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The possibilities of using sulphate-reducing bacteria for phenol degradation

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Abstract: Steel production processes generate the coke wastewaters contaminated predominantly by highly toxic phenol. Numbers of physical, chemical and physicochemical methods have been developed for the removal of phenol from coke wastewaters. Biological methods are eco-friendly and present appropriate alternative of conventional processes. Various microorganisms are able to degrade phenolic compounds including sulphate-reducing bacteria (SRB). In this work, we study the adaptation of SRB isolated from natural source to phenol and consequently the application of the adapted bacterial cultures for the biodegradation of phenol from model solutions. Two types of bacterial culture were used (monoculture containing SRB genera *Desulfovibrio* and mixed bacterial consortium containing SRB genera *Desulfovibrio*). In experiments the modified Postgate's medium C was used - sodium lactate as the original energy and carbon source was replaced by phenol with concentration 10 mg/dm^3 for adaptation and 50 mg/dm^3 for biodegradation. The mixed bacterial consortium has been shown as more capable to be adapted and grow on phenol and it has the better potential for phenol biodegradation as the bacterial monoculture of SRB genera *Desulfovibrio*.

Keywords: adaptation, biodegradation, phenol, sulphate-reducing bacteria

1. Introduction

Phenol is a highly toxic organic compound with mutagenic, carcinogenic and teratogenic properties. It was one of the first compounds listed into The List of Priority Pollutants by the U.S. Environmental Protection Agency (Si et al., 2013). Phenol is present in the effluents from different industries, such as chemical industry, oil refineries, pharmaceutical industry, pulp and paper industry, textile industry, tanneries, metallurgical and steel industry, coke manufacture or coal conversion plants (Pishgar et al., 2011). Production of coke wastewaters from metallurgical industry represents typical example from many harmful anthropogenic activities. Coke industrial wastewaters are generated in many steel production processes, including processes of making coke, purifying coal gas or recovering coke products (Collins et al., 2005; Gao et al., 2016). Annual worldwide production of coke at the level of 670 million Mg, comes out to 402 million m^3 of wastewaters (Kwiecińska et al., 2016). Coke wastewaters are called as phenolic wastewaters because predominantly consist of phenol, on an average of 60%, also contain cyanide, thiocyanate, ammonia and polyaromatic hydrocarbons (Janosz-Rajczyk et al., 2014; Pillai and Gupta, 2016). Due to toxic nature of phenol, it is necessary to treat phenolic wastewaters before they are discharged into the environment. The impact of phenol pollution on the aquatic environment improves scientific interest and it has led to intensive scientific investigation and research.

Numbers of physical, chemical and physicochemical methods have been developed for the removal of phenol from wastewaters. On the one hand, these processes are effective but, most of them are expensive, highly energy consuming and generate toxic waste as secondary pollution. Biological treatment methods of wastewater containing phenol are promising alternative and generally they are more cost-effective and eco-friendly than conventional treatment processes (Pishgar et al., 2011; Si et al.,

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2013). The microbial biodegradation of phenol is very problematic, because phenol may be toxic, even at low concentration. The microbial biodegradation depends on many factors, including adaptation of the biomass to the concentration of phenol (Sridevi et al., 2012). If bacteria are supposed to be able to degrade phenol with higher concentration, adaptation mechanisms responsible for degradation of phenol, have to be created (Kucerova and Fecko, 2006; Pishgar et al., 2011). Various microorganisms are able to degrade phenol under aerobic (Ahamad, 1995; Vázquez et al., 2006) and anaerobic conditions (Veeresh et al., 2005; Fang et al., 2006; Janosz-Rajczyk et al., 2014).

Sulphate-reducing bacteria (SRB) are anaerobic microorganisms that occur in oxygen-free environments and utilize sulphates as a terminal electron acceptor to produce hydrogen sulphide as one of the metabolic end products. To obtain energy for their growth and maintenance, SRB utilize a wide range of organic carbon substrates, including alcohols, fatty acids, lactate, hydrocarbons, etc. as electron donors (Wolicka and Borkowski, 2007; Mohanty et al., 2018). These bacteria are responsible for the process called microbial anaerobic sulphate reduction. In this process, organic compounds are oxidized in two ways, either completely to water and carbon dioxide, or incompletely to acetate and water as end products (Wolicka and Borkowski, 2007; Muyzer and Stams, 2008). Biodegradation of phenol under sulphate-reducing conditions has been studied using pure cultures (Bak and Widdel, 1986; Mort and Dean-Ross, 1994) and with mixed bacterial consortium containing SRB (Boopathy, 1997; Fang et al., 2004; Guo et al., 2015). However, recent research and studies have been focused mainly on the screening of degradation strains, their identification and description of enzymatic mechanism responsible for the biodegradation of phenol.

