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Thu, 26 Jan 2017 at 8:32 pm

26-Jan-2017

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Fri, 24 Feb 2017 at 3:09 pm

24-Feb-2017

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Thank you for reviewing manuscript # PRE-2017-011 entitled "Improvement of both lipid and biomass productivities of Qatar Chlorocystis isolate for Biodiesel production and food security" for Phycological Research.

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24-Mar-2017

Dear Referee,

Thank you very much for taking the time and making the effort to review the paper entitled "Improvement of both lipid and biomass productivities of Qatar Chlorocystis isolate for Biodiesel production and food security" for Phycological Research. A decision of 'Major Revision' has been rendered for the manuscript.

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Abstract
P1, line 16 – please write culture medium as "f/2 medium" not just "f/2". Please check throughout

Phycological Research / Volume 66, Issue 3 / p. 182-188

Research Article

Improvement of both lipid and biomass productivities of Qatar *Chlorocystis* isolate for biodiesel production and food security

Imen Saadaoui, Touria Bounnit, Maryam Muraikhi, Rihab Rasheed, Ghamza Alghasal, Hareb Al Jabri

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SUMMARY

Microalgae are considered a very promising alternative for biofuel production. Several strategies were developed to modulate and improve algae metabolites production to meet the requirements for biodiesel production. Most previous research evidenced that the increase of the lipid content is accompanied by a decrease of the biomass production, which increases the cost of the downstream processing. Hence, the challenge is to find special culture conditions that increase the lipid and the biomass productivities simultaneously. In the present work, we developed a strategy for the improvement of biomass and lipid productivities in a novel local microalga isolate, *Chlorocystis* sp. QUCCCM14, which was not previously known as a promising strain. Indeed, culturing QUCCCM14 using f/2 medium with 10× NaH_2PO_4 (0.15 g L^{-1} $NaNO_3$ and 5.6 mg L^{-1} NaH_2PO_4) resulted in an improvement of 3.178 folds the lipid productivity reaching 56.121 mg L^{-1} day^{-1} and enhanced the biomass productivity reaching 141.363 mg L^{-1} day^{-1}, simultaneously. Comparative analyses of the FAME profiles demonstrated that fed-batch culture with phosphate or nitrate separately leads to a high production of the omega 3 fatty acids (Linolenic acid), whereas fed-batch culture with phosphate and nitrate simultaneously increased the production of fatty acids suitable for biodiesel production.

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Improvement of both lipid and biomass productivities of Qatar *Chlorocystis* isolate for biodiesel production and food security

Imen Saadaoui ,* Touria Bounnit, Maryam Muraikhi, Rihab Rasheed, Ghamza Alghasal and Hareb Al Jabri

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SUMMARY

Microalgae are considered a very promising alternative for biofuel production. Several strategies were developed to modulate and improve algae metabolites production to meet the requirements for biodiesel production. Most previous research evidenced that the increase of the lipid content is accompanied by a decrease of the biomass production, which increases the cost of the downstream processing. Hence, the challenge is to find special culture conditions that increase the lipid and the biomass productivities simultaneously. In the present work, we developed a strategy for the improvement of biomass and lipid productivities in a novel local microalga isolate, *Chlorocystis* sp. QUCCCM14, which was not previously known as a promising strain. Indeed, culturing QUCCCM14 using f/2 medium with 10× NaH_2PO_4 (0.15 g L^{-1} $NaNO_3$ and 5.6 mg L^{-1} NaH_2PO_4) resulted in an improvement of 3.178 folds the lipid productivity reaching 56.121 mg L^{-1} day^{-1} and enhanced the biomass productivity reaching 141.363 mg L^{-1} day^{-1}, simultaneously. Comparative analyses of the FAME profiles demonstrated that fed-batch culture with phosphate or nitrate separately leads to a high production of the omega 3 fatty acids (Linolenic acid), whereas fed-batch culture with phosphate and nitrate simultaneously increased the production of fatty acids suitable for biodiesel production.

Key words: algal biomass, biofuel, metabolite improvement, nutritional potential.

INTRODUCTION

Microalgae are known as a promising alternative and sustainable feedstock for numerous applications, including biofuel, food, nutraceutical and pharmaceutical products (Zubia *et al*. 2009; Mimouni *et al*. 2012; Daroch *et al*. 2013). The rapid development of algae technology for high-value metabolite production faces many challenges, including the isolation and purification of algae strains that produce a large biomass with high levels of intrinsic metabolites of interest (e.g., lipid, protein and carbohydrates, pigments, etc.).

Different strategies have been adopted to optimize the balance between metabolite production and algae growth. These strategies are designed to manage environmental factors such as light intensity, temperature and nutrient availability, as well as molecular mechanisms involved in the cell division and metabolite synthesis pathways (Zucchi & Necchi Jr 2001). Valledor *et al*. (2014) reported that nitrate starvation led to an accumulation of oil bodies, changes in membrane structure and reduction of putative enzymes involved in carbon-concentrating mechanisms. Alternatively, researchers have demonstrated that growth-inhibiting conditions and an imbalance between carbon and some macro- and micro-elements, such as Fe, S, Zn, or N, cause metabolic rearrangements that modulate cell division, morphology, and photosynthetic capacity (Kropat *et al*. 2011) and enhance the accumulation of starch (Ball *et al*. 1990) and lipid (Wang *et al*. 2009). Recently, lipid accumulation was successfully enhanced through the heterologous expression of genes encoding key enzymes involved in the fatty acid synthesis (Yao *et al*. 2014; Ghosh *et al*. 2016; Tamayo-Ordóñez *et al*. 2017).

