BIODIVERSITAS
Volume 25, Number 9, September 2024
Pages: 3339-3349

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d250956

Evaluation of a newly isolated thermophilic microalga *Chlorella sorokiniana* UNM-IND1 as biodiesel feedstocks

NUR ZAKIYAH RAMADANI[1], INDRAYANI[2,♥], ELMI NURHAIDAH ZAINUDDIN[3]

[1]Department of Fishery Science, Faculty of Marine Science and Fishery, Universitas Hasanuddin. Jl. Perintis Kemerdekaan Km. 10, Tamalanrea, Makassar 90245, South Sulawesi, Indonesia

[2]Department of Agricultural Technology, Faculty of Engineering, Universitas Negeri Makassar. Jl. Mallengkeri Raya, Makassar 90224, South Sulawesi, Indonesia. Tel.: +62-878-40311101, ♥email: indrayani@unm.ac.id

[3]Department of Fishery Science, Faculty of Marine Science and Fishery, Universitas Hasanuddin. Jl. Perintis Kemerdekaan Km. 10, Tamalanrea, Makassar 90245, South Sulawesi, Indonesia

Manuscript received: 8 August 2024. Revision accepted: 27 September 2024.

Abstract. *Ramadani NZ, Indrayani, Zainuddin EN. 2024. Evaluation of a newly isolated thermophilic microalga* Chlorella sorokiniana *UNM-IND1 as biodiesel feedstocks. Biodiversitas 25: 3339-3349.* Microalgae contain lipids and fatty acids that can be used as an alternative energy for biodiesel. Efforts to increase lipid productivity in microalgae can be done by conditioning the microalgae under certain stress conditions. Temperature is one of the environmental factors that can stimulate lipid production in microalgae. This study aims to analyze the effect of different temperatures on the growth, biomass, lipid productivity and fatty acid composition of a newly isolated microalgae *Chlorella sorokiniana* Shihira & Krauss, 1965 UNM-IND1 and to evaluate the potential of the microalga as biodiesel feedstocks. The *C. sorokiniana* UNM-IND1 was initially cultured at three different temperature conditions namely culture room temperature (25-26°C), ambient room temperature (28-32°C) and drying room temperature (32-48°C) (phase I). In phase II, the cultures were transferred to lower temperature conditions and incubated for 3 days. The parameters measured were cell density, specific growth rate, biomass productivity, lipid content, lipid productivity and fatty acid composition. The results showed that all cultures can grow at all culture conditions tested. The highest specific growth rate and biomass productivity were achieved at drying room temperature treatment at 0.790 d^{-1} and 0,166 $g.L^{-1}.d^{-1}$, respectively. The highest lipid productivity (0.0674 $g.L^{-1}.d^{-1}$) was obtained at culture room temperature. After the temperature shift, the lipid yield, lipid content and lipid productivity of all cultures increased. Fatty acid analysis showed that the alga consists of mainly palmitic acid, linolenic acid and linoleic acid. Based on the growth, biomass, lipid and fatty acid composition, the *C. sorokiniana* UNM-IND1 has potential as a biodiesel feedstock.

Keywords: Biodiesel feedstocks, *Chlorella sorokiniana*, fatty acids, lipid, thermophilic

INTRODUCTION

Nowadays, the world is faced with a crisis of increasingly depleted petroleum availability, along with an increasing population and increasing energy needs. In addition, using fossil fuels as the main energy sourcing fossil fuels as main energy source negatively impacts has a negative impacts environmental quality. Therefore, there is an urgent need to develop alternative energy sources to mitigate the energy crisis through the development of sustainable and promising alternative energy (Makareviciene and Sendzikiene 2022). One of the alternative fuels that has the potential to overcome the problem of fossil fuels is biodiesel (Hossain et al. 2020). Biodiesel is a fuel made from vegetable oils and animal fat which have characteristics similar to diesel fossil fuel (Abbaszaadeh 2012). According to Hwang et al. (2022), biodiesel has great potential for its application in the industrial sectors due to its efficiency and environmentally friendly. It produces less CO_2, CO, SO_2, and hydrocarbon emissions compared to diesel fuel from petroleum fractions. Moreover, biodiesel does not exacerbate the greenhouse effect because the carbon chains involved in the cycle are short carbon chains that are easily degraded naturally (Pruvost et al. 2018).

Microalgae are currently receiving a lot of attention as a potential alternative energy source. They are known as one of the main sources of biodiesel due to their high lipid content that can be converted to biodiesel. According to Chisti (2007), microalgae with a lipid content of 70% can produce up to 136,400 L/Ha of vegetable oil. In addition, microalgae have advantages over higher plants, namely easy to grow, do not require fertile and large areas of land and they can be harvested daily throughout the year due to their fast growth. However, using microalgae as raw material for biodiesel still faces challenges in cost-effective large-scale production (Gaurav et al. 2024; Wang et al. 2024). Species or strain selection is the first and most important step in developing microalgae for any industrial applications including developing microalgae as biodiesel feedstocks. The search for oleaginous microalgae is an ongoing effort. In Indonesia, research and development of microalgae as a renewable fuel source continue to be carried out. Isolation and selection of microalgae from various aquatic habitats have been widely carried out including freshwater such as rivers, lakes or ponds/ponds as well as brackish and sea waters. However, there is no report on bioprospecting extremophile microalgae for biodiesel feedstocks in Indonesia.

One of the extreme environments that can be a habitat for microalgae is hot springs. Microalgae that can live in these conditions have advantages over other organisms, namely being able to produce heat-resistant enzymes and resistance to contaminants (Goecke et al. 2020). Thermophilic microalgae are of interest to biologists because of their biological genetic origins and perhaps some of these species are more valuable than commercially available strains. Currently, interest in microalgae that live in hot springs is increasing because several researchers have stated that isolating microalgae diversity from this habitat is important for understanding the role of microalgae in the biogeochemical cycle and for determining their biotechnological potential, one of which is their high lipid content (Ancona-Canche et al. 2017).

According to Gultom (2018) efforts to increase lipid in microalgae can be done by conditioning microalgae in certain stress conditions. Under stressful conditions, microalgae are stimulated to synthesize more lipids as a form of microalgae mechanism in protecting themselves and adapting to their environmental conditions (Widianingsing et al. 2011). Temperature has a significant effect on the lipid content of microalgae because it can affect the growth and productivity of biomass. Gómez-Loredo et al. (2021) revealed that higher temperatures can increase the growth rate of microalgae, such as *Chlorella sorokiniana* Shihira & Krauss, 1965 at a temperature of 30 -42°C, but can affect the balance of cell composition and decrease biomass quality. Shang et al. (2024) reported a significant increase in the biomass of Skeletonema dohrnii as the temperature increased but the total lipid content decreased as the temperature increased. The highest total lipid content was observed at 15°C (39.23±0.32 mg/g) compared to that of the control group at 25°C (30.97±0.06 mg/g). Indrayani et al. (2022) also reported that the highest lipid content of diatom *Amphora* sp. MUR258 (57.69±2.039%AFDW) was achieved at a lower temperature (25°C) and the lowest lipid content (34.43±3.955% AFDW) was obtained at 35°C.

Chlorella UNM-IND1 is a newly isolated microalga from the Waepella Hot Spring, Sinjai Regency, South Sulawesi, Indonesia (Indrayani et al. 2023). The previous study showed that the *Chlorella* sp. UNM-IND1 has fast growth ($0.77 d^{-1}$) and high biomass productivity ($0.166 g.L^{-1}.d^{-1}$) in urea + NPK media (Indrayani et al. 2023). Fast growth and high biomass productivity are the main characteristics needed to develop microalgae as raw materials for various industrial purposes. In addition, information on lipid content and fatty acid composition of microalgal species is very important to evaluate their potential as a raw material for biodiesel. Therefore, this study aims to analyze the effect of different temperatures on the growth, biomass, lipid and fatty acid composition of the newly isolated microalga *C. sorokiniana* UNM-IND1 and evaluate its potential as biodiesel feedstocks.

