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PRODUCTION AND OPTIMIZATION OF L-GLUTAMINASE FROM MANGROVE ISOLATE AND COMPARATIVE MUTATIONAL STUDIES

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ABSTRACT

Actinomycetes, isolated from marine and mangrove sediment samples, were evaluated for the production of L-glutaminase. All the isolates were screened for L-glutaminase production and the one exhibiting maximum glutaminolytic activity was selected as potential L-glutaminase producer and used for mutational studies. Both wild and mutant strains were used for L-glutaminase production by solid state fermentation using different agro- industrial by-products such as green gram, black gram, coconut oil cake, sesame oil cake, rice bran and wheat bran. Sesame oil cake was the best substrate for induction of L-glutaminase (153.066 U/gds by wild strain and 158.53 U/gds by mutant strain). Culture conditions like initial moisture content, inoculum volume, temperature and pH of the culture medium were optimized using a 2^4 full factorial CCD (Central Composite Design) and a second order polynomial model equation was obtained. The predicted optimum levels were as follows: temperature 36.21^0C, inoculum volume 1.87 ml, initial moisture content 69.47 % (v/w) and pH 8.2. Under these optimum conditions, the experimental yield of L-glutaminase was 203.86 U/gds, which was in close agreement with the value predicted by the model, 206.57 U/gds. In the present study the value of the regression coefficient R^2 = 0.9894 which indicates that 98.94% of the variability in the response could be explained by the model.

KEYWORDS: Actinomycete, L-glutaminase, mangrove, mutant, solid state fermentation, wild, central composite design

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1. INTRODUCTION

The enzyme L-glutaminase (L-glutamine amidohydrolase E.C 3.5.1.2) catalyze the hydrolytical deamination of L-glutamine to L-glutamic acid and ammonia[1]. This enzyme has received significant importance since it was reported as an effective agent in the treatment of leukaemia[2] and HIV[3,4]. L-glutaminase also has applications as an analytical agent[5,6], as a biosensor to monitor L-glutamine level[7], as a flavour-enhancing agent[8,9] and in the production of specialty chemicals like threonine by gamma glutamyl transfer reactions[10]. L-glutaminase is ubiquitous in terrestrial and marine microorganisms including bacteria, fungi and yeasts. L-glutaminase production was reported from various microorganisms such as *Escherichia coli*, *Pseudomonas species*, *Acinetobacter species*, *Bacillus species*, *Hansenula*, *Cryptococcus*, *Candida*, *Aspergillus oryzae* and *Beuveria bassiana*[11]. L-glutaminase activity was reported from few marine microorganisms such as *Pseudomonas fluorescens*, *Micrococcus luteus*, *Vibrio cholerae* and *Beuveria bassiana*[12]. Actinomycetes are aerobic gram positive filamentous bacteria with high G+C (Guanine + Cytosine) content which form asexual spores. The value of actinomycetes to society in terms of providinguseful drugs especially antibiotics and anticancer agents and to the pharmaceutical industry for revenue generating discovery platform, is indisputable[13]. Solid state fermentation has emerged as a promising bioprocess for production of enzymes and other economical products on a large scale. L-glutaminase production has been reported under solid-state fermentation using wheat bran, sesame oil cake and polystyrene beads[14-16]. Response surface methodology (RSM) consists of a group of statistical techniques for developing empirical model and its exploitation. It defines the effect of the independent variables, alone or in combination, on the process. In addition, this experimental methodology generates a mathematical model that accurately describes the overall process[17]. Through a careful design and analysis of experiments, RSM try to relate a response (or output variable), to the levels of a number of parameters (or input variables) that affect such response. The present investigation was designed for selective isolation of L-glutaminase producing actinomycetes from marine and mangrove soil samples and induction of mutation in the most active species. In addition, a sequential optimization strategy for L-glutaminase production through statistically designed experiments was carried out. First, one-variable-at-a time screening design was applied to address the most significant variables affecting L-glutaminase production. Secondly, a RSM technique named as the central composite design was used to investigate the interactive effect of temperature, inoculum volume, initial moisture content and pH on L-glutaminase production.

2. MATERIALS AND METHODS

2.1 Selective isolation of L-glutaminase producing actinomycetes

Marine and mangrove soil samples were collected from Krishna estuary (Lat 15°50' and 15°55'N; Long. 80°45' and 80°50'E), Andhra Pradesh. The pretreated samples were used for the isolation of actinomycetes[18]. Minimal glutamine agar (MGA) medium was used for the selective isolation of L-glutaminase producing actinomycetes[19]. L-glutaminase activity was identified by formation of a pink zone around colonies. The colony showing the largest zone diameter was selected as potent producer of L-glutaminase and subjected to UV irradiation to induce mutation for better yield of L-glutaminase. Spore suspensions of actinomycetes were irradiated using UV lamp at varying distances (5, 10, 15 and 20 cm) for 15 min in a dark room and the irradiated suspensions were protected from light until plating was done. Both the wild and the mutant strains were selected in the subsequent experimentation for L-glutaminase production using solid substrates. The cultures were maintained on MGA slants, incubated at 28°C for 7 days and then stored at 4°C until use.

