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Characterization and Biotechnological Potential of Two Native Marine Microalgae Isolated from the Tunisian Coast

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Featured Application: Newly isolated microalgae strains with potential applications in different fields, especially fish feeds and wastewater bioremediation.

Abstract: Microalgae are attracting considerable interest worldwide. In the present study, two native microalgae strains isolated from Tunisian cost were identified as *Chlamydomonas* sp. and *Navicula* sp. We characterized their pigment and protein contents, as well as their carbohydrate and lipid productivity. The predominant fatty acids were found to be α-linolenic acid (C18:3n-3) and palmitoleic acid (C16:1n-7) for *Chlamydomonas* sp. and *Navicula* sp. strains respectively. Microalgae methanol extracts showed important in vitro antibacterial activity against all tested gram negative bacteria. Antioxidant activities of methanol extracts were investigated by determining radical scavenging activity according to DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) methods. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay showed that the newly isolated microalgae were not toxic and have potential application in the fish feeds domain as a supplement in fish meal. Also, the biosorption of cadmium from aqueous solutions by microalgae living cells was evaluated. Large removal capacities were obtained with values ranging from 89.8% to 99.38%. These results are very promising as a starting point for a potential application of these new isolates for in situ bioremediation of heavy metals contaminating aqueous systems.

Keywords: antibacterial activity; antioxidant activity; cadmium bioremoval capacity; *Chlamydomonas* sp.; *Navicula* sp.; unsaturated fatty acids abundance

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1. Introduction

Microalgae comprise a vast group of both marine and freshwater habitats organisms. Based on DNA sequence data, they have been classified into ten major phyta which are Glaucophyta, Euglenophyta, Cryptophyta, Haptophyta, Dinophyta, Heterocontophyta (including diatoms), Rhodophyta, Chlorophyta, and the prokaryotic Cyanophyta (cyanobacteria) [1]. In recent years, a focus has been shifted towards these organisms due to their diverse metabolic contents. Indeed, they synthesize a large number of bioactive compounds, including pigments, sterols, polyphenols, fatty acids, proteins, vitamins, alkaloids, and sulfated polysaccharides with various chemical structures and biological activities [2,3]. These biomolecules are useful for microalgal-based feed and food, nutraceutical, pharmaceutical, and cosmetic industries through their antioxidant, antifungal, antibacterial,

antiviral, antienzymatic, anti-cancer, or anti-inflammatory activities [4–7]. Several studies have reported the therapeutic properties of *Spirulina* and *Nostoc* species and their ability to scavenge superoxide and hydroxyl radicals and inhibit lipid peroxidation [8,9]. Besides, due to their rich nutritional properties, some Chlorophycae and diatoms genera have been widely used as dietary supplements for both humans and animals [10,11].

Nowadays, there is a focus on using microalgae in renewable energy sources as well as environmental applications. Several studies reported the conversion of microalgae biomass into biofuels [12,13]. Also, microalgae environmental applications are mainly represented in CO_2 sequestration and wastewater treatment [14]. Recent studies have investigated microalgae capacities for the final polishing of municipal wastewater, or the direct treatment of a variety of industrial wastewaters [14,15]. They demonstrate that several microalgae species can uptake heavy metals with high efficiency [16]. Moreover, multiple metal-biosorption mechanisms have been reported that differ according to the species used, biomass kind, and adopted procedure [15]. These include ion exchange, chelation, complexation, and microprecipitation [16]. The goal of the present work was to isolate, identify, and characterize native microalgae strains from Tunisian coasts to study their biotechnological potentialities for future industrial-scale valorization in biotechnological fields.

2. Materials and Methods

2.1. Isolation of Axenic Uniclonal Cultures

Seawater samples were collected from the Tunisian Coast of Ksour-Essef (governorate of Mahdia) (Latitude: 35°23′32.85″ N; Longitude: 11°2′57.1704″ E) in February 2018. They were pre-filtered through a 60 µm pore size membrane then the flow-through fractions were transferred into sterile flasks containing f/2-medium [17]. Inoculated cultures were grown in continuous aeration and illumination of 2000 lux, 25 ± 1 °C until visible growth appeared in flasks. Afterwards, an aliquot of each flask was spread over a sterilized agar plate, incubated at 28 °C and continuously illuminated at a light intensity of 2000 lux until visible growth appeared in the plate. A single colony was transferred into sterile liquid f/2-medium aseptically. After 72 h, cultures were examined using inverted microscopy (Motic microscope AE2000, Barcelona, Spain) at 40× magnification. The pure cultures were obtained by performing the micromanipulation method after serial dilutions [18]. Cultures were maintained in the f/2 medium, at 25 ± 1 °C with continuous aeration and an illumination of 2000 lux.

