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USE OF RESPONSE SURFACE METHODOLOGY FOR OPTIMIZATION OF BASAL NUTRIENTS IN PRODUCTION OF AMYLASE BY *STREPTOMYCES GANCIDICUSASD*_KT852565

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ABSTRACT

Nutritional requirements at optimal level can contribute considerably to the production cost and the bioprocess economics. Media optimization using response surface methodology is one of the used methods to ameliorate the bioprocess economics. In the present study, Amylase production by Marine actinomycetes *Streptomyces gancidicus_ASD* was effectively enhanced by response surface methodology. An OFAT (one factor at a time) based statistical screening procedure was adopted to determine the most important factor affecting amylase production. The variables are screened and results show that sucrose and Beef extract concentrations influence the most as carbon and nitrogen source on amylase production respectively. A Central Composite Design was conducted to optimize the four selected factors. Statistical analyses of the data of model fitting were done by using Design expert 10.0(stat-Ease). Results show a maximum predicted Amylase yield of 11460.34 IU/ml when using 1.05 % sucrose, 0.608 % Beef extract, 7.1 pH and 40.35 °C Temperature. The predicted value is approximately 1.24 fold much higher than the original production (9248 IU/mL) determined by the conventional one-factor-at-a-time optimization method which can be applied in bioprocess for increased amylase yield.

KEYWORDS: **Marine actinomycetes, Streptomyces gancidicus ASD, Response surface Methodology, One Factor at a time method, Annova, Stastical method.**

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INTRODUCTION

Amylases are enzymes which hydrolyze starch molecules to give dextrins and sugars. This enzyme find number of applications in food, beverage, pharmaceutical anddetergent industries[1] Among all industrial enzymes, the global market for starch processing enzymes is around US$ 156 million and cost of enzymes used in the liquefaction process reported 24 % of the total process cost[2]. Though amylases originate from different sources including plants, animals and microorganisms, industrially they are mainly produced by employing microorganisms[3].The production of amylase is dependent on the strain, composition of media, methods of cultivation, cell growth, nutrient requirements, metal ions, pH, temperature, time of incubation and thermo stability[4]. Submerged fermentation by shake flask as well as stirred fermentor has been reported by many workers for large scale production[5]. Amylases are among the most important enzymes and are of great significance having approximately 25% of enzyme market. They find potential applications in food, pharmaceutical and fine chemical industries. Amylases derived from plants and mesophilic bacteria are very expensive and unstable. Therefore thermophilic organisms show promise for production of thermoactive and thermo-stable enzymes.Optimization of process variables following screening and isolation of potential amylase producer is a prerequisite to enhance production yields in order to suit the organism for industrial application. By optimization of process variables one can find out the significant parameters that enhance the yields[6]. The best fermentation technique for optimization involves submerged fermentation (SmF) [7]. The response surface method (RSM) is a statistical method that involves individual and interaction effects to account for curvature, to improve optimal process settings, and to troubleshoot process problems and weak points [8] and to build models, evaluating the effects of factors for desirable response[9]. Hence, statistically based experimental designs are preferred to evaluate the influence of medium components in fermentations for the production of industrially important enzymes, proteins[10].Therefore, a combination of 'one-at-a-time' approach and RSM is well suited for the study of main and interaction effects of distinct factors in amylase production. The main aim of this study is to increase the production of amylase production by *Streptomyces gancidicus_ASD* isolated from marine sediments with the accession no. KT852565 in NCBI. The two step optimization process, namely OFAT and Response surface methodology (RSM). These were being widely employed by most of the industries to scale up their production. The present study was carried out with an aim to investigate the optimum fermentation conditions for amylase production by *Streptomyces gancidicu*_ASD by response surface methodology statistical experimental designs.

MATERIALS AND METHODS

Culture Strain

Streptomyces gancidicus_ASD the amylase producer actinomycetes strain was isolated from marine sediment. The strain was identified by 16S rRNA gene followed by BLAST homology search. The nucleotide sequences have been deposited with NCBI database under accession number KT852565.

