

Screening and Isolation of *Bacillus subtilis* Exhibiting Protease Activity from *Thua Nao*

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Thua nao, a traditionally fermented soybean, is locally consumed by people in the Northern Thailand. In this study, eighty-two *Bacillus* species were isolated from *thua nao* and screened for their proteolytic activity using the azocasein as substrate. It was shown that the crude proteins of *B. subtilis* strain 38 produced the widest clear zone on skim milk agar with an average area of 480mm². Optimal pH and temperature of the proteases from *B. subtilis* strain 38 were found to be at 6.5 and 47°C, respectively. However, these proteases were not thermostable since their enzyme activity was rapidly decreased when incubated at 60°C. A variety of inhibitors, known to affect different classes of proteases, were tested but only 1, 10-phenanthroline affected the enzyme activity indicating the presence of metalloproteases. In addition, an attempt to produce *thua nao* using the *B. subtilis* strain 38 as a starter culture was undertaken.

Keywords: *thua nao*, fermented soybean, *Bacillus subtilis*, protease activity

Introduction

Thua nao is a traditional fermented soybeans locally produced and consumed by the people of the Northern Thailand (Sundhagul *et al.*, 1972). To produce traditional *thua nao*, soybeans are washed and soaked in water overnight. Soaked beans are then cooked by boiling for 3 – 4h, gently grounded, placed in the basket lined with banana leaves and left to ferment naturally for 2 – 3 days at ambient temperature. After the fermentation, fresh *thua nao* can be consumed directly by steaming. Besides, it is also popular—due to a long shelf-life—to store in a dried form by exposing to sunlight and these dried products are a major condiment in various kinds of local dishes.

Unlike other fermented soybeans (i.e., Indian *kinema* and Japanese *natto*), little information regarding microbial diversity of *thua nao* fermentation is known. In this study, the role of *Bacillus* species possibly the predominant microorganisms in the fermentation was investigated. Their protease activity was also described.

Materials and Methods

Thua nao samples were collected from local markets in different provinces of Northern Thailand. To screen for *Bacillus* bacterial strains, the samples (0.1g) were homogenised with 3.0ml of sterile water and incubated at 75°C for 20min. The suspension was then poured into nutrient agar plates and incubated at 37°C for 24h. The colonies which appeared were then isolated and examined by their morphological and biochemical properties.

Using crude extracts collected from an overnight culture, protease activity of each bacterial isolates was determined by azocasein method as described elsewhere (Moss *et al*, 1991). After initial screening, *B. subtilis* strain 38 was shown to produce proteases with strongest activity and thus chosen for further study in terms of their stability and activity at different temperature and pH. Besides, a variety of known protease inhibitors were also tested.

Thua nao was then prepared based on conventional method in the laboratory. However, an attempt to use *B. subtilis* strain 38 as a starter culture was performed in this investigation.

Results and Discussion

Several *thua nao* samples were obtained locally from provinces in the Northern Thailand. Of these *thua nao* products, eighty-two bacterial strains were isolated and further characterised as indicated by Norris *et al* (1981). Based on this key identification, thirty-nine (47.5%) were determined as *B. subtilis*. The isolation of *B. subtilis* species is of great significance due to its record of safe use in a variety of fermented food products. Besides, as the guideline of the USFDA, the *B. subtilis* species is considered as one of the GRAS microorganisms.

These *B. subtilis* isolates were then tested for their proteolytic activity using azocasein method with skim milk as a substrate. By this approach, the presence of a clear zone suggested the production of proteases from the bacterial strains tested. Preliminary results showed that *B. subtilis* strain 38 exhibited highest protease activity with a clear zone of approximately 480mm² (Figure 1). As a result, *B. subtilis* strain 38 was selected to study further regarding its protease property. Effect of pH and temperature on protease activity and stability was investigated. By varying pH and temperature profile, the optimum pH and temperature for the

enzyme activity were 6.5 and 47°C, respectively (Figure 2 and 3). Furthermore, the stability of the enzyme was assayed at different temperatures (40°C, 50°C and 60°C). Proteases from *B. subtilis* strain 38 became most unstable when incubated at 60°C considered from the rapid decrease of the enzyme activity (Figure 4). Additionally, a variety of protease inhibitors were also determined. For this case, only 1, 10-phenanthroline was able to inhibit the enzyme activity (Figure 5).

