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Short Communication
Unsaturated Fatty Acids in Antarctic Bacteria

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Abstract
Unsaturated Fatty Acids (UFAs) play a vital role in membrane lipids fluidity which maintains its structural integrity and also, are nutritionally and pharmaceutically important. Fish and vegetable oils are the major sources of the so-called essential fatty acids required for normal physiological activities of the body. However, the increasing demands for these fatty acids require more reliable, flexible and cheaper alternative sources. Antarctic bacteria can produce large amounts of unsaturated fatty acids which can be enhanced using simple biotechnological tools. To achieve this, five isolates of Antarctic bacteria were grown at low temperature and analysed for cellular fatty acids using Gas Chromatography Mass Spectrometry (GCMS). The results obtained revealed high amount of unsaturated fatty acids in virtually all the bacteria. Highest amount was recorded in *Arthrobacter* sp., 3B (47.24%) followed by *Pseudomonas* sp., A8 (45.09%), *Pseudomonas* sp., A3 (33.17%) and *Arthrobacter* sp., PB (31.92%). The results obtained suggest that the bacteria could be used to enhance essential fatty acids production through metabolic engineering for industrial applications.

Key words: *Arthrobacter* species, desaturase, *Pseudomonas* species, unsaturated fatty acids, GCMS

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Unsaturated Fatty Acids (UFA) account for the major components of membrane lipids and play vital roles to membrane physiology. The ratio of unsaturated to saturated fatty acids determines membrane fluidity which promotes several cellular activities such as membrane fusion and fission (Altabe *et al*., 2013). The desired membrane fluidity is maintained either by upward or downward regulation of unsaturated fatty acids synthesis in response to various environmental factors like temperature, hydrostatic pressure, osmotic, solvents, toxic compounds and external pH (Schweizer and Choi, 2011).

Certain groups of fatty acids are referred to as essential fatty acids such as long chain polyunsaturated fatty acids (PUFAs). These fatty acids cannot be synthesised by the body of man and animals and are very crucial both nutritionally and pharmaceutically. For example, unsaturated fatty acids are widely used to reduce symptoms of many diseases such as cardiovascular diseases, psoriasis, autoimmune diseases, neurological and inflammatory diseases (Xing *et al*., 2014). Fish and vegetable oils have been the major sources of essential fatty acids for the body (Adarme-Vega *et al*., 2014). However, the consumption of fish oil for PUFAs may have some drawbacks such as contamination of the fish oil by environmental pollution, problems of fishy smell and objectionable taste (Jadhav *et al*., 2010). Additionally, prior to application, fish oil may require expensive purification for the desired unsaturated fatty acids due to its complex nature of mixed fatty acids of different lengths with varying degrees of unsaturation (Jadhav *et al*., 2010).

Considering the rapid increasing demands for unsaturated fatty acids and undesirable features of fish oil, many alternative means of PUFAs production are currently being considered such as enhanced techniques for refining fish oil (Rubio-Rodriguez *et al*., 2010) and exploitation of microorganisms (Ratledge, 2001). It has been established that microbial PUFAs have several advantages including low cost of production and purification, high selling price, high oxidative stability and most importantly, their renewability compared to fish oil (Jadhav *et al*., 2010). The PUFAs producing isolates have been shown to thrive well in different extreme conditions of low temperature, high pressure, deep-sea habitats and permanently cold marine environments (Skerratt *et al*., 2002). Antarctic organisms, both prokaryotes and eukaryotes have certain characteristics that allowed them to thrive well in permanently cold seawater to about -1.8°C. Previous studies revealed that the rate of unsaturated fatty acids production in antarctic microorganisms is directly proportional to every decrease in growth temperature (Freese *et al*., 2009). This phenomenon was observed with many Antarctic organisms including bacteria such as *Pseudoalteromonas* sp., MLY15 (Li *et al*., 2009).

Various oleaginous microorganisms including microalgae, fungi and yeasts have been investigated for polyunsaturated fatty acids (Poli *et al*., 2013). However, reports from Antarctic bacteria that require cheaper growth requirements and simple handling processes coupled to availability of simple biotechnological tools that can be used to enhance unsaturated fatty acids production are very limited. In this study, five isolates of Antarctic bacteria were analysed for unsaturated fatty acids using gas chromatography-Mass spectrometry.

