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Research Paper

Statistical Optimization of Aspartase Production from *Aeromonas media* NFB-5 in a Stirred Tank Reactor

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Abstract

Optimization of process parameters for the production of aspartase by *Aeromonas media* NFB-5 was carried out at a laboratory scale bioreactor using response surface methodology (RSM). The effect of aeration (0.30-1.70, vvm), agitation (130-270, rpm) and incubation time (7-40, h) was studied on aspartase activity and biomass yield. The statistical assessment of the data was carried out by Fisher's statistical test for analysis of variance (ANOVA). The significance of each coefficient for aspartase production and biomass yield was determined by Fisher's F-test and P-values. Coefficient of determination of aspartase activity (R^2=0.9995) and biomass yield (R^2= 0.9973) depicted by the model was 99.95% and 99.73%, respectively of the total variation. At the same time, a relatively lower value of the coefficient of variation for aspartase activity (cv = 1.10%) and biomass yield (cv = 2.37%) indicated reliability of the conducted experiments. The model predicted optimal levels of agitation (250 rpm), aeration (1.30 vvm) and incubation time (24 h). Aspartase activity (171.42 U/g wet wt) and biomass yield (0.987 OD_{6005}) obtained under optimal conditions were in good agreement with the values predicted by statistical method.

Keywords: Aspartase, *Aeromonas media*, response surface methodology, process parameters, bioreactor

Aspartase (L-aspartate ammonia lyase, EC 4.3.1.1) catalyzes the reversible deamination of aspartate to produce fumarate and ammonia. The direction of reaction depends upon the pH of the medium, in alkaline pH L-aspartic acid is produced from fumaric acid and ammonia (Papierz *et al.*, 2007). Typically, aspartase is a bacterial enzyme extensively reported from *Escherichia coli*, *Pseudomonas flourescens*, *Bacillus cereus*, *Bacillus* sp. YM55-1, *B. staerothermophilus*, *B. subtilis*, *Propionibacterium* sp. and *Cytophaga* sp. KUC-1 (Singh and Yadav, 2012a). It is also reported from few yeasts, molds and some higher plants (Virtanen and Ellfolk, 1955). It has also been found in animal tissues, particularly in sharks and bony fishes (Salvatore *et al.,* 1965). Aspartase is intracellular and inducible in nature (Singh and Yadav, 2012a). It is the most specific enzyme known with extensive studies failing to identify any alternative amino acid substrate that can replace L-aspartic acid (Viola *et al.,* 2000).

Commercially, aspartase has gained importance due to its excellent catalytic efficiency for biological synthesis of L-aspartic acid which is commonly used in food and medicinal

industries. The aspartase producing cells and enzymatic preparations of aspartase are used for the industrial production of L-aspartic acid. To develop a new industrial bioprocess, optimization of production medium and process parameters at shake-flask and in a laboratory scale bioreactor are essential. At the bioreactor level aeration, agitation and incubation time strongly affect the production of biomass and product. The classical method of *one-variable-at-a-time* used for optimization of medium constituents and process parameters is very time-consuming and by this method, the effect of interactions among different variables could not be determined. Application of statistical and mathematical methods for experimental design allows adequate mathematical modeling on the basis of analysis and also decreases the number of experiments. Further, mathematical processing of experimental data allows prediction of values and conditions for targeted change in parameters and criteria (Bagdasaryan *et al.*, 2004). Response surface methodology (RSM) is a statistical strategy consisting of a particular set of mathematical and statistical methods. RSM can be used to establish the optimal levels of the primary ingredients and process parameters. This optimization procedure has been found to be time saving and cost effective. The present study reports the production of aspartase from *Aermonas media* NFB-5 at bioreactor level. Optimization of process parameters including agitation, aeration and incubation time at bioreactor level have been performed to obtain the maximal aspartase production. This is the first report of optimization of process parameters for enhanced production of aspartase from *Aeromonas media* NFB-5 at bioreactor level.

Materials and Methods

Bacterial strain and cultivation conditions

A. media NFB-5, an isolate from effluent of a fertilizer industry was used for aspartase production. The strain was maintained and cultivated as described previously (Singh and Yadav, 2012b).

