Research Article

Promising lipid source for biodiesel production from *Desmodesmus* sp. RMUO8 (Sphaeropleales: Scenedesmaceae)

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Abstract

Desmodesmus sp. RMUO8 strain was isolated from a freshwater pond. Identification was performed using physiological and molecular approaches determined by internal transcribed spacer (ITS) sequence analysis. The obtained DNA sequences were analyzed and identified by comparison with deposited sequences on NCBI. Based on BLAST searches against the GenBank database, these sequences were identical to *Desmodesmus communis* JQ922412, JX101322, JX101325 and JX101323 (99%). Gas chromatography analysis revealed that *Desmodesmus communis* RMUO8 contained unsaturated fatty acids including oleic and linoleic acid (47.03 and 16.76%), respectively, of total percent content. Thus, RMUO8 biomass could be used as a suitable feedstock for biodiesel production.

Keywords : Biodiesel; *Desmodesmus* sp., Microalgal oil and Green microalgae

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ศักยภาพของสาหร่ายสีเขียว *Desmodesmus* sp. RMUO8 (Sphaeropleales: Scenedesmaceae) สำหรับผลิตไบโอดีเซล

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สาขาวิชาชีววิทยา คณะวิทยาศาสตร์และเทคโนโลยี มหาวิทยาลัยราชภัฏมหาสารคาม อ.เมือง จ.มหาสารคาม 44000

บทคัดย่อ

Desmodesmus sp. RMUO8 ถูกคัดแยกมาจากบ่อน้ำจืด จำแนกลักษณะทางสัญฐานวิทยาและระบุเครื่องหมายยีนตาม internal transcribed spacer (ITS) นำลำดับยีนที่ได้ (DNA sequences) เก็บไว้ในฐาน NCBI จากการเปรียบเทียบกับฐานข้อมูลใน GenBank พบว่า *Desmodesmus* sp.RMUO8 มีลักษณะคล้ายกับ *Desmodesmus communis* (JQ922412, JX101322, JX101325 และ JX101323) (99%) ตามลำดับ จากการวิเคราะห์ด้วยแก๊สโครมาโทรกราฟีพบว่า *Desmodesmus* sp. RMUO8 มีกรดไขมันไม่อิ่มตัวประกอบด้วย oleic และ linoleic acid ร้อยละ 47.03 และ 16.76 ของน้ำหนักแห้งตามลำดับ ซึ่งเป็นปริมาณสูงพอ ดังนั้น *Desmodesmus* sp.RMUO8 สามารถเป็นทางเลือกหนึ่งในการนำมาผลิตไบโอดีเซล

คำสำคัญ : น้ำมันไบโอดีเซล, *Desmodesmus communis*, สาหร่ายขนาดเล็กที่น้ำมัน และสาหร่ายสีเขียว

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Introduction

Biofuel production from microalgae has been very important in recent years as an alternative fuel source (Carlos *et al*., 2014). Microalgae are exceedingly rich in lipids, which can be converted to biofuel. The use of algae for biofuel production has the great additional benefit as algae can sequester CO_2 and capture solar energy 10–50 times more efficiently than terrestrial plants (Snchez *et al*., 2003). Microalgae are the fastest growing photosynthesizing organisms, and many are exceedingly rich in oil. Approximately 46 tons of oil/ha/year can be produced from diatom algae; different algae species produce different amounts of oil, with some producing up to 50% oil by weight (Demirbas, 2009). Moreover, the yields from microalgae are approximately 10 times higher than those of land plants, so much less land is needed (Chisti, 2007). Several studies have indicated the good biodegradability of biodiesel fuels in aqueous milieu and soil, and this has also been observed in mixtures of biodiesel and diesel fuel (Pasqualino *et al*., 2006).

There have been several studies dealing with the feasibility of biodiesel production from different microalgae e.g. *Botryococcus*, *Chlorella*, *Dunaliella*, *Scenedesmus*, *Volvox* and *Chlamydo-monas* (Chisti, 2007; Sialve *et al*., 2009). Micro-algae have the potential to provide enough biodiesel to completely displace petroleum-derived transport fuels, such as gasoline, jet fuel and diesel. In a dramatic contrast with the best oil–producing crops, microalgal biodiesel has the potential for the complete displacement of petroleum–derived transport fuels without adversely affecting supplies of food and other agricultural products. It has been demonstrated that microalgal biodiesel is a better alternative than bioethanol from sugarcane, which is currently the most widely used transport biofuel (Gray, 2006).

