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PRODUCTION OF INTRACELLULAR L ASPARAGINASE FROM *ERWINIA CAROTOVORA* AND ITS STATISTICAL OPTIMIZATION USING RESPONSE SURFACE METHODOLOGY (RSM)

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ABSTRACT:

A Central composite Rotatable Design (CCRD) of Response Surface Methodology (RSM) was used to identify the interactive effect of the three variables viz. Yeast Extract, Maltose and L-asparagine which were identified earlier using 'one-factor-at-a-time' approach, on production of intracellular L-asparaginase from *Erwinia carotovora*. Yeast Extract was found to be the significant variable among three variables. Using Response Surface Methodology (RSM) it was found that a medium containing 11.33 g/L of Maltose, 17.44 g/L of Yeast Extract, 1.97 g/L of L-asparagine was optimum for production of intracellular L-asparaginase .The optimized medium was predicted to give 107.46 IU of L-asparaginase activity. The applied methodology was validated by using optimized medium and L-asparaginase activity of 108.06 IU was obtained with 1.32 fold increase in L-asparaginase activity

Keywords: Erwinia carotovora, L-asparaginase, media optimisation, Response Surface Methodology.

1. Introduction

L-asparaginase (L-asparagine amino hydrolase, EC 3.5.1.1) has long being considered in the management of childhood Acute Lymphoblastic Leukemia (ALL).Its antileukemic effect is attributed to the inability of neoplastic blast cells to synthesize asparagine from aspartic acid as they lack L-asparagine synthetase. However Lymphatic tumor cells need large amounts of asparagines in order to achieve rapid malignant growth. Therefore the commonest therapeutic practice to treat this condition is to intravenously administer L-asparaginase in order to deplete the blood L-asparagine level and exhaust its supply to selectively affect the neoplastic cells [1, 2]. Apart from its therapeutic application L-asparaginase is also being used in Food industry. Recently it has been found that L-asparaginase reduces the formation of carcinogenic acryl amides in deep fried potato recipes [3].

L-asparaginase is known to be produced from variety of organisms like *E.coli* [4], *Erwinia* species [5], *Proteus vulgaris* [6], *Serratia marcescens* [7] *Enterobacter aerogenes* [8], *Staplylococcus* species [9], *Pseudomonas aeruginosa* [10], and *Thermus thermophilus* [11]. Some filamentous fungi like *Aspergillus niger* [12] and yeast like *Saccharomyces cerevisiae* [13] also produce it.

Although L-asparaginase can be obtained from various microbial sources it is well demonstrated that only asparaginase obtained from *E.coli* and *Erwinia chrysanthemi* have been used in humans. Erwinase®, Kidrolase®, Crisantipase®, Oncospar® and Elspar® are some of the formulations containing L-asparaginase from *Erwinia chrysanthemi* and *E.coli*.

However it has been observed that the therapeutic effect of L-asparaginase from these two species is accompanied by side effects such as anaphylaxis, diabetes, leucopoenia pancreatitis, neurological seizures and coagulation abnormalities which may further lead to intracranial thrombosis or haemorrhage. These side effects are partially attributed to the glutaminase activity of asparaginase obtained from these sources [14]. It has been found that L-asparaginase from *Erwinia carotovora* posses lower glutaminase activity (2.6%) as compared to that from *Erwinia chrysanthemi*(10%) [15]. This has aroused an interest in L-asparaginase from *Erwinia carotovora* as it is expected to exhibit fewer side effects.

The conventional 'one-factor-at-a-time' approach for optimizing media components has certain drawbacks as this method is time consuming and often leads to confusion in understanding the process parameters. Also, this method neglects the interaction among variables and thus is incapable of reaching the true optimum. For production of particular compound by the bacterial cell, there are various combinatorial interactions of the media components with the cell metabolism. An experimental design based on statistical modeling is beneficial in evaluating the relationship between the set of controllable experimental factors and observed responses. Moreover, this method has an advantage of requiring few numbers of experiments and is time saving. Thus an optimum process can be developed using an experimental design procedure [16]. Generally, Response Surface Methodology (RSM) defines the effect of the independent process parameters, alone or in combinations, and generates a mathematical model that describes the overall process [17].To this aim RSM is widely used in order to improve product yield, reduce development time and overall process costs [18].

