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Effect of Time, Inoculum size (%) and Mass Substrate on succinic acid production from glycerol residue using immobilized *Escherichia Coli*

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ABSTRACT

The study on succinic acid production from glycerol residue using immobilized *Escherichia coli* in a simple fermentation process has shown a potentially low-cost production process. In this research, the bacterial cells were prepared by the entrapment method. This method was chosen since it was reported to be an effective method for large scale production of succinic acid. The effects of different times, inoculum size, and mass substrate values on succinic acid production were studied. Batch culture technique was employed to grow the *Escherichia coli* and the entrapment method for the immobilization of the cells. The optimum condition was observed as 30 g mass substrate (117.99 g/L succinic acid production), inoculum size 20 % working volume (102.30 g/L succinic acid production),and time of 4 h (110.20 g/L succinic acid production). This process condition gave the maximum succinic acid concentration. Preliminary characterization of the raw material was done by using the High-Performance Liquid Chromatography and Fourier Transform Infrared Spectrometry (FTIR). The results were then compared to the raw material (glycerol residue), pretreated glycerol (after pretreatment) and succinic acid concentrations. The effects of mass substrate, inoculums densities, and time were significant on the succinic acid production by immobilized cells.

INTRODUCTION

Nowadays, efforts to convert agricultural wastes, especially oil palm wastes to energy source are been widely investigated in Malaysia. In 2008, Malaysia was the second largest producer of palm oil with 17.7 million tons or 41% of the total world supply. On another note, palm kernel oil is normally been used as a raw material for the production of oleochemicals via transesterification to produce methyl esters. The oleochemical industries generate by-products that include glycerol residue (Ooi *et al*., 2001). Due to the availability of palm oil wastes and the distillation processes that generates much glycerol waste; it seems to be a very promising alternative source of renewable energy generation.

In general, esterification seems to be one of the pre-treatments methods for glycerol residue (Hayyan *et al*., 2011) in order to reduce the free fatty acids in oil and fats. Esterification is then followed by transesterification reaction by using an alkali catalyst to convert it to glycerol. Nevertheless, less attention has been paid to pre-treatment of agricultural wastes. In fact, limited data has been found for the pre-treatment of oleochemical wastes. In this study, the effect of glycerol residue pre-treatment on the production of succinic acid was investigated.

In the transition to a more sustainable renewable energy supply, glycerol from wastes has attracted wide attention across the world. Specifically, the glycerol residue is a promising alternative waste source that can be

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used to produce succinic acid in an environmentally friendly manner by simple pre-treatment and fermentation processes. Incidentally, in the future, this method could replace chemical synthesis plants for the production of succinic acid. Currently, succinic acid from glycerol residue has attracted wide attention across the world as a source of sustainable renewable energy source.

Nowadays, pre-treatment and immobilized fermentation have attracted research interest because of the potential applications. Succinic acid, also known as amber acid or butanedioic acid, is a member of the dicarboxylic acid family and having the molecular formula of $C_4H_6O_4$ (Isar *et al*., 2006b). After its first purification from amber by Georgius Agricola in 1546, it is been produced currently through simple microbial fermentation (Menzel *et al*., 1999). Succinic acid is used in a number of ways such as in the food, pharmaceutical and cosmetic industries (Zeikus *et al*., 1999). In addition, a pretreatment method is found to be an effective process to improve the properties and quantity of glycerol produced. The use of immobilized cell is also one of the methods to reduce the cost of processing in the succinic acid production. For instance, Table 1 tabulates the initial and final glycerol content recovered from biodiesel and Oleochemical wastes.

The focus of this study is on the fermentation of glycerol to produce succinic acid using immobilized Escherichia coli cells. The parameters screened during the pre-treatment process are the inoculum density, time, and mass substrate. The experiments were able to successfully explain the pattern of the succinic acid production, and its fermentative characteristics as discussed in the subsequent sections. Furthermore, a series of batch fermentation processes with different parameters were conducted and the experimental data was used to estimate the parameters and also to validate the experiments. In this research, the use of anaerobic fermentation for the conversion of glycerol to higher value products was also discussed. The succinic acid produced was quantitated by using the High-Performance Liquid Chromatography (HPLC) system.

