Isolation, Screening and Identification of Protease Producing Bacteria from Fish Sauce

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Abstract

Proteolytic microorganims in fish sauce are believed to play an important role in fish sauce fermentation. For better understanding their role in fish sauce fermentation and applying them back to the process, in this study the isolation and screening of protease-producing strain from fish mash was investigated. The results showed that the proteolytic bacteria could be isolated on skim milk salt agar at salt concentration 5%, 7,5% and 10% but not at 20% or 25%. 33 isolates with halo was selected and was screened further by spot inoculation on skim milk salt agar at 5% and 10% NaCl and by enzyme diffusion method on skim milk agar at salt concentration 10% All 9 isolates selected by high halo's diameter D-d were gram possitive spore forming rod and were identified as Virgibacillus halodenitrificants by 16s rDNA analysis with more than 99.2% homology. The strain Virgibacillus halodenitrificants CH201 possessed highest halo diameter 12.33 mm by enzyme diffusion method, following by CH322 9.33 mm. These two strains showed different profile of sugar utilisation on API kit CH50.

Keywords: fish sauce, *Virgribacillus halodenitrificants*, isolation, protease

1. Introduction

Proteolytic enzymes are produced by a variety of microorganisms and played an important role during fish sauce fermentation. Several protease-producing bacteria found in fish sauce fermentation, including halophilics, halotolerants bacteria. Protease-producing bacteria found in fish sauce are *Tetragenococcus halophililus* [1], *Virgibacillus* sp. [2, 3], *Halobacterium* sp.[4], *Halobacillus* sp. [5], *Filobacillus* sp. [6], *Staphylococcus* sp. [7], *Bacillus* sp. [8]. The protease produced by *Virgibacillus*, *Filobacillus* are proved to be stable in presence of NaCl up to 25%. The protease is believed to hydrolyze protein to peptide and amino acid during fermentation process and thus can apply as starter cultures to accelerate the fish sauce fermentation. *Bacillus* sp. was the most comment microorganism used for acceleration of fish sauce fermentation in Vietnam. However *Bacillus* can grow and synthesize protease in 4% salt medium [9], which is lower than required salt concentration 25% for fish sauce fermentation. Not only bacteria, fungi were also investigated as protease source for fish sauce application, however protease isolated from *Aspergillus oryzae* lost its activity at 25% NaCl concentration [10]. Thus the requirement of isolating new strains producing active proteases at a higher NaCl concentration is necessary.

In the present study the isolation of halophilic/halotolerant protease producing bacteria from Vietnam fish sauce was conducted. Promising 9 strains that can grow and produce an active protease at 10% NaCL concentration, are selected.

2. Materials and Methods

2.1. Materials

The fish sauce samples (500 mL including mash and liquid) were collected from Cat Hai factory at 1, 3, 6, 9 months of fermentation. The chemicals for medium preparation of technical grade were purchased from Sigma Aldrich (Germany).

2.2. Methods

2.2.1. Isolation of bacteria from fish sauce samples

Proteinase-producing bacteria were isolated using skim milk salt agar composed of [per L] 100 g NaCl, 10 g skim milk, 10g $MgSO_4.7H_2O$, 2 g KNO_3, 5 g peptone , 10 mL of glycerol, 20 g agar; pH7.2 with incubating 7 days at 30 °C [10]. The isolates with clear halos around the colony were selected for further purification.

2.2.2. Screening and selection of proteinase-producing bacteria

The positive isolates were spot inoculated on the same medium agar containing 5 and 10% NaCl at

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30 °C for 2 days. Proteolytic activity was tested using skim milk salt as a substrate for protease. A positive reaction for the proteolytic test was indicated by clear zone around the colony under high salt concentration of 10% NaCl. The isolates with large and clear halo were selected and purified for further studies. The selected isolates were purified again by streaking on agar plates, only single colonies with the clear halos were cultured on the liquid medium. For the protease diffusion method, the selected isolates were sub cultured with 10% inoculum on skim milk salt broth with 5% NaCl for 16h at 37 °C. The supernatants were collected by centrifugation (5000 xg, 10 mins, 4 °C) and were sterile filtrated (0.45 µm pore diameter). Then 200 µl filtrate samples were applying to the 10% NaCl skim milk agar plate holes and the plates were incubated at 30 ºC for 24 hours. The diameter of clear zone was measured.

