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Screening native isolates of cyanobacteria and a green alga for integrated wastewater treatment, biomass accumulation and neutral lipid production

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ABSTRACT

The value and efficiency of microalgal biofuel production can be improved in an integrated system using waste streams as feed-stock, with fuel-rich biomass and treated wastewater being key end-products. We have evaluated seven native cyanobacterial isolates and one native green alga for their nutrient removal, biomass accumulation and lipid production capacities. All native isolates were successfully grown on synthetic wastewater mimicking secondary treated municipal wastewater (without organic carbon). Complete phosphate removal was achieved by the native green alga, isolated from Tvärminne (SW Finland). Optimisation of the C:N ratio available to this strain was achieved by addition of 3% CO_2 and resulted in complete ammonium removal in synthetic wastewater. The native green alga demonstrated similar nutrient removal rates and even stronger growth in screened municipal wastewater, which had double the ammonium concentration of the synthetic media and also contained organic carbon. Sequencing of the genes coding for 18S small rRNA subunit and the ITS1 spacer region of this alga placed it in the Scenedesmaceae family. The lipid content of native isolates was evaluated using BODIPY (505/515) staining combined with high-throughput flow cytometry, where the native green alga demonstrated significantly greater neutral lipid accumulation than the cyanobacteria under the conditions studied.

1. Introduction

In order to preserve our current way of life, humans are now faced with the challenge of limiting the impact of our progress on the natural environment that supports us. This is important both in the treatment of waste products, such as contaminated water and waste CO_2 gases; and in the production of energy, particularly that which can be stored as fuel. In Finland, the importance of these issues is reflected in international agreements, including the Helsinki Convention on the Protection of the Marine Environment of the Baltic Sea Area (HELCOM), and Finland's national action plan for promoting energy from renewable sources (pursuant to 2009/28/EC). Both agreements set ambitious targets. HELCOM recommendation 28E/5 [1] stipulates 10% stricter wastewater treatment standards than the Urban Waste Water Treatment Directive 91/271/EEC [2], in setting wastewater phosphorus removal targets of 90% (population equivalents >10,000), whilst Finland's renewable action plan sets a target of 38% of (gross final consumption) energy to be supplied by renewable sources by 2020 [3]. Increasingly efficient conversion of solar energy to stored fuel is integral to meeting such targets.

Green algae and cyanobacteria are distinct photosynthetic organisms able to rapidly convert solar energy to carbon-based compounds. They are attractive raw materials for biofuel production due to their ability to capture CO_2 and the small impact that cultivation has on agriculture and land availability. Importantly, the economic value, energy, and resource efficiency of photosynthetic biofuels can be considerably improved when employing waste streams as feed-stocks [4,5]. Nutrient resource reuse is particularly important in the face of diminishing phosphorus reserves and threats to global food security (see, for example, [6]). Despite algal wastewater treatment being promoted as long ago as the 1950s [7], it has yet to be adopted as a conventional approach [4]. The phototrophic nature of these organisms presents both new opportunities and new challenges to standard process design used in conventional wastewater treatment. This, and the subsequent variety of strains and experimental conditions investigated thus far, have yielded a variety of nutrient removal efficiencies (see [8] for a brief summary), presenting large scope for optimisation.

Neutral lipids are accumulated as triacylglycerols (TAGs) in algae when the organism is under stress or other adverse environmental

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conditions, with levels of up to 50% of lipids per dry weight having been observed [9]. This tendency of algae to accumulate TAG has ensured a greater focus on their potential as a raw material for biodiesel production than cyanobacteria, which typically store carbon as glycogen and/or polyhydroxyalkanoates [10]. However, cyanobacteria do contain lipids available for direct conversion to biodiesel [11,12] and have previously been touted as suitable for high-rate lipid-production due to their accumulation of lipids in thylakoid membranes, their high levels of photosynthesis and rapid growth rate [13].

Whilst it is commonly stated that TAGs are not found in cyanobacteria, the converse has been sporadically reported since at least 1993 (see [14] and references therein). Moreover, TAGs found to accumulate under conditions of nutrient stress have recently been detected in lipid droplets of the cyanobacterium *Nostoc punctiforme* [15] and increases in lipid content have also been reported in nutrient starved cyanobacteria originating from Sweden [16]. However, the most well recognised advantages of cyanobacteria are the relative ease of their genetic manipulation and their ability to secrete fatty acids and other carbon-based products into their surrounding environment (see, for example: [10,17,18]). Thus, the identification and characterisation of unique isolates of both algae and cyanobacteria remains an important step in realising phototrophic bioenergy production.

The University of Helsinki Culture Collection (UHCC), containing more than 1000 isolates from the unique Finnish environment, presents great opportunity for exploring the biofuel potential of these native organisms. Employment of isolates native to a particular area may be advantageous in their inherent suitability to environmental conditions; a potential benefit in developing an energetically efficient biofuel production platform. We have already studied the biohydrogen photoproduction of isolates from the UHCC [19], and the extension and stabilization of production through improved light conversion efficiency of cells entrapped in thin alginate films [20,21]. We expect that native isolates may have an advantage at lower bioreactor temperatures and under lower light conditions. The current work investigates the potential for minimising the energy and fertiliser requirements of growing cyanobacteria and algae through the employment of an integrated system which simultaneously treats wastewater and generates biomass for use in biofuel (bio-hydrogen or biodiesel) production.

