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Research Paper

Isolation and screening of amylase producing fungi obtained from garri processing site

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Abbreviations: IU, International unit; **SSF,** solid state fermentation.

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Abstract. Amylase producing fungi were isolated from soil samples collected from garri processing site in Nsukka. The ability of the three fungal isolates (*Aspergillus niger, Penicillum* sp. and *Chrysosporium* sp) to degrade starch was determined. These fungal colonies which produced halo-zone on soluble starch agar medium were selected and subjected to various temperatures and pH ranges for amylase production using rice as the only carbon source. The results obtained showed that maximum amylase activity in all the isolate for temperature and pH variation were obtained at 30°C and pH 5, respectively. *A. niger* which produced 14.35 ± 1.23 IU/ml when subjected to a medium containing rice + rice bran in the ratio of 3:1 as the only carbon source. Thus, results of this work proved *A. niger* to be the best producer of amylase when compared with *Penicillum* sp. and *Chrysosporium* sp at 30°C and pH 5, while *Penicillum* sp and *Chrysosporium* is more efficient to *Aspergillus niger* on amylase yield comparism between rice and rice+rice bran. Isolation of amylase producing fungi from garri processing wastes site will help in the bioremediation of garri processing wastes environment which could have caused environmental pollution. This site can serve as a more reliable habitat for obtaining amylase producers.

Keywords: Amylase, *Aspergillus niger*, garri processing site.

INTRODUCTION

Enzymes are among the most important products obtained for human needs through plants, animals and microbial source. Nowadays, the use of enzymes in industrial sector is increasing due to increase in the number of industries especially food, beverages, textile, leather and paper industries (Reddy et al., 2003; Pandey et al., 2000; Romero et al., 1999).

Amylase is a group of enzyme whose catalytic function is to hydrolyze (breakdown) starch to give diverse products including dextrins, and progressively smaller polymers composed of glucose units. When they have broken down the insoluble starch, the soluble end products such as glucose or maltose are absorbed into their cells. They are important enzymes employed in the starch processing industries for hydrolysis of starch into simple sugars (Abu et al., 2005; Alva et al., 2007; George-Okafor, et al., 2013). Amylases from microbial sources especially fungi have gained much attention because of the availability and high productivity of fungi, which are also amenable to genetic manipulation (Pandey, 2003).

In other words, the major advantages of using microorganisms for production of amylases include economical bulk production capacity and easy manipulation of microbes to obtain enzymes of desired characteristics (Pandey, 2003). Although most of the biotechnology industries relied on submerged fermentation process (SmF), where the microorganisms are grown in liquid media, some of the biotransformation reactions can be carried out in a different fermentation process that is known as solid-state fermentation (SSF). SSF proves to be a better option than the SmF. Solid-state fermentation (SSF) has built up credibility in recent years in biotechnological industries due to its potential applications in producing enzymes, bio-pesticides, aroma

compounds, biopharmaceuticals, organic acids and other bioactive compounds.

Agro-industrial residues are processed using SSF because it has lower energy requirement, produce lesser waste water and are environment-friendly. The potential of SSF lies in bringing the cultivated microorganism in close vicinity of substrate and achieving the highest substrate concentration for the fermentation. SSF resembles the natural habitat of microorganism and is, therefore, preferred choice for microorganisms to grow and produce useful value added products. SmF can be considered as a violation to their natural habitat, especially the fungi (Reeta et al., 2009). The major difference between SSF and SmF is of the free water content in the substrate. As suggested by Moo-Young et al. (1983), the term solid-substrate fermentation is used in a more general sense to describe any process in which solid particles of substrates were involved, regardless of the amount of free water.

Therefore, SSF includes the processes in a solid matrix with an aqueous phase leaching through it, slurries of solid particles and solids suspended in an aqueous phase (Manpreet et al., 2005; Narahara, 1984). In the case of SmF, substrate is freely accessible to the microorganisms while in SSF the availability of substrate to the microorganisms may increase, decrease, or remain relatively constant during the fermentation Knapp and Howell (1985). SSF stimulates the growth of microorganisms in nature on the moist solids and has been credited to be responsible for the beginning of fermentation technique in ancient time Cannel and Moo-Young (1980). Enzymes produced in SSF have been reported to be extracellular, facilitating its recovery (Reeta et al., 2009).

