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Optimization of L-asparaginase production by *Serratia marcescens* (NCIM 2919) under solid state fermentation using coconut oil cake

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Abstract

Background: The present study focused on utilization of agrowaste byproducts generated from oil mill for L-asparaginase enzyme production using *Serratia marcescens* under solid state fermentation. Classical and statistical methods were employed to optimize the process variables and the results were compared.

Results: The classical one factor at a time (OFAT) and response surface methodology (RSM) are employed to optimize the fermentation process. When used as the sole carbon source in SSF, coconut oil cake (COC) showed maximum enzyme production. The optimal values of substrate amount, initial moisture content, pH and temperature were found to be 6 g, 40%, 6 and 35°C respectively under classical optimization method with maximum enzyme activity of 3.87 (U gds⁻¹). Maximum enzyme activity of 5.86 U gds⁻¹ was obtained at the predicted optimal conditions of substrate amount 7.6 g of COC, initial moisture content of substrate 50%, temperature 35.5°C and pH 7.4. Validation results proved that a good relation existed between the experimental and the predicted model.

Conclusions: RSM optimization approach enhances the enzyme production to 33% when compared to classical method. Utilization of coconut oil cake as a low cost substrate in SSF for L-asparaginase production makes the process economical and also reduces the environmental pollution by converting the oil mill solid waste into a useful bioproduct.

Keywords: Coconut oil cake, L-asparaginase, *Serratia marcescens*, Response surface methodology

Background

In recent years, utilization of agriculture based products and byproducts gains more importance in bioprocess industries because of high nutrient content and low cost. Agro byproducts such as oil cakes, peels of banana and citrus, apple pomace, jackfruit seed, wheat and rice bran, baggase have been reported in the literature for production of value added products. Transforming the waste byproducts to a useful bioproduct by fermentation not only reduces the process cost but also the risk of environmental pollution. Coconut oil cake (COC) is obtained as a waste byproduct after oil extraction from dried coconut. Generally COC is used as poultry and fish feed since it is rich in soluble sugars, soluble proteins and

lipids [1]. COC consists of large amount of short chain saturated fatty acids, crude protein (25.2%), crude fibre (10.8%), ash content (6%), phosphorus (0.67%) and calcium (0.08%) [2]. COC has been successfully utilized as a potential substrate in SSF for production of biomolecules, particularly enzymes [1,3-6].

L-asparaginase (EC.3.5.1.1) is a key enzyme mainly responsible for the hydrolysis of L-asparagine to L-aspartate and ammonia. In recent years, it has received importance for the treatment of various diseases like acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia and acute lymphoblastic leukemia [7-9]. L-asparaginase is present in a wide range of organisms including animals, microbes and plants. Microbial production of L-asparaginase has attracted more attention because of cost-effective and eco-friendly process. A wide range of microorganisms such as filamentous fungi [10,11], bacteria [12-14], yeast [15,16] and actinomycetes

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[17] have been reported in the literature for production of this enzyme. L-asparaginase production has been widely carried out by submerged fermentation (SmF) process. Though, SmF process is well established, it possesses few disadvantages like huge volume of waste water generation and difficulties in effluent treatment process [18]. Because of the above disadvantages, Solid state fermentation (SSF) has emerged as an alternate cost-effective process for bioproducts production by utilizing the agrowastes and byproducts [19]. It has been employed for production of various microbial metabolites such as enzymes [3,4,20], organic acids [21-23] and antibiotics [24,25]. Optimization of bioprocess by one factor at a time (OFAT) is a well-studied method but it has some disadvantages like more time consumption, more number of experimental runs and lack of knowledge about the interaction between the variables involved in the process [26]. Use of statistical experimental designs for screening and optimization of the process variables or conditions is well suited to study the interactive effects of the variables on the targeted response [10,13,27]. The main objective of this study was to produce L-asparaginase by selecting an appropriate low cost substrate and to determine the optimal level of the process variables by both OFAT and RSM methods.

Experimental

Microorganism and media

Bacterial strain *Serratia marcescens* (NCIM 2919) was procured from the National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. The stock culture was maintained at 37°C on Luria Bertani (LB) agar slants. Inoculum was prepared by adding a loopful of bacterial colonies in 500 mL Erlenmeyer flasks containing 100 mL of LB broth and incubated in a rotary shaker at 37°C for 24 h.

