Exploring Enzyme Diversity in Thraustochytrids: A Study of Multiple Enzyme Production.

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "Exploring Enzyme Diversity in Thraustochytrids: A Study of Multiple Enzyme Production" is based on the results of investigations carried out by me in the Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University under the supervision of Dr. Varada S. Damare and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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Akhila Anand Pednekar

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Date: 02/05/2024

Place: Goa University

COMPLETION CERTIFICATE

This is to certify that the dissertation report "Exploring Enzyme Diversity in Thraustochytrids: A Study of Multiple Enzyme Production" is a bonafide work carried out by Ms. Akhila Anand Pednekar under my supervision in partial fulfillment of the requirements for the award of the degree of M.Sc. in the Discipline Marine Microbiology at the School of Earth, Ocean and Atmospheric Sciences, Goa University.

Dr. Varada S. Damare

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PREFACE

The study explores the role of Thraustochytrids enzymes in marine ecosystems, focusing on their contribution to decomposition processes and nutrient cycling, and their potential environmental sustainability impact. Thraustochytrids are marine protists that play crucial roles in marine environments, yet their enzymatic capabilities and ecological significance remain relatively understudied. This research was motivated by the need to understand the enzymatic potential of Thraustochytrids and their impact on marine ecosystems. By investigating the production of multiple enzymes by Thraustochytrid isolates and assessing their enzyme activity under varying conditions, this study aims to shed light on the role of Thraustochytrids in marine nutrient cycling and ecosystem sustainability. The genesis of this study stems from the recognition of Thraustochytrids as key players in marine detritus degradation and their potential to influence marine carbon and nutrient dynamics. This research hopes to contribute to a better understanding of Thraustochytrids' ecological roles and their implications for marine ecosystem health and sustainability.

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AKHILA ANAND PEDNEKAR

TABLES

FIGURES

ABBREVIATIONS USED

ENTITY	ABBREVIATION
MV	Modified Vishniac
CMC	Carboxymethyl cellulose
R.T	Room temperature
DNSA	3,5-dinitrosalicylic acid

ABSTRACT

Thraustochytrids, marine unicellular microorganisms belonging to the phylum Heterokonta, are known for their diverse ecological roles and biotechnological applications. The role of Thraustochytrid enzymes in marine ecosystems focuses on their contribution to decomposition processes and nutrient cycling, and their potential environmental sustainability impact. This study aimed to investigate the production of multiple enzymes such as amylase, protease, pectinase, agarase, cellulase, lipase, gelatinase, and chitinase by Thraustochytrid isolates and assess their amylase activity under varying substrate concentrations and incubation times. Sixteen Thraustochytrid isolates were examined for multiple enzyme production potential. These were isolated previously from varied sources. The isolates were grown in Modified Vishniac (MV) broth for 3-4 days before analysis. Amylase and protease activity was observed on MV agar and in MV broth while the other enzymes were screened only for agar. Additionally, amylase production by Thraustochytrids isolate OMD4 was quantitatively estimated at various substrate concentrations (0.01%, 0.05%, 0.07%, 0.1%, 0.5%, and 0.8%) and incubation times (10, 20, and 30 minutes) and 0.5% starch concentration with an incubation time of 10 minutes was found optimum. These findings provide insights into Thraustochytrid enzymatic capabilities and their ecological significance in the marine environment.

Keywords: Thraustochytrids, Multiple enzymes, Substrate

CONTENTS

CHAPTER 1. INTRODUCTION

1.1 Background

Thraustochytrid is a marine unicellular, heterotrophic, osmo-heterotrophic protist. (Bongiorni et al.2005). They belong to the Phylum Heterokonta and Class Labyrinthulomycota of the Kingdom Stramenopila, of the SAR domain (Damare 2019). Thraustochytrids are found in a variety of habitats, such as rocky coastlines, coral reefs, salt marshes, sandy substrate, coastal waters, and deep sea. They are also found in plant detritus, such as brown algae and mangroves, and in zooplankton fecal pellets (Raghukumar and Raghukumar 1999). Particle-associated protists known as Thraustochytrids are common in the marine environment. Their relationship with phytoplankton cells is known to be unfavorable, but their relationship with phytoplankton cell exudates and detritus is known to be beneficial. Thraustochytrids are difficult to isolate and maintain in culture because environmental factors like temperature changes and salinity impact their growth (Damare et al. 2020).

Omega-3 PUFAs, especially docosahexaenoic acid, are produced in large quantities by Thraustochytrids, while certain strains also generate eicosapentaenoic acid (Gupta et.al., 2015). They have been shown to actively participate in the scavenging, breakdown, and mineralization of very refractory organic materials by enzymatic extracellular decomposition (Bongiorni et al.2005). The ability of Thraustochytrids to break down plant debris using extracellular hydrolytic enzymes, such as amylase, cellulase, lipase, protease, pectinase, chitinase, etc., has also led to the recognition of their significance in the degradation and mineralization of highly refractory organic matter in marine ecosystems (Taoka et al. 2009).

The Ectoplasmic net element (EN) is a branched network of plasma membrane extensions, linked to the bothrosome or sagenogenetosome organelle at the cell's periphery. EN increases cell surface area and contains hydrolytic enzymes which are surface-bound for organic material digestion. In the case of thraustochytrids, it penetrates organic particles (Raghukumar 2002). Thraustochytrids, through their saprophytic nutrition, play an ecological role as marine detritus degraders. They also produce exopolysaccharide (EPS), which helps in nutrient concentration and maintains exoenzyme activity through its extensive matrices (Jain et al. 2005). Thraustochytrids are believed to aid in the degradation of highly refractory organic compounds

by producing unique degradative enzymes, potentially overcoming bacterial competition (Bongiorni et al. 2005; Jain et al. 2005)

The many media that have been extensively utilized to isolate Thraustochytrids typically include peptone as a nitrogenous source, yeast extract, and glucose as a carbon source. Grown on modified Vishniac (MV) medium which contains 0.01% yeast extract, 0.15% peptone, 0.001% liver infusion broth, and 0.1% dextrose (Damare 2019).
Research on Thraustochytrids has mostly concentrated on their fatty acid accumulation in the cells, particularly on docosahexaenoic acid (DHA), their place in the marine environment, their pathogenicity against seaweed and seagrass, and their phylogenetic classification. However, there are few studies on the extracellular enzymes of Thraustochytrids. Thraustochytrids have been proposed as having a major part in the marine ecosystem's microbial loop carbon cycle (kimura et al.1999). They likely contribute to the microbial food chain in the marine ecology with the diverse enzymes they create (Taoka et al. 2009).

One of the enzymes produced by Thraustochytrids is amylase. The commonly utilized enzyme amylase, produced by Thraustochytrium, converts long-chain carbs into maltose, glucose, and limit-dextrin. The starch saccharification, food, beverage, textile, paper, textile, and detergent sectors all employ these special enzymes (Shirodkar et al. 2017).

Enzymes are biological catalysts that play essential roles in various biochemical processes. Among these, one of the most significant and often utilized enzymes is alpha-amylase. Amylase enzymes break down starch substrates through the process of hydrolysis, which involves the cleavage of D-(1-4) glycosidic bonds in the starch molecules. The simplest form of starch is Amylase polymer and the branched form is amylopectin. This initial hydrolysis of starch results in the formation of shorter oligosaccharides, including maltose, maltotriose, and branched oligosaccharides of 6–8 glucose units. To detect the presence of amylase iodine is used When iodine meets amylose, it forms a blue-black color complex, which is commonly used as a test for the presence of starch. This reaction occurs due to the helical structure of amylose, which allows iodine molecules to fit inside the helix, resulting in the characteristic colour change (de Souza et al. 2010).

Others include as follows Proteases which are enzymes that catalyse the hydrolysis of proteins. They are a diverse group of large and complex enzymes with highly specific protein hydrolysis capabilities. Proteolysis, the process of protein breakdown, occurs when proteases break the peptide bonds between amino acids, leading to the formation of smaller peptides and amino acids. Proteolytic enzymes are ubiquitous in all life forms and play crucial roles in cellular development and metabolism. Proteases act as hydrolytic catalysts, converting proteins into peptides and amino acids, which are essential for various biological processes (Zang et al.201, Purohot et al. 2021, Dhillon et al. 2016).

