OPTIMIZATION OF MODIFIED NINE MEDIA COMPOSITION FOR L-ASPARAGINASE PRODUCTION

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Abstract - This study was conducted with the aim to screen and optimize media composition to produce L-asparaginase enzyme with highest activity under optimal media composition. Selective medium was used for the production of L-asparaginase from bacteria locally isolates in Central Lab. Response surface Methodology (RSM) was employed to optimize the medium for the production of L-asparaginase at pH 8.6 and temperature of 25^0C. The two variables involves in this study were carbon source and nitrogen source. Primary screening resulted in Lactose and Tryptone are the best carbon and nitrogen sources with the highest enzyme activity of (2.6 IU/ml) and (2.7 IU/ml) respectively. The determination coefficient ($R^2$for full Quadratic Model) for L-arparaginase production implied that the model is significant with high satisfactory value of $R^2$of 90.10% which indicates that only 9.9% of total variation is not explained by the model. The optimum points were determined to be 0.189340Lactose and 8.34619 Tryptone that gave highest activity of 90.10U/ml of L-asparaginase.

Keywords - Carbon Sources, L-Asparaginase, Nitrogen Sources, Response Surface Methodology.

I. INTRODUCTION

Biotechnology is defined as “any technical application that uses biological systems, living organisms or derivatives thereof, to make or modify products or processes for specific use”. That biotechnology has existed since human first used fermentation to make bread, cheese and wine. Also modern biotechnology referred to" the understanding and application of genetic information of animal and plant species [1].

Microbial enzymes are considered as potential biocatalysts for a large number of reactions. They are widely used in medicines and industry because they are more active and stable than plants and animal enzymes, in additions that the microorganisms represent an alternative source of enzymes cause they can be cultured in large quantities in a short time by fermentation and they are of biochemical diversity and susceptibility to gene manipulation. Industries are looking for new microbial strains in order to produce different enzymes to fulfill the current requirement [2].

Asparaginase or colaspase is an enzyme that catalyzes the hydrolysis of asparagine to aspartic acid. Asparaginases are enzymes expressed and produced by microorganisms. They are used in food manufacture, and chemotherapeutic agent in the treatment of certain types of human cancer [3]. It is also active against non-Hodgkin’s lymphoma, pancreatic carcinoma and bovine lymphomosarcoma [4]. Asparaginase produces its anti-cancer effects by “breaking down” asparagine, a substance normally found in the body that is involved in biological processes that are essential for cells to maintain life. Healthy cells are able to create asparagine for themselves; however, cancer cells are not able to create asparagine. Therefore, the depletion of asparagine by asparaginase kills cancer cells, normal cells are not affected since they can synthesize L-asparagine [5]. The rationale behind asparaginase is that it takes advantage of the fact that acute lymphoblastic leukemia cells and some other suspected tumor cells are unable to synthesize the non-essential amino acid asparagine, whereas normal cells are able to make their own asparagine; thus leukemic cells require high amount of asparagine. These leukemic cells depend on circulating asparagine. Asparaginase, however, catalyzes the conversion of L-asparagine to aspartic acid and ammonia. This deprives the leukemic cell of circulating asparagine, which leads to cell death [6].

E.coli strains are the main source of medical asparaginase. Branded formulations (with different chemical and pharmacological properties) available in 1998 include Asparaginase, Medac, Ciderolase, and Oncaspar (Crasnitin has been discontinued) [7].

Response surface methodology (RSM) is powerful and efficient mathematical approach widely applied in the optimization of fermentation process eg. Media components on enzyme production [8], [9], [10], production of other metabolites [11], [12], [13], [14], spore product and biomass production optimization .It can give information about the interaction between variables information necessary for design and process optimization and give multiple responses at the same time.

II. MATERIALS AND METHODS

A. Study Design
This study was experimental design

B. Study period
This study was conducted from October 2016 to March 2017

C. Chemicals
All chemicals used in this study investigation were of analytical grade and procured from Sigma (USA), and Lab Rasayan (India).

