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OPTIMIZATION OF EXPERIMENTAL VARIABLES FOR THE PRODUCTION OF α- AMYLASE BY ASPERGILLUS ORYZAE USING RICE BRAN

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ABSTRACT

This investigation is made to optimize the experimental parameters for the production of α- amylase by Aspergillus oryzae MTCC-8624 on using rice bran as a cheaper substrate. pH, temperature, fermentation time, inoculum concentration and substrate using a Box–Behnken design under the response surface methodology. The optimum values of experimental parameters are found to be pH- 4.7; Temperature - 36.5^{o}C; Fermentation time - 66.6hr; Inoculum concentration - 5.7% and Substrate concentration - 18.9 g/L. At these optimized conditions maximum amylase activity is found to be 16.89 U/ml.

Key words: Alpha amylase, Aspergillus Niger, Rice Bran, Optimization, Plackett-Burman Design, Submerged fermentation

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1. INTRODUCTION

The potential application of amylase family enzymes is paramount in number of industrial processes such as bread making, brewing, starch processing, textile, paper industries [1, 2]. a-Amylases are extra-cellular enzymes that breaks down the 1, 4-α-D-glucosidic linkages between adjacent glucose units in the linear amylose chain. Also, an important fact is,

amylases are used to treat digestive disorders, cystic fibrosis and pancreatitis when a therapeutically effective amount of it is administered optionally together with a lipase. Vasudeo., [1] demonstrated that Aspergillus oryzae has the potential to utilize agricultural waste for production of enzyme. The investigation reports the glucoamylase productive ability of rice husk, wheat bran, rice bran, cotton seed powder, corn steep solid, bagasse powder, coconut oil cake, groundnut oil cake, corn steep liquor and soybean meal using Aspergillus oryzae. Further it is found that the optimized mineral supplements for all the substrates employed in this investigation are 50 – 110 % by v/m - initial moisture content; 1-10%, v/w - inoculum size; 0.25% w/w - inorganic nitrogen source; 1 % w/w- organic nitrogen source; 1 % - carbon at a pH of 3-9 and at a temperature of 20 - 400 ^{o}C. Wheat Bran inferred the highest enzyme production (1602U/gdfs) followed by Rice Bran (1271 U/gdfs).

The results are compared with earlier investigation done by Anto et al [2]., Kaur et al [3]., and Pandey et al [4]., Singh and Soni., [5]. The performance of Rice husk and cotton seed powder on enzyme yield are found to be the same as 875 U/gdfs. Enzyme production is found to be less with Bagasse Powder, Coconut Oil Cake, Coconut Oil Cake and Corn Steep Solid. Shivaramakrishnam et al., [6] reported that the agro residues for glucoamylase production from A. oryzae and obtained maximum production with wheat bran and significantly good production with oil cakes.

The investigation by Vasudeo., [1] reports the glucoamylase activity by A. oryzae of 1986 U/gdfs. It is achieved in 120 h at 30^{o}C on a wheat bran substrate having an initial moisture content 100% at a pH of 5.0, an inoculum level of 5% (v/w), and soluble starch (1% w/w) and urea (0.25%) as supplements in a per 10 g substrate. Mohamed and Hoda., [7] investigated the growth of nine Aspergillus and three of Trichoderma strains on wheat bran for production of amylase under solid state fermentation. The investigation reports that the *Aspergillus oryzae* has the highest level on amylase enzyme production. The maximum enzyme production is found to be of about 14249 IU/g WB under optimum conditions with an incubation period of 120 h, an initial moisture content of 54.5% and in the presence of sucrose (1 g/100g WB) at 30^{o}C. Of substrates tested, soluble starch was the best one hydrolysed by the crude enzyme. Corn starch, dextrin and potato starch were also hydrolysed to a lesser extent.

The enzyme exhibited maximum activity at 55^{o}C. Moreover, the enzyme was also able to hydrolyze wheat flour under optimized conditions with efficiency of 89%. The reports are compared with earlier investigation by Elegado and Fujio [8]., and Ghose et al [9]., Tada et al [10]., and Lachmund et al [11]. Elsa and Valentin., [12] explain the α - amylase production by mangrove fungi *Pestalotiopsis microspora* VB5 and *Aspergillus oryzae* strain VB6 at optimum pH of 6.4 and 6 respectively. This pH level is compared with the earlier investigation done by Ramachandran et al [13]., Negi and Banerjee [14], Chi et al [15]., Alamri [16] and Monga et al [17]., for the production of α- amylase. Mouna et al [18]., isolated *Aspergillus oryzae* strain from old sweet soy sauce to produce two extracellular α-amylases.

