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Original article

EFFECT OF ACIDS ON BIOMASS GROWTH AND DEVELOPMENT OF *CHLORELLA VULGARIS* CULTURE

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Background. Influence of dissolved nitrogen and sulfur oxides, as components of flue gases, on the cultivation of microalgae *Chlorella vulgaris*.
Objective. To study biomass production and changes in cells of the microalgae *Chlorella vulgaris* with the introduction of nitrogen and sulfate acids tor simulate the effects of dissolved nitrogen and sulfur oxides, aiming to develop biotechnology for the utilization of gas emissions by microalgae.
Methods. The effect of the constant introduction of small concentrations of nitrogen (up to 0.47%) and sulfate (up to 1.5%) acids on the development of *Chlorella vulgaris* cultures, pH changes in the cultural environment, and biomass growth were studied.
Results. The utilization of gaseous emissions by *Chlorella vulgaris* depends on the content of nitrogen and sulfur oxides that are constantly supplied to the cultivation medium, the initial biomass concentration, and pH. It was determined that for an initial cells concentration of $(85 \pm 5)\times10^4$ cells/ml in the culture medium, the threshold values of acids that do not cause significant changes in the cultivation process are 0.1% H_2SO_4 and 0.19% HNO_3. At a sulfuric acid concentration of 0.2%, the culture cells become discolored.
Conclusions. It is shown that with a constant supply of sulfuric or nitric acids above the threshold values of 0.1% H_2SO_4 and 0.19% HNO_3, the pH of the culture medium decreases, leading to the suspension of development and the death of the *Chlorella vulgaris* culture. Therefore, controling these parameters will improve the ecological state of the environment and form the basis for developing biotechnology for the utilization of gas emissions by microalgae.

Keywords: microalgae; acids accumulation; biomass production; *Chlorella vulgaris*; gas emissions utilization.

Introduction

Harmful gas emissions from production processes in various industries and energy sectors have compelled humanity to focus on significantly reducing CO_2 emissions to achieve a carbon-neutral society [1]. Various methods for capturing, storing, and disposing of gas emissions through different technological approaches have been proposed [2], including the use of sorbents and solvents [3]. However, these methods alone are insufficient to process the vast amount of existing gas emissions [4]. The biological utilization of gas emissions from thermal power plants using microalgae is considered a promising solution. Microalgae can grow by utilizing CO_2 from flue gases, enabling the simultaneous production of biomass for energy carriers and valuable products derived from microalgae cells [5].

In addition to the high CO_2 content, flue gas contains impurities such as SO_x (sulfur dioxide (SO_2) – 99% and sulfur trioxide (SO_3)) and NO_x (nitric oxide (NO) – 99% and nitrogen dioxide (NO_2)), which influence the microalgae used for its utilization [6]. Sulfur and nitrogen oxides can serve as nutrient sources for growing microalgae. Sulfur is essential for the biosynthesis of amino acids such as cysteine, methionine, and other biologically active substances. Nitrogen is a component of proteins, nucleic acids, and phospholipids.

The concentration of SO_2 in the flue gas of coal-fired power plants after desulfurization is 100−300 ppm. Typically, microalgae obtain sulfur by absorbing sulfate into the cytoplasm with the help of high-affinity transport systems. However, when bubbling the culture medium with gas emissions, an excessive amount of SO_2 can directly lead to decrease in pH value due to its high solubility in water [7]. Thus, as a result of the hydration reaction, sulfurous acid (H_2SO_3) is formed immediately after SO_2 dissolves in the culture medium:

$$SO_2 + H_2O \leftrightarrow H_2SO_3.$$

After, hydrogen ions (H^+), bisulfite (HSO_3^{2-}), sulfite (SO_3^{2-}), and sulfate (SO_4^{2-}) accumulate as a result of dissociation

$$H_2SO_3 \leftrightarrow HSO_3^- + H^+ \leftrightarrow SO_3^{2-} + H^+$$

and oxidation reactions [5, 8]

$$SO_2 + H_2O + \frac{1}{2}O_2 \rightarrow H_2SO_4 \leftrightarrow 2H^+ + SO_4^{2-},$$

