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Optimization of medium components for D-ribose production by transketolase-deficient *Bacillus subtilis* NJT-1507

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Abstract−Statistical experimental designs were used to optimize the composition of culture media for the production of D-ribose by *Bacillus subtilis*. A fractional factorial design $2^{(5-2)}$ was used to determine medium components that significantly affected D-ribose production. The concentrations of glucose and $(NH_4)_2SO_4$ were the significant factors. Central composite design and response surface methodology were then used to estimate the quadratic response surface and determine the factor levels for maximum production of D-ribose. Finally, the optimal medium composition was obtained (g/L): glucose, 172.75; $(NH_4)_2SO_4$, 13.2; yeast powder, 4; corn steep liquor, 8 and $MnSO_4$, 0.5. This optimization strategy increased D-ribose production from 73.21 g/L to 88.57 g/L, an increase of 22% compared with the original conditions. The D-ribose production yield to glucose concentration was also enhanced from 0.37 g/g to 0.52 g/g. Confirmatory experiments were also performed to demonstrate the accuracy of the model. Under the optimal medium using ammonia to control pH in a 5 L fermenter, the D-ribose yield was increased to 95.28 g/L after 3 days of cultivation at 37 °C.

Key words: *Bacillus subtilis*, D-ribose, Fermentation, Medium Optimization, Response Surface Methodology

INTRODUCTION

D-ribose is an important aldopentose present as the ribosyl residue of biomolecules such as ATP, RNA, NAD, NADP, FAD and coenzyme A [1]. D-ribose has been used for many decades to produce flavor enhancers (5′-nucleotides), riboflavin (vitamin B_2) [2], pharmaceuticals [3], cosmetics, food, animal feed additives [4], and antiviral and anticancer products [5,6]. Clinically, it has also been used to improve cardiac function and as a cure for cardiac ischemia and rare genetic diseases [7,8]. Recently, there has been increased interest in its role in multiple pharmacological actions [9], such as benefiting sleep duration and patterns and improving mental clarity in patients with fibromyalgia or chronic fatigue syndrome [10].

D-ribose was shown to be synthesized by enzymatic hydrolysis from yeast RNA or by chemical synthesis from arabinose, glucose, xylose and gluconic acid in the early part of the twentieth century [11]. However, these methods were associated with numerous problems such as low product recovery efficiency, insufficient amount and high costs, which led to development of the fermentation method for large-scale D-ribose production. Early in 1966, *Pseudomonas reptilivora* and *Candida pelliculose*, which have the ability to produce D-ribose from glucose, were isolated from soil [4]. Since then, several *Bacillus subtilis* and *Bacillus pumilus* strains accumulating D-ribose have been characterized as partial impairment of the transketolase (TKT EC 2.2.1.1) activity [12]. Recently, many researchers have focused on genetic engineering to develop a transketolase-negative system of *B. subtilis* strains for high-level D-ribose production [1]. These mutants convert D-glucose or other sugars to D-ribose-5-P through an oxidative pentose phosphate pathway, which is subsequently dephosphorylated to D-ribose in the cytoplasm.

To meet the increasing demands of commercial exploitation [4], methods capable of mass and cost-effective production of D-ribose from microorganisms should be developed and improved, and it is important to identify an effective medium formulation. The one-factor-at-a-time method cannot obtain sufficient information and does not consider the interactions among these factors. Response surface methodology (RSM) is an effective and quick experimental design tool that is widely used in many fields [13,14]. RSM is a combination of statistical techniques for designing experiments, building models, evaluating the effects of the factors, and searching for optimal conditions of factors for desirable responses [15,16]. However, utilizing these mathematical tools for optimization of D-ribose production has not yet been reported.

The objective of this study was to use a combination of statistical strategies involving fractional factorial design, central composite design and RSM to obtain the optimal medium for maximizing yield of D-ribose by a transketolase-deficient *B. subtilis* NJT-1507.

