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Two Stage Process of Microalgae Cultivation for Starch and Carotenoid Production

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Biotechnological processes based on microalgae cultivation are promising for several industrial applications. Microalgae are photoautotrophic microorganisms and can thus grow by using renewable and inexpensive resources as sunlight, inorganic salts, water and CO_2. They can store high amounts of neutral lipids (bioil), carbohydrates (mainly starch), carotenoids (such as lutein, astaxanthin, β-carotene), proteins and other molecules. Productions of lipids and carbohydrates have recently received an increasing interest for biofuel production, while proteins, carotenoids and other minor products are usable as feed additives and nutraceutical compounds. Biofuel production from microalgae is not yet economically sustainable, while there are different industrial plants in the world for the production of high values chemicals as carotenoids. Starch production from microalgae has been investigated mainly for the production of biofuels (e.g. bioethanol) by successive fermentation. However, purified starch can be used for other aims such as the production of bio-plastics. Superior plants as corn, potato and wheat are currently used for this purpose. However, there are different environmental and economic issues related to the use of fertile lands and edible plants for these kinds of productions. Microalgae can solve these social and ethical issues because they can grow on non-fertile lands and also reach starch productivity per hectare higher than plants. In this work, the production of starch and carotenoids from *Scenedesmus sp*. microalgal strain is reported. A two-stage process has been developed in order to reduce operative and investment costs. In the first stage, microalgae are cultivated in photoautotrophic conditions and then, when biomass concentration rises and light becomes a limiting factor for growth, microalgae are transferred to a heterotrophic reactor. In this reactor, microalgae are cultivated by using wastewaters as source of nutrients (mainly organic carbon). Microalgae use organic carbon to synthesize starch and simultaneously reduce the content of pollutants in the wastewater (codepuration). Biomass separated by the culture medium is treated for the extraction of lipids containing different antioxidant carotenoids (such as astaxanthin and lutein) and starch granules as raw material for biopolymers.

1. Introduction

The development of sustainable sources of energy and chemicals is necessary to reduce greenhouse emissions responsible for global warming. Microalgae are microorganisms which can grow in photoautotrophic conditions utilizing cheap renewable resources (as CO_2 and sunlight) to produce new biomass. This biomass consists mainly of lipids, carbohydrates, and proteins, which can be used to produce biofuels and fine chemicals. Microalgae can ensure biomass and bioproduct productivity per hectare, which are higher than vegetable cultivations due to high content and high growth rate (Oncel S.S. 2013). Nevertheless, microalgae-based productions still present two main limits: the high costs (both fixed and operative), and the unfavorable energy balance, which is of particular relevance if biofuels are target products. In addition, high consumption of fertilizers is required to support microalgal elevated productivities (Acién et al. 2012).
Development of an integrated process in the biorefinery view could be a valid way to overcome these limits. In fact, microalgae are able to use organic carbon as energy source in heterotrophic and mixotrophic conditions

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(Pagnanelli et al. 2013). This allows for their cultivation in media mainly made up of wastewaters that can be used to supply nutrients and reduce the global costs of the process (Zhou et al. 2014).
In the years 2011-2013, this research team designed and built a phototrophic pilot plant for microalgal cultivation within the project Alghe Energetiche, which was co-financed by the Italian Ministry of the Environment as reported on the Web (Ecoone).
The pilot plant is made up of 10 photobioreactors (25 L bubble column reactors) (Figure 1), a system for gas fluxing, an automatic control system for pH (by manipulating the flow of a CO_2-air mixture), and temperature (by water spray and by interception of solar light using a moving wall), a sedimentation unit, and solar tables for biomass drying.
The pilot plant was installed in Priolo Gargallo near Syracuse (Sicily, Italy) in order to evaluate technical feasibility of the microalgal cultivation in this region and the efficacy of the automatic control system to attenuate light and temperature fluctuations typical of the region, along with pH fluctuations due to algal metabolism.
Demonstration activity was performed using autochthonous strain of Scenedesmus sp., which demonstrated to survive and grow in outdoor conditions under the stress of high illumination and high temperature and in presence of contaminants. Biomass productivity estimated in phototrofic conditions (0.05-0.1 g/Ld) was in agreement with literature results (Mata et al. 2010). According to these data, a microalgal cultivation aiming at biodiesel production was proved to be uncompetitive in comparison with conventional vegetable cultivations used for the same aim such as coconut oil from Malasia cultivation (Oncel et al. 2013).
Pilot and laboratory scale results focused the research towards two concepts:

- heterotrophic growth using a waste stream, then integration of the cultivation plant with another plant producing the waste
- biorefinery approach, then exploiting all the different biomass fractions, not specifically only bioil but also starch and carotenoids

By joining these two concepts, the idea of “integrated biorefinery” emerged to pave the way of microalgal cultivation to sustainability and competitiveness.