The objective of this study was to isolate and determine the composition of fatty acid methyl esters derived from a naturally isolated strain of a *Desmodesmus communis* Moreover, the partial ITS sequences of the ITS rDNA gene of the naturally isolated *Desmodesmus communis* were sequenced to guide species identification.

Methods

1. Isolation, identification and cultivation of microalga

Microalgae were isolated using the capillary pipette method (Hoshaw and Rosowski, 1973) from a water sample collected from an artificial freshwater pond in Maha Sarakham Province, Northeastern Thailand in 2014. The *Desmodesmus communis* RMUO8 green colonies were separated and cultured in Erlenmeyer flasks containing 1 L BG–11 medium which contained 1,500 mg of $NaNO_3$, 40 mg of K_2HPO_4, 75 mg of $MgSO_4.7H_2O$, 36 mg of $CaCl_2.2H_2O$, 6 mg of citric acid, 6 mg of ferric ammonium citrate, 1 mg of EDTA-Na_2, 20 mg of Na_2CO_3, 2.86 mg of H_3BO_3, 1.86 mg of $MnCl_2$. $4H_2O$, 0.22 mg of $ZnSO_4.7H_2O$, 0.39 mg of $Na_2MoO_4.2H_2O$, 0.08 mg of $CuSO_4.5H_2O$, and 0.05 mg of $Co(NO_3)_2.6H_2O$ per liter. The initial pH value of medium was adjusted to 7.0 with 1 mol/L HCl. While being shaken at 125 rpm, 28±2°C and under 16:8 light:dark cycles with a light intensity of 33 $\mu Em^{-2}\ s^{-1}$ for 20 days. Cultures were harvested, and the dry biomass and lipid content were estimated at the station phase of growth. The strain was deposited in the Department of Biology, Faculty of

Science and Technology, Rajabhat Maha Sarakham University, Thailand.

2. DNA extraction and amplification

Genomic DNA was extracted using the Plant Genomic DNA Extraction Kit (RBC Bioscience Corp., Taiwan). Cells were ground into a powder in liquid nitrogen and DNA was extracted following the manufacturer's protocol. DNA samples were stored at –20°C. A fragment of the ITS region (internal transcribed sequence of ribosomal RNA gene) was amplified using the primers ITS1 and ITS4 (White *et al*., 1990). The PCR reaction was performed in a total volume of 50 µl containing 1x reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 2.5 µM of each primer, 0.4 units of *Taq* DNA polymerase (RBC Bioscience, Taiwan) and 2 µl of DNA sample (diluted 1:20 in ddH_2O). The temperature profile was 94°C for 2 min; followed by 36 cycles of denaturing at 94°C for 45 s, annealing at 50°C for 45 s and extension at 72°C for 1.30 min; and a final extension at 72 °C for 5 min. PCR products were checked on a 1% agarose gel containing 0.125 mg/L ethidium bromide. The PCR products were cleaned using the PCR purification kit (RBC Bioscience, Taiwan) and were sequenced using the same primers as in the PCR by the Macrogen DNA Sequencing Service (Seoul, Korea).

3. Data analysis of amplified sequence

Sequences were aligned using the Clustal W algorithm in BioEdit (Hall, 1999) followed by manual editing as appropriate. Sequences were deposited in GenBank under accession numbers KP797884. Neighbor-joining (NJ) was implemented in MEGA 6 Version 6.0.5 (Tamura *et al.*, 2013) and branch support for NJ was calculated using the bootstrapping method with 1000 replicates. For phylogenetic analyses, ITS sequences of *Neodesmus* sp. (HQ218940) in from the Genbank were used as an out group (Pan *et al*., 2011).

