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EFFECTS OF CULTURE CONDITIONS ON GROWTH AND PROTEASE PRODUCTION BY *Virgibacillus* STRAIN T1.6 ISOLATED FROM SHRIMP PASTE

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Abstract. A protease producing bacterium strain T1.6 isolated from shrimp paste was investigated in this study. The strain T1.6 was a Gram-positive, aerobic, spore forming, and rod-shaped bacterium. The analysis of 16S rRNA sequences demonstrated that this strain belonged to the genus *Virgibacillus*. The effect of culture conditions such as NaCl concentration, pH, and temperature on growth and protease production through shake-flask culture was investigated. Maximum protease activity of 83 IU/mL was obtained after 36 h of cultivation in LB medium containing 2.5% NaCl at 30 ºC and pH 7.0. The protease activity obtained in this study by strain *Virgibacillus* sp. T1.6 was higher than that obtained by other *Virgibacillus* species isolated from shrimp paste. Bacterial strain *Virgibacillus* sp. T1.6 could be a potential producer for the industrial production of protease.

Keywords: protease, *Virgibacillus*, shrimp paste.

1. Introduction

Today, fermented foods play a significant role in the food industry. About one-third of total food consumption worldwide is of fermented foods [1]. Shrimp paste is one of the traditional fermented foods that is consumed as a food condiment in many cuisines of the Vietnamese people. To produce shrimp paste, fresh shrimp (mainly *Acetes* sp.) are mixed with 10 - 30% (w/w) salt and ground into a fine paste. The mixture is then dried under sunlight and fermented for several months (1 - 6 months). During fermentation, the proteins were hydrolyzed by the action of endogenous and exogenous proteases into peptides and free amino acids, which contribute an essential role in the flavor and taste of the final product [2].

Although many microorganisms are known to produce protease, only a few of them are recognized as commercial protease producers. Proteases are normally produced by microorganisms under normal conditions, however, the application of proteases often takes place under extreme conditions such as high salt concentration and/or high temperature. For example, proteases are used in leather and detergent industries [3].

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Therefore, it is important to search for novel proteases with an industrial desired property. Halophilic and halotolerant bacteria are considered as a good source of such protease because their enzymes are not only salt tolerant but many of them are also thermotolerant [4]. In the present study, we isolated and identified protease producing bacteria from fermented shrimp paste collected from Nam Dinh province, the optimization of culture conditions for protease production by selected strain was also investigated.

2. Content

2.1. Materials and methods

2.1.1. Isolation of protease producing bacteria

The protease producing bacteria were isolated from shrimp paste samples (pH 7.4) collected from Nam Dinh province by dilution and spread on agar plates containing (g/L): NaCl, 100; peptone, 10; yeast extract, 5; skim milk, 10; the pH of this medium was initially adjusted to 7.0. After 48 h of cultivation, bacterial colonies exhibited a large clear zone and were collected for further study.

2.1.2. Screening of protease producing bacteria

The isolated strains were grown in 20 mL of liquid LB (Luria-Bertani) medium in 100 mL Erlenmeyer flasks. The medium contains (g/L) NaCl, 100; peptone, 10; yeast extract, 5; the pH of this medium was initially adjusted to 7.0. The cultures were incubated at 30 ^{o}C with rotary shaking at 180 rpm for 24 h. Screening of protease producing bacterial strain was then conducted using agar diffusion assay. Twenty milliliters of the agar medium containing 1% (w/v) gelatin was poured into each 15 × 100 mm Petri plate, and four wells were made with a cork borer after solidification. About 100 µL of the culture medium were pipetted into the wells, and the plates were incubated at 37 ^{o}C for 24 h. Undigested areas were visualized by staining with saturated $(NH_4)_2SO_4$ solution. The clear zone diameter was calculated by subtracting the diameter of the clear zone from the diameter of the well [5].

2.1.3. Phylogenetic characterization of the selected protease producing bacterium

The genomic DNA of the selected strain was extracted by Thermo Scientific GeneJET Genomic DNA Purification Kit according to the manufacturer's recommendations. The 16S rRNA gene was amplified using the universal primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACG ACTT-3'). Sequencing of the amplified DNA fragment was performed at 1st base (Singapore), and the GenBank database was used to search for 16S rRNA gene sequence similarities. Phylogenic analysis based on the 16S rRNA gene was performed with the aid of MEGA6 software [6] using the neighbor-joining distance correlation method [7].

