Jurnal Teknologi

Full Paper

ISOLATION AND IDENTIFICATION OF MICROALGAE FROM HIGH NITRATE LANDFILL LEACHATE

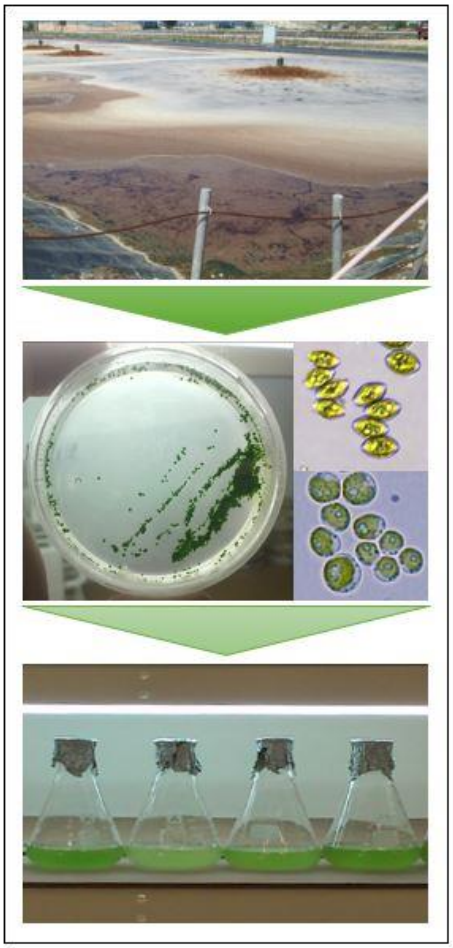
Norazela Nordin, Syakirah Samsudin*, Norjan Yusof

Department of Biology, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris, 35900, Tanjong Malim, Perak, Malaysia

Article history
Received
31 January 2019
Received in revised form
31 May 2019
Accepted
17 June 2019
Published online
26 August 2019

*Corresponding author
syakirah@fsmt.upsi.edu.my

Graphical abstract



Abstract

This study aims to isolate and identify microalgae capable of growing in high nitrate ($N\text{-}NO_3^-$) landfill leachate. Source of isolation was collected from a landfill leachate treatment plant and identified according to morphological characteristics and analysis of partial 18S and 28S rRNA genes. The isolates, identified as *Chlorella vulgaris* UPSI-JRM01 and *Tetradesmus obliquus* UPSI-JRM02 were capable of growing at high $N\text{-}NO_3^-$ concentration of nitrified landfill leachate, which is up to 1500 mg/L. The biomass productivities of *Chlorella vulgaris* and *Tetradesmus obliquus* were 36.28 mg/L/day and 40.49 mg/L/day, with 44% and 37% $N\text{-}NO_3^-$ removal, respectively. The biomass of *Chlorella vulgaris* and *Tetradesmus obliquus* consisted of 17.72% and 16.32% lipid and, 21.93% and 25.43% carbohydrate, respectively. The protein contents (>50%) were higher than lipid and carbohydrate contents for both microalgal species. The newly isolated microalgae species will be useful for future applications of high NO_3^- wastewater treatment and microalgae biomass production.

Keywords: Microalgae, *Chlorella vulgaris*, *Tetradesmus obliquus,* Microalgae Biomass

Abstrak

Kajian ini bertujuan untuk memencilkan dan mengenalpasti mikroalga yang mampu hidup di dalam air larut resap tapak pelupusan sampah yang berkepekatan nitrat ($N\text{-}NO_3$) tinggi. Sumber pemencilan diperolehi dari loji rawatan air larut resap dan dikenal pasti melalui ciri-ciri morfologi dan analisis gen separa 18S dan 28S rRNA. Isolat mikroalga yang telah dikenalpasti sebagai *Chlorella vulgaris* UPSI-JRM01 dan *Tetradesmus obliquus* UPSI-JRM02 didapati mampu berkembang biak di dalam air larut resap ternitrifikasi yang berkepekatan $N\text{-}NO_3^-$ tinggi sehingga mencapai 1500 mg/L. Produktiviti biojisim *Chlorella vulgaris* dan *Tetradesmus obliquus* adalah 36.28 mg/L/hari dan 40.49 mg/L/hari, dengan 44% dan 37% penyingkiran $N\text{-}NO_3^-$, masing-masing. Biojisim *Chlorella vulgaris* dan *Tetradesmus obliquus* mengandungi 17.72% dan 16.32% lipid dan, 21.93% dan 25.43% karbohidrat, masing-masing. Kandungan protein yang diperolehi adalah lebih tinggi (> 50%) daripada kandungan lipid dan karbohidrat untuk kedua-dua spesies mikroalga. Isolat mikroalga yang baru dipencilkan ini berguna untuk penggunaan di masa hadapan untuk rawatan air kumbahan $N\text{-}NO_3^-$ tinggi dan penghasilan biojisim mikroalga.

