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Cultivation of *Nannochloropsis* for eicosapentaenoic acid production in wastewaters of pulp and paper industry

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HIGHLIGHTS

- *Nannochloropsis* grows in a medium based on wastewaters of pulp and paper industry.
- The algal culture produces EPA in the wastewater based medium.
- Rates of growth and EPA production were similar in wastewater and in standard media.

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ABSTRACT

The eicosapentaenoic acid (EPA) containing marine microalga *Nannochloropsis oculata* was grown in an effluent from anaerobic digestion of excess activated sludge from a wastewater treatment plant serving a combination of a pulp and a paper mill and a municipality (digester effluent, DE), mixed with the effluent of the same wastewater treatment plant. The maximum specific growth rate and photosynthesis of *N. oculata* were similar in the DE medium and in artificial sea water medium (ASW) but after 7 days, algae grown in the DE medium contained seven times more triacylglycerols (TAGs) per cell than cells grown in ASW, indicating mild stress in the DE medium. However, the volumetric rate of EPA production was similar in the ASW and DE media. The results suggest that *N. oculata* could be used to produce EPA, utilizing the nutrients available after anaerobic digestion of excess activated sludge of a pulp and paper mill.
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1. Introduction

Production of pulp and paper requires vast amounts of water (in USA, 15–45 m^3 of water per ton of pulp was used in 2000; Anonymous, 2002). The water contains nutrients brought to the factory with the wood, and a wastewater treatment plant is an essential part of a pulp/paper factory. Algae might provide an economically and environmentally sustainable way of utilizing the nutrients. Unfortunately, it is not economically feasible to use wastewaters for cultivation of algal biomass for low-value end products like biodiesel oil (Medipally et al., 2015). However, the cultivation might become profitable if the biomass contains a high-value component like a polyunsaturated fatty acid.

Microalgae are a promising sustainable future source for edible oils (Klok et al., 2014). Species of the genus *Nannochloropsis* (Eustigmatophyceae, Monodopsidaceae) contain the long-chain polyunsaturated fatty acid eicosapentaenoic acid (EPA; 20:5n-3), that may be considered as an essential fatty acid for people with insufficient endogenous synthesis of EPA (Kris-Etherton et al., 2009). The most common nutritional source of long-chain polyunsaturated fatty acids is fish oil. Algal EPA can also be used in fish cultivation, and especially juvenile fishes may benefit from the presence of EPA in the diet. *Nannochloropsis* species can be included in the diet of cultivated fishes by feeding grazers like rotifers with the algae (Roessler, 1990; Sukenik et al., 1993). Algal biomass can be used, after extraction of long-chain fatty acids and other valuable compounds, also for production of methane, and algae may simultaneously contribute to wastewater treatment by taking up nitrogen and other nutrients (Park et al., 2011).

The fatty acid composition of the marine microalga *N. oculata* depends on growth conditions. Palmitic acid (14–29% of fatty acids), palmitoleic acid (15–32%) and eicosapentaenoic acid (EPA, 24–38%) have been found as the main fatty acids of *N. oculata* in

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most studies (Sukenik et al., 1993). In two studies (Vazhappilly and Chen, 1998; Ma et al., 2014), EPA was only found as a minor constituent, together with an unusually high proportion of either oleic acid (Ma et al., 2014) or linoleic (18:2n-6), α-linolenic (18:3n-3) and docosahexaenoic (22:6n-3) acids (Vazhappilly and Chen, 1998). The fatty acid profile may vary between the strains, but the unusual fatty acid profiles found by Vazhappilly and Chen (1998) and by Ma et al. (2014) must be ascribed to growth conditions, as both used the same strain as the present work. Vazhappilly and Chen (1998) grew the algae in photomixotrophic conditions and Ma et al. (2014) applied an extended batch culture period of three weeks, which led to an increase in neutral lipids and a decrease in the proportion of EPA.

In the present study, *N. oculata* was cultivated in nutrient-rich effluent water (DE) from anaerobic digestion of excess activated sludge of a plant treating wastewaters of a pulp and paper integrate (ca 90% of the volume) and a municipality (<10%). Our objective was to find economically viable uses for the wastewater nutrients and to simultaneously improve the wastewater management practices in the industry.

The results of the present study indicate that the use of growth media containing DE induces stress for *N. oculata*, but even then, the alga can grow and produce biomass. Furthermore, algae grown in a medium consisting of DE mixed with the effluent of the same wastewater treatment plant produced EPA at a high rate, suggesting that the algal biomass would act as a good source of EPA.

2. Methods

2.1. Wastewater based growth media

The activated sludge, used as the raw material of anaerobic digestion, originated from a wastewater treatment plant treating pulp and paper industry wastewater together with a minor amount (<10%) of municipal wastewater. Activated sludge was anaerobically digested as a sole substrate in a continuously stirred tank reactor (volume 5 L) at 35 °C. The digester was fed with activated sludge every weekday, operated with hydraulic retention time of 20 d and organic loading rate of 1.0–1.6 kg volatile solids m^{-3} d^{-1}, having stable performance with about 80 L methane per 1 kg of volatile solids fed (unpublished data). DE was collected during ca 50 days of operation and was stored at 4 °C until use.

