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Original Article

Effective Enzyme-Producing Bacteria Isolated from Diversified Thai Rice and Native Thai Bees (Cavity Nesting Honey Bees) and their Potential for Production of Protease Enzymes

Thapakorn Chumphon[1], Yaowanoot Promnuan[2], Sujinan Meelai[3], Saran Promsai[1,2,*]

ABSTRACT

Background: Thailand is one of the most bio-diversified countries in Southeast Asia, especially regarding plants, animals and microbes that impact directly on human and animal life. **Methods:** Of the 201 bacterial isolates that were screened from Thai rice and bees in Ratchaburi and Chiang Mai provinces, Thailand, most were classified in the genus *Bacillus*. **Result:** From the morphological test and 16S ribosomal DNA sequence study, three strains, *Bacillus flexus* KRptI_S2, *Microbacterium paraoxydans* C13HN2 and *Paenibacillus hunanensis* KRrb_T2, could produce amylase and protease enzyme and non-hemolytic activity at higher temperature. The highest enzymatic activity of protease was produced by *P. hunanensis* KRrb_T2 (378.9967±1.335 U.mL^{-1}) at 36 h. **Conclusion:** 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.

Key words: Bacteria, Bees, Biodiversity, Protease Enzyme, Rice.

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INTRODUCTION

Biodiversity refers to the presence of a diverse range of living organisms (plants, animals and microbes) in their natural habitat.[1] Not only is it the basis of life that underpins the important services provided by ecosystems but also it supports peoples' livelihoods and sustainable development in all areas of activity, including industry, agriculture, livestock and mining.[2]

Thailand is a country in Southeast Asia and at 513,120 km^2 it is the 50th largest in the world.[3] Thailand is divided to six geographical regions, based on natural features including landforms and drainage, as well as human cultural patterns. There are north, northeast, central, east, west and south regions.[4]

The west region of Thailand borders Myanmar and its geography is distinguished by towering mountains and steep river valleys.[5] Water and minerals are essential natural resources as well. The west area of 53,769 km^2 consists of 5 provinces: Kanchana-buri, Phetchaburi, Prachuap Khiri Khan, Tak and Ratchaburi.[6]

Thailand's agriculture is extremely competitive, diverse and specialized, and its exports are quite successful on a global scale. The most important crop in Thailand is rice (*Oryza sativa*),[7] with 60 % of 13 million farmers producing it on more than half of all farmed land making a significant exporter in the global rice market. The largest rice-producing regions are the central and Ratchaburi province. In 2019, 513,398 acres farmland were used for rice cultivation in Thailand.[8] Thai rice is not only a main food ingredient, it also is a pivotal part of Thai culture.

Ratchaburi is full of various geographical, mostly mountainous terrain and little lowland that is diverse and provides habitats for many species of plants and animals. Its land area is 1,284,074 acres, with a forest area of about 489,955 acres or 38.16 % of the total area. Forests also provide habitat for a vast array of animals and insects.[9] Bees (*Apis* spp.) are flying insects and important pollinators for many plants in forests. In Thailand, there are various bee species, especially native Thai bees such as *A. andreniformis, A. florea, A. mellifera, A. dorsata* and *A. cerana*.[10] Bees are good pollinators as part of their visits to flowers to seek nectar or pollen and this results in them transferring pollen grains between flowers, leading in terms of pollination. Honeybees are particularly efficient pollinators because to their hairy bodies, which allow them to gather up significantly more pollen as they move about inside flowers. Bees are found in rice fields and adjacent areas. Honeybee production offers many health benefits for human body such as food, beverages, cosmetics and medicines.[11,12]

Microorganisms are organisms (bacteria, yeasts and molds) that spend their life at a size too tiny to be seen with the naked eyes.[13] They can be found in all environments, including rice and bees. Many of the microorganisms are considered beneficial for humans and some microorganisms are able to produce high value substances such as enzymes. Enzymes are naturally occurring catalysts created by living organisms, plants, animals and microbes,[14] as part of the life need a vast and diversified collection of chemical processes. They are engaged in all life-sustaining activities such as DNA replication and transcription, as well as protein synthesis, metabolic and signal transduction. Their capacity to highly selective chemical reactions has improved their industrial utility. Enzymes are the catalytic cornerstone of a metabolism, and they are the subject of extensive international research, not only

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in the scientific community, but also in a variety of sectors particularly in industrial sector. Enzymes are well-known biocatalysts that carry out a wide range of chemical reactions and they're employed in the detergent and food sectors commercially, wine, beer, cheese and chemical substance production.[15]

Currently, many products include organic acids, amino acids, vitamins and enzymes. Enzymes are utilized for a variety of purposes, particularly as an intermediate in the 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.[16] 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.[17]

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.[18] 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.[19]

Proteases are employed in a diverse selection of beverage and food applications,[20] 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 examine the diversity of bacteria in Thai rice and bees from Ratchaburi and Chiang Mai provinces and to screen them for beneficial bacteria capable of producing protease enzymes. The application of effective microorganisms can enhance the development of a thriving commercial brewing industry and also help to explore the development of biodiversity in Thailand.