Aspartase production at laboratory scale bioreactor

Aspartase production was carried out in a laboratory scale bioreactor (1L, Biolab, B. Braun, Germany). The bioreactor was equipped with a ruston turbine impeller with six blades and automatic control of aeration, agitation and temperature. Inoculum preparation and production media used for aspartase production was the same as described earlier (Singh and Yadav, 2012b). Production media was inoculated with 10% (v/v) inoculum under aseptic conditions. Sterilized silicon antifoaming agent (0.002%, w/v) was added at the beginning of fermentation to control foam formation.

Experimental design and response surface analysis

Response surface methodology was used to obtain the optimal levels of process parameters for aspartase production. Experimental runs were designed using Design Expert version 7.1.2 (State-Ease Inc., Minneaopolis, MN, USA). Three independent variables (Agitation, A; Aeration, B and Incubation time, C) were optimized using central composite rotatable design (CCRD) of RSM. Each variable was studied at five coded levels i.e. -1.821, -1.000, 0.000, 1.000, 1.821 as shown in Table 1. Aspartase activity was taken as desired response. Since aspartase is an intracellular enzyme and its production depends on biomass yield (Singh and Yadav, 2012c), therefore, biomass yield was considered as second targeted response. To study the proposed second order polynomial model, CCRD was used with linear regression to estimate the model coefficients. Analysis of variance (ANOVA) was performed to evaluate the statistical significance and accuracy of the model. Optimal combination of process parameters was determined on the basis of graphical and numerical analysis.

Aspartase assay

Aspartase activity was determined as described earlier (Singh and Yadav, 2012c). Briefly, increase in absorbance due to production of fumarate was measured spectrophotometrically at 240 nm. Standard assay mixture contained Tris-HCl buffer (0.5 M, pH 8.8), sodium L-aspartate (0.5 M), $MgCl_2$ (0.1 M) and appropriate amount of crude enzyme in a total volume of 2 ml. The reaction was carried out at 30ºC in a UV-Visible Spectrophotometer (Pharmaspec 1700, Shimadzu, Japan) equipped with a Peltier-type cell temperature control unit. One unit of aspartase was defined as the amount of enzyme producing 1µmol of fumaric acid per minute. Molar coefficient of 2.53×10^3 $M^{-1}.cm^{-1}$ was used to determine the aspartase activity (Tokushige, 1985). Aspartase activity was expressed in units per gram of wet weight (U/g wet wt).

Determination of Biomass

Biomass yield was determined by taking the optical density of the cultured broth at 600 nm using UV-visible spectrophotometer (Pharmaspec 1700, Shimadzu, Japan) against the harvested cell free extract as blank. Cultured

broth was diluted five times with cell free extract to determine the optical density.

Results and Discussion

Initial design and mathematical modelling

A five coded level with CCRD (Table 1) was employed to optimize the levels of agitation, aeration and incubation time in submerged batch cultivation of *A. media* NFB-5 in a laboratory scale bioreactor. Second order quadratic design model was adopted to execute the experimental runs. A total of 15 experiments with appropriate combinations of the variables were conducted. The experimental design and the results obtained for aspartase activity and biomass yield are presented in Table 2. The experimental results of the CCRD were fitted with a second order polynomial equation. Linear regression was used for the prediction of results.

Second order polynomial equation and improvement of model

The values of regression coefficients were calculated and the fitted equations (in terms of coded values) for predicting aspartase activity (X) and biomass yield (Y) are as given below, regardless of the significance of the coefficients:

$$X = + 154.19 + 14.23 * A + 17.86 * B + 3.03 * C - 4.48 * A * B + 2.82 * A * C - 5.81 * B * C - 0.68 * A^2 - 9.77 * B^2 - 41.44 * C^2 \quad (1)$$

$$Y = + 0.89 + 0.073 * A + 0.083 * B + 0.25 * C + 0.054 * A * B + 0.076 * A * C + 0.033 * B * C - 0.050 * A^2 - 0.037 * B^2 - 0.14 * C^2 \quad (2)$$

Where A is agitation; B, aeration and C, incubation time. The statistical significance of equation 1 and 2 was checked by ANOVA for response surface quadratic model and is summarized in Table 3.

