

Production, Optimization and Characterization of Amylase Using Solid State Fermentation Method

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Abstract: Amylases are enzymes produced by a wide range of organisms. The applications of these biomolecules cut across different industries such as detergent, brewing and food industries. *Aspergillus flavus* has been well documented as one the microbes capable of degrading starch-based agricultural waste materials. This study aimed at optimizing and characterizing amylase produced using agro-waste (potato peels) as a sole carbon source. *A. flavus* was obtained from the Department of Microbiology laboratory, Adekunle Ajasin University, Akungba-Akoko, Ondo State, Nigeria. The fungus was preserved on PDA and stored at 4°C throughout the study period. This was followed by characterization and purification. Preliminary screening result showed that *A. flavus* had a clear zone of 13.33 mm (diameter of hydrolytic zone) and amylase activity of 545 U/ml. Stability assay revealed that the enzyme was stable at 50°C and 60°C by retaining 91% and 84% residual activities respectively after 4 h of pre-incubation period, while residual activities of 80% and 90% were observed at pH 6.0 and 8.0 respectively. Protein band analysis showed that the amylase from *A. flavus* had molecular weight of 35 kDa. These results indicated that the amylase produced from *A. flavus* had good catalytic properties and compared well with those from other known fungi. This study therefore recommends industrial production of amylase from *A. flavus* using potato peels as a substrate.

Keywords: Agro-Waste, Amylases, *Aspergillus flavus*, Extracellular Enzyme, Potato Peels

1. Introduction

Enzymes are important proteins because they typically act as catalysts in biological and chemical processes without being changed [1]. The drawbacks of this technology include the necessity for high temperatures and pressures for catalysis and the modest specificity of chemical catalysts, despite their widespread use. These limitations are resolved by the use of enzymes [2]. There are some advantages of enzymatic hydrolysis compared to chemical methods, these includes: biocatalysts which act under mild conditions of pH, temperature, reducing energy consumption, equipment corrosion and elimination of neutralization steps. The fundamental benefit of using enzymes, however, can be regarded as the specificity of enzymatic catalysis. Additionally, it stops the development of unwanted

byproducts that are frequently seen in catalyst-assisted reactions and makes chemical processes proceed more quickly than when chemical catalysts are employed [2].

There are several different sources of amylases, including plants, mammals, various fungi, yeast, bacteria, and actinomycetes [3]. In the industries that process starch, microbial amylases have successfully displaced chemical starch hydrolysis. The greater yields produced in the shortest fermentation period, inexpensive bulk production capacity, and ease of manipulation of microbes to produce enzymes with desired properties are few of the primary benefits of using microorganisms for amylase production [3].

For the highest possible product concentration to develop throughout the fermentation processes, it's crucial to optimize the fermentation settings, especially with regard to physical and chemical characteristics [4]. Submerged fermentation (SmF) can be used to produce α -amylases in a liquid-based

medium, whereas solid state fermentation (SSF) uses a medium based on a wet substrate that behaves as a solid that is insoluble in water and may or may not contain soluble nutrients [5]. The cost of enzyme production in submerged fermentation is high, which necessitates the use of alternative method (SSF). Also, SSF method shows high volumetric productivity with lower capital cost and energy requirements, followed by increase concentration of product, less effluent and water output and very handful fermentation equipment with easier product recovery [6]. The selection of an appropriate substrate, the substrate's particle size, the microorganisms, the concentration of the inoculum, and the substrate's moisture content are the main variables that determine the microbial production of enzymes in an SSF system. As a result, it entails checking a variety of agro-industrial materials for microbial development and product production [7].

The potential for using agricultural wastes as renewable energy and carbon sources is immense. Agriculture wastes can be used primarily in the food, feed for animals, biofuel, and medicinal industries. The initial step in the bioconversion of organic material into reducing sugars, such as glucose and xylose, is the saccharification of agricultural leftovers by microbial hydrolytic enzymes [8]. Agricultural byproducts, which are widely regarded as the best substrate, are among the most promising residues for SSF, making it an appealing alternative process. It has been claimed that an effective, low-cost fermentation medium for the manufacture of amylase utilizing agricultural byproducts exists [8]. Paddy husk, wheat bran, cassava peels, potato peels, rice processing waste and other starch containing wastes have gained importance as they support microbial growth during enzyme production.

