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Full Length Research Paper

Optimization of amylase production by *Aspergillus niger* cultivated on yam peels in solid state fermentation using response surface methodology

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The study involved the production of amylase from *Aspergillus niger* grown on yam peels in solid state fermentation. The process parameters: temperature, pH (initial) and incubation time were optimized for maximum amylase production using central composite design (CCD) of response surface methodology (RSM). Temperature was the most significant ($p<0.05$) parameter and the maximum interaction occurred between temperature and incubation time. The results of the study indicated that amylase is maximized (30.95 U/ml-min) at optimized levels of 49.53°C, 5.95 and 104 h for temperature, pH (initial) and incubation periods, respectively.

Key words: Response surface methodology (RSM), *Aspergillus niger*, central composite design, solid state fermentation (ssf), amylase, production, optimization.

INTRODUCTION

Amylase is one of the most important industrial enzymes that have found applications in the brewing, starch processing, textile, baking, pharmaceutical and detergents industries (Johnson et al., 2014). Amylase hydrolyzes starch molecules to yield various products, comprising dextrins and progressively smaller polymers composed of glucose units (Reddy et al., 2003). Amylase can be obtained from plants, animals and microorganisms (Saranraj and Stella, 2013); however, industries have much preference for enzymes from microbial sources (Xu et al., 2008).

Amylases for industrial activities are relatively non-existent in local Ghanaian industries, as a result, some of these businesses have stopped production and the few that are still in business utilize the staple foods such as maize, millet and sorghum as sources of amylase (Nyamful et al., 2014), which negatively impact on food security. Some local industries that are able to import these enzymes incur high cost in production. The commercial enzyme when stored for some time also tend to lose its stability (Dzogbefia et al., 2001) due to the uneven power fluctuations in the country. This has called

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Figure 2. *A. niger* area of hydrolysis on starch amended PDA.

for the local production of amylase to be produced commercially and supplied to these local industries upon demand.

The economic bulk production of enzymes on cheap substrates such as agro wastes in solid state fermentation using *Aspergillus niger* has been reported (Bhargav et al*.,* 2008; Pandey et al., 1999). These agro wastes are abundant in Ghana and underutilized; they could be put to an alternative use; as solid support (substrate) for amylase production. Maximal production of amylase at reduced cost is realized either by strain improvement or optimization of process parameters (Prajapati et al*.,* 2014). With respect to this, the current study aims to optimize the effect of pH, temperature and incubation time on the production of amylase by *A. niger* cultivated on yam peels in solid state fermentation using central composite design (CCD) of response surface methodology (RSM).

Response surface methodology (RSM) is a collection of statistical and mathematical techniques convenient for developing, improving and optimizing processes in which several variables influence the response of interest (Myers et al., 2009; Baş and Boyacı, 2007). Traditionally, optimization of processes involved varying one factor while the other factors were kept at their constant levels, that is, one-variable-at-a-time (OVAT). This procedure has received much criticism in the sense that, it is unable to consider the interactive effect among all the factors on the final outcome (Baş and Boyaci, 2007). The OVAT approach has also been found to be time wasting, laborious and waste of chemicals (Prajapati et al*.,* 2014). Response surface methodology can be used to overcome these limitations since its application can identify and quantify the various interactions among several parameters with less experimental runs (Shankar et al., 2015; Francis et al., 2003). There has been an extensive use of RSM for optimizing amylase productions (Hassan and Karim, 2015; Kalaiarasi and Parvatham, 2013; Tamilarasan et al*.,* 2012).

MATERIALS AND METHODS

Substrate (yam peel) preparation

Tubers of yam were purchased from the local market (Kejetia, Kumasi). These tubers were carefully washed under running water and peeled. The peels were oven dried at 60°C and milled to a particle size of about 0.3 mm mesh using Retsch KG Hammer miller (SKI, 23008, West Germany).

