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Cometabolic degradation of *para*-nitrophenol and phenol by *Ralstonia eutropha* in a Kissiris-immobilized cell bioreactor

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Abstract–*Ralstonia eutropha* was able to degrade *p*-nitrophenol (PNP) at concentrations ranging from 5 to 15 mg l^{-1} in the presence of phenol which was kept at the constant concentration of 200 mg l^{-1}. More than 90% of phenol was degraded within 30 h and in the absence of PNP. While in this time period and in the presence of 15 mg l^{-1} less than 30% of phenol was degraded and PNP removal ability of the test bacterium was about 20%. Kissiris as a natural source of silicon dioxide having a very rigid structure with many micropores irregularly distributed throughout its surface was used to evaluate effectiveness of the cell immobilization using a Kissiris-immobilized cell bioreactor [ICB]. By applying phenol-feeding regime in the ICB operated in a batch recycling mode, simultaneous degradation of phenol in total amount of 1,000 mg l^{-1} with 15 mg l^{-1} PNP was achieved within 40 h.

Key words: Cometabolic Degradation, Kissiris-immobilized Cell Bioreactor [ICB], *p*-Nitrophenol (PNP), Phenol, *Ralstonia eutropha*

INTRODUCTION

Applications of nitroaromatics as phenol derivatives in the manufacture of a variety of chemicals such as dyes, pigments, polymers, plastics, explosives, pharmaceuticals, pesticides, and fungicidal agents have generated attention towards ways of reducing environmental impacts of these hazardous compounds [1-3]. According to the United States Environmental Protection Agency (EPA) regulations, *p*-nitrophenol (PNP) concentration in natural waters should be less than 10 mg l^{-1} [4,5]. Different physical and chemical methods are available for the pollutants removal, but the use of biotechnological techniques has gained popularity mainly on the basis of the process cost and friendliness for the ecosystems. The first step in the bio-oxidative cleavage of the hydrocarbons (consider the ring structure of the aromatics for instance) is the hydroxylation reaction catalyzed by multienzyme system, in which cooperative actions of several coenzymes are of great importance: heme coenzymes, iron-sulfur clusters, flavin coenzymes, and nicotineamide coenzymes [6]. Uniqueness of flavoproteins relies on the ability of these flavin-dependent enzymes in serving as the switch point from the two-electron transfer systems to the one-electron transfer processes, important in oxygenases which incorporate oxygen atoms from O_2 into the relevant substrate [7]. In aerobic microorganisms the performance of these enzymes-coenzymes in the carbon metabolisms and in the generation of energy through the terminal electron transport processes is decisive [6].

Dissociation of the electron transport system from generation of ATP (an example of the membrane transport) has been found to be the dominating mode of toxic action of nitrophenols as well as chlorophenols in aquatic organisms [4]. Studies on these uncouplers of oxidative phosphorylation have shown that the uncoupling activity may be masked through the lipophilicity and acid characters of these phenolic derivatives, described as the octanol/water partition coefficients in logarithmic form (log K_{ow}) and the solution phase acidity (pK_a). The nonspecific toxicity of these compounds was the result of combinations of log k_{ow} and pK_a and has been demonstrated with the use of pollen (tobacco plant) tube growth test [4].

The integrity of the cytoplasmic membrane (lipophilic in nature) changes upon cellular passage of the nonpolar compounds and study on effects of tetrachlorobiphenyl and biphenyl on the composition of the cell membrane of *Ralstonia eutropha* showed increase in the saturated fatty acid content of the membrane upon transferring bacterial cells from fructose-containing medium to the media having these nonpolar test substances, and this negatively affected the membrane fluidity character [3]. Further work by the researchers on these effects showed that at least 25 generations were needed for the cells to recover themselves and gain enzymatic ability to change the fatty acids composition of the membrane to the original pattern.