Screening of substrates

Three natural substrates Coconut oil cake (COC), Cottonseed oil cake (CSOC) and Groundnut oil cake (GOC) used in this study were purchased from the local market in Chennai. 2 mL of overnight grown culture was inoculated into the Erlenmeyer flask containing 10 g of the respective substrate. The flasks were incubated at 37°C in temperature - controlled incubator and the contents mixed at regular intervals. Fermented samples were taken at 24 h intervals and assayed for enzyme activity.

Analytical methods

Enzyme extraction and assay Extraction of crude enzyme was done by adding 100 mL of sodium phosphate buffer (pH 7) to the fermented substrate, and kept in rotator shaker for 45 min. One milliliter of the extract was transferred to the eppendorf tube and centrifuged at 10000 rpm for 10 minutes. L-asparaginase activity was

determined by measuring the amount of ammonia released by nesslerization according to the method described by Wriston and Yellin [28]. 0.2 mL of cell free supernatant was mixed with 0.8 mL of 0.1 M sodium borate buffer (pH 8.5) and 1 mL of 0.04 M L-asparagine and incubated for 10 min. 0.5 mL of 15% TCA was added to stop the reaction and again centrifuged for 10000 rpm for 10 minutes. 0.2 mL of the supernatant was taken in a test tube in which 3.6 mL distilled water and 0.2 mL Nessler's reagent were added. The optical density was measured at 480 nm in UV spectrophotometer. One Unit (U) of L-asparaginase is defined as the amount of enzyme required to liberate one μmol of ammonia per min at 37°C.

Optimization studies

Classical optimization The optimization process was 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 substrate was studied by varying the amount (2 g, 4 g, 6 g, 8 g, and 10 g). The initial moisture content was varied over the range of 30%, 40%, 50%, 60% and 70%. Optimum pH and temperature were determined by studying a range of 6 to 8 and 25 to 45°C respectively, 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.

Statistical optimization

Response Surface Methodology (RSM) was employed to identify the optimized conditions for the enzyme production. Box-Behnken Design [29] of RSM was used to design the experiment using Design Expert software. Four process variables namely substrate amount (A), initial moisture content (B), temperature (C) and pH (D) were studied at three levels (+1,0,-1) and the experimental range is given in Table 1. Twenty nine experiments were carried out according to Table 1 and the enzyme activity was calculated after 96 h of incubation. A second order polynomial model which describes the relation between the response and the chosen variables was developed and given in Eqn 1.

$$Y = \alpha_0 + \sum_{j=1}^k \alpha_j X_j + \sum_{j=1}^k \alpha_{jj} X_j^2 + \sum \alpha_{ij} X_i X_j \quad (1)$$

where Y is the response, α_0 , α_j , α_{jj} , α_{ij} are the regression coefficients for the intercept, linear, quadratic and interaction effects, respectively and X_i and X_j are coded independent variables.

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

Process variables	Symbol	Low (-1)	Middle (0)	High (+1)
Amount of substrate (g)	A	4	6	8
Initial moisture content (%)	B	30	40	50
Temperature (°C)	C	30	35	40
pH	D	6	7	8

Results and discussion

Screening of carbon substrate

In SSF process, the selection of appropriate substrate should be based on nutrient value, availability and cost. The solid substrate not only provides nutrients for growth and metabolic activities but also provides anchorage to microbes for growth.

