

Comparison of Glucose, Glycerol and Crude Glycerol Fermentation by *Escherichia Coli* K12

Chaudhary N¹, M.O. Ngadi¹ and B. Simpson²

¹Bioresource Engineering Department, McGill University, 21111 Lakeshore Road, Ste-Anne-De-Bellevue, Quebec, Canada ²Food Science and Chemistry Department, McGill University, 21111 Lakeshore Road, Ste-Anne-De-Bellevue, H9X 3V9, Quebec, Canada

Abstract

Hydrogen and ethanol production from glucose, glycerol and crude glycerol fermentation using *Escherichia coli* was investigated. Crude glycerol used in this study contained 80% glycerol, 2.6% ash, 12.3% moisture, 1.7% free fatty acid, 3.4% MONG (matter organic non-glycerol), 2519 mg/kg sulphur and 9000 ppm sodium. The maximum yield of ethanol from crude glycerol of 0.36 g/g, corresponding to an ethanol concentration of 3.6 g/L was obtained at 10 g/L initial glycerol concentration, 5 g/L tryptone concentration and 100 rpm mixing speed. Comparable yields were obtained at the mixing speeds of 150 and 200 rpm. On comparison, this yield corresponded to 105% of the yield (0.34 g/g) obtained from pure glycerol at the same conditions and 85% of the maximum yield (0.42 g/g) of ethanol obtained from pure glycerol at 10 g/L initial glycerol concentration, 10 g/L tryptoneconcentration and 200 rpm mixing speed. Additionally, *Escherichia coli* growth for glycerol was characterized and compared to that for glucose with 10 % substrate concentration at 37°C and 200 rpm mixing speed. The net growth rate for glucose and glycerol were 0.43 and 0.26 h⁻¹, respectively. The maximum dry weight attained for glucose and glycerol were 0.12 and 0.04 g/L, respectively.

Introduction

Fuel ethanol is currently produced in large scale using yeast fermentation of glucose obtained from corn crops and sucrose from sugar cane, the second mainly in Brazil [1]. There has been much interest inimproving ethanol yield during fermentation as well as increasing throughput and decreasing costs [2]. The environmental advantages of ethanol over traditional fuels such as gasoline stem from its renewable nature since it is generated from plants that were recently fixing carbon dioxide, thus negating the greenhouse gas effect [2]. However, regardless of process improvement, there remains a need to find non-edible feedstock for ethanol production.

Present day interest in biodiesels has caused significant growth in the sector of biodiesel production. This has, in turn significantly increased glycerol production, as it is the major by-product of biodiesel production [3]. For every 100 lbs of biodiesel produced from transesterification of oil, 10 lbs of glycerol is produced [4]. As a result of this surplus of glycerol, there has been a 10 fold decrease in glycerol price, creating the necessity for a method of treatment for this waste stream of biodiesel production [4]. Glycerol has become an abundant source of carbon that is now equivalent in price to traditional fermentation sugar stocks. A cost analysis of ethanol production from glycerol demonstrates that ethanol from glycerol costs 0.66\$/gallon versus 1.05\$/ gallon from corn [4]. This is due mainly to complexity of the ethanol production process when corn is used as substrate versus the relative simplicity of the process when glycerol is used as substrate.

The capacity to ferment glycerol has been reported in species of multiple genera, including *Klebsiella*, *Citrobacter*, *Enterobacter* and *Bacillus* [5]. Microbial production of H_2 and ethanol from crude glycerol has been successfully carried out using *E*. *Aerogenes* [6]. However, the use of these micro-organisms on an industrial scale could pose several issues, including the requirement of strict anaerobic conditions, requirement of supplementation with rich and cost ineffective nutrients and high sensitivity to contamination [5]. It has recently been reported that *Escherichia coli* (*E. coli*) produces ethanol and H_2 from glycerol in a pH dependent manner, with an optimal pH for ethanol production of 6.3 [7]. However, the use of crude substrates, in this case glycerol, has not been explored in a comprehensive manner [8].

