JCEIB

Journal Chemical Engineering and Industrial Biotechnology
(JCEIB)
Open Access
Volume 2 pp. 27-36; September 2017

DOI: https://doi.org/10.15282/JCEIB-V2-01.29/9/2017/2.2

OPTIMIZATION OF GLYCEROL RECOVERY FROM PRETREATMENT PROCESS TO PRODUCE SUCCINIC ACID VIA ANAEROBIC FERMENTATION

Nik Nor Aziati Abdul Aziz[a], Mimi Sakinah Abdul Munaim[b*]
[a] Faculty of Engineering Technology, Universiti of Malaysia Pahang, Lebuhraya Tun Razak, 26300 Kuantan, Pahang, Malaysia
[b*]Faculty of Chemical and Natural Resources Engineering, Universiti Malaysia Pahang, Lebuhraya Tun Razak, 26300Kuantan, Pahang, Malaysia
∗ Corresponding author: E-mail: mimisakinah@gmail.com
Tel: +609 5492031/2825; Fax: +609 5492662

ABSTRACT

This study aims to produce succinic acid from glycerol residue through anaerobic fermentation process. The optimum condition of pH, mass substrate, and temperature was determined using response surface methodology (RSM). The concentration of glycerol and succinic acid were determined using HPLC analysis. The FTIR spectrometry was applied to examine the adsorption of organic element. Optimization of fermentation condition using RSM yielded glycerol of 159.312 g/L at pH 2, 35 °C, and 110.36 g of substrate. Succinic acid was the product of fermentation by *Escherichia coli* type k-12 under 19.67 g/L initial glycerol concentration, 200 rpm rotational speed, and 37 °C. The fermentation with treated glycerol generated 0.66983 g/L of succinic acid, compared to commercial glycerol which generated 0.73337 g/L of succinic acid. Overall, this research show that glycerol waste can be used as the carbon source to produce succinic acid by implementing fermentation of *Escherichia coli* type k-12. From the fermentation process, succinic acid was not the major product. Further study in genetic modification of *Escherichia coli* is highly suggested to produce succinic acid as the major product, as well as to improve the fermentation process.

Keywords: Glycerol, Glycerol residue, Glycerol pitch, Recovered glycerol production; Glycerol waste pre-treatment, pre-treatment process.

1.0 INTRODUCTION

Over the last decade, succinic acid has attracted attention worldwide with its excellent characteristic as an additive in products (Agarwal *et al*., 2005). Succinic acid is commonly produced from liquefied petroleum gas or petroleum oil as the starting material via chemical processes (Song and Lee, 2006). Therefore, it can cause potential hazard and environmental pollution as the impact of succinic acid production via chemical synthesis route. Succinic acid can also be produced through fermentation process using microorganism and glycerol as the substrate (Lee *et al*., 2000).

The fermentation process utilises glycerol as the carbon source, thereby reducing the potential of by-products generation such as acetic acid that will negatively affect the purification process (Lee *et al*., 2000). The production of succinic acid using glycerol

waste from palm-based oleochemical industry is attractive due to the abundance of glycerol source (Hazimah *et al*., 2003).

Furthermore, by utilizing renewable resources as fermentation medium is more cost-effective as it does not involve petroleum-based processes (Zeikus *et al*., 1999). In an anaerobic fermentation, succinic acid is one of the by-products. Hence, attempts have been made worldwide to screen anaerobic microorganisms for succinic acid production in large scale (Dikshit and Moholkar, 2016). The study is focus on the pre-treatment and fermentation process to produce succinic acid. The experiment developed will able to explain the process of succinic acid production. This study also discusses the conversion of glycerol into high value products under anaerobic condition.

2.0 MATERIAL AND METHOD

Glycerol residue was obtained from Emery Oleochemical Sdn. Bhd. Analytical grade of sulfuric acid, sodium hydroxide, methanol (98% purity), and glycerol were used in this research.

