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Hydrolytic enzymes-producing ability of species of actinomycetes and bacteria associated with wilted banana plants (*Musa* sp.)

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Abstract. *Ardhi A, Ahmad KC, Novrianti H, Husna EY, Yulis M, Pratiwi NW, Saryono. 2019. Hydrolytic enzymes-producing ability of species of actinomycetes and bacteria associated with wilted banana plants* (Musa *sp.). Biodiversitas 20: 1147-1153.* Banana plants contain many nutrients that enable microbes to grow and attack them, causing wilting disease. Microbes growing on the stumps and soil around banana plants are believed to have the ability to produce hydrolytic enzymes. The purpose of this study was to determine the ability of actinomycetes and bacteria isolated from the stumps and soil of wilted banana plants in producing hydrolytic enzymes, namely cellulase, inulinase, amylase, and protease. The confirmation tests of hydrolytic enzymes-producing ability were conducted by inoculating the microbes into media containing CMC, inulin, starch, and skim milk, using the method of paper disc diffusion. From the subculture results, there were 18 isolates of actinomycetes which have been identified as *Nocardia*, *Actinobiospora*, *Nocardiopsis*, *Streptomyces*, *Streptoverticillium*, *Streptosporangium*, and *Microbiospora*, as well as 40 bacterial isolates with 9 genera of bacteria, namely *Xanthomonas*, *Erwinia*, *Pesudomonas*, *Proteus*, *Ralstonia*, *Escherichia*, *Staphylococcus*, *Caulobacter*, and *Neisseria*, were found. As many as 8 actinomycetes and 40 bacterial isolates indicated the ability to degrade amylum, 39 bacterial and 18 actinomycetes isolates could degrade cellulose, 34 bacterial and 13 actinomycetes isolates could degrade inulin. The highest cellulase ratio was shown by *Nocardia* sp. LBKURCC101 (3.43) and *Ralstonia* sp. LBKURCC112 (3.90). The actinomycetes isolate of *Nocardia* sp. LBKURCC104 and bacteria *Pseudomonas* sp. LBKURCC133 gave the highest inulinase ratio of 3.36 and 3.47 respectively. In selective amylase media, the highest ratio of 3.10 and 3.80 was found in actinomycetes *Nocardia* sp. LBKURCC104 and bacteria *Erwinia* sp. LBKURCC125.

Keywords: actinomycetes, bacteria, banana plants, hydrolytic, wilting

INTRODUCTION

Banana is one of the horticultural products having high economic value. Indonesia is among the largest banana producers in Asia, and banana production continues to rise annually. Statistically, the productivity of banana in Indonesia increased between 1980 and 2013. Overall, it has been a significant contributor for banana production as indicated by its total production of 7.299.275 tons in 2015 (MoA 2014; BPS 2015). However, banana crop productivity development is hindered by wilting disease, characterized by several features: the dark brown and blackened inner parts of stump, the cracked and darkening stem, the withered leaves, and the rotten fruits that failed to mature. Some wilted banana plants wither and die before or in bearing fruit. The extent of this disease continues to increase every year (Apriyanto et al. 2007; Msogoya et al. 2012).

Bacterial diseases of bananas have not received the attention it needs compared to other major threats such as the fungal disease of Fusarium wilt (*Fusarium oxysporum* f. sp. cubense). However, they cause significant impacts on bananas globally. Mbaka et al. (2009) and Blomme et al. (2017) stated that bacterial diseases in bananas can be divided into three groups: (i) *Ralstonia*-associated diseases; (ii) *Xanthomonas* wilting of banana caused by *Xanthomonas campestris* pv. *musacearum* and (iii) *Erwinia*-associated diseases. Other less prominent bacterial diseases include: bacterial wilt of abaca, Javanese vascular wilt, and bacterial fingertip rot.

In addition, according to Nurkanto et al. (2010), actinomycetes can also excrete extracellular enzymes by taking nutrients and degrading natural polymers from their host plants to meet their needs. Actinomycetes are important soil microbes, known for their ability to produce antibiotics. Evidence indicates that actinomycetes are quantitatively and qualitatively important in the rhizosphere, where they may influence plant growth and protect plant roots against invasion by root pathogenic fungi (Crawford et al. 1993).

