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Statistical Optimization based Production of L-Glutaminase (EC.3.5.1.2) by *Serratia marcescens* under submerged Fermentation

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Abstract

L-Glutaminase majorly produced by microorganism including bacteria, yeast and fungi.L-Glutaminase mainly catalyzes the hydrolysis of γ-amido bond of L-Glutamine. In this report medium components optimization was through one-factor-at-a-time approach for the submerged production of L-Glutaminase by serratia marcescens using different additional carbon, nitrogen, amino acids, mineral salts and was treated with different concentration of sodium chloride. A significant influence of medium components (g/L) Galactose 10.0, Yeast extract 10.0, L-Glutamine 10.0, Manganous sulphate 0.5, KH_2PO_4 0.5, K_2HPO_4 0.5, NaCl 7.5 on L-Glutaminase production was noted. After medium components optimization, a face centered central composite design was used to identify the interactive effects of five independent variables,viz., Temperature , pH, Time, different concentration of Galactose and L-Glutamine on L-Glutaminase production. A significant influence of variables on L-Glutaminase production was noted. Response surface methodology predicted that a production containing Temperature 36.31 ^{0}C, pH 7.34, Time 67.63 h, Galactose 40.20 g/L and L-Glutamine 19.09 g/L to be optimum for the production of L-Glutaminase.This medium components and parameters was projected theoretically to produce an L-Glutaminase activity of 2676.15 IU/ml.The used methodology was validated using this optimized media components and parameters, the L-Glutaminase activity 2670.01 IU/ml was obtained.

Keyword: L-Glutaminase, one-factor-at-a-time, central composite design, serratia marcescens, response surface methodology.

Introduction

L-Glutaminase or Glutaminase (L-glutamine amido hydrolase EC.3.5.1.2) have identified applications in many fields. This enzymes that catalyze the deamidation of L-Glutamine to L-Glutamic acid and Ammonia. L-Glutaminase plays an important role in plants, animal tissues and microorganism including bacteria, fungi and yeast. L-Glutaminase has an important role in cellular nitrogen metabolism[1-4]. This enzyme widely used in industrial and pharmaceutical sector as an effective therapeutic agent in the treatment of HIV[5-6] and acute lymphocytic leukaemia[7]. The L-Glutaminase causes selective death of glutmine dependent tumor cells by blocking these cells of glutamine. The use of L-Glutaminase to blocking neoplasms of essential nutrients helps in the treatment of malignancies[7] and also used as an analytical reagent in the determination of glutamate and glutamine[8-9], as a biosensing agent in biosensor[10]. L-Glutaminase used in the food industries to enhances the flavour and aroma of fermented foods by increasing their glutamic acid level and thereby imparting a delicious taste[11-12]. L-Glutaminase has replaced the use of monosodium glutamate to imparting the flavour in Chinese foods[13] and also used in the manufacture of threonine by gamma glutamyl transfer reactions[14]. Its commercial demands gives much attention to search the viable bio processing technology for its large scale production[15].

Hence researchers are concerned in the identification of microbial strains and developing the viable bio processing technique to improved productivity. Bioprocess Engineering takes a role in enhancing the metabolite productivity under a given set of fermentation environment[16]. Enhancement of metabolite production is generally attempted by manipulating the dependent and independent variable of the process.

Generally interactions of medium components and incubational parameters with the cell metabolism to the production of the required compound are plentiful, so the optimum process conditions may be developed using an effective experimental design tools. In recent year, Response Surface Methodology (RSM) has been used to media components and parameter optimization for their large scale production. These designs show the effect of individual factors and their approach with neighbourhood factors of the optimum production. In general practice approach for identification of medium components and parameter does not show the net effect of total interactions between various medium components and parameters[17].

Response Surface Methodology not only allows the quick screening of a large experimental data, but also shows the each and every variable interaction in the processes. RSM provides an important relationship regarding the optimum level of each factor along with its interactive effects with other factors and

their effects on the metabolite yield[18]. Hence statistical method is suitable to show a near optimum conditions and for exact conditions in a multi factorial designs. RSM decreases the number of experiments without neglecting the interaction among the dependent and independent variables[19]. This design approach improves statistical interpretation possibilities and identify the significance of all affecting factors even in the presence of complex interactions.

To our knowledge reports on the production of L-Glutaminase from *serratia marcescens* is scanty. It's an aerobic a gram-negative bacillus classified as a member of the Entero-bacteriaceae. In the present investigation.

One-factor-at-a-time approach was used to select the best interaction of carbon, nitrogen, amino acids, mineral salts and different concentration of sodium chloride sources and validated the enhancement of mixed sources on L-Glutaminase production and A RSM technique, a face centered central composite design was used to investigate the interactive effect of five variables viz., Temperature, pH, Time, different concentration of Galactose and L-Glutamine on L-Glutaminase production by *serratia marcescens* under submerged fermentations.

Material and Methods

Medium components: Nutrient broth, L-Glutamine, Nesslers's reagent and other media components and chemicals were procured from Hi-Media Limited, Mumbai, India. For optical density measurements, the absorbance was read using UV/Vis Bio Spectrophotometer (EliCo Pvt.Ltd., India).

