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Contributed Paper

Optimization of Xylitol Production by *Candida tropicalis* A26

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Abstract

Factors affecting xylitol production by *Candida tropicalis* A26 was investigated using Plackett-Burman design. According to 5 variables; media ingredients (3 variables), inoculums and pH; xylose and peptone were critical parameters affected on the xylitol production. Central composite design (CCD) was applied to screen for an optimal concentration of xylose and peptone. The highest xylitol production in flask scale was 42.52 g/l when xylose and peptone were 80 g/l and 4 g/l, respectively. The statistical regression model predicted the maximum xylitol production with 43.27 g/l, while the observed one was 42.52 g/l. Moreover, the production was scale up to 5 L by using stirred fermenter under the optimized condition. The highest xylitol production and xylitol yield after 42 h cultivation were 71.59 g/l and 0.89 g/g xylose, respectively. The results of statistical model and 5 L fermenter indicated that the optimized medium increased xylitol yield up to 1.45 folds compared with an un-optimized condition. Experimental design was successful by applied for improvement of xylitol production by *C. tropicalis* A26.

Keywords xylitol, *Candida tropicalis*, optimization, experimental design

1. Introduction

Xylitol is an attractive sugar substitute due to its high sweetening power and unique pharmacological properties. It is commercially used in candy, chewing gum and ice-cream to promote oral health [1, 2]. Xylitol is found in nature such as in fruits and vegetables, but in low concentration [2, 3]. Presently, xylitol is produced by chemical reduction of D-xylose in a presence of nickel catalyst at high temperature and pressure. By this method, separation and product recovery is expensive [3, 4]. Microbial xylitol production is an alternative process which renewable resource of agricultural residues such as hemicelluloses. Bacteria, filamentous fungi and yeasts were known to produce xylitol. Various yeast species such as *Candida boidinii*, *C. guilliermindii, C. shehatae, C. parasilosis, C. peltata, C. mogii, C. maltosa C. tropicalis, Scheffersomyces stipitis,*

Pachysolen tannophilus, Debaryomyces hansenii and *D. nepatensis* were reported as xylitol producers [4-9]. However, the xylitol production yields reported required improvement through an optimization for economical viable. Conventional optimization procedure, one variable at a time, is effective in some situation but fails to consider the combined effects of the entire factors involved. In addition, it is time consuming and cannot provide an interaction of parameters on the desired outcome [11]. Statistical optimization procedure has an advantage over the conventional procedure for rapidity, reliability, and understanding of interaction among parameters at various concentrations [12].

Plackett-Burman design is a statistical design in which two levels of high and low variables are used to identify critical parameter of fermentation process [13]. Central composite design (CCD) is one of the popular mathematical and statistical methodology, response surface methodology (RSM), used to screen for an optimal concentration of the critical parameters of a process [14]. The aim of this study was to use the Plackett-Burman design and the Central composite design to screen for critical parameters and optimized condition for xylitol production of *C. tropicalis* A26.

2. MATERIALS AND METHODS

2.1 Yeast Strain

C. tropicalis A26 was isolated from cow feces, in Khonkaen province, Thailand and maintained on Yeast -Malt extract (YM) slant [0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose and 1.5% agar (w/v), pH 5.5]at 4°C and long time maintained in YM broth containing 10% (w/v) glycerol at -80°C.

2.2 Inoculum and Cultivation

One loopful of *C. tropicalis* A26 was transferred to 5 ml of Yeast Peptone-Xylose (YPX) medium (1% yeast extract, 2% peptone and 2% D-xylose (w/v), pH 5.5) then incubated at 30°C, 200 rpm for 24 h-48 h. The 1% seed culture (v/v) was subsequently transferred into 50 ml fresh YPX broth in 250 ml erlenmeyer flask and inoculated at the condition as described above. After 24 h, the yeast cells were collected by centrifugation at 4°C, 8000 rpm for 10 min, washed twice with sterile distilled water and used as inoculum.

Composition and concentration of fermentation medium; xylose, peptone, yeast extract, including pH and inoculum volume were varied according to Plackett-Burman design and central composite design. All fermentation runs were performed in 250 erlenmeyer flask containing 50 ml medium at 30°C (200 rpm) for 24 h. Cultures were centrifuged at 4°C, 8000 rpm for 10 min and obtained supernatants were analyzed for residual xylose and xylitol produced [15].

