Screening of Protease-producing Fungi from Soil Samples by Conventional and Molecular Methods

Author name: Singh M.

Author affiliation: ADI Intellect, 600, North Avenue, #107, Wakefield, MA 01880.

Corresponding author details

Name: Bragta S.

Address: ADI Intellect, 600, North Avenue, #107, Wakefield, MA 01880.

Phone: +91-9805433607

Email: sophiabragta16@gmail.com

Abstract

Purpose: To isolate protease-producing fungi from soil samples by conventional and molecular methods. Other objectives included comparing isolated fungi for protease production and identifying them molecularly using 18s RNA sequencing.

Methods: The soil samples were rich in organic matter and were used for the isolation of proteolytic fungi. The soil samples were collected from three sites, viz., gardens, crop fields, and ponds, in different localities of Punjab and Chandigarh. The molecular identification of the fungus was carried out using 18s RNA sequencing after the purification and identification of isolated fungi.

Results: During the study, it was found that a total of 9 samples were found positive for the occurrence of fungi. The percentage frequency of positive samples in different areas was found to be 50%, 33.3%, and 50% in samples collected from crop fields, garden soil, lake soil and pond soil. In the present study, from the 9 positive fungal samples, a total of 3 fungal forms were obtained that produce the protease enzyme. These were identified and found to belong to *Alternaria* and *Penicillium sp.* The samples belonging to garden soil yielded only one fungal species, *Alternaria solani*, and the samples of pond and lake soil yielded *Cladosporium sp.* A total of two *Penicillium sp.* have been isolated from Mohali samples.

Conclusion: The isolated strain of *Penicillium sp.* was found to be a potential producer of extracellular protease rather than *Alternaria*. The enzyme yield was greater in *Penicillium sp.* than in *Alternaria sp.* The findings highlight the importance of a local isolate of *Penicillium sp.* because it is particularly good at producing extracellular protease during fermentation.

Keywords: Fungi, Protease, Soil, *Penicillium*, *Alternaria*

Introduction

The soil is a dynamic medium for microbial and biological activities, and the number and kind of microorganisms present in a particular soil depend on many environmental factors, such as the amount and type of available nutrients, moisture, and the degree of aeration, pH, temperature, etc.[1] They play a pivotal role in various biochemical processes and, thus, are responsible for recycling organic compounds in nature.[2]

Fungi are an important part of the microbiota, and depending on soil depth and nutrient circumstances, they can make up more of the biomass in the soil than bacteria.[3] It is estimated that there are 1.5 million fungal species on earth, of which only 74,000 have been described up to now, which presents a potential source of novel organisms.[4]

Enzymes are among the most significant products from microbial sources for human requirements. Enzymes are finding new uses thanks to recent advances in biotechnology. A protease is an enzyme that initiates protein degradation by hydrolyzing the peptide bonds that connect amino acids in a polypeptide chain to produce the protein. Proteases work best in acidic conditions.[5]

As obligatory heterotrophs, fungi naturally break down organic matter and produce a variety of enzymes. Fungal enzymes comprise more than half of industrial enzymes and are effectively utilized in various industrial processes and products. Pulp and paper, clothing, detergents, food, feeds, nutraceuticals, and pharmaceuticals are well-known industries. *Aspergillus* is the most widely used fungus genus in manufacturing industrial enzymes. Other than protease, phytase, L-

asparaginase, and a few others, glycosyl hydrolases (such as cellulases, xylanases, mannanases, amylase, pectinases, and others) make up the majority of commercial fungal enzymes.[6]

A study demonstrated that the proteolytic fungus could be isolated from soil samples and grown on Reese agar plates with casein as the protein substrate. An investigation was carried out in the various regions of the Madhya Pradesh districts of Sagar and Jabalpur. There were 50 soil samples obtained and a total of 38 of those samples tested positive for the presence of fungi. The positive soil samples yielded 141 different fungus types. These comprised 38 from crop field soils, 47 from garden soil, and 56 from poultry farms' fungi. *Fusarium* and *Aspergillus* were the two most common genera.[7] In a different study, microorganisms that produce proteases were identified from the soil samples. *Aspergillus*, *Rhizopus*, *Fusarium*, and *Mucor sp.* were the isolates that produced a high percentage of positive screening results. *Aspergillus sp.* 14L3S, an isolate from the soil surface, has the highest protease activity of these isolates, as shown by the clear zone surrounding the colony. According to the findings of these investigations, fungi have immense potential to be used in commercial manufacturing and for the production of protease enzymes.[8]

