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Bioprospecting of Marine Fungi for Production of Extracellular Protease

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ABSTRACT: Proteases from microorganisms have attracted much attention in the last decade because of their gigantic potential in various industrial processes due to their proteolytic activity such as detergent, textile, leather, dairy, and pharmaceutical preparations. However, proteolytic enzymes from microorganisms are preferred in the industrial application of enzymes due to their technical and economic advantages. In the present research work, protease-producing microorganisms were isolated from marine soil samples and screened for protease-producing ability using skim milk agar. Among twenty-three isolates, nine isolates were protease positive indicated by the clear zone around the colony. In quantitative screening, the highest protease activity was observed with JML 15 (144.72 U/ml) and JML 8 (107.92 U/ml) and selected for further investigation. Based on the molecular characterization and phylogenetic studies, the selected isolates were identified as *Penicillium lagena* JML 8 (PP987316.1) and *Penicillium oxalicum* JML 15 (PQ160092.1). The results showed that JML 15 and JML 8 had great potential to be used for the production of protease enzymes and upscale for industrial production.

KEYWORDS: Marine fungi, protease, skimmed milk agar, penicillium.

INTRODUCTION

Proteases, also known as peptidases or proteinases, are enzymes that catalyze the hydrolysis of peptide bonds in proteins. Proteases are classified based on their catalytic mechanisms into four main types: serine proteases, cysteine proteases, aspartic proteases, and metalloproteases (1). Due to their diverse functionalities and stability under extreme conditions, proteases have extensive applications in industries such as pharmaceuticals, food processing, detergents, leather, and waste management (2-7). Proteases can be derived from animals, plants, and microbes. Microbial proteases are more commonly used for commercial purposes than those from plants and animals because microbes grow rapidly, require minimal cultivation space, and can be genetically modified easily for specific characteristics.

Filamentous fungi are well known for producing a range of hydrolytic enzymes, with proteases being one of the most common types. Numerous fungal strains from the genera *Penicillium* (8-10), *Aspergillus* (11,12), *Fusarium* (13), *Rhizopus* (14), *Mucor* (15,16), *Alternaria* (17), *Acremonium* (18) have been employed to produce proteases (8 – 19). Because marine fungi have evolved specifically to thrive in the complex marine environment, they may possess unique enzymes like proteases, which are highly salinity-tolerant, thermostable, and active across a broad pH range, making them particularly valuable for industrial applications (19).

The objective of this study is to isolate and screen marine fungi from marine soil samples for the production of extracellular protease enzymes. By exploring the proteolytic capabilities of these marine fungal isolates, we aim to identify strains that produce proteases with desirable industrial properties.

MATERIALS AND METHODS

Sample collection

The soil samples were collected from the seashores of Manginapudi Beach, Bapatla Beach, and Velankanni Beach. At a depth of 10 cm, marine soil samples were collected using surface-sterilized spatulas and placed in polythene bags. Each sample was labelled with the date and collection site before being brought to the laboratory. The soil samples were then stored at 4°C until they were processed.

Isolation of Fungi

A serial dilution plate technique was used for the isolation of the fungal species. The marine soil samples were serially diluted to obtain 10^{-1} to 10^{-10} dilutions. 0.1 ml of each suspension was drawn and spread over the surface of Sabourads dextrose agar (SDA)

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medium plates. The media was prepared with seawater and supplemented with streptomycin (50 µg/ml) to avoid bacterial contamination. The inoculated plates were incubated at 28ºC for 10 days. Later incubation, isolated fungi were sub-cultured on SDA agar plates until pure cultures were obtained. The pure cultures were transferred to SDA slants to achieve good growth and then preserved at 4°C.

Qualitative screening for protease activity

Fungal isolates obtained on SDA plates were screened for proteolytic activity according to the method of *Josephine Jenitta and Joel Gnanadoss* (20) using skimmed milk as the substrate. The medium was prepared with 1 g of skimmed milk and 1 g of agar, where each was dissolved in 50 ml of distilled water separately and autoclaved at 121°C for 15 min. Both the solutions were mixed aseptically and streptomycin (1 mg/100ml) was added to restrict bacterial growth. The media was poured into the sterile petri plates and allowed for solidification. After solidification, the pure culture isolates were spot inoculated at the center of media plates and incubated at 28ºC for 5 days. The clear zone around the colony indicates protease production. The fungal colony's diameter and complete zone of enzyme activity, including growth diameter, were measured. The 'Relative Enzyme Activity' (REA) was estimated using the following formula.

$$REA = Diameter\ of\ hydrolysis\ zone\ /\ Diameter\ of\ colony$$

Quantitative Screening

The fungal isolates producing clear zones in qualitative screening were selected and cultured in Protease specific fermentation broth containing (% w/v): yeast extract 0.5, $MgSO_4$ 0.1, glucose 1.0, K_2HPO4 0.2, KH_2PO4 0.2, Casein 0.5, pH 9.0 (21). For protease production, mycelia plug about 8mm from a 5-day-old culture was transferred to 100 mL of production medium and incubated at 28ºC for 10 days in a rotatory shaker at 120 rpm. After the incubation period, the broth was filtered through Whatman filter paper and the clear culture filtrate containing extracellular enzyme was used for protease assay.

