


ORIGINAL ARTICLE

Nutritional conditions of the novel freshwater *Coccomyxa* AP01 for versatile fatty acids composition

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Abstract

Aims: This study was to analyse the biomass production and fatty acids (FAs) profiles in a newly isolated chlorophyte, namely *Coccomyxa* AP01, under nutritionally balanced (NB) conditions (comparing nitrate and urea as nitrogen sources) and nitrogen or phosphate deprivation.

Methods and Results: Lipid yields was about 30%–40% of dried biomasses in all examined nutritional conditions. Under NB conditions, lipids were principally constituted by monounsaturated FAs, mainly represented by oleic acid, and saturated and polyunsaturated FAs at similar concentrations. Nutrients deprivation induced remarkable changes in FAs profiles, with the highest amounts of saturated (42%–46%), followed by similar amounts of monounsaturated and polyunsaturated, and the emergence of rare long-chain FAs. Under phosphate deprivation, biomass yield was similar to NB conditions, with the highest yield of saturated (mainly palmitic acid) and of polyunsaturated FAs (33%) (mainly linoleic and linolenic acids).

Conclusions: Balanced or deprived nutritional conditions in *Coccomyxa* AP01 induced a selective production and composition of FAs. The phosphate-deprivation condition concomitantly provided high biomass yield and the production of high value saturated and polyunsaturated FAs with industrial interest.

Significance and Impact of the Study: *Coccomyxa* AP01 could be considered a promising source of different FAs, including also docosapentaenoic acid, for several commercial purposes spanning from biodiesel production, pharmaceutical and cosmetic applications to innovative aquaculture fish feeds.

KEYWORDS

Coccomyxa, fatty acids profile, microalgae, nutrient starvation, rare long-chain polyunsaturated fatty acids (LC-PUFAs)

INTRODUCTION

Microalgae are a group of micro-organisms widely distributed in terrestrial and aquatic environments, which comprises a variety of prokaryotic and eukaryotic organisms, mainly autotrophic, including chlorophytes, diatoms, prasinophytes, haptophytes, rhodophytes, dinoflagellates and cyanobacteria. Because microalgae possess a fast growth cycle, independent on changing of the seasons, they are more competitive than plants in terms of value-added products, such as phycobiliproteins (e.g. phycocyanin or phycoerythrin), carotenoids, polysaccharides, peptides, vitamins and omega 3 and omega 6 fatty acids (FAs) (Chisti, 2007; Christaki et al., 2011). According to their carbon number, microalgal lipids are classified into FAs having 14–20 carbon atoms, used for the production of biodiesel (Chisti, 2007), and polyunsaturated fatty acids (PUFAs) with more than 20 carbon atoms, used for food and feed industries (Varfolomeev & Wasserman, 2011), aquaculture (Patnaik et al., 2006), nutraceuticals (Christaki et al., 2011) and cosmeceutical industry (Ryu et al., 2015).

Recently, the researcher's interest has focused on lipids produced by microalgae, because their content is usually in the range of 20%–50% of the dry cell weight (DCW) and can be as high as 80% under stress conditions, such as high salt, high light or nutrient limitation (Chisti, 2007; Singh et al., 2016).

FAs biosynthesis can be also triggered under nutrient starvation, when nitrogen (Wang et al., 2015; Zhu et al., 2014) or phosphorus (Roopnarain et al., 2014; Satpati et al., 2016) is depleted from the culture medium. For instance, in *Auxenochlorella pyrenoidosa*, oleic acid was reported to increase up to 60 times under nitrogen starvation, with respect to control cultures (Zhang et al., 2018), and it was suggested that nitrate could induce desaturation of C18 FAs.

Members of the genus *Coccomyxa*, within the Trebouxiophyceae class of Chlorophyta (Zhu et al., 2018), are ubiquitous inhabitants of different ecosystems as free-living cells in terrestrial habitats, or planktonic in freshwater ecosystems, and as lichen photobionts, even occurred as a contaminant in the cooling water of a nuclear power plant (Rivasseau et al., 2013), as well as in chemical solutions and distilled water in laboratories (Braune, 1964).

Recently, several species and strains belonging to the genus *Coccomyxa* have been intensively studied to identify the nutritional parameters for growth, which led to an increase in lipid yields (Allen et al., 2017; Guschina et al., 2003; Maltsev et al., 2019). However, the total FAs yield under nutritional stress often implies low biomass yield and significant variations in FAs profiles, as reported previously in *Coccomyxa elongata* MZ-Ch64 under different nutrient deficiencies (nitrogen and combined nitrogen and phosphorus starvation) (Maltsev et al., 2019).

Therefore, approaches for optimizing the production of lipids could necessarily include the selection of novel stress-tolerant strains to contextually achieve a low-cost biomass production, suitable for industrial purposes.

Recently, a novel freshwater oligotrophic strain of genus *Coccomyxa*, namely *Coccomyxa* AP01, has been isolated (Nicolò et al., 2010).

In this study, we analyse the biomass production and the FAs yield and composition in *Coccomyxa* AP01 under both nutritionally balanced (NB) conditions of growth, comparing nitrate (NB-NO₃) and urea (NB-U) as distinct nitrogen sources, and under nitrogen- or phosphate-deprived conditions in a novel formulated medium, named PMG. Interestingly, rare long-chain PUFAs (LC-PUFAs), with more than 18 carbon atoms (eicosatrienoic and docosapentaenoic acids), unreported previously for *Coccomyxa* genus, were specifically synthesized under deprived conditions.

