Indian Journal of Experimental Biology
Vol. 60, September 2022, pp. 681-688
DOI: 10.56042/ijeb.v60i09.65145

Bioconversion of glycerol waste to ethanol by *Escherichia coli* and optimisation of process parameters

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Received 15 February 2022; *revised 10 April 2022*

Biofuel is one of the best ways to reduce our dependence on fossil fuels. Ever since commercial biodiesel production began, waste glycerol, the biodiesel byproduct, has gained researchers' interest, especially its recycling. Here, we explored using glycerol residue (carbon source) as a substrate in the fermentation process for ethanol production by *Escherichia coli* K12 in anaerobic conditions. The factors affecting the ethanol production was optimised by response surface methodology (RSM). Significant variables that impact the ethanol concentration were pH, temperature and the substrate, with a statistically significant effect (P <0.05) on ethanol formation. The significant factor was analyzed by the Box-Behnken design. The optimum conditions for bioethanol formation using glycerol as substrate was obtained at pH 7 and temperature 37°C. The ethanol productivity was 0.77 g/L/h. The ethanol concentration of 9.2 g/L achieved from glycerol residue was close to the theoretical value with the fermentation achieved at optimised terms.

Keywords: Biodiesel, Box-Behnken design, Response surface methodology (RSM)

The high demand for transportation fuels leads to a faster depletion of petroleum reserves, depleting petroleum resources due to their non-renewability. Therefore, there is a need for renewable energy resources that should be used sustainably to partially or fully substitute petroleum-based fuels that also boost environmental concerns. The production of alternative renewable fuels from numerous biological sources is considered one of the priority areas in many countries[1]. Biodiesel is one of the most effective alternate liquid fuels produced from plant and animal fats[2]. It is also eco-friendly, and the only alternative fuel with the prospective to completely displace its oil counterpart[3]. Traditional diesel motors can utilise 100% biodiesel with only small adjustments to sustain the system and little influence on performance. Although biodiesel stands for a safe, renewable, and ecologically risk-free choice to fossil fuels, its economic practicality is a major problem. At the same time, biodiesel's enhanced manufacturing remarkably affected the glycerol market due to generating an excess of crude glycerol generated as a spin-off at about 10% in the biodiesel manufacturing process. Malaysia is one of the top ten countries producing biodiesel[4].

Glycerol is obtained as major byproduct during saponification and hydrolysis in oleochemical plants and the transesterification process in biodiesel plants. Disposal of crude glycerol is pricey; it comes to be helpful to an experience sorting process to generate revenue. The need for biodiesel production is increasing consistently has caused an enormous surplus of glycerol and partially detoxified glycerol in the setup. To raise the business economics of biodiesel production, unrefined glycerol usage and its disposal have become an issue of concern[5]. One way to add value to crude glycerol is to use refining procedures to make pure glycerol. "Universal Recovery Strategy" was established to recover important compounds from industry spin-offs. Pure glycerol is utilized for various value-added products[4].

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Nevertheless, small and medium-sized biodiesel producers face considerably high expenses in refinement processes. Supplying raw glycerol is only practical when biodiesel plants are located within the limited area. However, a conventional technique for glycerol purification requires high expense and facility processes. Existing glycerol purification methods have drawbacks such as high energy demand and high maintenance. If glycerol is used for purification, the process fails to eliminate additional contaminants. Using chemical process also has the drawback of low glycerol yield due to repeated acidification procedure.

Many wastes are converted into value added products by researchers[6,7]. The glycerol byproduct from the biodiesel industry is also converted to valuable compounds like propanol, acrolein, polyhydroxy butyrate (PHB), lipids, carotenoids and glyceric acid and dihydroxyacetone (DHA)[8]. For every 10 kg of biodiesel produced, about 1 kg of crude glycerol is produced. The vast amounts of crude glycerol formed annually have influenced the glycerol market, resulting in low cost of polished glycerol as well as unrefined glycerol. Unrefined glycerol has been a financial and ecological obligation of the biodiesel sector. Thus, it is crucial to transform unrefined glycerol into higher-value items to enhance the biodiesel sector's economic sustainability and reduce the ecological effects of crude glycerol garbage disposal.

Several research initiatives are committed to the microbial conversion of low-cost commercial wastes into bioenergy. Using unrefined glycerol could be an ideal source for commercial fermentation. As biodiesel is a widely approved eco-friendly fuel, glycerol bioconversion into necessary chemicals like citric acid, succinic acid, 1,3-propanediol and butanol, etc., will further add worth to the biodiesel sector. Another path is transforming crude glycerol into useful products straight using biological or chemical paths. Several bacteria like *Clostridium pasteurianum*, *Enterobacter aerogenes* and *Klebsiella pneumonia* undergoes fermentation of glycerol substrate in anaerobic conditions. The conversion of low-cost glycerol streams to more excellent worth items has been recommended as a path to economic practicality for biofuels. While the accessibility of glycerol is an attractive carbon resource for fermentation processes, there is yet another benefit being used this substance fuels as well as lowered chemicals can be created from glycerol. However, the capacity for utilising these microorganisms at the industrial level could be limited as a result of problems that consist of pathogenicity, the requirement for stringent anaerobic problems and supplementation with rich nutrients, as well as unavailability of the genetic tools as well as physiological knowledge needed for their efficient manipulation. Using microbes such as *Escherichia coli*, an organism extremely amenable to industrial applications that might assist overcome the problems on a large scale.

