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Amylase production by *Aspergillus niger* in submerged cultivation using cassava

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ABSTRACT

α-amylase can be produced from cassava using *Aspergillus niger* MTCC-282 in submerged state which is studied in this investigation. It reveals the possible use of cassava for a large-scale production of α-amylase substantially decreasing the organic wastes. Using central composite design (CCD), every separate and interactive effect of experimental factors such as pH, temperature, fermentation time, and substrate concentration can be found from central composite design (CCD). Furthermore, inoculum concentration is inferred for the α-amylase production. The optimum values are pH – 4.8; temperature – 32.4°C; fermentation time – 79.5 h; inoculum concentration – 5.07%; and substrate concentration – 18.2 g/L for α-amylase production using *Aspergillus niger* from cassava. Maximum amylase activity was found to be 14.01 U/ml under optimum conditions.

1. INTRODUCTION

Amylases play a pivotal role in various industrial processes [1,2]. α-amylase and glucoamylase are two major types of amylase which breaks the glycosidic linkages between adjacent glucose units in a linear amylose chain [3]. α-amylase has extensive applications in many fields such as clinical, medicinal, and analytical chemistry under various extracellular enzymes [4]. Apart from its use in starch saccharification, it has major application in baking, brewing, detergent, textile, and paper industries as well as distilleries [5]. The high production cost of enzymes indicates that the production cost can be reduced by identifying suitable substrates and methods. Agriculture wastes are promising substrate for enzyme production. Several research findings show that coconut oil cake, sugarcane bagasse, wheat bran, rice husk, and corn cob are major agriculture wastes for the production of amylase [5-8]. Different kinds of significant industrial enzymes can be produced from *Aspergillus* species [8]. Conventional method to optimize the experimental parameter involves more time and the experimental parameter interactions are not considered. Contradictorily optimization by statistical method has several advantages than conventional one. Such a way, Placket and Burman design is a opt one to screen several parameters. Response surface methodology (RSM) is a tool to find the significant factors and helps to build models to appraise the several parameter interactions [9]. In statistical method of optimization, the 3D plots would provide the clear anatomy about the interactions between experimental parameters [10]. It is used to select suitable conditions to reach the maximum yield [11].

In this investigation, it is aimed to study the effective utilization of cassava as substrate for the production of α-amylase by *Aspergillus niger* MTCC-282 with submerged state. The individual and interactive effects of experimental parameters: pH, temperature, fermentation time, inoculum concentration, and substrate concentration are also aimed to investigate on the α-amylase production using central composite design. Furthermore, it is aimed to report the optimum condition of experimental parameters for enhances α-amylase production.

2. MATERIALS AND METHODS

2.1. Microorganism and Maintenance

Aspergillus niger MTCC-282 is acquired from the MTCC, Institute of Microbial Technology, Chandigarh, India. Potato dextrose agar slants maintain the culture at 4°C [12]. The culture is initially screened on standard media by starch agar plate assay [13].

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2.2. Inoculum Preparation

Inoculum is equipped by transferring 2 ml of 72 h old slant culture in 100 ml of medium composed by glucose – 20 g/l; KH2PO4 = 1.9 g/l; MgSO4 = 2.06 g/l; NaCl = 1.21 g/l; $MnSO_4$ = 0.5 g/l, (NH4)2SO4 = 2.78 g/l, and mycological peptone – 3.0 g/l at pH 5. The culture is incubated at 25°C for 3 days at a rotation speed of 230 rpm [12,13].

2.3. Fermentation Medium

Cassava which is utilized as substrate in this investigation is collected from nearby areas of Chidambaram, Tamil Nadu, India. The cassava is heated in an oven at 80°C for 12 h. Subsequently, it is powdered in a laboratory grinder and sieved using a 40 mm sieve [14]. Passable amount of this powdered substrate is mixed with 100 ml of the corresponding mineral salt media in a 250 ml Erlenmeyer flask. The pH is adjusted to 5. The mixture is sterilized in an autoclave at 121°C and 15 psi for 15 min. Then, it is cooled to the room temperature. Proper volumes of inoculums are added with this flask [15]. All the experiments for media optimization are carried out with a substrate concentration of 20 g/L, inoculum size of 5% (v/v), and fermentation time of 72 h. The pH and temperature are maintained at 5 and 25°C [16].