MATERIALS AND METHODS

1. Strain and Medium

Transketolase-deficient *B. subtilis* NJT-1507 cells were maintained at −50 °C in glycerol-based (25%, v/v) inoculation medium. The strain maintained on the usual agar slant was inoculated in 5 mL of the inoculation medium in a test tube. The inoculation medium was composed of 5 g/L D-sorbitol, 10 g/L peptone, 2 g/L yeast extract, 2 g/L NaCl, and 20 g/L agar, at an initial pH was 7.0. The cells were cultured for two days at 30 °C, then stored at 4 °C and subcultured every two months.

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2. Inoculum Preparation and Shake Flask Culture

Culture was as follows unless otherwise stated. The strain was transferred from a slant culture into an Erlenmeyer flask (500 mL) containing 30 mL seed medium, which was the same composition as the above slant medium without agar, then was incubated at 37 ºC on a reciprocal shaker for 18 h. Two milliliters of the inoculum were transferred into an Erlenmeyer flask containing 20 mL fermentation medium. The fermentation medium contained glucose, corn steep liquor, yeast powder, $(NH_4)_2SO_4$ and $MnSO_4$, whose concentrations were varied based on the experimental designs. The pH of the medium was adjusted to 7.0 with 1 M NaOH before autoclaving (121 ºC, 10 min). Flasks were incubated at 37 ºC on a rotary shaker for 3 days.

3. Analytical Methods

The optical density was measured at 600 nm by using a UV-visible spectrophotometer (DU-640, Beckman, USA). Glucose and the metabolites of D-ribose were measured by high-performance liquid chromatography (Summit P 680 HPLC, Dionex, USA; Shodex RI-101 Refractive Index Detector, Showa Denko, Japan; Aminex HPX-87P Ion Exclusion Column 300 mm×7.8 mm, Bio-Rad, USA) under the following conditions: sample volume 10 μl; mobile phase 0.005 M H_2SO_4; flow rate 0.6 ml/min and column temperature 85 ºC.

4. Experimental Design and Data Analysis

4-1. Fractional Factorial Design (FFD)

It is well known that the FFD method can be used to estimate the main effects of factors [17]. It is particularly useful in the initial stages of medium optimization. In the present study, a $2^{(52)}$ fractional factorial design with five parameters at two different levels was used to determine the key factors that significantly affect D-ribose production. The experimental design is shown in Table 1. The linear model obtained was expressed as follows:

$$Y = \beta_0 + \sum_{i=1}^{5} \beta_i X_i \quad (1)$$

where Y is the predicted response, β_0 is the intercept, and β_i is the linear coefficient.

The most important factors influencing D-ribose production by *B. subtilis* were chosen to evaluate the maximum D-ribose by the *P* values.

4-2. Steepest Ascent Path

If the mean of the center points is less than the mean of the factorial points, the optimum will be outside the experimental design space and the method of steepest ascent should be applied [18]. This direction was parallel to the normal to the fitted response surface and passed through the center point of FFD. Increment was correlated with regression coefficients β_i.

Table 1. Factors and coded values of FFD

Factor	Levels		
	−1	0	+1
Glucose (X_1, g/L)	150	200	250
Corn steep liquor (X_2, g/L)	6	8	10
Yeast powder (X_3, g/L)	3	4	5
$(NH_4)_2SO_4$ (X_4, g/L)	5	7	9
$MnSO_4$ (X_5, g/L)	0.3	0.5	0.7

4-3. Central Composite Design and Response Surface Methodology

Central composite design and RSM were used to investigate the influence of the two most important factors affecting D-ribose production. In the regression equation, the test variables were coded according to the equation:

$$x_i = \frac{X_i - X_0}{\Delta X}, \quad i = 1, 2 \ldots k \quad (2)$$

where x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_0 is the value of X_i at the center point and ΔX is the step change.

This methodology allowed the formulation of a second-order equation that described the process. The concentration of D-ribose was analyzed by multiple regression analysis using the least squares method to fit the equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_{ii}^2 + \sum \beta_{ij} x_i x_j, \quad i = 1, 2 \ldots\ldots, k \quad (3)$$

where Y is the predicted response variable, β_0 is the intercept, β_i is the first-order coefficient, β_{ij} is the interaction coefficient and X_i and X_j are the coded forms of the input variables.

