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Relationship between microbial biomass and extracellular enzymes: a study of Pachamalai forested streams

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Abstract

In forest environments, aquatic micro fungi play a critical role in organic matter breakdown. These microorganisms break down refractory substances like lignin, allowing the microbial population to effectively use organic material. In Pachamalai rainforest streams, the primary inflow of allochthonous organic materials occurs in the autumn and is enriched with luxurious microbiota.Our study aimed to determine the relationship between microbial biomass and extracellular enzymes from the streams of Pachamalai forests. The physicochemical properties of leaves and the activity of the microbial flora on the organic matter degradation were also determined.The C: N, C: P, and N: P biofilm molar ratios were calculated based on the total N, P, and C contents of two leaves species viz. Morinda tinctoria and Pongamia pinnata eaves. The hydrolytic and oxidative enzyme activity of the leaf substrata was analysed by earlier know methods.Morinda tinctoria leaves had a faster breakdown with a decrease in leaf toughness relative to *P. pinn*ata. Extracellular enzyme assays revealed that *M. tinctoria had higher hydrolytic enzyme activity* when compared to *P. pinn*ata.From our study, it is conclusive that the microbiota associated with both the leaf species have significant extracellular enzymatic activity in degrading the polysaccharides and lignin. This plays a significant role in the stream biota and influences the ecosystem.

Keywords: Extracellular enzymes, forested stream, sporulation, microbial biomass, Morinda tinctoria, Pongamia pinnata

1. Introduction

Small streams to huge rivers, running waters usually occur in a wide range of climate, vegetation, terrain, and geological conditions (Allan J et al., 1995). Several studies have found persistent variations in biogeochemical features, hydrological parameters (Gasith et al., 1999) and biological structure and function across streams from different biomes and ecoregions. One of the most intriguing topics in earth sciences is the close interaction between abiotic conditions and the soil biosphere, which has enormous consequences for both environmental and human health (Van Elsas et al.,2008). It is not astonishing that the soil formation with a high degree of fertility is the consequence of hundreds of

years evolution of the soil due to the complex interactions between various factors (Harrison et al.,2008). The soil matrix, as well as chemical and physical characteristics of soils, such as the quality and amount of soil organic matter, pH, and redox conditions, have a significant impact on the form and function of microbial communities in soils (Lombard et al.,2011) . The size of the channel, the flow of water, and the form of the banks influence the development of biological communities(Allan J et al., 1995). Microbial communities may form in both organic (wood and leaves) and inorganic (coobles, gravel, rocks and sand) stream benthic substrata, and are comprised of algae, bacteria, fungi, and protozoa embedded in an extra polymeric material matrix(Locket al.,1984). The community structure and function may change depending on the presence of various microbial species in the substratum (Romani et al., 2000). One of the primary factors of stream water chemistry may be the chemical composition of particles in the watershed(Berner et al.,1987) . Microbial diversity in lotic settings is less often researched than in marine and lake ecosystems, according to a recent report of microbial diversity studies in aquatic habitats(Zinger et al.,2011) . With landscapes, streams and rivers are the foci of microbially mediated carbon (C) and nutrient metabolism(Valarmathy et al.,2017). Before the advent of molecular techniques, bacterial diversity was particularly difficult to characterize, beyond distinguishing gram-negative from gram-positive organisms or doing plate counts on selective medium(Milner et al.,1984) . In the stream ecosystem, fungi and bacteria are the primary producers of extracellular enzymes responsible for polysaccharides hydrolysis, lignin oxidation, breakdown of peptides, and organic phosphorus compounds peptides(Romani et al., 2014) . Physical forces, as well as the activities of microbes such as aquatic hyphomycetes, promote leaf degradation in streams(Barlocher,1992) . The activities of these organisms accompanied by chemical reactions, cause changes in litter quality (e.g., increases in N and P concentrations) and loss of litter mass. To evaluate microbe colonization patterns (Gessner et al.1993) , to assess the ecological state of the stream ecosystem(Panet al., 1996) , and to study the interactions between microbial groups and species in the group, a community composition investigation is essential(Rieret al., 2001 ; Gulis et al., 2003) . It seems easier to identify algal and fungal species up against the bacterial diversity in the streams.

