2006). Citric acid production from raw glycerol by acetate mutants of *Yarrowia lipolytica*. *Chemical Papers*, 60, 301-304.

Schröder, A. and Südekum, K.H. (1999). Glycerol as a by-product of biodiesel production in diets for ruminants. In: *Proceedings of the 10th International Rapeseed Conference*. Canberra, Australia: The Regional Institute, Ltd.

Thompson, J.C. and He, B.B. (2006). Characterization of crude glycerol from biodiesel production from multiple feedstocks. *Applied Engineering in Agriculture, 22,* 261-265.

Yazdani, S.S. and Gonzalez, R. (2007). Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. *Current Opinion in Biotechnology, 18,* 213-219.