D. Screening of isolates for L-asparaginase production
Strains were screened on the basis of good pink zone of lysis formed around microbial colonies in medium M9 containing phenol red as colour indicator (0.3ml from 2.5% phenol red stock solution in ethanol), which results in the formation of pink zone. The selected strains are maintained on asparagine rich agar slant at 40 C [15].

E. Inoculum Preparation
One single slant of culture (24h old slant culture for bacteria) is transferred to 50ml sterile M9 medium in a 250 ml Erlenmeyer flask and incubated for 24h at 37 °C. 2ml of this cell suspension (2x106 cells/ml) is used as inoculum. Submerage fermentation was carried out in a 250ml Erlenmeyer flask containing moistened with 10ml of mineral salt solution containing (g/l): $Na_2HPO_4.2H_2O$, 6.0; $KH_2PO4.7H_2O$, 3; 2ml of 1M $MgSO_4.7H_2O$; 1.0 ml of .1M $CaCl_2.2H_2O$; 1.0ml of 20% glucose stock; corresponding to 60% moisture level and at pH 6.0 then sterilized at 1210 C for 15min, cooled to desirable temperature, addition of 2ml (2x106 cells/ml) inoculum and incubated for 48h at 37^0 C [15].

F. Enzyme Preparation
The enzyme was extracted at the end of the fermentation period by the addition of 90ml of 0.01 M phosphate buffer of pH 7.2 to the fermented medium, shaking for 15 min followed by centrifugation at 8,000 rpm for 20min. The cell free supernatant was used for the estimation of the crude enzyme [15].

G. Experimental design

1- Screening
Various Carbon compounds (Sucrose, Lactose, Glucose, Raffinose, Xylose, Sorbitol, Gluctose, Manitol, Glyerol, Starch, Esculin, Fructose, Mannous, Arabnous, Inalim, Salicin, Trehalose) were added at same concentration of 1% to the M9 medium after 18 hours of fermination, while various nitrogen compounds (Urea, Aspergine, Glutamine, Gletin, Beef, Arginine, Casein, Wheat, NaNO3, Beenut. Tryptone, Tryptonewater, Peptonewater, Malt extract, Tryptone, D-analine, Yeast extract) were added at same concentration of 1% to the M9 medium after 18 hours of fermentation.

2- Optimization
Two level fractional factorial design with two variables consisting of one blocks with 13 runs were used. The Minitab was describe the response surface method (RSM).

The optimization of some medium composition including nitrogen source (Tryptone), carbon source (Lactose), 1g of K_2HPO_4nd3g of Asparagine.
The levels used for the experiment according to literature review as actual is low to high (1-7) for Tryptone and (0.5-1) for Lactose and coded form was listed in (Table 1).

Stander Order	Run Order	Pt. Type	Blocks	Lactose	Tryptone
7	1	-1	1	1.25000	0.846
6	2	-1	1	2.31066	3.75000
10	3	0	1	1.25000	3.75000
12	4	0	1	1.25000	3.75000
4	5	1	1	2.0000	7.0000
11	6	0	1	1.25000	3.75000
1	7	1	1	0.5000	0.5000
5	8	-1	1	0.18934	3.75000
13	9	0	1	1.25000	3.75000
3	10	1	1	0.5000	7.0000
9	11	0	1	1.25000	3.75000
2	12	1	1	2.0000	0.5000
8	13	-1	1	1.25000	8.34619

Table (1) Variable in experimental plan

H. Enzymatic assay
L-Asparaginase activity was measured by direct Nesslerization of ammonia. The activity of L-Asparaginase was measured employing the modified method of Wriston [16].The L-Asparaginase catalyzes L-asparagine to Laspartic acid and ammonia and the latter react with the Nessler's reagent to produce an orange colored product. The enzyme assay mixture consisted of 100 µL of freshly prepared L-asparagine (189 mmol/L) in Tris-HCl buffer (pH 8.6) and 100 µL of crude extract of the enzyme. The reaction mixture was incubated at 37 °C for 30 min and the reaction was stopped by adding 100 µL of 15% trichloroacetic acid (TCA). The reaction mixture was centrifuged at 6000×g for 5 min at 4 °C to remove the precipitates. The ammonia released in the supernatant was determined using colorimetric technique by adding 500 µL Nessler's reagent into the sample containing 200 µL supernatant and 4.3 ml distilled water. The contents in the sample were vortexed and incubated at room temperature for 10 min, scanned for emax. OD was measured at ë396 nm against the blanks that received TCA before the addition of crude enzyme. The ammonia produced in the reaction was determined based on the standard curve obtained with