Banana tubers are composed of cellulose, amylum, protein, inulin, lipid, and some minerals (Kesumaningwati 2015), which can produce some hydrolytic enzymes related to their activities in banana stumps and soil around the plants. In our previous studies, fungal isolates from wilting banana plants showed cellulase-degrading abilities

(*Fusarium* sp. and *Penicillium* sp.); *Aspergillus* sp., *Penicillium* sp., and *Trichoderma* sp were able to produce inulinase; amylase producing abilities were found in *Penicillium* sp. and *Trichoderma* sp; two fungal isolates of *Aspergillus* sp. and *Trichoderma* sp.produced lipase (Saryono et al. 2018). Additionally, endophytic fungi isolated from dahlia tubers produced anti microbes and also hydrolytic enzymes such as inulinase and β-galactosidase which could be utilized in various industries (Saryono et al. 2015a; 2015b; 2017; Silvera et al. 2018).

Based on our preliminary research, there were 18 isolates of actinomycetes and 40 isolates of bacteria from the tuber and soil of banana affected by wilting rot. To obtain the activity of bacteria, the qualitative and semiquantitative activity tests of hydrolytic enzyme (cellulase, amylase, protease, and inulinase) were carried out. The genera of these isolates were identified, but the ability to produce extracellular hydrolases was still unknown. The purpose of this study was to determine the ability of actinomycetes and bacteria isolated from the stumps and soil of wilted banana plants in producing hydrolytic enzymes, namely cellulase, inulinase, amylase, and protease. These potencies in producing extracellular hydrolases can be utilized in the field of biotechnology research and industry.

MATERIALS AND METHODS

Preparation of inocula

As many as 18 actinomycetes and 40 bacterial isolates were in the culture collections of Biochemistry Laboratory of Universitas Riau, isolated from wilted banana tuber and soil around the plants. Subculture of the isolates was obtained by taking 1 dose of the culture from the stock culture, then streaking it on a petri dish containing Starch Casein Agar (SCA) for actinomycetes and incubated for 5 days at 37°C and Nutrient Agar (NA) for bacteria and incubated for 24 hours at 37°C. The subcultured isolates were used as work stock inocula. Afterward, inoculum was obtained by taking 1 dose of actinomycetes or bacteria for inoculation on medium Starch Casein Broth (SCB) and Nutrient Broth (NB). The inocula on NB and SCB medium were then used for hydrolytic enzyme activity tests.

Activity tests

The ±4 mm-diameter paper discs were immersed in SCB and NB medium containing inocula, then placed on a solid medium with a certain composition for each enzymatic activity test (Table 1). The medium was incubated for 5 days (actinomycetes) and 24 hours (bacteria) at 37°C. After that, the media were added with iodine solution for enzymatic activity confirmation.

Data analysis

A positive result was marked by the formation of clear zone around the colonies and the width of clear zone formed was measured. The data were carried out in triplicate. Hydrolytic enzyme activity ratio was calculated using the following formula:

$$R = \frac{X1 - X2}{X2}$$

Where:
R: Activity ratio
X1: Clear zone diameter
X2: Colony diameter

Table 1. Media composition for enzymatic activity tests

Activity tests	Media composition
Cellulase	NaNO3 2 g/L; KCl 5 g/L; K2HPO4 1 g/L; MgSO4.7H2O 0.5 g/L; CMC 2 g/L; agar 17 g/L
Inulinase	(NH4)2SO4 0.5 g/L; KH2PO4 3 g/L; MgSO4 0.2 g/L; inulin 2 g/L; agar 20 g/L
Amylase	peptone 0.5 g/L; (NH4)2SO4 0.1 g/L; NaH2PO4 0.1 g/L; MgSO4.7H2O 0.5 g/L; KCl 0.1 g/L; starch 20 g/L; agar 8 g/L
Protease	skim milk 20 g/L; agar 20 g/L

RESULTS AND DISCUSSION

In this study, 18 actinomycetes and 40 bacterial isolates were tested for their ability to produce extracellular hydrolytic enzymes in the forms of cellulase, inulinase, amylase, and protease. Based on the results obtained, almost all isolates were able to produce cellulase, but some isolates did not produce inulinase and amylase. No isolates showed protease activity. The qualitative results can be seen in Table 2.