Most bioethanol manufacturing is from food crops such as corn, sugarcane, wheat and soy. This has resulted in unfavourable effects relative to food production, consisting of boosts in food prices, a lack of fodder, as well as expanding competition for land. The use of glycerol waste from the biodiesel industry to produce bioethanol has considerable capacity to ease these unwanted impacts on food manufacturing[8]. Bioethanol is one of the fermentative products generated from glycerol via anaerobic fermentation. Bioethanol has been deemed an option for biofuels due to its nature as a renewable biobased source and because it offers the potential to reduce particle discharges.

The capability of *E. coli* to transform crude glycerol waste from biodiesel production into ethanol will enable a no-waste process stream, causing a boost in the financial viability of biofuels market. The bioconversion of crude glycerol to ethanol without pretreatment or purification has been proposed as promising approach for economic viability in biofuel industry. Numerous methods for optimisation have been tested, with the one variable at a time (OVAT), Response surface methodology (RSM)[9,10]. An RSM is a statistical model that examines the impact of the stated parameters on the output of many experiments, looking at both the individual and interacting effects. RSM models have previously been used to optimise bioethanol production processes[11]. Improvement in process design and output can be achieved by better understanding dynamics of cell development and product generation[12]. The process parameters like solid substrate, temperature, and pH affect ethanol production. The process efficiency will be improved by lowering the process costs and maximising productivity and yield when these parameters are optimised. Substrate (15-25 g), starting pH (5-9), and temperature (31-42°C) have previously been investigated. Here, we studied bioconversion of glycerol waste from

the biodiesel market to ethanol using *Escherichia coli*. The bioethanol production was optimised with the input parameters of pH, temperature and glycerol residue.

Materials and Methods

Microorganisms and maintenance

Escherichia coli K12 is obtained from the genetic laboratory, University Technology Malaysia, Johar. *E. coli* preserved in 80% glycerol medium stored at –80°C was taken for the fermentation process. A single loop of *E. coli* was streaked on the LB agar plate. The plate was incubated overnight in an anaerobic jar placed with the aenoro pack for the generation of anaerobic conditions for 24 h. A single colony of *E.coli* was used to inoculate in LB medium and is purged with inert atmosphere for anaerobic conditions. The growth of *E. coli* in the medium was incubated at 37°C until 0.4 optical density (OD) at 550 nm was reached[13].

Inoculum and culture medium

The substrate used is the glycerol residue obtained from Malaysia's Oleochemical biodiesel industry. It was characterised as follows, 70% of glycerol content, 10% of ash, 1[4]-15% moisture content, 5-6% of matter organic non-glycerol (MONG) and a pH is 6.5-7. The glycerol residue is used as the carbon source for the culture medium. Ethanol formation is performed in 250 mL Erlenmeyer flask in anaerobic conditions. The fermentation medium for enrichment and cultivation of glycerol fermenting bacteria that contained 3.4 g $K_2HPO_4.3H_2O$, 1.3 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$,0.2 g $CaCl_2.2H_2O$, 0.5 g $FeSO_4H_2O$ in 1 litre of distilled water[14]. All the medium is autoclaved at 121°C for 20 min. The use of complex medium was avoided. For cultivating the medium was sterilised and 20% of inoculum was added to the glycerol residue (substrate) was purged with argon gas before being incubated at 37°C with continuous stirring at 120 rpm with pH at 7. The medium for optimisation experiments were carried out by varying the different glycerol concentration (15 to 25 g), pH 5 to 9 and temperature 31 to 42°C according to the experimental design.

Analytical methods

Scanning electron microscopy (SEM) was used to capture micro photos. The surface morphology of *E. coli* cells was also examined by the Hitachi TM303plus, Japan. Fourier transform infrared (FTIR) was used to identify the functional groups of glycerol residue using thermos scientific Nicolet iS5 spectrometer equipped with deuterated triglycine sulphate (DTGS) as detector and OMNIC software. For content analysis, HPLC 1200 agilent technologies with reflective index detector (RI) and Rezex TM ROA-organic acid column (300×7.8 mm, 9 μm) at 60°C, 0.005 M H_2SO_4 as mobile phase 0.60 mL per min flowrate was used. After analysis, the equipment was flushed with acetonitrile: water (70:30) for an hour. With UV Visible spectrophotometer, the optical density (OD) was measured at 550 nm.

