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METABOLITE PROFILING OF *Scenedesmus regularis* USING NUCLEAR MAGNETIC RESONANCE (NMR)

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ABSTRACT

Metabolites represent the end product of gene function and it reflects direct response of cells under certain conditions. Hence, metabolite profiling has become a powerful tool in examination of plant cell's response under certain conditions either *in vitro* or from tissue extract. This study aims to demonstrate how metabolite fingerprinting in microalgae in unfractionated extract. The results revealed the identification of 55 metabolites in unfractionated *Scenedesmus regularis* extract by using ^{1}H-NMR coupled with correlation spectroscopy (COSY).

Key words: Metabolites profiling, nuclear magnetic resonance (NMR)

INTRODUCTION

NMR is a branch of spectroscopy that bases its power on the fact that the atomic nucleus of each molecule resonates at different spin states. NMR spectra produces a breakdown of all the detectable proton signals contained in a single metabolite (Holtin *et al*., 2009; O'Connell & Jia, 2012). 2D NMR combines 1D NMR into a 2-planar analysis thus producing a spectrum which consists of a diagonal and contour patterns (Mahrous & Farag, 2015) and helps overcome spectral overlap by increasing signal dispersion and explicates connectivity between signals. When overlapping signals can be suppressed, certain peaks, which were hidden in 1D NMR can be revealed and identified.

For identification of 1D NMR spectra, a few important things to note are its; i) chemical shift, ii) signal multiplicity arising from spin-spin coupling of H atom and iii) signal intensity/ integration. All these points are important factors in determining the identity of the metabolites responsible for each peak. Chemical shift of each metabolite arises as relative to the chemical shift of the reference compound. In most cases, the standard reference compound is either tetramethylsilane (TMS), 3-(trimethylsilyl)-2,22,3,32-tetradeutero-propionic acid (TSP) or deuterated 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) in reference to the projected chemical resonance at 0 ppm. Peak patterns follow a spin-spin system which splits into either a singlet, doublet, triplet or so forth depending on the number of atoms that contribute to its neighbouring bonds while the concentration of each respective nuclei is translated into peak intensity. The splitting pattern and peak integration is good for identifying compound structure, however in metabolomics, both points represent an integral clue to a metabolite's identity. Due to the reliable and high throughput screening, metabolites profiling has become one of the important tools in omics study of algae (Ma *et al.*, 2015). Microalgae from the *Scenedesmus* genus is known for a high ability to produce high α-linoleic content thus making their oil content suitable for conversion to biodiesel (Fan *et al*., 2014; Sharma & Chauhan, 2016). Apart from

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being good biofuel feedstock candidates, several species from the genus has also been reported with high efficiency in removing pollutants and nutrients such as excess organic, inorganic nitrates and phosphates from wastewater making them potential candidates in bioremediation. Not much is known regarding *S. regularis* while use of the species in the literature has not been widely reported apart from studies involving carbon dioxide mitigation (Hazlina *et al*., 2016) and in evaluation of disruptive techniques (Ma *et al*., 2015).

MATERIALS AND METHODS

Microalgae extraction and data acquisition

Scenedesmus regularis was cultured in Guillard's F2 medium (Cha *et al*., 2011) and was harvested by centrifugation at 5000 g at 4°C for seven minutes. The concentrated pellet was rinsed once with 0.5 M of ammonium bicarbonate and again with distilled water. Ammonium bicarbonate was used to ensure removal of excess salts from the culture medium. A total of 0.215 g of sample was extracted using chloroform and aqueous solvent in 1:1 ratio (Ma *et al*., 2018). ^{1}H-NMR profile was obtained using Bruker DRX-400 advance (400 MHz) spectrometer (Bruker, Fremont, CA) with temperature maintained at 300k, parameter set at 9-Is (60) pulse, 6-kHz spectra width and 2.5-s relaxation delay. A total of 300 transients were collected in ^{1}H and 80 transients for COSY experiment (Ma *et al*., 2015).