Aspergillus niger isolation

A piece of bread purchased was kept in a moist environment for 4 days for it to grow mouldy. 10 g of the mouldy bread was serially diluted and plated on potato dextrose agar (Sigma – Aldrich) in Petri dishes for 4 days at room temperature. Growths observed on the plates were randomly selected and 5 different colonies were selected after the spore colour and morphology confirmed that they were *A. niger.* The isolates were designated KV1B to KV5B. These were sub-cultured onto PDA slants and kept in the refrigerator for further studies.

Lactophenol cotton blue staining

A drop of 70% alcohol and lactophenol cotton blue stain were centrally placed onto a glass slide. Fragments of about 2 to 4 mm were isolated from the colonies edge and placed on the stain with gentle teasing. A coverslip was gently placed on and viewed under a microscope to ascertain the morphology of the fungal strain.

Determination of *A. niger* spore concentration

The spore concentration of *A. niger* was determined using the method outlined by Bentil et al. (2015) with little modifications. The spore concentration obtained was 3.96×10^6cells/ml.

Confirmation of amylase activity

The ability of the isolated *A. niger* to hydrolyze starch was investigated using the procedure outlined by Uguru et al. (1997). A zone of clearance around the fungal growth indicated amylase activity. The isolate KV5B had the largest area of clearance of 29 mm (Figure 2) and thus selected for further studies.

Preparation of inoculum

Spore suspension of *A. niger* KV5B was prepared by scrapping off fungal spores within a 1 cm corkborer with 40 ml of distilled water. This was made up to a 60 ml mark. Two (2) ml of this suspension was used to inoculate the substrate.

Enzyme production

Five grams of the yam peels was weighed into a 100 ml Erlenmeyer flask and moistened with 5 ml of fermentation medium (KCl – 1.25 g, KH_2PO_4 – 0.35 g, $MgSO_4.7H_2O$ – 0.025 g, NH_4NO_3 – 2.5 g, $FeSO_4.7H_2O$ – 0.0025 g, soluble starch – 5, distilled water – 250 ml

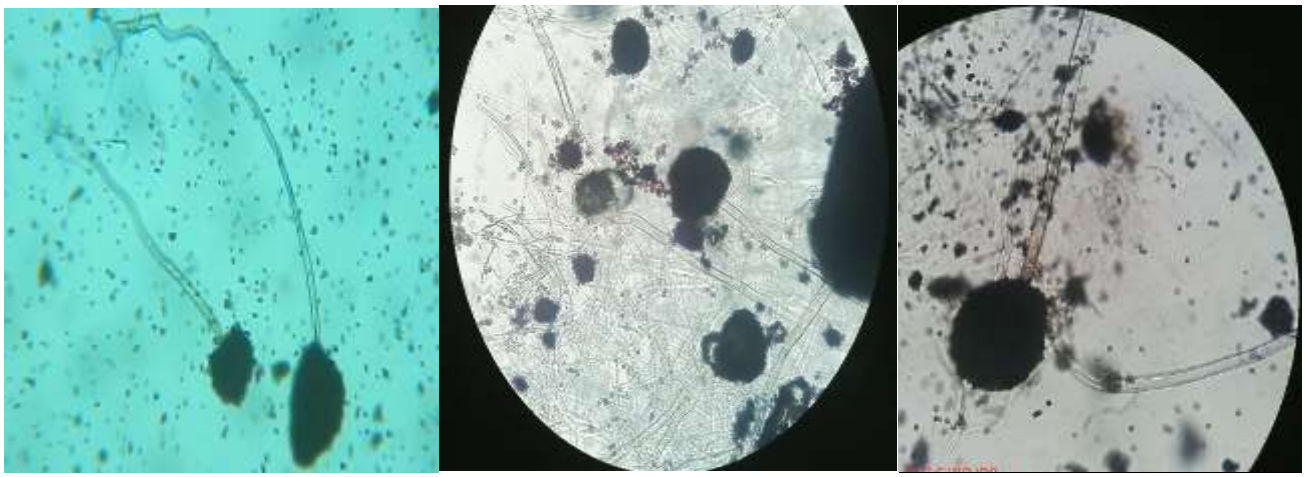

Figure 1. *A. niger* (40X) displaying conidiophores and conidia.

at pH 6.5) (Sethi and Gupta, 2015). The mixture was sterilized at 121°C for 15 min and cooled, after which each Erlenmeyer flask containing the substrate was inoculated with 2 ml of the spore suspension. Experiments were performed as outlined in Table 2.