2.2. DNA Extraction, PCR Amplification, Sequencing and Phylogenetic Analysis

The genomic DNA was extracted using phenol/chloroform extraction standard method followed by ethanol precipitation [19]. The 18S rRNA encoding gene of each isolated strain was amplified by PCR using the EukA—EukB primer pair [20] according to the procedure reported by Ben Amor et al. [21]. The PCR products obtained were purified from agarose gel using MiniElute Gel Extraction Kit (Qiagen S. A. Courtaboeuf, France) by following the instruction manual. The purified products were sequenced by Sanger sequencing using 3500 Series Genetic Analyzer by Thermo Fisher Scientific, USA. The sequences obtained were compared with the sequences available in GenBank using the BLAST server from the NCBI website (www.ncbi.nlm.nih.gov/BLAST, 26 June 2020).

2.3. Pigments Contents

Pigments contents were determined spectrophotometrically, in ethanol extracts, as previously detailed by Fazeli et al. [22]. Briefly, the cell density of microalgae cultures were adjusted to 5×10^9 cell/L, then 2 mL of each culture were centrifuged at 5000× *g* for 10 min. The pellet was suspended in ethanol 100% and sonicated at 65 °C for 30 min. After sonication, the solution was centrifuged at 5,000× *g* for 10 min then the absorbance was measured at 666, 653, and 470 nm using a UV/VIS spectrophotometer (Pharo 300; Merck, NJ, USA). Chlorophyll *a*, Chlorophyll *b*, and carotenoids amount were calculated according

to the formula reported by Lichtenthaler [23]. Pigments contents were expressed in mg/L of microalgae culture. All assays were set up in triplicates.

2.4. Proteins Contents

Freeze-dried samples (50 mg) were incubated at 4 °C for 12 h with 4 mL ultra-pure water then ground with a mortar and pestle. Homogenates were collected and centrifuged at 15,000× *g* for 20 min at 4 °C to recover the supernatants, which were transferred to fresh centrifuge tubes and kept at 4 °C. The pellets were re-extracted with 1 mL of NaOH (0.1 M) under the same conditions. Collected supernatants from both extractions were mixed and proteins were precipitated using cold trichloroacetic acid as detailed by Barbarino and Lourenço [24]. Precipitated proteins were suspended in 0.5 mL NaOH (1 M) and quantified with the Bradford method [25]. Absorbance was recorded at 595 nm using a UV/VIS spectrophotometer (Pharo 300; Merck, NJ, USA). Samples were calibrated against the bovine serum albumin (Sigma) curve. Assays were made in triplicate.

2.5. Carbohydrates Contents

The carbohydrate content (poly- and oligo-) of the studied microalgae was assessed by the phenol sulfuric-acid colorimetric method described by Dubois et al. [26]. In brief, fresh microalgae biomass was hydrolyzed using 1 mL phenol 5% (*w/v*) and 5 mL concentrate sulfuric-acid. The mixture was vortexed and incubated for 10 min at room temperature. The absorbance was measured at 490 nm with a UV/VIS spectrophotometer (Pharo 300; Merck, NJ, USA). The concentration of carbohydrates was determined based on the dextrose standard curve. Three replicates were made for each assay.

2.6. Lipids Contents and Fatty Acid Profiles

Total lipids were prepared according to the method reported by Fendri et al. [27]. Briefly, microalgae fresh biomass (2 g) was homogenized with 20 mL of chloroform/methanol (2:1, *v/v*) using a tissue disrupter (IKA ULTRA-TURRAX T 25 digital; IKA-WERKE, Staufen, Germany). Non-lipid impurities were removed by washing with KCl (0.88%, *w/v*). Lipid weight was determined gravimetrically after evaporating the solvent under a stream of nitrogen and overnight vacuum drying. Fatty acid methyl esters (FAME) were prepared by acid-catalyzed trans-esterification of total lipids using 2 mL of 1% sulfuric acid (*v/v*) in methanol, at 50 °C for 24 h. Methyl esters were extracted twice in 5 mL of hexane–diethyl ether (1:1, *v/v*) after neutralization with 2 mL of 2% $KHCO_3$, dried under a stream of nitrogen, then dissolved in 1 mL of iso-hexane. The obtained FAME were analyzed through gas-liquid chromatography in an SPTM 2560 flexible fused silica capillary column (length 100 mm, internal diameter 0.25 mm, film thickness of 0.20 mm SUPELCO) in a Hewlett–Packard 5890 gas chromatograph. The oven temperature program was set according to the following. The initial temperature was 140 °C, then it increased at a rate of 3 °C/min to 230 °C, followed by 2 °C/min to reach 240 °C and then it was held there for 12 min. The injector and flame ionization detector were set at 260 °C. Helium was used as the carrier gas at a column pressure of 300 kPa. Peaks were identified by comparing their retention times with appropriate FAME standards from the Sigma Chemical Company (St. Louis, MO, USA). Data for the individual components are expressed as a percentage of total content. Each extraction experiment was performed in triplicate, and each extract was independently analyzed three times.