SRB play an important role in different wastewater treatment and bioremediation technologies (from treatment of acid mine drainage to removal of sulphates and heavy metals from wastewaters). As a part of the basic research presented in this work, the authors have been studying the environmental and industrial significance of SRB for a long time. So far, the removal of inorganic pollutants from industrial wastewaters by SRB, which have been isolated from the mineral water Gajdovka (Luptakova et al., 2009; Macingova and Luptakova, 2010; Luptakova et al., 2012) have been dealt with. According to literature, SRB are also capable to degrade more than 100 different organic substrates (Wolicka and Borkowski, 2007; Tsai et al., 2009; Grossi et al., 2011). However, no application has been made by applying SRB isolated from mineral water Gajdovka for biodegradation of phenol. On demand from commercial field, the attention was paid to remediation of phenolic wastewaters.

The aim of present study is to investigate adaptation of SRB isolated from natural source and consequently their use for biodegradation of phenol on the purpose of treatment of coke wastewaters from metallurgical company U. S. Steel Kosice.

2. Materials and methods

2.1. Isolation of sulphate-reducing bacteria

SRB utilized in experiments were obtained from potable mineral water (Gajdovka spring, located in Kosice, Slovakia). The bacterial culture of SRB (BC1) was selected by the modified dilution method (Postgate, 1984) using the selective nutrient Postgate's medium C with sodium lactate as a carbon and energy source and sodium sulphate as a sulphate source. 20 cm^3 of the natural mineral water Gajdovka was added into the 250 cm^3 flask containing 180 cm^3 of medium with pH 7.5. The bottles were incubated at 30 °C without shaking under anaerobic conditions for 2 weeks. Then periodically, each two weeks in next 2 months, 20 cm^3 of bacterial culture was transferred into fresh cultivation medium under the same conditions. Chemical composition mainly high concentration of sulphates and pH/Eh of this water complies with the occurrence of SRB. Microbiological and biological analysis confirmed the presence of sulphate reducing bacteria and micromycetes, did not confirm the presence of pathogenic microflora.

2.2. Adaptation of bacterial culture to phenol

The base of bacterial culture adaptation was the replicate cultivation to the phenol presence. Postgate's medium C was modified for these experiments - sodium lactate as the original energy and carbon source was replaced by phenol with concentration 10 mg/dm^3. In view of the initial inoculation, i.e. the initial addition of the bacterial culture at the beginning of the adaptation, two types of the bacterial culture of

SRB were used. During adaptation the first bacterial culture (BC1) prepared by modified dilution method (see section 2.1.) was inoculated into modified Postgate's medium C classically (adding a bacterial cell suspension) and the inoculum volume was 10%. The second bacterial culture (BC2) was obtained by filtration 500 cm^3 of mineral water Gajdovka using 0.23 µm HPTFE filter. In this case, the initial inoculation was performed by the filter rinsing in modified Postgate's medium C. In all, 3 transfers were carried out into fresh modified medium containing 10 mg/dm^3 of phenol. After four months of adaptation period, adapted bacterial cultures (A-BC1 and A-BC2) were obtained.

2.3. Batch biodegradation study

Phenol biodegradation was studied in two consecutive cycles. For biodegradation experiments of Cycle I, as well as Cycle II, two biotic samples and one abiotic sample were prepared. All biotic biodegradation experiments were performed in 250 cm^3 batch flasks containing 180 cm^3 of modified Postgate's medium C and 20 cm^3 inoculum of adapted bacterial cultures (A-BC1 and A-BC2). Initial concentration of phenol was 50 mg/dm^3. Phenol was used as the sole energy and carbon source. Sodium sulphate was used as the sulphate source. Control abiotic set (K) without bacterial culture contained only 200 cm^3 of modified medium with concentration of phenol 50 mg/dm^3. All experimental samples were prepared under strictly anaerobic conditions. Initial value of pH was 7.5. Samples were incubated at 30 °C under anaerobic conditions without shaking. Cycle I was finished when phenol concentration of biotic samples decreased. Then followed by Cycle II, i.e. bacterial culture was transfer into fresh modified medium with the same concentration (50 mg/dm^3) of phenol. Experiments were carried out in duplicate under identical conditions. Samples labelling and liquid phase composition of Cycle I and Cycle II experiments are summarised in Table 1.