Extraction parameters are studied to improvement the extraction of -amylase production in submerged cultivations of A. oryzae CBS 819.72 strain by Radhouane et al., [19]. The research findings inferred that optimization of enzyme extraction from fermentation broth is an important tool to reduce costs of downstream processing. Radhouane et at., [20] demonstrated the optimization of A. oryzae culture conditions and media composition by a factorial experimental design leading to a substantial increase in a-amylase production yield.

The results clearly indicate that phosphate and magnesium play an important role on the enzyme expression. The produced amylase activity reaches 148 U/ml and 5920 U/g of gruel at an optimum condition. The results show a close concordance between the expected and obtained activity level. Optimization of experimental variables is of great importance in the

development of fermentation process, due to its influence on the economy and practicability of the process. Conventional optimization method comprises the single parameter variation, keeping the other parameters constant. This method is unfit for multiple experimental parameters due to the paramount error. Apart from that the interactive effect between the experimental parameters cannot be investigated [20, 21, 22]. On the other hand, Statistical design is the promising one to overcome such a limitation. It infers the anatomy of interaction effect between the essential experimental variables [23, 24, 25].

Analysis on literature infers that, the application of *Aspergillus oryzae* species is reported to be the non-toxigenic and non-pathogenic organism. It is extensively applied in traditional fermentation industries, including soy sauce, sake, bean curd seasoning, miso, shochu, and vinegar production. Further it is noticed form the literature, *Aspergillus oryzae* is easy to cultivate. Also it is known to be good producers of glucoamylase and alpha-amylases used in starch processing, brewing, and more importantly, bread making processes.

Considering the promising opportunities that Aspergillus oryzae species, the present study is aimed to investigate the production of α- amylase production using Aspergillus oryzae from rice bran as cheaper substrate through submerged fermentation. Using the statistical tool, Central Composite Design (CCD), the effect of individual and interactive effects of experimental variables such as pH, temperature, fermentation time, inoculum concentration and substrate concentration on the α- amylase production using Aspergillus oryzae from rice bran through submerged fermentation the are aimed to investigate.

2. MATERIALS AND METHODS

2.1. Chemicals Used

All the reagents used in this study were of analytical grade. Solutions are prepared using deionised distilled water.

2.2. Microorganism and Maintenance

The strain of *Aspergillus oryzae* MTCC-8624 is purchased from the MTCC, Institute of Microbial Technology (IMTECH), Chandigarh, India. It is maintained on potato dextrose agar slants at 4^{o}C [26]. The culture is initially screened for amylase production by starch agar plate assay on standard media [27]. The inoculated plates, containing media, supplemented with starch are stained with Gram's iodine reagent, after 72 hr of incubation. The plates are then flooded with iodine solution for 15 minutes and washed with warm water to remove the excess colour.

2.3. Inoculum Preparation

Inoculum is prepared by transferring 2-mL of 72-h old slant culture in 100 mL of medium (250 mL Erlenmeyer flask) composed by: glucose – 20 g/L; KH_2PO_4 = 2.69 g/L; MgSO4 = 1.70 g/L; $CaCl_2$ = 0.53 g/L; $FeSO_4$ = 0.5 g/L, $(NH4)_2SO_4$ = 4.95 g/L and mycological peptone - 3.0 g/L at pH 5. The culture is incubated at 25^{o}C for 3 days at a rotation speed of 230 rpm.

2.4. Fermentation Medium

Rice Bran is utilized as substrate in this investigation. It is collected from nearby areas of Chidambaram, Tamilnadu, India. The collected substrate is dried by keeping them in oven at 80^{o}C for 12 hr. subsequently it is powdered in a laboratory grinder and sieved using a 40mm sieve [5]. Passable amount of this powdered substrate is mixed with 100ml of the corresponding mineral salt media in a 250ml Erlenmeyer flask. The pH is adjusted to 5. The mixture is sterilized in an autoclave at 121^{o}C and 15 psi pressure for 15 minutes. Then it is

cooled to the room temperature. Proper volumes of inoculums are added with this flask [14]. All the experiments for media optimization are carried out with a substrate concentration of 20g/L, inoculum size of 5% (v/v) and fermentation time of 72 hr. The pH and temperature are maintained at 5 and 25^{o}C [23].

2.5. Amylase Extraction

The contents of the flask are filtered using a Whatman No.44 filter paper followed by filtration through a muslin cloth, at the end of the fermentation period. The filtrate is then centrifuged at 10,000 rpm for 10 min and the supernatant was used as the source of enzyme for assay [18].