DE was treated in two steps to remove solids (total solids ~2.8%). Firstly, the solids were separated by addition of 1 mL of SuperFloc C-581 flocculant (Kemira Oy, Helsinki, Finland) per 50 mL of effluent. The supernatant was collected after 20 min of settling. Secondly, the supernatant was run through a mesh filter with pore size of 5 μm. Before using the supernatant for cultivation of algae, it was sterilized with a 500 mL bottle top polystyrene filter with pore size of 0.22 μm (Corning Inc., Corning, NY, USA).

Wastewater treatment plant effluent (WWTPE; i.e. the purified wastewater) from the same pulp/paper/municipality wastewater treatment plant was used for dilution of DE after similar sterilization as applied for DE. The DE medium is defined as a mixture of 25% of raw DE and 75% of WWTPE.

2.2. Cultivation of algae

The *N. oculata* strain UTEX LB 2164 was used for the experiments. The strain originating from the same isolation (Droop, 1955) is also known as CCAP 849/1, CCMP 525, CCMP 66, CSIRO CS-189, NCPCC 631, SAG 38.85, SMBA 66, WHOI 66 and Millport 66. For culture maintenance, *N. oculata* was grown in 250 mL Erlenmeyer flasks in artificial seawater medium (ASW, UTEX, USA), pH 8.1, under continuous light at the photosynthetic photon flux density (PPFD) of 25 ± 5 μmol m^{-2} s^{-1} at 20 °C. For growth and EPA production experiments, the algae were grown in batch mode in a 400 ml flat panel photobioreactor, model FMT-150 (Photon Systems Instruments, Brno, Czech Republic). During the first 24 h, PPFD was 50 μmol m^{-2} s^{-1}, then 100 μmol $m^{-2}s^{-1}$ for 24 h, and thereafter 200 μmol m^{-2} s^{-1}, and the culture was bubbled with air for the first 72 h (ASW medium) or 100 h (DE medium), then with 0.3% CO_2 up to 132 h, and then again with air for 24 h, and for the last 12 h with 2% CO_2 (Hoshida et al., 2005).

2.3. Optical density and biomass measurements

Growth of *N. oculata* was monitored by the increase in optical density of the culture at 680 and 735 nm and by the accumulation of dry biomass. For biomass measurements, a 10 mL aliquot of the culture was filtered through a pre-dried and weighed 1.6 μm glass microfiber filter (Model 691, VWR, Leuven, Germany). The filter with algae was weighed after drying at 105 °C for 3 h.

2.4. Chlorophyll a and carotenoids

Chlorophyll (Chl) *a* and carotenoids were extracted with methanol (99.9%) at room temperature for 3 h. The extract was centrifuged (6500 *g*, 5 min), and the concentrations of Chl *a* and carotenoids were determined spectrophotometrically according to Ritchie (2006) and Strickland and Parsons (1968), respectively.

2.5. Lipid extraction

Lipids were extracted from the cultures after 7 days of growth. Algal cells were isolated by centrifugation at 1100*g* for 10 min at room temperature, and lipids were extracted following a modified Folch procedure (Folch et al., 1957). Accurately weighed samples (0.2–0.4 g) of algal biomass were extracted with 20 mL of chloroform/methanol (2:1, v/v) in an ultrasonic bath (Branson 5510, Branson Ultrasonics Danbury, CT, USA) for 20 min. The chloroform/methanol mixture was filtered through Whatman No. 1 paper and the filtered supernatant containing the extracted lipids was transferred to a separation funnel. The extraction container and the filter paper were rinsed first with 20 mL and then with 10 mL of pure chloroform/methanol (2:1, v/v), and the collected and filtered solvent batches were combined. Aqueous potassium chloride solution (12.5 mL, 0.88%, w/v) was added to the funnel and the mixture was shaken repeatedly. The lower organic phase was collected and the remaining small amount of solvent in the valve of the separation funnel was collected after addition of 3 mL of pure chloroform. The solvents were removed by a rotary evaporator (Heidolph 2, Heidolph Instruments GmbH & Co.KG, Schwabach, Germany) and the residue was transferred quantitatively into weighed vials with chloroform. The yield of raw extract was determined gravimetrically after evaporation of chloroform under a nitrogen stream. Raw extracts were dissolved in chloroform and stored at −80 °C.

2.6. Lipid class separation with solid phase extraction

In order to identify the main EPA storage forms, cholesteryl esters (CEs), TAGs, glycolipids (GLs) and phospholipids (PLs) were separated from raw extracts by solid phase extraction using a vacuum extraction manifold (Waters Corporation, Milford, MA, USA) (Hamilton and Comal, 1988). All extractions were performed in duplicate. The raw extract (600 μg) and internal standards, glyceryl triheptadecanoate (5 μg), cholesteryl pentadecanoate (5 μg), and 1,2-nonadecanoyl-*sn*-glycero-3-phosphatidyl choline (50 μg), were added to a clean glass test tube and the solvents were evaporated under a nitrogen stream. The residue was dissolved in

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