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Research Article

PECTINASE PRODUCTION BY *Aspergillus niger* AR-2 USING ORANGE PEELS AS SUBSTRATE

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ABSTRACT

Pectinases are the group of enzymes that catalyzes the degradation of pectic substances. Pectinase are enzymes that are remarkably employed in most industries because they have a large variety of applications, such as increasing juice extraction by decreasing the viscosity of concentrates in cellulose fibre preparation, coffee/tea fermentation and also for the clarification of juices and wines**.** This research was carried out for pectinase production by *Aspergillus niger* AR2 using local orange peel as substrate. *Aspergillus niger* was isolated from spoiled orange fruit and identified using phenotypic and molecular techniques. The isolate was screened for pectinase production on pectin agar. Fermentation was carried out by *Aspergillus niger* AR2 at fermentation days 3, 4 and 5; the amount of substrate (15g), pH (5), temperature (30^0C) and inoculum density of 10^6 (spore/mL). After fermentation, crude pectinase was extracted for enzyme assay. Enzyme activity was measured based on the amount of D-galacturonic acid released by the crude enzyme. Pectinase produced was used for orange juice extraction from mashed oranges and compared with juice with no enzymes. The results have shown that maximum pectinase production by *A. niger*AR2 was found to be at a temperature of 30^0C, inoculum density of 10^6 (spore/mL), pH 5 at day 5 using orange peels as substrate with an activity of 3.04±0.024 (U/ml). The result shows that the pectinase produced was effective in orange juice extraction from mashed oranges due to the increase in the volume of orange juice.

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INTRODUCTION

Enzymes are catalysts that bring about specific biochemical reactions in cellular metabolism. More than 3000 enzymes have been identified, but only about 5% are exploited industrially, (Patel *et al.,* 2017). The industrial application of enzymes has substantially abbreviated the ordinate dictations for energy in many industries and the wastes engendered from the application of enzymes in industries are biodegradable and non-toxic wastes that are cordial to the environment. Additionally, the utilization of industrial enzymes is more cost-efficacious and the possibility of genetically engineering microbes to engender more stable and ameliorated enzymes at an industrial scale can be achieved (Patel *et al.,* 2017). Pectinases are a group of enzymes that catalyze the breakdown of pectic substances by depolymerization and de-esterification reactions (Danielle *et al.,* 2009). Pectin was first isolated and described by Henri Braconnot in 1825 (Braconnot, 1825). The pectin matrix provides an environment for the deposition, sliding, and expansion of cellulose glycan networks and is the primary adhesive material between cells (Willats *et al.,* 2001). Degradation of pectin causes the degradation of cellulose and hemicellulose networks and plays an important role in fruit ripening (Lohani *et al.,* 2004). Pectinolytic enzymes are classified according to their mechanism of action on the galacturonan portion of the pectin molecule. The pectinase group of enzymes includes pectin methylesterase, lyase, and polygalacturonase. Pectin methylesterase acts on pectin, removing the methoxyl group from the C-6 carboxyl group of the galacturonic acid moiety by hydrolysis, and pectin lyase cleaves (1->4)-α-D-galacturonan methyl ester. Form an oligosaccharide with a 4-deoxy-6-O-methyl-alpha-D-galacto-4-enuronosyl group. On the other hand, polygalacturonase hydrolyzes α-1,4 glycosidic bonds between galacturonic acid residues (Castilho et al., 1999; Castilho et al., 2000). These enzymes are used to increase juice extraction by reducing concentrate viscosity in cellulosic fibre production, coffee/tea fermentation, and juice and wine clarification (Phutela *et al.,* 2005). . Pectinases form a group of enzymes that degrade pectin, which is present in most plants. Several types are found, but the most abundant and studied is polygalacturonase, which accounts for about 25% of industrial enzyme turnover (Jayani *et al.,* 2005).

