

Cellulase, Pectinase, and Xylanase Production by *Listeria* sp. ISH 16 using Coffee Pulp Waste Medium

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Abstract—In this study, microbial utilization of coffee pulp waste to produce three carbohydrases (cellulase, pectinase, and xylanase) has been investigated. An isolate *Listeria* sp. ISH-16 which grown in liquid medium containing 0.5% coffee pulp alkali extract can grow well with density of reach $10^7 \times 58.5$ cell/mL. Further, the culture can optimum produced of pectinase after 72 hours incubation at 37°C, but for cellulase and xylanase longer 84 hours incubation needed. The harvested of crude pectinase, cellulase and xylanase had activity 0.15, 0.23, and 0.61 unit/ ml after dialysis, respectively. All enzymes stable at a range of pH 4-5.5, but they work optimum at different pH. Of each enzyme pectinase, cellulase, and xylanase work optimum at pH 5, 4.5, and 5.5. based on these results, it may be adopted as a strategy to produce enzymes with low cost so that further investigation such as large scale and purification enzyme is needed.

Keywords—Carbohydrase, Cellulase, Coffee Pulp, Pectinase, Xylanase.

I. INTRODUCTION

THE use of enzymes in industries such as food, textile, detergent, agriculture, chemicals, and pharmaceuticals is increasing rapidly because of low cost, fast process, nontoxic and eco-friendly characteristics. According to industrial enzyme market global market demand for industrial purposes, estimated about USD 5.9 billion in 2020 and projected to reach USD 8.7 billion by 2026 and is expected to expand at a compound annual growth rate (CAGR) of approximately 6.5% over the period from 2020 to 2026 [1]. Further, the microbial enzyme is preferred, since this simplifies downstream processing, hence further lowering costs, so that the selection of substrate is important to produce an enzyme easily and at a low-cost [2]. The utilization of coffee pulp biomass can be an alternative.

It was known that coffee pulp waste is potential biomass which the main polysaccharide components are 57.9% cellulose, 21, 63% hemicellulose, 5.21% lignin, and 2.28% pectin, 12.4% reducing sugar and 2% non-reducing sugar [3-4]. These polysaccharides can be used as a bacterial growth media for enzyme production. Previous studies reported that coffee pulp can be used as a substrate to produce α -amylase from *Bacillus amyloliquefaciens* yielding 550 U/g after 120 hours of incubation [5]. According to Ubaidillah *et al*, the coffee pulp is a good material for *Pestalotiosis* sp. VM9 and *Aspergillus* sp. VTM5 to produce sugars which can be used as substrate for single cell production [6].

It was reported that carbohydrase group is the most enzyme frequently used in food industry [7]. More over, predicted in period of 2020-2026 the demand of the enzyme will increase significantly so that some efforts and low cost or cost-effectiveness strategy must be highly considered [1]. The utilization of a huge source coffee pulp waste is may one important thing to reduce cost production. In the other hand, to discover a new-sources of carbohydrases, investigation is needed.

In this paper, the microbial utilization of coffee pulp to produce carbohydrase (cellulase, pectinase and xylanase) using cellulolytic bacteria *Listeria* sp [8-9].

II. METHOD

A. Preculture and Optimum Growth Analysis of *Listeria* Sp. ISH 16 in Coffee Pulp Alkali Extract Medium

The *Listeria* sp. ISH 16 was obtained from previous research, grown in nutrient agar containing 0.5% coffee pulp alkali extract (CPAE) without any nutrient added as preculture [8, 10]. CPAE was prepared from 100 grams powdered coffee pulp suspended to distillate water containing 1 M NaOH. The suspension was stirred for 24 hours at 25°C. The filtrate was obtained by filtering on buchner using filter paper. The filtrate was adjusted to pH 7 using acetic acid. The filtrate was made to 60% in ethanol and the precipitate as CPAE was obtained after centrifugation at 10.000 rpm for 10 minutes. This pellet dried at 55°C and was used for *Listeria* sp. ISH 16 medium to growth and produce carbohydrase. To analyse optimum growth of *Listeria* sp. ISH 16, the examination was done by culturing of preculture to broth medium containing 0.5% CPAE. for this step, one ose was inoculated to medium. Then the culture incubated at 25°C on a shaker with speed 120 rpm, and growth of cell density (cell/mL) monitored and calculated periodically every 24 hours.

