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Antibacterial Activity of Fine Chemicals from *Scenedesmus acuminatus* (Scenedesmaceae, Chlorococcales, Chlorophyta)

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ABSTRACT Microalgae, *Scenedesmus acuminatus* was batch-cultured in a photo-bioreactor to facilitate better culture control and higher productivity. Each batch was cultured for 100 hours; final cell density and specific growth rate were 5.0 x 10^7 cell / ml and 0.028 $hour^{-1}$, respectively. The harvested biomass produced 70.80 mg crude extract with antibacterial activities. Bioassay guided separation and spectroscopy data revealed a mixture of fatty acid methyl esters (FAME) (C16:0, C16:1, C18:1n9c, C18:2n6c and C20:0) as the active principal. Further antibacterial bioassay with commercially available individual FAME revealed C18:1n9c methyl ester and C18:2n6c methyl ester, as the active compounds in the mixture. The active methyl esters, C18:1n9c and C18:2n6c, inhibited 48% (MIC: 0.3 $\mu gdisc^{-1}$) and 100% (MIC: 0.3 and 1.0 $\mu gdisc^{-1}$) of tested bacteria, respectively.

ABSTRAK Microalga, *Scenedesmus acuminatus* dikultur secara "batch culture" dalam foto-bioreactor untuk memastikan kawalan pengkulturan dan produktiviti yang tinggi. Setiap "batch" dikultur selama 100 jam dengan kepadatan akhir sel sebanyak 5.0 x 10^7 sel / ml dan kadar pertumbuhan setinggi 0.028 jam^{-1}. Biomass terkumpul menghasilkan 70.80 mg ekstrak mentah dengan aktiviti antimikrob yang ketara. Pemencilan berasaskan bioesei and data spektroskopi telah memberikan identiti sebatian aktif sebagai campuran metil ester asid lemak (FAME) (C16:0, C16:1, C18:1n9c, C18:2n6c and C20:0). Bioesei antibakteria dengan FAME komersial telah membuktikan metil ester C18:2n6c and metil ester C18:2n6c sebagai sebatian aktif dalam campuran FAME. Metil ester aktif, C18:1n9c dan C18:2n6c, menunjukkan perencatan terhadap 48% (MIC: 0.3 $\mu gdisc^{-1}$) dan 100% (MIC: 0.3 dan 1.0 $\mu gdisc^{-1}$) bakteria.

(Microalgae, *Scenedesmus acuminatus*, antibacterial activity, FAME)

INTRODUCTION

Freshwater microalgae of the genus *Scenedesmus* is an important microorganism in aquaculture, pharmaceuticals, food industry and bioremediation. It is an ideal organism in any biotechnological application due to its relatively short life-cycle, high contents of natural chemical and ability to use excess nutrients during bioremediation [1, 2, 3, 4]. There are about 200 species of phytoplankton in this genus [5], and known as ideal organisms in treatment of wastewater and removal of pollutants from waterways. Some of the biomass produced from treatment of organic waste can be used as a cheap source of protein and aquaculture feeds [6, 7, 8, 9]. In addition, some species has the potential to be a source of biologically active primary and secondary metabolites [10].

Although numerous species of *Scenedesmus* has been used in various research applications, but there are still some species whose potential as producers of fine chemicals are not fully investigated. One such species is *Scenedesmus acuminatus,* commonly found growing in fresh water ponds that are moderately undergoing "eutrophication". Most of the available data are in relation to their taxonomy, culture parameters and nutrient contents [11, 12, 13, 14], and almost none on its fine chemicals [15]. With the advent of high density microalgae culture using "photo

bio-reactor" and commercialization of high quality nutraceuticals, intensive culture of bioactive producing microalgae could pave the way to offset the increasing cost of production. Hence, present investigation involves culture, extraction, isolation and structure elucidation of compounds with antibacterial properties from the green microalga, *Scenedesmus acuminatus*.

MATERIALS AND METHODS

Microalgal Strain and Culture Media Composition

A pure strain of *Scenedesmus* acuminatus (UMS0132/02 was obtained from the School of Science and Technology, Universiti Malaysia Sabah microalgae stock collection. Culture and maintenance of the strain was done using Bristol medium (modified version of Bolds basal medium) [16]. Modification of the medium composition was done to obtain optimum culture condition.