Micro organism and Culture maintenance: *Serratia marcescens* NCIM 2919 procured from NCIM, National Chemical Laboratory, India, was used in the study. The culture was maintained on Nutrient agar medium slants. Inoculated slants were grown in an incubator at 33^0C for 4 days. After that the slants were stored at 4^0C in a refrigerator for short term preservation and sub cultured every 15 days in the above-mentioned media.

Inoculum preparation: Inoculum was prepared in 250 ml Erlenmeyer flasks containing 100 ml of nutrient broth liquid medium (pH 7). Prepared medium was autoclaved at 121^0C (15 lb) for 20 min and then inoculated with *Serratia marcescens* raised from Nutrient agar slants. The inoculated flasks were kept on a shaker at 150 rpm for 24 h and used as the inoculum.

Identification of medium components: Initially optimization of medium components required for maximum L-Glutaminase production by *Serratia marcescens* was evaluated in 100 ml of 250 ml Erlenmeyer flasks at 33^0C for 24 h at 150 rpm by adding 0.002% of inoculum. The L-Glutaminase production on nutrient broth was used as a control. Consequently the medium component studied included the effect of different additional carbon sources (Malt extract, D-glucose, Sucrose, Starch soluble, Tri sodium citrate, Cellobiose Cellulose, D-mannitol, Lactose, Galactose, D-fructose, Maltose) at 10 g/L,effect of different additional nitrogen sources (Peptone, Sodium sulphite, Yeast extract, Urea, Tryptone, Gelatin, Sodium nitrate) at 10 g/L,effect of different additional amino acids (L –Glutamic acid, Glycine, L –Ascorbic acid, L –Glutamine, Cysteine, Alanine) at 10 g/L,effect of different additional minerals (Zinc sulphate, Mercuric sulphate, Manganous sulphate, Copper sulphate, Ferrous sulphate, Magnesium sulphate , Potassium di hydrogen phosphate, Di potassium hydrogen phosphate, Calcium chloride) at 0.5 g/L. After identifying the nutrients improving L-Glutaminase production by one factor-at-a-time approach, the six most important nutrients ,viz., Galactose, Yeast extract, L-Glutamine and Manganous sulphate, Potassium di hydrogen phosphate, Di potassium hydrogen phosphate was selected as a medium components and finally the effect of sodium chloride concentration (10 -50 g/L) on above said medium was studied. All the fermentation experiments were carried out in triplicate.The optimum media was identified (g/L) Galactose 10.0, Yeast extract 10.0, L-Glutamine10.0, Manganous sulphate 0.5, Potassium di hydrogen phosphate 0.5, Di potassium hydrogen phosphate 0.5 and Sodium chloride 7.5, on L-Glutaminase production was observed at 24 h.

Statistical experimental design: After identifying the nutrients improving L-Glutaminase production by one factor-at-a-time approach, the five most important factor, viz., Temperature, pH, Time, Galactose and L-Glutamine were selected. The basal medium contained (g/L) Yeast extract 10.0, Manganous sulphate 0.5, Potassium di hydrogen phosphate 0.5, Di potassium hydrogen phosphate 0.5 and Sodium chloride 7.5, at 150 rpm by adding 0.002% inoculum.Response Surface Method using face centered central composite design (FCCCD) was used to improving L-Glutaminase production using the software Design-Expert Version 8.0.7.1, Stat-Inc.Minneapolis, USA to find the interactive effects of five variables. Central composite design at the given range of the above variables in terms of coded and actual values is presented in table 1. The average maximum L-Glutaminase activities (IU/ml) were taken as responses *Y*. Regression analysis was performed on the resulted data. A second order polynomial equation was then fitted to the data by multiple regression method. This resulted in an empirical model that related the responses measured to the independent factors of the experiment. For a five variable system, the model equation is

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_5 E + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{15} AE + \beta_{23} BC + \beta_{24,} BD + \beta_{25} BE + \beta_{34} CD + \beta_{35} CE + \beta_{45} ED + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{55} E^2$$

Where Y is the predicted response in the design ; β_0 is the intercept in the design ; β_1 , β_2 ,β_3 ,β_4 and β_5 are the linear coefficients in the design ; β_{12} ,β_{13} ,β_{14} ,β_{15} ,β_{23} ,β_{24} ,β_{25} ,β_{34} ,β_{35} and β_{45} are the interaction coefficients in the design ; β_{11} ,β_{22} ,β_{33} ,β_{44} and β_{55} are the squared coefficients in the design ; and A, B, C, D, E, AB, AC, AD, AE, BC, BD, BE, CD, CE, ED, A^2, B^2, C^2, D^2 and E^2 are independent variables in the design. Analysis of variance (ANOVA) was performed. The quantity of variance

explained by the polynomial models obtained was given by the multiple coefficient of determination R^2. The fixed polynomial equation was expressed as three-dimensional response contour and surface plots to discover the interaction of each variable for maximum L-Glutaminase production and picture the correlation between the responses and the experimental levels of each variable used in the design. To optimize the level of each variable for maximum response, 'Response optimizer' process using statistical software package MINITAB (Release15, PA, USA) was employed. The combination of different optimized parameters, which gave maximum L-Glutaminase responses, was tested experimentally to validate the model. All experiments were done in triplicate.

