CIBTech Journal of Microbiology ISSN: 2319-3867
An Online International Journal Available at http://www.cibtech.org/cjm.htm
2021 Vol.10 pp.1-7/Nguyen
Research Article (Open Access)

OPTIMIZATION OF PROTEASE PRODUCTION BY *BACILLUS THURINGIENSIS* STRAIN FDAARGOS_791 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 protease production and optimization by bacterial strain FDAARGOS_791 isolated from waste water. The bacterial isolate was selected and identified by microscopic, macroscopic, biochemical and 16S rRNA phylogenetic analyses using universal primers has 99.72% sequence identity with corresponding gene sequence of *Bacillus thuringiensis* strain FDAARGOS_791. The crude enzyme extract of the strain was also characterized with respect to temperature, pH, incubation period and two different enzyme substrates. High protease activity produced by *Bacillus thuringiensis* strain FDAARGOS_791 was observed at 24h of incubation at 40°C with the initial pH of 9.0 by using gelatin as its substrate. This study also showed that there was no significant difference in enzyme activity of isolated by using casein and gelatin substrates in nutrient medium.

Keywords: *Protease, Bacillus thuringiensis, Waste Water*

INTRODUCTION
Proteases play an important role in everyday life that constitute about 60% of the total enzyme market (Cui *et al*., 2015). Proteases 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 (Akbar *et al*., 2017; Ramkumar *et al*., 2016).
Protease are classified into two groups including exo-peptidases and endo-peptidases based on position of cleavage of peptide bonds (Ramkumar *et al*., 2016). 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 (Gupta *et al*., 2002).
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 (Rao *et al*., 1998). *Bacillus* is one of the most vital genera that have been used for alkaline proteases production (Cui *et al*., 2015).
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 (Huang *et al.*, 2003).
Bacillus thuringiensis subspecies produce metalloproteases and serine alkaline proteases (endogenous) which affect sporulation and entomotoxicity against different insect orders (Satinder *et al.,* 2007).
This study deals with identification and optimization of *Bacillus thuringiensis* strain FDAARGOS_791 isolated from waste water and its protease. The activity of proteases produced by researched strain was determined by varying different parameters including pH, temperatures, incubation time and substrates for maximum enzyme activity.

MATERIALS AND METHODS
Isolation and identification of microorganism
Bacillus thuringiensis strain FDAARGOS_791 used for this study was obtained from non-treatment

industrial waste water in Hue, Viet Nam. Sample was heated at 80°C in shaking water bath 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°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 bacteria was isolated by CTAB method. 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. Nucleotide sequence of 16S rRNA was analyzed by Bio-Edit program and the phylogeny and family of the bacterial strain was accessed using BLAST search with sequences which were published on GenBank.

Enzyme assay

Five milliliters of an overnight culture of *Bacillus thuringiensis* 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 was kept for shaker fermentation (160rpm) 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 (Ponnuswamy *et at.,* 2013).

The graph of tyrosine and enzyme activity was determined according to Sigma protocol for enzymatic assay of protease (Folin *et al.,* 1929). 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)

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 parameters tested by the protease assay method.

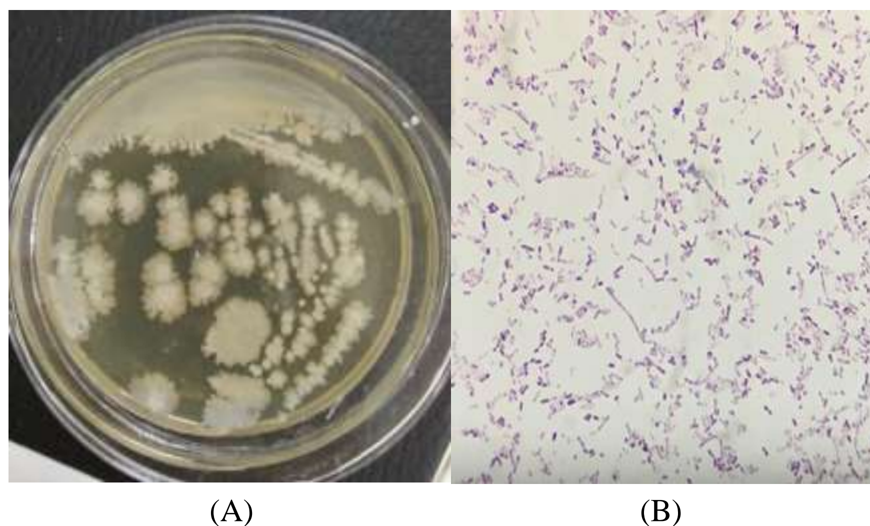
The influence of physical parameters of protease activity from *Bacillus thuringiensis* strain FDAARGOS_791 was investigated by using gelatin as its substrate. The effect of temperature on the enzyme activity was determined by performing the standard assay procedure at pH 7.9 within a temperature range from 25 to 50°C. For optimizing pH, the medium was prepared by varying the pH

range from 4 to 11 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 time 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 in to initial broth medium before fermentation.