2.7. Determination of Phenols Contents

Phenolic content was determined according to Folin–Ciocalteu method using a gallic acid standard (10–200 mg/mL). The absorbance was measured at 720 nm [28,29]. Phenols contents were expressed in mg/mL of microalgae culture using an initial cell density of 5×10^9 cell/L. All measurements were performed as independent triplicates.

2.8. Crude Extracts Preparation

Microalgae biomass (1 g) was collected from 15-day-old cultures by centrifugation at 5000× *g* for 15 min. Then, it was extracted twice using 15 mL methanol, with shaking for 20 min at the room temperature. The obtained extracts were dried in a rotary evaporator at 40 °C and under reduced pressure, then stored at −20 °C for further studies.

2.9. DPPH Free-Radical Scavenging Assay

The 2, 2 diphenyl-1-picrylhydrazyl (DPPH) tests were performed as previously detailed by Yan et al. [30]. An aliquot (1 mL) of algal extracts dissolved in dimethylsulfoxide (DMSO) (100 µg/mL) was mixed with DPPH (0.02%, *w/v*) in methanol. After incubation for 1 h in the dark at 25 °C, the absorbance was measured at 517 nm using a UV/VIS spectrophotometer (Pharo 300; Merck, NJ, USA). Ascorbic acid was used as positive controls. The percentage of inhibition was calculated according to the following formula:

$$\% \text{ DPPH radical scavenging} = [(A_{control} - A_{test})/A_{control}] \times 100 \quad (1)$$

where $A_{control}$ = Absorbance of DPPH and A_{test} = Absorbance of sample (extract/ascorbic acid). All tests were carried out in triplicates.

2.10. ABTS Radical Scavenging Assay

ABTS tests were carried out according to the method described by Arnao et al. [31]. Briefly, 50 µL of microalgae extracts dissolved in DMSO (100 µg/mL) were added to 950 µL of cation ABTS+ and the absorbance was measured at 734 nm using a UV/VIS spectrophotometer (Pharo 300; Merck, NJ, USA). Ascorbic acid was used as positive control. The antioxidative activity was calculated using the formula reported by Re et al. [32].

$$\%\text{ABTS radical scavenging} = [(A_{control} - A_{test})/A_{control}] \times 100 \quad (2)$$

where $A_{control}$ = absorbance of ABTS and A_{test} = absorbance of sample (extract/ascorbic acid).

The ascorbic acid equivalent was computed by extrapolating the results with the standard pattern of decay using four different concentrations of ascorbic acid [33]. All analyses were performed in triplicates.

2.11. Antioxidant Enzyme Assays

The catalase activity was determined following the method proposed by Aebi [34]. The assay mixture consisted of 30 mM 30% H_2O_2 and 10 µL microalgae enzyme extract. Absorbance was immediately determined at 240 nm using a UV/VIS spectrophotometer (Pharo 300; Merck, NJ, USA). The rate of H_2O_2 decomposition is directly proportional to the catalase activity. One unit of catalase activity is defined as the amount of enzyme required to decompose 1 µM H_2O_2. The enzyme activity was expressed as U/mg protein.

Peroxidase activity was assayed according to established protocol [35]. Briefly, the reaction mixture consisted of 100 µL guaiacol (34 mM), 100 µL H_2O_2 (100 mM) and 100 µL of microalgae enzyme extract in phosphate buffer (100 mM, pH 7). The rate of formation of oxidized guaiacol was followed spectrophotometrically at 470 nm using a UV/VIS spectrophotometer (Pharo 300; Merck, NJ, USA). Enzyme activity was calculated using the extinction coefficient of 26.6 mM^{-1} cm^{-1}. All measurements were made in triplicates.

2.12. Antibacterial Activities

The Gram-negative bacteria *Vibrio harveyi*, *Vibrio Anguillarum*, *Photobacterium damselae*, *Aeromonas salmonicida*, *Shewanella putrefaciens* (Pdp11), and *Tenacibaculum mesophyllum* were used as test organisms [36]. Antibacterial tests were performed in 96-well-flat-bottomed plates (Nunc, Thermo Fisher Scientific, United States), following the method of Stevens et al. [37] with slight modification. 20 µL of test extracts were added to 20 µL of the previously cultured bacteria (log phase) adjusted to 10^8 c.f.u/mL. After incubation for 5 h

at 25 °C, 25 µL of MTT (1 mg/mL) were added to each well, and incubated for 10 min at 25 °C to allow the formation of formazan. Then, the plates were centrifuged at 2000× *g* for 10 min. The supernatants were removed and the precipitates were dissolved in 200 µL DMSO. Dissolved formazan absorbance was measured at 570 nm. Bacteria plus sterile PBS or methanol served as negative controls. The antibacterial activities were expressed as a percentage of non-viable bacteria, calculated as the difference between absorbance of surviving bacteria compared to the absorbance of bacteria from positive control (100%). Control and treatments were performed in triplicate.