Our study aimed to determine the relationship between microbial biomass and extracellular enzymes from the streams of Pachamalai forests. It will also give an insight into the physicochemical properties of leaves and the activity of the microbial flora on organic matter degradation.

2. Material and Methods

2.1 Estimation of physicochemical properties of leaves

The microbial communities like fungi are usually found in leaf substrate and leaf tissues. Therefore, the physical and chemical properties of the leaves are largely responsible for the development of the

microbial community on organic substrata. In our study, chemical properties such as Carbon (C), Nitrogen (N) and Phosphorus (P), and Lignin content were determined.

2.1.1 Determination of Carbon, Nitrogen and phosphorus content

Leaf circles were dried and the weights of subsamples were recorded. These leaf circles were placed in the tin foil crucibles and then inside the CN Elemental Analyzer. The phosphorus content of the leaf subsamples was determined by basic digestion using NaOH by autoclaving(Grasshoff et al., 1980) at 110ºC for 90 min. Further the C: N, C: P, and N: P biofilm molar ratios were calculated based on the total N, P, and C contents of the leaves.

2.1.2 Estimation of Lignin Content

Pongamia pinnata and Morinda tinctoria species were considered for the estimation of Lignin content. According to the method of Iiyama & Wallis (1990), the leaf circles were first digested with a mixture of 4% perchloric acid and 25% acetyl bromide (in acetic acid medium) at 70ºC for 30 min. The equation of Morrison(Morrison et al., 1972) was used to determine the lignin content of the samples.

2.2 Enzyme activity

The enzyme activity of the microbial flora associated with leaves was tested to determine the activity of hydrolytic and oxidative enzymes.

2.2.1 Estimation of Hydrolytic enzyme assays

The extracellular enzyme activity was measured using the methodology described by Romaní & Sabater(Romani et al.,2001) . Six diverse hydrolytic enzyme assays were performed in this study namely -glucosidase, β-xylosidase, cellobiohydrolase, phosphatase, leucine-aminopeptidase, and β-glycosaminidase activity. These assays determined the decomposition rate of compounds viz. polysaccharides, organic phosphorus, peptides, and chitin respectively. All these assays were performed at the Indian Institute of Food Processing Technology (IIFPT), Tanjore, Tamilnadu and the obtained results were utilized for further studies.

2.2.2 Estimation of Oxidative enzymes assay

The microbiological colonies on stream substrata were also analyzed for the phenoloxidase and peroxidase enzyme activities. Fungi produce ligninolytic peroxidases and phenol oxidase which oxidize lignin polymer to generate aromatic radicals(Hammel et al.,1997) . In this study, L-3,4-dihydroxyphenylalanine (L-DOPA) is an electron-donor substrate used for the detection of phenoloxidase activity. The oxidative enzyme activity assay was carried out at IIFPT, Tanjore, Tamilnadu and the results were further utilized for the study.

