Optimization of Succinic Acid from Fermentation Process by Using Immobiized *Escherichia Coli* Cells Via Anaerobic Fermentation

Nik Nor Aziati Abdul Aziz
Faculty of Chemical Enginnering and Natural Resources,
University of Malaysia Pahang (UMP)
Kuantan Pahang, Malaysia
Tel: +6016-6668486, Fax: +60-095492825, E-mail: *atie_encat@yahoo.com*

Mimi Sakinah Abdul Munaim
Faculty of Chemical Enginnering and Natural Resources
University of Malaysia Pahang (UMP)
Kuantan Pahang, Malaysia
Tel: +60-095492825, Fax: +60-095492825, E-mail: mimi@ump.edu.my

Abstract— Studies on succinic acid production by using immobilized cells are essential in the optimization and development of anaerobic fermentation process while glycerol treated used as substrate. One factor at one time (OFAT) method was performed as preliminary studies for selection of parameters range and subsequently determine its optimum condition using response surface methodology (RSM). The optimum parameter combination was found at time 3.31 hour, 40 gram mass substrate (glycerol residue treated) and 15 % inoculums. Under these optimum conditions, 125.059 g/L succinic acid concentration was obtained. The succinic acid was found linearly dependent on time, mass substrates and inoculums. Multiple regression models with high R^2 values were developed to correlate the succinic acid concentration as a function of time, mass substrate and inoculums. An analysis of variance (ANOVA) showed that the inoculums had slightly higher significant effect on succinic acid concentration if compared to time and mass substrates. Overall, this research showed that by using immobilized cells of *Escherichia coli* the production of succinic acid can be optimize and higher production of succinic acid. From this situation, the further study should be taken in genetic modification of *Escherichia coli* in immobilized cells to produce succinic acid as a major product to improve the fermentation process in producing the succinic acid for the future.

Keywords— Succinic acid, immobilized cells, fermentation process, glycerol residue.

1. INTRODUCTION

The palm oil industry is one of the major agricultural industries in Malaysia since 1975. The glycerol waste normally can be obtained from palm based Oleochemical industry and 70 % can be recovered from the waste[1]. Glycerol residue is a palm waste by product and has been implemented in application of soaps, fatty acids, waxes and surfactants production. It can be utilized by a number of microorganisms and is available from renewable resources in large amounts.

These days, succinic acid produced from microbial fermentation has been attracting interests of researchers because of its potential applications. Previously, research has revealed the effects of various microorganisms on succinic acid production [2]. To produce succinic acid in an environmentally friendly manner, glycerol waste can be used as a base in the fermentation process by microorganisms.

However, the engineering aspects of succinic acid production involving heat and mass transfer are challenging and need to be overcome and modeled with a suitable bioreactor. Immobilized fermentation and optimization and scale up design techniques are well developed[3]. The objective of this study is thus to optimization process for succinic acid production from glycerol residue as a carbon sources for the immobilized fermentation process.

2. MATERIAL AND METHODS

A.Preparation of material

Glycerol residue was obtained from Emery Oleochemical, Malaysia. The glycerol residue was treating by using pre-treatment method to get the treated glycerol before fermentation process. The glycerol treated was used as a substrate and carbon sources to produced succinic acid production using immobilized cell.

B.Experimental Design

A complete factorial experimental design was chosen to determine the succinic acid production. The experiment was start with one factor at one time (OFAT) which is to find the range of parameters.

C.Pre-treatment method

The materials used for the pre-treatment process are sulphuric acid, natrium hydroxide and silicon oil bath.The first step is dilution, followed by filtration, neutralization, separation, evaporation and decanting [4].

D.Preparation of immobilized cell

The bacteria culture in 250 ml Lb broth and the medium. The cells were added with 250 ml sodium alginate solution with 2% concentration in 500 ml beaker.The sodium alginate (mix with cell) was drop into the 0.1 M calcium chloride ($Cacl_2$) with maintain high of syringe and surface of $Cacl_2$ solution. The solution of $Cacl_2$ was stirrer continuously. After formation of bead the solution of 0.1 M cacl2 was replace with 0.05 M $Cacl_2$ then allow to harden for 12 hours. After 12 hour, the bead was wash with sterile 0.85 % of NaCl solution which is to remove adherent cells and $Cacl_2$ ions[5] .

