Recycling of Aqueous Phase from Hydrothermal Liquefaction and Municipal Wastewater by Microalgae

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Abstract. The search for alternatives to hydrocarbon fuels remains an actual task. Microalgae (MA) as raw material for the production of biofuel remain an urgent object of research among other types of biomass, and the cultivation of MA is constantly growing. One of the promising technologies for biofuel production from MA is hydrothermal liquefaction (HTL) that allows processing wet biomass and turning all carbon-containing components (lipids, carbohydrates, proteins) into fuel. However, the hydrothermal liquefaction process leads to the formation of a significant amount of the aqueous phase, which is a by-product with low energy value and also needs to be processed. It is important to consider the possibility of using the aqueous phase after HTL, as well as municipal wastewater, for the cultivation of MA in combination with the production of biofuels. The MA strains capable to grow in a dilute aqueous phase after HTL (Galdieria sulphuraria rsemsu G-1, Chlorella vulgaris rsemsu Chv-20/11-Ps, Arthrospira platensis rsemsu Bios) and in wastewater (Arthrospira platensis rsemsu Bios, Chlorella ellipsoidea rsemsu Chl-el) were experimentally selected from collection of RSE Laboratory at LMSU. The article is devoted to the experimental study of the degree of nutrients utilization by MA from wastewater and an HTL-aqueous phase.

1. Introduction

Bioenergy remains the largest renewable energy industry. At the same time, microalgae (MA) are a promising object of research among other types of biomass and the scale of MA using for energy purposes is increasing [1]. On the other hand, the growth of global energy production and urbanization, which results in high rates of generation of municipal waste and wastewater, requires integrated waste management technologies with the complete waste utilization. Utilization of organic waste with energy production by various technologies is one of the methods for solving this complex problem.

In this work, we consider the cultivation of MA using wastewater and liquid phase from the HTL of MA. MA for liquid waste treatment are selected because:

- wastewater has a complex composition, the purification of wastewater from nitrogen and phosphorus and other nutrients, as well as from heavy metals using biological objects is optimal;
- MA are considered as a promising raw material for the biofuels production (third-generation biofuels), since they have higher growth rates than terrestrial biomass, and at the same time do not compete with the production of food and feed;
MA cultivation in wastewater reduces the use of fresh water, nutrients (fertilizers), which generally leads to a reduction in the cost of biofuels obtained from them.

2. The use of MA for wastewater treatment: existing approaches and results
The development of wastewater treatment technologies using MA is quite an urgent task [2-4]. Real or model wastewater with various COD and BOD indicators as well as the concentrations of nitrogen, phosphorus, and dissolved carbon compounds have been used. The consumption efficiency of these substances by MA is estimated by the rate and total amount of nitrogen absorbed from wastewater (in the form of NH$_4^+$, NO$_3^-$, NO$_2^-$, total nitrogen), phosphorus (PO$_4^{3-}$, total phosphorus), dissolved and total carbon, etc. [5]. At the same time, it is taken into account that in addition to organic and inorganic chemicals, bacteria and protozoa are also present in the wastewater, which compete for nutrients with MA and even eat them. MA is cultivated on wastewater of various compositions. According to the results of experiments, strains of MA are selected that demonstrate a significant growth rate and, accordingly, the absorption of nutrients and the most resistant to toxic components of wastewater [6]. A number of studies also set the task of improving methods for the cultivation of MA in wastewater [7], studying the effectiveness of consortia of MA and bacterial organisms [5, 8], and analyzing the possibility of cultivating MA in wastewater in open cultivators in a wide range of climatic conditions [2, 9].

3. Technologies for obtaining biofuel from MA
The MA biomass can be processed into a wide range of biofuels: bioethanol, biodiesel, bio-oil, biohydrogen, etc. A traditional technology is the transesterification of MA lipids into biodiesel. The obvious disadvantages of this method are the high energy costs in the process of separation of MA from the culture medium and drying, as well as the use of unsafe organic solvents (such as methanol) for the extraction of lipids from MA biomass. In addition, in the production of biodiesel, only the lipid part is converted to fuel, while the rest part (most of the MA biomass), including proteins and carbohydrates, is not used to produce liquid biofuel.