Each isolate showed different diameter ratio of clear zone and colony. Figures 1-3 reveal the ratio of actinomycetes and bacterial isolates on selective medium of cellulose, inulinase, and amylase.

The clear zones were formed with different ratios on each selective medium. The highest cellulase ratio was shown by *Nocardia* sp. LBKURCC101 (3.43) and *Ralstonia* sp. LBKURCC112 (3.90). The actinomycetes isolate of *Nocardia* sp. LBKURCC104 and bacteria *Pseudomonas* sp. LBKURCC133 gave highest inulinase ratio of 3.36 and 3.47 respectively. In selective amylase media, the highest ratios of 3.1 and 3.8 were found in actinomycetes *Nocardia* sp. LBKURCC104 and bacteria *Erwinia* sp. LBKURCC125. The comparison in the selective media between control, negative test results, and positive test results of isolates with the largest clear-zone ratios can be seen in Figure 4-7.

Actinomycetes are one of the endophytic microbes isolated from banana plants. Previous research (Cao et al. 2004) reported that a number of actinomycetes strains were isolated from both healthy and wilting banana plants. Most actinomycetes isolated from healthy trees were Streptomyces strains (94.7%), other strains belonged to Streptoverticillium (3.7%) and Nocardia (1.5%) genera. The dominant actinomycetes genera isolated from wilted banana plants were Streptomyces (83.6%), Actinomadura (8.2%), Streptoverticillium (6.4%) and Streptosporangium (1.8%). Each actinomycete isolated with a particular genus has the ability to produce some enzymes (Nurkanto 2010).

Gangwar et al. (2014) added that *Streptomyces* sp., *Nocardia* sp., *Micromonospora* sp., and *Saccharopolyspora* sp. showed the ability to solubilize phosphate, while producing some bioactive compounds such as indole-3 acetic acid (IAA), hydroxamate-type of siderophore, and catechol-type of siderophore which may be utilized as fungal pathogen biocontrol in wilting banana plants. Some genera of actinomycetes, for instance, Nocardiopsis, were reported to be able to become multifaceted and release an assortment of extracellular hydrolytic enzymes, such as cold-adapted α-amylases, thermotolerant α-amylases and xylanases, thermoalkalotolerant cellulases and β-1,3-glucanases, alkalitolerant thermostable inulinases, acid-stable keratinase, and alkalophilic serine proteases (Bennur et al. 2014).

Bacteria were also reported to be the cause in the wilting of banana plants. The basic abilities of microbes in producing endo- and exo-hydrolytic enzymes, namely cellulase, inulinase, amylase, lipase, and protease were confirmed, enabling microbes to degrade the compounds in banana stumps. On the other hand, the extracellular hydrolytic enzymes produced could be utilized in daily life. In this research, *Ralstonia* sp. produced cellulase with the largest ratio. This microbe was often associated with this wilting disease, for instance, Moko/Bugtok disease which was caused by *Ralstonia solanacearum* and banana blood disease caused by *Ralstonia syzygii* subsp. celebesensis (Mbaka et al. 2009 and Blomme et al. 2017). Blood disease is one of the important diseases in banana plants in Indonesia, especially in Lumajang Regency. In addition, Banana *Xanthomonas* Wilt (BXW) is named after the bacterium that infects the plant and eventually kills it (Blomme et al. 2014; Lestari et al. 2015). Degradation of CMC medium by cellulase of isolates occurred due to the cellulose existing in the media that had been degraded to glucose. Thus, the iodine was not bound and this impacted the clear zone formed. The purple media part is the result of a complex reaction between cellulose and iodine (Figure 4). Ningsih et al. (2014) isolated 8 genera of cellulose-degrading bacteria in which one of its genera was *Neisseria* sp. On the other hand, actinomycetes *Nocardia* sp. performed the greatest cellulase activity. On a previous study, nine isolates of cellulose-degrading actinomycetes were isolated from different sediment samples from the Bhitarkanika Mangrove Forest (Mohanta 2014).