Analytical Experiments: Enzyme separation: At proper time intervals the fermentation broths were harvested for the L-Glutaminase enzyme. The broth was centrifuged at 10000 rpm for 20 min at 4°C in a refrigerated centrifuge and the supernatant collected was used for further enzyme assay procedures.

Determination of Enzyme activity: L-Glutaminase was assayed according to Imada et al[20]. The reaction mixture, containing 0.5ml of an enzyme preparation ,0.5 ml of L-Glutamine(0.04 M), 0.5 ml of phosphate buffer 0.1 M (pH 8.0), and 0.5 ml of distilled water to a total volume of 2 ml solution was incubated at 37°C for 30 min. The reaction was stopped by addition of 0.5 ml of 1.5 M Trichloro acetic acid. Then to 3.7 ml of distilled water, 0.1 ml of the above mixture and 0.2 ml of Nessler's reagent were added and colour developed was read after keeping the mixture at 20°C for 20 min at 450 nm in a spectrophotometer. Enzyme and substrate blanks were used as controls. One unit of L-Glutaminase activity was defined as the amount of enzyme that liberated 1µmol of ammonia per 1 min under optimal assay conditions. Assays were done in triplicate and the mean enzyme activity was expressed as International unit per ml (IU/ml).

Results and Discussion

Identification of medium components: L-Glutaminase production by a *Serratia marcescens* under submerged fermentation state was observed during the course of study and the examination led to an investigation of the potential of L-Glutaminase synthesis in the direction of developing an ideal bioprocess for industrial production of this enzyme. Hence initially the various medium components and process parameters, which influence L-Glutaminase production by *Serratia marcescens* under submerged fermentation conditions, were optimized.

Effect of additional carbon sources: Carbon source represents the energy source that will be accessible for growth of the microorganism. Carbohydrates and related components are finer carbon sources for many microbes [21]. However, in some cases, accumulation of a small amount of outside carbon may lead to an increase in enzyme production. Figure 1 showed the effect of additional carbon source for yield of L-Glutaminase from *Serratia marcescens* was variably changed, when the carbon source changed. In this work, we found yield of L-Glutaminase was high as 38.8 IU/ml by utilized the Galactose as the carbon source.

Table-1
Experimental range and levels of the independent factors used in RSM in terms of actual and coded factors working in the FCCCD

Factors	Levels					
	Code	-2	-1	0	1	2
Temperature(°C)	A	25	30	35	40	45
pH	B	5	6	7	8	9
Time (h)	C	24	48	72	96	120
Galactose(g/L)	D	20	30	40	50	60
L-Glutamine(g/L)	E	10	15	20	25	30

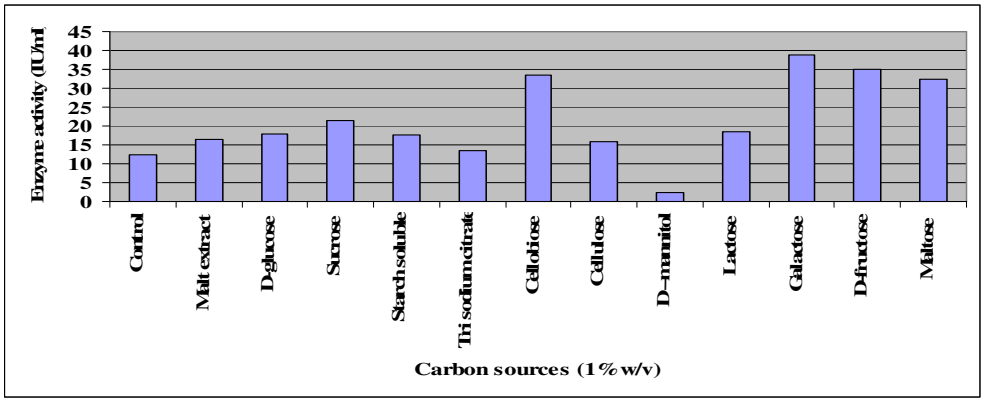

Figure-1
Yield of L-Glutaminase in different carbon sources

Effect of additional Nitrogen sources: Effect of different nitrogen sources, figure 2 showed that the maximum yield was obtained as 25 IU/ml in presence of yeast extract, because the yeast extract serves as complex Nitrogen source for the metabolic activity. Universal ingredient yeast extract was normally added to media for routine growth and amino acid supplementation was not required in complex media containing yeast extract.

Effect of additional amino acids sources: Amino acids were common growth factor required for the production of enzyme as major nitrogen source [22], hence the yield of L-Glutaminase was varied, when the amino acid was changed. Even though each and every amino acid was interchanged by other amino acids, the L-Glutaminase yield was varied according to the environment of amino acids figure 3. Yield of L- Glutaminase from the *Serratia marcescens* was high as 44 IU/ml in L-Glutamine. Since L-Glutamine is the substrate of L-Glutaminase, the addition to fermentation medium might stimulate enzyme production. It also serves as source of energy and carbon.

Effect of additional mineral salt sources: Generally living organisms need some inorganic nutrient for their growth, that do not usually contain the element carbon and when it dissolve in water they separate into ions. L-Glutaminase yield obtained from *Serratia marcescens* in the existence of different mineral salts, figure 4 showed that the maximum yield was 29-31.5 IU/ml in the presence of K_2HPO_4, KH_2PO_4 and Manganous sulphate which is supported both enzyme production and the bacterial growth[23-27].

