Optimization potential of anaerobic biocatalytic processes using intracellular ATP concentration as the main criterion for decision making

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Abstract. The effective use of such an analytical indicator as the concentration of intracellular adenosine triphosphate (ATP) in the process of anaerobic transformation of sulfones was shown. The bioluminescent method for determining ATP evaluates to quickly evaluate the state of biocatalysts in such processes, to select the conditions for effective conducted of biotechnological processes and the functioning of cells, as well as media compositions used in the conversion of sulfur-containing compounds.

1. Introduction

Today, in connection with the presence of global environmental problems, research related to the environmentally friendly development of the chemical industry, the development of the best and most feasible technologies, in particular those that could improve the quality of hydrocarbon desulfurization, is relevant [1]. Low sulfur petrochemical feedstocks with sulfur content less than 10 ppm are promising for inclusion in the green schemes for the production of various compounds and materials [2-3]. A promising approach to sulfur recovery from oil content is currently the process of oxidative desulfurization of hydrocarbons [4]. During its implementation, after the stage of chemical oxidation of the feedstock, the oxidized forms of sulfur in the form of polar compounds, including sulfones, are separated from the feedstock by different methods, most often by extraction using different solvents [5]. The question of further rational use of sulfones is still open. In this case sulfones, obviously, can be considered as a source of raw materials for the production of sulfur, necessary for the production of various fertilizers, as well as new polymer materials and composites [6-8]. Today the volumes of “recovered” sulfur produced during the processing of oil and gas are approximately equal and in total make up more than 50% [9]. Organic hydrocarbon skeleton is also valuable in organic sulfones.

Recently, taking into account all mentioned factors, the process of biotransformation of sulfones into commercially valuable products (an inorganic sulfide and biogas) under the action of an anaerobic consortium artificially immobilized in cryogel poly(vinyl alcohol) (PVA) was proposed and successfully tested with model media [5]. It was revealed that the following factors have a significant influence on the productivity of biocatalysts and the implementation of this process: the initial concentration and chemical structure of the substrate (sulfone), and the nature of the solvent, in which organic sulfone is initially placed in the bioreactor. It should be noted that, depending on
the chemical structure, the oxidized forms of sulfur in organic compounds, as well as solvents, can have an inhibitory effect on biocatalysts, but at the same time, under optimal conditions, complete biotransformation of sulfones into target products is possible [5]. During the implementation of such anaerobic processes, the duration of which is usually more than 24 hours [10], it is important to conduct a periodic assessment of the state of the biocatalyst in the reactor in order to, if necessary, timely adjust the process parameters and maximize its final productivity for the target products. Such monitoring is also necessary at the end of each working cycle, which allows us to draw conclusions about the possible reuse of biocatalysts.

The concentration of intracellular adenosine triphosphate (ATP) is a universal analytical signal that allows us to assess the viability and metabolic activity of microorganism cells [11-12]. This indicator allows to monitor the inhibitory effect of system components on the target activity of biocatalysts. Determining the value of this parameter to control the viability of biocatalysts during their storage and use in numerous aerobic processes in general has shown its high efficiency [10, 11, 13]. The concentration of intracellular ATP can also be proposed as a universal indicator of the metabolic activity of living cells during the development of methods for anaerobic biological destruction of organic sulfur-containing compounds. Since, when choosing a method for determining this parameter, one should take into account the fact that the ATP concentration in anaerobic cells is much lower as compared to aerobic cells. So, in this study, the ATP analysis was performed using the luciferin-luciferase bioluminescent method, which is characterized by high sensitivity and specificity of determination [14]. It is important to note other advantages of this method: possible usage of portable devices, simplicity of sample preparation, efficiency of measurements [15]. For now the method has been successfully adapted for the analysis of biocatalytic systems based on both free and immobilized cells of various microorganisms. By using this method it was repeatedly confirmed that the immobilization of cells in PVA cryogel makes them more tolerant to the presence of toxicants. Immobilized biocatalysts can be successfully used for biotransformation of renewable waste materials into various commercially significant products (organic acids, biofuels, etc) [16-18].

