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Statistical screening of medium components for lactic acid production from tapioca starch hydrolysate by *Lactobacillus casei* TISTR 453 using Plackett–Burman design

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Abstract

Plackett-Burman design was employed for efficiently screening and selection of medium components for lactic acid production by *Lactobacillus casei* TISTR 453 using tapioca starch hydrolysate as a carbon source in a minimal numbers of experiments. A total of 16 experiments were conducted to determine the significance of 13 nutrient parameters on lactic acid production. All experiments were carried out in a submerged fermentation for 96 h at 34 ^{o}C under static condition. An initial level of reducing sugar from tapioca starch hydrolysate was applied at 120 g/l. From the results, the highest lactic acid concentration at 73.00 g/l was obtained. From the regression analysis output, 10 out of 13 parameters studied were found to influence lactic acid production significantly. However, too many parameters would lead to a difficulty on medium optimization in the subsequent steps. Therefore, only 4 medium components namely winery yeast disposal, peptone, meat extract and dipotassium hydrogen phosphate were chosen for further optimization since they were previously reported as important factors on lactic acid fermentation. The other 4 components which showed significant results including calcium carbonate, triammonium citrate, manganese sulfate monohydrate, and sodium acetate were to be assigned as fixed variables of fermentation medium in medium optimization steps. On the other hand, corn steep liquor and ammonium sulfate were disregarded, although they provided significant results on lactic acid production, since their main effects were negative.

Keywords: *lactic acid, Lactobacillus casei, Plackett-Burman design, tapioca starch hydrolysate*

1. Introduction

Lactic acid is a valuable industrial chemical. It has wide applications in foods and beverages, pharmaceuticals, textiles, tanning, cosmetic industries, etc. Recently, it is also used for the preparation of polylactide which is used in the manufacture of biodegradable, biocompatible plastics and coatings. In addition, polylactide finds applications in biocompatible artificial organs, self-dissolving sutures and drug release (1,2).

Industrial production of lactic acid is possible either by microbial fermentation or chemical synthesis. However, the later method provided only racemic mixture of the L(+) and D(-) enantiomer of lactic acid. On the other hand, microbial fermentation has an advantage of producing optically pure product or DL lactic acid (3), depending on the strains chosen. The other major advantage of lactic acid fermentation over chemical synthesis is that cheap raw materials such as whey, molasses, starch, beet, cane sugar and other carbohydrate rich materials can be used. This allows an economic production of lactic acid (4). Among many raw materials available, tapioca starch is considered an interesting resource for lactic acid fermentation as it is cost-effective and its' availability is satisfactory. After being hydrolyzed, a hydrolysate of tapioca starch containing high concentrations of glucose and a few other fermentable sugars was obtained. This is feasible for an efficient lactic acid production (5).

Productivity of microbial metabolites mainly depends on 3 factors: nutritional compositions, physical parameters and genotypic characteristics of the producing strains. Nutritional compositions can be optimized either by conventional or statistical method. The later provides rapid and reliable results as well as reduces the number of nutrient to be studied which lead to more economic output in terms of time, glassware, chemicals and manpower (1). The Plackett-Burman statistical method offers a design where n variables are studied in n+1 experimental runs. The design is useful for preliminary selection of variables that should be studied in further optimization processes. (2,6).

The aim of this study was to apply Plackett-Burman design for screening medium components that significantly influenced lactic acid production by *Lactobacillus casei* TISTR 453, when using tapioca starch hydrolysate as a carbon source.

2. Materials and methods

2.1 Microorganism

L. casei TISTR 453, a homo-fermentative lactic acid bacterium, was obtained from the Bangkok MIRCEN, Thailand. The strain was maintained in MRS broth (Lab M Ltd., United Kingdom) with 15% (v/v) glycerol at -80 °C. A short-term preserved culture was stored in MRS agar slant at 4 °C with monthly subculturing.

