Jurnal Teknologi

Full Paper

PROTEOLYTIC FUNGI FROM VIRGIN FOREST

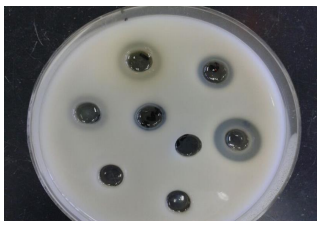
Zaidah Zainal Ariffin[a,b*], Mohd Sidek Ahmad[a], Rosalia Pepi[a], Zainon Mohd Noor[a]

[a]School of Biology, Faculty of Applied Sciences, Universiti Teknologi MARA 40450 Shah Alam, Selangor, Malaysia
[b]Atta-ur-rahman Institute for Natural Products Discovery, Universiti Teknologi MARA, Puncak Alam Campus, 42300 Puncak Alam, Selangor, Malaysia

Article history
Received
26 June 2015
Received in revised form
2 September 2015
Accepted
19 December 2015

*Corresponding author
drzaidah@salam.uitm.edu.my

Graphical abstract



Abstract

Microbial enzymes have continued to assist diverse reactions as biocatalysts. Soil derived microbes offer a prospective resource for such enzymes. Screening and isolation of proteolytic fungi were carried out from soil sample of a Malaysian virgin forest. Four isolates showed clear zone of protein hydrolysis on skim milk agar representing proteolytic activity. Aspergillus flavus UOA/HCPF 5774 exhibited the highest proteolytic activity with a clear zone diameter of 21 mm followed by Aspergillus niger and Trichoderma harzianum both with a clear zone of 16 mm, and Penicillium simplicissimum strain LP42 with a13 mm clear zone. Crude protease activity of 0.230 – 0.277 Units / ml for each fungus was seen after 24 hours incubation. A decline of protease production was observed after 48 hours incubation except for Aspergillus flavus UOA/HCPF 5774 which showed a drop only after 72 hours incubation. The protease producing fungi were partially identified based on their morphological characteristics, macroscopic and microscopic identification. The identification was confirmed by 18S rRNA Sequence Analysis. The four fungi protease producers were Aspergillus niger, Penicillium simplicissimum strain LP42, Aspergillus flavus UOA/HCPF 5774 and Trichoderma harzianum.

Keywords: Proteolytic activity, soil borne fungi, virgin forest

1.0 INTRODUCTION

Proteolytic enzyme or protease, proteinase, or peptidase belongs to a group of enzymes that break the long chainlike molecules of proteins to shorter fragments (peptides) and eventually into their monomers or amino acids. Proteolytic enzymes play an important part in the metabolism of almost all organisms (plants, animals, fungi, bacteria, and viruses). Proteases have a wide range of applications and products in the form of food, leather and detergents [1]; the detergent industry being the major consumer of proteases. In other industries, proteases are used in food and leather processing [2]. Protease's importance in commerce and industry can be seen in its market share of over 60% of total enzyme sales, where two-thirds of it was produced from microbial origin [4]. Protease production by microbes is influenced by physical factors (pH, inoculums density, incubation time, agitation and temperature), and by media components (carbon and nitrogen sources) [2].

Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Studies on microbial proteolytic enzymes are rapidly being developed due to their applications in various industries. Proteolytic enzymes from microbial sources are preferred to enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications.

Fungi exhibit a wider variety of proteases than bacteria, even though most commercial proteases originated from microorganisms belonging to the genus *Bacillus*. Fungi are normally regarded as safe strains and produce extracellular enzymes, which are easier to be recovered from fermentation broth

[5]. The microbial proteases of *Aspergillus* species, in particular, have been studied in detail since they are known for their capacity to secrete high levels of enzymes in their growth environment. Several of these secreted enzymes, produced in a large-scale submerged fermentation, have been widely used in the food and beverage industry for decades [6].

Despite more than 3000 different enzymes having been identified and many of them having found their way into biotechnological and industrial applications, the present enzyme toolbox is still insufficient to meet the demands of these industries. Therefore, the search for proteolytic enzymes from microbial sources especially fungi still continues.

2.0 MATERIALS AND METHODS

2.1 Collection of Soil Samples

A subsurface soil sample, 5 to 10cm in depth was collected at 900 feet above sea level from the forest floor of a virgin forest in Malaysia. A 1000-g sample of freshly collected soil was placed in a sterile plastic bag and kept in a container with ice during transportation from the collection area to the laboratory. Later, the soil samples were stored in the 4°C chiller.

