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RESEARCH ARTICLE

COST EFFECTIVE PROCESS FOR THE PRODUCTION OF FUNGAL L-ASPARAGINASES FROM *PENCILLIUM* SPS. ISOLATED FROM LOCAL SOIL SAMPLE

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ABSTRACT

L – asparaginase is known to have a therapeutic function and was used for the patients alleged from acute lymphoblastic leukemia (ALL). Out of different micro organisms, fungal samples isolated from a local soil sample, *Pencillium sps. 02* have utmost L-asparaginase activity. In the present study, *Pencillium* sp. 02 was isolated and studied for enhanced enzyme production with the help of statistical methods. Initially, all nutritional factors were selected by one factor- at-a-time method (171 IU/ml), then the consequence of every factor with respect to production of L-asparaginase was identified by taguchi design and the enzyme yield was increased to 1.073 folds (171 IU/ml to 183.5 IU/ml). Later, final optimization was carried out with response surface methodology (Central Composite Design) increased the yield of 1.065 folds (183.5 IU/ml to 195.5 IU/ml).

Keywords: Acute lymphoblastic leukemia, *Pencillium,* L-asparaginase, one factor at a time method, taguchi design, response surface methodology.

INTRODUCTION

L – asparaginase having therapeutic value to use in cancer therapies mainly focused on acute lymphoblastic leukemia (Athale *et al.,* 2003 & Narta *et al.,* 2007). In the United States, 5000 patients with ALL (acute lymphoblastic leukemia) were diagnosed annually (Greenlee *et al.,* 2001 & Cortes *et al.,* 1995). 80% of children were cured from ALL and 30 – 40% of adults achieve long-term disease-free survival (DFS) where as long-term chemotherapy effects several complications like anxiety (Ng *et al.,* 2000). Medical approach towards leukemia is based on metabolic deficiency in L – asparagine synthesis of several malignant cells (Broome, 1981; Ravindranath *et al.,* 1992). L-asparaginase also inhibits the protein production by L-asparagine hydrolysis (Marlborough *et al.,* 1975; Ravindranath *et al.,* 1992; Moola *et al.,* 1994) and its action on single and double strand nucleic acid synthesis has not been entirely elucidated yet, but it is thought to be G1 phase specific (Broome, 1981). For the past 25 years, an anti-neoplastic agent, L – asparaginase has been widely used to treat acute lymphoblastic leukemia (ALL) and lymphoma due to its specific action of mechanism (Evans *et al.,* 1982). L – asparaginase converts L – asparagine to L – aspartic acid and ammonia (Wriston & Yellin 1973, Capizzi *et al.,* 1971). The anti leukemic effect of L-asparaginase was due to result from the rapid and complete depletion of the circulating pool of L – asparagine. L – asparagine is needed for survival of the cancer cells, as an external source due to the lack of L-asparagine synthetase.

Now a day's most of the research is focused on production of glutaminase free L-asparaginase has attracted lot of attention by using microbial systems. Most of the micro organisms like bacteria, yeast and fungi are used as a source for the production of L-asparaginase. The production of L-asparaginase has been studied in *Serratia marcescens* (Khan *et al.,* 1970 & Heinemann *et al.,* 1969), *Erwinia carotovora* (Howard *et al.,* 1972), *Erwinia aroideae* (Peterson and Ciegler, 1969), *E. coli* (Mashburn *et al.,* 1964; Howard *et al.*, 1968), *Enterobacter aerogenes* (Mukherjee *et al.,* 2000), *Pseudomonas aeruginosa* (Abdel-Fattah and Olama, 2002), *Thermus thermophilus* (Pritsa *et al.,* 2001), *Thermus aquaticus* (Curran *et al.,* 1985), *Vibrio succinogenes* (Kafkewitz and Goodmanet, 1974), *Citrobacter freundi* (Davidson *et al.,* 1977), *Streptomyces griseus* (Dejong, 1972), *Proteus vulgaris* (Tosa *et al.,* 1972) *Bacillus subtilis* (Fischer *et al.,* 2002), *Staphylococcus* sp. (Prakasham *et al.,* 2007), *Zymomonas mobilis* (Pinheiro *et al.,* 2001) and *Bacillus licheniformis* (Golden and Bernlohr, 1985) with different carbon, nitrogen and nutrient sources. The enzyme was produced by both submerged and solid state cultures. Bacterial L-asparaginase can cause hypersensitivity in the long-term usage leads to allergic reactions and anaphylaxis (Reynolds & Taylor 1993). But production of L – asparaginase from eukaryotic micro organisms like yeast and fungi has less side effects when compared to the bacterial L – asparaginases.

