

Research Article

Glycerol Waste to Bio-Ethanol: Optimization of Fermentation Parameters by the Taguchi Method

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Received 29 June 2022; Revised 12 September 2022; Accepted 27 September 2022; Published 12 October 2022

Academic Editor: Ashanul Haque

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Global attention caused by pollutants and greenhouse gas emissions leads to alternative fuels that decrease the dependence on fossil fuels and reduce the carbon footprint that preceded the development of biodiesel production. Glycerol residue is generated more significantly from the biodiesel industry as a byproduct and is left as waste. In this study, we utilized glycerol residue from the biodiesel industry as an excellent opportunity to convert ethanol by bioconversion. The waste glycerol was used as a good and cheap carbon source as a substrate to synthesize ethanol by immobilizing *E. coli* cells. The screening of parameters such as mass substrate, temperature, inoculum size, and fermentation time was carried out using the one-factor-at-a-time (OFAT) technique. The Taguchi model employed optimization of fermentation parameters. The process parameters showed the mass substrate glycerol of 20 g with an inoculum size of 20%, and 12 hours yielded the ethanol concentration of 10.0 g/L.

1. Introduction

Biomass and bioenergy have recently gained increased attention because of the depletion of crude oil and environmental issues created by the growing usage of oil and its derivatives [1, 2]. As a result, research and development efforts should focus on ecologically friendly and renewable alternatives [3]. Maximizing renewable energy sources as a replacement for fossil fuels is becoming increasingly vital for several reasons, including lowering GHG emissions and ensuring reliable energy supplies [4]. Thus, biodiesel has increased commercial production and usage as a substitute for diesel. Biofuels are categorized into three groups based on the source. Food crops such as wheat, corn, potatoes, and

sugarcane are the first-generation biofuel sources. They are made up of starch and sugar, respectively. As a result of overfertilization due to food crops, the land becomes infertile, thus raising the cost of production. Nonedible sources such as agricultural, urban, and industrial wastes are included in the second generation of biofuels. They are made from lignocellulosic biomass, a renewable carbon source. In addition to agricultural and horticultural waste, wastes from parks, gardens, and forests also contribute to biomass. They are commonly available and inexpensive. Currently, the use of microalgae and macroalgae as the third generation of biofuels is being developed [5, 6].

Biodiesel is widely used as a renewable energy source that can substantially contribute to a country's long-term

viability and economic development. Biodiesel, a blend of fatty acid alkyl esters, has been recognized as a renewable fuel that can help reduce greenhouse gas emissions by partially replacing fossil fuels in the transportation sector [7]. Transesterification of vegetable or animal fats yields biodiesel, a desirable fuel due to its renewable nature, environmental friendliness, and lack of toxicity. About 10% of glycerol is a significant byproduct in biodiesel production. Glycerol production has increased due to an increase in biodiesel production worldwide. Glycerol waste overproduction has been identified as a serious concern to the biofuel sector in terms of disposal and purification costs. However, the combustion and uncontrolled burning of glycerol waste produce unsaturated aldehydes, which seriously threaten human health [8].

Crude glycerol from the biodiesel industry must be purified because it contains methanol, salts, water, acylglycerols, and fatty acids. The purification process is expensive for small- and medium-sized plants because of the restricted storage space required for the separation and refinement phase. Large-scale producers can readily convert glycerol into valuable compounds for commercial use. However, operational technique directly impacts the cost [9].

Green energy can be generated from biomass waste to assure economic, environmental, and social sustainability in many research projects [10]. To boost the economy's value-added development, wastes must be upgraded to biodiesel byproducts. In the biodiesel sector, glycerol waste byproduct is used as the best feedstock. Malaysia has an ongoing initiative to convert the enormous waste from oil palm into biofuels. Malaysia is the second largest producer of palm oil in the world. Palm kernel oil is primarily employed in oleochemical synthesis through the transesterification process with the generation of glycerol residue as waste. Using the abundant distillation plants in Malaysia, where glycerol is a significant waste product of the oleochemical industries, bioenergy can be generated from this industrial waste product [11].

Various researchers used waste to convert it into value-added products [12, 13] and focused on converting low-cost glycerol into valuable products [14, 15]. One of them is bioethanol production from glycerol using the fermentation process. Recent decades have seen the significance of developing new technologies that can boost ethanol production efficiency. Several investigations have shown that immobilized cells can be used to produce ethanol more cheaply [16]. This technology is appealing and promising since they make more than free cells. There has been an increased interest in immobilizing microorganisms due to many advantages, including high biomass, high metabolic activity, and excellent resistance to hazardous substances.

Furthermore, immobilized microbes could be cost-effective because they can be employed multiple times without considerable activity loss. Therefore, in recent decades, immobilized microbe technology has been examined as a potential method for wastewater treatment and for producing chemicals and fuels [17]. Using the design of the

experiment (DOE), the best configurations for maximizing yield can be found faster. A statistical model can be developed to predict the results as a function of the two factors and their combined effect. The current research study focuses on cell immobilization by *Escherichia coli* to produce bioethanol utilizing the glycerol waste residue from the biodiesel industry by fermentation. Optimization of fermentation parameters such as inoculum densities, fermentation time, and the mass substrate that affect ethanol formation was studied using one factor at a time (OFAT) and the Taguchi model.

2. Materials and Methods

2.1. Growth Profile of *Escherichia coli*. *E. coli* K12 was obtained from the genetic laboratory of the University of Technology Malaysia, Johar. *E. coli* K12 strain growth kinetics were investigated using batch fermentations. A medium comprising 10% (v/v) of the organism was prepared and was followed by incubation for 24 hours at 37°C. For every 3-hour interval, the samples were taken for optical density at 550 nm.

