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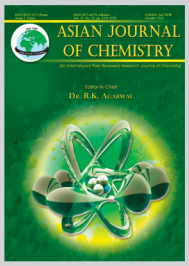

Isolation and Identification of a Novel *Aspergillus sydowii* F5 Producing α-Galactosidase and Statistical Optimization for the Enzyme Production

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A novel strain F5, producing α-galactosidase, was isolated from Chinese wheat Qu. According to the morphological characteristics and ITS gene sequence analysis, it was identified as *Aspergillus sydowii* F5. Statistically based experimental designs were applied to the optimization of cultural medium for the α-galactosidase production by *A. sydowii* F5. Plackett-Burman design was used to evaluate the effects of variables of the fermentation medium. Among these variables, soybean meal, $MgSO_4$ and KH_2PO_4 were identified to have the significant effects. Subsequently, the concentrations of soybean meal, $MgSO_4$ and KH_2PO_4 were optimized using central composite design. The optimum concentration of soybean meal, $MgSO_4$ and KH_2PO_4 was 22.4 g/L, 2.23 g/L and 0.91 g/L, respectively. The optimization strategy led to a 5.1-fold increase in the α-galactosidase production.

Key Words: α-Galactosidase, *Aspergillus sydowii*, Isolation, Optimization.

INTRODUCTION

α-D-galactose-galactohydrolase, commonly referred to as α-galactosidase, catalyzes cleavage of the terminal galactosyl residues from a wide range of substrates, including raffinose, stachyose, galactomannans and glycolipids[1]. It has a number of biotechnological applications. In beet sugar industry, it has been used to increase the sucrose yield by eliminating raffinose, which prevents the crystallization of beet sugar[2]. It is also applicable to enzyme replacement therapy for fabry disease[3,4] and blood group conversion[5]. In addition, its applications in legume food and feed processing have been impressed, because of lack of α-galactosidase in human and animals' intestinal tract, the microbial flora metabolizes raffinose family sugars in leguminous plant seeds to induce gastric distress[6-8].

α-Galactosidase has been reported to occur widely in plants[9,10]. The concentration of α-galactosidase in plants is low and choice of plants as a source of α-galactosidase is limited by seasonal conditions. Microbial production of α-galactosidase is more feasible than plant sources at industrial level. Several microorganisms including *A. foetidus*, *A. oryzae*, *A. terreus*, *Penicillium* sp., *B. stearothermophilus* and yeasts were reported to exhibit α-galactosidase activity, they vary in enzyme activity from 0.4 to 64.75 U/mL[11-15]. However, reports on isolation and identification of a new α-galactosidase producing strain are scanty.

In this present study, we investigated the isolation of a novel strain producing α-galactosidase from Chinese wheat Qu and identification of the strain. We also reported the optimal conditions for the production of α-galactosidase.

EXPERIMENTAL

Isolation of strains producing α-galactosidase: In order to isolate α-galactosidase strains, sample from Chinese wheat Qu was collected, 10 g of sample was mixed with 100 mL enrichment medium consisting of yeast extract 10 g/L, melibiose 10 g/L, KH_2PO_4 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L and $FeSO_4 \cdot 7H_2O$ 0.01 g/L, at 30 ºC for 2 days with shaking at 150 rpm. Obtained cultures were appropriately diluted, plated on agar enrichment medium containing 20 mg/L chromogenic X-α-D-Gal and incubated for 5 days at 30 ºC for isolation of pure cultures. Visibly different blue colonies were picked up from the plates and inoculated onto potato dextrose agar (PDA) slants for growth up, then checked for α-galactosidase production using enrichment medium in a shake flask culture at 30 ºC for 5 days. The strain secreting highest amount of galactosidase was used for further work.

Morphological and phylogenetic analysis of the isolated strain: The morphological characterization of the chosen strain was performed according to the methods described by Abrusci[16]. Genomic DNA extraction, PCR-mediated amplification of the ITS and sequencing of the PCR product were performed as described by Xie *et al.*[17]. The ITS sequence was aligned against representative sequences of members of related strains in the GeneBank database, using the facilities in the

Clustal X program. A phylogenetic tree was constructed using the neighbor-joining method by Mega program.

Optimization production of α-galactosidase: Erlenmeyer flasks (500 mL) containing 100 mL fermentation medium inoculated with 1 mL of 10^6 spores/mL suspension prepared from a potato dextrose agar slant of the culture, then incubated at 30 ºC for 5 days with shaking at 150 rpm for α-galactosidase production. The optimization of culture medium components for α-galactosidase production by *A. sydowii* F5 was carried out in three stages.