Esterification

Esterification process is the conversion of carboxylic acid to ester using acid and alcohol. The amount of glycerol residue (30g, 60g, 90g, 120g and 150g) was diluted with 150 mL of distilled water. Then, the mixture was heated and stirred in a reaction flask at the same speed for all test runs for 5 min to ensure complete dilution of glycerol residue and water. After that, the pH of the solution was adjusted to desired pH in the range of 1 to 5 using concentrated sulphuric acid (H_2SO_4). Then, the mixture was allowed to settle until two distinct layers were visible. The top layer of fatty acid containing tar such as solid waste and salt residue was removed using slow decantation. The aqueous layer was filtered to further remove any solid materials and other charred substances that remained in the mixture.

Alkali-Catalysed Transesterification

Alkali-catalysed transesterification using alkali to catalyst the products. By removing the residue salts from the glycerol-rich layer, the solution was neutralised by adding 5M NaOH until pH 7.0 was obtained. Later, the solution was left for a while prior to filtering once again to eliminate the precipitated salt. After the separation process, ether extracts were combined and concentrated using a rotary evaporator to remove residual water. During this extraction process, the temperature of the water bath was set to 105°C for 2 h. After the evaporation process, the methanol-to-crude glycerol ratio (v/v) were added at a ratio of 2:1to the mixture. The mixture was allowed to stand at room temperature for 30 min to cool, followed by refrigeration for another 30 min to ensure a complete precipitation of the salt in the mixture. The precipitated inorganic salt was filtered and washed with chilled methanol. After the crude glycerol was recovered, the methanol in the mixture was eliminated using a rotary evaporator and heated in a silicon oil bath at 80 °C for 20 min to recover a pure crude glycerol (Darnoko and Cheryan, 2000).

Fermentation Process

Succinic acid is the principal product in the anaerobic fermentation of glycerol. In the preliminary study, the pre-treatment processes (esterification and alkali-catalysed transesterification) were utilised to recover glycerol, and subsequently the process continued with the fermentation process to produce succinic acid. This fermentation

process was tested with two different substrates which were the treated glycerol from the oleochemical company and a commercial grade glycerol. The results were compared with a treated glycerol from a biodiesel company which had undergone fermentation in a similar manner. The profile growth of *Escherichia coli* type K-12 cultured in 20 g/L of treated glycerol as the carbon source under anaerobic condition and the medium was supplemented with 10 g/L of tryptone. About 10% of working volume of inoculum was added to the medium for profile growth process. Table 1 shows the reaction variables for the fermentation process.

Table 1: The reaction variables for the fermentation process.

Numbers	Reaction Variables	Value
1	Glycerol concentration (g/l)	19.67
2	Tryptone concentration (g/l)	12.19
3	Na_2SO_3 concentration (g/l)	1.0
4	Incubation period (h)	63.8
5	Inoculum density (%)	4.0
6	pH	6.88

HPLC analysis

The glycerol and succinic acid was determined by using high performance liquid chromatography (HPLC) analysis. The type of high performance liquid chromatography equipment used was the HPLC-1200 Agilent Technologies with Reflective Index detector (RI) and the type of column was Aminex HPX-87H; 300 mm x 7.8000 mm, 9 µm; manufactured by Bio-Rad Chemical Division, CA., USA. The operating temperature was about 50 °C and the mobile phase used was 0.0005 µm sulfuric acid (H_2SO_4) solution. The flow rate was controlled at 0.6000 ml /min (Agarwal *et al*., 2005).

Optimization of Parameters

Response surface methodology based on face-centred central composite design (FCCCD) under Design-Expert software (version 6.0.8, Stat-Ease, Minneapolis, USA) was used to optimise the fermentation condition (pH, temperature and mass substrate (glycerol residue)to obtain the highest amount of glycerol. The data was fitted to a polynomial equation to obtain a regression equation. The statistical significance at 95% confidence in terms of regression equation was examined by analysis of variance (ANOVA). Response surface plots were generated by the software. The average concentration of sugar mixture from a duplicate determination was used as a response(Rashid and Anwar, 2008).

3.0 RESULTS AND DISCUSSION

Response surface methodology (RSM) based on face-centred central composite design (FCCCD) was applied to determine the effects of operational parameters (pH, temperature and mass substrate) on the recovery of glycerol from its residue in the pre-treatment process. Twenty-eight experimental runs which correspond to varying parameters (pH, temperature and substrate concentration) were carried out in the pre-treatment process. The effects of these parameters on glycerol recovery from its residue were evaluated.

