The Isolation of Microorganisms Capable of Phenol Degradation

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Abstract

The results of a study on the composition of microflora settling the pilot biofilter bed that purifies the exhausting gases from a cable factory's coil-wire varnishing division are presented in this study. The ability of isolated bacterial strains to biodegrade phenol was also evaluated using culture media of various compositions. Phenol was introduced into the medium at the following concentrations: 0.25, 0.5, 0.75 and 1 $g \times dm^{-3}$. In addition, air in desiccators, where microorganisms grew, was saturated with phenol. The isolated microorganisms were graded by the phenol decomposition rate using gas chromatography. The beds of biofilters utilized in industry appeared to be the source of microorganisms capable of degrading phenol. The most active were: *Rhodococcus rhodochrous*, *Gordonia sputi*, *Pseudomonas putida.* Their mixture showed higher degradation activity than the particular isolates. Isolated and identified bacteria metabolized phenol at high rate (about 14 to 42 $g \times m^{-3} \times h^{-3}$).

K e y   w o r d s: microorganisms isolation, kinetic of phenol biodegradation

Introduction

Phenols are one of many commonly occurring organic pollutants in the environment. These compounds are stable and even at low concentrations they may be toxic towards living organisms and cause unfavorable chemical changes in waters and soils. Among others, biotechnological methods are applied for their removal; they usually are the most ecological and the most economic. These techniques are based on degradation using specially selected microorganisms, which utilize the pollutants as their energy, carbon, nitrogen, and phosphorus or sulfur sources. Therefore, microorganisms capable of completing a fast decomposition of xenobiotics, including phenols are still searched for Baek *et al*., 2003, Murialdo *et al*., 2003, El-Sayed *et al*., 2003.

The aim of present paper was to evaluate the quantitative composition of the microorganism consortium settling the biofilter's bed, to isolate bacteria able to utilize phenol as their only carbon and energy source, as well as to compare the degradation activity of isolated strains towards the compound in question.

Experimental

Material and Methods

The source of the samples for this study was a pilot biofilter bed purifying exhausted gases from the "Załom" cable factory's coil-wire varnishing division near Szczecin. This prototype biofilter was installed for an earlier study project (Wieczorek, 2005). The exhaust gases subjected to purification contained phenol and cresols among several different chemical agents (Baran and Wieczorek, 2000). The biofilter filling was a compost composed of a municipal and industrial waste mixture. Samples of the biofilter bed were taken for analyses from an upper layer about 10 cm deep (GI layer), and a deeper layer at about 30 cm level (GII layer). The number of bacteria in these samples was estimated based on the MPA medium, actinomycetes according to Cyganov and Zukovr (1964) and fungi on Czapek-Dox medium (Johnson *et al*., 1960). In the next phase of studies, microorganism isolation was performed using a sample consisting of a mixture of two compost layers. Thus, after selection based on literature data, two

media of various compositions were tested: medium according to Kojima *et al.* (1961) containing in 1 dm^{-3} of demineralized water: 3.78 g Na$_2$HPO$_4 \times$12 H$_2$O, 0.5 g KH$_2$PO$_4$, 5.0 g NH$_4$Cl, 0.2 g MgSO$_4 \times$7H$_2$O and 0.1 g of yeast extract and mineral medium (I) containing in 1 dm^{-3} of demineralized water: 4.35g K$_2$HPO$_4$, 1.7g KH$_2$PO$_4$, 0.2g MgSO$_4$, 2.1g NH$_4$Cl, 0.05g MnSO$_4$, 0.01g FeSO$_4 \times$H$_2$O, 0.03g CaCl$_2 \times$H$_2$O and 20g of agar. The following phenol concentrations were applied to microbial media: 0.25, 0.5, 0.75 and 1 g$\times$dm^{-3} of substrate. A study was also conducted on the influence of an additional phenol source in a gaseous state on microorganism growth in two used media at an optimized concentration in liquid (0.5 g$\times$dm^{-3}). Open tubes with phenol (10 g) were put into the desiccator where a culture was being grown. Incubation was carried out at 25°C for 2 weeks.