B. Cellulase, Pectinase and Xylanase Production using CPAE based Broth Medium

In this step, time of incubation for carbohydrase (cellulase, pectinase and xylanase) production based on broth CPAE medium must be first optimized. For this purpose, 45 mL broth CPAE 0.5% was inoculated with 5 mL *Listeria* sp. ISH 16 (58.5×10^7 cell/mL), incubated for 7 days at 25°C, and shaken 120 rpm. And then, optimum enzymes production was measured periodically every 12 hours base on enzyme

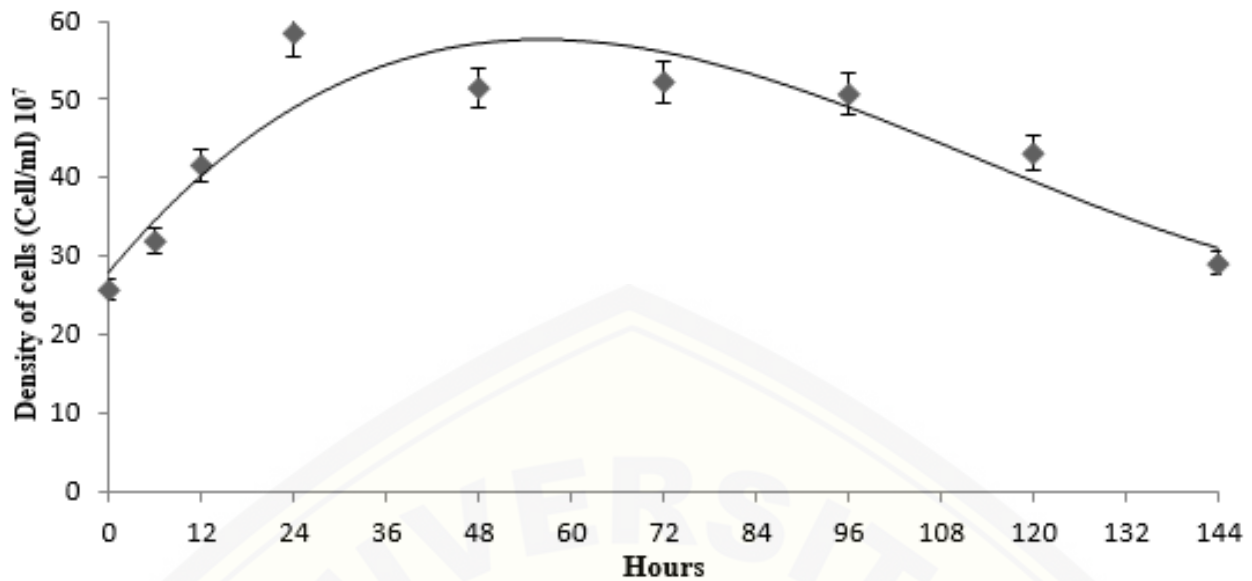


Figure 1. The growth curve of *Listeria* sp. ISH-16.