NMR spectra processing

Analysis and data processing (phase correction, baseline correction, and reference of NMR peaks) was carried out in Mnova (Ver 9.0 – Mestrelab Research, Escondido, CA, USA) and Topspin (Ver 3.2 – Bruker) software. NMR peaks were then identified according to integration, assigned chemical shifts and peak multiplicity by using online NMR depositories and databases (Human Metabolome Database; HMDB (http://www.hmdb.ca), Biological Magnetic Resonance Bank; BMRB (http://www.bmrb.wisc.edu), Yeast Metabolome Database; YMDB (http://www.ymdb.ca/)).

RESULTS AND DISCUSSION

Metabolites extracted from aqueous solvent

A total of 23 metabolites were detected and identified from aqueous extraction (Figure 1). Spectra was run with water suppression parameters to increase visualisation of low frequency metabolites. Residual lipoprotein signals were detected at δ 0.88 as a dishevelled multiplet. The residual lipoprotein signals did arise from the hydrophilic part of lipoproteins.

Eight protons (two from methyl CH_3 and two from CH groups) gave rise the characteristic signal of valine with visible doublets at δ 0.98 and δ 1.03 which slightly overlapped with leucine as leucine also produced doublets at δ 0.95 (Govindaraju *et al*., 2000). The difference for leucine is a multiplet at δ 1.72. However, the remaining valine multiplets at δ 2.2 and δ 3.63 were not visible in the 1D spectra due to spectral overlap with gamma-Aminobutyric acid (GABA) and sucrose respectively (Figure 1a) but was identified from cross-peaks in COSY spectra (Figure 1b).

Alanine signals arised from CH_3 and CH proton groups resulting in a visible doublet at δ 1.48 and

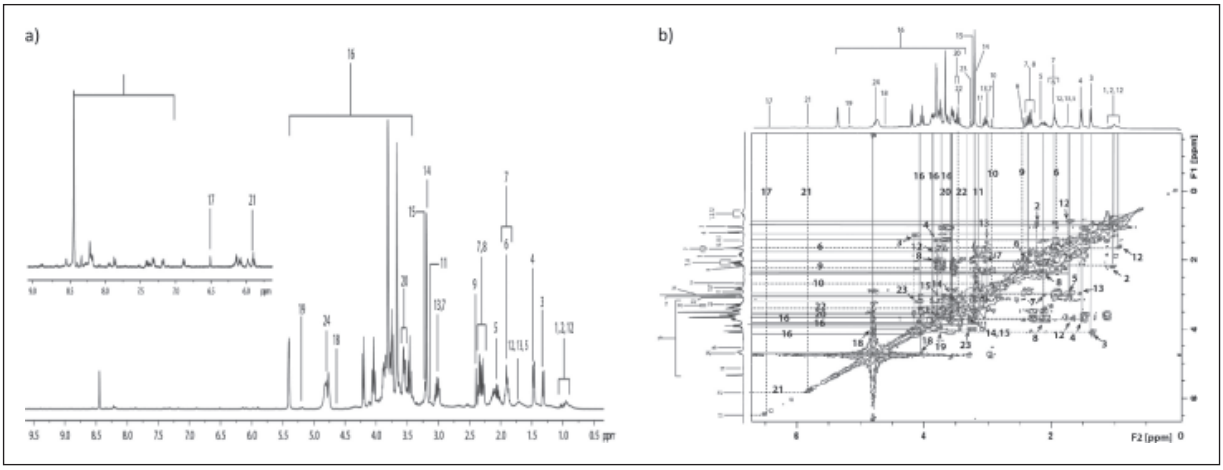

Fig. 1. Representative 1D ^{1}H-NMR spectra of aqueous extracts obtained from aqueous *S. regularis* samples; b) 2D COSY spectra of aqueous *S. regularis* extract.