The aim of this work was to study the possible application of bioluminescent method for determining the concentration of ATP in the cells of an anaerobic consortium during the development and monitoring of the biotransformation of organic sulfones. As the organic sulfone, dibenzothiophenesulfone (DBTO2) was chosen, which is one of the most commonly found components in various hydrocarbon feedstocks, for the purification of which from sulfur can be realized by the method of oxidative desulfurization with the separation of sulfones by extraction [19-20].

2. Materials and methods

2.1. Microorganisms

Anaerobic sludge sample was formed using the Bogaty biopreparation (Moscow, Russia). Its characteristics were described previously [21].

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 as additives 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 [14]. 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. Anaerobic fermentation

An artificial anaerobic consortium containing (wt.%): anaerobic sludge - 80%, Clostridium acetobutylicum B1787 - 10% and Desulfovibrio vulgaris. B4053 - 10% was used as a biocatalyst in
the study. Its composition was previously optimized [2, 5, 22]. Each culture was separately immobilized in PVA cryogel [10, 17-18]. The granules of each biocatalyst were mixed in the required proportions and the resulting consortium was used in experiments. When assessing the energetic state of each type of cells in the consortium, the granules were selected by color and analyzed (Figure 1).

**Figure 1.** The used anaerobic consortia was a mixture of granules with different cells immobilized in PVA cryogel:

A – anaerobic sludge,
B – *C. acetobutylicum* B1787,
C – *D. vulgaris* B4053.

The initial inoculum concentration in batch reactors was 10% (v/v) for immobilized biomass as part of the sum biocatalyst. The anaerobic incubation was carried out at 32°C in all the experiments. Solution of 0.1 M phosphate buffer with the addition of 1 g/l glucose was used as a nutrient medium.

2.2.2. **Determination of adenosine triphosphate (ATP) and sulfide concentration**

The concentration of intracellular ATP in immobilized cells was determined by the bioluminescent luciferin–luciferase method using cell-containing granules immediately after sampling. 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 [21, 22].

Sulfide ions in liquid phase was monitored spectrophotometrically at 660 nm using Shimadzu UV-1202 (Japan) [23].

2.2.3. **Calculations**

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**

To assess the feasibility of using the bioluminescent method for determining intracellular ATP in the process of anaerobic transformation of sulfones into inorganic sulfide, three model parts of experimental study were carried out to monitor the metabolic activity of the artificial anaerobic consortia.

3.1 **The choice of the initial concentration of sulfone for transformation under the action of anaerobic consortium**

Several initial DBTO₂ concentrations in the range from 0.5 mM to 2.5 mM initially dissolved in ethanol were tested in an anaerobic reactor (methane tank) for their effective conversion to sulfide. This range of sulfone concentrations was selected from the following considerations: the possibility of efficient transformation of DBTO₂ at a concentration of 0.15-0.45 mM under the influence of anaerobic sludge has already been established [5]. The choice of the upper limit of the tested concentration range (2.5 mM) was determined by the analysis of possible initial sulfur concentrations in real raw materials (vacuum gas oil ~ 14,800 ppm) [25] and the final concentrations
of oxidized forms of sulfur-containing compounds in ethanol extracts, taking into account their dilution with the extractant.

DBTO$_2$ at the indicated concentrations was introduced into a nutrient medium with an anaerobic biocatalyst, the mixture was exposed for 24 hours at 35 °C, and then the change in the concentration of intracellular ATP in the cells of the consortium was evaluated (Table 1).

