Polysaccharide-degrading enzymes from the marine protists, thraustochytrids

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ABSTRACT

Thraustochytrids form a unique group of ubiquitous marine protists of profound ecological importance. This study dwells on their multiple polysaccharide-degrading activities. Isolates from mangrove and coastal ecosystems of Goa, India were studied for the production of eight different extracellular polysaccharide-degrading enzymes. Significant agarase and amylase activities were observed by the standard plate clearing assays. Agarase activity in thraustochytrids has not been reported to date and this study is therefore the first of its kind. The production of agarase, amylase and cellulase was also monitored by growing the isolates in the presence of different carbon sources. Significantly, the enzyme production appeared to be constitutive in most cases. In view of the fact that reports on quantitative estimation of enzyme activities from thraustochytrids (whether polysaccharide-degrading or otherwise) are scarce, investigations to this end have also been carried out and specific activities of the extracellularly produced carboxymethyl cellulase, xylanase and pectinase enzymes recorded.

Keywords: Thraustochytrid enzymes, marine protists, polysaccharide-degrading activity, agarase, cellulase, xylanase, pectinase

INTRODUCTION

Thraustochytrids, a salient group of marine protists, have been in the limelight owing to their remarkable ability to produce high levels of ω-3-polyunsaturated fatty acids, docosahexaenoic acid, astaxanthin, carotenoids and other lipids of pronounced biotechnological importance [1]. They are characterized by the presence of ectoplasmic net (EN) elements which spread over as extensive networks. The EN is thought to contain hydrolytic enzymes that are surface bound or secreted into the surrounding medium, helping in digestion of food [2]. The saprophytic mode of nutrition of thraustochytrids confers upon them the ecological role of marine detritus degraders. Thraustochytrids also produce exopolysaccharide (EPS). One of the many functions of these extensive matrices of EPS is said to be in concentration of nutrients and in helping to localize and maintain exoenzyme activity [3]. Thraustochytrids are believed to contribute to the degradation of highly refractory organic compounds [4] and are said to probably overcome competition with bacteria by producing several unique degradative enzymes [1].

Thraustochytrids secrete a wide variety of extracellular enzymes *in vitro* [5] including a few polysaccharases. It still however remains to be ascertained whether and how exactly these enzymes could be used in industrial processes. None of the studies undertaken to date has identified their potential in any detail on the magnitude or mechanism of enzyme action, except for the report by Bremer et al. on cellulases [6] and a more recent one from our laboratory on lipases [7]. While enzymes continue to find use in most if not all industries, the mounting market value for polysaccharide-degrading enzymes is of relevance in the present context. The demand for novel enzymes with more practical usability in industrial processes (due to superior properties such as

high specific activities, tolerance to harsh conditions of pH and temperature or resistance to catabolite repression) only increases by the day. In view of these, it was thought appropriate to conduct a study on polysaccharases from the thraustochytrids, a ubiquitous group of marine protists. Such studies would add to the knowledge of marine enzymes, especially their mode of production and action in the oceanic water columns. It would also help to understand the role of degradative enzyme production in the nutrition and survival strategies of thraustochytrids [5].

This study dwells on the broad spectrum of polysaccharide degrading enzymes produced by thraustochytrids, many of which have not so far been reported to be produced by this group of organisms. It also suggests the constitutive nature of enzyme production in the isolates obtained and the requirement of minimum amounts of glucose for cellulase and xylanase production. The study also gives a comparative analysis of enzyme activities for industrially important enzymes such as cellulases, xylanases and pectinases from these organisms.

MATERIALS AND METHODS

Sample collection

Samples were collected during low tide from the beaches at Anjuna, Vagator, Bambolim, Dona Paula and the mangrove ecosystems at Marcela, Chorao, Divar, Kamurli, Cumbharjua, Banastarim and Ribandar of Goa, India. Decaying mangrove leaf, sediment and water samples from these coastal and mangrove habitats were collected in sterile glass vials. Physico-chemical parameters of the environment such as pH and salinity were recorded each time.

