HYDROLYTIC ENZYMES PRODUCING CAPABILITIES OF MICROORGANISMS FROM SOIL AND DECAYED YAM TUBERS IN UYO, NIGERIA

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ABSTRACT: Aliquots of ten-fold serially diluted samples were streak-plated on nutrients agar and Sabouraud dextrose agar for the isolation of bacteria and fungi respectively. The isolates were purified by repeated plating on the respective media and maintained as slant cultures at 4^{o}C. The isolated organisms were characterized and screened for the elaboration of hydrolytic enzymes following the hydrolysis of specific growth media. The hydrolytic enzymes producing cultures were characterized by their growth and elaboration of amylolytic, proteolytic, lipolytic as well as celloulolytic activities on nutrient-starch agar, nutrient-gelatin and nutrient-casein agar, Sierra-Tween 80 agar, and carboxymethyl cellulose-agar respectively. Elaboration of hydrolytic enzymes by the isolates was evident by the appearance of clear zone around the positive colonies in the respective media. All the isolates (*Bacillus* sp., *Streptomyces* sp., *Aspergillus* sp., *Rhizopus* sp., and *Mucor* sp.,) tested showed evidence of amylase, protease and lipase activities; while only *Streptomyces* species and all the fungal isolates demonstrated cellulase activity. Enzyme activities and production kinetics were assayed using standard procedures.

INTRODUCTION

Enzymes are proteins acting as hydrolytic organic catalysts to speed up various biochemical reactions without being changed (Nelson and Cox, 2000). The reactions always involve the coupling of enzyme to a specific substrate, resulting in an enzyme-substrate specificity complex, which at the completion of the reactions are released unchanged for catalyzing another reaction of same specificity. Thus, enzymes are bio-chemically active compounds, principally made up of a protein-apoenzyme, and a non-protein cofactors or coenzymes and are therefore sensitive to different physiological and environmental conditions (Nelson and Cox, 2000).

Enzymes complexes which are very useful and essential building blocks of life are generated by all living cells. Microbial enzymes are notably produced by moulds, yeasts, bacteria and actinomycetes. These microorganisms are adapted to produce diverse and large amounts of hydrolytic enzymes *in situ* when introduced to an appropriate system. Hydrolytic enzyme include the amylases, proteases, lipases, pectinases, pullulanases, amyloglucosidases, glucomylases, cellulases, xylanases, chitnases and keratinases (Cowan, 1991; Toye, 2002; Sanchez-Porro *et al.* 2003). Thus, microbial enzymes are responsible for the diverse array of substrate-specific biochemical reactions occurring during the growth and subsequent biodegration of organic matter by microorganisms, resulting from the liberated extracellular hydrolytic enzymes. These enzymes catalyzed the breakdown of different organic polymers such as starch, cellulose, protein, lipids, xylan, chitin and pectin into simpler monomeric

compounds such as glucose, maltose, limit-dextrin, amino-acids and esters for their nutritional, biogeochemical recycling and diverse industrial benefits (Berry and Patterson, 1990; Crueger and Crueger, 1990; Grab and Mitchison, 1997; Gupta *et al.* 2002).

The industrial and biotechnological applications of the extracellular hydrolytic enzymes are carried out under different and specific physicochemical conditions which cannot always be adjusted to the optimal values required for the activity of the available enzymes. This informed the need for the continuous search and isolation of extracellular hydrolytic enzymes producing microorganism. This preliminary study describes the isolation and screening of extracellur hydrolytic enzymes such as amylase, protease, lipase and cellulase produced by microorganisms from soil and decayed yam in Uyo, Nigeria.

MATERIALS AND METHODS

Collection and Processing of Samples

Soil samples from different areas of compost, refuse dumpsite as well as palm-oil processing sites were randomly collected in sterile polyethene bags and stored in the laboratory at 4^oC until processed, by sorting and sieving to remove debris. Yam tubers were also harvested from Uyo soil, sized into tiny pieces and kept in a dark place for decomposition by microbial activities within 7 days. The decayed yam samples were then macerated and homogenized. One gramme of the processed samples were respectively weighed and dissolved in 1 ml of sterile distilled water in 100 ml Erlenmeyer flasks. The prepared stock solutions were vigorously shaken to release the vegetative cells and spores and kept for 5 minutes to settle. Thereafter, 1 ml of these stock samples were serially diluted and used for the isolation of microorganisms.

