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BIOLOGICAL CHARACTERISTICS OF THE HETEROTROPHIC MARINE MICROALGAE *THRAUSTOCHYTRIUM PACHYDERMUM* TSL10 ISOLATED FROM THE SEA AREA OF TRUONG SA ARCHIPELAGO, VIETNAM

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SUMMARY

The sea area of the Truong Sa Archipelago, Vietnam - outpost island region of the country has a special geographical location and plays an important role in economic development as well as security and defense of Vietnam. Here, the first time for isolation of heterotrophic marine microalgae is precious. In this paper, the results on isolation, identification, and selection of suitable culture conditions of the strain *Thraustochytrium pachydermum* TSL10 isolated from the sea area of Spratly Island, Truong Sa Archipelago, Vietnam in 2021 were presented. The optimal culture conditions for the growth of *T. pachydermum* TSL10 in the flask were M1 medium, 3% glucose, 1% yeast extract and 25 - 28 °C temperature. Dry biomass and lipid content reached the highest levels of 12.54 ± 1.23 g/L and 28.65 ± 1.11 % dry cell weight (DCW), respectively. In a 30 L fermentor, *T. pachydermum* TSL10 grew well in a medium containing 9% glucose and 1% industrial extract yeast. After 120 hours of cultivation, dry biomass, lipid, protein, carbohydrate, carotenoids, and astaxanthin reached the highest levels of 30.76 ± 1.32 g/L; 22.95 ± 0.28% DCW; 6.65 ± 0.19% DCW, 15.96 ± 0.021% DCW, 178.00 ± 2.43 µg/L, and 8.30 ± 0.28 µg/L, respectively. The fatty acid composition contains mainly omega - 6 and omega - 9 fatty acids, accounting for 21.69% of total fatty acids (TFA) and 34.56% of TFA. The fatty acid profile was mainly dominated by linoleic acid and oleic acid, accounting for 21.54% of TFA and 34.23% of TFA, respectively. The results showed that TSL10 biomass was suitable raw material for screening bioactive compounds.

Keywords: *Thraustochytrium pachydermum* TSL10, Omega- 6, Omega- 9, Spratly.

INTRODUCTION

Microalgae in general and marine microalgae, in particular, have high nutritional value, rich in metabolites which exhibit different biological activities (Sathasivam *et al*., 2019). In recent decades, marine microalgae have become increasingly popular for their economic benefits, such as providing food for humans and in terms of aquaculture, they provide valuable active ingredients for many fields, namely medicine - pharmacy, cosmetics, fertilizers, environmental treatment, and renewable energy (biodiesel, biogas, bio-alcohol) (Hassi, Alouani, 2020).

Microalgae are established commercial sources of high-value chemicals such as polyunsaturated fatty acids (PUFAs), carotenoids, phycobiliproteins, polysaccharides, and phycotoxins (Sathasivam *et al*., 2019). The consumption of carotenoids has beneficial effects on the treatment of various types of cancers occurring in the breast, liver, bowel, and prostate as well as diabetes and obesity (Sathasivam, Ki, 2018; McClinton *et al*., 2019). Bio-oil isolated from oleaginous microorganisms, which has a high biomass yield with short growth time, and high lipid content, can not only live independently of the soil but is also considered raw material for biodiesel production and has a fatty acid profile similar to that of vegetable oil (Patel *et al*., 2020). Microalgae, bacteria, and yeasts are oleaginous microorganisms that can accumulate intracellular lipids accounting for over 20% of dry cell weight (DCW) (Papanikolaou, 2012). Microalgae are a good food source of PUFAs such as docosahexaenoic acid (C22:6 ω-3; DHA) and eicosapentaenoic acid (C20:5ω-3; EPA), alpha-linolenic acid (C18: 3 ω-3; ALA), linoleic acid (C18:2 ω-6; LA) for humans (Patel *et al*., 2020). The primary omega-9 fatty acid found in the brain is oleic acid (C18:1 ω-9), which is an essential fatty acid for the protection of the brain and cardiovascular system and can be synthesized by the human body or obtained from a regular diet (Bourre *et al*., 2009). Several species can synthesize lipid content of more than 70% of DCW, especially under a high C/N ratio (Papanikolaou, Aggelis, 2011).

Genus *Thraustochytrium* is heterotrophic marine microalgae belonging to the family Thraustochytriidae, class Labyrinthulomycetes. The difference between 16 species belonging to *Thraustochytrium* was proven by the absence or presence of one or some proliferation of the mode of discharge of fully motile or quiescent zoospores from the parent cell. This can be explained due to partial or complete disintegration of the sporangial wall (Dick, 2001). There are described 16 species, including *T. proliferum* Sparrow (1936), *T. multirudimentale* Goldstein (1963), *T. motivum* Goldstein (1963), *T. aureum* Goldstein (1963), *T. kinnei* Gaertner (1967), *T. rossii* Bahnweg & Sparrow (1974), *T. kerguelensis* Bahnweg & Sparrow (1972), *T. antarcticum* Bahnweg & Sparrow (1974), *T. benthocola* Raghukumar (1980), *T. globosum* Kobayashi & Ookubo (1953), *T. pachydermum* Schneid (1958), *T. roseum* Goldstein (1963), *T. aggregatum* Ulken (1965), *T. striatum* Schneider (1967), *T. arudimentale* Artemchuck (1972) and *T. indicum* D. K. Chakrayarty (1979) (Porter, 1990; Dick, 2001; Bongiorni *et al*., 2005a).

The genus *Thraustochytrium* may encompass sub-genera because it does not form a monophylogenetic group based on 18S rRNA, and does not show common PUFAs or carotenoid profiles among species. Therefore, it is expected that new genera will be proposed in the future (Marchan *et al*., 2017). The morphological characteristics, biomass and nutritional compositions, as well as fatty acid content in the biomass of thraustochytrids, were changed by the culture conditions, including nutrient composition, temperature, pH and salinity, especially carbon and nitrogen sources (Dang Diem Hong, Hoang Thi Lan Anh, 2016; Sahin *et al*., 2018; Barros *et al*., 2019).

The sea area of Truong Sa Archipelago, Vietnam is rich in species of seaweed, photoautotrophic, and heterotrophic marine microalgae with the extremely diverse distribution. Species of marine microalgae grow or survive in harsh conditions of the archipelago, such as high temperature, excess light, nutrient-deficient habitat, high salinity, frequent storms, etc., have already proved to exhibit valuable biological properties and have high-value active substances. Therefore, the study of marine microalgae in Truong Sa Archipelago has an important scientific significance. This publication will present research results on isolation, identification, and influence of different cultural conditions on the growth of strain *Thraustochytrium* sp. TSL10 is isolated from the sea area of Spratly Island belonging to Truong Sa Archipelago, Vietnam. Up to now,

there has been no publication of heterotrophic marine microalgae isolated in the sea waters mentioned above.

MATERIALS AND METHODS

Specimen collection

Specimens of leaves, seaweed, moss, seagrass, sand, rotten firewood, hard coral, seabed sediments etc. at the edge of sandy beaches around Spratly Island, Vietnam were collected from April to June and from September to November 2021. Obtained specimens were placed in plastic bags and processed on the same day for algae isolation. Samples were collected in the surface aquifer, where the light intensity was 80 Klux (measured at 8:00 am), 117 Klux (measured at 13:00 on the same day), the temperature was 33°C, and the salinity of seawater was 33‰ and pH 7. Spratly Island, belonging to Truong Sa Archipelago, Vietnam is located at 08°38'30"N North Latitude - 111°55'55"E Longitude.

