

MICROBIAL ENZYME ACTIVITY IN DECOMPOSING LEAVES OF MANGROVES

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ABSTRACT

The present work analysed the enzyme activities of the predominant microbial species associated with decomposing mangrove leaves. The microbes were isolated from decomposing leaves of mangroves and the extracellular enzymes such as amylase, protease, cellulase, chitinase and lipase were determined. Senescent leaves of two mangrove species (*Rhizophora mucronata* and *Avicennia marina*) kept in nylon bags, were separately in two tanks immersed in tidal water for 40 days situated along the intertidal area of the Vellar estuary, south east coast of India. Decomposing leaves were collected every eight days from each tank for isolation and enumeration of different groups of microorganisms. Two genera of total heterotrophic bacteria (THB), four species of *Lactobacillus*, two species of *Azotobacter*, two species of *Actinobacteria*, three isolates of fungus, four species of yeasts, two species of *Thraustochytrids* and four species of *Trichoderma* were identified and tested further for their enzyme activity. Fungal isolates, especially *Trichoderma* species, were found more efficient in producing the extracellular enzymes than the Bacterial isolates, revealing the significance of fungi in detritus-based mangrove systems.

Key words: Mangroves, Microbes, *Rhizophora*, Enzymes, *Avicennia*, Decomposition,

Introduction

Microorganisms play an important role in decomposing organic matter and producing protein-rich detritus that serves as a food for fishes, especially in detritus-based aquatic ecosystems like mangrove systems [1-2]. Fungi are particularly important in the marine environment as decomposers of dead organic substrates [3]. Marine straminipilous organisms (*Oomycetes* and *Thraustochytrids*) are associated with decomposition of leaf materials in the aquatic habitats. In the mangrove

environment, fallen leaves are colonized and decomposed by microorganisms. The

undecomposed leaves are poor in nutrients, and they become nutritious due to the microbial enrichment process during decomposition process [4]. The microbial decomposition of mangrove litter has been extensively studied [5-6]. However, very little information is available about the microbial enzymes produced during the mangrove litter decomposition. Hence, the present study was made on microbial counts and their ability for enzyme production

during leaf litter decomposition in two mangrove species, *Rhizophora mucronata* and *Avicennia marina*.

MATERIALS AND METHOD

Isolation of microbes from decomposing leaf samples

Fresh senescent leaves of *Rhizophora mucronata* Poir. (Rhizophoraceae) and *Avicennia marina* Forsk. (Vierh) (Avicenniaceae) were collected by gentle shaking the healthy trees in the mangrove forest of the Vellar estuary in southeast coast of India (Lat. 26°49'N; long. 46°96'E). One kg collected leaves was taken in a nylon bag (30 × 50 cm with mesh size of 2mm) for each species and submerged in pits with 1m × 0.5m x 1m dimension, constructed along the intertidal area of the estuary. In order to allow the nylon bag to sink in water, a stone weighing approximately 250 grams was put in each bag. This experiment was conducted for two months (January and February, 2011). The leaf samples were drawn from the bag at the intervals of 8, 16, 24, 32 and 40 days of experiment and brought to laboratory immediately for the microbial examination. The decomposing leaf samples were cleaned and washed with sterilized seawater to remove the debris present on the leaves. One gram of the sample was aseptically ground by using a pestle and mortar, then it was transferred to a sterile 250 ml conical flask containing 99ml of sterile distilled water and serial dilution was made to get different diluents: 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶ and 10⁻⁷ for the isolation of microbes. The nutrient media suitable for different microbial groups were prepared in 50% seawater, Zobell marine agar for total heterotrophic bacteria, yeast extract malt extract medium for yeasts, MRS (de man rogosa sharps) medium for *Lactobacilli*, yeast peptone agar medium for

Thraustochytrids, casein agar medium for *Actinobacteria*, marine nutrient medium for *Cyanobacteria*, Winogradsky's medium for *Azotobacters*, potato dextrose medium for fungi and selective medium for *Trichoderma* (Askew and laing,1993) For plating, 1 ml of the serially diluted samples of leaf extract was pipetted out into sterile Petri dish. Then sterile media was poured into dishes aseptically and swirled for a thorough mixing. After solidification the plates were incubated for 2-7 days in an inverted position at 27°C. All the determination was carried out in duplicates. The colonies were counted. The microbial counts were expressed as the number of colony forming units (CFU) per gram of wet leaf tissue.