Thermochemical conversion technologies don’t have these disadvantages; they are carried out by heating and decomposing biomass in the presence or absence of air (oxygen). Types of thermochemical conversion are direct combustion (the main product is electric energy or heat), gasification (synthesis gas), pyrolysis (synthesis gas, bio-oil, coal), and liquefaction (bio-oil, synthesis gas, coal). Nowadays pyrolysis and hydrothermal liquefaction technologies of MA biomass have been actively promoted.

Under the MA pyrolysis in order to obtain solid, liquid, and combustible gaseous products, the biomass is heated to temperatures of 400–600°C; in some cases the temperature can reach 800°C. The MA pyrolysis which is aimed at producing combustible gases with a high hydrogen and methane content and high calorific value is less studied. MA cells are small in size, the dry biomass of MA is loose, and therefore it does not require additional grinding, like other types of biomass. However, for effective pyrolysis, the MA biomass must contain a minimum amount of moisture, which significantly increases the cost of technology implementing.

Hydrothermal liquefaction (HTL) is a method of thermochemical conversion of wet MA, which produces a liquid biofuel referred to as “bio-oil” or “biocrude”, as well as gaseous, water-containing (aqueous phase) and solid by-products (“biochar”). The biomass processing by the HTL method does not require preliminary drying of the feedstock: MA can be fed to the hydrothermal liquefaction reactor in the wet state, for example, in the form of an aqueous suspension. In addition, when producing fuel by the HTL method, not only lipids, but also carbohydrates and proteins contribute to the bio-oil yield, which increases the total yield of the fuel. However, the hydrothermal liquefaction process leads to producing the significant amount of the aqueous phase, which is a by-product, has limited energy value and also needs to be utilized.

The cultivation of MA biomass using the municipal wastewater and its conversion by the HTL method with the subsequent utilization of the aqueous phase is the subject of this study.
4. Materials and methods
The following MA strains with a high growth rate and capability of responding to change in environmental conditions (a decrease / increase of the main nutrient elements concentration, toxic elements, etc.) from the collection of the Renewable Source Energy Laboratory at Lomonosov Moscow State University (RSEL MSU) were used for experiments:

- for recycling waste water - *Arthrospira platensis* rsemsu *Bios (P)*, *Gloeotila pulchra* rsemsu *Pz-6*, *Galdieria sulphuraria* rsemsu *G-1*, *Chlorella ellipsoidea* rsemsu *Chl-el*;
- for recycling HTL-aqueous phase - *Arthrospira platensis* rsemsu *Bios (P)*, *A. platensis* rsemsu 1/02, *A. platensis* rsemsu 1/02-T, *Galdieria sulphuraria* rsemsu *G-1*, *Chlorella vulgaris* rsemsu *Chv-20/11-Ps*.

All of these MA strains, excepting the *Arthrospira platensis* rsemsu *Bios (P)* culture, are algologically pure. They are maintained in the RSEL MSU collection on sterile nutrient media (*Chlorella ellipsoidea* and *Chlorella vulgaris* - on Tamiya medium; *Galdieria sulphuraria* on Allen medium; *Gloeotila pulchra* – on BG-11 medium) [10]. The culture of *Arthrospira platensis* rsemsu *Bios (P)* is grown in the RSEL MSU using Zarrouk’s medium [11] in open planar cultivators (volume is about 1000-500 l) by the semi-continuous method for more than 30 years and is represented by a stable consortium of MA *Arthrospira platensis* with heterotrophic bacteria-associates that were identified as representatives of the genera *Pseudomonas* and *Bacillus*. The latter are able to assimilate dissolved organic matter and toxic elements and thus provide a more efficient municipal wastewater treatment.

