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Lactic Acid Fermentation from Tapioca Starch by *Lactobacillus casei* TISTR 453 using Simultaneous Saccharification and Fermentation Process

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Abstract

Lactic acid and its derivatives have wide applications in various industries. One of the most interesting applications of lactic acid is to serve as a monomer for polylactide synthesis. In the current research, lactic acid production from tapioca starch by lactic acid bacterium, *Lactobacillus casei* TISTR 453, was studied using a simultaneous saccharification and fermentation process. We focused on investigating optimum temperature and medium compositions. Tapioca starch was supplied at 150 g/L and starch hydrolysis was initiated by the addition of 0.10 % (v/starch wt.) α- amylase. Subsequently, liquefied starch was used to prepare a fermentation medium (modified MRS formula). Afterwards, 0.10% (v/starch wt.) glucoamylase as well as lactic acid starter at 10^6 CFU/ml were simultaneously induced and lactic acid fermentation was allowed for 72 h. The results revealed that 40° C was an optimum temperature. For the optimization of glucoamylase and medium compositions when response surface methodology (RSM) with centrl composite design (CCD) was employed, the results suggested that 0.10% (v/starch wt.) glucoamylase, 87.5 g/L calcium carbonate, and 10.0 g/L yeast extract were optimal. The model generated from RSM showed a predicted lactic acid concentration at 131.50 g/L when fermentation was conducted using *Lb. casei* TISTR 453 with the optimized medium and incubated at 40 ℃ for 48 h. Eventually, the results were validated using the optimized medium resulting in 130 g/L lactic acid, which corresponds to 0.87 g lactic acid/g starch.

Keywords: lactic acid, tapioca starch, simultaneous saccharification and fermentation

1. Introduction

Lactic acid is a valuable organic acid discovered by a Swedish scientist, C.W. Scheele, in 1780 (1). Lactic acid and its derivatives have versatile applications. Of which, the applications of being an acidulent, a preservative, and a taste-enhancer in food industry have played the most important role (2). Furthermore, a special application of lactic acid as a raw material in biodegradable plastic manufacturing has expanded the global demand for this acid to an estimate of 150,000 metric tons per year (1). Although lactic acid can be produced chemically or biotechnologically, the later is a principal process. It was estimated that about 90% of lactic acid consumed worldwide were produced by lactic acid bacterial fermentation (3).

Utilization of cheap and renewable raw materials in substitution of refined sugars will bring an economic success. A number of literatures reviewed that the raw materials for lactic acid production usually relied on products and by-products from agricultures and industries (1, 4, 5, 6).

Among those, starch from cassava (*Manihot esculenta* Crantz) is one of the most interesting resources for lactic acid fermentation since it is cost-effective and available throughout the year. However, a bioconversion of starch into lactic acid by lactic acid bacteria can be obstructed since most of high potential lactic acid producing bacteria lack of amylolytic enzymes. Therefore, processes of starch hydrolysis usually are incorporated for substrate preparation (7). Enzymatic starch hydrolysis requires α amylase (EC 3.2.1.1) and amyloglucosidase or glucoamylase (EC 3.2.1.3) (7, 8). The α-amylase is an endo-enzyme which randomly hydrolyzes α-1,4 glycosidic linkages in starch molecule to release dextrins and oligosaccharides. Glucoamylase is a debranching enzyme which hydrolyzes α-1,4 and α-1,6 glycosidic bonds (8). In general, starch hydrolysis process will take at least 48-72 h until glucose is completely released. The disadvantages of two-step fermentation, a fermentation process which includes a step of starch hydrolysis into glucose followed by a step of fermentation by certain microorganisms (9), are that it requires relatively long time, which means a high cost, for overall process and that the enzymes will be inhibited during starch hydrolysis when a high glucose concentration is produced (10).

