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An Extracellular Pectinase from ISH16 Bacteria Isolated Induced by Coffee Pulp Waste Substrate

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Abstract. *An α-1,4-glycosidic bonds galactoses pectin, mainly composed of a D-galacturonic acid chain, are important biomaterial widely used in industries. Utilizing this material, a bioprocess, including the biocatalysis pectinase, is often needed. Pectinase production was optimized in 7 days SSF at 37°C, and the pectinase activities were daily measured by the method of Somogy-Nelson. The optimum pectinase production was 0.166 U/ml on the fourth day SSF. Purification using open column ion exchange chromatography DEAE cellulose DE-52 resulted in 1030.9 folds of pectinase purity with a yield of 25.9%. The enzyme was at optimal activity at pH six and attended stable in the pH range of 5.5-8, while optimal activity at a temperature of 50°C and was stable in the range of 30-45°C. The pectinase activity increased by 120% with the addition of 10 mM Mg^{2+}, and 95% retained when 10 mM Ca^{2+} was added. The presence of 10 mM Na^{+}, K^{+}, and Fe^{2+} resulted in a slight effect of activity at 85%, 83%, and 78%. However, it was strongly inhibited by 10 mM Al^{3+} and retained 25%. Based on the results above, the microbial utilization of coffee pulp waste by ISH16 bacteria pectinolytic is one opportunity to produce valuable pectinase with low-cost production, so comprehensive examination in large-scale production is needed too. In this paper, all research detail steps were described.*

Keywords: *coffee pulp, ISH16 bacteria isolate, pectinase, purification, solid state fermentation*

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INTRODUCTION

Pectinase is one of the biomolecules widely used in various industries, such as tea and coffee fermentation, fruit juice processing, vegetable oil extraction, paper bleaching, alcoholic beverage, food industrial processing, wastewater treatment, et cetera. (Kaur & Gupta, 2017). Pectinase hydrolysis of α-1,4-glycosidic bonds galactoses pectin which is mainly composed of D-galacturonic acid chain to be monomer or oligosaccharides galactoses. Therefore, to suffice this high demand, it is important to produce pectinase enzymes on a large scale at a low cost and environment-friendly.

Solid state fermentation (SSF) is defined as the growth of microbes in wet solid media with deficient moisture or water content (Pandey, 1992). The advantages of SSF

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are low cost and the controlled availability of nutrients. Further, utilizing agro-industrial waste as a substrate in the solid fermentation process provides an alternative route and added value for underutilized or unused waste (Pandey, 2003; Sadh et al., 2018; Šelo et al., 2021).

Indonesia is the 4th largest coffee-producing country after Brazil, Vietnam, and Colombia, with around 685,980 tons per year (International Coffee Organization, 2021). Every 5 tons of wet processing of coffee will produce about 3 tons of coffee pulp by-products (Chanakya and De Alwis, 2004; Fan et al., 2003), and at least 6.5-8% of it is pectin (Murthy and Madhava Naidu, 2012). The pectin content of dry coffee pulp can increase up to 20.5% after being treated with AIS (insoluble solid alcohol) by precipitation of polysaccharides on the coffee pulp with high concentrations of alcohol (Frómeta et al., 2020), which is expected to increase the amount of enzymes released during the production process.

The production of pectinase from using agricultural waste can be carried out by microorganisms such as fungi, yeast, and bacteria (Haile and Kang, 2019; Kaur and Gupta, 2017). An endosymbiotic bacterium ISH16 from the intestine of the coffee berry borer pest *Hypothenemus hampei* Ferr. is potential pectinolytic-producing bacteria that have been previously isolated. Under submerged fermentation in the alkaline extract of the coffee pulp at 10%, the pectinase activity reaches 0.52 U/ml (Azizah, 2019; Wasilah et al., 2020). Utilizing biomass by involving microbes is one of the strategies to effectively reduce costs and be environmentally friendly so that there is an opportunity for further research on producing pectinase from ISH16 bacteria isolate based on coffee pulp waste. In this paper, optimization of pectinase production under SSF, purification, and characterization was reported.