$$H_2SO_3 + SO_2 + H_2O + O_2 \rightarrow 2H_2SO_4.$$

When the cultural medium becomes acidified (1 pH drop for every 30 ppm approximately, at 1 atmosphere of CO_2, the equilibrium pH is about 5.2), the solubility and supply of CO_2 to the cells of microalgae decrease, which leads to the photosynthesis inhibition and consecutive decrease in biomass accumulation [7]. Today, in the technologies of gas emmissions utilization, the consistent use of *Galdieria sulphuraria* is proposed to increase the pH value due to the consumption of sulfur compounds in addition to *Chlorella vulgaris* for CO_2 utilization [9].

Research [8] shows that during the cultivation of *Chlorella vulgaris*, the constant introduction of SO_2 affects the reproduction of algae by decreasing carbon fixation rate. Specifically, the rate of CO_2 fixation drops down by 30% under the influence of 300 ppm SO_2, which leads to a decrease in biomass yield by 58% [8]. Under the action of an oxide concentration of 400 ppm, the pH drops to 3 within a day, and microalgae reproduction stops [8]. It was noted in other works that maximum admissible concentrations (MAC) reach NO 100 ppm and SO_2 60 ppm [10]. Based on the above, to use singular algae culture for gas emissions utilization, it is necessary to determine the maximum content of SO_2 at which the microalgae development is not inhibited and to determine the range of concentrations that can lead to an increase in biomass yield.

Microalgae usually assimilate inorganic nitrogen in form of nitrates, nitrites, and ammonium [11]. The content of nitrogen oxides in flue gas emissions reaches 90–95% NO and 5–10% NO_2, regardless of the combustion process [12, 13]. When the oxygen level exceeds 2% v/v, NO partially is oxidized to NO_2 [13]. Further oxidation can occur through chemical oxidation or nitrification [13]. In the work [9] the effect of oxides on the development of the *Chlorella sp.* ABC-001 strain was studied, there the authors concluded that NO had no significant effect. However, it was shown that NO_x due to reactions [5]

$$2NO + O_2 \leftrightarrow 2NO_2,$$

$$2NO_2 + H_2O \leftrightarrow HNO_2 + HNO_3$$

harmfully affect cell growth, causing a decrease in pH at a concentration of 100–200 ppm [9, 13–16].

Therefore, the amount of NO_x provided to the culture medium should be lower than the maximum tollerance value of microalgae.

The analysis of literary sources does not provide a clear answer to the question of how nitrogenous and sulfur acids formed from oxides affect the development of microalgae. Limiting concentrations at which biomass growth is maximal have yet to be established.

The aim and task of the work is to determine the effect of different concentrations of nitric and sulfuric acids on the *Chlorella vulgaris* cultivation process. There is a need to assess the effect of systematic acids accumulation in the nutrient medium on the efficiency of biomass growth and changes in the component composition of microalgae cells because taking into account these parameters will help in creating biotechnology for the utilization of gas emissions of microalgae and improve the ecological state of the environment.

Materials and Methods

The cultivated *Chlorella vulgaris* was taken from the culture collection of the Department of Environmental Engineering (University of Warmia and Mazury in Olsztyn).

The cultivation of microalgae was carried out in column photobioreactors (AB Aqua Medic GmbH, Germany) (Fig. 1) with an active volume of 2 liters and was equipped with a lighting and aeration system. *Chlorella* culture Illuminated continuously (24 h/day) with Aquael LED lamps Leddy Slim 10W, 650 lumens (AQUAEL sp.zo.o., Poland), 8000K. The temperature in the photobioreactors was maintained at 25 ± 1.0 °C.

For the cultivation of microalgae, a modified Tamiya nutrient medium was used [17], the composition of which is: KNO_3 – 5.0 g/l, $MgSO_4 \cdot 7H_2O$ – 2.5 g/l, KH_2PO_4 – 1.25 g/l, EDTA – 0.037 g/l, $FeSO_4 \cdot 7H_2O$ – 0.009 g/l, H_3BO_3 – 2.86 mg/l, $MnCI_2 \cdot 4H_20$ – 1.81 mg/l, $ZnSO_4 \cdot 7H_2O$ – 0.22 mg/l, MnO_3 – 0.018 mg/l, NH_4VO_3 – 0.023 mg/l. The initial pH was 6. The pH value was determined using a potentiometric method by pH-meter (AquaMedic GmbH, Bissendorf, Germany).

