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ISOLATION AND CHARACTERIZATION OF PROTEASE WITH COLLAGENOLYTIC ACTIVITY PRODUCED BY BACTERIA FROM DIFFERENT SOURCES FOUND IN PHRA NAKHON SI AYUTTHAYA PROVINCE

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Abstract – Collagen is generally extracted by non-environmentally friendly chemical methods, with occurrence restricted to several proteases due to its complex structure with hydrogen bond binding. Discovery of an enzyme with the potential to extract collagen would be beneficial. This research isolated and characterized 52 proteases with bacterial collagenolytic activity, which produced a clear zone surrounding colonies on Basal medium skim milk agar and Davis minimal gelatin agar. Two potential isolates as BS25 and BS44 showed maximum protease production in Basal medium gelatin broth, with specific activities of 158.30 and 167.25 Umg^{-1}, respectively. They were identified as *Chryseobacterium* sp. BS25 and *Pseudomonas aeruginosa* BS44. The optimum pH of crude enzyme from BS25 and BS44 isolates was 7.0, while optimum temperatures were 50 and 60ºC, respectively. The BS25 enzyme was stable in pH range 5.5 to 8.0, whereas the BS44 enzyme showed higher stability in a wider pH range (5.5 to 11.0). The BS25 enzyme was stable at 30ºC, while the BS44 enzyme retained activity over a wider range of 30 to 50ºC. Both the BS25 and BS44 isolates showed high potential to produce protease with collagenolytic activity and enabled efficient extraction of collagen from protein waste or crocodile cartilage for commercial production.

INTRODUCTION

Collagen is the major fibrous element found in connective tissues of skin, scales, bones, tendons, cartilages, blood vessels and teeth of multicellular organisms including bovine, porcine, fish and other marine-derived animals (Suphatharaprateep *et al*., 2011; Savita and Arachana, 2015; Gautam and Azmi, 2017). Collagen has a triple-helical structure, with three polypeptide chains intertwining to form collagen molecules that are stabilized by hydrogen bonds. With high amounts of glycines (1,000 amino acids), prolines (360 amino acids) and hydroxyprolines (300 amino acids), collagen degradation is restricted to several proteases (Li *et al*., 2013). Extraction methods are generally based on the solubility of collagen in acid (Sinthusamran *et al*., 2013; Wang *et al*., 2014; Nagai, 2015) or alkaline solution (Kaewdang *et al*., 2014). Collagen extraction processes require large amounts of energy to maintain the high temperatures required, and also produce varying qualities of collagen products and toxic wastes that pollute the environment (Rochima *et al*., 2017). Microbial proteases with collagenolytic activity have recently attracted increased attention as a resource for collagen extraction because they offer relatively mild and more controllable reaction conditions, more substrate specificities and are environmentally friendly. Identification, isolation and characterization of novel strains for the production of enzymes at economical levels are ongoing processes (Adrio and Demain, 2014). Protein-rich wastes are suitable sources of proteolytic agents such as soil/sewage from local fish markets and slaughterhouse areas (Gautam and Azmi, 2017) and reservoir waste as skins, scales and fins (Mahboob, 2015; Rochima *et al*., 2017). Several bacterial isolates produce protease with collagenolytic activity including *Microbacterium liquefaciens* (Kanayama and Sakai, 2005), *Bacillus licheniformis* (Baehaki *et al*., 2012), *Bacillus* sp. DPUA 1728 (Lima *et al*., 2015), *B. megaterium* KM369985

(Savita and Arachana, 2015), *B. subtilis* ATCC6633 (Rochima *et al*., 2016) and *Pseudomonas* sp. CS-20 (Gautam and Azmi, 2017).

This study isolated selected protease-producing bacteria from different naturally rich sources of collagen. Proteases obtained from bacteria possessing high collagenolytic activity were characterized in terms of optimum pH and temperature, and pH and thermal stability.

MATERIALS AND METHOD

Sample collection

Samples for isolation of protease producing bacteria were collected from seven sources in Phra Nakhon Si Ayutthaya Province including pig and chicken skins and fish wastes from Chao Phrom Market, water from a frog pond, soil around the frog pond, crocodile cartilage buried in soil at Rajamangala University of Technology Suvarnabhumi and water from the crocodile pond at Si Ayutthaya Crocodile Farm.

