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Full Length Research Paper

Response surface methodological approach to optimize the nutritional parameters for enhanced production of α-amylase in solid state fermentation by *Thermomyces lanuginosus*

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Response surface methodology was used to study the cumulative effect of the nutritional parameters and to enhance the production of extracellular α-amylase in solid-state fermentation by *Thermomyces lanuginosus* ATCC 58157. These nutritional parameters considered include carbon source (soluble starch), nitrogen source (peptone) and a concentrated mineral medium. For obtaining the mutual interaction between the variables and optimizing these variables, a 2^3 factorial central composite design using response surface methodology was employed. The optimal calculated values of tested variables for maximal production of α-amylase were: soluble starch, 71.10 g/Kg; peptone, 91.97 g/Kg and mineral salts solution, 175.05 ml/Kg with a predicted α-amylase activity of 5.085×10^5 U/Kg of wheat bran. These predicted optimal parameters were tested in the laboratory and the final α-amylase activity obtained, 4.946×10^5 U/Kg of wheat bran, was very close to the predicted value.

Key words: α-Amylase, *Thermomyces lanuginosus*, solid state fermentation, optimization, response surface methodology.

INTRODUCTION

α-Amylase have most widely been reported to occur in microorganisms, although they are also found in plants and animals. α-Amylase (endo-1,4-alpha-D-glucan glucohydrolase, EC 3.2.1.1) are extracellular enzymes that randomly cleave the 1,4-α-D-glucosidic linkages between adjacent glucose units in the linear amylose chain. These are endoenzymes that split the substrate in the interior of the molecules and are classified according to their action and properties. For example, amylases that produce free sugars are termed 'saccharogenic' and those that liquefy starch without producing free sugars are known as 'starch-liquefying'. α-Amylase may be derived from several bacteria, yeasts and fungi. *Thermomyces lanuginosus*, a thermophilic deuteromycete fungus, is an excellent source of amylases (Haasum et al., 1991) that have been purified and characterized (Jensen et al., 1987). The glucoamylase from *T. lanuginosus* can quantitatively convert starch into glucose (Jensen et al., 1988; Rao et al., 1981), whereas the α-amylase yields maltose as the principal final product of raw potato starch hydrolysis (Mishra and Maheshwari, 1996). The enzymes are reported to be thermostable, with the half-life of α-amylase and glucoamylase being 0.6 and 4 h, respectively at 70°C. Due to their increased thermostability, these enzymes are potentially useful in the starch industry for production of maltose and glucose

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(Mishra and Maheshwari, 1996; Jensen and Olsen, 1992). However, *T. lanuginosus* is a poor producer of amylase when compared to the commercial strains of *Aspergillus* sp. and, therefore, requires improvement by strain manipulation methods like mutagenesis, protoplast fusion, cloning and transformation, as well as media optimization (Lamsa and Bloebaum, 1990; Monaghan et al., 1989) to achieve higher enzyme titers. Addition of a low molecular weight dextran and Tween-80 to the culture medium results in increased production of α-amylase by *T. lanuginosus* (Jensen et al., 1987; Arnesen et al., 1998). Thermostable α-amylase are generally preferred as their application minimizes contamination risk and reduces reaction time, thus providing considerable energy saving. Hydrolysis carried out at higher temperatures also minimizes polymerization of D-glucose to iso-maltose.

Solid-state fermentation has gained renewed interest from researchers for the production of these enzymes in view of its several economic and engineering advantages and has been often employed to produce amylases (Pandey, 1992; Pandey et al., 1999). Selvakumar et al. (1996) reviewed microbial synthesis of starch-saccharifying enzymes in solid cultures. Since thermostability is a feature of most of the enzymes sold in bulk for industrial application, thermophilic microorganisms are of special interest for the production of thermophilic amylases.