Table 1: The initial and final content of glycerol and impurities at different sources of glycerol waste.

Sources	Glycerol (%)		Ash(%)		MONG(%)		Water(%)		References
	A	B	A	B	A	B	A	B	
Transesterification of waste used-oil (Biodiesel)	36.7000±0.4900	96.2000±0.0300	4.3100±0.0027	2.0800±0.0600	44.0000±0.4400	1.5000±0.0700	14.7000±0.9000	0.0006±0.0200	(Manosak *et al*., 2011)
Transesterification of palm kernel oil (Oleochemical)	17.7000	51.4000	58.7000	13.8000	5.9000	8.9000	17.7000	25.9000	(Yong *et al*., 2001)
Transesterification of waste used-oil (Biodiesel)	28.5600	93.3400	2.6500	0.00045	56.1300	5.1600	6.7000	1.5000	(Kongjao *et al*., 2010)
Palm kernel oil (Oleochemical)	-	20.2000	-	64.3000	-	12.4000	-	3.0000	(Yong *et al*., 2001)

A: Initial content of glycerol and impurities in the crude glycerol
B: Final content of glycerol and impurities in the refined crude glycerol

MATERIAL AND METHOD

Materials:

The glycerol residue was obtained from Emery Oleochemicals, Malaysia. Pre-treatment of the wastes was carried out to recover the glycerol which was the substrate for the fermentation process.

Pre-treatment process:

The waste was analyzed for the functional groups by using the Fourier Transform Infra-Red (FTIR). After the fermentation process, the amount of glycerol produced was estimated using the High-Performance Liquid Chromatography (HPLC). The pre-treatment was done using the decantation, filtration, and evaporation processes.

Fermentation process:

A single colony of *E. coli* was used to inoculate a 50 mL medium (Medium supplemented with 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L glycerol) in a 100 mL conical flask. The medium was incubated at 37 ^{o}C until an optical density (OD) of 0.4 was reached at a wavelength of 550 nm. The inoculated media were incubated at 37 ^{o}C for 24 hour. The same method was used for the pre-culture method. An appropriate volume of the actively growing pre-culture organism was centrifuged at 10000 rpm for 10 minutes and the pellet was later washed sterilized water and re-suspended. The re-suspended free cells were used at an optical density of 0.050 at 550 nm to inoculate fresh medium. For the immobilized cells, the pallets were used to produce beads of immobilized cells.

Immobilization of the cells:

The entrapment method was used to immobilize the cells before fermentation. The *E. coli* was grown and maintained in a 250 mL LB broth from which the pallet (cells) of *E. coli* were later extracted. The cells were added to a 250 mL solution of Sodium alginate at a concentration of 2% and mixed properly for about 1 h. After 1 h, the Sodium alginate containing the cells was added drop-wise into a 0.1 M Calcium chloride ($CaCl_2$) solution with continuous stirring which resulted in the formation of the beads.

After the formation of beads, the 0.1 M $CaCl_2$ solution was replaced with 0.05 M $CaCl_2$and then allowed to harden for 12 h. After 12 h, the beads were washed with sterile 0.85 % NaCl solution to remove the adherent cells and $CaCl_2$ ions.

RESULTS AND DISCUSSION

Stability study:

In order to test the stability of the beads at different temperatures, the bacterial activity was measured by using the pour plate count method. This method was to show the stability of the *E.coli* after been stored at low temperature (-4^{0}C) and at 37 ^{0}C for 1 week. The data was collected daily and the number of immobilized bacteria counted using the total plate count method.

Storage Stability (Temperature (-4 ^{0}C)):

The main goal of this study is to determine the prolonged and higher activity of *E.coli* in producing the desired products. The total bacteria count from day one to day seven after incubation at -4 ^{o}C was performed using the total plate count method. The results of the count are shown in Figure 1.