MATERIALS AND METHODS

Maintainance of ISH16 Isolate Preculture for Pectinase Production

The preculture was maintained at optimum conditions, as mentioned in our previous research (Wasilah et al., 2020). A nutrient agar broth medium was used, and the cell density reached 10^8/ml after being shaken at 100 rpm, 24 hours incubation at 30°C. This culture was used as an inoculum in SSF for pectinase production.

Solid State Fermentation and Pectinase Production

Two media, coffee pulp powder, and alcohol insoluble solid (AIS) of coffee pulp were used for the optimization of pectinase production. Five grams of each medium was autoclaved at 121°C and 1.5 atm for 25 minutes, inoculated with 1 ml of 10^8/ml of ISH16 in a 250 ml Erlenmeyer flask at 37°C. The AIS medium is prepared by following the steps. Ten grams of coffee pulp powder was suspended in boiled water 15 ml for 15 minutes, homogenized, and 96% ethanol was added with a ratio of 1:2=coffee pulp suspension: ethanol. Then, AIS was obtained by 70% ethanol extraction at 40°C for 30 minutes in a filter paper, re-washed three times in ethanol 96% and once in absolute acetone. The precipitate was dried in an oven at ±50°C for 3 days (Apolinar-Valiente et al., 2010; De Vries et al., 1981). Solid state fermentation for pectinase production was monitored for a period of 1-7 days by measuring the pectinase activity against 1% pectin substrate in 20 mM acetate buffer pH5. Optimizing water content during SFF in pectinase production is also carried out using the same previous research method (Wasilah et al., 2020).

Harvesting of Crude Pectinase

The crude pectinase in cultures was extracted daily by adding distilled water containing 0,01% NaN3 and 1% NaCl with ratio culture: solution (1:2) and then shaken at 120rpm for 12 hours. The suspension was filtered and centrifugated at 8000 rpm for 5 minutes, and the supernatant containing crude pectinase was kept at 4°C for further analysis (Dewi and Muzakhar, 2018; Khofiya et al., 2019; Ubaidillah & Muzakhar, 2019).

Pectinase Activity Assay

Pectinase activity was employed by the method of Somogy Nelson (Nelson, 1944) with a few modifications (Roy et al., 2018; Ubaidillah and Muzakhar, 2019). The reaction mixture of 500µl pectin substrate 1% and 100 µl crude pectinase containing 20 mM acetate buffer at pH 5 of was incubated at 37°C for 2 hours. Then, 500 µl Somogyi reagent was added to stop the reaction, boiled for 15min., and cooled. An amount of 500 µl Nelson reagent and 2.5 ml distilled water were added. The reducing sugar was separated from the remaining substrates by centrifugation at 8000 rpm for 10 minutes and then measured at absorbance 500 nm.

Pectinase Purification Steps

Purification was done by removing the debris (sugars and soluble remaining substrates) from the crude enzyme through dialysis in 10 Kda and 50 Kda cellulose membrane tubes against 20 mM acetate buffer pH5. The dialyzed crude pectinase was precipitated using saturated ammonium sulfate 40%-90% in 20 mM acetate buffer at pH 5. The pellets were recovered and suspended with the same buffer, followed by dialysis to remove the remaining ammonium sulfate using 10Kda and 50 Kda. The dialyzed pectinase was purified under an open column using DEAE Cellulose DE-52 anion exchange chromatography with the gradient stepwise 0.05 M NaCl in the same buffer, from 0 M to 0.5 M NaCl. Each fraction was assayed for pectinase activity, and all active fractions were pooled to quantify the yields and total pectinase-specific activity.

Optimum Temperature and pH of Purified Pectinase Activity

The optimum temperature and pH of purified pectinase were analyzed under temperatures 30-70°C and pH 3-8, respectively. For analysis at pH 3 to 5 and pH 6 to 8, 50 mM acetate and 50 mM phosphate buffers were used. The pectinase activity was quantified with the same procedure using the Somogy-Nelson method.

Temperature and pH Stability of Purified Pectinase Activity

The stability of purified pectinase on temperature and pH were analyzed after the enzyme exposure at temperature 30 to 70°C and pH 3-8 for 4 hours. The remaining activity was assayed using Somogy-Nelson method as described above.