2.2.3. Identification of selected isolates

The isolates were identified by 16S rDNA gene sequence analysis. For that genomic DNA was extracted by genomic DNA isolation Kit (ZYMO Research, USA) and used for PCR using pair of primers 16s rDNA 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 16s rDNA 1392R (5' GGTTACCTTGTTACGACTT 3'). The PCR products were purified by using DNA Purification Kit (ThermoFisher, Germany) and sent to GATC-Biotech AG (Konstanz, Germany) for sequencing. The isolation strains were identified by the blast program 16s rRNA sequences and phylogenetic tree was constructed by the Maximum Likelihood method using MEGA 6 software.

3. Results and disscution

3.1. Isolation and screening of protease-producing isolates

The fish sauce samples were diluted at three dilutions of 10^{-1}, 10^{-2} and 10^{-3} and 100 µl were spread on the skim milk salt agar medium at 5%, 7.5%, 10%, 20% and 25% NaCl. The growth seemed to be retarded at higher NaCl concentration as the colonies became smaller for the same time of incubation. No single colony could be detected at 20% and 25% NaCl after 3 days. The positive isolates formed a clear zone around the colony suggesting that these strains containing the active released protease by digesting the protein in skim milk (Fig.1).

From 4 fish samples, 33 isolates with clear zone on 10% NaCl were selected for further study. These isolates were spot inoculated on skim milk agar plate medium with 5 and 10% NaCl (Fig.2).

The diameters of clear halos were measured and results of selected isolates were presented on Table 1. It could be seen that the diameter of clear zone decreased at higher NaCl concentration 10% (D_{10}) compared to 5% (D_5) for all the isolates (Fig.2). However the decreased ratio (D_5/D_{10}) was various by different isolates (Table 1). The decreased ratio (D_5/D_{10}) of diameter was moderate by some isolates such as isolates CH205 and CH111 but was relative stronger by some others such as CH201, CH231 indicating the various ability of isolates to tolerate with high salt concentration. Low value of D_5/D_{10} implied the grow and/or protease production were only moderately inhibited at 10% NaCl compared to 5%. Isolates that possessed the high diameter of clear halo on 10% NaCl could be of interest since they can grow and produce protease at high NaCl concentration. Therefore 9 isolates exhibited large and clear halo on 10% NaCl CH111, CH201, CH204, CH205, CH207, CH214, CH231, CH304, CH322 were selected. Among 9 selected isolates, isolates CH322 (not presented here) and CH304 showed high hydrolytic diameter on both 5% and 10% NaCl suggesting their good growth and/or high production of protease on agar at both NaCl concentration.

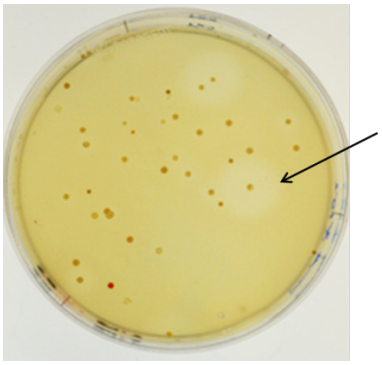

Fig. 1. Isolation of protease producing isolates on skim milk agar 10% NaCl at 10^{-2} dilution. The arrow indicated the isolate with proteolytic activity.

Table 1. Diameter of clear halo by spot inoculating method

Isolates	Diameter D (mm)		D_5 / D_{10}
	D_5 (5% NaCl)	D_{10} (10% NaCl)	
CH304	12.5	7.0	1,79
CH322	12.0	7.0	1,72
CH111	9.0	6.0	1,50
CH207	10.5	6.0	1,75
CH214	10.5	6.0	1,75
CH204	10.5	5.5	1,91
CH201	11.0	5.0	2,20
CH231	10.5	5.0	2,10
CH205	7.0	5.0	1,40