The objective of our study was to understand the relationship between various factors and the response values and to determine the optimum medium components for maximum production of intracellular L-asparaginase form *Erwinia carotovora* MTCC 1428 using Response Surface Methodology (RSM).

2. Materials and methods:

2.1 Medium components:

The media components like Yeast extract, Peptone, Proteose Peptone, Beef extract, Tryptone, Malt extract, Soya bean meal, Ammonium sulphate, Sodium chloride were procured from Hi Media Limited, Mumbai, India. The nitrogen sources Proflo® and Pharmamedia® were obtained as a gift sample from Traders Protein, Southern Cotton Oil Company, Memphis, Tennessee, USA. The buffer salts and assay reagents like Tris buffer, Nessler's reagent was procured from S.D.Fine Chem Ltd., Mumbai. The standard substrate L-asparagine monohydrate chromatographic grades were purchased from Sigma Aldrich Co., USA.

2.2 Microorganism and culture maintenance condition:

Erwinia carotovora MTCC 1428 was procured from Institute of Microbial Technology (IMTECH), Chandhigarh, India. The culture was maintained on YBP medium containing Yeast extract 2.0 g/l, Beef extract 1 g/l, Peptone 5 g/l, sodium chloride 5 g/l and Agar-Agar type-l

powder 20 g/l(pH 7.0).The microorganism was grown in an incubator at 30ºC for 24 h. The slants were kept at 4 ºC and sub-cultured every 30 days.

2.3 Inoculum preparation:

Inoculum was prepared in 250ml Erlenmeyer flasks containing 50 ml of YBP liquid media of pH 7.0. The media was autoclaved at 121 ºC (15lbs) for 20 min and inoculated with *Erwinia carotovora* MTCC 1428. The inoculated Erlenmeyer flasks were kept on an orbital shaker (Remi make) at 180 rmp for 24 h and were used as inoculum. The inoculum size of 4 ml was used throughout the study for subsequent inoculations unless otherwise specified.

2.4 Cell disruption for release of intracellular L asparaginase.

The use of ultrasonication for release of L asparaginase has been reported earlier (H. Geckil et al.,2005).In the present study cells were harvested from the production medium and centrifuged at 10,000 rpm for 10 min at 4 ºC. Cells were washed once with 50mM Tris–HCL buffer pH 8.6 and were resuspended in the same buffer to make a cell suspension of 2%w/v. Cells were disrupted using ultrasonication probe (Dakshin probe sonicator) with 30s pulses at a 30s interval for 10 min. Disrupate thus obtained was centrifuged at 12,000 rpm for 15 min at 4 ºC. The cell free supernatant was subjected to enzyme assay immediately.

2.5 L -asparaginase assay:

L-asparaginase is an enzyme that catalyses the hydrolytic reaction of L-asparagine to form L-aspartic acid and ammonia. Enzyme activity was measured by using Nesslerization reaction. In this method the ammonia liberated from L asparagine in enzyme reaction is quantified by using Nessler's reagent .In the assay procedure this reaction is initiated by adding 0.1 ml of cell free supernatant to a mixture of 0.1 ml of 189mM L asparagine solution (prepared in 50mM Tris–HCL buffer pH 8.6) ; 1.0 ml of 50mM Tris–HCL buffer (pH 8.6) and 0.9 ml of deionized water, prewarmed to 37 ºC. The reaction mixture is incubated at 37 ºC for 30 min after which the reaction was stopped by addition of 0.1 ml of 1.5 M Trichloroacetic acid (TCA). The ammonia released in the supernatant was determined by adding 0.5 ml of Nessler's reagent to tubes containing 0.20 ml supernatant in 4.30 ml of dH_2O.The absorbance was measured at 436 nm against the blanks which received TCA before the addition of 0.1 ml cell free supernatant; using UV-VIS Spectrophotometer (Hitachi, U-2800,Tokyo, Japan).

One unit of L-asparaginase is defined as the amount of enzyme that liberates 1.0 µmole of ammonia from L asparagine per minute at pH 8.6 at 37 ºC.

