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Fatty acid profiles of Antarctic cyanobacteria *Leptolyngbya*

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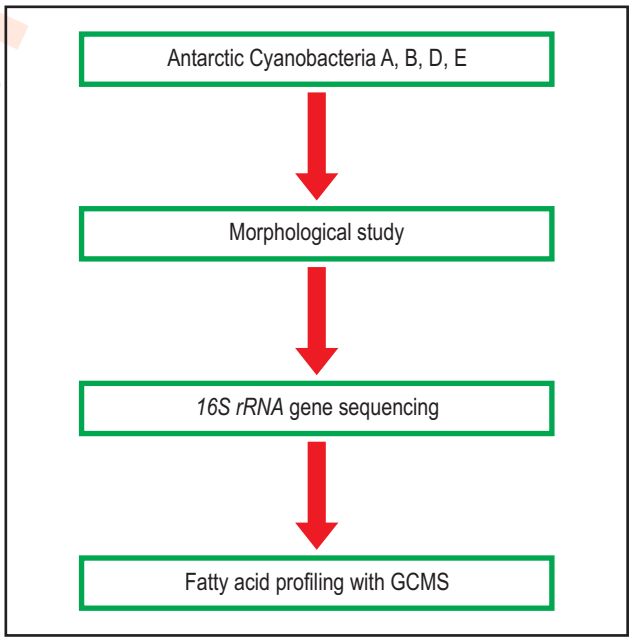
Abstract

Aim: Antarctic cyanobacteria may represent a potential resource of new and unique compounds with interesting capabilities. Profiling of fatty acids in Antarctic cyanobacteria can provide an overview of potential fatty acids present in them, that can be utilised in future applications.

Methodology: In total, 4 cyanobacteria previously isolated from Antarctic polar ice was used in this study. Molecular identification using *16S rRNA* gene was used to ascertain their identities as *Leptolyngbya spp.* and their fatty acid profiles were determined using GCMS.

Results: Morphologically, these cyanobacteria were found similar to *Leptolyngbya sp.* Analysis of *16S rRNA* gene sequences amplified further confirmed their identity and were designated as *Leptolyngbya* sp. A, B, D and E. Following fatty acid analysis using GCMS, it was determined that unsaturated fatty acids predominated the fatty acid profiles for *Leptolyngbya* sp. A, B and D, while saturated fatty acid was found dominant in *Leptolyngbya* sp. E. *Leptolyngbya* sp. D contained almost 100% of linoleic acid, whilst *Leptolyngbya* sp. A and *Leptolyngbya* sp. B contained 59.35% and 83.33% of linoleic acid, respectively. Besides linoleic acid, palmitoleic acid (18.45%), oleic acid (19.45%) and lauric acid (2.74%) were also present in *Leptolyngbya* sp. A. As for *Leptolyngbya* sp. B, other than linoleic acid, only oleic acid (16.67%) was detected.

Interpretation: Findings from this study demonstrate that the Antarctic *Leptolyngbya* spp. A, B and D identified in this study possess high content of unsaturated fatty acids, while only saturated fatty acid was present in *Leptolyngbya* sp. E. Fatty acid profiles revealed the potential of these Antarctic *Leptolyngbya* species to be further exploited for other applications.



Keyword: 16S rRNA, Antarctic region, Cyanobacteria, Fatty acid profiles, *Leptolyngbya*

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Introduction

Cyanobacteria are prokaryotes that are capable of photosynthesis and mostly exist in illuminated environments. These microorganisms contribute oxygen to the earth due to their photosynthetic property and presence of chlorophyll a (Sharma *et al*., 2013; Bela and Malliga, 2015). Cyanobacteria are predominantly known as blue-green algae due to the presence pigments phycobilin and phycocyanin (Schopf, 2012). Cyanobacteria exist in a wide-ranging shapes and sizes. The basic morphological shapes are spherical, rod or spiral, and they can also grow in colonies or filaments (Singh and Montgomery, 2011). Unicellular cyanobacteria exists as single cells, suspended or benthic, or aggregates, while filamentous type may be thin or thick, single trichome or bundles either with or without a sheath (Abed *et al*., 2009). Due to their vast availability throughout cold habitats, cyanobacteria are model microorganisms for the reconstruction of microbial life on the primitive earth.

