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THE POTENTIAL OF PHOTOSYNTHETIC BIOMASS RESULTED FROM SYNTHETIC WASTEWATER TREATMENT AS RENEWABLE SOURCE OF VALUABLE COMPOUNDS

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Abstract
The new trend in using photosynthetic microorganisms for the epuration of wastewater in recirculating aquaculture systems (RAS) opens a new question concerning the economic significance of this newly synthesized biomass. In this paper, we present our original results concerning the production of photosynthetic biomass (both prokaryotes and eukaryotes) and associated non- photosynthetic microbiota during the purification process of artificial wastewater. The results present the wet and dry weight quantities of weekly synthesized photosynthetic biomass (with 3 harvesting processes per week). The obtained biomass is analysed with respect to the lipid content, total proteins, as well as carotenes. As our main task is to use this microbial biomass as valuable substrate for fish growth and not for the trivial usage as material for biogas production or fertilizer in agriculture, these results could make a big impact for a better utilization of natural resources, including in the rather new context of circular economy.

Keywords: immobilization, lipid, photosynthetic microorganisms, protein and pigment concentrations, wastewater

1. INTRODUCTION

The ability of photosynthetic microorganisms to use inorganic nitrogen and orthophosphate as well as some organic substances as nutrients to sustain their growth has retained attention in the last six decades for the use of these cells as biocatalysts in wastewater treatment and for the synthesis of useful chemicals (Oswald, 1988; Benemann, 2013; Borowitzka, 2013; Ardelean and Manea, 2016; Tiron et al., 2017; Velea et al., 2017; Vuppaladadiyam et al., 2018). Much attention was focused on the raw materials from wastewater treatment (but not directly RAS) to be further used toward a circular economy. Firstly, specialized methods and techniques have been developed to obtain biomethane, biodiesel, and biofertilizer from fish biomass. Also, using physical, biochemical, and thermochemical processes, relevant substances (such as fish protein hydrolysate, natural pigments, chitosan, and collagen) can be obtained (Ward and Løes, 2011; Ehime et al., 2013; Krishna et al., 2013; Koszel and Lorencowicz, 2015).

There is the new trend to use the microbial biomass formed during the process of water cleaning as a source of nutrient for fish or a source of valuable chemical elements. For example, Tapa et al. (2015) used a fine powder of dried algae *Rhizoclonium* spp. (a green seaweed obtained as a waste product from a local algal-based wastewater treatment system) as improver to milled *Miscanthus* sp. (a perennial C4 grass). They found that *Miscanthus* discs mixed with algae had significantly greater

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compressive strength at blends at or above 20% algae content compared to pellets made from 100% *Miscanthus* (39N). In conclusion, they argue for the use of algae as a binding agent for biomass destined for bioenergy and bioproduct processes, and highlight an additional end use for algal biomass (Tapa et al., 2015). Ometto et al. (2014) showed that *Scenedesmus obliquus* and *Chlorella* sp. take up nitrogen and phosphorus at rates higher than 90%, the biomass being converted to biogas after thermal hydrolysis (used as a pre-treatment to improve biogas production during anaerobic digestion) by a threefold increase in methane yield. Interestingly, compared to a traditional activated sludge process, the additional tertiary microalgal treatment generates an integrated process potentially able to achieve up to 76% energy efficiency (Ometto et al., 2014).

It is well known that microalgae species can be used as bio-fertilizer as an alternative to the use of synthetic fertilizers, thus diminishing for example the aggressive synthetic fertilizer use in the paddy field (Dineshkumar et al., 2018). Microalgae are a cheap source of nitrogen, ensuring eco-friendly environment by avoiding chemical pollution especially when wastewaters can be used for their growth (Dineshkumar et al., 2018). Furthermore, wastewaters derived from municipal, agricultural, and industrial activities could provide cost-effective and sustainable means of algal growth for biofuels, especially by combining wastewater treatment by algae (including pollutants or nutrient removal) with biofuel production (Pittman et al., 2011) or special compounds production (i.e. lipids) for biodiesel or for fish foods supplements.

There are also pioneering papers showing their results concerning the use of green algae both for nutrient removal and lipid synthesis (Xin et al., 2010; Feng et al., 2011)

In agreement with the tasks of Institute of Biology within the ABAWARE Project and with our previous work (Ardelean, 2015; Ardelean and Manea, 2016; Ardelean et al., 2017; 2018; Moisescu et al., 2018) the aims of this paper are: i) the growth of previously selected mixed populations of photosynthetic microorganisms (both cyanobacteria and green microalgae) able to take up organic and inorganic pollutants from synthetic wastewater mimicking outlet water from RAS, in order to obtain newly synthesized biomass; ii) the use of newly synthesized biomass as a source of valuable compounds such as lipids, proteins and pigments (chlorophyll a and carotenes); iii) the development of valuable methods to harvest the immobilized photosynthetic biomass, able to be used at large scale and long term applications in real life.

