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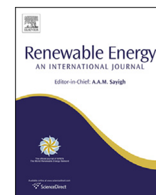

Optimization of bioethanol production from glycerol by *Escherichia coli* SS1

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A B S T R A C T

Bioethanol is a promising biofuel and has a lot of great prospective and could become an alternative to fossil fuels. Ethanol fermentation using glycerol as carbon source was carried out by local isolate, ethanologenic bacterium *Escherichia coli* SS1 in a close system. Factors affecting bioethanol production from pure glycerol were optimized via response surface methodology (RSM) with central composite design (CCD). Four significant variables were found to influence bioethanol yield; initial pH of fermentation medium, substrate concentration, salt content and organic nitrogen concentration with statistically significant effect ($p \le 0.05$) on bioethanol production. The significant factor was then analyzed using central composite design (CCD). The optimum conditions for bioethanol production were substrate concentration at 34.5 g/L, pH 7.61, and organic nitrogen concentration at 6.42 g/L in which giving ethanol yield approximately 1.00 mol/mol. In addition, batch ethanol fermentation in a 2 L bioreactor was performed at the glycerol concentration of 20 g/L, 35 g/L and 45 g/L, respectively. The ethanol yields obtained from all tested glycerol concentrations were approaching theoretical yield when the batch fermentation was performed at optimized conditions.

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1. Introduction

Many valuable chemicals can be produced from the microbial fermentation of glycerol, including 1,3-propanediol, dihydroxyacetone, ethanol and succinate. In this context, glycerol is used as a substitute for common, traditional substrates such as sucrose, glucose and starch [1]. Importantly, the fuels and reduced compounds from these glycerol fermentations can be produced at higher yields than those obtained from common sugars [2]. This is possible because the degree of reduction per carbon, k [3], is significantly higher for glycerol ($C_3H_8O_3$: $k = 4.67$) than for sugars such as glucose ($C_6H_{12}O_6$: $k = 4$) or xylose ($C_5H_{10}O_5$: $k = 4$). The conversion of glycerol to phosphoenolpyruvate or pyruvate produces twice the amount of reducing equivalents than the same conversions from glucose or xylose. Glycerol is produced by microbial fermentation and chemical synthesis [4]. It is also produced abundantly as a by-product of both soap manufacturing and biodiesel production. Due to these advantages, glycerol has become a potential feedstock in the production of various chemicals via fermentation processes.

Bioethanol is one of the fermentative products that can be generated from glycerol via anaerobic fermentation. Nakas et al. [5] described the ethanol productivity of a soil bacterium tentatively classified as a member of the genus *Bacillus* that produced ethanol with final concentration of 7.0–9.6 g/L from a glycerol-enriched algal mixture. Jarvis et al. [6] found that formate and ethanol were the major products of glycerol fermentation by *Klebsiella planticola* isolated from the rumen. Bioethanol is viewed as an alternative to biofuels because of its nature as a renewable biobased resource and because it provides the potential to reduce particulate emissions [7]. Currently, the majority of bioethanol production is from food crops such as corn, sugarcane, wheat and soy. This has led to undesirable effects with respect to food production, including increases in food prices, a shortage of fodder, and growing competition for land [8–10]. The utilization of biomass or glycerol-containing-waste for the production of bioethanol therefore has considerable potential to alleviate these undesirable effects on food production.

According to studies of the conversion of glycerol into ethanol, hydrogen and other chemicals [11,1,12–14], glycerol can be used as a source for producing biofuels. The ethanol produced in these

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reactions is affected by the glycerol concentration [12]. The maximum yield achieved by *Enterobacter aerogenes* HU-101, *Klebsiella* sp. HE1 and *Escherichia coli* was 0.6–1 mol ethanol/mol glycerol at a relatively low glycerol concentration of 10 g/L. In a study by Suhaimi et al. [1], the yield observed in *E. coli* SS1 was 0.8 mol ethanol/mol glycerol using 20 g/L of glycerol. To our knowledge, the majority of the reported ethanol fermentations using glycerol as a substrate were conducted at laboratory scale using a serum bottle and 500 mL flasks. Although the ethanol yield achieved in these settings was quite promising, studies of the conversion of glycerol into bioethanol in a bioreactor at relatively high glycerol concentrations are rare.

E. coli SS1 is a potential ethanol producer that can consume glycerol at concentrations over 10 g/L with high yields, as reported by Suhaimi et al. [2012]. This study describes the optimization of ethanol production from glycerol using a statistical tool, Response Surface Methodology (RSM). The usage of central composite design (CCD) is advantageous as it is an efficient design that is ideal for sequential experimentation and provides a reasonable amount of information to test lack of fit while not requiring an excessive number of design points. In fact, CCD is the most popular class of second-order design and consists of: (1) a full factorial (or fractional factorial); (2) an additional design (often a star design in which experimental points are at a distance from the center) and (3) a central point [16]. In this context, CCD is well suited for fitting the complicated surfaces that were selected for the experimental design in this study. In addition, CCD works well for process optimization and is an effective design that is ideal for chronological experimentation. The parameters involved in this optimization process were initial pH of the fermentation medium, substrate concentration, salt content and organic nitrogen sources. The effect of glycerol concentration on ethanol fermentation was further investigated by performing a series of fermentations in a 2-L bioreactor under optimum conditions. The tested glycerol concentrations were relatively high, i.e., 20 g/L–45 g/L.

2. Materials and methods

2.1. Strain and culture media

E. coli SS1 obtained from a stock culture was streaked on a Modified-Luria–Bertani (LB) plate containing, per liter, 5 g of yeast extract, sodium chloride; 5 g of peptone; 10 g of 20 g/L glycerol and 1.5% (w/v) technical agar. Chemical grade pure glycerol was used in this experiment (unless otherwise mentioned) as the sole carbon source to favor the growth of glycerol-utilizing bacteria [11]. The strain was incubated at 37 °C for 24 h. A single colony was then inoculated into a flask containing Modified Luria–Bertani media supplemented with 20 g/L glycerol and incubated in a rotary shaker at 37 °C at 120 rpm until it reached the active state ($OD_{600} = 1.0$). This culture was then used as the inoculum for fermentation. The medium for the inoculum preparation was transferred into screw-capped shake flasks, flushed with nitrogen gas and then sterilized at 121 °C for 15 min.