Chemicals and equipments

Chemicals used include: Glucose (Binh Minh Joint Stock Company, Vietnam), Industrial yeast extract (Institute of Food Technology, Vietnam), artificial sea salt (Japan), polypeptide (Merck, Germany), pure yeast extract (Merck, Germany), tris base (Merck, Germany), CHAPs (Merck, Germany), Dichloro-Diphenyl-Trichloroethane (Merck, Germany), $(NH_4)_2SO_4$, CH_3COONH_4, $NaNO_3$, maltose, maltodextrin, starch, acetone, n-hexane, NaOH, HCl, urea and thiourea made in Xilong Scientific Co., Ltd., China).

Equipments used include: Olympus CX21 light microscopes (Olympus, Japan), scanning electron microscopy - SEM (model JSM-5410L; Jeol Company, Tokyo, Japan), transmission electron microscope - TEM (JEM1010; Jeol Company, Tokyo, Japan), filter (Advantec, JP020AN, Japan), 30 L fermenter (handmade, Vietnam), 250 mL Erlenmeyer flask and other common equipments.

Methods

Method of sample isolation

Strain *Thraustochytrium* sp. TSL10 was isolated as described by Dang Diem Hong and Hoang Thi Lan Anh (2016). Obtained specimens (as leaves, seaweed, moss, and seagrass) washed with sterilized seawater and cut into 0.5 x 0.5 cm pieces and transferred into 30‰ sterilized seawater test tubes (containing 5 mL of sterilized seawater and supplemented with pine pollen) were sterilized at 121°C, at 1 atm for 30 min. After sterilization, the test tubes were allowed to cool at room temperature. Then, obtained specimens of 0.5 x 0.5 cm added to above test tubes were incubated in the dark for 3-5 days. Afterwards, pile pollen from tubes was collected with an inoculation loop and was spread on the surface of the agar plates containing GPYc medium, including glucose (0.2%), polypeptide (0.1%), yeast extract (0.05 %), artificial sea salt (1.75%), agar (1.5%), and chloramphenicol (50 mg/L). The Petri dishes were examined under Olympus light microscope (Japan) to detect colonies. Pure colonies were subcultured on a GPYc medium until pure strains were obtained.

Scientific name identification of the strain Thraustochytrium sp. TSL10

The marine microalgae strain of *Thraustochytrium* sp. TSL10 was identified scientific name by comparing the nucleotide sequences of the 18S rRNA gene. Specific primer pair of FA1/RA1 was used to amplify the partial 18S rRNA gene of the marine microalgae genera of *Aurantiochytrium*, *Schizochytrium*, and *Thraustochytrium* with the predicted size of 650 bps with the sequence: FA1: 5' - AAAGATTAAGCCATGCATGT-3' and RA1: 5'-AGCTTTTTAACTGCAACAAC-3' as published by Mo *et al.* (2002). The PCR reaction mixture had a total volume of 20 µL consisting of 2 µL Green buffer (2X, Thermo Fisher Scientific, USA), 1 µL each primer (10 pmol/µL), 2 µL DNA template (50-100 ng/µL), 0.5 µL Taq polymerase (2 u/µL), 1.5 µL dNTP, 0,5 µL $MgCl_2$ (50 mM) and 11.5 µL deionized water. PCR amplification was performed on

Veriti®96-Well Thermal Cycler (Applied Biosystems®, Life technology, Thermo Fisher Scientific, USA) with the following cycling program: denaturation for 3 min at 95ºC, 35 amplification cycles of 1 min at 94ºC, 1 min at 50ºC and 1 min at 72ºC, with a 10 min extended elongation step. PCR production was visualized on an ethidium bromide-stained 1.2% agarose gel. The 18S rRNA gene sequence of species belonging to the genera of *Aurantiochytrium, Schizochytrium, Thraustochytrium, Monohizochytrium,* and species belonging to the genus of *Diplophylus* was used as outgroups which were registered in the GeneBank database used to construct the phylogenetic tree (Hassett, 2020). PCR products were precipitated by ethanol and amplified with the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) according to the manufacturer's recommendations. Sequence analysis was made using Standard Nucleotide BLAST, blastn (https://blast.ncbi.nlm.nih.gov/), nucleotide collection (nr/nt) database and using ClustalX (1.81) and MEGA X software (Hoang Thi Lan Anh *et al*., 2010).

Determination of algal growth

Determination of algal growth through dry cell weight (DCW): At certain experimental times, 10 mL of culture broth are centrifuged to obtain biomass. After centrifugation, cell biomass was transferred to a cup with identified weight mass and dried at 105ºC until constant weight for three consecutive weighing times as described in the report of Dang *et al.* (2011). The following formula determined the DCW of the sample:

DCW (g) = Weight (cup + algal biomass) – Weight (cup)

Morphological observation

Thraustochytrium sp. TSL10 strains were maintained on slant culture with GPY⁄agar medium at 28 ºC. Living cells in the colony on an agar plate and M1 liquid medium were observed with Olympus CX21 light microscopes and scanning electron microscopy - SEM (Dang *et al.,* 2011).

Transmission electron microscopy (TEM)

After cultivation, the cell suspension was centrifuged at 3,000 × g for 5 min. The cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and kept overnight at 4°C. The sample was then rinsed carefully in 0.1 M cacodylate buffer (pH 7.2) several times. The last cells were fixed with 1% OsO_4 (osmium tetroxide) in the same buffer for 60 min, then rinsed carefully in 0.1 M cacodylate buffer (pH 7.2) several times, subsequently dehydrated in graded ethanol (50 to 100%), washed in propylene oxide, and infiltrated for 6 h in a 1:1 mixture of propylene oxide and epoxydic resin. The cells were then embedded in the resin. Thin sections were obtained with an ultramicrotome (Ultracut UC6; Leica Microsystems GmbH, Germany) and stained with uranyl acetate and lead citrate. The ultrathin section was then observed through a transmission electron microscope operating at 80 kV (Inagaki *et al*., 2020).

Scanning electron microscopy (SEM)

The prepared samples were examined by scanning electron microscopy FE-SEM S4800 (Nguyen Thanh Thuy *et al*., 2013). The specimens were fixed in glutaraldehyde 2.5% in cacodylate 0.1 M pH 7.2 for 1 h or overnight. Then the samples were washed three times in cacodylate 0.1 M; followed by post-fixing treating in cacodylate 0.1 M with OsO_4 1% for 20 min. The samples then were washed three times in cacodylate 0.1 M and dehydrated in 50º, 70º, 90º and 100º alcohol. After dehydration, the samples were underwent the critical dying point process and were mounted on to metal stub and sputter coated with gold by ion- coater.

