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Mangrove microflora as potential source of hydrolytic enzymes for commercial applications

Bhavya Kachiprath, Solly Solomon, G. Jayanath, & Rosamma Philip*

Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, Kochi, Kerala, India.

[E-mail: rosammap@gmail.com]

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The purpose of this study was to isolate and characterize mangrove microflora based on their hydrolytic enzyme production. A collection of 100 microorganisms including bacteria, actinomycetes, fungi and yeasts was isolated. The ability of microbial isolates to degrade hydrolytic enzymes such as amylase, cellulase, chitinase, glutaminase, laccase, ligninase, lipase, protease and tyrosinase were tested and the potent strains were identified based on 16S rRNA and ITS sequencing. More than 90% of the isolates exhibited amylolytic and proteolytic activity. Potent isolates were identified as: *Bacillus subtilis* (MB1), *Bacillus amyloliquefaciens* (MB11), *Bacillus megaterium* (MB23), *Bacillus mojavensis* (MB28), *Streptomyces galbus* (MA7), *Streptomyces* sp. (MA3), *Candida parapsilopsis* (MY6), *Candida etchellsi* (MY1), *Penicillium citrinum* (MF5), *Aspergillus stellifer* (MF12) and *Emericella* sp. (MF18). These microbes as well as the enzymes are of potential importance for commercial applications as bioremediators, detergent additives and nutritional supplements.

[**Keywords:** Mangrove; Microbial enzymes; Yeast; Actinomycetes; Fungus]

Introduction

Mangrove ecosystem is a reservoir of diverse microbial communities (bacteria, fungi, algae, plankton and viruses) which play a crucial role in biogeochemical processes[1]. In view of the fact that microbial populations are exceptionally diverse in terms of their genetic and biochemical properties, they are considered as a promising source of enzymes with prospective technological applications[2,3]. In starch process industry, chemical hydrolysis of starch has been completely replaced by microbial enzymes[4,5]. Microbial enzymes are used for various industrial applications *viz*., (i) Lipases for the production of cosmetics, detergents and as components of chemical and pharmaceutical reagents[6,7]; (ii) Proteases for the development of leather, detergents, digestive and anti-inflammatory drugs and additives in bioremediation processes[8,9]; (iii) Ligninases in feed, fuel, food, agricultural, paper, textile and cosmetics industries[10,11] and (iv) Amylases in food, fermentation, textile and paper industries[12,13].

Unlike other expensive chemical methods, microbial remediation enables the degradation of toxic contaminants into simpler compounds without causing any harm to the natural environment. Bioremediation acts as a low-cost, *in situ* alternative method, which is non-carcinogenic, non-combustible, widespread and eco-friendly in nature as compared to other conservative methods. Fungi, as a part of mycoremediation, also play a significant role in natural remediation of metal and aromatic pollutants by removing or degrading toxicants from natural environment.

In our study, we isolated and explored bacteria, fungi, actinomycetes and yeasts from mangrove ecosystem which showed significant potential for production of hydrolytic enzymes that can be used as potential candidates for bioremediation.

Materials and Methods

Sample collection

Sediment samples were collected from a mangrove ecosystem in Malippuram (Lat. 9°59'44.9"N; Long. 76°14'12.6"E), Ernakulam, Kerala, using Van-veen grab. Samples labelled, kept in sterile containers and transported to the laboratory in icebox for further processing.

Isolation of microorganisms

Approximately 10 g sediment mixed with 90 ml distilled water and 10^{-1} to 10^{-5} dilutions were prepared for bacterial isolation. ZoBell's marine agar (peptone – 5 g, yeast extract – 1 g, ferric phosphate – 0.02 g, agar – 20 g, sea water - 1000 ml; pH – 7 to 7.4) was