Design of experiment (DOE)

The Design of Experiment (DoE) technique allows for thorough investigation of all the factors affecting a certain process with the least amount of testing possible. The optimisation process begins with the selection of variables and their range of variation, selection of responses, selection of the optimal design, execution of statistically designed experiments in a randomized order, and estimation of the coefficients in a mathematical model. The statistical methods, including RSM with various designs, were effectively optimised for bioprocess, including medium components[15].

The RSM was utilised to define and forecast the ideal circumstances in the experimental area. The Box-Behnken design selected significant parameters that were optimised in the screening design. The ethanol production by modeling and optimisation was done using the RSM (Box-Behnken design). The pH, temperature, and substrate were the individual variables as input parameters for ethanol concentration as output response. A range of 5.0 to 9.0 pH, 31 to 42°C temperature, and 15 to 25 g substrate were used for the input parameters, is shown in Table 1. The input data ranges and parameters were selected based on past research. The experimental information was incorporated in the quadratic polynomial prototype concerning the input parameters for ethanol concentration using design expert software (version 13, State-Ease, USA). The significance of the model was assessed by the Analysis of Variance (ANOVA).

Results and Discussion

Glycerol residue was anaerobically fermented to ethanol by *E.coli* K12. Glycerol residue obtained from the biodiesel company was tested for the functional group analysis. The results show that the functional group appear for commercial glycerol and glycerol residue are alcohol, carbonyl and hydroxyl group. The O-H hydroxyl group appeared at the spectra value of 3279.64 cm^{-1} for commercial glycerol and 3346.31 cm^{-1}

Table 1 — Box-Behnken design in various process parameters affecting bioethanol concentration

Std	Run	pH A:A	Temp. (°C) B:B	Substrate (g) C:C	Ethanol (g/L) Response
5	1	5	36.5	15	6.5
1	2	5	31	20	7
6	3	9	36.5	15	6
7	4	5	36.5	25	7
2	5	9	31	20	6
12	6	7	42	25	5.5
16	7	7	36.5	20	9.5
15	8	7	36.5	20	9.5
14	9	7	36.5	20	9.5
3	10	5	42	20	5
13	11	7	36.5	20	10
4	12	9	42	20	6
17	13	7	36.5	20	10
10	14	7	42	15	6
11	15	7	31	25	6.5
8	16	9	36.5	25	6
9	17	7	31	15	6.5

for glycerol residue[16]. The appearance of absorbance frequency of C-H stretching at 2935.17 cm^{-1} and 2950 cm^{-1}, respectively for commercial glycerol and glycerol residue. The functional group with oxygen were carbonyl (C=O) and alcohol (C-OH) with bonding present with each activity based on location and hybridisation of C-O bond. The carbonyl C=O appeared in the range of 1648.39 cm^{-1} for glycerol and 1637.60 for glycerol residue however the sharp peak describes the impurity (MONG) which was similar to finding of Maru *et al*.[17]. The commercial glycerol and glycerol residue also noticed peak at 1031.52 cm^{-1} and 1039.25 cm^{-1} were due to alcohol C-O group stretching. The presence of C-O-H bending was observed at the frequency 1412.81 cm^{-1} (glycerol) and 1407.55 (glycerol residue). The FTIR spectra of glycerol residue is shown in Fig. 1.

The glycerol residue and ethanol confirmed by HPLC. The retention time of glycerol residue was 14.22 similar to pure glycerol. Therefore, it is apparent that glycerol content in the glycerol residue. However, another peak was observed at 7.332 and 12.88 in glycerol residue due to the impurities. The peak of ethanol was obtained at retention time of 20.55.

Before the fermentation process, *E. coli* growth profiling was checked with the optical density. Hence, batch fermentation was carried out to profile the growth kinetics of *E. coli* K12 strain. A medium containing 10% (v/v) of the organism was prepared

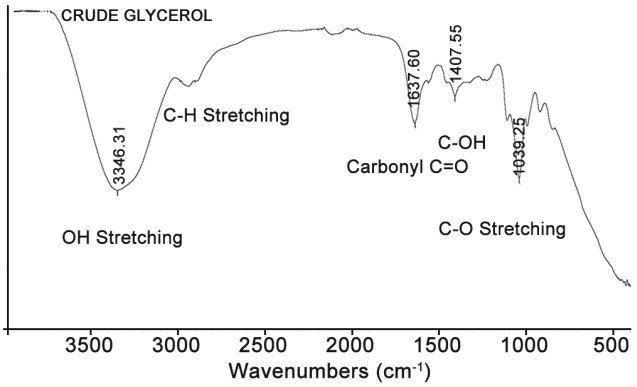

Fig. 1 — FTIR analysis of glycerol residue.

