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Original Research Article

Optimization of L-asparaginase production form *Pseudomonas fluorescens* by Response Surface Methodology

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Keywords

L-asparaginase, *Pseudomonas fluorescens*, Submerged fermentation, Optimization, RSM, Kinetic studies

A B S T R A C T

The current study is carried out on statistically-based experiments for the optimal production of L-asparaginase enzyme from *Pseudomonas fluorescens* by submerged fermentation. The parameters like pH, temperature and media concentration were considered for improving enzyme production process. The Response Surface Methodology (RSM) was used to study the effect and interaction between parameters for optimal production. Maximum enzyme activity (227 U/ml) has been obtained under the optimal values of process parameters. RSM proved to be a powerful method which increased the production by 2.14 folds. The optimization method described here is effective for determining the optimum parameters for maximum production of L-asparaginase. Kinetic studies on growth of organism with respect to production of enzyme indicated associated growth form of organism with respect to enzyme production. The values of kinetic parameters – μ_{max} 0.023 h^{-1} and K_s 0.41g/100ml. This model provided an excellent prediction of the growth kinetics and the interactions between the substrate.

Introduction

Therapeutic enzymes are those enzymes which can be safely used in medicine either alone or in adjunct with other therapies so that different diseases and medical conditions can be cured effectively and safely. L-asparaginase enzyme is used in cancer therapeutics and has shown effective results for Acute Lymphoblastic Leukemia (ALL) treatment of children (Marshall Shannon *et al.,* 2003). Tumor cells are unable to synthesize asparagines, thus leukemic cells require high amount of asparagine. These leukemic cells depend on circulating asparagines (Broome, 1981). L-asparaginase transforms L-asparagine into L-aspartate and ammonia via covalently bound intermediate involving a β-aspartyl enzyme (Bessoumy Ashraf *et al.,* 2004).

This deprives the leukemic cell of circulating asparagine, which leads to cell death. The following microorganisms have been reported for production of L-asparaginase: *Escherichia coli* (Derst *et al.,* 1994); *Serratia marcescens* (Heinemann and Howard, 1969); *Enterobacter cloacae* (Nawaz *et al.,* 1998); *Pseudomonas stutzeri* (Manna *et al.,* 1995); *Erwinia* species (Kotzia Georgia *et al.,* 2007); *Capsicum annum* (Bano Mozeena and Sivaramakrishnan, 1980). Continuous therapy with the enzyme from *E. coli* source has commonly resulted in problems of toxicity, including immunological sensitivity to the foreign protein, thus resulting in a neutralization of drug effect (Allison James *et al.,* 1971). Therefore, the use of new serologically different L-asparaginase with a similar therapeutic effect is highly desirable (Saleem Basha *et al.,* 2009). Production of L-asparaginase enzyme by submerged fermentation has been reported of low yield as compared to solid state fermentation (Saleem Basha *et al.,* 2009). Optimization of growth parameters increases the yield of enzyme activity in fermentation. Optimization by one factor at a time (OFAT) is a well studied method. This method is time consuming but it helps in finding high and low values of process variables. Use of statistical experimental designs for optimization of the process variables is well suited to study the interactive effects of the variables (Sangita Ghosh *et al.,* 2013).

The study on interacting variables is important as it has a major impact on production. Here an attempt was made to study the production of L-asparaginase by selecting an appropriate medium and to determine the optimal conditions with their interactions to obtain the maximum activity using Response surface methodology. Kinetic studies were performed for the growth of microorganism on substrate and product formation to obtain the growth rate of product formation.

Materials and Methods

Microbial strains: *Pseudomonas fluorescens* NCIM 2639 was procured from National Collection of Industrial Microorganisms (NCIM). It was maintained on Nutrient agar, pH 7.4. The slants were incubated at 25 ± 2°C for 24 hrs and the sub-culturing was carried out after every 20 days.