Key for spectra: *1 lipoproteins, 2 Valine, 3 Lactate, 4 Alanine, 5 Spermine, 6 Acetic acid, 7 GABA, 8 Glutamate, 9 Succinate, 10 Trimethylamine, 11 Ethanolamine, 12 Leucine, 13 Lysine, 14 Choline-1, 15 Choline -2, 16 Sucrose, 17 Furmarate, 18 α-glucose, 19 β-glucose, 20 Glycerol, 21 Uridine, 22 Glycine, 23 Betaine, 24 Water, 25 Nucleoside.*

a highly overlapped multiplet at δ 3.76. GABA produced resonances at δ 1.91, δ 2.31 and δ 3.03 due to three CH_2 groups of protons arising from α-CH_2, β-CH_2 and γ-CH_2 atomic nuclei. The signal at δ 1.91 corresponding to α-CH_2 was visible but was overlapped with a singlet from acetic acid (Figure 1a). The identification was resolved with coordinates of GABA cross-peaks (δ 2.1, δ 2.3; δ 2.1, δ 3.03) (Figure 1b). Glycine produced a single visible peak at δ 3.55 which did arise from a two methylene group protons which co-resonated at the same chemical shift (Govindaraju *et al.*, 2000). Glutamate is an amino acid possessing an acidic side chain with signals arising from two methylene groups and a methine group that are strongly coupled (Govindaraju *et al*., 2000). Glutamate resonances were highly overlapped in Figure 1 but were identified from its cross-peaks at (δ 2.1, δ 3.77; δ 2.1, 2.33) on the COSY spectra.

Amine containing compounds detected included spermine, choline, ethanolamine, betaine and trimethylamine (Figure 1). In *S. regularis*, the prevailing polyamine was spermine, producing multiplet signal at 2.12 ppm (Figure 1). Cross-peaks confirming presence of two more resonances were observed at (δ 1.71, δ 3.14; δ 1.71, δ 2.12) (Figure 1b). Spermine is involved in nucleic acid synthesis and cell division (Kneifel, 1979). Choline produced two split signals at δ 3.19 and δ 3.21. Choline-2 produced a prominent singlet at 3.19 ppm and is related to resonance from free choline while Choline-1 singlet at 3.20 ppm. Spectra did show a singlet at δ 3.25 corresponding to betaine and was confirmed by the cross-peak at (δ 3.25, δ 3.89). Ethanolamine peaks were not very clear in 1D spectra however cross-peaks were present at (δ 3.14, δ 3.85) (Figure 1b). Trimethylamine produced resonance of a singlet at δ 2.89 which resulted from three methyl groups.

Sucrose, a main carbon storage in microalgae has observed resonances at δ 3.46, δ 3.54 and δ 3.66 from glucose moiety and δ 3.81, δ 4.05, δ 4.21, δ 5.4 from fructose moiety. High intensity at the sugar region predominantly coincides with resonance of sucrose therefore establishing sucrose as the major constituent of sugar produced in microalgae cells for carbon storage. Sugars form many resonances due to their lengthy carbon chain but type of sugar from a mixture may still be identified from 1D and 2D spectra based on diagnostic signals specific to each sugar compound (Bubb, 2003). Minor sugars such as α-glucose and β-glucose were observed to have small diagnostic resonances at δ 3.81 and δ 4.62 and cross-peaks corresponding to OH groups were observed in 2D COSY (Figure 1b).

Tricarboxylic (TCA) cycle intermediates, fumarate and succinate were also detected. Succinate had signals arising from four protons, which coupled to form a singlet resonating at δ 2.40. Fumarate had a single singlet, which was raised from CH group that resonates at δ 6.5. Glutamate can also be indirectly linked related to TCA cycle through its conversion to α-ketaglutarate (a TCA cycle intermediate).

