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Short Communication

Comparative study of proteolytic activity of protease-producing bacteria isolated from *thua nao*

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Abstract: *Thua nao* is a Thai traditional fermented soya bean product with distinct flavour and aroma. This study was carried out to screen the potential protease-producing bacteria during the fermentation process. Of 171 strains, there were 169 bacterial isolates (98.8%) exhibiting proteolytic activity. Two potential isolates namely TN51 and TN69 showing highest activity as indicated by the widest clear zones (2.73 and 2.65 cm, respectively) were selected for further study. Using five different protein-based media, we found that the medium composition had an influence on the enzyme activity.

Keywords: *thua nao*, fermented soya bean, protease-producing bacteria, *Bacillus*

Introduction

There are various types of fermented soya bean from different parts of the world such as Indian *kinema*, Nigerian *dawadawa* (or *daddawa*), Japanese *natto*, and Thai *thua nao*. Typically, these soya

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bean foods are produced in similar manner: soya beans are washed, soaked overnight and cooked by boiling or steaming. The boiled or steamed soya beans are then fermented naturally at ambient temperature for 2-3 days. Among these fermented soya bean foods, Japanese *natto* is one of the best characterised products and, at present, has been produced commercially using the pure starter culture of *Bacillus subtilis natto* strain [1].

Thua nao is an alkali fermented soya bean widely consumed in the northern part of Thailand. Its use is versatile ranging from making ready-to-eat products after steaming or roasting to producing essential condiments in a variety of traditional dishes. Several studies have shown that, like other fermented soya beans, *Bacillus* species are responsible for the fermentation process of *thua nao* and they are abundantly isolated from the products [2-4]. In this present study, we aim to investigate the proteolytic activity of *Bacillus* species isolated from *thua nao* products. Further experiment was also undertaken to determine whether different protein-based media could have an effect on their protease activity.

Materials and Methods

Bacterial cultures

The bacterial cultures used in this study were isolated by Chukeatirote et al. [4]. They were initially characterised by morphological, physiological, and biochemical properties. These included Gram-staining, presence of spore, oxygen requirement, catalase test, ability to grow in 5 and 7 % NaCl, VP (Voges-Proskauer) test, and citrate and starch utilisation [5,6]. Additionally, *B. natto* ASA isolated from Asachiban *natto*, *B. natto* BEST195 [7], *B. subtilis* TISTR008 and *B. licheniformis* TISTR1109 obtained from Thailand Institute of Scientific and Technological Research (TISTR) were also used as reference strains in proteolytic activity study.

Determination of proteolytic activity

Initially, the bacterial strains isolated from *thua nao* were screened for their proteolytic activity. For this, a single colony of these bacteria was inoculated into a test tube containing 3 ml of nutrient broth containing 1 % peptone, 0.5 % beef extract, and 0.5 % NaCl. The culture was then grown at 37 ^{o}C for 24 h. Fifty microlitres of the overnight culture was subsequently transferred to a new test tube containing 3 ml of the nutrient broth and the culture was further incubated at 37 ^{o}C for 24 h. After incubation, the bacterial cells were harvested at 14,000 rpm for 15 min at 4 ^{o}C. The supernatant was collected for further use as the source of the protease enzymes and thus referred to the "crude extract". For preliminary assay, 5 µl of the crude extract were used to spot on a skim milk agar plate (containing 0.5 % peptone, 0.3 % beef extract, 0.5 % skim milk, and 1.5 % agar). The inoculated plate was then incubated at 37 ^{o}C for 24 h. The presence of a clear zone was recorded and used to indicate the bacterial ability to produce proteases.

In addition, further investigation was carried out using five different protein-based media: i) skim milk agar (SK: 1 % skim milk, 2 % agar); ii) skim milk agar supplemented with 0.02 % sodium azide (SK + SA); iii) soya protein agar (SPA: 1 % soya protein, 2 % agar); iv) nutrient agar

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supplemented with 1 % soya protein (NA + SP); and v) nutrient agar supplemented with 1 % gelatin (NA + G). For this, the selected bacteria were initially cultured in these media, centrifuged, and the supernatant was then used to determine the proteolytic activity as mentioned above. In general, the proteolytic activity was carried out at least in triplicate. The value of the relative index of enzyme activity (i.e. protease activity in this case) was also introduced in order to express appropriate results using the ratio between the diameter of the clear zone and that of the bacterial colony.