2.1.4. Effect of different culture conditions on the growth and protease production of selected bacterial strain

The selected strain was first grown in LB solid medium at 30^{o}C for inoculum preparation. A loopful of bacterial strain was then transferred to a 100 mL flask containing 25 mL LB liquid medium. The flask was incubated overnight at 30 ^{o}C and 180 rpm in a rotary shaking incubator. After that, 1 mL of culture broth was inoculated in 100 mL flasks containing 25 mL of LB medium. The effect of different culture conditions on

growth and protease activity was tested. To test the effect of NaCl, different NaCl concentrations (0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20%, w/v) were added to the culture medium. The effect of different temperatures (25, 30, 35, 40, and 45 ^{o}C), different pH values (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0), and different cultivation times (0, 18, 24, 30, 36 and 42 h) were also tested. The bacterial cells were collected by centrifugation for cell dry weight (CDW). The supernatant of the culture broth after centrifugation (10,000 rpm for 10 min) at 4 ^{o}C was used for determining extracellular protease activity.

2.1.5. Analytical methods

CDW was determined by centrifuging 3 ml of the culture samples at 4 000 *g* for 10 min in a pre-weighed centrifuge tube, the pellet was washed once with 3 mL distilled water, centrifuged, and dried at 105 ^{o}C until constant weight was obtained. The centrifuge tube was weighed again to calculate the CDW.

The protease activity was determined by a modification of Anson's method described by Keay and Wildi (1970) [8]. The supernatant (100 μL) was mixed with 100 μL casein solution (0.5%, w/v, pH 7.6), and the reaction mixture was incubated at 37 ^{o}C for 20 min. The reaction was stopped by adding 200 μL of 10% TCA solution and allowed to stand at room temperature for 10 min and then centrifugated at 10,000 rpm for 10 min. The supernatant (100 μL) was transferred to another clean test tube, 500 μL of 0.5M sodium carbonate solution was added, followed by 150 μL of Folin-Ciocalteu's phenol reagent: water (1:3, v/v) and incubated at room temperature for 30 min in dark condition. The optical density of solutions was determined at 650 nm. Tyrosine solution was used as the calibration standard. One unit of enzyme activity was defined as the amount of the enzyme that liberates 1 μg of tyrosine per minute under standard assay conditions.

All experiments and analyses were performed in triplicate.

2.2. Results and discussion

2.2.1. Screening of protease producing bacterium

Shrimp paste samples were serially diluted and spread on an LB medium containing 1% skim milk. After 48 h of cultivation, bacterial colonies exhibited a large clear zone and were collected by plating on a fresh LB medium. The bacterial strains were then grown on liquid LB medium at 30^{o}C for 24 h. The supernatant was collected and used for protease analysis. As shown in Figure 1, bacterial strain T1.6 exhibited the highest clear zone around the well on gelatin agar indicating the high protease activity in the hydrolysis of gelatin.

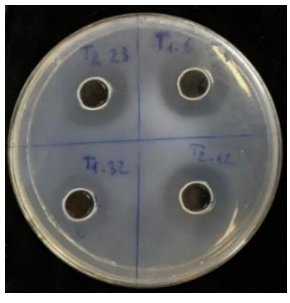

Figure 1. Protease activity of selected bacterial colonies on agar medium with 1% gelatin

2.2.2. Identification of selected protease producer

Strain T1.6 was a Gram-positive, aerobic, spore forming, and rod-shaped bacterium (Figure 2). The phylogenetic characterization of the strain T1.6 was analyzed using its 16S rRNA gene partial sequences. The results showed that strain T1.6 belonged to the genus *Virgibacillus*, and showed the closest similarity with *V. pantothenticus* OL03 and *V. pantothenticus* (100%), *V. dokdonensis* H-61 and *V. dokdonensis* JAN-2 (99.9%) (Figure 2).