2.2. Bacterial Cell Culturing. The *Escherichia coli* K12 strain was used for the immobilization method in this experiment. The growth of stock culture was maintained on a Luria Bertani (LB) medium and agar slants at 37°C. In 50 ml of the medium in the flask, a single colony of the organism was seeded and purged with nitrogen gas for anaerobic conditions. Supplemented media were incubated at 37°C, and optical density (OD) was examined by a spectrophotometer at 550 nm [18]. At 10,000 rpm of culture, the medium was centrifuged for cell pellets. These cell pellets were isolated from the medium and washed with water. These washed-free cells were used for immobilization and were entrapped in sodium alginate.

2.3. Immobilization of *E. coli* Cells. The cells were precultured in the LB broth and were centrifuged for 10 minutes at 10,000 rpm at 4°C. Further, these cell pellets were added to a 250 ml solution containing 2% Na alginate. The mixture was thoroughly mixed for an hour to achieve homogeneity. This mixture of cell-Na alginate was dropped into a 0.1 M CaCl₂ solution using a syringe in a fixed position. The calcium chloride solution was uninterruptedly stirred to ensure the formation of the bead. Subsequently, beads were replaced with 0.05 M calcium chloride solution intact for 12 hours to harden the beads. A sterilized 0.85% sodium chloride solution was used to remove untrapped cells and free calcium chloride ions after the beads hardened after 12 hours [19]. The activity of bacterial cells of the beads was used to evaluate the stability test by pour plate procedure at various temperatures. For seven days, bead cell samples were taken daily from the freezer at -4°C and an incubator at 37°C. Every day, these samples were tested for the total bacterial count.

2.4. Optimization and Fermentation Process. The design of the experiment (DOE) was performed by optimizing fermentation parameters. Taguchi is a statistical model that examines the individual and interdependent effects of the specified parameters on the output of numerous experiments. Various parameters like time, mass substrate, and inoculum density were used for OFAT screening. The medium was maintained at pH 7 and incubated at 37°C with an agitation speed of 120 rpm throughout the fermentation process. About 20% of *Escherichia coli* was used. The solid substrate used was glycerol residue, which acted as the carbon source for fermentation. Finally, the culture was centrifuged at 10,000 rpm for 10 minutes and the supernatant was taken to assay ethanol concentration using HPLC.

2.5. Analytical Methods. The glycerol residue obtained as a major byproduct from the oleochemicals biodiesel industry in Malaysia was used as the substrate for ethanol fermentation. Fourier transform infrared (FTIR) was used to identify the functional groups using a ThermoScientific Nicolet iS5 spectrometer equipped with deuterated triglycine sulfate (DTGS). The spectrum in the transmittance mode was recorded using OMNIC software. FTIR scanning was performed in the 4000–500 cm^{-1} spectral region. Scanning electron microscopy (SEM) of Hitachi TM303plus, Japan, was used to examine the morphology of immobilized *E. coli* cells. HPLC 1200 Agilent technologies with reflecting index detector (RI) and Rezex TM ROA-organic acid column (300 × 7.8 mm, 9 μm) at 60°C, 0.005 N H_2SO_4 as the mobile phase, and 0.60 ml/min flow rate were used for high-performance liquid chromatography.

2.6. Statistical Analysis. The variance analysis of variance (ANOVA) by the experiment design using Minitab (version 19) was used for statistical analysis. *P* value was used to determine whether the results were statistically significant ($P < 0.05$).

3. Results and Discussion

3.1. Characterization of the Glycerol Waste and Bio-Ethanol. The glycerol residue obtained from the biodiesel industry was characterized as follows, 70% of glycerol content, 10% of ash, 14–15% moisture content, 5–6% of matter organic nonglycerol (MONG), and a pH is 6.5–7. Infrared spectroscopy assigns distinct frequencies to functional groups and chemical bonds in different molecules. FTIR was employed to assess changes in biological composition before and after fermentation. The infrared spectra of glycerol (commercial), glycerol residue, and ethanol are shown in Figure 1. The FTIR showed the intensity of the major peaks at 3500–3000 cm^{-1} for the hydroxyl group and 2900–2960 cm^{-1} for C-H stretching. The peak at 1600–1650 cm^{-1} was due to OH bending [20]. The presence of C-O-H bending at 1400–1450 cm^{-1} , CCO stretching at 1100–1150 cm^{-1} , and C-O stretching at 1000–1050 cm^{-1} were observed for commercial glycerol and glycerol residue

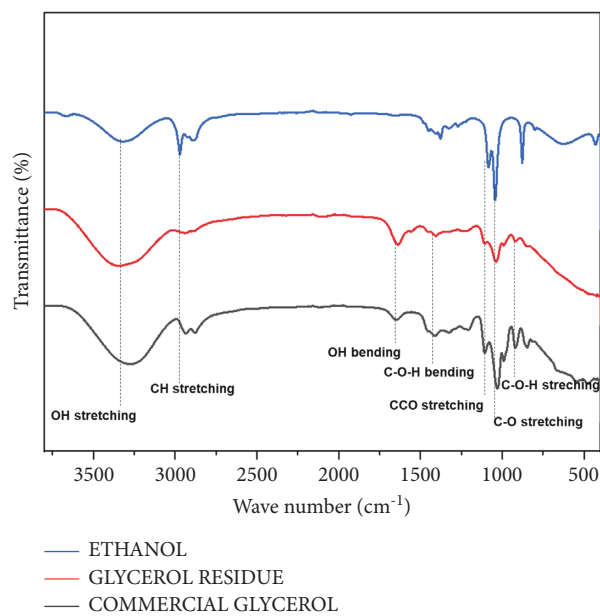


FIGURE 1: FTIR spectrum of glycerol (commercial), glycerol residue, and ethanol.

[21]. Specifically, in ethanol, the peak intensity at 3500–3000 cm^{-1} decreased sharply for the OH group, and an increase in the peak at 2972 cm^{-1} was due to the methyl group (C-H stretching) [22], and 1044 cm^{-1} for C-O stretching was noticed.