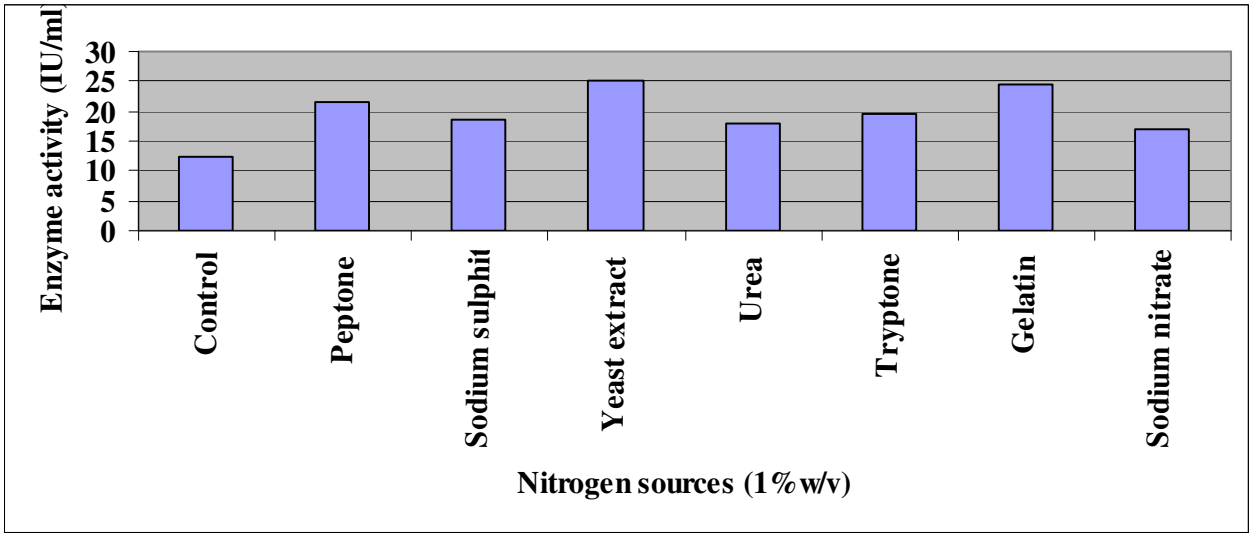

Figure-2
Yield of L-Glutaminase in different Nitrogen sources

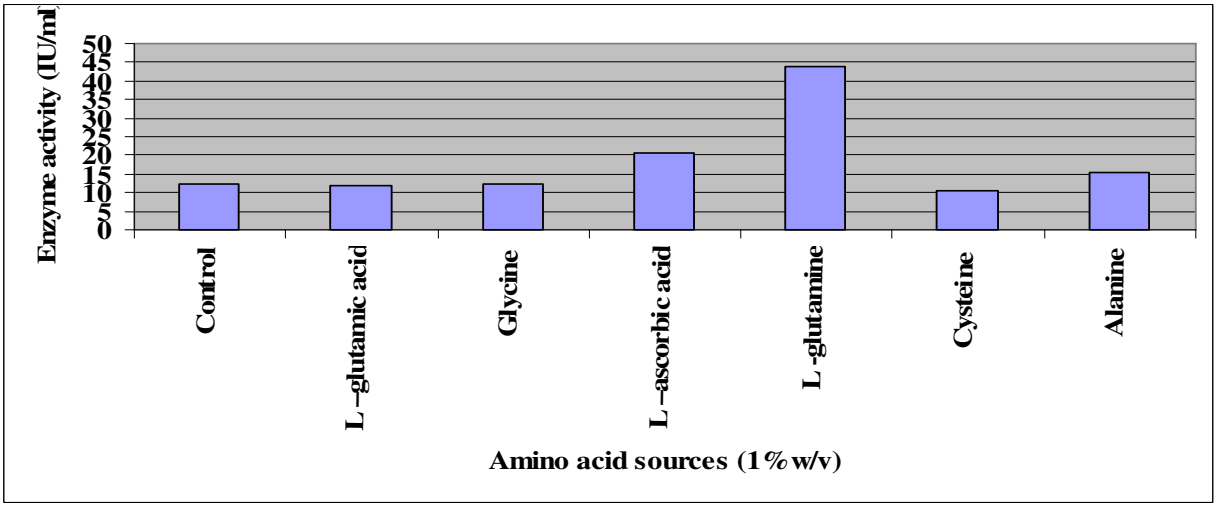

Figure-3
Yield of L-Glutaminase in different Amino acid sources

Effect of additional sodium chloride: Yield of L-Glutaminase was increased, when increased the NaCl concentration up to 0.75 % as maximum as 49.5 IU/ml. Yield was suddenly decreased, figure 5 when the concentration was increased above the 0.75 %.Hence, 0.75 % of NaCl concentration was the optimum for the production of L-Glutaminase from *Serratia marcescens*. The bacteria didn't produce more L-Glutaminase without the NaCl because the *Serratia marcescens* were halophilic, the bacteria were unable or try to grow in the low NaCl concentration, hence there was very low L-Glutaminase production and also the high concentration of NaCl was also affect the growth of bacteria.