Actinomycetes and bacteria can break up the inulinase. Inulin is one of the compositions contained in the banana tuber. The results showed that from 40 bacterial isolates tested, there were only six isolates that did not produce clear zone. Inulinase test was performed on isolates using inulin from the dahlia tuber extract as a carbon source. This is because the dahlia tuber has a large percent of inulin (Saryono 2008). The clear zone was visible after the addition of iodine solution. According to Azhar et al. (2017), undirect isolation from dahlia tuber found 5 isolates which could degrade inulin and grow well at room temperature and 40ºC. The endo- and exo-inulinase bonding termination reaction is shown in Figure 8.

Table 2. Qualitative tests of hydrolytic enzyme-production ability

Genera	Code (LBKURCC)	Activity tests			
		Cellulase	Inulinase	Amylase	Protease
Actinomycetes					
Nocardia sp.	88	+	+	+	-
Actinobiospora sp.	89	+	+	-	-
Actinobiospora sp.	90	+	+	-	-
Nocardiopsis sp.	91	+	+	+	-
Nocardiopsis sp.	92	+	+	+	-
Streptomyces sp.	93	+	+	+	-
Streptoverticillium sp.	94	+	+	-	-
Nocardiopsis sp.	95	-	+	+	-
Actinobiospora sp.	96	+	+	+	-
Nocardiopsis sp.	97	+	-	-	-
Streptosporangium sp.	98	+	-	-	-
Streptomyces sp.	99	+	-	-	-
Nocardiopsis sp.	100	+	-	-	-
Nocardia sp.	101	+	+	-	-
Streptoverticillium sp.	102	+	+	-	-
Microbiospora sp.	103	+	-	+	-
Nocardia sp.	104	+	+	+	-
Streptomyces sp.	105	+	+	-	-
Bacteria					
Xanthomonas sp.	106	+	+	+	-
Erwinia sp.	107	+	+	+	-
Pseudomonas sp.	108	+	+	+	-
Proteus sp.	109	+	+	+	-
Xanthomonas sp.	110	+	+	+	-
Xanthomonas sp.	111	+	+	+	-
Ralstonia sp.	112	+	+	+	-
Ralstonia sp.	113	-	+	+	-
Xanthomonas sp.	114	+	+	+	-
Ralstonia sp.	115	+	+	+	-
Pseudomonas sp.	116	+	+	+	-
Pseudomonas sp.	117	+	+	+	-
Ralstonia sp.	118	+	+	+	-
Erwinia sp.	119	+	+	+	-
Erwinia sp.	120	+	+	+	-
Xanthomonas sp.	121	+	+	+	-
Xanthomonas sp.	122	+	+	+	-
Xanthomonas sp.	123	+	+	+	-
Xanthomonas sp.	124	+	+	+	-
Erwinia sp.	125	+	+	+	-
Escherichia sp.	126	+	-	+	-
Staphylococcus sp.	127	+	-	+	-
Proteus sp.	128	+	+	+	-
Caulobacter sp.	129	+	-	+	-
Caulobacter sp.	130	+	+	+	-
Proteus sp.	131	+	+	+	-
Xanthomonas sp.	132	+	+	+	-
Pseudomonas sp.	133	+	+	+	-
Escherichia sp.	134	+	+	+	-
Neisseria sp.	135	+	+	+	-
Erwinia sp.	136	+	+	+	-
Neisseria sp.	137	+	-	+	-
Caulobacter sp.	138	+	-	+	-
Pseudomonas sp.	139	+	-	+	-
Pseudomonas sp.	140	+	+	+	-
Xanthomonas sp.	141	+	+	+	-
Pseudomonas sp.	142	+	+	+	-
Xanthomonas sp.	143	+	+	+	-
Erwinia sp.	144	+	+	+	-
Pseudomonas sp.	145	+	+	+	-

