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Effect of different nitrogen sources on the growth of microalgae Chlorella vulgaris cultivation in aquaculture wastewater

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Department of Biology and Aquaculture, Faculty of Agriculture, Trakia University, 6000 Stara Zagora, Bulgaria

Abstract. Nitrogen is one of the most important limiting nutrients and nitrogen control is critical for the intensive cultivation of algae. The aim of the present study was to explore the effect of different nitrogen sources on biomass accumulation in microalgae C. vulgaris during its cultivation in aquaculture wastewater. Microalgae cultivation was initiated in a bioreactor from 500 ml Erlenmeyer flask containing 250 ml wastewater from fish-ponds, “Getov” – Pleven, Bulgaria. The cultures were kept at room temperature (25–27°C) at fluorescent light with a light:dark photoperiod of 12:12 h. The experiment was conducted in variants with urea (1.125 g.l⁻¹) and ammonium nitrate (1.125 g.l⁻¹). The growth of the strain was checked for a 96-hour period. In the present study C. vulgaris showed better growth in wastewater from aquaculture with urea utilization as nitrogen source than as a source of nitrogen ammonium nitrate.

Keywords: aquaculture, biomass, Chlorella vulgaris, nitrogen sources, wastewater

Introduction

Algae cultures have been basically developed as an important source of many products, such as aquaculture feeds, human food supplements, pharmaceuticals and for biofuel (Apt et al., 1999; Pulz and Gross, 2001). Chlorella vulgaris refers to a globular green algae and cosmopolitan in occurrence. C. vulgaris is useful in biomass production on a commercial level because it is a rich source of protein, carbohydrates, and especially essential fatty acids (Sankar and Ramasubramanian, 2012). Microalgae optimize the suitable culture media for mass cultivation of microalgal biomass, which is very important for industrial production.

The generation of biomass by photosynthetic microalgae cultures varies depending on the environmental factors – temperature, nitrogen concentration, light intensity (Chen and Johns, 1991;Jimenez and Niell, 1991; Hu et al., 1998). The essential nutrients that contribute to biomass production are carbon and nitrogen (Prabakaran and Ravindran, 2012). Nitrogen is one of the most important limiting nutrients and nitrogen control is critical for the intensive cultivation of algae due to its role in growth and regulation of metabolism (Smit et al., 1997; Edwards et al., 2006). A wide variety of nitrogen sources, such as ammonia, nitrate, nitrite and urea can be used for growing microalgae. Among the organic nitrogen sources, urea is the best nitrogen source for culturing Chlorella (Becker, 1994). Nitrogen in the form of nitrate, ammonia and urea are the most common nitrogen sources. Ammonia is the chemical form of nitrogen, most readily taken up and assimilated by microalgae (Lourence et al., 1997).

Microalgae have also been used for wastewater treatment (Sirakov and Velichkova, 2014). Microalgae can mitigate the effects of sewage effluent and industrial sources of nitrogenous waste such as those originating from water treatment or fish aquaculture and at the same time contribute to biodiversity (Atanasov et al., 2013). Moreover, removing nitrogen and carbon from water microalgae can help reduce eutrophication in the aquatic environment. The interaction of nitrogen on their removal by microalgae should be of great concern when microalgae are used as a biological treatment in wastewater. Effluents from aquaculture are rich in solids and dissolved nutrients (Hii et al., 2011).

The aim of the present study was to explore the effect of different nitrogen sources on biomass accumulation in microalgae C. vulgaris during its cultivation in aquaculture wastewater.

Material and methods

Microalgae strain, medium and cultivation

C. vulgaris (SKU: 100-CVC00-50) was supplied from the Algae depot – USA (www.algaedepot.com). The wastewater used as a medium for the tested algae cultivation originates from fish-ponds “Getov” – Pleven, Bulgaria. Algae cultivation was initiated in a bioreactor of 500 ml Erlenmeyer flask containing 250 ml wastewater and added carbon dioxide (2%, v/v). The experiment was conducted in variants with urea (1.125 g.l⁻¹) and ammonium nitrate (1.125 g.l⁻¹) as nitrogen sources. Three luminescent lamps Sylvia Aqua Star – 18 w, 10 000 K were placed at a distance of 30 mm from the flasks. The light regime was adjusted at 12:12 h light:dark cycle in an illumination incubator until the end of the experiment. The temperature was kept between 25 and 27°C. The strains were checked for a 96-hour growth period. In the laboratory, the samples of wastewater were filtered through a 25mm, 3μm glass microfiber filters (GF/C) mounted on a Millipore filtration unit. The cells in the exponential period were inoculated (10%, v/v) in a liquid medium.

