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Original Research

Production and characterization of amylase by mixed cultures of *Aspergilllus flavus* and *Aspergillus tamarii*

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ABSTRACT: This study evaluated the potentials of mixed cultures of *Aspergillus flavus* and *A. tamarii* used for enhanced amylase production. Amylase producing moulds were screened from the soil by plating on Remazol Brillant Blue-Starch agar. Out of the 800moulds screened, studies were conducted on amylase production of monocultures and mixed cultures of non-aflatoxigenic *Aspergillus flavus*(A) and *Aspergillus tamarii*(C) by growing them on rice bran solid media at 30°C for 72h. The synergy between the two moulds was pronounced at 70°C and pH 6.0, 7.0 where the enzyme activity of the mixed culture(E) was 2.5times higher than that of the monocultures. Storage stability with Cassava starch and Soyabean flour showed that the maximal enzyme stability of 95% was obtained with 3% (w/v) of Cassava starch at 4°C while 96% enzyme stability was achieved with 4% (w/v) Soyabean flour at 30°C over a period of 8weeks. Thin Layer Chromatography of starch hydrolysates showed a mixture of glucose and maltose from extracts of A with only maltose from C suggesting that A produced glucoamylase and α-amylase while C produced only α-amylase. This study shows that extracts of the mixed cultures contain enzyme complex that can be of high importance in the starch industry.

KEYWORDS: Microorganisms, amylase, pure Culture, mixed Culture.

INTRODUCTION

Microorganisms including bacteria, fungi and yeast had made significant contributions to the production of Amylases over the years (Pandey, 1994). Amylases can also be commercially obtained from fungal species of *Aspergillus* and *Rhizopus* species (Godfrey and Reichett, 1983). Filamentous ascomycete, *Aspergillus niger* is well known for its ability to produce and secrete a variety of hydrolytic enzymes because they have a number of features that make them interesting organism for industrial application such as fermentation capabilities and high level of enzyme secretion (Evstatieva *et al.*, 2010).

Solid Substrate (State) fermentation (SSF) can be simply defined as any fermentation process allowing the growth of microorganisms on moist solid materials in the absence of free flowing water (Pandey *et al.*, 2000). The advantages of SSF include higher productivity levels (Jain, 1995), low level of catabolic repression (Favela *et al.*, 1998) and increased stability of the excreted enzymes (Minjares *et al.,* 1997). Amylases have been produced commercially for several years using fungal monocultures. The application of pure cultures dominates in biotechnological processes but through the application of mixed cultures their combined metabolism results in higher yields especially in ethanol and acetic acid production (Szambelan *et al.*,2004 and Kondo *et al.*,1996). Special attention has been given to the mixed cultures used for increasing enzyme production, such as amylase (Abate *et al.,*1999), inulase (Ongen-Baysal and Sukan,1996),xylanase, endoglucanase and ß-glucosidase

(Gutierrez-Correa and Tengerby,1998). Mixed cultures of amylolytic yeast and *Zymomonas mobilis* have been used to convert starch or disaccharides to ethanol and proteins (Abate *et al.*, Gonzalez *et al.*, 1998). Also a 2.5 fold increase in amylases by mixed cultures of *Bacillus* and *Zymomonas mobilis* has been reported.(Abate *et al.*, 1999) There are available reports on amylase production using a combination of bacteria, yeast or mould and any of bacteria or yeast, however reports on amylase production using mixed cultures of moulds is still very scanty. Hence the need to investigate the potential of mixed cultures of moulds for enhanced amylase production. In addition, strategies adopted for increased enzyme yield such as mutation, genetic manipulation are expensive, time consuming and too sophisticated for a developing economy. The study is therefore aimed at exploring a mixed culture of *Aspergillus tamarii* and *A. flavus* for an increased amylase yield.