High-performance liquid chromatography revealed the presence of glycerol and ethanol. Glycerol residue contains glycerol, and the peak was confirmed at a retention time of 14.218, whereas the commercial glycerol peak was noticed at 14.234. The presence of glycerol in glycerol residue was similar to commercial glycerol. However, due to contaminants, another peak was identified in the glycerol residue at 7.332 and 12.88. The ethanol peak was obtained at a retention time of 20.55. The HPLC chromatograms of commercial glycerol, glycerol residue, and ethanol are shown in Figure 2. Bacteria such as *Escherichia coli* have been found to thrive in even the most depleted environments. Different species of *E. coli* have different optimal development circumstances, such as temperature, salt concentration, pH, and the source of nutrients. For *E. coli* to grow, a carbon source must be present in the medium. Sulfur, magnesium, phosphorus, and nitrogen are the essential elements that are found in abundance in carbon-based compounds. Sugars and certain salts are commonly employed as carbon sources for synthesizing various critical cell components of bacteria. In addition to glycerol as a carbon source, other parameters like temperature and pH are also responsible for the increased production of ethanol.

The source of nitrogen is a mixture of constituents that aids in the growth of bacterial cells in a culture medium. The most common source of nitrogen is tryptone which increases enzyme activity. In most cases, it offers the essential amino acids that bacteria need to grow and thrive. Transport processes and the osmotic equilibrium are supported by sodium chloride, which acts as an additive. The medium's

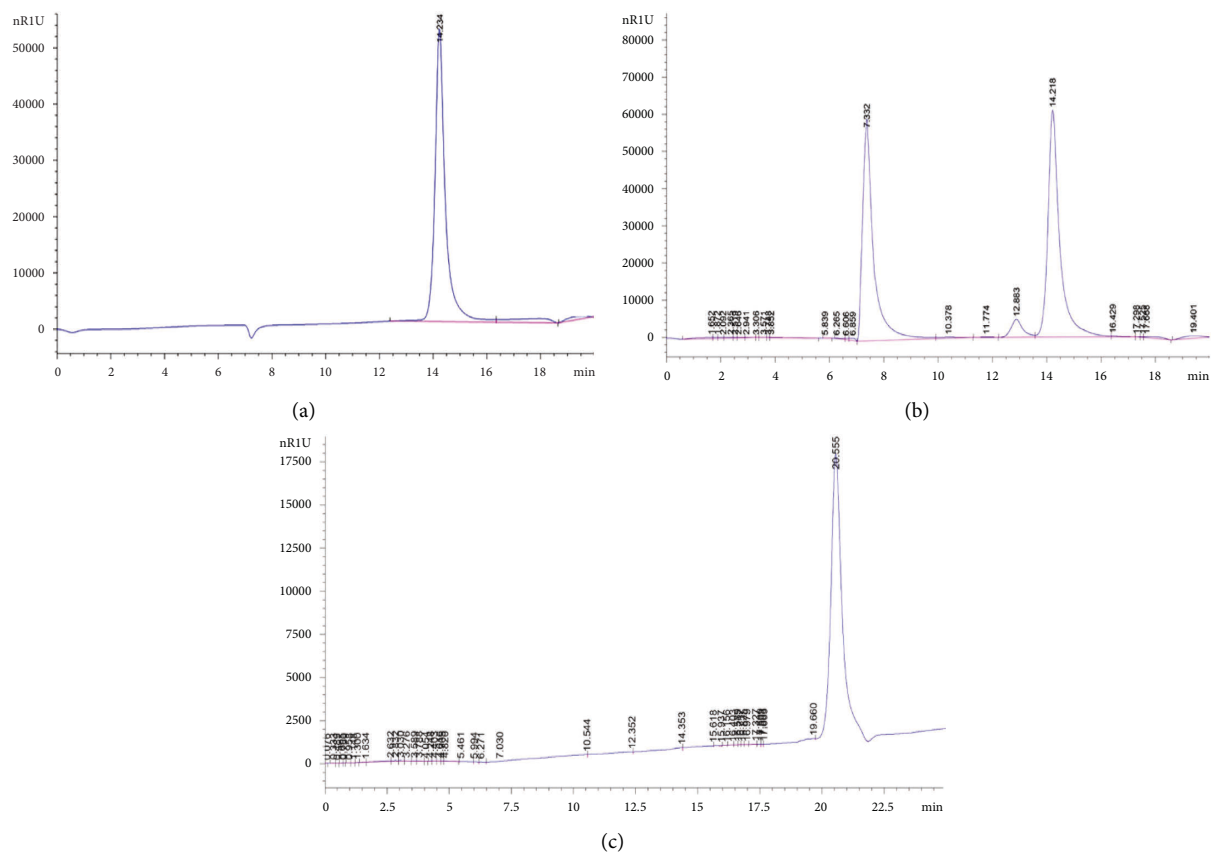


FIGURE 2: HPLC analysis of (a) glycerol (commercial), (b) glycerol residue, and (c) ethanol.

pH and temperature are critical for *E. coli* growth and must always be maintained. A pH of 7 and a temperature of 37°C are the ideal conditions for *E. coli* growth. The organism would not be able to grow at higher temperatures. Ethanol can be produced by fermentation using *E. coli* at 37°C within 24 hours. The rate at which ethanol is produced is inversely proportional to the temperature influence on the enzymes involved in its formation.

3.2. Optimization of Process Parameters. Improvement in process design and output can be achieved by a better understanding of cell development and product generation dynamics. The process parameters such as pH, temperature, substrate, inoculum, and time play a significant role in ethanol formation. The experiment was designed to optimize ethanol production to define and forecast the ideal circumstances in the experimental area. The Taguchi models were used to select important parameters to optimize. Modeling and optimization of ethanol production using Taguchi were accomplished. Based on previous research studies, the input data ranges and parameters were chosen. Using Minitab for the design of the experiment, the experimental data for ethanol concentration were incorporated into the quadratic polynomial prototype with input parameters. The analysis of variance (ANOVA) was used to determine the model's significance. Before utilizing statistical approaches to optimize media components, actual tests

and a literature study were conducted to assess the potential range of each factor.