MATERIALS AND METHODS

Microalgal strain and media

Coccomyxa AP01 was isolated previously from a rainwater open tank in Sicily (Italy) in the BG11 culture medium (Rippka et al., 1979) commonly used for microalgae growth and morphologically identified (Darienko et al., 2015). The culture was incubated at 25°C temperature, under continuous illumination and a light intensity of 25 μmol photons m⁻² s⁻¹.

To optimize biomass yields and growth rate, a modified BG11 medium was used, namely PMG, containing 204 μmol L⁻¹ K₂HPO₄; 300 μmol L⁻¹ MgSO₄·7H₂O; 24 μmol L⁻¹ CaCl₂·2H₂O; 3.1 μmol L⁻¹ citric acid; 2.3 μmol L⁻¹ ferric ammonium citrate; 0.27 μmol L⁻¹ EDTA; 18.8 μmol L⁻¹ Na₂CO₃; 0.1 ml L⁻¹ of trace elements solution (per litre: 46 mmol L⁻¹ boric acid; 9 mmol L⁻¹ MnCl₂·4H₂O; 0.77 mmol L⁻¹ ZnSO₄·7H₂O; 1.6 mmol L⁻¹ Na₂MoO₄·2H₂O; 0.31 mmol L⁻¹ CuSO₄·5H₂O; and 0.17 mmol L⁻¹ Co(NO₃)₂·6H₂O), plus 1.764 mmol L⁻¹ NaNO₃.

Coccomyxa AP01 was inoculated at OD₅₄₀ = 0.1, corresponding to a DCW of about 0.04 g, into 5-L flasks containing 1 L of each different medium.

To determine the optimal pH value for growth, *Coccomyxa* AP01 cultures were inoculated in PMG medium at four distinct pH values (from 5 to 8), by adding an adequate synthetic buffer and specifically 10 mmol L⁻¹ MES (pH 5 and 6), 10 mmol L⁻¹ MOPS (pH 7) and 10 mmol L⁻¹ tricine (pH 8). As control, one *Coccomyxa* AP01 culture was inoculated in PMG medium (pH 6.7) without buffer addition.

The optimal temperature of incubation was evaluated by incubating *Coccomyxa* AP01 cultures in PGM medium at five distinct temperatures (15, 20, 25, 30 and 35°C).

In all the subsequent experiments, no buffer was added to the medium, in that none of the tested pH values influenced the growth efficiency. All the cultures were incubated at the optimal temperature of 25°C. All the reagents used were purchased by Sigma Aldrich, Milano, Italy.

Nutritionally balanced conditions

Other than nitrate (PMG), urea (882 $\mu\text{mol L}^{-1}$) was tested as sole nitrogen source and added instead of nitrate to PMG medium (PMG+Urea). The initial pH value was about 6.7, and it was not further adjusted; then, the medium was sterilized by filtration through 0.22- μm pore size membrane (Sartorius). The PMG medium and PMG+Urea were used both in growth and in lipid synthesis experiments.

Nutrient-starved conditions

Biomass recovered from PMG was previously washed in nitrogen- or phosphate-free medium, centrifuged and then inoculated in nitrogen-deprived PMG (PMG-N) or in phosphate-deprived PMG (PMG-P).

All the cultures, in triplicate for each nutritional condition, were incubated for 14 days under continuous shaking (Stuart Scientific Gyrorocker SSL3, 70 rpm), with photons flux density, measured at air-liquid interface, of 25 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The light sources were white fluorescent tubes (Philips TLD 18 W) held horizontally, with respect to cultures.

Weekly, sterile MilliQ water was eventually added to counteract loss due to evaporation.

All reagents were purchased by Sigma-Aldrich.

Growth measurements and growth kinetics

After inoculation in the different conditions, growth measurements were performed throughout a period of 14 days. Daily, 10 ml from all the replicates of each cultural condition were withdrawn and filtered through previously heat-dried glass fibre 0.7 μm membranes (Millipore), dried in oven at 105°C for at least 15 h until weight was kept constant. Results were expressed as the mean \pm standard deviation (SD) of the DCW values obtained from the three replicates and graphically plotted as growth curve.

The specific growth rate (μ) was calculated as follows:

$$\mu = \frac{\ln\left(\frac{N_2}{N_1}\right)}{t_2 - t_1} \quad (1)$$

where N_1 and N_2 are biomass (DCW, mg L^{-1}) at time 1 (t_1) and time 2 (t_2), respectively, with t_1 the day at which growth started and t_2 at which growth stopped (Paredes-López et al., 1976).

The biomass productivity (DCW, $\text{mg L}^{-1} \text{d}^{-1}$) was calculated according to the following equation:

$$P = \frac{N_i - N_0}{t_i - t_0} \quad (2)$$

where N_i and N_0 are the biomass values (DCW, mg L^{-1}) at time t_i and t_0 , respectively (Griffiths et al., 2014).