Enzyme extraction

Fifty (50) ml of 0.1 M phosphate buffer (pH 6) was poured on each substrate bed and agitated for 30 min at 250 rpm using a rotary shaker. The suspension was filtered using a cheese cloth and the filtrate was centrifuged at 3600 g for 15 min. The decanted supernatant was used as the crude enzyme.

Assay for amylase activity

Amylase activity was determined using the method described by Sindiri et al. (2013). The reaction mixture consisted of 0.5 ml of the crude enzyme, 0.5 ml of 1% soluble starch in 0.02 M phosphate buffer with 0.06M NaCl, pH 6.9. The mixture was incubated for 3 min at room temperature, the reducing sugars liberated were estimated using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). Colour development was read at 540 nm with a UV – mini spectrophotometer (SHIMADZU). One unit of amylase (U) was defined as the amount of enzyme that released 1 µmol glucose equivalent per minute under assay conditions using glucose. Protein concentration (Lowry et al.,1951) was determined using bovine serum albumin as a standard curve.

Optimization using response surface methodology (RSM)

Production of amylase was optimized using a 2^3- factorial central composite design (CCD) with six star – (α) – points (α = ±1.682), six replicates at the center points and eight cube points. Independent variables (initial pH (A), temperature (B) and incubation time (C)) were optimized by assessing each factor at 5 different levels as shown in Table 1. In all, a total of 20 different experimental runs were carried out. A central coded value taken as zero was used to set all the variables. The maximum and minimum ranges of each variable was determined based on perusal of literature and the full experimental plan showing the actual values are shown in Table 2. The average amylase activity (U/ml-min) of each run was taken as the dependent variable (y) for that experimental run.

Statistical analysis

The effect of each of these variables in amylase production was analyzed using the analysis of variance (ANOVA), after which regression analysis was performed on the data obtained. The results obtained from the CCD were used to fit a second – order polynomial equation of the form:

$$y = \beta o + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} \beta^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 1$$

since this appropriately represented the behavior of such a system (Sun et al., 2011).

where y = predicted amylase response
βo = intercept
$\beta_1, \beta_2, \beta_3$ = variables linear effect
$\beta_{11}, \beta_{22}, \beta_{33}$ = variables squared effects
$\beta_{12}, \beta_{13}, \beta_{23}$ = interaction effect of variables
A, B, C, A^2, B^2, C^2, AB, AC, BC = independent variables

Fischer's test was used to test significance of variables and multiple coefficient of determination R squared (R^2) value was used to explain the proportion of variance by the model. Triplicate determinations were carried out for all the experimental runs and the averages reported. Design Expert V. 7.1 software (Stat-Ease Inc., Minneapolis, USA), was used for these analyses.

RESULTS AND DISCUSSION

Serial dilution from bread sample and subsequent plating on potato dextrose agar aided in the random selection of 5 different fungal colonies which were designated KV1B – KV5B. Lactophenol cotton blue staining ascertained that these colonies were *A. niger*. The conidiophores were smooth and about 2 to 4 nm long; spherical vesicles were observed on the top of globose conidia (Figure 1). Ogbonna et al. (2015) isolated *A. niger* from soil sample which exhibited similar characteristics. The easy identification and isolation of *A. niger* from bread sample confirm reports by Oyedeji (2016) and Okoko and

Table 1. Experimental range and levels of the three factors used in the central composite design (CCD) for amylase production by *A. niger.* KV5B