Kata kunci: Mikroalga, *Chlorella vulgaris*, *Tetradesmus obliquus,* biojisim mikroalga

1.0 INTRODUCTION

Removing ammonium (NH_4^+) is principally important in the tertiary treatment of landfill leachate in order to comply with the imposed discharge limit (10 mg/L N-NH_4^+) and to mitigate the toxic effects of ammonia (NH_3) on aquatic life. The biological treatment of landfill leachate using the nitrifying activated sludge system (NASS) has proven to be efficient for the bioconversion of high concentrations of NH_4^+ to NO_3^- [1]. Usually, the nitrification is followed by the denitrification process in which NO_3^- is reduced to nitrite (NO_2^-), nitric oxide (NO) nitrous oxide (N_2O), and finally to nitrogen gas (N_2) with the presence of carbon sources as electron donor. Unfortunately, the elimination of NO_3^- from mature landfill leachate through this process is challenging due to the lack of biodegradable carbon sources. Therefore, an alternative to denitrification is necessary for the removal of high NO_3^- levels from landfill leachate after the nitrification process.

The high level of NO_3^- in nitrified landfill leachate requires the development of a simple, efficient, and cost-effective nitrogen removal technique. Although a NO_3^- parameter has not yet been included in the landfill leachate discharge standards, its elimination is crucial as some river waters have already been contaminated with NO_3^- from mature landfill leachate [2]. The high concentration of NO_3^- in nitrified landfill leachate provides an opportunity for the production of value-added products. This NO_3^- can be assimilated by microalgae for the production of biomolecules such as lipid, carbohydrate, and protein. The microalgae biomass produced has various applications including bio-fertilizers, animal feeds, and feedstock for biofuels such as biodiesel, bioethanol, biogas, and biohydrogen via thermochemical and biochemical conversion processes [3].

Other than landfill leachate, high nitrate wastewaters (>1000 mg/L N-NO_3^-) were also generated from agro-industrial waste, effluent of explosives, fertilizers, and metal finishing industries [4]. To date, the utilization of microalgae in the wastewater treatment has been widely reported [5] and a number of microalgae strains have been previously isolated, preserved, and stored. However, not all strains of microalgae are able to grow in wastewater with high concentrations of NO_3^-.

Therefore, local microalgae strains isolated from wastewater would be the best candidates for both landfill leachate treatment and biomass production since they are more likely to adapt to local temperature, weather, light regimes, and more importantly, extreme environments such as waters with high concentrations of NO_3^- and other pollutants. This study describes the isolation and identification of microalgae obtained from a landfill leachate treatment plant in order to assess their potentials for the treatment of high NO_3^- in nitrified landfill leachate and for the production of biomass.

2.0 METHODOLOGY

2.1 Microalgae Collection

The microalgae samples were collected from a sequencing batch reactor at Jeram sanitary landfill leachate treatment plant, Selangor, Malaysia. The landfill leachate is considered mature with N-NH_4^+, COD and pH levels of 520 mg/L, 1530 mg/L and 7.8, respectively. Microalgae were collected from approximately 0 – 1.5 meters from the water surface, and half-filled in 500-mL sterile HDPE bottles to ensure sufficient oxygen concentration for microalgae growth. The epipsammic and epipelic microalgae from soft substrate were collected using sterile petri dishes that were held upside down and pressed lightly onto the substrate.

2.2 Culture Media, Microalgae Screening and Isolation

The collected microalgae samples were screened and selected based on highest specific growth rate obtained and potential to survive in nitrified landfill leachate with concentrations of N-NO_3^- up to 3000 mg/L. The microalgae samples were streaked on agar plates containing two growth media with the addition 1% agar; BG-11 plus (250 mg/L N-NO_3^-) and synthetic nitrified landfill leachate medium (3000 mg/L N-NO_3^-). The synthetic nitrified landfill leachate medium was prepared following the composition of synthetic leachate by Yusof *et al*. [2], except the total N-NH_4^+ was replaced by N-NO_3^- in the form of KNO_3 to resemble NO_3^- composition in nitrified landfill leachate. The composition of landfill leachate was based on one year monthly leachate characteristic determined from a selected landfill in Malaysia [2]. During screening, 15 microalgae isolates were obtained. However only 2 strains showed higher specific growth (>0.4/day) and were further investigated. The microalgae strains obtained were then purified through a serial dilution process, followed by a streak plating technique [6]. Purity of the culture was established by repeated streaking and routine microscopic examinations.

2.3 Microalgae Culturing

The isolated microalgae were inoculated and pre-cultured in an Erlenmeyer flask containing liquid BG-11 medium for maintaining their growth and establishing a stock culture collection. The flasks were placed on a culture rack and light was provided by using a cool, white fluorescent lamp with the light intensity adjusted to 2000-3000 lux and 12:12 light: dark cycles. The pH level of the cultures was maintained between 7 and 7.5 while the temperature of the culture room was monitored in the range of 25 °C to 30 °C [7]. The cultures were aerated by an aquarium pump filtered by a 0.2-µm syringe filter. The microalgae cells were harvested on day 7 (exponential phase) by centrifugation (4000 rpm x 5 min) and transferred to 1

L Erlenmeyer flask containing 750 mL synthetic nitrified landfill leachate (1500 mg/L $N\text{-}NO_3^-$) with initial microalgae biomass concentration about ~20 mg/L (dry cell weight). In addition, the BG-11 medium was used as a positive control [8]. The growth of microalgae strains in $N\text{-}NO_3^-$ concentration of 250 mg/L (BG-11) and in 1500 mg/L $N\text{-}NO_3^-$ concentration (nitrified landfill leachate) was compared. The isolated microalgae were cultured for 30 days under the same conditions as the pre-culture except that the pH of the culture was maintained at 8.45 (pH of actual leachate) by the addition of NaOH and HCl. Microalgae growth and $N\text{-}NO_3^-$ removal were examined every two days. The experiments were performed for three replications with at least three analytical repetitions of measurements.