Oil synthesis estimation by Nile red staining

Intracellular lipid bodies were stained with Nile red according to the procedure previously described and microscopically observed (Rizzo et al., 2017). Daily, 1 ml aliquots from each culture were withdrawn and centrifuged at 6000 g for 10 min at room temperature, and supernatants were discarded. Pellets were resuspended in 990 μl of sterile phosphate-buffered solution (Sigma-Aldrich) and then stained with 10 μl of Nile red (Sigma-Aldrich) stock solution (1 mg ml^{-1} in absolute ethanol). Samples were incubated for 20 min in the dark at 50°C, then cells were recovered by centrifugation (6000 g for 10 min) and resuspended in 100 μl of phosphate-buffered solution. Ten microlitres of stained cells were spotted on microscope glass slides and observed at $\times 63$ or $\times 100$ through an epifluorescence microscope (Leica DMR) equipped with an excitation filter BP 450–500 nm, a 510-nm centre wavelength chromatic beam splitter and a long pass barrier suppression filter LP 528 nm. For each sample, at least 10 optical fields were considered, each containing at least 20 cells. Images were kept by a COHU high-performance CCD camera. The number of oil-producing cells was expressed as a percentage related to the total number of cells present in the microscopic field evaluated by ImageJ (Abràmoff et al., 2004) image analysis software.

Lipid extraction and yield

Cells from all the cultures were harvested by centrifugation at 6000 g for 10 min at room temperature and dissolved in a minimal volume of sterile MilliQ water, kept at -80°C for 15 h and then lyophilized (Labco LB4 freeze dryer). Aliquots of 1 g were subjected to dynamic lipid extraction in *n*-hexane with a Soxhlet apparatus. Lyophilized cells were packed in a cellulose thimble inside the extraction chamber of the Soxhlet unit. Pure *n*-hexane of 300 ml (Romil Ltd) was used to extract the lipids for 10 h at the rate of 10 refluxes per hour. *N*-hexane was eliminated by rotavapor, and total lipid extract

was weighted and then resuspended in 1 ml of *n*-hexane and investigated for FA composition.

Lipid yield was calculated as the ratio between the extracted lipid amounts and biomass yield (DCW) and expressed as percentage.

FAs composition

The FAs composition was released as methyl ester by the official GU of the CEE methylation procedure (European Community Regulation 1991) and analysed by gas chromatography (GLC) as reported by Leu and Boussiba (2020). A Shimadzu GC 17A (Milano, Italy) instrument, equipped with a split/splitless injector (split ratio 70:1) and flame ionization detector, was used.

A Mega 10 fused silica capillary column, 50 m × 0.32 mm i.d., 0.25 μm film thickness (Legnano), was employed.

The following chromatographic conditions were used: column temperature, 170°C; injector and detector temperatures, 250 and 280°C, respectively; and carrier gas, hydrogen and linear velocity, 50 cm s⁻¹.

Peak areas of 13 FAs (C14:0, C16:0, C16:1, C17:0, C17:1, C18:0, C18:1, C18:2, C18:3, C20:0, C20:1, C22:0 and C24:0) were calculated using a HP 3394A integrator. The obtained results were expressed as mean ± SD of three replicates. Fatty acid methyl esters (FAMES) of nutritional interest were identified by direct comparison with the retention times of reference compounds present in the standard mixture (Sigma-Aldrich). The percentage of individual FAME was calculated in relation to the total area of the chromatogram.

Statistical analysis

Data from the different experimental groups were compared using the one-way analysis of variance and Tukey-*b* test for post hoc analysis. All statistical values were considered significant at the *p* level of 0.05. Statistical analysis was performed using Instat version 2.10 for Microsoft Windows (GraphPad Software Inc.).

RESULTS

Growth kinetics

Effects of different temperatures and pH values are reported in Figure 1.

Growth curves of *Coccomyxa* AP01 are plotted in Figure 2, and values of growth kinetics variables, that is, specific growth rates (μ), biomass productivities and final yields (DCWs), are summarized in Table 1.

In NB conditions, urea or nitrate was compared as nitrogen sources. The cell growth started at third day and proceeded until 10th day, then entered stationary phase. Growth rate (μ) was higher in the presence of urea than nitrate (0.08 and 0.052 d⁻¹, respectively), and DCWs were higher of about 30% than in nitrate conditions (2.3 and 1.78 g L⁻¹, respectively), as biomass productivities (0.161 and 0.124 g L⁻¹ d⁻¹, respectively).

Under nitrogen starvation, cultures showed a limited growth until the third day (DCW = 0.12 g L⁻¹); then, they entered stationary phase. μ value was -0.388, and biomass productivity and yield were 0.0025 and 0.075 g L⁻¹, respectively.

