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EXTRACTION AND CHARACTERIZATION OF PECTINASE FROM *ASPERGILLUS* SPECIES USING MANGO BIOMASS AS A CARBON SOURCE

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ABSTRACT

Pectinases are enzymes that catalyze the breakdown of pectic sugars. This research focused on isolating potential pectinase-producing pectinolytic fungi from decomposing mango peels, characterization of precipitated pectinase activity concerning a few physicochemical parameters, and using extracted mango pectin as a growth substrate under submerged fermentation. Pectin was extracted from waste generated by the consumption and processing of mango fruits. Using Pectin agar media, the pectinolytic fungi *A. niger* and *A. fumigatus* produce pectinase. By inoculating the two isolates and culturing them for seven days, the pectinase production medium was later employed for lab-scale production. After seven days of fermentation, the pectinase was extracted and pectinolytic activity was measured every day for seven days. *A. fumigatus* has a stronger pectinolytic activity than other strain, hence it was chosen for future research. In a submerged fermentation system, *A. fumigatus* were inoculated into a broth containing mango pectin. With 80% ammonium sulphate saturation, a crude enzyme from *A. fumigatus* was precipitated. At 70 °C, mango peels yielded 18% pectin at pH 2.2. The precipitated pectinase was characterized and found to have the highest activity at 40 °C, 35 min of reaction time, 25 mg mL^{-1} substrate concentration, and a pH of 5.0. According to the findings, mango peels have a high pectin concentration for the production of pectinase. *A. Fumigatus* produced most pectinase on the fourth day (96 hours) of incubation period and more effective pectinase producer.

Key-words: Characterization, Mango peels, Pectinase, Pectin, Submerged fermentation

INTRODUCTION

Pectinases are a group of enzymes that breaks down pectin, a polysaccharide found in plant cell walls. The most studied pectinases are polygalacturonase, pectate lyase, pectin lyase, and pectinesterase (Palagiri *et al*., 2019). Jayani., *et al* (2005) reported that the group of enzymes is classifed based on their mode of action on substrates, and the major classifed pectinase is polygalacturonase (PG), pectin lyase (PL), and pectinesterase (PE). Pectinase has various biotechnological applications in a variety of fields, including those in food, agriculture, industry, environment, and medicine. In the food sector to make fruit juices, coffee, cocoa, tea, jams, and jellies. Furthermore, pectinase, an enzyme supplement included in animal feed, improves nutritional absorption. In the agricultural sector, pectinase has been used for plant virus purification, oil extraction, retting and degumming processes, and cotton fiber bio-scouring. They aid in wastewater treatment by facilitating pectin breakdown in the environmental sector. Pectinases are mostly used in the wine and paper industries in the industrial sector (Mulluye and Atnafu, 2022).

Fungi are rich in diverse metabolites and have been frequently used to manufacture powerful pectinolytic enzymes in either solid or submerged state fermentation (John *et al*., 2020). Among the documented fungal strains, Aspergillus species are the most prolific producers of pectinases with industrial and biotechnological applications(Sudeep *et al*., 2020). The use of non-food pectin-rich wastes to generate pectinase of commercial significance by a fungal isolate native to the terrestrial environment is appealing from both an ecological and an industrial standpoint. Some of the waste materials generated by agroinductrial processing include rice husk, groundnut husk, orange peel, banana peel, and mango peel; however, they could be used by microbes to produce high-value products. (Elegbede and Lateef, 2018).