Results and Discussion

In this study, 171 bacterial strains previously isolated [4] were screened for their protease production. Using skim milk agar, the proteolytic activity could be determined by observing the presence of the clear zone. Preliminary results showed that, of 171 strains tested, 169 isolates representing approximately 99 % could produce protease enzymes. However, it was also evident that these bacteria had different proteolytic activity. To group these protease-producing bacteria, the size of the diameter of the clear zone was used as an indicator as illustrated in Table 1. Only two isolates did not show proteolytic activity on skim milk agar. The majority of the testing bacteria (119 isolates representing ~70 %) produced clear zones between 1.51 and 2.00 cm. The two bacterial isolates, namely TN51 and TN69, which exhibited highest proteolytic activity as indicated by the widest clear zones of 2.73 and 2.65 cm respectively, were selected for further experiment.

Table 1. Distribution and proportion of protease-producing bacteria isolated from *thua nao* based on the size of the clear zone diameter

Width of clear zone (cm)	Number of isolates	Proportion (%)
0 – 1.00	2	1.17
1.01 – 1.50	30	17.54
1.51 – 2.00	119	69.59
2.01 – 2.50	16	9.36
> 2.50	4	2.34

Based on cell morphology, 158 isolates (92.34 %) were rods and only 13 isolates were cocci. In addition, it was found that the endospore-forming bacteria were a major group representing ~76 % (120 isolates) of the rod-shaped bacterial group. According to Chukeatirote et al. [4], the endospore-forming bacteria were present throughout the 72-h fermentation period, whereas those non-sporeforming bacilli were found only at the beginning of the fermentation (less than 24 h). The bacteria TN51 and TN69, which had the strongest proteolytic activity were Gram-positive endospore-forming, rod-shaped bacteria (Figure 1) and thus were referred to as *Bacillus* spp. strains TN51 and TN69 respectively. Their detailed characteristics are given in Table 2.

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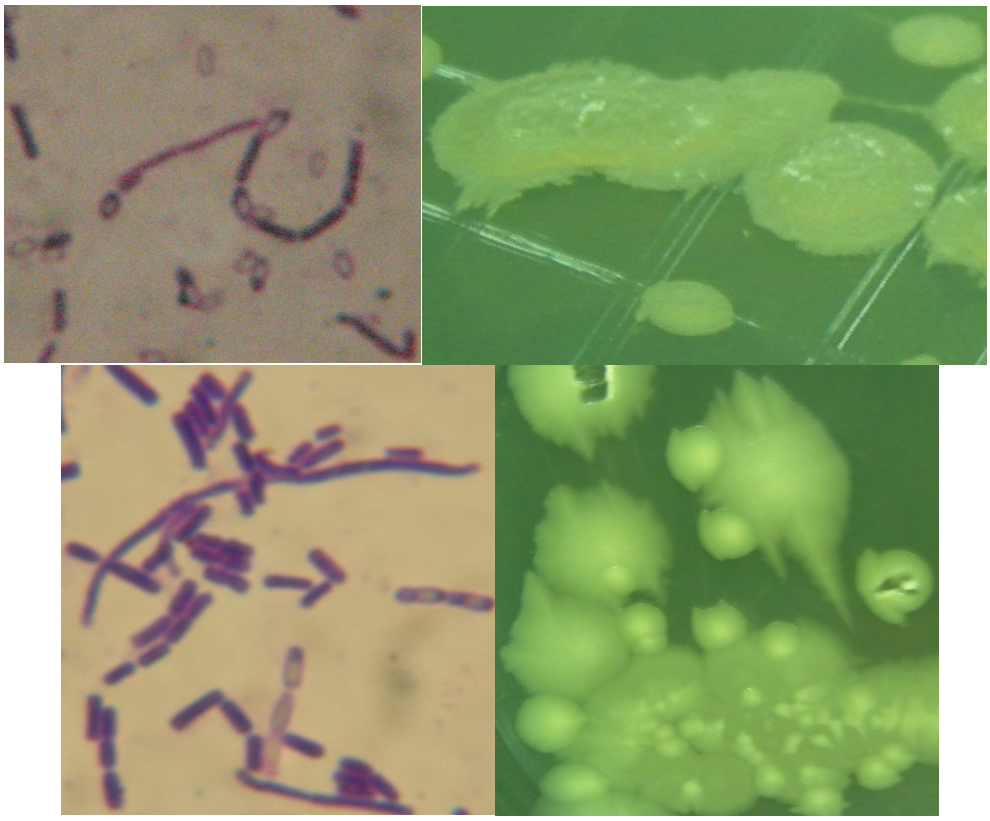