Estimation of protease activity

Protease activity in the crude enzyme extract was determined according to the modified method of Sedrah *et al.* by using casein as substrate (21). The reaction mixture containing 1 mL of 2% casein solution in 0.2M Na2CO3 and 1 mL of crude enzyme extract was incubated at 37 ºC for 10 minutes and the reaction was stopped by adding 2 ml of 10% Trichloro acetic acid. This mixture was allowed to stand for 20 minutes and was then filtered using Whatman's No 1 filter paper. Later 1 ml of filtrate mixed with 5 ml of 0.4M Na_2CO_3 and 1 ml of 0.5N 1 ml of two-fold diluted Folin Ciocalteu (FC) reagent was added. The resulting mixture was placed in the dark at room temperature for 30 minutes for the appearance of blue colour. The concentration of liberated tyrosine in the filtrate was measured at 660 nm against a reagent blank. Tyrosine was used as a standard. One protease unit was defined as the amount of enzyme required to release 1 µM of tyrosine per minute per ml.

Protease activity [U/mL] = (µmoles of tyrosine equivalents released × Total volume of assay [mL] /(Volume of enzyme used in the assay [mL] × Time of assay [min] × Volume in cuvette [mL].

All the experiments were done in triplicates and the mean value was presented.

Estimation of total protein content

Total protein content in the crude enzyme extract was determined by Folin- Lowry method using Bovine Serum Albumin (BSA) as standard (22). To 1ml crude enzyme, 5ml alkaline copper solution was added and allowed to stand for 10 minutes. After that 0.5ml of Folin's reagent was added and incubated for 30 minutes at room temperature. After incubation, the absorbance was measured at 600nm against blank. The total protein content was expressed as mg/ml of crude enzyme.

Morphological identification

Morphological identification of the most promising isolates was done by studying the macroscopic characteristics of the fungi such as colony size, appearance, mycelia texture, and pigmentation on both obverse and reverse sides. For microscopic observation, the fungal hyphae were mounted with lactophenol cotton blue, and species characteristics such as conidia, conidiophores, branching patterns, and chlamydospores were observed.

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Molecular identification

The molecular identification of potent fungal strains was done using the 18s r RNA sequencing technique. Fungal genomic DNA was extracted using genomic DNA kit (GENEI, INDIA). The 18S ITS region was amplified by PCR using universal fungal primers ITS1 and ITS4 (23). The primers were synthesized at an oligonucleotide synthesis facility (Macrogen, Seoul, Korea). After the sequencing, DNA similarity search was performed using the BLAST N of the National Centre for Biotechnology Information website (NCBI) (http//www.ncbi.nlm.nih.gov). Similar sequence alignment was carried out using Clustal W. The phylogenetic tree was constructed using the neighbor-joining method using the MEGA 11 software (24,25). The fungal ITS ribosomal region gene sequences determined in this study were deposited in GenBank and accession numbers were obtained.

RESULTS

Isolation and screening of proteolytic fungi

A total of 23 morphologically different fungi were isolated and named as JML 1, JML 2, JML 3 to JML 23 respectively. Table 1 summarises the preliminary screening results for protease production. The clear zone around the colony indicates protease production and the absence of a clear zone indicates a negative for protease production (Figure 1). Among 23 isolates, 9 isolates were found to be protease positive and the 'Relative Enzyme Activity' (REA) was calculated. The isolate JML 8 has shown significant relative enzyme activity of 1.85 followed by JML 15 (1.39), and JML 20 (1.27), while the isolate JML 2 showed the lowest activity (1.10) as shown in Table 2.

