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Phylogenetic and morphological characterization of *Chlorella pyrenoidosa* from the Java Sea, Indonesia

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Abstract. *Pane EP, Risjani Y, Yunianta, Belghoul Z, Rahmawati K, Maulana GD, Nugraha R. 2025. Phylogenetic and morphological characterization of* Chlorella pyrenoidosa *from the Java Sea, Indonesia. Biodiversitas 26: 3061-3070.* Pathek Beach in Situbondo, East Java, Indonesia, is a site of particular interest due to its richness in marine biodiversity and offers promising sites for microalgal bioprospecting. This beach is distinguished by its dark sand and natural rock wave breakers, contributing to its unique geomorphological features. This study explores and identifies green microalgae, specifically a strain suspected as *Chlorella pyrenoidosa*, namely UB02 PATHEK PSAL. This strain is part of the microalgae collection maintained by the Research Center on Algae and Environment (ALGAEN), where it was maintained after being isolated from the Pathek coastal area. The isolation phase of this study utilized capillary techniques for the specific morphological identification of the microalgae strain. Single-cell isolation was achieved using a capillary micropipette technique under the Olympus IX 53 inverted microscope, followed by cultivation in sterile F/2 Walne medium. The study objective was to describe the morphology features using light microscopy and Scanning Electron Microscopy (SEM) and conduct molecular identification using the rbcL gene to confirm the species identity. The rbcL gene was selected as a reliable molecular marker for low-level plant phylogenetics in this study due to its effectiveness in analyzing evolutionary relationships across different taxonomic groups. The isolated strain's genetic material was sequenced, and a comparative analysis was performed using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI). The results revealed a 99.48% similarity with known *C. pyrenoidosa* strains, specifically Wu-G3H3-1-2 and strain 820, confirming the species identity. This research enhances understanding of the biodiversity of East Java's marine ecosystems and highlights the potential for discovering new microalgae species, sparking excitement and hope for future discoveries. *C. pyrenoidosa* is widely recognized for its highly nutritional and bioactive compounds, including lipid and protein, which are valuable for use in biofuel production and functional foods, and pharmaceuticals.

Keywords: *Chlorella pyrenoidosa*, isolation, Pathek Beach, rbcL marker, SEM

INTRODUCTION

Indonesia is the largest archipelagic country in the world and consists of approximately 17,508 islands from Sabang in the west to Merauke in the east (Djunarsjah and Putra 2021; Andréfouët et al. 2022), as home to a rich marine biodiversity (Lusiana et al. 2023) consisting of different biological systems in the ocean, such as macroalgae (Risjani and Abidin 2020) and microalgae, including diatoms (Risjani et al. 2021; Arsad et al. 2022). Microalgae are microscopic photosynthetic organisms that play an essential role in marine ecosystems. Examples of microalgae include phytoplankton (Caroppo and Pagliara 2022; Molina-Grima et al. 2022). Furthermore, these microorganisms are gaining recognition for their economic value beyond their ecological roles, and as a third-generation feedstock for biodiesel production (Mubarak et al. 2019). In addition, microalgae have been utilized with their high protein content in aquaculture feed, particularly in the larval rearing of shrimp, fish, and molluscs (Duong et al. 2015). Likewise, these strains offer considerable promise in the pharmaceutical as evidenced by the production of bioactive compounds with antioxidant, anti-inflammatory, or immunostimulatory properties (Gürlek et al. 2020; Xiong et al. 2023). Microalgae show an elevated degree of efficiency in the fixation of carbon dioxide, thereby positioning them as natural agents for climate change mitigation through carbon sequestration (Ighalo et al. 2022; Elghazy et al. 2025). The fatty acid content in selected microalgae species ranges from 0.11% to 42.32% of dry weight, indicating the potential for biofuel production (Prartono et al. 2010). Phytoplankton consists of organisms such as diatoms (Bacillariophyta), dinoflagellates (Dinophyta), green flagellates (Chlorophyta), yellow-brown flagellates (Prasinophyta, Prymnesiophyta, Cryptophyta, Chrysophyta, and Raphidophyta), and blue-green flagellates (Cyanophyta) (El Gamal 2010).

Indonesia's tropical climate and abundant sunlight create ideal conditions for microalgae cultivation, with species such as *Chlorella* widely distributed across its islands (Jumiarni and Anggraini 2021; Purbani et al. 2021). Some new species have been described from islands in the Indonesian archipelago (Kryk et al. 2021; Rybak et al. 2021). Despite this potential, the development of microalgae biodiesel candidates and other biotechnological applications in Indonesia remains limited, necessitating further research to optimise cultivation and application strategies (Veza et al. 2021).