In Nigeria, most of the enzymes of industrial importance are bought from outside Nigeria using scarce foreign exchange. Production of enzymes in Nigeria using locally available substrates will go a long way in saving the country from this scarce foreign exchange and at the same time create an avenue for disposing of waste materials and creating wealth from them. It is given the foregoing, this research was set up to produce pectinase enzyme using waste materials sourced locally from the environment.

MATERIALS AND METHODS

Sample collection and identification

Orange fruit specimens used for the isolation of *Aspergillus niger* were collected from various suppliers and identified at the Herbarium of Gombe State University, Gombe State, Nigeria.

Substrate collection and preparation

Orange peels (Benue and Ibadan varieties) were collected in Gombe Metropolitan City, Gombe state. The peels were washed with water and cut into small pieces. The peels were washed again with water and dried in the sun for 7 days. The dried peels were ground into a powder using a mortar and pestle and stored in a clean container at room temperature.

Isolation and Identification of Fungal Isolates

Aspergillus niger isolated from spoiled orange fruits. Samples were cut with a sterile knife into very small pieces approximately 2 mm in diameter and aseptically inoculated with forceps

onto solidified potato dextrose agar plates containing chloramphenicol and incubated at 28°C for 5 days (Bukar *et al.*, 2009). Observed growth was further subcultured until pure colonies of the organisms were obtained and identified morphologically and microscopically (Ajayi and Adedeji, 2014).

Screening of *Aspergillus niger* for Pectinase Production

Screening of *Aspergillus niger* for pectinase production was performed on pectinase screening agar. A pectin medium was prepared according to the procedure by Reda *et al.* (2008).

Molecular Identification of Isolates

The identity of pectin degradation A. niger genomic DNA was extracted, verified, purified with the Wisard Genomic DNA Purification Kit (Promega), and purity was determined by agarose gel electrophoresis. *A. niger* was identified using the fungal primer 18S rDNA. The 18S rDNA and its region were subjected to PCR amplification using her fungal-specific 18D primers.

5'CCGTTGATCCTGCCAGTA3', 18R:5'GCTTGATCCTTCTGCAGGTT-33', ITSI: 5'TCCTCCGCTTATTGATATG3' (White *et al.*, 1990).

***Aspergillus niger* Inoculum Standardization**

The inoculum was prepared by dispersing the spores of *A. niger* slant culture in 0.1% Tween-80 solution with a sterile inoculation loop. Fungal spore counts were performed using a hemocytometer. A 1ml spore suspension with an inoculum density of 10^6 (spore/mL) was used per flask during the fermentation process (Ellaiah *et al.*, 2002).

Pectinase Production by Solid-State Fermentation

Five grams (5 g) of orange peels were placed in another Erlenmeyer flask (5 g/L) containing yeast extract (2 g/L), potassium dihydrogen phosphate (1 g/L), and magnesium sulfate (~0.5 g/L) was autoclaved. The flask was then cooled to room temperature and inoculated with 1 ml of spore suspension of inoculum density of 10^6 spores/ml and then incubated at 30°C, pH 5 and incubation time of 3, 4 and 5 days (Singh and Sharma, 2009).

Enzyme Extraction

This was done according to the method of Xirox *et al.* (2008). After the fermentation period, crude pectinase was obtained by taking 1 ml of fermentation material with 10 ml of buffer. It was then shaken for 30 minutes using an electric shaker. The mixture was then filtered through Whatman No. 1 filter paper to obtain a clear filtrate, the filtrate was centrifugation at 5000 rpm for 20 minutes. It was then filtered again using Whatman No. 1 filter paper the filtrate was used as a source of crude pectinase for enzyme assay.

