

# First report of microalgae *Rhexinema paucicellulare* (Ulvophyceae) in Mauritius and its biochemical evaluation as a source of fatty acids

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## Abstract

Mauritius is a tropical island with a very rich biodiversity of endemic organisms with an unexplored pool of microalgae, especially in the Ulvophyceae class. From freshwater sources, an isolate was characterised using morphological traits and 18S rDNA region. It was determined to be *Rhexinema paucicellulare* and was further evaluated for its biochemical content against commercially available *Spirulina* sp. This study evaluates the lipids content and offers a first report on the carbohydrate and protein contents of this species. The biochemical profile of *Spirulina* sp. differed significantly from *R. paucicellulare* with respect to carbohydrate (17.36% vs. 20.31%); protein (45.09% vs. 25.71%) and lipids (5.86% vs. 13.88%). Further exploration of the fatty acids profile

through GC analysis revealed high presence of alpha-linolenic acid at 26.48% and linoleic acid at 6.31%. The presence of important omega-3 FA was evidenced through GC/MS analysis with eicosapentaenoic acid and docosahexaenoic acid, which could make this isolate a potential candidate in the field of aquaculture.

## Introduction

Mauritius is a tropical island with a very rich biodiversity of native and endemic organisms. Several of them have been studied for their biochemical content with the potential for exploitation in various economic sectors such as pharmaceuticals.<sup>1</sup> However, it is not the same case for microalgae, which represent a very promising source of amino acids, proteins, lipids, essential fatty acids, carbohydrates and secondary metabolites.<sup>2</sup> Their culture in Mauritius is limited to few organisations such as the Albion Fisheries Research Centre, which cultures *Nannochloropsis* sp. and *Chaetoceros calcitrans* (both imported from Japan) for aquaculture practices and Green Create Nutra, which cultures *Spirulina* sp. (of foreign origin) as a nutraceutical product and as feed additives. The culture of local microalgae for commercial application remains inexistent. Although a few research have been carried out, they mostly covered aspects of identification and population density evaluations with cyanobacteria, dinoflagellates and diatoms being dominant species.<sup>3,4</sup> Therefore, based on these findings, other classes such as *Ulvophyceae* have been left completely unexplored.

This class of algae is globally dominated by marine species but recent studies have shown the occurrence of filamentous and sarcinoid freshwater species. The taxonomic positioning of some freshwater ulvophytes is, however, unstable based on recent research and revisions that emerged. A specific case of taxonomic instability is with the genus *Rhexinema*, in which *Helicodictyon* and *Pleurastrum sarcinoideum* would fall into, along with two new species *R. edaphica* and *R. sancta-tomeai*.<sup>5</sup> This claim is currently refuted due to a lack of type species for *Pleurastrum* and the new species being phylogenetically unrelated to the type species of *Rhexinema paucicellulare*.<sup>6</sup>

The stability of taxonomic nomenclature is important especially when species or strains of microalgae are cultured with the intention of commercialisation. As part of an investigation of the Mauritian microalgal biodiversity for commercial use in aquaculture practices, we describe here the phylogenetic and biochemical characterisation of an isolate from the genus *Rhexinema* (Ulvophyceae), isolated from freshwater sources. The carbohydrates, protein, lipids and fatty acid profiles were experimentally compared to locally commercialised *Spirulina* sp., as a means to evaluate the possible applications of the isolate.

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## Materials and Methods

### Sample collection, culture and morphological identification

Water samples were collected from Midlands Dam (MD) reservoir by filtering 50L of water through a 5 $\mu$ m mesh net. For inoculation, 100 $\mu$ L of sample was aseptically spread on Bold's Basal Medium (BBM) agar. The plates were incubated in a culture room with a light intensity of  $\sim$ 50 $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup> at 24°C with a photoperiod of 14:10 hours (light:dark) for 14 days and then inoculated in 200mL BBM supplied with filtered air at a rate of 10L/min. The cultures were allowed to mature for 10 days before initiating microscopic analysis for cellular measurements of 100 cells (along length and width).

