Advances in Bioscience and Biotechnology, 2014, 5, 957-965
Published Online October 2014 in SciRes. http://www.scirp.org/journal/abb
http://dx.doi.org/10.4236/abb.2014.512109

Optimization of Nutrients in Fermentative Lactic Acid Production Using Oil Palm Trunk Juice as Substrate

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Received 14 August 2014; revised 18 September 2014; accepted 7 October 2014

Abstract

Complex nutrients e.g. carbon, nitrogen, peptides, vitamin and salts significantly play important roles in lactic acid production efficiency depending on types of microorganism and sources of raw material. In this study oil palm trunk juice and *Lactobacillus rhamnosus* TISTR 108 (ATTC 10863) were utilized for lactic acid production. Additional nutrients including peptone, yeast extract and mixed salts were tested. Response surface methodology involving Box Behkhen Design (BBD) was applied to examine the optimal condition. Prediction of optimization was performed using full quadratic regression equation. The predicted maximum lactic acid concentration was obtained at 64.05 g·l^{-1} within a period of 48 h under an optimal condition of 10 g·l^{-1} peptone with mixed salts containing 0.4 g·l^{-1} $MgSO_4 \cdot 7H_2O$, 0.1 g·l^{-1} $MnSO_4 \cdot 4H_2O$, 3 g·l^{-1} K_2HPO_4, 3 g·l^{-1} KH_2PO_4 and 3 g·l^{-1} $CH_3COONa \cdot 3H_2O$ in 250 ml shake flask using $CaCO_3$ as a titrant. Verification of optimization condition was performed in 2 l fermenter using $Ca(OH)_2$ as neutralizing agent. Increase in lactic acid fermentation was achieved at 92.81 g·l^{-1} at 48 h cultivation. The lactic acid yield and volumetric productivity were 0.94 g·g^{-1} and 1.91 g l^{-1}·h^{-1}, respectively. This suggests that OPT juice is potentially used as carbon and nutrient sources for lactic acid production.

Keywords

Lactic Acid, Oil Palm, Oil Palm Trunk Juice, OPT, *Lactobacillus rhamnosus*, Box Behkhen Design, BBD

1. Introduction

Oil palm, *Elaesis guineensis* is an important crop in Southeast Asia accounting for a total plantation area of

How to cite this paper: Saelee, N. and Sriroth, K. (2014) Optimization of Nutrients in Fermentative Lactic Acid Production Using Oil Palm Trunk Juice as Substrate. *Advances in Bioscience and Biotechnology*, **5**, 957-965.
http://dx.doi.org/10.4236/abb.2014.512109

proximately 9,746,666 - 14,620,000 hectares in 2013/2014. Nevertheless, an enormous number of old oil palm trees (20 - 25 years) were cut down for replanting due to their low productivity [1] [2]. Considering a significant number of abandoned oil palm trunks (OPTs) are discarded daily or every year. Many researchers have attempted to utilize OPT waste as a raw material to produce value-added products. In general, OPTs are industrially used to produce plywood [3] in which a large amount of juice squeezed from the OPT is the by-product. The squeezed juice containing high sugar contents has been used as a substrate for ethanol and lactic acid fermentation [4] [5].

Lactic acid and its derivatives have been widely used in industrial applications [6] [7]. In 2017, the annual worldwide lactic acid production is expected to reach 367,000 tons and the demand for lactic acid has been estimated to grow 5% - 8% yearly [8]. Manufacturing of lactic acid can be done using various methods; fermentation technique has been paid more attention according to the advantages in terms of stereo isomer selection, low substrate costs, mild conditions, low energy consumption and environmental friendliness [9]-[11]. However, efficient lactic acid fermentation by lactic acid bacteria requires complex nutrients depending on microorganism capabilities and growing environments such as temperature, pH, oxygen, substrate and ongoing product concentration. Accordingly, the cost of raw materials is one of the limiting factors of the feasible economic production of lactic acid. Practically manufacturing sugars are utilized as carbon sources which are not cost-benefit for producing a cheap product of lactic acid. Using pure sugars for lactic acid production in industrial scale also requires supplementation including nitrogen sources, vitamins, minerals and growth factors.