Aspergillus species are filamentous fungi, commonly occurring in the soil, decaying plant materials and seeds. There are about 180 species of *Aspergillus*. *Aspergillus* spp. have a variety of functions. Spores of some *Aspergillus* species can cause lungs infection (CDC, 2021). Some species are known to Produce different beneficial compounds like citric acid, cyclosporine A, asperfuranone, gluconic acid, itaconic acid and kojic acid (Sun *et al.,* 2018; Chigozie *et al.,* 2022). Some species such as *Aspergillus versicolor* and *Aspergillus favipes* have shown biocontrol efficacy against soil-borne fungal pathogens such as *Macrophomina phaseolina* (Khan and Javaid, 2021, 2022). Likewise, *Aspergillus niger* has the ability to

produce herbicidal compounds for the control of noxious weed *Parthenium hysterophorus* (Bashir *et al*., 2018). Moreover, many *Aspergillus* species can produce a number of industrial enzymes such as amyloglucosidase, glucosidases, amylase, hemicellulase, protease, xylanase and cellulase (Moo, 2019).

Agroindustrial processing generates a large amount of biomass waste. Small fruit processing enterprises thrive in numerous cities across the world, and after separating the flesh for mango juice, the peels are frequently discarded. These generate a significant quantity of waste throughout the year. Instead of being disposed of in the environment, where they may create pollution, these waste materials might be used to make valuable items like pectin and pectinases. This demonstrates one approach of transforming waste into wealth (Mulluye *et al*., 2021). The objective of this study is to isolate and screen for effective pectinase producers *Aspergillus* species from decomposing mango fruit under a submerged fermentation condition using extracted mango pectin as a carbon source, one step purification of crude pectinase, and characterization of one step purify pectinase activity in terms of a few physicochemical parameters.

MATERIALS AND METHODS

Substrate preparation: Tsega Fruit and Juice House in Harar City, Ethiopia provided mango (Mangifera indica) peels. Before being crushed into a powder and stored at room temperature in a milling machine, the peels were treated with 97% ethanol.

Extraction of Pectin from Mango Peels: The procedure described was used to extract pectin by Mc Cready and Pectin (1970).

$$\%\text{Yield of pectin} = \frac{\text{Amount of pectin obtained(g)}}{\text{The total amount of mango peel powder used (g)}} \times 100$$

Isolation of Pectinolytic Fungi

Collection of Soil Samples: Soil samples were collected from decomposing waste dumping places in Addis Ababa, Ethiopia's Koshe massive garbage landfill, using the methods prescribed by Martin *et al.* (2004).

Media for Isolation of Microorganisms: Soil samples (3 g) were homogenized in a sterilized pectin agar medium (Udenwobele *et al*., 2014). The medium was autoclaved at 121°C for 15 min. It was allowed to cool to around 45 °C before being placed in sterile Petri plates to gel. To assure sterility, the plates were incubated overnight at 37 °C in an incubator.

Inoculation of plates and sub-culturing: The solid media was streaked with a loop of homogenized culture from the broth medium. After that, the plates were incubated at 35 °C until visible colonies appeared. Sub-culture was used to purify all morphologically dissimilar colonies. Potato dextrose agar was used to keep the isolates alive. Identification of microscopic and macroscopic aspects of isolated fungi was done by comparing features and micrographs to the identification of common *Aspergillus* species (Klich, 2002).

Fermentation: Submerged fermentation was used to make pectinase according to the method Martin *et al*. (2004). Flasks were picked from the appropriate groups on each day of the extract, and mycelia biomass was isolated using the filtration process. Until the seventh day of fermentation, the filtrate was tested for pectinase activity.

Pectinase Assay: The activity of pectinase was determined by measuring the enzyme's polygalacturonase activity. This was accomplished by employing the 3, 5-dinitrosalicylic acid reagent test method reported by Miller (1959) to measure the release of reducing groups from pectin.

Ammonium Sulphate Precipitation: In each test tube, pectinases were precipitated with moderate stirring at 20-100% saturation of solid ammonium sulphate at a 10% interval. The ammonium sulphate-crude pectinase solutions were kept at 4°C for 30 hours until the supernatant could be gently decanted. The test tubes were centrifuged for 30 min. at 3500 rpm (Leelamani *et al*., 2018).