Assay of asparaginase: Asparaginase activity was by determined by method reported by (Shirfrin *et al.,* 1974). In brief, 0.1 ml of 189 mM L-asparagine was added to 1.0 ml of 50 M Tris-hydrochloride buffer (pH 7.4), the volume was made up to 2ml by water. To this mixture 0.1 ml of sample was added and incubated for 30min at 37^{o}C. The reaction was quenched by addition of 0.1 ml 1.5M Trichloroacetic Acid (TCA). 0.2ml of supernatant from the centrifuged reaction mixture was diluted by 4.3 ml of double distil water followed by addition of 0.5ml of Nessler's reagent. The liberated ammonia was determined spectrophotometrically at 436nm. The concentrations of ammonia were determined from a standard curve with ammonium sulfate as the source of dissolved ammonia. 1 IU (International Unit) of L-asparaginase is equal to amount of ammonia liberated from L-asparagine per minute.

Site of enzyme production

To determine the site of L-asparaginase production i.e. extracellular or intracellular, *Pseudomonas fluorescens* was grown in nutrient broth medium and incubated for 24 hrs at 25^{0}C. After 24 hrs, 50ml of culture medium was centrifuged at 10,000rpm for 10 min. Enzyme assay for production of L-asparaginase was carried on supernatant.

The pellet was re-dissolved in phosphate buffer pH 6.8 solution and was subjected to sonication for 5min. The sonicated sample was centrifuged at 10,00 rpm for 5 min and the supernatant was subjected for enzyme assay with appropriates blank samples.

Screening of medium

Type of conventional medium which enhances optimum activity was screened from Nutrient broth, Luria bertani broth and MGYP broth (Malt extract, Glucose, Yeast extract and Peptone). 1mL of overnight grown culture was inoculated into the Erlenmeyer flask containing 1g of the respective medium in 100ml water. The flasks were incubated at 25°C and fermented samples were taken at 24 h intervals and assayed for enzyme activity

One factor optimization

The initial batch studies were carried out by one factor at a time (OFAT) method by varying only a single factor and keeping the remaining factors constant. The optimal level of nutrient broth concentration was studied by varying the amount as 0.5g/l, 1g/l, 1.5g/l and 2g/l. The initial pH was varied over the range of 2–10 and the effect of incubation temperature was determined by varying the range of 15–30°C, while all other factors were kept constant. Samples were drawn continuously at 24 h interval and the enzyme assay was carried out to calculate the enzyme activity.

Factorial Design

Response Surface Methodology (RSM) was employed using Minitab14 to identify the optimized conditions for the enzyme production. The OFAT study of parameters gave the high and low value for each parameter. These values were used in the RSM design and are as shown in Table 1. For each run triplicate study was carried out. The 20 set of batch experiments designed by software are as given in Table 2**.**

Kinetic studies of bacterial growth

A growth kinetic model for the growth of any microorganism on substrate and product formation is important for development of bioprocess technology and for designing industrial scale bioreactors. The concentration of substrate controls growth rate of product formation. A plot of biomass concentration vs. time was obtained. Variation in biomass concentration was obtained at different initial substrate concentrations for different runs, thus obtaining various Specific growth rates for different substrate concentration. Monod's model has been used to study the growth of *Pseudomonas fluorescens* for production of l-asparaginase. Growth rate of microorganism during exponential growth phase can be represented in equation 1

$$\frac{dx}{dt} = \mu X \qquad (1)$$

μ=Specific growth rate (1/hr), X=biomass concentration.

Monod's equation is given by equation 2

$$\mu = \frac{\mu max * S}{Ks + S} \qquad (2)$$

A graph of 1/ μ Vs 1/S for respective media concentration was plotted; the slope gives 1/ μ_{max} and the intercept gives K_s.

Results and Discussion

Site of production

Enzyme analysis of extracellular and intracellular fluid of *Pseudomonas fluorescens* for production of L-asparaginase indicated that the enzyme is produced

extracellular. Extracellular production would eliminate the interaction of intracellular components, thus affecting less on the stability of enzyme. It would also easy the load on purification.

Screening of medium

Experiments were carried out by using nutrient broth, Luria bertani broth and MGYP broth for L-asparaginase production. The experimental results for screening of conventional mediums are graphically shown in Figure 1. All three mediums showed positive results for the production of the enzyme with yields varying in the range of 59 to 106 (U/ml) (Figure 1). Nutrient broth showed maximum enzyme activity of 106 (U/ml) as compared to Luria bertani and MGYP broths.