2.2 Isolation of Soil Fungi

Isolation of fungi from the soil was done by serial dilution plate technique. For soil dilution technique, 5 g of soil was suspended in 50 mL sterilized distilled water and shaken vigorously on a magnetic stirrer for 20 minutes to obtain uniform suspension of soil fungi. Each test tube was filled up with 9 mL of sterile distilled water and labelled to reflect a ten - fold dilution (tube 1 = 10^{-1}, tube 2 = 10^{-2}, Tube 3 = 10^{-3}, tube 4 = 10^{-4}). From the 10^{-4} diluted solution, 0.5 mL soil solution was pipetted onto Potato Dextrose Agar (PDA) and was evenly distributed with a sterilized L-shaped glass spreader. Triplicates were made. The plate was incubated at 30°C for four days. Each of the fungi species was sub cultured onto a new potato Dextrose Agar (PDA). Single spore isolation was then carried out on new Potato Dextrose agar (PDA) to obtain pure cultures [7]. The plate was incubated again at 30°C for four days. The purification of soil fungi was done by needle inoculation; pure cultures were sub cultured on PDA slants and maintained at 4°C in a refrigerator.

2.3 Screening for Proteolytic Activity

Skim milk agar was used to detect the proteolytic activity by measuring the clear zone of protein hydrolysis on agar plates containing protein substrates. Casein was used as protein substrate. For this, 5 g of Sigma skim milk powder was dissolved in 50 mL of distilled water to produce 10% w/v of skim milk solution. Wells were made on the skim milk agar by using a 1 cm diameter cork borer. A 1 mL of each sample was placed in these wells, then incubated at 37°C for 24 to 48 hours. *Fusarium* species was used as positive control while sterile distilled water was used as the negative control. The formation of clear zone around the colony was measured and recorded [8]. Isolated fungi that produce clear zone were sub cultured again on PDA and incubated at 30°C.

2.4 Protease Production

The culture medium used in this study was Bacto Tryptic Soy Broth maintained at 30°C for 24 to 120 hours in a rotary shaker (180rpm). At the end of each fermentation period, 5 ml of fermentation broth was taken out and centrifuged at 8000 rpm at 4°C for 20 mins and the clear supernatant was used as crude enzyme preparation.

2.5 Determination of Crude Protease Activity

Protease activity was determined in triplicates. In this assay, casein was used as a substrate. Universal bottles were prepared and labelled as Iso-1, Iso-2, Iso-5, Iso-6 and blank. To each universal bottle, 5 ml of 0.65% casein solution was added and equilibrated in a water bath at 37°C for 5 mins. Some 500 ml of the crude enzyme extract from four positive protease producers were added to the four labelled bottles. The solutions were mixed by swirling and incubation at 37°C in water bath for 10 mins. After 10 mins incubation, 5 ml of the TCA reagent was added to each bottle to stop the reaction. Then, an appropriate volume of enzyme solution was added to each bottle even to the blank to ensure a final volume of enzyme solution in each tube is 1 ml. The solution was incubated at 37°C in a water bath for 30 mins. Then, the solution was filtered using 0.2 µm syringe filter to remove any impurities. The filtration of 2 ml standard solution and blank standard solution was added to another universal bottle. Then, 5 ml of sodium carbonate was added to each bottle. These solutions became cloudy. One (1) ml of Folin's & Ciocalteu's reagent was added immediately. Folin's reagent will react primarily with free tyrosine. The solution was mixed by swirling and incubation at 37°C for 30 mins. A colour change was observed. 2 ml of these solutions were filtered using 0.2 µm syringes filter into cuvettes. The absorbance of the standard solutions was measured by a spectrophotometer using a wavelength of 660nm [9]. One unit of protease activity is defined as the amount which liberates 1 µg of tyrosine per ml per minute under experimental condition [10].

Calculation on activity of enzyme in Units / mL was performed based on:

$$\text{Units / ml of Enzyme} = \frac{(\mu\text{mole tyrosine equivalents released}) \times (11)}{(1) \times (10) \times (2)}$$

11 = Total volume (in ml) of assay
10 = Time of assay (in minutes) as per the unit definition
0.5 = Volume of enzyme (in ml) of enzyme used
2 = Volume (in ml) used in colorimetric determination

2.6 Identification of Fungi Isolates by 18S rRNA Sequence Analysis

Isolated fungi with positive proteolytic activity were characterized by 18S rRNA gene and internal transcriber spacer region sequence analysis. DNA extraction was carried out using QIAGEN plant tissue kit. The fungi species confirmation was performed by 18S rRNA sequencing using an internal transcriptional spacer (ITS) fragment, ITS1 (5'-TCCGTAGGTGAACCTGCG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The purified PCR samples were then sent to a third party (MyTACG Sdn Bhd) for sequencing. A sequence similarity search was performed using the National Centre for Biotechnology (NCBI) database and the Basic Local Alignment Search Tool (BLAST) (URL:http://www.ncbi.nih.gov/BLAST/). Sequences with identity match of 98% and above were retrieved.

