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Isolation and characterization of Docosahexaenoic acid-producing Thraustochytrids from Indonesian mangrove ecosystem

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ABSTRACT

Polyunsaturated Fatty Acids (PUFAs) are unsaturated fatty acids which include docosahexaenoic acid (DHA). The conventional source of DHA is derived from various species of fish in the form of fish oil. DHA content in fish oil is relatively low, ranging from 7-14%, which necessitates the use of large quantities of fish, potentially leading to overfishging. Alternative source of DHA can be obtained from thraustochytrids. Thraustochytrids can produce relatively higher levels of DHA and does not require extensive land areas for cultivation. This study aims to identify applicable techniques for isolating and identifying thraustochytrid strains local to mangrove areas in East Java, to examine the growth and general fatty acid profile, and to identify the most effective methods for controlling contamination during growth . The isolation involved several steps, including sample preparation, isolation, pure culture development, and media control by adjusting pH and temperature. Visual observations were made using an inverted microscope to confirm the presence of Thraustochytrids followed by genetic analysis to identify the strain. The optimal growth was observed in L3P sample collected from brown mangrove leaves. The highest specific growth rate was 0.146 h-1 (L3P). DHA content was 172 mg/g from the dried cell weight biomass. The most effective method for isolation involved a pre-treatment step consisting of soaking the samples in pine pollen for 8-10 days. Thraustochytrid isolates were able to grow optimally in media with the pH of 7 and at the temperature of 25°C. This study represents a first successful step toward sustainable DHA production using thraustochytrids isolated from Indonesian mangrove ecosystem.

1. Introduction

In addition to being a valuable natural resource, marine areas present significant management challenges. Mangrove forests provide numerous benefits to both terrestrial and marine ecosystems. In terrestrial ecosystems, they prevent abrasion, erosion, hurricanes, and tidal waves, while in marine ecosystems, they supply organic material for marine life. The primary food source for marine organisms is detritus particles from fallen mangrove leaves (Audina et al., 2023). Mangrove forests, also known as mangrove swamps, are a type of plant that forms communities in intertidal areas. Mangrove ecosystems serve as a buffer for the life of both aquatic and terrestrial organisms, providing feeding grounds and spawning grounds. Ecologically, mangrove ecosystems are unique and rich in biodiversity potential (Zalni et al., 2020).

Thraustochytrids are abundant in marine and estuarine environments, including the water column, sediment, algae, mangroves, particulate detritus, and invertebrates. These unicellular, heterotrophic protists dominate the decomposition of leaf litter in mangrove ecosystems. Thraustochytrids attach to and penetrate substrates with the support of ectoplasmic networks, and they are capable of producing various hydrolytic enzymes such as amylase, protease, phosphatase, pectinase, lipase, esterase, cellulase, and xylanase. These hydrolytic enzymes dissolve nutrients, which can be absorbed by the cells through their ectoplasmic networks. Therefore, Thraustochytrids play an essential role in litter decomposition, nutrient enrichment, and the enhancement of food webs in mangrove ecosystems (Kalidasan et al., 2021).

Thraustochytrids are microalgae that produce lipids and thrive in marine and mangrove habitats. Indonesia has ideal climatic conditions for the growth of marine microalgae, supported by factors such as temperature, salinity, water supply, and pH, particularly in mangrove forests and surrounding waters, which aid the reproduction and development of these microalgae (Suhendra et al., 2023). The potential for developing Thraustochytrids in Indonesia is considered high. Although Indonesia's maritime regions are well known, studies on the biodiversity of microalgae in various Indonesian mangrove forests have not been thoroughly researched (Suhendra, 2022), let alone the utilization from the cultivated biomass (Mirzayanti et al., 2024).

Thraustochytrids are among the fastest-growing microorganisms, capable of producing dry biomass exceeding 200 g/l in just three days under optimal conditions. Another compelling reason for exploiting Thraustochytrids is their high

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economic potential, allowing them to compete with fish oil as a source of docosahexanoic acid (DHA), and they are capable of producing beta-carotene, DHA-rich animal feed, and compounds with anticancer properties (Hutari et al., 2022).