### DNA extraction and PCR amplification

DNA extraction was done using a protocol devised for microalgae<sup>7</sup> after 10 days of culture to ensure cells are harvested before the lag phase the growth pattern and free from contaminants. Due to unfavourable results obtained in initial trials with the original protocol, modifications were introduced, such as replacing TE buffer with CTAB buffer (2% CTAB, 2% PVP, 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0) at the lysis step. The PCR analysis was performed using the primers P45 (5'-ACCTGGTTGATCCTGCCAGT-3') and P47 (5'-TCTCAGGCTCCTCTCCGGA-3') for the amplification of the region 18S rDNA.<sup>8</sup> Successful amplicons were sequenced and then compared on NCBI BLASTn (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) against reference strains. The sequence was submitted under the accession number MT632094.1 in GenBank NCBI.

### Phylogenetic analysis

The closest sequences obtained for the isolates from BLASTn for the 18S gene were retrieved from GenBank (NCBI). Multiple sequence alignments were performed online using the MAFFT web server (version 7),<sup>9</sup> and then manually cleaned using the software MEGA 6.06 (build 6140226). The evolutionary models that fit best for the analysis were calculated using the program JModelTest (v2.1.10 v20160303).<sup>10</sup> The best model generated from the program was then selected using the Akaike Information Criterion (AIC).

For the phylogenetic analysis, the Maximum Likelihood (ML) method and Bayesian inference (BI) were used with the programs PhyML 3.0 and MrBayes 3.2.7, respectively.<sup>11,12</sup> The model "GTR+I" was selected with the following parameters: nucleotide frequencies A=0.2741, C=0.2001, G=0.2296, T=0.2962; substitution rates AC=0.2251, AG=1.8029, AT=1.0000, CG=0.2251, CT=1.8029, GT=1.0000 and P-invar =0.56204. Bootstrap support was calculated with 1000 replicates. For MrBayes, the evolutionary model GTR+I was used with 3,000,000 generations and a Markov Chain Monte Carlo sampling at a rate of 100. The Maximum Parsimony (MP) was performed using the software PAUP\* v.4.0a and the Neighbour Joining (NJ) analysis were performed in MEGA 6.06.

### Biomass production

The isolate, designated as strain MD1, was mass cultured with BBM in three 5L plastic bottles under a light intensity of 64.17 $\pm$ 7.50  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup>. The culture was supplied with air, at a rate of 6L/min filtered through a 0.2 $\mu$ m filter, and was exposed to a photoperiod of 14:10 hours (light:dark) at room temperature (27-29°C) for a period of 22 days. The culture growth was measured through spectrophotometric analysis at 680 nm every 24 to 48

hours.<sup>13</sup> The values were recorded in triplicate for statistical analysis. The biomass was harvested and oven-dried for 24 hours at 80°C, ground to powdered form and stored at 4°C for further use.<sup>14</sup>

### Biochemical analysis of biomass

To compare the carbohydrate, protein and lipids content of isolate MD1, powdered *Spirulina* sp. was purchased from a local microalgae farm, Green Create Nutra, to be used as reference material for the evaluation of the current isolate. For each test, triplicates were performed with statistical analysis using one-way ANOVA.

### Total carbohydrates analysis

To extract the carbohydrates from the biomass, acid hydrolysis was used as follows: 0.1g of biomass was hydrolysed with 5mL of sulphuric acid (2N) and autoclaved at 120°C for 30 min.<sup>15</sup> The mixture was neutralised with solid sodium carbonate, diluted, centrifuged and filtered with a 0.2 $\mu$ m syringe filter. The total carbohydrates analysis was then performed using the phenol-sulphuric acid procedure.<sup>16</sup>

### Protein extraction and analysis

Dried biomass (5mg) was weighed and mixed with 0.2mL of 24% (w/v) TCA in a microcentrifuge tube.<sup>17</sup> The suspension was vortexed and incubated at 95°C for 15 min. After cooling down to room temperature, 0.6mL of distilled water was added. The tubes were centrifuged at 12,000 rpm for 20min. The pellet was retained and resuspended in 0.5ml of Lowry reagent D, prepared as 48:1:1 ratio of Lowry reagents A [2% (w/v) Na<sub>2</sub>CO<sub>3</sub> (anhydrous) in 0.1 N NaOH]; B [1% (w/v) NaK tartrate tetrahydrate] and C [0.5% (w/v) CuSO<sub>4</sub>.5H<sub>2</sub>O in H<sub>2</sub>O], respectively, and incubated for 3 hours at 55°C. The suspension was centrifuged at 12,000 rpm for 20 min and the supernatant was retained for protein quantification.<sup>18</sup>

