

NOTE

Enzymatic activities of epiphytic and benthic thraustochytrids involved in organic matter degradation

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ABSTRACT: Thraustochytrids are marine osmo-heterotrophic protists which have been isolated from different habitats and substrates. These organisms are typically encountered in association with refractory substrates, but the extent of their role in organic matter decomposition is still unknown. We isolated 11 thraustochytrid strains from different substrates and tested all species for their potential constitutive ecto- (cell-surface associated) and exo- (free released) enzymatic activities. Our results indicate that the investigated strains exhibited a wide spectrum of enzymes involved in the hydrolysis of all classes of organic compounds, suggesting that thraustochytrids are capable of degrading a large variety of substrates. The enzymatic pools were similar among all strains, and exhibited a good production of lipase, a selection of protease and a poor pool of carbohydrate degradation enzymes. However, different isolates displayed different spectra and intensities of enzymatic activities. The comparison of enzymatic activities of 2 thraustochytrid strains and the total enzymatic activities measured in their natural substrates suggested that thraustochytrids, although representing a minor fraction of the total benthic microbial biomass, are contributors to the degradation of highly refractory organic compounds.

KEY WORDS: Thraustochytrids · Enzymatic activities · Organic matter degradation · Marine sediments

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INTRODUCTION

The role of fungi and osmo-heterotrophic protists in benthic detrital food webs has received little attention to date (Benner et al. 1984, Wong et al. 1998, Raghukumar 2002). Thraustochytrids are osmo-heterotrophic protists, which are widely distributed in most marine habitats (from coastal to deep seas), reaching high densities in substrates characterised by a highly refractory composition (Raghukumar 2002). Thraustochytrids are particularly abundant in marine sediments typically characterised by the accumulation of large amounts of organic matter recalcitrant to enzymatic decomposition (Santangelo et al. 2000, Bongiorno & Dini 2002) and have also been observed to grow opportunistically in sediments characterised by fish-farm biodeposition (Bongiorno et al. 2005).

Thraustochytrids generally represent a negligible fraction of microbial abundance and a minor fraction of the total benthic microbial biomass (Bongiorno & Dini 2002, Bongiorno et al. 2005). However, their role in the degradation of different substrates and mineralisation of organic matter has been hypothesised as significant, with regard to their ability to pervade various solid substrates (Raghukumar 2002). At present, the only available information on their enzymatic activities deals with a few species isolated from the water column, mangrove leaves, brown algae and faecal pellets for a limited number of enzymes (Bahnweg 1979a,b, Raghukumar et al. 1994, Sharma et al. 1994, Bremer & Talbot 1995, Raghukumar & Raghukumar 1999).

In the present study, the enzymatic profiles of 11 strains isolated from different marine substrates were investigated under fixed culture conditions. We then

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measured the exo- (free released) and ecto- (cell-surface associated) enzymatic activities of a subset of enzymes produced by all strains, to quantify the potential capability of thraustochytrids in degrading attached and free substrates. Additional measurements of total enzymatic activities in origin sediments of 2 of the 11 cultured strains were also carried out in order to provide a first estimate of the potential thraustochytrid contribution to organic matter degradation in marine sediments.

MATERIALS AND METHODS

Strain isolation and culture. Thraustochytrids were isolated from a variety of substrates (see Table 1 for details). After accurate washing with sterile artificial seawater, aliquots of the substrate and/or sediments were plated on Modified Vishniac's Medium (0.1% glucose, 0.01% yeast extract, 0.01% peptone, 0.1% gelatine hydrolysate, 1.2% agar in seawater, pH 7.0) or baited with sterile pine pollen (Raghukumar 2002). Strains were incubated at $20 \pm 1^\circ\text{C}$ until the appearance of colonies (within 3 to 7 d). Cells were then transferred to fresh agar plates or pine pollen suspensions, and supplemented with antibiotics (1 mg ml⁻¹ of penicillin G and streptomycin sulphate) to suppress bacterial growth.