Table 1. ATP concentration ($\times 10^{-12}$ mol/g biomass) of cells composing anaerobic artificial immobilized consortium (biocatalyst) in the process of DBTO$_2$ transformation (24h).

| DBTO$_2$ concentration (mM) | Anaerobic sludge | C. acetobutilycum | D. vulgaris |
|-----------------------------|-------------------|-------------------|-------------|
| 0                           | 57.2±2.2          | 66.4±3.1          | 17.3±0.8    |
| 0.5                         | 56.2±2.1          | 64.2±2.6          | 15.8±0.8    |
| 1.0                         | 54.7±2.1          | 63.4±2.4          | 18.1±0.8    |
| 1.5                         | 56.1±2.9          | 61.1±2.1          | 16.7±0.8    |
| 2.0                         | 53.8±2.7          | 54.3±2.2          | 16.8±0.8    |
| 2.5                         | 54.1±2.8          | 51.3±2.4          | 17.7±0.8    |

Analysis of the results allows us to conclude that the most resistant to the toxic effects of DBTO$_2$ are immobilized cells D. vulgaris. At a concentration of 2.5 mM DBTO$_2$, all cells as components of the immobilized anaerobic consortium did not show a significant decrease in intracellular ATP, therefore, for this sulfone, it is acceptable as the initial concentration. It is obvious that in the transformation of other sulfones, as well as a mixture of oxidized forms of sulfur-containing compounds in the case of transformation of real raw materials, to select the initial concentration of the substrate or the degree of dilution, it is possible to conduct a similar experiment based on the analysis of changes in the concentration of ATP in cells.

3.2. The choice of extractant for the extraction of oxidized forms of sulfur-containing compounds from the organic phase

A number of solvents that mix well with water and are characterized by a different toxicity class have been tested as candidate extractants, the use of which is potentially possible for extraction of oxidized forms of organic sulfur-containing compounds (sulfones and sulfoxides) from the organic phase. To determine the effect of these potential extractants on the viability and metabolic activity of the cells inside the artificial anaerobic immobilized consortium, the consortium was exposed to presence of the studied substances (50 mL/L) (Table 2).

A slight positive change in the level of ATP in anaerobic sludge was observed as compared with other extractants in the presence of ethyl and isopropyl alcohols (Table 2). This was because of anaerobic sludge cells can use these substances as a substrate, as it is well known [25]. In the presence of water, changes in the concentration of intracellular ATP were within the measurement errors provided by the method. The concentration of intracellular ATP changed most significantly when acetonitrile, dimethylformamide and N-methylpyrrolidone were added to the media with anaerobic consortium.

Thus, it was found that analysis of changes in the concentration of intracellular ATP allows to make conclusions about the possibility and advisability of using one or another extractant for the implementation of the developed biocatalytic processes with the usage of anaerobic consortia, including those based on anaerobic sludge, to assess the energy status of cells, including immobilized cells used as biocatalysts for the conversion of organic sulfones to inorganic sulfide.
Table 2. The concentration of intracellular ATP (× 10^{-12} mol/g biomass) in the immobilized cells inside the artificially created anaerobic consortium, after their exposure for 24 hours in media with different extractants.

| Extractant         | Immobilized cells inside consortia | Anaerobic sludge | C. acetobutylcum | D. vulgaris |
|--------------------|-----------------------------------|------------------|------------------|-------------|
| Acetonitrile       | 35.8±1.6                          | 32.5±1.4         | 9.2±0.2          |
| N-methylpyrrolidone| 21.2±1.2                          | 27.8±1.9         | 14.4±0.7         |
| Dimethylformamide  | 32.4±2.1                          | 25.4±1.1         | 11.8±0.4         |
| Water              | 55.4±2.3                          | 62.3±2.9         | 16.3±0.5         |
| Isopropanol        | 57.4±2.5                          | 64.9±2.0         | 15.8±0.6         |
| Ethanol            | 64.3±2.9                          | 64.7±2.8         | 16.2±0.5         |
| Control\(^a\)      | 56.1±2.3                          | 65.6±3.0         | 17.3±0.8         |

\(^a\) Control was based on 0.1 M potassium phosphate buffer (pH 7.2).

In this case, during the selection of extractants for the extraction of sulfones from the initial sulfur-containing media, from the point of view of the minimum inhibitory effect on the activity of biocatalysts, the use of ethanol, isopropanol or water turned out to be the most appropriate (Table 2).

3.3. Monitoring the state of the biocatalyst in the process of transformation of oxidized forms of sulfur-containing compounds

Initially dissolved in ethanol, DBTO\(_2\) was introduced into a nutrient medium with an anaerobic consortium at a concentration of 2.5 mM, and then the concentration of intracellular ATP was monitored every 24 hours.