Among various nitrogen sources, yeast extract leads to the highest lactic acid concentrations in a variety of nitrogen sources due to a wide range of growth factors including amino acids, vitamins, specific minerals, fatty acids, purines, and pyrimidines [12]. Cost of yeast extract is accounting for proximately 38% of the total production cost which is relatively high for economic benefit in industrial scale processes [13]. The alternative, cheaper nitrogen sources from organic nitrogen sources and agricultural by-products have been used as partial or total replacement of yeast extract, including peptone, soybean hydrolysate and soytone [14], corn steep liquor (CSL) [12], rice bran [15] [16], and wheat bran [17].

Considerably, addition of vitamins and mineral salts is required to enhance the lactic acid production [13]. Mineral salts such as phosphorous, magnesium, manganese, zinc, and iron are provided in the form of salts in a wide range of concentrations in the medium. In general, concentrations of 0.03 - 2.5 $g \cdot l^{-1}$ KH_2PO_4, 0.1 - 5 $g \cdot l^{-1}$ $MgSO_4$, 0.03 - 0.2 $g \cdot l^{-1}$ $MnSO_4$, 0.02 - 0.03 $g \cdot l^{-1}$ $FeSO_4$ and 0.01 - 5 $g \cdot l^{-1}$ NaCl have been used for lactic acid fermentation [13]-[16] [18]-[22].

According to our preliminary studies, OPT juice is considered suitable to be employed as high carbon and nutrient sources. However, nitrogen and salts content are considering insufficient in OPT juice in providing growth and lactic acid production by *Lactobacillus rhamnosus* TISTR 108 (ATTC 10863). This study investigated lactic acid production processes using OPT juice as a base medium. Response surface methodology (RSM) involving Box Behkhen design (BBD) and analysis of variance (ANOVA) were applied to find the appropriate components of yeast extract, peptone and salts solution in order to achieve maximum lactic acid production.

2. Materials and Methods

2.1. Oil Palm Trunk Juice Preparation

OPTs were obtained from local area in Nakhonsithammarat province, Thailand. OPTs were cut into small pieces and immediately squeezed using sugarcane press to collect OPT juice. The juice was centrifuged at 4000 rpm for 40 min to remove the cellulosic debris. The clear supernatant of undiluted OPT juice was stored at −18˚C and further used as a fermentation base medium for lactic acid production.

2.2. Microorganism and Culture Conditions

The lactic acid bacteria used in this study was *Lactobacillus casei* subsp. *rhamnosus* TISTR 108 (ATTC 10863), purchased from Thailand Institute of Scientific and Technological Research (TISTR). The culture was grown and maintained on MRS broth (de Man Rogosa and Sharpe, Difco, USA) with 30% glycerol. The strain was activated at 40˚C for 48 h in MRS broth. The fermentation inoculum was prepared in a medium that contained, per liter of distilled water, 20 g glucose, 10 g bactotryptone, 10 g yeast extract, 2 g K_2HPO_4, 5 g $CH_3COONa \cdot 3H_2O$, 0.2 g $MgSO_4 \cdot 7H_2O$ and 5 mg $MnSO_4 \cdot 4H_2O$ [4]. The initial pH of the medium was adjusted to 7.0 with 6 N NaOH and conc. HCl. After sterilization, the flask was inoculated and incubated at 40˚C for 18 h with shaking

speed of 150 rpm. The absorbance at 620 nm was adjusted to 4.0 as constant value before used as fermentation inoculum.