Different nitrogen sources and sugars added to the substrates have an impact on the productivity of the enzymes because they promote the production of the enzymes and create optimal circumstances for mycelial colonization and biomass development. Additionally, ideal growing conditions for microorganisms were crucial for the highest secretion of amylase [9]. Hence, the present study was to purify and characterize amylase produced on agricultural by-product (potato peels).

2. Materials and Methods

2.1. Culture

A fungal isolate (*Aspergillus flavus*) used for this study was obtained from the Department of Microbiology laboratory, Adekunle Ajasin University, Akungba-Akoko, Ondo State, Nigeria. The isolate was preserved on PDA and stored at 4°C throughout the study period.

2.2. Amylase Production by Solid State Fermentation

Amylase enzyme was produced using a modified method of Akpan and Kareem [10]. This was carried out in 250 ml Erlenmeyer flasks containing the inoculum and 100 ml liquid Amylase Activity Medium (AAM) consisting potato peels

30g/L; peptone 5 g/L; yeast extract 5 g/L; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g/L; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01 g/L; NaCl 0.01 g/L; and 2.5 g of potato peels. The contents were thoroughly mixed and sterilized at 121°C for 15 min. The sterilized medium was inoculated with a disc of 7 mm mycelial mat of *A. flavus*. Flask containing mineral medium without inoculum was used as control. The medium was incubated in a rotary incubator shaker (150 rpm) at 28°C for 7 days [11, 12].

2.3. Enzyme Extraction

The extraction of amylase was carried at room temperature. The liquid medium was filtered through Whatman No. 1 filter paper to obtain a mycelia-free filtrate. It was centrifuged at 10000 x g for 20 min using a TGL-16 high speed at 4°C. The supernatant of the crude enzyme extract was used to assay for amylase activity [11, 12].

2.4. Enzyme Assay

Amylase was assayed by adding 1 ml of enzyme to 1 ml soluble starch in 0.1 M sodium acetate buffer (pH 5.5), and incubated at 37°C for 30 min. By adding 1 ml of 3,5-dinitrosalicylic acid reagent to 1 ml of the enzyme-substrate solution, the process was stopped. The mixture was then heated in water bath set at 100°C for 5 min and cooled on ice for 5 min after which 5 ml of distilled water was added. The color formed was read in a spectrophotometer at 540 nm against a blank containing only buffer solution. The quantity of enzyme required to release 1 μ mole of maltose is called an enzyme unit (U/ml). A unit of enzyme activity per milligram of protein was used to indicate specific activity [11, 12].

2.5. Effect of Temperature and pH on Amylase Activity

The effect of temperature on amylase activity was determined at a temperature between 25°C and 80°C using soluble starch (1%, w/v) in 0.1 M sodium acetate buffer (pH 5.5) as the substrate. Sodium acetate buffer (0.1M) was used to conduct the reactions in order to test the impact of pH on enzyme activity (pH 5 - 5.5), 0.1 M sodium phosphate buffer (pH 6.0 - 7.5) and 0.1 M tri-glycerine buffer (pH 8 - 9) at 37°C for 1 h [11, 12].

2.6. Effect of Incubation Periods on Amylase Activity

The effect of incubation period on amylase activity was carried out at different incubation periods of 24, 48, 72, 96, 120 and 144 h, after which amylase production was determined by standard assay method [13].

2.7. Effect of Substrate Concentration on Amylase Activity

Enzyme activity was determined in the reaction medium (100 ml) using either 1 g, 2 g, 3 g, 4 g or 5 g potato peel concentration [14]. Potato peels substrate was selected because of it had a high amylase activity.