Most of the *Pencillium sps.* produce various types of enzymes, such as protease, lipase, chitinase, alkaline protease and alginate lyases. *Pencillium* sps. also serve as a good source of L-asparaginase. The objective of the study was to enhance the production of native L-asparaginase from *Pencillium* species by different optimization studies like classical method one-factor-at-a-time, Orthogonal matrix method and response surface methodology (RSM).

MATERIALS AND METHODS

Media Components

Various carbon sources like glucose, xylose, fructose, arabinose, lactose, rhamnose, maltose, inositol, sucrose and nitrogen sources like sodium nitrate, yeast extract, ammonium nitrate, corn steep liquor, meat peptone, mycological peptone and other chemicals like L-asparagine, L-proline, potassium chloride, magnesium sulfate, ferrous sulfate, di-potassium hydrogen phosphate, zinc sulfate, copper sulfate were procured from Hi-Media Limited, Mumbai, India.

Preparation of media and culture conditions

All experiments were carried out in 250 ml conical flasks with a working volume of 50 ml. The potato dextrose agar medium was used for the propagation and maintenance of culture. The optimization studies were carried out in production medium (modified Czepek-Dox medium) containing L-proline – 5 g/L, L-asparagine – 10 g/L, sodium nitrate – 10 g/L, potassium chloride – 5 g/L, magnesium sulfate – 0.5 g/L, ferrous sulfate – 0.05 g/L, di-potassium hydrogen phosphate – 5 g/L, zinc sulfate – 0.5 g/L, copper sulfate – 0.05 g/L, glucose – 30 g/L. The medium was autoclaved at 121^{0}C for 20 min. Then inoculated aseptically and was incubated on shaker at 30°C for 2 days.

Fungal Strain

Out of 15 different fungal strains, *Pencillium* sps. 02 was isolated from local soil samples according to the protocol of Seifert (1990), identified in the laboratory as described by Rapper and Fennell (1965) and cultivated in Czapek Dox agar plates at 30 ^{0}C for 2 days, stored at -20 °C as 50 % glycerol stocks and periodically sub-cultured.

Production of L-asparaginase

The isolate was screened for L-asparaginase production using modified CD medium incorporated with phenol red as a pH indicator (Gulati *et al.,* 1997). The activity of L- asparaginase was identified by the formation of pink zone around colonies.

Assay of L-asparaginase

The enzyme activity was determined by taking 10 ml of the culture broth aseptically from the flasks, for every 12 hrs and was filtered using Whatman filter paper No.1. The filtrate was centrifuged at 10,000Xg for 10 min (Ding *et al.*, 2003). Then the supernatant was used for L-asparaginase assay (Imada *et al.*, 1973) and the enzyme activity was expressed in International Unit (IU). One IU of L-asparaginase is the amount of enzyme which liberates 1 µM of ammonia per ml per min (µM/ml/min).

Optimization by using one Factor-at-a-time

Different nutritional factors such as carbon and nitrogen (organic and inorganic sources) were investigated for the optimization of medium components. Likewise, pH and temperature was also studied for better expression of enzyme.

Effect of carbon source

To study the effects of carbon in production medium, glucose was substituted with nine different carbon sources like xylose, fructose, arabinose, lactose, rhamnose, maltose, inositol, sucrose. All carbon sources were used at 3 % concentration.

Effect of nitrogen source

In the production medium, sodium nitrate was substituted with organic sources like yeast extract, ammonium nitrate, corn steep liquor, meat peptone, mycological peptone, soya peptone and beef extract, inorgonic nitrogen sources like ammonium nitrate and potassium nitrate were used at 1% concentration.

Effect of pH

To study the effect of pH on asparaginase production different types fermentation runs were carried out at different pH values between 3 and 9. The effect of pH on biomass production and enzyme production was studied.

Effect of temperature

Different temperatures viz., 20 ^{0}C, 25 ^{0}C, 30 ^{0}C, 35 ^{0}C and 40 ^{0}C were studied for the maximum enzyme production.