Batch fermentation was also performed in 5L stirred-vessel bioreactor (model MDL-8C, B.E. Marubishi, Japan) at 2.5 L working volume. The bioreactor temperature was controlled at 30°C using water jacket. Agitation speed and aeration were 200 rpm and 1 vvm, respectively.

During the fermentations, samples were taken at 6–42 h intervals for determine cell growth , xylose and xylitol concentration.

2.3 Analytical Procedures

Concentration of xylose and xylitol were determined by HPLC (Varian, Prostar, USA) using Lichrospher®100 NH_2 (4-250 mm) column (Merck, Germany) and evaporative light scattering detector (Alltech, USA). Mobile phase was acetonitrile : water (91:9) at 1.5 ml/min flow rate. Cell growth was monitored by optical density (OD) at 600 nm.

2.4 Experimental Design

2.4.1 Plackett-Burman design

Plackett-Burman design was used to screen for critical parameters with respect to their main

effects, but not an interaction effect [16] on xylitol production by *C. tropicalis* A26. Five parameters including fermentation composition; xylose, yeast-extract and peptone; pH and inoculum volume were selected. Each parameter was examined at two levels: -1 for low level and +1 for high level (Table 1). Plackett-Burman factorial design of the 5 independent factors in 8 run is shown in Table 2. All experiments were performed in triplicates and average values of observations were used.

Statistical procedure was used to calculate limitation to which effects the key independent variables assigned. Significant level (*p*-value) of each main effect was determined using *F*-test.

2.4.2 Central composite design

Response surface methodology was used to optimize the variables enhanced xylitol production. In this study, central composite design (CCD) with two variables (xylose and peptone), five levels and three replicates at the center point was used for fitting a secondary-order response surface [17]. A CCD always contains twice as many star points as factors in the design. The star points represent low and high values for each variable in the design. To maintain rotatability, the value depends on the number of experimental runs in the factorial portion of the CCD.

In this study, where k (variables number) was 2 (xylose and peptone) "α" could be written as

$$\alpha = (2^k)^{1/4} = (2^2)^{1/4} = 1.414 \quad (1)$$

Table 4 and Table 5 show factors, their values, and the experimental design. The variables were xylose and peptone. The responses were xylitol production. A second-degree quadratic model was estabilished as Eq. (2) by using the method of least squares as follows:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_{12}X_1X_2 + a_{11}X_{12} + a_{22}X_{22} \quad (2)$$

Table 1. Variable selected for Plackett-Burman factorial design.

Variables	Parameter	Units	Low level (-1)	High level(+1)
X1	Xylose	g/l	20	80
X2	Yeast extract	g/l	5	20
X3	Peptone	g/l	10	40
X4	Inoculum volume	%	5	20
X5	pH	-	4	6

Table 2. Experimental design and results of the Plackett-Burman factorial design.

Run no.	X1	X2	X3	X4	X5	xylitol (g/l)
1	1	1	1	-1	1	20.01
2	-1	1	1	1	-1	6.96
3	-1	-1	1	1	1	8.25
4	1	-1	-1	1	1	30.12
5	-1	1	-1	-1	1	10.84
6	1	-1	1	-1	-1	22.82
7	1	1	-1	1	-1	25.74
8	-1	-1	-1	-1	-1	9.36

X1 xylose (g/l); **X2** yeast extract (g/l); **X3** peptone (g/l); **X4** inoculum volume (%); **X5** pH.

Where Y is the predicted response (xylitol production, g/l); X_1 and X_2 are the codes forms of the input variables, xylose and peptone, respectively; a_0 is constant; a_1 and a_2 are the linear coefficients; a_{12} is cross-product coefficient; a_{11} and a_{22} are the quadratic coefficients. The relation between the codes forms of the input variable and actual value of xylitol production is described by Eq. (3):

$$X_i = (x_i - X_0)/ \Delta X \quad (3)$$

Where X_i is dimensionless code value of the variable x_i, X_0 is the value of x_i at the center point, and ΔX is the step change.

The data from the experimental design were subjected to second-order multiple regression analysis using the least square regression method to obtain the parameter estimator of the mathematic model. SPSS Statistics 17.0 and Statistica 5.0 software (Statsoft, USA) were used for regression analysis and graphical analysis of the data, respectively.