Culture Conditions and Biomass Production

Batch culture of *S. acuminatus* was carried out in a 1L photo-bioreactor with aeration, and temperature (25 - 26°C) regulated using a circulating cooler (Protech Model 632D, Malaysia), and lighting regulated (10 000 lux, 12:12h (L.D) [17, 18]. Aeration was provided by bubbling the inoculated media with filtered plain air, at a constant flow rate of 22.5 Lh^{-1}, duly monitored by flow meter (Aalborg). Each batch of Bristol medium was inoculated with 1.0×10^5 cells / ml and cell growth was quantified every 8 hours. Cell density was determined by removing triplicates of 1.0 ml culture medium and evaluated using a haemocytometer under compound microscope at 40x magnification (Carl Zeiss, Germany). Biomass was regularly harvested before the stationary phase, at approximately 72 hours after inoculation. Collected cultures were centrifuged at 6000 rpm for 20 minutes; resulting pellets were kept frozen until extraction and chemical analysis.

Extraction and Chemical Analysis

Frozen pellets (5.547g w/w) were soaked in 200 ml of methanol for four days, filtered with 0.45 µm Whatman filter, evaporated, and dissolved in diethyl ether and partitioned with distilled water. The resulting diethyl ether solution was dried over Na_2SO_4 anhydrous and concentrated to yield 70.8mg of dark green crude extract. Isolation of antibacterial bioactive constituents was carried out based on the "Bioassay Guided Isolation" protocol. Crude extract, approximately 50 mg was spotted on 20 x 20 cm SiO_2 Gel $F_{254\ nm}$ Preparative Thick Layer Chromatography, developed in Hexane: Ethyl Acetate (3:1) and visualized using UV light (254 nm) and molibdo-phosphoric acid spray. Visualized compounds were isolated, concentrated, and were further spotted on analytical SiO_2 F_{254} TLC to confirm their purity [19]. Pure compounds were further tested for their antibacterial activity *via* AA-disc antibacterial method; the resulting bioactive isolate was subjected to ^{1}H-NMR measurements to predict the tentative identity of the compound.

Fatty Acid Methyl Ester Analysis

FAME mixture (4 mg) was diluted in 2 ml of GC Grade (Merck) hexane prior to analysis. The conditions for Gas Chromatography measurements were as follows: Gas Chromatography, Shimadzu 2010 with FID detector; auto injector, AOC-20i (Shimadzu, Kyoto, Japan), column, BPX70 (0.25 mm i.d. x 60 m) (SGE, Australia); injection and detector temperature, 260 °C; column temperature 140 °C (4 min) – 4 °C/min (25 min) – 240 °C (30 min). The injection volume was 1 µl, the injection temperature 260 °C, split ratio 1:100, carrier gas helium and the detector temperature was set at 260 °C. The column temperature regime was 140 °C for 4 min, followed by 4 °C/min. FAME peaks were identified by comparison of their retention times with those of a standard mixture (Spelco 37 mixture FAME) (Spelco, UK). Shimadzu Class VP software (version 2.1) was used to integrate peak areas.

Antibacterial Bioassay

Antibacterial bioassay was carried out using seven types of marine environmental bacteria: *Clostridium cellobioparum* (MBSP231), *Clostridium sordelli* (MBSP232), *Clostridium novyi* (MBSP233), *Proteus mirabilis* (MBSP234), *Proteus vulgaris* (MBSP235), *Vibrio parahaemolyticus* (MBSP236), and *Vibrio cholerea* (MBSP237). Marine bacterial strains were isolated from diseased seaweed surface/lesions from the coastal waters of Sabah. Bacteria were isolated, purified, identified and stocks made for bioassay experiments and other applications at the Laboratory of Natural Products Chemistry, Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah. One loopful of each bacteria was taken and inoculated into nutrient broth and incubated

at 28 °C for about 24 hours. AA paper discs were prepared by introducing 30 µl of test extract/fraction and pure compound at 200 g/mL (crude extract) and 30 µg/mL of pure compounds into sterile 6mm Whatman AA disc, respectively. Since the isolated natural bioactive compound consisted of inseparable mixtures of FAME, further bioassay to identify the active FAME was carried out using commercially available pure single C16:0, C16:1, C18:1n9c, C18:2n6c and C20:0 (Sigma, Germany). Impregnated AA discs were then placed on sterile nutrient agar petri dishes that were lawn with the respective bacterial suspension at a McFarland Index optical density of 0.5. These plates were incubated at 28 °C for 48 hours and their inhibition zones measured.