ammonium sulfate. One International Unit (IU) of L-Asparaginase activity was defined as the amount of the enzyme that liberates 1 µmol/L of ammonia/ min at 37 °C

III. RESULTS AND DISCUSSION

A.Screening

I. Effect of carbon source

Various carbon compounds were added at same concentrations 1% to the M9 medium. All tested carbon source showed approximately activity ranged from 1.9891-2.6056U/ml and 1% lactose exhibited highest L-asparaginase activity (2.605IU/ml) after 18 hours of fermentation. Other carbon sources in the medium also showed L-asparaginase activity but the yield was lower with respect to lactose. As maximum enzyme activity was obtained with lactose as carbon source, for all subsequent experiments lactose was used.

Carbon source	Enzyme activity IU/ml
Lactose	2.6056
Glucose	2.5497
Sucrose	2.6031
Raffinose	1.9891
Xylose	2.2641
Sorbitol	2.4010
Gluctose	2.5977
Manitol	2.2328
Glycerol	2.4880
Lactose monohydrate	2.5406
Starch	2.5126
Esculin	2.2569
Fructose	2.4155
Mannous	2.5870
Arabnous	2.2373
Inalin	2.5124
Salicin	2.6006
Trehalose	2.5206

Table (2): Effect of different carbon sources on L-asparaginase activity. E. coli was cultured in the basal medium.

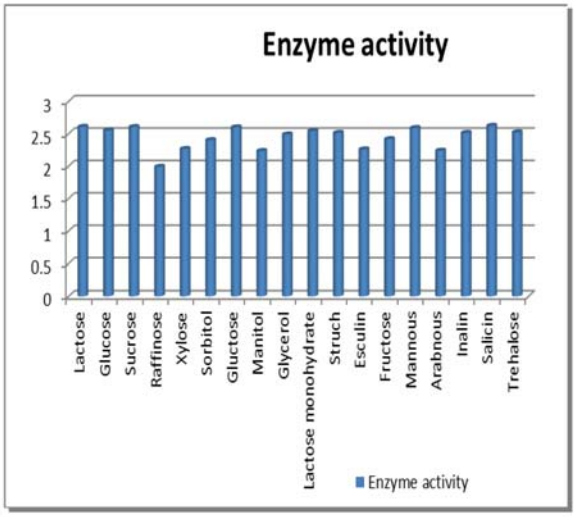


Figure (1): Effect of different carbon sources on L-asparaginase activity.

2. Effect of nitrogen sources:

Sixteen different nitrogen sources (L-asparagine, yeast extract, beef extract, peptone, glutamine. Gelatin, Beef, Arginine, Wheat, Tryptone. Tryptone water, peptone water, Benut, malt extract. Yeast extract, NaN03, D-analine and urea) were used at same concentration 1 % to the M9 medium containing lactose as carbon source. Among all the organic & inorgairic nitrogen sources used, the result showed urea was the most effective but did not use because when asparagine's hydrolyzed convert to L- aspartic acid and ammonia also E.coli have enzyme urease ,so found so found 1% Tryptone was the most effective in L-asparaginase production (2.7034IU/ml).

Nitrogen source	Enzyme activity IU/ml
Urea	3.7649
Aspergine	0.8556
Glutamine	1.1170
Gletain	1.735
Beef	2.5081
Arginine	1.7411
Casein	1.5403
Wheat	1.5975
NaNO3	0.8619
Beenut	1.7355
Tryptone water	2.6433
Peptone water	2.2994
Malt extract	0.4667
Tryptone	2.7034
D.analine	1.1321
Yeast extract	1.7107

Table (3): Effect of different N sources on L-asparaginase activity. E.coli was cultured in the basal medium.