Screening of isolates for collagenolytic activity

Proteases with bacterial collagenolytic activity were isolated from all samples by serial dilutions and the spread plate technique using two selective media. The first was Basal medium skim milk agar (BMSM agar) (wv^{-1}): 1.0% glucose, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4$, 1% skim milk and 1.5% agar, while the second was Davis minimal gelatin agar (DMG agar) (wv^{-1}): 0.7% K_2HPO_4, KH_2PO_4, 0.01% $MgSO_4$, 0.1% gelatin and 1.5% agar. The pH of each medium was adjusted to 7.0, and the plates were incubated at 37ºC for 48 hr. After incubation, DMG agar plates were flooded with 15% (wv^{-1}) $HgCl_2$ solution. Isolates that generated clear zone colonies on BMSM and DMG agars were selected for primary screening.

Primary screening of efficient proteases with bacterial collagenolytic activity

Selected isolates from the screening step were spotted on Basal medium gelatin agar (BMG agar) (wv^{-1}): 1% glucose, 0.5% yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4$, 1% gelatin and 1.5% agar. These plates were incubated at 37ºC for 48 hr. The ratio of the clear zone diameter to colony diameter was measured to select isolates with highest collagenase activity. The largest ratio was assumed to reflect the highest activity. Hydrolysis capacity (HC value) was calculated as described by Sreeja *et al.* (2013). Isolates with the highest ratio were selected for secondary screening.

Secondary screening of efficient protease-producing isolates

Isolates with the highest HC value on BMG agar were inoculated into 50 ml of BMG broth, pH 7.0. The culture was incubated at 37ºC on an incubator shaker at 200 rpm for 24 hr. The cell-free supernatant was recovered by centrifugation (8,000 rpm for 30 min at 4ºC) and used to measure enzyme activity.

Protease assay

Protease activity of the crude enzyme was assayed using casein as the substrate following the method of Alencar (2003). The activity was performed in a total volume of 1,000 µl containing 250 µl of a suitable concentration of enzyme and 250 µl of 2% (wv^{-1}) casein (Sigma, USA) in 50 mM phosphate buffer pH 7 (PB), incubated for 10 min at 37°C and then terminated by adding 500 µl of 10% (wv^{-1}) trichloroacetic acid (TCA). The sample was mixed and centrifuged at 8,000 rpm for 30 min. Then, 100 µl of supernatant was determined following the Lowry method *et al.* (1951) and measured for absorbance at 650 nm. One unit of enzyme activity was defined as liberating 1 µg of tyrosine in 1 min of hydrolysis condition.

Identification of bacterial strains

Isolated bacteria that showed the highest enzyme activity were identified based on morphological and biochemical characteristics and 16S rDNA sequencing. Single colonies obtained were sequenced by Macrogen, Inc. South Korea. DNA was analyzed using forward primer 785F (5'-GGATTAGATACYCYWGT-3') and reverse primer 1492R (5'- CCGTCAATTCMTTTGAGTTT-3'), and resulting sequences were compared with other DNA sequences deposited in the GenBank database using the BLAST program.

Optimum pH and pH stability

Optimum pH of the protease was determined by measuring the casein hydrolyzing activity ranging from 5.5 to 11.0 at 37ºC. The appropriate pH was performed using the following buffers: 50 mM acetate buffer (5.5 and 6.0), 50 mM phosphate buffer (7.0 and 8.0) and 50 mM glycine-NaOH buffer (9.0 to 11.0). The pH stability of the protease was determined after incubating the enzyme mixture in

different pH buffers for 2 hr at room temperature. Then, the residual activity was measured under optimum conditions of pH and temperature. The enzyme activity before incubation was regarded as 100% activity. Triplicate samples were used for each treatment.

Optimum temperature and thermal stability

The optimum temperature was investigated by incubation of the reaction mixture at pH 7 for 10 min at 30 to 80ºC. The thermal stability of the protease was determined by incubating the enzyme in the appropriate buffer within the temperature range 30-80ºC for 2 hr. Then, the residual activity was measured under standard assay condition of pH and temperature. Triplicate samples were used for each treatment.

