

**CELLULOLYTIC ENZYMES PRODUCED BY
AEROBIC FUNGI ISOLATED FROM THE GUT
OF *PANAQUE MACCUS*.**

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EXTENDED ABSTRACT ONLY-DO NOT CITE

The family Loricariidae is restricted to the Neotropics, yet contains at least 70 genera with more than 600 described species (Isbrücker 1980) and many additional undescribed species. An evolutionary success of this magnitude in such a restricted geographic region demands an explanation, however the biology of the loricariids is virtually. This radiation of the Loricariidae occurred almost entirely at the trophic level of primary consumer and entirely since the Cretaceous separation of South America from Africa, *circa* 80 million years ago. Novel arrangements of the jaw musculature and new and variable tooth morphology are thought to have contributed to the unprecedented evolutionary success of the loricariids (Schaefer and Lauder 1986; Schaefer and Stewart 1993), but the importance of other biological factors to this radiation are unknown. Besides being mostly herbivorous, some loricariids may be specialists for terrestrial plant material that falls into the water (allochthonous carbon). The few loricariids that have been analyzed derive most of their carbon from allochthonous sources (Araujo-Lima *et al.* 1986). One genus of loricariid catfish, *Panaque*, even has representatives that have been shown from field collections and laboratory experiments to be utilizing wood in their diet. Seven species of *Panaque* have been found to consume wood in their native habitats and two species will consume, extract energy from, and grow on a wood-only diet in the laboratory (Schaefer and Stewart 1993; Nelson *et al.* 1999). This is the first and

only suggestion of wood-eating in bony fishes (class Osteichthyes), a taxonomic grouping of more than 25,000 species.

The purpose of the present work was to see whether enzymes involved in the break down of complex carbon polymers found in wood are made by several species of fungi recently found in *Panaque* guts.

Methods

Specimens of *Panaque maccus* (Schaefer & Stewart, 1993) were obtained from aquarium wholesalers as they arrived from South America and were either sacrificed immediately or held in untreated water in aquaria well inoculated with the water they were transported in. These steps were taken to maximize the chance that the fungi we were working with were actually native gut flora.

Microbial cultures

Animals were killed by an overdose of the anaesthetic 3-amino benzoic acid ethyl ester (MS-222). Gastrointestinal tracts were isolated under sterile conditions, divided into sections and inoculated into broth cultures. Aerobic isolations took place on a sterile bench top while anaerobic cultures were started in a sterile, anaerobic glove box (Coy Laboratories) under an atmosphere of 10% hydrogen, 10% carbon dioxide, and 80% nitrogen. The methods of Wubah *et al.*, (1991) were used to start fungal cultures. Briefly, an antibiotic cocktail solution (0.1 ml per tube) containing streptomycin sulfate (20 µg/ml), penicillin G (80 µg/ml), chloramphenicol (30 µg/ml) and oxytetracycline (15 µg/ml) were added to each Hungate tube to inhibit bacterial growth. After 48 h, the tubes were checked for fungal growth. When fungal thalli and zoospores were observed, 0.5 ml. of the culture supernatant was inoculated onto a 100 x 15 mm Petri plate and covered with BAM containing antibiotics and 2% agar. Further purification was carried out until axenic cultures were obtained.

Enzyme assays

Broth cultures were dialyzed against 0.1M citrate buffer (pH 5.0) containing 0.01% sodium azide prior to being assayed. Carbon polymer degradative capacity was measured with two broad types of enzyme assay:

1) various glycosidase activities *e.g.* β-glucosidase (β-D-glucoside glucohydrolase E.C.3.2.1.21) and β-xylosidase (β-D-xylanoxylhydrolase

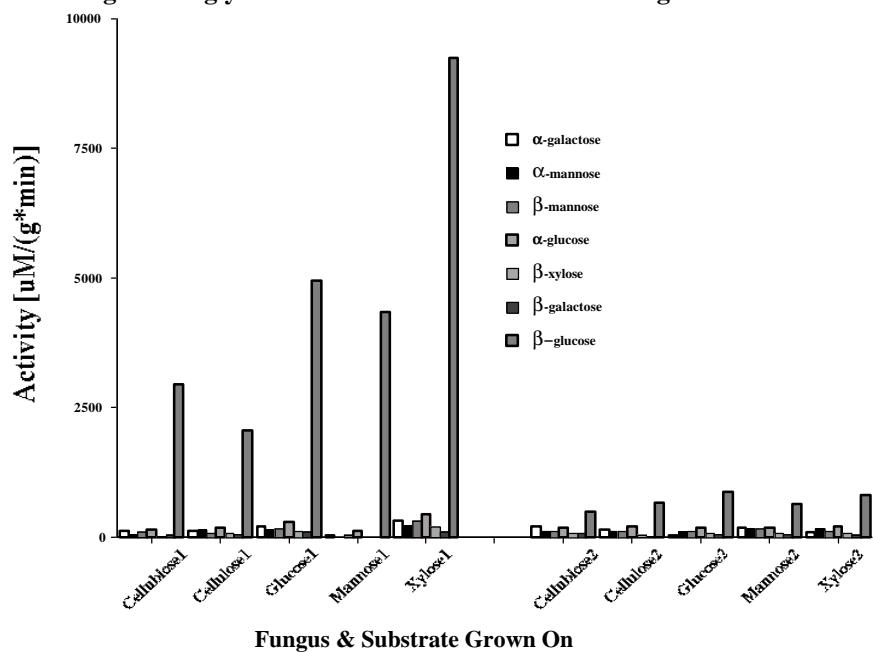
E.C.3.2.1.37) etc. were assayed using a variation Agrawal & Bahl (1968). The appropriate *p*-nitrophenyl conjugated substrate (Sigma® 0.5g•cm⁻³ in 0.1M citrate buffer pH 5.0) was incubated with diluted gut contents (volume fraction 0.33) for 2 hours at 40°C. After terminating the reaction with Na₂CO₃, the liberated *p*-nitrophenol was quantified by recording optical density (OD) in excess of a heat-inactivated control at 425nm. Concentrations were calculated by referencing the OD to a standard curve prepared with pure *p*-nitrophenol (Sigma®)

