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PRODUCTION OF EXTRACELLULAR POLYSACCHARASES BY THE MARINE PROTISTS, THRAUSTOCHYTRIDS, WITH SPECIAL REFERENCE TO α-AMYLASE ACTIVITY

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ABSTRACT

Thraustochytrids, a once obscure group of straminipilan protists, are now increasingly coming into the limelight by virtue of the diverse industrial potential of their cell products which include polyunsaturated fatty acids (PUFAs) and hydrolytic enzymes. The present study dwells on production of extracellular polysaccharide-degrading enzymes by isolates from various coastal and mangrove habitats of Goa. Extensive screening has yielded strains that produce enzymes with multiple hydrolytic activities and potential in diverse industrial applications. They produced a plethora of enzymes that included agarases, amylases, pectinases, chitinases and carrageenases, many of which appeared to be secreted constitutively. Agarase and amylase activities predominantly observed in most isolates from mangrove habitats had special characteristics that would favor commercial applications. This therefore stands as the first detailed report on extracellular amylase production by thraustochytrids. Amylases produced by two isolates, *viz.,* TZ (ATCC#PRA-295) and AH-2 (ATCC#PRA-296) were confirmed to be α-amylases, enzymes of great significance in present day biotechnology.

KEYWORDS: Thraustochytrids, polysaccharide-degrading enzymes, α-amylases, multiple hydrolytic activities

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INTRODUCTION

Thraustochytrids are marine, osmo-heterotrophic straminopilan protists with about 40 identified species. [1-4] They occur in marine and estuarine waters [5,6] and also in association with plant detritus (such as mangrove leaves and brown algae) and fecal pellets of zooplankton. [7-10] They have been demonstrated to be actively involved in the breakdown, scavenging and mineralization of highly refractory organic matter by producing a variety of extracellular enzymatic activities. [3] They form ectoplasmic nets around the cell, which harbor hydrolytic enzymes that are either surface bound or secreted into the surroundings, thus helping them to degrade organic matter [2,11] and raising the likelihood of their being an important component of the microbial food webs of marine ecosystems. [10,12] Studies on thraustochytrids have been largely focused on their high accumulation of polyunsaturated fatty acids, chiefly docosahexaenoic acid. Investigations on extracellular enzymes produced by six strains of the thraustochytrids, *Schizochytrium, Thraustochytrium* and *Aurantiochytrium* producing five to eight kinds of the extracellular enzymes have been reported. [13] Only the genus *Thraustochytrium* produced amylase. Given the prevalence of thraustochytrids as saprobes in the marine environment, it is likely that they harbor unique degradative enzymes, despite competition with bacteria. There are several reports on extracellular production of enzymes but detailed studies on such enzymes from thraustochytrids are numbered. Optimization of cultivation conditions for the production of alkaline lipases by thraustochytrids was first reported from our own laboratory [14], closely followed by a study on extracellularly produced cellulase, xylanase and pectinase enzymes.[15] Overall, however, specific information on kinds of enzymes produced by each strain of thraustochytrids is lacking. Alpha amylases, one of the most important and widely used enzymes, were first isolated and identified in the year 1894 from a fungal source used as additives in pharmaceutical digestive formulations.[16] Ever since and with the advent of new frontiers in biotechnology, their ambit of applications has gone a long way in many sectors such as medicinal and analytical chemistry. Amylases are produced by a wide spectrum of organisms and each source produces biochemical phenotypes of the enzyme that significantly differ in parameters such as pH and temperature optima as well as metal ion requirements. α-Amylases specifically act on long-chain carbohydrates, yielding the breakdown products maltose (from amylose) or maltose, glucose and limit-dextrin (from amylopectin). Of late, the interest and demand for enzymes with novel properties has recorded an upsurge in various industries, encouraging the discovery of various types of the amylases with unique properties. Besides a major application in starch saccharification, they also find significant use in the food, brewing, baking, distilling, textile, detergent as well as the pulp and paper industries. [17]

MATERIALS AND METHODS

Sample Collection

Samples were collected at low tide from various coastal and mangrove habitats of Goa during the period February to December 2013. Decaying mangrove leaves, water, macroalgae and sediment samples were collected in sterile glass vials and immediately transferred to the laboratory. Physico-chemical parameters of the environment such as pH and salinity were recorded each time.

Isolation of thraustochytrids

The pine pollen baiting method [18,19] was adopted for isolation of thraustochytrids. Samples were inoculated in vials containing sterile sea water, followed by dusting with pine pollen. They were then incubated at room temperature for 3-4 days and examined under the microscope. Those found to contain thraustochytrids were then streaked onto Modified Vishniac's (MV) agar medium plates (0.15% peptone, 0.1% yeast extract, 0.4% glucose, 3.4% crude salt and 0.8% agar) containing 1% antibiotics (streptomycin and penicillin). Thraustochytrid colonies observed under the microscope were purified by repeated transfer on fresh solid MV medium to obtain axenic cultures.

