Scaling up of Lactic Acid Fermentation using *Enterococcus faecalis*

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Abstract. Lactic Acid bacterium (LAB) *Enterococcus faecalis* was used with the aim of scaling up a lactic acid fermentation (LAF) from 3L jar fermenter to pilot scale up to 100-L in an open fermentation. Batch mode in Lab and pilot scale fermentations were compared in terms of productivity, overall yield, cell growth, lactic acid production, and glucose consumption. Fermentations were performed at pH 6.86, 45 oC, and 100 rpm. Process simulation was conducted by using SuperPro Software and the data obtained from pilot scale. The results were compared using t-test and ANOVA. The results showed that the production rate of Lab scale was 4.96±0.09 g/Lh with a yield of 0.93 ±0.005. While for pilot scale, the production rate was 3.91 g/Lh with a yield of 0.915 ±0.005. There was no significant difference between both processes, regarding the yield, but there was a difference regarding the production rate, being slowly lower for pilot scale, but still high for industrial scaling up. Contamination was controlled fairly because *Enterococcus faecalis* is a thermophilic strain and then, proved to have industrial potential to produce Lactic Acid (LA).

1.0 Introduction

Lactic acid (LA) is an important global commodity due to its diverse industrial application and mainly because of its use as a feedstock for the synthesis of poly-Lactic acid (PLA), a biodegradable plastic. In the last decade, world LA production has expanded 10 times due to the higher market demand in the

food industry and green products [1, 2]. The continuous research of LA is mainly focusing on seeking a better cost-effective substrate [3], improvement of process engineering and the screening or isolation of potential microorganisms which can boost the productivity [4, 5]. It has been reported that improving and further development of the LA technology will not bring any benefit if it involves higher raw material prices. Cost constraint and substrates availability have encouraged the company Purac from Holland to build a sub-company in Thailand to seek for cost effective substrates. One of the disadvantages of PLA is it cannot compete with conventional plastics derived from petrochemicals because it is heavily relying on its production cost [6]. It has been reported that Sago palm can be easily found in Malaysia and Indonesia and it is one of the cheaper crops to produce fermentable sugar [7]. Sago palm contains a lot starch in its trunk and this industry has been a well-established in Malaysia since 1980-an [8]. There were researches conducted on the conversion of the sago palm starch to glucose by enzymatic method and subsequently applied the obtained syrup for fermentation [9]. In order to reduce the production cost of the substrates, the screening and isolation of microorganisms with high LA productivity is an important task. Lactic Acid Bacteria (LAB) has been selected as the preferable microorganism for LA. Some drawbacks of these microorganisms were reported such as their fastidious nutrition requirements and the side effects of end-product inhibition [10, 11, 12]. The most commonly used LAB is the genus Lactobacillus because it provides higher LA concentration and volumetric productivity [13]. The LAB in the group of Enterococci offers good prospects as some strains are thermophilic and this can maintain a high concentration of LA [14, 15]. The thermotolerance confers a unique advantage and this property can avoid or reduce the risk of contamination from competitive microorganisms. Thus, the feasibility of scaling up the isolated microorganism and substrate for the industrial application needs to be tested at laboratory scale. In this paper, an isolated strain of LAB identified as *Enterococcus faecium*, was used to scale up the Lactic Acid Fermentation (LAF) and it was conducted at laboratory scale. Also, the scale- up was done by a geometric laboratory fermenter and the yield of LA was defined as the production response variable.

2.0 Materials and methods

2.1. Sago starch hydrolysis

Industrial grade sago starch was obtained from Nitsei Sago Industries, Kampung Teh, Mukah, Sarawak. The hydrolysis of sago starch was done with the method reported by Novozymes and elsewhere [7]. Briefly, for liquefaction, 400 g of sago starch (dry basis) was suspended in tap water and the final volume was adjusted to 1 litre. The pH of the suspension was diluted to 4.5. A thermostable α-amylase (1,4-α-D-glucan glucanohydrolase) (EC 3.2.1.1). from *Bacillus licheniformis*, 240 KNU-S/g of starch, (10 µl, 3000 U/ml) (Novozyme Co.) were added to liquefy the starch at 95-100℃ for 2 hours. The liquefied starch was left to cool and further treated with 0.5 µl (0.23 amyloglucosidase units AGU) of enzyme dextrozyme DX (Novozyme, Co). The syrup was then cooled to 60℃ and 0.5 µL of α-amyloglucosidase per gram of starch was added into the solution. The pH was adjusted to 5.5 and the syrup was incubated for 24 h with the agitation rate of 100 rpm. The final obtained product is glucose syrup.