Levels	Independent variables		
	pH	Temperature (°C)	Time (h)
α (+ 1.682)	7.7	70.2	168.5
+1	7.0	60.0	144.0
0	6.0	45.0	108.0
-1	5.0	30.0	72.0
-α (-1.682)	4.3	19.8	47.5

Table 2. Full factorial central composite design (CCD) matrix with their corresponding experimental and predicted responses for amylase production by *A. niger.* KV5B

Run	Factor 1:A: pH (initial)	Factor 2: B: Temperature (°C)	Factor 3: C: Time(h)	Y: Observed response (activity U/ml-min)	Y: Predicted response (activity, U/ml-min)
1	6.00	19.77	108.00	5.95	2.76
2	5.00	30.00	72.00	15.74	17.04
3	7.00	30.00	72.00	15.45	17.00
4	5.00	30.00	144.00	5.60	7.90
5	7.00	30.00	144.00	5.71	7.96
6	6.00	45.00	47.46	18.75	18.00
7	6.00	45.00	108.00	34.90	30.16
8	6.00	45.00	108.00	35.85	30.16
9	6.00	45.00	108.00	28.27	30.16
10	6.00	45.00	108.00	20.63	30.16
11	7.68	45.00	108.00	30.03	28.14
12	6.00	45.00	108.00	32.33	30.16
13	4.32	45.00	108.00	29.96	28.31
14	6.00	45.00	108.00	28.36	30.16
15	6.00	45.00	168.54.	14.09	11.31
16	5.00	60.00	72.00	20.81	21.06
17	7.00	60.00	72.00	20.61	20.81
18	5.00	60.00	144.00	21.20	22.15
19	7.00	60.00	144.00	20.78	21.99
20	6.00	70.23	108.00	18.28	17.94

Ogbomo (2010) that *A. niger* is the most common microorganism associated with the spoilage of bread.

Response surface methodology

Response surface methodology of central composite design was employed to investigate the interactive effects of the factors that affected maximal production of the amylase from a fungal source in solid state fermentation. The factors that were considered: initial pH (A), temperature (B) and incubation time (C) have been reported as the most influential factors to a noticeable extent, that affected enzyme production in solid state fermentation (Bhimba et al., 2011). Table 2 shows the CCD experimental plan with the observed and predicted response for the experimental runs. Determination of the optimum levels of each selected factor was made possible by solving the regression equation and by analysis of the response surface and contour plots. The effect of the variables (initial pH, temperature and incubation time) on amylase production was expressed in a second order polynomial regression of the form (2). The regression equation provided the level of amylase production () as a function of initial pH, temperature and incubation time.

Amylase activity (U / ml-min) $y = 30.16 - 0.049A + 4.5B - 1.99C - 0.68A^2 – 7.00B^2 - 5.48C^2 - 0.054AB + 0.022AC + 2.56BC$ …… (2)

Table 3. Analysis of variance (ANOVA) for amylase production by *A. niger* KV5B

Source	Sum of squares	Df	Mean square	F Value	p- value Prob > F	
Model	1427.69	9	158.63	7.97	0.0016	Significant
A-pH(initial)	0.033	1	0.033	1.65E-03	0.9684	
B-Temp	278.15	1	278.15	13.97	0.0039	
C-Time	54.01	1	54.01	2.71	0.1306	
AB	0.023	1	0.023	1.16E-03	0.9735	
AC	3.92E-03	1	3.92E- 03	1.97E-04	0.9891	
BC	52.28	1	52.28	2.63	0.1362	
A^2	6.73	1	6.73	0.34	0.5739	
B^2	706.98	1	706.98	35.51	0.0001	
C^2	432.92	1	432.92	21.74	0.0009	
Residual	199.1	10	19.91			
Lack of Fit	41.94	5	8.39	0.27	0.9133	Not significant
Pure Error	157.16	5	31.43			
Cor Total	1626.79	19				

Table 4. Summary of the ANOVA for amylase production by *A. niger.* KV5B

Std. Dev . = 4.46	R – squared (R^2) = 0.8776
Mean = 21.17	Adj R – squared = 0.7675
C.V (%) = 21.08	Predicted R – squared = 0.6661
PRESS = 543.22	Adeq. Precision = 8.684

Where y=predicted amylase activity; A, B and C are the coded values of initial pH, temperature and incubation time, respectively. The statistical significance of each of these factors were evaluated using the analysis of variance (ANOVA).