2.3. Optimization Condition Using the Box Behkhen Design

The fermentation conditions were optimized by response surface methodology (RSM) through the Box Behnken design (BBD) to fine the appropriate yeast extract (Difco, USA), peptone (Bacto Peptone, Difco, USA) and salts solution (all salts; $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, K_2HPO_4, KH_2PO_4, $CH_3COONa \cdot 3H_2O$ were analytical grade) concentrations to improve the efficiency of lactic acid fermentation on undiluted OPT juice by *L. rhamnosus* TISTR 108 (ATTC 10863). Three factors, namely peptone (*A*), yeast extract (*B*), and salts (*C*) in three coded levels (−1, 0, +1) were evaluated. The factors *A*, *B* and *C* represent the independent variables. The lactic acid (*Y*) is the response. Summary of variables, coded and uncoded values were shown in **Table 1**.

According to the design, 15 experiments were conducted in duplicates. The experiments were performed in 250 ml Erlenmeyer flasks containing 100 ml fermentation media with the addition of nutrients depending on the experiment design. One gram of $CaCO_3$ was added to maintain pH 6.5. The fermentation experiments were conducted in 250 ml Erlenmeyer flasks on the rotary shaker at 150 rpm for 48 h. The fermentation broth was heat at 70˚C for 30 min and centrifuged at 1000 rpm for 3 min to remove the $CaCO_3$ pellet. Ten ml of the clear supernatant was measured and 16 ml of 2 M H_2SO_4 was added to neutralize. The reaction tubes were left for complete precipitation and centrifuged at 10,000 rpm for 5 min. The clear supernatant was diluted and filtered through 0.45 µm (Millipore) before determination of sugars and lactic acid by HPLC.

Statistical analysis of the data was analyzed using the MINITAB statistical package. A full quadratic model was used to predict the optimal point, as the following equation:

$$Y = b_0 + b_1A + b_2B + b_3C + b_{12}AB + b_{13}AC + b_{23}BC + b_{11}A^2 + b_{22}B^2 + b_{33}C^2$$

where Y is predicted response (lactic acid concentration ($g \cdot l^{-1}$)), b_0 is intercept; b_1, b_2 and b_3 are linear coefficients, b_{12}, b_{13} and b_{23} are interaction coefficients, b_{11}, b_{22} and b_{33} are squared coefficients, *A*, *B* and *C* are the coded levels of the independent variables.

2.4. Batch Fermentation Using Optimized Conditions

The lactic acid fermentation under the optimization conditions of media obtained by response optimization was conducted in 2 l fermenter containing 1.2 l OPT juice based medium. The medium was autoclaved at 110˚C for 10 min and 10% of *L. rhamnosus* TISTR 108 (ATTC 10863) was inoculated. The temperature and agitation speed were controlled at 40˚C, 200 rpm, respectively. The pH was controlled by the automatic addition of 6 N $Ca(OH)_2$ at pH 6.5. Samples were taken every 6 h intervals for 48 h to determine viable cells, lactic acid and sugar concentration.

2.5. Analytical Methods

Lactic acid and sugars contents in fermentation broth were analyzed by high pressure liquid chromatography (HPLC) system with Animex HPX 87H column, 300 × 7.8 mm (BioRad, USA) with a refractive index detector (Waters, USA). The analytical condition was 5 mM H_2SO_4 mobile phase with a flow rate of 0.6 $ml \cdot min^{-1}$ and column temperature of 50˚C. Bacterial growth in term of the number of viable cells was estimated using spreading plate technique on MRS agar. The incubation was performed in an anaerobic condition at 40˚C in an anaerobic jar.

Table 1. Experimental design variables used in the Box Behkhen design.

Factors	Coded Units		
	−1	0	1
Peptone ($g \cdot l^{-1}$)	0	5	10
Yeast extract ($g \cdot l^{-1}$)	0	10	20
Salts concentrations[a]	(a)	(b)	(c)

[a]Salts concentration (per liter). (a) Without salt addition; (b) 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $MnSO_4 \cdot 4H_2O$, 1.5 g K_2HPO_4, 1.5 g KH_2PO_4 and 1.5 g $CH_3COONa \cdot 3H_2O$ [23] and with (c) = 2× (b).