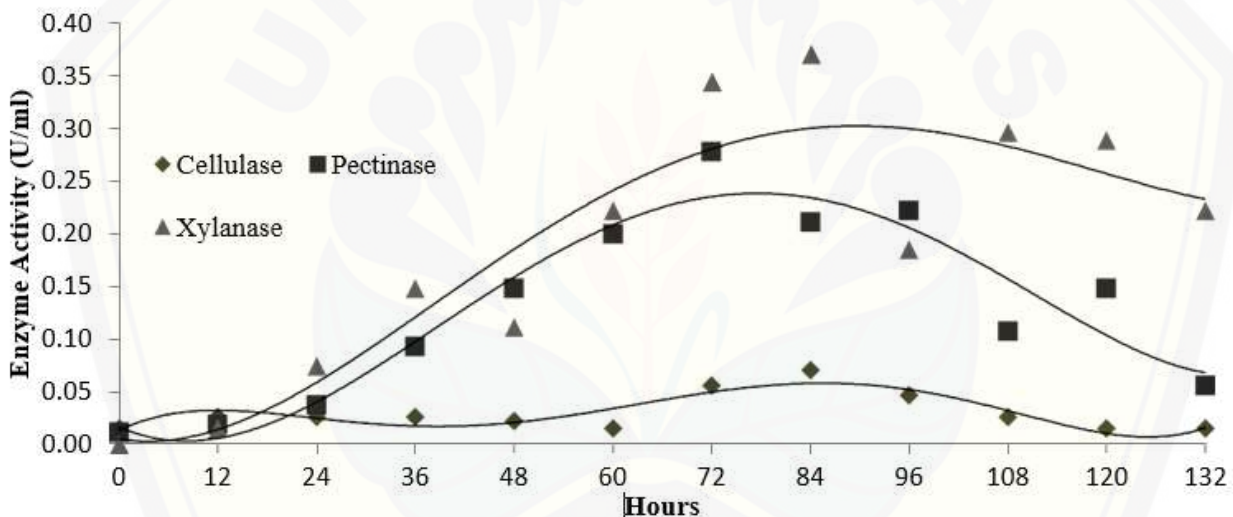


Figure 2. Optimization of the production of cellulase, pectinase, and xylanase by *Listeria* sp. ISH-16.

activity. For measuring the activities of enzymes, the reducing sugar produced as were quantified described by Somogy-Nelson method [11].

The enzymes activity was assayed in 1% substrate containing acetate buffer 20 mM of pH 5. One hundred microliter of enzyme was added and incubated at 37°C for 2 hours enzymes reaction was stopped by adding 500 μ L somogy and than boiled for 15 minutes. After cooling at room temperature, 500 μ L Nelson solution and 2.5 mL H₂O were added, centrifuged at 8000 rpm for 10 minutes. And then, the reducing sugar content of supernatant was measured at absorbance 500 nm compared with control.

C. Stability and Optimum pH

Before examine the stability and optimum pH the crude enzyme was dialysed in 20 mM acetate buffer pH 5. This step was done on cellulose dialysis membrane tube 14 kDa for 24 hours at 4°C. The buffer was changed every 12 hours.

Optimum and stability pH of cellulase, pectinase and xylanase on activities were determined at a range pH 3 to 8

using 20 mM acetate and phosphate buffer. Acetate buffer at a range pH 3 to 5.5 and phosphate buffer 6 to 8 were used. The optimum pH of enzymes activity analysis was assayed by measuring of sugar reducing as describe above. At concentration 1% of carboxymethyl cellulose (CMC), citrus pectin and xylan substrate on 20 mM buffer were used for assaying of cellulase, pectinase and xylanase activities.

III. RESULT AND DISCUSSION

A. Optimum Growth Analysis of *Listeria* sp. ISH 16 in Coffee Pulp Alkali Extract Medium

The investigation showed that an isolate *Listeria* sp. can grow on broth medium containing 0.5% coffee alkaline extract. The growth of microorganisms depends on the source of nutrients in the form of carbon and nitrogen, founded in the environments [12]. Shown at Figure 1, an exponential growth starting from the 12 to 24 hours incubation was achieved by *Listeria* sp. The increasing growth with density reached up to

