Screening For Extracellular Enzyme Production In Endophytic Fungi Isolated From Green And Brown Algae

A K Ramya*,Dr. R Devika*[2]andDr. K Sethumadhavan[3]

[1] ResearchScholar, [3]Professor Department of Microbiology, AarupadaiVeedu Medical College, Vinayaka Missions Research Foundation, Pondicherry, India

[2]Professor,Department of Biotechnology, AarupadiVeedu Institute of Technology, Vinayaka Missions Research Foundation ,Paiyanoor, Chennai, India

ORCID ID: A K RAMYA,0000-0002-5680-2235

DR R DEVIKA,0000-0002-1914-1378

DR K SETHUMADHAVAN,0000-0002-7990-7689

***Corresponding Author: vineethdevika@gmail.com ; akramya@gmail.com**

Abstract

Aim: The present investigation focuses on the screening of various enzymes produced by fungal endophytes isolated from various marine algae. **Materials & Method:**Five different types of green algae and two brown algae were collected from Thoonithurai, Mandapam, and Rameswaram regions and subjectedfor isolation of endophytic microorganisms as per standard protocols. The marine endophytic fungi were isolated and purified by continuous streaking.The purifiedendophytes were morphologically identified andwere screened for multi-enzyme production system. During enzyme screening around 10 isolates were identified. Fungal strains7, 8 and 9 showed themaximum and proved to be multi-enzyme producer comprising amylase, protease, tyrosine and phosphatase and fungal strains 6 and 10 registered moderate production of enzymes. Thus, the endophytic fungal isolates 7,8,9 were subjected 18s DNA sequencing and identified to be C***urvilariaplatzii, Chaetomiumperlucidum***,***Corynascussepedonium***,***Talaromycesaurantiacu, Scopulariopsisgracilis,Amesiaatrobrunnea***respectively, and deposited in NCBI.

Keywords:endophytic fungi,enzymes, marine algae, amylase, protease, phosphatase

Introduction

The marine environment acts as a harbour for many microorganisms andserves as an important source for a new novel biological product. Common endophytes isolated from marine algae and marine sediments are ***Bacillus sps., Pseudomonassps., Enterobactersps., Klebsiella sps.,*** etc. and fungi are ***Aspergillus sps., Penicillium sps.,***etc.The marine ecological system provides the greatest diversity of fungi and it has been reported from different sources like micro and macro algae; sponges, corals sediments etc. All these fungi are reported in many applications including antimicrobial, antidiabetic, antifungal, and antitumor activities. The by-products of marine microorganisms have high potentialfor developing industrially utilized products and act as a catalyst for certain reactions. Marine microbial isolates produce extra-cellular and intra-cellular enzymes which are commercially utilized in many industrial processes widely. The most commonly used enzymes are amylase,

cellulase, and protease which are helpful for the breakdown of starch, cellulose, and protease,respectively. Among these amylases from a microbial source,are extensively utilized due to their cost effectiveness, eco-friendly, short time for production,and easy gene manipulation etc. Industrially used amylase production is carried out using ***Bacillus*** strains like ***Bacillus licheniformis*,*Bacillus stearothermphilusand Bacillus amyloliquefaeciens***.Amylase hasa wide range of applicationsin the food, paper, and textile industries[13]. More often cellulose is also used in the textile industryand isadded inthe laundry process and they are proved to be bestantioxidant and stabilizer in food processing. Microbes from a marine source can produce extracellular enzymes (amylase, cellulase, protease) are highly important in industries and the protease enzyme helps to regulate the metabolic process which are of therapeutic value. Marine system provides the greatest diversity of endophytic fungiisolated from different marine microalgae, sponges, corals, sediments etc.Theseendophytic fungihave broad applications including antibacterial, antidiabetic, antifungal and antitumor activities. So, in thepresent investigation,endophytic microbes are isolated from marine algal sources which are capable of producing amylase, cellulase and protease, respectively.

MATERIALS AND METHODS

Collection of Algae

Fresh algae samples (Brown and green algae)were collected from Thoonithurai, Mandapam, Rameshwaram, India. Samples were collected and differentiated in their morphological appearances and colour that are transferred in to sterile plastic containers with sea water and kept in ice box during transportation to lab. The algae samples were surface washed to remove all debris and dirt using sea waterand processed for endophytic fungi isolation as per standard protocols. After completion of sterile water wash, the samples are kept in filter paper to remove excess water. The identification of macro algae was carried out in the presence of Prof. P. Jayaraman, Plant Anatomy Research Centre, Tambaram, Chennai and placed in the Herbarium for record.