MATERIALS AND METHODS

Sample collection

Thai rice

Rice (*Oryza sativa*) cultivar Pathum Thani I, Suphan Buri I and Riceberry samples were collected from different rice fields located in Pho Tharam district, Ratchaburi province, Thailand (13°51′49.1″N, 99°51′18.2″E). Generally, the mature rice crops from the field were to be harvested at 105–150 days after crop establishment depending on type of rice crop, geography and climate. Thai farmers in the western region usually cultivate rice in May and the rice is harvested in September. A total of 50 plants were pulled out at random in each sampling field (stems, roots and seeds, including soil in the planting plot) from 7 spots of 39.536 acres to guarantee that the greatest amount of plant material was gathered.

Bees

Bees, honeybees and nests of Thai-native bees (*Apis cerana*) were collected many parts from Chiang Mai province, Thailand (18°59′49.0″N 98°55′48.2″E). In total, 17 samples were collected on July 2016 and stored at -20°C until use.

Sample preparation

The samples of Thai rice were rinsed properly with water to eliminate any dirt from the root materials. All leaves, stems, leaf sheaths and roots were sorted. The samples were cut into about 5 cm lengths and cleaned with a three-step surface sterilized method that modified from Kampapongsa and Kaewkla (2015).[21] The cleaning process was divided into 3 steps. First the sample was washed with 70% alcohol for 60 sec and then with 3% sodium hypochlorite, with the final washing with sterile deionized water. The cleaned samples were divided into 1 cm length pieces. The samples from bees were classified into bee larvae, bees, bee pollen and honey prior to bacterial isolation and observation of diversity in the next step.

Isolation of bacteria from Thai rice and bees

Bacterial strains employing the serial dilution procedure, were extracted from 1 g of each sample as (modified from Promsai *et al*., 2018).[22] The samples of Thai rice and bees, including pollens and brood cells were ground, added with 10 mL sterile water and vortexed for 1 min, before being diluted in about 10 times until the suitable dilution was achieved. The diluted samples were then spread on nutrient agar (NA: Merck®, Germany) agar containing 25 mg mL^{-1} cycloheximide and then incubated at 37°C for 24-48 h. The different-shaped colony was randomly picked for streaking on agar media.

Morphological 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).[23]

Screening for enzyme-producing bacteria

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), protease from gelatin (beef extract 3 g, peptone 5 g, gelatin 5 g and agar 20 g 1 liter of deionized water), lipase (peptone 10 g, sodium chloride 5 g, calcium chloride 0.1 g, Tween 80 10 mL and agar 20 g 1 liter of deionized water) and amylase (soluble starch 2 g, nutrient medium 8 g and agar 20 g in 1 liter of deionized water).[24-27] 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.

Molecular identification

The genomic DNA of 15 bacterial isolates capable of enzyme production was extracted.[22] 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′).[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/).

Phylogenetic analysis

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.

Hemolytic activity

Bacterial isolates from Thai rice and bees were investigated for hemolytic activity using the stab inoculation method described by Chumphon *et al.* (2016).[29] The strains were cultured on bovine blood agar consisting of 0.5% sodium chloride, 1% tryptose and supplemented with 7% bovine blood for 48 h at 37 °C. *Bacillus cereus* was used as the positive control and *Lactobacillus plantarum* as the negative control. Strains showing green-hued zones around the colonies were classified as α-hemolysis. Strains which had not changed were classified as γ-hemolysis. Both α-hemolysis and γ-hemolysis were classified as displaying non-hemolytic activity. The clearing around colony was indicated that strains had hemolytic activity or were β-hemolysis.

Tolerance to high temperature

The tolerance to high temperature of bacteria was tested by their production of protease enzyme. The bacterial isolates were cultured in NB and then incubated at 40, 45, 50 and 55 °C for 48 h. After that, the survival of bacteria was examined using the streak plate technique on NA and incubated at 37 °C for 48 h. The tolerance of high temperature was observed for the colony on the surface of the medium.[30]

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 (1995).[31] 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.

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

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

Statistical analysis

All experiments were statistically designed.[32] All data were analyzed by one-way ANOVA and the treatment means were separated using Duncan's test at $P \leq 0.05$ considering as significant difference (SPSS V.22 software, SPSS Inc.; IBM®; USA).

RESULTS AND DISCUSSION

Isolation of bacteria and screening for enzyme-producing strains

In total, the Thai rice and bees produced 201 bacterial isolates that were classified as Gram-positive bacteria (62.2%) and Gram-negative bacteria (37.8%), as shown in Table 1 Among these bacteria, 15 isolates (7.5%) had the ability to produce amylase and protease (Table 2). None of the isolates exhibited lipase production.

The biodiversity was evaluated of microorganisms isolated from Thai rice and bee sources in Ratchaburi province, Thailand. The results discovered that the vast majority of isolated bacteria belonged to the genus *Bacillus* and the remainder to other genera. Notwithstanding there were no data on yield for the biodiversity of all genera, the results showed a high number of *Bacillus* (80%). Thailand has an average annual daytime temperature in range 30 °C (86° Fahrenheit) to 37 °C (98.6° Fahrenheit); the results of the isolation implied that *Bacillus* spp. can survive and grow in this environment. There are uniquely specialized endospores the in *Bacillus* that can resist the humid tropical climate and still grow actively, belonging to the family *Bacillaceae*, Gram-positive bacteria are rod- shaped, like a stick and the dominant organelle moves using flagella. They are facultative anaerobes. *Bacillus* spp. have been isolated from a variety of natural habitats across the world, including soil, water, insects, dust, trees and animals.