Chlorella pyrenoidosa is classified as a green microalgae (Chlorophyceae) with high biotechnology potential (Garrido-Cardenas et al. 2018; Mathur et al. 2021; Baldisserotto et al. 2022). It is characterized by spherical cells measuring 2-10 µm and chlorophyll content (Daliry et al. 2017). Optimal growth requires specific conditions such as adequate CO_2 supply, nutrient availability, temperature, pH, and light intensity (Blair et al. 2014). Indeed, *Chlorella* sp. KR-1 has been observed to thrive at temperatures between 25°C and 30°C (Hwang et al. 2013), with selected media that include Walne, Guillard, and agriculture fertilizer (Urea) (Mardalisa et al. 2022). Although microalgae diversity is well documented in culture collections globally (Sehgal et al. 2019), a considerable number of marine zones in Indonesia have yet to be thoroughly explored, as evidenced by Pathek Beach in Situbondo District. Referring to location, it exhibits a coastal area that presents a coastal area with limited prior microalgal identification, underlining the importance of targeted biodiversity surveys to uncover novel strains with potential biotechnological applications. The microalgae isolated from the sampling results in these aquatic areas were subsequently cultivated in axenic culture. Further characterization and identification are required, utilizing morphological and molecular analyses. The objective of this study was to obtain data about the characterization and molecular identification of *C. pyrenoidosa* using rbcL. According to Saputro et al. (2019), rbcL genes are universal genes that can be found in almost all plants, making them ideal markers for phylogenetic studies. They are the code of the subunit of ribulose-1,5-bisphosphate carboxylase (RuBisCO) enzymes in chloroplast genomes. The rbcL gene encodes the large subunit of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) enzyme, which plays a vital role in the process of photosynthetic carbon fixation. Accordingly, the optimization of its performance is central to numerous strategies that aim to enhance crop photosynthesis (Zhu et al. 2025). Several comparative studies have used the rbcL gene in similar research, including Chlorophyta (Leliaert et al. 2012) and diatoms (Theriot et al. 2010). These findings have shown that the rbcL gene is a reliable molecular marker in the study of algal systematics and taxonomy.

MATERIALS AND METHODS

Sampling location

Sampling was conducted at Pathek Beach, Gelung Village, Panarukan Sub-district, Situbondo District, East Java, Indonesia, from April to December 2022 (Figure 1). Pathek Beach is characterized by dark sand and natural rock wave breakers, contributing to its distinctive landscape. The sampling of microorganism populations was conducted through the implementation of vertical and horizontal screening methods on planktonic surfaces; furthermore, other sampling sites include rattle rocks near the planktonic samples area (Witkowski et al. 2014). Next, a plankton net with a mesh size of 20 µm was utilized to collect the samples, which were subsequently transferred into multiple 50 mL Falcon tubes for further analysis. The samples were subsequently isolated and analyzed at the Hydrobiology Laboratory of the Fishery Resources Division, Universitas Brawijaya. Scanning Electron Microscopy (SEM) analysis was conducted at the Materials Laboratory of the State Universitas Malang, Indonesia, while PT Genetika Science Indonesia performed DNA barcoding.

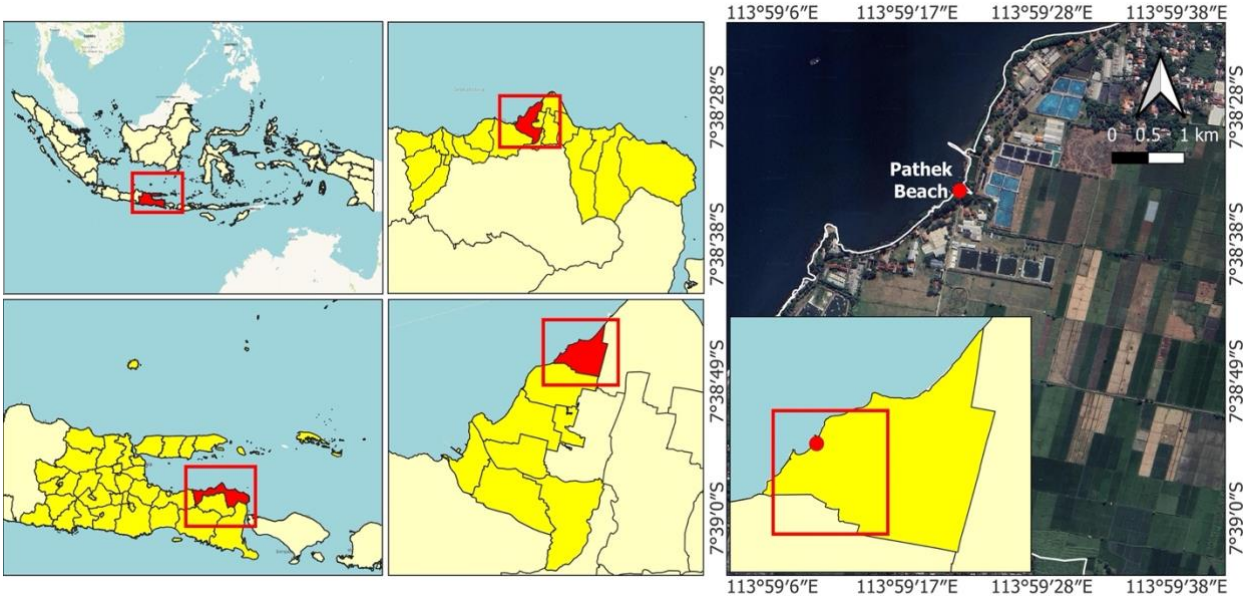

Figure 1. Sampling site of *Chlorella pyrenoidosa* isolated in Pathek Beach, Gelung Village, Situbondo District, East Java, Indonesia

Isolation stage

The isolation stage of this study uses capillaries to identify the strain morphologically. The prepared sample was transferred into a petri dish and placed under a microscope, with a volume of approximately ±5 mL (Pane et al. 2024). The utilized isolation equipment included an Olympus IX 53 inverted microscope, which provided a minimum magnification of 400x, and glass pipettes for isolation to form a small diameter, enabling the collection of monospecific isolates.