The demand for animal feed requires ingredients with high energy content, such as fish oil, which helps meet the energy needs of farmed animals and increases their feed intake (Sukerta et al., 2020; Humaidah et al., 2020). To meet the projected global fish demand, over 164 million tons per year is required (Allan, 2010). According to the Indonesian Ministry of Maritime Affairs and Fisheries Decree No. 19/2022 on the Estimation of Fishery Resource Potential, the allowable catch (JTB), and the Utilization Rate of Fishery Resources in the Republic of Indonesia's Fisheries Management Area, the total fish catch is 12.01 million tons per year, with an allowable catch of 8.6 million tons per year (Kementerian Kelautan dan Perikanan, 2022). The DHA content in fish oil is relatively low, ranging from 7-14%, which necessitates a large amount of fish to produce high levels of DHA. This can result in overfishing across the globe.

Numerous studies have been conducted to find alternative sources of DHA other than fish, one of which is microalgae. Compared to plants or other DHA sources, microalgae have relatively high nutritional lipid content and do not require much land for cultivation, while vegetal sources command better yield for energy uses such as fuel oils and industrial usage (Madina et al., 2013; Nadir et al., 2013; Erliyanti et al., 2021). Furthermore, the lipid profile of microalgae is simpler than that of fish oil, thus limiting the separation steps required for obtaining the desired fatty acids (Ryckebosch et al., 2014). This study will focus on the isolation of DHA-producing Thraustochytrid microalgae from Probolinggo and Situbondo.

2. Material and Methods

The materials used in this study were Polypepton, D-Glucose, Yeast Extract, Agar, Penicillin, Streptomycin, and Fluconazole. The equipment utilized included Erlenmeyer flasks, a shaker, an analytical balance, beaker glasses, graduated cylinders, a microscope, a laminar air flow, an autoclave, filter paper, GFF (Glass Microfiber Filter), micropipettes and tips, petri dishes, plastic wrap, and a reagent bottle.

2.1. Sample preparation

The initial phase of this study involved collecting fallen mangrove leaves, twigs, and coral samples. These samples were obtained from three locations: Bama Beach within Baluran National Park, Sumberwaru, Banyuputih, Situbondo, East Java; Beejay Bakau Resort (BJBR) located at PPP Mayangan Port, Wisata Primadona, Mangunharjo, Mayangan, Mangunharjo, District of Mayangan, Probolinggo City, East Java; and the Kampung Kerapu Tourism site located in Gundil Hamlet, Klatakan Village, Kendit District, Situbondo Regency, East Java.

The samples collected from Bama Beach were: leaves (LB), coral (CB), and twigs (BB). The samples from Beejay Bakau Resort (BJBR) were: leaves (LP), and the samples from Kampung Kerapu Tourism were: leaves (LK) and twigs (BK). The area around the sample collection sites was dusted with pine pollen. The collected samples were placed in ziplock bags and stored in a cooler box.

2.2. Soaking pretreatment

In this isolation process, prior to direct plating, a pre-treatment was conducted where the samples were first soaked in pine pollen. Each sample was provided with 25-30 mg of pine pollen powder and incubated at 25°C for eight to ten days as shown in Figure 1. After five days of incubation, colonization was checked daily under a microscope. Once the pollen grains were colonized, 20 µL of the solution was taken and spread onto agar media containing antibiotics. Pine pollen was added to induce the chemotactic movement of zoospores and their attachment to the edges of the pine pollen. Pine pollen has been widely used in the isolation of *Thraustochytrids* from marine samples. Pine pollen contains significant monosaccharides such as glucose, mannose, and xylose (4-10%), polysaccharides like starch and pectin (30-44%), proteins (6-30%), amino acids such as glutamic acid (14-22%), and flavonoids (2-4%). Pectin and glutamic acid have been reported to induce a strong chemotactic response in *Thraustochytrids* zoospores (Gupta et al., 2015).