2.2. Fermentation procedure

The fermentation media used in this experiment was modified according to Ito et al. [11] and contained, per liter, 7.0 g of K_2HPO_4, 5.5 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.021 g of $CaCl_2 \cdot 2H_2O$, 0.12 g of $Na_2MoO_4 \cdot 2H_2O$, 2.0 mg of nicotinic acid, 0.172 mg of Na_2SeO_3, 0.02 mg of $NiCl_2$ and 10 mL of a trace element solution containing 0.5 g of $MnCl_2 \cdot 4H_2O$, 0.1 g of H_3BO_4, 0.01 g of $AlK(SO_4)_2 \cdot H_2O$, 0.001 g of $CuCl_2 \cdot 2H_2O$ and 0.5 g of Na_2EDTA per liter. Tryptone and yeast extract were added at the desired concentrations to the medium, in which pure glycerol was used as the sole carbon source. Comparison fermentation was conducted using crude glycerol obtained from a biodiesel production plant (Carotech Bhd, Perak, Malaysia). The crude glycerol consisted of glycerol (50%–80%), alkaline compounds such as soaps and hydroxides (3%–5%), methyl esters, methanol, water and other components. The crude glycerol was alkaline and had a pH value in the range of 9–11, while the moisture content was approximately (2%–20%).

The fermentation was performed in a 120 mL serum bottle with a total working volume of 50 mL. Anaerobic conditions were created by flushing the serum bottle with nitrogen gas or argon gas. The preparation and inoculation were performed in an anaerobic chamber to maintain anaerobic conditions. The sealed serum bottle was incubated at 37 °C with an agitation speed of 120 rpm. The sampling of the fermentation broth was performed at 12-h intervals, and the samples were subjected to further analysis.

2.3. Experiment design and statistical analysis

In this experiment, six quantitative variables that were expected to influence ethanol production were selected. These variables were determined by employing a two-level factorial design that included initial pH of fermentation medium, incubation temperature (°C), substrate concentration (g/L), organic nitrogen sources (g/L), salt content (g/L), and trace element solution (ml/L). The real and coded values of these variables are presented in Table 1. This design considered the interaction effects among the variables that affected the response based on the contribution percentage of the tested variables. The experimental data analyses were performed using Design Expert® software version 7.0 (STAT-EASE Inc., Minneapolis, USA). All experiments were conducted in triplicate to reduce variability in the data collection. The software was designed with 32 experimental runs and 3 center points, providing a total of 35 experimental runs. The variables that significantly affected ethanol production were determined using a confidence level above 95% or a p-value less than 0.05.

The significant factors identified in the 2-level factorial experiment were employed in CCD. The optimum conditions for maximum ethanol production were calculated and evaluated using Design Expert® software version 7.0. For each variable studied, the high and low levels were selected according to the results obtained from the 2-level fractional factorial design (Table 2). All experiments were performed in triplicate with five center points to verify the accuracy of the model predicted by the software. Three-dimensional plots and their respective contour plots were obtained based on the effects of the levels of two parameters (at five different levels each) and their interactions on the maximum ethanol production by fixing the other parameters at their optimal conditions. From these contour plots, the interaction of one parameter with another parameter was studied. After the optimum conditions were identified, a validation experiment was performed to verify the predicted values for maximum ethanol production obtained from the software.

Table 1
2-Level fractional factorial design for bioethanol production.

Variables	Unit	Low level (−1)	0	High level (+1)
A: pH	–	5	7	9
B: Substrate concentration	g/L	5	37.5	70
C: Temperature	°C	25	35	45
D: Salt content	g/L	0	25	50
E: Trace element solution	ml/L	0	10	20
F: Organic nitrogen concentration	g/L	0.5	5.25	10

Table 2
Coded Values for each variable of the Central Composite Design (CCD) for bioethanol production.

Variables	Unit	$-\alpha$	-1	0	$+1$	$+\alpha$
A: pH	–	5	6	7	8	9
B: Substrate concentration	g/L	5	16.25	27.5	38.75	50
C: Organic nitrogen concentration	g/L	1	3.25	5.5	7.75	10
D: Salt content	g/L	0	7.5	15	22.5	30

2.4. Effect of pure glycerol concentration in a 2 L bioreactor

The batch fermentation experiment was initiated by inoculating a 10% volume of cells into a 2 L bioreactor with a working volume of 800 mL that contained the production media described above [11]. Fermentations were conducted using pure glycerol at concentrations of 20 g/L, 35 g/L and 45 g/L. The bioreactor was flushed with nitrogen gas to provide anaerobic conditions. The fermentation was performed at 37 °C with an agitation speed of 50 rpm for 120 h. The samples were withdrawn periodically for the determination of the ethanol and glycerol concentrations.

2.5. Analytical methods

2.5.1. Cell determination

The relative biomass density was measured via optical density at a wavelength of 600 nm. The cell dry weight was determined by sedimenting the cell using centrifugation at 8000 rpm and 4 °C for 10 min. Subsequently, the correlation between the relative biomass density and the cell dry weight was determined as 1 OD = 0.29 g-CDW/L. This correlation was used throughout the experiment to determine the biomass density, expressed as g/L. The cell count was performed by transferring 1 mL of broth to a universal bottle containing 9 mL of 0.85% sterile saline water. Appropriate dilution was performed, and a total of 100 µl of the diluted sample was then transferred to LB containing glycerol agar plate. The plate was then incubated for 24 h at 37 °C incubator. The number of colony formed was counted at average 3 plates containing 30–300 colonies per plate. The unit used is expressed as CFU/ml.

2.5.2. Ethanol analysis

Ethanol concentration was determined by using gas chromatography GC-17A (Shimadzu, Japan) using BP 21 column (25-m length × 0.53-mm internal diameter × 0.5-µm film thickness), helium gas as the carrier gas and flame ionization detection (FID) at temperatures of 150 °C and 200 °C. The oven temperature was initially maintained at 40 °C for 1 min and then increased to 130 °C at a gradient of 20 °C/min. 1-Propanol was used as the internal standard.

2.5.3. Glycerol assay

The glycerol content was measured using a free glycerol reagent, Cat. No F6428 (Sigma, USA), and indicated by an increase in the absorbance at 540 nm, which is directly proportional to the free glycerol concentration of the sample.