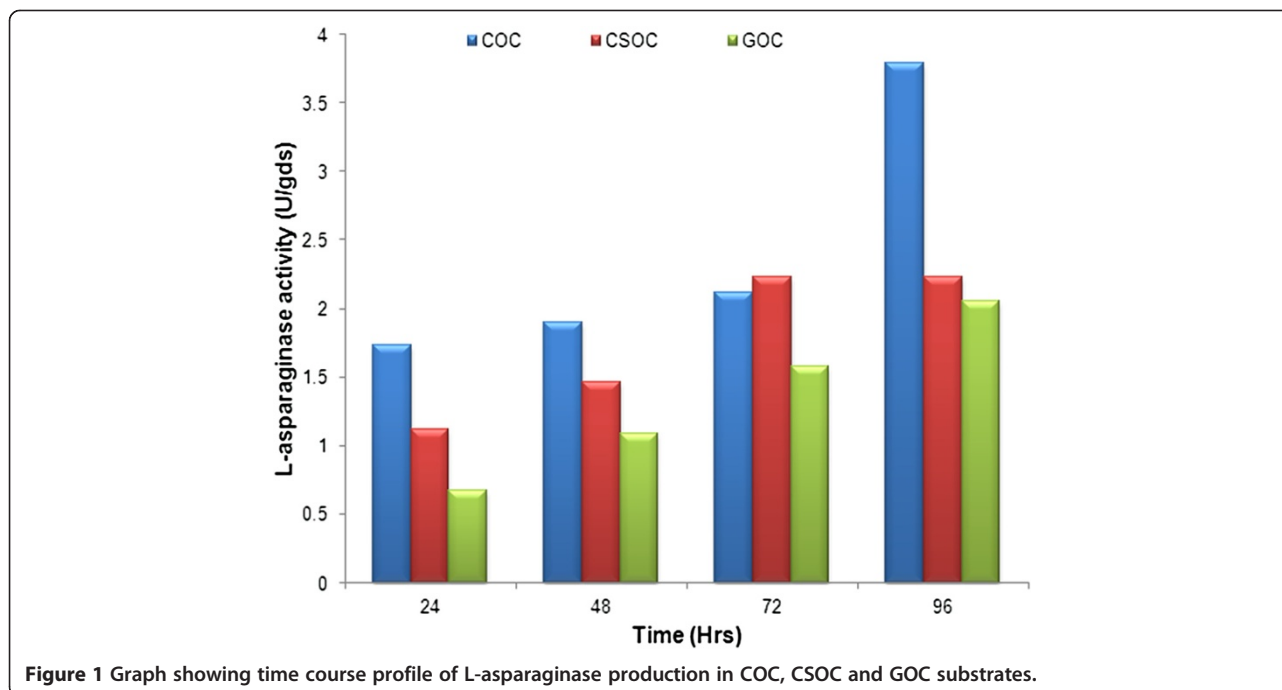
A series of experiments were carried out by using Coconut oil cake (COC), Cottonseed oil cake (CSOC) and Groundnut oil cake (GOC) as carbon source for L-asparaginase production by *Serratia marcescens*. The experimental results for screening of carbon source is graphically shown in Figure 1. All the four substrates showed positive results for the production of the enzyme with yields varying in the range of 0.6 to 3.70 (U/gds) (Figure 1). COC showed maximum enzyme activity of 3.79 (U/gds) as compared to CSOC and GOC. In our study coconut oil cake (GOC) showed better results than the other solid substrates. As a waste product of oil industries, the use of GOC as a carbon source may reduce the cost of the enzyme production process.