Simultaneous saccharification and fermentation (SSF) process has been widely reviewed as an efficient tool for the production of microbial products. It is a process that glucoamylase and microbial starters are applied at the same time to allow the steps of saccharification of dextrins into glucose and fermentation of glucose into final product to occur simultaneously (9, 11). Therefore, the released glucose will be gradually fermented into lactic acid and the problem of enzyme inhibition will be minimized. In our present research, lactic acid production from tapioca starch by lactic acid bacterium, *Lb. casei* TISTR 453, was carried out using SSF process. Optimum temperature, glucoamylase calcium carbonate and yeast extract were studied.

2. Materials and Methods

2.1 Microorganism and inoculum preparation

Lb. casei TISTR 453, a homofermentative lactic acid bacterium, was obtained from the Bangkok MIRCEN, Thailand. The strain was maintained in de Man Rogosa and Sharpe (MRS) broth (HIMEDIA, HiMedia Laboratories, Mumbai, India) with 15% glycerol at -80 °C. Working culture was maintained on MRS agar at 4 °C with monthly subculturing. The inoculum was prepared in a 125 ml Erlenmeyer flask containing 100 ml MRS broth and incubated at 37 °C for 24 h. A 10% (v/v) inoculum was used to provide approximately 10^6 CFU/ml initial count of starter culture in fermentation medium.

2.2 Materials and chemicals

Tapioca starch was purchased from local store. 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 starch hydrolysis

Starch hydrolysis was carried out using two commercially available enzymes: 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 and glucoamylase (EC 3.2.1.3), Spirizyme® Fuel (Novozymes A/S, Denmark) with a declared activity of 750 Units/g.

2.4 Preparation of liquefied starch for SSF

Tapioca starch (150 g/L in distilled water) was hydrolyzed following the conditions recommended by the enzyme manufacturer: 0.10% (v/starch wt.) α-amylase at 80°C, pH 6.0 for 90 min.

2.5 Optimization of incubation temperature

Fermentation medium was made using the prepared liquefied product that was supplemented 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 75 g/L $CaCO_3$. Then, the medium was sterilized at 121°C, 15 psi for 15 min. After the medium was cooled down, 0.10% (v/starch wt.) glucoamylase and 10% (v/v) inoculum were applied. Incubation temperature for the saccharification and lactic acid fermentation was varied at 30, 34, 37, 40, and 43°C and incubation periods were 72 h with 150 rpm agitation in 125 ml Erlenmeyer flasks.

2.6 Optimization of glucoamylase, $CaCO_3$ and yeast extract using RSM

Fermentation medium was prepared mostly the same way as mentioned in the optimization of incubation temperature experiment, except the amounts of glucoamylase, $CaCO_3$ and yeast extract which were varied. The three components were supplied at different levels to investigate the optimal conditions for lactic acid production. Experimental designs were conducted using CCD (Table 1).

Dependent variable was lactic acid concentration. 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:

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

Where Y is the amount of lactic acid, 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 three variables, twenty runs were created (Table 3).

2.7 Sample analysis

At the end of fermentation, the pH level of fermentation medium was determined using a pH meter (Metrohm Siam, Thailand). Then, 4 M HCl was added until the sample pH dropped to 1.8-2.0 to release free lactic acid from calcium lactate salt. Thereafter, distilled water was added to adjust the volume to make each sample equal volume. Subsequently, samples were centrifuged at 6,000 rpm for 15 min and supernatant was collected. Lactic acid concentration was determined by the colorimetric method (12). Concentrations of reducing sugars and glucose were analyzed by DNS method (13) and PGO enzyme preparation, respectively. Briefly, 1 ml of sample was mixed with 1 ml of DNS reagent, boiled for 10 min, cooled down and added with 10 ml distilled water. Then, the absorbance at 540 nm was measured 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 mixture was incubated at 37°C, 30 min and the absorbance was measured at 425 nm.

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

Independent variables	Range and levels				
	-2	-1	0	1	2
Glucoamylase (% v/starch weight), x_1	0.06	0.10	0.14	0.18	0.22
$CaCO_3$ (g/L), x_2	50.0	62.5	75.0	87.5	100.0
yeast extract (g/L), x_3	2.5	5.0	7.5	10.0	12.5

Table 2. Effect of temperature on pH, lactic acid production, yield and productivity after fermentation by *Lactobacillus casei* TISTR 453 for 72 h with 150 rpm agitation.