Analysis of variance (ANOVA)

Statistical analysis of the results shows that the optimum parameter combination is at 35 °C, 110g of substrate (glycerol residue), and pH 1.5. Under this optimum condition, 159.414 g/L of glycerol concentration was obtained. The second order regression model shows the relationship between the response (glycerol concentration) and operating parameters of pH (A), temperature (B), and substrate (C). The regression model analysis is shown in Eq. (1):

Final Equation in Terms of Actual Factors:

$$\begin{aligned}\text{Glycerol Concentration} = {} & + 46.0394 + 80.9362*\text{pH} + 5.2028*\text{ Temperature} \\ & -1.15086*\text{ Substrate (Glycerol residue)} + 0.0802* \\ & \text{Temperature}_2 + 0.0198*\text{ Substrate (Glycerol residue)}_2 \\ & - 0.6268*\text{ pH} * \text{Substrate (Glycerol residue)} - \\ & 0.0788*\text{ Temperature} * \text{Substrate (Glycerol residue)}\end{aligned} \quad (1)$$

In order to establish a good response from the model, three tests were performed which were the significance of regression model, significance on individual coefficient, and the lack-of-fit. Fisher's statistical test for ANOVA was employed to verify the determination of coefficient (R^2) and adequacy of the model by determining the significance of variable according to the value of F-ratio. It was found that the confidence level was greater than 95% ($p<0.05$) for glycerol recovery. The *F*-value of the model was 12.26 ($p< 0.0001$) which implies its significance. In the similar manner, the main effects of pH (A), temperature (B), substrate (C), second order effect of temperature (B^2), second order effects of substrate (C^2), the two-level interaction of pH and substrate (AC), and the two-level interaction of temperature and substrate (BC) were also significant as model factors.

The determination of coefficient (R^2) for this model was 0.8346 indicating that 83.46% variation could be explained by the model equation. A better correlation between the observed and the predicted value was indicated by the nearest R^2value to 1. R^2 is used to decide whether the regression model is appropriate or not by measuring the amount of variation around the mean being explained by the model. If the residual value increased, the R^2 decreased in the range 1 to 0. Residual is the difference between the observed value and the fitted value. The value adjusted R^2 (Adj R^2) of this model was 0.7665 (76.65%) while Pre R-squared was 0.6202 (62.02%). Adj R^2 is a measure of the amount of variation in the dependent variables for which the model took into account, and it will adjust the R^2 based on the number coefficient in the model.

In general, the value of lack-of-fit is to indicate the insignificance in the pure error (Isar *et al*., 2006a). It is calculated using the ratio between the mean square of the model error. For this model, the lack-of-fit is 0.8786 which implies 87.86% chance that lack-of-fit would occur due to the noise. It appears that the represented model is desirably fit. Significant effect on the response was further analysed and diagnosed.

The normal probability plot of residual and the plot of residual versus predicted response for glycerol recovery are shown in Figures 1.0 and 2.0. Based on Figure 1.0, the residual falls on the straight line implying that the errors are distributed normally and support the adequacy of least-square fit. In addition, Figure 2.0 reveals no obvious pattern and

unusual structure. It shows that the plots are almost equally scattered above and below the x-axis and uniformly tabulated within the red lines of the x-axis. This means that the proposed model is adequate and there is no reason to suspect any violation of the independence or constant variance assumption. Figure 3.0 shows the outlier-t plot response for glycerol concentration. The data plots reveal that the plots are within the red lines of the x-axis, thereby indicating that the data obtained are located within the prediction range.