2.2. Pre-inoculum preparation

Stock culture of *Enterococcus faecalis* was isolated in the laboratory of Biochemistry of the faculty of Resource Science and Technology at UNIMAS and kept at -10˚C in the medium containing 5 g/L yeast extract (YE) and 30 g/L glucose in 2 ml Eppendorf vial. The stock culture was thawed at room temperature and activated in 5 ml culture medium containing 5 g/L YE and 30 g/L glucose. The culture medium was then incubated in a static condition at 37 ˚C for 24 h using Shel Lab SL incubator.

2.3. Inoculum preparation

The activated pre-inoculum culture of *Enterococcus faecalis* after 24 h incubation was inoculated into 200 ml of culture medium containing 5 g/L YE and 30 g/L glucose in 250 Erlenmeyer flasks. The culture medium was cultivated in Shel Lab SL incubator at static condition at 37 ˚C for 24 h. The culture was then centrifuged at high-speed centrifuge Kubota model CR21G at 6000gc, 37°C for 5 minutes in order to harvest the cells.

2.4. Main media preparation for fermentation

The medium for the fermentation consisted of glucose and YE only at level of 100 g/L and 5 g/L respectively. The medium was heated for 12 minutes in the microwave to ensure homogenous mixing and poured in the 3-L jar fermenter to be autoclaved at 121˚C for 20 minutes.

2.5. Fermentation of Enterococcus faecalis in batch reactor

The harvested pellet of cells from the inoculum preparation was inoculated in the 3L bioreactor with a working volume of 2L. The parameters of the fermentation process such as pH and temperature and NaOH consumption were controlled automatically and monitored in real time. The fermentation was carried out at 45˚C, agitation rate of 100 rpm and the pH was adjusted automatically at 6.86 by adding 10 M NaOH. The process was carried out until the 10 M NaOH was depleted. During the fermentation, the consumed weight of 10 M NaOH was recorded. After the completion of the fermentation, the broth in the bioreactor was centrifuged to harvest the cell. The harvested cell was used as inoculum for the next fermentation.

2.6. Pilot scale fermentation

For pilot scale an inoculum of 10L was prepared in a 10 L jar fermenter. The inoculum was transferred to the 100 L STR for pilot scale fermentation. The culture medium of fermentation containing 72 g/L glucose and 5 g/L yeast extract. The fermentation process was the same as laboratory scale where the parameters such as pH and temperature were monitored in real time. The fermentation was stopped when there was no NaOH being consumed. The weight of 10 M NaOH consumed was recorded.

2.7. Simulation using SuperPro software for commercial scale

The mass stoichiometry from the pilot scale experiment was used for simulation using SuperPro Software to determine the yield parameter for LAF. The kinetic parameters used in the simulation were K_s and μ_{max}. The constant parameters used in the simulation were temperature, pressure and substrate concentration (glucose). The simulation was conducted in the first-order reaction for batch fermentation for 48 h.

2.8. Analytical method

The analysis was conducted to determine the LA yield, glucose concentration and dry cell weight (DCW) analysis at the end of experiment. Reducing sugar was analysed using dinitrosalicylic acid (DNS) method. For DCW, a volume of broth was centrifuged in high-speed centrifuge Kubota model CR21G at 6000gc, 37°C for 10 minutes to collect the pellet of cells. The pellet was rinsed with 0.2M HCl and centrifuged. Then, the cell weight was determined by drying the pellet for 3 days in 60°C. The LA was analyzed based on its direct titration by using 10M NaOH throughout the experiments. NaOH was then pumped automatically using a peristaltic pump controlled by the computer based on the setpoint controlled at 6.86. The computer registered the variation of weight of NaOH throughout the experiment. The total amount of LA produced was determined by multiplying the amount of NaOH consumed at any time by the conversion factor, F=0.69. The conversion factor (F) was obtained from the stoichiometric of the reaction:

