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Optimization of L-Asparaginase Production by Isolated *Aspergillus niger* using Sesame Cake in a Column Bioreactor

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Acute lymphocytic leukemia is a common leukemia characterized by frequent infections and anemia. Thousands of new cases are diagnosed each year worldwide. L-Asparaginase (E.C.3.5.1.1), also known as L-asparagine amino hydrolase, is a potential anti-tumor enzyme that catalyses the hydrolysis of L-asparagine into L-aspartic acid and ammonia. L-Asparaginase production was investigated in the isolated filamentous fungi on sesame cake using solid state fermentation (SSF). L-Asparaginase production using isolated *Aspergillus niger* grown on sesame cake has been optimized in a column bioreactor using a statistically-based method. The 23 BHH design having eight different fermentation conditions was applied to evaluate their significance on L-Asparaginase activity, where the three independent variables evaluated were Aeration, Thickness of Solid Substrate Bed and Fermentation Temperature. Aeration and Temperature were identified to be important variables and had a positive effect on responses, however, the thickness of solid substrate had an insignificant effect on L-Asparaginase activity within the tested range. The optimum fermentation condition for L-Asparaginase activity consisted of high aeration (0.4 vvm), deeper thickness of bed (22 cm) and high fermentation temperature (32°C), respectively. Under optimum levels, a maximum L-Asparaginase activity of 344.2132 IU was predicted which is slightly less than the activity at batch level.

Key words: L-Asparaginase; *Aspergillus niger*; Sesame cake; Solid-state fermentation, column bioreactor, Box Hunter & Hunter design.

L-Asparaginase, the enzyme which catalyses the hydrolysis of asparagine to aspartic acid and ammonia, is an important natural enzyme that possesses a broad spectrum of anti-tumor activity. It has been successfully applied to the treatment of cancers such as lymphocyte sarcoma and leukemia[1-3]. Literally called as L-Asparagine amino hydrolase E.C 3.5.1-1, the enzyme has been successfully applied to various applications. Clinical studies have demonstrated that the leukemia patients treated with L-Asparaginase have shown relatively higher recovery efficiency when compared to the recovery efficiency of leukemia patients treated with IL-2[4]. L-Asparaginase is an important chemotherapeutic

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agent used for the treatment of a variety of lymphoproliferative disorders and lymphomas, acute lymphoblastic leukemia (ALL) in particular. It has been a mainstay of combination chemotherapy protocols used in the treatment of pediatric ALL for almost 30 years[5]. Based on this reason, L-Asparaginase has also been included in most contemporary, multi-agent regimens for adult ALL[6,7]. The pioneering work by Clementi[8] in 1922, which also revealed the presence of high L-Asparaginase activity in the serum of guinea pig, has led to the development of L-Asparaginase as a potential antineoplastic agent. Interestingly, only guinea pigs have shown L-Asparaginase activity whereas other mammals have shown to be devoid of this enzyme. In 1953, Kidd[9] described the regression of transplanted lymphomas in mice and rats by the administration of guinea pig serum. Similar cytotoxic activity was not evident in horse or rabbit serum.

It was Broome in 1961, who compared Kidd's findings of growth inhibition with the earlier work of Clementi and succeeded in concluding that the antilymphoma activity in guinea pig sera was due to L-Asparaginase[10]. Further investigations by the same author confirmed its therapeutic potential[11]. Yellin and Wriston in 1966, succeeded in partial purification of two isoforms of L-Asparaginase from the serum of guinea pig[12]. As expected, only one isoform exhibited antilymphoma activity *in vivo*[13]. Since the extraction of this enzyme from the guinea pig serum in sufficient amounts was difficult, other sources such as microbes were explored. Mashburn and Wriston in 1964[14] and Campbell and Mashburn in 1969[15] reported the purification of L-Asparaginase from *E. coli*, and demonstrated its tumoricidal activity similar to that seen from guinea pig serum. Those findings provided a practical base for large-scale production of the enzyme for pre-clinical and clinical studies[16]. Tumor cells, more specifically lymphatic cells, require large amounts of asparagine for their rapid malignant growth. Normally, cancer cells get asparagine from the diet (blood serum) as well as by endogenous synthesis (which is very limited) to satisfy their large L-asparagine requirement. L-Asparaginase as a drug exploits this unique requirement of tumor cells by catalyzing the hydrolysis of L-Asparagine to L- Aspartic acid and ammonia (Fig. 1) there by depriving the cancer cell of the necessary L-Asparagine.