Effect of medium concentration on production of L-asparaginase

The amount of medium concentration plays a vital role for the enhancement of the enzyme production. Four different initial medium concentration (nutrient broth) amounts (0.5 g, 1 g, 1.5 g and 2 g per 100 ml) were considered for optimization studies. Figure 2 shows a constant increase in enzyme activity with a maximum value of 135 (U/ml) for 1.5g of substrate per 100 ml. Further increase in substrate concentration is observed to be inhibitory for production of l-asparaginase. Reduction in the enzyme yield is due to substrate inhibition. Thus, an optimal amount of substrate should be used for better activity and 1.5g/100ml of nutrient broth was found as optimum substrate amount for L-asparaginase production in the present study.

Effect of pH on production of L-asparaginase

Initial pH of the medium is necessary for growth and production of enzyme. It reduces lag phase and also enhances the production. Buffers with pH of 2, 4, 6, 8, and 10 were taken into observation. Highest enzyme activity of 129 (U/ml) was observed at a pH of 8 after 96 h of incubation. Figure 3 shows the enzyme activity for the various pH conditions. Maximum enzyme activity was also observed near pH 8 for *Pseudomonas aeruginosa* (Abdel-Fattah and Olama, 2002)

Effect of incubation temperature

The incubation temperature has a direct effect on the growth of the bacteria and thus affects the enzyme production. A range of temperature varying from 25°C to 35°C was taken into study. Maximum enzyme activity of 150 (U/ml) was recorded at 30°C after 96 h of incubation as represented in Figure 4. Further increase in the temperature resulted in reduction of enzyme activity.

An experiment was carried out at the optimum values of medium concentration 1.5 gm/100 ml, initial pH 8 and temperature (30°C). A maximum enzyme activity of 165 U/ml was found after 96 h of fermentation.

Optimization of process variables using Response Surface Methodology

To determine the optimal level of the three variables which influences the enzyme production OFAT design is used. The 20 set of experiments were performed as per the software. The response was activity of enzyme. The response of L-asparaginase activity ranged from 77(U/ml) to 150(U/ml). The RSM design for L-asparaginase is shown in Table 3. The actual values of enzyme activity obtained from the design of experiments versus the predicted values by the software are shown in Figure 5. It is seen that the model predicts most of the experimental data reasonably well with an R^2 value of 0.994.

Analysis of Variance (ANOVA) was performed to obtain the interaction between the process variables and the response. ANOVA statistics results and the estimated regression coefficients are given in Table 4. In this case the process variables and their interaction with each other have P-values less than 0.05, indicating that they are significantly different from zero at the 99.4% confidence level. A second order polynomial equation gives the coefficients for variable effect on production. The values of coefficients were generated from the model. It can be concluded that when the effect of a factor is positive, an increase in the value of the enzyme activity efficiency is observed. It can be observed from equation 3.

Enzyme activity(Y) = 115.16 + 9.32 A + 32.4 B + 12.41 C -16.24 A^2 +18.52B^2 -14.52 C^2 + 1.13 A B -1.88A C +3.03 BC (3)

Where Y is the predicted response; A=pH, B=Temperature, C=media conc.;

The *P*-values were used as a tool to check the significance of each of the interaction among the variables. To make other effect values to less that 0.05 some of the combinations of parameters were eliminated in the software. Equation 4 gives the coefficients for the parameters effect on production.

Enzyme activity(Y) = 115.6 + 9.32 A + 32.4 B + 12.41 C -16.24 A^2 +18.52B^2 -14.52 C^2+ 3.03 BC (4)

This model can be used to navigate the design space. Values of "Prob> F" less than 0.0500 indicate model terms are significant.

Surface plots

The contour and 3D surface plots are generally used to represent the interaction effects between the process variables (Bas and Boyaci, 2007). 3D graphs generated for the pair-wise interaction of the three factors explain the role played by factors affecting L-asparaginase production. From Figure 6, it can be observed that with increase in temperature, concentration of medium from 1 gm/100 ml to 1.25 gm/100 ml and pH from pH 7 to pH 7.5, the yield of L-asparaginase increases considerably. Further increase in media concentration and pH decreases the activity. The contour plots indicate the levels of temperature, pH and media concentration that have to be used to get optimum conditions.