$$\boldsymbol{C_3H_6O_3 + NaOH \rightarrow C_3H_5O_3Na + H_2O} \qquad (1)$$

Where,

$NaOH_{mw}$ = 40 g/mol, Lactic $acid_{mw}$ = 90 g/mol

NaOH density, $\boldsymbol{\rho}$ = 1.3 g/mL

Thus, 1 g of 10 M NaOH equivalent to 0.7692 mL

Gram of NaOH per mL of 10M NaOH = 400g/1000mL = 0.4

Equivalent factor NaOH to Lactic acid = 90/40 = 2.25

Conversion factor NaOH to Lactic acid:

$$= \left(\mathbf{0.7692}\ \frac{\boldsymbol{mL}}{\boldsymbol{g}}\right) \times \left(\mathbf{0.4}\frac{\boldsymbol{g}}{\boldsymbol{mL}}\right) \times \left(\mathbf{2.25}\ \frac{\boldsymbol{g/mol}}{\boldsymbol{g/mol}}\right) = \mathbf{0.692} \qquad \mathbf{(2)}$$

3.0 Results and discussion

3.1. Lactic acid fermentation in laboratory scale

According to the stoichiometric of the reaction where 2L fermentation broth containing 100 g/L of glucose concentration, a theoretical consumption of 10M NaOH must be 213 mL for the titration of the LA produced. The volume of NaOH consumption is equivalent to 277 g of 10M NaOH. The average of the data for NaOH consumption and its conversion to LA production rate (volumetric productivity for the laboratory scale) are shown in Table 1. The amount of the NaOH consumption was 270 ±1.0g, where this value is near to the theoretical calculation 277g and clearly illustrated the evolution of the metabolism of the microorganism. In the last hours of the fermentation, a deceleration was observed due to the concentration of LA reaching its optimum and the glucose being fully consumed. The effect of LA concentration was widely studied. LA is commonly recognized as the growth inhibitory due to the solubility of the undissociated LA within the cytoplasmic membrane and insolubility of dissociated lactate, which will lead to acidification of cytoplasm and failure of proton motive forces. Consequently, the transmembrane pH gradient was distorted and decreased the amount of energy available for cell growth [15]. This phenomenon can be avoided via direct LA titration by alkali addition. The use of the type of alkali is controversial and some researchers used NaOH and some claimed that combination of ammonium hydroxide (NH_4OH) and buffering by calcium carbonate ($CaCO_3$) will further enhance the productivity [16]. However, the production rate of LA does not increase significantly by using alkaline substance because the control of the productivity is a series of parameters rather than pH. In this research, a higher concentration of NaOH, e.g. 10M NaOH was employed to avoid dilution of the fermentation broth, especially during the operating condition of high substrate concentration.

Table 1. Kinetics of the titration of LA in laboratory scale fermentation and LA production rate

Time (h)	NaOH Consumption (g)	LA Production Rate (g/L h)
0	0.00±0	0.00±0
4	38.7±6.4	3.34±1.2
8	114. ±2.5	4.96±0.1
12	168.3±3.5	4.85±0.2
16	200.0±8.2	4.33±0.2
20	225.0±3.6	3.89±0.1
24	240.0±6.2	3.46±0.1
28	253.0±1.0	3.12±0.1
32	262.0±2.0	2.83±0
36	268.0±1.0	2.58±0
40	269.0±1.0	2.33±0
42	270.0±1.0	2.24±0.1

3.2. Lactic acid analysis

Figure 1 shows LA concentration and residual glucose concentration during the fermentation in 3-L jar fermenter (Lab scale). The trend of the LA production increased rapidly during the first 8th hour, which indicated the exponential phase and decreased after 8 hours. No lag phase was noticed. The LA concentration reached 93.4±0.82 g/L at the end of the fermentation. Meanwhile, the glucose concentration decreased from 100 g/L to 2.7±0.3 g/L. The LA yield was achieved 0.93±0.004 g/g. Although high concentration of LA,182 g/L was reported, it was produced in a jar fermenter at laboratory scale [17]. Figure 2 shows the NaOH consumption (g) and LA production rate (g/Lh). The higher rate was reached (4.95±0.09 g/Lh) at 8-10 h of fermentation. The stability of the fermentation was defined using the small standard deviation in the kinetics model via statistical analysis (one-way ANOVA) (IBM SPSS Statistic software). It was determined that there was no significant difference between the experiments and the generated model as shown in Table 2 where p-value (significant value) is greater than 0.05. Computer control was a good tool to maintain the variation of pH at a minimum level so that *Enterococcus faecalis* was not altered to maintain its internal rate of metabolism. Similarly,

controlling the temperature at minimum variation has helped to maintain the metabolism rate of the strain. A mild agitation speed (100 rpm) was used to ensure a gentle homogeneous mixing during the fermentation process.