2.4. Amylase Extraction

The contents of the flask are filtered using a Whatman No. 4 filter paper followed by filtration through a muslin cloth. Then, the filtrate is centrifuged at RPM of 10,000 for 10 min and the supernatant was used as the source of enzyme for assay [17].

2.5. Estimation of Amylase Assay

Estimation of amylase activity is done by determining the amount of reducing sugar with the DNS method [14,15]. A mixture of 1 ml aliquots of each enzyme source and 1% soluble starch dissolved in 0.1 M phosphate buffer was incubated at 55°C for 15 min at a pH of 7 to enhance consciousness. Add 1 ml 3,5-DNS acid to stop the reaction, then boil for 10 min. The final volume was made up to 12 ml with distilled water and the reducing sugar released was measured at 540 nm.

$$\text{Enzyme acitivity ((IU/mL)/min)} = \frac{\text{(absorbance of enzyme solution)}}{\text{(time of incubation)}} \text{ x standard factor}$$

One unit of amylase activity is defined as the amount of enzyme that releases 1 µmol glucose equivalent per minute under the measurement conditions. Under same condition, reducing sugar concentration is determined using glucose [18]. Figure 1 shows the calibration chart for glucose concentration using biospectrophotometer. Dry cell mass of the fungal culture is determined by filtering the culture broth with a pre-weighed Whatman No. 44 filter paper. Mycelia are carefully eroded with distilled water and warmed in oven at 105°C for 2 h. The dry cell mass was obtained by subtracting the initial weight from the final weight and represented as g/L.

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

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

2.6. Determination of Starch

For the determination of starch, 0.2 g 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 color with anthrone reagent. To the residue, 5 ml of distilled water is added, cooled in ice water bath with the addition of 6.5 ml 52% perchloric acid on occasional stirring. 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°C. Pipetted out 0.2 ml of the filtered supernatant and make to 1 ml of water in a test tube. Also add 4 ml of anthrone reagent and placed in boiling water bath for 8 min. The contents are chilled and the intensity of green color is recorded at 630 nm [19,20].

3. RESULTS AND DISCUSSION

The medium components are optimized by Placket-Burman design. It is an active method for the medium optimization. It is necessary to incorporate significant factors and eliminates the insignificant one to get smaller set of factors. Fifteen different mineral salt medium components have been chosen separately for the three strains to evaluate their effect on amylase production. The selection of the components was based on the works reported previously. The significant components obtained are KH2PO4 = 1.9 g/l; MgSO4 = 2.06 g/l; NaCl =1.21 g/LlMnSO4 = 0.5 g/l; and (NH4)2SO4 = 2.78 g/l for cassava.

To study the interaction as well as the optimum levels of the significant factors, central composite design plays a key role in the production of

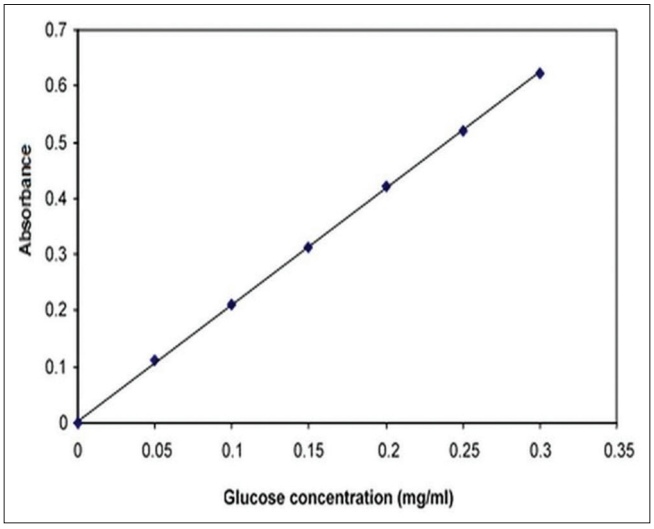

Figure 1: Calibration chart for glucose concentration using biospectrophotometer.

Table 1: Coded and uncoded 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 (°C)	B	24	27	30	33	36
Fermentation Time (h)	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 design with five factors for parameter optimization of *A. niger* MTCC-282 utilizing cassava as substrate.

