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Isolation and characterization of extremophile bacteria for hydrolytic enzyme production from Waepella Hot Spring, Sinjai, Indonesia

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Abstract. *Indrayani, Putra RP, Hambali A, Ardiansyah. 2022. Isolation and characterization of extremophile bacteria for hydrolytic enzyme production from Waepella Hot Spring, Sinjai, Indonesia. Biodiversitas 23: 6345-6351.* Extremophiles are organisms that have adapted to live in extreme environments. Therefore, they have huge potential for various industrial applications, specifically enzyme production. The aim of this study was to isolate and screen the potential of extremophile bacteria for hydrolytic enzyme production from the Waepella hot spring in Sinjai District, South Sulawesi, Indonesia. The water samples were collected from the hot spring at three different locations. The method of bacterial isolation was the agar plating technique using Tryptic Soy Agar media. The obtained isolates were characterized by examination of colony colors, cell shapes, gram staining, endospore, catalase, and enzymatic activity (amylase, cellulose, protease, lipase, and pectinase). The result showed that a total of eighteen isolates were successfully isolated from Waepella hot springs. Eight isolates showed amylolytic activity, and the highest (22.6± 0.44 mm) activity was observed in isolate BHSS10. Nine isolates had a cellulolytic activity with the highest clear zone of 11.6±0.4 mm (isolate BHSS7). Sixteen isolates had a pectinolytic activity with the highest clear zone of 12±0.24 (isolate BHSS16). Only 3 isolates showed proteolytic activity, and the highest (9.83±0.40 mm) was observed in isolate BHSS15, while none showed lipolytic activity. That is the first report on extremophile bacterial isolation and screening for enzyme production from the hot spring in South Sulawesi, Indonesia.

Keywords: Amylolytic activity, extremophile bacteria, hydrolytic enzymes, Waepella Hot Spring

INTRODUCTION

Microorganisms can be found in almost all habitats, including extreme habitats, which shows that microorganisms have extensive adaptability to various environmental parameters (Merino et al. 2019). Extreme habitat is an environment with physicochemical conditions that are beyond normal limits to support the life and growth of most organisms, such as the environment that has very high temperatures (hot springs), very low temperatures (snow), high salinity (hypersaline ponds/lakes), very high pH or very acidic (Varshney et al. 2015).

Hot springs are an extreme environment characterized by high water temperatures above 98°F (36.7°C) (Jiang et al. 2018). Thermophilic organisms, including bacteria, inhabit this habitat. The thermophilic bacteria are of great interest as they are cell factories of industrially important biomolecules, including thermostable enzymes such as Taq-polymerase enzyme used in Polymerase Chain Reaction (PCR) from *Thermus aquaticus* and *Pyrococcus furiosus* (Bruins et al. 2001; Irwin and Baird 2004). In addition, thermophilic bacteria have important applications for bioremediation. For example, thermophilic isolated from geothermal sites in Antarctica with temperatures between 50 and 100°C showed the capability to degrade petroleum hydrocarbons. Therefore, there is a potential application in the petroleum industry, such as bioremediation in extreme environments and microbial-enhanced oil recovery (MEOR) in reservoirs (Schultz et al. 2022). Thermophilic bacteria also have interesting applications for the bioremediation of heavy metals (Rakhmawati et al. 2021) and the removal of dyes from textile industry wastewater (Nzila 2018; Baker et al. 2021; Aragaw et al. 2022). Most importantly, thermophilic bacteria have the ability to produce hydrolytic enzymes, including amylase, protease, lipases, cellulase, laccases, and xylanases (Damiano et al. 2003; Alrumman et al. 2018; Atalah et al. 2019; Geraldi et al. 2019; Sahoo et al. 2020; Khadka et al. 2022). Hydrolytic enzymes have potential applications in almost all industrial processes (Manisha and Yadav 2017).