2.8. Enzyme Stability Profile

The thermostability of the enzyme was determined by pre-

incubating a solution of enzyme (5 ml) for 6 h at various temperatures (25 - 80°C), with or without substrates (1% w/v). The pH stability of the enzymes were determined by mixing the enzyme solution with 0.1 M sodium acetate buffer with pH range from 5 to 9 and kept at 37°C. The residual activity was determined at regular intervals [11].

2.9. Enzyme Purification

Imarsil (1.0 g) was mixed with 100 ml crude amylase (pH 5.0) and kept at 4°C for 4 h according to the method of [15]. The supernatant was gently siphoned and further purified by adding activated charcoal (3% w/v) at 50°C for 20 min as described by Kareem *et al.* [16, 17] and then centrifuged at 2,500 rpm for 15 min. The supernatant, which contains the crude enzyme, was introduced into Sephadex G-100 column (1.5 cm x 25 cm) which had been equilibrated with 0.2 M sodium acetate buffer (pH 5.0). The column was eluted with the same buffer at a flow rate of 1 ml/min. Fractions that show amylase activity were qualitatively determined using the starch-layer plate method described by [10]. Standard assay methods were thereafter used to quantify amylase activity. In 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), the protein profile, molecular weight of the purified protein was determined; mini gels were thereafter stained with Coomassie brilliant blue to visualize the protein [11].

2.10. Data Analysis

Results of each experiment was subjected to analysis of variance (ANOVA) using general linear model option SAS. Test of significance was determined by Duncan's multiple range test at 0.5% level of probability. Error bars were represented as the standard errors of the means (\pm SEM).

3. Results

The result indicated that amylase activity observed was 545 U/ml with potato peels as agro-waste substrate (Figure 1).

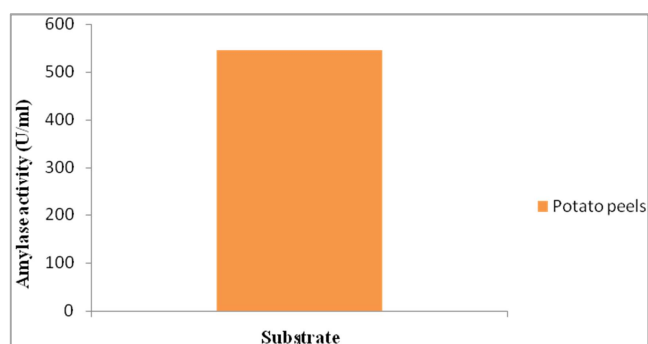


Figure 1. Production of amylase using potato peels as substrate.

3.1. Effects of pH and Temperature on Amylase Activity of *Aspergillus flavus*

The effect of pH on amylase activity of *A. flavus* was shown in Figure 2. There was gradual increase in enzyme activity with increase in pH values until an optimum pH was reached at pH 6 with 567 U/ml before a gradual decrease in enzyme activity was observed. The lowest activity was recorded at pH 2 (102 U/ml). Temperature played a crucial role in amylase activity. The result in Figure 3 shows the effect of temperature on amylase activity of *A. flavus*. There was a gradual increase in amylase activity with increased in temperature from 25°C to 50°C where the optimum temperature was observed with 583 U/ml, and then there was a sharp decline after the optimum temperature. This indicated that the enzyme is sensitive to high temperatures, where the lowest amylase activity was observed was 80 U/ml at 80°C.