Figure 1: Photobiorectors used for phototrophic growth of Scenedesmus sp. strain during the demonstration activity performed in Sicily (Italy) in the ambit of the project Alghe Energetiche (Ecoone).

In heterotrophic conditions, light is not a limiting factor and then also conventional closed reactor can be used reducing both fixed and operating costs. In addition, in heterotrophic growth conditions biomass productivity can be enhanced up to two orders of magnitude (Richmond 2004). Using a wastewater as carbon source a double advantage can be achieved of reducing costs of chemicals for growth and obtaining a codepuration of the wastewaters. Then, reducing cultivation costs and increasing biomass productivity can significantly improve the final product yields. Finally, bioil is just one of the possible products of microalgal cultivation. In fact, microalgae can produce even high percentage of starch granules (40-50% as dry weight), which can be used as fermentation feedstock or as raw material for bioplastics. In addition, in stress conditions of temperature and light, microalgae can produce carotenoids, a very wide family of compounds with antioxidants properties finding application in different nutraceutical formulations.
This integrated biorefinery vision is the basic idea of the project just founded by BioP srl in the ambit of a collaboration between Sapienza University and Maire-Tecnimont.
The general idea of the project is performing a two stage cultivation (first in autotrophic and then in heterotrophic conditions) using an agro-industrial wastewater as organic carbon source; produced biomass is treated for recovery of carotenoid-containing bioil and carbohydrates as raw materials for bioplastic production. In Figure 2, a schematic block diagram is reported describing the process flowsheet, the input materials and the main products.
Microalgae are initially cultivated in photoautotrophic conditions until reaching a concentration which limits light penetration through the reactor. After this precultivation, microalgae are used in a second step as inoculum for a cultivation in heterotrophic conditions. In this second stage, a wastewater or a mixture of different wastewaters is used as carbon source. In heterotrophic conditions, the codepuration of the wastewater is achieved as a result of biomass growth along with an increased productivity in biomass and starch content. After biomass harvesting, the extraction of the different components is performed in order to obtain a carotenoid-containing bioil (Di Caprio et al. 2015a, Di Caprio et al. 2015b) and starch as raw material for new bioplastics. Actually starch is about 85-90% of raw material produced from renewable resources. Superior plants as corn, potato and wheat are currently used for this purpose. However there are different environmental and economic issues related to the use of fertile lands and edible plants for these kinds of productions. Microalgae can solve these social and ethical issues because they can grow on non-fertile lands and also reach starch productivity per hectare higher than plants. Preliminary estimates considering conservative productivities of biomasses also for the heterotrophic growth (0.1 g/Ld) gave starch productivity by microalgae which are at least one order of magnitude larger than those from vegetables like corn or potatoes.

