

PRODUCTION VALUE-ADDED PRODUCTS FROM BIODIESEL DERIVED CRUDE GLYCEROL

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ABSTRACT: Crude glycerol, a by-product of biodiesel production which contain impurities such as methanol, triglycerides and catalysts, would represent a huge challenge to the environment if disposed. Thus the best opportunity is to convert crude glycerol into valuable products. While a number of microorganisms are able to utilize glycerol as a sole carbon source to yield a range of organic acids and alcohols, the fermentation process has not been optimized. The main objective of the study was to evaluate the application of ultrasound to enhance the production of value-added acids and alcohols by anaerobic fermentation of crude glycerol. By a method of screening, *Clostridium diolis* was selected and used in batch fermentation experiments at 37 °C, pH of 6.8 - 7 and 150 rpm on anaerobic environment by N₂ sparging. Prior to each experiment, crude glycerol was washed three (3) time with diethyl ether and a series of glycerol concentrations (50-150 g/L) was fermented for 48 hours with samples taken every 3 hours for analyses. The high-performance liquid chromatography (HPLC) and a light microscopy were used to analyze samples before and after the fermentation process. Interestingly, very low amounts of methanol and free fatty acids were observed in the washed glycerol after HPLC analysis compared to crude glycerol. Results also demonstrated the production of 1, 3-Propanediol (1, 3-PD) and acetic acid when the fermentation liquor was analyzed by HPLC after 48 hours. Moreover, other solvents such as butanol and lactic acid were obtained in very low quantities. Subsequently, the effect of ultrasound on the growth of *Clostridium diolis* was evaluated and lead to enhanced yields by promoting rapid cellular growth rate. The results thereby indicate that better product yields can be achieved when microorganisms are stimulated with low intensity ultrasonic irradiations.

Keywords: crude glycerol, *Clostridium diolis*, anaerobic fermentation, value-added products

1 INTRODUCTION

Biofuels can be defined as forms of energies such as solid (biochar), liquid (bioethanol, vegetable oil and biodiesel) or gases (biogas, biosyngas and biohydrogen) that are primarily produced from biomass resources. Biomass is the largest renewable energy source; about 77.4 % of global energy supply and 10.4% of the total primary energy supply [1]. There are two worldwide known and mostly used biofuels (biodiesel and bioethanol) which show potential to replace diesel and gasoline fuels, respectively. Recently, biodiesel has become more attractive because it has greater environmental benefits that include reduced harmful emissions, low sulphur content, improved lubricity and requires less energy in its production [2]. Glycerol is the main by-product of biodiesel production process, stoichiometrically, for every 10 kg of biodiesel produced 1 kg of crude glycerol is generated [3]. Glycerol is common reagent in foods, cosmetics and pharmaceuticals industries [4]. For crude glycerol to be used for any industrial processes it needs to be purified, normally requiring complex and expensive methods [5]. Moreover, crude glycerol cannot be deposited to the environment without treatment and the cost for treatment is excessive [6]. Currently different methods for the utilization of crude glycerol into industrial chemicals exist, these include biological and chemical pathways [7].

Although chemical catalysed conversion of crude glycerol was preferred for many years, it has more operational disadvantages including use of high pressure or temperature, low production specificity and inability to use contaminated crude glycerol [8]. However, biochemical conversion *via* anaerobic fermentation has many advantages ranging from less energy use to lower capital and operation costs [4].

Various microorganism are able to grow anaerobically on glycerol as sole carbon source, these includes *Enterobacter spp*, *Klebsiella spp*, *Citrobacter spp*

and *Clostridia spp* to produce broad variety of chemicals [9]. Microorganisms metabolise glycerol through oxidation and reduction pathway and the usage of glycerol is proportional to the production of solvents such as 1, 3-propanediol [10]. *Clostridiaceae* family had been anaerobically tested and assessed to produce different chemicals and alcohols such as 1, 3-propanediol (1, 3-PD), butanol, ethanol and others [11].

Since by 2016 the world's biodiesel market is estimated to generate 37 billion gallons, this indicates that about 4 billion gallons of crude glycerol will concurrently be produced [12]. These developments in biodiesel industry has already resulted in drop of glycerol prices and has negatively affect the economically feasibility of biodiesel industry [13]. Thus the promising option is to convert glycerol into value-added products which will alongside stimulate biodiesel economic value.

The present study investigates the potential of producing value-added product (1, 3-propanediol) by fermentation of *Clostridium diolis* from biodiesel-derived crude glycerol. To achieve the aims of this research, parameters such the inhibition rate of crude glycerol, crude glycerol purification by petroleum ether, concentration of crude glycerol will be studied. Eventually, the degree of enhancement of glycerol conversion with ultrasound irradiation and optimize major products will be evaluated.