Cyanobacterial populations are expanding and dominating many environments, particularly freshwater lakes, basins, rivers, irrigation channels, brackish, sea waters and salty lakes (Manganelli *et al.,* 2012). In extreme cold temperature of Antarctic, Arctic and alpine environment, cyanobacteria are of basic ecological importance as primary producers and ecosystem engineers (Chrismas *et al*., 2018). Cyanobacterial mats are common in Antarctic lakes, pond and on moist soils. The extreme condition in Antarctic makes it a non-conducive habitat for living things and requires remarkable adaptive mechanism to survive in harsh environment. Such includes the non-stop cold temperature, accompanied by freeze-thaw cycles, irradiation with extreme fluctuation as well as differences in supply of nutrients and salinity. However, cyanobacteria found in ice and cold desert habitats are equipped with mechanisms to survive the harshness of surroundings. For example, some dwell in rocks where humidity is high, and thermal differences are not that significant. They also form dark mats on or within ice where temperature is high enough to be able to obtain water as well as absorbing sunlight. Interestingly, most cyanobacteria in cryosphere are actually psychrotrophic (cold tolerant), instead of psychrophiles (Oliver *et al.*, 2012). This is reinforced by Üveges and co-workers (2012) that polar cyanobacteria has originated from temperate regions of warmer temperature due to the fact that Antarctic cyanobacteria cannot grow at temperatures lower than 5ºC but can grow between 5º- 30ºC . Although cyanobacteria do not have high metabolism rate and grow slowly, they have broad tolerance to polar condition.

They are able to survive dormancy and are resistant to natural loss which make cryosphere conducive habitat for them. Even with optimum conditions, they take longer time to grow as compared to psycrophilic algae (Vincent, 2007). Some of the species found in the polar region include *Anabaena* sp., *Leptolyngbya* sp., *Nostoc* sp., *Synechococcus* sp., and *Oscillatoria* sp. (Oliver *et al.*, 2012). *Leptolyngbya* sp. are filamentous cyanobacterium clustered together with the genus *Plectonema*, *Lyngbya* and *Phormidium* (Rippka *et al*., 1979). This cyanobacterium is widely distributed in different habitats such as marine, deserts and freshwater environments (Wilmotte and Herdman, 2001). Morphological characteristics of genus comprises of thin trichomes and sometimes sheaths (Castenholz, 2001). Due to the small size and simple morphology, it complicates the taxonomic classification of *Leptolyngbya* sp. Some species of *Leptolyngbya* from Antarctic have been identified as *L. vincentii* sp. nov, *L. nigrescens* sp. nov, *L. fritschiana* sp. nov and *L. borchgrevinkii* sp. nov. (Komárek, 2007).

Cyanobacteria have substantial amount of fatty acids which consequently aid in their survival in cold habitats. The adaptive mechanism is influenced by the composition of membrane fatty acids whereby it controls the fluidity and metabolic functions (Králová, 2017). It is proved that low chain lengths of polyunsaturated fatty acids are integrated in the cell membranes in order to retain the fluidity of membrane at low temperatures (Zakhia *et al*., 2008). A comparison between Antarctic cyanobacteria and the ones from New Zealand have shown similarities in the abundance of palmitic, stearic and oleic acid, where the latter is unsaturated (Martineau *et al*., 2013). Fatty acid composition is important as it elucidates the potential sources of commercially intriguing fatty acids which could drive big industries such as food and biofuel. One of the potential applications of palmitic acid is lowering fatty acid oxidation and increased daily energy expenditure, helps in reducing weight and total lipid content as well as circulating the insulin level (Kien *et al*., 2018). It has been demonstrated that palmitic acid induced degeneration of myofibrils and modulate apoptosis in adult rat cardiomyocytes where it could open the pathway for further research to be implemented on humans (Dyntar *et al*., 2001). Oleic acid is omega-9 unsaturated fatty acid, commonly found in various types of plants including olive oil and almond oil. It helps in reducing high blood pressure (Terés *et al*., 2008). It was reported that the fatty acid methyl ester (FAME) content of *Leptolyngbya* BL0902 obtained from microalgae culture plant is 18.9% which comprises mainly of palmitic acid (Taton *et al*., 2012). In *Leptolyngbya* sp. KIOST-1 (isolated from an open pond), constitutes 21.4% of stearic acid (Kim *et al*., 2015). To date, fatty acids profile of polar *Leptolyngbya* sp. is yet to be explored.