Screening for polysaccharase activities in thraustochytrids

Preliminary screening for enzyme activities was carried out by qualitative plate assays. The isolates were tested for amylase, agarase, alginate lyase, chitinase, pectinase and carrageenase activities, using starch, agar, sodium alginate, chitin, pectin and carrageenan, respectively, as substrates. Two methods were used: (a) Culture plates were prepared with the respective substrates and the isolates were spot inoculated on these plates. They were then incubated at room temperature for 3-4 days. Amylase activity was checked for by flooding the assay plates with Grams Iodine [20], agarase activity with Lugol's iodine [21], alginate lyase activity by using 10% sulphuric acid, chitinase and pectinase with Congo Red [22] and carrageenase by using Phenol Red; halo formation around the spotted culture indicated the production of the respective enzyme. (b) Plates were prepared with the respective substrates as described above. Culture supernatant of the isolates grown for 3-4 days in broth culture was used to analyze the polysaccharide-degrading activities by agar cup diffusion method, for which plates were incubated at room temperature or at 50^{o}C for 24 h. They were then stained for the respective enzyme activities. All assays results are the mean of replicate analyses in a single experiment and the data presented are representative of two/three independent experiments.

Enzyme production

The culture medium used for α-amylase production contained (as %, w/v): yeast extract (0.1), peptone (0.15), glucose (0.4) and sea salt (3.4) at a pH of 6.8-7.0. A 1% inoculum of a 3-day old broth culture was added and incubated at 25 – 28ºC in a rotary shaker at 120 rpm for 96 h. The turbidity of the cultures was gauged at regular time intervals by measuring the optical density at 660 nm. The cells were separated by centrifugation for 10 min at 10,000 rpm and 4ºC; the clear supernatant served as the crude enzyme preparation.

Effect of substrate added in the growth medium

Amylase activity was analyzed after growing the isolates in medium containing varying combinations of glucose and starch, or glucose and maltose at pH 7.

α-Amylase Assay

The activity of α-amylase was assayed by incubating 0.5 mL of suitably diluted enzyme with 1.0 mL soluble starch (0.3 mg/mL) prepared in 0.1 M acetate buffer, pH 5. After incubation at 40ºC for 10 min the reaction was stopped by adding 0.2 M NaOH and the rate of starch disappearance was measured spectrophotometrically at 578 nm after the addition of 1% iodine solution. One unit of alpha amylase activity is defined in terms of milligram starch digested per min by 1 mL of the enzyme.[23] Protein concentration was measured in triplicate, following the procedure of Lowry *et al.* [24] using bovine serum albumin as standard.

Analysis of hydrolysis products of α-amylase

Thin layer chromatography (TLC) was carried out to identify the products of starch hydrolysis by the crude enzyme extract. The silica plates were developed in a solvent system consisting of *n*-butanol:acetic acid (1:1). Reducing sugars were determined by spraying a solution consisting of 0.5 g aniline hydrochloride, 0.5 g diphenylamine in 50 mL acetone and 5 mL *ortho*-phosphoric acid, followed by baking at 150°C for 5 min.

Determination of α-amylase by $CNPG_3$ method

For confirming the presence of α-amylase in the crude enzyme extract, 2-chloro-4-nitrophenyl-α-D-maltotrioside ($CNPG_3$) was used as substrate. The release of 2-chloro-4-nitrophenol (CNP) and the resulting absorbance increase at 405 nm per minute is directly related to the α-amylase activity in the sample (Thermoscientific Protocol). [25]

Effect of pH on activity and stability of α-amylase

Effect of pH on α-amylase activity was measured using various buffers: acetate buffer (0.5 M, pH 3.5 – 5.0), phosphate buffer (0.1 M, pH 6.0 -8.0) or borate buffer (12.5 mM, pH 8.0 -10.0). Stability of the enzyme at different pH values was studied by incubating the enzyme with an equal volume of the respective buffer at pH 3-9 for 10 min at room temperature (25± 2°C) and then estimating the residual activity as measured under the standardized reaction conditions.

Effect of temperature on amylase activity and stability

To determine the optimum assay temperature, amylase activity was measured at different temperatures for a 10 min reaction at pH 7.0. For thermal stability studies, the enzyme solution was pre-incubated at different temperatures for 10 min in phosphate buffer (0.1 M, pH 7.0) before measuring the residual activity under the optimum conditions.

RESULTS AND DISCUSSION

Thraustochytrids isolated from various coastal habitats of Goa, India (mangrove area, seawater and sediment) were screened for their potential for extracellular production of six different polysaccharide-degrading enzymes. A total of 18 thraustochytrid isolates were obtained by the pine pollen baiting technique (Fig 1a) of which only one (isolate TPU 8) was from coastal waters while all the others were from mangrove sites in Goa. The various sampling locations (between 15.3-15.6 °N and 73.8-73.9 °E) are depicted in Fig 2. The salinity of the sites varied from 10 to 32 psu and pH from 6.7 to 7.6. Of the 18 isolates, 12 were obtained from decaying mangrove leaves while six were from sediment samples (Table 1). Axenic cultures obtained by repeated streaking on MV agar medium were then observed for their colony characteristics.