3. Results and discussion

3.1. Screening of the significant factors affecting ethanol production from glycerol by E. coli SS1 using 2-level factorial design

Glycerol was fermented anaerobically to produce ethanol by *E. coli* SS1 [11,17,18]. However, a number of variables could potentially restrict the effective fermentation activity and thus affect ethanol yields. Greater ethanol yields could therefore potentially be obtained by optimizing the fermentation parameters. A two-level factorial design was used to screen for significant variables that affected bioethanol production. The six variables hypothesized to influence the ethanol production from glycerol, with respective runs and responses, are shown in Table 3. The total number of runs was 35, with three center points generated from these six variables. As displayed in Table 4, only four of the six factors were significant, including pH, substrate concentration, salt content and organic nitrogen concentration. The significance of these factors, indicated by *p*-values of less than 0.05, demonstrates that these factors affect ethanol production. In contrast, ethanol production was less effected by temperature and trace elements, as indicated by the *p*-values of over 0.05. Generally, the *p*-value of lack of fit was not significant ($p = 0.992$), and the regression model was strongly significant ($p < 0.0001$, $R^2 = 0.9133$). The design indicates that the second-order model was fitted to the data using Equation (1)

$$Y = 1.49 + 0.16 \times A + 0.14 \times B + 0.083 \times C - 0.18 \times D - 0.078 \times E + 0.51 \times F - 0.11 \times B \times D + 0.12 \times B \times F - 0.26 \times C \times D + 0.19 \times C \times F - 0.12 \times A \times C \times F + 0.17 \times A \times D \times E + 0.24 \times A \times D \times F \quad (1)$$

where Y is the ethanol production (g/L) and A, B, C, D, E, and F represent initial pH, substrate concentration, temperature, salt content, trace element and organic nitrogen concentration, respectively.

3.2. Optimization of bioethanol production from glycerol by E. coli SS1

The effects of initial pH, glycerol concentration, salt content and organic nitrogen concentration on ethanol production were investigated. Regression analysis of the data from Table 2 resulted in the following quadratic Equation (2)

Table 3
Experimental data and results of CCD for ethanol production.

Run	A	B	C	D	Ethanol production (g/L)	
					Experimental	Predicted
1	6	16.25	3.25	7.5	6.65	7.24
2	8	16.25	3.25	7.5	6.72	5.76
3	6	38.75	3.25	7.5	4.2	4.60
4	8	38.75	3.25	7.5	10.48	9.85
5	6	16.25	7.75	7.5	7.45	7.80
6	8	16.25	7.75	7.5	6.78	7.26
7	6	38.75	7.75	7.5	6.78	8.35
8	8	38.75	7.75	7.5	14.23	14.54
9	6	16.25	3.25	22.5	5.16	4.36
10	8	16.25	3.25	22.5	5.84	4.82
11	6	38.75	3.25	22.5	4.23	4.30
12	8	38.75	3.25	22.5	12.32	11.48
13	6	16.25	7.75	22.5	4.41	5.59
14	8	16.25	7.75	22.5	7.86	6.98
15	6	38.75	7.75	22.5	8.25	8.72
16	8	38.75	7.75	22.5	16.87	16.83
17	5	27.5	5.5	15	3.39	1.51
18	9	27.5	5.5	15	6.33	8.15
19	7	5	5.5	15	2.89	3.46
20	7	50	5.5	15	11.31	10.68
21	7	27.5	1	15	4.05	5.68
22	7	27.5	10	15	13.28	11.59
23	7	27.5	5.5	0	12.91	11.88
24	7	27.5	5.5	30	10.34	11.30
25	7	27.5	5.5	15	15.62	15.41
26	7	27.5	5.5	15	15.47	15.41
27	7	27.5	5.5	15	15.45	15.41
28	7	27.5	5.5	15	15.44	15.41
29	7	27.5	5.5	15	15.52	15.41
30	7	27.5	5.5	15	15.48	15.41

Table 4
Analysis of variance (ANOVA) in 2-level fractional factorial design.

Source	Sum of squares	Mean square	*F*-Value	*P*-Value prob > *F*
Model	18.85	1.35	14.30	<0.0001
A	0.86	0.86	9.11	0.0071
B	0.62	0.62	6.56	0.0191
C	0.22	0.22	2.36	0.1410
D	1.01	1.01	10.72	0.0040
E	0.19	0.19	2.07	0.1669
F	8.45	8.45	89.68	<0.0001
Lack of fit	0.77	17	0.090	0.992
Pure error	1.02	2		
Cor total	158.30	34		

R-squared: 0.9133, Adjusted *R*-squared: 0.8494.

$$Y = 15.41 + 1.66 \times A + 1.81 \times B + 1.48 \times C - 0.15 \times D + 1.68 \times A \times B + 0.23 \times A \times C + 0.48 \times A \times D + 0.80 \times B \times C + 0.64 \times B \times D + 0.17 \times C \times D - 2.65 \times A^2 - 2.09 \times B^2 - 1.70 \times C^2 - 0.96 \times D^2 \tag{2}$$

where *Y* is ethanol production, A is pH, B is substrate concentration, C is organic nitrogen concentration and D is salt content. The quadratic model was selected to provide the best fit with the experimental results.

The model presented in Table 5 exhibits a high determination coefficient ($R^2 = 0.9474$), explaining 94.74% of the variability in the response, as well as a high value of the adjusted determination coefficient (adjusted $R^2 = 0.8861$), suggesting a high significance of the model. A very low probability ($p < 0.0001$) obtained from the regression analysis of variance (ANOVA) demonstrated that the model was significant. In this study, the factors of pH (A), substrate concentration (B) and organic nitrogen concentration (C) were the significant model terms, as the *p*-values calculated for these factors were less than 0.05. Therefore, changes in these parameters could significantly impact ethanol production from glycerol fermentation. The lack of fit *p*-value of 0.1585 implies that the lack of fit is not significant relative to the pure error. The non-significant lack of fit is positive because it demonstrates a good fit of the model to the data.

