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Identification and Biochemical Composition of a Green Microalgae

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ABSTRACT

Microalgae are being studied aggressively lately as a potential feedstock for biofuel and other valuable compound production. In this study, marine microalgae were collected at Fisheries Department of Terengganu in Malaysia which were then being isolated, purified and identified using rDNA approach. For the molecular identification analysis, several DNA extraction methods were investigated in order to obtain high quality algal DNA. Chloroplast genomes were amplified successfully using eight sets of universal chlorophyte primers from the genomic DNA extracted. These amplified fragments were then purified and ligated into pGEM-T vector which was subsequently cloned into *E. coli* competent cell. Plasmids DNA of the selected transformed cells were extracted and prepared for sequencing. The sequences were analyzed and compared with other strains in NCBI database. Based on the chloroplast sequences, the collected strain is identified as *Parachlorella kesslari*. As for the analysis of biochemical composition of this strain, the result revealed that it composed of 30% lipid, followed by 25% carbohydrate, 15% protein and 8% starch, respectively. Forty percent of the extracted lipid was composed of PUFAs through GC-MS analysis.

Key words: Microalgae, *Parachlorella kessleri*, extraction method, fatty acid, linoleic acid, α-linolenic acid

INTRODUCTION

Microalgae, being at the base of the food chain, are contributing to the critical components of many habitats on the planet. Most importantly, they are also the major O_2 producer. Moreover, they have been ascertained as a promising and commercially importance in the food industry and aquaculture, as a natural source of high-value products such as fatty acids, carotenoids, steroids and etc. (Cardozo *et al*., 2007).

Furthermore, the whole world is recently facing with several problems such as soaring of oil prices, diminishing world oil reserves and the environmental deterioration affiliated with fossil fuel consumption. All these have risen and renewed the interest in using microalgae as an alternative and renewable feedstock for fuel production (Saha *et al*., 2003). However, before this concept can become a commercial reality, many fundamental biological questions relating to the lipid biosynthesis and regulation in microalgae need to be resolved. Deeper understanding of the biological fundamental on microalgae lipid biosynthesis and regulation are essential for the algae-based biofuel production to be an economically viable business.

Cultivation of microalgae as a research tool is expanding rapidly worldwide and is attracting some major companies to explore into it. There are few chemicals that has been commercialized and successfully isolated from microalgae such as β-carotene (Olmos-Soto *et al*., 2002).

Many studies claimed that a number of green microalgae strains has high potential to growth in mass culture and can accumulate large quantities of lipids under nutrient stress. Some species of green microalgae, such as *Chlorella* sp., has rapid growth rate and is able to be cultured on a large scale. All these benefits which include cost efficiency, easy cultivation, have similar glycosylation patterns with higher plants and biologically safe make it one of the potential candidate of the biofuel sources (Wang *et al*., 2005).

Abundance of algae resources available in Malaysia including freshwater and marine algae lead to intensive and wider studies to be done. The identification of microorganisms has been long performed using taxonomic characteristics. However, traditional methods that base on morphological examinations using light and scanning electron microscopy are not only time consuming but also require taxonomic expertise Taxonomic characteristics that based on morphological examinations has been frequently used for microorganism identification. Those methods are not only time consuming but also require a professional taxonomist to distinguish among closely related species. This causes many of the strains in the culture collections are misnamed and lead to confusions and difficulty in making comparison of the results. Thus, the development of modern biotechnological tools, such as PCR and analysis of rDNA/rRNA based technologies make identifying small number of microorganisms in complex populations easier and more precise (Olmos *et al*., 2000).

The objective of this study was to isolate and identify the local available strain. Therefore, several isolation and purification methods, followed by molecular tools to identify one of the marine green microalgae strains were being studied. Besides that, gas chromatography-mass spectrometry (GC/MS) was used to identify and quantify the fatty acids of the identified strain.

MATERIALS AND METHODS

This study was carried out during the period of Dec. 2009 and Dec. 2010 at Sime Darby Technology Centre, Malaysia.