Temperature (ºC)	pH	Lactic acid (g/L)	Yield (g LA/g starch)	Productivity (g/L h)
30	5.33	$116.73^{bc} \pm 2.96$	0.78	1.62
34	5.06	$121.40^{b} \pm 2.24$	0.81	1.69
37	5.16	$120.67^{b} \pm 1.78$	0.80	1.68
40	4.79	$128.27^{a} \pm 2.85$	0.86	1.78
43	5.31	$118.17^{bc} \pm 3.11$	0.79	1.64

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

Table 3. Experimental designs for an optimization of glucoamylase, $CaCO_3$ and yeast extract and results

Run	x_1	x_2	x_3	Lactic acid (g/L)		Yield (g LA/ g starch)*	Productivity (g/L h)*
				Experimental	Predicted		
1	-1	-1	-1	105.06	103.41	0.70	2.19
2	1	-1	-1	106.33	107.72	0.71	2.22
3	-1	1	-1	119.61	120.06	0.80	2.49
4	1	1	-1	121.61	120.51	0.81	2.53
5	-1	-1	1	107.06	109.76	0.71	2.23
6	1	-1	1	113.00	114.16	0.75	2.35
7	-1	1	1	131.28	131.50	0.88	2.74
8	1	1	1	128.78	132.04	0.86	2.68
9	-2	0	0	117.17	117.11	0.78	2.44
10	2	0	0	123.50	121.95	0.82	2.57
11	0	-2	0	91.89	90.89	0.61	1.91
12	0	2	0	126.05	125.43	0.84	2.63
13	0	0	-2	107.33	108.59	0.72	2.24
14	0	0	2	129.34	126.47	0.86	2.69
15	0	0	0	126.44	124.36	0.84	2.63
16	0	0	0	120.00	124.36	0.80	2.50
17	0	0	0	115.94	124.36	0.77	2.42
18	0	0	0	126.11	124.36	0.84	2.63
19	0	0	0	130.50	124.36	0.87	2.72
20	0	0	0	128.78	124.36	0.86	2.68

Note: x_1, x_2, x_3 represented glucoamylase, $CaCO_3$ and yeast extract, respectively. Values of the three variables presented were coded values.
*Yield and productivity were calculated from experimental values of lactic acid of each runs.

3. Results and Discussion

3.1 Optimization of incubation temperature

Incubation temperature is known as one of the key factors for the success or failure of microbial fermentation process (14). During SSF process, glucoamylase and starter culture work concomitantly to hydrolyzed dextrins and generate the required product. Therefore, incubation temperature must be able to support the functions of both glucoamylase and starter culture. However, the temperature recommended by the enzyme manufacturer for glucoamylase was 60°C which was too high for lactic acid starter (the product data sheet of Novo Nordisk A/S, Denmark). Therefore, an optimum incubation temperature was determined. When SSF process was carried out for lactic acid production by a selected strain of lactic acid starter culture, *Lb. casei* TISTR 453, at the temperature ranges from 30-43°C, the results were given in Table 2. Obviously, the starter culture produced higher lactic acid when incubated at 34-40°C than at 30 or 43°C, indicating that the starter culture is a mesophilic bacterium that tolerates slightly elevated temperature. At 40°C, a highest lactic acid level (128.27 g/L) along with maximum yield and productivity (0.86 g LA/g starch and 1.78 g/L h, respectively) were observed. The results were in an agreement with the information reported by Hofvendahl and Hägerdal (3) that *Lb. casei* had optimum temperature for lactic acid production between 37 and 44°C. Therefore, SSF process was run at 40°C in following experiments.