2.2 Inoculum preparation

Actinomycete spore suspension was prepared from a freshly raised 7 days old culture on MGA slants by suspending in 10 ml of 0.85% sterile saline solution. One ml of spore suspension contained about 10^6 spores/ml.

2.3 Fermentation medium

Several agro-industrial by-products namely green gram, black gram, coconut oil cake, sesame oil cake, rice bran and wheat bran were utilized as substrates for L-glutaminase production by isolated wild and mutant actinomycete strains. 5 g of each substrate was taken separately in a 250 ml Erlenmeyer conical flask and moistened with 2 ml of moistening medium (distilled water). The flasks were autoclaved at 121°C for 20 min, cooled to room temperature and inoculated with 2 ml of wild and mutant actinomycete spore suspensions. The inoculated flasks were mixed thoroughly and incubated at 28°C for 7 days in static incubator.

2.4 Enzyme extraction and assay

Fermented substrate containing crude enzyme was mixed with 41 ml of 0.1M phosphate buffer (pH 8) and the flasks were kept on a rotary shaker at 150 rpm for 30 min. The contents of the flasks were centrifuged at 10,000 g for about 10 min at 4°C in a cooling centrifuge. Supernatant was collected and used for enzyme assay[20]. The activity of L-glutaminase was determined by estimating the amount of ammonia liberated from L-glutamine using Nessler's reagent[21]. Reaction mixture containing 0.5 ml of 0.04M L-glutamine in 0.1M Tris-HCl buffer was made to react with 0.5 ml of crude enzyme solution and incubated at 37°C for 30 min. The reaction was stopped by adding 0.5 ml of 1.5 M Trichloro-acetic acid (TCA). To 3.7 ml distilled water, 0.1 ml of the above mixture and 0.2 ml of Nessler's reagent were added.

After keeping the mixture for 20 min, the extinction at 450 nm was measured with a UV-spectrophotometer. One unit (U) of L-glutaminase activity was defined as the amount of enzyme that liberates one µmole of ammonia per ml per minute. Enzyme yield was expressed as the activity of L-glutaminase per gram dry sesame oil cake (U/gds).

2.5 Optimization of the culture conditions for L-glutaminase production

The different physicochemical parameters to maximize the yield of L-glutaminase by wild and mutant actinomycete strains under solid state fermentation were investigated based on one-variable-a-time approach. The impact of initial moisture content (20–80%), incubation temperature (25–50°C), size of inoculum (1-5 ml) and pH (6-10) on L-glutaminase production was evaluated. All the experiments were conducted in duplicate and the mean values were considered.

2.5.1 Central composite design

Response surface methodology and central composite design were widely applied as an integral part of data analysis in optimization and data interpretation. In this study, central composite quadratic design was employed to optimize the most significant factors for enhancing L-glutaminase production. The central composite design was conducted in the optimum vicinity to locate the true optimum concentrations for the 4 factors, viz. pH, temperature, inoculum volume and initial moisture content. Twenty six experiments were conducted with 16 factorial points (2^4), 8 axial points (2×4) and 2 replications at the centre points (n_o=2) according to CCD and L-glutaminase yield was measured in each case. The range and centre point values of 4 independent variables were based on the results of preliminary experiments. The second-order polynomial coefficients were calculated to determine the role of each variable, their interactions and statistical analysis to obtain predicted yield of L-glutaminase.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2$$

where Y is the predicted response, β_0 is the intercept, β_1, β_2, β_3 and β_4 are the linear coefficients, β_{11}, β_{22}, β_{33} and β_{44} are the squared coefficients, β_{12}, β_{13}, β_{14}, β_{23}, β_{24} and β_{34} are the interaction coefficients and A, B, C, D, A^2, B^2, C^2, D^2, AB, AC, AD, BC, BD and CD are independent variables. The design matrix generated by CCD was shown in Table 1.

