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Medium optimization for the production of a metagenome-derived β-galactosidase by *Pichia pastoris* using response surface methodology

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To investigate the optimal medium for the production of a metagenome-derived β-galactosidase (ZQ114) by *Pichia pastorisi*, Plackett and Burman design was applied to screen the significance of eight medium compositions, and three factors (methanol, sorbitol and $MgSO_4.7H_2O$) were found to have significant effect on the production of ZQ114. Then, the steepest ascent method was further used to determine their optimal levels, and Box-Behnken design and response surface methodology were eventually adopted to derive a statistical model for optimizing the medium. The experimental results indicate that the optimum of three main factors was 10.43 g/L (methanol), 33.98 g/L (sorbitol), and 0.52 g/L ($MgSO_4.7H_2O$), respectively. The highest yield of ZQ114 using the optimal medium reached up to 14.94 U/mL with lactose as substrate, which was 2.57 times higher than that unoptimized. Also, the experimental data of 14.94 U/mL is basiclly accordant with the predicted value (14.68 U/mL).

Key words: β-Galactosidase, enzyme production, medium optimization, response surface methodology.

INTRODUCTION

β-Galactosidase (EC 3.2.1.23), which hydrolyzes lactose to glucose and galactose, is a relatively costly enzyme. This enzyme has two main applications in food industry, including production of low lactose milk (and dairy products made from it) for lactose intolerant people and production of galactosylated products (Neri et al., 2008). Traditionally, commercial β-galactosidase is produced from fungi of the genus *Aspergillus* and yeasts of the genus *Kluyveromyces*. Recently, producing β-galactosidase in mesophilic microorganisms by using recombinant techniques has become very attractive due to its low production cost and high yield, and several bacterial β-galactosidases have been successfully cloned and expressed in *Escherichia coli* (Hung and Lee, 2002; Kang et al., 2005) or *Pichia pastoris* (Yuan et al., 2008; Hildebrandt et al., 2009).

The methylotropic yeast *P. pastoris* is widely being used as a host for the production of various recombinant heterologous proteins. It is a useful system for the expression of milligram-to-gram quantities of proteins for both basic laboratory research and industrial manufacture (Cereghino et al., 2002). The composition of the fermentation medium plays a very significant role in the production of β-galactosidase by high cell density fermentation of *P. pastoris*.

Traditionally, the most commonly used medium for the high cell density fermentation of *P. pastoris* is the basal salt medium (BSM) as mentioned in *Pichia* fermentation

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process guidelines proposed by Invitrogen (USA). However, this medium is only a standard one, and it is not the optimal medium for production of every heterologous protein due to some drawbacks like precipitation, unbalanced composition and high ionic strength, etc (Cos et al., 2006). Therefore, some investigations should be performed to optimize the medium compositions for production of β-galactosidase in *P. pastoris*. The classical method for medium optimization is the "one-factor-at-a-time" method, and it involves changing one factor at a time and maintaining the rest factors at a fixed level. This method is extremly time consuming and is unable to detect the effect of interaction of various factors (Cochran and Cox, 1992). To overcome this difficulty, response surface methodology (RSM), the statistical method, can be used to optimize the medium composition. RSM is a collection of statistical techniques including designing experiments, building models, evaluating the effects of factors, and analyzing optimum conditions of factors for desirable responses (Box and Wilson, 1951). It has been successflully utilized to optimize fermentation medium components of several industry-potential enzymes, such as β-glucanase (Tang et al., 2004) and carbonyl reductase (Soni et al., 2006). Nevertheless, no investigation has yet been performed on the optimization of medium compositions for β-galactosidase production in *P. pastoris* using response surface methodology so far according to the literature report.

Recently, a novel metagenome-derived β-galactosidase ZQ114 has been isolated and characterized in our laboratory, and the enzyme displayed some unusual properties: good thermostability, high enzymatic activity to its natural substrate lactose, pH optimum of 6.8, and highly expression in *P. pastoris*, which make ZQ114 an interesting enzyme in scale production and applications of food industry. In the present study, the experiments of medium optimization for ZQ114 production by *P. pastoris* were carried out. Plackett and Burman design was firstly applied to screen the significance of eight components based on improved BSM medium of *P. pastoris*. Then, the steepest ascent method was used to determine the level of experimental factor and experimental center point of the main factors to lead the experimental values closely toward maximal response surface region. Finally, Box-Behnken design and response surface methodology were further adopted to derive a statistical model for optimizing the medium components for ZQ114 production in *P. pastoris.*

To our best knowledge, it is the first report of medium optimization for β-galactosidase production in *P. pastoris* using response surface methodology. The highest yield of ZQ114 using the optimal medium reached up to 14.94 U/mL with lactose as substrate, which was 2.57 times higher than that unoptimized. Furthermore, β-galactosidase production with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate was also investigated using the optimized medium, and result showed an average value of 518.74 U/ml, which is far higher than that of other reports of β-galactosidase production in *P. pastoris.*

MATERIALS AND METHODS

Chemicals and regents

All chemicals and reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, CA, USA), unless otherwise stated.

