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Research Article

Lactic Acid Production from Sequential Inorganic Salt Pretreated Oil Palm Empty Fruit Bunch via Simultaneous Saccharification and Fermentation

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ABSTRACT

Lactic acid is produced from inorganic salt pretreated oil palm empty fruit bunch (OPEFB) for the first time through simultaneous saccharification and fermentation (SSF). OPEFB is an agricultural waste that can be turned into lactic acid (LA), a highly desired chemical product. An inorganic salt ($Na_3PO_4.12H_2O$-$FeCl_3$) pretreatment precedes SSF with *Bacillus coagulans* DSM2314. The effect of solid loading, concentration $CaCO_3$ and enzyme loading on LA generation is studied using design of experiments. The results show that solid loading and concentration $CaCO_3$ affect LA yield. 50 g/L biomass, 50 g/L $CaCO_3$ concentration, and 50 FPU/g cellulase enzyme yield maximal LA (46.66/L) and yield (0.93/g OPEFB). The model created to predict LA production was then validated.

INTRODUCTION

Lactic acid is utilised in the production of food, chemicals, and pharmaceutical products throughout the world. Recently, there has been a surge of interest in biodegradable polylactic acid as a substitute for petroleum-derived plastic. Lactic acid is commercially produced through microbial fermentation of glucose, sucrose, and lactose using feedstocks such as corn stover, corncob, and lignocellulosic biomass such as empty fruit bunch, EFB (Hassan et al., 2020). The feedstock is selected on the basis of its price, availability, and recovery and purification costs.

In comparison to these traditional feedstocks, lignocellulosic biomass is a low-cost, widely available renewable carbon source with no competing food value. Lignocellulose is mainly composed of cellulose, hemicellulose, and lignin. EFB can be used to hydrolyse cellulose into glucose subunits at a low cost. However, a pretreatment process is required as the presence of hemicellulose and lignin inhibited enzymatic hydrolysis and reduced sugar yield. But the current pretreatment processes are not environmentally friendly, tedious, expensive and chemicals cannot be recycled. To tackle these issues, inorganic salt was chosen as the pretreatment method in this study because it is less corrosive and can be recycled as metal hydroxide using ultrafiltration.

Currently, there are two process routes to produce lactic acid; SHF (2 steps process) and SSF (1 step process). The one-step SSF technique is gaining popularity and research due to its low cost and high yield. Because it is anaerobic, the current SSF technique generates low yields and is not economical. Anaerobic processes that produce high yields at a cheap cost are needed. To counteract this problem, *Bacillus coagulans* DSM 2314 was used as a microbial biocatalyst in this study to generate lactic acid from salt- inorganic treated EFB in SSF because it is an anaerobic process that can give high yields at low cost.

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According to Hassan et al. (2020), inorganic salts were used to pretreat EFB in order to increase the delignification and saccharification yield of EFB. With a yield of 0.97 g/g EFB, a high amount of total reducing sugars was produced. This led to the current study, which will investigate the possibility of producing lactic acid utilising pretreated EFB as a substrate via SSF. The objectives of this research were to utilise $Na_3PO_4.12H_2O$-$FeCl_3$ pretreatment method on EFB and evaluate its effect on sugar production and to optimise SSF of pretreated EFB for lactic acid production.

MATERIALS AND METHOD

Pre-processing of biomass

Biotrade Noh Resources, Bandar Penawar, Johor, Malaysia, provided the ground oil palm empty fruit bunches (OPEFB) fibre. These ground materials are 3–5 mm long. The OPEFB fibres were washed thoroughly before being dried at 60 °C for 48 hours to a consistent weight. The dried processed OPEFB was sealed in a container for further use.

Pretreatment of OPEFB

OPEFB fibres were pretreated sequentially with inorganic salt ($Na_3PO_4.12H_2O$ and $FeCl_3$) under optimum conditions as suggested by Hassan et al. (2020) with a solid to liquid ratio of 1:10. The raw OPEFB fibres were treated in the first stage with a 15% (w/v) $Na_3PO_4.12H_2O$ solution at 121 °C for 30 minutes using an autoclave (ALP CL-32LDP, Japan). Following that, the solution was allowed to cool to room temperature before being washed with distilled water to remove any remaining Na_3PO_4. The second stage included mixing the $Na_3PO_4.12H_2O$ pretreated OPEFB with a 5% (w/v) $FeCl_3$ solution. The mixture was then autoclaved for 30 minutes at 121 °C. After that, the OPEFB solid fraction was filtered and washed with distilled water until the pH reached 7. The samples were then dried in an oven for 24 hours at 60 °C.