MATERIALS AND METHODS

Isolates of antarctic bacteria: The Antarctic bacteria used in this study were obtained from Enzyme and Microbial Technology Research Centre, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia. The bacteria included *Pseudomonas* sp., A3 (GenBank accession No: KR821141), *Pseudomonas* sp., A8 (GenBank accession No: KC977308), *Arthrobacter* sp., 1B (GenBank accession No: KT124223), *Arthrobacter* sp., 3B (GenBank accession No: KT072051) and *Arthrobacter* sp., PB (GenBank accession No: KT124224).

16S rRNA identification of the bacteria: The bacteria were grown on nutrient agar plates at 15°C for 3 days. A loop of the colonies was transferred to 10 mL nutrient broth (Difco™ USA) and grown at 15°C, 200 rpm for 24 h. Genomic DNA was extracted using DNeasy(R) genomic DNA extraction kits (Qiagen) according to manufacturer's instructions and analyzed on 1% (w/v) agarose gel. The DNA size was estimated using *Hind*III DNA maker (Fermentas) (Sambrook and Russell, 2001).

Polymerase Chain Reaction (PCR) was performed on PCR thermocycler (G-Storm) using 50 μL reaction mixture which contained 2 μL genomic DNA, 19 μL dH_2O, 2 μL each for forward and reverse universal primers and 25 μL of 2x PCR master mix (Thermo scientific, 0.05 u μL^{-1} taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$ and 0.4 mM of dNTP). The PCR was incubated at 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 58°C for 30 sec and 72°C for 1 min and a final extension at 72°C for 10 min. The PCR

product was purified using gel purification kits (GeneAll® kits) and sequenced at 1st BASE (Sdn, Bhd, Malaysia). The sequences were submitted to National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast) for nucleotide blast search, aligned and used for phylogenetic tree analysis. In addition to 16S identification, the bacteria were subjected to some morphological, cultural and biochemical studies.

Preparation of fatty acid methyl esters: The bacteria were grown on nutrient agar plates (Liofilchem, Italy) at 4°C for 7 days. Fatty acid methyl esters were prepared according to MIDI protocol (Microbial Identification system, Microbial ID Inc., Newark, DE, USA). Approximately 40 mg of weight cells was suspended in 1 mL NaOH (15% w/v) in methanol/water (1:1, v/v) and saponified at 100°C for 30 min. Methylation was performed by adding 2 mL of 6N hydrochloric acid in methyl alcohol (13:11, v/v) at 80°C for 10 sec. The FAMEs were extracted using 1.25 mL of hexane (SupraSolv®, Germany) in tert-butyl-methyl ether (1:1, v/v) (Sigma, Aldrich) and vortex mixed for 10 min. Sample wash was carried out using 3 mL of sodium hydroxide in distilled water (1.2%, w/v) and vortex mixed for 5 min. An aliquot of organic phase was placed in a GC vial for analysis.

Gas Chromatography Mass Spectrometry (GCMS) analysis: The FAMEs were analysed by GCMS (QP 2010 GCMS, Shimadu, Japan) equipped with 30-m HP-5 column (0.25 mm×0.25 μm) using helium as a carrier gas and a linear velocity of 30 cm sec^{-1}. The column temperature was initially kept at 40°C for 1 min with ramping at 2°C min^{-1} and then raised to 240°C. Then, the heating temperature increased to 300°C with ramping at 30°C min^{-1} and finally held at 300°C for 6 min whereas, the injector temperature was held at 250°C (Li *et al*., 2008).

Statistical analysis: The data was analysed using a one-way ANOVA of variance which showed significant difference with $p<0.05$ (Mohamed *et al*., 2016).

