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Isolation and characterization of acid-tolerant *Stichococcus*-like Microalga (*Tetratostichococcus* sp. P1) from a tropical peatland in Malaysia

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Abstract

Tropical peat swamp forests are unique ecosystems that are found in the tropical regions of Southeast Asia. Since tropical peatlands have high sunlight penetration, high temperature, and low pH (2.9–3.5), unique microorganisms can survive under certain environmental conditions. Acid-tolerant microalgae, S*tichococcus*-like species from a peatland in Malaysia, were isolated and identified as *Tetratostichococcus* sp. P1. This strain showed a high growth rate ($\mu = 1.47$ day^{-1}) with an optical density (OD_{730}) of 9.25 in 7 days. *Tetratostichococcus* sp. P1 shows four primary fatty acids (C16:0, C18:1, C18:2, and C18:3) at different pH values. The highest total amount of fatty acids was at a pH of 5, followed by at a pH of 3 and a pH of 7 with 102.88 μg mg^{-1}, 75.8 μg mg^{-1}, and 67.77 μg mg^{-1}, respectively. *Tetratostichococcus* sp. P1 reached an OD_{730} of 2.85 ± 0.40 at 12 days with a growth rate of $\mu = 0.22$ day^{-1} when peat water was used as a nutrient source and produced a significant amount of C20:0. This result indicated that tropical peat water could be applicable for the mass growth of *Tetratostichococcus* sp. P1 to reduce cost for biodiesel production.

Keywords *Tropical microalga* · *Tetratostichococcus* · Acid-tolerant · Fatty acid production

Introduction

Peatland is defined as an ecosystem where organic matter from decaying plants accumulates under wet conditions. The high water level creates waterlogged conditions on land and causes incomplete decay of organic matter (Dise 2009). The main components of peat are water, organic matter, gases, and minerals (Ritzema et al. 2014). More than 98% of the original peat consists of organic matter and a mixture of semidecomposed plant biomass gradually accumulates

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underwater saturation conditions (Zhong et al. 2020). The formation of peat is prolonged and the rate of peat formation is approximately 0.5–1 mm per year (Parish et al. 2008). Peatlands are unique and complex ecosystems. They are essential for biodiversity conservation at the genetic, species, and ecosystem levels, even though peatlands cover only 3% of the Earth's land surface (Mazei et al. 2020). Peatlands are essential carbon reservoir ecosystems; they store 68.5 Gt of carbon, accounting for 11–14% of the sum of global carbon (Müller et al. 2015). Most carbon in the soil exists below the waterlog, where it is subject to anaerobic decomposition by microbes, which generates carbon dioxide (CO_2) and methane (CH_4) as the end products (Keller and Bridgham 2007). These massive deposits of carbon indicate that peatlands have acted as sinks of atmospheric carbon dioxide (CO_2) for millennia. Peatlands are mainly located in high latitude regions, such as in Northern Europe, Siberia, and North America, and these occupy over two-thirds of the peatlands in the world (Jackson and Raub 2010). The slow decomposition of the organic matter in the northern peatlands is due to the low temperature (Bubier et al. 2006). However, peatlands are also found in tropical regions, known as peat swamp forests, such as peat swamp forests in Southeast Asia, Africa, the Caribbean, and Central South America (Yule and Gomez 2009). These have very acidic conditions with pH values ranging from 2.9 to 3.5 and are regarded as crucial terrestrial carbon sinks in the biosphere (Pattanop et al. 2011).

Microalgae can grow in many ecosystems ranging from marine and freshwater environments to desert sands and hot springs to snow and ice. Microalgae belong to many groups and species, with more than a hundred thousand strains of microalgae constantly being studied and explored (Fistarol et al. 2018). Eukaryotic microalgae have a more complex cell structure and they are more tolerant to acidic conditions (Visviki and Santikul 2000). Some microalgae have been successfully isolated, such as the acidophilic green alga *Chlamydomonas eustigma* (isolated from sulfuric acid mine drainage; pH 2.13), which only grows under acidic conditions ($pH \leq 4$), and the acid-tolerant green algae *Chlamydomonas applanata* (isolated from acid mine streams; pH 2.6–3.1), which can grow under both acidic and alkaline conditions from pH 3.4–8.4 (Visviki and Santikul 2000; Hirooka et al. 2017). Extremophile algae growth is sometimes slower than that of "common" microalgae (Pulz and Gross 2004). However, microalgae that grow in extremely acidic environments benefit from preventing contamination; they are also believed to have unique characteristics compared to common microalgae, such as *Pseudochlorella* sp. YKT1 accumulates high lipid content under low pH (Hirooka et al. 2014).

Microalgae accumulate large amounts of storage lipid, primarily in triacylglycerols (TAG). TAG contain saturated and unsaturated fatty acids, mainly having 14–20 carbons in their structure find their application in biodiesel production. TAG can be converted into biodiesel through transesterification by converting it into FAME (Kumar and Bharadvaja 2020). Studies have indicated changes in biomass lipid synthesis and fatty acid composition in diverse environments due to different nutrition, pH, light intensity, and temperature (Grace et al. 2022). For example, freshwater green algae have FA compositions similar to vascular plants, with C16 PUFAs higher than C18 PUFAs. In contrast, marine species have PUFAs greater than C18, with green algae being rich in C18 PUFAs (ALA, STA, and LA) and red algae being rich in C20 PUFAs (AA and EPA), while brown algae have a mixture of both (Domínguez 2013).

Peat waters are rich in carbon and organic acids such as ferulic acid, synapic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, vanillic acid, and syringic acid. That compound might be useful for the growth and fatty acid elongation as well as desaturation of microalgae (Fernandes et al. 2016; Mazei et al. 2020). Therefore, bioprospecting extremely acidic tropical ecosystems (pH 3) can provide axenic unique native microalgae strains that can grow under low pH to prevent unwanted contaminants in open pond culture. In addition, acidic microalgae can facilitate triacylglycerols (TAGs) production for high-quality liquid fuel products, such as aviation fuel.

Microalgal research on tropical peat swamp forests worldwide is still scarce and unexplored. Consequently, isolation and identification are fundamental processes to obtain microalgae cultures for further study to determine the potential of peatland microalgae for biodiesel production and applications. This study reports the isolation and characterization of the *Stichococcus-like* microalga *Tetratosticho-coccus* sp. P1 from a Malaysia peat swamp forest with very acidic conditions and their fatty acid information for biofuel.

