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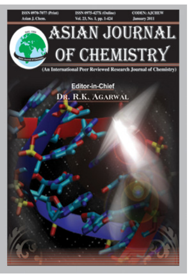

Medium Optimization of the Production of Fructooligosaccharide by *Aspergillus japonicus* zz-1 Using Response Surface Methodology

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This study leads to find the optimum compositions of medium for fructooligosaccharide production by *Aspergillus japonicus* zz-1 using the Plackett-Burman design and response surface methodology. The yield of fructooligosaccharide production determined by HPLC-ELSD was chosen as the response variable, while sucrose, yeast extract and $NaNO_3$ were chosen as independent variables. The optimum medium condition was as follows: sucrose 140 g kg^{-1}, yeast extract 50 g kg^{-1} and $NaNO_3$ 3 g kg^{-1}. Under that condition, the yield of fructooligosaccharide reached 633 g kg^{-1}. The Plackett-Burman design and response surface methodology were found to be useful and possessed advantage in optimizing the culture medium of fructooligosaccharide production comparing with traditional methods. The optimum medium condition was accurate and reliable.

Key Words: Fructooligosaccharide, Medium, Plackett-Burman design, Response surface methodology.

INTRODUCTION

Fructooligosaccharide (FOS) is a common name only for fructoseoligomers that are mainly composed of 1-kestose (GF_2), nystose (GF_3) and 1^F-fructofuranosyl nystose (GF_4)[1]. Fructooligosaccharide has become significant in both research and economy due to its active effects of physiological function in promoting the growth of bifidobacteria in the intestinal tract, relieving constipation, enhancing mineral absorption decreasing total cholesterol and lipid in serum. Besides, FOS is a good sweetener with non-cariogenic and non-calorific value. Due to these characters, FOS is widely used in various foodstuffs, such as beverages, dairy products, confectionery, jams, as a functional sweetener[2-6].

Fructooligosaccharide is now produced commercially through the enzymatic synthesis from sucrose using β-fructofuranosidase of microbial origin. Fructooligosaccharide can be produced by transferring 1-3 units of fructose bound to β-1,2 position of sucrose through the action of β-fructofuranosidase. Generally, it is reported that there are some producing strains of FOS, such as *Aspergillus niger*, *Aspergillus oryza*, *Aureobasidium pullulans*, *Arthrobacter sp* and so on[7-9].

At present, the yield of FOS produced by microbial fermentation maintained 500-600 g kg^{-1} [10]. The components of medium are the most important influence factor to the FOS production, because many factors could affect microbial growth and enzyme production during the process of fungus fermentation. Carbon source and nitrogen source which have a significant influence on fermentation production are the most important components of medium. Sucrose and peptone were commonly used by researchers for producing β-FFases and biomass[11-13]. But yeast powder was also a good nitrogen source. According to literature, Chen found that sucrose and yeast extract were the key nutritional factors in a vecting enzyme production and the improved media composition of sucrose and yeast extract increased enzyme production significantly by *Aspergillus japonicus* TIT-90076[14].

Optimizing the condition of fermentation medium of the strain is one of the important ways to increase the production of FOS and reduce the production cost. So far, the conventional method such as the single-factor experiment and orthogonal test design method have been used for optimizing the fermentation condition. As a result, a large number of experiments are needed to find out the effect of individual factors and none of them has an effective and reasonable way to find out the best combination of experimental factors and the optimal value. This is not only laborious and time-consuming, but also suffers from major drawbacks of giving unreliable results and less accurate conclusions since it is unable to determine interactive effects among the factors[15-17]. On the other hand, using response surface methodology (RSM) to optimize the fermentation condition has much more advantages, compared with the conventional method. Response surface methodology is a useful group of mathematical and statistical technique, used for analyzing the influence of several independent factors on one or more characteristics of the processes or production. It requires less numbers of experiments and has a high fitting precision of regression equation. This method has been widely and successfully applied for optimizing microbiological media composition, enzymatic synthesis conditions and food processing[18,19]. To the best of our knowledge, there were rarely reports about the optimization of culture medium for FOS production by *Aspergillus japonicus* using Plackett-Burman design and RSM.