Table 1. Details of phenol biodegradation study for Cycle I and Cycle II, initial phenol concentration: 50 mg/dm^3

Cycle I			Cycle II		
Sample	Inoculum (from adaptation process)	Modified Postgate's medium C	**Sample**	Inoculum (from Cycle I)	Modified Postgate's medium C
	(cm^3)			(cm^3)	
I. A-BC1	20	180	**II. A-BC1**	20	180
I. A-BC2	20	180	**II. A-BC2**	20	180
I. K	0	200	**II. K**	0	200

2.4. Methods

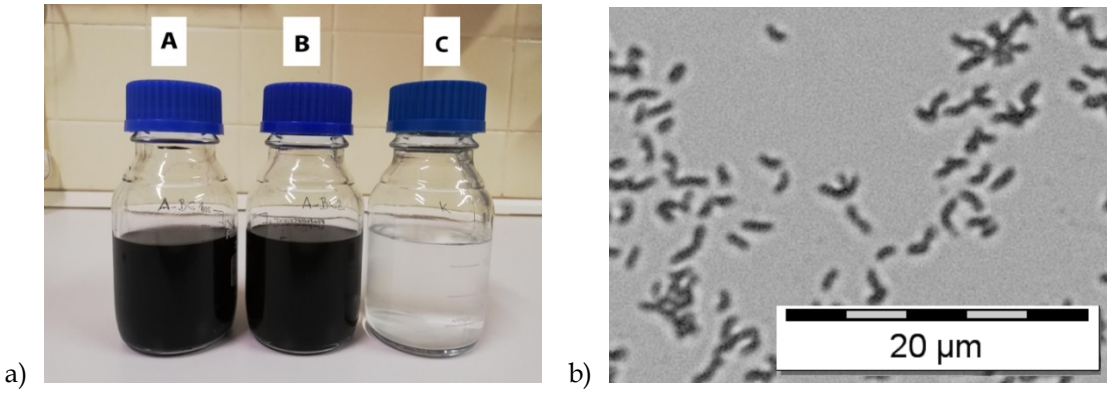
Samples of liquid phase were collected for determination of sulphate and phenol concentration every 7 days. 2 cm^3 of samples were centrifuged at 10 000 rpm for 10 minutes and filtered onto 0.23 µm HPTFE filter before analyses. Sulphates were determined by Ion Chromatography using Dionex ICS 5000 instrument with an IonPac AS11-HC anion column and suppressed conductivity detector. Phenol concentration was analytically estimated by High Performance Liquid Chromatography with Dionex UltiMate 3000 system using diode array detector and Acclaim C18, 150 mm x 2.1 mm column. At the beginning and at the end of isolation and adaptation processes the presence of bacteria was monitored by the microscopic observation (after the gram stained by oil immersion with the magnification x1200) using the light microscope Nikon Eclypse 400.

3. Results and discussion

3.1. Isolation of sulphate-reducing bacteria

Monoculture SRB genera *Desulfovibrio* BC1 was isolated from the mineral water Gajdovka by the modified dilution method using the selective liquid Postgate's medium C. The growth of SRB was detected by the formation of black precipitates at the bottom of the flasks and flask walls (Fig. 1a) (Postgate, 1984; Luptakova et al., 2002). For the confirmation of SRB presence and identification of SRB the microscopic

observation of the bacterial morphology (after the gram stained) (Fig. 1b.) and Bergey's Manual of Determinative Bacteriology were used (Bergey and Holt, 2000).



a) b)

Fig. 1. a) Formation of black precipitates in biotic samples is typical sign of presence of the growth of SRB in the selective liquid Postgate's medium C. Samples A and B – biotic samples with SRB. Sample C – control; b) the microphotograph of BC1 (SRB genera *Desulfovibrio*)

3.2. Adaptation of bacterial culture to phenol

Biodegradation of phenol is not easy process because of the toxicity of phenol for the microorganisms (Srivedi et al., 2012). Marrot et al. (2006) reported that phenol can cause the inhibition of the microbial growth at concentration lower than 200 mg/dm^3. According to Bak and Widdel (1986) concentration higher than 200 mg/dm^3 inhibited the growth significantly. Garcia-Cruz et al. (2010) reported that acute inhibition to the sulphate reduction was observed at 143.8 mg/dm^3 of phenol. One of the strategies how to overcome phenol inhibition is sequential adaptation of microbial culture to higher concentration of phenol (Basha et al., 2010). Adaptation of the SRB culture makes the bacteria compatible to take up phenol as the sole carbon and energy source for their growth and further possible degradation with phenol concentration higher as 200 mg/dm^3 (Fang et al., 2004; Srivedi et al., 2012; Guo et al., 2015).