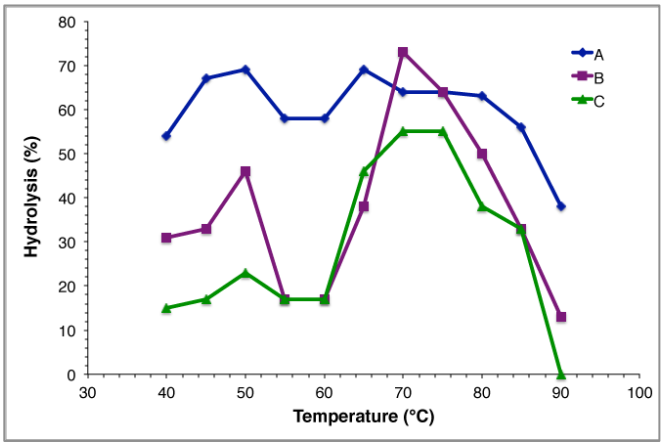

Figure 1: Temperature versus rate of hydrolysis of crude amylase of *Aspergillus sp (A,C)* and its mixed culture (B).

A= *Aspergillus flavus*, C= *Aspergillus tamarii*, B= Mixed cultures of A and C

MATERIALS AND METHODS

Screening of Amylase positive moulds

Screening for amylase positive moulds was carried out by using the method of Akpan *et al.* (1999). The plate medium is made up of nutrient agar containing 0.2% Remazol Brillant Blue-Starch (RBB-starch). The medium was autoclaved at 121 °C for 15 minutes at a pH range of 4.5. Decimal dilution (0.1ml) of a suitably diluted soil sample was plated on RBB-starch agar medium and incubated at 35 °C for 48 h. Amylase production was detected by the disappearance of the colour of the blue starch around microbial colonies. The clear zones were measured with calipers by taking two perpendicular measurement of diameter.

Amylase production by Solid State Fermentation

Solid state production of amylase was carried out by using the method of Akpan and Adelaja (2003). The medium is made up of Rice bran, Soyabean flour and Starch formulated in the ratio 10:3:1 w/w respectively .The mixture in a petridish was moistened with distilled water to 55% moisture content and sterilized at 121 °C for 15 minutes. The sterilized medium was inoculated with a loopful of spores of the two molds as single and mixed cultures. These were incubated at 30 °C for 72 h.

Effect of temperature on crude amylase activity and thermostability profiles

The effect of temperature was studied by assaying the enzyme from the mixed and monocultures at temperatures 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90 °C and pH 4.5. The thermostabilities of the enzyme from the single and mixed cultures were tested by pre-incubating the enzyme preparation for 4 h at different temperatures (30, 40, 50, 55, 60, 65 and 70 °C) with or without $CaCl_2.2H_20$ (10 mM). The residual enzyme activity was then determined.

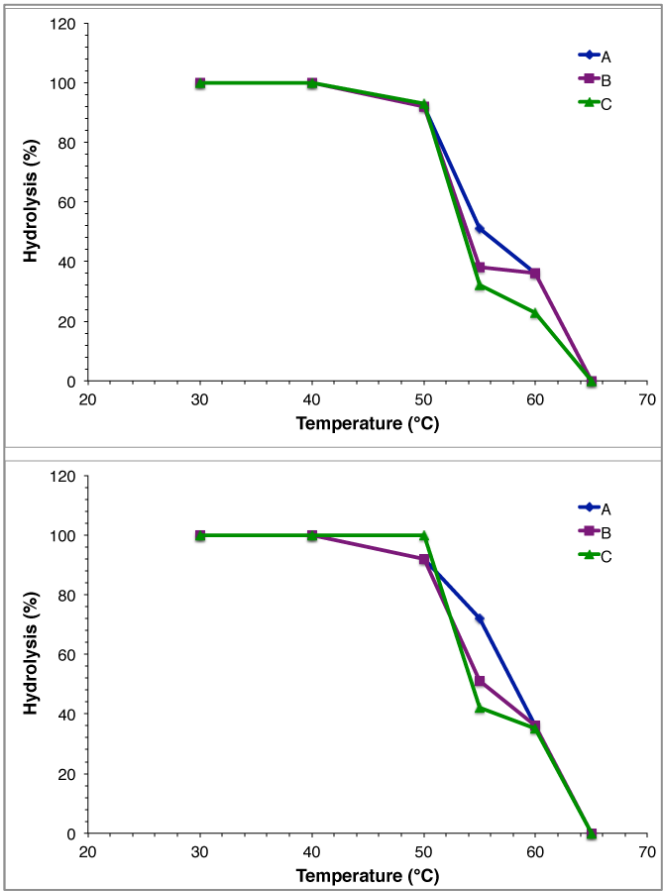

Figure 2: Thermal stability of crude amylase without additive at different temperatures (A) and with $CaCl_2.2H_2O$ at different temperatures (B).