2.3 Estimation of Fungal Sporulation Rates

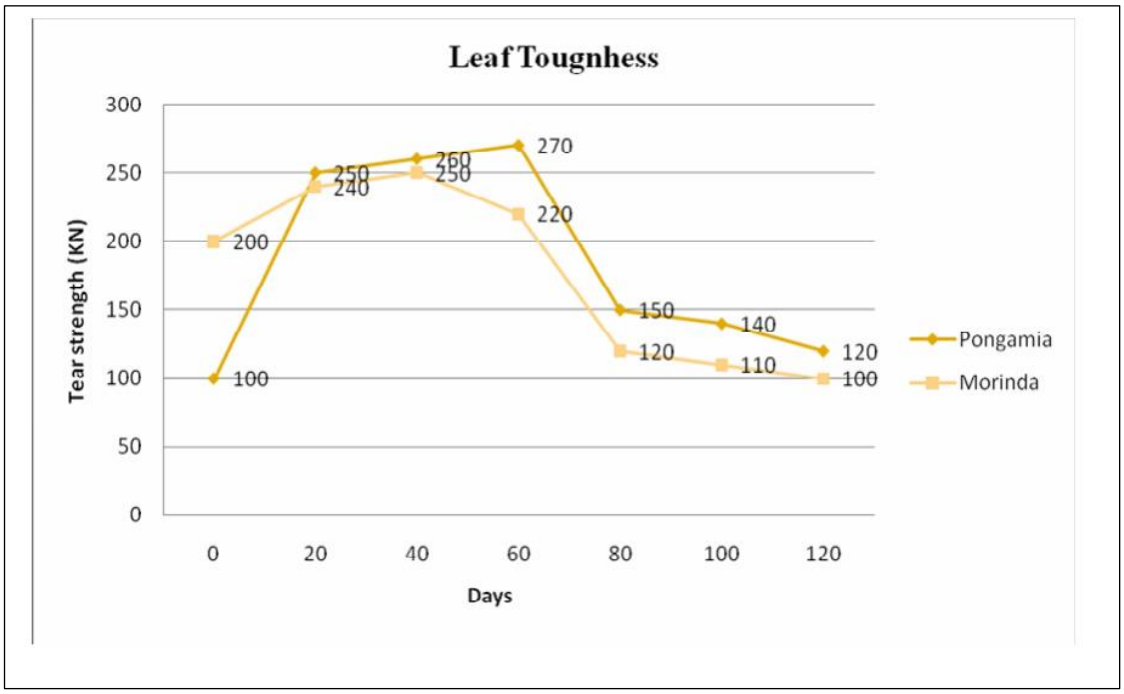
Leaf discs of P. pinnata and M. tinctoria were used to measure the sporulation rates of aquatic hyphomycetes. Sand samples, tiles, and leaves were washed with sterile water before incubation to remove the surface deposits of biofilms. Pyrex flasks of 250ml containing 100ml sterilized steam water were used to incubate the substratum samples. The samples were all incubated in a shaking bath at 80rpm, 10ºC for 48 h to induce sporulation. Later, 25µL of 0.5% (w/v) Triton X-100 solution was added and 20ml of the conidial suspension was passed through Nitrocellulose membranes (5µm pore size, Whatman). The membranes retained the conidial spores which were later stained with 0.1% Trypan Blue(Baldy et al., 2002) . The retained conidial spores were stained with 0.1% Trypan blue (dissolved in 60% lactic acid). Leaf samples were counted at 400× magnification, whereas the fine and coarse samples of substrata were counted using the whole filter. Sporulation rates were expressed as below:

$$\text{Sporulation rate} = \frac{\text{number of conidia produced}}{\text{unit surface area and time}}\ \text{cm}^2\text{.day}$$

3. Results and Discussion

Field observations suggest that M. tinctoria leaves had a faster decomposition rate when compared to P. pinnata. It was also found that P. pinnata only remained in the litter bags after 58 days. The faster breakdown was seen in M. tinctoria accompanied by a decrease in leaf toughness on Day 7 while compared to P. pinnata (Figure 1).

Figure 1. **Leaf tear strength of the two-leaf species, M. tinctori and P. pinnata in the litter bag experiment.**



Values are represented as mean (n=3) and standard errors of each sampling date.

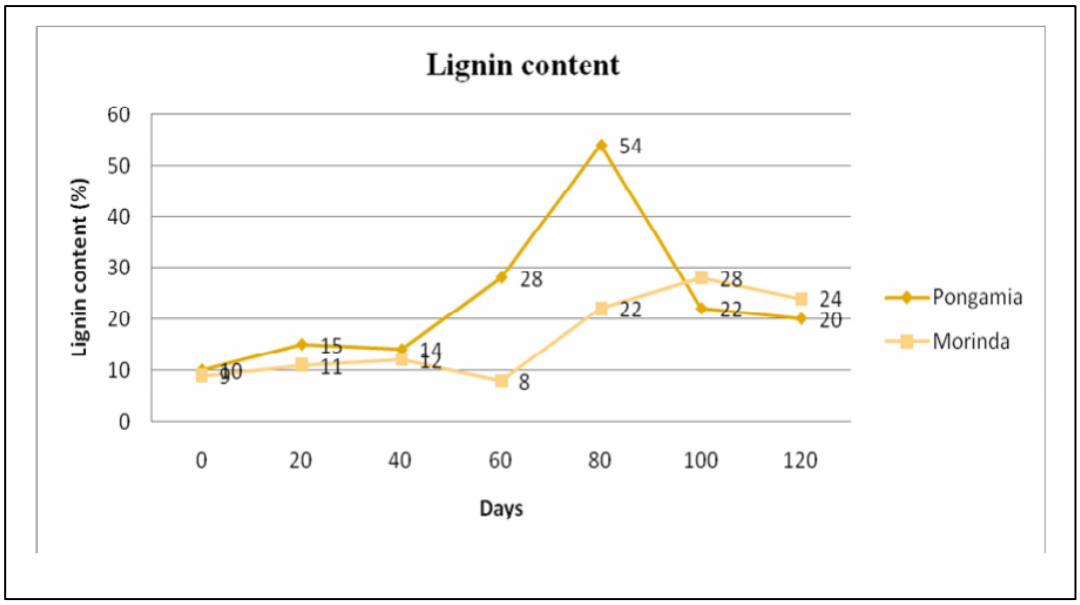
Experimentally, it was observed that the nutrient molar ratios C:N and C:P in leaves showed a drastic decrease on Day 7 and Day 17. Statistical significance was observed only in the decreasing trend of C:N ratio. Meanwhile, no drastic changes were observed in the N:P ratio during the experiment. Table 1 explains the difference in the nutrient molar ratios between M. tinctoria and P. pinnata.