Two types of the bacterial culture (BC1 and BC2) were used for the adaptation. Monoculture (BC1) was obtained by isolation from mineral water Gajdovka by the modified dilution method. Mixed bacterial consortium (BC2) was obtained by filtration of the mineral water. Because bacterial cultures used in the experiments were isolated from natural source without any organic contamination, it was necessary to give the sufficient time to bacteria for adaptation. For this reason, adaptation with low concentration of phenol 10 mg/dm^3 was started. At the end of the third sub-cultivation two types of adapted bacteria A-BC1 and A-BC2 (Fig. 2) were obtained. Mixed bacterial consortium (A-BC2) has been shown as more capable to be adapted and grow on phenol as pure bacterial culture (A-BC1) (Rudzanova et al., 2018).

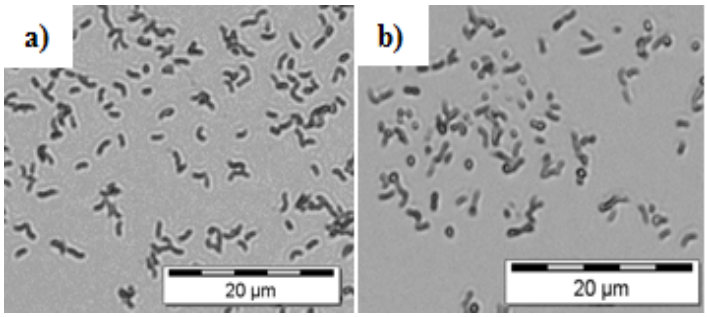


Fig. 2. The microphotograph of bacterial cultures after adaptation to phenol: a) A-BC1 (SRB genera *Desulfovibrio*); b) the microphotograph of A-BC2 (mixed bacterial consortium with SRB genera *Desulfovibrio*)

3.3. Biodegradation study of phenol

After adaptation phase, adapted bacterial cultures were transferred into modified Postgate's medium C with higher initial concentration of phenol (50 mg/dm^3), for the purpose of examination of phenol biodegradation. Results of sulphate reduction summarized in Fig. 3a shows the changes of sulphate

concentration during Cycle I of phenol biodegradation. The experimental results confirm that higher bacterial sulphate reduction was observed in biotic sample containing adapted mixed bacterial culture (A-BC2). The concentration of sulphates decreased from 1554 ± 3.5 mg/dm^3 to 1382 ± 7.0 mg/dm^3. In sample with adapted mono bacterial culture of genera *Desulfovibrio* (A-BC1) was recorded the decrease in the sulphates concentration, from 1572 ± 4.0 mg/dm^3 to 1506 ± 14.0 mg/dm^3. The minor decrease of sulphate concentration was observed in abiotic control from 1568 ± 1.41 mg/dm^3 to 1520 ± 2.12 mg/dm^3 (I.K). Results of phenol biodegradation, for the Cycle I, are shown in Fig. 3b. During Cycle I in the case of adapted mixed bacterial culture (A-BC2), the strong reduction in phenol concentration was remarked after 80 days. Consequently, after 97 days the phenol concentration decreased to 0.143 ± 0.09 mg/dm^3. In case of samples with mono culture (A-BC1) and abiotic control, concentration of phenol allocated minimal changes (± 2 mg/dm^3) in respect of initial phenol concentration 50 mg/dm^3.

Results of the sulphates reduction from Cycle II of phenol biodegradation study are shown in Fig. 4a. Trends in sulphate concentration changes in the studied samples were similar to those of the first cycle. Also, a higher bacterial sulphate reduction was observed in the biotic sample containing adapted mixed bacterial culture (A-BC2) where concentration of sulphates decreased from 1552 ± 0.70 mg/dm^3 to 1357 ± 4.60 mg/dm^3. In samples with mono bacterial culture (A-BC1) and abiotic control, the concentration of sulphates negligible declined from 1572 ± 1.08 mg/dm^3 to 1537 ± 7.07 mg/dm^3 and from 1568 ± 2.12 mg/dm^3 to 1526 ± 2.12 mg/dm^3, respectively.