Isolation of thraustochytrids

Isolation of thraustochytrids was attempted by two methods, *viz.*, direct plating [4] and pine pollen baiting methods [2]. Pine pollen baiting method was carried out by inoculating the samples into vials containing sterile sea water supplemented with antibiotics (streptomycin and penicillin) followed by dusting with small amounts of pine pollen and incubation at room temperature. After 3-5 days, the vials were examined under an inverted microscope and samples found to harbor thraustochytrids were streaked onto Modified Vishniac's (MV) agar medium plates (0.15% peptone, 0.1% yeast extract, 0.4% glucose and 0.8% agar). Ensuing colonies of thraustochytrids were purified by repeated transfer on plates of the same medium. In the direct plating method the samples were rinsed with sterile sea water and spot-plated onto MV agar medium fortified with antibiotics. In case of water samples, a minimum amount (0.1 ml) was spread-plated on MV agar plates. The plates were routinely examined under an inverted microscope for growth of thraustochytrids. The isolates thus obtained were maintained and stored at 4°C as stabs in MV medium.

Screening for different polysaccharide-degrading activities

Initial screening was carried out by qualitative plate assays in order to check for the presence of eight polysaccharide-degrading activities, *viz.*, those of xylanases, cellulases, amylases, pectinases, carrageenases, chitinases, alginate lyase and agarases. The qualitative assays were carried out by spot inoculating the culture onto the prepared assay plates containing 0.5% of the respective substrate and flooding with a suitable dye after 5 days of incubation at 28°C. Formation of a zone of clearance around the spotted culture indicated the production of the respective enzyme. For the detection of cellulases, xylanases and chitinases, the plates were flooded with Congo Red dye (0.1%) for 30 min followed by washing twice with 1M NaCl for 5 min each [8]. Agarase and amylase activities were detected by flooding the assay plates with Lugol's Iodine [9]. Cetyl

pyridinium chloride (0.5% w/v) was used for the detection of carrageenase [10]. Pectinase activity was detected by flooding the plates with conc. HCl for 5 min while alginate lyase activity was visualized by the use of 0.5M H_2SO_4.

Quantification of enzyme activities

The isolates were grown for 5 days in MV broth medium and then centrifuged at 10,000 rpm for 30 min at 4°C. The culture supernatant thus obtained was stored at -20°C for use as the source of crude enzyme for all the enzymatic studies. Quantitation of agarase, cellulase, xylanase [11,12] and pectinase [13] activities was carried out at 50°C at pH 7. One unit of cellulase, xylanase, pectinase, or agarase activity was defined as the amount of enzyme that released, respectively, 1µmole of glucose, xylose, D-galacturonic acid or D-galactose equivalents per min.

Effect of different carbon sources on enzyme production

The requirement of glucose (in the growth medium) for eliciting different polysaccharide-degrading activities was ascertained by spot inoculating the culture on the substrate-containing agar plates with/without 0.4% glucose. The presence of a zone of clearance was used as a determinant of the respective enzyme activities. The culture supernatants obtained after growing the isolates in MV broth medium with or without 0.4% glucose were used for quantitative estimation of cellulase and xylanase activities. The effect of different carbon sources on the production of three enzymes, *viz.*, cellulase, amylase and agarase was analyzed by the agar cup diffusion plate assay, using the culture supernatant obtained after growing the isolates in the presence of cellobiose, sucrose, glycerol, agarose, maltose, lactose, sorbitol, mannose or oat spelts xylan.

Induction by substrate

All of the 8 different polysaccharide-degrading activities were analyzed after growing the isolates in the presence or absence of the respective polysaccharide substrates (0.5%) in MV medium. The enzyme activities in the culture supernatants were monitored by the agar cup diffusion plate assay at 37°C at pH 7. Quantitation of cellulase and xylanase activities was also carried using the culture supernatant obtained after growing the isolate in the presence or absence of 0.5% substrate, *viz.*, CMC and oat spelts xylan, respectively.