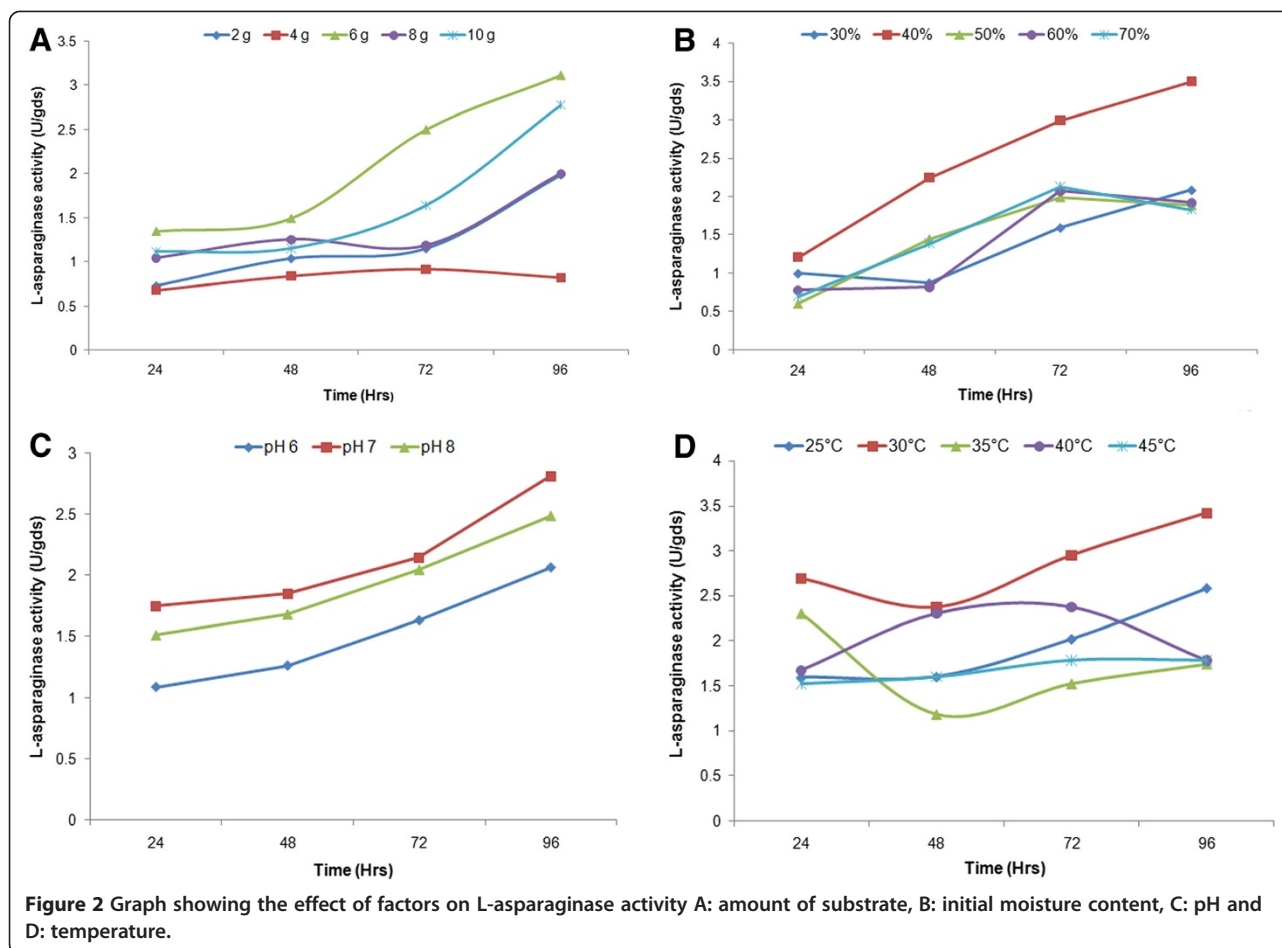
Effect of carbon source on L-asparaginase production

The amount of substrate also plays a vital role for the enhancement of the enzyme production. Five different initial substrate (COC) amounts (2 g, 4 g, 6 g, 8 g and 10 g) were considered for optimization studies. Figure 2A shows a constant increase in enzyme activity with a maximum value of 3.12 (U/gds) for 6 g of substrate. The optimum substrate amount is mainly required for growth and production of metabolites by microorganisms in SSF. High and low levels of the substrate may lead to decrease the yield of the process. Reduction in the enzyme yield may be due to substrate inhibition when substrate used in high level whereas low level of substrate may decrease the yield because of less nutrient availability for microbial growth and metabolite synthesis. Thus, an optimal amount of substrate should be used for the better results and 6 g of COC was found optimum substrate amount for L-asparaginase production in our study.

Effect of initial moisture content on L-asparaginase production

Initial moisture content is a very important parameter in solid state fermentation and directly affects the enzyme production and its activity. Five different moisture contents were taken into account i.e. 30%, 40%, 50%, 60% and 70%. Figure 2B shows that maximum activity of 3.49 (U/gds) was attained with 40% initial moisture content after 96 h of incubation. In this study, decrease in enzyme activity was observed at low and high level of moisture content. This can be attributed to the fact that higher moisture level decreases porosity, promotes





development of stickiness, and increases the chances of contamination [30].

Effect of pH on L-asparaginase production

The pH of the extraction buffer of the enzyme is yet another important factor. Buffers with pH of 6, 7 and 8 were taken into observation. Highest enzyme activity of 2.81 (U/gds) was recorded at a pH of 7 after 96 h of incubation. Figure 2C shows the enzyme activity for the various pH conditions. L-asparaginase from *Serratia marcerens* showed high stability and maximum activity at neutral pH in our study. Maximum enzyme activity was observed near neutral pH for the strain *Serratia marcerens* whereas the optimum pH value was varied for different strains employed in L-asparaginase production *E.coli* (pH 9) and *Pseudomonas aeruginosa* (pH 8) [26,31].