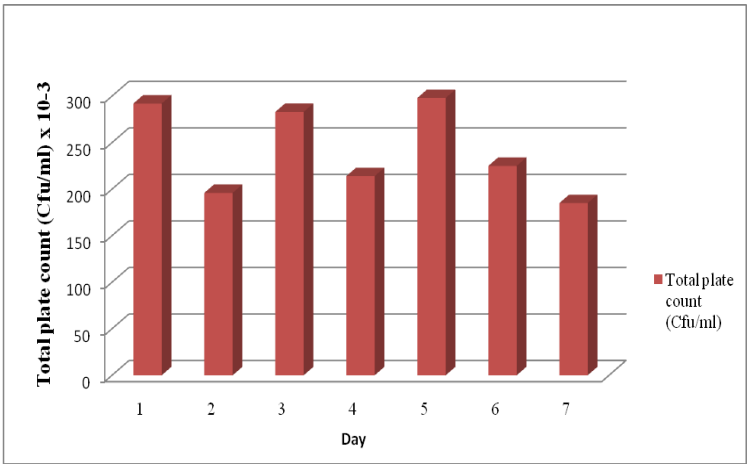

Fig. 1: The total plate count of the *Escherichia coli* from day one to seven.

This study was conducted to investigate whether immobilization can preserve the fermentation ability of *E.coli* after a long storage at low temperature since it has been suggested that immobilization can confer protection to cells when exposed to an unfavorable environment. From the results, the number of *E. coli* in the beads on the first day was approximately 292 x 10^{-3}CFU/mL and the range was maintained at about 10^{-3}CFU/mL for 1 week. The number of *E. coli* decreased on day two, then increased on day three (283 x 10^{-3}CFU/mL), and after day five (298 x 10^{-3}CFU/mL), started decreasing once more. After day seven, the cell count was still high though there was a slight decrease in the viable count due to the death or inactivation of some of the cells occasioned by exposure to unfavorable conditions such as temperature, handling, and the bead properties. Furthermore, the study recommends a maximum storage period of five days for the immobilized cells in order to achieve a better activity of the cells.

Actually, there were not many changes on the pattern of the activity of the bacteria when stored at low temperature as could be observed in the pattern of the changes in the bacterial count from the day one to day seven of storage. Bisping and Rehm (1982) studied the storage stability of a mixed culture of *Cryptococcus elinovii H1*and *Pseudomonas putida P8* adsorbed on activated carbon. From the results, there was quite a little variance in the results obtained after the first fermentation process and those obtained during the second fermentation after storage. Chávarri *et al*. (2010) studied the stability of microencapsulated probiotic bacteria with or without quercetin during storage at 4 ^{o}C for 4 weeks. The results showed a statistically significant difference in the stability of the bacteria stored at the studied conditions.

Incubation period Stability (Temperature (37 ^{0}C)):

The aim of this investigation is to study the total number of viable *E. coli* when stored at 37 ºC for seven days. The *E. coli* in the beads after the immobilization process were stored in an incubator at 37 ºC without the addition of the fermentation broth. The number of *E. coli* growth was calculated and the results are shown in Figure 2.

