Chapter 17
Enzyme Activity Analysis of Protease Produced by Marine Bacteria

Qi Zhang, Xihong He and Hao Liu

Abstract Proteolytic enzymes are ubiquitous in nature, found in all living organisms, and have been applied to many aspects of industry. In this study, 19 strains hydrolyzing protein were screened from deep-sea sediments, of which No. 9 strain has the largest protease activity. Based on morphological characterization and 16S rDNA gene sequences analysis, the strain was identified to be *Bacillus subtilis*. The optimum medium for producing protease was determined through orthogonal test as follows: 2.5 % xylose, 3.5 % peptone, 1.6 % yeast extract and 1 % NaCl, the highest protease activity reached 215.95 U/mL, and increased by 128.06 % than the origin.

Keywords Marine microorganisms · 16S rDNA · Protease · Enzyme activity

17.1 Introduction

Proteases are one of the most important industrial enzymes, accounting for nearly 60 % of all enzyme sales [1], and have been used in various industrial processes, such as pharmaceutical, food, leather, silver recovery, and textiles [2, 3]. However, the use of protease in industrial application is limited by the low activity and instability of the enzymes [4]. In order to improve the stability of the enzyme, the technique of random mutagenesis and site-directed mutagenesis emerged. Pantoliano et al. [5] reported that the rate of inactivation of the combination variant was 300 times slower than that of wild-type subtilisin by six individual amino acid substitution at separate positions. Narhi et al. [6] reported the stabilization of subtilisin from *Bacillus subtilis* by modification of two Asn-Gly sequences. However, this approach is dependent on the number of structural and biochemical data, so the number of variants is rare. While, the microorganisms from exotic

Q. Zhang · X. He · H. Liu (✉)
MOE Key Laboratory of Industrial Fermentation Microbiology, College of Biotechnology, Tianjin University of Science & Technology, Tianjin 300457, China
e-mail: liuhao@tust.edu.cn

T.-C. Zhang and M. Nakajima (eds.), *Advances in Applied Biotechnology*,
Lecture Notes in Electrical Engineering 333, DOI 10.1007/978-3-662-46318-5_17

environment are considered to be an important source of enzymes, and they remain catalytically active at the extremes of salinity, pH, and pressure [7, 8]. Therefore, screening of producing stable protease strains and studying the optimum medium for protease production are becoming increasingly important.

In this study, we screened the strains producing protease from deep-sea sediment, and studied the factors on enzyme production to improve the protease activity, so as to provide theoretical support for the screening strains producing stable protease.

17.2 Materials and Methods

17.2.1 Materials and Reagents

Sterile taken deep-sea sediments, R2A Agar (Oxoid) [containing protease peptone 0.5 g, starch 0.5 g, glucose 0.5 g, yeast extract 0.5 g, casein hydrolysate 0.5 g, potassium hydrogen phosphate 0.3 g, sodium pyruvate 0.3 g, magnesium sulfate anhydrous 0.024 g, bacteriological agar 15 g, 1 L distilled seawater], 2216 marine agar [9] (Difco Laboratories), artificial seawater (sodium chloride 24 g, magnesium chloride 10.88 g, sodium sulfate 4 g, calcium chloride 146 g, potassium chloride 0.7 g, sodium bicarbonate 0.2 g, potassium bromide 0.1 g, boric acid 0.027 g, strontium chloride 0.04 g, sodium fluoride 0.003 g, 1 L distilled water) [10].

17.2.2 Isolation and Screening [11]

The strains producing protease were isolated from deep-sea sediments through plate screening. In detail, 1 g of sediments sample was added to 9 mL of sterile artificial seawater, and inoculated, respectively, on R2A and 2216 marine agar plate containing sterile skimmed milk with final concentration of 1 % after gradient dilution (10^{-2}–10^{-5}), then cultured for 2 days at 28 °C. Finally, the single colonies that grew well, and obviously formed proteolytic circles were retained for subsequent screening.

The strains with big proteolytic circles were inoculated in fermentation medium, respectively, and cultured in shaken flasks with rotational speed of 200 r/min for 48 h at 28 °C. The enzyme activity was measured by Folin-phenol method [12], and the strain with the highest enzyme activity was as candidate strain.