MATERIALS DAN METHODS

Microalgal species

Microalga *C. sorokiniana* UNM-IND1 is a newly isolated species from the Waepella Hot Spring in Sinjai Regency, South Sulawesi Indonesia. The species was isolated following the agar plating technique in Guillard's f/2 agar medium (2% w/v) (Andersen and Kawachi 2005). The alga is maintained in the Agricultural Technology Laboratory, Faculty of Engineering, Universitas Negeri Makassar, Indonesia.

Microalgae identification

Identification of microalgae isolates was carried out morphologically and molecularly. Morphological identification was based on pigmentation, cell shapes (coccoid, filamentous), cell size, the presence or absence of flagella and other distinctive features. Morphological observations were carried out using a light microscope (Olympus BX40) and a scanning electron microscope (SEM).

For molecular identification, the DNA isolation process was carried out using the Genomic DNA Mini Kit (Plant) (Geneiaid Biotech Ltd, Taiwan). The kit consists of GP1 buffer, GPX1 buffer, GP3 buffer (plus isopropanol), W1 buffer, Wash buffer (plus ethanol), elution buffer (pre-heat at 60°C), RNase A, filter column, GD column, and 2 mL collection tube. DNA isolation consists of five stages, namely cell dissociation, lysis, DNA binding, washing, and DNA elution.

After the DNA extraction stage, the DNA quantity was analyzed using Nanodrop 2000 (Thermo Fisher Scientific, USA) to determine the concentration and purity of DNA (Cheng et al. 2021). DNA concentration was determined by the absorbance value at a wavelength of 260 nm (A260). Meanwhile, the purity of DNA from protein, polyphenol, and polysaccharide contamination was assessed by estimating the absorbance ratio at A260/A280 and A260/A230. The A260/A280 ratio approaching 1.8 is generally accepted as pure DNA. The A260/A230 ratio generally accepted for dsDNA is 1.8-2.2. The results of DNA quantity analysis were used as a guideline for DNA dilution used for the PCR process. DNA amplification was carried out using a PCR machine (Bio-rad, USA). The total volume of amplification reaction in this study was 40 µL with details of 20 µL My Taq HS Red Mix 2X (Meredian Bioscience, USA), 0.8 µL of each forward and reverse primer (10 µM), 16.4 µL dH2O, and 2 µL DNA template (92 ng/µL). The amplification program on the PCR machine was determined as follows: initial denaturation (95°C for 2 minutes), 35 cycles (95°C for 15 seconds, 54°C for 15 seconds, and 72°C for 1 minute), then continued with a final extension at 72°C for 5 minutes.

The amplicons produced from the reaction were electrophoresed in a 1% agarose gel soaked in 1x TAE buffer for 55 minutes at 100V. Then the gel was stained using DiamondTM Nucleic Acid dye. The DNA bands in the gel were photographed using a gel documentation system.

The DNA sequence was analyzed for base sequence homology by utilizing DNA sequence information available

in the GenBank database (http:/www/ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST) and the creation of a phylogenetic tree using analysis from the Clustal X application, Neighbor-Joining (NJ-Plot).

Microalgae cultivation

Microalgae were cultured using a 300 mL Erlenmeyer flask containing 150 mL of urea + NPK enriched culture media (Indrayani et al. 2023). The study was conducted in two phases. In Phase I, microalgae were cultured at three different temperature conditions, namely culture room temperature (25-26°C), room temperature (30-34°C), drying room temperature (37-48°C) at a light intensity of 3500 lux with a light-dark cycle of 12 hours. The initial culture density for all treatments was the same at around 100×10^4 cells.mL^{-1}. The cultures were maintained for 10 days. After that, it was continued to phase II where the cultures were transferred to a lower temperature condition, namely the cultures in the drying room were transferred to ambient room temperature, the cultures at ambient room temperature were transferred to the culture room temperature while the cultures in the culture room were transferred to a showcase at a temperature of 11-12°C. In phase II, the culture was maintained for 3 days. During the culture period, the cultures were stirred manually at least 3 times a day and rotated every day. Sampling for cell density calculations was carried out every day. Sampling for biomass and lipids was carried out in the exponential and stationary phases in phase I while in phase II, sampling for biomass and lipids was carried out every day.

Lipid extraction

Lipid extraction refers to the research of Indrayani (2017). Lipid extraction follows the method of Bligh and Dyer (1959) as modified by Kates and Volcani (1966), which was adapted by (Mercz 1994). Namely, 5 mL of microalgae culture was harvested and then filtered with 25 mm Whatman GF/C paper. Whatman GF/C paper containing microalgae was put into a tube and crushed using a glass rod until it formed a smooth paste. One mL of a mixed solution (methanol + chloroform + aquabides) at a ratio of 2:1: 0.8 v/v/v was added into the tube and then transferred into a centrifuge tube. Another 1 mL of the mixed solution was added to clean the remaining extract in the tube and then transferred into the centrifuge tube. Next, 3.7 mL of the mixed solution was added into the centrifuge tube, so the total extract was around 5.7 mL. After that, the extract was centrifuged for 20 minutes at 3000 rpm. The supernatant was then transferred into a 30 mL clean glass tube. The pellet was resuspended by adding 5.7 mL of the mixture solution, then vortexed and centrifuged again. The supernatant was then combined into the glass tube. About 3 mL of aquabidest and 3 mL of chloroform were added to the glass tube and vortexed. The extract in the glass tubes was then incubated uninterrupted for 24 hours in the refrigerator until two phases were formed. The next day, the top phase was removed using a fine syringe and the bottom phase was transferred into a pre-weighed vial. The lipid sample in the vial was evaporated until completely dry at a temperature of 40°C using a hot plate. The vial containing lipids was then re-weighed.

Fatty acid analysis

The fatty acid analysis follows the method adapted by Christi (1989). Where 1 mL of toluene was added to the lipid sample before adding 2 mL of 1.5% sulfuric acid in methanol. The sample was then incubated in a water bath at 50°C overnight. After incubation, about 5 mL of 5% NaCl in deionized water was added to the sample to dissolve the water-soluble compounds. The top organic phase was transferred into a clean vial and the bottom phase was washed 2 times with 5 mL of hexane then the washing was combined in a vial then washed with 4 mL of 2% NaHCO3 in deionized water. The top layer was then transferred into a clean and dry vial. The sample was evaporated using a hot plate at 40°C until the sample volume was about 0.5mL. The sample was then transferred to a smaller vial ready for analysis using gas chromatography (GC-MS Ultra QP 2010 Shimadzu).

For GC-MS analysis, a total of ±0.1 mL of sample was added with 5 mL of methanol: chloroform (1:1). The sample was extracted using a sonicator for 20 minutes at 40°C. The sample was then inserted into the GC-MS vial. GC-MS instrument conditions injector temperature 250°C with splitless mode, pressure 76.9 kPa and flow rate 14 mL/min and ratio 1:10. Ion source and interface temperature 200 and 280°C, solvent cut time 3 minutes, 400-700 m/z. Column type SH-Rxi-5Sil MS column length 30 m with inner diameter 0.25 mm. The initial temperature of the column was 70°C with a holding time of 2 minutes and the temperature was increased to 200°C at a rate of 100°C/min and the final temperature was 280°C with a holding time of 9 minutes at a rate of 50°C/min so that the total analysis time was 36 minutes. The chromatogram data obtained were read using the NIST 17 and Wiley 9 libraries.

Data analysis

Cell density

Culture cell density was calculated daily during the culture period using a Neubauer haemocytometer (Moheimani et al. 2013). The cell density was calculated using the formula:

$$\text{Cell density (cells.mL}^{-1}) = (nA+nB+nC+nD)/4 \times 10^4$$

Where: nA, nB, nC, nD = number of cells in square A, B, C, D; 4 = number of squares counted.

Specific Growth Rate (SGR)

Specific growth rate is a parameter that describes the rate of increase in microalgae cells per unit of time. The specific growth rate (μ) is calculated using the following equation (Moheimani et al. 2013):

$$\mu = \frac{\mathbf{Ln}\,(N_2 \,/\, N_1)}{t_2 - t_1}$$

Where: N_2 is the cell density at time t_2 and N_1 is the cell density at time t_1 within the exponential phase.

