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DISCOVERY OF COLD-ACTIVE PROTEASE FROM PSYCHROPHILIC BACTERIA ISOLATED FROM ANTARCTIC REGION FOR BIO-PROSPECTING

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ABSTRACT

The Antarctic region is a new frontier as natural sources for bio-prospecting purposes. Its extreme cold temperature may provide unique enzyme characteristics that have valuable potential for industrial and biotechnological applications. This study was designed to discover proteases that are activate and can work at very low temperatures. Soil samples from the Antarctic region were screened for protease activity on skim milk agar at 4°C. Bacteria that showed clear halo zone around the colonies were selected and identified through 16S rDNA sequencing. Out of 35 bacteria, 10 bacteria that showed rapid halo zone formation were selected and further analyzed by enzymatic assay. By using azocasein as a substrate, the reaction was measured using spectrophotometer at OD340 nm. Based on the 16S rDNA sequence, phylogenetic analysis showed that 88% of the bacteria producing protease were from *Pseudomonas* sp., 9% from *Arthrobacter* sp. and 3% from *Paenibacillus* sp. For enzymatic assay analysis, sample SC8 showed the highest protease activity compared to other 10 samples. This preliminary study successfully demonstrated cold active protease producers that can be further investigated for bioprospecting. In future, purification and characterization of this enzyme is required in order to optimize the enzyme activity.

Key words: Cold-active protease, Antarctica, bio-prospecting, biotechnology

INTRODUCTION

Antarctica is a pristine place with very cold temperatures. Although it seems not to be livable, there is a diverse microorganism community that can thrive in these extreme conditions. Their survival at these very low-temperature conditions is very fascinating. Many researchers have studied their unique features on how they can work at such low temperatures (De Maayer *et al.*, 2014; Elleuche *et al*., 2014). Among the unique features that psychrophiles possess, one is the production of enzymes that are active in low temperatures. Recently, novel cold-active and alkaline pectate lyase has been characterized and extracted from Antarctica bacterium (Tang *et al.,* 2019). Cold-active enzymes originated from these psychrophilic microbes have shown a lot of advantages either in industrial processing or biotechnological applications. With lower activation energy, it can economically save the energy and reduce the cost of production (Siddiqui & Cavicchioli, 2006). Besides, compared to the chemical catalysts this biocatalyst is more environmental friendly (Barroca *et al*., 2017). As for food industries, it can maintain the taste and food structure while reducing the risk

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of spoilage or contamination. This experiment was carried out to identify Antarctic bacteria that can produce cold-active extracellular protease for bioprospecting.

MATERIALS AND METHODS

Sample collection

Soil samples utilized in this study were collected from the coastal environments of the Southern Victoria Land and Ross Island (Ross Sea region, continental Antarctic) in 2012 from the project of J. Smykla. The field survey and soil sampling strategy included a range of different terrestrial environments and was described in detail by Smykla *et al*. (2015). Briefly, the samples were collected from the upper soil layer (0-10 cm deep) using a sterile scoop, and then placed into sterile polyethylene bags (Whirl-Pack®). Within a few hours after collection, the samples were transported to the US Antarctic Station McMurdo and frozen by reducing the temperature over 48 hours period from 1 to -20°C. The samples were then shipped and stored in a frozen state for processing and analysis to the National Antarctica Research Center (NARC), University of Malaya.

Screening on skim milk agar

Proteolytic activity was screened on skim milk agar. A mass of 5 g Antarctic sediment was diluted with 10 mL of sterile distilled water. The sample was mixed by vortexing and centrifuged at 5000 X g for 10 minutes. The supernatant was used for ten-fold serial dilution. Then, 200 mL from the serial dilution were spread on the skim milk agar and incubated at 4°C. The colonies that showed halo zone (Figure 1) were selected and transferred into LB broth. Then, from the broth, the bacteria were stored at -80°C with 25% glycerol.

DNA-based bacterial identification

DNA extraction was done by heat and boiled techniques. Each colony was cultured on LB agar overnight and transferred into 200 µL of sterile distilled water. The bacterial suspension was boiled at 100°C for 15 minutes and centrifuged at 2000 X g. The supernatant was used as DNA sample for PCR amplification. PCR amplification was performed according to the standard protocol of BIOTAQ™ DNA Polymerase using primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Chen *et al.,* 2015). Purified PCR products were sent for sequencing and analyzed by blasting in NCBI website.

