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Research Article



Amylase production from *Aspergillus flavus* using millet pomace by Solid State Fermentation (SSF)

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Abstract

The industrial importance of amylase in the hydrolysis of starch cannot be overemphasized. In this study, amylase was produced from *Aspergillus flavus* by solid state fermentation using millet pomace induced with soluble starch. This amylase was further characterized and precipitated with ammonium sulphate. The specific activity of crude amylase was 19.12 ± 1.83 . Ammonium sulphate fractionation of 80 % resulted in enhanced specific activity of 39.49 ± 7.73 . Optimum pH, temperature, and substrate concentration of the amylase activity were 6, 50 °C and 1 % respectively. Amylase was activated in the presence of Ca^{2+} , Mn^{2+} and Co^{2+} , with Ca^{2+} having the highest activity of 124 %. Zn^{2+} , Cu^{2+} , and K^{2+} inhibit amylase activity. The result indicated that amylase produced from this *Aspergillus flavus* can be used industrially at a relatively high temperature, slightly acidic and in the presence of some divalent ions.

Keywords: Millet pomace, amylase, *Aspergillus flavus*, activity, ammonium sulphate, solid state fermentation (SSF).

Introduction

Amylases are enzymes which catalyse the hydrolysis starch into sugars such as glucose and maltose or specific malto-oligosaccharide or mixed maltooligosaccharides. Isolated enzymes were first used in detergents in the 1914, their protein nature proven in 1926 and their large scale microbial production started in 1960s. A large number of industrial processes in the areas of industrial environmental and food technology utilize enzymes at some stage or the other. Current advancements in biotechnology are yielding new application for enzymes (Pandey *et al.*, 1992). Now a day, microbial enzymes are produced in industry and these include lipase, protease, amylase, cellulase, pectinase e.t.c. The most widely used enzyme in the industry for starch hydrolysis is amylase. Sources of amylases in bacteria, yeast and other fungi have been reported and their properties described by (Liu and Xu, 2008).

Due to their diversity, fungi have been recognized as a source of new enzymes with useful and/or novel characteristics (Bakri *et al.*, 2009). Microbial enzymes are widely used in industrial processes and amylase is one of the most important industrial enzymes, having applications in industrial processes such as food, brewing, baking, textiles, pharmaceuticals, starch processing, and detergents. With the advent of new frontiers in biotechnology, the spectrum of amylase applications has expanded into many new fields such as clinical, medicinal and analytical chemistry (Pandey *et al.*, 1999).

Amylases are some of the most versatile enzymes in the industrial enzyme sector and account for approximately 25% of the enzyme market. The morphology and physiology of the molds used enable them to penetrate and colonize various solid

substrates. Solid State Fermentation (SSF) employs the use of various agro industrial wastes as substrate for both physical support and source of nutrients (Sidhu *et al.*, 1997). Owing to the increasing demand for amylase and its magnificent potentiality in the industrial sector, this study was done to produce amylase from *Aspergillus flavus* using millet pomace by solid state fermentation.

Materials and Methods

Isolation

Soil sample from cassava waste dump site was collected in a sterile container. Ten fold serial dilution of the sample was carried out and inoculation of a prepared Potato Dextrose Agar (PDA) medium containing 0.1 % (w/v) streptomycin was done in duplicate. The inoculated plates were incubated at 25 °C for 5 days.

Lactophenol cotton blue staining

A speck of fungal cultures was placed on a clean glass slide, a drop of lactophenol cotton blue stain was then mixed with the culture. A clean cover slip was placed over the culture and viewed under the microscope ($\times 40$) and the morphology of mold isolates was observed.

Screening for amylase production

The amylase screening of *A. flavus* was determined using the starch agar plate method as described by Bertrand *et al.*, (2004). This was done by inoculating the identified organism into Potato Dextrose Agar medium which was supplemented with 2 % of soluble starch. The agar plates were then incubated at 25 °C for 2 days. After the incubation period, Lugol's iodine solution was added to the culture plate to identify the zones around the cultures. The diameter formed after the addition of iodine solution was measured to represent the amylase activity.