Finally, we also attempted to use *B. subtilis* strain 38 as a starter culter for *thua nao* fermentation. The fermentation was performed in both traditional Thai and *natto* styles at 37°C and 45°C. During the fermentation, pH changes were monitored every 6h. In general, the pattern of the pH change was quite similar with initial pH of 6.0 and gradually increased to ~8.0 after 3-day fermentation (see Figure 6). Increase of pH was expected due to proteolysis and ammonia release (Sakar *et al.*, 1993). As shown in Figure 5, difference in pH change was highest at 12h. The differench in pH value was also clearly observed for both fermentation styles. The more alkaline pH values at 45°C were likely to occur from the higher activity of proteases. Consequently, an alternative for rapid fermentation may be possible using high incubating temperature. It would be worthwhile for future study regarding use of starter culture in *thua nao* fermentation in terms of nutritional value and consumers' acceptability.

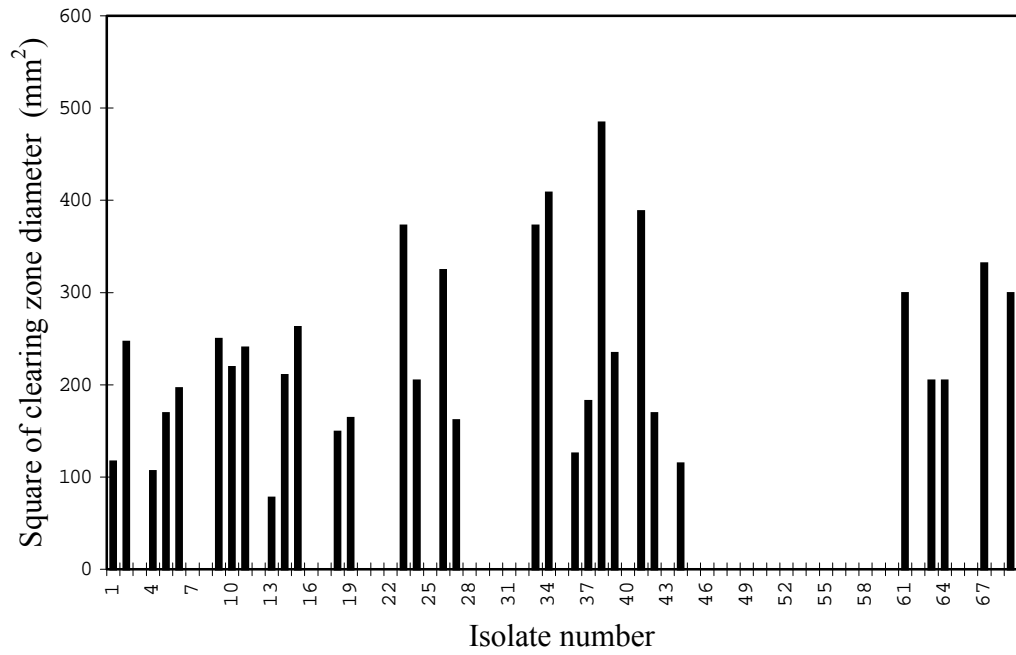


Figure 1 Proteolytic activity on skim milk agar of *Bacillus subtilis* isolated from Thai fermented soybeans. Bars indicate means of triplicates.