Optimization of concentrations of the production medium components using the L_{12} – orthogonal array

L_{12} - orthogonal design was developed and analyzed by using ''MINITAB 14.00'' software. The following Table – 1 represents the fermentation conditions and the L_{12} – orthogonal array, which was used in this study. All experiments were performed in duplicates.

Table 1: L_{12}-orthogonal array for asparaginase production.

S. No	Glu-Cose (g/L) [a]	Mg-SO_4 (g/L)[a]	Proline (g/L) [a]	Zn-SO_4 (g/L) [a]	K_2H-PO_4 (g/L) [a]	Cu-SO_4 (g/L) [a]	Fe-SO_4 (g/L) [a]	Aspar-agine (g/L) [a]	Na-NO_3 (g/L) [a]	K-Cl (g/L) [a]	Yield (IU/mL)[b]
1	1(25)	1(0.3)	1(3)	1(0.3)	1(4)	1(0.04)	1(0.04)	1(8)	1(8)	1(4)	138.6 ± 1.5
2	1(25)	1(0.3)	1(3)	1(0.3)	1(4)	2(0.06)	2(0.06)	2(12)	2(12)	2(6)	142.5 ± 1.1
3	1(25)	1(0.3)	2(7)	2(0.7)	2(6)	1(0.04)	1(0.04)	1(8)	2(12)	2(6)	157.6 ± 3.3
4	1(25)	2(0.7)	1(3)	2(0.7)	2(6)	1(0.04)	2(0.06)	2(12)	1(8)	1(4)	167.5 ± 2.1
5	1(25)	2(0.7)	2(7)	1(0.3)	2(6)	2(0.06)	1(0.04)	2(12)	1(8)	2(6)	172.9 ± 4.7
6	1(25)	2(0.7)	2(7)	2(0.7)	1(4)	2(0.06)	2(0.06)	1(8)	2(12)	1(4)	146.5 ± 1.9
7	2(35)	1(0.3)	2(7)	2(0.7)	1(4)	1(0.04)	2(0.06)	2(12)	1(8)	2(6)	183.5 ± 0.4
8	2(35)	1(0.3)	2(7)	1(0.3)	2(6)	2(0.06)	2(0.06)	1(8)	1(8)	1(4)	178.6 ± 3.8
9	2(35)	1(0.3)	1(3)	2(0.7)	2(6)	2(0.06)	1(0.04)	2(12)	2(12)	1(4)	173 ± 2.2
10	2(35)	2(0.7)	2(7)	1(0.3)	1(4)	1(0.04)	1(0.04)	2(12)	2(12)	1(4)	164.7 ± 3.3
11	2(35)	2(0.7)	1(3)	2(0.7)	1(4)	2(0.06)	1(0.04)	1(8)	1(8)	2(6)	158.4 ± 2.3
12	2(35)	2(0.7)	1(3)	1(0.3)	2(6)	1(0.04)	2(0.06)	1(8)	2(12)	2(6)	158.4 ± 4.1

[a] Values in curved brackets are uncoded variables,[b] Results are mean ± SD of two determinations.

Optimization of the medium components by RSM

To investigate the effect of four important variables viz, glucose, L-asparagine, dipotassium hydrogen phosphate and sodium nitrate on production of L-asparaginase, media optimized by orthogonal design array was used. Each variable was studied at two different levels, with all variables taken at a central coded value of zero. The experiments were designed using the software, Design Expert Version 6.0.10 version (Stat Ease) which gave 30 experiments (24 plus 6 centre points). The CCRD matrix in terms of coded and actual values of independent variables is given in Table – 1 and response surface plots were obtained. The quadratic model suggested by RSM was validated by using the optimized medium composition shown in Table 2.

Table 2: The CCRD matrix of independent variables in coded form with their corresponding response from experiments.

