IDENTIFICATION AND OPTIMIZATION OF PROTEASE PRODUCTION BY *Bacillus* sp. B9 ISOLATED FROM WASTE WATER

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Abstract: Proteases have high commercial value and find multiple applications in various industrial sectors in detergent, leather, textile, food and pharmaceutical industry. This study investigated the protease production and optimization by bacterial strain which named *Bacillus* sp. B9 isolated from waste water. The bacterial isolate was identified by morphological, biochemical and 16S rRNA phylogenetic analysis using 27F and 1492R primers has 99.64% sequence identity (100% query cover) with corresponding gene sequence of *Bacillus horneckiae* strain OOM25 (MH542294.1) on GenBank. The crude enzyme extract of the strain was also characterized the activity with respect to temperature, pH, incubation period and two different enzyme substrates. High protease activity produced by *Bacillus* sp. B9 was observed at 36h of incubation at 45ºC with the initial pH of 9.0 by using gelatin as its substrate. Additionally, when using gelatin substrate, the protease activity was much higher than in the case of casein.

Keyworks: Protease activity, Bacillus sp. *Bacillus horneckiae*, Waste water.

1. INTRODUCTION

Proteases play an important role in everyday life that are constitute about 60% of the total enzyme market [6]. Proteases are possessed a wide range of application in physiology and in industries such as detergent, leather, waste treatment, therapeutics, diagnostics, silk degumming, silver recovery, peptide synthesis, baking and brewing [2]; [18].

Protease was classified into two groups including exo-peptidases and endo-peptidases based on position of cleavage of peptide bonds [18]. Based on pH, proteases can be classified as alkaline, neutral and acidic. Alkaline proteases are quite important in industries as they have the capability to withstand higher pH conditions [10].

Nowadays, microbes are more propitious source of proteases than Plant and animal because of their huge diversity, expeditious growth, requirement for limited space during cultivation and easy genetic manipulation [19]. *Bacillus* is one of the most vital genera that have been used for alkaline proteases production [6].

The optimization of different fermentation parameters like nitrogen and carbon source, media pH, incubation temperature, agitation and incubation time can enhance the yield of industrially useful enzymes [12].

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This study deals with identification and optimization of *Bacillus* sp. B9 isolated from waste water and its protease. The proteases produced by researched strain was determined by varying different parameters such as pH, temperatures, incubation time and substrates for maximum enzyme activity.

2. MATERIALS AND METHODS

2.1. Isolation and identification of microorganism

Bacillus sp. B9 used for this study was obtained from non-treatment industrial waste water in Hue, Viet Nam. Sample was heated at 80^{o}C in shaking water bath in order to eliminate the vegetative cells before using. This strain was maintained on LB nutrient agar (10g tryptone; 5g yeast extract; 10g NaCl and 1,5% agar) having initial pH 7.9 at 30 ± 2^{o}C slants. The pure culture was stored as glycerol stocks and sub-cultured for every 2 weeks.

The bacterial strain was identified by morphological and biochemical characteristics as well as comparison of 16S rRNA gene. Morphological characterization was done using stereomicroscope and Gram staining method using bright field microscope. The bacterial isolate was characterized biochemically by performing oxidase and catalase activities. Genomic DNA of the Ibacteria was isolated by CTAB method. The partial fragment of 16S rRNA was amplified by PCR using genomic DNA as template with two primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). PCR products were sequenced by Sanger sequencing method at Apical Scientific Sdn Bhd company (Selangor, Malaysia). Nucleotide sequence of 16S rRNA was analyzed by Bio-Edit program and family of the bacterial strain was accessed using BLAST search with sequences which were published on GenBank. Phylogenetic tree was built by The Molecular Evolutionary Genetics Analysis X (MEGAX) (https://www.megasoftware.net). The tree was constructed using the neighbor-joining method and genetic distances were generated using the Kimura 2-parameter method [13].