Effect of harvesting time on cellulase activity

Culture supernatants obtained after 2, 5 and 6 days of growth in MV medium at 28°C were assayed qualitatively to determine the optimum time for harvesting the cells so as to favor maximum enzyme production. These extracts were tested for zone of clearance on agar plates containing 0.5% CMC at pH 7. Quantitation of the cellulase activity was also carried out.

Protein estimation

Protein estimation of the enzyme preparations was carried out by the method of Lowry et al. [14], basing bovine serum albumin as the standard.

RESULTS AND DISCUSSION

A total of 32 thraustochytrid isolates were obtained by the pine pollen baiting method. Figure 1 depicts one such representation of thraustochytrid cells growing on pine pollen. Out of the 32 isolates, 10 were isolated from along the coastline of Goa whereas 22 were isolated from mangrove ecosystems. The salinity at the site of sample collection ranged from 5 to 35 psu. Five isolates were obtained as associated with seaweeds. Among the 32 isolates, 19 tested positive for multiple polysaccharide degrading activities as per the qualitative plate assays. Fourteen isolates were found to produce cellulase, 15 produced agarase, 10 amylase and 8 xylanase (Table 1). One isolate (DS1) produced chitinase, carrageenase, pectinase and alginate lyase in small amounts, besides possessing cellulase and agarase activities. To the best of our knowledge, this is the first ever report of agarase activity from thraustochytrids. Representative qualitative assay plates for cellulase, amylase, carrageenase, chitinase, alginate lyase and agarase are depicted in figure 2. The ability of microorganisms to degrade polysaccharides such as cellulose is a characteristic of considerable interest both in terms of microbial ecology and from the viewpoint of industrial microbiology. Taoka et al. [15] investigated extracellular enzyme production in different strains of thraustochytrids and reported amylase in three strains of the genus *Thraustochytrium* while chitinase was found to be produced in small amounts in *T. striatum*.

Table 1. Detection of polysaccharide-degrading activities in different isolates.

Isolate	Location	Source of the sample	pH	Salinity (psu)	Polysaccharide-degrading activity (Extent of zone clearance)							
					Cellulase	Agarase	Xylanase	Amylase	Chitinase	Carrageenase	Alginate lyase	Pectinase
RS1	Ribandar	Sediment	8.0	10	+	+	+	+	-	-	-	-
RL1	Ribandar	Leaf	8.0	10	+	+	+	-	-	-	-	-
AA3	Anjuna	Algae	8.0	10	+	-	-	-	-	-	-	-
VS1	Vagator	Sediment	7.3	10	+	-	-	-	-	-	-	-
NW	Nerul	Water	8.3	35	-	+	-	+	-	-	-	-
SS	Sao Pedro	Sediment	6.7	5	-	+	-	+	-	-	-	-
BS1	Banastarim	Sediment	7.3	10	++	++	+	+	-	-	-	-
BS2	Banastarim	Sediment	7.3	10	-	+	-	-	-	-	-	-
BS3	Banastarim	Sediment	7.1	5	+	++	-	+	-	-	-	-
BS5	Banastarim	Sediment	7.3	10	+	++	-	+	-	-	-	-
RW	Ribandar	Water	8.7	5	++	++	++	+	-	-	-	-
CH1	Chorao	Water	7.4	20	+	+		-	-	-	-	-
CL1	Chorao	Leaf	7.4	20	+	++	+	-	-	-	-	-
CL2	Chorao	Leaf	7.4	20	++	++	++	+	-	-	-	-
CH2	Chorao	Water	7.6	20	-	+	+	+	-	-	-	-
DH2	Diwar	Water	7.5	20	+	-	-	-	-	-	-	-
DH1	Diwar	Water	7.5	20	+	+	-	+	-	-	-	-
DL1	Diwar	Leaf	7.5	20	-	-	+	-	-	-	-	-
DS1	Diwar	Sediment	7.5	20	+	+	-	-	+	+	+	+

