Biogas production from biomass of microalgae Chlorella vulgaris in the presence of benzothiophene sulfone

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Abstract. The possibility of methanogenesis of microalgae Chlorella vulgaris biomass under the action of natural and artificially created anaerobic consortia in the presence of sulfur-containing xenobiotic benzothiophene sulfone demonstrated. It has been shown that, despite a slight decrease in the concentration of intracellular ATP in the used biocatalysts, the process of methanogenesis proceeded quite effectively. It has been established that the components of hydrolyzate of microalgae Chlorella vulgaris biomass, remaining after the extraction of lipids, are excellent substrate for the production of biogas.

1. Introduction
Microalgae biomass has long established itself as a promising substrate for the production of various products under the influence of microbial biocatalysts [1-5]. Its use was also effective as a co-substrate (an additional source of proteins and carbon) in the processes of biocatalytic decomposition of xenobiotics [6].

Currently, the possibility of biocatalytic utilization of sulfur compounds using aerobic bacterial cells, in particular, obtained during the desulphurisation of petroleum products, has been shown. The research in the field of alternative technologies desulfurization during the last 30 years was mainly focused in the development of biotechnological strategies to remove sulfur via a serial of reactions realized under aerobic conditions, known as 4S path-way [7-8]. This path was first described for gram-positive bacteria Rhodococcus erythropolis IGTS8 [9], but later it was found in other bacteria [10-11]. This multistage process provides a biocatalytic transformation of heterocyclic sulfur-containing compounds dibenzothiophene through a 4-step sequential education dibenzothiophene-sulfoxide (catalyst - monooxygenase DszC), dibenzothiophene sulphone (catalyst - monooxygenase DszC) and 2-hydroxydiphenyl-sulfinate (catalyst - monooxygenase DszA) 2-hydroxybiphenyl and sulfite (the catalyst - desulphenasa DszB) [9]. Then sulfite is assimilating by its oxidation to sulfate under the action of sulfite oxidoreductase [12]. In addition, the reduced form of flavin mononucleotide is consumed at three stages of oxidation. Its presence in the medium is ensured by the functioning of flavin reductase (DszD) [13].

The main disadvantages of the 4S path-way are the duration of its implementation even at the stage of transition to dibenzothiophensulfone (up to 1 month) and low yields of each intermediate product due to the inhibitory effect of sulfur-containing compounds on the catalytic activity of cells. It is known that the formation and accumulation of sulfones leads to inhibition of whole biocatalytic oxidation of sulfur-containing compounds [14]. Genetic modification and immobilization of cells
allow intensifying 4S path-way, however the optimal solution is still not found. There are a few studies about the effect of such compounds on anaerobic cells metabolism.

Biocatalytic consortia containing sulfate-reducing bacterial strains that can utilize sulfur compounds under anaerobic conditions and its transformation to the same final product as in hydroconversion - to hydrogen sulfide is known [15]. However, such processes are characterized by long time-consuming costs. The speed of the process significantly slows down as hydrogen sulfide accumulates, that imposes restrictions on the possibility of increasing the process speed by increasing biocatalyst concentration.

There are studies describing the effect of other sulfur-containing xenobiotics, most often dimethyl sulfoxide, on the metabolism of anaerobic cultures [16]. However, specially allocated or adapted anaerobic bacterial cells used at such studies.

As a promising and original solution associated with environmentally safely destruction of sulfones - waste of oxidative desulfurization of petroleum products, it would be possible to consider their biotransformation combined with the methanogenesis processes. However, no data have been presented about the effect of the presence of sulfur-containing xenobiotics (sulfones) at the metabolism of producers during methanogenesis.

So, the aim of this work was to assess the effect of sulfones presence at methanogenesis efficiency of Chlorella vulgaris microalgae biomass under action of natural and artificially created anaerobic consortia.

2. Materials and methods

2.1. Microorganisms

Green microalgal cells Chlorella vulgaris [Beier.] rsemsu Chv-20/11-II from IBCP RAS collections. Wastewater from Ostankino milk processing plant “OOO Ostankinsky Molochny Kombinat” (Moscow, Russia) was used to accumulate C. vulgaris biomass. The cultivation was performed in Erlenmeyer flasks (750 mL) containing 100 mL of cultivation medium. The cells were harvested after cultivation using Avanti J 25 centrifuge (Beckman Coulter, USA) at 8,000 rpm for 10 min.

Anaerobic sludge sample formed using the Bogatyr biopreparation (Moscow, Russia). The dry matter (56.4±2.6 g/L), ash content (38.7±1.8 %), and volatile suspended solids (VSS) (34.6±1.6 g/L) were determined according to the published procedure [17-18]. The both acidogenic (1560±30 mg COD/g VSS/d) and methanogenic (80±1 mg COD/g VSS/d) activity of the anaerobic sludge samples were evaluated according to the previously described techniques [17-18]. Nutrition media based on glucose and acetate, respectively, were used in these experiments.