Dry weight (biomass)

Dry Weight (DW) was determined following the method of Moheimani et al. (2013). Briefly, five mL of culture was filtered through pre-weighed Whatman GF/C 25 mm filter paper. The filters were then dried in an oven at 75°C for 5 hours. The filters were then removed from the oven and cooled in a desiccator before weighing. The DW was determined by the following formula (Moheimani et al. 2013):

DW ($g.L^{-1}$) = (weight of filter containing algae cells) - (weight of filter)

Biomass productivity (g L-1 d-1)

Biomass productivity was calculated based on the specific growth rate and biomass. Determination of biomass productivity followed the formula of Indrayani (2017):

Biomass productivity ($g\ L^{-1}\ d^{-1}$) = SGR × DW

Lipid yield ($g.L^{-1}$)

Lipid yield was calculated using the following equation (Indrayani 2017):

Lipid yield ($g.L^{-1}$) = weight of vial containing lipid - weight of vial

Lipid content (%)

Lipid content is the percentage of lipid accumulation based on dry weight biomass. The lipid content was calculated using the following formula (Indrayani 2017):

Lipid content (%) = (lipid yield/biomass yield) × 100%

Lipid productivity ($g.L^{-1}.d^{-1}$)

Lipid productivity expresses the formation of lipids in microalgae in mass units per volume per unit time. To obtain lipid productivity, use the formula (Indrayani 2017):

Lipid productivity ($g.L^{-1}.day^{-1}$) = SGR × lipid yield

Statistical analysis

One-way Analysis of Variance (ANOVA) was used to analyze significant differences between treatments and the Pairwise multiple comparison procedure (Holm-Sidak Method) was used to precisely test differences between conditions. All statistical analysis was performed using Sigma-Plot 14.5 Systat Software Inc., USA.

RESULTS AND DISCUSSION

Microalga idenfication

The microalga species used in this study is a newly isolated microalga from Waepella Hot Springs, Sinjai District, South Sulawesi, Indonesia. Morphologically, microalga has a solitary round cell shape with a diameter ranging from 2-4 µm and is green in color (Figure 1).

To confirm the species of microalga studied, molecular identification was carried out. The molecular identification process begins with the isolation of microalgae DNA, electrophoresis amplification of PCR products, and then purification and sequencing. The results of the amplification of the genomic DNA of the UNM-IND1 isolate using 18S rDNA PCR produced a single band at 3000 bp with relatively high intensity (Figure 2).

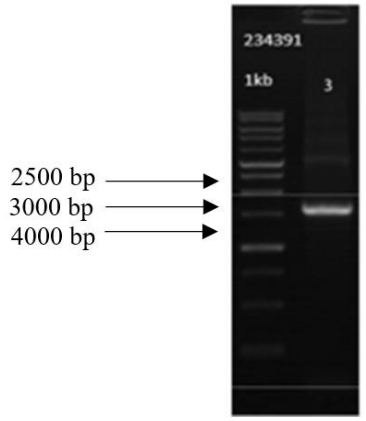
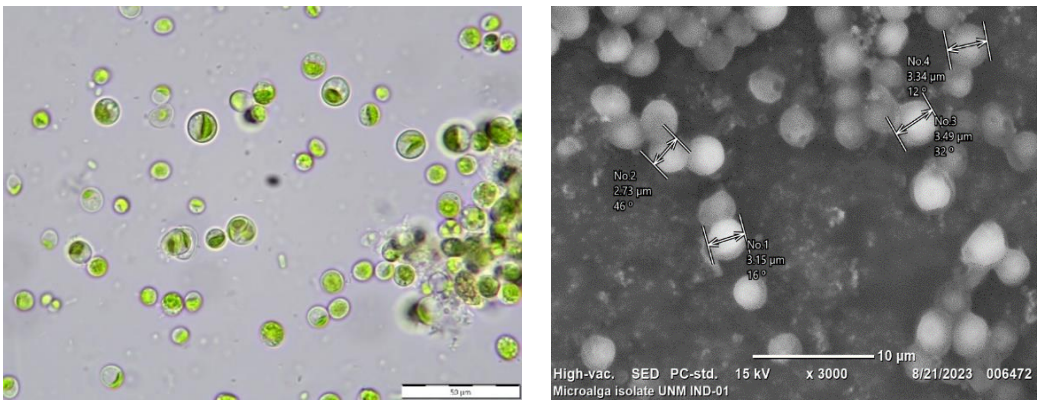


Figure 2. Electropherogram of 18S rDNA PCR product of isolate UNM IND1



A **B**

Figure 1. Microalgae *Chlorella sorokiniana* UNM IND-01 under light microscope (Olympus BX40) (A) and Scanning Electron Microscope (SEM) at 3000x magnification (B)

To determine the species of the isolate UNM-IND1, the Basic Local Alif=gnment Search Tool (BLAST) was performed. The BLAST results of UNM-IND1 DNA sequence with data available in the NCBI GeneBank showed that the UNM-IND1 isolate had a very close relationship with *C. sorokiniana* with a similarity level of 96.57% (Figure 3). The phylogenetic tree construction of microalga isolate UNM-IND1 showed that the isolate UNM-IND1 is in a monophyletic group with other Chlorella. The branch length of UNM-IND1 was 0.000, indicating that the UNM-IND1 is identical to *C. Sorokiniana* (Figure 4). The branch length of 0.000 indicates that there are no sequence changes as stated by Hall (2001) that the greater the branch length value, the more sequence changes occur.

Growth and biomass of *Chlorella sorokiniana* sp.UNM-IND1

Based on the results of temperature measurements for each treatment for five consecutive days, it was found that the culture room had a temperature ranging between 25-27°C, the ambient room temperature ranged between 27-34°C, while in the drying room, the temperature ranged between 32-48°C (Figure 5).

Sequences producing significant alignments Download Select columns Show 100

select all *100 sequences selected* GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Chlorella sorokiniana NKH6 gene for 18S ribosomal RNA, partial sequence	Chlorella sorokini...	2015	2015	99%	0.0	96.57%	1731	LC505542.1
Micractinium sp. KSF0094 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8...	Micractinium sing...	2015	2015	99%	0.0	96.57%	2387	MN414469.1
Micractinium sp. KSF0085 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8...	Micractinium vari...	2015	2015	99%	0.0	96.57%	2385	MN414468.1
Micractinium pusillum isolate Zhalong Salt Lake 1 small subunit ribosomal RNA gene, partial sequence	Micractinium pusi...	2015	2015	99%	0.0	96.57%	1688	MK764917.1
Chlorella sp. YACCYB105 18S ribosomal RNA gene, partial sequence	Chlorella sp. YAC...	2015	2015	99%	0.0	96.57%	1695	MH619552.1
Chlorella sp. YACCYB102 18S ribosomal RNA gene, partial sequence	Chlorella sp. YAC...	2015	2015	99%	0.0	96.57%	1699	MH619549.1
Chlorella sp. YACCYB100 18S ribosomal RNA gene, partial sequence	Chlorella sp. YAC...	2015	2015	99%	0.0	96.57%	1698	MH619547.1
Chlorella sp. YACCYB196 18S ribosomal RNA gene, partial sequence	Chlorella sp. YAC...	2015	2015	99%	0.0	96.57%	1704	MH636653.1

Figure 3. BLAST search result of DNA sequence of *Chlorella sorokiniana* UNM-IND1

