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Waste-free technology of wastewater treatment to obtain microalgal biomass for biodiesel production

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ABSTRACT

Five axenic cultures of microalgae were isolated from the wastewater of Almaty city and identified as *Chlorella vulgaris* strain № 1, *Chlorella* sp. strain № 3, *Scenedesmus obliquus*, *Phormidium foveolarum* and *Lyngbya limnetica*. Among these strains, *C. vulgaris* strain № 1 was characterized by the maximum growth rate and the highest productivity. Mass cultivation of this strain in wastewater resulted in accumulation of 5×10^7 cells per ml in 16 days, and in the removal of ~95% of pollutants from water. Cells of *C. vulgaris* consisted of ~35% proteins, 29% carbohydrates, 30% lipids, and 6% ash, as calculated on a dry weight basis. The major fatty-acids of *C. vulgaris* were represented by palmitic, *cis*-7,10-hexadecenoic acid, linoleic, and α-linolenic acids. Culturing in wastewater decreased the unsaturation index of FAs. Thus, *C. vulgaris* cells are suitable for both waste water purification and accumulation of biomass for further biodiesel production.

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Introduction

The development of industry, growth of cities, and other human activities lead to the pollution and collection of wastes that are harmful for biosphere. One of the main problems of big cities is the pollution of natural waters with hazardous elements, which are present in domestic wastewater. Such pollutants cause eutrophication of natural ponds together with a decrease in oxygen concentration, which may finally lead to an increase in proportion of pathogenic organisms over natural non-pathogenic inhabitants [1]. To prevent this, multiple steps of wastewater treatment are undertaken, which include the use of heterotrophic organisms, mechanical and pneumatic aeration, ozonation and UV treatment [2]. The problem of finding an effective way to protect the environment, to remove the pollutants, and to increase the quality of life is emerging nowadays.

Modern biotechnological methods based on the ability of living organisms to accumulate and to degrade dangerous pollutants proved to be most effective, harmless, and profitable ways to solve ecological problems [3,4].

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Wastewaters are usually bioremediated with microalgae that have the ability to degrade harmful organic wastes (nitrogen/phosphate, organic carbons, pharmaceutical or textile dye compounds, heavy metals, *etc.*), produce oxygen, and accumulate biomass [5–9]. Until recently, such an approach had been applied with limitations, because of technological difficulties in conversion of the accumulated biomass. However, the technologies of algal biomass conversion into bio-oil or biofuels are now rapidly developing [5,6]. Therefore coupling of biofuel production system with wastewater treatment may significantly reduce the high cost of microalgal cultivation [8]. The conversion of biomass into biofuel sounds economically and environmentally advantageous, because such fuel is fully renewable. Thus, algal biotechnology can provide multiple contributions into bio-based economy: purification of wastewaters, oxygen enrichment, and biomass for biodiesel production [10,11].

Here we describe the biological technology of wastewater treatment with the use of microalgae, which implies cultivation on wastewaters and optimization of lipid accumulation and fatty acid (FA) composition for further production of biodiesel.

Experimental section

Determination, isolation and cultivation of axenic cultures of microalgae

The species of microalgae in water samples have been identified according to the following guides [12–14]. To eliminate bacteria, samples were plated on Petri dishes with Tamiya solid medium. Green colonies, which appeared after several weeks of incubation on light, were transferred on fresh agar medium with ampicillin at 100 μg ml^{-1} for suppression of bacterial growth. Isolated colonies were passed several times on a fresh nutrient medium. Their purity was confirmed by cultivation on meat peptone broth.

Collected samples of water were cultivated in flasks in Tamiya media in the light at 6×10^3–10×10^3 lux at 25–28 °C.

The number of cells was determined in Gorjaev's count chamber. The growth rate coefficients were determined according to the equation [12].

$$k = \frac{1}{t} \ln \frac{Nt}{N0}$$

where N_0 – the density of the initial culture; N_t – the density of the culture after a certain time of cultivation (t).