4. Extraction of total lipids and transesterification of fatty acids

Lipids were obtained by extracting the freeze dried algal biomass with hexane in a Soxhlet extractor (Becker, 1994). Fatty acid methyl ester (FAME), production was obtained via esterification of the extracted algal lipid using sulfuric acid as the catalyst, and the reactions were performed in 50:1 molar ratio (methanol: oil). H_2SO_4 (100% based on oil weight) was used as the catalyst at 30°C, and it was spun at 130 rpm for 4 h (Miao and Wu, 2006). The fatty acid compositions in the FAME were analyzed using a HP6850 Gas Chromatograph equipped with a cross–linked capillary FFAP column (FFAP, a reaction product of polyethylene glycol 20000 and 2–nitrophthalic acid, Crompton, 2000) length 30 m, 0.32 mm I.D., 0.25 µm film thickness and flame ionization detector. Operating conditions were as follows: inlet temperature 290°C, initial oven temperature 210°C held for 12 min then ramped to 250°C at 20°C/min, hold for 8 min and the detector temperature was 300°C. Fatty acids were identified by comparing their retention times with standards based on their respective peak areas and normalized.

Results and Discussion

The ITS sequence of the *Desmodesmus* sp. RMUO8 isolate had 99% similarity to the ITS sequences in Genbank from several *Desmodesmus communis*: JQ922412, JX101322, JX101325 and JX101323 (Table 1) and lower similarity to other *Desmodesmus* species, such as *Desmodesmus*

asymmetricus (DQ417549). These results suggest that the RMUO8 isolate belongs to *Desmodesmus communis*, or a closely related species. The multiple sequence alignments as well as the similarities between the studied sequences and conserved domains are shown with a color scale (Fig. 1). The ITS sequences of the *Desmodesmus communis* RUM08 from Thailand indicated that it was genetically distinct from morphologically closely related taxa from other geographic regions, The morphology of the *Desmodesmus communis* RUM08 included spines extending from the cell (Pan, 2011; Lurling, 2003; Vanormelingen *et al*., 2007). The *D. communis* clade was divided into two major clades. Samples from Thailand and *D. communis* from the Romania (Genbank accession number JQ922412) formed a clade with *D. communis* from the Romania (Genbank accession number JX101322, JX101325 and JX101323) (Fig. 2).

The Gas chromatography (GC) analysis revealed that the lipids from *Desmodesmus* sp. RMUO8 contained oleic, palmitic and linoleic acid fatty acids (47.03, 34.61, and 16.76%, respectively, of total fatty acids) which are comparable to conventional vegetable oil used for biodiesel production (Miao and Wu, 2006). Some fatty acids such as capric, myristic, arachidic and linolenic were found less than 1%. The fatty acids suitable for biodiesel derived from *Desmodesmus communis* RMUO8 were the unsaturated fatty acid (64.2%) (Table 2). In addition, *Desmodesmus* sp. showed high contents of oleic acid, about 47.03 % C18:1 and a very low content of capric acid. It was also reported from the previous study which found that *Desmodesmu*s spp. can synthesize lipids with the main fatty acids were palmitic, linoleic and oleic (70–80% of total fatty acids) (Pan, 2011). Also, Mata *et al.* (2010) found fatty acids from *Desmodesmus* sp. in the range C14–C22, with the major acids being palmitic, oleic and linoleic acid. Oleic acid predominates in *Desmodesmus* sp. (more than 80% of the total fatty acids) (Kaur *et al.*, 2012).

The fatty acids of microalgae isolated from lakes and freshwater ponds in Southern Thailand were mostly represented by saturated and monoenoic acids with chain lengths ranging from C12 to C22; the dominant fatty acids were palmitic and oleic acids (Yeesang and Cheirsilp, 2011). The amount of unsaturated fatty acids from the *Desmodesmus* sp. RMUO8 were higher than those found from *Chlamydomonas* sp. JSC4 which has only 46.3% of the total unsaturated fatty acids.

The fatty acid composition of the *Desmodesmus* sp. RMUO8 isolated from a freshwater pond in Northeastern Thailand is mostly unsaturated fatty acids (>50% total fatty acid). The high content of unsaturated fatty acids was shown by the low oxidative stability, but high fuel properties at low temperatures. Total percentage of unsaturated fatty acid from *Desmodesmus* sp. RMUO8 showed higher amount than those produced from crude palm oil, crude coconut oil, palm stearin, and palm olein but lower than those produced from soybean oil and sunflower oil (Nakpong and Wootthikanokkhan, 2010). In the US biodiesel was mostly produced from corn oil and soybean oil. Soybean oil contains linoleic and oleic acids at 53.7% and 23.3%, respectively (Yeesang and Cheirsilp, 2011; O'Brien, 1988). Therefore, the biodiesel from *Desmodesmus* sp.RMUO8 also a good resource to produce biodiesel as from soybean oil.