Amylase production medium

Two hundred grammes of flowered millet pomace was moistened with 280 ml of mineral salt medium containing: 0.35 g, $MgSO_4 \cdot 7H_2O$; 0.32 g, CaCl; 0.18 g, $FeSO_4 \cdot 7H_2O$; 0.36 g, $(NH_4)_2SO_4$; 0.34 g, KH_2PO_4 ; 0.84 g, KNO_3 ; 5.8 g, soluble starch, at pH 7.2. The

medium was sterilized by autoclave (Dixon portable autoclave DA 025T model) at 121 °C for 60 minutes and allowed to cool. Spores of 3 days young culture of *Aspergillus flavus* was aseptically transferred into the millet pomace medium with 10 ml sterile distilled water containing 2 drops of tween 80. This was incubated for 5 days at 25 °C under aseptic condition.

Enzyme extraction

At the end of incubation period, the sporulated medium was dissolved in phosphate buffer, pH 4.5 for 60 minutes. The mixture was sieved with muslin cloth. Filtrate obtained was separated from fungi spores using centrifuge (Hettich Zentrifugen D-78532 Tuttlingen model) at 6000 rpm for 20 minutes; supernatants were decanted and used as crude amylase.

Enzyme activity

One milliliter of amylase extract was added to 1ml of 1 % soluble starch in citrate-phosphate buffer (pH 6.5) and incubated in a water bath at 40 °C for 30 minutes. Blank consisting of 2 ml of the enzyme extract that was boiled for 20 minutes (boiling inactivates the enzyme) and starch solution was added and treated with the same reagent as the experimental tubes. The reaction was stopped by adding 2 ml of DNSA reagent (1.0 g of 3,5, dinitrosalicylic acid, 20 ml of NaOH and 30 g of sodium potassium tartarate in 100 ml), boiled for 5 minutes at 100 °C and allowed to cool (Bhimba *et al.* , 2011). Absorbance of the color development was read at 540 nm against a blank using the spectrophotometer (Unispec 23D model). One unit of enzyme activity (U) was defined as the amount of the enzyme that liberated 1.0 μ mole of maltose from starch in 1.0 μ L reaction mixture under the assay conditions.

Protein determination

Protein content of the amylase was determined following the method describe by Lowry *et al.*, (1951) using Bovine serum albumin as standard. Amylase, 0.2 ml was measured into tubes and 0.8 ml distilled water was added to it. Distilled water was used as blank while BSA standard curve was equally set up, (10 mg/ml), 1-10 mg/ml, 5.0 ml of alkaline solution was added to all the tubes, mixed thoroughly and allowed to stand for 10 minutes, 0.5 ml of Folin- C

solution was added to all the test tubes and left for 30 minutes after which the optical density was read at 600 nm wavelength in a spectrophotometer (Unispec 23D model). The protein concentration was estimated using values obtained from the standard graph.

Ammonium sulfate fractionation

Adequate amount of 200 ml crude amylase extract were first brought to 20 % saturation with solid ammonium sulfate (enzyme grade) as mentioned by Dixon and Webb (1964). The precipitated proteins were regimented by centrifugation for 15 minutes at 500 rpm. The resulted pellet was dissolved in 5 ml of 0.2 M phosphate buffer at pH 6.2. The left supernatant was applied again with ammonium sulfate to achieve 60, 70, 80, 90 and 100% saturation. Both enzyme activity and protein content were determined for each separate fraction.

Effect of pH

The effect of pH on amylase activity was determined using citrate-phosphate buffer with the pH values of 3, 4, 5, 6, 7, 8 and 9. Reaction mixture was incubated at 37 °C for 15 minutes. The reaction was stopped by adding 2 ml of DNSA reagent, boiled for 5 minutes at 100 °C and allowed to cool (Bhimba *et al.*, 2011). Absorbance of the color development was read at 540 nm against a blank using the spectrophotometer (Unispec 23D model). One unit of enzyme activity (U) was defined as the amount of the enzyme that liberated 1.0 µmole of maltose from starch in 1.0 µL reaction mixture under the assay conditions.