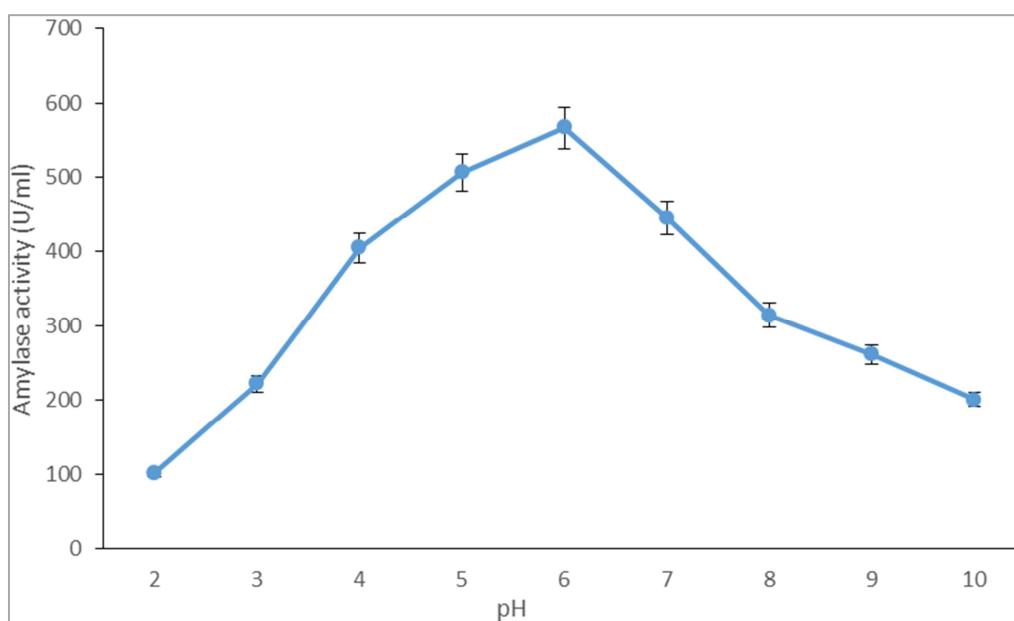


Figure 2. Effect of pH on amylase activity of *Aspergillus flavus*.

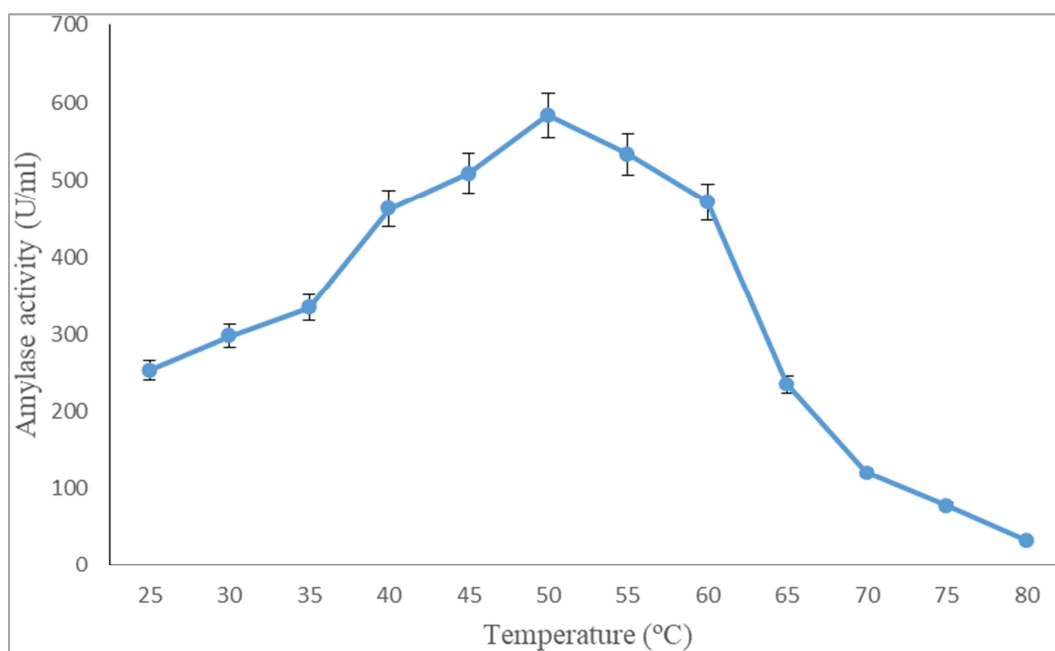


Figure 3. Effect of temperature on amylase activity of *Aspergillus flavus*.

3.2. Effect of Incubation Period on Amylase Activity

The activity of fungal amylase at different time intervals ranging from 24, 48, 72, 96, 120 and 144 hours of incubation showed that as the incubation time increase amylase activity

also increase and maximum amylase production was obtained after 72 hours of incubation. Beyond this period relative activity of enzyme was decreased (Figure 4).