### Lipids extraction

The process of lipids extraction from the biomass was performed by solvent extraction for the treatment of microalgae biomass.<sup>19</sup> The process was performed by mixing 1g of dried biomass with 10mL of chloroform:methanol (2:1v/v) and incubated in a shaker for 4 hour at 150rpm. The suspension was then centrifuged for 5min at 12,000 rpm. The supernatant was further filtered and then 4mL of distilled water were added. The oil was separated as an organic phase in the bottom layer, which was collected in a bottle. A rotary evaporator was used to make the oil extract become solvent free at 60°C and the productivity in % was calculated.<sup>19</sup> The crude oil was resuspended in 1mL of methanol and stored at 4°C for further analysis.

### Fatty acids profiling

Fatty acid quantification of the crude microalgae oil was determined by Gas Chromatography with Flame Ionization Detection (GC-FID) (Shimadzu model GC 2010) using the saponification process to get solvent-free methyl fatty acid sample.<sup>19</sup> Exactly 1 $\mu$ L of the sample was injected in the GC column (SP<sup>®</sup>-2560; fused silica, 100m x 0.25mm x 0.2 $\mu$ m, Sigma-Aldrich). Helium was used as carrier gas at a split flow of 25.5mL/min. The injector and detector temperatures were both at 250°C. The injection took place at an initial oven temperature of 60°C maintained for 5 min and raised at a rate of 15°C/min till 165°C. This was maintained for 1 min and then raised to 225°C for 20 min at a rate of 2°C/min. Fatty Acid Methyl Esters (FAMES) in the samples were identified through the comparison of their retention times against those of the standard FAME Mix 37 (Sigma-Aldrich).

## Gas chromatography–mass spectrometry (GC/MS) analysis of fatty acid profiles

Some of the signals from the GC analysis, which could not be identified, were further analysed by their mass spectra. The equipment and column used were Shimadzu and Phenomenex – ZB-5MS (phenyl arylene polymer, 30m x 0.25mm x 0.25µm), respectively. The GC/MS was performed using the selected ion monitoring mode. The temperature monitoring program was oven temperature of 60°C maintained for 3 min and raised at a rate of 10°C/min till 250°C. Then raised to 300°C at a rate of 5°C/min. The identification was performed through comparison to the Wiley GC-MS Database library.

## Results

### Microscopical diagnosis and phylogenetic analysis

Isolate MD1 presented spherical/ovoid to cylindrical elongated cells, green in colour. The cells would form filaments consisting of 2 to 8 cells that could be broken easily (Figure 1). A pair of cells would share common mucilage presenting a parietal chloroplast each with one pyrenoid. During cell division, the elongating cells make a slight curvature. In terms of size, the cells would measure  $6.47\mu\text{m}\pm 1.42 \times 5.00\mu\text{m}\pm 0.90$  (length x width).

Following the BLAST analysis, the isolate was found to be closely related to *Rhexinema paucicellulare* MF000570.1, MF034645.1 and MF034601.1 (100% sequence similarity). The phylogenetic analysis, using sequences retrieved from NCBI, revealed that isolate MD1 clustered in the *Rhexinema* clade with strong bootstrap values (Figures 2 and 3). The phylogenetic analysis using the different methods also indicated 2 different clades in the *Rhexinema* genus corresponding to 2 different species (*R. paucicellulare* and *R. sancta-tomeum*).

### Analysis of growth pattern and biochemical composition of biomass

The microalgae growth pattern was observed for a period of 22 days for 3 batches (replicates). A mean curve was drawn to illustrate the lag, exponential and early stationary phase (Figure 4). The culture was stopped at the late log phase at D21 resulting in a mean biomass production of  $0.27 \pm 0.03 \text{ g/L}$ . The carbohydrate yield between MD1 and *Spirulina* sp. was found to be significantly different ( $p < 0.05$ ). The content of MD1 was recorded at  $20.31\% \pm 3.09$  while that of *Spirulina* sp. was recorded at  $17.36\% \pm 1.17$ .

