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PSEUDOMONAS PUTIDA P67.2 AND *PSEUDOMONAS FLOURESCENS P75* BASED MICROBIAL SENSORS FOR BIOCHEMICAL OXYGEN DEMAND (BOD) MEASUREMENTS IN PHENOLIC WASTEWATERS OF OIL SHALE INDUSTRY

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This study evaluates applicability of microbial sensors based on Pseudomonas putida P67.2 (Ps. p.) and Pseudomonas fluorescens P75 (Ps. fl) for BOD measurements in the phenol-containing wastewaters that mimic the wastewaters from oil shale industry. Sensors are calibrated with OECD synthetic wastewater. Linear range in calibration solution for Ps. p. and Ps. fl. sensors is BOD 0-50 mg/L and 0-65 mg/L, respectively. The steady state response time for both sensors is 15-45 min. Measurements show that although Ps. fl. has better sensitivity to calibration solution, the concurrence between sensor-BOD and BOD_7 in phenol-spiked wastewater is better for Ps. p. sensor. In linear range the error is only 1.2–13%. For Ps. fl. the error in linear range is 17–55%. Preconditioning in phenol solution (BOD = 5 mg/L) increases sensor's sensitivity to phenol considerably (52%). This study demonstrates that preconditioned microbial sensors with specifically selected cultures can give better results for fast BOD determination in specific wastewaters.

Introduction

Oil shale industry is the most prominent industry in Estonia. It has enormous influence to local economy. But with this industry some specific environmental problems arise as well. One of the problems is resulting wastewater that contains phenolic compounds. Phenol and phenolic compounds are toxic to most types of living organisms including aquatic life and human organism, also they cause disagreeable taste and odor of water [1, 2]. Therefore, it is important that this kind of organic pollution (phenolic compounds)

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can be detected and quantified in order to avoid overwhelming the biotreatment plants and control its presence in the effluent as well [1–4].

Widely used parameter for the determination of biodegradable organic compounds in wastewater is biochemical oxygen demand (BOD). The conventional test method for the BOD estimation requires 5 or 7 days for incubation [5]. This time is too long to effectively control any bioprocess, therefore, a more rapid method is needed. A BOD biosensor based on immobilized microorganisms has been proposed for that purpose. That kind of biosensor consists of a combination of an oxygen electrode as a transducer and immobilized microorganisms as a recognition element and measures the respiration activity of cells. Microbial biosensors can estimate the rate of pollution much faster (in minutes instead of days) and carry out the on-line monitoring.

Both pure and mixed cultures have been used in number of BOD biosensor fabrications as a biological sensing material [6, 7]. Utilization of mixed cultures has its advantages like better agreement with BOD_7 values. However, it also has several disadvantages. Mixed cultures are unstable, have shorter lifetime, and results are often irreproducible. These disadvantages are diminished by using pure cultures as a biological detection material [6, 7].

Unfortunately most of the microbial sensors account only for easily biodegradable organic compounds while in the case of bigger organic molecules and aromatic compounds they greatly underestimate the BOD values. To ensure better response of the BOD sensor in phenolic wastewaters of oil shale industry it was crucial that the microorganisms were specifically cultivated in order to obtain culture in which the enzymatic pathways required for degradation of phenol and phenolic compounds were present. Therefore, heterotrophic bacteria like *Pseudomonas putida P67.2* (*Ps. p.*) and *Pseudomonas fluorescens P75* (*Ps. fl.*) were selected as basis for the microbial membranes**.** Both *Ps. p.* and *Ps. fl.* are aerobic bacteria and have remarkably wide substrate spectrum that includes sugars, alcohols, organic acids, and, most importantly, phenolic compounds [4]. In order to test their ability to degrade phenol, bacteria were tested in 2.5-mM phenolic minimal medium (the only source of carbon and energy was 2.5 mM phenol solution). Fast biomass formation was considered a prerequisite to successful culture choice. Prepared phenol-sensitive BOD biosensors were calibrated using Organization for Economic Cooperation and Development (OECD) synthetic wastewater (with BOD_7 value 2000 mg/L) [8].