METHODOLOGY

From the literatures[11-14] explained the developments and current practices in the area of process improvement recommend employing RSM for expressing the output parameters (responses), in terms of input variables.

Response Surface Methodology (RSM)

RSM is a collection of statistical and mathematical methods that are useful for the modelling and analysing engineering problems. In this technique, the main objective is to optimize the response surface that is influenced by various process parameters[15-17]. RSM also quantifies the relationship between the controllable input parameters and the obtained response surfaces. The design procedure is as follows

(i) Designing of a series of experiments for adequate and reliable measurement of the response of interest.
(ii) Developing a mathematical model of the second order response surface with the best fittings.
(iii)Finding the optimal set of experimental parameters that produce a maximum or minimum value of response.
(iv)Representing the direct and interactive effects of process parameters through two and three dimensional plots.

Design of Experiments for RSM

RSM designs allow us to estimate interaction and even quadratic effects, and therefore give us an idea of the (local) shape of the response surface under investigation. Box-Behnken designs and central composite designs are efficient designs for fitting second order polynomials to response surfaces, because they use relatively small number of observations to estimate the parameters. Rotatability is a reasonable basis for the selection of a response surface design. The purpose of RSM is optimization and the location of optimum is unknown prior to running the experiment, it makes sense to use a design that provides equal precision of estimation in all directions. For such purposes, Central Composite Design (CCD) - spherical or face centered and Box – Behnken design are the commonly used experimental design models for three level three factor experiments.

ANOVA

Analysis of variance, ANOVA, is a statistical decision making tool used for detecting any differences in average performances of tested parameters[18]. It employs sum of squares and F statistics to find out relative importance of the analysed processing parameters, measurement errors and uncontrolled parameters. Analysis of variance (ANOVA) was used to check the adequacy of the model for the responses in the experimentation.

Primary optimization stage: One-Factor-at-a-time method study

Various cheap carbon and nitrogen sources were selected for one factor- at-a-time method (OFAT) study using *Streptomyces gancidicus*_ASD-KT852565 for amylase production**.** To test the effect of carbon sources, each carbon source (sucrose, fructose, dextrose, lactose, glucose, galactose, mannose, trehalose, melibose, xylose purchased from Himedia, Mumbai) were added at 1 % (w/v) , by replacing starch and the influence of nitrogen sources by replacing casein with each nitrogen source such as (peptone, yeast extract, ammonium sulphate, beef extract, sodium acetate, meat extract, urea, nutrient broth, potassium nitrate, sodium citrate from Himedia, Mumbai) at 0.3% level.

Central Composite Design (CCD)

RSM was carried out using CCD design, optimize for further process to identify the interactions between the significant factors obtained from OFAT. The 4 variables chosen in this experiment were (C source) Sucrose, (N source) Beef extract, pH and temperature with 5 coded levels (-α,-1, 0, +1, + α) were used for their combined influence on amylase production. 30 experimental trials were carried out with 16 factorial points, 8 axial points with α = 2 and 6 replication of central points. In developing regression equation, the test factors were coded according to the Equation (1).

$$X_i = (X_i - X_0)/\delta X_i \quad (1)$$

Where x_i is the dimensionless coded value of the i^{th} independent variable; X_i the natural value of the i^{th} independent variable; X_0 the natural value of the i^{th} independent variable at the centre point and $\delta X_i$is the difference in effect.The response data obtained from the design were fitted with a second order polynomial. The general polynomial equation is as follows in Equation (2).

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 \quad (2)$$

Where, Y is the predicted response, β_0 is the model constant, β_1, β_2, β_3, $\beta_4$the linear co-efficient, β_{11}, β_{22}, β_{33}, β_{44} the squared co-efficient and β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34} the interaction co-efficient.