Lactic acid yield ($Y_{P/S}$) and lactic acid productivity (Q_P) were calculated as follow:

$$Y_{P/S} = \frac{\Delta P_{la}}{\Delta S_{total}} = \frac{P_{la}^{f} - P_{la}^{i}}{S_{total}^{i} - S_{total}^{f}}$$

$$Q_P = \frac{\Delta P_{la}}{t} = \frac{P_{la}^{f} - P_{la}^{i}}{t}$$

where, P_{la}^{f} is the lactic concentration at the end of fermentation ($g \cdot l^{-1}$). P_{la}^{i} is the lactic concentration at the beginning of fermentation ($g \cdot l^{-1}$). S_{total}^{i} is total sugars concentration at the beginning fermentation ($g \cdot l^{-1}$). S_{total}^{f} is total sugars concentration at the end of fermentation ($g \cdot l^{-1}$). t is the fermentation time (h).

3. Results and Discussions

3.1. Media Optimization of Lactic Acid Production by Box Behkhen Design

Box Behkhen design (BBD) showed to effectively describe the fermentation optimal conditions of *L. rhamnosus* TISTR 108 (ATTC 10863) using OPT juice as a base medium. The design matrix of the variables in coded units and responses of lactic acid concentration are shown in **Table 2**. The analysis of variance (ANOVA) for the quadratic model of lactic acid production is shown in **Table 3** and the multiple regression analysis for lactic acid concentration is exhibited by the following equation.

Table 2. Observed and predicted lactic acid concentrations obtained from undiluted oil palm trunk juice fermentation with various nutrient supplementations by Box Behkhen design.

Run	Coded Units			Lactic Acid ($g \cdot l^{-1}$)	
	A (Peptone, $g \cdot l^{-1}$)	*B* (Yeast Extract, $g \cdot l^{-1}$)	*C* (Mineral Salts)	Observed	Predicted
1	−1	0	1	37.75	38.27
2	−1	0	−1	37.23	34.16
3	0	−1	−1	41.27	43.18
4	1	0	1	53.52	56.50
5	−1	−1	0	36.65	38.15
6	−1	1	0	38.63	41.03
7	0	1	−1	47.75	49.69
8	0	0	0	43.72	42.14
9	1	−1	0	53.03	51.16
10	0	−1	1	58.47	57.29
11	1	0	−1	39.90	40.80
12	0	0	0	42.04	42.14
13	0	1	1	55.92	55.38
14	1	1	0	54.18	52.89
15	0	0	0	40.12	42.14
16	−1	0	1	38.94	38.27
17	−1	0	−1	37.54	34.16
18	0	−1	−1	42.15	43.18
19	1	0	1	53.05	56.50
20	−1	−1	0	36.14	38.15
21	−1	1	0	40.33	41.03
22	0	1	−1	48.29	49.69
23	0	0	0	42.82	42.14
24	1	−1	0	52.39	51.16
25	0	−1	1	59.46	57.29
26	1	0	−1	41.55	40.80
27	0	0	0	43.05	42.14
28	0	1	1	57.79	55.38
29	1	1	0	55.10	52.89
30	0	0	0	41.07	42.14

Table 3. ANOVA results for nutrient optimization in lactic acid production from undiluted oil palm trunk juice by Box Behkhen design.