Enzymatic assays

From the skim milk agar screening, ten bacteria strains were selected to proceed with enzymatic assay. The reaction mixture contained 250 µL of bacteria culture with OD600 0.1, 250 µL of 1% Azocasein and 250 µL of 0.5 M Tris. The mixture was incubated for 24 hours at 4°C and 200 rpm. The reaction was stopped by adding 25% of trichloroacetic acid (TCA) and centrifuged at 11 000 X g for 30 minutes at room temperature. The supernatant was transferred into a new cuvette and read at OD340 nm and 37°C. *Pseudomonas aeruginosa* ATCC 27853 was used as positive control, because of its known proteolytic activity on skim milk agar (Brown & Foster, 1970). The data was represented as enzyme unit mean ± standard error mean (SEM). Statistical analysis was calculated

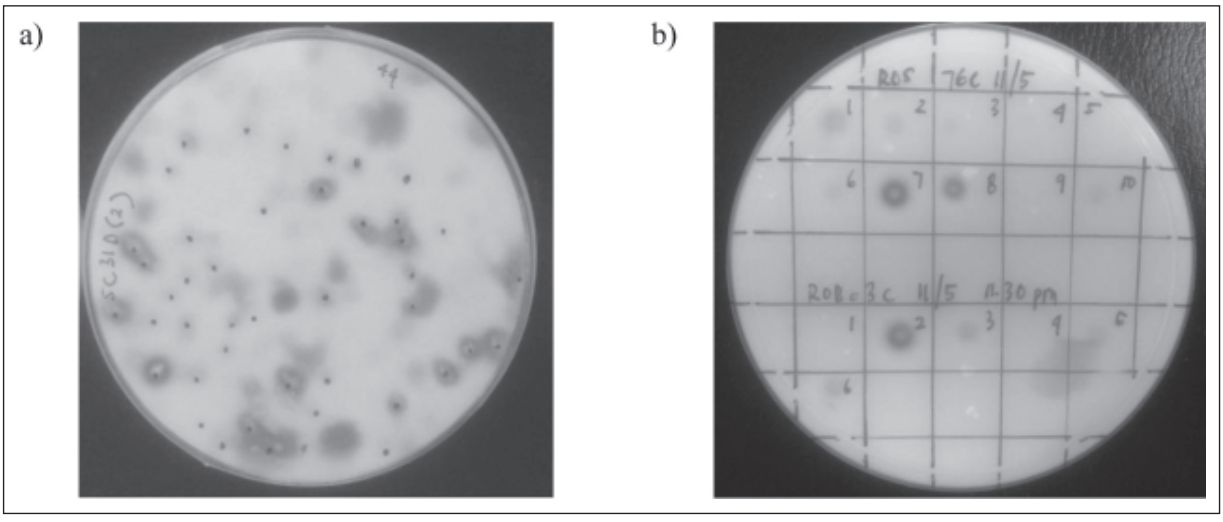

Fig. 1. a) Halo zone formation around the colonies indicated proteolytic activity. b) Each strain was re-screened to identify colony with clear halo zone formation after 24 hours.

using one-way analysis of variance (ANOVA), SPSS 26.0. The results were considered significant, if the p-value was < 0.05.

RESULTS AND DISCUSSION

Skim milk agar method was generally used to screen protease activity (Harer *et al*., 2018). Clear halo zone formation around the colony showed that the bacteria produced extracellular protease. These bacteria excreted the enzyme out of the membrane cell to digest casein around the colony. From this agar screening, 35 colonies showed positive proteolytic activity, thus were selected and patched onto new Luria-Bertani (LB) agar. Based on Table 1, phylogenetic analysis showed that 31 out of 35 (88%) colonies were from *Pseudomonas* sp., and another three (8%) and one (3%) colonies were from *Arthrobacter* sp. and *Paenibacillus* sp. respectively. *Pseudomonas* sp. is a Gram-negative strain that belongs to the class Gammaproteobacteria. This species colonized various type of environments including soil, water and plant. Besides, it can adapt with their surrounding environment very well. It also has been reported to inhabit the extreme cold Antarctic region. Many strains of *Pseudomonas* have been isolated from this extreme cold region (Reddy *et al*., 2014). *Pseudomonas antarctic* was one of the strains that have been characterised and isolated from this polar region. Besides, proteolytic activity of this species has been reported by Martínez-Rosales and Castro-Sowinski (2011).