Table 2. One-way ANOVA of Lactic acid production rate between three repetitions of laboratory scale

Groups	∑squares	df	Mean Square	F	*p*
Between	0.12	2	0.06	0.032	0.97
Within	60.92	33	1.85	-	-
Total	61.04	35	-	-	-

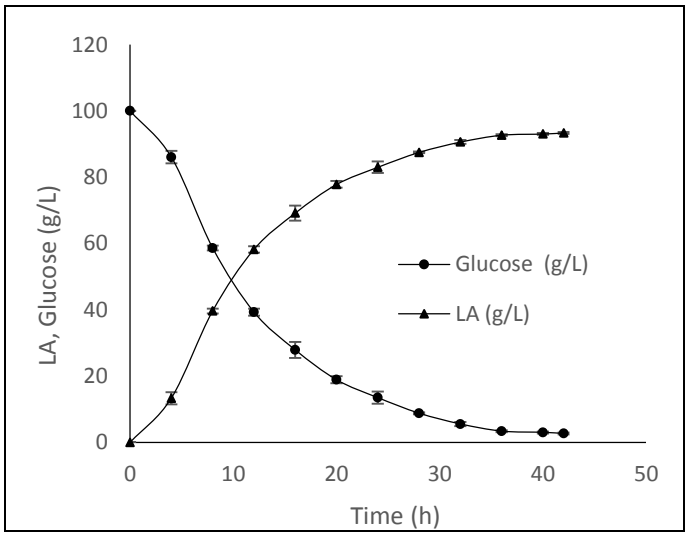

Figure 1. Kinetics of glucose consumption and Lactic acid production in 3L jar fermenter.

3.3. Pilot scale lactic acid fermentation

The LAF was scaled up to 110L using a working volume of 100L. The fermentation data for the pilot scale for two repetitions are shown in Table 3. Based on the theoretical calculation, every 100-L of LAF containing 71±1 g/L of glucose concentration will consume 7470 mL of 10M NaOH during the titration stage in order to synthesize LA (by considering an efficiency of 95% to convert glucose to LA). The volume of NaOH is equivalent with 9711g of 10M NaOH (density 1.3 g/L). The concentration of 71±1 g/L of glucose was chosen to be controlled. Figure 1 shows that the concentration of LA beyond this level will result in a lower production rate because at this concentration inhibit the reaction as reported by Nolasco-Hipolito, et al., (2002) for *Lactococcus lacti*s IO-1 [18]. Other strains show similar behavior, that is further increasing the concentration of LA slow-down the reaction. The completion of fermentation process took 38 h. It was found that increasing the concentration of inoculum from 1% to 5%, increases the DCW of the viable cells. The metabolic activity decreased as the pH decrease [19]. Inoculum size will affect the performance of the fermentation. This is due to the addition of fresh bacteria in higher volume speeding up the fermentation process and increasing the reaction rate [20]. Besides pH, the temperature is also one of the factors that affects the metabolic activity. Incubation condition must be set at optimum temperature to obtain a maximum production rate for the fermentation [21]. *E. faecalis* is one of the LAB which is able to withstand high temperature during the fermentation process [22].

Table 3. Kinetics of glucose consumption, lactic acid and sodium hydroxide used for the direct titration of lactic acid.