Nitric and sulfuric acids were used as a source of nitrogen and sulphur coresponding to action of sulfur and nitrogen oxides from emissions. Acids were applied using Shenzhen peristaltic pumps LabV I-III (Baoding Shenzhen Precision Pump Co., Ltd., China). The sulfur content of sulfuric acid, which could be formed at the input of different concentrations of sulfur dioxide, was calculated (Table 1)

based on the conditions of the gas supply rate of 0.5 l/min for the working volume of the reactor of 2 l. Recalculation by nitrogen of the probable content of nitrogen oxides in the gas to a similar amount of nitric acid (Table 2) is made based on the gas supply rate is also 0.5 l/min per working volume of the reactor of 2 l. The acid injection rate in both cases was 0.03 ml/min, corresponding to the gas supply rate and its corresponding concentration. The calculated data are given in the Tables 1 and 2.

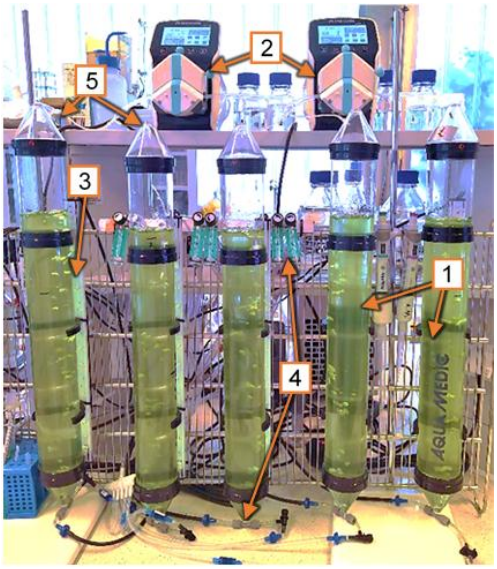

Figure 1: Experimental installation: 1 – column of photobioreactors, 2 – peristaltic pumps, 3 – LED lamp, 4 – rotameter and air injection, 5 – acid injection

Table 1: Correspondence of concentrations of sulfur oxides and sulfuric acid

Concentration of SO_x in flue gases	Concentration of H_2SO_4, %
0 ppm	0
15 ppm	0.1
30 ppm	0.2
45 ppm	0.3
50 ppm	0.33
60 ppm	0.4
100 ppm	0.73
150 ppm	1.1
200 ppm	1.5

Table 2: Correspondence of concentrations of nitrogen oxides and nitric acid

Concentration of NO_x in flue gases	Concentration of HNO_3, %
0 ppm	0
40 ppm	0.19
60 ppm	0.28
80 ppm	0.37
100 ppm	0.47

Carbon dioxide was provided from atmospheric air supplied by a Hailea compressor ACO-208 (Hailea Group Co., Ltd., China) in vertical column photobioreactors. The bubling air feed rate ensured the mixing and adequate mass exchange for the cultures, gas flow rate was 0.5 l/min and regulated by an Oxygen rotameter Air Flow Meter with a range of 0–1.5 l/min (Shanwen, China).

Taxonomic analysis and cell count of algae cells in a hemocytometer (cell counting chamber Thoma, Germany) was carried out using an MF 346 microscope with an Optech 3MP camera (Delta Optical, Warsaw, Poland) at a total microscope magnification of 1000× or 400×. Determination of the biomass concentration during the cultivation time was carried out by determining the chlorophyll content using a BBE algae analyzer (Moldaenke, Schwentinental, Germany). The specific growth rate was determined according to the standard method [18]. The initial concentration of cells in the study of nitrogen compounds was $(85 \pm 5)\times10^4$ cells/ml; in the study of sulfur compounds, it was $(75 \pm 5)\times10^4$ cells/ml, which is 4.5 times higher than the suggested initial concentrations of other researchers [12].