Isolation and purification: Water samples as an isolating source of microalgae were collected from Fisheries Department of Terengganu. A few isolation and purification techniques (Robert, 2005) were used to isolate and purify the strain. The mix cultures contained of different types of microorganism were diluted in 1/10 dilution series with sterile F/2+Si medium (UTEX) prepared with ASW (Artificial sea water, SUE) and 0.1 mL of appropriate dilutions was spread on F/2+Si agar plates. The agar plates were incubated at ambient temperature for a period of time. The individual colonies were isolated and inoculated in both liquid and agar slants of F/2+Si medium, incubated at ambient temperature under Fluorescence light with 12:12 h light dark cycle. The axenic of the culture was ensured by the filtration technique and antibiotic treatment. The antibiotic mixture yielded 100/25/25 mg L^{-1} of ampicillin, streptomycin and gentamycin. One of the isolates was proceed with the subsequent analysis on identification based on rDNA approach along with fatty acid profiling and biochemical analysis.

DNA extraction method: The algal was harvested during exponential growth in sterile 50 mL centrifuge tubes. Cells were collected by centrifugation at 10,000 rpm for 5 min and the pellets were stored at -80°C until further analysis. Total genomic DNA was extracted using different methods. A commercial available kit (QlAamp DNA micro kit, Qiagen) and a method based on hexadecyl-trimethyl-ammonium bromide (CTAB) (Hilario and Mackay, 2007) with and without sonication were compared.

Extraction with the QlAmamp DNA micro kit was performed according to the manufacturer's instructions. For sonication step, in brief, cell culture was centrifuged at 5000 g for 5 min and washed twice with sterile distilled water. This followed by adding in 500 μL of the extraction buffer and 10 μL of lysozyme. The mixture was sonicated using a probe sonicator (Misonix) for 90 sec at 10 Amplitude, 15 sec pulse on and 15 sec pulse off. The sample was cooled on ice for 1 min and the subsequently steps of the commercial kit and CTAB method were performed. The genomic DNA collected would be used as a template in downstream PCR analysis.

Phylogenetic analysis of PCR-amplified SSU rDNA: Polymerace Chain Reaction (PCR) amplification of SSU rDNA of this strain was performed using 8 sets of universal primers for Chlorophyta which reported by Provan *et al.* (2004). PCR was performed in 25 μL reactions volumes and the master mix was followed as described by Provan. Amplification was performed using an C1000 Thermal Cycler (BioRad) as follows: initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 48-56°C (different temperature in each set of universal primers) for 1 min. Extension at 72°C for 1 min and a final extension at 72°C for 5 min. PCR products were later resolved on 1.5% agarose gels at 95V for 25 min and visualized by ethidium bromide staining.

The resulting products were ligated into pGEM-T Easy vector (Promega) and transformed into competent JM 109 *E. coli* cells. After extraction and purification of plasmids using AccuPrep Plasmid Mini Extraction Kit (Viogene), Nucleotide sequences were blast through the NCBI database. Alignment of the sequence was performed.

Biochemical analysis

Carbohydrate content: The carbohydrate content was analyzed based on the procedure published by Miao and Wu (2004) but with some modification. The freeze-dried algal sample was hydrolyzed with 2.5 M HCl at 105°C for 30 min. This followed by the neutralization using 1.25 M NaOH and 1.25% (w/v) KH_2PO_4. The neutralized algae would be subjected to 3,5-dinitrosalicylic acid (DNS) assay (Miao *et al.*, 2003).

Protein content: The protein content of the cells was determined by the dye-binding method after extraction with 1 M NaOH at 90°C for 1 h (Bradford, 1976).

Starch content: A known amount of freeze-dried algal was transferred to a tube containing 1.5 mL of 80% ethanol and the mixture was sonicated with an probe sonicator (Misonix) for 10 min to dissolve the soluble sugars. Then the mixture would be centrifuged at 13,000 rpm for 10 min at 4°C, the supernatant would be removed and the pellet would be air-dried for several minutes. One mL of water, 50 μL of 2 M sodium acetate solution (pH 5.0) and 50 μL of 10-fold heat resistant amylase (A7505, Sigma) were added and a brief vortex was introduced. This followed by incubating the mixture at 80°C for 30 min. The supernatant after centrifugation was subjected to DNS assay (Jeong *et al.*, 2010).