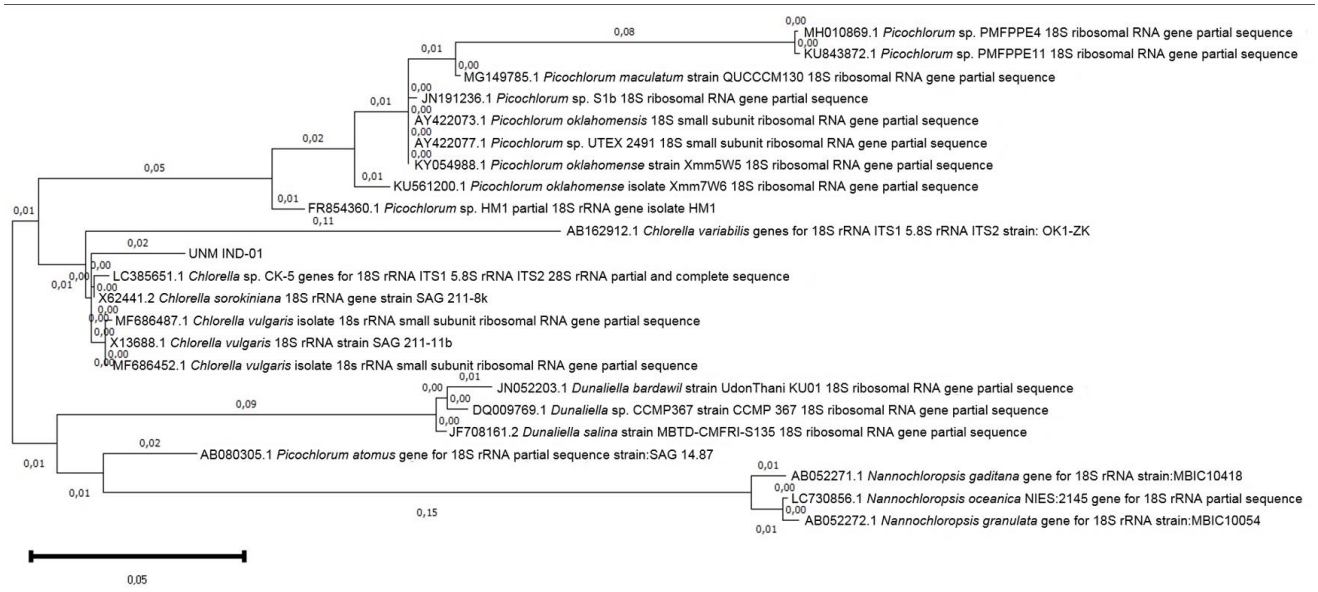


Figure 4. Phylogenetic tree depicting the relationship between isolate UNM IND 1 with *Chlorella sorokiniana* and other sequences from GenBank using the Neighbor-Joining model

The cell growth curve is formed by plotting the cell density of microalga culture against time. The results showed that the microalgae *C. sorokiniana* UNM IND1 could grow in all culture conditions tested but the rate and the cell density were different. The initial cell density in all treatments at the time of inoculation was the same at around 100×10^4 cells.mL^{-1}. One day after inoculation, the increase in the number of cells for all treatments was still small, indicating that the culture was in the lag phase. In the 2nd day, the culture at room temperature culture treatment experienced an exponential increase in cell density until it reached the maximum cell density on the 6th day at an average of 1729×10^4 cells.mL^{-1} before finally experiencing a decrease in cell density on the 7th day. In the room ambient temperature treatment, the lag phase lasted until the 2nd day before experiencing an exponential increase in cells on the 3rd day and reaching the maximum cell density on the 5th day at aroun 1104×10^4 cells.mL^{-1}. In contrast, in the drying room temperature treatment, the lag phase lasted longer until the 3rd day before entering exponential phase on the 4th day and reaching a maximum density on the 5th day at about 747×10^4 cells.mL^{-1} (Figure 6).

The results of the determination of the Specific Growth Rate (SGR) of *C. sorokiniana* UNM-IND1 at different temperature treatments can be seen in Figure 7. The highest specific growth rate was obtained at the drying temperature treatment with an average value of 0.790±0.096 day^{-1} and the lowest at room temperature treatment with an average of 0.689±0.066 day^{-1}. The results of the one-way ANOVA analysis showed that different temperature treatments did not significantly affect the specific growth rate of microalgae (p=0.277).

The results of the determination of the dry weight of the microalga biomass *C. sorokiniana* UNM-IND1 obtained in the exponential phase and stationary phase can be seen in the Figure 8. In general, there was an increase in the biomass of microalgae cultures for all treatments along with the increasing age of the culture. The highest biomass in both the exponential and stationary phases was obtained in the drying room temperature treatment with an average biomass of 0.160±0.02 g.L^{-1} and 0.233±0.11 g.L^{-1}, respectively. One way ANOVA analysis showed that there was no significant difference in the biomass of the microalga *C. sorokiniana* UNM-IND1 under different temperature treatments tested (P=0.178).

The biomass productivity of *C. sorokiniana* UNM-IND1 cultured at different temperatures can be seen in Figure 9. The highest biomass productivity was obtained at the drying room temperature treatment with an average biomass productivity of 0.184±0.021 g.L^{-1}.h^{-1} while the lowest biomass productivity was at the ambient room temperature at an average of 0.156±0.012 g.L^{-1}.h^{-1}. One way ANOVA analysis showed that different temperature treatments did not significantly affect the biomass productivity of *C. sorokiniana* UNM-IND1 (P=0.239).

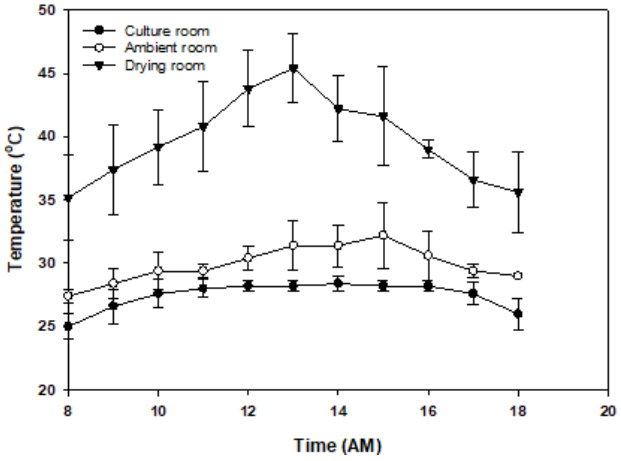


Figure 5. Temperature of each culture condition

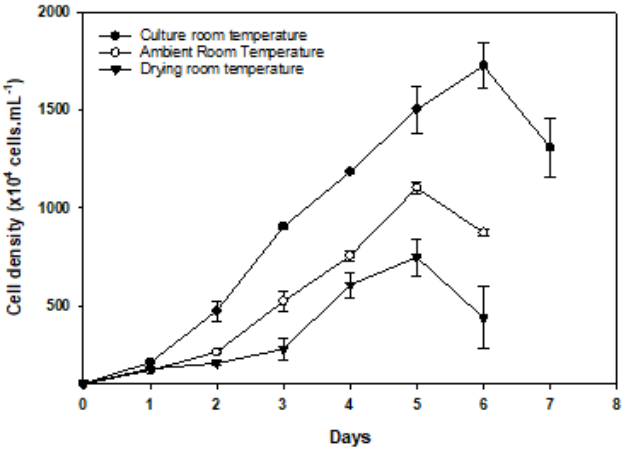


Figure 6. Growth curve of microalga *Chlorella sorokiniana* UNM-IND1 cultured at different temperatures

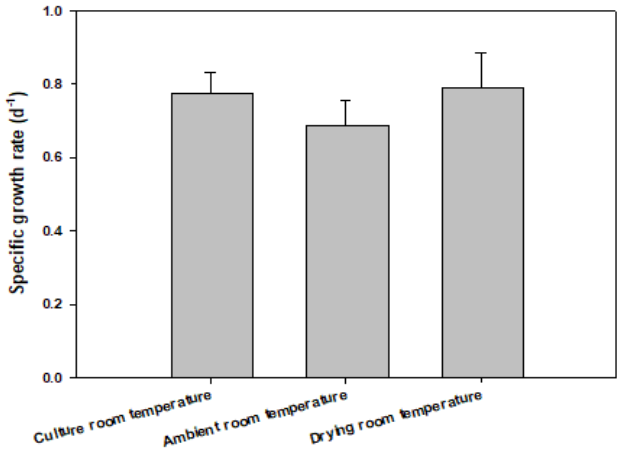


Figure 7. Specific Growth Rate (SGR) of *Chlorella sorokiniana* UNM-IND 01 at different temperatures