Pectinase is an enzyme complex that catalyzes the breakdown of pectin into simpler compounds, primarily galacturonic acids. Pectinases are widely used in various industries, including food and medicine, as probiotic enzymes (Mulluye et al. 2022).

Agarase is an enzyme that degrades agar, a polysaccharide derived from seaweed, into oligosaccharides and neoagarooligosaccharides. Agar consists mainly of agarose, a linear polymer, and agaropectin. The agarolytic activity of agarase can be detected using Lugol's solution which is visualized by pale yellow zones surrounding colonies on a reddish-brown background (Panchal et al. 2019).

Cellulose biodegradation is mediated by enzymes called cellulases, which catalyze the bioconversion of cellulose to soluble sugars, including glucose. The substrate used for cellulase activity is a cellulose derivative that is carboxymethyl cellulose (CMC), also known as cellulose gum (Niranjan et al. 2017; Bhat et al.1997).

Lipases are another important group of enzymes. Polyoxyethylene sorbitan monooleate, commonly known as Tween 80, is a non-ionic surfactant that contains a mono-unsaturated fatty acid. Tween 80 is known to interfere with the permeability of cell membranes, making them more permeable, which can enhance the nutritional input from the surroundings to the cell body. Additionally, Tween 80 is used as a substrate for lipase activity in various enzymatic assays. Lipases are enzymes that hydrolyze fats into fatty acids and glycerol, and Tween 80 can serve as a source of fatty acids in these assays. The presence of Tween 80 can increase the solubility of lipids and aid in the activity of lipases, making it a commonly used substrate in lipase assays (Taoka et al. 2011).

Gelatinases hydrolyze gelatine through a two-step process, first breaking it down into polypeptides and then further degrading these polypeptides into amino acids. Gelatine is commonly used as a substrate to assay for gelatinase activity (Ekpenyong et al. 2016).

Chitin is the second most abundant linear polymer composed of β-1,4-linked N-acetylglucosamine (GlcNAc) units. It is present in all major groups of microorganisms, including bacteria, fungi, plants, and animals. Chitinase is an enzyme that contains glycosyl hydrolases and catalyzes the degradation of chitin to its oligomers (chitooligosaccharides) and monomers (N-acetylglucosamine). Chitin is the major structural component in the exoskeletons of crustaceans, crabs, and shrimps (Thirumurugan et al. 2015).

1.2 **Aim and Objectives**

Aim: This thesis aims to investigate the multiple enzyme production by Thraustochytrid isolates and to evaluate the enzyme activity of Thraustochytrids.

Objectives:

- To check the multiple enzyme production by Thraustochytrid isolates.
- To check the enzyme activity of Thraustochytrids using crude enzymes with different substrate concentrations and at different time intervals.

1.3 Hypotheses

- Thraustochytrid can produce a diverse range of enzymes.
- The enzyme activity of Thraustochytrid varies significantly with different substrate concentrations or at different time intervals

1.5 Scope

- This study will help to understand the multiple enzyme profiles of thraustochytrids from different habitats and help unravel their functional diversity.

CHAPTER 2. LITERATURE REVIEW

Thraustochytrids are marine protists classified under the phylum Heterokonta and Class Labyrinthulomycota of the Kingdom Stramenopila, within the SAR domain (Damare 2019). They are found in diverse marine habitats, including rocky coastlines, coral reefs, and coastal waters, as well as in plant detritus and zooplankton fecal pellets (Raghukumar and Raghukumar 1999). Thraustochytrids play a vital role in the marine ecosystem by producing omega-3 polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid (DHA), and participating in the breakdown and mineralization of organic materials through extracellular enzymatic decomposition (Gupta et al. 2015; Bongiorni et al. 2005).

These organisms have a specialized structure known as the Ectoplasmic Network (EN), which enhances the cell surface area and contains surface-bound hydrolytic enzymes for digesting organic material (Raghukumar 2002; Jain et al. 2005). Thraustochytrids also produce exopolysaccharides (EPS), which aid in nutrient concentration and maintain exoenzyme activity through extensive matrices, contributing to their role as marine detritus degraders (Jain et al. 2005). The ability of Thraustochytrids to break down highly refractory organic compounds using unique degradative enzymes highlights their ecological significance in marine ecosystems (Bongiorni et al. 2005; Jain et al. 2008).

Isolation of Thraustochytrids for research purposes typically involves media containing peptone as a nitrogen source, yeast extract, and glucose as a carbon source, with the modified Vishniac (MV) medium being commonly used (Damare 2019). While much research has focused on the fatty acid production and ecological roles of Thraustochytrids, studies on their extracellular enzymes, including amylase, are relatively limited (Bongiorni et al 2005, Damare & Raghukumar 2006 ; Taoka et al. 2009; Damare et al. 2015; Shirodkar et al. 2017).

These enzymes have potential applications in various industries due to their ability to convert long-chain carbohydrates into simpler sugars, highlighting the importance of further research into the enzymatic capabilities of Thraustochytrids (Shirodkar et al. 2017).

Thraustochytrids, known for their production of extracellular enzymes, including protease, amylase, lipase, and chitinase, in marine ecosystems, focusing on their enzymatic capabilities and their role in the marine food chain (Taoka et al. 2009; Damare and Raghukumar 2006).

Various thraustochytrid strains examined extracellular enzyme production in different thraustochytrid strains, detecting enzyme activities like protease, lipase, phosphatase, urease, and -glucosidase. Agar plates were used to compare enzyme activity, showing varying strengths among strains. Enzyme assays were conducted for protease, amylase, lipase, chitinase, and -glucosidase, with positive results determined by specific criteria (Taoka et al. 2009).

Thraustochytrids were also studied for their enzymatic activities, observing their production of protease, lipase, amylase, and chitinase enzymes by examining the zone of clearance after 5 days of inoculation (Damare and Raghukumar 2006). The study found that Thraustochytrids produce various enzymes, with amylase only found in the *Thraustochytrium* genus. Enzyme activities vary among strains, showcasing their diverse capabilities. Cellulase was not detected in any tested strains, suggesting a potential inability to utilize cellulose as a carbon source. Chitinase was only detected in *T. striatum* (Taoka et al. 2009). Thraustochytrids lack lipase, amylase, or chitinase enzymes (Damare and Raghukumar 2006).

Thraustochytrid enzymatic activities and their potential contribution to organic matter degradation in marine environments highlight the wide range of ecto- and exo-enzymatic activities involved in protein, carbohydrate, and lipid breakdown, as well as the potential effects on organic matter cycling in benthic environments. The study also examines the enzymatic profiles of 11 thraustochytrid strains isolated from different marine substrates, indicating That enzymatic pools were similar among all strains, with a good production of lipase and a selection of protease, but a poor pool of carbohydrate degradation enzymes. The study also found that different strains displayed different activities for the investigated enzymes and that the ecto-enzymatic activity per thraustochytrid cell was higher than that of marine bacteria. The study concluded that thraustochytrids are contributors to the degradation of highly refractory organic compounds in marine environments (Bongiorni et al. 2005)

One of the species of Thraustochytrids that is *Thraustochytrium aureum* exhibited enhanced growth in the presence of Tween 80, leading to an increase in lipid accumulation. The aerobic desaturase-elongase pathway catalyzes fatty acid metabolism in *T. aureum*. Two biosynthetic pathways for DHA production exist in Thraustochytrids, including *T. aureum*. The DHA yield per culture broth increases significantly with Tween 80, possibly due to the activation of fatty acid synthesis pathways (Taoka et al. 2011).

Another study aimed to identify enzymes secreted by *T. striatum*, quantify cell growth and enzyme production, and correlate enzyme production with extracellular polymeric substances (Bongiorni et al. 2005; Taoka et al. 2009).

T. striatum produces a complex enzyme system with multiple polysaccharides. The study monitored EPS production alongside enzyme production to understand the relationship between enzyme and EPS production, providing insights into *T. striatum's* metabolic activities. The study quantified the daily enzyme activities of T. striatum during cultivation, induced by six enzyme-specific polysaccharides and carbon-starvation conditions. The daily activities and dissolved cell mass were determined over 7 days. The detection of extracellular enzymes was done using agar plates with specific enzyme assays. The study quantified T. *striatum* was found to produce a complex enzyme system, including amylase, lipase, cellulase, xylanase, chitinase, pectinase, and carrageenase, capable of degrading macromolecules in marine environments. Its extracellular enzymes are constitutive, with glucose being more effective than polysaccharides. No significant enzyme activity was observed for xylanase under high salinity conditions, suggesting a limitation in enzyme production (Xia et al 2018).