From the ANOVA (Table 3), the model had an F –value of 7.97. Statistically, this implied that the model was significant and there was only 0.16% chance that the model F –value could occur due to error. Probability values (Prob>F) < 0.05 specified model terms were significant. Thus, B, B^2 and C^2 were significant terms in maximizing amylase production since their Prob>F values were 0.39, 0.01 and 0.09% respectively (Table 3). Statistically, temperature affected the enzyme production in SSF more than any of the other two factors ($p<0.05$) (Table 3). Lokeswari (2010) reported that temperature was the most significant factor amongst other two factors when *Bacillus subtilis* in SSF was used to optimize amylase production by employing RSM. Incubation time was also observed to be the most significant factor when Sun et al. (2011) used RSM to optimize amylase produced by *Bacillus subtilis* ZFJ-1A5 in SSF. These results are similar to the current study as temperature was found to be the most influential factor, therefore indicating that temperature plays critical role in enzyme production. An F – value of 0.27 for lack of fit (Table 3), implied the lack of fit was insignificant relative to the pure error. Prob>F value of lack of fit indicated that the quadratic model was valid and adequate for the optimization of the parameters that obtained optimum production of the amylase enzyme. Closeness of the multiple correlation coefficient value (R^2) (Table 4) to 1 indicated better correlation between the predicted and actual values. The model offered a relatively high determination coefficient with an R^2 value of 0.8776 (Table 4), indicating the model could explain 87.6% of the responses variability. Predicted R^2 (0.666) was in reasonable agreement with the adjusted R^2 (0.767) (Table 4) since the difference between both R^2 was < 0.200 (Hassan and Karim, 2015). The degree of accuracy with which the experiments were associated was indicated by the coefficient of variation (CV); a high value of CV usually indicated a lower reliability of the experiment (Prajapati et al., 2014). This study showed a low CV of 21.08% (Table 4), signifying that the experiments performed were dependable. The signal to noise ratio is measured by the adequate precision (Hassan and Karim, 2015). The study showed a ratio of8.684 (Table 4) for adequate signal (a ratio greater than 4 is desirable), implying that the model was reliable in optimizing the chosen process variables for maximum amylase production.