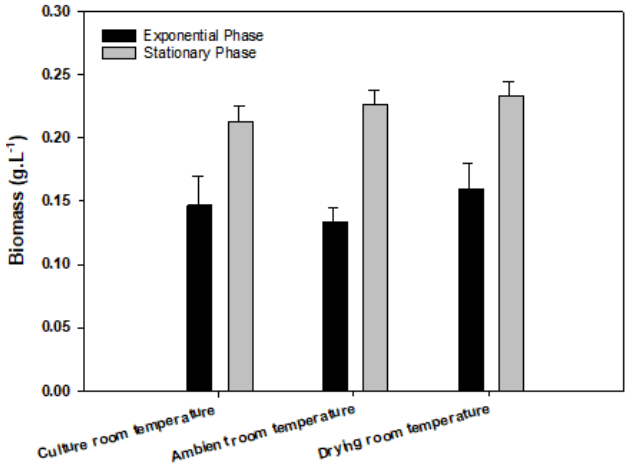


Figure 8. Biomass of microalga *Chlorella sorokiniana* UNM-IND01 at different temperature treatments

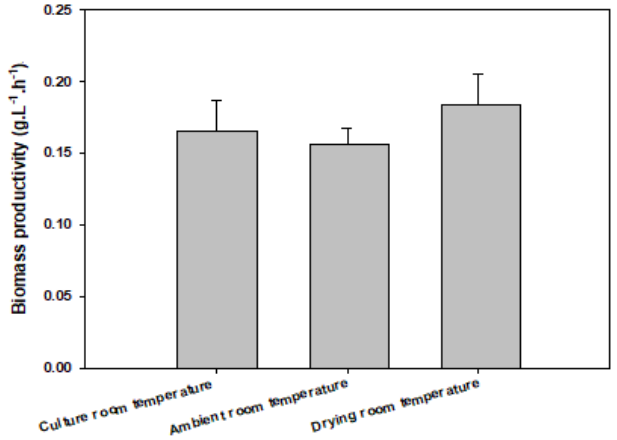


Figure 9. Biomass productivity of microalgae *Chlorella sorokiniana* UNM-IND 01 under different temperatures

Lipid of *Chlorella sorokiniana* UNM-IND1

To determine the effect of temperature stress on lipid accumulation of microalgae *C. sorokiniana* UNM-IND1, the experiment was conducted in two phases where in phase I, the culture was maintained at different temperature conditions, namely culture room temperature, room ambient temperature and drying room temperature for approximately a week. Entering phase II, the culture was transferred to a lower temperature, namely the cultures at culture room temperature were transferred to showcase temperature, the cultures at room ambient temperature were transferred to culture room temperature and the cultures in the drying room were transferred to room ambient temperature and incubated for 3 days. The results showed that the lipid yield and lipid content of microalgae in phase I for all treatments increased from the exponential phase to the stationary phase where the lipid yield in the exponential phase ranged from an average of 0.0467-0.0533 $g.L^{-1}$ increasing to an average range of 0.080-0.0867 $g.L^{-1}$ in the stationary phase. Likewise, the lipid content (%) for all treatments increased from an average range of 33.13-36.11% dry weight biomass in the exponential phase to an average range of 34.09-40.61% dry weight biomass in the stationary phase. One day after the temperature shift treatment in phase II, all treatments experienced an increase in both lipid yield and lipid content where lipid yield increased to an average range of 0.087-0.100 $g.L^{-1}$ and lipid content to an average range of 36.11-42.93% dry weight biomass. In the 2nd and 3rd days, in general, all treatments experienced a decrease in both lipid yield and lipid content (Figure 10).

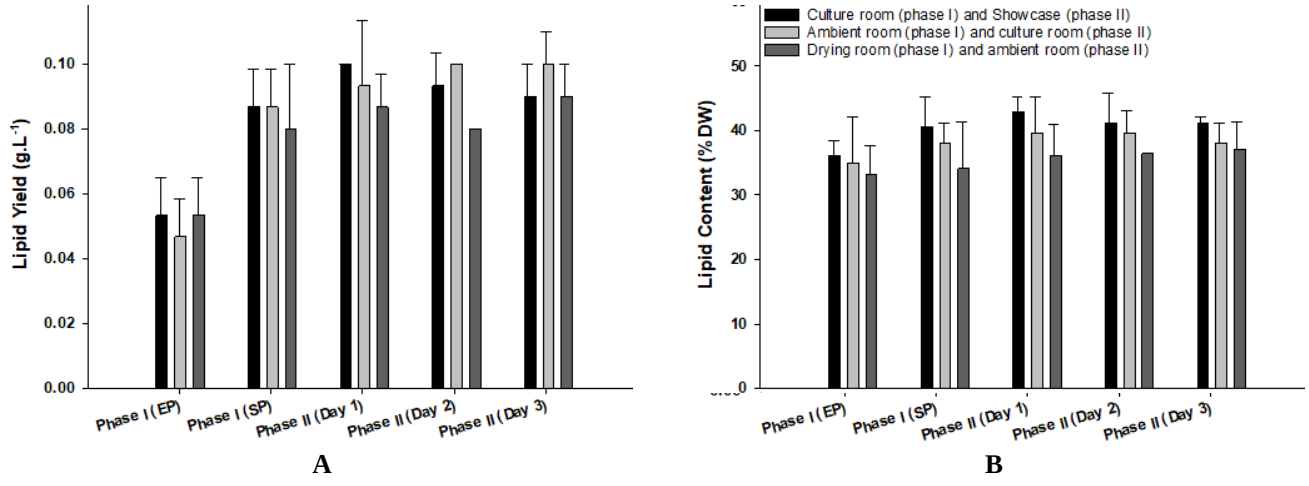


Figure 10. Lipid yield ($g.L^{-1}$) (A) and lipid content (% DW biomass) (B) of microalga *Chlorella sorokiniana* UNM-IND1

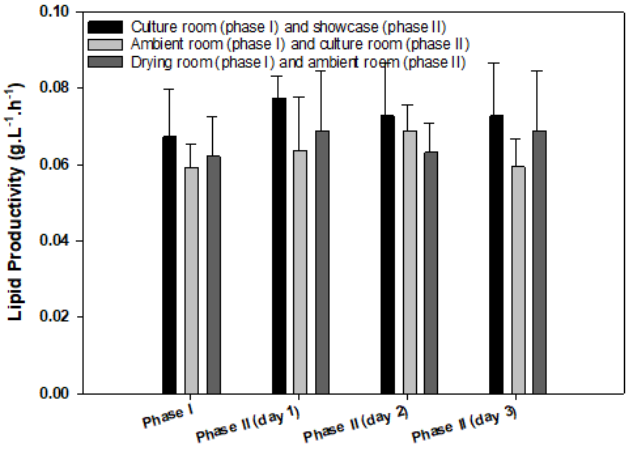


Figure 11. Lipid productivity of microalga *Chlorella sorokiniana* UNM-IND1

The highest lipid productivity of microalgae *C. sorokiniana* UNM-IND1 during phase I was obtained in the culture room treatment with an average biomass productivity of 0.067±0,012 $g.L^{-1}.d^{-1}$ while the lowest lipid productivity was in the ambient room temperature with an average of 0.0595±0.006 $g.L^{-1}.d^{-1}$. After the temperature shock treatment, lipid productivity on day 1 for all treatments increased where the treatment at showcase temperature had the highest lipid productivity with an average of 0.078±0.006 $g.L^{-1}.d^{-1}$, then the treatment at room temperature increased to 0.638±0.014 $g.L^{-1}.d^{-1}$ and the treatment at room temperature increased to 0.068±0.015 $g.L^{-1}.h^{-1}$. On day 2, lipid productivity in the treatment of showcase temperature and room ambient temperature decreased while lipid productivity at room temperature increased to 0.069±0.007 $g.L^{-1}.d^{-1}$. On day 3, lipid productivity in the showcase temperature and culture room temperature treatments decreased while the ambient room temperature increased to 0.069±0.016 $g.L^{-1}.d^{-1}$ (Figure 11).