It identified various enzymes, including alkaline phosphatase, esterase, lipase, and leucine arylamidase, contributing to a deeper understanding of their potential in marine ecosystems Thraustochytrids were screened for enzymatic activities using a commercial enzyme kit, identifying valine arylamidase, cysteine arylamidase, acid phosphatase, napthol-AS-BI-phosphohydrolase, a-galactosidase, and b-galactosidase. Additional enzyme activities like b-glucuronidase, a-glucosidase, and a-mannosidase were also screened. Thraustochytrids display various enzymatic activities, including alkaline phosphatase, esterase, lipase, and leucine arylamidase. They also have valine arylamidase, cysteine arylamidase, acid phosphatase, napthol-AS-BI-phosphohydrolase, a-galactosidase, and b-galactosidase. However, certain enzyme activities, such as b-glucuronidase, a-glucosidase, and a-mannosidase, were found to be negative (Byreddy 2016).

Moreover, the study on Thraustochytrids examined their production of eight extracellular polysaccharide-degrading enzymes, including agarase, amylase, cellulase, xylanase, and pectinase. It monitored these enzymes' activities and quantified their capabilities. The findings offer insights into Thraustochytrids' enzymatic potential and their ability to degrade polysaccharides, potentially benefiting biotechnological applications. Isolates were grown in MV broth medium, and culture supernatants were used for crude enzyme extraction. Enzyme

activities like agarase, cellulase, xylanase, and pectinase were quantified under specific conditions. Various carbon sources were used for analysing enzyme production. Some isolates showed no activity in cellulose powder (Devasia and Muraleedharan 2012).

One study focused on enzymes like alkaline phosphatase, phytase, and urease, highlighting their potential roles and characteristics The study found that all strains of thraustochytrid bacteria had lipase, protease, and alkaline phosphatase, while amylase, cellulase, and phytase were not detected. Urease activity was found only extracellularly, and L-asparaginase and a-glucosidase were observed in crude cell lysates. (Lin et al. 2020).

Furthermore, a study explored the influence of a molasses-based medium on extracellular enzyme production in Thraustochytrids, focusing on protein digestion enzymes. Results showed a significant influence on enzymes like leucine aryl-amidase, valine aryl-amidase, acid phosphatase, and naphthol AS-BI phosphohydrolase, suggesting the need for optimized culture conditions for enhanced enzyme production. However, Thraustochytrids strains experienced significant inhibition of growth and lipid production in molasses medium but regained post-acclimatization. Enzyme activities, including leucine aryl-amidase and valine aryl-amidase, were suppressed in molasses medium. The medium's lower sugar content affected enzyme activities, while its high organic-nitrogen and total nitrogen content influenced enzyme production (Taoka 2023).

Another study explores the production of extracellular polysaccharide-degrading enzymes by Thraustochytrids from coastal and mangrove habitats, focusing on α-amylase activity. It confirms the production of α-amylases by specific isolates, highlighting their importance in coastal and mangrove habitats in Goa to produce various hydrolytic enzymes, including α-amylases, significant in biotechnology. These unique characteristics suggest potential commercial applications, without any negative results (Shirodkar et al.2017).

Lastly, a study aimed to identify and characterize extracellular proteases in Thraustochytrids induced by nitrogen-limited conditions. The primary trigger for protease activity was nitrogenous nutrition, not carbon sources. The study identified a peptidase S8 family protein and its paralogous protein. Multiple types of proteases can be induced, secreted, and degraded without suitable substrates, leading to decreased proteolytic activity over time (Man et al. 2022).

CHAPTER 3. METHODOLOGY

1. Source of Thraustochytrids Cultures

Thraustochytrid cultures were provided by Dr. Varada Damare. The cultures were grown in Modified Vishniac (MV) broth at room temperature on a shaker for 3-4 days at 105 rpm. After growing the cultures, they were checked under a microscope for purity and then used for further analysis.

Table 3.1: Thraustochytrid isolates

OMD 2	OMD-4
OMS 2	MC-4
5 LONG PADINA	DB SARG
RD 1	OMD-1
8 B RED	OMD-3
OGS 2	A 3 BROWN
ZB 6	9 B
OMS-4	MC-1

2. Multiple Enzyme Screening of Thraustochytrid isolates

i. Amylase Activity

A) On MV Agar

The MV agar was prepared and autoclaved, Once cooled, 1% starch solution (separately autoclaved) and 1 ml chloramphenicol were added to the agar, which was then poured into petri dishes and left to solidify. The plates were streaked with previously grown culture in MV broth of 3-4 days and then incubated at room temperature for 3-4 days. After incubation, the plates were observed for growth. Iodine solution was then added to the plates to visualize starch hydrolysis, indicated by clear zones around the growth where the starch had been hydrolyzed.

B) In MV Broth

In MV broth, 10 ml of autoclaved MV broth was placed in a 50 ml centrifuge tube. To this, 1% starch solution, 0.1 ml of chloramphenicol, and 0.1 ml of culture previously grown in MV broth of 3–4 days. The contents were thoroughly mixed and incubated at room temperature for 3-4 days at 105 rpm. After the incubation period, iodine solution was added to the tube. The color of the solution was observed, colorless indicating a positive result for amylase activity. A control tube with no culture was also prepared and treated in the same manner to serve as a comparison for the results (Damare et al. 2006).

ii. Protease Activity

A) On MV Agar

The MV agar was prepared and autoclaved, Once cooled, 1% milk powder solution (separately autoclaved) and 1 ml chloramphenicol were added to the agar, which was then poured into petri dishes and left to solidify. The plates were streaked with previously grown culture in MV broth of 3-4 days and then incubated at room temperature for 3-4 days. After incubation, the plates were observed for growth. Congo red solution was then added to the plates to visualize the zone of clearance of milk around the colony.

B) In MV Broth

In MV broth, 10 ml of autoclaved MV broth was placed in a 50 ml centrifuge tube. To this, 1% Milk solution, 0.1 ml of chloramphenicol, and 0.1 ml of culture previously grown in MV broth of 3–4 days. The contents were thoroughly mixed and incubated at room temperature on a shaker for 3-4 days at 105 rpm. After the incubation period, congo red solution was added to the tube. The color of the solution was observed. A control tube with no culture was also prepared and treated in the same manner to serve as a comparison for the results (Damare et al.2006 ; Bhat et al. 2014).

iii. Pectinase Activity

The MV agar was prepared and autoclaved, Once cooled, 1% pectin solution (separately autoclaved) and 1 ml chloramphenicol were added to the agar, which was then poured into Petri dishes and left to solidify. The plates were streaked with previously grown culture in MV broth of 3-4 days and then incubated at room temperature for 3-4 days. After incubation, the plates were observed for growth. Congo red solution was then added to the plates to visualize the zone of clearance around the colony (Shirodkar et al. 2017).

iv. Agarase Activity

The MV agar was prepared and autoclaved, 1 ml chloramphenicol were added to the agar, which was then poured into petri dishes and left to solidify. The plates were streaked with previously grown culture in MV broth of 3-4 days and then incubated at room temperature for 3-4 days. After incubation, the plates were observed for growth. Lugol's iodine solution was then added to the plates to visualize zone of clearance around the colony (Shirodkar et al. 2017).

v. Cellulase Activity

The MV agar was prepared and with 1% CMC (prepared in 30ml of 95 % ethanol) was added and then autoclaved, and 1 ml chloramphenicol were added to the agar, which was then poured into petri dishes and left to solidify. The plates were streaked with previously grown culture in MV broth of 3-4 days and then incubated at room temperature for 3-4 days. After incubation, the plates were observed for growth. Congo red solution was then added to the plates to visualize zone of clearance around the colony (Devasia et al. 2012).

vi. Lipase Activity

MV agar was prepared and 1% Tween 80 with 0.1g $CaCl_2$ was added and then autoclaved, and 1 ml chloramphenicol were added to the agar, which was then poured into petri dishes and left to solidify. The plates were streaked with previously grown culture in MV broth for 3-4 days and then incubated at room temperature for 3-4 days. After incubation, the plates were observed for growth. Slight precipitation around the colony indicates a positive result (Damare et al. 2006).

vii. Gelatinase Activity

MV agar was prepared and autoclaved, Once cooled, 1% gelatin solution (separately autoclaved) and 1 ml chloramphenicol were added to the agar, which was then poured into

sterile petri dishes and left to solidify. The plates were streaked with previously grown culture in MV broth of 3-4 days and then incubated at room temperature for 3-4 days. After incubation, the plates were observed for growth. The plates to visualize clear zones around the growth (Taoka et al. 2014).

viii. Chitinase Activity

Shrimp exoskeleton was dried for 6-7 days. Seven grams of the dried shrimp exoskeleton were weighed and treated with 50% sulfuric acid, allowing the mixture to cool down to room temperature. Next, 110 ml of cold distilled water was added to the acid-treated mixture, which was then refrigerated overnight. The suspension was centrifuged at 4500 rpm for 20 minutes at 15°C. After centrifugation, the precipitate was divided into two centrifuge tubes and centrifuged again. The pH of cold distilled water was adjusted to 7, and the precipitate was suspended in the adjusted water. Centrifugation was repeated until the pH of the supernatant reached 7.

