BIODEGRADATION POTENTIAL OF *PSEUDOMONAS PUTIDA* TO PHENOL COMPARED TO *XANTHOBACTER AUTOTROPHICUS GJ10* AND *PSEUDOMONAS DENITRIFICANS* STRAINS

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ABSTRACT

Phenol is a waste product from petroleum, pharmaceutical and plastic industries. It is a major environmental pollutant. There is a variety of methods for treatment of waste water containing phenol. The applied physico-chemical methods are often economically unfeasible and may cause onset of other toxic products. For this reason, microbiological methods are preferred because the microorganisms present use phenol as the sole source of carbon and energy. After preliminary adaptation of Pseudomonas putida strain, a degradation of 1,9 g/l of phenol is achieved over a period of 23 days using a laboratory bioreactor and a feed process. The experiments carried out prove also the tolerance of Xhantobacter autotrophicus GJ10 and Pseudomonas denitrificans to the presence of 0.3 g/l of phenol in the culture medium.

Keywords: phenol, biodegradation, tolerance, feeding processes.

INTRODUCTION

Phenol and its derivatives are classified as some of the most dangerous organic pollutants [1]. They can reach the environment following two main ways - through industrial wastewater and natural events such as the occurrence of forest fires, or water running out of the town areas. Every year, different chemical and woodworking plants, coal mines generate large amounts of phenol in their wastewater. Various techniques are used to reduce its concentration to acceptable and harmless values including biodegradation, adsorption, ion exchange and sorption on activated carbon. The biological treatment is the most promising and cost-effective method. It is believed that it leads to a complete phenol mineralization [3]. Over the last three decades, the attention of a large number of researchers is focused on the degradation of phenol by different types of microbial cultures. Annadurai et al. [2] report biodegradation and adsorption of phenol using immobilized *Pseudomonas* cells on chitin. Their research contributes to the more economical treatment of industrial waste water of low phenol concentrations of 0.200 mg/l. Many types of aerobic bacteria can consume aromatic compounds as an only source of carbon and energy. It is known that phenol is successfully degraded by the strain *Pseudomonas putida*. Many authors have publications on this topic [4 - 9]. The biodegradation occurs through adaptation of the culture to the phenol's impact. Wang et al. [10] conduct a preliminary cold-adaptation of *Pseudomonas putida* in presence of 4-chlorophenol and phenol initial concentrations of 20 mg/l - 400 mg/l. El-Naas et al. [11] gradually activate and adapt the microbial culture to reach 300 mg/l of phenol. The glucose concentration is brought to zero as a result of its reduction starting with 10 mg/l/day phenol to reach a phenol concentration of 300 mg/l.

The strain *Pseudomonas putida* is a rod-shaped gram-negative bacterium known for its ability to degrade

organic solvents with a particularly high efficiency in respect to phenol removal [12]. The degradation of this hazardous pollutant by many other bacteria such as *Bacillius* sp. [13], *Alcaligenes faeca*lis [14], *Candida tropicalis* [15], *Nocardia hydrocarbonoxydans* [16], *Ralstonia taiwanensis* [17], and active sediment [18] is reported. Under aerobic conditions, phenol is decomposed to carbon dioxide passing through various intermediates. Factors such as its concentration, the medium temperature, the presence of other biogenic elements have a significant effect on the successful phenol degradation.

The main purpose of this study is to prove the tolerance of *Xanthobacter Autotrophicus GJ 10*, *Bradyrhizobium japonicum 273* and *Pseudomonas denitrificans* to the presence of phenol and the latter biodegradation by *Pseudomonas putida*.