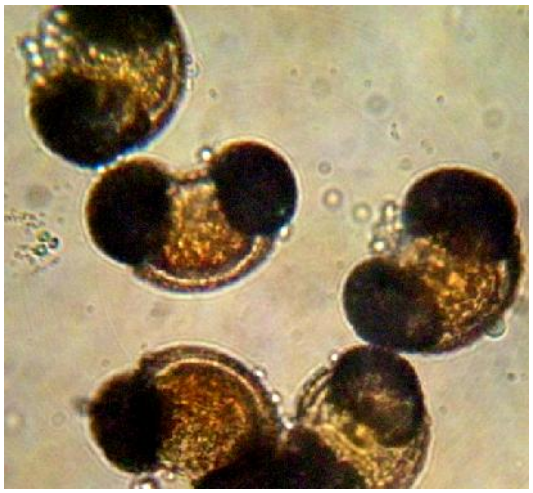


Figure 1. Thraustochytrids growing on pine pollen as seen under the compound microscope.

Table 2. Comparison of 4 polysaccharide-degrading activities from 12 isolates.

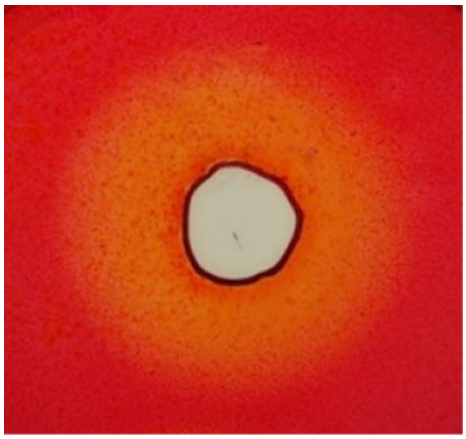
Isolates	Enzyme activity (U/ml)			
	Xylanase	Cellulase	Pectinase	Agarase
RL1	0.094	0.297	0.027	N.D
RL3	0.106	0.192	0.168	N.D
RS1	0.066	0.254	0.020	N.D
MW	0.060	0.176	0.017	N.D
BW	0.063	0.150	0.021	N.D
VS1	0.048	0.225	0.020	N.D
AH2	0.077	0.298	0.025	0.018
TZ	N.D.	0.316	0.039	N.D.
AA3	0.097	0.150	0.003	N.D.
DPW2	N.D.	0.236	0.028	N.D.
NW	0.023	-	0.017	N.D.
SS	-	-	0.028	N.D.

*N.D = not detectable

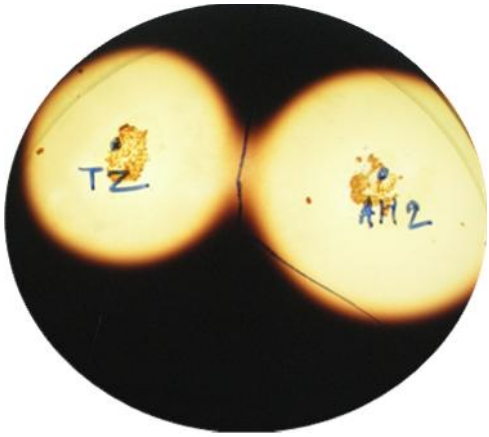
Table 3. CM-cellulase activities of isolates.