Metabolites indicative of fermentative pathways were also identified. Lactate and acetic acid were found to produce a signal at δ 1.33 and δ 1.91 respectively. Signal from CH group in lactate was not visible in spectra due to overlap but cross-peak with CH group nuclei was present in 2D COSY (Figure 1). Glycerol produced an obvious doublet of doublets centred at δ 3.55 and δ 3.65 and multiplet at δ 3.77 was visible at 2D COSY (Figure 1b)

Metabolites extracted from chloroform solvent

There were 22 metabolites that can be detected in chloroform extract (Figure 2). Long hydrocarbon chains possessing a multitude of signals tend to overlap and crowd at similar ppm making chemical identification complicated. Fatty acid chains produce signals of specific type of protons that can be characterized according to distinct regions found along the fatty acyl chains (Figure 2).

Fatty acids can be un-/saturated or branched/ straight and are basically made up a long aliphatic hydrocarbon chain with a methyl group and carboxylic group attached at either end of the chain. The signals produced by the protons linked to the double bonds from polyunsaturated fatty acids (PUFA) formed a multiplet signal at δ 5.4. The allylic chain protons gave rise to signals at δ 2.06 and δ 2.81 while protons located near the carboxylic end of the chain produced signals at δ 1.61 and δ 2.34. Protons situated along the long saturated carbon chain gave a very obvious multiplet at δ 1.25. The methyl-end gave rise to signals at δ 0.98 and signal at δ 0.88. Connections between the alkyl chains of fatty acid can also be observed from 2D COSY (Figure 2b).

Signals related to carotenoid are formed at the region of δ 6.14 – δ 6.64. Signals from a common structure in all carotenoids are attributed to a fragment of conjugated trans double bonds, represented chemically as –(CH_3-)-C=C**H**-C**H**-C**H**-C–(CH_3-)- (Sobolev *et al*., 2005). The protons (in bold) give rise to signals of multiplets at δ 6.14, δ 6.64 and δ 6.35 in the literature. The presence of lutein was not easily determined from 1D spectra. However, diagnostic cross-peaks related to signals of lutein were observed in 2D COSY spectra (Figure 2b). Concentration of a certain metabolite in the extract may sometimes influence the intensity of signals on both 1D and 2D. Since 2D is more sensitive due to the higher number of scans than 1D experiments, it is sometimes possible to detect molecules that do not show up on 1D spectra. *S.*