Data Analysis

Design expert 10.0 (Stat-Ease, Inc., Minneapolis, USA) was used for the regression analysis of the experimental data obtained. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2 and analysis of variance (ANOVA). The significance of the model, an optimum value of parameters was assessed by the determination coefficient, correlation coefficient and statistical testing of the model was made by Fisher's test[19].

RESULTS

Primary optimization stage: One-Factor-at-a-time method study

The maximum amylase activity was determined as 9248 IU/ml for sucrose and fructose as carbon and nitrogen source respectively with control medium (traditional actinomycetes medium-starch casein agar) observed to be 394 IU/mL.Fig.1. When compared to the other Nitrogen sources beef extract enhanced the amylase activity given in Fig.2.Ours is the first study on enhanced amylase production using sucrose and beef extract as simple source in modified actinomycetes media. Hence sucrose and beef extract was selected as the potential carbon and nitrogen source respectively for production of amylase from *Streptomyces gancidicus*_ASD.

Experimental design of RSM: Central Composite Design (CCD)

Based on the result of OFAT approach optimum levels of significant factors and the effect of their interactions on amylase production was determined by CCD experiments. The following variables sucrose, Beef extract was selected based on result of the OFAT method further pH and temperature were added for the optimization by RSM. The experimental design was carried out to determine the parameter ranges together with coded and actual values of the 4 independent variables for amylase production presented in Table 1 and Table 2 shows the results of 30 run from CCD experiments for studying the effects of 4 independent variables on amylase production. The maximum experimental value for amylase production was 11450 IU/mL based on RSM. The regression analysis data were fitted to a quadratic model and the second order regression equation obtained was full actual model on amylase production is shown in Equation (3).

$$Y = +11450 + 441.67 * A + 316.67 * B + 150.00 * C + 58.33 * D - 237.50 * AB + 100.00 * AC + 125.00 * AD - 650.00 * BC + 75.00 * BD - 212.50 * CD - 2352.08 * A^2 - 2614.58 * B^2 - 2252.08 * C^2 - 2614.58 * D^2 \quad (3)$$

Where Y is enzyme (amylase) activity IU/mL, A is sucrose (g/L), B is Beef extract (g/L), C is temperature (ºC) and D is pH. The statistical significance of Eq.3was checked by an F-test; and the analysis of variance (ANOVA) for response surface quadratic model is given in Table 3. ANOVA for amylase production from the Table 3 the Model F-value of 19.03 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. Values of "Prob> F" less than 0.0500 indicate model terms are significant. In this case A^2, B^2, C^2, D^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The goodness of the model can be made by the detremination coeffecient (R^2) and the correlation coeffecient (R)[22-23].The R^2 value of 0.9467 gives 94.67% variability in the production of amylase, and about 5.3% total variation cannot explained by the model. The close the value of R (R= multiple correlation coeffecient) to 1, the better the correlation between the experimental and predicted values [24]. The adequate precision is used to measure the ratio of signal to noise, it is believed to be desirable greater 4. Here the value 12.171 shows that the polynomial quadratic model is of an adequate signal, and can be used to navigate the design space. The "Pred R-Squared" of 0.6930 is not as close to the "Adj R-Squared" of 0.8969 as one might normally expect as presented in Table 4**.**The coefficient estimates of Eq. (3), along with the corresponding *P* values are given in Table 3.The *P* values implied the significance of each coefficient and it is important in turn to indicate the pattern of mutual interaction between the coefficients. The smaller the value of *P*, the more significant is the corresponding coefficient [25]. It can be seen from the Table 3 that the 3 linear coefficient (A, B and C), all quadratic coefficients are highly significant. The insignificant coefficients were not omitted, since it is a hierarchical model.Fig.3a-3b. Response surface representation provides a method to show the relation between the response and experimental levels of each variable. These figures are useful in understanding the kind of interaction among test variables to deduce the optimum conditions. This technique has been widely adopted for optimizing the process of enzymes and peptides [26]. Fig. 3a, 3b and 3c depicts the contour plot showing the effects of sucrose and beef extract to have a significant effect on amylase production, and it is positively interacted with the other 2 factors. As seen in Fig. 3d and 3e, the optimum activity was obtained at higher Beef extract concentration with pH and temperature at the same range as mentioned earlier. Further, Fig. 3f shows that pH at the range of 5-7, temperature at the range of 30 – 40ºC increases the amylase production. Therefore the results apparently prove that sucrose and beef extract was found to be the important component interacted with pH and temperature for the enhanced amylase activity. The effect of variation in level of all 4 independent variables on amylase production has been shown in the perturbation graph Fig. 4.From the graph it can be concluded that the sucrose plays an important role in enhanced activity and production, followed by temperature with beef extract and temperature. Normal probability versus residuals was plotted in a graph Fig. 5which showed that data were close to the straight line and situated at both sides indicating the model is fairly good.