After adjusting the pH of the suspension to 7, the suspension was autoclaved separately. Following autoclaving, 1% of the suspension was incorporated into autoclaved MV agar along with 1% chloramphenicol. The agar was then poured into plates and allowed to solidify. The plates were streaked with previously grown culture in MV broth of 3-4 days and then incubated at room temperature for 3-4 days. After incubation, the plates were examined for the zone of clearance (Damare et al. 2006).

Fig.3.1: After treating dried shrimp with acid

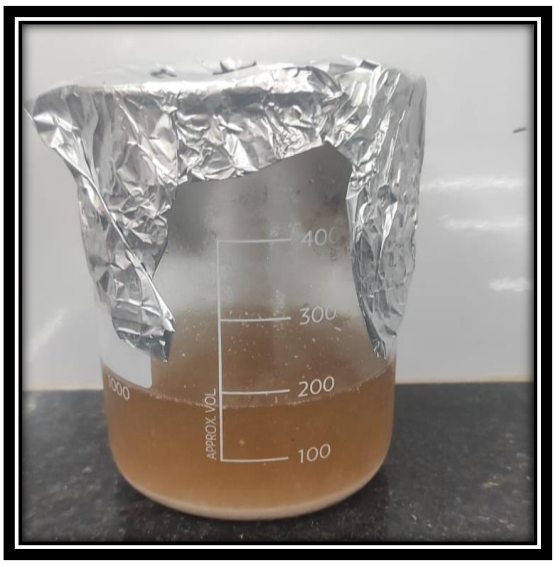


Fig.3.2: After adding cold distilled water

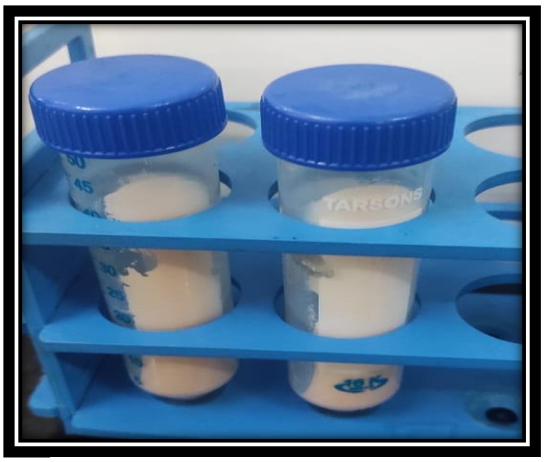


Fig.3.3: After centrifugation

2. Amylase production by thraustochytrid isolate (OMD4)

3A. Growth of the isolate in the presence of starch

1% starch solution and 0.1 ml of the culture previously grown in MV broth were added to 10 ml of autoclaved MV broth. The contents were mixed thoroughly and incubated at room temperature at 105 rpm for 3-4 days to allow for enzyme production.

3B. Quantitative estimation of amylase production (Enzyme activity of the crude enzyme at various substrate concentrations and at different incubation times)

A. Extraction of Crude Enzyme

After the incubation period, the broth was centrifuged at 4500 rpm for 20 minutes at 15°C to separate the supernatant containing the crude enzyme from the cell debris. The supernatant was then carefully collected and stored in a freezer for further use. Prior to use, the frozen supernatant was thawed to room temperature.

B. Quantitative estimation of amylase production

Various substrate concentrations (0.01%, 0.05%, 0.07%, 0.1%, 0.5%, and 0.8%) also control of 0.01% and 0.8% (without enzyme) were prepared, and Phosphate buffer (pH 7.3) was added to each to create the reaction mixtures. The enzymatic reactions were initiated by adding the enzyme to each reaction mixture, and a timer was set immediately. After 10, 20, and 30 minutes of incubation, 1 ml of DNSA reagent was added to each reaction tube to stop the reaction. The reaction tubes were then placed in a water bath and incubated for an additional 5 minutes in the boiling water bath to develop color. Subsequently, the absorbance of each reaction mixture

was measured at 540 nm using a spectrophotometer to determine the rate of the enzyme-catalyzed reaction at different substrate concentrations and time points. A standard curve of DNSA was prepared prior to this for the estimation of sugar to find out the residual sugar after the enzyme reaction. Maltose was used to prepare the standard.

3C. Standard curve of DNSA using Maltose

Seven sterile test tubes were prepared, and six different concentrations of maltose stock solution (5%) were prepared by diluting it with distilled water to achieve concentrations of 0.01%, 0.05%, 0.07%, 0.1%, 0.5%, 0.8%, and control. One ml of DNS (3,5-dinitrosalicylic acid) reagent was transferred to a test tube. The test tubes were then placed in a water bath at 100°C for 5 minutes. After incubation, the tubes were cooled, and the absorbance of each solution was measured at 540 nm using a spectrophotometer (Kiran et al. 2005).

CHAPTER 4. ANALYSIS AND CONCLUSIONS

2. Multiple Enzyme Screening of Thraustochytrid isolates

i. AMYLASE ACTIVITY

Amylases are enzymes that catalyze the hydrolysis of starch into simpler sugars such as maltose and glucose. The amylase activity of 16 cultures was assessed using plate and broth assays to determine their starch-degrading capabilities.

a) On MV agar

Of the 16 Thraustochytrid cultures tested for amylase activity using the plate assay method, 13 exhibited positive results. A positive result was indicated by a clear zone around the colony, indicating starch hydrolysis. This suggests that these cultures can produce amylase enzymes

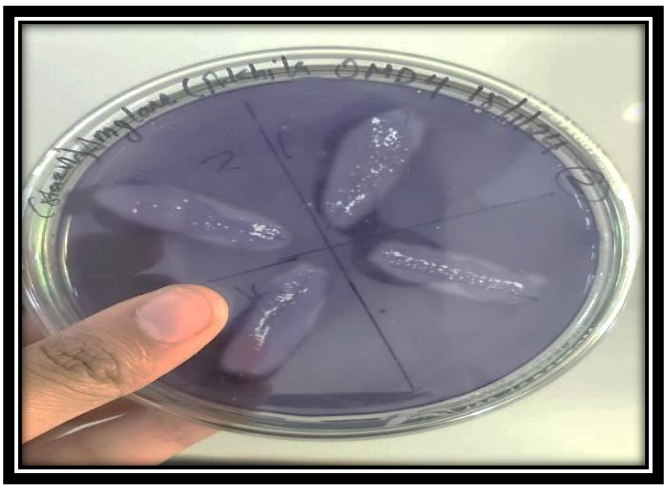


Fig.4.1: Positive Amylase activity for OMD4 after 3 days of incubation at R.T.

Fig.4.2: Positive Amylase activity for OMS4 after 3 days of incubation at R.T.

b) In MV broth

In the MV broth assay, 9 Thraustochytrid cultures tested positive for amylase activity. A positive result was observed as the solution was colourless after the addition of iodine. This further confirms the amylase-producing capability of these 9 cultures.