The nitrogen and phosphorous are considered as the major factors influencing the algae growth and lipid accumulation (Yeh & Cheng 2011). Indeed, it was reported that several researchers adopted the strategy of nutrient stress (especially nitrogen (N) or phosphorus (P)) limitation to enhance ultimately the neutral lipid accumulation (Li *et al*. 2008; Converti *et al*. 2009; Dean *et al*. 2010). Wan *et al*. (2013) proved that adding nitrogen depleted f/2 growth medium to *Nannochloropsis oceanica* culture to promote long-term cell growth increased the lipid content with high abundance of saturated fatty acids. However, the nutrient stress approach, which leads to a high lipid accumulation, is mainly limited by the very low algae biomass productivity associated with low lipid productivity (Saraf & Thomas 2007; Ramos *et al*. 2009; Wan *et al*. 2013). Consequently, it is of a great importance to optimize the growth conditions leading to the greatest lipid productivity (Hu *et al*. 2008; Griffiths & Harrison 2009).

The Qatar University Culture Collection of Cyanobacteria and Microalgae (QUCCCM) maintains a diverse assembly of living microalgae. Strains were collected from local regions, and this collection represents an untapped resource of primary and secondary metabolites with a large spectrum of bioactivity. Our previous studies showed that *Chlorocystis* sp. is the most abundant marine algae genus found along the Qatar coastline and naturally adapted to the harsh local Qatar environmental conditions (Saadaoui *et al*. 2016). On the other

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hand, *Chlorocystis* isolates showed interesting growth rate, final biomass and lipid accumulation (Saadaoui *et al*. 2016). Accordingly, *Chlorocystis* can be considered as a good alternative for algae lipid production. Hence, the current work aims to improve *Chlorocystis* metabolite and biomass productivities, by managing nitrate and phosphate concentrations and cultivation method for an economic and feasible biodiesel production.

MATERIALS AND METHODS

Algae culture and growth rate analysis

A *Chlorocystis* sp. microalga isolate from Qatar University Culture Collection of Cyanobacteria and Microalgae (QUCCCM) was used in the current research since it corresponds to the most dominant microalgae in the Qatar marine environment and it showed a good potential in biomass production and lipid accumulation (Saadaoui *et al*. 2016). A single colony of QUCCCM14 was used to inoculate 3 mL of modified f/2 (f/2m) medium growth medium (Guillard & Ryther 1962) f/2 medium containing 0.15 g L^{-1} $NaNO_3$ and 5.6 mg L^{-1} of NaH_2PO_4. After 10 days of incubation at 30°C, pH 8, 50% humidity, a photon flux density of 100 μmol photons m^{-2} s^{-1} and a 12:12 h dark : light cycle with 150 rpm agitation using illuminated shaker (Innova 44R, New Brunswick Scientific, USA), the culture was scaled up to 50 mL of appropriate media with an initial $OD_{750\ nm}$ of 0.1. This culture was then incubated for 10 days under the same conditions as above. Finally, an adequate volume was used to inoculate a DASGIP parallel 1L bioreactor system for phototrophic cultivation (#76DG08PBBB, Eppendorf, USA). This culture was grown at 30°C, pH 8, at 300-rpm agitation to avoid settling of the *Chlorocystis* isolate, with 100 μmol photons m^{-2} s^{-1}, a 12:12 h dark : light and 5% CO_2 during the light phase. Seven different conditions were tested: (i) the culture control; f/2m; (ii) f/2 medium with 10× $NaNO_3$ (1.5 g L^{-1}); (iii) Fed-batch culture with $NaNO_3$ to reach 10× (1.5 g L^{-1}); (iv) f/2 medium with 10× NaH_2PO_4 (56.5 mg L^{-1}); (v) Fed-batch culture with NaH_2PO_4 to reach 10× (56.5 mg L^{-1}); (vi) f/2 medium with 10× $NaNO_3$ (1.5 g L^{-1}) and 10× NaH_2PO_4 (56.5 mg L^{-1}); and (vii) Fed-batch culture with $NaNO_3$ and NaH_2PO_4 to reach a $NaNO_3$ concentration of 1.5 g L^{-1} and 10× NaH_2PO_4 (56.5 mg L^{-1}). Growth rates for each condition were determined through optical density (OD_{750nm}) analysis and cell dry-weight determination. All conditions were tested in duplicate, and the entire experiment was performed twice (resulting in four values for each condition). Growth rate and doubling time were calculated with the following equations as it was described by Schoen (1988) and Guillard (1973).

Growth rate (u) is: μ = lnX2 - lnX1 t2-t1 where X1 and X2 are optic densities at times t1 and t2.

Doubling time is calculated with the following equation: dt = 0.6931/μ.

Cell dry-weight determination

Dry weight determinations were done in triplicate as described by Zhu and Lee (1997). After reaching stationary phase, the culture was collected from the photobioreactor. Then, a 10 mL aliquot was filtered through 0.47 mm cellulose nitrate membrane filter microfiber filters (Whatman, Germany). The filtered sample was washed with 0.5 M ammonium formate then dried at 80°C for 24 h, after which it was transferred to desiccators over silica gel for dehydration until a stable weight was obtained.

Total lipid extraction

Total lipids were extracted from the algae biomass using the method of Folch *et al*. (1957) with modifications (Saadaoui *et al*. 2016). The total lipid content was determined gravimetrically, subsequently, the lipid content (%) and lipid productivity (mg L^{-1} d^{-1}) were determined as per it was described by Arora *et al*. (2016) using the following equations:

Lipid content (%) = Total lipids (g)/Dry biomass (g).

Lipid productivity = Biomass productivity * lipid content (%)/100.

