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Pectinase Production by *Rhizopus stolonifer* A3 Isolated from Apple Peels

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Abstract

The application of pectinase in industries will continue to be developed. To broaden the applicability of enzyme manufacturing in new industries, more research to investigate pectinolytic microorganisms with high activity and stability is still required. Therefore, this study aimed to obtain pectinolytic fungi that have the potential as pectinase producers. Pectinolytic fungi were isolated from rotten apple peels and selected based on clear zone formation on pectic agar media after cetyl trimethyl ammonium bromide (CTAB) staining. Pectinolytic fungi were identified based on 18S rRNA partial gene sequences and morphological characteristics. Pectinase production used Mandels and Weber medium with citrus pectin 20 g l^{-1} addition. Pectinase activity was determined based on the measurement of reducing sugars by a colorimetric method. A total of 5 fungal isolates were successfully isolated. All of the isolates had pectinolytic activities with clear zone diameters ranging between 0.99 to 7.32 mm. The isolate A3 showing the highest pectinolytic activity was identified as *Rhizopus stolonifer*. Microscopically, *R. stolonifer* A3 showed typical characteristics for *Rhizopus*, characterized by the presence of rhizoids, stolons and sporangiophores bearing a single spherical sporangium. The pectinase production of *R. stolonifer* A3 was optimum at initial pH 5.0, temperature 35 °C and incubation period of 3 days with pectinase activity of 14.75 U ml^{-1}. *R. stolonifer* A3 produced acidic pectinase having optimum activity at pH 5.0 and temperature 50 °C. Thus *R. stolonifer* A3 has the potential to be used as a producer of acidic pectinase appropriate for use in the processing of fruit products.

Keywords: apple peel; pectinase; pectinolytic fungi; *Rhizopus stolonifer*

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INTRODUCTION

Pectin is a heteropolysaccharide present in plant cell walls especially between the middle lamella. The basic structure of pectin consists of a long chain of α-D-galacturonate, which are linked together by α (1 → 4) bonds and 2 to 3% of L-rhamnose units that associate with the galacturonate units through β (1 → 2) and (1 → 4) bonds (Kameshwar and Qin, 2018). Pectin side chains can be L-rhamnose, arabinose, galactose and xylose (Oumer, 2017). In plant cell walls, pectin is associated with proteins and other structural polysaccharides to form insoluble protopectins (Flutto, 2003). Pectin is found in the cell walls of many fruits and vegetables and is particularly abundant in citrus, beet, carrots, apples and tomatoes (Haile and Ayele, 2022). Mechanical processing of fruits will produce viscous puree-like jelly, making it difficult to get clear juice by filtering and pressing (Kumar, 2015; Javed et al., 2018). The high viscosity and cloudiness affect the market value of fruit and vegetable juice. Therefore, the removal of pectic

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constituents improves the appearance, texture and taste of the final product (Patel et al., 2022).

Pectinase or pectinolytic enzyme is one of the enzymes having commercial value and it is reported to share 25% of the global sales of food enzymes (Oumer and Abate, 2018). It has long been used for juice processing to reduce viscosity and turbidity, and increase the brightness of color, clarity, filterability and taste (Praveen and Suneetha, 2016). Pectinase can be a valuable tool in sustainable agriculture by contributing to the wine industry; food industry; bleaching of pulp, treatment of pectic wastewater; processing of fruit and vegetables, fermentation of coffee and tea, animal feed; extraction of vegetable oil and scouring of plant fibers, bioethanol production, extraction of DNA and protoplast isolation from a plant (Kashyap et al., 2001; Garg et al., 2016; Shrestha et al., 2021). Application of pectinase will reduce environmental hazards and serious health disorders due to the use of chemical and mechanical methods employed in industrial processes (Kaur et al., 2023).

Pectinases degrade pectic substances, either by depolymerization (hydrolases and lyases) or de-esterification (esterases) reactions (Singh et al., 2019). Based on the cleavage site, pectinases are divided into three groups: (1) hydrolases, including polygalacturonase PG (EC 3.2.1.15), catalyzing the hydrolysis of polygalacturonic acid chains; (2) pectin lyase (EC 4.2.2.10), catalyzing the random cleavage of pectin, and pectate lyase (EC 4.2.2.2), selectively cleaving the glycosidic bonds of polygalacturonic acid to produce an unsaturated product by metabolic removal process, forming the lyase/trans-eliminates; and (3) pectin esterase (EC 3.1.1.11), catalyzing the de-esterification of the methoxyl group of pectin to form pectic acid and methanol (Kashyap et al., 2001; Yadav et al., 2009; Oumer, 2017).