EXPERIMENTAL

Strains of bacteria and culture media

The strains *Pseudomonas denitrificans* (NBIMCC 1625) and *Pseudomonas putida* (NBIMCC 1046) were obtained from the Bulgarian National Bank for Industrial Microorganisms and Cell Cultures. The *Xanthobacter autotrophicus GJ 10* strain was obtained from the Department of Biochemistry of the University of Groningen (The Netherlands) through the Bulgarian National Bank for Industrial Microorganisms and Cell Cultures. These strains were cultured in the following media in absence of phenol:

Pseudomonas denitrificans: peptone - 10 g/l, yeast extract - 1 g/l, NaCl - 10 g/l. The inoculum developed for up to 24 h at 30°C per shaker at 50 rpm to avoid unwanted aeration;

Pseudomonas putida: peptone - 10 g/l, meat extract - 10 g/l, NaCl - 5 g/l. The inoculum developed up to 24 h at 30°C per shaker and 100 rpm;

Xanthobacter autotrophicus GJ10 was cultured in a mineral media (MMY) containing (per liter): 5.37 g $Na_2HPO_4.12H_2O$, 1.36g KH_2PO_4, 0.5g $(NH_4)\ 2SO_4$, 0.2g. $MgSO_4\ .7H_2O$ and 0.015 g, 0.25 yeast extract, 2 g. $C_{12}H_{22}O_{11}$ supplemented with 1 ml of microelement solution containing (per liter): 2.5 mg of $CaCl_2.2H_2O$, 1 mg of $FeSO_4.7H_2O$, 0.05 mg of $ZnSO_4.7H_2O$, 0.05 mg of H_3BO_4, 0.02 mg of $CoCl_2.\ 6H_2O$, 0.015 mg of $Na_2Mo_4.2H_2O$ and 0.02 mg of $NiCl_2.6H_2O$. The inoculum developed up to 24 h at 30°C per shaker and 100 rpm.

The studies of the bacterial strains tolerance were conducted in flasks in a rotary shaker under batch conditions at a low revolution speed (100 rpm) at a temperature of 30°C. The initial concentration of phenol was 0.3 g/l. A mineral medium containing glucose or phenol was the only carbon source.

The phenol biodegradation experiments by *Pseudomonas putida* were carried out in a 0.500 ml fermenter (Bioflo, New Brunswick Scientific, Edison, NJ) with continuous stirring (150 rpm) at an operating temperature of 30°C. The stirring speed was low to avoid the formation of air bubbles or undesirable suction of air. The bio-reactor was sterilized prior to the experiments. After cooling it was charged using the working media and 10 % (vol.) of inocula.

The initial phenol concentrations, which were between 0.06 g/l and 0.3 g/l, were selected on the ground of the literature [10, 11].

Analyses

Phenol concentration

Phenol was determined photometrically by a standard colorimetric method based on the formation of a red color dye by treating the phenol-containing sample with 3.5 % 4 aminoantipyrine and 20 % ammonium persulfate in presence of a buffer (50 g of NH_4Cl in 900 ml of distilled water). Ammonia was used to adjust pH to 9.3. The samples were centrifuged and then treated by the above mentioned reagents. After 15 min their light absorbance was measured at a wavelength of 540 nm. The concentration of phenol was calculated on the ground of a calibration line.

Biomass

The biomass concentrations read as optical densities were determined spectrophotometrically by a Specol spectrophotometer (Carl Zeiss, Jena, Germany) at a wavelength of 610 nm.

RESULTS AND DISCUSSION

Glucose is the most preferred carbon source for microbial growth because it is readily biodegradable by many microbial strains [10]. Fig. 1 shows how the optical density of the microorganisms' biomass increases with time increase using a mineral medium containing glucose (1 g/l). A pronounced exponential phase with almost absent lag phase is seen in this case. The highest biomass concentrations are attained in presence of *Pseu-*