Most members of the genus *Virgibacillus* have been isolated from saline environments such as marine sediment, soil, lake, fish sauce fermentation, and shrimp paste fermentation [4, 9-16]. Many of them showed the ability to produce extracellular proteases such as *Virgibacillus* sp. strain TKNR13-3 [4], *V. natechei* strain FarD [11], *Virgibacillus* sp. strain CD6 [13], *V. halodenitrificans* strain ST-1 [15], and *Virgibacillus* sp. strain SK37 [16]. Some of the protease producing bacteria have been used as starter cultures in seafood fermentation. For example, *Virgibacillus* sp. SK37 has been applied as a starter cultures in shrimp paste production [16].

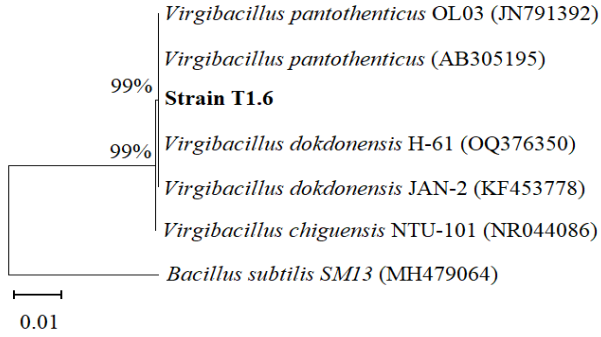

Figure 2. Neighbor-joining phylogenetic tree based on the comparison of 16S rDNA sequences, showing the relationships between the selected strain and other strains of the genus Virgibacillus. Bar, 0.01 substitutions per nucleotide position

2.2.3. Effects of culture conditions on bacterial cell growth and protease activity

The effect of culture conditions including salt concentration, pH value, incubation temperature, and cultivation time on bacterial cell growth and protease activity was investigated.

**** Effect of salt concentration***

The effect of different salt concentrations on bacterial growth and the production of protease by *Virgibacillus* sp. T1.6 after 30 h of cultivation at 30 ^{o}C and pH 7.0 is shown in Figure 3. Figure 3 indicates that *Virgibacillus* sp. T1.6 was able to grow and produce protease in all tested salt concentrations. The highest CDW of 4.03 g/L was observed at 2.5% NaCl, followed by 5%, 7,5%, 10%, and 0% NaCl. The growth of *Virgibacillus* sp. T1.6 was drastically decreased at the concentration of 12.5% NaCl or higher. The effect of the salt concentration on the production of protease was in agreement with the growth of the bacterium. The highest protease activity of 70.69 IU/mL was obtained at 2.5% NaCl. The protease activity was still high in higher salt concentrations (5 to 12,5% NaCl).

The results obtained in this study indicated that salt is a prerequisite for the growth of the bacterial strain T1.6. The strain T1.6 can be classified as a member of moderately

halophilic bacteria. Previous studies showed that *Virgibacillus* spp. species isolated from shrimp paste are also moderately halophilic bacteria. For example, *Virgibacillus* strain TKNR13-3 isolated from shrimp paste in Thailand was able to grow in various NaCl concentrations (0 to 20%, w/v), and the highest enzyme activity was obtained at 5% NaCl [4]. Recently, *V. halodenitrificans* ST-1 isolated from shrimp paste in China can also be grown in a medium containing 1 to 15% NaCl, and the optimum growth was in a 7% NaCl medium [15].

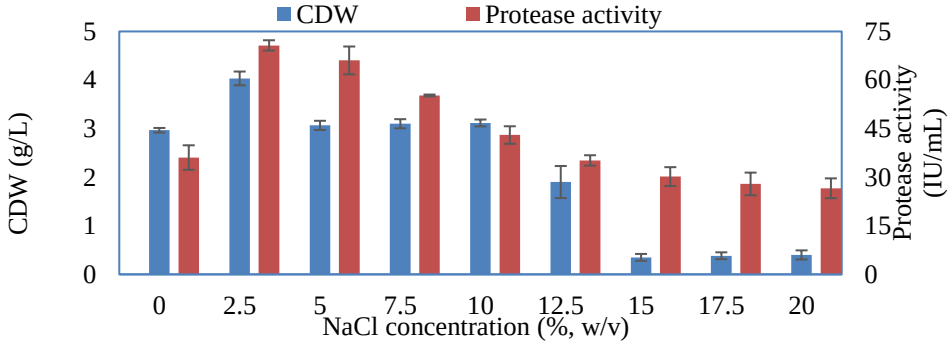

Figure 3. Effect of salt concentration on the growth and protease activity of Virgibacillus sp. strain T1.6