L-Asparaginase has also been produced by *Escherichia coli*, *Serratia marcescens*, *Erwinia carotovora* , *Pseudomonas acidivoras* and *P. geniculata* under aerobic conditions[17,18]. However, it is highly desirable to discover new types of L-Asparaginase that may be serologically different but possesses similar therapeutic effects. Normally, L-Asparaginase is produced by a submerged fermentation (SF) method[19-21]. However, the SF method is known to be a cost-intensive, highly problematic and poorly understood unit operation[22] and usually gives low yields of the product in addition to some problems such as handling, reduction and disposal of large volumes of water during down stream processing. In contrary, the solid state fermentation (SSF) process has been proved to be a very effective technique as the yield of the product is many fold higher when compared to that obtained in SF[23]. The SSF method also offers many other advantages such as resistance to contamination, ease of product extraction (i.e., does not require complicated methods of treating the fermented residue), etc. [24]. Also, in comparison with SF, SSF offers a better opportunity for the biosynthesis of low-volume-high cost products[25]. Our objective in this study therefore was to determine the best conditions for the production of extracellular L-Asparaginase from the isolated *Aspergillus niger* by taking advantage of SSF method, especially in resisting bacterial contamination, ease of purification and the use of cheap natural materials like Sesame cake.

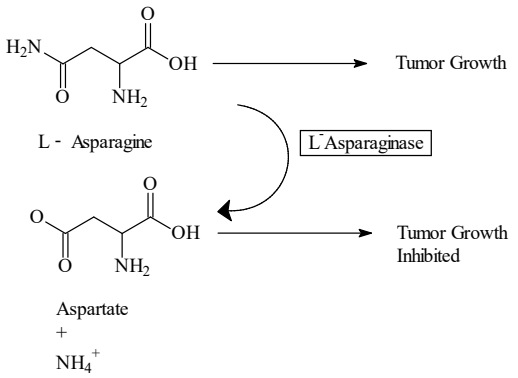

Fig. 1. Schematic representation of mechanism of action of L-Asparaginase

The objective of the present study was, therefore, to evaluate L-Asparaginase activity by isolated *A. niger* when grown using a semi-continuous system consisting of a column bioreactor holding the Sesame cake supplemented with nutrients. The physical fermentation parameters optimized were: aeration, thickness of solid substrate and temperature. The present study used a 23 Box, Hunter & Hunter (BHH) to identify, for each variable or physical fermentation parameter, the level optimizing L-Asparaginase activity and to evaluate the interactions between the variables.

MATERIAL AND METHODS

Microorganism

The fungal culture used in this study was isolated from soil sample collected from a small town named Bapatla in the state of Andhra Pradesh, India, using Czapek Dox agar by the serial dilution method. The inoculated agar plates were incubated at 30°C in an incubator. After purification, the culture was identified as *Aspergillus niger* by using standard microscopy (Light and electron Microscopy) and biochemical tests.

Assay for screening of L-Asparaginase activity

The screening methodology was based on Gulati et.al (1997) with the incorporation of phenol red in a stock solution prepared in ethanol with L-Asparagine incorporated in the medium for the selection of the microorganism that has the ability to produce L-Asparaginase[26].

Raw material

Local Sesame (Black) oil cake, after extracting the oil from sesame, was collected and grounded to obtain a coarse powder. The coarse powder thus obtained was passed through a sieve to get a homogeneous powder containing 0.6cm size particles.

Cultivation conditions and crude enzyme extraction

After isolation of the pure culture of *Aspergillus niger*, a stock culture of *Aspergillus niger* was grown on Potato dextrose agar slants, and was stored at 4°C. Cultivation was achieved by solid-state fermentation as reported previously[27]. Dry Sesame sample of 150 g (0.6 cm particle size) was placed in 0.5 l autoclave bags (Himedia, banglore) and wetted using 150 ml of 0.1 M sodium phosphate buffer (pH 6.5) with an initial pH adjusted to 6.5. Each wetted sesame sample was supplemented with nutrients, Fructose (3.98% w/w) and ammonium sulphate (3.19% w/w) and Asparagine (0.2% w/w)[28]. Once supplemented with nutrients and stimulators, the wet Sesame samples were autoclaved for 30 minutes at 121°C for thrice with an interval of 6 hrs. Once cooled to room temperature, all samples were inoculated with 3%v/w of inoculum containing 1.6×10^2 spores/ml for every 20g Sesame cake by thorough mixing with sterile glass rods under aseptic conditions. The prepared wet Sesame samples were removed from the autoclave bags before being placed in the column Bioreactors.