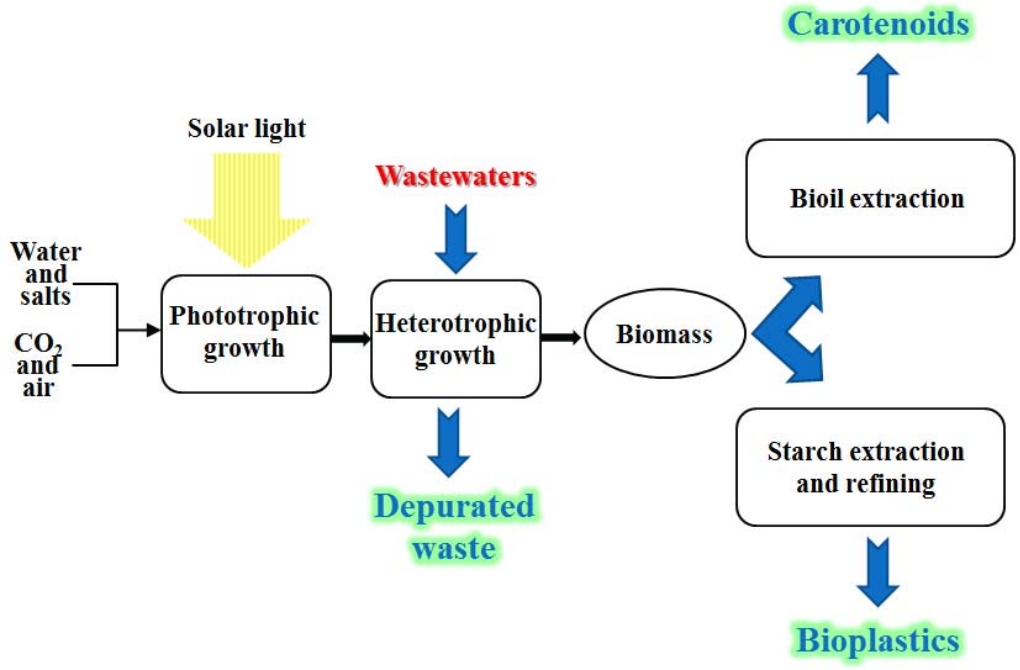

Figure 2: Schematic representation of the two stage cultivation of microalgae for the production of carotenoid-containing bioil and starch for biopolymer production.

2. Materials and Methods

2.1 Microalgae cultivation

A strain of *Scenedesmus sp*. was selected in Siracusa (Sicily, Italy) and maintained in Petri dish in MBG11 (modified BG11 medium, with a reduced $NaNO_3$ concentration of 0.3 g/L) solid medium (Di Caprio et al. 2015b). Microalgae were firstly transferred from the Petri dish to 500 mL flasks in MBG11 liquid medium and then inoculated in 4000 mL column reactors using 1:10 dilution ratio. In the first photoautotrophic step of cultivation microalgae were cultivated under constant illumination (24 h) with 80 ± 10 μE m^{-2} s^{-1} and feed with 0.5 L/min of CO_2/air (0.05/1 v/v). In the second stage, 9% v/v of Olive Mill Wastewater (OMW) was added to the reactors for heterotrophic cultivation. OMW was pre-treated by a centrifugation at 3000 rpm for 5 min in order to remove sedimentable solids. During the growth transmittance (I/I_0*100) was determined by measuring the incident light intensity (I_0) and the light passing behind the reactor (I).

2.2 Determination of microalgae concentration

Microalgae concentration was determined both spectrophotometrically and by dry weight. For the spectrophotometer determination of the microalgae suspension, the sample was washed three times by centrifugation at 8000 rpm and re-suspended in distilled water. The absorbance was then measured at 690 nm by the UV-Visible spectrophotometer (Varian Cary 50 Scan). Dry weight of the suspension was measured by filtration of 10 mL of sample on 0.45 μm acetate cellulose filter. The filters were dried at 105 °C and then weighted. All the measures were made in duplicate.

2.3 Determination of carbohydrate content

Samples of microalgal suspensions were centrifuged at 8000 rpm for 5 min, washed with distilled water and then dried at 105 °C until reaching a constant weight. 100 mg of dried microalgae were put in a Pyrex tube with 1 mL of concentrated sulphuric acid (95-97%) at 30 °C for 1 hour. Afterwards the sample was put in a glass vial with 28 mL of distilled water and kept for 1 hour at 120 °C in autoclave. The sample was then quickly cooled and 1 mL was centrifuged at 8000 rpm for 5 minutes. The sugar concentration in the supernatant was then analysed by Dubois method (Dubois et al. 1951).

2.4 Lipid determination

Dried microalgae samples were put in a mortar and pre-treated by hand milling in order to obtain a homogeneous granulometry. The extractions were carried out by putting 200 mg of biomass in a 100 mL Soxhlet extractor loaded with 90 mL of chloroform and 40 mL of methanol. Extractions were carried out for seven hours until a complete discoloration of solvent mixture. Solvent was then removed by a rotary evaporator (IKA RV 10 Digital) and the lipid concentration was gravimetrically determined.