The classical method for the optimization of medium and cultural conditions involves one variable at a time, while keeping the other parameters at fixed levels. This method is generally time consuming and requires a considerable number of experiments to be carried out and does not include interactive effects among the variables (Dey et al., 2001). Response surface methodology (RSM) is the most widely used statistical technique for bioprocess optimization (Francis et al., 2003; Liu et al., 2003). It can be used to evaluate the relationship between a set of controllable experimental factors and observed results. The interaction among the possible influencing parameters can be evaluated with limited number of experiments (Francis et al., 2003). It has been successfully employed for optimization in many bioprocesses (Dey et al., 2001; Francis et al., 2003; Liu et al., 2003). The aim of the work includes the statistical optimization of supplementary nutrients predicted to play a significant role in enhancing α-amylase production in solid-state fermentation.

MATERIALS AND METHODS

Microorganism

T. lanuginosus strain ATCC 58157 was maintained on potato dextrose agar (PDA) at 4°C and transferred every 6-7 weeks. PDA plates were incubated at 50°C for 4 to 5 days and stored at 4°C until use.

Seed inoculum

Inocula were prepared by transferring 5 ml of suspension prepared from 5 days old slant culture, into 250 ml Erlenmeyer flasks containing 45 ml sterile inoculum medium. The composition of the inoculum medium was (g/L): soluble starch, 15.0; yeast extract, 5.0; K_2HPO_4, 5.0; and $MgSO_4$, 5.0 with a pH of 6.0. The flasks were incubated in a shaker incubator at 50°C for 4 days.

Solid state fermentation

The static experiments were conducted in Erlenmeyer flasks containing 10 g of wheat bran. Distilled water was added in such a way that the final substrate moisture content was 90%. After sterilization by autoclaving, the flasks were cooled and inoculated with 1 ml of mycelial inoculum. The contents were mixed thoroughly and incubated at 50ºC. Samples as whole flasks in triplicate, were removed after 5 days incubation.

Selection of a suitable substrate: Commercial quality millet cereal, wheat bran, crushed wheat, crushed maize, corncobs, wheat flakes, maize meal, molasses bran, barley bran, and rice bran were purchased from local markets and used as solid substrates and were investigated for their effect on the production of α-amylase. The solid substrate that induced highest enzyme activity was used in further experiments.

Effect of additional nutrients on enzyme production: The effects of various additional nutrients (carbon sources, nitrogen sources and mineral salt solution) on α-amylase production were studied by adding these to the wheat bran substrate in separate experiments. Various carbon sources (soluble starch, sucrose, lactose, maltose, dextrose, fructose and glucose) and nitrogen sources (peptone, tryptone, meat extract, ammonium sulphate, yeast extract, soybean meal, urea, ammonium sulphate and sodium nitrate) were added at a 1% (w/w) concentration to the substrate. To study the effect of mineral salts solution on α-amylase production, 1 ml salt solution was added to the substrate. The mineral salts solution comprised (g/L): K_2HPO_4, 2; KH_2PO_4, 3 and $MgSO_4.7H_2O$, 5 and pH 6.0. Fermentation studies were carried out for 5 days.

Optimization procedure

The optimization of nutritional parameters for α-amylase production by *T. lanuginosus* was carried out by central composite design and response surface methodology.

Central composite design: A 2^3 factorial central composite experimental design with six start points and six replicates at the central point, resulting in 20 experiments [generated by Design Expert, Version 6.0, Stat-Ease Inc., Minneapolis, MN) statistical software and the contour response surface generated using STATISTICA (StatSoft Inc., Tulsa, USA] was used to optimize the screened variables grouped as soluble starch (X_1), peptone (X_2) and mineral salts solution (X_3). The experimental design is shown in Table 1 and the coded variables are reflected in Table 2 according to the equation (1):

$$X_i = (X_i - X^x_i) / \Delta X_i \qquad i = 1,2,3\ldots,j \qquad \text{---------} \quad (1)$$

Where X_i = coded (dimensionless) value of the variable Xi,
X_0 = the value of X_i at the center point,
ΔX = the step change.

Table 1. Central composite design consisting of 20 experiments for the study of three experimental factors in coded units.