Materials and methods

Sample collection and isolation

The algal strain was isolated from Raja Musa Peat Swamp Forest Reserve, Hulu Selangor, 0"N 101°26′31, 3°27′58, 5. Samples were collected on 22 June 2016 when the temperature of the peatland was 30 °C and the pH was 3.0–3.5. The algae were grown at the acidic condition from pH 3 to 5 on modified BG11 medium (Stanier et al. 1971) plates containing 1.5% Bacto-agar by serial dilution and colony pick up to obtain a single colony, where repeated dilution on BG11 and routine microscopic examination ensured the culture contained a single species. The morphology of the strain was identified based on size, shape, and color. For selection, single-cell colonies appearing (12–14 days) on agar plates were inoculated into a 50 mL culture flask containing 30 mL of modified BG11

liquid medium at pH of 3–5 and incubated in a shaker cabinet at 120 ± 1 rpm under continuous light at 70 µmol photons m^{-2} s^{-1} at 30 °C for 10 days. Bacterial contamination was eliminated by the addition of 10–14 µg mL^{-1} ampicillin. Contamination was tested by growing 300 µL of cells on 3000 µL of standard LB medium at 37 °C for 3 days (Sarsekeyeva et al. 2014). No bacteria or fungi were observed after 3 days of incubation. To obtain the optimal temperature, microalgae were incubated at three different temperatures: 25 °C ± 1 °C, 30 °C ± 1 °C, and 35 °C ± 1 °C and cultured in an aeration system. The experiments were repeated at least three times.

Culture conditions and growth measurement for microalgal isolation

The strain was grown in 100 mL glass test tubes (diameter 30 mm) containing 50 mL BG11 medium. The culture was incubated at 30 °C under continuous illumination (70 µmol photons m^{-2} s^{-1}) by fluorescent lamps and aerated with 1% (v/v) CO_2-enriched air. The growth of the culture was measured at an optical density (OD) of 730 µm and chlorophyll α content every 24 h at 665 µm followed the previous method (Asada et al. 2019). The growth rate of the microalgae was observed by fitting the OD at the exponential phase of the isolate to an exponential function as follows:

$$\mu = \frac{\ln OD_2 - \ln OD_1}{\Delta t} \quad (1)$$

where OD_1 is the initial optical density measured at the start of the exponential stage, OD_2 is the optical density measured on the last exponential phase, and Δt is the total days of the exponential stage (t_2-t_1). Each experiment and measurement were repeated at least three times.

Species identification

DNA extraction

Genomic DNA was extracted and followed the protocol of the FastDNA SPIN Kit (Funakoshi, Japan). Microalgal cells were harvested from 10 mL of liquid culture by centrifugation at 14,000 × *g* at 4 °C for 5 min. One milliliter of sample was mixed with 1 mL of lysis solution "CLS-Y" in a bead beating tube. The algal cells were broken with bead beating at 4,200 strokes/40 s. The supernatant was collected by centrifugation at 12,000 × *g* at 4 °C for 5 min. The remaining solid particles were removed by centrifugation at 14,000 × *g* at 4 °C for 2 min. Then, 700 µL of supernatant was mixed with binding matrix (1:1 v/v) and incubated for 5 min. After centrifugation at 14,000 × *g* at 4 °C for 2 min the supernatant was discarded and the DNA was washed with 500 µL of washing solution "concentrate SEW-S". DNA samples were obtained by transferring the column into a new tube and 100 µL of H_2O was added to the white area and then incubated for 2 min. DNA was collected by centrifugation at 14,000 × *g* at 4 °C for 2 min. The genomic DNA quality and quantity were assessed by standard absorbance measurement (absorbance 280 nm/260 nm and absorbance 230 nm/260 nm) using a Nanodrop UV–Vis spectrophotometer.

PCR amplification and sequencing

18S rDNA was amplified by PCR using the following primers: forward primer SR1 (5'-TACCTGGTTGATCCTGCC AG-3') and reverse primer SR12 (5'CCTTCCGCAGGT TCACCTAC-3') (Nakayama et al. 1996). Direct sequencing was performed with 12 primers (SR1-S12, **Supplemental Table** S1) (Nakayama et al. 1996). The sequencing reaction followed the instructions of the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Japan). The sequencing products were purified by precipitation with ethanol/EDTA, denatured, and resolved on a Genetic Analyzer SeqStudio (Thermo Fisher Scientific). The BLAST program (Altschul et al. 1990) searched the homologous sequences from the database. The sequences were deposited into the National Center for Biotechnology Information (NCBI) [GenBank: MT053478].

Phylogenetic analysis

Most related species in NCBI and previous reports of *Stichococcus*-like microalgae sequences (Pröschold and Darienko 2020) were selected for phylogenetic analysis. The *Pseudostichococcus* sequence was used as an outgroup. The phylogenetic tree was inferred using four different methods: the neighbor-joining (NJ) method (Saitou and Nei 1987), maximum likelihood (ML) method, minimum evolution (ME) method (Rzhetsky and Nei 1992), and maximum parsimony (MP) method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was determined (Felsenstein 1985). The distances were determined according to the Kimura 2-parameter method and are in the units of base substitutions per site (Kimura 1980). The analysis involved ten nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary studies were conducted in MEGA X (Sudhir et al. 2018).

Acidic growth conditions in BG11 media and peat water

The ability of the selected strain to grow under acidic conditions was observed. The strain was cultured in BG11 medium at pH values of 3.0, 4.0, and 5.0. Trisodium citrate buffer (20 mM) was used to maintain the pH (Fuji Film Wako). The

growth of the strain was also assayed under neutral and alkaline pH conditions (6–8). To examine the ability of the microalga to utilize peat water as a medium, peat water was collected on 5 March 2020 from a peat water river, Hulu Selangor, near the sample collection. The pH of the peat water was 4.5. Sterilization of the peat water was performed by vacuum filtration (Millipore Sigma Stericup Quick Release-HV Vacuum Filtration System 0.2 µm). The strain was cultivated in 100 mL bubbling tubes containing 50 mL pure peat water medium and BG 11 as control (pH 4.5) and aerated with air only or 1% (v/v) CO_2-enriched air at 30 °C. To measure the growth, the optical density (OD) of the culture was measured at 730 µm every 24 h based on (i) calculation analysis. Each experiment and measurement were repeated at least three times (n = 3).