17.2.3 Bacterial Cultivation and Identification

The candidate strains were cultured in 2216 marine agar plates at 28 °C, and separated single colonies were selected for PCR amplification. The 16S rDNA gene

was amplified by the forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and the reverse primer 1492R (5′-TACGGCTACCTTGTTACGACTT-3′). The amplification procedure included 5 min at 95 °C, 50 s at 95 °C, 50 s at 52 °C, followed by 30 cycles of 2 min at 72 °C and extend for 10 min at 72 °C. The PCR products were purified and linked with pMD18-T and sequenced with both the forward and reverse primers. The initial nearest neighbor sequences were made by the online BLAST program to the NCBI GenBank database. Sequences were aligned using the CLUS-X program, and phylogenetic tree was constructed with MEGA program.

17.2.4 Protease Activity Analysis

The protease activity was determined by the method of Kembhavi et al. [13]. One unit of protease activity was defined as the amount of enzyme that liberated 1 μmol of tyrosine in 1 min.

17.2.5 Effects of Different Factors on the Ability of Candidate Strain Producing Protease

Only one factor was changed in each experiment keeping all others constant. The salt content, different carbon and nitrogen sources, and their different concentrations were initially studied at single factor test.

17.3 Result

17.3.1 Screening of Strains Producing Protease and Preliminary Identification of the Ability of Enzyme Production

As shown in Fig. 17.1, four stains displayed relatively larger zone of proteolytic activity when grown on skimmed milk agar medium. Then, by using Folin-phenol method, the ability of enzyme production was measured to screen the candidate strain. The result revealed that the ability of enzyme production of No. 9 is the highest (94.69 U/mL), so No. 9 strain was the candidate strain.

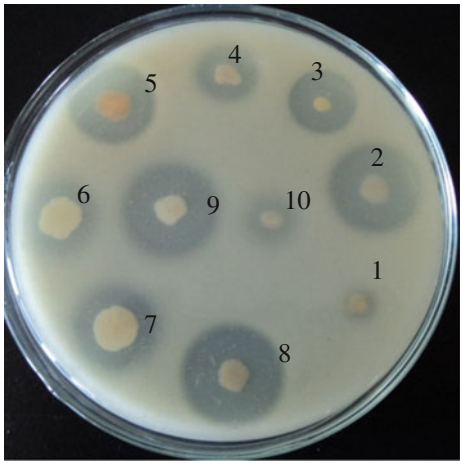

Fig. 17.1 Different strains were inoculated on skimmed milk plate. Different numbers represent different strains, Nos. *2*, *5*, *8*, and *9* strains displayed relatively larger proteolytic *circles* and defined as primarily screening strains

17.3.2 Analysis of 16S rDNA and Physiological and Biochemical Characterization of Candidate Strain

A maximum-likelihood phylogenetic tree (Figs. 17.2 and 17.3) showed that candidate strain (No. 9) had quite high sequence identity to the *B. subtilis* strain Acj 115, and further the result of Gram's method showed that No. 9 strain was a Gram-positive bacterium (Fig. 17.4). Identification of physiological and biochemical characterization of candidate strain revealed that V.P. test was positive, while methyl red test was negative, and most of the carbon source could be used. So the candidate strain was identified to be *B. subtilis.*

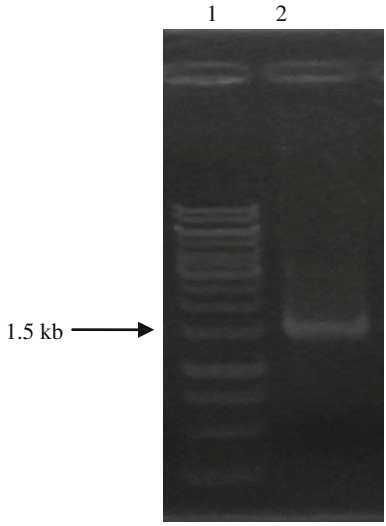

Fig. 17.2 The amplification of 16S rDNA. *Lane 1* 1 kb DNA ladder, *Lane 2* PCR product of 16S rDNA fragment of 1,465 bp

