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Solid-State Fermentation and Characterization of α-Amylase from a Rhizospheric Isolate of *Aspergillus flavus* associated with *Mangifera indica*

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ABSTRACT

Agronomic wastes have attracted worldwide attention as these can act as potential raw materials which may be utilized microbiologically for conversion into bio-based products and bioenergy. Amylases are industrially important enzymes that can be produced from different sources including microorganisms. Solid-state fermentation (SSF) was employed to produce amylase from the rhizosphere isolate of Aspergillus flavus and the amylase assay was performed by the dinitrosalicylic acid method with absorbance at 540 nm. Among the several agronomic wastes, sugarcane bagasse supported the highest yield of amylase. Maltose and yeast extract (1% w/w) when used as supplements enhanced the enzyme production. Optimization of the physical parameters revealed the optimum pH, temperature and incubation period for amylase production by the isolate as 6.0, 30°C and 120 h, respectively. The apparent molecular weight of the enzyme following sodium dodecyl sulphate polyacrylamide gel electrophoresis was found to be 55 kDa. The partially purified enzyme was optimally active at 80°C and pH 7.0. The enzyme being stable at elevated temperatures emphasizes that it may meet the requirements of thermostable amylase in starch processing industries, where extremes of temperatures are often involved.

Keywords: *Aspergillus flavus*, amylase, solid-state fermentation, sugarcane bagasse, enzyme activity.

INTRODUCTION

Starch-degrading enzymes such as amylases are of great significance in industrial applications like food, pharmaceutical, textile and paper industries [1]. Although amylase can be obtained from several sources such as plants, animals and microorganisms, the enzyme from microbial sources generally meet industrial demand and have almost completely replaced chemical hydrolysis of starch in starch processing industry [2].

Amylase has been derived from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungi and bacteria still dominate in industrial sectors. Fungal amylase is preferred for use in formulation for human or animal consumption involving application under acidic condition and around 37°C. Studies on fungal amylase especially in the developing countries have concentrated mainly on *Rhizopus* sp. and *Aspergillus* sp., probably because of the ubiquitous nature and non-fastidious nutritional requirements of these organisms [3].

Amylases can be produced by several strains of *Aspergillus* under both submerged fermentation (SmF) and solid-state fermentation (SSF) utilizing various food and agronomic wastes [4]. Over SmF system, SSF is reported to be the most appropriate process for developing countries in terms of less space needed for fermentation, usage of agronomic wastes as substrates, lesser operational complexity, lesser water output, better product recovery and lack of foam build up [5].

Roots of plants have great influence on the soil that surrounds it (rhizosphere) because they release upto 40% of total dry matter produced during photosynthesis in the form of organic carbon as root exudates. Rhizosphere microorganisms draw nutrients from these root exudates and participate in the nutrient cycling [6].

The objectives of the present study were isolation of amylolytic fungi from the rhizosphere, optimization of process parameters to achieve maximum yield of amylase using solid-state fermentation, partial purification and characterization of the enzyme.

MATERIALS AND METHODS

Isolation and screening of fungi producing amylase

Fungal forms were isolated from the *Mangifera indica* rhizosphere soil sample by serial dilution agar plate technique and screened for amylase production using a minimal agar medium containing (g/l): K_2HPO_4, 7; KH_2PO_4, 2; $(NH_4)_2SO_4$, 1; sodium citrate, 0.5; $MgSO_4$, 0.1; agar, 20 and distilled water at pH 7, supplemented with soluble starch (1% w/v). The medium was point inoculated with the spores of the individual fungal isolate and incubated at 27°C. Following incubation, the plates were flooded with 1% (w/v) iodine solution to determine the amylolytic potency of the isolates. The fungal isolate demonstrating the highest zone of starch hydrolysis was identified as *Aspergillus flavus* on the basis of colony morphology and microscopy [7] and was selected for further studies.

Optimization of cultural conditions

Four agronomic wastes (corn cob, rice bran, sugarcane bagasse and rice straw) were procured from the local market of Bangalore city and used for the production of amylase. 5 g of individual substrate was moistened with distilled water, autoclaved, cooled and inoculated with 1 ml of fungal spore suspension (10^7spores/ml). Following incubation at 27°C for 5 days, the solid substrate yielding the highest amylase titre was supplemented with different carbon sources such as 1% (w/w) (maltose, sucrose, soluble starch, glucose and fructose) and 1% (w/w) nitrogen sources (peptone, yeast extract, tryptone, beef extract, ammonium chloride, ammonium sulphate, urea and sodium nitrate). Various physical parameters such as pH (4, 5, 6, 7, 8, 9 and 10), temperature (25, 30, 35, 40 and 45°C) and incubation time (24, 48, 72, 96, 120, 144, 168 and 192 h) were optimized by conventional methods for maximal enzyme production. All the experiments were conducted in triplicates.