Isolate	Specific activity (U/mg)
BS1	0.22
PW2	0.29
BS2	0.45
BS5	0.57
CH3	0.67

Sharma et al. [16] investigated the production of 6 polysaccharide degrading activities in thraustochytrids from Goa, India but failed to find any. Taoka et al. [15] used insoluble cellulose powder as a substrate in the plate assays and found no cellulase producers whereas Bremer et al. [6] used CM-cellulose in their study and reported that thraustochytrids produced cellulases. In our study

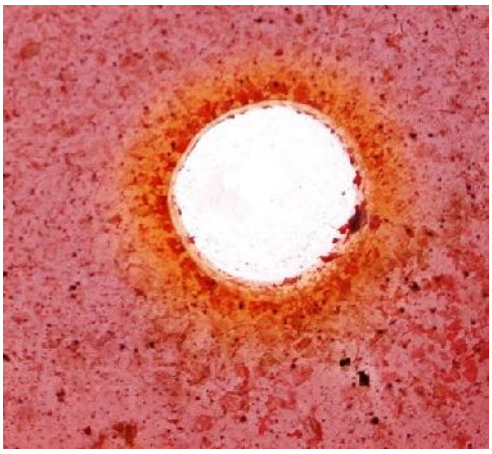


A. Cellulase activity



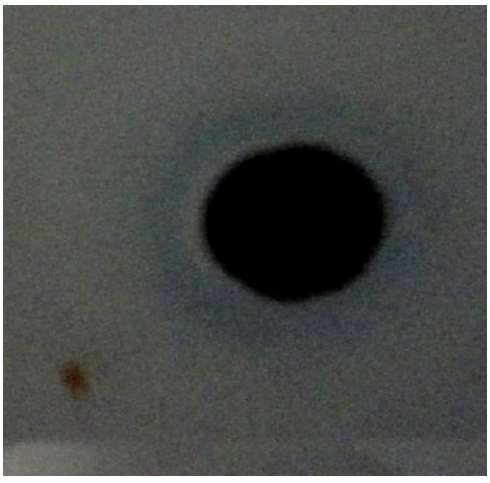
B. Amylase activity

C. Carrageenase activity



D. Chitinase activity

E. Agarase activity



F. Alginate lyase activity

Figure 2. Isolates showing different polysaccharide-degrading activities in plate assay.

also, no clear zone was obtained when cellulose powder was used in the plate assay. This could be due to the fact that insoluble cellulose powder did not get evenly distributed and tended to settle down when used as the substrate for detection in plate assays, probably leading to 'negative' results. The absence of clearance zones with the use of cellulose powder in this study therefore need not necessarily imply the inability of the organism to degrade insoluble cellulose.

Earlier workers have reported the presence of different hydrolytic enzymes in thraustochytrids [4,6,15,17] and/or the semi-quantitative analysis of cellulase [18] but without reference to quantification of the polysaccharide-degrading activities. Quantitative studies on lipases from thraustochytrids as first reported from our laboratory [7] have confirmed the potential industrial applications of these enzymes. In view of this, activities of cellulase, xylanase, pectinase and agarase from a few isolates (including two isolates AH2 and TZ from the laboratory culture collection) were quantified (Table 2 and 3). Activities in the crude enzyme extracts from a few promising isolates were of the order of 1.5U/mg, 0.53U/mg and 0.16 U/ml for CM-cellulase, xylanase and pectinase, respectively. Carboxymethyl cellulose was used as the substrate for quantitation of the "cellulase" activity. Such an enzyme is often referred to as CM-cellulase which, however, is an endo-1,4-β-glucanase. The cellulase sytems of most fungi are said to have synergistic action of three different enzymes, *viz.*, endo-1,4-β-glucanase, exo-1,4-β-glucanase and cellobiase. Previous workers [6] have reported CM-cellulase activity from thraustochytrids and it was assumed that the other two activities too were present. There appears, however, no further report as yet that confirms the same.