Separation of biomass from the culture liquid was carried out by centrifugation (2 min, 11000 rpm) with cell-washing in distilled water) in a laboratory centrifuge 5804 R (Eppendorf, Hamburg, Germany).

The content of dry mass, organic and mineral component was measured by the gravimetric method using a laboratory dryer FED (Binder, Germany) and a laboratory furnace Carbolite ELF 11/14B, drying was carried out at a temperature of 105 °C, ashing – for 550 °C.

The content of total nitrogen, total carbon and organic carbon was determined using the Multi-analyzer NC 3100 (Analytik Jena, Jena, Germany). The content of sulfate (SO_4^{2-}) was determined in the supernatant using a Hach spectrophotometer DR 5000 (Hach, Loveland, CO, USA) with mineralizer HT200S (Hach-Lange, Germany).

Experiments were conducted in four replications. Statistical data analysis was performed using MSExcel (Microsoft Corporation) and Statistica 14.01 (TIBCO Software Inc.) software. The results were subjected to a one-way analysis of variance (ANOVA) and considered significant at $p < 0.05$; differences between mean values were determined with the Tukey test (HSD) [19].

Results

When gaseous emissions enter the culture medium, gradual oxidation of SO_2 occurs in the photobioreactor [5, 8, 20]. Accordingly, SO_4^{2-} accumulates in the culture medium with constant bubbling.

In Fig. 2, the dependence of the change in the biomass growth rate during cultivation with the constant introduction of sulfuric acid of different concentrations is given.

In Fig. 3, the change in pH during cultivation under the constant action of sulfuric acid is given.

Table 3 shows the change in the components of the culture medium during the introduction of sulfuric acid on the fourth day of the experiment.

Fig. 4 shows the visual changes of the culture before and after exposure to sulfuric acid, mainly the discoloration of the cells (Fig. 4b).

In the aqueous phase, NO_2 continuously and irreversibly reacts with water to form nitrous and nitric acids [11]. In Fig. 5. the dependence of the change in the biomass growth rate during cultivation with the constant introduction of nitric acid of different concentrations is given.

In Fig. 6, the change in pH with the constant introduction of nitric acid of different concentrations is shown.

Table 4 shows the change in the components of the culture medium during the introduction of nitric acid on the fourth day of the experiment.

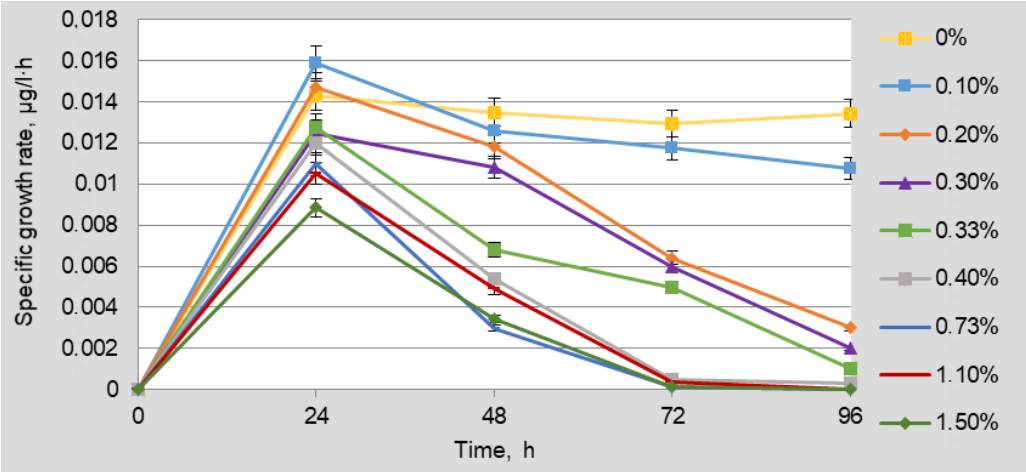

Figure 2: Dynamics of the specific growth rate of biomass from the time of cultivation at different concentrations of sulfuric acid