Other than playing a prominent role in survival, fatty acids also contribute in identification of cyanobacteria from the patterns formed by fatty acid profiles. Further analysis via molecular identification helps in documentation of species, based on the conserved region in the fragment of 16S rRNA gene from the records in NCBI GenBank. Hence, this study was conducted with the aim to identify four samples of cyanobacteria collected from Antarctic, and to profile their fatty acid composition.

Materials and Methods

Cyanobacteria: *Leptolyngbya* sp. samples were isolated from polar ice in Antarctic by the members of Universiti Sains Malaysia (USM) and British Antarctic Survey (BAS). The samples were labelled as *Leptolyngbya* sp. A, B, D and E. These strains were maintained in BG-11 media agar. *Leptolyngbya* sp. A, B, D and E were identified by using a light microscope at 40-100x magnification. The morphological features such as size, shape of colonies and filaments were noted as proposed by Komárek (2007).

Molecular identification: Genomic DNA extraction was performed on *Leptolyngbya* sp. A, B, D and E using the conventional phenol-chloroform method. Next, PCR amplification of *16S rRNA* gene was conducted on the extracted genomic DNA using two universal primers: 5'-AGAGTTTGATCCTGGC TCAG-3' (forward) and 5'-GGTTACCTTGTTACGACTT-3' (reverse). The reaction mixture was prepared in a total volume of 50 µl containing 5 µl of extracted DNA, 1 µl of each primer, 25µl of Mastermix containing *Taq* polymerase, dNTPS, BSA and reaction buffer. PCR temperature profile was as follow: 94ºC for 5 min, 30 cycles of 95ºC for 30 sec, 60ºC for 30 sec, 72ºC for 2 min 30 sec and finally an extension step at 72ºC for 10 min. Amplification products were analyzed by electrophoresis in 1% (w/v) agarose gels stained with 1µl Midori Green staining solution. After visualization with Alpha Imager, single bands of 1500 bp were cut and inserted into 1.5 ml microcentrifuge tubes. The samples were sent to Apical Scientific Sdn. Bhd. for purification and sequencing. The resultant *16S rRNA* gene sequences were manually verified and edited using BioEdit Sequence Alignment Editor. Partial nucleotide sequences were compared with GenBank database via BLASTn search tool.

Fatty acid analysis: Fatty acid profile of Leptolyngbya A, B, D and E were estimated by the method described by Sasser (1990). The isolates were grown in BG-11 agar for 14 days at 23ºC. Approximately, 40 mg of bacterial cells were placed in 15 ml centrifuge tube. To this tube, 1 ml of Reagent 1 (45 g sodium hydroxide, 150 ml methanol, 150 ml distilled water) was added, and the tubes were vortexed and heated in a boiling water bath for 5 min, and vigorously vortexed for 5-10 sec and returned to water bath to complete the 30 min heating. Then, 2 ml of Reagent 2 (325 ml 6.0N hydrochloric acid, 275 ml methyl alcohol) was added. After vortexing, the tubes were heated for 10 ± 1 min at 80º ± 1ºC. Next, 1.25 ml of Reagent 3 (200 ml hexane, 200 ml methyl tert-butyl ether) were added to cooled tube followed by recapping and gentle tumbling on a clinical rotator for about 10 min. The aqueous (lower) phase were pipetted out and discarded. About 3 ml of Reagent 4 (10.8 g sodium hydroxide dissolved in 900 ml distilled water) was added to organic phase remaining in the tube. The tubes were recapped, and then tumbled for 5 min. About 2/3 of organic phase was pipetted into a vial and sent for analysis. Determination of fatty acid profiles of cyanobacteria was carried out with gas chromatography mass spectrometer (GC-MS) with run time of 40 min, temperature of 30ºC per minute and increased in 5ºC per minute until it reached 200ºC with holding time of 5 min.