Time (h)	NaOH Consumed (g)	Glucose (g/L)	LA (g/L)	LA (g/L h)

0	0	72.2	0.00±0	0.00
4	2070	56.8	13.9±0.9	3.7
8	4389	39.6	33.9±2.6	3.9
12	5824	28.9	42.2±0.6	3.5
16	6935	20.6	48.7±0.7	3.1
20	7673	15.2	54.1±0.6	2.7
24	8278	10.6	59.5±0.5	2.5
28	8599	8.3	62.2±0.9	2.2
32	8920	5.9	64.0±0.4	2.0
36	9165	4.0	65.4±0.0	1.8
38	9238	3.5	66.2±0.3	1.7

3.4. Lactic acid production in pilot plant

Figure 3 shows the increasing LA concentration increasing with time. In the first repetition, the graph shows the LA concentration increased rapidly in the range of 5th hour to 28th hour and slightly increased from 28th hour to 60th hour indicating that the exponential phase occurs. While Figure 2 shows NaOH consumption increased rapidly till 8th hour and started to decrease after 8th hour. There was no visible lag phase noticed as there was no delay from the inoculation to pilot scale fermentation. The age of the inoculation will affect the overall performance of the fermentation. An experiment was conducted on lipase activity and reported the longer the inoculation age, the lower the lipase activity [23]. This issue was minimized by monitoring the optical density and controlling the temperature and the agitation rate. Applying the suggested method, the lag phase of inoculum was observed and ready to continue performing exactly in the pilot test. The fermentation reached a LA concentration of 66.2g/L at the end of the fermentative process. Long fermentation process will result in higher LA concentration [24]. Under this condition a residual, glucose of 3.5 g/L was determined and LA yield of the fermentation was 0.91. Fermentation at 8th hour showed the maximum production rate, 3.9 g/L h. After 8th hour, the LA production rate started to decrease when the glucose concentration was 39.6 g/L. Increasing the concentration of the LA will inhibit the solubility of the associated form within the cytoplasmic membrane of bacteria and impacts the LA production rate. The LA will acidify the cytoplasm membrane and lead to the malfunction of proton motive force, thus, decreasing the nutrient transport across the membrane [25]. Figure 4 and Figure 5 show a higher performance observed in every parameter for laboratory scale than the pilot scale. The data of LA production rate in the laboratory and pilot scale in Figure 4 were compared using one-way ANOVA and the result was presented in Table 4. From the result, there is no significant difference between the data, as the p-value ($p<0.05$) is greater than 0.005. From this analysis, it validates the null hypothesis in the objective. An amount of 3.15 g/L of cells and a final residual glucose of 3.5 g/L were obtained during the completion of the fermentation. Table 5 shows the kinetic and yield parameter of the laboratory and pilot scale of each repetition.

Table 4. Kinetic and yield parameter of laboratory and pilot scale of LAF

	LA (g/L)	Yield (g/g)	Overall Productivity (g/L·h)	Maximum Productivity (g/L·h)
Lab.	93.4	0.93	2.242	4.96
Pilot	66.2	0.91	1.7	3.9

Table 5. One-way ANOVA of Lactic acid production rate between laboratory and pilot scale

Groups	∑ Squares	df	Mean square	F	*p*
Between	4.237	3	1.412	0.828	0.486
Within	73.320	43	1.705	-	-
Total	77.557	46	-	-	-

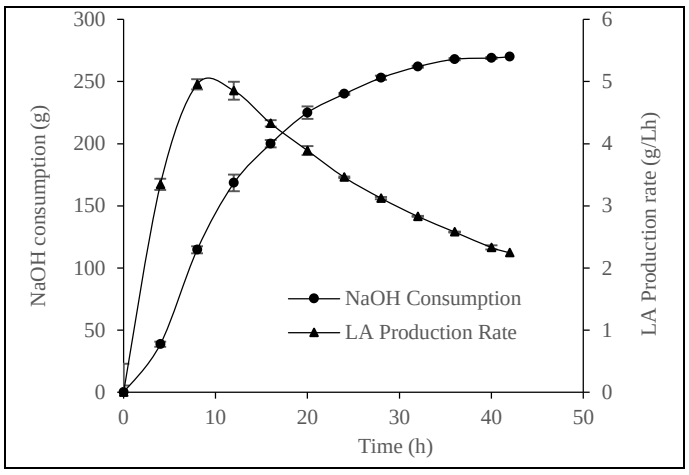

Figure 2. Kinetics of 10 M NaOH consumption (g) and volumetric productivity (g/Lh). The data is the average of three replicate experiments in 3L jar fermenter.