Pectinases are produced by a wide variety of species, including bacteria, fungi, yeasts, insects, nematodes, protozoans and plants (Islam and Roy, 2018); however, practically all commercial pectinase preparations come from fungi (Oumer and Abate, 2018). Fungal pectinase is generally acidic so it is appropriate for use in the processing of fruit products, which are usually acidic (Kashyap et al., 2001). The quick growth, brief lifespan and ease of genetic modification of microbes make it more profitable to produce enzymes from them as well (Haile and Ayele, 2022). Microbial pectinases will continue to be used in various sectors. To broaden the applicability of enzyme manufacturing in new industries, more research is still required (Suhaimi et al., 2021). It is important to emphasize various investigations to explore pectinolytic microorganisms with high activity and stability over a broad range of temperatures and pH ranges for longer periods. Therefore, the objective of this work was to identify pectinolytic fungus that could potentially create acidic pectinase.

MATERIALS AND METHOD

Media

Isolation and screening of pectinolytic fungi used pectin agar medium with the composition (g l^{-1}) of citrus pectin 5.00, K_2HPO_4 0.50, $MgSO_4.7H_2O$ 0.10, NaCl 0.20, $CaCl_2.2H_2O$ 0.20, $FeCl_3.6H_2O$ 0.01, yeast extract 1.00, agar 20.00 (Amilia et al., 2017). Pectinase production used Mandels and Weber medium modified by Ellouz Chaabouni et al. (1995) with the composition (g l^{-1}) of KH_2PO_4 2, $(NH_4)2SO_4$ 1.4, $MgSO_4.7H_2O$ 0.3, $CaC1_2.2H_2O$ 0.3, yeast extract 1, tween 80 1 ml^{-1}, trace element solution 1 ml l^{-1} (g l^{-1} $COC1_2$ 2, $MnSO_4H_2O$ 1.6, $ZnSO_4.H_2O$ 1.4, $FeSO_4.7H_2O$ 0.5) and citrus pectin 20 g l^{-1}.

Isolation and screening of pectinolytic fungi

Rotten apple peels (*Malus sylvestris* Mill.) collected from Batu, Malang, East Java, were used to isolate pectinolytic fungi. Isolation and screening of pectinolytic fungi were done by a method described by Amilia et al. (2017). A slice of rotten peel was placed on a pectic agar medium and incubated for 72 hours. The various fungal colonies were purified by repeated streaking on a potato dextrose agar (PDA) medium. The pure culture was kept in a PDA medium and kept at 4 °C. Pectinolytic activities were screened based on the ability to produce a clear zone on pectic agar medium after staining by Cetyl trimethyl ammonium bromide (CTAB).

Identification of pectinolytic fungi

Fungal DNA extraction was performed using a Presto™ Mini gDNA Yeast Kit (Geneaid) following the manufacturer's instructions. The partial sequence of the fungal 18S rDNA was amplified using ITS1 and ITS4 primers and Toyobo KOD FX Neo. DNA from fungi were amplified in 25 µl PCR mixtures containing: 5 µl of ddH_2O, 12.5 µl 2x PCR Buffer KOD FX neo, 5 µl 2 mm dNTPs, 0.5 µl 10 pmol μl^{-1} ITS1 Primer, 0.5 µl 10 pmol μl^{-1} ITS4 Primer, 1 µl DNA template and 5 µl KOD FX Neo (1.0 U μl^{-1}).

The amplification was performed as follows: initial denaturation for 3 minutes at 94 ℃, 35 cycles each of denaturation for 10 seconds at 98 ℃, annealing for 30 seconds at 52 ℃ and elongation for 45 seconds at 68 ℃. PCR product sequencing was done by 1st BASE (Singapore). PCR product purification was performed using the Zymoclean Gel DNA Recovery Kit (Zymo Research) based on the manufacturer's instructions. Partial sequences of 18S rRNA were compared to the database available in the GenBank using the BLAST program at the National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/. Macroscopic features such as colony morphology and color were examined. The color and nature of mycelia or spores were observed under the light microscope at 400 X magnification. Mycelia and spore were colored with lacto-phenol blue.

Pectinase production

Fungal isolates were grown on PDA slant media at 30 ℃ for 3 days. The spore suspension was made by adding 3 ml sterile water into the 3-day culture. The number of spores was counted by using a hemacytometer. A total of 10 ml of spore suspension was transferred into 90 ml of pectinase production medium in a 500 ml Erlenmeyer flask and then incubated for 2 days in an incubator shaker at 30 ℃ with an agitation of 120 rpm. Mycelium was separated from the medium by filtering using Whatman filter paper No.1 previously weighed. The mycelium was dried at 60 ℃ until it reached a constant weight to determine the dry weight of the mycelium. The supernatant was used as the source of enzyme and stored at 4 ℃.