Strain and plasmid

The *P. pastoris* strain GS115 (*his4*) was purchased from Invitrogen (Carlsbad, CA, USA) and used as a host for expression of β-galactosidase gene *zq114*. Recombinant plasmid pPICZαB (Invitrogen) carrying z*q114* was integrated into the *Pichia* genome and used as an expression vector to produce β-galactosidase. The GS115-*zq114* strain (methanol utilization slow, Mut^S) was used for this study and the promoter was AOX1, induced by the methanol.

Media and culture conditions

The medium optimization studies were investigated using 1-L Erlenmeyer flask containing 200 mL medium, and here the production of β-galactosidase by *P. pastoris* in shake flask involves two stages. The first stage is the cell growth phase in which the maximum biomass is obtained in a component-fixed basic medium on a non-fermentable carbon source, glycerol; The second phase is the target protein induction phase, the medium composition used in this phase is similar with that of cell growth phase, except using methanol to substitute glycerol. Moreover, the medium formula used in the induction phase was changeable, and was specified according to experimental design of medium optimization. The basic medium for the cell growth phase based on a basal salt medium for *P. pastoris* fermentation, followed with the enhancement of four components (seven vitamins, two trace elements, sorbitol and yeast extract) according to three reports (Boze et al., 2001; Inan and Meagher, 2001; Sreekrishna et al., 1997). The composition of the improved basic medium was 20 g/L glycerol (or 7.50 g/L methanol), 20 g/L sorbitol, 7.50 g/L ammonium sulfate [$(NH_4)_2SO_4$], 5 g/L yeast extract, 3.50 g/L potassium dihydrogen phosphate (KH_2PO_4), 0.70 g/L magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), 1 mL/L vitamins solution and 10 mL/L trace elements solution. Composition of vitamins solution used was D-biotin 0.05 g/L, Ca D-panthothenate 1.00 g/L, nicotinic acid 1.00 g/L, myo-inositol 25.00 g/L, thiamin hydrochloride 1.00 g/L, pyridoxol hydrochloride 1.00 g/L and *p*-amino benzoic acid 0.20 g/L. The trace elements solution contained Ethylene Diamine Tetraacetie Acid (EDTA) 3 g/L, zinc sulfate heptahydrate 0.90 g/L, manganese chloride dehydrate 155 mg/L, cobalt (II)-chloride dehydrate 60 mg/L, copper (II)-sulfate pentahydrate 60 mg/L, disodium molybdenum dehydrate 80 mg/L, calcium chloride dihydrate 0.90 g/L, iron sulfate-heptahydrate 0.60 g/L, boric acid 200 mg/L, and potassium iodide 20 mg/L. For medium preparation, glycerol, sorbitol, yeast extract, $(NH_4)_2SO_4$, along with KH_2PO_4 and $MgSO_4 \cdot 7H_2O$ were sterilized by autoclaving at 121°C. Vitamins and trace elements solutions were sterilized with filter separately and then the whole medium was aseptically reconstituted. Finally, the pH was adjusted to 5.5 using 1 N NaOH prior to inoculation. *Pichia* fermentation experiments were performed by inoculating a single colony to 10 mL BMGY medium (1% yeast extract, 2% peptone, 40 mg/L L-histidine, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 0.4 mg/L biotin,

Table 1. Level of variables used for the production of β-galactosidase using Plackett–Burman design.

Code	Variable	Level (g/L)	
		-1	+1
A	Methanol	5	10
B	Sorbitol	10	30
C	$(NH_4)_2SO_4$	5	10
D	Yeast extract	2.5	7.5
E	KH_2PO_4	2.5	5
F	$MgSO_4 \cdot 7H_2O$	0.5	1.0
G	Vitamins solution	0.5 mL/L	2 mL/L
H	Trace elements solution	5 mL/L	15 mL/L
J	Dummy 1	-1	1
K	Dummy 2	-1	1
L	Dummy 3	-1	1

J, K, L are three dummy variables.