Enzymatic profiles. We grew 11 axenic strains in the liquid medium (see 'Strain isolation and culture' above

for description) under continuous gentle shaking (150 rpm), till the late exponential growth phase was reached (ca. 96 h). Bacterial contamination was checked by epifluorescence microscopy after DAPI staining (Porter & Feig 1980). Cells were harvested by centrifugation ($1000 \times g$), washed with sterile artificial seawater and then resuspended to be tested for enzymatic activities. First, all strains were tested for the presence/absence of 19 different enzymes using the semi-quantitative API-ZYM system (Biomérieux; Tiquia 2002). Small sub-aliquots (65 μl), corresponding to a cell density varying between 2.0 and 4.0×10^5 cells ml⁻¹, were inoculated in the API-ZYM strip wells. Wells were checked under a stereomicroscope to ensure the presence of cells and then incubated overnight at $20 \pm 1^\circ\text{C}$. Presence/absence of the different enzymes and intensity of each reaction were detected according to a colorimetric scale (Tiquia 2002).

Enzymatic activities. Ecto- and exo-enzymatic activities of isolated strains were quantified fluorometrically by the cleavage of fluorogenic substrates for L-aminopeptidase, β -D-glucosidase, β -D-galactosidase, alkaline-phosphatase, and lipase (Hoppe 1993). Saturating substrate concentrations were determined using increasing concentrations of L-leucine-4-methylcoumarinyl-7-amide, 4-methylumbelliferone β -D-glucopyranoside, 4-methylumbelliferone β -D-galactoside, 4-methylumbelliferone phosphate (20, 50, 100, 200 and 400 μM) and 4-methylumbelliferone stearate (8, 20, 40,

Table 1. Environments and substrates from which thraustochytrids were isolated

Strain	Habitat	Date	Location	Forms on pollen
MAN2	Senescent mangrove leaves (<i>Bruguiera</i> sp.)	Apr 2004	Bunaken, north Sulawesi (Indonesia)	Clustered cells
MAN6B	Senescent mangrove leaves (<i>Bruguiera</i> sp.)	Apr 2004	Bunaken, north Sulawesi (Indonesia)	Clustered cells
FAN5	Senescent seagrass leaves (<i>Enhalus acoroides</i>)	Apr 2004	Bunaken, north Sulawesi (Indonesia)	Clustered cells
ULV B	Green algae <i>Ulva rigida</i>	May 2004	Palombina Beach, Ancona (Italy)	Single round cells
ULV R	Green algae <i>Ulva rigida</i>	May 2004	Palombina Beach, Ancona (Italy)	Single round cells, pink pigmentation
CR2	Dead crab exoskeleton	May 2004	Palombina Beach, Ancona (Italy)	Single round cells
GR2-1	Dead crab exoskeleton	May 2004	Palombina Beach, Ancona (Italy)	Single round cells
Sar3	Sand	Jan 2003	Poetto Beach, Sardinia (Italy)	Clustered cells
GORO2	Mud	May 2004	Goro Lagoon, mussel farm (Italy)	Single round cells
PAL	Sand	Feb 2004	Palombina Beach, Ancona (Italy)	Single round cells
POR	Sand–mud	Feb 2004	Ancona Port (Italy)	Single round cells

Chitin occurs commonly as an exo- and endoskeletal material in many marine organisms, representing a dominant fraction of particulate carbon and nitrogen in the marine environment (Gooday 1990, Place 1996). This enzyme was detected in 8 of the 11 strains, indicating that thraustochytrids can play an important role in the process of degradation of this biopolymer. The present study is also the first to reveal the thraustochytrids' ability to degrade lipids and organic P. In fact, all screened strains exhibited lipase, alkaline phosphatase and acid phosphatase activities. The isolated strains displayed a certain selection in production of peptidases. Among peptide-degrading enzymes, leucine- and valine-arylamidase were present in almost all strains, whereas α -chymotrypsin and trypsin were observed in only 2 and 5 of the 11 strains, respectively. Carbohydrate degrading enzymes were poorly represented and, among these, only β -D-galactosidase and β -D-glucosidase were present in all the screened strains, while α -fucosidase was not detected at all.

The cluster analysis revealed that the enzymatic pools of thraustochytrids were rather homogeneous, with a similarity close to 85% for most strains (Fig. 1). Therefore, all tested thraustochytrid species had the potential to degrade a wide variety of organic substrates, confirming that thraustochytrids play a role in the decomposition of organic detritus. In this regard, it is worth noting that the production of enzymes has occasionally been reported for thraustochytrids (Bahnweg 1979a,b, Raghukumar et al. 1994, Sharma et al. 1994, Bremer & Talbot 1995, Raghukumar & Raghukumar 1999) and their role in the decomposition of organic matter has been postulated in both benthic and pelagic environments (Kimura et al. 2001, Bongiorno & Dini 2002, Bongiorno et al. 2005).