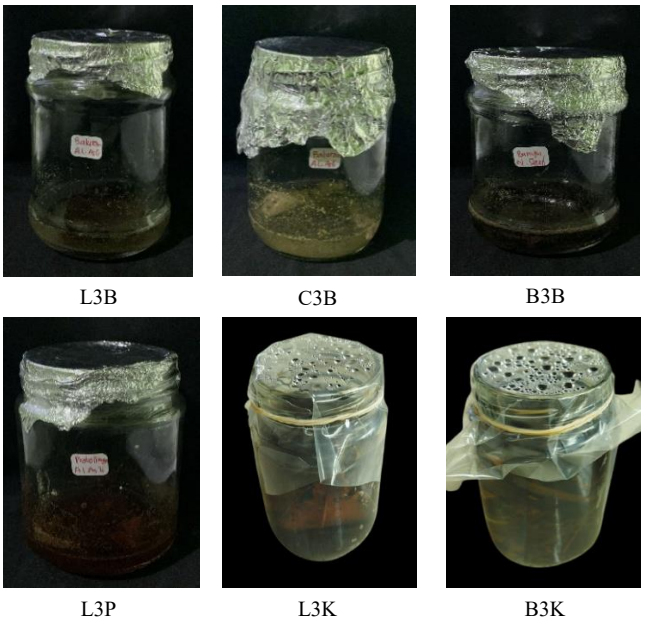


Fig. 1. Samples along with pine pollen soaked in sterilized seawater

2.3 Direct plating isolation

Before isolation, the samples were soaked in antibiotics and antifungal agents and then rinsed with sterile distilled water. The samples were subsequently immersed in pine pollen powder (25-30 mg) and incubated at 25°C for eight to ten days. After 8 days of incubation, colonization was checked daily under a microscope. When Thraustochytrids were observed adhering to the pine pollen under the microscope, direct plating isolation was performed by taking 20 µL of the solution and spreading it onto agar media containing antibiotics and antifungal agents. The plates were incubated at 25°C for 2-3 days in a dark environment. Microscopic observation was conducted to pick a colony suspected to be Thraustochytrids. If Thraustochytrids were identified, similar colonies were streaked onto fresh media until a pure isolate was obtained.

Observations were made after 8 days using a microscope at 40x and 1000x magnification. In Figure 2, it can be observed that *Thraustochytrids* attach to the pine pollen. Pine pollen

releases saccharides and proteins that can attract *Thraustochytrids* (Gupta et al., 2013). Once *Thraustochytrids* were identified as being attached to the pine pollen, direct plating isolation was performed by pouring the sample soaking solution onto agar media (ATCC 790 By+ Thraustochytrids). The samples were then incubated on the agar media at 20°C for 2-3 days.

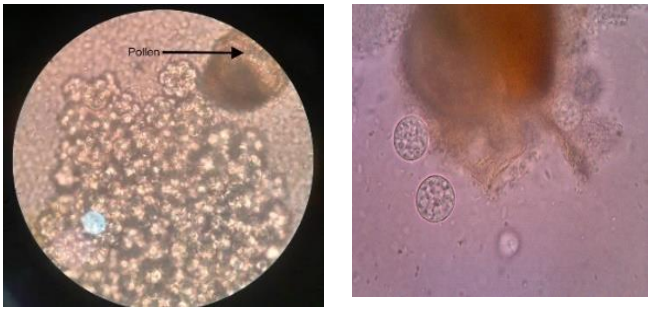


Fig. 2. Thraustochytrids cells forming a colony attached to pine pollen grain obtained from microscope observations after 8 days of soaking