3. Results and Discussion

3.1 Screening of Factors Affecting Xylitol Production

A total of 5 parameters; xylose, yeast-extract, peptone, pH and inoculum volume were screened for their effects on xylitol production by*C. tropicalis* A26 using Plackett-Burman design. Table 1 represented the independent variables and their respective high and low levels used in the optimization study. Plackett- Burman Design of 5 variables investigated at two levels of each variable for optimization of xylitol value production in 8 runs is shown in Table 2. Based on the obtained data (Table 3), the effect of each variable was calculated and significance of each variable was determined by *F*-test. The *F*-values of 2 variables; xylose and peptone were found to have higher significant values and represented a confidence level of ≥ 90%. It means that xylose and peptone significantly affected on xylitol production. An effect of yeast extract and inoculum volume or pH were insignificant and its confidence levels were less than 90%. Yeast extract, inoculum volume and pH at these levels studied did not improve xylitol production. Levels of them according to run No.4 were used in further optimization study. The run No. 4 was achieved the highest xylitol production with 30 g/l when xylose and peptone concentration were used at higher(+1) and lower (-1) level, respectively. From this

Table 3. Effect estimated for xylitol production from the results of Plackett-Burman design.

Variables	Parameter	Effect	*F*-value	*P*-value
X_1	Xylose	-15.825	143.257	0.007
X_2	Yeast extract	1.755	1.753	0.317
X_3	Peptone	4.500	11.617	0.076
X_4	Inoculum value	-2.005	2.313	0.268
X_5	pH	-1.080	0.678	0.498

Table 4. Experimental variables, parameters, range and level of the independent variables in the central composite design.

Variables	Parameter (g/l)	Range and levels				
		-1.414	**-1**	**0**	**1**	**1.414**
X_1	Xylose	51.72	60	80	100	108.2
X_2	Peptone	0.2	1	3	5	5.8

Table 5. Experimental design with real values and predicted values of xylitol production.

Run no.	X_1	X_2	Xylitol production	
			Experimental	Predicted
1	1	1	32.21	34.60
2	1	-1	27.05	28.21
3	-1	1	33.99	33.96
4	-1	-1	31.83	30.61
5	1.414	0	37.81	27.19
6	0	1.414	46.20	39.51
7	-1.414	0	30.05	28.35
8	0	-1.414	37.94	32.70
9	0	0	44.87	42.92
10	0	0	45.82	42.92
11	0	0	45.69	42.92

X_1 xylose (g/l); X_2 peptone (g/l)

result showed that initial xylose concentration was found to be the most important effect on xylitol production due to was the main substrate for xylitol production. Silva and Roberto [8] optimized xylitol production by *C. guilliermondii* FTI 20037 and reported that high initial xylose concentration increased final xylitol production. Maximum xylitol (52 g/l) was produced from rice straw hemicellulose hydrolysate by adjustment of initial xylose concentration to 80 g/l and of inoculum level to 3 g/l. *C. boidinii* and *D. hansenii* produced high xylitol yield (0.47 g/g and 0.77 g/g) when initial xylose concentration was increased to 150 g/l and 156 g/l, respectively [15,18]. High concentration of xylose (279 g/l) enhanced xylitol production of *D. hansenii* to 221 g/l [19]. Xylitol production of *D. nepalensis* was increased from 27 g/l to 36 g/l after using 100 g/l xylose, 10.6 g/l K_2HPO_4 and 8.9 mg/l $ZnSo_4$ [7]. A similar effect was found in *C. tropicalis* A26 that maximal level of xylose produce xylitol higher than minimum level of xylose (Table 2). Peptone and yeast extract were good sources of organic nitrogen. However, increasing of peptone and yeast extract concentrations resulted in decrease of xylitol production yield. Conversion of D-xylose to xylitiol by *C. tropicalis* DMS 7524 was blocked at high concentration of yeast extract (15 g/l) [18]. Increasing of yeast extract concentration from 5 g/l to 10 g/l, biomass of *C. guillermondii* FTI20037 increased but its xylitol production decreased [22]. Similarly, the addition of yeast extract and peptone to defined medium enhanced biomass production of *C. mogii* ATCC 1834 but had no significant effect on yield and specific productivity of xylitol [6]. Maximum xylitol yield of *C. tropicalis* OMV5 (0.9007 g/g) was obtained by using 1.32% peptone and 0.48% yeast extract [23]. Peptone was reported as only one organic nitrogen source that had significantly effect on xylitiol production of *C. tropicalis* [23]. Increasing of xylitol yield resulted in lower level of peptone because peptone was a pool of carbon, nitrogen and growth factors for microorganisms [23]. According to the result of Plackett-Burman design using 2 variables (xylose and peptone) on xylitol production (Table 2), concentration of xylose and peptone were further optimized by central composite design (CCD) .