Identification of microbes

Bacteria were identified by using Bergey's manual (Buchanan, and Gibbons, 1974) fungi by following the keys given by Ainsworth *et al* (1973) [7] Other groups of microbes were identified adopting standard keys: yeast [8] *Thraustrochytrids* [9], *Trichoderma* ([http://nt.ars-grin.gov/taxadescriptions/keys/Trichoderma Index.cfm](http://nt.ars-grin.gov/taxadescriptions/keys/TrichodermaIndex.cfm)).

Extracellular production of enzymes by microbes

The microbial strains isolated from the decomposing leaves were tested for their ability of extra cellular enzyme production by using the streak plate method (Maria *et al*, 2005).

Amylase Assay: Amylase activity was assessed by growing the selected strains on glucose yeast extract peptone (GYP) agar medium (glucose 1 g; yeast extract 0.1g; peptone 0.5g; agar 16 g; 50% seawater 1000 mL) with 2% soluble starch. After incubation, the plates were flooded with 1% iodine in 2% potassium iodide. The clear

zone formed surrounding the colony was considered positive for amylase activity.

Cellulase assay: For cellulase, the strains were cultured on yeast extract peptone agar medium (yeast extract 0.1g, peptone 0.5 g, agar 16 g and 50% seawater 1000 mL) supplemented with 0.5% Na-carboxymethyl cellulose (CMC). After incubation, the plates were flooded with 0.2% aqueous Congo red and destained with 1M NaCl for 15 minutes. The clear zone surrounding the colony indicated the cellulase activity.

Chitinase assay: For chitinase activity, the strains were grown on colloidal chitin agar medium (colloidal chitin 2 g; agar 16 g; 50% seawater 1000 mL). The clear zone surrounding the colony after incubation was considered positive for chitinase activity.

Lipase assay: For lipase activity, the strains were grown on peptone agar medium (peptone 10 g; NaCl 5 g; CaCl₂.2H₂O 0.1 g; agar 16 g; 50% seawater 1000 mL) supplemented with Tween 20 (separately sterilized and added 1 mL to 100 mL medium). A clear zone around the colony indicated lipase-positive strain.

Protease assay: Protease assay was performed by growing the strains on GYP agar medium amended with 0.4% gelatin (gelatin 8 g/100 mL distilled water, sterilized separately and mixed with sterile GYP agar medium) adjusted the pH to 6. After incubation, plates were flooded with saturated aqueous ammonium sulphate. The undigested gelatin precipitated with ammonium sulphate and digested area around the colony was clear.

The values were treated with two way analysis of variance (ANOVA) to find out the significance between the microbial counts and other variables. Correlation analysis between the variables were also analyzed [10].

RESULTS:

BACTERIA

The total heterotrophic bacterial (THB) counts were high in decomposed leaves. The counts reached a peak between 16 to 24 days of decomposition and declined thereafter. In *Rhizophora mucronata*, THB counts ranged from 0.24×10^8 to 0.95×10^8 CFU.g⁻¹ in the leaves decomposed for 8 and 40 days respectively (Fig. 1). In *Avicennia marina*, the counts ranged from 0.28×10^8 to 0.99×10^8 CFU.g⁻¹ in the leaves decomposed for 8 and 40 days respectively (Fig. 2). The THB counts between days were significant, but not between the mangrove species. The bacteria identified were *Bacillus* species which exhibited maximum amylase activity and minimum or no activity for cellulase (Table 1).