2.5 Astaxanthin extraction and analysis

Milled biomass (100 mg) was put in a glass vial and 2 mL of solvent mixture (25 % dichloromethane and 75 % methanol) were added. Astaxanthin hydrolysis was carried out by adding 0.4 mL of a methanol solution with NaOH concentration equal to 0.02 M to 2 mL of solvent mixture obtained from extraction. The reaction mixture was maintained in dark, under constant agitation and nitrogen atmosphere for 2 hours. Analysis of astaxanthin was carried out by using a reverse-phase high performance liquid chromatography (HPLC) with a C_{18} Hypersil GOLD column and a UV-Vis detector (Spectra System UV1000). The mobile phase consisted of a solvent mixture of dichloromethane: methanol: acetonitrile; water, in the ratio of 5.0:85.0:5.5:4.5 ($v \cdot v^{-1}$). The flow rate was maintained at 1 $mL \cdot min^{-1}$ and the absorbance was detected at 480 nm. Quantification was carried out by a standard curve obtained through astaxanthin standard purchased from Sigma Aldrich.

3. Results and discussion

3.1 Microalgae cultivation

In Figure 3, the biomass concentration inside the reactor in the two stages of cultivation is reported. Microalgae cultivated in photoautotrophic conditions grow by using the light, which passes through the reactor. However, when biomass concentration increases, the large part of the light cannot penetrate inside the suspension. As a consequence, the growth rate is limited inside the reactor. This is shown in Figure 3, where the grey triangles indicate percentage of the light transmitted through the reactor. In the used reactor, transmittance decreased to zero after about 10 days due to the increment of biomass concentration. This phenomenon is one of the main issues in the development of process based on photoautotrophic microalgae cultivation. The idea of the process reported in this work aims to maintain high productivities by a second stage of cultivation in heterotrophic conditions. As showed in Figure 3 organic substrates in the wastewater allow for biomass growth also without light. Moreover, the degradation of organic molecules by microalgae to obtain energy and mass from them leads to the reduction of pollutant content.

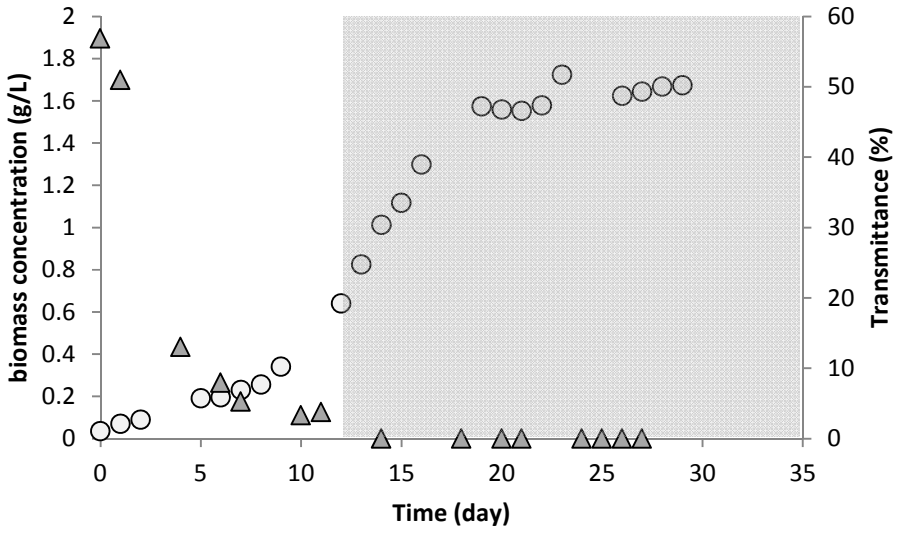

Figure 3: Cultivation of microalgae in two stage process; grey triangles indicate transmittance values of the reactors; white circles indicate the concentration of biomass. The grey square area in the graph indicates heterotrophic conditions induced by the addition of 9% OMW.