Another study showed that the amount of fungi in the soil was isolated using the soil-plate method. Researchers used this method to show that fungi can be found in various environments, including rhizosphere soils.[9] Proteolytic fungi were recovered from a variety of sources, including garden soil and alkaline soil, and soil samples.[10] In Coimbatore, Tamil Nadu, Kalpana Devi isolated a proteolytic *Aspergillus niger*.[11] Charles et al. isolated *Aspergillus nidulans* HA-10, a proteolytic fungus, from poultry farm soil.[12]

The main focus of the present study was to isolate the protease-producing fungi from soil samples by conventional and molecular methods. The other objectives included comparing isolated fungi for protease production and identifying them molecularly using 18s RNA sequencing.

Methodology

Sample collection

The soil samples were rich in organic matter and were used to isolate proteolytic fungi. The soil samples were collected from three sites, viz., gardens, crop fields, and ponds, in different localities of Punjab and Chandigarh. The samples were randomly collected from the above habitats from the superficial layer of soil not exceeding 5-6 cm in depth using a pre-sterilized spatula. Then, the soil was transferred into sterilized polythene bags. The samples were then brought to the laboratory and kept at 4˚C until processed. The general study design and sample collection are detailed in **Figure 1**.

Determination of pH of the soil samples

To check the pH of the soil samples, 10 g of each soil sample was suspended in 100 mL of double distilled water and shaken vigorously for 30 minutes, then checked the pH using a pH meter.

Screening of fungi from the soil sample

Sabouraud dextrose agar (SDA) medium and potato dextrose agar (PDA) medium were used as growth media. Initially, the pH of the medium was adjusted to 9.0 with a solution of 1 N NaOH. The serial dilution method isolated the fungal strain from the soil[13] on SDA and PDA media. Then, 1 g (dry weight equivalent) of soil sample was suspended in 10 mL of sterile distilled water. Then, 1 mL of the soil suspension was serially diluted (six-fold) to estimate the fungal population. Incubation of the plates took place at ± 28°C for 4-5 days.

Purification of isolated fungi and identification

The isolated fungi were purified by point-inoculating them on plates that contained a PDA medium. Repeated point inoculations purified the fungi. The purity of the isolated fungus was confirmed by microscopic examination of the culture at 400X magnification using a light microscope. After ensuring purity, the culture was subcultured on PDA slants, allowing it to grow for 5-7 days. The stock cultures were stored at 4˚C, the worked and stock cultures were maintained, and the worked cultures were transferred to fresh PDA slanted at regular intervals of 3 months. The isolated fungi were subcultured on PDA, allowing them to grow and sporulate. Based on their colony and morphological characteristics, the fungi were identified. As a mounting fluid, a lactophenol cotton blue stain was used. The slides were observed under a microscope, and the fungi were identified using the mycological literature.

The following morphological characteristics were evaluated: colony growth (length and width), presence or absence of aerial mycelium, colony color, presence of wrinkles and furrows, pigment production, etc.[14-16]

Production of protease

Submerged fermentation (SmF)

The 3% spore suspension (106 spores/mL) prepared from the PDA slant was inoculated in 200 mL of nutrient broth (NB) with a pH of 7 and then incubated in the flask at 35˚C 120 hours on a rotary shaker at a speed of 150 rpm. Then, the NB culture was centrifuged at 10,000 rpm for 15 minutes, and the supernatant thus obtained was used as crude enzyme extract. Then, protease activity in the supernatant was detected.