The mean protein content by dry weight was calculated at  $25.71\% \pm 2.96$  for MD1 as opposed to the  $45.09\% \pm 3.13$  for *Spirulina* sp. They were found to be significantly different between the isolate MD1 and *Spirulina* sp. ( $p < 0.005$ ). Based on the statistical analysis of the lipids content, it was observed that the content varied significantly between the two samples ( $p < 0.05$ ). The mean lipids content for MD1 was  $13.88\% \pm 3.86$  and was  $5.86\% \pm 1.74$  for *Spirulina* sp. The overall biochemical composition of the isolate *R. paucicellulare* MD1 and *Spirulina* sp. indicated significant differences between their primary metabolite contents (Figure 5).

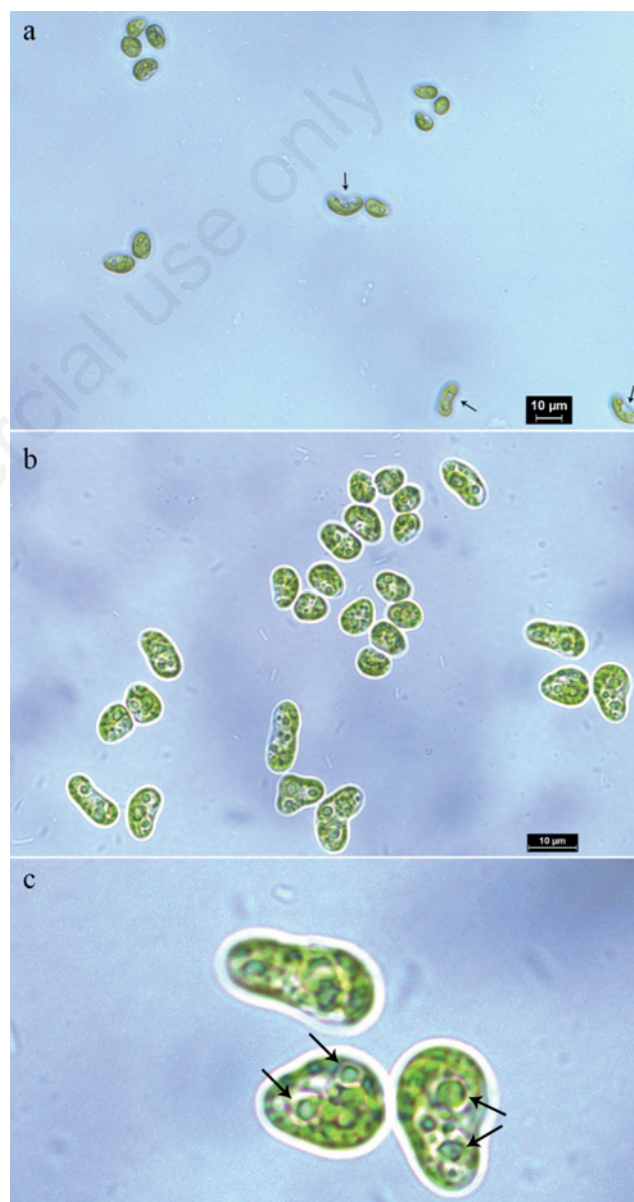
### Analysis of fatty acids through GC and GC/MS

The GC analysis of the extracts from MD1 and *Spirulina* sp. gave different FAME profiles and of different quantities (Table 1). However, certain compounds were common between them such as palmitic acid and linoleic acid based on the chromatograms obtained from each sample (Figure 6). From the MD1 lipid extract, the per-

centage composition of the fatty acid (FA) groups varied considerably such as saturated FA (20.31%), mono-saturated FA (5.69%), polyunsaturated fatty acids (PUFAs) (32.79%) and trans FA (12.90%). Only a total composition of 71.69% of the compounds could be identified. The main FA in the lipids extracts were methyl esters of C16:0, C18:0, C18:1, C18:2n-6t, C18:2n-6 and C18:3.

The *Spirulina* sp. sample displayed a much higher content of saturated FA (63.64%), mono-saturated FA (2.86%) and PUFAs (30.84%). Out of 100%, only a total composition of 97.34% of the compounds could be identified. The main FA in the lipids extracts were methyl esters of C16:0, C16:1, C18:0, C18:1, C18:2n6 and C18:3. The individual profiles, compound identities and percentage composition are presented in Table 1.