RESULTS AND DISCUSSION

The 16S rRNA identification which was supported by cultural, morphological and biochemical tests confirmed that the bacteria belonged to *Pseudomonas* and *Arthrobacter* species based on nucleotide blast search at National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/

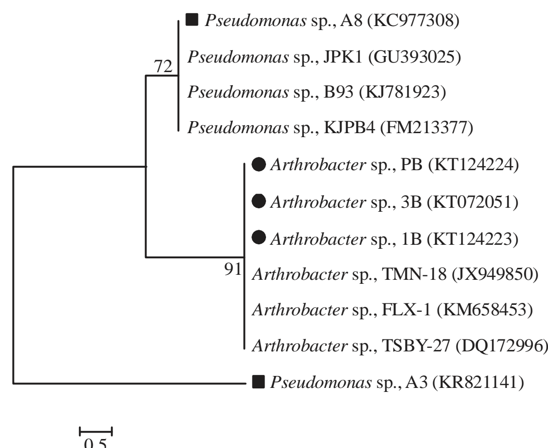
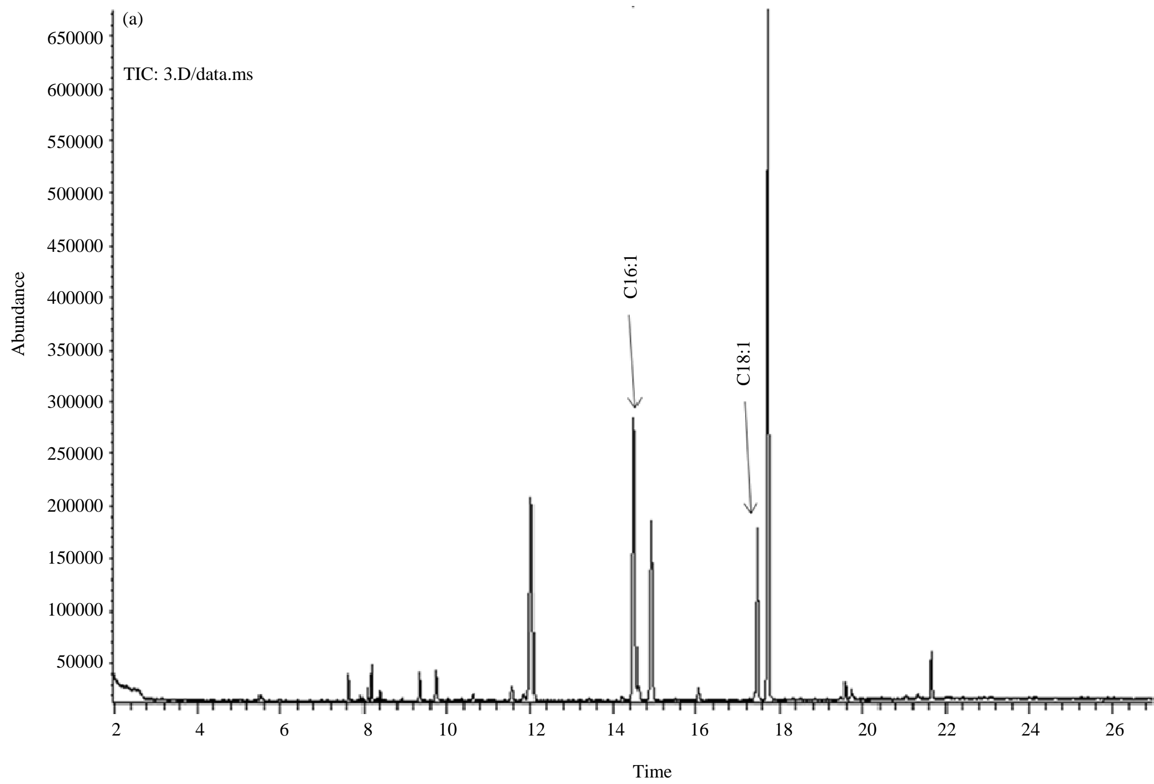


Fig. 1: Phylogenetic analysis of *Arthrobacter* and *Pseudomonas* species constructed using neighbour-joining method (Saitou and Nei, 1987)

blast). Therefore, the *Pseudomonas* species were named as *Pseudomonas* sp., A3 and *Pseudomonas* sp., A8 whereas the *Arthrobacter* species were named as *Arthrobacter* sp., 1B, *Arthrobacter* sp., 3B and *Arthrobacter* sp., PB (Fig. 1). Phylogenetic analysis revealed a far distant relationship between the *Pseudomonas* sp., A3 and *Pseudomonas* sp., A8 whereas a close relationship was observed among the *Arthrobacter* species. However, the bacteria reported in this study revealed a close relationship with their respective family members available in the public database (http://www.ncbi.nlm.nih.gov/blast).

