ISOLATION, IDENTIFICATION AND SCREENING OF MICROBES FROM THE FEDERAL POLYTECHNIC, ILARO'S BOTANICAL GARDEN FOR AMYLASE

ODUWOBI, O. O.

Science Laboratory Technology Department,
The Federal Polytechnic, Ilaro, Ogun State
E-mail: dwheazy2012@gmail.com; 08057954550

Abstract

Amylases are enzymes that catalyze the hydrolysis of starch or glycogen into simple sugar units of maltose and glucose. Amylases are among the most important enzymes widely studied in biotechnology. The aim of this study is to isolate and screen microbes from The Federal Polytechnic, Ilaro's botanical garden for amylase production. The isolates were all screened for amylase by growing their axenic cultures on starch agar medium. Three fungi belonging to three genera and two bacteria belonging to two genera were repeatedly isolated; Fusarium oxysporum, Malassezia globosaAspergillus niger,Staphylococcus aureus and Bacillus subtilis respectively, from soil samples collected from three different points. All the isolated fungi are amylase producers, though at varied degrees with Aspergillus niger being the most impressive producer by recording significant clear zones on starch agar medium while Malassezia globosa was the least. The former recorded a mean value of 34.33mm while the latter recorded an 11.67mm mean value. Fusarium oxysporum recorded a mean value of 24.67mm. B. subtilis is an amylase producer while S. aureus is not. The former recorded a mean value of 18.3mm. The control experiment was negative for the presence of starch and amylase. It could be deduced that the Aspergillus niger and Bacillus subtilis microbial strains isolated from the soil sample both have greater potentialities for the production of amylase than the other microbial strains. More attention and funding should be given to the research of the use of indigenous microbes with the potential ability to produce heat-stable amylase en masse.

Keywords: Enzymes, catalyze, hydrolysis, indigenous, heat-stable

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 the increase in the number of industries, especially food, beverages, textile, leather and paper industries (Pandey *et al*., 2000). Amylases are enzymes that catalyze the hydrolysis of insoluble starch or glycogen into soluble simple sugar units of maltose and glucose. Starch, a main component of our daily diet, is frequently found not only in food residues on dishes but also in food stains on clothes. Soil is one of the richest sources of starch-degrading microorganisms, as it contains abundant starchy substances (Pandey *et al*., 2000).

Amylases are important enzymes employed in the starch processing industries for hydrolysis of starch into simple sugars (George-Okafor, Tasie, &Anyamene, 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). Amylase can be obtained from several fungi, yeast, bacteria and actinomycetes. Many fungi have been found to be good sources of amylolytic enzymes. Many studies indicated that amylases of fungal origin are more stable than those of bacterial origin (Sanghvi, Koyani, & Rajput,2011). The major advantage of using microorganisms for the production of amylase is in economical bulk production capacity and microbes are manipulated to obtain enzymes of desired characteristics (Aiyer, 2005). Although, many microorganisms produce this enzyme, the most commonly used for their industrial application are *Bacillus licheniformis*, *Bacillus amyloliquifaciens* and *Aspergillus niger*,as theystand out as a class of enzymes which have 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 malto-tetraose syrup. In detergent production, they are applied to improve cleaning effect and are also used for starch de-sizing in textile industry (Vidyalakslimi, Pratithaman, & Indhumathi, 2009). The capacity of *Bacillus* strains to produce large

quantities of enzymes has placed them among the most important industrial enzymes producers. Indeed they produce about 60% of commercially available enzymes (Burhan *et al*., 2003).

Amylases are among the most important enzymes widely studied in biotechnology. They constitute a class of industrial enzymes having approximately 25% of the enzymes in the market (Rajagopalan, & Krishnan, 2008). They have a wide area of potential applications in baking and bread industry, paper industry, textile desizing, detergent industry, starch liquefaction and saccharificaiton, food and pharmaceutical industries and also in medical and clinical chemistry. In addition, they play an important role in the biochemical cycle of carbon (Gupta, Gigras, Mohapatra, Goswami, & Cahuhan, 2003). One of the best characteristics of industrial enzymes is stability at high temperature. Therefore, thermostability is a desired characteristic of most of the industrial enzymes. Thermostable enzymes isolated from thermophilic organisms have a number of commercial and industrial applications because of their stability. Amylases working at high temperature are important for industrial application. This high temperature helps to decrease viscosity of the medium, increase substrate solubility and reduce risk of microbial contamination (Kichiner, & Arnold, 1997). Thermostable amylases have been reported from several bacteria strains and have been produced through the use of submerged fermentation as well as solid state fermentation (Teodoro, & Martin, 2002).