The key media components affecting bioethanol production were glycerol waste (mass substrate), inoculum density, and time. The various ranges for each element were approximately 5 to 25 g/L for glycerol residue (mass substrate), 10 to 30% of inoculum density, and 6 to 30 hours used for the experiment design. However, the range of input parameters was finalized with 18–22 g/L, 18–22% inoculum, and 10–14 hours. Higher-order response surfaces are generated using Taguchi, which requires fewer runs than a traditional factorial technique. The main effects of data means of time, mass substrate, and inoculum concerning ethanol are shown in Figure 3. The results of each media component on bioethanol production were investigated using Taguchi, and data were used to analyze variance (ANOVA).

Various values (*P* value, *f* value, coefficient of variation, and determination coefficient) obtained from ANOVA demonstrate that the selected model is significant. The *P* value was less than 0.002, which is essential for understanding the pattern of mutual interactions between the variables. When the *P* value is less than 0.05, the variables are usually far more variable and statistically significant. The analysis of the variance of signal-to-noise (SN) ratio with the degree of freedom (DF), the sequential sum of squares (Seq SS), the adjacent sum of squares (Adj SS), adjacent mean square (Adj MS), and probability (*P*) are shown in Table 1.

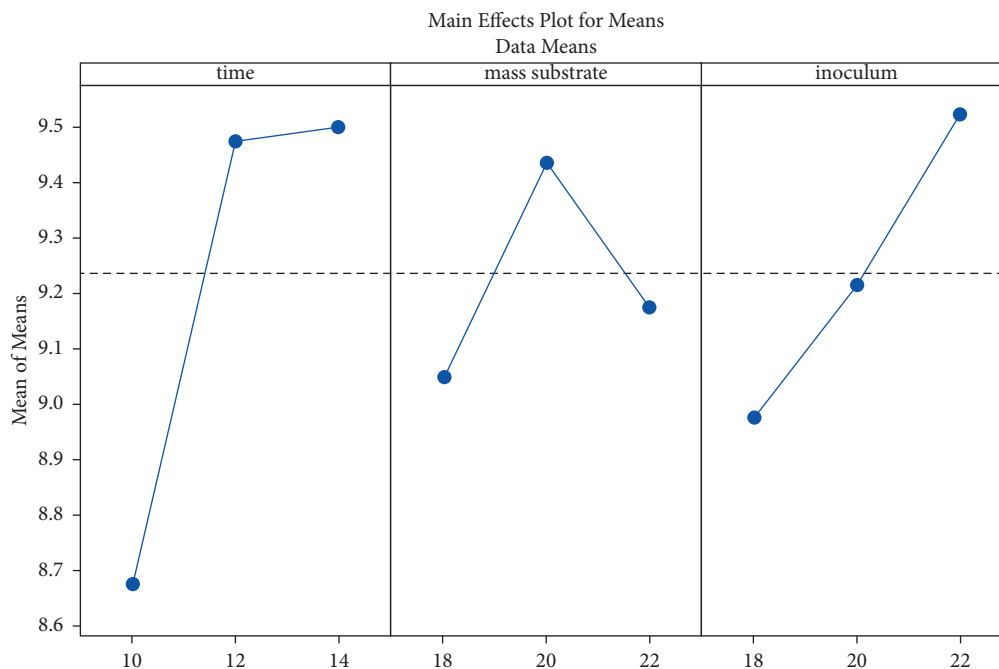


FIGURE 3: The main effects for means of time, mass substrate, and inoculum.

TABLE 1: Analysis of variance for signal-to-noise (SN) ratios.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	2	1.6761	2.1854	1.09272	22.39	0.002
Mass substrate	2	0.5879	0.8034	0.40169	8.23	0.019
Inoculum	2	0.7707	0.7707	0.38536	7.90	0.021
Residual error	6	0.2928	0.2928	0.04880		
Total	12	3.3275				

The F-test resulted in a statistically significant model. The F value is a statistically reliable indicator of how well the factors account for variation in the data around their mean. The model has a high determination coefficient (Rsq 0.8240), accounting for 82.40 percent of the response variability. A more significant determination coefficient suggests that the relationship between the experimental and anticipated data is highly reliable. A model's Rsq should be greater than 0.80 to indicate a good fit. As a result, these factors' changes could significantly impact glycerol fermentation and ethanol production. The *P* value of 0.001 for lack of fit suggests that the lack of fit is insignificant compared to the pure error. The nonsignificant lack of fit is good because it shows that the model fits the data well [23]. The significance of time, mass substrate, and inoculum contour plots for ethanol concentration are shown in Figure 4.

3.2.1. Substrate Concentration. Glycerol residue, a carbon source, was used as the substrate for ethanol production. The concentration of glycerol considerably influenced ethanol formation. The various carbon source concentrations affect the growth rate of microorganisms and the metabolic product. *E. coli* was used to test the effect of various glycerol concentrations on ethanol production. The relation between

the substrate concentration and ethanol formation is given in Figure 5.

A gradual increase in substrate concentration preceded to rise in the rate of ethanol formation when it reached 20 g/L of the substrate with maximum ethanol production at 10.0 g/L. The rise in the substrate to 25 g/L resulted in a steady decrease in ethanol concentration. The Monod equation explains the relationship between substrate concentration and the rate of chemical or enzymatic reaction [24].

$$\mu = \mu_{\max} \frac{S}{K_S + S} \quad (1)$$

Here, μ is the specific growth, μ_{\max} is the maximum growth rate, *S* is the substrate (concentration), and K_S is the concentration of substrate at μ equal to 0.5 μ_{\max} . It is explained by saturation kinetics in which the microbial growth rate depends on the maximum growth rate (μ_{\max}) and limiting constant (K_S). Moreover, the organism's growth relies on the uptake of substrate rate, which is directly proportional to substrate concentration.