**** Effect of pH***

Figure 4 shows the growth and the production of extracellular protease by *Virgibacillus* sp. T1.6 after 30 h of cultivation at 30°C in LB medium containing 2.5% NaCl, and in a pH range of 5.0 - 8.0. The bacterium was unable to grow at pH 5.0. *Virgibacillus* sp. T1.6 was able to grow well in the pH range of 6.5 - 8.0 with the optimum at pH 7.0. The highest CDW of 4.1 g/L was obtained at pH 7.0 after 30 h of cultivation in the medium containing 2.5% NaCl. Protease activity was also high at pH values 6.5 and 7.0. The highest enzyme activity of 80.13 IU/mL was obtained at pH 7.0. This was similar to findings relating to other *Virgibacillus* species isolated from shrimp paste. *Virgibacillus* strain TKNR13-3 showed maximum growth and protease activity at pH 6,5 - 7.0 [4]. The optimal pH for the growth of *Virgibacillus halodenitrificans* ST-1 was in the pH range of 7.0 - 8.0 [15].

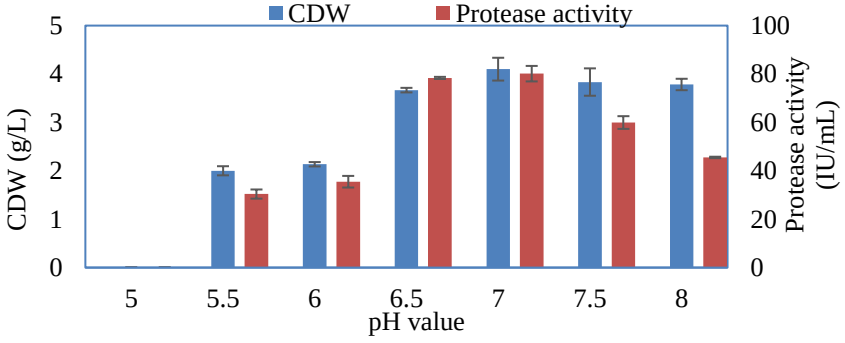

Figure 4. Effect of pH on the growth and protease activity of Virgibacillus sp. strain T1.6

**** Effect of temperature***

The effect of temperature on bacterial cell growth and extracellular protease production by *Virgibacillus* sp. T1.6 was investigated. Samples were collected after 30 h of cultivation in an LB medium containing 2.5% NaCl, pH 7.0. The optimal temperature for growth of *Virgibacillus* sp. T1.6 was in a range of 25 - 30 ^{o}C. The highest CDW of 4.2 g/L was obtained at 30 ^{o}C, followed by 3.82 g/L at 25 ^{o}C. The growth of the bacterium was decreased when the incubation temperature increased, and at 45 ^{o}C the growth decreased by more than 50% as compared to that at the optimal temperature. Strain *Virgibacillus* sp. T1.6 was also produced protease in a temperature range of 25 - 45 ^{o}C. The highest protease activity of 80.5 IU/mL was obtained at 30 ^{o}C. The production of protease was decreased at temperatures higher than 35 ^{o}C, which was in accordance with the decreased bacterial growth (Figure 5).

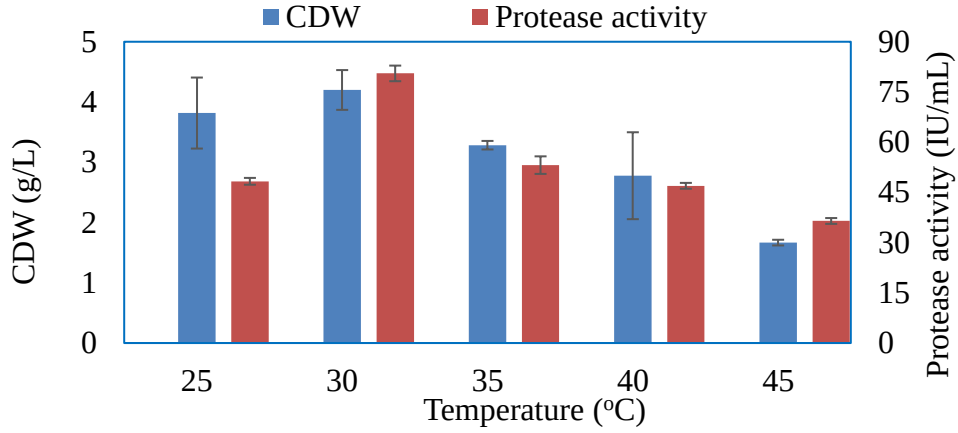

Figure 5. Effect of pH on growth and protease activity of Virgibacillus sp. strain T1.6