4-4. Statistical Analysis

The medium composition was selected according to the experimental designs. The combinations of substrate and concentrations were selected by using the design module program of the Statistica 6.0 package (SoftStat Inc., 1984-2001, USA).

4-5. Experimental Validation of the Optimal Medium Composition

To validate the optimization of the culture medium, three tests were performed, using the optimal conditions, to confirm the response surface analysis results.

RESULTS AND DISCUSSION

1. Fractional Factorial Design

Although no information was obtained on how medium components affected the mechanisms of D-ribose production, the FFD proved to be a valuable tool for screening important medium components. Table 2 shows the considerable variation in the yields of D-ribose with different medium compositions. According to the analysis by Statistica 6.0, a linear regression equation could be obtained from the regression results of the fractional factorial experiment:

$$Y = 75.61 - 2.79X_1 - 1.14X_2 + 0.38X_3 + 6.59X_4 + 1.92X_5 \quad (4)$$

Table 2. Experimental design and results of the $2^{(5-2)}$ design

Run	X_1	X_2	X_3	X_4	X_5	D-ribose (g/L)	
						Observed	Predicted
1	−1	−1	−1	1	1	86.30	87.68
2	1	−1	−1	−1	−1	66.01	65.05
3	−1	1	−1	−1	1	71.37	72.21
4	1	1	−1	1	−1	80.70	81.55
5	−1	−1	1	1	−1	85.85	84.61
6	1	−1	1	−1	1	74.15	69.68
7	−1	1	1	−1	−1	73.75	69.14
8	1	1	1	1	1	78.86	80.58
9	0	0	0	0	0	73.21	75.61

Table 3. Regression results of the FFD

Term	Coefficient	*T*-value	*P*-value
Intercept	75.61444	131.9699	0.000001
X_1	−2.79750	−4.6032	0.019276*
X_2	−1.14250	−1.8800	0.156700
X_3	0.38750	0.6376	0.569050
X_4	6.59500	10.8520	0.001674*
X_5	1.92500	3.1676	0.050576

R^2=0.98076

*Statistically significant at 95% of confidence level

Statistical analysis of the data (*t*-test) (Table 3) showed that, in the concentration range tested, only glucose and $(NH_4)_2SO_4$ had a significant effect on D-ribose production (P<0.05), and the coefficient of determination (R^2) was 98.08%, which indicated that the accuracy and general ability of the polynomial model was good. Using the model analysis of the response trends was therefore considered to be reasonable.

The negative effect of glucose might be caused by the "glucose effect," by which the activities of several catabolic enzymes, such as glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase can be influenced, and PFK activity can be adjusted rapidly in response to changes in glucose transport. This appeared to be a key step in the energy downshift and catabolite repression mechanism [19]. $(NH_4)_2SO_4$ has been shown to excellently shift D-glucose towards the pentose phosphate pathway, leading to a high D-ribose yield, and low concentrations of biomass and glycolytic end-products [4,20]. The pH of the medium should preferably be controlled, although pH-free processes, performed in a sufficiently buffered medium, may be appropriate [21].

However, these approaches could not be used to predict the optimal levels of the medium components that significantly affected D-ribose production. This information was obtained via the following optimization steps.

2. Steepest Ascent Path

The path of the steepest ascent was determined by Eq. (4) and the proper direction of changing variables was determined. Glucose and $(NH_4)_2SO_4$ were significant factors and the coefficient of X_1 was negative, whereas the coefficient of X_4 was positive, meaning that increasing the concentration of X_4 while decreasing the concentration of X_1 had a positive effect on the level of D-ribose production. Experimental design of the steepest ascent determination and corresponding results are shown in Table 4. Run 1 shows a maximum of the D-ribose, which suggests that this point was near the maximum D-ribose production response level and this point was selected for further optimization.