Despite these toxic characteristics of nitroaromatics, several microorganisms have been identified as nitroaromatics degraders, and at least four enzymatic mechanisms have been suggested in this area: oxidative removal of the nitro group from the aromatic nucleus with production of nitrite, initial reduction of the nitro group with production of an aminoaromatic product, complete reduction of the nitro group and its removal with the formation of a hydride-Meisenheimer complex, and use of the nitro reducing enzymes and partial reduction of nitro group to a hydroxlamin yielding ammonia upon the hydrolysis [8,9].

As mentioned above, several components are involved in the enzymatic hydroxylation system, oxygenases (monooxygenases and dioxygenases) as the flavin-dependent enzymes play crucial roles in oxidative removal of the nitro group from PNP [6,7,10-13]. Aerobic removal of nitro group and formation of nitrite is shown to occur either at the start of the oxidation process with formation of hydroquinone (*Pseudomonas* sp., *Moraxella* sp.) or after substitution

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of hydroxyl group into PNP structure followed by formation of 2-hydroxy-1,4-benzoquinon intermediate (*Rhodococucus opacus*) [13, 14]. Formation of the latter compound has also been shown in *Bacillus sphaericus* in which release of nitro group as the nitrite at the beginning of the reaction was followed by substitution of hydroxyl group into *p*-benzoquinon intermediate [11,12].

Co-existence of the pollutants with each other has raised questions about probable interactions between these toxic/hazardous substances, which could affect the microbial cells growth and metabolic activities in degrading the compound of the interest. According to the reported studies in the literature, several environmental pollutants insensitive to ordinary biological degradation could be subjected to cometabolism, in which a growth supportive substrate plays a crucial role biochemically in the degradation of the nongrowth substrate. The growth substrate apparently has two effects on cometabolism process: cell growth stimulation and increasing the nongrowth transformation through inducing certain enzymatic systems that are usable by the microorganism in degrading both the growth and nongrowth substrates [15]. Adaptation of *Pseudomonas putida* to biodegradation of PNP was found to be highly dependent on the cell density [16]. Growth inhibition by either growth or nongrowth substrate is considered as a reasonable approach for describing the mechanisms involved in the kinetics of cometabolisms [17]. Although PNP biodegradation has been extensively studied, works on cometabolism of PNP with phenol as the growth substrate using *R. eutropha* are limited.

Performance of microbial cells would be improved by fixing in a predetermined matrix, so the immobilized cells can be repeatedly used in the bioprocess of the interest. The subject of cometabolism biotransformation in the environmental biotechnology becomes even more important considering cell immobilization concept. The challenging point in immobilization of living cells is not only finding an appropriate immobilization technique but also using a biocompatible material as the cell support. The given definition for the latter is that substance which does not cause any damage to cells genetic machinery and cells functions [18]. One-fourth of the mass of the earth's crust is composed of silicon atoms, which its binary compound with oxygen called silica (SiO_2) comprises more than 70% of the Kissiris contents [19,20]. Formation of Kissiris is through the foam thickening of volcanic lava (natural glass) and the volume of the gas released during this process is almost equal to the glass mass and this characteristic has made Kissiris in having many micropores, which are irregularly distributed throughout the surface [20]. This natural silicate mineral with highly porous structure, good mechanical strength, stability towards chemical agents, and no wettability by water could be very attractive biomaterial for the microbial cells attachment and bioreactor studies.

An immobilized cell bioreactor [ICB] operating with medium recycle and without separation of the microorganism could be an effective reactor configuration in keeping the biodegradation process stable since this cell cycling mode facilitates physiological adaptation of the microbial cells to the test pollutant [21]. Thus, concentrations of the organic pollutant could be increased without expressing significant growth inhibitory behavior towards the test microorganism.

In this work, cometabolism of PNP on phenol by the phenol-adapted *R. eutropha* was studied using phenol at either of two constant concentrations 200 and 500 mg l^{-1} and PNP at three concentrations of 5, 10, and 15 mg l^{-1}. Performance of the test bacterium in a Kissiris-immobilized cell bioreactor was evaluated where the operation mode in this ICB was in the form of the repeated batch recycling considering application of phenol-feeding regime.

