PROTEASE-PRODUCING BACTERIA ISOLATED FROM THAI RICE AND NATIVE THAI BEES (APIS CERANA)

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Abstract - Microbial enzyme production from bacteria, yeasts and molds is an essential event in the industries that consume approximately 90% of the world's total enzyme usage. Many microbial enzymes such as protease are also widely used in the manufacturing of various products such as cosmetics, pharmaceuticals, agricultural food and beverages. Two bacterial isolates were isolated from Thai rice and bees in Ratchaburi and Chiang Mai provinces, Thailand. From the morphological test and 16S ribosomal DNA sequence study, the isolate C13HN2 and KRrb_T2 were identified as Microbacterium paraoxydans and Paenibacillus hunanensis, respectively. All isolated bacteria could produce protease enzymes. The highest enzymatic activity of protease was produced by P. hunanensis KRrb_T2 (378.9967±1.335 U/mL) at 36 h. In summary, the bacteria from Thai rice and bees could be potential suitable sources of protease production for development by the food and beverage industries globally.

Keywords - Bacteria, Bees, Protease, Rice.

I. INTRODUCTION

Currently, many products include organic acids, amino acids, vitamins and enzymes. Enzymes are utilized for a variety of purposes, particularly as an intermediate in food and pharmaceutical manufacturing. Natural enzymes are expensive and scarce, thus they are used sparingly and a move toward manufacturing from alternative sources has occurred[1]. The second part of the twentieth century saw an unparalleled increase in our understanding of the application of microbes (bacteria and yeasts), their metabolic products and enzymes in fundamental study, as well as their potential industrial uses [2].

Microbial enzyme production from bacteria, yeasts and molds is an essential event in the industries that consume approximately 90% of the world's total enzyme usage [3]. Many microbial enzymes such as amylase, lipase and protease, are also widely used in the manufacturing of various products such as cosmetics, pharmaceuticals, agricultural food and beverages [4].

Proteases are employed in a diverse selection of beverage and food applications [5], including milk-clotting enzymes for cheese manufacturing, proteolysis is used to alter wheat gluten in bread and to degrade protein turbidity complexes in fruit juices and alcoholic beverages. In the brewing industry, proteases can reduce the turbidity in beer that appeals to more savvy food and beer customers. The purposes of this research were to investigate beneficial bacteria capable of producing protease enzymes that were isolated from Thai rice and bees from Ratchaburi and Chiang Mai provinces. The application of effective microorganisms can enhance the development of a thriving commercial brewing industry and help to explore the development of biodiversity in Thailand.

II. MATERIALS AND METHODS

A. Bacterial Cultures

Bacterial isolates C13HN2 and KRrb_T2 were stored in our culture collection at the Division of Microbiology, Department of Science and Bioinnovation, Faculty of Liberal Arts and Science, Kasetsart University. The isolate C13HN2 was isolated from Thai rice in the rice fields located in Pho Tharam district, Ratchaburi province, Thailand. The isolate KRrb_T2 was isolated from honey of native Thai bees (Apis cerana).

B. Morphological and Molecular Identification

The bacterial strains that were isolated from Thai rice and bees were incubated at 37°C for 24 h using nutrient broth (NB). They were basically characterized for surface, color, Gram staining, cell arrangement and spore forming under a light microscope (CX31; Olympus®, Japan) as described by Halebian et al. (1981) [6].

The genomic DNA of 2 bacterial isolates capable of enzyme production was extracted [7]. Almost complete 16 Svedberg units ribosomal ribonucleic acid (16S rRNA) gene (1.5 kb) was amplified using

the universal primer pair 20F (5′AGTTTGATCCTGGCTC-3′) and 1540R (5′-AAGGAGGTGATCCAGCC-3′) [8].[28] The 16S rDNA gene was amplified using polymerase chain reaction (PCR; MULYIGNE OPTIMAX; Labnet®; USA). The PCR products were purified using Nucleo Spin® Gel and a PCR Clean-up Kit (Invitrogen; USA). The purified PCR products were subjected to the FIRST BASE Company, Malaysia for sequencing of 16s rDNA using primers 20F and 1540R. The identities of nucleotide sequences of the 16S rRNA gene obtained were performed with BLAST analysis using the NCBI database (http://www.ncbi.nlm.nih.gov). The sequence information was provided regarding the deposition of DNA sequences. The 16S rRNA genes sequences were accessible via GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

The relationships and diversity were studied in the evolutionary lines of the protease-producing bacteria isolated from Thai rice and bee samples. The bacterial sequences were studied using the Bio Edit Sequence Alignment Editor version. 7.0.5.3 Primer using Clustal_W multiple alignment and the number of bootstraps was 1,000. The phylogenetic tree was developed by calculating the distance between the molecular sequences using the maximum likelihood (ML) method with the MEGA X program, version 10.1.8 to represent an evolution chart.