Cell size determination using MapInfo Professional software 7.5

The standard image (with the known actual size of 25 µm) was taken at a particular magnification. Photographs of cells were taken at the same magnification as the standard image. All images were transferred to the computer. Then, the ruler tool in MapInfo Professional 7.5

software was used to determine the size of the standard and cell images. Determining the cell size is calculated as follows:

$$\text{Cell size (µm)} = \frac{\text{cell size (ft) x 25 µm}}{\text{cell size sr (ft)}}$$

Where: Cell size (ft): Cell size was measured on the image by software; Cell size sr (ft): The measured size on the image of the standard ruler segment, the actual size is 25 µm.

Analysis of total lipid content

Total lipid content was analyzed according to the method of Bligh and Dyer (1959) with some modifications to suit the conditions of Vietnam laboratories as published by Dang *et al*. (2011). The total lipid content is calculated by dry biomass using the following formula:

$$\text{Total lipids (\%)} = (m2/m1) \times 100$$

Where: m1 (g) is DCW used for lipid analysis; m2 (g) is the total lipid obtained from m1 g DCW.

Extraction and determination of total carotenoid and astaxanthin

The total carotenoid content was determined as described by Furlan *et al.* (2019). Isolated, the total carotenoid content (µg/g of DCW) was calculated by the following formula:

$$\text{Total carotenoid content} = \frac{A477nm \cdot Vextrac \cdot DF}{0.2.Wsample}$$

Where: A 477nm is the optical density absorbed at the wavelength of 477 nm; $V_{extract}$ is extract volume (mL); DF is the dilution factor (final volume divided by the initial volume); 0.2 is the value of the carotenoid solution of 1 µg/mL was measured at A 477nm; W sample is sample weight (g).

The astaxanthin content (µg/g DCW) was calculated by the formula (Strickland, Parsons, 1972)

$$\text{Astaxanthin content} = \frac{4.0 \cdot A_{480nm} \cdot}{m}$$

Where: A_{480nm} is the optical density absorbed at the wavelength of 480 nm; m is sample weight (g).

Extraction and determination of total protein and carbohydrates contents

Total protein content was determined according to Cakmak *et al*. (2012), Bradford (1976).

Carbohydrate content was determined according to Sun *et al*. (2014).

Analysis of fatty acid composition in algae biomass

The composition of fatty acid in algae biomass was determined by method of LFOD-TST-8444 (GC-FID) conducted at SGS Vietnam Ltd. Company.

Methods for determining optimal culture conditions in Erlenmeyer flask

In order to determine the optimal culture conditions of strain TSL10 in 250 mL Erlenmeyer flask, the effects of nutritional media such as Bajpai, M1, and GPY; carbon sources as glucose, maltose, maltodextrin, and starch; glucose levels (1, 3, 5, 7, and 9%); nitrogen sources as yeast extract, $(NH_4)_2SO_4$, CH_3COONH_4, and $NaNO_3$; concentrations of yeast extract (0.5; 1; 1.5, and 2%); temperatures (15, 20, 25, 28, 30, and 35 ^oC) on the algal growth were studied. After 120 h of cultivation, samples were taken to evaluate the parameters of DCW and lipid content.

Cultivation of T. pachydermum TSL10 in 30 L fermentor

An aeration rate when growing biomass was always maintained at 0.5 L air/L/min after the air was filtered through a 0.2 mm filter (Advantec, JP020AN, Japan). Unlike cultivation in the Erlenmeyer flasks, technical parameters for biomass cultivation have changed on a large scale of 30 L fermentor: while the dissolved oxygen was maintained above 10% with the manual stirring speed from 300 to a maximum of 400 rpm. In the 30 L fermentor, the effects of glucose concentrations (3, 6, 9, and 12%), and yeast extract (pure yeast extract and industrial yeast extract) on the growth of strain TSL10 were studied. During the fermentation process,

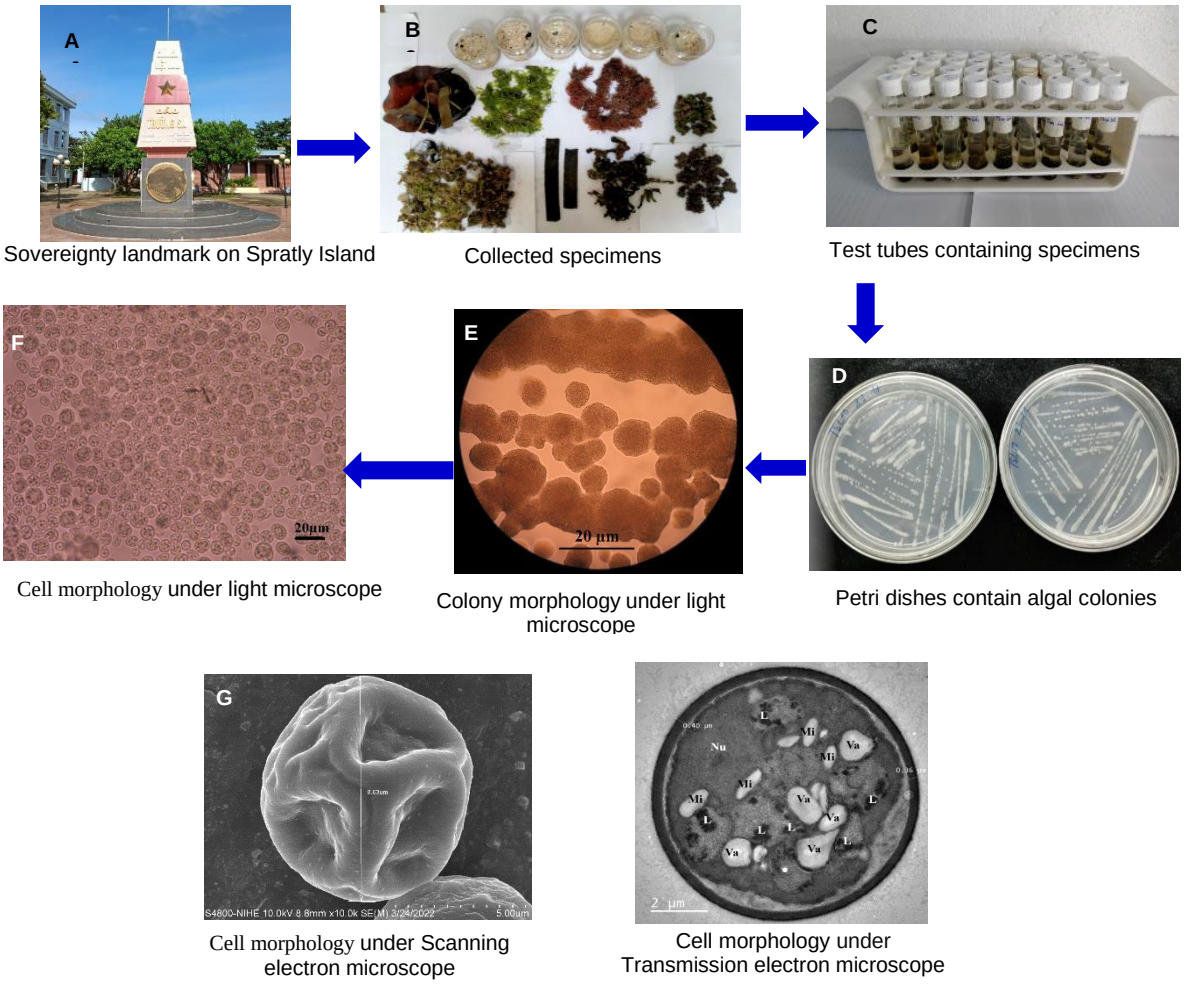
samples were taken at 24, 72, 120, and 168 h to determine DCW and after 120 h of culture to determine lipids, proteins, carbohydrates, carotenoids, and astaxanthin contents and analyze fatty acid composition.