Table 1. The molar ratios C: N, C: P and N: P of the two-leaf species as obtained in the litter bag experiment.

	C: N		C: P		N: P	
Days	**P. pinnata Mean(SE)**	**M. tinctoria Mean(SE)**	**P. pinnata Mean(SE)**	**M. tinctoria Mean(SE)**	**P. pinnata Mean(SE)**	**M. tinctoria Mean (SE)**
0	75.5 (0.5)	90.4 (2.4)	838.7(120.4)	710.4 (68.2)	70.5 (11.5)	50.0 (2.5)
1	64.5 (0.8)	95.5 (12.2)	864.2 (116.1)	2245.5 (490.6)	94.2(26.2)	148.6(12.0)
2	92.1 (10.9)	96.6 (16.4)	838.5 (260.4)	1875.2(782.2)	65.1 (24.1)	128.1 (41.0)
4	81.5 (1.0)	91.1 (4.4)	1344.4 (92.8)	1306 (198.4)	106.7 (7.5)	95.4 (15.0)
7	92.3(0.4)	68.2(5.7)	986.0(102.6)	1072.1 (95)	71.1(9.6)	100.6 (4.8)
17	56.9 (0.5)	45.7 (0.8)	564.2 (58.2)	390 (30.3)	62.1 (2.2)	52.4 (1.4)
28	38.4 (1.4)	36.1 (1.0)	451.4(26.5)	386.2 (34.0)	74.5(4.2)	64.2 (5.4)
44	52.4 (0.2)	45.7(0.9)	506.1 (61.3)	403.2 (62.4)	62.2(7.8)	56.4 (8.6)
58	44.6 (5.5)	42.4 (2.6)	492.2 (124.2)	621.1 (136)	65.6 (10.6)	92.1 (12.4)
73	49.5 (2.1)	656.1 (24.3)	85.1(2.2)			
93	35.1 (2.6)	322.5(34.2)	60.2 (10)			
112	54.2 (1.0)	476.2(28.4)	56.1(4.3)			

Our study showed that the content of lignin was in a similar range between different leaf species, however, during breakdown the changes in the percentage of lignin were noticed (Figure 2).

Figure 2. Percentage of lignin content in the leaves of M. tinctori and P. pinnata during the degradation procedure.



Values are represented as mean (n=3) and standard errors of each sampling date.

Hydrolytic and oxidative enzyme assays revealed that the polysaccharide and lignin degradation increased during the breakdown experiment (Table 2). The polysaccharide degrading activities in M. tinctoria showed an increasing trend during week 2 (Day 7- Day 17), a sharp peak was noted on Day 44 and then decreased on day 56 (Figure 3.2a). A similar trend was observed in the activities of P. pinnata leaves. The evaluation of phenol-oxidase assay exposed that a common pattern was noticed among the two leaf species i.e., an increase after Day 7, a plateau on Day 17, and further extended until the end of the experiment (Figure 3.2b). It is evident from hydrolytic enzyme activity that M. tinctoria had higher activity when compared to P. pinnata, however, the other activities had many similarities between the two species. Biomass-specific enzyme assays showed higher activity in P. pinnata leaves than in M. tinctoria (Figure 3.2c). This suggests that the microbial biomass on P. pinnata had better decomposing activity than that of M. tinctoria. Moreover, a sharp peak was observed in the enzyme activity per unit of microbial carbon at the onset of the experiment that gradually decreased later (Figure 4).