Experimental

Cultivation

Microorganisms were cultivated in 2·2.5 ml (5 ml) Luria-Bertani (LB) [9] liquid culture medium. Cultivation took place under aerobic conditions and

lasted for 12 hours at the constant temperature of 30 °C. To ensure aerobic conditions of the solution, a shaker was used to agitate it. In the second step the grown bacterium suspension was inoculated in 100–200 ml LB liquid culture medium for 8 hours under the same conditions. The cells in the stationary growth phase (*Ps. p.* OD_{590} = 4.69 and *Ps. fl.* OD_{590} = 4.55) were gathered by centrifuging them for 15 min at speed of 4000 rpm at 4–11 °C and washed twice with 10 ml of phosphate buffer (pH = 6.86) for the purpose of cleaning.

Immobilization

The biomembrane was made of the 7% (180 mg in 7.5 ml buffer) solution of agarose (type I-A: Low EEO, Sigma) in the phosphate buffer (pH = 6.86) and of the suspension of the bacteria. The agarose mixture was heated to the boiling point in order to dissolve agarose completely. Then the solution was cooled down to 45 °C, and 900 µl of the bacterial suspension was added to it. The resulting mixture was spread on the polymer net (800 µm, polypropylene. InterNet incorporated, Netting, Mesh and Screens for Industry) in order to give membrane physical strength and to gain a certain thickness. Resulting membranes were cooled down to the room temperature to gain gel form. Between all measurements membranes were stored in the phosphate buffer at 4 °C.

Adaptation

All microorganisms have the ability to adapt to new conditions. This property is often put to use in biosensor's development. Adaptation period is used to induce the necessary enzymes for assimilation of desired compounds. As the same solution is used for calibration of the microbial sensor the biodegradation capacity increases and thus improves agreement between the BOD_7 and BOD_5 [10, 11].

For adaptation purposes the prepared membranes were put directly into OECD synthetic wastewater (peptone 1.6%, meat extract 1.1%, urea 0.3%, NaCl 0.07%, $CaCl_2 \cdot 2H_2O$ 0.04%, $MgSO_4 \cdot 7H_2O$ 0.02% and K_2HPO_4 0.28% in 1 L of water) [8] (BOD_7 = 170 mg/L) for 2-2.5 days. After the adaptation period the membranes were placed in phosphate buffer to recover. In order to increase their specificity to phenolic compounds, membranes were kept in phenol solution (made in phosphate buffer) with a BOD value of 5 mg/L after the second month of usage.

Measurements with the biosensor

The previously prepared biomembrane was placed on top of the conventional Clark-type oxygen electrode (CellOx 325, WTW, Germany). BOD biosensor is shown schematically in Fig. 1.

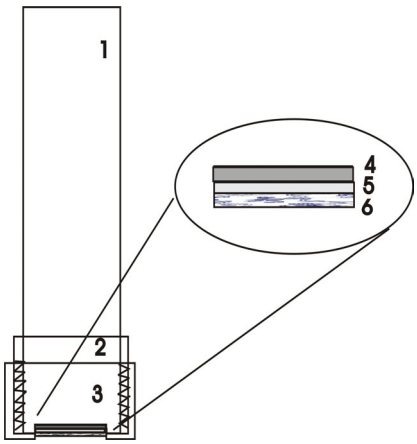

Fig. 1. Schematic drawing of the used BOD biosensor: 1 – sensor body, 2 – attached head-cap, 3 – threaded holder, 4 – cathode, 5 – Teflon film, 6 – membrane with immobilized bacteria.

It is appropriate to point out the advantage of the biosensor's design. The fitted head-cap gives an opportunity to change membranes quickly and makes handling of the biosensor easier and more effective.

All the measurements were carried out at the room temperature in the beaker, wherein was 50 ml phosphate buffer (pH = 6.86). Solution was saturated with air oxygen using an air pump and mixed with a magnetic stirrer. In this study the steady state signal was used.

When the signal of the oxygen electrode stabilized in the 50 ml phosphate buffer solution, the certain amounts of calibration solution were added to achieve known BOD_7 values to the measurement solution. Change in the signal was recorded. The response was taken as the difference between the steady state output signal in the buffer solution and the steady state output signal after addition of the OECD synthetic wastewater solution. This value is a measure of the oxygen consumed by the microorganisms during assimilation of substrate in the solution.