E. coli is highly amenable to use in industrial applications. For ethanol production, advantages include its capability to ferment a wide range of sugars and previous industrial use for the production of recombinant proteins. The fermentation of glucose by *E. coli* for the production of ethanol has been extensively studied. The fermentative pathway for glycerol is similar to that of glucose. In addition, the theoretical yield of ethanol from glycerol is higher than that from other traditional sugars, such as glucose [4]. Chemical equations of ethanol production from glucose and glycerol are given in Equations (1) and (2), respectively.

$$\frac{1}{2}C_6H_{12}O_6 \to CH_3CH_2OH + CO_2 \tag{1}$$

$$\overline{C_3H_8O_3} \rightarrow CH_3CH_2OH + CH_2O_2/(H_2 + CO_2)$$
⁽²⁾

The ability of *E. coli* to transform crude glycerol, waste of biodiesel production, into ethanol will allow for a zero waste process stream, leading to an increase in the economic viability of biofuels industry. The main aspect of this investigation is to study and compare the use of glucose, glycerol and crude glycerol as a carbon source for anaerobic growth of *E. coli* in order to produce ethanol and H_2 . The comparison was carried out in two separate experiments. For glycerol and glucose, the effect of carbon source is investigated by a comparative growth analysis of *E. coli* in the two substrates under anaerobic conditions. For glycerol and crude glycerol, the effects of initial glycerol concentration, supplement concentration and agitation speed on final ethanol concentration and dry weight were tested.

*Corresponding author: M.O. Ngadi, Bioresource Engineering Department, McGill University, 21111 Lakeshore Road, Ste-Anne-De-Bellevue, Quebec, Canada; E-mail: michael.ngadi@mcgill.ca

Received October 19, 2011; Accepted March 13, 2012; Published March 15, 2012

Citation: Chaudhary N, Ngadi MO, Simpson B (2012) Comparison of Glucose, Glycerol and Crude Glycerol Fermentation by *Escherichia Coli* K12. J Bioprocess Biotechnig S1:001 doi:10.4172/2155-9821.S1-001

Copyright: © 2012 Chaudhary N, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Microorganism and innoculum

E. coli MG1655 (ATCC 700926) was obtained from Cedar Lane Labs. M9 minimal medium containing 990 ml distilled water, 2 ml of MgSO₄, 10 ml of 20% glucose, 6.0 g Na₂HPO₄, 3.0 g KH₂PO₄, 0.5 g NaCl and 1.0 g NH₄Cl was mixed; 3.6 g of agar was added to 240 ml of this medium, autoclaved and poured into petri dishes. 60 ml of the medium was used to rehydrate the bacteria pellet. The bacteria was then transferred to the prepared plates and grown overnight at 37°C. Single colonies were then transplanted onto Luria Bertani agar plates and incubated overnight at 37°C. Single colonies selected from these agar plates were used to generate 80% glycerol stock solutions which were then stored at – 80°C.

The innoculum for experiments was prepared in hungate tubes (Bellco Glass, 2047-16125) filled with MOPS minimal media [9] supplemented with 1% 1.32 mMN₂HPO₄ and 0.1% 1 µmol sodium selenite. The amount of 10% glycerol was added as carbon source and the pH adjusted to 6.3 with 1 M NaOH. Bacteria from the glycerol stock was plated and grown on Luria Burtani agar incubated for 24 hours in an Oxoidanaerobic jar with CO₂ generation kit (Oxoid Ltd.). The prepared media inside hungate tubes was then inoculated with a single colony from the agar plate. The hungate tubes were incubated at 37°C in a shaker at 200 rpm until the initial optical density of 0.1 at 550 nm (OD₅₅₀) was reached.

Experimental medium

Glycerol vs. Glucose: For comparison of glycerol and glucose, the medium consisted of MOPS minimal media made from 10X MOPS modified rich buffer minimal media kit (Teknova, M2106) [9] supplemented with 1% 0.132mM N₂HPO₄ and 0.1% 1 µmol sodium selenite. The media was also supplemented with 5 g/L yeast extract and 10 g/L tryptone and 20% glucose or 20% glycerol, as required for the given experiment.

Glycerol vs. Crude Glycerol: For comparison of glycerol and crude glycerol, the medium consisted of MOPS minimal media made from 10X MOPS modified rich buffer minimal media kit (Teknova, M2106) [9] supplemented with 1%vol. 0.132 mol N_2 HPO₄ and 0.1%vol. 1 µmol sodium selenite for every litre. Crude glycerol was used as the carbon source for the culture medium. The crude glycerol was obtained from a local biodiesel production company. It was characterized as follows: 80% glycerol, 2.6% ash, 12.3% moisture, 1.7% free fatty acid, 3.4% MONG, 2519 mg/kg sulphur, 9000 ppm sodium, 2.4 pH and 6591 BTU/lb. The pH was adjusted to pH 6.3 by addition of 1 M HCl. The media was also supplemented with 5 g/L yeast extract. As required par the experimental plan, the culture medium contained 10, 25 and 50 g/L of crude glycerol and 0, 5 and 10 g/L of tryptone. Mixing speed was set at 100, 150 and 200 RPM.