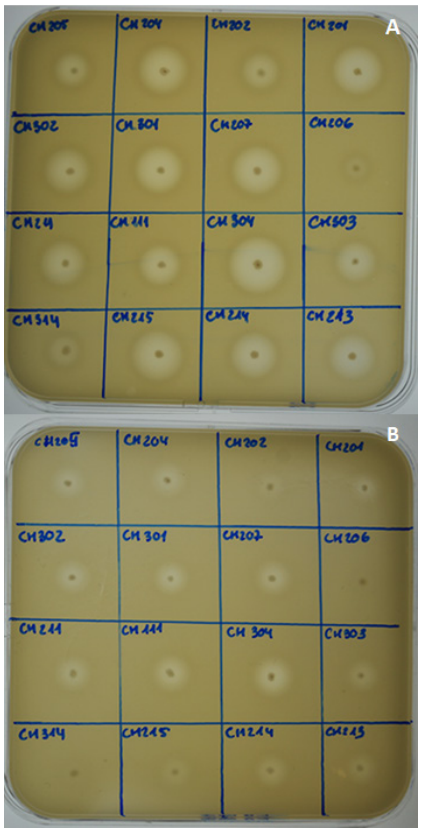

Fig. 2. Screening of protease producing isolates. The isolates with clear zone from isolation plate were spot inoculating on skim milk salt agar plates with 5% NaCl (A) and 10% NaCl (B).

The proteolytic activities of 9 isolates were tested further by enzyme diffusion method in triplicate and the results of halo's diameters were averaged and summarized on Table 2. These 9 isolates were cultured in skim milk salt liquid medium with 5% NaCl for 16 h at 37 ºC. The supernatant were collected and filtered prior to apply on the skim milk agar 10% NaCl. Fig.3 represented the clear halos by diffusion of supernatants of isolates CH201, CH304 on skim milk agar. The isolate CH201 possessed the largest and clearest halo 12.33 mm, which was almost two times higher than the lowest one isolate CH231 and higher than isolate CH304 or CH322. Similar pattern were also observed for these isolates growing on liquid skim milk 10% NaCl.

The different results between spot inoculation and diffusion methods are always observed and could be explained by different growth condition that impacted growth and protease production. Isolate CH322 possessed the secondary high diameter of clear zone (Table 2) and such seemed to grow good on both agar and liquid skim milk medium.

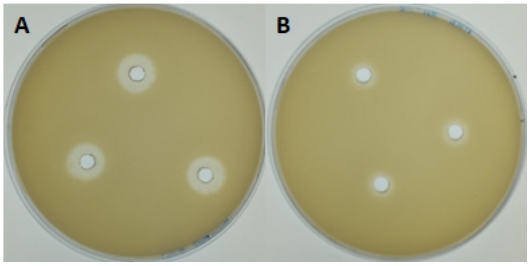

Fig. 3. Clear halos of diffusion of 16h incubation broth of isolates CH201(A), CH304 (B) on skim milk agar 10% NaCl.

Table 2. Diameters of clear halos of 9 selected isolates by enzyme diffusion method

Isolates	D (mm)
CH231	6.33±0.94
CH304	8.00±0.00
CH111	8.67±0.47
CH204	8.33±0.47
CH205	8.00±0.00
CH207	9.33±0.47
CH214	9.33±0.47
CH322	9.33±0.47
CH201	12.33±0.47

From the results of Table 1 and Table 2, isolates CH201 and CH322, showing the good hydrolyzing ability, could be chosen for the next study.

3.2. Identification of selected isolates

The colony's morphology of 9 selected isolates was presented on Fig.4. They are circular, raised, and white to cream color with 1-4 mm diameter on skim milk salt agar medium 10% NaCl after 2 days incubation at 30 °C.

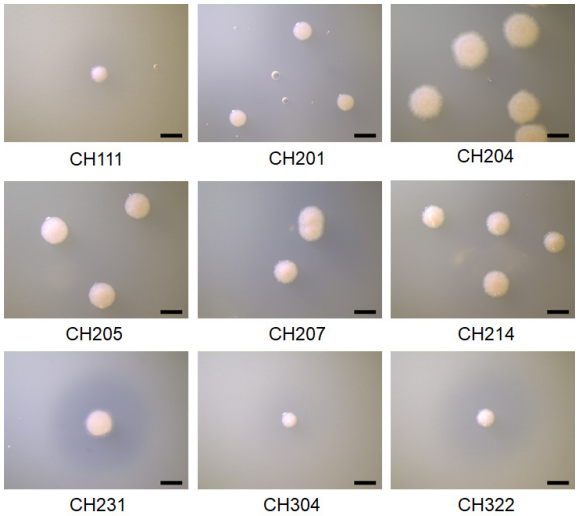

Fig. 4. Colonies of protease-producing bacteria from fish sauce on skim milk salt agar containing 10% NaCl (The bar presents 2mm diameter).