Ecto- and exo-enzymatic activities of β -D-glucosidase, L-aminopetidase, β -D-galactosidase, lipase and

alkaline phosphatase, expressed under fixed culturing conditions, are reported in Table 3. Density of cultured thraustochytrids ranged between 1.6 and 36.6×10^5 cells ml^{-1} . Thraustochytrid strains exhibited very high ecto-enzymatic activities per cell, particularly for the enzymes alkaline phosphatase and aminopeptidase, which displayed the highest activities in all strains. This result suggests that thraustochytrids are implicated in N and P cycling.

Different strains displayed different activities for the investigated enzymes. For example, ecto- β -D-glucosidase and lipase activities per cell were highest in strains isolated from the algae *Ulva rigida*, and from tropical seagrass leaves (HSD-Tukey, $p < 0.05$). These strains also exhibited the highest values of ecto- β -D-galactosidase (HSD-Tukey, $p < 0.05$). The relationship between enzymatic activity and substrate availability may be very close (Misic & Fabiano 2005), so our results would support the hypothesis that thraustochytrid enzymatic pools are related to natural organic substrates. However, in the present study, all thraustochytrids were grown on the same medium, so the enzymes expressed by the tested strains should be regarded as constitutive rather than inducible. Therefore, our hypothesis needs further study and should only be considered with caution.

Exo-enzymatic activities of aminopeptidase accounted, on average, for more than 30% (range: 4 to 69%) of the total enzymatic activity (measured as ecto-plus exo-enzymatic activities) of tested thraustochytrids, whereas the exo-enzymatic activity of β -D-glucosidase and alkaline phosphatase represented on average 49% (range: 0 to 88%) and 52% (range: 23 to 67%) of their total pools, respectively. Exo-enzymatic activities of β -D-galactosidase and lipase represented, on average, 61% (range: 0 to 89%) and 64% (range: 0 to 93%), respectively, of the total activities.

These results led us to hypothesise that the exo-enzymatic activities produced by thraustochytrids may contribute to the total extracellular enzymatic activities of marine sediment, with possible effects on organic matter cycling in benthic environments, and to the release of low molecular weight compounds available for thraustochytrids and other benthic heterotrophic microorganisms (Vetter & Deming 1999).

As expected from differences in cell size, the results presented here indicate that the ecto-enzymatic activity per thraustochytrid cell was as much as 2 to 3 orders of magnitude higher than the activity per cell of marine bacteria reported by Martinez & Azam (1993) and Martinez et al. (1996). However, assuming a mean bacterial biomass of 5×10^{-8} $\mu\text{g C cell}^{-1}$, and the thraustochytrid biomass (range 2.0 to 6.9×10^{-5} $\mu\text{g C cell}^{-1}$) estimated from cell size measurements, the enzymatic activities per unit of biomass of bacteria were up to 18

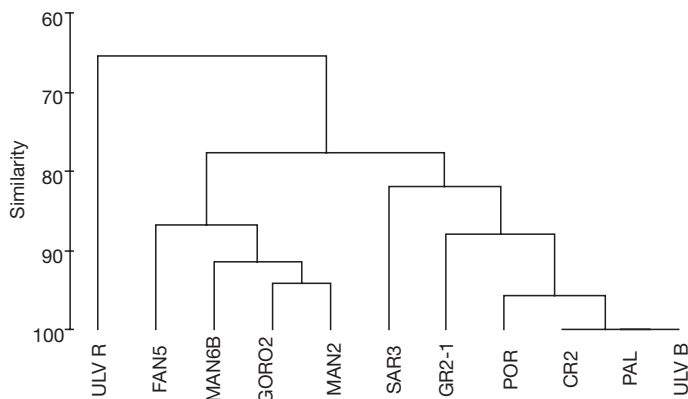


Fig. 1. Cluster analysis of similarities among the 11 thraustochytrid strains based on presence/absence matrix of the 19 tested enzymes

Table 3. Average ecto- and exo-enzymatic activities ($\text{nmol ml}^{-1} \text{h}^{-1}$) of the 11 thraustochytrid strains ($\pm \text{SD}$). nd: not determined; ecto-enzymatic activities are also expressed as $\text{fmol cell}^{-1} \text{h}^{-1}$ (values within parentheses)