Enzyme Assay

Pectinase activity was determined by determining the reduced end product released and galacturonic acid as a standard. Enzyme activity was measured based on the amount of D-galacturonic acid released by the crude enzyme (Miller, 1959). The enzyme was assayed using 1 ml crude enzyme (culture filtrate), 1 ml buffer solution (0.1 M citrate buffer pH 5) and 1 ml 1% soluble starch. This mixture was incubated at 45°C for 30 minutes. The enzymatic reaction was stopped by the addition of 3 ml of 3, a 5-dinitrosalicylic acid reagent containing 10 g of 3, 5-DNSA and 300 g of potassium sodium tartrate. 2 ml of buffer (0.1 M citrate buffer pH 6) and 1 ml of 1% soluble starch solution were used as reference blanks. All tubes containing 3, 5-DNSA-treated reaction products were heated in a boiling water bath for 15 minutes. The final volume was brought to 7 ml in each case by adding 1 ml of distilled water. Absorbance was measured at 575 nm

using a UV-Vis spectrophotometer and compared to a standard curve. Enzyme activity was expressed in international units (IU). (Silva *et al.*, 2005)

Effect of Crude Pectinase on Orange Juice Extraction Based on Retention Time

Different times (15, 30, 45, and 60 min) were used to determine the optimal reaction time for juice extraction (measured in ml). In this process, the orange fruit was washed with clean water, peeled, sliced and crushed, releasing more juice by making it easier for enzymes to break down the pectin in the fruit (Shefali and Sudhir, 2013). Two hundred grams of pureed orange was weighed separately into a labelled beaker of crude pectinase and 1 mL of concentrated protein (10 mg/mL) was added to activate the enzyme. At the end of each reaction period, the contents were filtered using a funnel and Whatman No. 1 filter paper and the volume of juice obtained were compared to a control (orange pulp without enzyme).

RESULTS

Screening of Pectinase Producing by *Aspergillus niger*

Figure 1 shows the result of the screening of fungal isolates for pectinase production. Out of the eight that were screened for pectinase production, *Aspergillus niger*AR2 had the highest zone of hydrolysis (74mm).

Identification of Isolates

Table 1 shows the cultural and morphological characteristics of Fungi isolated from spoiled oranges Fruits. Eight (8) isolates were confirmed to be *A. niger* using cultural and morphological characteristics. Plate I shows the 18S RNA amplified fragments for two selected isolates of *A. niger*. Molecular Characterization (Sequencing and BLAST) revealed the isolate with a higher zone of hydrolysis during screening to be 96% similar to *A. niger* AR2 (Table 2).

Table 1: Cultural and Morphological Characteristics of Fungi isolated from Spoiled Oranges Fruits

Sample	Cultural	Morphological	Inference
B1	Dark brown	HC	*A. niger*
B2	✓	✓	✓
G	✓	✓	✓
M1	✓	✓	✓
P	✓	✓	✓
R	✓	✓	✓
T1	✓	✓	✓
T2	✓	✓	✓

Keys:

✓=Similar

HC-Hyphae are septate, conidiospore terminate in a swollen vesicle; B1-sample from Bolari quarters; P- sample from Pantami; G-sample from Gombe State University; T2- sample from Tsohuwar Kasuwa; M1- sample from Main market 1; B2-sample fromBagadaza; R- sample from Riyal quarters; T1- sample from Tudun wada