Screening carbon and nitrogen source for α-galactosidase production: Basal fermentation medium contained 1 g of KH_2PO_4, 0.5 g of $CaCl_2$, 0.5 g of $MgSO_4{\cdot}7H_2O$ and 0.01 g of $FeSO_4{\cdot}7H_2O$ per liter. A number of carbon and nitrogen sources were tested in order to determine the effects on growth and α-galactosidase production of *A. sydowii* F5. After the screening, inexpensive soybean meal was tested to completely replace the carbon and nitrogen source for α-galactosidase production.

Plackett-Burman design: Plackett-Burman experimental design was used to screen the important variables that influence α-galactosidase production and not the interaction effects between various factors. Each parameter was tested at high level and low level. Concentration ranges for the variables were decided by extensive literature survey. All experiments were performed in duplicate and the average of α-galactosidase activity after 5 days was taken as the response. The effects of variables on enzyme production were calculated by SAS statistical package (version 8.1, SAS Institute, Cary, NC, USA). The variables whose confidence levels were greater than 95 % were considered to significantly influence α-galactosidase production.

Central composite design: A central composite rotary factorial design with five-coded levels, including six replicates at the centre point was used for fitting a second order response surface. Twenty experiments were performed to optimize the screened variables grouped as X_1, X_2 and X_3. The results obtained from central composite rotary design on α-galactosidase production were used to fit the following second order polynomial eqn. 1, as it represents the behaviours of such a system more appropriately.

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 \qquad (1)$$

where, Y is the measured response variable; β_0, β_i, β_{ij} and β_{ii} are constant and regression coefficients of the model and x_i, x_j represent the independent variables in coded values.

The SAS statistical package (version 8.1, SAS Institute, Cary, NC, USA) was used for regression analysis of the data obtained and to estimate the coefficients of the regression equation. Canonical analysis, which is used to predict the shape of the curve generated by the model and a representative contour plot were carried out as well.

Enzyme activity assay: α-Galactosidase activity was measured by a modified *p*NPG method described by Dey[18] using a final concentration of 1 mM *p*NPG as substrate. Aliquots of 0.1 mL of *p*NPG (10 mM) and 0.8 mL of acetate buffer (0.1 mM and at pH 5) were pre-incubated at 37 ºC for 2 min before adding 0.1 mL of suitably diluted enzyme to initiate the reaction. This was terminated after 10 min by adding 3 mL of 0.5 M Na_2CO_3 and the released *p*-nitrophenol was determined spectrophotometrically at 405 nm. One unit of α-galactosidase activity was defined as the amount of enzyme liberating 1 µmol *p*-nitrophenol in 1 min under the assay conditions.

RESULTS AND DISCUSSION

Isolation of strains producing α-galactosidase: Various microbial strains were isolated from Chinese wheat Qu sample by the enrichment culture. X-α-gal is an important chromogenic substrate, which can be used to demonstrate α-galactosidase activity[19]. With the aid of X-α-gal, the deep blue colonies growing on the plates were assayed for the α-galactosidase activity in a second screening by fermentation. Under the conditions used, the strain F5 with the maximum α-galactosidase activity was isolated and selected as the best strain for further studies.

Characterization and identification of strain F5: The ITS gene sequence of strain F5 was amplified and detected (GeneBank accession no. EF151430). Blast analysis showed that it similar to *Aspergillus sydowii* NRRL 250. Through the alignment and cladistic analysis of homologous nucleotide sequences of known fungi, phylogenetic relationships could be inferred, the approximate phylogenetic position of the strain F5 was shown in Fig. 1. The morphological characterization of the strain F5 was also investigated. The colony was radially and concentrically sulcate and the reverse side was yellowish brown. Mycelium and soluble pigment was yellow coloured. Conidial heads was coloured brown with compact column arrangement. According to the morphological properties and comparison of ITS gene sequence, the strain F5 was identified as A*spergillus sydowii* F5.