Preparation for axenic culture

Glass sterilization was performed using infrared light at a temperature of 130°C, while disinfection was conducted by soaking the equipment in a chlorine solution at a concentration of 1 mL-1L of seawater. The tools were soaked for over 24 hours, as described by Yustinadiar et al. (2020), to ensure thorough removal of potential contaminants. The seawater utilized as the culture medium underwent a two-phase sterilization process: the first phase involved chemical treatment with chlorine, followed by neutralization using sodium thiosulfate ($Na_2S_2O_3$) to remove the chlorine; the second phase involved heat sterilization. The sterilized seawater was subsequently enriched with F/2 Walne and vitamin B12. The microalgal cultures were cultivated at 22-25°C and placed approximately 30 cm under tube lamps, and the F/2 Walne nutrient medium (Table 1) was thoroughly homogenized in the sterile seawater.

DNA isolation

DNA isolation was conducted using the Quick-DNA™ Plant/Seed Miniprep Kit. To optimize the procedure, 0.5% (v/v) beta-mercaptoethanol was added to the Genomic Lysis Buffer, with a final concentration achieved by adding 250 µL of beta-mercaptoethanol to 50 mL or 500 µL to 100 mL of buffer. Up to 150 mg of finely cut plant or seed sample was placed into a ZR BashingBead™ Lysis Tube (2.0 mm), followed by 750 µL of BashingBead™ Buffer. The tube was tightly capped and processed in a bead beater at maximum speed for at least 5 minutes following the manufacturer's protocol. The sample was then transferred to a 1.5 mL microcentrifuge tube and centrifuged at 16,000 × g for 3 minutes to obtain the purified DNA. Electrophoresis on a 1% agarose gel was used to characterize the DNA isolation results of *Chlorella pyrenoidosa* qualitatively. The DNA concentration was measured by the absorbance value at λ 260 nm, while the A260:A280 ratio determined the purity.

Table 1. Composition of F/2 Walne for microalgal culture

Chemical components	Dose
NH_4NO_3	100 ppm
NaH_2PO_4	20 ppm
H_3BO_3	33.6 ppm
NaEDTA	45 ppm
$FeCl_3$	1.3 ppm
$MnCl_2$	0.36 ppm
Vitamin B12	0.001 ppm

Source: Center for Brackish Water Aquaculture, Situbondo, East Java, Indonesia

The lysis buffer is enhanced with β-mercaptoethanol, a compound that has been shown to improve the isolation of DNA by denaturing proteins, inhibiting nucleases, and preventing oxidative damage from polyphenols. Enhancing DNA yield and purity is crucial for successful molecular analyses. In this capacity, it functions as a strong reducing agent, catalyzing the cleavage of disulfide bonds within proteins, which effectively denatures the proteins, thereby preventing contamination of the DNA extract (Yu et al. 2017; Schenk et al. 2023).

Identification of gradient PCR conditions using the rbcL marker

PCR amplification was performed using (2x) MyTaq HS Red Mix (BIO-25048). The PCR reactions were prepared by combining 10 µM/µL RBCL-F primer (5'-ATG TCA CCA CCA ACA GAG ACT AAA GC-3') at a final concentration of 0.4 µM, 10 µM/µL RBCL-R primer (5'-GTA AAA TCA AGT CCA CCA CG-3') at 0.4 µM, 12.5 µL of MyTaq HS Red Mix (2X), 9.5 µL of ddH_2O, and 100.7 ng/µL of DNA concentration with a volume of 50 µL. The PCR cycling conditions consisted of an initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 15 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 45 seconds. A final extension was carried out at 72°C for 3 minutes, followed by a holding step at 4°C. The amplified samples were stored at -20°C.

Detection of gradient PCR results in the electrophoresis method

A 1 µL sample of the PCR product, handled with utmost care, was mixed with 2.5 µL of loading dye and loaded into the well of an agarose gel. Electrophoresis was performed using a 1% TBE agarose gel, and the molecular weight marker was a 100 bp DNA ladder. The agarose gel was dissolved in TBE to ensure the electric current flowed through the gel and allowing nucleic acid to move through the agarose matrix. 1% agarose is considered optimal for shorter DNA fragments. A 100 bp ladder was considered because it contains the range of DNA target length, which is around 500-600 bp. The final assembled sequence of the sample was 588 bp. The PCR products were then subjected to visualization using a UV transilluminator (Uvitec, Cambridge, UK) after undergoing electrophoresis in a horizontal agarose gel system (HU6, SCIE-PLAS, Cambridge, UK) powered by Peqlab power supply (Erlangen, Germany).