The growth of the cell is associated with substrate concentration, shown by equation 1 which describes the link between substrate concentration and its uptake. While the carbon source is critical for heterotrophic organisms and

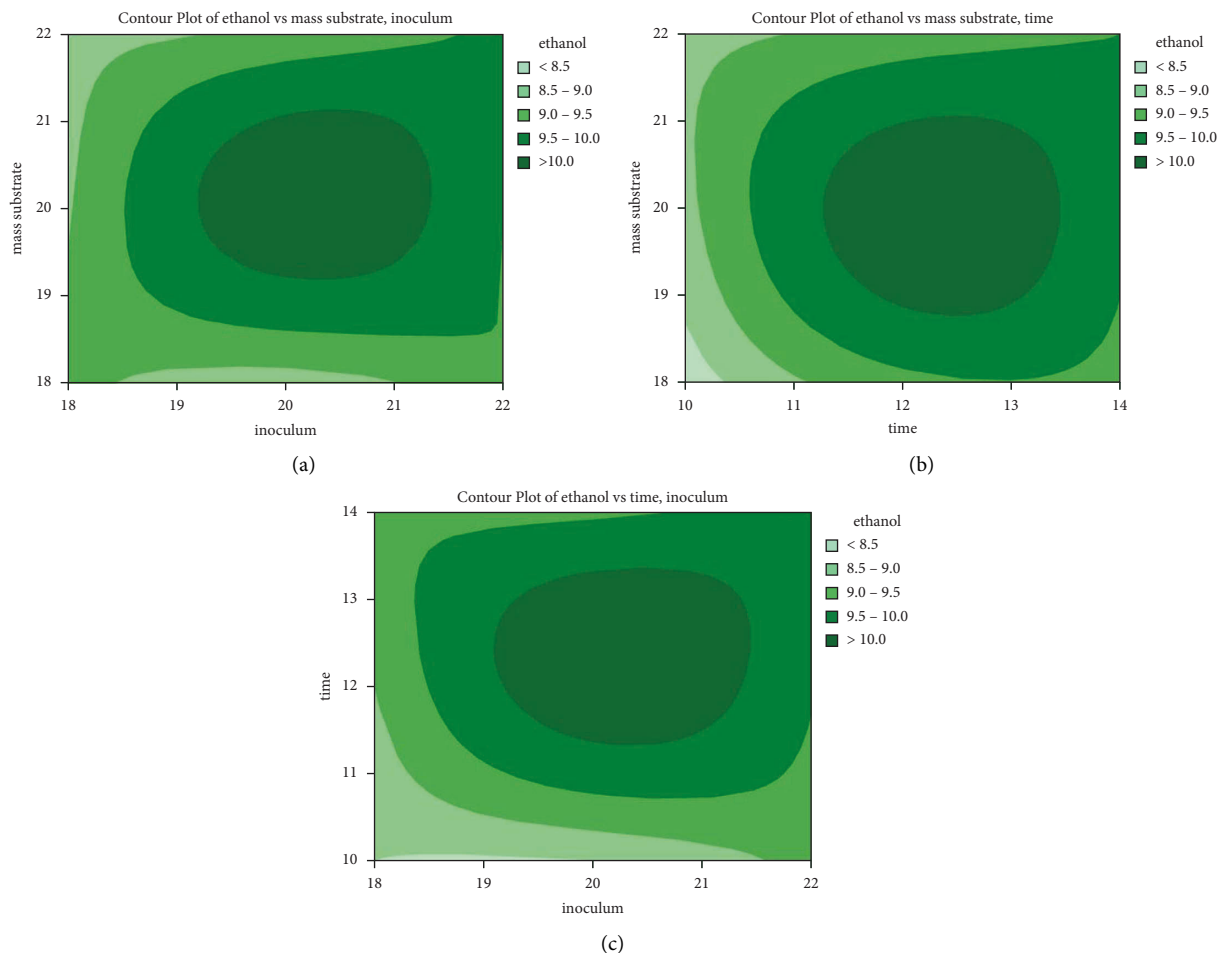


FIGURE 4: The contour plots of ethanol with two variables. (a) Mass substrate and inoculum, (b) mass substrate and time, and (c) inoculum and time.

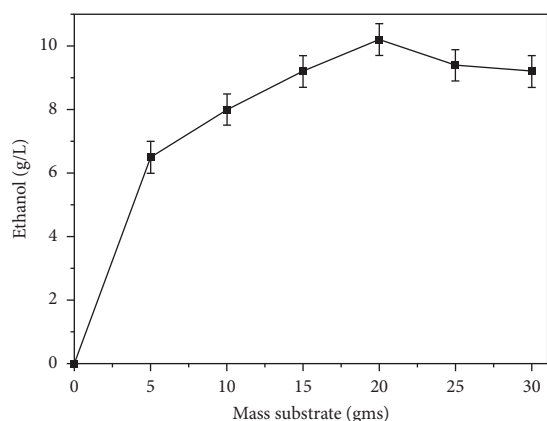


FIGURE 5: The relation of mass substrate for ethanol formation.

anaerobic fermentation, the rate of anaerobic uptake and generation of energy from organic substrate plays a significant role in limiting or enhancing the growth rate of an organism. Growth is regulated by limiting nutrient uptake, known as energy-limited growth. Butanol of 7.2 g/L was produced from 20 g/L of crude glycerol by *C. pasteurianum*