RESULTS AND DISCUSSION

Culture of *S. acuminatus*

Figure 1 shows the growth curve for *S. acuminatus* cultured in photo-bioreactor. Culture duration of the each batch was less than 100 hours, the culture started to enter the stationary phase after 70 hours. Final cell density and specific growth rate were calculated to be 50.0 x 10^6 cell ml^{-1} and 0.028 $hour^{-1}$, respectively. Close system photo-bioreactor was used as it provided greater control of culture conditions to facilitate higher productivity, higher quality and contaminant free biomass. High quality biomass is often needed for the isolation of valuable chemicals like high-value bioactive and various commercially important fatty acids [1, 20]. Multi-directional illumination created by vertical fluorescent tubes surrounding the culture flask supplied optimum light radiation [21]. The constant aeration also created a well-mixed culture to ensure constant and even photon flux density throughout the culture duration. Temperature was regulated between 25 - 26 °C as the optimum temperature for the culturing of *Scenedesmus* was in the range of 25 - 28 °C [17].

Other than temperature and light intensity, medium selection is also an important aspect. In this case, Bristol medium was chosen as it provides essential nutrients in certain ratios that ensure good growth with similar cell coenobia as that occurring in nature [5]. Normal cell morphology was observed in all the batch cultures, cells were observed to form from four or eight-celled coenobia and very seldom sixteen-celled coenobia. The eight-celled coenobia were often observed during the exponential phase while as the culture enters stationary phase most cells are either single or in pairs. This is due to the abundance of nutrients during the exponential phase and this enables auto-coenobium reproduction [22] while at the stationary or death phases, most nutrients are used up and toxicity levels are higher.

Each batch culture was harvested at the exponential phase as in this phase the cells are still active and healthy. The recovery of biomass was done through centrifugation as it was suitable for high-value products where the biomass collected remain fully contained and intact with minimum loss [23].

Isolation and Identification of Bioactive Metabolites

A total of 5547 mg of wet biomass was collected during the course investigation. Upon extraction, concentration and partition with diethyl ether and water, 70.80 mg of greenish paste like crude extract was obtained. Isolation of antibacterial metabolites was done based on the "Bioassay Guided Separation" methodology. First, stock crude solution of 200 µg/mL was prepared in MeOH and each AA disc was impregnated with 30µL of stock. Each AA disc contains 6.0 µg $disc^{-1}$. Crude extract exhibited potent inhibition (> 25 mm) against all the test bacteria, *Clostridium cellobioparum, Clostridium sordelli, Clostridium novyi, Proteus mirabilis, Proteus vulgaris, Vibrio parahaemolyticus, and Vibrio cholerea.* Upon Preparative Thick Layer Chromatography separation, seven isolates were diluted to a concentration of 100 µg/mL solution; discs of 3.0 µg $disc^{-1}$ were prepared and tested against all the seven bacterial strains. Out of the seven isolates only isolate 3 showed antibacterial activities against the tested bacteria. Isolate 3 represented 12.5 mg (17.6 %) of the crude extract, obtained from 5547 mg of wet micro-alga biomass. Isolate 3 was dissolved in $CDCl_3$ and subjected to ^{1}H-NMR measurement. As shown in Figure 2, presence of at least two well separated triplet peaks attributing to terminal methyl proton at δ 0.98 and δ 0.81 was observed. Other prominent peaks were; 1) a singlet peak at δ 3.78, attributing to methoxy proton, and 2) a multiplet peak at δ 5.42 that could be assigned for olefin protons. Furthermore, FTIR measurements of isolate 3 revealed the presence of carbonyl functional group at 1700 cm^{-1} and the absence of OH functionality.