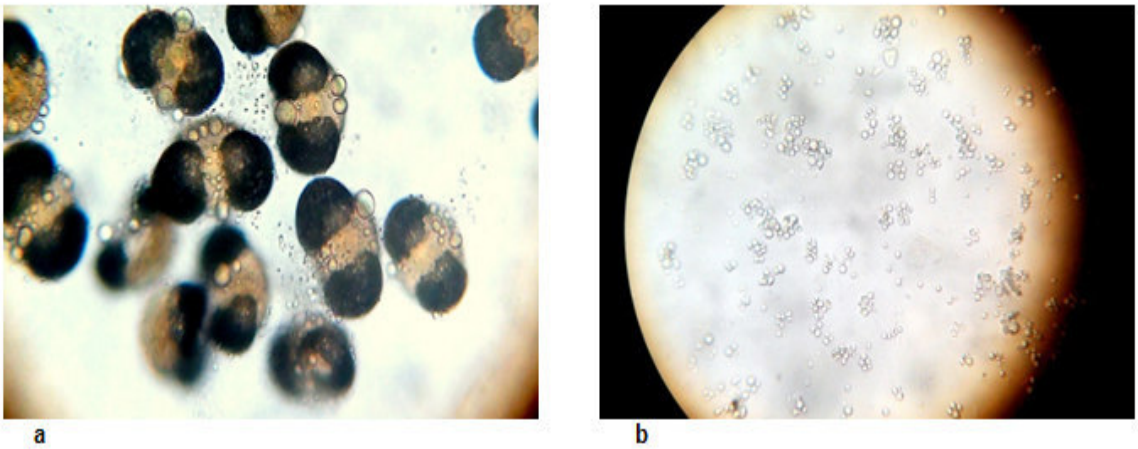

Figure 1
Thraustochytrids growing *(a) on pine pollen and (b) in broth, as seen under the compound microscope (40X)*

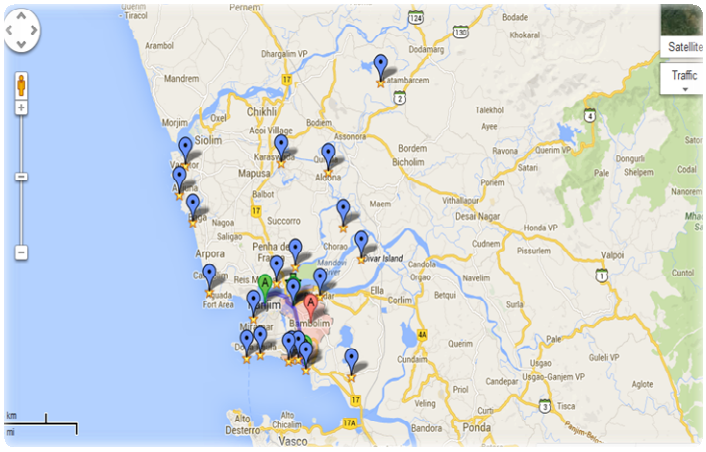

Figure 2
Sampling sites in Goa, India

Table 1
Thraustochytrid isolates obtained primarily from mangrove habitats of Goa, India

Isolate No.	Location	Source	pH	Salinity (psu)
TPU 1	Sao Pedro	Leaf	7.0	10
TPU 2	Sao Pedro	Leaf	6.7	10
TPU 3	Ribandar	Leaf	6.9	10
TPU 4	Ribandar	Sediment	6.7	10
TPU 5	Diwar	Leaf	7.3	25
TPU 6	Panaji	Leaf	7.4	29
TPU 7	Betim	Leaf	7.3	31
TPU 8	Dona Paula	Sediment	7.6	32
TPU 9	Moira	Sediment	7.2	13
TPU 10	Moira	Sediment	7.2	13
TPU 11	Moira	Sediment	7.2	13
TPU 12	Moira	Leaf	7.2	13
TPU 13	Aldona	Sediment	7.1	11
TPU 14	Aldona	Leaf	7.1	11
TPU 15	Poira	Leaf	7.0	10
TPU 16	Poira	Leaf	7.0	10
TPU 17	Chodan-Madel	Leaf	7.2	12
TPU 18	Chodan-Madel	Leaf	7.2	12

In addition to the 18 isolates listed in Table 1, seven others from our laboratory collection were also screened for their polysaccharide-degrading ability. All 25 isolates were tested for amylase, agarase, chitinase, pectinase, carrageenase and alginate lyase activities using both spot inoculation as well as culture supernatant assays. Of these, 10 isolates tested positive for multiple polysaccharase activities (Fig 3). In all, 10 isolates were found to produce amylase, 15 agarase, 7 pectinase and one, low amounts of chitinase, based on the spot inoculation technique (Fig 4). The above activities were elicited in the presence of the respective polysaccharide substrate, raising a possibility that the systems might be inducible in nature.