In order to evaluate the degradation activity of isolated strains, microorganism suspensions were prepared by washing out the cultures from slants using 3 ml of 0.85% NaCl solution. One-milliliter aliquots of inoculum were taken from the suspension and introduced into 100 ml of liquid mineral medium. Then 10-ml samples of culture were transferred into sterile "Supelco" 125 ml flasks. 0.5-ml aqueous solutions of phenol at a 5 g$\times$dm^{-3} concentration were added into every flask and the vessels were hermetically sealed using caps with a hole and a teflon-silicon gasket below. Blank control samples, where a sterile medium was added instead of bacterial suspension, were prepared in an analogous way. After preparation the sample's starting phenol concentration was 238 mg$\times$dm^{-3}. Samples were shaken for the whole incubation period at 25°C. Directly after sample preparation and later at changed time intervals, 1 µl aliquots of the liquid phase were taken to gas chromatography analysis by puncturing the septum with a syringe needle (Hamilton). Samples were taken and analyzed every 1 hour during the initial measurement period. Strains showing the highest activity (No 21, 26 and 28) were mixed at 1:1:1 ratio and the mixture's degradation ability was compared with that of individual strains.

Measurements of the phenol concentration as a function of time, performed to monitor the course of biodegradation, were weakly repeatable in subsequent measurement series. Literature data (Łabużek *et al.*, 1996; Kim, 2002; Mrozik and Łabużek, 2002) as well as study observations pointed out that diminished repeatability might be, among others, the result of the decrease or loss of the degradation ability of passaged strains and those strains stored on media with an easily available carbon source – glucose. In order to reduce the influence of these factors, in the second part of kinetic tests with the most active bacteria, strains were conditioned before a test. In the experiment 0.5-ml aliquots of an aqueous solution of phenol at 50 g·dm^{-3} concentration were introduced into flasks containing 3 ml of washed cultures and 180 ml of mineral medium once a day. These procedures were performed for several days until establishment of stable strain degradation activity. Afterwards, those cultures were diluted with mineral medium and after addition of phenol were used for kinetic studies as was earlier written. The statistical significance of received microorganisms counts were determined with help of ANOVA calculation. For the calculation of LSD the Duncan test was used.

Identification of the three most active cultures was done using gas chromatography fatty acid methyl ester analysis performed by Microbial ID (Newark, DE, USA).

Results and Discussion

A comparative analysis of microflora composition and the number of microorganisms in both layers of the biofilter showed substantial differences (Tab. I). The deeper layer appeared to be microbiologically richer, which makes it possible to draw a conclusion that better conditions for microbial growth and development occurred there. Perhaps this was due to factors such as humidity, temperature, nutrient contents (nitrogen and phosphorus compounds) as well as the concentration of xenobiotics supplied along with the gas (during biofiltration, gas was supplied from below the bed). It was found that the humidity of the studied filtration material was 66% on average and was slightly higher in the deeper layer. According to the literature the humidity range of compost biofilters accepted by numerous authors is between 20–70% (Bezborodov *et al.*, 1994). Thus the humidity of the studied bed samples may be considered as close to optimum. It can be assumed that the optimum value of this parameter and a higher concentration of pollutants accelerated the process of the biological purification of gases, which was confirmed by a higher number of microorganisms found in a deeper part of biofilter's bed.

In most studies evaluating the biodegradation properties of microorganisms, the culture is carried out on mineral media amended with a proper amount of xenobiotics (Łabużek *et al.*, 1992a; Łabużek *et al.*, 1992b;

Table I
Number of microorganisms settling the bed of biofilter

Group of microorganisms	Number in 1 g of compost dry matter Bed layer	
	Surface-GI	Deep-GII
Bacteria	5367$\times$10^3	6041$\times$10^3
	LSD = 25951.0	
Fungi	167$\times$10^3	368$\times$10^3
	LSD = 15238.0	
Actinomycetes	462$\times$10^3	604$\times$10^3
	LSD = 2165.8	

Table II
Number of microorganisms on mineral media with phenol addition

Phenol concentration in medium (g$\times$dm^{-3})	Number in 1 g of compost dry matter LSD = 3836.2	
	Mineral medium	Kojim's medium
0.25	281$\times$10^3	1338$\times$10^3
0.5	850$\times$10^3	1992$\times$10^3
0.75	266$\times$10^3	1373$\times$10^3
1	0	75$\times$10^3

Łabużek et al., 1996; Hannaford and Kuek, 1999; Mordocco et al., 1999; Mrozik and Łabużek, 2002). The number of microorganisms found grown on Kojim's medium in the experiment (regardless the phenol concentration in medium) was significantly higher than in the case of a mineral substrate (I), (Tab. II). Therefore it may be considered as better-balanced with respect to the quantity and composition of macro- and microelements necessary for the growth and development of the microorganisms tested.