Based on these data, it could be deduced that isolate 3 could be a mixture of fatty acid methyl ester (FAME). Further analysis of isolate 3 was carried out using Shimadzu GC 2010 under conditions described earlier. Figure 3 shows the Gas Chromatography spectrum obtained, describing the presence of five peaks corresponding to five species of FAME; C16:0 (18 %), C16:1(32 %), C18:1n9c (10 %), C18:2n6c (18 %) and C20:0 (22 %). Since the antibacterial activities of isolate 3 could have been exhibited by anyone of the available saturated and unsaturated FAME, commercially available corresponding fatty acids were purchased and tested. The purchased FAMEs were first analyzed using GC under the same condition to ensure their retention time corresponds to the natural compounds, 24.0, 25.1, 29.2, 30.6 and 32.5s. This confirms the similarity in the stereochemistry of natural and purchased FAME. Individual FAMEs were then tested for their antibacterial activities against the test bacteria at the concentration of 0.9 μg $disc^{-1}$. As shown in Table 1, only C18:1n9c and C18:2n6c showed antibacterial activities at 43 % and 100 % inhibition against the tested bacteria, respectively. Their Minimum Inhibition Concentrations were also comparable to natural metabolites that have been reported from our studies [24].

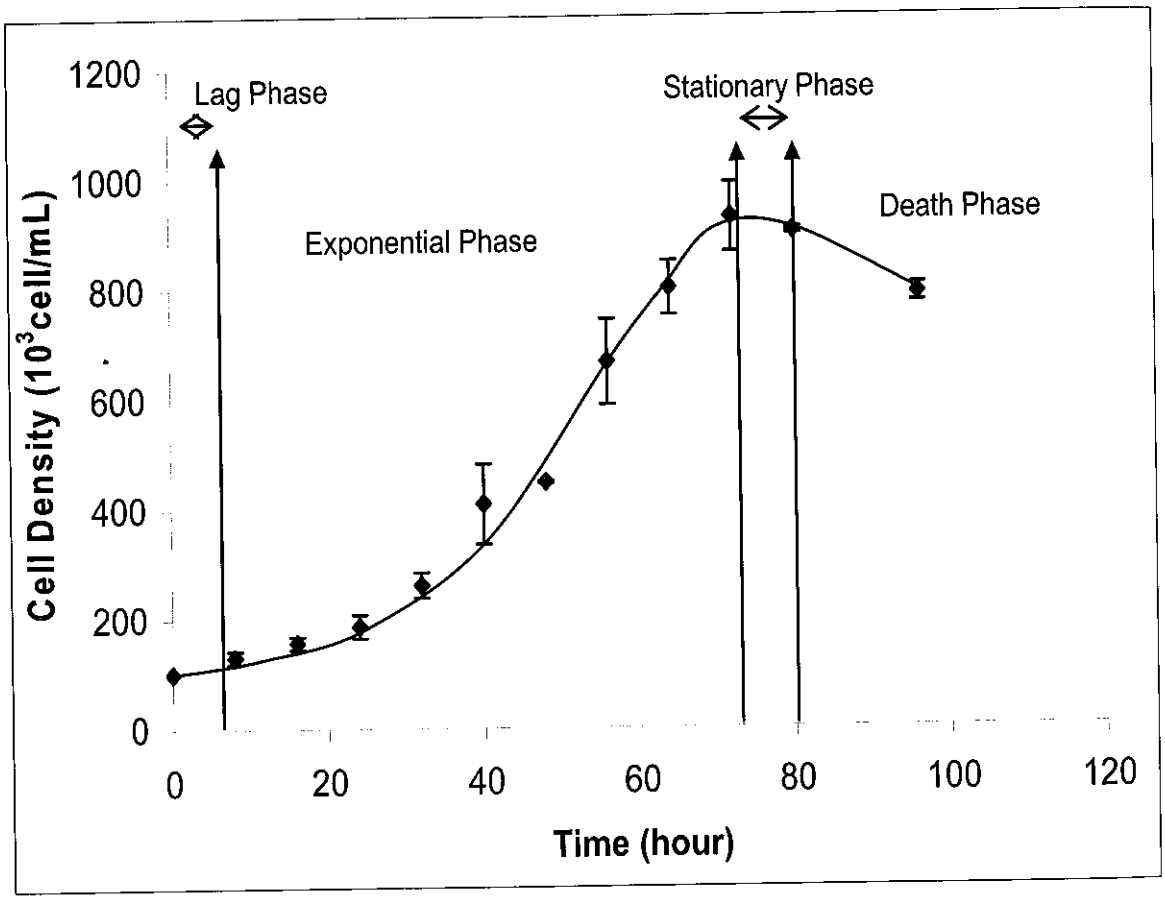

Figure 1. Growth curve of *S. acuminatus* cultured in Bristol media in a photobioreactor.