Fatty acid analysis

To determine the lipid content and fatty acid composition samples were harvested at the stationary phase of culture. The algae were concentrated to a minimum optical density of 5.0 mL^{-1} at OD_{730} by centrifuging at 1400 × *g* at 4 °C for 1 min. Fatty acid methyl esters (FAMEs) were prepared by transesterification (Machida et al. 2016). Cells were harvested by centrifugation and resuspended in 2 mL methanol and transferred to glass test tubes. Drying was performed by a concentrating centrifuge (CC-105, Tomy Seiko, Japan). Then the pellet was resuspended in 0.1 M hydrochloric acid methanolic solution (Wako Pure Chemicals, Japan) and the tubes were incubated at 10 °C for 1 h to allow saponification of the acyl groups in microalgal lipids and conversion into FAMEs. The resultant FAMEs were recovered using n-hexane. The hexane phases recovered were evaporated and the residues containing FAMEs were dissolved in 100 µL of n-hexane. In this study, the C17 internal standard was used.

To identify and quantify the FAMEs, the method of Machida et al. (2017) was followed. Briefly, 1 µL of hexane was applied to a GC-FID 2014 gas chromatograph equipped with a flame ionization detector (Shimadzu, Japan). Helium was used as a carrier gas at a constant flow rate of 1.25 mL min^{-1} in the splitless mode. A CP-Sil5 CB column (Agilent Technologies, USA) was used at the following temperatures: 60 °C for 1.5 min, then 130 °C at 20 °C. per min, and a further increase to 230 °C at 4 °C per min. FAMEs were provisionally identified based on the retention time and mass spectra of commercial FAME standards (Nu-Chek Prep, USA). The experiments were repeated at least three times.

Draft genome sequence, assembly, and annotation of isolates

The DNA of the microalga was extracted by a FastDNA SPIN Kit (Funakoshi, Japan) to obtain good-quality genomic DNA. The quality and quantity of DNA extraction was assessed by measuring absorbance ratio 280/260 and absorbance 230/260 using a NanoDrop 2000 UV–Vis spectrophotometer (Thermo Fisheries Scientific, Japan). The quality of the genomic DNA for NGS library preparation was measured under an Invitogren Qubit 3 fluorometer (Thermo Fisheries Scientific). DNA fragmentation and library preparation followed the Ion Xpress Plus gDNA Fragment Library preparation protocol (Fisher Scientific 2016). Size selection was conducted using a Pippin PrepTM Quick Guide 2% Agarose Gel Cassette targeting 400 bp. The library was amplified and purified using Agencourt AMPure XP Reagent. The size of the final library was analyzed with an Agilent High Sensitivity DNA Bioanalyzer Kit (Agilent Technologies 2013). Template preparation was performed using Ion Chef according to the manufacturer's protocol. Sequencing was performed using Ion S5 XL. The draft genome sequence was then deposited into NCBI [GenBank: JAAIKU000000000.1].

Assembly was performed using Genomics Workbench CLC 11.0 (CLC Bio 2010) and annotation was performed by Augustus programs with parameters trained by BUSCO (Simão et al. 2015). Briefly, repeat sequences in contigs were masked using RepeatMasker and RepeatModeler (Tarailo-Graovac and Chen 2009) implicated in the Funannotate program. 1.0.1. Training parameters for genome annotation were performed using BUSCO software with a "long" option based on the Chlorella dataset. Gene models were constructed using Augustus with the parameters. Functional annotation was performed using an Eggnog mapper (Jaime et al. 2016). The KEGG Orthology ID assignment (Kanehisa et al. 2016) was performed to predict the genes.

Statistical analysis

Statistically significant differences between means were determined using one-way ANOVA Post-Hock Turkey's test ($p \leq 0.05$) following IBM SPSS Statistical Software (ver.25).

Results

Isolation, identification, and characterization of acidic microalgae from peatlands

The isolation objective was to find an alga that can grow fast under a low pH. In this study, 6 out of 37 samples were able to grow under acidic conditions. A *Stichococcus*-like species showed the fastest growth in 8 days of cultivation at pH 3, with optical densities at OD_{730} of 2.00 ± 0.2 (µ = 0.28 day^{-1}) in shaking culture and 5.70 ± 0.4 (µ = 1.05 day^{-1}) in bubbling culture aerated with 1% (v/v) CO_2-enriched air; thus, it was chosen for further study.

Based on the distinguishable morphological characteristics under light microscopic examination (Fig. 1), the strain was preliminarily described as a *Stichococcus*-like species that was green in color, rod shape, and single cells were 3.5–8 µm in length and 2–3 µm in width under shaking culture (Fig. 1A). The algal morphology changed under the air bubbling system with CO_2 (Fig. 1C). It showed a more extended size of 30 µm in length and 3–4.5 µm in width, and some of the microalgae had irregular shapes after 7 days of cultivation. The algae from peatland showed a larger vacuole than those without CO_2, and chloroplast bleaching occurred.

18S rRNA and Phylogenetic tree

For further characterization 18S rDNA sequences of the isolated potential microalga were obtained. The amplification target of 18S rDNA using SR1 and SR12 was 1,781 bp (Nakayama et al. 1996). However, the amplicon of the candidate strain from peatland showed that a DNA fragment of 2,672 bp was successfully amplified from the genomic DNA with six pairs of 18S primers SR1/SR12. Based on the phylogenetic tree analysis, the isolated strain showed high homology and was closely related to *Tetratostichococcus* sp. (Fig. 2). Therefore, the strain from peatlands was named *Tetratostichococcus* sp. strain P1.

Growth under acidic conditions

To examine the capacity of *Tetratostichococcus* sp. P1 to grow under acidic conditions, the strain was cultured in BG11 media at various pH (Fig. 3). *Tetratostichococcus* sp. P1 showed high growth rates ($\mu > 1.1$ day^{-1}) under acidic pH conditions (pH 3 – pH 5), where a pH of 3 was the highest with $\mu = 1.46 \pm 0.19$ day^{-1}. *Tetratostichococcus* sp. P1 also showed the ability to grow in a wide range of pH values from acidic to alkaline conditions (2 to 8) (Fig. 3a) suggesting that *Tetratostichococcus* sp. P1 is closer to acidic-tolerant microalgae than acidophilic algae. *Tetratostichococcus* sp. P1 did not grown well under extremely acid conditions, but it can survive at a pH of 2 with a growth rate of 0.23 ± 0.05 day^{-1}; this result was supported by the chlorophyll content of *Tetratostichococcus* sp. P1. A pH of 3 was the best condition for growth, with 1.47 day$^{-1} \pm 0.02$, followed by a pH of 4 and a pH of 5, with growth rates of 1.36 ± 0.01 day^{-1} and 1.10 ± 0.05 day^{-1}, respectively. The strain also showed the ability to grow in neutral conditions (pH of 7) and alkaline conditions (pH of 8) with growth rates of 0.89 ± 0.01 day^{-1} and 0.70 ± 0.01 day^{-1}, respectively (Fig. 3b). Statistical analysis showed that the growth rate at pH 3 was significantly higher ($p < 0.05$) than at other pH.