E.Fermentation Process

Series of batch fermentations were conducted for immobilized fermentation process.The profile growth of *Escherichia. coli (E-coli)* type K-12 was conducted to monitor the growth of e-coli. The immobilized cell of e-coli was prepared. The fermentation was conducts in incubator for temperature 37°C and the sample were taken to measure the wavelength (OD 550 nm) and succinic acid production.

3. RESULTS AND DISCUSSION

The optimization of succinic acid was done by using the immobilization cell. The range input variables was studied for the inoculums, time and mass substrate. The succinic acid production for the mass substrate, inoculums densities and times was quadratic graph and the range of value are used for optimization process[6] .

The second order regression model shows the relationship between the response (succinic acid concentration) and the operating parameters A, B, C (time, mass substrate, inoculum) obtained by multiple regression analysis of the experimental data which is to identify the optimum conditions deduced by the second order regression model. The regression model analysis are shown as follows:

Table 1: ANOVA table (partial sum of squares) for quadratic model (response: concentration of succinic acid)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1780.47	9	197.8303467	14.711361	< 0.0001	significant
A-time	13.0473	1	13.04728217	0.9702419	0.3377	
B-mass substrate	3.35509	1	3.355089435	0.2494963	0.6235	
C-inoculum	1102.52	1	1102.51937	81.987224	< 0.0001	
AB	38.0742	1	38.07420858	2.8313323	0.1097	
AC	86.8342	1	86.83420784	6.4572975	0.0205	
BC	48.8235	1	48.82345442	3.630684	0.0728	
A^2	286.038	1	286.0380857	21.2708	0.0002	
B^2	87.1509	1	87.15089168	6.4808473	0.0203	
C^2	18.5901	1	18.59005682	1.3824221	0.2550	
Residual	242.054	18	13.44745341			
Lack of Fit	71.1712	5	14.2342431	1.0828767	0.4144	not significant
Pure Error	170.883	13	13.144842			

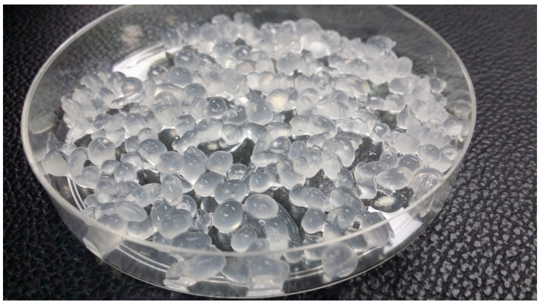

Figure 1: *The Escherichia coli* in bead for immobilization process

Final Equation in Terms of Coded Factors:

succinic acid concentration = +110.91-0.74* A+ 0.37* B - 6.78* C -1.54 * A * B + 2.33 * A * C -1.75 * B * C - 3.34 * A^2 + 1.85* B^2 + 0.85 * C^2

The analysis of variance in Table 1 indicates that the effect of mass substrate, time and inoculums is significant on succinic acid concentration (highest F-value). The behavior of response surface in Figure 1 also confirmed this inferences.

Further analysis is to validated the model. The normal plot of residual graph showed the normal plot and also the normal probability of the model for succinic acid production [6]. The graph showed the normality of the model and for the graph residual versus predicted the there are no obvious patern and and the plots was in the line and there are no point exits the red line. Then, if the pattern is increase from start to the end plots it was called downword trend. Normally can cause because of the stability of the systems and also merit investigation.

The three-dimensional response surface curve is to study the interaction among different factors used and to find the optimum condition for each factor to produce the maximum succinic acid production. Figure 1 shows the interaction effect graph between time and mass substrate on succinic concentration which is succinic acid production. The interaction between A (time) and B (mass substrate) showed the relationship between these factors. From the 3D surface response the time range is 3 to 5 hours and mass substrate range is 20 to 40 grams and the maximum concentration of succinic acid response is 127.103 g/L. Same studies done by Malvessi *et al*., that studies about the effect of substrate in immobilized cell on the activity of the complex glucose-fructose oxidoreductase/glucocno-δ-lactonaseby using *zymomonas mobilis* cells[7] .

It is observed that succinic acid concentration increased if the inoculums and time increased. . The higher production succinic acid is 127.10 g/L and the lowest is about 94.35 g/L by using this two interaction parameters. Therefore this fermentation process by immobilized cells shows that time and inoculum plays an important role in the succinic acid production and this can be explain in fermentation phase[8]. For the exponential phase, increased the time will increased the number of cells and also increased the growth rate (μ) and automatically when higher in growth rate also higher production of succinic acid. In fact, at certain time when the fermentation process have inhibits factors the μ which is growth rate will decreased and the time and number of cell also and increased because of production of succinic acid decreased.