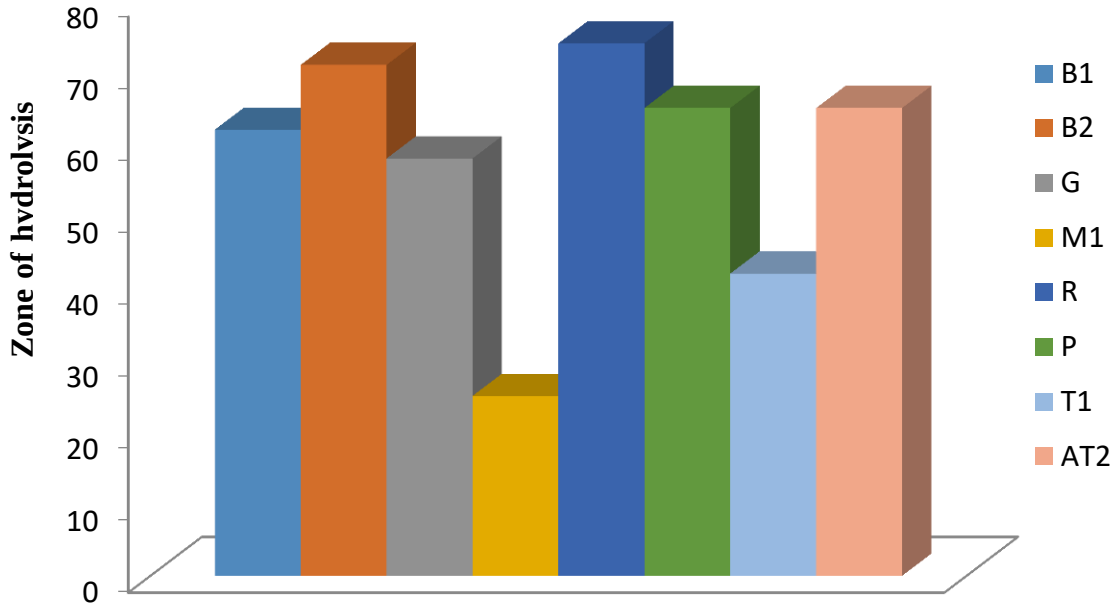

Figure 1: Zone of hydrolysis of the different *Aspergillus niger* isolates screened