Determination of proteolytic activity

The protease activity was tested using skim milk agar. For this, 0.01 mL (using a graduated micropipette) of crude enzyme produced from a local isolate of *Penicillium sp.* was added very carefully inside the wells in the center of the plates so as not to overflow the wells. Then the plates were incubated at 30˚C for 24 hours. The proteolytic activity was detected by observing the presence of clear zones. After initial screening for proteolytic activity, the *Penicillium sp.* strain exhibited activity for proteases and was used throughout the experiment.

Enzyme assay

At 50˚C, a reaction mixture containing 0.1 mL of an enzyme and three mL of 0.5% casein in 2.95 mL of 0.1 M Tris-HCl buffer (pH 8.5) was incubated. After 10 min, the reaction was stopped by adding 3 mL of cold 10% trichloroacetic acid (TCA). After 1 hour, the culture filtrate was centrifuged at 8,000 rpm for 5 minutes to remove the residue, and the absorbance of the supernatant was read spectrophotometrically at 280 nm. The enzyme activity was calculated by measuring the unit (mg) of tyrosine equivalent released and comparing it with the standard.

Effect of pH on protease production

The effect of pH on the protease activity produced by *Penicillium* was carried out using different pH ranges like 3, 4, 5, 6, 7, 8, 9, and 10.

Effect of temperature on protease production

Several temperature ranges, including 30˚C, 35˚C, 40˚C, 45˚C, 50˚C, and 55˚C, were used to study the impact of temperature on the activity of protease produced by *Penicillium sp.*

Molecular identification of fungus

The fungus samples were identified using 18s RNA sequencing from B.R. Biochem Life Sciences (New Delhi).