Effect	DF	seq. SS	adj. SS	adj. MS	f Value	p Value
Blocks	1	3.01	3.01	3.010	0.59	0.451[c]
Regression	9	1556.14	1556.14	172.904	34.06	0.000[a]
Linear	3	1032.43	285.82	95.272	18.77	0.000[a]
A	1	619.04	7.60	7.602	1.50	0.236[c]
B	1	21.21	244.71	244.713	48.20	0.000[a]
C	1	392.18	12.45	12.481	2.16	0.133[c]
Square	3	420.33	420.33	140.109	27.60	0.000[a]
*A***A*	1	81.42	51.52	51.525	10.15	0.005[a]
*B***B*	1	275.14	294.20	294.203	57.95	0.000[a]
*C***C*	1	63.77	63.77	63.769	12.56	0.002[a]
Interaction	3	103.38	103.38	34.459	6.79	0.003[a]
*A***B*	1	0.67	0.67	0.668	0.13	0.721[c]
*A***C*	1	67.24	67.24	67.238	13.24	0.002[a]
*B***C*	1	35.47	35.47	35.472	6.99	0.016[b]
Residual Error	19	96.46	96.46	5.077		
Lack of Fit	15	87.62	87.62	5.842	2.64	0.179[c]
Pure Error	4	8.83	8.83	2.209		
Total	29	1655.61				
		R^2 = 94.17, adj. R^2 = 91.11, pred. R^2 = 84.06				

Note: the analysis was done using the coded units; [a]Significant at $p \leq 0.01$; [b]Significant at $p \leq 0.05$; [c]Non-significant; SS, sum of square; MS, mean square; DF, degree of freedom; *A*, *B* and *C* represent yeast extract, peptone and salt concentration, respectively.

$$Y = 42.14 + 6.22A + 1.15B + 4.95C - 2.64A^2 + 6.31B^2 + 2.94C^2 - 0.29AB + 2.90AC - 2.11BC$$

where *Y* is the lactic acid production ($g \cdot l^{-1}$), *A*, *B* and C is the coded levels for peptone, yeast extract and salts solutions ($g \cdot l^{-1}$), respectively.

The full quadratic correlation for the estimated regression equation had R^2 of 94.17% and the predicted R^2 for lactic acid production was 84.06%. The R^2 value of 94.17% (close to 100%) indicated that the variables including peptone, yeast extract and mineral salts contributed to a highly positive response and only about 5.83% cannot explained by this equation. The adjusted R^2 of 91.11% indicated that the equation was highly accurate. The linear and quadratic effects of variables were significant at level of $p \leq 0.01$. The linear effect of yeast extract was significant at the level of $p \leq 0.01$, whereas the linear effect of peptone and mineral salts was not significant. The square terms of peptone (A^2), yeast extract (B^2) and salts (C^2) were highly significant ($p \leq 0.01$). The interaction terms (*A***C*, *B***C*), were significant ($p \leq 0.05$) while the interaction terms between *A* and *B* was not significant. Lack of fit of this equation was not significant which indicated the good predictability of the equation. The significant linear and quadratic terms of parameters suggests that peptone, yeast extract and mineral salts can act as limiting nutrients for lactic acid production.

Response optimization condition of three coded variables using 1, −1, 1 for peptone, yeast extract and mineral salts was performed by the MINITAB program. The highest lactic acid concentration of 64.05 $g \cdot l^{-1}$ was predicted at the optimum condition of peptone 10 $g \cdot l^{-1}$ and mineral salts containing 0.4 $g \cdot l^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.1 $g \cdot l^{-1}$ $MnSO_4 \cdot 4H_2O$, 3 $g \cdot l^{-1}$ K_2HPO_4, 3 $g \cdot l^{-1}$ KH_2PO_4 and 3 $g \cdot l^{-1}$ $CH_3COONa \cdot 3H_2O$. The 3D plots for the interactions between peptone (*A*) and mineral salts (*C*) of lactic acid fermentation are shown in **Figure 1**. It can be noticed that by increasing the peptone and mineral salts, the lactic acid production from undiluted OPT juice was increased. Furthermore, the fermentation of undiluted OPT juice under the optimized condition of nutrient supplementation obtained by BBD were performed to validate the empirical equation. The maximum lactic acid concentration of 63.2 ± 3.45 $g \cdot l^{-1}$ was obtained experimentally and this was close to the predicted valve of 64.05 $g \cdot l^{-1}$. However, it is important to note that this experiment was performed in 250 ml Erlenmeyer flask which $CaCO_3$ was used as a titrant. This optimized nutrient condition was then further evaluated in a 2 l fermenter (with 1.2 l working volume), using $Ca(OH)_2$ as a neutralizing agent.

