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Statistical Optimization of α-Galactosidase Production in Submerged Fermentation by *Streptomyces griseoloalbus* Using Response Surface Methodology

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Summary

α-Galactosidase production by a novel actinomycete strain *Streptomyces griseoloalbus* in shake flask culture was optimized using response surface methodology. Screening of variables to find their relative effect on α-galactosidase production was done using Plackett--Burman design. Out of the eleven factors screened, salinity, magnesium sulphate and temperature were found to influence the enzyme production significantly. The optimal levels of these variables and the effect of their mutual interactions on enzyme production were determined using Box-Behnken design. The interaction between salinity and magnesium sulphate concentration was found to enhance α-galactosidase production, whereas temperature exhibited an influence independent of the other two factors. Using this statistical optimization method, the α-galactosidase production was increased from 17 to 50 U/mL.

Key words: α-galactosidase, response surface methodology, *Streptomyces griseoloalbus*, submerged fermentation

Introduction

α-Galactosidase or melibiase (α-D-galactoside galactohydrolase, EC 3.2.1.22) is an exoglycosidase that cleaves the terminal non-reducing α-1→6-linked galactose residues from α-D-galactosides, including galactose oligosaccharides such as melibiose, raffinose and stachyose, and branched polysaccharides such as galactomannans and galactoglucomannans (*1*). α-Galactosidases are of particular interest in view of many potentials of their biotechnological and medicinal applications. They play a crucial role in improving the nutritional value of legume-based food. They can be applied for the reduction or removal of antinutritive galactooligosaccharides such as raffinose family sugars that cause flatulence (*2*,*3*). Microbial α-galactosidases are useful enzymes in sugar--making industry, where they eliminate raffinose and/or stachyose that negatively affect the crystallization of sucrose (*4*). Transglycosidase activity was also demonstrated in some of the α-galactosidases (*5*). The galactooligosaccharides produced by the transferase action of α-galactosidases can be used as a probiotic in functional food (*6*). α-Galactosidases have interesting applications in the pulp and paper industry (*7*). Although the existence of α-galactosidase in various organisms like plants (*8*), animals (*9*) and microorganisms such as fungi (*10*,*11*), yeast (*12*), bacteria (*13*,*14*) and actinomycetes (*15*–*17*) has been known for a number of years, mangrove actinomycetes, which have immense potential as a source of exoenzymes, are yet to be harnessed as a source of α-galactosidase for commercial application.

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The production and characterization of an enzyme are necessary for its industrial application. The first step in achieving this goal is the establishment of a suitable enzyme production technology. The conventional method of medium optimization involves changing one independent variable at a time while fixing all the others at a certain level. This method is very time-consuming and requires a large number of experiments to determine the optimum levels. It does not include the interactive effects among the variables and is therefore unreliable (*18*). Statistical optimization is preferable because it is helpful in evaluating the interactions among the possible influencing parameters with limited number of experiments (*19*). It involves a specific design of experiments, which minimizes the error in determining the effect of parameters, and the results are achieved in an economical manner. Plackett-Burman design and Box--Behnken design are among the most widely used statistical techniques for optimization of biological processes.

The Plackett-Burman experimental design is a two--level factorial design, which identifies the critical physicochemical parameters required for elevated enzyme production by screening *N* variables in *N*+1 experiments (*20*), but it does not consider the interaction effect among the variables. The variables that are found significant in this initial screening can be further optimized using response surface methodology (RSM). To the best of our knowledge, there are no reports on the application of statistical methods for the optimization of α-galactosidase production in submerged fermentation. Here we attempt to increase the production of α-galactosidase by using *Streptomyces griseoloalbus* through the optimization of process parameters applying statistical methods.

Materials and Methods

Microorganism

The actinomycete *Streptomyces griseoloalbus* producing large amounts of extracellular α-galactosidase was isolated in our laboratory from a soil sample collected from the mangrove regions along the West Coast of India and deposited in Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India with accession number 7447. The organism was maintained at 4 °C on starch casein agar slants and was subcultured fortnightly.