Effect of incubation temperature on L-asparaginase production

The incubation temperature has a direct effect on the growth of the bacteria and thus affects the enzyme production and its activity. A range of temperature varying

from 25°C to 45°C was taken into study. Maximum enzyme activity of 3.42 (U/gds) was recorded at 30°C after 96 h of incubation as represented in Figure 2D. Further increase in the temperature resulted in reduction of enzyme activity. An experiment was carried out at the optimum values of substrate amount (6 g), initial moisture content (40%), pH (7) and temperature (30°C) obtained from OFAT method and maximum enzyme activity (3.87 U/gds) was found after 96 h of fermentation. In this study, *Serratia marcerens* strain showed maximum enzyme activity at optimum temperature of 30°C. The optimum temperature for enzyme production varies with bacterial strains. Maximum enzyme activity was expected at 20°C for *Chrombacteriaceae* [32] and optimum temperature of 37°C was reported for various bacterial species [8,33,34].

Optimization of process parameters using response surface methodology

Response surface methodology is one of the statistical techniques to evaluate the relationship between the experimental variables and the measured responses and

to find the optimal range of the process variables within the design [35]. In our study, we used Box-Behnken design to determine the optimal level of the four variables which influences the enzyme production. The response, L-asparaginase activity ranged from 2.82 (U/gds) to 6.18 (U/gds) as tabulated in Table 2. 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 3. A second order polynomial equation given in Eqn. 2 was constructed by using the estimated coefficients (in coded units).

Table 2 Box-Behnken Design matrix with experimental and predicted L-asparaginase activity

Std order	A	B	C	D	L-asparaginase activity (U/gds)	
					Experimental	Predicted
1	4	30	35	7	4.710	4.688
2	8	30	35	7	4.655	4.860
3	4	50	35	7	2.912	3.729
4	8	50	35	7	4.790	5.829
5	6	40	30	6	3.458	3.802
6	6	40	40	6	3.493	3.647
7	6	40	30	8	3.352	4.220
8	6	40	40	8	3.534	4.212
9	4	40	35	6	3.827	3.279
10	8	40	35	6	3.504	3.512
11	4	40	35	8	3.305	2.866
12	8	40	35	8	4.790	4.907
13	6	30	30	7	5.354	4.922
14	6	50	30	7	6.182	5.370
15	6	30	40	7	4.902	5.284
16	6	50	40	7	4.843	4.846
17	4	40	30	7	3.264	3.813
18	8	40	30	7	4.338	4.058
19	4	40	40	7	2.823	2.840
20	8	40	40	7	5.683	4.869
21	6	30	35	6	4.174	4.579
22	6	50	35	6	4.679	4.634
23	6	30	35	8	5.342	5.120
24	6	50	35	8	5.747	5.075
25	6	40	35	7	5.858	5.169
26	6	40	35	7	5.013	5.169
27	6	40	35	7	4.802	5.169
28	6	40	35	7	4.855	5.169
29	6	40	35	7	5.049	5.169

Table 3 ANOVA statistics for L-asparaginase production by *Serratia marcescens*

Source	Sum of squares	Degrees of freedom	Mean square	F value	p-value	Significance
A	3.989	1	3.989	7.966	0.014	significant
B	2.130	1	2.130	4.260	0.995	
C	0.037	1	0.037	0.074	0.789	
D	0.717	1	0.717	1.433	0.251	
AB	0.934	1	0.934	1.865	0.193	
AC	0.797	1	0.797	1.592	0.228	
AD	0.817	1	0.817	1.631	0.222	
BC	0.196	1	0.196	0.392	0.541	
BD	0.002	1	0.002	0.004	0.945	
CD	0.005	1	0.005	0.010	0.919	
A ²	4.169	1	4.169	8.326	0.012	significant
B ²	1.086	1	1.086	2.170	0.162	
C ²	1.455	1	1.455	2.906	0.110	
D ²	3.421	1	3.421	6.832	0.020	significant
Residual	7.010	14	0.500			
Lack of Fit	6.278	10	0.627	3.429	0.123	not significant
Pure Error	0.732	4	0.183			
Total	24.558	28				

$$\begin{aligned}
 Y_{L\text{-asparaginase activity}(U/gds)} = & 5.12 + 0.58^*A + 1.33E \\
 & -0.03^*B - 0.056^*C + 0.24^*D + 0.48^*AB \\
 & +0.45^*AC + 0.45^*AD - 0.22^*BC \\
 & -0.025^*BD + 0.037^*CD - 0.80^*A^2 \\
 & +0.41^*B^2 + 0.47^*C^2 - 0.73^*D^2
 \end{aligned}
 \tag{2}$$