3.0 RESULTS AND DISCUSSION

Six isolates were obtained and identified based on their morphological characteristics, macroscopic and microscopic identification and also comparison of these isolates with Bergey's manual. Two of the isolates were confirmed to be *Aspergillus niger* (Iso-1) and *Trichoderma harzianum* (Iso-6). The other four isolates were identified as *Penicillium* sp. (Iso-2), *Rhizopus* sp. (Iso-3), *Mucor* sp. (Iso-4) and *Aspergillus flavus* (Iso-5).

3.1 Screening for Proteolytic Activity

The fungi isolates were screened for proteolytic activity using skim milk agar plate assay and expressed as clear zone diameter and in mm. Principally, the assay allows qualitative determinations of protease activity by the formation of clear zones around samples cultured on Skim Milk Agar which could be related to the amount of protease produced by the fungus [11]. In this study, proteolytic activities were observed in *Aspergillus niger* (Iso-1), *Penicillium* sp. (Iso-2), *Aspergillus flavus* (Iso-5) and *Trichoderma harzianum* (Iso-6) after 24 and 48 hours incubation at 37°C (Figures 1 and 2) and the clear zone diameters were tabulated in Table 1. The positive isolates exhibited bigger clear zones after 48 hours incubation compared to 24 hours. *Aspergillus flavus* exhibited the highest proteolytic activity with a clear zone diameter of 21 mm followed by *Aspergillus niger* of 16 mm, *Trichoderma harzianum* (Iso-6) of 16 mm and *Penicillium* sp. (Iso-2) of 13 mm, thus, indicating that these four species are capable of producing the protease enzyme; so these four species were then selected for further studies.

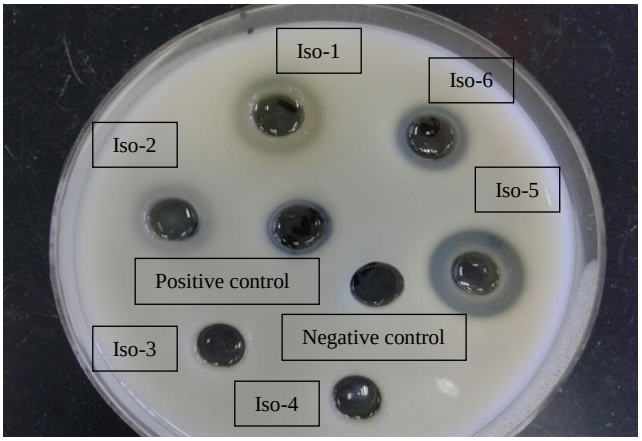


Figure 1 Protease present on the skim milk agar after 24 hours incubation

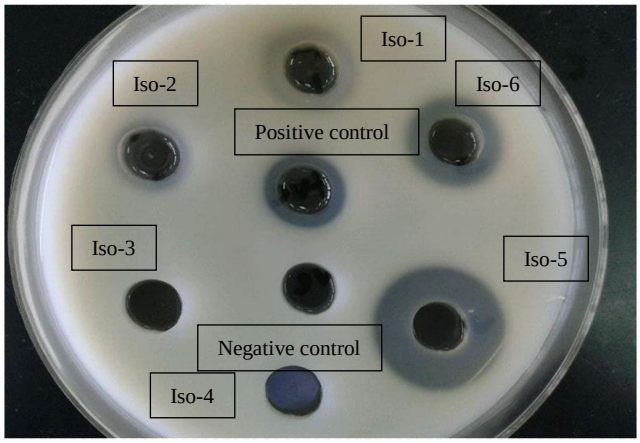


Figure 2 Protease present on the skim milk agar after 48 hours incubation

Table 1 Clear Zone Diameter of Samples (Iso-1, Iso-2, Iso-3, Iso-4, Iso-5, Iso-6) on Skim Milk Agar

Diameter (mm) / Time (hrs)	1	2	3	4	5	6	Positive control	Negative control
24	13	13	-	-	16	12	12	-
48	16	13	-	-	21	16	13	-

*Standard deviation for all readings is close to zero.

In another study, [5] identified fungi of genera *Aspergillus* and *Penicillium* which are mainly used for the protease production. Previous study on the proteome analysis of *Trichoderma* spp revealed that *Trichoderma* spp may have one of the largest sets of protease among fungi [12]. These results are agreeable with the results of this study where

Aspergillus spp., *Penicillium* spp. and *Tricorderma* spp. were able to produce protease.

3.2 Determination of Protease Activity

Positive samples (Iso-1, Iso-2, Iso-5 and Iso-6) with protease activities were subjected to a protease enzyme assay to determine their enzyme activities in Units / mL. All four isolates were cultured in 100 ml over a period of 120 hours. The protease activity exhibited from each isolate is tabulated in Table 2.