2.6. Estimation of Amylase Assay

Estimation of amylase activity is done by measuring the amount of reducing sugar according to the DNS method [28, 29]. It is strong minded by incubating a mixture of 1 ml of aliquot of each enzyme source and 1% soluble starch dissolved in 0.1 M phosphate buffer, at pH 7, at 55oC for 15 min. The reaction is stopped by adding 1 ml of 3, 5 DNS Acid and then followed by boiling for 10 min. The final volume is made up to 12 ml with distilled water and the reducing sugar released is measured at 540 nm. One unit of amylase activity is defined as the amount of enzyme that releasing 1µmol glucose equivalent per minute under the assay conditions. Reducing sugar concentration is determined from a standard curve under same condition using glucose [18]. Figure 1 shows the calibration chart for glucose concentration using Bio-spectrophotometer.

Dry cell mass of the fungal culture is determined by filtering the culture broth through a pre-weighed What man No. 44 filter paper. Mycelia is carefully washed with distilled water and dried in oven at 105°C for 2 hr. The Equation 1 is used to obtain the dry cell mass by subtracting the initial weight from the final weight and represented as g/L.

$$M_d = \frac{M_f - M_i}{V} - - - -\rightarrow (1)$$

where, Md is the dry cell mass (g/L), Mf is the final mass of filter paper with dried mycelium (g), Mi is the initial mass of the filter paper (g) and V is the volume of fermentation media (L).

2.7. Determination of Starch

For the determination of starch, 0.2g of the homogenized sample is initially treated with 80% ethanol to remove sugars. Centrifuge the mixture and the residue collected is repeatedly washed with 80% hot ethanol till the washing does not give colour with an throne reagent. To the residue, 5ml of distilled water is added, cooled in ice water bath followed by addition of 6.5 ml 52% perchloric acid with occasional stirring [30]. After 20 min 20 ml of water is added, centrifuged and collected the supernatant. The extraction process is repeated using fresh perchloric acid and the collected supernatant is made up to 100 ml. The extract is then filtered and stored at 0^{o}C. Pipetted out 0.2 ml of the filtered supernatant and made up to 1 ml with water in a test tube. 4 ml of anthrone reagent is added and the reaction mixture is kept in boiling water bath for 8 min. The contents are cooled and the intensity of green colour is recorded at 630 nm [31, 32].

3. RESULTS AND DISCUSSION

The process parameters pH, temperature, fermentation time, inoculum concentration and substrate concentration for amylase production using A. oryzae MTCC-8624 were optimized

with substrate rice bran using CCD. The actual and coded values of the process parameters selected for A. oryzae MTCC-8624 are given in Table 1. The 52-run design matrix using the five independent variables with the experimental and predicted responses is shown in Table 2.

The second order polynomial Equation 2 for amylase production (Y) for A. oryzae MTCC-8624utilizing rice bran as substrate is obtained as follows:

$$Y = 16.0267 - 0.11165A + 0.0381942B - 0.50028C + 0.15453D + 0.123293E - 0.36812A2 - 0.580252B2 - 0.580252C2 - 0.227583D2 - 0.571413E2 - 0.404062AB + 0.538438AC - 0.224062AD + 0.254062AE + 0.152813BC - 0.0334375BD - 0.166563BE + 0.0790625CD + 0.0046875CE - 0.156562DE - - - -\rightarrow (2)$$

where A - pH; B - Temperature (^oC); C - Fermentation time (hr); D - Inoculum concentration (%) and E - Substrate concentration (g/L).

The estimated parameter and the corresponding P-values are shown in Table 3 for rice bran. From the data the terms except B, BD, CD and CE were found to be influential on the production of α-amylase. Here the R^2 value of 0.9697 indicates a good agreement between the experimental and predicted values for rice bran respectively. The R^2 predicted values of 0.8848 were also in good agreement with R^2 adjusted values of 0.9502. The parity chart was represented in Figure 2.

The minimum and maximum amylase production for rice bran was 11.32 U/ml and 16.02 U/ml respectively for Run No.36 and Run No.6. The results obtained by CCD were analyzed by standard analysis of variance (ANOVA) and are shown in Table 4.57.

The three-dimensional response surface curves constructed by the regression model are shown in Figure 3.1 to Figure 3.10. The optimum values of the variables are found from the equations derived by the differentiation of the obtained second order polynomial equations. The optimum values are found to be pH- 4.7; Temperature - 36.5oC; Fermentation time - 66.6hr; Inoculum concentration - 5.7% and Substrate concentration - 18.9 g/L were shown in Table 5.

3.1. Validation of the Experiment

Validation of the experimental model is tested by carrying out the batch experiment under optimal operation conditions. Three repeated experiments are performed and the results are compared. The amylase activity obtained from the experiments is very close to the actual response credited by the regression model which proved the validity of the model. At these optimized conditions maximum amylase activity is found to be 16.89 U/ml.

