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Isolation and Characterization of Heterotrophic Marine Protists for Potential PUFA Production from Nothern coast of East Java and Madura Island

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Isolation and Characterization of Heterotrophic Marine Protists for Potential PUFA Production from *Nothern coast of East Java* and Madura Island

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Abstract. Polyunsaturated Fatty Acids (PUFAs), particularly omega-3s, are essential for human health, yet traditional fish oil sources face critical sustainability challenges. This study explores alternative omega-3 sources from marine oleaginous protists isolated from mangrove ecosystems on the *Nothern coast of East Java* (Probolinggo/Situbondo) and Madura Island (Bangkalan), Indonesia. Using the pine pollen baiting method, the research aimed to isolate, identify, and compare the lipid-producing capabilities of indigenous strains. While initial morphological screening suggested the presence of Thraustochytrids in all locations, 18S rRNA gene sequencing revealed distinct taxonomic identities. The isolate from the *Nothern coast of East Java* (ZN2L) was identified as Thraustochytrids, whereas the isolate from Madura (ZN7L) was identified as *Crypthecodiniaceae sp.* (heterotrophic Dinoflagellates). Despite their evolutionary divergence, both strains exhibited functional convergence with rapid heterotrophic growth and significant lipid accumulation on agro-industrial waste. Quantitative analysis revealed that the Java isolate produced 17.95 mg/g of Docosahexaenoic Acid (DHA), a yield comparable to the 18.25 mg/g achieved by the Madura isolate when cultivated on lemon peel waste substrate. Notably, the Madura isolate also demonstrated the highest specific growth rate (0.127 h−1). These findings highlight the effectiveness of the baiting method in capturing diverse lineages of the SAR supergroup and confirm that Indonesian mangroves harbor a rich biodiversity of metabolically robust protists suitable for sustainable omega-3 production.

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Keywords: Thraustochytrids, Crypthecodiniaceae, Docosahexaenoic Acid (DHA), Mangrove Ecosystems, Agro-industrial Waste

Introduction

Polyunsaturated Fatty Acids (PUFAs), particularly omega-3 fatty acids such as Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA), play a vital role in human ealth, including brain development and the prevention of cardiovascular diseases (Alagawany, 2022). Traditionally, the primary source of omega-3 has been extracted from marine fish oils such as herring, mackerel, and salmon (Tocher, 2015). However, reliance on fish oil faces significant challenges regarding ecosystem sustainability due to overfishing, marine pollutant accumulation, and fluctuating and relatively low omega-3 content (around 7–14%), which leads to high purification costs (Adarme-Vega, 2012).

Therefore, the exploration of non-animal alternative sources has become a global priority. Oleaginous (oil-producing) marine microorganisms offer a promising solution due to their rapid growth rates, lack of requirement for extensive agricultural land, and simpler fatty acid profiles compared to fish oil, facilitating the purification process (Ratledge, 2004). Among various microorganisms, marine heterotrophic protists have been identified as primary producers of DHA in the marine food web. Two of the most dominant taxonomic groups in lipid production are Thraustochytrids such as the genus *Aurantiochytrium* and heterotrophic Dinoflagellates such as the family Crypthecodiniaceae (Mendes, 2009).

Thraustochytrids, which are often found associated with mangrove litter, have long been studied for their ability to produce dry biomass of up to more than 200 g/L with high lipid content (Raghukumar, 2008). On the other hand, Dinoflagellates of the Crypthecodiniaceae family, although phylogenetically distinct, also occupy a similar ecological niche as decomposers in marine environments and are known as high producers of pure DHA, often used in infant formula (Barclay, 1994; Sullivan, 2003). Although both groups have immense biotechnological potential, comparative studies regarding their presence and growth characteristics from the same habitat, particularly in tropical mangrove ecosystems, remain limited.

Mangrove forests generate a continuous supply of organic matter through fallen leaf detritus, which serves as the primary substrate for heterotrophic microorganisms (Kristensen, 2008; Kalidasan, 2021). As home to some of the world's most extensive and dense mangrove coastlines, Indonesia offers an ideal habitat for these marine protists. However, despite this ecological significance, research into microbial diversity within Indonesian mangrove ecosystems remains notably limited.

In the North Coast of East Java, existing mangrove restoration efforts predominantly emphasize physical rehabilitation (Rudianto, 2022) , economic aspects (Utami, 2022; Ramadesta, 2022), and macroscopic flora and fauna (Dewi, 2023), leaving microbial communities virtually uncharacterized. Given the environmental differences between the *Nothern coast of East Java* and Madura Island, these regions are likely to harbor distinct and previously undiscovered microbial diversity. This study provides one of the first reports on microbial biodiversity in East Java mangrove ecosystems, highlighting oleaginous marine protists with significant biotechnological potential.

This study aims to isolate, molecularly identify, and compare the growth characteristics of lipid-producing marine protists from the mangrove areas of the *Nothern coast of East Java* and Madura Island. Furthermore, a comparative analysis of the effectiveness of isolation

methods using pine pollen, specific growth rates, phylogenetic identification, and the quantitative fatty acid content of both isolates will be conducted to determine their viability as sustainable alternative sources of Omega-3 fatty acids.

Materials and Method

Materials

The ingredients used in the studies are polypepton, D-glucose, yeast extract, agar, penicillin, streptomycin, and fluconazole. Polypepton and D-glucose provide the essential nitrogen and carbon required for energy and cellular synthesis, while yeast extract acts as a vital nutrient supplement offering complex vitamins and minerals. The addition of agar provides the necessary solid structure for colony observation. Most critically, the simultaneous inclusion of penicillin and streptomycin provides broad-spectrum antibacterial coverage against both Gram-positive and Gram-negative bacteria, while fluconazole serves as a potent antifungal to inhibit yeasts and molds. Together, these selective agents ensure that only the resistant target of interest survives.

The equipment used in the following research includes Erlenmeyer flasks, a shaker, an analytical balance, glass beakers, a measuring cylinder, a microscope, laminar air flow, an autoclave, filter paper, GF/F (glass microfiber filter), micropipettes and tips, petri dishes, plastic wrap, and a conductor. Furthermore, this is divided into two special sub-sections, namely Sample Preparation and Direct Plating Isolation.