C. Preliminary screening for protease production

All the isolated bacteria were screened for their enzyme production ability on agar plates using protease from skimmed milk (skimmed milk 2 g, glucose 1 g, potassium phosphate 0.2 g, magnesium sulfate 0.2 g and agar 20 g in 1 liter of deionized water) and protease from gelatin (beef extract 3 g, peptone 5 g, gelatin 5 g and agar 20 g 1 liter of deionized water) [9,10]. The enzyme production was observed via the clear zone around the point inoculation on the agar plate. The proportion of clear zone (d1/d2) was then calculated where d1 was diameter of the clear zone and d2 was diameter of the bacterial colony, with both measurements in millimeters.

D. Determination of Protease Enzyme Activity

The bacterial strains were overnight cultured to perform bacterial inoculum and then added in the NB at pH 7.0 prior to incubation at 37°C for 0, 12, 24, 36 and 48 h under shaking of 150 rpm. The bacterial culture was then centrifuged at 3,300×g for 15 min at 4°C. The protease measurement was conducted using the obtained cell-free supernatant according to Han and Shahidi [11]. One milliliter of crude enzyme was added to 1 mL of azocasein (1% w/v in deionized sterile water, pH 7.5) and the mixture was then incubated for 10 min at 40°C. The reaction was stopped by adding 2 mL of 0.4 M trichloroacetic acid (TCA) reagent, incubated at 40°C for 30 min (left until settled), incubated at room temperature and then centrifuged at 3,300×g for 15 min. One milliliter of supernatant was added with 5 ml 0.4 M Na_2CO_3, and Folin's reagent in the ratio 1:1. The mixture was then incubated for 10 min at room temperature. The enzyme activity was observed, and the absorbance (OD_{660}) was measured using a spectrophotometer (G10S UV-Vis; Thermo scientific®; USA) and non-enzyme sample was used as blank. One unit of protease was calculated as the amount of the enzyme yielding the equivalent of 1 µmol of tyrosine per minute under the conducted assay conditions.

$$(OD_{660} / \text{Slope} \times 1 / 10) \times 5$$

Where OD_{660} = Optical density at 660 nm
L-tyrosine standard = Slope standard graph (y = 0.015)

1= Reaction enzyme volume
10= Time reaction enzyme
5 = Total volume enzyme activity

III. RESULTS AND DISCUSSION

A. Bacterial Identification

The morphological characteristics of all isolates were examined using Gram staining and observed under a microscope. It was found that the growth characteristics on culture medium showed the colony sizes in the range 2–8 mm, the colony shape was round, and amorphous. Cell shapes were round and rod-shaped. The isolate KRrb_T2 produced endospores inside cells.

After the 16s RNA gene analysis of selected bacteria, it was found that the isolates C13HN2 and KRrb_T2 had similarity with Microbacterium paraoxydans and Paenibacillus hunanensis of 100, and 96%, respectively (Table 1). Subsequently, the 16s RNA gene sequences of the selected bacteria were evaluated closely using phylogenetic tree construction (Fig. 1,2). Based on the phylogenetic tree construction, the maximum likelihood tree confirmed the placement of the isolate C13HN2 to M. paraoxydans, while the isolate KRrb_T2 was P. hunanensis.

Bacterial isolates	Accession number	Closest species	Similarity
C13HN2	MZ081649	Microbacterium paraoxydans	100%
KRrb_T2	MZ081650	Paenibacillus hunanensis	96%

Table 1 Molecular identification of selected bacteria that were isolated from rice and bee samples using amplification of 16S rRNA gene.

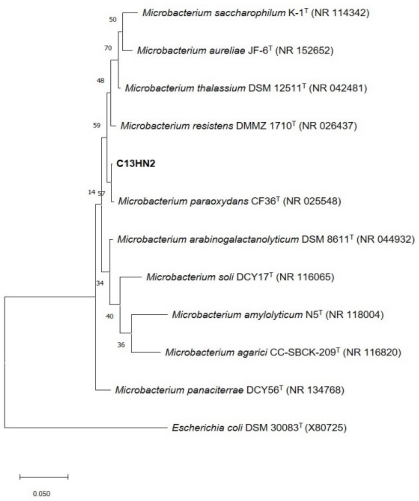

Fig. 1 16S rRNA gene-based maximum likelihood tree showing phylogenetic relationships of C13HN2 relatives to type strains of other Microbacterium species. E. coli DSM 30083^T was used as an outgroup. Numbers on branches indicate percentage bootstrap values of 1,000 replicates. The scale bar represents 0.050 changes per nucleotide.