Preculture of *B. coagulans* DSM2314

B. coagulans DSM2314 (DSMZ, Germany) was suspended for 30 minutes in 5ml Tryptic Soy Broth (TSB) media, which had been pre-sterilised at 121 °C for 20 minutes. Then, the cells were transferred into 60 ml anaerobic flasks containing 50ml TSB medium and incubated for 16 hours to achieve an optical density (OD660) of about 2 without shaking as suggested by van der Pol et al. (2016). The cells were stored in 1.5 ml aliquots in cryovials at -20°C after the addition of glycerol to achieve a concentration of 15% v/v in the sample until further use. Next, the anaerobic flasks with 250 µl *B. coagulans* freezer stock were inoculated in 50 ml TSB media. Then, the seed culture was cultivated in an incubator at 50 °C at 180 rpm. The seed culture was added into SSF process when an OD660 of 1 was reached.

Fermentability of OPEFB

Fermentation media was prepared which consists of 25 g/L of yeast extract, 5 g/L of $(NH_4)_2HPO_4$, 8.75 g/L $(NH_4)_2SO_4$, 0.05 g/L of $MgCl_2.6H_2O$ and 0.25 g/L of $CaCl_2.2H_2O$. Next, the concentration of $CaCO_3$ varied from (20,40,60) g/L was added into the medium for a stable pH 6.0 in fermentation and formed an anaerobic condition for bacteria growth. Then, 5% of the preculture *B. coagulans* DSM 2314 was added to the medium and inoculated. After inoculating the *B. coagulans* DSM2314, cellulase varied from (10,30,50) FPU/g was added into fermentation media. The solids loading was varied from (50-100) g/L by adding an equivalent amount of EFB into the medium. The SSF experiment was performed in a 100 ml serum bottle at 50 °C and 200 rpm of agitation of operation conditions for incubator shaker with a final volume of 50 ml. The concentration of lactic acid was determined by collecting samples at 72 hours.

Total Reducing Sugar (TRS) analysis

DNS assay was used to quantify TRS of OPEFB yields during the enzymatic hydrolysis process. 2 ml of DNS reagent was added in a centrifuge tube containing 1 ml of enzymatic hydrolysate and heated in a water bath for 5 minutes at 100 °C for colour changes to reddish-brown. After this, the solution was cooled to room temperature and a UV-Vis spectrophotometer (LAMBDA 465, PerkinElmer, Malaysia) to measure absorbance at 540nm wavelength. The yield of TRS was calculated using Eq. 1;

$$TRS\ yield = \frac{sugar\ recovered\ \left(\frac{g}{L}\right) \times working\ volume\ (L)}{Mass\ of\ OPEFB\ used\ (g)} \quad (1)$$

Lactic acid analysis

Samples for the lactic acid fermentation performance was analysed using UV-Vis spectrophotometer (LAMBDA 465, PerkinElmer, Malaysia) as suggested by Borshchevskaya et al. (2016). A filtered test solution containing 80 µl lactic acid was added to 3.2 ml of 0.2% solution of $FeCl_3.6H_2O$. The absorbance was measured at wavelength of 390 nm with $FeCl_3.6H_2O$ as a blank. Next, the result was compared with the standard calibration curve of lactic acid.

RESULTS AND DISCUSSION

Factors influencing lactic acid production

Among the three factors chosen, a two-level factorial design was used to find the most significant factors affecting lactic acid production as illustrated in **Table 1**.