In relation to *Arthrobacter* sp., *Arthrobacter antarcticus* has been isolated from Southern Ocean sediment off the Antarctic (Pindi *et al*., 2010). This species has been reported to produce cold active protease (Kim *et al*., 2010). In this study, there were three strains of *Arthrobacter* sp. that have been

Table 1. Highest sequence similarity of isolated bacteria that showed proteolytic activity on skim milk agar based on BLAST sequence alignment

Isolates	Possible organism	Sequence Length (bp)	Max Score/ Total Score	Max. Identity (%)	GenBank accession number
BB1	*Pseudomonas brassicacearum*	1051	1230	94	NR_116299.1
BB8	*Pseudomonas gessardii*	723	1033	97	NR_024928.1
BB16	*Pseudomonas silesiensis*	566	737	97	NR_156815.1
CR25	*Paenibacillus xylanexedens*	618	863	98	NR_044524.1
DI23	*Pseudomonas mandelii*	1199	2049	99	NR_114216.1
DI25	*Pseudomonas mandelii*	1185	1661	95	NR_114216.1
MP1	*Pseudomonas marginalis*	718	989	96	NR_112072.1
MP2	*Pseudomonas mandelii*	1216	2122	98	NR_114216.1
MP4	*Pseudomonas baetica*	1194	1724	95	NR_116899.1
MP7	*Pseudomonas prosekil*	625	939	99	NR_132724.1
MP8	*Pseudomonas corrugata*	1447	758	95	NR_117826.1
MP9	*Pseudomonas canadensis*	595	758	98	NR_156852.1
MP12	*Pseudomonas prosekil*	638	941	99	NR_132724.1
MP13	*Pseudomonas migulae*	905	1181	93	NR_114223.1
ROB1	*Pseudomonas helmanticensis*	1260	2189	98	NR_126220.1
ROB6	*Pseudomonas arsenicoxydans*	1717	2303	97	NR_117022.1
ROS1	*Pseudomonas azotoformans*	1476	2283	96	NR_113600.1
ROS2	*Pseudomonas azotoformans*	803	1384	99	NR_113600.1
ROS3	*Pseudomonas lactis*	1150	1557	95	NR_156986.1
ROS4	*Pseudomonas marginalis*	1208	2106	98	NR_117821.1
ROS5	*Pseudomonas marginalis*	1210	2021	99	NR_117821.1
ROS8	*Pseudomonas lactis*	627	601	99	NR_156986.1
SC1	*Arthrobacter globiformis*	426	254	87	NR_112192.1
SC2	*Arthrobacter globiformis*	676	1919	97	NR_026236.1
SC3	*Arthrobacter oxydans*	1260	950	96	NR_112192.1
SC6	*Pseudomonas mandelii*	1172	1775	98	NR_114216.1
SC8	*Pseudomonas mandelii*	1218	1459	96	NR_114216.1
SC10	*Pseudomonas mandelii*	1177	1655	96	NR_114216.1
SC11	*Pseudomonas prosekii*	580	883	99	NR_132724.1
SC12	*Pseudomonas meridiana*	610	944	99	NR_025587.1
SC13	*Pseudomonas prosekii*	653	1016	99	NR_132724.1
SC14	*Pseudomonas mandelii*	1202	2021	99	NR_114216.1
SC15	*Pseudomonas meridiana*	1713	1858	95	NR_025587.1
SC17	*Pseudomonas meridiana*	1168	1777	96	NR_025587.1

isolated from Spike Cape, Scott Coast, Southern Victoria Land. Regarding *Paenibacillus* sp., only one strain has been isolated from soil sample originated from Cape Royds, Ross Island that showed proteolytic activity. This bacilli class strain has been detected in a various type of environment including soil, water and plant. Several novel *Paenibacillus* sp. have been isolated from Antarctic samples such as *Paenibacillus antarcticus* (Montes *et al*., 2006) and *Paenibacillus wynnii* (Rodríguez-Díaz *et al.*, 2005). Besides, several researchers have reported proteolytic activity of this species previously (Li *et al*., 2013).