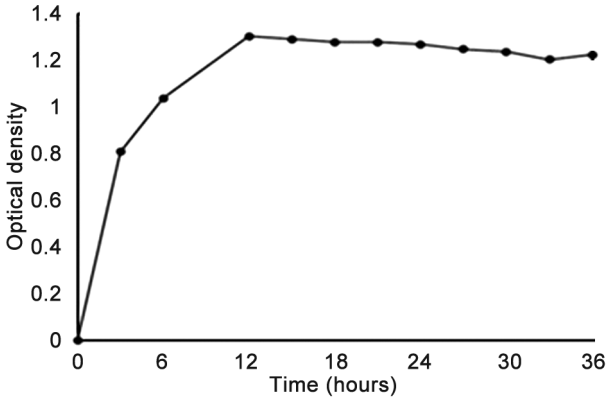

Fig. 2 — Profile growth of *E. coli* by optical density.

and incubated at 37°C for 36 h. Aliquots of samples were withdrawn at 3 hours for OD measurement at 550 nm using UV-Visible spectrophotometer is shown in Fig. 2. The OD, measured with a transmission densitometer represents the actual light blocking ability of the material. The higher cell activity is manifested in higher ethanol concentration.

The morphology of *E. coli* K12 strain is captured by SEM with different magnification. The *E. coli* cells were completely dried before taking the SEM images. The *E. coli* K12 strains are rod shaped and are agglomerated. The average size of the *E. coli* strains of 6-10 micrometres was observed. The *E. coli* strains grown in anerobic conditions in the LB medium with various magnifications at 1000, 2000, 3000 and 5000 are shown in Fig. 3. SEM depicted the growing cluster of Gram negative bacteria of *E. coli* of K12 strain. The *E. coli* K12 strains used for ethanol generation were cultured anaerobically at 37°C for 24 h. The initial measurement of ethanol production was made after 6 h, and the fermentation process was then prolonged until it reached a stationary phase. The

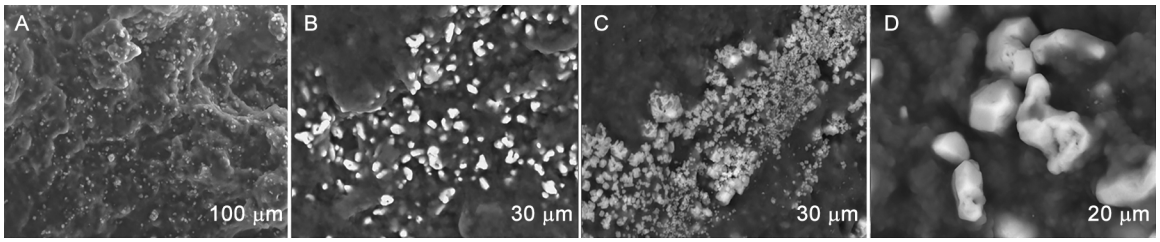

Fig. 3 — (A-D) SEM images of *E. coli* at 1 to 5k magnification

maximum ethanol formation was observed at 12 h. Microorganisms' specific needs for nutrients influenced ethanol generation, which in turn was influenced by media composition.

Ethanol production from glycerol with diverse strains in rich medium composition was reported by Ito *et al.*[18] and Jarvis *et al.*[19]. Microbial growth in rich medium was superior than growth in low-nutrient medium. The microbial growth was aided by the rich medium's nutrients and minerals, which ultimately led to the production of ethanol. However, some microorganisms does not sustain to rich medium.

The glycerol fermentation route used to produce ethanol from glycerol is depicted in following equation. The carbon present in this process, pyruvate can be converted to carbondioxide and ethanol by pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH)[20].

Glycerol concentration, pH, and temperature had a statistically significant effect on response. In order to increase ethanol production, it may be necessary to fine-tune fermentation parameters. Significant factors were screened for using a two-level factorial design that impacted the production of bioethanol. The authenticity of the fitted patterns was analysed with the help of a variance analysis (ANOVA) is shown in the Table 2. The ethanol concentration displayed high F-value of 49.87 with low p-value of 0.0001. The high F values and low p-values (<0.05) indicate the model significance[21].