Effect of temperature

Effect of temperature on amylase activity was determined by incubating reaction mixture for 15 minutes at temperature of 20, 30, 40, 50, 60 and 70 °C for 15 minutes. The reaction was stopped by adding 2 ml of DNSA reagent, boiled for 5 minutes at 100 °C and allowed to cool (Bhimba *et al.*, 2011). Absorbance of the color development was read at 540 nm against a blank using the spectrophotometer. One unit of enzyme activity (U) was defined as the amount of the enzyme that liberated 1.0 µmole of maltose from starch in 1.0 µL reaction mixture under the assay conditions.

Effect of substrate concentration

Effect of substrate concentration was determined at different percentage of soluble starch at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 % in citrae-phosphate buffer (pH 6.5). The reaction was stopped by adding 2 ml of DNSA reagent, boiled for 5 minutes at 100 °C and allowed to cool (Bhimba *et al.*, 2011). Absorbance of the color development was read at 540 nm against a blank using the spectrophotometer. One unit of enzyme activity (U) was defined as the amount of the enzyme that liberated 1.0 µmole of maltose from starch in 1.0 µL reaction mixture under the assay conditions.

Effect of activators and inhibitors

Effect of activators and inhibitors on amylase activity was investigated using the following chemicals in concentrations of 10 mM: Zn²⁺, Ca⁺², Cu²⁺, Mn⁺², Mg⁺², Co²⁺, K²⁺, and Na²⁺. The samples with the presence of various metal ions was incubated for 15 minutes at 37 °C The reaction was stopped by adding 2 ml of DNSA reagent, boiled for 5 minutes at 100 °C and allowed to cool (Bhimba *et al.*, 2011). Absorbance of the color development was read at 540 nm against a blank using the spectrophotometer. One unit of enzyme activity (U) was defined as the amount of the enzyme that liberated 1.0 µmole of maltose from starch in 1.0 µL reaction mixture under the assay conditions.

Results and Discussion

The ability of some microorganisms to grow in various environments may be due to the presence of unusually large number of genes for catabolism, metabolic regulation, nutrient transport and efflux of organic molecules in their genome. Naessens, (2003), report that production of multiple forms of enzymes improve microorganisms ability to adapt to environmental modifications. In this study, amylase producing *Aspergillus flavus* was isolated from cassava waste dump site. Qualitative screening of the *Aspergillus flavus* on PDA fortified with 2 % soluble starch showed a clear zone of 23.5±0.71 against the blue-black background. This indicated hydrolysis of starch by amylase in the area of the clear zone. Alhassan *et al.*, (1992) who used *A. flavus* and *A. niger* for amylase production reported the highest amylase activity for *A. flavus*.

Promising result was obtained using dried millet pomace induced with soluble starch was used as substrate for amylase production by Solid State Fermentation (SSF). This observation agrees with the work of, Fadahunsi and Garuba (2012) who investigated various carbon sources for amylase production reported that starch stimulated the highest amylase. For the crude enzyme, amylase activity,

protein concentration and specific activity was 242.05±0.11, 12.66±0.06 and 19.12±1.83 respectively (Table 1). Ammonium sulphate precipitation at 60 to 100% concentration yield higher specific activities as shown in Table 1. However, concentration of 80% showed a gradual enhanced activity of 39.49±7.73 (Table 1).

Table 1: Ammonium sulphate fractionation of amylase

Ammonium sulphate concentration	Activity (U) ± SD	Protein concentration (mg/ml) ± SD	Specific activity (U/ml) ± SD
Crude	242.05±0.11	12.66±0.06	19.12±1.83
60%	209.45±0.95	6.52±0.06	32.12±15.83
70%	212.23±2.44	6.30±0.09	33.69±27.11
80%	225.87±0.85	5.72±0.11	39.49±7.73
90%	183.36±0.42	5.36±0.23	34.21±1.83
100%	166.27±0.53	5.24±0.06	31.80±8.83