Methods

Sample Preparation

The selection of the sampling locations was based on the existence of coastal areas dominated by fishery activities and traditional ponds that provide an abundant source of detritus. Detritus serves as the main substrate for the growth of marine heterotrophic protists. In addition to ecological factors, these locations were chosen due to the lack of research regarding the exploration of local marine microorganisms, opening opportunities to find new or superior strains for omega-3 fatty acid (DHA) production.

The surrounding sampling area was sprinkled with pine pollen. The samples collected were placed in a ziplock bag and stored in a refrigerated box. Mangrove forests are known habitats for diverse microorganisms, including Thraustochytrids and other oleaginous protists, which serve as alternative raw materials for fatty acid production.



Figure 1. Sites for samples collection from mangrove parks and beaches in East Java

The initial stage of this study involved collecting samples of fallen mangrove leaves, twigs, and corals. Samples were obtained from eight locations along the northern coast of East Java (Situbondo & Probolinggo) and Madura Island as depicted in Figure 1: Bama Beach Baluran National Park, Sumberwaru, Banyuputih (1); Kerapu Beach, Kendit (2); Tambak Beach, Probolinggo (3); Beejay Bakau Resort BJBR (4); Labuhan Mangrove, Sepulu (5); Mangrove Botanical Garden, Lembung Paseser (6); Martajasah Beach (7); and Gebang Beach (8).

Sample treatment was not conducted at the sampling site to ensure sterility. Further processing was performed in a controlled environment using a laminar air flow cabinet and autoclave.

Direct Platting Isolation

The process of isolating lipid-producing marine protists began by cutting small sections of leaves, twigs, and coral samples. The samples were rinsed with sterile seawater containing antifungal (fluconazole) and antibiotics (streptomycin and penicillin) to prevent the growth of contaminants. The isolation process employed two direct plating treatments: without pre-treatment and with pine pollen pre-treatment.

In the direct plating without pre-treatment, sample pieces were placed directly onto agar media (1.5 g agar PDA) supplemented with nutrients suitable for heterotrophic marine protists (0.5 g glucose, 0.1 g polypeptone, 0.1 g yeast extract in 100 mL of sterile seawater). The samples were incubated at 20°C for 1–3 days in dark conditions.

Meanwhile, the isolation with pre-treatment involved soaking the samples with pine pollen prior to plating. Each sample was sprinkled with pine pollen powder (25–30 mg) and incubated at 25°C for 8–10 days until colonization was observed at the periphery of the pollen grains. Colonization was monitored daily under a microscope after the fifth day. Once the pollen was colonized by zoospores or motile cells, 100 µL of the water was carefully dispersed onto the antibiotic-containing agar medium.

Strain Identification

To confirm the presence of target protists, initial observations were carried out using an inverted microscope. Furthermore, to determine the specific taxonomy (morphology and homology), samples were sent to Genetica Company for strain identification analysis.

The identification stage began with centrifugation at 3000 rpm for 20 minutes, followed by lyophilization. Next, PCR analysis (using PrimeStar HS DNA Polymerase primer and BigDye Terminator v3.1 Sequencing Kit) and electropherogram analysis were conducted using Chromas Pro 1.7 to obtain the 18S rRNA sequence. The resulting sequences were then compared with the BLASTN (Basic Local Alignment Search Tool) database to identify the species, distinguishing between Thraustochytrid and non-Thraustochytrid strains.

PUFA Quantifiacations

To assess the biotechnological potential of the isolated strains, a quantitative lipid analysis was performed. Biomass from both the Java isolate and the Madura isolate was cultivated on lemon peel waste hydrolysate (5 g/L) for 5 days. The harvested biomass was dried, trans esterified, and analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) to determine the fatty acid composition and quantify Docosahexaenoic Acid (DHA) yield.

Results and Discussion

Isolation of Marine Heterotrophic Protists

The isolation of oleaginous marine protists in this study was carried out using direct plating techniques with two main approaches: without pre-treatment and with pine pollen pre-treatment. To optimize the recovery of target strains, each approach was further evaluated based on the use of microbial suppression agents, specifically the presence or absence of antifungal and antibiotic mixtures. This dual-approach strategy was implemented to evaluate the effectiveness of the isolation method and to mitigate the proliferation of contaminants such as terrestrial fungi and bacteria that can inhibit the growth of slower-growing protists like Thraustochytrids and heterotrophic Dinoflagellates (Raghukumar, 2008; Burja, 2006).

Direct Plating Isolation Without Pre-Treatment

In the direct plating method without pre-treatment, sample fragments (mangrove leaves and twigs) were cut into approximately 1 cm^2 sections and placed directly onto the agar surface. In the experimental setup where no antimicrobial agents (antifungals and antibiotics) were added to the media, the results indicated a failure to isolate the target protists. After an incubation period of 1 to 3 days, the media was overgrown by dominant fungal colonies. This overgrowth occurs because mangrove litters are naturally rich in lignocellulolytic fungi which possess faster growth kinetics compared to marine protists, allowing them to outcompete other microorganisms for nutrients when suppression agents are absent (Burja, 2006). The dominant fungal overgrowth observed in this treatment is illustrated in **Figure 2**.

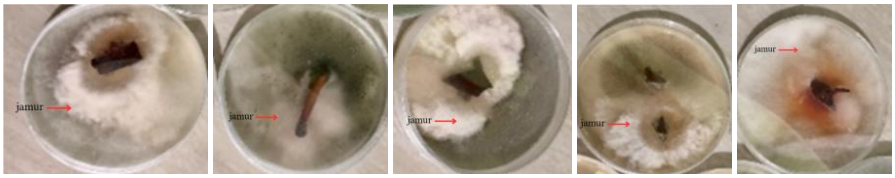


Figure 2. Culture plate showing extensive fungal overgrowth

In contrast, when antifungal and antibiotic supplements were added, the sample was incubated at 25°C for two days in dark conditions. Results after incubation are shown in **Figure 3**. These selective agents successfully inhibited the proliferation of non-target organisms; specifically, fungal overgrowth was suppressed effectively, allowing for the clear isolation and observation of the target microbial colonies without interference from mycelial expansion.