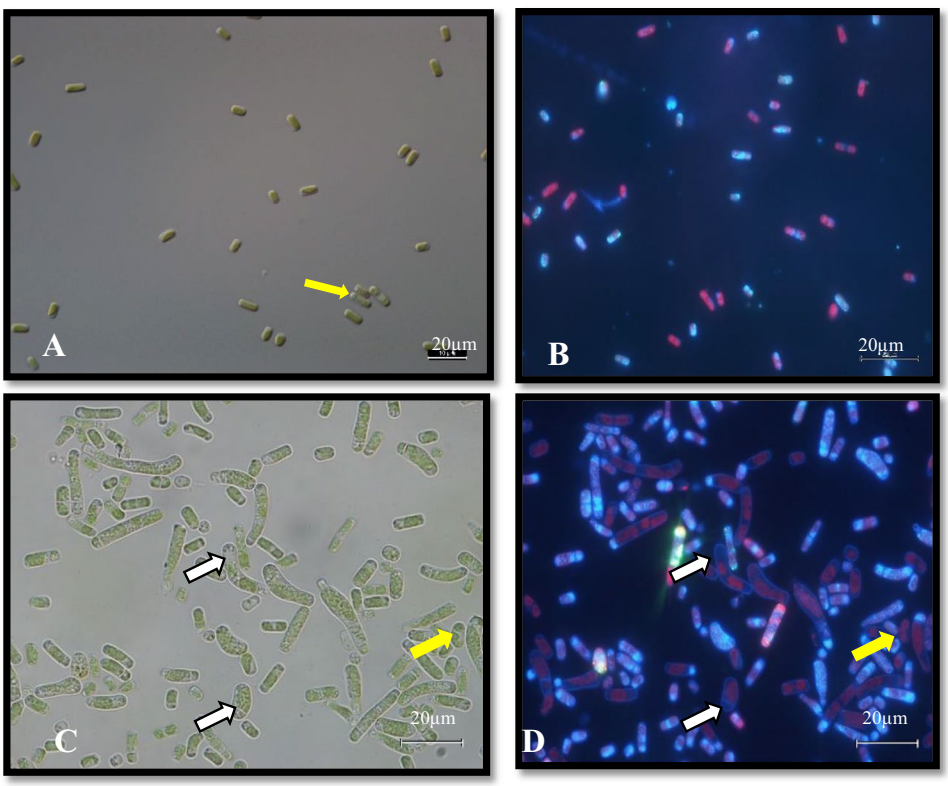

Fig. 1 Morphological image of *Tetratostichococcus* sp. P1 under a microscope at 100× magnification. **A** Morphology under shaking system cultivation. **B** DAPI staining strain under shaking culture. **C** Morphology under an air bubbling system with 1% CO2. **D** DAPI staining strain under bubbling culture. White arrows show chloroplast bleaching; yellow arrows show the accumulation of storage vacuoles

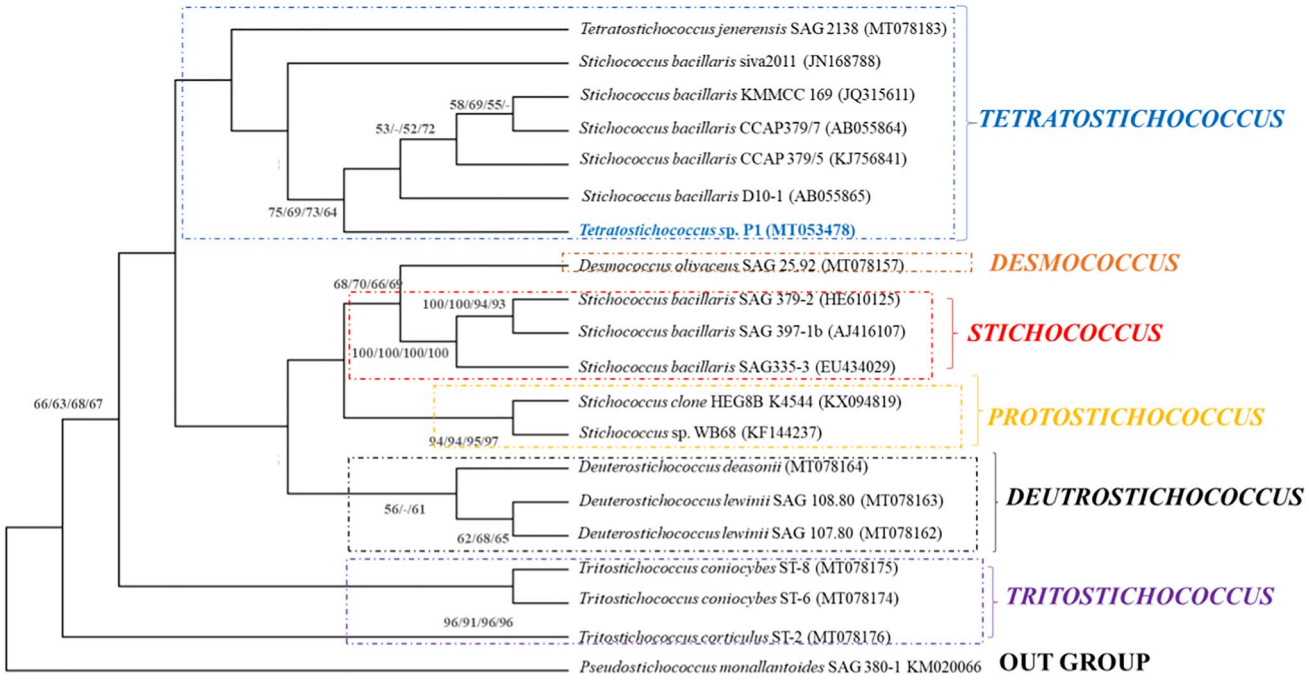

Fig. 2 Phylogenetic tree of *Tetratostichococcus* sp. P1. The tree was inferred using the neighbor-joining (NJ) method, maximum likelihood (ML) method (Kimura 1980), minimum evolution (ME) method (Rzhetsky and Nei 1992), and maximum parsimony (MP) method in the bootstrap test (1000 replicates) (Felsenstein 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980); analyses were conducted in MEGA X (Sudhir et al. 2018). The values indicate bootstrap points of NJ, ML, ME, and MP

