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Simultaneous saccharification and fermentation for lactic acid production from sweet potato

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Abstract

Simultaneous saccharification and fermentation (SSF) process has been employed in numerous of recent researches since it is an efficient process for the production of a wide range of valuable products. In our present research, sweet potato (SP) was converted into lactic acid by SSF process, which involved a liquefaction step with α-amylase followed by the SSF step in which glucoamylase and lactic acid bacterial starter were incorporated. Optimal conditions for a hydrolysis of SP by α-amylase were investigated using response surface methodology (RSM) with central composite design (CCD). SP was applied at 50% (w/v) in distilled water and incubation time was allowed for 90 min. The results suggested that 0.155% α-amylase (% v/SP weight), 70°C incubation temperature, pH 6.5, and 0.75 g/L $CaCl_2$ were optimal for liquefaction of SP and the high concentration of reducing sugars at 60.32 g/L was predicted. Subsequently, the liquefied product was applied for lactic acid production using SSF process by a lactic acid bacterium, FAG 302, which was eventually identified as *Lactobacillus* sp. and optimum temperature was determined. From the results, optimal temperature for SSF step was selected at 34°C and 105.52 g/L lactic acid was yielded at 72 h.

Keywords: lactic acid, sweet potato, simultaneous saccharification and fermentation

1. Introduction

Lactic acid was first discovered in sour milk in 1780 by Scheele. Ever since, its extensive applications have been recognized in abundant purposes including in food, chemicals, pharmaceutical, cosmetic, and other industries. Recently, lactic acid has gained more interest as being a good feedstock for the biodegradable polymer, polylactic acid (PLA) [1, 2, 3]. It was estimated that the world market of PLA could be as high as 500,000 metric tons per year in the near future (2). This leads to a rapid growth for lactic acid global demand.

Microbial fermentation of lactic acid usually relies on cheap and renewable resources i.e. cassava powder (4), cellulose, corncob, wood (3), barley starch (5), sweet potato (6), potato, other products and by-products from agricultures and industries (2, 7). Microbial fermentation has advantages over chemical synthesis since its issues are more environmental friendly and the production of stereo-specific acid is possible when appropriate microbial strains are carefully selected while a racemic mixture form is usually yielded via chemical synthesis (1, 8). However, a bioconversion of starchy raw materials into lactic acid requires steps of starch hydrolysis to

release glucose since most of high potential lactic acid bacteria lack of amylolytic enzymes. Enzymatic starch hydrolysis usually involves at least two enzymes; α-amylase (EC 3.2.1.1) and amyloglucosidase or glucoamylase (EC 3.2.1.3) [9, 10] and overall process requires at least 48-72 h. The incorporation of starch hydrolysis as a part of lactic acid production process causes obvious drawbacks of being time and cost-consuming as well as the inhibition of enzymes involved in starch hydrolysis due to the production of high glucose concentration (11). The mentioned obstacles can be overcome by an operation of simultaneous saccharification and fermentation (SSF) process, which is a process that combines the steps of saccharification by glucoamylase in starch hydrolysis and fermentation by microbial starters (3, 5). With SSF, the saccharification and fermentation take place simultaneously and the released glucose will be gradually converted into lactic acid.

Sweet potato (SP), *Ipomoea batatas* L., is the world's seventh most important food crop, which has a short cultivation period of 3-4 months. Tubers of SP are rich in carbohydrates and many other nutrients (12, 13). Therefore, SP is an interesting choice of substrate for lactic acid fermentation. In this research, lactic acid production from SP was studied using SSF by lactic acid bacterial starter. Optimal conditions for a hydrolysis of SP by α-amylase and an optimum temperature for SSF step were investigated.

2. Materials and Methods

2.1 Microorganism and seed culture preparation

The lactic acid bacterium strain FAG 302, eventually identified as *Lactobacillus* sp., which was isolated in our laboratory from Thai Northeastern style fermented sausage was used in this study. The strain was cryo-preserved under liquid nitrogen in de Man Rogosa and Sharpe (MRS) broth (HIMEDIA, HiMedia Laboratories, Mumbai, India) with 15% glycerol. Working culture was maintained on MRS agar at 4°C with monthly subculturing. The seed culture was prepared by cultivating the strain in a 125 ml Erlenmeyer flask containing 100 ml MRS broth and incubated for 24 h at 37°C. A 10% (v/v) inoculum was applied to make approximately 10^6 CFU/ml of initial bacterial count in fermentation medium.