Hot springs are widespread in Indonesia, with a total of 256 hot springs (Darma et al. 2010). Several studies have been done on the isolation and characterization of thermophilic bacteria in Indonesia. Murtiyaningsih et al. (2022) have successfully isolated and screened thermophilic bacteria from Ijen Crater, which have the potential as DNA polymerase enzyme producers. Thermophilic bacteria *Bacillus licheniformis* isolated from rom Bukit Gadang Hot Spring, West Sumatra, Indonesia,

showed potential as an amylase enzyme producer (Ardhi et al. 2020). *Bacillus cereus* isolated from the Way Panas hot springs, Kalianda, South Lampung, has high amylolytic activity (Mahestri et al. 2021). Several other studies have successfully isolated hydrolytic enzymes from thermophilic bacteria (Zilda et al. 2012; Yohandini et al. 2015; Laras et al. 2017; Ifandi and Alwi 2018; Ningsih et al. 2020; Lischer 2021; Geraldi et al. 2021; Ginting et al. 2021). However, it is the first study on bioprospecting thermophilic bacteria for hydrolytic enzyme production from hot springs in South Sulawesi Province. Species and strain selection are the first and most important steps in the bioprospecting activities of organisms for commercial applications (Indrayani 2017). The selection and screening processes involve stages such as sample collection, isolation, purification, identification, maintenance, and characterization (Gong and Jiang 2011). Microbial selection and screening activities can be done in two ways: selecting and screening microbes from the collection center and the natural environment. Species selection through a culture collection center can be easily accessed but only represent a small portion of the microbial species in nature (Borowitzka 2013).

Conversely, the microbes' resources that exist in nature are numerous and under-explored (Indrayani 2017). Therefore, the aim of this study was to isolate and screen thermophilic bacteria from Waepella Hot Spring in Sinjai District, South Sulawesi, Indonesia, for biotechnological application, specifically for hydrolytic enzyme production. Furthermore, this research is expected to be the basis for developing local extremophile microorganisms for commercial applications.

MATERIALS AND METHODS

Sampling sites

The sampling sites were in Waepella hot spring, Kampala Village, Sinjai Tengah Sub-district, Sinjai District, South Sulawesi, Indonesia (05º08'35.22" S, 120º12'07.67" E). Water quality parameters, including temperature, pH, and salinity, were measured onsite. The water samples were collected using sterile glass bottles.

Procedures

Isolation of thermophilic bacteria

The bacteria were isolated using the agar plating technique (Andersen and Kawachi 2005). About 0.2 mL of the water samples were plated on TSA medium and incubated at ambient room temperature (30±2℃). The grown colonies on the plate were then re-streaked to obtain pure colonies.

Characterization of isolates

The obtained isolates were characterized by examination of colony colors, cell shapes, Gram staining, endospore test, catalase test, and enzymatic activity (amylase, cellulose, protease, lipase, and pectinase).

Gram staining

In Gram staining, a smear was prepared on the surface of a sterile slide before adding 1 drop of crystal violet on the bacterial layer and allowed to stand for 1 minute. After 1 minute, the slide was rinsed with water until the dye faded. Next, the slide was dried over a Bunsen burner before adding 1 drop of iodine solution to the surface of the slide and allowed to stand for 1 minute. Next, the slide was rinsed with water, followed by 70% alcohol, rinsed until all the dye had faded, and then washed again with water. Next, the slide was dried over a Bunsen burner before adding 1 drop of safranin and allowed to stand for 45 seconds. The slide was then washed with water and dried. Then the slide was observed under a microscope at (100x magnification).

Catalase test

For the catalase test, a loopful of the bacterial isolate was placed on a slide before adding 1- 2 drops of 3% H_2O_2 solution. The presence of bubbles means catalyzing positive bacteria, and the absence means catalyzing negative bacteria.

Endospore test

For endospore staining, a bacterial smear was made on the surface of a sterile slide then fixation was carried out by adding 1-2 dopped of malachite green and heating for 2-3 minutes. If evaporation occurred, malachite green was again dropped on the glass slide. Afterward, the glass slides were rinsed with distilled water and dried before adding 1 drop of safranin to the slide. The slide was then allowed to stand for 1 minute. Next, the slide was washed and then observed using a microscope.

Enzyme activity tests

All the procedures were the same for enzyme activity tests except for the media used. A proteolytic activity test was carried out on agar media using 1% skim milk (Nespolo et al. 2010). An amylolytic activity test was carried out on agar media with 1% starch (Fossi et al. 2005). A lipolytic activity test was carried out on MRS agar media with 2 ml of olive oil (Svetlitshnyl et al. 1996). A cellulolytic activity test was carried out on solid media containing 1% Carboxymethylcellulose (CMC). Finally, a pectinolytic activity test was carried out on solid media containing 1% pectin and 0.1% Congo red indicator. Briefly, 48 hours aged 20 μL bacterial culture was pipetted onto a paper disc (5.5 mm diameter), then placed on agar media and incubated at 30°C for 3 days. The formation of a clear zone around the colony indicates that bacteria could hydrolyze the media. The enzyme activity tests were carried out for 3 days. The clear zone diameter was calculated by subtracting the diameter of the clear zone from the diameter of the paper disc.