Optimization of the incubation period, pH and temperature for pectinase production

Optimization of pectinase production was carried out in stages including incubation periods (1, 2, 3, 4, 5, 6 and 7 days), temperature (27, 30, 35, 40 and 45 ℃) and initial pH of the medium (4, 5, 6, 7, 8). Fungal growth was determined based on biomass production.

Measurement of pectinase activity

Pectinase activity was determined by the colorimetric method (Miller, 1959) as described by Takcı and Turkmen (2016) with modification. A total of 0.5 ml of crude enzyme extract was added to 0.5 ml of 1% citrus pectin in 0.1 M acetate buffer (pH 5.0). This mixture was incubated at 50 ℃ for 10 minutes and then 1 ml 3.5–dinitro salicylic acid (DNS) reagent was added to stop the reaction. Tubes were kept in boiling water for 5 minutes. After cooling, the absorbance was measured at 540 nm using a UV-visible spectrophotometer. The amount of released reducing sugar was quantified using D-galacturonic acid as a standard. One unit of enzyme activity was determined as the amount of enzymatic (U) required to release one micromole equivalent of D-galacturonic acid per minute under assay conditions.

RESULTS AND DISCUSSION

Isolation and screening of pectinolytic fungi

Five distinct fungal isolates, designated isolates A1, A1, A3, A4 and A5, were successfully isolated from rotten apple peels. Pectinolytic fungi or pectin-degrading fungi are commonly found in materials that contain a lot of pectin, such as fruits and vegetables (Sandhya, 2013). The ability of fungi to produce plant cell wall degrading enzymes including pectinase is an important mechanism for obtaining nutrients from plants (Choi et al., 2013). Apple peels contain 1.21% pectin (Virk and Sogi, 2004) so it is a good substrate for pectinolytic microbes. According to the screening results based on clear zone formation on pectic agar plates following staining with CTAB, all of the isolates demonstrated pectinolytic capabilities (Figure 1). The sizes of the clear zones ranged from 0.99 to 7.32 mm (Table 1). The clear zone around the colony indicates the production of extracellular pectinase, which depolymerizes pectin in the medium. Depolymerization of pectin inhibits precipitation by CTAB and results in a clearer appearance (Hadj-Taieb et al., 2011; Amilia et al., 2017). Differences in the diameter of the clear zone indicate differences in the ability of the isolate to degrade pectin in the environment. The formation of a large clear zone shows that the isolate produced extracellular pectinase with good substrate hydrolyzing ability (Singh et al., 2015).

Table 1. Pectinolytic activities of fungi isolated from rotten apple peels based on clear zones formation on pectic agar media after staining with CTAB

Code of isolate	Clear zone diameter (mm)
A1	0.99
A2	1.36
A3	7.32
A4	1.41
A5	1.52

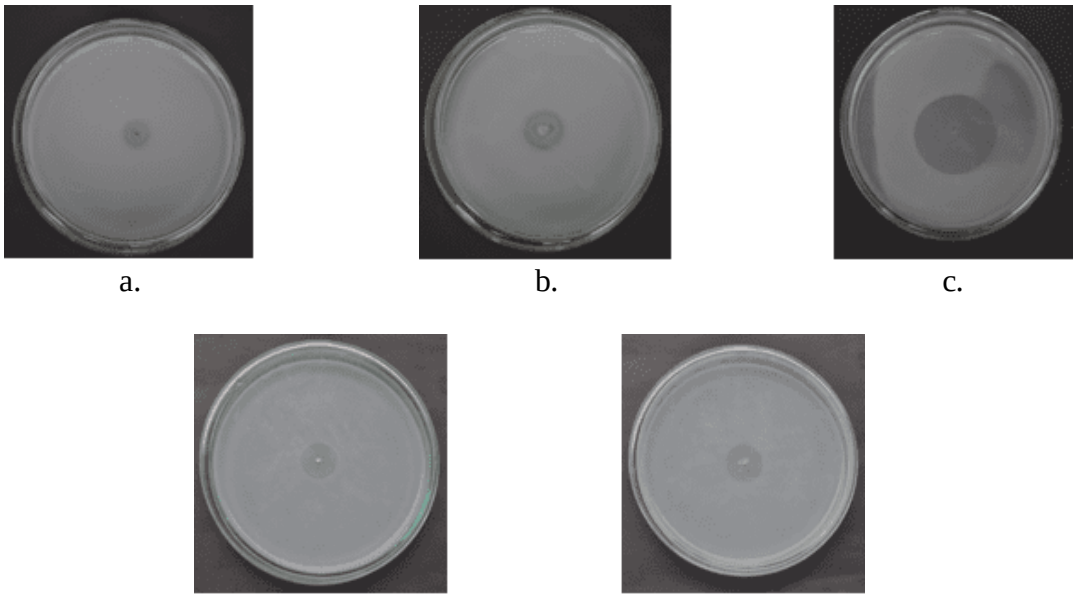

Figure 1. Isolate A1 (a), A2 (b), A3 (c), A4 (d) and A5 (e) on pectic agar media (aged 24 hours) after staining by CTAB, clear zones showing pectinolytic activities