Run No.	Coded values					Amylase activity (U/ml)	
	A	B	C	D	E	Exp.	Pred.
1	1	1	1	1	1	10.54	10.290
2	1	1	−1	1	1	8.69	9.035
3	0	0	0	0	0	13.32	13.171
4	−1	1	−1	1	1	10.02	10.282
5	−1	−1	1	1	−1	8.01	8.108
6	−1	1	1	1	1	10.79	10.404
7	0	0	0	0	0	13.28	13.171
8	−1	1	−1	−1	1	9.62	9.217
9	0	2.38	0	0	0	10.22	9.876
10	1	−1	1	1	1	9.46	9.763
11	1	−1	1	1	−1	9.94	10.004
12	−2.38	0	0	0	0	8.66	8.871
13	0	0	0	-2.38	0	9.58	9.823
14	1	−1	1	−1	−1	10.75	10.609
15	0	0	0	0	0	13.00	13.171
16	2.38	0	0	0	0	8.16	7.972
17	0	0	0	2.38	0	10.59	10.37
18	−1	−1	−1	1	1	8.91	8.940
19	1	1	1	1	−1	10.75	10.973
20	−1	1	1	−1	1	9.86	10.249
21	1	1	1	−1	−1	9.86	9.980
22	−1	1	1	−1	−1	11.87	11.91
23	−1	−1	−1	−1	−1	9.62	9.921
24	0	0	0	0	0	13.00	13.171
25	−1	1	−1	−1	−1	10.19	10.108
26	0	0	2.38	0	0	8.61	8.644
27	0	−2.38	0	0	0	8.66	9.027
28	1	−1	−1	1	−1	9.58	9.584
29	0	0	0	0	0	13.00	13.171
30	0	0	0	0	0	12.88	13.171
31	1	−1	−1	−1	1	9.26	9.241
32	1	−1	1	−1	1	10.06	9.800
33	1	1	−1	−1	1	6.48	6.565
34	1	−1	−1	−1	−1	9.10	9.279
35	−1	−1	1	−1	−1	10.46	10.118
36	−1	−1	−1	−1	1	9.66	9.473
37	1	1	−1	−1	−1	7.20	7.046
38	−1	−1	−1	1	−1	9.21	8.821
39	−1	−1	1	−1	1	9.16	8.900
40	0	0	0	0	0	13.30	13.171
41	0	0	0	0	−2.38	10.54	10.359
42	0	0	0	0	0	13.30	13.171
43	0	0	0	0	0	13.30	13.171
44	−1	1	−1	1	−1	10.38	10.606
45	0	0	−2.38	0	0	6.93	6.918
46	0	0	0	0	2.38	8.81	9.013
47	1	1	−1	1	−1	8.81	8.949
48	−1	−1	1	1	1	7.40	7.457
49	1	−1	−1	1	1	10.46	10.113
50	0	0	0	0	0	13.32	13.171
51	1	1	1	−1	1	8.61	8.730
52	−1	1	1	1	−1	11.39	11.497

Table 3: Results of the regression analysis of the second-order polynomial model for parameter optimization of *Aspergillus niger* MTCC-282 utilizing cassava as substrate.

Term constant	Regression coefficient	T-statistics	P-value
Intercept	13.1715	147.029	0.000
A	−0.1891	−4.366	0.000
B	0.1785	4.121	0.000
C	0.3628	8.378	0.000
D	0.115	2.656	0.012
E	−0.2829	−6.533	0.000
A2	−0.8397	−22.538	0.000
B2	−0.6576	−17.651	0.000
C2	−0.9528	−25.575	0.000
D2	−0.5436	−14.591	0.000
E2	−0.6161	−16.536	0.000
A.B	−0.605	−12.007	0.000
A.C	0.2831	5.619	0.000
A.D	0.3512	6.971	0.000
A.E	0.1025	2.034	0.051
B.C	0.4012	7.963	0.000
B.D	0.3994	7.926	0.000
B.E	−0.1106	−2.196	0.036
C.D	−0.2275	−4.515	0.000
C.E	−0.1925	−3.82	0.001
D.E	0.1419	2.816	0.008

R-Sq = 98.51%: R-Sq (pred) = 94.84% : R-Sq(adj)=97.55%

Table 4: ANOVA for the fitted polynomial model for parameter optimization of *A. niger* MTCC-282 utilizing cassava as substrate.