Using the 'Statistica 6' software, the data obtained was analyzed and response surface plots were constructed which indicated the possibility of enhancement in the production of L-glutaminase. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

Table 1

Central composite design consisting of 26 experiments for the study of four experimental factors in coded values

Runs	A	B	C	D	Final response
1.	-1	-1	-1	-1	_
2.	-1	-1	-1	1	_
3.	-1	-1	1	-1	_
4.	-1	-1	1	1	_
5.	-1	1	-1	-1	_
6.	-1	1	-1	1	_
7.	-1	1	1	-1	_
8.	-1	1	1	1	_
9.	1	-1	-1	-1	_
10.	1	-1	-1	1	_
11.	1	-1	1	-1	_
12.	1	-1	1	1	_
13.	1	1	-1	-1	_
14.	1	1	-1	1	_
15.	1	1	1	-1	_
16.	1	1	1	1	_
17.	-2	0	0	0	_
18.	2	0	0	0	_
19.	0	-2	0	0	_
20.	0	2	0	0	_
21.	0	0	-2	0	_
22.	0	0	2	0	_
23.	0	0	0	-2	_
24.	0	0	0	2	_
25(c)	0	0	0	0	_
26(c)	0	0	0	0.	_

3. RESULTS AND DISCUSSION

3.1 Effect of substrates

The economical advantages of solid state cultures include reduced thermal processing requirements, reduced energy requirement for agitation[22]. The critical factor in solid state fermentation is the choice of a suitable substrate for the fermentation process[23]. In the present study, six substrates such as green gram, black gram, sesame oil cake, coconut oil cake, rice bran, wheat

bran were screened. The screening for L-glutaminase production by wild and mutant actinomycete strains using different solid substrates (Fig.1) show a detectable variation of L-glutaminase yield. The maximum L-glutaminase yield for wild strain (153.066 U/gds) and mutant strain (158.53 U/gds) was observed using sesame oil cake followed by coconut oil cake, wheat bran, rice bran, black gram and green gram. Sesame oil cake has highest crude protein content (32%) and essential amino acids namely methionine and cystine[24].

3.2 Effect of Temperature

In order to determine optimum temperature for L-glutaminase production, flasks were incubated at various temperatures such as 25, 30, 35, 40, 45 and 50°C. Incubation temperature has a profound effect on L-glutaminase production under solid cultural conditions (Fig.2). The maximum enzyme yield of 157.44 U/gds for wild and 180.4 U/gds for mutant strain was obtained when fermentation was carried out at 35°C. However, the enzyme production reduced gradually above 35°C. This may be due to the denaturation of microbial strain at higher temperatures. It has been suggested that at high temperatures, microorganisms may synthesize only a reduced number of proteins essential for growth and other physiological processes[25]. These results were in coincidence with L-glutaminase production by *Rhizopus oligosporus*[26] and *Vibrio costicola*[14].

3.3 Effect of inoculum volume

Fermentation was carried out with different inoculum volumes varying from 1-5 ml to study its effect on the production of L-glutaminase (Fig.3). Maximum L-glutaminase yield of 194.62 U/gds for wild and 196 U/gds for mutant strain was obtained using 2 ml spore suspension of 7 days old culture. With further increase in inoculum volume, there was a gradual decrease in the enzyme production and microbial activity which might be attributed to the nutrient limitations[16] or accumulation of some non-volatile self inhibiting substance. On the other hand, lower inoculum levels are unsuitable because they contain less number of cells which require long time to grow and form desired products[27].

3.4 Effect of initial moisture content

To investigate the influence of initial moisture content of the substrate on L-glutaminase production, fermentation was carried out with various initial moisture content levels such as 20, 30, 40, 50, 60, 70 and 80 (% v/w) of sesame oil cake (Fig.4). Maximum enzyme yield of 168.37 U/gds for wild and 174.93 U/gds for mutant was achieved at 70% initial moisture content. A further increase in the initial moisture content resulted in a significant reduction in the enzyme production. The inhibitory effect on enzyme production at higher moisture content may be due to substrate particle agglomeration, lower O_2 transfer, decrease in porosity and enhancement of bacterial growth. A reduction in the solubility of nutrients of the substrate and a low degree of swelling are the disadvantages of low moisture content[23]. These results were in coincidence with L-glutaminase production by *Vibrio costicola*[14] and *Trichoderma koningii*[20].

3.5 Effect of pH

Experiments were performed to find out the optimum pH in order to maintain the favourable conditions for increased L-glutaminase production. This was established by carrying out the fermentation by varying the pH from 4-9 (adjusted with 1N HCl or 1N NaOH). The results were shown in Fig. 5. The maximum L-glutaminase production of 184.77 U/gds and 186.96 U/gds for wild and mutant were obtained at pH 8.0 .This may be attributed to the balance of ionic strength of plasma membrane[20].These results were in coincidence with L-glutaminase production by *Beauveria* sp[7].