Out of all the groups of fungi, the filamentous fungi are the major group of micro organisms, which predominate in the SSF process. Filamentous fungi are widely distributed and typically grow on/in solid materials such as plants, grains and animals. These organisms efficiently produce several different enzymes to degrade these solid materials and utilize the nutrients for survival. Thus, SSF process has been harnessed for the production of traditional foods and beverages for many years. In Japanese traditional food and beverage industries, koji, which is prepared by solid-state fermentation (SSF) using *Aspergillus* spp, is sufficient to supply these hydrolytic enzymes (Alva et al., 2003; Kazunari et al., 2011; Akpan et al., 1999). Different genera of fungi used in SSF process include many species of *Aspergillus, Rhizopus, Alternaria, Fusarium, Monilia, Mucor, Trichoderma* and some species of *Penicillium*. Most of the species belong to filamentous fungi, as these are best suited because of their ability to spread over and to penetrate inside the solid-substrate. The other advantage of using filamentous fungi is that the fungal mycelia synthesize and release large quantity of extra-cellular hydrolytic enzymes (Manpreet et al., 2005; Kim et al., 2003). Among a large number of non- pathogenic microorganisms capable of producing useful enzymes, *Aspergilli* are particularly interesting due to their easy cultivation, and high production of extracellular enzymes with potential industrial exploitation. The use of starch degrading enzymes was in fact the first large-scale application of microbial enzymes in the food industry (Pandey et al., 2000; Abe et al., 1988).

The aim of this study is to isolate and screen for the amylase-producing fungi from garri processing site, as part of bioremediation and still produces amylase at minimal cost.

MATERIALS AND METHODS

Sample collection

Soil samples were collected in containers under sterile conditions from garri processing site in Ogige market, Nsukka, Enugu State, Nigeria.

Inoculation of PDA plates

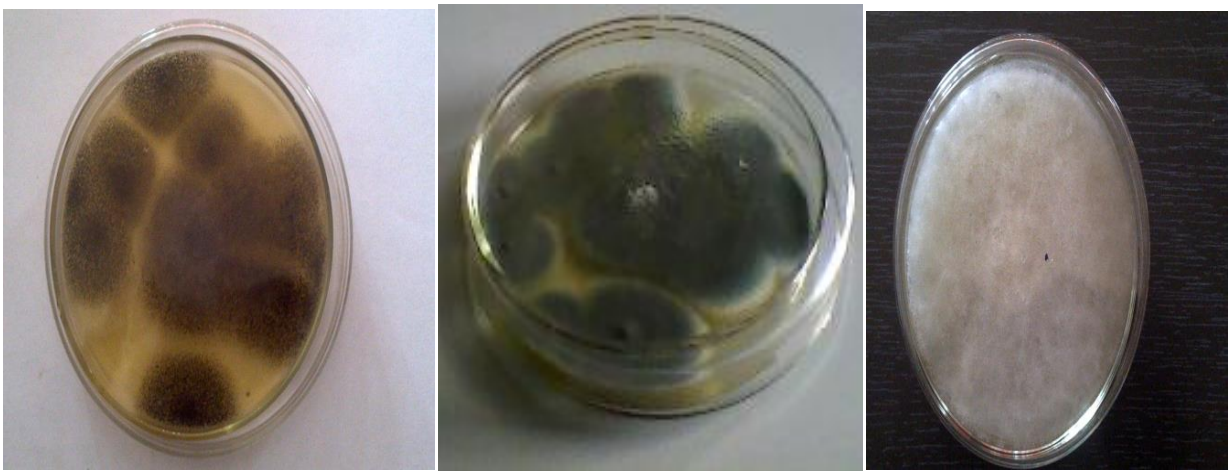
One milliliter (1 ml) each from the above dilutions was transferred into the PDA plates with the aid of a pipette. The plates were observed for 72 h at room temperature. Morphologically, distinct colonies were observed and were purified by repeated sub culturing on separate PDA plates.

Screening for amylase activity and identification of the isolates

The colonies on PDA plates were transferred onto soluble starch agar medium flooded with iodine. Isolates with clear zone are selected. The selected amylase producing fungal isolate were characterized and identified on the basis of morphological feature and microscopy using lactophenolcotton-blue stain. Among the characteristics used were colonial characteristics such as surface appearance, texture and colour of the colonies. In addition, microscopy revealed vegetative mycelium including presence or absence of cross-walls, diameter of hyphae. Appropriate references were made using mycological identification keys and taxonomic descriptions (David et al., 2007).