Optimum levels of these significant nutrients and the effect of their interactions on L-Glutaminase productions were determined by the one-factor- at -a- time. The optimized medium components (g/L) Galactose 10.0, Yeast extract 10.0, L-Glutamine 10.0, Manganous sulphate 0.5, KH_2PO_4 0.5, K_2HPO_4 0.5, NaCl 7.5 on L-Glutaminase production was noted, which gave the maximum enzyme yield of 49.5 IU/ml.

Statistical Optimization of screened 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 face centered central composite design of Response Surface Methodology. Table 1 shows the information of the actual and coded values working in the FCCCD. The results obtained by FCCCD were analyzed by standard analysis of variance and the mean predicted and observed responses are presented in table 2.

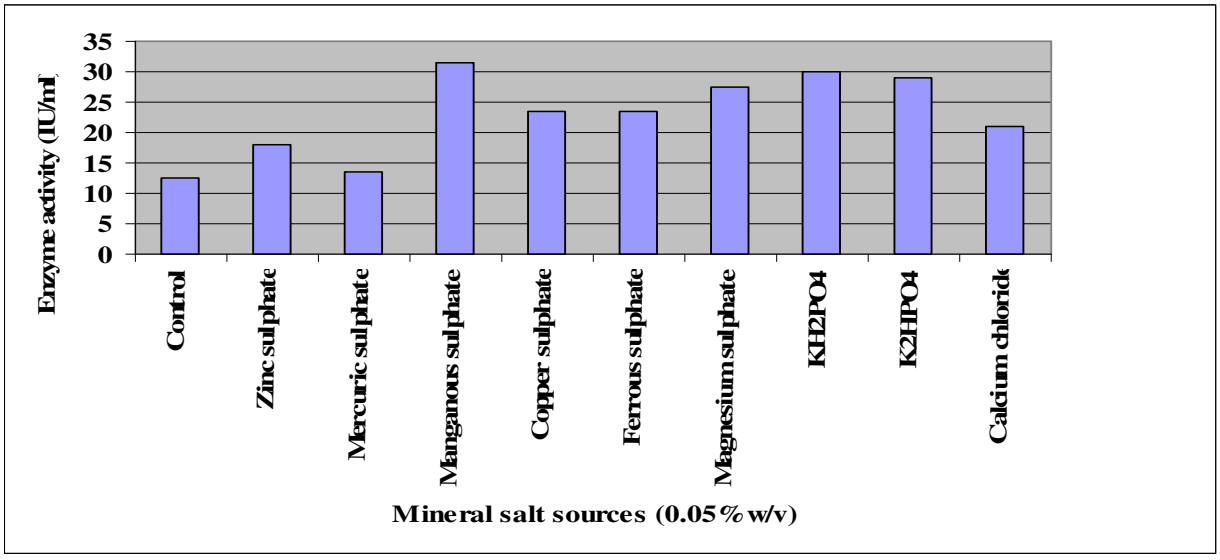

Figure-4
Yield of L-Glutaminase in different Mineral salt sources

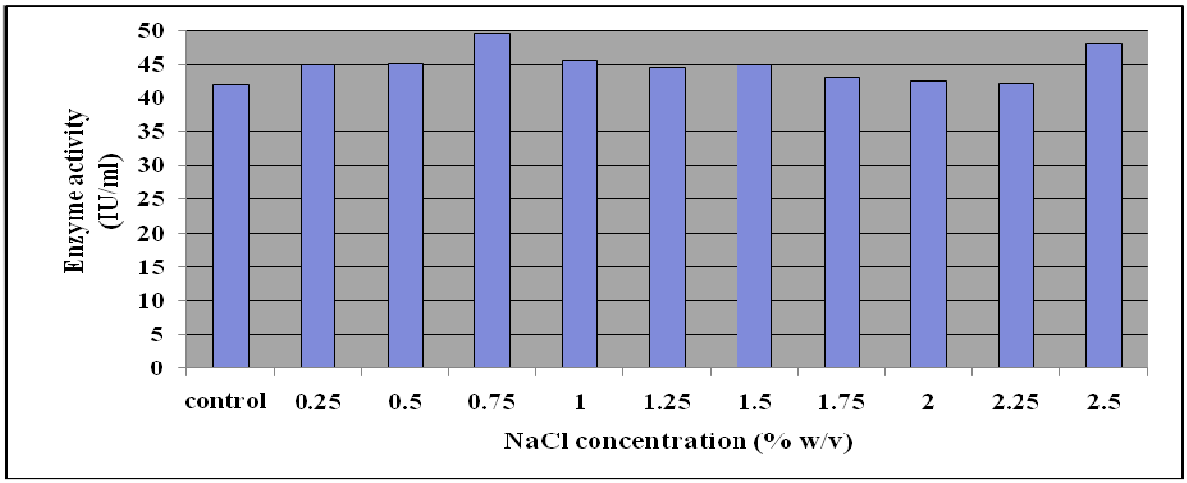

Figure-5
Yield of L-Glutaminase in different concentration of Sodium chloride

Table-2
Results of FCCCD using five independent factors and eight centre points showing observed and predicted responses