Identification of pectinolytic fungi

According to BLASTN analysis, all of the pectinolytic fungi had similarities with the GenBank database, with a similarity degree of 98 to 100% and query cover of 99 to 100% (Table 2). Isolates are categorized into one species if it has ≥ 97 to 100% similarity level and ≥ 80% query coverage (Raja et al., 2017). Thus, isolates A1, A2, A3, A4 and A5 were identified as *Fusarium lateritium* A1, *Colletotrichum carthami* A2, *Rhizopus stolonifer* A3, *Diaporthe longicolla* A4 and *Aspergillus flavus* A5 respectively.

The macroscopic and microscopic characteristics of pectinolytic fungi are shown in Figure 3. The colony of *F. lateritium* A1 on PDA media aged 5 days was yellow. After more than a week, the colony turned red with a yellow color in the middle (Figure 2a). The reverse color of the colony was red (Figure 2b). Vitale et al. (2011) have succeeded in isolating *F. lateritium* grouped into 2 based on the morphology of the colony, namely colonies with dark grayish-olive and orange-yellow colors. The microscopic characteristics of *F. lateritium* A1 showed the characteristic feature of Fusarium, namely boat-shaped macroconidia with 3 to 6 septa (Figure 2c). Species of Fusarium are frequent plant infections. In order to successfully weaken and invade the host plant, they produce a variety of cell wall-degrading enzymes, such as amylase, cellulase, glucosidase, pectate lyase and xylanase (Sharafaddin et al., 2019; Perincherry et al., 2020). Fusarium species such as *F. oxysporum* (Bravo-Ruiz et al., 2017), *F. proliferatum* (Sharafaddin et al., 2019) and *F. graminearum* (Paccanaro et al., 2017) have demonstrated the ability to produce pectinase.

The colony color of *C. carthami* A2 was white and gray on the edge of the colony (Figure 2d). The opposite color was also white but with brownish to orange color in the center of the colony (Figure 2e). Previous research by Uematsu et al. (2012) showed that colonies of *C. carthami* that were isolated from living

Table 2. Result of BLASTN analysis of 18S rRNA partial gene sequence of pectinolytic fungi isolated from rotten apple peels

Code of isolate	Description	Query cover (%)	E value	Ident (%)	Accession number
A1	*Fusarium lateritium*	100	0.0	98	KJ816862.1
A2	*Colletotrichum carthami*	100	0.0	100	KM510463.1
A3	*Rhizopus stolonifer*	100	0.0	99	JN543963.1
A4	*Diaporthe longicolla*	99	0.0	100	JQ754023.1
A5	*Aspergillus flavus*	100	0.0	99	MF767617.1

