Original Research Article

Optimization of Solid State Production of Antileukemic Enzyme (L-Asparaginase) by *E. coli* K-12 using Taguchi Doe and Three Phase based Recovery

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A B S T R A C T

Keywords	
L-Asparaginase, Acute Leukemia, Taguchi DOE Methodology, Three Phase Partitioning.	The production of an antileukemic enzyme, L-asparaginase by the *E. coli* K-12 under solid-state fermentation may be promising alternative to produce it economically. The production level of the enzyme by the bacterial sources is very low and hence the factors have to be distinguished which effect the growth of cells and production of enzyme directly or indirectly. The production of L-Asparaginase was optimized by the use of Taguchi DOE methodology. L-18 array was selected for the purpose of optimization. The six factors at three levels were considered for the optimization. L-asparaginase production of 4.32 IU/ml was observed on wheat bran supplemented with 1.25%, (w/w) yeast extract, 0.75%, (w/w) L-asparagine 80%, (v/w) initial moisture and 37°C after incubation of 24 hrs. The L-asparaginase is partially purified from the three phase based partitioning (TPP) method by using t-butanol followed by ammonium sulphate (45%) precipitation at pH 6.7. After complete optimization approximately 2.88 fold increase in enzyme yield was achieved.

Introduction

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1.) has been widely found in biological world. In recent years, the use of L-asparaginase in the treatment of leukemia and other lymphoproliferative disorders has expanded immensely. For these reasons L-asparaginase has established itself to be an indispensable component in medicine [1]. Many research groups have studied asparaginase production and purification in an attempt to minimize impurities that produce allergenic reactions

[2] Moreover, L-asparaginase degradation gives rise to free amino acid, which is a precursor of acrylamide before baking. It is also used in the food industry to reduce the acrylamide formation in food products. L-asparaginase production using microbial systems has attracted considerable attention, owing to the cost-effective and eco-friendly nature. Asparaginase is an enzyme which converts L-asparagine to L-aspartic acid and ammonia [3].

In recent years, SSF has shown much promise in development of several bioprocesses and products. Solid-state fermentation (SSF) is defined as the fermentation process in which microorganisms grow on solid materials without the presence of free liquid.The concept of using solid substrates is probably the oldest method used by man to make microorganisms work for him. SSF has several advantages such as lesser energy requirements, very low risk of bacterial contamination, lower need of water and less environmental concerns regarding the disposal of solid waste. Additionally, the utilization of agro-waste solid as a substrate for carbon and energy requirement under SSF makes this approach environmental friendly. Usually substrates used for solid state fermentation are water insoluble polymer of starch or cellulosic materials. [4] In statistical method sets of experiments can be designed by Taguchi DOE method. It involves establishment of large number of experimental situation described as orthogonal arrays (OA) to reduce experimental errors and to enhance their efficiency and reproducibility of the laboratory experiments. [5, 6].

There are a few reports on the production of extra-cellular L-asparaginase under solid state fermentation by microbial strains. In present study the optimization of L-asparaginase was carried out by using substrates of wheat bran, rice bran and vegetable wastes under solid state fermentation. The partial purification of L-asparaginase was carried out by using three phase partitioning (TPP) technique. TPP is a cost effective method for the purification of enzymes. The TPP technique is based on the principles of salting out, co-solvent precipitation, isoionic precipitation, and kosmotropic precipitation of proteins. It is easily scalable and can be used directly with crude suspensions [7]

Materials and Methods

Microorganism

E.coli K-12 was used in this study. The bacterium was confirmed for L-Asparaginase activity on the media agar plate having phenol red as an indicator. Change in color due to increase in pH on the release of aspartic acid and ammonia was observed [8]. The microorganism was sub cultured every 30 days. All the chemicals used in the study were analytical grade.