The GC/MS analysis revealed the presence of more FA from both extracts. Sample MD1 displayed various FA from the omega-



**Figure 1. Morphology of isolate MD1: (a) Formation of elongated and slightly curved cells during division at x400 (small arrows); (b, c) Distinct singular pyrenoid observed per cell at x1000 magnification (larger arrows) with a parietal chloroplast.**



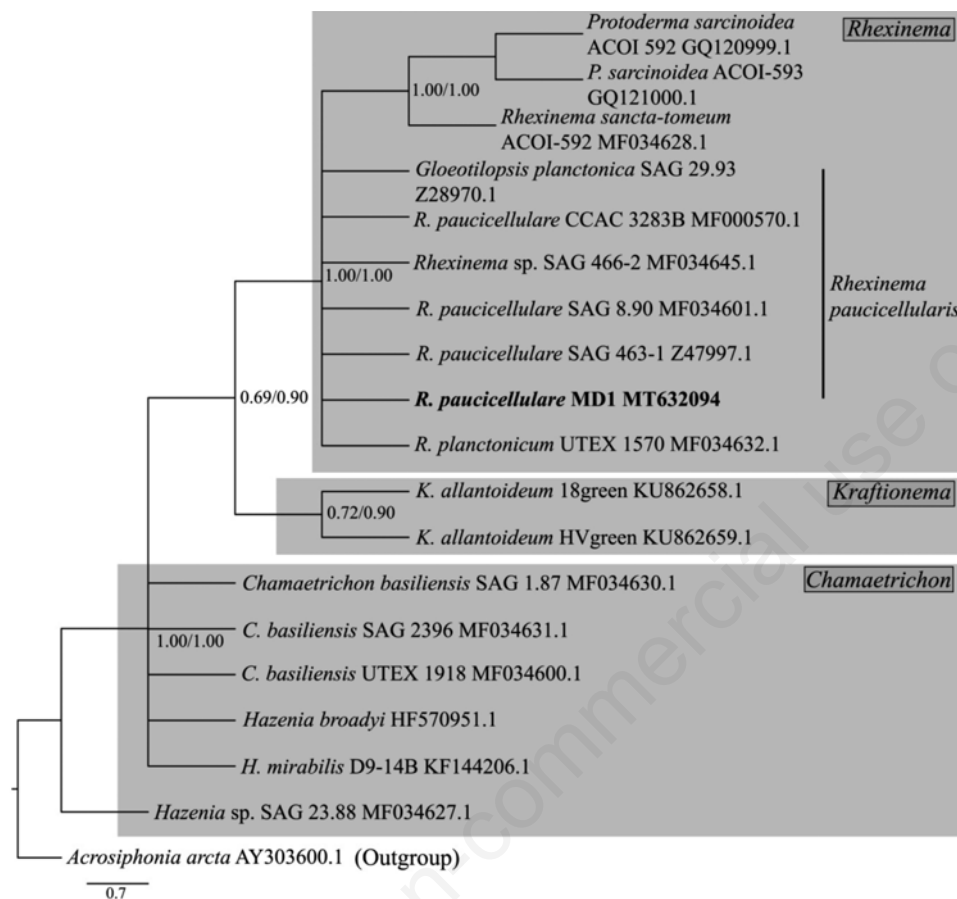


respectively. However, the *Rhexinema* and *Gloeotilopsis* genus are very unstable due to various proposed combinations that are put forward following recent investigations. It appears that *G. planctonica* SAG 29.93 (Vischer) has been wrongly diagnosed.<sup>22</sup>

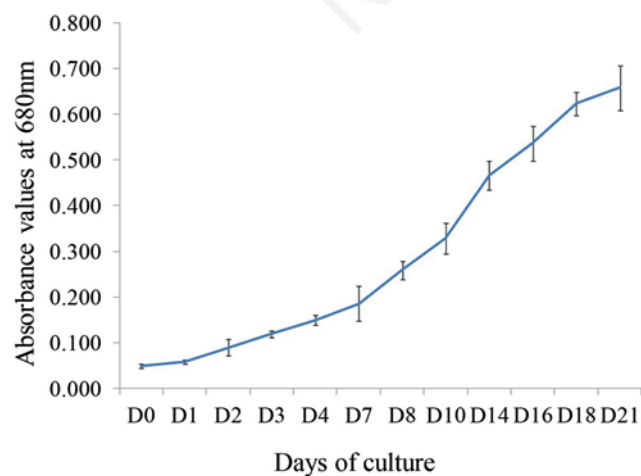
Recent research with the use of molecular tools coupled with microscopy, indicated that *G. planctonica* SAG 29.93 was confirmed to be *R. paucicellulare*.<sup>5</sup> Our phylogenetic analysis indicat-

