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Selection of Factors by Plackett-Burman Design for Production of Lactic Acid by Lactobacillus rhamnosus B14-2 in Cassava Fermentation

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***Abstract*— Lactic acid is one of the functional and valuable compounds utilized in food, pharmaceutical, and chemical industries while Poly lactic acid (PLA) is a biodegradable polymer that has a variety of applications. In recent years, microbial conversion of renewable raw materials has become an important objective in industrial biotechnology. 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 hydrolyte of cassava (tapioca starch) containing high concentrations of glucose and a few other fermentable sugars was obtained. This is feasible for an efficient lactic acid production. The objective of this study was to screen and analyze important nutrient constituents was carried out using Plackett-Burman experimental design for production of lactic acid by *Lactobacillus rhamnosus* B14-2 grown in cassava fermentation. Plackett-Burman experimental design was used to evaluate ten factors added to cassava. Three variables, namely Seed culture (10%), Peptone (10 g/L). and pH (6.5) of 10 were significantly and highest contribution increased lactic acid production. The concentrations of these three variables as well as the cassava were further optimized using the response surface method.**

Keywords*— *Lactic acid, lactobacillus rhamnosus B14-2, cassava, poly lactic acid, renewable raw material

1. Introduction

Lactic acid has numerous applications in food, chemical, textile, pharmaceutical, and other industries. Recently, there has been a great demand for lactic acid, as it can be used as a monomer for the production of the biodegradable polymer Poly Lactic Acid (PLA), which is an alternative to synthetic polymers derived from petroleum resources. While only racemic DL-lactic acid is produced through chemical synthesis, a desired stereoisomer (i.e. an optically pure L- or D-lactic acid) could be produced through a fermentative production of renewable resources if the proper microorganisms are chosen for lactic acid fermentation [1]. The other major advantage of lactic acid fermentation over chemical synthesis is that cheap raw materials such as whey, molasses, starch, beet, sugarcane and other carbohydrate richmaterials can be used. This allows an economic production of lactic acid [2]. 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 [3,4]. The Plackett–Burman statistical method offers a design where n variables are studied in n+1 experimental runs. These experimental designs are available in multiples of four runs and hence they are excellent screening methods, as the number of experimental runs required are very few, leading to saving of time, chemicals, glassware and manpower. Moreover, the design is orthogonal in nature, implying that the effect of each variable worked out is pure in nature and not confounded with interaction among variables. Experimental design and data analysis using appropriate software makes the analysis easier as observed in the present study [5]. In the present study, it was described screening and analysis of important nutrient constituents was carried out using Plackett-Burman experimental design for production of lactic acid by *Lactobacillus rhamnosus* B14-2 grown in cassava fermentation.

2. Material and Methods

2.1 Microorganisms, medium and culture conditions

A lactic acid producing strain of *Lactobacillus rhamnosus* B12-4 used in this study was identified by 16S rDNA technique and the cultures were maintained at -80°C in 20% glycerol stocks and grow in Man Rossa de Sharpe (MRS) broth. The inoculum was prepared by transferring glycerol stock culture (1 mL) to an Erlenmeyer flask containing 20 mL of MRS medium and incubated at (37±1)°C for 24 h. Initial pH of the medium was adjusted to 6.5±0.2. Then, the mediums were autoclaved at 15 psi and 121°C for 15 min. The composition of MRS medium was (in g/L): Peptone 10, Yeast extract 5, Meat extract 10, Glucose 20, Sodium acetate 5, K_2HPO_4 2, Tween 80 1, $(NH_4)2HC_6H_5O_7$ 2, $MgSO_4$•$7H_2O$ 0.1 and $MnSO_4$•$4H_2O$ 0.05. Erlenmeyer flasks containing the production medium were inoculated with 10% inoculum grown in the modified MRS medium at (37±1)°C for 12 hrs. The total values of modified MRS medium were 50 mL in 250 mL Erlenmeyer flask.

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2.2 Substrate (cassava preparation)

The cassava (tapioca starch) was obtained from Rose brand, Thai-wah company Bangkok, Thailand and this substrate was hydrolyzed by adding 3 mL of H_2SO_4 (20%) to 100 mL of cassava solution. The acidified cassava solution was heated in a boiling water bath for 60 min. The pH of the medium was adjusted to 6.5 with 4.0 M KOH prior to sterilization [6].

2.3 Reducing Sugar Analysis

Reducing sugar was determined by the DNS (Di-nitrosalisylic) method while sugar glucose was calculated according to the method describe by [7,8].

