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PHENOL BIODEGRADATION BY *PSEUDOMONAS PUTIDA* PCM2153

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COMMUNICATION

Keywords: 16S rRNA, aromatic hydrocarbons, degradation, phenolic compounds.

Abstract: Phenol degradation efficiency of *Pseudomonas putida* PCM2153 free cells was experimentally studied. Bacterial cells were acclimatized to phenol what relied on gradually increasing the phenol concentration in the medium. The highest phenol degradation rate was calculated as approximately 15.2 $mg \cdot dm^{-3} \cdot h^{-1}$. Investigated strain degraded the phenol at the concentration of 400 $mg \cdot dm^{-3}$ in 24 h. The result of toxicity analysis showed that acclimatized cells of *P. putida* PCM2153 are able to survive even at as high concentration of phenol as 3000 $mg \cdot dm^{-3}$. The obtained result suggests that the analyzed strain can be used for effective treating of high strength phenolic wastewater. Due to resistance of the strain to high phenol concentration it may be applied in bioremediation of exceedingly contaminated sites, especially where dilution of pollutants cannot be implemented.

INTRODUCTION

One of the main human impacts on the natural environment is pollution of soil and ground water by inorganic and organic chemicals. Among these chemicals, low molecular weight aromatic hydrocarbons such as phenols, the simplest structurally aromatic compounds, can be found. Phenolic compounds are extensively used in polymer resin production, oil refinery, dye industry, pharmaceutical and pesticide industry, semi conductor industry etc [15, 16]. Phenolic compounds are very stable in the environment; therefore various chemical and biological methods for their removal have been extensively investigated. The concentration of phenols in effluents has been observed to vary from 10 to 104 $mg \cdot dm^{-3}$ [18], but microorganisms can survive even at higher concentrations and efficiently biodegrade this hazardous compound [1]. Traditionally, activated sludge process has been used to remove that pollutant from industrial wastewater but this method does not appear to be absolutely satisfactory, because this system is known to be very sensitive to high phenol loading rates [10]. An alternative approach applies pure cultures that are physiologically adapted to metabolize such as contaminant even in very high concentrations. This approach is based on degradation using specially selected microorganisms, which utilize xenobiotics as their energy, carbon, nitrogen and phosphorus or sulfur sources. For this purpose, numerous bacteria mainly from the genera *Pseudomonas*, *Acinetobacter*, *Kleb-*

siella and *Bacillus* are used for phenols degradation. Application of pure cultures is a very promising strategy; therefore finding new microorganisms capable of fast decomposition of phenols is still a hot research topic [3–8, 14].

Among others, the pseudomonads are renowned for their abilities to degrade compounds which are highly refractory to other microorganisms, including aliphatic and aromatic hydrocarbons. *Pseudomonas* metabolism has been the subject of intensive biochemical research, and many of the unique catabolic pathways found in *Pseudomonas* have been described. Studies on the microbial degradation of naturally and artificially synthesized pollutants have been exploited in order to solve the problems of environmental pollution. The ability of *Pseudomonas* strains to degrade various substrates results mainly from their genetic and physiological diversity. The high degree of their evolvability makes a chance for finding new strains that could be applied for pollutants decay during bioremediation processes.

The main goal of this work was to estimate the rate of phenol decomposition by pure culture of *Pseudomonas putida* PCM2153 and to determine the inhibitory effect of high phenol concentration on this species strain.

MATERIALS AND METHODS

Culture media and microorganism

Pseudomonas putida PCM2153 was obtained from the Polish Collection of Microorganisms (Polish Academy of Science, Wroclaw, Poland). The stock culture was stored at 4°C. The cells were cultivated in mineral salt medium (MSM) supplemented with glucose (1 $g \cdot dm^{-3}$). The composition of modified MSM was: $(NH_4)_2SO_4$ (0.5 $g \cdot dm^{-3}$), $MgSO_4 \cdot 7H_2O$ (0.4 $g \cdot dm^{-3}$), KH_2PO_4 (2.65 $g \cdot dm^{-3}$) and $Na_2HPO_4 \cdot 12H_2O$ (9.65 $g \cdot dm^{-3}$). In addition, 1 cm^3 of a trace element solution was added to the medium. This solution contained (g): 20 $FeCl_3 \cdot 6H_2O$, 10 $CaCl_2 \cdot H_2O$, 0.03 $CuSO_4 \cdot 5H_2O$, 0.05 $MnCl_2 \cdot 4H_2O$, and 0.1 $ZnSO_4 \cdot 7H_2O$ in 1 dm^3 0.5 N HCl.

25 cm^3 of inoculum from the late exponential growth phase was transferred aseptically to flask with 475 cm^3 of MSM supplemented with phenol up to concentration of 100 $mg \cdot dm^{-3}$ at the beginning of the culture. After 24 and 48 hours the concentration of phenol was increased up to 200 and 400 $mg \cdot dm^{-3}$, respectively. Cells were grown in 1000 cm^3 flask temperature of 30°C with shaking at 200 rpm. A flask with the same amount of bacterial inoculum and without phenol was used as control. Samples were withdrawn periodically for cell density and phenol concentration analyses. From 48 until 72 hour the culture was monitored more frequently in order to determine the rate of phenol decay. Cell growth was monitored spectrophotometrically by measuring absorbance at 600 nm after 24, 48, and 72 h of cultivation.