Extraction of amylase

The fermented substrate along with the fungal mycelia were mixed thoroughly and homogenised in sterile pestle and mortar using sterile distilled water (5 ml/g). The extract was filtered through Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England) and the filtrate was centrifuged at 5000 rpm for 30 min at 4ºC. The clear supernatant was subjected to amylase assay and further purification.

Assay of amylase

The reaction mixture containing 1 ml of soluble starch (1% w/v), 0.5 ml acetate buffer (0.1 M, pH 5.0) and 0.5 ml of crude enzyme extract was incubated at 37°C for 30 min. The amount of reducing sugar released during the enzymatic activity was estimated by 3, 5- Dinitrosalicylic acid (DNS) method [8] and glucose as standard. A UV-VIS spectrophotometer (Sanyo Gallenkamp, Germany) was used to read the colour development at 540 nm. One unit (U/gds) of amylase activity was defined as the amount of enzyme that releases 1 µg of reducing sugar as glucose per gm of dry substrate per minute, under standard assay conditions.

Estimation of protein content

The soluble protein content of the enzyme sample was determined by Lowry's method [9] using crystalline bovine serum albumin as the standard.

Characterization of amylase

Amylase was purified by 40% ammonium sulphate precipitation method. The precipitate obtained by centrifugation was dissolved in 0.1 M phosphate buffer (pH 7) and dialyzed overnight against 0.01 M phosphate buffer at 4°C to prevent enzyme denaturation. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was employed for the molecular weight determination of the dialyzed sample, using a broad range pre-stained protein marker (New England Biolabs, UK).

Factors affecting enzyme activity

Optimum temperature for the enzyme activity was determined by incubating the enzyme substrate reaction mixture at different temperatures (30, 40, 50, 60, 70, 80, 90 and 100ºC) for 30 min and then the enzyme assay was carried out. The effect of pH on amylase activity was determined by incubating the reaction mixture with different buffers of 0.1 molarity - citrate buffer (pH 4, 5 and 6); phosphate buffer (pH 7 and 8), glycine-NaOH buffer (pH 9 and 10).

Statistical analysis
Effect of each parameter was studied in triplicate and the data have been graphically represented as the mean ± S.D. of triplicates (n = 3). ANOVA was performed using Microsoft Excel 2007. *P* values < 0.05 were considered significant with a confidence limit of 95%.

RESULTS AND DISCUSSION

Amylases are one of the most important enzymes used in industrial processes. Although amylases in general and α-amylases in particular, are in use in starch liquefaction and other starch based industries for many decades and a number of microbial sources exists for the efficient production of this enzyme, the commercial production is limited to only a few selected strains of fungi and bacteria [10].

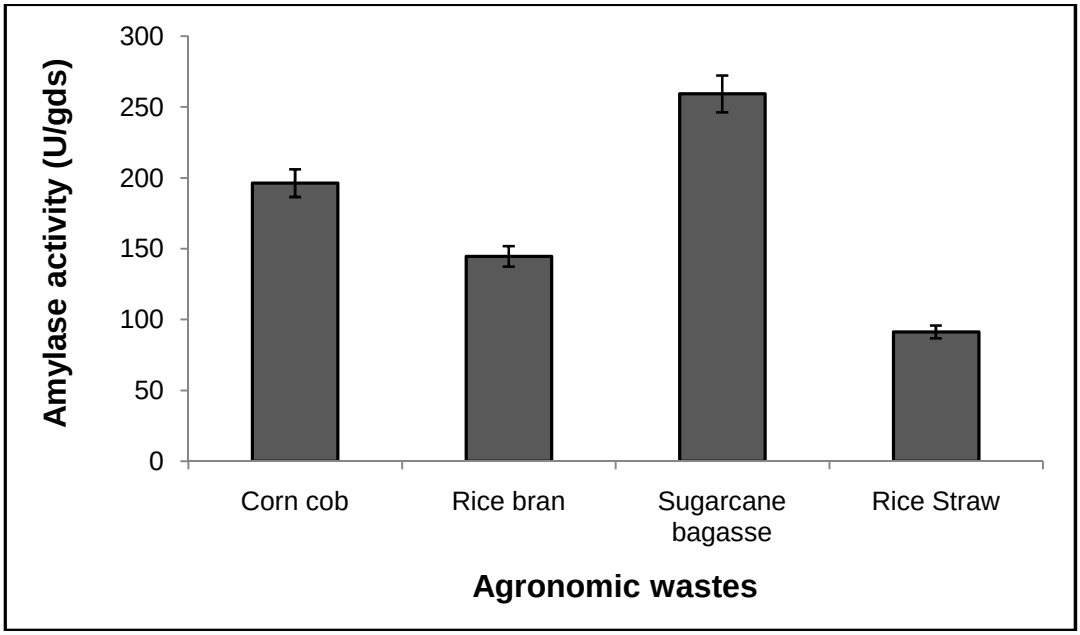

Fig. 1: Effect of different agronomic wastes on α-amylase production. Data represent mean ± S.D. (n=3); *P* < 0.05