Determination of optimum temperature and ph range for amylase production

Fermentation medium comprising of starch 20 g/L, yeast extract 0.5 g/L, KH_2PO_4 10 g/L, $(NH_4)_2SO_4$ 10.5 g/L, $MgSO_4.7H_2O$ 0.3 g/L, $CaCl_2$ 0.5 g/L, $FeSO_4.7H_2O$ trace element, $MnSO_4.7H_2O$ 0.004 g/L and $ZnSO_4.H_2O$ trace element were prepared with incubation temperature



FS 1 FS 2 FS 3

Figure 1. Isolate FS1 identified as *Aspergillus niger*; isolate FS 2 as *Penicillium* sp. and isolate FS 3 as *Chrysosporium* sp.

variations at 25, 30, 35 and 40°C . The optimum ph was determined by adjusting the ph of the medium at ph 4, 5, 6, 7 and 8 which was achieved by the addition of 0.2 M of citrate phosphate with either HCl or NaOH prior to sterilization

Enzyme assay

Amylase activity was assayed for by the Dinitrosalicyclic acid (DNS) method. In a test tube, the reaction mixture (containing 1 ml of soluble starch solution mixed with 1 ml of potassium phosphate buffer, pH 6.9) was mixed with 0.1 ml of the crude enzyme source from one of the labeled conical flasks and incubated for 15 min at room temperature. After the incubation, 2 ml of the DNS reagent was added and the reaction terminated by immersing the tube in a boiling water (100°C) for 10 min. The same technique was simultaneously applied to the rest of the crude enzyme sources. The reducing sugars given off were estimated by the DNS methods (Miller, 1959). One unit of enzyme activity has been defined as the amount of enzyme that hydrolyses 1 mg of starch/min under assay conditions (Monga et al., 2011).

Optimization of carbon source for crude enzyme (amylase) production

Effect of carbon sources was carried out. Twenty grams (20 g) of rice grains only and 15 g of rice grains mixed with 5 g of rice bran (3:1) were used as carbon sources, and their enzyme yield compared.

Preparation of glucose calibration curve

The glucose stock was pipette in the following order 1, 2, 3, 4, 5, 6, 7, 8 and 9 ml into 9 appropriately labeled 100ml beakers. These were subsequently diluted with 99, 98, 97, 96, 95, 94, 93, 92 and 91 ml of distilled water respectively. One milliliter (1 ml) of each concentration was added into 9 appropriately labelled test tubes. Also, 1 ml of DNS was added to the solution in each of the 9 test tubes. A tenth test tube containing a solution of 1ml of distilled water and 1ml of DNS served for control. To avoid the loss of liquid due to evaporation, these tubes were lightly plugged with cotton wool and the mixture were heated at 90°C for 5 to 15 min to develop a red-brown color. Then, 1 ml of a 40% potassium sodium tartrate solution was added after heating to stabilize the color. After cooling to room temperature in a cold water bath, the absorbance was taken at 540 nm. The blank (control) was used to zero the spectrophotometer. In this experiment, a standard curve for the analysis of glucose was constructed by plotting absorption on the Y axis, and increasing concentrations of standard along the X axis.

RESULTS

All the isolates were screened for production of amylase using starch plate method resulting in clear zone of starch hydrolysis in the Petri dishes after KI/I_2 treatment. The preliminary amylase production analysis of all the isolates revealed that a total of three isolates were able to produce amylase under solid state fermentation process.

The three fungal isolates were characterized based on the morphological and microscopical method as was shown in Figure 1 and Table 1 where isolate FS1 were identified as *Aspergillus niger*; isolate FS2 as *Penicillium* sp. and isolate FS3 as *Chrysosporium* sp.

Tables 2 and 3 showed the effects of pH and temperature on amylase activity using rice as the only

Table 1. Identification of isolated fungal amylase producers.

Isolate code	Colony morphology	Microscopic observation	Probable identity
FS 1	Velvety, Black creamy and white with time	Septate hyphae, brush-like conidiophores chain conidia	*Aspergillus niger*
FS 2	Green colour colonies on PDA plates and yellowish with time	Mycelium septate, conidiophores with vesicle-like tips	*Penicillum* sp.
FS 3	Colonies are fast growing white to cream- coloured with a very granular surface	Conidia are numerous, hyaline, single-celled, clavate to pyriform, smooth, slightly thick-walled and broad truncate base	*Chrysosporium* sp.

Table 2. Effect of pH on amylase production, using rice as carbon source.

Microorganisms	Amylase activity (IU/ml)				
	pH 4	pH 5	pH 6	pH 7	pH 8
Aspergillus niger	5.11 ± 0.20	12.38 ± 0.12	12.15 ± 0.18	12.05 ± 1.01	10.47 ± 0.58
Penicillum sp.	4.23 ± 0.18	13.02 ± 0.17	10.05 ± 0.26	5.83 ± 0.05	5.14 ± 0.27
Chrysosporium sp.	3.17 ± 0.05	12.53 ± 0.11	11.40 ± 1.40	7.51 ± 0.28	3.56 ± 0.19

Table 3. Effect of temperature on amylase production, using rice as carbon source.