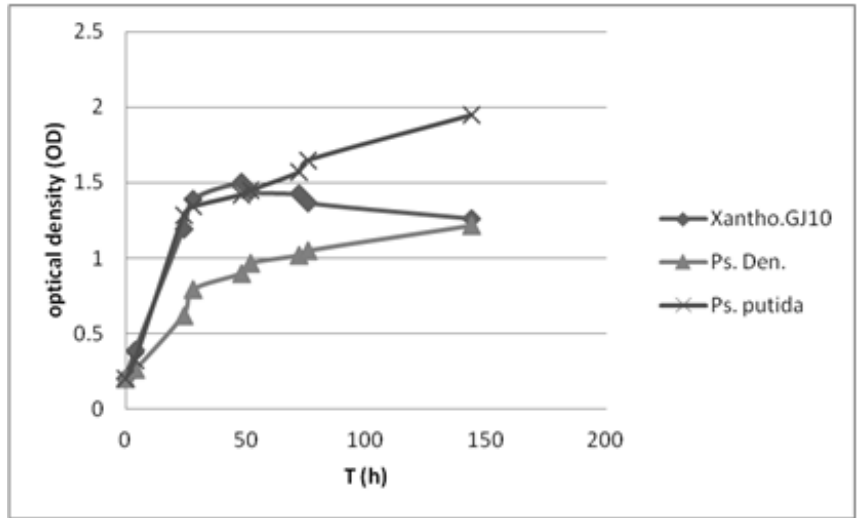

Fig. 1. Cultivation of bacterial X*anthobacter autotrophicus GJ10*, *Pseudomonas denitrificans* and *Pseudomonas putida* strains in mineral medium and absence of phenol and present of glucose.

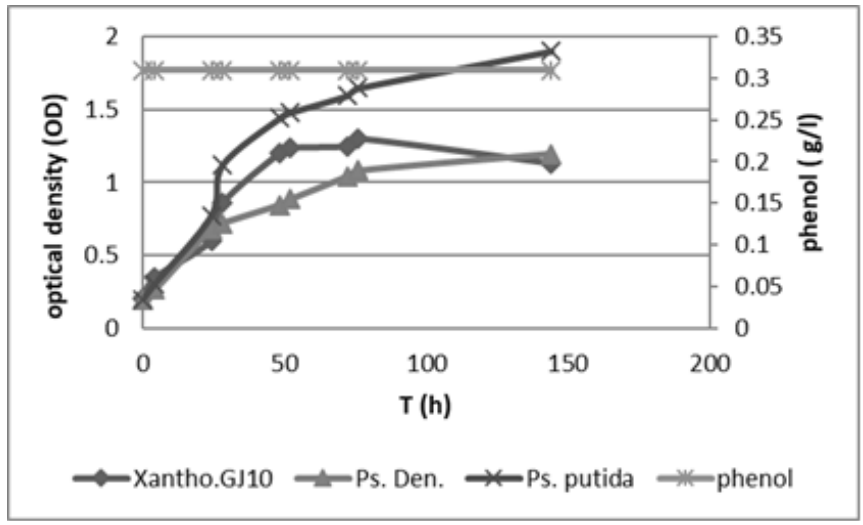

Fig. 2. Cultivation of bacterial X*anthobacter autotrophicus GJ10*, *Pseudomonas denitrificans* and *Pseudomonas putida* strains in a mineral medium and presence of phenol (0.3 g/l) and glucose (1 g/l).

domonas putida. Fig. 2 illustrates the microorganisms' cultivation in presence of 0.3 g/l phenol and 1 g/l glucose for the three different strains. The graph shows that the bacteria are tolerant to the presence of the pollutant in the medium and accumulate almost the same amount of a biomass as when grown in a phenol-free medium although the phenol concentration is relatively high. In all three cases the phenol concentration decreases slightly, i.e. glucose is the only carbon source used for the bacterial growth.