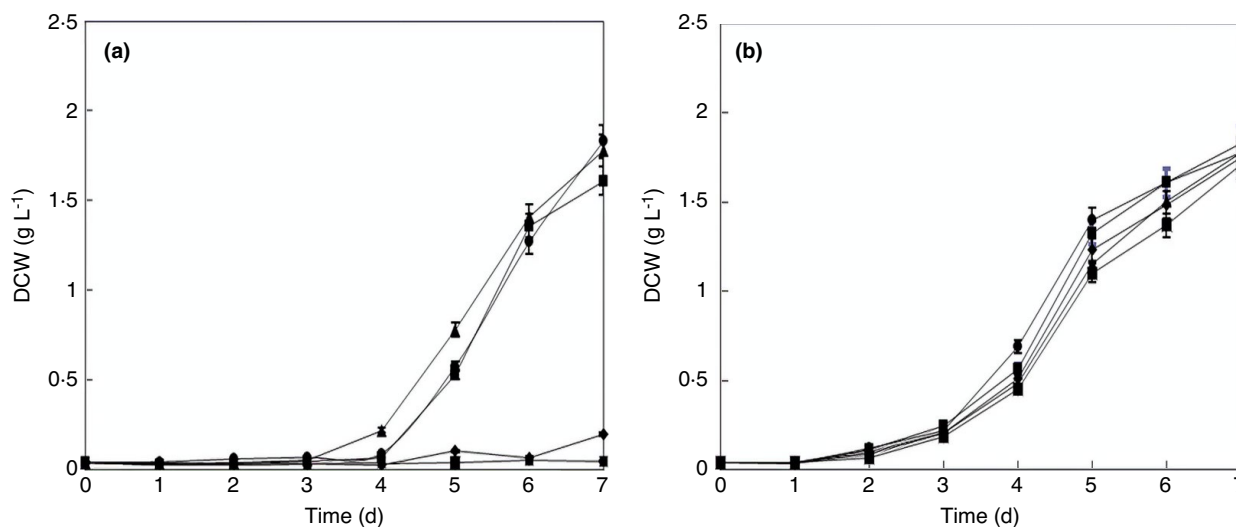


FIGURE 1 Growth of *Coccomyxa* AP01 in PMG medium under different values of (a) temperature, that is, 15°C (◆), 20°C (■), 25°C (▲), 30°C (●) and 35°C (✱), and (b) pH, that is, pH 5 (✱), pH 6 (▲), pH 7 (■), pH 8 (◆) and unbuffered medium PMG (●). The graphs show data from triplicate experiments (mean ± standard deviation) [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 2 Growth of *Coccomyxa* AP01 under different cultural conditions, that is, PMG (●), PMG+Urea (■), PMG-N (◆) and PMG-P (▲). The graph shows data from triplicate experiments (mean \pm standard deviation)

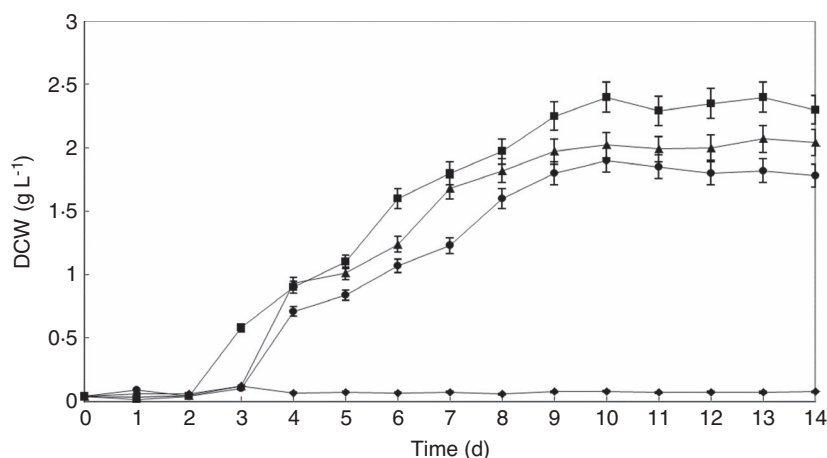


TABLE 1 Values of specific growth rate (μ), biomass productivity and biomass yield (expressed as dry cell weight, DCW) in *Coccomyxa* AP01 under nutrient-balanced (PMG and PMG+Urea) and deprived (PMG-N and PMG-P) conditions. The values are data from triplicate experiments (mean \pm standard deviation)

	Growth condition			
	PMG	PMG+Urea	PMG-N	PMG-P
Specific growth rate (μ)	0.052 \pm 0.0021	0.08 \pm 0.004	0.39 \pm 0.001	0.06 \pm 0.002
Biomass productivity (g L ⁻¹ d ⁻¹)	0.124 \pm 0.014	0.161 \pm 0.005	0.002 \pm 0.0007	0.143 \pm 0.004
Biomass yield (DCW) (g L ⁻¹)	1.78 \pm 0.0011	2.3 \pm 0.031	0.075 \pm 0.001	2.04 \pm 0.019

On the contrary, under phosphate starvation, growth was observed until 10th day (DCW = 2 g L⁻¹); thereafter, no further increase in biomass was detected. μ was 0.06, and biomass productivity and yield were 0.143 and 2.04 g L⁻¹, respectively.

These results suggest that differently for nitrogen, phosphate is not a limiting factor for cells growth.

Intracellular lipids

Nile red-stained intracellular lipids appeared as distinct yellow droplets (Figure 3).

In NB conditions, lipidic droplets were observed at third day in the middle-exponential phase. The number of oil producing cells increased until 10th day (about 45%) and then remained constant until 14th day (Figure 4a).

Under nitrogen and phosphate starvation, droplets were observed after 24 h of incubation. Although without cellular biomass production, under N starvation, the number of oil producing cells progressively increased throughout the whole period of incubation (maximum value of 83.3%) (Figures 3 and 4b). Differently, under P starvation, lipid accumulation increased from 6th to 10th day of incubation (69% of oil producing cells), and thereafter, no further variation was observed until the end of the incubation.

Lipid yield and FAs composition

Lipids from all the cultures were extracted from 1 g of each dried sample, and total lipids yield was expressed as percentage of biomass dry weight (Table 2).

FAs characterized by gas chromatography are reported in Table 3.

In cultures with nitrate as nitrogen source, lipid yield was of about 30% of DCW. Saturated FAs (SFAs, 28.01%) were mainly composed of palmitic (18.86%) and stearic acids (7.61%). Monounsaturated FAs (MUFAs) accounted for 44.28% and were mainly composed of oleic acid (42.43%), which was the most abundant FA present in the lipid extract. PUFAs had a percentage similar to SFAs (27.71%) and were represented by linoleic (21.87%) and linolenic (5.84%) acids.