ed that both strains SAG 463-1 and SAG 29.93 strongly clustered together, in accordance to other studies,<sup>5,6</sup> with isolate MD1, which was thus confirmed to be *R. paucicellulare*. However, the analysis also indicated a separate clade containing *R. santa-tomea* Darienko et Pröschold sp. nov. and *Protoderma sarcinoidea* (Groover et Bold).

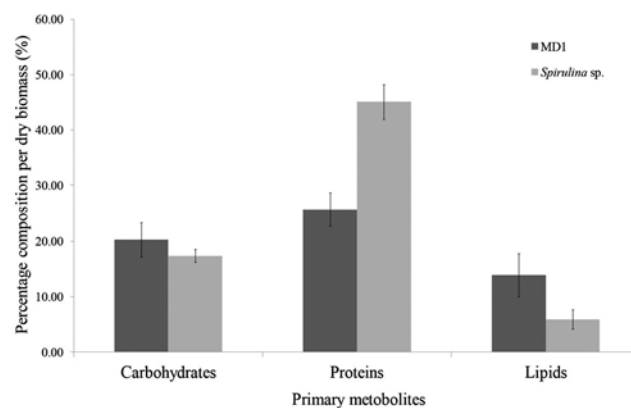
Darienko and Pröschold,<sup>5</sup> have recently proposed *R. santa-*



**Figure 3. Molecular phylogeny of isolate MD1 based on 18S rDNA sequences using maximum parsimony and neighbour joining analysis with support values indicated at the nodes (from left to right; values <0.60 are not shown). The species in bold represent the newly sequenced isolate from Mauritius.**



**Figure 4. Average growth curve for isolate MD1 for a period of 22 days.**



**Figure 5. Overall biochemical composition of *R. paucicellulare* MD1 and *Spirulina* sp.**

*tomea* as a new species and transferred *Protoderma sarcinoidea* (Groover et Bold) to *Rhexinema sarcinoidea* (Groover et H. C. Bold) Darienko et Pröschold combinatio nova. Škaloud *et al.*<sup>5</sup> do not accept these new proposals as taxonomically, *Pleurastrum* was named before *Rhexinema* and therefore considered the need for revising the type strains for both *Pleurastrum* and *Rhexinema*. This kind of situation highlights the significance of identification of microalgae through DNA based markers along with morphological characterisation.

### Carbohydrate content

*Spirulina* sp. was used as a positive control to be able to determine the biochemical value of the isolated microalgae as this particular strain was intensively cultured to be used as a nutraceutical product and feed additive. Other *Spirulina* sp.-based studies have shown levels as low as 14.94%±3.20 and as high as 58.17%±3.70 under specific conditions.<sup>23</sup> However, for isolate MD1 which was better than *Spirulina* sp., it is difficult to compare it to other members of its genus due to a lack of data in this regard but was found to be satisfactory in comparison to other microalgae such as *Dunaliella tertiolecta*, *Chlorella vulgaris* and *Nannochloropsis* sp. (46.5%±4.5, 55.6%±4.3 and 54.9%±5.0, respectively).<sup>24</sup>

Furthermore, light intensity is also important in the production and accumulation of carbohydrates.<sup>25</sup> The contents of *Chlorella* sp. PTCC 6010 increased from 10.69% to 49.82% when the light intensity was increased from 60 to 255  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ . In the

current study, the light intensity could have led to a lower carbohydrate content than the true potential of the isolate. Therefore, isolate *R. paucicellulare* MD1 should not yet be ruled out as a potential carbohydrate enriched microalgae.