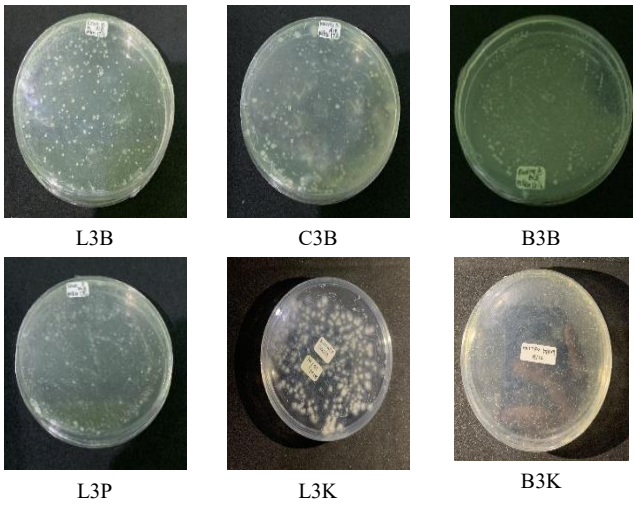


Fig. 3. Colonies grown on agar dish from direct plating

The culture was monitored daily over the course of 2-3 days to prevent fungal growth. Figure 3 shows that the colonies that grew were round, slightly convex, and white or cream-colored. These characteristics are consistent with the findings of Sirirak et al. (2020), who reported that *Thraustochytrids* colonies are white, opaque, and have an irregular, spreading shape. After 2 days, a pure streaking was performed on both agar and liquid media with the same composition as before. This media was used to carry out new streaks after the incubation process of the samples. To rejuvenate the culture or to separate the microorganisms from their mixture, the streak plate method was repeated onto fresh media (Pakaya et al., 2022). Once the culture had grown on the first agar plate, it was then streaked onto another media to obtain a pure isolate.

3. Results and Discussion

3.1. Identification

From the six samples observed under the microscope, the highest growth of *Thraustochytrids* was found in the L3P sample. In Figure 4(a), 4(b), 4(c), contamination is still present, so to obtain a pure *Thraustochytrids* isolate, further pure streaking was performed on both solid and liquid ATCC 790 By+ media. After several rounds of pure streaking, *Thraustochytrids* grew most abundantly and purely in the L3P sample, which is a leaf sample from Beejay Bakau Resort (BJBR), Probolinggo City. The location of the sample collection also affected the growth of *Thraustochytrids*. The collection of brown leaves also influenced the presence of *Thraustochytrids*, as they serve as decomposers (Suhendra et al., 2019). Meanwhile, in Figure 4(e), 4(f), no growth of *Thraustochytrids* was observed. This was due to the sample collection being carried out after rainfall, which caused sudden changes in the salinity and temperature of the surrounding waters. These environmental changes disrupted the habitat balance required by *Thraustochytrids*, and it is likely that contaminants from the seawater were introduced, leading to inhibited growth on solid media (Raghukumar, 2002).

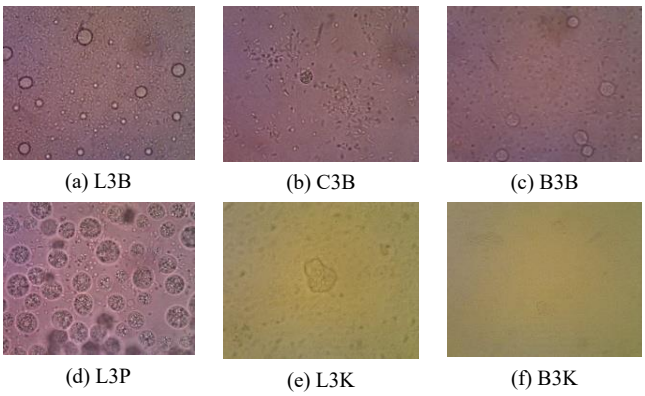


Fig. 4. Microscope observations to identify the pure Thraustochytrids culture

After repeatedly performing the purification process of the microalgal isolate until a single colony of the microalga was obtained, a microscopic observation was conducted again. This was done to ensure that the microalgal isolate obtained was truly pure. Based on Figure 5(b), at 40x magnification, *Thraustochytrids* can be clearly observed. These *Thraustochytrids* originated from Beejay Bakau Resort (BJBR), Probolinggo. Figure 5 shows that the culture is now pure and sterile. L3P notably showed the largest cell size and less amount of contamination.

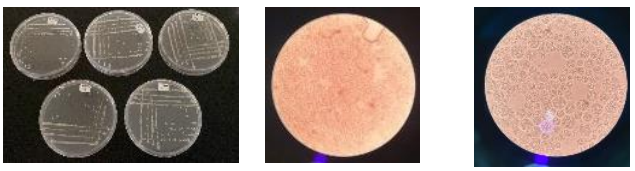


Fig. 5. Pure isolates of L3P showing large globular cells of Thraustochytrids with the characteristic oil blob inside