2.2 Starch hydrolysis

Tapioca starch was purchased from local stores in Nakhon Pathom, Thailand. The process of enzymatic starch hydrolysis was allowed in a 1 L Erlenmeyer flask containing 280 g dry tapioca starch and 600 ml of distilled water. An initial pH level was adjusted to 5.5. Liquefaction was started up by the addition of Liquozyme® SC DS (Novozymes A/S, Denmark), an alpha-amylase (EC 3.2.1.1) produced by *Bacillus licheniformis* with a declared activity of 240 KNU-S/g. The level used was 0.23 g enzyme/kg starch. Temperature was maintained at 70 °C for 90 min. Then, saccharification process was needed. Prior to an addition of gluco-amylase (EC 3.2.1.3; Spirizyme® Fuel, Novozymes A/S, Denmark), temperature was decreased to 55 °C whereas the pH level required was still at 5.5. Gluco-amylase

activity was 750 AGU/g and the dosage applied was 1.0 g enzyme/kg starch. After 48 h, the enzymes were inactivated by heating the starch hydrolysate at 100 °C for 5 min. Subsequently, a refrigerated centrifugation at 5000xg was applied for 15 min to get rid of starch residuals. The clear starch hydrolysate was kept refrigerated until use.

2.3 Materials

Winery yeast disposal was obtained from Siam Winery CO., Ltd. Other chemicals used in this work were of analytical grade. Meat extract was purchased from HiMedia Laboratories, India. Peptone was obtained from Biomark Laboratories, India. Corn steep liquor, magnesium sulfate monohydrate and sodium acetate were obtained from Fluka, Sigma-Aldrich, Germany. Calcium carbonate, ammonium sulfate, dipotassium hydrogen phosphate and potassium dihydrogen phosphate were provided by UNIVAR, Ajax Finecem, Australia. Triammonium citrate was obtained from BHD Laboratory Supplies Poole, England. Manganese sulfate monohydrate was obtained from J. T. Baker, J. T. Baker chemicals B.V., Holland. Tween 80 was obtained from RANKEM, RFCL Limited, India.

2.4 Cultivation

Seed culture was prepared by inoculating one loopful of *L. casei* TISTR 453 from MRS slant into 125 ml Erlenmeyer flask containing 200 ml MRS broth. The culture was incubated at 37 °C for 24 h without agitation. Then, centrifugation was applied to the seed culture (6,000 rpm for 15 min at 4 °C) to remove supernatant. Cells were re-suspended in 500 ml sterile 0.1% peptone to achieve an optical density (660 nm) at 1.0. Subsequently, 10 ml of cell suspension was inoculated into 125 ml Erlenmeyer flask containing 90 ml of fermentation medium which consisted of 120 g/l reducing sugar from starch hydrolysate. The fermentation of lactic acid was carried out at 34 °C for 96 h under static condition.

2.5 Screening of important nutrient components

The Plackett-Burman design was applied to screen significant parameters from 13 nutrient components including an acid neutralizing agent, nitrogen sources, minerals and a surfactant. All components were tested at two different concentrations, high (1) and low (-1), as shown in Table 1. In addition, 2 dummy variables were incorporated to increase the accuracy of the results. Therefore, 16 combinations of medium components were generated according to the method of Plackett-Burman (7) (Table 2). In Table 2, each row represents an experiment and each column represents a variable. All experiment used initial level of reducing sugar from tapioca starch hydrolysate at 120 g/l. Statistical analysis was performed on the data of lactic acid production using regression analysis.

Table 1. Concentrations of nutrient components at low and high levels in Plackett-Burman design for lactic acid production.

Variable No.	Variables with designate		Low level (-1)	High level (1)
1	X_1	Calcium carbonate (g/l)	0	60.0
2	X_2	Winery yeast disposal (g/l)	0	15.0
3	X_3	Peptone (g/l)	0	15.0
4	X_4	Meat extract (g/l)	0	12.25
5	X_5	Corn steep liquor (ml/l)	0	15.0
6	X_6	Triammonium citrate (g/l)	0	8.7
7	X_7	Ammonium sulfate (g/l)	0	7.1
8	X_8	Manganese sulfate monohydrate (g/l)	0	0.05
9	X_9	Tween 80 (ml/l)	0	1.0
10	X_{10}	Dipotassium hydrogen phosphate (g/l)	0.2	2.0
11	X_{11}	Potassium dihydrogen phosphate (g/l)	0.2	2.0
12	X_{12}	Magnesium sulfate monohydrate (g/l)	0	0.2
13	X_{13}	Sodium acetate (g/l)	0	5.0
14	X_{14}	Dummy 1	-	-
15	X_{15}	Dummy 2	-	-

