

ISOLATION, SELECTION AND IDENTIFICATION OF *Aspergillus oryzae* FROM TRADITIONAL FERMENTED FOODS PRODUCING HIGH SALT TOLERANT NEUTRAL PROTEASE

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ABSTRACT

This study aimed to isolate, select, and identify *Aspergillus oryzae* producing high salt tolerant neutral protease from traditional fermented foods which could potentially be used for food fermentation and other relevant applications under the high salt conditions. Twelve of 23 isolates were primarily assumed to be *Aspergillus oryzae* by morphological observation. Of which, TB1 from soy sauce revealed the highest protease activity with 49.26 U/l, corresponding to a 17mm diameter clear zone on a BCG casein agar plate, and was identified using molecular biology methods and named *Aspergillus oryzae* TB1. Protease activity of this strain was highly active in the pH range of 5.0 - 8.0 and was optimal at pH 7.0. The enzyme activity remained 70% after 8 hours of incubation at pH 7.0 and 37°C. The protease activity of TB1 was reduced when the sodium chloride concentration increased from 0% to 16%, and relative activity was 51.8% at 16% NaCl. In the salt tolerance test, the results indicated that the protease tolerated 16% NaCl and residual activity was 49.2% after 9 hours of incubation at 37°C.

Keywords: *Aspergillus oryzae*, protease activity, salt tolerance

Phân lập, tuyển chọn và định danh *Aspergillus oryzae* có khả năng sinh protease trung tính và chịu mặn cao từ một số thực phẩm lên men truyền thống

TÓM TẮT

Nghiên cứu này nhằm phân lập, tuyển chọn và định tên *Aspergillus oryzae* từ một số thực phẩm lên men truyền thống có khả năng sinh protease trung tính và chịu mặn cao, có tiềm năng ứng dụng trong lên men thực phẩm và các ứng dụng khác ở điều kiện muối cao. Mười hai trong 23 chủng được định danh sơ bộ là *Aspergillus oryzae* bằng phương pháp quan sát hình thái. Trong đó, chủng TB1 phân lập từ tương bần sinh protease cao nhất với 49,26 U/l, tương ứng với đường kính 17mm của vòng phân giải trên đĩa thạch chứa BCG được định danh bằng phương pháp sinh học phân tử và đặt tên là *Aspergillus oryzae* TB1. Protease của chủng này có hoạt độ cao trong khoảng pH 5,0 - 8,0 và tối ưu ở pH 7,0. Hoạt độ enzyme vẫn còn 70% sau 8 giờ ủ ở pH 7,0 và 37°C. Nhìn chung, hoạt độ protease giảm khi nồng độ muối natri clorua tăng từ 0 đến 16%, hoạt độ tương đối là 51,8% ở 16% NaCl và nồng độ muối này được sử dụng để xác định khả năng chịu mặn của protease. Kết quả cho thấy hoạt độ enzyme còn lại là 49,2% sau 9 giờ ủ ở 37°C.

Từ khóa: *Aspergillus oryzae*, hoạt độ protease, chịu mặn.

1. INTRODUCTION

Proteases are multifunctional enzymes and extremely important in the pharmaceutical, medical, biotechnology, and, particularly, food industries, accounting for nearly 60% of the whole enzyme market (Ramakrishna *et al.*,

2010). Proteases are ubiquitous, however, high salt tolerant neutral proteases are receiving considerable attention as these enzymes are currently commercially limited.

Proteases can be classified into three types based on their optimal pH. Neutral proteases are more important for the food industry because

they can hydrolyze the proteins of raw materials thoroughly and reduce the bitterness. They are mainly used in the industries of food fermentation, brewing and feed additives, etc. In addition, some kinds of food are unique due to their high concentration of sodium chloride. Higher sodium chloride contents provide a lower degree of protein degradation. The salt resistant proteases are used in fermented food production, antifouling coating preparation, and waste treatment, especially in marine habitats (Gao *et al.*, 2016). The protease activity and stability are decreased sharply when the materials are mixed with sodium chloride at a high concentration, which is used for inhibiting spoilage bacteria, selectively retaining the slow growth of osmotolerant yeast and lactic acid bacteria, as well as prolonging the preservation time. Consequently, a protease that could tolerate a high concentration of sodium chloride is important in order to improve food quality, to shorten the time for the maturation process, and to improve the efficiency of raw material utilization (Wang *et al.*, 2013). In food fermentation, such as in koji or sauce production, a protease capable of high salt concentration tolerance is very necessary. A recent study by Mueda (2015) revealed that the sodium chloride content found in commercial fish sauce was 20 - 25%. It was considered as high salt product due to its 20 - 25% salt content.