In urea-fed cultures, lipid yield was of about 40% of biomass (DCW). SFAs represented 27.70%, and palmitic (18.6%) and stearic (8.18%) acids were the most abundant. MUFAs were the most present fraction (56.26%), which were essentially composed of oleic acid (54.44%), as seen in cultures with nitrate. PUFAs (16.04%) were mainly represented by linoleic (10.60%) and linolenic (5.44%) acids.

In N-starved cultures, lipid yield was of about 40% of DCW. SFAs were the most abundant (41.55%), with palmitic and stearic acids representing 30.45% and 6.53%, respectively. MUFAs (30.43%) consisted principally of oleic acid

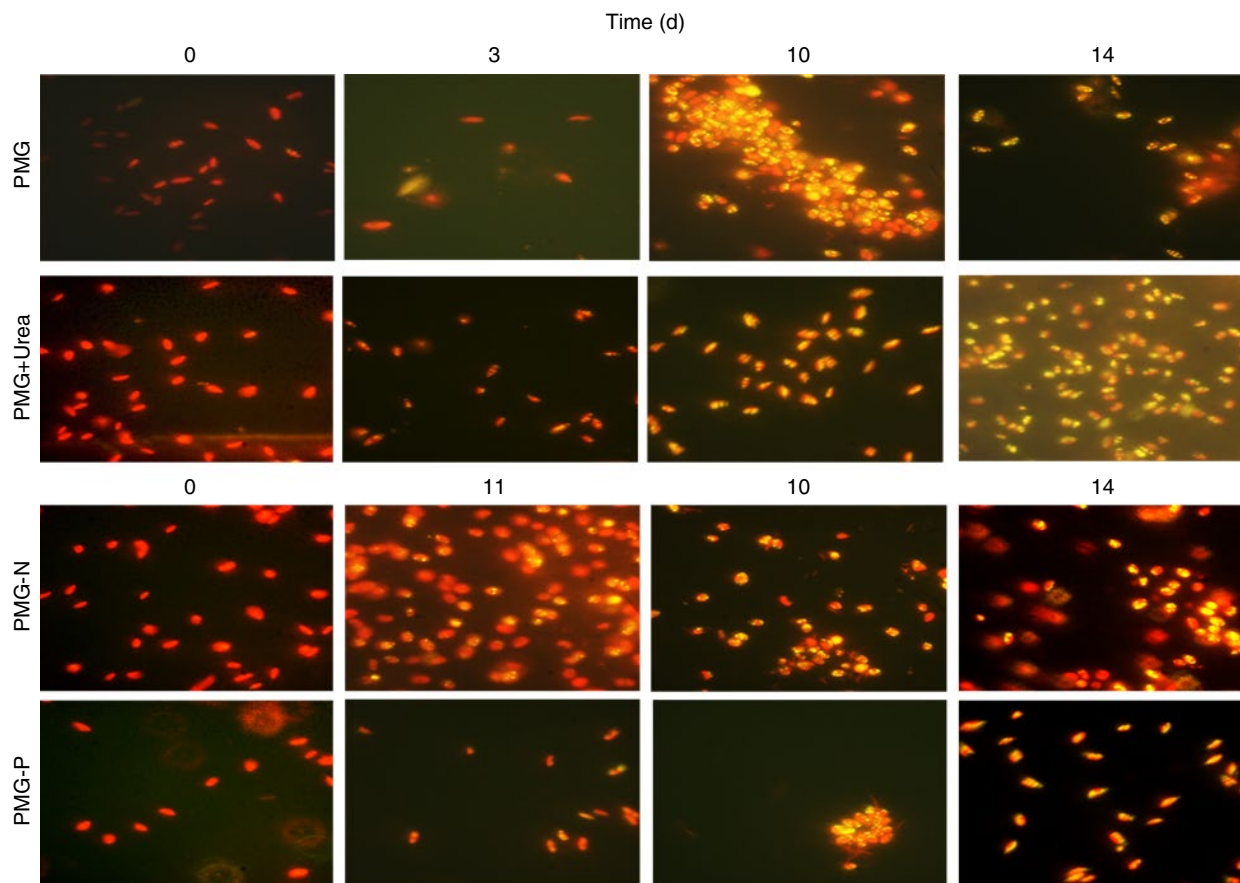


FIGURE 3 Nile red-stained cells of *Coccomyxa* AP01 under nutritionally balanced (PMG and PMG+Urea) and starved (PMG-N and PMG-P) conditions at different times of incubation. Oil droplets appeared in orange yellow, whereas oil-free cells were homogeneously red stained [Colour figure can be viewed at wileyonlinelibrary.com]

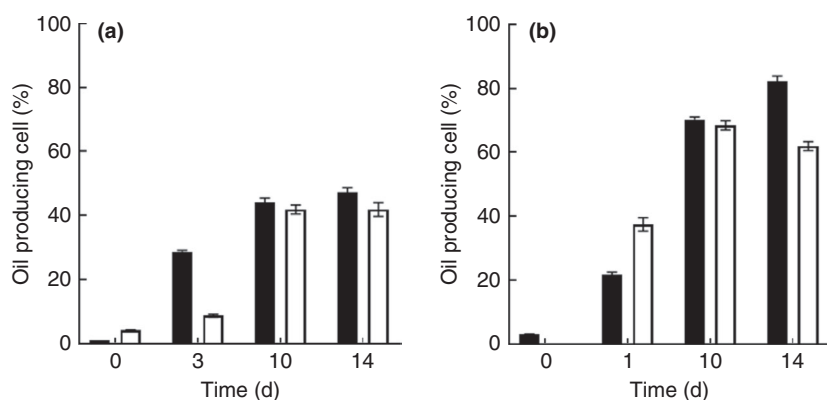


FIGURE 4 Oil producing cells of *Coccomyxa* AP01 under (a) nutritionally balanced (PMG and PMG+Urea) (■) and (b) starved (PMG-N and PMG-P) (□) conditions at different times of incubation. The number of oil producing cells was expressed as percentage related to the total number of cells present in the microscopic field