Table 1 Lactic acid production of factorial design

		Factors			Response
Std	Run	A: solid loading (g/L)	B: concentration $CaCO_3$ (g/L)	C: enzyme loading (FPU/g)	lactic acid (g/L)
5	1	50	20	50	30.71
4	2	100	60	10	14.62
1	3	50	20	10	19.84
11	4	75	40	30	30.38
6	5	100	20	50	17.05
9	6	75	40	30	25.50
7	7	50	60	50	53.42
3	8	50	60	10	35.88
10	9	75	40	30	27.79
8	10	100	60	50	16.09
2	11	100	20	10	14.81

ANOVA was used to interpret the study. Based on **Table 2**, the model p-value of 0.0013 is significant. The coefficient's significance increases with decreasing p-value. Model terms with p-values less than 0.05 are significant, while those with p-values more than 0.10 are not. It was found that factors A, B, C, AB and AC were significant, but factors BC and ABC were not. The curvature is not significant at 0.1876. So, the linear model could describe the lactic acid production relationship. The p-value for the lack of fit is not

significant at 0.5141. The determination coefficient (R^2) was 0.982, indicating 98.2% response variability. The predicted R^2 of 0.848 was close to the adjusted R^2 of 0.961. The predicted R^2 value was used to assess the model's predictive accuracy for new data sets. The range of predicted values at design points is compared to the average prediction error. A ratio of at least four was desired, and the obtained ratio of 19.431 was adequate.

Table 2 ANOVA for lactic acid production

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1327.47	5	265.49	45.79	0.0013	significant
A-Solid Loading	746.52	1	746.52	128.75	0.0003	
B-Concentration $CaCO_3$	176.72	1	176.72	30.48	0.0053	
C-Enzyme loading	128.96	1	128.96	22.24	0.0092	
AB	199.00	1	199.00	34.32	0.0042	
AC	76.26	1	76.26	13.15	0.0222	
Curvature	14.61	1	14.61	2.52	0.1876	not significant
Residual	23.19	4	5.80			
Lack of Fit	11.27	2	5.64	0.95	0.5141	not significant
Pure Error	11.92	2	5.96			
Cor Total	1365.27	10				
Std. Dev.	2.41		R^2	0.982		
Mean	26.01		Adj R^2	0.961		
C.V. %	9.26		Pred R^2	0.848		
PRESS	207.15		Adeq Precision	19.431		

The final equation in terms of coded and actual factors for the lactic acid model was shown as follows Eq. 2 and Eq. 3, respectively:

$$\text{Lactic acid} = 25.30 - 9.66A + 4.7B + 4.02C - 4.99AB - 3.09AC \quad (2)$$

$$\text{Lactic acid} = -4.96 + 0.20A + 0.98B + 0.66C - 0.01AB - 0.01AC \quad (3)$$

Path of steepest ascent

The ANOVA earlier demonstrated that the optimal zone was not attained due to insignificant curvature. As a result, the steepest ascent method was employed to swiftly move from the current operating condition to the optimum zone. See **Table 3** for details. Solid loading was given a coded step size of 0.5 based on Eq. 2. Since enzyme loading was not a significant factor, it remained at 50 FPU/g as, during the initial experiment, 50 FPU/g of enzyme gave the most lactic acid of 53.42 g/L.

Table 3 Lactic acid production of steepest ascent

Run	Step	Coded		Natural		Response lactic acid (g/L)
		Solid loading (g/L)	Concentration $CaCO_3$ (g/L)	Solid loading (g/L)	Concentration $CaCO_3$ (g/L)	
	Origin	0	0	75	40	
	Step size, Δ	-0.5	(4.7/9.7)/2 = 0.24	-12.5	0.24 * 20 = 4.8	
1	Origin + 0.5Δ	-0.5	0.24	62.5	44.8	28.10
2	Origin + Δ	-1	0.48	50	49.6	44.78
3	Origin + 1.5Δ	-1.5	0.72	37.5	54.4	30.05
4	Origin + 2Δ	-2	0.96	25	59.2	18.55
5	Origin + 2.5Δ	-2.5	1.2	12.5	64	9.22

The results showed that lactic acid concentration consistently increased when the solid loading decreased and the concentration of $CaCO_3$ increased. High solid loadings hindered proper mixing due to increased viscosity of fermentation medium and high inhibitor concentrations reduced cell viability, resulting in low lactic acid yield (Varga et al. 2004; Koppram and Olsson 2012; Hassan and Idris, 2016). However, further reductions in solid loading below 50 g/L of EFB caused drop in lactic acid concentration as less carbon source. Maximal lactic acid production of 44.78 g/L was obtained at a condition of 50 g/L of EFB and 49.6 g/L of $CaCO_3$ concentration.