Effect of Metals on Pectinase Activity

The purified pectinase activity was tested on 10 mM metal ions Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, and Al^{3+} in 20 mM acetate buffer pH 5. An amount of 100 µl of pectinase enzyme was incubated containing 100 µl of 10 mM metal ions for 1 hour at 37°C. An amount of 500 µl of 1% pectin substrate in 20 mM acetate buffer pH 5 was added to the sample and incubated at 37°C for 2 hours. The activity of the pectinase was further tested using the same Somogyi-Nelson method.

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RESULTS AND DISCUSSION

Optimum Pectinase Production

The optimization of pectinase production by ISH16 isolate has been carried out using two kinds of coffee-pulp-based substrates (Figure 1). ISH16 could produce and release pectinase when grown in coffee pulp without either AIS coffee-pulp medium. The pectinase production was initially detected on day 2 SFF in all media used, and the pectinase activity continued increased maximum at day four incubation. Based on Figure 1, the maximum pectinase activity in coffee pulp media was 0.14 U/ml. In contrast, the AIS coffee pulp media was only 0.106 U/ml. A similar result was when *Bacillus subtilis* SAV-21 inoculated in a mixture of orange peel and coconut fiber medium in a ratio of 4:1 could produce optimum pectinase in 4 days SSF (Kaur and Gupta, 2017). Further, *Bacillus cereus* secreted pectinase in the solid fermentation using orange peel and wheat bran (Gophane et al., 2016). The investigation found a significant decrease of pectinase activity at the 5th to 7th-day SSF by ISH16. It would be due to the depletion of nutrients in the coffee pulp substrate required for bacterial growth and reproduction as well as the accumulation of toxic by-products (Aslam et al., 2020). The pectinase activity in the AIS coffee pulp medium used was lower than that of the coffee pulp without the AIS treatment. This may be because AIS coffee pulp medium was low in the form of simple oligosaccharides pectic, so that may be difficult for ISH16 to use this complex pectin as a carbon source for their growth. As a result, the pectinase enzyme produced was also less when compared with the coffee pulp medium without AIS treatment.

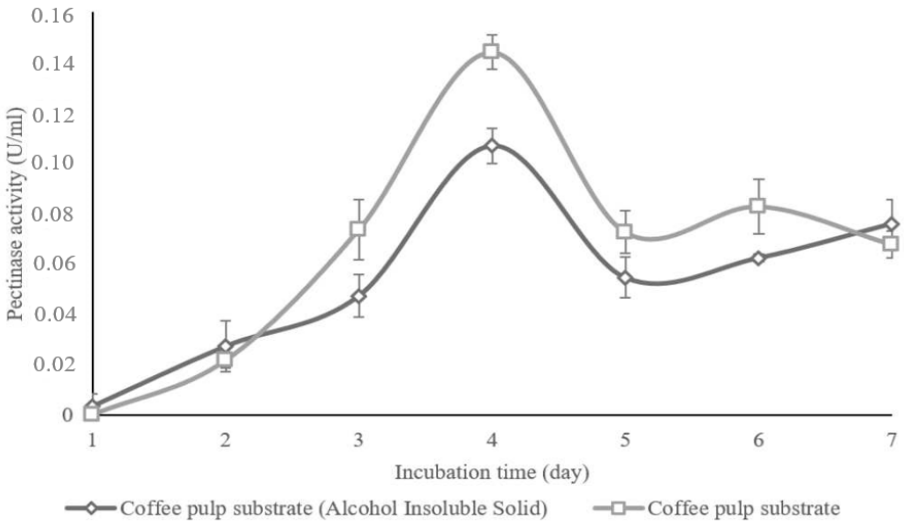

Figure 1. Cross section of the cerebellum of mice exposed to permot leaf extract at a dose of 4000 ppm (x450, HE) showing an increase in white matter thickness (WM) compared to the control group, a nucleus of the Purkinje cell changes shape to become flat and even damaged (PC, arrows), without typical changes in the granular architecture of the cell layer (GL) and molecular layer (ML)