The three dimensional response surface plot between pH and temperature (Figure 7A) shows that the enzyme activity increased towards the higher temperature and reached a maximum (225 U/ml) at highest-value region whereas, in case of pH, a maximum of enzyme activity (200 U/ml) was observed at the mid value of pH (pH 7). Figure 7B represents the combined effect of the media concentration and pH. A maximum activity of 116.90 U/ml is noted at the mid value of media concentration and pH. The interaction between the media concentration and temperature is shown in Figure 7C. It showed that while mid value of media continued to give the maximum activity, high level of temperature was seen to increase the activity to a maximum (218 U/ml). According to Muralidhar *et al.*, 2001, strong interactions between the variables are expected if the contour lines are elliptical in shape. In the present study, strong interaction existed between the media concentration and pH (Figure 7B) for L-asparaginase production since the contour lines were elliptical in nature.

Validation of model

Minitab14 software was used to predict the optimum values of the variables. Theoretical maximum enzyme activity (227U/ml) was

obtained at the optimal values of media concentration 1.075 g/100ml, Initial pH 7.17 and Temperature 35°C. Validation of model was carried out with the optimum values predicted by the software. Results showed that experimental value of enzyme activity (217 U/ml) was very closer to the predicted response (227U/ml) and the predicted model fitted well with 95.59% of experimental results. The preset study showed significant increase in results for L-asparaginase production by in SmF by *Pseudomonas fluorescens.*

Kinetic studies

During the fermentation, L-asparaginase production appears to parallel the growth of the organisms Figure 8. The growth rate rises and decreases but the rate of L-asparaginase production rises to a maximum value and then attains a constant phase. The specific rates, both of growth and of L-asparaginase production, are in a sense measure of the metabolic activity of the individual cells. It is seen, that the specific rates are high in the early part of the fermentations and that they decline steadily as the fermentation proceeds due to the disappearance of nutrients and the accumulation of toxic products. In order to design a fermentor and establish a relationship between substrate and organism, the value of μ_{max} and K_s are required. Increase in μ was observed with increase in the substrate concentration, later increase in substrate concentration is found to be inhibitor which results in decrease in μ as shown in Table 5. The plot of 1/μ vs. 1/s Figure 9 gives a negative slope validating theoretically the above result for decrease in μ. The low value of μ_{max} 0.023 h^{-1} indicates a slow specific growth rate and K_s 0.41g/100ml indicates that the organism requires a low concentration of substrate for growth and product formation. As product formation is growth associated.

Fig.1 Screening of conventional mediums for production of L-asparaginase production