RESULTS AND DISCUSSIONS

Screening of isolates to identify proteases with collagenolytic activity

Screening was based on the clear zones on BMSM and DMG agars from 52 bacterial isolates that could produce proteases with collagenolytic activity, as shown in Table 1. Crocodile cartilage buried in the soil was the richest source in 32 isolates (61.54%) followed by pig skins with 7 isolates (13.46%), fish wastes with 6 isolates (11.54%), water from the frog pond with 4 isolates (7.69%), water from the crocodile pond with 2 isolates (3.85%) and chicken skins with 1 isolate (1.92%). No isolates were obtained from soil surrounding the frog pond (Table 1). BMSM agar using skim milk as a selected protein is a good method for rough screening of protease-producing bacteria because its structure is easily hydrolyzed by protease. Thus, the number of proteolytic bacteria obtained from BMSM agar was than higher from DMG.

Primary screening of efficient protease-producing isolates

Agar medium containing gelatin for screening protease-producing bacteria through the zone of hydrolysis has been reported by many researchers (Suphatharaprateep *et al*., 2011; Ahmed *et al*., 2017; Gautam and Azmi, 2017). Among the 52 isolates that showed protease activity, 13 exhibited HC values ranging 3.50-6.00 (Table 2). The BS25 and BS44 strains isolated from crocodile cartilage buried in the soil showed the highest HC values (6.00 and 5.00, respectively). Soil samples contain thousands of different species of bacteria (Wawrik *et al*., 2005). Crocodile cartilage provided the opportunity to investigate bacteria showing the ability to produce protease with collagenolytic activity. However, the HC value is only a rough method and may not accurately reflect the true collagenase activity. Thus, 13 isolates with the highest HC values were retested by the colorimetric method using casein as the substrate.

Secondary screening of efficient proteases with bacterial collagenolytic activity

The two bacterial isolates BS25 and BS44 exhibited highest specific activity at 158.30 and 167.25 Umg^{-1}, respectively (Table 2). This result corresponded with HC values in the previous section, indicating that these isolates had the capacity to hydrolyze collagen. Therefore, the crude enzymes obtained

Table 1. Screening of protease collagenolytic producing bacteria from samples of waste, water and soil grown on BMSM and DMG agar

Source	Medium		Isolates number (%)
	BMSM	DMG	
Chao Phrom Market			
Pig skins	4	3	7 (13.46)
Chicken skins	1	0	1 (1.92)
Fish wastes	1	5	6 (11.54)
Rajamangala University of Technology Suvarnabhumi			
Water from frog pond	3	1	4 (7.69)
Soil around the frog pond	0	0	0 (0.00)
Cartilage crocodile buried in the soil	29	3	32 (61.54)
Sri Ayutthaya Crocodile Farm			
Water pond	2	0	2 (3.85)
Total 40	12	52 (100.00)	

Table 2. Evaluation of protease activity of some isolates on BMG agar plate through HC value and specific enzyme activity

Isolates	Isolation source	HC value	Specific activity (Umg^{-1})
BP1	Pig skin	3.75	40.80±2.11
DF2	Fish waste	4.00	10.58±0.94
DF7	Fish waste	3.75	56.05±4.81
BMF1	Water from the frog pond	3.50	49.37±4.22
GMDB1	Crocodile cartilage buried in the soil	3.50	6.30±1.11
GMDB3		3.50	14.40±1.53
BS18		4.29	30.01±1.93
BS25		6.00	158.30±1.57
BS221		3.67	45.43±0.56
BS33		3.50	16.83±2.14
BS44		5.00	167.25±1.42
BS514		3.88	17.80±0.78
BS516		3.57	9.65±1.84

from both isolates were selected to further identify and characterize enzyme properties.

Identification of bacterial strain

Biochemical and morphological characteristics and analysis of 16S rDNA sequencing identified the BS25 and BS44 strains as *Chryseobacterium* sp. BS25 (100% similarity, GenBank accession number MT254825.1) and *Pseudomonas aeruginosa* BS44 (100% similarity, GenBank accession number MT254826.1), respectively.

Collagenase production was previously reported by *Chryseobacterium* sp., isolated from ayu (*Plecoglossus altivelis*) affected by cold-water disease (Nakayama *et al*., 2015) and by *Pseudomonas aeruginosa* from soil/sewage samples collected from local fish markets and slaughterhouse areas of Bilaspur and Shimla, India (Gautam and Azmi, 2017). The *Pseudomonas* family can cause infectious disease in humans (Gautam and Azmi, 2017). Therefore, the BS44 strain was more suitable for further application in collagen extraction than BS25. This is the first report of the isolation of BS25 and BS44 strains from crocodile cartilage buried in soil.