MATERIALS AND METHODS

1. Microorganism and Culture Conditions

The bacterium *R. eutropha* (PTCC 1615) was obtained from Persian Type Culture Collection (Iranian Research Organization for Science and Technology "IROST") in lyophilized form. The bacterial culture was maintained in yeast extract pepton [YEP] growth medium with the following composition (g l^{-1}): glucose, 3; yeast extract, 2; peptone, 3; KH_2PO_4, 1; K_2HPO_4, 1; and $(NH_4)_2SO_4 \cdot 7H_2O$, 0.05 and $MgSO_4 \cdot 7H_2O$, 0.05. In preparing solid growth media, agar (15 g l^{-1}) was added. The pH of the medium was adjusted to 7 using 1 N NaOH. The medium was sterilized in an autoclave (121 °C for 20 min). Growth of the bacterial culture was measured spectrophotometrically (absorbance at 600 nm) and the culture (10% v/v having absorbance equal to 600 nm) was then inoculated into 100 ml of the YEP growth medium in a 250 ml conical flask and incubated at 30 °C in a shaker incubator (150 rpm) for 24 h. The YEP-grown *R. eutropha* culture was then transferred to a medium which was prepared with glucose and phenol but without yeast extract and peptone [22]. Removal of glucose from this culture medium containing the *R. eutropha* cells was gradual while it was being substituted with phenol; further details of this phenol adaptation process are given elsewhere [23,24]. The biodegradation experiments were performed using an appropriate volume of the phenol-grown culture (10% v/v).

2. PNP Cometabolic Degradation with Phenol

The media used for the phenol degradation studies were prepared by diluting 1,000 mg l^{-1} of the stock phenol solution to the desired concentrations (200, 500 mg l^{-1}) with use of the mineral salt medium (MSM). The MSM had the following composition (g l^{-1}): KH_2PO_4, 0.5; K_2HPO_4, 0.5; $(NH_4)_2SO_4$, 0.5; and $MgSO_4 \cdot 7H_2O$, 0.05. For having 200 mg l^{-1} phenol as the initial concentration for instance, 20 ml of 1,000 mg l^{-1} phenol solution was added to 80 ml of the MSM. Thereafter, the pH of the test solution was adjusted to 7 with 1 N NaOH and the medium was sterilized in an autoclave at 121 °C for 20 min.

On the basis of the preliminary works, PNP at the three different concentrations of 5, 10, and 15 mg l^{-1} was used in PNP cometabolic degradation experiments. With use of mortar and pestle PNP was completely ground and these fine particles then readily became solubilized in water.

The biodegradation studies were performed batchwise using either freely suspended cells (shake flask experiments) or Kissiris immobilized cells (ICB experiments).

3. Shake Flask Experiments (Freely Suspended Cells)

Initial phenol concentrations 200 and 500 mg l^{-1} without or with PNP at the three selected concentrations (5, 10, and 15 mg l^{-1}) were used in shake flask experiments. In each of these experiments, a 250 ml conical flask containing 100 ml of the test solution (MSM having phenol with or without PNP, each at the selected concentration) was inoculated with the culture (10% v/v having absorbance equal to 0.6 at 600 nm) of the freely suspended cells. The flasks