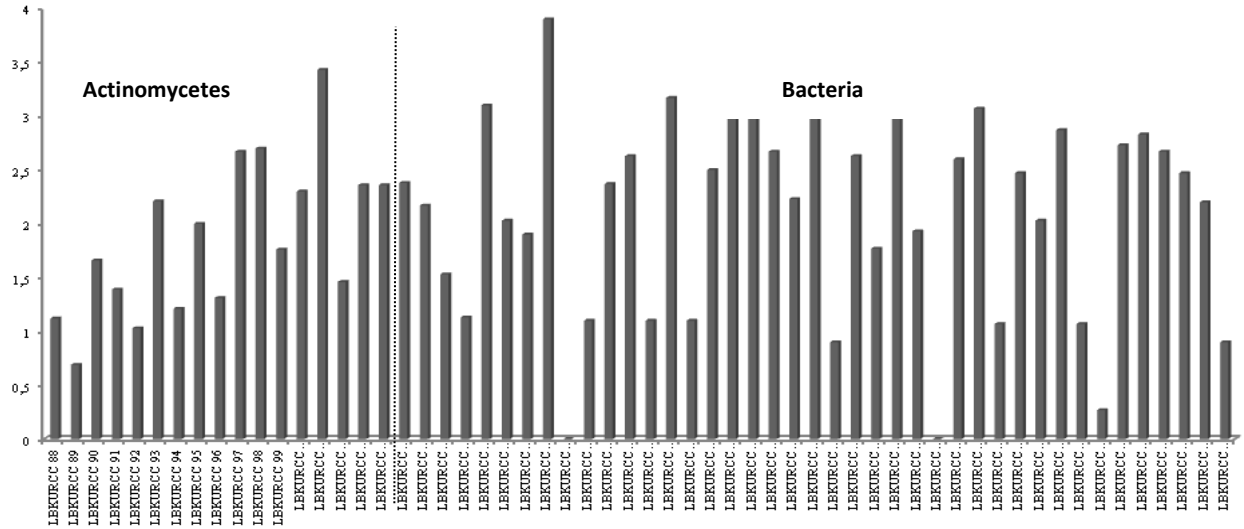

Figure 1. Diameter ratio for cellulose activity

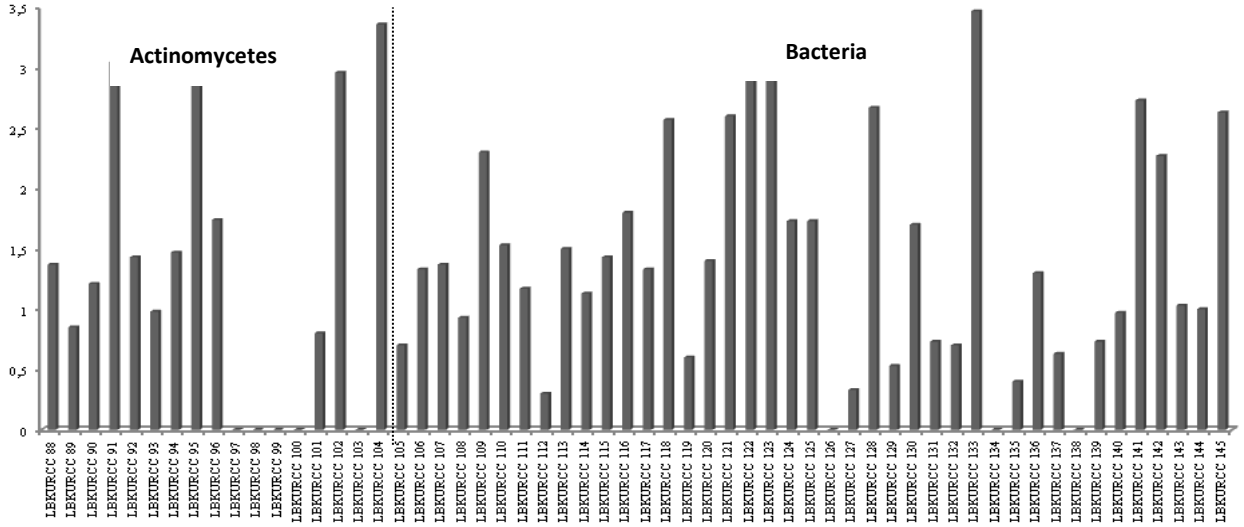

Figure 2. Diameter ratio for inulinase activity

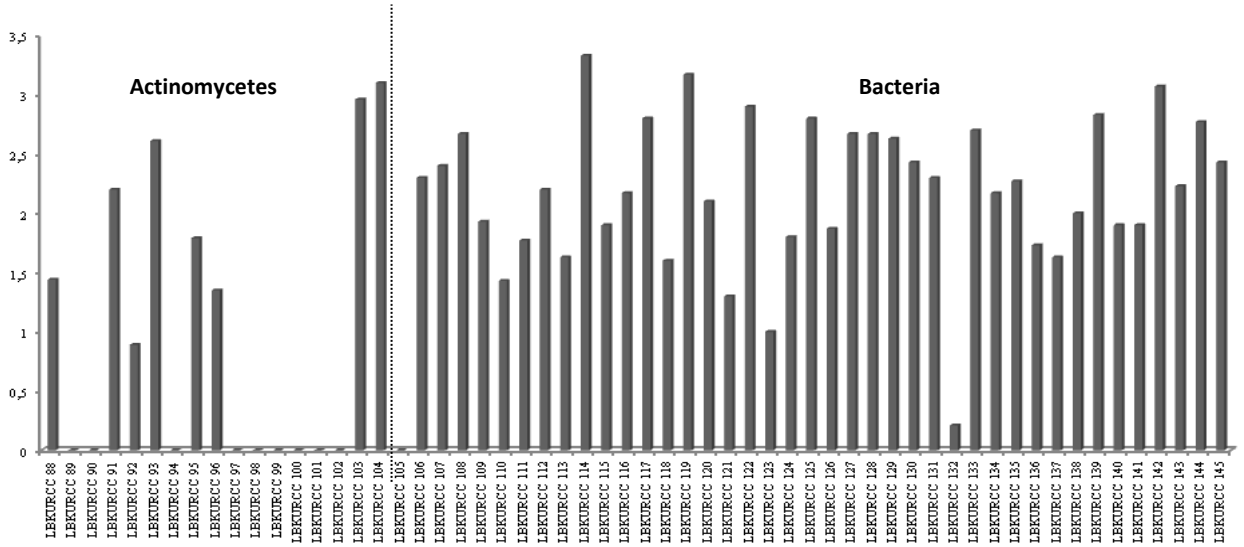