In order to calibrate the microbial sensors, the calibration solution is required. It is reasonable to choose the calibration solution whose composition is close to actual wastewater. In the present study we used OECD synthetic wastewater (composition see above), as it is more similar in composition to sewage than commonly used GGA (1:1 glucose and glutamic acid) [11, 12].

Measurements were also conducted in phenol-spiked OECD synthetic wastewater (OECD synthetic wastewater (BOD_7 = 2000 mg/L) plus addition of phenol with BOD_7 = 400 mg/L) and in pure phenol solutions. The conventional BOD_7 test was performed with all the solutions used to compare BOD_7 values with the values obtained using BOD biosensor. All calibration data were results of at least five parallel measurements. The averaged curve

was used. GraphPad Prism 4 and Microsoft Office was used to analyze the data.

Results and discussion

Calibration of the microbial sensor

The biosensor response depends on the amount of the substrate added. The values of the stabilized output signal in the substrate solutions of different concentrations were registered. As mentioned above, the microbial sensor was calibrated using OECD synthetic wastewater. Calibration curves were plotted as shown in Fig. 2. Depending on the BOD value of the particular solution it took 15-45 min to achieve a stabilized output. The higher the BOD value of the solution, the longer the time required for stabilization of the signal.

Recovery time is defined as the time required for the sensor output signal to reach the initial value after experiment [13, 14]. During the first month after the immobilization of bacteria the recovery times of the microbial membranes were up to a week. After a month of usage the membranes reached a condition were recovery times were in hours instead of days. Therefore, in the future longer adaptation periods are considered for this kind of membranes to ensure full adaptation of the immobilized bacteria.

The detection limit for both bacterial cultures was below 5 mg/L as the measurements at this concentration gave always a valid change in sensor signal (Fig. 2).

Figure 2 shows averaged calibration curves (calibration curves were calculated on basis of Michaelis-Menten equation) for *Ps. p.*-based sensor and *Ps. fl.*-based sensor, where stabilized output signal of the respective sensor is plotted against the BOD_7 value of the calibration solution of OECD synthetic wastewater. The BOD_7 value of OECD wastewater was also tested

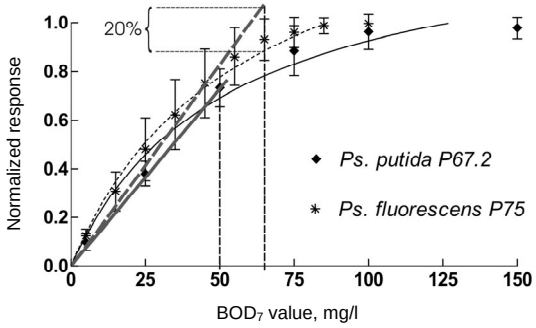

Fig. 2. Calibration curves for *Ps. p.* BOD biosensor and *Ps. fl.* BOD biosensor in OECD synthetic wastewater. Straight lines show the linear approximation of the Michaelis-Menten curves in the region were deviation is less than 20%.

using the standard method for BOD_7 determination. The results remained within the limits of the allowed experimental error (15–20%). The results of parallel measurements coincided in the range 2–15%.

The linear range of the biosensor is defined as concentration range, where the dependence of the biosensor response from the substrate concentration is linear, allowing 20% deviation. Linear range shows the concentration range in which the biosensor can be used [13, 14]. The slope of the linear range of calibration curve is used to characterize biosensor's sensitivity [15].

Although both bacteria *Ps. p.* and *Ps. fl.* have similar substrate spectrum, the comparison of their calibration curves shows that *Ps. fl.* BOD biosensor is slightly more sensitive to OECD synthetic wastewater. Still, the difference between the slopes of calibration curves is only 1%.

Values of linear ranges and corresponding slopes of calibration curves for *Ps. p.*- and *Ps. fl.*-based microbial sensors are given in Table 1.