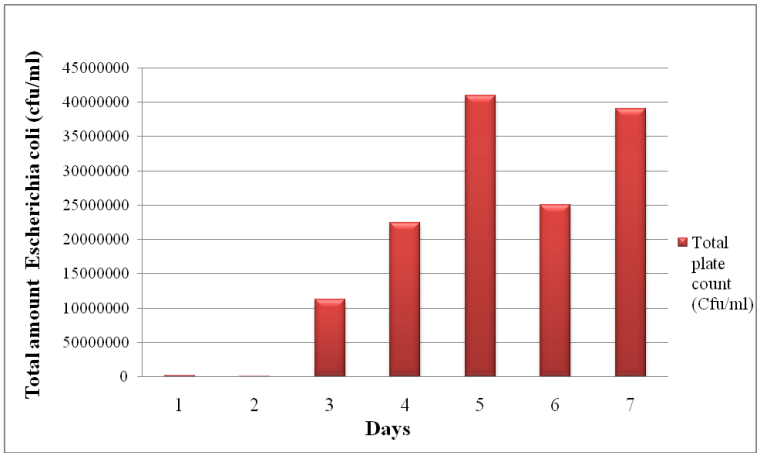

Fig. 2: The total plate count of the *Escherichia coli* from day one to seven in temperature 37 ^{0}C.

The incubator temperature (37 ºC) was used to check the stability of the beads for one week. From the results, the bacterial activity increased with time possibly due to the fact that the optimum temperature required for the growth of *E. coli* is 37 ºC. The number of bacteria was initially 28 x 10^{-4} CFU/mL on the first day of incubation and later increased continuously until day six after which the bacteria count decreased to 25 x 10^{-7}CFU/ mL. The decrease could be due to the fact that the death phase of the bacteria has been reached. The maximum growth of 41 x10^{-7}CFU/mL was achieved at day six.

Among the different temperatures studied, maximum growth was achieved at 37 °C. However, the organism failed to grow at 50 °C because its maximum thermal tolerance limit is 37 °C. The effect of temperature on the activities of the bacterial enzymes via the reverse TCA cycle is at a maximum at 37°C. Based on this, different bacteria mostly grow at different temperatures, with many organisms tolerating a maximum temperature range of 20 to 30°C although the thermophiles can grow at higher temperatures (50 to 55°C) while the thermo-phobic ones prefer colder temperatures (15 to 20°C). Furthermore, some lactic acid producing bacteria grow best at 18 to 22°Cwhile *Leuconostoc* species which initiates fermentation have an optimum of 18 to 22°C. The *Lactobacillus* species have temperature optimum above 22°C.

The growth activity of *E.coli* which is temperature dependent can affect the production of the product and the best temperature is believed to enhance the production of the product. Corona-González *et al*. (2008) used *E. coli* for anaerobic fermentation at 37 ºC but Isar *et al*. (2006a) used *E. coli* to produce succinic acid at a temperature of 39 ºC. For other bacteria, Zheng *et al*. (2009), used *Actinobacillussuccinogenes* to produce succinic acid from straw hydrolysate at temperature 37 ºC. Bacteria have varying requirements in terms of the range of temperature at which they grow.

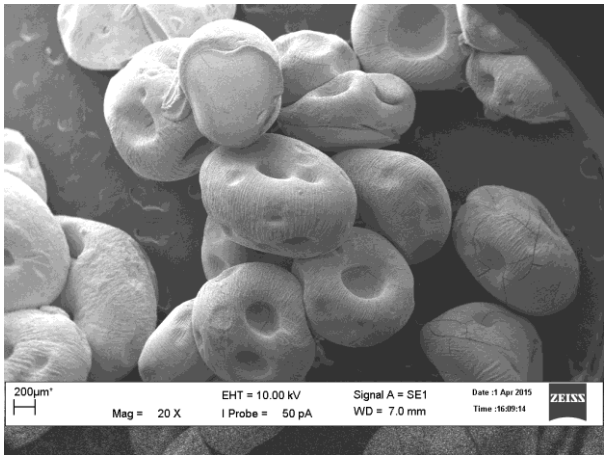

Fig. 3: SEM micrographs of *Escherichia coli* cells immobilized on bead (alginate).

Effect of Inoculum:

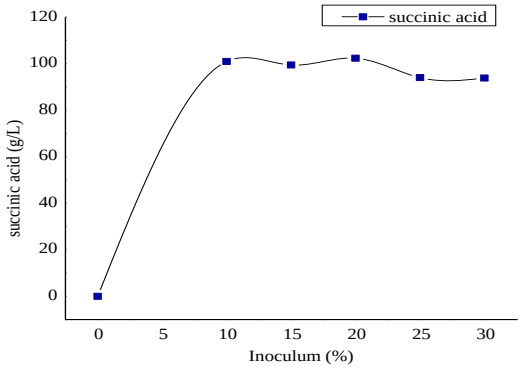

Fig. 4: The graph showed the succinic acid production versus the inoculums.