3.2 Optimization of glucoamylase, $CaCO_3$ and yeast extract using RSM

According to CCD experimental design with 5 levels of the three variables described in Table 1, twenty runs were generated to determine the effects of glucoamylase, $CaCO_3$ and yeast extract on lactic acid production as well as to optimize the conditions. Lactic acid produced from each run was analyzed and the results were shown in Table 3. Then, experimental data were subjected to multiple regression analysis and the outputs were provided in Table 4.

According to Box and Draper (15), Myers and Montgomery (16), the significance of variable coefficients was determined by the probability value (p-value). If the p-value less than 0.05, the variables significantly influence lactic acid production. From the output in Table 4, the p-values of $CaCO_3$ and yeast extract were less than 0.05, interpreting that the two variables were important for lactic acid production. $CaCO_3$ is an acid neutralizing agent commonly used in various organic acid fermentation processes to maintain the medium pH in acceptable ranges for starter cultures (14, 17). The addition of $CaCO_3$ provided more preferable pH environment for lactic acid starter culture during lactic acid fermentation. The result was in accordance with the work of Kotzamanidis et al. (5), Panda and Ray (18) which reported that $CaCO_3$ efficiently increased lactic acid production when appropriate amounts were used. In general, the optimum pH for lactic acid production by lactic acid bacteria lies between 5.0 and 7.0 (3). Yeast extract is reported to enhance lactic acid fermentation since it is rich in amino acids, B vitamins, and other growth factors which are beneficial to lactic acid bacteria (19). In contradictory, the p-value of glucoamylase was higher than 0.05 which implied that the amounts of glucoamylase in the studied ranges (0.06-0.22%v/w) did not give a significant difference on lactic acid production.

From the results in Table 4, a quadratic model for lactic acid prediction was generated.

$$Y = 124.360+1.211x_1+8.635x_2+4.470x_3-1.208x_1^2-4.049x_2^2 -1.708x_3^2-0.964x_1x_2+0.022x_1x_3+1.272x_2x_3 \quad \ldots\ldots\ldots(2)$$

Table 4. Estimated regression coefficients, t-value and P-value

Term	Coefficient	SE Coefficient	t-value	P-value
Constant	124.360	1.7546	70.875	0.000
Glucoamylase (x_1)	1.211	1.0998	1.101	0.297
$CaCO_3$ (x_2)	8.635	1.0998	7.851	0.000
Yeast extract (x_3)	4.470	1.0998	4.065	0.002
x_1^2	-1.208	0.8773	-1.376	0.199
x_2^2	-4.049	0.8773	-4.615	0.001
x_3^2	-1.708	0.8773	-1.946	0.080
x_1x_2	-0.964	1.5553	-0.620	0.549
x_1x_3	0.022	1.5553	0.014	0.989
x_2x_3	1.272	1.5553	0.818	0.433

R^2 = 91.1% R^2 (adj) = 83.1%

Table 5. Analysis of variance

Source	Df	Seq SS	Adj SS	Adj MS	F-value	P-value
Regression	9	1988.230	1988.230	220.915	11.420	0.000
Linear	3	1536.100	1536.100	512.035	26.460	0.000
Square	3	431.750	431.750	143.917	7.440	0.007
Interaction	3	20.380	20.380	6.792	0.350	0.789
Residual Error	10	193.520	193.520	19.352		
Lack-of-Fit	5	39.420	39.420	7.885	0.260	0.920
Pure Error	5	154.100	154.100	30.820		
Total	19	2181.750				

df = degree of freedom, Seq SS = sum of squares, Adj SS= Adjusted sum of squares, Adi MS= Adjusted mean sum of squares, F-value = Variance ratio, P-value=Probability

Where Y is the predicted lactic acid concentration, x_1, x_2, x_3 are the coded values of glucoamylase, $CaCO_3$ and yeast extract, respectively. The R^2 value (91.1%) indicates a good agreement between experimental and predicted values.