2. MATERIALS AND METHODS

Photosynthetic microorganisms. The cells were selected as previously shown (Ardelean and Manea, 2016; Ardelean et al., 2017; 2018; Moisescu et al., 2018), grown immobilized on a hydrophobic support and harvested weekly by two different methods. The immobilized photosynthetic microorganisms were immersed in two vessels containing 500 mL of synthetic wastewater. Every two days the water was removed by filtration and the dry weight biomass production was determined gravimetrically after incubation at 60°C until constant weight between two consecutive drying periods.

Synthetic wastewater composition was according to Takaya et al. (2003) diluted 4 times.

Lipid content was estimated by the phosphor vanillin method (Park et al., 2016). Microalgal paste was resuspended in 2:1 parts of chloroform: methanol (v/v) by manually shaking the tube vigorously for a few seconds or until the biomass was dispersed in the solvent system. Finally, a 0.73% NaCl water solution was added to produce a 2:1:0.8 system of chloroform: methanol: water (v/v/v). The phospho-vanillin reagent was prepared by dissolving 0.75 g vanillin in 0.125 L distilled water and mixed with 500 mL of 85% phosphoric acid solution. The final concentration of reagent was 1.2 mg vanillin per mL of 68% phosphoric acid. Sunflower oil was dissolved in chloroform (10

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mg in 10 mL for a final concentration of 1 mg mL^{-1}), and different concentrations (10-150 µg) of standard lipid samples were prepared in clean glass vials. The vials were incubated at 90°C for 10 min to evaporate the chloroform. Concentrated sulphuric acid (0.1 mL) was added to each vial, and then heated at 90°C for 10-20 min. After cooling on ice for about 5 min, 2.4 mL of phospho-vanillin reagent was added and allowed to develop for 10 min, until the colour of the sample turned pink (Park et al., 2016)

Chlorophyll a was extracted in 90 % methanol and the concentration calculated using the following equations: [Chl a] = 16.29 E665.2 – 8.54 E652.0 (Pora, 2002).

Carotenoids were measured spectrophotometrically using the modified method of Mackinney (1941) as presented by (Boyer, 2006). Briefly, a known volume of culture was centrifuged at 4000×g for 10 min. The supernatant was decanted and the same volume of methanol was added to the pellet. The mixture was incubated in a water bath at 55°C for 15 min and then centrifuged at 4000×g for 10 min. The absorbance of the extract (A) was measured against blank of free methanol at 650, 665, and 452 nm. Carotenoids were estimated as mg/mL of culture suspension using the following equation: Carotenoids (µg/ mL) = 4.2 A452 – [0.0246 (10.3 A665 – 0.918 A650)].

Total soluble proteins were estimated using the method of Boyer (2006). After carotenoids extraction, residual cells were extracted using 1 N NaOH in a boiling water bath for 2 h. The ratio of absorbances at 280 nm vs. 260 nm was used to estimate the protein concentration using the following formula: mg protein/mL = A280 x correction factor (Boyer, 2006).

3. RESULTS AND DISCUSSIONS

In Table 1 there are presented the results concerning the production of wet and dry biomass by photosynthetic microorganisms grown in synthetic residual wastewater. The excess of wet biomass was harvested and collected by centrifugation every two days (or three days over the weekend) and the overall dry biomass collected during 7 days.

Table 1. Biomass weekly collected by centrifugation from 3L of artificial wastewater

Harvesting	Time	Wet biomass (g)			Total wet biomass (g)	Total dry biomass (g)
		2 days	2 days	3 days		
Centrifugation	7 days	10.426	3.974	3.471	17.871	0.843
	7 days	4.27	2.744	3.297	10.311	0.735
	7 days	3.546	3.293	5.995	12.834	0.736

In Table 2 there are presented the results concerning the production of dry biomass by photosynthetic microorganisms grown in synthetic residual wastewater. The excess of wet biomass was harvested and collected by filtration each two days (or three days over the weekend) and the overall dry biomass collected during 7 days.

Table 2. Biomass weekly collected by filtration from 3L of artificial wastewater

Harvesting	Time	Dry biomass (g)			Total (7 days) dry biomass (g)
		2 days	2 days	3 days	
Filtration	7 days	0.093	0.073	0.117	0.283
	7 days	0.099	0.072	0.107	0.278
	7 days	0.074	0.069	0.08	0.223
	7 days	0.053	0.103	0.108	0.264

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One can see that by using two different methods of harvesting, the quantity of the collected biomass is different. One should take into account that the growing medium is limited in inorganic nitrogen, the growth being far away from optimum. This limitation in nitrogen source could nevertheless be an advantage for lipid synthesis (Li et al., 2008).
This dry biomass has been analysed with respect to lipid, carotenes, proteins, and chlorophyll a content. In Figure 1 there are presented the concentrations of these compounds.