DNA sequencing

The DNA fragment was sequenced using a Bi-Directional Sequencing machine to determine the DNA base sequence of the *C. pyrenoidosa* microalga. The sequencing results were analyzed for homologous sequences by comparing the obtained DNA sequence with those in the GenBank database (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool for nucleotides (BLASTn). Phylogenetic relationships with other species were determined using the MEGA 11 (Molecular Evolutionary Genetic Analysis) software with the Maximum likelihood method (Tamura 3-parameter model) with 1000 times bootstrap,

which facilitated the construction of a phylogenetic tree (Tamura et al. 2021).

Scanning Electron Microscope (SEM)

The biomass harvested during the exponential growth phase of the cultivation process at the Pathek Beach site was utilized for morphological identification purposes. The biomass was collected, separated by centrifugation at 559 × g for 10 minutes, and dried in an oven for 18 hours. The sample to be processed was inserted into a holder with a maximum allowable deviation of ±10 mm. The test sample was coated with a gold-palladium alloy to ensure its suitability for the analysis. The sample was then transferred into the SEM chamber, and after the chamber was pumped and thoroughly vacuumed, the SEM machine was prepared for operation (beam on).

RESULTS AND DISCUSSION

Morphology of *Chlorella pyrenoidosa*

Chlorella pyrenoidosa (UB02 PATHEK PSAL) is a species of algae found in the Java Sea and is distinguished by several unique characteristics, including a relatively small cell size and a round or slightly oval shape. The colonies formed by this species typically consist of individual cells, though they may occasionally form small groups. All observed isolates showed thick cell walls and well-developed chloroplasts, resulting in a bright green coloration (Figure 2). Comparisons with specimens of *C. pyrenoidosa* from other regions revealed minor variations in cell size, but the overall morphology remained consistent. The strain under consideration had an average diameter of 4.433-6.972 µm (Table 2). The SEM observations (Figure 3) yielded substantial insights into the surface structure of the cells, showing smooth, compact surfaces without any apparent irregularities or rib-like features, and showed a distinct rough or wrinkled texture, forming a reticulated pattern. Additionally, no external flagella or colonial structures were detected, further supporting the non-motile and unicellular nature of the organism. Its morphology initially led to its classification within Chlorellaceae, order Chlorellales, and genus *Chlorella*. Notably, the genetic stability of the microalgae population ensures its survival in the face of external influences, providing a reassuring aspect of its resilience.

Scanning Electron Microscopy (SEM) analysis identified *C. pyrenoidosa* as a spherical or oval, unicellular microalga with a cell diameter ranging from 2 to 8 µm (Pane et al. 2024). It appears as a circular microalga that lacks flagella, resulting in a relatively slow movement. Its cell walls primarily comprise cellulose and pectin (Saputro et al. 2019). Additionally, *Chlorella* sp. features a stratified cell wall structure comprising layers of inorganic salts, proteins, and polysaccharides, with a thickness ranging from 100 to 200 nm (Ahmed and Kumar 2022). According to Wang et al. (2023a), the measurement of *Chlorella* appeared to be 3-10 µm (Figure 3), which is similar to the "globular cells" formed by the assembling of several *Chlorella*. The microalgae genus *Chlorella* is classified as a member of Chlorophyta or Trebouxiophyceae and is found in a range of natural environments, including rivers, lakes, and marine and stream waters (Li et al. 2016). Most *Chlorella* forms have a spherical morphology, while some appear to be in a state of shrinkage, likely attributable to inadequate hydration during the harvesting process.

Chlorella sp. have a rigid cell wall due to a sporopollenin layer, which may be observed through the trilaminar layer. The cytoplasm consists of water, proteins, and minerals and is surrounded by the cell membrane. Pyrenoids, containing high levels of ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), are the center for fixing carbon dioxide. The chloroplasts contain fused thylakoid sheets that synthesize the dominant chlorophyll pigment, which masks the color of other pigments, such as lutein (Safi et al. 2014). The cell protoplast is surrounded by a specific membrane, with a thick cellulose and pectin wall outside the membrane. Inside the cell is a thin protoplast in a cup or glockenspiel shape, with the position facing upward. Physiological changes in microalgae can significantly affect entire biological systems, as phytoplankton serve as the primary level in the collection of nanoparticles within the food chains of ocean ecosystems (Romero et al. 2020).