Agarase was not detected in the enzyme extracts obtained after growing the cultures in MV medium in the absence of the substrate (0.5% agar). Large zones of clearance obtained when the cultures were grown on agar plates (Figure 3) would however suggest that the agarases in these systems might be inducible enzymes. The plate assay carried out using enzyme preparations obtained every successive day after inoculation showed that the CM-cellulase production first observed after day 2 increased up to the day 5, after which it stabilized by the day 7 (Figure 4). Most of the thraustochytrid isolates exhibited maximum cellulase activity at 5 days of incubation, as shown in figure 5. On the basis of these results, a harvesting time of 5 days was opted for, for all the preliminary studies. Cellulase and xylanase activities were found to be higher in the enzyme preparations obtained after growing the isolates in the presence of added glucose in the medium as compared to when grown in the absence of glucose, indicating a requirement of glucose for maximal enzyme production (Table 4 and 5). Interestingly, in a study on the efficacy of different carbon sources in the growth medium, higher enzyme activities were elicited during growth in the presence of maltose rather than glucose (data not shown). More detailed investigations are underway to ascertain the effect of different substrates on enzyme production.

Figure 3. Agarase activity as shown by isolates RS1 and AH2 on medium containing 0.8% agar and no glucose.

Table 4. Cellulolytic activity (U/mg protein) of isolates grown in the presence/absence of substrate and glucose.

Isolate	- Glucose		+ Glucose	
	0% CMC	0.5% CMC	0% CMC	0.5% CMC
RL1	0.039	N.D.	0.466	0.119
RS1	0.023	N.D.	0.158	0.004
AH2	0.062	N.D.	1.076	0.082
TZ	0.077	N.D.	1.503	0.197
NW	0.034	N.D.	0.402	0.028
SS	0.054	N.D.	0.589	N.D
BW	0.006	N.D.	0.239	0.148
MW	0.019	N.D.	0.198	0.017
VS1	0.029	N.D.	0.266	0.106
DPW2	0.044	N.D.	N.D.	0.006
VA1	0.047	N.D.	N.D.	N.D.

*N.D. = not detectable

Table 5. Xylanolytic activity (U/mg protein) of isolates grown in the presence/absence of substrate and glucose.

Isolate	- Glucose		+ Glucose	
	0% xylan	0.5% xylan	0% xylan	0.5% xylan
RL1	0.004	N.D.	0.215	0.009
RS1	0.003	N.D.	0.082	N.D.
AH2	0.006	N.D.	0.077	0.010
TZ	N.D.	0.007	0.187	0.062
NW	0.022	0.018	0.522	N.D.
SS	N.D.	N.D.	0.022	0.094
BW	N.D.	0.04	0.008	N.D.
MW	N.D.	N.D.	0.228	N.D.
VS1	0.003	0.012	N.D.	0.045
AA3	N.D	N.D.	N.D.	0.073
DPW2	N.D.	N.D.	N.D.	0.046
VA1	N.D.	0.003	N.D.	0.126