Effect of pH on amylase activity was studied by varying the phosphate buffer pH between 3 to 9. The result presented in Figure 1 showed that the optimum pH was 6 for amylase activity produced by *A. flavus* using millet pomace. Deb *et al.*, (2013) who produced and characterized microbial amylase also reported an optimum pH of 6.5. Previous reports from Okolo *et al.* (2006) and Nagamine *et al.* (2003) explained that temperature is one of the environmental conditions affecting amylase. This study agreed to this previous report. Studies on the effect of temperature between 20 to 70 °C showed an enhanced amylase activity as the temperature was raised (figure 2). Optimum

temperature of 50 °C was observed for amylase activity produced by *A. flavus* using millet pomace. Decrease in amylase activity at temperature above 50 °C may probably be due to denaturation of enzyme protein at extremes of temperature. The effect of substrate concentration on amylase activity was investigated at range of 0.5 to 3.0 % soluble starch in citrate-phosphate buffer. Concentration of 1.0 % gave the highest activity as shown in figure 3. Kuiper *et al.*, (1978) reported that the maximum activity of amylase enzyme was obtained at 1.67 % of substrate (starch) concentrations.

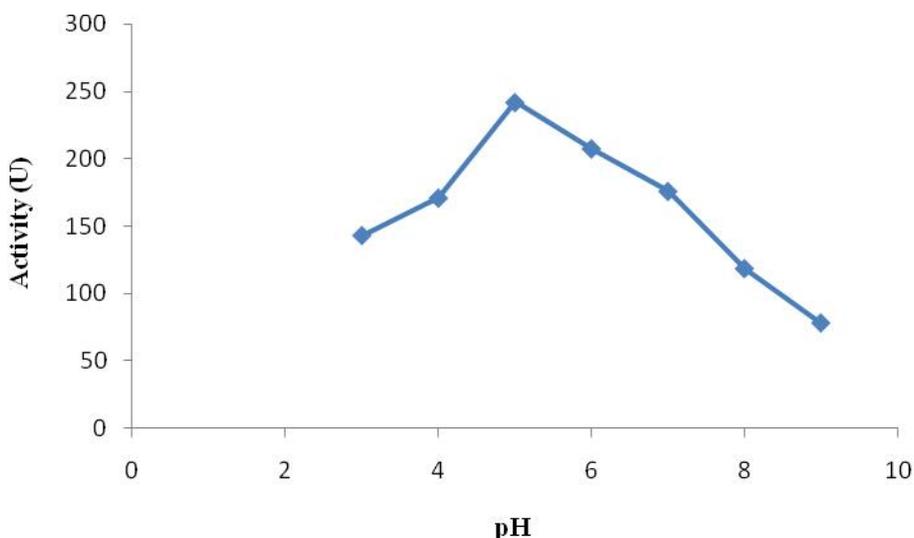


Figure 1: Effect of pH on amylase activity

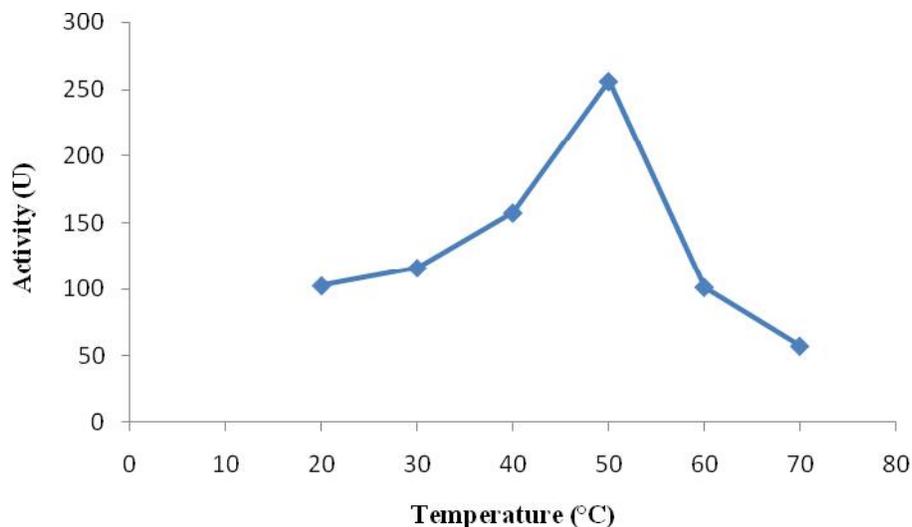


Figure 2: Effect of temperature on amylase activity

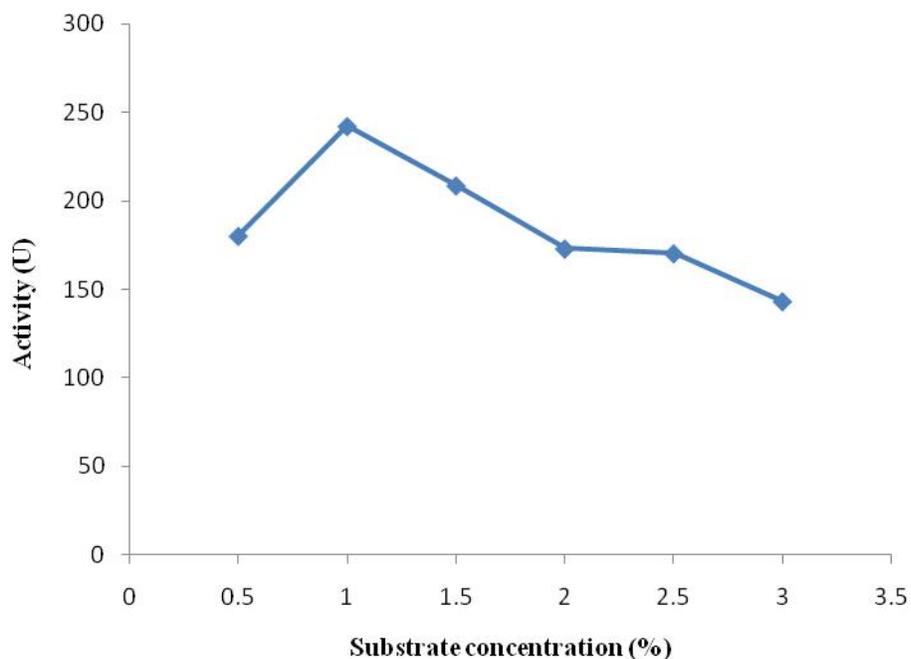


Figure 3: Effect of substrate concentration on amylase activity

Pandey *et al.*, (2000), reported that most of amylases are known to be metal ion-dependent enzymes, namely divalent ions like Ca^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} , Fe^{2+} etc. Effect of metal ions on amylase activity was estimated in the presence of 10 mM of various metal ions. The amylase activity was activated in the

presence of Ca^{2+} , Mn^{2+} , Mg^{2+} , Na^{2+} and Co^{2+} . Strong inhibitory effect was however observed for Zn^{2+} , Cu^{2+} , and K^{2+} on amylase activity. This result indicated that amylase may require Ca^{2+} , Mn^{2+} and Co^{2+} for increased activity, with relative activity of 124 %, 116 % and 118 % respectively (figure 4).