Experimental data are processed by Excel software and statistically analyzed by one-way ANOVA with Duncan's post hoc at a significant level of $P < 0.05$ level.

RESULTS

Isolation of the strain *Thraustochytrium* sp. TSL10

From samples collected in May 2021 in the sea area of Spratly Island belonging to Truong Sa Archipelago, Vietnam, we have successfully isolated and stably cultured strain *Thraustochytrium* sp. TSL10 under laboratory conditions.



Sovereignty landmark on Spratly Island

Collected specimens

Test tubes containing specimens

Petri dishes contain algal colonies

Colony morphology under light microscope

Cell morphology under light microscope

Cell morphology under Scanning electron microscope

Cell morphology under Transmission electron microscope

Figure 1. Some images of isolated samples from Spratly Island (A, B, C, D). Cell morphology was observed under microscope with a magnification of 400X (E, F). Cell morphology was observed under Scanning electron microscope (SEM) with a magnification of 20.000X (G). Cell morphology was observed under Transmission electron microscope (TEM) with a magnification of 20.000X (H). *Nu: Nucleus, Va: Vacuole, L: Lipid, Mi: Mitochondria.*

The morphological characteristics of colonies of strain TSL10 is a red-yellow colour that is not smooth. It showed that the algal cells of strain TSL10 are single cells, not connected, and contain endoplasmic reticulum and reproduce by spores (Bongiorni *et al*., 2005b). *Thraustochytrium* has spherical cells with or without a proliferous body and is characterized by the release of zoospores caused by partial disintegration of the cell wall of sporagania (Yokoyama, Honda, 2007). The sporangium divides into sporophytes with a deviated flagellum with a long flagellum on the apex and a shorter posterior flagellum. The sporophytes are formed by mitotic division of multinucleated sporangia and then released nuclei pass through the hole or partially or wholdissolve the sporangium's cell wall. Species can vary in the thickness of the sporangium, the spore colour, the move or immobility of the sporophyte after release (Bongiorni *et al*., 2005b). When cultured on liquid medium, the cells of strain TSL10 exhibited smaller cell size (8 µm x 9 µm in size) so the preliminary isolation was named *Thraustochytrium* sp. TSL10. In order to confirm the scientific name of the isolated TSL10 strain, the scientific name identification by reading and comparing the nucleotide sequences of partial 18S rRNA gene was conducted. Figure 1 illustrates an isolated sample in Spratly Island, Vietnam.

Identification of the scientific name of the strain *Thraustochytrium* sp. TSL10

We performed phylogenetic analysis using *Thraustochytrium* sp. TSL10 18S rRNA gene sequence. Twenty-two 18S rRNA genes of 5 genera were found using BLAST. The results of phylogenetic analysis are shown in Figure 2. A part of the nucleotide 18S rRNA gene sequence of the TSL10 strain was 543 bp. The comparison of a partial nucleotide sequence of the 18S rRNA gene was shown that the TSL10 strain exhibits a high identity of 99.1% with sequences belonging to *T. pachydermum* (accession number AB022113.1). Genus *Thraustochytrium* clusters more compactly but does not form a separate monophyletic group. Our phylogenetic reconstruction of the Thraustochytriaceae species agrees with the previous data published (Caamaño *et al*., 2017)**.** The partial 18S rRNA gene sequence of *Thraustochytrium pachydermum* TSL10 was published and is available in GenBank (with accession number OL721615). Therefore, based on the morphological characteristics and 18S rRNA gene sequence, we can conclude that sample TSL10 belong to the species of *Thraustochytrium pachydermum*.

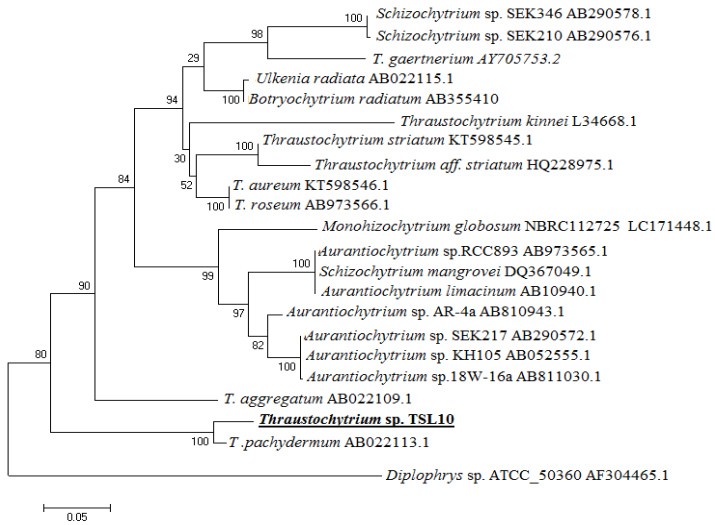


Figure 2. Phylogenetic tree of species belonging to genera of *Thraustochytrium, Aurantiochytrium*, *Monohizochytrium* and *Schizochytrium* based on 18S rRNA gene sequences

Selection of the optimal culture conditions

The effect of the culture medium

Culture medium is an important factor that determines the growth, development, and lipid accumulation of cells. After isolation and stable cultivation on agar medium, strain of *T. pachydermum* TSL10 was cultured on different media including GPY, M1, Bajpai. The results on the effects of 3 nutrient media on the growth of strain TSL10 after 120 h of cultivation were presented in figure 3.

The obtained results in figure 3 indicated that strain TLS10 was grown in all three media after 120 h of cultivation. However, the DCW and lipid content of this strain reached highest values in M1 medium of 11.89 ± 0.21 g/L and 25.09 ± 1.13% DCW, respectively, followed by Bajpai medium, and finally in GPY medium. In M1 medium, algal cells had large size and clear morphology. There was a statistically significant difference between different culture media ($p < 0.05$). Therefore, in the subsequent experiments, M1 medium was selected as the suitable medium for growth of strain TSL10.

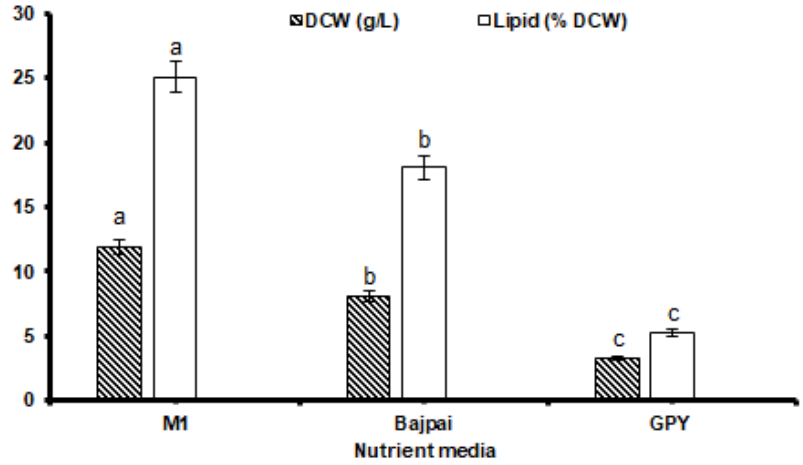


Figure 3. Growth, lipid content of strain TSL10 cultured in different media after 120 hours of cultivation. (Note: The different letters ($^{a, b, c}$) exhibit difference between mean value of the sample (One-way ANOVA with Duncan's post hoc, $p < 0.05$).