2.4 Determination of pH and Titratable Acidity (% lactic acid)

The pH value was detected by SensIonTM+ pH meter. Before use, the pH-meter was calibrated using standard buffer solutions of pH 4.0, 7.0, and pH 10.0. Titratable acidity expressed as percent lactic acid was determined by titrating samples using 0.1 N NaOH solution and phenolphthalein as indicator according to the Association of Official Analytical Chemists [9].

2.5 High Performance Liquid Chromatography (HPLC) analysis

The quantification of lactic acid concentrations was determined using a High Performance Liquid Chromatography (Hitachi, L-7100 PUMP 220V 60HZ, Japan), equipped with a tunable UV detector set at 210 nm. RP-18 column (244 mm x 4 mm, Lichrocart) was eluted with 60% MeOH as a mobile phase at a flow rate of 0.7 mL/min. The injection volume was 20 µL for analysis, which was maintained at room temperature.

2.6 Plackett-Burman experimental design

The statistically planned experimentation is to identify the significant variables and their corresponding coefficients, so that the levels of variables can be managed to obtain a desired output. Using the Design Expert (version 7.0, Stat-Ease, Inc., Minneapolis, USA) statistical was used for the experimental design and regression analysis of the experimental data. The purpose of this first step of the optimization was to identify the medium components with a significant effect on lactic acid production by *L. rhamnosus* B14-2 using modified MRS medium of cassava. The reducing sugar concentration from cassava was maintained constant (60±2 g/L) and twelve experiments were generated from ten factors: pH, speed shaking (rpm), temperature (°C), % seed culture, % cassava, meat extract, calcium carbonate ($CaCO_3$), yeast extract, peptone and di-potassium hydrogen phosphate. The variables with a confidence level greater than 95% were considered to have a significant influence on lactic acid production. The Plackett-Burman experimental design (PBD) was based on the first-order model, with no interaction among the factors [10]. The concentrations used for each variable are displayed in Table 1. The experiments were done in 250 mL Erlenmeyer flasks containing 50 mL of production medium within 12 hrs.

Table 1. Variables and levels used in Plackett-Burman design

No	Variables	Codes	Range and levels	
			-1	+1
A	pH	X_1	5.5	6.5
B	Speed shaking (rpm)	X_2	0	100
C	Temperature (°C)	X_3	37	40
D	% Seed culture	X_4	5	10
E	% Cassava	X_5	5	10
F	Meat extract (g/L)	X_6	0	10
G	Calcium carbonate (g/L)	X_7	0	5
H	Yeast extract (g/L)	X_8	0	5
I	Peptone (g/L)	X_9	0	10
J	Di-potassium hydrogen phosphate (g/L)	X_{10}	0	2

3. Results and Discussion

Table 2 displays Plackett-Burman design matrix (real and coded values) of the twelve experiments with ten variables added to cassava (X_1: pH, X_2: Speed shaking (rpm), X_3: Temperature (°C), X_4: % Seed culture, X_5: % Cassava, X_6: Meat extract (g/L), X_7: Calcium carbonate (g/L), X_8: Yeast extract (g/L), X_9: Peptone (g/L), and X_{10}: Di-potassium hydrogen phosphate (g/L)) and the respective results (lactic acid) (Table 2). Seed culture (D) was the most influential variable in the production of lactic acid, followed by Peptone (I) and pH (A). All three variables had a significant positive effect on lactic acid (%) production at a 95% confidence level. Figure 1 (Pareto chart) illustrates the effects of these variables, which were therefore used to optimize the production of lactic acid (%). In the present study *L. rhamnosus* B14-2 produced high lactic acid production in combinations run 3 (Table 2) and the experimental analysis were shown in Table 3. Three variables namely Seed culture (D), Peptone (I) and pH (A) of 10 variables form 95% sum of squares (Table 2) implying that these variables influenced the fermentation process significantly.

Table 2. Plackett-Burman design (real and coded values) with the respective results