Table 2 Crude protease activity in Units / mL by four protease producing fungi

Isolate	Day 1/ 24 hrs	Day 2 / 48 hrs	Day 3 / 72 hrs	Day 4 / 96 hrs	Day 5 / 120 hrs
***Aspergillus niger* (Iso-1)**	0.2772	0.2596	0.2299	0.1029	0.1106
***Penicillium* sp. (Iso-2)**	0.2574	0.1276	0.1029	0.0930	0.0627
***Aspergillus flavus* (Iso-5)**	0.2271	0.2382	0.0974	0.0347	0.0341
***Trichoderma harzianum* (Iso-6)**	0.2343	0.2167	0.1722	0.0721	0.0622

*Standard deviation for all readings is close to zero.

Enzyme production by microorganisms is greatly influenced by media components especially carbon and nitrogen sources and other physical factors such as temperature, pH and incubation periods and inoculums density. In this study, the fermentation medium was inoculated with the protease producing fungal isolate and incubated at various incubation hour intervals (24 – 120) hours. The temperature was maintained at 37°C. Amount of µmoles tyrosine released by each strain for 120 hours were obtained and the crude protease enzyme activity was calculated.

Aspergillus niger (Iso-1), production of enzyme was gradually decreased after 24 hours. This strain showed the highest enzyme activity among the other three species. The protease enzyme activity decreased after 24 hours. Previous study [13] on the protease production by *Aspergillus niger* using solid state fermentation, showed that the maximum activity of protease was achieved after 72 hours of incubation. But the enzyme activity was slightly decreased after 96 hours incubation. In a similar study, [14] reported that maximum protease activity with *Aspergillus oryzae* was obtained after 72 hours incubation. When the incubation period was increased up to 96 and 120 hours, enzyme activity also decreased. The incubation period was found to be directly proportional to the production of enzymes and other metabolites although only to a certain extent. After that, enzyme production and growth of the microorganism also starts to decline which can be attributed to the decrease in supply of nutrients to the microorganism and accumulation of toxic metabolites [15].

Penicillium sp. (Iso-2) produced 0.257 Unit / ml of protease enzyme activity after 24 hours. However, the enzyme activity declined after 24 hours. Previous study [16] reported that protease production from *Penicillium chrysogenum* gradually increased with time and the highest enzyme activity was obtained after 72 hours incubation. After prolonged incubation, the enzyme activity showed a decreasing rate.

Trichoderma harzianum (Iso-6) yields 0.234 Unit / ml of protease enzyme activity which is the third highest among the four isolates. However, the enzyme decreased gradually after 24 hours. Previous study [17] showed that the maximum proteolytic activity of *Trichoderma viride* was on the fifth day but the activity increased after adding a protein inducer into the growth medium.

Aspergillus flavus Iso-5 only yields 0.227 Unit / ml of protease enzyme activity for the first 24 hours. Then, the enzyme produced gradually increased with time and the highest amount of activity was obtained in 48 hours. It was also observed that prolonged incubation decreased the enzyme activity, maximum protease enzyme production occurred after 7 days incubation by using *Aspergillus flavus* [18]. Similar study conducted [19], reported that the maximum protease produced was also seen after 7 days incubation.

3.3 Identification of Fungi Isolates by 18S rRNA Sequence Analysis

Results of sequences were analysed using DNA Base software and identification was done by homology search of the ITS region sequence using BLASTN on DNA database GenBank. Based on sequence alignment, the two protease producing fungi were identified as *Penicillium simplicissimum strain* LP42 (Iso-2) and *Aspergillus flavus* UOA/HCPF 5774 (Iso-5) The BLASTN showed that the sequence of the fungal isolate, Iso-2 have 99% homology with *Penicillium simplicissimum* strain LP42. Iso-5 has 97% homology with *Aspergillus flavus* UOA/HCPF 5774. Isolates with ITS region sequences ≥ 99% with the closest species is the same species [20]. These results are summarised in Table 3.

Table 3 Fungi isolate confirmation based on 18S rRNA Sequence Analysis

Fungi Isolate	Size(bp)	Homology	Accession
***Penicillium simplicissimum strain* LP42**	576	99%	HQ392489.1
***Aspergillus flavus* UOA/HCPF 5774**	592	97%	FJ878681.1

4.0 CONCLUSION

In conclusion, the four isolated fungi, *Aspergillus niger*, *Penicillium simplicissimum strain* LP42, *Aspergillus flavus* UOA/HCPF 5774 and *Trichoderma harzianum* are promising strains for producing proteolytic enzymes. Purification and further investigation on strain improvement studies will provide better yield of this enzyme.

Acknowledgement

The authors would like to thank Universiti Teknologi MARA (UiTM) Malaysia and *Ministry of Higher Education, Malaysia for the financial support under the Fundamental Research Grant Scheme;* FRGS/2/2014/STWN10/UITM/02/1.

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