Materials and Methods

Sample Collection

Soil samples (sub-surface soil) were collected from three different points, using a hand trowel, at The Federal Polytechnic, Ilaro's botanical garden, after removing the surface debris. They were collected on the 4th of October, 2021, into clean polythene bags. The Federal Polytechnic, Ilaro's botanical garden is situated in the polytechnic itself in Ilaro town, Ogun State, with the coordinates latitude 6.8863^0N and longitude 2.9967^0E.The appropriate precautionary measure was adopted such as the use of a pair of hand gloves.

Media Preparation

The total viable bacterial count was carried out on nutrient agar. The nutrient agar (NA) was prepared according to the manufacturer's instruction. Approximately 12ml of the molten medium was aseptically dispensed into sterile disposable 90mm Petri plates and then allowed to gel (Cheesbrough, 2000). Potato dextrose agar (PDA) was the medium for the growth and maintenance of the fungal isolates.The PDA was also prepared according to the manufacturer's instruction. The medium was allowed to cool and streptomycin was incorporated to suppress bacterial contamination. Approximately 12ml of the molten PDA was aseptically dispensed into the sterile disposable 90mm Petri plates and then allowed to gel (Okigbo, & Emeka, 2009). Soluble starch agar was the medium used for the screening of the bacterial and fungal isolates for amylase production. It was likewise prepared according to the manufacturer's instruction.

Isolation

Pour plate method, as described by Chukwu *et al*. (2000), was employed to isolate bacteria and fungi from the soil samples. A six serial ten-fold dilution from 1g of the sample was conducted using 1ml stock homogenate. 1ml stock homogenate was aseptically introduced into the 10^{-1} test tube; the serial dilution was carried out up to 10^{-6}. 10^{-2} and 10^{-4} diluents were used for the bacterial culturing (Hassan, 2014). Distinct colonies were sub-cultured to obtain pure cultures. The plated pure cultures were then incubated appropriately after repeatedly streaking. The microbial cultures in agar slants were preserved in McCartney bottles and incubated until the appearance of distinct colonies. The pure cultures were kept refrigerated as stock cultures. Labeling of the samples was done accordingly.

Cultural Identification

The fungal isolates were identified on the basis of their morphological and cellular characteristics, as described by Barnet and Hunter (1999). The isolates were identified by first mounting each fungal mycelium on a glass slide and then stained with lactophenol-in-cotton blue dye, covered with a cover slip and then mounted on a compound microscope. The slides were viewed under x10 and x40 objective lenses. The physical microscopic characteristics (hyphae, spores, conidial heads and arrangement of conidia of the fungi) were observed and related to a Fungi Atlas for identification.The bacteria isolated were identified based on their Gram's status, colonial characteristics and various biochemical tests such as urease, catalase, citrate, indole, methyl red, coagulase, oxidase etc., according to

Cheesbrough (2000) and Silverton and Pallister (2001). Bergey's Manual of Systematic Bacteriology was used as a reference for the identification of the bacteria isolated.

Screening of the Microbial Isolates for Amylase Production

The isolates were all screened for amylase production potentiality by growing their axenic cultures on starch agar medium comprising the following in gm/l; yeast extract 1.5g, peptone 0.5g, sodium chloride 1.5g, starch 10g and agar 15g (Lingappa, & Vivekbabu, 2005). The pH of the medium was made similar with the pH of the soil sample. By streaking the cultures unto the media, the plates containing the inocula were incubated appropriately. After the incubation, iodine solution was flooded onto the cultured plates with a dropper for 30 seconds and observed for zone of hydrolysis around the colonies. The iodine solution indicates that starch is indeed present in the growth medium, as iodine solution is commonly used for the qualitative analysis of starch. The presence of blue colour around the growth indicates negative result and a clear zone of hydrolysis around the growth indicates a positive result. The isolates that produced clear zones of hydrolysis were considered as amylase producers. The diameter formed after the addition of iodine solution was measured in millimetres using a transparent ruler, to represent the degree of amylolytic activity (Oyeleke, Oyewole, & Egwim, 2011).A control experiment with nutrient agar and potato dextrose agar media both with iodine solution instead of the starch agar medium with iodine solution as an indicator was set up to confirm the presence of starch and the ability of the pure isolates for amylase production.

