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**CULTIVATION OF MICROALGAE (*Chlorella vulgaris*)
FOR BIODIESEL PRODUCTION**

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Abstract

Production of biofuel from renewable sources is considered to be one of the most sustainable alternatives to petroleum sourced fuels. Biofuels are also viable means of environmental and economic sustainability. Biofuels are divided into four generations, depending on the type of biomass used for biofuels production. At present, microalgae are presented as an ideal third generation biofuel feedstock because of their rapid growth rate. They also do not compete with food or feed crops, and can be produced on non-arable land. Cultivation conditions (temperature, pH, light, nutrient quantity and quality, salinity, aerating) are the major factors that influence photosynthesis activity and behaviour of the microalgae growth rate. In this paper, we present an overview about the effect of cultivation conditions on microalgae growth.

Key words

microalgae, biofuels, cultivation conditions, growth rate, Chlorella vulgaris, Chlorella sp.

INTRODUCTION

Since humanity necessarily needs energy for its existence, it must look for the sources which are renewable and inexhaustible. In recent years, biomass-derived fuels have received increasing attention as a solution to our nation's continued and growing dependence on imported oil, which exposes the country to the risk of critical disruptions in fuel supply, creates economic and social uncertainties for businesses and individuals, and impacts our national security.

MICROALGAE

Algae are a very diverse group of predominantly aquatic photosynthetic organisms that account for almost 50 % of the photosynthesis that takes place on Earth. Algae have a wide range of antenna pigments to harvest light energy for photosynthesis giving different types of

algae their characteristic colour. Algae are proposed to play a role in the global carbon cycle by helping remove excess carbon dioxide from the environment. Recently, algae are recognized as a promising biodiesel source due to their efficient absorption and conversion of solar energy into chemical energy (1).

Depending on size, algae are classified as microalgae or macroalgae (seaweed). Microalgae are unicellular micro-organisms. Microalgae are categorized as prokaryotes and eukaryotes. Organelles are the major difference between prokaryotes and eukaryotes. Prokaryotes do not possess chloroplast, mitochondria and nuclei but they contain chlorophyll a and high protein contents. Microalgae are further divided into different groups based on their taxonomy, including blue-green, green, yellow-green, red, brown, and golden algae. There are more than 50,000 species of microalgae (2).

Microalgae can also be categorized based upon carbon supply. Some microalgae use inorganic carbon such as CO₂, are known as autotrophs. Autotrophs perform photosynthesis using light as energy source while heterotrophic microalgae use organic carbon as sugars. There are some species which can use both, organic and inorganic carbon sources, are called mixotrophs (2, 3).

Chlorella vulgaris (Table 1) has a great potential as a resource for biodiesel production due to faster growth and easier cultivation. However, lipids content in *Ch. vulgaris* under general growth conditions is up to ~20 % by weight of dry biomass, which cannot meet the standard industrial requirements (4, 5).

CLASSIFICATION OF CHLORELLA VULGARIS (6)

Table 1

Microalgae	<i>Chlorella vulgaris</i> Beyerinck (Beijerinck)
Empire	Eukaryota
Kingdom	Plantae
Phylum	Chlorophyta
Class	Trebouxiophyceae
Order	Chlorellales
Family	Chlorellaceae
Genus	Chlorella
Type species	This is the type species (lectotype) of the genus Chlorella
General environment	This is a freshwater/terrestrial species

BIODIESEL PRODUCTION

Biodiesel can be derived from edible oil seed crops such as sunflower, palm, rapeseed, soybean, coconut, etc. which are considered as the first generation biodiesel feedstocks. However, use of such feedstocks for biodiesel production has faced problems as they disturb the overall worldwide balance of food reserves and safety. The non-edible seed crops of jatropha, karanja, jojoba, mahua and waste cooking oil, grease, animal fats, etc. have gained importance in the last few years as the second generation feedstocks for biodiesel production. However, these second generation feedstocks are not sufficient to entirely substitute the present transportation needs. Recent focus is on microalgae as the third generation feedstock (7).