Table 1 shows the overall fatty acids of different chain lengths and degrees of unsaturation analysed from the bacteria. Both saturated and unsaturated fatty acids were identified from all the bacteria except *Arthrobacter* sp., 1B which contained only saturated fatty acids. The *Arthrobacter* sp., 1B had the highest saturated fatty acids contents of 98.03% followed by *Arthrobacter* sp., PB (61.27%), *Pseudomonas* sp., A3 (53.50%), *Arthrobacter* sp., 3B (52.01) and *Pseudomonas* sp., A8 (47.30%). Moreover, in terms of total unsaturation, *Arthrobacter* sp., 3B had the highest percentage (47.24%) followed by *Pseudomonas* sp., A8 (45.09%), *Pseudomonas* sp., A3 (33.17%) and *Arthrobacter* sp., PB (31.92%). The GC-MS analysis confirmed palmitoleic acid (C16:1) and oleic acid (C18:1) as the only unsaturated fatty acids observed in the bacteria (Fig. 2). Although, there was no evidence of polyunsaturated fatty acids production based on the GCMS analysis, the C16:1 and C18:1 observed in this study constitute the main ingredients of polyunsaturated fatty acids synthesis including



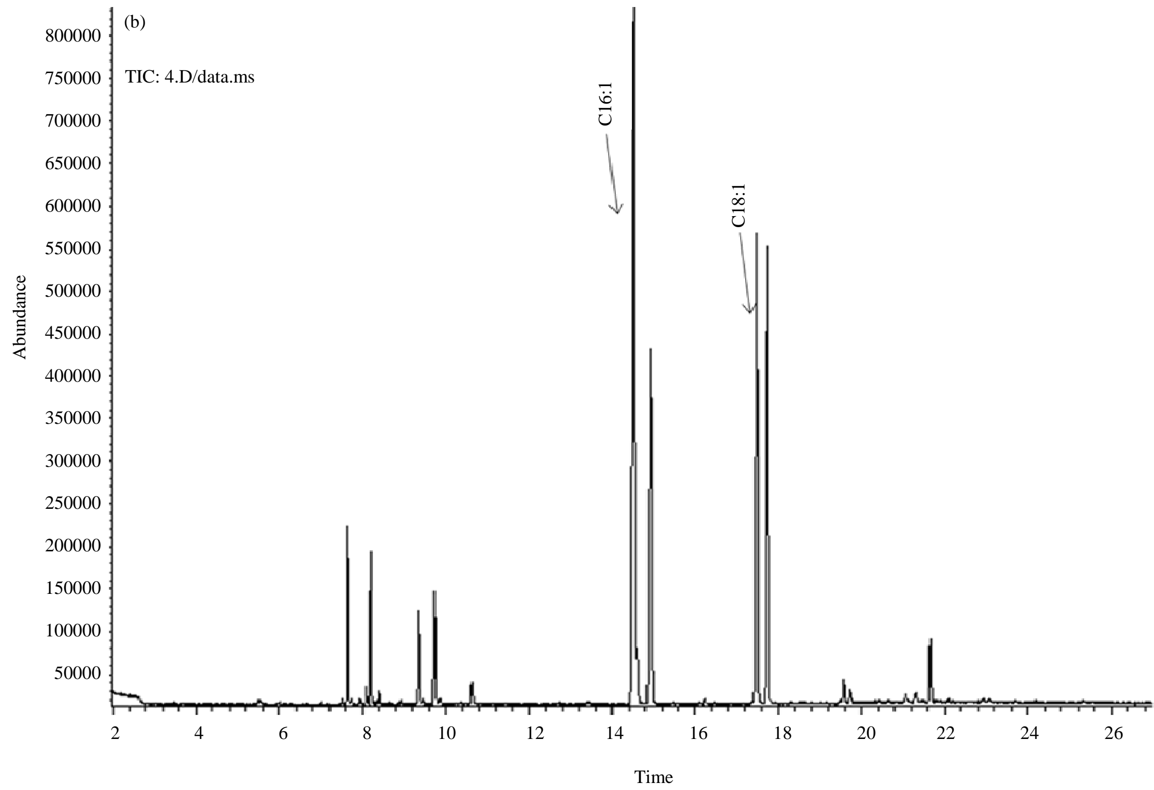


Fig. 2: Continue

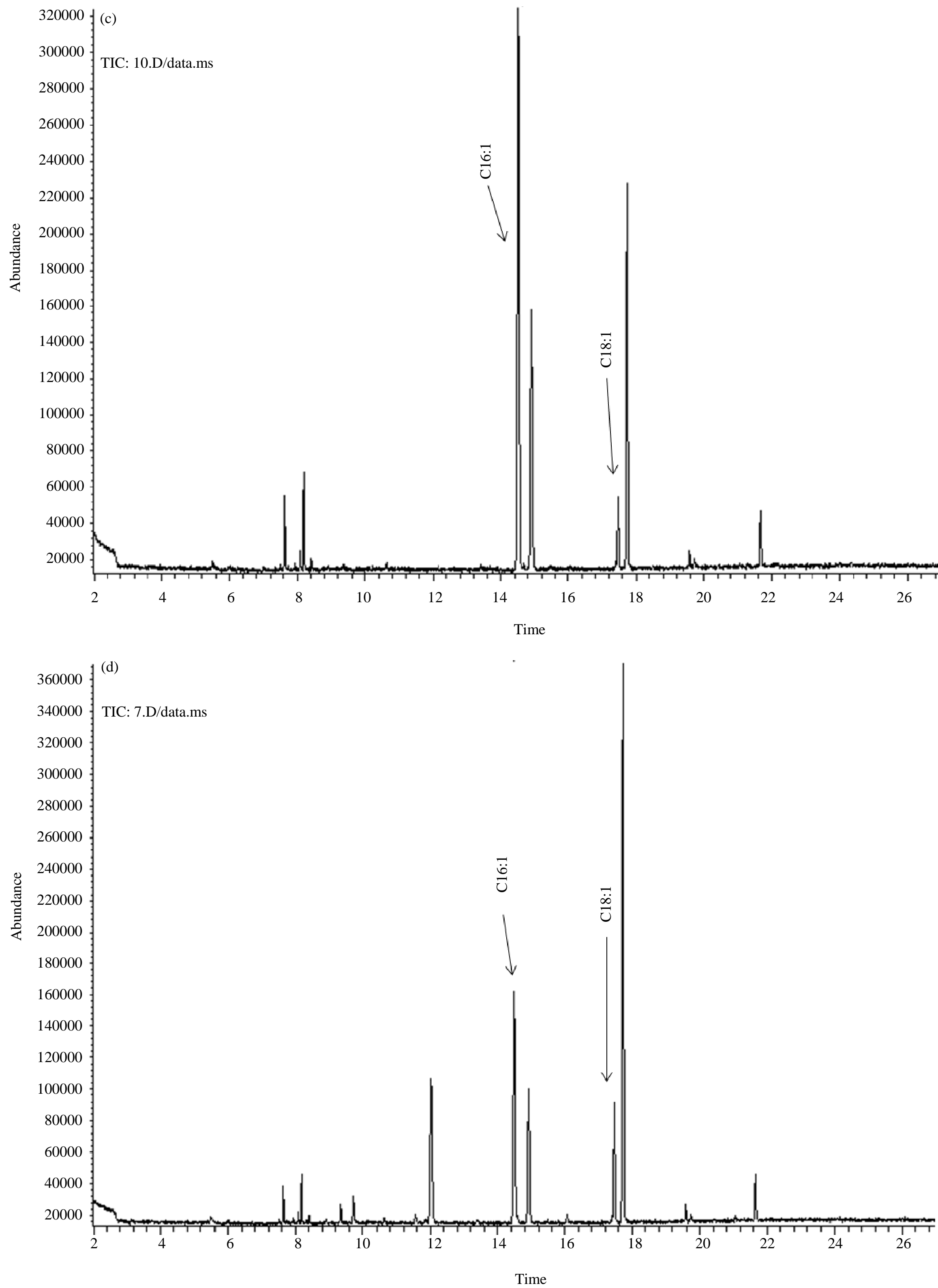


Fig. 2(a-d): Gas chromatograms of monounsaturated fatty acids. Peaks representing Palmitoleic (16:1) and stearic acids (18:1) are indicated with an arrow on each GC chromatogram for (a) *Pseudomonas* sp., A3, (b) *Pseudomonas* sp., A8, (c) *Arthrobacter* sp., 3B and (d) *Arthrobacter* sp., PB as identified using MS library