A= *Aspergillus flavus*, C= *Aspergillus tamarii*, B= Mixed cultures of A and C

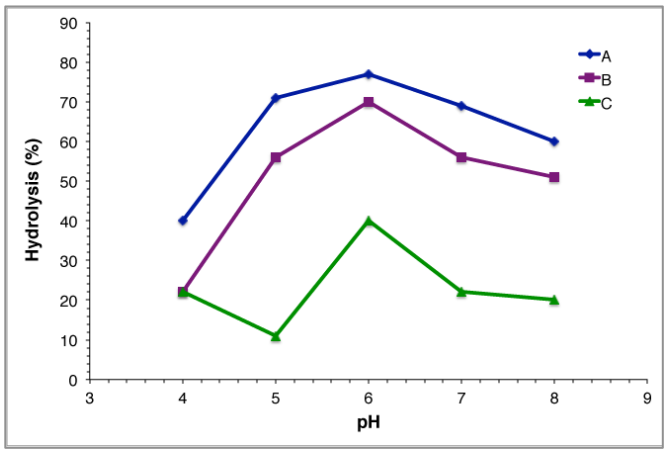

Figure 3: Effect of pH on hydrolysis of cassava starch by crude amylase of *Aspergillus sp (A,C)* and its mixed culture (B) at 65 ºC

A= *Aspergillus flavus*, C= *Aspergillus tamarii*, B= Mixed cultures of A and C .