The effect of carbon sources

The effects of four carbon sources including glucose, maltose, maltodextrin, and starch on the growth and lipid accumulation of strain TSL10 were studied. The results in Figure 4 showed that the highest DCW and total lipid content were 12.20 ± 1.31 g/L and 25.35 ± 1.01% DCW, respectively, with using glucose as a source carbon. Carbon sources such as maltose and starch are not good for growth and lipid accumulation in the strain TSL10. There was a statistically significant difference between different carbon sources ($p < 0.05$).

The culture conditions affect biomass yield and fatty acid composition of the strain (Chatdumrong *et al*., 2007). According to Ye *et al.* (2020), based on the transcriptome analysis, it was showed that glucose can participate in regulation of some genes involved in fatty acid synthetic pathway, while glycerol regulates some genes of polyketides synthetic pathway in *Thraustochytrium* sp. PKU#Mn16. Therefore, among 4 carbon sources used in our experiment, glucose was selected as a carbon source for TSL10 strain cultivation on larger scales to provide raw materials for screening activities and studying chemical nutritional compositions.

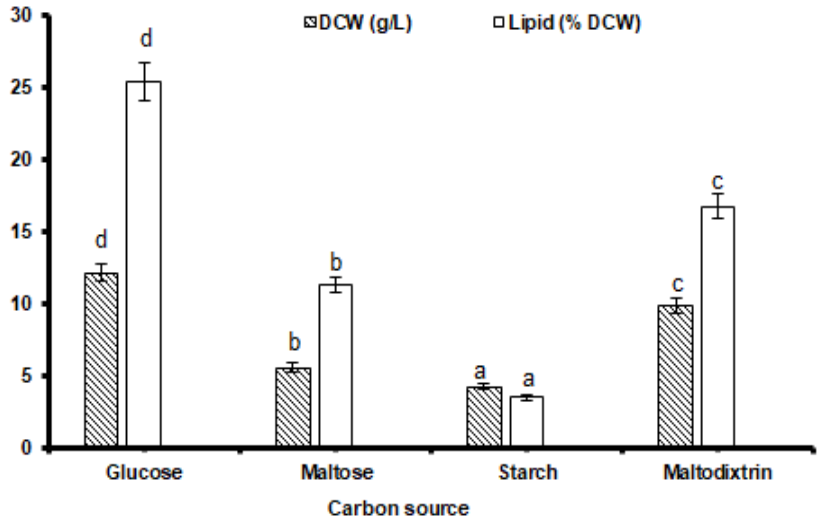


Figure 4. Growth, lipid content of strain TSL10 cultured with different carbon sources after 120 hours of culture. (Note: The different letters ($^{a, b, c}$) exhibit difference betweeb mean value of the sample (One-way ANOVA with Duncan's post hoc, $p < 0.05$).

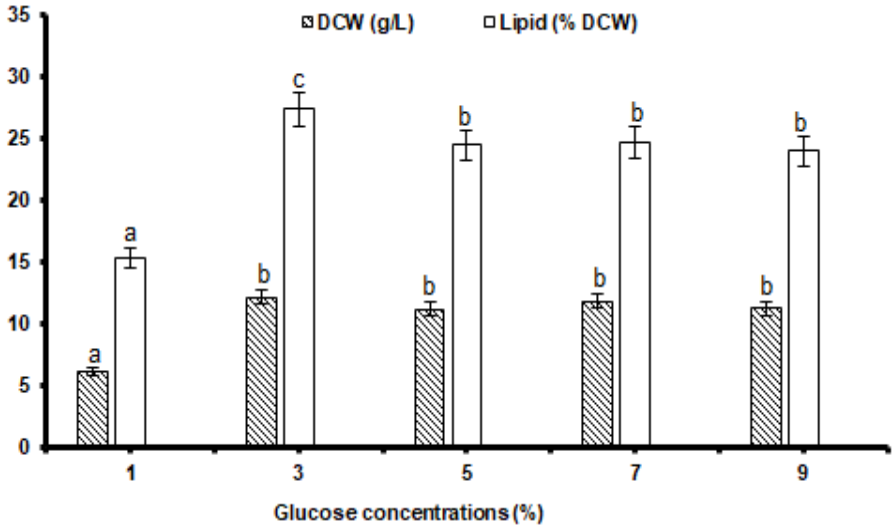


Figure 5. Growth, lipid contents of strain TSL10 cultured with different glucose concentraions after 120 hours of culture. (Note: The different letters ($^{a, b, c}$) exhibit difference betweeb mean value of the sample (One-way ANOVA with Duncan's post hoc, $p < 0.05$).

The effect of glucose concentration

The glucose concentrations were investigated including 1, 3, 5, 7, and 9%. The results in Figure 5 showed that DCW increased from 6.1 ± 0.15 g/L to 12.17 ± 1.35 g/L when the glucose concentration increased from 1% to 3%. However, the biomass and lipid content did not change significantly when the glucose concentration was higher (as 5, 7, and 9%). At the glucose concentration of 3%, strain TSL10 reached highest DCW and lipid content of 12.17 ± 1.35 g/L and 27.38 ± 0.16 % DCW,

respectively. In term of lipid content, there was statistically significant difference ($p < 0.05$) between glucose concentration of 3% and other concentrations. Our research results are completely consistent with those of Min *et al*. (2012), Lida *et al.* (1996), and Burja *et al.* (2006). For *Thraustochytrium aureum* strain ATCC 34304, the maximum dry biomass yield was 7.9 g/L on culture medium containing 30 g/L glucose and 2.5 g/L yeast extract (Min *et al*., 2012). Meanwhile, the maximum biomass reached 5.7 g/L when cultured *Thraustochytrium striatum* on medium containing 10.25 g/L glucose and 1g/L yeast extract (Lida *et al*., 1996). However, when Burja *et al.* (2006) cultured *Thraustochytrium* sp. ONC-T18 on the medium containing 40 g/L glucose and 10 g/L yeast extract, the maximum growth reached 13.73 g/L. The difference in growth among the strains was due to the different C/N ratios leading to difference in growth and lipid accumulation ability among strains (Burja *et al*., 2006). Therefore, glucose concentration of 3% was selected in next experiments.

The effect of nitrogen sources

Nitrogen is an important factor that affects the growth of thraustochytrids (Chen *et al*., 2010). The effect of four sources of organic and inorganic nitrogen including yeast extract, $(NH_4)_2SO_4$, CH_3COONH_4, and $NaNO_3$ on the growth and lipid accumulation of strain TSL10 was studied (Table 1).