Analytical methods

For the phenol concentration determination samples were initially diluted with distilled water to obtain OD value at the level of 0.6. An amount of 1.0 cm^3 of diluted culture medium was collected in Eppendorf tube and centrifuged at 13 000 rpm for 5 min, and supernatants were used for further analysis. The residual phenol concentration was determined in triplicate using standard colorimetric assay, where phenolic compounds react with 4-aminoantypiryne dye. The absorbance of color solution was measured at 550 nm [11].

The toxic effect of phenol on *P. putida* PCM2153 cells was studied by cells cultivation at various phenol concentrations. After 72 hours of acclimatization, the culture was divided into six 50 ml samples and transferred to 250 cm^3 flasks. The cultivation in medium supplemented with phenol up to 500, 1000, 1500, 2000, 2500, and 3000 $mg \cdot dm^{-3}$ were conducted for 24 hours. After that, the cells growth was estimated spectrophotometrically.

RESULTS AND DISCUSSION

Accordingly to our knowledge, even bacteria possessing necessary genetic ability to phenol degradation must be initially adapted to the environment containing such compound. During acclimatization process certain enzymes are induced, what is needed for their usefulness in the metabolism reaction. At the beginning of acclimatization process, the stock culture was transferred into flasks with phenol concentration at the level of 100 $mg \cdot dm^{-3}$. After 24 and 48 hours the concentration of phenol was increased up to 200 and 400 $mg \cdot dm^{-3}$, respectively. This procedure was necessary to prepare the cells for effective phenol biodegradation. The low concentration of pollutant in the first stage of culturing helped to perform this process easily without the risk of inhibition of the bacterial growth [9].

In this study, after acclimatization period, the culture of *P. putida* PCM2153 was supplemented with phenol at concentration of 400 $mg \cdot dm^{-3}$, as it turned out such concentration was best for microorganism's growth. What is worth of indicating, very short lag phase was observed (Fig. 1). Margesin *et al.* [13] studied the efficiency of phenol degradation by cold-tolerant *Arthrobacter* sp. AG31 and mesophilic *Pseudomonas putida* DSM6414. Both strains degraded 200 and 400 mg phenol$\cdot dm^{-3}$ within 48–72 h. Kumar *et al.* [12] studied phenol and catechol degradation by *P. putida* MTCC1194. The strain utilized 1000 mg phenol$\cdot dm^{-3}$ within 162 hours and 500 mg catechol$\cdot dm^{-3}$ within 94 hours. Annadurai *et al.* [2] tested the ability of phenol degradation by *Pseudomonas putida* ATCC31800 as well as by mixture of this strain cells with activated sludge. The best results were achieved using the mixed liquor (activated sludge and *P. putida* cells). When the initial phenol concentration in medium was 0.2 $g \cdot dm^{-3}$, the maximum phenol degradation, at the level of 80% was reached within 48 h. Comparing the phenol degradation ability of strains mentioned above and strain used in our studies, *P. putida* PCM2153 exhibits better phenol degradation ability because it could degrade ca. 400 mg phenol$\cdot dm^{-3}$ within 24 h with the efficiency of phenol decay at the level of 96% (Fig. 1).

In this study phenol degradation rate reached 15.2 $mg \cdot dm^{-3} \cdot h^{-1}$. Comparing this value with other strains of *Pseudomonas* e.g. *P. pseudomallei* and the *P. putida* strains or mixed liquor of activated sludge and *P. putida* ATCC31800 it places *P. putida* PCM2153 slightly above them [2, 12, 17]. This value shows that this strain has great potential in terms of biodegradation of phenol.

During the toxicity test, any of applied concentrations did not influence the survival of bacterial cells. Growth of bacteria and phenol degradation was observed in a medium containing 1000 $mg \cdot dm^{-3}$ (data not shown). At higher phenol concentrations (up to 3000 $mg \cdot dm^{-3}$) the decrease of cells number was not observed after 24 hours of culturing. This observation could suggest that the analyzed strain is extremely resistant to high phenol concentration and could be used for bioremediation of exceedingly contaminated sites, where dilution of pollutants cannot be implemented.