The pH of the medium decreased as the concentration of lactic acid increased. Lactic acid generation would be inhibited at low pH levels due to the increased amount of free lactic acid. To avoid reducing the pH of the medium, fermentation in the presence of excess $CaCO_3$ is utilised. During the SSF, $CaCO_3$ reacts with the lactic acid to form calcium lactate, which keeps the pH of the fermentation broth around 6.0. Thus, $CaCO_3$ protected *B. coagulans* cells against free lactic acid inhibition. Fermentation tests with $CaCO_3$ concentrations of more than 30 g/L showed higher lactic acid yields (Cunha et al., 2018). Therefore, the ability of neutralising agents to adjust the pH of the medium is a critical aspect in the generation of lactic acid.

Analysing the optimum region

In the second stage, central composite design (CCD) was used for the optimum lactic acid production using two significant factors; solid loading and concentration $CaCO_3$. To fit the response surface experiments data, regression analysis was performed based on the second-order and the responses of lactic acid concentration were evaluated by ANOVA (**Table 4**). Based on the data obtained, run 9 gave the highest concentration of lactic acid which was 46.66 g/L at condition of 50 g/L of solid loading and 50 g/L of $CaCO_3$ concentration.

Table 4 Lactic acid production of CCD design

Std	Run	Factor 1 A: Solid Loading (g/L)	Factor 2 B: Concentration $CaCO_3$ (g/L)	Response Lactic Acid Concentration (g/L)
9	1	50.0	50.0	45.98
10	2	50.0	50.0	45.02
8	3	50.0	56.8	37.77
1	4	37.5	45.2	33.95
6	5	67.7	50.0	26.16
5	6	32.3	50.0	25.12
7	7	50.0	43.2	36.3
4	8	62.5	54.8	32.05
11	9	50.0	50.0	46.66
2	10	62.5	45.2	30.27
3	11	37.5	54.8	28.76

The ANOVA was used to summarise the data of the entire CCD test performed. Based on **Table 5**, the model p-value of less than 0.0001 implies the model was significant. The quadratic model for lactic acid production shows that the main effect of AB, A^2 and B^2 were the significant model terms. However, factors A and B were not. The lack of fit is not significant relative to the pure error where the p-value is 0.3424 and there was a 34.24% chance that a model with an F-value this large could occur due to noise.

Furthermore, the R^2 value obtained is 0.991, indicating that 99.1% of the variation in the response could be explained. The modified R^2 of 0.982 corresponds to the projected R^2 of 0.946. The range of projected values at the design points is compared to the average prediction error to determine adequate precision. Model discrimination is adequate when the ratio is greater than 4. In this example, the value is significantly higher than 4.

Table 5 ANOVA of CCD design

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	606.75	5	121.35	108.94	< 0.0001	significant
A-Solid loading	0.15	1	0.15	0.13	0.7321	
B-Concentration $CaCO_3$	0.22	1	0.22	0.20	0.6743	
AB	12.15	1	12.15	10.90	0.0214	
A^2	581.01	1	581.01	521.60	< 0.0001	
B^2	111.62	1	111.62	100.20	0.0002	
Residual	5.57	5	1.11			
Lack of Fit	4.21	3	1.40	2.07	0.3424	not significant
Pure Error	1.36	2	0.68			
Cor Total	612.31	10				
Std. Dev.	1.06		R^2	0.991		
Mean	35.28		Adj R^2	0.982		
C.V. %	2.99		Pred R^2	0.946		
PRESS	33.00		Adeq Precision	26.271		