Table 4. Experimental design and results of the steepest ascent path

Run	Glucose (g/L)	$(NH_4)_2SO_4$ (g/L)	D-ribose (g/L)
Origin	200	7.00	74.7
1	150	11.12	85.77
2	100	15.24	81.81
3	50	19.36	46.99
4	0	23.48	9.65
5	0	27.6	6.40
6	0	31.72	5.12

Table 5. Levels of factors tested in the central composite design

Factor	Levels				
	−1.41	−1	0	+1	+1.41
Glucose (A, g/L)	135.25	125	150	175	185.25
$(NH_4)_2SO_4$ (B, g/L)	3.95	6	11	16	18.05

Table 6. Experimental design and results of the central composite design

Run	A (glucose)	B $(NH_4)_2SO_4$)	D-ribose (g/L)	
			Observed	Predicted
1	−1	−1	82.24	70.90
2	−1	1	86.21	84.92
3	1	−1	45.49	39.46
4	1	1	60.79	56.81
5	−1.41	0	77.04	81.44
6	1.41	0	45.34	39.46
7	0	−1.41	48.39	54.72
8	0	1.41	77.71	76.84
9	0	0	77.67	77.90
10	0	0	78.00	77.90

3. Response Surface Methodology

The optimal concentrations of medium components were determined by using a central composite design (CCD) with two variables: glucose and $(NH_4)_2SO_4$. The ranges of the coded levels for the factors are shown in Table 5. The levels of the two variable factors and experimental results are presented in Table 6.

The coefficients of the regression equation were calculated by using Statistica 6.0, and the following regression equation was obtained:

$$Y=77.83-13.37A+7.59B-6.68A^2-5.75B^2+2.83A*B \quad (5)$$

where Y is the predicted response, and A and B are coded values of glucose and $(NH_4)_2SO_4$ concentrations, respectively.

The analysis of variance (ANOVA) of the regression model given in Table 7 gives a satisfactory value for the coefficient of determination R^2 which was calculated as 0.9215, indicating that the statis-

Table 7. Analysis of variance (ANOVA) for the second-order polynomial model

Source	SS	DF	MS	F-value	Prob>F
A (L)	1431.140	1	1431.140	30.88596	0.005132
A(Q)	204.103	1	204.103	4.40481	0.103798
B(L)	461.089	1	461.089	9.95092	0.034365
B (Q)	151.241	1	151.241	3.26400	0.145111
A(L)by B(L)	32.092	1	32.092	0.69259	0.452083
Error	185.345	4	46.336	30.88596	
Total SS	2360.483	9	1431.140		

R^2=0. 9215; SS, sum of squares; DF, degrees of freedom; MS, mean square

Table 8. Regression results of the central composite design

Factor	Coefficient	*P*-value
Intercept	77.8350	0.000086
A	−13.3751	0.005132[a]
B	7.5918	0.103798
A^2	−6.6819	0.034365[b]
B^2	−5.7519	0.145111
AB	2.8325	0.452083

[a]Statistically significant at 99% of confidence level
[b]Statistically significant at 95% of confidence level

tical model could explain 92.15% of variability in the response The R^2 value was always between 0 and 1 and the closer the R^2 value was to 1.0, the stronger the model and the better it predicted the response [22].

The regression coefficients and corresponding *P*-values for the model are presented in Table 8. The *P*-value was used as a tool to determine the significance of each coefficient, which was necessary to understand the pattern of the mutual interactions among the most important factors [23]. A small *P*-value indicates that the corresponding coefficient had a significant effect [24].

The 3D response surface plots described by the regression model were drawn to illustrate the effects of the independent variables and interactive effects of each independent variable on the response variables. .ncreasing predicted the respons The response surface based on the independent variables glucose and $(NH_4)_2SO_4$ is shown in Fig. 1. It is clear that D-ribose production was sensitive to even small changes in the concentrations of glucose and $(NH_4)_2SO_4$. The model predicted that the optimal values of test factors in the coded units were X_1=0.90, X_2=0.43. At these values, the concentrations of glucose and $(NH_4)_2SO_4$ were 172.75 g/L and 13.2 g/L, respectively. The maximum predicted value of D-ribose was 86.30 g/L.