Table 1:

List of Identified and Authenticated algal Samples.

S. No	Voucher No.	Family	Binomial
1	PARC/2022/4830	Gigartinaceae	Chondrus crispus Stackh
2	PARC/ 2022/4829	Dictyotaceae	Padina boergesenii Allender and Kraft
3	PARC/2022/4828	Dictyotaceae	Padina gymnospora (Kuetzing) Vickers
4	PARC/2022/4827	Caulerpaceae	Caulerpa racemose (Forsskal) J Agardh
5	PARC/2022/4826	Caulerpaceae	Caulerpa sertularioides (S G Gmel) M Howe
6	PARC/2022/4825	Ulvaceae	Ulva intestinalis L.

7	PARC/2022/4824	Ulvaceae	Ulva lactuca L.
8	PARC/2022/4823	Caulerpaceae	Caulerpa taxifolia (M Vahl) C. Agardh
9	PARC/2022/4822	Ceratophyllaceae	Ceratophyllum submersum L.

Surface Sterilization

The dried algae samples were rinsed with 70 % ethanol for 60 seconds followed by 0.4 % sodium hypochlorite for 30 seconds to remove the epiphytic micro-organism from outer surface of algae. Finally, after two washes with sterile distilled water, final wash was collected in a beaker to screen for endophytic microorganisms. The water washed algae was placed in the filter paper to remove excess water for 10 to 20 minutes using sterile blade and the surface samples were cut in to small segments (2.0 cm) and fine pieces were placed into Potato Dextrose Agar Nutrient Agar and Actinomyces agar separately, respectively. The plates are prepared using seawater and streptomyces in PDA to reduce bacterial growth and nystatin in starch casein agar to suppress fungi isolates. Then the algal-pressed plates were incubated for 7 days in dark conditions at 28 $\pm$ 2 ^{0} C and AA media plates were incubated for 10 to 15 days in 37^0 C. The colonies grown around the segments were isolated and sub cultured in slants for further studies. The pure endophyte cultureswere preserved in glycerol and photographed for colony morphological studies.

MOLECULAR IDENTIFICATION OF ENDOPHYTES

The isolated fungi were identified by 18 S rDNA ITS sequencing. The fungal cultures were grown in PDA slant for 7 days at 28 $\pm$ 2 ^{0} C. After incubation, the fungal mat were taken and suspended in lysis buffer and the DNA isolation were done using Expure Microbial DNA isolation kit. After DNA isolation ITS 1 (5^1 TCC GTA GGT GAA CCT GCG G 3^1) and ITS 4 (5^1 TCC TCC GCT TAT TGATAT GC 3^1) primers were used for DNA amplification of the fungal genome. The pure PCR product were used for Sanger Sequencing in Reginal Facility for DNA fingerprinting, Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, India. From the raw data (ATB file), the FASTA sequence collected and the Basic Local Alignment Search Tool (BLAST) were carried out for identification of fungal species.

Screening of various enzymes for isolated strains

All the isolated strains were tested for the production of enzymes viz, amylase, protease, and tyrosinase phosphatase enzymes as per standard procedures.

Screening of amylase activity

Amylase activity was tested on the agar medium supplemented with starch as substrate. The fungi were steaked on the medium and incubated in an appropriate medium. After incubation, all the plates were flushed with Gram's Iodine solution for 5 – min to produce deep blue colour Starch-

Iodine complex for 5 – min. If the organism is capable of producing amylase enzyme it will produce a clear zone in blue colour background. This helps to identify the detection of amylolytic strain.

Screening of protease Activity

The isolated microbes were screened to find out the best strain for protease activity in skim milk agar. After 24 Hours, of incubation clear zone (diameter), was measured.

Screening of tyrosinase enzyme activity

Tyrosine activity was screened using tyrosine agarwhere the selected cultures were streaked and incubated at 30 ^{o}c for 2 – 3 days. After incubation, brown pigmented colonies gradually changed toblack coloured colonies gradually due to melonin pigment.

Screening of phosphatase enzyme activity

Pikovskayas agar is used to screen phosphate-stabilizing organisms. The isolates were loaded in a well and incubated at 35-37 ^{o}C for 48 hours. The isolates which have solubilizing potential will produce clear zone around colonies.