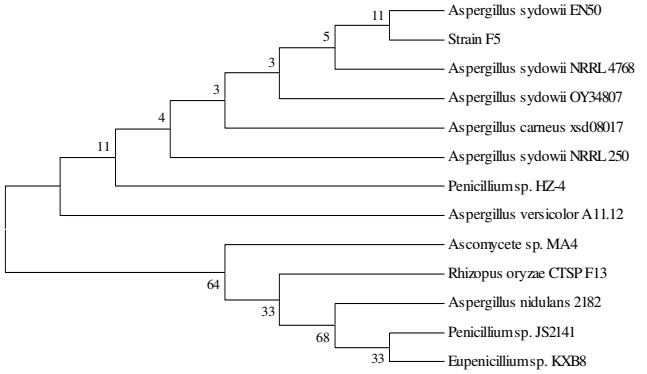

Fig. 1. Phylogenetic tree based on the ITS region among strain F5 and related strains

Influence of the carbon source on growth and α-galactosidase production: Different carbon sources were added at 1 % (w/v) level into basal fermentation medium supplemented with tryptone (1 %, w/v). The results were summarized in Fig. 2 and showed that *A. sydowii* F5 was able to grow in all carbon sources tested, but that grown in culture medium with lactose was less than the mean value. The greatest production of α-galactosidase occurred when stachyose was present in the culture medium. In contrast to this, glucose yielded the lowest level of enzyme activity. These results are in agreement with those of LeBlanc *et al.*[20] who found induction of α-galactosidase from *Lactobacillus fermentum*.

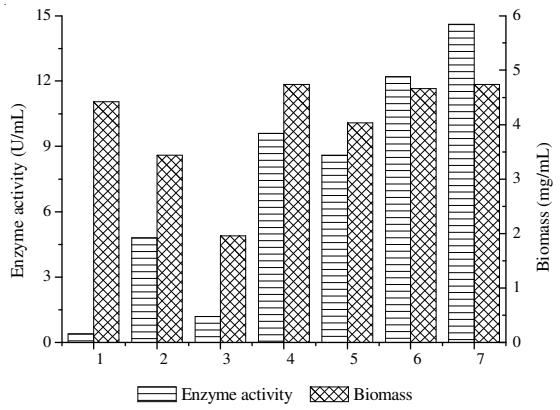

Fig. 2. Effects of carbon source on growth and a-galactosidase production of *A. sydowii* F5 (1: Glucose; 2: Galactose; 3: Lactose; 4: Sucrose; 5: Melibiose; 6: Raffinose; 7: Stachyose)

Influence of the nitrogen source on growth and α-galactosidase production: Using stachyose as carbon source, the effects of seven inorganic and organic nitrogen sources were examined on the growth and α-galactosidase production of *A. sydowii* F5. The results were summarized in Fig. 3 and showed that tryptone and soy protein isolate were the best nitrogen sources for α-galactosidase production. Better growth and high-yield α-galactosidase activity were obtained on organic nitrogen whereas poor growth and low-yield α-galactosidase activity were observed on inorganic nitrogen sources.

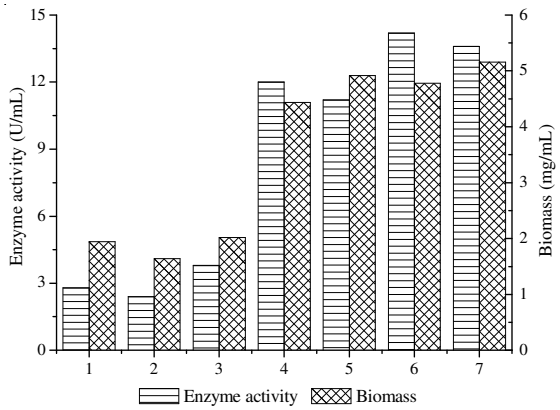

Fig. 3. Effects of nitrogen source on growth and a-galactosidase production of *A. sydowii* F5 (1: $(NH_4)_2SO_4$; 2: $NaNO_3$; 3: Urea; 4: Beef extract; 5: Yeast extract; 6: Tryptone; 7: Soybean protein isolate)

Effect of soybean meal on α-galactosidase production: The use of stachyose enhances the cost of enzyme production and is a major limitation to the economic feasible of bioprocess. Therefore, an easily available agricultural residue such as soybean meal, which is composed of 44 % protein, 25-30 % carbohydrate (Appr. 6 % stachyose) and other minor nutrients, was used as main substrate to completely replace of the stachyose and tryptone. The results were summarized in Table-1. When the basal medium supplemented with soybean meal (2 %, w/v), 108 % α-galactosidase activities of that control was obtained. Therefore, soybean meal, completely replaced of stachyose and tryptone was used in the further experiment to obtain optimum levels of α-galactosidase by *A. sydowii* F5.