2.2 Materials and chemicals

SP was obtained from Phichit Agricultural Research and Development Center. Sodium hydroxide and potassium tartrate were purchased from UNIVAR (Ajax Finechem, Australia). D-glucose was obtained from Biomark™ Laboratory, India. Yeast extract was purchased from Lab M Limited, UK. PGO Enzyme Preparation, *ortho*-Dianisidine dihydrochloride, and 3,5-dinitrosalicylic acid were provided by SIGMA-ALDRICH, USA.

2.3 Enzymes for sweet potato hydrolysis

Hydrolysis of sweet potato in liquefaction step was carried out using commercially available bacterial thermostable α-amylase (EC 3.2.1.1), BAN™ 240L (Novozymes A/S, Denmark), produced by *Bacillus amyloliquefaciens* with a declared activity of ≥ 250 Units/g. The saccharification step was functioned by glucoamylase (EC 3.2.1.3), Spirizyme® Fuel (Novozymes A/S, Denmark) with a declared activity of ≥ 750 Units/g.

2.4 Preparation of sweet potato

Tubers of SP were washed in running tap water, peeled, and steamed for 30 min. Then, the tubers were ground and mixed together until a homogeneous mash was obtained. To avoid microbial contamination, the prepared SP mash was autoclaved for 15 min at 110°C on two consecutive days. After the mash cooled down, it was stored under refrigeration until use.

2.5 Optimization of SP liquefaction hydrolysis by α-amylase using RSM

A 50% (w/v) of the prepared SP mash in distilled water was subjected to liquefaction by α-amylase in

Table 1. Experimental range and levels of independent variables used in CCD

Independent variables	Range and levels				
	-2	-1	0	1	2
α-amylase (% v/ SP weight), x_1	0.020	0.065	0.110	0.155	0.200
incubation temperature (ºC), x_2	65	70	75	80	85
pH, x_3	5.0	5.5	6.0	6.5	7.0
$CaCl_2$ (g/L), x_4	0.00	0.25	0.50	0.75	1.00

250 ml Erlenmeyer flasks for 90 min. The optimal conditions were investigated and studied parameters included the amounts of α-amylase and $CaCl_2$, pH and incubation temperature. CCD was implemented for experimental design and each variable was studied at five levels (Table 1).

Dependent variable was the amount of reducing sugars released that was estimated by DNS method (15). The data were analyzed according to RSM methodology. The relationships and interrelationships of the variables were determined and the quadratic model was generated following the quadratic equation below:

$$Y = b_0 + \Sigma b_i x_i + \Sigma b_{ii} x_i^2 + \Sigma b_{ij} x_i x_j \quad \ldots\ldots\ldots(1)$$

Where Y is reducing sugars released, b_0, b_i, b_{ii}, b_{ij} are regression coefficients: b_0 is the offset term, b_i is the linear effect, b_{ii} is the squared effect, b_{ij} is the interaction effect, x_i and x_j are independent variables. According to the CCD with five levels of four variables, thirty one runs were created (Table 2).

2.6 Optimization of incubation temperature in SSF step

Optimal conditions obtained for SP hydrolysis in liquefaction step by α-amylase from previous experiment was applied to the prepared SP mash. Then, liquefied product was added with following components: 10.0 g/L bacteriological peptone, 8.0 g/L meat extract, 4.0 g/L yeast extract, 1.0 ml/L Tween 80, 2.0 g/L $(NH_4)_3C_6H_5O_7$, 5.0 g/L CH_3COONa, 0.2 g/L $MgSO_4.7H_2O$, 0.05 g/L $MnSO_4.H_2O$, 2.0 g/L K_2HPO_4 and 60 g/L $CaCO_3$. The fermentation medium was sterilized at 121°C, 15 psi, 15 min. After the medium cooled down, 0.10% glucoamylase (%v/SP weight) as well as 10% (v/v) inoculum were introduced. Incubation temperature for the saccharification and lactic acid fermentation was varied at 34, 37, 40, and 43°C and the process was allowed for 72 h with 150 rpm agitation.