Table 1: Values of coded levels used for the experimental design in optimization of process parameters.

Factors	Symbols	Actual levels of coded values				
		-1.821	-1.000	0.00	1.000	1.821
Agitation (rpm)	A	130	150	200	250	270
Aeration (vvm)	B	0.30	0.50	1.00	1.50	1.70
Incubation time (h)	C	7.00	12.00	24.00	36.00	40.00

Table 2: Central composite rotatable design consisting of various experiments for study of 3 experimental factors in actual units and results.

Run No.	Factors[a]			Experimental results		Predicted results	
	A	B	C	Aspartase activity (U/g fresh wt)	Biomass ($OD_{600/5}$)[b]	Aspartase activity (U/g fresh wt)	Biomass ($OD_{600/5}$)[b]
1.	250	0.50	36.00	113.87	0.901	114.82	0.896
2.	200	1.00	40.00	76.56	0.943	75.61	0.965
3.	200	1.00	24.00	153.81	0.897	154.19	0.902
4.	150	0.50	12.00	58.77	0.438	59.72	0.447
5.	150	1.50	36.00	103.87	0.833	104.82	0.823
6.	250	1.50	12.00	128.91	0.531	129.86	0.527
7.	200	1.00	24.00	153.81	0.897	154.19	0.902
8.	200	1.70	24.00	160.85	0.923	159.90	0.941
9.	270	1.00	24.00	143.91	0.883	142.96	0.908
10.	200	0.30	24.00	110.34	0.689	109.39	0.710
11.	200	1.00	07.00	67.98	0.249	67.03	0.266
12.	200	1.00	24.00	153.81	0.897	154.19	0.902
13.	200	1.00	24.00	153.81	0.897	154.19	0.902
14.	129	1.00	24.00	133.67	0.676	132.72	0.697
15.	200	1.00	24.00	153.81	0.897	154.19	0.902

[a] Symbols A, B, C are the same as mentioned in Table 1.
[b] Sample was diluted five times for determination of OD.

Table 3: Analysis of variance (ANOVA) for response surface quadratic model obtained from experimental design.

Source[a]	Aspartase activity			Biomass		
	Sum of squares	DF	Prob.>F	Sum of squares	DF	Prob.>F
Model	18642.98	9	<0.0001	0.62	9	< 0.0001
A	809.63	1	<0.0001	0.021	1	0.0005
B	1275.63	1	<0.0001	0.027	1	0.0003
C	36.81	1	0.0074	0.24	1	< 0.0001
A^2	3.52	1	0.2371	0.019	1	0.0006
B^2	736.83	1	<0.0001	0.010	1	0.0025
C^2	13244.76	1	<0.0001	0.16	1	< 0.0001
AB	40.17	1	0.0062	5.857E-003	1	0.0085
AC	15.94	1	0.0355	0.012	1	0.0019
BC	67.47	1	0.0020	2.170E-003	1	0.0510
Residual	9.77	5		1.663E-003	5	
Lack of fit	9.77	1		1.663E-003	1	
Pure error	0.000	4		0.000	4	
Core total	18652.75	14		0.62	14	

[a] Symbols A, B, C are the same as mentioned in Table 1.

Table 4: Regression coefficients and significance of quadratic model for aspartase activity and biomass yield.

Model[a]	Aspartase activity			Biomass yield		
	Coefficient estimate	Standard error	F value	Coefficient estimate	Standard error	F value
Intercept	154.19	0.60	1060.54	0.89	7.847E-003	207.31
A	14.23	0.70	414.51	0.073	9.118E-003	64.43
B	7.86	0.70	653.10	0.083	9.118E-003	82.34
C	3.03	0.70	18.85	0.25	9.118E-003	724.22
A^2	-0.68	0.50	1.80	-0.050	6.565E-003	58.15
B^2	-9.77	0.50	377.24	-0.037	6.565E-003	31.44
C^2	-41.44	0.50	6781.05	-0.14	6.565E-003	466.58
AB	-4.48	0.99	20.57	0.054	0.013	17.61
AC	2.82	0.99	8.16	0.076	0.013	35.18
BC	-5.81	0.99	34.54	0.033	0.013	6.52

[a] Symbols A, B, C are the same as mentioned in Table 1.