One of the factors that affect the production of succinic acid is the inoculum size. Figure 4 shows the effect of inoculum size on the succinic acid production. A series of inoculum range was studied to determine the highest percentage of inoculums that could give the highest production of succinic acid. This study aimed to observe the relationship between the percentage of inoculums and the succinic acid production. From the graph shown (Figure 4), the optimum inoculum for succinic acid production was 20% of inoculum and the succinic acid production was 102.30 g/L. The bacteria were entrapped in the immobilized cells before usage and this showed that *E. coli* vary in growth rate and succinic acid production at different inoculums sizes (immobilized cells). From the graph, the concentration of succinic acid started to increase (100.90 g/L) at an inoculum size of 10% until 20% but later decreased (93.79 %) when the inoculum size was increased to 30 %.

Increasing the inoculums size will increase the number of *E. coli* cells in the medium which will considerably affect the growth rate. The equation (Eq. 1) describes the relationship between the biomass (X), the cell number (N) and the growth rate (μ) which is time dependent. Increasing the cell number (N) or biomass (X) will increase the growth rate of the *E.coli* thereby increasing the rate of succinic acid production. This production can decrease with time as a result of the accumulation of factors such as increased waste product concentration, reduced surface area and death of some cells due to the toxicity of the environment.

$$X(t) = \mu (t) \, . \, X (t) \quad (a)$$
$$N (t + t_d) = 2.N(t) \quad (b)$$
$$t_d = \ln 2 / \mu \quad (c) \qquad (1)$$

The biomass concentration has the same effect as the growth of the organism on the production of succinic acid. From the equation (b), the doubling time t_o of the cell population which commonly describes the cell number or cell mass was described and for the equation c, the relationship to the number or mass based on doubling time to specific growth rate was described. Several studies have reported studying free bacteria or immobilized bacterial cells as one of the parameters. Ercan *et al*. (2013) got results by varying the amount of yeast cells entrapped in beads by using RSM to optimize the process. From the results, using 5% yeast cells entrapped in beads gave the optimum concentration of ethanol after fermentation. The amount of cells entrapped in the beads had a significant effect on the production of the product.

Effect of Time:

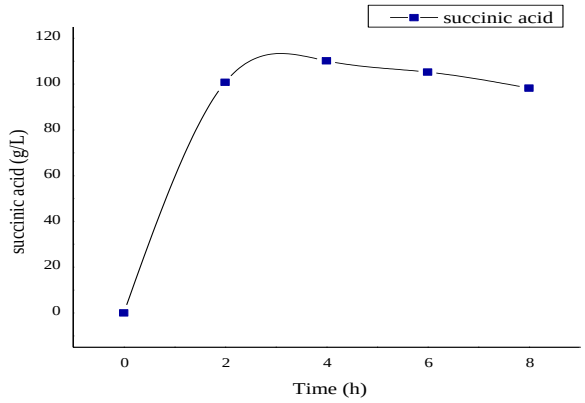

Fig. 5: The graph showed the succinic acid production versus the time.

The time as a parameter was selected and studied so as to determine the optimum time on the production of succinic acid from glycerol using immobilized *E. coli*. Figure 5 shows the effect of time on succinic acid production and from the result, a maximum succinic acid production was achieved after 4 h after which the production decreased with time. The succinic acid production increased with time until 4 h where a concentration of 110.20 g/L was achieved but after 4 h, the production decreased with time. This is because after 4 h the *E. coli* started a new phase which is the accelerated phase, followed by the exponential death phase and then the death or survival phase. For the batch culture fermentation, there are different growth stages including the lag phase, acceleration phase, and exponential growth.