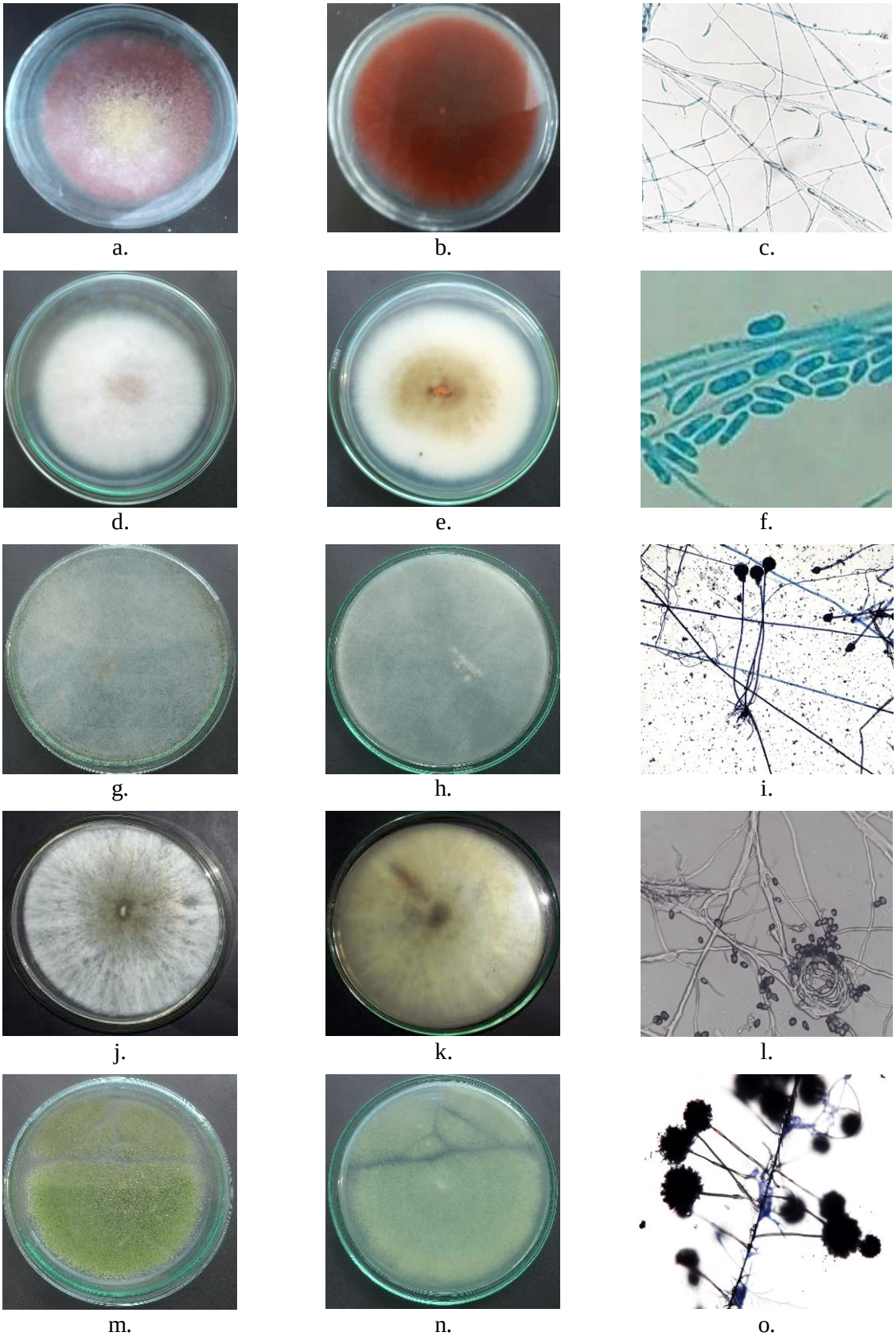

Figure 2. The colony from the upper site and reverse site and microscopic characters of pectinolytic fungi. i.e., *F. lateritium* A1 (a, b, c), *C. carthami* A2 (d, e, f), *R. stolonifer* A3 (g, h, i), *D. longicolla* A4 (j, k, l) and *A. flavus* A5 (m, n, o)

plants were greyish to dark olive in color and the reverse color was dark olive to dark brown. Orange conidial masses occasionally appeared close to the colony's beginning. Microscopically, the *C. carthami* A2 showed cylindrical conidia, sympodulosporous mainly two-celled and septated hyphae (Figure 2f). The genus Colletotrichum contains several significant plant pathogens that infect a wide range of woody and herbaceous plants, as well as plant saprobes and endophytes from a variety of substrates (Cannon et al., 2012). The ability of Colletotrichum to create various symptoms may be related to variations in the extracellular enzymes that the fungi produce, including pectin lyase, polygalacturonase and laccase (Velho et al., 2018).

Macroscopically, the colony of *R. stolonifer* A3 was cottony, white with grey to black at the top (due to sporulation) (Figure 2g). Colonies were fast growing and covered an agar surface within 3 days. The opposite color was also white (Figure 2h). Microscopically, *R. stolonifer* A3 showed typical characteristics for Rhizopus, shown by the appearance of nonseptate hyphae, rhizoids and stolons (Figure 2i). According to Bautista-Baños et al. (2014), *R. stolonifer* has whitish aerial hyphae, which then turn into black spots due to sporulation. Each sporangiophore has a spherical sporangium, which contains many spores. Rhizopus is the only fungus yet known to produce rhizoids and is characterized by the presence of stolons and sporangiophores, and bears a single spherical sporangium (Omoifo, 2011; Bautista-Baños et al., 2014). *R. stolonifer* has been reported to be a fruit pathogen (Hartanti et al., 2020). Rhizopus exhibits a complex metabolism and produces a variety of extracellular enzymes, including amylases, pectinases, cellulases, proteases and phytases (Lennartsson et al., 2014). Some commercial pectinases are produced from Rhizopus. Rhizopus pectinase producers include *R. oryzae* (Chowdhury et al., 2017), *Rhizopus* sp. (Jaworska et al., 2022) and *R. microspores* (Abd El-Rahim et al., 2020).