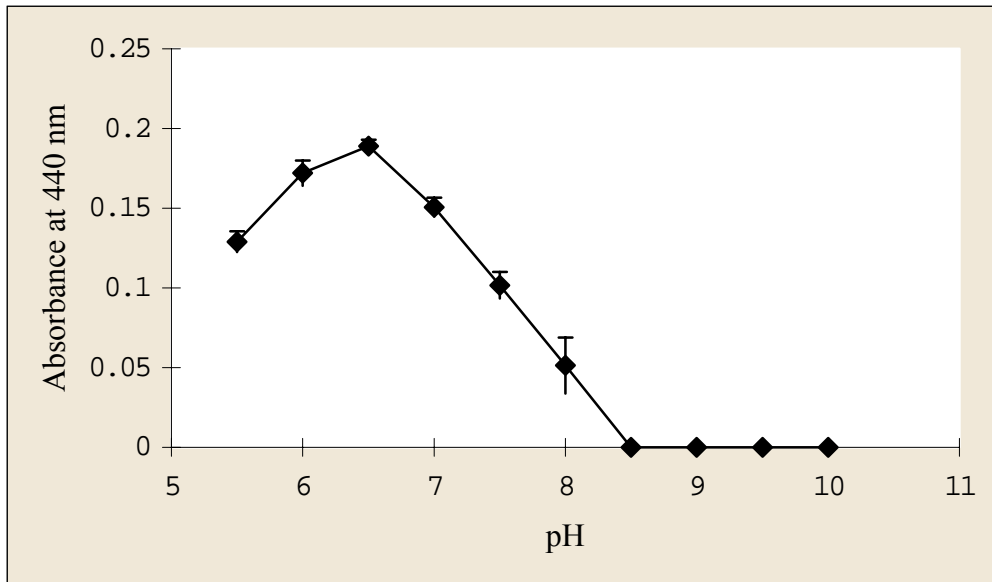


Figure 2 pH optimum of *Bacillus subtilis* 38 determined determined by Azocasein method (Error bars indicate the standard deviation of triplicates).

Figure 3 Temperature optimum of *Bacillus subtilis* 38 determined by Azocasein method (Error bars indicate the standard deviation of triplicates)

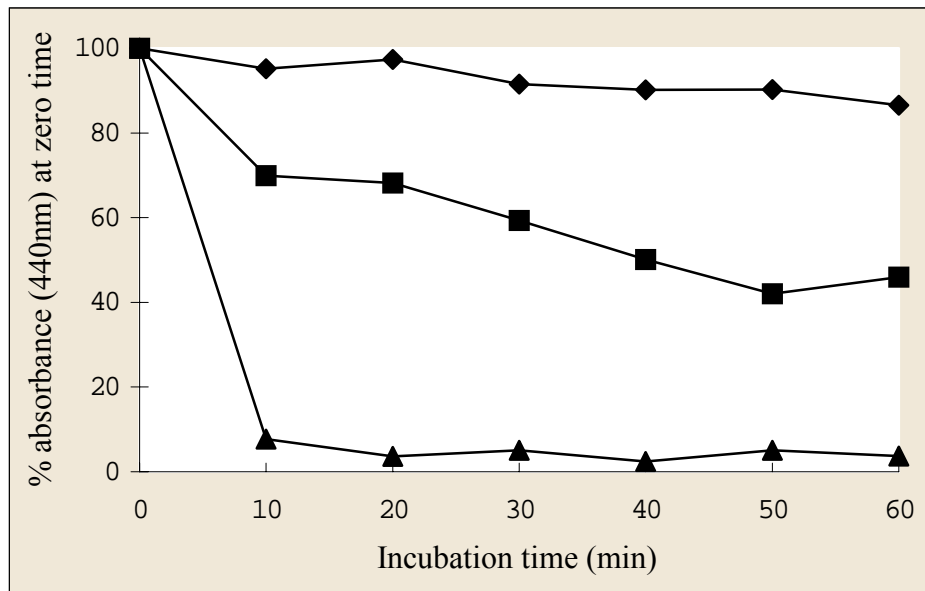


Figure 4 Heat stability of proteases produced from *B. subtilis* 38. The supernatant sample were held at 40,50, and 60 °C and at intervals subsamples were removed and assayed by the azocasein method at 37 °C. a (◆:40 ,■: 50 ▲: 60 °C respectively).