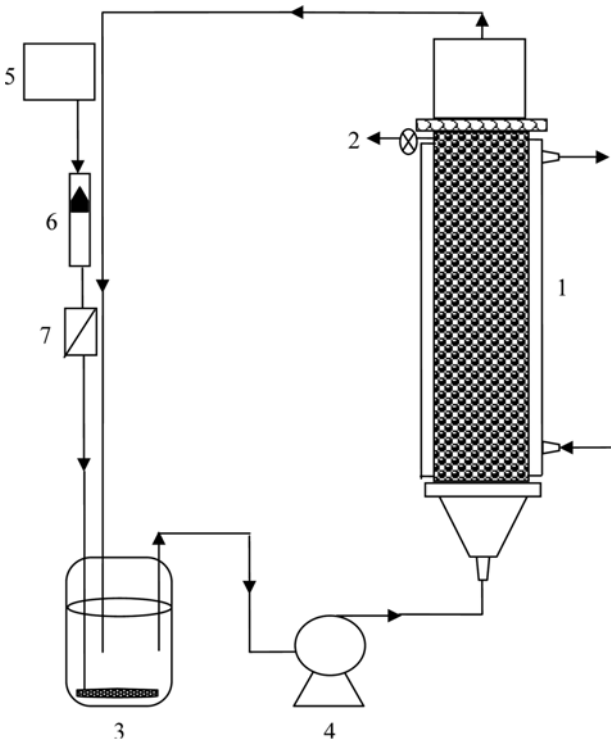


Fig. 1. Experimental setup.

1. ICB with tempering jacket
2. Samplingport
3. Aeration tank containing test substrate solution
4. Prestatic pump
5. Air pump
6. Flowmeter
7. Air filter

were incubated at 30 °C in a shaker incubator (150 rpm). Samples were taken out regularly for phenol, PNP, and biomass analyses.

4. ICB Experiments: Construction and Inoculation

The ICB, in the form of a packed bed reactor (PBR) used in the present study consisted of a vertical glass jacketed column with a diameter of 4 cm, height of 30 cm, ending with a cone-shaped bottom (Fig. 1) and total volume of 500 ml (constant temperature of 30 °C inside the ICB was provided). The column was filled with 150 g of Kissiris pieces (1 cm diameter) as the cells support material and the height of the packed section was 25 cm. The Kissiris pieces were purchased from the local market; these material originally are from Qazvin which is located in northwest of Tehran. An aeration tank with a 12 cm diameter and 30 cm height was situated externally to the column and contained 500 ml of the test solution, which was aerated using an air diffuser and air pump [RESUN AC-9603, China] with an aeration rate of 0.5 vvm and 1,000 ml as the total volume of the test solution was recirculated through this tank (Fig. 1).

Based on our previous experimental works, Kissiris was used as the support material for the cell immobilization and by circulating the freely suspended cells of the phenol-adapted *R. eutropha* through the packed bed the cell immobilization was complete within approximately two days [20,25]. In this stage of the immobilization the mineral salt solution containing 200 mg l^{-1} phenol was used as the growth medium. Feeding the system with PNP (one liter of 15 mg l^{-1}) as cometabolic substrate was performed with phenol as the growth substrate, where these two solutions were placed in the container as shown in Fig. 1. With a peristaltic pump [Heidolph 510] a flow rate of 25 ml min^{-1} was provided for this feeding system and the effluent solution was recirculated back to the test solution container using the same flow rate; this mode of recycling gave a total of 36 cycles in 24 h (operation was in a repeated batch recycling mode). The process was continued until the phenol biodegradation was nearly complete (≤10 mg l^{-1}). Next, the liquid was removed and the whole system was washed thoroughly with mineral medium and the column was filled with a fresh test solution containing phenol at 200 mg l^{-1}, and the process was repeated four more times (40 h).

5. Analytical Methods

The measurement of phenol concentration was performed spectrophotometrically (JASCO V-550 UV-Vis Spectrophotometer) using the Folin-Ciocalteau reagent as per standard procedure [26]. At regular time intervals, an appropriate aliquot was withdrawn from the test culture system (growing culture), and after centrifugation (8,000 rpm for 15 min), the amount of PNP was determined in the supernatant. The PNP measurement also was performed spectrophotometrically by reading the absorption maximum at 400 nm after adding 5 ml of 0.1 N NaOH to the 1 ml of the supernatant. A yellow color was observed after standing the treated sample for 5 min [27].

Biomass concentration was measured spectrophotometrically by reading optical density (OD) at 600 nm, while OD values were converted into the dry cell weight (DCW) with use of a calibration curve (OD versus DCW).