The coefficient estimates of equation 1 and 2 are presented in Table 4. The quadratic model in equation 1 and 2 contain three linear, three quadratic and three two-factorial interactions. Values of "Prob > F" less than 0.0500 indicates that model terms are significant (Pan *et al.* 2010; Ottoni *et al.* 2012). For aspartase activity, A, B, C, AB, AC, BC, B^2 and C^2 are significant model terms. In case of biomass yield, model terms A, B, C, AB, AC, A^2, B^2 and C^2 are significant. Insignificant terms (on the basis of "Prob > F" values, which are more than 0.0500 for each response) were neglected (Table 3) and model equation 1 and 2 were modified to reduced fitted model equation 3 and 4:

$$X = + 154.19 + 14.23 * A + 17.86 * B + 3.03 * C - 4.48 * A * B + 2.82 * A * C - 5.81 * B * C - 9.77 * B^2 - 41.44 * C^2 \quad (3)$$

$$Y = + 0.89 + 0.073 * A + 0.083 * B + 0.25 * C + 0.054 * A * B + 0.076 * A * C - 0.050 * A^2 - 0.037 * B^2 - 0.14 * C^2 \quad (4)$$

Statistical significance of the design

The model F-value of 1060.54 and 207.31 for aspartase activity and biomass yield, respectively implies that the model is significant (Table 4). "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable

(Rajasimman and Subathra, 2009). Ratio of 99.235 and 46.612 for aspartase activty and biomass yield, respectively indicates an adequate signal. The models can be used to navigate the design space. The lack of fit value relative to pure error are shown in Table 3. Lack of fit value indicates that the obtained experimental data fitted well with the model.

Analysis of variance (ANOVA) and accuracy of the design

ANOVA indicated R^2 value of 0.99 for aspartase activity. This value showed satisfactory adjustment of the quadratic model to the experimental data and indicated that this model could explain 98% response variability. R^2 value obtained for biomass yield was 0.9973 which also suggested the satisfactory adjustment of the quadratic model to the experimental data. R^2 value for both aspartase activity and biomass yield are very close to one which indicates an excellent correlation between predicted and experimental values. The "Predicted R-squared" is in good agreement with "Adjusted R-squared" value for both aspartase activity and biomass yield. This reflects applicability and accuracy of the CCRD used for the experimental design (Sawale and Lele, 2009). The value of CV for aspartase activity and biomass yield was 1.10% and 2.37%, respectively. Low CV values indicate that deviations between experimental and predicted values are low (Rajasimman and Subathra, 2009).

Effect of process parameters on aspartase production and biomass yield

The response surfaces based on the final model were obtained by holding one variable at its middle level while varying the other two within their experimental range. The response surfaces for aspartase production and biomass yield depict interaction among aeration, agitation and incubation time (Fig. 1-3) which affect production of aspartase and biomass yield as well. Aspartase activity increased considerably when agitation was increased up to 200 rpm with an appropriate combination of aeration (Table 2; experimental run No. 3 and 8). Thereafter, an increase in agitation resulted in decreased aspartase activity (Table 2). This decrease may be due to shear stress, oxidative stress, disruption and physiological disturbance of cells (Açikel *et al.*, 2011; Abdullah *et al.* 2011). Adverse effect of higher agitation on production of penicillin G acylase has been reported (Kheirolomoom *et al.*, 2001). Agitation below 200 rpm also resulted in loss of aspartase activity (Table 2; experimental run No. 14) which was probably due to improper mixing of air and lack of homogeneous suspension of fermentation medium constituents. Improper balance of agitation and aeration negatively affected aspartase activity. This can be concluded from experimental run No. 5 (Table 2) where a higher aeration rate was used with lower agitation rate. Decreased aspartase activity was observed in this experimental run.