DISCUSSION

Marine actinomycete streptomyces showing 391.45 IU/ml as its amylase activity with starch-casein agar Similar results was reported by [20] and also It is an evident that sucrose was found to be the best carbon source for the enhanced amylase activity when compared to starch and other sugars tested. Similar result was obtained by [21].

Validation of the optimum condition

In order to verify the optimization results and to determine the accuracy of the experiment run by RSM (activity of 11450.34 IU/ml), Laboratory test was conducted in duplicate with the optimized media containing 1.054% sucrose, 0.573% beef extract, 6.9pH and 40.4º C. On experimentation, observed response of amylase yield from the *Streptomyces gancidicus*_ASD KT852565 was 11460.00 IU/mL strongly proves the suitability of the developed model.

Table 1
Range of value for response surface method

Factors	Independent Variables	Coded levels				
		-α	**-1**	**0**	**+α**	**+1**
A	**Sucrose %**	0	0.5	1	1.5	2.0
B	**Beef extract %**	-0.35	0.1	0.5	1	1.45
C	**Temperature(degree)**	20	30	40	50	60
D	**pH**	3	5	7	9	11

Table 2

Coded experimental design and results for the response surface of maximum Amylase production of S.gancidicusas a function of Sucrose(A), Beef extract (B), Temperature (C), pH(D)

Rows		Coded values				
Numbers	order	A	B	C	D	Amylase activity IU/ml
9	1	0.5	0.1	30	9	1200
21	2	1	0.55	20	7	1100
8	3	1.5	1	50	5	700
15	4	0.5	1	50	9	1200
11	5	0.5	1	30	9	700
20	6	1	1.45	40	7	2100
29	7	1	0.55	40	7	11450
26	8	1	0.55	40	7	11450
27	9	1	0.55	40	7	11450
2	10	1.5	0.1	30	5	700
13	11	0.5	0.1	50	9	1200
1	12	0.5	0.1	30	5	300
7	13	0.5	1	50	5	1200
12	14	1.5	1	30	9	4200
4	15	1.5	1	30	5	1200
16	16	1.5	1	50	9	700
6	17	1.5	0.1	50	5	4200
23	18	1	0.55	40	3	300
25	19	1	0.55	40	7	11450
10	20	1.5	0.1	30	9	900
22	21	1	0.55	60	7	4200
18	22	2	0.55	40	7	4200
14	23	1.5	0.1	50	9	900
5	24	0.5	0.1	50	5	700
17	25	0	0.55	40	7	300
28	26	1	0.55	40	7	11450
19	27	1	-0.35	40	7	300
30	28	1	0.55	40	7	11450
3	29	0.5	1	30	5	4200
24	30	1	0.55	40	11	2100

Table 3

Analysis of variance (ANOVA) for the quadratic modal of Amylase Production obtained from the experimental results