Sources of variation	Sum of squares	Degrees of freedom (DF)	Mean square (MS)	*F*-value	*P*-value
Regression	166.337	20	8.3169	102.37	0.000
Linear	12.67	5	2.5341	31.19	0.000
Square	120.971	5	24.1942	297.8	0.000
Interaction	32.696	10	3.2696	40.24	0.000
Residual error	2.519	31	0.0812	-	-
Lack of fit	2.24	22	0.1018	3.29	0.061
Pure error	0.279	9	0.031	-	-
Total	168.856	51	-	-	-

Table 5: Optimum values of the process parameters obtained from regression equation for *Aspergillus niger* MTCC-282 utilizing cassava as substrate.

Independent variables	Optimum value (coded)	Optimum value (real)
pH	−0.216219	4.8
Temperature (°C)	0.360366	32.4
Fermentation time (h)	0.264268	79.5
Inoculum concentration (%)	0.0720732	5.07
Substrate concentration (g/L)	−0.312317	18.2

amylase by *A. niger* MTCC-282 utilizing the substrate cassava. Table 1 gives coded and actual values. Table 2 shows 52 run design matrix along with the experimental and the predicted responses for cassava.

The results of the regression analysis of the second-order polynomial model are given in Table 3 for cassava. The second-order polynomial equation derived from the regression analysis for amylase production (Y) using cassava was as follows:

$$Y = 13.1715 - 0.189067A + 0.178473B + 0.362835C + 0.115026D - 0.282928E - 0.839711A2 - 0.657631B2 - 0.952848C2 - 0.543610D2 - 0.616089E2 - 0.605AB + 0.283125AC + 0.35125AD + 0.1025AE + 0.400125BC + 0.399375BD - 0.110625BE - 0.2275CD - 0.1925CE + 0.141875DE \quad ®.141$$

Where, A, B, C, D, and E are pH, temperature, fermentation time, inoculum concentration, and substrate concentration, respectively.

ANOVA was used to check the model adequacy and the results are shown in Table 4 for cassava. From the results of ANOVA, the model terms except AE were found to be influential for the production of α-amylase. R^2 value 0.9851 indicates the corresponding to cassava which indicates good relations of predicted and experimental values. The predicted R^2 values 0.9484 for cassava are also in good agreement with the corresponding R^2 adjusted values of 0.9755. The predicted and experimental values of parity plots are indicated in Figure 2. Figure 3.1–3.10 for cassava represents the major interaction effects

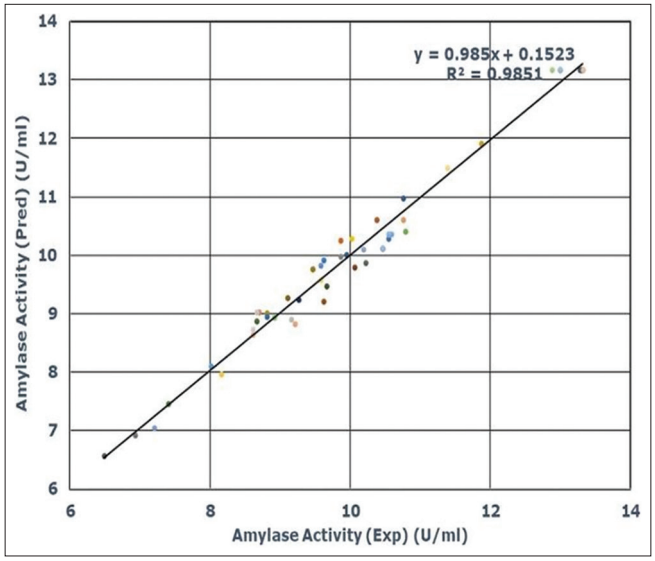

Figure 2: Parity plot between the experimental and predicted values of process parameters for *A. niger* MTCC-282 utilizing cassava.