2) β-endoglucanase (endo 1,4,-D-glucan 4-glucanohydrolase(E.C.3.2.1.4) activity was assayed by incubating diluted gut contents with either a 1% solution of carboxymethylcellulose in 0.1M pH 5.0 citrate buffer, or a suspension of 20μ crystalline cellulose (Sigmacell 20) in the same buffer, all to a final sodium azide concentration of 0.01% to prevent bacterial activity during the assay. Hemicellulolytic activity was assayed analogously, except that mannan and xylan were the substrates. Reducing sugar groups were assayed after 24 hours of incubation at 40 C, using a modification of the Somogyi method (Nelson, 1944). Optical density at 500nm, in excess of a control blank which had gut contents added after the 24 h incubation, was measured and recorded on a Zeiss™ PM6 spectrophotometer. Concentrations were calculated by reference to a standard curve prepared with glucose. All activities are expressed per gram of soluble protein as determined by the bicinchoninic acid method (Pierce®).

Results and Discussion

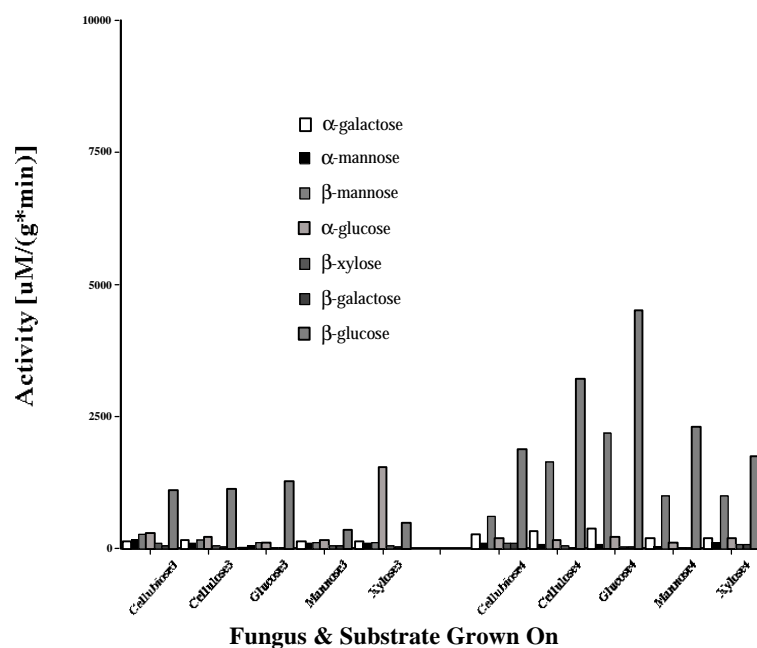
We were able to develop pure cultures of four separate fungi from the gastrointestinal tracts of *Panaque maccus*. Two of the pure fungal cultures (1 & 4) exhibited substantial hydrolytic activity against the β-glycosidic linkages connecting the monomers of cellulose (β-glucosidase activity) and, to a lesser extent, the various disaccharide linkages found in hemicellulose (Fig. 1).

Figure 1a: glycosidase activities of unidentified fungi # 1 and 2



The other two pure fungal cultures (2 & 3) had some activity against β -glycosidic linkages and one culture of fungus # 3 grown on xylan had substantial α -glucosidase activity (Fig. 1b). Interestingly, except for this one culture, substantial α -glycosidic activity, like that common to vertebrate intestines, was virtually absent from these fungal cultures (Fig. 1). Extracellular enzyme activity was relatively independent of the media upon which the fungi grew.

