Amylolytic Properties of Fungi Associated with Spoilage of Bread

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Abstract: An amylolytic property of fungi associated with spoilage of bread was conducted in Abuja, Nigeria. A total of twenty bread samples were purchased from Zuba, Gwagwalada, Tungamaje and Anagada in Gwagwalada Area Council and screened for the presence of amylolytic fungi by using Spread Plate Technique. Eighteen (18) fungal isolates belonging to three genera and four species of amylolytic fungi were observed, *Aspergillus niger* (38.89 %) was the most prevalent species, followed by *Aspergillus flavus* (33.33 %), *Penicillium chrysogenum* (16.67 %) and *Rhizopus stolonifer* (11.11 %) being the least prevalent. The highest amylolytic activities were recorded in *Rhizopus stolonifer* (15.19±1.3 Amylase unit (Au)/ml), *Penicillium chrysogenum* (14.02±0.3 Au/ml), *Aspergillus niger* (12.13±0.5 Au/ml) and *Aspergillus flavus* (10.05±1.4 Au/ml). The amylolytic activities as well as the frequencies of occurrence of the fungal isolates were not significantly different at p = 0.05 level of significant. These amylolytic fungi have the potential to be a useful tool in biotechnological processes involving starch hydrolysis. The results of this work show that amylolytic activity is relatively widespread among common fungi and may have an important role in starch degradation in natural environment.
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1.0 Introduction

Amylases are among the most important enzymes and are of great significance in present day biotechnology, having approximately 25% of the enzyme market (Sakthi *et al.,* 2012). Although amylases can be obtained from several sources, such as plants and animals, the enzymes from microbial sources generally meet industrial demand. Amylases stand out as a class of enzymes, which are of useful applications in the food, brewing, textile, detergent and pharmaceutical industries. They are mainly employed for starch liquefaction to reduce their viscosity, production of maltose, oligosaccharide mixtures, high fructose syrup, and maltotetraose syrup. In detergents production, they are applied to improve cleaning effect and are also used for starch de-sizing in textile industry (Aiyer, 2005; Radley, 1976). Fungi are ubiquitous in nature and are used extensively to produce industrial chemicals like citric, gluconic, lactic, and malic acids, (Joseph *et al*., 2008) and industrial enzymes, such as lipases used in biological detergents, (Kumar *et al*., 2008) cellulases used in making cellulosic ethanol and stonewashed jeans, amylases, invertases, proteases and xylanases (Kumar *et al*., 2008). The first enzyme produced industrially was an amylase from a fungal source in 1894, which was used for the treatment of digestive disorder (Sakthi *et al.,* 2012). Alpha amylase has been derived from several fungi, yeasts, bacteria and actinomycetes, fungal sources are mostly terrestrial isolates such as *Aspergillus* species and Rhizopus species (Sakthi *et al.,* 2012). Ugoh and Ijigbade (2013) reported the production and characterization of amylase by fungi isolated from soil samples at Gwagwalada. Tokhadze *et al*., (1975) isolated 86 strains of the *Aspergillus* producing maximum alpha-amylase. Mahmoud *et al*. (2007) reported the use of different agricultural by-products and wastes such as wheat bran, rice bran, cane molasses, corn bran, glucose syrup, cornstarch as a substitute of original carbon source in the fermentation medium for the synthesis of alpha amylase by *Aspergillus niger*. Tsekova *et al*., (1993) studied the ability of *Aspergillus* genus for alpha amylase production. This study was therefore carried out to determine the amylolytic properties of fungi associated with spoilage of bread.

2.0 Materials And Methods

2.1 Study area

This research work was carried out at the Laboratory of the Department of Biology, School of Sciences, Federal Capital Territory College of Education, Zuba-Gwagwalada, Abuja, Nigeria.

2.2 Collection of samples

A total of twenty (20) bread samples were collected randomly with five (5) loaves of bread each from four (4) different bakeries in Gwagwalada FCT-Abuja. Bread was purchased from Zuba, Gwagwalada, Tungamaje and Anagada. The bread was collected with sterile polythene bags and brought to the Biology Laboratory of Federal Capital Territory College of

Education, Zuba, Abuja, for determination of the amylolytic properties of fungi associated with spoilage of bread using the method of Sakthi *et al*., 2012 with some modifications.

2.3 Preparation and sterilization of media

Sabraud's Dextrose Agar and Starch Agar were used in this study and they were prepared according to the manufacturer's instructions thus, 65g of SDA was dissolved in 1000ml of sterile water and then sterilized (autoclaved) at 121ºC and pressure of 15Pa for 15 minutes, while Starch Agar was prepared by adding 2% (20g) of starch with 1.5% (15g) of agar in 1000ml of sterile distilled water and then boiled for 30minutes. Sabraud's Dextrose agar was used for the isolation and maintenance of pure cultures of fungi and the starch agar was used for the screening of amylolytic fungi (Sakthi *et al*., 2012).