Upon completion of the biodegradation experiments samples of Kissiris pieces having attached biomass were taken from different sections of the ICB and the pieces then were washed with distilled water, dried in an oven (105 °C for 24 h) and weighed. These pieces were heated using a laboratory heater in a 0.25 N NaOH solution for 10 min, and the obtained samples were washed with distilled water, dried in an oven (105 °C for 24 h), and weighed. Determination of the biomass content was on the basis of the difference in weight between the dried Kissiris immobilized cells and dried bare Kissiris pieces; the attached biomass dry weight in the entire reactor then was calculated. All the biological treatments as the independent experiments have been performed in duplicate and final measurements (analytical methods) were reported as the mean of two tests. The standard deviation for all these measurements was too low to show as bars in the relevant graphs.

Scanning Electron Microscopy ('SEM' Philips XL-30) was used to obtain SEM images by coating the test kissiris pieces with a thin layer of gold under vacuum and with following the standard procedure.

For the analyses of phenol and PNP by high pressure liquid chromatography (HPLC) the test culture sample was centrifuged at 8,000 for 15 min (Heraeus, Germany) and the supernatant containing intermediates were used. On the basis of comparing retention times and UV visible spectra with those of the external standards, the intermediates in the supernatant were identified by using isocratic reverse-phase HPLC (Waters 2795, Milford, MA, USA) equipped with a Nova pack C_{18} column (Waters) at 280 nm. The mobile phase consisted of methanol-water-acetic acid (100 v : 100 v : 2 v). The injection volume was 40 µl.

RESULTS AND DISCUSSION

1. Free Cells and PNP Cometabolic Degradation on Phenol

In the present study, phenol at the two concentrations of 200 and

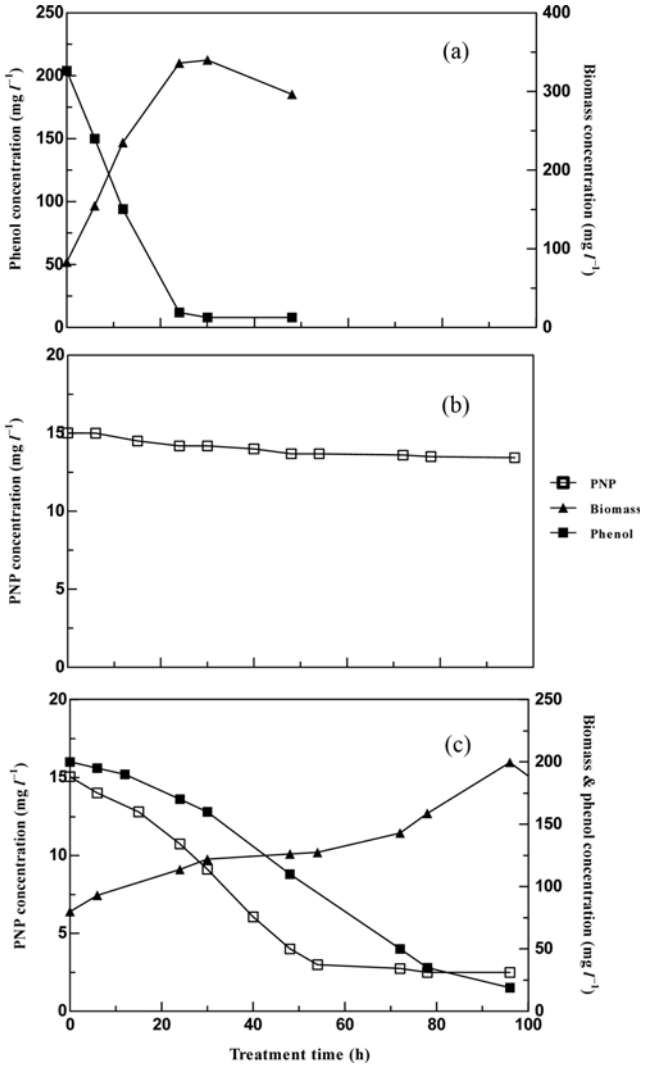


Fig. 2. Cometabolic degradation of PNP at 15 mg l^{-1} with phenol at the initial concentration of 200 mg l^{-1} used as the growth substrate by *R. eutropha*.