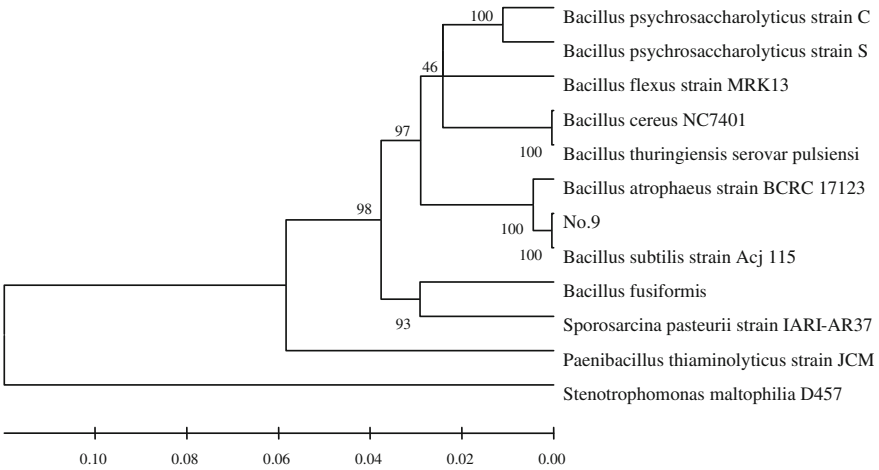

Fig. 17.3 The phylogenetic tree resulting from analysis of the 16S rDNA sequences of candidate strain (No. 9). The tree was created using maximum-likelihood distance clustered by the neighbor-joining method and with MEGA program

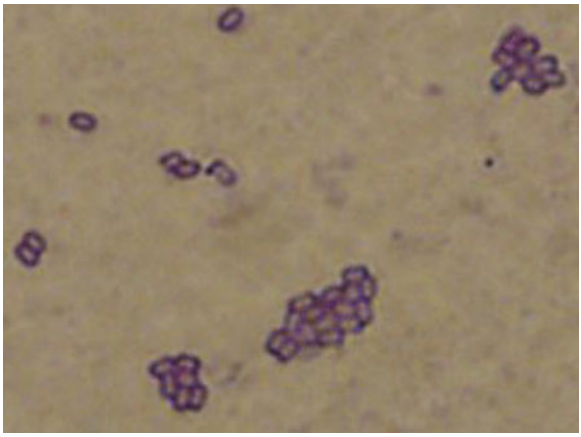

Fig. 17.4 The result of Gram's dye of bacterium. The strain cultured in the 2216 E medium for 2 days, then Gram's staining and oil microscopy observation, the result showed that the candidate strain is a Gram-positive bacterium

17.3.3 Effect of Different Factors on the Ability of Candidate Strain Producing Protease

17.3.3.1 Carbon Sources

After carbon sources were added to the fermentation medium, compared with the control containing 2 % glucose, the ability of candidate strain producing protease increased by 1.37 fold with xylose (Fig. 17.5). In addition, xylose was added with

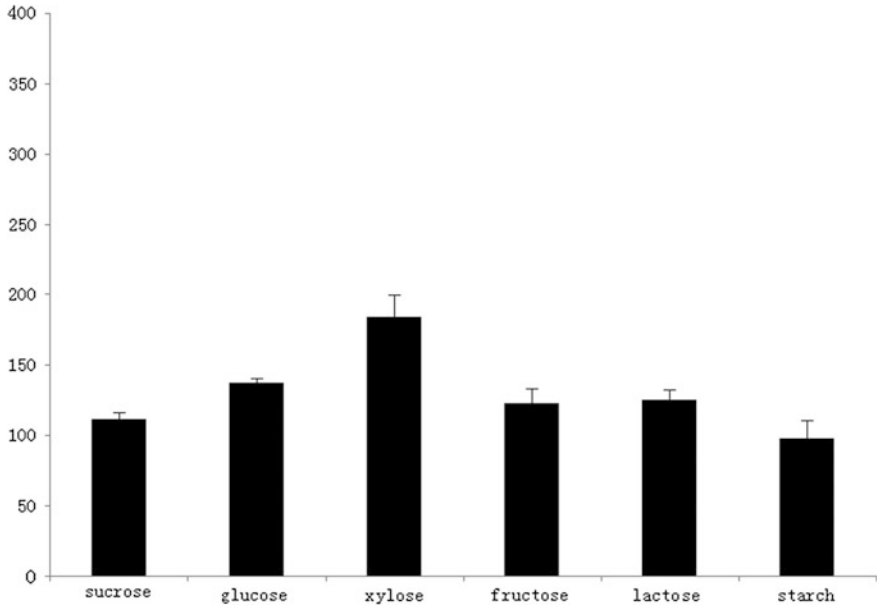

Fig. 17.5 Effects of carbon sources on protease activity. The candidate strain was grown on 2216 E medium at 28 °C and 200 rpm. The protease activity was detected by Folin-phenol method. The highest enzyme activity was 185.78 U/mL, when the carbon source was xylose

concentration of range from 1 to 5 % respectively, and the result showed that the protease activity improved obviously with 2 % of xylose in the medium (185.78 U/mL).