Strain	β -D-glucosidase		β -D-galactosidase		Lipase		Alkaline phosphatase		L-aminopetidase	
	Ecto	Exo	Ecto	Exo	Ecto	Exo	Ecto	Exo	Ecto	Exo
GORO 2	2.0 \pm 0.9 (0.7 \pm 0.3)	0.0 \pm 0.0	19.8 \pm 1.8 (0.1 \pm 0.1)	0.0 \pm 0.0	2.3 \pm 1.0 (0.8 \pm 0.0)	0.0 \pm 0.0	57.5 \pm 48.8 (19.8 \pm 17.7)	68.1 \pm 0.8	0.4 \pm 0.4 (6.6 \pm 1.1)	9.4 \pm 0.0
PAL	3.5 \pm 2.0 (13.0 \pm 2.0)	25.8 \pm 4.0	46.9 \pm 23.3 (10.0 \pm 7.0)	5.6 \pm 3.4	20.4 \pm 9.9 (52.8 \pm 28.6)	258.1 \pm 100.4	52.9 \pm 3.2 (49.2 \pm 29.7)	82.7 \pm 5.0	4.3 \pm 0.5 (147.8 \pm 19.3)	12.3 \pm 4.0
POR	7.4 \pm 0.6 (3.6 \pm 1.0)	5.4 \pm 2.9	44.0 \pm 30.7 (8.0 \pm 4.4)	14.9 \pm 16.7	36.9 \pm 30.0 (39.5 \pm 24.1)	125.6 \pm 97.8	340.0 \pm 16.3 (488.4 \pm 334.0)	368.1 \pm 396.7	4.0 \pm 1.3 (45.7 \pm 5.0)	2.6 \pm 0.7
SAR3	4.8 \pm 3.5 (6.0 \pm 1.4)	7.1 \pm 0.0	45.1 \pm 17.3 (2.4 \pm 0.1)	2.1 \pm 0.0	17.7 \pm 22.2 (5.3 \pm 1.3)	nd	255.4 \pm 24.3 (372.5 \pm 344.3)	478.0 \pm 92.6	5.3 \pm 2.3 (25.8 \pm 12.6)	101.8 \pm 30.9
MAN2	2.9 \pm 1.6 (1.0 \pm 0.4)	0.1 \pm 0.1	48.4 \pm 22.9 (1.2 \pm 1.5)	0.2 \pm 0.2	15.2 \pm 4.0 (5.6 \pm 1.8)	4.0 \pm 0.7	115.1 \pm 95.1 (58.5 \pm 3.7)	201.7 \pm 1.9	0.2 \pm 0.2 (48.7 \pm 21.4)	16.3 \pm 3.2
MAN6B	3.7 \pm 2.3 (3.5 \pm 1.5)	1.6 \pm 2.0	45.8 \pm 6.9 (4.0 \pm 4.0)	5.4 \pm 6.4	1.2 \pm 0.3 (0.9 \pm 0.3)	0.8 \pm 0.1	257.4 \pm 48.3 (180.3 \pm 62.1)	75.5 \pm 6.8	5.3 \pm 8.1 (35.2 \pm 15.1)	2.0 \pm 0.9
FAN5	10.7 \pm 10.0 (27.7 \pm 19.7)	49.6 \pm 13.5	47.3 \pm 10.1 (20.7 \pm 5.4)	12.4 \pm 0.1	26.9 \pm 26.2 (55.5 \pm 42.9)	283.1 \pm 304.5	297.6 \pm 56.9 (591.7 \pm 150.0)	402.3 \pm 289.5	5.9 \pm 4.6 (112.6 \pm 49.0)	19.3 \pm 23.0
ULV B	12.7 \pm 5.5 (17.4 \pm 20.6)	1.7 \pm 0.6	17.5 \pm 2.7 (2.4 \pm 1.6)	6.7 \pm 9.1	1.9 \pm 1.0 (0.8 \pm 0.5)	17.6 \pm 3.3	229.9 \pm 64.3 (293.8 \pm 331.9)	193.1 \pm 69.0	3.0 \pm 1.5 (6.8 \pm 2.0)	2.4 \pm 0.7
ULV R	5.6 \pm 1.0 (23.4 \pm 16.1)	17.1 \pm 17.2	51.3 \pm 8.9 (0.6 \pm 0.3)	7.6 \pm 4.6	35.5 \pm 9.0 (158.2 \pm 95.2)	44.5 \pm 27.8	190.8 \pm 81.7 (148.0 \pm 45.7)	383.6 \pm 264.6	1.0 \pm 0.5 (260.3 \pm 173.9)	22.4 \pm 18.8
CR2	3.9 \pm 1.2 (1.9 \pm 0.7)	9.2 \pm 3.2	20.8 \pm 5.9 (9.4 \pm 11.8)	30.7 \pm 26.6	35.1 \pm 36.6 (22.3 \pm 29.5)	160.8 \pm 2.3	52.2 \pm 19.1 (30.5 \pm 11.6)	63.5 \pm 6.4	4.6 \pm 4.6 (8.9 \pm 4.8)	43.7 \pm 22.3
GR2-1	4.7 \pm 2.5 (5.5 \pm 1.6)	13.6 \pm 4.7	17.3 \pm 5.0 (3.4 \pm 0.7)	5.2 \pm 1.2	20.6 \pm 4.6 (37.4 \pm 8.3)	122.0 \pm 135.7	49.8 \pm 19.7 (73.0 \pm 27.1)	24.53 \pm 8.1	1.3 \pm 0.7 (31.4 \pm 19.2)	11.7 \pm 3.0