Table 2. Extracellular enzyme activities in P. pinnata and M. tinctoria leaves, and leaf physical and chemical properties during the breakdown experiment.

	C: N	C: P	Toughness
Pongamia (n=12)			
β-glucosidase	-0.825**	-0.786**	-0.821**
β-xylosidase	-0.75**	-0.742**	-0.810**
cellobiohydrolase	-0.650**	-0.756**	-0.614**
phenol oxidase	-0.784**	-0.736**	-0.768**

bacterial biomass	-0.690**	-0.712**	-0.784**
fungal biomass	-0.612*	-0.625**	-0.822**
Morinda (n=12)			
ß-glucosidase	-0.910**	-0.748*	-0.878**
ß-xylosidase	-0.754*	ns	-0.836**
cellobiohydrolase	-0.716*	ns	-0.824**
phenol oxidase	-0.798**	-0.677*	-0.796*
bacterial biomass	-0.886**	-0.693*	-0.948**
fungal biomass	ns	ns	Ns

Note: Significant correlations are indicated by asterisks [(*) for P<0.05 and (**) for P< 0.005] while the not significant are indicated by (ns).

Figure 3. Graphical representation of enzyme activities during the breakdown of the two leaf species (a) β-glucosidase, (b) β-xylosidase, (c) cellobiohydrolase and (d) Phenol oxidase.