Municipal wastewater taken from the observation well in Moscow in October 2018 was used as a model medium for MA cultivation. The cultivation was carried out in Erlenmeyer flasks with a volume of 250-500 ml, the initial MA concentration was about 0.16 g (dry weight) / l. The cultivation was carried out in undiluted wastewater, as well as in wastewater diluted with freshly boiled tap water (dilution range used: 5 ... 50 times). The reference represents the growth of *A. platensis* biomass on 1/2 or 1/3 of Zarrouk’s medium; for *Gloeotila pulchra* - on the 1/2 medium BG-11; *Chlorella ellipsoidea* and *Chlorella vulgaris* – Tamiya medium; for *Galdieria sulphuraria* – Allen medium. Each experiment was carried out three times. Biomass growth was monitored colorimetrically. All flasks were arranged on a shaker (rotation 120 rpm; T = 33°C; lighting 30μE/m²s during 24 h). The cultivation of MA in wastewater was carried out for 10 to 47 days. The concentration of nutrients in wastewater was studied by chemical analysis of the nutrients in the culture fluid at different stages of MA cultivation. COD, BOD₅, nitrates (NO₃⁻), phosphates (PO₄³⁻), ammonium (NH₄⁺) were considered as indicators. The state of MA cultures at different stages of the experiments was visually monitored by microscopy of culture fluid samples (LOMO Mikmed-2 and Axioplan 2 Imaging microscopes with an AxioCam MBm camera and AxioVision 3.1. Carl Zeiss).

Hydrothermal processing experiments were performed at a laboratory reactor (Figure 1). HTL was carried out at the installation of the Joint Institute for High Temperatures of the Russian Academy of Sciences. Hydrothermal liquefaction experiments were conducted at T = 270°C and 300°C.

The yields of bio-oil, gaseous products, solid residue and aqueous phase were 34-46%, 12-18%, 12-18% and 10-24%, respectively. The molecular formulas of the substances in the composition of the final products were determined. It has been established that compounds containing one and two nitrogen atoms dominate in bio-oil, while ON2, N3 classes are also present. Bio-oil from MA with a high content of lipids and carbohydrates is close to traditional oil [12]. The aqueous phase was used for cultivating the MA and thus for recirculation of this by-product.

5. Results and discussion
5.1. Composition of municipal wastewater
Due to the autumn period with high precipitation, municipal wastewater samples were significantly diluted (Table 1). The amount of nutrients (nitrogen ammonia, nitrates and orthophosphates) are present in a small amount - 110, 2.4 and 15 mg/l, respectively. COD (total organic matter in heavily polluted effluents) 140 mgO/l and BOD₅ (readily oxidizable organic matter concentration in effluents)
85 mgO₂ / l indicate that the sample contains a small amount of dissolved organic matter. According to the value of the hydrogen index (pH = 8.2), the wastewater is alkaline. The amount of oil products (2.7 mg/ l) and total aromatic substances (0.668 mg/l) exceeds the average for this class of wastewater. A comparison of the wastewater composition with the culture media composition for the growth of MA shows that the wastewater contains all the trace elements necessary for the growth and development of MA. An analysis of published data on the composition of wastewater showed that the wastewater used in the study is typical, but with a low COD value and high nitrogen ammonia content.

Figure 1. Scheme of the experimental plant: R₁, R₂ – reactors-autoclaves with agitators and cooling/heating coils, E₁ – tank for receiving the raw feedstock, E₂ – tank with water, E₃ – tank for receiving the products of hydrothermal liquefaction, N₁ – pump for the loading of MA suspension into the reactor, N₂ – high-pressure pump for circulating water, N₃ – reverse pump for heat recuperation, C – air compressor, B – buffer tank, T – heat exchanger, Q – heater, N₂ – high-pressure tank with nitrogen, M – agitators, P – pressure sensors, V – valves, V₅ – safety valves, L – level sensor.

Table 1. The chemical composition of the municipal wastewater sample from the observation well of Moscow.

| Chemical Composition, units | Value | Chemical Composition, units | Value |
|-----------------------------|-------|-----------------------------|-------|
| Hydrogen indicator (pH), units pH | 8,2 | Hydrocarbons, mg / l | 820 |
| COD (bichromate oxidation), mgO₂ / l | 140 | Carbonates, mg / l | <6 |
| BOD₅, mgO₂ / l | 85 | Ammonium, mg / l | 110 |
| Nitrates, mg / l | 2,4 | Phosphates, mg / l | 15 |
| Potassium, mg / l | 20 | Magnesium, mg / l | 14 |
5.2. Determination of the wastewater dilution degree for microalgae cultivation

Since wastewater contains a significant amount of substances toxic to MA, an important step of the study was the selection of dilution degree. On the one hand, the toxicity of dissolved substances should be reduced, and on the other hand, the nutrients concentration should not be significantly reduced. The experimental results of the MA cultivation at different waters dilution degree are shown in Figure 2.