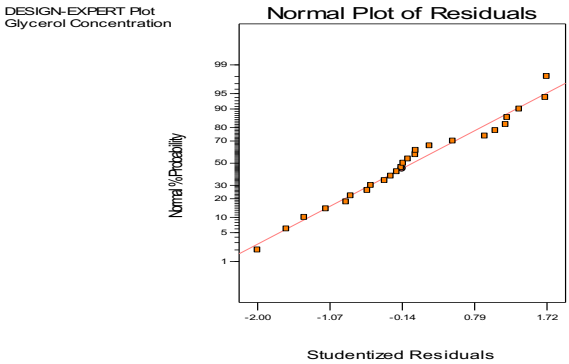

Figure 1: Normal probability plot of residual for glycerol concentration

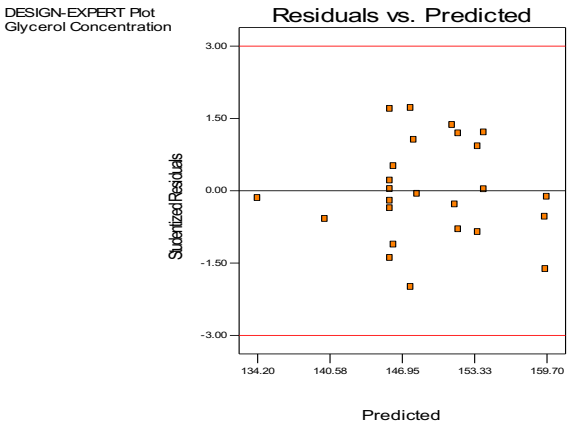

Figure 2: Residual versus predicted response for glycerol concentration

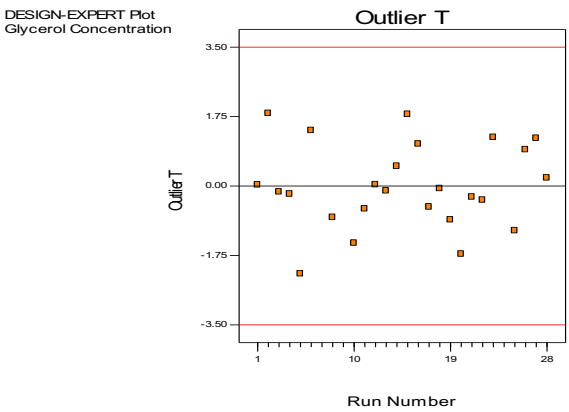

Figure 3: Outlier-t plot response for glycerol concentration

Interaction of different operating parameters

The three-dimensional response surface curve is used to study the interaction among different factors used and to find the optimum condition for maximum recovery of glycerol (Isar *et al*., 2006b). Figure 4.0 shows the 3D response surface graph for the interaction between pH and temperature in glycerol recovery. Meanwhile, Figure 5.0 shows the interaction effect graph between pH and temperature. It can be seen that the temperature range was from 25 to 35 °C and the pH was from 1 to 2 which corresponds to a maximum glycerol concentration of 153.552 g/L. Jansri *et al*. (2011), had evaluated the temperature as a parameter for methyl ester production from mixed crude palm oil by using acid-alkali catalyst. The temperatures used for their study were 55, 60, and 65 °C. Another work by Stamenkovitc *et al*. (2008) reported the use of low temperature (10–30 °C) in sunflower oil methanolys is process.

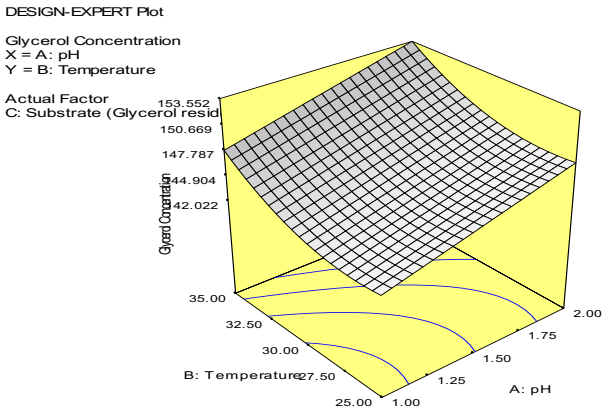

Figure 4: 3D surface response graph for interaction between pH and temperature

The interaction between pH and substrate analysis in terms of the 3D response surface curve is shown in Figure 5.0, and the interaction effect graph is exhibited in Figure 6.0. The A plot represents pH and C plot represents the substrate. The 3D response surface curve implies a relative interaction between pH and substrate (glycerol residue) which corresponds to 151.02 g/L of glycerol concentration. Yong *et al*.(2001) used pH less than 5 to avoid foaming in refining of crude glycerine recovered from glycerol residue.