Figure 1. Cell and colony morphology of the bacteria TN51 (top) and TN69 (bottom)

Table 2. Morphological, physiological and biochemical characteristics of the bacteria TN51 and TN69

Characteristic	Bacteria TN51	Bacteria TN69
Gram-staining	Positive	Positive
Presence of spore	+	+
Shape	Rod	Rod
Catalase test	+	+
Voges Proskauer test	+	+
Starch hydrolysis	+	-
Citrate utilisation	-	-
Nitrate reduction	+	+
Oxygen requirement	Facultative anaerobe	Facultative anaerobe
Growth in 5% NaCl	+	+
Growth in 7% NaCl	-	-

Note: + = positive; - = negative

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Subsequently, we used five different protein-based media to evaluate whether this might affect the proteolytic activity. For this, the bacterial cells were cultured in these media, centrifuged and their supernatants collected and used as crude enzymes for evaluation of activity. As shown in Table 3, our results clearly suggest that the protein substrate can significantly affect the production of proteases. Sodium azide, known to prevent microbial growth, is shown to reduce (or even inhibit) the protease production, possibly as a result of inhibition of bacterial growth. Skim milk agar and NA supplemented with soya protein or gelatin are shown to be the appropriate media for induction of proteases enzyme of the tested bacteria. However, it should be noted that the bacterial cell growth could still be observed despite the fact that the crude extracts were used (Figure 2). Besides, it was clear that the growth of the bacterial colony had a major effect on the size of the clear zone; a large bacterial colony tended to induce a wide clear zone. The proteolytic activity could then be misinterpreted unless the size of the bacterial colony was taken into account. Consequently, to provide a better interpretation, the relative index of enzyme activity was calculated as the ratio of diameter (mm) of the clear zone to that of the bacterial colony and was used for this comparative analysis. According to Table 4, *Bacillus* spp. strains TN51 and TN69 exhibit highest activity of proteases when cultured in skim milk agar (SK) and nutrient supplemented with gelatin (NA + G), in which their relative indices of enzyme activity, respectively, are 2.0 and 1.8 for SK, and 1.9 and 1.6 for NA + G. The difference in the ability to utilise different protein sources is likely due to a difference in substrate specificity of the enzymes produced.

Table 3. Influence of different protein-based media on the production of extracellular proteases. (Figures represent mean width of clear zone diameter (mm) ± SD from three replicates.)

Bacterial isolate	SK	SK + SA	SPA	NA + SP	NA + G
Bacillus subtilis	16.0 ± 0	6.0 ± 0	0	20.0 ± 0	24.0 ± 0
B. licheniformis	3.8 ± 0.58	0	0	0	ND
B. natto ASA	17.0 ± 0	7.0 ± 0	11.67 ± 0.58	20.0 ± 0	27.0 ± 1.22
B. natto BEST195	17.0 ± 0	10.0 ± 0	10.67 ± 0.58	18.33 ± 1.53	24.0 ± 0.71
Bacillus TN51	16.67 ± 0.58	7.67 ± 0.58	10.33 ± 0.58	18.33 ± 0.58	24.4 ± 0.89
Bacillus TN69	22.67 ± 0.58	0	17.67 ± 0.58	19.0 ± 1.00	19.0 ± 1.00

Note: SK = skim milk agar; SK + SA = skim milk agar supplemented with 0.02% sodium azide; SPA = soya protein agar; NA + SP = nutrient agar supplemented with soya protein; NA + G = nutrient agar supplemented with 1% gelatin; ND = not determined.