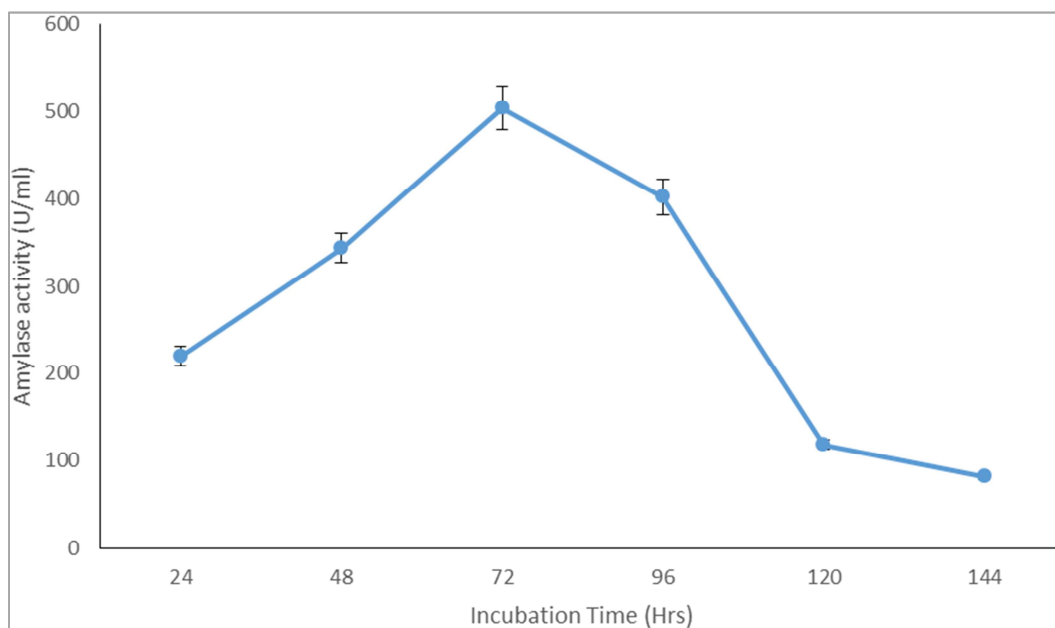


Figure 4. Effect of incubation time on amylase activity of *Aspergillus flavus*.

3.3. Effect of Substrate Concentration on Amylase Activity

Amylase was produced by *A. flavus* at varied substrate concentrations (1 g, 2 g, 3 g, 4 g and 5 g) of potato peels. At 1 g concentration, the enzyme activity was 190 U/ml. there was a gradual increase in the enzyme activity until the optimum concentration was reached at 3 g with 520 U/ml

after which a gradual decline in enzyme activity was observed (Figure 5).

3.4. Stability Profile of Amylase from *Aspergillus flavus*

The effect of pH on the stability of amylase showed that the enzyme is more stable at pH values 6.0 - 8.0 while retaining substantial enzyme activities of 85%, 90% and

80% at pH values 6.0, 7.0 and 8.0 respectively after 4 h of incubation (Figure 6). The result of thermal stability of amylase showed that it was stable at 50°C and 60°C by

retaining 91% and 84% residual activity respectively after 4 h of pre-incubation period (Figure 7).