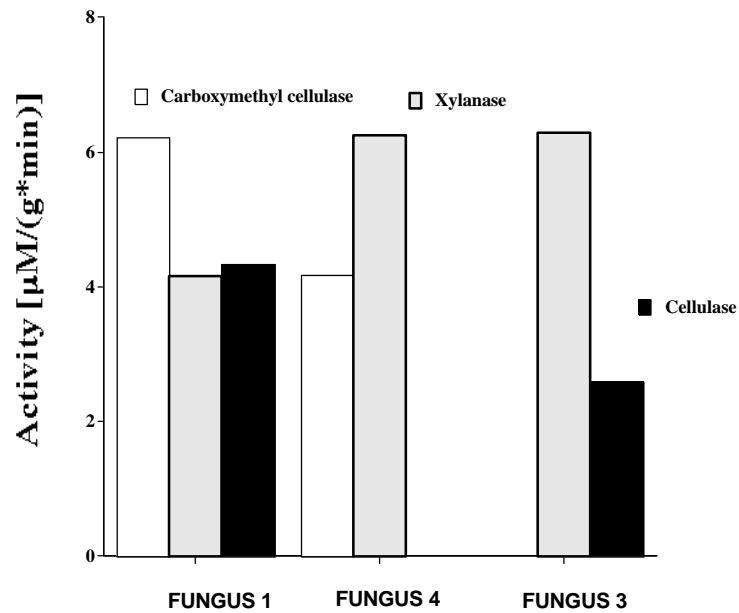
Figure 1b: glycosidase activities of unidentified fungi # 3 and #4



There are several possible explanations for why these fungi only made extracellular enzymes directed against the more difficult to break β -glycosidic linkages and not the easier α -glycosidic ones. The simplest is that these fungi are descended from wood-decomposition specialists that stopped producing α -glycosidases because of the paucity of α -linkages in the carbon polymers of wood. A second possibility is that because there are bacteria present in *Panaque* guts that produce α -glycosidases (Nelson *et al.* 1999), and because the fish undoubtedly also produce α -glycosidases, the fungi are merely "avoiding" the waste of energy inherent in competing for these resources. A third possibility is that the fungi are quite capable of producing α -glycosidases, but our culture conditions did not induce them to do so. The production of α -glucosidase by fungus #3 when grown on xylan supports this latter interpretation.

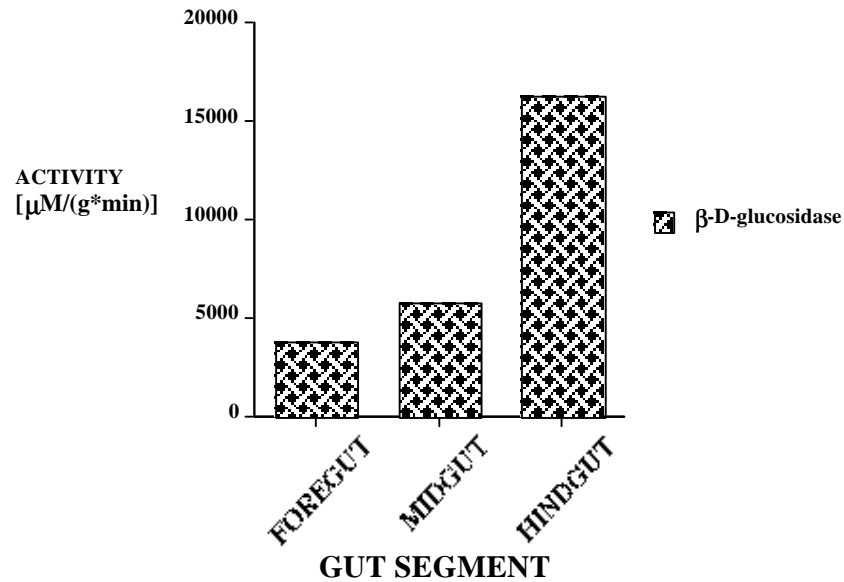
Three of the fungal cultures had substantially lower, but still measurable, enzymatic activity in their gut contents against the larger carbon polymers that were tested (Fig. 2). Only positive results are shown. There was insufficient material to run this assay based upon the substrate that the fungi grew on.

Figure 2: Endoglucanase activities of fungal cultures



There appears to be some localization of these fungi within the gastrointestinal tracts of *Panaque*. For instance, for two separate fish, the initial cultures were begun from approximate "one thirds" of the GI tract. The extracts from these initial cultures of both fish showed a trend towards increasing β -glucosidase activity as one proceeded through the GI tract (*e.g.* Figure 3). Again, one interpretation of these results is that the fungi aren't competing with the animal or the bacteria for the "easy energy" to be obtained in the foregut; instead the fungi are focusing on the more difficult to obtain carbon as it appears in later gut segments. Confirmation of this idea awaits more thorough experimentation.

Figure 3 β D-glucosidase activity from cultures of different gut segments



Cultures of fungi have also been analyzed for biochemical growth characteristics, colony morphology and spore morphology. To date, we have been unable to identify them as any described species. Interestingly, none of the four fungal species were found on the surface of the animal. Light micrographs of the four fungi will be presented.

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