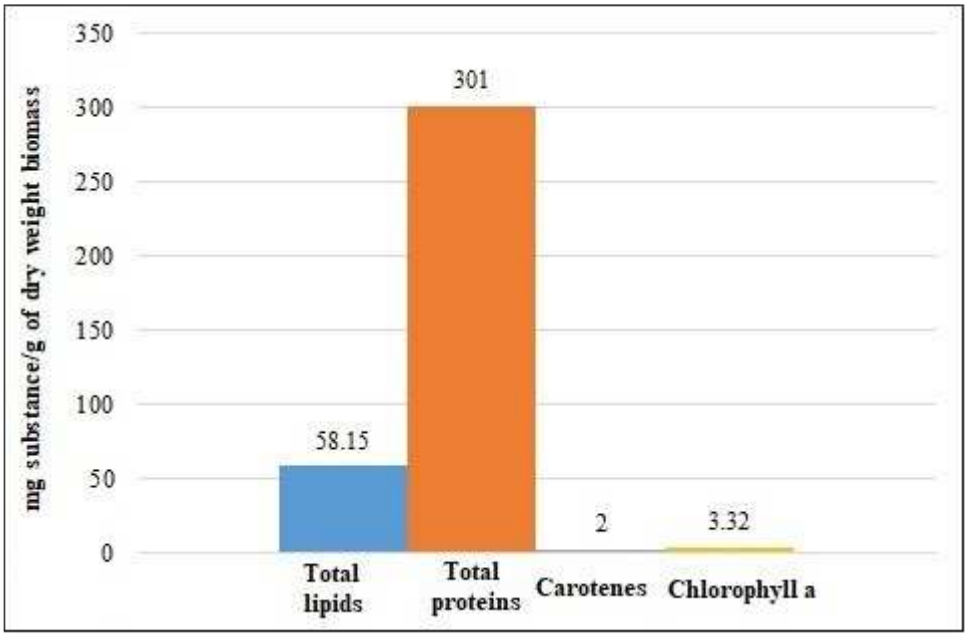

Figure 1. Some chemical components within the dry biomass produced during artificial wastewater treatment by previously selected photosynthetic microorganisms

In this paper, there were evaluate only the content of biomass with respect to total lipids, proteins, carotenes and chlorophyll a but the list of potential valuable compounds is not restricted to the ones mentioned above.
However, our reported results with previously selected photosynthetic microorganisms (both prokaryotes and eukaryotes) are modest as compared with results reported in literature using green microalgae with more complex growing conditions. In a pioneering paper, Xin et al. (2010) investigated the freshwater microalga *Scenedesmus* sp. LX1 for its ability to both remove the nutrients and to accumulate lipids during its growth in the secondary effluent. During this experiment, the dry weight reached 0.11 g L^{-1} and, after a trigger of nitrogen deficiency on day 10, the lipid content reached 31–33% of dry weight, accumulating at a rate of 8 mg lipid/L/day. Interestingly, all these events occurred while the culture was removing inorganic nutrients by over 98% in 10 days (Xin et al., 2010). *Chlorella vulgaris* was also used for simultaneous artificial wastewater treatment and lipid biosynthesis in a column aeration photobioreactor (CAP), under batch and semi-continuous cultivation, with various daily culture replacements (Feng et al., 2011). Their results are very promising, the highest lipid content was 42% (average value of the phase) and the lipid productivity was of 147 mg/L/day. Furthermore, in these conditions the nutrient removal efficiency was 86% (COD), 97% (NH_4^+), and 96% (TP), respectively. The authors concluded that, according to their results, this will lead to an economical technology of algal lipid production.

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Our reported results with respect to lipid content are much lower that the above data from literature. Trying to understand these large differences, possible ways to overcome them in the future, one possible explanation is based on the fact that these photosynthetic microorganisms were previously selected mainly for their capacity to use nitrate, ammonium, phosphorous, and organic compounds as nutrients, thus assimilating them in the cell biomass. However, other analysed components (i.e. total protein content) open the possibility to use the obtained biomass as potential food for herbivorous fish, for example.

4. CONCLUSIONS

The growth of previously selected consortia of photosynthetic microorganisms (both prokaryotes and eukaryotes) in artificial wastewater mimicking the composition of outlet water from RAS allowed a weekly harvest of around 0.27 and 0.75 g dry biomass by filtration and centrifugation, respectively from 3L of wastewater

Chemical analysis of the harvested biomass shows the following concentrations: lipids – 58.15, proteins – 301.0, chlorophyll a – 3.32 and carotenes – 20 mg substance/g dry weight biomass.

These results argue the potential of using the biomass of selected consortia of photosynthetic microorganisms (both prokaryotes and eukaryotes) as a renewable source of lipids, proteins, and pigments and, potentially, for other compounds.

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