2.4 Measurement of Microalgae Growth

Cell densities were measured by spectrophotometry at 680 nm (OD_{680}) using a Visible Spectrophotometer (Secomam Prim Visible Light Spectrophotometer, France). Proper dilution of samples yielding optical density readings greater than 1.0 was carried out to ensure an absorbance in the range of 0.1-1.0. The growth curves of the microalgae in BG-11 plus $N\text{-}NO_3^-$ were plotted from the ln OD_{680} readings of the samples. The dry cell weight (DCW, mg/L) was measured according to the method as described by Taher *et al.*, 2014 [9]. Specifically, 20 mL of cultures was harvested by centrifugation at 5000 rpm for 5 min and each pellet was washed at least twice with distilled water. The washed samples were filtered using a pre-weighed Whatman filter paper (Germany). The DCW was determined gravimetrically after constant weight was achieved after drying at 105 °C.

The relationship between optical density and the microalgae's DCW was established by linear regression using SPSS Ver. 16. The value *y* (dependent variable) referred to the DCW (mg/L) and the value x (independent variable) signified the value of optical density (OD_{680}). Accordingly, the optical density can be used to precisely calculate the biomass concentration. Equations 1 and 2 show the regression equations generated by linear regression between DCW (mgL^{-1}) and optical density (OD_{680}) for these microalgae isolates.

$$\text{DCW (Isolate 1)} = 138.214 \times OD_{680} - 0.479 \quad (R^2 = 0.997) \tag{1}$$

$$\text{DCW (Isolate 2)} = 179.382 \times OD_{680} - 1.992 \quad (R^2 = 0.994) \tag{2}$$

The specific growth rate (μ, /day) was determined at the exponential phase as in Eq. 1, where N_1 and N_2 are defined as DCW at time 1 (t_1) and time 2 (t_2).

$$\mu = \text{Ln}(N_1/N_2) / (t_2 - t_1) \tag{3}$$

The biomass productivity (BP, $mgL^{-1}day^{-1}$) was calculated according to Equation 4 where DCW_1 and DCW_2 represent the biomass density at the time, T (days) during exponential growth phase [10].

$$BP = (DCW_2 - DCW_1) / T \tag{4}$$

2.5 Chemical Analyses

Chemical analyses were performed at the initial and the end of 30 days culturing period. $N\text{-}NO_3^-$ concentration was analysed using Dionex ICS-1100 Ion Chromatography (Dionex ICS-1100, USA) and the 7-anion standard II (Dionex P/N 057590, USA) as a standard. The total carbohydrate content was determined using a modified phenol-sulphuric acid method [11]. Meanwhile, the total protein analysis was performed using Pierce BCA Protein Assay Kit (ThermoScientific, USA). The carbohydrate and protein contents present were determined using the standard graph prepared using D-glucose and bovine serum albumin, respectively. Furthermore, the total lipid was analysed gravimetrically using a modified Bligh & Dyer method [12]. The total carbohydrate, protein, and lipid content (%) were expressed as % dry cell weight (weight/dry weight biomass).

2.6 Microscopic Examination and Preliminary Microalgae Species Identification

The cell morphology and colony characteristics of microalgae were observed under an inverted microscope (Nikon Eclipse TE 2000-U, UK). The microalgae cultures were preliminarily identified by using the Key for Identification of Freshwater Algae Common in Water Supplies and Polluted Water 10900 C. [13]. The cell diameters and areas were measured using NIS-Element Software.

2.7 DNA Extraction

DNA of fresh microalgae cells from the liquid culture were used for DNA extraction using Qiagen DNeasy Plant Mini Kit (Germany) following the manufacturer's instructions [14]. The quality and size of the extracted DNA were checked by electrophoresis using Bio-Rad electrophoresis cells (USA) with Bio-Rad PowerPac Basic power supplies (USA). The DNA bands were visualized under a UV lamp using a Bio-Rad Gel Doc EZ Imager (USA) [15].

2.8 Polymerase Chain Reaction (PCR) Amplification

18S rRNA and 28S rRNA gene-based molecular species identification was performed using the protocols and primers as listed in Table 1 [16, 17]. Polymerase chain reaction (PCR) was performed on the whole genomic DNA extracts using the primers and KAPA Taq ReadyMix PCR kit (US) according to the manufacturer's instructions. Amplifications were performed in a Bio-Rad MyCycler Thermal Cycler (USA) starting with a hot start for 5 min at 95°C and followed by 35 cycles of denaturation for 1 min at 9 °C, annealing for 1 min at 49.8°C (18S rRNA)/ 48.6°C

(28S rRNA), and polymerization for 1 min at 72 °C. Another 10 min of extension step was added at 72°C.