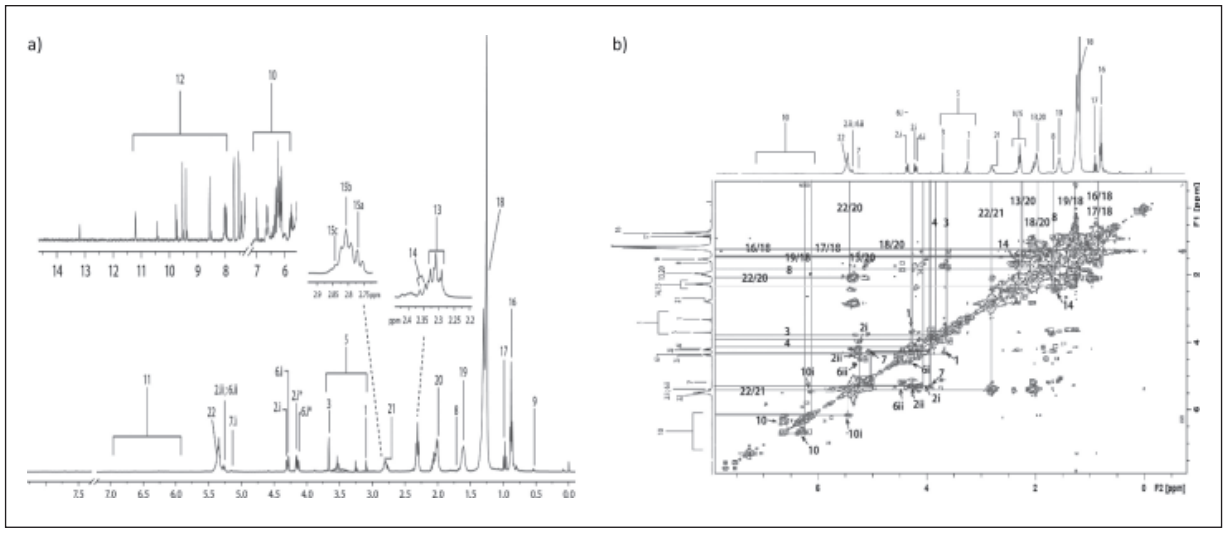

Fig. 2. Representative 1D ^{1}H-NMR spectra of chloroform extracts obtained from *S. regularis* samples; b) 2D COSY spectra of chloroform *S. regularis* extract.
Key for spectra: *1 Phosphatidylcholine, 2 Glyceroglycolipids, 3 Phosphatidylglycerol, 4 Cardiolipin, 5 Phospholipid, 6 Triacylglycerol, 7 Diglyceride, 8 Squalene, 9 Sterol, 10 Carotinoids, 10i Lutein, 11 Olefinic proteins (alkene), 12 Aldehyde, 13 Ester, 14 Free fatty acid, 15 PUFA, 16 Methyl end, 17 PUFA methyl end, 18 Alkyl chain, 19 Carboxylic end, 20 Alkyl chain (allylic), 13* Carboxylic end, 21 Alkyl chain, 22 Double bond.*

regularis is a species markedly known as a major synthesiser of lutein carotenoid (Guedes *et al*., 2011; Yen *et al*., 2012). Literature review has pointed out the diagnostic cross-peak for lutein to be at (δ 5.46, δ 6.16) (Sobolev *et al*., 2005). In this study, cross-peaks corresponding to (δ 5.46, δ 6.16) were detected therefore proving the presence of lutein in the extract (Figure 2).

As a general rule, the proton attached to C-18 moiety of methyl groups belonging to sterol compounds will resonate at δ 0.6 – δ 0.8 (Sobolev *et al*., 2005). However, methyl group of C-18 sterol structures of different sterols often resonate at different chemical shifts. This study produced a signal at δ 0.53, pointing to presence of ergosterol-like sterols in the mixture (Bonzom *et al*., 1999).

Chemical shifts coinciding with signals related to phospholipids are usually detected in the region δ 3.0 – δ 4.05 (Stringer *et al*., 2011; Sarpal *et al*., 2015). Signals corresponding to phosphate head groups (CH_2OP) connected to phosphatidylcholine were detected δ 3.23 in 1D spectra. Diacylglycero-phospholipids (glyceroglycolipids), another major component in ensuring permeability of the plasma membrane (Fan *et al*., 2014) presented signals at δ 4.16, δ 4.38 and δ 5.26. Triacylglycerols (TAG), which are made up of glycerol group attached to three fatty acids present signals from glycerol moieties. Glycerol moieties were detected along different *sn1* positions along the chain at δ 4.31, δ 4.14 and δ 5.26 with cross-peaks similarly detected between the resonances in 2D COSY (Figure 2b).

Alkenes / fatty alcohols (olefinic protons) and aldehydes produced characteristic signals at δ 5.8 – δ 6.8 and δ 8.0 – δ 11.0 respectively (Sarpal *et al*., 2015). These signals are a mixture of signals from fatty hydrocarbons and conjugated or non-conjugated protons of oxylipins of PUFAs such as DHA (Sarpal *et al*., 2015).

CONCLUSION

NMR is proven to be powerful, unbiased and non-destructive method that enable the identification of metabolites from microalgae. There are 55 metabolites identified from *S. regularis* and can be grouped into 4 important pathways, namely calvin cycle, TCA cycle, lipid biosynthesis and amino acid biosynthesis cycle. The complexity of lipid fraction extracted using chloroform should be further examined using other 2D method such as Total Correlation Homonuclear Spectroscopy (TOCSY), Heteronuclear Multiple Bond Correlation (HMBC) and Homonuclear J-Resolved Spectroscopy (JRES). High content of lipid fractions identified from chloroform extract suggests high oil body in *S. regularis* and hence suitable for biofuel application.

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