Using microalgae has several advantages, like microalgae have ability to fix atmospheric CO₂ and convert it into sugars, which are then converted into fuel after biochemical processing (2), microalgae do not compete for land and can grow anywhere - freshwater, brackish water, and also in wastewater (7), they demand less water and nutrients for their

growth as compared to terrestrial crops and microalgae have high growth rate and accumulate lipids up to 70 % in their cell body (2) - potential oil yields from certain algae strains are projected to be at least 60 times higher than from soybeans, approximately 15 times more productive than jatropha, and approximately 5 times that of oil palm per acre of land on an annual basis (8). Despite these advantages, the scale-up applications of microalgae biofuels have some technical limitations (2).

A process of biodiesel production from microalgae can include cultivation, harvesting/dewatering, extraction, and conversion to biodiesel.

1. *Cultivation*: Microalgae and cyanobacteria can be cultivated via photoautotrophic methods (where algae require light to grow and create new biomass) in open or closed ponds or via heterotrophic methods (where algae are grown without light and are fed a carbon source, such as sugars, to generate new biomass). Designing an optimum cultivation system involves leveraging the biology of the algal strain used and integrating it with the best suited downstream processing options. Choices made for the cultivation system are key to the affordability, scalability, and sustainability of algae to biofuel systems.
2. *Harvesting/dewatering*: Some processes for the conversion of algae to liquid transportation fuels require pre-processing steps such as harvesting and dewatering. Algal cultures are mainly grown in water and can require process steps to concentrate harvested algal biomass prior to extraction and conversion. These steps can be energy-intensive and can entail siting issues.
3. *Extraction*: Three major components can be extracted from algal biomass: lipids (including triglycerides and fatty acids), carbohydrates, and proteins. While lipids and carbohydrates are fuel precursors (e.g., gasoline, biodiesel and jet fuel), proteins can be used for co-products (e.g., animal/fish feeds).
4. *Conversion*: Conversion technology options include chemical, biochemical, and thermochemical processes, or a combination of these approaches. The end products vary depending on the conversion technology utilized (8).

CULTIVATION - PHYSICAL AND CHEMICAL CONDITIONS OF ALGAE PRODUCTION

Temperature, pH, light, nutrient quantity and quality, salinity and aerating/mixing (CO_2 flow rate) are the major factors that influence the photosynthesis activity and the behaviour of microalgae growth rate (9, 10).

Light

Light is arguably one of the most important factors for microalgae growth. As an endothermic reaction, carbon fixation requires energy and this is mainly provided by light. This energy enables microalgae cells to undergo the photosynthesis process which converts carbon dioxide (CO_2) into organic compounds such as carbohydrate and protein while releasing oxygen as waste. Microalgae cells cultivated under limited light conditions assimilate carbon towards the synthesis of amino acids and other essential cell constituents, but under saturated light conditions, sugars and starch are formed via the pentose phosphate-reducing pathway, suggesting the dependence of the biomass composition with the light availability. In reality, cultures under continuous light are often used in research and the industry because this condition generally achieves the maximum growth rate recorded (10). However, some findings suggest the use of light/dark cycles instead of continuous light as it allows for either an increase in final concentration or a lowering of production costs. This is because cell division occurs

under dark conditions for many unicellular photosynthetic cultures while for others, cell division occurs both in the dark and the illuminated phase. For the latter, cell divisions are more frequent after the interruption of the illuminated phase. In addition, some enzymes of the pentose cycle of photosynthesis and CO₂ fixation are inactive during illumination (10, 11).

Light intensity plays an important role, but the requirements vary greatly with the culture depth and the density of the algal culture: at higher depths and cell concentrations the light intensity must be increased to penetrate through the culture (e.g., 1,000 lux is suitable for erlenmeyer flasks, 5,000–10,000 is required for larger volumes). Light may be natural or supplied by fluorescent tubes. Fluorescent tubes emitting either in the blue or the red light spectrum should be preferred as these are the most active portions of the light spectrum for photosynthesis. While light is essential for algae growth, too much light, or certain wavelengths of light, can damage the algae and inhibit growth (9).