RESULTS AND DISCUSSION

Sampling sites

The Waepella hot spring was a freshwater hot spring (salinity 0 ppt) located in Kampala Village, East Sinjai

Sub-district, Sinjai District. The water temperature and pH ranged from 49-55°C and 7.28-7.61, respectively (Table 1).

Table 1. Water quality parameters at the Waepella Hot Spring, Sinjai, Indonesia

Parameters	Stations		
	Station 1	Station 2	Station 3
Temperature (°C)	55	53	49
Salinity (ppt)	0	0	0
pH	7.43	7.61	7.28

Morphological characterization

Bacterial isolation was carried out by the agar plating method on Tryptic Soy Agar (TSA) media. The results showed that a total of 18 isolates of thermophilic bacteria were successfully isolated from Waepella Hot Spring. All isolates had round colonies with milky white color except the isolates BHSS8 and 18, which were yellow, and isolate BHSS16 was brown. Based on the Gram staining test, most isolates were Gram-negative (15 isolates), and only 3 were Gram-positive. The catalase test result showed that 14 isolates showed positive tests and only 4 negative ones. In the endospore test, only 4 isolates exhibited positive results (Table 2).

Table 2. Characteristics of thermophilic bacteria isolated from Waepella Hot Spring, Sinjai, Indonesia

Isolate codes	Colony color	Gram staining	Cell shapes	Catalase	Endospore
BHSS1	White	Positive	Rod	-	+
BHSS2	White	Negative	Rod	-	-
BHSS3	White	Negative	Cocci	+	+
BHSS4	White	Positive	Cocci	+	-
BHSS5	White	Negative	Cocci	+	-
BHSS6	White	Negative	Cocci	+	-
BHSS7	White	Negative	Rod	+	-
BHSS8	Yellow	Negative	Cocci	+	-
BHSS9	White	Negative	Cocci	+	-
BHSS10	White	Positive	Cocci	+	+
BHSS11	White	Negative	Rod	-	-
BHSS12	White	Negative	Rod	+	-
BHSS13	White	Negative	Cocci	+	-
BHSS14	White	Negative	Cocci	+	+
BHSS15	White	Negative	Cocci	+	-
BHSS16	Brown	Negative	Cocci	+	-
BHSS17	White	Negative	Rod	+	-
BHSS18	Yellow	Negative	Cocci	+	-

Note: + = Presence, - = Absence

Enzyme production

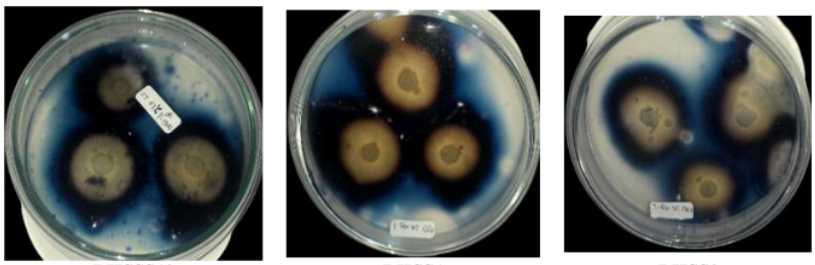
All isolates obtained were further screened for producing various hydrolytic enzymes, including amylase, lipase, protease, pectinase, and cellulase. The results exhibited that eight out of 18 isolates showed amylolytic activity with the clear zone ranging from 5.73±0.15 to 22.6±0.44 mm (Table 3). The highest (22.6±0.44 mm) amylolytic activity was observed from the isolate BHSS10, followed by isolate BHSS1 (19.6±0.62 mm) and BHSS2 (18.57±0.60 mm) (Figure 1).