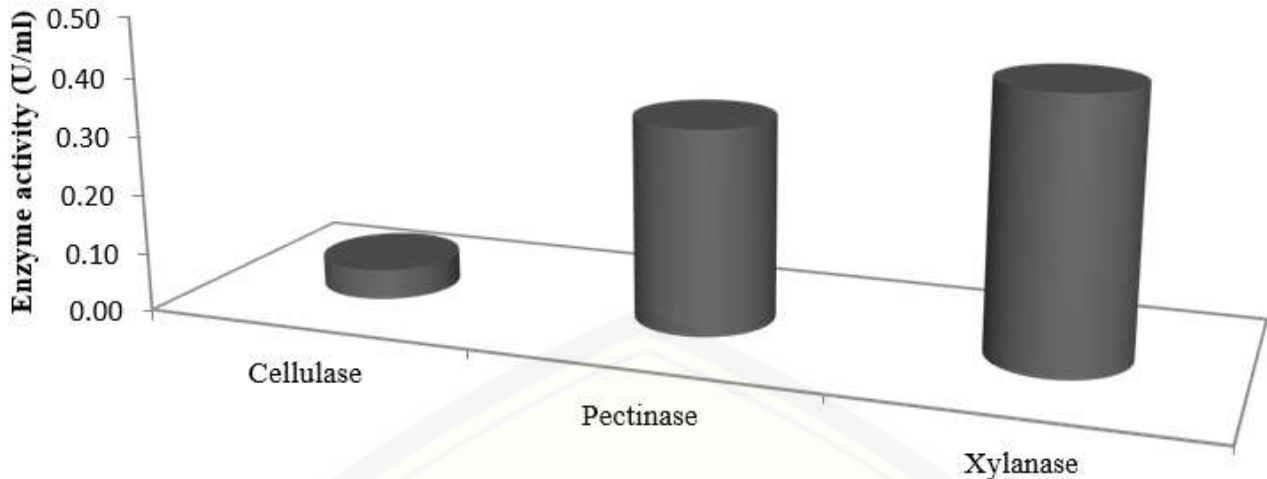


Figure 3. Enzyme activity after 72 hours incubation.

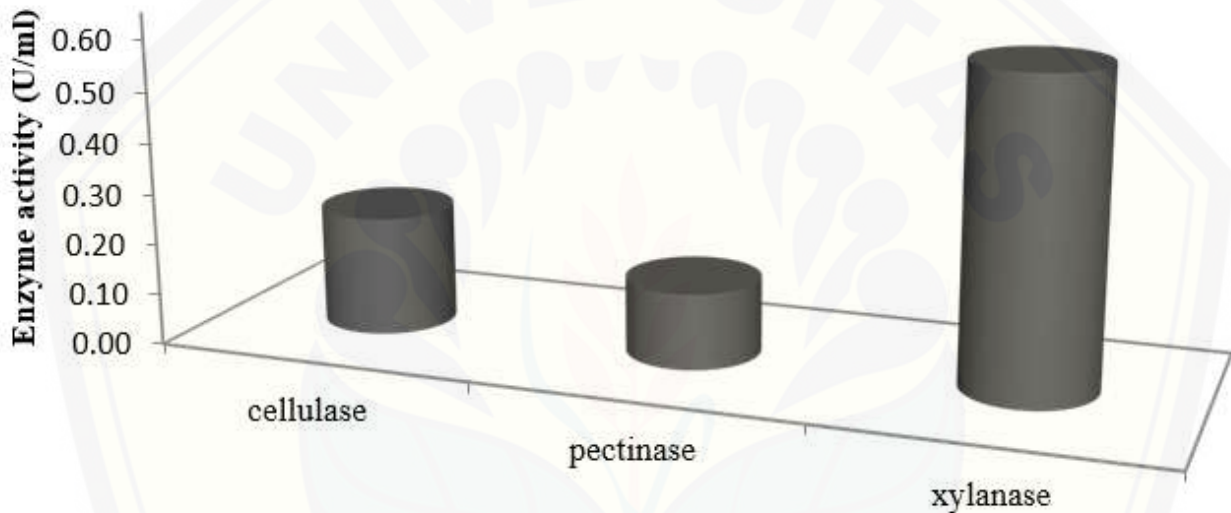


Figure 4. Enzyme activity of cellulase, pectinase and xylanase after dialysis.