FAME profile determination

Fatty acid methyl esters (FAMEs) were extracted using a one-step transesterification method (Saadaoui *et al*. 2016). Briefly, 100 mg of dried biomass that was collected during stationary phase was added to an adequate volume of sulfuric acid (95%) and methanol (H_2SO_4:CH_3OH, 1:10). Then, a 10 min sonication was performed (Branscon 1510, Mexico). After 2 h of heating at 80°C, the mixture was transferred to a centrifuge tube containing distilled water and a mixture of hexane : chloroform (4:1). Finally, the FAME fraction was stabilized by the addition of BHT, followed by filtration, prior to GC-FID (Shimadzu, Japan) analysis.

Protein and carbohydrate extraction

Total proteins were extracted from 100 mg of dry algae biomass using a Sigma kit (Plant Total Protein Extraction PE0230, USA). The extracted proteins were quantified using the Bradford assay (Bradford 1976).

The carbohydrate extraction as performed as per Dubois *et al*. (1956), 100 mg of dried biomass was first subjected to glacial acetic acid treatment followed by incubation at 80°C. The sample was then washed with acetone and centrifuged. The pellet was subjected to acid hydrolysis using 4 M HCl at 90°C for 2 h. An equal volume of water was added to the acid mixture, then 20 μL of the sample was removed and mixed with 900 μL of phenol sulfuric acid reagent. The mixture was boiled for 20 min and the absorbance was read at 490 nm.

Statistical analysis

Statistical analyses of all data presented were performed with Microsoft Excel. Test Student *t*-tests (α = 0.05) were used to evaluate significant differences between experimental groups. Means are considered significantly different when *P*-value (α) < 0.05).

RESULTS

Effect of the Nitrate and Phosphate on *Chlorocystis* growth rate

The growth rate as well as the biomass production of *Chlorocystis sp.* strain QUCCCM14 was investigated under seven different conditions (Fig. 1, Table 1). Under control conditions of f/2 medium alone, the strain presented a slow growth rate (0.126 ± 0.025 day^{-1}) and a very low biomass productivity of 87.5 ± 14 mg L^{-1} day^{-1}. To improve the growth rate, as well as the metabolite productivity, six other culture conditions described above were investigated.

Comparative analyses of the seven culture conditions showed that the $NaNO_3$ and NaH_2PO_4 concentrations, as well as the cultivation method, affected the growth rate and the amount of biomass produced. The highest growth rate and biomass production were observed in the case of f/2 medium with 10× $NaNO_3$ and 10× NaH_2PO_4 (1.275 ± 0.19 day^{-1} and 178.55 ± 10 mg L^{-1} day^{-1}) and f/2 medium with 10× $NaNO_3$ (1.125 ± 0.08 day^{-1}, 176 ± 40 mg L^{-1} day^{-1}) in a very short doubling time of 0.617 ± 0.045 day and 0.549 ± 0.08 day, respectively. These conditions all yielded very similar effects, namely, a 2× improvement in biomass productivity compared with f/2. Fed-batch culture with $NaNO_3$ and Fed-batch culture with NaH_2PO_4 led to a slight improvement.

Assessing the effects of $NaNO_3$ and NaH_2PO_4 on metabolite production

To assess the effect of the $NaNO_3$ and NaH_2PO_4 on metabolite production by the *Chlorocystis* sp. strain, lipid, protein, and carbohydrates were extracted using 100 mg of dry biomass from each photobioreactor since these metabolites are considered as the main components of the algae biomass (Lv *et al.* 2010). Figure 2 shows all the findings related to the seven conditions tested. Our results evidenced that batch culture using f/2 medium with 10× NaH_2PO_4; fed-batch culture with NaH_2PO_4 and fed-batch culture with $NaNO_3$ and NaH_2PO_4 doubled the lipid production, with no major differences between all the other conditions. Lipid production of the previously cited conditions was between $36.8\% \pm 0.84$ and $39.7\% \pm 0.8$, with the highest percentage observed in f/2 medium with 10× NaH_2PO_4.

Table 1. Growth-rate study of QUCCCM14 under seven different conditions

Condition	Growth rate μ (day^{-1})	Doubling time (day)	Max biomass (g L^{-1})
f/2m medium modified	0.12 ± 0.02	5.68 ± 1.03	1.22 ± 0.20
f/2m medium with 10× $NaNO_3$	1.12 ± 0.08	0.62 ± 0.04	3.52 ± 0.80
Fed-batch culture with $NaNO_3$	0.98 ± 0.31	0.74 ± 0.24	2.34 ± 0.10
f/2m m medium with 10× NaH_2PO_4	0.83 ± 0.45	0.83 ± 0.08	3.11 ± 0.15
Fed-batch culture with NaH_2PO_4	1.20 ± 0.06	0.57 ± 0.03	2.77 ± 0.12
f/2m medium with 10× $NaNO_3$ and 10× NaH_2PO_4	1.27 ± 0.19	0.55 ± 0.08	3.57 ± 0.21
Fed-batch culture with $NaNO_3$ and NaH_2PO_4	1.14 ± 0.42	0.65 ± 0.24	3.04 ± 0.36

Chlorocystis QUCCCM14 was not a protein-rich strain, presenting 13.3% when cultivated using f/2 medium growth medium. Our results showed that culture using 10× $NaNO_3$ (1.5 g L^{-1}), 10× $NaNO_3$ and 10× NaH_2PO_4, as well as Fed-batch culture with $NaNO_3$ led to high levels of protein production of between $53.8\% \pm 0.8$ and $59.6\% \pm 0.9$ (approximately 6× that of f/2 medium alone). Culture using f/2 medium with 10× NaH_2PO_4; f/2 medium with 10× $NaNO_3$ and 10× NaH_2PO_4, as well as Fed-batch culture with NaH_2PO_4, led to protein production between $35.5\% \pm 0.8$ and $40.5\% \pm 0.7$, an approximate fourfold improvement. However, a comparative analysis of carbohydrate production revealed a slight effect under the different conditions. The highest production was observed after simultaneous Fed-batch culture with $NaNO_3$ and NaH_2PO_4 (Fig. 2).