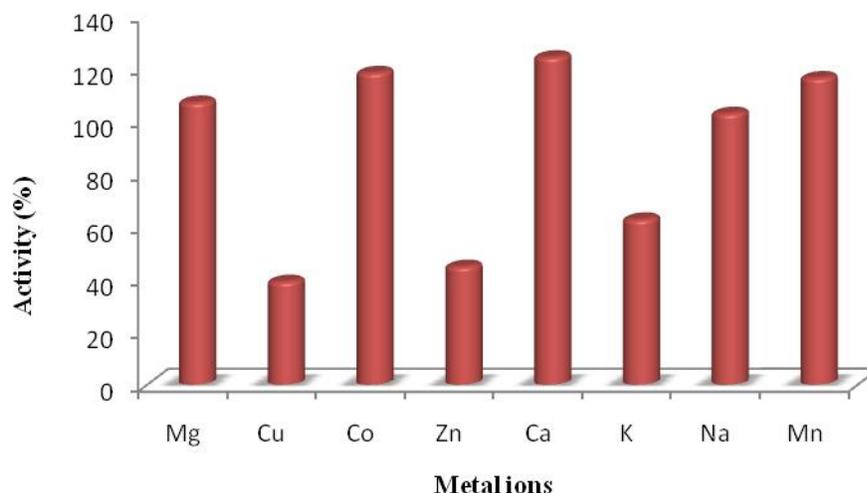


Figure 4: Effect of activators and inhibitors

Conclusion

Contents of synthetic media are very expensive and the cost of enzyme production by submerged fermentation (SmF) is high. These necessitate the use of more economically available agro waste to reduce cost of enzyme production by SSF. Production of amylase by SSF has several advantages in the area of high enzyme titres, stability of temperature and pH. These make SSF the ideal choice for amylase production. Utilization of agro industrial wastes such as millet pomace may be a cheap eco-friendly substrate for amylase production. Because of the availability of agro waste as a cost effective and eco-friendly substitute even affluent nations, which can afford the luxury of expensive synthetic media, can adopt agro waste as low cost enzyme production technology.

References

- Alhassan Sanni, Francis A. Awe and Joseph A. Akinyanju (1992). Amylase synthesis in *Aspergillus flavus* and *Aspergillus niger* grown on cassava peel. *Journal of Industrial Microbiology* 10 (1): 55-59.
- Bakri, Y., Magali, M. and Thonart, P., (2009). Isolation and identification of a new fungal strain for amylase biosynthesis. *Pol. J. Microbiol.* 58(3): 269-73.
- Bertrand, T.F., Frederic, T. and Robert, N. (2004). Production and Partial Characterization of a thermostable amylase from Ascomycetes yeast strain isolated from starchy soil. *McGraw-Hill Inc.*, New York. pp. 53-55
- Bhimba, Valentin, B., Yeswanth, S. and Naveena, Edhaya, B., (2011). Characterization of extracellular amylase enzyme produced by *Aspergillus flavus* MV5 isolated from mangrove sediment. *Indian Journal of Natural Products and Resources.* 2(2): 170-173.
- Deb, Promita, Saimon Ahmad Talukdar, Kaniz Mohsina, Palash Kumar Sarker and S. M., Abu Sayem (2013). Production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001. *Springeropen Journal* 2:154
- Dixon, M. and webb, E. G., (1964). Enzymes, 2nd Edit. *Academic Press. Inc.* New York.
- Fadahunsi, Ileasanmi F. and Garuba, Oluwaseun, E. (2012). Amylase Production by *Aspergillus flavus* Associated with the Bio-deterioration of Starch-Based Fermented Foods. *New York Science Journal*; 5(1).
- Kuiper, J., Roels J. A. and Zuidwedg M. H. J., (1978). Flow through viscometer for use in the automated determination of hydrolytic enzyme activities: Application in protease, amylase, and pectinase assays. Gist-Brocades N. V. research and development, Delft, the Netherlands Analytical biochemistry 90: 192-203.
- Liu, X. D. and Xu, Y. (2008). A novel raw starch digesting α -amylase from a newly isolated *Bacillus* sp. YX-1: Purification and characterization. *Biores. Technol.* 99: 4315-4320.

- Naessens, M. and Vandamme, E. J. (2003). Multiple forms of microbial enzymes. *Biotechnol Lett* ; 25: 1119-24.
- Nagamine, K., Murashima, K., Kata T, Shimoi H, Ito K. (2003). Mode of alpha amylase producing the Schochu Koji mold *Aspergillus Kawachii*. *Bioscience Biotechnology and Biochemistry*; 67: 2194-2202.
- Okolo, B. N., Ezeogwu, L. I., and Mba, C. N. (2006). Production of raw starch digesting amylase by grown on native Starch sources. *Journal of Science Food and Agriculture*; 69: 109-115.
- Pandey, A., Nigam, P., Soccol, C. R., Soccol, V. T., Singh, D., and Mohan, R. (2000). Advances in microbial amylases. *Biotechnol Appl Biochem* 31:135–152
- Pandey A., Selvakumar P., Soccol C. R. and Nigam P., (1999). Solid state fermentation for the production of industrial enzymes, *Curr Sci*, 77: 149-162.
- Pandey, A. P., Selvakumar, C. R., Soccol and Poonam Nigam, (1992). Solid State fermentation for production of industrial enzyme, 27:109- 117.
- Sidhu, G. S., Sharma, P., Chakrabart T *et al.*, (1997). Strain improvement for the production of a thermostable -amylase. *Enzyme and Microb Technol* 21: 525-530.