0.5% glycerol) in a 100 mL shake flask and growing at 30°C and 250 rpm until the cultures reached an OD_{600}=3 (approximately 16 to 18 h). Then, 2 mL of cultures were inoculated to 200 mL component-fixed basic medium in a 1-L Erlenmeyer flask with vigorous agitation (300 rpm) at 30°C. When the OD_{600} of the cultures reached 6.0, they were centrifuged at 3,000×*g* for 5 min at room temperature. To induce β-galactosidase expression, the supernatant was decanted, and cell pellet was resuspended in induction medium using one fifth of the original culture volume (40 mL) and 1-L Erlenmeyer flask. Then, fermentation was performed at 30°C and 250 rpm, and 100% methanol was added to a final concentration of 0.5% every 24 h to maintain induction. At each of time points (0, 24, 48, 72, 96, 120 and 144 h), 1 mL of the culture was transferred to a 1.5 mL microcentrifuge tube and used to analyze enzymatic activity (U/mL) of the supernatant, and determine the optimal post-induction time to harvest. After about 120-h induction, the highest enzyme activity of the supernatant was reached, and thus 120 h was considered as the optimal post-induction time in this study.

Analysis of enzyme production and enzymatic activity

At abovementioned each time point, the culture was collected into microcentrifuge tubes and centrifuged to separate the cells from the supernatant. The supernatant was used to determine the yield of the β-galactosidase according to its enzymatic activity in 1 mL of the culture. The β-galactosidase activity was measured according to the method of Wang et al. (2010) using two substrates including ONPG and lactose. The activity of β-galactosidase for ONPG was measured by following the amount *o*-nitrophenol released from ONPG. The reaction mixture (1 mL) included 10 µL of enzyme solution (the supernatant) and 990 µL of 100 mM Tris-HCl buffer (pH 6.8) containing ONPG at a concentration of 22 mM. The mixture was incubated at 52°C for 15 min, and the reaction was terminated by adding an equal volume of 1.0 M Na_2CO_3. The released *o*-nitrophenol was quantitatively determined by measuring A_{420}. One unit of activity was defined as the amout of enzyme liberating 1 µmol of *o*-nitrophenol per minute. Assays for enzymatic activity towards lactose were carried out in the same buffer containing 10 µl enzyme solution and 5% lactose, and the reaction was stopped by boiling for 10 min, and the concentration of glucose was determined using a glucose oxidase-peroxidase assay kit (Sigma-Aldrich). The released glucose was quantitatively determined by measuring A_{492}. One unit of enzyme activity was defined as the amount of activity required to release 1 µmol of glucose per minute.

Experimental design

In order to obtain the highest β-galactosidase production, the medium composition used for the target protein induction phase was optimized using a series of statistically design. First, Plackett and Burman design was applied to screen the significance of eight medium components (methanol, sorbitol, $(NH_4)_2SO_4$, yeast extract, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, vitamins solution and trace elements solution). Second, the steepest ascent method was used to determine the level of experimental factors and experimental center point of the main factors to lead the experimental values closely toward maximal response surface region. Finally, Box-Behnken design and response surface methodology were further adopted to derive a statistical model for optimizing the medium for ZQ114 production by *P. pastoris*. All experiments were performed in triplicate and response values were the averages of the corresponding results.

Plackett-Burman design

For the selection of various variables, “Design Expert 8.05” (Stat-Ease Inc, Minneapolis, USA) was used to generate and analyze the experimental design of Plackett-Burman. According to this program, abovementioned eight factors were chosen, and three dummy variables (J, K, L) were used to evaluate experimental error. Each parameter was tested at two levels, high (+1) and low (-1). Concentration ranges for the variables were decided by extensive literature survey (Table 1). The induction-medium optimization was performed in a 1-L Erlenmeyer flask containing 40 mL medium following the instruction of design matrix (Table 2). The effects of variables on enzyme production were measured in terms of β-galactosidase activity of enzyme solution, all these data were analyzed using “Design Expert 8.05” program, and the results of variance analysis for Plackett-Burman factorial model are shown in

Table 2. The Plackett –Burman design matrix for screening significant variables for β-galactosidase production.

Run	A	B	C	D	E	F	G	H	J	K	L	Enzyme production (U/mL)
1	1	1	-1	-1	-1	1	-1	1	1	-1	1	12.19±0.42
2	-1	-1	1	-1	1	1	-1	1	1	1	-1	7.84±0.26
3	-1	1	1	1	-1	-1	-1	1	-1	1	1	10.18±0.19
4	1	-1	-1	-1	1	-1	1	1	-1	1	1	11.51±0.35
5	1	1	1	-1	-1	-1	1	-1	1	1	-1	13.65±0.44
6	-1	-1	-1	1	-1	1	1	-1	1	1	1	8.05±0.31
7	-1	1	-1	1	1	-1	1	1	1	-1	-1	9.84±0.27
8	1	-1	1	1	1	-1	-1	-1	1	-1	1	11.72±0.35
9	-1	1	1	-1	1	1	1	-1	-1	-1	1	8.74±0.29
10	1	1	-1	1	1	1	-1	-1	-1	1	-1	12.45±0.41
11	1	-1	1	1	-1	1	1	1	-1	-1	-1	9.64±0.27
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	8.74±0.25

The experiments were performed in triplicates and the mean values were presented, and enzyme production was measured using lactose as substrate.