Analytical methods

J Bioproces Biotechnig

Biomass was measured by correlating optical density with dry weight (1 $OD_{550} = 0.34$ g/L dw). A 15 ml sample was collected and the optical density measured at 550 nm in spectrophotometer zeroed with sterile broth. The sample was then centrifuged at 10000 RPM for 10 min. The pellet was collected and washed twice with 35 ml distilled water. To verify the correlation between absorbance and dry weight, the pellet was then dried at 105°C for 24 hrs and then weighed. The supernatant was collected and stored at -20°C for analysis. Glycerol concentration was determined using the glycerol assay kit (Megazyme,

K-GCROL). Ethanol concentration was measured using the ethanol assay kit (Megazyme, K-ETOH). H₂ concentration of the headspace was measured by taking samples through the septa with a syringe and direct injection into an HP Packard Series II 5890 GC with TCD detector with a Porapak Q Molecular Sieve 5A column with Ar carrier at 75 psig. A one point calibration was carried out to determine the relationship between peak size and H₂ concentration. The H₂ peak eluted at approximately 1.066 minutes (Figure 1).

The experiment design was in line with Statistical Analysis Software (SAS*). Experimental data was analyzed by SAS.

Fermentation

Glycerol vs. Glucose: Batch fermentation was carried out following a modified hungate tube method [10] in sealed 20 ml test tubes in 24 hr runs. The tubes capped, flushed with argon and maintained at 37°C and placed in a shaker set at 200 rpm. The initial pH of the media was adjusted to 6.3 prior to inoculation.

Glycerol vs. Crude Glycerol: Batch fermentation was performed anaerobically in sealed flasks following a modified hungate method [10]. The flasks were initially flushed with argon and then placed in a shaker set at agitations of 100, 150 and 200 rpm, as required for the experiment. Table 1 shows the experimental design and selected levels for each factor studied. The temperature was controlled at 37°C and initial pH of 6.3. Samples were collected at intervals of 2 hours. After recording absorbance at 550 nm, the samples were centrifuged for 10 minutes and the supernatant stored for glycerol and ethanol content analysis. The headspace of the flasks was sampled at the same interval time and the sample analyzed for H_2 content.

Results and Discussion

Glycerol vs. Glucose

The batch kinetics of bacteria growth with glycerol and glucose as carbon substrates is shown in Figure 2. It is typical growth kinetics as





Factors		Levels	
Glycerol Concentration	10 g/L	25 g/L	50 g/L
Tryptone Supplement Concentration	None	5 g/L	10 g/L
Mixing Speed	100 RPM	150 RPM	200 RPM

 Table 1: Experimental levels for glycerol type, glycerol concentration, tryptone supplement concentration and mixing speed.

Page 3 of 5

have been observed in several other different fermentation processes [7,11,12] (Figure 2).

For batch fermentation, the exponential growth rate is first order kinetics as shown in Equation (3):

$$\frac{dX}{dt} = \mu_{net} X \tag{3}$$

Integration of Equation (3) yields Equation (4):

$$\ln\left(\frac{X}{X_o}\right) = \mu_{net}t\tag{4}$$

Therefore, the growth rate is evaluated by linearizing the exponential portion of the growth curve shown in Figure 2. The net growth rate for glucose was 0.43 h⁻¹ and a maximum dry weight of 0.12 g/L was reached at 14 hours. The net growth rate for glycerol was 0.26 h⁻¹ and a maximum dry weight of 0.04 g/L was reached at 20 hours. Thus the growth yield for glucose as substrate is 0.012 and the growth yield for glycerol is 0.004. As a result, E. coli growth with glycerol is slower than with glucose as carbon source. Although, the results of this study cannot be compared directly with other literature reports where different substrate and microorganisms have been used, it is known that carbon source is an important parameter for microbial growth [12]. Fermentation of glucose or glycerol by E. coli produces succinate, lactate, formate and acetate as by-products and ethanol as principal product. Buildup of these pH altering products apparently quickly dropped the pH below the initial value of 6.3 which is the ideal glycerol fermentation pH [7]. Since E. coli ferments glycerol in a pH dependant manner, pH adjustment by addition of a base will be necessary in order to increase ethanol production.