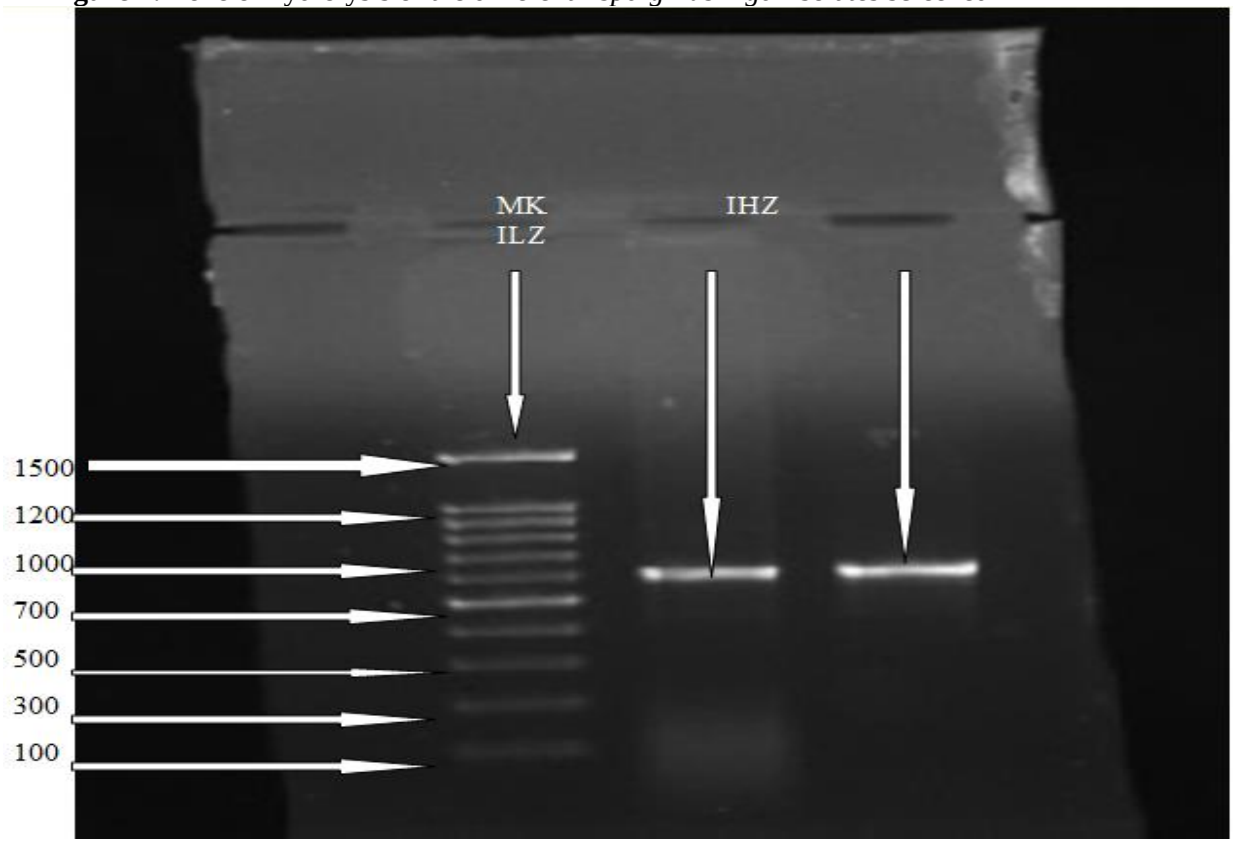

Plate I: Agarose (1.5%) 18S RNA amplified fragments for two isolates of *A. niger*.
IHZ; Isolate with higher zone of hydrolysis
ILZ; isolate with lower zone of hydrolysis.
MK; Markers

Table 2: Sequence Similarity of Pectonolytic Fungal Isolate to Nearest Relatives

Description	Max score	Total score	Query cover	F value	Percentage	Accession
Aspergillus niger strain AR2 internal transcribed spacer partial	1013	1013	96%	0.0	100%	MK633878.1

Pectinase Production under controlled conditions

Table 3 shows the result of pectinase production based on inoculum density of 10^6 (CFU/mL), the temperature of 30^{o}C, pH5 and substrate amount of 15g by *A. niger* AR2 using a different variety of substrates oranges peels (Ibadan and Benue variety) The result of the pectinase production using *A. niger* AR2 has shown that highest pectinase activity was produced on oranges peels (Ibadan variety) as substrate with an activity of 3.04±0.024 (U/ml) at day 5 while orange peels (Benue variety) with an activity of 2.39±0.024 (U/ml) has least pectinase activity. There is a significant difference ($P<0.05$) between the means of pectinase produced by *Aspergillus niger*AR2 on different varieties of substrates across the fermentation days 3, 4 and 5.

Table 3: Effect of fermentation days on pectinase enzymes production

	Pectinase Activity (U/ml)		
	Days		
Samples	**3**	**4**	**5**
OPI	2.42±0.03 [a]	2.57±0.02 [a]	3.04±0.02 [a]
OPB	1.80±0.067 [a]	2.12± 0.02 [a]	2.39±0.02 [a]

Values with the same letter superscript are significantly different at 0.05 level of significance

Key:
± represents standard error
OPI=orange peel (Ibadan variety) OPB=orange peel (Benue variety)

Effect of Crude Pectinase on Orange Juice Extraction Process

Table 4 shows the effect of crudes pectinase on orange juices using crude pectinase with highest activity by enzymes produced by *A. niger* AR2.The results shows that highest volume of juice (5mL) was obtained at 60 minute compared to control which extracted 2.1mL of the juice.

Table 4: Effect of Crudes Pectinase on Orange Juice Extraction Process

Volume (mL) of Juice Extracted Using Crude Pectinase	Volume (mL) of Juice Extracted without Enzyme (Control)	Time (minutes)
2.0	1.0	15
2.5	1.5	30
3.4	1.8	45
5.0	2.1	60

DISCUSSION

Fungi associated with fruit spoilage have been isolated and identified. In this study, ten different specimens were used to isolate his *Aspergillus niger*, and eight isolates were found to be his *Aspergillus niger* based on cultural, morphological, and microscopic characteristics. *Aspergillus niger* was isolated from rotting orange fruit as it is a major cause of fruit rot. This confirms previous studies where fungi have been found to cause fruit rot (Singh *et al.*, 2007). Fruits are high in sugar and nutrients and have a low pH that favours fungal growth (Singh and Sharma, 2009). It is also suitable for fungi in the fruit environment due to the high nutrient content and water content of the fruit. It has been found that most fruit spoilage is caused by fungi (Singh and Sharma, 2009). Isolation and enzyme production of *Aspergillus niger* from fruits have been reported by many researchers (Bukar *et al.*, 2016; Darah *et al.*, 2013; Oyeleke *et al.*, 2012).