Fatty acids composition of *Chlorella sorokiniana* UNM-IND1

Analysis of the fatty acid composition of *C. sorokiniana* UNM-UND1 lipid was carried out using Gas Chromatography-Mass Spectrophotometry (GC-MS) aiming to determine the type of fatty acids contained in microalgal lipids and their suitability as raw materials for biodiesel. The chromatogram of the fatty acid analysis revealed about 16 major peaks (Figure 12). Detailed information on the chromatogram can be seen in Table 1. The fatty acid analysis showed that the largest fatty acid components are palmitic acid (28.13%) followed by linolenic acid (23.47%) and linoleic acid (21.64%) (Table 1).

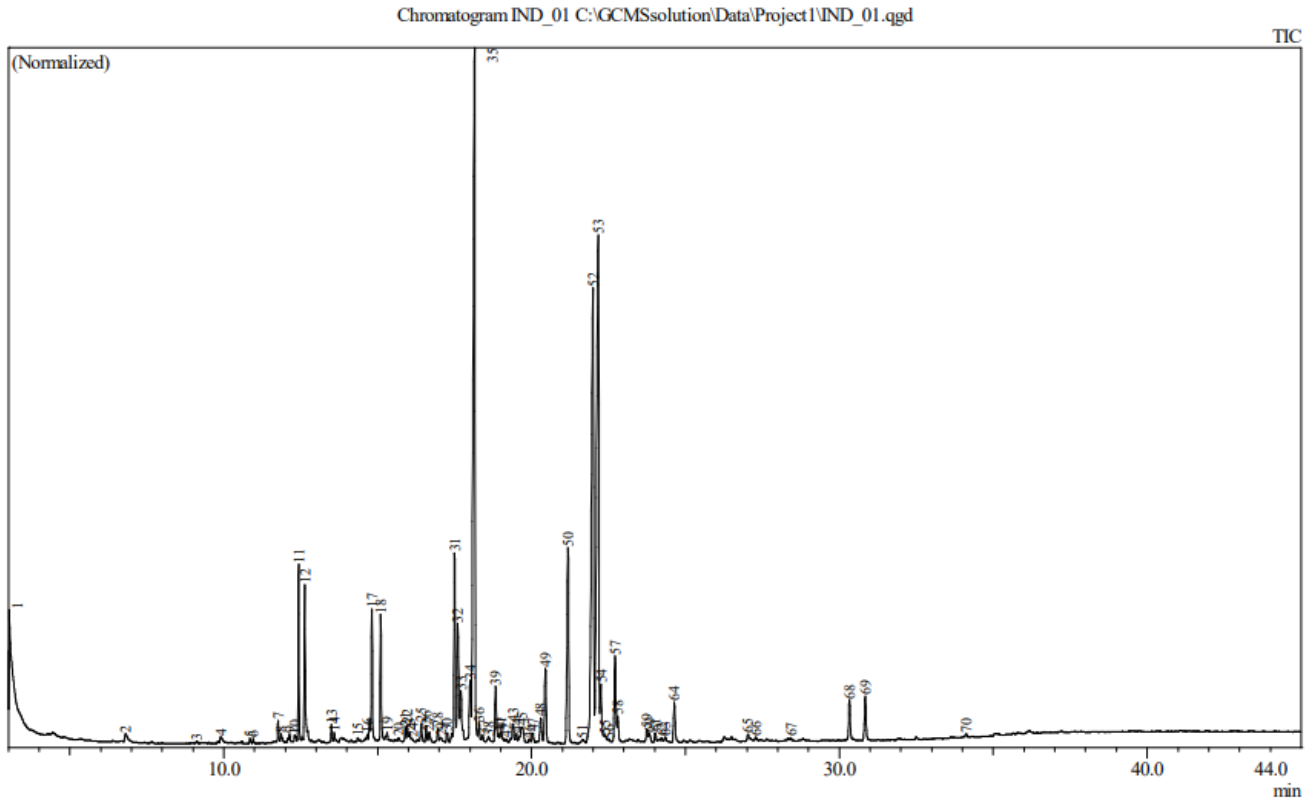


Figure 12. Chromatogram of fatty acid analysis of *Chlorella sorokiniana* UNM-IND1 using GC-MS

Table 1. Fatty acids compositions of microalga *Chlorella sorokiniana* UNM-IND1

Peak	Retention time	Fatty acids	Area (%)
2	6.811	Octanoic acid, methyl ester (C9:0)	0.49%
4	9.907	Decanoic acid methyl ester, Capric acid methyl ester (C10:0),	0.28%
12	12.629	Lauric acid (C12:0)	4.34%
18	15.100	Methyl Tetradecanoate, Methyl myristate, Myristic acid methyl ester (C14:0)	2.86%
20	15.678	Tridecanoic acid (C13:0)	0.13%
21	15.900	Pentadecanoic acid, Pentadecylic acid (C15:0)	0.66%
31	17.503	7,10-Hexadecadienoic acid (C16:2)	5.43%
32	17.604	9,12,15-Octadecatrienoic acid, Methyl linolenate, Linolenic acid methyl ester (C18:3)	4.23%
33	17.704	Palmitoleic acid (C16:1)	1.94%
35	18,293	Palmitic acidMethyl palmitate, Methyl hexadecanoate (C16:0)	28.13%
36	18.293	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-	0.61%
43	19.400	Hexadecanoic acid(C16:0)	0.80%
45	19.684	8,11-Eicosadienoic Acid, Methyl 8,11-Eicosadienoate	1.09%
48	20.300	Heptadecanoic acid (C17:0)	1.109%
52	21.996	Linoleic acid (C18:2)	21.64%
53	22.165	Linolenic acid (C18:3)	23.47%
54	22.250	Oleic acid, methyl oleate, (C18:1)	1.97%
64	24.639	Cis-10-Nonadecenoic acid (C19:1)	1.39%
65	27.036	Eicosanoid Acid, Arachidic Acid Methyl Ester, Methyl Icosanoate (C20:0)	0.18%
66	27.286	Cyclopropanedecanoic acid, 2-hexyl-.alpha.-hydroxy-, methyl ester	0.11%
68	30.328	13-Docosenoic acid, erucic acid (C22:1)	1.26%
70	34.117	Lignoceric acid, tetracosanoic acid (C23:0)	0.11%
		Total	100%

Discussion

Molecular identification results confirmed that the isolate UNM-IND1 is a microalgae species *C. sorokiniana* strain UNM-IND1. *Chlorella* sp. is a microscopic unicellular microalgae that can be found in fresh water and has structural elements similar to plants (Yamamoto et al. 2005). *Chlorella* sp. has a high growth rate and is capable of reproducing very quickly within 24 hours (Safi et al. 2014; Ullah et al. 2014). In addition, microalga from the genus *Chlorella* is one of the most widely studied genera of microalgae about their potential use as raw materials for biodiesel (Khorramdashti et al. 2021; Khalaji 2022) because this microalga can accumulate large amounts of lipids and has suitable fatty acid profile for biodiesel (Converti et al. 2009; Zheng et al. 2011). According to Ullah et al. (2014), Chlorella has the potential to produce 8-36 times more oil per hectare per year than palm oil. Based on the numerous studies on Chlorella and its wide industrial applications potential including as raw material for biodiesel, it is therefore important to explore the potential of the newly isolated *C. sorokiniana* UNM-IND1 as biodiesel feedstocks.

In this study, it was found that *C. sorokiniana* UNM-IND1 can grow well over a very wide range of temperatures starting from the lowest temperature, the showcase temperature of 11°C to the highest temperature in the drying room of 48°C. The ability to tolerate wide range temperatures, especially high temperatures, is understandable considering that the origin of this microalgae is the Waepella Hot Spring which has a temperature ranging from 45-55°C (Indrayani et al. 2022; Indrayani et al. 2023).

From the growth curve, it can be seen that the cultures in the culture room have a short lag phase and the cell density increases exponentially on the 2nd day whereas the other two conditions have a longer lag phase. The reason for this is that the inoculum used was cultured in the culture room, while the other two treatments had temperature conditions far above the initial culture temperature conditions, so they took a longer time to adapt. The growth curves also showed that the culture at the culture room temperature has a higher cell density than the other treatments. This is because the cultures can adapt quickly so that their cell density is higher in a short time. In addition, temperature plays an important role in increasing the density of culture cells. At lower temperatures, the solubility of gases, especially CO_2 is higher, so that more CO_2 is available to be utilized by microalgae. The cell density will continue to increase until it reaches a point where nutrients and other limiting factors including CO_2 are no longer able to support the growth of microalgae cells. In contrast, at higher temperatures, the solubility of CO_2 is limited which in turn leads to reduced growth (Duan et al. 2006; Indrayani et al. 2020).