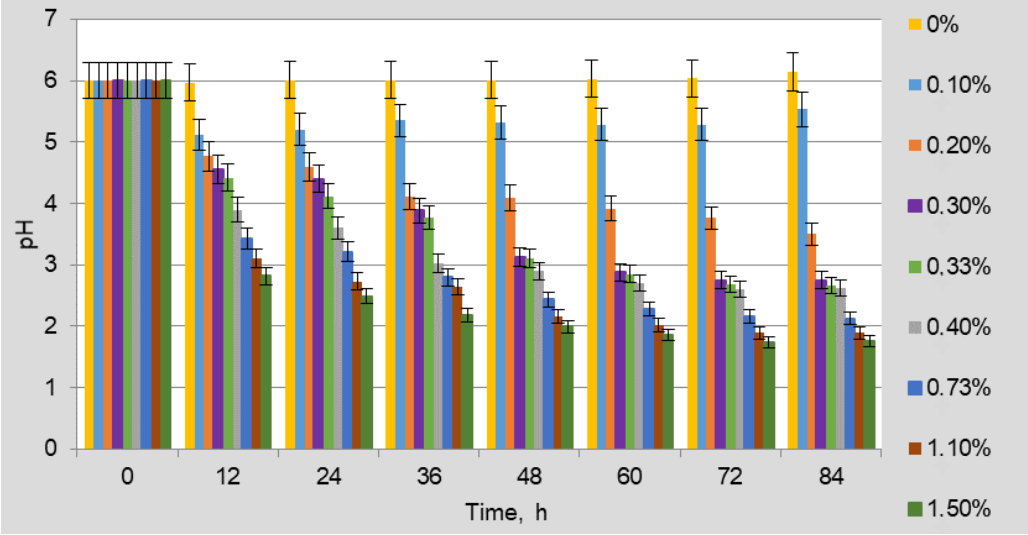

Figure 3: Change in pH during cultivation under the action of sulfuric acid

Table 3: Changes in the components of the culture medium over four days effects of sulfuric acid

Concetration H_2SO_4	Total carbon, mg/l	Total organic carbon, mg/l	Inorganic carbon, mg/l	Total intake H_2SO_4 (4 d.), mg/l	Increase of SO_4^{2-} content, %	Final SO_4^{2-} content, mg/l
0%	114.3 ± 5	69.6 ± 3	44.7 ± 2	0	0	983 ± 50
0.1%	56.2 ± 2	28.9 ± 1.5	27.2 ± 1,3	86.3 ± 4	8.8	1140 ± 57
0.2%	66.9 ± 3	41.5 ± 2	25.3 ± 1,3	172.6 ± 8	17.6	1240 ± 62
0.3%	71.8 ± 3	37.8 ± 2	33.9 ± 1,6	258.9 ± 12	26.3	1300 ± 65
0.4%	69.9 ± 3	36.5 ± 2	33.4 ± 1	345.2 ± 17	35.1	1330 ± 66

Table 4: Changes in the components of the culture medium over four days effects of nitric acid

Concetration HNO_3	Total carbon, mg/l	Total organic carbon, mg/l	Inorganic carbon, mg/l	Initial content NO_3^-, mg/l	Total intake HNO_3 (4 days), mg/l	Final total HNO_3 content, mg/l
0%	33.7 ± 1.6	23.5 ± 1	10.2 ± 0.5	690 ± 34	0	635.3 ± 31
0.19%	32.9 ± 1.6	22.6 ± 1	10.3 ± 0.5	690 ± 34	163 ± 8	668.1 ± 33
0.28%	31.2 ± 1.5	21.4 ± 1	9.8 ± 0.5	690 ± 34	240.2 ± 10	667.2 ± 33
0.37%	28.2 ± 1.4	19.2 ± 1	9.0 ± 0.5	690 ± 34	317.4 ± 15	662.0 ± 33
0.47%	23.6 ± 1	14.8 ± 0.7	8.9 ± 0.5	690 ± 34	403.2 ± 20	686.7 ± 34

a b c

Figure 4: Culture cells before (a) and after (b) exposure to sulfuric acid at a concentration of 0.4% (magnification 400×). Comparison of the appearance of cultures (c)