Medium composition and culture conditions

The basal medium used for α-galactosidase production contained (in g/L): locust bean gum 10, yeast extract 3, $(NH_4)_2HPO_4$ 3.03, KH_2PO_4 1.0, $MgSO_4{\cdot}7H_2O$ 0.49, and 1 mL of trace elements solution. The trace elements solution was composed of (in g/L): $FeSO_4{\cdot}7H_2O$ 0.1, $MnCl_2{\cdot}4H_2O$ 0.1 and $ZnSO_4{\cdot}7H_2O$ 0.1. The pH of the medium was adjusted to 6.0. The 250-mL Erlenmeyer flasks containing 90 mL of the medium were inoculated with 10 mL of 48-hour grown inoculum containing $3{\cdot}10^6$ CFU/mL and were incubated in a rotary shaker for 7 days at 30 °C and 150 rpm. Aliquots were withdrawn at regular time intervals according to the experimental set-up and the culture supernatant obtained after centrifugation at 10 000 rpm for 20 min was used as the enzyme preparation.

Optimization of enzyme production

The optimization of physicochemical factors for α-galactosidase production by *S. griseoloalbus* was carried out in two stages.

Screening of physicochemical factors using Plackett-Burman design

Plackett-Burman experimental design consisting of a set of 12 experiments was used to determine the relative significance of 11 factors that influenced α-galactosidase production by *S. griseoloalbus* in submerged fermentation. The complete experimental design is shown in Table 1. The factors or independent variables considered for study included 6 physical factors (X_1 to X_6, representing pH, temperature, inoculum size, inoculum age, incubation period and agitation, respectively) and 5 nutritional factors (X_7 to X_{11}, representing carbon source, yeast extract, $MgSO_4$, $FeSO_4$ and salinity, respectively). All variables except the carbon source were numerical factors and were investigated at two widely spaced levels designated as –1 (low level) and +1 (high level). The carbon source was studied as a categorical factor and the two carbon sources studied were locust bean gum (LBG) and chickpea flour (CPF). The level of salinity was varied by varying the amount of sea water (having a salinity of 3.5 mg/mL) and distilled water in the medium.

Optimization of significant variables using Box-Behnken design

Response surface methodology using Box-Behnken design (*21*) was adopted to find the optimum levels of the significant variables (salinity, $MgSO_4$ and temperature) and the effects of their mutual interactions on enzyme production. A total of 17 experiments were carried out. Each independent variable was studied at three different levels (low, medium and high, coded as –1, 0 and +1, respectively). The center point of the design was replicated five times for the estimation of error. The experimental design used for the study is shown in Table 2. The software Design-Expert (Version 6.0.6, Stat-Ease, Inc., Minneapolis, USA) was used for experimental design, data analysis and quadratic model building. Each run was performed in triplicate and the average of α-galactosidase yield obtained was taken as the experimental values of the dependent variable or response (*Y*), while predicted values of the response were obtained from quadratic model fitting techniques. A multiple regression analysis of the data was carried out to define the response in terms of the independent variables. The response surface graphs were obtained to understand the effect of variables individually and in combination, and to determine their optimum levels for maximum α-galactosidase production.

Validation of the model

The statistical model was validated with respect to all three significant variables within the design space. A random set of six experimental combinations under the optimized conditions was used for validation of the statistical model.

Table 1. Plackett-Burman experimental design matrix with α-galactosidase production by *S. griseoloalbus*

Trial no.	Variables											α-Galactosidase yield
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	U/mL
1	+1	+1	–1	+1	–1	–1	LBG	+1	+1	+1	–1	0.96
2	–1	–1	–1	+1	+1	+1	LBG	+1	+1	–1	+1	2.75
3	+1	+1	–1	+1	+1	–1	CPF	–1	–1	–1	+1	23.70
4	–1	+1	+1	–1	+1	–1	LBG	–1	+1	+1	+1	14.10
5	–1	–1	–1	–1	–1	–1	LBG	–1	–1	–1	–1	2.30
6	–1	+1	–1	–1	–1	+1	LBG	+1	–1	+1	+1	25.08
7	–1	–1	+1	+1	+1	–1	CPF	+1	–1	+1	–1	3.06
8	–1	+1	+1	+1	–1	+1	CPF	–1	+1	–1	–1	6.20
9	+1	+1	+1	–1	+1	+1	CPF	+1	–1	–1	–1	13.10
10	+1	–1	+1	+1	–1	+1	LBG	–1	–1	+1	+1	24.40
11	+1	–1	–1	–1	+1	+1	CPF	–1	+1	+1	–1	4.60
12	+1	–1	+1	–1	–1	–1	CPF	+1	+1	–1	+1	8.90