Response surface plots based on (Equation (1)), with the relationships between the response and variables, are presented in Fig. 1(a–f). The plots were constructed by plotting the response (ethanol production) on the *Z*-axis against any two dependent variables while maintaining the other variables at their optimal values. Fig. 1a depicts the interaction between the pH and the substrate concentration. High ethanol production was observed at middle pH ranges and relatively high substrate concentrations. The ethanol production was relatively high at pH 7.61. Reungsang et al. [19] and Varrone et al. [17] reported that ethanol production was greatly affected by pH and identified an optimum pH value of 8. Further increases in the pH resulted in lower ethanol production. The initial pH is an important factor that influences the NADH to NAD^+ ratio, which greatly affects the metabolic flux under anaerobic conditions [20]. Hence, to obtain optimal ethanol production, it is necessary to control the initial pH under optimum conditions [19].

Fig. 1b illustrates the interaction between pH and organic nitrogen concentration, which reflects the mixture of yeast extract and peptone at the same 1:1 ratio. High ethanol production was observed at near-neutral pH ranges and a relatively high concentration of organic nitrogen. A similar pattern was observed in Fig. 1d and f, where the ethanol yield was greater at higher organic nitrogen concentrations coupled with a high substrate concentration and a middle range of the salt content, respectively. As a result, the ethanol production was greatest at an organic nitrogen concentration of 6.42 g/L. Organic nitrogen is needed as an important supplement for microbial growth as well as for its alcohol dehydrogenase activity [20]. The important elements in yeast extract, such as Mg^{2+}, Fe^{2+}, Zn^{2+}, Cr^{2+}, and Pb^{2+} [21], are important cofactors in the metabolic pathways of microorganisms and influence substrate uptake by the microorganisms, which affects product yield [22].

Fig. 1a,d,e shows the effect of the substrate concentration in relation to the pH, nitrogen source and salt content, respectively. High ethanol production was achieved at a high substrate concentration of 34.53 g/L. The observed increase in ethanol production with increasing substrate concentration is in agreement with other research findings [19,23,24]. However, ethanol production ceased when the glycerol concentration was further increased to approximately 48 g/L due to substrate inhibition [25]. In addition, higher glycerol concentrations are believed to produce osmotic pressure inside the cell, causing cell damage due to the purging of water molecules from the cells [26].

Crude glycerol contains salts such as NaCl and KCl due to the addition of NaOH and KOH as catalysts in the biodiesel production process. The salt content in crude glycerol typically ranges from 2.5 to 20 g/L [27]. In this study, salt was added to the fermentation to mimic the characteristics of crude glycerol from biodiesel plants. Some studies have suggested that salt contents higher than 10 g/L are toxic to microorganisms [28]. Previous studies have reported that high salinity in growth media inhibited cell growth and substrate utilization by reducing respiratory activities [29]. Additionally, similar inhibitions were reported for halotolerant bacteria when the cells were incubated in the presence of high concentrations of NaCl or KCl [30]. Therefore, high salinity causes cell damage, resulting in the inhibition of glycerol utilization and ethanol production. Because the salinity of the medium could inhibit cell growth and ethanol production, the inhibitory effects of salts on ethanol production by *E. coli* SS1 were investigated at salt concentrations in the range of 0–22.5 g/L. However, the results from ANOVA indicated that the effects of the salt concentration on the fermentation were not significant as long as the concentration did not inhibit cell growth. In this study, *E. coli* SS1 was able to tolerate a high salt content of 15 g/L without reducing its ethanol production.

Table 5
Analysis of variance (ANOVA) for the experimental results of the CCD.

Source	Sum of squares	df	Mean square	*F*-Value	*p*-Value Prob > *F*
Model	458.33	14	32.74	15.45	<0.0001
A	66.17	1	66.17	31.22	0.0001
B	78.23	1	78.23	36.91	<0.0001
C	52.48	1	52.48	24.76	0.0003
D	0.51	1	0.51	0.24	0.6334
AB	45.26	1	45.26	21.35	0.0006
AC	0.87	1	0.87	0.41	0.5339
AD	3.72	1	3.72	1.75	0.2102
BC	10.19	1	10.19	4.81	0.0488
BD	6.64	1	6.64	3.13	0.1020
CD	0.44	1	0.44	0.21	0.6572
A^2	149.40	1	149.40	70.49	<0.0001
B^2	92.86	1	92.86	43.81	<0.0001
C^2	61.30	1	61.30	28.92	0.0002
D^2	19.46	1	19.46	9.18	0.0105
Residual	25.43	12	2.12		
Lack of fit	24.57	10	2.46	5.70	0.1585
Pure error	0.86	2	0.43		
Cor total	483.76	26			

$R^2 = 0.9474$; Adjusted $R^2 = 0.8861$; Predicted $R^2 = 0.7034$; Adequate precision = 14.125; PRESS = 143.48.

3.3. Validation of the model

The reproducibility of the model was tested by performing the fermentation under the optimal conditions obtained from the CCD.