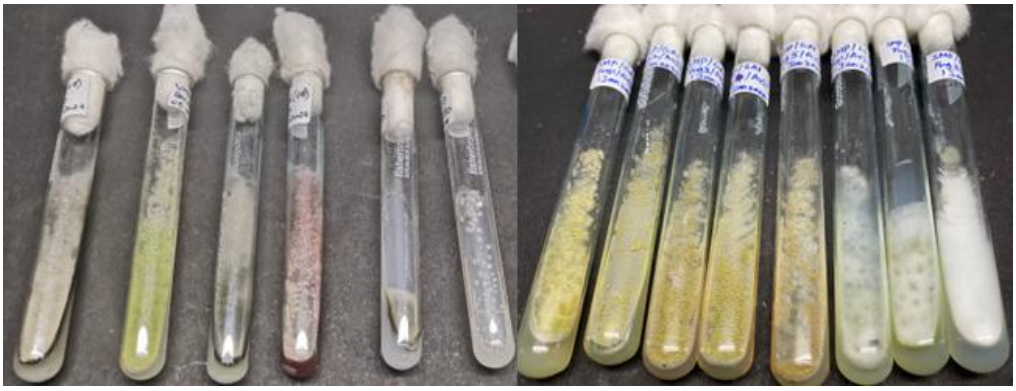

Figure 1 Pure cultures of Endophytic fungi isolated from green and brown marine algae

S. No	Name of the fungi	Amylase	Protease	Tyrosinase	Phosphatase
1	**FUG_01**	--	--	--	--
2	**FUG _02**	+	++	--	--
3	**FUG _03**	++	+++	+	+
4	**FUG _04**	--	++++	--	--
5	**FUG _05**	--	++	--	--
6	**FUG _06**	--	++++	+	+

7	**FUG _07**	+++	++++	+++	+
8	**FUG _08**	++++	++++	++	++
9	**FUG _09**	++++	++++	++	++
10	**FUG _10**	++	++	++	++

Table 1 interpretation of various enzyme production observed in specific media using 10 fungal isolates (Note : -- Negative; + Low ; ++ Moderate; +++High; ++++ Very High)

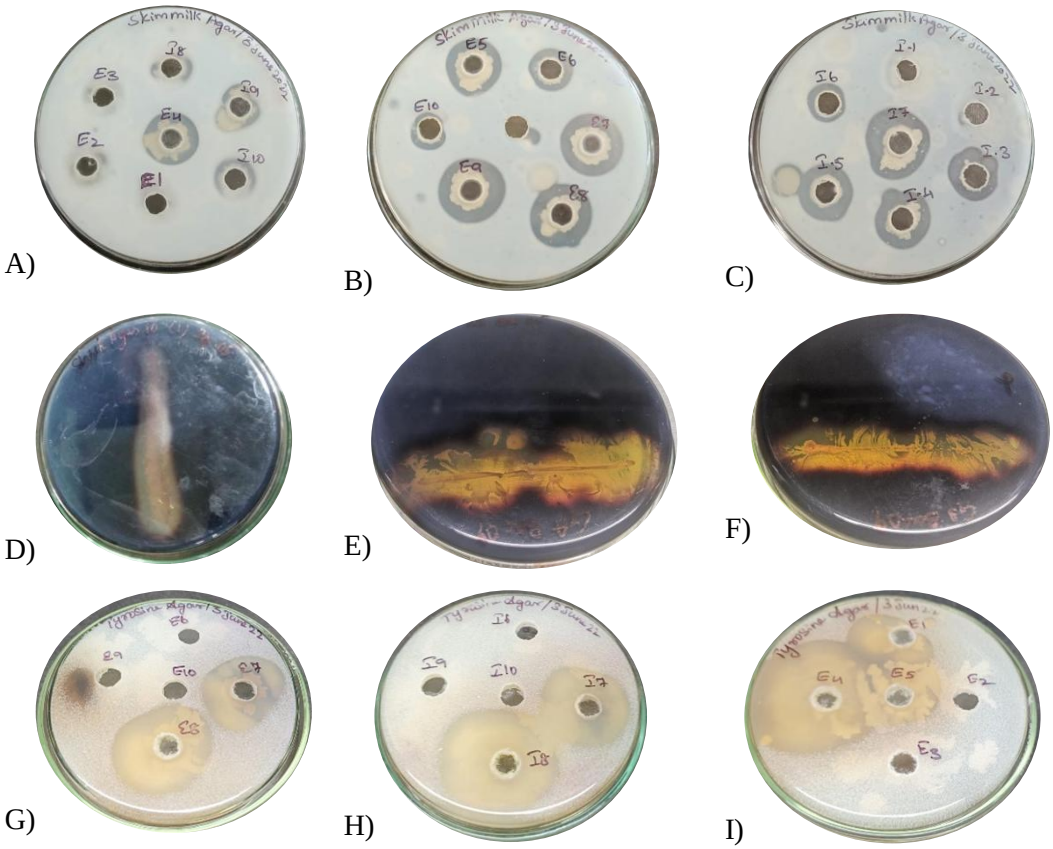

Figure 2 various enzyme activity (Note: A, B, C show protease enzyme production; D, E and F show amylase enzyme production; G,H and I show tyrosinase enzyme production)