+1, high level; –1, low level
LBG, locust bean gum; CPF, chickpea flour
X_1, pH at a high level of 7.0 and low level of 5; X_2, temperature at a high level of 33 °C and low level of 27 °C; X_3, inoculum size at a high level of $4.5 \cdot 10^6$ CFU and a low level of $1.5 \cdot 10^6$ CFU; X_4, age of inoculum at a high level of 96 h and a low level of 48 h; X_5, incubation period at a high level of 120 h and a low level of 72 h; X_6, agitation at a high level of 175 rpm and a low level of 125 rpm; X_7, carbon source as a categorical variable; X_8, yeast extract at a high level of 4 g/L and a low level of 2 g/L; X_9, $MgSO_4$ at a high level of 0.98 g/L and a low level of 0 g/L; X_{10}, $FeSO_4$ at high level of $2 \cdot 10^{-5}$ g/L and a low level of 0 g/L; X_{11}, salinity at a high level of 1.92 mg/mL and a low level of 0.96 mg/mL

Table 2. Box-Behnken design matrix with biomass, experimental and predicted values of α-galactosidase production by *S. griseoloalbus*

Trial no.	Variables						γ(biomass)	α-Galactosidase yield	
	γ(salinity)		γ($MgSO_4$)		Temperature				
	mg/mL		g/L		°C		mg/mL	U/mL	
	Coded	Actual	Coded	Actual	Coded	Actual		Experimental	Predicted
1	0	1.92	–1	0	–1	27	1.7	27.8	28.5
2	0	1.92	0	0.13	0	30	2.9	35.6	35.6
3	0	1.92	+1	0.25	+1	33	2.6	33.8	33.1
4	+1	2.49	+1	0.25	0	30	3.1	35.9	36.1
5	0	1.92	0	0.13	0	30	2.9	35.8	35.6
6	+1	2.49	0	0.13	–1	27	1.8	28.6	28.7
7	–1	1.34	0	0.13	–1	27	1.8	28.1	27.5
8	0	1.92	0	0.13	0	30	2.9	35.7	35.6
9	0	1.92	0	0.13	0	30	2.9	35.5	35.6
10	–1	1.34	0	0.13	+1	33	2.1	31.4	31.2
11	–1	1.34	–1	0	0	30	2.6	34.3	34.1
12	0	1.92	0	0.13	0	30	2.9	35.2	35.6
13	+1	2.49	0	0.13	+1	33	2.5	33.4	34.0
14	0	1.92	–1	0	+1	33	2.3	32.6	33.0
15	+1	2.49	–1	0	0	30	2.7	35.2	34.3
16	–1	1.34	+1	0.25	0	30	2.2	31.4	32.4
17	0	1.92	+1	0.25	–1	27	2.0	29.1	28.6

Enzyme assay

The activity of α-galactosidase was routinely determined according to the method of Dey and Pridham (*22*) using *p*-nitrophenyl-α-D-galactopyranoside (*p*NPG), with minor modifications. The *p*NPG hydrolyzing activity was estimated by incubating 100 μL of enzyme with 50 μL of 2 mM *p*NPG and 850 μL of 0.1 M McIlvaine buffer (citrate-Na_2HPO_4, pH=7.0) at 55 °C for 10 min. The reaction was terminated by the addition of 2 mL of 1 M sodium carbonate. The *p*-nitrophenol released was estimated spectrophotometrically by absorbance at 400 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated one μmol of *p*-nitrophenol per minute under the assay conditions.

Analytical procedures

Total soluble protein in the culture filtrate was determined by the method of Lowry *et al.* (*23*) using bovine serum albumin as standard. The biomass was estimated by determining the dry mass of the mycelium after it had been dried to constant mass in an oven at 80 °C. The salinity of sea water was measured using salinometer. All experiments were carried out in triplicate to check the reproducibility of the results.

Results and Discussion

Screening of parameters using Plackett-Burman design

S. griseoloalbus produced 17 U/mL of α-galactosidase in the basal medium (*24*). The Plackett-Burman experimental design used for the screening of physicochemical factors influencing α-galactosidase production by *S. griseoloalbus* along with the corresponding experimental and predicted values of response is shown in Table 1.