(22.73%). PUFAs were present as 28.02% of lipid extract, with similar quantities of linoleic (11.93%) and linolenic (12.14%) acids as the more abundant. Higher levels of miristic and eptadecanoic acids were present.

In *P*-starved cultures, lipid yield was about 30% of DCW. SFAs were the most abundant fraction (45.92%) of the lipid extract, mainly consisting of palmitic acid (32.83%), which had the highest concentration recorded in all the cultural conditions. MUFAs were the least abundant (21.35%), and oleic

acid was largely represented (14.98%). PUFAs represented the 32.73% of the lipid extract, and linolenic acid reached the highest concentration detected. Limited quantities of miristic, pentadecanoic and eptadecanoic acids were present, but at higher concentration than NB cultures.

Moreover, pentadecanoic, vaccenic and LC-PUFAs, with more than 18 carbon atoms, as *cis*-11,14,17-eicosatrienoic and *cis*-7,10,13,16,19-docosapentaenoic acids were synthesized in *N*- and *P*-starved cultures but not in NB cultures.

TABLE 2 Characteristics of the strain *Coccomyxa* grown in control and nutritionally balanced (NB) conditions and nitrogen-depleted (PMG-N) and phosphorus-depleted (PMG-P) conditions. Data are reported as the mean \pm standard deviation from three independent replicates

	NB conditions		Depleted conditions	
	PMG	PMG+Urea	PMG-N	PMG-P
Biomass dry weight (g L ⁻¹)	1.78 \pm 0.01	2.30 \pm 0.03	0.08 \pm 0.001	2.04 \pm 0.02
Total lipids (% of biomass)	30	40	40	30
Total lipids (g L ⁻¹)	0.53	0.92	0.03	0.61

TABLE 3 Fatty acids (FAs) composition (expressed as percentage of total lipids) in saturated FAs, monounsaturated FAs and polyunsaturated FAs produced by *Coccomyxa* AP01 after growth in different nutritional conditions. The values, expressed as percentages, are data from triplicate experiments (mean \pm standard deviation) (n.d., not detected)

Fatty acids composition (% of total lipids)	PMG	PMG+Urea	PMG-N	PMG-P
Saturated FAs				
C14 Miristic acid	0.01	0.01	1.8	2.83
C15 Pentadecanoic acid	n.d.	n.d.	1.64	2.24
C16 Palmitic acid	18.86	18.6	30.45	32.83
C17 Eptadecanoic acid	0.34	0.32	1.12	3.39
C18 Stearic acid	7.61	8.18	6.53	4.63
C20 Arachidic acid	1.17	0.6	n.d.	n.d.
C22 Behenic acid	0.01	0.01	n.d.	n.d.
C24 Lignoceric acid	0.01	0.02	n.d.	n.d.
Total	28.01	27.7	41.55	45.92
Monounsaturated FAs				
C16:1 Palmitoleic acid	0.32	0.86	1.14	0.87
C17:1 Eptadecenoic acid	1.12	0.52	2.45	1.71
C18:1n9 Oleic acid	42.43	54.44	22.73	14.98
C18:1n7 Vaccenic acid	n.d.	n.d.	3.71	2.83
C20:1 Eicosenoic acid	0.41	0.4	0.4	0.97
Total	44.28	56.26	30.43	21.35
Polyunsaturated FAs				
C18:2n6 Linoleic acid	21.87	10.6	11.93	15.8
C18:3n3 Linolenic acid	5.84	5.44	12.14	15.03
C20:3n3 <i>cis</i> -11,14,17-Eicosatrienoic acid	n.d.	n.d.	1.36	0.56
C21:5n3 Heneicosapentaenoic acid	n.d.	n.d.	0.81	n.d.
C22:5n3 <i>cis</i> -7,10,13,16,19-Docosapentaenoic acid	n.d.	n.d.	1.77	1.33
Total n3	5.84	5.44	16.09	16.93
Total n6	21.87	10.6	11.93	15.8
Total n3 + n6	27.71	16.04	28.02	32.73