3.2. Growth characteristics

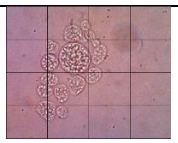
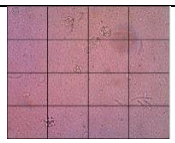
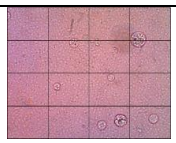
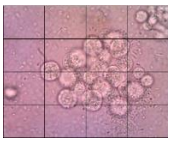
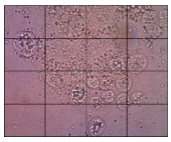
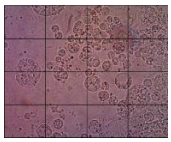
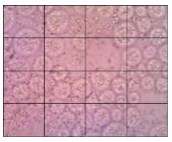
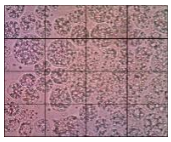
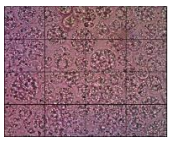
A sample control was then performed by preparing a control solution using ATCC 790 By+ media (0.5 grams of D+-glucose, 0.1 grams of polypepton, 0.1 grams of yeast extract, and 100 mL of sterilized seawater). A colony from the pure culture was then taken and introduced into the control solution. Microscopic observations and cell counting using hemocytometer were made at 24, 48, and 72 hours to assess the

growth of *Thraustochytrids* and to determine how much *Thraustochytrids* had developed. Isolates from L3B and L3P were chosen as the two best growing samples.

Based on the microscopic observations and the count of *Thraustochytrids* in Table 1 and 2, Figure 6 summarizes the estimate number of *Thraustochytrid* cells in the control samples from Baluran and Beejay increased significantly over the 72-hour period. The rapid growth phase (logarithmic phase) of *Thraustochytrids* occurred at 24 hours, while the phase where the number of growing cells equaled the number of dead cells (stationary phase) took place between 48 to 72 hours, and the death phase (dead phase) occurred after 72 hours and beyond (Humaidah et al., 2020). This is due to limiting factors such as nutrients, carbon dioxide, and others. Growth rate then slows to zero during the stationary phase as the nutrients in the culture medium are depleted. During this phase, storage carbon products, such as starch, and neutral lipids accumulate. The concentration of microalgal cells decreases rapidly during the death phase due to nutrient depletion, excess heat, pH disturbance, or contamination (Lee et al., 2015).

The results indicate that the growth of *Thraustochytrids* was more optimal in the leaf sample from Beejay Probolinggo (L3P) compared to the *Thraustochytrids* from the leaf sample of Bama Baluran (L3B). This is likely because the leaves collected from Beejay were more brown compared to the leaves from Pantai Bama Baluran. The collection of brown leaves also influences the presence of *Thraustochytrids*, as they act as decomposers (Suhendra et al., 2019).

Table 1. Hemocytometer observations on L3P (triplicate)

Time	(1)	(2)	(3)
24 h			
	$\frac{3+3+4}{3} \times 16 \times 10^4$ = 53,3×10⁴ cell/mL	$\frac{1+2+1}{3} \times 16 \times 10^4$ = 21,3×10⁴ cell/mL	$\frac{2+2+1}{3} \times 16 \times 10^4$ = 26,6×10⁴ cell/mL
48 h			
	$\frac{4+6+3}{3} \times 16 \times 10^4$ = 69,3×10⁴ cell/mL	$\frac{3+6+2}{3} \times 16 \times 10^4$ = 58,67×10⁴ cell/mL	$\frac{4+6+4}{3} \times 16 \times 10^4$ = 74,67×10⁴ cell/mL
72 h			
	$\frac{5+6+4}{3} \times 16 \times 10^4$ = 80×10⁴ cell/mL	$\frac{5+3+4}{3} x16 \times 10^4$ = 64×10⁴ cell/mL	$\frac{5+6+5}{3} \times 16 \times 10^4$ = 85,3×10⁴ cell/mL