Lipid content: The lipids were extracted from the biomass by using a modified version of the procedure described by Bligh and Dyer (1959). Briefly, the freeze-dried algal were blended with chloroform/methanol (1:1) for 24 h. Followed by adding in water and the solvent phase was recovered by centrifugation. The solvent was then dried under nitrogen stream. The extract was kept at -80°C for subsequently fatty acid analysis.

Fatty acid: Fatty acid methyl esters (FAMEs) were prepared dissolving the dried extracts in 100 μL chloroform and 300 μL methanol containing 1.25 M HCl and then incubated at 50°C for 24 h. Samples were then dried under a stream of nitrogen for 2 h. Dried transmethylated samples were resuspended in 70 μL pyridine and derivatized in 30 μL N-methyl-N-trifluoroacetamide (MSTFA) with 1% Trichloromethylsilane (TMCS). It would be incubated at 50°C for 2 h. FAME later would be analyzed by using an Agilent 5973 series high-temperature gas chromatography-mass spectrometer (GC-MS), fitted with an auto injector. The main focus of using it was exclusively for lipid identification. For GC-MS analysis, a capillary column (DB-5 ms, 30 m×0.25 mm×0.25 μm film thickness) (J and W Scientific) was used. The injector and detector temperatures were set at 280°C while the initial column temperature was set at 80°C. A 1 μL sample volume was injected into the column and ran using a 100:1 split ratio. After 3 min, the oven temperature was raised to 315°C at a ramp rate of 5°C min^{-1} and finally maintained at this temperature for 12 min. The helium carrier gas was programmed to maintain a constant flow rate of 2 mL min^{-1} and the mass spectra were acquired and processed using both Agilent ChemStation (Agilent, USA) and AMDIS32 software.

RESULTS AND DISCUSSION

DNA extraction: From the studies, several methods were investigated in order to obtain high quality of genomic DNA from this microalgae strain. High quality and purity DNA with approximately 150 ng μL^{-1} was obtained using sonication incorporated into CTAB method. The results of gel visualization clearly showed bright bands of successfully extracted genomic DNA (Fig. 1).

The results proved that the sonication step does assist in the extraction by increased the degree of lysis on cell wall and cell membrane. However, there is no significantly different on the results when the Qiagen kit with and without sonication were used. The use of commercialized kit did significantly shorten the duration of the DNA isolation procedure even though it didn't appear to

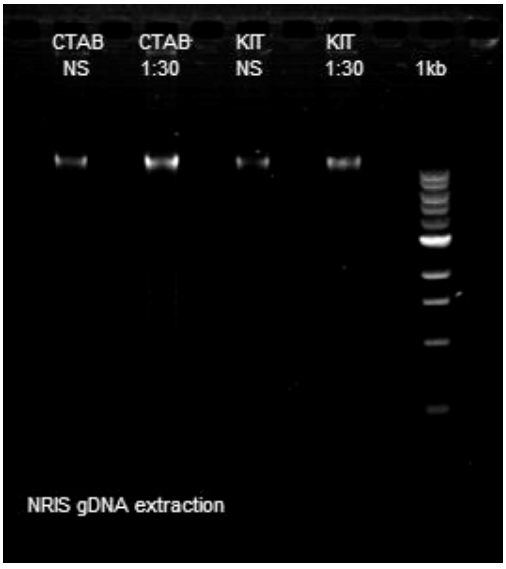

Fig. 1: gDNA extracted from the strain using CTAB and commercial kit without and with sonication process