TABLE-1
EFFECT OF SOYBEAN MEAL ON α-Galactosidase PRODUCTION FROM *A. sydowii* F5

Supplementary nutrients	Concentrations (w/v) (%)	Relative activity (%)
Control (Stachyose and tryptone)	1	100
Soybean meal	1	44
Soybean meal	2	108
Soybean meal	3	90
Soybean meal	4	72

Plackett-Burman design: A Plackett-Burman design was performed when the carbon and nitrogen sources were replaced with soybean meal (2 %, w/v). The experimental design and corresponding α-galactosidase production were shown in Table- 2. When the sign of the effect Ex_i of the tested variable is positive, the influence of the variable on α-galactosidase production is greater at a high level. And when negative, the effect of the variable is greater at a low level. The effect of variables was showed in Table-3. With the help of relative ranking of Ex_i, variability in three factors (soybean meal, $MgSO_4$ and KH_2PO_4) significantly affected enzyme production, as their confidence levels above 99 %. The positive effect of soybean meal was 6.9, may be caused by the requirement of a large quantity of substrate to synthesize α-galactosidase. Therefore, high soybean meal concentration would lead to higher α-galactosidase production in the tested range. $MgSO_4$ at high concentration would also enhanced α-galactosidase production. This result coincided with the case of *Absidia sp*. WL511

TABLE-2
PLACKETT-BURMAN DESIGN OF α-galactosidase PRODUCTION FROM *A. sydowii* F5

Runs	Variables (g/L)								α-Galactosidase (U/mL)
	$ZnSO_4$ X_1	Soybean meal X_2	$MgSO_4$ X_3	$FeCl_3$ X_4	$MnSO_4$ X_5	$CaCl_2$ X_6	K_2HPO_4 X_7	KH_2PO_4 X_8	
1	1	10	3	0	0	0.5	1	1.5	17.62
2	1	30	1	1	0	0.5	0.5	1.5	18.00
3	0	30	3	0	1	0.5	0.5	0.5	25.70
4	1	10	3	1	0	1	0.5	0.5	19.10
5	1	30	1	1	1	0.5	1	0.5	22.76
6	1	30	3	0	1	1	0.5	1.5	22.94
7	0	30	3	1	0	1	1	0.5	26.80
8	0	10	3	1	1	0.5	1	1.5	16.60
9	0	10	1	1	1	1	0.5	1.5	11.86
10	1	10	1	0	1	1	1	0.5	14.76
11	0	30	1	0	0	1	1	1.5	19.10
12	0	10	1	0	0	0.5	0.5	0.5	13.96

as $MgSO_4$ increased the α-galactosidase production[21]. It is possible that the high concentration within tested range of KH_2PO_4 could cause acidification of the culture, resulting in low-yield α-galactosidase production.

TABLE-3
RESULTS OF THE SCREENING EXPERIMENTS FOR α-Galactosidase PRODUCTION FROM *A. sydowii* F5

Variables	Ex_i	t	Pr>F	Ranking of Ex_i
$ZnSO_4$	0.19	0.49	0.6571	5
Soybean meal	6.9	17.52	0.0004**	1
$MgSO_4$	4.72	11.99	0.0012**	2
$FeCl_3$	0.17	0.44	0.6896	6
$MnSO_4$	0	0.02	0.9876	8
$CaCl_2$	-0.01	-0.03	0.9751	7
K_2HPO_4	1.01	2.57	0.0823	4
KH_2PO_4	-2.83	-7.18	0.0056**	3

Significant levels of regression coefficient are given as (**) 99% by t-test

Central composite design: Based on the Plackett-Burman design, where soybean meal, $MgSO_4$ and KH_2PO_4 were selected for their significant effect on the α-galactosidase production, a central composite design was used for further optimization. The coded values of those major nutrients used in central composite design were presented in Table-4. Other nutrients concentrations were set at their centre point tested in the Plackett-Burman design. For RSM based on the central composite rotary design, 20 experimental runs with different combinations of three factors were carried out. For each run, the experimental responses along with the predicted response obtained from the regression equation for the 20 combination were shown in Table-5. It could be seen from Table-5, there was a considerable variation in the α-galactosidase production depending on the concentration of soybean meal, $MgSO_4$ and KH_2PO_4 in the medium. The maximum α-galactosidase production was 31.22 U/mL in run number[20], which corresponded to the central points. The centre point in the design was repeated for six times for estimation of error. The replication at the centre point conditions resulted in higher α-galactosidase production than at other levels, the average value was 30.93 U/mL.

