Доклади на Българската академия на науките
Comptes rendus de l'Académie bulgare des Sciences

Tome 62, No 8, 2009

BIOLOGIE
Biotechnologie

PHENOL BIODEGRADATION BY TWO XENOBIOTICS-TOLERANT BACTERIA IMMOBILIZED IN POLYETHYLENE OXIDE CRYOGELS

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(*Submitted on April 24, 2009*)

Abstract

Biofilms were formed on poly(ethylene oxide) (PEO) cryogels by using bacteria cultured from xenobiotics polluted environments and their phenol biodegrading capability was studied. PEO cryogels were synthesized via UV irradiation cross linking of moderately frozen aqueous system. Two xenobiotics tolerant bacterial isolates KCM R_5 and KCM RG_5 were used to construct the biofilms on the cryogels.

Obtained PEO-biofilms were assessed for their ability to remove phenol at concentrations 300, 400, 600 and 1000 mg L^{-1} for 28 days. The biofilm PEO-KCM RG_5 removed phenol up to 600 mg $L^{-1}/24$ h, whereas the biofilm PEO-KCM R_5 was able to degrade up to 1000 mg $L^{-1}/24$ h. The high content of free-water in the cryogels allowed reproduction of the used bacteria. Short initial adaptation of the PEO-biofilms with 100 mg $L^{-1}/24$ h phenol was crucial for protecting the bacterial cells from dead. The obtained results showed that the liquid debit through the biofilms on the 28th day of the experiments was lower than at the beginning. The cryogels demonstrated non-toxicity, high biocompatibility with bacteria and excellent mechanical characteristics. After aggressive phenol treatment the PEO-biofilms remained compact, porous and elastic. The investigated new biological materials demonstrate potential for application in the industrial wastewater treatment technologies.

Key words: polluted environments, xenobiotics, biodegradetion, bacteria

Introduction. In the last decade widening and reorientation of immobilized cells and enzymes application was recorded. It comes out the previous frames of

This work was supported by grant VUH – No 302 financed by the National Science Fund, Ministry of Science and Education, Bulgaria.

food and pharmaceutical industry [1,2] and expands in wastewater treatment and detoxification technologies.

Among the monocyclic aromatic substances phenol and its derivatives are some of the most dangerous environmental pollutants. Because of its long term application in chemical-, wood-, textile- and oil processing industry the above mentioned compounds persist in the environment in high concentrations.

Many bacteria from various genera are capable of phenol degradation. Representatives of *Pseudomonas* [3–5], *Acinetobacter* [6], *Alcaligenes* [7], and *Bacillus* [8] are among them. Pseudomonades, especially their environmental isolates were reported as most numerous and active. The biodegradation of phenol in wastewater by immobilized cells of *Pseudomonas putida* was described by Loh et al. [9].

In recent years different organic carriers for bacterial immobilization were investigated [10,11]. Among the synthetic polymers, poly(ethylene oxide) hydrogels are excellent candidates because they are nontoxic biocompatible materials which meet all of the requirements of strength, absorbency, flexibility and adhesiveness [12]. Firstly, PEO hydrogels have been obtained in situ by γ-irradiation of dilute aqueous solutions [13], and two decades later, via methods based on chemical cross linking [14]. Hydrogels of high-molecular-weight poly(ethylene oxide) have also been synthesized in situ by applying a facile procedure that involves UV crosslinking of PEO in aqueous solution. Moreover, when the UV cross linking is performed in a frozen aqueous system, macroporous hydrogels (cryogels) with very high yield of gel fraction and high crosslinking density are obtained [15,16].

In the present study, macroporous PEO hydrogels were investigated as carriers of xenobiotics degrading bacteria and the obtained PEO-biofilms were investigated for their ability for phenol biodegradation.