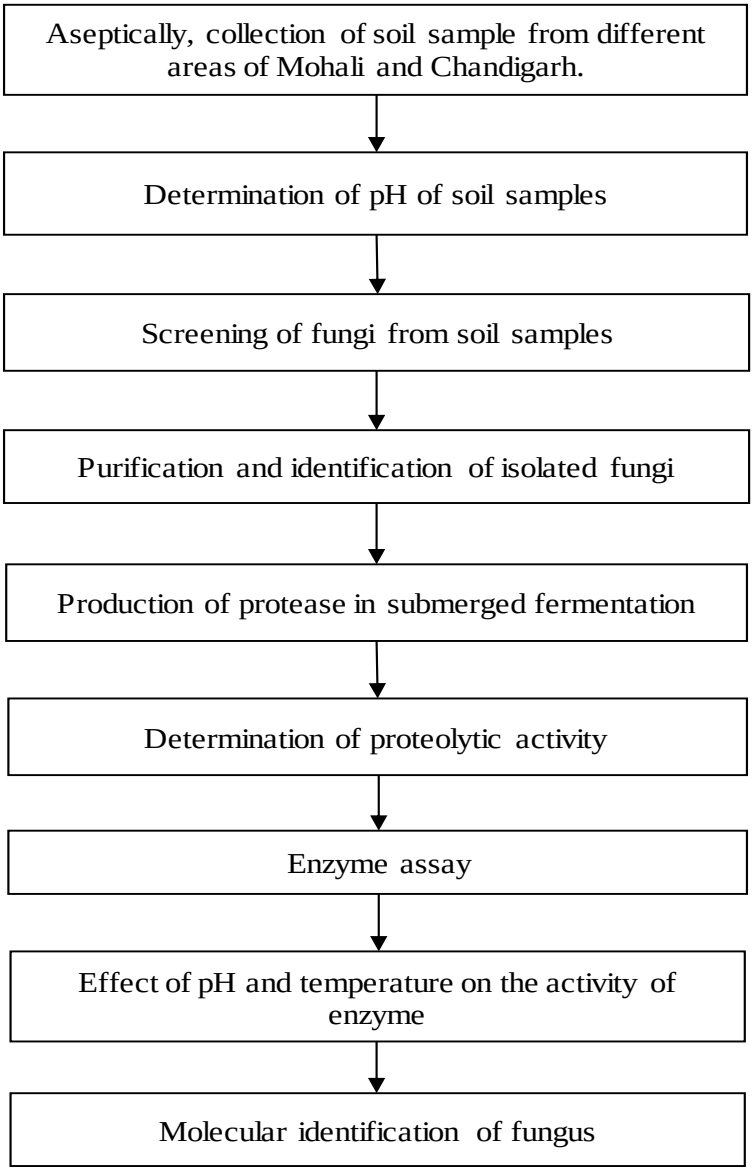

Figure 1 Flow chart depicting the study design

Results

In the present study, a total of 5 fungal forms were obtained from the positive samples. These included 1 fungal forms from garden soils, 2 from crop field, 1 from lake soils and 1 from pond soil (**Table 1**). The soil samples of crop field yielded maximum number of fungi.

Table 1 Distribution of positive samples in different areas

Habitats	Place of sample collection	No. of samples	No. of positive samples	No. of fungi isolated
Garden soil	Chandigarh	3	2	1
Crop field (Rice)	Mohali	3	2	2
Crop field (Spinach)	Mohali	2	-	-
Lake soil	Sukhna lake	2	1	1
Pond soil	Punjab	2	1	1

The data in **Table 2** indicated the dominance of Genus *Penicillium* in the soils of surveyed habitats. Members of *Penicillium* have been widely reported in the soil since their nature allows them to have nutrients and moisture for all different life cycle studies.

Table 2 Percentage frequency of occurrence of fungal isolates

Fungal species	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Percentage frequency
Penicillium brevicompactum	+	+	+	-	-	-	50%
Penicillium islandicum	-	-	+	+	-	-	33.3%
Alternaria solani	-	+	-	-	+	+	50%

Effect of temperature

The medium was incubated at various temperatures (20-55°C). *Penicillium* showed maximal protease production at 35°C (**Figure 2**). A significant range of protease was produced at temperatures ranging from 30-55°C. Temperatures below 30°C, as well as above 55°C, harm enzyme production.

Effect of pH

Penicillium showed maximal protease production at pH 7, although significant levels of protease could be recorded at other pHs (**Figure 3**). The strain grew in the pH range of 3.0 to 9.0, with better protease yield. The pH values below 3.0 and above 9.0 harmed enzyme production.