Isolation, Purification and Characterization of Microorganisms from Soil and Yam Samples.

Aliquots of the appropriately diluted samples were respectively streak-plated on sterile nutrient agar (NA) and Sabouraud dextrose agar, (SDA) and incubated at 37^oC for 48h and 28^oC for 72 h for the isolation of bacteria and fungi respectively. Pure cultures of the microbial isolates were obtained by repeated subculturing and maintained as slants in the respective media at 4^oC. The microbial isolates were characterized and identified based on standard taxonomic, morphological, biochemical and physiological indices typical of bacteria and fungi taxa. Bacteria were identified following the method of Collins and Lyne (1991). Fungal isolates were identified following the slide culture protocols of Evans and Richardon, (1989).

Extracellular Hydrolytic Enzymes Assay

Prevalent bacterial and fungal species isolated from the soil and decayed yam samples were screen for hydrolytic enzymes producing potential on different agar- plate using the solid state fermentation (SSF) as follows:

Amylase Assay

Amylase production and amylotytic activity was assayed on plates of nutrient-starch agar, NSA (soluble starch, 2 g, nutrient-agar, 3 g, distilled water 100 ml) following starch hydrolysis (Cowan, 1991; Sanchez-Porro *et al*. 2003). After incubation for 7 days at 37^oC for bacteria and at 28^oC for fungi, the plates were flooded with 0.3 % 1_2- 0.6 % KI solution and kept for 5 min. Clear zones around the growth of positive colonies indicated starch hydrolysis. The zones were measured and the positive colonies isolated as alpha-amylase producers.

Protease Assay

Protease production and proteolytic activity was assayed on plates of both nutrient-casein agar, NCA (skim milk 10 g, nutrient agar 3 g, distilled water 100 ml) and nutrient-gelatin agar, NGA (8 % gelatin 50 ml: nutrient agar 3 g, distilled water 100 ml) following either casein

hydrolysis, or gelatin liquefaction (Sanchez-Porro *et al.*, 2003). The appearance of zone of casein precipitation around the positives colonies after 48 h incubation at 37°C for bacteria and 28°C for fungi; or the appearance of clear zone around the positive colonies following the flooding of NGA plates with a solution of saturated ammonium sulphate after 7 days incubation at 37°C for bacteria and 28°C for fungi was taken as evidence of proteolytic activity.

Lipase Assay

Lipase production and lipolytic activity of isolates was detected on plates of Sierra-Tween 80 agar, STA (peptone 10 g, NaCl 0.5 g, $CaCl_2$ $2H_2O$ 0.05 g, 10 % Tween 80 10 ml, agar 2 g, distilled water 100 ml). The appearance of zones of hydrolysis around the positive colonies grown on STD plates after 48 h incubation at 37^0C for bacteria and 28°C for fungi, indicated lipolytic activity.

Cellulase Assay

Cellulase production and cellulolytic activity by the isolates was detected on plates of carboxymethyl cellulose (CMC) agar, CMC-A (for bacteria: CMC 2 g, NH_4CI 0.5 g, NaCl 0.5 g, K_2HPO_4 0.3g KH_2PO_4 0.4 g, $NgSO_4$ 0.1 g, yeast extract 1g, agar, 2 g, distilled water 100 ml; for fungi-CMC 2g $(NH_4)_2SO_4$ 4.5 g, $MgSO_4$ 0.1 g, KH_2PO_4 2.5 g, urea 1.0 g, agar 2 g, distilled water 100 ml; for fungi-CMC 2 g $(NH_4)SO_4$ 4.5 g, $MgSO_4$ 0.1 g, KH_2PO_4 0.1 g, KH_2PO_4 2.5 g, urea 1.0 g, agar 2 g, distilled water 100 ml). Cellulolytic activity of positive colonies after 7 days of growth on CMC – A at 37^0C for bacteria and 28 ^{0}C for fungi, was detected by the degradation of CMC-A, indicated by the appearance of clear zone of hydrolysis, after the flooding of the CMC-A plates with Congo red solution and later with 1 M NaCl for 15 minutes, and later with 1 m dilute hydrochloride for 15 minutes, to stabilized the zones of hydrolysis.