Std	Run	Block	Glu-Cose [a]	Aspa-ragine [a]	K_2HPO_4 [a]	$NaNO_3$ [a]	Experi-mental [b] (IU/mL)	Predicted (IU/mL)
1	16	Block 1	-1(20)	-1(7)	-1(1)	-1(5)	139.2	137.4792
2	12	Block 1	1(50)	-1(7)	-1(1)	-1(5)	143.4	146.0958
3	2	Block 1	-1(20)	1(17)	-1(1)	-1(5)	164.3	165.9458
4	28	Block 1	1(7)	1(17)	-1(1)	-1(5)	157.6	155.0125
5	15	Block 1	-1(20)	-1(7)	1(7)	-1(5)	153.1	153.0125
6	4	Block 1	1(50)	-1(7)	1(7)	-1(5)	168.2	167.4792
7	11	Block 1	-1(20)	1(17)	1(7)	-1(5)	174.4	173.0292
8	13	Block 1	1(50)	1(17)	1(7)	-1(5)	168.6	167.9458
9	1	Block 1	-1(20)	-1(7)	-1(1)	1(11)	169.8	171.4958
10	19	Block 1	1(50)	-1(7)	-1(1)	1(11)	182.7	181.2125
11	22	Block 1	-1(20)	1(17)	-1(1)	1(11)	184.5	182.3625
12	30	Block 1	1(50)	1(17)	-1(1)	1(11)	171.4	172.5292
13	3	Block 1	-1(20)	-1(7)	1(7)	1(11)	179.6	179.3292
14	18	Block 1	1(50)	-1(7)	1(7)	1(11)	195.5	194.8958
15	14	Block 1	-1(20)	1(17)	1(7)	1(11)	183.4	181.7458
16	5	Block 1	1(50)	1(17)	1(7)	1(11)	178.9	177.7625
17	8	Block 1	-2(5)	0(12)	0(4)	0(8)	187.7	188.7417
18	6	Block 1	2(65)	0(12)	0(4)	0(8)	192.6	193.375

19	24	Block 1	0(35)	-2(2)	0(4)	0(8)	164.5	163.8417
20	10	Block 1	0(35)	2(22)	0(4)	0(8)	172.7	175.175
21	20	Block 1	0(35)	0(12)	-2(-2)	0	148.4	147.875
22	26	Block 1	0(35)	0(12)	2(10)	0	166.3	168.6417
23	7	Block 1	0(35)	0(12)	0(4)	-2(2)	135.6	136.0917
24	9	Block 1	0(35)	0(12)	0(4)	2(14)	178.6	179.925
25	17	Block 1	0(35)	0(12)	0(4)	0(8)	175.3	175.3333
26	25	Block 1	0(35)	0(12)	0(4)	0(8)	175.1	175.3333
27	29	Block 1	0(35)	0(12)	0(4)	0(8)	175.7	175.3333
28	21	Block 1	0(35)	0(12)	0(4)	0(8)	175	175.3333
29	23	Block 1	0(35)	0(12)	0(4)	0(8)	175.4	175.3333
30	27	Block 1	0(35)	0(12)	0(4)	0(8)	175.5	175.3333

Negative values in RSM are considered as zero

[a] Values in curved brackets are uncoded variables , [b] Results are mean ± SD of two determinations.

RESULTS

One Factor-at-a-time Method

Carbon source act as a major nutrient for cell proliferation and for the production of L-asparaginase, The following figure shows the different carbon sources on the production of asparaginase at the end of 24 hours fermentation. Glucose, sucrose and fructose are found to be promising carbon sources. Out of all these glucose supports for the maximum enzyme production (171 IU/mL). These results were similar to the results of Mohsin (2012).

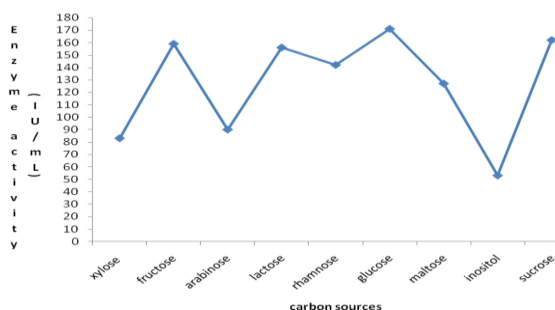

Figure 1: Effect of different carbon sources on asparaginase production

Following figure shows the effect of different organic and inorganic nitrogen sources on asparaginase production. Among different nitrogen sources sodium nitrate gave maximum enzyme production among mycological peptone and yeast extract.