RESULTS

Tables 1 and 2 show the probable identities of the fungi and bacteria isolated from the soil samples collected from the three different points at the botanical garden of The Federal Polytechnic, Ilaro. Three fungi belonging to three genera were repeatedly isolated and were conspicuously differentiated by the variation in their colonial appearance and colour. They include *Fusarium oxysporum*, *Malassezia globosa* and *Aspergillus niger*. Two bacteria belonging to two genera were isolated and were identified based on standard tests for the identification of bacteria. They are *Staphylococcus aureus* and *Bacillus subtilis.*

Table 1: Cultural and Microscopic Characteristics of the Fungal Isolates

S/N	Colonial appearance	Microscopy	Organisms
1	Beige mould	Mono-polar budding, cream to yellowish to brown colonies, smooth and hypha absent	*Malassezia globosa*
2	White mould	Multicellular distinctive sickle-shaped macroconidia, aseptate, floccose with sparse mycelia	*Fusarium oxysporum*
3	Black mould	Colonies are jet-black, globular or spherical profuse conidia, conidia are biseriate	*Aspergillus niger*

Table 2: Bacterial Strains Isolated from the Soil Samples

Characteristic test/Probable identity	*S. aureus*	*B. subtilis*
Gram stain	+	+
Morphology/shape	Coccus	Rod
Motility	-	+
Indole	-	-
Citrate	-	+
H_2S	+	-
Urease	-	-
Spore	-	+
Catalase	+	-

Oxidase	-	-
Methyl Red	+	+
Voges-Proskauer	-	-
Capsule	-	-
Coagulase	+	-
Starch hydrolysis	+	+
Nitrate reduction	-	+
	Sugar Fermentation Test	
Glucose	+	+
Fructose	+	-
Lactose	+	-
Sucrose	+	-
Raffinose	-	+
Maltose	+	-
Mannose	+	-
Xylose	-	+
	Sugar Alcohol Test	
Mannitol	+	+
Sorbitol	+	+
Inositol	+	-

Keys: + = Positive, - = Negative

Table 3: The Fungal Load of the Soil Samples

Isolate	**Point A (cfu/g)**	**Point B (cfu/g)**	**Point C (cfu/g)**	**Mean value (cfu/g)**
Malassezia globosa	18.00×10^4	10.00×10^4	12.00×10^4	13.33×10^4
Fusarium oxysporum	28.00×10^4	30.00×10^4	22.00×10^4	26.73×10^4
Aspergillus niger	35.00×10^4	38.00×10^4	39.00×10^4	37.33×10^4
Total	81.00×10^4	78.00×10^4	73.00×10^4	77.39×10^4

Table 4: The Bacterial Load of the Soil Samples

Isolate	**Point A (cfu/g)**	**Point B (cfu/g)**	**Point C (cfu/g)**	**Mean value (cfu/g)**
S. aureus	$21.00 \pm 0.58 \times 10^4$	$22.10 \pm 0.50 \times 10^4$	$18.25 \pm 0.48 \times 10^4$	20.42×10^4
B. subtilis	$39.30 \pm 0.35 \times 10^4$	$38.00 \pm 2.71 \times 10^4$	$30.10 \pm 4.33 \times 10^4$	35.80×10^4
Total	60.03×10^4	60.10×10^4	48.35×10^4	56.16×10^4

From the investigation, as shown in tables 3 and 4,there is a relatively high population of these isolated bacteria at the botanical garden site, particularly *Bacillus subtilis*; a well-known soil bacterium and also a relatively high population of those fungi, particularly *Aspergillus niger*. Out of the three different points from which the soil debris was obtained at the dump site, point A recorded the highest fungal load of 81.00×10^4 cfu/g while points B and C

recorded lower values in terms of the fungal load; 78.00 x 10^4 cfu/g and 73.00 x 10^4 cfu/g respectively. *Aspergillus niger* was the most prevalent fungus at the three points.Point B recorded the highest bacterial load of 60.10 x 10^4 cfu/g while points A and C recorded lower values in terms of the bacterial load; 60.03 x 10^4 cfu/g and 48.35 x 10^4 cfu/g respectively. *Bacillus subtilis* was more prevalent at the three points.