Since proteases are physiologically necessary for living organisms, they are found in a wide diversity of sources including plants and animals, but commercial proteases are produced exclusively from microorganisms. Fungi of the genera *Aspergillus*, *Penicillium* and *Rhizopus* are especially useful for producing proteases, as

several species of these genera are generally regarded as safe, of which, *Aspergillus oryzae* is of particular interest (Chutmanop *et al.*, 2008). This fungus is a potential source of proteases due to its high proteolytic activity, broad biochemical diversity, susceptibility to genetic manipulation, high productivity, and is extracellular and thus is easily recoverable from the fermentation medium (De Castro and Sato, 2014).

2. MATERIALS AND METHODS

2.1. Materials

Soy sauce, fish source, Miso sauce, Com ruou, Dau xi sauce, and fermented shrimp (Table 1) were used to isolate, select, and identify *Aspergillus oryzae* producing high salt tolerant neutral protease. The salt content in each sample was indicated by the label or by producers.

2.2. Methods

Isolation of Aspergillus oryzae from some fermented foods

The isolation was performed using the method of Nevalainen *et al.*, (2014) with several modifications. A small amount of each sample was aseptically placed onto the central part of several prepared Petri plates containing PDA medium. These plates were sealed and labeled by sample codes and incubated at 30°C. After 3 days of incubation, fungal colonies were observed growing on the surface of the medium plates, and colonies that differed in terms of appearance, size, color, morphological shape, and spread rate of mycelium were recorded.

Table 1. Fermented food samples were used to isolate *Aspergillus oryzae*

Samples	Location of samples	Symbol of sample	Salt concentration (%)
Soy sauce	Hung Yen	TB	16
Fish sauce	Hanoi	NM	25
Miso sauce	Hanoi	MS	22
Com ruou	Nam Dinh	CR	4
Dau xi sauce	Nam Dinh	DX	20
Fermented shrimp	Hue	TC	10

Morphological identification

The morphological identification of colonies as *Aspergillus oryzae* was performed by the slide culture method as described by Leck, 1999.

Identification of Aspergillus oryzae by molecular biology method

Genomic DNA was extracted using a CTAB (Cetyltrimethyl ammonium bromide) method (Doyle & Doyle, 1987).

PCR of ITS region: Two primers, ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White *et al.*, 1990), were used to amplify the complete ITS region.

Sequencing: The PCR products were purified from agarose gel using the PureLink Quick Gel Extraction Kit (Invitrogen). The mixture of purified PCR product and primer was sent to First-base, Malaysia for sequencing and reading.

Sequencing analysis: After being assembled using the SeqMan program (DNASTAR, Madison, WI), the sequences were initially compared to known ITS sequences using a BLAST search. The sequences were aligned using the ClustalX program.

Preparation of crude enzyme

Protease production followed the methods of Fernandes *et al.*, 2010 with slight modifications. After 4 days on PDA plates, 3 mycelium plugs (5 mm diameter) of actively growing fungi were taken out to inoculate PD broth medium. After 3 days of shaking incubation, the cultures were filtered and centrifuged at 4°C with 6000 rpm for 20 min. The supernatants were used for determination of enzyme activity and further characterization of the enzyme.

Determination of protease activity

Protease activity was qualitatively determined according to the well diffusion method described by Vijayaraghavan and Vincent, 2013. Agar culture was prepared along with Bromocresol green (BCG) and 1% (w/v) casein and poured in the Petri dishes. The

plates were solidified for 30 min and wells (5mm diameter) were punched on each plate. One-hundred µl of crude enzyme was pipetted into each well, and then the plates were incubated at a temperature 30°C for 2 - 3 days. A zone of proteolysis was measured on the casein agar plates.