The colony of *D. longicolla* A4 had similar characteristics to *D. longicolla*, which had been isolated by Petrovic et al. (2018) from soybean seeds. These isolates on PDA had white, compact aerial mycelium with a typical yellowish-green ring around the center of the colony (Figure 2j). The opposite color of the colony was yellowish green (Figure 2k). Microscopically, it was characterized by coiled structure and ellipsoidal conidia with 2-guttules (Figure 2l). According to Gao et al. (2014) Diaporthe (including its Phomopsis form) has been reported as one of the most prevalent genera of endophytic fungi discovered on a variety of plant hosts. This endophytic fungus produces intriguing secondary metabolites and enzymes. Several species of Diaporthe were known to produce extracellular pectinase, among others *Phomopsis sapotae* and *P. psidii* (Khare et al., 1994), *P. cucurbitae* (Zhang et al., 1997), *Diaporthe helianthi* (Catalano et al., 2012) and *P. azadirachtae* (Vedashree et al., 2013).

The *A. flavus* A5 colony had similar characteristics to *A. flavus*, which had been isolated by Okayo et al. (2020) from groundnut. These isolates on PDA had a greenish colony with concentric zones and the texture of the colony was powdery (Figure 2m and 2n). Microscopic characters showed the presence of conidiophores, sub globose or globose vesicles of variable size and metulas (Figure 2o). *A. flavus* is an opportunistic plant pathogen that is widely found in various host plants and as a soil fungus. This fungus produces aflatoxins, which are harmful to animals and humans (Klich, 2007). Members of the Aspergillus are widely used as producers of polysaccharide hydrolyzing enzymes because of their ability to produce broad-spectrum hydrolytic enzymes (Anisa et al., 2013). Several species of Aspergillus were known as pectinase producers including *A. flavus*, *A. niger* and *A. ochraceous* (Panda et al., 2012; Anisa et al., 2013).

Effect of incubation time, pH and temperature on *R. stolonifer* A3 pectinase production

Screening results based on the formation of clear zones on pectin agar media showed that the *R. stolonifer* A3 had the highest pectinolytic activity with a clear zone diameter of 7.32 cm. Therefore, *R. stolonifer* A3 was used to produce pectinase. Growth and pectinase production of *R. stolonifer* A3 reached a maximum at 3 days of incubation with a dry weight gain of 0.23 g and pectinase activity of 10.45 U ml^{-1} (Figure 3a). Pectinase production was correlated with biomass production. The results were similar to Chowdhury et al. (2017). They reported that the pectinase production capacity of *R. oryzae* was optimal after an incubation period of 72 hours (3 days) and that the highest enzyme yield was achieved at the end of the log phase. Therefore, the faster the fungus grows, the higher the enzyme yield will be. Different results were reported by

Sethi et al. (2016), that pectinase production from *Aspergillus terreus* NCFT4269.10 was optimal after 96 hours incubation period and that pectinase production was not strongly correlated with biomass production.

The maximum pectinase production from *R. stolonifer* A3 was at an initial pH of 5 with a pectinase activity of 12.5273 U ml^{-1} (Figure 3b). This result was similar to the report of previous research that pectinase production by *A. terreus* NCFT 4692.10 and *A. flavus* was optimum at pH 5.0 (Sethi et al., 2016; Begum, 2020). Chowdhury et al. (2017) reported that *R. oryzae* exhibited maximum pectinase production at an initial pH of 6.0. Optimal fungal pectinase production is known to occur at acidic pH. Increasing or decreasing the pH beyond the optimal value will reduce enzyme production. However, the mechanism of pH impact on pectic enzyme production is still unidentified. According to Sethi et al. (2016), the pH of the medium regulates the growth of the culture or has an impact on the catalytic activity of enzymes.