3.2 Optimization of Xylitol Production by Central Composite Design

Variables used for CCD optimization were xylose (X_1) and peptone (X_2). Correspondence between the coded levels and real levels of the variables are shown in Table 4. Structure of experiment, results observed and results predicted according to second-order model obtained are shown in Table 5. Xylitol production was selected as the response of different cycle of the runs. Eleven experiments were performed in triplicates and central point was repeated three times (Run 9-11). Repetition at the center point allows determination of standard error. Experimental results of central composite design (CCD) and regression analysis were followed secondary-order polynomial equation. The xylitol production was an empirical function of test variables in coded unit as shown in the following equation:

$$Y= -83.832+2.961X_1+4.911X_2-0.019X_1X_2 -0.869X_1^2+0.019X_2^2 \quad (4)$$

Where Y is the predicted response (xylitol production); and X_1, X_2 are coded values of xylose and peptone. Statistical significance of Eq.(4) evaluated by Fisher's *F*-test analysis of variance (ANOVA) for response surface quadratic model is shown in Table 6. It was evident that the model was significant ($p<0.05$) at 95% of confidence level. The *p*-value of the model was used as tool to check significance of each coefficient, and indicated that the model was suitable to use in the experiment [17]. The model did not show lack of fit and presented a determination coefficient (R=0.852). The closer the value of R (correlation coefficient) to 1, the better is the correlation between experimental and predicted values [24]. According to R^2 value of 0.727, the model could explain an actual value at about 72.7%. Student t-distribution and corresponding *p*-value, along with parameters estimated, is given in Table 7. It could be concluded that X_1 had significant effect for linear regression on the response. The quadric effect of X_1^2 and an interaction effect of $X_1X_2$was significant at 95% confidence level. This indicated that X_1 (xylose), X_1^2 and interaction of X_1X_2 had a significant effect on xylitol production.

Normal P-P Plot of regression standardized residual (Figure 1) demonstrated actual xylitol production from experimental design versus the predicted production from an empirical model, Eq (4). The predicted data of response from the experimental model were in agreement with observed data in the range of the operating variables.

The contour plot and Response surface plot in Figure 2 present effect of xylose and peptone on xylitol production, while the other two variables were held at constant level. The statistical optimal value of variable was investigated by carefully considering of major and minor axis of response and contour plots at center point yield of xylitol production. Optimum values of xylose and peptone for xylitol production were 80 g/l and 4 g/l, respectively.

Table 6. Analysis of variance (ANOVA) for the regression model representing xylitol production.

Model	SS	df	MS	*F*-value	*P*-value
Model	749.364	5	149.873	8.507	.000[b]
Residual	281.897	16	17.619		
Total	1031.261	21			

R^2 =0.727; R=0.852; SS, sum of squares; df, degrees of freedom; MS, mean square; Significance level =95%

Table 7. Results of regression analysis of the central composite design.

Term	Coefficients	t-statistic	Significant
(Constant)	-83.137	-3.758	0.002*
X_1	2.961	5.751	0.000*
X_2	4.911	1.378	0.187
X_1X_2	-.019	-5.980	0.000*
X_1^2	-.869	-2.742	0.014*
X_2^2	.019	.504	0.621

*Significant at 5% level.

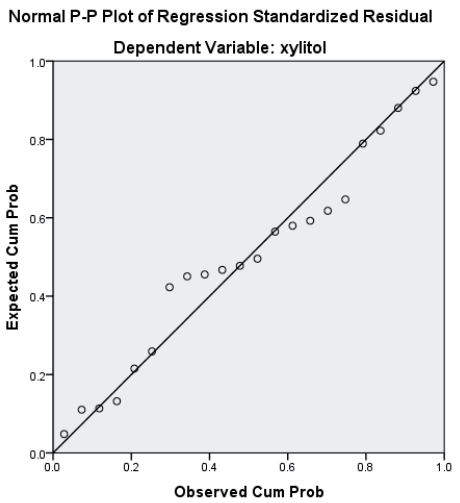

Figure 1. Observed xylitol production vs predicted xylitol production under optimum fermentation condition.