Lactobacillus counts were high in decomposed leaves. The counts reached a peak between 8 and 24 days of decomposition and declined thereafter. In *Rhizophora mucronata*, lactobacillus counts ranged from 0.3×10^8 to 0.95×10^8 CFU.g⁻¹ in the leaves decomposed for 8 and 40 days respectively (Fig.1). In *Avicennia marina*, the counts ranged from 0.3×10^8 to 0.85×10^8 CFU.g⁻¹ in the leaves decomposed for 8 and 40 days respectively (Fig.2). The lactobacillus counts between days were significant, but not between the mangrove species. The lactobacillus identified were *Lactobacillus brevis*, *L. fermentum*, *L. buchneri* and *L. lactis* exhibiting the maximum amylase activity and the minimum activity of chitinase and protease (Table 1).

Azotobacter counts increased with a peak between 8 and 16 days of decomposition and declined thereafter. In *Rhizophora mucronata*, azotobacter counts ranged from 0.018×10^8 to 0.074×10^8 CFU.g⁻¹ in the leaves decomposed for 8 and 40 days respectively (Fig. 1). In *Avicennia marina*,

the counts ranged from 0.05×10^8 to 0.083×10^8 in the leaves decomposed for 8 and 40 days respectively (Fig. 2). The *Azotobacter* counts between days were significant, but not between the mangrove species. The selected *Azotobacter* isolates exhibited the maximum chitinase and the minimum amylase activity (Table 1).

Actinobacteria counts increased with decomposing leaves, reached a peak between 32 and 40 days of decomposition and declined thereafter. In *Rhizophora mucronata*, the *actinobacteria* ranged from 0.001×10^8 to 0.364×10^8 CFU.g⁻¹ in the leaves decomposed for 8 and 40 days respectively (Fig.1). In *Avicennia marina*, the counts ranged from 0.001×10^8 to 0.426×10^8 CFU.g⁻¹ in the leaves decomposed for 8 and 40 days respectively (Fig. 2). The *Actinobacteria* counts between days were significant, but not between the mangrove species. The selected *Actinobacteria* isolates exhibited the maximum cellulase and the minimum lipase activity (Table 1).

FUNGI

In *Rhizophora mucronata*, fungal counts ranged from 0.012×10^8 to 0.045×10^8 CFU.g⁻¹ in the leaves decomposed for 8 and 40 days respectively (Fig.1). In *Avicennia marina*, the counts ranged from 0.012×10^8 to 0.084×10^8 CFU.g⁻¹ in the leaves decomposed for 8 and 40 days respectively (Fig.2). The fungal counts between days were significant, but not between the mangrove species. The fungi identified were *Trichosporon* sp., *Aspergillus* sp., and *Fusarium* sp. They exhibited the maximum cellulase and protease; and the minimum lipase activity (Table 2).

Thraustrochytrids counts were less abundant compared to other microbes in decomposed leaves. In *Rhizophora mucronata*, *Thraustrochytrids* counts ranged from

0.0003×10^8 to 0.0934×10^8 CFU.g⁻¹ in the leaves decomposed for 8 and 40 days respectively (Fig.1). In *Avicennia marina*, the counts ranged from 0.0005×10^8 to 0.0742 CFU.g⁻¹ in the leaves decomposed for 8 and 40 days respectively (Fig. 2). The *Thraustrochytrids* counts were significant between days and also mangrove species. The *Thraustrochytrids* isolates exhibited maximum in amylase and protease and minimum in lipase activities (Table 2).

Yeasts counts were less abundant as compared to other microbes in decomposed leaves. In *Rhizophora mucronata*, yeasts counts ranged from 0.001×10^8 to 0.364×10^8 CFU. g⁻¹ in the leaves decomposed for 8 and 40 days respectively (Fig.1). In *Avicennia marina*, the counts ranged from 0.001×10^8 to 0.426×10^8 CFU.g⁻¹ in the leaves decomposed for 8 and 40 days respectively (Fig. 2). The yeasts count was significant between days and also mangrove species. The yeasts identified were *Pichia salicaria*, *Geotrichum* sp., *Pichia fermentans*, *Cryptococcus dimenna*. They exhibited high activity of cellulase, amylase and protease and low in lipase activity (Table 2).