The model in Table 2 shows a high determination coefficient (R^2) value 0.984 explaining 98% of the variability in the response and also high value of the adjusted determination coefficient (adjusted R^2) suggesting a high significance of the model. In this study, the factors pH, temperature and substrate (glycerol residue) were the significant model terms as the p values calculated for these factors were less than

Table 2 — ANOVA model for ethanol

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	47.29	9	5.25	49.87	<0.0001*
A-A	0.2813	1	0.2813	2.67	0.1463
B-B	1.53	1	1.53	14.53	0.0066
C-C	7.105E-15	1	7.105E-15	6.744E-14	1.0000
AB	1.00	1	1.00	9.49	0.0178
AC	0.0625	1	0.0625	0.5932	0.4664
BC	0.0625	1	0.0625	0.5932	0.4664
A^2	12.53	1	12.53	118.92	<0.0001
B^2	16.42	1	16.42	155.89	<0.0001
C^2	10.78	1	10.78	102.31	<0.0001
Residual	0.7375	7	0.1054		
Lack of Fit	0.4375	3	0.1458	1.94	0.2643**
Pure Error	0.3000	4	0.0750		
Cor. Total	48.03	16			

[*significant; **not significant]

0.05. This indicates the model terms are significant. Hence, the changes in these parameters significantly impact ethanol production from glycerol fermentation. The lack of fit F-value of 1.94 implies the lack of fit is not significant relative to the pure error. There is a 26.435 chance that a lack of fit F-value significant lack of fit is due to noise. Non-significant lack of fit is good. Lower temperature and pH may result in lowering the cell growth of bacteria.

However, the microbial cells are thermally lysed if temperature are raised too high[22]. Higher and lower pH also effect the cell growth of bacteria[23]. The substrate is also a parameter that impact on efficiency in conversion to ethanol.

Response surface plots predicated on the interdependence of response and variables are depicted in Fig. 4. The response (ethanol production) on the Z-axis were plotted against with any two related variables by retaining another variable at their ideal values. Fig. 4A shows the interaction between pH and temperature. The temperature at 36.5°C shown higher ethanol formation. Fig. 4B shows the interaction between the pH and the glycerol (substrate). Elevated ethanol formation was reported at middle pH ranges and relatively with substrate

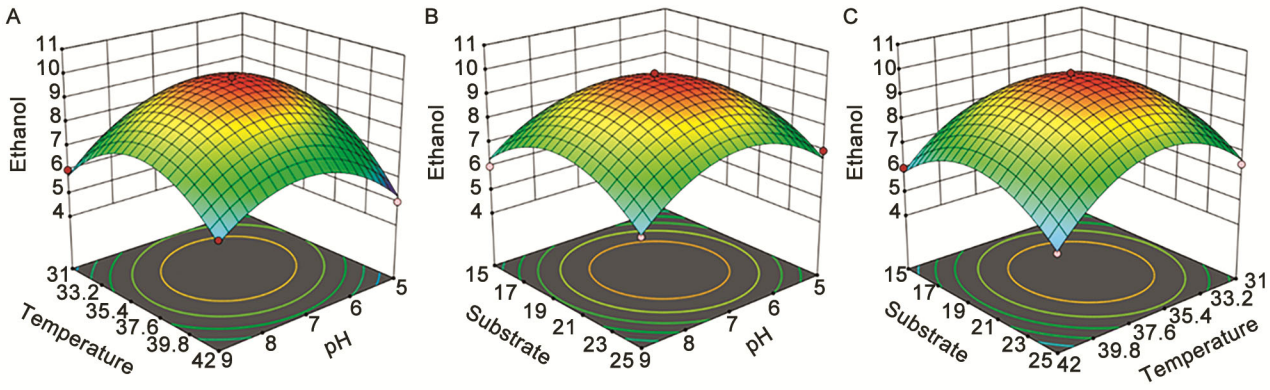

Fig. 4 — Response surface plots showing interface with variables in the ethanol formation. (A) pH and Temperature; (B) pH and substrate; and (C) Temperature and substrate.

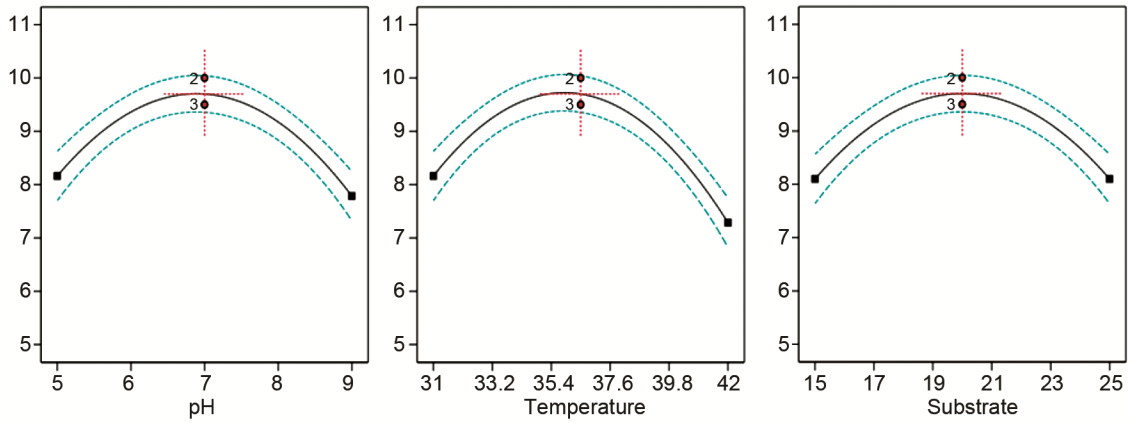

Fig. 5 — Interaction of variables pH, temperature and substrate affecting ethanol concentration.