$10^7 \times 58.5$ cell/mL proved that this isolate able to utilize carbon and nitrogen source from coffee pulp waste without any nutrient added. Stationary phase was seen at 48 to the 96 hours and growth decreased after 120 to 144 hours (Figure 1). In this research it has been found that the best growth is at 24-hour incubation, where an exponential growth phase occurs. This incubation period is used to determine the amount of inoculum needed in enzyme production.

B. Optimization of The Production of Cellulase, Pectinase, and Xylanase by *Listeria sp.* ISH 16

Optimization of enzyme production is carried out by cultivating microorganisms in liquid nutrient broth. This involves growing carefully selected microorganisms in closed vessels that contain containing a high concentration of oxygen and a rich of nutrients. As the microorganisms break down the nutrients, they release the enzymes into a solution [13]. When an organism grows in liquid media, it utilizes the components of the medium and excreted by-products of bacterial metabolism into the medium such as various enzymes [14]. This technique was best for microorganisms such as bacteria which required high humidity and was easier product purification [15]. Many industrial enzymes can be

produced using this process. According to Manpreet, bacteria would grow better in media had high water content [16]. This was related to the growth of bacteria on the surface while the fungus could penetrate the substrate using mycelium. Thus, it would be more suitable to be grown on solid media.

The method used to measure reducing sugars in enzyme activity was using the Somogy-Nelson method. The principle of the Somogy-Nelson method was that reducing sugars would reduce Cu^{2+} into Cu^{+} , Cu^{+} would reduce arsenomolybdate compounds to form a greenish-blue color [11]. The incubation time affected the number of isolates ISH- 16 on the alkaline media of coffee pulp to produce crude enzymes. Enzyme activity produced by a microorganism was affected by the incubation time depending upon the type of microorganism and other culture conditions such as inoculum size, pH, and temperature [17-18]. Other studies mention that fermentation time has a profound effect on the formation of microbial products [19]. The optimum time for enzyme production was used as the time for large-scale enzyme production.

Optimization of enzyme crude production was conducted to find out optimum incubation duration to produce cellulase,