Table 1. **Linear ranges and corresponding slopes of calibration curves for *Ps. p.*- and *Ps. fl.*-based microbial sensors in OECD synthetic wastewater and phenol-spiked OECD synthetic wastewater**

	Ps. putida P67.2		*Ps. fluorescens P75*	
	Linear range, mg/L	Slope	Linear range, mg/L	Slope
OECD	0–50	0.0124	0–65	0.0156
OECD + Phenol	0–70	0.0136	0–85	0.011

Measurement with phenolic wastewaters and comparison with BOD_7 test

As the main goal of this research was to find a suitable BOD sensor for measurements in phenolic wastewaters, it was necessary to test the sensors in a medium that contains phenolic compounds. For that purpose we composed a solution that consisted of OECD synthetic wastewater (BOD_7 = 2000 mg/L) and phenol so that phenol added to the whole BOD_7 value 400 mg/L. This way obtained phenol-spiked solution (BOD_7 = 2400 mg/L) was used to mimic the phenolic wastewater that could be found in the regions of oil shale industry.

In order to characterize suitability of a biosensor for a specific task, the results from the measurements with the microbial sensor (sensor-BOD) in phenol-spiked OECD wastewater and phenol solution were compared to BOD_7 test values [8]. Sensor-BOD values were obtained from calibration curve. In the ideal case the two would coincide. In the most cases, though, the sensor-BOD tends to be less than expected from the calibration. BOD underestimation is often reported in literature [11] and is attributed to the shorter measurement times of the BOD sensors compared to conventional BOD_7 measurement. Microorganisms can not achieve degradation of poly-

meric and other slowly biodegradable compounds within such short time constraints. Therefore, the sensor signal is mainly based on the compounds with lower molecular weight and faster biodegradation rates [11].

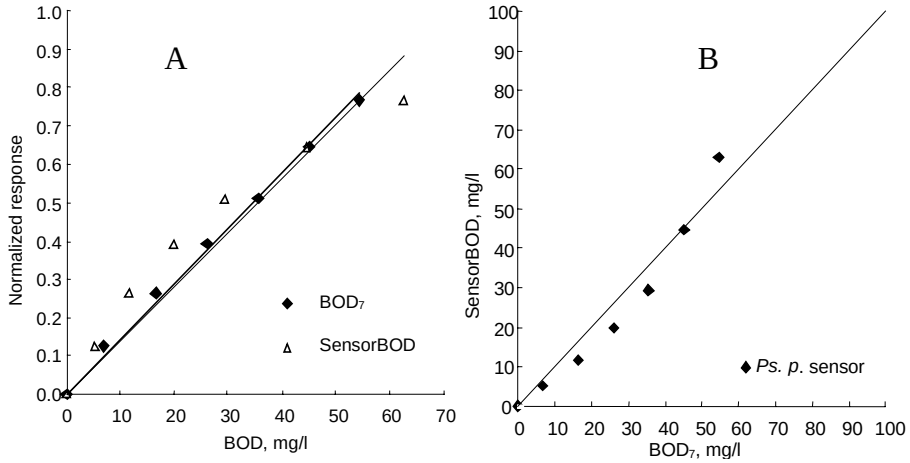

Fig. 3. (A) Comparison of linear range of *Ps. p.* biosensor measurements with phenol-spiked OECD synthetic wastewater, where BOD_7 values for each dilution were calculated from the results obtained from standard BOD_7 method and sensor-BOD values were obtained from the calibration curve. (B) Correlation of *Ps. p.* biosensors sensor-BOD to BOD_7 in phenol-spiked OECD synthetic wastewater, where dotted line represents the 1-1 correlation line.

Figure 3A shows comparison of linear range of *Ps. p.* biosensor measurements with phenol-spiked OECD synthetic wastewater to sensor-BOD values. BOD_7 values were calculated from the dilutions made and the standard BOD_7 measurements with the phenol-spiked OECD synthetic wastewater. Sensor-BOD values were obtained from the calibration curve of the respective microbial sensor. As can be seen from Fig. 3, in the case of *Ps. p.*-based microbial membrane, sensor underestimates the BOD at lower values (below BOD value 45 mg/L) and at the BOD values above that it overestimates the BOD. The deviation from the sensor-BOD value is in the range of 1.2–13% that stays well inside the measurement error of 20% allowed in the standard method for BOD_7. Parallel measurements with phenol-spiked OECD synthetic wastewater resulted in reproducible curves with deviation of 1–5%.