2.9 PCR Purification and Sequencing

The PCR products were purified with the Qiagen Qia-quick Purification Kit (Germany) following the manufacturer's instructions and viewed by electrophoresis before being sent to First Base Laboratories Sdn. Bhd., Malaysia for sequencing using the selected primers. The sequences were compared with the sequences in GenBank using standard nucleotide BLAST to determine the genus and species of the organisms. Aligned sequences were checked manually and were edited with BioEdit Sequence Alignment Editor (Version 7.1.3.0) and CLC Sequence Viewer (Version 6.7.1). Evolutionary analyses were conducted in MEGA7 software by using the Neighbour-Joining method.

Table 1 18S rRNA and 28S rRNA primers used for microalgae species identification using PCR

Target Gene	Forward Primer	Reverse Primer	Ampli-fication	Ref.
18S rRNA	Universal eukaryotic primers: 5'-GTC AGA GGT GAA ATT CTT GGA TTT A-3'	Universal eukaryotic primers: 5'-AGG GCA GGG ACG TAA TCA ACG-3'	~700 bp	[16]
28S rRNA	DIR: 5'-CCT TGG TCC GTG TTT CAA GA-3'	D2C: 5'-ACC CGT GAT TTT AAG CAT A-3'	~700 bp	[17]

3.0 RESULTS AND DISCUSSION

3.1 Microalgae Screening and Isolation

In this study, only microalgae strains that grew in the synthetic nitrified landfill leachate were purified and cultured axenically. Two microalgae isolates that survived in a high N-NO_3^- nitrified landfill leachate were selected amongst other microalgae strains collected, each having maximum specific growth rates (μ) of 0.67 day^{-1} and 0.40 day^{-1}, respectively.

Figure 1 shows the morphological characteristics of the microalgae isolates. Isolate 1 was a unicellular, green, spherical alga, approximately 3.2 μm to 8.5 μm in diameter. The isolate occurred as solitary cells without flagella and was free floating. The isolate also contained green photosynthetic pigments (chlorophyll a and b) and had an asexual reproductive cycle. From microscopic observation, the isolate reproduced through the production of autospores from the mature large cells that divided into smaller units. A mature cell typically divided into four new cells. Based on APHA's Key for Identification of Freshwater Algae Common in Water Supplies and Polluted Waters 10900 C [13], the observed characteristics of this isolate suggest that it is classified under Chlorellaceae family. However, the coccoid microalgae with simple morphologies cannot be easily identified up to genus or species levels using identification keys.

Meanwhile, Isolate 2 occurred as either single ellipsoidal cells or as green colonies or coenobia, containing 4 cells arranged in a row. Coenobia consisted of four cells that are formed inside the mother cell wall and released to form either a new cell colony or dispersed as unicells. The cell area ranged from 29 μm^2 to 120 μm^2 with the cell length ranging from 6 μm to 13 μm. The cells were nonmotile and had a smooth cell wall. The organelles were visible and included in each cell a single nucleus, a single parietal chloroplast and a single pyrenoid. The isolate reproduced by nonmotile autospores. The observed morphological characteristics of the isolate suggest that it is classified as *Scenedesmus*.

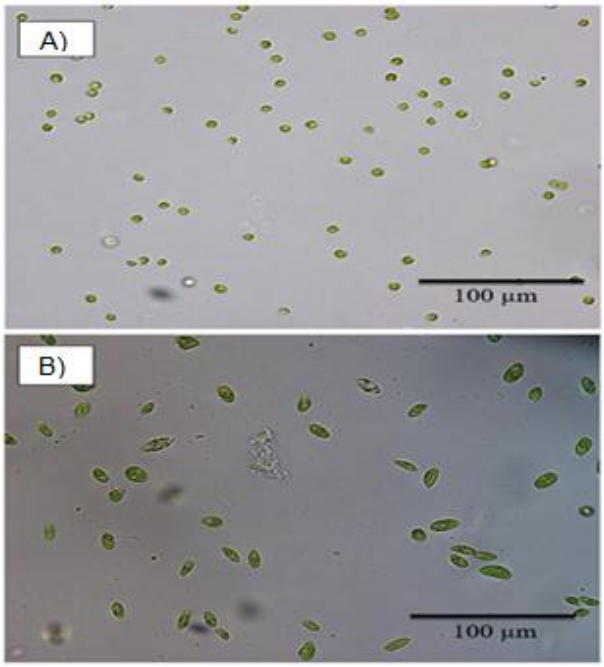


Figure 1 Micrograph of microalgae under 400x magnification. Isolate 1 (A) and Isolate 2 (B)

3.2 Molecular Identification of the Microalgae

Microalgae DNA were successfully extracted, and yielded single, clear bands without smearing upon electrophoresis. The size of each extracted DNA was more than 10000 bp. The 18S and 28S rRNA gene fragments of the selected microalgae species were successfully amplified, yielding PCR products that were ~700 bp for both 18S rRNA and 28S rRNA genes. The 18S and 28S rRNA gene sequences of both microalgae isolates were compared to the complete GenBank nucleotide database. Table 2 shows the maximum identity results obtained from BLAST.