The growth or exponential phase is described using Eq. 2 only if all the cells have naturalized in the medium and are able to divide and grow. This process can only be favored in the presence of nutrients and absence of inhibitors. The duration of the batch fermentation should not be studied only at the lag phase where the growth rate is maximum but also have to be considered at the beginning of the fermentation or growth phase.

$$X(t) = X_0 . e^{\mu max.t} \quad (2)$$

The growth of *E. coli* started to decrease after six hours and this phase was the deceleration or retardation phase where growth is retarded due to exhaustion of nutrients and accumulation of inhibitors. After this phase, the specific growth rate becomes the function of the nutrient, product concentration and time. The cell may have morphological changes because of the increase in cell population but the specific growth rate at certain process times could be reduced. At this phase, the cell net growth rate is zero with time because of exhaustion of nutrients in the medium. Furthermore, certain products like intracellular materials could still promote the proliferation of some cells while others may have died already. Lastly, the specific growth rate is usually negative and the cells could be unable to maintain their physiological activities.

There have been scientific studies on the effect of time on immobilized cells such as described by Ghorbani *et al*. (2011) who used immobilized cells to produce ethanol from cane molasses. Based on the study, one of the parameters studied was time and the results showed that time had a significant effect on the production of ethanol with retention times of 5.21, 6.94, 10.42 and 15.63 h. Also, Zhao and Xia (2010) used immobilized cells to produce ethanol from corn stoverhemicellulosic hydrolysate and the results showed that fermentation using immobilized cells was more effective after 72 h. In addition, the research by Khanna *et al*. (2013) who studied the time when using biodiesel-derived crude glycerol to produce n-ethanol through immobilization showed that the maximum yield of n-butanol was achieved after 120 h. The effect of *E. coli* on the production of succinic acid by immobilized fermentation process is inthe range of two to six hours for the maximum production of succinic acid. The effect of time was first screened using One-factor-at-a-time (OFAT) to find the best time before proceeding to the optimization process where the optimum time for maximum succinic acid production will be achieved.

Effect of Mass Substrate:

Glycerol residue is the raw material used after the pre-treatment process for succinic acid production. Glycerol was used as the carbon source during the study and it had a significant effect on the production of succinic acid. The study showed the effect of varying concentration of the carbon source on the microbial growth as well as on the rate of production of succinic acid. Different substrates masses were used during the study to observe the effect of substrates used for the production of succinic acid. Figure 6 shows the concentration graph for succinic acid production versus the mass of treated glycerol residue which is known as the substrate. Based on the graph, the rate of succinic acid production increased with increase in the substrate mass. The increase was sustained until a substrate concentration of 40 % of treated glycerol residue after which

the rate of production of succinic acid started to decrease. The result showed that the maximum concentration of succinic acid (117.99 g/L) was achieved at a substrate concentration of 30 g.

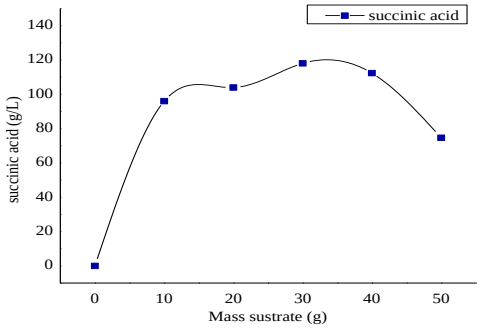

Fig. 6: The graph showed the succinic acid production versus mass substrates.