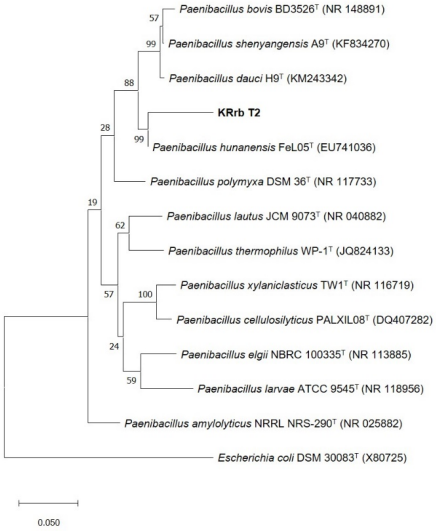

Fig. 2 16S rRNA gene-based maximum likelihood tree showing phylogenetic relationships of KRrb_T2 relatives to type strains of other Paenibacillus species. E. coli DSM 30083^T was used as an outgroup. Numbers on branches indicate percentage bootstrap values of 1,000 replicates. The scale bar represents 0.050 changes per nucleotide.

B. Screening of protease enzyme-producing bacteria

Table 2 showed that the isolated bacteria had potential to produce protease enzymes and that the protease could be catalyzed by a variety of raw materials. Moreover, protease can reduce the turbidity in beer that is a unique characteristic of this enzyme. Skim milk and gelatin are the best nitrogen source for protease enzyme synthesis [12]. Several reports revealed that Bacillus sp. could use skim milk, yeast extract, casein, and peptone for increasing the yields of protease enzyme [13,14]. Protease enzymes are synthesized by microorganisms using precursors that are inactive to avoid undesired protein breakdown [15].

Isolate	Skimmed milk agar	Gelatin agar
M. paraoxydans C13HN2	11.66±4.93	4.06±1.07
P. hunanensis KRrb_T2	3.11±0.68	5.45±1.43

Table 2 Enzyme transparent circle diameter (mm) of bacteria displayed on skimmed milk and gelatin agar. Values represent the mean ± SD of three independent experiments.

C. Protease enzyme activity

The two isolates of promising bacteria were assayed for protease enzyme activity (Table 3). Interestingly, the production of protease by P. hunanensis KRrb_T2 was highest at 36 h (378.9967±1.33 U/ml). Indeed, M. paraoxydans C13HN2 also exhibited high activity of protease production.

The study demonstrated that P. hunanensis KRrb_T2 was the best performer with the highest protease activity at up to 378.99±1.33 U/mL at 36 h. The study of Asha & Palaniswamy [16] reported that Bacillus cereus FT that was isolated from soil could exhibit protease activity at up to 165 U/mL at 48 h. However, the study of Singh and Bajaj [17], revealed the protease activity of Bacillus licheniformis K-3 was up to 1321 U/mL at 24 h which was about 4 times higher than for the P. hunanensis KRrb_T2 in the current study. Therefore, further research is needed to optimize the key factors for even higher production of protease.

Protease enzymes, also known as peptidyl-peptide hydrolases, are valuable enzymes for industrial sector that catalyze the breakdown of the protein molecule's peptide link. Protease enzymes form 50–65 % of the worldwide industrial enzyme market, with alkaline protease accounting for most of this share [18]. Protease enzymes are a commercially important class of extracellular microbial enzymes employed widely in a variety of manufacturing, particularly in the fermentation industry and pharmaceutical production [19].

Time of incubation (h)	Isolate	
	M. paraoxydans C13HN2	P. hunanensis KRrb_T2
0	0±0.0	0±0.0
12	338.21±1.50	338.99±1.33
24	287.66±1.20	364.66±1.66
36	290.66±0.66	378.99±1.33
48	318.55±1.54	320.66±0.87

Table 3 Enzyme activity (U/mL) of protease produced by two promising bacteria. Values represent the mean ± SD of three independent experiments.

IV. CONCLUSION

Thai rice and bees are promising sources for diversified microorganisms of which the current study indicated that bacterial strains are beneficial microorganisms. Two isolated strains had high ability to produce protease enzymes. These prominent strains were non-pathogenic and have high potential for cost-effective production of protease. Thus, effective bacterial strains could be developed for novel environmental-benign production in the food and beverage industries.

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