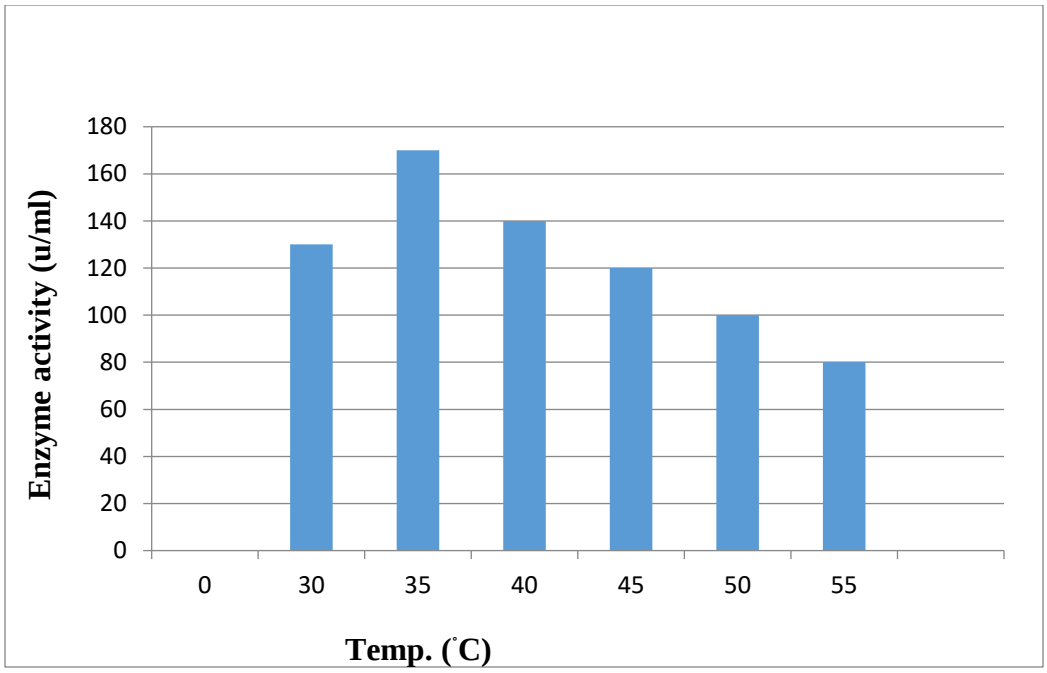

Figure 2 Effect of temperature on enzyme activity

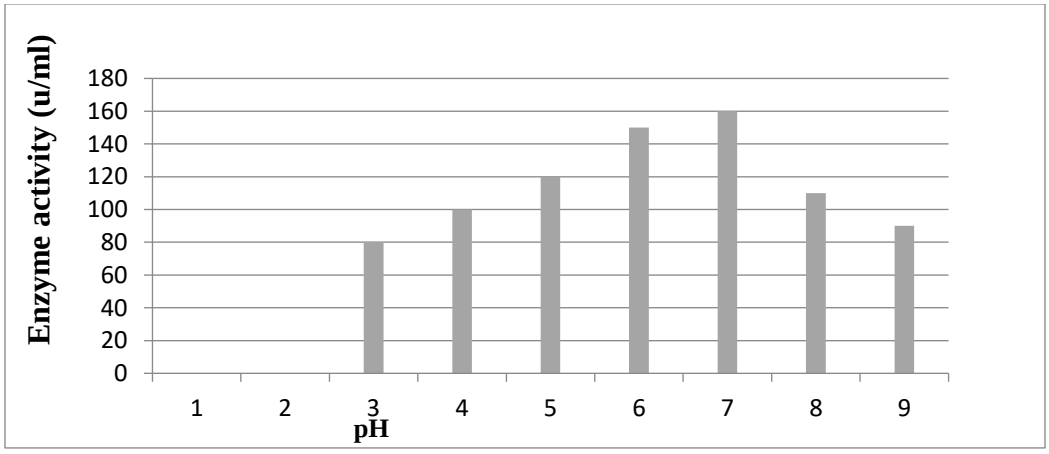

Figure 3 Effect of pH on enzyme activity

Effect of incubation time

The SmF medium was inoculated with the fungal strain and incubated at various time intervals (120 hrs). **Figure 4** shows the effect of incubation time on protease production. *Penicillium* produced the highest amount of enzyme on the fifth day of incubation.

