



## Process parameters optimization for amylase production through rice bran bioconversion by *Aspergillus niger*

Daniel Thakuma Tizhe<sup>1</sup> and Jáafar Nuhu Jáafar<sup>1,2\*</sup>

<sup>1</sup>Department of Biotechnology, Modibbo Adama University of Technology, P.M.B. 2076, Yola, Adamawa State, Nigeria. E-mail: [danielthakumatizhe@gmail.com](mailto:danielthakumatizhe@gmail.com)

<sup>2</sup>Chevron Biotechnology Center, Modibbo Adama University of Technology, P.M.B. 2076, Yola, Adamawa State, Nigeria. E-mail: [jnjaafar@mautech.edu.ng](mailto:jnjaafar@mautech.edu.ng)

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### Abstract

Enzymes are produced by a wide range of living things including bacteria, fungi, plants and animals. Fungal enzymes are preferred over other microbial sources owing to their widely accepted Generally Regarded As Safe (GRAS) status. Amylase is one of the most widely used enzymes in the biotechnology industry with a global market of over \$4 bn as at 2015. However, the synthetic media used for the production of this enzyme is becoming costlier and has become a subject of concern for many food-processing industries. Hence, manufacturers are now in search for alternative approaches to cut down the cost of production. In addition, the Raw Materials Research and Development Council, Nigeria ([rmrdc.gov.ng](http://rmrdc.gov.ng)) indicated that the country spends about \$200 bn annually on importation of industrial enzymes. Therefore, this study was designed with the aim of utilizing agro-industrial waste (rice bran) as the sole carbon source for the production of amylase in a Solid-State Fermentation (SSF) culture by optimizing the major process parameters. A 19-run experiment generated by Design Expert™ software was carried out to identify the optima for pH, temperature and substrate concentration that would yield the highest amount of amylase. The results of the current study showed that significant interaction effect exists between temperature and pH. However, no interaction was observed with substrate concentration and pH depicting the sensitivity of the enzyme to varying pH conditions. Similarly, a statistically significant 2FI model ( $p < 0.0022$ ) that could be used to predict the yield of amylase was identified. Conclusively, the findings of this study suggests that the feasibility of using rice bran as an alternative carbon source in the production of amylase calls for its wider utilization in the food processing industries, thus, alleviating the cost concern.

**Keywords:** Amylase, *Aspergillus niger*, Solid state fermentation

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## 1. Introduction

Enzymes are biological molecules responsible for metabolic activities in living cells. The unique ability of enzymes to carry out specific biochemical functions in isolation has led to their increase demand in industries

\* Corresponding author: Jáafar Nuhu Jáafar, Chevron Biotechnology Center, Modibbo Adama University of Technology, P.M.B. 2076, Yola, Adamawa State, Nigeria. E-mail: [jnjaafar@mautech.edu.ng](mailto:jnjaafar@mautech.edu.ng)

(Ajay and Farhath, 2010). Amylases (E.C.3.2.1.1.) are one of the most widely used enzymes in biotechnological industries by catalyzing the breakdown of starch into simple sugars. They are widely distributed in microbial, plant and animal kingdoms (Banks and Satyanarayana, 1975) accounting for approximately 25% of the enzyme market covering most biotechnological industrial processes such as sugar, textile, paper, baking, brewing, detergent, distilling and pharmaceutical industries (Mamo *et al.*, 1999; Pandey *et al.*, 2000; Oudjeriouat *et al.*, 2003; Ramachandran *et al.*, 2004; and Kathiresanand Manivannan, 2006). Similarly, they have gained spectrum of application in many sectors such as clinical, medical and analytical chemistry.

Microbes are considered the most convenient sources of commercial enzymes due to efficient production processes (Sodhi *et al.*, 2005). Specifically, microbial sources of enzymes are preferred due to their economic feasibility, high production yield, consistency, ease of genetic modification and optimization, regular supply due to absence of seasonal and weather fluctuations, rapid proliferation of microbes on inexpensive substrate, greater stability and catalytic activity (Neelam *et al.*, 2013). The advantage of using microbes for the production of enzymes is that bulk production is economical and easy to manipulate to obtain enzymes with desired characteristics. Recovery, isolation and purification process of microbial enzymes are easier than that from other sources. Fungal enzymes are preferred over other microbial sources owing to their widely accepted Generally Regarded As Safe (GRAS) status (Sindhu *et al.*, 2009).