Glycerol vs. Crude glycerol

A comparison was made to evaluate the effect of utilizing crude glycerol in place of pure glycerol. In addition, the effect of glycerol concentration, mixing speed and tryptone supplement concentration was also evaluated. The experiment was conducted in 2 blocks with pure and crude glycerol as the carbon source. For each block, a full factorial design for 3 remaining factors at 3 levels with 3 replicates was employed.

Figures 3 to 8 show the final dry weight and final hydrogen and ethanol concentrations for pure and crude glycerol substrate with 0, 5 and 10 g/L tryptone supplement and 100, 150 and 200 rpm.



Final dry weight concentrations are higher with the slower speed mixing, 100 and 150 rpm, than with 200 rpm for media that contains













g/L tryptone. At mixing speed of 200 rpm, there is less biomass and ethanol production; this high mixing rate promotes a more uniform solution, dispersing the scare nutrients to reduce localized growth observed at lower mixing speeds.

When tryptone supplementation was increased, a mixing speed of 200 rpm produced beneficial results, as shown in Figure 7. The uniform distribution promotes overall growth when there are substantial nutrients available even after dispersion.

The Figures also show that generally the final ethanol concentration is lower for an initial glycerol concentration of 25 g/L as compared to that with 10 or 50 g/L at the same conditions with respect to tryptone supplement and mixing speed. However, it is to be noted that although the absolute ethanol concentration is lower, the yield for 25 g/L initial glycerol concentration is higher than that of 50 g/L as ethanol yield is calculated as final ethanol concentration divided by initial glycerol concentration.

Use of crude glycerol instead of pure generally increases the final dry weight concentration if all other conditions are constant. The effect of crude glycerol on hydrogen and ethanol production is variable and demonstrated in the Figures. Figure 6 shows that for mixing speed of 150 rpm, the final dry weight, ethanol and hydrogen concentrations for crude glycerol are similar to those for pure glycerol at the same conditions, as shown in Figure 5. In addition, for initial glycerol concentration of 10 and 25 g/L, there is significant ethanol production for 5 g/L tryptone at both 100 and 150 rpm. This demonstrates that when crude glycerol is utilized, there is less need for high supplementation of the media. This effect is not present at 200 rpm, thus lower mixing speeds are required for crude glycerol. However, as mixing speed increases, the final dry weight for 25 g/L initial glycerol concentration with 5 g/L tryptone decreases. This implies that less carbon is utilized for the production of biomass and minimizes waste biomass requiring treatment at the completion of fermentation.

Table 2 shows the effects of glycerol type and concentrations, tryp-







Figure 7: Final H_2 concentration (\square), ethanol concentration (\square) and dry weight (\square) for pure glycerol for 0, 5 and 10 g/L tryptone at 200 RPM.



Page 4 of 5



		Glycerol type, glycerol concentra- tion (g/L)					
		Pure			Crude		
Mixing speed	Tryptone concentration (g/L)	10	25	50	10	25	50
100 rpm	0	0.26	0.09	0.06	0.25	0.08	0.08
	5	0.34	0.08	0.07	0.36	0.12	0.09
	10	0.32	0.05	0.06	0.25	0.07	0.03
150 rpm	0	0.33	0.10	0.09	0.26	0.08	0.07
	5	0.41	0.12	0.09	0.32	0.10	0.07
	10	0.34	0.11	0.08	0.27	0.09	0.07
200 rpm	0	0.11	0.07	0.07	0.20	0.06	0.08
	5	0.31	0.11	0.08	0.35	0.07	0.08
	10	0.42	0.14	0.09	0.21	0.10	0.07

 Table 2: Ethanol yield for glycerol type, glycerol concentration, tryptone concentration and mixing speed.

tone concentrations and mixing speeds on ethanol yield. Feedstock usually account for a third of the production costs, making the ethanol yields a focus for optimization [13]. A maximum ethanol yield overall of 0.42 was observed with 10 g/L pure glycerol, 10 g/L tryptone and 200 rpm. For crude glycerol, the maximum yield observed was 0.36 for 10 g/L crude glycerol, 5 g/L tryptone and 100 rpm. However, the yields for 10 g/L crude glycerol with 5 g/L tryptone were comparable for all mixing speeds.