2.13. Cytotoxicity and Antitumor Assay

Cytotoxicity effects of microalgae methanol extracts were evaluated against SAF-1 (ECACC nº 00122301) and PLHC1 (ATCC® CRL2406™) cell lines, using the standard MTT method, as previously described by Espinosa Ruiz et al. [38]. The tested concentration of each microalgae extract was 0.1 mg/mL. Cells incubated with medium with methanol served as a negative control. All tests were performed in five replicates.

2.14. Cadmium Removal Capacity

The capacity of both studied strains to remove metal was determined in batch assays, by culturing microalgal biomass (0.02 g/L) in f/2 medium, containing cadmium at initial concentrations of 0.5, 2.5, and 5 mg/L, under the previously mentioned conditions. After incubation for 5 days and 10 days, culture samples were collected, centrifuged, and residual cadmium concentration (in the supernatant) was determined via atomic absorption spectrophotometry (BMG LABTECH) according to the method described by Travieso et al. [39]. Then, percentages of removed cadmium were calculated. Experiments were run in triplicate. Blank controls (containing f/2 medium plus cadmium) and negative controls (containing f/2 medium and microalgae biomass) were also carried out.

2.15. Statistical Analysis

Statistical differences between antioxidant assay results were performed by Student's *t*-test, $n = 3$, using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). One-way ANOVA followed by Duncan multiple range tests were used to compare antibacterial activities, cytotoxicity assays, and cadmium removal capacities results. Data were considered significant when at least p was < 0.05.

3. Results

3.1. Isolation and Identification of the Microalgae Strains

Sea water samples were taken from Ksour-Essef (governorate of Mahdia) and two microalgae strains were isolated (MA_{a1} and MA_{n1}). Different selective nutrient media were used; f/2 medium provided the optimal culturing conditions. Using the EukA and EukB universal primer pairs, the amplified products of the 18S rDNA genomic region, of approximately 1.8 kb for both isolated microalgae strains were sequenced. Based on sequence comparison against DNA sequences already available in GenBank database, the isolated strains, MA_{a1} and MA_{n1}, were found to be affiliated to *Chlamydomonas* and *Navicula* genus, respectively, with more than 98% similarity. These results demonstrated that MA_{a1} and MA_{n1} belong to the *Chlorophyceae* and *Bacillariophyceae* classes, respectively.

3.2. Biochemical Composition

The biochemical composition of each native microalgae isolate was evaluated. The whole algal biomass compositions of newly isolated strains, MA_{a1} and MA_{n1}, were summarized in Table 1.

Table 1. Microalgae biomass composition.

	Chlamydomonas sp. MA_{a1}	*Navicula* sp. MA_{n1}
Proteins (%)	47.22 ± 0.19	34.16 ± 0.05
Lipids (%)	25.55 ± 0.2	15.00 ± 0.1
Carbohydrates (%)	27.23 ± 0.05	50.83 ± 0.2
Chlorophyll (mg/L)	1.5 ± 0.3	0.95 ± 0.05
Phenols (mg/mL)	40.5 ± 0.15	11.32 ± 0.05
Carotenoid (mg/L)	3.81 ± 0.3	4.72 ± 0.15

Proteins and carbohydrates were the most abundant compounds for both studied strains, which could be of interest for several biotechnological applications. As shown in Table 1, *Chlamydomonas* sp. MA_{a1} strain accumulated a significantly higher quantity of proteins (47.22%), lipids (25.55%), chlorophyll (1.5 mg/L), and phenolic compounds (40.5 mg/mL) than *Navicula* sp. MA_{n1} strain. Besides, an important amount of carbohydrates (50.83%) has been found in the algal biomass of *Navicula* sp. MA_{n1} strain.

Fatty acid profiles were determined for each isolate via fatty acid methyl esters analysis (Table 2).

Table 2. Fatty acid compositional profiles of lipid extracts from microalgae isolates.