Isolates Name	Closest Relative[a]	Accession No[b]	% Identity[c]
FUG_05	***Corynascussepedonium***	ONO59588.1	99.78
FUG_07	***Talaromycesaurantiacus***	ONO59708.1	98.84
FUG_08	***Amesiaatrobrunnea***	ONO63018.1	100
FUG_09	***Microascusgracilis***	ONO63045.1	100
FUG_10	***Curvulariaplatzii***	ONO63065.1	99.77
FUG_06	***Chaetomium perlucidum***	ON350775.1	93.55

Table 2 Six endophytic fungal strains identified by 18 s r DNA Sequencing

[a]Closest species which high % identity in BLAST Analysis, [b]NCBI Gene bank accession number in website (http://www.ncbi.nlm.nih.gov/pubmed),[c]GenBank accession no. of our strains deposited on NCBI website (http://www.ncbi.nlm.nih.gov/pubmed),[d]% identiy of strain based on BLAST Analysis

RESULT

Around 15 fungal endophytes were isolated from different green and brown algae collected from Thoonithurai, Mandapam, and Ramesmeshwaram. Nearly 15 endophytic fungi were isolated and purified cultures were identified microscopically. From these 15 isolates, only 10 different isolates(Figure 1) were inoculated in PD Broth and kept for incubation at 37^0 C for 3 – 5 days. Mycelium developed in the broth was filtered and centrifuged at 5000 rpm for 15 minutes and the supernatant was used to inoculate in specific media. The multienzyme screening was carried out using agar plate method either streaking or well method. Enzymes like amylase, protease, tyrosin, and phosphatase were screened using starch agar, skim milk agar, tyrosin agar and Pikovskayas agar. In skim milk agar, out of 10 fungal isolates, 8 fungal strains were able to produce protease enzyme on the plate and formed halo around the colonies. Likewise, in starch agar out of 10 isolates Fug 7,8,9showed positive results(Figure 2. D,E,F) when the plate was washed with grams Iodine solution. In tyrosine agar plates Fug 7,8,9 showedbrown pigmented colonies which changed in to black colour colonies due to melanin pigment (Figure 2. G,H,I). In proteasescreening, fungal strains FUG 2,3,7,8,9,10 produced positive result (Table 1; Figure 2. A, B, C) which was able to produce protease enzyme by utilizing the protein source in the media.The fungal strains FUG 7,8,9 showed positive result and were able to produce phosphatase enzyme. These potentialfungal strains which showed a high yield of enzymes were further identified by 18 s DNA sequencing and submitted in NCBI and the list of accession nos. and percentage of identity are listed in the Table 2. The fungal strains FUG 5,6,7,8,9,10 were identified as ***curvilariaplatzii, Chaetomium perlucidum***,***Corynascussepedonium***,***Talaromycesaurantiacu, Scopulariopsisgracilis,Amesiaatrobrunnea.***

DISCUSSION

Enzymes like amylase, protease, tyrosinase are commonly used in various application such as detergent formulation, bioleaching of pulp, textile fibre recovery, food industries. Globally demand on enzyme production are increased and the import rate also increased by 9.40% per year. Due to lack of production of commercial enzyme leads to focus on microbial source for multienzyme production system.Isolates obtained from starch-rich sources have a higher ability to produce amylase.According to Luang et al, nearly 13 isolates showed higher amylase production efficiency, similar to previous reports. The present study investigated the multi-enzyme activity of different endophytic fungi of *Ceratophyllum submersum*, resulting in the 10 endogenous fungal isolates, 3 showed positive results in the production of some enzymes such as amylases, protease, tyrosinase and phosphatase. Similarly,

isolated endophyte of *Cymbidium aloifolium* and endogenous *Strptomyces* from *Vanda spathulate* showed high production of various enzymes such as amylase, protease, lipase and cellulase.

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AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Not available

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript. NCBI Bank, Accession numbers:

ONO59588.1; ONO59708.1; ONO63018.1; ONO63045.1; ONO63065.1; ON350775.1

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