Under experimental conditions, the complete transformation of DBTO\(_2\) into inorganic sulfide was achieved in 12 days. The level of ATP concentration in the cells as parts of sum anaerobic biocatalyst gradually decreased. At the end of the process, the residual ATP level in anaerobic sludge cells was 62 ± 3% of the initial level, in C. acetobutylcum and D. vulgaris cells it was 38.2 ± 1.9% and 70.1 ± 3.5% respectively. In this regard, it was decided to activate biocatalysts by exposing them for 24 hours in a nutrient medium of optimal composition. After activation, the concentration of ATP in cells increased to 90-95% of the initial level. After activation, the artificial biocatalyst was reused for biotransformation of the oxidized forms of sulfur-containing organic compounds. It was found that the 24-hour activation of the biocatalyst ensured its reuse in no less than 5 cycles of 12-15 days each, in which a 100% reduction of the oxidized form of sulfur in the form of 2.5 mM DBTO\(_2\) to sulfide was observed.

Thus, monitoring the concentration of intracellular ATP allows to quickly respond to observed changes in the energy state of cells and the metabolic activity of the anaerobic biocatalyst used, as well as to evaluate the possible long-term and effective use of the biocatalyst under periodic conditions.

4. Conclusion

It was shown that the bioluminescent method for determining the concentration of intracellular ATP, as a way to control the energy state and metabolic activity of biocatalysts, can be effectively used in the development and implementation of new anaerobic biocatalytic processes.

The following parameters were selected for control within the process of anaerobic transformation of oxidized forms of sulfur-containing compounds using this method: allowable
initial substrate (DBTO₂) concentration, extractant being non-toxic for the biocatalyst and suitable for extracting oxidized sulfur-containing compound from the organic phase for its transferring to the anaerobic reactor, the favorable mode of the biocatalyst use under periodic conditions (activation within 24 hours after each 12-15 days-cycle).

The results of the study seem to be useful to researchers involved in solving complex problems in the direction of the transition of existing production cycles to green chemical technologies based on the use of various biocatalysts in the form of whole cells.

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References
[1] Barthe P, Chaugny M, Roudier S and Delgado Sancho L 2015 Best available techniques (BAT) reference document for the refining of mineral oil and gas. European Commission. 754 p
[2] Maslova O, Senko O, Stepanov N and Efremenko E 2019 Perspective approaches with the use of biocatalysts for improving the processes of polyaspartic acid production from oil benzene fraction after oxidative desulfurization. IOP Conf Ser Mater Sci Eng 525(1) 012037
[3] Rana M S 2017 Heavy oil refining processes and petrochemicals: A role of catalysis. RAPSci 2(1) 555-580
[4] Koseoglu O R, Bourane A and Kressmann S 2018 U.S. Patent No. 10,087,377. Washington, DC: U.S. Patent and Trademark Office.
[5] Senko O, Maslova O, Gladchenko M, Gaydamaka S, Akopyan A, Lysenko S, Anisimov A and Efremenko E 2019 Prospective approach to the anaerobic bioconversion of benzo-and dibenzothiophene sulfones to sulfide. Molecules 24(9) 1736
[6] Zhang Y, Glass R S, Char K and Pyun J 2019 Recent advances on the polymerization of elemental sulphur, inverse vulcanization and methods to functional chalcogenide hybrid inorganic/organic polymers (CHIPs) Polym Chem 10 4078-4105
[7] Oishi S, Oi K, Kuwabara J, Omoda R, Aihara Y, Fukuda T, Takahashi T, Choi J-C, Watanabe M and Kanbara T 2019 Synthesis and characterization of sulfur-based polymers from elemental sulfur and algae oil. ACS Appl Polym Mater 1(5) 1195-1202
[8] Gomez I, Leonet O, Alberto Blazquez J, Grande H J and Mecerreyes D 2018 Poly (anthraquinonyl sulfides): high capacity redox polymers for energy storage. ACS Macro Letters 7(4) 419-424
[9] Sulfur: history, technology, applications & industry. Ed G Kutney (Toronto ChemTec Publishing) 2007. 260 p
[10] Senko O, Gladchenko M, Maslova O and Efremenko E 2019 Long-term storage and use of artificially immobilized anaerobic sludge as a powerful biocatalyst for conversion of various wastes including those containing xenobiotics to biogas. Catalysts 9(4) 326
[11] Efremenko E N and Tatarinova N Y 2007 The effect of long-term preservation of bacterial cells immobilized in poly (vinyl alcohol) cryogel on their viability and biosynthesis of target metabolites Microbiology 76(3) 336-341
[12] Efremenko E N, Azizov R, Raeva A and Abbasov V 2005 An approach to the rapid control of oil spill bioremediation by bioluminescent method of intracellular ATP determination Int Biodeterior Biodegradation 56(2) 94-100
[13] Stepanov N, Senko O, Perminova I and Efremenko E 2019 A new approach to assess the effect of various humic compounds on the metabolic activity of cells participating in methanogenesis *Sustainability* **11** 3158