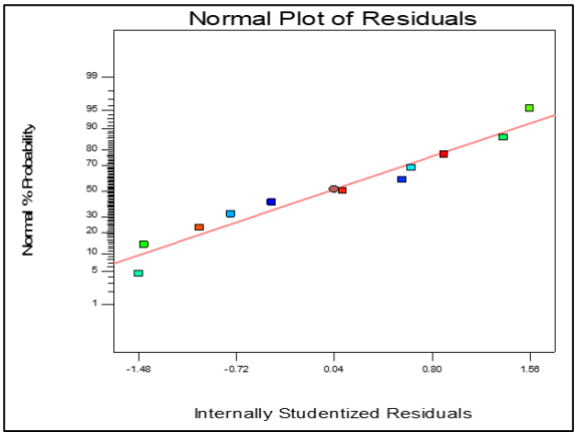

Figure 1 Residuals normal probability plot

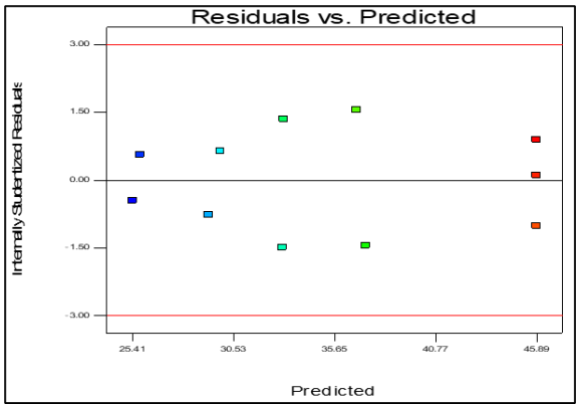

Figure 2 Residuals vs predicted values

The normal probability plot of the effects depicts the standardised effects in relation to a distribution fit line in the absence of any effects. Effects that deviate from zero on the normal probability plot are statistically significant. According to **Figure 1**, the residuals of the central composite design lie on the line indicating that the error distribution is normal. **Figure 2** shows the residuals versus predicted response plot. There was no distinctive structure or pattern that indicated the model was adequate. Thus, there was no reason to suspect any violation of the independence or constant variance assumptions.

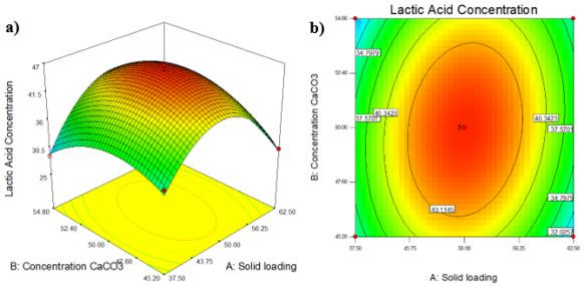

Figure 3 Response surface (a) and contour plot (b) for lactic acid production via CCD as a function of AB interactions

The response surface and contour plots for lactic acid generation are shown in **Figure 3**. These figures illustrated the link between independent variables (solid loading and $CaCO_3$ concentration) and dependent variables (lactic acid concentration). Both the response surface and contour plot bear a strong resemblance to the quadratic model proposed. It was found that low solid loading and high $CaCO_3$ concentration gave maximal lactic acid

concentration (46.66 g/L) according to the response surface and contour plot. However, decreasing the solid loading below 50 g/L did not influence lactic acid production. Based on the results, it is indicated that the optimal point of lactic acid can be attained by decreasing the solid loading and increasing the $CaCO_3$ concentration while maintaining the enzyme loading at 50 FPU/g.

The final equation in terms of coded and actual factors for the lactic acid model was shown as follows Eq. 4 and Eq. 5, respectively:

$$\text{Lactic acid} = 45.89 + 0.14A - 0.17B + 1.74AB - 10.14A^2 - 4.45B^2 \quad (4)$$

$$\text{Lactic acid} = -525.01 + 5.05A + 17.81B + 0.03AB - 0.06A^2 - 0.19B^2 \quad (5)$$

Model validation test

The confirmation runs for validating the acceptability of the generated model are shown in **Table 6**. The confirmation runs consisted of three runs in which the enzyme loading was held constant while the substrate and CaCO3 loading was adjusted within the range examined. Confirmation runs demonstrated that the created models matched well with the experimental results, with errors of less than 5% for lactic acid generation.

Table 6 Confirmation runs for model validation

Run	Factor A: solid loading (g/L)	Factor B: concentration $CaCO_3$ (g/L)	Factor C: enzyme loading (FPU/g)	Actual lactic acid value (g/L)	Predicted lactic acid value (g/L)	Error (%)
1	50.0	50.0	50	44.85	45.89	2.26
2	42.0	53.0	50	37.99	39.11	2.86
3	56.5	48.2	50	44.35	42.31	4.81