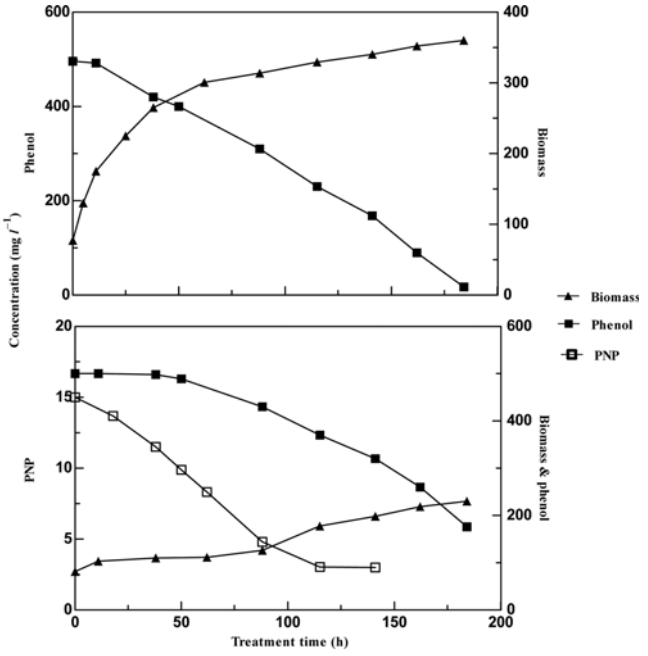


Fig. 3. Cometabolic degradation of PNP at 15 mg l^{-1} with phenol at the initial concentration of 500 mg l^{-1} used as the growth substrate by *R. eutropha*.

500 mg l^{-1} was used. At this level no growth inhibition for the phenol-adapted *R. eutropha* was observed [23,24]. Similar initial levels of phenol also have been used by others in studying the cometabolic degradation of chlorophenols and nitrophenols with phenol [28-30]. Results of the earlier literature show that phenol substrate for *R. eutropha* is deficient in energy, i.e., the amount of energy generated during the phenol dissimilation is not enough to assimilate carbon consumed by the cells [31]. *R. eutropha* used in the present study was able to consume phenol as the sole source of carbon and energy. Fig. 2(a) shows that at 200 mg l^{-1} as initial phenol concentration, the biomass level increased rapidly and reached about 350 mg l^{-1} in 30 h without any lag time. The biomass decreased sharply thereafter and its amount reached to the level below 300 mg l^{-1} within the next 10 h. The bacterial cells were not able to consume PNP when it was the sole source of carbon and energy (Fig. 2(b)). As seen in Fig. 2(c), the biomass formed at the expense of phenol was responsive to PNP as the cometabolic substrate and decreasing trend of PNP reached to a palatue. The likely explanation is that phenol when it decreased below 100 mg l^{-1} was not supportive for the PNP cometabolism, while the increasing trend of the biomass formation was not as much as that observed in Fig. 2(a) where phenol was the only source of the cells growth. Apparently the growth inhibitory function of PNP resulted in slowness of the active primary substrate consumption. By increasing concentration of phenol from 200 to 500 mg l^{-1}, increase of the cells was slowed indicating appearance of lag phase as shown in Fig. 3 and by considering this gradual biomass growth, 80% of the maximum biomass was attainable within less than 65 h. As pointed out in the literature, there are situations in which the amount of cells affects the enzyme induction. For instance, a particular intermediate product formed during metabolic activities may induce an enzyme, and if the cells cannot retain the intermediate and the metabolite is diffused out of the cells, then the small bacterial populations are not capable of producing enough quantity of this compound of interest and the cells do not respond effectively in the enzyme induction process [16].

Study on cometabolism of phenol and PNP substrate in the present work was based on the results of our previous works, in which phenol utilization capability of *R. eutropha* was considerable and activity of the bacterium towards PNP was low [22,23]. Suggestion in the literature in cases of low concentrations of the cometabolic substrates is to use less complicated kinetic expressions mainly because of non-dominance of the transformation capacity and product inhibition terms which are usually included in the models that have been developed in describing the cometabolic activities [17,32]. Regeneration of the reductants, mostly in the form of coenzyme molecules, is highly dependent on the availability of the growth substrate, and competition between growth and nongrowth substrate for the active site of the some enzymes which can react with more than one substrate (such as oxygenases enzymes), decreases the transformation rate of each of these substrates. Sequential utilization of growth and nongrowth substrate is found to be preferred mode of

the microbe's metabolic activities [17].