Fatty acid accumulation profile under different pH values in BG11 medium

To measure the fatty acid profile, cultures were harvested after seven days in BG11 medium at pH values of 3, 5, and 7 and aerated with air supplied with 1% (v/v) CO_2 at 30 °C. The results showed that *Tetratostichococcus* sp. P1 contained four primary fatty acids (Fig. 4 and **Supplemental Table** S2), i.e., palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (LA) (C18:2), and α-linolenic acid (ALA) (C18:3), which are known as ω3 fatty acids. Other types of fatty acids, including palmitoleic acid (C16:1), hexadecatrienoate acid (C16:3), and stearic acid C18:0, were found to be minor compounds. The long carbon chains of fatty acids C20 and C24 were also detected in *Tetratostichococcus* sp. P1.

The highest total amount of FAMEs (μg mg^{-1}) was observed at a pH of 5, followed by a pH of 3 and a pH of 7, with 102.88 ± 2.40 μg mg^{-1}, 75.8 ± 2.30 μg mg^{-1}, and 67.77 ± 4.70 μg mg^{-1}, respectively. The fatty acid content at pH 5 was significantly higher ($p < 0.05$), relative to the total FAMEs at pH 3 and 7. The main unsaturated FAMEs detected under all pH conditions are consistent, i.e., C18:1, C18:2, and C18:3, and the predominant saturated FAME is C16:0. Based on the mol percentage results of FAMEs, polyunsaturated fatty acids (PUFAs) were directly proportional to the pH level (Fig. 4), where at a higher pH, PUFA has a more significant percentage. For example, the rate of ALA at a pH of 7 was significantly higher ($p < 0.05$) than those at a pH of 5 and a pH of 3 (i.e., 32.1 ± 1.3%, 24.37 ± 0.9%, and 23.66 ± 1.0%, respectively). This result indicated that FA desaturation was related to the pH, where Δ15 was more highly expressed under alkaline conditions than acidic conditions. In FA pathway Δ15, desaturase converts LA to ALA. There was no significant difference in the LA percentage, but C18:1 was highest at a pH of 5 (29 ± 0.9%), followed by at a pH of 3 (24 ± 1.0%), and the percentage of C18:1 at a pH of 7 was the lowest at 14.98 ± 1.20%.

Application of peat water as a medium for *Tetratostichococcus* sp. P1 growth and the fatty acid accumulation profile after cultivation.

To examine whether peat water can be a potential nutrient source for *Tetratostichococcus* sp. P1, the alga was cultivated in peat water in an aeration system with air and 1% CO_2 enriched air (**Supplementary Fig.** S2.a). The growth rate under the bubbling system with air only was significantly higher ($p = 0.019$) than the growth rate with the addition of 1% CO_2 ($\mu = 0.19 \pm 0.05$ day^{-1} and $\mu = 0.26 \pm 0.1$ day^{-1}, respectively) (**Fig.** S2.b). In a bubbling system aerated with air the alage reached an OD_{730} of 1.33 ± 0.3 at 8 days, while with additional CO_2, the strain reached an OD_{730} of 1.10 ± 0.3

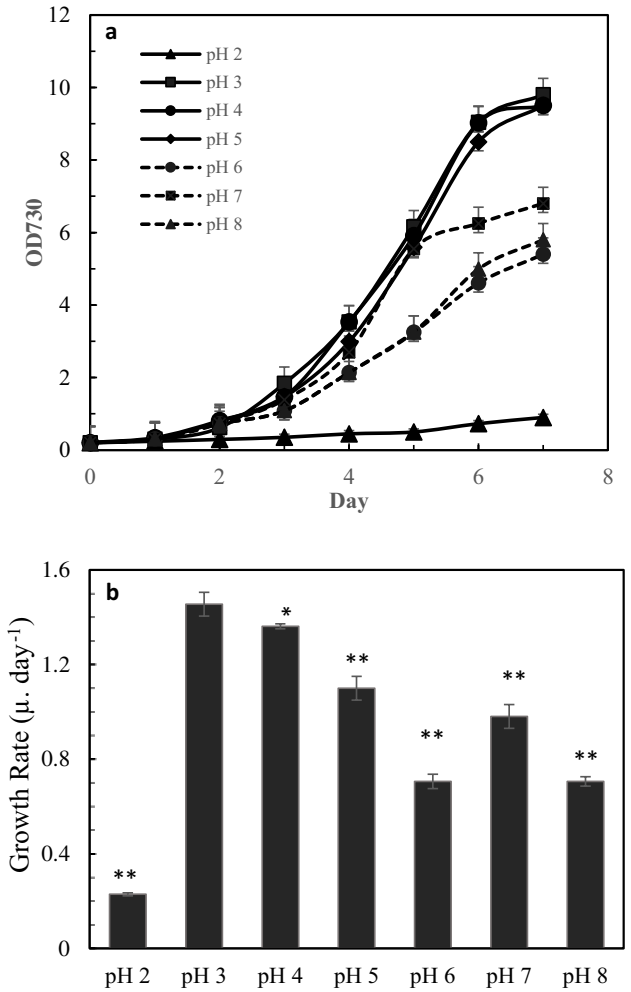

Fig. 3 Growth curve of *Tetratostichococcus* sp. P1 under different pH with 1% CO_2. **a** Growth based on OD_{730}. **b** Growth rate ($\mu.day^{-1}$) after seven days of cultivation (*$p<0.05$; **$p<0.01$); Trisodium citrate buffer was used for pH 3 to pH 5, MES buffer for pH 6, HEPES buffer for pH 7, and Tricine buffer for pH 8. The error bar indicates the standard deviation (n = 3)

at 8 days of cultivation. At 12 days of cultivation, the OD_{730} values were 2.87 ± 0.3 and 2.21 ± 0.3, respectively.