As can be seen, a positive growth dynamic of the *Arthrospira platensis* Bios (P) biomass in diluted wastewater was observed at all dilution degrees during the first 7–9 days. Moreover, a 2-fold increase in biomass occurred on the 5th day of the experiment. With all wastewater dilution degrees, the growth of biomass lagged behind the reference medium (the MA cultivating on the 1/3 of the Zarrouk’s nutrient medium composition, without wastewater). Apparently, toxic substances (ammonium compounds, oil products and aromatic compounds) in wastewater without dilution suppressed the growth of MA. The optimal wastewater dilution degree is not higher than 5 x. And with dilutions of more than 5x, the biomass growth stopped after 5–7 days due to the complete exhaustion of nutrients. Thus, the need for wastewater dilution for sustainable growth of the *Arthrospira platensis Bios* biomass was determined and the dilution limits (no more than 5x) were found. In addition, it was found that for the arthrospira, sodium bicarbonate must be added to the wastewater, as arthrospira grows only in alkaline waters in the presence of bicarbonate anion as a carbon source.

5.3. Characteristics of microalgae growth in wastewater

Three clonal algologically pure cultures of *A.platensis* did not grow well in weakly diluted wastewater, and by 5-7 days the cultures died. On the contrary, the culture of *A.platensis Bios (P)* with associative bacteria showed good growth in wastewater diluted even 2 times. Therefore, experiments with the *A.platensis Bios (P)* culture with associative bacteria were carried out in 2-3 times diluted wastewater. The growth of this culture not only does not lag behind the reference medium, but in the period of 7–12 days, its growth in wastewater with dilution degree 2x even exceeds the reference medium (Figure 3).

After 12 days of the experiment, the growth of cultures slowed down, and all biomass accumulation curves reached a plateau. Subsequently, portions of wastewater equal to their volume at the beginning of the experiment were added to these experimental cultures. In this case, an additional
dilution of wastewater (1.3x and 1.75x, respectively) occurred. The results of further cultivation of MA cultures under modified experimental conditions are presented in Figures 3, 4.

**Figure 3.** Dynamics of Arthrospira platensis Bios (P) biomass density in wastewater with 2x and 3x dilution.

**Figure 4.** Continuation of the experiment: the growth of Arthrospira platensis Bios (P) biomass in wastewater with the addition of a portion of wastewater after 12 days of growth.

On the 19th day of the experiment, the cultures density in both cases reached the same values as in the previous stage of the experiment. Then, the growth of culture was continued up to 47 days with the addition of the same wastewater portions. At such conditions the growth of MA occurred at approximately constant speed. After 47 days, the growth of A. platensis Bios (P) slowed down and reached a plateau, and a significant increase cannot be achieved without a periodic selection of the grown biomass from the culture medium. Apparently, at a biomass density of more than 1 g / l on dry matter, the culture reaches the maximum density at which illumination and nutrients are limited, and exametabolites accumulate that adversely affect cell growth (Figure 4).

Similar experiments with the periodic wastewater and nutrient medium addition were carried out with other strains of MA (Chlorella ellipsoidea, Gloeotila pulchra and Galdieria sulphuraria). The reaction of various MA to the periodic wastewater and nutrients addition was strain-specific. For example, chlorella unlike other strains in wastewater without the addition of nutrients in the first 12 days showed a significant increase in biomass, while the galdieria and gleotila did not give biomass increase, although their cells remained in a viable state (Figure 5).

Additions of wastewater on the 12th day of experiments caused a significant growth of chlorella cells until the maximum density of 0.8 g / l on dry matter was reached on the 20th day. The next addition of Tamiya medium (25% by volume) stabilized the growth of chlorella cells at a level of 0.6 g / L dry matter for a further 20 days. But the culture of Chlorella ellipsoidea has significant adhesive properties to the inner surface of glass flasks, which undoubtedly reduced the real values of the culture density. MA Gloeotila and Galdieria reacted differently to the wastewater and culture media addition. Gloeotila increased biomass by 5 times on the 47th day of growth, adapting to growth conditions in wastewater. For the extremophilic alga Galdieria, the growth conditions were unsuitable (Figure 5). Thus, for the effective cultivation of specific MA in wastewater, the search for specific key factors for their growth and development is needed.

