STUDIES ON PECTINOLYTIC AND PROTEOLYTIC ENZYMES FROM DETERIORATED GRAPES (*Vitis vinifera*)

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***Abstract*:** The ability of microorganisms to cause grape (*Vitis vinifera*) fruit deterioration by production of pectinolytic and proteolytic enzymes to degrade the plant cell was carried out in the Microbiology laboratory of the Department of Biological Sciences, Covenant University, Ota, Ogun State, Nigeria. A bunch of grape fruit was purchased from a local market in Ikeja, Lagos, South West, Nigeria. These samples were allowed to rot for five days in a clear packaging bag. The grape samples were disinfected and cultured on nutrient agar and potato dextrose agar for bacterial and fungal isolates respectively. The morphological and microscopic characteristics of the isolates in combination with a series of biochemical tests were used to identify them. The isolates were tested for their ability to produce pectinolytic and proteolytic enzymes. *Aspergillus* sp, *Mucor* sp, *Rhizopus* sp., *Micrococcus* sp., and *Bacillus* sp. were the organisms isolated from this study. The results of this investigation revealed appreciable pectinolytic, and proteolytic enzymes by *Aspergillus niger and Bacillus* sp. These organisms can be utilized as good sources of industrial enzymes.

Keywords: Grape (*Vitis vinifera*) fruits; Pectinase, Protease, Fungal isolates and Bacterial isolate.

Introduction

Grape (*Vitis vinifera*) fruits are native to the Mediterranean region and Central Asia (Bertelli and Das, 2009). They are non-climacteric fruits, specifically berries that grow in clusters on the perennial and deciduous woody vines of the genus *Vitis (Turgut* et al., *2011)*. There are three main species of grapes, European (*Vitis vinifera)* North American (*Vitis labrusca and Vitis*

rotundifolia) and French hybrids (Bertelli and Das, 2009). They are small round or oval berries that feature semi-translucent flesh encased by a smooth skin with some containing edible seeds while others are seedless (Nature and More, 2012). Grapes are in a variety of colours such as blue, red, purple, black, golden, and green which are the most common (Walker et al.,2002). The colour of the fruit is due to the presence of poly-phenolic pigments in them (Jensen et al., 2008; Nature and More, 2012). Red or purple berries contain anthocyanin while white-green berries contain more of tannins especially, catechin (Versari et al., 2007). Grapes rank with blueberries and blackberries as excellent sources of antioxidants (Bauer, 2009; Superfoods: Grapes, 2012). Interestingly, these antioxidant compounds are densely concentrated in the skin and seeds (Parry et al., 2006). Grapes can be eaten raw or they can be used for making jam, juice, jelly, vinegar, wine, grape seed extracts, raisins, molasses and grape seed oil (Health Benefits,2013). Commercially, many cultivars of grapes are grown for different purposes (Health Benefits, 2013). Grapes like most other fruits can be infected by microorganisms that secrets a variety of extracellular enzymes such as protease and pectinase which act on different substrates and can be extracted for various uses (Kumar and Takagi, 1999). Yeast, one of the earliest domesticated microorganisms, occurs naturally on the skins of grapes, leading to the innovation of alcoholic drinks such as wine (Cawineries, 2014).

In nature, microorganisms have been endowed with vast potentials. They produce an array of enzymes, which have been exploited commercially over the years (Kalisz, 1988). They are currently the primary source of industrial enzymes with about 50% from fungi and yeast, 35% from bacteria, while the remaining 15% are either of plant or animal origin (Satyanarayana, 2009). Pectinolytic enzymes, also called pectinase, are a group of enzymes that catalyzes pectic substance degradation through depolymerisation and deesterification reactions (Naglaa, 2012). These pectinase have wide application in fruit juice and wine industries (Adrian *et al*., 2002; Ajayi *et al*., 2011). Proteolytic enzymes referred to as proteinases or proteases, are any of a group of enzymes that break the long chainlike molecules of proteins into shorter fragments (peptides) and eventually into their components, amino acids (Bond and Lopez-otin 2009). Proteases are used in food industries such as in bread baking and detergent industries where they form part of many laundry

detergents (Gupta *et al*., 2002). This research work was therefore carried out to isolate, characterize spoilage microorganisms from deteriorated grapes and detect the ability of the isolated organisms to produce pectinolytic and proteolytic enzymes.