In the case of *Ps. fl.*-based microbial sensor when measuring in the same phenol-spiked OECD synthetic wastewater solution we could see a different picture (Fig. 4.). Unlike *Ps. p.*-based sensor, this sensor underestimates the BOD value in the solution at all concentrations. That is in accordance with literature [11]. Overall, both bacterial cultures showed high reproducibility and little deviance from the theoretical values.

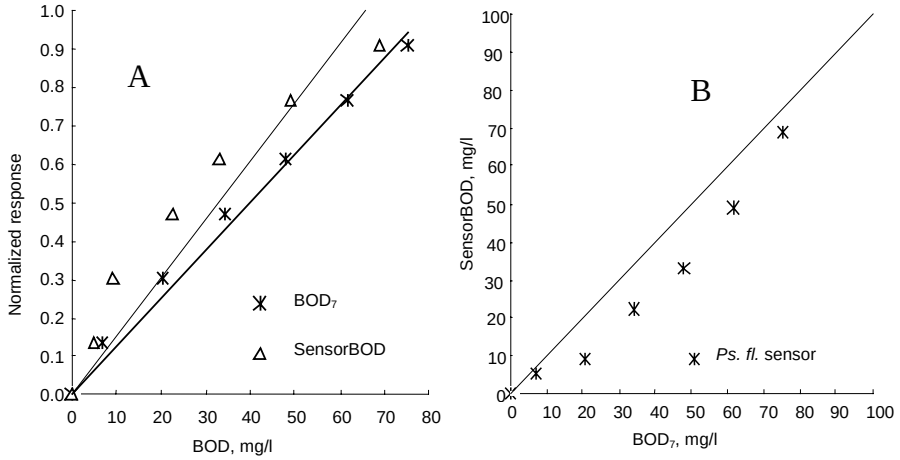

Fig. 4. (A) Comparison of linear range of *Ps. fl.* biosensor measurements with phenol-spiked OECD synthetic wastewater, where BOD_7 values for each dilution were calculated from the results obtained from standard BOD_7 method and sensor-BOD values were obtained from the calibration curve. (B) Correlation of *Ps. fl.* biosensors sensor-BOD to BOD_7 in phenol-spiked OECD synthetic wastewater, where the dotted line represents the 1-1 correlation line.

In order to examine sensors' specificity to phenol, the measurements in phenol solution were carried out. Sensitivity to the phenol was examined for both *Ps. p.* and *Ps. fl*. BOD biosensors. Three measurements were carried out with both cultures, once a day. After the first measurement the membranes were stored in phenol solution with BOD value of 5 mg/L in order to condition membranes to phenol degradation. As can be seen from Table 2, *Ps. p*. based membranes performance improved over time. In the first experiments the slopes between two cultures differed from one another just by 25%, while in the later experiments performance of *Ps. fl.*-based membranes stayed the same whereas *Ps. p.*-based membranes' performance improved. Compared to the first day, in the second experiment sensitivity had increased by 37% and on the third day the increase was 52%.

Although the response we obtained from both cultures was below theoretical, we could clearly see increase in response from the precondition ing of the *Ps. p.* membranes. In the Figures 5 and 6 the comparison is

Table 2. **Effect of preconditioning in phenol solution on sensitivity to phenol of *Ps. p.* and *Ps. fl.* BOD biosensors**

Day	Slope of calibration curve *Ps. putida*	Slope of calibration curve *Ps. fluorescens*
1	0.0056	0.0042
2	0.0077	0.0042
3	0.027	0.0042