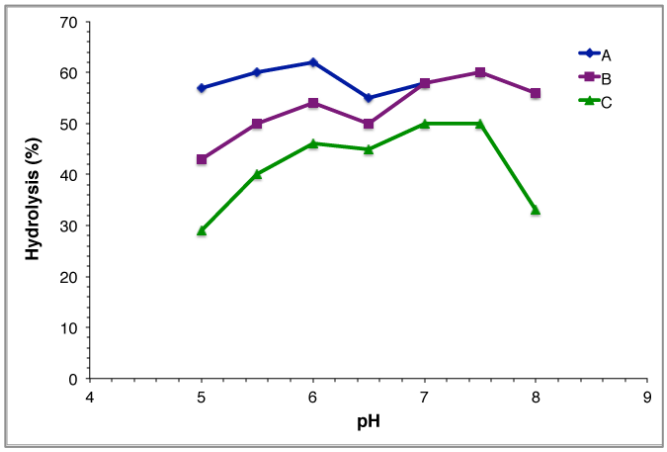

Figure 4: pH stability profile of crude amylase of *Aspergillus sp*

A= *Aspergillus flavus*, C= *Aspergillus tamarii*, B= Mixed cultures of A and C

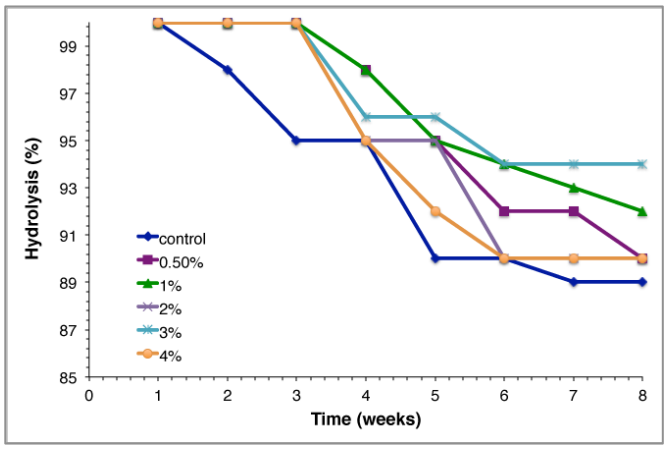

Figure 5: Effect of soyabean flour as stabilizer on amylase activity at 4 ^{0}C

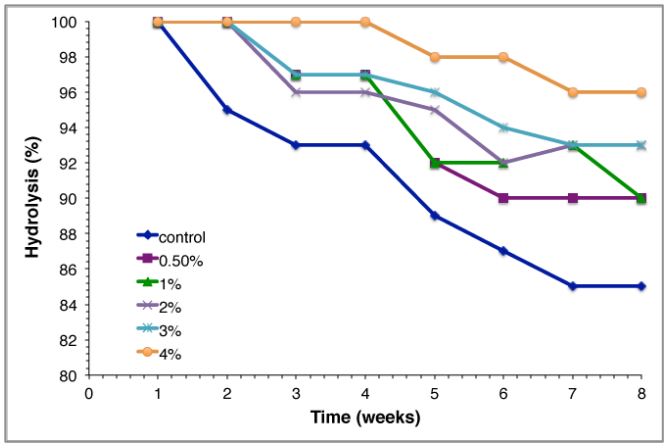

Figure 6: Effect of soyabean flour as stabilizer on amylase activity at 30 ^{0}C

Effect of pH on crude amylase activity and pH stability profiles

The pH activity profiles were studied in 0.1 M sodium phosphate buffer with a pH range of 4.0–8.0 (pH 4.0, 5.0, 6.0, 7.0 and 8.0) and sodium acetate buffer with a pH range of 4.0–8.0. In the pH stability studies, the enzyme was mixed with buffers in the range 5.0–8.0 (pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0) and incubated up to 6 h. The residual enzyme activity was determined.

Storage stability

Studies on the storage stability of the enzyme produced by the mixed and single cultures was carried out using 0.5-4 % Soyabeans flour and cassava starch as additives at two different temperatures of 30 °C and 4 °C. Enzyme activities of the stabilized samples was determined at weekly intervals for a period of two months.

Identification of End products

Thin Layer Chromatography (TLC) was carried out on the starch hydrolysate of 2% starch solution by the crude amylase recovered from the mixed and single cultures. Aliquots (10 μL) of each starch hydrolysate were spotted on the TLC plates (POLYGRAM, UK) using capillary tubes. The sugars spotted as standards were Glucose and Maltose (1%w/v). The plates were then developed at room temperature (30 ºC) for 90 minutes and the chromatogram was removed from the tank, air dried and spots were located by dipping in 4-amino benzoic Acid. After dipping, it was air dried and placed in an oven at 100 ºC for 15 minutes to develop the colour characteristics of the sugars in the hydrolysate.The relative fraction (R_f) values of the samples were recorded and compared with that of the standards.

RESULTS AND DISCUSSION

Screening and Identification of amylase positive moulds

Out of 800 moulds screened from the soil environment in Abeokuta, Ogun state, 2 moulds were selected based on their amylase activity, thermostability and ability to retain their activity after prolonged storage. The moulds selected were identified based on standard microbiological methods of Fassatiova (1986). Further identification using standard microbiological methods was carried out at International Institute for Tropical Agriculture (IITA) Oyo state. The organisms were identified as *Aspergillus flavus* **(A)** and *Aspergillus tamarii* **(C**). As shown on Plate 1, the clearance exhibited around colonies of the two moulds identified as *Aspergillus flavus* and *A. tamarii* showed their amylolytic potentials. The non-amylase producing mould gave no zone of clearance around its colony because amylase was not produced.