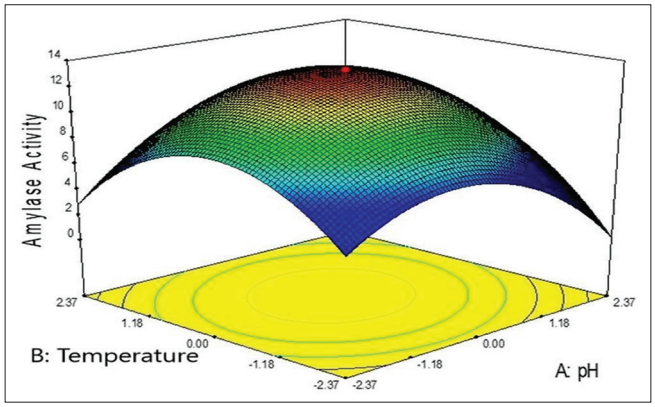

Figure 3.1: 3D plot shows pH and temperature interactions for *Aspergillus niger* using cassava.

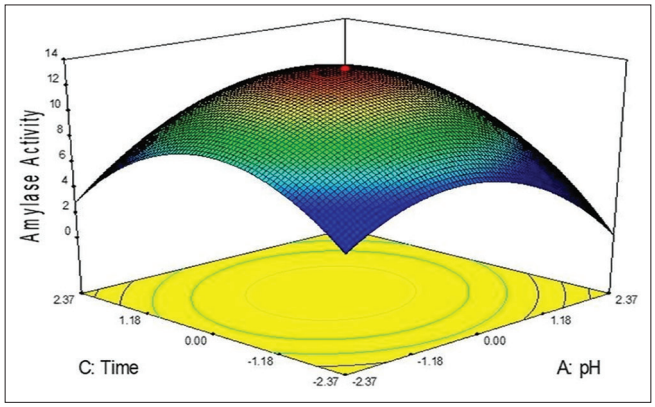

Figure 3.2: 3D plot shows pH and time interactions for *Aspergillus niger* using cassava.

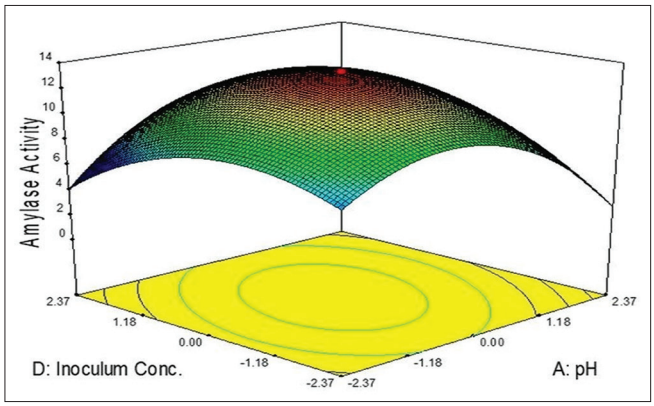

Figure 3.3: 3D plot shows pH and inoculum conc. interactions for *Aspergillus niger* using cassava.

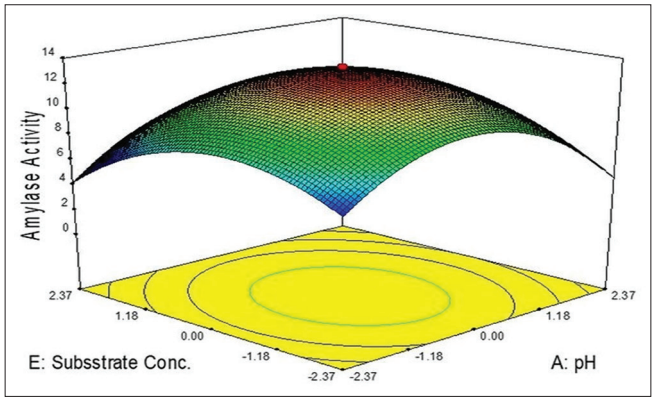

Figure 3.4: 3D plot shows pH and substrate conc. interactions for *Aspergillus niger* using cassava.

