Chemical mutation of *Stichococcus* sp. for the selection of strains with reduced chlorophyll content

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Microalgae are unicellular photosynthetic microorganisms which can grow in various qualities of freshwater, including saline water and wastewater. Microalgae are classified according to their pigmentation, arrangements of photosynthetic membranes or other morphological features, as diatoms, green algae, golden algae, and cyanobacteria (Anastopoulos and Kyzas, 2015). They share similar growth mechanisms as terrestrial plants, without having roots, stems or leaves. Thus, microalgae use sunlight to sequester CO_2 (which dissolves in the growth medium from the atmosphere) for the production of cell mass and O_2. Hence, they contribute to the reduction of CO_2 concentration and the mitigation of greenhouse effect. Microalgae can be cultivated in open ponds or in various set ups of photo-bioreactors (such as tubular or flat-panel) and may implement different light sources, such as sunlight, fluorescence light lamps, LED lamps. The most common limiting factor for microalgae growth is light, as the chlorophyll content of microalgae cells, creates a "shadow effect" to the microalgae which grow further away from the light source.

Microalgae produce a number of high added value products, such as, proteins, lipids, hydrocarbons, fatty acids, pigments (chlorophyll a, b, carotenoids), vitamins, biopolymers, and biodiesel (Costa *et al*., 2019), which may be used in pharmaceutical and industrial applications. The oil content of microalgae is usually higher than most terrestrial plants, which may account for up to 50-70% of dry weight (Chisti, 2007). Lately, the potential for capturing the carbon dioxide from flue gases in industrial scale is under exploration. Moreover, as wastewater contains nutrients and micronutrients in significant concentrations, microalgae growth may be combined with wastewater treatment, thus achieving a triplet of wastewater remediation, production of high added value products and capture of CO_2.

Our laboratories are working on a project aiming to capture CO_2 from flue gasses of hydrothermal plants, with parallel production of high added value products. The scope of the present study is the maximization of microalgae growth, by reducing the "shadow effect" created by high concentration of chlorophyll in the cellular mass of microalgae. To achieve the above, mutagenic factors have been applied to *Stichococcus* sp. and the strains with low chlorophyll content have been selected. Those strains will then be tested for growth efficiency and for their ability to produce oil and other high added value products.

Stichococcus, is a rod-shaped green microalgae, which belongs in the taxon of Chlorophyta. It is resistant in extreme pH, high temperatures, salinity and nutrients in its growing environment. It mainly grows in saline water, but researchers have encountered it growing in fresh water, soil, and even in the extreme environment of Antarctica. The mutation of *Stichococcus* sp. was achieved by using Ethyl Methyl Sulphonate (EMS) as a random chemical mutagen. EMS is an ethylating agent, which induces changes in the DNA of microalgae. Other mutation methods that have been used in previous studies are: UV radiation and heavy ion irradiation (Beacham *et al*., 2015; Ma *et al*., 2013). Perin et al. (2017) compared the chlorophyll content of a wild type of *Nannochloropsis* gaditana with its mutant strain (mutated by explosion to EMS). The results showed that chlorophyll content per cell was successfully reduced in the mutated strain. Beacham et al. (2017) stated that reduced chlorophyll content minimizes the shadow effect of microalgae, cultivated in photobioreactors.

Stichococcus sp. was grown in sterile seawater enriched with F/2 medium (starting concentration: 2% (v/v)). F/2 medium consists of salts (mainly $NaNO_3$), vitamins and trace metals. To increase inorganic nutrients, additional $NaNO_3$ was supplemented into the culture. To generate mutants, the wild cells were cultivated until they reached log phase, then they were harvested and were treated successively, with aqueous solutions of EMS with five different concentrations: 1%, 1.25%, 1.5%, 1.75%, and 2%, followed by mild shaking for 2 h, in a dark room. The EMS was then inactivated by the addition of sodium thiosulfate. The mutants were then plated at various appropriate dilutions on solid medium, in order to form visible colonies. Wild *Stichococcus* sp. was also plated on solid media, to be compared with the mutant colonies (Fig 1 top). The petri dishes were placed for approximately two weeks under the light of fluorescent lamps with total power of 72W and radiation at 3000 K and 6000 K. Subsequently, the colonies were counted and compared with the colonies of wild *Stichococcus* sp. to establish a survival rate of 5% in the plates, according to Mehtani *et al*., 2017. The colonies with the lighter green color were cultured in petri dishes for three generations to confirm mutation. For every mutant, individual colonies were

picked up and incubated for about two weeks in autoclaved seawater with F/2 medium. The biomass production of wild and mutant strains was monitored by filtering 10 ml from each culture. The optical density of the cultures (with the same biomass concentration) was measured every 24 h at a wavelength of 750 nm (Fig 1 bottom). Chlorophyll was extracted using methanol and its concentration was determined spectrophotometricaly.

Mutagenesis of *Stichococcus* sp. using EMS was proved to be successful (Figure 1). Moreover, biomass yield was greater in the mutant strains, than in the wild strain of *Stichococcus* sp. Specifically, the maximum biomass production observed in the wild strain was 8.4 mg/ml against 8.8 mg/ml for the mutated strain. On the other hand, chlorophyll content was decreased in mutant strains. The total chlorophyll (chlorophyll a and chlorophyll b) in the wild *Stichococcus* sp. was 0.84 μg/mg biomass while in the mutant strain contained approximately 15% less chlorophyll.

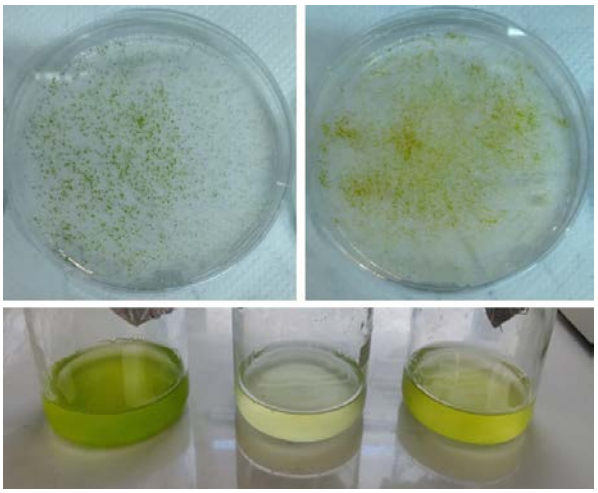

Figure 1: Top: Petri dish cultivations of wild Stichococcus sp.(left) and mutant strain (right). Bottom: Liquid cultures with biomass concentration 7.6 mg/L, of wild Stichococcus sp. (left) and mutated species with reduced chlorophyll content (middle and right).

In conclusion, the mutant strain appears advantageous over the wild strain of *Stichococcus* sp., in terms of biomass yield, while it contains significantly less chlorophyll. Thus, the mutant strain will be used in the photoreactors in our subsequent trials.

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