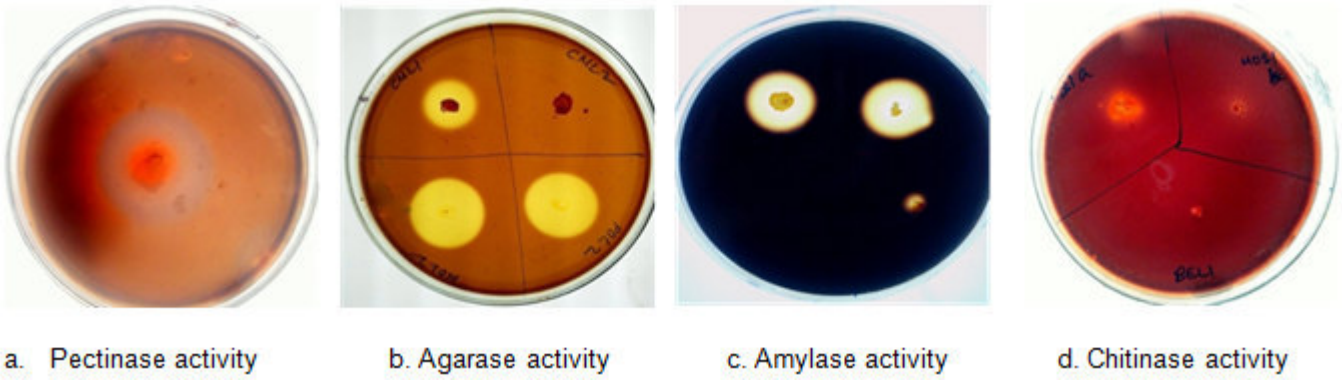

Figure 3
Plate assay showing multiple polysaccharide-degrading activities of the isolates by the spot inoculation technique

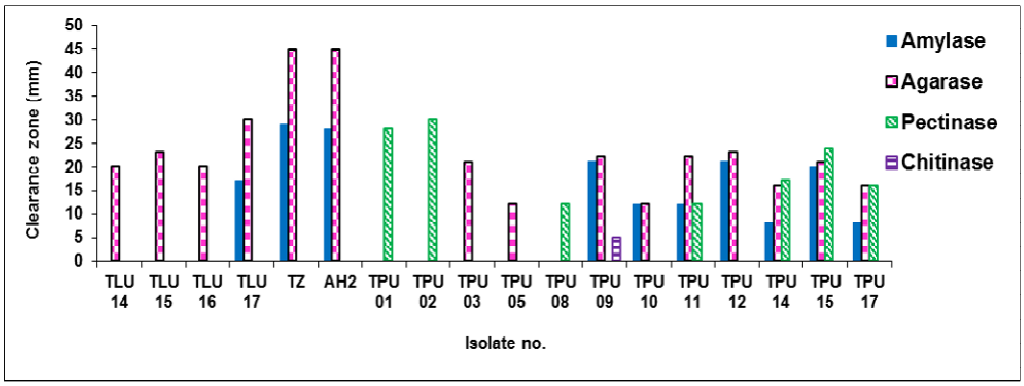

Figure 4
Polysaccharide-degrading activities from different isolates as determined by the spot inoculation technique

During qualitative analysis of the activities using culture supernatant, however, all the isolates that showed polysaccharase activity as depicted in Figs 5 and 6 did so when grown in the absence of the polysaccharide substrate also, indicating the constitutive nature of the enzyme production in these cases: Ten isolates were found to produce agarase and nine produced chitinases. There were two amylase, two pectinase and two carrageenase producers while two isolates produced small amounts of alginate lyase. In particular, chitinase production in the above isolates, with the exception of isolate TPU 9, was observed to be constitutive rather than inducible. This feature could well afford a competitive advantage to isolates for immediate substrate utilization during their encounter with such substrates in nature.