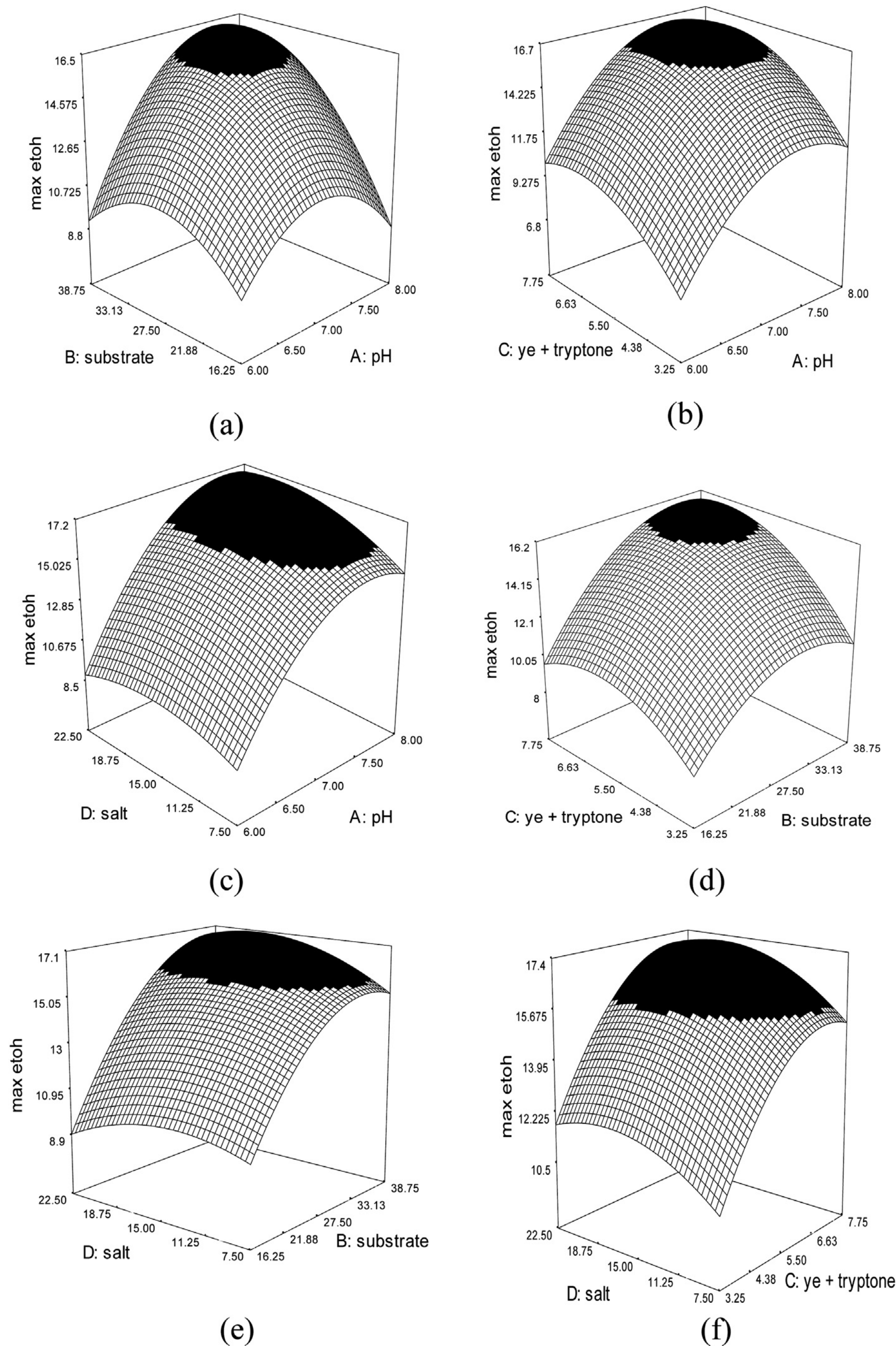

Fig. 1. Response surface plots depicting the interaction between variables in the production of ethanol from glycerol: (a) pH and substrate concentration, (b) pH and nitrogen source, (c) pH and salt content, (d) substrate concentration and nitrogen source, (e) substrate concentration and salt content, (f) nitrogen source and salt content.

This validation was also used to verify the accuracy of the model. The ethanol production model suggested that optimum ethanol production could be achieved at initial pH of 7.61, glycerol concentration of 34.53 g/L and organic nitrogen concentration of 6.42 g/L. The predicted ethanol concentration under these optimum conditions was 17.05 g/L. Three replicates of the batch fermentation using pure glycerol under the optimized conditions were conducted in serum bottles to confirm the model validity. A maximum ethanol production of 15.72 $\pm$ 0.26 g/L was obtained from the confirmation test. This actual value is in close agreement with the predicted value, with a difference of only 7.8%. Hence, we confirmed that the model developed from the response surface methodology could reliably predict ethanol yields. According to Levin et al. [31], differences between experimental and predicted values of less than 10% confirm the validity of a model.

Table 6 outlines the ethanol production from different microorganisms under various fermentation conditions. Comparing the results of different studies and drawing proper conclusions is challenging due to differences in operational conditions and parameters, such as reactor type and size, substrate concentration, inoculum, use of additional nutrients. Therefore, it is necessary to consider the role of operational conditions when comparing the

Table 6
Comparison of the ethanol production by glycerol-fermenting bacteria under various fermentation conditions.

Culture	Microorganism	Fermentation scale	Glycerol (g/L)	Glycerol conversion (%)	Initial pH	Organic nitrogen concentration (g/L)	Ethanol production (g/L)	References
Mixed	Mixed culture	Serum bottle (45 mL working volume)	15*	97.7	8	–	7.9	[17]
	Enterobacter agglomerans, Klebsiella pneumoniae ATCC, *Citrobacter freundii* ATCC	120 mL flask (60 mL working volume)	20	100	8	2 (YE)	0.1–1.50	[18]
Recombinant	*Klebsiella pneumoniae* DSM 2026	250 shake flask (100 mL working volume)	20*	>70	7	1.5 (YE)	10.3	[43]
	Klebsiella oxytoca (deleted dehydrogenase gene)	500 mL shake flask (100 mL working volume)	60	ND	7	1 (YE)	15.6	[32]
	Recombinant *Escherichia coli*	300 mL working volume vessel	20	80	6.3	5 (YE) + 10 (T)	6.60	[44]
Wild type	*Enterobacter aerogenes* KKU-S1	Serum bottle (30 mL working volume)	37*	ND	8.14	1 (YE)	5.53	[19]
	Clostridium pasteurianum	75 mL culture vial (40 mL working volume)	5	80	7	1 (YE)	1.80	[45]
	Enterobacter aerogenes HU-101	Serum bottle (50 mL working volume)	25*	80	6.8	5 (YE) + 5 (T)	10.25	[11]
	Escherichia coli	500 mL working volume vessel	10	80	6.3	2 (T)	4.60	[14]
	Escherichia coli MG1655	Reactor (500 mL working volume)	10	84.46	ND	4.25 (YE)	4.53	[46]
	Escherichia coli SS1	2 L bioreactor (800 mL working volume)	34.5	>90	7.61	6.42 (YE + T)	15.72	This study

YE = yeast extract; T = tryptone; ND = Not determine (*) = crude glycerol.