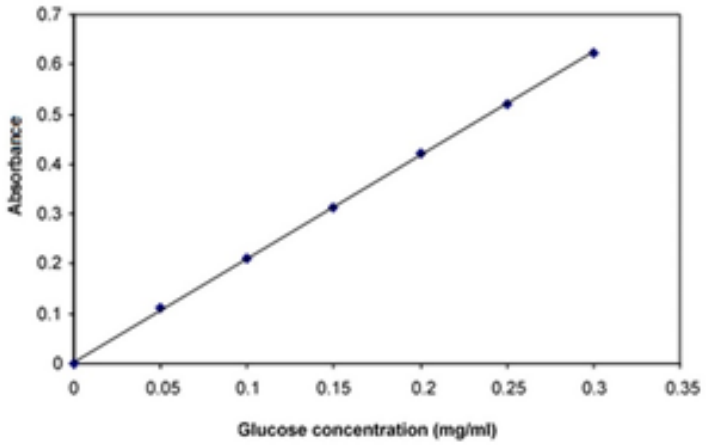

Figure 1 Calibration chart for glucose concentration using Bio-spectrophotometer

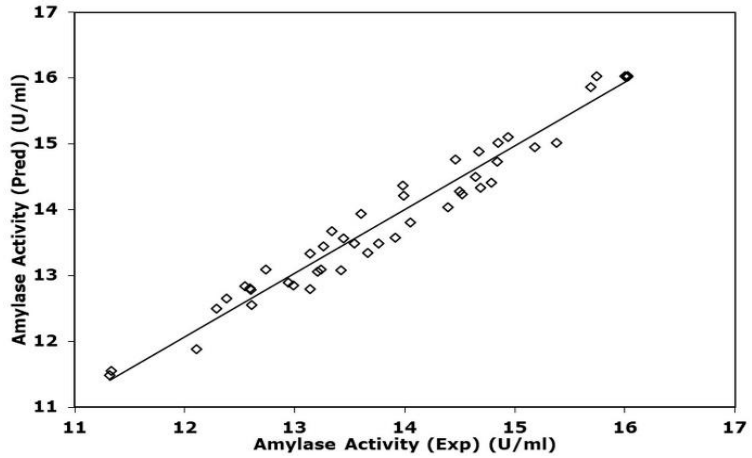

Figure 2 Parity plot between the experimental and predicted values of process parameters for *A. oryzae* MTCC-8624 utilizing rice bran

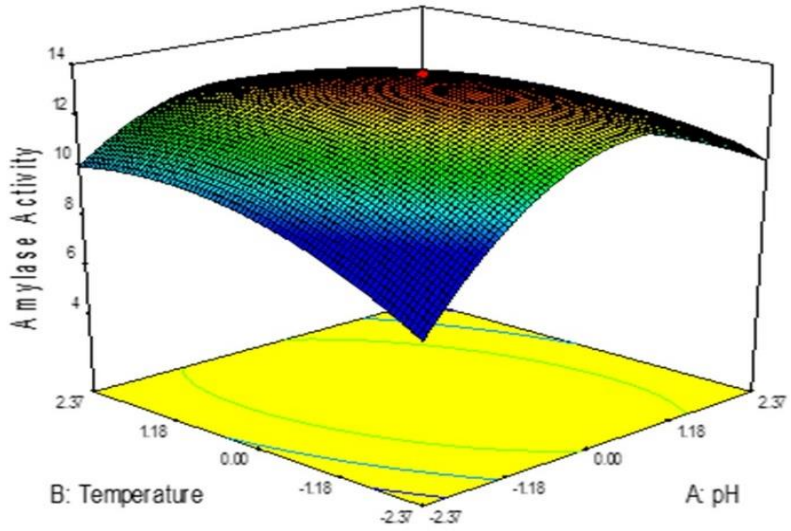

Figure 3 3D Plot shows the interaction between the process parameters pH and Temperature for *Aspergillus awamori* using rice bran

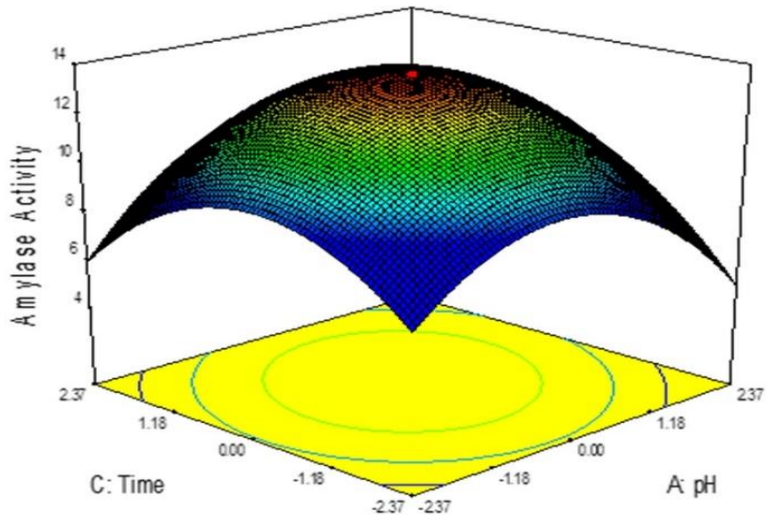

Figure 4 3D Plot shows the interaction between the process parameters pH and Time for *Aspergillus awamori* using rice bran