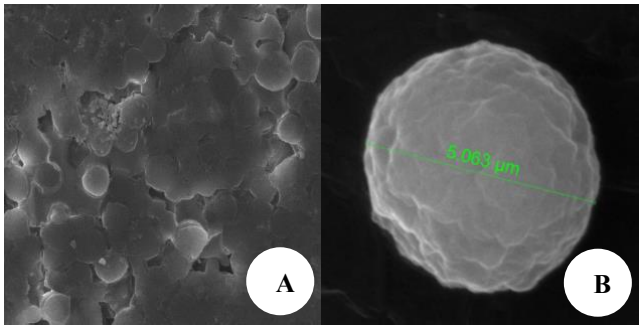

Figure 2. A. The SEM identification of *Chlorella pyrenoidosa* isolated from Pathek Beach, Situbondo District, East Java, Indonesia, SEM with mag 5,000×. B. Scale bar: 20 µm SEM with mag 30,000×. Scale bar: 3 µm

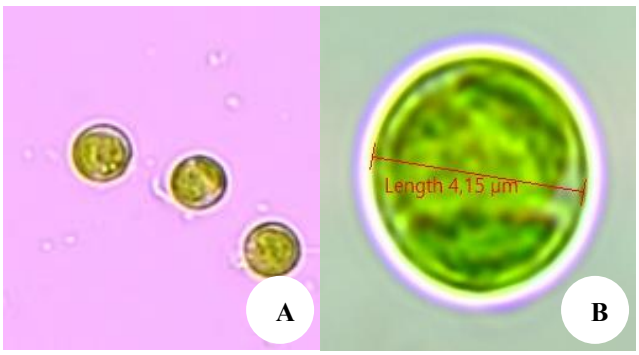

Figure 3. A. Light Microscope of this study with Olympus CX21 Microscope (magnification 400×). Scale bar: 10 µm. B. Light Microscope of this study with Olympus CX21 Microscope (magnification 1000×). Scale bar: 5 µm

Table 2. Identified morphological characteristics of *Chlorella pyrenoidosa*

Species	Strain origins	Measurement (µm)	Description	Reference
Chlorella pyrenoidosa (this study)	Pathek Beach, East Java, Java Island, Indonesia	4.433-6.972	Spherical or round shape	This study
Chlorella pyrenoidosa	Part of the UTCC (University of Toronto Culture Collection) collection, numbered UTCC 89	2-10	Round or oval shape	Campbell et al. (1997)
Chlorella pyrenoidosa	Brackish water near Liaodong Bay	5.404	Round or oval shape	Yi et al. (2019)
Chlorella pyrenoidosa	Freshwater bodies located in Haryana, Punjab, Rajasthan and Uttarakhand	2	Round shape; surface is smooth and compacted, as well as covered with an irregular network of subtle ribs	Bajwa et al. (2018)
Chlorella pyrenoidosa	Freshwater specimen from the Algae Culture Collection at the Institute of Hydrobiology, Chinese Academy of Sciences (China)	2.5-3	Spherical shape	Feng et al. (2023)

The strain isolated from Pathek Beach, Indonesia, had sizes between 4.433 and 6.972 µm, which was more significant than strains from other regions, such as Haryana, Punjab, Rajasthan, and Uttarakhand, India, for which the cells are only 2 µm. The habitat of the strain also appeared to influence its morphology. In contrast, strains from freshwater habitats, such as those from Haryana and the Algae Culture Collection at the Institute of Hydrobiology in China, tend to be smaller, with the latter ranging from 2.5 to 3 µm and exhibiting a spherical shape (Table 2). Strain UB02 PATHEK PSAL has a bigger diameter than freshwater green microalgae from this study.

Chlorella pyrenoidosa is a commercialized microalga for food, animal feed, bioenergy, and related co-products (Das et al. 2015; Chen et al. 2022). It can be cultivated worldwide in Indonesian aquatic areas and tropical regions. This vast array of microalgae has a cosmopolitan appeal, which allows microalgae to be grown widely across the globe, making it convenient to cultivate (Jiménez-Llanos et al. 2020; Hadiyanto et al. 2021). Due to its unique biochemical composition and environmental resilience, Isolated *C. pyrenoidosa* exhibits significant potential for diverse applications. *C. pyrenoidosa* contributes to nutrient cycling in marine ecosystems primarily as a photosynthetic microalga that assimilates inorganic nutrients, including nitrogen and phosphorus, from the water. *C. pyrenoidosa* has been demonstrated to contribute to mitigating nutrient pollution and eutrophication in aquatic environments. Mechanistically, this occurs through efficiently removing nitrogen compounds, including ammonia, nitrate, total nitrogen, and phosphorus during the growth phase. The metabolism process in these organisms transforms nutrients into algal biomass, which is characterized by a high concentration of proteins and other valuable compounds. These compounds subsequently enter the food web, serving as a nutritional source for aquatic organisms (Tan et al. 2021; Wang et al. 2023b). In nutrition, *C. pyrenoidosa* is a rich source of high-quality protein, essential amino acids, vitamins, bioavailable vitamin B12, and antioxidants, making it an excellent candidate for dietary supplements and functional foods, particularly benefiting vegetarian and vegan populations (Wu et al. 2023). Additionally, it is a promising feedstock for sustainable biofuel production due to its high lipid content and biomass productivity. Cultivating *C. pyrenoidosa* in nutrient-rich wastewater has enhanced biomass yield and contributed to wastewater nutrient removal (Zhang et al. 2019). The strain identified in this study could be subjected to further evaluation for these traits under applied conditions to determine its commercial viability. The findings from this study may be beneficial for developing large-scale cultivation systems for sustainable biomass production. Due to its cultivation capacity and rapid growth, the *C. pyrenoidosa* UB02 Pathek PSAL strain under consideration could be utilized in photobioreactor systems to facilitate continuous biomass production.