Run no.	X_1	X_2	X_3	Coefficients assessed by
1	1	1	1	Fractional 2^{3-1} factorial design
2	1	-1	-1	
3	-1	1	-1	
4	1	1	-1	
5	1	-1	1	
6	-1	1	1	
7	-1	-1	1	
8	-1	-1	-1	
9	-2	0	0	Star points (6 points)
10	0	-2	0	
11	0	0	-2	
12	2	0	0	
13	0	2	0	
14	0	0	2	
15	0	0	0	Central points (6 points)
16	0	0	0	
17	0	0	0	
18	0	0	0	
19	0	0	0	
20	0	0	0	

Table 2. Boundaries of experimental domain and spacing of levels expressed in coded and natural units.

Code unit	Experimental factor		
	Soluble starch (g/Kg of substrate) (X_1)	Peptone (g/Kg of substrate) (X_2)	Mineral medium (ml/Kg of substrate) (X_3)
-2	20	25	50
-1	40	50	100
0	60	75	150
1	80	100	200
2	100	125	250
ΔX	20	25	50

ΔX is the increment of the experimental factor natural values corresponding to one unit of the coded variable.

The behavior of the system was explained by the following second order polynomial equation:

$$Y = b_o + \sum_i b_i x_i + \sum_i \sum_y b_{ij} x_i x_j + \sum b_{ii} x^2_i + e. \quad \text{---- (2)}$$

Where y = predicted response, b_0 = offset term, b_i = linear effect, b_{ii} = squared effect, b_{ij} =interaction effect.

The Design Expert was used for regression analysis of the data obtained and to estimate the coefficients of the regression equation. Isoresponse contour plots were also obtained by using Design Expert.

Analytical methods

Determination of α-amylase activity: α-Amylase activity was determined according to the method of Okolo et al. (1995). The reaction mixture consisted of 1.25 ml 1% (w/v) soluble starch (Merck) solution, 0.25 ml, 0.1 M sodium acetate buffer (pH 6.0), 0.25 ml of distilled water, and 0.25 ml of properly diluted crude enzyme extract (10-320x). After 10 min of incubation at 50°C, the liberated reducing sugars (glucose equivalents) were estimated by the dinitrosalicylic acid method (Miller, 1959). Appropriate blanks were used. One unit (U) of α-amylase is defined as the amount of enzyme releasing 1 μmol glucose equivalent per ml per min under the assay conditions. All the experiments were conducted in

triplicate and the mean of the three with standard deviation (S.D) is represented as number of units of enzyme produced per gram of wheat bran.

Estimation of moisture content: Moisture content of the substrate was estimated by drying 10 g of substrate to constant weight at 105°C and the dry weight was recorded. To fix the initial moisture content of the solid medium, the substrate was soaked with the appropriate quantity of distilled water. The sample was then dried as described above and moisture content (%) was calculated as follows:

Percent of moisture content (initial) of solid medium = (wt. of the substrate - dry wt.) × 100/dry wt.

Table 3. Effect of different substrates on α-amylase production by *T. lanuginosus* under solid state fermentation.

Substrate	α-amylase activity (U/g)) ± S.D.
Wheat bran	261.0 ± 6.0
Millet cereal	196.0 ± 5.8
Crushed wheat	170.0 ± 5.5
Crushed maize	162.5 ± 5.6
Corncobs	147.5 ± 4.6
Wheat flakes	118.5 ± 3.7
Maize meal	96.0 ± 3.4
Molasses bran	85.0 ± 2.5
Barley bran	67.0 ± 2.6
Rice bran	43.0 ± 2.1

RESULTS AND DISCUSSION

Selection of a suitable substrate

In solid state fermentation, the selection of a suitable solid substrate for a fermentation process is a critical factor and thus involves the screening of a number of agro-industrial materials for microbial growth and product formation. In our studies, all the substrates supported growth and enzyme formation by the fungus, with wheat bran being the best substrate. A high titre of α-amylase activity (261.0 U/g) was obtained in a medium containing wheat bran as the substrate (Table 3) followed by millet cereal. Significant levels of α-amylase have previously been produced in wheat bran (Babu and Satyanarayana, 1995). Other authors have also reported wheat bran as the best substrate (Ellaiah et al., 2002) and suitable for necessary manipulation (Beckord et al., 1945).