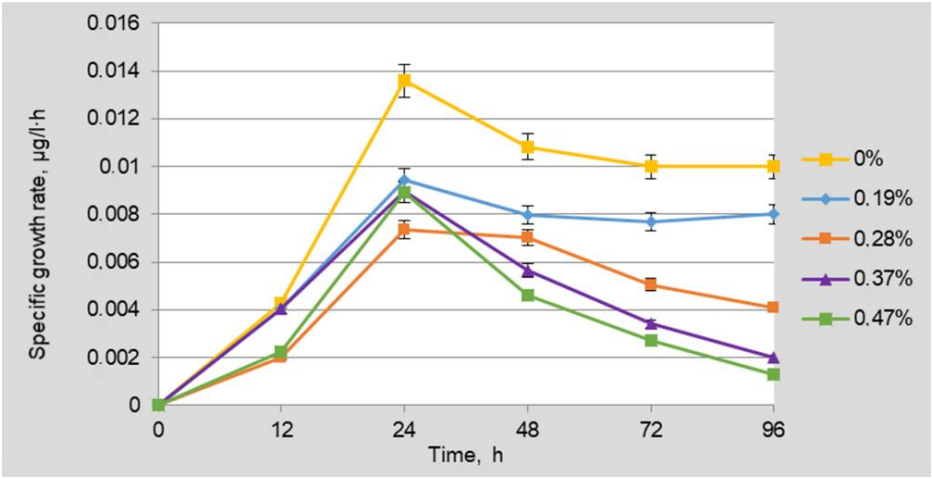

Figure 5: The dynamics of the specific growth rate of biomass for the time of cultivation under the action of nitric acid of different concentrations

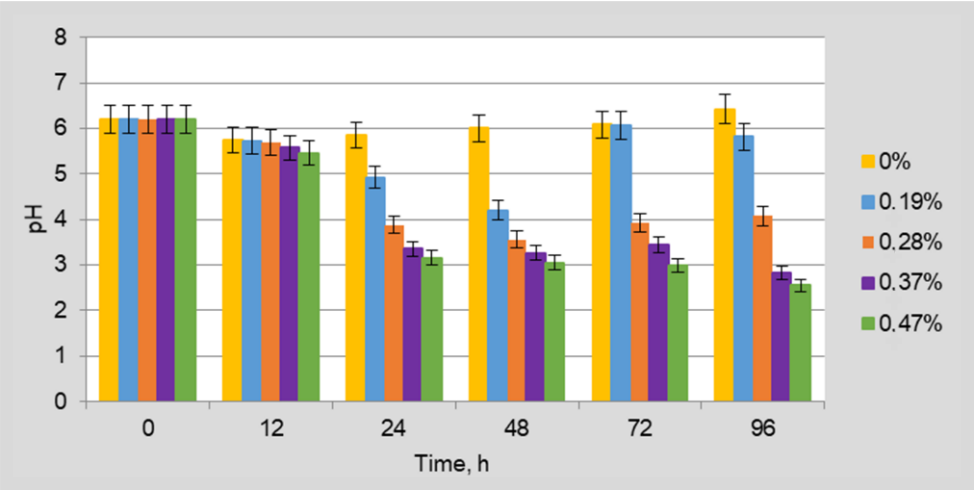

Figure 6: Change in the pH of the medium during cultivation under the influence of nitric acid

Discussion

The proposed research method allows us to achieve the goal and solve the problem of determining the limited concentrations of sulfuric and nitric acids that do not affect the growth of biomass and the development of the *Chlorella vulgaris* culture. The concentration of acids, which were continuously added to the culture medium, corresponded to the concentration of sulfur and nitrogen oxides in gas emissions. The results indicate using microalgae to utilize gas emissions is possible using small concentrations of the obtained acids. Also, as a result of photosynthesis, microalgae assimilate carbon dioxide, turning it into biomass, which in our study was observed as an increase in the optical density of the medium.