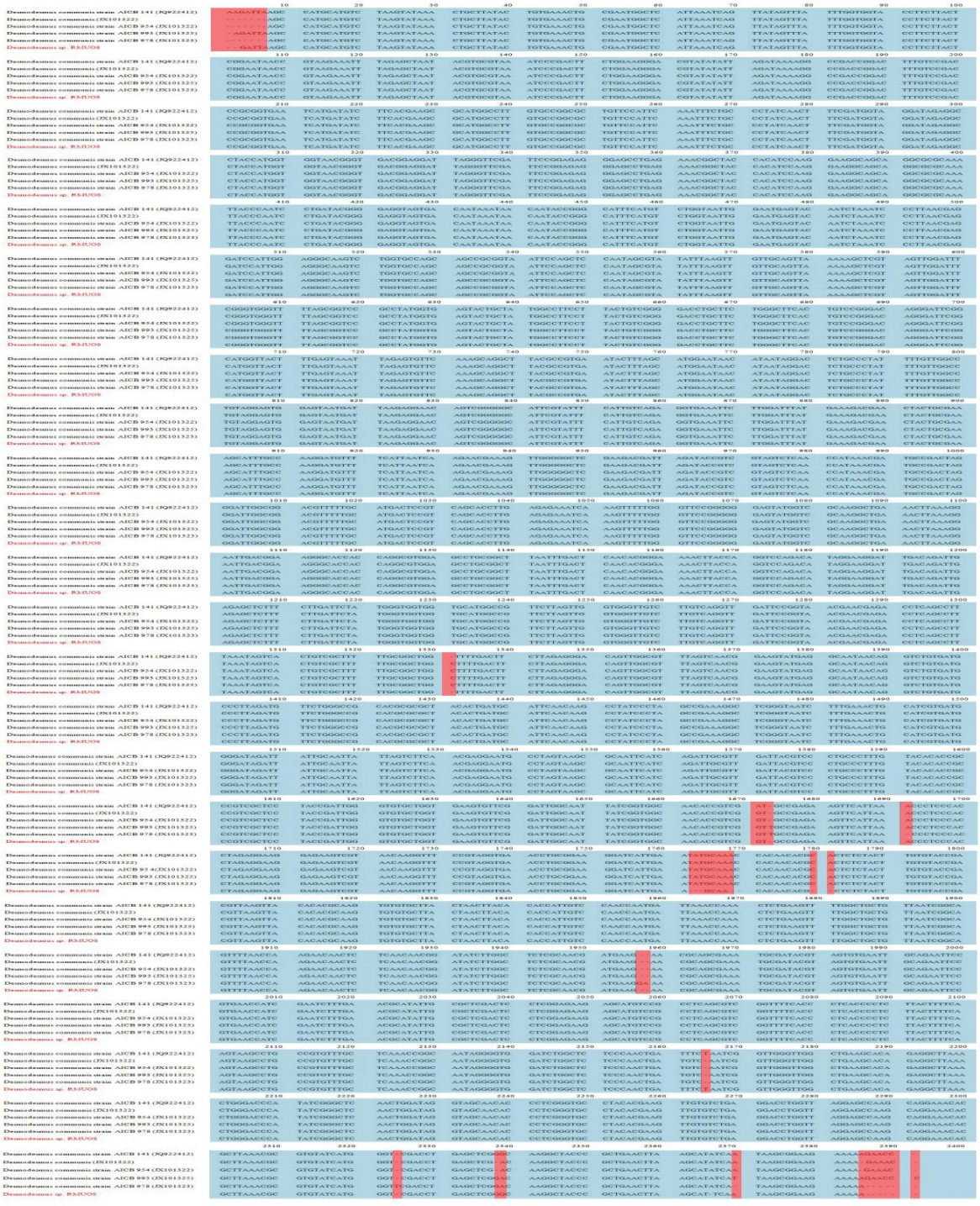


Figure. 1 Tabular format of multiple alignments from seven ITS rDNA genes of *Desmodesmus* strains from the Genbank database using the CLC sequence viewer software, version 5.1.1. Sequence names appear at the beginning of each row and the residue position is indicated by the numbers at the top of the alignment columns. The level of sequence conservation is shown on a color scale with red residues being the least conserved and blue residues being the most conserved. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