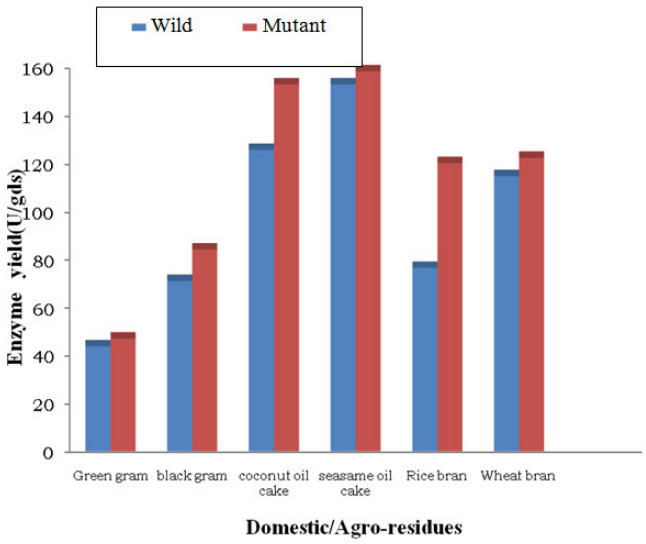

Figure 1
Effect of substrates on L-glutaminase production

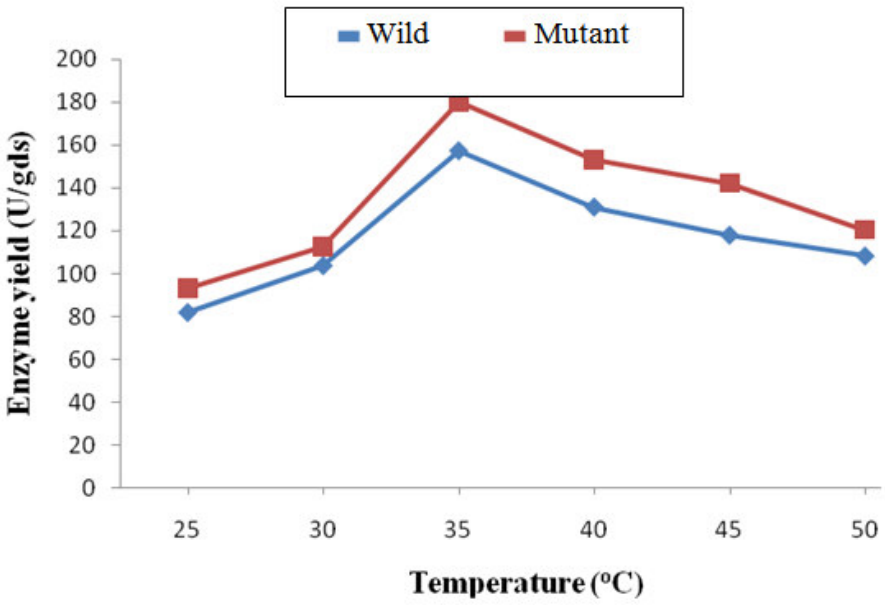

Figure 2
Effect of temperature on L-glutaminase production

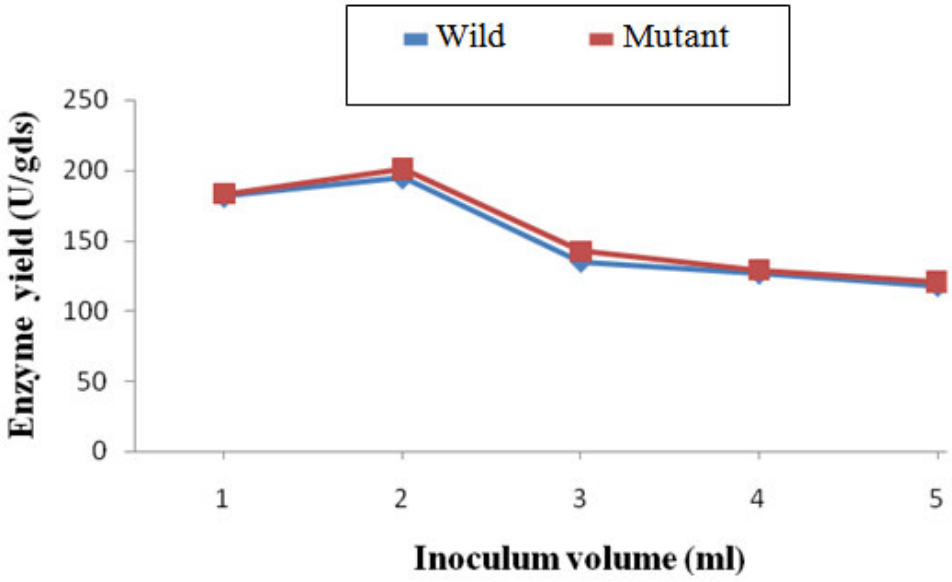

Figure 3
Effect of inoculum volume on L-glutaminase production

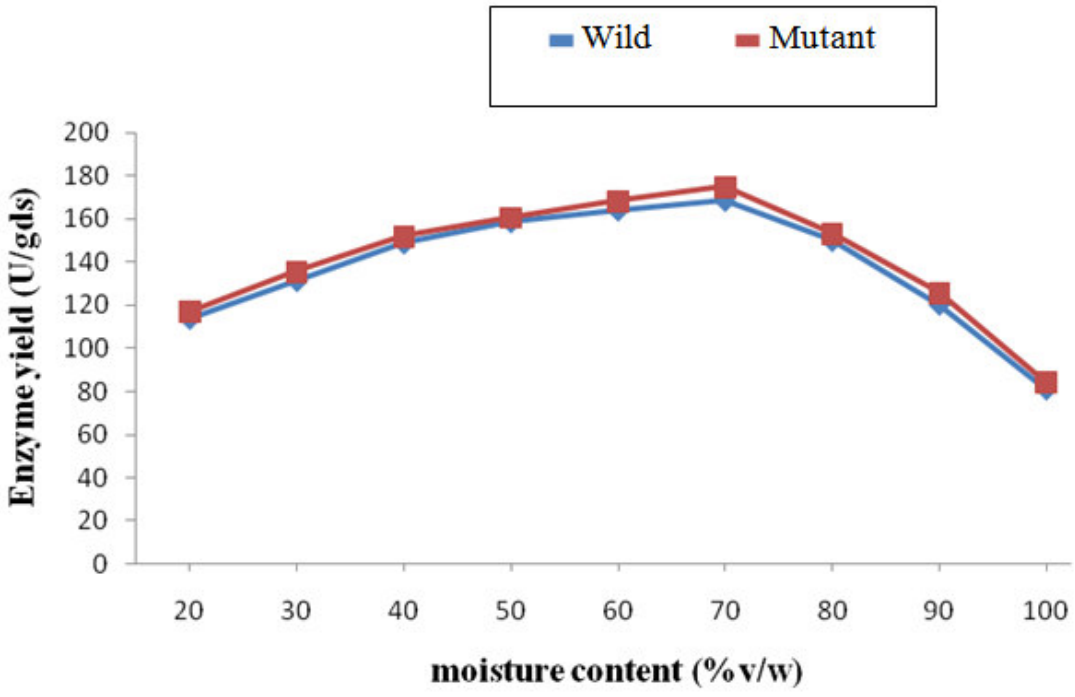

Figure 4
Effect of initial moisture content on L-glutaminase production

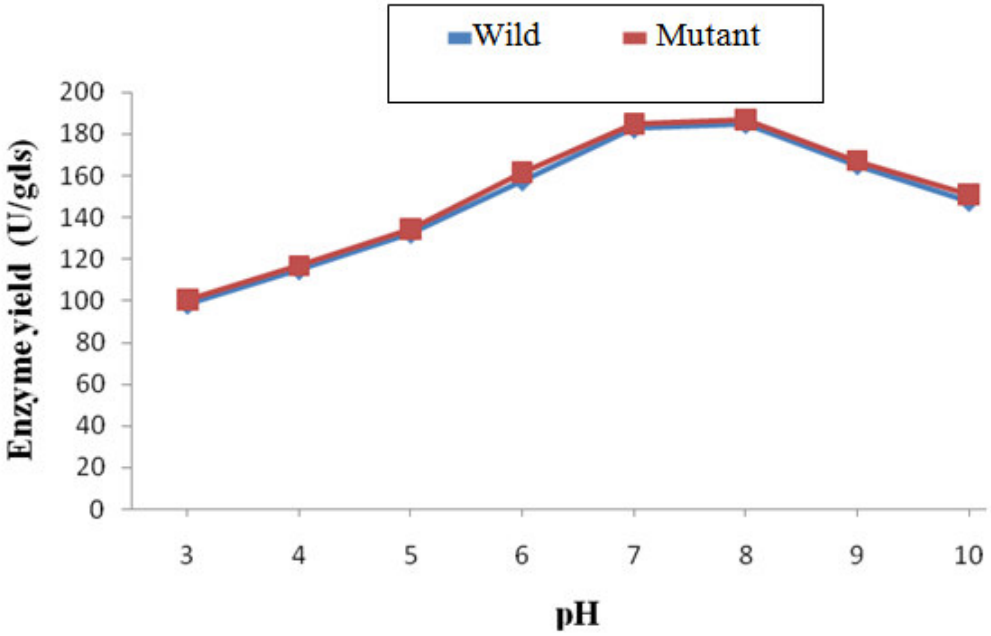

Figure 5
Effect of pH on L-glutaminase production

3.6 Statistical optimization of variables and their interaction analysis

Optimum levels of the above mentioned important variables and the effect of their interactions on L-glutaminase production were determined by the central composite design of Response Surface Methodology. Table 2 shows the information of the actual and coded values working in the CCD. The results obtained by CCD were analyzed by standard analysis of variance and the mean predicted and observed responses were presented in Table 3.