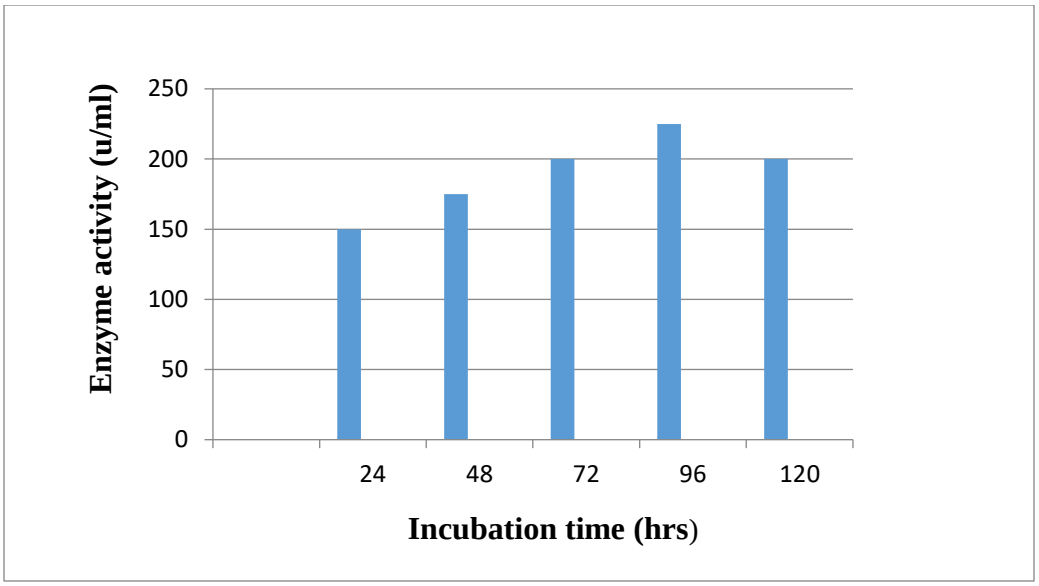

Figure 4 **Effect of incubation time on enzyme activity**

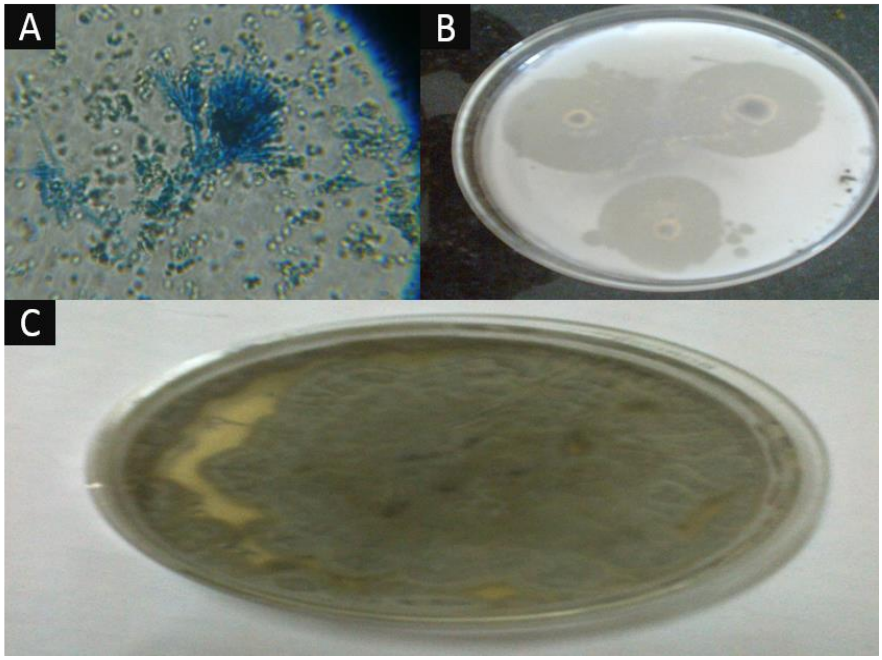

Figure 5 **Microscopic view of a structure of A) *Penicillium sp.*, B) zone of inhibition shown on the skimmed milk agar plate, and C) pure culture of *Penicillium sp.* on PDA plates**

Discussion

Fungi occurring in natural habitats with changing environmental conditions are important from an industrial point of view. These microorganisms are known to produce new metabolites or enzymes with enhanced catalytic characteristics. Therefore, an effort has been made in the current work to isolate the proteolytic fungi from soils obtained from various habitats. Twelve soil samples collected from different areas have been used to isolate fungi. These include 3 samples of garden

soil, 5 crop fields, 2 samples of lake soil, and 2 samples of pond soil from Punjab and Chandigarh. Out of these, a total of 9 samples were found to be positive for the occurrence of fungi.

The percentage frequency of positive samples in different areas was 50%, 33.3%, and 50% in samples collected from crop fields, garden soil, lake soil and pond soil. In the present study, a total of 3 fungal forms that produce the protease enzyme was obtained. These were identified and found to belong to *Alternaria* and *Penicillium sp*.

In the present study, 5 fungal forms were obtained from the positive samples. These include 1 fungal form from garden soils, 2 from crop fields, 1 from lake soils, and 1 from pond soil. Soil samples from a crop field yielded the maximum number of fungi. These were identified and found to belong to 2 species of a single genus, including *Penicillium brevicompactum* and *Penicillium islandicum.* In the present study, the samples belonging to garden soil yielded only one fungal species, *Alternaria solani,* and *Cladosporium sp.* were isolated from pond and lake soil samples. A total of two Penicillium *sp.* fungus forms have been isolated from Mohali samples.