Generally, microalgae use light of wavelengths from 400 to 700 nm for photosynthesis. The wavelengths absorbed by microalgae differ depending on the species. For instance, green microalgae absorb light energy for photosynthesis through chlorophylls as a major pigment absorbing light energy in the range of 450–475 nm and 630–675 nm and carotenoids as an accessory pigment absorbing light energy of 400–550 nm. Several studies reported the growth of microalgae in different light wavelengths. Red (600–700 nm) and blue lights (400–500 nm) stimulate the growth of microalgae, and the growth rates and lipid content of the microalgae differ with light intensity (12).

Culture medium/nutrients

The correct maintenance of algal strains is dependent on the choice of growth media and culture parameters. Two approaches are possible for selection of media composition:

- In theory it is best to work on the principle that if the alga does not need the addition of any particular chemical substance to the culture media (i.e., if it has no observable positive effect on growth rate), it does not add it.
- In practice it is often easier to follow well-known (and presumably, therefore, well tried) media recipes, and safer to add substances “just in case” (providing they have no observable detrimental effect on algal growth).

When choosing a culture medium, the natural habitat of the species in question should be considered in order to determine its environmental requirements. It is important to know whether the environment is eutrophic, hence nutrient rich, or oligotrophic, hence nutrient poor, and whether the algae belong to a r-selected or a k-selected species. r-selected species are characterized by a rapid growth rate, autotrophic metabolism, and a wide environmental plasticity, whereas k-selected species shows a slow growth rate, mixotrophic or photoheterotrophic metabolism, and a low environmental tolerance.

The media recipes currently available are not always adequate for many species, and the exact choice for a particular species therefore is dependent on trial and error. It must be remembered that in culturing in general there are (within limits) no right and wrong methods; culture media have only developed trying out various additions, usually based on theoretical considerations. Refinement of media composition for laboratory-maintained algal cultures have been the object of research for several decades, resulting in many different media recipes being reported in the literature and being used in different laboratories (13).

Media are usually prepared from stock solutions of macronutrients, trace metals, and vitamins which are added to a large proportion of the final volume of water in order to avoid precipitation.

Salinity

Marine phytoplankton are extremely tolerant to changes in salinity. Most species grow best at a salinity that is slightly lower than that of their native habitat, which is obtained by diluting sea water with tap water. Salinities of 20–24 g/L have been found to be optimal (9).

pH

The pH range for most cultured algal species is between 7 and 9, with the optimum range being 8.2–8.7, though there are species that dwell in more acidic/basic environments. Complete culture collapse due to the disruption of many cellular processes can result from a failure to maintain an acceptable pH. The latter is accomplished by aerating the culture. In the case of high-density algal culture, the addition of carbon dioxide allows to correct for increased pH, which may reach limiting values of up to pH 9 during algal growth (9, 13).

Aeration/mixing

Mixing is of paramount importance to microalgal cultures. There are various methods of inducing mixing in microalgal cultures; however, the type of mixing to be adopted would depend on various factors such as, the type of microalgal strain, type of culture system (i.e., open ponds or photobioreactors), scale of culture systems (i.e., small or large-scale cultures), as well as, on the environment where the culture is operated (i.e., indoor or outdoor type) (14).

Mixing is necessary to prevent sedimentation of the algae, to ensure that all cells of the population are equally exposed to the light and nutrients, to avoid thermal stratification (e.g., in outdoor cultures) and to improve gas exchange between the culture medium and the air. The latter is of primary importance as the air contains the carbon source for photosynthesis in the form of carbon dioxide. For very dense cultures, the CO₂ originating from the air (containing 0.03 % CO₂) bubbled through the culture is limiting the algal growth and pure carbon dioxide may be supplemented to the air supply (e.g., at a rate of 1 % of the volume of air). CO₂ addition furthermore buffers the water against pH changes as a result of the CO₂/HCO₃⁻ balance. Depending on the scale of the culture system, mixing is achieved by stirring daily by hand (test tubes, erlenmeyers), aerating (bags, tanks), or using paddle wheels and jetpumps (ponds). However, it should be noted that not all algal species can tolerate vigorous mixing (9).