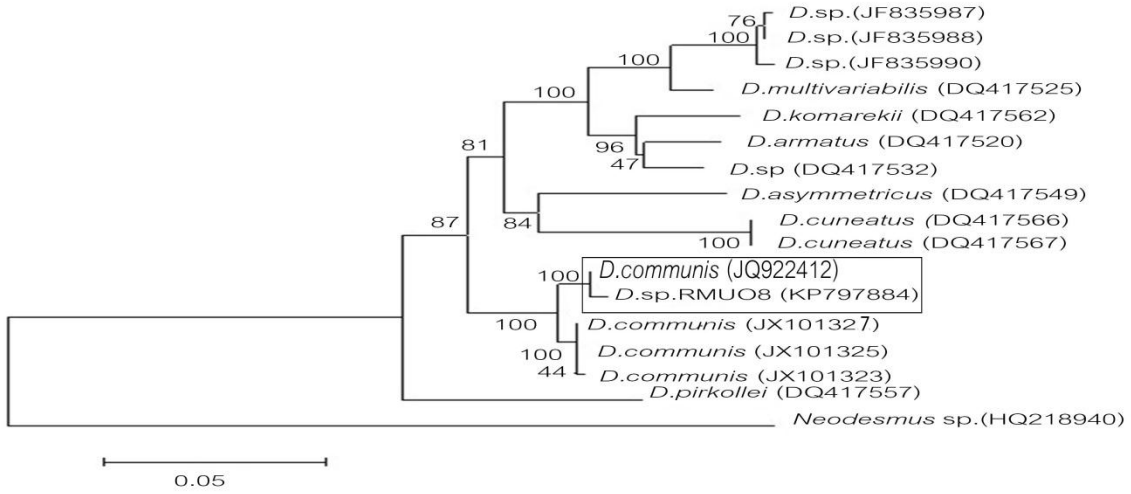


Figure. 2 Phylogenetic tree of *Desmodesmus communis* RMO8 (KP797884), and its closely related species. This tree shows that the four strains are closely related to species in the family of Scenedesmaceae. *Neodesmus* sp. (HQ218940) serves as an outgroup in this analysis. The numbers in the parenthesis are the accession numbers of the respective ITS sequences in GenBank. The scale bar measures the distance between species. Numbers at the nodes indicate bootstrap values (expressed as a percentage) with 1,000 replicates, which sequences were used to construct the Clustal W alignment.

Table 2. Fatty acid composition of lipid from *Desmodesmus* sp. RMUO8.

Fatty acid	Total (% total fatty acids)
Saturated fatty acid	
Capric acid, C8:0	0.14
Myristic acid, C14:0	0.41
Palmitic acid, C16:0	34.61
Stearic acid, C18:0	0.34
Arachidic acid, C20:0	0.29
Unsaturated fatty acid	
Oleic acid, C18:1	47.03
linoleic acid, C18:2	16.76
linolenic acid, C18:3	0.41

There have been several studies dealing with the feasibility of biodiesel production from microalgae (Chisti, 2007; Sialve *et al*., 2009; Ho *et al*., 2014) such as screening Microalgae strains for biodiesel production found that the highest lipid yields were observed for *Botryococcus braunii* and *B. terribilis* (Nascimento *et al*., 2013). The *Desmodesmus* sp. RMUO8, a fresh water green algae, unifies several properties that is an ideal candidate for biodiesel production because it easily to handle and fast growing. Genetic knowledge and mutant collection are also available thus provides the basis for this choice (Morowavat *et al*., 2010; Rupprecht, 2009). Furthermore, the advantage of using algae is that they were prior use in industrial processes

thus, experience is available to perform at larger scale (Rupprecht, 2009).

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