TRS yield and lactic acid production

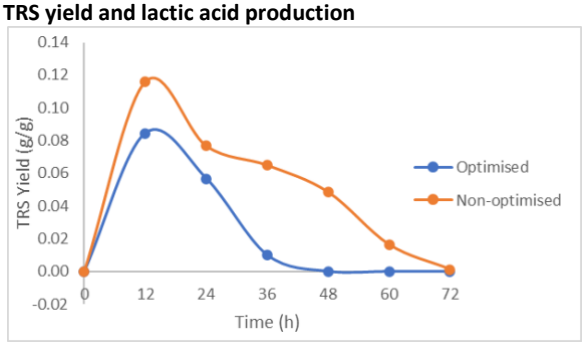

Figure 4 Total reducing sugar yield profile

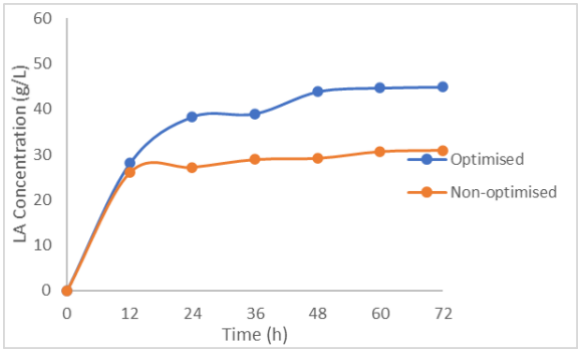

Figure 5 Lactic acid concentration profile

The experimental procedure was conducted under SSF condition at 50℃ for 72 hours. **Figure 4** depicts the amount of TRS yield during the 72 hours. There was no sugar released at the beginning of SSF yet the concentration increased exponentially when it reached 12 hours of fermentation. As the fermentation duration approached 24 hours, the sugar yield has slowly reduced because *B. coagulans* started consuming the sugar as a carbon source to produce lactic acid.

The optimised condition has lower TRS yield compared to non-optimised condition. $CaCO_3$ concentration was critical in the fermentation process because pH decreased quickly to 4.5 after 6 hours and stayed around 4.0–4.5 for the next 24 hours (Panesar et al., 2007; da Silva et al., 2021). pH regulates cellular metabolism and so has an impact on *B. coagulans* enzyme activity. Low pH slows *B. coagulans* cell development and denatures cellulase. Optimised media had more $CaCO_3$ than non-optimised media, indicating greater pH stability. The carbonate source provided carbon dioxide for lactic acid generation and pH buffering. A constant pH in optimised media boosted *B. coagulans* sugar intake, lowering TRS yield and finishing first.

As indicated in **Figure 5**, optimised medium produced more lactic acid (44.85 g/L) than non-optimised medium (30.90 g/L). The results matched prior research where $CaCO_3$ increased lactic acid concentrations (Yang et al., 2015; Saavedra et al., 2021). Cunha et al. (2018) found that increasing the $CaCO_3$ concentration enhanced lactic acid production (15 g/L, 30 g/L, 45 g/L), with a maximum output of 180.45 g/L, 2.506 g/L.h productivity, and 96.7% yield.

Solid loading influenced lactic acid generation. In SSF, glucose was produced from cellulose and hemicellulose, with larger solid loading indicating more bioconversion. However, according to Martinez et al. (2013), a low initial substrate concentration is required to obtain a high lactic acid concentration. High substrate concentration reduced saccharification rate and consequently lactic acid output (Stenberg et al. 2000; Varga et al. 2004). Due to decreased solid loading, optimised media produced more lactic acid than non-optimised media. Similarly, Hassan and Idris (2016) found that a solid loading of 15 g/L produced a maximum lactic acid yield of 7 g/L, while solid loadings of 20 and 30 g/L produced only 3.5 and 1.8 g/L, respectively.

CONCLUSION

The sequential pretreatment of $Na_3PO_4.12H_2O$-$FeCl_3$ had a significant effect on lignin removal with 1.27 g/g of TRS yield achieved. This inorganic salt pretreatment increased the TRS yields, which in turn increased the LA production via SSF. The ANOVA findings showed that solid loading and $CaCO_3$ concentration have a significant effect on LA production. This is achieved by reducing the solid load and raising the $CaCO_3$ concentration, whereas the enzyme loading has no effect. This study revealed that 50 g/L of solid loading, 50 g/L of $CaCO_3$ concentration and 50 FPU/g of enzyme loading had the highest LA production which was 46.66 g/L with a yield of 0.93 g/g EFB. An extensive study is required to employ OPEFB as a carbon source for lactic acid production on a large scale in the near future.

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