The aim of this work is to demonstrate the availability of using the Plackett-Burman design and RSM to optimize the culture medium composition of *Aspergillus japonicus* zz-1, in order to increase the yield of FOS production.

EXPERIMENTAL

Microorganisms and medium: *Aspergillus japonicus* zz-1 isolated from Saccharum officinarum planting soil was grown on potato dextrose agar slants at 30 ºC for 3 days and then stored at 4 ºC, pH 7. The medium composed of sucrose, yeast powder, $NaNO_3$, K_2HPO_4 1 g kg^{-1} and $MgSO_4{\cdot}7H_2O$ 2 g kg^{-1}. The pH was 7.

Fructooligosaccharide production: The inoculum was prepared by transferring 1.0 mL spore suspension in 250 mL shake flasks containing 100 mL of medium. The shake flasks were agitated in an orbital shaker, at 120 rev min^{-1} for 48 h at 28 ºC. At the end of 48 h of culture fermentation, broth was collected and the cells were disrupted, followed by centrifugation (4 ºC, 8000 rev min^{-1}) for 10 min. Then the supernatant liquid was collected which was raw enzyme. The reaction mixture contained 10 mL of raw enzyme and 100 mL of 500 g kg^{-1} sucrose solution in citrate buffer (0.1 M, pH 5.4) and reacted in water bath at 50 ºC. After 1 h, the reaction was stopped by treatment in boiling water for 10 min.

Analytical method: The reaction products of FOS were analyzed by HPLC-ELSD, with an NH_2 column (4.6 mm × 250 mm) (waters). The mobile phase were acetonitrile and water (75:25, v v^{-1}). The flow rate was 1.0 mL min^{-1}. The ELSD drift tube temperature was 85 ºC, the carrier air pressure was 0.5 MPa, the flow rate was 2.0 L min^{-1}. The amount of FOS produced were calculated by peak area normalization method. In this study, we take the yield of FOS as evaluation standard for convenience. The formula was as follows:

FOS yield (g kg^{-1}) = FOS produced (g)/initial sucrose (kg)

Design of Plackett-Burman: The experiment of Plackett-Burman is a method to confirm the change of step length, according to the influence level of various factors on preparation of the target product, in order to approach the neighbourhood of the optimum response of the FOS production rapidly and directly[20-22]. The direction is determined based on the gradient variation direction of response value. In this work, the concentrations (g kg^{-1}) of sucrose (Z_1), yeast powder (Z_2), $NaNO_3$ (Z_3) and $MgSO_4{\cdot}7H_2O$ (Z_4) were taken as independent functions and the yield of FOS (Y) was regarded as the target function. On the basis of Plackett-Burman design software, the design level of independent functions was translated into linearity encodes, so the regression correlation between the target function (Y) and the independent functions was transformed into the relationship between Y and the coded values which were the independent functions coded on space coordinate axis-X. The experiment scheme was studied out according to the coded values. The formula was as follows:

$$Z_0 = \frac{(Z_{1j} + Z_{2j})}{2} \quad (1)$$

$$\Delta j = \frac{(Z_{2j} - Z_{1j})}{2} \quad (2)$$

$$A = \Delta j \times e \times b_j \quad (3)$$

$$Zk_j = Z_{0j} + K \times A \quad (4)$$

*"Z_{2j}", "Z_{1j}", "Z_{0j}" and "Δj" denoted respectively the high level, low level, zero level and transformation interval of Z_j; "Zk_j" denoted the value of the factors in further experiment k; the "e" denoted coefficient; the "A" denoted step length; the "b_j" denoted regression coefficient.

According to the result of our previous work, it was found that the concentrations (g kg^{-1}) of sucrose (Z_1), yeast powder (Z_2), $NaNO_3$ (Z_3) and $MgSO_4{\cdot}7H_2O$ (Z_4) were the main influence factors of FOS production. The zero level and a correspondence stenotic transformation interval were chosen, the level of empirical factor and experimental arrangement were shown in Tables 1 and 2, based on the formula (1) and (2).