α-amylase (endo-1, 4, α-D- glucan glucohydrolase) is an extracellular enzyme that randomly cleaves the 1, 4, α-D glucosidic linkages between adjacent glucose units and hydrolyzes single glucose units from the non-reducing ends of amylose and amylopectin in a stepwise manner. α-amylase is secreted as a primary metabolite and its secretion is growth associated [11].

Screening of solid substrate for α-amylase production
Sugarcane bagasse proved to be the best solid substratum for the production of α-amylase although the other agronomic wastes supported considerable growth and enzyme production (Fig. 1). *Aspergillus niger* strain UO- 01 when inoculated on different solid substratum, sugarcane bagasse resulted in the highest amylase production [12].

Moisture is essential for the ramification of the fungal hyphae into any substratum and acts as the prime factor to maintain the turgor pressure at the tip of the fungal hyphae. The air pockets in between the fibres of bagasse trap air, thus preventing oxygen tension to develop in the later stages of fermentation. These above factors alone or collectively could have played significant role in determining sugarcane bagasse as the best solid substratum.

Effect of carbon sources on amylase production
Enzyme substrates are typical inducers with different potentials for enzyme induction. Hence, the exogenous addition of various carbon sources to the solid medium may improve the growth of the participating microorganism and thus the product yield [13].

While studying the effect of carbon sources on α-amylase production, compared to other carbon sources, maltose was found to enhance amylase production (98.14 U/gds) and was selected as an inducer for rest of the study (Fig. 2). Maltose acted as the best carbon supplement possibly because of its interaction as an inducer with the repressing protein. Such an interaction might have prevented its further interaction with the operator gene, which in turn favoured the occurrence of both the transcription process and enzymatic synthesis [11].

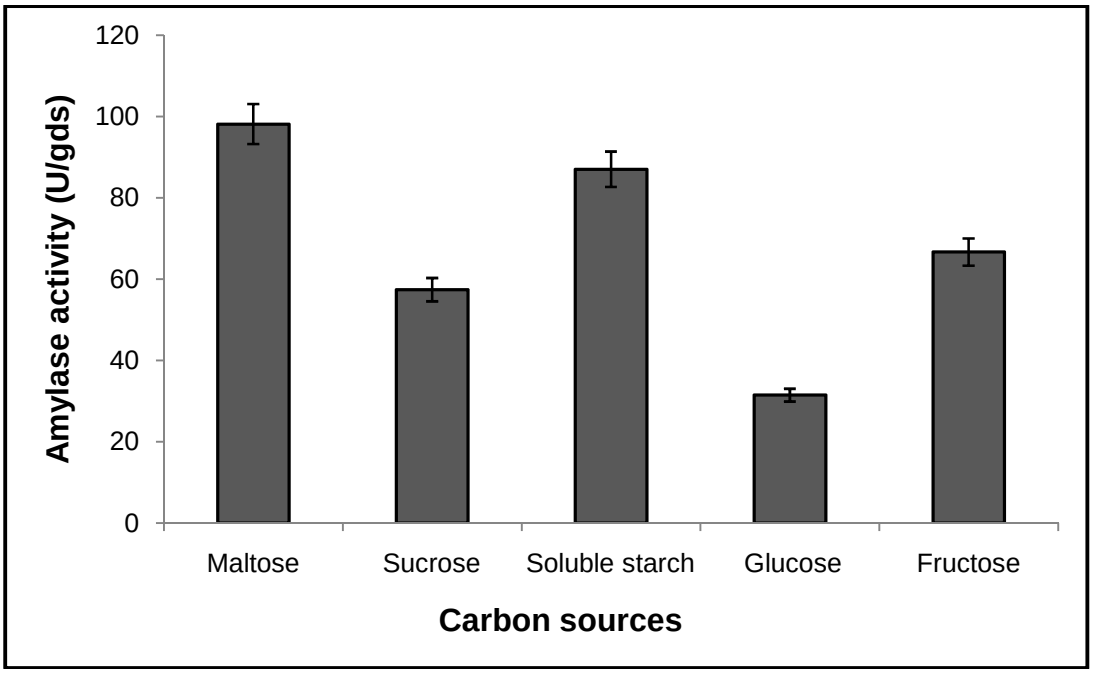

Fig. 2: Effect of different carbon sources on α-amylase production. Data represent mean ± S.D. (n=3); *P* < 0.05