2 MATERIALS AND METHODS

2.1 Raw material

Crude glycerol used in this study was generated as a by-product in biodiesel synthesis within the Bioenergy research group at the North-West University, Potchefstroom campus. Biodiesel was produced via the transesterification of sunflower oil in the presence of methanol in a ratio of 1:6 (oil: methanol) and 0.5% (w/v) of KOH as a catalyst. The transesterification reaction was

conducted in a 200 L stainless-steel reactor with monitored pressure and at 60 °C for 12 hours.

2.2 Partial purification of crude glycerol

Crude glycerol obtained from biodiesel preparation was washed with petroleum ether, a non-polar solvent. The method is adapted and modified from previous literature [14] and involved using 1:1 volume ratio of crude glycerol to petroleum ether, shaking at 200 rpm at room temperature for 3 hours. The mixture was then left to stand in a separating funnel for two hours, the lower phase is the washed crude glycerol and the upper phase is the solvent with impurities. After separation, the mixture was centrifuged at 4000 rpm for 2 min to separate into two distinct phases. The filtered sample was then dried at 95 °C for 12 hours to evaporate free water.

2.3 Microorganism and culture condition

Clostridium diolis (*C. diolis*) DSM 1541 was obtained from German Collection of microorganisms stored as freeze-dried in a sterile environment; bacterial was rehydrated for 30 minutes with 0.5 ml of the Reinforced Clostridium Medium (RCM). The content was mixed gently with an inoculation loop and transferred to a test tube containing 5 mL of RCM. Approximately 100 µl of the mixture was used to streak into an agar plate to prepare a stab culture in an anaerobic chamber containing Quantum-biotech anaerobic sachets.

Colonies formed after 72 hours of incubation were stored at 4 °C and long-term storage of microorganisms was done at -80 °C by freezing with 15 % (v/v) reagent grade glycerol in distilled water.

2.4 Fermentation

Fermentation of glycerol was supplemented with media containing the following per litre (L): 3.21 g KH_2PO_4 , 2.75 g K_2HPO_4 , 2 g CaCO_3 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.005 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g $(\text{NH}_4)_2\text{SO}_4$, 2 g yeast extract and 2 ml SL7 trace element solution. The composition of SL7 trace element solution is as follows: 0.1 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.07 g ZnCl_2 , 0.06 g H_3BO_3 , 0.04 g $\text{Na-MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.02 g $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$, 0.02 g $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.02 g $\text{NiCl}_2 \cdot 2\text{H}_2\text{O}$, 1 ml of 25% HCl. The media components and culture conditions were adapted from literature [15].

Fermentation was carried out by inoculation of the suitable amount (50 g.L⁻¹ and 100 g.L⁻¹) of the pre-culture to the fermentation media. All experiments were run in 250 mL modified Erlenmeyer flasks with a working volume of 100 mL sparged with nitrogen gas. Incubation was carried for 48 hours with 3 hour interval for sampling and analysis. Glycerol was autoclaved prior to being used in the fermentation at 121 °C for 15 minutes.

2.5 Ultrasonic-assisted fermentation

Ultrasound assisted fermentation of glycerol was conducted in an Elma sonic bath at a frequency of 35 kHz and 35 W power input as described in previous literature [15]. During the sonication the bath was filled with water for better transmission of waves. The experiments were carried in a 250 mL flask with 100 mL of the sample. The flasks with sample were placed ultrasonic bath for 2 minutes prior fermentation under conditions previously described in Section 2.4.

2.6 Analytical methods

2.6.1 Ultraviolet spectroscopy

Spectrometer (SHIMADZU) at a wavelength of 600 nm was used to quantify cell growth in sample drawn from fermentation flask. Distilled water was used as the blank. The absorbance for each time interval was analysed three and the average values were recorded.

2.6.2 High performance Liquid chromatograph

Residual glycerol and a variety of organic products were identified and quantified using a high performance liquid chromatography (Agilent 1200 Series) equipped with a HPX-87H column (100 x 7.7 mm, Bio-Rad) which is known to detect glycerol, sugars, certain organic acids and alcohols with a Refractive-index (RI) detector. Fermentable sample were taken every three hour interval from the broth filtered through a 0.45 µm membrane filter into HPLC vials. Prior to analysis, standard solutions for calibration were prepared by carefully weighing 10 g each standard compound and placed into a 1000 mL volumetric flask and dissolved in deionised water. The standard were further diluted 5-folds and filtered using 0.45 µm membrane filters into HPLC vials. The HPLC was operated with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.7 mL/min and a temperature of 65 °C.