The gene sequence for the 18S rRNA of the small subunit of the eukaryotic ribosome is usually used to determine the phylogeny of eukaryotic organisms including eukaryotic microalgae [18]. However, in this study, the partial 18S rRNA gene sequence amplified by universal primers found that Isolate 1 was closely related with 99% similarity to several species including *Chlorella vulgaris, Planktochlorella nurekis, Masaia oloidia,* and *Parachlorella beijerinckii*. In addition, sequence comparisons of the Isolate 2 18S rRNA gene found that it was closely related with 99% similarity to several strains under genus *Scenedesmus* (*Scenedesmus regularis*, *Scenedesmus obliquus*, *Scenedesmus raciborskii*) and *Tetradesmus obliquus.* The species of Isolate 1 and 2 could not be identified based on 18S rRNA gene sequence alone, because all the microalgae species mentioned earlier had 99% sequence similarities with the isolated microalgae.

Therefore, the results suggested that the data provided by 18S rRNA gene sequences were not adequate for species identification up to species level, thus the gene sequence of the 28S rRNA of the large subunit of the eukaryotic ribosome was required to provide additional information regarding the species identities. Results of BLAST comparisons of the 28S rRNA gene sequences indicated that Isolate 1 and Isolate 2 were 99% similar to *Chlorella vulgaris* strain KZN 23 and 99% similar to *Tetradesmus obliquus* isolate SGM19. By comparing and analysing the 18S and 28S rRNA genes, Isolate 1 and Isolate 2 were identified as *Chlorella vulgaris* UPSI-JRM01 and *Tetradesmus obliquus* UPSI-JRM02, respectively. *Chlorella vulgaris* and *Tetradesmus obliquus* (previously known as *Scenedesmus obliquus*) are well-known species utilized in many studies for lipid and biodiesel production [19]. The 18S and 28S rRNA gene sequences of *Chlorella vulgaris* UPSI-JRM01 and *Tetradesmus obliquus* UPSI-JRM02 were deposited into GenBank database under accession no. KY174321, KY174322, KY174323, and KY174324. The evolutionary history inferred using the Neighbour-Joining method is shown in Figure 2, with the optimal sum of branch lengths of 0.0589 and 0.3509 for 18S rRNA and 28S rRNA genes, respectively.

Table 2 BLAST Maximum Identification

Isolates	RNA gene	Max. Ident.	Description	Accession
Isolate 1	18S rRNA	99%	*Chlorella vulgaris* isolate BDUG 10021 18S ribosomal RNA gene	KT726935.1
	28S rRNA	99%	*Chlorella vulgaris* strain KZN 23 28S ribosomal RNA gene	HM063008.1
Isolate 2	18S rRNA	99%	*Scenedesmus obliquus* genomic DNA containing 18S rRNA gene	FR865738.1
	28S rRNA	99%	*Tetradesmus obliquus* isolate SGM19 28S rRNA gene	MG760624.1

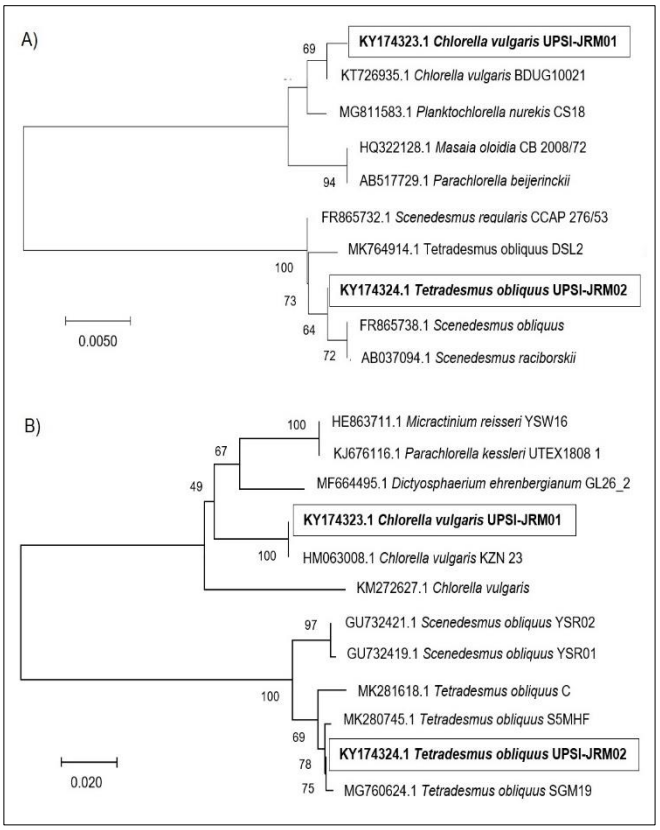


Figure 2 Neighbour-joining tree for 18S rRNA gene (A) and 28S rRNA (B) sequences of *Chlorella vulgaris* UPSI-JRM01, *Tetradesmus obliquus* UPSI-JRM02, and other microalgae species

3.3 Biomass Accumulation and N-NO_3^- Removal

Figure 3 depicts the DCW, specific growth rate, and percentage N-NO_3^- removal of the cultures, while Table 3 summarizes the biomass productivity, percentage of N-NO_3^- removal and biochemical properties of the microalgae biomass produced. At the end of 30 days of cultivation period, the DCW produced by both strains in landfill leachate were higher than recorded in BG-11, suggested that the suitability of using landfill leachate as an economic culture medium for microalgae cultivation. Figure 2b showed that the specific growth rate of *Chlorella vulgaris* cultured in landfill leachate was the highest amongst all other cultures, suggested that this strain highly tolerance to N-NO_3^-, and could be used for high nitrate wastewater treatment.