Results and Discussion

Leptolyngbya spp. A, B and E showed similar morphology of dark green thin filamentous morphology (Fig. 1) whereas *Leptolyngbya* sp. D was particularly different due to the growth of colonies that were contoured in shape and had more compact morphological feature. Further inspection under microscope revealed all samples were found to be filamentous (Fig. 1). *Leptolyngbya spp.* A, B and E all were similar in size while

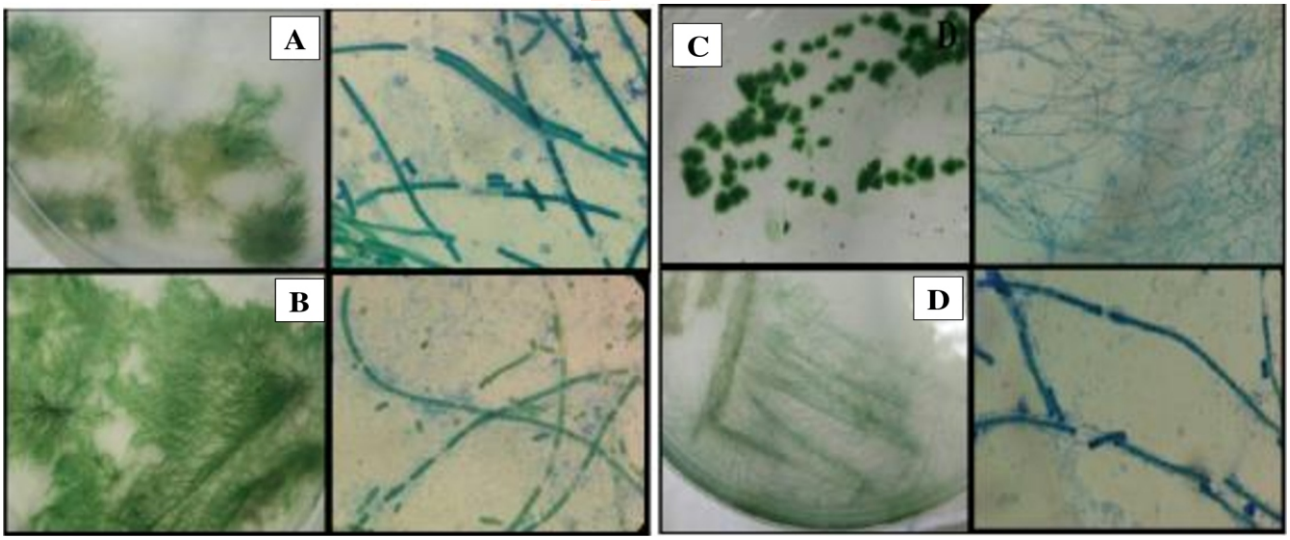


Fig. 1: Morphological images of colonies and filaments of (A) *Leptolyngbya* sp. A; (B) *Leptolyngbya* sp. B; (C) *Leptolyngbya* sp. D and (D) *Leptolyngbya* sp. E (1000x).

Table 1 : Comparison of partial *16S rRNA* gene sequences with NCBI GenBank Database

Isolate	Closest match	Similarity (%)
A	*Leptolyngbya valderiana* BDU 30711	97
B	*Leptolyngbya valderiana* BDU 30711	98
D	*Leptolyngbya* sp. O-77	98
E	*Leptolyngbya valderiana* BDU 140081	98

Table 2 : Composition of different type of fatty acids of *Leptolyngbya* spp. A, B, D and E detected by GC-MS