Many studies have been conducted to evaluate the potential utilization of microalgae *Chlorella* sp. for biodiesel, but there is not much information available regarding the evaluation of the potential development of thermophilic microalgae species as biodiesel feedstock. Besides fast growth and ease of culture, lipid quantity and quality are important parameters to consider for the development of microalgae as biodiesel feedstocks. The quantity and quality of microalgae lipids are influenced by environmental conditions including temperature (Pasquet et al. 2014; Pandey et al. 2024). In this study, it was found that the lipid content of *C. sorokiniana* UNM-IND1 was higher at low temperatures. This is in line with previous studies (Chaisutyakorn et al. 2018; Indrayani et al. 2020). The high lipid content at low temperatures can be caused by low temperatures being suboptimal for the growth of *C. sorokiniana* UNM-IND1 which prefers higher temperatures for optimal growth so that at lower temperatures the microalga will modify its lipid biosynthesis pathways by

accumulating more neutral lipids act as energy reserves under suboptimal conditions (Hu et al. 2008). The lipid content of *C. sorokiniana* UNM-IND1 ranged from 33.13-42.93% dry matter weight of biomass which is comparable with the lipid content of other *Chlorella* species. For example, Ferreira et al. (2019) reported the lipid content of the *Chlorella* sp. ranged from 28-52% dry matter with lipid productivity of 42.1 $mg.L^{-1}.d^{-1}$ whereas Li et al. (2014) reported the lipid content of *C. sorokiniana* UTEX 1602 of 31.5% dry matter. *Chlorella* sp. S5 cultured on BG11 and agricultural runoff had lipid content ranging from 15.8-16% DW, and lipid productivity of 41-44 $mg.L^{-1}d^{-1}$ (Rana et al. 2024).

Another aspect of important consideration for developing a microalgae species as a biodiesel raw material is the composition of lipid fatty acids. Microalgae lipids can be converted into biodiesel by a transesterification reaction involving the reaction between triglycerides and alcohol to form fatty acid methyl esters and glycerol. Fatty acid composition greatly affects the quality of biodiesel. Fatty acids dominated by long-chain fatty acids (C16-C18) are desirable as the Cetane Number (CN), heat of combustion, and viscosity will increase with increasing chain length (Saber et al. 2024). However, high CN values have poor cold flow characteristics. Compared to sturated fatty acids, unsaturated fatty acids especially polyunsaturated fatty acids have lower melting points which are preferred for improved low-temperature characteristics but are not preferred for fuels due to low CN and decreased oxidative stability (Knothe 2009; 2013). Therefore, good quality biodiesel must have an optimal balance of saturated and unsaturated fatty acids (Islam et al. 2013). In the present study, it was found that the fatty acid composition of *C. sorokiniana* UNM-IND1 was dominated by palmitic acid (C16: 0) as much as 28.13%, linolenic acid (C18: 3) 23.47% and linoleic acid (C18: 2) 21.64%. The fatty acid composition of *C. sorokiniana* is almost the same as the fatty acid composition of palm oil which has palmitic acid as the largest component of about 44% (Mancini et al. 2015). Based on its fatty acid composition, *C. sorokiniana* has the potential as a biodiesel raw material because C16 and C18 fatty acids are the main fatty acids in *C. sorokiniana* UNM-IND1. As stated by Knothe (2013), C16 and C18 fatty acids are the most common fatty acids contained in biodiesel.

Conclusion, microalga *C. sorokiniana* UNM-IND1 has the ability to grow well over a wide range of temperature ranging from 11-48°C. The highest specific growth rate (0.790 ± 0.096 day^{-1}) and biomass productivity (0.184 ± 0.021 $g.L^{-1}.h^{-1}$) were obtained at higher temperatures (drying room temperature), whereas, high lipid content (42% dry weight) and lipid productivity (0.078 ± 0.006 $g.L^{-1}.h^{-1}$) were achieved at lower temperatures (showcase temperature and culture room temperature). The fatty acid composition of microalgae is dominated by palmitic acid (C16: 0) 28.13%, linolenic acid (C18: 3) 23.47% and linoleic acid (C18: 2) 21.64%. This study indicated that the microalga *C. sorokiniana* UNM-IND1 can be regarded as a potential source of biodiesel due to its high growth rate, biomass productivity, lipid content and lipid productivity as well as fatty acids composition suitable for biodiesel.

REFERENCES

Abbaszaadeh A, Ghobadian B, Omidkhah MR, Najafi G. 2012. Current biodiesel production technologies: A comparative review. Energy Conversion Manag 63: 138-148. DOI: 10.1016/j.enconman.2012.02.027.

Alavijeh RS, Karimi K, Wijffels RH, van Den Berg C, Eppink M. 2020. Combined bead milling and enzymatic hydrolysis for efficient fractionation of lipids, proteins, and carbohydrates of *Chlorella vulgaris* microalgae. Bioresour Technol 309: 123321. DOI: 10.1016/j.biortech.2020.123321.

Ancona-Canché K, López-Adrián S, Espinosa-Aguilar M, Garduño-Solórzano G, Toledano-Thompson T, Narváez-Zapata J, Valdez-Ojeda R. 2017. Molecular phylogeny and morphologic data of strains of the genus *Coelastrella* (Chlorophyta, Scenedesmaceae) from a tropical region in North America (Yucatan Peninsula). Bot Sci 95 (3): 527-537. DOI: 10.17129/botsci.1201.

Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911-917. DOI: 10.1139/y59-099.

Chaisutyakorn P, Praiboon J, Kaewsuralikhit C. 2018. The effect of temperature on growth and lipid and fatty acid composition on marine microalgae used for biodiesel production. J Appl Phycol 30: 37-45. DOI: 10.1007/s10811-017-1186-3.

Chisti Y. 2007. Biodiesel from Microalgae. Biotechnol Adv 25 (3): 294-306. DOI: 10.1016/j.biotechadv.2007.02.001.

Christie WW. 1989. Gas Chromatography and Lipids: A Practical Guide. The Oily Press Ltd, UK.

Converti A, Casazza AA, Ortiz EY, Perego P, Borghi MD. 2009. Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculata* and *Chlorella vulgaris* for biodiesel production. Chem Eng Process: Process Intensif 48 (6): 1146-1151. DOI: 10.1016/j.cep.2009.03.006.

Duan Z, Sun R, Zhu C, Chou I-M. 2006. An improved model for the calculation of CO2 solubility in aqueous solutions containing Na+, K+, Ca2+, Mg2+, Cl-, and SO42-. Mar Chem 98: 131-139. DOI: 10.1016/j.marchem.2005.09.001.

Ferreira GF, Rı´os Pinto LF, Filho RM, Fregolente LV. 2019. A review on lipid production from microalgae: Association between cultivation using waste streams and fatty acid profiles. Renew Sustain Energy Rev 109: 448-466. DOI: 10.1016/j.rser.2019.04.052.

Gaurav K, Neeti K, Singh R. 2024. Microalgae-based biodiesel production and its challenges and future opportunities: A review. Green Technol Sustain 2 (1): 100060. DOI: 10.1016/j.grets.2023.100060.

Goecke F, Klemetsdal G, Ergon Å. 2020. Cultivar development of kelps for commercial cultivation—past lessons and future prospects. Front Mar Sci 8: 110. DOI: 10.3389/fmars.2020.00110.

Gómez-Loredo A, Benavides J, Rito-Palomares M. 2016. Growth kinetics and fucoxanthin production of *Phaeodactylum tricornutum* and *Isochrysis galbana* cultures at different light and agitation conditions. J Appl Phycol 28: 849-860. DOI: 10.1007/s10811-015-0635-0.