The isolated bacteria were able to produce a variety of enzymes, especially protease. Therefore, determination of their ability to produce protease was tested using 2 culture media (skimmed milk and gelatin medium). The results showed that the isolated bacteria had potential to produce protease enzyme 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.[33] Several reports revealed that *Bacillus* sp. could use skim milk, yeast extract, casein and peptone for increasing the yields of protease enzyme.[34,35] Protease enzymes are synthesized by microorganism using precursors that are inactive to avoid undesired protein breakdown.[36]

Product cost depends on the prices of raw materials and these may fluctuate. Therefore, it is necessary to identify lower priced new raw materials that provide a stable enzyme product. Several studies have reported on the production of enzymes in other raw materials such as skimmed milk, beer, waste material and agricultural waste. The current screening of protease-producing microorganisms in Ratchaburi and Chiang Mai province, Thailand suggested that bacteria can grow and survive at high temperatures and in alternative raw materials. In further studies, the effective bacteria from Thailand will be widely applied in a variety of industries.

Biodiversity and relative abundance of genera and species

From the results of enzyme production, 15 isolates could produce amylase and protease enzyme. Most of the selected bacteria (80%) were molecularly identified as *Bacillus* spp. and determined as spore-forming bacteria (Table 3, Figure 1 to 4).

In addition, an evolutionary tree could explain the variety of bacteria from Thai rice and bee sources that could produce protease. All regions of Thailand have high numbers of native bees and the information of enzyme-producing microorganisms from native bees is scarce. The pollen of many plant species serves as the principal food source for developing bee larvae, while honeybees and other bees are also important pollinators. In uncommon environments, spore-forming bacteria were found in high numbers.

This study revealed that the biodiversity of microorganisms from Thai

Table 1: Microscopic characterization of bacterial isolates.

Taxon		Isolates	%
Gram-positive			
	Short rod shaped	64	31.84
	Long rod shaped	14	6.96
	Coccus shaped	47	23.38
Gram-negative			
	Short rod shaped	49	24.37
	Long rod shaped	3	1.49
	Coccus shaped	24	11.94
	Total	201	100

Table 2: Screening of potential enzyme-producing bacteria.

Isolate	Enzyme transparent circle diameter (mm)			
	Protease (skimmed milk)	Protease (gelatin)	Amylase (starch)	Lipase (Tween 80)
C13HN3	1.88±0.62^{b}	3.46±1.21^{cd}	2.11±0.19^{de}	0
RICE3	2.53±1.00^{b}	1.59±0.12^{e}	1.58±0.14^{ef}	0
RICE13	1.73±0.63^{b}	1.51±0.30^{e}	2.06±0.31^{de}	0
KFRRC1	1.11±0.51^{b}	1.35±0.04^{e}	1.42±0.02^{ef}	0
KFR4	3.08±1.02^{b}	2.32±0.26^{de}	2.32±0.66^{de}	0
KRrb_P5	3.53±1.27^{b}	5.41±1.02^{ab}	1.38±0.12^{ef}	0
KRptI_T5	2.62±0.67^{b}	5.71±1.36^{a}	3.52±0.79^{bcd}	0
C1PN2	1.58±0.52^{b}	2.76±0.31^{de}	0^{f}	0
C3AN5	1.32±0.13^{b}	1.36±0.11^{e}	1.40±0.17^{ef}	0
C13HN2	11.66±4.93^{a}	4.06±1.07^{bc}	4.16±2.56^{bc}	0
C15AN1	1.79±0.05^{b}	1.74±0.14^{e}	36.66±1.52^{a}	0
KRrb_T2	3.11±0.68^{b}	5.45±1.43^{ab}	4.96±1.42^{b}	0
KRspI1	2.83±0.76^{b}	5.92±0.18^{a}	4.24±1.20^{bc}	0
KRptI_S2	1.21±0.08^{b}	2.51±0.13^{de}	2.52±0.75^{cde}	0
KRptI_R3	1.94±0.53^{b}	4.03±0.70^{bc}	2.1 1±0.19^{de}	0

Values represent the mean ± SD of three independent experiments. A different lowercase, superscript letter displays a significantly different value using Duncan's new multiple range test *(*DMRT*)* with a confidence level of 95%.

Table 3: Molecular identification of enzyme-producing bacteria isolated from rice and bee samples using amplification of 16S rRNA gene.

Bacterial isolates	Accession number	Closest species	Similarity
C13HN3	MZ130461	*Bacillus subtilis*	98%
RICE3	MZ130462	*Bacillus subtilis*	98%
RICE13	MZ130463	*Bacillus subtilis*	98%
KFRRC1	MZ130464	*Bacillus subtilis*	99%
KFR4	MZ130465	*Bacillus flexus*	98%
KRrb_P5	MZ130466	*Bacillus flexus*	97%
KRptI_T5	MZ149259	*Bacillus flexus*	96%
C1PN2	MZ149260	*Bacillus safensis*	98%
C3AN5	MZ149261	*Bacillus thuringiensis*	97%
C13HN2	MZ081649	*Microbacterium paraoxydans*	100%
C15AN1	MZ149262	*Bacillus thuringiensis*	99%
KRrb_T2	MZ081650	*Paenibacillus hunanensis*	96%
KRspI1	MZ149263	*Exiguobacterium indicum*	98%
KRptI_S2	MZ081651	*Bacillus flexus*	98%
KRptI_R3	MZ149264	*Bacillus flexus*	96%

rice and bee sources in Ratchaburi and Chiang Mai provinces, Thailand tends to be more valuable for development in variety of industries, especially the food and beverage industries globally.