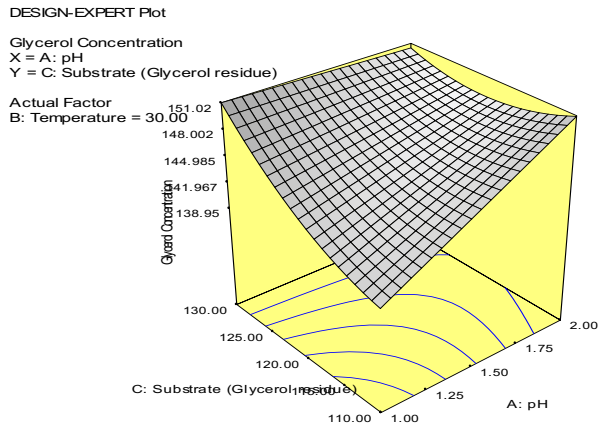

Figure 5: 3D surface response graph for interaction between pH and substrate

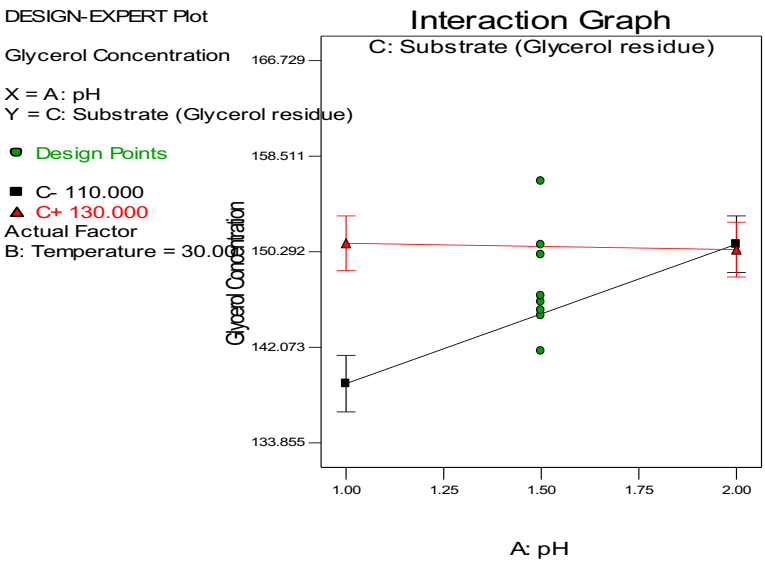

Figure 6: Interaction effect graph between pH and substrate

The interaction between temperature (B) and substrate (C) was examined from 25 to 35 °C and 110 to 130 g of glycerol residue as the substrate. Figure 7.0 shows the 3D surface response graph for the interaction between temperature and substrate in glycerol recovery, while Figure 8.0 displays the interaction effect between the two parameters. From the 3D response curve, the maximum glycerol concentration was found to be 153.881 g/L. Hayyan *et al*.(2011) studied the reduction of high content of free fatty acid in palm oil sludge via acid catalyst to produce biodiesel and found that the temperature was played an important role in the transesterification process. Their optimum temperature for the study was 60 °C.

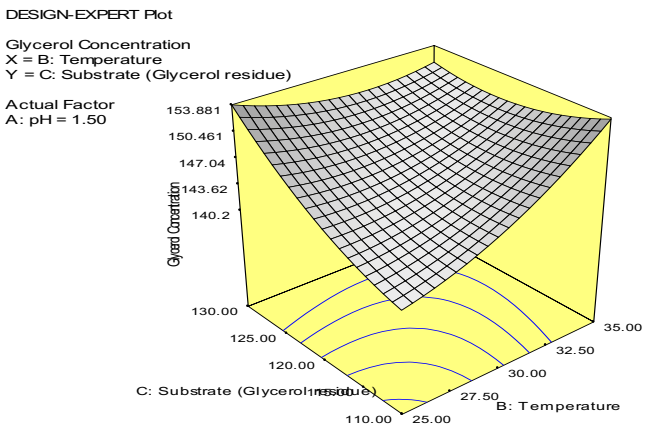

Figure 7: 3D surface response curve for the interaction between temperature and substrate