Trichoderma counts were less abundant in decomposed leaves. In *Rhizophora mucronata*, *Trichoderma* counts ranged from 0.015×10^8 to 0.122×10^8 CFU.g⁻¹ in the leaves decomposed for 8 and 40 days respectively (Fig.1). In *Avicennia marina*, the counts ranged from 0.013×10^8 to 0.035×10^8 in the leaves decomposed for 8 and 40 days respectively (Fig. 2). *Trichoderma* counts between days were significant, but not between the mangrove species. The species identified were *T. asperellum*, *T. aaggerssivum*, *T. spirale*, *T. polysporum*. They were capable of producing maximum activities of cellulase, amylase and protease and minimum of lipase (Table 2).

DISCUSSION

The role of microorganisms and their ecological factors that affect the decomposition processes of mangrove leaf litter has been well-documented by a number of researchers [13]. In the decomposition of organic matters, three phases are involved leaching of soluble compounds, microbial oxidation of refractory compounds such as cellulose and lignin and physical and biological fragmentation [11]. In all these phases, microbes especially saprophytic fungi play an important role as the primary decomposers of mangrove leaf litter [12]. This is substantiated in the present study that saprophytic fungi such as *Trichoderma* and *Thraustochytrids* were found efficient because of their enzymatic activity and ability to degrade cellulose, starch, lipid, protein and lignin (Table 3, Fig 1,2). The role of fungi in the enzyme production through their degradative activities as found by Ulken (1981) [14] is also in accordance with our results. The enzyme activity varies between types of microbes and enzymes produced. In general, fungi including *Trichoderma* and yeasts, azotobacters, *Actinobacteria* were found more efficient in enzyme production than total heterotrophic bacteria and *lactobacillus*, whereas *Thraustochytrids* were least efficient in enzyme production (Table 3).

While studying the green and senescent leaves of *Rhizophora apiculata* and *Sonneratia alba* in the mangroves forests of Malaysia, the microbial counts have been observed initially to increase with days of decomposition and decrease in later stages of decomposition. However, the microbial counts do not vary significantly between the mangrove species but with the days of decomposition, as observed in the present

study. This differential leaf litter decomposition can be attributed to the microbial counts and their enzyme activity. However, the enzyme activity of each group of microbes showed a different trend of enzyme production. For example, *Trichoderma*, were found to be more efficient than other fungi, whereas *Actinobacteria*, total heterotrophic bacteria and *lactobacilli* were more efficient than azotobacters.

Thraustochytrids are also known to be colonizing on fallen senescent leaves of *Sonneratia* and *Rhizophora* [13]. Results from the present study showed that *Thraustochytrid* species can also colonize and help in mangrove leaf decomposition in mangrove environment (Table 1). The early colonizers are fungi which degrade a wide variety of organic compounds [15]. The periods during which the microbes lead to maximum extracellular enzyme activity is of newly submerged leaves of red mangrove [16]. In the present study the maximum bacterial count was recorded during the 24th day of decomposition. A similar observation has been made by Benner *et al* (1988).

In present study during the decomposition the microbes produced extra cellular enzymes including cellulolytic enzyme. The cellulolytic enzymes have extreme capacity to degrade the cellulolytic materials thus enriching the nutrient of the leaves with their biomass [17]. This process changes the plant debris into a nutritious form for consumption of organisms in higher trophic levels. Association of microbes and their enzyme activity has a vital role to play in decomposition of mangrove leaves and palatability of detritus food to marine organisms.