Table 3 shows the coefficient of each variable (indicative of its effect), degree of freedom, standard error, and Prob>F value. The positive or negative sign of the coefficient of a tested variable indicates whether an increase in the level of that tested variable enhanced or inhibited α-galactosidase production within the tested limits. The variables X_1, X_2, X_3, X_6, X_7, X_{10} and X_{11} had a positive effect on α-galactosidase production, while the rest had a negative influence. The Prob>F value is used as a tool to check the significance of each variable. A Prob>F lower than 0.0500 indicates that the effect of the parameter in question can be considered as significant at 95 % confidence level. Therefore, the variables X_2, X_9 and X_{11} were found to be statistically significant in affecting α-galactosidase production, whereas all the other variables were insignificant. The magnitude of the coefficient of each variable indicated the intensity of its effect on the studied response. The greater the magnitude, the higher was the significance of that variable. Thus salinity had the highest influence on α-galactosidase production compared to the other factors. This is probably because higher salinity is essential for the normal growth of the actinomycete *S. griseoloalbus*, which was originally isolated from mangrove soil sample. Salinity of the medium was adjusted by varying the amount of sea water and distilled water rather than adding NaCl, because the sea water is rich in numerous micronutrients other than NaCl, which can promote the growth of mangrove and marine microorganisms.

Optimization of significant variables using Box-Behnken design

Based on the results of screening experiments by Plackett-Burman design, those variables with Prob>F value lower than 0.0500 were selected and further optimized using Box-Behnken design. The Box-Behnken design along with the corresponding experimental and predicted values of the α-galactosidase yield and biomass is given in Table 2. The data were analyzed by multiple regression analysis using the Design-Expert software and the following equation was obtained:

$$Y=35.6285+0.9827x_1+0.0297x_2+2.2344x_3-0.9096x_1^2-0.4694x_2^2-4.298x_3^2+0.8991x_1x_2+0.3866x_1x_3-0.0113x_2x_3 \quad /1/$$

where Y is the predicted value of α-galactosidase yield and x_1, x_2 and x_3 are the coded values for salinity, $MgSO_4$ concentration and temperature, respectively.

The experimental data were statistically analyzed by the analysis of variance (ANOVA) and the results are

Table 3. Results of screening experiments using Plackett-Burman design

Factor (variable)	Coefficient estimate	Degree of freedom	Standard error	Prob>F
Intercept	10.7730	1	0.9333	0.0055
pH (X_1)	1.8405	1	0.9333	0.1057
Temperature (X_2)	3.1055	1	0.9333	0.0208
Inoculum size (X_3)	0.8745	1	0.9333	—
Inoculum age (X_4)	–0.5811	1	0.9333	—
Incubation period (X_5)	–0.5471	1	0.9333	—
Agitation (X_6)	1.9311	1	0.9333	0.0933
Carbon source (X_7)	1.1628	1	0.9333	—
Yeast extract (X_8)	–1.7945	1	0.9333	0.1125
$MgSO_4$ (X_9)	–4.5028	1	0.9333	0.0048
$FeSO_4$ (X_{10})	1.2655	1	0.9333	—
Salinity (X_{11})	5.7195	1	0.9333	0.0017

shown in Table 4. The ANOVA of the quadratic regression model indicated that the model was highly significant, as the F-value for the model was 24.67. There was only 0.02 % chance that the 'model F-value' this large could occur due to noise. The Prob>F value of the model was 0.0002, which also confirmed that the model was highly significant. The coefficient estimate and the corresponding Prob>F values (Table 4) suggested that among the independent variables studied, salinity and temperature as well as the squared terms of these two variables had a significant effect on α-galactosidase production by *S. griseoloalbus*.

The coefficient of variation (CV), indicative of the degree of precision with which the treatments are compared, had a lower value (2.38 %), showing greater reliability. Also, the multiple regression coefficient (R^2) had a value of 0.9694, indicating that the model could explain up to 96.94 % of the variability of the response. The value of R^2 (0.9694) indicated a good agreement between the experimental and predicted values of α-galactosidase yield. The signal to noise ratio (adequate precision) for the model was higher than 4 (14.21), indicating a good fit.