Effect of additional nutrients on α-amylase production

Of the carbon sources tested, soluble starch increased α-amylase production (388 U/g) followed by sucrose (Table 4). Earlier workers reported similar findings wherein soluble starch as the best carbon supplement for amylase production in *Myceliophora thermophila* D14 (Sadhukhan et al., 1990) and *Aspergillus fumigatus* (Goto et al., 1998).

Table 4. Effect of potential nutrients on α-amylase production by *T. lanuginosus* under solid state fermentation.

Supplementary nutrients	α-Amylase activity (U/g) ± S.D.
Soluble starch	388.0 ± 9.0
Sucrose	362.0 ± 8.5
Lactose	326.2 ± 7.8
Maltose	294.0 ± 7.2
Dextrose	278.0 ± 6.7
Fructose	266.6 ± 5.6
Glucose	192.0 ± 5.0
Peptone	414.0 ± 11.6
Tryptone	356.7 ± 10.4
Meat extract	338.0 ± 9.0
Ammonium sulphate	310.0 ± 8.5
Yeast extract	306.4 ± 8.0
Soyabean meal	294.0 ± 7.3
Urea	280.0 ± 6.7
Ammonium nitrate	272.0 ± 6.0
Sodium nitrate	264.9 ± 5.3
Mineral salts solution	332.0 ± 7.9
Control (wheat bran alone)	261.0 ± 5.4

Among the nitrogen sources, peptone increased α-amylase production (414 U/g) followed by tryptone and meat extract (Table 4). Previous findings have shown that peptone, sodium nitrate and casein hydrolysate are good nitrogen supplements for amylase production in *Aspergillus fumigatus* (Goto et al., 1998), *Aspergillus niger* (Pandey et al., 1994) and *Aspergillus oryzae* (Pederson and Neilson, 2000).

Supplementation with a mineral salt solution also showed enhanced α-amylase production (Table 4). This shows that the minerals have a role in the biosynthesis of α-amylase. The requirement of the metal ions like iron, zinc, magnesium, phosphate and calcium for optimum production of alkaline protease has been described by Ellaiah et al. (2002).

Optimization of the nutritional parameters for α-amylase production

Three variables viz., soluble starch, peptone and mineral salts solution were selected on the basis of highest induced α-amylase activity and optimized using a central composite design. The experimental and predicted response of α-amylase production after 5 days of

Table 5. Observed responses and predicted values.

Run no.	α-amylase yield (U/g)		Residual value
	Observed response	Predicted value	
1	457.0	484.0	-27.0
2	196.0	231.7	-35.7
3	220.0	269.7	-49.7
4	382.0	369.5	12.5
5	301.0	306.2	-5.2
6	348.0	367.2	-19.2
7	199.0	266.5	-67.5
8	181.0	209.0	-28.0
9	262.0	207.2	54.7
10	159.0	118.2	40.7
11	205.0	182.0	23.0
12	347.0	346.7	0.2
13	371.0	356.7	14.2
14	386.0	354.0	32.0
15	475.0	469.5	5.5
16	486.0	469.5	16.5
17	480.0	469.5	10.5
18	471.0	469.5	1.5
19	482.0	469.5	12.5
20	478.0	469.5	8.5

cultivation by *T. lanuginosus* are shown in Table 5. By applying multiple regression analysis on the experimental data, the following second order polynomial equation explains α-amylase production:

$$Y = 469.500 + 34.875X_1 + 59.625X_2 + 43X_3 + 19.250X_1X_2 + 4.250X_1X_3 + 9.999X_2X_3 - 48.125X_{11} - 58X_{22} - 50.375X_{33} \quad (3)$$

where, Y = predicted response, X_1, X_2 and X_3 are the coded values of soluble starch, peptone and mineral salts solution, respectively.