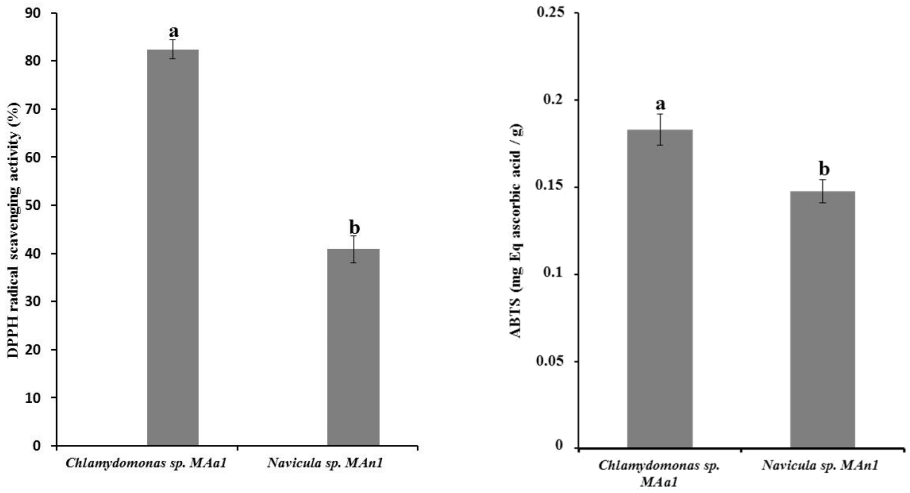
	Fatty Acid (*Relative Percentage*)	
	***Chlamydomonas* Sp. MA_{a1}**	***Navicula* Sp. MA_{n1}**
C14:0 Myristic acid	0.69	4.96
C15:0Pentadecylicacid	0.13	1.62
C16:0 Palmitic acid	23.87	10.80
C18:0 Stearic acid	4.98	0.45
C20:0 Arachidic acid	0.27	0.00
C22:0 Behenic acid	0.41	0.15
Total saturated (%)	**30.35**	**17.98**
C16:1n-7 Palmitoleic acid	1.14	43.48
C18:1n-9 Oleic acid	12.50	5.04
C18:1n-7 Vaccenic acid	10.45	2.07
C20:1n-9 Gondoic acid	1.00	0.14
C22:1n-9 Erucic acid	0.00	0.08
C24:1n-9 Nervonic acid	0.21	0.07
Total monounsaturated (%)	**25.30**	**50.88**
C18:2n-6 Linoleic acid	5.82	2.91
C18:3n-6 γ-linoleic acid	0.33	1.46
C20:3n-6 Dihomo-γ-linoleic acid	0.00	0.56
C20:4n-6 Arachidonic acid	1.41	5.64
C22:2n-6 Docosadonic acid	0.36	0.14
C22:4n-6 Adrenic acid	0.00	0.14
Total n-6 PUFA (%)	**7.92**	**10.85**
C18:3n-3 α-linolenic acid	30.08	0.00
C18:4n-3 Stearidonic acid	0.00	0.05
C20:3n-3 Dihomo-α-linolenic acid	0.20	0.00
C20:5n-3 Eicosapentaenoic acid	6.15	19.99
C22:6n-3 Docosahexanoic acid	0.00	0.25
Total n-3 PUFA (%)	**36.43**	**20.29**
Total PUFAs (%)	**44.35**	**31.14**
n-3/n-6	**4.59**	**1.87**

Eighteen and twenty fatty acids have been identified in the present study for *Chlamydomonas* sp. MA_{a1} and *Navicula* sp. MA_{n1} isolates, respectively. *Chlamydomonas* sp. MA_{a1} strain, showed the highest portion of polyunsaturated fatty acids (PUFAs) 44.35% with

7.92% of n-6 PUFA and 36.43% of n-3 PUFA. Regarding MA_{n1} isolate, belonging to *Navicula* genus, MUFAs was the major portion of the total fatty acids (50.88%) with a dominance of palmitoleic acid (C16:1n-7) (43.48%).

3.3. Antioxidant Activity

Antioxidant activities of *Chlamydomonas* sp. MA_{a1} and *Navicula* sp. Ma_{n1} isolates methanol extracts were performed by two spectrophotometric methods: the 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH) assay and the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay (Figure 1a,b).

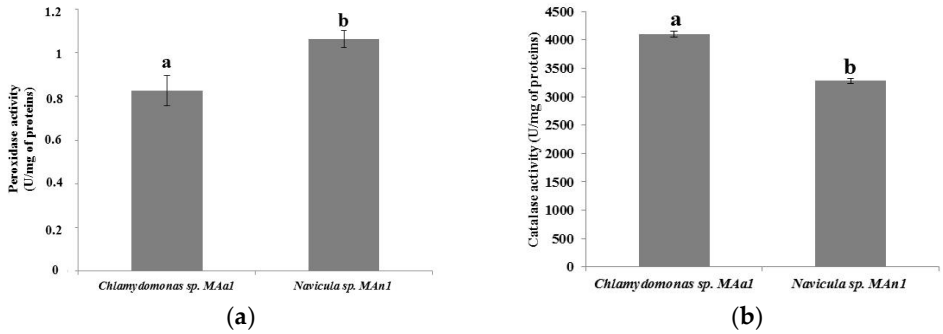


(**a**) (**b**)

Figure 1. Non enzymatic antioxidant activities of *Chlamydomonas* sp. MA_{a1} and *Navicula* sp. MA_{n1} methanol extracts. (**a**) DPPH scavenging activities; (**b**) ABTS scavenging activities. Data show means ± SD (n = 3). Statistical significance was determined by a Student's t test; significant difference ($p < 0.05$) is indicated by different lowercase letters.

The results obtained showed that the green microalgae isolate *Chlamydomonas* sp. MA_{a1} exhibited a higher antioxidant activity compared to *Navicula* sp. Ma_{n1} strain using both tests.

The enzymatic antioxidants from *Chlamydomonas* sp. MA_{a1} and *Navicula* sp. Ma_{n1} isolates were investigated (Figure 2).