2.6.3 Fourier Transform Infra-red spectroscopy (FTIR)

The functional groups of glycerol and products were estimated with FTIR spectrophotometer (SHIMADZU). The transmittance of the sample was measured in the range of 400 cm⁻¹ to 4000 cm⁻¹ with 45 scans per sample. Ultrasound and non-ultrasound sample were also compared by analysis using the FTIR. Upon completion, a software (IRAffinity) was used to interpret the spectrum.

2.6.4 Microscope analysis

The microbial microstructure was analyses by using Primo Star light microscope with Zeiss camera to take images of microorganism. The pictures were analyzed at different magnification to compare the effect of ultrasound during fermentation.

3 RESULTS AND DISCUSSIONS

3.1 Characterization of crude and washed glycerol

Since the raw crude glycerol in this study was a by-product from the production of biodiesel using Sunflower oil, KOH and methanol, washing crude glycerol with the aim to lowering the impurities was essential. The physical characterization of raw and washed glycerol was compared to the commercial pure glycerol. Pure glycerol has a pH of 6.41, velocity of 11191 (mPa*s) and density of 1.28 (g/mL). Raw was a dark liquid in colour with pH reading of 10.48, low density and low viscosity compared to pure glycerol. The high (alkalinity) pH value associated with raw are caused by residual KOH left from the biodiesel production process and low density was due to the influenced of the present of impurities that include fatty acids, soap, ester, water and methanol. A summary of the physical characteristic of the three classes of glycerol is shown in Table I.

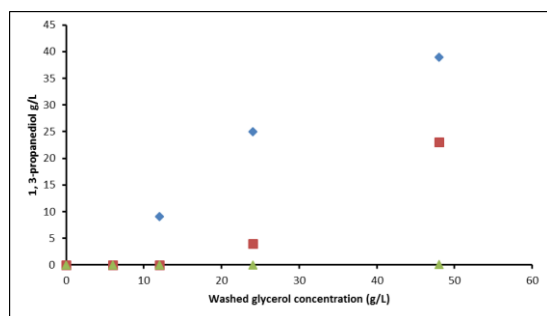
Table I: The physical characterization of crude, washed and pure glycerol

Parameter	Crude glycerol	Washed glycerol	Pure glycerol
pH	10.84	8.94	6.41
Viscosity(mPa*s)	967	1002	1191
Density (g/mL)	1.05	1.15	1.28
Glycerol (g/L)	148.12	185.23	~
Methanol (g/L)	309.9	0.23	~

Results in Table I shows that washed crude glycerol had a pH of 8.94 which is lower than that of raw crude glycerol but higher than that of pure glycerol. Similarly, viscosity and density of washed crude glycerol was lower than that of crude glycerol but higher than that of pure glycerol. The improved properties of washed crude glycerol were more influenced and associated with the extraction of some of the impurities by petroleum ether. The physical characteristic of the washed crude glycerol could then be utilize as the sole carbon source for the production of organic solvents. It can be concluded that value-added utilisation opportunities for crude glycerol are in terms of improving its quality using a simple and cheap approach to reduce the impurity contents.

3.2 Effect of glycerol concentration on product formation

The effect of substrate concentration on the formation of products and fermentation of *C.diolis* was studied by using 50, 100, and 150 g/L of washed crude glycerol for 48 hours at 37 °C. Results showing effect of substrate concentration on the production of 1, 3-PD as a sole product is represented in Figure 1.

**Figure 1:** The effect of washed glycerol concentration (◆ - 50 g/L, ■ - 100 g/L, ▲ - 150 g/L) on the production of 1, 3-Propanediol during *C. diolis* fermentation.

Utilization of washed crude glycerol is seen to increase steadily as fermentation time increased. Washed crude glycerol concentration of 50 g/L recorded the best results and demonstrated production of higher quantities of 1, 3-PD throughout the incubation period. The highest 1, 3-PD yield (31.1 g/L) was obtained in 48 hours. Compared to glycerol concentration of 100 g/L and 150 g/L, the concentrations of 50 g/L is the optimal total glycerol required for utilization to give highest quantity of 1,3-PD. At substrate concentration of 100 g/L showed less utilization of substrate and relatively lower production of 1, 3-PD was observed (Figure 1). Interestingly, at a further higher substrate concentration of 150 g/L shows no utilization of substrate was seen, hence no the products formed either. These results demonstrated a principle called 'growth inhibition'