Protease activity was quantitatively assayed according to the modified method of Sigma's non-specific assay. One mL of the enzyme was incubated with 5.0 mL 0.65% casein solution at 37°C. The reaction was terminated by adding 5.0 mL trichloroacetic acid (TCA) after 10 minutes. The suspension was allowed to settle for 30 min at 37°C to precipitate the protein. The precipitate was removed by centrifuging at 6000 rpm for 10 min. Two mL of supernatant was mixed with 5 mL 0.5 M Na₂CO₃ and 1.0 mL of 1.0 M Folin reagent and incubated at 40°C for 20 min. The absorbance of the supernatant was measured with a spectrophotometer at 660 nm. One unit (U) of protease activity was defined as the amount of enzyme releasing 1.0 mmol of tyrosine equivalent per min.

Effect of incubation time on protease activity and stability

The effect of incubation time on protease production was carried out by the method of Pant *et al.*, 2015 with slight modifications. The protease production was carried out in 100-mL conical flasks with agitation. Every 12 h, each flask was filtered, followed by centrifugation at 4°C and 6000 rpm for 20 min. The culture filtrates obtained were tested for protease activity.

Effect of pH on protease activity and stability

Protease activity was determined as described above but by varying the pH between 3.0 and 9.0 using Britton-Robinson buffer. To determine pH stability of the protease, enzyme samples were incubated in buffer with varied pHs at 37°C for 12 hours, and at interval times, enzyme was withdrawn to test the residual enzyme activity.

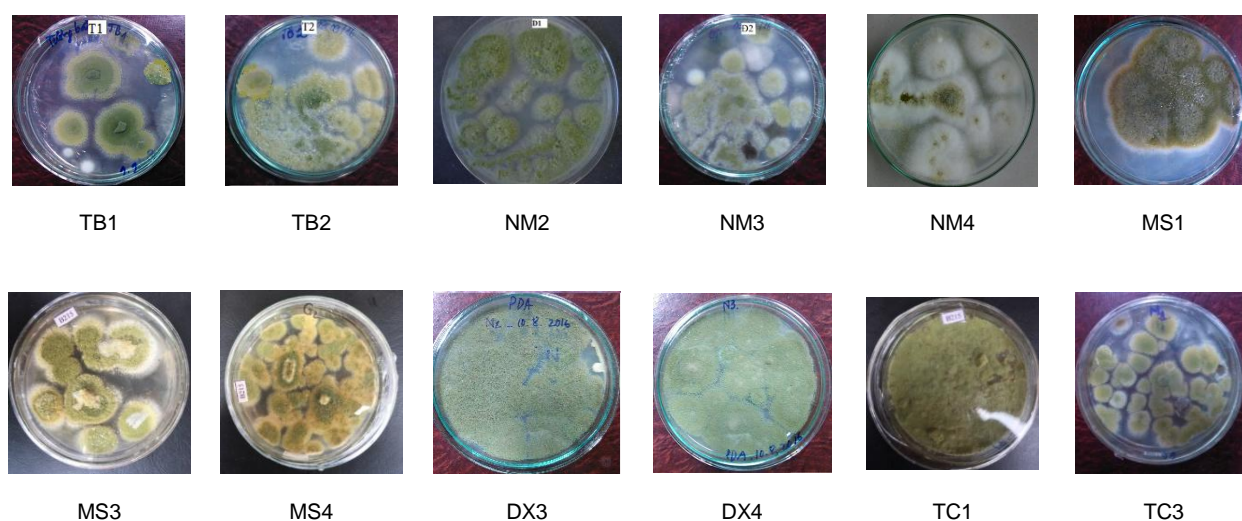


Figure 1. Isolation of *A. oryzae* from fermented foods

Effect of NaCl on the protease activity and stability

In order to study the effect of NaCl concentration, enzyme activity was measured in 50 mM potassium phosphate buffer (pH 7.5) by adding 1 mL of the crude enzyme to 1 mL of 0.65% casein in the presence of NaCl varying from 0 to 20%.