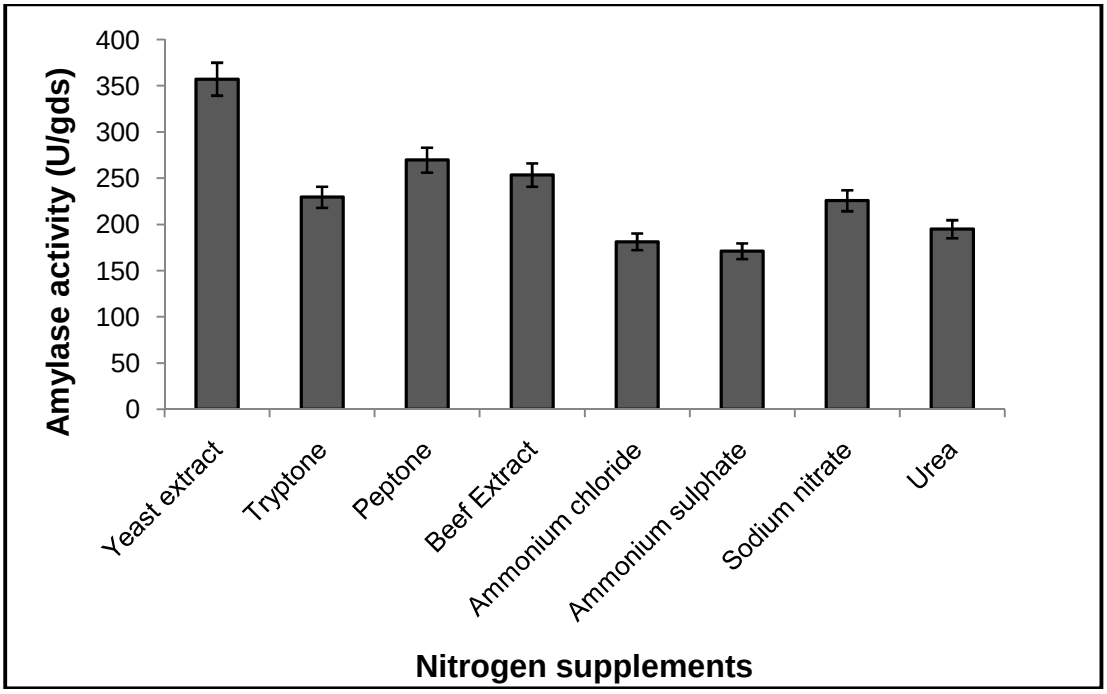

Fig. 3: Effect of nitrogen supplements on α-amylase production. Data represent mean ± S.D. (n=3); *P* < 0.05

Identical observations were reported earlier for *Bacillus cereus* [14]. Likewise, while studying the effect of various carbon sources on amylase production by *Penicillium fellutanum*, maltose supported highest amylase activity of 146 U/mL, which was 7% higher than sucrose [2]. It was reported that maltose was the best inducer of α-amylase of *Aspergillus oryzae* among the carbon sources tested [15]. Previous workers have reported that induction of amylase requires substrates having α-1,4 glucoside bond, including starch or its hydrolytic product like maltose [16]. Whereas, in *Humicola* sp, the extracellular amylase activity was induced by maltose and cellobiose, suggesting that the mechanisms of induction of amylase were not specific for α-1,4 glucoside bonds [17].

At par with our result, earlier studies suggest that addition of different carbon sources like glucose, sucrose and fructose inhibited the growth and amylase production in case of *Aspergillus awamori* [18]. The inhibitory effect of

glucose is due to catabolic repression, where the cell blocks the enzyme synthesis when substrates of easier assimilation are available [19].