Materials and Methods

Sources of Grape Samples

Fresh grapes samples were purchased from Ikeja, Lagos state, South Western Nigeria and they were left to deteriorate in a clear packaging bag for five days

Materials

The media used for this research work are:

Nutrient agar: Lab M Limited Topley House 52 Wash Lane, Bury, Lancashire BL9 6AS United kingdom.

Simmon's Citrate agar: Biomark laboratories PUNE 41101, India

Culture media preparation

The media used for the isolation, cultivation and identification of isolates were prepared according to manufacturers' instructions. All media were sterilized at 121^{o}C and 15 pounds per square inch pressure for 15 minutes.

Sterilization of grape samples

Surface sterilization or disinfection was carried out on all grape samples by soaking the deteriorated grapes in 10% (v/v) sodium hypochlorite solution for 15minutes. The grapes were rinsed with several changes of sterile distilled water to remove the residual effect of sodium hypochlorite solution.

Isolation of organisms

The grape samples were cut with a sterile knife into very small portion of about 2mm in diameter and inoculated aseptically with forceps on Nutrients agar and Potato dextrose agar plates using the pour plate method. Incubation was carried out at 37^{o}C for bacteria and 25^{o}C for fungi. It was left for a period of 24-48 hrs. The observed growth was further sub-cultured until pure colonies of the organisms were obtained. The pure cultures were identified using morphological, microscopic and biochemical characteristics.

Bacterial identification (Cultural characteristics)

This include amount of growth, cell morphology, surface appearance, size, shape, pigmentation, edge as described by Olutiola *et al*. (1991)

Microscopy

Gram's reaction

This was carried out according to the Gram's staining technique. Thin smear were made with a loopful of pure culture of bacteria isolates of 18-24 hrs on clean grease free slides. The slides were then air dried and heat fixed. The slides were stained with already prepared Gram stain reagents. The primary stain,

crystal violet solution was used to flood the slides for one minute. The slides were then rinsed off under running water before being flooded with Lugol's iodine which is the mordant for another 1 minute. Excess iodine was poured off and rinsed off with water before decolourization with 95% ethanol for 30 seconds. It was then counterstained with Safranin dye. The slides were washed with running water and left to dry on a staining rack; filter paper was used to blot them dry. The slides were observed with a compound microscope using oil the oil immersion lens (x100).

Biochemical tests

Biochemical tests were used to identify the isolates based on their distinguishing characteristics such as colour change, gas or acid production, production of bubbles or coagulation. The tests made use of enzymatic activities to differentiate among bacteria. Fresh cultures of the isolates were used to carry out all the biochemical tests.

Indole production test, citrate utilization test, urease test, MRVP (Methyl Red-Vogues Proskauer), catalase test, coagulase test, oxidase test, starch hydrolysis, sugar fermentation were carried out by techniques described by Ajayi *et al*. (2007).

Fungal identification (Morphology and Microscopy)

The identification of the fungal isolates was based on a combination of morphological and cultural characteristics with special reference to their sporulation. The two methods used as direct observation of the plates and the slide culture technique. Physical appearance such as the colony colour (black to brown), shape and texture (moist mycelia) were examined first as well as abundance of growth.

Slide Culture Technique

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

Extracellular Enzyme Activity

Pectinolytic enzyme production

The medium used was described by Hankin *et al*., (1975) and it contained: Yeast extract- 1g, mineral salt solution- 500ml, Agar-15g, pectin-5g, and distilled water – 500ml. The composition of the mineral salt solution per litre of distilled water was as follows: Ammonium sulphate ($NH_4)SO_4$ - 2.0g, potassium dihydrogen phosphate KH_2PO_4 -4.0g, Disodium hydrogen phosphate Na_2HPO_4 -6.0g, Ferrous Sulphate (hydrated) $(FeS)_4.7H_2O$ -0.2g, Calcium

chloride (anhydrous) CaCl -1.0mg, Hydroboric acid (H_3B_3) -10.0µg, Manganese Sulphate ($MnSO_4$) - 10.0µg, Zinc Sulphate($ZnSO_4$) – 70.0µg, Copper Sulphate (hydrated) ($CuSO_4$) -78.0µg, and Molybdenum Oxide (MoO_3) – 10.0 µg.

Proteolytic enzyme production

This was carried out according to the method of Berkenkamp (1973) and Hankin and Anagnostakis (1975). The medium used contained: Nutrient agar -1.5%, Gelatin - 0.4%, pH-6.0. The medium used contained gelatin water as substrate. It was sterilized in the autoclave at 121^o C for 15 minutes. It was added to pre sterilized nutrient agar at the rate of 5ml per 100ml. It was mixed thoroughly and poured into plates to set. The plates were then inoculated and incubated for three days.