The effect of the interaction of various physicochemical parameters on α-galactosidase production by *S. griseoloalbus* was investigated by plotting the response surface curves against any two independent variables while keeping the third independent variable at the '0' level. Thus three response surfaces were obtained by considering all the possible combinations. The interactive roles of salinity, $MgSO_4$ concentration and temperature on α-galactosidase production by *S. griseoloalbus* are illustrated in the three-dimensional curves of the calculated response surface shown in Figs. 1–3. An increase in α-galactosidase production was observed when the salinity was increased together with an increase in the concentration of $MgSO_4$ (Fig. 1), and the highest enzyme activity (35.9 U/mL, Table 2) was obtained when the salinity and $MgSO_4$ concentration were 2.49 mg/mL and 0.25 g/L, respectively. Within the tested limits, when the salinity was low, the α-galactosidase yield was also low, regardless of the $MgSO_4$ concentration and *vice versa*. In the initial screening experiments using Plackett-Burman design, $MgSO_4$ was found to exhibit a negative effect on α-galactosidase production at the higher tested level (0.98 g/L). The results of final optimization with RSM

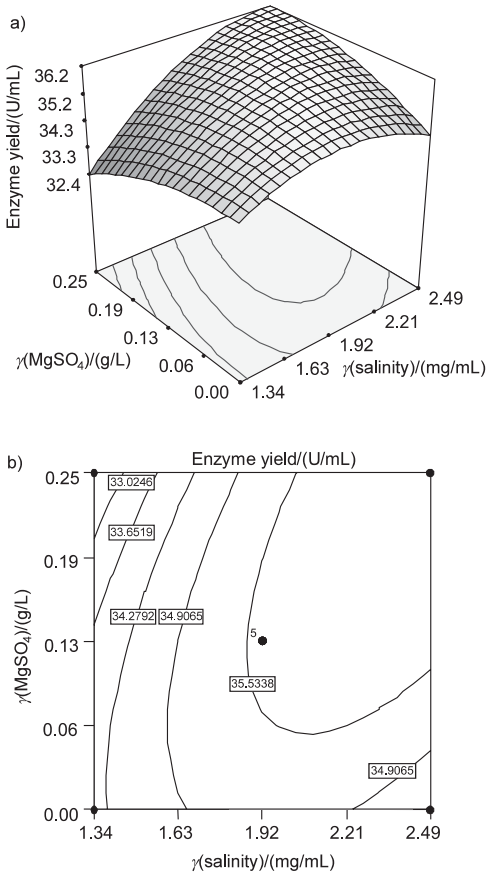

Fig. 1. The three-dimensional response surface (a) and the contour plot (b) showing the relative influence of salinity and $MgSO_4$ on α-galactosidase production by *S. griseoloalbus*

Table 4. Analysis of variance (ANOVA) for the response surface quadratic model

Source	Sum of squares	Degree of freedom	Mean square	F-value	Prob>F
Model	137.1738	9	15.241530	26.015930	0.0002
x_1	7.7261	1	7.726184	13.187900	0.0095
x_2	0.0070	1	0.007057	0.012045	0.9179
x_3	39.9407	1	39.940790	68.175340	<0.0001
x_1^2	3.4836	1	3.483672	5.946315	0.0493
x_2^2	0.9278	1	0.927831	1.583724	0.2600
x_3^2	77.7820	1	77.782040	132.766900	<0.0001
$x_1\,x_2$	3.2340	1	3.234063	5.520254	0.0560
$x_1\,x_3$	0.5979	1	0.597993	1.020720	0.3580
$x_2\,x_3$	0.0005	1	0.000518	0.000883	0.9777
Residual	4.1009	7	0.585854		
Lack of fit	4.1009	3	1.366993	24.440000	0.0049
Pure error	0	4	0		
Corrected total	141.2748	16			

suggest that a concentration of at least 0.25 g/L of $MgSO_4$ is necessary for enhanced α-galactosidase production, as the enzyme activity decreased in the absence of $MgSO_4$. In the present study the α-galactosidase production was found to be growth associated since higher activities correlated with higher biomass (Table 2). Thus it was further emphasized that a relatively higher salinity was essential for the better growth of *S. griseoloalbus*. It has already been established that the microbial production of α-galactosidase varies with growth rate (*25*) and its activity increases with the increase in biomass concentration (*11*).

The response surfaces in Figs. 2 and 3 show the effect of temperature on α-galactosidase production in combination with the other two variables, where it can be seen that the influence of temperature is independent from the other two variables. Growth of the organism and α-galactosidase production increased with the increase in temperature up to the optimum level, after which growth declined and the associated enzyme production also dropped. The effect was the same at all concentrations of sea water and $MgSO_4$. The results indicate that the independent influence of temperature on α-galactosidase production masked the effects of the other two variables.