TABLE-1
LEVEL OF EMPIRICAL FACTOR OF PLACKETT-BURMAN DESIGN

Empirical factor	Encoding level			
	Low level (-1)	Zero level (0)	High level (+1)	Transformation interval (Δj)
Z_1	150	200	250	50
Z_2	30	40	50	10
Z_3	1	2	3	1
Z_4	1	2	3	1

TABLE-2
ARRANGEMENT OF STEEPEST ASCENT EXPERIMENT

Serial number	Empirical factor			
	Z_1	Z_2	Z_3	Z_4
1	-1	-1	-1	-1
2	1	-1	-1	1
3	-1	1	-1	1
4	1	1	-1	-1
5	-1	-1	1	1
6	1	-1	1	-1
7	-1	1	1	-1
8	1	1	1	1

Design of response surface methodology: On the basis of the result of Plackett-Burman design experiment, a central composite Box-Behnken was adopted to evaluate the combined effects of sucrose, yeast powder and $NaNO_3$. The experiment of three factors and three levels was designed according to the model provided by SAS V8.1 software. There were 15 testing points to the experiment of RSM. They were divided into two groups. Group 1 was composed of 12 testing points called factorial points. They were the 3D vertex of the model composed of the data of each independent variable. There were 3 testing points in group 2 called zero points. They were central points of data area. Three replications were carried out in order to estimate the experimental error[23,24]. The concentrations (g kg^{-1}) of sucrose, yeast powder and $NaNO_3$ were taken as independent variable X_1, X_2, X_3. The yield of FOS was regarded as the value of response Y. The data of three factors and three levels and the experiment arrangement for RSM were shown in Tables 3 and 4.

TABLE-3
INDEPENDENT VARIABLES AND THEIR CODED VALUES OF RSM

Independent variable	Symbol	Coded levels		
		-1	0	1
Sucrose	X_1	140	150	160
Yeast powder	X_2	40	50	60
$NaNO_3$	X_3	2.5	3.5	4.5

TABLE-4
ARRANGEMENT OF RSM

Serial number	Empirical factor		
	X_1	X_2	X_3
1	-1	-1	0
2	-1	1	0
3	1	-1	0
4	1	1	0
5	0	-1	-1
6	0	-1	1
7	0	1	-1
8	0	1	1
9	-1	0	-1
10	1	0	-1
11	-1	0	1
12	1	0	1
13	0	0	0
14	0	0	0
15	0	0	0

RESULTS AND DISCUSSION

Plackett-Burman design: By using the Plackett-Burman design software, the main effect of the factors can be estimated as precisely as possible, by much less times of experiments. It can screen the more important ones from the investigated factors quickly and effectively for the further research of optimization. The experiments were carried out according to Table-2. Based on the result of the experiment, the regression equation and coefficient of regression (bj) was obtained. The step length (A) of each factor was worked out through the formula (3), as follows in Table-5.

The magnitude of absolute value of each coefficient of regression (b_j) obtained from the regression equation reflxed the influence of each factor (Zj) in the experiment. It was obtained that the influence of sucrose (Z_1), yeast powder (Z_2) and $NaNO_3$ (Z_3) was more outstanding on the FOS production. So they were selected for the further experiment of optimizing. The signs of each coefficient of regression (b_j) expressed the characters of the influence, videlicet the direction of the up rise. Plus sign expressed that the changing trend of the factor was going to a higher level, while minus sign expressed that the changing trend of the factor was going to a lower level. The change of each factor was worked out according to the

TABLE-5
RESULT OF PLACKETT-BURMAN EXPERIMENT

Serial number	Empirical factor				Yield of FOS
	Z_1	Z_2	Z_3	Z_4	Y
1	-1	-1	-1	-1	470.6
2	1	-1	-1	1	501.8
3	-1	1	-1	1	483.0
4	1	1	-1	-1	513.3
5	-1	-1	1	1	491.5
6	1	-1	1	-1	521.6
7	-1	1	1	-1	541.2
8	1	1	1	1	518.0
b_j	-17.7	15	34.2	-7.8	
Primitive A	-8.85	1.5	0.342	-0.078	
A	-10	2	0.3	-0.08	

formula (4) and further seven experiments were carried out. Because the step length of $MgSO_4{\cdot}7H_2O$ (Z_4) was very tiny, it could be neglected. 2 g kg^{-1} was chosen for the concentration of $MgSO_4{\cdot}7H_2O$ (Z_4). The result of further experiment was given in Table-6.