3.2. Batch Fermentation of Lactic Acid Production under Optimized Conditions

The profiles of cell growth, sugar and lactic acid concentration during lactic acid production from undiluted OPT juice with optimized nutrient supplementation in a fermenter are presented in **Figure 2** and some kinetic parameters are summarized in **Table 4**. The high lactic acid concentration of 92.81 $g \cdot l^{-1}$ was obtained with yield of 0.94 $g \cdot g^{-1}$ and productivity of 1.91 $g \cdot l^{-1} \cdot h^{-1}$ at 48 h fermentation. The highest cells of *L. rhamnosus* TISTR 108 (ATTC 10863) of 1.35×10^{11} $cfu \cdot ml^{-1}$ was achieved at 30 h cultivation in the optimized medium compositions. However, some residual sugars were present at the end of fermentation time. The incomplete sugar consumption might be caused by some inhibitory effect as indicated by lower viable cells ($<10^{11}$ $cfu \cdot ml^{-1}$). In this study, the statistical approaches were successfully applied and overcame the limitation of empirical method. The use of peptone and salts could improve lactic acid production by *L. rhamnosus* TISTR 108 (ATTC 10863). Study of Abdul Karim, *et al.* [24] indicated that peptone provided the best lactic acid production by *L. rhamnosus*

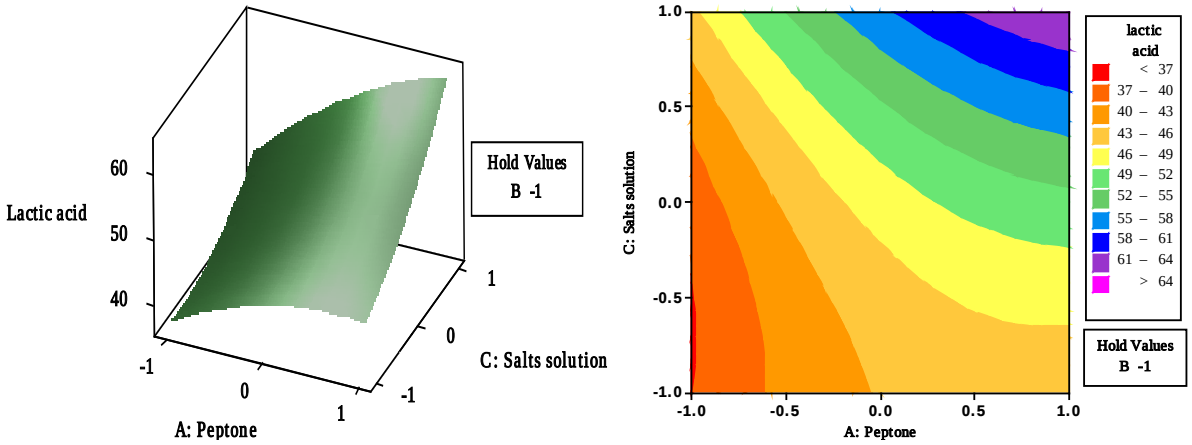

Figure 1. Response surface and contour plots of lactic acid concentration during fermentation of undiluted oil palm trunk juice by *Lactobacillus rhamnosus* TISTR 108 (ATTC 10863), showing the interaction between peptone (*A*) and mineral salts (*C*).