used for bacterial isolation. Medium used for the isolation of actinomycetes was ISP4 medium (soluble starch – 10 g, K_2HPO_4- 1 g, $MgSO_4.7H_2O$ – 1 g, NaCl – 1 g, $(NH_4)_2SO_4$ – 2 g, $CaCO_3$ – 2 g, agar- 20 g, sea water- 1000 ml; pH – 7 to 7.4) with Bavistin as antifungal agent. Fungi were isolated using Rose Bengal agar (31.55 g/l seawater) with chloramphenicol (100 mg/l) as antibacterial agent. Wickerham's medium (MYGP medium – yeast extract – 3 g, malt extract – 3 g, peptone – 5 g, glucose – 10 g, agar - 20 g, sea water - 1000 ml, pH – 7 to 7.4) was used for the isolation of yeasts with chloramphenicol (200 mg/litre) as antibacterial agent. Pour plate technique was employed and incubation was done at 28 °C for 5 days. Single colonies were isolated, purified and stored in nutrient agar slants (bacteria and actinomycetes) and malt extract agar (fungi and yeasts) slants for further screening, (Figure 1).

Screening for extracellular enzymes

Isolates were screened for hydrolytic enzymes *viz*., amylase[14], cellulase[15], chitinase[16], glutaminase, laccase, ligninase[17], lipase[18], protease[19] and tyrosinase. Table 1 showed substrates and reagents used for enzyme assays. Based on the enzyme

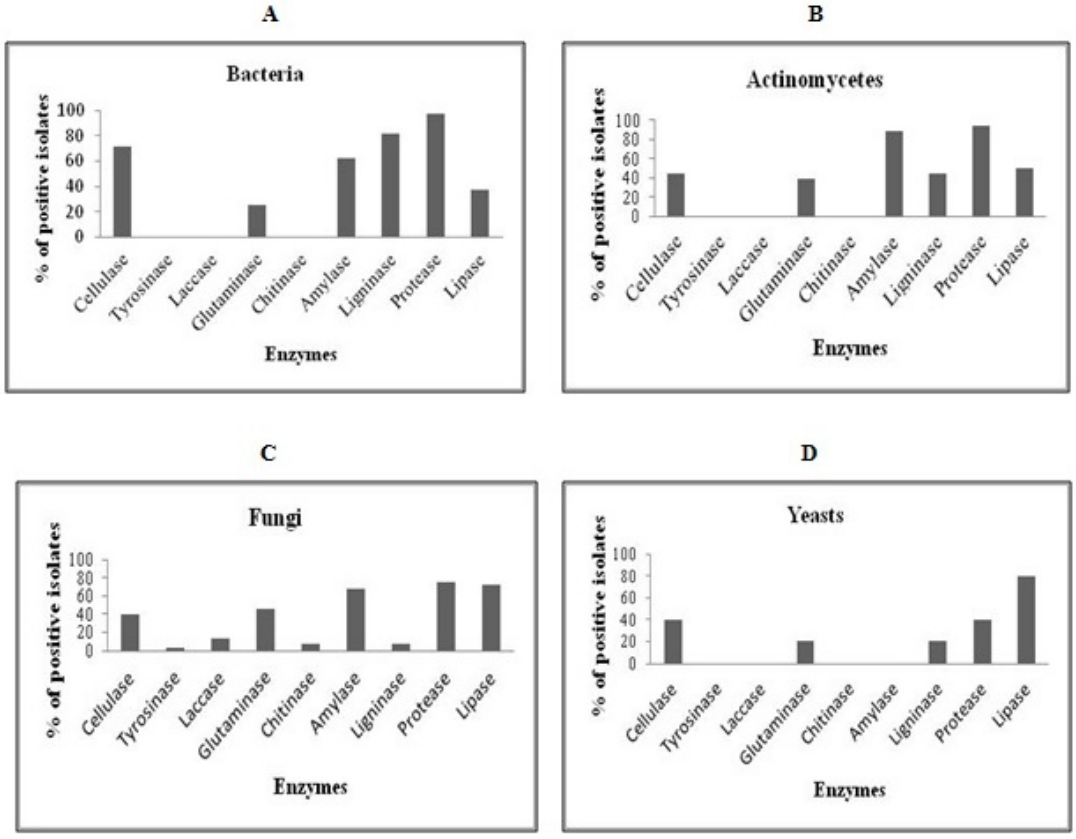

Fig.1 — Enzyme production by mangrove microflora (A - Bacteria, B - Actinomycetes, C - Fungi, D - Yeasts)