Table 3. Amylolytic activity of bacterial isolates

Isolate codes	Clear zone diameter (mm)		
	Day 1	Day 2	Day 3
BHSS1	3.97±0.76	12.27±0.6	19.6±0.62
BHSS2	11.1±0.15	12.23±0.38	18.57±0.60
BHSS5	0	5.77±0.75	11.47±0.74
BHSS7	0	8.3±0.7	15.63±0.76
BHSS10	9.77±0.21	15.60±0.52	22.6±0.44
BHSS12	0	4.2±0	5.73±0.15
BHSS16	6.97±0.81	11.43±0.57	14.5±0.78
BHSS18	4.53±0.32	7.77±0.31	11±0.36

The cellulolytic activity test was performed to determine the activity of the extracellular cellulolytic enzyme on agar media containing CMC 1%. The results showed that nine out of 18 isolates exhibited cellulolytic activity (Table 4). The highest cellulolytic enzyme activity was observed from the isolate BHSS7 with a clear zone diameter of 11.6±0.4 mm.

The pectinolytic enzyme activity test was conducted to determine the activity of the extracellular pectinolytic enzyme on agar media containing 1% pectin. The clear zone formed in the pectin medium indicated that the isolate could degrade the pectin compounds contained in the media (Figure 2). The results showed that sixteen out of 18 isolates showed pectinolytic activity (Table 5). The highest pectinolytic enzyme activity was observed from the isolate BHSS16 with a diameter of clear zone, i.e., 12±0.24 mm, followed by isolate BHSS12 and BHSS2 with a clear zone diameter of 11.43±0.15 mm and 10.53±0.49 mm, respectively.



BHSSS10 BHSS1 BHSS2

Figure 1. The formation of a clear zone around the paper disc containing bacteria on agar media with 1% starch

The proteolytic activity test was carried out by placing a paper disc containing bacteria on a medium containing 1% skimmed milk agar. The clear zone formed in the skimmed milk medium indicated that the isolate could degrade the protein contained in the media (Figure 3). The formation of a clear zone around the disc was observed for 3 consecutive days. The result revealed that only 3 isolates showed proteolytic enzyme activity (Table 6). The larger the clear zone formed, the greater the ability of the isolate to produce protease enzymes. Isolate BHSS15 showed the highest proteolytic enzyme activity with a clear zone diameter of 9.83±0.40 mm.

The proteolytic activity test was carried out by placing a paper disc containing bacteria on a medium containing 1% skimmed milk agar. The clear zone formed in the skimmed milk medium indicated that the isolate could degrade the protein contained in the media (Figure 3). The formation of a clear zone around the disc was observed for 3 consecutive days. The result revealed that only 3 isolates showed proteolytic enzyme activity (Table 6). The larger the clear zone formed, the greater the ability of the isolate to produce protease enzymes. Isolate BHSS15 showed the highest proteolytic enzyme activity with a clear zone diameter of 9.83±0.40 mm.

In the lipolytic enzyme activity test, no clear zone was formed in any of the plates after 3 days of incubation, indicating that none of the isolates could hydrolyze lipids.

Discussion

Hot spring is one of the extreme environments formed naturally. This environment is an interesting research object as it has very high temperatures far exceeding the optimum temperature to support the life of most living organisms. Microorganisms that live and reproduce in a hot spring with temperatures higher than 45°C are known as thermophiles (Sarmiento et al. 2015). Microorganisms that live in this condition have certain mechanisms to survive, including the presence of thermostable proteins, enzymes, and cell membranes that remain stable at high temperatures (Sarmiento et al. 2015). The Waepella hot spring is one of the hot springs in South Sulawesi Province in Indonesia. However, it has not been studied concerning microbial biodiversity and the exploration of their potential biotechnological applications. Waepella hot spring has a temperature ranging from 49-55°C and is therefore classified as thermophiles with an optimum growth temperature of 45°C or above (Mohammad et al. 2017).