TABLE-4
CODED VALUES OF THE INDEPENDENT VARIABLES USED IN CENTRAL COMPOSITE ROTARY DESIGN

Independent variables	Level				
	-1.68	-1	0	1	1.68
Soybean meal (X_1) (g/L)	3.2	10	20	30	36.8
$MgSO_4$ (X_2) (g/L)	0.32	1	2	3	3.68
KH_2PO_4 (X_3) (g/L)	0.18	0.5	1	1.5	1.82

By applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to explain the α-galactosidase production:

$$Y = 30.88 + 4.49X_1 + 2.86X_2 - 1.84X_3 - 5.43X_1^2 - 3.52X_2^2 - 3.24X_3^2 - 0.29X_1X_2 - 0.07X_1X_3 + 0.14X_2X_3 \quad (2)$$

where, Y is the predicted response; X_1, X_2, X_3 are coded values of soybean meal, $MgSO_4$, KH_2PO_4, respectively.

Statistical testing the model was done by the Fisher's statistical test for analysis of variance (ANOVA) and the results were shown in Table-6. Analysis of variance (F-test) showed that the second model is well adjusted to the experimental data. The determination coefficient (R^2 = 98.06 %) implies that the sample variation of 98.06 % for α-galactosidase production was attributed to the independent variables. Here the value of R (0.9910) for eqn. 2 being close to 1 indicated a close agreement between the experimental results and the theoretical values predicted by the model equation. The coefficient of variation (CV) is the ratio of the standard error of estimate to the mean value of the observed response. A model can be considered reasonably reproducible if the coefficient of variation is not greater than 10 %. Usually, the higher the value of coefficient of variation, the lower is the reliability of experiment. Here, a lower value of coefficient of variation (6.70 %) indicated a greater reliability of the experiments performed.

TABLE-5
CENTRAL COMPOSITE ROTARY DESIGN MATRIX WITH EXPERIMENTAL AND PREDICTED VALUES OF α-galactosidase PRODUCTION BY *A. sydowii* F5

Trial number	Variables			α-Galactosidase production (U/mL)	
	X_1	X_2	X_3	Experimental	Predicted
1	-1	-1	-1	14.10	12.96
2	-1	-1	1	10.68	9.14
3	-1	1	-1	19.28	18.98
4	-1	1	1	15.88	15.72
5	1	-1	-1	22.40	22.66
6	1	-1	1	18.18	18.56
7	1	1	-1	25.88	27.52
8	1	1	1	22.76	23.98
9	-1.68	0	0	6.08	8.01
10	1.68	0	0	25.14	23.10
11	0	-1.68	0	14.88	16.14
12	0	1.68	0	27.12	25.75
13	0	0	-1.68	25.04	24.83
14	0	0	1.68	18.56	18.64
15	0	0	0	30.56	30.88
16	0	0	0	31.08	30.88
17	0	0	0	30.84	30.88
18	0	0	0	31.02	30.88
19	0	0	0	30.86	30.88
20	0	0	0	31.22	30.88

TABLE-6
ANALYSIS OF VARIANCE FOR THE RESPONSE OF α-galactosidase PRODUCTION[a]

Source	Degree of freedom	Sum of squares	Mean square	F-value	*P*>F
Linear	3	432.87	-	65.72	<0.0001
Quadratic	3	567.03	-	86.09	<0.0001
Cross product	3	0.86	-	0.13	0.9391
Total model	9	1000.76	-	50.65	<0.0001
Total error	9	19.76	2.20	-	-

[a]Coefficient of variation (CV) = 6.70; coefficient determination (R^2)=0.9806;

The Student *t*-distribution and the corresponding *P*-values, along with the parameter estimate, were given in Table-7. The *P*-values are used as a tool to check the significance of each of the coefficients which, in turn, are necessary to understand the pattern of the mutual interactions between the best variables. The smaller the magnitude of the *P*, the more significant is the

corresponding coefficient. The parameter estimate and the corresponding *P*-values suggested that the independent variables X_1, X_2 and X_3 had a significant effect on α-galactosidase production. Positive coefficient of X_1 and X_2 indicated a linear effect to increase α-galactosidase production, while negative coefficient of X_3 revealed the opposite effect. The quadratic term of them also had a significant effect, but there were no interactions.