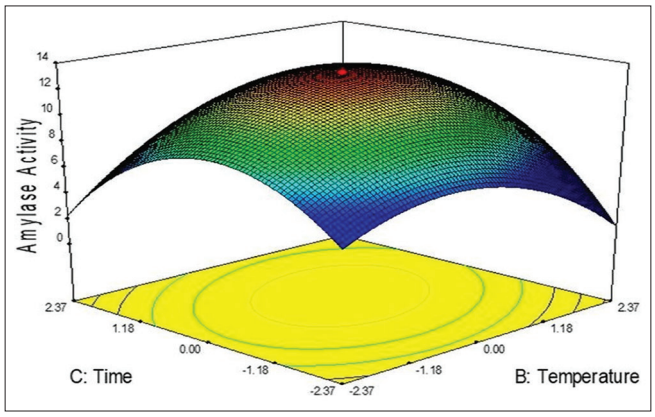

Figure 3.5: 3D plot shows temperature and time interactions for *Aspergillus niger* using cassava.

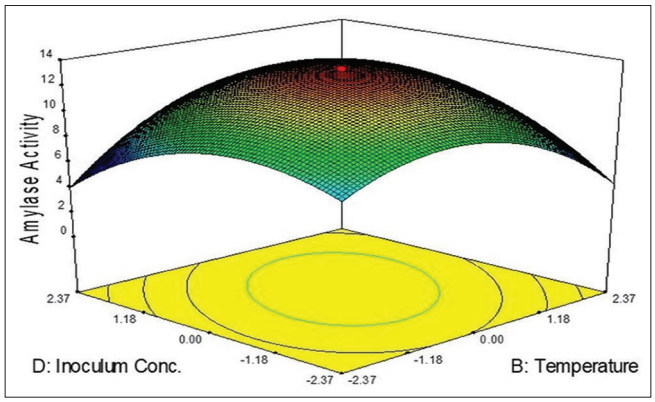

Figure 3.6: 3D plot shows temperature and inoculum concentration interactions for *Aspergillus niger* using cassava.

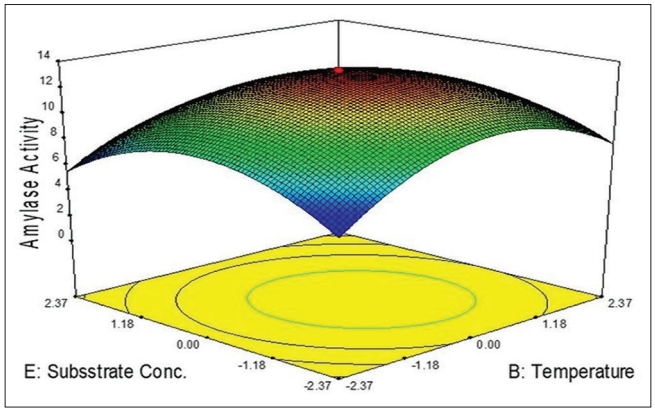

Figure 3.7: 3D plot shows temperature and substrate concentration interactions for *Aspergillus niger* using cassava.

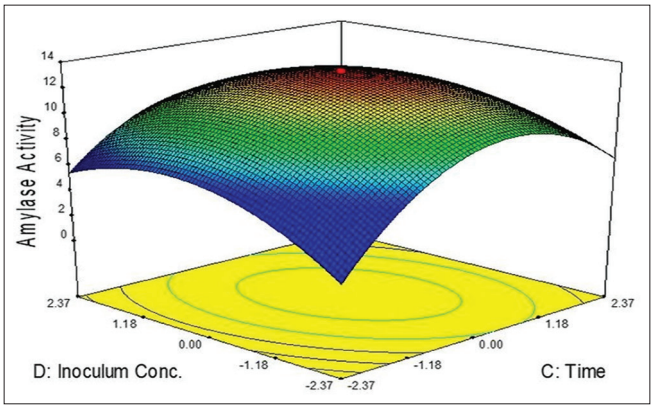

Figure 3.8: 3D plot shows time and inoculum concentration interactions for *Aspergillus niger* using cassava.