Aspergillus niger isolated from spoiled orange fruit was screened for its ability to produce pectinase. In this study, eight isolates were screened for pectinase production. *Aspergillus niger* AR2 has the highest zone of hydrolysis (74 mm), indicating a high potential for pectinase production. The results of this study are consistent with those of Yogesh *et al.* (2009) worked on two different strains of *Aspergillus niger* to determine their ability to produce pectinase. *Aspergillus niger* (NCIM 543) exhibited large hydrolysis zones around large colonies on pectin agar, while *Aspergillus niger* (MTCC 1344) exhibited low hydrolysis zones around colonies on pectin agar.

A wide variety of substrates have been used for the production of *Aspergillus niger* pectinase. Based on this finding, *Aspergillus niger* AR2 produced maximal pectinase activity on day 5 using orange peel (Ibadan variety) as substrate. This means that orange peel (Ibadan variety) is the most suitable for pectinase production. The reason for the highest pectinase activity when using orange peel (variety Ibadan) as the substrate may be due to the higher percentage of pectin found in the cell walls of the peel. The results of this study are consistent with those of Ezugwu *et al.* (2014). He worked on pectinase production using two types of agricultural waste they reported that *Aspergillus niger* produced the highest pectinase activity using orange peel as substrate. He suggests that orange peels can be used as a source of pectin. Pectin can be used to induce the production of pectinase by *Aspergillus* strains. Pectinases are key enzymes in the clarification of fruit juices and the biological washing of textile materials.

Several researchers have proposed the use of solid-state fermentation for pectinase production using various solid agricultural and agro-industrial residues as substrates (Castilho *et al.*, 1999).

The results of this study showed that pectinase enzyme production was highest at 30°C. A temperature of 30°C is therefore the optimum temperature for pectinase production by *Aspergillus niger* using different types of substrates (orange peels). Temperature is a very important factor in microbial growth and product formation and varies from microbe to microbe. Slight changes in growth temperature can affect pectinase production (Shefali and Sudhir, 2013). The results of this study are consistent with those of Banu *et al.,* (2010) reported that 30 °C is the optimum temperature for pectinase production by *A. niger* NCIM548. This is also consistent with a study (Javed *et al.,* 2010) which reported that 30°C was the optimum temperature for pectinase production and showed higher pectinase activity.

The results obtained showed that the highest pectinase activity was at pH 5. Therefore, this finding suggests that the optimal pH for pectinase production by *A. niger* using orange peel as a substrate is pH 5. Indeed, the medium of the environment is acidic in nature, which makes the environment favourable for fungal growth (Singh and Sharma, 2007). This acidic condition during solid-state fermentation is acceptable because the orange peel used as a substrate in this study is acidic in nature. In general, pH changes the enzyme structure, recognition site, active site and substrate conformation (Palaniyappan *et al.,* 2009); therefore, it is important to determine the best pH for maximum pectinase activity. In addition, the optimal pH is important for the growth of microorganisms and their metabolic activities. Since the metabolic activities of microorganisms are quite sensitive to changes in pH, pectinase production by *A. niger* is affected by the changing pH values of the medium. The results of this finding are consistent with the work of Darah *et al.,* (2013) Ellaiah *et al.,* (2002) and Muthuprakash and Abraham (2011). They reported that pH 5 was one of the optimal culture conditions for enzyme production by fungal strains, including *Aspergillus* species, in a solid-state fermentation system. Jayani *et al.* (2005) also obtained similar results for polygalacturonase from *Aspergillus* species. Ramakrishna *et al.* (1982) also reported that the optimal pH for pectinase activity of heat-resistant Aspergillus species is pH 5. The results of this finding are also in agreement with the work of Javed *et al.,* (2010).), who reported that the optimum pH condition for pectinase production by *Aspergillus niger* was found to be pH 5. Coral *et al.,* (2003) reported that fungal acid enzymes had an optimal pH between 4.0 and 5.0. A similar study by Jayani *et al.* (2005), studied pectinase production using different fungal strains, in which the optimum pH was observed to be 5.0 and there were significant differences in pH below and above 5. Pectinases Acids are commonly produced by fungi, especially *Aspergillus* spp (Pedrolli *et al.,* 2009). Therefore, the pectinase of *A. niger* could be one of the candidates for acid pectinase, which can be used to extract pectin from fruit juice. This result shows that pH 5.0 is the optimal condition for pectinase production and can be applied in the industry, especially the fruit juice industry.