(**a**) (**b**)

Figure 2. Enzymatic antioxidant activities of *Chlamydomonas* sp. MA_{a1} and *Navicula* sp. MA_{n1} methanol extracts. (**a**) Peroxidase activities; (**b**) catalase activities. Data show means ± SD (n = 3). Statistical significance was determined by a Student's t test; significant difference ($p < 0.05$) is indicated by different lowercase letters.

The obtained peroxidase activities were 1.0625 U/mg of proteins and 0.8 U/mg of proteins for *Chlamydomonas* sp. MA_{a1} and *Navicula* sp. MA_{n1} isolates, respectively. Also, 4101.27 U/mg of protein of catalase activity was recorded for *Chlamydomonas* sp. MA_{a1} and 3281.01 U/mg of protein for *Navicula* sp. MA_{n1}.

3.4. Antibacterial Activity

Antimicrobial activities of methanol extracts from both isolates were determined by MTT method against six Gram negative pathogenic bacteria strains for fish aquaculture: *V. harveyi*, *V. anguillarum*, *P. damselae*, *A. salmonicida*, *S. putrefaciens* (Pdp11), and *T. mesophyllum* (Figure 3).

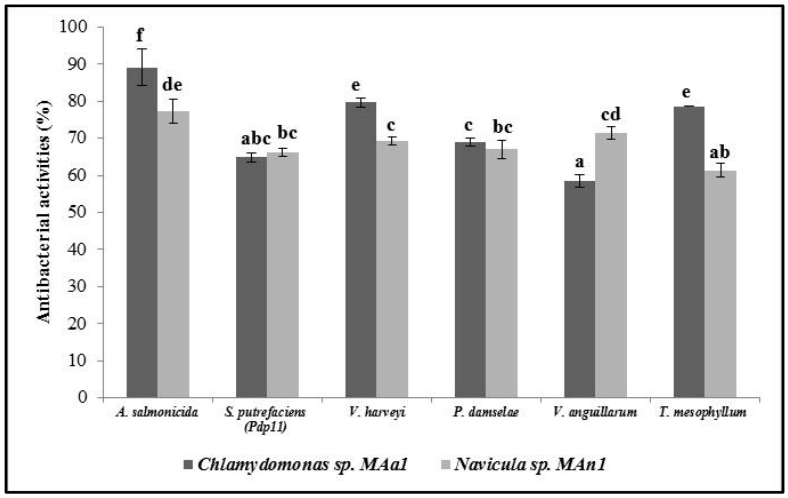


Figure 3. Antibacterial activities of *Chlamydomonas* sp. MA_{a1} and *Navicula* sp. MA_{n1} methanol extracts against *V. harveyi*, *V. anguillarum*, *P. damselae*, *A. salmonicida*, *S. putrefaciens* (Pdp11) and *T. mesophyllum*. Data show means ± SD (n = 3). Statistical significance was determined by one-way ANOVA followed by Duncan multiple range test; significant difference ($p < 0.05$) is indicated by different lowercase letters.

Methanol extracts of *Chlamydomonas* sp. MA_{a1} and *Navicula* sp. MA_{n1} exhibited interesting antibacterial activities against tested pathogenic bacteria strains. The obtained inhibition percentages were greater than 60% against all tested Gram negative pathogenic bacteria. In fact, methanol extracts of both microalgae isolates showed comparable antibacterial activities against *P. damselae* and *S. putrefaciens* (Pdp11) with inhibition percentages of around 65%. *Navicula* sp. MA_{n1} extract was the most active against *V. anguillarum*. Interestingly, inhibition percentages greater than 80% were obtained against *V. harveyi*, *A. salmonicida*, and *T. mesophyllum* using *Chlamydomonas* sp. MA_{a1} extract.

3.5. Cytotoxic Assay

Cytotoxic effect *Chlamydomonas* sp. MA_{a1} and *Navicula* sp. MA_{n1} methanol extracts were tested against SAF-1 and PLHC-1 cells cultures using MTT colorimetric assay (Figure 4).

The obtained results showed that *Navicula* sp. MA_{n1} extract has no significant effect on cell viability of SAF-1 tested cell lines (96%). However, it caused a slight decrease in cell viability (83%) of PLHC-1 tumor cell lines. Regarding *Chlamydomonas* sp. MA_{a1} extract, the obtained cell viabilities were 70.4% and 91.4% using SAF-1 and PLHC-1 cell lines, respectively.