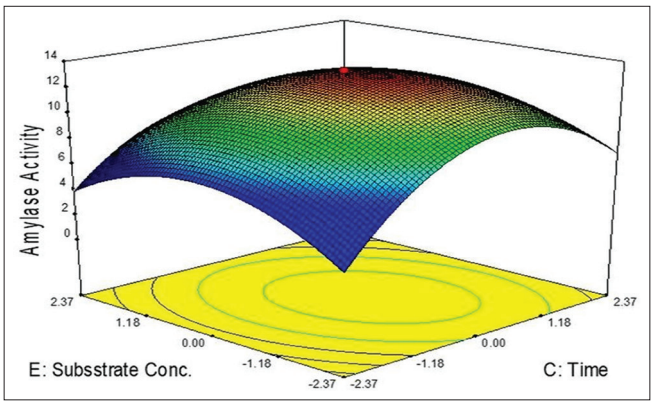

Figure 3.9: 3D plot shows time and substrate concentration interactions for *Aspergillus niger* using cassava.

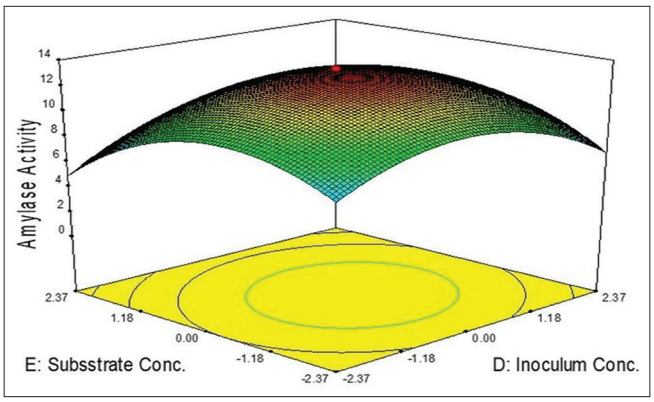

Figure 3.10: 3D plot shows inoculum conc. and substrate concentration interactions for *Aspergillus niger* using cassava.

and also the optimum levels of selected variables in response surface curve. The optimum values obtained were pH – 4.8; temperature – 32.4°C; fermentation time – 79.5 h; inoculum concentration – 5.07%; and substrate concentration – 18.2 g/L for cassava, as shown in Table 5. In all the three cases, the pH optimum is in the range of 4.5–5 and temperature around 30°C. The results obtained have good agreement with the works reported previously with *Aspergillus niger* [15–18, 21] The inoculum concentration of 5% was also reported previously. Experiments are conducted 3 times and the obtained results are in close agreement with the value of regression model which shows the validity of the experiment. Amylase activity found from the experiments is very near to the actual response credited by the regression model which proved the validity of the model [22–24]. At these optimized conditions, maximum amylase activity is found to be 14.01 U/ml.

4. CONCLUSION

The data exhibited the possible use of cassava as substrate for a large-scale production of α-amylase considerably decreases unwanted

wastes.. The individual and interactive effects of experimental factors of pH, temperature, fermentation time, inoculum concentration, and substrate concentration are studied for the α-amylase production. The optimum values are pH – 4.8; temperature – 32.4°C; fermentation time – 79.5 h; inoculum concentration – 5.07%; and substrate concentration – 18.2 g/l for αfamylase production using *Aspergillus niger* from cassava. Maximum amylase activity is found to be 14.01 U/ml.

5. ACKNOWLEDGMENTS

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6. CONFLICTS OF INTEREST

Authors declared that they do not have any conflicts of interest.

7. FINANCIAL SUPPORT AND SPONSORSHIP

None.