Statistical analysis

Results are represented as mean ± standard error of three replicates. Data was processed and analyzed by R software version 3.6.2 (R Core Team, 2019). Additionally, T-test also was used to compare the mean of two groups and "agricolae" package (Mendiburu, 2020) was the tool for conducting Duncan Test.

RESULTS AND DISCUSSION



(A) (B)

Figure 1: *Bacillus thuringiensis* strain FDAARGOS_791 colony morphology in LB agar (A) and Gram staining of it showing Gram-positive rods (B)

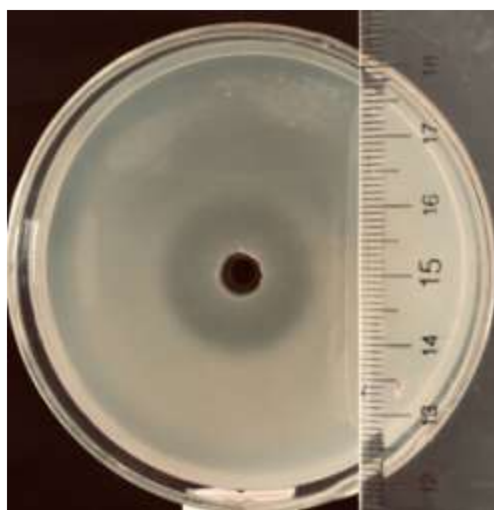


Figure 2: Clear zone surrounding colony of *Bacillus thuringiensis* strain FDAARGOS_791 showing protease activity on LB agar stained with $HgCl_2$ 10%

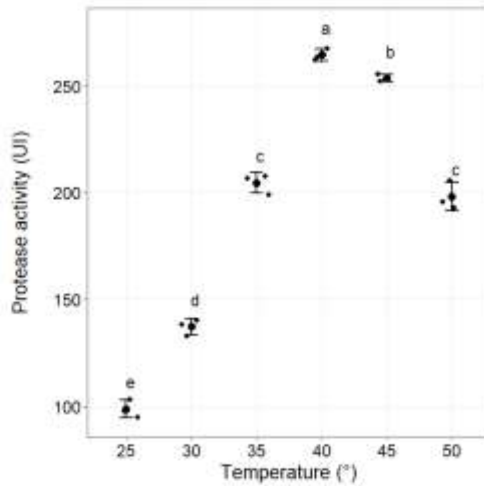


Figure 3: Effect of different temperature on protease activity in *Bacillus thuringiensis* strain FDAARGOS_791

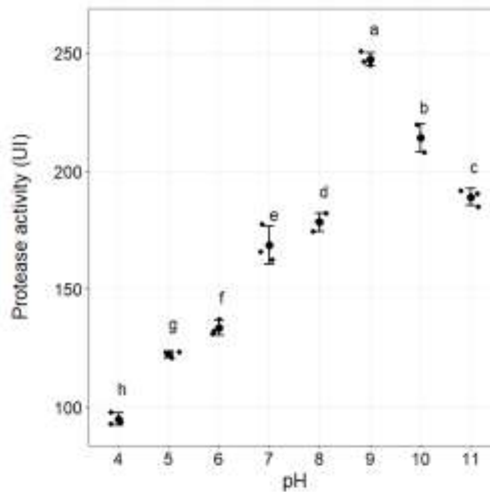


Figure 4: Effect of different pH on protease activity in *Bacillus thuringiensis* strain FDAARGOS_791

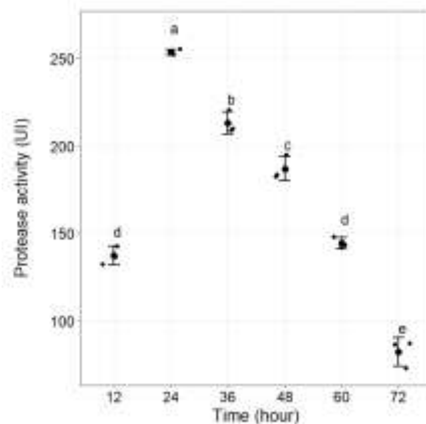


Figure 5: Effect of different incubation periods on protease activity in *Bacillus thuringiensis* strain FDAARGOS_791

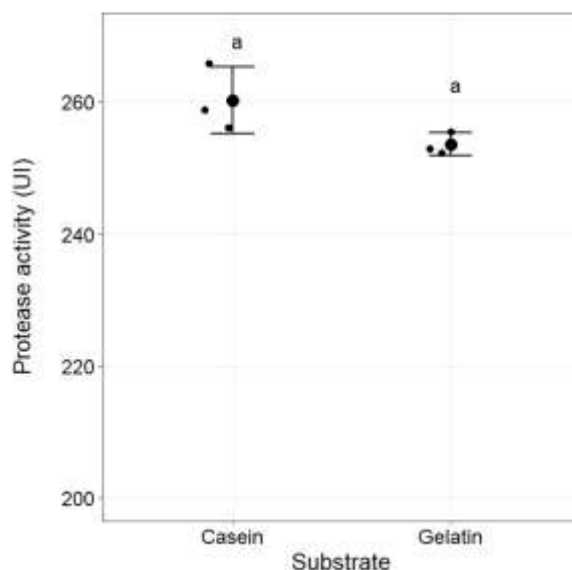


Figure 6: Effect of casein and gelatin substrates on protease activity in *Bacillus thuringiensis* strain FDAARGOS_791