Inoculum and Media Preparation

E. coli K-12 was cultivated in Luria broth medium 20g per liter of distilled water (pH 7.0). The cells were cultivated in this medium at 37°C on a rotary shaker at 160 rpm for 24 h. Then 10g of substrate was weighed into a 250 ml Erlenmeyer flask into which a supplemental salt solution was added to get the desired moisture level. The composition of the salt solution was as follows (%w/w): $Na_2HPO_4.2H_2O$: (0.6); KH_2PO_4: (0.3); $MgSO_4.7H_2O$: (0.05); $CaCl_2.2H_2O$: (0.00015); NaCl: (0.05). L-asparagine was supplemented as inducer for synthesis of enzyme L-asparaginase [8].The contents were thoroughly mixed and autoclaved at 121☐ C (15 psi) for 20 min.

Design of Experiment (DOE) by Taguchi Methodology

The method followed for the optimization is based on the Taguchi orthogonal array (OA) experimental design (DOE) [9,10,11]. Optimization has been done for six factors *i.e.* five different components of media and incubation time (Table-1) by considering their concentrations at three different levels. L-18 array was constructed. 18 different trial experiments were designed on this basis at 3 different levels: maximum, minimum and

intermediate level with same composition. The result of different trial conditions have been used to estimate the optimum condition for the enhanced production of L-Asparaginase. Further analysis has been done to find out all the possible interactions between the different factors participating in the L-asparaginase production. QUALITEK-4 software was used for the designing of OAs and for the optimization based analysis of the media for the better production. [12]

Solid state fermentation and extraction of L-asparaginase

The sterilized substrate including other media components were inoculated with 2 ml of pre grown microbial culture. The contents were mixed thoroughly and incubated at 37 □C. All designed experimental conditions were carried out and samples were withdrawn after 24hrs of incubation. The crude enzyme from the fermented material was recovered by simple extraction method. The fermented substrate was mixed thoroughly with 50 ml of 50 mM phosphate buffer (pH 7.0) and the contents were agitated in a rotary shaker (160 rpm) for 60 min at 37°C, a temperature high enough to increase the extraction efficiency without causing enzyme denaturation. The raw extract was obtained by pressing the mixture and subsequent centrifugation. The resulting clear filtrate was further purified by three phase partitioning technique.

Three Phase Partitioning (TPP) Technique

The clear filtrate of fermentation broth after centrifugation was purified by using TPP technique as proposed by Jha et al 2012 [13]. Precipitation of proteins was carried out by salting out by adding desired level of ammonium sulphate in fermentation broth. Then 1:1 t-butanol-fermentation broth was

used to further precipitation of enzyme. The phase separation was carried out after the centrifugation (at 2000g at 4^0C) of mixture followed by incubation. The enzymatic activity was assayed in interfacial precipitate, aqueous phase and organic phase.

Standardization of Enzyme Assay

L-Asparaginase assay has been started by the Worthington manual protocol but few modifications have been done to optimize it in the laboratory conditions. The rate of hydrolysis of asparagine is determined by measuring the released ammonia. One unit of L-Asparaginase releases one micromole of ammonia per minute at 37°C and pH 8.6 under the specified conditions. The standardization of the method has been done by the pure L-Asparaginase purchased from SIGMA-ALDRICH. Enzymatic assay through Nesslerization was continued with very small amount of supernatant. O.D. was taken at 480 nm to define the activity of enzyme [14].

Results and Discussion

Maximum production of L-Asparaginase has been observed after 24 hours of inoculation of media. The yield of enzyme in all 18 trial conditions (Fig-1) were used for the analysis of solid state fermentation and identifying optimum levels of proposed factors.

Optimization was done on the basis of the results of these 18 experimental trials. The interaction and severity index (Table-2) between two factors may be provide an understanding of overall process analysis. It has been observed that Moisture level and Yeast extract has maximum severity index (74.46) on enzyme production, rather yeast extract and L-asparagine has the minimum (12.32) in case of *E. Coli* K-12. Rest of the

factors has intermediate severity index on enzyme production. Moreover, after optimizing the different factors in 18 trial conditions it has been analysed that different factors affect the enzyme production at different levels. The favourable levels of different factors can be evaluated from the value of severity index. Different factors at their best levels and their respective contributions are mentioned in table 4. Graphical representation of favourable levels can be seen in fig.2.