during the anaerobic fermentation microorganisms at higher substrate amounts. The theory of having too much concentration of substrate a culture adversely affect the formation rate of fermentation products since the cells are believed to experience severe osmotic pressure. Similar results of high glycerol concentrations inhibiting fermentation was previously reported by Khanna [15]. In that study glycerol concentrations were increased from 150 mmol/L to 300 mmol/l which resulted in a 26 % decrease on the production of 1, 3-PD. Compared to our study, 1, 3-PD also decreased from 31.4 g/L, 19.9 g/L and 0.034 g/L at 48 hour when substrate concentrations was increased from 50, 100, and 150 g/L, respectively. Therefore, amount of glycerol is one of the vital parameter needed to be considered during fermentation of glycerol with *C. diolis*.

3.3 Influence of type of glycerol on anaerobic fermentation and product yield

The influence of different glycerol sources at 50 (g/L) on the fermentation of *C.diolis* and product formation was investigated. Three classes of glycerol including crude glycerol, washed crude glycerol and pure glycerol were used in the study. Results showing the effect of glycerol type on formation of 1, 3-PD is shown in Figure 2.

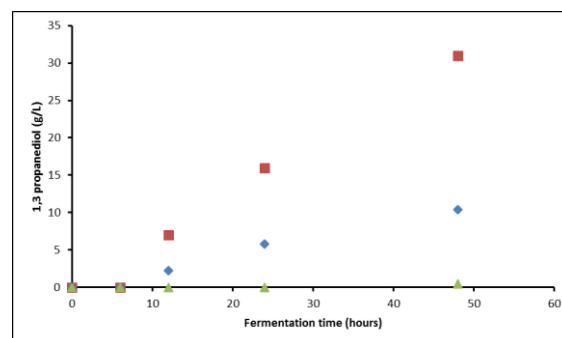
**Figure 2:** The influence of glycerol type (■ - pure glycerol, ◆ - washed glycerol, ▲ - crude glycerol) on the fermentation of *C.diolis* and the production of 1, 3-Propanediol with 50 (g/L) of three classes of glycerol was incubated at 37 °C at pH 7.0 for 48 h.

Figure 2 shows the overall yields from the fermentation of crude, pure and washed glycerol. The amounts of 1,3-PD and its volumetric production can be seen as directly influenced by the presence of impurities in the feedstock. The lowest 1, 3-PD yields was obtained for crude glycerol and could be observed throughout the 48 hour fermentation period. This was due to the higher contents of methanol and traces of KOH and free fatty acids (Table I) compared with washed crude glycerol, which gives a slightly higher average 1,3-PD yields. In case of washed crude glycerol, the highest amount of 1, 3-PD was found to be 10.8 g/L. Pure glycerol is the highest among all three cases and gave about 32 g/L of 1, 3-PD after a fermentation for 48 hours, which however still can compare well with most values reported in previous literature. For example, Powalowska [16] had reported yields of 1,3-PD concentrations between 43.2 g/L and 54.2 g/L. This was conducted under repeated batch method with the use of *Clostridium butyricum* DSP1 in which three cycles of fermentation medium replacement were carried out.

3.4 Influence of ultrasound on anaerobic fermentation and cell biomass growth

The anaerobic digestion of washed crude glycerol was conducted with *C. diolis* in which the influence of applying ultrasonic radiation as a biological pretreatment methods prior to the washed glycerol digestion was investigated. Results showing the effect of ultrasonic stimulation of *C. diolis* are described in Figure 3 below.

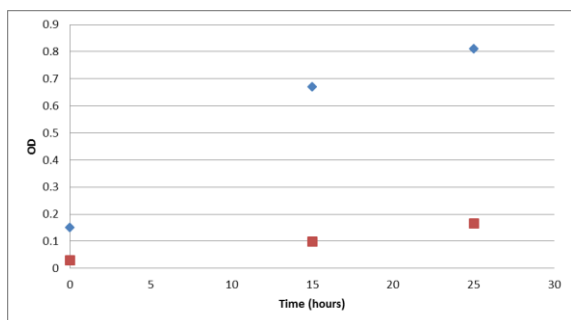


Figure 3: Growth profile for *C. diolis* on washed crude glycerol after fermentation for 25 hours. The graph shows ◆ - with ultrasound, ■ - without ultrasound.

It can be seen from Figure 3 that, ultrasonic-assisted fermentation could significantly improve the cell mass yield and concentrations at short time of 25 hours compared to over 48 hours of normal fermentation without ultrasound. The low power ultrasound (35 W) and frequency of 20 kHz have been found to facilitate the rapid growth of *C. diolis* and also reduce the fermentation time by approximately 50%. Ultrasound is found to increase the microbial cell growth by two way, either via mass transfer enhancement of air intake by the microorganism or thermal effects.