Table 3. Analysis of variance for Plackett-Burman factorial model.

Source	Sum of square	Degrees of freedom	Mean square	F-value	*P*-value Prob>F
Model	38.46	8	4.81	14.34	0.026
A-Methanol	26.31	1	26.31	78.49	0.003
B-Sorbitol	7.60	1	7.60	22.67	0.018
C-$(NH_4)_2SO_4$	0.085	1	0.085	0.25	0.649
D-Yeast extract	0.052	1	0.052	0.16	0.720
E-KH_2PO_4	0.010	1	0.010	0.030	0.873
F-$MgSO_4{\cdot}7H_2O$	3.77	1	3.77	11.26	0.044
G-Vitamins solution	0.24	1	0.24	0.71	0.461
H-Trace elements solution	0.39	1	0.39	1.15	0.362
Residual	1.01	3	0.34		
Cor total	39.47	11			

Table 3.

Steepest ascent method

Frequently, the initial estimate of the optimum conditions for the system will be far from the actual optimum. To move rapidly towards the neighborhood of the optimum response, variables that significantly influenced β-galactosidase activity were optimized with respect to enzyme activity by applying a single steepest ascent experiment (Montgomeryd and Runger, 2002). The experiments were performed following the instruction of Table 4.

Response surface methodology

Based on the selection of the significant variables for β-galactosidase production by Plackett-Burman design experiment and the ranges of these variables, the response surface methodology, using a Box-Behnken design, was used to determine the optimum concentration of these variables for the improvement of β-galactosidase production. The central values of all variables were coded as zero, and the minimum values (-1) and maximum values (+1) of these variables are shown in Table 5. A total of 17 experiments were formulated according to the design of the statistical software package “Design Expert 8.05”, and the full experimental plan with regard to their values in actual and coded form was also provided in Table 5. The response value in each trial was the average of the triplicates. The program of “Design Expert 8.05” was used to analyze the experimental design and results.

Nucleotide sequence accession number

The β-galactosidase gene *zq114* reported here was derived from a metagenomic library of dairy-plant soil samples, and its nucleotide

Table 4. Experimental design of steepest ascent and corresponding response.

Experiment number	Methanol (g/L)	Sorbitol (g/L)	$MgSO_4.7H_2O$ (g/L)	Enzyme production (U/mL)
1	7.5	20	0.75	12.25±0.28
2	9.0	25	0.65	13.44±0.35
3	10.5	30	0.55	13.79±0.37
4	12	35	0.45	12.77±0.29
5	13.5	40	0.35	12.33±0.31

The experiments were performed in triplicates and the mean values were presented, and enzyme production was measured using lactose as substrate.

Table 5. Box-Behnken design with three independent variables.

Run	Methanol (X_1) (g/L)		Sorbitol (X_2) (g/L)		$MgSO_4.7H_2O$ (X_3) (g/L)		Enzyme production (U/mL)	
	Coded value	Actual value	Coded value	Actual value	Coded value	Actual value	Observed value	Predicted value
1	0	10.5	1	35	-1	0.40	14.27±0.28	14.17
2	-1	8.5	1	35	0	0.55	13.24±0.29	13.32
3	0	10.5	1	35	1	0.70	13.65±0.24	13.68
4	0	10.5	0	30	0	0.55	14.49±0.27	14.54
5	0	10.5	-1	25	1	0.70	12.81±0.32	12.91
6	1	12.5	1	35	0	0.55	13.43±0.29	13.42
7	0	10.5	0	30	0	0.55	14.33±0.24	14.54
8	1	12.5	-1	25	0	0.55	12.49±0.25	12.41
9	1	12.5	0	30	-1	0.40	12.71±0.28	12.82
10	0	10.5	0	30	0	0.55	14.65±0.31	14.54
11	-1	8.5	-1	25	0	0.55	12.28±0.26	12.29
12	0	10.5	0	30	0	0.55	14.63±0.29	14.54
13	-1	8.5	0	30	1	0.70	12.57±0.17	12.46
14	-1	8.5	0	30	-1	0.40	12.66±0.25	12.68
15	0	10.5	-1	25	-1	0.40	12.95±0.29	12.92
16	1	12.5	0	30	1	0.70	12.55±0.27	12.53
17	0	10.5	0	30	0	0.55	14.58±0.32	14.54

The experiments were performed in triplicates and the mean values were presented, and enzyme production was measured using lactose as substrate.

seqence data have been deposited in the nucleotide sequence databases (GenBank) under accession number (HQ909087).