2.6 Optimization of fermentation medium using one-factor-at-a-time method:

The various physicochemical parameter and media components required for production of maximum L-asparaginase by *Erwinia carotovora* MTCC 1428 were studied in 250 ml Erlenmeyer flasks at 180 rpm containing 50 ml of production medium. The media components and incubation parameters were optimized by using the 'one factor-at-a-time' approach wherein independent parameters were evaluated at a time keeping other parameters constant .The optimized parameter obtained (based on highest enzyme activity achieved) was

incorporated in the next experiment while optimizing the next parameters The parameters studied included seed age (12 h to 60 h), batch time (24 h to 36 h), inoculum size (1 ml to 5 ml) of 24 h old culture, pH of the medium (pH 3 to pH 10) and incubation temperature (20 ºC to 37 ºC). Effect of different carbon sources was studied using Sucrose, Dextrose, Fructose, Maltose, Galactose, Lactose, Sorbitol and Soluble Starch in concentration of 10g/L.All the carbon sources were separately autoclaved. The effect of nitrogen sources was studied using Beef Extract, Tryptone, Malt Extract, Yeast Extract, Soyabean meal, Peptone, Corn steep liquor, Urea, Pharmamedia®, Proflo® in the concentration of 8g/L and in-organic nitrogen sources such as ammonium sulphate and ammonium nitrate were used in the concentration of 2 g/L . Effect of different inducers such as Citrate, Pyruvate, Oxalate, Acetate, Lactate, L-aspartic acid, Succinate, L-aspargine were studied in concentration of 1 g/L.

2.7 Statistical Experimental Design:

On identification of the variables affecting L-asparaginase production by 'one-factor-at-time' approach , three most important variables were selected . These were Yeast Extract , Maltose and L-asparagine. While the basal medium contained 5g/L of NaCl . Central composite Rotatable Design (CCRD) of response surface analysis was adopted for improving the L asparaginase production. The independent variable Yeast Extract, Maltose and L-asparagine were studied for interactions in concentration range of 12-18 g/L, 5-15 g/L and 1-3 g/L respectively. In this regards, a set of twenty experiments was carried out with triplicates at the center of the design. The range and central point values of the independent variables **(Table 1)** were assorted based on the results of preliminary experiments. Average maximum L-asparaginase activity was taken dependent variable (Y_1) . The full experimental design, with respect to the real values of the independent variables and attained values for the response (L-asparaginase activity IU) is presented in Table 2.

2.8 Statistical analysis:

Regressional analysis was performed on the data obtained .The data was then fitted to a Second-order polynomial model using multiple regression procedure. This gave us an empirical model that related the response measured to the independent variables of the experiment. For a three factor system, the model equation is:

$$\mathbf{Y = \beta_0 + \beta_1 A + \beta_2 B - \beta_3 C - \beta_{11} A^2 - \beta_{22} B^2 - \beta_{33} C^2 + \beta_{12} A B - \beta_{13} A C - \beta_{23} BC} \quad \mathbf{(1)}$$

Where Y is the predicted response ; β_0 is the intercept ; β_1 , β_2, β_3 are the coefficients; β_{11}, β_{22}, β_{33} , are squared coefficients ; β_{12} , β_{13} , β_{23} are interaction regression coefficients A, B, C, A^2, B^2, C^2 , AB, BC and AC are independent variables . The proposed model equation predicts the response as a function of the different levels of independent variables. Student t-test and p-value was used to determine the significance of each coefficient of the resulted model. Analysis of variance (ANOVA) was performed .Adequacy of the polynomial model was determined by proportion of variance which is expressed by the coefficient of multiple determinations R^2 . In order to visualize the relationship between the response and experimental levels of each factor and to find the concentration of each factor for

maximum L asparaginase production, the fitted polynomial equation was presented as surface plots. The Software Design Expert Version 6.0.10, Stat-Ease Inc. Minneapolis, USA was used to study interactions of the three variables. Surface plots were drawn using the same software. 'Numerical optimization' process was employed to optimize the level of each factor for maximum response .Combination of different optimized parameters which gave maximum L-asparaginase was tested experimentally to validate the model.