Temperature

The optimal temperature for phytoplankton cultures is generally between 20 and 24 °C, although this may vary with the composition of the culture medium, the species and strain cultured. Most commonly cultured species of microalgae tolerate temperatures between 16 and 27 °C. Temperatures lower than 16 °C will slow down growth, whereas those higher than 35 °C are lethal for a number of species. If necessary, algal cultures can be cooled by a flow of cold water over the surface of the culture vessel or by controlling the air temperature with refrigerated air - conditioning units (9).

DISCUSSION AND CONCLUSION

The growth rate and content of lipid depend on the optimal cultivation conditions specific species of algae, e.g. concentration of CO₂, temperature, light conditions, pH, design of photobioreactor for culturing (15, 16, 17).

Effect of temperature

The optimum range of temperature for *Chlorella vulgaris* is about 30 °C. Results in (18) showed that the optimum temperature for biomass production was 30 °C under elevated CO₂ (6 %). In (19) reported that *Ch. vulgaris* at 35 °C exhibit a 17 % decrease in its growth rate when compared to 30 °C. Further increase in temperature (38 °C) led to an abrupt interruption of microalgal growth, and later the cells dead. The (20) found that the maximum growth rate was obtained at pH of about 6.31 to 6.84, and the optimum temperature was 32.4 °C. By (21) *Ch. vulgaris* had the best growth rate with urea growth medium by optimum temperatures of 30 °C. The growth rate increases with increasing of temperature to 30 °C and then decreases with increasing of temperature to 35 °C. Results of (22) indicate that the highest biomass yield was obtained at 30±2 °C, after which an increase in the temperature (to 35±2 °C) resulted in a drop in the biomass yield.

Effect of light

The optimal light wavelengths for best growth rate of algae is white or yellow light, but some researchers considered the red light as optimal. Therefore, light intensity is probably a factor explaining the differences in the results other studies.

Results in (12) have shown that light wavelength has a noticeable effect on the algal growth rates. Red and green light did not show a positive trend in the growth rates compared to clear and blue light wavelengths. Authors of (23) reported that the clear (white) and red light had shown higher growth rates of *Ch. vulgaris*, however, their study was conducted between 6 and 10 days and their growth medium was synthetic high-strength wastewater. In (22) *Ch. vulgaris* indicated the best rate under yellow light with low energy (high yield of photosynthesis). Other colours (blue, white and red) resulted in somewhat less biomass production. Results of (24) indicate there was higher amount of biomass (measured as dried biomass) when use yellow, white and red light after 7 days of growth than in blue, green and purple light (light intensity 100 µmol/m²/s). By (25), the red light is commonly suggested as optimal for biomass productivity in *Ch. vulgaris* (this microalga has a high concentration of chlorophyll and hence absorbs efficiently in the red wavelength area). However, another study (26) reported that red light was less efficient in converting light energy into *Ch. vulgaris* biomass than violet, green and orange light (250 µmol/m²/s). Therefore, light intensity is probably a factor explaining the differences in the results reported in other studies.

Effect of CO₂concentration

Chlorella sp. shows great potential for capturing carbon dioxide. It will grow at a fast rate and tolerate higher concentration CO₂ in air. Optimal concentration CO₂ is about 10 %. *Chlorella sp.* can be grown under 50 % CO₂ conditions, but higher concentration is not acceptable.