The results of the orthogonal array (OA) experiments were analysed by the use of ANOVA (Table 3). The F-ratio was used to determine the degree of variation contributed by each factors (Armstrong and Hilton, 2004). The all factors and their respective interactions considered in the experimental design were statistically significant effects at 95% confidence limit. By study of main effect of each factor the general trends of the influence of the factors towards the process can be characterized. Analysis of variance has given the

percentage contribution of all factors on the performance of the process. In case of *E. coli* K-12, agrowaste has contributed 12.075%, moisture level 24.112%, yeast extract 21.43%, L-asparagine 10.272%, inoculum 23.01% and incubation time 8.001% (Table-3). The expected result calculated by the designing experiment was 4.670 IU/ml.

The proposed optimized conditions by factorial design were validated by estimating L-Asparaginase activity according to designed conditions. After validation the enhanced activity of 4.34 IU/ml was found in the solid state fermentation by *E. coli* K-12. There was 2.88 fold increase in the production level after the Taguchi DOE optimization of the process.

The three phase partitioning of L-asparaginase recovery yield the maximum activity after the 45% ammonium sulphate precipitation. The best partitioning was observed at 1:1 ratio of t-butanol and fermentation broth at pH 6.7.
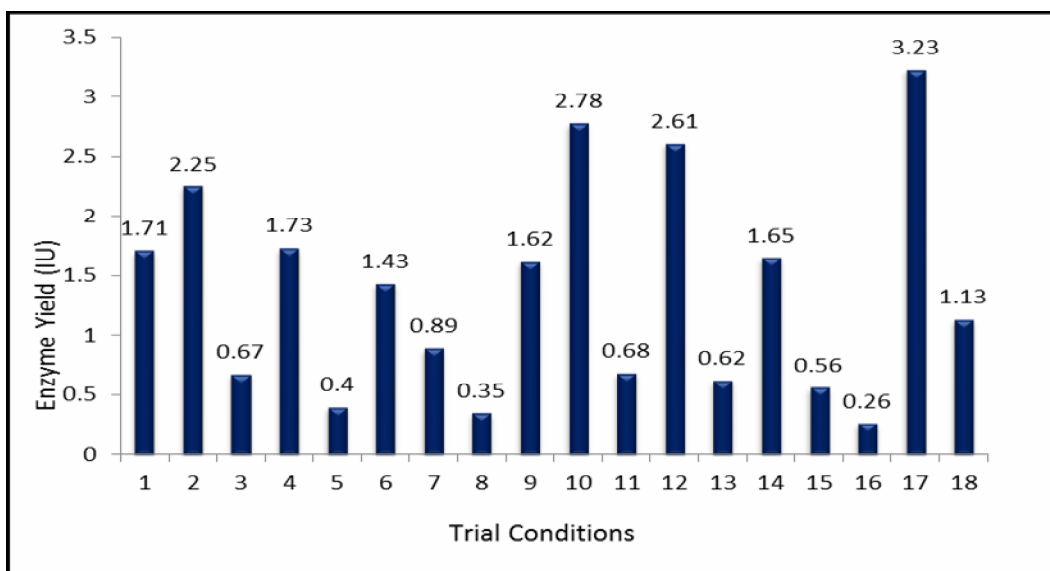
Fig.1 Enzyme yield on different trial conditions

Table.2 Estimated interaction between the factors (Severity Index)

S.NO.	Interacting Factor Pairs (order based on SI)	Columns	SI%	Optimum levels
1	Moisture level x Yeast extract	2x3	74.46	1,3
2	L-asparagine x Incubation time	4x6	73.51	3,2
3	Yeast extract x Incubation time	3x6	63.99	3,2
4	Agrowastes x Incubation time	1x6	57.02	1,2
5	Agrowastes x Yeast extract	1x3	48.94	1,3
6	Moisture level x Incubation time	2x6	36.27	1,2
7	Inoculum level x Incubation time	5x6	27.68	2,2
8	Agrowastes x L-asparagine	1x4	26.76	1,3
9	L-asparagine x Inoculum level	4x5	17.92	3,2
10	Moisture level x L-asparagine	2x4	12.55	1,3
11	Yeast extract x L-asparagine	3x4	12.32	3,3