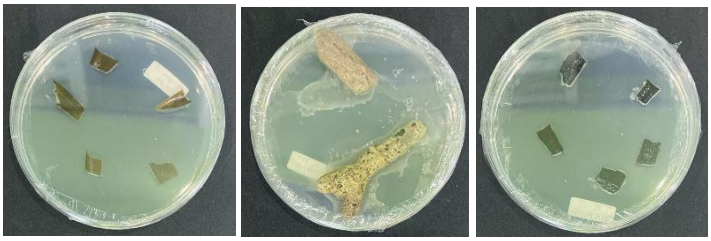


Figure 3. Samples after incubation for two days showing minimal fungal contamination

During the incubation process, cultures were observed daily for three days to monitor the emergence of target colonies and prevent the overgrowth of contaminants, specifically mold. Between the first and third day, no growth of the target marine protist colonies was observed. This absence is attributed to the lack of specific stimulation for the release and aggregation of motile cells. Thraustochytrids (zoospores) and heterotrophic Dinoflagellates (dinospores) rely on chemotactic cues to thrive and colonize new substrates.

The release and concentration of these motile cells are effectively triggered by the addition of pine pollen. In the baiting method, pine pollen serves as a nutrient source to induce chemotactic movement, facilitating the attachment of cells to the periphery of the pollen grains (Byreddy, 2015). Consequently, the direct plating method without pre-treatment failed to yield the expected colonization of oleaginous protists. Due to its ineffectiveness in competing with faster-growing fungi, this approach was discontinued, and the study proceeded with the pine pollen baiting method.

Isolation with Pine Pollen Pre-Treatment

In this isolation procedure, a pre-treatment step was conducted prior to direct plating by soaking the samples with pine pollen. The samples were sprinkled with pine pollen to induce chemotactic movement of motile cells (zoospores and dinospores) and facilitate their attachment to the periphery of the pollen grains. Pine pollen has been widely utilized in the isolation of various marine heterotrophic protists, including Thraustochytrids and chytrids. This effectiveness is due to its significant monosaccharide-like composition, including glucose, mannose, and xylose (4–10%), polysaccharides such as 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 the motile stages of oleaginous microorganisms (Byreddy, 2015). The pine pollen soaking process and microscopic observation are illustrated in **Figure 4**.

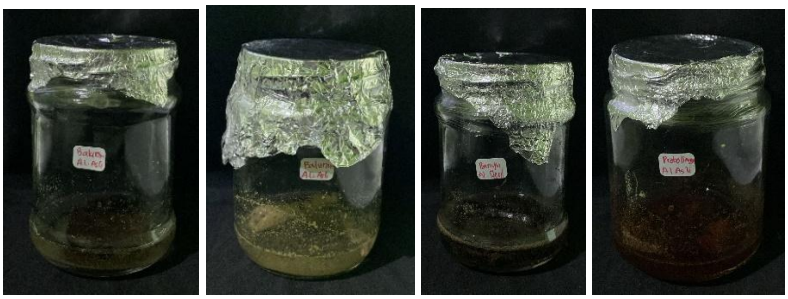


Figure 4. Samples with soaked pine pollen

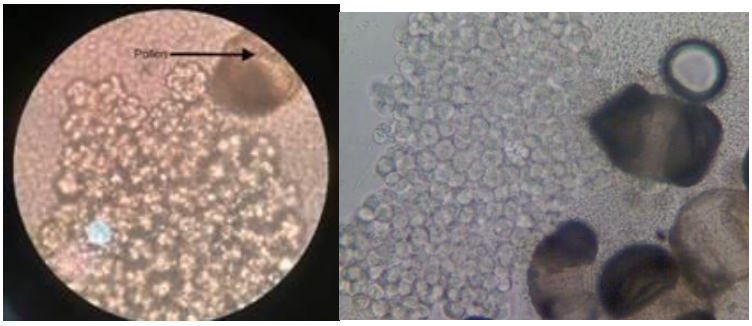


Figure 5. Microscopic Observation After 8 Days of Soaking

After 8–10 days of incubation, observations were conducted using a microscope at 50x magnification. **Figure 5** demonstrates the colonization of target protists on the pine pollen grains. The pollen releases saccharides and proteins into the water, creating a nutrient gradient that attracts these microorganisms. Once the attachment of cells to the pine pollen was confirmed, direct plating isolation was performed by transferring aliquots of the soaking water onto agar media (ATCC 790 By+). The inoculated plates were then incubated at 20°C for 2–3 days.