In the opinion of many scientists, it is possible to adapt microorganisms to high phenol concentrations ranging from 0.75 up to 5 mg×dm^{-3}. However, the achievement of such results requires an earlier adaptation to increasing amounts of the compound, immobilization procedures or changing the culture medium composition. The modification of mineral media most often consisted of the introduction of an additional conventional carbon source in the form of glucose, mannose or yeast extract (Łabużek et al.,1992a, b; Annadurai et al., 2000; Silva et al., 2002; Baek et al., 2003; El-Sayed et al., 2003). Nevertheless, most microorganisms adapted relatively easily to the presence of lower concentrations of the compound in medium, such as 0.25, 0.5 and 0.75 g×dm^{-3}. In this study, a concentration of 0.5 g×dm^{-3} appeared to be the best for microorganism growth. Such concentration is accepted as optimal for biodegradation processes by many authors (Hannaford and Kuek, 1999; Mordocco et al., 1999; Annadurai et al., 2000).

In literature, no studies in which an additional carbon source was applied in form of gaseous phenol to microbial cultures were found. A biodegradation process of gaseous phenol was observed only during the biofiltration of a gas polluted with it (Łabużek and Składzień, 1997). A higher number of microorganisms in the experiment on mineral media amended with an additional (gaseous) source of the studied compound was achieved due to the great volatility of phenol and a possibility of its intake by microorganisms. The number of microorganisms increased up to 100% as opposed to the culture where phenol was introduced only into the medium (Fig. 1a).

In total, during this study 29 strains capable of growth on mineral media with the only carbon source in phenol form were isolated. Among them, 6 strains (No 1, 3, 11, 21, 26 and 28) were characterized by a relatively high rate of the compound decomposition (Fig. 1b). Strains were included in the active group on the basis of differences in curve inclination representing phenol concentration changes in time for the tested samples and the control, as well as the time necessary for the decrease of phenol concentration to its detection limit (about 5 mg×dm^{-3}).The inclination of the curve is a graphical representation of biodegradation rate changes, and the slope is the reaction rate. Therefore, the higher the slope, the higher the biodegradation rate, and thus a shorter time for phenol consumption in a sample.

The three most active strains (No 21, 26 and 28) were identified and together with their mixtures at equal quantities were used for further tests. These strains were respectively: No 21- *Rhodococcus rhodochrous*, No 26- *Gordonia sputi* or similar *Gordonia* species, No 28- *Pseudomonas putida*. Sample results for one of these measurements are presented in Figure 2.