2.2. Enzyme assay

Five milliliters of an overnight culture of *Bacillus* sp. B9 strain was used to inoculate a 250 mL Erlenmeyer flask containing a 100 mL aliquots of LB Broth (combine 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 1 liter of distilled water) and 1% of enzyme substrate (casein or gelatin) was kept for shaker fermentation (160 rpm) at different research condition of initial pH, temperature and incubation time period. The bacterial cells were harvested by centrifugation at 10.000 rpm for 10 min at 4°C. The obtained supernatant, named crude extract (CE), was filtered using a 0.22 μm filter (Merck Millipore, Millipak 40) and used for further optimization studies to enhance the protease production.

The extracellular protease production capacity of *Bacillus* strains was determined by using well diffusion method [16].

The graph of tyrosine and enzyme activity was determined according to Sigma protocol for enzymatic assay of protease [8]. This protocol is based on protein hydrolysis with protease, followed by stopping the reaction with 110 mM trichloracetic acid (TCA) solution. Quantify the product formed in the hydrolysis reaction by coloring with Folin 0.5 M reagent. Then, based on the standard graph of tyrosine to calculate the amount of product created by the enzyme catalyzed. Each unit of protease activity (UI) is the minimum amount of enzyme under experimental conditions hydrolyzed gelatin (0.65%) for 1 minute to form a soluble product in TCA, reaction Folin reagent gives an OD absorbance at 660 nm corresponding to 1M tyrosine in the calibration curve. The protease activity is determined by the formula:

$$Enzyme\ activity\ (UI/ml) = \frac{T * 11}{1 * 10 * 2}$$

In which:

T: the corresponding 1M tyrosin is released

11: total reaction volume (ml)

1: volume of enzyme used for the reaction (ml)

2: volume used to measure optical intensity after reaction with Folin reagent (ml)

10: reaction time (minutes)

2.3. Optimization of cultural conditions for protease production

Different cultural conditions including incubation temperature, broth pH, incubation period and enzyme substrates were optimized for maximum protease production based on one parameter at a time approach. Protease activity was determined for different ranges of each parameter tested by the protease assay method.

The influence of physical parameters of protease activity from *Bacillus* sp. B9 was investigated by using gelatin as its substrate. The effect of temperature on the enzyme activity was determined by performing the standard assay procedure at pH 7.9 within a temperature range from 25 to 50^{o}C. For optimizing pH, the medium was prepared by varying the pH range from 4.0 to 11.0 using the buffer systems including citrate phosphate, pH 4-6, sodium phosphate, pH 7.0, Tris-HCl, pH 8.0 and glycine NaOH, pH 9-11. Similarly, protease production was determined at various incubation times from 12 to 72h. For every 12h, each flask was filtered, followed by centrifugation to collect crude protease. In order to determine the effect of enzyme substrate, either 1% gelatin or 1% casein (using phosphate buffer) was added into initial broth medium before shaking fermentation.

2.4. Statistical analysis

Results are represented as mean ± standard error of three replicates. Data was processed and analyzed by R oftware version 3.6.2 [17]. Additionally, T-test also was used to compare the mean of two groups and “agricolae” package [7] was the tool for conducting Duncan Test.

3. RESULTS AND DISCUSSION

3.1. Results

Bacillus sp. B9 was isolated from wastewater was identified by 16S rARN sequencing, the results showed that 99.64% similar to the sequence of *Bacillus horneckiae* strain OOM25 (MH542294.1) (Figure 1-4). In this study, *Bacillus* sp. B9 demonstrated the protease production ability (Figure 5), and 36h incubation time, pH 9.0 and 45^{o}C was optimum growth parameters for maximum enzyme activity (Figure 6-8). Additionally, the protease activity was much higher when using gelatin compared with casein as substrate (Figure 9).