The FAME content after growth with peat water at the original pH (pH of 4.5 ± 0.15) (Fig. 5) showed some differences in FAME proportions under BG11 and peat water. One of the clearest differences in the FAME percentage under BG11 and peat water with the addition of 1% CO_2 was C18:0 and C18:3, where C18:0 of *Tetratostichococcus* sp. P1 was very high under peat water (22.20 ± 1.05%) compared to BG11 (2.29 ± 0.20%), but C18:3 showed the opposite effect, where C18:3 was significantly higher ($p<0.05$) in BG11 than in peat water (24.37 ± 0.9% and 4.5 ± 1.0%, respectively). In cultivation under air only (-CO_2), a high percentage of C18:3 was shown under BG11 with 32.2 ± 1.0%, while peat water was only 13.8 ± 1.5%. Surprisingly, long carbon chains of fatty acids and long PUFAs (i.e., C20:0 and C20:3n6) were present under peat water with air only, with 23.6 ± 1.2% and 2.5 ± 0.7%, respectively. These FAMEs were not detected or found in trace amounts under CO_2. The difference in the percentage of C20:0 under BG11 with CO_2 or air was not significant. However, the result was unique under peat water, where C20:0 was very high under peat water without adding 1% CO_2. It can be concluded that CO_2 inhibited unsaturated fatty acids and elongated enzyme activity in peat water. Therefore, peat water can be used as a growth medium without additional CO_2.

Draft genome sequence analysis of *Tetratostichococcus* sp. P1

To evaluate the genomic contents of *Tetratostichococcus* sp. P1 for fatty acid production and adaptation to low pH conditions, draft genome sequencing analysis was conducted. A total of 51 Mbp of the sequence was obtained from 15,474 contigs. *Tetratostichococcus* sp. P1 had a high number of exons and introns, 95,289 and 77,707, respectively (**Table** S3), and a total of 17,582 genes in 5,534 clusters were predicted from the annotation results.

Discussion

Characteristic of microalgae from tropical peatland under CO_2

Peat swamp forest is a unique tropical wetland due to its lowland and acid condition (Lo and Parish 2013). The acidic conditions of tropical peatland are due to the organic acid and waterlogged conditions (Sabiham 2010). This study successfully isolated an acidic tolerant, *Stichococcus*-like microalga from peatland. The strain was able to grow on BG11 agar medium pH 3. *Stichococcus-like* microalgae are euryhaline algae primarily found in the Arctic and Japan. It has a short life cycle and can survive in a wide range of pH conditions (Sivakumar et al. 2014). Our research showed that CO_2 induces a morphological change in the strain. Under CO_2 the cell size was bigger by eight times than their usual size (3.5 μm) to 30 μm. The morphology shape was changing under CO_2 also from rod to irregular shape. CO_2 might affect the alga membrane and cytoplasm. Iwasaki et al. (1998) reported that the sudden rise in the CO_2 concentration affected the chlorophyll and D1 protein in thylakoid membranes, damaging photosystem II (PS II). High CO_2 concentration has an acidifying impact in *Stichococcus*-like cytoplasm. A previous study also showed the changing morphology of S*tichococcus*-like species under saline stress. This morphological change might result from responses to environmental stress (Van et al. 2021). S*tichococcus*-like

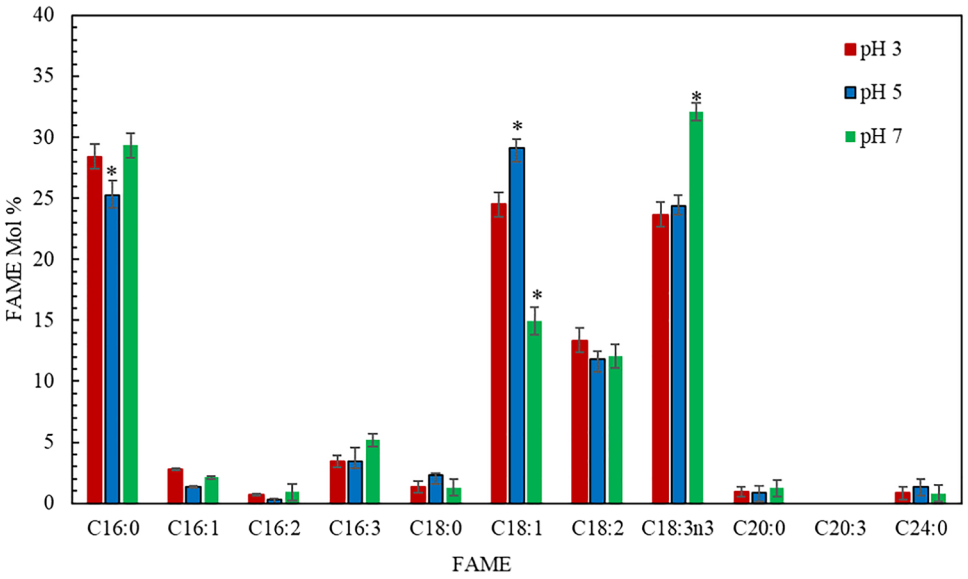

Fig. 4 The molar percentage of fatty acid methyl esters (FAMES) contained in *Tetratostichococcus* sp. P1 under different pH conditions with BG11 media. Asterisk indicates statistical significance ($p < 0.05$) of FAME against FAME at pH 3. The bar with red color indicates FAMEs contained at a pH of 3, blue indicates FAMEs contained at a pH of 5, and green indicates FAMEs contained at a pH of 7. The error bar indicates the standard deviation (n = 3)