With the constant introduction of sulfuric acid at a concentration of 0.1% (which corresponds to a sulfur oxide concentration of 15 ppm in gas emissions) during the first day, there is a slight increase in biomass compared to the control (see Fig. 2). During the next day, the increase in biomass in the control sample exceeds the increase in the introduction of any concentration of sulfuric acid. At the same time, pH value drops under 4 for all samples, except for samples with the introduction of sulfuric acid concentrations of 0.1 and 0.2% (see Fig. 3). At such pH values, a sharp decrease in biomass growth is observed. When pH drops down to 3 or less, culture development stops, which is observed on the third day of cultivation (see Fig. 2). The obtained pH values at which cell death occurs correspond to the values obtained in the work [21], where gradual cessation of photosystem II function at low pH and possible cell damage was observed. A slight increase in pH value from 5 to 5.5 during the 3.5 days of cultivation for the sample with the introduction of 0.1% sulfuric acid (15 ppm SO_2) is explained by the increase in microalgae biomass.

In the case of sulfuric acid, a sharp decrease in acidity from the first day for ranges higher than 0.73% (100 ppm SO_2) was observed. When the content of sulfuric acid increases, the release of metabolites into the culture medium decreases, which indicates a change in the metabolism of microalgae cells (see Table 3). The increase in carbon content with increasing sulfuric acid content can be explained by the decomposition of *Chlorella vulgaris* cells [7]. With the constant introduction of sulfuric acid at a concentration of 0.4%, when the pH value reached 3.5, the cells lost chlorophyll, which is evidenced by their discoloration (see Fig. 4). However, when the supply of acid was stopped at a concentration that did not exceed 0.73% (100 ppm SO_2), regeneration of the culture was observed after 7–10 days.

The acidity of the environment directly affects various physiological and biochemical processes occurring in the cells of microalgae, as well as on solubility and availability of CO_2, so maintaining an optimal pH range is essential for cell growth, enzyme activity, and nutrient uptake [22]. Thus, for utilizing gas emissions with the help of *Chlorella vulgaris*, it is necessary to constantly introduce sulfur oxide in a concentration that does not exceed 15 ppm based on the initial number of cells of $(75 \pm 5)\times10^4$ cells/ml. With a higher initial concentration of cells, the culture is more resistant to slight increases in the content of sulfur oxides in gas emissions, which is explained by better regulation of the acidity of the environment due to the

vital activity of the culture itself and an increase in its need for nutritional components [8].

The concentration of nitrate ions as a nitrogen source in the nutrient medium affects both biomass growth and metabolite biosynthesis [23]. In the process of nitrate assimilation, it is transferred through the cell membrane, converted to nitrite, and subsequently to ammonium. All processes are energy-consuming. Flavoprotein enzymes namely nitrate reductase, located in the cytoplasm and the pyrenoid of microalgae, activates nitrate reduction to nitrite using pyridine nucleotides (ATP, GTP) as electron donors. Nitrite reductase is a chloroplast enzyme that uses ferredoxin as an electron donor in the 6-electron transfer reaction in the process of nitrite reduction [24]. The significant energy costs for nitrogen recovery can explain the lower growth of microalgae biomass when an excess amount of nitric acid is introduced. The decrease in biomass growth rises with an increase in the content of nitric acid introduced into the culture medium (see Fig. 5). At the same time if only nitrate ions are used as a nitrogen source, the nutrient medium's pH value increases during its consumption. It is typical for the additional introduction of nitric acid with a concentration of 0.19% (40 ppm of nitrogen oxides) on the third day of cultivation (see Fig. 6).

The pH value of the medium in the first two days when nitric acid was applied with concentrations up to 0.28% is explained by the microalgae adaptation process to such environmental conditions. For all excess amounts of nitric acid introduced, its consumption is observed, as evidenced by the almost identical nitrogen content in the culture medium on the fourth day of cultivation (see Table 4). This does not contradict the data obtained in the work [23].

When the concentration of nitric acid in the medium increases, the cells' metabolism changes, evidenced by a decrease in the carbon content in the culture medium (see Table 4). When bubbling the culture medium with gas emissions with a concentration of nitrogen oxides of more than 60 ppm, the synthesis and release of metabolites from the cell decreases.

