Available online at http://ajol.info/index.php/ijbcs

Int. J. Biol. Chem. Sci. 7(2): 641-648, April 2013

International Journal of Biological and Chemical Sciences

ISSN 1991-8631

Original Paper

http://indexmedicus.afro.who.int

Isolation, characterization and extracellular enzyme detection of microbial isolates from deteriorated apple (*malus domestica*) fruits

O.I. AYANDA, A.A. AJAYI*, G.I. OLASEHINDE and O.T. DARE

Department of Biological Sciences, Covenant University, Ota, Ogun State, Nigeria.
**Corresponding author, E-mail: adesola.ajayi@ymail.com; Tel: +2348095492372*

ABSTRACT

Studies were carried out on deteriorated apples obtained from the Just Rite supermarket, Ota, Ogun State, Nigeria, to isolate microorganisms associated with post-harvest deterioration of apple (*Malus domestica*) fruits. The bacterial species were identified using microscopy, morphology and various biochemical tests while macroscopy and morphology was used to identify the fungi isolated. The ability of the isolate to elaborate extracellular amylase and protease were tested for. Results revealed *Aspergillus niger* and *Aspergillus flavus* as the fungal species while the bacterial species isolated were Bacillus spp and Micrococcus spp. All the isolates except *Aspergillus niger* produced amylase in substantial amount. *Micrococcus spp, Aspergillus niger* and *Aspergillus flavus* produced protease. The results of this investigation if combined with further studies can be used in identifying organisms which could be used as a biological method in the control of apple pathogens.

Keywords: *Aspergillus niger, Aspergillus flavus, Bacillus* spp, *Micrococcus* spp, agar.

INTRODUCTION

Food spoilage is a metabolic process that causes foods to be undesirable or unacceptable for human consumption due to changes in sensory characteristics. Spoiled foods may be safe to eat, i.e. they may not cause illness because there are no pathogens or toxins present but changes in texture, smell, taste, or appearance cause them to be rejected. Some ecologists have suggested these noxious smells are produced by microbes to repulse large animals, thereby keeping the food resource for themselves (Burkepile et al., 2006; Sherratt et al., 2006)

Spoilage is manifested by a variety of sensory cues such as off-colours, off-odours, softening of vegetables and fruits, and slime. However, even before it becomes obvious, microbes have begun the process of breaking down food molecules for their own metabolic needs. Sugars and easily digested carbohydrates are used first, plant pectins are degraded. Then proteins are attacked, producing volatile compounds with characteristic smells such as ammonia, amines, and sulfides. (Ellis and Goodacre, 2006)

The apple is the pomaceous fruit of the apple species *Malus domestica* in the rose family Rosaceae and it is a perennial fruit (Potter et al., 2007). The apple tree originated from central Asia. There are more than seven thousand five hundred (7500) cultivars of apples (Elzebroek and Wind, 2008). Apples

DOI : http://dx.doi.org/10.4314/ijbcs.v7i2.20

play a vital role in human nutrition by supplying the necessary growth factors such as vitamins and essential minerals in human daily diet and this helps to keep a good and normal health and one of the limiting factors that influence the economic value of apple fruits is the relatively short life period caused by pathogens attack. An estimate of about 20 – 25% of the harvested apple fruits are decayed by pathogens during post-harvest handling even in developed countries (Droby, 2006; Zhu, 2006). In developing countries, postharvest losses are often more severe due to inadequate storage and transportation facilities and so fungal fruits infections may occur during the growing season, harvesting, handling, transport, post-harvest storage and marketing conditions, or after purchasing by the consumers and the fruits contain high levels of sugars and nutrient element and their low pH values make them particularly desirable to fungal decay (Singh and Sharma, 2007). Proteases have been extensively studied as potential virulence factors in pathogens of animals, whose intercellular matrix, in contrast to plants, is mainly protein. Also protease is an enzyme that breaks down proteins and peptides by catalyzing the hydrolysis of peptide bonds (Showalter, 1993). Amylases are starch degrading enzymes which are widely distributed in microbial, plant and animal kingdoms (Bernfeld, 1955; Fisher and Stein, 1960; Myrback and Neumuller, 1950). They act by hydrolyzing bonds between adjacent glucose units, yielding product characteristics of the particular enzyme involved. One of the factors influencing virulence of pathogens is their ability to produce enzymes capable of degrading their host's tissue. Microorganisms especially bacteria and fungi have been identified as major organisms causing deterioration of various fruits by the secretion of extracellular cell wall degrading enzymes. This research work was therefore carried out to isolate and identify spoilage fungi and bacteria from the deteriorated apple fruits. The production of protease and amylase from the deteriorated apple *(Malus domestica)* fruits by the microbial isolates is described.