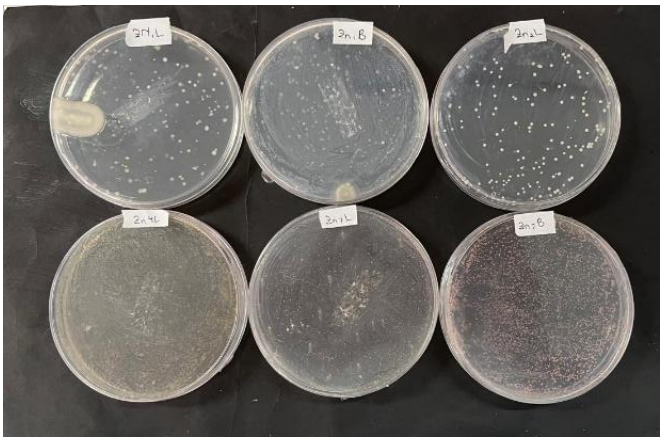
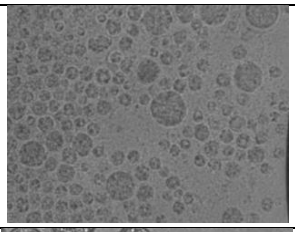
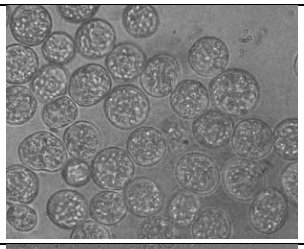
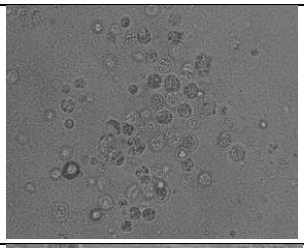
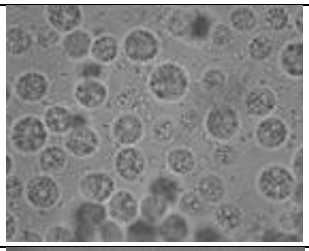
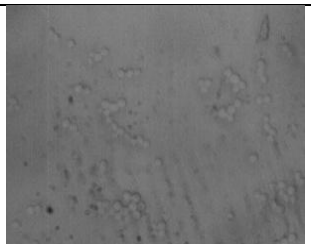
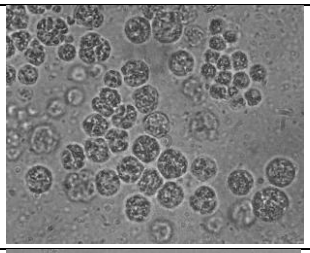
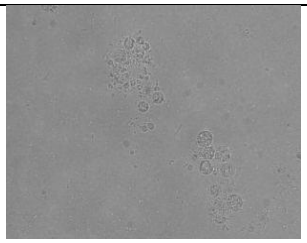
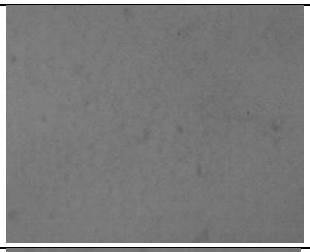
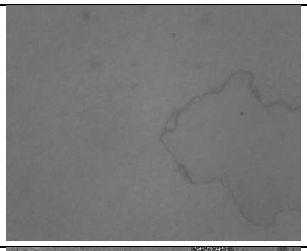
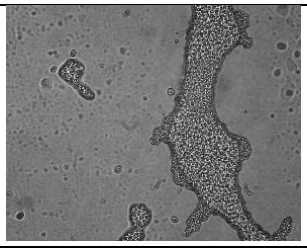


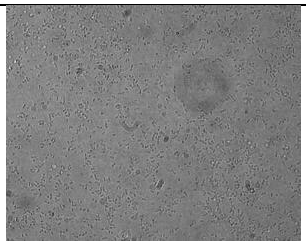
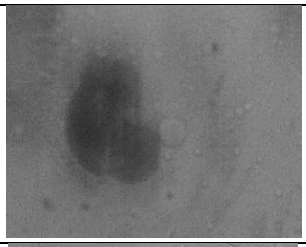
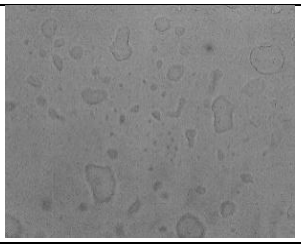
Figure 6. Direct Platting Isolation with Pre-Treatment

Cultures were observed daily for 2–3 days to monitor growth and prevent mold contamination. On the first day, minimal colony emergence was observed. However, by the second and third days, significant growth of protist colonies was evident. Figure 6 shows that the growing colonies exhibited a flattened, slightly convex morphology with a white and/or beige coloration. After three days, a verification check was performed using a microscope to confirm the purity of the isolates before proceeding to the identification stage.

Table 1. Results of Microscope Isolation with Pre-Treatment

Sample	Microscope observation	Explanation
ZN_4L		Marine protists were detected.
ZN_1L		Marine protists were detected.
ZN_1B		Marine protists were detected.
ZN_2L		Marine protists were detected.
ZN_3L		No indication of marine protists

ZN_6L		No indication of marine protists
ZN_7L		Marine protists were detected.
ZN_7B		Marine protists were detected.
ZN_8L		No indication of marine protists
ZN_8B		No indication of marine protists
ZN_8C		No indication of marine protists

ZN_5L		No indication of marine protists
ZN_5B		No indication of marine protists
ZN_5C		No indication of marine protists

Based on the microscopic observations summarized in Table 1, the presence of oleaginous marine protists was successfully confirmed in several isolates, specifically samples ZN4L, ZN1L, ZN1B, ZN2L, ZN7L, and ZN7B. These samples exhibited robust colonization, characterized by the typical morphological development of globular thraustochytrid-like cells on the pine pollen substrate. Larger cell sizes were found in ZN1L, ZN2L, and ZN7L.

In contrast, samples ZN3L, ZN6L, ZN8L, ZN8B, ZN8C, ZN5L, ZN5B, and ZN5C remained devoid of growth, with no detectable cellular expansion or metabolic activity observed throughout the incubation period. The absence of growth could be attributed to a low amount of specific initial cell abundancefrom the source environment at negligible densities. Furthermore, the selective nature of the pine pollen baiting and antibiotics and antifungal regimet method may have excluded more fastidious strains that possess complex nutritional dependencies or require environmental triggers.

Unlike photosynthetic microalgae, the target microorganisms in this study (Thraustochytrids and heterotrophic Dinoflagellates) are obligate heterotrophs or saprophytes. Therefore, their propagation does not rely on photosynthesis but rather on the assimilation of organic nutrients from their environment. According to Perez-Garcia et al. (2011) and Marchan et al. (2018), the critical macronutrients for sustaining heterotrophic growth are Organic Carbon (such as glucose or glycerol) which serves as the primary energy source, Nitrogen (essential for protein and nucleic acid synthesis), and Phosphorus (vital for energy transfer via ATP). Furthermore, the manipulation of the Carbon-to-Nitrogen (C/N) ratio is the key factor in

triggering the lipid accumulation pathway in these microorganisms (Perez-Garcia, 2011; Marchan, 2018).

Growth Characteristics of Marine Isolates

To evaluate the growth performance of the obtained isolates, a controlled cultivation assay was conducted using ATCC 790 By+ liquid medium (0.5 g D+-glucose, 0.1 g polypeptone, 0.1 g yeast extract, and 100 mL sterile seawater). Pure colonies from the isolation stage were inoculated into the medium, and cell proliferation was monitored via microscopic observation at 24-hour intervals (24, 48, and 72 hours). The cell density was calculated using a Neubauer haemocytometer according to the standard formula (Humaidah, 2020).