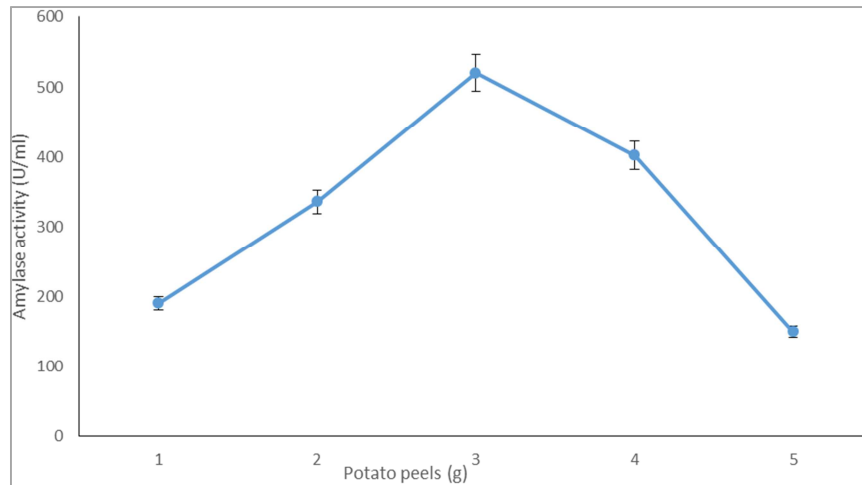


Figure 5. Effect of substrate concentration on amylase activity of *Aspergillus flavus*.

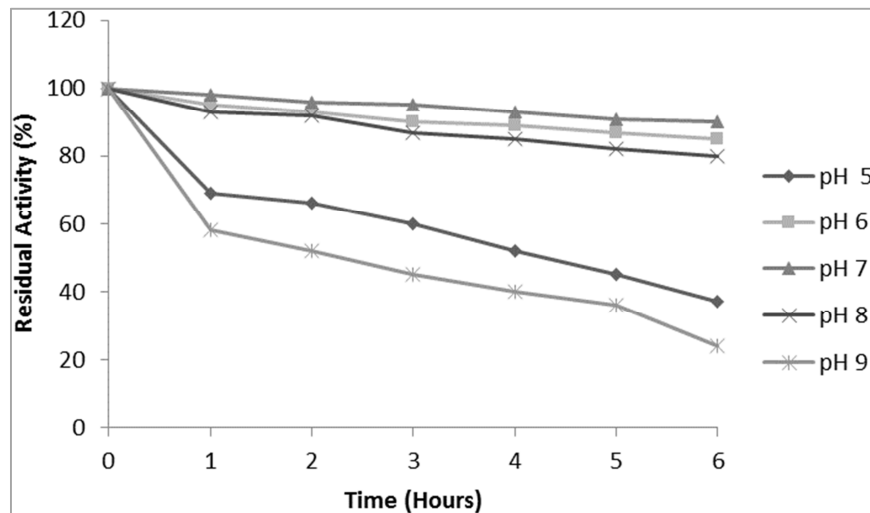


Figure 6. pH stability profile of amylase from *Aspergillus flavus*.

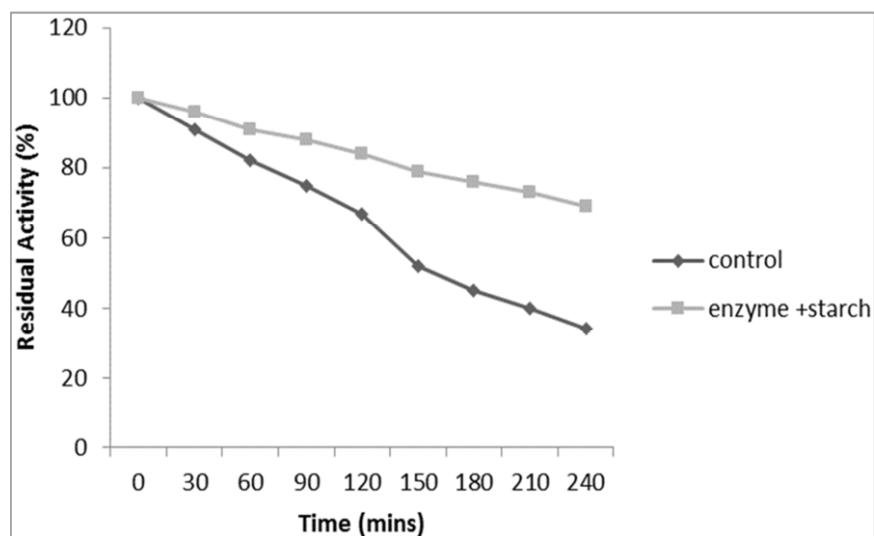


Figure 7. Thermostability profile of amylase from *Aspergillus flavus*.