Evaluating the effects of nutrients on Lipid productivity

Compared to the standard cultivation condition (f/2 medium), f/2 medium with 10× $NaNO_3$ (1.5 g L^{-1}) led to increasing in

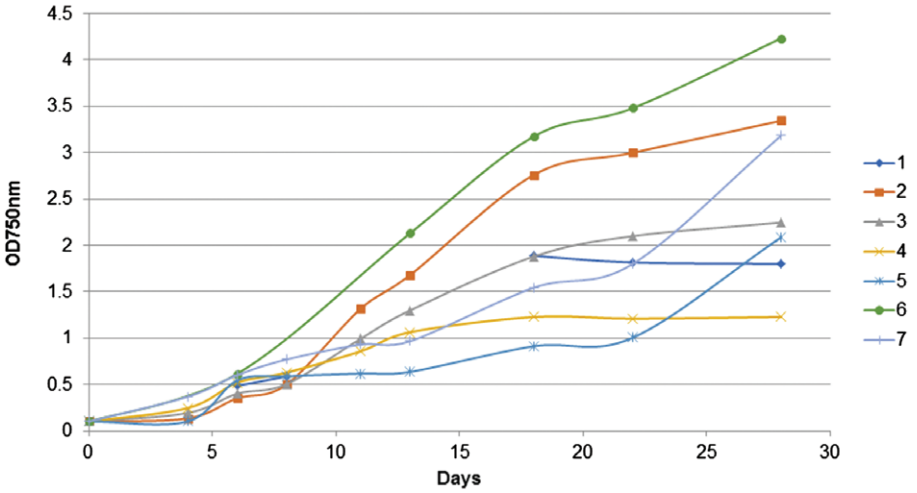

Fig. 1. Growth rate analysis of the seven different conditions: 1: f/2m; 2: f/2m medium with 10× NaNO3; 3: Fed-batch culture with NaNO3; 4: f/2m medium with 10× NaH2PO4; 5: Fed-batch culture with NaH2PO4; 6: f/2m medium with 10× NaNO3 and 10× NaH2PO4; 7: Fed-batch culture with NaNO3 and NaH2PO4. [Color figure can be viewed at wileyonlinelibrary.com]

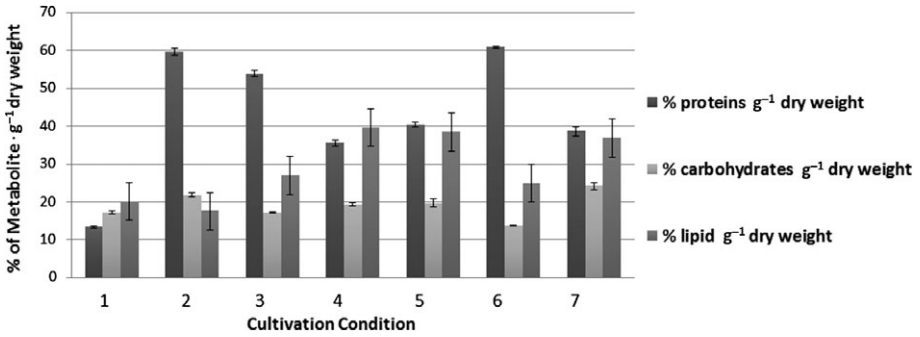

Fig. 2. Effect of nitrate and phosphate concentrations on metabolite production by *Chlorocystis* sp. isolate QUCCCM14. 1: f/2m; 2: f/2m medium with 10× $NaNO_3$; 3: Fed-batch culture with $NaNO_3$; 4: f/2m medium with 10× NaH_2PO_4; 5: Fed-batch culture with NaH_2PO_4; 6: f/2m medium with 10× $NaNO_3$ and 10× NaH_2PO_4; 7: Fed-batch culture with $NaNO_3$ and NaH_2PO_4.

the biomass productivity reaching up to 176 ± 40 mg L^{-1} day^{-1} while fed batch culture using $NaNO_3$ led to a slight improvement of the biomass productivity along with improving its lipid productivity. However, both these conditions resulted in similar lipid productivity which was 1.74 folds higher than the standard condition (using f/2 medium modified) (Fig. 3).

The cultivation using f/2 medium with 10× NaH_2PO_4 (0.15 g L^{-1} $NaNO_3$ and 0.056 g L^{-1} NaH_2PO_4) resulted in an improvement of lipid productivity up to 56.121 mg L^{-1} day^{-1} which is 3.178 folds higher.

The cultivation under 10× $NaNO_3$ and 10× NaH_2PO_4 (1.5 g L^{-1} $NaNO_3$ and 0.056 g L^{-1} NaH_2PO_4) led to the highest biomass and lipid productivities with 178.55 ± 10 mg L^{-1} day^{-1}; 65.715 mg L^{-1} day^{-1}, respectively. The fed batch cultivation led to less biomass and lipid productivities.