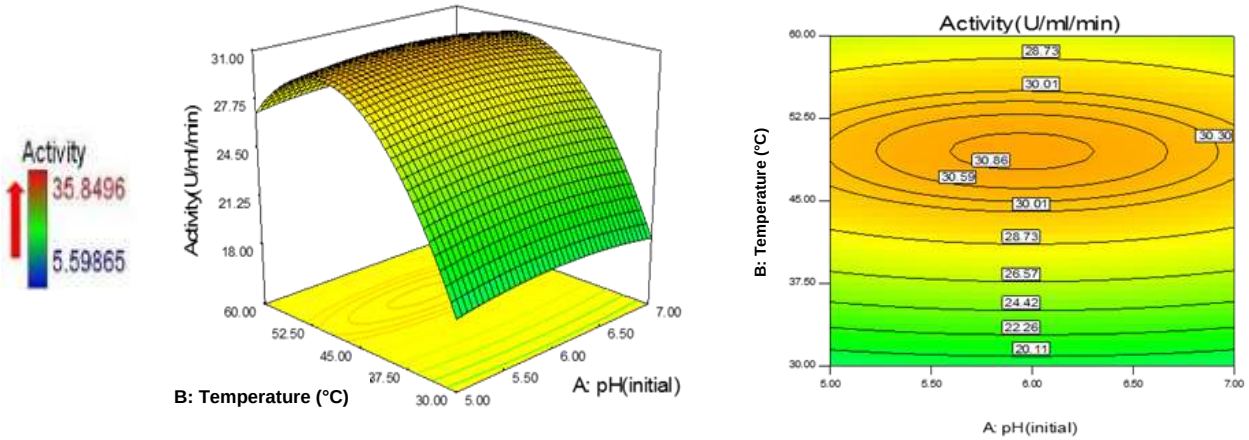

Figure 3. Three dimensional (3D) response surface and contour plot showing the effect of temperature and initial pH on amylase production when incubation time was kept at an optimized level of 104 h.

Usually, the optimal levels and interaction effects of the factors are analyzed using 3 –dimensional (3D) contour plots. Interaction between variables are considered negligible when circular contour plots are observed, however, a perfect interaction among the variables are indicated by the development of elliptical contours of which the smallest eclipse in the contour represent the maximum/optimum level (Hassaïne et al., 2014).

Interaction effects of the variables in maximizing amylase production were studied among any two independent variables, while the other independent variable was kept at its optimized level. The possible combination of the variables in maximizing amylase production is shown in Figures 3 to 5. Figure 3 shows the interaction between temperature and pH (initial), while incubation time was kept at the optimized level. Low and high levels of temperature did not result in maximum amylase production. Maximum amylase production was recorded in the middle level of temperature (45 to 52.50°C). Variations in pH (5 to 7) were only marginal in the production of amylase. However, a slight increase was observed at pH value of about 6.

The shape of the response curve also indicated a moderate interaction between these tested values. The reduction in metabolism of the microorganism and the concomitant reduction in enzyme synthesis (Roses and Guerra, 2009) may account for the low activities at low temperatures. Higher temperatures may cause inactivation or halting of cell viability and enzyme denaturation (Hassan and Karim, 2015; Roses and Guerra, 2009), this may be the reason for the low activities observed at high temperatures (60°C). Optimum temperature ranges between 50 to 55°C have been reported, especially, for the thermophilic fungal cultures of *Thermomyces lanuginosus*, *Talaromyces emersonii* and *Thermomonospora fusca* (Haasum et al., 1991). Temperatures of 70 (Uguru et al., 1997), 45 (Sethi and Gupta, 2015) and 40 and 45°C (Suganthi et al*.,* 2011) have also been reported of amylases from *A. niger*. It therefore appears that the particular strain of *A. niger* used also influenced the optimum temperature for enzyme production. Figure 3 also depicts that the *A. niger* had preference for pH within the acidic and neutral levels as the chosen levels (5 to 7) supported enzyme production. pH optima of 5.5 (Uguru et al., 1997) and 7.2 (Tamilarasan et al., 2012) have been reported for *A. niger* and *A. oryzae*, respectively.

Figure 4 shows the interaction between incubation time and initial pH while temperature was held at the optimized level. Low and high incubation periods recorded low amylase activities. The highest activity was recorded in the middle levels (90 to 108 h) of incubation. Short incubation period offers potential for inexpensive production of enzymes (Vishnu et al*.,* 2014). Low activity at longer incubation period may be as a result of the depletion of nutrients and sugar content in the media and indirectly resulting in the generation of secondary metabolites by the fungus, thereby inhibiting enzyme production (Alnour et al., 2015; Abdullah et al., 2014). Optimum incubation periods of 5 (Ruban et al., 2013) and 4 days (Uguru et al., 1997) for *A. niger* have been reported. Thus, the incubation period obtained in this study is consistent with others. Effect of pH levels also seemed minimal when it interacted with incubation time as both high and low levels of pH caused only a slight decrease in amylase activity as compared to the high activity round pH 6. As indicated earlier, initial pH levels posed minimal influence on the amylase activity as the chosen levels (pH 5 to 7) have been reported to favour the growth of *A. niger* (Gowthaman et al., 2001).