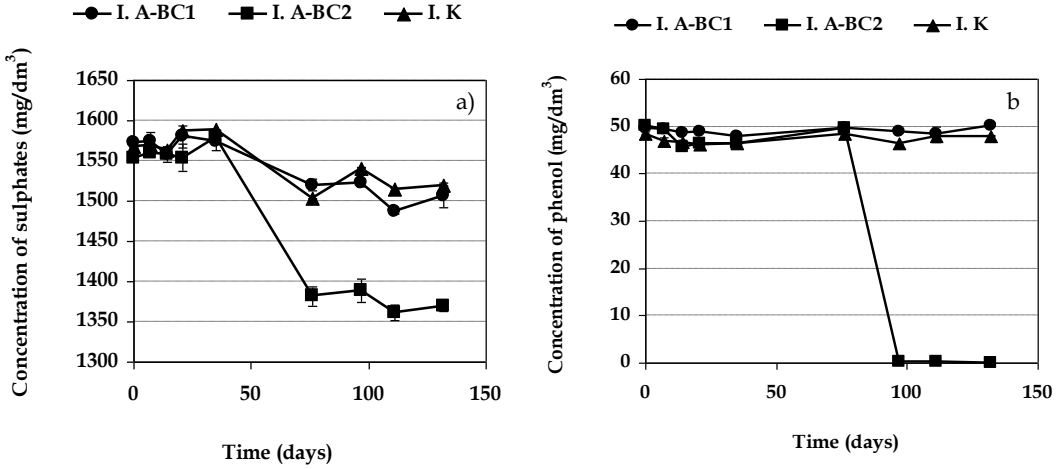


Fig. 3. Cycle I: a) changes in the sulphate concentration and b) phenol concentration during phenol biodegradation by adapted SRB genera *Desulfovibrio* (A-BC1) and the adapted mixed bacterial consortium with genera *Desulfovibrio* (A-BC2). Abiotic control (K). Absolute error has been marked

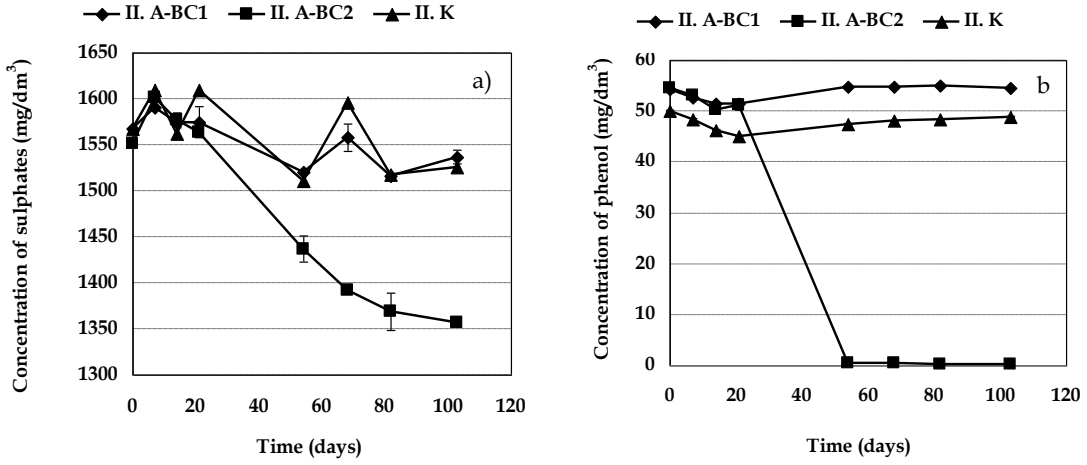


Fig. 4. Cycle II: a) changes of the sulphates and b) phenol concentration during the phenol biodegradation by adapted SRB genera *Desulfovibrio* (A-BC1) and the adapted mixed bacterial consortium with genera *Desulfovibrio* (A-BC2). Abiotic control (K). Absolute error has been marked

Fig. 4b shows biodegradation of phenol, during Cycle II, by two types of studied adapted bacterial cultures. The expressive utilization of phenol was observed after 54 days only at application of the adapted bacterial consortium, i.e. adapted mixed bacterial culture (A-BC2). After 54 days of biodegradation experiments phenol concentration decreased from initial concentration 50 ± 0.48 mg/dm^3 to 0.29 ± 0.02 mg/dm^3. Biodegradation of phenol in samples with adapted pure culture (A-BC1) was not observed.