TABLE-7
LEAST-SQUARE FIT AND PARAMETERS
(SIGNIFICANT OF REGRESSION COEFFICIENT)

Model term	Degree of freedom	Estimate	Standard Error	t value	*P* >|t|
Intercept	1	30.88	0.66	46.65	<0.0001*
X_1	1	4.49	0.40	11.20	<0.0001*
X_2	1	2.86	0.40	7.13	<0.0001*
X_3	1	-1.84	0.40	-4.58	0.0013*
X_1^2	1	-5.43	0.40	-13.52	<0.0001*
X_1X_2	1	-0.29	0.52	-0.55	0.5933
X_1X_3	1	-0.07	0.52	-0.12	0.9040
X_2^2	1	-3.52	0.40	-8.77	<0.0001*
X_2X_3	1	0.14	0.52	0.27	0.7953
X_3^2	1	-3.24	0.40	-8.06	<0.0001*

*Significant at 1 % level (P<0.01)

A representative contour curve indicating the levels of X_1 and X_2 for optimal process was shown in Fig. 4, when X_3 was fixed in the middle level. The contour plot affirms that the objective function is unimodal in nature, which show an optimum in the boundaries. It could be seen from Fig. 4, when the X_1 was at a high level (0.3-0.5, coded value), α-galactosidase production increased gradually with the increasing X_2 up to 0.3-0.5 (coded value). In this case, the yield of α-galactosidase production could keep a higher level that was simply over 30 U/mL. However, when the X_2 was enhanced further more, much higher than 0.3-0.5 (coded value), α-galactosidase production decreased with the increasing X_2.

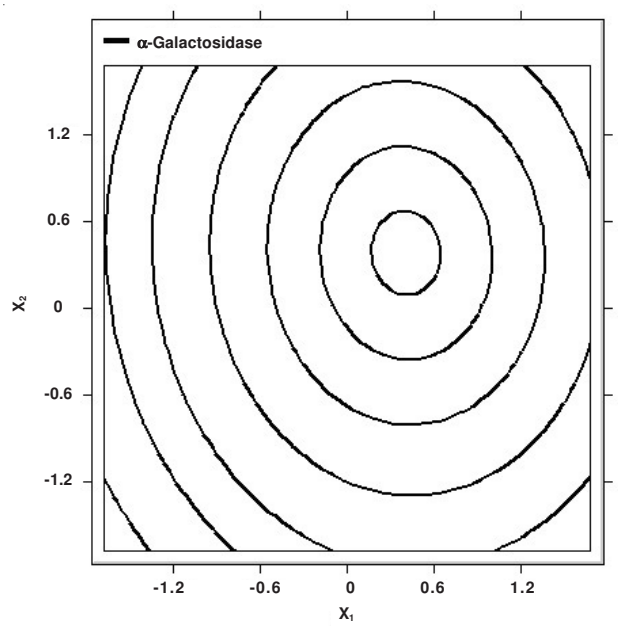

Fig. 4. Contour plot of α-galactosidase production by *A. sydowii* F5 for soybean meal and $MgSO_4$

The optimum culture medium components to obtain the greatest production of α-galactosidase by *A. sydowii* F5 of 32.60 U/mL, was obtained in a culture medium containing soybean meal 22.4 g/L, $MgSO_4$ 2.23 g/L, KH_2PO_4 0.91 g/L.

A repeated fermentation of α-galactosidase under optimal conditions was carried out. The maximal α-galactosidase level obtained was 31.88 U/mL. This value was found to be 2.20 % lower than the predicted value. This discrepancy might be due to the slight variation in experimental conditions. The optimization resulted in 5.1-fold increase of α-galactosidase production.

Conclusion

A novel strain F5, producing α-galactosidase, was isolated from Chinese wheat Qu. According to the morphological characteristics and its ITS gene sequence analysis, it was identified as *Aspergillus sydowii* F5. Statistical optimization method for fermentation medium could overcome the limitations of classic empirical methods and was proved to be a powerful tool for the optimization of α-galactosidase production by *A. sydowii* F5. A high significant quadratic polynomial obtained by the central composite design was very useful to determine the optimal concentrations of constituents that had significant effects on α-galactosidase production. Under optimal condition (soybean meal 22.4 g/L, $MgSO_4$ 2.23 g/L, KH_2PO_4 0.91 g/L), the predicted α-galactosidase activity was 32.60 U/mL. Validation experimentations were also carried out to verify the availability and accuracy of the model and results showed that the predicted value agreed with the experimental value well.

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