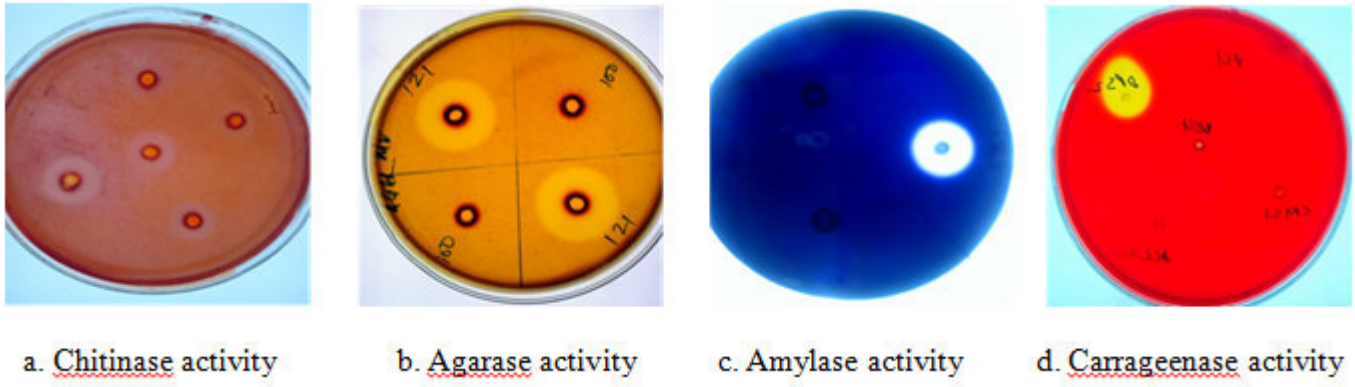

Figure 5
Multiple polysaccharide-degrading activities of the isolates in plate assay by culture supernatant analysis

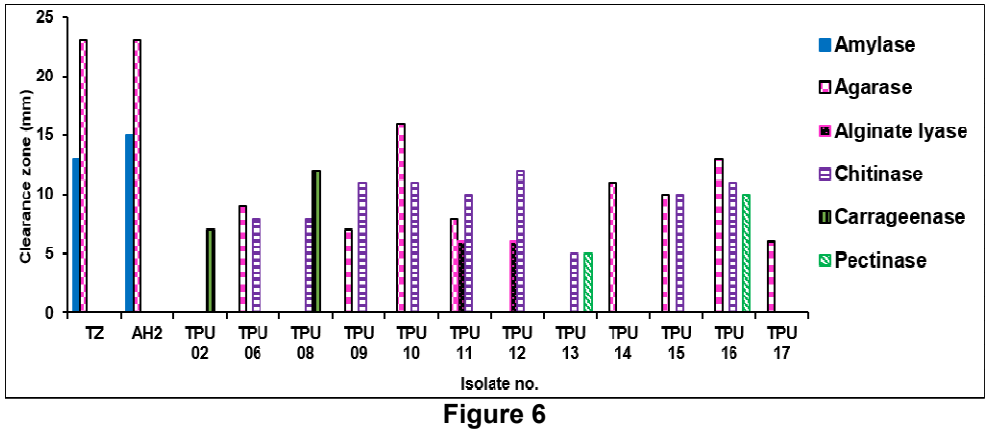

Figure 6
Polysaccharide-degrading activities from different isolates by culture supernatant analysis on plates

When all the 10 isolates chosen (on the basis of size of clearance zones in plate assays) were grown in MV medium for 96 h, highest activity (5.02 and 6.62 U/mg, respectively) was recorded in culture supernatants from our isolates TZ (ATCC #PRA-295™) and AH-2 (ATCC® PRA-296™), under the assay conditions of pH 5 and 40°C (Fig 7). These isolates were hence selected for further optimization studies.

Amylase activity was significantly higher in both the isolates TZ and AH-2, when a combination of starch and glucose (1:1) served as carbon source in the growth medium (Fig. 8). Replacement of starch by maltose also

yielded similar results (Fig. 9). Thin layer chromatographic separation of products of soluble starch hydrolysis by the crude enzyme extract showed maltose as the end product in reaction mixtures containing culture filtrates from isolates TZ as well as AH-2 (Fig. 10). The $CNPG_3$ assay measurements at 37°C and pH 6 recorded α-amylase activities of 36 and 47 U/L, respectively, from isolates TZ and AH-2, confirming the enzyme activity in the crude enzyme extracts as due to α-amylase.