Microorganisms	Amylase activity (IU/ml)			
	25°C	30°C	35°C	40°C
Aspergillus niger	7.75 ± 0.14	13.49 ± 0.16	12.76 ± 0.13	12.05 ± 1.10
Penicillum sp.	7.38 ± 0.20	13.26 ± 0.11	11.83 ± 0.50	5.81 ± 0.05
Chrysosporium sp.	9.34 ± 0.53	12.87 ± 0.08	12.61 ± 0.02	7.51 ± 0.28

Table 4. Amylase production using rice as carbon source.

Microorganisms	Amylase activity (IU/ml)
Aspergillus niger	13.49 ± 1.37
Penicillum sp.	13.26 ± 0.93
Chrysosporium sp.	12.87 ± 1.42

Table 5. Amylase production using rice + rice bran in the ratio of 3:1 as carbon source

Microorganisms	Amylase activity (IU/ml)
Aspergillus niger	14.35 ± 1.23
Penicillum sp.	14.27 ± 0.97
Chrysosporium sp.	13.93 ± 0.78

carbon source. The results indicated that the best yields in all the three isolates tested were produced at pH 5 and 30°C.

Table 4 showed that *Aspergillus niger* produces more amylase activity under rice carbon source environment, when compared with *Penicillum* sp and *Chrysosporium* sp but when rice+rice bran were used as carbon source in the ratio of 3:1, *Aspergillus niger* still proved best by yielding 14.35 ± 1.23 (IU/ml) amylase activity as was shown in Table 5, although, *A. niger* made higher yield than the other two, yet *Penicillum* sp and *Chrysosporium* sp produced more amylase than *Aspergillus niger* when compared with the yield between the two carbon sources used as was shown in Table 6.

DISCUSSION

The occurrence of amylolytic organisms in the soil, especially starch processing sites, agrees with earlier reports by Omemu *et al.,* (2005) that soil is known to be a repository of amylase producers. The use of starch nutrient agar and iodine for detecting amylase (hydrolytic enzyme) producing microorganisms have been reported by Forgarty and Kelly (1979) and also by Iverson and Millis (1974) that starch hydrolysis can be detected on plates as a clear zone surrounding a colony.

The three fungal isolates were selected by starch hydrolysis in starch yeast extract media after KI/I_2 treatment produced detectable quantities of amylase in solid state fermentation process.

Results on the effect of temperature and pH on amylase production using rice as carbon source showed

Table 6. Amylase yield comparison between rice and rice+rice bran.

Microorganisms	Amylase activity (IU/ml) (Amylase yield on rice+ricebran minus amylase yield on rice alone)
Aspergillus niger	0.86
Penicillum sp.	1.01
Chrysosporium sp.	1.06

that amylase were best produced in the three isolates tested at 30°C and pH 5. But when subjected to rice + rice bran in the ratio of 3:1 as the only carbon source result showed that the crude enzyme yields were significantly better when compared with only rice as carbon source.

Emphasis today is gearing towards the possibility of generating wealth from wastes-agro-industrial-by-products like rice bran among many others. In support of this innovation of amylase production using wastes-agro-industrial-by-products, our work showed that *A. niger, Chrysosporium* sp. and *Penicillium* sp. are lucrative isolates-producing-amylase using rice-bran, even though *Chrysosporium* sp and *Penicillium* sp produced 1.06 and 1.01 IU/ml amylase activity respectively for against 0.86 IU/ml amylase activity produced by *A. niger.* Although *A. niger* produces higher yield in both carbon sources, yet *Chrysosporium* sp. and *Penicillium* sp. produces more amylase activity when compared with their individual performances using the two carbon source, which shows that *Chrysosporium* sp. and *Penicillium* sp. can be more efficient, using agro waste rice bran than with rice alone.

This supported the work done by Kathiresan and Manivannan (2006) and Jarosalava et al. (2014) which proved that *Penicillium fellutanum* and *Chrysosporium tropicum* respectively can produce high yield of amylase using wastes-agro-industrial-by-products. Why we referred *Chrysosporium* sp. and *Penicillium* sp as more lucrative is based on the utilization and conversion of agro waste to products of higher value.

Solid-state fermentation has emerged as a potential technology for the production of microbial products such as enzymes, feed, fuel, food, industrial chemicals and pharmaceutical products. Thus, channeling such agro-industrial residues to products of higher value will not only reduce wastage but will also create employment opportunities to people and expand our horizon for further exploration regarding industrial applications.

In conclusion, garri processing site is a proper habitat that harbors amylase producing microorganisms and can be explored the more on whether these isolates can proffer help in the area of bioremediation of starch polluted environment.

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