Table 2
Coded and real values of medium components used for central composite design

Independent variables	Coded factors				
	-2	-1	0	1	2
(A) Temperature (^{0}C)	25	30	35	40	45
(B) Inoculum volume (ml)	0	1	2	3	4
(C) Initial moisture content (%v/w)	50	60	70	80	90
(D) pH	6	7	8	9	10

Table 3

Central composite design consisting of 26 experiments for the study of four experimental factors and comparison of experimental and predicted values of L-glutaminase yield

Run No.	Temperature (^{0}C) (A)	Inoculum volume (ml) (B)	Initial moisture content (%v/w) (C)	pH (D)	L-glutaminase yield (U/gds) (Y)		
					Experimental	Predicted	Residual
1	30 (-1)	1 (-1)	60 (-1)	7 (-1)	203.2100	203.2229	-0.012917
2	30 (-1)	1 (-1)	60 (-1)	9 (1)	204.1700	204.0858	0.084167
3	30 (-1)	1 (-1)	80 (1)	7 (-1)	203.8900	204.1175	-0.227500
4	30 (-1)	1 (-1)	80 (1)	9 (1)	204.3100	204.2279	0.082083
5	30 (-1)	3 (1)	60 (-1)	7 (-1)	203.7300	203.5908	0.139167
6	30 (-1)	3 (1)	60 (-1)	9 (1)	204.7100	204.8762	-0.166250
7	30 (-1)	3 (1)	80 (1)	7 (-1)	203.4500	203.5929	-0.142917
8	30 (-1)	3 (1)	80 (1)	9 (1)	204.2500	204.1258	0.124167
9	40 (1)	1 (-1)	60 (-1)	7 (-1)	204.3300	204.3258	0.004167
10	40 (1)	1 (-1)	60 (-1)	9 (1)	204.8800	204.8113	0.068750
11	40 (1)	1 (-1)	80 (1)	7 (-1)	204.8700	204.7779	0.092083
12	40 (1)	1 (-1)	80 (1)	9 (1)	204.500	204.5108	-0.010833
13	40 (1)	3 (1)	60 (-1)	7 (-1)	203.8900	204.0462	-0.156250
14	40 (1)	3 (1)	60 (-1)	9 (1)	205.3100	204.9542	0.355833
15	40 (1)	3 (1)	80 (1)	7 (-1)	203.6500	203.6058	0.044167
16	40 (1)	3 (1)	80 (1)	9 (1)	203.700	203.7612	-0.061250
17	25 (-2)	2 (0)	70 (0)	8 (0)	204.6400	204.5529	0.087083
18	45 (2)	2 (0)	70 (0)	8 (0)	205.1500	205.2912	-0.14250
19	35 (0)	0 (-2)	70 (0)	8 (0)	205.2100	205.2229	-0.012917
20	35 (0)	4 (2)	70 (0)	8 (0)	204.800	204.8413	-0.041250
21	35 (0)	2 (0)	50 (-2)	8 (0)	202.3100	202.4412	-0.131250
22	35 (0)	2 (0)	90 (2)	8 (0)	202.2200	202.1429	0.077083
23	35 (0)	2 (0)	70 (0)	6 (-2)	204.0600	203.9029	0.157083
24	35 (0)	2 (0)	70 (0)	10 (2)	204.7100	204.9213	-0.211250
25	35 (0)	2 (0)	70 (0)	8 (0)	206.500	206.5067	-0.006667
26	35 (0)	2 (0)	70 (0)	8 (0)	206.500	206.5067	-0.006667

The second order regression equation provided the levels of L-glutaminase production as a function of initial values in Table 4 of temperature, inoculum volume, initial moisture content and pH, which can be predicted by the following equation,

Enzyme Yield $Y = 72.9225 + 1.51675\,A + 3.22917\,B + 1.74021\,C + 10.39917\,D - 0.03238\,AB - 0.00221\,AC - 0.01888\,AD - 0.02231\,BC + 0.10562\,BD - 0.01881\,CD - 0.01585\,A^2 - 0.36865\,B^2 - 0.01054\,C^2 - 0.52365\,D^2$