Evaluating the effects of nutrients on FAME profile

The FAME profile of *Chlorocystis* QUCCCM14 cultivated at different nitrogen and phosphate concentrations under different growth conditions was also analyzed (Table 2). The results showed heterogeneity in the FAME profiles. The lowest relative percentage of unsaturated fatty acid was observed in the case of culture batch-fed with $NaNO_3$ and NaH_2PO_4, simultaneously (73.26%). The other conditions showed the presence of high and similar percentages of unsaturated fatty acids, ranging between 94.12 and 99.58%. Batch culturing with 1.5 g L^{-1} nitrate (10× $NaNO_3$) showed a high percentage of mono-unsaturated fatty acids (MUFAs) such as paullinic acid (C20:1, W7) and DHA (C22: 6, W3), with relative percentages of 51.43% and 18.42%, respectively. Similar results were

Table 2. Percentage relative fatty acid FAME/total FAME extracted from 100 mg of dry microalgae biomass by *Chlorocystis* sp. isolate QUCCCM14

Fatty acid methyl ester	1	2	3	4	5	6	7
Myristic acid (C14: 0)	0.33	0.06	0.04	0.1	0.15	0	0.78
Myristoleic acid (C14: 1)	0.82	0.35	0.44	0	0	0.79	0.32
Palmitic acid (C16: 0)	0	0	0.14	0.65	0.02	1.05	2.53
Palmitoleic acid (C16: 1)	0.53	0.23	0.35	0.44	0.1	0.18	0.79
Stearic acid (C18: 0)	1.33	0.15	3.01	5.02	0.24	0	23.27
Elaidic acid (C18: 1n9t)	0.05	9.31	0	2.63	0.13	13.94	0
Oleic acid (C18: 1n9c)	8.38	2.59	16.23	33.36	7.94	13.41	39.2
Linoleic acid (C18: 2n6c)	4.04	2.9	3.93	5.13	0.4	0.26	0
Linolenic acid (C18: 3n6) ALA	0.46	0.51	0.54	0.52	71.98	25.46	0
Arachidic acid (C20: 0)	0	0.02	0.01	0.01	0.04	0.02	0.02
Paullinic acid (C20: 1)	73.15	51.4	44.18	43.75	9.85	12.72	18.48
Cis11, 14-Eicosadienoic acid (C20: 2)	0.09	0.06	0.07	0.14	0	0	0.02
Cis-8, 11, 14-Eicosatrienoic acid (C20: 3n6)	0	0	0	0	0.08	0	0
Arachidonic acid (C20:4)	3.13	1.59	2.41	2.99	7.99	0.82	0.83
Cis-5, 8, 11, 14, 17 Eicosapentaenoic acid (C20: 5n3 EPA)	0	0	0	0	0	23.45	0.4
C22:0	0.11	0.17	0.12	0.09	0	0.08	0.11
Erucic acid (C22:1)	0.01	0.03	0	0.02	0.01	0.71	1.46
Cis-4,7,10,13,16,19-docosahexaenoic acid (C22: 6 DHA)	0	18.4	19.47	0	0	0	0.59
C24: 1 nervonic acid	7.48	12.1	8.99	5.11	0.88	7.05	11.13
Saturated fatty acids	1.8	0.41	3.34	5.87	0.47	1.15	26.73
Monounsaturated acids (MUFA)	90.45	76	70.21	85.33	18.94	48.83	71.4
Polyunsaturated (PUFA)	7.74	23.5	26.43	8.78	80.58	50.01	1.85
C16-C18	14.81	15.7	24.22	47.78	80.85	54.31	65.81

Conditions: 1: f/2m medium modified (Control); 2: f/2m medium with 10× $NaNO_3$; 3: Batch fed with $NaNO_3$; 4: f/2m medium with 10× NaH_2PO_4; 5: Batch fed with NaH_2PO_4; 6: f/2m medium with 10× NaH_2PO_4 f/2m medium with 10× $NaNO_3$ and 7: Batch fed with $NaNO_3$ and NaH_2PO_4.

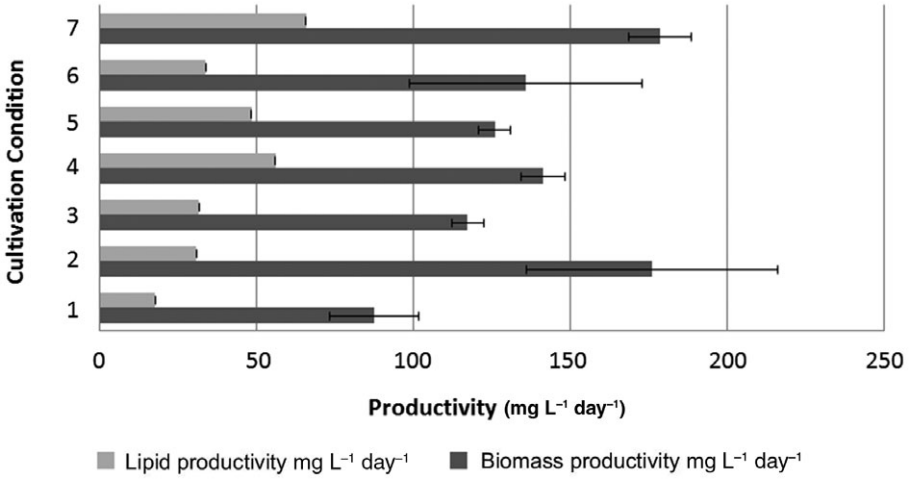

Fig. 3. Effect of nitrate and phosphate concentrations on Biomass and Lipid productivities of *Chlorocystis* sp. isolate QUCCCM14. 1: f/2m; 2: f/2m medium with 10× $NaNO_3$; 3: Fed-batch culture with $NaNO_3$; 4: f/2m medium with 10× NaH_2PO_4; 5: Fed-batch culture with NaH_2PO_4; 6: f/2m medium with 10× $NaNO_3$ and 10× NaH_2PO_4; 7: Fed-batch culture with $NaNO_3$ and NaH_2PO_4.

shown for f/2 medium with 10× phosphate (44.18% paullinic acid (C20:1) and 19.47% DHA (C22:6)) in addition to a good relative percentage of oleic acid (C18:1n9c) (16.23%). Culture using f/2 medium with 10× $NaNO_3$ and 10× NaH_2PO_4 led to an FAME profile dominated by oleic acid and paullinic acid with relative percentages of 33.36% and 43.75%, respectively.