17.3.3.2 Nitrogen Sources

After nitrogen sources were added to the fermentation medium, the result (Figs. 17.6 and 17.7) shows that the protease activity is higher with organic nitrogen sources than inorganic nitrogen sources, and the control has the highest protease activity (123.15 U/mL). Moreover, the supplementation of yeast extract (1.6 %) and peptone (3 %), respectively, resulted in maximal protease activity.

17.3.3.3 NaCl Concentration

Different concentrations of NaCl were added to the fermentation medium, the result showed that the ability of candidate strain producing protease reduced compared with the control containing 2 % NaCl (125.81 U/mL).

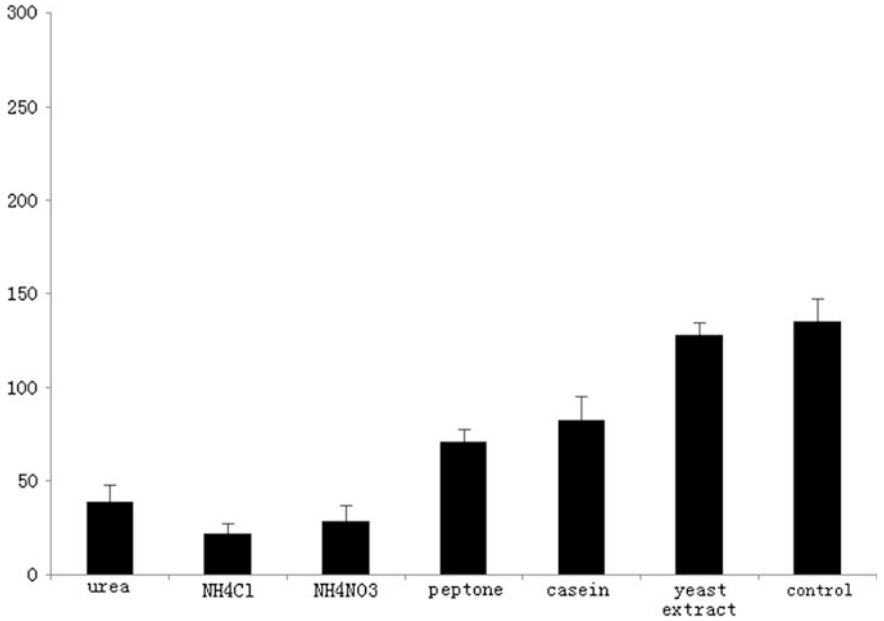

Fig. 17.6 Effects of nitrogen sources on protease activity. The strain grown on 2216 E medium at 28 °C and 200 rpm with 2 % glucose. The protease activity reached the maximum (123.15 U/mL), when peptone and yeast extract are the complex nitrogen sources

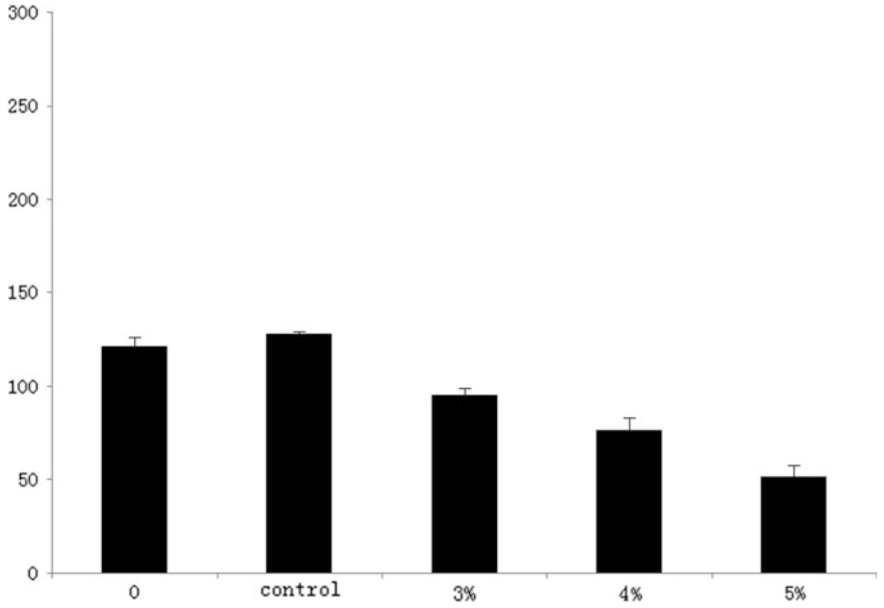

Fig. 17.7 Effects of different concentrations of NaCl on protease activity. The strain grown on 2216 E medium at 28 °C with 2 % glucose, 1 % peptone and 0.5 % yeast extract