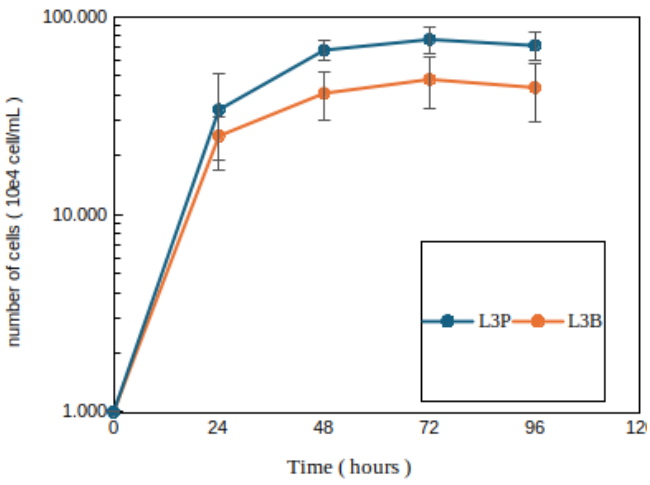
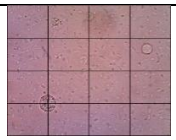
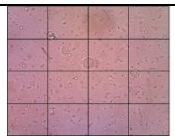
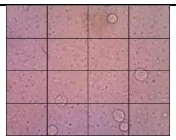
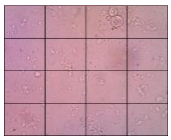
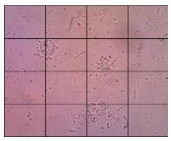
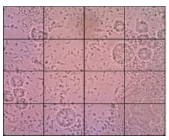
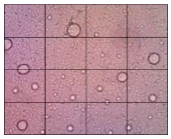
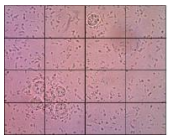
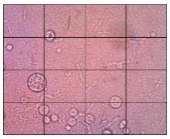


Fig. 6. Growth curve of two best performing isolates

Table 2. Hemocytometer observations on L3B (triplicate)

Hour	(1)	(2)	(3)
24 h			
	$\frac{2+1+1}{3} \times 16 \times 10^4$ = 21,3 x 10^4 cell/mL	$\frac{1+2+1}{3} \times 16 \times 10^4$ = 21,3 x 10^4 cell/mL	$\frac{2+2+2}{3} \times 16 \times 10^4$ = 32 x 10^4 cell/mL
48 h			
	$\frac{5+1+1}{3} \times 16 \times 10^4$ = 37,3 x 10^4 cell/mL	$\frac{4+1+1}{3} \times 16 \times 10^4$ = 32 x 10^4 cell/mL	$\frac{4+2+4}{3} \times 16 \times 10^4$ = 53,3 x 10^4 cell/mL
72 h			
	$\frac{2+3+3}{3} \times 16 \times 10^4$ = 42,67 x 10^4 cell/mL	$\frac{3+3+1}{3} \times 16 \times 10^4$ = 37,3 x 10^4 cell/mL	$\frac{5+5+2}{3} \times 16 \times 10^4$ = 64 x 10^4 cell/mL

3.3. DHA content and cell adaptability

Based on the effects of temperature, salinity and pH on the biomass and lipid productions by *Aurantiochytrium* sp. L3P, *Aurantiochytrium* sp. L3P was cultivated under the optimal condition of 25°C, 30 PSU, and pH7, and resultant biomass was analyzed for FAs. The biomass of *Aurantiochytrium* sp. L3P contained FAs at 270 mg/g, and DHA accounted for a half of its FAs content. The second abundant fatty acid was palmitic acid, and together with DHA it accounted for the total fatty acid content at 83%. The DHA concentration of 135 mg/g was within the range of that in other strains of *Aurantiochytrium* spp. reported before (Table 3). Although the DHA content of *Aurantiochytrium* sp. L3P is not high, optimization of culture condition may allow this strain to produce more DHA. For example, about four times increase in the DHA production was observed for *A. limacinum* mh0186 (Nagano et al., 2009; Taoka et al., 2009). In addition, a fed-batch or continuous fermentation strategy may be used to improve the DHA productivity (Fossier-Marchan et al., 2018).