The data indicated the dominance of Genus *Penicillium* in the soils of surveyed habitats. Members of *Penicillium* have been widely reported in the soil since their nature allows them to have nutrients and moisture for all different life cycle studies.

Isolates of *Alternaria solani* have been recorded only from the soils of gardens. Gardens, crop fields, and other types of soil are regarded as rich environments for fungi with a variety of metabolic functions. In recent years, microbial proteases have replaced traditional proteases from animal and plant resources and have found various applications in recent eras. Proteases are widely distributed among microorganisms, including fungi, bacteria, and actinomycetes.[7] Proteases are well-known producers among fungi species such as *Aspergillus* and *Penicillium*[11, 17-19] and are reported active over a wide pH range.[5]

With at least 150 species, many of which have a similar shape, *Penicillium* is a wide genus. Many species also have great variability; at least 1,000 recognizably different phenotypes may eventually be cataloged. Even from common sources, only 70–80% of isolates are easily distinguishable because of the genus' inherent diversity. Many taxonomic keys to identifying *Penicillium sp.* are based primarily on morphological criteria. There are several classifications based on the micromorphology, macromorphology, and colors produced in the mycelium or diffused into the growing medium of the organism.[20] *Penicillium sp.* forms a well-developed septate mycelium with distinctive colors such as yellow, orange, red, or purple. The tips of unbranched conidiophores may terminate in a cluster of phialides or metulae. Each branch of branched conidiophores ends in a single or several metulae. The conidia are spherical to ellipsoidal and aseptate, and they may be blue-green, grey-green, or yellow, according to the species shown in Figure 5A.

Protease activity was tested using skimmed milk agar. For this, 0.01 mL of crude enzyme made from a local strain of *Penicillium sp.* was put within the wells (by employing a graduated micropipette). The proteolytic activity was detected by observing the presence of clear zones. The zone of inhibition of 24 mm and 25 mm was formed due to the hydrolysis of milk proteins shown in Figure 4.

Conclusion

The present study obtained an extracellular protease from *Penicillium* and *Alternaria sp*. Instead of *Alternaria*, the isolated strain of *Penicillium sp.* was discovered to be a possible producer of extracellular protease. The enzyme yield was greater in *Penicillium sp.* than in *Alternaria sp.*

As a result of this fungus's high productivity in manufacturing the extracellular protease SmF, the data presented here point to the significance of a local isolate of the *Penicillium sp.* Also, this study indicates the importance of the SmF technique since it is very simple in application and yields high-yield products. This strategy might be considered for large-scale applications for the production of industrial enzymes. Penicillium *sp.* growth in SF for large-scale protease production can be experimentally optimized using the study's large-scale protease production as a reference point.

Conflict of interest

The authors whose names are listed in the paper have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria, educational grants, participation in speakers' bureaus, membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements) or non-financial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the paper's subject matter or accoutrements.

Data availability statement

The data can be made available upon request from the author.

Ethics statement

Not applicable.

Acknowledgement

Not applicable.

References

1. Sachidanand B, Mitra NG, Kumar V, Roy R, Mishra B. Soil as a huge laboratory for microorganisms. *Agric Res Technol*. 2019;22(4):556205. doi:10.19080/ARTOAJ.2019.22.556205
2. Bot A, Benites J. The importance of soil organic matter: Key to drought-resistant soil and sustained food production. Food & Agriculture Org.; 2005. Accessed on May 17, 2023. https://www.fao.org/3/a0100e/a0100e00.htm
3. Frąc M, Hannula SE, Bełka M, Jędryczka M. Fungal Biodiversity and Their Role in Soil Health. *Front Microbiol*. 2018;9:707. doi:10.3389/fmicb.2018.00707
4. Hawksworth DL. The magnitude of fungal diversity: the 1· 5 million species estimate revisited. *Mycol. Res*. 2001;105(12):1422-32. doi:10.1017/S0953756201004725\
5. Rao MB, Tanksale AM, Ghatge MS, Deshpande VV. Molecular and biotechnological aspects of microbial proteases. *Microbiol Mol Biol Rev*. 1998;62(3):597-635. doi:10.1128/MMBR.62.3.597-635.1998
6. Kango N, Jana UK, Choukade R. Fungal enzymes: sources and biotechnological applications. Advancing Frontiers in Mycology & Mycotechnology: Basic and Applied Aspects of Fungi. 2019. Accessed on May 23, 2023. doi: 10.1007/978-981-13-9349-5_21
7. Choudhary V, Jain PC. Isolation and identification of alkaline protease producing fungi from soils of different habitats of Sagar and Jabalpur District (MP). *J. Acad. Indus. Res*. 2012;1(3):106.