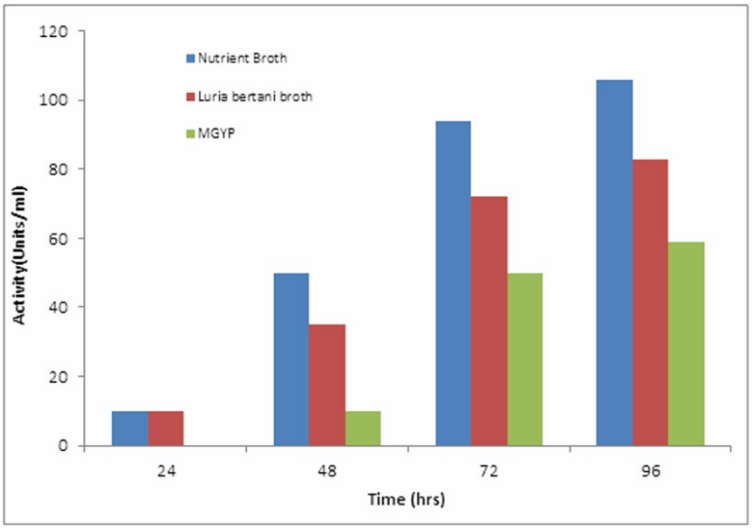

Fig.2 Effect of temperature on production of L-asparaginase

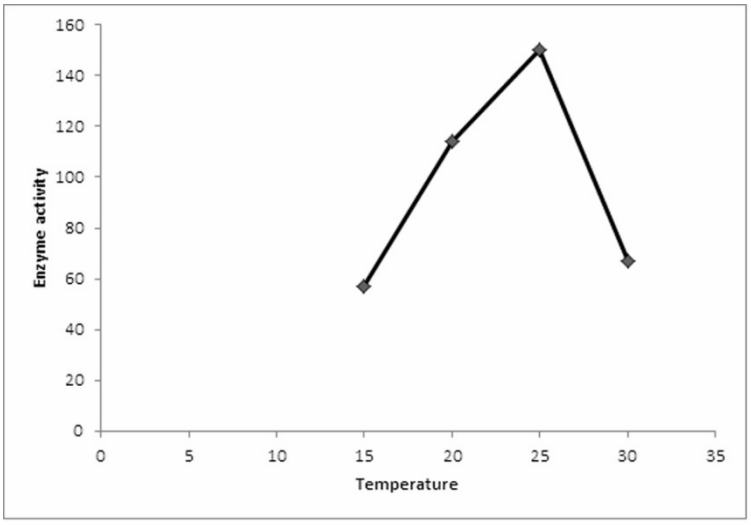

Fig.3 Effect of Media concentration on production of L-asparaginase

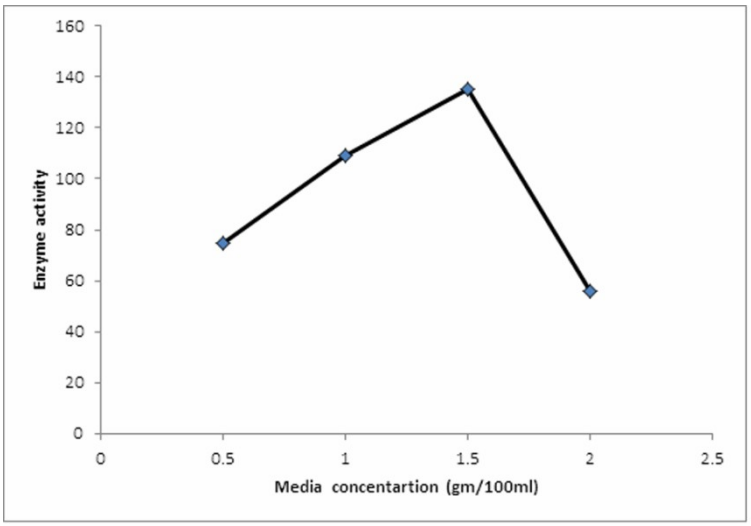

Fig.4 Effect of pH on production of L-asparaginase

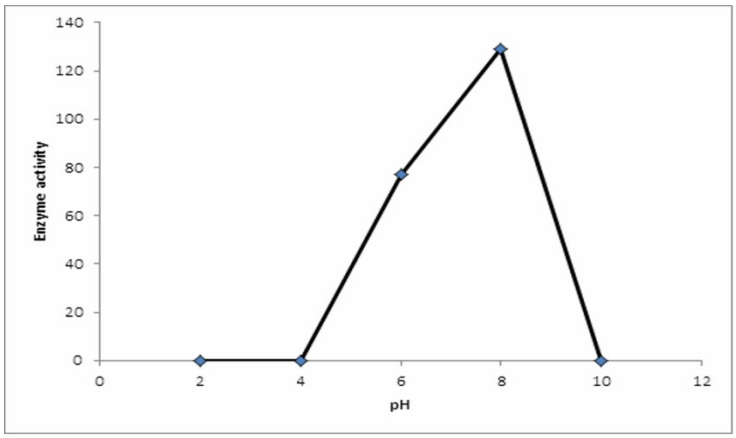

Fig.5 Validation of optimization model by evaluation of Actual Vs Predicted enzyme activity