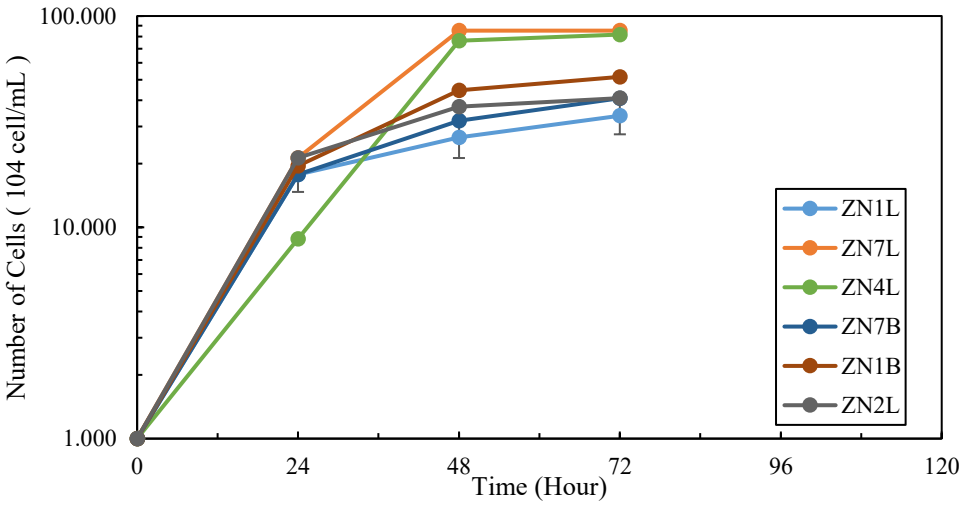


Figure 7. Growth Chart

Based on the microscopic observations and cell counts shown in Figure 7, the isolates from both locations demonstrated significant growth over the 72-hour period. The growth kinetics followed the typical microbial growth curve, consisting of distinct phases:

- Log Phase (0–24 h): A rapid increase in cell number was observed, indicating that the isolates successfully adapted to the liquid medium and utilized glucose for cell division.
- Stationary Phase (48–72 h): The growth rate eventually reached an equilibrium where cell proliferation was balanced by cell death. This phase typically occurs due to the depletion of essential nutrients (limiting factors) and the accumulation of metabolic byproducts (Lee, 2015). During this stage, oleaginous microorganisms generally shift their metabolism from replication to lipid accumulation.
- Death Phase (>72 h): A decline in viable cell concentration was observed after the peak, often referred to as the "crash phase," caused by nutrient exhaustion, pH changes, or potential contamination (Ren, 2009).

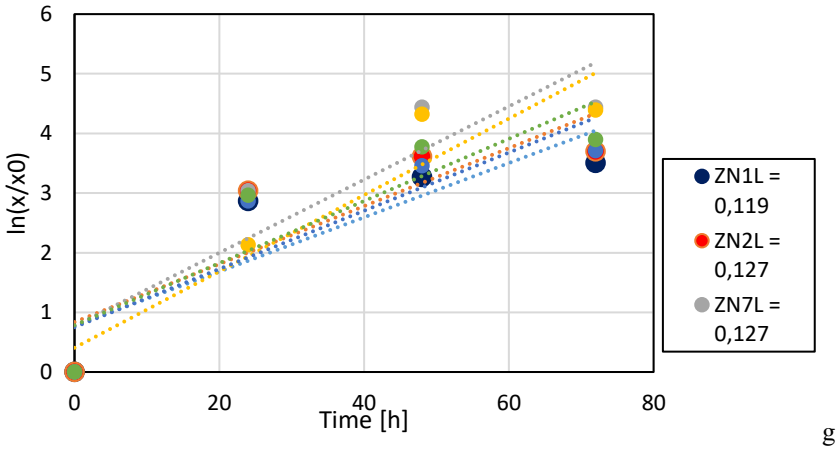
Among all samples, the isolate from Martajasah Beach, Madura (ZN7L) exhibited the most optimal growth performance. This superior growth can be attributed to the origin of the

sample, which was isolated from brown, decomposing mangrove leaves. Ecologically, brown leaves represent a substrate in an advanced stage of decomposition, providing a rich source of dissolved organic carbon and detritus. Since the targeted marine protists function as decomposers in the mangrove food web, isolates derived from such nutrient-dense substrates often exhibit more robust saprophytic capabilities and faster growth rates compared to those from green or less decomposed samples (Raghukumar, 2008).

The Specific Growth Rate (Sg or μ) is defined as the rate of increase in cell population biomass per unit of time. In the microbial growth curve, this maximum rate occurs during the exponential (log) phase, located between the lag phase and the stationary phase (Lee, 2015). The cell growth kinetics follow the first-order reaction equation:

$$\mu = \frac{ln\, W_t - ln\, W_0}{t}$$

Based on the calculation, the specific growth rates forg[the isolated strains are as follows: ZN2L (0.127 h^{-1}), ZN7L (0.127 h^{-1}), ZN1B (0.123 h^{-1}), ZN1L (0.119 h^{-1}), ZN7B (0.119 h^{-1}), and ZN4L (0.090 h^{-1}).



g

Figure 8. Specific Growth Rate

The results indicate that ZN2L (from Beejay Mangrove Resort, *Nothern coast of East Java*) and ZN7L (from Martajasah Beach, Madura) exhibited the highest specific growth rates compared to other isolates as shown in Figure 8. The observed specific growth rate (μ=0.127 h^{-1}) indicates a doubling time of approximately 5.4 hours, which is highly competitive for wild-type marine protists isolated from mangrove ecosystems. The specific growth rate of thraustochytrids and similar oleaginous protists typically ranges from 0.04 to 0.15 h^{-1} depending on the strain and glucose concentration (Gupta, 2012a). The fact that ZN2L and ZN7L reached the upper tier of this range suggests that these indigenous strains possess an efficient glucose transport system and metabolic adaptation to the provided culture conditions.

Furthermore, a high specific growth rate is directly correlated with volumetric productivity, a critical parameter for industrial scale-up. Selecting strains with rapid cell division in the exponential phase is crucial to shortening the fermentation cycle and minimizing production costs (Ren, 2010). The superior performance of ZN2L and ZN7L suggests they are not only ecologically robust but also biotechnologically promising candidates.