2.4 Isolation of amylolytic fungi

The bread was exposed for 7 days by placed at different locations in the Laboratory and observed daily for spoilage. The fungi were isolated from the spoiled bread using the spread plate technique (Sakthi *et al*., 2012). One gram (1 g) each, of spoiled bread was dissolved in 10 ml sterilized distilled water. The suspensions were diluted serially up to 10^5. The samples were inoculated on already prepared Sabraud's dextrose agar plates. The inoculated plates were incubated at ambient temperature (25 ± 2^0C) for 5 days. Colony developments were observed after incubation period.

2.5 Preparation of pure cultures of fungal isolates

The young fungal colonies were aseptically picked up and transferred to fresh sterile SDA plates to obtain pure cultures. The pure cultures on SDA plates were grown at 25 ± 2^0C for 7 days and stored in the refrigerator at 4^0 C until required for further use. The isolates were sub-cultured to obtain young cultures for further studies (Tokhadze *et al*., 1975).

2.6 Identification of amylolytic fungal isolates

Isolates obtained were characterized and identified on the basis of their colonial and morphological characteristics which include macroscopic and microscopic examinations. Among the characteristics used were colonial characteristics such as size, surface appearance, texture, reverse and pigmentation of the colonies (Sharma and Rajak 2003).

2.7 Screening of fungal isolates for amylase production

Primary screening was done by starch agar Plate method. The isolated fungi were inoculated on the agar plates amended with 2% of starch with 1.5% of agar (Sakthi *et al*., 2012). The plates were incubated at 25 ± 2^0C, 37 ^{0}C and 40 ^{0}C for 7 days. The plates were flooded with iodine solution and clear zones around the colonies were observed. The plates that showed a maximum hydrolysis halo on the medium was selected for further investigations.

2.8 Determination of amylase potentiality of fungal isolates

A modified medium of Ali *et al*., (1989) consisting of soluble starch; 2%, peptone; 0.2%, ammonium sulphate; 0.3%, potassium dihydrogen phosphate; 0.1%, magnesium sulphate heptahydrate; 0.03% and calcium chloride; 0.03% (w/v) was sterilized and employed as the substrate for fungal isolates. The fungi spores of seven (7) days old culture of each isolate were diluted serially up to 5-fold dilution and the spore suspension containing 10^5 spores/ml was aseptically introduced into each tube of fermentation medium (Metwally, 1998). Cultures were incubated at 25±2^0C, 37^0C and 40^0C for 7 days.

2.9 Extraction of amylase enzyme

Two milliliter of 0.1 M phosphate buffer (pH-6.5) was added to cultures, the mixtures were agitated for 30 min at 19^0C and 140 rpm on a rotary shaker. The mixture was filtered using whattman No1 filter paper and the filtrate was centrifuged at 4000 rpm for 10 min. The supernatant was used as the crude enzyme preparation (Mahmoud *et al*., 2007).

2.10 Assay of amylase enzyme

Amylolytic products in the supernatant were determined by reading absorbance at 280 nm against basal medium using UV-Spectrophotometer (JENWAY 6305). An increase of 0.100 in the absorbance was considered as equivalent to 1 unit of AU (amylase unit).

2.11 Plate assay

The Plate assay was performed using agar plates amended with starch (Sakthi *et al*., 2012). The agar plates were amended with 2% of starch and 1.5% of agar. After the agar solidification, about 10 mm diameter of well was cut out aseptically using a 6 mm cork borer. The well was filled with the culture filtrate and incubated at 25 ± 2^0C, 37 ^{0}C and 40 ^{0}C for 72 hours. 1% of iodine solution was over layered on the agar and was observed for zones of hydrolysis around the wells (Tsekova *et al*., 1993). The control was maintained by adding sterile water in separate well. All the examinations were replicated thrice.

2.12 Statistical analysis

The results obtained were analyzed using one-way ANOVA and the F- test statistic at P = 0.05 level of significant.

3.0 Results

3.1 Prevalence of amylolytic fungi

Isolation rate of amylase producing fungi from bread samples at Gwagwalada FCT-Abuja shows that *Aspergillus niger* was the highest and higher in bread collected from the Zuba and Angwandodo (Figure 1 and Table 1). *Aspergillus niger* is the most prevalent

amylolytic fungus and also dominant species that was isolated from 7 (38.89%) bread samples of the five different bakeries, followed by *Aspergillus flavus* 6 (33.33 %), *Penicillium chrysogenum* 3 (16.67%) and *Rhizopus stolonifer* 2 (11.11%) being the least prevalent.