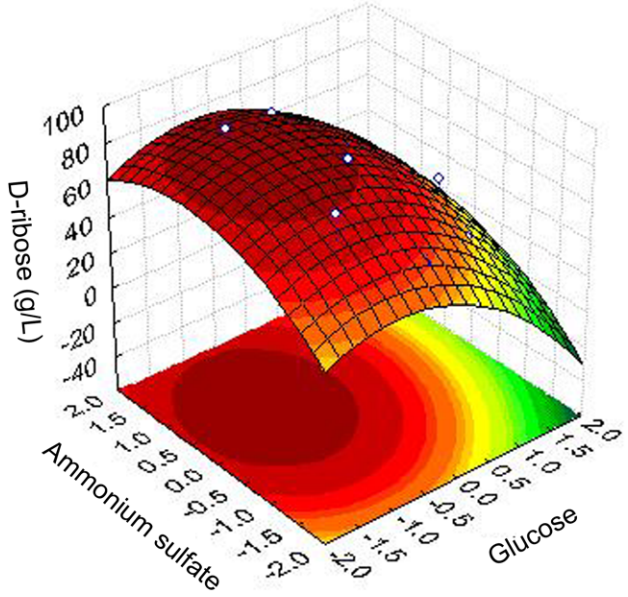

Fig. 1. Surface plot of the combined effects of glucose and $(NH_4)_2SO_4$ on D-ribose production in *B. subtilis* NJT-1507.

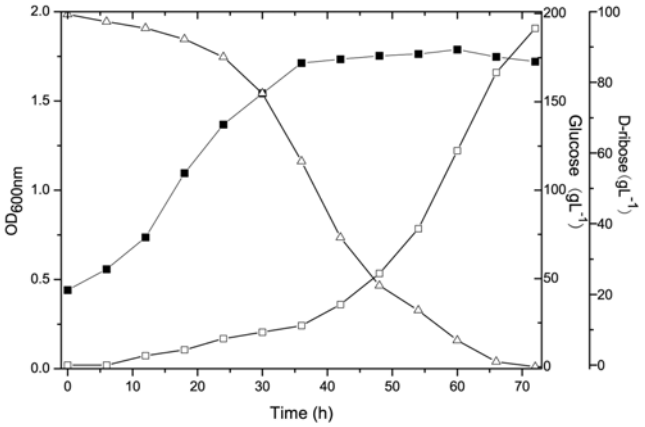

Fig. 2. Time profile of D-ribose batch fermentation in a 5-L fermenter using the optimized medium conditions and using ammonia to control pH. Glucose (unfilled triangle), dry cell weight (filled square), D-ribose (unfilled square).

4. Experimental Validation of the Optimized Condition

The model was validated by performing three experiments in shake flasks under the predicted conditions. The predicted response for D-ribose production was 86.30 g/L, whereas the actual (experimental) response was 88.57 g/L. The experimental values were close to the predicted values, and hence the model was successfully validated.

5. 5-L Fermenter Experiment with Ammonia Controlling the pH

The feasibility of the regression models to predict the outcomes in a 5-L fermenter using ammonia to control the pH was tested under the optimal medium condition. Fig. 2 shows the batch profile of cell growth and D-ribose production. The D-ribose concentration was 95.27 g/L. The ammonia provided nitrogen for growth and also prevented the introduction of other ions when control the pH, which usually restrained the growth of the strains.

CONCLUSIONS

A statistical optimization method for fermentation was shown to overcome the limitations of classic empirical methods and to be adequate for the design and optimization of a *B. subtilis* NJT-1507 bioprocess. Using the method of experimental FFD and response surface analysis, it was possible to determine the optimal cultivation conditions for high D-ribose production. The final optimized medium composition was (g/L): glucose, 172.75; $(NH_4)_2SO_4$, 13.2; yeast powder, 4; corn steep liquor, 8, and $MnSO_4$, 0.5. This optimization strategy increased D-ribose production from 73.21 to 88.57 g/L, an increase of 22% compared with the original conditions. The D-ribose production yield to glucose concentration was also enhanced from 0.37 g/g to 0.52 g/g. Thus, the relatively high D-ribose yield demonstrates the potential of using this strain to optimize bioprocesses for the commercial production of D-ribose.

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