Table 1. The growth and lipid accumulation of strain TSL10 cultured in medium containing different nitrogen sources after 120 hours of cultivation

Nitrogen sources	DCW (g/L)	Lipid content (% DCW)
Yeast extract	12.29 ± 0.43^{d}	27.56 ± 1.31^{d}
$(NH_4)_2SO_4$	5.34 ± 0.09^{a}	12.24 ± 0.65^{a}
CH_3COONH_4	8.91 ± 0.42^{b}	18.59 ± 0.73^{b}
$NaNO_3$	10.12 ± 0.14^{c}	21.21 ± 1.26^{c}

(Note: The different letters ($^{a, b, c}$) exhibit difference between mean values of the sample (One-way ANOVA with Duncan's post hoc, $p < 0.05$)

The obtained results, as shown in Table 1, indicated that the highest DCW and total lipid content of strain TSL10 reached 12.29 ± 0.43 g/L and 27.56 ± 1.31% DCW, respectively, with using yeast estract as a nitrogen source. Besides yeast extract, the strain TSL10 can also use $NaNO_3$ as a nitrogen source for growth. However, in terms of lipid content, when using yeast extract, TSL10 strain had the highest lipid content and was significantly higher than those from other nitrogen sources ($p < 0.05$). According to Chen *et al*. (2017), nitrogen is one of the most critical nutrients affecting cell growth and lipid accumulation. Nitrogen starvation is employed condition granting high lipid content in species of *Thraustochytrium*. When cultured *T. roseum* ATCC28210, Singh and Ward (1996) used yeast extract as alternative nitrogen source to $(NH_4)_2SO_4$ that significantly increased in DCW from 6.1 g/L to 8.6 g/L. Zhang *et al.* (2022) added yeast extract into culture medium of *Thraustochytrium* sp. ATCC 26185 to reach maximum biomass yield of 5.12g/L. Therefore, in the next experiments, yeast extract should be selected as the nitrogen source for the growth of the strain TSL10 in order to provide raw materials for screening activities and studying the chemical nutritional compositions.

The effect of yeast extract concentrations

The effect of yeast extract concentrations in the range of 0.5 - 2% on the growth of strain TSL10 was investigated. The results in Table 2 showed that the DCW and lipid content

increased linearly when the yeast extract concentration increased from 0.5 to 1.5%. The DCW and lipid content of strain TSL10 reached the highest values at yeast extract concentration of 1% with values of 12.36 ± 1.19 g/L and 28.43 ± 1.16 % DCW, respectively, followed by yeast extract concentration of 1.5%. There was no statistically significant difference between the in terms of growth and lipid content at yeast extract concentrations of 1 and 1.5% ($p > 0.05$). Heggeset *et al.* (2019) have demonstrated that under excess nitrogen, the productivity of fatty acids has been found to drop. However, in order to save chemical, yeast extract concentration of 1% should be chosen to culture strain TSL10 at fermentor scales for obtaining enough biomass for nutrient composition analysis and screening.

Table 2. The growth and lipid content of strain TSL10 cultured in media containing yeast extract with different concentrations after 120 hours of cultivation.

Yeast extract concentration (%)	DCW (g/L)	Lipid content (% DCW)
0.5	8.69 ± 0.28^{a}	20.15 ± 0.13^{a}
1.0	12.36 ± 1.19^{b}	28.43 ± 1.16^{c}
1.5	11.98 ± 1.34^{b}	27.87 ± 1.93^{c}
2.0	9.76 ± 0.47^{a}	25.45 ± 0.18^{b}

(Note: The different letters ($^{a, b, c}$) exhibit difference between mean values of the sample (One-way ANOVA with Duncan's post hoc, $p < 0.05$)

The effect of culture temperatures

According to Caamaño *et al*. (2017), biomass yield, carbon consumption, and lipid content can be changed according to the modification of culture temperature of strains belonging to the genus *Thraustochytrium*. The effect of temperature in range of 15 - 35°C on the growth and lipid accumulation of strain TSL10 was investigated (Table 3).

The results obtained in Table 3 indicated that the temperature has a significant effect on the growth of strain TSL10. The optimal temperature for growth of strain TSL10 was 25 – 28°C. At 28°C, the DCW and lipid content reached highest values of 12.54 ± 1.23 g/L and 28.65 ± 1.11 % DCW, respectively, followed by the temperature of 25°C. There was no statistically significant difference ($p > 0.05$) when compared at the temperature of 25°C and the temperature of 28°C. At low temperature of 15°C or high temperature (above 30°C), it was found to be the clear effect on the growth and lipid accumulation of strain TSL10. Smaller cell size and crumbling cells are common phenomena observed at above mentioned temperatures. This obtained result is also completely consistent with the results of Kalidasan *et al*. (2021) who showed that the biomass yield of *T. kinnei* reached 13.53 g/L at the temperature range of 25 – 28°C. Therefore, the suitable temperature for the growth of strain TSL10 are in the range of 25 – 28°C. This is also completely consistent with the isolation sites of strain TSL10 - specimens of leaves, seaweed, and seagrass collected from bottom sea areas surrounding Spratly Island where the temperature are usually lower than air tempeature.

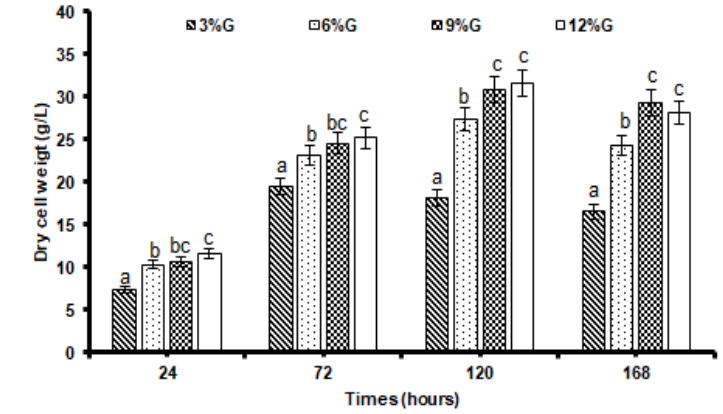
The effect of glucose concentration on the growth of strain TSL10 cultivated in 30 L fermentor

According to Chang *et al*. (2014), the glucose is the carbon source required for growth of thraustochytrids, in which different genera use different glucose sources. Therefore, at fermentation scale of 30 L, it is recommended to study the effect of glucose concentrations and nitrogen sources as industrial yeast extract 1% (produced by Food Industries Research Institute, Vietnam) instead of pure yeast extract 1% (pure yeast extract made by Merck, Germany) in order to reduce costs of biomass cultivation on large - scale.

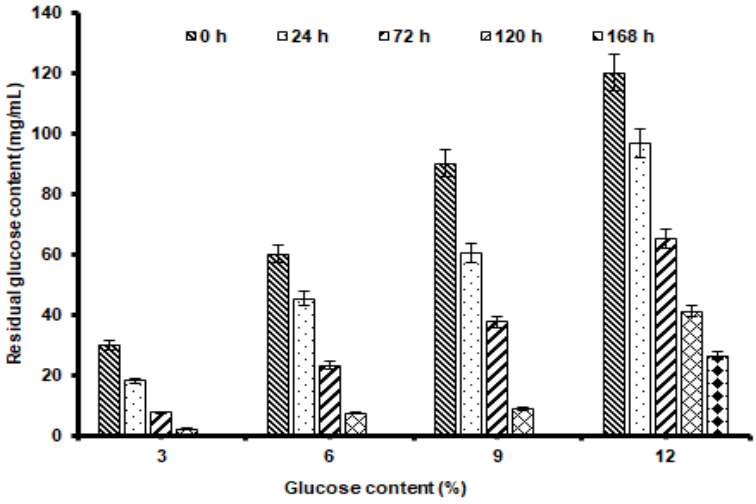
Table 3. The growth and lipid accumulation of strain TSL10 cultured at different temperature conditions after 120 hours of culture.