Overall, the biomass productivity of *Tetradesmus obliquus* (40.49 mg/L/day) was higher than *Chlorella vulgaris* (36.28 mg/L/day). The biomass produced for both microalgae species had significant protein content (>50%), suggesting that they may have commercial value as animal feeds and fertilizers after toxicological verification [20]. The carbohydrate and lipid contents, respectively, were 21.93% and 17.72% for *Chlorella vulgaris* and, 25.43% and 16.32% for *Tetradesmus obliquus*.

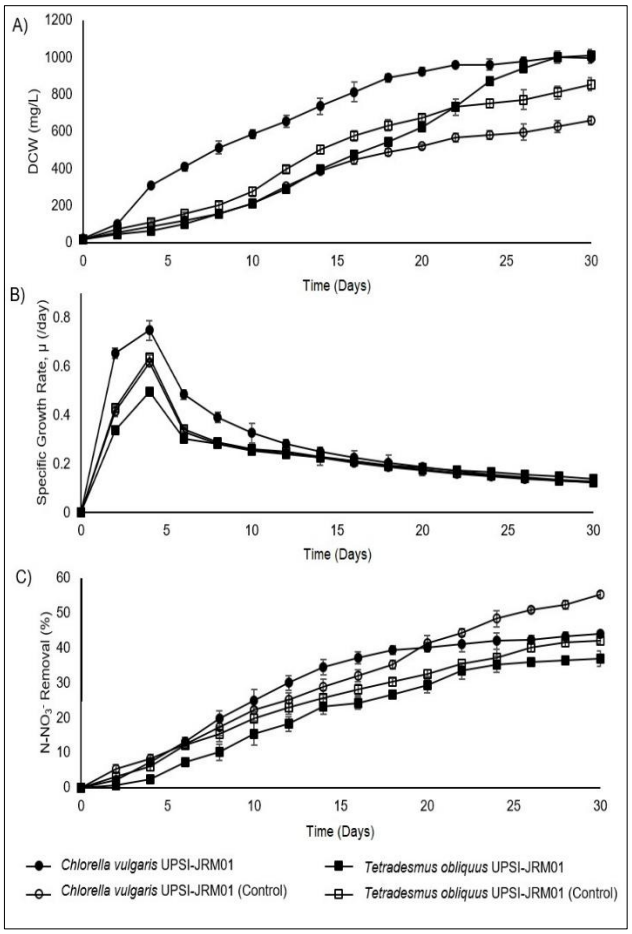


Figure 3 The DCW (A), specific growth rate (B), and N-NO_3^- removal percentage (C) of *Chlorella vulgaris* UPSI-JRM01 and *Tetradesmus obliquus* UPSI-JRM02

Table 3 Biomass productivity, N-NO_3^- removal percentage and biochemical properties of the microalgae biomass

Microalgae Isolates	*Chlorella vulgaris* UPSI-JRM01	*Tetradesmus obliquus* UPSI-JRM02
Biomass productivity (mg/L/day)	36.28 ± 0.45	40.49 ± 0.53
N-NO_3^- removal (%)	43.96 ± 1.49	36.96 ± 2.51
N-NO_3 influent (mg/L)	1500.00 ± 24.52	1500.00 ± 24.52
N-NO_3^- effluent (mg/L)	840.32 ± 11.46	945.23 ± 25.48
Lipid content (%)	17.72 ± 1.21	16.32 ± 1.74
Carbohydrate content (%)	21.93± 0.83	25.43 ± 0.73
Protein content (%)	57.52 ± 0.75	54.14 ± 0.52

*Number of measurement repetition (n)=3

It seems that *Chlorella vulgaris* tends to produce more lipids than *Tetradesmus obliquus*, while the latter produced more carbohydrates. The results of the biomass accumulation experiments are in accordance with previous findings suggesting that microalgae biomass is composed of 40-60% protein, 20-30% carbohydrate and 10-20% lipid [21]. However, the biomass contents of microalgae grown in wastewater are highly dependent on the environmental condition and nutrients availability [22]. Previous research reported that nutrient stress could lead to high lipid accumulation as the cells switch its main energy storage in the form of carbohydrate (starch) to lipid (triacylglycerol) [23, 24].

This study also examined the feasibility of removing N-NO_3^- from landfill leachate. The N: P ratio of the synthetic leachate used was 380: 1, far exceeding the optimal 16:1 N: P Redfield ratio [25]. Under this condition, it was found that the N-NO_3^- removal percentage of *Chlorella vulgaris* (44%) was higher than *Tetradesmus obliquus* (37%). These N-NO_3^- removal efficiencies may have been limited by the initial P-PO_4^{3+} concentration (7.89 mg/L) in both microalgae species. However, N-NO_3^- removal efficiency corresponded to the protein contents of both *Chlorella vulgaris* and *Tetradesmus obliquus*. Nitrogen is known to be assimilated by microalgae in the form of NO_3^-, NO_2^- or NH_4^+. It is believed that nitrogen in the form of N-NO_3^- is assimilated into proteins of the microalgae biomass. Although the N-NO_3^- removal efficiency for both microalgae was less than 50%, significant amounts of N-NO_3^- (>500 mg/L) were removed from synthetic leachate (Table 3). The amount of N-NO_3^- removed in this study higher than reported from previous study, in which only 336 mg/L was removed from wastewater with 800 mg/L N-NO_3^- concentration [4]. Higher percentages of nitrogen assimilation by microalgae might be achieved if the N: P ratio in the culture is adjusted to a favourable range. However, the optimal N: P ratio differs among cultures due to strain-specific metabolic pathways. The N: P ratio in healthy freshwater can be up to 250: 1 [26].