Figure 1. *Bacillus sp. B9 colony morphology in LB agar (A) and Gram staining of it showing Gram-positive rods (B)*

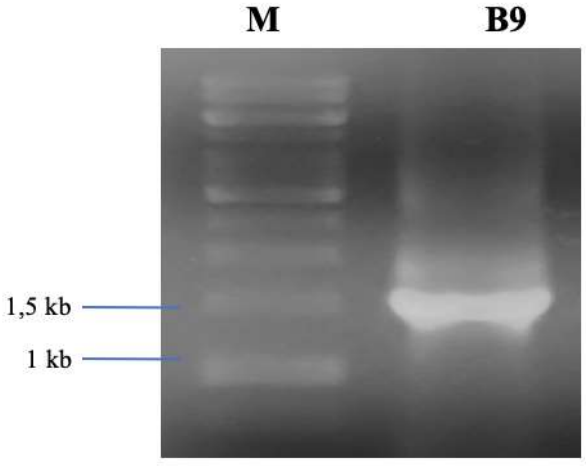

Figure 2. *PCR product electrophoresis of 16S rRNA gene amplification of Bacillus sp. B9 (M: Marker; B9: PCR product of Bacillus sp. B9*)

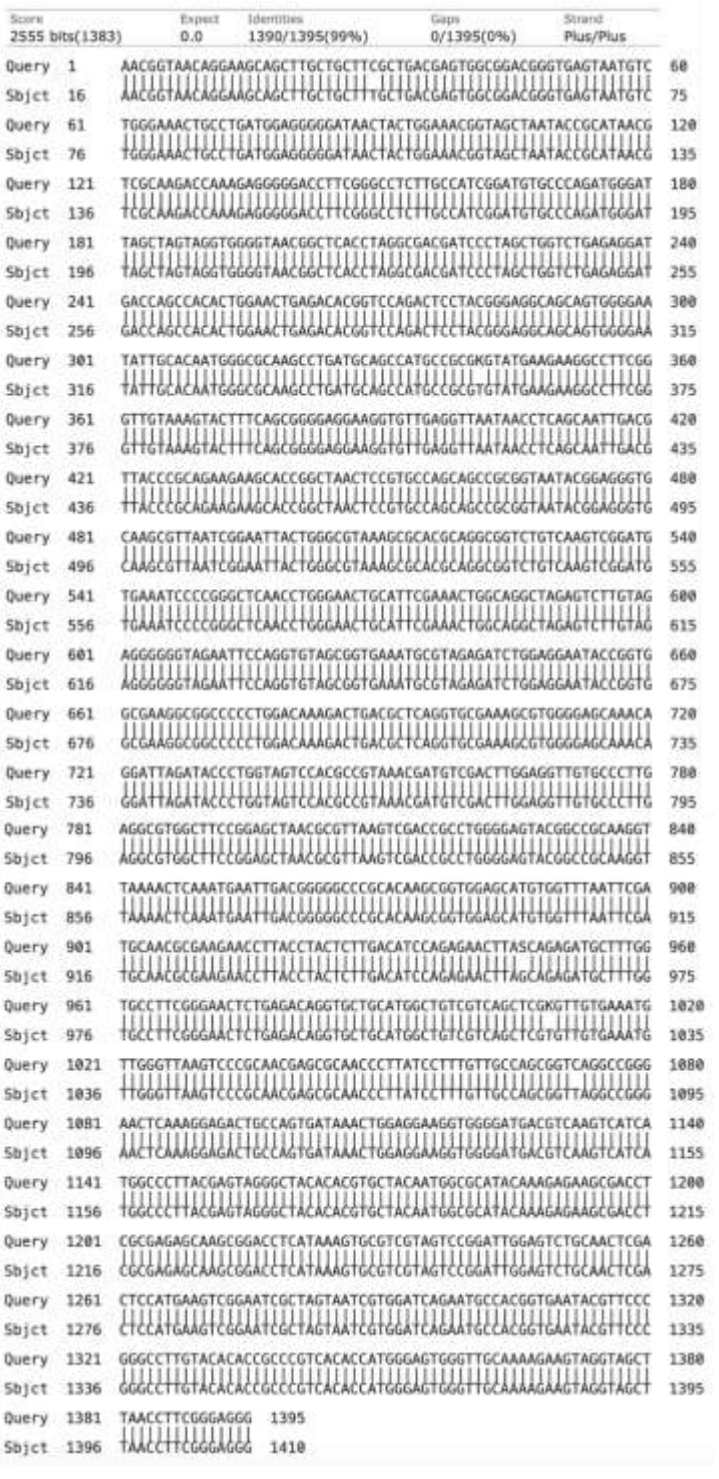

Figure 3. *Results comparing 16S rRNA gene sequence of Bacillus sp. B9 with Bacillus horneckiae strain OOM25 on GenBank*

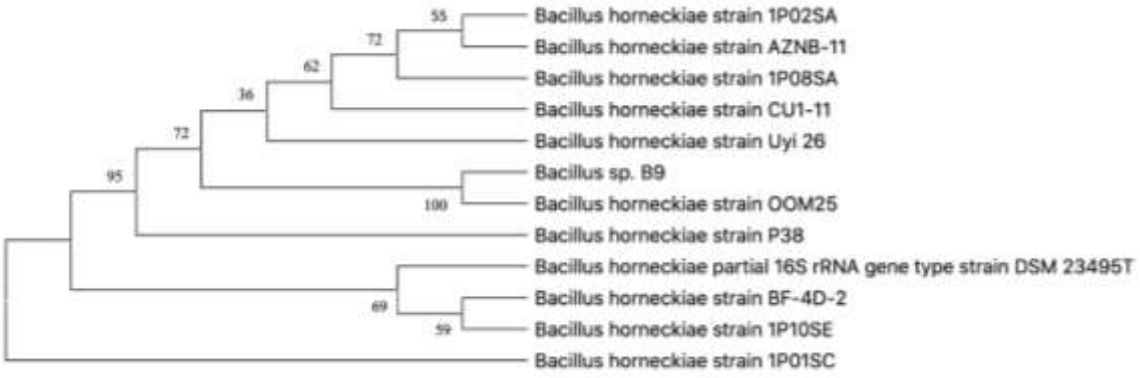