Optimum pH

The optimum pH of the crude enzymes BS25 and BS44 exhibited a similar trend with values of 7.0. However, relative activity of the BS25 enzyme rapidly reduced at pH 7.0-11.0, whereas the BS44 enzyme was highly active over a broad range of pH from 5.5-10.0 (at 76.67-79.70%) (Fig. 1). These results indicated that conditions used for initial isolation of the bacteria affected the behavior of the enzyme because BS25 and BS44 were isolated under neutral conditions (pH 7.0). Beside BS25 and BS44 enzymes, other protease with collagenolytic activity have been previously reported to have optimum pH of 7.0 such as proteases from *Photorhabdus luminescens* (Marokhazi *et al*., 2004), *Bacillus cereus* CNA1 (Suphatharaprateep *et al*., 2011) and *Bacillus licheniformis* F11.4 (Baehaki *et al*., 2012). However, the BS25 enzyme optimum pH differed from *Chryseobacterium* sp. P1-3 (8.0) (Hong *et al*., 2015), while optimum pH of the BS44 enzyme obtained from *P. aeruginosa* was different from those of the same genus as *Pseudomonas* sp. SUK (8.0) (Bhagwat *et al*., 2016), *Pseudomonas aeruginosa* MTCC 7926 (9.0), (Patil and Chaudhari, 2011) and *Pseudomonas* strain DY-A (10.0) (Zeng *et al*., 2003).

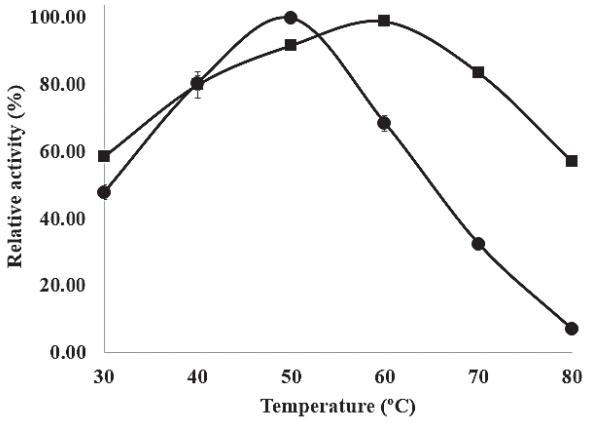

Fig. 1. Optimum pH of (l) BS25 and (%) BS44 enzymes

Optimum temperature

Relative activities of the crude enzyme BS25 and BS44 gradually increased with temperature, and exhibited optimum activity at 50 and 60ºC,

respectively. When the reaction temperature exceeded 50ºC, relative activity sharply decreased to 68.37% and 32.37% at 60ºC and 70ºC, respectively with loss of almost all activity at 80ºC (6.86%) for the BS25 enzyme. By contrast, the BS44 enzyme was active over a greater temperature range (40-70ºC), with relative activity ranging from 79.94 to 83.53% (Fig. 2). An optimum pH of 50ºC was previously reported for *Bacillus licheniformis* F11.4 from Palembang South Sumatra, Indonesia (Baehaki *et al*., 2012), *Bacillus* sp. DPUA1728 from Amazonian soil (Lima *et al*., 2015) and *Bacillus thuringiensis* from the Cirata Reservoir, Indonesia (Rochima *et al*., 2016). The BS25 enzyme revealed an optimum temperature that differed from *Chryseobacterium* sp. P1-3 (30ºC) (Hong *et al*., 2015), while optimum temperature of the BS44 enzyme was different from *P. aeruginosa* MCM B-327 (30ºC) (Zambare *et al*., 2011), *P. aeruginosa* (40ºC) (Yadav *et al*., 2010) and *P. aeruginosa* RGSS-09 (75ºC) (Gaur *et al*., 2015).