Growth measurements, chlorophyll and carotenoid content of microalgae culture

Algal growth curves and biomass concentrations were determined by measuring the absorbance at 450 nm and dry cell weight, respectively. Optical densities of microalgae cultures were measured at 0, 24, 48, 72 and 96 hours after start of the experiment in three replicates. The sample with volume one ml was appropriately diluted with deionized water and the average value was recorded by absorbance at 450 nm with the help of spectrophotometer DR 2800 (Hach Lange). For dry weight
determination, culture samples (5 ml) taken at different times were centrifuged at 5000 rpm for 10 min. They were rinsed twice with distilled water and dried at 70°C for 24 h to give the dry cell weight (mg.l⁻¹).

The isolation of pigments from algae cells included the following procedures: harvesting 2 ml of microalgae cells by centrifugation at 10000 rpm, two times for 3 min and discarding the supernatant, suspension of cells in 2 ml methanol/water 90:10 v/v and mixing Vortex for 1 min, heating the suspension for half an hour in a water bath at 60°C, cooling the samples at room temperature, centrifuging the suspension (10000 rpm for 3 min) and discarding the supernatant with dissolved pigments. The absorbance of the pigments extract (665, 652 nm for chlorophyll content (a+b) and 470, 666 nm for carotenoids content) was recorded by using a spectrophotometer. The chlorophyll content was computed (mg.l⁻¹) according to Lichtenthaler (1987) and carotenoid content was computed (mg.l⁻¹) according to Lichtenthaler (1987).

Data analyses were conducted by using one-way Analysis of Variance ANOVA (MS Office, 2010).

Results and discussion

The optical density of C. vulgaris cultivation in wastewater from aquaculture used as growing media with nitrogen sources increased twice every 24 hours from the beginning to the end of the experiment. A better algal density (0.8) was measured in wastewater from aquaculture with nitrogen source – urea in 96 hours in the present study (Figure 1). It had 5% higher optical density compared to nitrogen source ammonium nitrate for the same strain (Table 1). The algal density starts from 0.1 and increases to 0.76 in 96 hours of the cultivation of C. vulgaris in wastewater with ammonium nitrate. Under the influence of urea as nitrogen source the optical density increases from 0.12 to 0.8 under the same condition.

The concentrations of both used nitrogen sources had strong influence on cell division during the cultivation of C. vulgaris in the present study. We receive the best algal optical density with 1.125 g.l⁻¹ urea concentrations on the fourth day of the cultivation. The results obtained correspond to those of other authors using urea as nitrogen source in concentrations of 1 – 1.2 g.l⁻¹ during the cultivation of microalgae (Weena et al., 2010; Danesi et al., 2002).

Maximum dry biomass (1.2 g.l⁻¹) of C. vulgaris was obtained in wastewater enriched with urea as an nitrogen source, in comparison to its dry weight in wastewater with ammonium nitrate (0.98 g.l⁻¹) (Figure 2). From the beginning of growing up to 72 hours biomass doubled. From 72 hour to 96 hour a four-fold increase of the algal biomass was observed by the cultivation of C. vulgaris in wastewater from aquaculture with urea as a nitrogen source. The received results showed that the maximum vegetative growth was reached approximately 96 hours of incubation. Shi et al. (2000) receive the maximum biomass concentrations (dry cells) in the cultures with urea as nitrogen source compared to ammonium nitrate. In this study we received 18.3% lower dry weight of C. vulgaris grown with ammonium nitrate compared to a cultivate with urea as a nitrogen source.

The quantity of chlorophyll starts from 0.4 mg.l⁻¹ and increases to 4.8 mg.l⁻¹ in 96 hours of the cultivation of C. vulgaris in wastewater with urea as a nitrogen source and correspondingly from 0.3 mg.l⁻¹ to 4.2 mg.l⁻¹ with ammonium nitrate. The obtained results showed that the amount of chlorophyll from the beginning to the end of the experiment increased four-fold. The highest chlorophyll content was determined in C. vulgaris cultivated in wastewater from aquaculture with nitrogen source – urea (Table 1). It was 12.5% higher than the chlorophyll content for C. vulgaris cultivated under the same conditions, but with ammonium nitrate as a nitrogen source (Figure 3). Similar results presented authors who cultivate other green microalgae with urea and ammonium nitrate as sources of nitrogen. Prabakaran and Ravindran (2012) tested two nitrogen sources (urea and ammonium nitrate) for Scenedesmus and Chlorococcum cultivation. The best results – maximum amount of chlorophyll content were recorded in the treatments with 0.02% urea. According to Kong et al. (2011) with respect to dry cell weight, total chlorophyll yields, as well as cost, urea is the best nitrogen source for culture of C. vulgaris in their study. The results for dry weight and chlorophyll content in our study of C. vulgaris proved a better growth effect of urea used as nitrogen source compared to ammonium nitrate.