DISCUSSION

There is an increasing demand for novel natural products based on microalgal metabolites, including FAs, in many commercial applications, such as food and feed industries, biodiesel, cosmetic and cosmeceutical market (Joshi et al., 2018). In this study, we report the variation of growth, biomass production and FAs profiles under different

nutritional conditions in a newly isolated chlorophyte, namely *Coccomyxa* AP01. *Coccomyxa* AP01 is able to grow in wide ranges of temperature and pH, which are highly desirable features in biotechnological processes. Growth curves of *Coccomyxa* AP01 showed that under NB conditions, both nitrate and urea were suitable nitrogen sources, with urea being more efficient in producing the highest biomass yield. Similar results have been previously reported for *Coccomyxa*

acidophila (Casal et al., 2011), suggesting that urea can be degraded releasing CO₂ directly into cell cytoplasm (Krausfeldt et al., 2019; Leftley & Syrett, 1973). The biomass yield of *Coccomyxa* AP01 was the lowest in nitrogen-deprived condition, presumably as a cellular survival mechanism as well as it occurs in other microalgae (Kraft et al., 2008). However, the low biomass yield obtained in this condition precludes any prospective for industrial purposes. Differently to other microalgae, biomass yield of *Coccomyxa* AP01 under phosphate deprivation was high and comparable to NB conditions. Growth in phosphate-depleted conditions has also been reported for *C. elongata* (Maltsev et al., 2019) and *Coccomyxa peltigerae-variolosae*, which are able to use cytoplasmic polyphosphate granules as internal phosphate source for growth (Powell et al., 2009). Moreover, cellular-membrane phospholipids are replaced by neutral, phosphate-free lipids, as observed in several microalgae (Cañavate et al., 2017a, 2017b; Iwai et al., 2015; Mühlroth et al., 2017).

Lipid yield from *Coccomyxa* AP01 was about 30%–40% of dried biomass in all examined growth conditions. However, different FAs profiles were obtained by varying the nutritional conditions, such also previously observed in different microalgae (Amin et al., 2013; Lin & Lin, 2011; Nigam et al., 2011; Zhang et al., 2013). Urea stimulated the synthesis of oleic acid (more than 50% of total FAs), while nitrate increased the synthesis of linoleic acid (about 20% of total FAs) and reduced that of oleic acid, probably because of the different redox states of nitrogen source (Nigam et al., 2011). The high content of oleic acid produced by *Coccomyxa* AP01 under NB conditions might be of interest in both traditional and innovative applications. Also, it is noteworthy that the MUFAs, SFAs and PUFAs relative percentages of *Coccomyxa* AP01 under NB conditions may be suitable for biodiesel production (Puhan et al., 2010), because of the structure of the FAs affecting some critical parameters of the biodiesel, such as cetane number, iodine values and cold flow properties. Cetane number is widely used as diesel fuel quality parameter related to the ignition delay time and combustion quality. Iodine value is a measure of total unsaturation within a mixture of FAs, related to the polymerization of glycerides, induced by high temperature. This can lead to the formation of deposits or to deterioration of the lubricating (Mittelbach, 1996). Cold filter plugging point indicates the influence of cold temperature on biodiesel flow properties (Knothe, 2005). *Coccomyxa* AP01 oil under NB conditions possesses a good cetane number and iodine value, which depend on high concentration of MUFAs, and a good cold filter plugging point, which depends on the low concentration of PUFAs (Ramos et al., 2009). Furthermore, oleic acid is widely used in the realization of various products, such as cleansing agents and texture enhancers or to allow the penetration in the skin of other beneficial substances (Čižinauskas et al., 2017) also against skin inflammation (Chen et al.,

2019) or acting as anti-oxidative agent. In recent years, a number of studies have found that oleic acid has protective effects against human diseases, such as colorectal cancer and obesity; moreover, it prevents the nucleotide-binding oligomerization domain-like receptor 3/caspase-1 inflammasome pathway (Tutunchi et al., 2020), and it shows anti-inflammatory properties (Pegoraro et al., 2021).

Similarly, to other microalgae (Nigam et al., 2011; Yang, Chen, et al., 2018), FAs profiles from *Coccomyxa* AP01 under nutritionally deprived conditions differed remarkably from cultures in NB conditions, because SFAs (mainly represented by palmitic acid) and PUFAs (linolenic acid) increased, while MUFAs, and particularly oleic acid, decreased. This last appears in contrast with results reported for *Coccomyxa* sp. C-169 that showed a marked increase in monounsaturated oleic acid under nitrogen limitation (Msanne et al., 2012). The reduction of unsaturation degree of FAs induced in nitrogen-starved cultures was also reported in several microalgae species (Lv et al., 2016; Zhang et al., 2018). Because SFAs (i.e. palmitic acid) are less susceptible to oxidative degradation than their less saturated counterparts, the increase in SFAs could be considered as a defence mechanism against oxidative damage induced by nitrogen starvation, as demonstrated in *Chlorella sorokiniana* C3 (Zhang et al., 2013), as well as the increase in PUFA linolenic acid production (Richard et al., 2008; Tzovenis et al., 1997).

Microalgal biomass usually decreases under phosphate starvation, while lipid production increases with remarkable variations in FAs composition. In contrast, the biomass obtained from *Coccomyxa* AP01 was high under phosphate starvation with yield similar to those in balanced cultures, whereas lipid yield did not increase. When compared with NB cultures, FAs profiles from *Coccomyxa* AP01 showed that oleic acid was drastically reduced in nutrient-depleted conditions, while SFAs (mainly represented by palmitic acid) and PUFAs (in particular linolenic acid) increased of about three times. Under phosphate starvation, a general increase in SFAs and PUFAs was observed as described previously in several microalgal species (Huang & Cheung, 2011; such as *Chlamydomonas reinhardtii* (Qari & Oves, 2020), *Scenedesmus* sp. (Yang, Xiang, et al., 2018), *Phaeodactylum tricornerutum* and *Dunaliella tertiolecta* (Siron et al., 1989). Under both phosphate and/or nitrogen deprivation, the increase in both SFAs and PUFAs could be considered as a cellular response to cope oxidative stresses, as reported previously in *Chlorococcopsis minuta* (Hamid & Sibi, 2018; Richard et al., 2008; Tzovenis et al., 1997).