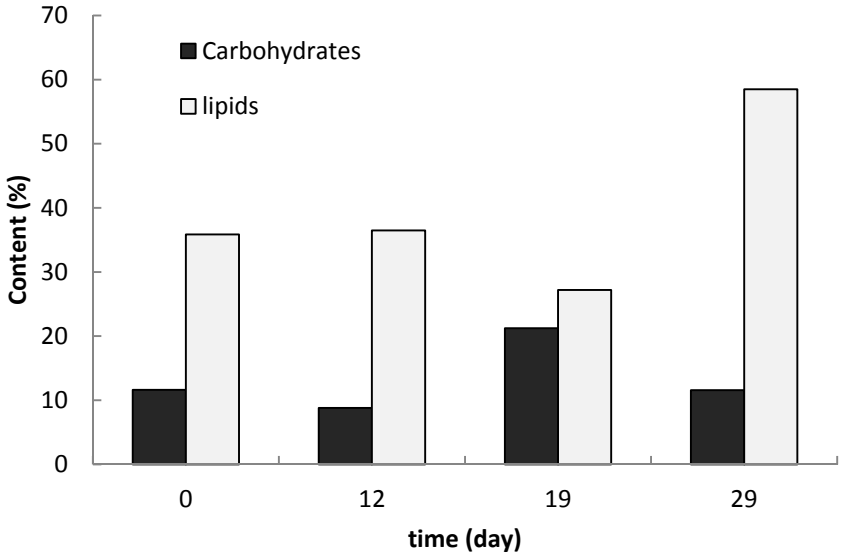

Figure 4: Evolution of the contents of starch and lipids in the microalgal biomass during the different stages of growth: during phototrophic growth a constant composition was observed (until day 12); when OMW is added heterotrophic growth started and after accumulation of starch (day 19) a switch to lipid accumulation is observed (day 29).

3.2 Starch production

In Figure 4, the content of total lipids and starch in microalgal biomass during the different phase of cultivation is reported. In a first stage of phototrophic growth, microalgal biomass reproduced in a balanced way presenting a constant composition in the biocomponents of interest, i.e. total lipids and starch.

In the second stage of heterotrophic growth an increment in starch content was observed due to the stimulation of glucose assimilation determined by organic carbohydrates present in OMW (Di Caprio et al., 2015b).

As the concentration of promptly metabolizable carbohydrates in the medium decreased, a metabolic switch is observed inside the cells and stored carbohydrates are converted to lipids (starch-lipid switch). This is a quite

generally observed behavior denoting how starch is the first polymer stored in presence of an excess of organic carbon, and that successively the stored starch is gradually converted to bioil and/or used for energy requirement in biosynthetic activity. The regulation of this switch, which appears of paramount importance for the optimization of bioproduction, is still object of research and not completely defined.

3.3 Astaxanthin production

Carotenoids such as astaxanthin can be also recovered from microalgal cultivation according to the traditional use of these biomasses. *Scenedesmus* strain demonstrated to be able to produce astaxanthin in phototrophic growth conditions. Solvent extraction procedure gives a content of about 0.2 mg/g in these conditions. Preliminary characterization tests evidenced also the presence of other interesting carotenoids such as loroxanthin, zeaxanthin, lutein and β-carotene (experimental results not reported here). Further tests are now in course to quantify all these fine chemicals and evaluate the effect of type of growth (phototrophic or heterotrophic) on the production of these substances.

4. Conclusions

Interest towards microalgae cultivation seems to be periodically synchronized with the different energetic crisis occurred during last decades. Nevertheless, microalgal cultivations are not yet economically competitive with fossil fuels or other biofuels. The application of biorefinery concept and then the exploitation of all possible bioproducts given by microalgae can help the achievement of economic sustainability. As a proof microalgal cultivation in open ponds and photobioreactors are already commercially efficient for nutraceutical applications (i.e. fine chemical production such as astaxanthin). The integration of microalgal cultivation with an agro-industrial plant generating carbon-rich wastewaters can be a further improvement due to biomass productivity stimulation in heterotrophic growth. The concept of integrated biorefinery then arises in perfect agreement with the new visions of industrial symbiosis and circular economy (reuse of wastes and by products of a plant as raw materials for other productions).

Experimental results reported in this paper evidenced the capacity of a wild strain of Scenedesmus (selected in outdoor plant conditions) to grow in heterotrophic conditions using carbohydrates of OMW as carbon source. A two stage cultivation strategy was then applied and lipid and starch content within the cells monitored during time showing typical starch-lipid switch. The selected strain is also able to produce other interesting fine chemicals such as astaxanthin, lutein, loroxanthin, zeaxanthin and β-carotene.

Further tests will be developed in order to optimize cultivation conditions in both phototrophic and heterotrophic growth.

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