In order to determine the protease stability at a high concentration of NaCl, the crude enzyme was incubated in 16% NaCl at 30°C and pH 7.5 (phosphate buffer) for 24 hours. At interval times, enzyme was withdrawn to test the residual activity.

protease produced by the fungus (Vermelho *et al.*, 1996). After 3 days of incubation at 30°C, the clear zones were observed (Figure 2) and quantification of the protease activity was completed as indicated in Table 2. The clear distinct zones were observed after the addition of BCG reagent on the casein agar plates. The zones were distinct and the surrounding areas were greenish blue in color, but the color of the plates strongly depended on the pH value of the agar medium. In this study, the plates appeared blue in color due to the pH of the culture medium being maintained at 8.0. The TB1 isolate with highest diameter of the clear zone and enzyme activity was selected for further study.

3. RESULTS AND DISCUSSION

3.1. Isolation of *Aspergillus oryzae* from the fermented foods

Based on colony and mycelium color, only 12 fungal colonies of 23 isolates were assumed to be *Aspergillus oryzae*. The isolates with the codes in Figure 1 were used to test protease activity.

3.2. Determination of protease activity produced from the isolates

The casein agar plate assay allows principally for qualitative determination of protease activity. The hydrolysis zones on the casein agar could be related to the amount of

3.3. Molecular identification of the TB1 fungus

From the qualitative protease activity test, TB1 showed the highest enzyme activity and was selected for identification using molecular biology methods. The ITS (Internal Transcribed Spacer) region of the TB1 strain was used for identification. Two primers, ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC 3') were used to amplify the complete ITS region of TB1. The expected PCR product, around 700 bp as shown in Figure 3, was purified and directly sequenced using the ITS1 and ITS4 primers.

Table 2. Diameter of clear zones of protease produced from isolates

Isolates	Samples	Diameter of clear zones (mm)	Enzyme activity (U/L)
TB1	Soy sauce	17.0	49.26
TB2		11.0	29.10
NM2	Fish sauce	9.5	13.73
NM3		10.4	14.21
NM4		8.9	12.89
MS1	Miso sauce	8.5	16.90
MS3		8.2	16.20
MS4		7.6	15.74
DX3	Dau xi sauce	8.5	14.31
DX4		6.4	11.31
TC1	Fermented shrimp	6.5	13.49
TC3		5.1	7.24

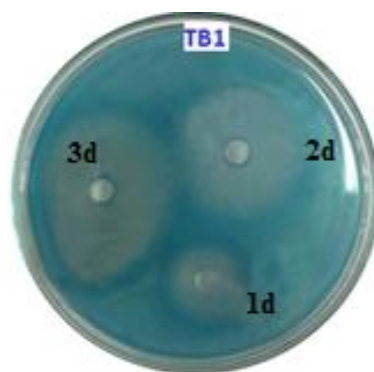


Figure 2. Casein degradation of TB1 protease on an agar plate flooded with BCG reagent after 3 days of incubation (1 d, 2d, 3d: after 1 day, 2 days, 3 days of enzyme incubation, respectively)

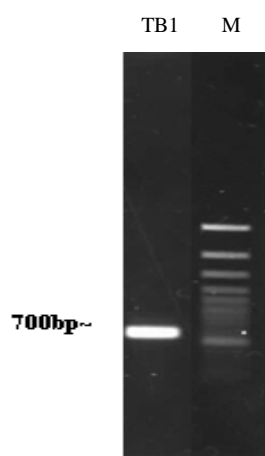


Figure 3. PCR product of the ITS region from strain TB1, showing a 700bp fragment. Lane M, DNA ladder (100bp DNA ladder, New England Biolabs)

Homologous sequences of the ITS sequence of the strain TB1 were searched for in the GenBank database using the BLAST program. The BLAST searches showed that the TB1 strain was most closely related to an *A. oryzae* originating from a wide range of sources throughout the world. Based on the analysis of the ITS sequence, TB1 was identified and named to be *Aspergillus oryzae* TB1 (*A. oryzae* TB1).

3.4. Determination of suitable cultivation time on protease production

In tests to find out the suitable cultivation time to harvest protease of *A. oryzae* TB1, protease activity increased gradually and

reached a maximum value at 72 hours of incubation, and then decreased with time (Fig. 4). A 72-hour cultivation time was used to produce enzyme extract for further study.