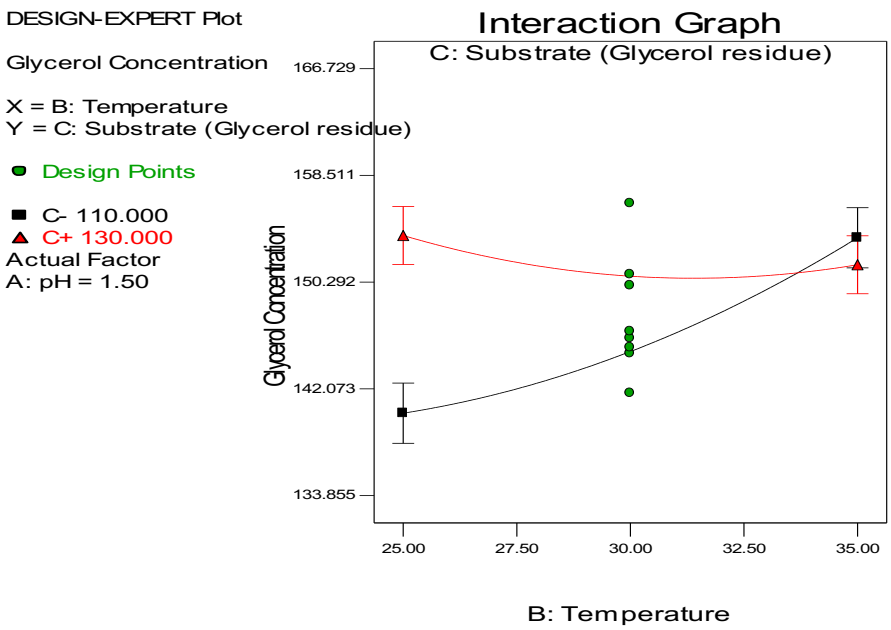

Figure 8: Interaction effect graph between temperature and substrate

Validation

In order to validate the adequacy of the model, five confirmation runs were performed and the observed results were compared with the predicted results. The condition for each run is listed in Table 2.0.

Table 2: The percentage error between the actual and predicted value for treated glycerol.

Number of run	Run Factor			Glycerol production (g/L)			
	pH	Temp	Substrate	Actual	Predicted	Residual	Error (%)
1	2	35	110.36	159.414	159.312	0.102	0.064
2	2	35	110.28	159.473	160.107	-0.634	0.398
3	2	35	110.06	159.652	160.317	-0.665	0.417
4	2	35	110.00	159.622	161.617	-1.995	1.250
5	2	35	110.59	159.231	152.388	6.843	4.298

It appears that out of five runs, three runs were favoured the goal of the response (glycerol concentration) than the experimental results (159.414g/L, 159.473g/L and 159.652 g/L). Hence, the optimum glycerol recovery from the pre-treatment process was at pH 2, 35 °C, and 110.36 g of substrate. Under this condition, 159.312 g/L of glycerol was recovered. The acidic condition is better for esterification process to get high concentration of glycerol. The best amount of mass substrate is important because high concentration of substrate can have saturated the solution and affects the reaction in esterification process for recovery of glycerol.

Succinic acid production via anaerobic fermentation

The effect of different substrate sources in the anaerobic fermentation of glycerol was investigated with batch fermentation using the medium that was supplemented with 19.67

g/L of glycerol for each sources of glycerol. The sample of succinic acid production was measured using high performance liquid chromatography (HPLC).

The fermentation result in terms of average succinic acid production from using treated glycerol from oleochemical sources was 0.66983 g/L. Meanwhile, fermentation using commercial glycerol produced 0.73337 g/L of succinic acid. It can be concluded that the treated glycerol was suitable asthe medium for the growth of *E.coli* with comparable amount of succinic acid produced with commercial glycerol. Both treated and commercial glycerol offer carbon source for the microorganism in the medium. The use of commercial glycerol has the advantage by having higher purity of which resulted in greater production of succinic acid. HPLC results detected two unknown component or composition at 7.868 minute and 10.233 minute. Thus, reduces the purity of treated glycerol, yet, the difference of succinic acid generated between treated glycerol and commercial glycerol was only approximately 10%. The study showed that the other composition besides glycerol did not affect the fermentation process to produce succinic acid and the difference error was less than 10%.