Determination of Time Course of Enzymes Production

Estimation of time course of hydrolytic enzymes production by the isolates was carried out in submerge fermentation (SMF) using the basal fermentation medium and protocols of Achi and Njoku-obi (1992). The medium comprised K2HPO_4 1 g, NaCl 4 g, $MgSO_4$. $7H_2O$ 1g, $CaCl_2$ 3 g, tryptone 1 g, yeast extract 2 g, sodium citrate 5 g, cowblood meal 20 g, and distilled water 1000 ml. the basal fermentation medium was modified by supplementing with specific substrates as follows: amylase-soluble starch 20 g; protease-1 % azocasein 25 ml; 8 % gelatin 2 ml; lipase-10%Tween 80 10 ml, and cellulose-CMC 20 g.

2 ml of 48 h old bacterial cultures and 72 h old fungal cultures of the hydrolytic enzymes producers previously grown on NA and SDA respectively were aseptically incubated in 50ml of the differently modified production media contained in 100 ml Erlenmeyer flasks. The flasks were incubated into 50 ml of the different modified production media contained in 100 ml Erlenmeyer flasks. The flasks were incubated in a Gallenkamp rotary incubator operating at 200 rev/min at 37 ^{0}C for bacteria, and 28^0C for fungi for 8 days for the elaboration of the hydrolytic extracellular enzymes. Cells were harvested daily in the composite reaction mixture by adding 20 ml of 0.1 % TCA, to stop enzymes synthesis, and extracted by aseptical filtration with Whatman No.1 filter paper. The crude culture filtrates were centrifuged at 400 rem for 15 min, and the culture supernatants assayed for enzymes activity following standard procedures.

Measurements of Enzymes Activity

Amylase and cellulase activities were calculated by measuring the amount of reducing sugars liberated from soluble starch (Bernfeld, 1955: Obi and Odibo, 1984); and CMC (Mandels *et. al.*, 1976). The culture supernatants were incubated with the substrate solutions [(1 % ($^w/_V$) soluble starch; 1 % ($^w/_V$) CMC, all in 50 mM acetate-buffer, pH 6.51] at 37^0C for 1 h. Dinitrosalicyclic acid, DNS 2.0 ml was added to stop the reaction. The mixture was boiled for 5 min., and allowed to cool naturally. Reducing sugars liberated were determined by measuring the

absorbance at 550 nm. One unit of enzymes activity was defined as the amount of enzyme producing 1ml of reducing sugars (maltose of glucose) per min at 37 ^{0}C.

Protease activity was determined by the hydrolysis of azocasein (Sigma) following the modification of the method of Lowry *et. al.,* (1951), by Lama *et. al.,* (2004). The reaction mixture consisted of 2 ml of culture supernatants, 25 ml of 1 % ($^w/_v$) azocasein, and 25 ml of 50 mM Tris-HCl pH 8.0, containing 2.0 mM $CaCl_2$ and 2.0 % NaCl. The reaction mixtures were incubated at 60 ^{0}C for 10 min, and enzymes activity stopped by addition of 10 % ($^v/_v$) TCA and refrigerated for 15 min. Thereafter, the mixtures were centrifuged for 10 min at 9000 g, and 1 ml of 1:3 folin – ciocalteau reagent was added to the supernatants to measure the amount of peptides liberated by the proteinase action, with bovine serum albumin (BSA) as standard (Lowry *et. al.,* 1951). The supernatants absorbances were measured spectrophotometrically at 360 nm against a blank (complete reaction mixture stopped before incubation). One unit of protease activity was defined as the amount of the enzyme giving an absorbance change of 0.1 in 10 min.