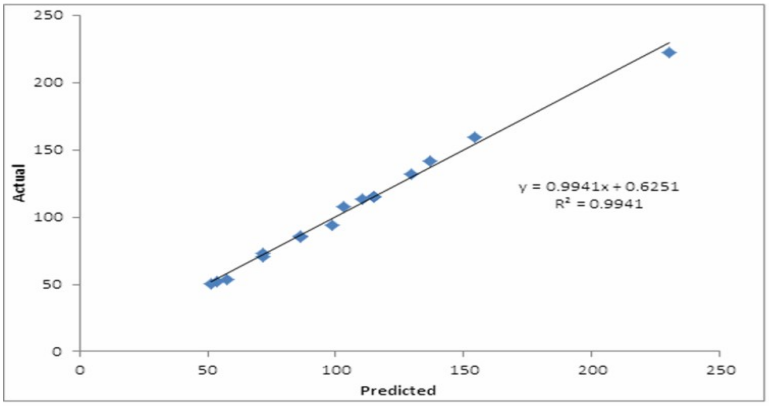

Fig.6 Contour plots for L-asparginase production.These plots repesent the interaction of process parameters

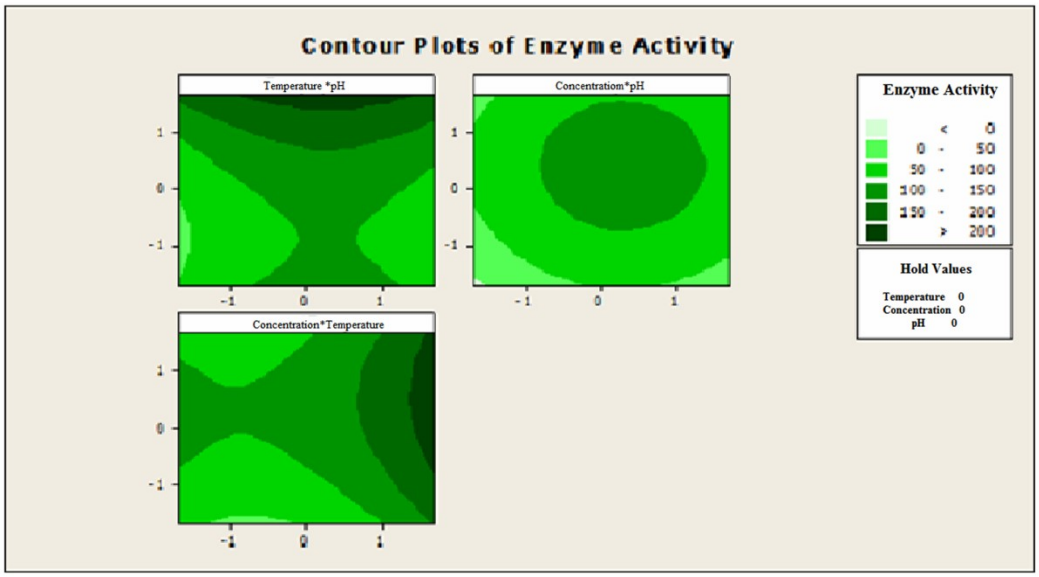

Fig.7 Surface plots for production of L-asparaginase

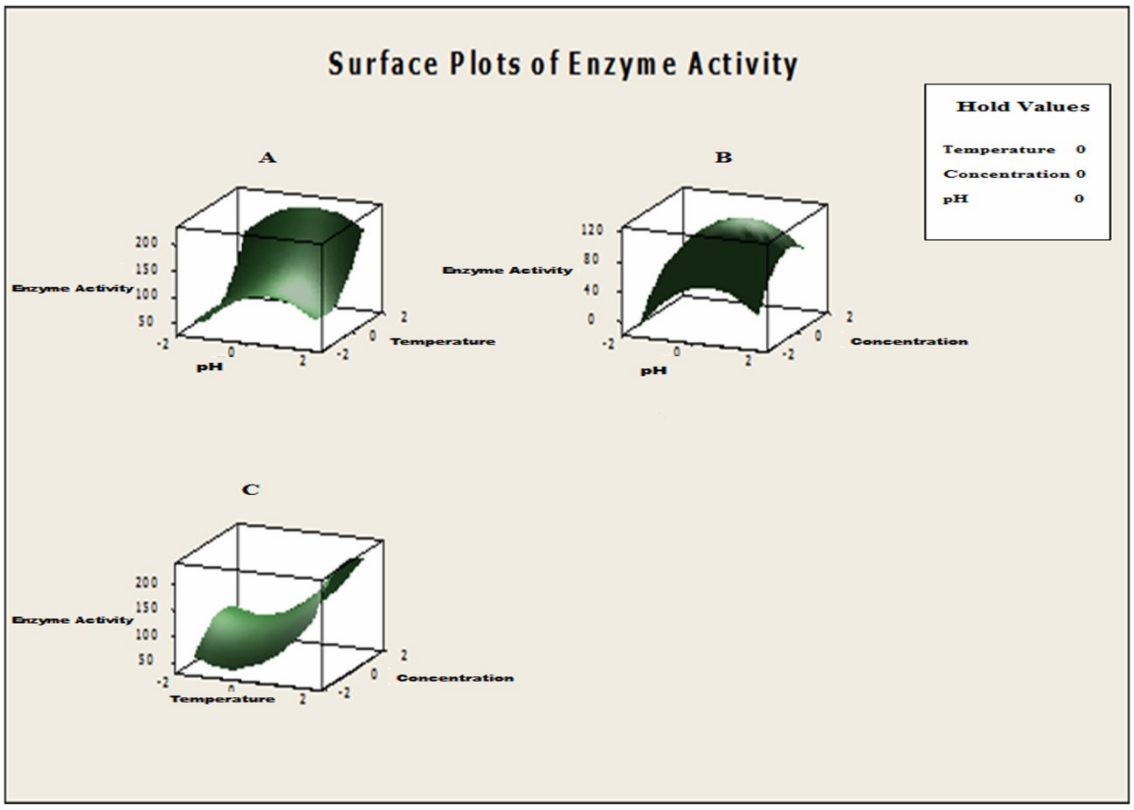