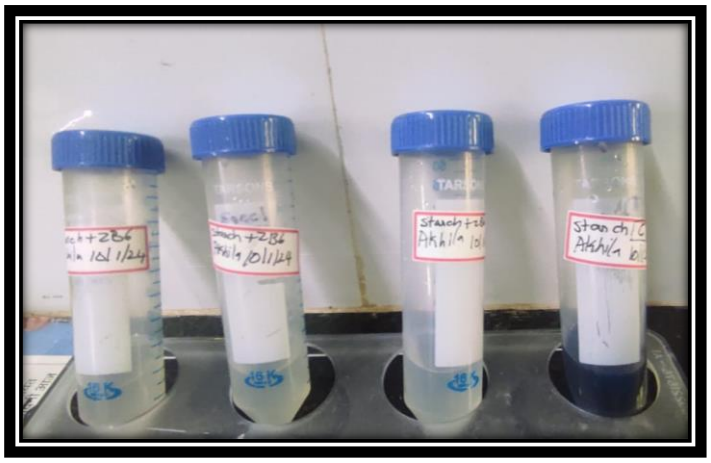


Fig.4.3: Positive results for amylase activity in broth for ZB 6 after 5 days of incubation at R.T

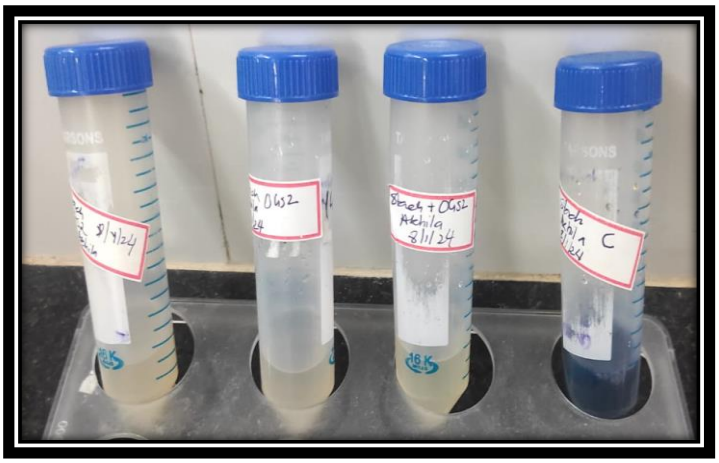


Fig.4.4: Positive results for amylase activity in broth for OGS 2 after 3 days of incubation at R.T

ii. Protease activity

Proteases are enzymes that hydrolyze peptide bonds in proteins, leading to their degradation. In this study, the protease activity of 16 cultures was assessed using plate and broth assays to determine their protein-degrading capabilities.

a) On MV agar

Out of the 16 cultures tested, 11 exhibited positive results for protease activity on the plate assay. A zone of clearance of milk around the colony was observed indicating protein hydrolysis.

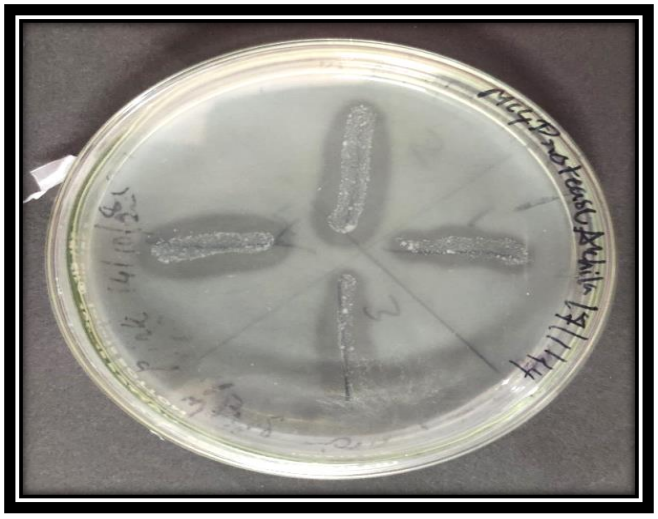


Fig.4.5: Positive result for protease activity for MC 4 after 4 days of incubation at R.T

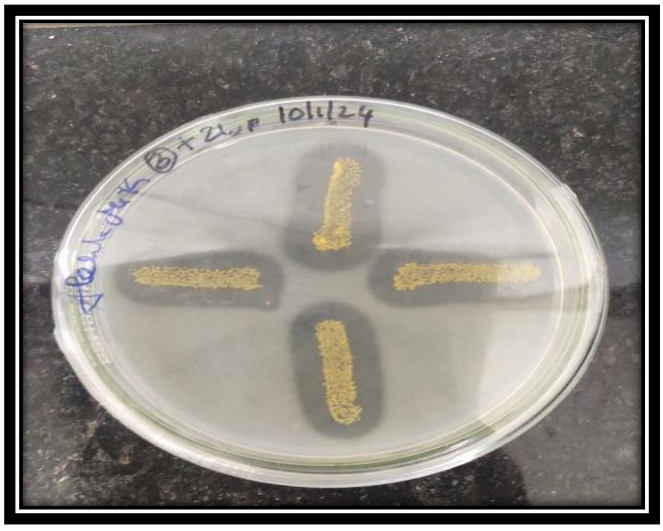


Fig.4.6: Positive results for protease activity for ZB6 after 5 days of incubation at R.T

b) In MV broth

In the MV broth assay, 9 Thraustochytrids culture tested positive for protease activity. The cultures showed variations in colors, indicating protein degradation. Notably, culture ZB6 showed colorlessness after the addition of Congo red, suggesting strong protease activity.

Fig.4.7: Positive result for protease activity in broth for ZB6 after 5 days of incubation at R.T.

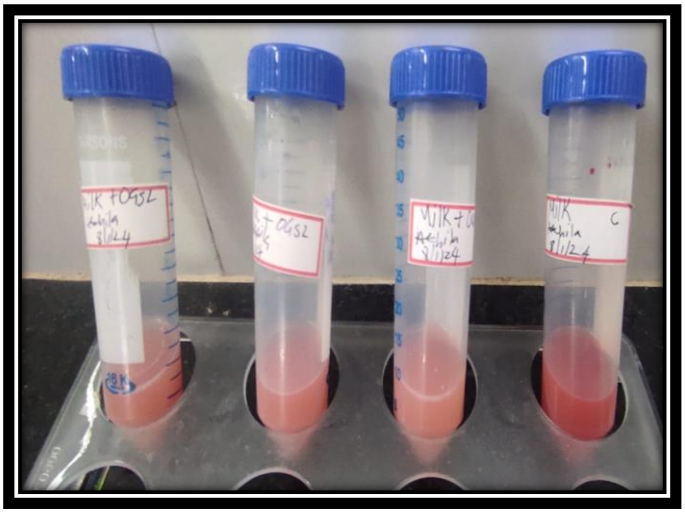


Fig.4.8: light pink colouration positive results for protease activity in broth for OGS2 after 3 days of incubation at R.T.

iii. Agarase Activity

- Agarase is an enzyme that hydrolyzes agarose, the main component of agar, into oligosaccharides. The agarase activity of 11 cultures was assessed using plate assays.
- Out of the 11 cultures tested, cultures OMS4 and OMD3 showed positive results for agarase activity on the plate assay. The presence of pale-yellow zones around colonies against a reddish-brown background after adding Lugol's iodine was considered indicative of agarolytic activity**.**

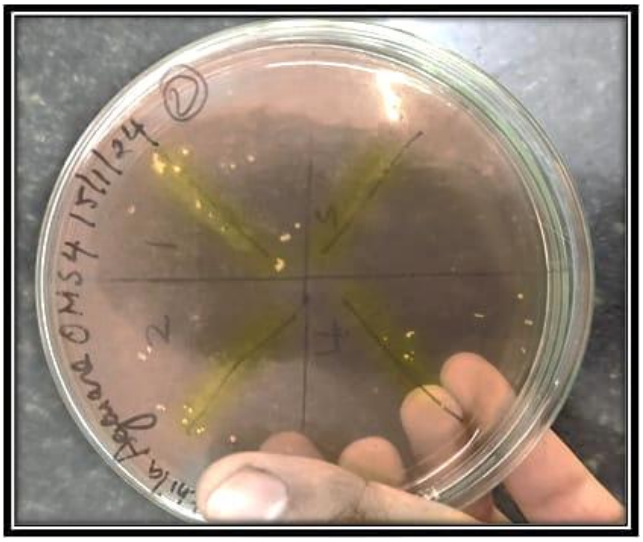


Fig.4.9: Positive results for agarase activity for OMS 4 after 5 days of incubation at R.T