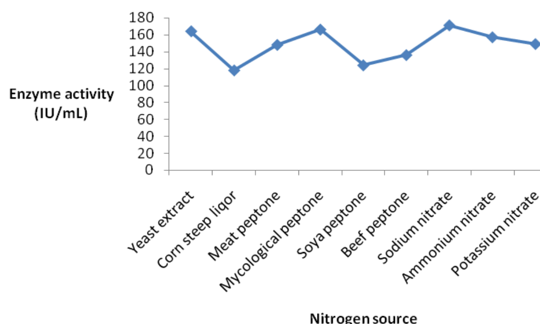

Figure 2: Effect of different organic and inorganic nitrogen sources on asparaginase production

The effect of different pH values on the production of L-asparaginase was shown in the figure. pH 7.0 supported the maximum enzyme production and previous reports described the maximum production at acidic pH 6.0 (Mohsin *et al.*, 2012).

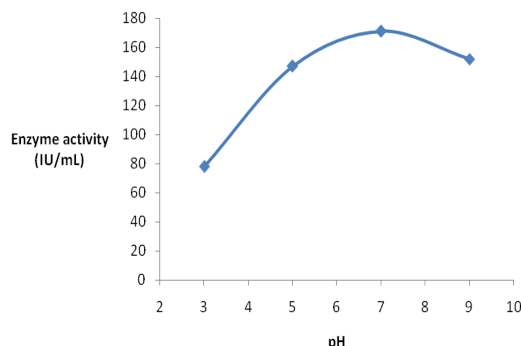

Figure 3: Asparaginase production at different pH

The effect of different temperatures on the production of L-asparaginase was shown in the figure. 30^{0}C was supported for the maximum enzyme production and previous reports described the maximum production at 35^{0}C (Mohsin S *et al.*, 2012).

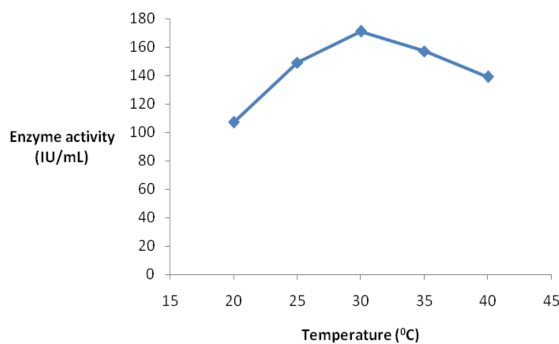

Figure 4: Effect of different temperatures on asparaginase production

Optimization by using L_{12} – orthogonal array

The medium was subjected to L_{12} – orthogonal array after selection of carbon and nitrogen sources by one factor at a time. The optimized parameters involved the concentrations of all components for better expression of asparaginase. Table – 3 & 4 response table for signal to noise ratio and mean obtained with the design. The delta values and ranks in the two tables helps to evaluate which factors have maximum effect on response. The size of the effect was measured with delta by taking the difference among highest and lowest average for a factor. A higher value indicates greater effect of that component. Rank orders the factors from the greatest effect (based on the delta values) to the least effect. Out of all the ten factors glucose has major effect and KCl had least effect on asparaginase production. Figs. 5 & 6 characterize the main effect plots for the means and S/N ratio.

MINITAB version 14.00 creates the main effects plot by plotting the characteristic average for each factor level. A horizontal line connects the points for each factor indicates there is no main effect present. Each level of the factor affects the characteristic in the same way and the characteristic average is the same across all factor levels. The larger the difference in the vertical points of the plotted axis shows the greater main effect. In this study it can be seen that for each of the ten variables at two levels, factors showing different ranking system among the mean and s/n ratio.

Table 3: Response Table for Signal to Noise Ratios

Level	Glucose	$MgSO_4$	Proline	$ZnSO_4$	K_2HPO_4	$CuSO_4$	$FeSO_4$	Asparagine	$NaNO_3$	KCl
1	43.7318	44.1484	43.8796	43.9490	43.8247	44.1135	44.0821	44.0821	44.3888	44.1320
2	44.5372	44.1205	44.3893	44.3199	44.4442	44.1554	44.1868	44.4368	43.8801	44.1369
Delta	0.8054	0.0279	0.5096	0.3709	0.6195	0.0419	0.1048	0.6047	0.5088	0.0049
Rank	1	9	4	6	2	8	7	3	5	10