Because of the high demand of amylases by industries, cost effective production medium is required (Aliyu *et al.*, 2011). The components of this synthetic media such as nutrient broth, soluble starch, as well as other components are very expensive and these could be replaced with cheaper agricultural by-products that are usually considered as waste in the environment to minimize the cost of production process (Solange and Jose, 2010).

The commercial production of industrial enzymes has been reported through Submerged Fermentation (SmF) and Solid-State Fermentation (SSF) (Sodhi *et al.*, 2005; and Perez-Guarre *et al.*, 2003). SSF is considered more suitable to SmF for commercial enzymes production due to the simplicity of the technique, cost effectiveness, semblance of microbe's natural habitat, ease of product recovery with stable physiochemical properties and high quality of the product (Baysal *et al.*, 2003; and Ajay and Farhath, 2010). More so, SSF technique is generally confined to processes involving fungi (Kiran *et al.*, 2010).

Research on the selection of suitable substrates for SSF has been centered on agro industrial residues due to their potential advantages for filamentous fungi, which are capable of penetrating into the hardest of these solid substrates (Maryam *et al.*, 2010). Substrates such as, rice husk, soybean meal, cotton seed meal, wheat bran, pearl millet and rice bran have been tried for SSF (Maryam *et al.*, 2010). In addition, the utilization of these agro industrial wastes, not only provides alternative substrates but also helps in solving pollution problems (Priya *et al.*, 2011).

However, the production of enzymes of microbial origin for example, is influenced by optimal process parameters such as temperature, pH, ideal substrate selection and microorganism, size of the substrate, concentration of the inoculum and the moisture content of the substrate (Sodhi *et al.*, 2005). Hence, this study was aimed at optimizing some key process parameters using a mathematical model while utilizing agro-waste as the alternative substrate.

## 2.. Materials and methods

### 2.1. Microorganism

*Aspergillus niger* (*A. niger*) was obtained from the Department of Microbiology, Modibbo Adama University of Technology (MAUTECH), Yola and maintained on potato dextrose agar slant at 4 °C.

### 2.2. Determination of fungal isolate for amylase activity

The isolates obtained were tested for extracellular amylase production on starch agar media fortified with 1% soluble starch as described by Bertrand *et al.* (2004). Briefly, the fortified media were inoculated with the fungal isolate and incubated at 28 °C for 48 h. The plates were flooded with iodine solution and zone of hydrolysis were observed.

### 2.3. Preparation of inoculum

Selected *A. niger* from the step above was sub cultured into potato dextrose broth and incubated at 28 °C for 48 h.

### 2.4. Experimental design

A mathematical approach using Design Expert® version 9.0.5 was employed to generate a central composite design. Independent factors; temperature, pH and substrate varied at five levels generated 19 runs of experiments with five replicated center points (Table 1).

Table 1: A five-level central composite design generated using Design Expert®					
Variables	-2	-1	0	1	2
Temp (°C)	6	20	40	60	74
pH	1	3	6.5	10	12
Substrate (g)	1	5	12.5	20	24

### 2.5. Substrate collection, pre-treatment and fermentation

Rice bran collected from a local rice processing plant in Mubi, Adamawa State, Nigeria was solely used as the substrate. The bran was pre-treated by washing with 1 M HCl solution, rinsed thoroughly with distilled water and allowed to air-dry. It was then weighed into 19 Erlenmeyer flasks as in the experimental design and each mixed with distilled water in a substrate to moisture ratio of 1:4. The pH of respective flask was adjusted accordingly and autoclaved at 121 °C for 15 min. Following sterilization, all flasks were uniformly inoculated with 2 ml of fungal broth and incubated at their respective temperatures for five days.

### 2.6. Crude enzyme extraction

Following fermentation, the product was processed as described by Kheng and Omar (2005). Briefly, the product was diluted with 0.1 M phosphate buffer (pH 7) at a solid to moistening ratio of 1:2. It was incubated at room temperature in an incubator shaker set at 250 rpm for 30 min. This extract was filtered through Whatman No.1 filter paper to obtain a clear filtrate. The filtrate was spun at 5000 rpm for 20 min and re-filtered. This filtrate was used as the crude enzyme extract for further analysis.