Effect of pH on pectinase activity: At intervals of 0.5, the optimum pH was established using 0.05 M sodium acetate buffer (pH 3.5-5.5), phosphate buffer (pH 6.0-7.5), and Tris-HCl buffer (pH 8.0-10.0). By dissolving 0.5 g pectin in 0.05 M of the relevant buffers and marking up to 100 mL, a 0.5 % pectin solution

was created. For pectinase experiments, precipitated pectinase was disseminated in the various buffers and 0.5 mL of the enzyme was combined with 0.5 mL pectin solution at the appropriate pH (Ubani *et al*., 2015).

Effect of temperature on pectinase activity: By incubating the precipitated enzyme with pectin solution at 25-55 0C for 1 hour and at pH 5 intervals of 50C, the optimal temperature was identified. After then, the activity was evaluated (Sudeep *et al*., 2020).

Effect of reaction time on pectinase activity: At pH 5 and 40 ^{0}C, multiple reaction periods ranging from 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min. were used to determine the best reaction time for precipitated pectinase test.

Effect of substrate concentration on pectinase activity: By incubating the enzyme with 5, 10, 15, 20, 25, 30, 35, 40, and 45 mg mL^{-1} of mango pectin at pH 5 and 40 ^{0}C, the effect of substrate concentration on the activity of ammonium sulphate precipitated pectinase was determined (Ubani *et al*., 2015).

Data Analysis: The data were entered into the computer and statistical analysis was done using ANOVA provided by the SAS version 9.1 and significant difference examined for different parameters and between pectinolytic fungal isolates at 95% confidence level of significance and at $p < 0.05$.

RESULTS AND DISCUSSION

The yield of Extracted Mango Pectin: Using the ethanol-HCl method described by McCready (1970), pectin was extracted from mango peel with a yield of 18% at pH 2.2, 70 °C, and an extraction time of 60 min. Rehman *et al*. (2004) Mango pectin extraction yields of 13.45%, 21%, and 15.1% were reported using hydrochloric acid, sulphuric acid, and nitric acid at pH 2.5, 80 °C, and 120 min., respectively. The differences in the yields could be as a result of differences in the sources of pectin and other factors such as extraction technique, changes in pH, temperature, extraction time and environmental factors. This result proves mango peel a good source of pectin for a carbone source of the production pectinase use for several biotechnological applications.

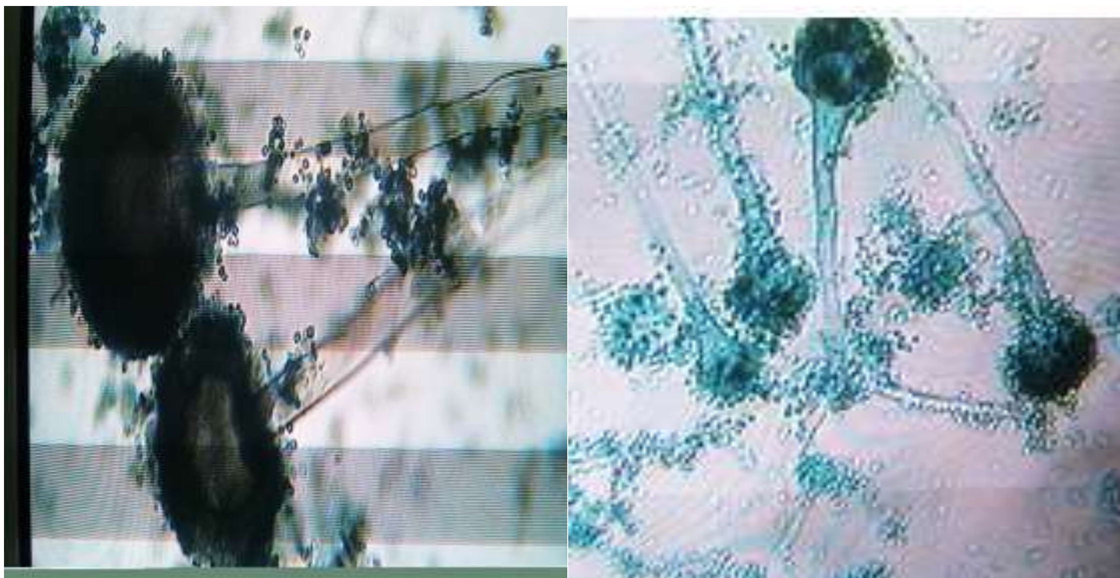

Fig. 1. Micrograph of pectinolytic fungal isolates (40X)