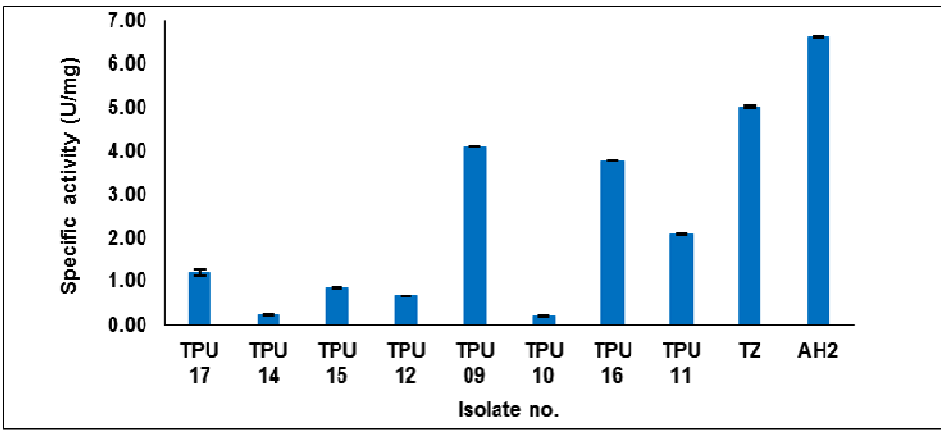

Figure 7
Specific activities of *α*-amylases produced by different isolates

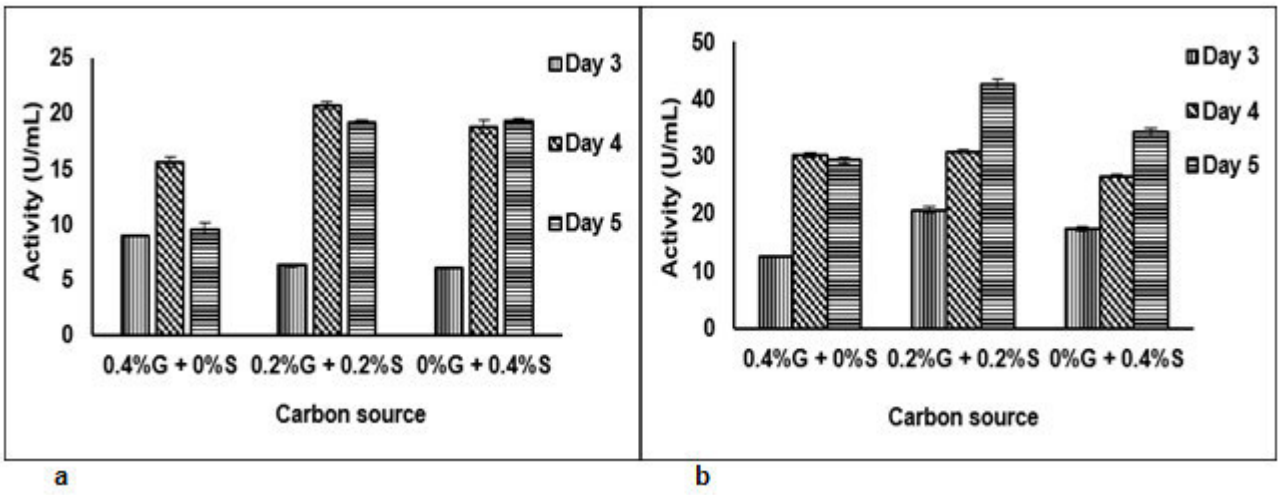

Figure 8
Effect of starch on *α*-amylase production by isolates (a) TZ and (b) AH-2