Table 4

Regression data of the model

	Coefficient	Regression	Std .Error	t-value	p-value
Constant	β_0	72.9225	5.414473	13.4681	0.000000
A	β_1	1.51675	0.130535	11.6195	0.000000
A^2	β_{11}	-0.01585	0.001309	-12.1044	0.000000
B	β_2	3.22917	0.561976	5.7461	0.000039
B^2	β_{22}	-0.36865	0.032727	-11.2641	0.000000
C	β_3	1.74021	0.065268	26.6627	0.000000
C^2	β_{33}	-0.01054	0.000327	-32.1946	0.000000
D	β_4	10.39917	0.680227	15.2878	0.000000
D^2	β_{44}	-0.52365	0.032727	-16.0002	0.000000
AB	β_{12}	-0.03238	0.008570	-3.7777	0.001825
AC	β_{13}	-0.00221	0.000857	-2.5817	0.020846
AD	β_{14}	-0.01888	0.008570	-2.2024	0.043692
BC	β_{23}	-0.02231	0.004285	-5.2071	0.000106
BD	β_{24}	0.10562	0.042850	2.4650	0.026257
CD	β_{34}	-0.01881	0.004285	-4.3903	0.000527

The *p*-values were 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 test variables. If the model is a good predictor of the experimental data, the computed F-value would be higher than the tabulated F-value. From the calculated F value (F model = 1736.909), the model is generously significant and a very low probability value ($p<0.0001$) (Table 5). Also Table 5 shows that pH and temperature influence were significant ($p<0.0001$) for the L-glutaminase production.

Table 5
ANOVA for the model

Source of variation	SS	df	Mean square(MS)	F-value	*P*> F
Model	41.14155	14	51.02747	1736.909	< 0.0001
A	0.81770	1	0.81770	27.834	< 0.0001
A^2	4.30441	1	4.30441	146.516	< 0.0001
B	0.21850	1	0.21850	7.438	0.015585
B^2	3.72754	1	3.72754	126.880	< 0.0001
C	0.13350	1	0.13350	4.544	0.049973
C^2	30.45036	1	30.45036	1036.491	< 0.0001
D	1.55550	1	1.55550	52.947	< 0.0001
D^2	7.52105	1	7.52105	256.007	< 0.0001
AB	0.41926	1	0.41926	14.271	0.001825
AC	0.19581	1	0.19581	6.665	0.020846
AD	0.14251	1	0.14251	4.851	0.043692
BC	0.79656	1	0.79656	27.114	0.000106
BD	0.17851	1	0.17851	6.076	0.026257
CD	0.56626	1	0.56626	19.275	0.000527
Error	0.44068	15	0.02938		
Total	41.58223	29			

df, degree of freedom; SS, sum of squares; F, factor F; P, probability.

Table 6
ANOVA for the design

Mean	51.02747
R- square	0.9894
Adjusted R- square	0.9751
F-value	1736.909

The correlation coefficient, R^2 value (Table 6) being the measure of the goodness of fit of the model, indicates that 98.94% of the total in the dependent variable (response) could be explained by the model. The adjusted R^2, which is more suited for models with different numbers of independent variables, was 0.9751. Normally, a regression model is considered to have a very high correlation when its R^2 value is higher than 0.9. The closer the value of R (correlation coefficient) to 1, the better is the correlation between the experimental and predicted values[28]. A good association between observed and predicted results reflected the exactness and applicability of the central composite design for process optimization.

3.7 Interaction effects of fermentation variables

The yield of L-glutaminase over different combinations of independent variables was visualized through three-dimensional view of response surface plots in Fig 6 to 11. Response surface plot is a function of two factors at a time maintaining all other factors at a fixed level (zero for instance) which is more helpful in understanding both the main and interaction effects of the two factors. All the response surface plots revealed that at low and high levels of the variables, the L-glutaminase yield was maximal, however, it was noted that there existed a region where there was neither an increasing nor a decreasing trend in the enzyme yield. This phenomenon confirmed that there was an existence of optimum for the fermentation variables in order to maximize L-glutaminase yield.