### 5.4. Effectiveness of wastewater treatment using microalgae

Inorganic nitrogen and phosphorus are particularly difficult to remove from wastewater. A comparative analysis of the wastewater main chemical indicators before and after growing MA showed that *A.platensis Bios (P)* is capable of efficient growth in slightly diluted wastewater. However, a prerequisite for growth is the creation of alkaline conditions in a nutrient medium by
adding NaHCO3 in an amount of at least 6 g / l (Table 2). *A. platensis Bios (P)* cells completely consumed phosphorus from the wastewater in the form of orthophosphates and nitrogen in the form of ammonium and nitrates, the concentration of which decreased by 4-30, 58-73 and 24 times, respectively.

![Figure 5](image.png) **Figure 5.** Dynamics of the MA *Chlorella ellipsoidea*, *Gloeotila pulchra* and *Galdieria sulphuraria* biomass density. The legend indicates the initial and final dilution of wastewater. The cultivation of MA in flasks with a volume of 250 ml, the initial volume of the nutrient medium is 100 ml: ⭐ - the addition of 50 ml of wastewater for chlorella, the addition of 50 ml of the BG-11 medium for gleotila, the addition of 50 ml of the Allen medium for galdierium, ✡ – addition 34 ml ¼ Tamiya medium for chlorella.

In experiments with *Chlorella ellipsoidea*, due to the low cell growth rate at the beginning of the experiment and exhaustion of nutrients from wastewater, addition of Tamiya nutrient medium was carried out on the 27th day of the experiment with a potassium nitrate salt content of 5 g/l, which increased the nitrate content in the nutrient medium to 807.7 mg/l. At the end of the experiment (47 days), nitrates were assimilated by more than 70%, and phosphates and ammonium were assimilated completely by chlorella cells.

**Table 2.** Comparative analysis of the wastewater main chemical indicators before and after growing MA (growing time 47 days).

| Composition indicator, units | Source wastewater | Experimental results | Relative error, % |
|-----------------------------|------------------|---------------------|------------------|
|                             | *A. platensis Bios* | *A. platensis Bios* | *Chlorella ellipsoidea* |                   |
|                             | 2x-1,2x           | 3x-1,5x             | 3x-1,8x           |                   |
| COD (bichromate oxidation), mgO / l | 140                 | 140                 | 120               | 140               | 15                  |
| BOD5, mgO2 / l             | 85                | 68                 | 53               | 72               | 13                  |
| Potassium, mg / l          | 15                | <0,5               | 4                | 0,95             | 10                  |
| Nitrates, mg / l           | 2,4               | <0,5              | <0,1             | 235              | 20                  |
| Ammonium, mg / l           | 110               | 1,9               | 1,5              | 1,3              | 12                  |
Gloeotila pulchra adopted to growth conditions by 25 days; as a result, for 47 days of cultivation in diluted wastewater with the addition of BG-11 medium at intermediate stages, it assimilated about 67% of the main biogenic elements from wastewater. Galdieria sulphuraria did not adopt to cultivation on wastewater.

After growing MA in wastewater, BOD₃ values significantly decreased, which indicates a decrease in the readily soluble organics in the wastewater.

5.5. Efficiency of purification of the HTL aqueous phase using microalgae
As mentioned above, in the production of biofuels from MA by the hydrothermal liquefaction method, a significant amount of the aqueous phase is formed as a by-product that has no energy value. Therefore, the study of the biofuel production cycle including the cultivation of MA using aqueous phase is relevant.

The possibility of the nutrients utilization from an HTL aqueous phase (AP) obtained in the process of A. platensis 1/02-P cultivation has been studied. It was found that the aqueous phase contains a large amount of nutrients, which are necessary for the MA cultivation, as well as trace elements (58 compounds were identified). The nutrients content in AP is orders of magnitude higher than the standard nutrient media required for the MA cultivation. However, an aqueous phase is characterized by a toxic effect on MA. Growth inhibition is associated with the presence of a large number of toxic compounds (phenols, cyclic nitrogen compounds, heavy metals, ammonium ions). Intensive dilution of AP is necessary to prevent the toxic effect of growth inhibitors. It was done by using a mixture of aqueous phase and standard nutrient medium or only distilled water. MA strains that could grow for a long time (more than a month) on diluted HTL aqueous phase have been experimentally selected: Galdieria sulphuraria, Chlorella vulgaris and A. platensis Bios (P) (collection of Renewable Source Energy Laboratory at LMSU).