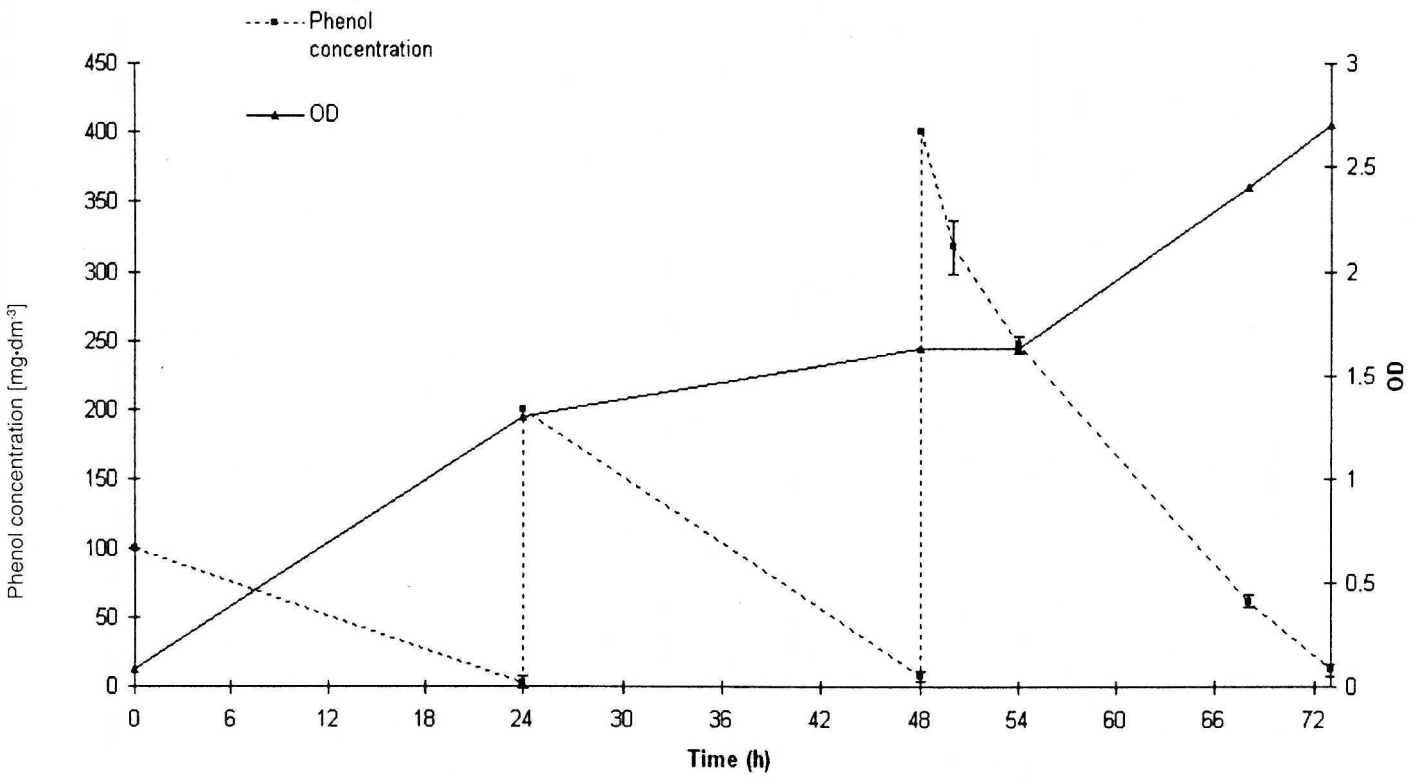

Fig. 1. Efficiency of phenol biodegradation by *Pseudomonas putida* PCM2153 (OD – optical density measured at λ = 600 nm)

The results of this study suggest that investigated strain can be applied for effective removing of phenolic compounds from wastewater. The results presented here, indicate that acclimatization is required for successful decay of phenol by studied *P. putida* strain. Due to the high phenol utilization rate it is worth to consider the designing of degradation system with periodical loading of phenol. We propose adding previously diluted phenol-containing wastewater periodically after every 24 hours, what guarantees efficient phenol removal as well as the proper bacterial growth. The phenomenon of the cell survival at concentrations as high as 3 g phenol·dm^{-3} shows a great potential of this strain in bioremediation.

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BIODEGRADACJA FENOLU PRZEZ *PSEUDOMONAS PUTIDA* PCM2135

W doświadczeniu analizowano efektywność rozkładu fenolu przez szczep *Pseudomonas putida* PCM2135. Komórki bakteryjne zostały poddane adaptacji do wysokich stężeń fenolu, która polegała na stopniowym zwiększaniu jego stężenia w pożywce. Maksymalna szybkość rozkładu fenolu wyniosła 15,2 $mg \cdot dm^{-3} \cdot h^{-1}$. Badany szczep usunął 400 mg fenolu w ciągu 24 godzin. Przeprowadzone testy toksyczności wykazały, że zaadaptowane komórki *P. putida* PCM2153 są zdolne do przeżycia w roztworze fenolu nawet o stężeniu 3000 $mg \cdot dm^{-3}$. Uzyskane wyniki sugerują, że badany szczep może być wykorzystany do efektywnego oczyszczania ścieków charakteryzujących się wysokim stężeniem fenolu. W zawiązku z odpornością szczepu na wysokie stężenia fenolu, może on być wykorzystywany do bioremediacji terenów silnie zanieczyszczonych fenolem, gdzie rozcieńczanie zanieczyszczeń jest niemożliwe.