Consequently, due to their highest biomass production and growth performance during the isolation phase, ZN2L and ZN7L were selected as the representative strains for the *Nothern coast of East Java* and Madura Island, respectively. These two strains were then subjected to further molecular identification (18S rRNA sequencing) and lipid profiling to determine their taxonomy and Omega-3 content.

Molecular Identification and Phylogenetic Analysis

To determine the precise taxonomic identity of the isolated strains, molecular analysis targeting the 18S rRNA gene was conducted. The BLASTN (Basic Local Alignment Search Tool for Nucleotide) analysis revealed that the isolates obtained from the pine pollen baiting method belong to two distinct taxonomic groups, despite their similar morphological appearance. The specific genetic sequence homologies for isolates ZN2L and ZN7L are detailed in Tables 2 and 3, respectively."

Table 2. Search Results for the top four BLAST Libraries with the Highest Homology for ZN2L sequence which is amplified using Euka-550R and SR4-950 primers.

Registered Name	***Accession Number***	***Homology***	***E-value***	***Query Code***
Aurantiochytrium sp.	MH595611.1	99.81%	0.0	Euka-550_B
Schizochytrium sp.	PQ512232.1	99.81%	0.0	
Aurantiochytrium sp.	MH319325.1	99.81%	0.0	
Schizochytrium sp.	PQ512305.1	99.81%	0.0	
Schizochytrium sp.	PQ512231.1	98.92%	0.0	SR4-950_B
Aurantiochytrium sp..	KX688806.1	98.92%	0.0	
Schizochytrium sp.	PQ512232.1	98.92%	0.0	
Schizochytrium sp..	PQ512246.1	98.92%	0.0	

Table 3. Search Results for the top four BLAST Libraries with the Highest Homology for ZN7L sequence which is amplified using Euka-550R and SR4-950 primers.

Registered Name	***Accession Number***	***Homology***	***E-value***	***Query Code***
Crypthecodinium sp.	MG96451.1	99.10%	0.0	Euka-550_M
Crypthecodinium sp.	OQ184974.1	98.80%	0.0	
Crypthecodinium sp.	KY263646.1	99.10%	0.0	
Crypthecodinium sp.	OQ184975.1	98.80%	0.0	
Crypthecodinium sp.	OQ184975.1	98.49%	0.0	SR4-950_M
Crypthecodinium sp.	AB811790.1	98.59%	0.0	
Crypthecodinium sp.	OQ184974.1	98.38%	0.0	

Crypthecodinium sp.	DQ241737.1	98.38%	0.0	

The BLAST results (Table 2) demonstrated that the isolates from Baluran, Java (Code: Euka-550R B / SR4-950R B) exhibited a high degree of homology with the Thraustochytrid group. The sequence alignment showed a Query Coverage of 100% and a Percent Identity nearing 100% with an E-value of 0.0, confirming that the Java isolates are indeed Thraustochytrids (specifically closely related to *Aurantiochytrium* sp.).

In contrast, the isolates from Madura (Code: Euka-550 M / SR4-950 M) showed a different genetic profile. The BLAST analysis identified these strains as members of the Crypthecodiniaceae family, a group of heterotrophic Dinoflagellates. Similar to the Java samples, the Madura isolates also showed high confidence levels with 100% Query Coverage and an E-value of 0.0 as shown in Table 3. This finding corrects the initial assumption based on morphology and confirms that the organism isolated from Madura is a heterotrophic dinoflagellate, not a Thraustochytrid.

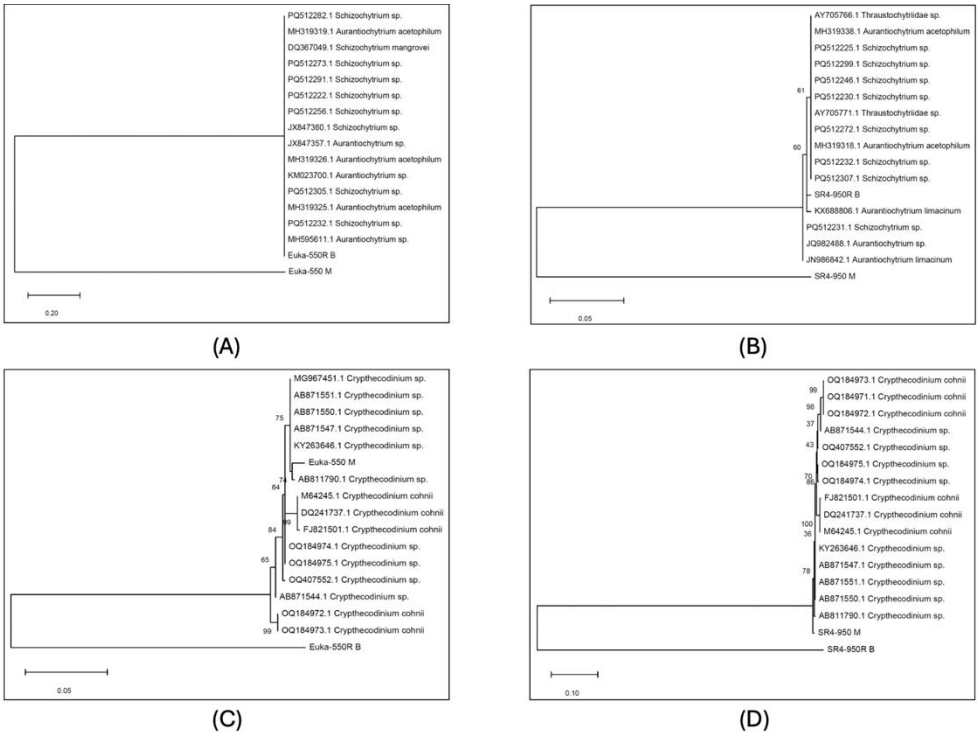


Figure 9. Maximum Likelihood phylogenetic tree inferred from 18S rRNA gene sequences. The sequences were amplified using Euka-550R and SR4-950R primers. The tree topology reveals two distinct evolutionary lineages: the Java isolates (ZN2L) cluster tightly within the Thraustochytriidae clade (*Aurantiochytrium sp.*), while the Madura isolates (ZN7L) form a distinct branch within the Crypthecodiniaceae family (Dinoflagellata), despite sharing the same ecological niche.