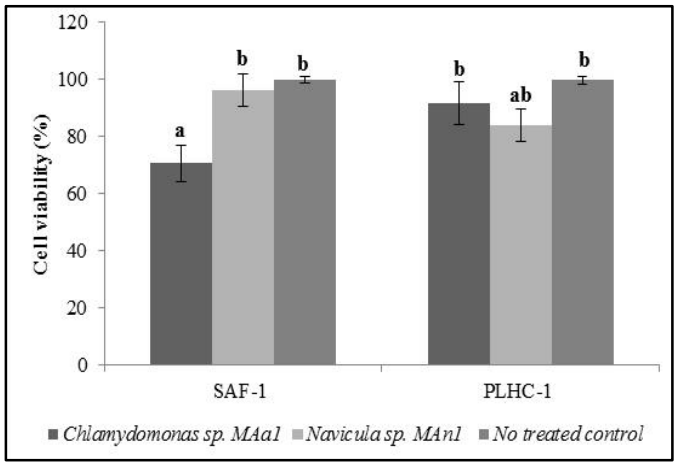


Figure 4. Cytotoxic activities of *Chlamydomonas* sp. MA_{a1} and *Navicula* sp. MA_{n1} methanol extracts against SAF-1 and PLHC-1 cell lines. Data show means ± SD (n = 5). Statistical significance was determined by one-way ANOVA followed by Duncan multiple range test; significant difference ($p < 0.05$) is indicated by different lowercase letters.

3.6. Capacity of Microalgae to Remove Heavy Metals

The capacity of *Chlamydomonas* sp. MA_{a1} and *Navicula* sp. MA_{n1} strains to remove cadmium were tested (Figure 5).

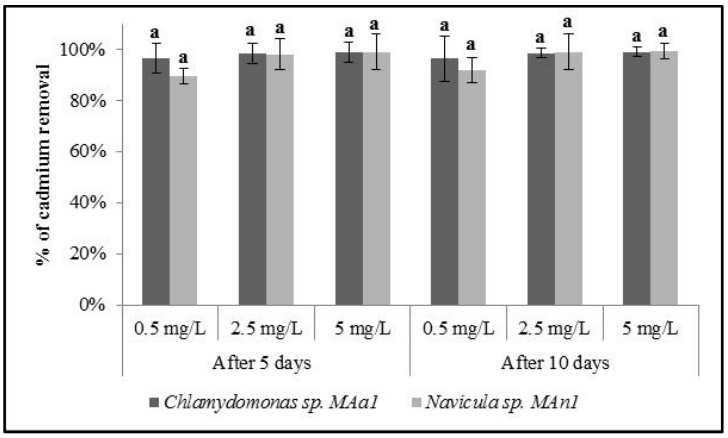


Figure 5. Capacities of *Chlamydomonas* sp. MA_{a1} and *Navicula* sp. MA_{n1} to remove cadmium from aqueous solutions. Data show means ± SD (n = 3). Statistical significance was determined by one-way ANOVA followed by Duncan multiple range test; no significant difference ($p < 0.05$) is indicated by the same lowercase letters.

Both tested strains exhibited high removal efficiencies of cadmium from water at all tested concentrations. In fact, the obtained percentages of removed cadmium vary between 89.8% and 99.38%.

4. Discussion

The bio-prospecting of microalgae from local biotopes has been considered an efficient alternative to identify species of different genera that exhibit a wide of biotechnological activities [40,41]. In this context, two microalgae strains, MA_{a1} and MA_{n1}, were isolated from local marine habitat and identified as microalgae belonging to *Chlamydomonas* and *Navicula* genera of *Chlamydomonadaceae* and *Naviculaceae* families using the nuclear 18S rDNA gene as a universal DNA barcode marker [42]. Microalgae biomasses were characterized and a genus-dependent biochemical composition was observed, as previously

reported [43]. The obtained composition of the green microalgae isolate *Chlamydomonas* sp. MA_{a1} was in agreement with that reported by Behl et al. [44], with a remarkable abundance of proteins of about 47.22%, while *Navicula* sp. MA_{n1} strain biomass was richer in organic compounds especially carbohydrates (50.83%) than previously studied *Navicula* isolates [45,46]. The growth pattern and biochemical composition of microalga cells are closely related to biotic and abiotic culture parameters such as cultivation conditions and nutrient media composition and concentrations [44].

Both newly isolated strains exhibited moderate lipid contents. However, they were found to exhibit important proportions of mono-unsaturated and polyunsaturated fatty acids; 69.65% and 82.02% for *Chlamydomonas* sp. MA_{a1} and *Navicula* sp. MA_{n1}, respectively. Fatty acid profiles have been considered as chemotaxonomical characteristics, since a comparatively higher homology of fatty acid profiles is always found within the strains with closer phylogenic relationships [47]. *Chlamydomonas* sp. MA_{a1} strain is particularly rich in monounsaturated especially oleic acid (C18:1n-9), vaccenic acid (C18:1n-7), and polyunsaturated α-linolenic acid (C18:3n-3) accounting for approximately 12.5%, 10.5%, and 30.08% of the total *Chlamydomonas* sp. MA_{a1} oil, respectively. The main abundant fatty acid in *Navicula* sp. MA_{n1} isolate is palmitoleic acid (C16:1n-7) representing 43.48% of the total fatty acids. The obtained results agree with multiple previously-published reports showing that almost all diatoms contained high proportions of (C16: 0) and (C16: 1n-7) fatty acids [48]. Unsaturated fatty acid production should be of great importance in different fields such as special human or animal dietary, nutraceuticals, and/or cosmeceuticals given the functional properties of PUFAs and their health benefits [44]. In fact, PUFAs, especially n-3 PUFAs, have many potential applications including disease prevention and treatment [49].