TABLE-6
RESULT OF FURTHER EXPERIMENT

Serial number	Empirical factor				Yield of FOS
	Z_1	Z_2	Z_3	Z_4	Y
1	190	42	2.3	2	502.1
2	180	44	2.6	2	493.6
3	170	46	2.9	2	531.7
4	160	48	3.2	2	552.1
5	150	50	3.5	2	578.6
6	140	52	3.8	2	542.2
7	130	54	4.1	2	500.8

According to Table-6, it was found that the yield of FOS was much higher on condition 5. So the center point of RSM was selected around condition 5.

Response surface methodology: The experiments were carried out based on Table-4 and the result was shown in Table-7.

TABLE-7
RESULT OF RSM

Serial number	Empirical factor			Yield of FOS
	X_1	X_2	X_3	Y
1	-1	-1	0	560.6
2	-1	1	0	566.1
3	1	-1	0	443.9
4	1	1	0	501.5
5	0	-1	-1	491.7
6	0	-1	1	500.8
7	0	1	-1	475.3
8	0	1	1	489.6
9	-1	0	-1	615.6
10	1	0	-1	457.2
11	-1	0	1	505.4
12	1	0	1	472.2
13	0	0	0	628.9
14	0	0	0	626.8
15	0	0	0	630.7

According to the experimental result in Table-7, taking the yield of FOS production as response value, the experimental data were analyzed by commonly used statistical analysis software SAS v8.1. The analytic results were shown in Table 8 and 9.

TABLE-8
ESTIMATE VALUE OF REGRESS EQUATION
PARTIAL REGRESSION COEFFICIENT

Parameter	DF	Estimate	Pr > |t|
X_1	1	-46.61250	0.002581**
X_2	1	4.437500	0.619001
X_3	1	-8.975000	0.333
$X_1 \times X_1$	1	-43.76250	0.016408*
$X_1 \times X_2$	1	13.02500	0.321707
$X_1 \times X_3$	1	31.30000	0.045875*
$X_2 \times X_2$	1	-67.01250	0.002862**
$X_2 \times X_3$	1	-1.300000	0.916896
$X_3 \times X_3$	1	-72.43750	0.002029**
Root MSE		23.69573	
R-square		95.56 %	

*** < 0.001 %, ** < 0.01, * < 0.05.

TABLE-9
VARIANCE ANALYSIS OF REGRESSION EQUATION

Regression	DF	F value	Pr > F
Linear	3	8.65	0.0319*
Quadratic	3	12.86	0.0160*
Cross product	3	2.19	0.2317
Total Model	9	7.90	0.0309*

***< 0.001 %, **< 0.01, * < 0.05.

According to Table-8, the t-test was used to determine the significance of the regression coefficients of the variables. It was found that the effects of quadratic term were all significant, while in linear term, only X_1 was significant; reciprocation term was not significant except $X_1 \times X_3$. So the concentration of sucrose was the most important factor of medium in FOS production.

To determine the optimal condition of FOS production and the relationship between the response and the significant variables, statistical analyses of variance (ANOVA) were performed through the F-test shown in Table-9. It was summarized that the influence of linear term and quadratic term of the equations were also significant as it was found in Table-8. So it was demonstrated that the relationship between response value (Y) and each experimental factor in this model were correct and reliable. Data obtained from the experiments (Table-7) were analyzed by linear multiple regression using software SAS V8.1. The corresponding regression equation was as follows:

$$Y_1 = 628.8 - 46.6125X_1 + 4.4375X_2 - 8.975X_3 - 43.7625X_1X_1 + 13.025X_1X_2 + 31.3X_1X_3 - 67.0125X_2X_2 + 1.3X_2X_3 - 72.4375X_3X_3$$

According to this regression equation, the linear relationship between response value (Y) and independent variable (X) was conspicuous. The coefficient of determination was 95.56 %, which demonstrated a high goodness of fit of the mode in all cases. On the basis of the regression equation, the 3D response surface curves and the 2D contour plots were then generated to explain the interaction of variables and the optimum levels of each variable required for the maximum production of FOS, as followed in Figs. 1-3.