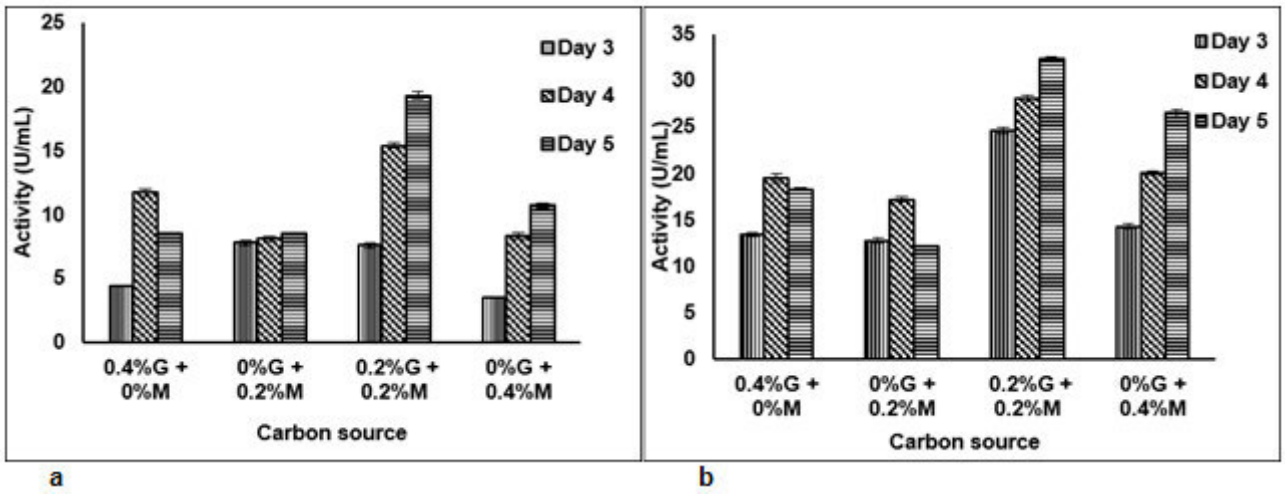

Figure 9
Effect of maltose on amylase activity of isolate (a) TZ and (b) AH-2

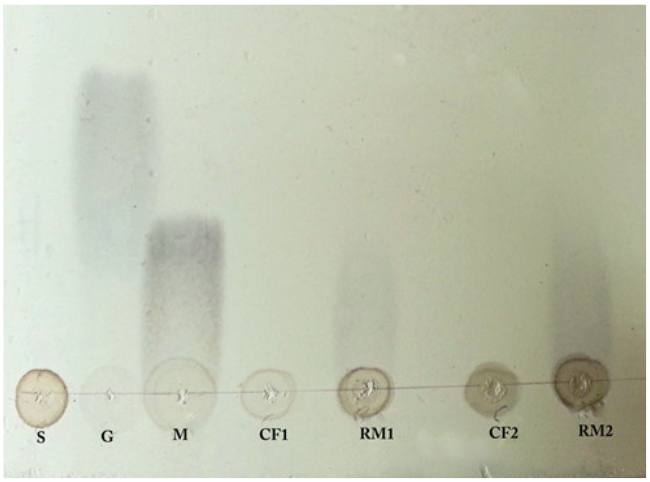

Figure 10
TLC based identification of products of starch hydrolysis by crude enzyme extracts from isolates AH-2 & TZ.
Lane S: soluble starch substrate, Lane G: glucose, Lane M: maltose, Lane CF1: culture filtrate of AH-2, Lane RM1: reaction mixture with CF1, Lane CF2: culture filtrate of TZ and Lane RM2: reaction mixture with CF2

The enzymes from both the isolates were optimally active at pH 7 (Fig 11). Two peaks were observed in both cases, one at pH 5 and the other at pH 7. Although enzyme activity diminished beyond pH 7, isolate AH-2 showed significant activity (60 % of the highest activity) even at pH 10. While the amylases exhibited maximum stability as well as activity at pH 7, they were significantly stable over a wide pH range (Fig 12). At pH 7 the activity from isolate AH-2 increased by 1.43-fold over the control while that from isolate TZ was unchanged by the treatment. More than 50% activity was retained after treatment at all pH values ranging from 3 to 9. Such amylases with wide pH stability ranges would have huge potential in the detergent industry.

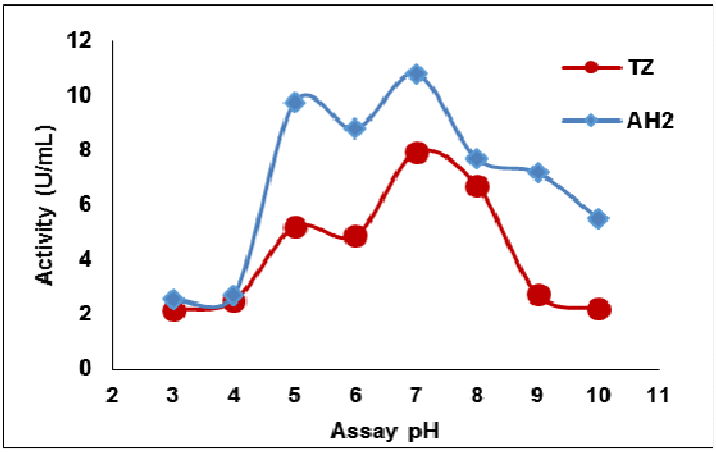

Figure 11
Dependence of assay pH on α-amylase activity from isolates AH-2 and TZ

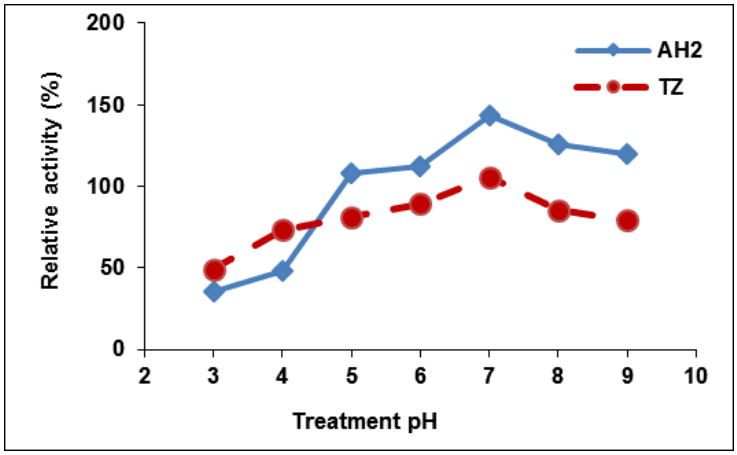

Figure 12
pH stability of the enzymes from isolates AH-2 and TZ

Amylases are the second type of enzymes used for formulation of enzymatic detergents [26,27] and 90% of all liquid detergents contain α-amylase. [28] Currently, such enzyme formulations are widely used in laundry and automatic dish washing for removal of starchy food substances derived from gravies, potatoes, chocolate, custard and smaller oligosaccharides [29]. When assayed at pH 7.0 over a temperature range of 25-80ºC the optimum activity of the enzyme from both isolates was found to be at 50ºC (Fig 13). That the decline in activity beyond this temperature was due to enzyme protein degradation was supported by thermal stability studies (Fig 14a). The enzyme was stable at 50°C for up to 60 min in case of isolate AH-2 and up to 30 min in case of isolate TZ; the stability decreased upon further incubation. From Fig 14b, it was evident that a 50°C treatment for 10 min elevated the activity by 1.9- and 1.33- fold, respectively, for the enzymes from isolates AH-2 and TZ. Mohamed et al.[30] reported that some wheat α-amylases were stable up to 50°C and some at 40°C after incubation for 15 min, whereas in *P. erosus* tubers α-amylase was stable up to 40°C for 30 min incubation, beyond which there was rapid inactivation.