Statistical testing of the model was done by the Fisher's statistical test for analysis of variance (ANOVA) and the results are shown in Table 6. The analysis of variance of the quadratic regression model demonstrates that the model is highly significant, as the computed *F* value is much greater than the tabular *F10,9* value (=5.05E-05). The closer the value of *R* (multiple correlation coefficient) to 1, the better the correlation between the observed and predicted values. Here the value of *R* (=0.967) indicates an excellent correlation between the experimental and predicted values. The coefficient of variation (CV) indicates the degree of precision with which the treatments are compared. Usually, the higher the value of CV, the lower is the reliability of experiment. In this experiment, a lower value of CV (=11.16 %) indicates a greater reliability of the experiments performed.

The Student *t* distribution and the corresponding *P* values, along with the parameter estimate, are 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 *P*, the more significant is the corresponding coefficient. The parameter estimate and the corresponding *P* values (Table 7) suggest that among the independent variables starch (X_1) and peptone (X_2) have a significant effect on α-amylase production. The quadratic term of these two variables also have a significant effect. But the independent variable, mineral medium (X_3), has no effect. And there is no interaction. The effect of peptone (X_2) is greater than that of starch (X_1). It was also found that α-amylase production increased with the increase of starch concentration and with the increase of peptone concentration. A representative response surface plots, which are more or less surface confined, is shown in Figures. 1 to 3.

The response surface plot obtained as a function of starch concentration versus peptone concentration and other variables are held at zero level. A maximum increase in α-amylase yield with increase in starch concentration versus peptone concentration was observed (Figure 1). The response surface plot obtained as a function of starch concentration versus mineral salts solution concentration and other variables are held at zero level. An α-amylase yield with increase in starch concentration versus mineral salts solution concentration was observed (Figure 2). The effect of peptone concentration versus the mineral salts solution concentration and other variables are held at zero level. An increase in α-amylase yield with peptone concentration versus mineral salts solution concentration was observed (Figure 3). The regression equation [equation (3)] was solved by using Design Expert. The optimal values of test variables in the coded units were $X_1 = 0.555$, $X_2 = 0.679$ and $X_3 = 0.501$. At these values, the concentration of soluble starch, peptone and mineral salts solution were 71.10 g/Kg, 91.97 g/Kg and 175.05 ml/Kg of wheat bran, respectively. The maximum predicted value of α-amylase yield obtained was 5.085×10^5 U/Kg of wheat bran.

A repeated fermentation of α-amylase under optimal conditions was carried out. The maximal α-amylase level obtained was 4.946×10^5 U/kg of wheat bran. This value was found to be 2.74 % less than the predicted value. This discrepancy might be due to the slight variation in experimental conditions.

The use of an experimental design allowed the rapid screening of a large experimental domain in search of the best nutritional parameters for optimisation of α-amylase production. A comparison of the results obtained both theoretically and experimentally revealed

Table 6. Analysis of variance (ANOVA) for the three factorial design.

Sources of variation	Sum of squares	Degrees of freedom	Mean square	*F* value	Prob (*P*) >*F*
Regress.	242755.8	9	26972.7	17.7	5.05E-05
Residual	15183.3	10	1518.3		
Total	257939.1	19			