Out of 35 colonies, 10 colonies that showed clear halo zone formation within 24 hours were further analyzed by enzymatic assay. Azo-casein enzymatic assay was performed in order to quantify the proteolytic activity of these bacteria. Azo is a synthetic dye that attaches with the casein molecule and can be measured through absorbance 340 to 440 nm. When this substrate and the bacteria culture was incubated together, protease broke down the Azo-casein and thus release the dye into reaction supernatant. Thus, higher absorbance reading implies higher proteolytic activity. This technique was an adaptation of the previous studies (Martínez-Rosales & Castro-Sowinski, 2011). Results for the enzymatic assay was illustrated in the graph (Figure 2). From the graph, we can conclude that sample SC8 showed the highest protease activity with reading 0.2012 ± 0.003, followed by sample ROS8 (0.1953 ± 0.002) and sample SC11 (0.1843 ± 0.002). Only SC8 showed statistically significant difference with p-value less than 0.05. It showed higher proteolytic activity compared to *P. aeruginosa* at 20°C.

In nature, protease plays an important role as a tool for degradation of sedimentary nitrogenous compounds. These compounds were abundantly precipitated in sediments providing nitrogen sources for the microorganisms including in Antarctic regions (Dall'Osto *et al.,* 2017; Vero *et al.,* 2019). Evolution of the enzyme sequence and structure enable it to work at lower temperature. Some of the findings displayed that amino acid composition contributes to the flexibility of the enzyme structure and unique loop formation providing more accessible active site (Latip *et al.*, 201; Hashim *et al.*, 2018).

In industrial enzymes, the protease is one of the components used to tenderize the meat, but commercial tenderizer enzyme cannot work at a lower temperature (Naveena *et al*., 2004; Gokoglu *et al*., 2017). This cold active protease has the potential to be applied in the poultry industries. This is because, after processing the meat, they will be stored in the freezer for long-term storage or for export purposes. In order to take advantage while storing, this enzyme can be applied to the meat. Therefore, once the customer buys it, it will be ready for cooking. With this initiative, it will be time and energy saving. Besides, the end user also can apply this cold active protease when they want to cook the meat, after taking out of cold storage. This cold-active protease also has been studied on their potential for detergent industries (Park *et al.,* 2018).

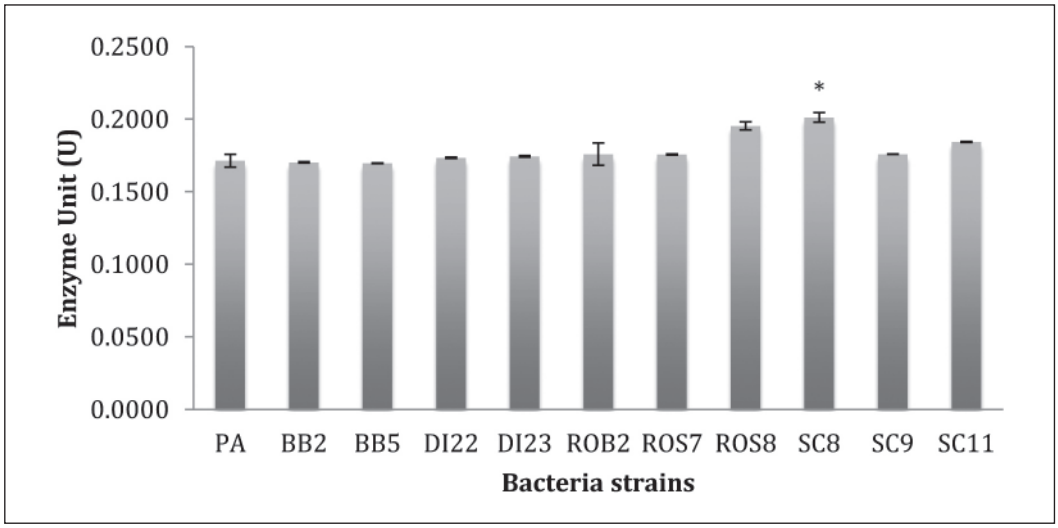

Fig. 2. Protease activity of ten selected bacteria strains after 24 hours incubation period at 20°C and 200 rpm. *P. aeruginosa* (PA) was used as positive control. Results were expressed as mean ± SEM. * $p < 0.05$.

CONCLUSION

Enzymes have been widely accepted as substitute to the chemical catalysts. This biocatalyst has played a major role not only at industries level but also in our daily life. Overall, we have successfully discovered cold active protease activity produced by psychrophiles isolated from Antarctic region. In future, purification and characterization of this enzyme is essential in order to identify the optimum conditions for enzyme activity. Once the optimum conditions meet the market demand, production of this enzyme can be scaled up for commercialization purpose.

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