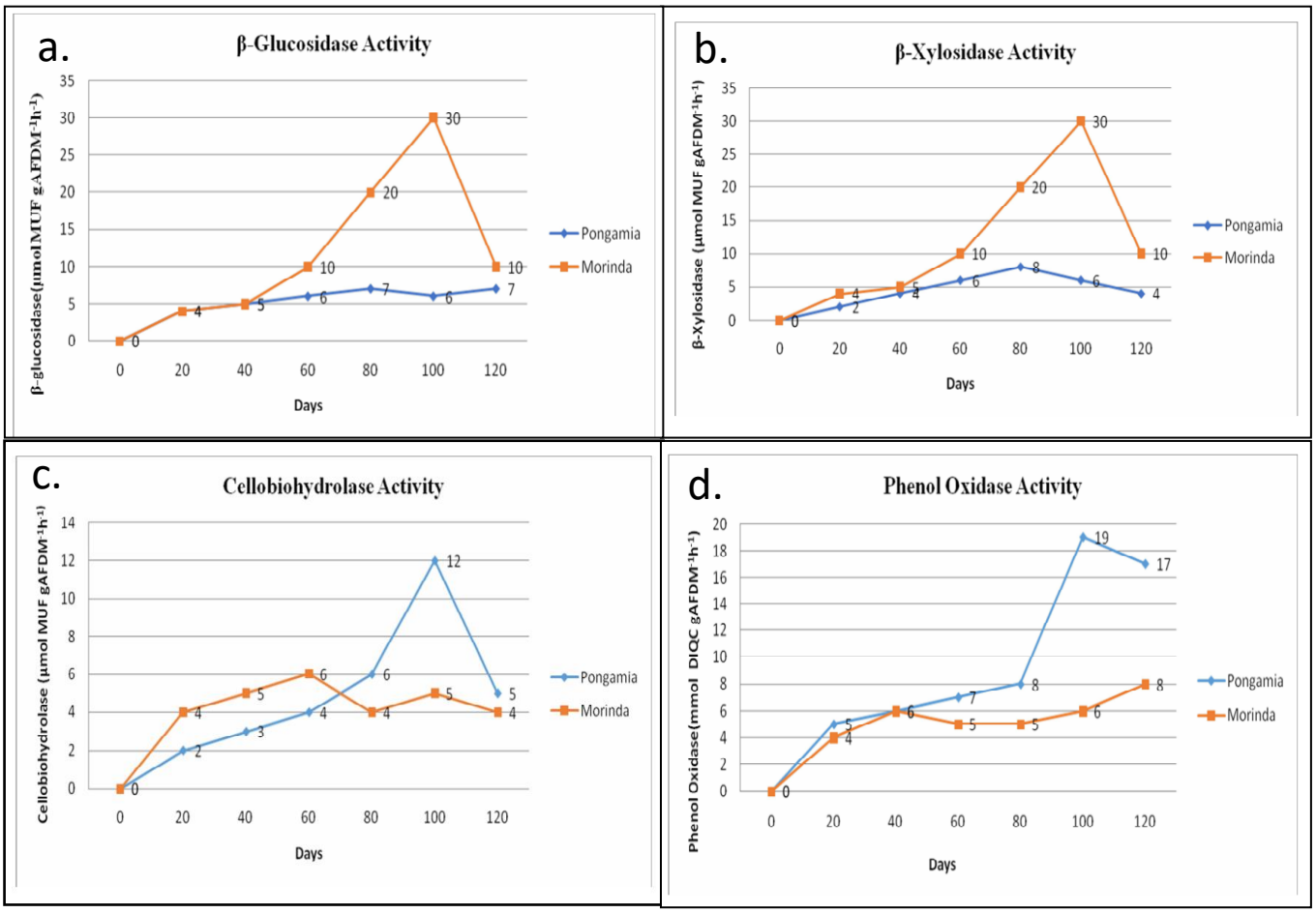
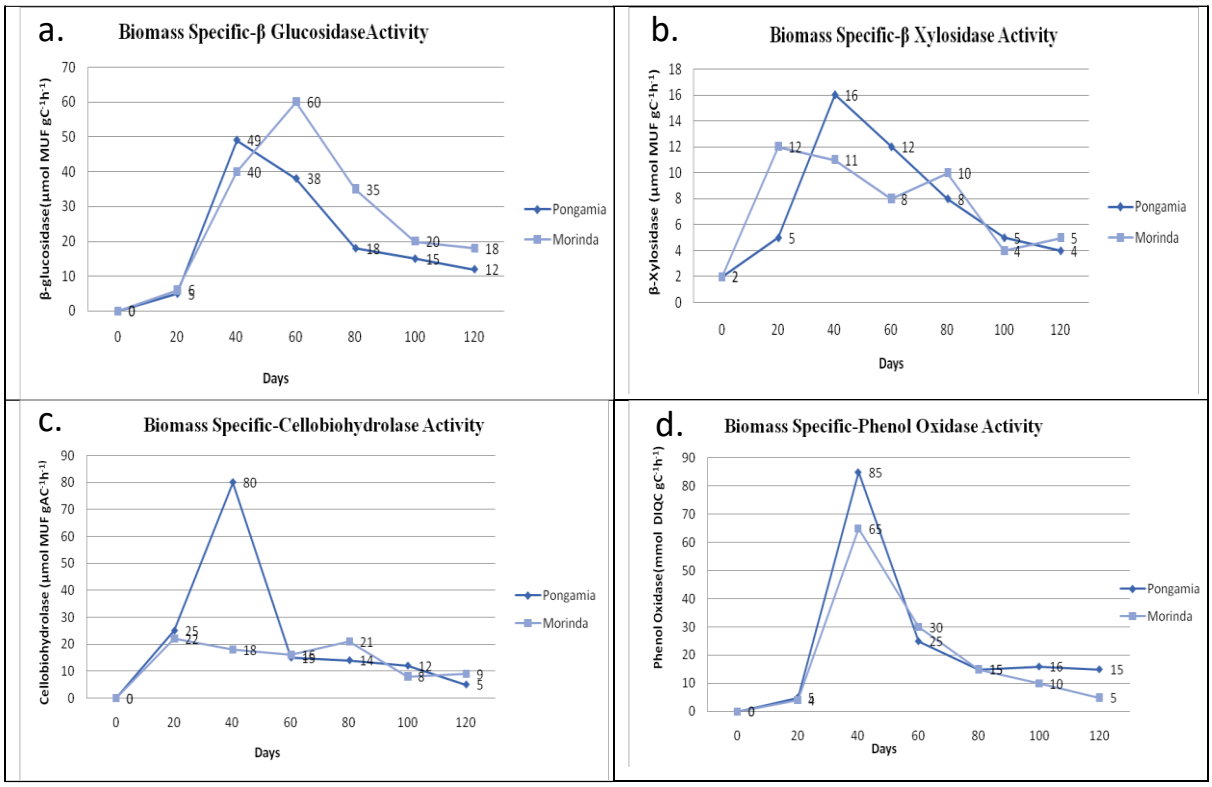


Figure 4. Biomass specific enzyme activities of (a) β-glucosidase, (b) β-xylosidase, (c) cellobiohydrolase and (d) Phenol oxidase on the two-leaf species during the experiment.