Effect of temperature on amylase activity and thermostability profiles

Studies on the effect of temperature on enzyme activity of the mixed and monocultures showed that the enzyme produced by *A. Flavus* had two optimum temperatures of 50 °C and 65°C with 69% efficiency while that of *A. tamarii* only exhibited 23% and 31% efficiency at these temperatures. However, the enzyme of the mixed cultures exhibited 73% efficiency at 70 °C. The synergy between the monocultures was observed in the mixed cultures producing the highest amylase activity of 73% at 70 °C and pH 4.5 (Figure 1). The report agreed with the findings of Verma *et al.* (1999) that a mixed culture of yeasts resulted in an increased yield of ethanol production. The fact that they exhibit peak activities at two different temperatures shows that the mixed culture comprise of an enzyme complex of which amylase has been identified and studied. Further study is ongoing to investigate the other enzymes present in the enzyme complex with a view to characterizing them. What is novel about the amylase produced by the mixed cultures of the organisms under study is that the activity of the enzyme of the mixed culture increased with temperature peaking at 70°C and an efficiency of 73% as compared to the monoculture with a maximal efficiency of 69% at 65°C by *Aspergilus flavus.* This showed that these isolates might have acted synergistically in their enzyme activities (Ueda, 1981). The implication of this is that the enzyme produced by the mixed cultures ensure a direct conversion of starch to glucose against the conventional method of cooling the starch slurry from high temperature of the α-amylase to a temperature of 65 °C of which glucoamylase functions optimally. Also, the enzyme of the mixed culture shows an increased efficiency at high temperature; an important breakthrough in the starch industry because the cost incurred in temperature regulation during enzymatic hydrolysis of starch is drastically reduced. In the thermostability studies, the addition of $CaCl_2.2H_2O$ had no

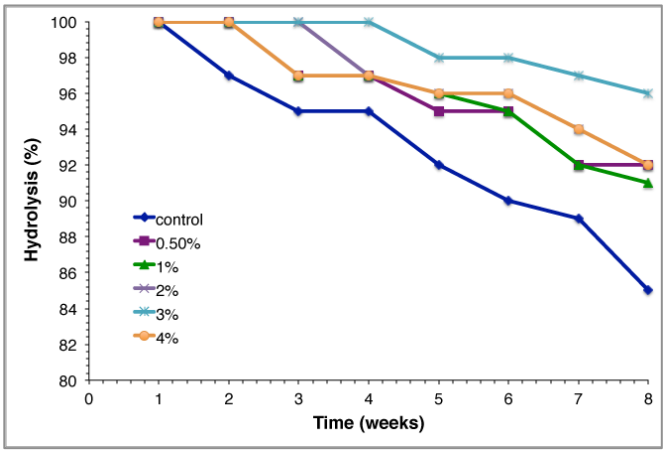

Figure 7: Effect of Cassava Starch as Stabilizer on Amylase Activity at 4 ^{0}C

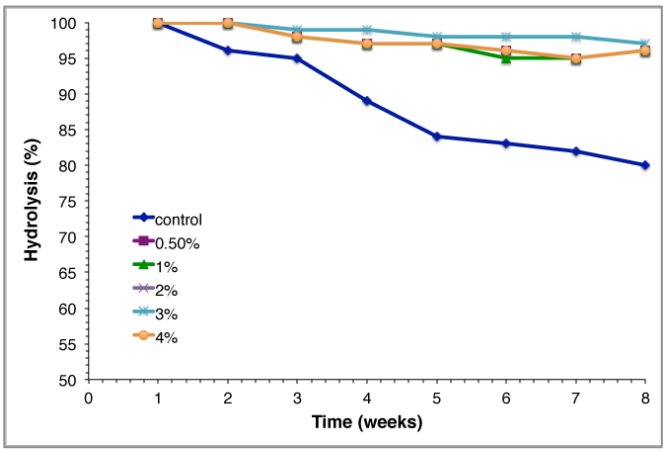

Figure 8: Effect of Cassava Starch as Stabilizer on Amylase Activity at 30 ^{0}C.