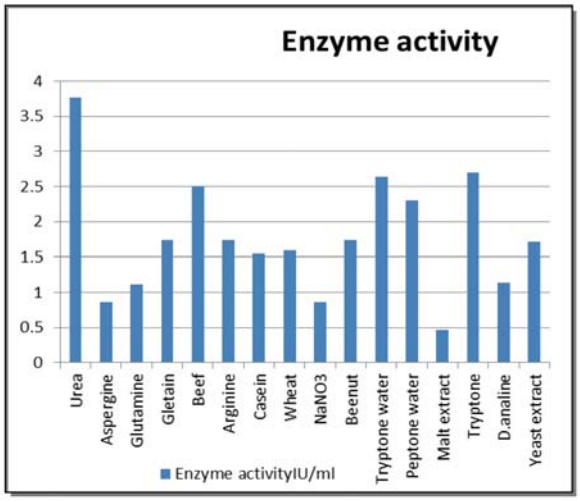


Figure (2): Effect of different nitrogen sources on L-asparaginase activity.

B.Optimization

Central composite design (CCD) allowed calculation of maximum production based on a sets of experiments in which all the factors were varied with in chosen ranges. This method had been successfully applied in

the optimization of medium composition. The experiment design and result shown in table (4). The highest L-asparaginase activity (0.203, 0.875, and 0.2768) were observed at runs numbers 2,5,10 respectively.

Stander Order	Run Order	Pt. Type	Blocks	Lactose	Tryptone	Enzyme activity IU/ml
7	1	-1	1	1.25000	-0.846	0.4974
6	2	-1	1	2.31066	3.75000	1.0106
10	3	0	1	1.25000	3.75000	0.2768
12	4	0	1	1.25000	3.75000	0.3904
4	5	1	1	2.0000	7.0000	1.3244
11	6	0	1	1.25000	3.75000	0.345
1	7	1	1	0.5000	0.5000	0.2242
5	8	-1	1	0.18934	3.75000	0.8751
13	9	0	1	1.25000	3.75000	0.2495
3	10	1	1	0.5000	7.0000	1.3958
9	11	0	1	1.25000	3.75000	0.455
2	12	1	1	2.0000	0.5000	0.2057
8	13	-1	1	1.25000	8.34619	0.8665

Table (4): Experimental design and result of factorial design, L-asparaginase activity

The regression coefficient, T value, P value and determination coefficient (R^2) for full Quadratic model for L-asparaginase production are presented in table (5). This model was tested for adequacy by analysis of variance implied that the model is significant with high of satisfactory value of R^2 of 90.10% which indicates that only 9.9% of total variation is not explained by the model. This indicated that the variation in those selected factors could explain the variation in L-asparaginase activity up to 90.10%.These made the fitted second order polynomial more acceptable.

Term	Coef	SE Coef	T	P
Constant	0.3434	0.04129	8.317	0.000
lactose	-0.1951	0.04617	-4.226	0.004
Tryptone	0.2901	0.04617	6.284	0.000
lactose*lactose	0.4147	0.07001	5.923	0.001
Tryptone*Tryptone	0.2310	0.07001	3.299	0.013
lactose*Tryptone	-0.3607	0.09233	-3.907	0.006

Table (5): Regression Result of a full Quadratic model for L-asparaginase production (using encoded unit)

T=coef/standard error
P=the effect in the model is statistical significant
S = 0.0923300 PRESS = 0.270311
R-Sq = 94.22% R-Sq (pred) = 73.84% R-Sq (adj) = 90.10%