RESULTS AND DISCUSSION

Evaluation of effect of medium components by Plackett-Burman design

In the present study, we choose an improved basal salt medium (eight components) as the basic medium of *P. pastoris* fermentation. Compared to conventional basal salt medium (four components) of *P. pastoris*, this medium supplies four new components including seven vitamins, two trace elements, sorbitol, and yeast extract based on undermentioned three reports: (1) Boze and co-workers found that supplementation of a basal medium with seven vitamins and two trace elements increased production of a recombinant porcine follicle-stimulating hormone from 93 mg/L (basal medium) to 187 mg/L (supplemented medium), showing that vitamin and trace element requirements have an important effect on cell growth and recombinant protein production in *P. pastoris* (Boze et al., 2001); (2) non-expression-repressing carbon source sorbitol increased the production of a recombinant β-galactosidase as compared to cells grown with glycerol or glucose, as well as reduce the amount of methanol required for induction of protein expression (Inan and Meagher, 2001); (3) other nitrogen sources affect recombinant protein production in *P. pastoris*, and the use

of yeast extract increased protein secretion and accumulation (Sreekrishna et al., 1997). Then, Plackett-Burman design was used to analyze the effect of 11 variables (eight components from improved basal salt medium and three dummy variables) on β-galactosidase production by *P. pastoris*, and the design matrix selected for the screening of significant variables for β-galactosidase production and the corresponding responses were shown in Table 2. To find significant components from eight variables, the adequacy of the model was tested, and the variables evidencing statistically significant effects were identified via Fisher's test for analysis of variance (ANOVA) (Table 3). Factors evidencing probability values (*P*-values) of less than 0.05 are considered to have significant effects on the response, and the lower *P*-value indicates the more significant factor on β-galactosidase production. The *F*-value of 14.34 implied that the model was significant, and the *P*-value of 0.026 (less than 0.05) further substantiated that model terms were significant (Table 3). Furthermore, methanol, with a *P*-value of 0.003, was determined to be the most significant factor, followed by sorbitol (0.018) and $MgSO_4.7H_2O$ (0.044) (Table 3). Among three significant variables identified, methanol and sorbitol displayed positive effects on β-galactosidase production, whereas $MgSO_4.7H_2O$ exerted a negative effect on the production of β-galactosidase. Plackett-Burman design results suggested that a combinated induction of methanol and sorbitol would favor maximum β-galactosidase production, which was accordant with the report of Inan and Meagher (2001). However, yeast extract displayed indistinctive effects on β-galactosidase production, the reason was probably that yeast extract was not always a crucial medium component for product of every heterologous protein by *P. pastoris.* According to the results of Plackett-Burman design, abovementioned three significant variables were therefore selected for further optimization studies, and all other insignificant variables were fixed at their low level (-1) for later experiments.

Optimization of significant variable levels by the path of the steepest ascent experiment

Although the most significant variables affecting β-galactosidase production were screened by Plackett-Burman design, it was unable to predict the optimum levels of the variables. Therefore, based on Plackett-Burman design results, the path of the steepest ascent was further employed to find the proper direction to change the variables by increasing methanol and sorbitol and decreasing $MgSO_4.7H_2O$ concentration to improve β-galactosidase production. The center point (zero point) of the Plackett-Burman design was considered as the origin of the steepest ascent experiment, and results are shown in Table 4. The maximum enzyme production (13.79 U/mL, with lactose as substrate) was obtained when 10.5 g/L methanol, 30 g/L sorbitol, and 0.55 g/L $MgSO_4.7H_2O$ were used. It suggested that this point might be near the region of the maximum β-galactosidase response. Thus, this point was chosen for further optimization.

Medium optimization by response surface methodology

According to the results of Placket-Burman design and the method of the steepest ascent, three variables, including methanol, sorbitol, and $MgSO_4.7H_2O$, which significantly influenced the β-galactosidase production, were used to determine their optimum levels. The Box-Behnken design with three factors and three levels, was used for fitting a second-order response surface. A total of 17 experiments with different combinations of methanol (X_1), sorbitol (X_2), and $MgSO_4.7H_2O$ (X_3) were performed (Table 5), and the results were analyzed by ANOVA (Table 6). Regression analysis of the data was performed for testing the adequacy of the proposed quadratic model-A, and the following second-order polynomial equation was derived:

$$Y=14.54+0.054X_1+0.51X_2-0.13X_3-(5.000E-003)X_1X_2-0.018X_1X_3-0.12X_2X_3-1.24X_1^2-0.44X_2^2-0.68X_3^2 \quad (1)$$