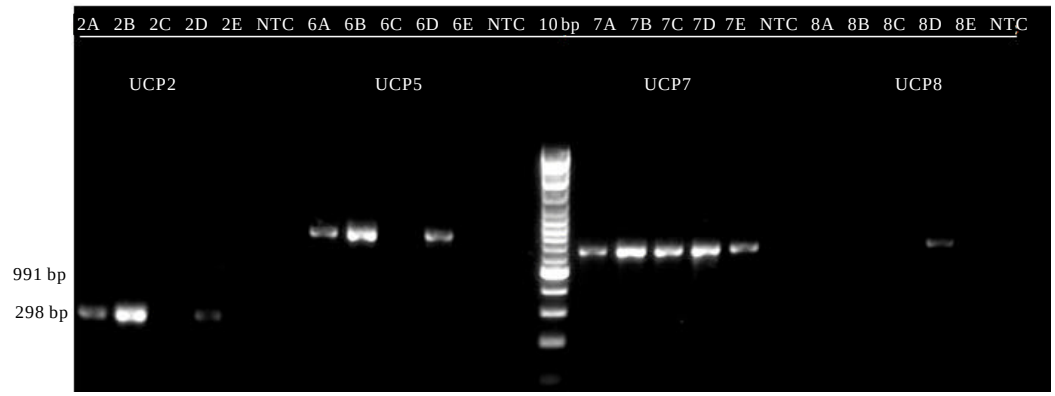

Fig. 2a: Amplification with universal chlorophyte primers (UCP2, UCP6, UCP7, UCP8). Lane 12 molecular weight marker. Lanes 1-5 DNA amplified with UCP2. Lanes 7-11 DNA amplified with UCP6. Lanes 13-17 DNA amplified with UCP7. Lanes 18-22 DNA amplified with UCP8. NTC is non template control

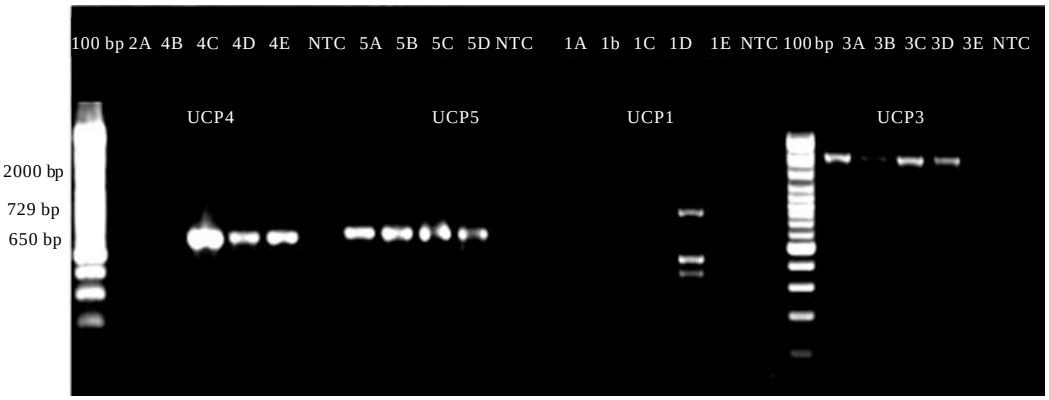

Fig. 2b: Amplification with universal chlorophyte primers (UCP4, UCP5, UCP1, UCP3). Lane 1 and 19 molecular weight marker. Lanes 2-6 DNA amplified with UCP4. Lanes 8-12 DNA amplified with UCP5. Lanes 13-17 DNA amplified with UCP1. Lanes 20-25 DNA amplified with UCP8. NTC is non template control

be the best extraction method. Furthermore, by using commercial kit, extensive purification steps could be avoided (Borneman *et al*., 1996; Volossiouk *et al*., 1995). The CTAB method alone appeared to be the least efficient methods to extract the genomic DNA from the strain. This could be due to this microorganism has relatively thick cell walls which contributes to its rigidness and making it difficult to break the cell walls. Hence, by incorporating the sonication step did provide the solution for this problem.

Amplification of chloroplast genomes from DNA: Identification of the strain was made on the chloroplast genome fragments. 8 sets of Universal Chlorophyte Primers (UCP) obtained from Provan *et al*. (2004) were used to amplify the chloroplast fragments in a phylogenetic study. This set of primers was reported to be useful in wide range of algal species. Amplification with these primers resulted in 8 different sizes of PCR products which amplified the ~5kb *rps* 11 to *rpl* 12 gene cluster conserved in green algae chloroplast genome (Fig. 2a, b).