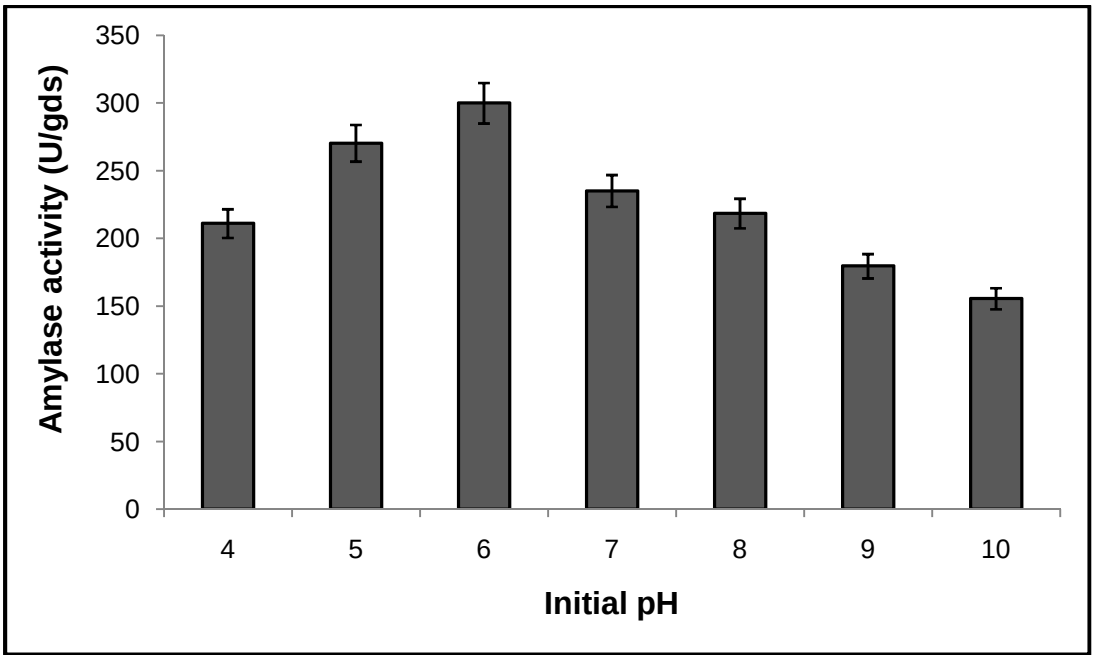

Fig. 4: Effect of initial pH on α-amylase production. Data represent mean ± S.D. (n=3); *P* < 0.05

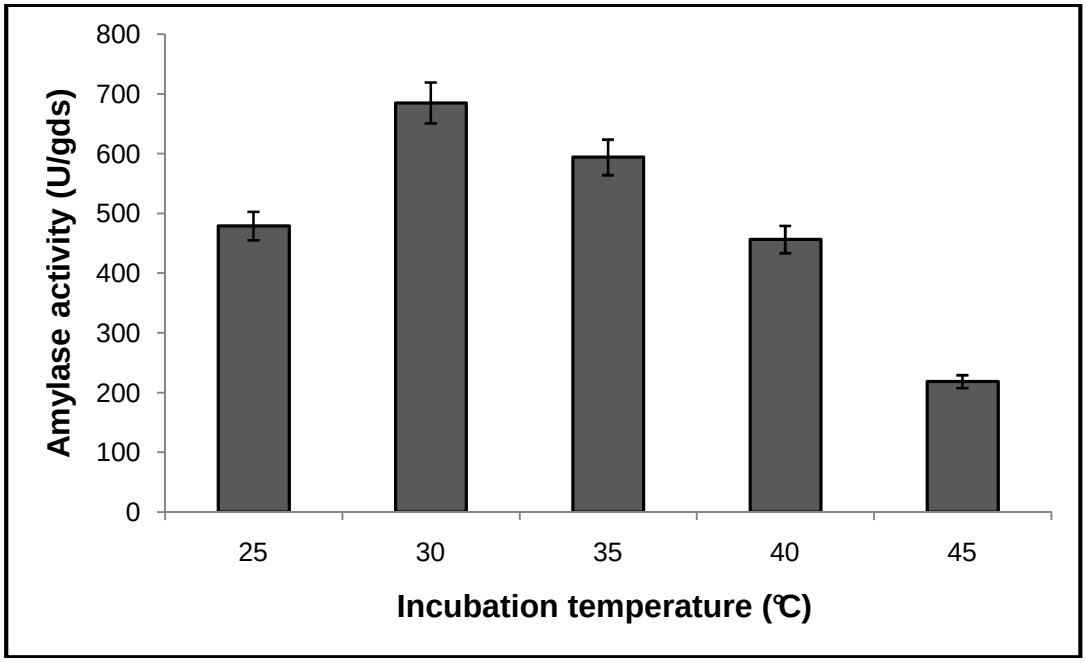

Fig. 5: Effect of incubation temperatures on α-amylase production. Data represent mean ± S.D. (n=3); *P* < 0.05

Effect of nitrogen supplementation on amylase production

Next to the carbonaceous nutrients, nitrogen compounds are secondary energy sources for organisms and play an important role in growth and production of metabolites. In the present study, effect of nitrogen supplementation on α-amylase production under SSF, showed that yeast extract supported the highest production of α-amylase by the rhizosphere isolate of *A. flavus* (1071.66 U/gds) (Fig. 3).