The quantity of lipase produced in the culture supernatants was assayed titrimetrically by the modifications of several methods, Das and Bernejee (1977); Hellgreen and Vincent (1980); Deploey (1981); Okafor and Gugnani (1990). The reaction mixture consisted of 1 ml of crude enzymes supernatant, 2 ml of substrates and 5 ml of sodium acetate buffer incubated in a water bath at 45 ^{0}C for 3 h using 100 ml Erlenmeyer flasks. The reaction mixture was stopped by addition of 16 ml 1:1 ($^v/_v$) of acetone-ethanol, and back titrated with 0.05 M KOH against the liberated fatty acids, using two drops of phenolphthalein as end point indicator. The amount of lipase produced was expressed as units of enzymes activity. One unit of lipase activity was taken as the amount of enzymes necessary to liberate fatty acids equivalent to 0.01 ml of 0.05 M KOH from the triglycerides (Tween80).

RESULTS AND DISCUSSION

A total of 36 aerobic microorganisms comprising 22 (61.1%) bacterial and 14 (38.9%) fungal species were isolated and characterized. The bacterial isolates were all Gram-positive species belonging to the genera: *Bacillus* (8 isolates), and *Streptomyces* (14 isolates). The fungal isolates were members of the genera *Aspergillus* (7 isolates), *Rhizopus* (4 isolates) and *Mucor* 3 isolates) (Table 1) The spatial distribution of the isolates indicated that bacteria accounted for the highest frequency (61.1 %), while fungal species were less (38.9 %). Amongst the bacterial isolates; Streptomyces species occur more frequently (63.6%) compared to species in the genus *Bacillus* (34.4 %); while the frequency of occurrence of the fungal species was in the descending order *Aspergillus* (50 %), *Rhizopus* (28.6 %) and *Mucor* (21.4 %). This observed intra and inter species distribution differential could be attributed to the nutritional, physiological as well as the genetic makeup of the organisms. All the microbial species were isolated from the different samples, indicating the *in situ* presence of these heterotrophic saprophytes in the environment and involved in the biodegradation and biogeochemical recycling of the abundant organic matter.

Of the 36 microbial isolates, 16 strains were most outstanding in the growth and elaboration of the hydrolytic extracellular enzymes on plates of specific substrates, detected by the appearance of clear zone of hydrolysis (Table 2). The results indicate no observable significant differences in the hydrolytic activities of the isolates with respect sources of sample.

Nevertheless the plate assay method (solid state fermentation, SSF) employed in the study provided convenient, rapid and sensitive preliminary screening test for the isolation of the extracellular hydrolytic enzymes producing microorganisms (Krishna, 2005). The SSF only permitted the effective use of soluble substrates. However the formation of clear zones of

hydrolysis by positive colonies may not be an indication of production of all enzymes of the specific enzymes-system complexes. This may be particularly related to the activities of lipolytic and cellulolytic enzymes. In the former, the hydrolysis of the soluble Tween 80 in STA by the isolates was indicative of an esterase and not lipase activity.

Table 1: Spatial Distribution, Frequency of Occurrence and Prevalence of Microbal Isolates in the soil and Decayed Yam Samples.