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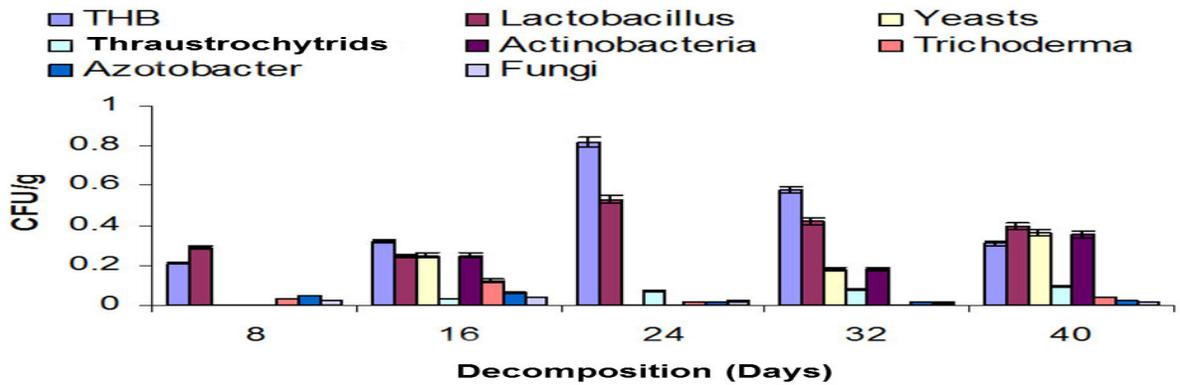


Fig 1. Colony forming units (CFU.g⁻¹) of different microbial groups associated with decomposing leaf litter of *Rhizophora mucronata*

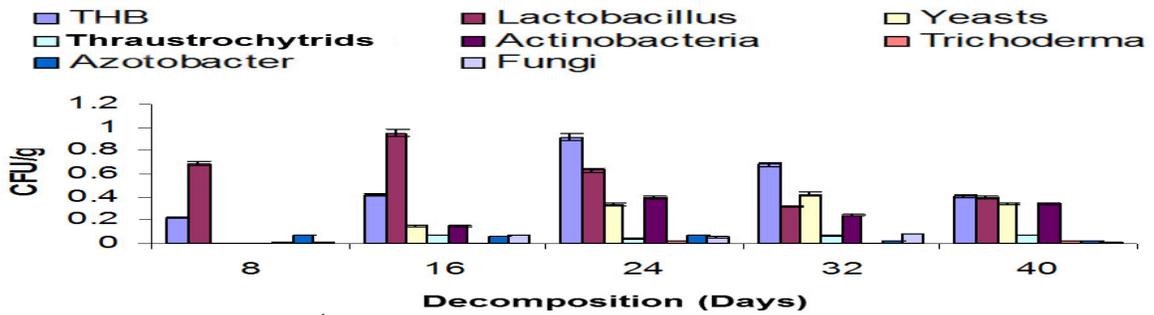


Fig 2. Colony forming units (CFU.g⁻¹) of different microbial groups associated with decomposing leaf litter of *Avicennia marina*

Bacterial strain	Enzyme activity (diameter of clearing zone in cm)				
	Amylase	Cellulase	Lipase	Chitinase	Protease
Total Heterotrophic Bacteria					
<i>Bacillus</i> sp	2.9±0.3	0	0.3±0.1	2.1±0.2	1.5±0.3
Lactobacillus					
<i>Lactobacillus brevis</i>	2.3±1.2	1.2±0.3	1.2±0.1	0.2±0.1	2.1±0.2
<i>Lactobacillus fermentum</i>	2.1±0.6	1.3±0.1	1.2±0.2	0.1±0.05	2.1±0.3
<i>Lactobacillus buchneri</i>	2.5±0.2	1.5±0.3	0.2±0.3	0.5±0.02	2.4±0.3
<i>Lactobacillus lactis</i>	1.6±0.6	0.5±0.2	0.5±0.1	0.4±0.1	1.2±0.2
Actinobacteria					
<i>Actinobacteria</i> sp	1.6±0.2	2.1±0.1	1.2±0.2	3.5±0.2	1.2±0.2
Azotobacter					
<i>Azotobacter</i> sp	0.2±0.1	1.5±0.2	1.5±0.6	2.3±0.6	1.2±0.2