Fig.8 Relationship between enzyme production and biomass formation. (◆) Indicates the production of biomass and (▲) indicates enzyme production

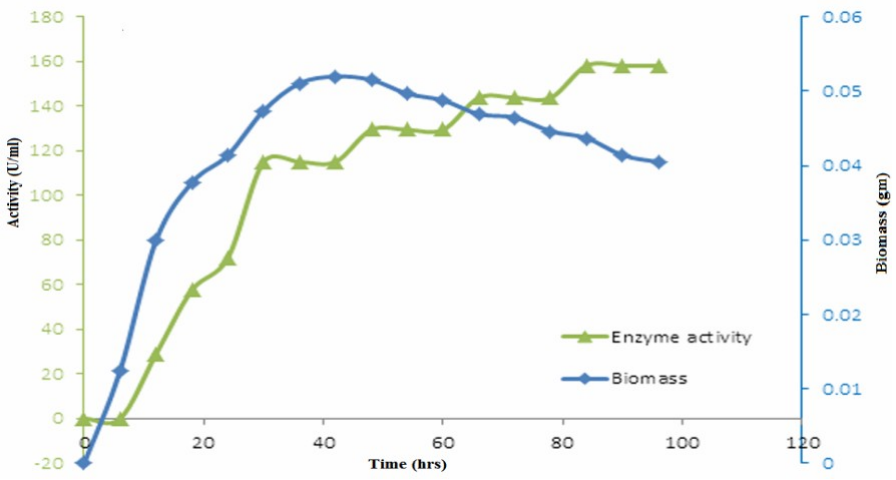

Fig.9 Reciprocal plots of specific growth rate versus reciprocal of limiting substrate for *Pseudomonas fluorescens*