Figure 4. *Phylogenetic tree of Bacillus sp. B9 based on 16S rRNA gene sequences (the numbers at the branches are bootstrap confidence percentages from 1000 bootstrapped trees)*

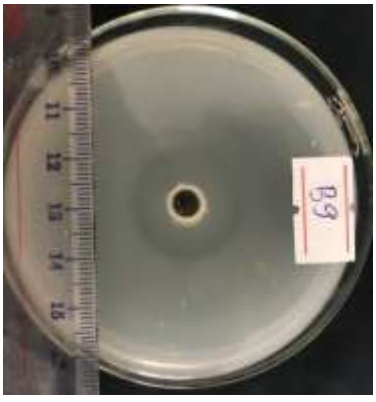

Figure 5. *Clear zone surrounding colony of Bacillus sp. B9 showing protease activity on LB agar stained with $HgCl_2$ 10%*

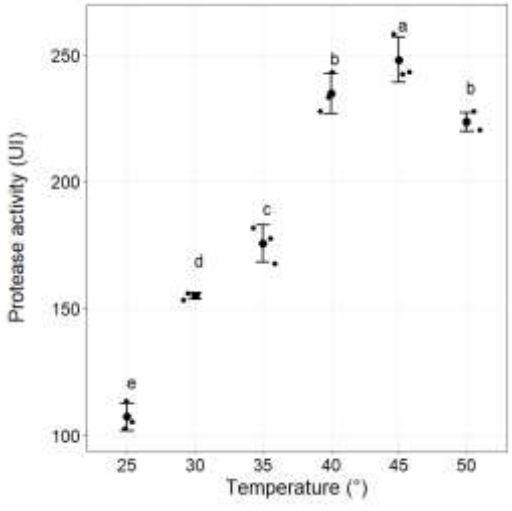

Figure 6. *Effect of different temperature on protease activity in Bacillus sp. B9*