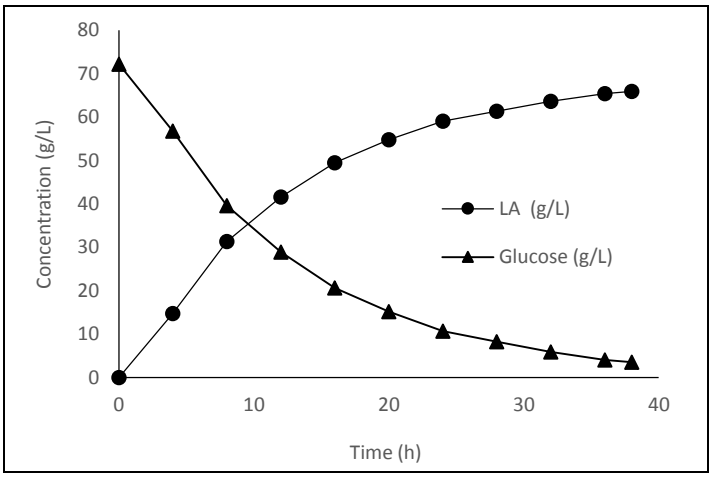

Figure 3. Lactic acid concentration and glucose concentration in the STR fermentation.

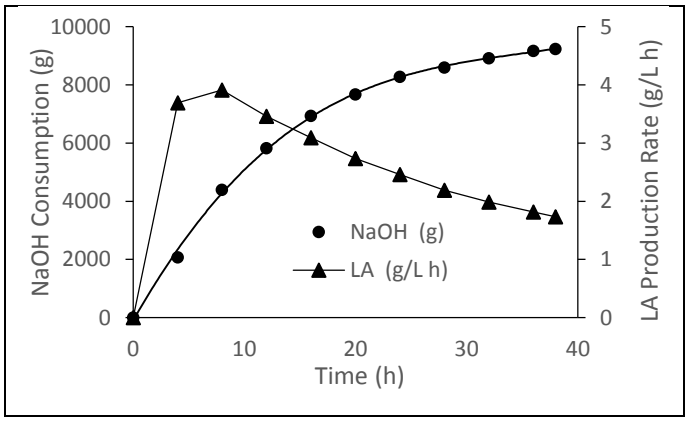

Figure 4. Kinetics of NaOH consumption and lactic acid production rate.

3.5. Fermentation simulation using SuperPro software

Figure 6 and Figure 7 show the simulation and simulation result of LAF using SuperPro software based on Equation 3 (Figure 8). The laboratory scale showed a higher performance in every parameter than the pilot scale. The stoichiometry mass of overall fermentation reaction was obtained from the fermentation of pilot scale. The kinetic properties of μmax and Ks value were 1.0 h-1 and 70 g/L

respectively. The final LA, glucose and biomass concentration is 68.2 g/L, 3.05 g/L, and 3.60 g/L respectively. The biomass concentration is expressed as the DCW of the *E. faecalis*. These results show a strong agreement of pilot scale with final LA, glucose and biomass concentration of 66.2 g/L, 3.5 g/L, and 3.15 g/L respectively. The strong fitness between simulation and experimental results indicated the simulation was able to predict the optimum operating conditions for achieving the maximum production rate.

7000 Glucose → Bio + 6392 LA + 294 RG + 9 others (3)

Fermentation of LA using *Enterococcus faecalis* could be scaled up from laboratory scale to pilot scale whereas the LA production rate of pilot scale was approximately performing the same as laboratory scale. The LAF of laboratory and pilot scale were developed successfully using *Enterococcus faecalis*. After comparing the production rate between laboratory and pilot scale using ANOVA, there was no significant difference between them. The simulation of LAF was also conducted using SuperPro software. The result of fermentation simulation showed the same LA yield as experimental scale where the data from pilot scale was used.