Furthermore, the phylogenetic tree constructed using the Maximum Likelihood algorithm (**Figure 9**) clearly visualizes this divergence. The tree topology separates the isolates into two distinct clades: the Java isolates cluster tightly with the Thraustochytriidae lineage, while

the Madura isolates form a distinct clade within the Crypthecodiniaceae (Dinoflagellata) lineage. Interestingly, although they differ at the phylum level, both groups belong to the same SAR (Stramenopiles, Alveolates, and Rhizaria) supergroup, which explains their shared ecological niche and functional capability in accumulating omega-3 fatty acids.

The discovery of two genetically distinct lineages (*Aurantiochytrium* and Crypthecodiniaceae) occupying the same ecological niche underscores the limitations of morphological identification in detecting marine microbial diversity. The phenotypic resemblance between these two isolates can be attributed to convergent evolution, where distinct taxonomic groups evolve similar traits such as heterotrophic mode, cell shape, and lipid accumulation in response to similar environmental pressures in mangrove ecosystems. This phenomenon aligns with the modern eukaryotic classification which places both Stramenopiles (Thraustochytrids) and Alveolates (Dinoflagellates) within the SAR (Stramenopiles, Alveolates, and Rhizaria) supergroup (Adl, 2019). Despite diverging millions of years ago, members of the SAR supergroup often share homologous metabolic pathways, including the Polyketide Synthase (PKS) gene clusters responsible for the synthesis of Long-Chain Polyunsaturated Fatty Acids (LC-PUFAs) (Collier, 2020). Consequently, this study confirms that mangrove habitats harbor "cryptic biodiversity" of high-value oleaginous protists that can only be resolved through molecular phylogenetic analysis, not microscopic observation alone.

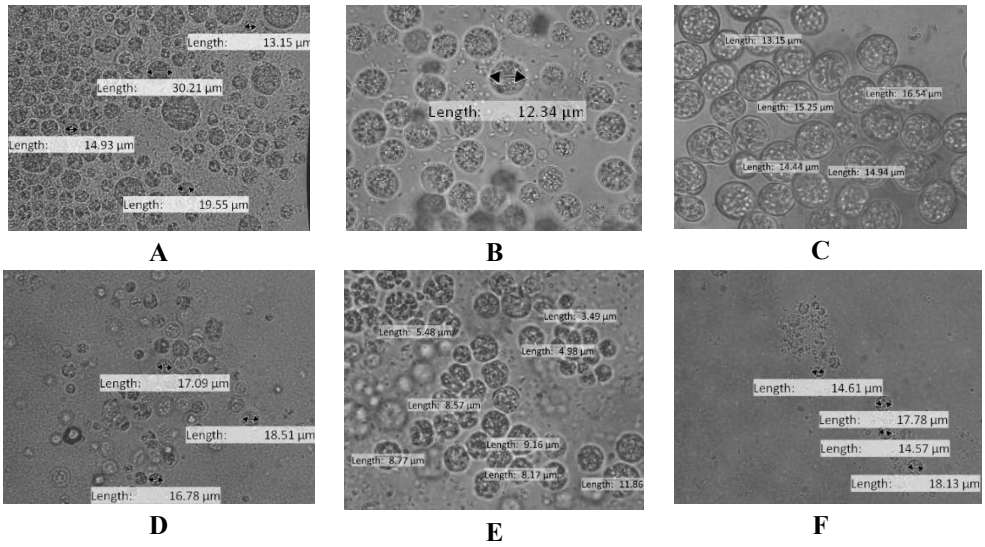


Figure 10. Figure 6. Micrographs of isolated oleaginous marine protists. (A-D) Thraustochytrid strains (*Aurantiochytrium* sp.) from the *Nothern coast of East Java*: (A) Bama Beach, ZN4L; (B) Beejay Mangrove Resort, ZN2L; (C) Tambak Beach (Leaf), ZN1L; (D) Tambak Beach (Stem), ZN1B. (E-F) Heterotrophic Dinoflagellate strains (Crypthecodiniaceae sp.) from Madura Island: (E) Martajasah Beach (Leaf), ZN7L; (F) Martajasah Beach (Stem), ZN7B.

Figure 10 illustrates the morphological diversity of lipid-producing protists isolated from various mangrove habitats in Indonesia. Figures A–D display the isolates from the *Nothern coast of East Java* (Situbondo and Probolinggo), which were molecularly identified as

Thraustochytrids (*Aurantiochytrium sp.*). These cells appear spherical with diameters ranging significantly from 10 µm to 30 µm (Figure A) and 12–19 µm (Figures B, C, D). This observation is consistent with the findings of Dellero et al. (2018), who described *Aurantiochytrium sp.* as having vegetative cells typically ranging from 10–20 µm, which can enlarge significantly prior to zoospore formation at zoosporangium phase (Dellero, 2018).
In contrast, subfigures E and F show the isolates from Martajasah Beach, Madura (ZN7L and ZN7B), which were identified as Heterotrophic Dinoflagellates (Crypthecodiniaceae). The isolate from the leaf sample (ZN7L, Figure E) exhibited a smaller cell diameter ranging from 4 µm – 11 µm, while the stem isolate (ZN7B, Figure F) ranged from 12 µm – 20 µm. The smaller size observed in ZN7L likely corresponds to the vegetative motile stage or early cyst formation characteristic of Crypthecodiniaceae.

Heterotrophic dinoflagellates such as *Crypthecodinium cohnii* exhibit a complex life cycle where cells can vary from small swarmers (approx. 5–8 µm) to larger non-motile cysts (20–50 µm) depending on nutrient availability and growth phase (Sui, 2020). Therefore, the size discrepancy between the Java and Madura isolates is not merely due to growth phases but reflects the distinct taxonomic characteristics between Labyrinthulomycetes and Dinophyceae.