Figure 3. Diameter ratio for amylase activity

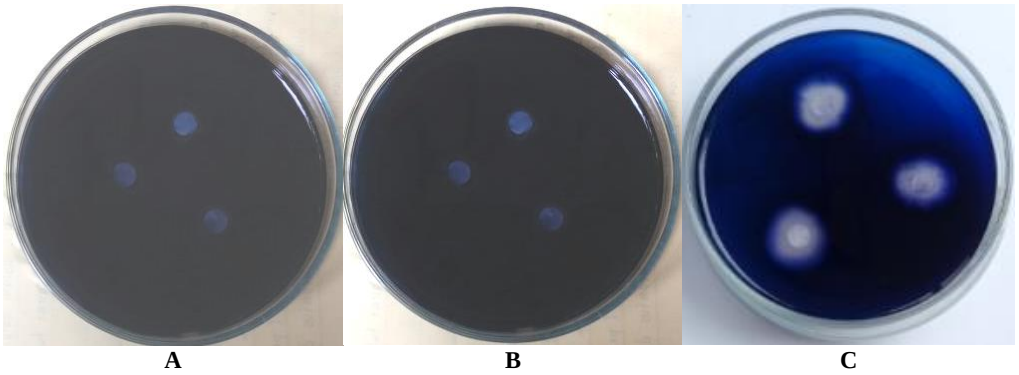

Figure 4. Test of amylase activity: A. Control, B. Isolate with negative result, C. Isolate with positive result