Materials and methods. ***1. Cryogels preparation.*** High molecular weight poly(ethylene oxide) (Union Carbide; MW = 2.10^6 g/mol) was dissolved in distilled water (5 wt.%) and then a photo initiator (4-benzoylbenzyl) trimethylammonium chloride (Aldrich; 2 wt.% with respect to the polymer) was added under stirring at room temperature. The resulting homogeneous solution was poured into Teflon dishes (50 mm diameter) forming a 2.5 mm thick layer, which was then kept in a freezer at a temperature of $-20\,^{\circ}$C for 2 h. The dishes were quickly placed in a thermostatic open chamber connected with a Julabo cryostat apparatus. The frozen system was irradiated with full spectrum UV-VIS light at $-20\,^{\circ}$C with a Dymax 5000-EC UV curing equipment with 400 W metal halide flood lamp for 2 min (dose – 11.4 J/cm^2; input power – 93 mW/cm^2).

The PEO cryogels were extracted in distilled water for seven days, quickly frozen in liquid nitrogen and freeze dried in an Alpha 1-2 Freeze Drier (Martin Christ) at $-55\,^{\circ}$C and 0.02 mbar for 24 h. The gel fraction (GF) yield was calculated by the following equation: GF yield [%] = (weight of dried sample/initial weight of polymer) $\times$ 100.

2. Microbial strains and inoculants. The studied microbial isolates were

cultured from polluted soil near the Pb-Zn smelter called KCM (strain KCM R_5) and from groundwater collected near the pesticide producing factory AGRIA (KCM RG_5). Both manufactories are situated at a distance of about 6 km from the town of Plovdiv, Bulgaria. The Pb-Zn smelter is the biggest one on the Balkan Peninsula. AGRIA factory is manufacturing more than 50 various pesticides. Most of them contain in their structure monocyclic aromatic rings.

The bacterial isolates KCM R_5 and KCM RG_5 were initially cultured in sterile 200 ml synthetic mineral media according to Furukawa and Chakrabarty [17] containing per 1 L: 5.6 g $K_2HPO_4 \times 3H_2O$, 3.4 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.34 g $MgCl_2 \times 6H_2O$, 0.001 g $MnCl_2 \times 4H_2O$, 0.0006 g $FeSO_4 \times 7H_2O$, 0.026 g $CaCl_2 \times 2H_2O$ and also 0.002 g $Na_2MoO_4 \times 2H_2O$. The substances $K_2HPO_4 \times 3H_2O$ and $CaCl_2 \times 2H_2O$ were added at the end in order to avoid precipitation. The medium was autoclaved at 0.8–1 atm for 30 min. To the medium 0.1% sterile glucose and 100 mg L^{-1} phenol were added. The bacterial strains were cultivated in Erlenmeyer flasks for 24 h/180 rpm/28 °C to reach OD of 0.300. The OD was measured spectrophotometrically at $\lambda = 450$ nm using 10 mm cuvettes. Afterwards the bacterial strains used were adapted to low phenol concentrations. For this in 300 mL sterile synthetic mineral medium 100 mg L^{-1} phenol and 20 ml of the pre-cultivated strains KCM R_5 and KCM RG_5 were added and incubated for 72 h/180 rpm/28 °C until reaching an OD of 0.550.

3. Cryogels adaptation and biofilms construction. PEO cryogels were adapted for 24 h in 100 mL synthetic mineral medium without shaking. To each one of the adapted bacterial cultures KCM R_5 and KCM RG_5 grown in a volume of 300 mL an equal volume sterile mineral medium was added and the resulting mixture was then divided in three equal parts each of 200 mL. The bacterial cultures were transferred in vessels, tightly locked and shaken with the added PEO-cryogels gently at 100 rpm for 48 h. The resulted PEO-biofilms were placed on the membrane filter of reusable bottle-Top Filter 47 mm, 500 mL (Nalgene, Rochester, USA). The locking ring was screwed up softly in order to avoid cutting of the biofilm. The media run through the PEO-biofilms by its gravity forces. The biofilms were prepared in triplicates.

4. Phenol removal. In order to clean the constructed PEO-biofilms from residual compounds 250 mL synthetic mineral medium with no phenol added was run through them for 24 h. The PEO-biofilms were then treated for 28 days consequently with sterile mineral medium containing increasing concentrations of phenol as follows: 7 days with 300 mg L^{-1}, 5 days with 400 mg L^{-1}, 4 days with 600 mg L^{-1} and 12 days with 1000 mg L^{-1}. The phenol amount in the filtered liquid was measured every 24 h. The phenol concentration was determined colorimetrically following the pyramidone based method [18]. In 12.375 mL distilled water were added 0.125 mL filtrate sample, 0.250 mL ammonia chloride buffer, pH 9.3 (adjusted with ammonia), 0.125 mL 3.5% pyramidone and 0.375 ml ammonia persulfate, pH 7.0 (adjusted with ammonia). After mixing the reaction was

kept at room temperature for 45 min. The absorption was measured spectrophotometrically at $\lambda = 540$ nm against the control in which distilled water instead of filtered liquid was added. The phenol amount was determined according to a standard curve.