Source	Sum of Squares	df	Mean Square	F Value	p-value Probability> F
Model	4.840E+008	14	3.457E+007	19.03	< 0.0001*
A-Sucrose	*4.682E+006*	*1*	*4.682E+006*	*2.58*	*0.1293*
B-Beef extract	*2.407E+006*	*1*	*2.407E+006*	*1.32*	*0.2678*
C-temperature	*5.400E+005*	*1*	*5.400E+005*	*0.30*	*0.5937*
D-pH	*81666.67*	*1*	*81666.67*	*0.045*	*0.8350*
AB	*9.025E+005*	*1*	*9.025E+005*	*0.50*	*0.4917*
AC	*1.600E+005*	*1*	*1.600E+005*	*0.088*	*0.7707*
AD	*2.500E+005*	*1*	*2.500E+005*	*0.14*	*0.7159*
BC	*6.760E+006*	*1*	*6.760E+006*	*3.72*	*0.0729*
BD	*90000.00*	*1*	*90000.00*	*0.050*	*0.8269*
CD	*7.225E+005*	*1*	*7.225E+005*	*0.40*	*0.5378*
A^2	*1.517E+008*	*1*	*1.517E+008*	*83.52*	*< 0.0001**
B^2	*1.875E+008*	*1*	*1.875E+008*	*103.20*	*< 0.0001**
C^2	*1.391E+008*	*1*	*1.391E+008*	*76.57*	*< 0.0001**
D^2	*1.875E+008*	*1*	*1.875E+008*	*103.20*	*< 0.0001**
Residual	2.725E+007	15	1.817E+006		
Lack of Fit	*2.725E+007*	*10*	*2.725E+006*		
Pure Error	*0.000*	*5*	*0.000*		

Significant

Table 4

R-quared, Adj R-Squared, Pred R-Squared, and Adeq Precision value of the model

Std. Dev.	1347.92	R-Squared	0.9467
Mean	3583.33	Adj R-Squared	0.8969
C.V. %	37.62	Pred R-Squared	0.693
PRESS	1.570E+008	Adeq Precision	12.171

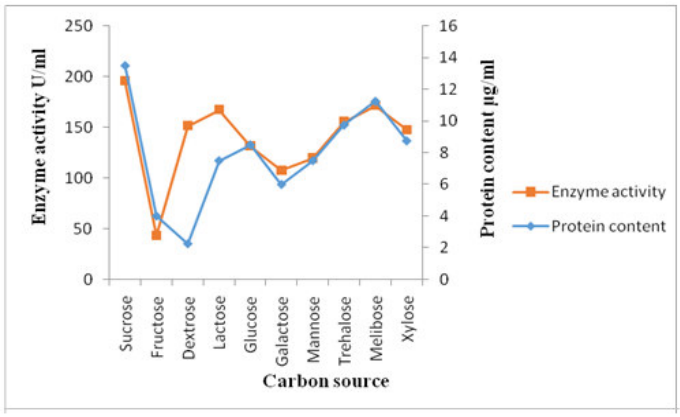

Figure 1
Effect of different Carbon sources on amylase production by Streptomyces gancidicusASD KT852565

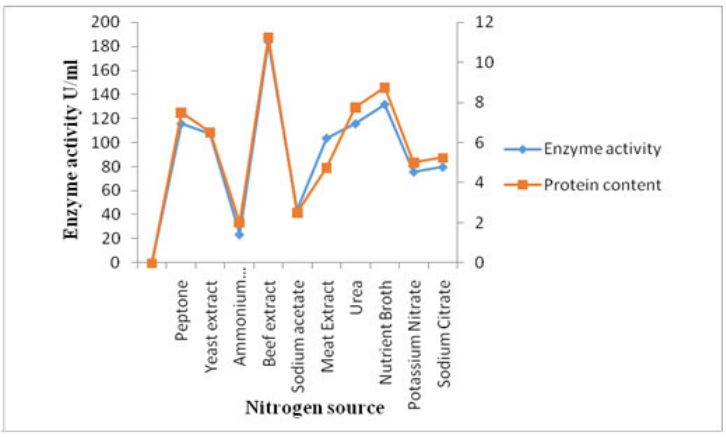

Figure 2
Effect of different Nitrogen sources on amylase production by Streptomyces gancidicus_ASD KT852565