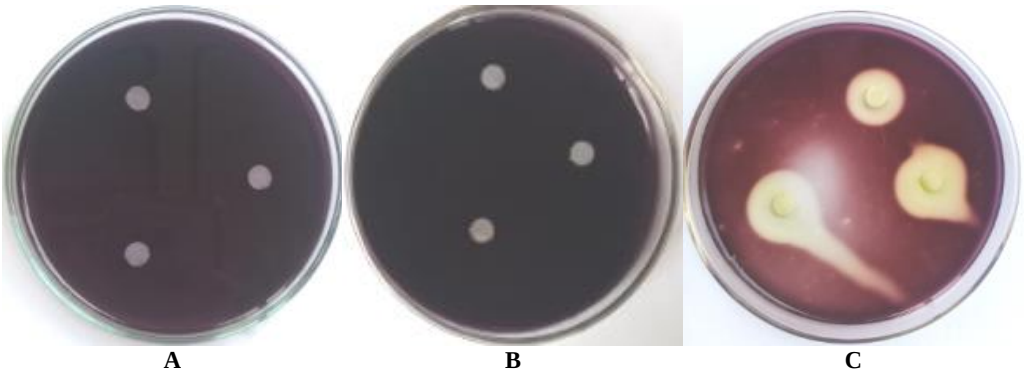

Figure 5. Test of cellulase activity: A. Control, B. Isolate with negative result, C. Isolate with positive result

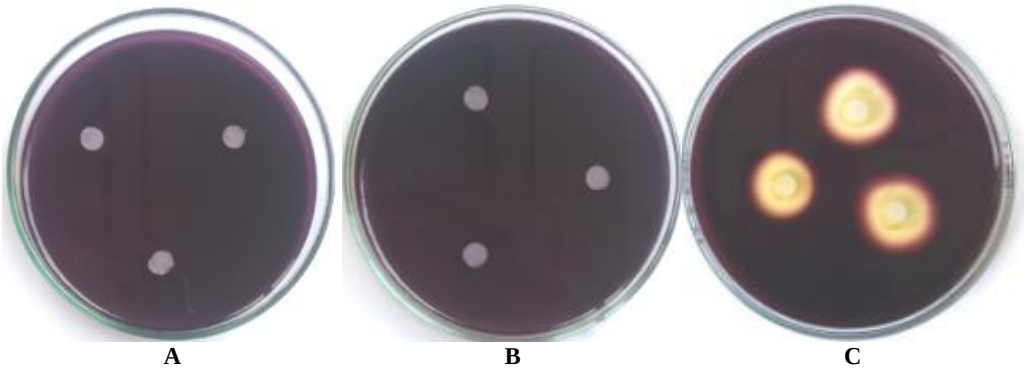

Figure 6. Test of inulinase activity: A. Control, B. Isolate with negative result, C. Isolate with positive result

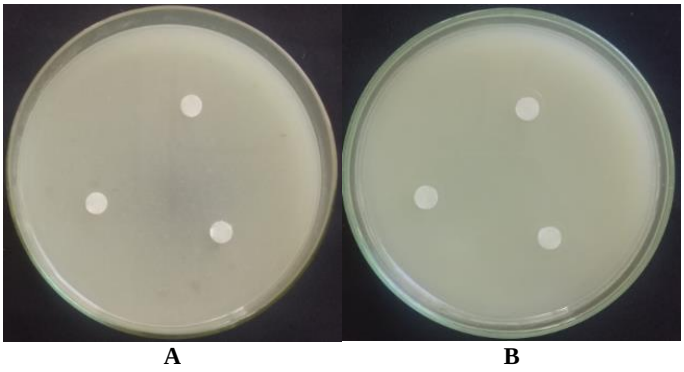

Figure 7. Tests of protease activity: A. Control, B. Isolate with negative result