Table 1 — List of substrates and reagents used for enzyme assays

Enzyme	Substrate	Reagents used for detection	Observation
Gelatinase	Gelatin -1%	Mercuric chloride	Clear Zone
Lipase	Tributyrin-1%	-	Clear zone
Amylase	Starch-0.2%	Gram's iodine	Clear zone
Chitinase	Colloidal chitin-1%	-	Clear zone
Cellulase	Cellulose-0.5%	-	Clear zone
Glutaminase	Glutamine-1%	Phenol red	Pink coloration
Ligninase	Methyleneblue-0.02%	-	White zone
Tyrosinase	Tyrosine-0.5%	-	Clear zone
Laccase	α α - Naphthol-0.005%	-	Purple colour zone

production, potent strains were segregated and subjected to molecular identification.

Molecular identification of selected strains

Bacteria/actinomycetes were inoculated into nutrient broth and fungi/yeasts into malt extract broth and incubated at 28 ^{0}C at 120 rpm overnight. The cells were pelletized by centrifugation at 12,000 rpm for 15 min. DNA from this pellet was isolated using salting out method in which the pellet was thoroughly mixed with 500 µl solution I (20 mM EDTA (pH - 8), 50 mM Tris-HCl (pH - 8), 20% SDS). Then 5 µl proteinase K (20 mg/ml) added to it, mixed well and incubated at 55 °C for 2 hrs. After that the samples were kept in ice for 10 min. 200 µl of solution II (6M NaCl) was added, incubated in ice for 5 min and centrifuged at 8000 rpm for 15 min. Supernatant was collected, equal volume of isopropanol was added to it and the samples were incubated at 4 °C overnight. Samples were centrifuged at 11,000 rpm for 20 min. The supernatant was discarded and the pellet was washed in 70% ethanol thrice followed by 100% ethanol. Pellet was air-dried and dissolved in 30 µl TE buffer. Purity of the isolated DNA was checked using spectrophotometric methods by measuring 260/280 ratio. DNA concentration and yield were also determined. DNA was visualised on 0.8% agarose gel and was shown in Figure 2.

Primers used for the amplification of 16S rRNA gene of bacterial and actinomycetes and ITS region of fungi and yeasts were given in Table 2. PCR conditions for 16S gene amplification included an initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, extension at 72°C for 1 min and a final extension at 72 °C for 10 min. PCR conditions for ITS amplification included an initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 45 s, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. Amplicons were visualised on 1% agarose gel using a gel documentation system (Syngene) and are shown in Figure 3 (A, B, C & D). PCR products were sequenced at SciGenom, Kochi, India.

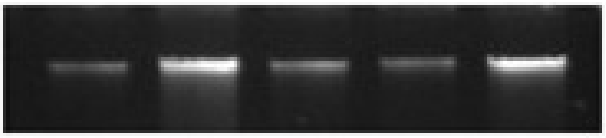

Fig. 2 — Gel image of isolated DNA from potent isolates using salting out method

Table 2 — Primer sets used for the identification of microorganisms

Sl. No	Microorganisms	Primers	Sequences (5'-3')
1	Bacteria/Actinomycetes	27F	5'-AGAGTTTGATCMTGGCTCAG-3'
		1492R	5'-TACGGYTACCTTGTTACGACTT-3'
2	Fungi/Yeasts	ITS1	5′-CCGTAGGTGAACCTGCGG-3'
		ITS4	5'-TCCTCCGCTTATTGATATGC-3'

Results

Screening for extracellular enzymes

We have tested the ability of 32 bacteria, 20 actinomycetes, 4 yeasts and 41 fungi for enzyme production. In the case of bacteria, 90% exhibited protease production and 70% cellulase production. Among actinomycetes, 95% showed protease activity, 90% amylase, 58% lipase, 50% cellulase, 50% ligninase, and 40% glutaminase production. More than 50% of the fungal isolates showed amylase, protease and lipase activity. Among yeasts, 80% showed lipase production, 40% showed cellulase and protease production and 20% strains exhibited glutaminase and ligninase production (Fig. 1).

