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Conversion of biowaste leachate to valuable biomass and lipids in mixed cultures of *Euglena gracilis* and chlorophytes

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ABSTRACT

Microalgae are a sustainable alternative for production of valuable omega −3 fatty acids (FAs), but high production costs limit commercialization. Utilization of waste as a nutrient source increases the economics of the cultivation process. Additionally, using mixed algal cultures instead of monocultures makes the cultivation process more flexible and can increase biomass and lipid production. Here, the growth and lipid production of microalgae *Euglena gracilis*, *Selenastrum* sp. and, *Chlorella sorokiniana* were studied in mono- and mixed cultures in small and pilot scale experiments in biowaste leachate. In pilot scale, also nutrient reduction and the number of bacteria were analyzed. Biomass production in the most productive mixed cultures was similar, but not higher than in most productive monocultures. The lipid production was highest in the small-scale monoculture of *Selenastrum* (10.4% DW) and in the pilot scale culture of *Selenastrum* with *E. gracilis* (11.1% DW). The content of alpha-linolenic acid (ALA) increased and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) remained stable during the cultivation period in all pilot scale cultures. However, increases in biomass and lipid production toward the end of the cultivation resulted in higher EPA and DHA yields in the well growing monoculture of *E. gracilis* and in the mixed culture of *E. gracilis* with *Selenastrum*. Co-cultivation of *E. gracilis* and *Selenastrum* also had a positive influence on nutrient uptake and resistance against bacteria. This type of mixed culture may be a good option for commercialization. However, as shown here, minor changes in cultivation conditions can rapidly result in dominance of a subdominant strain, and thus the stability of strain performance and production of desired FAs needs further investigation.

1. Introduction

Sustainable production practices and alternatives for fish oils as a source of essential long chain polyunsaturated fatty acids (LC-PUFAs) are needed to satisfy nutritional needs of a growing world population and to protect endangered aquatic ecosystems [1]. Concerns have been raised on pollution effects of growing fish farming and over-exploitation of wild fish populations as fish-feed ingredients [1,2]. Microalgae produce several health promoting, essential omega −3 FAs such as ALA (18:3n3), EPA (20:5n3) and DHA (22:6n3). The polyunsaturated fatty acid (PUFA) ALA is a common C_{18} FA (18 carbon atoms in FA chain) in plant oils, while LC-PUFAs EPA and DHA, (C_{20} and C_{22}) have traditionally been obtained from marine and fresh water food [3,4]. ALA is classified as an essential FA, since it is a precursor of LC-PUFAs in the omega −3 family [5]. Most animals cannot synthetize precursor FAs de novo, whereas conversion efficiency to LC-PUFAs varies between

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organisms and taxa; for example among the zooplankton, conversion of precursor FAs has been found in freshwater copepods but not in cladocerans [6]. However, conversion of ALA to EPA and DHA in the human body is limited [7] and thus, it is necessary to have additional dietary sources. EPA and DHA have anti-inflammatory properties [8], and reduce the risk for cardiovascular diseases [9] while DHA is essential for the development and function of the brain, nervous system and retinas [5,10].

In comparison to fish oils, production costs of microalgae based LC-PUFAs are still high. In a techno-economic analysis (TEA) by Chauton et al. [11], costs of EPA and DHA production in flat-panel photobioreactor (PBR) in high irradiance conditions was estimated to be 39.10 USD per kg of EPA/DHA equivalents, whereas the current production costs of fish oil based EPA and DHA are 8 USD per kg of EPA/DHA equivalents. Biorefinery concepts utilizing waste for cost-effective and environmentally friendly production of lipid rich algal biomass mostly focus on biofuel production. However, converting waste nutrients to biomass makes algal cultivation more sustainable, reduce cultivation costs and converts waste to valuable biomass. Based on TEA by Chauton et al. [11], utilization of waste nutrients and CO_2 can reduce the EPA/DHA production costs by approximately 25%, whereas combining waste stream utilization, reduction of energy consumption, and doubling of EPA and DHA production from 6% to 12% (biomass DW) can decrease the total production cost to 11.90 USD per kg of EPA/DHA equivalents. Among the microalgae, euglenoids (Euglenophyta) have raised interest as producers of LC-PUFAs [12,13] whereas green algae (*Chlorophyceae*) are rich in ALA [14]. Furthermore, euglenoids and green algae have high capacity for production of biomass and removal of nutrients and dissolved organic matter from wastewaters [15–18].

Most studies so far have focused on algae cultivation in monocultures, although cultivation of algal strains in mixed cultures, in some circumstances, is known to enhance biomass [19,20] and lipid production [19,21]; transgressive overyielding refers to higher productivity in mixed cultures than in most productive single strain cultures [21]. Mixed culturing, where strains with differing growth requirements and potentials are combined, can also enhance nutrient uptake [21] and increase the biomass stability over time [22] and flexibility of the cultivation process [15]. In comparison to monocultures, mixed cultures can better tolerate potentially stressful changes in growth conditions, such as modifications in the growth medium, while maintaining continuous biomass production. They can also be more competitive and thus tolerant of invasive algal species [22] or other contamination [23]. Alternatively, the advantage of mixed cultures can be “multifunctionality” i.e. to have a good performance in several functions, instead of transgressive overyielding or superiority in some specific parameter [22]. On the other hand, variable algal characteristics in mixed cultures can make production of specific valuable compounds unpredictable [15].