Table 1: Fatty acids profiles of Antarctic *Arthrobacter* and *Pseudomonas* species

	Bacteria				
FAME (%)	*Pseudomonas* sp., A3	*Pseudomonas* sp., A8	*Arthrobacter* sp., 1B	*Arthrobacter* sp., 3B	*Arthrobacter* sp., PB
C8:0	1.73	nd	nd	nd	nd
C9:0	2.12	nd	nd	nd	nd
C10:0	2.16±0.04	nd	nd	10.59	7.1
C12:0	3.89	9.88±0.19	23.06	nd	nd
C14:0	2.20	nd	20.19	nd	nd
C15:0	nd	nd	26.68±0.54	nd	14.86
C16:0	16.28±5.15	14.51±1.01	3.60±1.85	23.36	13.63±3.66
C18:0	32.12±1.86	29.91±3.68	24.50±0.29	25.06	32.78±3.54
ΣSFA[(a)]	60.50±7.05	54.3±4.88	98.03±2.68	59.01	68.37±7.20
C16:1	22.16±4.50	31.23±1.79	nd	43.12	22.06±2.46
C18:1	11.01±3.02	13.86±3.34	nd	4.12	9.86±2.04
ΣUFA[(b)]	33.17±7.52	45.09±5.13	0.0	47.24	31.92±4.50

The overall fatty acids identified from the bacteria according to MS library. The bacteria were grown at 4°C for 1 week and analysed using gas chromatography-mass spectrometry as explained in the methodology, the results are means of independent experiments±standard deviation, data highlighted in bold represent the [(a)]Total saturated, [(b)]Unsaturated fatty acids from each bacterium are significantly different with p<0.02, nd: Not determined

Omega 3, 6 and 7 fatty acids, very important fatty acids for healthy growth and physiological activities. Moreover, C16:1 and C18:1 are the most abundant unsaturated fatty acids found in many Antarctic bacteria (Moyer and Morita, 2007). Furthermore, microbial lipids could be in the form of saturated and straight-chain, straight monounsaturated, branched-chain and cyclopropane (Mishra *et al*., 2015).

It has been shown that the chances of polyunsaturated fatty acids production such as 22:6 and 20:5 by Antarctic bacteria become higher at very low temperature approaching 0°C (Moyer and Morita, 2007) which is contrary to the incubation temperature used in this study and could be the main reason for the lack of polyunsaturated fatty acids in this findings. According to many studies, monounsaturated fatty acids were the predominant fatty acids observed from various Antarctic bacteria including *Micrococcus cryophilus* (Li *et al*., 2008), *Pseudoalteromonas* sp., MLY15 (Li *et al*., 2009), *Halomonas* species (Jadhav *et al*., 2013), *Flavobacterium* and *Flectobacillus* species (Dobson *et al*., 1991, 1993) and many other bacterial species (Jadhav *et al*., 2010; Russell and Nichols, 1999). Our findings are in line with these. Despite differences in cultivation temperatures and possibly other related factors, the high amount of saturated fatty acids observed in this study (50-100%, Table 1) is comparable to those revealed by many Antarctic bacteria such as isolates of BRI 1 (94.6%) and slightly disagrees with the amounts produced by isolates of BRI 7 and 28 (40-47%) (Jadhav *et al*., 2010). Generally, higher amount of unsaturated fatty acids and/or a shorter acyl-chain length in Antarctic bacteria correlates highly with lower growth temperatures (Chintalapati *et al*., 2004; D'Amico *et al*., 2006).

CONCLUSION AND FUTURE RECOMMENDATION

Palmitoleic acid (C16:1) and stearic acid (C18:1) were the major unsaturated fatty acids identified from all the bacterial isolates. Further study need to be carried out on the bacteria to produce polyunsaturated fatty acids using metabolic engineering.

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