All 9 selected isolates were Gram-positive endospores forming rod. On Fig.5 presented the picture of Gram staining of cell of isolate CH201 and CH322.

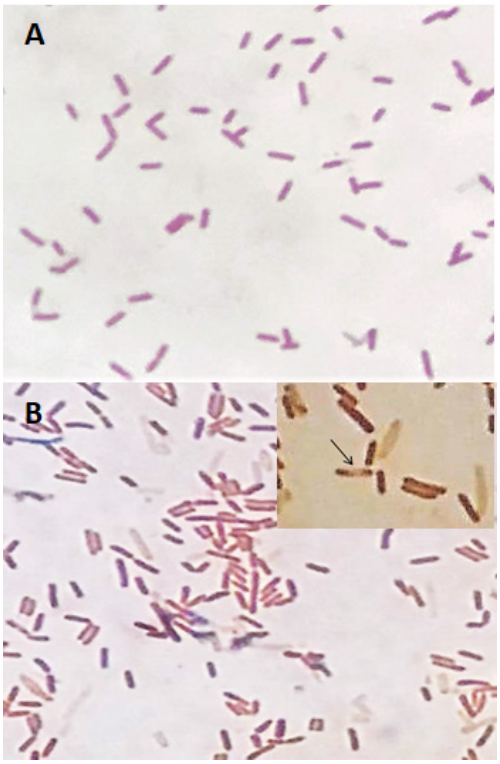

Fig. 5. Gram stain of the isolates CH201 (A) and CH322 (B) growing on skim milk medium at 10% NaCl at 30 ˚C for 2 days (Magnification: x 1000). Arrow indicated the spore.

The genomic DNA was extracted from 9 bacterial isolates and used for 16S rDNA amplification. The size of amplified DNA fragments was about 1300-1400 bp. (Fig.6). Results of % similarity and strain homology are shown in Fig.7.

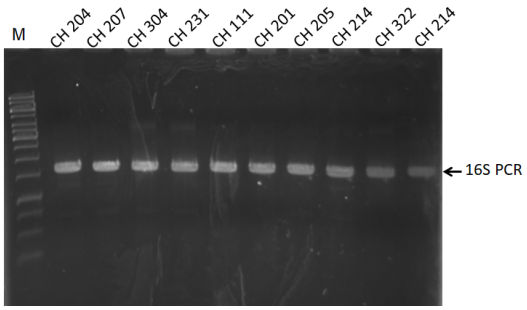

Fig. 6. Gel electrophoresis of PCR products obtained from the amplification of bacterial 16S rRNA. M-Molecular weight marker of 1kb DNA ladder from Thermofisher.

They had 99.2 to 99.4 % 16S rDNA gene sequences similarity when compared to *Virgibacillus halodenitrificans* DSM 10037, NBRC102361 and ATCC49067. Such all 9 selected isolates belong to genus *V. halodenitrificans* (Fig.7), which is recorded as halophic protease-producing bacteria [11]. *Virgibacillus* sp. SK37 from Thai fish sauce showed only 96% of 16S rDNA sequence similarity to the members of *V. halodenitrificans* ATCC49067. Phylogenetic analyses provided the similar result (Fig.7). The biochemical test on ability of using different sugars from API kit CH50 showed the total different profile of CH201 from *Virgibacillus* sp. SK37. The *V. halodenitrificant* CH201 could use several sugars like glucose, fructose, mannose, Methyl-β-D-glucopyranoside, maltose, D-trehalose and Amidon whereas *Virgibacillus* sp. SK37 could use salicin, cellobiose only. The *V. halodenitrificant* CH322 did not use mannose, Methyl-β-D-glucopyranoside but used saccharose and thus might belong to other group.