Identification of Pectinolytic Fungi: Table 1 shows pectinolytic fungus isolates identification based on colony morphology, growth characteristics, microscopic features, and 35°C growth.

Isolation and Screening of Pectinase Producing Fungi:

A. fumigatus was the isolate that secreted more pectinase during the screening process. The result is in good agreement with Ezugwu *et al*. (2013) which reported that *A. fumigatus* can produce large quantities of extracellular pectinase enzyme.The total *Aspergillus* species isolates from soil samples containing decomposed mango peel were evaluated for extracellular production of pectinase in this study. Only two *Aspergillus* species isolates were identified as pectinase producers and were thus subjected to colony morphological identification, microscopic features, and growth characteristics on the selected growth media. *A*. niger and *A*. fumigatus were the two isolated *Aspergilus* species as shown (Table 1). Figure 1 displays microscopic pictures of the two pectinolytic fungus isolates. The isolates were identified using microscopic characteristics.

Table 1. Identification of pectinolytic fungal based on colony morphology, microscopic features, and a growth temperature (35 ^{0}C)

On pectin agar media, colony characteristics	Growth on Petri plate(85 X 15mm diameter)	Features at a microscopic level	Identity
A submerged form of mycelium was developed by moderately developing colonies with carbon black/deep brownish black color conidia; reverse-colorless to pale yellow, exudates absent.	In 8 days, I had completely covered the plate.	Conidiophores up to 3 mm in length, phialides biseriate, conidial heads radiate, vesicles nearly globose, conidiophores nearly globose	*Aspergillus niger*
Colonies were colorless to yellow or velvety, dull blue-green, reverse colorless to different colors, and create tufted aerial mycelium up to felted floccose forms, and they spread quickly.	Covered the plate in 7 days	Conidial heads were columnar, densely crowded, with flask-shaped vesicles, short conidiophores, and uniseriate phialides.	*Aspergillus fumigatus*

Production of Pectinases by Submerged Fermentation: On the fourth day (96 h) of incubation, both isolates produce the most. Table 2 shows *A. niger* and *A.fumigatus* produced pectinase with higher enzyme activity of 0.0276 and 0.0290 U/mL, respectively. *A*. fumigatus has higher pectinolytic activity of the two isolates, hence it was chosen for future pectinase characterisation research.

The duration of fermentation is determined by the nature of the medium, the fermenting organisms, the concentration of nutrients, and the physiological process. In the solid state fermentation of polygalacturonase, a 96-hour incubation duration was similarly obtained (Martin *et al*., 2004). The pectinase activity decreased after day four. This decline in pectinase synthesis could be ascribed to a high concentration of aging fungal cells, a reduction in some critical nutrients, and an accumulation of inhibitory chemicals in the fermentation medium. The composition of the substrates used in the fermentation medium has a substantial impact on the cost of enzyme production (Mohammadi *et al*., 2020). This finding implies that the pectinase expression pathway was best up-regulated in the presence of agro-residues, particularly mango peel. The ability of mango waste to support pectinase production could be attributed to the presence of high concentrations of pectin and other sugars in mango peels (da Câmara Rocha *et al*., 2020; Wongkaew *et al*., 2021), which could have positively induced pectinase biosynthesis for subsequent degradation.