Experimental columns

The Batch fermentation with 150 g wet Sesame cake was carried out in a glass column bioreactor with the following dimensions: internal diameter 7 cm, height 23 cm and internal volume 0.89 l. The cylindrical bioreactor consisted of a 3 mm thickness Plexiglas with top and bottom made up of acrylic material. Non-sterile air was saturated with water by passing through humidifiers before it entered in to the bioreactor from the bottom. Outlets on the top and bottom allowed for air exhaustion and excess water drainage. After inoculation, the bioreactor was placed in an incubator for 7 days under sterile conditions.

The procedure was designed to evaluate the effect of the following fermentation parameters on L-Asparaginase activity: aeration level, thickness of Sesame bed and fermentation temperature. These parameters were varied simultaneously according to factorial design matrix. Glass column could hold up to 300 g of wet Sesame cake, wet Sesame depth tested corresponded to 20 ± 0.5 cm (150 g of wet Sesame). The effect of temperature was tested by incubating the column bioreactors for 7 days, in a closed chamber maintained at either 22 or 32°C. The effect of aeration was tested by bubbling humidified non-sterile air at the base of the reactors. The aeration rates tested were 0.2 and 0.4 vvm (volumes of air per minute per working volume of reactor). On day 7, the entire content of column bioreactor was harvested for

L-Asparaginase activity by adding 0.01 M sodium phosphate buffer (90 ml for 20 gms at pH 7.0) to the medium followed by centrifugation at 8000 rpm for 20 min. The cell-free supernatant was used as crude enzyme preparation.

Protein determination

The protein content was determined according to the modified Lowry's method[29].

Determination of L-Asparaginase activity

L-Asparaginase activity was assayed by quantifying the ammonia formation in a spectrophotometric analysis[30]. One mole of Ammonium sulphate liberates two moles of Ammonia. Based on this, a standard curve for Ammonia generation was prepared. One L-Asparaginase unit (IU) is defined as that amount of enzyme which liberates 1 µmole of ammonia per min under the optimal assay conditions.

Statistical Procedure

A 23 Box, HUNTER & HUNTER method was applied to determine the effect of fermentation parameters on L-Asparaginase by A. *niger grown* in column reactors under semi-continuous fermentation. The 23 BHH method needed that two levels of each parameter, where each high and low levels were coded +1 and –1. Thus, 8 combinations of fermentation conditions were tested. The design and coded values were shown in Tables 1 and 2. The performance of the 8 different conditions could be used to produce a behavior model expressed using the following equation, which included all linear and interaction terms:

$$Y = \beta_o + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{23} x_2 x_2 + \beta_{13} x_1 x_3 + \beta_{123} x_1 x_2 x_3$$

where Y = predicted response; β0, intercept; β1, β2, β3 are linear coefficients; β11, β22, β33 are squared coefficients and β12, β13, β23, β123 are interaction coefficients; STATISTICA 6.0 was used for the regression analysis of the experimental data obtained. All experimental designs were randomized to exclude any bias. Experiments were performed in duplicate and mean values are given.

Statistical analysis of the data

The significance and adequacy of the second-order equation was measured using analysis of variance (ANOVA), defined as "a method for estimating the amount of variation within all treatment and comparing it to the variables between treatments" (Berthouex and Brown, 1994)[31]. The data on enzyme activity was subjected to multiple linear regressions using STATISTICA 6.0 to estimate t -value, P -value and confidence level. The significance level (P -value) was determined using the Students t -test. The t -test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. Confidence level is an expression of the P - value in percent. Critical values of Variables are estimated using the STATISTICA 6.0 tools. The *t*-test was applied to evaluate the statistical significance of the model. High *t* values imply that models were significant and was possible accurately predict the experimental results. The importance of the *t* values was interpreted as a level of provability (*P),* where a level under 0.05 implies a level of confidence of over 95%. The coefficients of all linear terms of the model equation provided a measure of the effect of the level of the independent variable on the response[32].