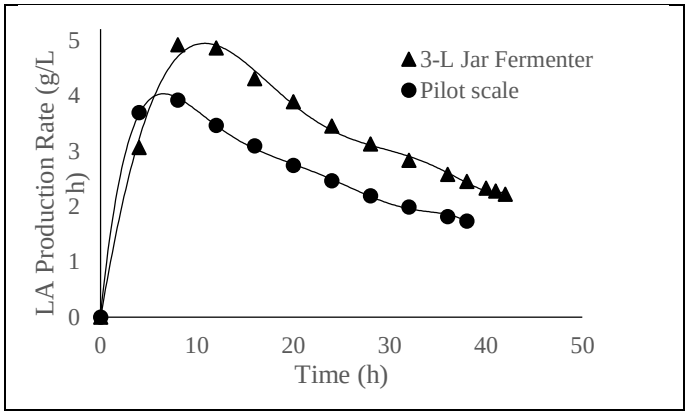

Figure 5. Kinetics of lactic acid production rate of in -L jar fermenter and Pilot scale levels.

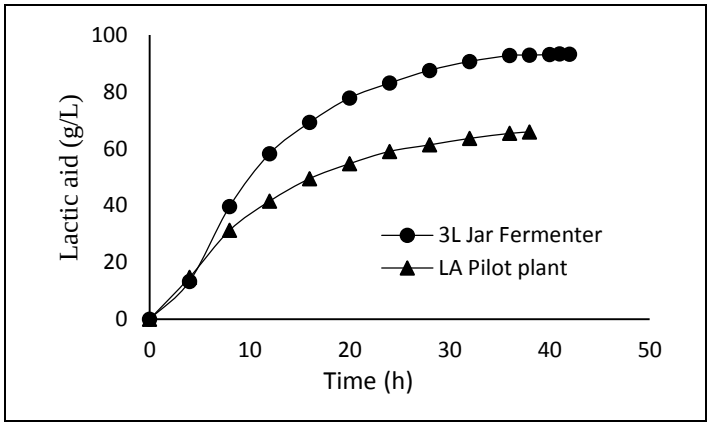

Figure 6. Kinetic of lactic acid fermentation from experimental results.

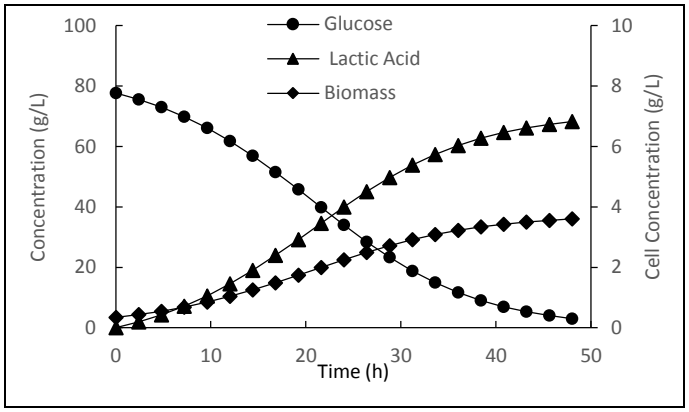

Figure 7. Kinetic of lactic acid fermentation from process simulation using SuperPro Software.

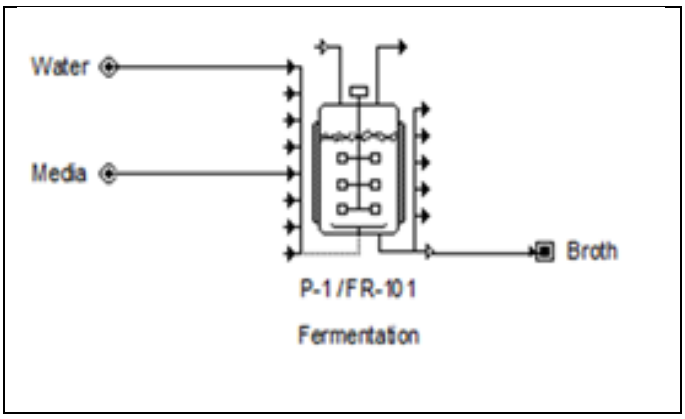

Figure 8. Process simulation using SuperPro software was used for the scaling up the lactic acid fermentation.

4.0 Conclusions

In conclusion, through the comparison and analysis of the experimental results, it was found that *Enterococcus faecalis* bacterium is able to scale up to industrial plant as the behaviour of the *Enterococcus faecalis* bacterium in lab fermenter and pilot plant was similar. The simulation has proven that the productivity of the system is reproducible and very close to those reported in the literature. The thermophilic character of the strain also contributed to maintaining the stability of the system because opportunistic microorganisms were kept to a low level.

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