Ammonium Sulphate Precipitation Profiling of Pectinases: At 80% ammonium sulphate saturation, the crude enzyme produced from *Aspergillus fumigatus* had the maximum activity (0.024501 U/mL); this percentage was employed for the precipitation of pectinases in subsequent experiments (Fig. 2). Joshi *et al*. (2011) Verified the result, claiming that pectinase from apple pomace was saturated with 80% ammonium sulphate. The enzyme being protein can be precipitated by the addition of ammonium sulphate, thus increasing the purity of the enzyme in the precipitates.

Table 2. Incubation time has an effect on enzyme synthesis.

The incubation period (days)	Isolated pectinolytic fungi enzyme activity U/ Ml	
	Aspergillus niger	*Aspergillus fumigatus*
1	0.0144 ± 0.0012[d]	0.0145 ± 0.0009 [c]
2	0.0170 ± 0.0011[dc]	0.0209 ± 0.0021[abc]
3	0.0177 ±0.0014[c]	0.0219 ± 0.0020[abc]
4	0.0276 ±0.0015[a]	0.0290 ± 0.0107[a]
5	0.0258 ± 0.0006[a]	0.0259 ± 0.0076[a]
6	0.0218 ± 0.0029[b]	0.0259 ±0.0076[ab]
7	0.0171± 0.0018[dc]	0.0162 ±0.0005 [bc]

- Values are mean ± S.D. of 3 replicates
- [a,b,c,d]Means within a column with values followed by different superscripts are significantly different at ($p < 0.05$).
- Values followed by same superscripts are not significantly different at ($p < 0.05$).
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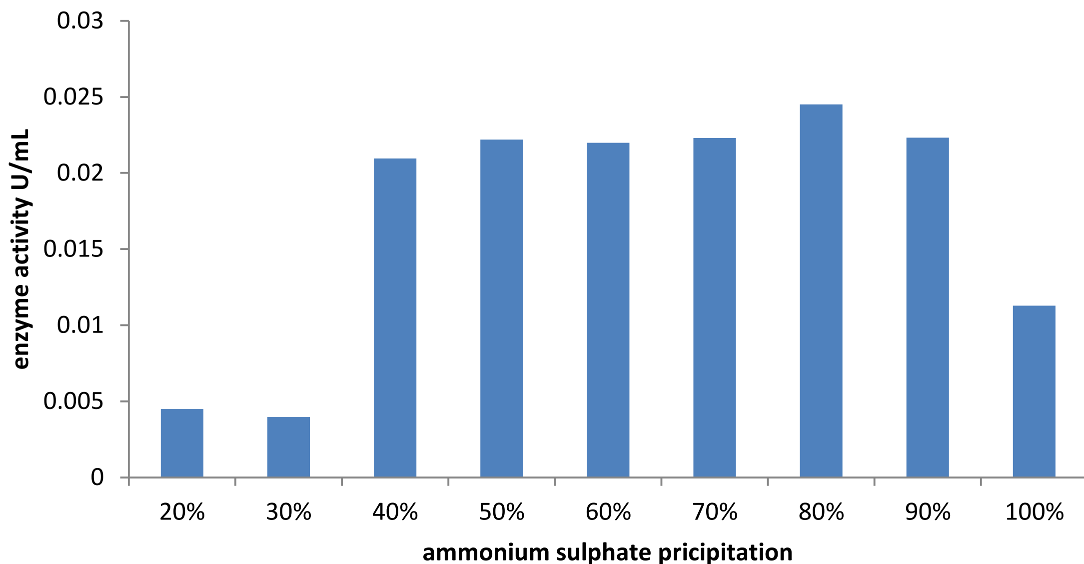

Fig. 2. Ammonium sulphate precipitation profiling of pectinase.

Characterization of Ammonium Sulphate Precipitated Pectinase

The findings of the characterisation of precipitated pectinase for various factors such as temperature, pH, substrate concentration, and reaction time are reported and discussed below.