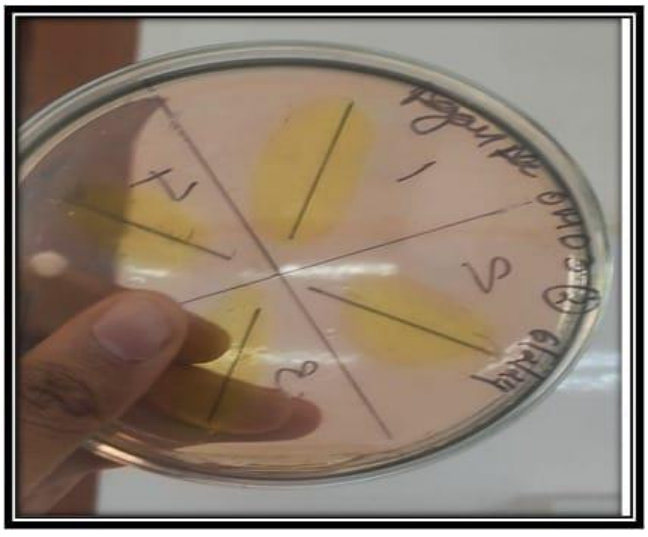


Fig.4.10: Positive results for agarase activity for OMD3 after 3 days of incubation at R.T

Table. 4.1: Multiple Enzyme Screening of Thraustochytrids Cultures

key: -

"-" - indicates that the corresponding isolate has not been tested for the enzyme

sr.no.	thraustocytrid isolates	RESULTS									
		AMYALSE		PROTEASE		PECTINASE	AGARASE	GELATINASE	LIPASE	CELLULASE	CHITINASE
		MV TUBES	PLATES	MV TUBES	PLATES						
1	OMD 2	POSITIVE	POSITIVE	POSITIVE	POSITIVE	-	-	-	-	-	-
2	OMS 2	POSITIVE	POSITIVE	POSITIVE	POSITIVE	-	-	-	-	-	-
3	5 long padina	-	POSITIVE	-	POSITIVE	-	-	-	-	-	-
4	RD1	POSITIVE	POSITIVE	POSITIVE	POSITIVE	-	-	-	-	-	-
5	8BRED	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NO GROWTH	NEGATIVE	NEGATIVE	NO GROWTH	NEGATIVE	-
6	OGS 2	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	-	NEGATIVE	-	-	-
7	ZB6	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	NO GROWTH	NO GROWTH	NEGATIVE	NO GROWTH	-
8	OMS4	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	POSITIVE	-	-	-	-
9	OMD4	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE
10	MC4	POSITIVE	POSITIVE	POSITIVE	POSITIVE	NEGATIVE	NEGATIVE	-	-	-	-
11	DB SARG	-	NO GROWTH	-	NO GROWTH	NO GROWTH	NO GROWTH	NO GROWTH	NO GROWTH	NO GROWTH	-
12	OMD 1	-	POSITIVE	-	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	-
13	OMD 3	-	POSITIVE	-	NEGATIVE	NEGATIVE	POSITIVE	NEGATIVE	NEGATIVE	NEGATIVE	-
14	A3 BROWN	-	POSITIVE	-	POSITIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	-
15	9B	-	NO GROWTH	-	NO GROWTH	NO GROWTH	NEGATIVE	NEGATIVE	NEGATIVE	NEGATIVE	-
16	MC1	-	NO GROWTH	-	NO GROWTH	NO GROWTH	NO GROWTH	NEGATIVE	NEGATIVE	NEGATIVE	-

3. Amylase production by thraustochytrid isolate (OMD4)

B. Quantitative estimation of amylase production

- Using the following formula enzyme activity was calculated of amylase using maltose as standard.

$$\textbf{Enzyme activity } \mu\text{mol /min} = \frac{\mu\text{g of product released x 1000}}{\text{Molecular weight of product x incubation time}}$$

Maltose m.w. (342.3g/mol)

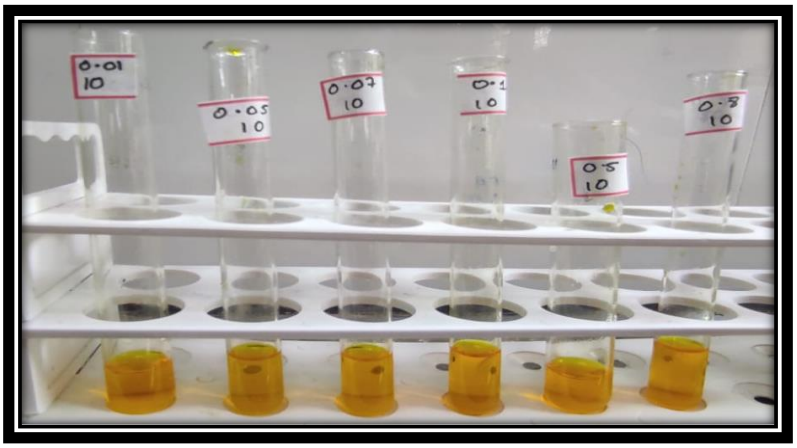


Fig.4.11: Amylase activity of different substrate concentrations for 10 min.

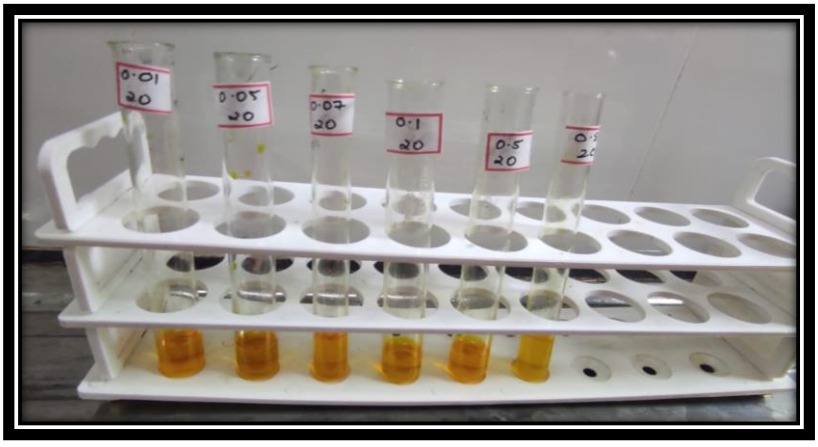


Fig.4.12: Amylase activity of different substrate concentrations for 20 min.

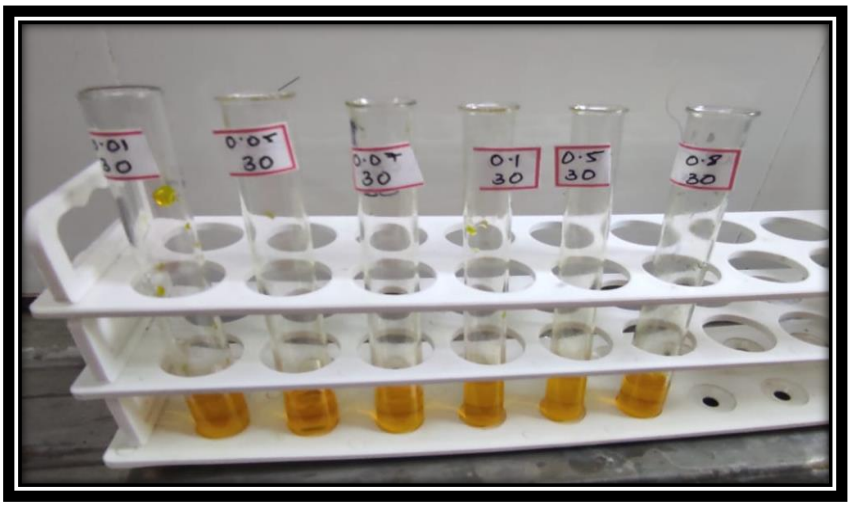


Fig.4.13: Amylase activity for different substrate concentrations for 30 min.