Yeast extract was the best nitrogen source for α-amylase production, probably because it comprise of lysed yeast cells, abundant in minerals, vitamins, coenzymes and nitrogen components [20]. Earlier workers reported that,

amylase production by *Aspergillus oryzae* and a mangrove isolate of *Aspergillus flavus* under SSF using sugarcane bagasse was greatly influenced by organic nitrogen sources especially yeast extract [11, 21].

Certain literatures reported that apart from yeast extract, other organic nitrogen sources supported amylase production by various *Aspergillus* species [22]. In other studies, inorganic nitrogen sources such as NH_4NO_3 yielded the highest amylase titre [23].

Effect of initial pH on amylase production

Since microorganisms are sensitive to the concentration of hydrogen ions present in the medium, pH is considered an important factor that determines the growth, morphology and product formation [10]. Each organism possesses a characteristic pH range for its growth and activity with an optimum value in between the range [11].

Fig. 4 demonstrates that α-amylase production was significant over a wide range of pH values and was maximum at pH 6.0 (900.00 U/gds). Similar results were obtained for other fungi such as *Thermomyces lanuginosus, A. niger*, *Penicillium janthinellum* NCIM 4960 where they yielded significant levels of α-amylase at pH 5.0-6.0 [10, 24, 25].

Effect of temperature on amylase production

Among the fungi, most amylase production studies have been conducted with mesophilic fungi within the temperature range of 25-37°C [26]. Fig. 5 demonstrates the effect of temperature on α-amylase production, where the optimum temperature for maximum α-amylase production was 30°C (685.00 U/gds). Being a mesophilic fungus, the membrane of the *A. flavus* isolate was stable when the incubation temperature fell in the mesophilic range resulting in the maximum conversion of the substrate to reducing sugars. Decrease in enzyme yield at lower or elevated temperatures resulted from the reduced metabolic activity and impaired action of the cell membrane of the fungus.

Our result is in perfect accordance with the results obtained by other workers, where raw starch degrading α-amylase was produced respectively by *Penicillium fellutanum* and *A. flavus* at 30°C [2, 11].

Effect of incubation period on amylase production

Enzyme production by each strain is based on the specific growth rate. Incubation time influences the growth rate of the culture and its enzyme synthesis ability, the two main aspects of fermentation process.

The enzyme activity was determined after every 24 h of incubation in order to determine the optimum incubation period for maximum production of extra cellular α-amylase. Increase in the incubation period beyond 120 h resulted in a decrease in the production of α-amylase by the isolate (Fig. 6). It may be due to the fact that beyond this time period the production of by-products and toxic metabolites resulted in the depletion of nutrients essential for the growth and enzyme production.

Similarly, the maximal productivity of amylase, was achieved after 120 h at 30°C on a wheat bran substrate using *A. flavus, A. oryzae* and *Aspergillus awamori* respectively [11, 25, 27]. However, the purified α-amylase isolated from *Aspergillus falvus var. columnaris* had a maximum activity at pH 6.2, after 30 h of incubation [28].

Characterization of α-amylase

Effect of pH on amylase activity

A change in the pH of the reaction mixture results in the change in the ionic character of the amino and carboxylic acid components of the enzyme. This in turn affects both the catalytic site and conformational status of the enzyme thereby altering its activity [29].

The effect of pH on the enzyme activity indicated that the amylase was more active over a wide range of pH, with the highest activity (461.85 U/gds) at pH 7 (Fig. 7). Studies on amylolytic enzyme synthesized by *Aspergillus flavus* associated with mouldy bread revealed that the pH of the reaction mixture influenced the activity of the enzyme, optimum activity being at pH 7.0 [30].

Effect of temperature on amylase activity

The insoluble nature of starch is lost when gelatinization is adopted, a process where starch is heated at temperatures around 100°C. The resulting viscous slurry poses a problem during mixing and pumping. To check this, gelatinization is coupled with liquefaction which results in partial hydrolysis and loss of viscosity. This action is mediated by thermostable α-amylases which remain active at temperatures around 70-100°C [25].