RESULTS AND DISCUSSION

The measured L-Asparaginase activity obtained with the 8 different variable combinations of parameters were reported in Table 3. L-Asparaginase activity ranged from 77.088 to 350.984 IU. The highest level of production was obtained using the aeration rate of 0.4 vvm, the Sesame cake thickness of 22 cm and a temperature of 32°C. L-Asparaginase activity at 32°C was three times as high as those measured at 22°C. Aeration was another parameter having a major impact, as an air flow rate of 0.4 vvm increased L-Asparaginase activity by a factor of 3, as compared to no aeration. Also Cake thickness was the parameter which had the effect, as doubling its thickness increased L-

Asparaginase activity

The measured levels were used to generate a second-order equation for predicting L-Asparaginase activity, by computing the significant linear, squared and interaction coefficients. Neglecting the term, $x_1 x_2 x_3$, which was insignificant, the result of 23 BHH produced the following equation which describes the effects of the variables on the response. The equation shows that L-Asparaginase is a function of the coded levels of the three tested input variables.

Table 1. Full factorial design matrix for the evaluation factors effecting L-Asparaginase activity by column reactor

Run No	Aeration	Thickness of solid bed	Temperature	L-Asparaginase activity
1	-1.00000	-1.00000	-1.00000	134.4952
2	1.00000	-1.00000	-1.00000	97.7032
3	-1.00000	1.00000	-1.00000	77.088
4	1.00000	1.00000	-1.00000	97.0316
5	-1.00000	-1.00000	1.00000	142.4668
6	1.00000	-1.00000	1.00000	276.5532
7	-1.00000	1.00000	1.00000	105.996
8	1.00000	1.00000	1.00000	350.984

Table 2. Full factorial design matrix Design: Coded Values

Level	Aeration (vvm)	Thickness of solid bed (cm)	Temperature °C
+1	0.4	22	32
-1	0.2	11	22

Table 3. Full Factorial Design : Observed Vs. Predicted Values

Run NO	L-Asparaginase activity (observed)	L-Asparaginase activity Predicted		Residuals	
		From the BHH	From the Equation	From the BHH	From the Equation
1	134.4952	141.2660	134.495	-6.77	0.0002
2	97.7032	90.9325	97.7032	6.77	0
3	77.088	70.3173	77.088	6.77	0
4	97.0316	103.8024	97.0316	-6.77	0
5	142.4668	135.6961	142.467	6.77	-0.0002
6	276.5532	283.3239	276.553	-6.77	0.0002
7	105.996	112.7668	105.996	-6.77	0
8	350.984	344.2132	300.984	6.77	50

Table 4. BHH Design: Effect Estimates

Var.:Var4; R-sqr = 0.99466; Adj : 0.96261 2**(3-0) design; MS Residual=366.7444

	Effect	Std.Err.	t(1)	p	-95.%	+95.%
Mean/Interc.	160.2898	6.77075	23.673	0.0268	74.259	246.320
(1)Aeration	90.5565	13.5415	6.687	0.0944	-81.505	262.617
(2)Thickness of solid bed	-5.0297	13.5415	-0.371	0.5735	-177.091	167.031
(3)Temperature	117.4205	13.5415	8.671	0.0730	-54.641	289.481
1 by 2	41.9093	13.5415	3.094	0.1989	-130.152	213.970
1 by 3	98.9807	13.5415	7.309	0.0865	-73.080	271.041
2 by 3	24.0097	13.5415	1.773	0.3269	-148.051	196.070

The second-order equation used to predict those optimal fermentation conditions for best L-Asparaginase activity[33].

$$Y = -2.3015 + 0.6807x_1 - 0.0137x_2 - 0.0180x_3 + 0.0216x_1x_2 + 0.834x_1x_3 + 0.002x_2x_3$$

where the coefficients x_1, x_2 and x_3 correspond to the coded value of fermentation parameters such as aeration, thickness of solid substrate bed and incubation temperature.