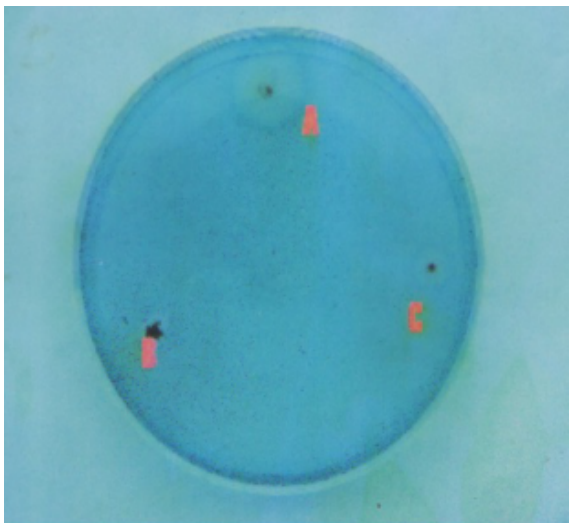

Plate 1: Amylase and non-amylase producing mould

A, C (Amylase positive); B (Non-amylase producing mould)

significant effects on the monocultures but led to the mixed culture retaining its initial activity of 100% up to 50 °C (Figures 2A and 2B). The stabilizing effect of Ca^{2+} on thermostability of the enzyme can be explained due to the salting out of hydrophobic residues by Ca^{2+} in the protein, thus causing the adoption of a compact structure (Volkin and Klibanov, 1989)

Effect of pH on amylase activity and pH stability profiles

Studies on the effect of pH on enzyme activity as indicated in Figure 3 showed that enzyme activity for both mixed and monocultures increased progressively and are optimum at pH 6.0 where they produced different amylase activities. Enzyme extracts of *A. flavus* gave an activity of 77%, *A. tamarii* 40% and the mixed culture 70% as a result of the synergy between the two organisms. After pH 6.0, a decrease in enzyme activity was observed in all the enzyme extracts. The pH stability studies (Figure 4) showed the enzyme extracts of *A. flavus* to be stable at pH 6.0 with 62% activity, that of *A. tamarii* was stable at pH 7.5 with 60% activity and the mixed culture at pH 7.0 and 7.5 with 50% activity after 6 h of incubation.

Storage Stability

Stability studies of the enzyme extracts of the mixed and monocultures using soyabean flour and cassava starch was investigated at 4 °C and 30 °C (Figure 5–8). It was observed that after storage for the first four weeks there was a decline in the enzyme activity of extracts without stabilizer whereas enzyme extracts with stabilizers (soyabean) at 30 °C retained its activity of 100%. On the average, after storage for eight weeks at 4 °C and 30 °C, enzyme extracts without any stabilizer retained only 80% and 85% of their activity. On the other hand, maximal enzyme stability was produced by 3% (w/v) cassava starch with an activity of 95% at 4 °C.This was followed by 4% (w/v) soyabean flour producing an activity of 96% at 30 °C. This stability could be due to the carbohydrate or glycoprotein components of the stabilizers and is in agreement with the findings of Srivastava (1991) who reported that glycoprotein often exhibit increased stability towards proteolysis, heat and storage. Therefore, the production of enzymes that can be stabilized at room temperature is an advantage especially where power supply for the storage of enzyme extracts is erratic.

Identification of end product

Thin Layer Chromatography of starch hydrolysates of the monocultures showed the different sugars produced after hydrolysis for 6h. Hydrolysis of extracts of *A. flavus* contains maltose and glucose, which indicate the presence of α-amylase and glucoamylase. While *A. tamarii*, which contains only maltose, indicates the presence of α-amylase. Hence,the hydrolysates of the mixed culture contains both glucose and maltose The enzyme of the mixed culture encourages one-step starch hydrolysis as against the conventional two steps. This is a biotechnological advantage because it is envisaged to shorten the time and reduce labour cost.

Conclusion

In conclusion, this study describes the production of amylase from mixed culture of *Aspergillus sp* under SSF using cheap and cost effective substrate, rice bran and some supplements. The enzyme of the mixed culture was thermostable and pH stable showing maximum activity of 73% at 70 °C and pH 6.0 and 7.0 due to the synergy between the two moulds. This showed that there is a synergy between the mixed cultures of moulds which led to the production of a more pH and thermostable enzyme preparation with an increased amylase yield.

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