Table 1: Biochemical composition of *Parachlorella kessleri*

Component	Carbohydrate	Protein	Lipid	Starch
Percentage	25±0.25	15±0.12	30±0.2	8±0.05

GTGGCAGAATAACTTATCGCCTTCGAACCGTAAAATNTTNATAAATATTT
TTGATAAAAAATTATGAAAGTTCGCCCTTCAGTTCGAAAAATGTGTGATA
AATGTCGATTAATTCGACGTAACAGGAACTTTACGTGTTATTCTGTCAAA
ATCCAAAACATAAACAAAGTCAAGGAATCACA-86% similarities with *Parachlorella kesslari*, chloroplast complete genome.

Fig. 3: UCP amplified sequences of the strain

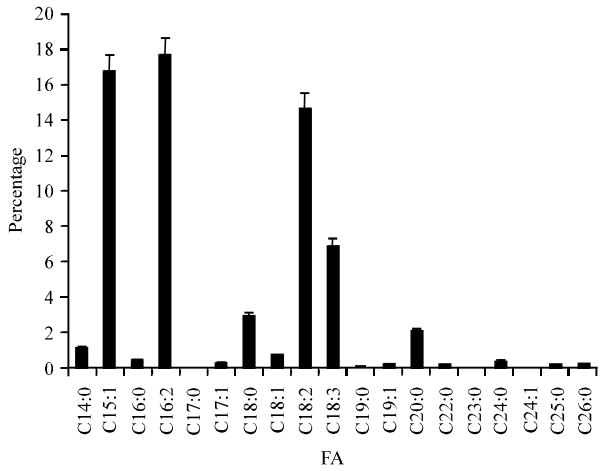

Fig. 4: Type of fatty acid in *Parachlorella kessleri*

All 8 amplified fragments were then were sequenced and analyzed against NCBI database. In comparison, it was clearly observed that the sequences have a high percentage similarity which was approximately 86% with *Parachlorella kessleri* culture-collection SAG: 211-11 g chloroplast, complete genome (Accession No: FJ968741.1) (Fig. 3). Thus based on the analysis, it can be concluded that the isolated strain is identified as *Parachlorella kesslari*.

Biochemical composition: The biochemical composition of this strain which grown in F/2+Si medium under autotrophic condition was shown in Table 1.

Approximately of 30% was lipid, followed by carbohydrate which was 25%. Protein only showed to be 15% and starch was 8%, respectively. From the result, it showed that lipid content was apparently higher and the amount is reasonably high in the strain that could make it as a potential biofuel producer.

Fatty acid profile: The predominant fatty acid of *Parachlorella kessleri* was 16:2 (Fig. 4) which was approximately 18%. This strain also contains of relatively high amount of C18:2 (linolenic acid, also known as LA) and C18:3 (α-linolenic acid, also known as ALA) which are approximately 15 and 7%, respectively.

Both of these are essential fatty acids. They are required by the human body due to the fact that humans lack of the ability in introducing double bonds in fatty acids beyond carbons 9 and 10, as counted from the carboxylic acid side. ALA is the precursor for other long-chain n-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which is normally, can be obtained from fish.

However, the safety issues have been raised because of the possible accumulation of toxins in fish (Apt and Behrens, 1999). Moreover, fish oil is not suitable due to the presence of mixed fatty acid for certain application (Medina *et al*., 1998). It is believed that the PUFAs that found in fish are originating from microalgae that consumed in oceanic environment. Therefore, it is logical to consider microalgae as potential sources of PUFAs (Jiang *et al*., 1999).

CONCLUSION

The isolated strain was successfully identified as *Parachlorella kessleri* and is found to be a potential candidate for biofuel or efficiency fatty acid producer since its lipid content and the respectively FA is quite high. The economic feasibility of algal mass culture for biofuel or other product production is greatly depends on the high biomass productivity and appreciable lipid yields. Hence, further investigation need to be carried out on this strain in order to produce the products that would be in the way of more diversified and economically competitive.

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