The efficiency of the phenol removal was calculated according to the formula

$$\text{Efficiency in (X) hour (\%)} = \frac{C_i - C_f}{C_i} \times 100,$$

where C_i is the initial phenol concentration in the filtration system, and C_f is the phenol concentration in the filtrate.

Results and discussion. PEO cryogel of high gel fraction yield (93%) was synthesized via UV irradiation of moderately frozen semi-diluted solution of PEO and photo initiator for extremely short time and subsequent thawing as described elsewhere [15,16]. The cryogel obtained was heterogeneous, supermacroporous material with large interconnected pores (50–100 µm) surrounded by dense thin walls (ca. 1–2 µm). Importantly, more that 80% of the water in the cryogel is capillary-bound water that fills the space of the macro pores. The freeze-dried PEO cryogel possesses the original macro porous structure (Fig. 1) and, once immersed in the suspension, the liquid fills the channels (interconnected pores) of the cryogel.

The isolate KCM R_5 was determined as Gram(−), motile and oval-shaped bacterium while the isolate KCM RG_5 as a Gram(+), motile and a rod-shaped one. KCM R_5 was affiliated with high extent of 16S rRNA gene similarity to *Pseudomonas rhodesiae* and KCM RG_5 – to *Bacillus subtilis*. KCM R_5 and KCM RG_5 demonstrated tolerance to heavy metals such as Pb, Mn and Cd and could grow in the presence of phenol, *ortho*-nitrophenol, 2,4-dinitrophenol, 2,5-nitrophenol, pentachlorophenol, and 2,4-dichlorophenoxiacetic acid as a sole carbon and energy source [19,20]. The morphology of the isolates is shown in Fig. 2.

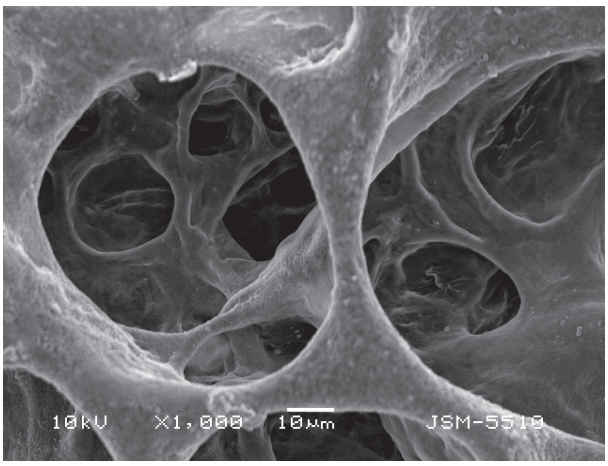

Fig. 1. SEM micrograph of PEO cryogels, ×1000

Both isolates were immobilized in the cryogels of poly(ethylene oxide) and two PEO-biofilms were obtained.

Phenol biodegradation by constructed PEO-biofilms was studied at 28 °C. According to Polymenakou and Stephanou [4] the optimal phenol degradation at concentrations