times higher than those of thraustochytrids.

Additional results were obtained from sediments collected in the Adriatic Sea, on which we carried out the thraustochytrid counting and the fluorimetric measurements of total enzymatic activities. In these sediments thraustochytrid abundance and biomass ranging from 6.7 to 7.4×10^3 cells g^{-1} and from 29.4 to 32.5 ng C g^{-1} , respectively, were similar to those reported elsewhere in coastal marine sediments (Bongiorni et al. 2005). Based on the activities per cell measured on the strains isolated from these sediments, we estimated the thraustochytrid contribution to the total enzymatic activity for β -D-glucosidase, aminopeptidase and alkaline phosphatase. Our results revealed that thraustochytrids may contribute up to 4, 1 and 1.4% of the total β -D-glucosidase, aminopeptidase and alkaline phosphatase activities in the sediment, respectively (Table 4). Although these values are low, it is likely that the isolated culturable strain represents a minor fraction of the entire thraustochytrid community. This leads us to the hypothesis that thraustochytrids might actually contribute to organic matter degradation processes in marine sediments.

Thraustochytrids have been repeatedly isolated from senescent seaweed, seagrass and mangrove leaves (Sharma et al. 1994, Bremer & Talbot 1995, Raghukumar et al. 1995). Bongiorni et al. (2005) found higher abundance and biomass of thraustochytrids in sediments characterised by the presence of the Mediterranean seagrass *Posidonia oceanica* than in surrounding unvegetated sediments, and very high biomass values in sediments impacted by fish-farm biodeposition. In marine sediments characterised by the presence of seagrasses, structural carbohydrates represent the dominant biochemical component (Lawrence et al. 1989, Pusceddu et al. 2003). Accordingly, our investigation revealed that the highest levels of β -D-glucosidase and β -D-galactosidase activities were measured in strains isolated from macroalgae and tropical seagrasses, further confirming the potential of thraustochytrids to degrade refractory organic compounds

Table 4. Contribution ($\text{nmol g}^{-1} \text{h}^{-1}$) of total β -D-glucosidase, L-aminopeptidase and alkaline phosphatase activities of thraustochytrids to total enzymatic activities in the sediments of Palombina Beach and in the sediments of the Ancona Port. nd: not determined

	β -D-glucosidase		L-aminopeptidase		Alkaline phosphatase	
	Palombina Beach	Ancona Port	Palombina Beach	Ancona Port	Palombina Beach	Ancona Port
Thraustochytrids	0.05	0.73	0.36	1.25	nd	7.54
Sediments	18	31.4	400	35.1	nd	526.4
Contribution (%)	4	0.1	1.0	0.3	nd	1.4

(Raghukumar & Shaumann 1993, Raghukumar & Raghukumar 1999).

The results of the present study open new perspectives on patterns of organic matter degradation processes in marine sediments. Further studies are required to better assess the quantitative role of thraustochytrids in this key step of the benthic microbial food chain.

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