Molecular identification

The method of the Quick-DNATM Plant/Seed Miniprep Kit was used to isolate DNA from *C. pyrenoidosa* with good quality, as shown by the electrophoresis results: the DNA bands were separated and were of different brightness (Figure 4). The isolation of DNA from *C. pyrenoidosa* was performed using the Quick-DNA™ Plant/Seed Miniprep Kit, yielding genomic DNA of good quality as illustrated by the results of the subsequent electrophoresis, in which DNA bands of varying thickness were observed (Figure 4). The amplification of the rbcL gene resulted in the formation of a distinct PCR band with an approximate length of 600 base pairs, as illustrated in Table 3. The amplification of the rbcL gene resulted in an amplification band with a base length of ±600 bp (Table 3).

A similar finding was reported in previous studies that used the rbcL gene as a molecular marker for planktonic samples from a Florida reservoir and from algal isolates. Accordingly, Paul et al. (1990) reported the amplification of rbcL fragments from various *Chlorella minutissima* and *Synechococcus*, with product sizes depending on the primer set used, typically ranging from 498 base pairs (bp) to 621 bp. In addition, the research conducted by Fitriyah et al. (2021) yielded PCR results for six isolates of *Chlorella*, obtained using the rbcL gene. These results demonstrated the presence of an amplified band with a size of approximately 615 base pairs.

The gene amplification results of the microalgae isolates were confirmed by sequencing. A machine sequencing readout of approximately 600 bp is considered to be good, and BLAST was used to determine if the gene was similar to the data in the GenBank. Subsequent sequence similarity assessments were conducted using the Basic Local Alignment Search Tool (BLAST) against the GenBank database to confirm the identity of the amplified genes. The genomic DNA extraction process yielded samples of high purity and quality, as demonstrated by the obtained integrity and concentration metrics. In this study, the genomic DNA obtained an A260/280 ratio of 1.92 and a DNA concentration of 100.7 ng/µL in the isolates obtained from Pathek Beach, Situbondo District, East Java, indicating that the DNA was of acceptable purity with minimal protein or RNA contamination. An A260/A280 ratio of 1.7 to 2.0 is generally considered indicative of acceptable purity. The ratio of absorbance at 260 and 280 nm is an indicator of DNA purity. A ratio of ~1.8 is widely interpreted as "pure" for DNA. However, if the ratio is substantially lower (≤1.6), it may suggest the presence of proteins, phenol, or other contaminants that exhibit strong absorption at or near 280 nm. However, the presence of RNA can lead to an increase in the ratio, as the readings are unable to differentiate between DNA and RNA. This possibility must be considered to avoid an inaccurate quantification of DNA (Glasel 1995; Bruford et al. 1998; Lucena-Aguilar et al. 2016). The purity of DNA affects the outcome of amplification, since a high purity value makes the resulting DNA bands more visible under UV light. Falcon (1982) states that the range of these numbers is adequate for advanced molecular testing.

Genetic distance and phylogenetic analysis based on the rbcL gene

Based on the BLAST results obtained from GenBank, the species *C. pyrenoidosa* Wu-G3H3-1-2 and *C. pyrenoidosa* strain 820 were identified using the rbcL marker with a similarity percentage of 99.48% (Table 4) to the *C. pyrenoidosa* isolated from Pathek Beach. *C. pyrenoidosa* Wu-G3H3-1-2 is a marine green microalga from Taiwan cultivated in modified Walne medium, and *C. pyrenoidosa* strain 820 is a marine microalga stored in the Key Laboratory of Marine Biotechnology in the Province of Zhejiang in Ningbo University, China. Molecular identification of the Chlorophyta members showed that they were included in a group with the genus *Chlorella*, as shown by the phylogenetic tree of the construction results using the maximum likelihood tree method (Figure 5). The sample UB02 Pathek PSAL is closely related to *C. pyrenodoisa* strain 820 with a distance of 0,0048, which indicates the same ancestor. The phylogenetic tree was constructed using the find best DNA model, with the Tamura 3-parameter was chosen based on the highest score, with the rate variation among sites modeled with a gamma distribution. The identification of *C. pyrenoidosa* as a new record species for Pathek Beach enriches the local microalgal biodiversity inventory and provides an initial basis for further ecological and biotechnological investigations. Genetic diversity confers an evolutionary distinctiveness, enabling disparate strains to adapt to variable environmental conditions or selective pressures. Future studies involving molecular characterization or whole-genome sequencing of this isolate could provide insights into its unique traits and guide selective culture or genetic modification steps.