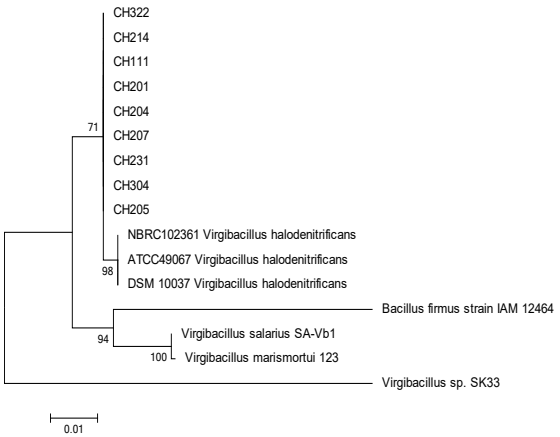

Fig. 7. Phylogenetic tree of 9 selected isolates based on 16S rDNA gene sequence data (1438 bp). The scale bar represents 0.01 substitutions per base position. Bootstrap values above 70 from 1000 replicates are shown for each node.

4. Conclusion

All 9 halophilic protease producing bacteria isolated from Cat Hai fish mash at various time of fermentation belonged to group *Virgibacillus halodenitrificant* with more than 99% sequence homology based on 16 S rDNA sequence analysis. CH201 and CH322 showed the best hydrolyzing ability on skim milk agar and had different profile of sugar utilization. According to our data, these are new strains with uncharacterized protease activity. Therefore, a further study is needed to investigate protease activity and stability of CH201 and CH322 at higher NaCl concentration as well as their using as starter for fish sauce fermentation.

Acknowledgments

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References

[1]. N. Udomsil, S. Rodtong, S. Tanasupawat, and J. Yongsawatdigul.; Proteinase-producing halophilic lactic acid bacteria isolated from fish sauce fermentation and their ability to produce volatile compounds; International Journal of Food Microbiology. 141 (2010) 186-194.

[2]. S. Sinsuwan, S. Rodtong, and J. Yongsawatdigul; Evidence of cell-associated proteinases from Virgibacillus sp. SK33 isolated from fish sauce fermentation; J Food Sci. 76 (2011) C413-9.

[3]. S. Sinsuwan, S. Rodtong, and J. Yongsawatdigul; Hydrolytic activity of Virgibacillus sp. SK37, a starter culture of fish sauce fermentation, and its cell-bound proteinases; World J Microbiol Biotechnol. 28 (2012) 2651-9.

[4]. T. C., M. T.J., S. P., and G. W.D; Isolation and characterization of an extremely halophilic archaeobacterium from traditionally fermented Thai fish sauce (nam pla); Letters in Applied Microbiology. 14 (1992) 111-114.

[5]. S. Chaiyanan, S. Chaiyanan, T. Maugel, A. Huq, F.T. Robb, and R.R. Colwell; Polyphasic taxonomy of a novel Halobacillus, Halobacillus thailandensis sp. nov. isolated from fish sauce; Systematic and applied microbiology. 22 (1999) 360-365.

[6]. K. Hiraga, Y. Nishikata, S. Namwong, S. Tanasupawat, K. Takada, and K. Oda; Purification and Characterization of Serine Proteinase from a Halophilic Bacterium Filobacillus sp. RF2-5. Bioscience, Biotechnology, and Biochemistry. 69 (2005) 38-44.

[7]. N. Udomsil, S. Rodtong, S. Tanasupawat, and J. Yongsawatdigul; Improvement of Fish Sauce Quality by Strain CMC5-3-1: A Novel Species of Staphylococcus sp.; Journal of Food Science. 80 (2015) M2015-M2022.

[8]. W.J. KIM and S.M. KIM; Purification and characterization of Bacillus subtilis JM-3 protease from anchovy sauce Journal of Food Biochemistry. 29 (2005) 591-610.

[9]. N.V. Lâm, N.P. Nhuệ, and T.T. Ngọc; Phân lập và nghiên cứu vi khuẩn ưa muối, ưa kiềm sinh enzyme protease và bước đầu thử nghiệm để sản xuất nước mắm ngắn ngày; Tạp chí khoa học và phát triển. 9 (2011) 352-463.

[10]. Le Van Viet Man, Tran Thi Anh Tuyet; Characterization of protease from Aspergillus oryzae surface culture and application in fish sauce processing; Tạp chí phát triển KH&CH, 9 (2006) 53-58.

[11]. J. Yongsawatdigul, S. Rodtong, and N. Raksakulthai; Acceleration of Thai Fish Sauce Fermentation Using Proteinases and Bacterial Starter Cultures; Journal of Food Science. 72 (2007) M382-M390.