The plates were observed for colour changes and clear zones. The isolates were classified as either a potential amylase producer or not. Table 5 shows the amylase-production potential of the three fungal isolates and its degree is depicted by the diameters of the clear zones. From the investigation, all the isolated fungi are amylase producers, though at varied degrees with *Aspergillus niger* being the most impressive producer by recording significant clear zones on starch agar medium while *Malassezia globosa* was the least impressive producer but nevertheless, still is an amylase producer. The former recorded a mean value of 34.33 mm while the latter recorded an 11.67 mm mean value. *Fusarium oxysporum* recorded a mean value of 24.67 mm.

Table 5: The Amylase-production Potentiality of the Fungal Isolates

	Zones of Hydrolysis (mm)		
Location	***Malassezia globosa***	***Fusarium oxysporum***	***Aspergillus niger***
Point A	11.00	24.00	32.00
Point B	10.00	22.00	38.00
Point C	14.00	28.00	33.00
Mean Value	11.67	24.67	34.33

Table 6: The Amylase-production Potentiality of the Bacterial Isolates

	Zones of Hydrolysis (mm)	
Location	***B. subtilis***	***S. aureus***
Point A	20.0	-
Point B	17.0	-
Point C	18.0	-
Mean Value	18.3	-

Table 6 shows the amylase-production potential of the two bacterial isolates, as the two plates showed a blue-back colour change which is a strong indication of the presence of starch. The control experiment was negative for the presence of starch and amylase production (plates not presented). From the investigation, *B. subtilis* is an amylase producer while *S. aureus* is not. The former recorded a mean value of 18.3 mm while the latter recorded a nil mean value.

Discussion

From this study, *Bacillus subtilis* not only recorded a higher frequency, it was also established to have the potentiality of producing amylase enzyme, unlike *Staphylococcus aureus* which recorded no amylolytic activity and a lower frequency of occurrence. *Aspergillus niger*toonot only recorded the highest frequenc, it was also established to have the best potentiality of producing amylase enzyme, unlike *Fusarium oxysporum* and *Malassezia globosa* which both recorded lower amylolytic activity and frequency of occurrence. Perhaps, the former, in each case, has a shorter generation time as compared with the latter, hence, their ability to quickly proliferate or they are both able to survive better or are better suited to adapt to the environmental conditions present at the botanical garden at that time.

The occurrence of these amylolytic organisms in the soil samples obtained from the botanical garden agrees with an earlier report by Omemu, Akpan, Bankote, &Tenioda (2005) that soil is known to be a repository of amylase producers. The result of this investigation is in agreement with Pandey *et al.* (2000) who reported *Bacillus subtilis* to be the most prominent among various amylase-producing bacteria. *Bacillus* species are considered to be the most

important sources of amylase and have been used frequently for enzyme production. This result is also similar with the findings of Ubalua (2014) who reported a high occurrence of *B. subtilis* from garden soil. Starch degrading enzymes called amylases have received a great deal of attention because of their perceived technological significance and economic benefits. Amylases have got increasing demand and potential application in a wide number of industrial processes. This is because of the enormous interest in developing enzymes with better properties such as raw starch degrading amylases suitable for industrial applications and their cost-effective production techniques (Sharma, & Shukla, 2008). The amylases produced by microorganisms have a broad spectrum of industrial applications as they are more stable than plant and animal amylases. Due to its promising applications, the search for new source of thermostable amylase continues. Bacteria have become important sources thermostable amylases for industrial application because they produce amylases with greater thermostability properties as compared to fungal amylases (Prakash, & Jaiswal, 2009).

Conclusion

By this investigation, it could be deduced that four of the five microorganisms isolated from the soil samples from the botanical garden have potentialities for the production of amylasebut *Aspergillus niger* and *Bacillus subtilis* microbial strains both have very greater potentialities, judging by their abilities to generate zones of clearance on starch agar medium incorporated with iodine. Although, *Aspergillus niger* was a better amylase producer between the two, *Bacillus subtilis* was also significantly remarkable as an amylase producer.

Recommendations

Based on the promising lead from this investigation, it is therefore recommended that;

- More attention and funding should be given to the research of the use of indigenous microbes with the potential ability to produce heat-stable amylase en masse.
- On the basis of biotechnology, focus may be given to the possibility of producing amylase synthetically by DNA recombinant technology to boost amylase yield.
- Due to high industrial and commercial demand for amylase, it can be recommended that readily-made and cheap amylase could be obtained from microbes isolated from botanical gardens.
- The upstream and downstream processing of amylase should be adequately scaled-up and the production of amylase optimized to meet the growing demand for amylase for commercial use and also ensure its affordability.

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