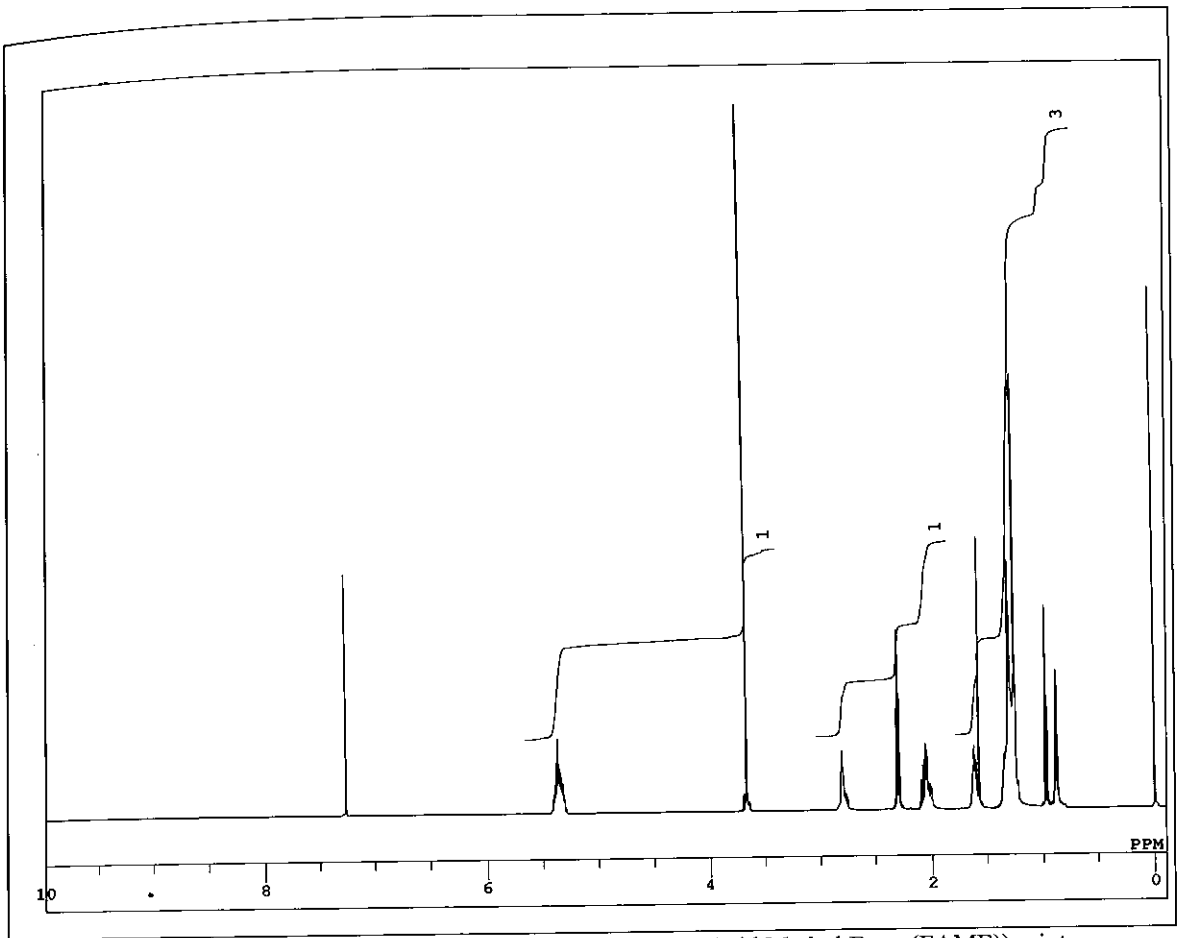

Figure 2. ^{1}H-NMR spectrum of the bioactive compound (Fatty Acid Methyl Ester (FAME)) mixture.