*N.D. = not detectable

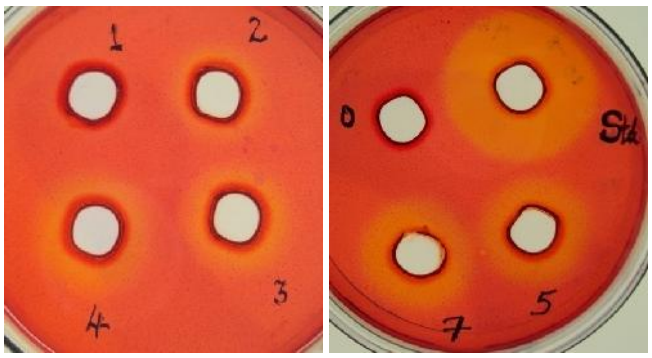
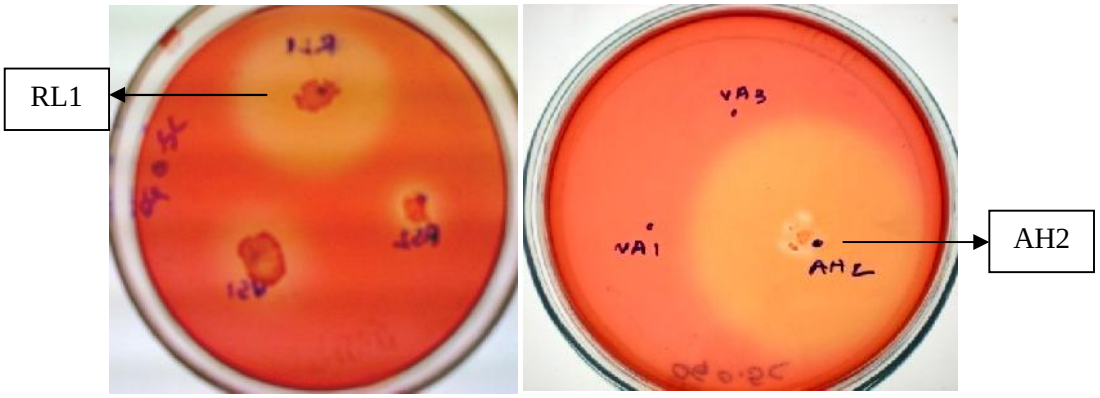
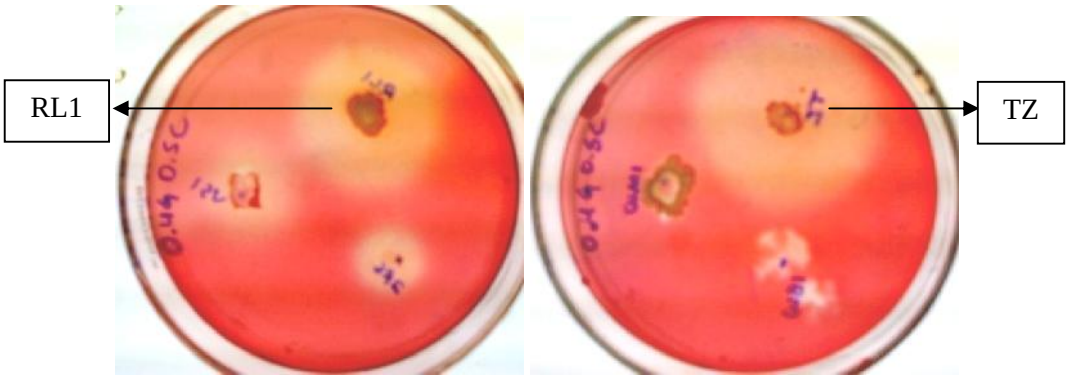


Figure 4. Agar cup diffusion assay depicting CMCase activity of a potential cellulolytic isolate each successive day after inoculation. The numerals in the figure represent the day of harvesting.



Isolates showing activity on medium with 0.5% CMC and no glucose.



Isolates showing activity on medium with 0.5% CMC and 0.4% glucose.

Figure 5. CMCase activity of selected isolates.

Polysaccharase activity was also detected when some isolates were grown in the absence of the respective substrates (Table 4 and 5). Cellulase as well as xylanase activities were noted to be higher in culture filtrates obtained after growing such isolates in the absence of substrate, suggesting the constitutive nature of the enzyme production in these cases. It was, however, interesting to note that the cultures produced cellulase on agar plates when grown in the presence of 0.5% CM-cellulose and in the absence of glucose (Figure 6), contrary to the "non-detectable" activity in broth culture. This may be attributed to the presence of multiple polysaccharide-degrading activities in many of the isolates, especially that of agarase, leading to the release of specific monosaccharides that would favor growth and enzyme production by the isolates.

In summary, the present work shows that most of the thraustochytrid isolates studied exhibited all of the eight polysaccharide-degrading activities that were tested for, the prominent activities being those of cellulase, agarase and amylase. Most of the enzymes appeared to be constitutive in nature and one isolate in particular was shown to be able to produce 6 out of the 8 polysaccharases screened for. By virtue of the production of various degradative enzymes and their absorptive mode of nutrition, these organisms may play an important role as remineralizers in the ocean [5]. Faced with the fact that they do not occupy any special spatial niche in relation to bacteria, the strategy of production of a multitude of enzymes does seem to go well with the cause of scavenging for nutrients on substrates, especially after cessation of associated bacterial growth, as suggested by the previous laboratory based studies [5].