The graph shows the monod relationship which stipulates that growth rate or chemical reaction depends on the concentration of the chemical nutrient.

$$\mu = \mu \max \frac{S}{Ks + S} \quad (3)$$

From the equation above, the relationship with the Monod kinetic graph is shown where μ and μmax are the specific and maximum growth rate while S is the substrate concentration and Ks is the value of the substrate concentration at μ = 0.5 μmax. The model suggested that the formulation based on the analogy with the saturation kinetics and the model with the dependence of growth on chemical concentration can be described by two constants which are the maximum growth rate and the limitation constant (ks). Furthermore, using only two parameters does not significantly explain the Monod equation for the description of the substrate dependent. In addition, growth is dependent on the substrate concentration and it reflects what is known as the mechanism of substrate uptake.

$$r_s = \frac{1}{Yx,s} . \mu + m \quad (4)$$

Normally, the growth rate will be dependent on the substrate concentration and the graph of the growth will show a straight line for the Monod equation above which is a linear relationship between the substrate uptake or product formation and the growth rate. In fact, for heterotrophic organism the organic sources play an important role as energy sources. In addition, normally for anaerobic fermentation, one of the factors that limit the growth rate is the anaerobic uptake rate and the energy generation from the organic substrate, while the growth is controlled by limited substrate up. This is referred to as energy limited growth.

The substrate affected product formation just as Khanna *et al*. (2013) who used biodiesel derived from crude glycerol and immobilized *Clostridium pasteurianum*to produce n-butanol using different concentrations of glycerol. From the research, they found that the production of butanol was higher at a substrate concentration of 25 g/L while the yields of 1-2-Propanediol and ethanol were higher at substrate concentrations of 10 g/L and 150 g/L, respectively. Lin *et al*. (2008) studied the substrate and product inhibition kinetic in succinic acid production by using *Actinobacillussuccininogenes*. The carbon sources used in the study was glucose and the results showed that the substrate and product inhibition have a significant effect on the cell growth and succinic acid production using *Actinobacillussuccininogenes*. Ghorbani *et al*. (2011) also studied the effect of different substrate concentration on the production of ethanol using immobilized cells. They used immobilized *Saccharomyces cerevisiae* to produce ethanol from cane molasses and the results showed that the ethanol production was affected by the concentration of molasses (50, 100 and 150 g/L) in the fermentation broth.

From this study, we found that an increase in the substrate concentration will increase the production of succinic acid but at one point, the succinic acid concentration will decrease with the increase of the substrate. This is due to the increase in the phase of the fermentation broth from semi-solid phase to the solid phase which does not promote the activity of the fermentation organisms. When the fermentation broth is saturated with the substrate, it tends to affect the reaction in this phase solution whereby the reaction becomes low and cannot progress smoothly. This is generally referred to as substrate inhibition. Furthermore, all nutrients have an upper concentration limit above which further increase will cause a decrease in the growth rate. The increasing solute concentration causes partial dehydration of the cell as the cells were starved of moisture due to the solidity of

the medium, thereby resulting in either death or reduced growth of the cells (Heipieper *et al*., 1991). This happened in the fermentation of the glycerol for succinic acid production using immobilized cells, where at a substrate concentration of 40 g, the reaction was slowed due to the saturation of the medium with the substrate which led to a decreased succinic acid production. Table 2 show the comparison with different substrate for succinic acid production using batch fermentation method.

Table 2: Comparison results of difference studies that produce succinic acid production with different substrate

Substrate	Method	Microorganism	Succinic acid production	References
Carob pods (30 g/L substrate)	Batch fermentation	*Actinobacillus succinogenes*	1.67 g / L	(Carvalho *et al*., 2016)
Cellobiose (50 g/L)	Batch fermentation	*Actinobacillus succinogenes*	30.3 g/L	(Jiang *et al*., 2013)
Crude and Purified Glycerol (30 gram)	Batch fermentation	*Escherichia coli*	117.99 g/L	This study

Conclusion:

Based on the experimental data presented in this study, the immobilized cells could be reused for up to five cycles and this can reduce the cost of production of succinic acid from biowastes. The effect of time, mass substrate, and inoculum size (densities) were pronounced on the concentration of succinic acid produced. The plate count of bacteria analysis showed that the immobilized cells had a good chemical stability within the beads. Based on the results of this study, it could be said that immobilized *E. coli* were able to produce succinic acid from glycerol residue in a submerge fermentation process.

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