Growth analysis of candidate-strains (Galdieria sulphuraria, Chlorella vulgaris) in AP after the dilution with distilled water in 500 times showed that the most active growth is in case of Galdieria. Its growth in the first 25 days of cultivation exceeds that in the nutrient media. This result confirms the ability of Galdieria to mixotrophy. Chlorella had a steady growth also. However, it was still lower than in reference media. Consortia of A. platensis Bios with associate bacteria showed steady growth in 300-400 times diluted HTL aqueous solution. Cultivation of this consortia required the bicarbonate ions addition to create more favorable conditions for the growth and development of arthrospira. It allows partially recycling the by-product of bio-oil from MA (aqueous phase) [13].

6. Conclusion
The methods of MA cultivation in municipal wastewater and the aqueous phase after hydrothermal liquefaction of MA were experimentally selected. The effectiveness of wastewater and aqueous phase treatment were analyzed.

Based on experiments with seven candidate MA strains, two promising MA cultures (Arthrospira platensis Bios (P) and Chlorella ellipsoidea rsems Chl-el), which are capable to grow efficiently in wastewater of different dilutions for a long time, were selected.

It was shown that the consortium of A. platensis rsems Bios (P) with associative bacteria can grow efficiently in wastewater, diluted by 5 times and assimilate nutrients within 8 days. At higher dilutions, due to lack of nutrients, cell growth ceased for 7 days. The addition of 6 g/l NaHCO₃ to wastewater created more favorable conditions (pH>8.5; a sufficient concentration of HCO₃⁻ anions) for the growth of arthrospira. When the wastewater was diluted 1.2-1.5 times the addition of NaHCO₃ provided the effective growth of this MA consortium with associative bacteria for 47 days, as a result of which the concentration of nitrogen and phosphorus ions in the wastewater decreased to trace values. In addition, the amount of readily soluble organic matter (BOD₃) decreased from 85 to 68-53 mgO₂/l, which indicates the ability of the MA to switch to mixotrophic growth with the consumption of short-chain organics.

Chlorella ellipsoidea in wastewater without adding nutrients in the first 12 days showed a significant increase in biomass (biomass density increased by 3.5 times). The addition of wastewater
portion and ¼ Tamiya medium on the 12th day of the experiment, as in the case of arthrospira, led to a
significant growth of chlorella cells until their maximum density (0.8 g/l dry matter) was reached.

It was found that for more efficient wastewater utilization and treatment by MA growth, it is
necessary to select specific biomass growth key factors. The experiments duration (47 days) made it
possible to identify the adaptive abilities of some MA to wastewater conditions and to obtain an algo-
bacterial culture capable of "excessive" absorption of wastewater nutrients.

The aqueous phase (AP) after hydrothermal liquefaction of the MA biomass contains a wide
variety and a large concentration of nutrients and trace elements necessary for the MA cultivation.
Moreover, the aqueous phase can have a toxic effect on MA due to the high content of phenols, heavy
metals, etc., which requires, as in the case of wastewater, intensive AP dilution for MA cultivation.
MA strains that can grow for a long time in diluted HTL aqueous phase (Galdieria sulphuraria and
Arthrospira platensis Bios (P) from the collection of Renewable Source Energy Laboratory at LMSU) have been experimentally selected. The most active growth was demonstrated by the consortium A. platensis Bios (P) with heterotrophic associative bacteria, which grew well on
less diluted AP (300x–400x), and when sodium bicarbonate ions were added in an amount of 6 g / l,
their growth was enhanced due to more favorable conditions for the arthrospira growth. Galdieria sulphuraria also grew well for more than a month in the aqueous phase, which confirms
its ability to mixotrophy, but with a dilution of 500x.

The obtained results confirm that the technology based on wastewater treatment and aqueous phase
after HTL by MA is technically feasible and promising. It allows partial processing of a by-product of
the bio-oil production from MA by hydrothermal liquefaction technology.

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