Under oxidizing conditions, microalgae cells produce various antioxidants [50]. There are several reports on the evaluation of the antioxidant activity of many genera belonging to Cyanobacteria [51,52], Chlorophycae [53–55] and some diatoms [56]. The antioxidant and antibacterial properties investigation of newly isolated strains showed high potential of in vitro activities. In fact, methanolic extracts from both studied strains exhibited interesting antibacterial activities against six Gram negative pathogenic bacteria strains. These results seem to be in perfect agreement with the data of biochemical compositions since a significant relationship between total antioxidant activity and total phenolic compounds has been reported [57,58]. It is worth noting that microalgae methanolic extracts seem to be particularly rich in flavonoids, and that terpenes and carbohydrate were present in moderate amount and alkaloids were also found at trace levels in [59,60]. Previously, a correlation between metabolites like flavonoids, terpenes and carbohydrates presence and antibacterial activities was established [59,60].

Additionally, important antimicrobial activities of both microalgae extracts were obtained against tested Gram- bacteria pathogen for vertebrates and invertebrate marine animals, especially fish, bivalves, and crustaceans. Those activities could be due to the presence of bioactive compounds that may include isoprenoids, polyketides, polyunsaturated fatty acids, and alkaloids which may affect the growth and metabolism of bacteria [61,62]. It has been reported that antibacterial activities of microalgae extracts, against both Gram+ and Gram- bacteria, were attributed to eicosapentaenoic acid and palmitoleic acid, particularly produced by diatoms [63,64]. Interestingly, no antibacterial activities of *Chlamydomonas* or *Navicula* species against aquatic animal pathogen have been previously reported. However, antibacterial activities were described for *Chlamydomonas reinhardtii* and *Navicula* sp. against human pathogenic bacteria such us *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhi*, and *Bacillus* sp. [65,66]. Darah and Lim [67] reported the mechanisms of action involved in the bacterial killing process. Among them are the interactions of the antimicrobial compounds with the cell membrane and intracellular accumulation through the cell wall and membrane permeability [68].

To ensure algae biomass safety for potential feed supplement applications, the toxicity of microalgae extracts was studied against fish cell lines. Hepatocytes, such as PLHC1 cells,

are good models for studying toxicity since the liver is the primary site for drug metabolism and biotransformation [69]. According to Abdillahi et al. [70], treated concentrations of *Chlamydomonas* sp. MA_{a1} and *Navicula* sp. MA_{n1} methanol extracts are considered non-toxic since the cell viability is greater than 70%. In recent years, many studies have reported the in vivo immunostimulant effect of different microalgae such as *Nannochloropsis gaditana*, *Tetraselmis chuii*, and *Phaeodactylum tricornutum* on fish production [71,72].

Microalgae have been gaining attention in the treatment of wastewater, especially those contaminated by heavy metal. Interestingly, the newly isolated strains showed large cadmium removal capacities, with values ranging from 89.8% to 99.38%. Previously, rapid cadmium bioremoval by diatom cells has been observed in several other studies using cadmium concentration lower than that tested in the present study. In fact, *Navicula incerta* showed linear removal of cadmium using initial ion metal concentrations of 5 mg/L [73]. Cherifi et al. [74] also reported a cadmium removal capacity of *Navicula subminuscula* in aqueous solution containing 8 mg/L cadmium.

Chlamydomonas genus has a great potential in metal remediation [75]. Mera et al. [76] reported that *Chlamydomonas moewusii* remove up to 8 mg/L of cadmium. Also, *Chlamydomonas reinhardtii* remove up to 42.6 mg/L 67.4 mg/L of cadmium using fresh cells and lyophilized biomass, respectively [77,78]. Several other microalgae have shown an important ability to remove cadmium ions, especially *Spirulina platensis and Chlorella vulgaris* [79–81]. It is worth noting that the initial concentration of metal ions, contact time, pH, temperature, biosorbent concentration, and agitation rate are key factors affecting biosorption efficiency [82,83].

5. Conclusions

Two microalgae strains MA_{a1} and MA_{n1} were isolated from seawater of Tunisia costs and molecularly identified as *Chlamydomonas* sp. and *Navicula* sp., respectively. Both strains were initially characterized and exhibited significant heterogeneity in key parameters, such as carbohydrate productivity, lipid productivity, and fatty acids composition. Investigation of their biological activities showed that the newly isolated strains exhibited interesting antioxidant and antibacterial activities, particularly *Chlamydomonas* sp. MA_{a1}. *Chlamydomonas* sp. MA_{a1}, and *Navicula* sp. MA_{n1} biomass safety was verified against fish cell lines which support their potential application in the fish feeds domain. Furthermore, both isolated strains were capable of removing cadmium from aqueous solution, opening the horizon towards their application in the wastewater treatment domain.

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