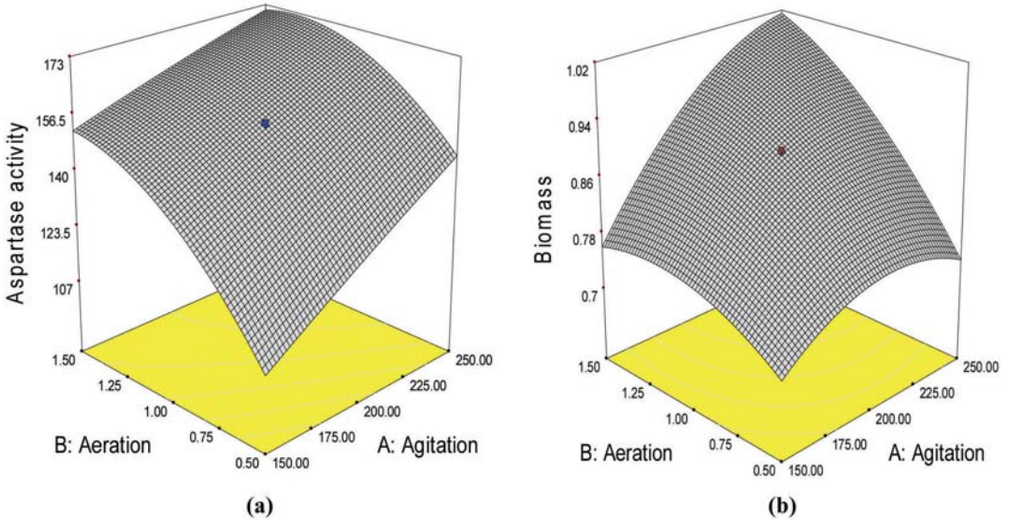

Fig. 1: The combined effect of aeration and agitation, as predicted by response surface, on (a) aspartase activity and (b) biomass yield.

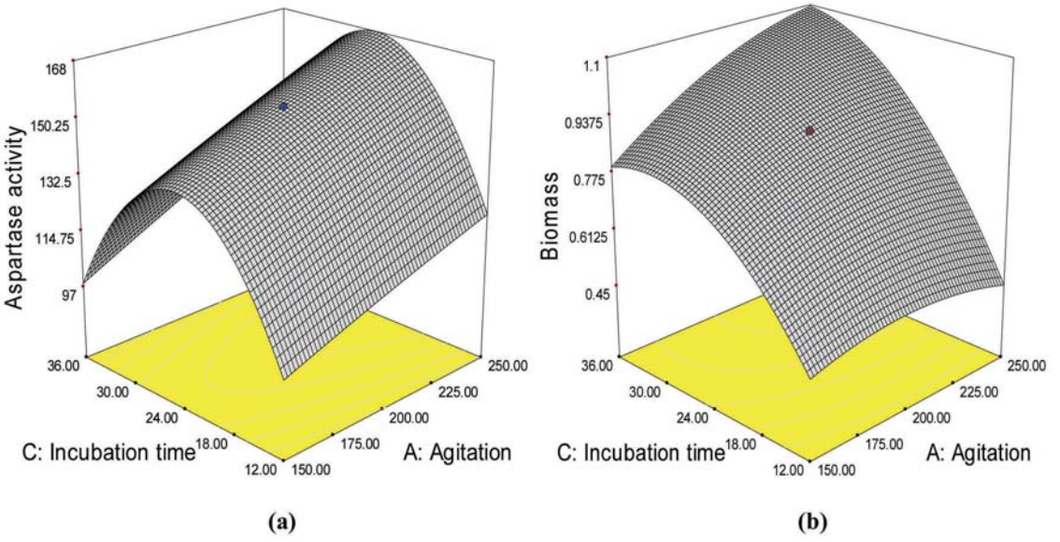

Fig. 2: The combined effect of incubation time and agitation, as predicted by response surface, on (a) aspartase activity and (b) biomass yield.