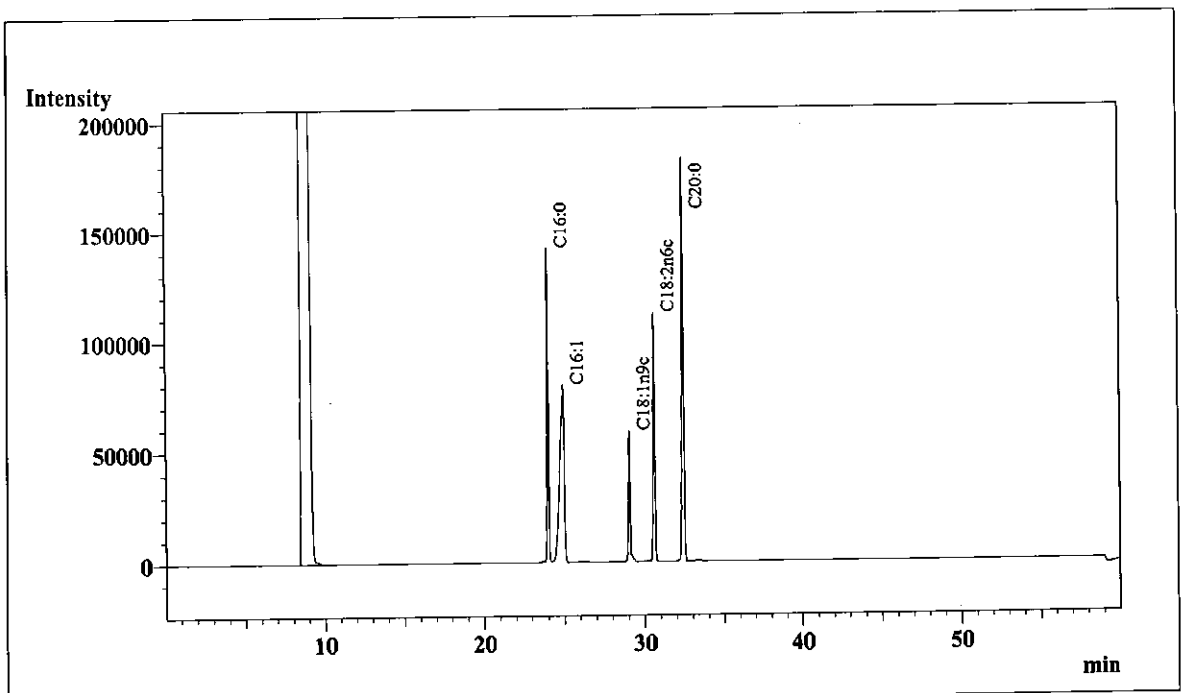

Figure 3. Gas-Chromatography separation profile of 5 bioactive FAME types (C16:0, C16:1, C18:1n9c, C18:2n6c and C20:0).

Table 1. Antimicrobial bioassay activity of the related commercial FAME standards against seven strains of marine bacteria.

BACTERIA SPECIES	ANTIBACTERIAL ACTIVITY					MIC VALUE (µg/DISC)				
	C16:0	**C16:1**	**C18:1n9c**	**C18:2n6c**	**C20:0**	**C16:0**	**C16:1**	**C18:1n9c**	**C18:2n6c**	**C20:0**
Clostridium cellobioparum	-	-	++	+++	-	-	-	0.20	0.10	-
Clostridium sordelli	-	-	-	+	-	-	-	-	0.30	-
Clostridium novyi	-	-	-	+	-	-	-	-	0.30	-
Proteus mirabilis	-	-	+	++	-	-	-	0.30	0.30	-
Proteus vulgaris	-	-	++	+++	-	-	-	0.20	0.10	-
Vibrio parahaemolyticus	-	-	-	+	-	-	-	-	0.30	-
Vibrio cholerae	-	-	-	+++	-	-	-	-	0.10	-