4.0 CONCLUSION

Process optimisation has shown that the optimum condition for glycerol recovery was at pH 2, 35 °C, and 110.36 g of substrate. With this optimum condition, 159.312 g/L of glycerol was recovered. The use of treated glycerol and commercial glycerol significantly produced succinic acid by *Escherichia coli* type k-12. The highest amount of succinic acid obtained from treated glycerol was0.66983 g/L while commercial glycerol produced0.73337 g/L. The percentage error between treated glycerol (glycerol residue) and commercial glycerol with regards to succinic acid production was 9.48%, and the error between treated glycerol residue and treated glycerol pitch was 9.82%.

5.0 REFERENCES

Agarwal, L., Isar, J., Meghwanshi, G.K., Saxena, R.K., 2007. Influence of environmental and nutritional factors on succinic acid production and enzymes of reverse tricarboxylic acid cycle from Enterococcus flavescens. *Enzyme Microbial* Technolology 40, 629–636.

Agarwal, L., Isar, J., Saxena, R.K., 2005. Rapid screening procedures for identification of succinic acid producers. *J. Biochem. Biophys. Methods* 63, 24–32.

Corona-González, R.I., Bories, A., González-Álvarez, V., Pelayo-Ortiz, C., 2008. Kinetic study of succinic acid production by Actinobacillus succinogenes ZT-130. *Process Biochemistry* 43, 1047–1053.

Darnoko, D., Cheryan, M., 2000. Kinetics of palm oil transesterification in a batch reactor. *Journal of American Oil Chemist' Society*. 77, 1263–1267.

Dikshit, P.K., Moholkar, V.S., 2016. Kinetic analysis of dihydroxyacetone production from crude glycerol by immobilized cells of Gluconobacter oxydans MTCC 904. *Bioresource Technology* 216, 948–957.

Hayyan, A., Alam, M.Z., Mirghani, M.E.S., Kabbashi, N.A., Hakimi, N.I.N.M., Siran, Y.M., Tahiruddin, S., 2011. Reduction of high content of free fatty acid in sludge palm oil via acid catalyst for biodiesel production. *Fuel Process Technology* 92, 920–924.

Hazimah, a H., Ooi, T.L., Salmiah, a, 2003. Recovery of Glycerol and Diglycerol From Glycerol Pitch Recovery of Glycerol and Diglycerol From Glycerol Pitch. *Journal Oil Palm Residue*. 15, 1–5.

Isar, J., Agarwal, L., Saran, S., Saxena, R.K., 2006a. A statistical method for enhancing the production of succinic acid from Escherichia coli under anaerobic conditions. *Bioresource Technology*. 97, 1443–1448.

Isar, J., Agarwal, L., Saran, S., Saxena, R.K., 2006b. Succinic acid production from Bacteroides fragilis: Process optimization and scale up in a bioreactor. *Anaerobe* 12, 231–237.

Lee, P.C., Lee, W.G., Lee, S.Y., Chang, H.N., Chang, Y.K., 2000. Fermentative Production of Succinic Acid from Glucose and Corn Steep Liquor by Anaerobiospirillum succiniciproducens. *Biotechnology Bioprocess Engineering*. 5, 379–381.

Rashid, U., Anwar, F., 2008. Production of biodiesel through optimized alkaline-catalyzed transesterification of rapeseed oil. *Fuel 87*, 265–273.

Song, H., Lee, S.Y., 2006. Production of succinic acid by bacterial fermentation. *Enzyme Microbioly Technology*. 39, 352–361.

Zeikus, J.G., Jain, M.K., Elankovan, P., 1999. Biotechnology of succinic acid production and markets for derived industrial products. *Applied Microbiology Biotechnology*. 51, 545–552.