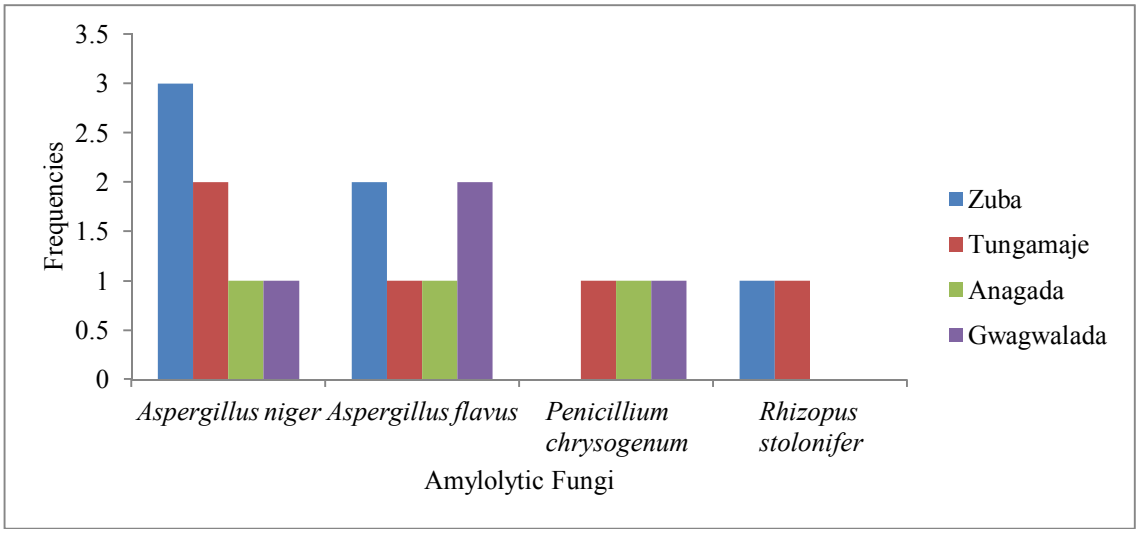

Figure1: Frequencies of occurrence of amylolytic fungi isolated from bread

Table1: Frequencies and Percentages of amylolytic fungi isolated from bread

Fungal isolates	Number of isolates	Percentages (%)
Aspergillus niger	7	38.89
Aspergillus flavus	6	33.33
Penicillium chrysogenum	3	16.67
Rhizopus stolonifer	2	11.11
Total	**18**	**100**

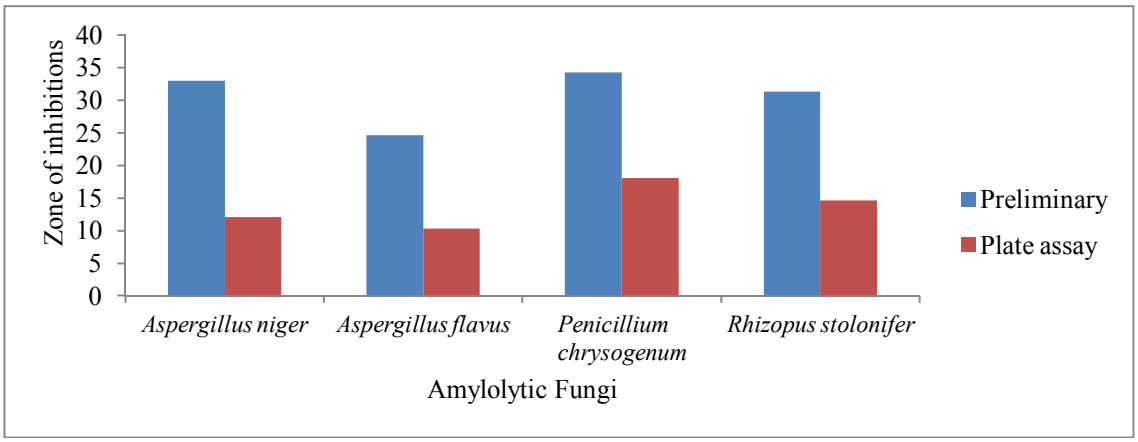

Figure 2: Preliminary screening of fungal isolates and plate assay of enzyme amylase

3.2 Preliminary screening and plate assay of enzyme amylase

The results for the primary screening of the fungal strains by starch agar plate method are shown in Figure 2. The results showed the fungi involved in bread spoilage in this study were *Aspergillus niger, Aspergillus flavus, Penicillium chrysogenum* and *Rhizopus stolonifer* with the following zone diameter 33 mm, 24.67 mm, 34.33 mm and 31.33 mm respectively.