Since the pH tolerance of the strain L3P is a potential unique property, we investigated an effect of pH on production of FAs by *Aurantiochytrium* sp. L3P. In a previous paper, a range of pH from 5 to 8 was reported as one of essential criteria for production of DHA by thraustochytrids (Raghukumar et al., 2008). Additionally, we confirmed production of DHA, pentadecanoic, and margaric acids by *Aurantiochytrium* sp. L3P at pH range from 3 until 9. As the pH value decreased or increased from 7, the abundance of FAs in the biomass of *Aurantiochytrium* sp. L3P became lower. On the other hand, the lipid content was not affected by pH at the tested range.

Some previous studies confirmed production of squalene in addition to DHA by *Aurantiochytrium mangrovei* (Fan et al., 2010) and *Aurantiochytrium* sp. T66 (Patel et al., 2019) and xanthophylls by *Aurantiochytrium* sp. KH105 (Yamasaki et al., 2006; Iwasaka et al., 2013). Based on these finding, alteration of the lipid composition in the biomass might be the plausible explanation for reduction of the FAs content by the change of pH. Our future study will address the effects of pH on lipid composition of valuable lipids.

Table 3. Comparison of the DHA content in Thraustochytids biomass across various strains

Strain	DHA (mg/g-dry cell)	Medium	Ref.
Aurantiochytrium limacinum mh0186	111	Artificial	Yokochi et al., 1998
Aurantiochytrium sp. KH105**	115	Shochu wastewater	Yamasaki et al., 2006
Aurantiochytrium mangrovei Sk-02**	214	Coconut water	Unagul et al, 2007
Aurantiochytrium limacinum mh0186*	121	Artificial	Taoka et al., 2009
Aurantiochytrium limacinum mh0186	467	Artificial***	Nagano, 2009
Aurantiochytrium sp. KRS101	169	Glucose, corn steep	Hong et al., 2011
Aurantiochytrium mangrovei Sk-02	126	Artificial	Chodchoey et al., 2012
Aurantiochytrium sp. KH105	32	Waste syrup	Iwasaka et al., 2013
Aurantiochytrium sp. SD116	247	Artificial	Gao et al., 2013
Aurantiochytrium limacinum ATCC MYA-1381	150	Artificial	Abad et al., 2015

Table 3 (continued). Comparison of the DHA content in Thraustochytids biomass across various strains

Strain	DHA (mg/g-dry cell)	Medium	Ref.
Aurantiochytrium sp. SW1	250	Artificial***	Nazir et al, 2018
Aurantiochytrium sp. T66	124	Forest biomass hydrolysates	Patel et al., 2019
A. limacinum ICTSG-17	188	Artificial (glucose)	Pawar et al., 2020
Aurantiochytrium sp. YB-05	140	Date fruit	Abdel-Wahab et al., 2022
Aurantiochytrium sp. PKU#Mn16	93	Artificial****	Liu et al., 2023
	239	Artificial	Yin et al., 2023
Aurantiochytrium sp. L3P (this study)	172	Artificial	This study

* The strain was once reported as *Aurantiochytrium* sp. mh0186.
** The strain was once reported as *Schizochytrium mangrovei*.
*** Optimization of the culture condition was carried out.
**** Reduced oxygen saturation at the medium

4. Conclusions

Several conclusions can be drawn regarding the optimal conditions and methods for isolating and cultivating thraustochytrids. The most effective isolation method involved pre-treatment by immersing pine pollen for 8–10 days before performing direct plating, which significantly enhanced the growth of thraustochytrids. Among the samples tested, the Probolinggo leaf sample (L3P) showed the highest growth performance. The growth pattern of thraustochytrids followed a typical microbial growth curve: a rapid growth (log) phase occurred within the first 24 hours, followed by a stationary phase between 48–72 hours, and a death phase beginning after 72 hours. The specific growth rate of L3P was 0.146 h^{-1}, higher than that of L3B, which was 0.133 h^{-1}, indicating that L3P grew more rapidly. Optimal cultivation conditions were found to be at a pH of 7 and a temperature of 25°C. To prevent contamination, antifungal (fluconazole) and antibiotic agents (streptomycin and penicillin) were added to both liquid and solid media. In addition, all procedures were carried out using sterilized equipment and in a sterile environment to prevent contamination, notably from yeast. These findings provide valuable insights into the optimal conditions and techniques for isolating and cultivating *Thraustochytrids*, which could contribute to future research and commercial applications, particularly in the production of DHA fatty acids.

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