results of different studies. As depicted in Table 6, glycerol fermentations have primarily been performed at relatively small scales with working volumes less than 500 mL. In addition, the majority of studies focused on glycerol concentrations ranging from 5 to 20 g/L. Only a few reports describe ethanol production at glycerol concentrations greater than 20 g/L. Furthermore, the glycerol conversion achieved in several studies was above 70%, but the yield of ethanol per glycerol was low compared to our study, which achieved a nearly theoretical yield. It can be observed that even though genetically modified microorganisms have been employed at high glycerol concentrations of 60 g/L [32] and 37 g/L [19], they achieved lower ethanol yields of only 15.6 g/L and 5.53 g/L, respectively. Interestingly, our current study demonstrates the potential of *E. coli* SS1 to produce 15.72 g/L of ethanol from a relatively high glycerol concentration of 34.5 g/L. In terms of the initial pH, ethanol production from fermentation generally requires neutral to alkaline conditions [17,18,43,32,19,42,11], as observed in Table 6. In addition, Barbirato et al. [18] observed that the amount of glycerol consumed increased with increasing pH and that the exhaustion of glycerol occurred at pH 8. Meanwhile, when pH levels are low and slightly acidic, the co-production of hydrogen is likely to occur because metabolic pathways are altered by changing NADH to NAD^+ ratios, which affect the distribution of the carbon flux through the metabolites' routes under anaerobic conditions. Nakashimada et al. [20] demonstrated the relationship between the culture pH and NADH to NAD^+ ratios at various pH ranges using *E. aerogenes*. At a pH range of 6.0–6.7, the NADH to NAD^+ ratios were higher than at other pH values, and this affected hydrogen production. In contrast, Zhang et al. [33] reported that ethanol production was enhanced by the addition of NADH. It can therefore be concluded that ethanol fermentation favors alkaline conditions.

The use of organic nitrogen sources in fermentation can enhance microbial growth [34]. Some studies have demonstrated that the addition of yeast extract to crude glycerol [11] and glucose fermentation [35] resulted in increased product yield as well as biomass growth. Suitable nitrogen sources can repress the formation of by-products and increase ethanol yields [36]. As outlined in Table 6, yeast extract and tryptone are the primary nitrogen sources in ethanol production. However, there are no general rules of thumb to guide the selection of the added quantities. The ranges used in previous studies for both yeast extract and tryptone were less than 10 g/L (Table 6). As a control, a fermentation was performed using media supplemented with yeast extract and peptone under optimized conditions without any added glycerol. Only trace amounts of ethanol were detected during fermentation, indicating that yeast extract and peptone are not suitable substrates for ethanol production but rather enhance biomass growth (data not published). Nikel et al. [37] found that yeast extract provides D-pantothenate, which is the precursor in the synthesis of acetyl-coA in *E. coli*, hence explaining why ethanol production might be enhanced.

Compared to the other studies listed in Table 6, this study was among the best in terms of ethanol production. Substrate concentrations below 35 g/L were used in the majority of previous studies, in contrast to the relatively high substrate concentration used in our study. In addition, *E. coli* SS1 was able to utilize approximately 35 g/L of glycerol to produce nearly 16 g/L of ethanol, corresponding to a yield of 0.9 mol/mol. Yang et al. [32] utilized high glycerol concentrations (60 g/L); however, the ethanol yield was relatively low, and the microbe used was genetically modified. In this study, the organic nitrogen concentration required for the fermentation process was considered in the middle range compared to other studies. Moreover, the ethanol production under the optimized conditions (15.72 ± 0.26 g/L) was approximately 1.7-fold higher than that obtained under non-optimized conditions (9.23 ± 0.6 g/L).

A comparison of the fermentation using either pure glycerol or crude glycerol under the optimized conditions was performed. This study was conducted to evaluate the feasibility of using crude glycerol as a substrate for bioethanol production by *E. coli* SS1. Fig. 2a and b displays the fermentation profile using pure and crude glycerol by *E. coli* SS1, respectively. Similar fermentation trends were observed for the two fermentations. The pure glycerol was converted to 15.68 ± 0.62 g/L ethanol at 96 h, resulting in 2.95 ± 1.18 g/L unfermented glycerol. This represents a maximum ethanol yield of 0.994 mol/mol, with a glycerol consumption of 91.4%. On the other hand, the fermentation of the crude glycerol produced a maximum ethanol concentration of 16.46 ± 0.44 g/L at 120 h with 3.06 ± 0.34 g/L of unfermented glycerol remaining, corresponding to a yield of 1.0 mol/mol. The glycerol consumption achieved was 91.1%. These results demonstrate that the ethanol production was higher for crude glycerol than for pure glycerol. This difference could be due to the presence of various carbon sources or electron sources in the crude glycerol, which might have contributed to the ethanol yield [27]. Matter of organic non-glycerin (MONG), such as fatty acids contained in the crude glycerol, might also influence cell growth and ethanol production. Thompson and He [38] reported that carbon, nitrogen, and some metals are present in the crude glycerol derived from biodiesel production and that the ethanol yield from crude glycerol (1.07 mol/mol) exceeds the theoretical yield (1.0 mol/mol) at 20 g/L concentrations. Jitrwung and Yargeau [23] reported that a small amount of glycerol was produced as a by-product from both pure glycerol and crude glycerol by *E. aerogenes*, so that glycerol by-products might be converted to ethanol and lead to ethanol yields greater than the theoretical yield. These results demonstrate that crude glycerol is a promising and feasible substrate for use in ethanol fermentation. Table 7 provides a comparison of the ethanol yield obtained in this study with those of other studies using crude glycerol. The ethanol yield obtained in this study is among the best in terms of the yield obtained at high glycerol concentrations. There is considerable interest in using crude glycerol for fine chemical production due to its many advantages, including low price and ample supply. Furthermore, no pre-treatment or purification is necessary prior to fermentation, and the direct conversion of this substrate to other fuels seems promising [19,13].