Following the confirmation of colony colonization, a purification process was conducted using the streak plate method on solid agar media with the same nutrient composition. This step aims to separate the target protists from bacterial contaminants and obtain axenic (pure) cultures. Repeated streaking on antibiotic-containing media is the most effective standard protocol for isolating pure strains of marine protists for biotechnological applications (Gupta, 2012b).

Quantitative Analysis of Fatty Acid Profiles
The GC-MS analysis revealed that both isolates (ZN2L and ZN7L) produced a lipid profile dominated by saturated fatty acids, particularly palmitic acid (C16:0) when cultured using 5 g-dry/L lemon peel dried at 60°C as the growth stubtrate, along with a significant proportion of polyunsaturated fatty acids, mainly docosahexaenoic acid (DHA, C22:6), as presented in Table 4. The DHA content in ZN7L (18.341) was slightly higher than that in ZN2L (17.998), indicating comparable omega-3 production capacity between the two strains when cultivated on lemon peel substrate.

Tabel 4. Fatty Acid Composition Based on GC-MS Analysis Using Lemon Peel Substrate

Fatty acids components	Concentration (mg/L)		
	ZN2L	ZN7L	Control (ATCC 790 By+)
Lauric acid (C12:0)	22.760	-	-
Tetradecanoic acid (C14:0)	23.406	0.100	5.374
Palmitic acid (C16:0)	29.467	27.429	111.255
Linoleic acid (C18:2)	19.451	5.425	3.472
Strearic acid (C18:0)	22.437	20.180	-
Arachidic acid (C20:0)	6.053	3.023	-
Docosahexanoic acid (C22:6)	17.998	18.341	51.030
Tricosanoic acid (C23:0)	2.179	1.057	1.083
Lignoceric acid (C24:0)	5.085	0.293	-

Lipid Profile of the Java Isolate (Aurantiochytrium sp.)

The GC-MS chromatogram for the Java isolate (Strain ZN2L/L3T) revealed a lipid profile characteristic of the Thraustochytrid family. The fatty acid composition was dominated by two distinct groups: Saturated Fatty Acids (SFAs), primarily Palmitic Acid (C16:0), and Polyunsaturated Fatty Acids (PUFAs), exclusively dominated by DHA.

Quantitative analysis indicated that the Java isolate produced a DHA concentration of 17.95 mg/g dry cell weight (DCW). The absence of significant amounts of structural PUFAs such as Eicosapentaenoic Acid (EPA) suggests that this strain possesses a highly selective Polyketide Synthase (PKS) pathway, which bypasses the desaturation-elongation steps typical of microalgae, directly synthesizing long-chain DHA ().

Lipid Profile of the Madura Isolate (Crypthecodiniaceae sp.)

Interestingly, the isolate from Madura Island (Strain ZN7L), identified as a heterotrophic Dinoflagellate, exhibited a metabolic performance that rivaled the Thraustochytrid strain. The GC-MS analysis detected a high peak retention time corresponding to DHA standards.

Quantitative results showed that the Madura isolate achieved a DHA yield of 18.25 mg/g DCW on the lemon peel substrate. Additionally, FTIR spectroscopy analysis performed on this biomass confirmed the presence of functional groups associated with polyunsaturated lipids, specifically the cis-alkene (=C-H) stretching at 3004 cm^{-1} and carbonyl ester (C=O) stretching at 1736 cm^{-1}. The alignment of GC-MS and FTIR data confirms that the *Crypthecodiniaceae* sp. from Madura is a potent oleaginous protist capable of converting low-cost organic waste into high-value lipids.

The ability of both strains to synthesize DHA using lemon peel waste is a significant finding. Citrus waste contains limonene and phenolic compounds, which are known antimicrobial agents that often inhibit microbial fermentation. The successful lipid accumulation in *Crypthecodiniaceae* sp. (ZN7L) suggests that this wild mangrove strain possesses robust stress-response mechanisms. *Aurantiochytrium* species can metabolize crude glycerol and waste residues, but the ability of wild Crypthecodiniaceae to thrive on citrus hydrolysate suggests a broad enzymatic capability for degrading complex carbohydrates found in mangrove detritus (Ethier, 2011).

The lipid profile of the Madura isolate, which is rich in DHA but low in EPA, is consistent with the metabolic signature of the Crypthecodinium genus. Heterotrophic dinoflagellates are unique because they do not accumulate intermediate fatty acids; they channel metabolic flux directly into DHA storage lipids (triacylglycerols) as a survival mechanism against fluctuating salinity in intertidal zones (Mendes, 2009). This contrasts with many photosynthetic microalgae that produce a mix of EPA and DHA. The purity of the DHA profile in the Madura isolate makes it a highly desirable candidate for downstream processing, as it simplifies the purification of single-cell oils.

While industrial strains of Schizochytrium can achieve higher yields under optimized fermenter conditions, the performance of these wild isolates on unoptimized waste substrates is promising. Recent report on the isolation of a new *Crypthecodinium* sp. from a mangrove that achieved high lipid content, may bring about a competitive challenge the monopoly of *Aurantiochytrium* in the industry (Sui, 2020). Our results support this emerging view, indicating that Indonesian mangroves harbor a complex diversity of dinoflagellates (like strain ZN7L) that are equally competent compared to Thraustochytrids for sustainable omega-3 production.

Conclusion

This study provides new insights into the diversity of oleaginous marine protists in the northern coast of East fJava's mangrove ecosystems. The results confirm that geographical differences correlate with distinct microbial community structures. Molecular identification successfully differentiated the isolates into two separate lineages: Thraustochytrids from the northern coast of East Java and Crypthecodiniaceae sp. (Heterotrophic Dinoflagellates) from Madura Island. This finding corrects the initial assumption based on morphology and highlights the presence of "cryptic" biodiversity within the mangrove microbial loop.

Despite belonging to different phyla, both strains demonstrated high metabolic efficiency in converting organic substrates into biomass rich in omega-3 fatty acids. The Crypthecodiniaceae strain from Madura showed superior adaptation to decomposing organic matter, evidenced by its robust growth on fruit peel waste and high specific growth rate. Therefore, both strains are identified as promising indigenous candidates for the industrial production of microbial oils, offering a sustainable, non-animal alternative to fish oil. Future research should focus on bioprocess optimization to maximize the volumetric productivity of these novel isolates.

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