2.6 Sample analysis

At 96 h of fermentation, 20 ml of samples were collected and analyzed for pH level, lactic acid produced, and reducing sugar remained. The pH measurement was conducted by a pH meter (Metrohm Siam, Thailand). Then, lactic acid was released from calcium lactate salt by lowering the pH level of fermentation medium to 1.8-2.0 by 4 M HCl. Subsequently, the volume of each sample was adjusted to 40 ml by distilled water to make the volume equal. To get rid of cells, samples were centrifuged (6,000 rpm, 15 min) and supernatant was collected. Finally, the concentrations of lactic acid and reducing sugar were determined following the method of Barker and Summerson (1941) and DNS method (8), respectively.(9)

3. Results and discussion

In the present study, screening of important nutrients for lactic acid production by *L. casei* TISTR 453 was carried out using Plackett-Burman design.(7) From the results in Table 2, maximum lactic acid production (73.00 g/l) was observed in run 1, whereas minimum lactic acid concentration (4.67 g/l) was revealed in run 16 which contained only tapioca starch hydrolysate. Furthermore, the concentrations of lactic acid from different medium combinations were greatly different. The results suggested that *L. casei* TISTR 453 required complex nutrient compositions and the levels of variables studied influenced lactic acid formation significantly.

Table 2. Plackett-Burman experimental design of 15 variables for the production of lactic acid by *L. casei* TISTR 453 when tapioca starch hydrolysate was supplied to provide 120 g/l reducing sugar.

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	Lactic acid (g/l)*
1	1	1	1	1	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	73.00 ± 4.72
2	1	1	1	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	1	37.01 ± 0.65
3	1	1	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	33.68 ± 2.38
4	1	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1	29.34 ± 1.41
5	-1	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1	1	16.96 ± 1.02
6	1	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	45.86 ± 2.65
7	-1	1	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	1	12.59 ± 1.28
8	1	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1	14.98 ± 0.90
9	1	-1	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1	1	41.45 ± 5.85
10	-1	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1	1	1	7.85 ± 0.19
11	-1	1	-1	-1	-1	1	1	1	1	-1	1	-1	1	1	-1	5.35 ± 0.66
12	1	-1	-1	-1	1	1	1	1	-1	1	-1	1	1	-1	-1	16.05 ± 0.92
13	-1	-1	-1	1	1	1	1	-1	1	-1	1	1	-1	-1	1	14.07 ± 0.70
14	-1	-1	1	1	1	1	-1	1	-1	1	1	-1	-1	1	-1	19.94 ± 2.44
15	-1	1	1	1	1	-1	1	-1	1	1	-1	-1	1	-1	-1	13.88 ± 0.05
16	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	4.67 ± 0.55

*The values of lactic acid concentration given above are average of three experiments. Symbols 1 and -1 refer to high and low levels of each variable, respectively.

The results of regression analysis with respect to main effect, standard error, t-value and P-value of each component were revealed in Table 3. Any components that showed the P-value below 0.05 were significant parameters for lactic acid production. If their main effects were negative, the amount required during further optimization must be lower than that indicated as low concentrations (-1) in Plackett-Burman design. On the other hand, if the main effects were positive, higher concentrations than that indicated as high values (1) were needed.

From Table 3, P-values of 3 components namely tween 80, potassium dihydrogen phosphate and magnesium sulfate monohydrate were greater than 0.05, implying that those 3 components were insignificant on lactic acid fermentation by *L. casei* TISTR 453. Hence, they were excluded from parameters of concern in further optimization. The rest of the components which were calcium carbonate, winery yeast disposal, peptone, meat extract, corn steep liquor, triammonium citrate, ammonium sulfate, manganese sulfate monohydrate, dipotassium hydrogen phosphate and sodium acetate showed P-values smaller than 0.05 and were considered significant. Among the significant components, corn steep liquor, ammonium sulfate, dipotassium hydrogen phosphate and sodium acetate provided negative effects on lactic acid production, suggesting that the amounts required were less than that used at their low concentration (-1). However, apart from dipotassium hydrogen phosphate, the low concentration of the rest 3 components showing negative main effect was 0 g/l (Table 1). Thus, corn steep liquor and ammonium sulfate were disregarded in further steps of medium optimization whereas sodium acetate was assigned as a fixed variable.