The quantity of carotenoids starts from 0.06 mg.l⁻¹ and
increases to 1.0 mg.l⁻¹ in 96 hours of the cultivation of C. vulgaris in wastewater with ammonium nitrate as a nitrogen source and correspondingly from 0.07 mg.l⁻¹ to 1.2 mg.l⁻¹ with urea (Figure 4). Here the same trend was observed as in chlorophyll – the content of carotenoids in C. vulgaris was higher in cultures grown in wastewater with urea nitrogen source, compared to the carotenoids of wastewater with ammonium nitrate. The quantity of carotenoids in C. vulgaris cultivated in aquaculture wastewater is four times less in comparison to that of the chlorophyll in the same culture at 96 hours. Anontho (2011) established for industrial application purposes, utilization of wastewater with a source rich in nitrogen such as urea CO(NH)₂, ammonia NH₃ or other excess nitrogen substances which make biomass production and photosynthetic pigments more economical on Chlorella’s growth at the first 72 hours of cultivation. In our study at 72 hours of the Chlorella cultivation the biomass, chlorophyll and carotenoid quantities doubled. Also the carotenoid biosynthesis depends on the increase in biomass content of the microalgae. Anitha et al. (2009) reveals that at decreasing the concentration of nitrogen sources there was a decreased growth, chlorophyll and biomass. Nitrogen starvation also triggered a rapid decline in nitrogen containing compounds such as photosynthetic pigments causing complete loss of photosynthetic efficiency.

The pH increased with the culture time and exceeded 10 at the end of cultivation (Kong et al., 2011). During our trial the measured pH varied from 7.0 to 8.96 in tested algae strain and the pH value increased mostly in cultivation with ammonium nitrate (Figure 5). The pH values in the cultures with potassium nitrate and urea fluctuated around 7.2. The microalgae C. vulgaris maintained the maximum growth rate in a wide range of pH between 6.0 and 9.0, but started to be inhibited from pH 5 (Yun et al., 1996). The reason of the

**Table 1.** Optical density, dry weight, chlorophyll and carotenoid of C. vulgaris grown of wastewater with different nitrogen sources

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<th>Parameters</th>
<th>C. vulgaris urea</th>
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<td>min</td>
<td>max</td>
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<tr>
<td>Optical density</td>
<td>0.12</td>
<td>0.80</td>
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<td>Dry weight (g.l⁻¹)</td>
<td>0.20</td>
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<td>Chlorophyll a+b (mg.l⁻¹)</td>
<td>0.40</td>
<td>4.80</td>
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<tr>
<td>Carotenoid (mg.l⁻¹)</td>
<td>0.07</td>
<td>1.20</td>
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* – p≤0.05, ns – p≥0.05

**Figure 3.** Chlorophyll (mg.l⁻¹) of C. vulgaris cultivated in wastewater from aquaculture with different nitrogen sources

**Figure 4.** Carotenoid (mg.l⁻¹) of C. vulgaris cultivated in wastewater from aquaculture with different nitrogen sources

**Figure 5.** pH of C. vulgaris cultivated in wastewater from aquaculture with different nitrogen sources
drop of pH values in the mixotrophic cultures might attributedo the increase of releasing H+5 with the utilization of ammonium ion and the metabolism of organic acids during aerobic respiration by the algae. In our study the pH was relatively stable in both nitrate sources used for the cultivation of C. vulgaris in the wastewater from aquaculture as a nutrient medium.

In consideration of the specific growth rate, biomass content and productivity, potassium nitrate, ammonium nitrate or urea is the suitable nitrogen source for cultivation of C. vulgaris, urea gained important generally in large-scale algal cultivation, because the cost of urea is lower than the others (Kong et al., 2011).

Conclusion

The study of the cultivation conditions of C. vulgaris in wastewater from aquaculture with nitrogen sources urea and ammonium nitrate has shown that the best results of the algae biomass growth and photosynthetic pigments could be achieved when cultivation is with urea. Besides, wastewater from aquaculture could be used as cultivation medium for microalge strains C. vulgaris.

Acknowledgments

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