3.3 Statistical Verification

The statistical validation of the model was investigated. The maximum xylitol concecntration with 43.27 g/l was predicted by using regression equation obtained from statistical model. Verification of calculated optimum conditions for xylitol production was done by performing an experiment at optimized conditions. Under this condition, *C. tropicalis* A26 produced the highest xylitol concentration of 42.52 g/l with yield of 0.77 g/g at 24 h (Table 8) which agreed well with the predicted value from statistical model (98.27% of prediction value). This statistical design could improve xylitol production yield from 0.53 g/g xylose (un-optimization) to 0.77 g/g xylose (optimization) by using CCD in flask scale (Table 8). Moreover, xylitol

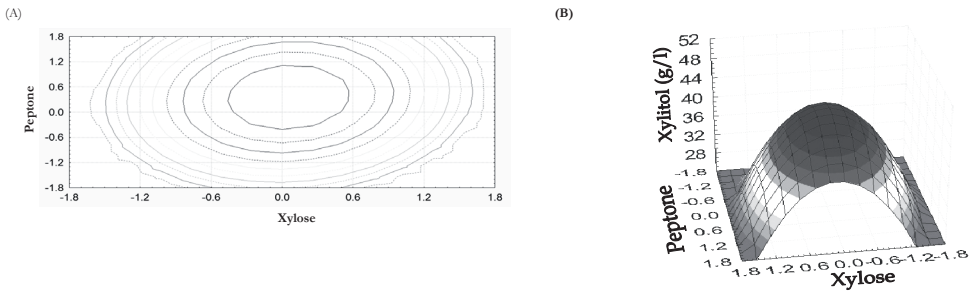

Figure 2. Contour plot (A) and response surface (B) described by the model, representing xylitol production as function of xylose and peptone.

Table 8. Summary of xylitol production improvement of *C. tropicalis* A26.

Step	Method	Condition	Xylitol production (g/l)	Xylose consumed (g/l)	Xylitol yield (g/g xylose)
1.	Un-optimized medium/ condition	40 g/l xylose, 20 g/l peptone, 10 g/l yeast extract	21.30	40	0.53
2.	Optimization of medium composition using CCD in skake flasks	80 g/l xylose, 4 g/l peptone, 5 g/l yeast extract	42.52	55	0.77
3.	Validation of the model in 5L stirred-vessel fermenter	80 g/l xylose, 4 g/l peptone, 5 g/l yeast extract, aeration rate of 1 vvm, initial pH 5.5 (uncontrol), temperature at 30 °C	71.59	80	0.89

production was validated in 5 L fermenter by batch fermentation using optimized medium composition. Figure 3 demonstrated that 80 g/l of initial xylose was completely consumed by strain A26 with the highest xylitol concentration of 71.59 g/l after cultivation 42 h, 32 °C, agitation speed of 200 rpm and aeration at 1 vvm. The xylitol production by *C. tropicalis* A26 showed growth associated metabolism which the concentration of xylitol was increased with cell growth. Table 8 concluded the process of xylitol production, maximal yield of xylitol was 0.89 g/g xylose when the fermentation was performed using 5 L stirred bioreactor under the optimized medium composition.

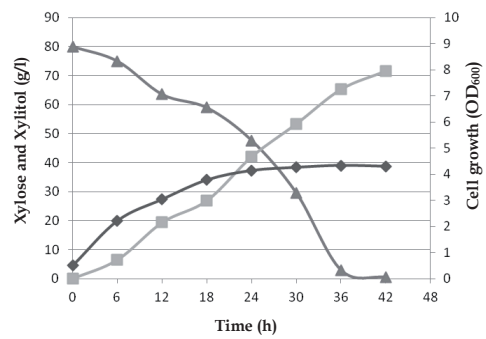

Figure 3.Time course of xylitol batch fermentation by *C. tropicalis* A26 at optimum condition and initial pH 5.5. ▲, Xylose; ■, Xylitol; , ■, Cell growth (OD_{600}).

4. Conclusion

The xylitol production of *C. tropicalis* A26 was optimized by using Plackett-Burman design and central composite design (CCD) model. The critical parameters screened by the Plackett-Burman design experiments were xylose and peptone. Interactions between these two parameters were further studied by the CCD. Optimal conditions for xylitol production by *C. tropicalis* A26 were; xylose (80 g/l), peptone (4 g/l) and yeast extract (5 g/l), inoculum (20% v/v) and initial pH 5.5. The xylitol production and yield at the optimized condition were 2 and 1.45 folds higher than un-optimized condition, respectively. Batch fermentation at the optimized condition, highest xylitol yield

was 0.89 g/g xylose which was 1.67 folds higher than at un-optimized condition. The results indicated that the statistical design was useful information for optimization of fermentation products including xylitol of *C. tropicalis* A26.

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