Here we grew algae in diluted biowaste leachate and hypothesized that lipid production of algae and thus the yield of ALA, EPA and DHA is boosted in mixed cultures. Biomass production, nutrient removal capacity, and tolerance of bacterial contamination were hypothesized to be higher in mixed cultures than in monocultures. Chemical oxygen demand (COD) predicts both biodegradable and biologically inert organic matter in wastewater [24]. Heterotrophic bacteria as well as mixo- or heterotrophic microalgae can participate in COD removal and thus we assumed that COD removal by algae in mixed cultures is more efficient than in monocultures. Experiments with the euglenoid *E. gracilis*, and the green algae *Selenastrum* sp. and *Chlorella sorokiniana*, were carried out both in small laboratory scale and in pilot scale experiments using monocultures as well as mixed cultures. First, lipid and biomass production was tested in small scale units. Second, upscaling tests of the lipid and biomass production and nutrient removal capacity were carried out in pilot scale PBRs with a monoculture of *E. gracilis* and in mixed cultures of *E. gracilis* with *Selenastrum* sp. or *C. sorokiniana*. Since the long time maintenance of axenic algal cultures in commercial scale is not cost-effective or even plausible, we used non-axenic algal cultures and determined the number of bacterial cells in pilot scale cultures. The overall aim of this research was to evaluate the potential of mixed cultivation for commercial production of omega −3 FAs in combination with nutrient removal capacity.

2. Materials and methods

2.1. Strains and growth media

Before the experiments, the three microalgae, *E. gracilis* (CCAP 1224/5Z), *Selenastrum* sp. (SCCAP K-1877) and *C. sorokiniana* (UTEX 1230), were cultured for two months in mixed cultures to ensure co-existence of strains and to stabilize the relative abundance of strains in mixed cultures. Mixed cultures were prepared by combining equal amounts (based on cell numbers) of strains from EG-medium [25] grown stock cultures in the following combinations: *E. gracilis* with *Selenastrum*, *E. gracilis* with *C. sorokiniana*, *Selenastrum* with *C. sorokiniana* and all three strains together. These pre-cultures as well as monocultures were regularly transferred to fresh EG-medium and later used as inocula in small and pilot scale batch culture experiments carried out in composting leachate. In small scale experiments, monocultures were used as controls when evaluating influence of mixed cultivation on biomass and lipid production. The amounts of inoculated cell suspensions in experiments were 8 mL in small scale and 5 L in pilot scale (1.6 and 2.8% of cultivation volume). In small scale experiments, biomass DW was analyzed from pre-cultures before inoculation, and in pilot scale DW was determined after inoculation to the biowaste leachate, i.e. from the first sampling point for growth determination. Cell concentrations in mixed pre-cultures in small scale and in all pre-cultures were counted before inoculation for further evaluation of strain composition. Selection of strains was based on our earlier experiments showing, that monocultures of *E. gracilis*, *C. sorokiniana* (unpublished) and *Selenastrum* [26] grow well in composting leachate. For pilot scale testing, a monoculture of *E. gracilis* and mixed cultures of *E. gracilis* with *C. sorokiniana* and *E. gracilis* with *Selenastrum* were selected based on the results of the small scale experiments (see the Section 3.1).

At both experimental scales diluted (1%) leachate from a mixture of municipal organic waste and partly composted garden waste originating from a composting plant (Labio Oy, Lahti, Finland) (hereafter called “biowaste leachate”) was used for cultivation. Suitability of diluted biowaste leachate for algal cultivation was confirmed in an earlier study [26]. Prior to the experiments solid particles were removed from the biowaste leachate by centrifugation and filtering, and the supernatant was collected and sterilized by autoclaving. A detailed description of the pre-treatment process was published earlier [26]. In the small-scale experiments, biowaste leachate was diluted with distilled water and pH adjusted to 7 using NaOH, while tap water was used for dilution in the pilot scale and pH was kept below 7 by CO_2 feeding.

2.2. Growth conditions

The small scale experiments were carried out with five replicates in a growth chamber (SANYO growth cabinet MLR-350 H; 294 L) at 20 °C, in plastic tissue culture flasks containing 500 mL of the diluted (1%) biowaste leachate. In the pilot scale test (without replication) algae were grown at 25 ± 2 °C in 180 L of the same medium in transparent polycarbonate PBRs (99 × 24.5 × 84.5 cm, 200 L). The light and dark cycle was adjusted to 16:8 in both experiment types. At both scales light entered from the sides with a photon flux density of 150 μmol $m^{-2} s^{-1}$ in the small scale and 230 μmol $m^{-2} s^{-1}$ in the pilot scale cultivations. In the PBRs, where mixing was achieved by pumping air, pH was controlled continuously by feeding CO_2 (99.8%) to cultures when pH rose above 7. Installation and buildup of PBRs is described in detail by Tossavainen et al. [26]. The pH of the small-scale cultures was