The pectinase production from *R. stolonifer* A3 increased as the temperature increased from 25 to 35 °C and exhibited maximum production

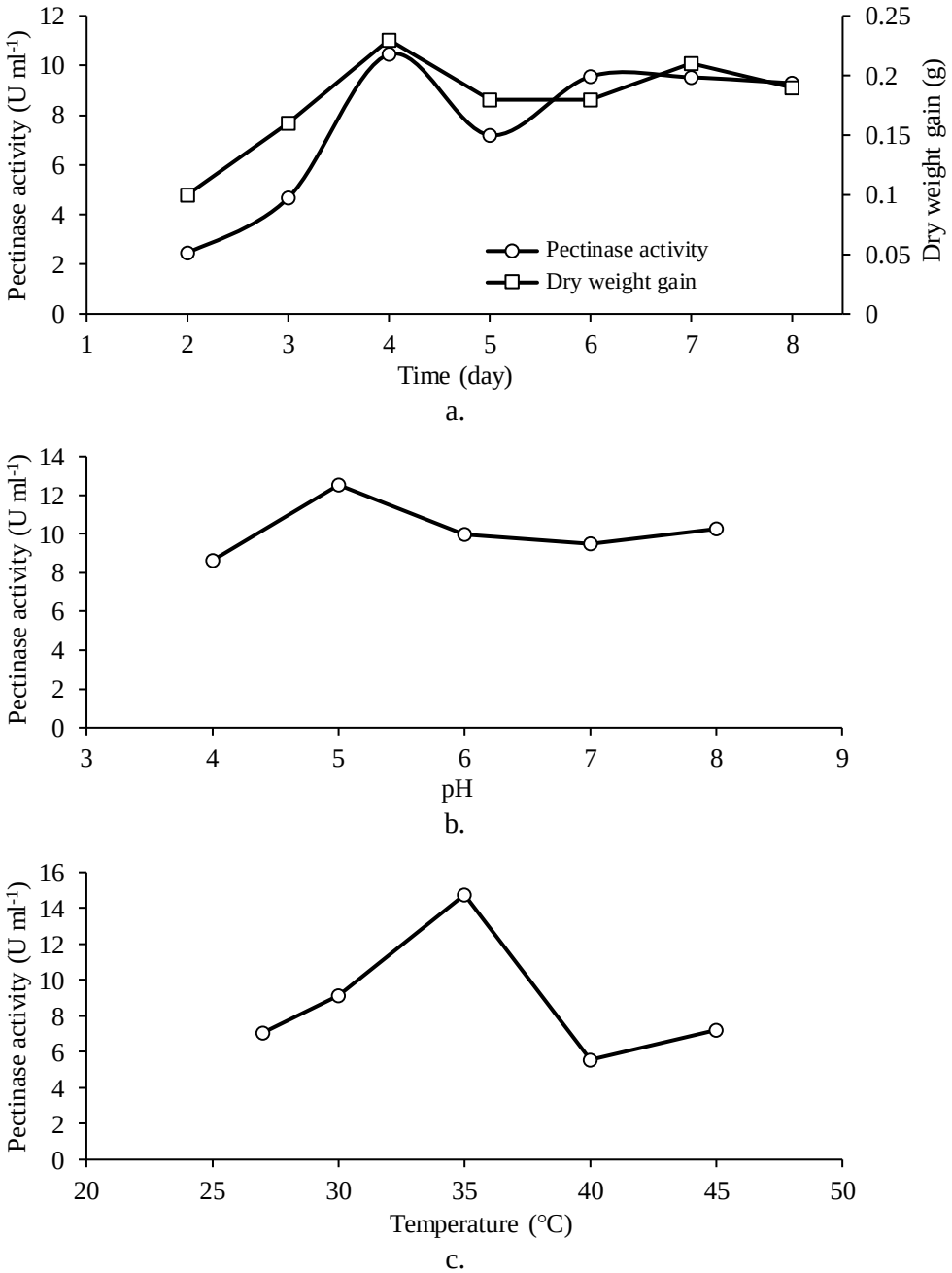

Figure 3. Effect of incubation period (a), pH (b) and temperature (c) on pectinase production from *R. stolonifer* A3

Table 3. Pectinase production of *R. stolonifer* A3

Before optimization (U ml^{-1})	After optimization (U ml^{-1})	Increment (%)
10.45	14.75	41.15