The shapes of phenol concentration-time curves for active strains were typical for periodic batch experiments although it did not follow the typical Michaelis-Menten reaction kinetic, or a similar kinetic. Perhaps the cultures were taken before the exponential growth phase. In the case of the control samples, a slight
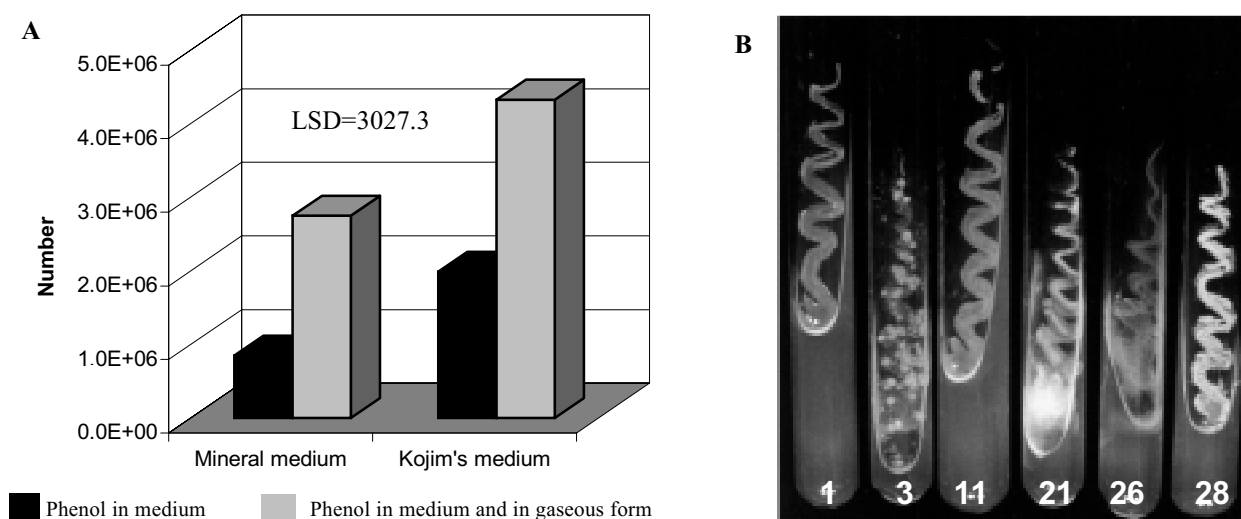

Fig 1. Number of bacteria (in 1 g of DM) on different media with optimum phenol amount (0.5 g×dm^{-3}) and with addition of gaseous phenol (A) and strains with the highest biodegradation activity (B)
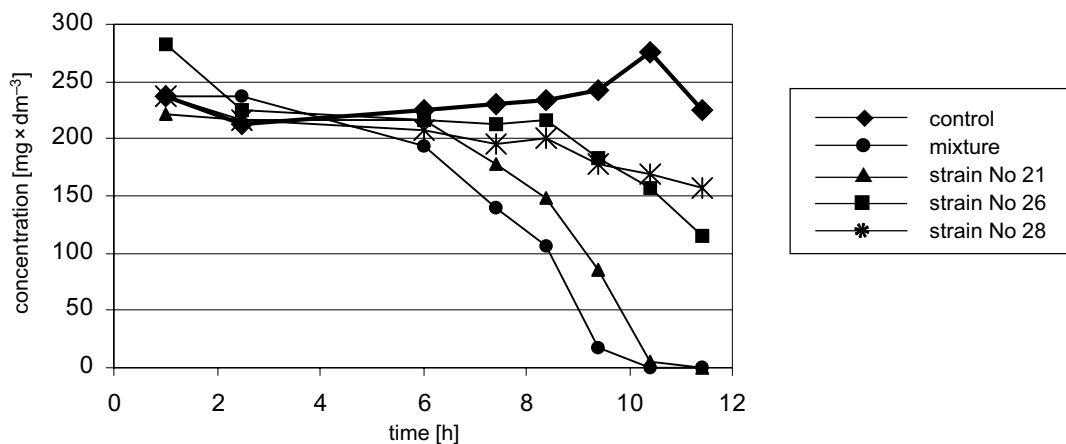
Fig 2. Decrease of phenol concentration in time on a sample of strains *Rhodococcus rhodochrous* (No 21), *Gordonia sputi* (No 26) and *Pseudomonas putida* (No 28) and their mixture as compared to the control.

linear decrease of phenol concentration was usually observed. Taking into account that phenomenon, the loss of phenol from the medium after 11 hours due to the activity of particular strains reached up 38–98% depending on the strain.

Comparing the rate of phenol decomposition by single strains Nos 21, 26 and 28, as well as their mixture, it was found that the mixture showed the highest rate, although the difference between the most active strain, No 26, and the mixture was small. In this case, the decrease of phenol concentration below a detection threshold occurred after 10.4 hours. These results are consistent with those achieved by Wang and Loh (1999). Also Bieszkiewicz *et al.* (1997) have found great xenobiotic biodegradation capability of mixtures of various active microorganisms. The degradation of phenol by adapted microorganisms originating from active sediment is twice as fast as the case of single pure cultures, which is confirmed by the results of Buitron and Gonzalez (1996) and Murialdo *et al.* (2003).