The nature of the solid substrate is one of the essential factors in solid fermentation because the substrate not only provides nutrients for the microbial culture growing on it but also serves as a shelter for the growth of only microbial cells (Kaur and Gupta, 2017). It is necessary to select a solid substrate for optimal production. Therefore, the next production of pectinase uses a coffee pulp medium (without AIS treatment) because it is considered more efficient, economical, and environmentally friendly. The research, as reported by Frómeta et al. (2020), stated that the pectin content in the coffee pulp increased by 20.5% after being treated with AIS. Another critical factor to consider in the SSF process is the sufficient water content in the medium (Sella et al., 2009). The results proved that the optimum pectinase activity of ISH16 was 0.166 U/ml when SSF was done at 90% water content in the substrate (Figure 2). ISH16 isolates are motile bacteria, so they need sufficient water for their growth. Similar results occurred in *Bacillus* sp. MG-cp-2 produces optimal pectinase activity on wheat bran and flax fiber substrates with 90% water content (Kapoor et al., 2000).

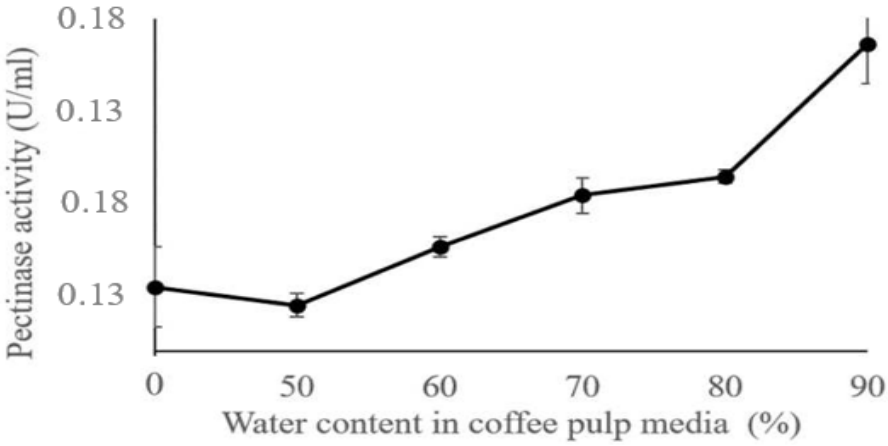

Figure 2. Optimization for pectinase production in 4 days at 37°C with various water content (40-90% moisture content in coffee pulp solid fermentation media.

Purification of Pectinase

The first step of purification was done by ammonium sulfate precipitation. As shown in Figure 3, the optimum to precipitate crude pectinase was obtained at 40% ammonium sulfate saturation with pectinase activity of 0.21 U/ml. However, when the ammonium sulfate saturation of 50% to 90% was used, the pectinase activity tended to decrease, and at 90% saturation, the activity was sharply decreased to 0.058 U/ml. This is thought to occur because the non-target proteins and impurities present in the crude extract enzyme are completely precipitated, thereby disrupting pectinase activity. In addition, a high percentage of ammonium sulfate saturation may be caused pectinase to be denatured (Duong-Ly and Gabelli, 2014; Li et al., 2014).

The precipitated crude pectinase was dissolved and dialyzed in a cellulose membrane tube against 20 mM acetate buffer at pH 5 to remove the remaining ammonium sulfate. As shown in Figure 4, When 50 kDa of a cellulose membrane tube was used, the total pectinase activity was lost 75% and the specific activity 0.05 U/ml only. This indicates

that almost all pectinase enzymes diffuse out of the 50 kDa dialysis membrane tube. In contrast, when a 10 kDa cellulose dialysis membrane tube was used, pectinase activity of 0.2 U/ml while 91% of its activity remained. This evidence proved that this pectinase has a molecular weight of between 10-50 kDa. Similar results found below 50 kDa that pectinase from *Bacillus bataviensis, Bacillus megaterium,* and *Paenibacillus* sp. were 32.4, 25.1, and 29.6 kDa (Akinyemi et al., 2017).