Results

Bacillus thuringiensis strain FDAARGOS_791 was isolated from waste water which has circle colonies, white, wrinkled edges, slippery, elevation raise in LB agar. The vegetative cells contain endospore and gram staining of it showed gram-positive rods. The strain isolated also indicated positive catalase and negative oxidase. In this study, *Bacillus thuringiensis* strain FDAARGOS_791 demonstrated the protease production ability, and 24h incubation time, pH 9.0 and 40°C was optimum growth parameters for maximum enzyme activity. Additionally, the protease activity was not changed when using gelatin or casein as substrate. The obtained results were indicated in Figures (1-6).

Discussion

In the present study, gelatin was used as the substrate in nutrient agar medium for screening and showed the maximum zone of protease activity (22 mm) after overnight incubation and addition of 10% $HgCl_2$ solution as the indicator (Figure 2). This result was similar with zone of activity of *Bacillus subtilis* (Gaurav *et al.,* 2014) 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 (Haddar *et al*., 2009). In this study, the temperature effect on the activity of protease was analyzed between 25 and 50°C (Figure 3). The result showed that an optimal protease activity at 40°C (264 ± 2.8 UI) while the temperature below or above 40°C exhibited lower activities of protease. Thus, the protease obtained from *Bacillus thuringiensis* strain FDAARGOS_791 can be considered a thermostable enzyme. When the incubated temperature was reached at 50°C, there was a rapid enzyme activity decrease and < 25% of the maximum activity was retained. *Bacillus thuriginesis* also was reported highest protease activity at 47°C (Agasthya *et al.,* 2013). The current strain experienced same optimum temperature (40°C) for enzyme activity with *Bacillus infantis* (Saguu *et al.,* 2017) and *Bacillus cereus* (Catalina Kotlar *et al.,* 2915) while 45°C was recorded as suitable temperature for protease activity of *Bacillus subtilis* (Gaurav *et al.,* 2014). 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 (Haddar *et al*., 2009).

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 (Maghsoodi *et al.,* 2013). The optimum pH for alkaline proteases of *Bacillus* sp. has been reported to vary from 8 to 11 and they are species specific (Bajaj *et al.,* 2013). To determine the optimal H+ concentration for protease activity, *Bacillus thuringiensis* strain FDAARGOS_791 was cultivated over the pH range 4-11. The influence of pH on the enzyme activity was shown in Figure 4. 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 (247± 2.9 UI) before gradually decreasing. This result indicated the alkaline source of the enzyme which was similar with *Bacillus cereus* (Catalina Kotlar *et al*., 2015); *Bacillus licheniformis* (Lakshmi *et al.,* 2014). On the other hand, Agasthya and his partner (2013) showed that the optimum pH for the protease activity of *Bacillus thuringiensis* was 8.

Effect of incubation periods 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 thuringiensis* strain FDAARGOS_791, protease activity increased rapidly from 12 to 24 hours. At 24 hours, the protease activity was maximal (253 ± 1.7 UI) which was double from previous 12 hours; it then decreased with time (Figure 5). *Bacillus subtilis* (Yossan *et al.,* 2006) and *Bacillus cereus* FT1 (Asha *et al.,* 2018) was reported that 48 hours was suitable time for maximal enzyme activity.

Effect of substrates on protease activity

To estimate the effect of the kind of enzyme substrates on protease activity in *Bacillus thuringiensis* strain FDAARGOS_791, 1% casein and gelatin were added to culture medium (pH 7.9). After 24 hours shaking fermentation at room temperature (30°C), there was no significant difference between two kind of substrates on protease activity with (Figure 6) in which the result was 260 ± 5.0 and 254 ± 1.7 UI for casein and gelatin, respectively.

CONCLUSION

Maximum enzyme activity is aimed while selecting an microoganism for enzyme production for commercial uses. Present study reported the increase in protease activity by the waste water isolated *Bacillus thuringiensis* FDAARGOS_791 under optimized cultural conditions. The result determined the optimum growth parameters for cultivating researched strain for maximum activity of protease which was observed at pH 9, 24hour incubation and a temperature of 40°C. The data also indicated that protease activity was almost unchanged in case of using gelatin instead of casein as enzyme substrate.

ACKNOWLEDGMENT

We would like to acknowledge Hue University of Education, Hue city, Viet Nam for funding the study.

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