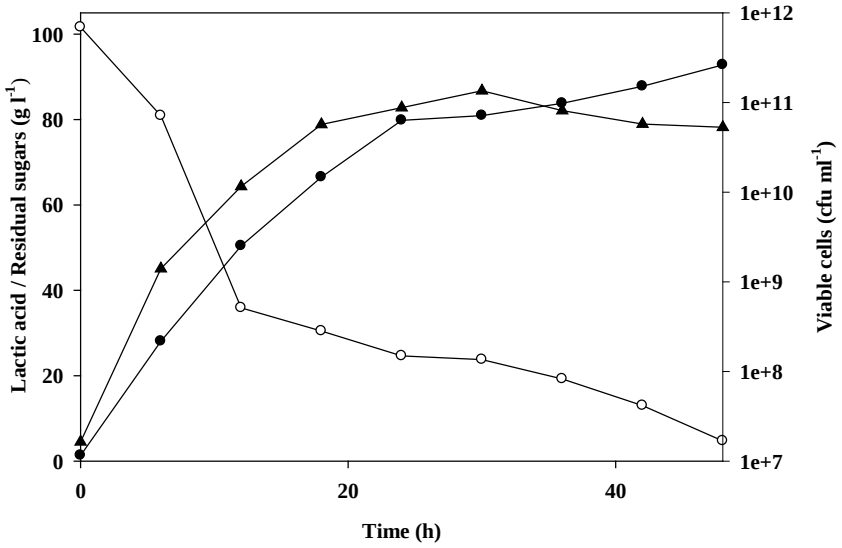

Figure 2. Profiles of cell growth, sugar and lactic acid concentrations during lactic acid fermentation of undiluted oil palm trunk juice with statistically optimized nutrient supplementation by *Lactobacillus rhamnosus* TISTR 108 (ATTC 10863). The batch fermentation was performed in 2 l fermenter with 1.2 l working volume at 40˚C, pH 6.5 and the agitation speed of 200 rpm, using 10% inoculum; lactic acid (●), residual sugars (o) and viable cells (▲).

Table 4. Kinetic parameters of lactic acid fermentation on undiluted oil palm trunk juice by *Lactobacillus rhamnosus* TISTR 108 (ATTC 10863) with nutrient supplementation, optimized by Box Behkhen design.

Parameters	Values
Fermentation time (h)	48
Lactic acid ($g \cdot l^{-1}$)	92.81
$Y_{P/S}$ ($g \cdot g^{-1}$)	0.94
Q_P ($g \cdot l^{-1} \cdot h^{-1}$)	1.91
Residual sugars ($g \cdot l^{-1}$)	4.71

Note: the fermentation was conducted in 2 l fermenter using undiluted OPT juice, at 40˚C, pH 6.5 with 10% inoculums and agitation speed of 200 rpm.

comparing to yeast extract in MRS medium containing the same amount of glucose. Even though, OPT juice was rich in carbon source but the amount of nitrogen and salts were low and not enough for efficient lactic acid production. Many studies reported high lactic acid yields and productivities by statistical optimization [25] [26]-[30]. John *et al*. [31] used Box Behnken design to optimize the production of L(+)-lactic acid by *Lactobacillus casei* and *Lactobacillus delbrueckii* and lactic acid yield was obtained at 81 $g \cdot l^{-1}$.

4. Conclusion

The predicted equation has confirmed that the proper amount of peptone and salts supplementation was essential for lactic acid production by *L. rhamnosus* TISTR 108 (ATTC 10863) on OPT juice fermentation. The findings in this study demonstrate the potential use of undiluted OPT juice as a cheap carbon source for lactic acid production. In term of economic benefits, the fermentative lactic acid production cost varies depending on various factors and raw material cost is considerably high. The OPT is generally an agricultural waste, and OPT juice extract is an industrial by-product when fiber was separated for plywood processing. According to lab-scale process based on the juice production per one trunk, approximately 19.7 - 22.7 tons of OPT juice could be obtained from a hectare of plantation area. Considering a vast area of oil palm plantation in Southeast Asian region, a large amount of OPT juice can inundate the industry demand for fermentative lactic acid production. However, fermentation process techniques in large scale have yet to be improved.

Acknowledgements

This work was supported by Kasetsart University and Walailak University, Thailand.

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