Temperatures (ºC)	DCW (g/L)	Lipid content (% DCW)
15	5.07 ± 0.08^{a}	7.12 ± 0.09^{a}
20	10.23 ± 0.89^{b}	22.13 ± 1.35^{c}
25	11.67 ± 1.17^{c}	27.81 ± 1.15^{d}
28	12.54 ± 1.23^{c}	28.65 ± 1.11^{d}
30	9.08 ± 0.24^{b}	19.18 ± 1.58^{b}
35	4.13 ± 0.18^{a}	6.12 ± 0.53^{a}

(Note: The different letters ($^{a, b, c}$) exhibit difference between mean values of the sample (One-way ANOVA with Duncan's post hoc, $p < 0.05$).



A



B

Figure 6. Effect of glucose concentrations on DCW (A) and residual glucose (B) of strain TSL10 in a 30 L fermentor. (Note: The different letters ($^{a, b, c}$) exhibit difference between mean values of the sample (One-way ANOVA with Duncan's post hoc, $p < 0.05$).

The effect of glucose concentrations including 3, 6, 9, 12% on the growth of strain TSL10 was shown in Figure 6. The results indicated that in the 30 L fermentor, strain TSL10 grew well at all glucose concentrations. At 9% glucose concentration, the biomass of this strain increased rapidly in the initial 24 hours and reached highest value after 120 hours of culture with 30.76 ± 1.32 g/L. By increasing culture time to 168 hours, the growth tended to decrease. The algal growth at glucose concentration of 9% was similar to algal growth at glucose concentration of 12%. There was no statistically significant difference observed in growth at glucose concentrations between 9% and 12% ($p > 0.05$).

With the increase in cell biomass, residual glucose concentration in the culture medium also decreased inversely proportion to biomass. The obtained results, as shown in Figure 6B, indicated that at glucose concentration above 9% (90 mg/mL), residual glucose concentration in the culture medium was 8.76 ± 0.09 mg/mL after 120 hours of culture. However, when the glucose concentration was increased to 12%, the residual glucose concentration in the medium was only about 40 mg/mL after 120 hours of culture. This suggested that using glucose concentration of 12% may inhibit the growth of algae. Therefore, when cultured strain TSL10 in 30 L fermentor, in order to deliver good growth and reduce cost of culture medium, glucose concentration of 9% was selected for further experiments.

The effects of pure yeast extract and industrial yeast extract on the growth of strain TSL10 in 30 L fermentor

In 30 L fermentor, the change in the DCW of strain TSL10 cultured in medium containing nitrogen sources as pure yeast extract and industrial yeast extract was presented in Figure 7. The results showed that there was no difference in the DCW when cultured the strain in medium containing nitrogen sources of pure yeast extract and industrial yeast extract with 31.02 ± 1.24 and 28.68 ± 1.13 g/L after 120 hours of cultivation, respectively. Therefore, in the 30 L fermentation system, it is recommended to choose industrial yeast extract for biomass cultivation as a raw material for screening active compounds and reasearch chemical nutritional compositions.

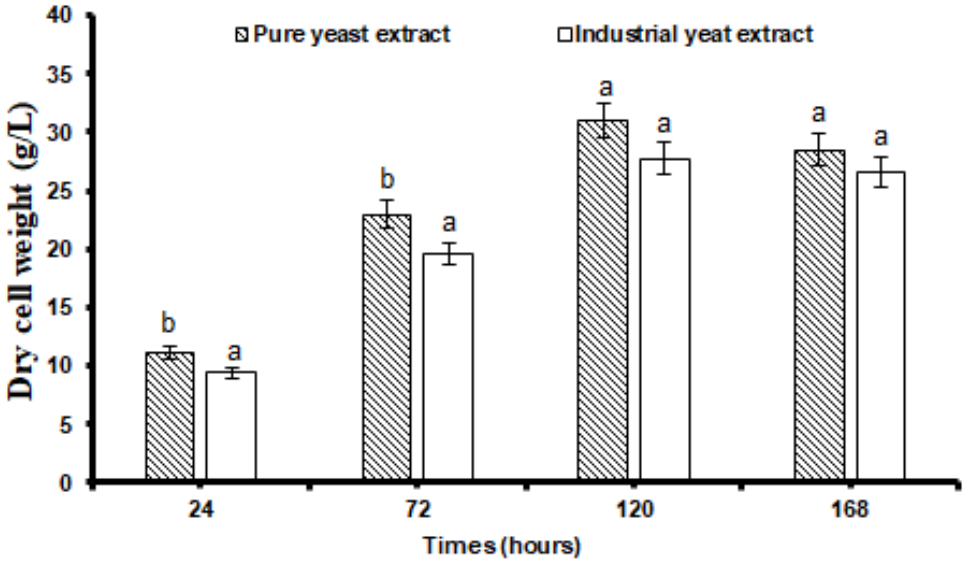


Figure 7. Effect of pure yeast extract and industrial yeast extract on DCW of strain TSL10 in 30 L fermentor. (Note: The different letters ($^{a, b, c}$) exhibit difference between mean values of the sample (One-way ANOVA with Duncan's post hoc, $p < 0.05$).

Table 4. Fatty acid composition in dry biomass of strain TSL10 cultured in 30 L fermentor after 120 hours of culture.