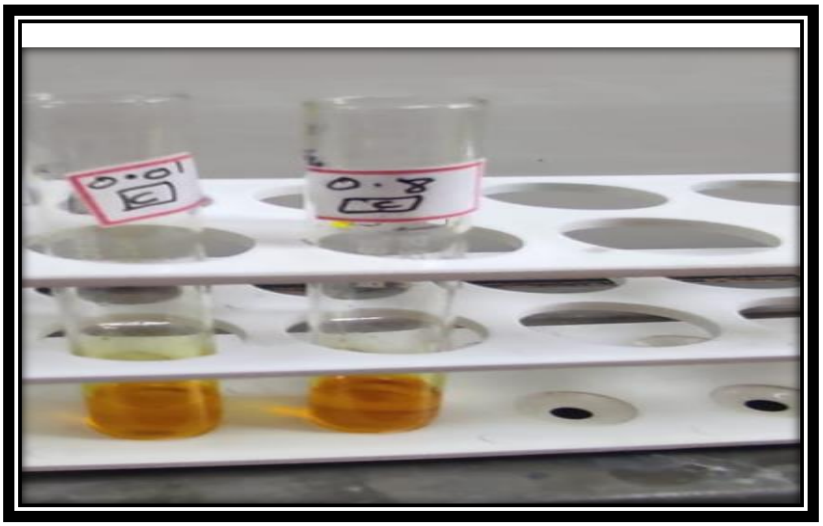


Fig.4.14: Controls of 0.1% and 0.8% substrate concentration without enzyme.

Table 4.2: Amylase activity at different substrate concentrations after 10min.

Substrate concentration (%)	Absorbance at 540 nm after 10 min	Using the standard of maltose	Enzyme activity µmol/min
0.01	0.3317	0.298263279	0.087135051
0.05	0.2234	0.189963279	0.055496138
0.07	0.2903	0.256863279	0.075040397
0.1	0.2944	0.260963279	0.076238177
0.5	0.3394	0.305963279	0.08938454
0.8	0.2974	0.263963279	0.077114601

Table 4.3: Amylase activity at different substrate concentrations after 20min.

Substrate concentration (%)	Absorbance at 540 nm after 20 min	Using the standard of maltose	Enzyme activity µmol/min
0.01	0.3199	0.286463279	0.041843891
0.05	0.3781	0.344663279	0.050345206
0.07	0.3163	0.282863279	0.041318037
0.1	0.3068	0.273363279	0.039930365
0.5	0.3372	0.303763279	0.044370914
0.8	0.3758	0.342363279	0.050009243

Table 4.4: Amylase activity at different substrate concentrations after 30 min

Substrate concentration (%)	Absorbance at 540 nm after 30 min	Using the standard of maltose	Enzyme activity µmol/min
0.01	0.2136	0.180163279	0.017544384
0.05	0.1709	0.137463279	0.013386238
0.07	0.1879	0.154463279	0.015041706
0.1	0.1838	0.150363279	0.014642446
0.5	0.2538	0.220363279	0.021459079
0.8	0.1594	0.125963279	0.012266363

Table 4.5: Control of 0.001% and 0.8%

Substrate concentration (%) controls	Absorbance at 540 nm	Using the standard of maltose
0.01	0.2987	0.265263
0.8	0.4411	0.407663

3C. Standard curve of DNSA using Maltose

A standard curve was constructed using absorbance readings at 540 nm to quantify the amylase activity. A linear regression analysis was performed on the absorbance values of the standards to generate a standard curve equation ($y = 5.5777x + 0.1865$ ($R^2 = 0.9814$)). This equation was then used to calculate the enzyme activity of OMD4 isolates based on their absorbance readings.

Table 4.6: The absorbance of different maltose concentrations for standard

Maltose concentration (%)	Absorbance at 540nm
0.01	0.0079
0.05	0.7632
0.07	0.6991
0.1	0.4651
0.5	3.202
0.8	4.5155

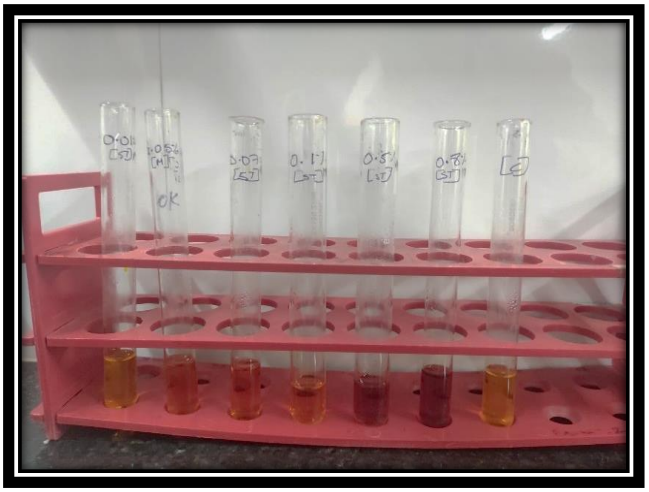
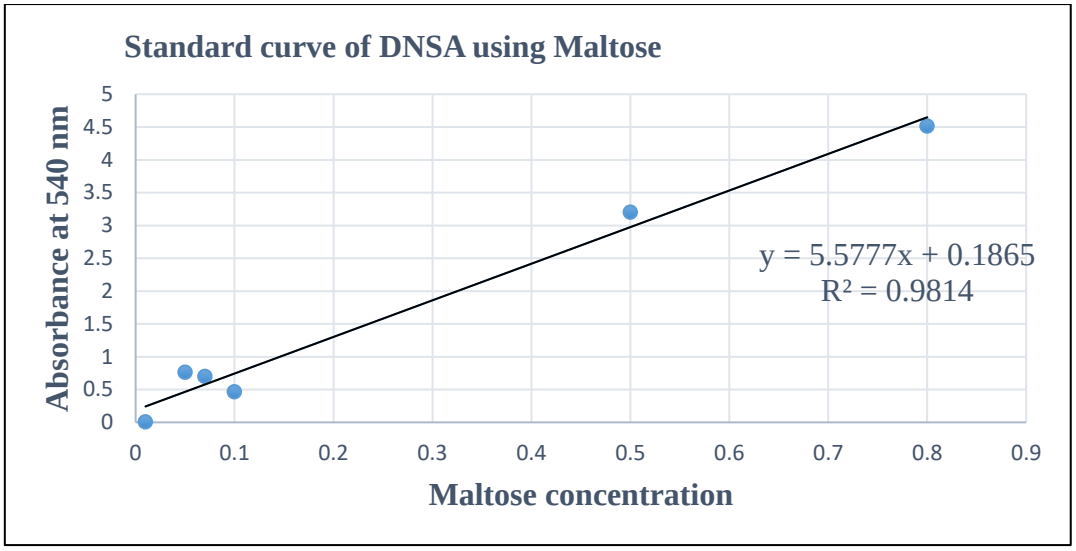


Fig.4.15: Different maltose concentrations for the standard curve.

Graph 4.1: Standard curve of DNSA using maltose



- For all the different substrate concentration the enzyme's activity peaked at 10 minutes, then decreased at 20-30 minutes, suggesting an optimal reaction time of 10 minutes. Depletion of the substrate could indicate active substrate conversion, reducing the reaction rate.
- At 0.5% substrate concentration, the highest amount of product was released at 10 minutes, with decreasing amounts at 20 and 30 minutes. Additionally, the enzyme activity was highest at 10 minutes and then decreased at 20 and 30 minutes. This suggests that the enzyme was most active or efficient at 10 minutes and that its activity decreased over time.
- Therefore, it would be appropriate to conclude that 0.5% is the most optimal substrate concentration at 10 min for amylase under the conditions tested.

CONCLUSION

The screening of Thraustochytrid isolates for multiple enzymes revealed a predominant production of amylase and protease enzymes on MV agar and MV broth, with limited agarase activity detected in OMD 3 and OMS 4 cultures on MV agar. Conversely, there was an absence of activities for lipase, gelatinase, cellulase, chitinase, and pectinase among the studied isolates. Additionally, the study of amylase production by Thraustochytrid isolate OMD4 demonstrated an optimal reaction time of 10 minutes, with the highest enzyme activity and product yield observed at 0.5% substrate concentration providing valuable insights into the biotechnological capabilities of Thraustochytrids. The present study provides valuable insights into the enzymatic diversity of Thraustochytrids and highlights the need for further exploration to understand their metabolic potential fully.