Study of physical and chemical properties of water

The important indicator of water quality in ponds is the value of BOD_5 (biological oxygen demand), which characterizes the level of water pollution. For its determination water samples were incubated in the dark at 20 °C during 5 days following determination of concentration of dissolved oxygen in water before and after cultivation of microalgae. Oxygen concentration was determined with a dissolved oxygen meter (Model YSI 5100; YSI Inc., Yellow Springs, OH, USA) [15]. Physical and chemical composition of wastewater determined according to the Lurie and Alekin [16,17].

Estimation of biochemical composition of biomass

Total protein content in biomass was determined according to Lowry. The content of carbohydrates was determined by the phenol-sulfur method [18]. Extraction and determination of total lipids from the preprocessed biomass was carried out according to Folsh [19].

Fatty acid composition of biomass

Fatty acid methyl esters (FAMEs) were prepared by transesterification of the stored materials in a mixture of methanol and acetyl chloride (9:1) for 60 min at 70 °C. Analysis of the resulting mixture of FAMEs and quantitative content of total lipids in terms of esterified fatty acids was performed with GC–MS Agilent 7890A gas–liquid chromatography system with the mass spectrometric detector Agilent 5975S (Agilent Technology Systems, Santa Clara, CA, USA). The 60-m capillary column DB-23 (∅ 0.25 mm; Fischer Scientific, Loughborough, UK) was filled with 50% cyanopropyl methylpolysiloxane. The details of fatty acid analysis have been described earlier [20].

Results and discussion

Sample collection

Modern technologies of wastewater treatment demand a search of the organisms that utilize pollutants most effectively. This is required for the design of stable remediation systems, which could ensure a steady process of biodegradation of all pollutants produced by human.

Wastewaters already contain a number of macro- and microscopic organisms adapted to these adverse conditions. Such organisms may be employed for bioremediation. We, therefore, collected water samples from wastewater treatment facilities of Almaty (water utility) in order to estimate the biological diversity of algal strains and their potential ability for bioremediation. As a result, 16 species of microalgae have been isolated, among which 56% species were attributed to green algae (Chlorophyta) 13% – to diatoms (Bacillariophyta), 4% – to Euglenophyta, and 27% – to Cyanophyta. Among green algae, the dominating species were *Chlorella*, *Scenedesmus*, and *Ankistrodesmus*. Euglenophyta was represented by only one species. Cyanobacteria were mainly represented by *Phormidium*, *Oscillatoria*, and *Lyngbya*.

Algal diversity in waste channels varies upon organic and mineral composition of water. Prevalence of one taxon of algae over another depends on a type of sewage and on the presence of toxicants, on the technological scheme of water cleaning, and on climatic conditions. Some sources point to representatives of Oscillatoriaceae as dominating species at the initial steps of water purification [21]. Nevertheless, it's hard to imagine the existence of a universal bioremediation system, which would fit to any sewage purification facility. It would be rather reasonable to create a system based on local properties of wastewaters and their inhabitants.

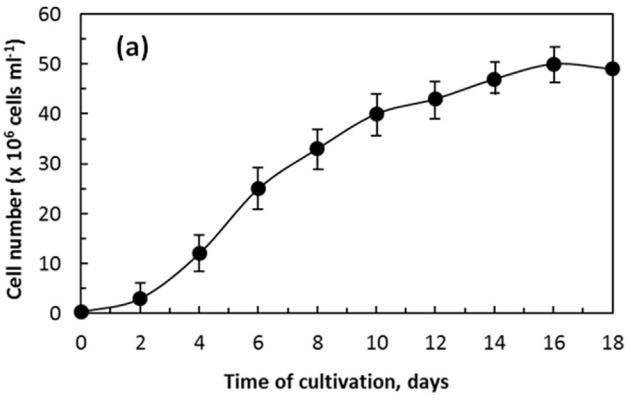
Isolation and cultivation of axenic microalgae cultures

As a result of numerous passages on various selective nutrient media, five axenic cultures of eukaryotic microalgae and cyanobacteria have been isolated. Green algae were identified as *Chlorella vulgaris* strain №1, *Chlorella* sp. strain №3, and *Scenedesmus obliquus*, whereas cyanobacteria were identified as *Phormidium foveolarum* and *Lyngbya limnetica*.