The biodegradation of phenol is accomplished by gradually adapted bacteria from the strain *Pseudomonas putida* as shown in Fig. 3. It starts at a sufficiently low pollutant concentration of 0.06 g/l and proceeds within 4 days. When the adaptation is performed with higher phenol concentrations, the time of the lag phase increases, i.e. the adaptation period is prolonged [10]. Under the conditions of the experiments carried out a biodegradation rate of 50 % is achieved. The gradual increase of the phenol concentration shortens drastically the periods of degradation as a result of the adapted culture presence: five cycles of phenol addition are made

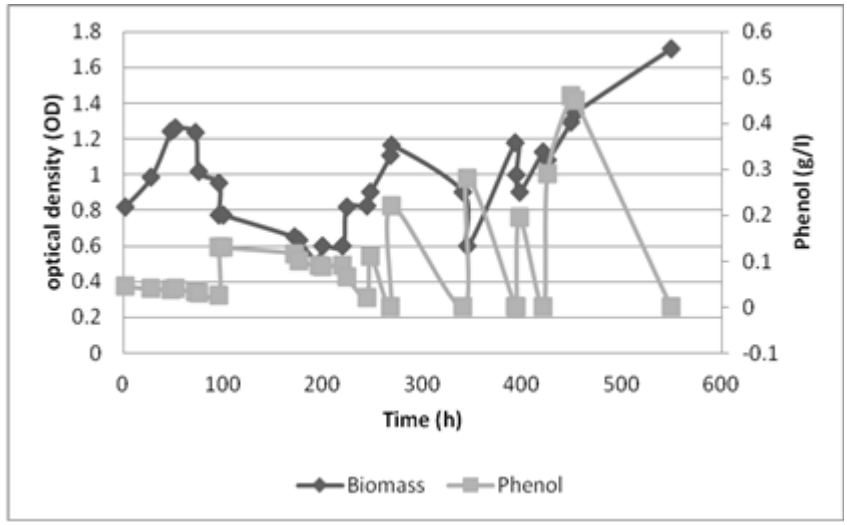

Fig. 3. Biodegradation of 1.9 g/l phenol from the *Pseudomonas putida* strain.

within the period from the 250th to 550th hour. The last cycle of phenol biodegradation starts at 0.4 g/l phenol and ends successfully within about 100 h. The amount of the degraded phenol after the culture adaptation over a period of 23 days is 1.9 g/l. A comparative experiment of abiotic chemical degradation of 0.2 g/l phenol in a 500 ml flask is performed. It is found that there is no chemical degradation within 72 h. This confirms the conclusion that the main degradation of phenol is due to the enzymatic activity of the bacterial cells.

The calculations of the specific growth rate of the bacteria from the strains *Xanthobacter autotrophicus GJ10, Pseudomonas denitrificans* and *Pseudomonas putida* show that they are similar and independent on the composition of the media for the whole concentration range studied (Table 1).

Table 1. Specific cell growth rates of *Xanthobacter autotrophicus GJ10*, *Pseudomonas denitrificans* and *Pseudomonas putida.*

. Types of media	Specific cell growth rates, μ (h^{-1}) of *Xanthobacter Autotrophicus GJ 10*	Specific cell growth rates, μ (h^{-1}) of *Pseudomonas denitrificans*	Specific cell growth rates, μ (h^{-1}) of *Pseudomonas putida*
Poor media (mineral) without phenol	0.1045	0.046	0.0810
Poor medium (mineral) with phenol 0.3g/l	0.1051	0.0449	0.0858
Rich media (with glucose) without phenol	0.1038	0.0459	0.0819
Rich media (with glucose) with phenol 0.3g/l	0.1063	0.0443	0.0843
Rich media (with glucose) with phenol 0.06g/l	0.1063	0.0472	0.0863

CONCLUSIONS

The investigation carried out provides the following conclusions:

● The specific cell growth rate of strains *Xanthobacter autotrophicus GJ 10*, *Pseudomonas denitrificans* and *Pseudomonas putida* is independent on the composition of the medium and is identical in the phenol concentration range from 0.06 g/l to 0.3 g/l.

● *Xanthobacter autotrophicus GJ 10*, *Pseudomonas denitrificans, Pseudomonas putida* are shown to grow irrespectively of 0.3 g/l presence of phenol in the medium.

● After an adaptation period of 4 days *Pseudomonas putida* strain degrades successfully 1.9 g/l of phenol for 23 days.

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