Gultom SO. 2018. Microalgae: A future source of renewable energy. Jurnal Kelautan: Indonesian J Mar Sci Technol 11 (1): 95-103. DOI: 10.21107/jk.v11i1.3802. [Indonesian]

Hall BG. 2001. Phylogenetic Trees Made Easy: A How - To Manual for Moleculer Biologists. Sinauer Associates, Inc, Sunderland, Massachusetts, USA.

Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A. 2008. Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances. Plant J 54 (4): 621-639. DOI: 10.1111/j.1365-313X.2008.03492.x.

Hwang TY, Kin CM, Shing WL. 2021. Potential of microalgae for biofuel and food production. INTI J 2022: 37. DOI: 10.61453/INTIj.202237.

Indrayani. 2017. Isolation and Characterization of Microalgae With Commercial Potential. [Dissertation]. Murdoch University, Australia.

Indrayani I, Haslianti H, Asriyana A. 2018 Isolation and screening of marine microalgae from Kendari Waters, Southeast Sulawesi, Indonesia suitable for outdoor mass cultivation in hypersaline media. AACL Bioflux 11 (5): 1445-1455.

Indrayani I, Haslianti H, Asmariani A, Muskita WH, Balubi M. 2020. Growth, biomass and lipid productivity of a newly isolated tropical marine diatom, *Skeletonema* sp. UHO29, under different light intensities. Biodiversitas 21 (4): 1498-1503. DOI: 10.13057/biodiv/d210430.

Indrayani I, Putra RP, Hambali A, Ardiansyah. 2022. Isolation and characterization of extremophile bacteria for hydrolytic enzyme production from Waepella Hot Spring, Sinjai, Indonesia. Biodiversitas 23 (12): 6345-6351. DOI: 10.13057/biodiv/d231231.

Indrayani I, Ramadani NZ, Mawaddah N, Kaseng ES, Sukainah A, Putra RP, Hambali A, Fadilah R, Nurmila N, Ardiansyah A. 2023. Influence of different culture media and light intensity on the growth and biomass productivity of a newly isolated *Chlorella* sp. UNM-IND1 from Waepella Hot Spring, South Sulawesi, Indonesia. AACL Bioflux 16 (3): 1508-1518.

Islam MA, Magnusson M, Brown RJ, Ayoko GA, Nabi MN, Heimann K. 2013. Microalgal species selection for biodiesel production based on fuel properties derived from fatty acid profiles. Energies 6 (11): 5676-5702. DOI: 10. 3390/ en611 5676.

Jin H, Chuai W, Li K, Hou G, Wu M, Chen J, Wang H, Jia J, Han D, Hu Q. 2021. Ultrahigh-cell-density heterotrophic cultivation of the unicellular green alga *Chlorella sorokiniana* for biomass production. Biotechnol Bioeng 118: 4138-4151. DOI: 10.1002/bit.27890.

Kates M, Volcani BE. 1966. Lipid components of diatoms. Biochim Biophys Acta 116: 264-278. DOI: 10.1016/0005-2760(66)90009-9.

Khalaji M. 2022. Evaluation of fatty acid profiles of *Chlorella Vulgaris* microalgae grown in dairy wastewater for producing biofuel. J Environ Health Sci Eng 20 (2): 691-697. DOI: 10.1007/s40201-022-00808-z.

Khorramdashti MS, Giri MS, Majidian N. 2021. Extraction lipids from *Chlorella vulgaris* by supercritical CO_2 for biodiesel production. S Afr J Chem Eng 38: 121-131. DOI: 10.1016/j.sajce.2021.03.008.

Knothe G. 2009. Improving biodiesel fuel properties by modifying fatty ester composition. Energy Environ Sci 2 (7): 759-766. DOI: 10.1039/B903941D.

Knothe G. 2013. Production and Properties of Biodiesel from Algal Oils. In: Borowitzka MA, Moheimani NR (eds.). Algae for biofuels and energy. Springer, Dordrecht. DOI: 10.1007/978-94-007-5479-9_12.

Makareviciene V, Sendzikiene E. 2022. Application of microalgae biomass for biodiesel fuel production. Energies 15 (11): 4178. DOI: 10.3390/en15114178.

Mercz TI. 1994. A study of high lipid yielding microalgae with potential for large-scale production of lipids and polyunsaturated fatty acids. Murdoch University, Perth, Australia.

Moheimani NR, Borowitzka MA, Isdepsky A, Fon Sing S. 2013. Standard methods for measuring growth of algae and their composition. In: Borowitzka MA, Moheimani NR (eds.). Algae for Biofuels and Energy. Springer, Dordrecht. DOI: 10.1007/978-94-007-5479-9_16.

Pandey S, Narayanan I, Selvaraj R, Varadavenkatesan T, Vinayagam R. 2024. Biodiesel production from microalgae: A comprehensive review on influential factors, transesterification processes, and challenges. Fuel 367: 131547. DOI: 10.1016/j.fuel.2024.131547.

Pasquet V, Ulmann L, Mimouni V, Guihéneuf F, Jacquette B, Morant-Manceau A, Tremblin G. 2014. Fatty acids profile and temperature in the cultured marine diatom *Odontella aurita*. J Appl Phycol 26: 2265-2271. DOI: 10.1007/s10811-014-0252-3.

Pruvost J, Goetz V, Artu A, Das P, Al Jabri H. 2018. Thermal modelling and optimizition of microalgal biomass production in the harsh desert conditions of State of Qatar. Algal Res 36: 101381. DOI: 10.1016/j.algal.2018.12.006.

Rana QUA, Latif S, Perveen S, Haq A, Ali S, Irfan M, Gauttam R, Shah TA, Dawoud TM, Wondmie GF, Bourhia M, Badshah M. 2024. Utilization of microalgae for agricultural runoff remediation and sustainable biofuel production through an integrated biorefinery approach. Bioresour Bioprocess 11 (1): 8. DOI: 10.1186/s40643-023-00720-w.

Saber H, Galal HR, Abo-Eldahab M, Alwaleed E. 2024. Enhancing the biodiesel production in the green alga *Chlorella vulgaris* by heavy metal stress and prediction of fuel properties from fatty acid profiles. Environ Sci Pollut Res Intl 31 (24): 35952-35968. DOI: 10.1007/s11356-024-33538-w.

Safi C, Zebib B, Merah O, Pontalier P-Y, Vaca-Garcia C. 2014. Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review. Renew Sustain Energy Rev 35: 265-278. DOI: 10.1016/j.rser.2014.04.007.

Shang X, Yang Y, Zan Y, Sun Z, Lu Z, Sun J. 2024. Effects of temperature on the growth, total lipid content and fatty acid composition of *Skeletonema dohrnii*. Front Mar Sci 11: 1361157. DOI: 10.3389/fmars.2024.1361157.

Ullah K, Ahmad M, Sharma VK, Harvey LA, Zafar M, Anyanwu CN. 2014. Algal biomass as a global source of transport fuels: Overview and development perspectives. Prog Nat Sci: Mater Intl 24 (4): 329-339. DOI: 10.1016/j.pnsc.2014.06.008.

Wang M, Ye X, Bi H, Shen Z. 2024. Microalgae biofuels: Illuminating the path to a sustainable future amidst challenges and opportunities. Biotechnol Biofuels Bioprod 17 (1): 10. DOI: 10.1186/s13068-024-02461-0.

Widianingsih W, Hartati R, Endrawati H, Yudiati E, Iriani VR. 2011. Pengaruh pengurangan konsentrasi nutrien fosfat dan nitrat terhadap kandungan lipid total *Nannochloropsis oculata*. Ilmu Kelautan: Indones J Mar Sci 16 (1): 24-29. [Indonesian]

Yamamoto M, Kurihara I, Kawano S. 2005. Late type of daughter cell wall synthesis in one of the Chlorellaceae, *Parachlorella kessleri* (Chlorophyta, Trebouxiophyceae). Planta 221 (6): 766-775. DOI: 10.1007/s00425-005-1486-8.

Zheng H, Yin J, Gao Z, Huang H, Ji X, Dou C. 2011. Disruption of *Chlorella vulgaris* cells for the release of biodiesel-producing lipids: A comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves. Appl Biochem Biotechnol 164 (7): 1215-1224. DOI: 10.1007/s12010-011-9207-1.