DSM 525 [25]. The crude glycerol of 34.5 g/L using *Escherichia coli* SS1 had 6.42 g/L of ethanol [23]. In another study, *Escherichia coli* MG1655 was utilized with 37.7 g/L of crude glycerol as a feed to produce 7.78 ± 1.52 g/L of ethanol [26]. Immobilized *G. oxydans* cells were used for dihydroxyacetone (DHA) production of 7.74 g/L, 6.84 g/L and 6.63 g/L by mechanical shaking with crude glycerol of 20 g/L, 30 g/L, and 50 g/L, respectively [27]. The cell growth and ethanol productivity were optimum with glycerol up to 24.3 g/L and 31.32 g/L using *E. aerogenes* TISTR 1468 [28]. Crude glycerol of 20 g/L produced 18.2 g/L of 1,3-propanediol using *Lactobacillus brevis* N1E9.3.3 [29]. The ethanol formation was influenced by the substrate concentration using immobilized *Saccharomyces cerevisiae* cells [30]. The immobilized cells of *Pachysolen tannophilus* were used to produce 6.2 g/L of ethanol from glycerol of 15 g/L, whereas the 50 g/L of glycerol produced 6.8 g/L of ethanol. The maximum ethanol of 8.3 g/L was made from the 25 g/L glycerol substrate. According to the findings, different products were affected by varying substrate concentrations. A substrate concentration of 25 g/L favored the maximum ethanol production [31]. The concentration of substrate had a considerable effect on the ethanol formation.

Glycerol concentrations in the range of 5 to 20 g/L were focused on in most studies for ethanol production, whereas concentrations greater than 20 g/L glycerol have only been described in very few reports [23]. However, the maximum ethanol was noticed with 25 g/L of crude glycerol, and the inhibition effect was detected at 40 g/L of crude glycerol using *E aerogenes* [32]. Increasing the glycerol concentration increases the ethanol formation rate up to a point before it begins acting as an inhibitor. The higher concentration of the glycerol residue led to substrate saturation and the cause of inhibition. Slower reaction rates can be observed at high levels of substrate concentrations, which can have an impact on phase solution reactions. A low conversion rate is caused by a high substrate saturation level i.e. oversupplied with the excess substrate may be toxic to the organisms [33]. Cell growth is reduced as a result of partial dehydration, which is caused due to an elevated substrate concentration [34]. In this study, substrate inhibition increased with a glycerol concentration of 25 g/L.

3.2.2. Inoculum. Inoculation is also one factor influencing the rate of reactions. Figure 6 depicts the impact of various inoculums on ethanol production. The correlation between the inoculum size and the amount of alcohol produced was observed in this study. Figure 6 shows the optimum inoculum size was 20% v/v for a maximum ethanol concentration of 10.0 g/L. *E. coli*, after being trapped in sodium alginates, were found to have different growth rates, which resulted in different production yields at different inoculum sizes. The ethanol concentration decreased when the inoculum size was reduced to 10% v/v. However, an increased inoculum size to 30% v/v resulted in lower ethanol formation due to the quick utilization of available nutrients in the medium, with higher cell numbers accompanied by cell starvation and death.

A preactivated bacterial seed culture at 10% v/v inoculum was added to the bioreactor for ethanol production [35]. *Klebsiella pneumoniae* of 2.5% v/v inoculum was used to transform crude glycerol into ethanol [36]. *Saccharomyces cerevisiae* of 5% which was entrapped in beads influenced the ethanol production rate. The amount of cell entrapment in the beads substantially impacts product formation [19]. The growth rate increases as the number of cells (N) or biomass (X) increases. The number of cells is directly proportional to the organism's growth rate. Ethanol production increased as a result of the rapid growth rate. Several factors could decrease production levels, such as reduced *E. coli* activity from an overabundance of cells and nutrient exhaustion in the medium.

3.2.3. Time. The glycerol to ethanol conversion was determined by measuring the fermentation time. The impact of fermentation time is depicted in Figure 7. The research study found that the maximum concentration of 10.0 g/L ethanol after 6 hours of fermentation tends to decrease over time. The decline was due to *E. coli* entering a growth phase involving idle activity after 12 hours. It was found that the organism's growth rate varied depending on its stages of growth, that is, lag, acceleration, and exponential growth

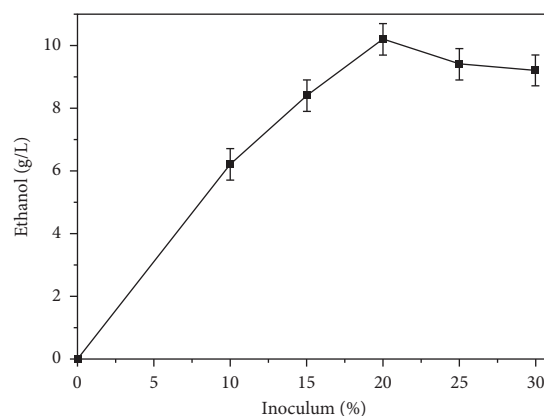


FIGURE 6: The effect of inoculum on ethanol formation.

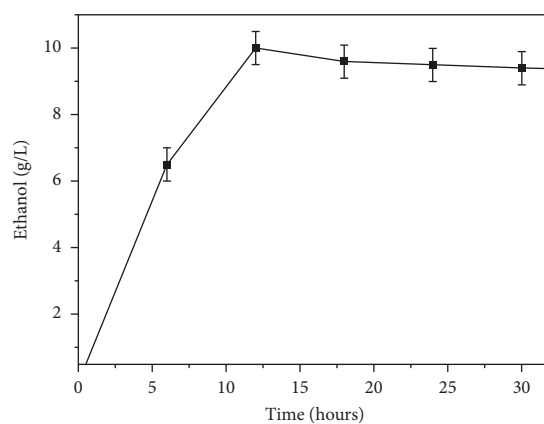


FIGURE 7: The effect of time on ethanol formation.

phase. In the exponential phase of bacterial growth, regardless of whether nutrients or inhibitors are present in the medium, all cells become accustomed to dividing and multiplying. Later, the growth phase did not end up with a lag phase, but only the starting of fermentation; other stages of the organism's growth profile must be taken into account. The organism's growth slowed after 12 hours, a symptom of the deceleration or retardation phase, during which nutrients are depleted, and toxins accumulate in the medium, preventing further growth. After this stage, the growth rate depends on the concentration of nutrients, products, and time. Due to the depletion of nutrients in the media, the specific growth rate eventually reaches zero. The constant rise in the number of cells may cause morphological changes around the cell, but the specific growth rate tends to decrease over time. Some products, such as intracellular materials, may allow the proliferation of a few cells, whereas others die. In the last phase, the cells can no longer maintain their physiological activities, and the specific growth rate decreases to zero.