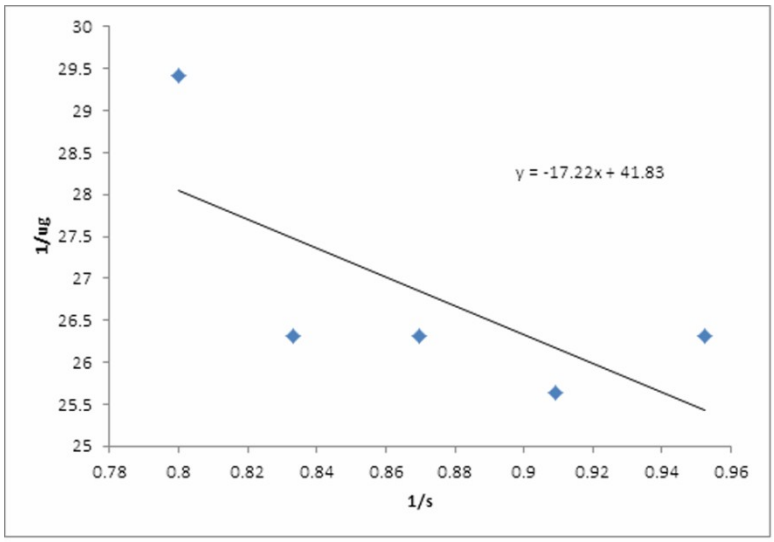

Table.1 Experimental range and level of the process variables for L-asparaginase production

Factor	Component	-	-1	0	+1	
A	pH	6	6.5	7	7.5	8
B	Temperature	20	21.23	22.5	23.75	25
C	Nutrient broth concentration	0.5	0.75	1	1.25	1.5

Table.2 RSM experimental design given by design software Minitab14 for L-asparaginase production

	Values		
	A	**B**	**C**
1	6.5	23.75	0.75
2	7	22.5	1
3	8	22.5	1
4	7	22.5	1
5	6.5	23.75	1.25
6	7	22.5	1.5
7	7	20	1
8	7.5	23.75	1.25
9	7	22.5	1
10	7	25	1
11	7.5	23.75	0.75
12	7	22.5	1
13	7	22.5	1
14	7	22.5	0.5
15	7.5	21.25	1.25
16	7.5	21.25	0.75
17	7	22.5	1
18	6	22.5	1
19	6.5	21.25	0.75
20	6.5	21.25	1.25

Table.3 RSM design for L-asparaginase production with experimental and predicted L-asparaginase activity

	A	**B**	**C**	**Enzyme activity(units/ml)**
1	6.5	23.75	0.75	103
2	7	22.5	1	115
3	8	22.5	1	86.5
4	7	22.5	1	115
5	6.5	23.75	1.25	137
6	7	22.5	1.5	98.5
7	7	20	1	110.63
8	7.5	23.75	1.25	154.38
9	7	22.5	1	115
10	7	25	1	230
11	7.5	23.75	0.75	129.4
12	7	22.5	1	115
13	7	22.5	1	115
14	7	22.5	0.5	53.5
15	7.5	21.25	1.25	86.25
16	7.5	21.25	0.75	71.88
17	7	22.5	1	115
18	6	22.5	1	57.5
19	6.5	21.25	0.75	51.5
20	6.5	21.25	1.25	71.88

Table.4 a,b ANOVA statistics results and the estimated regression coefficients

(a)

Term	Coef	SE Coef	T	P
Constant	115.158	1.738	66.256	0
pH	15.677	1.939	8.083	0
Temp	54.557	1.939	28.131	0
Conc	20.862	1.939	10.757	0
pH*pH	-45.927	3.175	-14.465	0
Temperature*Temperature	52.388	3.175	16.499	0
Concentration*Concentration	-41.927	3.175	-13.205	0
pH*Temperature	3.193	4.262	0.749	0.471
pH*Concentration	-5.314	4.262	-1.247	0.241
Temp*Conc	8.567	4.262	2.01	0.072

(b)

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	30786.6	30786.6	3420.73	188.36	0
Linear	3	17659.8	17659.8	5886.6	324.13	0
Square	3	13014.9	13014.9	4338.31	238.88	0
Interaction	3	111.8	111.8	37.27	2.05	0.17

Table.5 Specific growth rate with increasing substrate concentration

substrate concentration (S) in gm	Specific growth (μ_g)
1.05	0.038
1.1	0.039
1.15	0.038
1.2	0.038
1.25	0.034

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