From results it can be seen that the adapted mixed culture SRB containing genera *Desulfovibrio* (A-BC2), compared with monoculture SRB genera *Desulfovibrio* (A-BC1), is able to degrade phenol, completely. Similarly, Boopathy (1995) studied biodegradation of phenol with mixed bacterial culture containing SRB isolated from swine manure which could degrade 51.7 mg/dm^3. Bak and Widddel (1986) isolated and described *Desulfobacterium phenolicum* which was able to degrade phenol with initial concentration 47.05 mg/dm^3. Then *Desulfobacterium phenolicum* was transferred to fresh medium containing phenol with higher concentration 100 and 200 mg/dm^3. Phenol was completely degraded as long as the concentration of phenol did not exceed 200 mg/dm^3.

Senthilvelan et al. (2014) studied biodegradation of phenol using *Pseudomonas putida Tan-1* and *Staphylococcus aureus Tan-2*. These two cultures were studied for biodegradation of phenol as mono culture and also in the mixed culture form. Mixed bacterial cultures have been found to be more potent than monocultures and are more capable of degrading more than 90 % of phenol. The individual microbial cultures are able to metabolize only a limited range of substrates and degrade it with enzymes to specific products. Pishgar et al. (2011) reported that during the biodegradation of phenol using pure culture, toxic intermediates may be produced, what leads to the inhibition of biodegradation process. The solution can be in using mixed microbial consortium with wide spectrum of metabolic activity. Presumably, in mixed microbial cultures containing different bacterial species with broader enzymes can degrade different constituents of substrates to different products with greater capacity (Senthilvelan et al., 2014). At the biodegradation experiments, sulphates are utilized as final electron acceptor. The decline of sulphates throughout the experiment reflects the performance of the system. This proposition was confirmed by sulphate concentration analyses. Fig. 3a and 4a show that in sample with adapted SRB genera *Desulfovibrio* (A-BC1) the sulphate concentration was without significant change. The declined sulphate concentration was similar to that of abiotic control. The decrease in sulphate concentration was observed only in samples with adapted mixed bacterial culture (A-BC2), and the assumption is, that in this system biodegradation of phenol is ongoing. Better biodegradation of phenol by adapted A-BC2 culture probably can be caused by other bacteria (bacterial consortium) present in mineral water Gajdovka. For this reason, further research will be undertaken to study the bacterial composition of A-BC2 (detection of other bacterial consortium than SRBs) using classical microbiological methods and molecular microbiology.

4. Conclusions

In the present study, the possible adaptation of SRB and biodegradation of phenol under sulphate-reducing conditions was studied. Bacterial culture, used in our experiments, was isolated from natural source without any organic contamination, and it is necessary to adapt SRB to new organic substrate. The utilization of phenol as the substrate can be accomplished when the microbial biomass can be acclimatized to it for a sufficient time. If bacteria are supposed to be able to degrade phenol with higher concentration, adaptation mechanisms responsible for degradation of phenol have to be created. Biodegradation of phenol between pure and mixed bacterial culture was compared. The experimental results confirm that higher biodegradation of phenol was observed in biotic sample containing adapted mixed bacterial culture (A-BC2). During Cycle I, in the case of adapted mixed bacterial culture (A-BC2), the strong reduction in phenol concentration was remarked after 80 days. After 97 days the phenol concentration decreased to 0.143 ± 0.09 mg/dm^3. During Cycle II, the expressive utilization of phenol was observed after 54 days only at application of the adapted bacterial consortium (A-BC2). After 54 days of biodegradation experiments phenol concentration decreased from initial concentration 50 ± 0.48 mg/dm^3 to 0.29 ± 0.02 mg/dm^3. Biodegradation of phenol in samples with adapted pure culture (A-BC1) was not observed. The results are consistent with scientific literature suggesting that mixed bacterial culture is more effective in comparison with pure bacterial culture. For this reason, it is better to use mixed microbial

consortium with wide spectrum of metabolic activity. Results obtained provide bases for further biodegradation study working with higher phenol concentration and then with real wastewater samples.

Acknowledgments

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