Table 1. Preliminary screening of fungal isolates for protease activity

Isolate number	Source	Protease activity
JML 1	Manginapudi beach	-
JML 2	Manginapudi beach	++
JML 3	Manginapudi beach	-
JML 4	Manginapudi beach	-
JML 5	Manginapudi beach	+
JML 6	Manginapudi beach	+
JML 7	Bapatla beach	-
JML 8	Bapatla beach	+++
JML 9	Bapatla beach	-
JML 10	Bapatla beach	-
JML 11	Bapatla beach	+
JML 12	Bapatla beach	+
JML 13	Bapatla beach	-
JML 14	Bapatla beach	-
JML 15	Velankanni beach	+++
JML 16	Velankanni beach	-
JML 17	Velankanni beach	-
JML 18	Velankanni beach	-
JML 19	Velankanni beach	-
JML 20	Velankanni beach	++
JML 21	Velankanni beach	-
JML 22	Velankanni beach	+
JML 23	Velankanni beach	-

(Zone scale – absent; + 1- 3 mm; ++ 4- 7 mm; +++ 8-12 mm)

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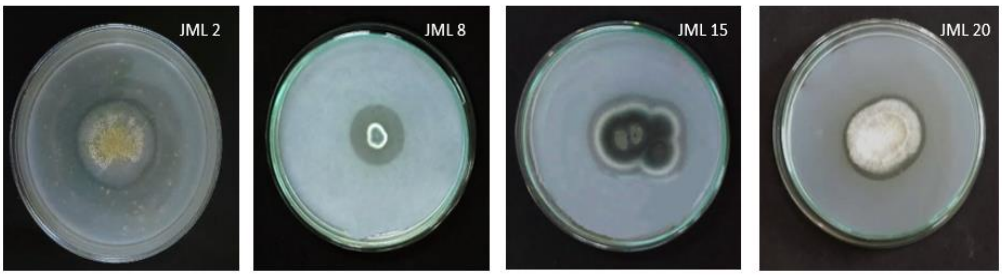

Figure 1. Potent fungal isolates showing clear zones on Skimmed milk agar plate

Table 2. Relative Enzyme Activity of fungal isolates

Isolate name	Hydrolysis zone diameter (mm)			Colony diameter (mm)			Relative Enzyme Activity (REA)		
	Incubation hours								
	48 hrs	72 hrs	96 hrs	48 hrs	72 hrs	96 hrs	48 hrs	72 hrs	96 hrs
JML 2	**21.2**	**26.5**	**33.7**	**19.5**	**24.2**	**28.5**	**1.08**	**1.09**	**1.18**
JML 5	8.0	10.5	13.5	7.8	9.8	12.0	1.02	1.07	1.12
JML 6	10.0	13.4	15.2	8.5	11.2	13	1.17	1.19	1.16
JML 8	**6.4**	**14.6**	**22.2**	**4.3**	**9.6**	**12**	**1.48**	**1.52**	**1.85**
JML 11	8.0	12.4	16.5	7.5	10.5	14.5	1.06	1.18	1.13
JML 12	7.25	11.2	13.5	6.5	9.8	12.0	1.11	1.14	1.12
JML 15	**17.5**	**26.2**	**32.4**	**13.2**	**19.2**	**23.3**	**1.32**	**1.36**	**1.39**
JML 20	**15.3**	**24.4**	**31.7**	**12.2**	**19.3**	**24.8**	**1.25**	**1.26**	**1.27**
JML 22	13.5	20.2	26	12.0	16.5	22.5	1.12	1.22	1.15

Quantitative screening

The high protease positive fungal isolates were inoculated into protease specific fermentation broth and analysed for protease production quantitatively. The isolate JML 15 showed maximum activity yielding 144.72 U ml^{-1} followed by JML 8 (107.92 U ml^{-1}), JML 2 (63.41 U ml^{-1}), JML 20 (52.25 U ml^{-1}) (Figure 2).