### 2.7. Enzyme assay

Amylase activity was determined according to the method reported by Okolo *et al.* (1995). The reaction mixture consisted of 1 ml 1% (w/v) soluble starch dissolved in 0.1 M phosphate buffer (pH 7) and 1 ml of crude enzyme extract. The tubes were incubated at 50 °C for 10 min and the released reducing sugars (glucose equivalents) were estimated by DNS method. Enzyme activity was expressed in International Units (IU) (Silva *et al.*, 2005).

### 2.8. Crude enzyme estimation

The concentration of crude enzyme was determined with nanodrop® in mg/ml.

## 3. Results and discussion

To identify the interactive effects of process parameters towards the production of amylase, a systematic analysis was conducted using modeling and Analysis of Variance (ANOVA). A second-order polynomial equation (2FI) that identifies and explains the interactive effects between the selected independent variables as a function of amylase production was generated as shown:

$$\gamma = \beta_0 - \beta_1\chi_1 - \beta_2\chi_2 + \beta_3\chi_3 + \beta_{12}\chi_1\chi_2 + \beta_{23}\chi_2\chi_3$$

where  $\gamma$  is the predicted dependent variable (amylase concentration);  $\beta_0$  is the intercept that measures the average response in the experiment;  $\chi_1$ ,  $\chi_2$ , and  $\chi_3$  are independent variables (temperature, pH and substrate). Furthermore,  $\beta_1$ ,  $\beta_2$ , and  $\beta_3$  are linear coefficients;  $\beta_{12}$ , and  $\beta_{23}$  are interaction coefficients.

The statistical model representing amylase production and the interactive effect of process parameters as a function of the independent variables within the region under investigation is expressed by the following 2FI model equation in coded form:

$$\text{Amylase concentration (mg/ml)} = 1.16 - 0.12A - 0.20B + 0.14C + 0.25AB + 0.13BC$$

where *A*, *B*, and *C* are the coded variables for temperature (°C), pH, and substrate (g), respectively. The model evaluation was checked using ANOVA and coefficient of determination (*R*<sup>2</sup>) that measures the goodness of fit of the regression model (Table 2). Adjusted *R*<sup>2</sup> is the correlation coefficient for the number of model parameters relative to the number of runs while predicted *R*<sup>2</sup> is a measure of the predictive capability of the model. In addition, adequate precision compares the range of predicted values at design points to the average prediction error (that is, signal to noise ratio). Ratios greater than four indicate adequate model discrimination (Stat-Ease user guide). The Predicted Residual Error Sum of Squares (PRESS) measures how the particular model fits each point in the design. It is used to calculate the predicted *R*<sup>2</sup>. The model *F*-value, 7.94 (*p* < 0.0022) indicate that the model was statistically significant (Table 2). In the same vein, the statistical insignificance of lack of fit 3.48 (*p* = 0.1227) (Table 2) signifies adequate fit of the model.

Table 2: ANOVA results for response surface reduced 2FI model table					
	Sum of Squares	DF	Mean Square	F-Value	*Prob. > F
2FI Model	1.11	5	0.22	7.94	<0.0022
Lack of Fit	0.26	7	0.038	3.48	0.1227
<i>R</i> <sup>2</sup> = 0.7831		Adjusted <i>R</i> <sup>2</sup> = 0.6845		Predicted <i>R</i> <sup>2</sup> = 0.3031	
Adequate Precision = 9.258		PRESS = 0.98			
<b>Note:</b> * <i>p</i> < 0.05.					