The estimate equation of model for activity showed significant positive linear effect for lactose and tryptone, which significantly this variable will significantly increase L-asparaginase activity. The interaction between Lactose and Tryptone showed highly significant positive effect, which indicates that interaction of both factors required for L-asparaginase activity. The model is represent by the equation below:

$$Activity=0.3434-0.1951X_1+0.2901X_2+0.4147X^2_1+0.231X^2_2-0.3607X_1X_2$$

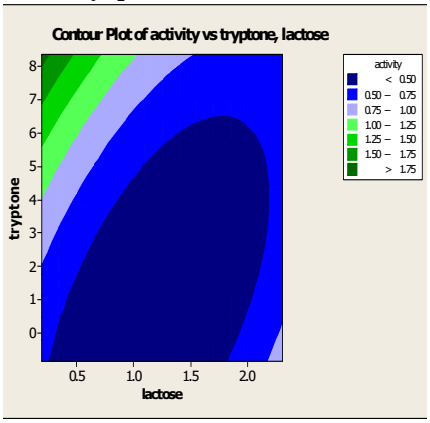


Figure (3): Contour Plot of activity versus tryptone, lactose

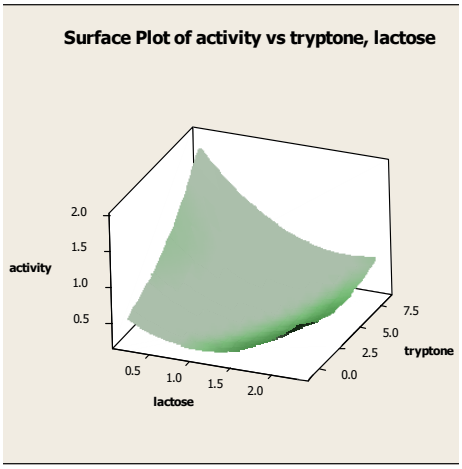


Figure (4): Fitted contour and surface plots of Lactose - Tryptone interaction on -asparaginase.

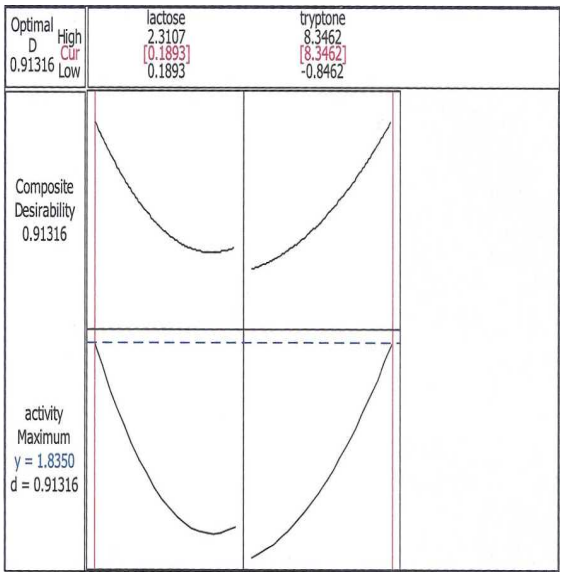


Figure (5): Mintab's response activity

By using the Mintab's response optimizer under global solution of desirability equal 0.91316.That is Lactose at 0.1893% and Tryptone 8.3462% the maximum response was 1.8350 IU/ml.

Response Optimization:
Local Solution
Lactose = 0.189340
Tryptone = 8.34619
Predicted Responses
Activity = 1.83501,
Desirability = 0.913164
Composite Desirability = 0.913164

IV. CONCLUSION

From this work we conclude that E.coli have potential for L-asparaginase production on M9 medium under submerged fermentation methodology. It was found that 2.6056 IU/ml lactose as carbon source, 2.7034 IU/ml Tryptone as nitrogen source.

The optimization of media compositions enhanced the production of L-asparaginase by E.coli the maximum optimum activity was obtain desirability equal to 0.913164. That is Lactose at 0.189340 and Tryptone 8.34619 the maximum response was 1.8350 IU/ml.

The determination coefficient (R^2) for L-asparaginase production implied that the model is significant with high satisfactory value of 90.10% which indicates that only 9.9% of total variation is not explained by the model.

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