concentrations. The ethanol generation was relatively high at pH 7.0 and at substrate 20 g. It was observed that increase in pH, temperature and substrate resulted in lower ethanol formation. Alternatively, lower range in pH, temperature and substrate also resulted in decrease in ethanol concentration were also shown in Fig. 5. Ethanol formation was significantly affected by pH and identified an optimum pH 7.0. However, lower ethanol formation was seen with rise in pH[24,25]. The initial pH greatly influence that impacts the NADH to NAD^+ ratio of the metabolic flux at anaerobic conditions[26]. Because of this, it is imperative that the initial pH be controlled to maximise ethanol production[25].

Substrate also plays an important role in the product formation. The increase in the glycerol concentration enhances the ethanol production. However, ethanol formation is ceased with the excess of glycerol concentration due to substrate inhibition and also the greater glycerol concentration produce osmatic pressure with cell damage by purging water molecules from the cells[27]. NaOH and KOH are used as catalysts in the biodiesel synthesis process, and as a result byproduct crude glycerol contains sodium chloride and potassium chloride[5].Crude glycerol normally has a salt concentration of 2.5 to 20 g/L. However, certain research suggested the salt concentration higher than 10 g/L are toxic to microorganisms. Excessive salt in growth media slowed cell growth and substrate utilisation by lowering respiratory activities[28]. Halotolerant bacteria treated with higher NaCl or KCl shown similar inhibition[29].

If we compare this study to the previous one, the conversion of glycerol using isolated *E. coli* SS1 produced ethanol as the main product. During ethanol production by fermentation process one molecule of ATP is generated from each molecule of glycerol into ethanol[30]. *E. coli* MG1655 can anaerobically convert 10 g/L glycerol into 4.5 g/L ethanol within 84 h of active growth [31]. Glycerol-rich biodiesel wastes can

be used as a carbon source for *E. aerogenes* HU101 to produce ethanol. Glycerol (5 g/L) was fermented in a bioreactor to produce 2.5 g/L of ethanol utilising an optimal fermentation process[18]. Under microaerobic conditions, the modified *E. coli* strain generated 21 g/L of ethanol from 60 g/L of pure glycerol with a volumetric productivity of 0.216 g/L/h[32].

Glycerol from biodiesel production was converted by *Kluyvera cryocrescens* S26 to the bioethanol by maintaining temperature at 30°C and pH 7. The glycerol fermentation in limited oxygen resulted in remarkable enhancement in cell growth, higher ethanol productivity and yield. In case of higher oxygen concentration, the cell growth is raised but the ethanol yield was lowered due to formation of byproducts (acetic acid and lactic acid). Adding oxygen to the culture medium could also eliminate the need for expensive nutrients, which could save money. Redox balance can be achieved using oxygen as an electron acceptor and eliminating the need for medium supplementation, but high oxygen levels would lead to low product yields because most of the carbon would be integrated into the cellular mass and converted to carbon dioxide[32]. The glycerol and crude glycerol were used as substrate for ethanol formation by *Enterobacter aerogenes* under 7 pH and 30°C. However, there was no negative influence on *E aerogenes* from the crude glycerol impurities, and in fact, the concentration and yield of ethanol were increased by 32 and 21%, respectively, in comparison to pure glycerol fermentation at the optimal glycerol concentration[35]. Various microorganisms with glycerol as substrate used to convert ethanol by fermentation process are shown in Table 3.

Conclusion

Here, we investigated the use of glycerol residue from the biodiesel industry for ethanol through anaerobic fermentation, making the waste a valuable product. The best fermentation conditions for ethanol production using experimental factorial design and response surface analysis was determined. Glycerol residue from biodiesel industry was successfully converted to bioethanol by *Escherichia coli* K12. Parameters like pH, temperature, and glycerol as a substrate significantly affect ethanol production. Optimisation of fermentation parameters with pH 7, temperature at 37°C and 20 g of glycerol residue produced 9.2 g/L of bioethanol. This study has demonstrated that anaerobic fermentation using crude glycerol as a substrate for ethanol generation in *E. coli* is feasible.