Inhibition Zone: + + +: > 25 mm, + + +: 19-24 mm, + +: 12-18 mm, +: 7-11 mm, -: No Inhibition

is not uncommon to find that primary ıetabolites such as fatty acids or derivatives of ıtty acids (in this case FAME) possess bioactive otentials against bacteria [25]. This approach ɔuld be a very simple and practical mechanism) protect themselves against infection or injuries. atty acids and FAMEs could be taken in through bsorption or produced within the cell via ıetabolism. But, it is close to impossible to esign an experiment to show this process ctually taking place in nature. Therefore, it is uggested that FAMEs such as C18:1n9c and :18:2n6c could have a role in microalgal defense ıechanism. Usually the FAMEs and fatty acids re not free chemicals but rather attached to ;licolipids such as MGDGs Monogalactopyranosyl diacylglycerols), MGMG Monogalactopyranosyl monoacylglycerols) .nd/or DGDG (Digalactopyranosyl liacylglycerols) [26]. They are the constituents ɔf microalgal cell walls and can be regarded as he first line of defense for these organisms.

)n the other hand, these bioactive FAMEs could ılso be utilized as nutraceutical supplements as ın addition to the present range of microalgae-lerived nutraceuticals. There was one reported esearch conducted in 1975 where crude extract ɔf *Scenedesmus oliquus* was observed to exhibit ɔotential pharmaceutical properties, which was ıttributed to the existence of carotene, vitamin B ınd chlorophyll [10].

CONCLUSION

Scenedesmus acuminatus was successfully mass-cultured in a temperature-controlled photo-bioreactor. Total culture duration per batch run was less than 70 hours with the specific growth rate of 0.028 hour^{-1}. The bioactive isolate 3 (17.6 %) was identified as a mixture of saturated and unsaturated FAME (C16:0, C16:1, C18:1n9c, C18:2n6c and C20:0). Based on the antibacterial results of commercial available corresponding fatty acids, it was proven that the antibacterial activity of isolate 3 was due to C18:1n9c and C18:2n6c methyl ester. These findings suggest that FAMEs and fatty acids could have a role in the microalgal defense mechanism. Other than that, these bioactive FAMEs may have potential to be utilized for nutraceutical purposes.

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REFERENCES

1. Olaizola, M. (2003). Commercial development of microalgal biotechnology: From the test tube to the marketplace. *Biomolecular Engineering* **20**: 459 - 466.
2. Becker, E. W. (1986). Nutritional Properties of Microalgae: Potentials and Constraints. In: *CRC Handbook of Microalgal Mass Culture* (ed. Richmond, A.). CRC Press Inc, Florida, pp. 339 - 408.
3. Venkataraman, G. S., Goyal, S. K., Keushik, B. D. and Roychoudhury, P. (1974). *Algae: Form and Function*. Today and Tomorrow's Printers and Publishers, New Delhi.
4. Shelef, G., Azov, Y., Moraine, R., and Oron, G. (1980). Algal Mass Production as an integral part of a Wastewater Treatment and Reclamation System. In: *Algae Biomass: Production and Use* (eds. Shelef, G. and Soedar, C. J.). Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 163 - 189.
5. Soeder, C. J., and Hegewald, E. (1988). *Scenedesmus*. In: *Micro-algal Biotechnology* (eds. Borowitzka, M. A. and Borowitzka, L. J.). Cambridge University Press, Cambridge, pp. 59 - 84.
6. Voltolina, D., Gomez-Villa, H., and Correa, G. (2005). Nitrogen removal and recycling by *Scenedesmus obliquus* in semicontinuous cultures using artificial wastewater and a simulated light and temperature cycle. *Bioresource Technology* **96**: 359 - 362.
7. Jimenez-Perez, M. V., Sanchez-Castillo, P., Romera, O., Fernandez-Moreno, D., and Perez-Martinez, C. (2004). Growth and nutrient removal in free and immobilized plaktonic green algae isolated form pig manure. *Enzyme and Microbial Technology* **34**: 392 - 398.
8. Omar, H. H. (2002). Bioremoval of zinc ions by *Scenedesmus obliquus* and *Scenedesmus quadricauda* and its effect on growth and

metabolism. *International Biodeterioration and Biodegradation* **50**: 95 - 100.