R^2 = 0.936, R = 0.967, Adjusted R^2 = 0.885

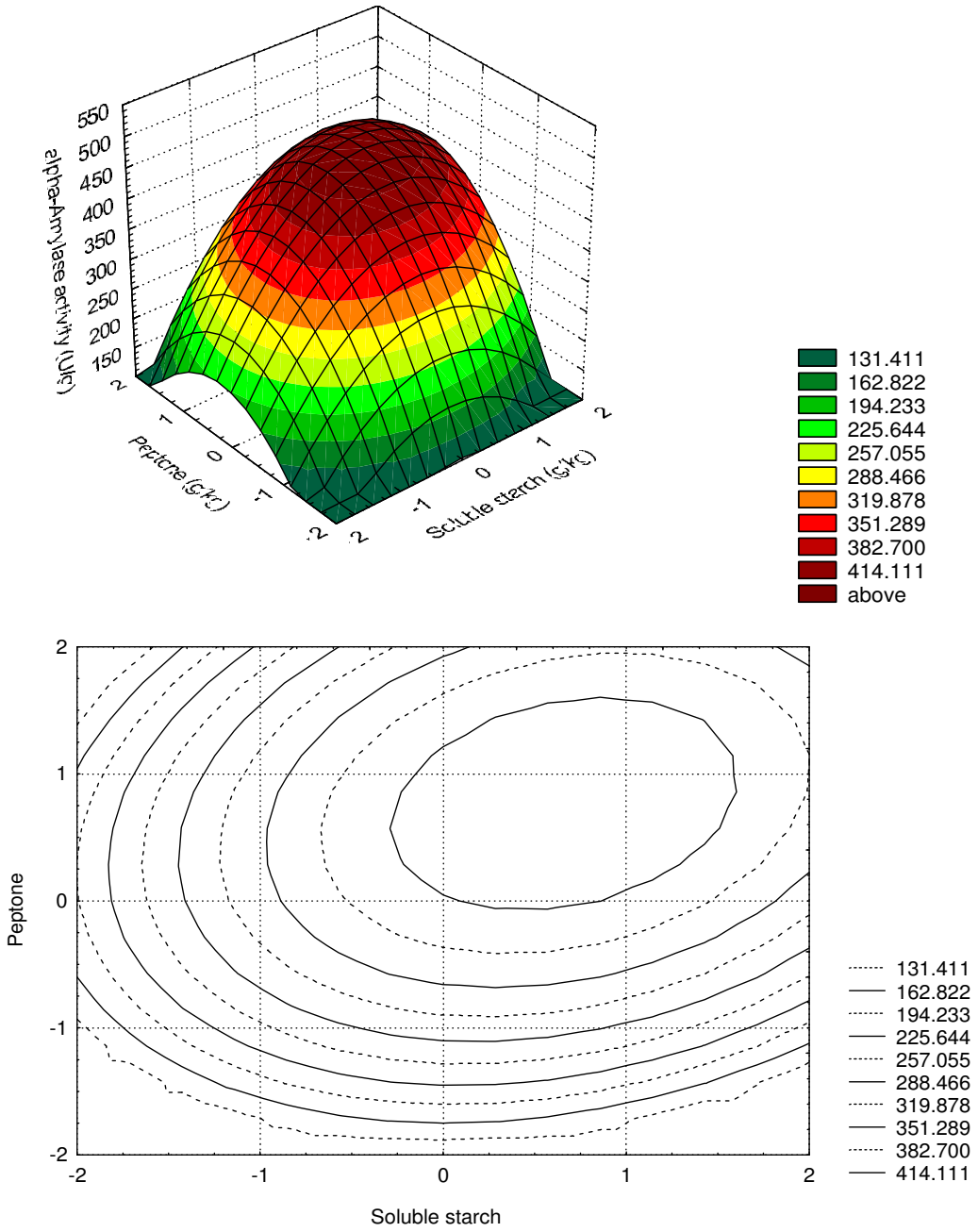

Figure 1. Response surface plot showing the effect on soluble starch concentration, peptone concentration and their mutual effect on the production of α-amylase. Other variables are held at zero level.