3.5. Enzyme Purification

In this study, purification of crude fungal amylase indicated that a 9.83-fold purification was obtained with a final yield of 45% of total amylase in a 3-step purification procedure (Table 1).

Table 1. Purification of amylase from *Aspergillus flavus*.

Steps	Total activity (U/mL)	Total Protein (mg/mL)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	64800	297	218.18	1	100
Imarsil	53820	27.9	1929.03	8.84	83
Activated charcoal	39480	18.9	2088.88	9.57	61
Sephadex G-100	28840	13.44	2145.83	9.83	45

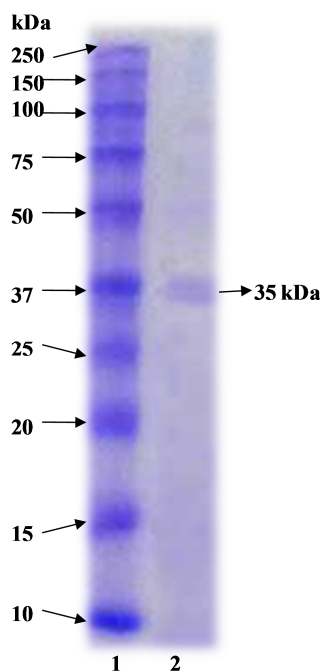


Figure 8. SDS-PAGE analysis of amylase from *Aspergillus flavus*. Lane 1: molecular weight standard markers and Lane 2: purified amylase band of molecular weight approximately 35 kDa.

4. Discussion

Fungi represent primary organisms for enzyme production and secondary metabolites. *Aspergillus* is the most prevailing amylolytic genus [18]. *Aspergillus* is suitable for solid state fermentation because its morphology facilitates colonization and penetration into solid substrates as posited by [19]. Microorganisms utilize various substrates as nutrient source for growth and metabolic activities and subsequently produce metabolism-related products such as enzymes [20]. Enzymes are relatively expensive to produce, while the cost of raw materials accounts for almost one third of such enzyme production costs. Agricultural wastes lessen the final fermentation media cost and have gained importance for bio-economic of amylase under solid state fermentation [20]. Moreover, these agro wastes can be employed in many large-scale industrial fermentation processes as an effective renewable bio-energy source. Furthermore; it can serve as the suitable substrate for growth of microorganisms and production of low-cost high-valued products such as enzymes [21].

Amylolytic microorganisms are present in soils and agricultural wastes [22, 40]. Potato peels has been reported as the best substrate for amylase production, this was contrary to the study of Khan and Yadav [23]; Nunez *et al.* [24] who reported wheat bran as the best substrate for amylase production by *Rhizopus microsporus* var. *oligosporus* and *A. niger*. Agro-industrial residues have been used to produce amylase in previous studies [18, 19].

The enzyme production by filamentous fungi is reported to be influenced by numerous factors, such as pH, temperature, carbon and nitrogen sources [25]. Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion [26]. This study revealed that as the pH value increases, amylase activity also increases with its highest value obtained at pH 6.0. This agrees with the findings of Shaliniet *al.* [27] and consistent with those obtained by Saxena and Singh [28]. Although the results were not the same with those reported by Vijayaraghavan *et al.* [29] who showed that amylase activity was optimum at pH 7.0. Similarly, Tochukwu and Bartholomew [30] revealed that optimum pH for amylase activity by *Fusarium* sp was 6.5.

The effect of temperature showed that the enzyme activity was greatly affected by high temperature. The inactivation at high temperature can be due to amino acid destruction or hydrolysis of the peptide chain [41]. Enzyme activity was optimum at 50°C with 583 U/ml, this agrees with the study of Vijayaraghavan *et al.* [29] and also with the results from the study of Saxena and Singh [28]. Further increase in temperature resulted in a decreased enzyme activity [40]. In addition, it may be due to the production of a large amount of metabolic heat, the fermenting substrate temperature shoots up, thereby inhibiting microbial growth and enzyme formation [40]. Temperature above 50°C led to reduced amylase production [41]. Furthermore, Tochukwu and Bartholomew [30] reported that *Fusarium* sp. amylase showed optimal activity at 50°C. As well as, Raul *et al.* [31] recorded maximum enzyme activity at 40°C and pH 7.1. These characteristics (pH and temperature) are good enough to make amylase from this study suitable for industrial purpose.