Effects of the linear terms, squared terms, and interaction terms were summarized in the analysis of variance for lactic acid production in Table 5. The p-value of linear term (p = 0.000) indicated the influence of studied variables on lactic acid production while the small p-value of the square term (p = 0.007) indicated the curvature in the response surface. However, the interaction effect was insignificant since the p-value was higher than 0.05 (p = 0.789).

From the model, lactic acid production was predicted (Table 3). A maximum value was predicted in run 8 (132.04 g/L) and the second most lactic acid level was predicted in run 7 (131.50 g/L). However, when the conditions of run 8 and 7 were compared, a higher level of glucoamylase (0.18%v/starch wt.) was required in run 8 while the amounts of the other two variables were equal. Since glucoamylase is a high-priced enzyme, the application at

less level is more satisfactory when the concentrations of final product are not very different. In addition, lactic acid yield and productivity in run 7 was higher than run 8. Thus, optimal conditions for lactic acid production by *Lb. casei* TISTR 453 using SSF process was 0.10% (v/starch wt.) glucoamylase, 87.5 g/L $CaCO_3$ and 10.0 g/L yeast extract. The optimized medium gave a predicted lactic acid at 131.50 g/L. Eventually, the results were validated using the optimized medium and fermentation was allowed at 40 ° C for 48 h. From the results, 130.00 g/L lactic acid was obtained, corresponding to the yield and productivity at 0.87 g LA/g starch and 2.71 g/L h, respectively. It was interesting that the result observed from the validatione xperiment was very close to the predicted lactic acid. This was a good evident to ensure the high efficiency of the model.

Figure 1 presented a correlation between experimental and predicted values of lactic acid. Since all points were scattered closely to the diagonal line, it was suggested that the model worked effectively.

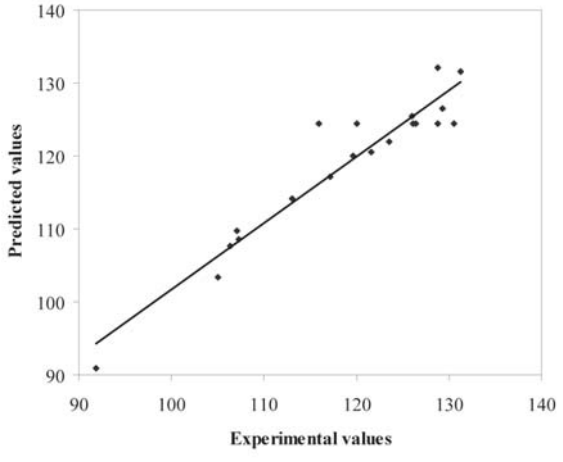

Figure 1. Experimental vs. predicted values plot of lactic acid (g/L)

From literature reviews, glucose was used for lactic acid production in some research works. A maximum D-lactic acid concentration at 58.9 g/L was reported by Bustos et al. (20) when glucose was used as a substrate and fermentation was allowed for 96 h by *Lactobacillus coryniformis*. Yu et al. (21) reported that 115.12 g/L lactic acid was obtained from glucose when fermentation by *Lactobacillus rhamnosus* CGMCC 1466 was conducted. In some publications, a complete starch hydrolysis was incorporated in the process to liberate glucose prior to fermentation. For example, Shamala and Sreekantiah (7) reported lactic acid yield produced by *Lactobacillus plantarum* at 21.8 g from 100 g reducing sugars used when tapioca starch hydrolysate was applied as a substrate. In this study, an efficient process and conditions for lactic acid production using tapioca starch was obtained. The amount of lactic acid as well as yield and productivity were satisfactory when compared with the results reported in other publications. In addition, tapioca starch is readily available at low cost in Thailand. Therefore, it is interesting that tapioca starch can be used efficiently for lactic acid production without the necessity to completely hydrolyze it before using.

4. Conclusion

Optimal conditions for efficient production of lactic acid from tapioca starch by *Lb. casei* TISTR 453 were achieved. The optimized conditions provided markedly higher lactic acid amount and productivity. With SSF process, overall processed time was reduced since another 72 h for the pre-starch hydrolysis in saccharification step were not required.

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