2.9 Production profile studies of L-asparaginase:

Production profile for *Erwinia carotovora MTCC 1428* was performed in RSM optimized media containing 11.33 g/L Maltose , 17.44 g/L of Yeast Extract and 1.97 g/L of L-asparagine in a basal medium containing 5g/L of NaCl. The pH of the medium was adjusted to 7.0 and inoculated with *Erwinia carotovora* MTCC 1428 inoculum, as mentioned earlier .The inoculated flasks were kept on a shaker at 180 rpm and three flasks were used every 12 h to determine intracellular L-asparaginase activity . All fermentation experiments were done in triplicates.

3. Result and Discussions :

Optimization of growth conditions:

During optimization of seed age it was found that a 24 h old seed gave maximum intracellular activity. Further optimization of batch time revealed that maximum productivity could be achieved at 36 h old batch. Studies in optimization of inoculum size, showed that 4 ml (8%v/v) of 24 h old culture gave maximum production of L-asparaginase after 36 h .When the growth medium of varying pH (pH 3 to pH 10) were inoculated with microbial culture it was observed that growth of microorganism increased as the pH was lowered from pH 8 upto pH 4.5 but there was a significant decrease in the L-asparaginase activity. The maximum L-asparaginase activity was obtained at pH 7 .The effect of temperature on production of L-asparaginase *E. carotovora* MTCC 1428 was observed in the temperature range 22°C–37 °C . The optimum temperature was found to be at 25°C which is in accordance to the growth temperature reported by Prita Borkar *et al.*, 2006. Among the various Nitrogen sources screened, Yeast Extract gave appreciable results than Beef Extract, Tryptone, Malt Extract, Soyabean meal, Peptone, Corn steep liquor , Urea, Pharmamedia®, Proflo®. The carbon sources were separately autoclaved and added to the medium containing nitrogen sources at the time of inoculation in order to avoid the untoward Milliard reaction. For the various carbon sources screened (Sucrose, Dextrose, Fructose, Maltose, Galactose, Lactose, Sorbitol and Soluble Starch), Maltose gave maximum of L-asparaginase activity .This could be because of the presence of extracellular maltose binding proteins present in *Erwinia carotovora*. Further supplementation of medium with L-aspargine increased the L-asparaginase activity. Thus after screening various variables, Yeast Extract, Maltose and L-asparagine were found to be significant variables in production of L-asparaginase. The results of One factor at a time experiments are summarized in Table 1.

Statistical analysis and optimization of variables and their interaction :

The Central Composite Rotatable Design (CCRD) of RSM determined the optimum levels and the effect of interactions on L-asparaginase production for the above mentioned significant factors . Values obtained from 'One-factor-at-a-time' approach were employed as center points in RSM CCRD design .Results obtained from RSM CCRD design were analyzed by performing analysis standard analysis of variance (ANOVA). The observed mean L-asparaginase activity and that predicted by the software are presented in Table 3 whereas the Table 2 gives the details regarding the actual and the coded values employed in the RSM CCRD design. The second degree regression equation provided the levels of L -asparaginase production as a functions of initial values of Maltose ,Yeast Extract , L-aspargine which is predicted by following equation .

Asparginase activity = 99.08+ 6.77 x A +16.35 x B – 1.41 x C -17.86 x A^2 – 11.34 xB^2-16.44xC^2+8.88 x AB -5.90 x AC -2.29 x BC (2)

Where *A:* Maltose , *B:* Yeast Extract , *C:* L-asparagine .

According to the present model A, B, A^2, B^2, C^2, AB, AC were found to be significant model terms .The values of "Prob > F" for all the above terms was less than 0.0500 indicate model terms are significant whereas values greater than 0.010 suggests insignficant model terms . Table 4 shows the details in this regards . ANOVA values for the response of L-asparaginase from the RSM experiments is given in Table 5. ANOVA values for L-asparaginase production (activity IU) indicated that the Model F-value of 40.31 which implies the model is significant at high confidence interval . There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

ANOVA indicated the R^2 value of 0.9784 for L-asparaginase activity which ensured that the quadratic model was satisfactorily adjusted in experimental data and indicated that model could explain 90-95% of variability in response .The term "Adequate Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The S/N ratio of 14.645 was obtained which indicated an adequate signal thus implying that this model can be used to navigate the design space for the response of L asparginase activity. The Prededicted R-squared of 0.7729 is in reasonable agreement with the Adjusted R-squared of 0.9542. The accuracy and applicability of the Central Composite Rotatable Design (CCRD) for process optimization was reflected through a good corelation between observed and predicted results.