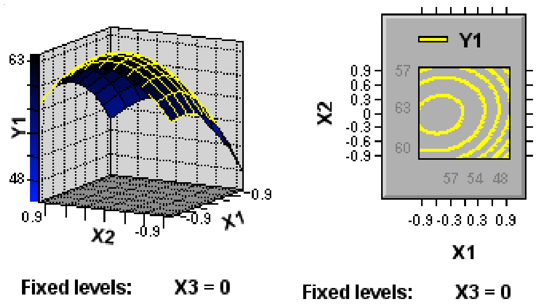

Fig. 1. Response surface and contour plots for the effects of sucrose (X_1) and yeast powder (X_2)

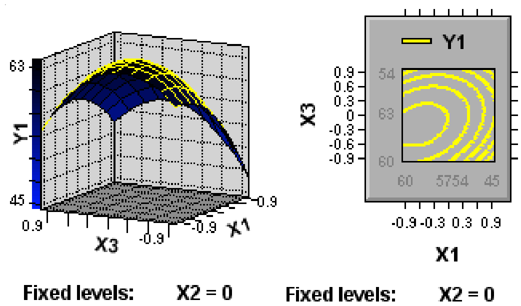

Fig. 2. Response surface and contour plots for the effects of sucrose (X_1) and $NaNO_3$ (X_3)

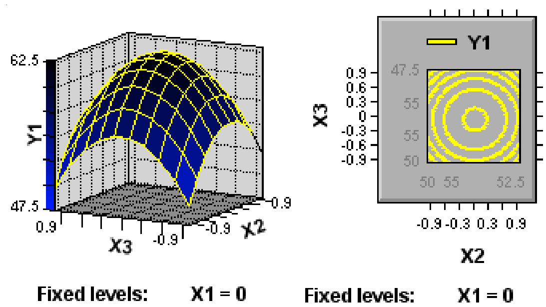

Fig. 3. Response surface and contour plots for the effects of yeast powder (X_2) and $NaNO_3$ (X_3)

Response surface curves and contour plots were generally the graphical representation of the regression equation. Response surface curves as a function of the effect of two factors varied at a time on the production of FOS, while maintaining the third factor at zero level, which was helpful in understanding both the main and interaction effects of two factors. These plots were easily obtained by calculating from the model values. The yield values for different concentrations of the variables are also predicted from the respective response surface plots (Figs. 1-3). The maximum predicted yield was indicated by the response surface diagram.

Optimal value: Through the analyzing by statistical analysis software SAS v8.1 and the response surface diagram, the peak of regression model was found. The max estimated value (Y) was 642.97 g kg^{-1}, while the code of (X_1, X_2 and X_3) was (-0.613595, -0.028626 and -0.196095). The practical value of each factor was: sucrose (X_1) = 143.864 g kg^{-1}, yeast powder (X_2) = 49.7137 g kg^{-1}, $NaNO_3$ (X_3) = 3.30394 g kg^{-1}. Considering the convenience of FOS production, the composition of medium was sucrose (X_1) = 140 g kg^{-1}, yeast powder (X_2) = 50 g kg^{-1}, $NaNO_3$ (X_3) = 3 g kg^{-1}.

Verification of the results: Verification experiments were carried out to confirm the correctness of this model for predicting the values of dependent variables. The results obtained under the fermentation condition which was optimized by RSM were listed in Table-10. The average practical value of FOS was 633 g kg^{-1}, which was very proximal to the maximum estimated value. This test was exact and reliable and the RSD was 0.69 %.

TABLE-10
VERIFICATION TEST

Number	Yield of FOS production	Average of FOS production	RSD (%)
1	628.4		
2	633.6	633	0.69
3	637.1		

Conclusion

The Plackett-Burman design and response surface methodology were found to be useful in optimizing the culture medium of FOS production. Second-order regression models were developed using statistical analysis software SAS v8.1 for predicting the responses in all experimental regions. The R^2 value showed a good fit of the model with experimental data. The best medium condition was selected as follows: sucrose (X_1) = 140 g kg^{-1}, yeast powder (X_2) = 50 g kg^{-1}, $NaNO_3$ (X_3) = 3 g kg^{-1}. The practical yield rate of FOS was 633 g kg^{-1}, which was better than the common level in fermental production of FOS (500-600 g kg^{-1}). The results were very reliable and also gave a basis for further study with large scale fermentation in industrialization for the production of FOS.

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