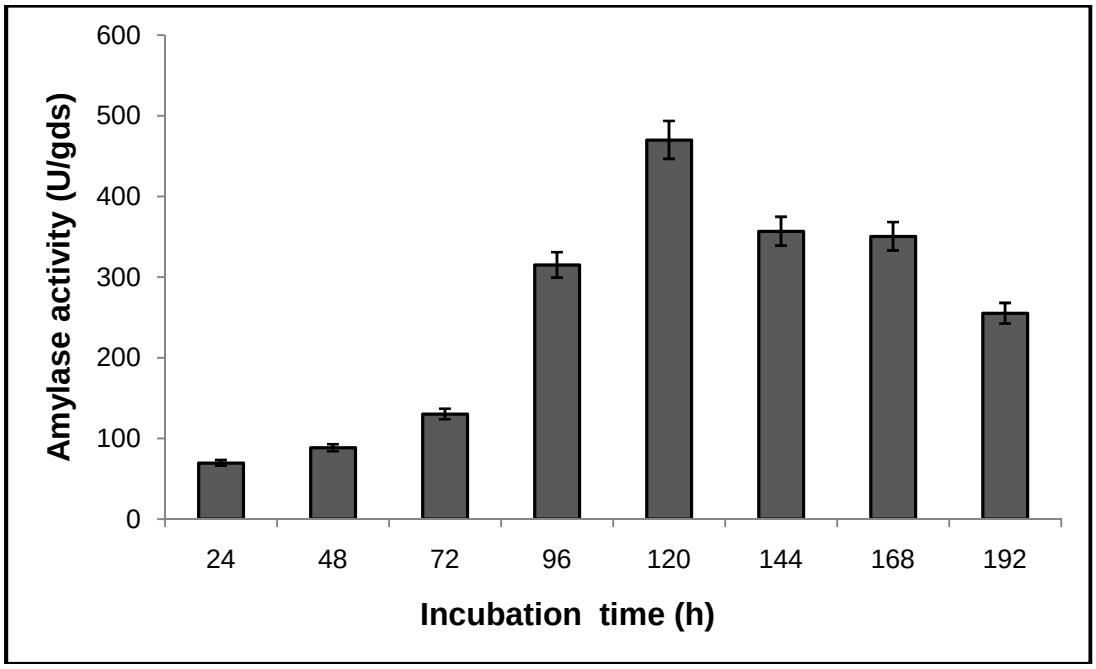

Fig. 6: Effect of incubation time on α-amylase production. Data represent mean ± S.D. (n=3); *P* < 0.05

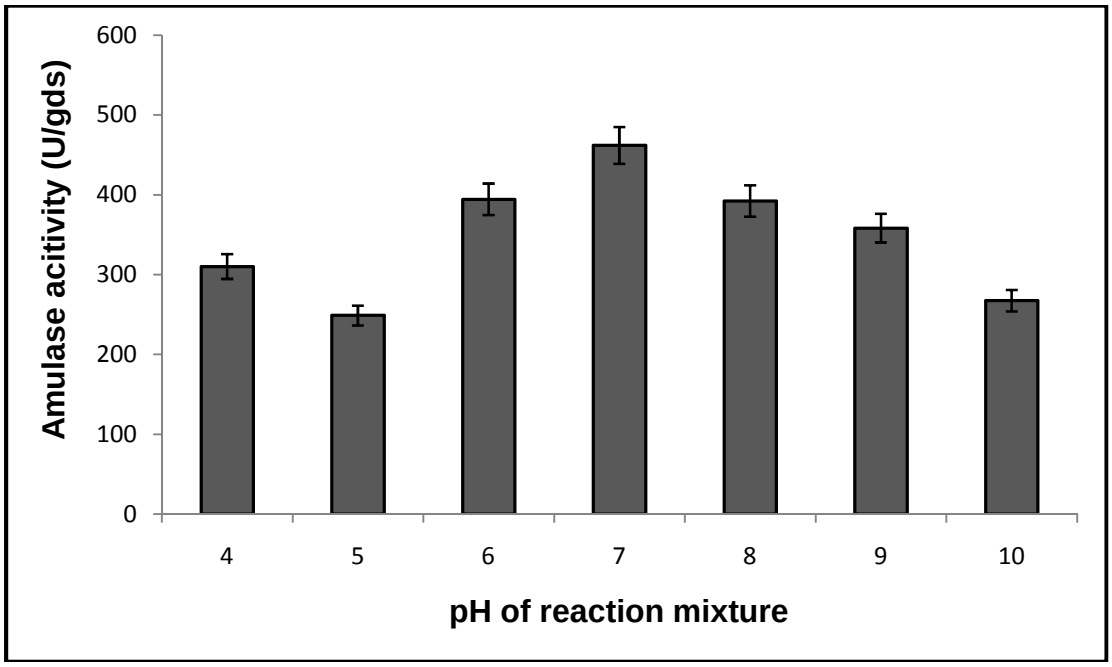

Fig. 7: Effect of different pH of reaction mixture on α-amylase activity. Data represent mean ± S.D. (n=3); *P* < 0.05

The influence of temperature on the activity of crude amylase showed that enzyme activity increased progressively with increase in temperature, reaching a maximum (629.07 U/gds) at 80°C (Fig. 8). Above 80°C, there was a reduction in the amylase activity. In support of our results are the observations of many workers. Effect of temperature and pH on the activity of purified amylase of *Aspergillus niger* sp. MK 07 was evaluated and the purified enzyme showed an activity till 75°C at a pH of 6.5 [31]. A promising producer of extracellular amylases, *Aspergillus flavipes* produced new, highly stable forms of amylase with pH optima of 5.5 and 7.5 and maximum activity at 60-80°C [32].