The biomass issued from fed-batch cultures showed different profiles. We observed the presence of paullinic acid but at (×4) lower percentages (9.85%–18.48%) than the previously cited conditions. The biomass produced under batch-fed with $NaNO_3$ was dominated by linolenic acid, ALA (C18:3n6), with a relative percentage of 71.98%, whereas NaH_2PO_4 fed-batch culture led to FAME profile rich in ALA, EPA, elaidic acid, oleic acid, and at 25.46, 23.45, 13.94, 13.41%, respectively.

Chlorocystis cultured using batch-feed with NaH_2PO_4 and $NaNO_3$ showed similar Oleic and Paullinic relative percentages than the previously cited conditions. Slight differences were observed in the latter conditions, with higher relative percentages of stearic acid (C18:0, 23.27%) and oleic acid (C18:1n9c, 39.2%). As per our findings, we observed that Fed-batch cultures with $NaNO_3$ and NaH_2PO_4 simultaneously increased the percentage of the saturated fatty acids.

Finally, we considered that cultivating *Chlorocystis* QUCCCM14 using f/2m with 10× NaH_2PO_4 (0.15 g L^{-1} $NaNO_3$ and 0.056 g L^{-1} NaH_2PO_4) is the best condition that can be adopted for biodiesel production since it is economically feasible. Such conditions led to high lipid productivity with less nutrient input.

DISCUSSION

The *Chlorocystis* slow growth rate of (0.126 ± 0.025 day^{-1}) confirmed that QUCCCM14 is not a suitable candidate for biomass production, which potentially explains why this species has not been very attractive for algal applications. This species is abundant in the Qatar Coastline and we want to make from it an interesting strain since it is naturally adapted to the local desert climate. To improve its potentials, several culture conditions were tested, and our results proved that providing f/2 growth media with 10× $NaNO_3$ and 10× NaH_2PO_4 or with 10× $NaNO_3$ doubled the biomass productivity of *Chlorocystis* sp. QUCCCM14. These findings demonstrate that favorable and rich growth media enhance algae growth and biomass accumulation (Chiu *et al*. 2009).

Algae metabolite production is dependent on the algae species (Ho *et al*. 2008) and several strategies were developed to enhance the special metabolite accumulation. Our results evidenced that batch culture using f/2m medium with 10× NaH_2PO_4; fed-batch culture with NaH_2PO_4 and fed-batch culture with $NaNO_3$ and NaH_2PO_4 doubled the lipid production. Such results converged with Hsieh and Wu (2009) proving that fed-batch cultivation increased the lipid productivity. Fed-batch culture with $NaNO_3$ and f/2m medium with 10× $NaNO_3$ and 10× NaH_2PO_4 yielded a slight increase in lipid production. This result correlates with the findings of Yen *et al*. (2013) proving that most oleaginous microalgae produced a small quantity of lipids under conditions favorable for their growth. Hu *et al*. (2008) proved that the nitrogen source is the most critical factor affecting lipid production in microalgae. Thus, Ho *et al*. (2008) demonstrated that the nitrogen depletion led to decrease of the lipid production. They are suggesting that under nitrogen depletion, the microalgae are transforming the proteins to lipids.

On the other hand, culture using f/2m medium with 10× NaH_2PO_4; f/2m medium with 10× $NaNO_3$ and 10× NaH_2PO_4, as well as Fed-batch culture with NaH_2PO_4, led to approximately 4-fold improvement of protein production, reaching approximately 40%, compared to the standard cultivation condition. Such findings could be explained by the fact that phosphate improves the absorption of the nitrate providing the nitrogen, which is an essential nutrient for the synthesis

of proteins (Singh *et al*. 2015). We noted that conditions improving lipid production led to lower protein productivity and vice-versa. The carbohydrate production is constant under the different tested conditions. Such results correlate with the findings of Wan *et al*. (2013) proving that when algae biomass composition is monitored throughout the culture, the increase of lipid is always associated with the decreasing protein content, while the carbohydrate content is maintained stable.

The cultivation of *Chlorocystis* sp. QUCCCM14 using f/2m medium with 10× NaH_2PO_4 resulted in 3.17-fold higher lipid productivity (56.12 mg L^{-1} day^{-1}) comparatively to the standard condition. Such an achievement was like what was described by Wan *et al*. (2013) (58.39 mg L^{-1} day^{-1}) after using a growth media deprived of Nitrogen and was Phosphorous sufficient and higher than the one achieved by Arora *et al*. (2016), stating that 49.1 ± 0.41 mg L^{-1} day^{-1} after using BBM growth medium limited on Phosphorous and Nitrogen. Feeding a batch culture with NaH_2PO_4 resulted in higher lipid content but led to lower biomass and lipid productivity. This is since the synergistic effect between both the macronutrients as phosphorous is essential for nitrate absorption, energy transfer, signal transduction and photosynthetic respiration (Singh *et al*. 2015).

Chlorocystis sp. cultivation under 10× $NaNO_3$ and 10× NaH_2PO_4 led to the highest biomass and lipid productivities of 178.55 ± 10 mg L^{-1} day^{-1}; 65.715 mg L^{-1} day^{-1}, respectively. Such findings confirmed the synergistic effect between both the macronutrients as phosphorous is essential for nitrate absorption, energy transfer, signal transduction and photosynthetic respiration (Singh *et al*. 2015). The best conditions leading to the highest lipid productivity will be studied further in detail for its quality via investigation of the FAME profile using GC-FID (Schimatzu, Japan). This approach will highlight the effect of NaNO3 and NaH2PO4 on the FAME production to choose the most suitable condition for biodiesel production.