Run order	Std order	Temp (^{0}C)	pH	Time (h)	Galactose (g/L)	L-Glutamine (g/L)	Enzyme Activity(IU/ml)	
							Observed	Predicted
1	50	-1	1	-1	1	-1	1045	1076.69
2	42	2	0	0	0	0	1341	1347.02
3	45	0	0	0	0	0	2711.5	2712.24
4	44	1	1	1	-1	-1	1363	1357.53
5	4	0	2	0	0	0	1541	1572.23
6	18	-1	1	1	1	1	2060	2071.85
7	16	0	0	0	-2	0	565	546.58
8	9	0	0	0	0	0	2711.5	2712.24
9	37	0	0	0	0	0	2711.5	2712.24
10	24	0	0	0	0	-2	1302	1294.54
11	2	-1	1	1	-1	1	1445	1449.35
12	34	0	0	2	0	0	657	639.27
13	8	0	0	0	0	0	2711.5	2712.24
14	33	-1	-1	-1	-1	-1	1434.2	1413.15
15	25	1	-1	-1	1	1	907	924.33
16	32	1	-1	1	1	-1	380	361.44
17	20	0	-2	0	0	0	754	731.83
18	5	-1	-1	1	1	1	1280.5	1282.41
19	19	1	1	-1	-1	-1	1945	1952.32
20	17	-1	-1	-1	1	-1	984	984.44
21	49	-1	-1	1	-1	1	1257.3	1261.41
22	29	1	-1	1	1	1	300	294.12
23	47	0	0	0	0	0	2711.5	2712.24
24	22	1	1	-1	-1	1	1269.6	1286.12
25	31	-1	1	-1	-1	-1	905	903.92
26	39	1	1	1	1	-1	1563.1	1575.14
27	43	-1	1	1	1	-1	1532	1530.84
28	26	0	0	0	0	0	2711.5	2712.24
29	10	-2	0	0	0	0	1272.5	1275.54
30	13	-1	1	-1	-1	1	833	843.83
31	36	1	-1	-1	-1	1	1376.1	1366.65
32	15	-1	-1	-1	1	1	1111.2	1117.76
33	30	0	0	0	0	2	1126.5	1143.02
34	3	-1	-1	-1	-1	1	1350	1350.84
35	27	0	0	0	0	0	2711.5	2712.24
36	40	1	1	1	1	1	1500	1510.04
37	7	-1	-1	1	1	-1	767.5	743.62
38	28	1	-1	-1	1	-1	1383.4	1397.11
39	14	1	-1	-1	-1	-1	2051	2035.07
40	46	0	0	-2	0	0	1124	1150.80
41	35	0	0	0	2	0	500.7	528.19
42	12	1	-1	1	-1	1	493.1	482.35
43	38	1	1	1	-1	1	1113.01	1096.78
44	21	-1	1	-1	1	1	1278	1212.23
45	1	0	0	0	0	0	2711.5	2712.24
46	41	1	1	-1	1	-1	1956	1915.85
47	48	1	1	-1	1	1	1475.7	1445.28
48	11	1	-1	1	-1	-1	698.6	745.31
49	23	-1	1	1	-1	-1	1137.5	1103.99
50	6	-1	-1	1	-1	-1	866.5	918.25

The second order regression equation provided the levels of L-Glutaminase production as a function of initial values in table 3 of temperature, pH, time, galactose and L-Glutamine, which can be predicted by the following equation,

Enzyme Activity Y = 2712.24+ 15.03x A + 176.67xB-107.54xC-3.87xD -31.85x E +106.62x A x B -198.72x A x C -52.31x A x D -151.53x A x E +173.74x B x C +150.37x B x D +0.55x B x E +63.52x C x D +101.37x C x E +48.91x D x E -247.66x A^2 -275.81x B^2-321.24x C^2 -384.46x D^2-264.01xE^2.

ccording to the present designed Model, table 4 shows the F-value of 1600.52 implies the model is significant. There is only a 0.01% chance that a "Model 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, B, C, E, AB, AC, AD, AE, BC, BD, CD, CE, DE, A^2,B^2,C^2,D^2,E^2 are significant model terms.Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

ANOVA indicated in table 5, the R^2 -value of 0.9991 for response *Y*. This again ensured a reasonable adjustment of the quadratic model to the experimental data, and indicated that the model could explain 95% of the variability in the response. The "Pred R-Squared" of 0.9967 is in reasonable agreement with the "Adj R-Squared" of 0.9985."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Here ratio of 137.449 indicates an adequate signal. This model can be used to navigate the design space. This model can be used to navigate the design space. A good association between observed and predicted results reflected the exactness and applicability of the central composite design for process optimization.

L-Glutaminase yield for different levels of factors was predicted from the relevant contour and surface plots figure 6 (A-J). Each contour curve represents an infinite number of combinations of two test factors with the other two maintained at their respective zero levels. Elliptical nature of the contour in 3D response surface graphs figure 6 (A-J) depicted the shared interactions of all the factors. There was a relative significant interaction between every two variables, and there was a maximum predicted yield as indicated by the surface confined in the smallest ellipse in the contour diagrams.

Maximum L-Glutaminase production was up to 2711.50 IU/ml when all the variables were kept at their central code. The model was used for optimization by Response optimizer. The model predicted maximum L-Glutaminase production up to 2676.15 IU/ml could be achieved using the medium (g/L) Galactose 40.20,Yeast extract 10.0,L- Glutamine 19.09, Manganous sulphate 0.5, KH_2PO_4 0.5, K_2HPO_4 0.5 at pH 7.34 in Temperature of 36.31°C for 67.63 h. Thus, L-Glutaminase production was being predicted after validation of RSM.