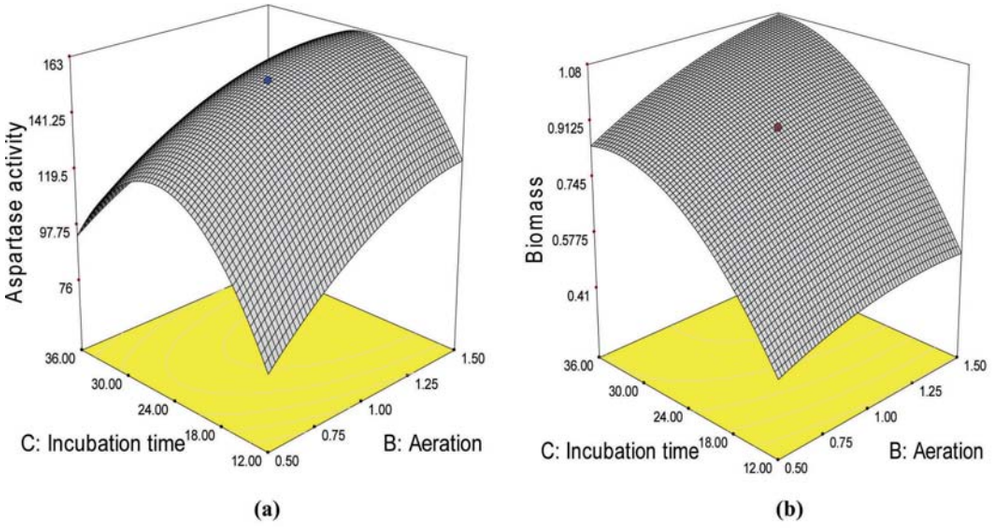

Fig. 3: The combined effect of incubation time and aeration, as predicted by response surface, on (a) aspartase activity and (b) biomass yield.

The lower agitation rate with higher aeration results an increased air flow along the stirrer shaft resulting the formation of an air column surrounding the impeller (Doran, 1995; Nadeem *et al.* 2009; Singh and Yadav, 2013). Flooding of impeller by air column does not allow proper contact of impeller with the nutrient medium resulting in poor mixing of nutrients and air (Doran, 1995; Nadeem *et al.*, 2009). Results of the present study and information given in literature suggests that balanced agitation and aeration is a critical parameter for homogenous distribution of air and media constituents and also for preventing the cell clumps formation (Kheirolomoom *et al.*, 2001; Abdullah *et al.*, 2011). Experimental run No. 2 and 11 (Table 2) indicate the effect of incubation time in combination with agitation and aeration. In these runs, agitation and aeration rates were similar as in experimental run No. 3 but

Table 5: Predicted values *vs.* experimental values for maximum aspartase activity and biomass yield

Variable	Aspartase activity (U/g fresh wt)		Biomass yield($OD_{600/5}$) [a]	
	Predicted value	Experimental value	Predicted value	Experimental value
Agitation 250 (rpm) Aeration 1.30 (vvm) Incubation 24.00 (h)Time	172.341	171.421	0.993	0.987

[a]Sample was diluted five times for determination of OD.

incubation time was different. Significant variation in aspartase activity was observed in these runs. Much poor aspartase activity was observed in both the cases when incubation time was very low and very high. Though increased incubation time with aeration beyond a value adversely affected the aspartase activity, but there was a considerable increase in biomass (Fig. 3).

Experimental validation of the model

Aspartase activity and biomass production as predicted by the final quadratic model along with the corresponding observed values are given in Table 5. Comparison of these values indicated excellent agreement between the predicted and experimental data. The location of optimum, obtained by differentiation of the quadratic model, for achieving maximum aspartase activity and biomass production was agitation 250 (rpm), aeration 1.30 (vvm) and incubation time 24 (h). The predicted optimal aspartase activity and biomass yield obtained with these values were 172.34 (U/fresh wt) and 0.993 ($OD_{600/5}$), respectively. To confirm the accuracy of the model, an additional experiment using the optimized values of process parameters was performed. The experiment resulted in maximum aspartase activity and biomass production of 171.42 (U/g fresh wt) and 0.987 ($OD_{600/5}$), respectively. A good agreement between the predicted and experimental values verifies the validity of the model as well as the existence of optimal point.

Conclusion

The optimal levels of agitation (250 rpm), aeration (1.30 vvm) and incubation time (24 h) at laboratory scale bioreactor resulted in 171 U (per g wet wt) of aspartase as compared to 136 U (per g wet wt) at shake-flask level with the same medium constituents. Aeration, agitation and incubation time were found to affect the fermentative production of aspartase in an interactive manner. This study will provide important insight to execute further investigations on L-aspartic acid production for scale-up studies.

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