Molecular identification of selected strains

The nucleotide sequences first edited with BioEdit software and compared with NCBI database through BLAST searches and the isolates were identified based on similarity. The sequences were aligned with multiple alignment and phylogenetic trees constructed by MEGA 5.2. Potent bacterial isolates were *Bacillus subtilis* (MB1), *Bacillus amyloliquefaciens* (MB11), *Bacillus megaterium* (MB23), and *Bacillus mojavensis* (MB28). Potent actinomycetes included *Streptomyces galbus* (MA7) and *Streptomyces* sp. (MA3). Yeasts were *Candida parapsilopsis* (MY6) and *Candida etchellsi* (MY1). Potent fungal strains included *Penicillium citrinum* (MF5), *Aspergillus stellifer* (MF12), and *Emericella* sp. (MF18).

Characteristics of the selected cultures

Hydrolytic enzyme production potential of selected cultures was shown in Table 3. Bacterial isolates, namely, *Bacillus subtilis* (MB1), *Bacillus amyloliquefaciens* (MB11), *Bacillus megaterium* (MB23) and *Bacillus mojavensis* (MB28) exhibited significant protease production; moderate production

Table 3 — Hydrolytic enzyme production by selected isolates

Sl. No	Microorganisms	Hydrolytic Enzymes								
		Cellulase	Tyrosinase	Laccase	Glutaminase	Chitinase	Amylase	Ligninase	Protease	Lipase
	Bacteria									
1	*Bacillus subtilis* (MB1)	*+++	-	-	+	-	+++	++	+++	++
2	*Bacillus amyloliquefaciens* (MB11)	+++	-	-	+	-	++	++	+++	++
3	*Bacillus megaterium* (MB23)	+	-	-	++	-	++	++	+++	++
4	*Bacillus mojavensis* (MB28)	+	-	-	+	-	++	++	+++	++
	Actinomycetes									
5	*Streptomyces galbus* (MA7)	++	-	-	-	-	++	-	++	+
6	*Streptomyces* sp. (MA3)	++	-	-	+	-	++	++	-	++
	Fungi									
7	*Aspergillus stellifer* (MF12)	++	-	-	+++	-	+	-	++	++
8	*Penicillium citrinum* (MF5)	+	-	-	++	++	+	-	-	-
9	*Emericella* sp. (MF18)	+	-	-	+++	-	+	-	++	-
	Yeasts									
10	*Candida parapsilopsis* (MY6)	+	-	-	+	-	-	+	++	++
11	*Candida etchellsi* (MY1)	+	-	-	-	-	-	-	++	++

*Clear zone is indicated by: (+) 0.5 -1cm; (++) 1-2cm; (+++) above 2cm; and (-) no activity

of amylase, ligninase and lipase and weak production of cellulase and glutaminase. *Streptomyces galbus* (MA7) and *Streptomyces* sp. (MA3) showed moderate production of all the tested enzymes except tyrosinase, laccase and chitinase. *Penicillium citrinum* (MF5), *Aspergillus stellifer* (MF12) and *Emericella* sp. (MF18) showed very high glutaminase production. Only *Penicillium citrinum* (MF5) exhibited chitinase activity. Yeasts, *Candida parapsilopsis* (MY6) and *Candida etchellsi* (MY1) exhibited protease, lipase, cellulase, glutaminase and ligninase production.