High temperature tolerance and pathogenicity

The overall growth at high temperature of selected isolates is shown in Table 4. Most bacteria that were spore-forming bacteria could grow at 40-50°C, whereas some isolates including *Microbacterium*, *Paenibacillus* and *Exiguobacterium* could grow at low temperature *(*Table 4*)*.

The results of haemolytic activity revealed that three strains *(B. flexus* KRptI_S2, *M. paraoxydans* C13HN2 and *P. hunanensis* KRrb_T2*)* had no hemolysin protein that could lyse human red blood cells *(*Table 4*)*. Therefore, these three non-pathogenic isolates are promising for use in food application and were selected for enzyme activity determination.

The energy consumption of food and beverage production results in accumulated heat from all processes. The high temperature in the process is not suitable for growing bacteria and so a cooling system

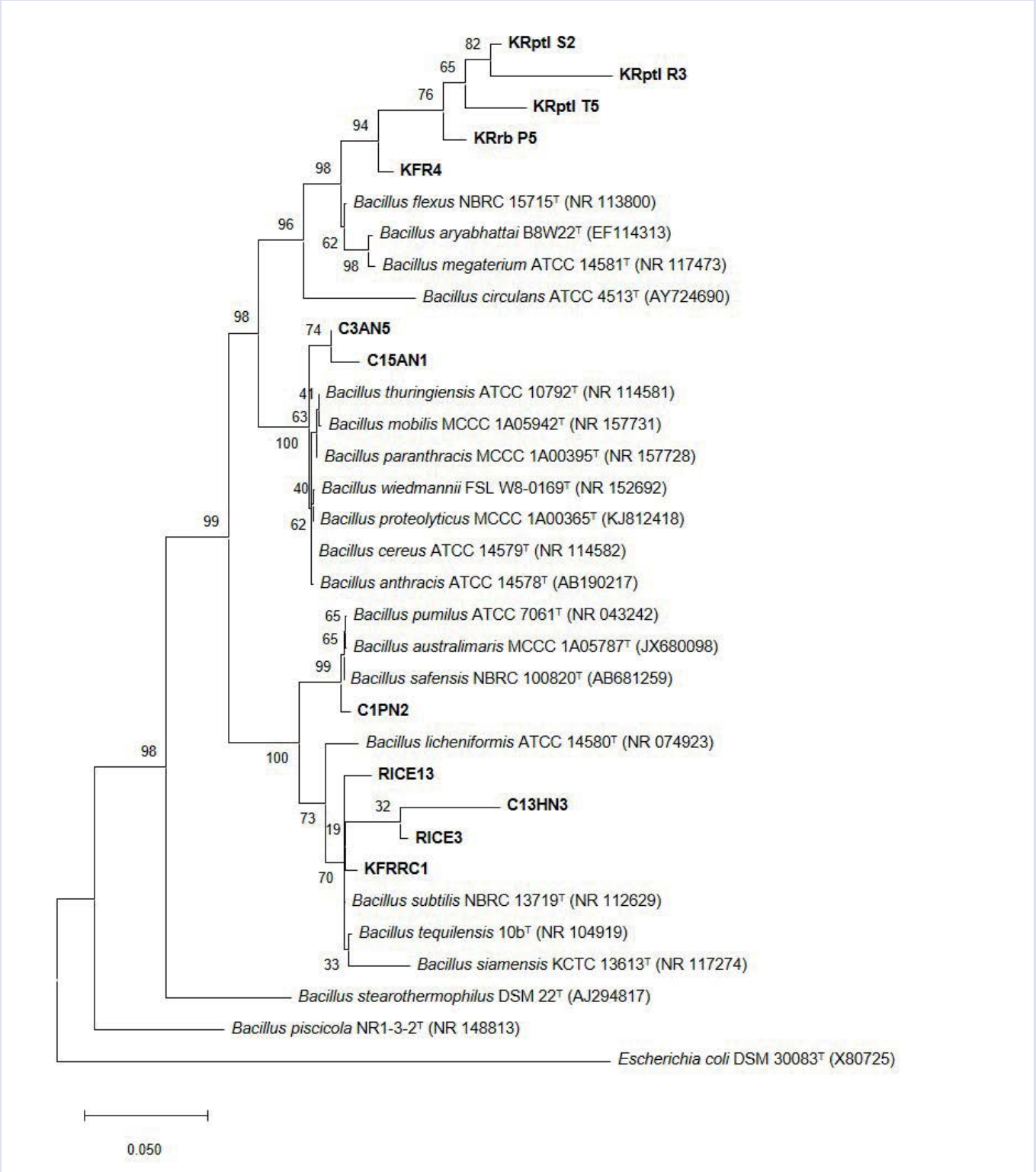

Figure 1: 16S rRNA gene-based maximum likelihood tree showing phylogenetic relationships of 12 isolates of bacteria from rice and bees relative to type strains of other *Bacillus* species. *E. coli* DSM 30083^T was used as an outgroup. Numbers on branches indicate percentage bootstrap values of 1,000 replicates. The scale bar represents 0.050 changes per nucleotide.