The probability value (p-value) is usually used to evaluate the statistical significance of each coefficient [29]. Low p-value ($p < 0.05$) indicates high significance of the corresponding coefficient and its effect on the L-asparaginase activity at 95% confidence level. The statistical significance of the model equation was evaluated by the F-test of ANOVA table. The model was found highly significant with the F-value of 2.50. From Table 3, the linear effect of substrate (COC) amount (A) and the square effect of substrate (COC) amount (A) and pH (D) were found to be more significant ($p < 0.05$) than the other linear and interaction effects between process variables. The correlation coefficient (R^2) is a tool to check the “goodness of fit” of the polynomial model and R^2 value near to unity is said to be perfect fit. In this study, R^2 value of 0.714 demonstrated that there was

good relation existed between the independent and the dependent variables.

Surface plots

The contour and 3D surface plots are generally used to represent the interaction effects between the process variables [36]. The fitted response for the regression model was plotted in Figure 3A-F. 3D graphs generated

for the pair-wise interaction of the four factors explain the role played by factors affecting L-asparaginase production. The three dimensional response surface plot between the amount of substrate and the initial moisture content (Figure 3A) shows that the enzyme activity increased towards the high value of the substrate amount and reached a maximum (4.60 U/gds) in the mid-value region whereas, in case of the initial moisture content, a