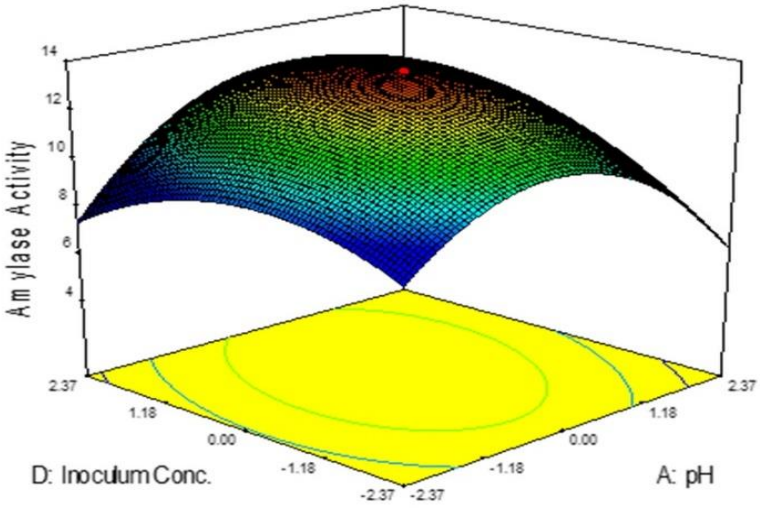

Figure 5 3D Plot shows the interaction between the process parameters pH and Inoculum Conc. for *Aspergillus awamori* using rice bran

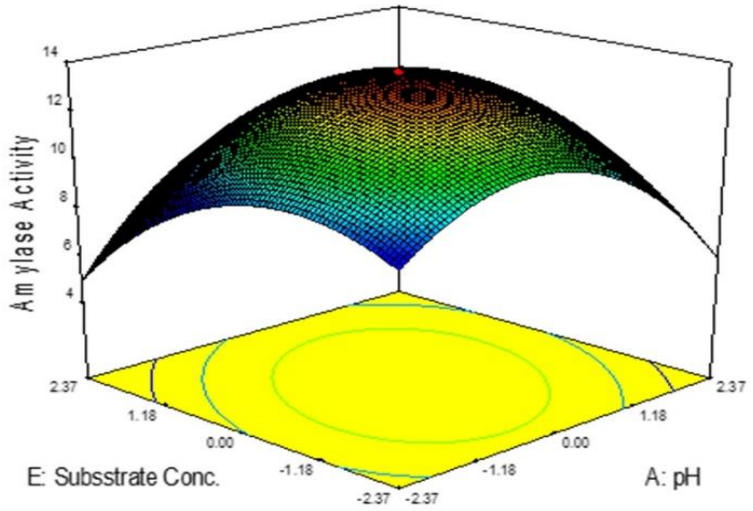

Figure 6 3D Plot shows the interaction between the process parameters pH and substrate Conc. for *Aspergillus awamori* using rice bran

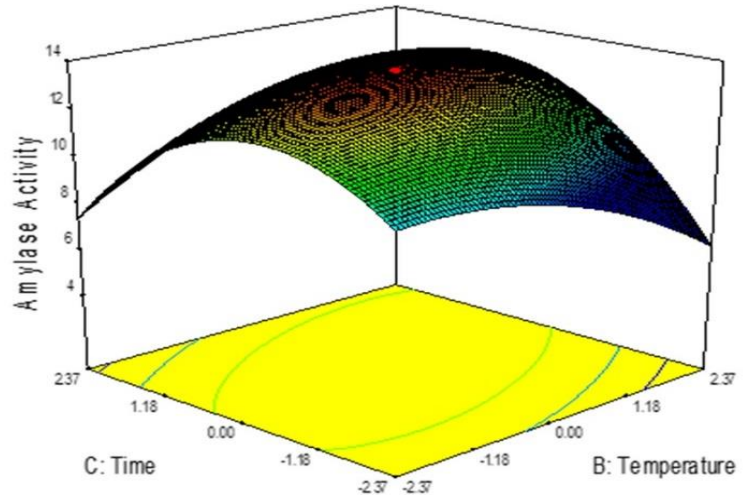

Figure 7 3D Plot shows the interaction between the process parameters Temperature and Time for *Aspergillus awamori* using rice bran