Effect of pH on the activity of precipitated pectinase

The influence of pH on precipitated pectinase activity (Table. 3). The data reveal that pectinase activity increased with increasing pH up to 5, then declined with rising pH beyond 5. (Table 3) at pH 5, the enzyme had the maximum activity (0.033439 U/mL). The decrease in enzyme activity at higher pH may be attributed to fungus (*A*.fumigatus) preferring lower pH for growth and metabolism (Baladhandayutham and Thangavelu, 2010). Also, Enzymes are amphoteric molecules with a significant number of acid and basic groups on their surface. The charges on these groups will change with the pH of their surroundings, according to their acid dissociation constants. The total net charge of the enzymes, as well as the distribution of charge on their outer surfaces, will be affected by an increase in pH, as will the reactivity of the catalytically active groups. The influence of pH on charge distribution on ionizable groups disrupts the enzyme's tertiary structure and hence accelerates denaturation (Reena *et al*., 2005, Eleonora *et al*., 2009).

Table 3. Effect of pH on pectinase activity.

pH	Enzyme activity U/ML
3.5	0.014091 ± 0.000379138[h]
4.0	0.013740 ± 0.000369270[h]
4.5	0.025973 ± 0.000860562[c]
5.0	0.033439 ± 0.000278371[a]
5.5	0.032212 ± 0.000437550 [b]
6.0	0.032036 ± 0.000843702[b]
6.5	0.023589 ± 0.000856321[d]
7.0	0.023694 ± 0.000264774[d]
7.5	0.017560 ± 0.000315500[e]
8.0	0.016404 ± 0.000585603[f]
8.5	0.015142 ± 0.000364308[g]
9.0	0.015072 ± 0.000539793[g]
9.5	0.015142 ± 0.000379138[g]
10	0.015177 ± 0.000529482[g]

- Values are mean ± S.D. of 3 replicates
- [a,b,c,d]Means within a column with values followed by different superscripts are significantly different at ($p < 0.05$).
- Values followed by same superscripts are not significantly different at ($p < 0.05$).

Effect of temperature on the activity of precipitated pectinase

The effect of temperature on the activity of *A*. fumigatus precipitated pectinase was investigated, and 40 °C was found to be the optimum temperature for pectinase activity (Fig. 3). The decrease in activity over 40 °C could be due to enzyme inactivation. These enzymes are composed of proteins and have relatively narrow substrate specificity. Temperature can control how active enzymes are, ranging from very active to very inactive. Although greater temperatures improve enzyme activity and reaction rates, enzymes are proteins, and like other proteins, temperatures beyond 40 °C begin to break them down and render them non-functional. These findings suggest that the optimal temperature for commercial application of *A. fumigatus* pectinase is 40°C.