However, the only drawback with the use of ultrasonic irradiation in fermentation is that it does not require prolonged usage. For example, Hong and co-workers [17] previously conducted evaluation of ultrasonic radiation and had shown a reduction on the cells and concluded that cells must not be subjected to ultrasound for period longer than 15 min.

Another similar study was done by investigating the effect of ultrasonic radiation on *E. coli* and *K. pneumonia* when subjected to ultrasonic radiation for more than 2 minutes. *K. pneumonia* showed a decrease in cell content from 93.2 % to 77.2% however *E. coli* shows the increase in cell content from 51% to 62.7%. Thus, microorganisms may behave differently on the contact with ultrasound at various stages of their growth. The growth profile of *C. diolis* was monitored by use of a light microscope and the diagram showing the behavior of the cells in represented in Figure 4.



Figure 4: Microscopic images showing the fermentation cell morphology of *C. diolis* in washed crude glycerol at varying time intervals; time 0 with ultrasonic radiation (A), time 24 h without ultrasonic radiation (B), time 24 h with ultrasonic radiation (C).

Microscopic observation of the *C. diolis* cell morphology at different time cycle was evaluated by photographic method and depicted in Figure IV. It shows apparent decreased amount of cell count at the start of fermentation (Figure IVA). Increased fermentation time (24 hours) caused an increase in viability of the cells with not high productivity (Figure IVB). At 24 hours fermentation time with ultrasonic stimulation, many cells were formed to have clearly grown rapidly (Figure IVC). Since ultrasonic stimulation of bacterial cell is found to increase cell concentration during fermentation as opposed to without sonication, this bound to improve 1, 3-PD production efficiency.

3.5 Influence of ultrasound on anaerobic fermentation product range

The influence of ultrasonic radiation on the production of 1, 3-PD, acetic acid, lactic acid, acetic and butyric acid by *C. diolis* in 50 g/L of washed glycerol. The results showing the formation of different products from ultrasound-assisted fermentation are depicted in Figure V below.

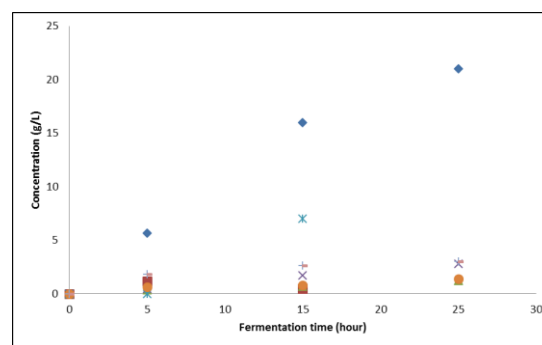


Figure 5: Effect of ultrasound on the fermentation of washed glycerol. Ultrasonic radiation was applied to 5 mL of inoculum for 2 min prior to addition of the seed culture to the fermentation broth. Results show four solvent products namely (◆ - 1, 3-propanediol, ■ - acetone, ▲ - lactate, x - butanol).

Figure 5 indicate the wide variation of end-products when *C. diolis* is subjected to ultrasound for 2 minutes. The main solvents that were obtained include 1, 3-PD and n-butanol after 24 hours of fermentation. Other products, such as acetone and lactate were in found in lower quantities after the fermentation period.

Product distributions under ultrasonic stimulation conditions differ substantially from growth conditions without ultrasound as observed in earlier results. It appears that the metabolic pathways may be modified during the growth phase of the *C. Diolis* when it was sono-stimulated. To date, no literature has been reported on this kind of observation involving the distribution of fermentation products after ultrasonic biological pretreatment. Potentially, 1,3-PD can be produced simultaneously with other solvents by fermentation of glycerol and that would be beneficial to the biodiesel industry.

4 CONCLUSION

- Crude glycerol contains 27. 41 % of glycerol content, 309 g/l methanol and traces of soap and

free fatty acids.

- Higher glycerol concentrations seem to inhibit *C. diolis* growth and subsequently slows down the production of 1, 3-PD and other by-products.
- The type of glycerol is important for 1, 3-PD production with pure glycerol giving the highest yields.
- However, washed crude glycerol can also be used because it provided relatively high product yields. This can be attributed to the reduction of impurities.
- Application of ultrasound to inoculum before fermentation enhanced the growth of *C. diolis* and shortened the fermentation time.
- Ultrasound irradiation seem to modify the bacterial pathways and lead to production of acetic acid, lactic acid and butanol.

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7 LOGO SPACE