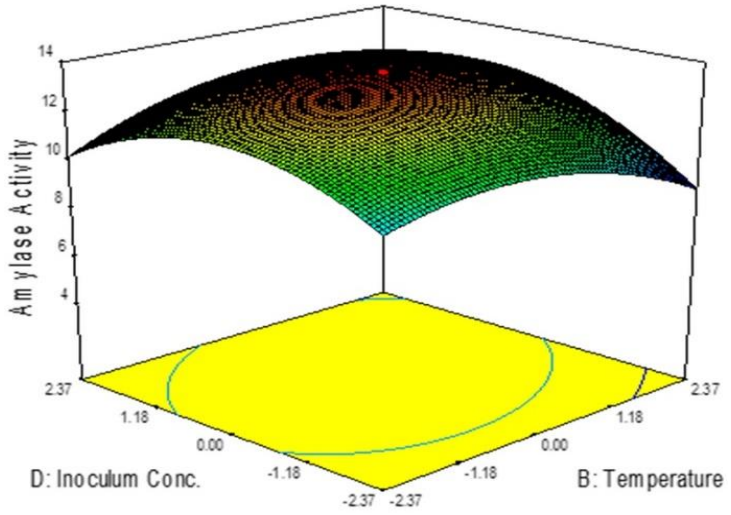

Figure 8 3D Plot shows the interaction between the process parameters Temperature and Inoculum Conc. for *Aspergillus awamori* using rice bran

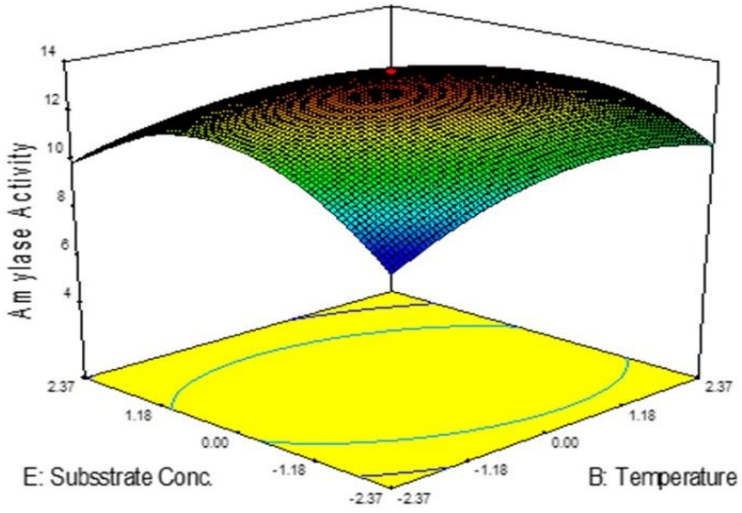

Figure 9 3D Plot shows the interaction between the process parameters Temperature and substrate Conc. for *Aspergillus awamori* using rice bran

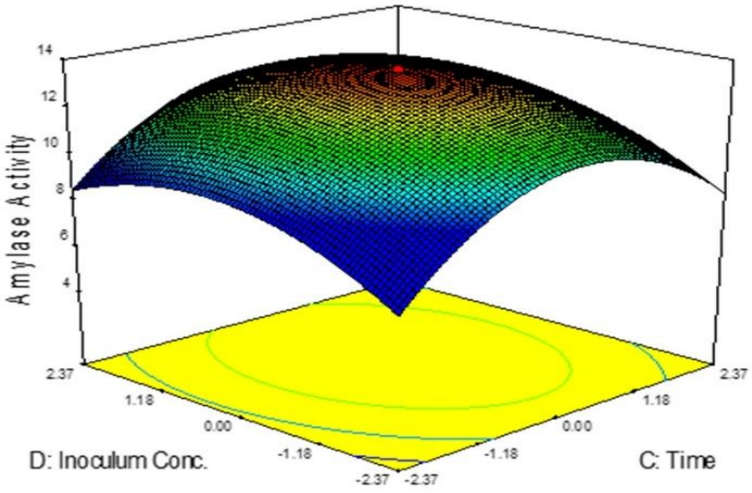

Figure 10 3D Plot shows the interaction between the process parameters Time and Inoculum Conc. for *Aspergillus awamori* using rice bran