Table1. Enzyme activity of bacteria associated with

Fungal strains	Enzyme activity (diameter of clearing zone in cm)				
	Amylase	Cellulase	Lipase	Chitinase	Protease
<i>Thraustrochytrids</i>					
<i>Thraustrochytrids</i> sp	3.5±0.5	2.6±0.3	0.1±0.1	1.2±0.3	3.5±0.3
Yeasts					
<i>Pichia salicaria</i>	2.5±0.3	2.6±0.3	0.2±0.1	1.3±0.2	2.1±0.2
<i>Geotrichum</i> sp	2.6±0.1	2.5±0.1	0.5±0.2	1.6±0.3	5.2±0.2
<i>Pichia fermentans</i>	3.6±0.2	2.9±0.3	0.6±0.2	1.5±0.3	2.6±0.1
<i>Cryptococcus dimennae</i>	2.1±0.3	1.5±0.2	0.9±0.2	2.5±0.1	2.5±0.2
<i>Trichoderma</i>					
<i>Trichoderma asperellum</i>	3.6±0.3	5.6±0.2	1.2±0.3	1.5±0.5	3.6±0.2
<i>T. aaggerssivum</i>	3.5±0.2	5.2±0.3	1.6±0.2	4.6±0.6	3.4±0.3
<i>T. spirale</i>	3.6±0.5	3.5±0.1	1.5±0.1	5.2±0.5	3.2±0.2
<i>T. polysporum</i>	2.5±0.4	2.6±0.2	1.4±0.3	2.6±0.4	2.6±0.1
Other fungi					
<i>Trichosporon</i> sp.	1.5±0.2	1.5±0.3	0.2±0.2	1.5±0.3	2.3±0.2
<i>Aspergillus</i> sp.	1.2±0.3	1.2±0.1	0.6±0.1	0.6±0.2	2.6±0.3
<i>Fusarium</i> sp.	1.6±0.1	1.6±0.2	0.9±0.1	0.9±0.1	2.4±0.3

decomposing leaf litter of *Avicennia marina* and *Rhizophora mucronata* (n=3; mean ± SE)

Table 2. Enzyme activity of fungi associated

Microbes	Enzyme activity (cm X 10 ⁸ / g of leaf tissue)				
	Amylase	Cellulase	Lipase	Chitinase	Protease
Total Heterotrophic Bacteria	0.93±0.01	0.37±0.01	0.1±0.01	0.28±0.01	0.41±0.01
Lactobacillus	0.64±0.05	0.33±0.02	0.23±0.002	0.09±0.02	0.58±0.02
Actinobacteria	0.24±0.04	0.52±0.05	0.20±0.02	0.37±0.01	0.22±0.001
Yeasts	0.43±0.05	0.30±0.02	0.081±0.02	0.27±0.02	0.49±0.01
<i>Trichoderma</i>	1.06±0.06	1.07±0.02	1.02±0.01	1.062±0.01	1.05±0.02
Fungi	1.04±0.01	1.04±0.01	1.01±0.001	1.02±0.01	1.07±0.01
Azotobacter	0.02±0.01	0.12±0.01	0.10±0.001	0.17±0.002	0.10±0.01
Thraustrochytrids	0.00012±0.00001	0.0001±0.00001	0.0005±0.00001	0.004±0.001	0.004±0.001

with decomposing leaf litter of *Avicennia marina* and *Rhizophora mucronata* (n=3; mean ± SE)

Table 3. Enzyme activity of decomposing leaf litter of *Avicennia marina* and *Rhizophora mucronata* (n=3; mean ± SE)