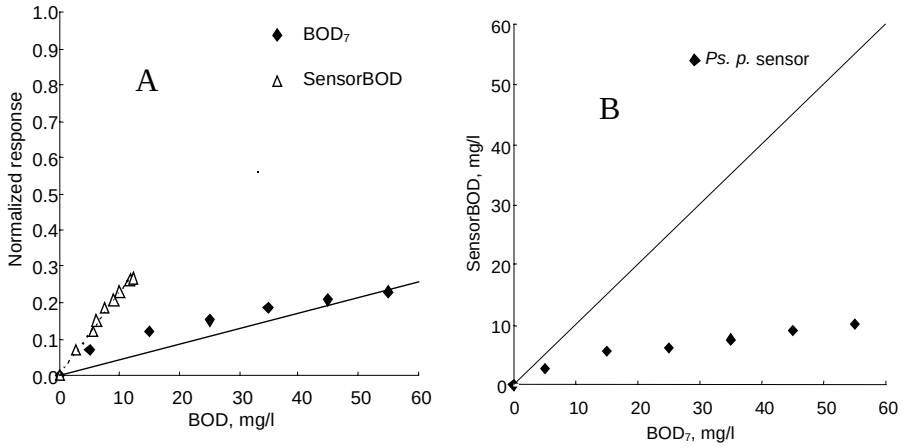

Fig. 5. (A) Comparison of linear range of *Ps. p.* biosensor measurements with phenol solution, where BOD_7 values for each dilution were calculated from the results obtained from standard BOD_7 method and sensor-BOD values were obtained from the calibration curve. (B) Correlation of *Ps. p.* biosensors sensor-BOD to BOD_7 in phenol solution, where the dotted line represents the 1-1 correlation line.

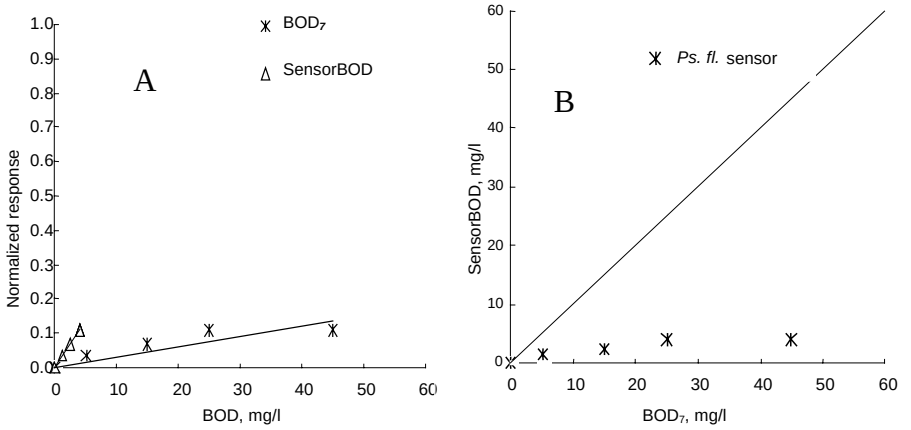

Fig. 6. (A) Comparison of linear range of *Ps. fl.* biosensor measurements with phenol solution, where BOD_7 values for each dilution were calculated from the results obtained from conventional method and sensor-BOD values were obtained from the calibration curve. (B) Correlation of *Ps. fl.* biosensors sensor-BOD to BOD_7 in phenol solution, where the dotted line represents the 1-1 correlation line.

made between the BOD_7 values for each dilution calculated from the results obtained from standard BOD_7 method and sensor-BOD values obtained from the calibration curves of both cultures. Figures show that even in the better adapted *Ps. p.* membranes the BOD values are greatly underestimated. Already at concentrations as low as BOD = 5 mg/L the deviation from the

theoretical values is 45%. At BOD = 15 mg/L the difference is already more than 100%. Still, for the *Ps. fl.*-based sensor the results are worse as they did not adapt to phenol degradation. This shows promise that with better and longer preconditioning *Ps. p.* microbial sensors could be adapted to an extent that phenol presence can be detected with certainty and reasonable quantifiability.

Conclusions

Pseudomonas putida- and *Pseudomonas fluorescens*-based microbial sensors were studied and tested for usability in monitoring of phenolic wastewaters. Choice of the microorganisms was based on the ability to biodegrade phenol. Microbial membranes reached the highest sensitivity after one month use and stayed stable for over 2.5 months.