Sporulation rates greatly varied among the two-leaf species as shown in Table 3. It was studied that the fungal sporulation rates were higher in organic substrata than those of the inorganic substrata. P. pinnata leaves recorded the highest fungal sporulation rates. Finally, we could conclude that a prolonged enrichment experiment affected the inorganic substrata sporulation meanwhile addition of nutrients could not generate any effect of significance (Table 3).

Table 3. Analysis of variance considering the effect of different factors on sporulation rates.

Sources	Sporulation rates
Substrate (S)	F3.32 = 162.50
	P< 0.001
Long - term Enrichment (E)	F1.32 = 8.04
	P< 0.01
Short-term Nutrient Addition (N)	F1.32 = 0.18
	P=0.626
S x E	F3.32 = 7.06
	P< 0.0001

S x N	F3.32 = 1.20
	P=0.302
E x N	F1.32 = 0.64
	P=0.410
S x E x N	F3.32 = 6.08
	P< 0.005

Berg (1986) proposed that the decomposition of fresh leaves begins with the easily mineralized fractions of non-lignified carbohydrates, followed by the mineralization of more refractory fractions of lignified carbohydrates in later stages. CBH activity was high for the entire period in the Koraiyaru stream after leaf fall peak (October), but P and PO activity increased only after several weeks. This finding suggests that OM decomposition began with cellulose degradation and progressed to the degradation of lignin-related chemicals. In our earlier study, rDNA methods revealed a higher bacterial species richness. The degradation of organic materials followed a distinct temporal pattern, with notable changes in enzyme activity and ergosterol levels between substrata. Berg(1986) proposed that the decomposition of fresh leaves begins with the easily mineralized fractions of non-lignified carbohydrates, followed by the mineralization of more refractory fractions of lignified carbohydrates in later stages. CBH activity was high for the entire period in the Koraiyaru stream after the leaf fall peak (October), but P and PO activity increased only after several weeks.

Physico-chemical factors, such as discharge and dissolved inorganic nitrogen DIN, might, nevertheless, influence the enzymatic activity time-pattern. Several studies in forested streams in the autumn or early winter suggest that the majority of the nitrate in the catchment of the streams is mobilized(Gasith et al.,1997; Bernal et al., 2002) . The weathering of dissolved and particulate organic matter deposited on the soil after the dry period (summer) caused N content in stream water to be positively connected with lignocellulosic activities (P and PO). As a result, in a system where N may be a scarce resource, nitrate availability boosted fungal activity (Romani et al., 2004). The enzymatic activity of biofilms varied between species' leaves, which could be due to changes in leaf composition (C:N ratio, lignin content, polyphenol content, leaf durability)(Griffin, 1994). Lower enzymatic activity for the biofilm on P. pinnata was identified in previous research on leaf decomposition in soil and other aquatic habitats, and similar results were observed in P. pinnata species of our study. The high C:N ratio recorded in this substratum could be the reason for the material's low mineralization(Bernal et al.,2003).

Baldy et al.,(1995) supported our current work by demonstrating the role of bacteria in the late phases of the leaf breakdown process. Throughout the study period, estimates of ergosterol

concentration per stream reach demonstrated a progression of fungal biomass, which dropped only after the majority of the material had been treated. In the forested stream of Pachamalai, rDNA methods confirmed a higher bacterial species richness(Valarmathy et al.,2017). In terms of selection and colonization of streambed substrata during the fall, the fungi may be called facultative microorganisms. They take advantage of a new allochthonous CPOM that was introduced to the system in the fall. Our work is a clear evidence of the influence of microbiota on the stream in aquatic environment.

Conclusion

In conclusion, our study have given an insight into the microbiota associated with both the leaf species and their role in extracellular enzymatic activity in degrading. Also, out of the two species P. pinnata showed significant content of polysaccharides and lignin degradation activity than that of M. tinctoria. This plays a significant role in the stream biota and influences the ecosystem.

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