Table 3 — Various microorganisms used for bioconversion of glycerol to ethanol

Substrate	Microorganism	Glycerol (g/L)	Ethanol (g/L)
Glycerol waste[25]	*E. aerogenes* KKU-S1	37	120 mmol/L
Pure glycerol[33]	*E. coli* SS1	45	18.55
Glycerol waste[34]	*K. variicola* TB 83	20	9.80
Glycerol waste[27]	*E. aerogenes* ATCC29007	12	5.29
Crude glycerol[24]	Mixed culture	15	7.92
Glycerol waste[35]	*E. aerogenes*	25	204 mM
Crude glycerol[36]	*E.aerogenes* TISTR 1468	21.43	6.72
Pure glycerol[37]	*E. coli* SS1	20	9.23
Glycerol residue*	*E. coli* K12	20	9.20

*Current study

Acknowledgement

The authors are grateful to University Malaysia Pahang for providing financial support through higher education Malaysia's ministry under the fundamental research grant scheme number FRGS/1/2019/STG05/UMP/01/1 (RDU 1901123).

Conflict of Interest

Authors declare no competing interests

References

1 Chilakamarry CR, Sakinah AMM, Zularisam AW, Sirohi R, Khilji IA & Panday A, Bioconversion of Glycerol into Biofuels—Opportunities and Challenges. *BioEnergy Res*, 15 (2022) 46. https://doi.org/10.1007/s12155-021-10353-6.

2 Ganguly A Das, Bhattacharya AD & Chatterjee PK, Enzymatic hydrolysis of water hyacinth biomass for the production of ethanol: Optimisation of driving parameters. *Indian J Exp Biol*, 51 (2013) 556.

3 Soccol CR, Vandenberghe LPS, Woiciechowski AL, Thomaz-Soccol V, Correia CT & Pandey A, Bioremediation: An important alternative for soil and industrial wastes clean-up. *Indian J Exp Biol*, 41 (2003) 1030.

4 Chilakamarry CR, Sakinah AMM, Zularisam AW & Pandey A, Glycerol waste to value added products and its potential applications. *Syst Microbiol Biomanufacturing*, 1 (2021) 378.

5 Chilakamarry CR, Sakinah AMM & Zularisam AW, Opportunities of biodiesel industry waste conversion into value-added products. *Mater Today Proc*, 57 (2021) 1014.

6 Chaitanya Reddy C, Khilji IA, Gupta A, Bhuyar P Mahmood S, Khater Ahmed SAJ & Chua GK, Valorisation of keratin waste biomass and its potential applications. *J Water Process Eng*, 40 (2021) 101707.

7 Chilakamarry CR, Mahmood S, Saffe SNBM, Arifin MA Bin, Gupta A, Mohamed YS, Sabarunisha B & Narasaiah B, Extraction and application of keratin from natural resources: a review. *3 Biotech*, 11 (2021) 220.