Based on the proportionality of phenol concentration *vs.* time (Fig. 2), an attempt to evaluate the rate of phenol decomposition was also undertaken. In order to estimate a biodegradation rate, data for phenol concentration *versus* time were correlated respectively using a multivariable regression technique in MS Excel to obtain the equations that best fit the degradation curves. A sample result is given on Fig. 3.

The equations (second order polynomials) were differentiated with respect to time. After subtracting the curve equation for control samples from the equation for the active samples the linear equation describing degradation rate was achieved (Fig. 4.)

Thus the highest biodegradation values were observed at the end of the experiments – in a range 14 to 42 $g \times m^{-3} \times h^{-1}$.

Conclusions. The concentration and form of phenol introduced into a culture significantly affected the number of achieved strains able to biodegrade the compound. The optimal phenol concentration in medium was 0.5 $g \times dm^{-3}$, and additional air saturation with phenol vapors caused an over two-fold increase in the
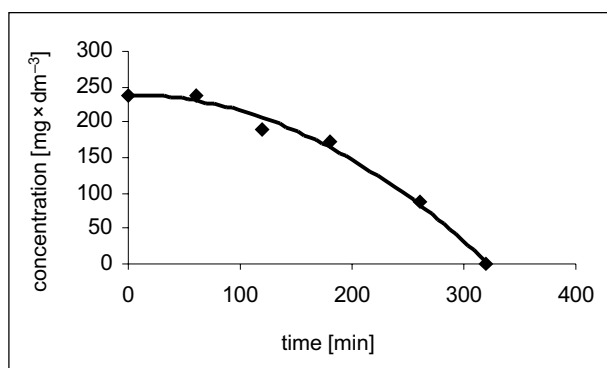

Fig 3. Correlation of phenol concentration with time on an example of strains *Rhodococcus rhodochrous* (No 21)
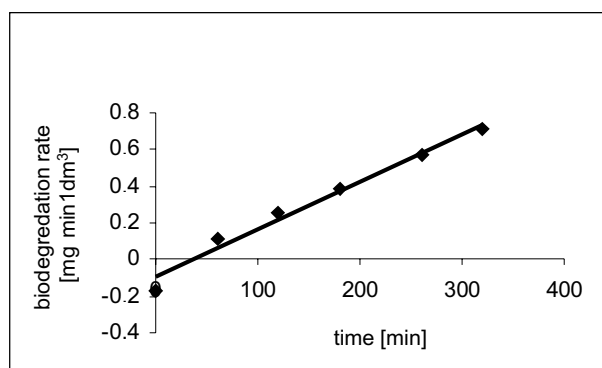$y = -0.0023x^2 + 0.0099x + 238.58$; $R^2 = 0.9894$.

Fig 4. Biodegradation rate of phenol by *Rhodococcus rhodochrous* (No 21)
$y = 0.0026x - 0.0993$; $R^2 = 0.9792$.

number of microorganisms forming colonies. Three of the 29 isolated strains *Rhodococcus rhodochrous, Gordonia sputi, and Pseudomonas putida* metabolized phenol at great rate (up to 40 $g \times m^{-3} \times h^{-1}$). In most cases, their mixture showed higher degradation activity than particular isolates.

Literature

A n n a d u r a i G., B. M a t h a l a i and T. M u r u g e s a n. 2000. Box-Behnken design in the development of optimized complex medium for phenol degradation using *Pseudomonas putida* (NICM 2174). *Bioprocess Biosyst. Eng.* **21**: 415–421.

B a e k S.H., K.H. K i m, C.R. Y i n, C.O. J e o n, W.T. I m, K.K. K i m, S.T. L e e. 2003. Isolation and Characterization of Bacteria Capable of Degrading Phenol and Reducing Nitrate under Low-Oxygen Conditions. *Curr Microbiol.* **47**(6): 462–6.

B a r a n J. and A. W i e c z o r e k. 2000. SPME Sampling and GC/MS analysis of Exhaust Gases from Thermal Treatment of Lacquer-Coated Surfaces. In: Proceedings of the 3rd International Scientific Conference "Air Protection in Theory and Application, Zabrze, 30 May-1 June.

B e z b o r o d o v A., E. K o z l y a k, M. Y a k i m o v, I. U t k i n and I. R o g o z h i n. 1994. Microbiological methods of air purification. *Biotechnologia* **24**: 95–108.