Fatty acid			Fatty Acid (%)			
			Leptolyngbya			
Common name	Molecular formula	Systemic name	A	B	D	E
		Saturated				
Lauric acid	$C_{12}H_{24}O_2$	Dodecanoic (12:0)	2.74	nd	nd	nd
Stearic acid	$C_{18}H_{34}O_3$	Octadecanoic (18:0)	nd	nd	nd	83.0
Heptatriacontanoic acid	$C_{37}H_{72}O_5$	Heptatriacontanoic (37:0)	nd	nd	nd	17.0
Total FA (%)			2.74			100
		Monounsaturated				
Palmitoleic acid	$C_{16}H_{30}O_2$	9-Hexadecenoic (9-16:1)	18.45	nd	nd	nd
Oleic acid	$C_{18}H_{34}O_2$	9-Octadecenoic (9-18:1)	19.45	16.67	nd	nd
		Polyunsaturated				
Linoleic acid	$C_{18}H_{32}O_2$	9,12-Octadecedienoic (9,12-18:2)	59.35	83.33	100	nd
Total FA (%)			97.26	100	100	nd

*nd: not detected

Leptolyngbya sp. D was comparatively smaller than the rest. *Leptolyngbya* sp. A had thin and straight filaments with distinct trichomes. The sheaths were thin and the content of cells was dark green. The apical cells were round in shape whereas. *Leptolyngbya* sp. B had long, thin and straight filaments. Trichomes were not as distinct as sample A and the cellular content which was also green, appeared to be a shade lighter than sample A. The sheaths were also thin and the end of cells were round. *Leptolyngbya* sp. E had long, straight filaments and clear trichomes. The cell content appeared to be dark green and the sheaths were thin. The ends of the filaments were also round. *Leptolyngbya* sp. D had very thin, long and straight, sometimes twisted filaments that clustered together. The trichomes were not very distinct due to small size. The cell content was also green in color and the apical cells seemed to be round with thin sheaths.

Although morphological appearances of *Leptolyngbya spp.* A, B, D and E suggested that all isolates belonged to genus *Leptolyngbya,* molecular identification is vital to ascertain their identities at species level. PCR amplification of *16S rRNA* gene was conducted on genomic DNA extracted from all samples and PCR product of ~1500 bp successfully amplified from all samples as depicted in Fig. 2. Comparison of partial sequences of 16S rRNA with NCBI database revealed that all samples belonged to genus *Leptolyngbya. Leptolyngbya spp.* A, B and E were closely