Conversely, Gupta *et al.* [32] reported that the production of α -amylase by *A. niger* was found to be the best at pH 5 and 30°C after 5 days incubation. Below and above this pH value, production of α -amylase was significantly lower. Also,

Oyelekeet *al.* [33] in their study found that *Bacillus megaterium* is a good producer of extracellular amylase at high temperature (60°C) and pH 7. Moreover, Zaferanlooet *al.* [34] reported that maximum production of α -amylase by fungus *Preussia minima* was obtained at 25°C and pH 9.

The amylase activity was affected by incubation time. It was observed that there is an increase in amylase activity as the incubation time increases up till 72 h (optimum activity). The maximum amylase activity (502 U/ml) was observed after 72 h with *A. flavus*. This finding corroborates the results of Ratnasriet *al.* [35] who recorded optimum incubation period for *A. fumigatus*. In like manner, other studies suggested that the highest amylase production was after 2–4 days of incubation time [36].

The result of thermal stability showed that the amylase was stable between 50°C and 60°C by retaining 91% and 84% residual activity respectively after 4 h of incubation, this agrees with the findings of Kareem *et al.* [37] that the enzyme was stable at 50°C and 60°C by retaining 92% and 82% residual activity respectively, after 2 h incubation period. The *A. flavus* retained 80% activity at 70°C after 30 min incubation period in the absence of substrate, this might be due to the affinity of these enzymes for metal ions in the production medium which has made the enzyme thermo tolerant. It has been reported that thermostability of amylase enhances bioconversion of starch by ensuring maximal efficiency and reduction of microbial contamination [38]. Since the industrial application of amylases require high reaction temperatures for optimal efficiency with minimal contamination, thermostable amylases are now of utmost importance in biotechnological processes.

The effect of pH on the stability of amylase showed that the enzyme was more stable at pH values 6.0–8.0 while retaining substantial enzyme activities of 85%, 90% and 80% at pH values 6.0, 7.0 and 8.0 respectively, after 6 h of incubation. Similar results had been reported by Kareem *et al.* [37]. At pH 9.0, the enzyme retained 58% activity after 1 h incubation. This property might offer *A. flavus* as a candidate in industrial application.

A. flavus was purified in a 3-step purification procedure using imarsil, activated charcoal and Sephadex-G-100 to achieve a 9.83-fold purification with a final yield of 45%. Kareem *et al.* [16] reported that partial purification of enzyme with activated charcoal prior to gel filtration will ensure a high purification fold by circumventing some associated problems such as difficulty of scaling up and plugging when treating crude extracts which often contain viscous and particulate materials.

The result of electrophoretic analysis of purified amylase showed that the purified amylase has a single major protein band with corresponding molecular weight of 35 kDa. Kareem *et al.* [38] had reported glucoamylases from *Rhizopus* species has two major protein bands with corresponding molecular weight of 36 kDa and 50 kDa. This finding is consistent with the previous reports on the molecular masses of amylases from *Penicillium janthinellum* (42.7 kDa) [39], in addition, molecular weights of amylases

from various microbial sources vary from 10 to 210 kDa.

5. Conclusion

Amylases have potential application in the food fermentation, textile, paper and pharmaceutical industries. Low yield of enzymes and availability of substrate have always been among the problems associated with commercial production of amylases. Solid state fermentation was carried out with potato peels to serve as a good and readily available substrate for a cost effective growth medium for *A. flavus*. Findings from this study revealed that this enzyme could reduce the process time required for starch hydrolysis, and the thermal stability of this enzyme is a desirable feature for the bio-economy. Therefore, amylase from *A. flavus* is suitable for application in starch, food and related industries.

Conflict of Interests

The authors declare that they have no competing interests.

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