2. Materials and methods

2.1. Strain selection and growth

Native isolates were obtained from the University of Helsinki Culture Collection (UHCC) and were maintained, along with model cyanobacteria strain *Synechocystis* PCC 6803, herein *Synechocystis*, and control alga *Chlorella vulgaris* (UTEX 265), herein *Chlorella*, in standard BG11 medium (buffered with 5 mM HEPES/NaOH, pH 7.4) under continuous low light (approximately 15 μmol photons m^{-2} s^{-1} photosynthetic active radiation (PAR)) at room temperature. For all experiments, cell cultures underwent an antecedent controlled growth period under exact experimental conditions. This was to allow poly-phosphate stores to reach levels that may be expected in a semi-continuous growth system. For experiments performed in municipal wastewater (mWW), antecedent growth was undertaken in synthetic media, with NH_4^+-N and PO_4^{3-}-P concentrations adjusted to match those of the mWW.

A guide to the UHCC identification code, taxonomy and place of isolation are provided for the native cyanobacteria and alga employed in this study (Table 1). To demonstrate the diversity of native cyanobacterial strains, a neighbour-joining tree was generated from the alignment of the available 16S DNA sequences [22,23] using MEGA 6 software (http://www.megasoftware.net/) with bootstrapping at 1000 replicates (Fig. S1). Included for reference are the 3 closest relatives of each native isolate (found using BLAST searches and excluding uncultured hits), *Synechocystis* sp. PCC 6803 (used as a control strain in this study) and the species employed in the study of Capella-Gutierrez et al. [24] to represent the four major phylogenetic groups of Cyanobacteria.

2.2. Screening for biomass accumulation and nutrient removal

Biomass and nutrient removal screening was performed in synthetic wastewater media (synWW), which was based on BG11 media with nitrogen and phosphorus concentrations adjusted to those of Shi et al. [25]. Concentrations of nitrate-N (NO_3^--N) as $NaNO_3$, ammonium-N (NH_4^+-N) as NH_4Cl, and phosphate-P (PO_4^{3-}-P) as K_2HPO_4 were approximately 3, 21, and 4 mg/L respectively. The pH of the media was adjusted to 7.4, buffered with 5 mM HEPES/NaOH, and autoclaved prior to use. Growth was performed in 500 mL batch culture from a low starting concentration ($OD_{750} = 0.1$) and under low energy conditions (40 μmol photons m^{-2} s^{-1} continuous light, 22 °C and mixing at 100 rpm) for 14 days. Conditions were chosen to target isolates best suited for generating biomass in synWW, without a large energy input. All isolates evaluated were unicellular to allow easy comparison of biomass accumulation using optical density (OD) at 750 nm, OD_{750}.

Samples were analysed approximately daily for: OD_{750}; Chlorophyll *a* (Chl *a*) (according to Meeks et al. [26]); NH_4^+-N (using Merck kit 100683, according to the phenate method); and PO_4^{3-}-P (using Merck kit 114848, according to the ascorbic acid method).

All experiments included a method blank, which consisted of the sample matrix but did not have cyanobacteria or algae added. At each time point, OD_{750} and Chl *a* were adjusted to account for any contribution from the method blank (sample − blank). In synWW, values of OD_{750} and Chl *a* from the method blank were negligible (i.e. no contaminating growth was observed). Nutrient removal was calculated at each time point relative to the blank control (at the same time point) using the formula below:

$$\%\,\text{removal}(t) = 100 * \left(([b]_t - [s]_t)/[b]_t\right) \quad (1)$$

where t is time, b is the method blank, and s is the sample.

Table 1
Native cyanobacteria and alga employed in this study.

UHCC identifier	Place and year of isolation	Taxonomy	Reference
1TU21S5	Tuusulanjärvi (2001)	*Synechococcus* sp.	Rajaniemi-Wacklin et al. [23]
1TU44S8	Tuusulanjärvi (2001)	Unknown	N/A
0TU37S4	Tuusulanjärvi (2000)	*Snowella litoralis*	Rajaniemi-Wacklin et al. [22]
SYKE695	Unknown	*Microcystis* sp.	Personal communication, Lyudmila Saari
UHCC0027	Tvärminne (2010)	*Scenedesmus* sp.	This study
1TU39S1	Tuusulanjärvi (2001)	*Synechococcus* sp.	Rajaniemi-Wacklin et al. [23]
0TU24S4	Tuusulanjärvi (2000)	*Synechococcus* sp.	Personal communication, Anne Ylinen
SYKE2088A	Unknown	*Microcystis* sp.	Personal communication, Lyudmila Saari

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