It is apparent that these microalgae isolates not only capable to remediate the landfill leachate by assimilating NO_3^- and thereby preventing eutrophication, but they also generate biomass with the potentials to be used for biofuel and animal feed. At the same time, the excessive nutrients in the landfill leachate can be used as a low-cost microalgae growth medium to ensure cost effective and profitable biofuel production.

4.0 CONCLUSION

In this study, two microalgae isolates capable of growing in high N-NO_3^- concentration up to 1500 mg/L were successfully isolated and identified using a combination of morphological and molecular characterizations of 18S rRNA and 28S rRNA. The isolated microalgae strains were identified as *Chlorella vulgaris* Isolate UPSI-JRM01 and *Tetradesmus obliquus* Isolate UPSI-JRM02. In the condition of high N-NO_3^- concentration of synthetic leachate and high N: P ratio, the microalgae biomass produced were significant and has commercial values. Further research will be conducted in a near future to

increase the N-NO_3^- removal efficiently and optimize the microalgae biomass production for biofuel feedstock.

Acknowledgement

The authors wish to thank Universiti Pendidikan Sultan Idris and The Ministry of Education Malaysia (MOHE) for financing this study through University Research Grant (Code: 2011-0056-102-01- Screening & Isolation) and Fundamental Research Grant Scheme (Code: 2015-01611-102-02 - Molecular Identification), respectively. We are also grateful to Worldwide Landfill Sdn. Bhd. for their technical support throughout the study.

References

[1] Yusof, N., Hassan, M. A., Phang, L. Y. Tabatabaei, M., Othman, M. R., Mori, M., Wakisaka, M., Sakai, K., & Shirai, Y. 2011. Nitrification of High-strength Ammonium Landfill Leachate with Microbial Community Analysis Using Fluorescence in Situ Hybridization (FISH). *Waste Management & Research*. 29(6): 602-611. DOI: 10.1177/0734242X10397581.

[2] Yusof, N., Hassan M. A., Phang, L. Y., Tabatabaei, M., Othman, M. R., Mori, M., Wakisaka, M., Sakai, K., & Shirai, Y. 2010. Nitrification of Ammonium-rich Sanitary Landfill Leachate. *Waste Management*. 30: 100-109. DOI: 10.1016/j.wasman.2009.08.018.

[3] Zhu, L. D., Hiltunen, E., Antila, E., Zhong, J. J., Yuan, Z. H., & Wang, Z. M. 2014. Microalgal Biofuels: Flexible Bioenergies for Sustainable Development. *Renew. Sust. Energ. Rev.* 30: 1035-1046. DOI: 10.1016/j.rser.2013.11.003.

[4] Taziki, M., Ahmadzadeh, H., & Murry, M.A. 2015. Growth of *Chlorella vulgaris* in High Concentrations of Nitrate and Nitrite for Wastewater Treatment. *Current Biotechnology*. 4(4): 441-447. DOI: 10.2174/2211550104666150930204835.

[5] Otondo, A., Kokabian, B., Stuart-Dahl, S., & Gude, V. G. 2018. Energetic Evaluation of Wastewater Treatment Using Microalgae. *Chlorella vulgaris*. *Journal of Environmental Chemical Engineering*. 6(2): 3213-3222. DOI: 10.1016/j.jece.2018.04.064.

[6] Parvin, M., Zannat, M., & Habib, M. 2007. Two Important Techniques for Isolation of Microalgae. *Asian Fish. Sci.* 20: 117-124.

[7] Serra-Maia, R., Bernard, O., Gonzalves, A., *et al*. 2016. Influence of Temperature on *Chlorella vulgaris* Growth and Mortality Rates in a Photobioreactor. *Algal Res.* 18: 352-359. DOI: 10.1016/j.algal.2016.06.016.

[8] Nordin, N., Yusof, N., & Samsudin, S. 2016. Biomass Production of *Chlorella* sp., *Scenedesmus* sp., and *Oscillatoria* sp. in Nitrified Landfill Leachate. *Waste and Biomass Valorization*. 8(7): 2301-2311. DOI: 10.1007/s12649-016-9709-8.

[9] Taher, H., Al–Zuhair, S., Al–Marzouqi, A. H., Haik, Y., & Farid, M. 2014. Effective Extraction of Microalgae Lipids from Wet Biomass for Biodiesel Production. *Biomass Bioenergy*. 66: 159-167. DOI: 10.1016/j.biombioe.2014.02.034.

[10] George, B., Pancha, I., Desai, C., Chokshi, K., Paliwal, C., Ghosh, T., & Mishra, S. 2014. Effects of Different Media Composition, Light Intensity and Photoperiod on Morphology and Physiology of Freshwater Microalgae *Ankistrodesmus falcatus*-a Potential Strain for Bio-Fuel production. *Bioresource Technology*. 171: 367-74. DOI: 10.1016/j.biortech.2014.08.086.