Run	Independent variables[a]										Response
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	Lactic acid (g/L)
1	6.5(1)[b]	100(1)	37(-1)	10(1)	10(1)	10(1)	0(-1)	0(-1)	0(-1)	2(1)	4.4
2	5.5(-1)	100(1)	40(1)	5(-1)	10(1)	10(1)	5(1)	0(-1)	0(-1)	0(-1)	1.0
3	6.5(1)	0(-1)	40(1)	10(1)	5(-1)	10(1)	5(1)	5(1)	0(-1)	0(-1)	6.2
4	5.5(-1)	100(1)	37(-1)	10(1)	10(1)	0(-1)	5(1)	5(1)	10(1)	0(-1)	4.4
5	5.5(-1)	0(-1)	40(1)	5(-1)	10(1)	10(1)	0(-1)	5(1)	10(1)	2(1)	3.5
6	5.5(-1)	0(-1)	37(-1)	10(1)	5(-1)	10(1)	5(1)	0(-1)	10(1)	2(1)	5.3
7	6.5(1)	0(-1)	37(-1)	5(-1)	10(1)	0(-1)	5(1)	5(1)	0(-1)	2(1)	1.8
8	6.5(1)	100(1)	37(-1)	5(-1)	5(-1)	10(1)	0(-1)	5(1)	10(1)	0(-1)	5.3
9	6.5(1)	100(1)	40(1)	5(-1)	5(-1)	0(-1)	5(1)	0(-1)	10(1)	2(1)	2.6
10	5.5(-1)	100(1)	40(1)	10(1)	5(-1)	0(-1)	0(-1)	5(1)	0(-1)	2(1)	3.5
11	6.5(1)	0(-1)	40(1)	10(1)	10(1)	0(-1)	0(-1)	0(-1)	10(1)	0(-1)	6.0
12	5.5(-1)	0(-1)	37(-1)	5(-1)	5(-1)	0(-1)	0(-1)	0(-1)	0(-1)	0(-1)	2.8

[a] X_1: pH, X_2: Speed shaking (rpm), X_3: Temperature (°C), X_4: % Seed culture, X_5: %Cassava, X_6: Meat extract (g/L), X_7: Calcium carbonate (g/L), X_8: Yeast extract (g/L), X_9= Peptone (g/L), and X_{10}= Di-potassium hydrogen phosphate (g/L)

[b] (-1) and (1) are coded levels

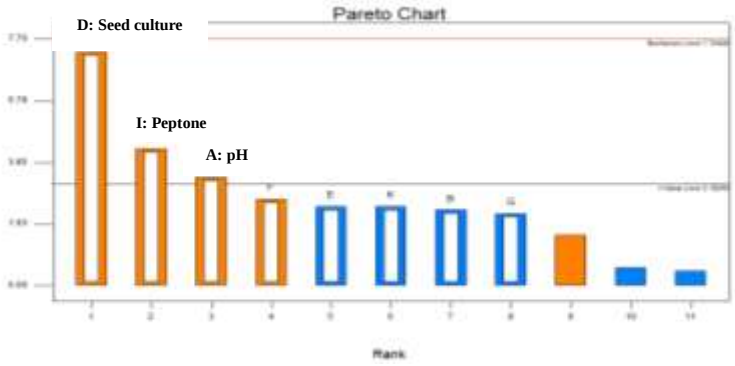

Figure 1. Pareto chart for lactic acid (g/L) with *L. rhamnosus* B14-2 fermentation from cassava medium

Table 3. Regression analysis output of the Plackett-Burman design for lactic acid (g/L) production by *L. rhamnosus* B14-2

Source	Sum of Squares	df	Mean Square	*F Value*	*p*-value Prob > *F*
Model	29.85	8	3.73	14.17	0.0259*
A-pH	3	1	3	11.39	0.0432*
B-rpm	1.47	1	1.47	5.582	0.0992
D-Seed culture	14.08	1	14.08	53.48	0.0053*
E-Cassava	1.613	1	1.61	6.13	0.0897
F-Meat extract	1.92	1	1.92	7.29	0.0738
G-Calcium Carbonate	1.33	1	1.33	5.06	0.1099
I-Peptone	4.81	1	4.81	18.28	0.0235*
K-Di-potassium hydrogen phosphate	1.61	1	1.61	6.13	0.0897
Residual	0.79	3	0.26		
Cor Total	30.64	11			

S= 0.9870, *R-Squared*= 0.9742, *Adj R-Squared* = 0.9054

*Variables showed significant effects on lactic acid (g/L) production

4. Conclusions

A Plackett-Burman experimental design was used to evaluate ten factors added to cassava (pH, speed shaking (rpm), temperature (°C), % seed culture, % cassava, meat extract, calcium carbonate ($CaCO_3$), yeast extract, peptone and di-potassium hydrogen phosphate. Three variables namely seed culture (10%), peptone (10 g/L) and pH (6.5) of 10 variables significant increased lactic acid production. The concentrations of these three components as well as the cassava were further optimized using the response surface method. Plackett-Burman design helps efficiently screening the important factors among a great deal of variables and statistically analyzing the results. However, if the number of variables is large, the work involved for real experiments will be burdensome in practice because the researchers have to add all screening components one by one into the media according to Plackett-Burman matrix. It not only allowed quick identification of the most significant medium components for lactic acid production, but also proved to be useful in saving the researchers' labor and reducing the errors occurred in the process of making medium.

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