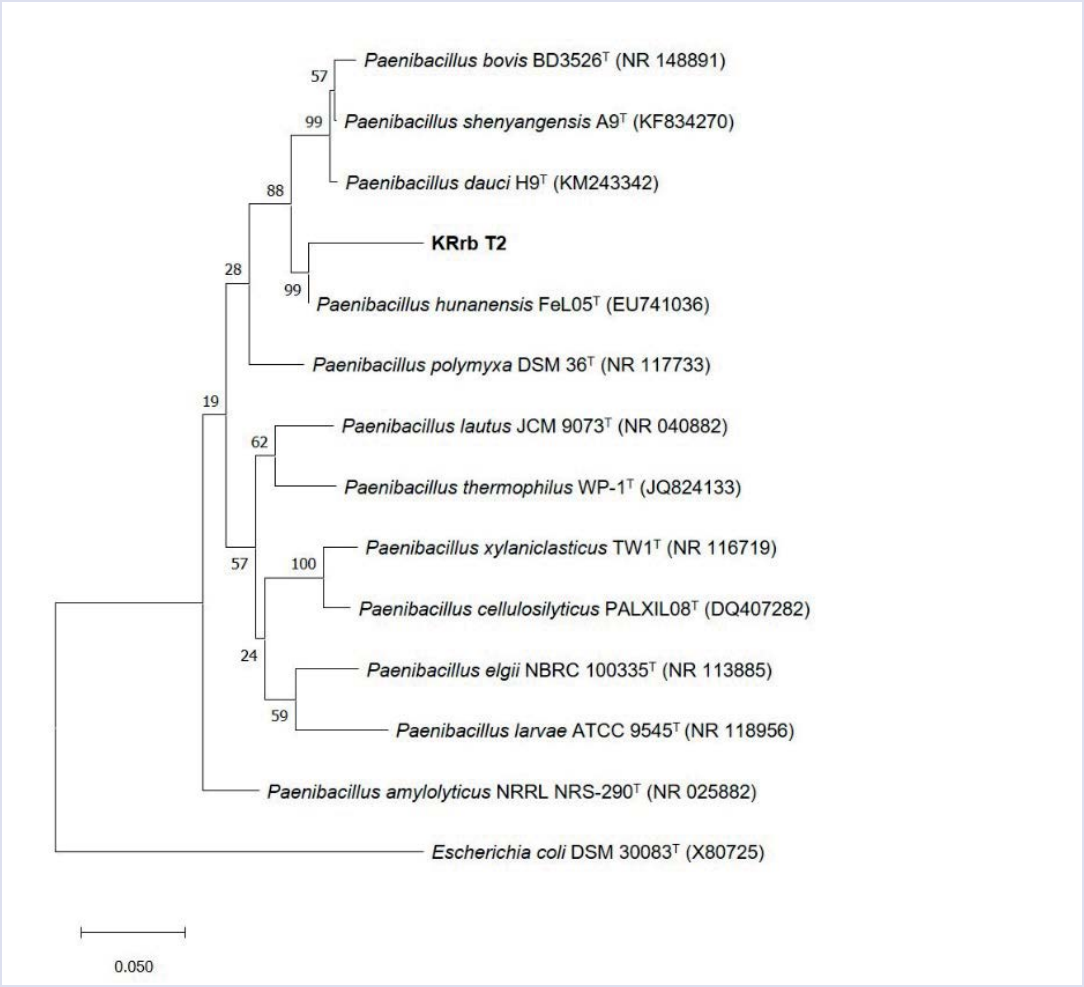

Figure 2: 16S rRNA gene-based maximum likelihood tree showing phylogenetic relationships of KRrb_T2 relatives to type strains of other *Paenibacillus* species. *E. coli* DSM 30083^T was used as an outgroup. Numbers on branches indicate percentage bootstrap values of 1,000 replicates. The scale bar represents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

Table 4: Tolerance to high temperature and hemolytic activity.

Isolate	Growth at (°C)				Hemolytic activity
	40	45	50	55	
B. subtilis C13HN3	++++*	++++	+++	-	β†
B. subtilis RICE3	++++	++++	+++	-	β
B. subtilis RICE13	++++	++++	+++	-	β
B. subtilis KFRRC1	++++	++++	+++	-	β
B. flexus KFR4	++++	++	++	-	α
B. flexus KRrb_P5	+++	+	-	-	α
B. flexus KRptI_T5	++++	++	-	-	α
B. safensis C1PN2	++++	++++	+++	-	β
B. thuringiensis C3AN5	+++	-	-	-	β
M. paraoxydans C13HN2	+++	-	-	-	-
B. thuringiensis C15AN1	++++	++++	+++	-	β
P. hunanensis KRrb_T2	+++	-	-	-	-
E. indicum KRspI1	+++	-	-	-	ϒ
B. fkexus KRptI_S2	++++	++	-	-	-
B. flexus KRptI_R3	+++	-	-	-	α

*++++, Very high growth; +++, High growth; ++, Moderate growth; +, Growth; -, No growth

†α, Alpha-hemolysis (greening around colony)

β, Beta-hemolysis (hemolytic activity)

ϒ, Gamma-hemolysis (unchanged colony)