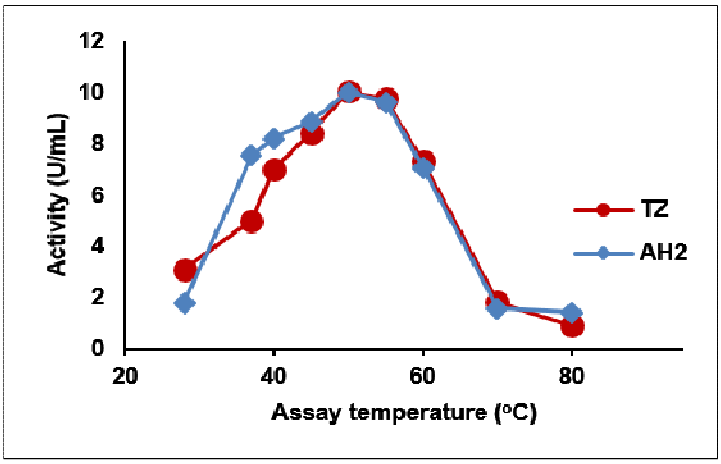

Figure 13
Effect of assay temperature on α-amylase activity from isolates AH-2 and TZ

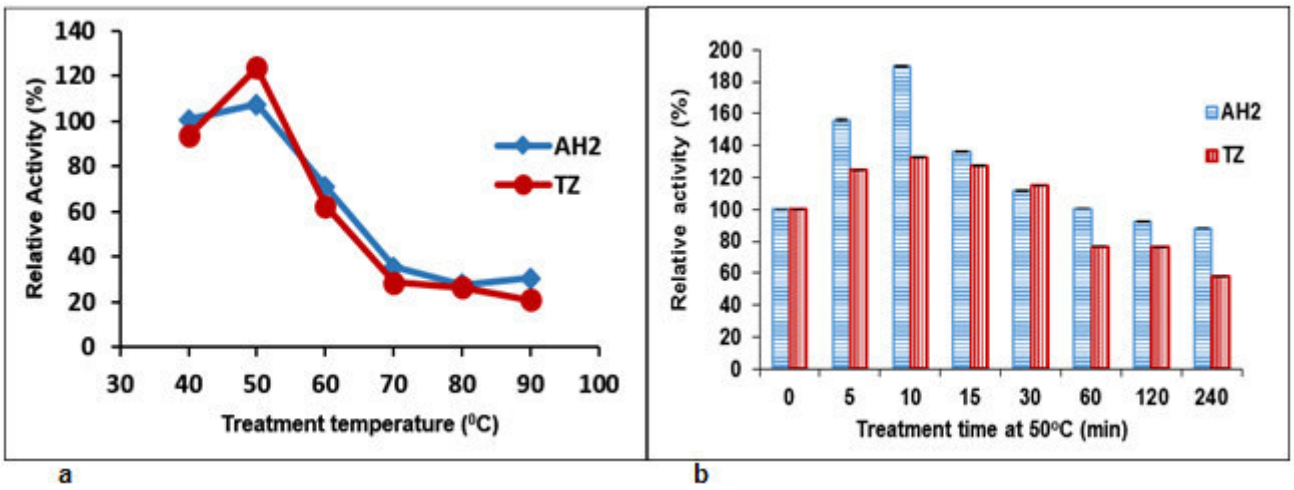

Figure 14
Thermal stability studies on the enzymes from isolates AH-2 and TZ when treated (a) for 10 min at various temperatures and (b) at 50°C for various time periods

CONCLUSION

In summary, this research throws light on the presence of multiple polysaccharide-degrading enzymes produced by thraustochytrid isolates. While there are several reports on the biological production of amylases from various microorganisms, the present study specifically reports production of amylases by the marine protists. Amylases are among the most important enzymes used in industrial processes, with their major application being in the starch industry, besides their well-known usage in the baking industry. They can also be of potential use in the food, pharmaceutical and fine chemical industries. The observed optimal thraustochytrid amylase activity at pH 7 and 50ºC from both the potential isolates AH-2 and TZ was found to be contributed by alpha amylases. Preliminary studies have indicated that the amylases from these isolates showed promise in degrading starch and that they could thus have practical applications in the starch industry in view of their favorable temperature and pH characteristics. Under optimum conditions, isolate TZ produced 20 U/mL and isolate AH-2 30 U/mL of α-amylase, respectively, which could probably be enhanced by further refinement of media components and conditions. Besides, several other isolates too exhibited starch degrading ability, projecting thraustochytrids as a novel potential source of industrial amylases.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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