The response surface model in Figure 2 also shows that succinic acid increased with the increase of mass substrate and fermentation time of immobilized cell. This happens because of a few factors such as the growth factors of bacteria Escherichia coli[9]. The succinic acid concentration was modeled through a quadratic multiple regression equation as a function of time (A) and mass substrate (B) as shown in the follows equation. Note that the A and B are normalized variables where A is -1 for 25 g and 1 for 35 g and B is -1 for 3.5 hour and 1 for 4.5 hour. Figure 2 shows that the values predicted by the model are very close to the actual values. The standard deviations are 3.67 and R^2 is 0.88 for all the data points.

The interaction between mass substrate (B) and inoculums (C) was examined with a mass substrate 20 to 40 gram and inoculums approximately 15 to 25 %. Figure 1 shows the 3D surface response graph and interaction effect graph for interaction between mass substrate and inoculums in succinic acid production. The results shows that the maximum succinic acid concentration that can be produced is 127.10 g/L. The 3D plots show the increasing the succinic acid production when increasing the mass substrates and decreasing the amount of inoculums at range 15 to 25 % of inoculums. The lowers succinic acid production 94.35 g/L. The interaction between the inoculums and mass substrate was explained in the 3D plot above. This one of parameters give significant effects on the product formation (ethanol production). Thus, from the results about 5% optimum of the yeast cell entrapped in the bead that could be high production of ethanol.

From the validation results, it was observed that the predicted results from three out of the five sets of experiments favour the goal of the response (succinic acid concentration) than the experimental results[3]. Hence, the optimum conditions observed for succinic acid fermentation process was at time 3.32 hour, mass substrate of 40 g and inoculums 15 %. At this optimum stage, 124.09 g/L of succinic acid was obtained.

4.CONCLUSION

The succinic acid can be optimize at time 3.32 hour, mass substrate of 40 g and inoculums 15 % for , 124.09 g/L of succinic acid production. The relationship between mass substrate, inoculums densities and time can be fitted by a quadratic multiple regression equation with high accuracy[10]. This can be utilized for bioreactor design.