Discussion

Mangrove ecosystem, located as a transition zone between land and sea, is one of the most dynamic and variable ecosystems due to its constantly fluctuating patterns of tidal flooding, salinity, oxygen, sea surface temperature, pH, muddy and anaerobic soil, wind pattern and nutrient availability. To exist in such a fluctuating environment, the mangrove microbial community possesses unique and specific adaptations like salt tolerance, biosynthetic potential and heavy metal resistance compared with their terrestrial and marine counterparts[20]. Many studies reported that microbial consortia with almost all enzymatic abilities are essential to degrade complex hydrocarbon mixtures such as crude oil from industrial waste water, fresh water and marine environments[21-23]. Bacteria are the most dynamic agents in petroleum degradation and they perform as primary degraders of spilled oil in various environments[24]. Several studies have proved the capability of microbial communities like *Bacillus cereus*[25], *Pseudomonas putida, Pseudomonas fluorescens*[26], *Pseudomonas desmolyticum*[27] and *Bacillus* sp.[28] on decolourization of pulp and paper industrial effluents and normalization of other physicochemical parameters, such as odour, temperature, pH, BOD, COD, calcium, magnesium, chlorine, total solids, total dissolved solids, total suspended solids and total hardness of textile dye effluents. They naturally biodegrade pollutants, several types of toxic, carcinogenic and mutagenic chemicals such as dispersants, levelling agents, acids, alkalies and various dyes and thereby remove them from the environment. In our study, 60-95% of isolated bacterial strains including *Bacillus subtilis* (MB1), *Bacillus amyloliquefaciens* (MB11), *Bacillus megaterium* (MB23) and *Bacillus mojavensis* (MB28) showed cellulase, amylase, ligninase and protease production and 20-40% showed lipase and glutaminase production. *Bacillus subtilis* is a biosurfactant producing microorganism whose decolourization ability was reported as 92-97% and has the ability to produce lipopeptide antibiotics[29]. Studies proved that *Bacillus subtilis* utilize the crude oil components as carbon and energy sources, degrade heavy metals like mercury, chromium, etc. and is a significant candidate for bioremediation in coastal and marine environment[30,20].

Out of more than 10,000 known antibiotics, 50–55% are reported to be produced by streptomycetes and are considered economically the most significant among the actinomycetes[31]. In the present study, we could get potent actinomycetes, *Streptomyces galbus* (MA7) and *Streptomyces* sp. (MA3). Actinomycete isolates were highly potent in terms of cellulase, glutaminase, amylase, ligninase, protease and lipase production. Streptomyces are reported to be involved in the breakdown and recycling of organic compounds and degradation of petroleum hydrocarbons[32].

Fungal strains, classified under basidiomycetes are reported as the best producers of ligninolytic enzymes. They can be considered as the ideal source in studies related to environmental and industrial applications including the biological treatment of lignocellulosic substrate for biofuel production[33]. Ligninolytic enzymes are able to degrade a broad range of compounds via free radical-mediated oxidizing reactions[34]. In the present study, we got two potent fungal strains *viz., Aspergillus stellifer* (MF12), *Penicillium citrinum* (MF5) and *Emericella* sp. (MF18). Industries targeted for chemical manufacturing, oil and gas production always generated hyper saline wastes containing high concentration of salts, oil, organic acids, heavy metals and radionuclides[35]. *Penicillium* sp. can tolerate and transform heavy metals and xenobiotic compounds into less mutagenic products and are potentially interesting to remediate pollutants in the presence of salt for biological treatment without damage[36,38]. The plant polymers, cellulose and lignin which are very sturdy compounds that give plants their structure are degraded by fungal enzymes[39,40]. In this study, 10% of the isolated fungal isolates showed chitinase and ligninase production and more than 50% fungal strains showed amylase, protease and lipase production.

Marine yeasts have been isolated from different sources such as seawater, hydrothermal vents, marine deposits, seaweeds, fish, marine mammals, sea birds and also from hypersaline habitats. Marine yeasts mostly included *Candida* spp., *Cryptococcus* spp., *Debaryomyces* spp., *Rhodotorula* spp., *Metchnikowia* spp., *Kluyveromyces* spp., *Rhodosporidium* spp., *Pichia* spp., *Hansenula* spp., *Saccharomyces* spp., *Trichosporon* spp., and *Torulopsis* spp.[41,42]. Several yeast strains possess filaments or pseudohyphae and produce hydrolytic enzymes to endorse efficient disintegration of substrates. Potent yeast strains obtained from this study included *Candida parapsilopsis* (MY6) and *Candida etchellsi* (MY1). Bioremediation of diary effluents by yeast strains including *Candida intermedia* and *Kluyveromyces marxianus* was already reported[43]. *Candida utilis*, *Candida albicans, C. tropicals,* and other *Candida* spp., were reported to have played prominent role in the control of algal blooms, indicating their potential use in the control of aquatic weeds, bio-ethanol production and bioremediation of environmental pollutants such as heavy metals[44-47]. This study indicated that the mangrove microflora, especially the actinomycetes are potential source of hydrolytic enzymes and can find varied applications in industries.

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