Further purification using DEAE Cellulose DE-52 anion exchange chromatography as shown in Figure 5, was found that the pectinase eluted at a concentration of 0.1-0.15M NaCl with the highest activity of 0.12 U/ml. As the NaCl concentration increases, Na^+ or Cl^- ions compete with the protein for binding to the charge on the surface of the DEAE matrix, and the protein will be released and eluted to the bottom of the column. The peak that was formed after the highest peak with an average activity of 0.04 U/ml was probably due to the remaining pectinase not being eluted at 0.1-0.15 M NaCl concentration. This may be because the molecules in the DEAE matrix are not only pectinase but also proteins and other impurities so the ions between pectinase and the DEAE matrix are slightly disturbed, as well during the elution process of pectinase by NaCl. Fractions number 1 and 14 showed a high protein absorbance value of 280 nm, which was 2.9 and 1.4, respectively, but had low pectinase activity, which was 0.01 and 0.028 U/ml, respectively. It was developed that the eluted protein was a non-target protein or an impurity substance (Nooralabettu, 2014). As shown in Table 1, the specific activity of pectinase and purity multiples increased at each step of purification. A significant increase in purity occurred after the pectinase enzyme was finally purified by DEAE-Cellulose DE-52 anion exchange chromatography. The purity increased to 1030.9 times, and the specific activity reached 0.49 U/mg with a yield of 25.9%. This significant increase occurred because the protein eluted in the DEAE cellulose anion exchange chromatography process DE-52 was pure pectinase with little or no other impurities. This also shows that the pectinase produced in the form of crude extract still contains a lot of protein and other impurities so a purification process is needed to separate pectinase from the protein and impurities so that the pectinase activity is not disturbed and can be maximally expressed (Reddy, 2007).

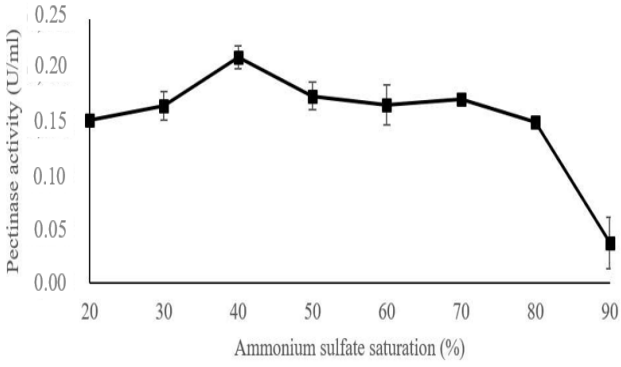

Figure 3. Optimization of ammonium sulfate saturation in crude pectinase

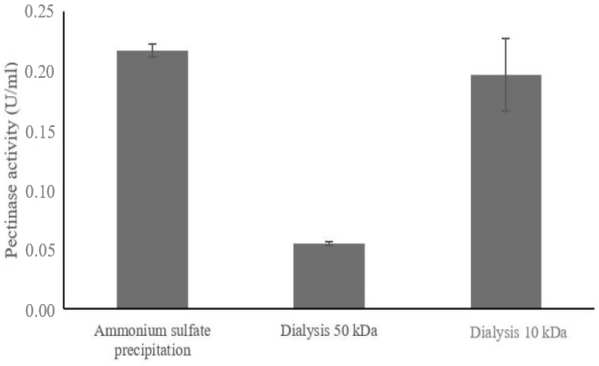

Figure 4. Comparison of pectinase activity after ammonium sulfate precipitation, dialysis at 50 kDa, and 10 kDa membrane dialysis tube