[11] Jia, J., Han, D., Gerken, H. G., Li, Y., Sommerfeld, M., Hu, Q., & Xu, J. 2015. Molecular Mechanisms for Photosynthetic Carbon Partitioning into Storage Neutral Lipids in *Nannochloropsis oceanica* under Nitrogen-depletion Conditions. *Algal Research*. 7: 66-77. DOI: 10.1016/j.algal.2014.11.005.

[12] Li, L., Cui, J., Liu, Q., Ding, Y., & Liu, J. 2015. Screening and Phylogenetic Analysis of Lipid-rich Microalgae. *Algal Research*. 11: 381-386. DOI: 10.1016/j.algal.2015.02.028.

[13] American Public Health Association (APHA). 2005. *Standard Methods for the Examination of Water and Wastewater*. 21st ed. Washington DC: American Public Health Association.

[14] Ho, S. H., Huang, S. W., Chen, C. Y., Hasunuma, T., Kondo, A., & Chang, J. S. 2013. Characterization and Optimization of Carbohydrate Production from an Indigenous Microalga *Chlorella vulgaris* FSP-E. *Bioresource Technology*. 135: 157-165. DOI: 10.1016/j.biortech.2012.10.100.

[15] Talebi, A. F., Tohidfar, M., Tabatabaei, M., *et al*. 2013. Genetic Manipulation, a Feasible Tool to Enhance Unique Characteristic of *Chlorella vulgaris* as a Feedstock for Biodiesel Production. *Mol. Biol. Rep.* 40: 4421-4428. DOI: 10.1007/s11033-013-2532-4.

[16] Rasoul-Amini, S., Ghasemi, Y., Morowvat, M. H., & Mohagheghzadeh, A. 2009. PCR Amplification of 18S rRNA, Single Cell Protein Production and Fatty Acid Evaluation of Some Naturally Isolated Microalgae. *Food Chem.* 116: 129-136. DOI: 10.1016/j.foodchem.2009.02.025.

[17] Bhola, V., Desikan, R., Santosh, S. K., Subburamu, K., Sanniyasi, E., & Bux, F. 2011. Effects of Parameters Affecting Biomass Yield and Thermal Behaviour of *Chlorella vulgaris*. *J. Biosci. Bioeng.* 111(3): 377-382. DOI: 10.1016/j.jbiosc.2010.11.006.

[18] Yi, L., Yuesong, X., Ying, P. Y., Rongqing, Y., Pei, L., Dairong, Q., Yi, C., & Yu, C. 2014. The Biodiversity of Oleaginous Microalgae in Northern Qinghai-Tibet Plateau. *Afr. J. Microbiol. Res.* 8(1): 66-74. DOI: 10.5897/AJMR12.806.

[19] Mishra, A., Medhi, K., Maheshwari, N., Srivastava, S., & Thakur, I. S. 2018. Biofuel Production and Phycoremediation by *Chlorella* sp. ISTLA1 Isolated from Landfill Site. *Bioresource Technology*. *253*: 121-129. DOI: 10.1016/j.biortech.2017.12.012.

[20] Roeselers, G, Loosdrecht, M. C. M., & Muyzer, G. 2007. Phototrophic Biofilms and Their Potential Applications. *J. Appl. Phycol.* 20: 227-235. DOI: 10.1007/s10811-007-9223-2.

[21] Singh, A. & Olsen, S. I. 2011. A Critical Review of Biochemical Conversion, Sustainability and Life Cycle Assessment of Algal Biofuels. *Appl. Energy*. 88(10): 3548-3555. DOI: 10.1016/j.apenergy.2010.12.012.

[22] De Francisci, D., Su, Y., Iital, A., & Angelidaki, I. 2018. Evaluation of Microalgae Production Coupled with Wastewater Treatment. *Environmental Technology (United Kingdom)*. 39(5): 581-592. DOI: 10.1080/09593330.2017.1308441.

[23] Li, T., Gargouri, M., Feng, J., Park, J. J., Gao, D., Miao, C., Dong, T., Gang, D. R., & Chen, S. 2015. Regulation of Starch and Lipid Accumulation in a Microalga *Chlorella sorokiniana*. *Bioresource Technology*. 180: 250-257. DOI: 10.1016/j.biortech.2015.01.005.

[24] Concas, A., Steriti, A., Pisu, M., & Cao, G. 2014. Comprehensive Modeling and Investigation of the Effect of iron on the Growth Rate and Lipid Accumulation of *Chlorella Vulgaris* Cultured in Batch Photobioreactors. *Bioresource Technology*. 153: 340-350. DOI: 10.1016/j.biortech.2013.11.085.

[25] Lynch, F., Santana-Sánchez, A., Jämsä, M., Sivonen, K., Aro, E. M., & Allahverdiyeva, Y. 2015. Screening Native Isolates of Cyanobacteria and a Green Alga for Integrated Wastewater Treatment, Biomass Accumulation and Neutral Lipid Production. *Algal Res.* 11: 411-420. DOI: 10.1016/j.algal.2015.05.015.

[26] Cai, T., Park, S. Y. & Li, Y. 2013. Nutrient Recovery from Wastewater Streams by Microalgae: Status and Prospects. *Renew. Sust. Energ.Rev.* 19: 360-369. DOI: 10.1016/j.rser.2012.11.03.