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Phototrophic culture of *Chlorella* sp. using charcoal ash as an inorganic nutrient source

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ABSTRACT

Although several studies have recognized the suitability of employing ashes as a component of a culture medium or fertilizer, the number of these studies remains limited. The use of biomass ash as a nutrient source for algal culture is an unexplored research topic that should be investigated to analyse the possibility of reducing the costs associated with commercial microalgae culture.

In this study, biomass ash from charcoal was used as a source of nutrients for the cultivation of *Chlorella* sp., and two alternative processes for nutrient supply were studied. First, various culture media containing the leachate stock solution from solid ash at different concentrations were prepared. Second, different amounts of solid ash were added directly to the culture media. The results indicated that the direct use of biomass ash mixed in water enables the formation of a more suitable medium compared with Guillard's f/2 medium because it promotes faster cell growth and higher biomass productivity. The higher biomass productivities were reached over the same period compared with those achieved with the culture media based on biomass ash leachates. Moreover, the nutrients in the media containing ash leachates are sufficient to maintain cell growth rates and biomass productivities that are comparable to those achieve with Guillard's f/2 medium.

1. Introduction

One of the primary objectives of research on microalgal biotechnology is to produce basic commodities in large-scale and cost-effective ways [1]. One of the key issues of algal cultivation is the employment of abundant and low-cost nutrient sources to reduce the production costs. Regardless of their form in the media, carbon, nitrogen, and phosphorus (macronutrients) are the three most important nutrients, and their uptake depends on the environmental conditions, species, nutrient ratios, and growth rates. It is also important to note that algae need other micronutrients, such as, K, Na, Fe, Mg, Ca, B, Cu, Mn, Zn, Mo, Co, V, and Se [1]. Many of these micronutrient elements are important in enzyme reactions and for the biosynthesis of many compounds.

At the bench scale, ordinary culture media are composed of highly purified and thus expensive chemical compounds. Nevertheless, the use of these supplies for large-scale cultivation is non-profitable, and thus, available alternatives may represent lower production costs and lower biomass yields. Ashraf et al. [2] suggested the replacement of pure and expensive nutrient media with low-cost commercial fertilizers used in current fish production and agriculture. Nevertheless, it is important to consider that the manufacturing of these fertilizers is based on an intense use of fossil fuels as a feedstock and energy source [3]. Hence, this alternative may be adequate in the short term but not in the mid to long term due to the scarcity and unstable prices of fossil fuels. As a result, the recycling of waste materials as a nutrient source for large-scale algal cultivation may be an interesting option that should be evaluated. Yang et al. [4] demonstrated that the recycling of harvest waste reduces the nutrient usage by 55% and that the use of sea/wastewater as the culture media eliminates the need of all of the nutrients with the exception of phosphate. Fenton et al. [5] showed that the nutrient content of agriculturally derived organic fertilizers, runoff and drainage waters has the potential to facilitate algal biomass growth. In particular, these researchers demonstrated that the surplus of pig and poultry manures or other bioproducts from anaerobic digestion are potentially viable sources of nutrients for algal growth. Skorupskaite et al. [6] investigated several potential inexpensive waste materials for microalgae *Chlorella* sp. biomass production rate, including technical glycerol and the liquid waste fraction of the digestate after biogas production. This research demonstrated that the microalgae growth was strongly dependent on the nitrogen concentration in the growth medium.

One alternative waste material that should be investigated as a nutrient source for algal growth is biomass ash (BA). In one scenario, the increasing number of combustion facilities of natural biomass for energy generation appears to be one of the main drivers for biofuel promotion in many countries worldwide in the near future [7,8,9], and one of the key issues associated with natural biomass facilities is the management of BA. In contrast, BA is a complex inorganic–organic mixture with a poly-component, heterogeneous and variable composition in the solid, liquid and gaseous phases [10]. According to Vasilev et al.

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[10], the chemical elements present in BA (in decreasing order of abundance) are commonly O > Ca > K > Si > Mg > Al > Fe > P > Na > S > Mn > Ti as well as some Cl, C, H, N and other trace elements, such as B > Au > Cd > (Cr, Mn) > Ag > Zn > (Be, Cu, Se) > Ni > Rb. These researchers also state that the water-soluble elements leached from BAs are commonly Cl > S > K > Na > Sr > Ni > Mn > Cd > Cr > Zn > Co > Si > Mo > Li > (Mg, Pb) > Ca > Cu > Ba > P > Se > Sb > Al > Fe > (Br, Hg) > (As, B, Sn, Ti, V).

These facts indicate that BA may be a nutrient source for algal cultivation. However, very few studies have evaluated the effects of BA on algal growth, and most of these have focused on the environmental impacts of using wood ash in aquatic systems [11,12,13]. Hence, it is clear that the use of BA as a nutrient source for algal cultivation needs to be investigated, which may allow reducing the costs of algal products in commercial microalgal culture and indicate that BA may be a suitable solution to the management of ash waste that would otherwise be disposed in landfills.

In this study, we investigated the effects of using charcoal ash as an inorganic nutrient source for the cultivation of *Chlorella* sp. We first analysed the chemical composition of the ash and its leaching properties. We then studied the effects of two alternative nutrient supplies on the growth of *Chlorella* sp. The first alternative nutrient supply involved the preparation of culture media with a leachate stock solution of solid ash at different concentrations, and the second involved the first addition of different amounts of solid ash to the culture media. We also compared the results obtained with those achieved with Guillard's f/2 as the reference medium.