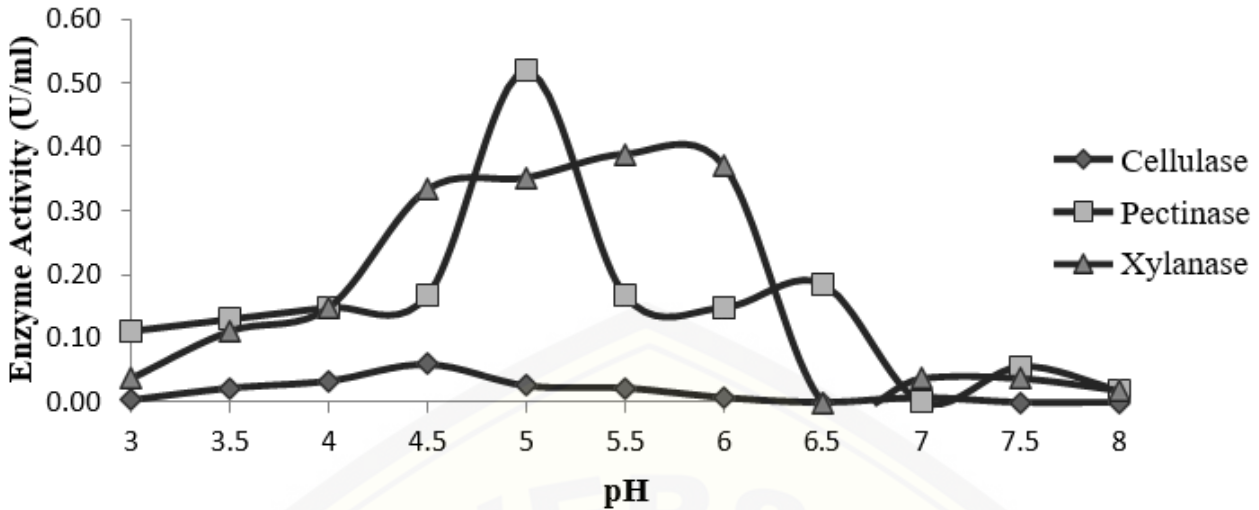


Figure 5. Enzyme activity of optimum pH.

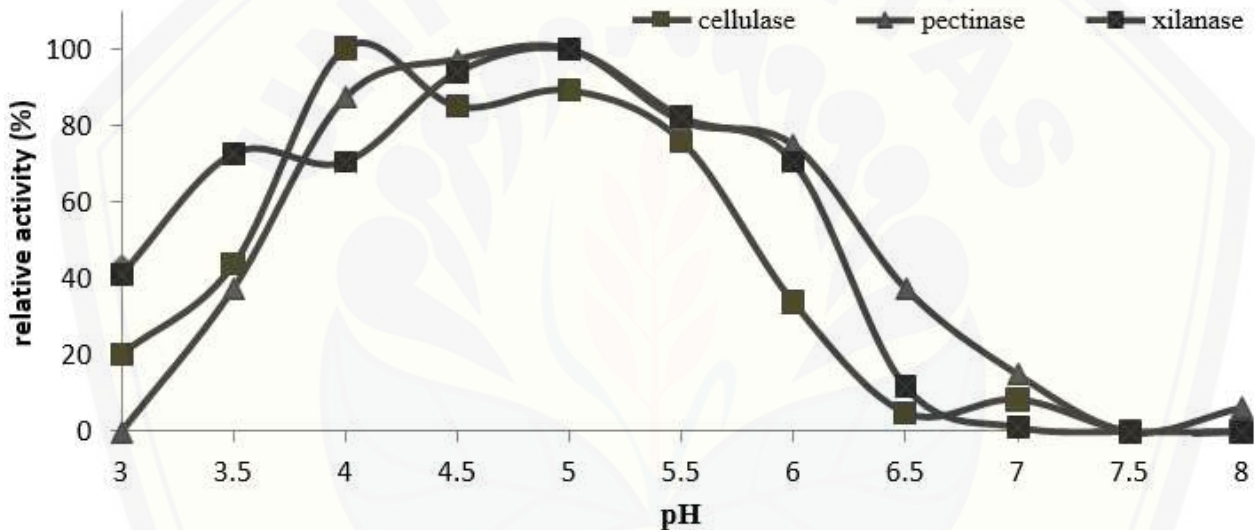


Figure 6. Relative activity of pH stability.

pectinase, and xylanase enzymes. The optimum incubation duration for each enzyme had a different time (Figure 2). In this research, an isolated ISH-16 could produce cellulase with optimum value at 84 hours with the highest activity of

0.07 U/mL. According to Sirisena and Manamendra, *Listeria* sp. could produce cellulase with a concentration of 1.8 mmol/l using cellulose substrate and 1.5 mmol/l with CMC substrate [9]. Crude enzyme activity produced by *Listeria* sp. was considered high if compared to other species. According to Asem, *A. aneurinilyticus* exhibited cellulase activity (FPase) 0.62 U/mL at 120 hours, while *Aneurinibacillus* sp. and *Serratia rubidaea*, which had activities of 0.53 and 0.5 U/mL at 48 and 96 hours, respectively [20].