-, No activity/Non hemolytic activity

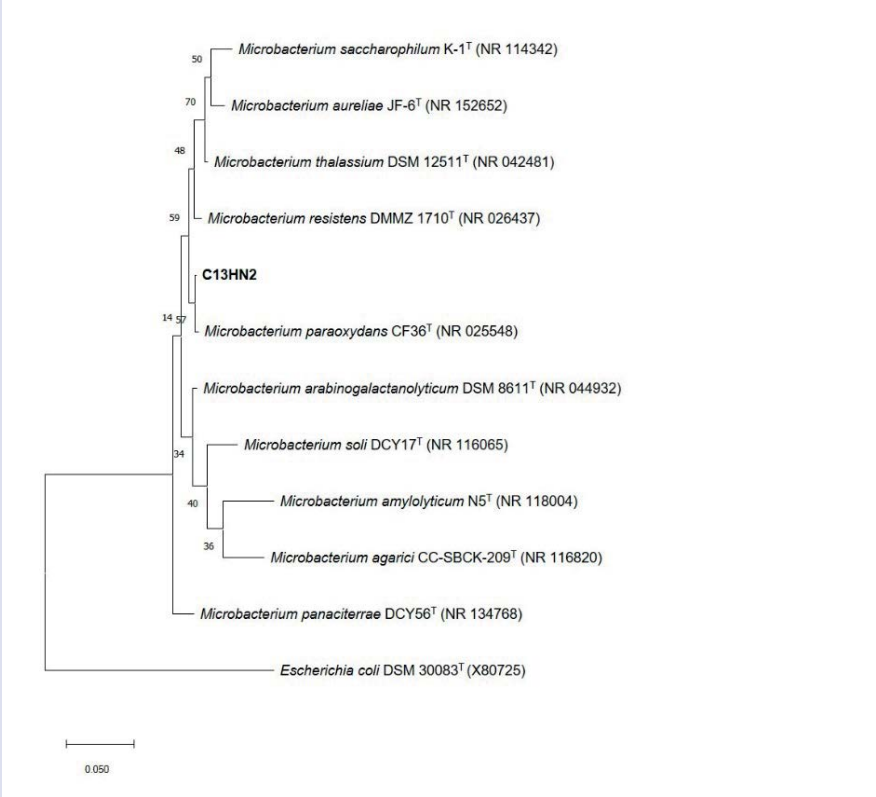

Figure 3: 16S rRNA gene-based maximum likelihood tree showing phylogenetic relationships of C13HN2 relatives to type strains of other *Microbacterium* species. *E. coli* DSM 30083^T was used as an outgroup. Numbers on branches indicate percentage bootstrap values of 1,000 replicates. The scale bar represents 0.050 changes per nucleotide.

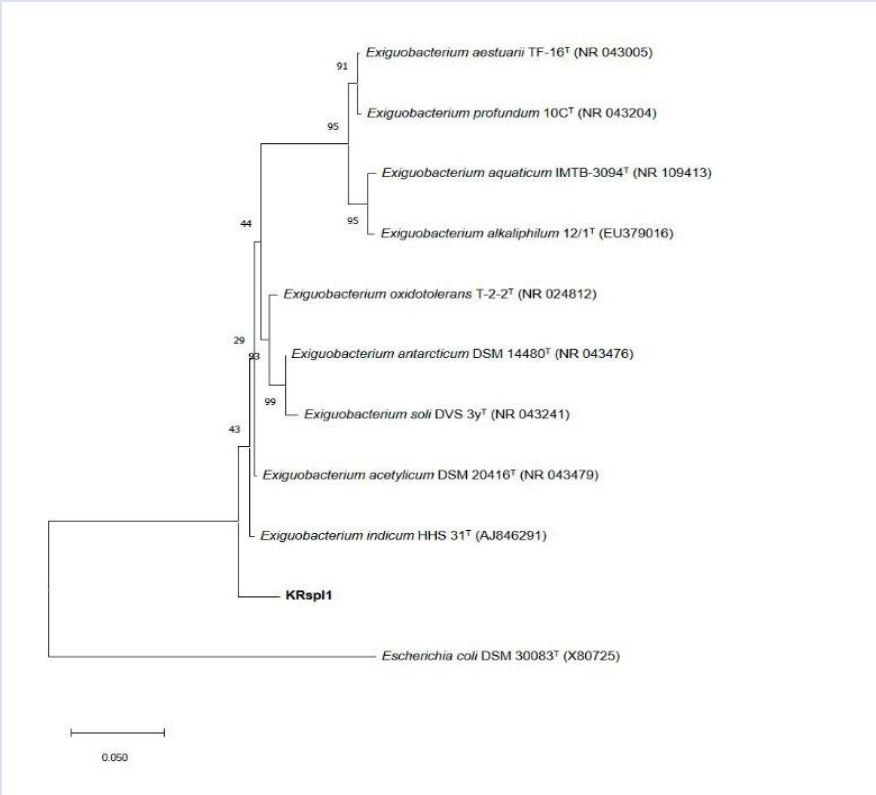

Figure 4: 16S rRNA gene-based maximum likelihood tree showing phylogenetic relationships of KRspI1 relatives to type strains of other *Exiguobacterium* species. *E. coli* DSM 30083^T was used as an outgroup. Numbers on branches indicate percentage bootstrap values of 1,000 replicates. The scale bar represents 0.05 changes per nucleotide.