Under nutrient-depleted conditions, SFAs produced by *Coccomyxa* AP01 are mainly constituted by palmitic acid that is known to be extensively used in cosmetics as basis for creams and ointments with soothing properties (Kramer & Thodos, 1988).

PUFAs possess anti-cancer action both in vitro and in vivo, and linoleic acid inhibited colorectal tumour cell growth at high concentrations ($\geq 300 \mu\text{mol L}^{-1}$) (Lu et al., 2010); moreover, it has been shown that it may have a role in reducing the risk of rheumatoid arthritis (de Pablo et al., 2018). On the other hand, linolenic acid has been reported to exert anti-inflammatory effect in inflammatory bowel disease and to have cardiovascular-protective, anti-cancer, neuro-protective, anti-osteoporotic, anti-inflammatory and anti-oxidative effects (Kim et al., 2014). Furthermore, linoleic and linolenic acids are employed as lightening agents in cosmeceutical applications, because these PUFAs were reported to inhibit melanin synthesis and to improve the cell turnover into the stratum corneum, increasing the removal of the pigment from the skin (Ando et al., 1998).

Because of the MUFAs and PUFAs composition under phosphate-deprived conditions, the oil produced by *Coccomyxa* AP01 could be used in aquaculture as innovative fish feeds, in substitution of other expensive natural sources (i.e. from grubs or squids) (Durmus, 2019). It has been reported that aquaculture is one of the fastest-growing sectors in food industry, with an expected growth at 2.5% compound annual growth rate until 2030 (FAO, 2016). On the parallel, the production of traditional ingredients for feeding, that is, fish meal and fish oil, has declined since 1997, reaching its lowest level in 2016 (Beal et al., 2018). Then, growth in aquaculture is limited by the supply of fishmeal and particularly fish oil used to feed carnivorous cultured species, such as salmonids, flatfish, groupers and breams (Pickova, 2009). For these reasons, alternative sources of proteins and FAs for aquaculture are actually highly required. Among these, bacterial and insect feed are very promising, but the major investments have focused on algae and microalgal oil, rich in PUFAs, is actually considered for salmon farming (Beal et al., 2018) and for Nile tilapia (Sarker et al., 2020).

Moreover, it must be outlined that both nitrogen- and phosphate-deprived conditions induced also the synthesis of rare LC-PUFAs, even if at low relative percentages, such as eicosatrienoic and docosapentaenoic acids, which have not reported for the genus *Coccomyxa* so far. Eicosatrienoic acid, produced by few microalgal species, such as *Galdieria* sp. USBA-GBX-832 (López et al., 2019), *Nannochloropsis oculata* (Gog et al., 2012), *Platymonas subcordiformis* and *Porphyridium cruentum* (Huang & Cheung, 2011), the heterotrophic *Aurantiochytrium limacinum* (Moran et al., 2018), *Pheodactylum tricoratum* and *Scenedesmus maximus* (Guedes et al., 2011), was shown to have an anti-ageing effect, as it reduces UV-induced skin damage (Jin et al., 2010). Docosapentaenoic acid was extracted from different microalgae belonging to the class of Chlorarachniophyceae (Leblond et al., 2005), the dinoflagellate *Schizochytrium* sp. (Wang et al., 2018) and also from traditional fish sources as anchovies and sardine, cod liver, farmed salmon and tuna (Harwood, 2019). Docosapentaenoic acid is involved in sev-

eral biological functions, as stimulator of the migration of endothelial cells (Kanayasu-Toyoda et al., 1996), inhibitor of platelet anti-aggregation and anti-thrombosis compound (Phang et al., 2009).

Our results appear relevant in that it is possible to differentiate the FAs composition, including also rare PUFAs, by imposing or not the phosphate deprivation as nutritional condition, that concomitantly provides high biomass yield. As producer of FAs at similar or even higher yields than other microalgae, *Coccomyxa* AP01 could be considered as a promising source of bioactive compounds, including also docosapentaenoic acid, for several commercial purposes, spanning from biodiesel to pharmaceutical and cosmetic applications and to innovative aquaculture fish feeds.

ACKNOWLEDGEMENTS

This work was funded by FFABR Unime 2019 Research Program, supported by the University of Messina. Open Access Funding provided by Università degli Studi di Messina within the CRUI-CARE Agreement. [Correction added on 10 June 2022, after first online publication: CRUI funding statement has been added.]

CONFLICT OF INTEREST

No conflict of interest declared.

AUTHOR CONTRIBUTIONS

Marco Sebastiano Nicolò (MN), Concetta Gugliandolo (CG) and Salvatore Pietro Paolo Guglielmino (SG) conceived and designed research. MN, Maria Giovanna Rizzo (MR) and Vincenzo Zammuto (VZ) conducted experiments. Nicola Cicero (NC) and Giacomo Dugo (GD) contributed experiment resources. MN, CG and SG analysed data. MN, CG, VZ and MR wrote the manuscript. All authors read and approved the manuscript.

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How to cite this article: Nicolò, M.S., Gugliandolo, C., Rizzo, M.G., Zammuto, V., Cicero, N., Dugo, G. & et al. (2022) Nutritional conditions of the novel freshwater *Coccomyxa* AP01 for versatile fatty acids composition. *Journal of Applied Microbiology*, 132, 401–412. <https://doi.org/10.1111/jam.15223>