Isolate/Codes	No. of Isolates	Occurrences in soil samples (n=3)	Occurrence in Yam Samples (n=1)	Prevalence Rate (%)
Bacteria (Isolates, 61.1%)				
Bacillus- BB1	3	2	1	75
Bacillus-BB2	2	1	1	50
Bacillus-BB3	1	1	0	25
Bacillus –BB4	3	2	1	75
Bacillus –BB5	3	2	1	75
Bacillus –BB6	2	2	1	50
Bacillus –BB7	2	1	0	50
Bacillus –BB8	4	3	1	100
Streptomyces-BS1	4	3	1	100
Streptomyces-BS2	3	2	1	75
Streptomyces-BS3	2	1	1	50
Streptomyces-BS4	4	2	0	50
Streptomyces-BS5	1	1	0	25
Streptomyces-BS6	2	1	1	50
Streptomyces-BS7	4	3	1	100
Streptomyces-BS8	3	2	1	75
Streptomyces-BB9	3	2	1	75
Streptomyces-BS10	4	13	1	100
Streptomyces-BS11	2	1	1	50
Streptomyces-BS12	2	1	1	50
Streptomyces-BS13	1	1	0	75
Streptomyces-BS14	2	2	1	50
Fungi (14 isolates, 38.9%)				
Aspergillus –FAI	3	2	1	75
Aspergillus- FA2	4	3	1	100
Aspergillus- FA3	2	1	1	50
Aspergillus- FA7	3	1	1	75
Rhizopus –FRI	4	3	1	100
Rhizopus-FR2	3	2	1	75
Rhizopus-FR3	2	1	1	50
Rhizopus- FR4	2	1	1	50
Mucor-FM1	4	2	1	75
Mucor-FM2	2	1	1	50
Mucor-FM3	2	1	1	50

This assertion is in line with previous reports that in lipolytic activity, esterase is produced by soluble emulsifible substrates and lipase by insoluble emulsifiable substrates (Jaeger *et. al* 1994; Arpigny and Jaeger, 1999; Sanchez-Porro *et al.*, 2003). Equally, the microbial formation of clear zones of cellulosis on the soluble CMC-A plates may not be indicative of production of all the enzymes of the cellulase complex, but rather it indicates the action of endoglucanases (Coughlan, 1985; Cowan, 1991; Pandey *et. al.*, 2000; Sanchez-Porro, *et. al.*, 2003).

Table 2: Hydrolytic Enzymes Producing Potential of the Active Microbial Isolates

Isolates	**Growth/zone of hydrolysis on media (mm)**				
	NSA	**NCA**	**NGA**	**STA**	**CMC-A**
Bacillus BB1	4.0	3.3	1.7	1.6	-
Bacillus BB4	4.1	3.5	2.6	1.4	-
Bacillus BB5	4.1	3.5	2.8	1.1	-
Bacillus BB8	4.4	3.8	2.8	1.9	-
Streptomyces BS1	4.3	3.7	2.0	1.2	-
Streptomyces BS2	5.2	3.0	2.0	1.3	
Streptomyces BS7	5.5	3.2	2.3	1.5	0.9
Streptomyces BS8	4.7	3.5	2.2	1.4	1.1
Streptomyces BS9	4.8	3.8	2.2	1.4	0.7
Streptomyces BS10	5.6	4.2	2.5	1.8	1.5
Aspergillus FA1	5.8	4.2	3.4	1.6	2.4
Aspergillus FA2	7.0	4.8	4.3	2.4	3.2
Aspergillus FA6	6.7	4.2	3.6	1.8	2.8
Rhizopus FR1	5.4	3.8	4.1	1.6	2.2
Rhizopus FR2	4.8	3.3	4.1	1.4	1.4
Mucor FMI	5.2	3.5	1.3	1.3	1.8

- Less microbial growth no zone of hydrolysis

Combined hydrolytic activities have been detected in a number of the isolates. Only eleven strains derived from the genera: *Streptomyces, Aspergillus, Rhizopus* and *Mucor* elaborated the four hydrolytic activities –amylase, protease, lipase and cellulase tested. Besides, all the 16 strains of *Bacillus, Streptomyces, Aspergillus, Rhizopus* and *Mucor,* showed combined activities only for three of the enzymes (amylase, protease and lipase), without any detectable cellulolytic activity on the plates of CMC-A, Presumably, this may not indicate a lack of activity, but that the released activity was not enough to cause noticeable zones clearing of the plates. It may possibly be associated with the scarcity or non-availability of the polymer in the habitat, thus cellulolytic bacteria are not normal inhabitants. Thus, the combined hydrolytic activities of the isolates is suggestive of the metabolism and assimilation of such complex substrate as their sole sources of carbon , nitrogen and energy (Sanchez-Porro, *et. al*, 2003, Favela-Torres *et. al.*, 2006). The lack of detectable cellulolytic activity by the *Bacillus* species and little activity by the Streptomyces species under the test conditions, compared with the much activity of the fungal isolates does not indicate that cellulolytic activity is exclusively produced by fungi. The cellulolytic activities of bacteria had previously been reported (Mandels *et. al.*, 1976).