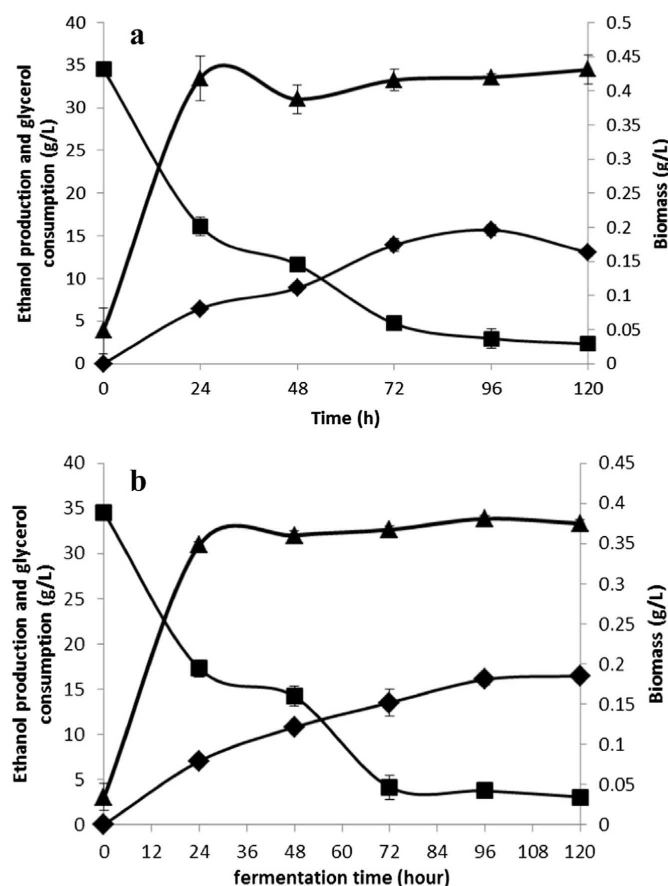

Fig. 2. a. Ethanol production using pure glycerol by *E. coli* SS1 under optimized conditions: glycerol (■), ethanol (◆) and biomass (▲). b. Ethanol production using crude glycerol by *E. coli* SS1 under optimized conditions: glycerol (■), ethanol (◆) and biomass (▲).

3.4. Batch fermentation

Batch ethanol fermentation was performed at 3 glycerol concentrations of 20 g/L, 35 g/L and 45 g/L. Although the optimum glycerol concentration obtained from RSM analysis was 34.5 g/L, it is valuable to investigate the effect of glycerol concentration on ethanol production in 2 L bioreactors as the performance may differ as a result of the scale up process. Moreover, the ability of *E. coli* SS1 to utilize higher glycerol concentrations will be beneficial in industrial applications using crude glycerol from biodiesel processing plants, as it will minimize the reactor size and running cost if modifications to the crude glycerol can be avoided [11].

Fig. 3 depicts the ethanol fermentation profile for the glycerol concentrations of 20 g/L, 35 g/L and 45 g/L. It is apparent that the ethanol fermentation occurred in two phases: a) the first phase, which is characterized by concomitant growth and ethanol production and b) the second phase, in which the growth remained constant but the cells continued to produce ethanol until reaching the maximum yield [39]. The uptake of nutrients and the replication of cells occurred rapidly, leading to rapid glycerol consumption and ethanol production and increases in cell numbers. The highest biomass growth was observed with cell dry weights ranging from 0.3 to 0.5 g/L, irrespective of the glycerol concentration. This observation is further supported by the viable cell numbers achieved in these three sets of experiments of 2.5×10^9, 2.08×10^9 and 1.87×10^9, respectively. Meanwhile, the kinetic parameters for the three experiments using 20 g/L, 35 g/L and 45 g/L glycerol are illustrated in Table 8. The maximum substrate consumption was determined using a glycerol assay kit and calculated based on the equation below:

$$S_{max}(g/L) = S_i - S_o$$

where S_{max} represents the maximum substrate concentration, S_i represents initial substrate concentration, and S_o represents final substrate concentration, at which the maximum product formation occurs. For example, the maximum product formation in this experiment occurred at 72 h. The quantities of consumed glycerol for the fermentations performed at 20 g/L, 35 g/L and 45 g/L glycerol were 18.23 ± 0.28 g/L, 30.31 ± 0.76 g/L and 40.72 ± 1.41 g/L,

Table 7
Comparison of ethanol production from crude glycerol.

Strain	Crude glycerol concentration (g/L)	Ethanol yield (mol/mol)	References
E. aerogenes HU-101	10	0.86	[11]
Mixed culture	15	1.00	[16]
Klebsiella sp. HE1	10–70	0.26 (70)– 0.80 (10)	[12]
E. aerogenes KKU-S1	31.2	0.83	[15]
E. coli SS1	34.5	1.00	This study

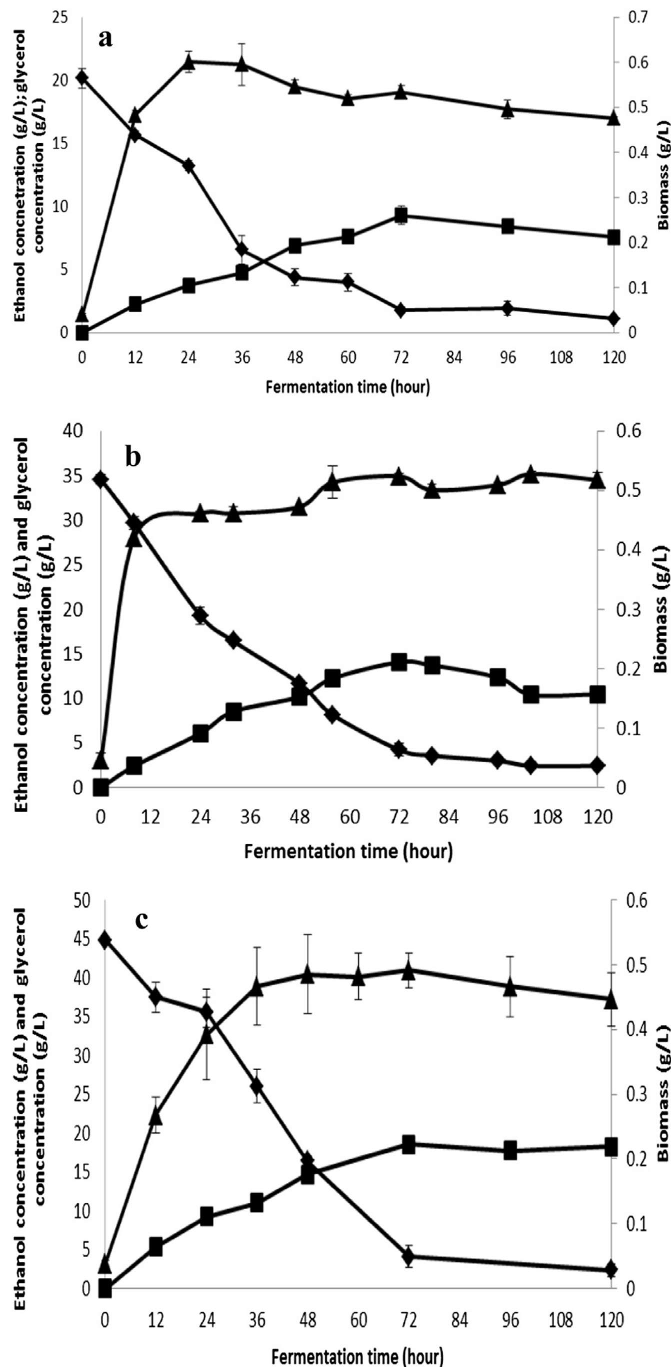

Fig. 3. a. Fermentation profile using 20 g/L glycerol substrate in a 2 L bioreactor: ethanol (■), biomass (▲) and glycerol (◆). b. Fermentation profile using 35 g/L glycerol substrate in a 2 L bioreactor: ethanol (■), biomass (▲) and glycerol (◆). c. Fermentation profile using 45 g/L glycerol substrate in a 2 L bioreactor: ethanol (■), biomass (▲) and glycerol (◆).