Overall, the ethanol yield is greatest for initial glycerol concentrations of 10 g/L. In addition, all cases but two, 100 rpm and 200 rpm, the yield at 10 g/L glycerol is higher for pure glycerol than for crude glycerol.

At 100 and 150 rpm, the yield is higher for 5 g/L tryptone but for 200 rpm, the yield is higher for 10 g/L tryptone. The higher agitation speed is thus necessary for the effective utilization of the extra supplements.

Conclusion

This work investigated the potential of crude glycerol used for production of ethanol and H_2 via anaerobic fermentation. The variables of interest were mixing speed, initial glycerol concentration and tryptone supplementation. Maximum yield of 0.36 for ethanol was obtained at 10 g/L glycerol, 5 g/L tryptone and 100 rpm; this corresponded to an ethanol concentration of 3.6 g/L and was 105% the yield obtained from pure glycerol at the same variable conditions. However, the maximum yield of ethanol for pure glycerol was 0.42, observed with 10 g/L pure glycerol, 10 g/L tryptone and 200 rpm; thus maximum yield for crude

Page 5 of 5

glycerol was 85% that of pure glycerol overall. Analysis of variance demonstrated that all three variables, as well as the interactions had an effect on the final dry weight, ethanol and H₂ concentrations; however, not all these effects were significant. This study demonstrates the feasibility of utilizing crude glycerol as substrate for anaerobic fermentation for the production of ethanol via *E. coli*.

Acknowledgement

The authors would like to acknowledge The Natural Sciences and Engineering Research Council of Canada (NSERC) for providing the funding for this research and Roth say Biodiesel for supplying the crude glycerol used.

References

- 1. Lin Y, S Tanaka (2006) Ethanol fermentation from biomass resources: current state and prospects. Appl Microbiol Biotechnol 69: 627-642.
- Wheals AE, LC Basso, DMG Alves, Amorim HV (1999) Fuel ethanol after 25 years. Trends in Biotechnology 17: 482-487.
- Pagliaro M, Ciriminna R, Kimura H, Rossi M, Della Pina C (2007) From glycerol to value-added products. Angewandte Chemie-International Edition 46: 4434-4440.
- Yazdani SS, and R Gonzalez (2007) Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. Curr Opin Biotechnol 18: 213-219.
- 5. Murarka A, Dharmadi Y, Yazdani SS, Gonzalez R (2007) Fermentative Utiliza-

tion of Glycerol by *Escherichia coli* and Its Implications for the Production of Fuels and Chemicals. Appl Environ Microbiol 74: 1124-1135.

- Ito T, Nakashimada Y, Senba K, Matsui T, Nishio N (2005) Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process. J Biosci Bioeng 100: 260-265.
- 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.
- Zafar S, M Owais (2006) Ethanol production from crude whey by Kluyveromyces marxianus. Biochemical Engineering Journal 27: 295-298.
- Neidhard Fc, PL Bloch, David FS (1974) Culture Medium for Enterobacteria. J Bacteriol 119: 736-747.
- Miller TL, MJ Wolin (1974) A Serum Bottle Modification Of Hungate Technique For Cultivating Obligate Anaerobes. Appl Microbiol 27: 985-987.
- Papanikolaou, S., Fakas, Fick M, Chevalot I, Galiotou-Panayotou M, et al. (2008) Biotechnological valorisation of raw glycerol discharged after bio-diesel (fatty acid methyl-esters) manufacturing process: production of 1,3-propanediol, citric acid and single cell oil. Biomass & Bioenergy 32: 60-71.
- Papanikolaou S, G Aggelis (2003) Modelling aspects of the biotechnological valorization of raw glycerol: Production of citric acid by *Yarrowia lipolytica* and 1,3-propanediol by *Clostridium butyricum*. Journal of Chemical Technology and Biotechnology 78: 542-547.
- Dien BS, Cotta MA, Jeffries TW (2003) Bacteria engineered for fuel ethanol production: current status. Appl Microbiol Biotechnol 63: 258-266.

This article was originally published in a special issue, **Biomolecular Engineering** handled by Editor(s). Dr. Yuhua Song, The University of Alabama at Birmingham, USA.