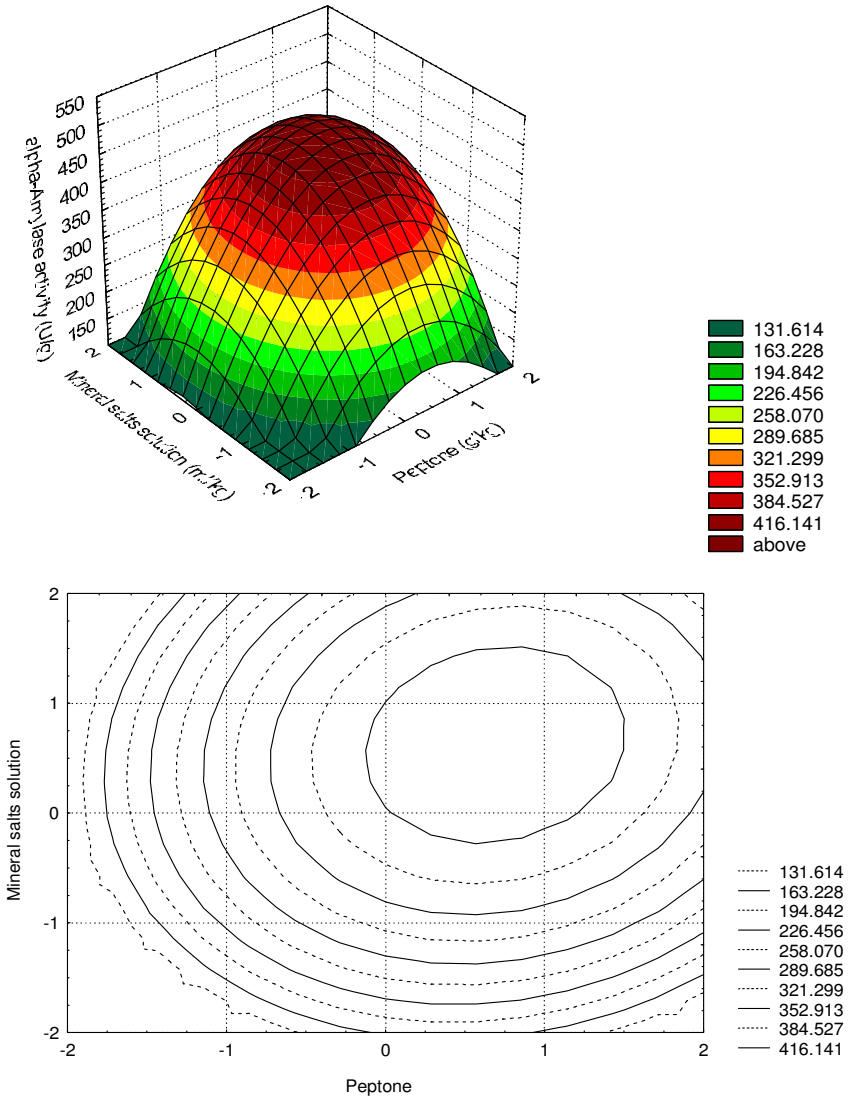

Figure 2. Response surface plot showing the effect on soluble starch concentration, mineral medium concentration and their mutual effect on the production of α-amylase. Other variables are held at zero level.

Table 7. Model coefficients estimated by multiples linear regression.

Factor	Coefficient	Computed t-value	*p*-value
Intercept	469.5	30.2	4.53E-11
X_1	34.9	3.6	0.004*
X_2	59.6	6.1	0.0001*
X_3	43.0	4.4	0.001*
X_1X_2	19.2	1.4	0.1
X_1X_3	4.2	0.3	0.7
X_2X_3	10.0	0.7	0.7
X_{11}	-48.1	-6.2	0.0001*
X_{22}	-58.0	-7.4	3.36E-05*
X_{33}	-50.4	-6.5	8.96E-05*