Table 3. Regression analysis output of the Plackett-Burman design for lactic acid production by *L. casei* TISTR 453.

Variables	Main effect	Coefficient	SE Coefficient	t-value	P-value
Constant		24.167	0.5240	46.12	0.000
Calcium carbonate	24.509	12.255	0.5240	23.39	0.000*
Winery yeast disposal	3.528	1.764	0.5240	3.37	0.002*
Peptone	11.531	5.765	0.5240	11.00	0.000*
Meat extract	16.376	8.188	0.5240	15.62	0.000*
Corn steep liquor	-7.774	-3.887	0.5240	-7.42	0.000*
Triammonium citrate	2.667	1.334	0.5240	2.55	0.016*
Ammonium sulfate	-4.899	-2.450	0.5240	-4.67	0.000*
Manganese sulfate monohydrate	6.069	3.035	0.5240	5.79	0.000*
Tween 80	1.644	0.822	0.5240	1.57	0.126
Dipotassium hydrogen phosphate	-8.233	-4.116	0.5240	-7.85	0.000*
Potassium dihydrogen phosphate	-0.519	-0.260	0.5240	-0.50	0.624
Magnesium sulfate monohydrate	2.006	1.003	0.5240	1.91	0.064
Sodium acetate	-2.962	-1.481	0.5240	-2.83	0.008*

S = 3.63 R-Sq = 97.04% R-Sq (adj) = 95.91%

*Variables showed significant effects on lactic acid production.

Calcium carbonate was used to neutralize lactic acid produced during fermentation. It controlled the pH of fermentation medium in the range of 5.0-7.0. This increased lactic acid production since lactic acid bacteria need buffering system to convert high substrate concentration into high acid level (1, 10). From the result presented in Table 3, calcium carbonate showed a positive effect on lactic acid formation and the P-value at less than 0.05, indicating that calcium carbonate significantly affected the production of lactic acid. However, calcium carbonate as well as triammonium citrate and manganese sulfate monohydrate was assigned as fixed variables in further optimization to reduce the number of parameters to be studied. The levels of calcium carbonate to be used were based on lactic acid concentration estimated (1 mole of calcium carbonate is required to neutralize 2 moles of lactic acid; Naveena *et al.*, (2)).

Effect of various nitrogen sources on lactic acid production was studied. Winery yeast disposal, peptone, and meat extract were significant for lactic acid production by *L. casei* TISTR 453 and were selected for further studies. Lactic acid bacteria are fastidious microorganisms which have complex nutrient requirement (11). Winery yeast disposal consisted of essential nutrients that the starter culture required. Moreover, it was much cheaper than yeast extract which incorporated in most medium formula for lactic acid fermentation by lactic acid bacteria. The other nitrogen source, triammonium

citrate, was significant for lactic acid production as well, implying that *L. casei* TISTR 453 was able to utilize both organic and inorganic nitrogen. However, the cost of triammonium citrate is high. Hence, triammonium citrate was to be supplied at a minimum level in our next experiment.

Manganese sulfate monohydrate had been proven to be beneficial for lactic acid production since manganese ions acts as a co-factor of lactate dehydrogenase (12). Optimal concentration of manganese sulfate monohydrate for lactic acid production from tapioca starch hydrolysate by *L. casei* TISTR 453 was previously reported at 0.05 g/l (1). Thus, the amount of manganese sulfate monohydrate would be fixed at 0.05 g/l in further optimization.

Dipotassium hydrogen phosphate was the source of phosphate frequently applied in lactic acid fermentation medium. From the result obtained, dipotassium hydrogen phosphate showed significant impact and negative effect on lactic acid production. Therefore, its' lower concentration was used for further studies.

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