From the response surface graphs it could be concluded that the optimum values of salinity, $MgSO_4$ and temperature for the maximum production of α-galactosidase by *S. griseoloalbus* were in the range of 1.92–2.49 mg/mL, 0.13–0.25 g/L and 30–31 °C, respectively.

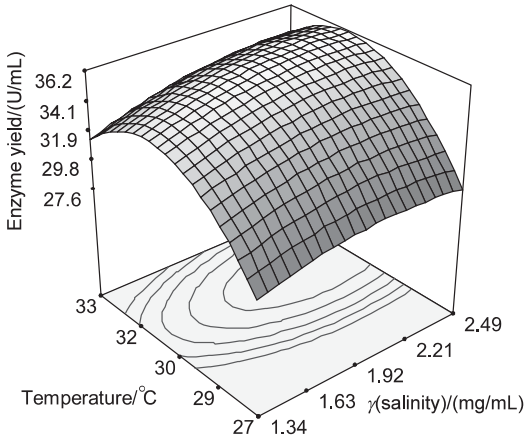

Fig. 2. The influence of temperature and salinity on α-galactosidase production by *S. griseoloalbus*

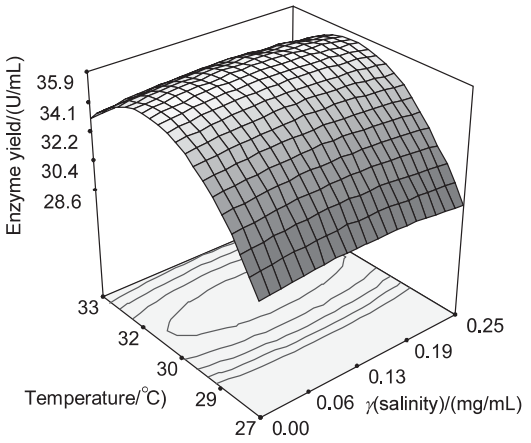

Fig. 3. The influence of temperature and $MgSO_4$ on α-galactosidase production by *S. griseoloalbus*

Validation of the model

In order to validate the adequacy of the model, a total of six verification experiments were conducted under various fermentation conditions within the experimental range. The design matrix showing the uncoded values of the independent variables along with the experimental and predicted values of α-galactosidase yield are given in Table 5. The results indicate that the model was satisfactory and could enhance the enzyme yield considerably.

Optimization of α-galactosidase production by *Streptomyces erythrus* under submerged fermentation was reported by Elshafei *et al.* (*17*), but it did not deal with statistical optimization of the process. Srinivas *et al.* (*26*) reported the use of Plackett-Burman design for rapid screening of nutrients for the production of α-galactosidase by *Aspergillus niger* MRSS 234 in solid-state fermentation system and a 73 % increase in enzyme production was achieved. Statistical optimization of α-galactosidase production by *S. griseoloalbus* resulted in 194 % increase compared to the unoptimized medium.

Conclusions

There is a growing acceptance for the use of statistical experimental designs in biotechnology. The application of statistical design for screening and optimization

Table 5. Validation experiments

Trial no.	γ(salinity)	γ($MgSO_4$)	Temperature	α-Galactosidase yield U/mL	
	mg/mL	g/L	°C	Experimental	Predicted
1	2.05	0.17	30	50.3	36.0
2	2.10	0.09	30	48.6	36.0
3	2.38	0.22	31	35.3	36.4
4	2.16	0.15	30	32.1	36.2
5	2.34	0.19	30	31.6	36.3
6	2.19	0.11	30	49.8	36.0

of process parameters allows quick identification of important factors and interactions between them. In the present study, Box-Behnken design was useful in studying the physicochemical factors that supported the enhanced production of α-galactosidase by *S. griseoloalbus* under submerged fermentation.

Currently, there is a lot of interest in the scientific community around the world in exploiting novel microorganisms. Marine and mangrove microorganisms, with their unique nature, differ very much in many aspects from their terrestrial counterparts and are known to produce diverse spectra of novel useful substances. In this context, the results obtained during the course of this study indicate the scope for utilization of mangrove actinomycetes for extracellular α-galactosidase production through submerged fermentation.

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