9. Terry, P. A., and Stone, W. (2002). Biosorption of cadmium and copper contaminated water by *Scenedesmus abundans*. *Chemosphere* **47**: 249 - 255.
10. Becker, E. W. (1994). *Microalgae: Biotechnology and Microalgal*. Cambridge Uni Press, Cambridge.
11. Takeda, H. (1996). Cell wall sugars of some *Scenedesmus* species. *Phytochemistry* **42**: 673 - 675.
12. Payer, H. D., Chemvichak, Y., Hosakul, K., Kongpanichkul, C., Kraidej, L., Nguitragool, M., Reungmanipytoon, S., and Buri, P. (1980). Temperature as an important climatic factor during mass production of microscopic algae. In: *Algae Biomass: Production and Use* (eds. Shelef, G. and Soeder, C. J.). Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 389 - 400.
13. Ooshima, K. (1981). Taxonomic studies on *Scenedesmus* in Japan 1. On *Scenedesmus acuminatus* (LAG) CHOD and it is varieties and *Scenedesmus Javanensis* CHOD. *Jap. J. Phycol.* **29** (2): 85 - 93.
14. Meeks, J. C., Willson, D. L. and Gaines, R. D. (1968). Triosephosphate isomerase from selected chlorophyta. *Phytochemistry* **7**: 2095 - 2101.
15. Lambardi, A. T., Hodalgo, T. M. R., and Vieira, A. A. H. (2005). Copper complexing properties of dissolved organic materials exuded by the freshwater microalgae *Scenedesmus acuminatus* (Chloropphyceae). *Chemosphere* **60**: 453 - 459.
16. Borowitzka, M. A. (1988). Algal Growth Media and Sources of Algal Culture. In: *Microalgal Biotechnology* (eds. Borowitzka, M. A., and Borowitzka, L. J.). Cambridge University Press, Cambridge, pp. 456 - 465.
17. Hanagata, N., Takeuchi, T., Fukuju, Y., Barnes, D., and Karube, I. (1992). Tolerance of microalgae to high CO_2 and high temperature. *Phytochemistry* **31**(10): 3345 - 3348.
18. Payer, H. D., Pabst, W., and Runkel, K. H. (1980). Review of the nutritional and toxicological properties of the green alga *Scenedesmus obliquus* as a single cell protein. In: *Algae Biomass: Production and Use*. (eds. Shelef, G. and Soeder, C. J.). Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 787 - 797.
19. Vairappan, C. S., Kawamoto, T., Miwa, H. and Suzuki, M. (2004). Potent antibacterial activity of halogenated compounds against antibiotic-resistant bacteria. *Planta Medica* **70**: 1087 - 1090.
20. Borowitzka, M. A. (1999). Commercial production of microalgae: ponds, tanks, tubes and fermenters. *Journal of Biotechnology* **70**: 313 - 321.
21. Myers, J. (1962). Laboratory Cultures. In: *Physiology and Biochemistry of Algae*. (ed. Lewin, R. A.). Academic Press, New York, pp. 605 - 615.
22. Richmond, A. (1986). Microalgae of economic potential. In: *CRC Handbook of Microalgae Mass Culture* (ed. Richmond, A.) CRC Press Inc, Florida, pp. 199 - 243.
23. Grima, M. E., Belarbi, E.-H., Acién Fernández, F.G., Robles Medina, A. and Chisti Y. (2003). Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnology Advances* **20**: 491 - 515.
24. Vairappan, C. S. (2004). Potent antimicronial activity of halogenated metabolites from Malaysian red algae, *Laurencia majuscula* (Rhodomelaceae, Ceramiales). *Biomolecular Engineering* **20**: 255 - 259.
25. Rosell, K. G. and Srivastava, L. M. (1987). Fatty acids as antimicrobial substances in brown algae. *Hydrobiologia* **151/152**: 471 - 475.
26. Vairappan, C. S. (2003). Bacterial dynamics associated with algal antibacterial substances during post-harvest desiccation process of *Sargassum stolonifolium* Phang *et* Yoshida. *Indian Journal of Experimental Biology* **41**: 837 – 845.