Figure 5 shows the interaction between incubation time and temperature, while pH was kept at the optimized

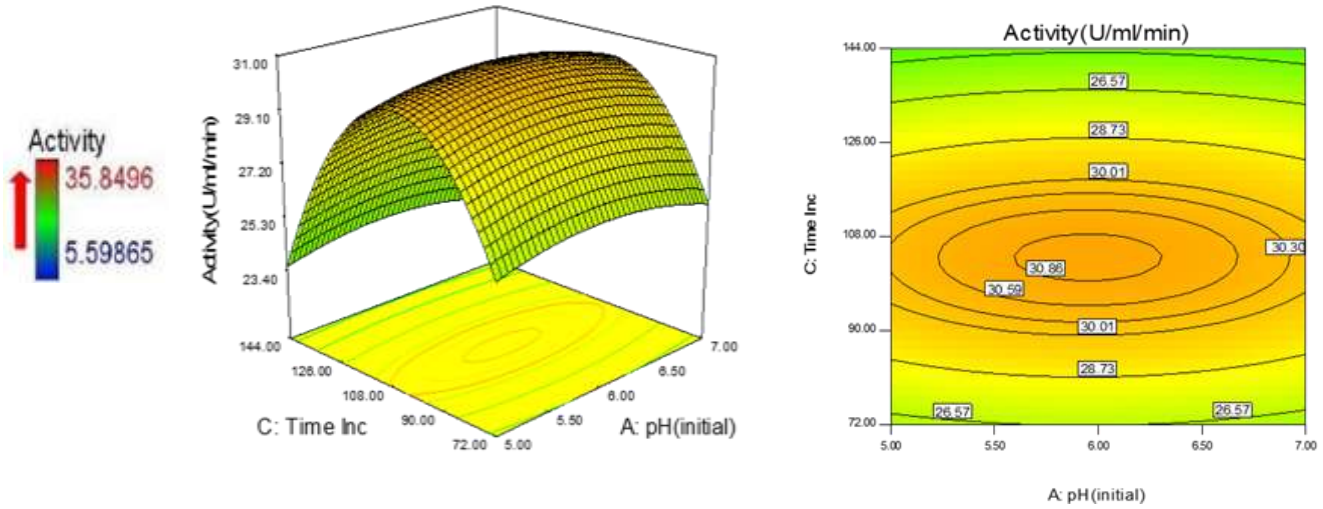

Figure 4. Three dimensional (3D) and contour plot showing the effect of incubation time and initial pH on amylase production when temperature was kept at an optimized level of 49.53°C.