DISCUSSION

Thraustochytrid is a marine osmo-heterotrophic protist (Bongiorni et al.2005) which is believed to aid in the degradation of highly refractory organic compounds by producing unique degradative enzymes (Bongiorni et al.2005; Jain et al.2008). Earlier studies examined extracellular enzyme production in different thraustochytrid strains, including protease, amylase, lipase, cellulase, gelatinase, agarase, pectinase, and chitinase, in marine ecosystems, focusing on their enzymatic capabilities and their role in the marine food chain (Taoka et al. 2009; Damare and Raghukumar 2006; Shirodkar et al. 2017). Contrary to these findings, the present study revealed that Thraustochytrid isolates primarily produce amylase and protease enzymes. Two cultures only exhibited agarase activity also. However, the activities for lipase, gelatinase, cellulase, chitinase, and pectinase were absent among the studied isolates isolated from different marine habitats.

Additionally, one of the studies showed that starch was the primary carbon source for maximum α-amylase production in *Bacillus* sp. K-12 leading to high enzyme activity levels (Kiran et al. 2015). In this study, it was shown that the enzyme activity of Thraustochytrids varies significantly with different substrate concentrations at different time intervals. The enzyme's activity peaked at 10 minutes, decreasing over 20-30 minutes, suggesting an optimal reaction time of 10 minutes. At 0.5% substrate concentration, the highest product was released at 10 minutes, with decreasing amounts at 20 and 30 minutes. Therefore, 0.5% is the optimal substrate concentration at 10 min for amylase.

REFERENCES

1. Bhat, M. K., & Bhat, S. (1997). Cellulose degrading enzymes and their potential industrial applications. Elsevier Science Inc.Vol 15, pp. 583-620.
2. Bongiorni, L., Pusceddu, A., & Danovaro, R. (2005). Enzymatic activities of epiphytic and benthic Thraustochytrids involved in organic matter degradation. Aquatic Microbial Ecology, 41, 299-305.
3. Byreddy, A. R. (2016). Thraustochytrids as an alternative source of omega-3 fatty acids, carotenoids, and enzymes. Lipid Technology, 28(3–4).
4. Damare, V. S. (2019). Advances in isolation and preservation strategies of ecologically important marine protists, the Thraustochytrids.In: Meena and Naik M.M. (Eds) Advances in Biological Science Research Elsevier Inc.pp.485-500.
5. Damare, V. S., D'Costa, P. M., Shivaramu, M. S., Borges, V., Fernandes, M., Fernandes, C., Cardozo, S. (2020). Preliminary study on the response of marine fungoid protists, the Thraustochytrids, to lipid extracts of diatoms. Aquatic Ecology, 54, 355-367.
6. Damare, V., & Raghukumar, S. (2006). Morphology and physiology of the marine straminipilan fungi, the aplanochytrids isolated from the equatorial Indian Ocean. Indian Journal of Marine Sciences, 35(4), 326-340.
7. Devasia, V. L. A., & Muraleedharan, U. D. (2012). Polysaccharide-degrading enzymes from the marine protists, Thraustochytrids. Biotechnology, Bioinformatics, and Bioengineering, 2(1), 617-627.
8. Dhillon, A., Sharma, K., Rajulapati, V., & Goyal, A. (2017). Proteolytic Enzymes. Current Developments in Biotechnology and Bioengineering, 149–173.
9. Ekpenyong, M., Asitok, A., Odey, A., & Antai, S. (2016). Production and activity kinetics of gelatinase by Serratia sp. SLO3. Nigerian Journal of Biopesticides, 1(1), 70-82.
10. Gupta, A., et al. (2015). Exploring omega-3 fatty acids, enzymes, and biodiesel-producing Thraustochytrids from Australian and Indian marine biodiversity. Biotechnology Journal, 11.pp.1-10.
11. Jain, R., Raghukumar, S., Tharanathan, R., & Bhosle, N. B. (2005). Extracellular polysaccharide production by Thraustochytrid protists. Springer Science Business Media, 7, 184–192.

12. Kimura, H., Fukuba, T., & Naganuma, T. (1999). Biomass of thraustochytrid protoctists in coastal water. Marine Ecology Progress Series, 189, 27-33.
13. Kiran, Ö., Çömlekçioğlu, U., & Arikan, B. (2005). Effects of carbon sources and various chemicals on the production of a novel amylase from a thermophilic Bacillus sp. K-12. Turkish Journal of Biology, 29, 99-103.
14. Lin, H., Li, W.-H., & Cheng, T.-H. (2020). Diverse enzymes with industrial applications in four Thraustochytrid genera. Frontiers in Microbiology,11.pp.1-11.
15. Man, C. H., Shimura, Y., & Suzuki, I. (2022). Identification of extracellular proteases induced by nitrogen-limited conditions in the thraustochytrids Schizochytrium aggregatum ATCC 28209. Marine Biotechnology, 24, 243–254.
16. Monteiro de Souza, P., & Oliveira e Magalhães, P. (2010). Application of microbial α-amylase in industry – A review. Brazilian Journal of Microbiology, 41, 850-861.
17. Mulluye, K., & Atnafu, Y. (2022). Biotechnological application of pectinase. IJPSR, 13(8), 3069-3077.
18. Niranjane, A. P., Madhou, P., & Stevenson, T. W. (2007). The effect of carbohydrate carbon sources on the production of cellulase by *Phlebia gigantea*. Enzyme and Microbial Technology, 40, 1464-1468.
19. Panchal, H. K. (2019). Isolation and characterization of agarase-producing bacteria from the marine ecosystem of South Gujarat. International Journal of Pharmacy and Biological Sciences (IJPBS), 9(2), 09-13.
20. Purohit, A., Andhare, P., Marchawala, F., Bhattacharya, I., & Upadhyay, D. (2021). A review on protease. IJBPAS, April, Special Issue, 10(4), 269-277.
21. Raghukumar, S. (2002). Ecology of the marine protists, the Labyrinthulomycetes (Thraustochytrids and Labyrinthulids). European Journal of Protistology, 38, 127–145.
22. Raghukumar, S., & Raghukumar, C. (1999). Thraustochytrid fungoid protists in faecal pellets of the tunicate Pegea confoederata, their tolerance to deep-sea conditions and implication in degradation processes. Marine Ecology Progress Series, 190, 133-140.
23. Shirodkar, P. V., Muraleedharan, U. D., & Raghukumar, S. (2017). Production of extracellular polysaccharases by the marine protists, Thraustochytrids, with special reference to α-amylase activity. International Journal of Pharmaceutical and Biological Sciences, 8(2), 453-462.
24. Taoka, Y., Nagano, N., Okita, Y., Izumida, H., Sugimoto, S., & Hayashi, M. (2009). Extracellular Enzymes Produced by Marine Eukaryotes, Thraustochytrids. Bioscience, Biotechnology, and Biochemistry, 73(1), 180–182

25. Taoka, Y., Nagano, N., Okita, Y., Izumida, H., Sugimoto, S., & Hayashi, M. (2011). Effect of Tween 80 on the growth, lipid accumulation, and fatty acid composition of *Thraustochytrium aureum* ATCC 34304. Journal of Bioscience and Bioengineering, 111(4), 420–424.
26. Taoka, Y., Tai, N., Matsumoto, T., Sakai, K., & Goto, T. (2023). Growth, fatty acid composition, and extracellular enzyme production in the thraustochytrid, *Aurantiochytrium* cultured in molasses-based medium.1,22-11.
27. Thirumurugan, D., Sankari, D., & Vijayakumar, R. (2015). Screening of chitinase production and antifungal activity of Streptomyces sp. ACT7 from east coast region, South India. International Journal of Pharmaceutical Sciences and Research, 7(5), 38-41.
28. Xiao, R., Li, X., & Zheng, Y. (2018). Enzyme production by a fungoid marine protist, Thraustochytrium striatum. European Journal of Protistology, 66, 136–148.
29. Zhang, X., Shuai, Y., Tao, H., Li, C., & He, L. (2021). Novel method for the quantitative analysis of protease activity: The casein plate method and its applications. ACS Omega, 6, 3675-3680.

Appendix

Composition of MV media

Composition	Concentration (g/100ml)
Liver infusion powder	0.001
Yeast extract	0.01
Peptone	0.15
Dextrose	0.4
Seawater	100 ml
Agar	0.9

CMC

Composition	Concentration
CMC	3g
95% of Ethanol	29ml
Distilled water	1ml

DNSA reagent

COMPOSITION
DNS
NaOH
Sodium potassium tartrate

Phosphate buffer

Composition	Concentration (40mM/400ml)
Potassium dihydrogen phosphate	2.17728g
Dipotassium hydrogen phosphate	2.78688g