MATERIALS AND METHODS

Cleaning and sterilization of materials

The glass wares were thoroughly washed with liquid soap, rinse thoroughly with distilled water and then allowed to dry. The glass wares were sterilized in the hot air oven at 160 ˚C for 1 hour. The inoculating loop was also sterilized by flaming.

Culture media preparation

The culture media used in this research work were nutrient agar, potato dextrose agar, simmons citrate agar, urease agar, amylolytic agar, proteolytic agar and peptone water and they were prepared according to manufacturer's instructions. The media dissolved in the required amount of distilled water. It is then heated on hot plate to homogenize the media and autoclave for sterilization.

Isolation and identification of organisms

The samples were disinfected using 10% (v/v) sodium hypochlorite solution. This was done by soaking the deteriorated apple in the sodium hypochlorite for 30 minutes and rinsed several times with distilled water to remove the residual effect of the sodium hypochlorite. The deteriorated part was cut aseptically in dimension (2x5 mm) and inoculated on sterile Petri dishes using sterile forceps. Nutrient agar and potatoes dextrose agar was then poured into various plates and incubated at 37 ˚C (bacteria) and 26 ˚C (fungi) for a period of 24 hours for fast growing organisms and 48 hours for the ones with a slower growth. Sub- culturing was carried out to obtain pure strains. The organism was identified using morphological, biochemical and cultural characteristics.

Colony morphology on nutrient agar

Colony morphology includes the type of pigment (if present), size of colony, texture (opaque, translucent, or transparent), adherence to agar and undulating/round/dentate edge.

Gram staining

Pure bacteria colony of the bacterium isolated from the apple sample was gram-stained and examined under oil immersion lens.

Biochemical tests

Biochemical tests were performed for identification purposes and confirmatory tests and the organisms used for inoculation of test media were from fresh cultures. Biochemical tests performed include Catalase test, Carbohydrate fermentation, Urease test, Indole Production test, Citrate Utilization test, Methyl Red test and Coagulase test.

Fungal identification

The morphological and cultural characteristics with special reference to the sporulation of the fungi isolates were used to identify them. The two methods that were used are direct observation of plates and riddle's slide culture technique.

Observation of fungal plates

The plates were observed daily for the rate of growth of each of the isolates. The colour and morphology of the colonies were noted. The base of the plate, odour, type of sexual and asexual reproduction were noted.

Slide culture technique

A wet mount of each fungus was prepared by suspending a loopful of fungal culture in a few drops of lacto-phenol cotton blue solution on a microscope slide and then cover with a slip then view under x40 magnification.

Extracellular enzyme detection

This experiment was carried out to detect if the extracellular enzymes amylase and protease were secreted by the isolate.

Amylolytic enzyme production

The ability of the various isolates to degrade starch following the production of amylolytic enzymes was the criterion used to detect the elaboration of the enzyme isolates.
The Amylolytic agar contained starch as the substrate and the medium contained

Nutrient agar 1.5% ;
Soluble starch 0.2% ;
pH 6.0 ;

After 2-5days of incubation, depending on the rate of growth, the plates were flooded with iodine solution for a few minutes.

Proteolytic enzyme production

The proteolytic medium used contained gelatin as a substrate and the medium contained 8% solution of gelatin water was prepared by dissolving 8 g of gelatin in 100 ml of distilled water and sterilized. This was added to pre-sterilized nutrient agar at the rate of 5 ml per 100 ml and mixed thoroughly. This was followed by inoculation and incubation. After incubation, complete degradation and the gelatin is shown by a clearing in the opaque medium around the colonies. However, if the plate are then flooded with an aqueous saturated solution of ammonium sulphate, a precipitate is formed which makes the agar more opaque and enhances clear zones formation around the colonies that produces the enzymes.

RESULTS

A total of four organisms were isolated from this research on deteriorated apples which includes two bacterial and two fungal isolates. The fungal isolates were identified to the species level while the bacterial isolates were identified to the generic level (Table 1).