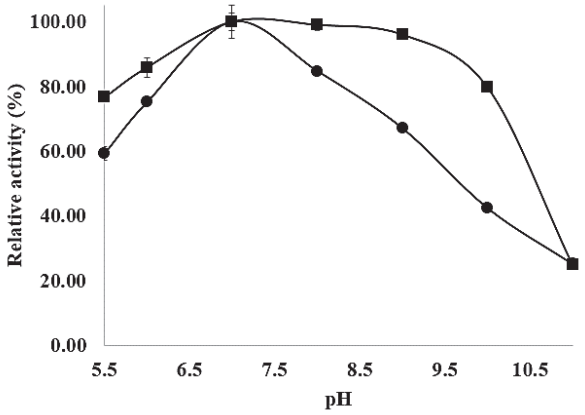

Fig. 2. Optimum temperature of (l) BS25 and (%) BS44 enzymes

pH stability

The BS44 enzyme was stable at a wide pH range (5.5-11.0), whereas a slight loss of enzyme activity was observed for the BS25 enzyme incubated at pH values above 6.0. (Fig. 3). The BS44 enzyme was more stable than other protease with collagenolytic enzyme from *Klebsiella pneumonia* CNL3 (5.0-7.0) and *Bacillus cereus* CNA1 (6.0-8.0) (Suphathrarateep *et al*., 2011), *Nocardiopsis dassonvillei* NRC2aza (Abdel-Fattah, 2013) (6.0-8.0) and *Bacillus* sp. DPUA1728 (7.2-10.0) (Lima *et al*., 2015).

Thermal stability

Thermal stability is a significant property of microbial enzymes for various applications. Mass

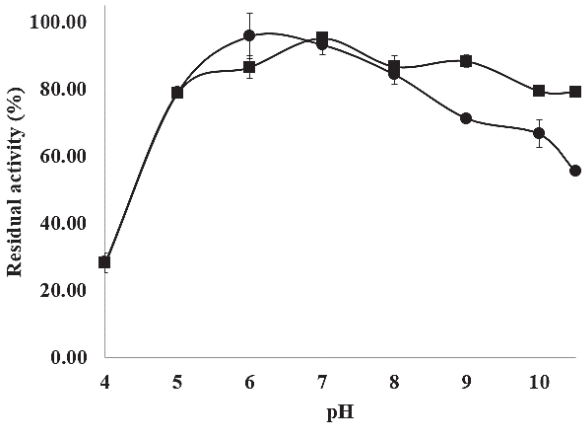

Fig. 3. pH stability of (l) BS25 and (%) BS44 enzymes

transfer increases and viscosity of the substrate decreases during hydrolysis at high temperature (Nigam, 2013). Activity of the BS25 enzyme was stable at 30ºC and rapidly decreased when the temperature increased (40-80ºC). The BS44 enzyme was stable between temperatures of 30-50ºC and lost almost all activity after incubation at 70ºC for 2 hr. (Fig. 4). Both enzymes were more stable than proteases from *Microbacterium liquefaciens* (0-20ºC) (Kanayama and Sakai, 2005), *Klebsiella pneumoniae* CNL3 (< 37ºC) and *Bacillus cereus* CNA1 (<40ºC) (Suphathrarateep *et al*., 2011) but less stable than *Bacillus* sp. UPUA1728 (45-60ºC) (Lima *et al*., 2015) and *Cynoscion leiarchus* (25-60ºC) (Oliveria *et al*., 2017).

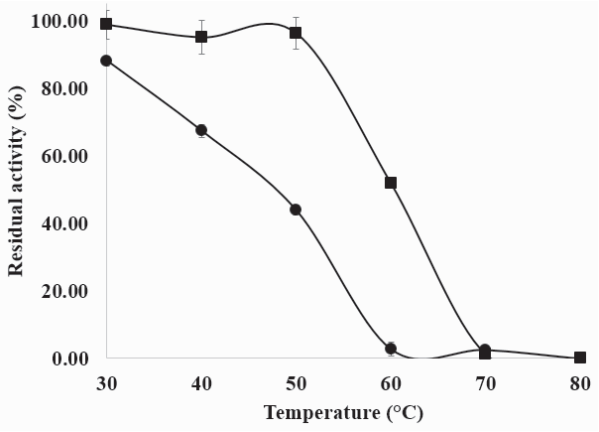

Fig. 4. Thermal stability of (l) BS25 and (%) BS44 enzymes

CONCLUSION

BS25 and BS44 enzymes were isolated from soil containing crocodile cartilage as the major source of proteases with bacterial collagenolytic activity. The

BS44 enzyme had a higher optimum temperature (60°C) than the BS25 enzyme (50°C), with optimum pH at 7.0. The BS44 enzyme showed pH and thermal stability than BS25 enzyme. Both isolates showed potential as alternative sources to extract collagen from marine waste and crocodile cartilage.

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Conflict of Interest

The author declare no conflict of interests regarding the publication of this paper.

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