Where, Y is the predict value of the experiment, X_1, X_2 and X_3 are the coded values of methanol, sorbitol, and $MgSO_4.7H_2O$, respectively. The results of Table 6 indicate that only X_2, X_3 X_1^2, X_2^2, and X_3^2 were significant variables, thus the model was remodified by taking off insignificant variables (P>0.05): X_1, X_1X_2, X_1X_3, and X_2X_3. The simplified second-order polynomial equation was:

$$Y=14.54+0.51X_2-0.13X_3-1.24\ X_1^2-0.44X_2^2-0.68X_3^2 \quad (2)$$

The data of simplified model-B were also analyzed by ANOVA (Table 6). As shown in Table 4, the F-value of the model-B was 110.04. It implied that the model was very significant, and there was only a 0.01% chance that a "Model F-Value" could occur due to noise. Moreover, the *P*-values (<0.001) of the model and the lack of fit (0.470) also suggested that the obtained experimental data was a good fit with the model. The value of determination coefficient R^2= 0.985 for β-galactosidase yield, ensured a satisfactory adjustment of the quadratic model to the experimental data, and also indicated a high correlation between the predict values and the practical values. Furthermore, three dimensional response surface plots (Figures 1a to 3a) and 2D contour plots (Figures 1b to 3b), which graphically represent regression equations, were used to demonstrate relationships between the response and experimental levels of each variables. Figures 1 to 3 show the response surface plots and their respective contour plots of β-galactosidase production. Each figure presents the effect of two factors while the other factor

Table 6. Analysis of variance for the response surface quadratic model-A and the simplified response surface quadratic model-B.

Source	Sum of square	Degrees of freedom	Mean square	F- value	*P-value Prob > F*
Model-A	12.29	9	1.37	75.65	<0.001
X1-typtone	0.023	1	0.023	1.28	0.295
X2-yeast extract	2.06	1	2.06	114.11	<0.001
X3- MgSO4.7H2O	0.13	1	0.13	7.06	0.033
X1X2	1.000E-004	1	1.000E-004	5.538E-003	0.943
X1X3	1.225E-003	1	1.225E-003	0.068	0.802
X2X3	0.058	1	0.058	3.19	0.117
X12	6.44	1	6.44	356.67	<0.001
X22	0.81	1	0.81	44.99	<0.001
X32	1.93	1	1.93	106.80	<0.001
Residual	0.13	7	0.018		
Lack of fit	0.058	3	0.019	1.13	0.436
Pure error	0.068	4	0.017		
Cor total	12.42	16			
Simplified model-B	12.24	6	2.04	110.04	<0.001
X_1-Methanol	0.023	1	0.023	1.25	0.290
X_2-Sorbitol	2.06	1	2.06	111.18	<0.001
X_3- $MgSO_4 \cdot 7H_2O$	0.13	1	0.13	6.88	0.026
X_1^2	6.44	1	6.44	347.52	<0.001
X_2^2	0.81	1	0.81	43.84	<0.001
X_3^2	1.93	1	1.93	104.06	<0.001
Residual	0.19	10	0.019		
Lack of fit	0.12	6	0.020	1.14	0.470
Pure error	0.068	4	0.017		
Cor total	12.42	16			