The optimum pectinase incubation duration was 72 hours with the highest activity 0,28 U/mL. Other research mentions that *Bacillus circulans* could produce total activity 256 U/mL pectinase by Submerged fermentation method using galactose substrate [21]. The optimum incubation duration for xylanase was 84 hours with 0.37 U/ml activity. There was no research on *Listeria* sp. to produce the enzymes pectinase

and xylanase. If it was compared to the research conducted by Asem using *S. Rubidaea*, *Aneurinibacillus aneurinilyticus*, and *Bacillus aureus* has xylanase activity of 6.15 U/mL at 72 hours, 4.12 U/mL at 96 hours and 3.05 U/ml at 48 hours, respectively [20].

C. Large Scale Production

Large scale production is obtained from optimization tests that have been done before. Large scale production uses the most optimum activity and with a relatively short time of incubation at 72 hours (Figure 3). Crude enzymes showed that the xylanase activity was higher than the others (0.44 U/mL with specific activity of 0.011 U/mg), followed by pectinase (0.34 U/mL with specific activity of 0.009 U/mg) and cellulase (0.05 U/mL) with specific activity of 0.0013 U/mg, respectively (Table 3). The results of large-scale production indicate that enzyme activity has increased compared to optimization.

D. Dialysis

The enzyme activity that has been dialyzed has increased compared to a crude enzyme. During the process of dialysis

Table 1.

Comparison of cellulase enzyme activity at various stages of purification								
Purification steps	Volume	Activity (U/mL)	Total (U/mL)	Activity Abs 280 nm	Total Abs. 280	Specific Activity/Abs. nm (U/mg)	280Yield (%)	Purification Fold
Crude enzyme	473	0,05	23,65	0,193	91,29	0,0013	100	1,00
Dialysis	207	0,23	47,61	0,14	28,98	0,0082	201,31	6,34

Table 2.

Comparison of pectinase enzyme activity at various stages of purification								
Purification Steps	Volume	Activity (U/ML)	Total (U/ML)	Activity Abs 280 nm	Total Abs. 280	Specific Activity/Abs. 280 nm (U/mg)	Yield (%)	Purification Fold
Crude enzyme	473	0,34	160,82	0,193	91	0,009	100	1,00
Dialysis	207	0,15	31,05	0,14	28,98	0,005	19,3	0,61

Table 3.

Comparison of xylanase enzyme activity at various stages of purification								
Purification steps	Volume	Activity (U/mL)	Total (U/mL)	Activity Abs 280 nm	Total Abs. 280	Specific Activity/Abs. 280 nm (U/mg)	Yield (%)	Purification Fold
Crude enzyme	473	0,44	208,12	0,193	91	0,011	100	1,00
Dialysis	207	0,61	126,27	0,14	28,98	0,022	60,67	1,91

in Figure 4, the unwanted protein in the dialysis tubing moves from high concentration to low concentration. As the volume of the solution in the dialysis tubing decreases, the enzyme activity is increased [22]. Cellulase activity was 0.23 U/mL with specific activity 0.0082 U/mg and purification fold 6.34 (Table 1). Pectinase activity was 0.15 U/mL with a specific activity of 0,005 U/mg and purification fold 0.61, respectively (Table 2). While xylanase activity was 0.61 U/mL with a specific activity 0.022 U/mg and purification fold 1.91 (Table 3). Dialysis can remove contaminants with lower molecular weight than enzyme molecules. The principle of dialysis is the transfer of smaller molecules by passing through a semi-permeable membrane by diffusion [23]. Sample molecules larger than the membrane pores will be retained on the sample side of the membrane, but small molecules and buffer pass freely through the membrane, reducing the concentration of these molecules in the sample [24]. Recent reports mention that *Listeria* sp. cellulolytic bacteria that are incubated in 72 hours could produce cellulase enzymes with an enzyme activity of 0.078 U/ml using CMC substrate [8]. There was no research on *Listeria* sp. to produce the enzymes pectinase and xylanase. when compared with the Navasivayam study using *Bacillus cereus*, Maximum pectinase enzyme production (44 U/mL) [25]. *B. cereus* produces a pectinase higher than this study because the purification used by the research is more complexly using ammonium sulfate precipitation and dialysis. Ho and Heng used *Bacillus* sp. bacteria and barley media husk enzyme activity produced were 4.947 U/mL at 48 hours incubation [26]. This proves that *Bacillus* sp. can produce xylanase enzymes higher than *Listeria* sp. ISH-16 using Barley husk media. A comparison of enzyme activities at each stage is presented in tables 1, 2, and 3.