Finally, our results proved that FAME profile is closely related to the growth condition and the nutrient concentrations. These findings converge with Saraf and Thomas's (2007) findings, who proved that the FAME profile depends on the organism and on the growth conditions. Moreover, Ramos *et al*. (2009) and Chen *et al*. (2015) demonstrated that varying the nutrients concentration significantly modulates the lipid content of the algae and the composition of the major FAMEs. The presence of the polyunsaturated fatty acids PUFA especially omega3 fatty acids in the algae biomass increased its nutritional value. The most important PUFAs are mainly eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) as an alternative to the fish oil due to their crucial role on animal and human health (Bandarra *et al*. 2003; Donato *et al*. 2003) Accordingly, biomass issued from QUCCCM14 culture using f/2m m medium with 10× $NaNO_3$ or 10× NaH_2PO_4 and batch fed with NaH_2PO_4 are considered to be very interesting to produce feed and food supplements and supporting food security due to their richness in DHA and EPA, respectively. Whereas QUCCM14 culture that was batch fed with $NaNO_3$ and NaH_2PO_4 simultaneously led to an abundance of monounsaturated fatty acids suitable for biodiesel production. Accordingly, QUCCCM14 is considered a good candidate to treat N and P in waste water to produce biodiesel in an economically feasible process.

ACKNOWLEDGMENTS

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Abstract: Diatoms have been emerging as a major source for the production of bioactive compounds. Marine diatoms can store high amount of lipids and grow quickly. Unfortunately, they are little studied and underexploited resources. The current work objective is to promote diatom strains from a new and original origin never investigated before: intertidal mudflats. Benthic diatom strains were isolated and hosted in the Nantes Culture Collection (NCC). Six strains known for their high biomass and/or lipid productivity: Amphora sp., E. paludosa , N. alexandrina , Nitzschia sp., Opephora sp. and Staurosira sp were cultivated in airlift photobioreactor for the first time. Their lipid class composition, fatty acid and sterol distribution were studied. Total lipid production varied from 11.4%DW (Amphora sp.) to 41%DW (Staurosira sp.). Neutral lipid amounts varied from 23% (Amphora sp.) to 76% (Staurosira sp.) of the total lipids (%TL). Glycolipids ranged from 18%TL (Staurosira sp.) to 59%TL (Opephora sp.) and phospholipids accounted for 6%TL (Staurosira sp.) to 26%TL (Amphora sp.). Some qualitative and quantitative differences were identified in fatty acid and sterol composition in the different analyzed strains. Staurosira sp. seems to be the most promising strain in terms of lipid production and most particularly in triacylglycerol production. E. paludosa produced phytosterols and eicosapentaenoic acid (EPA), compounds that could be recoverable in pharmaceutical industry. N. alexandrina produced squalene and low saturated fatty acids which could be interesting in nutraceutical industry as antioxidants.

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Mon, 23 Nov 2020 at 11:33 am

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JAPH-D-20-00738R1 - invitation to review revised ms

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Dear Dr Indrayani,

You may recall reviewing the manuscript Assessment of the lipid production potential of six benthic diatom species grown in airlift photobioreactors for us. I am herewith inviting you to take a look at the revised manuscript.

Manuscript Number: JAPH-D-20-00738R1

Abstract: In recent years, diatoms have been emerging as a major source for the production of bioactive compounds. Marine diatoms grow quickly and can store high amount of lipids. Unfortunately, they are little studied and underexploited resources. The current work deals with an original and rarely investigated source of diatoms: intertidal mudflats. It aims to evaluate the lipid production potential of some strains of benthic diatom species,isolated and hosted in the Nantes Culture Collection (NCC), when cultivated in an airlift photobioreactor. Six strains known for their high biomass and/or lipid productivity: Amphora sp. (NCC169), Entomoneis paludosa (NCC18.2), Nitzschia alexandrina (NCC33), Nitzschia sp. (NCC109), Opephora sp. (NCC366) and Staurosira sp. (NCC182) were cultivated in airlift photobioreactors for the first time. Their lipid class composition, fatty acid and sterol distribution were studied. Total lipid production varied from 11.4%DW (Amphora
sp.) to 41%DW (Staurosira sp.). Neutral lipid amounts varied from 23% (Amphora sp.) to 76% (Staurosira sp.) of total lipids (%TL). Glycolipids ranged from 18%TL (Staurosira sp.) to 59%TL (Opephora sp.) and phospholipids accounted for 6%TL (Staurosira sp.) to 26%TL (Amphora sp.). Some qualitative and quantitative differences were identified in both fatty acid and sterol composition in the different strains analyzed. Staurosira sp. seems to be the most promising species in terms of lipid production and most particularly in triacylglycerol production. E. paludosa produced phytosterols and eicosapentaenoic acid (EPA), compounds with potential for application in the pharmaceutical sector. N. alexandrina produced squalene and low levels of saturated fatty acids which could both be interesting in the nutraceutical industry as antioxidants.