Isolates with strong extracellular enzymes producing potential, were further assayed for enzymes production in submerged fermentation, SMF (Table 3). The result indicated that the supernatants of the test isolates elaborated the hydrolytic enzymes tested. However, *Bacillus-BB8* failed to produce any detectable cellulolytic activity. This may be attributed to the factors previously discussed, and not the bacterial inability to elaborate cellulase. In SMF, enzymes activities were observed to be minimal within the first day reaching peaks on 48 h for bacteria, and between 72 h and 96 for fungal isolates. This biosynthesis variation may be attributed to differences in composition of the fermentation media, incubation period, as well as physiological and genetic differences between bacteria and fungi in the metabolism and assimilation of the subtrates for growth and enzymes synthesis. The values of the extracellular hydrolytic enzymes activities obtained from solid-state fermentation, SSF (Table 2) were generally lower than values observed during the submerged fermentations SMF, (Table 3).

Table 3: Enzymes Assay and Time Course of Production

Isolates/Enzymes	**Enzymes activity (u mol^{-1})/Time (days)**				
	1	**2**	**3**	**4**	**5**
Amylase					
Bacillus BB8	0.85	1.37	1.05	0.53	0.21
Streptomyces BS 10	0.95	1.62	1.32	0.74	0.33
Aspergillus FA 2	0.53	1.36	1.89	1.74	1.56
Protease					
Bacillus BB8	0.07	0.15	0.04	0.03	0.015
Streptomyces BS10	0.07	0.14	0.05	0.04	0.02
Aspergillus FA 2	0.03	0.09	0.18	0.16	0.13
Lipase					
Bacillus BB8	4.0	6.4	3.4	2.2	2.1
Streptomyces BS10	3.8	6.8	3.6	3.3	2.6
Aspergillus FA2	2.6	5.6	7.8	7.6	7.4
Cellulase					
Bacillus BB4	-	-	-	-	-
Streptomyces BS9	0.09	0.18	0.07	0.04	0.015
Aspergillus FA2	0.13	0.39	0.64	0.82	0.78

-(not detected)

The observed differences between the two systems, corroborate several reports and may be attributed to the lack of catabolic repression in SSF as compared to that in the SMF; lower diffusion of substrates to the microorganisms in SSF; as well as higher oxygen levels in SSF at the solid-air interface which supports faster growth (Kapoor and Kuhad, 2002). However, the advantages of SSF over SMF, and the economic feasibility of adopting SSF technology in the commercial production of industrial enzymes have been recently evaluated (Krishna, 2005). In terms of economic feasibility of production steps, it is required to design process using inexpensive raw material that would enable high enzymatic concentration and productivities. Additionally, SSF is considered more suitable for moulds than for bacteria and yeasts growth (Raimbault, 1998; Pandey, 2003). This is justified in terms of the fact that moulds, as obligate aerobes prefer oxygenated substrates conditions which may not be provided by the comparatively more reduced submerged fermentation (SMF) technique. The low water activity status of SSF may counter bacterial contamination by improving the fungal metabolism (Favela-Torres *et .al.* 2006).

CONCLUSION AND RECOMMENDATION

It is obvious that microorganisms absorb nutrients through their cell wells by secreting extracellular enzymes to hydrolyze the food into small particles which are then absorbs into the cells and the concomitant biosynthesis of metabolites. Thus, heterotrophic saprophytes have the broadest spectrum of polysaccharides, proteins and lipids hydrolyzing enzymes to metabolise and assimilate available nutrients in the environment, owing to the large diversity of substrates large diversity of substrates in nature. However, studies to select the best hydrolytic enzymes producers, production optimization as well as detailed physicochemical characterization of the enzymes, cloning and characterization of the corresponding encoding genes are ongoing and recommended.

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