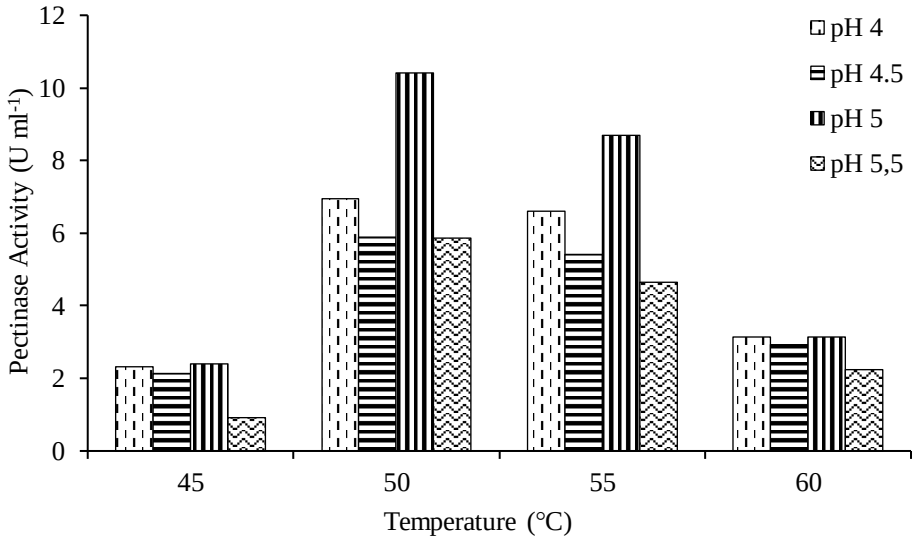

Figure 4. Effect of temperature and pH on activities of pectinase from *R. stolonifer* A3

at 35 ℃. Increasing the temperature above the optimal temperature reduced pectinase production (Figure 3c). The results were similar to those reported by Chowdhury et al. (2017), that the pectinase production of *R. oryzae* was optimal at 35 ℃. Temperature has a significant influence on enzyme production because growth, metabolism and product biosynthesis are influenced by environmental temperature and vary for each microorganism (Begum, 2020). Therefore, pectinase production from *R. Stolonifer* A3 was optimal at pH 5.0, temperature 35 ℃ and incubation time of 3 days with pectinase activity of 14.7484 U ml^{-1}. The pectinase yield under optimal conditions was 41.14% higher than the initial condition before optimization (Table 3).

Effect of pH and temperature on pectinase activities

Characterization results of the crude pectinase extract showed that the optimal conditions for pectinase activity were at 50 ℃ and an initial pH of 5, with a pectinase activity of 10.42 U ml^{-1} (Figure 4). Pectinase activity was strongly affected by temperature and pH. Pectinase activity increased when pH increased from 4 to 5, then decreased when pH increased to 5.5. Similarly, increasing the temperature intensified pectinase activity up to the optimal temperature, i.e., 50 ℃. However, increasing the temperature above the optimal temperature reduced pectinase activity. The pectinase activity decreased slightly from the initial pH of 5.0 to 5.5 at 50 ℃ but was still higher than the activity at other temperatures.

R. stolonifer A3 produced acidic pectinase, which had optimum activity at pH 5.0, lower than that of *R. oryzae*, which was optimal at pH 6.5 (Chowdhury et al., 2017). Acid pectinase is commonly produced by fungi. According to previous studies, pectinase from *R. oryzae* MTCC 1987 was most active at pH 5.0 and 50 ℃ (Yadav et al., 2012), *Aspergillus fumigatus* was most active at pH 5.0 and 60 ℃ (Okonji et al., 2019) and *Aspergillus* sp. pectinase was most active at pH 4.0 and 50 ℃ (Jaworska et al., 2022). Acid pectinase is commonly used in wine making, pectin extraction, clarification and removal from fruit juices, and in the maceration of fruits and vegetables to create pastes and purées. Due to their high ability to produce enzymes, especially pectinase, filamentous fungi are of great interest (Suhaimi et al., 2021). According to research by Lennartsson et al. (2014), Rhizopus is a widely used as food-processing organism that does not create aflatoxin. Therefore, *R. stolonifer* A3 has the potential to be used as an acidic pectinase producer. However, more research would be needed, including those to test enzyme stability, enzyme kinetics and its application in various bioprocesses. Enzyme production costs are very high, so further research to reduce production

costs needs to be carried out, for example, the use of cheap local substrates and the application of solid-state fermentation for pectinase production.

CONCLUSIONS

A total of 5 fungal isolates were successfully isolated from rotten apple peels. Screening results showed that isolate A3 had the highest pectinolytic activity. Based on 18S rRNA partial gene sequences, isolate A3 was identified as *R. stolonifer*. Microscopically, *R. stolonifer* A3 showed typical characteristics for Rhizopus, characterized by the presence of rhizoids, stolons and sporangiophores bearing a single spherical sporangium. The pectinase production of *R. stolonifer* A3 was optimum at initial pH 5.0, temperature 30 ℃, and incubation period of 3 days with pectinase activity of 14.75 U ml^{-1} and increment of 41.15%. *R. stolonifer* A3 produced acidic pectinase, which had optimum activity at pH 5.0 and temperature 50 ℃. Therefore, *R. stolonifer* A3 has the potential to be used as a producer of acidic pectinase. However, further research is required, including research that tests enzyme stability, enzyme kinetics and the application of *R. stolonifer* A3 in various bioprocesses.

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