Table 2 shows the morphological characteristics of the bacterial isolates. These

characteristics reveal circular appearance on agar plate, abundant growth, creamy pigmentation, raised and unraised elevation, mucoid and dry surfaces amongst others. The two microbial isolates also tested positive to Gram's staining.

The result of the biochemical tests carried out on the bacterial isolates obtained from deteriorated apple fruits is shown in Table 3. Isolates were positive to both catalase and coagulase. They were negative to citrate, indole, methyl red, oxidase and Voges Proskauer. Starch hydrolysis was positive in both isolates while urease was negative in both. It also shows the sugar fermentation tests which include sucrose, lactose, glucose, maltose and galactose. In the test for galactose, glucose and maltose, from both bacterial isolates, acid was produced while gas was not produced. In the test for lactose, acid was produced from both bacterial isolates; gas was produced from micrococcus but was not produced from bacillus. For sucrose, acid was not produced from both isolates; gas was also not produced from bacillus but was produced from micrococcus.

The result of the cultural characteristics and microscopic examination of the fungal isolate and the presumptive identification of the isolates were *Aspergillus niger* and *Aspergillus flavus* (Table 4). Table 5 showed the responses of all the isolates to the production of extracellular enzymes. From the result all the isolates were positive to the production of amylase however, the bacterial isolates produce more while the fungal isolates produced less. Bacillus spp did not produce protease while the other three isolates i.e. Micrococcus spp, *Aspergillus niger* and *Aspergillus flavus* produced protease.

Table 1: Identification of isolates from deteriorated apple fruits.

Isolate code	Identification
Y1	Bacillus spp
Y2	Micrococcus spp
Z1	*Aspergillus niger*
Z2	*Aspergillus flavus*

Table 2: Characterization of bacterial isolates on nutrient agar.

Morphological And growth characteristics	Y1	Y2
Cell morphology	Short rods in stain	Cocci in clusters
Grams reaction	positive	positive
Form on agar plate	circular	circular
Pigmentation	creamy	creamy
Growth	Abundant	Abundant
Edge	Entire	filamentous
Surface	Mucoid	Dry
Elevation	Raised	Slightly raised

Table 3: Biochemical identification of bacterial isolates.

Biochemical tests			**Sugar Fermentation Test**		
	Y1	**Y2**		**Y1**	**Y2**
Catalase	+	+	Galactose	A+ve G-ve	A+ve G-ve
Citrate	-	-	Glucose	A+ve G-ve	A+ve G-ve
Coagulase	+	+	Lactose	A+ve G-ve	A+ve G+ve
Indole	-	-	Maltose	A+ve G-ve	A+ve G-ve
Methyl Red	-	-	Sucrose	A-ve G-ve	A-ve G+ve
Oxidase	-	-			
Starch Hydrolysis	+	-			
Urease	-	+			
Voges Proskauer	-	-			
Identification	*Bacillus* spp	*Micrococcus* spp			

A+ve: Acid is produced; A-ve: Acid is not produced; G+ve: Gas is produced; G-ve: Gas is not produced; +: Produced; - : Not produced.

Table 4: Characterization of the fungal isolates on potato dextose agar.

Isolate identification code	**Cultural characteristics**	**Microscopic examination of slide culture**	**Organism**
Z1	White fluffy growth of colonies with elevated mycelia that turned black after 36 hours	Black with sulphur yellow area on the surface single celled spores (conidia) in chains developing at the end of the sterigma arising from the terminal bud of the septate hyphae.	*Aspergillus niger*
Z2	Colonies are granular, velvety or wooly and yellow brown	Conodiophores are long and rough just beneath the globolose vesicle. Philades are circumferential and are biseratial. Conidia are round, smooth or slightly rough and form long chains.	*Aspergillus flavus*

Table 5: Production of amylase and protease enzymes obtained from deteriorated apples.

ENZYMES	**Y1**	**Y2**	**Z1**	**Z2**
Amylase	+	+	+	+
Protease	-	+	+	+

+Positive; -Negative.

DISCUSSIONS

The result of this research work revealed *Bacillus* species and *Micrococcus* species as the microbial isolates obtained from post-harvest deterioration of apple fruits. The fungal isolates obtained include *Aspergillus niger* and *Aspergillus flavus.* Other researchers have reported the involvement of fungi and bacteria in the deterioration of apple fruits (Oelofse et al., 2006). Bali et al. (2008) reported that the black mold produced by *A. niger* caused post harvest spoilage in sweet orange and acid lime on the field. Okereke et al. (2010) reported fungi species *A. niger, Alternaria* sp., *Botryodiplodia theobromae* and *Colletotrichum gloeosporioides, Fusarium* spp, *A. flavus and Phoma spp*. from infected mango fruits.