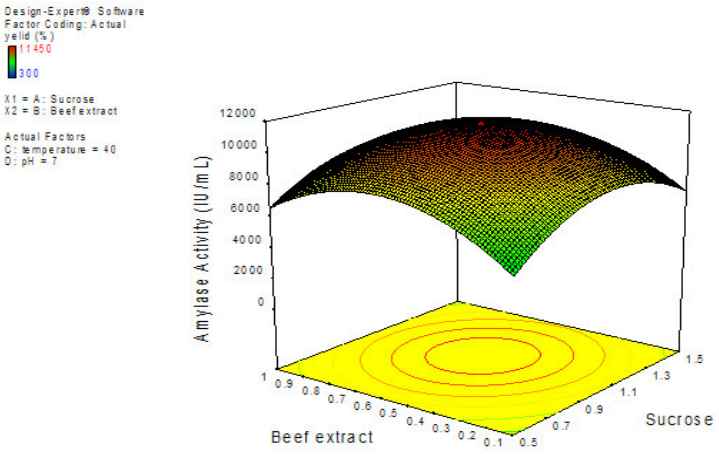

Figure 3a
3D surface showing the effects between sucrose and Beef extract

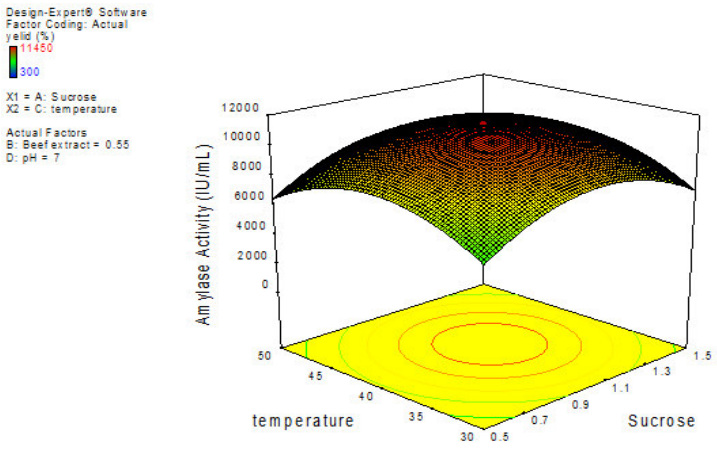

Figure 3b
3D surface showing the effects between sucrose and Temperature

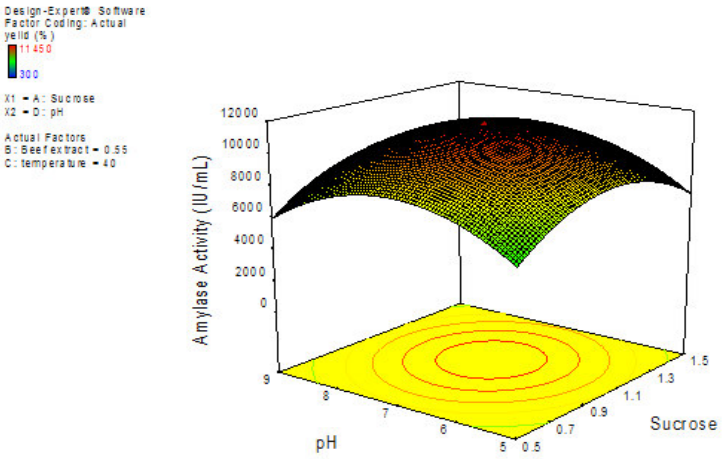

Figure 3c
3D surface showing the effects between sucrose and pH

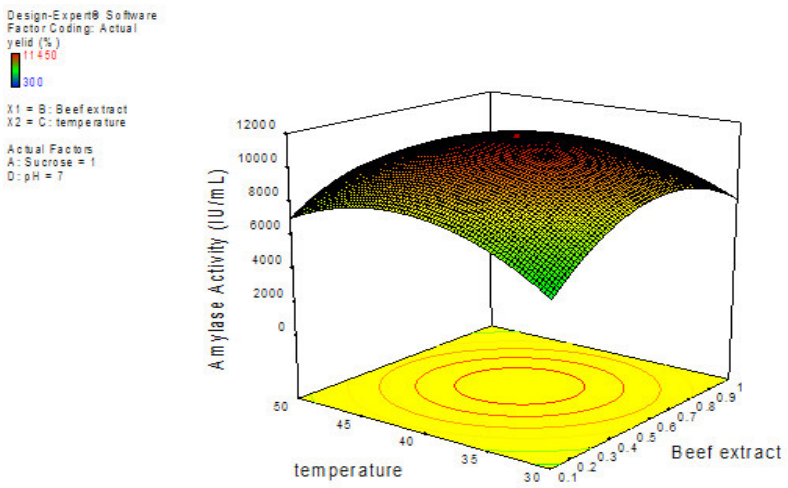

Figure 3d
3D surface showing the effects between Beef extract and Temperature

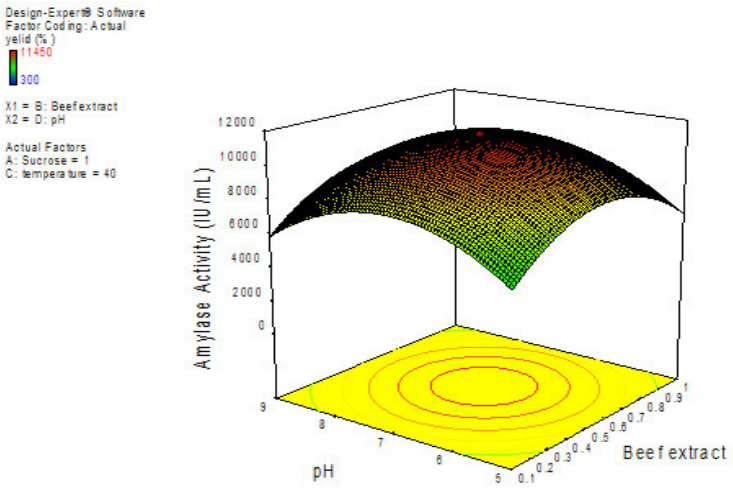

Figure 3e
3D surface showing the effects between Beef extract and pH

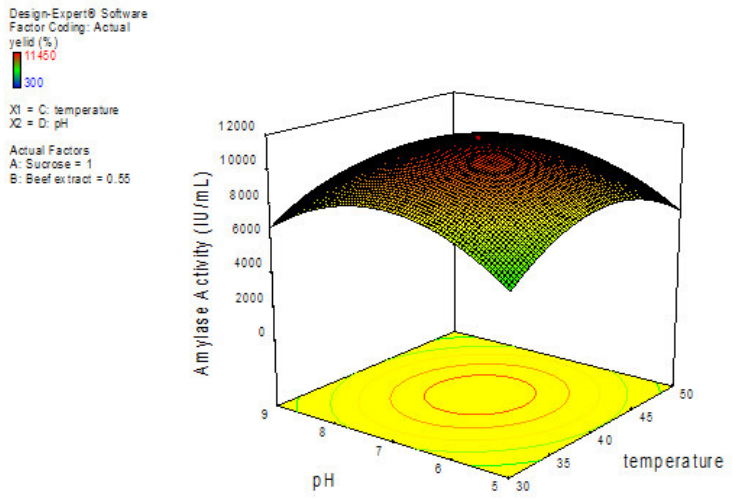

Figure 3f
3D surface showing the effects between pH and Temperature

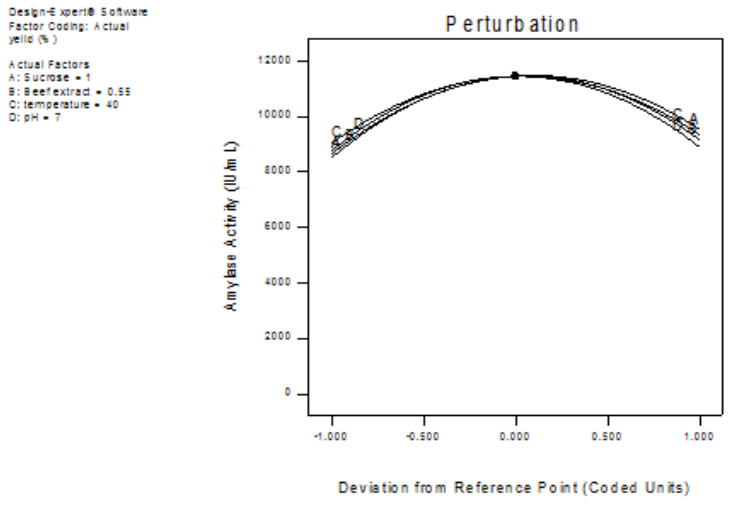

Figure 4