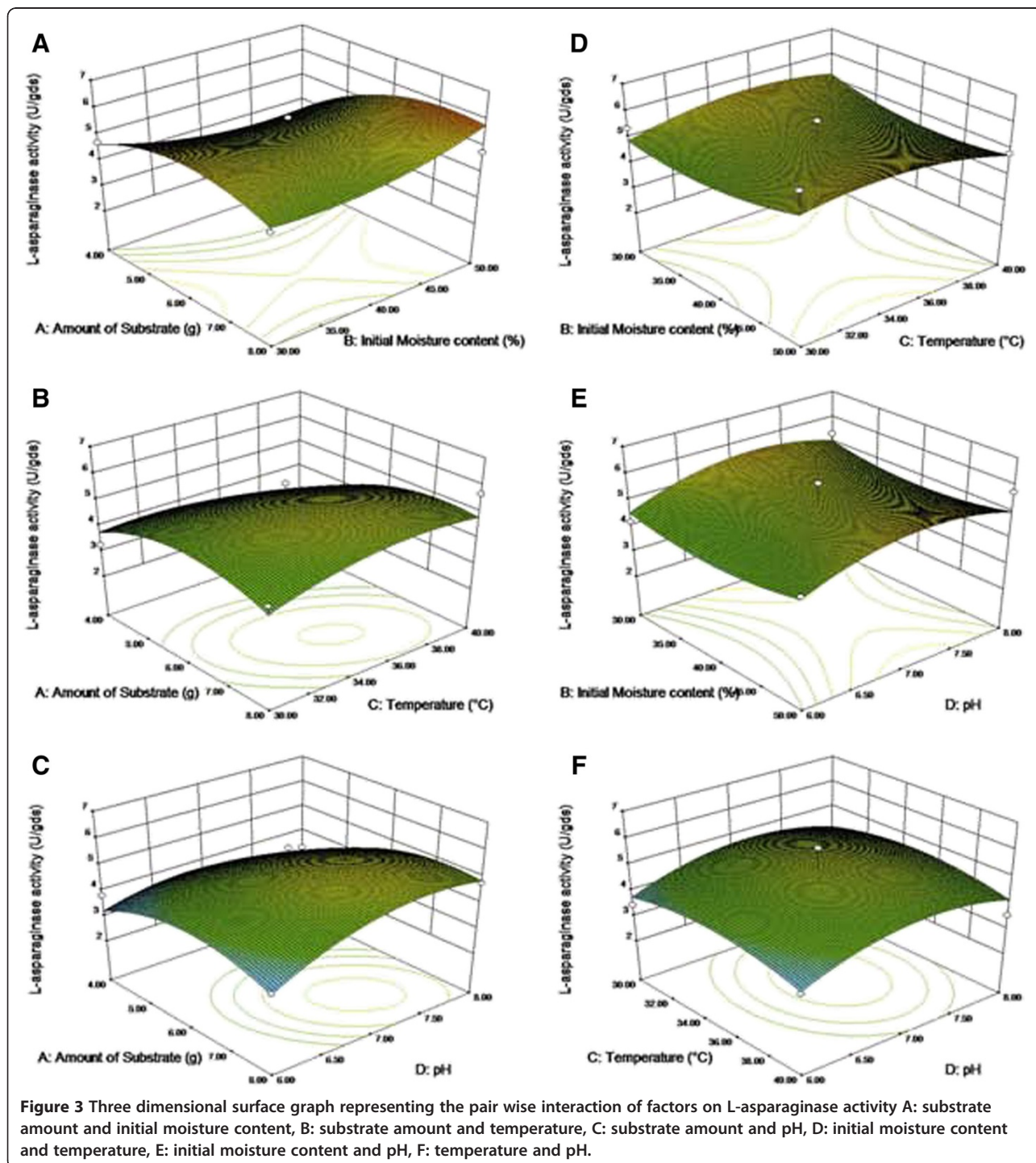


Figure 3 Three dimensional surface graph representing the pair wise interaction of factors on L-asparaginase activity A: substrate amount and initial moisture content, B: substrate amount and temperature, C: substrate amount and pH, D: initial moisture content and temperature, E: initial moisture content and pH, F: temperature and pH.

maximum of enzyme activity (5.80 U/gds) was observed at the high value of moisture content (50%). Figure 3B represents the combined effect of the substrate amount and temperature and maximum activity (3.90 U/gds) was noted at the mid value of substrate amount (6 g). The activity steadily increased with the increase in temperature and reached a maximum (4.90 U/gds) at the optimal value of temperature (50°C). The interaction between the substrate amount and pH (Figure 3C) showed that while mid value of substrate continued to give the maximum activity, high level of the pH 8 was seen to increase the activity to a maximum (4.95 U/gds). The response plot between the initial moisture content and temperature (Figure 3D) demonstrates that the activity was almost constant for the initial moisture content values with slightly high activity recorded for the low value. It remained constant for the temperature variations. The graph between initial moisture content and pH (Figure 3E) highlights a constant activity for the initial moisture content whereas a maximum activity (4.99 U/gds) was recorded for the high level of pH. The interaction plot between the temperature and pH (Figure 3F) implies that the enzyme activity remained constant for the temperature variations whereas in case of pH, there was an increase and a maximum activity (4.00 U/gds) was recorded at the high level. According to Muralidhar et al. [37], strong interaction between the variables are expected if the contour lines are elliptical in shape. In our study, strong interaction existed between the amount of substrate and temperature (Figure 3B), amount of substrate and pH (Figure 3C) and temperature and pH (Figure 3F) for L-asparaginase production since the contour lines were elliptical in nature.

Response optimization

Point Prediction tool of Design Expert software was used to predict the optimum values of the variables. Maximum enzyme activity (5.86 U/gds) was obtained at the predicted optimal values of substrate amount 7.6 g, Initial moisture content of substrate 50%, Temperature 35.5°C and pH 7.4.

Validation of model

An experiment was carried out with the optimum values predicted by the software to verify the accuracy of the model. Validation results showed that experimental value of enzyme activity (5.75 U/gds) was very closer to the predicted response (5.86 U/gds) and the predicted model fitted well with 97.4% of experimental results. Our study showed significant results for L-asparaginase production by *Serratia marcescens* in SSF, since there was no previous literature available for the enzyme production by using COC and *Serratia marcescens*.

Conclusion

Response surface methodology was successfully employed to optimize the process variables for L-asparaginase production. Four variables (substrate amount, Initial moisture content of substrate, Temperature and pH) were chosen for optimization studies by RSM. Under optimal conditions, L-asparaginase activity of 5.75 U/gds was obtained and it is closer to the predicted value. Application of RSM for optimization studies is an effective method for improving the enzyme production and also understanding the interaction effects between the variables with less number of experiments. The results of this study also revealed that utilization of coconut oil cake might be reduced the cost of therapeutically important L-asparaginase enzyme production.

Abbreviations

SSF: Solid state fermentation; COC: Coconut oil cake; CSOC: Cottonseed oil cake; GOC: Groundnut oil cake; GDS: Gram dry substrate; OFAT: One factor at a time; RSM: Response Surface Methodology; BBD: Box-Behnken Design; ANOVA: Analysis of variance; *p*: Probabililty value.

Competing interests

The authors declared that they have no competing interests.

Authors' contribution

SG & SM carried out the experimental work. GS & CM designed the study and involved in all discussions, data interpretation and manuscript drafting. All authors read and approved the final manuscript.

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