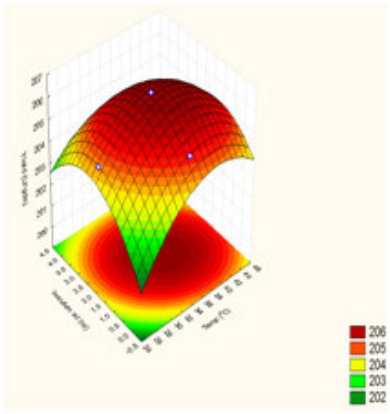

Figure 6
Effect of temperature and inoculum volume on L- glutaminase yield

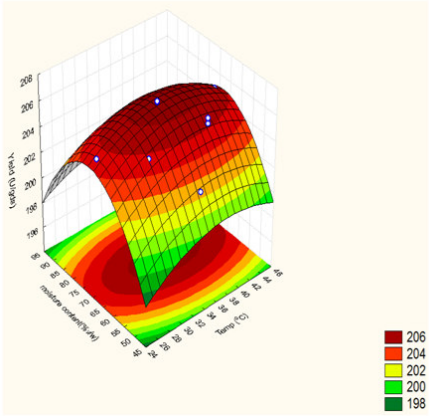

Figure 7
Effect of temperature and moisture content on L-glutaminase yield

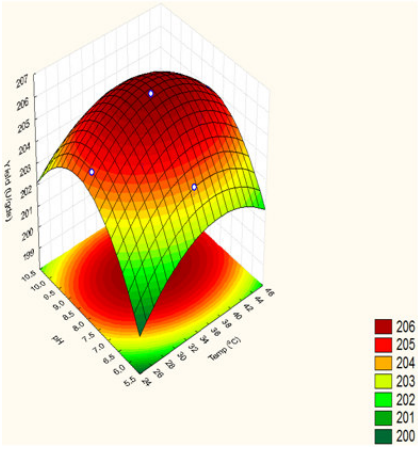

Figure 8
Effect of temperature and pH on L-glutaminase yield

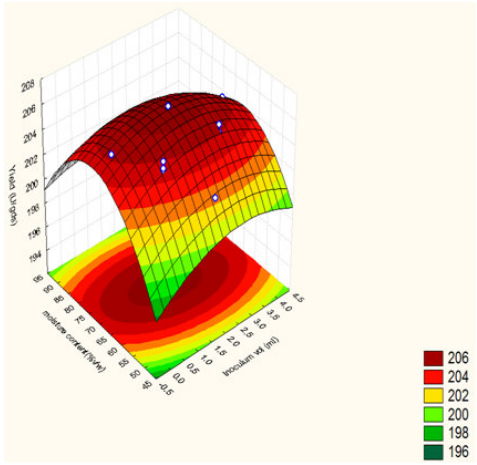

Figure 9
Effect of inoculum volume and moisture content on L-glutaminase yield

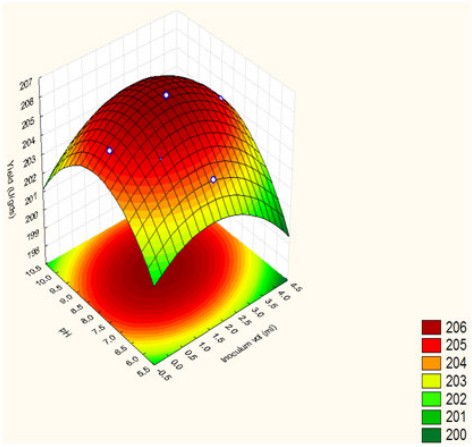

Figure 10
Effect of inoculum volume and pH on L-glutaminase yield

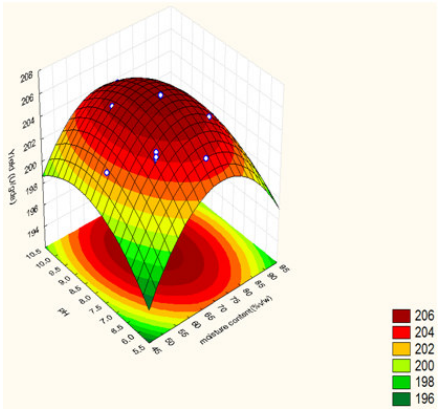

Figure 11
Effect of moisture content and pH on L-glutaminase yield

4. CONCLUSION

The important applications of L-glutaminase in the biotechnological industries and its production in large scale focussed to search high potential microorganisms. This study has been taken up with a view of isolating the actinomycetes producing L-glutaminase from marine and mangrove soil samples and comparison of yields of wild and mutant actinomycete strains. The present study shows that mutant actinomycetes culture can produce L-glutaminase with relatively good yield from sesame oil cake as a substrate which is easily available and economical, in solid state fermentation. In this work, medium components and process parameters for maximum L-glutaminase production were optimized by RSM. Central composite design was used to study the interactive effects of temperature, inoculum volume, initial moisture content and pH on L-glutaminase production. The optimal levels of medium components and parameters were obtained as temperature 36.21°C, inoculum volume 1.86 ml, initial moisture content 69.47 %v/w and pH 8.2. Using this optimized environment, the produced yield of L-glutaminase reached 203.86 U/gds. These results show a close agreement between the expected and obtained production levels.

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