8 Chilakamarry CR, Mimi Sakinah AM, Zularisam AW, Pandey A & Vo D-VN, Technological perspectives for

utilisation of waste glycerol for the production of biofuels: A review. *Environ Technol Innov*, 24 (2021) 101902.
9 Czitrom V, One-Factor-at-a-Time versus Designed Experiments. *Am Stat*, 53 (1999) 126.
10 Eshragh F, Pooyandeh M & Marceau DJ, Automated negotiation in environmental resource management: Review and assessment. *J Environ Manage*, 165 (2015) 148.
11 Hamouda HI, Nassar HN, Madian HR, Abu Amr SS & El-Gendy NS, Response Surface Optimization of Bioethanol Production from Sugarcane Molasses by *Pichia veronae* Strain HSC-22 . *Biotechnol Res Int*, 2015 (2015) 905792.
12 Almquist J, Cvijovic M, Hatzimanikatis V, Nielsen J & Jirstrand M, Kinetic models in industrial biotechnology – Improving cell factory performance. *Metab Eng*, 24 (2014) 38.
13 Murarka A, Dharmadi Y, Yazdani SS & Gonzalez R, Fermentative Utilization of Glycerol by *Escherichia coli* and Its Implications for the Production of Fuels and Chemicals. *Appl Environ Microbiol*, 74 (2008) 1124.
14 Barbirato F, Camarasa-Claret C, Grivet JP & Bories A, Glycerol fermentation by a new 1,3-propanediol-producing microorganism: *Enterobacter agglomerans*. *Appl Microbiol Biotechnol*, 43 (1995) 786.
15 Mandenius CF & Brundin A, Bioprocess optimisation using design-of-experiments methodology. *Biotechnol Prog*, 24 (2008) 1191.
16 Hazimah AH, Ooi TL & Salmiah A, Recovery of glycerol and diglycerol from glycerol pitch. *J Oil Palm Res*, 15 (2003) 1.
17 Maru BT, López F, Kengen SWM, Constantí M & Medina F, Dark fermentative hydrogen and ethanol production from biodiesel waste glycerol using a co-culture of *Escherichia coli* and *Enterobacter* sp. *Fuel*, 186 (2016) 375.
18 Ito T, Nakashimada Y, Senba K, Matsui T & Nishio N, Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process. *J Biosci Bioeng*, 100 (2005) 260.
19 Jarvis GN, Moore ER & Thiele JH, Formate and ethanol are the major products of glycerol fermentation produced by a *Klebsiella planticola* strain isolated from red deer. *J Appl Microbiol*, 83 (1997) 166.
20 Sawasdee V, Vikromvarasiri N & Pisutpaisal N, Optimisation of ethanol production from co-substrate of waste glycerol and acetic acid by *Enterobacter aerogenes*. *Biomass Convers Biorefinery*, 11 (2021) 1. https://doi.org/10.1007/s13399-021-01844-9
21 Hlangwani E, Doorsamy W, Adebiyi JA, Fajimi LI & Adebo OA, A modeling method for the development of a bioprocess to optimally produce umqombothi (a South African traditional beer). *Sci Rep*, 11 (2021) 20626.
22 Imtiaz U, Assadzadeh A, Jamuar SS & Sahu JN, Bioreactor temperature profile controller using inverse neural network (INN) for production of ethanol. *J Process Control*, 23 (2013) 731.
23 Chilakamarry CR, Mimi Sakinah AM, Zularisam AW, Sirohi R, Khilji IA, Noormazlinah A & Ashok Pandey, Advances in solid-state fermentation for bioconversion of agricultural wastes to value-added products: Opportunities and challenges. *Bioresour Technol*, 343 (2022) 126065.
24 Varrone C, Giussani B, Izzo G, Massini G, Marone A, Signorini A & Wang A, Statistical optimisation of biohydrogen and ethanol production from crude glycerol by microbial mixed culture. *Int J Hydrog Energy*, 37 (2012) 16479.
25 Reungsang A, Sittijunda S & Angelidaki I, Simultaneous production of hydrogen and ethanol from waste glycerol by *Enterobacter aerogenes* KKU-S1. *Int J Hydrog Energy*, 38 (2013) 1813.
26 Nakashimada Y, Rachman MA, Kakizono T & Nishio N, Hydrogen production of *Enterobacter aerogenes* altered by extracellular and intracellular redox states. *Int J Hydrogen Energy*, 27 (2002) 1399.
27 Lee SJ, Lee JH, Yang X, Yoo HY, Han SO, Chulhwan Park C & Kim SW, Re-utilization of waste glycerol for continuous production of bioethanol by immobilised *Enterobacter aerogenes*. *J Clean Prod*, 161 (2017) 757.
28 Nagata S, Wang Y, Zhang H, Sasaki H, Oshima A & Ishida A, Effect of moderate salinity stress treatment on the stimulation of proline uptake and growth in *Escherichia coli* CSH4 and its mutants under high salinity. *J Biosci Bioeng*, 108 (2009) 205.
29 Shkedy Vinkler C & Avi Dor Y, Betaine-induced stimulation of respiration at high osmolarities in a halotolerant bacterium. *Biochem J*, 150 (1975) 219.
30 Shams Yazdani S & Gonzalez R, Engineering *Escherichia coli* for the efficient conversion of glycerol to ethanol and co-products. *Metab Eng*, 10 (2008) 340.
31 Dharmadi Y, Murarka A & Gonzalez R, Anaerobic fermentation of glycerol by *Escherichia coli*: a new platform for metabolic engineering. *Biotechnol Bioeng*, 94 (2006) 821.
32 Choi WJ, Hartono MR, Chan WH & Yeo SS, Ethanol production from biodiesel-derived crude glycerol by newly isolated *Kluyvera cryocrescens*. *Appl Microbiol Biotechnol*, 89 (2011) 1255.
33 Adnan NAA, Suhaimi SN, Abd-Aziz S, Hassan MA & Phang LY, Optimisation of bioethanol production from glycerol by *Escherichia coli* SS1. *Renew Energy*, 66 (2014) 625.
34 Suzuki T, Nishikawa C, Seta K, Shigeno T & Nakajima-Kambe T, Ethanol production from glycerol-containing biodiesel waste by *Klebsiella variicola* shows maximum productivity under alkaline conditions. *N Biotechnol*, 31 (2014) 246.
35 Chanthoom K, Tanikkul P, Sirisukpoka U & Pisutpaisal N, Ethanol Production form Biodiesel-derived Crude Glycerol by *Enterobacter aerogenes*. *Chem Eng Trans*, 50 (2016) 211.
36 Sunarno JN, Prasertsan P, Duangsuwan W, Cheirsilp B & Sangkharak K, Biodiesel derived crude glycerol and tuna condensate as an alternative low-cost fermentation medium for ethanol production by *Enterobacter aerogenes*. *Ind Crops Prod*, 138 (2019) 111451.
37 Suhaimi SN, Phang LY, Maeda T, Abd-Aziz S, Wakisaka M, Shirai Y & Hassan MA, Bioconversion of glycerol for bioethanol production using isolated *Escherichia coli* ss1. *Brazilian J Microbiol*, 43 (2012) 506.