L-asparaginase yield for different levels of variables was predicted from the contour plots drawn using Software , 'Design Expert Version 6.0.10', Stat-Ease Inc. Minneapolis, USA. Each contour curve represents a finite number of combination for any two test variables with respect to third variable at its zero level .The elliptical nature of contour depicts the mutual interactions of all variables in a 3D- response surface graphs (Fig 1).There was a significant interaction between every two variables. The maximum predicted yield was indicated by surface

confined in smallest ellipse of the contour diagrams.

Maximum L-asparaginase total activity of 104.72 IU was achieved at the central values of the variables .Thus a 1.28 fold increase in L-asparaginase was observed in RSM optimized medium as compared to the growth medium optimized by 'One-factor-at-a-time' approach (Fig 2) .On further using this model for numerical optimization, the model predicted that maximum L-asparaginase activity of 107.46 IU could be achieved if 11.33 g/L of Maltose, 17.44 g/L of Yeast Extract , 1.97 g/L of L-asparagine was used as growth medium . By further numerical optimization, the L-asparaginase activity was predicted to increase by 1.31 fold

Validation was carried out in shake flasks under conditions predicted by model. On experimentation the yield obtained was 108.06 IU which suggested that there was about 1.32 fold increase in L-asparaginase activity. As the experimental values were close to the predicted values, the model is successfully validated.

Production profile studies of L-asparaginase:

Production profile studies were performed, where effect of inoculum size, temperature and batch time on newly optimized medium were studied. The inoculum size was varied from 6 to 10 %v/v and the optimum inoculum size was found to be 8% v/v (4ml) which was same as that obtained for one factor at a time experiments. Similarly, Studies on effect of temperature revealed that maximum activity was achieved at 25°C. However, studies on effect of newly RSM optimized medium on batch time showed that the batch time was reduced from 36 h to 24 h with higher activity (Fig 3). The L-asparaginase activity thus obtained was 110.92 IU showing 1.35 fold increases in L-asparaginase activity.

4. Conclusion :

The production of L-asparaginase was significantly enhanced by Maltose, Yeast Extract and L-asparagine and its interactions with other media components .Statistical optimization of media components using Central Composite Rotatable Design (CCRD) of RSM gave substantial increase (1.32 fold increase) in L-asparaginase yield. This suggests that *Erwinia carotovora* MTCC 1428 could be a promising source for production of L-asparaginase. However further work to understand the metabolism of L-asparaginase production and downstream processing for purification of L-asparaginase needs to be done.

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Table 1 : Results of *'one factor at a time'* experiments .

Factor	Result
Seed Age	24 h
Batch Time	36 h
Inoculum Size	4 ml
pH	7
Temperature	25ºC
Carbon Source	Maltose
Nitrogen source	Yeast Extract
Inducer	L asparagine

Table 2: Actual and Coded levels for independent variables used in RSM study for optimizing media components for intracellular production *Erwinia carotovora* MTCC1428.

Coded Values	Actual values of media components in (g/L)		
	Maltose	Yeast Extract	L - asparagine
-1	5	12	1
0	10	15	2
1	15	18	3

Table 3 : Central-composite experimental design of the independent variables along with the observed values for the response,(Yo).(*Results are mean ± SD of three determinations)