### Protein content

The protein content of *Spirulina* sp. was recorded at an appropriate, if not a minimum, amount for this microalgae as it is considered as one of the richest sources of protein. However in comparison to isolate MD1, the protein content was half that of *Spirulina* sp. Microalgae such as *Dunaliella* sp., *Tetraselmis* sp. and *Nannochloropsis* sp. were found to produce levels of proteins equivalent to 12.26%, 4.83% and 13.00% respectively and are actively used as sources of feed for aquaculture.<sup>17,26</sup> Microalgae from the genus *Rhexinema* have not yet been studied in terms of protein content, although MD1 showcases appropriate levels as a Chlorophyte.

### Lipid content analysis

Between the two investigated microalgae, *Spirulina* sp. showed the lowest lipid contents. Other research has indicated values of 4-9% for *Spirulina* sp.<sup>27</sup> More recent investigations have shown maximum yields of 9% from *Spirulina platensis* in an attempt of identifying potential candidates for biodiesel production.<sup>28</sup> In the case of isolate MD1, the lipid contents were significantly higher than that of *Spirulina* sp. However, lipid content

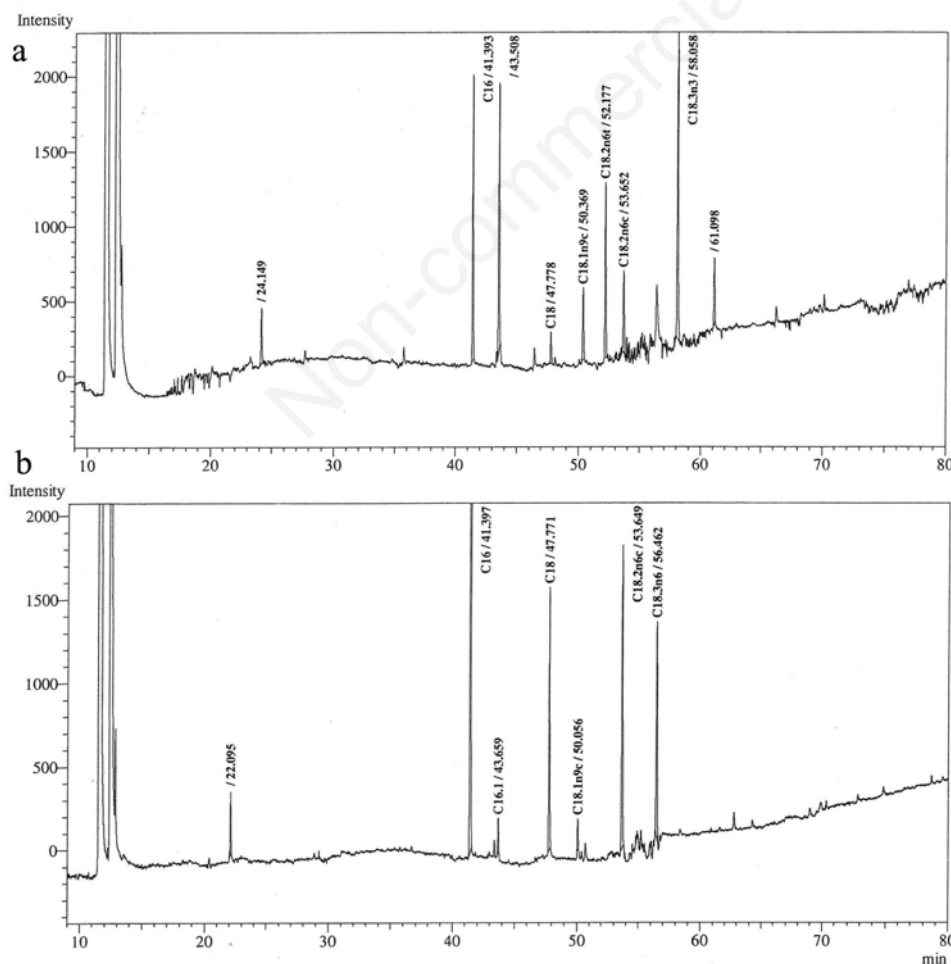


Figure 6. Fatty acid profile chromatograms of (a) isolate MD1 and (b) *Spirulina* sp.

analysis for this species or even genus is not available although only the fatty acid profile of *R. paucicellulare* SAG 463-1 and SAG 29.93 have been examined.<sup>20</sup>