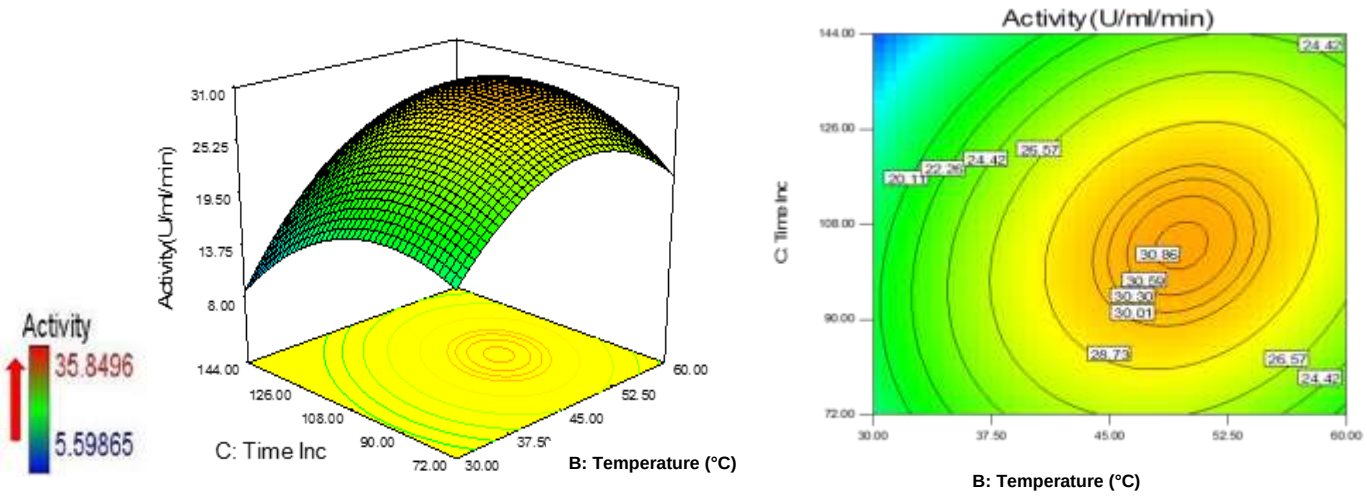

Figure 5. Three dimensional (3D) response surface and contour plot showing the effect of incubation time and temperature on amylase production when initial pH was kept at an optimized level of 5.95.

level. High and low levels of both factors resulted in low activities. The highest activity was recorded in the middle levels of both factors, that is, 90 to 108 h and 45 to 52.50°C for incubation time and temperature, respectively. Decrease in amylase activity at longer incubation periods and higher temperatures may be attributed to the evaporation of moisture in the substrate and amylase decomposition by the interaction with other components in the medium (Kalaiarasi et al., 2008). A similar simultaneous increase and decrease in amylase activity when incubation time and temperature were not in their optimal ranges have been reported (Sun et al., 2011). Temperature seems to be more significant than incubation time in enhancing amylase activity as the contours are heading towards the temperature level (Figure 5).

From the 3D surface plots, it has been deduced that the ranges of initial pH, temperature and incubation time that can result in maximum amylase production are 5 to 6, 45 to 52.50°C and 90 – 108 h, respectively. These ranges were further analyzed to determine the optimum value of each factor.

The solution for optimization of amylase production as illustrated from the RSM is shown in Table 5. The pH (initial), temperature and incubation time were set in range while amylase activity was set in the maximum yield. The optimum values predicted were pH – 5.95, temperature –

Table 5. Solution for optimizing amylase production by *A. niger.* KV5B

Name	Goal	Lower limit	Upper limit	Weight	Weight	Importance
pH(initial)	Is in range	5.0	7.0	1	1	3
Temp	Is in range	30.0	60.0	1	1	3
Time Inc	Is in range	72.0	144.0	1	1	3
Activity	Maximize	5.6	35.8	1	1	5
Solutions						
Number	**pH(initial)**	**Temperature**	**Time**	**Activity**	**Desirability**	
1	5.95	49.53	104	30.95	0.838	Selected

Table 6. Validation of amylase production by *A. niger KV5B* using the predicted optimized values of initial pH (5.95), temperature (59.53°C) and incubation time (104 h). KV5B

Run	Enzyme Activity (U/ml/min) (experimental)	Enzyme Activity (U/ml/min) (predicted)	Percentage difference (%)
1	30.41	30.95	1.15
2	32.05	30.95	6.24
3	29.66	30.95	4.15

49.53°C, incubation time – 104 h (4 days 8 h) and an amylase activity of 30.95 U/ml-min. The desirability value of 83.8% which is close to 100% indicated a high favorability of the response value and the tendency in obtaining the predicted enzyme activity value using these optimized values was high.

A triplicate experimental run was conducted using the optimized values to validate the predicted response as shown in Table 6. The results shown in Table 6 proved that these predicted values from RSM can be used in optimizing amylase production using *A. niger* in SSF as the predicted and experimental values were very close.

Conclusion

From the results obtained, it appears that the application of RSM in SSF to optimize process factors for amylase production in developing countries such as Ghana is feasible. This method can be applied to locally produce amylase for our industries, thus, cutting down the challenges associated with the acquisition of enzymes for industrial applications.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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