Results

Deterioration of Grapes

Wholesome grapes maintained their integrity for about five days after which they started to rot. Visible change in characteristics such as discolouration, softening of flesh with visible holes and foul odour were observed.

Identification of Isolates

This research revealed bacterial and fungal isolates from the spoilage of grape fruits (Table 1). The isolates were characterized and identified based on a variety of morphological, microscopic and biochemical characteristics (Tables 2, 3, 4 and 5). Some of the microorganisms isolated from the deteriorated samples produced pectinase, protease or a combination of both. Following incubation of the organism in the medium for detection of protease, a complete degradation of the gelatin is shown by a clearing in the opaque medium around the colonies. However when plates were flooded with an aqueous solution of ammonium sulphate, a precipitate which made the agar more opaque and enhanced clear zone formation around the colonies that produced the enzyme was formed. The medium used to detect pectinase was at pH 5.0 for polygalacturonase (Table 6).

Biochemical Tests

Gram's reaction: Purple colour of cells: Gram positive bacteria. Red or pink colour: Gram negative bacteria. Circular shape of cells (bacteria): Cocci and rod-like shape: bacilli

Indole Production: Red/pink: positive result (the bacteria can breakdown tryptophan to form Indole). No colour change: negative result (Indole was not formed from tryptophan).

Utilization of Citrate: Prussian blue colour: positive result. Media remained intermediate green colour: negative result.

Urease Production: colour change from yellow to bright pinkish-red: positive. Lack of colour change:

negative result.

Production of Catalase: Visible bubbles: positive test. A lack of bubbles indicated the absence of catalase.

Coagulase Production: Any degree of clotting within 24hours: coagulase positive.

Oxidase: A colour change to purple or blue at 30 seconds-1 minute was positive.

Hydrolysis of Starch: Reddish colour or clear zone around bacterial growth (starch has been hydrolysed): positive test. Black /blue area: negative (presence of starch).

Sugar Fermentation: Colour change from red to yellow or yellow colour with gas bubble: positive result. Red colour: negative result (no gas bubble).

MRVP: A pinkish-red to burgundy indicated a positive test and if culture appears yellowish-copper, it was negative.

Extracellular Enzyme Detection

Some of the microorganisms isolated from the deteriorated samples produced pectinase, protease or a combination of both. Following incubation of the organism in the medium for detection of protease, a complete degradation of the gelatin was shown by a clearing in the opaque medium around the colonies. However when plates were flooded with an aqueous solution of ammonium sulphate, a precipitate which made the agar more opaque and enhanced clear zone formation around the colonies that produced the enzyme was formed (Table 6).

Table 1: Isolates obtained from deteriorated grapes

Isolate Codes	Organism
B_1	*Micrococcus sp*.
B_2	*Bacillus sp*.
F_1	*Mucor sp*.
F_2	*Aspergillus sp*.
F_3	*Rhizopus sp.*

Table 2: Morphological properties of bacterial isolates after 24-28 hrs of incubation

Cultural Characteristics	**B_1**	**B_2**
Pigmentation	Creamy	Pink
Growth	Abundant/ Profuse	Abundant/ Profuse
Surface	Smooth	Glossy
Form on Agar Plate	Circular	Circular
Elevation	Slightly raised	Raised
Edge	Circular	Undulate

Table 3: Microscopic observation of bacterial isolates

Isolates/Media	**Cell Morphology**	**Gram's reaction**
B_1 /Nutrient Agar	Cocci in clusters	+
B_2/ Nutrient Agar	Short rods	+

Keys

Positive +

Negative -

Table 4: Biochemical characteristics of bacterial isolates

Organism/Test	Indole	Citrate	Urease	Methyl-red	Vogues Proskauer	catalase	coagulase	oxidase	starch hydrolysis	Sugar Fermentation: glucose	maltose	sucrose	lactose	galactose
	–	–	–	–	–	+	–	+	–	A+	A+	A-	A+	A+
B1										G-	G-	G-	G+	G-
B2	–	+	–	–	–	+	–	–	–	A+	A+	A-	A-	A+
										G-	G-	G-	G-	G-