The optimum temperature range of *Virgibacillus* sp. T1.6 was similar to that reported for strain *Virgibacillus halodenitrificans* ST-1. Strain ST-1 also grew well over a temperature range of 25 - 45 ^{o}C and exhibited the highest growth at 30 ^{o}C [15]. *Virgibacillus* strain TKNR13-3 was able to grow in a temperature range of 30 - 55 ^{o}C, and the highest protease activity was obtained at 37 ^{o}C [4].

**** Effect of cultivation time***

The effect of incubation time on the growth rate and protease production of T1.6 was then investigated. Figure 6 shows that both CDW and protease activity was increased during the fermentation process and reached maximum values of 5.1 g/L and 83 IU/mL after 36 h of cultivation, respectively.

The protease activity obtained in this study by *Virgibacillus* sp. T1.6 (83 IU/mL) is much higher than that obtained by other *Virgibacillus* species isolated from shrimp paste. For example, a maximum protease activity of about 1.7 IU/mL was obtained by strain *Virgibacillus* sp. TKNR13-3 after 3 days of cultivation [4]. Higher protease activity of 9.5 IU/mL in the case of *V. halodenitrificans* strain MSK-10P was achieved after 48 h of cultivation [14]. Based on the results obtained in this study, it can be concluded that *Virgibacillus* sp. T1.6 is a good candidate for protease production.

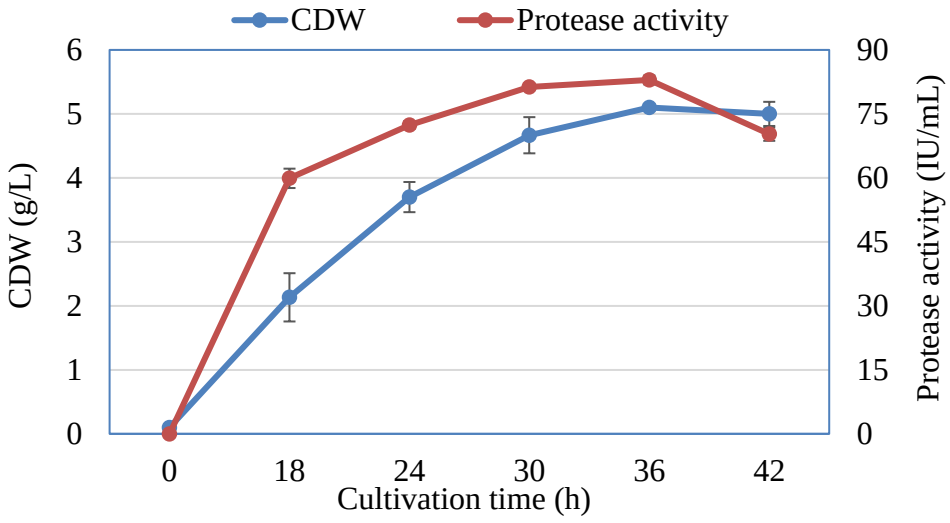

Figure 6. Time course of the growth and protease production by Virgibacillus sp. T1.6 at optimum conditions (2.5% NaCl. pH 7.0, and 30 ºC)

3. Conclusions

A halophilic bacterium strain T1.6 isolated from shrimp paste was identified as a strain of the genus *Virgibacillus*. The cultural conditions for the production of protease by *Virgibacillus* sp. T1.6 has been developed in this study. The optimum enzyme production by the bacterial strain was found at 30 ºC, pH 7.0, and with 2.5% NaCl. A high protease activity of 83 IU/mL was obtained under optimum cultural conditions after 36 h of cultivation. It can be concluded that *Virgibacillus* sp. T1.6 can be a potential producer of protease.

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