Microalgae appear in natural water systems in various combinations and quantities. Controlled mass cultivation, however, makes them suitable for sewage treatment. It is known that *Chlorella* species grow fast, accumulate considerable amounts of lipids, and may accumulate and decompose wastes [22–24]. In this regard, we investigated the strains of green algae, *C. vulgaris* strain №1, *Chlorella* sp. strain №3, and *S. obliquus*.

These strains were grown in Tamiya medium in laboratory photobioreactor during 14 days. The density of the initial culture was 1×10^6 cells ml^{-1}. During cultivation, the growth rate coefficients reached the following values: 0.24 – for *C. vulgaris* strain №1, 0.14 – for *Chlorella* sp. strain №3, and 0.19 – for *S. obliquus*. Thus, the highest growth rate was observed in case of *C. vulgaris* strain №1, which was selected for further studies.

We also tried to select an optimal proportion between sewage and a nutrient medium. For comparison to the selected *C. vulgaris* strain №1, two collection strains, *Chlorella pyrenoidosa* C-2m1 и *C. pyrenoidosa* C-2m2, which were previously characterized by high productivity accumulation of biomass and lipids [25], were included into the assays. These three strains were cultured in three different media: 1) wastewater; 2) wastewater + clean water at 1:1 ratio; 3) wastewater + Tamiya nutrient medium at 1:1 ratio. Cells were inoculated at the initial concentration of 1×10^6 cells per ml and incubated for 14 days at 6×10^3–10×10^3 lux light intensity and temperature 25–28 °C. All three cultures grew intensively in all variants, exhibiting a slight decrease when wastewater was diluted with clean water (Table 1), probably, due to a decrease in nutrient concentrations. These results show that wastewater itself is suitable for intensive cultivation of the examined algal strains.



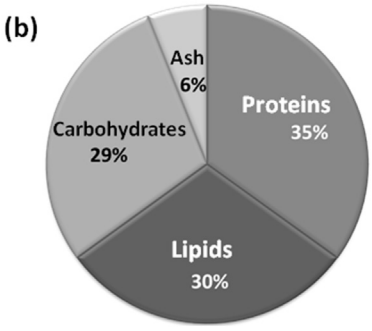


Fig. 1 – Cell growth curve (a) and biomass content (b) of *C. vulgaris* strain №1.

Table 1 – Growth coefficients of different *Chlorella* strains cultured in different media.

Algal strain	Culture medium		
	Wastewater	Wastewater/ clean water (1:1)	Wastewater/ Tamiya medium (1:1)
C. vulgaris strain №1	0.34 ± 0.02	0.31 ± 0.02	0.35 ± 0.03
C. pyrenoidosa C-2m1	0.25 ± 0.02	0.22 ± 0.02	0.26 ± 0.03
C. pyrenoidosa C-2m2	0.31 ± 0.02	0.28 ± 0.02	0.32 ± 0.02

The results represent an average of 3 independent experiments.

Table 2 – Physical and chemical parameters of wastewater before and after cultivation of *Chlorella* strains.

Parameter	Before	*C. vulgaris* strain №1		*C. pyrenoidosa* C-2m1		*C. pyrenoidosa* C-2m2		Measure unit
		After	Purification %	After	Purification %	After	Purification %	
pH	7.0	7.3		7.3		7.3		
Fresh weight	58	4	93	15	73	5	82	mg l^{-1}
Odor	5	0	100	1	80	0	100	points
BOD_5	57	9	97	8	86	2	96	mgO_2 l^{-1}
Oxidability	38	2	95	2	94	1	98	mgO_2 l^{-1}
Ammonia	9	0	100	0.5	95	0.3	97	mg l^{-1}
Nitrites	0.2	0	100	0.02	91	0	99	mg l^{-1}
Nitrates	0.8	0	100	0.02	97	0.02	97	mg l^{-1}
Phosphates	3.9	0	100	0.04	99	0.04	99	mg l^{-1}

BOD_5 – a test for Biochemical Oxygen Demand (BOD) or biological oxidation completed in 5 days. Oxidability represent the total amount of substances capable of being oxidized.