B i e s z k i e w i c z E., R. M y c i e l s k i and H. B o s z c z y k - M a l e s z a k. 1997. Biodegradation of the oily fraction of petrochemical wastewaters isolated from oily soil (in Polish). *Biotechnologia* **36**: 70–77.

B u i t r o n G. and A. G o n z a l e z. 1996. Characterization of the microorganisms from an acclimated activated sludge degrading phenolic compounds. *Water Sci. Technol.* **34**: 289–294.

C y g a n o w V. and A. Z u k o v r. 1964. Morfologobiochimiciskie osobennosti novowo vida actinomiceta (in Russian). *Mikroobiologija* **33**: 863–869.

E l - S a y e d W.S., M.K. I b r a h i m, M. A b u - S h a d y, F. E l - B e i h, N. O h m u r a, H. S a i k i, A. A n d o. 2003. Isolation and Identification of a Novel Strain of the Genus Ochrobactrum with Phenol-Degrading Activity. *J. Biosc. Bioeng.* **96**: 3, 310–312.

H a n n a f o r d A. and C. K u e k. 1999. Aerobic batch degradation of phenol using immobilized *Pseudomonas putida. J. Ind. Microbiol. Biotechnol.* **22**: 121–126.

J o h n s o n F., E. C u r l, J. B o n d and H. F r i b o u r g. 1960. Methods for studying soil microflora-plant disease relationships. Burgess Publ. Comp., Minneapolis, Minn.

K i m E. 2002. Alkylbenzene degradation by R*hodococcus. Appl. Environ. Microbiol.* **68**: 3270–3277.

K o j i m a Y., N. I t a d a and O. H a y a i s h i. 1961. Metapyrocatechase: a new catechol-cleaving enzyme. *J. Biol. Chem.* **236**: 2223–2229.

Ł a b u ż e k S. and J. S k ł a d z i e ń. 1997. Attempt at biological purification of coke plant wastes piles using biological preparations-laboratory scale (in Polish). *Biotechnologia* **36**: 92–107.

Ł a b u ż e k S., B. K ł a p c i ń s k a, A. M r o z i k, I. R a d e c k a and A. W o ź n i c a. 1996. Gaining of a highly-active mixed population of microorganisms capable of degrading phenolic compounds (in Polish). *Archiwum Ochrony Środowiska* **1–2**: 49–63.

Ł a b u ż e k S., I. L i p k a and A. M r o z i k. 1992a. Degradation of phenol and cyanide by *Pseudomonas* sp. strain – Part 2 (in Polish). *Biotechnologia* **18**: 42–49.

Ł a b u ż e k S., J. P a j ą k and I. P a j ą k. 1992b. Degradation of phenol and cyanide by *Pseudomonas* sp. strain (in Polish). *Biotechnologia* **16**: 28–38.

M o r d o c c o A., C. K u e k and R. J e n k i n s. 1999. Continuous degradation of phenol at low concentration using immobilized *Pseudomonas putida. Enzyme Microb. Technol.* **25**: 530–536.

M r o z i k A. and S. Ł a b u ż e k. 2002. A comparison of biodegradation of phenol and homologous compounds by *Pseudomonas vesicularis* and *Staphylococcus sciuri* strains. *Acta Microbiol. Pol.* **51**: 367–378.

M u r i a l d o S.E., R. F e n o g l i o, P.M. H a u r e, J.F. G o n z á l e z. 2003. Degradation of phenol and chlorophenols by mixed and pure cultures. *Water SA.* **29**, 457–463.

S i l v a M., M. C o e l h o and O. A r a u j o. 2002. Minimization of phenol and ammoniacal nitrogen in refinery wastewater employing biological treatment. *Engenharia Termica, Edicao Especial* **2**: 33–37.

W a n g S.-J. and K.-C. L o h. 1999. Modeling the role of metabolic intermediates in kinetics of phenol biodegradation. *Enzyme Microb. Technol.* **25**: 177–184.

W i e c z o r e k A. 2005. Pilot-Scale biofiltration of waste gazes containing aliphatic and aromatic hydrocarbons, phenol, cresols, and other volatile organic compounds. *Environmental Progress* **24**: 60–66