Table-3
Model coefficients estimated by multiple linear regression and significance of regression coefficient

Factor	Enzyme Activity (IU/ml)	
	Coefficient Estimate	Standard Error
Intercept	2712.24	9.52
A-A	15.03	4.12
B-B	176.67	4.12
C-C	-107.54	4.12
D-D	-3.87	4.12
E-E	-31.85	4.12
AB	106.62	4.80
AC	-198.72	4.80
AD	-52.31	4.80
AE	-151.53	4.80
BC	173.74	4.80
BD	150.37	4.80
BE	0.55	4.80
CD	63.52	4.80
CE	101.37	4.80
DE	48.91	4.80
A^2	-247.66	3.64
B^2	-275.81	3.64
C^2	-321.24	3.64
D^2	-384.46	3.64
E^2	-264.01	3.64

Among the five variables tested, Galactose and L-Glutamine was the most considerable factor influencing production of L-Glutaminase. It was found that high Galactose concentration was inhibitory to L-Glutaminase production while lower concentration decreased the production of L-Glutaminase. Thus a balance of carbon source enough to attain L-Glutaminase was required. As clear from figure 6 C, D, F-J, the minimum response for enzyme production occurred when L-Glutamine and Galactose both were in low concentration, while production increased considerably as concentration of L-Glutamine and Galactose was increased. This suggested the L-Glutamine to have a significant effect on enzyme production. L-Glutaminase being a primary metabolite its production was directly related to enzyme being generated. As the L-Glutamine concentration increased, the response indicated a maximum enzyme production of approximately 2711.50 IU/ml nearly at the middle of Galactose level. higher Galactose concentrations beyond this limit decreased L-Glutaminase titres showing a tendency towards nutrient limitation. A very high concentration of L-Glutamine is uneconomical in the production of enzyme[11]. The response also varied distinctly at different levels of L-Glutamine along the axis. Figure 6 D.G, I, J suggesting that there is a considerable interaction of L-Glutamine with Galactose, Temperature, pH and Time.

Table-4
ANOVA for Response Surface Quadratic Model

Source	Sum of Squares	df	Mean Square	F value	p-value prob> F
Model	2.359E+00720	20	1.179E+006	1600.52	< 0.0001
A-A	9779.39	1	9779.39	13.27	0.0010
B-B	1.352E+006	1	1.352E+006	1834.57	< 0.0001
C-C	5.009E+005	1	5.009E+005	679.69	< 0.0001
D-D	647.30	1	647.30	0.88	0.3564
E-E	43948.60	1	43948.60	59.64	< 0.0001
AB	3.638E+005	1	3.638E+005	493.65	< 0.0001
AC	1.264E+006	1	1.264E+006	1714.69	< 0.0001
AD	87561.71	1	87561.71	118.82	< 0.0001
AE	7.347E+005	1	7.347E+005	997.03	< 0.0001
BC	9.659E+005	1	9.659E+005	1310.77	< 0.0001
BD	7.236E+005	1	7.236E+005	981.87	< 0.0001
BE	9.80	1	9.80	0.013	0.9090
CD	1.291E+005	1	1.291E+005	175.21	< 0.0001
CE	3.288E+005	1	3.288E+005	446.18	< 0.0001
DE	76547.08	1	76547.08	103.87	< 0.0001
$\mathbf{A^2}$	3.408E+006	1	3.408E+006	4624.95	< 0.0001
$\mathbf{B^2}$	4.227E+006	1	4.227E+006	5736.17	< 0.0001
$\mathbf{C^2}$	5.734E+006	1	5.734E+006	7781.54	< 0.0001
D	8.214E+006	1	8.214E+006	11145.97	< 0.0001
$\mathbf{E^2}$	3.873E+006	1	3.873E+006	5255.85	< 0.0001
Residual	2.361E+007	29	736.93		
Lack of Fit	21371.02	22	971.41		
Pure Error	0.000	7	0.000		
Cor Total	21371.02	49			

Table-5
ANOVA for the design

Term	Response of L-Glutaminase activity(IU/ml)	Term	Response of L-Glutaminase activity(IU/ml)
Std. Dev.	27.15	R-Squared	0.9991
Mean	1418.74	Adj R-Squared	0.9985
C.V. %	1.91	Pred R-Squared	0.9967
PRESS	78125.15	Adeq Precision	137.449