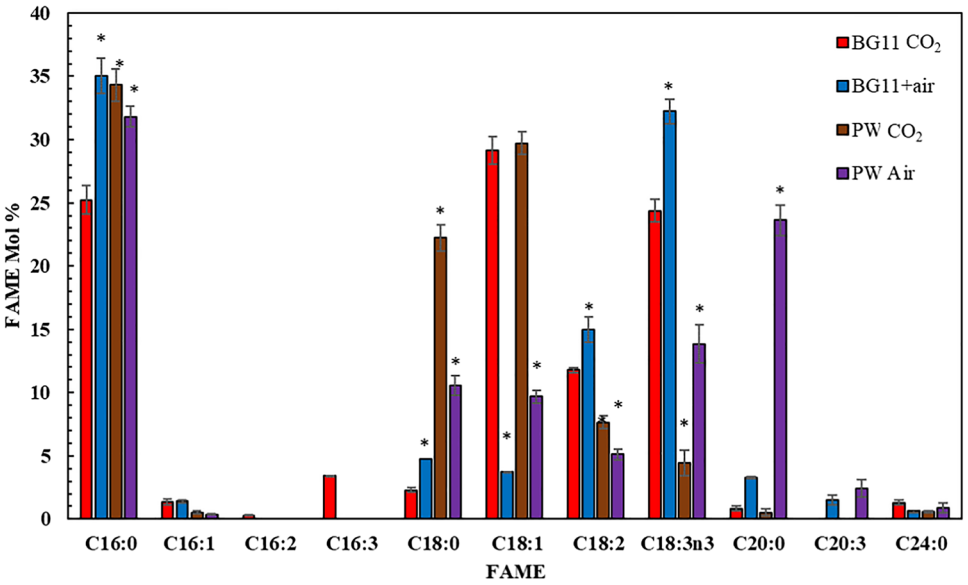

Fig. 5 FAME content of *Tetratostichococcus* sp. P1 under BG11 and peat water. Asterisk indicates statistical significance ($p < 0.05$) of FAME against FAME at BG11 + CO_2. The bar with the red color indicates FAMEs contained in BG11 with 1%CO_2 enriched air, blue indicates FAMEs in BG11 aerate with air, brown indicates FAMEs contained in peat water with 1%CO_2 enriched air, and the bar with purple indicates FAMEs contained in peat water + air. The error bar indicates the standard deviation (n = 3)

microalgae have low CO_2 resistance (Iwasaki et al. 1998; Sivakumar et al. 2014). Despite the morphological changes with an additional 1% CO_2 in this study, faster growth was observed under 1% CO_2 than that in air only **(Supplementary Fig.** S1**).** Low concertation of CO_2 has positively affected the alga growth based on the chlorophyll content and growth at OD_{730}. A low concentration of CO_2 can be utilized as a carbon source for the growth of *Tetratostichococcus* sp. P1.

Phylogenetic tree position of Tetratostichococcus sp. P1

The presence of introns caused a more extended sequence range than the 18S target (1,781 bp). The BLAST hits of partial 18S rDNA gene sequences of the microalga isolate indicated their phylogenetic identity with *Stichococcus bacillaris* D10-1 (99%). However, since *Stichococcus*-like microalgae have been divided into seven new lineages ((Pröschold and Darienko 2020), the phylogenetic tree was analyzed using the recent *Stichococcus*-like taxonomic revision. The phylogenetic tree shows that *Tetratostichococcus* sp. P1 was in one clade with *Stichococcus bacillaris* strains such as *Stichococcus bacillaris* D10-1, *Stichococcus bacillaris* Siva 2011 and *Stichococcus bacillaris* KMCC 169. This indicated that these strains should be renamed and moved to *Tetratostichococcus. Stichococcus*-like algae are primarily found in cold regions, such as in the Antarctic (Chen et al. 2012). There are no reports of this algal species from tropical peatland areas. Arctic *Stichococcus* UMACC could not

survive at temperatures higher than 20 °C, while *S. bacillaris* NIES-3639 Japan can grow at a maximum temperature of 23 °C (Yamamoto et al. 2016; Mutaf et al. 2019) (). Therefore, this is the first report about the isolation of a *Stichococcus*-like species from tropical peatland environments.

Genes involved under an acidic environment

The ecosystem of peatlands is extremely acidic, with a pH range of approximately 2.9–3.5 (Yule and Gomez 2009); another report mentioned a pH of 2.6–3.7 (Könönen et al. 2015). Therefore, microalgae from peatlands should have the ability to grow under low pH conditions. This study shows that the growth of *Tetratostichococcus* sp. P1 under low pH was very high compared to a previous report of *S. bacillaris*, which only reached the highest peak at OD_{682} of 2.0 in 7 days (Mutaf et al. 2019).

A draft genome was performed to understand the possibility of the strain adapting to acidic conditions through genetic information. A total of 826 unique clusters existed, and 826 clusters did not exist in *Tetratostichococcus* sp. P1 from draft genome sequence analysis when compared to the genome of the neutrophilic green alga *Chlamydomonas reinhardtii,* suggesting that *Tetratostichococcus* sp. P1 involves hundreds of different gene families with neutral microalgae. This result indicated that the unique gene might be responsible for the ability of *Tetratostichococcus* sp. P1 to survive under low pH conditions. Acidophilic green algae can maintain intracellular pH. This is supported by some genes that adapt to acidic conditions, such as plasma membrane H + -ATPase (PMA) and heat shock proteins (HSPs) (Hirooka et al. 2017). There are three essential HSPs related to pH stress: HSP60, HSP70, and HSP90. The acidophilic algae *Chlamydomonas eustigma* and *Chlamydomonas acidophilia* showed higher accumulation of HSP90, HSP70, and HSP60 at low pH, while the neutrophilic microalga *C. reinhardtii* showed higher expression of HSP60 at natural pH. It has been reported that the acidophilic green alga *C. eustigma* has four HSP90 genes, eight HSP70 genes, and three HSP60 genes in its nuclear genome (Gerloff-Elias et al. 2006; Hirooka et al. 2017). Meanwhile, *Tetratostichococcus* sp. P1 has six HSP90 genes, seven HSP70 genes and one HSP60 gene (**Table** S4). The ability of *Tetratostichococcus* sp. P1 to survive under acidic conditions is also supported by its ability to pump protons from the cytosol to the outside of the cell. This mechanism is supported by the presence of the protein PMA. PMA in *Tetratostichococcus* sp. P1 is present in two scaffolds. PMA consumes ATP to pump protons from the cytosol to outside of the cell to maintain a neutral pH inside the cell (Hirooka et al. 2017). *Tetratostichococcus* sp. P1 also lacks some enzymes involved in organic acid fermentation production pathways, such as proteins related to lactate and the format pathway (**Fig** S3.A), where those pathways were present in the neutrophilic microalga *C. reinhardtii.* Organic acid fermentation production pathways are not present in acidophilic microalgae as the mechanism of the strains to keep the neutral pH in the cytosol and survive in acidic conditions (Hirooka et al. 2017).