Water analysis after bioremediation with microalgae

The ability of all experimental strains for bioremediation was tested by the analysis of wastewater physico-chemical properties before and after algal cultivation (Table 2). Results show that all three tested strains of *Chlorella* are characterized by high integrated performance index of treatment: *C. pyrenoidosa* C-2m1 – 89%, *C. pyrenoidosa* C-2m2–96%, and *C. vulgaris* strain №1–98%. The latter strain displayed the maximal remediation activity: the concentration of organic contaminants (BOD_5) was greatly reduced, in average up to 97%, weight of substances in water – up to 93%, oxidability – 95%. An odor, ammonia, nitrites, nitrates, and phosphates have been completely eliminated from wastewater.

Estimation of growth parameters and biomass properties of C. vulgaris *strain* № 1

We determined the yield and chemical composition of biomass of *Chlorella* cultivated in the laboratory type photobioreactor in wastewater mixed with Tamiya medium at 1:1 ratio. Cells were grown for 18 days at 25 °C and continuous illumination of 6×10^3 lux. Initial number of cells was 0.3×10^6 cells ml^{-1} (Fig. 1a). During the experiment cell number increased and reached the maximum of 50×10^6 cells ml^{-1} on day 16th. Starting from day 17th of the experiment, a slight decrease in cell number was observed. Dry weight of biomass estimated at day 18th reached 8.5 $g\,l^{-1}$. That biomass consisted of 35% protein, 30% lipids, 29% carbohydrates, and 6% ash (Fig. 1b).

Fatty acid composition of C. vulgaris *strain* № 1

C. vulgaris strain № 1, grown in wastewater, is capable of accumulation of as much as 30% of total lipids in biomass as determined by crude lipid analysis. This makes the strain a suitable candidate for biofuel production. FA composition of these cells grown in laboratory conditions in Tamiya medium resembled that of other strains of *Chlorella* [26,27]. The major FAs were represented by palmitic (16:0), *cis*-7,10-hexadecenoic acid ($16{:}2\Delta^{7,10}$), linoleic ($18{:}2\Delta^{9,12}$), and α-linolenic ($18{:}3\Delta^{9,12,15}$) acids (Fig. 2). Lauric (12:0), myristic (14:0), and stearic (18:0) acids were present at low amounts (Table 3).

According to modern knowledge of algal species with the highest productivity of fatty acids relevant to transesterification reactions [28], *C. vulgaris* strain № 1 may be considered as a prospective candidate for biodiesel production. The standard FA composition (palmitic, linoleic, linolenic acids), however, may be improved to increase the yield of saturated fatty acids that are ideal substrates for biodiesel production [26,28]. One of the ways to adjust the FA composition towards saturated species is culturing in mixotrophic condition, for example, in Tamiya medium in the presence of glucose [27]. We cultured *C. vulgaris* strain № 1 in wastewater and observed the following changes in FA composition: 1) an increase in saturated FAs (16:0, 18:0); 2) a dramatic decrease in