The bacterial strains Clostridium acetobutylicum B1787 and Desulfovibrio vulgaris. B4053 were obtained from the Russian National Collection of Industrial Microorganisms (www.genetika.ru) for introduction into the methane tank in addition to the anaerobic sludge. The C. acetobutylicum strain B1787 was cultivated in the following medium (g/L): glucose – 20; triptone – 10; yeast extract – 5 (pH 6.8). The D. vulgaris. B4053 was cultivated in the Postgate medium [19]. Cells were cultivated under anaerobic conditions at 30°C for 1 week. Cultivation of C. acetobutylicum and D. vulgaris cells was performed under anaerobic conditions in an argon atmosphere at 37°C for 20–24 h and 48 h, respectively.

2.2. Methods.

2.2.1. Pretreatment and enzymatic hydrolysis of Chlorella biomass and lipids extraction

Pretreatment and enzymatic hydrolysis of Chlorella biomass for its microbial conversion into various products were described in previous work [20]. Biochemical composition of Chlorella biomass was done using standard methods described previously [1]. Extraction of lipids from biomass was carried out by the method describe Araujo et al. [21].
2.2.2. Anaerobic fermentation

The initial inoculum concentration in batch reactors was 10% (v/v) for suspended anaerobic sludge. The quantity of the artificially immobilized anaerobic sludge introduced into the medium was such as to ensure similar concentrations of sludge biomass in the liquid phase. The anaerobic incubation was carried out at 35°C in all the experiments.

To study the bioconversion of all the substrates to methane the 0.1 M phosphate buffer (pH 7.2) (55 mL) loaded into hermetically sealable vials (“anaerobic reactors”, 120 mL). The experiments were performed in triplicate.

A control experiment similar to that described above as usual was concurrently conducted to account for the biogas formation due to the possible lysis of the microbial inoculum [17]. The methane content in the biogas in the experimental control batches was subtracted from that obtained in the corresponding test batches to calculate the methanogenesis efficiency.

2.2.3. Accumulation of biogas and determination of its content.

After 2-5 days we measured the total pressure and biogas concentration in the gas phase of each reactor. Gas measurements were repeated until constant methane content was reached in the gas phase of the reactor.

The content of hydrogen, methane, and carbon dioxide in the gas phase was measured with an LKhM 8 MD chromatograph (Russia) Model 3 with a katharometer (the carrier gas was argon with 20 mL/min flow rate). 2 m long columns were filled with Q porapak [17]. Oven temperature was maintained at 50°C, the retention times of hydrogen, methane, and carbon dioxide were 43, 67, and 82 s, respectively.

2.2.4. Determination of adenosine triphosphate (ATP) concentration

The concentration of intracellular ATP in immobilized cells was determined by the bioluminescent luciferin–luciferase method using cell-containing granules immediately after thawing. For this purpose, granules were weighed (0.15 ± 0.05 g), transferred to dimethyl sulfoxide (1 mL) and allowed to stand at 25°C for 2 h to extract intracellular ATP [22].

2.2.5. Calculations

The efficiency of methanogenesis (E) was calculated using the equation:

\[ E = \left( \frac{Q}{Q_{\text{max}}} \right) \times 100 \text{ (%)}; \]

where \( Q \) (mL) is the volume of methane produced in a reactor with a test sample, (equation 2):

\[ Q = \left( \frac{C \times 100 \times P_{\text{tot}} T_0 V_{\text{gph}}}{(T_1 P_0)} \right) \times 1000, \]

where \( C \) is the methane content in the gas phase (%); \( V_{\text{gph}} \) is the volume of the gas phase in the reactor (L); \( T_0 \) is the temperature under normal conditions, 273 K; \( T_1 \) is the operating temperature in the reactor (K); \( P_0 \) is the pressure under normal conditions, 1 atm; and \( P_{\text{tot}} \) is the total pressure in the reactor (atm),

and \( Q_{\text{max}} \) (mL) is the theoretical maximum volume of CH\(_4\) (equation 3):

\[ Q_{\text{max}} = (C_1 V_{\text{ph}}) \times 0.35 \times 1000 \text{ (mL)}, \]

where \( C_1 \) is the initial concentration of the organic substances in the used waste sample, (g COD\(_{\text{tot}}\)/L); \( V_{\text{ph}} \) is the volume of the liquid phase in the reactor (L); and 0.35 is the volume of methane produced from 1 g COD at 273 K (L).

The specific productivity of methanogenesis was calculated as the volume of methane formed from 1.0 g of COD under the influence of anaerobic microbial inoculum introduced into the reactor (mL CH\(_4\)/g COD).

The data were shown as means of at least three independent experiments ± standard deviation (± SD). Statistical analysis was realized using SigmaPlot 12.5 (ver. 12.5, Systat Software Inc., San Jose, CA, USA). The significant (p≤0.05) differences between obtained results were estimated by one-way analysis of variance (ANOVA).