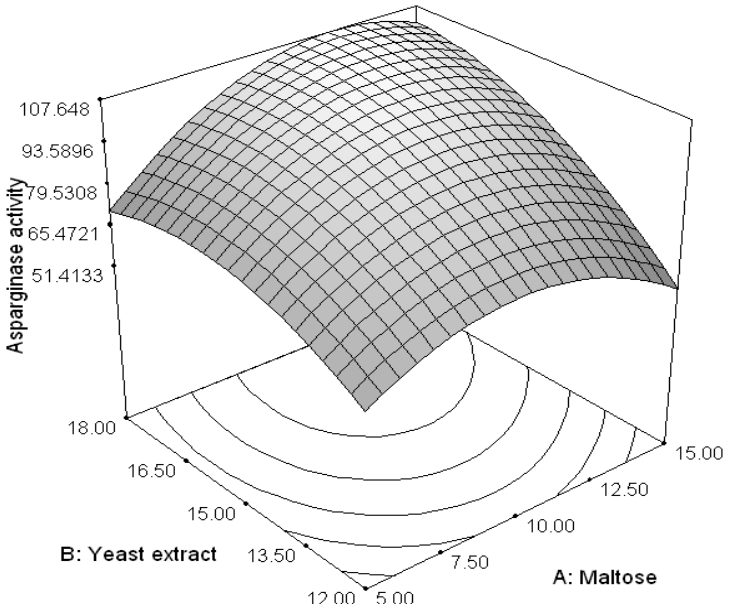
Standard order	Run order	Maltose g/L	Yeast Extract g/L	L-asparagine g/L	L-asparaginase Total Activity IU		Standard Deviation
					Observed*	Predicted	
1	6	5	12	1	36.24	37.46	± 0.58
2	5	15	12	3	41.06	35.01	± 1.76
3	4	5	18	3	59.96	61.39	± 0.58
4	1	15	18	1	99.56	100.08	± 1.17
5	3	10	15	2	100.51	104.13	± 1.19
6	2	10	15	2	104.86	104.13	± 0.58
7	11	5	12	3	44.16	44.37	± 1.76
8	12	15	12	1	39.08	38.38	± 0.58
9	9	5	18	1	43.56	50.34	± 1.76
10	10	15	18	3	74.71	74.22	± 0.58
11	7	10	15	2	95.83	97.47	± 2.65
12	8	10	15	2	104.91	97.47	± 2.64
13	19	1.6	15	2	39.13	33.75	± 0.64
14	20	18.41	15	2	52.16	56.51	± 0.64
15	13	10	9.95	2	32.54	36.05	± 0.66
16	15	10	20.05	2	95.60	91.05	± 0.64
17	18	10	15	0.32	55.83	51.52	± 0.68
18	17	10	15	3.68	43.50	46.77	± 0.59
19	14	10	15	2	98.20	95.64	± 0.53
20	16	10	15	2	89.97	95.64	± 0.59

Table 4: Model coefficients estimated by multiple linear regression and significance of regression coefficient for intracellular L-asparaginase activity.

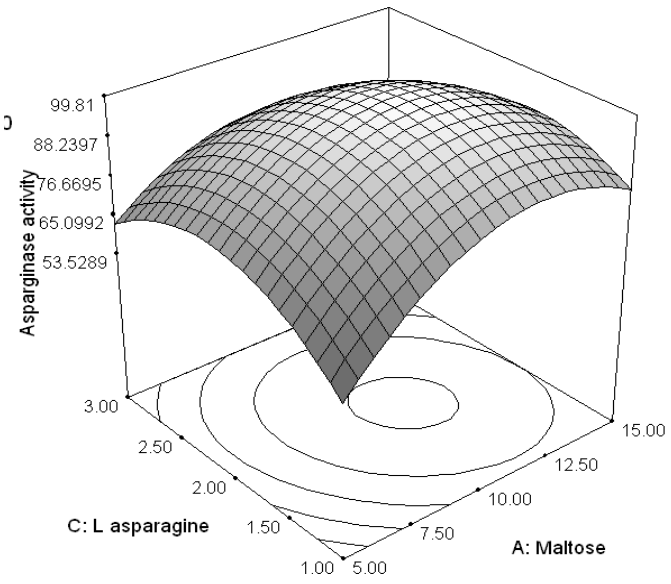
Factor	L-Asparaginase activity (IU)		Prob > F
	Coefficient Estimate	Standard Error	
Intercept	99.08	2.53	
A-Maltose	6.76	1.67	0.0038
B-Yeast extract	16.35	1.67	< 0.0001
C-L asparagine	-1.41	1.67	0.4245
A^2	-17.85	1.63	< 0.0001
B^2	-11.34	1.63	0.0001
C^2	-16.43	1.63	< 0.0001
AB	8.87	2.19	0.0037
AC	-5.89	2.19	0.0275
BC	-2.29	2.19	0.3263

Table 5: ANOVA for experiments.