2. Materials and methods

2.1. Microalgal isolation and initial culture conditions

Chlorella sp. (Chlorophyceae) was isolated from wastewater collected from a river near Quito, Ecuador. The strain was propagated and maintained under laboratory conditions in batch culture in Guillard's f/2 medium [14] with constant air bubbling. The temperature range was 25 to 28 °C, the light intensity was 3000 lx with a photoperiod of 12:12 h, and the pH was controlled between 8 and 10 (see Fig. 1).

2.2. Culture medium under phototrophic conditions

2.2.1. Charcoal ash preparation

Five hundred grammes of wood charcoal from a local Laurel tree (*Morella pubescens*) were placed in a porcelain crucible and burnt for 4 h in a Thermolyne FD1535M muffle furnace at 575 + 25 °C following the procedure described by Sluiter et al. [15]. The ash obtained was ground and sieved to 38 μm.

2.2.2. Charcoal ash thermogravimetric analysis (TGA)

A mass of 14.183 mg of charcoal ash was introduced into a Shimatzu TG60W thermogravimeter set to a constant heating rate of 35 °C/min. The analysis was initiated at ambient temperature and terminated at 900 °C. During the analysis, the sample was maintained in a nitrogen-inert atmosphere with a flow rate of 45 mL/min.

2.2.3. Leachate stock solution preparation

The leachate solution (LS) was prepared by adding 1 g of dried charcoal ash to a litre of ASTM Type II water (ASTM D 1193) following the Standard DIN 38414–4 [16].

2.2.4. Culture medium conditions

Culture media from leachates and with solid ashes, Guillard's f/2 medium and a blank medium without nutrients were prepared by triplicate in 500 mL flasks. Each flask was inoculated with *Chlorella* sp. strain to obtain a cell density of $1 \cdot 10^6$ cells mL^{-1}. The inoculum of native *Chlorella* sp. was previously filtered using N° 4 filter paper (pore diameter of 20 to 25 μm) and then washed with Type II water. The culture conditions for all of the media were 25 °C with a constant air flux of 2 L per minute, a white light intensity of 3000 lx and a photoperiod of 12:12 h. All of the culture media lacked buffer solution, and the pH was thus measured at the beginning and end of the runs.

2.2.4.1. Guillard's f/2 reference medium. Guillard's f/2 was chosen as the reference medium. Macronutrient, micronutrient and vitamin stock solutions were prepared using the formulation described in [14,17]. Table 1 shows the preparation of the Guillard's f/2 reference medium, including the volumes of inoculum and of the vitamin, macronutrient and micronutrient stock solutions.

2.2.4.2. Leachate medium. Different volumes of the stock leachate solution were mixed with 145 mL of the inoculum, 0.5 ml of the vitamin stock solution and the necessary volumes of ASTM Type II water to achieve concentrations of 70%, 35% and 18% to prepare the TM1, TM2 and TM3 culture media, respectively (see Table 2). A control blank culture was prepared by mixing 145 mL of the inoculum with 354 mL of ASTM Type II water and 0.5 mL of the vitamin solution.

2.2.4.3. Solid ash medium. To prepare the solid ash medium TM4 and TM5, 1 g and 0,5 g of charcoal ash were mixed with 145 ml of inoculum, 354 ml of ASTM Type II water and 0,5 ml of vitamin stock solution (see Table 2).

2.2.5. Charcoal ash, leachate stock solution and final TM4, TM5 culture media chemical analyses

The concentrations of several metals in the dried charcoal ash, leachate stock solution, Guillard's stock solutions and the final solutions of the TM4 and TM5 media after the experiment were determined through inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent ICP-MS equipment model 7700. The samples were prepared trough microwave digestion before elemental analysis by ICP-MS [18]. In addition, the concentrations of nitrates, sulphates and phosphates in the leachate stock solution were quantified through methods ion chromatography technique (Waters, LC 10 Ai). The electrical conductivity and pH of the leachate stock solution and Guillard's f/2 medium were measured with a multiparameter Edge HI 20X0-20 instrument.

2.3. Evaluation of cell density and biomass productivity

The cell density was estimated by counting directly using a Marienfeld Neubauer chamber with a depth of 0.1 mm and an area of 0.0025 mm^2. The cell density was estimated during 15 alternate days starting on the third day.

The kinetics growth curve, generation time (G_t), and specific growth rate (μ) were calculated for all of the batch cultures. The specific growth rate μ (in d^{-1}) and biomass productivity X (in g L^{-1} d^{-1}) were estimated using Eqs. (1) and (2), respectively [19]:

$$\mu = \frac{Ln(N_2/N_1)}{(t_2 - t_1)} \quad (1)$$

$$X = \frac{N_2 - N_1}{(t_2 - t_1)} \quad (2)$$

N_2 and N_1 describe the biomass concentration (in g L^{-1}) at times t_2 and t_1 (in d).

2.4. Statistical analyses

The biomass growth rate data were analysed by ANOVA with an α value of 0.05 to compare the results of the different leachate, solid ash and Guillard's f/2 culture stock solution. Tukey's test with a confidence level of 95% was applied to the experiments using the Microsoft Excel (2010) programme [20].