Effect of pH on protease activity and stability: The optimum pH for protease activity in *A. oryzae* TB1 was determined over a pH range from 3.0 to 9.0. The results, as shown in Figure 4a, showed that protease from *A. oryzae* TB1 was more active at a neutral pH than in acidic or alkaline conditions, and the optimum pH was revealed to be pH 7.0. This result was comparable with that of Sandhya *et al.*, 2005 and Wang *et al.*, 2013 who showed that protease from *Apergillus* sp. exhibited maximum activity in the pH range 7.0 - 8.

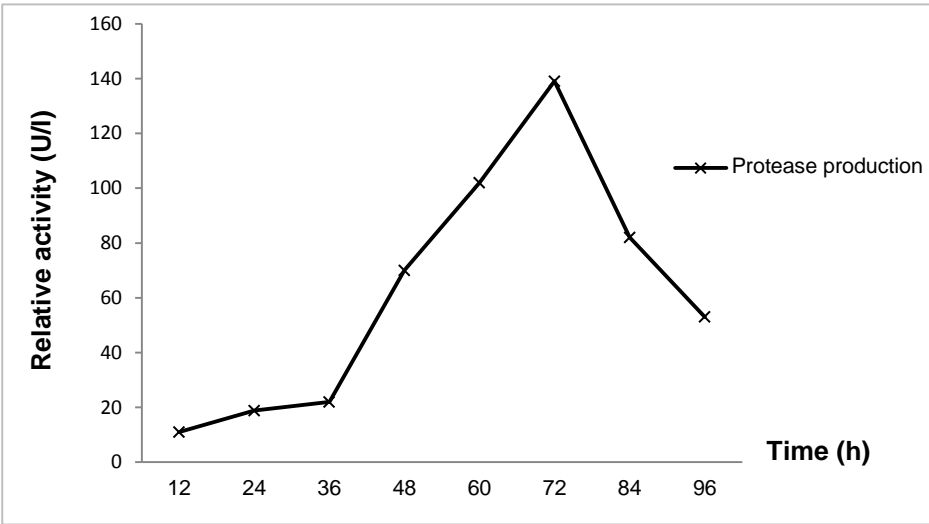
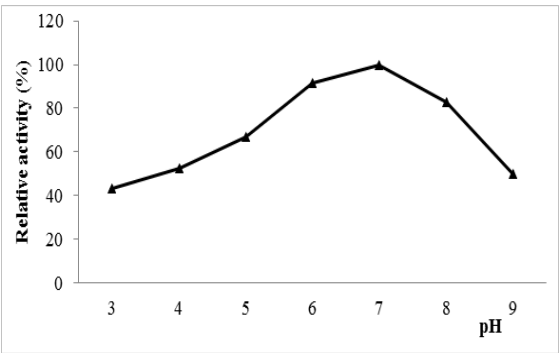
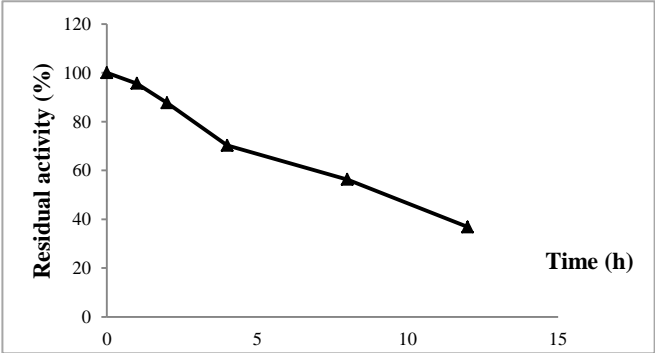


Figure 4. Effect of incubation time on protease production of *A. oryzae* TB1



(a)



(b)

Figure 4. Effects of pH on the activity (a) and stability (b) of the protease produced from *A. oryzae* TB1

Protease stability was expressed as residual activity after incubation at pH 7.0 for 12h at 37°C with 100% referring to the initial activity. In general, protease activity from TB1 tended to decrease under the mentioned conditions (Figure 4b). Protease activity was maintained over 50% and 37% of its original activity after 8 hours and 12 hours of incubation, respectively.