Ajayi et al. (2011) carried out a similar study on carrot and reported *A. niger*, *Penicillum chrysogenum*, *Mucor* sp, *Fusarium* sp as the fungal sp isolated. *Bacillus* sp, *Leuconostic* sp, *Xanthomonas* sp and *Klebsiella* sp were also reported as the bacterial species associated with post-harvest deterioration of carrots. *A. flavus* and *A. niger* produced extracellular enzymes when inoculated into fresh tomato fruits. *A. niger* seems to be a common fungal isolates from different fruits in different studies.

Previous studies had revealed that cell wall degrading enzymes secreted by pathogens can breach and use the plant cell wall as nutrient sources that reduced post-harvest life and finally lead to the fruits developing inedible, undesirable quality and soft rot spoilage (Al-Hindi et al., 2011). Extracellular enzymes function by breaking down plant cell material (Bateman, 1963). Pathogenic organisms therefore produce these extracellular enzymes which include amylase, protease, cellulose and pectinase. The two bacterial isolates *Micrococcus* spp and *Bacillus* spp produced amylase in large quantities while *A. flavus* produced minute quantities of amylase. Amylases are a group of enzymes used mainly in the starch processing industries for the hydrolysis of polysaccharides into simple sugars (Damien et al., 2010; Adejuwon et al., 2012). *A. niger* lacked amylase produced protease. Chimata et al. (2010) reported that extracellular amylase was produced from agricultural residues by a newly isolated *Aspergillus* species in solid state fermentation. Amylase was also produced from *A. niger* using a defined synthetic growth medium and rice (*Oryza sativa*) as growth substrate (Adejuwon et al., 2012).

Protease is an enzyme that breaks down protein into amino acids and amines which can easily be utilized. This can lead to increased proliferation and virulence (Kaur and Padmaja, 2009). Proteases attack and hydrolyze the peptide bonds of proteinous materials such as proteins and peptide bonds (Alexander, 1977). *Aspergillus niger*, *Aspergillus flavus* and *Micrococcus* sp were found to produce protease. It shows that they are serious pathogens of apples and therefore they cause fast proliferation and leads to damage of plant tissues.

Taragano et al. (1997) reported the production of extracellular protease by *A. niger*. *A. niger* was also identified as a producer of an extracellular bleaching stable acid protease (Siala et al., 2009). The invasion of the host by the pathogen may be aided by the production of bacterial extracellular substances which act against the host by breaking down primary or secondary defenses of the body. Medical microbiologists have long referred to those substances as invasions (Kenneth Todar, 2009).

Conclusion

Apples consumed in Nigeria are imported and it is possible that these apple fruits are infected by spoilage organisms during crop growth on the field, during harvesting, post-harvest handling, storage and distribution. These spoilage microbes which are soil-borne are typically found on harvesting and handling equipment, in storage facilities and on food contact surfaces. Safety of consumers may be better ensured if proper

handling and optimal storage conditions are practised.

REFERENCES

Adejuwon AO, Olajide O, Adewole O. 2012. Production of amylase from *Aspergillus niger* using a defined synthetic growth medium and also rice (*Oryza sativa*) as growth substrate. *E3 Journal of Medical Research*, **1**(7): 091-094.

Ajayi AA, Ubon-Israel NN, Olasehinde GI. 2011. Detection of extracellular enzymes in microbial isolates from diseased carrot (*daucus carota*) fruits. *International Journal of Advanced Biotechnology and Research*, **2**(2):244-249.

Alexander M. 1977. *Introduction to Soil Microbiology.* John Wiley and Sons, Inc.: New York.

Al-Hindi RR, Al-Najada AR, Mohamed SA. 2011. Isolation and identification of some fruit spoilage fungi: Screening of plant cell wall degrading enzymes. *African Journal of Microbiology Research*, **5**(4): 443-448.

Bali RV, Bindu MG, Chenga RV, Reddy K. 2008. Postharvest fungal spoilage in sweet orange (*Citrus sinensis*) and acid lime (*Citrus aurentifolia* Swingla) at different stages of marketing. *Agricultural Science Digest*, **28**: 265-267.

Bateman DF. 1963. The macerating enzyme of *Rhizoctonia solani. Phytopathology*, **53**:1178-1186.