The growth of *Chlorella sp.* increases with increasing the CO₂ concentration from 0.5 % to 5 % (27). The growth of *Ch. vulgaris* was enhanced when 2 % CO₂ enriched air was supplied compared with air without CO₂. Increase of CO₂ concentration in air on 4 % and 8 % did not show any increase in their final biomass concentrations compared with 2 % CO₂ in air (28). By (21), the *Ch. vulgaris* tolerates 10–15 % CO₂. According to (29), the maximum growth of *Chlorella sp.* was found with 10 % CO₂, but high growth were maintained with CO₂ concentration of 30 and 50 %. The growth was low with 80 and 100 % CO₂. The similar results were obtained in (30), where the maximum growth rate of algae was found in the culture 10 % CO₂, high growth with 30 and 50 % CO₂ and very low growth rate was with 70 % CO₂. By (31), standing biomass of *Ch. vulgaris* increased with increasing concentrations of CO₂ under

the four different light sources and the highest biomass production was found when the algal culture were supplied with 8.5 % CO₂ and exposed to white light. The growth potential of *Chlorella sp.* MTF-7 cultures aerated with flue gas from the coke oven of a steel plant, which contained approximately 25 % CO₂, 4 % O₂, 80 ppm NO and 90 ppm SO₂, was higher than that of the cultures aerated with 2 %, 10 % or 25 % CO₂-enriched gas without pH control (32).

Effect of cultivation medium

The cell growth and lipid accumulation are greatly affected by medium composition and cultivation conditions. Optimizing the growth media is a critical step to develop an economical route for sustainable algal biomass production. Nutrients are required in large quantities, therefore utilization of cheap nutrient sources such as municipal wastewater and other industrial co-products is ideal for algal growth economics. Utilizing wastewater resources to replace growth media may alleviate the cultivation costs to some extent (12).

Results in (12) showed that at the recommended (100 %) growth media composition (BBM medium), the algal growth was observed to be linear, however at 50 % composition; the growth followed an exponential trend. Further tests on nutrient concentration effect (nitrogen and phosphorous) have shown that low nitrogen concentrations can also stimulate algal growth. At 50 % the recommended growth media, algal growth is independent of phosphate concentration from 25 % to 100 % levels, (i.e., low or no effect) and severely delayed at 200 % Phosphorous concentration. Results of (22) show individual effects of NaCl, NaHCO₃ and Fe⁺² concentrations in medium on growth of *Ch. vulgaris*. Increasing the concentrations of NaCl and sodium bicarbonate resulted in corresponding decreases in the growth of algae. Media with 30.0 g/L NaCl did not indicate any algal growth. Also, with 9.0 g/L sodium bicarbonate, biomass production decreased. *Ch. vulgaris* showed different growing behaviours at the various concentrations of iron (Fe⁺²) and at the different temperatures. Maximum biomass production was obtained at the 0.0 g/L sodium bicarbonate, 10.0 g/L NaCl, 18.0 µmol/L iron and at 30±2 °C. By (21) *Ch. vulgaris* had the best growth rate with urea growth medium by optimum temperatures of 30 °C. In (33), the trends of the chlorophyll-a indicate that the modified basal medium had a lower growth capacity than the two others tested (modified Kolkwitz medium, modified BG-11 medium). Similar results were obtained as far as the number of cell trends was concerned. By [34], higher biomass production was obtained using nitrogen-rich medium under various growth conditions, whereas higher lipid content (20–53 %) was achieved by using a nitrogen-limiting MBL medium. The highest lipid productivity was obtained from mixotrophic cultivation on Modified Bristol's medium. Different nutritional conditions (Jaworski's medium, an enriched solution from modified Dual Solvay process, natural mineral water) were investigated in (35). The best growth resulted in an enriched solution from the modified Solvay process in a medium diluted in a 1:10 ratio. In (36) a KNO₃ based medium was found to work better for *Ch. vulgaris* compared with the urea based media.

Cultivation conditions (temperature, pH, light, nutrient quantity and quality, salinity, aerating) are the major factors that influence the photosynthesis activity and the behaviour of microalgae growth rate. It is important to find the best combination of these parameter for the best growth rate and lipid content for specific algae.

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