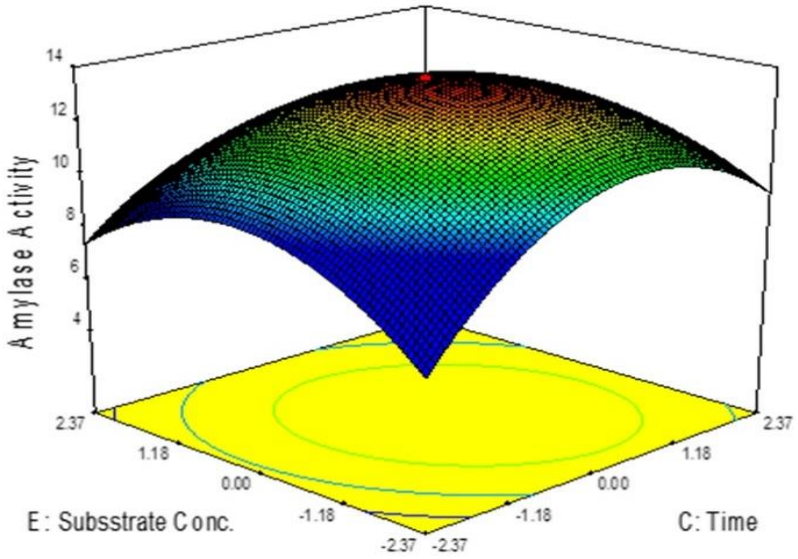

Figure 11 3D Plot shows the interaction between the process parameters Time and substrate Conc. for *Aspergillus awamori* using rice bran

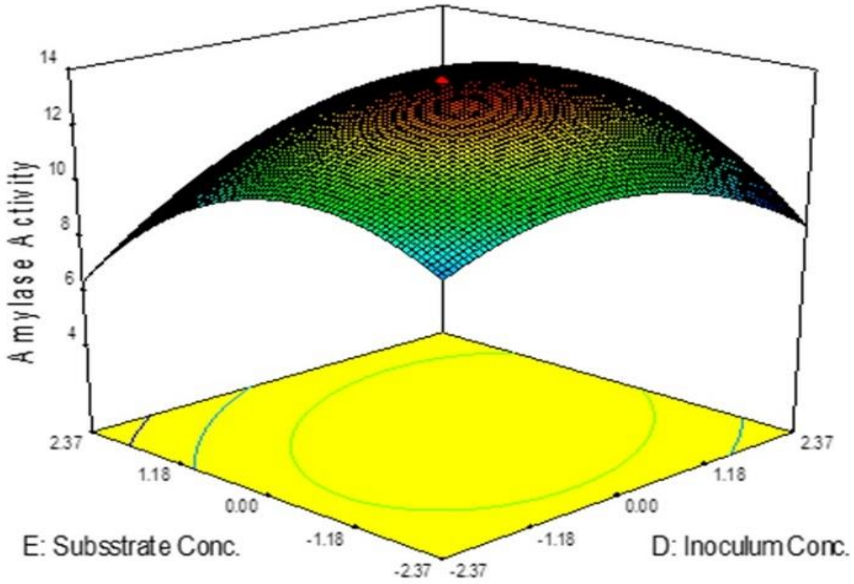

Figure 12 3D Plot shows the interaction between the process parameters Innoculum Conc. and substrate Conc. for *Aspergillus awamori* using rice bran

Table 1 Coded and un-coded values employed in CCD for parameter optimization of *Aspergillus niger* MTCC-104

Variables	**Symbols**	**Coded levels**				
		-2.38	**-1**	**0**	**+1**	**+2.38**
pH	A	4	4.5	5	5.5	6
Temperature (ºC)	B	24	27	30	33	36
Fermentation Time (hr)	C	66	72	78	84	90
Inoculum Concentration (%)	D	3	4	5	6	7
Substrate Concentration (g/L)	E	10	15	20	25	30

Table 2 The Central Composite experimental design with five independent variables for parameter optimization of *A. oryzae* MTCC-8624 utilizing rice bran as substrate