E. Characterization (Stability and Optimum pH)

Figure 5 depicts the effect of different pH on the production of enzymes target by isolate ISH-16. The maximum production of cellulase was obtained at pH 4.5 with activity 0.06 U/ml and stable more than 70% at pH 4.0-5.5 (Figure 6). These findings were similarly reported by

Yang *et al*, where a *Bacillus subtilis* isolate from Tibetan pig's intestine were exhibited when at pH 4 CMCase activity showed a minimum activity of 0.5 U/mL while at pH 5.5 maximum activity is obtained 2.41 U/mL [27]. Vatanparast *et al*, reported that optimum pH for cellulase activity from the larval midgut of *R. ferrugineus* was obtained at pH 5.0 and 6.0 [28]. The best pH for the pectinase producers was pH 5 with activity 0.52 U/mL and stable in a pH range 4.0-6 (Figure 6). Decrease enzyme activity on both sides of the optimum pH due to enzymes undergoing a change in secondary or tertiary structure caused by changes in pH that are too low or vice versa. Extreme pH will damage the catalytic site of the enzyme so that it cannot be bound to the substrate. Robinson mentions that enzymes have a characteristic optimum pH at which the speed of the catalyzed reaction is maximum, and above and below the speed decreases [29].

According Vatanparast *et al* reported that pectinase enzymes from the midgut of *R. ferrugineus*, hydrolyzed substrate in a pH range 6.0-7.0 [28]. Orange peel as a substrate at the concentration of 1% with a pH of 7.0 was found to be optimum for pectinase production by *Bacillus* sp. The xylanase was found to be active at range of pH values between 3.5 to 6.0 with maximum pH of 5.5 (0.41 U/mL). The enzyme activity gradually enhanced with increased pH from 3.5 to 6.0 and decreased in alkaline condition from pH 6.5-8.0. This study was similarly reported by Seo *et al*, where a *B. licheniformis* isolate from the rumen of a Korean goat exhibited a maximal xylanase activity of 1.08 U/mL at pH 5.0 [30]. Other studies mention that xylanase produced by *Aspergillus* sp. using coffee pulp waste substrate optimal at pH 5 and stable in the pH range of 3-6.5 [31]. This result indicates that the target enzyme can work actively in the acidic pH range and its activity decreases dramatically in alkaline or basic pH.

IV. CONCLUSION

Listeria sp. ISH-16 could has the highest xylanase and cellulase activity 0.37 U/mL, 0.07 U/mL in 84 hours of

incubation time, respectively. Whereas pectinase activity 0.28 U/mL in 72 hours of incubation time. The harvested of crude cellulase had activity was 0.23 U/mL with a specific activity of 0.0082 U/mg and purification fold 6.34, pectinase had highest activity 0.15 U/mL with a specific activity of 0,005 U/mg and purification fold 0.61. Xylanase had an activity of 0.61 U/mL with a specific activity 0.022 U/mg and purification fold 1.91 after dialysis. Cellulase worked optimally at pH at 4.5 and was stable at pH 4-5.5. Pectinase showed to be more stable at 4-5.5 with optimum pH was 5. Xylanase had an optimal pH at 6 and was stable at pH 4.5-5.5. These results could be as pre-indicator that *Listeria* sp. able to produce some target enzymes. However, to increase enzyme activity, further purification is needed.

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