respectively. More than 90% of the glycerol was consumed by *E. coli* SS1 using 20 g/L, 35 g/L and 45 g/L glycerol in the first phase. Ethanol was produced rapidly at the early stage of fermentation, and its formation rate depended on the glycerol concentration. As the initial glycerol concentration increased, the production of ethanol increased [27]. Ethanol was continuously produced even after the biomass reached the stationary phase, and the viable cell count was maintained at approximately 10^8–10^9 until the fermentation was complete. The highest ethanol concentration, designated as P_{max}, can be calculated as:

Table 8
Kinetic parameters for ethanol fermentation at various glycerol concentrations.

Kinetic parameters	Glycerol concentration, g/L		
	20	35	45
P_{max} (g/L)	9.3 ± 0.7	14.06 ± 0.36	18.55 ± 0.39
S_{max} (g/L)	18.23 ± 0.28	30.31 ± 0.76	40.72 ± 1.41
Cell growth, (cell/ml)	2.5×10^9	2.08×10^9	1.87×10^9
Ethanol yield, (mol ethanol/mol glycerol)	1.0	0.93	0.92
Productivity of ethanol, (g/l/h)	0.13	0.19	0.26

$$P_{max}(g/L) = P_{highest} - P_i$$

where P_{max} is the maximum ethanol concentration; $P_{highest}$ is the highest ethanol concentration and P_i is the initial ethanol concentration. In this experiment, the maximum ethanol yield of 18.55 ± 0.39 g/L was observed during the stationary phase at 72 h, yielding 0.92 mol ethanol/mol glycerol. 35 g/L and 20 g/L glycerol fermentations exhibited maximum ethanol concentrations of 14.06 ± 0.36 g/L and 9.3 ± 0.7 g/L, respectively. A similar fermentation profile was observed in the study by Yazdani and Gonzalez [13] using genetically engineered *E. coli*, where the maximum ethanol production achieved was approximately 5 g/L for 10 g/L glycerol. For wild type *E. coli*, the maximum ethanol production achieved was approximately 3 g/L when 10 g/L glycerol was used [40]. Both strains exhibited the same fermentation trend, in which ethanol was produced after the cells had stopped growing and reached the stationary phase.

The ethanol fermentation profile in the 2 L bioreactor exhibited a trend similar to that in the serum bottle for the 20 g/L and 35 g/L glycerol concentrations. Although the yield dropped slightly as the glycerol concentration increased, the yield was still acceptable, as the results obtained were close to the theoretical yield. The yield achieved in this experiment was 1.0 mol ethanol per mol glycerol from 20 g/L glycerol, 0.93 mol ethanol per mol glycerol from 35 g/L glycerol and 0.92 mol ethanol per mol glycerol from 45 g/L glycerol. Wu et al. [12] reported that the ethanol production decreased from 62 to 22% of the total soluble microbial product (SMP) as the glycerol concentration increased from 10 g/L to 70 g/L. Ito et al. [11] stated that decreases in hydrogen, ethanol and acetate were observed when the glycerol concentration was increased to 25 g/L. A study using a higher substrate concentration of 37 g/L [19] optimized the ethanol fermentation conditions using RSM from engineered *E. aerogenes* KKU-S1 and resulted in a yield of approximately 5.53 g/L ethanol.

In this study, it was observed that the productivity achieved by *E. coli* SS1 increased 2-fold when the glycerol concentration increased from 20 g/L to 45 g/L. This result indicates that substrate inhibition did not occur in this ethanol fermentation and that the SS1 strain is able to achieve close to theoretical yields. As displayed in Fig. 3a–c, the cultivation time required by *E. coli* SS1 to achieve maximum ethanol production was consistent at 72 h. This phenomenon could be due to the presence of sufficient viable cells up to 10^9 CFU/ml in the fermentation system to convert glycerol into ethanol. According to Kapu et al. [41], high cell density can provide effective fermentation at high substrate concentrations. A 77% ethanol yield was achieved using yeast strain LYCC 6469 after 48 h at high cell density. Meanwhile, Kleman and Strohl [42] investigated a method to increase cell density because they hypothesized that high cell densities could enhance the formation of important products. They found that a high cell density improved ethanol production even at high glycerol concentrations.

These findings have considerable impact because the strain used in this experiment was the wild type strain, did not undergo any

modification, and was able to consume up to 45 g/L glycerol without hindering the production of bioethanol.

4. Conclusions

Using the methods of experimental factorial design and response surface analysis, we determined the optimal fermentation conditions for ethanol production. Glycerol was successfully converted to ethanol with nearly theoretical yields. Our experimental results indicated that the initial pH, substrate concentration, and organic nitrogen concentration exert significant effects on bioethanol production yield. The optimum conditions for ethanol production comprised initial pH of 7.61, substrate concentration of 34.53 g/L and organic nitrogen concentration of 6.42 g/L and provided an ethanol yield of 15.72 g/L. Compared to the predicted maximum ethanol production of 17.05 g/L, only a small error exists between the predicted value and the actual experimental value.

The results obtained in this study are among the best reported to date for glycerol fermentation in terms of both substrate concentration and yield (see Table 6). This study has successfully demonstrated the feasibility of ethanol production by *E. coli* SS1 using high glycerol concentrations of up to 45 g/L in a 2-L bioreactor without any substrate inhibition. More than 90% of the initial glycerol was converted to ethanol as the main fermentation product.

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