The goodness of fit of the second-order regression equation was checked by determining its coefficient of determination (R2), where R measures the correlation between the measured and predicted L-Asparaginase activity. A value of 1.0 indicates perfect correspondence or goodness of fit between the two values. The R2 of equation 5 was found to be 0.99466 for L-Asparaginase activity, which indicates a good agreement between the quadratic model of BHH and the measured data. These values indicate that 99.46% of the variability in the response can be explained by equation 2.

The ANOVA procedure produced *t* value 23.67385 for L-Asparaginase activity, indicating a good fit between the equations and the actual values (Table 4). For the L-Asparaginase activity, ANOVA produced *P* levels under 0.09 for x_1 (aeration) and x_3 (temperature), indicating that these two variables have a highly significant effect, for the range tested [34-36].

Modeling the effects of the independent variables

The effect of the three independent variables on L-Asparaginase activity during semi-continuous fermentation were studied using equation 2 to plot response surface curves. The interaction between aeration and thickness of solid substrate was plotted for an incubation temperature fixed at 27°C. At a bed thickness of 22 cm, L-Asparaginase activity increased noticeably with increased aeration rate. However, at constant aeration rte, L-Asparaginase activity did not changed with a deeper solid substrate bed. As compared to a bed thickness of 22 cm, a fixed bed thickness of 11 cm demonstrated a less aeration effect. Under optimum levels, a maximum L-Asparaginase activity of 344.2132 IU was predicted. A similar response surface curve was obtained for L-Asparaginase activity, which also maximized at 12%, but varied little between bed thicknesses. Thus, thick beds of solid substrates need a forced aeration to support high L-Asparaginase activity. The effect of aeration has been intensively investigated by many researchers on citric acid production, who reported that the growth of fungi is aerobic, that forced aeration is indispensable for the over-production of citric acid (Abou-Zeid and Ash, 1984)[37]. The interactive effect of aeration and incubation temperature on L-Asparaginase activity and yield were plotted. At a high incubation temperature, aeration had a significant effect on increasing L-Asparaginase activity, at a constant solid substrate thickness of 16.5 cm, while at a low incubation temperature, aeration level had no significant effect. Thus, high L-Asparaginase activity rates obtained under the optimal incubation temperatures require forced aeration considering the effect of an incubation temperature.

As the cylindrical column bioreactor has a relatively small surface area compare to its bed volume, surface aeration leads to insufficient oxygen supply within the center of the solid substrate mass. At low incubation temperatures, cell growth and L-Asparaginase activity were lower and surface aeration suffices in supplying oxygen to the fungi. Plot the response surface curves L-Asparaginase activity as a function of the solid substrate thickness and incubation temperature. The two plots showed the dominant effect of the incubation temperature on L-Asparaginase activity and the limited effect of thickness. Optimum L-Asparaginase activity was achieved at an incubation temperature of 32°C and a bed thickness of 22 cm. The results also indicate that the statistically-based optimization procedure using a BHH method proved to be an effective method in optimizing fermentation conditions.

CONCLUSION

A Box, Hunter & hunter design was applied to the three chosen variables namely Aeration, Thickness of solid bed and the Temperature, as these are the most significant factors for the bioprocess in column reactor using a fungal species for the production of L-Asparaginase. The measured L-Asparaginase activity obtained with the 8 different variable

combinations of parameters are reported in Table 3. L-Asparaginase activity ranged from 77.088 to 350.984 IU. The highest level of production was obtained using the aeration rate of 0.4 vvm, the bed thickness of 22 cm and a temperature of 32°C. L-Asparaginase activity at 32°C was three times as high as those measured at 22°C. The goodness of fit of the second-order regression equation was checked by determining its coefficient of determination (R^2), where R measures the correlation between the measured and predicted L-Asparaginase activity. The R^2 of equation fitted from the experimental data was found to be 0.99466 for L-Asparaginase activity, which indicates good agreement between the quadratic model of Box, Hunter and Hunter design and the measured data. These values indicate that 99.46%. The results demonstrated that for L-Asparaginase activity by isolated *A. niger* , the semi-continuous fermentation process is slightly less activity than a batch process[28]. The values obtained from semi-continuous fermentation and other fermentation parameters need to be optimized by using sequential optimization for further improvement in L-Asparaginase activity.

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