Table 4: Response Table for Means

Level	Glucose	$MgSO_4$	Proline	$ZnSO_4$	K_2HPO_4	$CuSO_4$	$FeSO_4$	Asparagine	$NaNO_3$	KCl
1	154.150	162.142	156.825	158.175	156.025	161.158	160.425	155.892	166.400	161.533
2	168.908	160.917	166.233	164.883	167.033	161.900	162.633	167.167	156.658	161.525
Delta	14.758	1.225	9.408	6.708	11.008	0.742	2.208	11.275	9.742	0.008
Rank	1	8	5	6	3	9	7	2	4	10

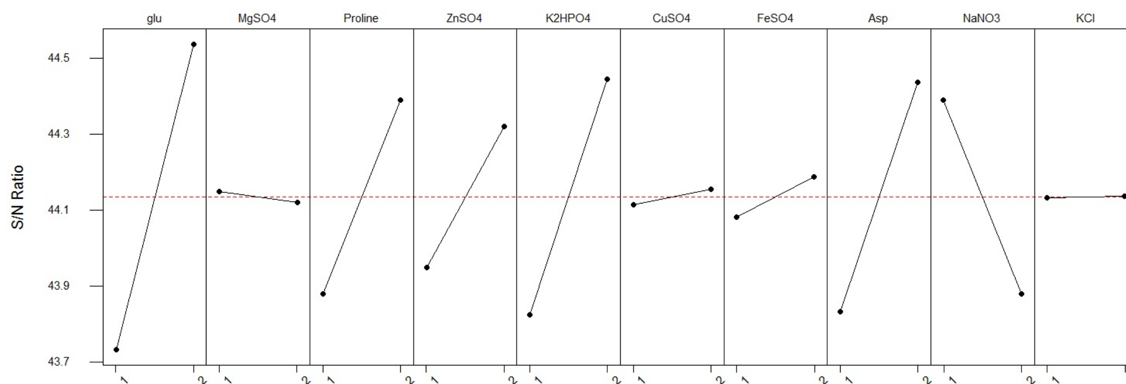

Figure – 5: Main Effects Plot for S/N Ratios

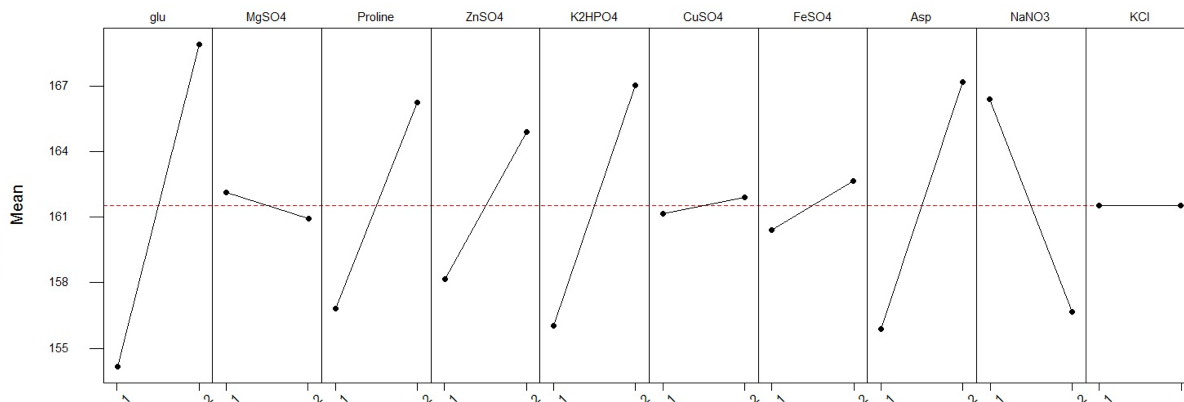

Figure 6: Main Effects Plot for Means

Optimization of concentrations of the selected medium components by RSM

The combined effect of four independent variables glucose, asparagine, K_2HPO_4 and $NaNO_3$ for production of asparaginase was studied by using RSM. CCRD gave quadratic model for the given set of experimental results. The experimental and predicted values for production of asparaginase were given in Table 2.