The degradation of proteins has been previously described as the most important biochemical change occurring during the soya bean fermentation [8-10]. In addition, it has been suggested that such proteolysis is strongly relevant to dominating *Bacillus* species, especially for the *dawadawa* case [8,11]. Previous investigations including this present study have also shown that the predominant microbes present in *dawadawa* and *thua nao* are capable of producing proteases [3,12].

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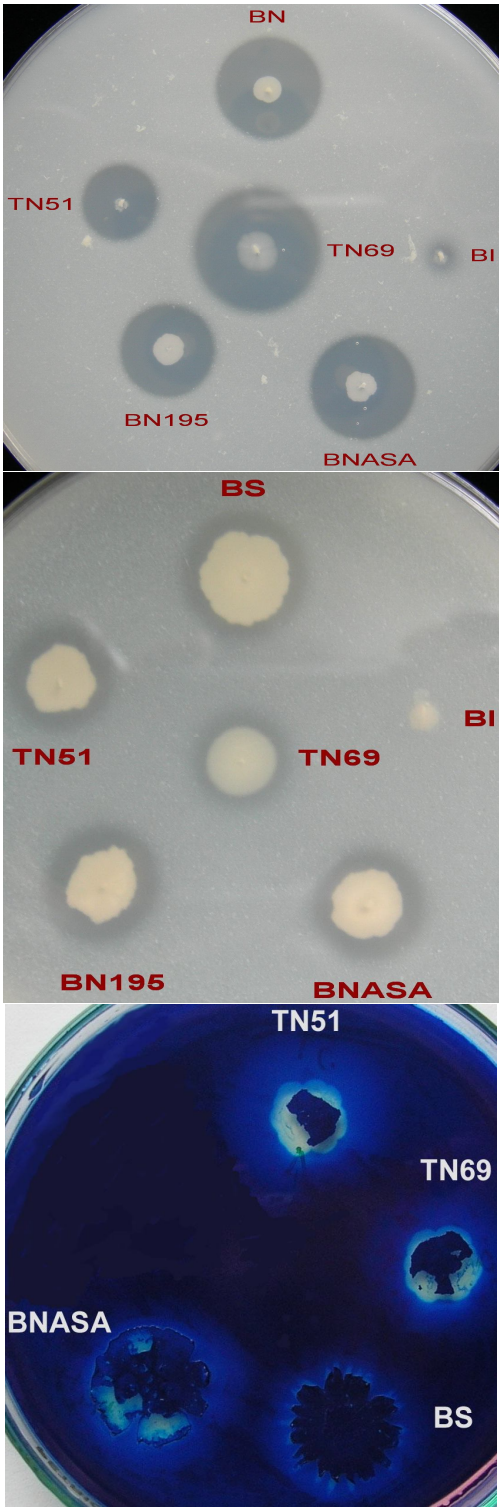

Figure 2. Proteloytic activity of *Bacillus* species when cultured in skim milk agar (top), NA supplemented with soya protein (middle), and NA with gelatin (bottom). BS = *Bacillus subtilis*; Bl = *B. licheniformis*; BNASA = *B. subtilis* (natto) ASA; BN195 = *B. subtilis* (natto) BEST195; TN51 and TN69 = *Bacillus* spp. strains TN51 and TN69

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Table 4. Relative index of protease activity of *Bacillus* species when cultured in skim milk agar (SK) and NA supplemented with 1% gelatin (NA + G)

Bacterial isolates	SK	NA + G
Bacillus subtilis	1.1 ± 0.1	1.6 ± 0.14
B. natto ASA	1.1 ± 0.04	1.4 ± 0.46
Bacillus TN51	2.0 ± 0.09	1.9 ± 0.08
Bacillus TN69	1.8 ± 0.09	1.6 ± 0.06

Note: SK = skim milk agar; NA + G = nutrient agar supplemented with 1% gelatin

Conclusions

The present study has shown that nearly all bacterial species isolated from *thua nao* are capable of producing protease enzymes. Furthermore, the production of extracellular proteases can be induced using appropriate media. The *Bacillus* species TN51 and TN69 previously isolated are of great importance due to their strong proteolytic activity. Both strains are now being studied for their potential as pure starter cultures in the improvement of *thua nao* fermentation process.

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