Fig.2 Effect of Various Factors on enzyme production by *E. coli* K-12 (a-Agro-wastes; b-Moisture content; c-Yeast extract; d-L-asparagine; e-Inoculum level; f-Incubation time)
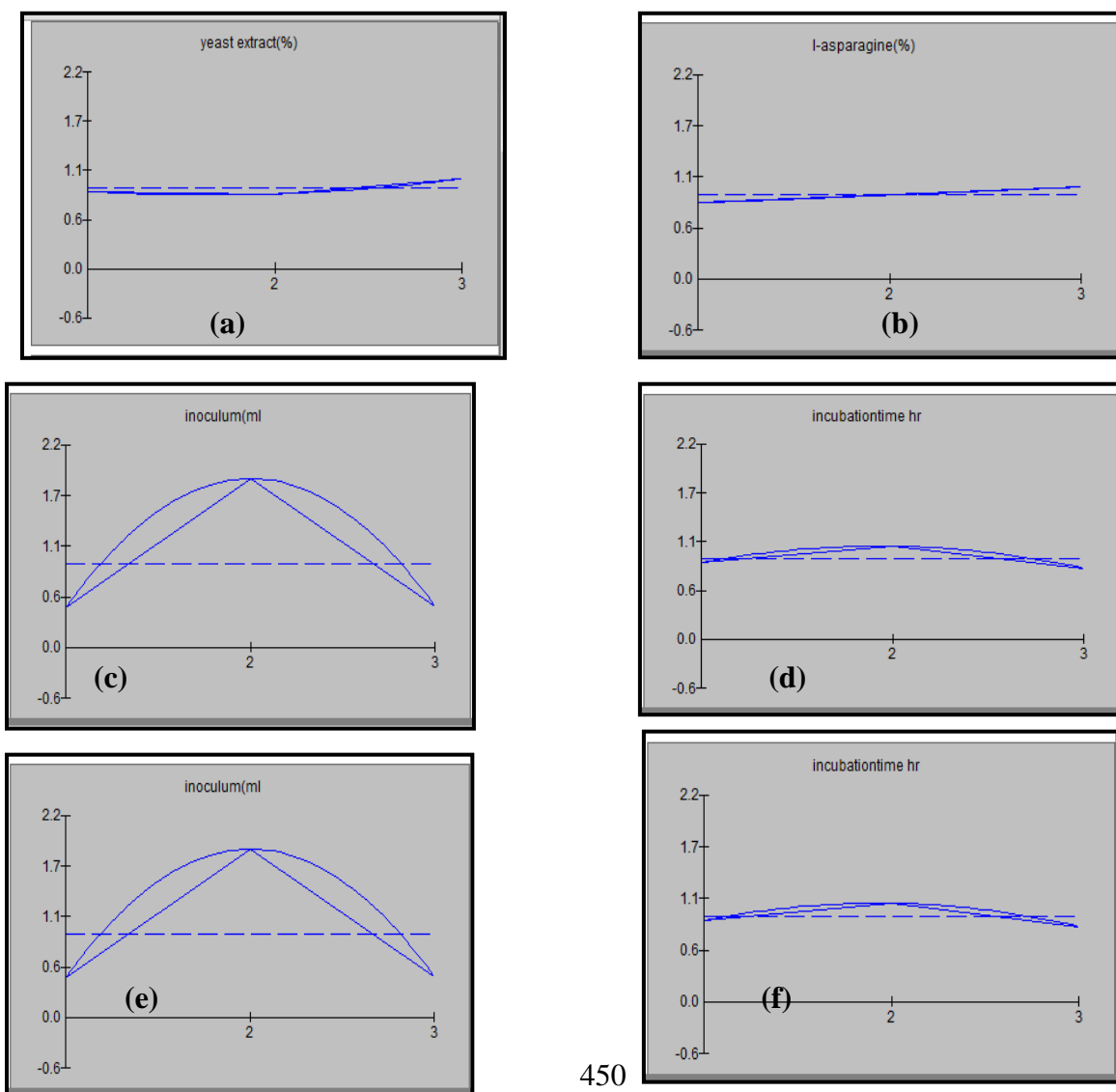
Table.3 Analysis of Variance (ANOVA)