The results were analyzed by using ANOVA. The ANOVA of the quadratic model indicated that the model was significant. The model F-value of 123.30 implies the model is significant. The Prob > F was very low and less than <0.0001, again indicating the model to be significant. The coefficient estimates and the corresponding P values suggests that, among the test variables used in the study, A, B, C, D, A^2, B^2, C^2, D^2, AB, AC, BC, BD and CD (where A = Glucose B = Asparagine, C = K_2HPO_4 and D = $NaNO_3$) are significant model terms.

The fit of the model was also expressed by the coefficient of regression (R^2), which was found to be 0.9914, indicating that 99.14% of the confidence level of the model to predict the response. The "Pred R-Squared" of 0.9506 is in reasonable agreement with the "Adj R-Squared" of 0.9833. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Here, the ratio of 43.748 indicates an adequate signal.

Final equation in terms of coded factors for Asparaginase production (IU/mL) = 175.33 + 1.16 X Glucose + 2.83 X Asparagine + 5.19 X K_2HPO_4 + 10.96 X $NaNO_3$ + 3.93 X $(Glucose)^2$ – 1.46 X $(Asparagine)^2$ – 4.27 X $(K_2HPO_4)^2$ – 4.33 X $(NaNO_3)^2$ – 4.89 X Glucose X Asparagine + 1.46 X Glucose X K_2HPO_4 + 0.28 X Glucose X $NaNO_3$ – 2.11 X Asparagine X

K_2HPO_4 – 4.40 X Asparagine X $NaNO_3$ – 1.92 X K_2HPO_4 X $NaNO_3$.

Final equation in terms of actual factors for Asparaginase production (IU/mL) = +175.33333 + 1.15833 X Glucose + 2.83333 X Asparagine + 5.19167 X K_2HPO_4 + 10.95833 X $NaNO_3$ + 3.93125 X $(Glucose)^2$ – 1.45625 X $(Asparagine)^2$ – 4.26875 X $(K_2HPO_4)^2$ – 4.33125 X $(NaNO_3)^2$ – 4.8875 X Glucose X Asparagine + 1.46250 X Glucose X K_2HPO_4 + 0.275 X Glucose X $NaNO_3$ – 2.1125 X Asparagine X K_2HPO_4 – 4.4 X Asparagine X $NaNO_3$ – 1.925 X K_2HPO_4 X $NaNO_3$.

Accordingly, three – dimensional graphs were plotted for the pair-wise combination of the four factors, while keeping the other two at their center point levels. From the central point of the contour plot or from the bump of the 3D plot the optimal composition of medium components was identified. The following figures (7 – 12) were illustrated the response surface plot for asparaginase production. Figure – 13 illustrated the parity plot for the distribution of predicted and experimental values of asparaginase production.

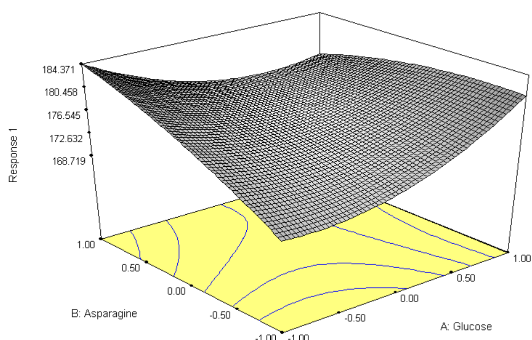

Figure 7: Response Surface Plot for Asparaginase production; Effect of Glucose and asparagine

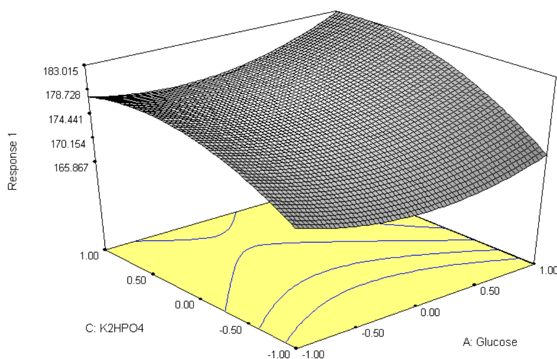

Figure 8: Response Surface Plot for Asparaginase production; Effect of Glucose and K_2HPO_4