In this study, fermentation time was considered to determine the optimal date for pectinase production. The results of this finding suggest that the optimal day for pectinase production is day 5. The results of this finding are consistent with the study by Adebare *et al.,* (2012), who reported that the maximum activity was the superiority of pectinase obtained on day 5 of fermentation of *Aspergillus* species using orange peel as a substrate. This is also consistent with the work of Oyeleke *et al..,* (2012) on the production of cellulase and pectinase enzymes

by *Aspergillus niger*. They reported that maximum pectinase activity was observed on day 5 of fermentation. Similar work for the remarkable and highly efficient production of pectinase and cellulase by commercial baker's yeast *Saccharomyces cerevisiae* using potato processing waste was performed for 5 days of solid-state fermentation (Magdy, 2011).

Based on these results, the culture density of the standardized strain 10^6 (CFU/mL) of the isolate was found to be the best for pectinase production using orange peel as a substrate. The study was conducted using different inoculum densities to determine the best inoculum for pectinase production and a culture size of 10^6 (CFU/mL) proved to be favourable for enzyme production (Darah *et al.,* 2013). Sen and Satyanarayana, (1993) Gajju *et al.,* (1996); Prakasham *et al.,* (2005) reported that a culture density of 2 × 108 CFU/mL of *B. licheniformis* produced the highest pectinase activity. The results of this study on pectinase production using 10^6 (CFU/mL) inoculum were different from the findings of Sen and Satyanarayana, (1993) and others, therefore, differences from the findings present may be due to differences between isolates and substrates used. In this study, different holding times and crude pectinase concentrations were used to determine the optimal time for orange juice extraction. A large amount of juice was extracted after 60 min and at 4ml/40g crude pectinase and protein respectively. In a similar study, 30 min was found to be the best reaction time for gout pineapple juice extraction using the same enzyme (Dzogbefia *et al.,* 2001). The difference in optimal reaction times in the present study may be due to differences in the conditions of use for pectinase production, concentration and purity of the enzyme, or may also be due to differences in the fruit from which the juice is extracted (Dzogbefia *et al.,* 2001).

The results of this study showed that *Apergergillus niger* AR2 produced the highest pectinase activity when using orange peel as a substrate under optimal conduction with an activity of 3.04 ± 0.024 IU. The results of this study are consistent with the work of Darah *et al.,* (2013), who reported that *Aspergillus niger* HFM-8 produced the highest pectinase activity of 3.0156 IU. it is also consistent with the work of Ezugwu *et al.* (2014), who investigated pectinase production by *Aspergillus niger* using two types of substrates, where higher pectinase activity was observed when using orange peels as the substrate. The results of this study confirm that orange peel can be used as a substrate for pectinase production by *Aspergillus niger*.

CONCLUSION

In this study, eight different strains of *Aspergillus niger* were isolated from spoiled oranges, but one strain of *A. niger* was identified at the molecular level. *Aspergillus niger* AR2 isolated from spoiled oranges (Ibadan Variety) was observed to have the highest hydrolysis area on pectin agar. Optimal conditions for pectinase production were found to be 15 g of substrate, inoculum density was 10^6 (CFU/mL), a temperature of 30°C, and pH of 5 on day 5 with activity 3.04 ± 0.024 (IU). The use of crude pectinase for juice extraction is recommended due to the increased yield of orange juice obtained compared with orange puree without enzyme (control).

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