3. Results and Discussion
The following experiment was carried out to assess the feasibility of methanogenesis under the action of anaerobic consortia in the presence of sulfones. Benzothiophenesulfone (BTO₂) at a concentration of 0.15 mM was used as the sulfur-containing compound. This concentration was chosen based on the analysis of known data on the decomposition of sulfur-containing xenobiotics by anaerobic bacterial cells. Various combinations of anaerobic sludge, sulfate-reducing bacteria *D. vulgaris* [19] and anaerobic producers of hydrogen, ethanol, cellulases *C. acetobutylicum* were used as anaerobic biocatalysts [1, 3, 5]. These cultures were introduced into artificially created anaerobic consortia to intensify the process of methanogenesis.

It is known that the processes of xenobiotics decomposition proceed much more efficiently in the presence of additional sources carbohydrates. [23]. Glucose is traditionally used as such carbon source. However, the transition to a cheaper source of raw materials could contribute to the implementation of such developments. It was decided to use hydrolysates of *C. vulgaris* biomass, containing polysaccharides and proteins, as an additional carbon source. Such substrate is a waste of lipid extraction from *C. vulgaris* biomass obtained during wastewater treatment. [3, 4, 24]. As a control similar process was performed using glucose as an additional source of carbon (Figure 1).

![Figure 1](image.png)

**Figure 1.** Basic characteristics of the methanogenesis with glucose (white bars) or hydrolysates of *C. vulgaris* biomass (grey bars) and different biocatalysts (1 - anaerobic sludge, 2 - anaerobic sludge + *C. acetobutylicum* cells, 3 - anaerobic sludge + *D. vulgaris* cells, 4 - anaerobic sludge + *C. acetobutylicum* cells + *D. vulgaris* cells) in the presence of 0.15 mM BTO₂

The transition from glucose use to hydrolyzed biomass of microalgae *C. vulgaris* as a carbon source does not reduce the main indicators of methanogenesis, carried out under the action of anaerobic consortia in the presence of sulfones. Obviously it is necessary to create anaerobic consortia, which contain additional cells, allowing intensifying the different stages of a complex biotechnological process with a lot of intermediate enzymatic reactions for the most effective methanogenesis in the presence of sulfones.

Evaluation of the energy status (viability) of anaerobic consortia components (Table 1), indicate that, in general, the concentration of intracellular ATP decreases in all cells used in the process in the presence of BTO₂. The degree of BTO₂ influence at various anaerobic cells is varied. The maximum negative effect from sulfones presence was noted for cells of sulfate-reducing bacteria *D. vulgaris*. At the same time, the presence of BTO₂ at a concentration of 0.15 mM practically did not negatively affect at viability of *C. acetobutylicum* cell (Table 1).
The observed decrease of the specific concentration of intracellular ATP is not a limiting factor for considering the process with the participation of anaerobes as a promising and further study and implementation of methanogenesis in the presence of sulfones, possibly simultaneously with their biological destruction. The decrease in the intracellular concentration of ATP even of anaerobes in the experiment conditions, particularly, may be due to including insufficient concentration of nutrients in the media, because in the control (medium without BTO2) also observed a decrease in the intracellular concentration of ATP compared with the initial level of this parameter.

### Table 1. The concentration of intracellular ATP (×10^{12}, mol/g biomass) in anaerobic bacterial cells used in methanogenesis of biomass C. vulgaris hydrolysate in the presence of BTO2.

| Biocatalyst        | Initial | At the end of the process | % reduction from initial |
|--------------------|---------|--------------------------|-------------------------|
|                    |         | without BTO2 | with BTO2 | without BTO2 | with BTO2 |
| Anaerobic sludge   | 50.2±2.1| 15.4±0.6      | 1.5±0.1   | 69.3         | 97.0      |
| C. acetobutylicum   | 66.4±3.1| 4.2±0.1       | 1.0±0.1   | 93.7         | 94.0      |
| D. vulgaris        | 27.3±0.3| 12.3±0.9      | 2.0±0.5   | 93.6         | 94.0      |

However, if it is necessary to produce a biotechnological process in the presence of sulfur-containing xenobiotics, then may be it is appropriate to use artificially immobilized microbial cells for reduce negative influence of xenobiotics on anaerobic cells. Such approach has long been positively proven itself in different biocatalytic transformation processes of renewable raw materials, in particular, waste [1-5, 22-23, 25].

### 4. Conclusion

The possibility of methanogenesis process in the presence of sulfur-containing xenobiotic BTO2 was demonstrated under the action of natural and artificially created anaerobic consortia. It has been shown that, despite a slight decrease in the concentration of intracellular ATP in the used biocatalysts, in general, the methanogenesis process proceeded quite effectively. It has been established that the components of microalgae *Chlorella vulgaris* biomass hydrolysate, remaining after the extraction of lipids, are an excellent substrate for the production of biogas.

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