S. No.	Factors	Sum of Squares	variance	F-Ratio	Pure sum	Percent
1.	Agrowastes	1.266	0.633	20.965	5.206	12.075
2.	Moisture level	5.171	1.085	92.834	9.11	24.112
3.	Yeast extract	3.103	0.951	71.717	6.043	21.43
4.	L-Asparagine	0.888	0.544	53.463	4.028	10.272
5.	Inoculum	4.999	1.009	80.424	7.939	23.01
6.	Incubation time	0.903	0.301	33.369	3.143	8.001
	Other errors					1.100
	Total	16.330				100.00%

Table.4 Optimum condition and Performance

S.No.	Factors	Level	Level Description	Contribution
1.	Agrowaste	1	Wheat bran	0.466
2.	Moisture level	1	80	0.964
3.	Yeast extract	3	1.25	0.706
4.	L-Asparagine	3	0.75	0.286
5.	Inoculum level	2	1.5	0.942
6.	Incubation time	2	24	0.146
	Total contribution from all factors			3.510
	Current Grand Average of			1.917
	Expected Result at Optimum			4.670

Fig.3 Percentage contribution of various factors on performance

Fig.4: Comparison of the enzyme yield
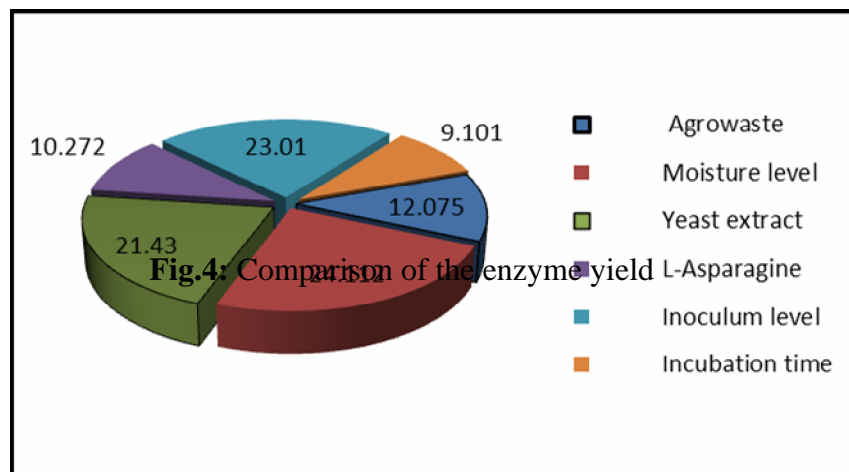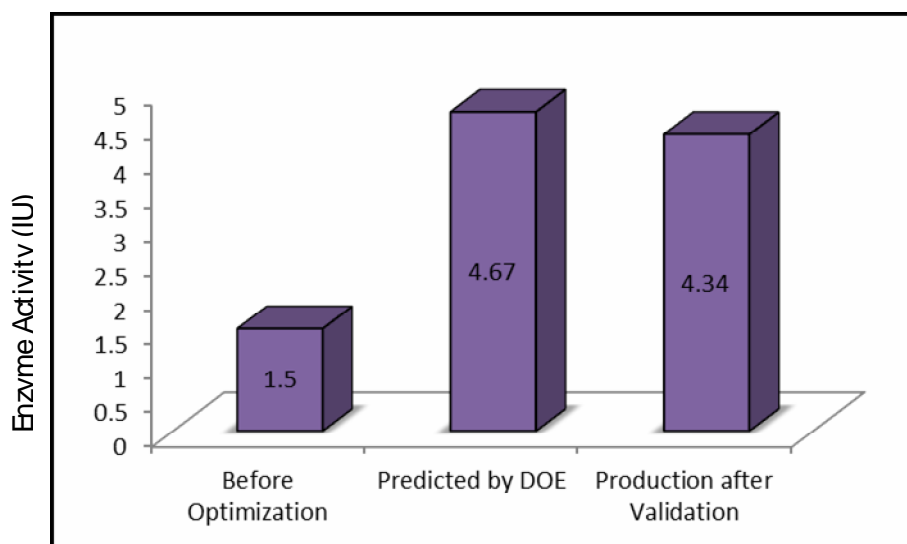
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