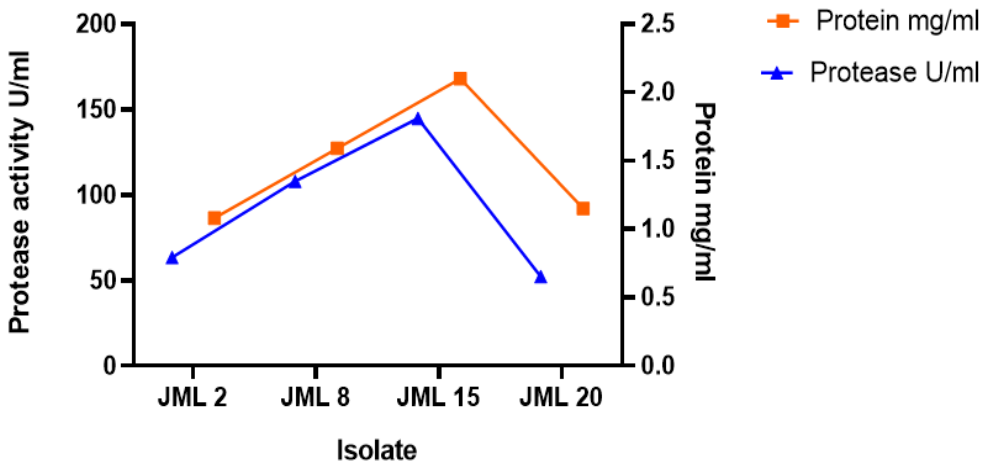

Figure 2. Quantitative screening of potent isolates

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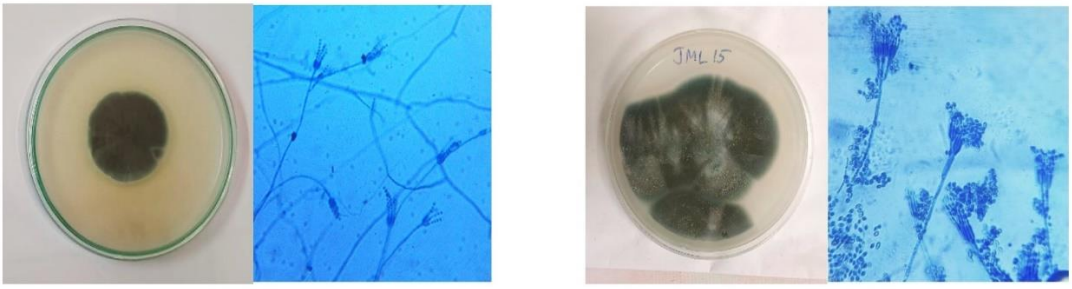
Morphological and Microscopic Identification of Fungal Isolates

Macroscopic characteristics of JML 8 on the SDA plate appeared as circular green, a velvety colony with pale yellow reverse colour whereas JML 15 has a circular flat bluish green, powdery appearance. Microscopic characteristics of JML 8 has long, monoverticillate conidiophore with globose conidia and JML 15 has shown long, Biverticillate conidiophore, cylinder phialides with ellipsoidal spores (Figure 3). Based on microscopic observation of spores and their arrangement, the protease-producing fungi JML 8 and JML 15 were identified as *Penicillium sp*.



JML 8 JML 15

Figure 3. Colony morphology and microscopic image of JML 8 and JML 15 on SDA plate

Molecular identification

Based on the results of similarity comparison of the ITS-5.8S gene sequences it is concluded that the isolate JML 8 is closely related to *Penicillium lagena* and identified as *Penicillium lagena* JML 8 with accession number PP987316.1. The isolate JML 15 has a similar percentage homology in gene sequence with *Penicillium oxalicum*, hence identified as *Penicillium oxalicum* JML 15 with accession number PQ160092.1. The neighbor-joining tree was constructed based on ITS rDNA region sequences of Penicillium sp. (Figure 4).

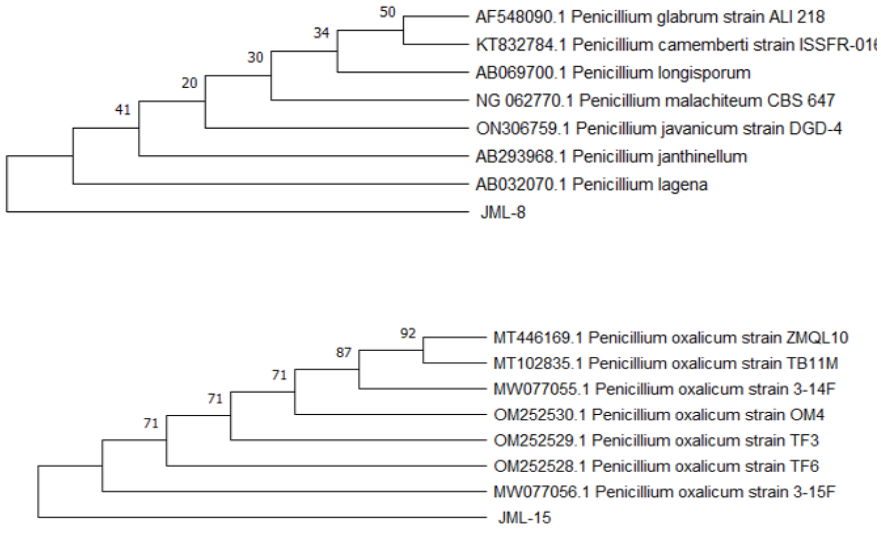


Figure 4. Phylogenetic tree analysis of JML 8 and JML 15

CONCLUSION

The study involved isolating and screening marine fungi from marine soil to find those capable of producing protease enzymes. A total of 23 isolates were obtained and screened for their ability to produce protease enzymes by observing zone formation in Skimmed milk agar plates. After the initial screening, 9 isolates were selected, and 4 of them were quantitatively tested for protease activity. The most significant protease activity was observed in JML 15 (144.72 U/mL), followed by JML 8 (107.92 U/mL). Using

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molecular techniques, the potent isolates were identified as Penicillium lagena JML 8 and Penicillium oxalicum JML 15. These isolates exhibited notable protease activity, and further optimization of culture conditions could enhance their production.

Aknowledgements

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