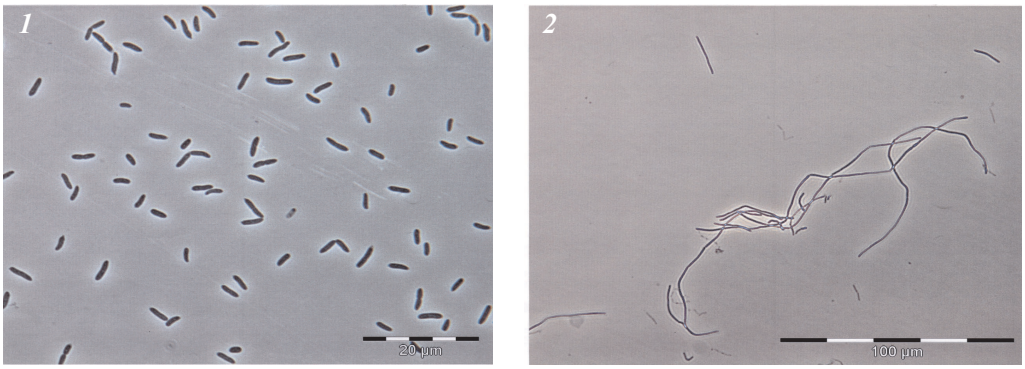

Fig. 2. Morphology of the environment bacterial isolates KCM-R_5 (*1*) and KCM-RG_5 (*2*)

of up to 500 mg L^{-1} was realized by a *Pseudomonas* sp. strain at 30 °C. Tsai and Yuang [5] described maximum rates of phenol degradation at 30 °C and at pH 7 and demonstrated that no considerable phenol depletion was performed at 10 °C or 40 °C. In addition to the temperature they described also a pH influence on the phenol biodegradation. Phenol biodegradation at a higher concentration of 1000 mg L^{-1} was studied for another member of the genus *Pseudomonadaceae* [21]. Only one case of phenol degradation at a higher concentration of 1600 mg L^{-1} was described up to date. It concerned a strain of *Alcaligenes faecalis* and was reported by [7]. In contrast to the bacterial strains studied in our work, this

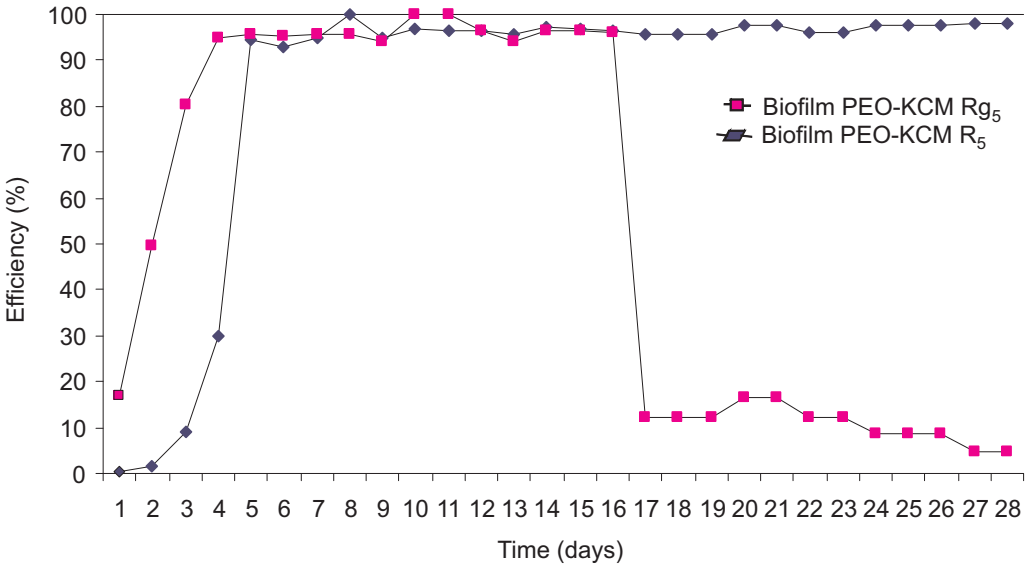

Fig. 3. Efficiency of the phenol biodegradation by biofilms PEO-KCM R_5 and PEO-KCM RG_5. Biofilms were treated for 28 days with different phenol concentrations: 300 mg L^{-1} (1–7 day), 400 mg L^{-1} (8–12 day), 600 mg L^{-1} (12–16 day) and 1000 mg L^{-1} (17–28 day)

bacterium was not isolated from an industrial area. Similar data about representatives of the genera *Pseudomonas* and *Bacillus* are not yet reported.