The relationship between the response (amylase concentration) and experimental levels of variables in the study were expressed in the form of linear interactive effect response surface plots (Figures 1 and 2). Statistical significant interaction (*p* < 0.05) was observed between temperature and pH (Figure 1), which showed an

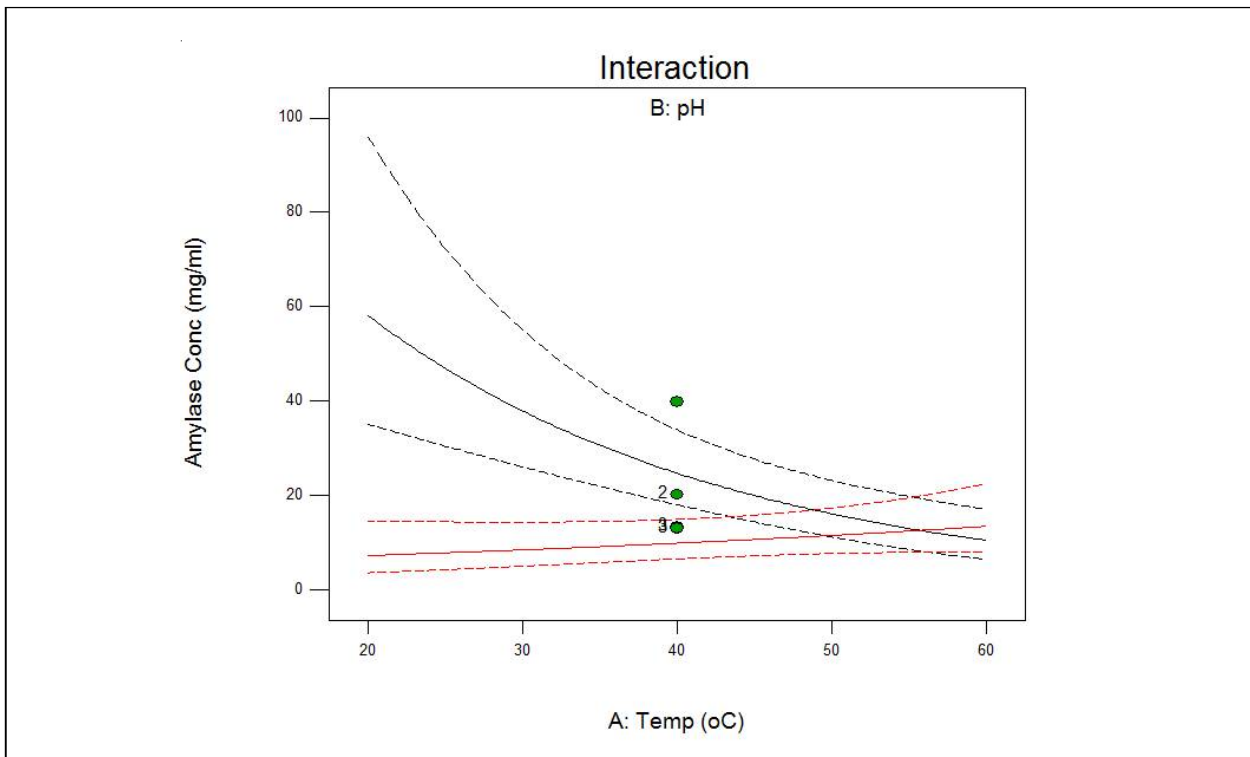


Figure 1: Interactive effect of pH and temperature in amylase yield

acidic environment yielding high amounts of amylase albeit at lower temperatures. However, increasing the temperature did not yield any significant increase of the product rather it decreases the yield. Although a non-statistically significant interaction existed between pH and substrate (Figure 2), a high yield of amylase was obtained in acidic environment and low substrate concentration.

Maximal yield of amylase from *A. niger* was in acidic environment as earlier reported (Ellaiah *et al.*, 2002; Hernandez *et al.*, 2006; Mitidieri *et al.*, 2006; and Suganthi *et al.*, 2011). In this study, both the highest and least amount of amylase produced were in the range of an acidic environment (pH 1 – 3). On the other hand, temperature requirement and other physiological properties is based on the origin of the microbe (Sethi and Gupta, 2015). Most amylase production studies have been with mesophilic fungi within the temperature ranges of 25-37 °C (Takahiro and Shigehiko, 2011).