The impact of time on immobilized cells has been reported in several studies. Sodium alginate immobilized *Saccharomyces cerevisiae* yeast which was used to produce ethanol from cane molasses. The effect of ethanol production is affected by time. The maximum ethanol production was

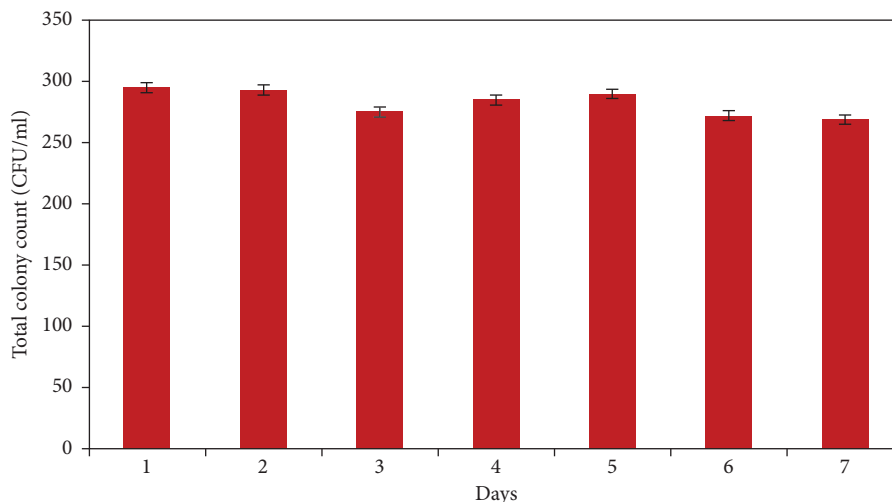


FIGURE 8: The total plate count of *E. coli* from day one to seven days at -4°C .

noticed with a hydraulic retention time of 15.63 hours [30]. The maximum ethanol production of 8.90 g/L was obtained after 48 h from sugarcane bagasse hydrolysate by immobilizing yeast *Scheffersomyces stipitis* in a calcium alginate matrix [37]. After a 12-hour preincubation period, 6.49 g/L of waste glycerol was converted to 2.18 g/L ethanol, yielding 0.67 mol-ethanol/mol-glycerol using *Enterobacter aerogenes* ATCC 29007 immobilized cells [16]. Immobilized *Saccharomyces cerevisiae* cells in calcium alginate beads fermented coffee mucilage into ethanol directly. The maximum ethanol of 16.87 ± 0.11 g/L was produced at 18 hours with a 2% calcium alginate concentration [38]. The maximum ethanol production by fermentation glycerol residue with immobilized *E. coli* was obtained at 12 hours.

3.3. Immobilized Cell Stability. Immobilization protects cells from harsh environments; hence, the temperature stability of the immobilized *E. coli* cells at -4°C was investigated and is shown in Figure 8.

The results show that the number of *E. coli* cells in a bead on the first day was 295×10^3 CFU/ml and they were retained in 10^3 CFU/ml for a week. The *E. coli* level remained almost the same on day 2 (293×10^3 CFU/ml) and it lowered on day three (275×10^3 CFU/ml), then it raised slightly to 285×10^3 CFU/ml and further increased to 290×10^3 CFU/ml on the 5th day. The cells remained almost high till five days of storage; however, the number of cells gradually reduced on the 6th and the 7th day as particular cells died due to temperature, handling, and bead characteristics. However, the new cells are preferred for the experiment and these cells can be stored for five days.

Morphological differences were not noticed even after a week of storage at low temperatures. Although slight changes in cell concentration within the beads were observed when stored at low temperatures for a week. *E. coli* was trapped in alginate beads by the 2% alginate solution mixed with bacteria dropped into the CaCl_2 solution. Uniform immobilized *E. coli* beads from calcium alginate were

obtained in the size of 2 mm. The beads formed were round, as shown in Figure 9(a). The beads were produced due to ionic interaction between Ca^{2+} ions and carboxylate groups of alginates. A higher concentration of alginates make smaller pore size of beads with reduced immobilization efficiency. A lower concentration of alginates results in fragile beads and large pores that lead to a release of bacterial cells from beads. The increased CaCl_2 concentration strengthens the calcium bond with alginate. Figures 9(a) and 9(b) depict the surface morphology of immobilized *E. coli* cells in calcium alginate beads at 60 and 100 magnification. According to Sar et al., for repeated batch culture, the immobilized bead cells were kept for 60 days at 4°C in the different storage solutions. One storage solution contained 0.2% of both glucose and yeast extract. Another storage solution contained only 2% CaCl_2 [39].

The stability of the bead was tested for one week in the incubator at 37°C . According to the findings, the bacteria's activity increased with time. The ideal temperature for growth was 37°C . Among various temperatures, the highest growth occurred at 37°C . However, the organism did not grow at 50°C . Similarly, the *E. coli* K12 strain thrived at 37°C . The influence of temperature affects the enzymes' activities, and it was noticed that the maximum is 37°C . Bacteria developed their growth at different temperatures for the most part [40]. Most have the optimum range between 20 and 30°C . However, some prefer warmer temperatures of $50\text{--}55^{\circ}\text{C}$, and others prefer cooler temperatures of $15\text{--}20^{\circ}\text{C}$. For instance, lactic acid bacteria and *Leuconostoc* species work best at $18\text{--}22^{\circ}\text{C}$. The *Lactobacillus* species grow at a temperature optimum above 22°C [41]. The product formation was affected by the growth activity of *Escherichia coli*, and the ideal temperature resulted in higher productivity and yield. Adnan et al. used a temperature of 37°C for the anaerobic fermentation of *E. coli* for ethanol production [23].