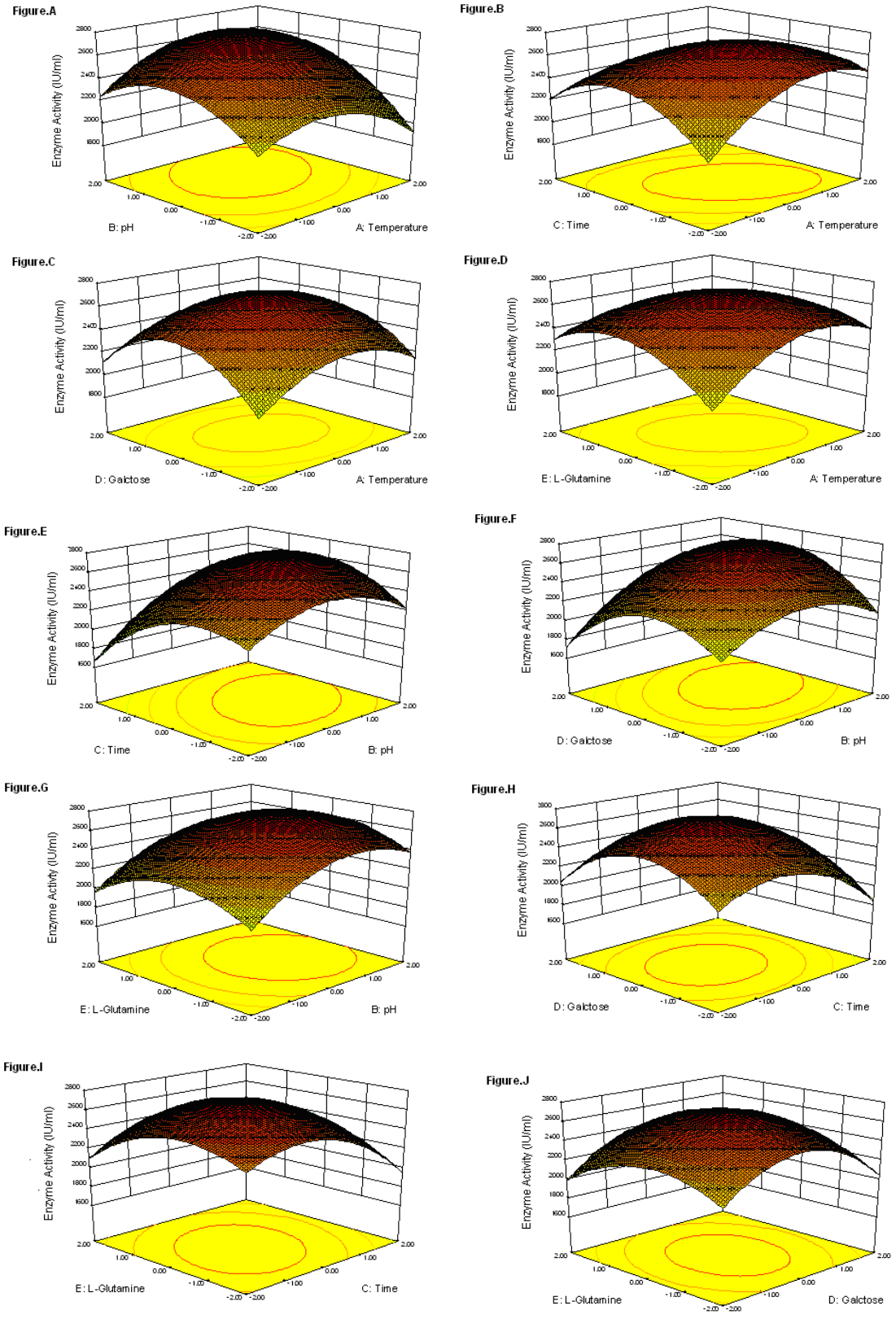

Figure-6 A-J
Three-dimensional response surface and contour plots for L-Glutaminase production showing the interactive effects of five selected variables

Almost every biological process was pH dependent; a small variation in pH had changed the rate of process. Hence, the optimal pH was very important for maximizing the yield of L-Glutaminase production. L-Glutaminase yield was in increasing trend by increased the pH as 5.0 to 7.0 and after that pH, the yield was decreased in higher pH. Figure 6 A, E-G showed the maximum and minimum responses on the production.

Incubation temperature also influenced the microbial metabolism, on incubated in different temperature. Figure 6 A-D showed the increased trend of yield from 25°C to 35°C and after that the yield was decreased from 35°C to 45°C. Incubation time was optimized on L-Glutaminase production because the yield of L-Glutaminase was specifically based on substrate utilization and generation time of bacteria. Thus the yield was increased randomly when the incubation time was increased up to 67.63 h, figure 6 A-D after that the yields become low due to the competitive between them for the substrate.

Validation was carried out under conditions predicted by the model. The predicted yield was 2676.15 IU/ml. On experimentation, the L-Glutaminase production was about 2670.01 IU/ml was obtained. The experimental values were found to be very close to the predicted values and hence, the model was successfully validated.

Conclusion

In this work medium components and process parameters for maximum L-Glutaminase production from *Serratia marcescens* were optimized by one- factor- at -a time approach and by RSM. Using one factor at a time approach (g/L) Galactose 10.0,Yeast extract 10.0,L-Glutamine 10.0, Manganous sulphate 0.5, KH_2PO_4 0.5 , K_2HPO_4 0.5,NaCl 7.5 were found to be the most considerable variables, which significantly enhanced L-Glutaminase production. Central composite design was used to study the interactive effects of Temperature, pH, Time, different concentration of Galactose and L-Glutamine on L-Glutaminase production. The optimal levels of medium components and parameters were obtained as Temperature 36.31°C, pH 7.34, Time 67.63 h, Galactose 40.20 g/L and L-Glutamine 19.09 g/L. Using this optimized environment, the produced enzyme activity of L-Glutaminase reaches 2670.01 IU/ml. The results show a close agreement between the expected and obtained production level.

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