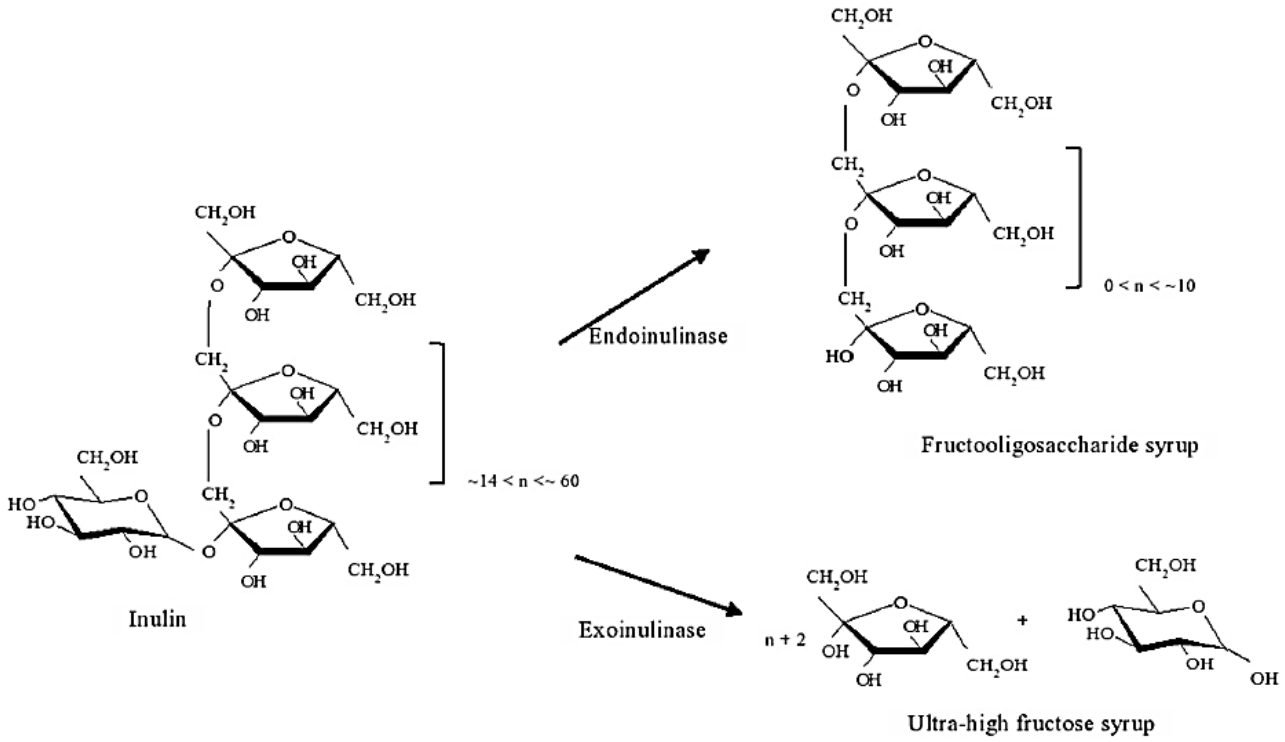

Figure 8. Reaction of inuline bonding termination by inulinase enzyme (Fernandes 2014)

In addition, actinomycetes and bacteria were also able to hydrolyze starch contained in Starch Agar medium, so the degraded starch cannot bind to iodine and produce a clear zone around bacterial colonies. The iodine-stained media portion indicated that the starch in the media had not been hydrolyzed. The purple color was from the adsorption of iodine into molecules of spiral-formed amylose, only if the bonds of amylose broke up (Juwita et al. 2013). Almost all isolates of bacteria could potentially produce amylase, and the largest amylase activity was found in *Erwinia* sp. LBKURCC135 whilst there were 9 actinomycetes isolates that did not degrade amylum. One type of wilting banana disease was caused by bacterial head rot or tip-over disease *Erwinia carotovora* subsp. carotovora and *Erwinia chrysanthemi* (Mbaka et al. 2009 and Blomme et al. 2017).

Similarly, the potential of protease was analyzed through observation of the clear zone resulting from the decomposition of proteins into amino acids by protease enzymes produced by bacteria on skim milk medium (Figure 4), which occurred due to protease activity that broke the peptide bond of casein in skim milk. The skim milk medium supported the growth of proteolytic microbes because it contained casein which functioned as a substrate for protease enzymes. Based on the results, all the isolates indicated no ability to produce clear zones., perhaps due to insignificant presence of protein content or different protein type than those provided in the medium which makes the isolates unable to process the substrate. Saryono et al. (2018) also stated that fungal isolates from wilting banana did not perform proteolytic activity.

It can be concluded that there were 7 genera of actinomycetes isolated from wilted banana plants and soil around the plants showing the ability to degrade amylum, 18 actinomycetes, and 39 bacterial isolates could degrade cellulose; 13 actinomycetes and 37 bacterial isolates could degrade inulin, and no isolates could degrade protein. The clear zones were formed with different ratios on each selective medium. The extracellular hydrolytic enzymes produced could hopefully be utilized in daily life. Overall, this result will have a great benefit for further research to find the optimum condition for production of these enzymes by actinomycetes and bacteria associated with wilted banana plants.

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