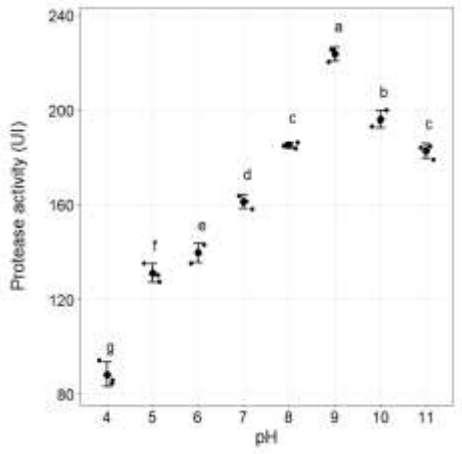

Figure 7. *Effect of different pH on protease activity in Bacillus sp. B9*

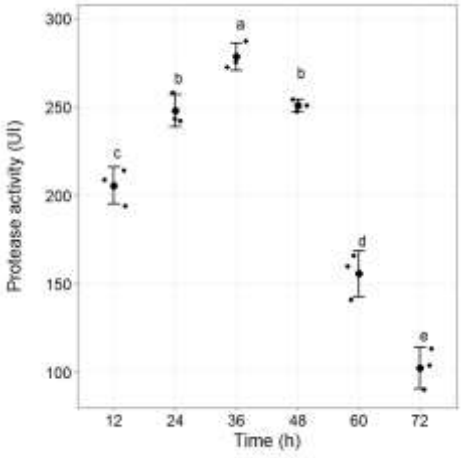

Figure 8. *Effect of different incubation periods on protease activity in Bacillus sp. B9*

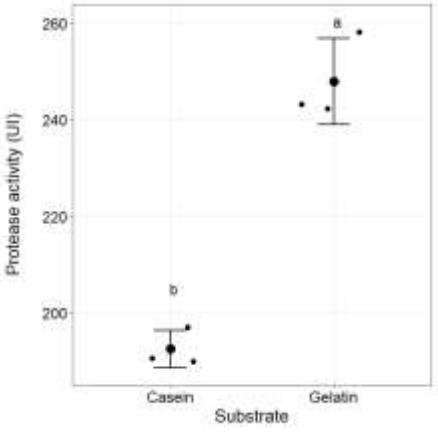

Figure 9. *Effect of casein and gelatin substrates on protease activity in Bacillus sp. B9*

3.2. Discussion

Identification of Bacillus strain isolated by 16S rRNA sequencing

After being isolated from wastewater, based on colony morphology and cell characteristics, the strain was preliminarily identified under the genus *Bacillus* and named *Bacillus* sp. B9. *Bacillus* sp. B9 has circle colonies, yellowish, irregular edges, elevation raise, 3-5mm diameters in LB agar. The vegetative cells are rod-shapes, motile and contain sub-terminal endospore and gram staining of it showed gram-positive rods (Figure 1).

The *Bacillus* sp. B9 was successtively identified by 16S rRNA gene sequencing. The 16S rRNA gene was amplified by PCR reaction with a pair of specific primers as shown in the methodology section. The product of the PCR reaction was electrophoresis on 1% agarose gel. Results of electrophoresis are shown in Figure 2. The electrophoresis shows that the PCR positive products has size of about 1436 bp as expected. Positive product of PCR reaction with primer pair recognizes 16S rRNA gene segment of *Bacillus* sp. B9 has been sequenced for the nucleotides. The BLAST result on GenBank showed the sequence of 16S rRNA gene segment of bacillus sp. B9 is 99.64% similar to the 16S rRNA gene sequence of *Bacillus horneckiae* strain OOM25 (MH542294.1) (Figure 3). This result shows strains of *Bacillus* sp. B9 belongs to the species of *Bacillus horneckiae* (Figure 4).