Key

Acid Production	**A**
Gas Production	**G**
Positive	**+**
Negative	**-**

Table 5: Characterization and Identification of the fungal isolates

Code	Morphological Characteristics	Microscopic Examination	Identification
F1	colonies c of Mucor grew rapidly at 25-30oC and quickly covered the surface of the ag , producing a fluffy appearance wity a height of several cm resembling cotton candy From the front, the colour was white initially and became grayish brown with time.	Non septate or sparsely septate, broad sporangiophores, sporangia, and spores were visualized. Sporangia are round, gray to black in colour, and are filled with sporangiophores. Sporangiophores are short, erect, taper towards their apices and form short sympodial branches.	*Mucor sp*
F2	Growth on agar plate was fluffy white which turned black producing large black conidial heads after 2-3 days of inoculation.	Possess hyphae that are colourless, septae and branched. A vesicle is borne at the end of each long conidiophore. On this vesicle, rows of sterigmata develop, that bear chains of yellow-green to blue-green conidia. The sterigmata are borne in single or double series on an elongated to subglobose vesicle	*Aspergillus niger*
F3	colonies were fast growing and covered the agar surface with a dense cottony that was at first white becoming grey or yellowish brown with sporation	Unbranched and mostly brown sporangiophores and spherical sporangium. Collumella is spherical or elongated with indistinct rhizoid.	*Rhizopus sp*

Table 6: Extracellular enzyme production by Bacterial and Fungal Isolates

Isolate Codes	Enzymes	
	Protease	**Pectinase**
B_1	+	-
B_2	+++	+++
F_1	-	-
F_2	+++	+++
F_3	-	+

Key

Positive +

Negative -

Discussion

This research work examined the ability of microorganisms isolated from deteriorated grapes to produce pectinolytic and proteolytic enzymes. The results of the investigation revealed a total of five isolates which were found to be pathogenic on the grape fruit. The organism isolated were *Micrococcus sp*., *Bacillus sp*., *Mucor sp*., *Rhizopus sp*., and *Aspergillus niger*. The organisms were cultured on appropriate media and showed rapid and profuse growth. These organisms also showed ability for enzyme production in varying degrees. Of the five isolates, active production of both enzymes was reported in *Bacillus sp.* and *Aspergillus niger*. The production of protease by *A.niger* from deteriorated apples had been reported (Ayanda *et al*., 2013). *Micrococcus sp.*, produced protease but lacked pectinase while *Rhizopus sp*. produced pectinase and lacked protease.While pectinase and protease production was absent in *Mucor sp.* This study corroborates the findings of previous studies where microorganisms have been found to produce extracellular enzymes (Semenova *et al*., 2006; Reddy and Sreeramulu, 2012). Bacteria are the most dominant source of alkaline protease (Gupta

et al., 2002). *Bacillus* being the most prominent can serve as an ideal source of these enzymes of biotechnological importance because of their rapid growth and small space required for their cultivation (Gupta *et al.,* 2002). Pectinase was produced from *Bacillus subtilis* isolated from soil (Tripathi *et al*., 2014). The enzyme production was significant when relevant substrate was added to the media. This ability and growth rate is also influenced by different factors. Studies have showed that nutritional factors including sources of carbon and nitrogen can influence protease enzyme production (Kezia *et al.,* 2011). Physical factors such as inoculum concentration temperature, pH and incubation time (Kaur et al., 1998; Yossan et al., 2006; Muthulakshmi *et al*., 2011; Mohammed *et al.,* 2012) can also significantly affect protease production. Ayanda *et al.* (2013) reported the production of microbial protease from deteriorated apples. The enzymes are produced by these organisms chiefly as part of survival instincts (Subhadeep and Pandey, 2013). The production of multiple forms of enzymes therefore improves the microorganism's ability to adapt to environmental modifications (Naessens and Vandamme, 2003).

Conclusion

Pectinolytic and proteolytic enzymes have been widely studied and these enzymes are of significant importance in the current biotechnological era with their all-embracing applications in scouring of cotton, degumming of plant fibres, waste water treatment, peptide synthesis, in poultry feed additives, in detergent, leather, photographic industry and food industries especially in fruit juice extraction and its clarification as well as in the production of alcoholic beverages.

Information on studies carried out on grapes, its application, enzymes isolated from grape fruits and other parameters is limited. This research work contributes to existing information on the ability of bacteria such as *Bacillus* and Fungi like *Aspergillus* isolated from grape fruits to produce these enzymes of great significance.

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