After 72 h of fermentation, the pH level of fermentation medium was measured using a pH meter (Metrohm Siam, Thailand). Then, free lactic acid was released from calcium lactate salt by adding proper amount of 4 M HCl to lower the sample pH level to 1.8-2.0. Subsequently, distilled water was added to make equal volume of each sample and samples were centrifuged at 6,000 rpm for 15 min. Lactic acid concentration in the supernatant was analyzed by the method described by Barker and Summerson (14) whereas DNS method (15) and PGO enzyme preparation were used to determine reducing sugars and glucose levels, respectively. Briefly, sample and DNS reagent (1 ml each) were mixed, boiled for 10 min, cooled down and added with 10 ml distilled water. Then, the solution absorbance was detected at 540 nm for reducing sugars determination. For glucose determination, 0.25 ml of sample was mixed with 2.5 ml of PGO enzyme reagent containing *o*-Dianisidine. The reaction was allowed at 37°C for 30 min before measuring the absorbance at 425 nm.

Table 2. Experimental designs for an optimization of SP liquefaction hydrolysis by α-amylase and results

run	x_1	x_2	x_3	x_4	Reducing sugars (g/L)	
					Experimental	Predicted
1	-1	-1	-1	-1	41.47	43.11
2	1	-1	-1	-1	47.41	46.74
3	-1	1	-1	-1	44.16	44.06
4	1	1	-1	-1	48.01	49.82
5	-1	-1	1	-1	49.13	48.78
6	1	-1	1	-1	55.88	54.99
7	-1	1	1	-1	45.69	47.38
8	1	1	1	-1	54.76	55.71
9	-1	-1	-1	1	58.39	57.24
10	1	-1	-1	1	59.18	57.29
11	-1	1	-1	1	53.49	54.17
12	1	1	-1	1	56.19	56.34
13	-1	-1	1	1	59.71	57.70
14	1	-1	1	1	60.42	60.32
15	-1	1	1	1	51.79	52.27
16	1	1	1	1	58.86	57.02
17	-2	0	0	0	56.18	55.54
18	2	0	0	0	62.88	63.92
19	0	-2	0	0	48.46	50.97
20	0	2	0	0	50.72	48.62
21	0	0	-2	0	48.19	47.76
22	0	0	2	0	53.28	54.11
23	0	0	0	-2	44.79	42.55
24	0	0	0	2	55.34	57.99
25	0	0	0	0	52.54	54.42
26	0	0	0	0	53.13	54.42
27	0	0	0	0	57.02	54.42
28	0	0	0	0	55.08	54.42
29	0	0	0	0	55.16	54.42
30	0	0	0	0	54.48	54.42
31	0	0	0	0	53.55	54.42

Note: x_1, x_2, x_3, x_4 represented α-amylase, incubation temperature, pH, and $CaCl_2$, respectively. Values of the fourvariables presented were coded values.

3. Results and Discussion

3.1 Optimization of SP liquefaction hydrolysis by α-amylase using RSM

Liquefaction is an early step of starch hydrolysis which was functioned by α-amylase, an endo-enzyme which randomly hydrolyzes α-1,4 glycosidic linkages in starch molecule leading to the release of dextrins and oligosaccharides. This renders the product viscosity to obviously be reduced (10). High levels of dextrins and oligosaccharides are required since they will be further hydrolyzed by glucoamylase to yield glucose, a substrate for fermentation of lactic acid and other valuable products. Glucoamylase is a debranching enzyme which hydrolyzes α-1,4 and α-1,6 glycosidic bonds (10). However, it is known that enzymes will fully function only under their optimal conditions. Various factors are realized to affect α-amylase function. Information obtained from the product data sheet of Novo Nordisk A/S, Denmark reviewed that the amounts of α-amylase and $CaCl_2$, pH and incubation temperature are among the most important factors. Calcium was said to improved α-amylase stability while pH and temperature are important physical environments.

According to CCD experimental design with 5 levels of the four variables described in Table 1, 31 runs were created and conducted. Reducing sugars level of each run was determined and the results were revealed in Table 2. Then, experimental data of reducing sugars obtained were subjected to multiple regression analysis and the results were indicated in Table 3.