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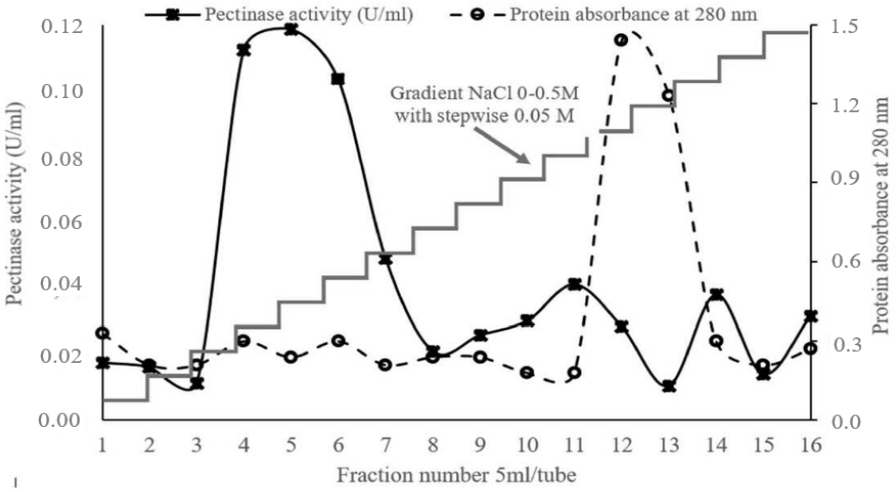

Figure 5. Purification profile of pectinase under an open column using DEAE Cellulose DE-52 with the gradient 0-0.5M NaCl and stepwise 0.05M.

Table 1. Pectinase purification steps

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude pectinase	1.15	2386.4	0.00048	100.0	1.00
$(NH_4)_2SO_4$ Precipitation	0. 94	569.6	0.00164	81.5	3.41
Dialysis	0.99	367.5	0.00268	85.8	5.57
DEAE cellulose DE-52	0.30	0.6	0.49583	25.9	1030.94

Characterization of Purified Pectinase

The catalytic activity of enzymes depends on pH, temperature, and cofactors. The pH conditions can affect enzyme activity in different ways, namely by ionization of groups at the active site of enzymes or ionization of groups in substrates and by affecting the conformation of enzymes or substrates. As shown in Figure 6, the purified pectinase of ISH16 isolate bacteria showed optimal activity at pH 6 (0.168 U/ml). It was also similarly found in the pectinase activity of *Bacillus firmus* and *Bacillus* sp. FW2 is isolated from soil optimum at pH 6 (Kaur et al., 2016). Further, ISH16 isolate bacterial pectinase was very stable in the pH range of 5.5-8, and the remaining relative activity was nearly 86% after the enzyme was incubated for 4 hours at 37°C. The pectinase showed instability in the pH range of 3.5-5, with relative activity values below 60%. The reduction in pectinase activity in this pH range may occur due to the breakdown of ionic bonds in the presence of a higher concentration of H^+ ions, thus affecting the functional form of the active site and resulting in lower yield products (Bisswanger, 2014). According to Oumer & Abate (2017), pectinase with this pH range has the potential to be applied in the alkaline pectin degradation process in coffee processing, paper, and pulp industries, as well as pectin wastewater treatment.

ISH16 isolate bacterial pectinase reached optimal activity (0.156 U/ml) at 50°C with an incubation time of 2 hours. The bacterial pectinase ISH16 isolate was stable in

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a temperature range of 30-45°C because its relative activity could be retained up to 80% after incubation for 4 hours at according temperatures (Figure 7). However, the pectinase activity showed instability at the temperature over 50°C. This is because pectinase undergoes denaturation due to interference with hydrogen bonds, ionic bonds, and other weak bond interactions that stabilize the active form of enzymes and protein molecules so that in the end, the enzyme will be denatured (Urry et al., 2017). The effect of temperature on enzyme activity is similar in several respects to the effect of pH, i.e., activity increases with increasing temperature, past a maximum point, followed by a decrease. Enzyme temperature profiles arise from two different processes, namely: increased activity with increasing temperature (as found in all chemical reactions) and inactivation at higher temperature conditions due to protein denaturation (Reddy, 2007). This condition can easily arise if a certain time is required to prepare and start the test, while the enzyme is already present in the thermostat test mixture. During this time, denaturation has taken place, and because the preparation time is not always the same, the loss of enzyme activity will also vary (Bisswanger, 2014).