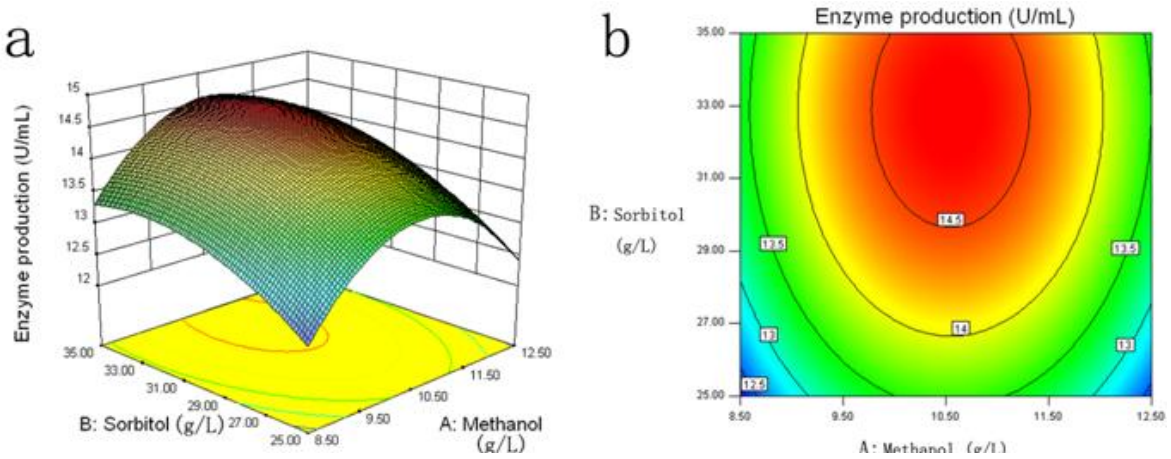

Figure 1. The response surface plot (a) and the corresponding contour plot (b) of the effect of methanol and sorbitol on β-galactosidase production by *P. pastoris* with 0.55 g/L $MgSO_4 \cdot 7H_2O$.

was held at zero level. As shown in Figures 1 to 3, there was significant interaction between each pair of variables. Moreover, The optimum values of each significant variable was also identified based on the hump in the three dimensional plot, or form the central point of the corresponding contour plot. The results predicted by the

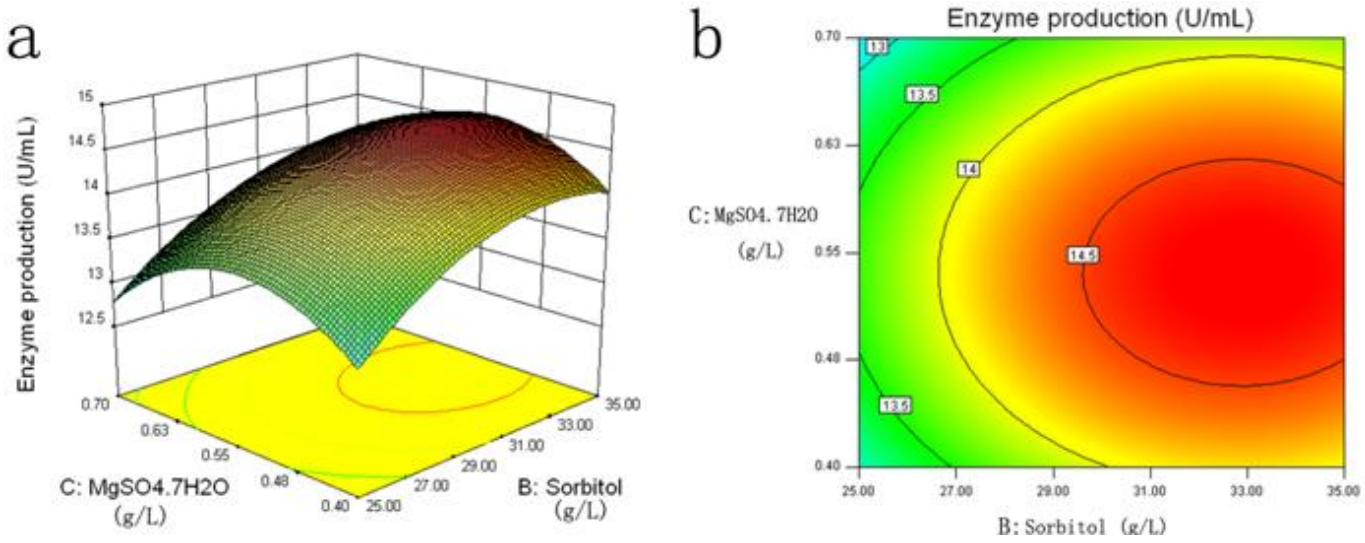

Figure 2. The response surface plot (a) and the corresponding contour plot (b) of the effect of sorbitol and $MgSO_4 \cdot 7H_2O$ on β-galactosidase production by *P. pastoris* with 10.5 g/L methanol.