Run No.	Coded Values					Amylase Activity (U/ml)	
	A	B	C	D	E	Exp.	Pred.
1	1	-1	1	-1	-1	13.24	13.090
2	-1	1	1	1	1	13.66	13.342
3	-1	-1	-1	1	1	14.46	14.768
4	0	0	0	0	-2.38	12.29	12.501
5	1	-1	-1	-1	1	14.67	14.879
6	0	0	0	0	0	16.02	16.027
7	-1	1	-1	-1	-1	14.85	15.018
8	1	-1	1	-1	1	14.64	14.501
9	1	-1	-1	1	1	14.69	14.335
10	0	0	0	2.38	0	14.94	15.107
11	1	1	1	-1	-1	13.21	13.064
12	1	-1	1	1	1	14.50	14.274
13	0	0	2.38	0	0	11.33	11.554
14	1	1	1	1	-1	13.14	13.330
15	0	0	0	0	2.38	12.74	13.088
16	-1	1	-1	1	-1	15.69	15.864
17	0	0	0	0	0	16.02	16.027
18	1	1	-1	1	-1	13.14	12.799
19	0	0	0	0	0	16.02	16.027
20	1	1	-1	-1	-1	12.99	12.850
21	2.38	0	0	0	0	13.34	13.679
22	1	1	1	-1	1	14.05	13.808
23	0	0	0	-2.38	0	13.98	14.372
24	-1	1	1	-1	1	12.59	12.807
25	-1	-1	1	1	1	12.61	12.552
26	-1	-1	1	1	-1	12.60	12.784
27	1	1	-1	-1	1	13.91	13.575
28	-1	-1	-1	-1	1	14.79	14.415
29	0	0	-2.38	0	0	13.60	13.934
30	0	0	0	0	0	16.00	16.027
31	-1	-1	1	-1	1	12.11	11.883
32	-1	-1	-1	-1	-1	14.39	14.039
33	-2.38	0	0	0	0	13.99	14.210
34	0	0	0	0	0	16.02	16.027
35	0	0	0	0	0	16.02	16.027
36	-1	-1	1	-1	-1	11.32	11.489
37	0	-2.38	0	0	0	12.38	12.653
38	-1	1	-1	-1	1	14.84	14.727
39	-1	-1	-1	1	-1	15.38	15.018
40	0	0	0	0	0	16.02	16.027
41	0	0	0	0	0	15.74	16.027
42	0	0	0	0	0	16.02	16.027
43	-1	1	1	1	-1	14.52	14.240
44	1	-1	1	1	-1	13.76	13.490
45	0	0	0	0	0	16.02	16.027
46	1	-1	-1	1	-1	13.44	13.570
47	1	1	1	1	1	13.26	13.448
48	1	1	-1	1	1	12.94	12.898
49	-1	1	-1	1	1	15.18	14.946
50	0	2.38	0	0	0	12.55	12.835
51	1	-1	-1	-1	-1	13.54	13.487
52	-1	1	1	-1	-1	13.42	13.079

Table 3 Results of the regression analysis of second order polynomial model for parameter optimization of *A. oryzae* MTCC-8624 utilizing rice bran as substrate

Term constant	Regression coefficient	T-statistics	P-value
Intercept	16.0267	171.48	0.000
A	-0.1116	-2.471	0.019
B	0.0382	0.845	0.404
C	-0.5003	-11.072	0.000
D	0.1545	3.42	0.002
E	0.1233	2.729	0.010
A^2	-0.3681	-9.471	0.000
B^2	-0.5803	-14.928	0.000
C^2	-0.5803	-14.928	0.000
D^2	-0.2276	-5.855	0.000
E^2	-0.5714	-14.701	0.000
A.B	-0.4041	-7.687	0.000
A.C	0.5384	10.243	0.000
A.D	-0.2241	-4.262	0.000
A.E	0.2541	4.833	0.000
B.C	0.1528	2.907	0.007
B.D	-0.0334	-0.636	0.529
B.E	-0.1666	-3.169	0.003
C.D	0.0791	1.504	0.143
C.E	0.0047	0.089	0.930
D.E	-0.1566	-2.978	0.006

R-Sq = 96.97% R-Sq(pred) = 88.48% R-Sq(adj) = 95.02%

Table 4 ANOVA for the fitted polynomial model for parameter optimization of *A. oryzae* MTCC-8624 utilizing rice bran as substrate

Sources of variation	Sum of squares	Degrees of freedom (DF)	Mean square (MS)	F- value	P-value
Regression	87.8512	20	4.3926	49.67	0.000
Linear	13.1364	5	2.6273	29.71	0.000
Square	53.885	5	10.777	121.88	0.000
Interaction	20.8298	10	2.083	23.56	0.000
Residual Error	2.7412	31	0.0884		-
Lack-of-Fit	2.6714	22	0.1214	15.66	0.003
Pure Error	0.0698	9	0.0078	-	-
Total	90.5925	51	-	-	-

Table 5 Optimum values of the process parameters obtained from regression equation for *A. oryzae* MTCC-8624 utilizing rice bran as substrate

Independent variables	Optimum value (coded)	Optimum value (real)
pH	-1.22524	4.7
Temperature (°C)	0.312317	36.5
Fermentation Time (hr)	-0.888902	66.6
Inoculum Concentration (%)	0.792805	5.7
Substrate Concentration (g/L)	-0.264268	18.9

4. CONCLUSION

This investigation infers the potential use of rice bran as cheaper substrate for a large-scale production of both α- amylase by Aspergillus oryzae MTCC-8624 which significantly reduce the organic load of agricultural wastes. The research finding reports optimized values of experimental parameters pH, temperature, fermentation time, inoculum concentration and

substrate using a Box–Behnken design under the response surface methodology. The maximum α- amylase activity is found to be 16.89 U/ml at an optimized value of pH- 4.7; Temperature - 36.5ºC; Fermentation time - 66.6hr; Inoculum concentration - 5.7% and Substrate concentration - 18.9 g/L.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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