Nonetheless, other microalgae such as *Scenedesmus obliquus* and *Chlorella vulgaris* are used in the production of lipids as they can yield up to 14% and 22% of lipids, respectively.<sup>29</sup> Strains of *S. obliquus* were found to produce 39.73% of lipids under normal culture conditions.<sup>30</sup> *N. salina* CCMP1176 yielded a lipid content of 36.95%±0.91 while *N. granulata* CCMP525 yielded a content of 60.35%±1.20.<sup>31</sup>

However, it should be noted that light intensity has considerable influence on how microalgae can produce and/or increase lipids.<sup>32</sup> *Scenedesmus* sp. was found to produce a lipid content of 41.1% and a neutral lipid content of 32.9% when the light intensity was increased from 250 to 400  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ .<sup>33</sup> Therefore, although every microalgae will have a certain upper and lower limit, hypothetically, the value reported in this project may not be representative of the real capacity of isolate *R. paucicellulare* MD1.

### Fatty acids profiles analysis

The fatty acid profile of isolate MD1 and *Spirulina* sp. displayed variations in terms of their content and composition. The FA found in *Spirulina* sp. was found to contain a high level of palmitic acid in comparison to the other FA. Among the PUFAs, the presence of linoleic acid and  $\gamma$ -linolenic acid was confirmed and this type of FA profile is typical in *Spirulina* sp. strains.<sup>34</sup> The GC/MS analysis did not indicate the presence of any omega-3 FA. However, the presence of other omega-6 fatty acids such as docosatetraenoic acid was revealed but was not detected in the GC analysis, which could be due to a low concentration.

Isolate MD1 presented a different profile with lower palmitic acid content. Furthermore,  $\alpha$ -linolenic acid was quantified at 26.48% instead of  $\gamma$ -linolenic acid in the GC although the latter was also detected through GC/MS, which was indicative of a very low concentration. The occurrence of these PUFAs is a known observation in freshwater microalgae. It should be noted that marine microalgae have a tendency to produce more eicosapentaenoic acid and docosahexaenoic acid while freshwater species produce more  $\gamma$ -linolenic acid and  $\alpha$ -linolenic acid.<sup>27</sup> Freshwater species such as *Chlorella vulgaris* and *Spirulina* sp. *platensis* were found to be abundant in  $\gamma$ -linolenic acid (1.9g/100g) and  $\alpha$ -linolenic (2.8g/100g), respectively, while marine species such as *Nannochloropsis oculata* and *Phaeodactylum tricornutum* possessed higher levels of eicosapentaenoic acid and docosahexaenoic acid.<sup>27</sup> Furthermore, data is scarce on the *Rhexinema* genus when it comes to fatty acids profiling.

The only study retrieved so far evaluated the strains for this genus, SAG 463-1 and SAG 29.93.<sup>20</sup> Their respective profiles were compared to isolate MD1 (Table 1). Strain 463-1 shared a close profile to MD1 than with SAG 29.93. Significant differences could be observed by the content of  $\gamma$ -linolenic acid and  $\alpha$ -linolenic acid. Additionally, the occurrence of linoleic acid was quite surprising as this trans fatty acid has only been detected in the microalgae *Trachelomonas similis*.<sup>20</sup> The same authors concluded that FA profiles of closely related species would show variations as shown here.<sup>20</sup> However, the presence of these PUFAs is still encouraging as they are highly required in aquaculture practices and feed diets, especially eicosapentaenoic acid and docosahexaenoic acid. Although they were detected at low levels in isolate MD1, it should be noted that the lipid content and FA profile resulted from culture under specific conditions and that the contents may change under stress conditions. Therefore, further experiments would be required to assess the maximum productivity of isolate *R. paucicellulare* MD1.

### Conclusions

The findings on *R. paucicellulare* MD1 showed adequate levels of carbohydrate and proteins in comparison to other microalgae and this data would be the first report of its kind for this particular species. The lipid and fatty acid profile also indicated suitable contents of  $\alpha$ -linolenic acid and the presence of other PUFAs such as eicosapentaenoic and docosahexaenoic, which are highly demanded in aquaculture practices. However, the current biochemical profile of this isolate was obtained under specific conditions where the light intensity was identified as a limiting factor, which could have influenced the current findings. Further investigations involving stress conditions such as high light conditions, influence of temperature and nutrient starvation would help understand the lower and upper limits of its biochemical composition.

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