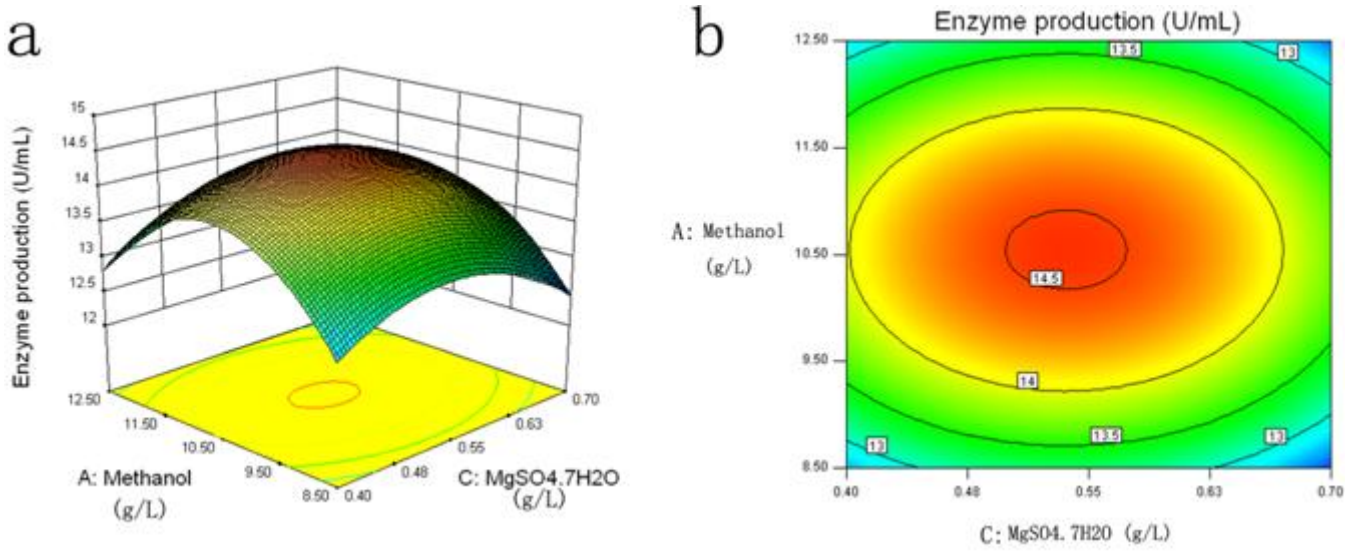

Figure 3. The response surface plot (a) and the corresponding contour plot (b) of the effect of $MgSO_4 \cdot 7H_2O$ and methanol on β-galactosidase production by *P. pastoris* with 30 g/L sorbitol.

model equation from RSM show that a combination of methanol (10.43 g/L), sorbitol (33.98 g/L), and $MgSO_4.7H_2O$ (0.52 g/L) would favor maximum β-galactosidase production, giving 14.68 U/mL (with lactose as substrate).

Validation of the optimized medium

To verify the predicted results, validation experiment was performed in triplicate tests. Using the optimized medium, the observed experimental value of average β-galactosidase production was 14.94 U/mL (with lactose as substrate), suggesting good agreement with the predicted value (14.68 U/mL). Also, β-galactosidase production with ONPG as substrate was investigated using the optimized medium, and result showed an average value of 518.74 U/ml. Moreover, β-galactosidase yield was only 5.81 U/mL(with lactose as substrate) in non-optimized media (the medium composition used in the cell growth phase and the target protein induction phase are both component-fixed basic medium except

using 7.5 g/L methanol to substitute 20 g/L glycerol during the target protein induction phase), the optimized maximum value of β-galactosidase production was 2.57 times higher than that unoptimized. This result thus corroborated the effectiveness of the model, indicating that the optimized medium favors the production of β-galactosidase. Nowadays, recombinant DNA technology has been used to express and optimize the production of interesting β-galactosidas from the most diverse sources in microbial hosts that are recognized for their higly efficient heterologous protein production. This possibility greatly expands the range of potential applications for β-galactosidase and their economically effective utilization in industrial processes. *P. pastoris* is a widely used host for production of heterologous proteins, and it has been employed to produce two bacterial β-galactosidases aiming at their industrial application, including a thermostable β-galactosidase from *Alicyclobacillus acidocaldarius* (Yuan et al., 2008) and a cold-adapted β-galactosidase from Antarctic *Arthrobacter* sp. 32c (Hildebrandt et al., 2009). The experimental results of Yuan et al (2008). indicated that the amount of thermostable β-galactosidase from *A. acidocaldarius* secreted in shake-flask cultures (90 mg/L) was higher than that expressed naturally, however, the enzymatic activity of the supernatant with ONPG as substrate was only 1.7 U/mL (Yuan et al., 2008), which is far lower than that of this study (518.74 U/mL); using constitutive or inducible *P. pastoris* expression systems, higher amounts (97 and 137 mg/L) of cold-adapted β-galactosidase from Antarctic *Arthrobacter* sp. 32c were obtained (Hildebrandt et al., 2009), and the authors considered the *P. pastoris* system as a high efficient and cheap way to produce this cold-adapted enzyme extracellularly. Nevertheless, the enzymatic activity towards ONPG of the supernatant in that study was not still high enough (97.58 U/mL) when compared with that of this study (518.74 U/mL). Therefore, this study highlights the utility of response surfact methodology in medium optimization for the β-galactosidase production by *P. pastoris*, and it also paved the way for further industrial scale production of β-galactosidase by *P. pastoris* in the future.

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