Experiments showed that both bacterial cultures gave good results in OECD synthetic wastewater (used for calibration) and phenol-spiked OECD synthetic wastewater. The deviation of the sensor-BOD from BOD_7 value in the linear range (BOD = 45 mg/L) was 1.2–13% and parallel measurements resulted in 1–5% deviation from each other.

Even though in the calibration solution *Ps. fl.* showed higher sensitivity, *Ps. p.* showed better results with phenol-spiked OECD synthetic wastewater and phenol solution. Preconditioning in the low concentration phenol solution of BOD = 5 mg/L gave noticeable rise in *Ps. p.* sensor's sensitivity to phenol. Nevertheless, the deviation from the sensor-BOD values was 45% even at very low BOD values of phenol solution. This shows that even more attention should be paid to adaptation and preconditioning of the microbial membranes.

In conclusion it can be said that both bacterial cultures can be used successfully for monitoring of wastewater BOD levels. Preconditioned *Pseudomonas putida*-based microbial sensor showed also promise to be used in the phenolic wastewaters of oil shale industry.

REFERENCES

1. *Handique, J. G., Baruah, J. B.* Polyphenolic compounds: an overview // React. Funct. Polym. 2002. Vol. 52, No. 3. P. 163–188.
2. *Amlathe, S., Upadhyay, S., Gupta, K. V.* Spectrophotometric determination of trace amounts of phenol in waste water and biological fluids // Analyst. 1987. Vol. 112, No. 10. P. 1463–1465.
3. *Rätsep, A., Toomik, A.* // Hydrotechnogenic influxes into the Purtse basin rivers in North-East Estonia / Energy, Environment and Natural Resoures Management in the Baltic Sea Region – 4th International Conference on System Analysis. Nordiske Seminar og Arbejdsrapporter, 1993. Vol. 653. P. 459–462.

4. *Timur S., Pazarlioglu, N., Pilloton, R., Telefoncu, A.* Detection of phenolic compounds by thick film sensors based on *Pseudomonas putida* // Anal. Chim. Acta. 2003. Vol. 61, No. 2. P. 87–93.
5. APHA. Standard Methods for Examination of Water and Wastewater. – Washington, DC: American Public Health Association, American Water Works Association, American Water Environment Federation. Washington, 1992.
6. *D'Souza, S. F.* Microbial biosensors review // Biosens. Bioelectron. 2001. Vol. 16, No. 6. P. 337–353.
7. *Riedel, K., Renneberg, R., Wollenberg, U., Kaiser, G., Scheller, W.* Microbial sensors. Fundamentals and application for process control // J. Chem. Technol. Biotechnol. 1989. Vol. 44. P. 85–106.
8. Organization for Economic Cooperation and Development (OECD), 1986. Standard Methods for the Examination of Waters and Wastewater. American Public Health Association, Washington, DC, 16th ed. P. 525–531
9. *Miller, J. H.* A short course in bacterial genetics. – New York, 1972.
10. *Kim, M.-N., Park, K.-H.* Klebsiella BOD sensor // Sens. Actuators. 2001. Vol. 4030, P. 1–6.
11. *Liu, J., Mattiasson, B.* Microbial BOD sensors for wastewater analysis // Water Res. 2002. Vol. 36, No. 15. P. 3786–3802.
12. *Liu, J., Björnsson, L., Mattiasson, B.* Immobilised activated sludge based biosensor for biochemical oxygen demand measurment // Biosens. Bioelectron. 2000. Vol. 14, No. 12. P. 883–893.
13. *Eggins, B.* Biosensors: An Introduction. – Wiley-Teubner, 1996. P. 1–10, 31–35, 133–135.
14. *Li, F., Tan, T. C., Lee, Y. K.* Effects of pre-conditioning and microbial composition on the sensing efficacy of a BOD biosensor // Biosens. Bioelectron. 1994. Vol. 9, No. 3. P. 197–205.
15. *Thévenot, D. R., Toth, K., Durst, R. A., Wilson, G. S.* Electrochemical biosensors: recomended definitions and classification // Biosens. Bioelectron. 2001. Vol. 16, No. 1–2. P. 121–131.

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