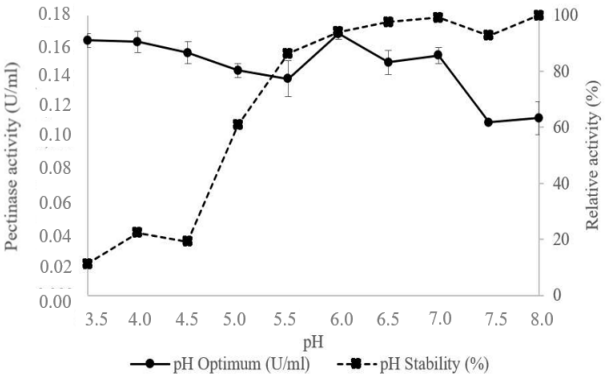

Figure 6. The effect of pH on optimum activity and stability of purified pectinase

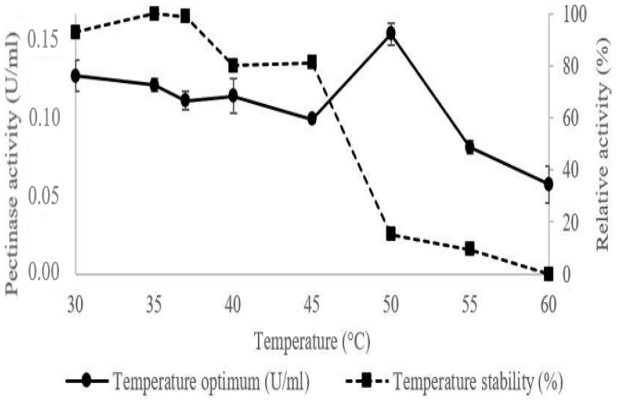

Figure 7. The effect of temperature on optimum activity and stability of purified pectinase

Metals Ions Effect on Pectinase Activity

Pectinase produced by bacteria ISH16 isolate increased activity by adding metal ion Mg^{2+} 10 mM (125.9%), and activity was nearly stable when the ions Ca^{2+}, Na^{+}, K^{+}, and Fe^{2+} with relative activities of 97.4%, 85.4%, 81.3%, and 76.6%. However, its activity was significantly inhibited by the addition of Al^{3+} metal ions with only 22.9% of pectinase activity remaining. This indicates that metal ions such as Mg^{2+} play an important role in maintaining the active conformation of pectinase to stimulate enzyme activity (Oumer and Abate, 2017). The results are the same as the increase in pectinase activity by Mg^{2+} ions, namely *Bacillus subtilis* isolated from kimchi (traditional Korean food) (Rahman et al., 2020), *Bacillus subtilis* strain Btk 27 (Oumer and Abate, 2017), and *Paenibacillus lactis* isolated from the coast (Sheladiya et al., 2022). In contrast, different results were reported that the pectinase activity of *Bacillus subtilis* isolated from the sea was inhibited by 10 mM Mg^{2+} ions, and the pectinase was increased when Fe^{2+} ions were added (Joshi et al. 2015). Different results were also found in *Penicillium chrysogenum* pectinase activity, which was inhibited by 5 mM Mg^{2+} ions and increased by Ca^{2+} ions (Banu et al., 2010). Na^{+} and K^{+} ions increase the pectinase activity of

Bacillus bataviensis, *Bacillus megaterium*, and *Paenibacillus* sp. isolated from the waters of the Lagos Lagoon polluted by industrial waste, while the Mn^{2+} and Zn^{2+} ions inhibited their activity (Akinyemi et al., 2017).

This difference in metal ion preference indicates that each enzyme may have different flexibility in its active site from other enzymes (Oumer and Abate, 2017). Metal ions can act as cofactors or inhibitors. These results indicate that enzymes from different sources require various metal ions at certain concentrations to carry out the activity. In addition, the evolution of enzymes also determines the need for metal ions at the active site because microorganisms evolve according to the surrounding environment to survive and grow well so they tend to modify their metabolic pathways or enzymes (Sheladiya et al., 2022).

CONCLUSION

Potential extracellular pectinase from endosymbiotic ISH16 bacteria could be optimum produced at four days under SSF using coffee pulp substrate. Purification yielded 25.9%, and the pectinase purity increased up to 1030.9 times, so this pectinase can have the opportunity to be applied in industry.

AUTHOR CONTRIBUTION

Besides as the corresponding author, K. M. participated in the research design, manuscript, and revision of article. All authors analyzed, drafted, wrote, read, and approved the final manuscript.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

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