Bernfeld P. 1955. Amylases α and β., In *Methods in Enzymology*, Colowick SP, Kalpan NO (eds). Academic Press: New York; 149-158.

Burkepile DE, Parker JD, Woodson CB, Mills HJ, Kubanek J, Sobecky PA, Hay ME. 2006. Chemically mediated competition between microbes and animals: microbes as consumers in food webs. *Ecology*, **87**: 2821–2831.

Chimata MK, Sasidhar P, Suresh C. 2010. Production of extracellular amylase from agricultural residues by a newly isolated *Aspergillus* species in solid state fermentation. *African Journal of Biotechnology*, **32**: 5162-5169.

Damien M, Catherine J, Patrice D, Christopher B. 2010. Enhanced mechanical properties of partially beta-amylase trimmed starch for material application. *Carbohydr. Poly*., **80**(3): 747-752.

Droby S. 2006. Improving quality and safety of fresh fruit and vegetables after harvest by the use of biocontrol agents and natural materials. *Acta Horticulturae*, **709**: 45–51.

Ellis DI, Goodacre R. 2006. Quantitative detection and identification methods for microbial spoilage. In *Food Spoilage Microorganisms*. Balakburn CW (ed). CRC Press LLC: Boca Raton FL; 3–27.

Elzebroek T, Wind K. 2008. In *Guide to Cultivated Plants*, Elzebroek T, Wind K (eds) . Cabi.: Wallingford, UK; 182-225.

Fisher EH, Stein EA. 1960. α –Amylase. In *The Enzyme* (2nd edn). Academic Press Inc.: New York; 313-143.

Kaur J, Padmaja V. 2009. Relationship among activities of extracellular enzyme production and virulence against *Helicoverpa armigera* in *Beauveria bassiana*. *Journal of Basic Microbiology*, **49**(3): 264-274.

Kenneth Todar. 2009. *The Microbial World: The Mechanisms of Bacterial Pathogenicity. http://textbookofbacteriology.net/themicrobialworld/pathogenesis.html*

Myrbrack K, Neumuller G. 1950. In *The Enzyme: Chemistry and Mechanisms of Action* (1st edn, vol. 1), Sumner JB, Myrbrack K (eds). Academic Press: New York.

Potter D, Eriksson T, Evans RC, Oh SH, Smedmark JEE, Morgan DR, Kerr M, Robertson KR, Arsenault MP, Dickinson TA, Campbell CS. 2007. Phylogeny and Classification of Rosaceae. *Plant Systematics and Evolution*, **266**(1–2): 5–43.

Siala R, Sellami-Kamoun A, Hajji M, Abid I, Gharsallah N, Nasri M. 2009.

Extracellular acid protease from *Aspergillus niger* II: purification and characterization. *African Journal of Biotechnology*, **8**(18): 4582-4589.

Sherratt TN, Wilkinson DM, Bain RS. 2006. Why fruits rot, seeds mold and meat spoils: a reappraisal. *Ecological Modelling*, **192**: 618–626.

Showalter AM. 1993. Structure and function of plant cell wall proteins. *Plant Cell*, **5**: 9-23.

Singh D, Sharma RR. 2007. Postharvest diseases of fruit and vegetables and their management. In *Sustainable Pest Managemen*, Prasad D (ed). Daya Publishing House: New Delhi, India.

Taragano V, Sanchez VE, Pilosof AMR. 1997. Combined effect of water activity depression and glucose addition on pectinases andprotease production by *Aspergillus niger*. *Biotechnol. Lett*., **19**(3): 233-236.

Oelofse D, Dubery IAM, Arendse S, Mm S, Gazendam I, Berger DK. 2006. Apple polygalacturonase inhibiting protein expressed in transgenic tobacco inhibits polygalacturonases from fungal pathogens of apple and the anthracnose pathogen of lupins, *Phytochemistry*, **67**: 255-263.

Okereke VC, Godwin-Egein MI, Arinze AE. 2010. Assessment of Postharvest Rot of Mango at Different Stages of Market in Port Harcourt, Nigeria. *Int. J. Curr. Res.*, **11**: 6-10.

Zhu SJ. 2006. Non-chemical Approaches to Decay Control in Postharvest Fruit. In *Advances in Postharvest Technologies for Horticultural Crops*, Noureddine B, Norio S (eds). Research Signpost: Trivandrum, India; 297–313.