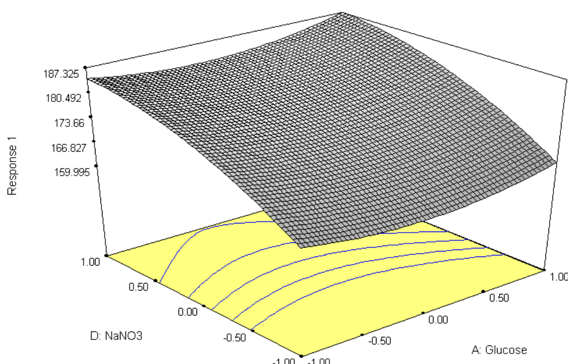

Figure 9: Response Surface Plot for Asparaginase production; Effect of Glucose and $NaNO_3$

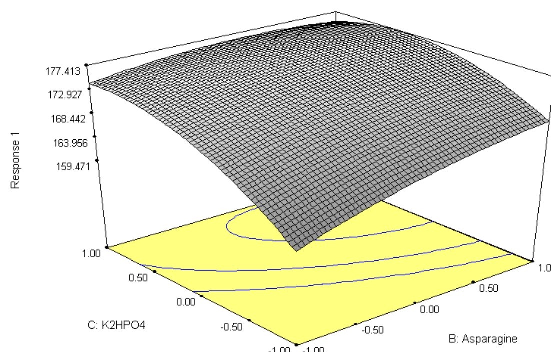

Figure – 10: Response Surface Plot for Asparaginase production; Effect of asparagine and K_2HPO_4

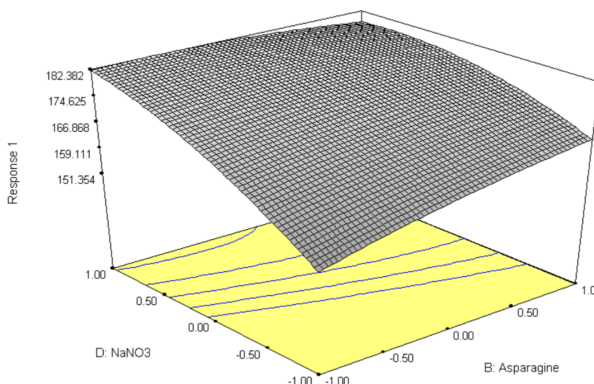

Figure 11: Response Surface Plot for Asparaginase production; Effect of asparagine and $NaNO_3$

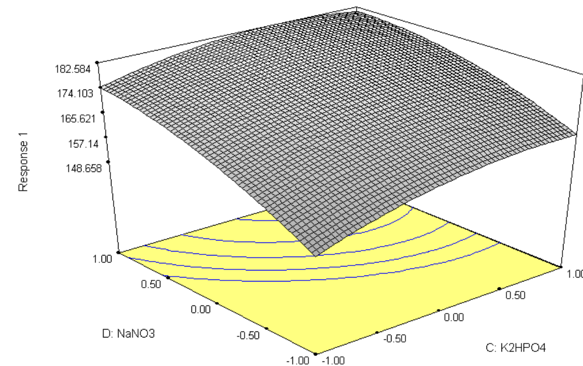

Figure 12: Response Surface Plot for Asparaginase production; Effect of $NaNO_3$ and K_2HPO_4

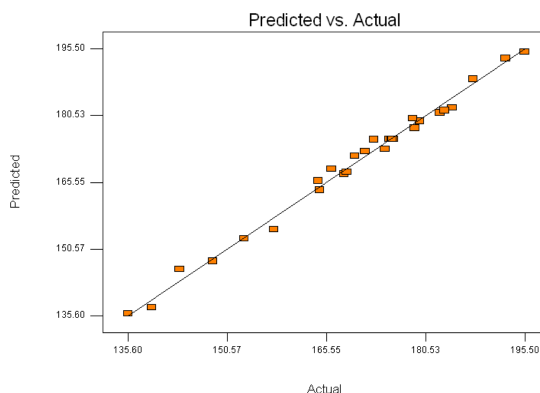

Figure 13: Parity plot for the distribution of predicted and experimental values of asparaginase production

CONCLUSION

The modified Czepek – Dox medium was used in this study for enhanced production of glutaminase free L-asparaginase from *Pencillium* sps. with conventional methods viz., one factor at a time and other statistical approaches taguchi and response surface methodology (CCRD). The enzyme production increased remarkably 1.14 folds over test tube results of native CD medium. So, in future production of low cost therapeutics might be possible to meet the industrial needs.

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