Genes related to fatty acid production

To confirm the related genes/enzymes for fatty acid production, KEGG analysis was performed using the KEGG Orthology (KO) number from the protein annotation of *Tetratostichococcus* sp. P1. *Tetratostichococcus* sp. P1 can convert C18:0 fatty acids to C18:2 (linoleic acid (LA)), 18:3ω3 (α-linoleic acid (ALA)) and 18:3ω6 (gamma-linolenic acid (GLA)). These begin with the presence of Δ9-desaturation, which produces 18:1 (oleic acid); oleic acid is then converted by Δ12-desaturation to LA, in which 15-desaturation then converts it to ALA or Δ6-desaturation converts it to GLA. Since the percentage of ALA was higher at a pH of 7 than at pH values of 3 and 5, it was indicated that the Δ15-desaturation metabolism was higher under high pH conditions than under low pH conditions. Δ9-desaturation and Δ12-desaturation metabolism increased at low pH, which increased the oleic acid and linoleic acid percentages at low pH. Even though fatty acid analysis in GC-FID showed that GLA was absent in the strain under BG11, KEGG pathway analysis showed the presence of GLA. This indicated that changing the pH condition did not affect the Δ6-desaturation metabolism that produces GLA. The GLA might be in trace amount or not produced under the experiment condition. In has been reported that long PUFA, such as GLA production by microalgae, was higher under the low temperature (≤ 20 °C) (Santin et al. 2021). *Tetratostichococcus* sp. P1 also showed the presence of C20 and C24. This result was supported by the elongation of very-long-chain fatty acid proteins from the draft genome (**Table** S5). The fatty acid profile of *Tetratostichococcus* sp. P1 is consistent with that of other *Stichococcus*-like algae, such as *S. bacillaris* strains NJ-10 and NJ-17 and *S. bacillaris* siva 2011, which were grown at pH values of 7.5 and 5.0, respectively (Chen et al. 2012) (Sivakumar et al. 2014). Since *Tetratostichococcus* sp. P1 produced a high percentage of C18:3, C20 and C24 were present; this result suggests that the strain has a unique fatty acid profile compared to other *Stichococcus*-like strains. In a previous study of *Stichococcus*-like microalgae, fatty acids longer than C18:0 were absent, such as C20:0 and C24:0. In addition the growth rate of *Tetratostichococcus* sp. P1 was very high ($\mu = 1.46\ d^{-1}$) under acidic conditions (pH of 3) compared to other acid-tolerant microalgae, where extremophilic microalgae mostly have a low growth rate at low pH ($\mu = 0.1691$) (Tan et al. 2020).

Some microalgae produce high amounts of C16:0, C18:1, and C18:3; these fatty acids are favorable for biodiesel

production as they have better strength to oxidation and fluidity (Pandey et al. 2019), but most microalgae have meager growth rates (Pan et al. 2011; Talebi et al. 2013). Meanwhile, *Tetratostichococcus* sp. P1 showed high amounts of those FAs together with a fast growth rate. The composition of fatty acids is crucial to determining the quality of biodiesel. Because unsaturated fatty acids increase cold flow characteristics and saturated fatty acids maintain oxidative stability, the unsaturation ratio is essential in determining the rate. Oleic acid is a desirable fuel oil component because it offers a good balance of cold flow and oxidation stability (Hoekman et al. 2012; Minhas et al. 2016).

In addition, *Tetratostichococcus* sp. P1 can utilize peat water for growth. According to Fernandes et al. (2016) nutrients are responsible for the growth rate, fatty acid elongation, and desaturation of microalgae. Selangor peat swamp forests are rich in phenolic acids such as ferulic acid and p-coumaric acid (Lim et al. 2017). In Sumatra and Borneo, Indonesia, tropical peatlands contain some organic acids, i.e., ferulic acid, synapic acid, p-coumaric acid, p-hydroxybenzoic acid, vanilic acid, and syringic acid. Ferulic acid and synapic acid had the highest concentrations (Sabiham 2010). Organic acids are needed for fatty acid biosynthesis. Organic acids will be conveyed to the citric cycle and oxidized for energy production by acetyl-CoA. Organic acids and acetyl-CoA are essential to producing energy and efficient lipid accumulation. The presence of ATP:citrate is crucial as a reductant used in fatty acid biosynthesis (FAS). A previous study showed that the addition of lactic acid significantly affects DHA production in microalgae (Ju et al. 2020). Other organic acids might also affect the production of PUFAs. *Tetratostichococcus* sp. P1 showed the presence of enzymes related to organic acid metabolisms, such as acetyl-CoA synthetase, and the presence of acetyl-CoA, which can convey the organic acid to the citrate cycle to produce energy used in fatty acid biosynthesis (**Supplemental** Fig. 3B). Therefore, peat water rich in organic acids can be used for algal media to increase the production of PUFAs and reduce the cultivation cost. In addition, supplementation of CO_2 did not significantly affect the growth rate of *Tetratostichococcus* sp. P1 in peat water indicating that the cultivation in peat water does not require additional CO_2.

To conclude, a unique microalga *Tetratostichococcus* sp. P1 from Malaysia's acidic peat swamp forest was isolated and characterized. The strain showed high growth rates under acidic and slightly alkaline pH conditions, with high C16 to C18 fatty acid accumulation. This study is the first report of the fatty acid profile of *Tetratostichococcus* sp. P1 at low pH from a tropical peatland. *Tetratostichococcus* sp. P1 can utilize peat water for cultivation. Therefore, large-scale *Tetratostichococcus* sp. P1 cultivation in open ponds under acidic conditions with peat water can be developed to prevent unwanted algae strains or other microorganisms and achieve low-cost production for the feasibility of biomass scale-up with possible fuel production in Malaysia.

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Author Contribution Eri Sahabudin: Conceptualization, Data curation, Methodology, Investigation, Writing Original draft preparation. Jinwoong Lee: Resources. Riyo Asada: Project administration, Formal analysis. Nurtasbiyah Yusof, Nurul Syazwani Ahmad Sabri, Ezzah Atikah Marsid, Hani Susanti: Data Curation, Software, Validation. Fazrena Nadia Md Akhir, Nor'azizi Othman, Zuriati Zakaria, Kengo Suzuki: Visualization, Investigation. Ali Muhammad Yuzir, Kengo Suzuki, Koji Iwamoto, Iwane Suzuki, and Hirofumi Hara: Supervisor, Conceptualization, Reviewing, Editing and Approving manuscript to be published.

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Data Availability All data generated or analyzed during this study are included in this published article (and its Supplementary Information files). The species sequence data used in this paper have been superseded by the 18S GenBank record [MT053478] (https://www.ncbi.nlm.nih.gov/nuccore/MT053478.1). The whole genome shotgun (WGS) project used in this paper was superseded by the complete genome in the (non-WGS) GenBank record [JAAIKU000000000.1] (https://www.ncbi.nlm.nih.gov/nuccore/JAAIKU000000000.1).

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