Table 4. Cellulolytic enzyme activity of bacterial isolates

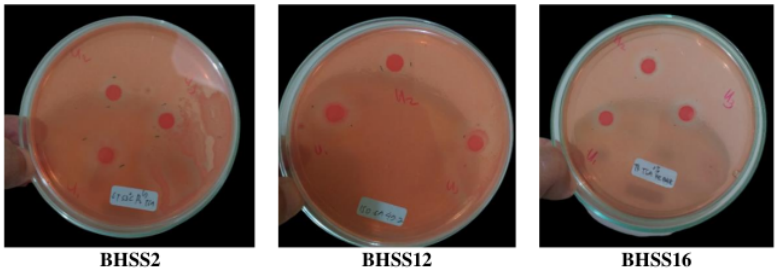
Isolate codes	Clear zone diameter (mm)		
	Day 1	Day 2	Day 3
BHSS1	2.23±0.32	3.63±0.47	3.93±0.67
BHSS4	0	0	4.03±0.15
BHSS5	0	0	3.13±0.15
BHSS6	0	2.57±0.29	3.67±0.15
BHSS7	4.5±0.3	7.73±1.01	11.6±0.4
BHSS11	0	1.73±0.40	2.33±0.32
BHSS12	0	0	2.8±0.2
BHSS15	0	0	3.5±0.44
BHSS17	1.6±0.2	2.13±0.23	2.4±0.26

Table 5. Pectinolytic enzyme activity of the isolates

Isolate codes	Clear zone diameter (mm)		
	Day 1	Day 2	Day 3
BHSS2	7.53±0.40	8.1±0.67	10.53±0.49
BHSS3	0	5.9±0.3	6.57±0.57
BHSS4	2.37±0.67	6.37±0.35	8.73±0.31
BHSS5	2.33±0.35	6.4±1.01	7.37±0.23
BHSS6	0	3.37±0.11	3.5±0.26
BHSS8	0	4.47±0.45	4.87±0.35
BHSS9.1	5.03±0.15	5.67±0.25	6.4±0.5
BHSS10	2.17±0.15	7.07±0.45	9.4±0.72
BHSS11	0	4.97±0.40	5.23±0.59
BHSS12	3.3±0.3	8.97±0.15	11.43±0.15
BHSS13	3.7±0.46	6.9±0.79	8.7±0.56
BHSS14	5.27±0.15	6.27±0.15	9.33±0.21
BHSS15	2.67±0.23	7.43±0.15	9.73±0.15
BHSS16	8.07±0.12	8.9±0.16	12±0.24
BHSS17	2.47±0.15	3.3±0.2	3.43±0.15
BHSS18	1.4±0.36	2.73±0.15	3.47±0.38

Table 6. Proteolytic enzyme activity of bacterial isolates

Isolate codes	Clear zone diameter (mm)		
	Day 1	Day 2	Day 3
BHSS13	0	5.57±0.72	6.67±0.60
BHSS15	0	2.77±0.15	9.83±0.40
BHSS17	0	5.43±0.25	6.53±0.35



BHSS2 BHSS12 BHSS16

Figure 2. The formation of a clear zone around the paper disc containing bacteria on agar media with 1% pectin

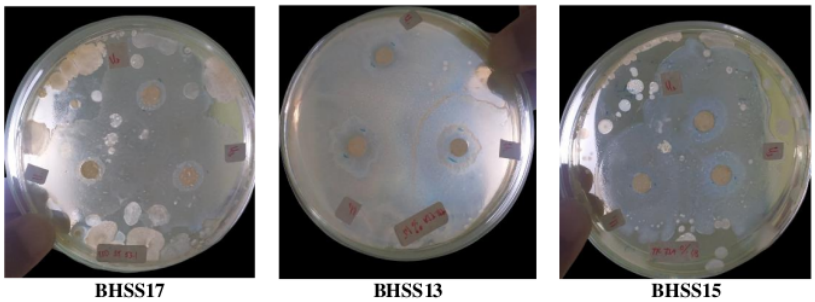


Figure 3. The formation of a clear zone around the paper disc containing bacteria on agar media with 1% skimmed milk

From this study, 18 bacterial isolates were successfully isolated from Waepella hot springs. Most isolates had a milky white colony, while only two showed a yellowish colony and one brown colony. The color of the bacterial colonies is caused by differences in pigment content, including carotenoid pigments, anthocyanins, and melanin (Savitri 2006). Gram staining procedures were performed to determine cell shape and bacterial cell structure. In Gram staining, 15 isolates were gram-negative bacteria, and only 3 were gram-positive. Differences in the structure of the cell walls of the bacterial isolates caused the difference in the results of the Gram staining of bacteria. Gram-positive bacteria have a cell wall structure that only comprises one relatively thick layer of peptidoglycan. In contrast, gram-negative bacteria have a cell wall composed of two cell wall layers: the outer layer composed of lipopolysaccharide and protein, and the inner layer composed of peptidoglycan but thinner than the peptidoglycan layer in gram-positive bacteria (Silhavy et al. 2010). In addition, results showed that 12 isolates had spherical shapes (coccus), and the other 6 isolates had rod shapes (bacillus). Bacterial cell shape is used as one of the characteristics to classify bacteria.