Protease biosynthesis in Bacillus sp. B9

In the present study, gelatin was used as the substrate in nutrient agar medium for screening and showed the maximum zone of protease activity (18.3 ± 1.53 mm) after overnight incubation and addition of 10% $HgCl_2$ solution as the indicator (Figure 5). The zone was distinct with the surrounding by the transparent white in which the pH of the culture medium was maintained as 7.9 ± 0.2.

Effect of temperature on protease activity

Temperature is a critical factor for maximum enzyme activity [11]. In this study, the temperature effect on the activity of protease was analyzed between 25 and 50^{o}C (Figure 6). The result showed that an optimal protease activity at 45^{o}C (247.8 ± 8.9 UI) while the temperature below or above 45^{o}C exhibited lower activities of protease. Thus, the protease obtained from *Bacillus* sp. B9 can be considered a thermostable enzyme. When the incubated temperature was reached at 50^{o}C, there was a gradual enzyme activity decrease and < 15% of the maximum activity was retained. *Bacillus thuriginesis* was reported highest protease activity at 47^{o}C [1]. The current strain experienced optimum temperature was 40^{o}C for enzyme activity with *Bacillus infantis* [20] and *Bacillus cereus* [5]; while the same temperature (45^{o}C) also was recorded as suitable temperature for protease activity of *Bacillus subtilis* [9]. High and low temperatures may affect structural and functional changes in proteins to modify biological behavior by altering rates of enzyme activities, which may have important consequences for the integration of biochemical pathways [11].

Effect of initial pH on protease activity

Protease yields vary considerably with pH which may be attributed to other components of the medium and its combined influence on the metabolism of the bacterial species [15]. The optimum pH for alkaline proteases of *Bacillus* sp. has been reported to vary from 8 to 11 and they are species specific [4]. To determine the optimal H^+ concentration for protease activity, *Bacillus* sp. B9 was cultivated over the pH range 4-11. The influence of pH on the enzyme activity was shown in Figure 7. The enzyme was active over the pH range studied. The activity of its protease was very low at a pH between 4 and 6 and finally increased sharply beyond pH 7. The protease had its maximum activity at pH 9 (223.5 ± 2.8 UI) before gradually decreasing. This result indicated the alkaline source of the enzyme which was similar data with *Bacillus cereus* [5]; *Bacillus licheniformis* [14].

Effect of incubation time on protease activity

Since microorganisms show considerable variation at different incubation periods, it was very essential to detect the optimum incubation time at which the organism showed highest enzyme activity. In the tests to optimize time of *Bacillus* sp. B9, protease activity increased rapidly from 12 to 36 hours. At 36 hours, the protease activity was maximal (278.4 ± 7.8 UI); it then decreased with time (Figure 8). *Bacillus subtilis* [21] and *Bacillus cereus* FT1 [3] was reported that 48 hours was suitable time for maximal enzyme activity.

Substrate optimization for protease activity

To optimize the substrates for protease activity in *Bacillus* sp. B9, 1% casein and gelatin were added to culture medium (pH 7.9). After 24 hours shaking fermentation at room temperature (30^oC), gelatin demonstrated higher protease activity than casein (Figure 9) in which the result was 192.4 ± 3.9 and 248.8 ± 5.9 UI for casein and gelatin, respectively.

4. CONCLUSION

Maximum enzyme activity is aimed while selecting an microorganism for enzyme production for commercial uses. The present study reported the increased protease activity by the waste water isolated *Bacillus* sp. B9 which has a 16S rARN sequence showing 99.64% similarity with *Bacillus horneckiae* strain OOM25 (MH 542294.1) under optimized cultural conditions. The result determined the optimum growth parameters for cultivating researched strain for maximum activity of protease was observed at pH 9, 36h incubation and a temperature of 45^oC. The data also indicated that protease activity was clearly higher in case of using gelatin instead of casein as enzyme substrate.

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