[14] Ismayilov I T, Stepanov N A, Efremenko E N and Abbasov V M 2015 Evaluation of biocidal properties of vegetable oil-based corrosion inhibitors using bioluminescent enzymatic method *Mosc Univ Chem Bull* **70**(4) 197–201

[15] Lomakina G Y, Modestova Y A and Ugarova N N 2015 Bioluminescence assay for cell viability *Biochemistry (Moscow)* **80**(6) 701–713

[16] Maslova O, Stepanov N, Senko O and Efremenko E 2019 Production of various organic acids from different renewable sources by immobilized cells in the regimes of separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) *Bioresour Technol* **272** 1-9

[17] Efremenko E N, Nikolskaya A B, Lyagin I V, Senko O V, Makhlis T A, Stepanov N A, Maslova O.V. and Varfolomeev S D 2012 Production of biofuels from pretreated microalgal biomass by anaerobic fermentation with immobilized *Clostridium acetobutylicum* cells. *Bioresour Technol* **114** 342-348

[18] Efremenko E N, Stepanov N A, Nikolskaya A B, Senko O V, Spiricheva O V and Varfolomeev S D 2011 Biocatalysts based on immobilized cells of microorganisms in the production of bioethanol and biobutanol. *Catalysis in industry* **3**(1) 41-46

[19] Akopyan A V, Fedorov R A, Andreev B V, Tarakanova A V, Anisimov A V, Karakhanov E A 2018 Oxidative desulfurization of hydrocarbon feedstock *Rus J Appl Chem* **91**(4) 529-542

[20] Yang Y, Lv G, Deng L, Lu B, Li J, Zhang J and Du S 2017 Ultra-deep desulfurization of diesel fuel via selective adsorption over modified activated carbon assisted by pre-oxidation. *J Clean Prod* **161** 422-430

[21] Senko O, Maslova O, Gladchenko M, Gaydamaka S and Efremenko E 2019 Biogas production from biomass of microalgae *Chlorella vulgaris* in the presence of benzo-thiophene sulfone. *IOP Conf Ser Mater Sci Eng* **525**(1) 012089

[22] Stepanov N and Efremenko E 2017 Immobilised cells of *Pachysolen tannophilus* yeast for ethanol production from crude glycerol. *New Biotech* **34** 54–58

[23] Trukhina A I, Gladchenko M A and Kalyuzhnyi S V 2011 Optimizations of sulphide and organic modifications of the DEAMOX process. *Appl Biochem Microbiol* **47**(9) 841-845

[24] Akopyan A V, Plotnikov D A, Polikarpova P D, Kedalo A A, Egazar’yants S V, Anisimov A V and Karakhanov E A 2019 Deep purification of vacuum gas oil by the method of oxidative desulfurization. *Petrol Chem* **59**(9) 975-978

[25] Immobilized cells: biocatalysts and processes. Ed. E. Efremenko (Moscow RIOR) 2018, 499 p