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Published: 11 May 2021

Assessment of the lipid production potential of six benthic diatom species grown in airlift photobioreactors

Eva Cointet, Elise Séverin, Aurélie Couzinet-Mossion, Vona Méléder, Olivier Gonçalves & Gaëtane Wielgosz-Collin

Journal of Applied Phycology **33**, 2093–2103 (2021)

Abstract

In recent years diatoms have emerged as a major algal source for the production of bioactive compounds. Marine diatoms grow quickly and can store high amount of lipids. Unfortunately, they are little studied and underexploited resources. The current work deals with an original and rarely investigated source of diatoms: intertidal mudflats. It aims to evaluate the lipid production potential of some strains of benthic diatom species, isolated and hosted in the Nantes Culture Collection (NCC) when cultivated in an airlift photobioreactor. Six strains known for their high biomass and/or lipid productivity: *Amphora* sp. (NCC169), *Entomoneis*

paludosa (NCC18.2), *Nitzschia alexandrina* (NCC33), *Nitzschia* sp. (NCC109), *Opephora* sp. (NCC366), and *Staurosira* sp. (NCC182) were cultivated in airlift photobioreactors for the first time. Their lipid class composition, fatty acid, and sterol distribution were studied. Total lipid production varied from 11.4 (*Amphora* sp.) to 41%DW (*Staurosira* sp.). Neutral lipid amounts varied from 23 (*Amphora* sp.) to 76% (*Staurosira* sp.) of total lipids (%TL). Glycolipids ranged from 18 (*Staurosira* sp.) to 59%TL (*Opephora* sp.) and phospholipids accounted for 6 (*Staurosira* sp.) to 26%TL (*Amphora* sp.). Some qualitative and quantitative differences were identified in both fatty acid and sterol composition in the different strains analyzed. *Staurosira* sp. seems to be the most promising species in terms of lipid production and most particularly in triacylglycerol production. *Entomoneis paludosa* produced phytosterols and eicosapentaenoic acid (EPA), compounds with potential for application in the pharmaceutical sector. *Nitzschia alexandrina* produced squalene and low levels of saturated fatty acids which could both be interesting in the nutraceutical industry as antioxidants.

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Contributions

Eva Cointet and Elise Séverin conducted experiments. Eva Cointet, Elise Séverin, Aurélie Couzinet-Mossion, Vona Méléder, Olivier Gonçalves, and Gaëtane Wielgosz-Collin analyzed and interpreted the data. Vona Méléder, Olivier Gonçalves, and Gaëtane Wielgosz-Collin designed and supervised the research. All the authors drafted the work and/or revised it critically and approved the final version of the manuscript.

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Ethics declarations

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The authors declare no competing interests.

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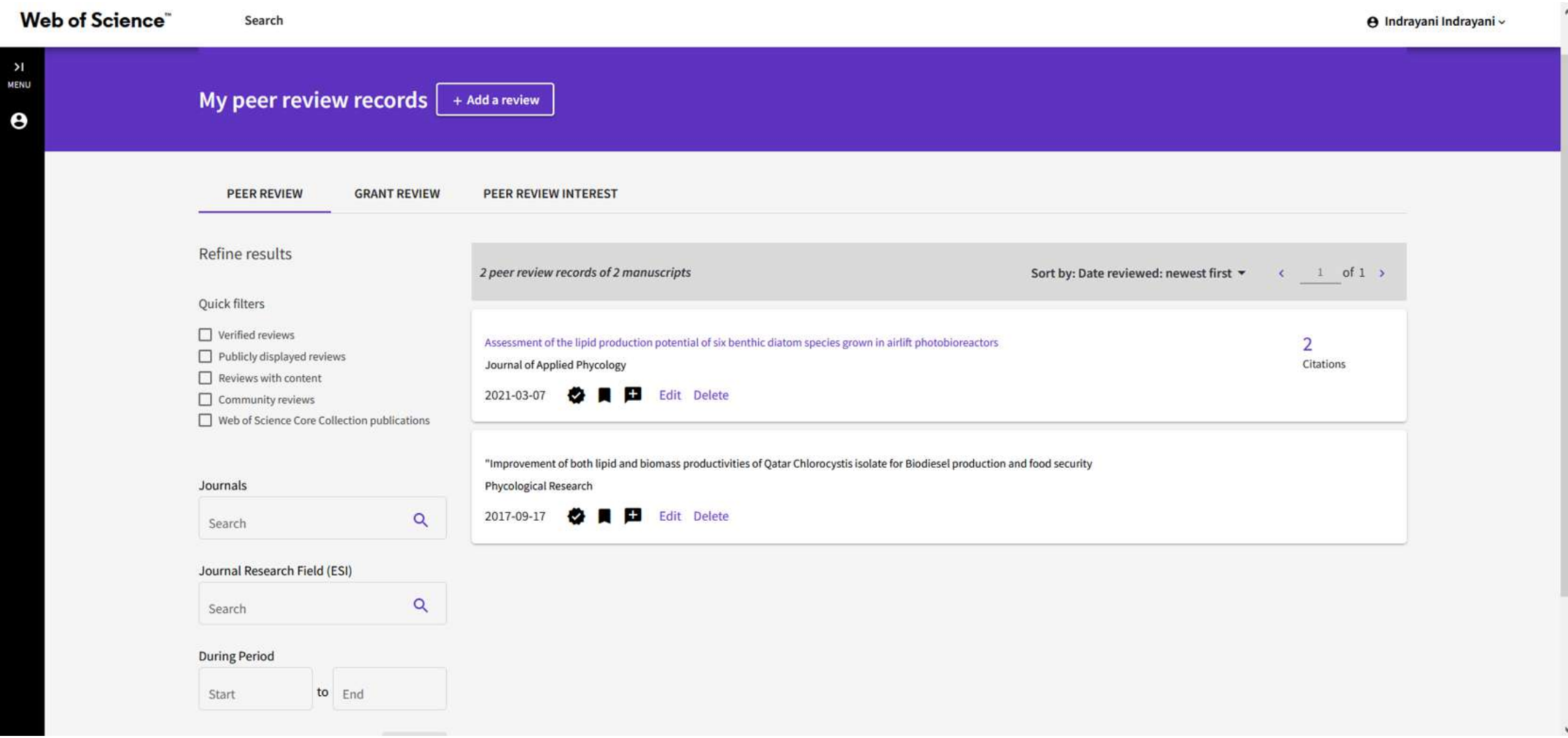
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