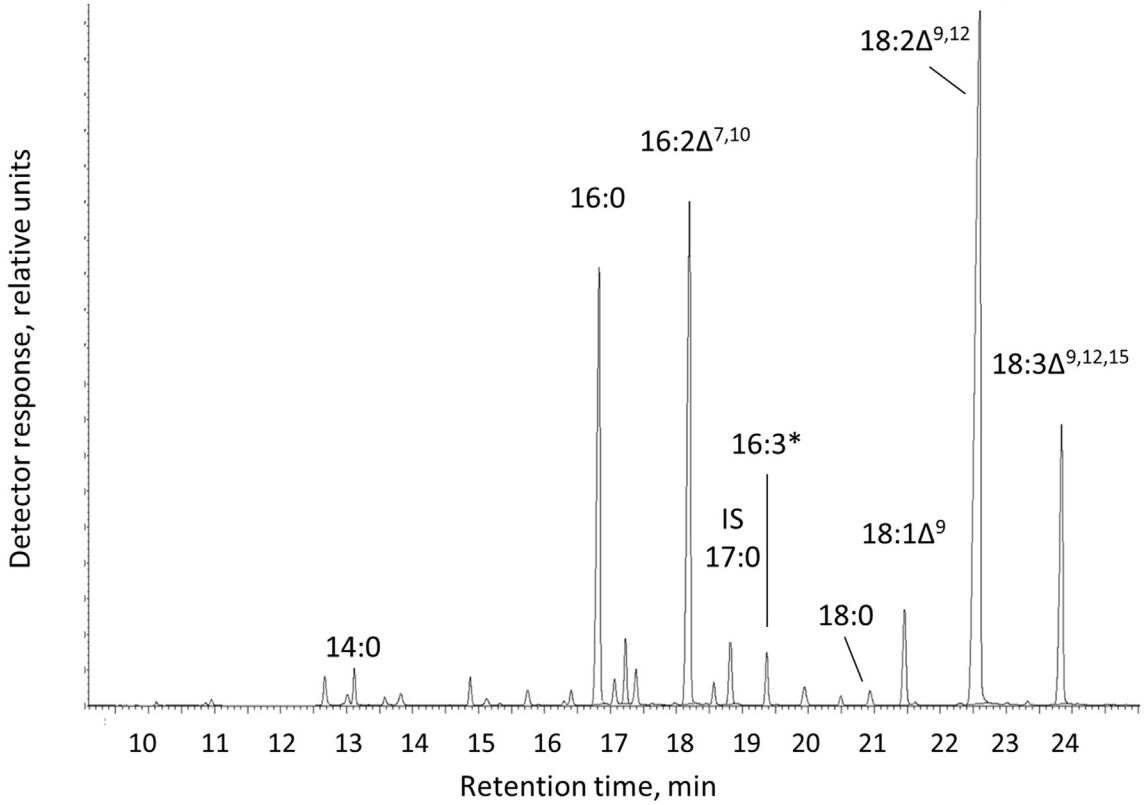


Fig. 2 – GC separation of fatty acids of *C. vulgaris* strain № 1. Margarinic acid (17:0) was used as the internal standard (IS). *The positions of double bonds were not precisely determined.

Table 3 – Fatty acid composition of *C. vulgaris* strain № 1 stores in Tamiya medium and grown in wastewater.

Fatty acid	Mass %	
	Tamiya medium	Wastewater
12:0	0.2	0.5
14:0	1.3	1.0
16:0	17.8	25.0
$16:1\Delta^{7}$	2.2	2.0
$16:1\Delta^{9}$	1.4	1.5
$16:2\Delta^{7,10}$	21.0	6.2
$16:2\Delta^{9,12}$	0.8	0.6
$16:3\Delta^{4,7,10}$[a]	1.7	0.5
18:0	0.5	6.6
$18:1\Delta^{9}$	3.3	8.0
$18:1\Delta^{11}$	0.1	0.1
$18:2\Delta^{9,12}$	39.7	46
$18:3\Delta^{9,12,15}$	10.0	2.0
UI, rel units	1.65	1.34
Lipid content, mg/g DW	85	290
Saturated FAs	20	33
Monoenoic FAs, mass %	7	12
Dienoic FAs, mass %	62	53
Trienoic FAs, mass %	11	3

Cells were grown in laboratory photobioreactor for 16 days in Tamiya medium or in wastewater. Before sampling for FA analysis cells were harvested by centrifugation and washed with distilled water 3–4 times. FAs – fatty acids; UI – unsaturation index. Major FAs are shaded.

[a] The positions of double bonds were not precisely determined.

$16:2\Delta^{7,10}$ and $18:3\Delta^{9,12,15}$; 3) a subsequent decrease in unsaturation index from 1.65 to 1.34 (Table 3). Thus, culturing in wastewaters leads to a decrease in unsaturation index, and makes FA composition of algal biomass more suitable for the production of biodiesel.

Conclusions

In total, sixteen species of microalgae and cyanobacteria have been determined in wastewaters of water purification facilities of Almaty, Kazakhstan. Among them, the strain of green algae, *C. vulgaris* strain № 1, was chosen as the candidate for water treatment and biomass production. The strain could successfully purify wastewater and accumulate up to 30% of lipids in the biomass. Analysis of fatty acid composition of total lipids revealed that the major fatty acids may be suitable for biodiesel production. Culturing in wastewater decreases the unsaturation index of FAs. Thus, these newly isolated fast growing cells of *C. vulgaris* strain № 1 are suitable for both waste water purification and accumulation of biomass for further biodiesel production.

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