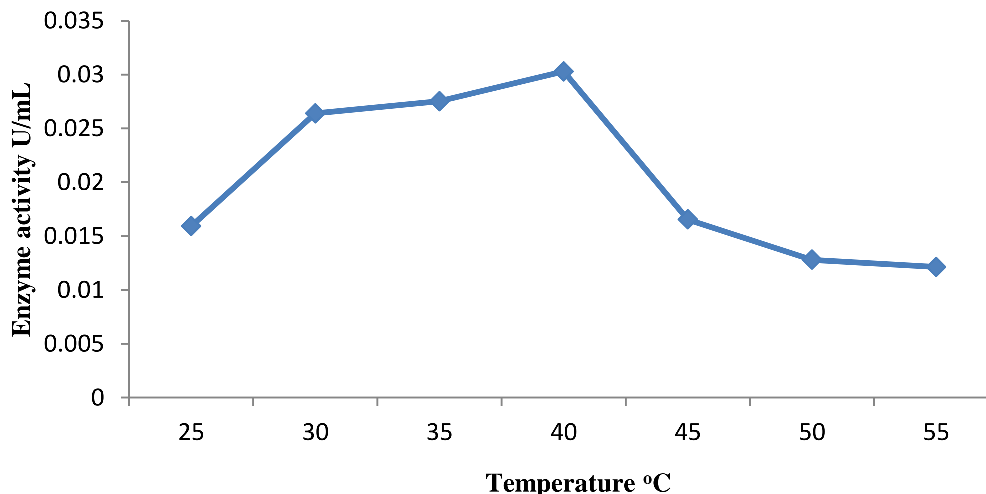

Fig. 3. Effect of reaction temperature on pectinase activity

Effect of reaction time on the activity of precipitated pectinase: The activity of enzymes increases as the reaction time grows until it reaches 35 min., at which point the reaction begins to drop. The optimum reaction time for A. fumigatus pectinase activity was found to be 35 min, as illustrated in (Fig. 4). Other research backs up this assertion (Torimiro and Okanji, 2013). For maximum catalytic activity, all enzymes have an optimal reaction time.

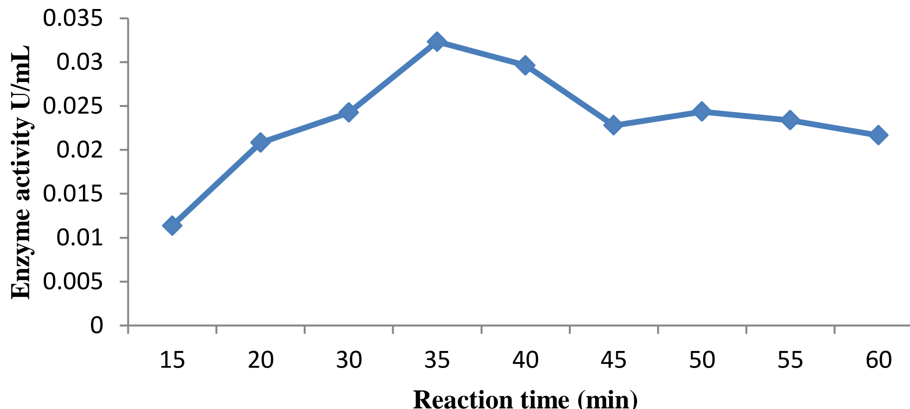

Fig. 4. Effect of reaction time on the pectinase activity.

Effect of substrate concentration on the activity of precipitated pectinase: The findings revealed that increasing the quantity of substrate increased enzyme activity up to a concentration of 25 mg mL^{-1}, after which pectinase activity declined (Table 4). Excess substrates in the enzyme active sites encircle substrate considerably attached to them. The enzyme molecules are saturated with the substrate in other ways. The extra substrate molecules are unable to react until the substrate that has already been bound to the enzymes has reacted and been released.

Table 4. Effect of substrate concentration on pectinase activity.

Substrate conc.(mg/mL)	Pectinase activity (U/mL)
5	0.0140 ± 0.0099^{c}
10	0.0190 ± 0.0109^{bc}
15	0.0205 ± 0.0044^{bc}
20	0.0265 ± 0.0006^{ab}
25	0.0311 ± 0.0010^{a}
30	0.0233 ± 0.0027^{ab}
35	0.0240 ± 0.0022^{ab}
40	0.0248 ± 0.0020^{ab}
45	0.0184 ± 0.0021^{bc}

- Values are mean ± S.D. of 3 replicates
- ^{a,b,c,d}Means within a column with values followed by different superscripts are significantly different at ($P < 0.05$).
- Values followed by same superscripts are not significantly different at $p < 0.05$.

Conclusions

These findings show that mango peels with a pectin content of 18% were effective in promoting the production of pectinase during submerged fermentation. The pectin derived from mango peels works as an inducer for pectinase production. A total of two pectinolytic fungus isolates were identified as efficient producers such as A. *niger* and A. *fumigatus*, with *A. fumigatus* being more efficient. The findings suggest that using pectin extracted from mango peels as a substrate for the production of pectinases in a submerged fermentation system using *A. fumigates* is highly promising.

Competing interests: The authors declare that they have no competing interests.
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