All isolates were subjected to catalase, endospore, and hydrolytic enzyme activity tests. In the catalase test, most of the isolates were catalase positive. Catalase-positive bacteria are indicated by the formation of air bubbles when 3% H_2O_2 is dripped, which means the formation of oxygen gas (O_2) due to the breakdown of hydrogen peroxide (H_2O_2) by the catalase enzyme produced by these bacteria. In contrast, no air bubbles will be formed in catalase-negative bacteria when dripped with H_2O_2. Catalase is an enzyme that catalyzes the decomposition of hydrogen peroxide into water and oxygen, where H_2O_2 is toxic to cells as it deactivates enzymes in cells (Locke et al. 2013). Catalase-positive bacteria are aerobic because hydrogen peroxide is formed during aerobic metabolism, so aerobic bacteria must decompose toxic H_2O_2 (Lay 1994). In the endospore test, only 4 isolates showed the formation of an endospore. According to (Oktari et al. 2017), endospore-positive bacteria can survive unfavorable environmental factors, such as heat, acid and salt for a long period until environmental conditions are suitable for their development. An enzyme is a natural product biologically used in various industrial applications, including agriculture, food, textiles, chemicals, pharmaceuticals, and biofuels (Sysoev et al. 2021). One enzyme group that has great benefits and is very important in industry is hydrolytic. Enzyme production and trade are dominated by hydrolytic enzyme groups, such as amylase, protease, cellulase, catalase, pectinase, and lipase (Gurung et al. 2013; Liu and Kokare 2017). The present research focused on the isolation and screening of thermophilic bacteria for their ability to produce hydrolytic enzymes. These enzymes have an extraordinary ability to catalyze the formation of various industrial products. However, most enzymes currently used for various industrial applications are limited by the narrow range of biocatalyst stability (Raddadi et al. 2015; Sysoev et al. 2021). The use of extremozymes derived from microorganisms that thrive under extreme conditions can overcome the limitation of the narrow range enzymatic stability for chemical reactions, i.e., temperature and the demand for these extremozymes is higher than ever (Singh et al. 2016a; Jorquera et al. 2019). It was observed that all the thermophilic bacteria isolated from the Waepella hot spring could produce at least one extracellular hydrolytic enzyme. Results showed that eight out of 18 isolates showed amylolytic activity. This enzyme plays important roles in hydrolyzing starch into maltose, glucose, and dextrin molecules (Benson 2001) that have applications in beverage, food, textile, pharmaceuticals, and distillation industries (Pandey et al. 2000). Nine isolates were the producer of the cellulolytic enzyme, which hydrolyze celluloses, used in various industrial application including the brewery, wine, textile, paper and pulp, food processing and biofuel industries (Goldbeck et al. 2012; Rodrigues and Odaneth 2021).

Pectinase enzyme activity was observed in almost all isolates. Pectinase is a group of enzymes capable of hydrolyzing pectin polymers and polysaccharides in plant cell walls. Pectinase plays an important role in the beverage industry because of its ability to increase clarity and reduce the viscosity of fruit juices. Pectinase is naturally found in animals, plants, and microbes. However, microbes are the main source of enzymes because they are easy to grow and harvest, and yield improvement can be done through genetic engineering and by producing enzymes under extreme conditions (Singh et al. 2016b). Only 3 isolates showed proteolytic activity. Wilson and Remigio (2012)

reported that bacterial isolates could hydrolyze natural peptides in the media into peptides and amino acids. Proteases have a wide range of usages, like cosmetics, detergents, leather, food, and medicine (Baltaci et al. 2017). At the same time, none of the isolates showed lipolytic activity. However, further studies are still needed to optimize enzyme production.

The results of the present study indicate that the bacteria isolated from the Waepella hot springs could produce various important hydrolysis enzymes. Furthermore, these thermophilic bacteria are known to produce thermostable enzymes that are very important for industrial applications owing to their stability against many solvents, detergents, acidic and alkaline pH, and high temperatures (Baltaci et al. 2017). Therefore, it is clear that this study is very important as a basis for developing thermophilic bacteria as a producer of hydrolytic enzymes for industrial application.

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