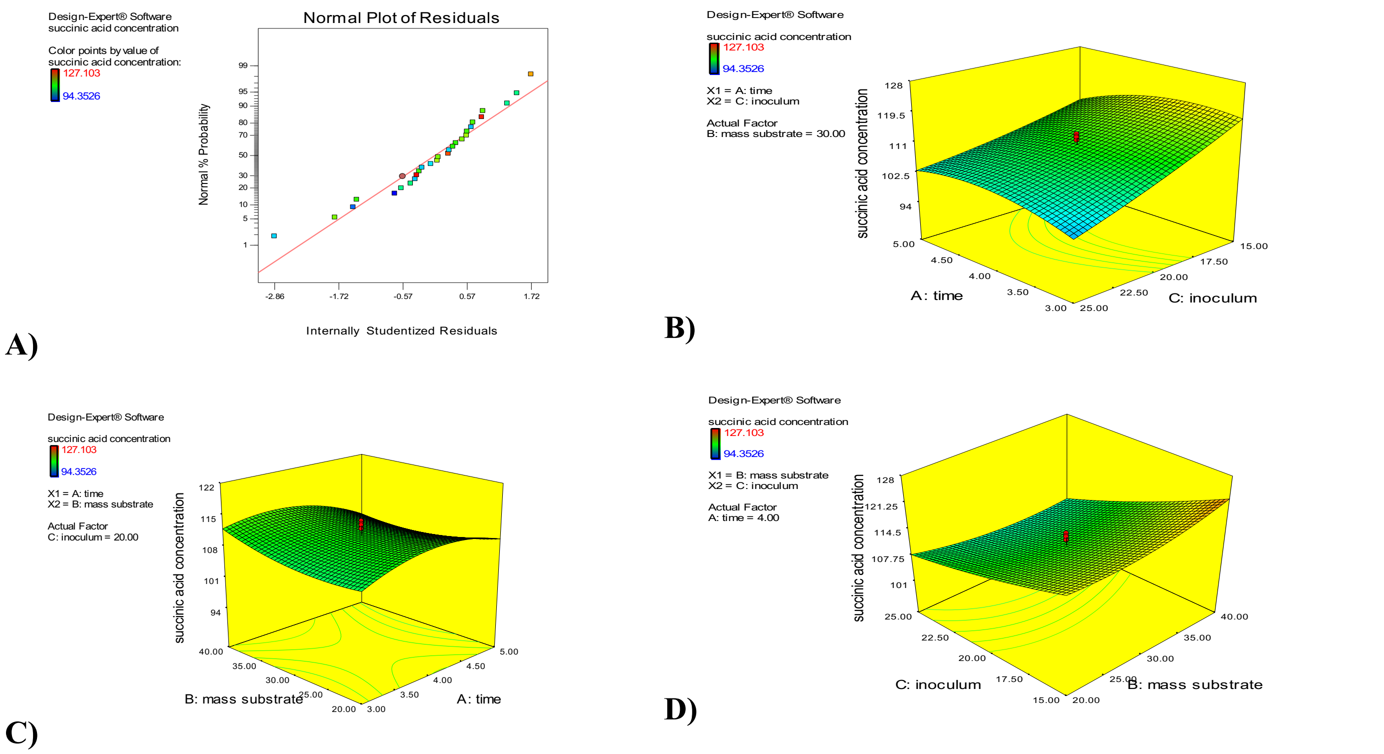

Figure 2: A) Normal probability plots of residuals for succinic acid concentration, B) 3D surface response graph for interaction between time and inoculum in succinic acid concentration, C) 3D surface response graph for interaction between time and mass substrate in succinic acid concentration, D) 3D surface response graph for interaction between mass substrate and inoculum in succinic acid production.

REFERENCE

[1] a H. Hazimah, T. L. Ooi, and a Salmiah, “Recovery of Glycerol and Diglycerol From Glycerol Pitch Recovery of Glycerol and Diglycerol From Glycerol Pitch,” *J. Oil Palm Res.*, vol. 15, no. 1, pp. 1–5, 2003.

[2] H. Song and S. Y. Lee, “Production of succinic acid by bacterial fermentation,” *Enzyme Microb. Technol.*, vol. 39, no. 3, pp. 352–361, 2006.

[3] J. Isar, L. Agarwal, S. Saran, and R. K. Saxena, “A statistical method for enhancing the production of succinic acid from Escherichia coli under anaerobic conditions,” *Bioresour. Technol.*, vol. 97, no. 13, pp. 1443–1448, 2006.

[4] A. Hayyan, M. Z. Alam, M. E. S. Mirghani, N. A. Kabbashi, N. I. N. M. Hakimi, Y. M. Siran, and S. Tahiruddin, “Reduction of high content of free fatty acid in sludge palm oil via acid catalyst for biodiesel production,” *Fuel Process. Technol.*, vol. 92, no. 5, pp. 920–924, 2011.

[5] B. Bisping and H. J. Rehm, “Glycerol production by immobilized cells of Saccharomyces cerevisiae,” *Eur. J. Appl. Microbiol. Biotechnol.*, vol. 14, no. 3, pp. 136–139, 1982.

[6] M. A. Dinamarca, A. Rojas, P. Baeza, G. Espinoza, C. Ibacache-Quiroga, and J. Ojeda, “Optimizing the biodesulfurization of gas oil by adding surfactants to immobilized cell systems,” *Fuel*, vol. 116, pp. 237–241, 2014.

[7] E. Malvessi, S. Carra, M. M. da Silveira, and M. A. Z. Ayub, “Effect of substrate concentration, pH, and temperature on the activity of the complex glucose-fructose oxidoreductase/glucono-??-lactonase present in calcium alginate-immobilized Zymomonas mobilis cells,” *Biochem. Eng. J.*, vol. 51, no. 1–2, pp. 1–6, 2010.

[8] P. C. Lee, W. G. Lee, S. Y. Lee, H. N. Chang, and Y. K. Chang, “Fermentative Production of Succinic Acid from Glucose and Corn Steep Liquor by Anaerobiospirillum succiniciproducens,” *Biotechnol. Bioprocess Eng.*, vol. 5, pp. 379–381, 2000.

[9] F. Chen, X. Feng, H. Xu, D. Zhang, and P. Ouyang, “Propionic acid production in a plant fibrous-bed bioreactor with immobilized Propionibacterium freudenreichii CCTCC M207015,” *J. Biotechnol.*, vol. 164, no. 2, pp. 202–210, 2012.

[10] L. Agarwal, J. Isar, and R. K. Saxena, “Rapid screening procedures for identification of succinic acid producers,” *J. Biochem. Biophys. Methods*, vol. 63, no. 1, pp. 24–32, 2005.