The perturbation graph showing the effect of all independent variables on Amylase production by S. gancidicus _ASD.

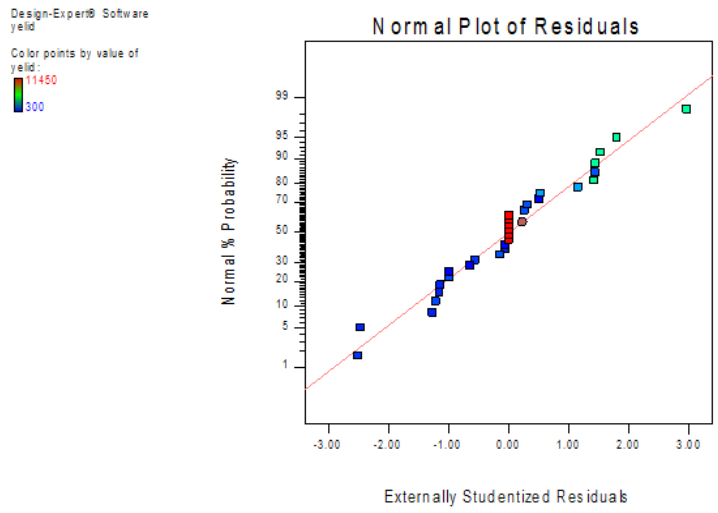

Figure 5
Plot between expected normal values versus residuals.

CONCLUSION

Amylase is a well-known enzyme for its application in various starch, textile, food, syrup and detergent industries. The present research was carried out to enhance amylase produced by the *S.gancidicus* Marine actinomycetes isolate which was isolated from Marine soil sediments, an-actinomycetes strain. In this study 2 step media optimization of amylase production was carried out using OFAT and RSM for various physical and chemical factors. It was observed that amylase produced by *S.gancidicus* was increased by 23.4 fold (IU/mL) for sucrose as carbon source and beef extract which acted as nitrogen source when compared to its normal amylase produced in the Starch-casein agar (394 IU/ml). Further studies were carried out to increase amylase production using RSM tool with optimum carbon and nitrogen sources as chemical factors and pH and temperature as physical factors in the modified media. At final, 23.4 fold increase in amylase production (9248 IU/mL) was observed from OFAT experiments and a total of 1.24 fold increase of amylase production was observed from RSM. This is the first report on amylase production from *S.gancidicus.*

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CONFLICT OF INTEREST

Conflict of interest declare none.

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