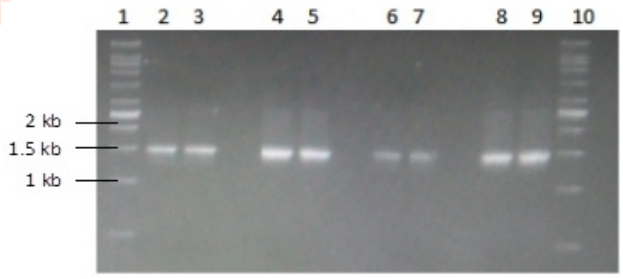
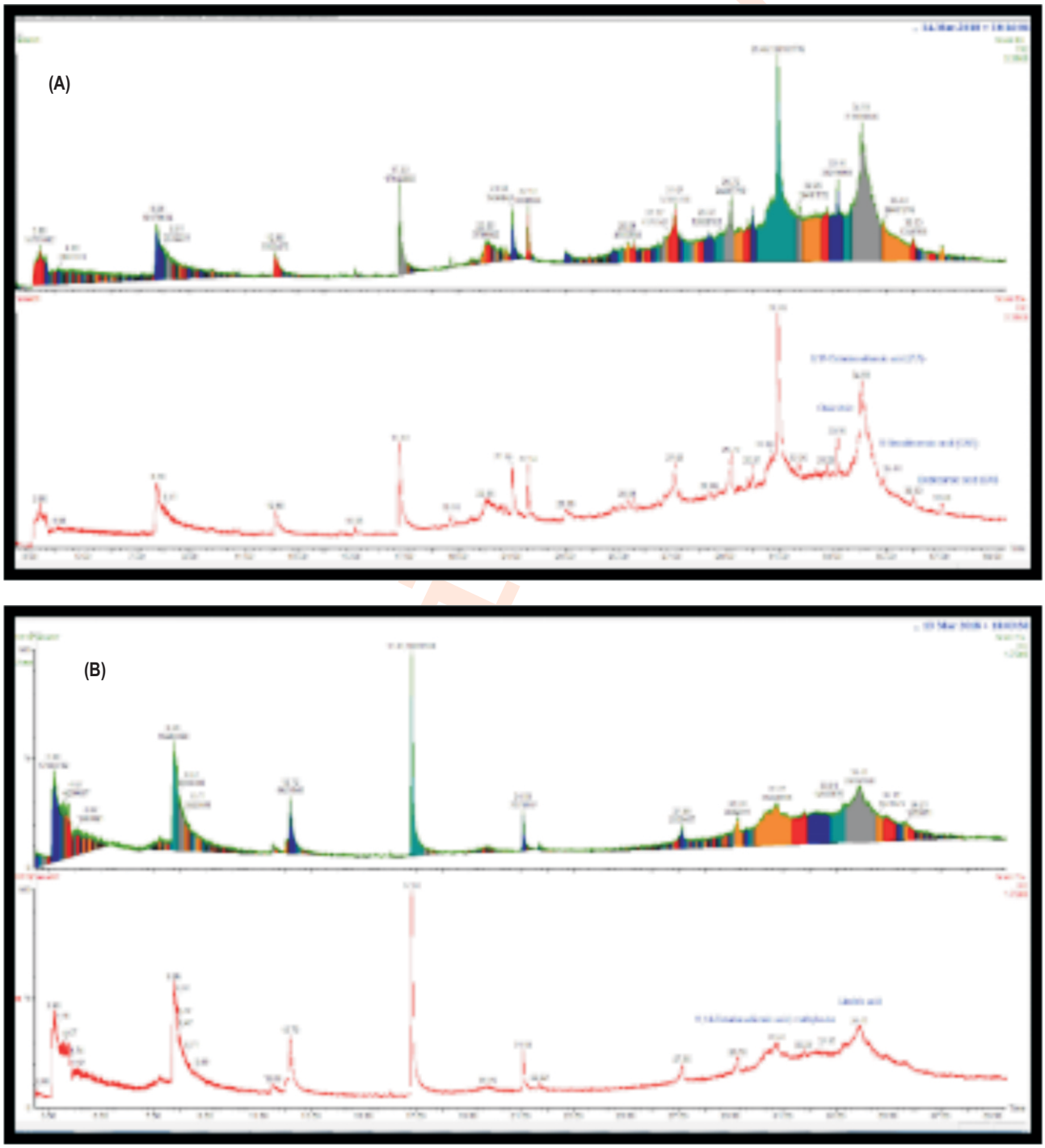


Fig. 2 : Agarose gel electrophoresis of PCR product of *16S rRNA* gene. Lane 1: 1kb ladder; Lane 2 and 3: Sample A; Lane 4 and 5: Sample B; Lane 6 and 7: Sample D; Lane 8 and 9: Sample E; Lane 10: 1kb ladder.

related to *Leptolyngbya valderiana* with 97%, 98% and 98% similarity, while *Leptolyngbya* sp. D was identified as *Leptolyngbya* sp. (Table 1) with 98% similarity. The absence of species level for *Leptolyngbya* sp. D suggested the possibility of new species of *Leptolyngbya* and this may explain the differences in their morphology compared to *Leptolyngbya spp.* A, B and E.