*Significant at $p < 0.01$

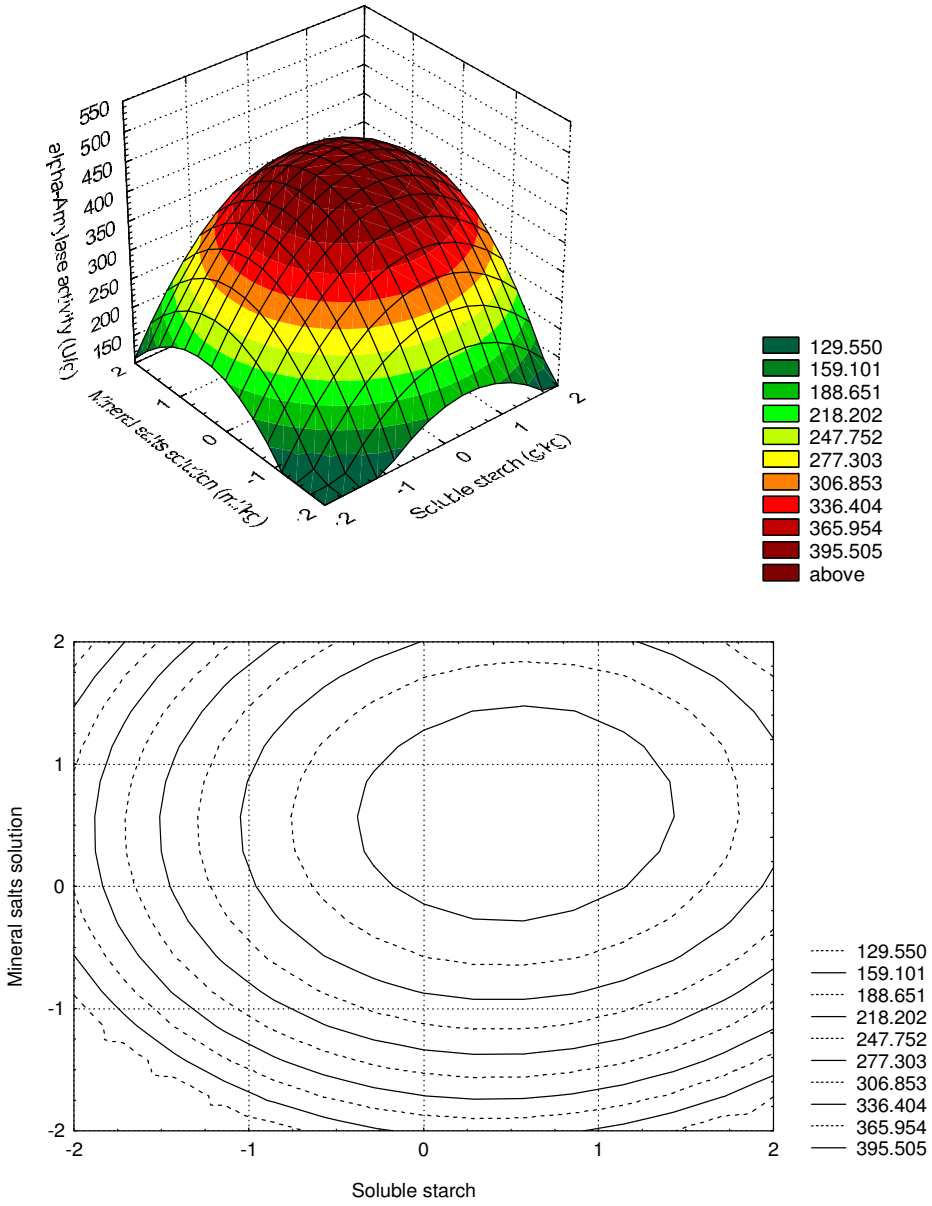

Figure 3. Response surface plot showing the effect on peptone concentration, mineral medium concentration and their mutual effect on the production of α-amylase. Other variables are held at zero level.

that α-amylase production increased with the increase of starch concentration and with the increase of peptone concentration.

The R^2 value of 0.936 shows a good fit of the model with the experimental data. To the best of our knowledge there are no reports of α-amylase production by *T. lanuginosus* by media engineering. This paper is mainly an attempt to demonstrate the applicability of statistical design to optimize α-amylase production. The next step, for improvement of α-amylase production, should be in a bioreactor under optimized conditions. We hope these results will show that this strategy (response surface methodology) can be applied to many similar settings for routine optimization.

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