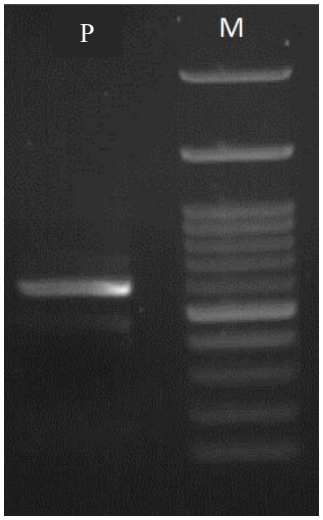

Figure 4: Gene amplification results of *Chlorella pyrenoidosa* on 1% agarose gel

Table 3. Sequence assembly result-PCR products rbcL primer

Isolate	Sequence
Chlorella pyrenoidosa (new record)	1 TGTCACCCCC AACAGAGACT AAAGCAGGTG CTGGATTTAA AGCAGGTGTT AAAGATTATC 61 GTTTAACATA CTATACTCCT GATTATCAAC CAAAAGAAAC TGATATTCTT GCAGCATTTC 121 GTATGACACC ACAACCAGGT GTACCACCAG AAGAAGCTGG TGCTGCTGTT GCTGCAGAAT 181 CTTCAACAGG TACTTGGACA ACTGTATGGA CAGACGGTTT AACTAGTTTA GATCGTTATA 241 AAGGTCGTTG TTATGATATT GAACCAGTTC CAGGCGAAGA AAATCAATAT ATTGCATATA 301 TTGCTTACCC TTTAGATCTT TTCGAAGAAG GTTCTGTAAC GAATTTATTT ACTTCAATTG 361 TAGGTAACGT TTTTGGTTTC AAAGCTCTTC GTGCATTACG TCTAGAAGAT CTTCGTATTC 421 CACCAGCATA TGTAAAAACT TTCCAAGGAC CTCCACACGG TATTCAAGTT GAACGTGATA 481 AACTAAACAA ATACGGTCGT GCGCTTTTAG GTTGTACTAT TAAACCAAAA TTAGGTCTTT 541 CTGCGAAAAA CTATGGTCGT GCAGTTTATG AGTGTTTACG CGGTGGAC

Table 4. Similarity of *Chlorella pyrenoidosa* UB02 Pathek PSAL to the other strains. BLAST result of the isolate taken from Pathek Beach, Situbondo District, East Java, Indonesia

Specimen	Max score	Total score	Query cover	E value	Perc. similarities	Accession number
Chlorella pyrenoidosa Wu-G3H3-1-2	1048	1048	97%	0.0	99.48%	KR069064.1
Chlorella pyrenoidosa strain 820	1048	1048	97%	0.0	99.48%	EU038284.1

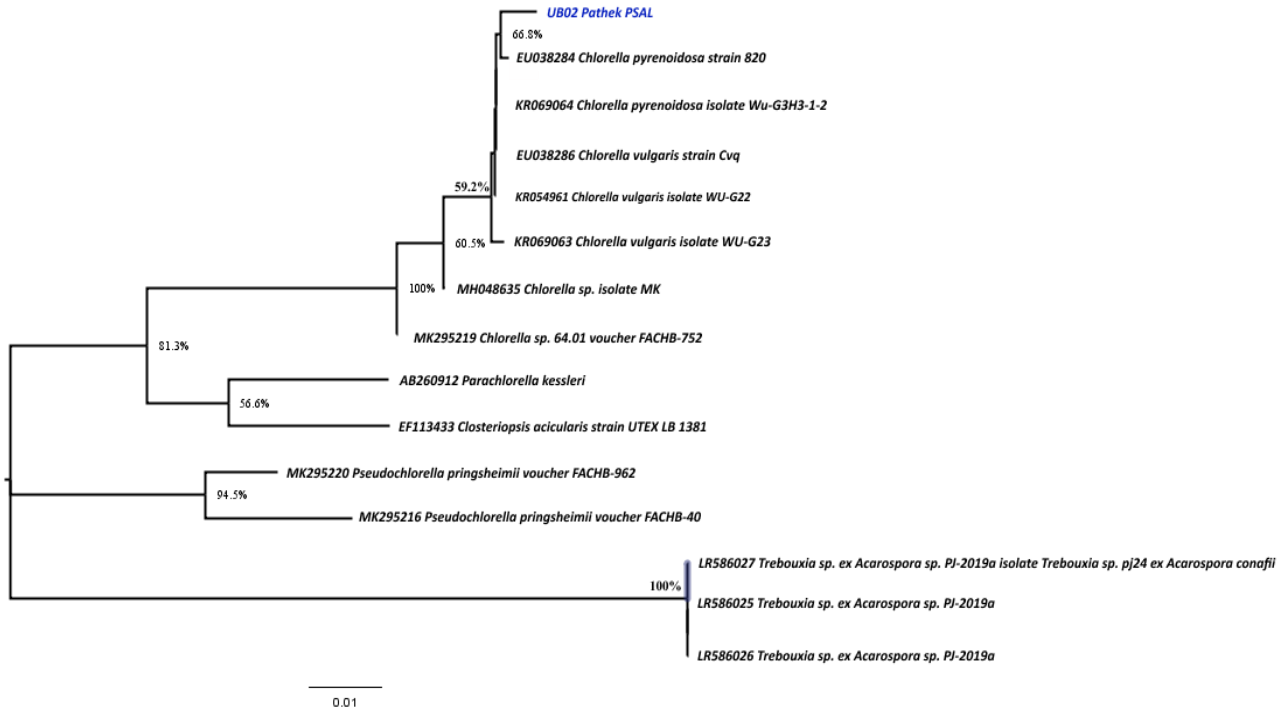

Figure 5. Phylogeny tree of the microalgal isolate, created by Maximum Likelihood (bootstrap of 1000) in the MEGA11 software