Table 17.1 The result of orthogonal test

Group number	Xylose (%)	Peptone (%)	Yeast extract (%)	NaCl (%)	Enzyme activity (U/mL)
1	1.5	2.5	1.2	1	174.13
2	1.5	3	1.6	2	190.35
3	1.5	3.5	2	3	192.87
4	2	2.5	1.6	3	174.13
5	2	3	2	1	212.07
6	2	3.5	1.2	2	184.87
7	2.5	2.5	2	2	200.87
8	2.5	3	1.2	3	164.98
9	2.5	3.5	1.6	1	215.95
K_1	557.35	523.98	523.98	602.15	
K_2	571.06	567.41	580.43	576.09	
K_3	581.81	620.89	605.81	531.98	
F	1.00	23.49	11.68	8.37	

F stands for analysis of variance values

17.3.3.4 Orthogonal Test

Orthogonal test with four factors and three levels was performed to analyze the optimal medium for producing protease. The result (Table 17.1) showed that the order of importance that influenced protease activity was found to be peptone > yeast extract > NaCl > xylose, and the optimal combination parameters were 2.5 % xylose, 3.5 % peptone, 1.6 % yeast extract, and 1 % NaCl. Under the optimal culture medium, the activity of protease increased by 128.06 % than the previous culture medium.

17.4 Discussion

By plate screening and enzyme activity determination, No. 9 strain was screened as the experimental strain, followed by construct phylogenetic tree and identification of physiological and biochemical characteristics showed that experimental strain was *B. subtilis*.

Further, through orthogonal test, the best culture medium was determined as: xylose 2.5 %, peptone 3.5 %, yeast extract 1.6 %, NaCl 1 %, and artificial seawater without NaCl. The activity of protease increased by 128.06 % compared to the origin, and peptone was the most important factor. The results of single factor experiment and orthogonal test were different may be due to the interaction between the factors. This paper has studied the conditions for improving the activity of protease, but the stability of protease needs further analysis.

Acknowledgments The authors gratefully acknowledge the support of the National Nature Science Foundation of China (No. 31201282) and the 863 project (No. 2012AA020403).

References

1. Orhan E, Omay D, Gvüenilir Y (2005) Partial purification and characterization of protease enzyme from Bacillus subtilis and Bacillus cereus. Appl Biochem Biotechnol 121(1–3):183–194
2. Basu SK, Govardhan CP, Jung CW (2004) Protein crystals for the delivery of biopharmaceuticals. Expert Opin Biol Ther 4(3):301–317
3. Kumar S, Sharma NS, Saharan MR (2005) Extracellular acid protease from Rhizopus oryzae: purification and characterization. Process Biochem 40(5):1701–1705
4. Gupta R, Beg QK, Lorenz P (2002) Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59(1):15–32
5. Pantoliano MW, Whitlow M, Wood JF et al (1989) Large increases in general stability for subtilisin BPN' through incremental changes in the free energy of unfolding. Biochemistry 28 (18):7205–7213
6. Narhi LO, Stabinsky Y, Levitt M et al (1991) Enhanced stability of subtilisin by three point mutations. Biotechnol Appl Biochem 13(1):12–24
7. Holmes ML, Scopes RK, Moritz RL et al (1997) Purification and analysis of an extremely halophilic β-galactosidase from Haloferax alicantei. Bba-Protein Struct M 1337(2):276–286
8. Synowiecki Z, Grzybowska B, Zdzieblo A (2006) Sources, properties and suitability of new thermostable enzymes in food processing. Crit Rev Food Sci 46(3):197–205
9. ZoBell CE (1941) Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. J Mar Res 4:42–75
10. Lyman J, Fleming RH (1940) Composition of sea water. J Mar Res 3(2):134–146
11. Zhou Y, He W, Fu S, Tian J, Ma T (2011) Screening of Strains Producing Alkaline Protease from Soil and Study on the Conditions for Enzyme Production. Meteo Envl Res 08:82–85
12. Folin O, Denis W (1915) A colorimetric method for the determination of phenols (and phenol derivatives) in urine. J Biol Chem 22(2):305–308
13. Kembhavi AA, Kulkarni A, Pant A (1993) Salt-tolerant and thermostable alkaline protease from Bacillus subtilis NCIM No. 64. Appl Biochem Biotechnol 38(1–2):83–92