Parameters	Response L-asparaginase activity IU
F-value	40.31
P>F	< 0.0001
Mean	67.57
R^2	0.978424056
Adjusted R^2	0.954151118
Adequate precision	14.6449746



[a]



[b]

Fig 1: 3D response surface curves : interactive effects of [a] varied Yeast extract and Maltose at 2g/L of L-asparagine.[b] varied L-aspargine and Maltose at 15g/L of Yeast extract. (L-asparginase activity measured in IU)

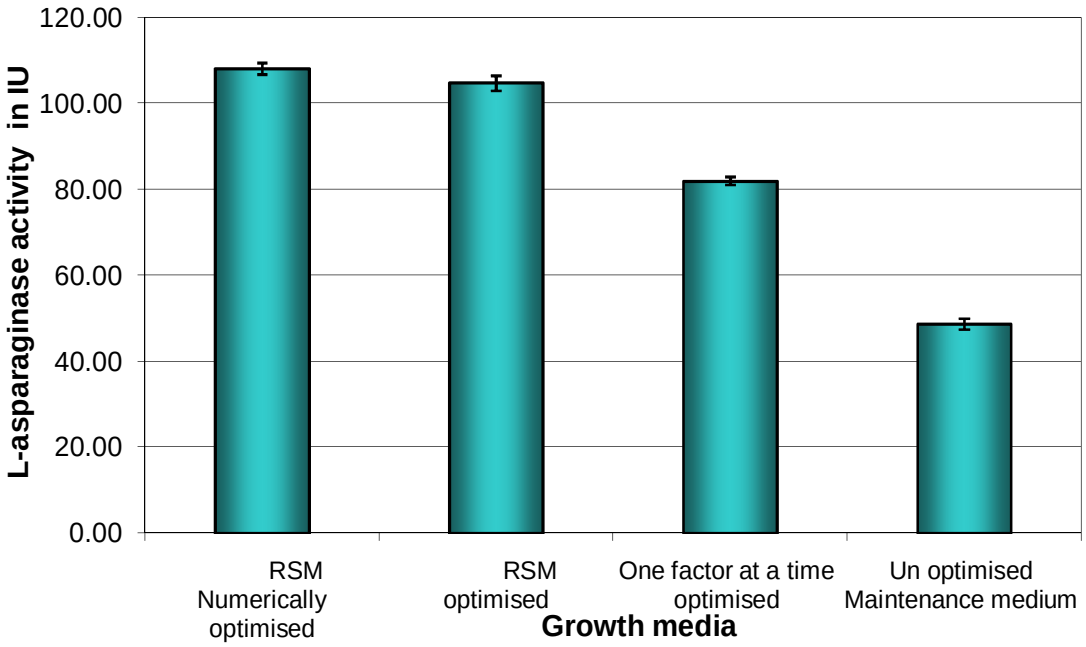


Fig 2: Comparison of RSM optimized medium with the 'one-factor-at-a-time' medium and Un-optimised maintenance medium.

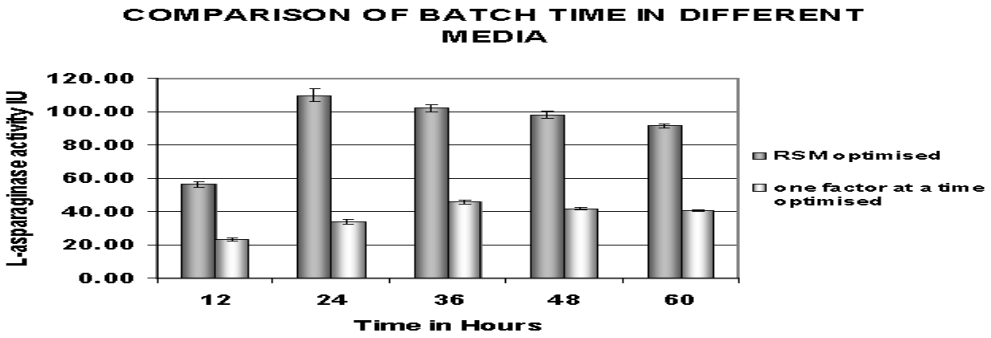


Fig 3: Comparison of batch time for production of L-asparaginase in RSM optimized media and media optimized by '*one factor at a time*'.