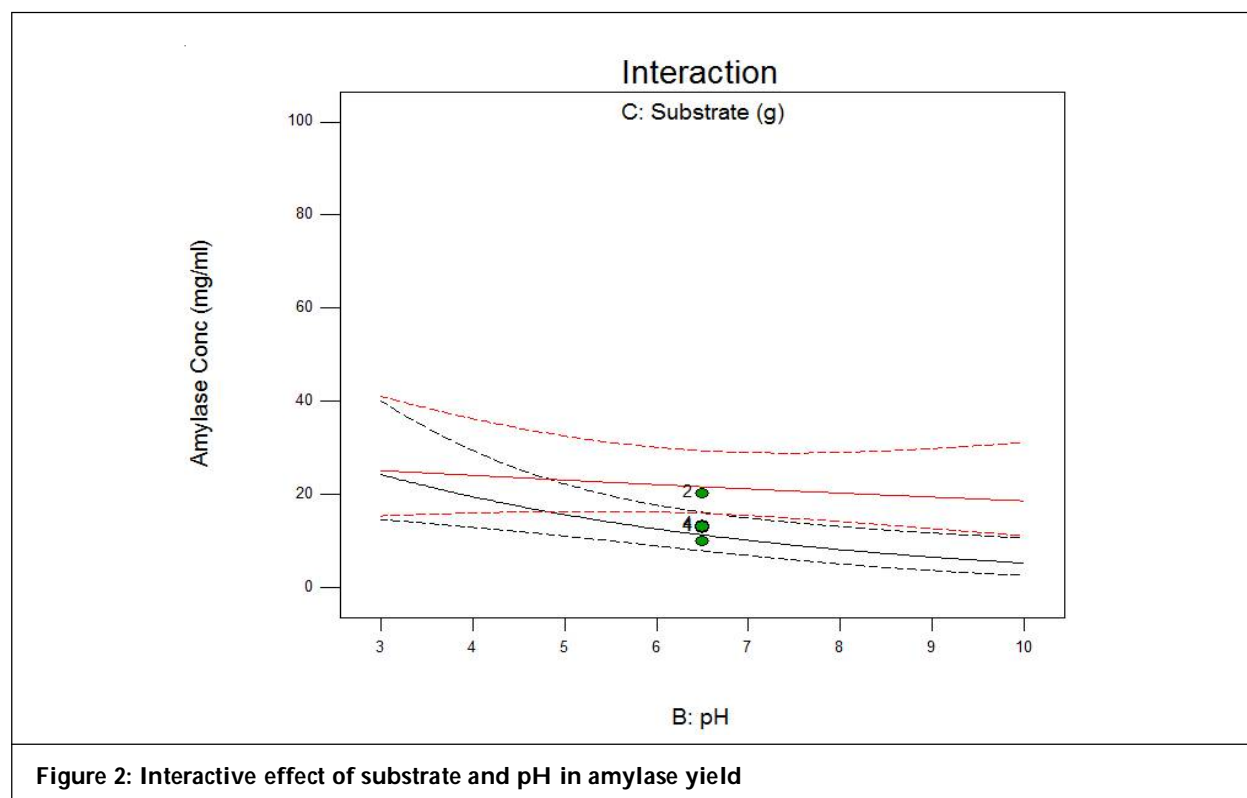


Figure 2: Interactive effect of substrate and pH in amylase yield

Similarly, as far back as 1986, a raw starch degrading amylase was produced by *A. ficum* at 30 °C by Hayashida and Teramoto (1986). In addition, yeast such as *Saccharomyces kluyveri* and *S. cerevisiae* were reported to produce alpha amylase at 30 °C (Moller *et al.*, 2004). However, Soni *et al.* (2003), Hernandez *et al.* (2006) and Ahmad *et al.* (2010) suggested an optimum temperature of 50-55 °C for the thermophiles. The results of this study agree with earlier reports that production of amylases from fungi differs in their temperature requirement. Here, we report a temperature range between 20-30 °C. In contrast, the concentration of substrate has no significant effect on the yield of amylase.

#### 4. Conclusion

The results of the current study show that the significant parameters in amylase production using *A. niger* while utilizing rice bran as an alternative substrate is pH and temperature. Therefore, the ability of using rice bran as an agricultural waste in the production of bio-product calls for further research in recycling other agro wastes in other biotechnology industries.

#### Conflicts of interest

The authors declare no conflict of interest.

#### Authors contribution

Daniel Thakuma Tizhe conducted the research and wrote the manuscript while Ja'afar Nuhu Ja'afar design and supervised the research work.

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