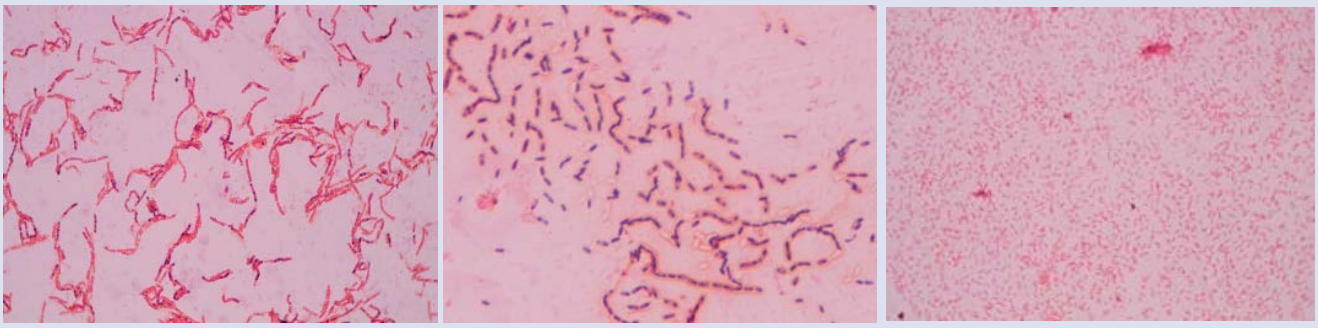

Figure 5: Cell morphology of strains under enzyme activity study: *(A) B. flexus* KRptI_S2, *(B) M. paraoxydans* C13HN2 and *(C) P. hunanensis* KRrb_T2.

Table 5: Enzyme activity (U/mL) of protease produced by three promising bacteria.

Isolates	Time of incubation (h)				
	0	12	24	36	48
M. paraoxydans C13HN2	0±0.0	338.21±1.50^{a}	287.66±1.20^{b}	290.66±0.66^{a}	318.55±1.54^{a}
B. flexus KRptI_S2	0±0.0	279.66±1.20^{b}	271.33±0.67^{b}	282.33±1.45^{a}	291.77±1.57^{b}
P. hunanensis KRrb_T2	0±0.0	338.99±1.33^{a}	364.66±1.66^{a}	378.99±1.33^{a}	320.66±0.87^{a}

Values represent the mean ± SD of three independent experiments. A different lowercase, superscript letter displays a significantly different value using Duncan's new multiple range test (DMRT) with a confidence level of 95%.

is provided. A cooling system is expensive and this is reflected in a higher production cost. In this study, the isolated bacteria that had ability to produce the desired enzyme could grow and survive at high temperatures. The application of these isolated bacteria in the production of beverage and food is an outstanding strategy that could reduce the production cost. Not only are the microorganisms from Thai rice and bees useful as a raw material in these industries, but they also contribute to the development of biodiversity in Thailand.

These endospores supported the long-term cell survival of bacteria in unsuitable conditions. Most of the bacteria contained the internal structure of an endospore. The endospore structure is a special structure that enhances bacterial survival and allows them to thrive in unusual environments. This reflects the fact that these bacteria can reduce the production cost because there is no need to increase the cooling system for growth in order to produce protease enzyme.

This study showed that the isolated 3 strains *Microbacterium paraoxydans* C13HN2, *Paenibacillus hunanensis* KRrb_T2 and *Bacillus flexus* KRptI_S2 are non-hemolytic based on hemolytic testing on blood agar. From the examination of their ability to lyse red blood cells and species-level information, it may be concluded that they are non-harmful bacteria for the consumer that can be used as raw material for production in the food and beverage industries. There are two groups and eight sub-categories in the beverage industry. Soft drinks and syrup, bottled water, vegetable and fruit juices, and tea and coffee are all included in the nonalcoholic category. The alcoholic group comprises distilled beverages (spirits, wine and beer). Enzymes (protease) are utilized as processing aids in breweries to generate uniform and high-quality output by digesting cell walls during the extraction of plant material to boost production, color, aroma and clearer products.[15]

The results of this study were consistent with other studies regarding the screening of bacterial isolates from other sources and the utilization of enzymes from other raw materials. *Bacillus pumilus* isolated from sea water[37] and *Bacillus* sp. isolated from fruits[38] had useful production of protease enzyme and were utilized as a detergent additive.

Based on their tolerance to high temperature and pathogenicity, it was concluded that *Bacillus flexus* KRptI_S2 would be most useful in indicating the potential of these bacteria for further studies.

Enzyme activity

The three isolates of promising bacteria were assayed for protease enzyme activity (Table 5). Interestingly, the production of protease by *P. hunanensis* KRrb_T2 was highest at 36 h (378.9967±1.33 U ml^{-1}). Indeed, *M. paraoxydans* C13HN2 and *B. flexus* KRptI_S2 also exhibited high activity of protease production.

The 201 bacterial isolates were screened for enzyme production. Among these, three strains (*Bacillus flexus* KRptI_S2, *Microbacterium paraoxydans* C13HN2 and *Paenibacillus hunanensis* KRrb_T2, as shown in Figure 5) could produce amylase and protease enzyme. 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^{-1} at 36 h. The study of Asha & Palaniswamy (2018)[39] reported that *Bacillus cereus* FT that was isolated from soil could exhibit protease activity at up to 165 U mL^{-1} at 48 h. In addition, *Aspergillus foetidus* that was isolated from soil demonstrated protease activity at up to 55.8 ± 1.1 U mL^{-1} at 96 h.[40] However, the study of Singh and Bajaj (2016),[41] revealed the protease activity of *Bacillus licheniformis* K-3 was up to 1321 U mL^{-1} 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 refers to a class of enzymes with the catalytic activity of hydrolyzing proteins. Proteolytic enzymes and proteinases are other names for them. Protease enzymes are categorised based on their structure or active site features. Protease enzymes are classified into numerous types, including alkaline serine-, neutral-, carboxyl-, metallo- and acidic proteases.[42] Proteases are the delicate protein molecules of enzymes which play a critical role in industry due to their vast range of uses. Among the various protease enzymes, when compared to animal, plant and fungal protease enzymes, bacterial proteases are the most important.[43] 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 the majority of this share.[44] Protease enzymes are a commercially important class of extracellular microbial

enzymes employing widely in a variety of manufacturing, particularly in the fermentation industry and pharmaceutical production.[45]