Improved stability is one of the significant goals of immobilization. Zhang et al. performed the thermal inactivation study at various temperatures (50 to 80°C) and

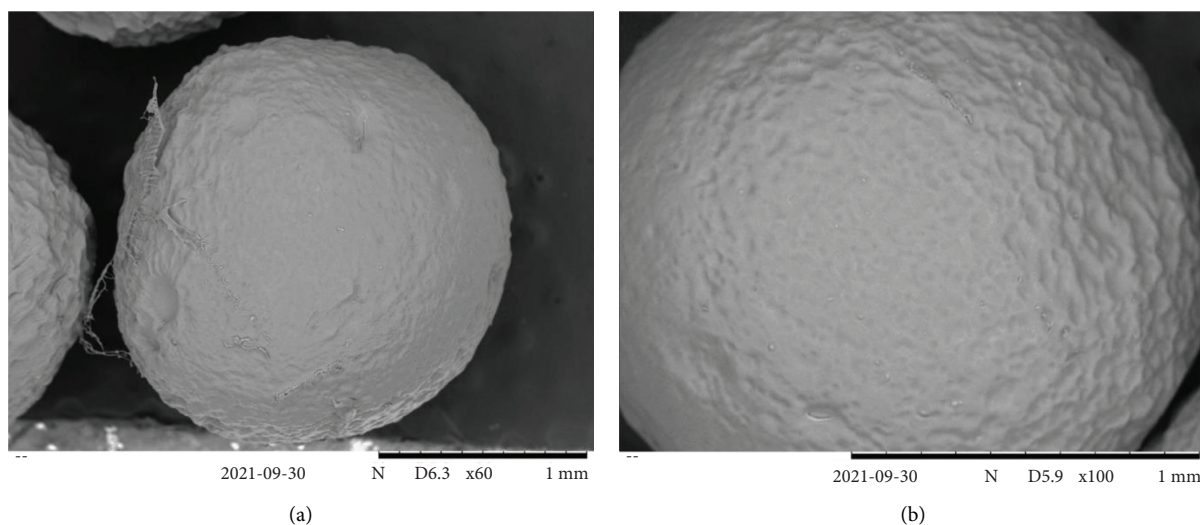


FIGURE 9: SEM of immobilized *Escherichia coli* cells. (a) 60K magnification and (b) 100K magnification.

noticed the immobilized cells showed high stability at 50°C and 60°C; however, there was little activity loss after 24 h incubation [42]. The optimal temperature for growth and ethanol productivity was found to be 30°C by immobilizing yeast cells using cane molasses [43]. Glucose and sucrose fermentation with immobilized *Saccharomyces cerevisiae* cells with chitosan-coated Ca alginate, and calcium alginate beads yielded ethanol at 30°C [44]. The immobilized *E. coli* strain TS3 was incubated at 37°C and produced ethanol from cheese whey powder [39].

3.4. Immobilized Cell's Reusability. The reusability of the cells is a significant advantage of the cell immobilization process. Cells can be easily separated from the slurry using the immobilization process. Immobilized cells could be reused up to eight times in a 10-hour fermentation cycle using chitosan-covered calcium alginate and alginate beads [44]. Sar et al. noticed higher ethanol production for immobilized *E. coli* TS3 than immobilized *E. coli* FBR5 strain for all 14 cycles [39]. A practical method for immobilization of *Saccharomyces cerevisiae* C12 was applied, and the entrapped cells produced high levels of ethanol for more than 42 days [45].

Immobilized cells were recycled for about six rounds of the fermentation process. The ethanol concentration was sustained after the sixth round of fermentation. After about six cycles, the immobilized cell beads can be reused with slight or no changes in the amount produced, as shown in Figure 10. In terms of sustainability, there is less waste of resources and time, which benefits the environment. As a result, the fermentation environment can maintain many viable cells.

Even though the first and the second fermentation processes produced slightly different results, the yield remained high after comparing the first round with fresh beads and the difference was insignificant. A high level of ethanol output was maintained throughout cycle six, despite minor fluctuations in values. As a result, it can be said that

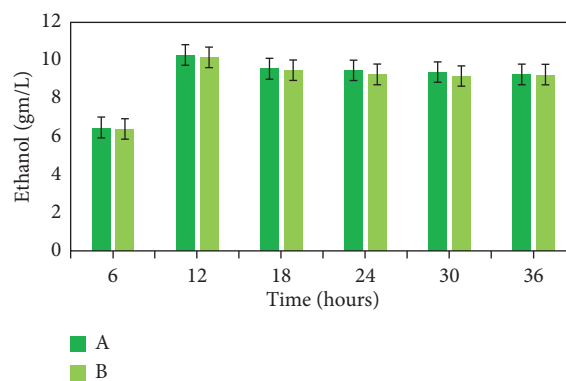


FIGURE 10: Ethanol production after (a) first and (b) second rounds of immobilized *E. coli* cells.

immobilizing *E. coli* during ethanol production is a better option, with the added benefits of being easy to handle and reusable.

4. Conclusion

Utilizing glycerol waste from the biodiesel industry and productivity of the desired product are the primary objectives of optimizing the fermentation process. There is a considerable interest in using crude glycerol for ethanol production. The present study shows the utilization of glycerol waste as a clean and green approach to produce ethanol by the fermentation process. To further improve and reduce the cost of ethanol production, the immobilization technique was employed with *E. coli* cells. There were pronounced effects with substrate concentration, inoculum size, and time using immobilized cells for bioethanol production. Finally, immobilized *Escherichia coli* cells can utilize glycerol residue as the carbon source to generate ethanol with the advantage of reusing the immobilized cells as biocatalysts and for easy usage.

Data Availability

The data used to support the findings of this study have not been made available because of the funding agency requirements.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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).

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