Fig. 3 depicts the fatty acid profiles of each *Leptolyngbya* spp. A, B, D and E using GCMS. Based on the peaks obtained, 6 types of fatty acids were identified namely lauric acid, stearic acid, heptatriacontanoic acid, palmitoleic acid, oleic acid and linoleic

acid. Table 2 illustrates the comparison of fatty acid percentages between *Leptolyngbya* spp. A, B, D and E. Though *Leptolyngbya spp.* A, B and E were closely related to *Leptolyngbya valderiana,* their fatty acid contents varied. Unsaturated fatty acids dominated the fatty acid composition of *Leptolyngbya spp.* A and B, but *Leptolyngbya sp. E contained* 83% of stearic acid and 17% of heptatriacontanoic, both belonging to saturated fatty acid (SFA). *Leptolyngbya sp.* A contained lauric acid (2.74%), palmitoleic acid (18.45%), oleic acid (19.45%) and linoleic acid (59.35%), while *Leptolyngbya* sp. B contained 16.67% of oleic acid and 83.33% of linoleic acid. None of saturated fatty acids was detected in *Leptolyngbya* sp. D, instead 100% of linoleic acid which is a polyunsaturated acid (PUFA) was present in *Leptolyngbya* sp. D. Pushparaj *et al.* (2008) reported that most of Antarctic cyanobacteria were high in unsaturated fatty acid with up to 67% of total fatty acids. Additionally, in *Leptolyngbya* sp., the