Table 3. Regression coefficients, t-value and P-value for reducing sugars concentration

Term	Coefficient	SE Coefficient	t - value	P - value
Constant	54.4229	0.7498	72.584	0.000
α-amylase (x_1)	2.0950	0.4049	5.174	0.000
Temperature (x_2)	-0.5883	0.4049	-1.453	0.166
pH (x_3)	1.5883	0.4049	3.922	0.001
$CaCl_2$ (x_4)	3.8592	0.4049	9.530	0.000
x_1^2	1.3274	0.3710	3.578	0.003
x_2^2	-1.1576	0.3710	-3.120	0.007
x_3^2	-0.8713	0.3710	-2.349	0.032
x_4^2	-1.0388	0.3710	-2.800	0.013
x_1x_2	0.5313	0.4959	1.071	0.300
x_1x_3	0.6450	0.4959	1.301	0.212
x_1x_4	-0.8963	0.4959	-1.807	0.090
x_2x_3	-0.5900	0.4959	-1.190	0.252
x_2x_4	-1.0063	0.4959	-2.029	0.059
x_3x_4	-1.3050	0.4959	-2.631	0.018

$R^2 = 92.3\%$ R^2 (adj) = 85.6%

From the results in Table 3, a model for the prediction of reducing sugars concentration of each run was generated.

$$Y = 54.4229+2.0950x_1 - 0.5883x_2+1.5883x_3+3.8592x_4 +1.3274x_1^2-1.1576x_2^2-0.8713x_3^2-1.0388x_4^2+ 0.5313x_1x_2+0.6450x_1x_3-0.8963x_1x_4-0.5900x_2x_3- 1.0063x_2x_4-1.3050x_3x_4 \ldots\ldots\ldots(2)$$

Where Y is the predicted reducing sugars concentration, x_1, x_2, x_3, and x_4 are the coded values of α-amylase, incubation temperature, pH, and $CaCl_2$, respectively. The R^2 value (92.3%) demonstrates a good agreement between experimental and predicted reducing sugars concentration.

The probability value (p-value) indicates the significance of each variable coefficient (16). The p-value at less than 0.05 indicated that the variables significantly affected reducing sugars concentration in liquefied product. From the output in Table 3, the p-values of α-amylase, pH and $CaCl_2$ were less than 0.05, which implied that the amount of α-amylase as well as pH and $CaCl_2$ were crucial for the efficiency of this liquefying enzyme. However, the temperature p-value was greater than 0.05, which interpreted that incubation temperature in the studied ranges (65-85°C) did not give a significant difference on α-amylase efficiency. This is probably because of the enzyme ability to work at wide temperature ranges (the product data sheet from Novo Nordisk A/S, Denmark). The squared terms of all variables were significant ($p<0.05$) suggesting the curvature in the response surface. The insignificance of the interaction effects, except for the interaction effect of pH and $CaCl_2$, was indicated by the high p-value.

From the model generated (equation 2), reducing sugars concentration of each run was predicted (Table 2). The highest predicted reducing sugars level (63.92 g/L) was shown in run 18. However, the predicted reducing sugars level in run 14 was only slightly less (60.32 g/L). The conditions in run 14 were very interesting when cost was one of the concerned factors since it required less α-amylase and lower temperature while the reducing sugars level was still high. Thus, optimal conditions for liquefaction of SP were 0.155% α-amylase (% v/SP weight), 70 °C incubation temperature, pH 6.5, and the supplement of 0.75 g/L $CaCl_2$. The results were in accordance with the conditions suggested by the enzyme manufacturer (0.02-0.15% α-amylase, 70-90°C, pH 6.0-7.0, with low amount of $CaCl_2$). The validation of the results using the optimal conditions was performed by repeating experiments of run 14 and reducing sugars obtained (60.54 g/L) was in close agreement with the value predicted by the model.

Figure 1 presented a correlation between experimental and predicted values of reducing sugars formation. All points were located around the diagonal line, indicating that the model worked efficiently.