Thai rice and bees are promising sources for diversified microorganisms of which the current study indicated that bacterial strains of genus *Bacillus* are dominant. Three isolated strains had high ability to produce several hydrolytic enzymes, especially protease. These prominent strains were able to grow at high temperature and were non-pathogenic and have high potential for cost-effective production of protease. Thus, the effective bacterial strains could be developed for novel environmental-benign production in the food and beverage industries.

CONCLUSION

Thai rice and bees are promising sources for diversified microorganisms of which the current study indicated that bacterial strains of genus *Bacillus* are dominant. Three isolated strains had high ability to produce several hydrolytic enzymes, especially protease. These prominent strains were able to grow at high temperature and were non-pathogenic and have high potential for cost-effective production of protease. Thus, the effective bacterial strains could be developed for novel environmental-benign production in the food and beverage industries.

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GRAPHICAL ABSTRACT

Effective Enzyme-Producing Bacteria Isolated from Diversified Thai Rice and Native Thai Bees (Cavity Nesting Honey Bees) and their Potential for Production of Protease Enzymes

Isolation of bacteria from Thai rice and bees

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Screening of enzyme-producing bacteria

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Molecular identification & Phylogenetic analysis

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Tolerance to high temperature & Hemolytic activity

↓

Enzyme activity

Isolate	Enzyme transparent circle diameter (mm)			
	Protease (skimmed milk)	Protease (gelatin)	Amylase (starch)	Lipase (Tween 80)
C13HN3	1.88±0.62^{b}	3.46±1.21^{cd}	2.11±0.19^{de}	0
RICE3	2.53±1.00^{b}	1.59±0.12^{e}	1.58±0.14^{ef}	0
RICE13	1.73±0.63^{b}	1.51±0.30^{e}	2.06±0.31^{de}	0
KFRRC1	1.11±0.51^{b}	1.35±0.04^{e}	1.42±0.02^{ef}	0
KFR4	3.08±1.02^{b}	2.32±0.26^{de}	2.32±0.66^{de}	0
KRrb_P5	3.53±1.27^{b}	5.41±1.02^{ab}	1.38±0.12^{ef}	0
KRptl_T5	2.62±0.67^{b}	5.71±1.36^{a}	3.52±0.79^{bcd}	0
C1PN2	1.58±0.52^{b}	2.76±0.31^{de}	0^{f}	0
C3AN5	1.32±0.13^{b}	1.36±0.11^{e}	1.40±0.17^{ef}	0
C13HN2	11.66±4.93^{a}	4.06±1.07^{bc}	4.16±2.56^{bc}	0
C15AN1	1.79±0.05^{b}	1.74±0.14^{e}	36.66±1.52^{a}	0
KRrb_T2	3.11±0.68^{b}	5.45±1.43^{ab}	4.96±1.42^{b}	0
KRspl1	2.83±0.76^{b}	5.92±0.18^{a}	4.24±1.20^{bc}	0
KRptl_S2	1.21±0.08^{b}	2.51±0.13^{de}	2.52±0.75^{de}	0
KRptl_R3	1.94±0.53^{b}	4.03±0.70^{bc}	2.11±0.19^{de}	0

Isolate	Growth at (°C)				Hemolytic activity
	40	45	50	55	
B. subtilis C13HN3	++++^{a}	++++	+++	-	β^{+}
B. subtilis RICE3	++++	++++	+++	-	β
B. subtilis RICE13	++++	++++	+++	-	β
B. subtilis KFRRC1	++++	++++	+++	-	β
B. flexus KFR4	++++	++	++	-	α
B. flexus KRrb_P5	+++	+	-	-	α
B. flexus KRptl_T5	++++	++	-	-	α
B. safensis C1PN2	++++	++++	+++	-	β
B. thuringiensis C3AN5	+++	-	-	-	β
M. paraoxydans C13HN2	+++	-	-	-	-
B. thuringiensis C15AN1	++++	++++	+++	-	β
P. hunanensis KRrb_T2	+++	-	-	-	-
E. indicum KRspl1	+++	-	-	-	Υ
B. flexus KRptl_S2	++++	++	-	-	-
B. flexus KRptl_R3	+++	-	-	-	α

Isolates	Time of incubation (h)				
	0	12	24	36	48
M. paraoxydans C13HN2	0±0.0	338.21±1.50^{a}	287.66±1.20^{b}	290.66±0.66^{a}	318.55±1.54^{a}
B. flexus KRptl_S2	0±0.0	279.66±1.20^{b}	271.33±0.67^{b}	282.33±1.45^{a}	291.77±1.57^{b}
P. hunanensis KRrb_T2	0±0.0	338.99±1.33^{a}	364.66±1.66^{a}	378.99±1.33^{a}	320.66±0.87^{a}

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