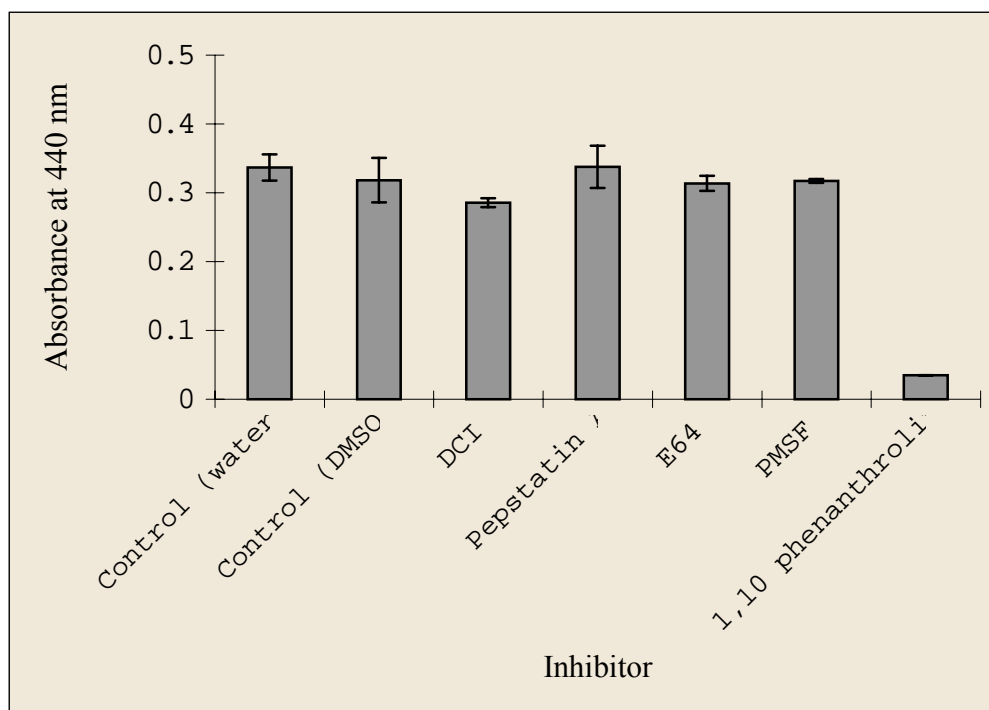


Figure 5 Effect of inhibitors on proteolytic activity of *B. subtilis* 38 (Error bars indicate the standard deviations of triplicates)

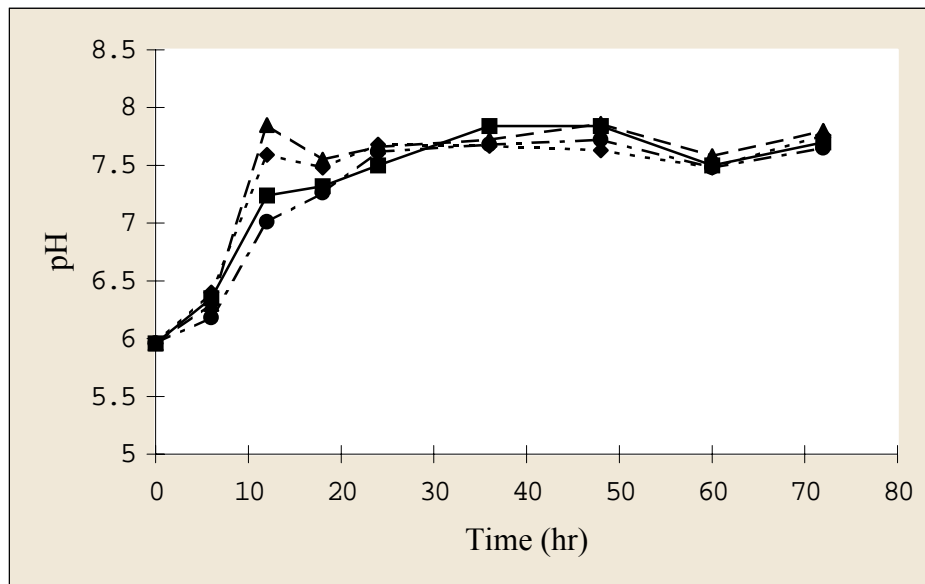


Figure 6 pH measurement of laboratory scale fermented soybeans (▲: Natto (45 °C),◆: Thua-noa (45 °C), ■: Thua-noa (37 °C), ● Natto (37°C))

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