The result for the determination of the amylase activity of the crude enzyme of the strains of fungi cultivated in basal medium are presented in Figure 2 as follow *Penicillium chrysogenum* 18.08 diameter

(mm), *Rhizopus stolonifer* 14.60 mm, *Aspergillus niger* 12.10 mm and *Aspergillus flavus* 10.30 mm. Amylolytic activity of the culture filtrates (Crude enzyme) were confirmed on starch agar Plates.

3.3 Amylase activities of the isolated fungi from bread

The quantities of the amylase enzyme produced by the fungi in the basal medium were measured using UV-Spectrophotometer (JENWAY 6305) and the result is shown in Table 2. The optimum time, pH and temperature on the amylase production was investigated in these fungi. The amylase activity was found to be 12.13±0.5, 10.05±1.4, 14.02±0.3 Au/ml and 15.19±1.3 Au/ml at 30 ^{0}C for *Aspergillus niger, Aspergillus flavus Penicillium chrysogenum* and *Rhizopus stolonifer* respectively. The activity was observed to decrease with an increase in temperature. Amylase activity was found to be highest at pH 6.5 in *Rhizopus stolonifer,* closely followed *Penicillium chrysogenum* at pH 6.5. This trend was observed in all the four organisms in this study.

Table 2: Amylase activities of the isolated fungi from bread

Fungal Species	Amylase Activity (AU/mL)	Optimum PH	Optimum Temp(^{o}C)	Optimum Time(days)
Aspergillus niger	12.13±0.5	6.5	30	7
Aspergillus flavus	10.05±1.4	6.5	30	7
Penicillium chrysogenum	14.02±0.3	6.5	30	7
Rhizopus stolonifer	15.19±1.3	6.5	30	7

Each value represents Mean ± Standard Deviation of three independent determinations

4. Discussion

This study showed *Aspergillus niger, Aspergillus flavus, Penicillium chrysogenum* and *Rhizopus stolonifer* as been responsible for the spoilage of bread. Their saprophytic mode of life may be one of the qualities that has confered this destructive property on these organisms. This is because many of them have the ability to elicite some very potent enzyme into their substrates.

It appears from this study that *Aspergillus niger* is the most prevalent amylolytic fungus and also dominant species that was isolated from 7 (38.89 %) bread samples of the five different bakeries, followed by *Aspergillus flavus* 6 (33.33 %), *Penicillium chrysogenum* 3 (16.67 %) and *Rhizopus stolonifer* 2 (11.11 %) being the least prevalent (Table 1 and Figure 1). This agrees with PiH and Hocking, 1985) which reported the same fungi in food spoilage. In this study, starch enhanced adequate production of amylase by the fungal isolates; alpha amylase can hydrolyze starch and is a key enzyme in metabolism of fungi which utilize starch as carbon and energy sources. This study revealed that *Penicillium chrysogenum, Aspergillus niger, Rhizopus stolonifer* and *Aspergillus flavus* were active amylolytic fungi isolated which agree with the report by Adeniran and Abiose (2009) that fungi demonstrated the greatest potential in the production of amylase. Research on amylase has progressed very rapidly over the last five decades and potential industrial applications of the enzyme especially in solid waste management have been identified. *Aspergillus flavus* is a good producer of amylase but cannot be regarded as "Generally Regarded As Safe" (GRAS) organism due to the possibility of liberation of aflatoxin by *Aspergillus flavus* which limits its application for commercial α-amylase production (although can be used for degradation of starch base waste in the environment as reported by Geweely and Ouf (2011). The degradative enzymes produced by *Aspergillus* species, Rhizopus species and *Penicillium* species are capable of breaking down complex starch in nature, and may be responsible for the biodegradation of starch substance in polluted habitats. In this study, *Aspergillus niger* also produced amylase in submerged medium, and therefore regarded as good source of amylase. Sakthi *et al*. (2012) also reported the evaluation of amylase activity of the amylolytic fungi *Aspergillus niger* using cassava as substrate. Major impediments to exploit the commercial potential of amylase are the yield, stability and cost of amylase production. Although amylase production by microbes have been extensively studied by many researchers. A thorough review of literature on microbial amylolytic shown that some of the fungi which were active in the characterization had been mentioned by some authors. However, the present study revealed that *Penicillium chrysogenum, Aspergillus niger, Rhizopus stolonifer* and *Aspergillus flavus* are good producers of amylase enzyme. These also agree with Ugoh and Ijigbade (2013) which reported the production and characterization of amylase by *Penicillium chrysogenum, Aspergillus niger,* and *Aspergillus flavus.*

5. Conclusion

The three fungi isolated in this study are capable of secreting the enzyme amylase into the bread which

is one of the factors responsible for bread spoilage and off-odour. It is therefore recommended that we avoid the exposure of our foods and other valuable materials to fungi and their spores as a means of protecting them from spoilage and damage.

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