Based on the above, it can be stated that the utilization of gas emissions by microalgae *Chlorella vulgaris* will be influenced by the content of sulfur and nitrogen oxides. Limiting concentrations of oxides in gas emissions, which do not lead to cell growth arrest, are 15 ppm SO_2 and 40 ppm nitrogen oxides for an initial cell concentration of $(85 \pm 5)\times10^4$ cells/ml. An increase in the content of oxides leads to a decrease in the growth of biomass, a decrease in the rate of purification of emissions, and, in the case of sulfur oxides, to the death of cells.

Conclusions

Threshold values of nitric and sulfuric acids for constant introduction into the culture medium, which do not adversely affect the development of the culture and growth of the biomass of microalgae *Chlorella vulgaris*, have been determined. For sulfuric acid – 0.1% H_2SO_4, which corresponds to a sulfur oxide concentration of 15 ppm in gas emissions flow 0.25 l/l·min, and 0.19% HNO_3, which corresponds to 40 ppm of nitrogen oxides in gas emissions flow 0.25 l/l·min for the initial cell concentration of $(85 \pm 5)\times10^4$ cells/ml.

An increase in the concentration of acids above the threshold value leads to a decrease in cell growth and a change in their metabolism due to a decrease in the pH of the culture medium.

Under the action of sulfuric acid, when pH 3.5 is reached, cells lose chlorophyll, which leads to cell death.

The use of gas emissions is possible at the initial stages, but it contributes to a rapid transition to the phase of cell death and a dynamic decrease in biomass.

Further research should clarify the simultaneous influence of sulfur, nitrogen, and carbon oxides on the development of microalgae culture, their influence on the biosynthesis of metabolites, and the degree of purification of gas emissions. It is also necessary to determine the dependence of the limited concentrations of oxides on the initial number of cells.

Understanding the parameters affecting microalgae growth and biomass yield, in particular, the concentrations of acid oxides in gas emissions, is crucial in scaling up the technology for utilizing gas emissions by microalgae, as only by optimizing the operating parameters and controlling the growth conditions of microalgae can effectively apply this technology for sustainable and environmentally friendly production.

Interests disclosure

Nataliia Golub is the member of the Editorial Council of *Innovative Biosystems and Bioengineering* and was not involved in the evaluation or decision to accept this article for publication. The other authors have no conflicts of interest to declare.

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ВПЛИВ КИСЛОТ НА ПРИРІСТ БІОМАСИ ТА РОЗВИТОК КУЛЬТУРИ *CHLORELLA VULUGARIS*

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Проблематика. Вплив розчинених оксидів азоту і сульфуру як компонентів димових газів на культивування мікроводоростей *Chlorella vulgaris*.
Мета. Досліджено продукування біомаси та зміни в клітинах мікроводоростей *Chlorella vulgaris* із введенням азотної та сульфатної кислот для симулювання дії розчинених оксидів азоту та сульфуру для подальшого створення біотехнології утилізації газових викидів мікроводоростями.
Методика реалізації. Вплив дії постійного введення малих концентрацій азотної (до 0,47 %) та сульфатної (до 1,5 %) кислот на розвиток культури мікроводоростей *Chlorella vulgaris*, зміну pH культурального середовища та приріст біомаси.
Результати. Утилізація газових викидів мікроводоростями *Chlorella vulgaris* залежить від вмісту оксидів азоту і сульфуру, що постійно подаються в середовище культивування, початкової концентрації біомаси та pH. Визначено, що для початкової концентрації клітин у культуральному середовищі $(85 \pm 5)\times10^4$ клітин/мл пороговими значеннями вмісту кислот, за яких не відбувається суттєвих змін у процесі культивування, є 0,1 % H_2SO_4 і 0,19 % HNO_3. За концентрації 0,2 % сульфатної кислоти відбувається знебарвлення клітин культури.
Висновки. При постійному надходженні сульфатної або нітратної кислот вище порогових значень 0,1 % H_2SO_4 і 0,19 % HNO_3 знижується pH культурального середовища, що призводить до призупинення розвитку та подальшої загибелі культури *Chlorella vulgaris,* тому контроль цих параметрів поліпшить екологічний стан довкілля і стане підґрунтям для створення біотехнології утилізації газових викидів мікроводоростями.

Ключові слова: мікроводорості; накопичення кислот; продукування біомаси; *Chlorella vulgaris*; утилізація газових викидів.