Our results showed that both biofilms removed phenol at almost equal amount at phenol concentration of 300, 400 and 600 mg L^{-1} for 24 h (17th day) reaching efficiency up to 100% (Fig. 3). When treating the PEO-biofilms with 1000 mg L^{-1}/24 h phenol the biofilm PEO-KCM R_5 kept its ability for phenol removal while the biofilm PEO-KCM RG_5 loose it and did not restore it up to the end of the experiment. As it is shown in Fig. 3, the biodegradation efficiency of the PEO-KCM R_5 biofilm remains high – 96–98%, in contrast to the biofilm PEO-KCM RG_5 which efficiency was reduced to 5%.

T a b l e 1

Volumes of the liquid filtered through the biofilms

Day	Influent (mineral media + phenol (ml))	Effluent of biofilm PEO-KCM-R_5 (ml)	Effluent of biofilm PEO-KCM-RG_5 (ml)
1st	250	212*	50*
2nd	250	224	45
3rd	250	148	39
4th	250	90	38
5th	250	56	91
6th	250	33	69
7th	250	24	58
8th	250	16	50
9th	250	17	43
10th	250	15	36
11th	250	15	35
12th	250	14	36
13th	250	14	36
14th	250	14	37
15th	250	12	35
16th	250	12	37
17th	250	12	37
18th	250	10	29
19th	250	10	29
20th	250	10	29
21st	250	12	30
22nd	250	12	30
23rd	250	19	30
24th	250	19	30
25th	250	14	25
26th	250	14	25
27th	250	14	25
28th	250	7	15

*Data are average value of three replicates

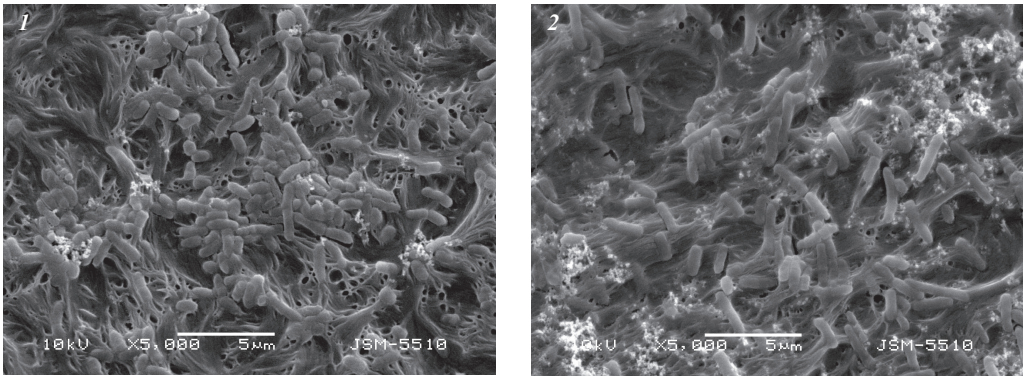

Fig. 4. SEM micrograph of biofilms after phenol treatment (*1*) biofilm PEO-KCM R_5 and (*2*) biofilm PEO-KCM RG_5

As it is evident from the results presented in Fig. 3 the first phenol portion of 1000 mg L^{-1}/24 h caused immediate decrease of the phenol biodegradation by the biofilm PEO-KCM RG_5. This biofilm was not able to bear higher phenol concentration than 600 mg L^{-1}/24 h.

It must be remarked that in the course of our experiments the volumes of the filtered liquid decreased up to 10 times in comparison to those of the initial days (see Table 1). The probable reason for the volume decreasing is the intensive bacterial reproduction in the cryogels.

SEM micrographs of two studied PEO-biofilms after 28 days of phenol treatment are shown in Fig. 4. Our results showed that biofilms remained compact and elastic, with no damages.

Conclusions. PEO cryogels showed high biocompatibility with xenobiotics degrading bacterial strains KCM R_5 and KCM RG_5. The biofilm PEO-KCM RG_5 utilized phenol up to 600 mg L^{-1}/24 h while the biofilm PEO-KCM R_5 was able to utilize phenol at 1000 mg L^{-1}/24 h. In the course of our experiments the volumes of the filtered liquid decreased up to 10 times compared to those of the initial days. The long term phenol treatment does not influence the compactness and mechanical strength of the constructed PEO biofilms.

Acknowledgements. We thank Mrs. Elmira Daskalova from Sofia University for her valuable technical assistance.

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