Chlorella sp. isolate MK is closely aligned with the clade comprising *Chlorella vulgaris* and *C. pyrenoidosa*, indicating a close phylogenetic relationship (Figure 5). Furthermore, *Parachlorella kessleri* and *Closteriopsis acicularis* are positioned in adjacent clades to the *Chlorella* species, reflecting their phylogenetic proximity. *Pseudochlorella pringsheimii*, as represented by voucher FACHB-962 and FACHB-40, was found to form a separate clade positioned close to the *Chlorella* clade. The species from the genus Trebouxia, including *Trebouxia* sp. ex *Acarospora*, constitute a distinct clade (Figure 5). Their position is separate from the primary *Chlorella* clade, signifying considerable genetic divergence while maintaining broader phylogenetic connections.

Tallei et al. (2016) stated that two organisms that have a closer family relationship have a smaller genetic distance between them on the phylogeny tree. Schneider and Cannarozzi (2009) stated that outgroups should be used to confirm distant relationships and increase the credibility of research findings. Based on the BLAST results, the isolates from Pathek Beach showed a close relationship with *C. pyrenoidosa*, with a similarity index of 99%. In addition, quality parameters of the medium culture, including temperature (24.3 to 25.4°C) and pH (7.64 to 7.84), were measured during this study. The presented data (Table 5) provides a quantitative representation of the genetic distance between the species "UB01 PATHEK PSAL" and two strains of *C. pyrenoidosa*, identified with accession numbers "KR069064" and "EU038286 strain 820", respectively. The minimal genetic distance of 0,00476 suggests a high genetic similarity between "UB01 PATHEK PSAL" and the mentioned strains. This similarity indicates a close phylogenetic relationship, suggesting that all three are from the same evolutionary lineage or have a relatively close ancestor. The phylogenetic analysis in this study shows that the new *Chlorella* strains cluster within established clades, with bootstrap support values around 66.8%. This aligns with prior investigations on *Chlorella* species, where similar moderate to high bootstrap values have been reported. The current findings align with the observations reported by Baytut et al. (2017) and Fitriyah et al. (2021), highlighting the *Chlorella* isolates into distinct clades with moderate to high bootstrap support. These studies demonstrated that *Chlorella* strains from diverse geographic regions formed close clusters, exhibiting high genetic similarity (up to 99%) and robust bootstrap values above 65% in rbcL and 18S rDNA phylogenies. These findings indicate the challenges of distinguishing closely related

Chlorella species, a phenomenon attributed to the limited genetic divergence and morphological similarity observed among Turkish and Indonesian freshwater isolates.

Chlorella genus from various geographic regions shows high genetic similarity and forms a closely related phylogenetic group. Therefore, their biotechnological potential may vary significantly due to differences in physiological and environmental adaptability. The selection of biodiesel feedstock is essential to ensure the competitiveness of biodiesel prices in the market. A multitude of factors are instrumental in the successful implementation of microalgae-based biodiesel production on a commercial scale. Therefore, the optimization of cultivation environment conditions must be carried out in conjunction with the optimization of nutrient composition, whether synchronously or sequentially. Praharyawan (2021) stated that, further to enhance the techno-economic feasibility of the microalgae propagation process, it is possible to carry out this process by utilizing various types of waste available in Indonesia. However, applying the optimum composition of growth media in microalgae cultivation should still be a primary focus. Concurrently, optimizing cultivation environmental conditions will provide microalgae with the most conducive conditions for growth and reproduction, thereby avoiding stress that can negatively affect their growth. Consequently, the optimization of environmental conditions for microalgae cultivation in Indonesia should be informed by the natural characteristics of the isolation site. Regarding Gaurav et al. (2024) stated the requirement for optimized cultivation conditions and technological enhancements to increase biomass and lipid productivity necessitates investment and infrastructure support.

In conclusion, this study provides the morphological and molecular characterization of *C. pyrenoidosa* isolated from Pathek Beach, Situbondo, East Java, Indonesia. The morphological analysis revealed the characteristics of small, spherical to round-shaped cells with thick cell walls and distinct chloroplasts, consistent with the established descriptions of *C. pyrenoidosa*. In addition, DNA barcoding using the rbcL gene marker confirmed the identity of the chlorophyte strain, thereby substantiating the species' presence in Pathek Beach, East Java, Indonesia. This finding provides a foundation for further research and applications in diverse biotechnological fields.

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