8. Maitig AM, Alhoot MA, Tiwari K. Isolation and screening of extracellular protease enzyme from fungal isolates of soil. *J Pure Appl Microbiol*. 2018;12(4):2059-67. doi:10.22207/JPAM.12.4.42
9. Kazerooni EA, Maharachchikumbura SSN, Rethinasamy V, Al-Mahrouqi H, Al-Sadi AM. Fungal Diversity in Tomato Rhizosphere Soil under Conventional and Desert Farming Systems. *Front Microbiol*. 2017;8:1462. doi:10.3389/fmicb.2017.01462
10. Nehra KS, Dhillon S, CHAUDHARY K, SINGH R. Production of alkaline protease by Aspergillus sp. under submerged and solid substrate fermentation. *Ind. J. Microbiol*. 2002;42(1):43-7.
11. Devi MK, Banu AR, Gnanaprabhal GR, Pradeep BV, Palaniswamy M. Purification, characterization of alkaline protease enzyme from native isolate Aspergillus niger and its compatibility with commercial detergents. *Ind. J. Sci. Technol*. 2008;1(7):1-6. Doi: 10.17485/ijst/2008/v1i7.8
12. Charles P, Devanathan V, Anbu P, Ponnuswamy MN, Kalaichelvan PT, Hur BK. Purification, characterization and crystallization of an extracellular alkaline protease from Aspergillus nidulans HA-10. *J Basic Microbiol*. 2008;48(5):347-352. doi:10.1002/jobm.200800043
13. Waksman SA. A Method for Counting the Number of Fungi in the Soil. *J Bacteriol*. 1922;7(3):339-341. doi:10.1128/jb.7.3.339-341.1922
14. Gilman JC. A manual of soil fungi (Second Indian Reprint). Accessed on May 17, 2023. https://www.scirp.org/(S(351jmbntvnsjt1aadkposzje))/reference/ReferencesPapers.aspx?ReferenceID=186457
15. Onions AH, Allsopp D, Eggins HO. Smith's introduction to industrial mycology. Edward Arnold (Publishers) Ltd.; 1981.
16. Domsch KH, Gams W, Anderson TH. Compendium of soil fungi. Volume 1. Academic Press (London) Ltd.; 1980. Accessed on May 17, 2023. https://www.cabdirect.org/cabdirect/abstract/19811962574
17. Agrawal D, Patidar P, Banerjee T, Patil S. Production of alkaline protease by Penicillium sp. under SSF conditions and its application to soy protein hydrolysis. *Proc. Biochem*. 2004;39(8):977-81. doi:10.1016/S0032-9592(03)00212-7
18. Sindhu R, Suprabha GN, Shashidhar S. Optimization of process parameters for the production of alkaline protease from Penicillium godlewskii SBSS 25 and its application in detergent industry. *Afr J Microbiol Res*. 2009;3(9):515-22.
19. Krishna VK, Gupta M, Gupta N, Gaudani H, Trivedi S, Patil P, Gupta G, Khairnar Y, Borasate A, Mishra D. Optimization of growth and production of protease by Penicillium species using submerged fermentation. *Int. J. Microbiol. Res*. 2009;1(1):14. doi:10.9735/0975-5276.1.1.14-18
20. Pianzzola MJ, Moscatelli M, Vero S. Characterization of Penicillium Isolates Associated with Blue Mold on Apple in Uruguay. *Plant Dis*. 2004;88(1):23-28. doi:10.1094/PDIS.2004.88.1.23