Bacillus amyloliquefaciens and *Lactobacillus fermentum* isolated from some Cameroonian soils contaminated by starchy residues when screened for thermostable amylases showed that the crude amylase retained 100% of original activity after been heated at 80°C for 30 min [33]. The α-amylase activity of *Geobacillus thermodenitrificans* HRO10 on potato starch was optimal at pH 5.5 and 80°C [34].

Determination of molecular weight of α-amylase

Molecular weights of α-amylases from various microbial sources vary from 10 to 210 kDa. However, α-amylases from fungal sources are usually about 41.5 to 76 kDa as shown directly by analysis of cloned α-amylase genes and deduced amino acid sequences [35].

The molecular weight of the partially purified amylase, as analyzed by SDS-PAGE, showed a single protein band of approximately 55 kDa. This observation is in corroboration with the molecular weight of α-amylase, extracted from *A. flavus*. The study reported the enzyme to be homogenous on SDS-PAGE with a molecular weight of 55 kDa [11]. The α-amylase from *Aspergillus flavus* F_2Mbb isolated from some enviro-agro-industrial wastes was found to have a molecular weight of 56 kDa which is in agreement with our result [36].

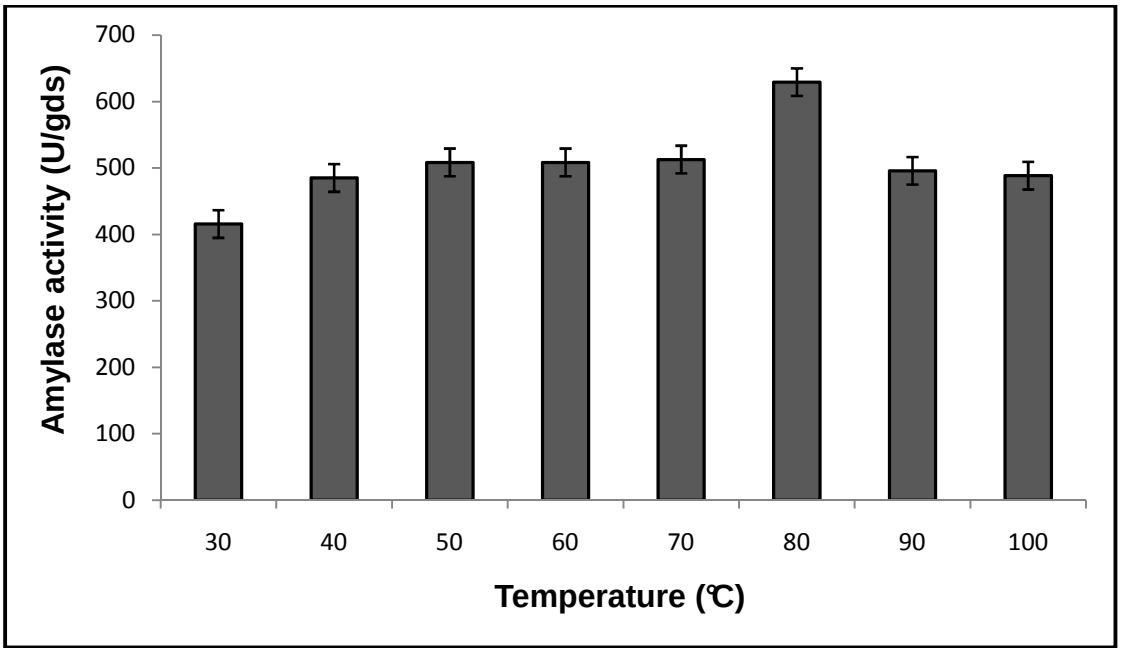

Fig. 8: Effect of temperature on α-amylase activity. Data represent mean ± S.D. (n=3); $P < 0.05$

CONCLUSION

The rhizosphere isolate of *A. flavus* proved to be an efficient producer of α-amylase under solid-state fermentation using sugarcane bagasse. The remarkable stability of the enzyme even at temperature as high as 80°C evokes the idea that it can be exploited in the starch processing industry.

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