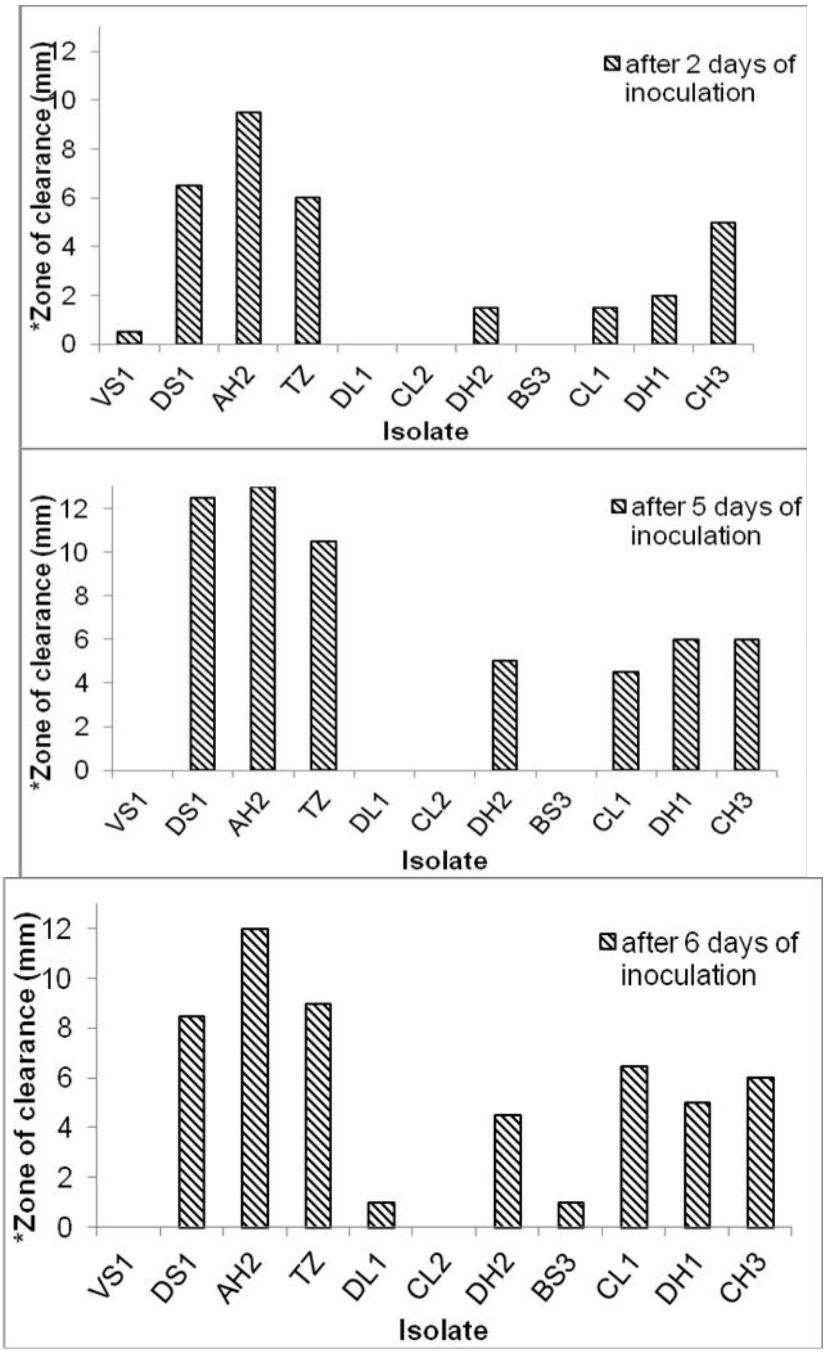


Figure 6. Comparative cellulase activities of isolates at different growth intervals.

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