No	Fatty acid composition	Fatty acid Content (g/100 g DCW)	No	Fatty acid composition	Fatty acid content (g/100 g DCW)
1	C4:0 Butyric acid	0.000	28	C20:1 Gondoic acid	0.014
2	C6:0 Caproic acid	0.004	29	C20:2 Eicosadienoic acid	0.009
3	C8:0 Capryllic acid	0.006	30	C20:3 gamma -Eicosatrienoic acid	0.000
4	C10:0 Capric acid	0.000	31	C20:3 Eicosatrienoic acid	0.000
5	C11:0 Undecanoic acid	0.000	32	C20:4 Arachidonic acid (ARA)	0.000
6	C12:0 Lauric acid	0.001	33	C20:5 Eicosapentaenoic acid (EPA)	0.000
7	C13:0 Tridecanoic acid	0.000	34	C21:0 Heneicosanoic acid	0.000
8	C14:0 Myristic acid	0.011	35	C22:0 Behenic acid	0.111
9	C14:1 Myristoleic acid	0.000	36	C22:1 Erucic acid	0.003
10	C15:0 Pentadecanoic acid	0.006	37	C22:2 Docosadienoic acid	0.000
11	C15:1 Pentadecenoic acid	0.000	38	C22:4 Docosatetraenoic acid	0.000
12	C16:0 Palmitic acid	1.153	39	C22:5 all cis-4, 7, 10, 13, 16	0.000
13	C16:1 cis-7-Hexadecenoic acid	0.003	40	C22:5 all cis-7, 10, 13, 16	0.000
14	C16:1 Palmitoleic acid	0.036	41	C22:6 Docosahexaenoic acid	0.000
15	C17:0 Heptadecanoic acid	0.039	42	C23:0 Tricosanoic acid	0.005
16	C17:1Δ 10-Heptadecenoic acid	0.000	43	C24:0 Lignoceric acid	0.101
17	C18:0 Stearic acid	0.441	44	C24:1 Nevonic acid	0.000
18	C18:1 Tran fatty acids	0.036	45	Other fatty acids	0.439
19	C18:1 Oleic acid	2.014	46	**Total fatty acids**	**5.883**
20	C18:2 Trans fatty acids	0.037	47	**Total fat (as triglyceride fat)**	**6.151**
21	C18:2 Rumenic acid (CLA)	0.000	48	**Saturated fatty acids**	**2.008**
22	C18:2 Linoleic acid (LA)	1.267	49	**Trans fatty acids**	**0.078**
23	C18:3 Trans fatty acids	0.005	50	**Monounsaturated fatty acids**	**2.069**
24	C18:3 Linolenic acid (ALA)	0.005	51	**Polyunsaturated fatty acids**	**1.289**
25	C18:3 gamma-Linolenic acid	0.000	52	**Omega-3**	**0.013**
26	C18:4 Octadecatetraenoic acid	0.007	53	**Omega-6**	**1.276**
27	C20:0 Arachidic acid	0.129	54	**Omega-9**	**2.033**

Fatty acid composition and lipid, protein, carbohydrate, carotenoid contents of the biomass cultured in 30 L fermentor

The fatty acid composition in the biomass of TSL10 strain cultured in 30 L fermentor after 120 hours of cultivation was shown in table 4. The result indicated that the fatty acid composition consisted mainly of Palmitic acids (19.60% of total fatty acids-TFA), Oleic (34.23% of TFA), Linoleic (21.54% of TFA). The fatty acid composition consisted mainly of

omega 3-6-9 fatty acids, accounting for 56.47% of TFA. In which, omega -3, omega- 6 and omega -9 fatty acids accounted for 0.22% of TFA, 21.69% of TFA and 34.56% of TFA, respectively. The main omega - 9 fatty acid in the brain is oleic acid (18:1, ω-9) - a very important fatty acid for the brain and cardiovascular system that can be synthesized or obtained from the diet (Bourre *et al*., 2009). According to Johnson and Bradford (2014), omega -3 fatty acids are often associated with the initiation of anti-inflammatory responses, while omega - 6 fatty acids associated with proinflammatory responses. Omega - 9 fatty acids serve as essential components for other metabolic pathways. These fatty acids act as an independent lipid, but they can interact with other biomolecules in the body to maintain homeostatis and ensure good health for humans. Simopoulos (2006) suggested that human beings envolved on a healthy diet with a ratio of omega - 6 to omega - 3 fatty acids of 4:1 - 5:1 and should not exceed 10:1. In addition, a study in rats indicated that a 25:1 omega-6/omega-3 fatty acid ratio will favorably modify the erythrocyte fatty acid profile of spontaneously hypertensive rats (Johnson *et al*., 2018). In the fatty acid composition of strain TSL10, the omega-6 to omega-3 ratio is 98:1. Therefore, when supplement the biomass of this strain in the diet, it should be calculated that a ratio of omega-6 to omega – 3 fatty acids is considered to be the best for humans and animal health.

The content of lipid, protein, carbohydrate, and carotenoid of the biomass of strain TSL10 cultured in a 30 L fermentor

The results on the analysis of lipid, protein, carbohydrate and carotenoid contents of the biomass of strain TSL10 cultured in a 30 L fermentor were shown in Table 5.

Table 5. Contents of lipid, protein, carbohydrate, and carotenoid of strain TLS10.

Parameter	Content
Lipid (% DCW)	22.95 ± 0.28
Protein (% DCW)	6.65 ± 0.19
Carbohydrate (% DCW)	15.96 ± 0.021
Carotenoid (µg/L)	178 ± 2.43
Astaxanthine (µg/L)	8.3 ± 0.28

The results showed that the highest contents of lipid, protein and carbohydrate were 22.95 ± 0.28; 6.65 ± 0.19 and 15.96 ± 0.021% DCW, respectively. In addition, the contents of carotenoid and astaxanthin reached 178 ± 2.43 and 8.3 ± 0.28 µg/L, respectively. The above obtained results are similar to the previous studies of Burja *et al.* (2006), Valadon (1976), Carmona *et al.* (2003), Park *et al.* (2018), Nham Tran *et al.* (2020), Leyton *et al.* (2021). Through the use of HPLC mass spectrometry, Burja et al. (2006) also demonstrated that *Thraustochytrium* sp. ONC-T18 contains antioxidants such as astaxanthin, zeaxanthin, canthaxanthin, echineone, and β-carotene (Burja *et al*., 2006). Echinenone and canthaxanthin were found in *Schizochytrium aggregatum* (Valadon, 1976), while *Thraustochytrium* sp. CHN-1 was demonstrated to contain astaxanthin, echinenone, canthaxanthin, phoenicoxanthin and β-carotene (Carmona *et al*., 2003). Several new strains isolated from Australian mangroves belonging to the family thraustochytriidae have lipid, carbohydrate, and protein contents ranging from 13 to 41%, 6 to 28%, and 3 to 15% of DCW, respectively, depending on the genetic characteristics of the strain and culture conditions (Nham Tran *et al*., 2020). In addition, several species of the genus of *Thraustochytrium* can accumulate substantial quantities of carotenoid. The amount and composition of carotenoids depends on the species and medium

conditions such as medium composition and temperature (Park *et al*., 2018). According to Leyton *et al*. (2021), some strains of the genus of *Thraustochytrium* had DCW of 1.9 - 7.2 g/L and carotenoid yield in the range of 52 - 460 µg/L after 5 days of culture. In which, the predominant carotenoids include the β-carotene and canthaxanthin. Thus, when comparing the carotenoid yield of strain TSL10 (178 ± 2.43 g/L) with publication of Leyton *et al*. (2021), it showed that it was shown to be equivalent to some strains but it had a value 2.5 times lower than that of other strains with the highest carotenoid yield (460 µg/L). This difference proved that the strains have different genetic characteristics as well as the cultivation conditions.

CONCLUSION

In this study, for the first time in Vietnam, in May 2021, we have successfully isolated one strain of heterotrophic marine microalga (TSL10) from seaweed/seagrass samples of Spratly Island belonging to Truong Sa Archipelago. Based on the morphological characteristics and the 18S rRNA gene sequence, the strain TSL10 was identified as *Thraustochytrium pachydermum* TSL10 (with accession number OL721615 in GenBank). This strain can produce high amounts of lipids, proteins, carbohydrates, carotenoids, and astaxanthine. In which, linoleic acid (21.54% of TFA) and oleic acid (34.23% of TFA) are mainly dominated polyunsaturated fatty acids in *T. pachydermum* TSL10. The finding of our study offers potential biotechnological applications of this strain such as production of health food for humans, feed for livestock and poultry as well as for the extraction of bioactive substances and biofuel production in the near future.

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