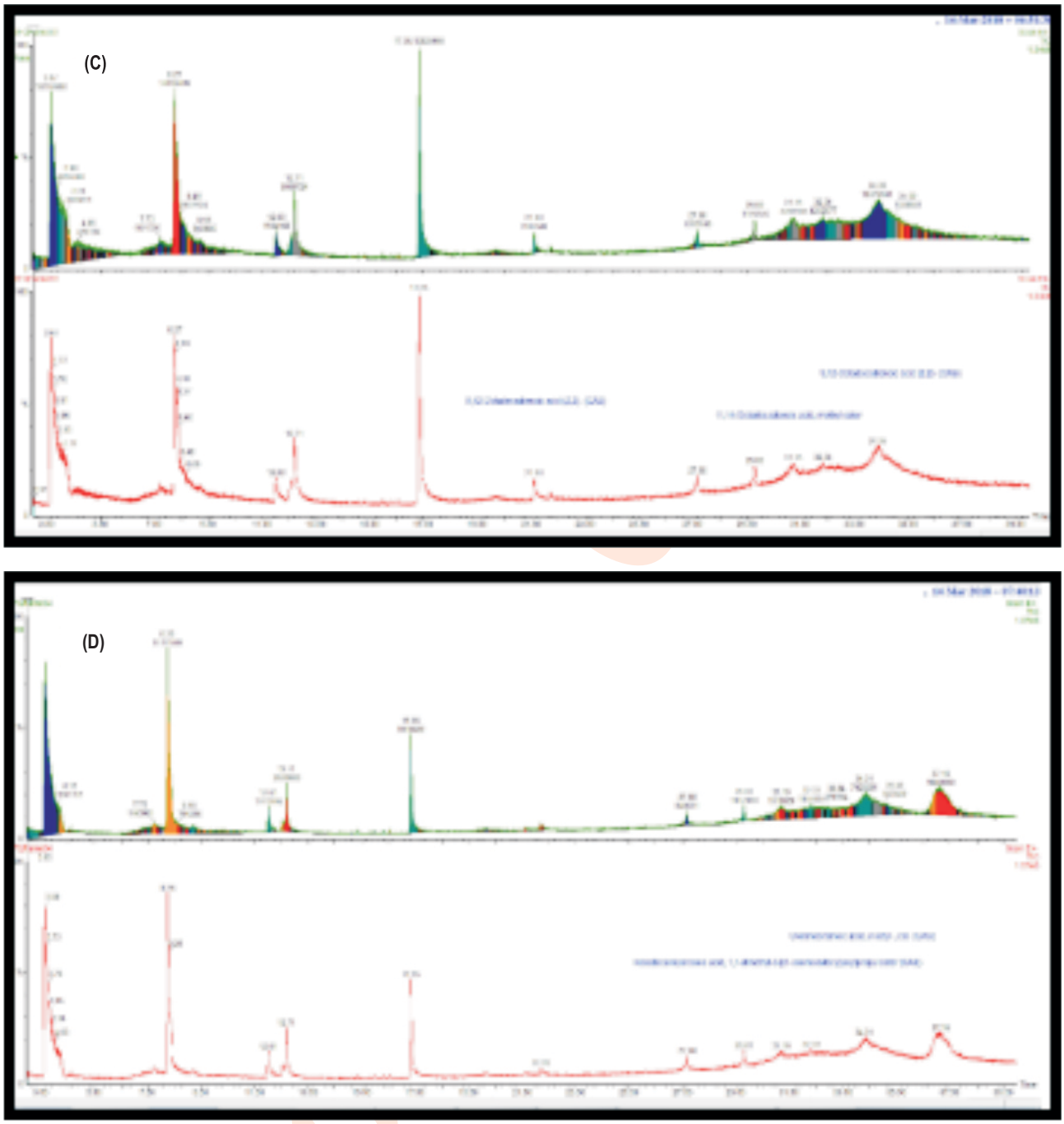


Fig. 3 : Peaks and corresponding obtained after 40 minutes of running time. (A) Peaks of fatty acid methyl ester (FAME) of *Leptolyngbya* sp. A; (B): Peaks of FAME *Leptolyngbya* sp. B; (C) Peaks of FAME *Leptolyngbya* sp. D and (D) Peaks of FAME *Leptolyngbya* sp. E.

percentage of PUFAs was higher than SFAs with the mean linoleic acid percentage being 16% of total fatty acids. Interestingly, *Leptolyngbya* sp. A contained 59.35% linoleic acid, *Leptolyngbya* sp. B contained 83.33% of linoleic acid and *Leptolyngbya* sp. D possessed up to 100% of linoleic acid, all of which were higher than reported by Pushparaj *et al*. (2008). High content of PUFAs in *Leptolyngbya* is a desirable trait because it can potentially be utilised as dietary supplement as well as lipid extract for medicinal purposes to treat cancer and other illness. The presence of PUFAs make these isolates valuable from nutraceutical point of view due to their health benefits, and increasing demand in the global market (Steinhoff *et al*., 2014).

Linoleic acid, is valuable in the medical field as it has potential to reduce coronary heart disease and help battle cancer cells in its conjugated form (Ramsden *et al.*, 2013). Besides, single fatty acid such as palmitoleic acid and oleic acid are of particular interest due to their health benefits. Duckett *et al*. (2014) reported that palmitoleic acid can promote the release of satiety hormones in rats, therefore it can open pathway for further research to implement on humans. Oleic acid helps to reduce low density lipoprotein and blood lipids and triglycerides (Lopez-Huertas, 2010). *Leptolyngbya* sp. E. containd only SFAs namely, stearic acid and heptatriacontanoic acid. This finding significantly differed with Martineau *et al.* (2013) who reported that SFAs are made up of 29.7-79% of total fatty acid in various strains of Antarctic cyanobacteria. Variations in the fatty acid composition is influenced by the difference and adaptations of each species to perform its own metabolism activities. Although SFAs seems to be underrated, SFAs do possess potential application, for example, stearic acid is commonly used as food additive and for the production of detergents, soaps and cosmetics. Heptatriacontanoic acid is a long chain saturated fatty acid with 37 carbons, an efficient compound in the neurite outgrowth activity which is highly significant in developing a novel anti-Alzheimer agent (Zhang *et al.*, 2014). Lauric acid has gained its name in the field of green technology due to significant involvement in electrospun ultrafine composite fibers that act as phase change material for storage and retrieval of solar thermal energy (Cai *et al.*, 2012). This study highlights the fact that although cyanobacteria isolates are closely related to one species as in the case of *Leptolyngbya* spp. A, B and E to Leptolyngbya valderiana, their fatty acids content showed variation among them. Moreover, high content of unsaturated fatty acids was found in *Leptolyngbya* spp. A, B and E in the range of 97%-100%, while only saturated fatty acids were detected in *Leptolyngbya* sp. D. Nevertheless, fatty acids are used in various fields, and in this study Antartctic *Leptolyngbya* was able to synthesize fatty acids in a significant amount. Therefore, their ability to produce fatty acids should be scaled up such as growing in bioreactor or manipulating through genetic engineering, which will lead them as renewable source of biomaterials.

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