Effect of sodium chloride (NaCl) on protease activity and stability: In food fermentation, such as in koji or sauce production, a protease capable of high salt concentration tolerance is very necessary. In a recent study, Mueda (2015) revealed that the sodium chloride content found in commercial fish sauce was around 16 - 20 %.

The effect of sodium chloride on protease activity of crude enzyme extracts from *A. oryzae* TB1 is shown in Figure 5a. The concentration of sodium chloride significantly affected protease activity of *A. oryzae* TB1. The higher the concentration of sodium chloride, the lower the enzymatic activity. These results were quite compatible with a study of Wang *et al.*, 2013 which revealed the residual activity of protease decreased sharply with the increase of NaCl concentrations from 0% to 16%.

The salt tolerance of protease is a very important property in soy sauce production, and it is related to the length of fermentation time and the utilization of raw materials. The tolerance of protease activity from *A. oryzae* TB1 was determined at 16% NaCl (w/v) concentration, pH 7.0 and 30°C. As shown in

Figure 5b, TB1 protease activity decreased slowly to 49.2% after 9 hours of incubation. The results showed that protease activity would decrease as the ionic strength increased. As a result, the protein had the tendency to change its conformation to reduce exposure of its surface by the salting out mechanism (Wang *et al.*, 2013). With 9 hours of salt tolerance, the protease in this study showed feasibility to be used in short-length fermented products such as koji. Many researchers have studied the fractionation of proteases from koji molds and demonstrated their roles in soy sauce manufacturing. However, all of these protease activities declined significantly when the harvested koji was incubated with a high level of sodium chloride (~16 - 18% NaCl) to make a mash (Su *et al.*, 2005).

4. CONCLUSIONS

In this paper, we described the identification and characterization of a crude protease from *A. oryzae* TB1 isolated from soy sauce. Protease activity of this strain was highly active in the pH range of 5.0 - 8.0, especially at pH 7.0. Furthermore, after 9 hours of enzyme incubation at a 16% NaCl concentration (w/v), 49.2% of residual activity still remained. Consequently, this high salt tolerant protease is a new potential enzyme for soy sauce production and other relevant applications under high salt conditions.

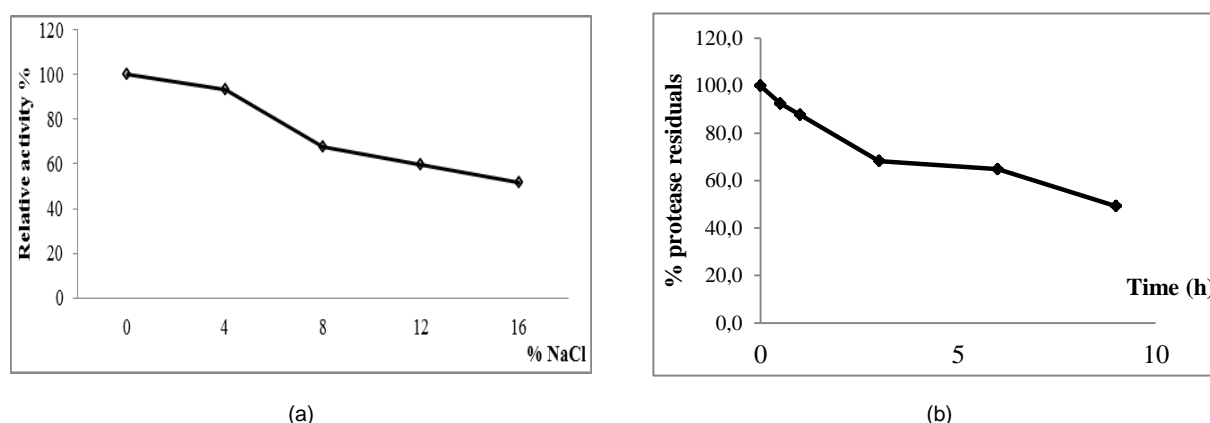


Figure 5. Effects of NaCl on TB1 protease activity (a) and stability (at 16% NaCl) (b)

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