REFERENCES

1. Mojumdar A, Deka J. Recycling agroindustrial waste to produce amylase and characterizing amylase-gold nanoparticle composite. Int J Recycl Org Waste Agric 2019;8:S263-9.
2. Taghreed N, Almanaa P, Vijayaraghavan S, Alharbi NS, Kadaikunnan S, Khaled JM, *et al*. Solid state fermentation of amylase production from *Bacillus subtilis* D19 using agro-residues. J King Saud Univ Sci 2019;32:1-7.
3. Sidkey NM, Abo-Shadi MA, Al-Mutrafy AM, Sefergy F, Al-Reheily N. Screening of microorganisms isolated from some enviroagro-industrial wastes in Saudi Arabia for amylase production. J Am Sci 2010;6:326-39.
4. Ramachandran S, Patel AK, Nampoothiri KM, Francis F, Nagy V, Szakacs G, *et al*. Coconut oil cake-a potential raw material for the production of alpha-amylase. Bioresour Technol 2044;93:169-74.
5. Ramachandran S, Singh SK, Larroche C, Soccol CR, Pandey A. Oil cakes and their biotechnological applications-a review. Bioresour Technol 2007;98:2000-9.
6. Anto H, Trivedi UB, Patel KC. Glucoamylase production by solid-state fermentation using rice flake manufacturing waste products as substrate. Bioresour Technol 2006;97:1161-6.
7. Baysal Z, Uyar F, Aytekin C. Solid-state fermentation for production of α-amylase by a thermotolerant *Bacillus subtilis* from hot-spring water. Process Biochem 2003;38:1665-8.
8. Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R. Advances in microbial amylases. Biotechnol Appl Biochem 2000;31:135-52.
9. Mohandas BS, Prabhakar A, Rao RR, Madhu GM, Rao GH. Statistical optimization and neural modeling of amylase production from banana peel using *Bacillus subtilis* MTCC 441. Int J Food Eng 2010;6:1-6.
10. Saran S, Isar J, Saxena RK. Statistical optimization of conditions for protease production from *Bacillus* sp. and its scale-up in a bioreactor. Appl Biochem Biotechnol 2007;141:229-39.
11. Reddy LV, Wee YJ, Yun JS, Ryu HW. Optimization of alkaline protease by batch culture of *Bacillus* sp. RKY3 through Placket-Burman and response surface methodological approaches. Bioresour Technol 2008;99:2242-9.
12. Ellaiah P, Adinarayana K, Bhavani Y, Padmaja P, Srinivasulu B. Optimization of process parameters for glucoamylase production under solid state fermentation by a new isolated *Aspergillus* species. Process Biochem 2002;38:615-20.
13. Hernandez MS, Rodrıguez M, Guerra NP. Amylase production by *Aspergillus niger* in submerged cultivation on two wastes from food industries. J Food Eng 2006;73:93-100.
14. Fogarty WM. Microbial amylases. In: Fogarty WM, editor. Microbial Enzymes and Biotechnology. London, UK: Applied Science Publishers Ltd.; 1983. p. 1-92.
15. Kalaiarasi K, Parvatham R. Optimization of process parameters for α-amylase production under solid-state fermentation by *Aspergillus awamori* MTCC 9997. J Sci Ind Res 2015;74:286-9.
16. Hayashida S, Teramoto Y. Production and characteristics of raw-starch-digesting α-amylase from a protease negative *Aspergillus ficuum* mutant. Appl Environ Microbiol 1986;52:1068-73.
17. Irfan M, Nadeem M, Syed Q. Media optimization for amylase production in solid state fermentation of wheat bran by fungal strains. J Cell Mol Med 2012;10:55-64.
18. Jamrath T, Lindner C, Popovic MK, Bajpai R. Production of amylases and proteases by *Bacillus caldolyticus* from food industry wastes. Food Technol Biotechnol 2011;50:355-61.
19. Carlsen M, Nielsen J, Villadsen J. Growth and α-amylase production by *Aspergillus oryzae* during continuous cultivations. J Biotechnol 1996;45:81-93.
20. Ashwini S. Isolation, process parameters for optimization and purification of alpha amylase for mass production from *Bacillus* species. Int J Ext Res 2015;5:65-71.
21. Sunitha VH, Ramesha A, Savitha J, Srinivas C. Amylase production by endophytic fungi *Cylindrocephalum* sp. Isolated from medicinal plant *Alpinia calcarata* (HAW.) Roscoe. Braz J Microbiol 2012;1:1213-21.
22. Zhu W, Lestander TA, Orberg H, Wei M, Hedman B, Ren J, *et al*. Cassava stems: A new resource to increase food and fuel production. GCB Bioenergy 2015;7:72-83.
23. Oboh G. Isolation and characterization of amylase from fermented cassava (*Manihot esculenta* Crantz) wastewater. Afr J Biotechnol 2005;4:1117-23.
24. Brisibe EA, Bankong H. Biotechnological potential of alpha amylase production by *Bacillus subtilis* using cassava peel powder as a substrate. Br Biotechnol J 2014;4:1201-11.

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