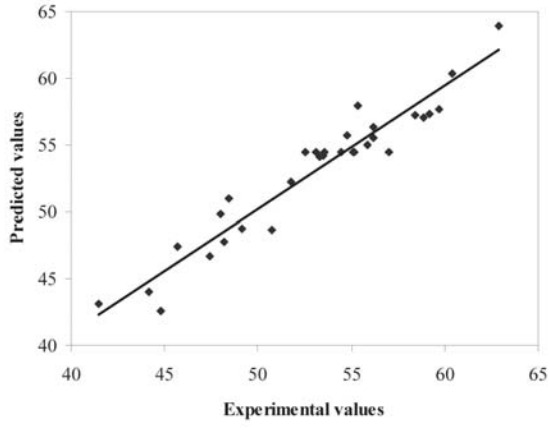

Figure 1. Experimental vs. predicted values plot of reducing sugars (g/L) from a liquefaction hydrolysis of SP by α-amylase

3.2 Optimization of incubation temperature in SSF step

After optimal conditions for the hydrolysis of SP by α-amylase were obtained, the conditions were applied and liquefied product was used as a substrate for lactic acid production by SSF process as mentioned in materials and methods. However, the temperature

recommended for glucoamylase was 60°C which was too high for lactic acid starter. Incubation temperature is very critical for microbial fermentation (17). Therefore, an optimum incubation temperature was determined and it was evident from the results that 34°C was optimal since a highest lactic acid concentration at 105.52 g/L was yielded (Table 4). When temperature was elevated above 34°C, lactic acid concentration significantly decreased whereas the amount of remaining sugars increased, suggesting that lactic acid starter became less efficient to convert sugars into lactic acid.

Starch content of SP tuber was determined by an enzymatic hydrolysis method using α-amylase and glucoamylase following AOAC method (18) with some modifications. SP tubers used in this research contained around 24% starch. Since 50% of SP mash in distilled water was applied, therefore the fermentation medium contained about 12% starch (120 g/L). From the results in Table 4, a maximum lactic acid obtained was 105.52 g/L, which corresponds to 87.9% conversion. Panda and Ray (12) reported 23.86 g lactic acid produced from sweet potato flour with 56% conversion based on starch content after 120 h of incubation by *Lactobacillus plantarum*. Bustos et al. (19) reported a maximum D-lactic acid concentration at 58.9 g/L after 96 h of fermentation by *Lactobacillus coryniformis* when glucose was used as a substrate. Yu et al. (20) reported that 115.12 g/L lactic acid was achieved from glucose when fermented by *Lactobacillus rhamnosus* CGMCC 1466. In conclusion, the amount and yield of lactic acid obtained from SP using the process and conditions described in the current research were satisfactory when compared to the results reported previously by other researchers. In addition, SP is abundantly available in Thailand and its cost is much cheaper than glucose. Therefore, lactic acid production from SP by SSF appears to be an interesting alternative.

Table 4. Effect of temperature on pH, lactic acid production, remaining reducing sugars and glucose after fermentation by *Lactobacillus* sp. for 72 h with 150 rpm agitation.

Temperature (ºC)	pH	Lactic acid (g/L)	Reducing sugars (g/L)	Glucose (g/L)
34	$5.04^{a} \pm 0.63$	$105.52^{a} \pm 2.81$	10.22 ± 1.56	8.95 ± 0.66
37	$5.20^{ab} \pm 0.06$	$100.22^{b} \pm 1.86$	13.73 ± 0.84	9.96 ± 1.43
40	$5.22^{ab} \pm 0.11$	$92.09^{c} \pm 1.56$	17.84 ± 1.79	11.67 ± 1.30
43	$5.30^{bc} \pm 0.02$	$88.31^{d} \pm 1.19$	21.41 ± 1.34	15.31 ± 1.81

[a.b.c,d] Values with different letters in the same column are significantly different ($p < 0.05$).

4. Conclusions

Sweet potato showed a high potential as a good resource for lactic acid production by *Lactobacillus* sp. An optimization of SP liquefaction hydrolysis by α-amylase was successfully achieved by using RSM. The optimal conditions obtained provided a liquefied product with high reducing sugars, which subsequently was a fermentation substrate for lactic acid production by an efficient process called SSF process. This process makes the production of lactic acid possible with at least 48-72 h faster since it is not necessary to completely hydrolyze the starch into glucose prior to the beginning of fermentation.

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