In the present study, thus, it was not expected to see competition between growth and nongrowth substrate for a particular enzyme system in the test concentration ranges herein (200 mg/5 mg; 200 mg/10 mg; 200 mg/15 mg). The overall rate of transformation of growth - as well as nongrowth - substrate could reasonably be free of the competition subject. For instance, study on malathion biodegradation by *Acinetobacter johnsonii* showed that complete degradation of malathion occurred in the presence of sodium acetate or sodium succinate, while cometabolism of glucose or fructose substrate with this pesticide resulted in only 26% and 30% of the degradation, respectively [15]. These researchers used a zero-order kinetics model to describe the process, and the highest rate constant at 3.2 mg l^{-1} was obtained at very high ratio of growth to nongrowth substrate (150 mg/mg), where the growth substrate concentration ranged from 500 to 25,000 mg l^{-1} and the nongrowth substrate concentration was 100 to 200 mg l^{-1} [15]. The ratios for phenol: PNP in the present study ranged from 40 to 70, where 200 mg/5 mg gave the highest ratio and 1,000 mg/15 mg in the [ICB] study gave the lowest ratio. A reasonable approach in treating these results thus was to describe a first-order kinetics model, in which dependency of the degradation rate on the growth and nongrowth substrate concentration could be seen. The following expression was used to quantify biodegradation rates (r_{phenol} and r_{PNP}, mg l^{-1} h^{-1}):

$$r = -\frac{dS}{dt} = K(s)$$

where S is the concentrations of the either primary or cometabolic substrate (mg l^{-1}). As shown in Table 1, the test bacterium was more efficient in utilizing phenol at the low initial level of PNP (r_{phenol}= 2.68 versus r_{phenol}=1.88 mg l^{-1} h^{-1}) while at these conditions; the cells were less active in PNP degradation (0.06 and 0.16 mg l^{-1} h^{-1}).

A simplified version of the PNP biodegradation pathway is given in Fig. 4. The detailed analyses of the pathway and intermediates involved were not carried out here because our primary concern was to find the capability of consuming PNP by the phenol-adapted *R. eutropha*. HPLC analyses of phenol and PNP but were performed in this study. The first step in phenol bio-oxidation following hydroxylation catalyzed by phenol hydoxylase as a monooxygenase enzyme, is the breakage of the ring structure of this hydroxylated intermediate [33]. A result of catalytic action of 2, 3-dioxygenase as the decyclizing enzyme on catechol (1, 2-benzenediol) is the formation of 2-hydroxymuconate semialdehyde (2-HMS) as the immediate product of catechol cleavage at the meta position [34]. The color intensity of 2-HMS was found to be directly related to the growth rate and/or the rate of phenol degradation by *R. eutropha* [35]. During PNP degradation by *Moraxella* sp. formation 2-HMS as a result of hydroquinone (1, 4-benzenediol) oxidation has also been reported [14]. Hydroquinone is the hydroxylated product of *p*-benzoquinone after -NO_2 removal during PNP degradation [13,14].

Table 1. Cometabolic degradation of PNP by *R. eutropha* (free cells experiments) using phenol as the growth substrate at 200 mg l^{-1} initial concentration

Concentration of cometabolic substrate (mg l^{-1})	Phenol removal (%)	r_{phenol} (mg l^{-1} h^{-1})	r_{PNP} (mg l^{-1} h^{-1})
Phenol only	96	6.53	-
+PNP (5)	41	2.68	0.06
+PNP (10)	30	2.37	0.10
+PNP (15)	20	1.88	0.16

Phenol removal (%) data obtained at 30 h (see Fig. 2 for the time of complete removal of phenol from the test solutions)

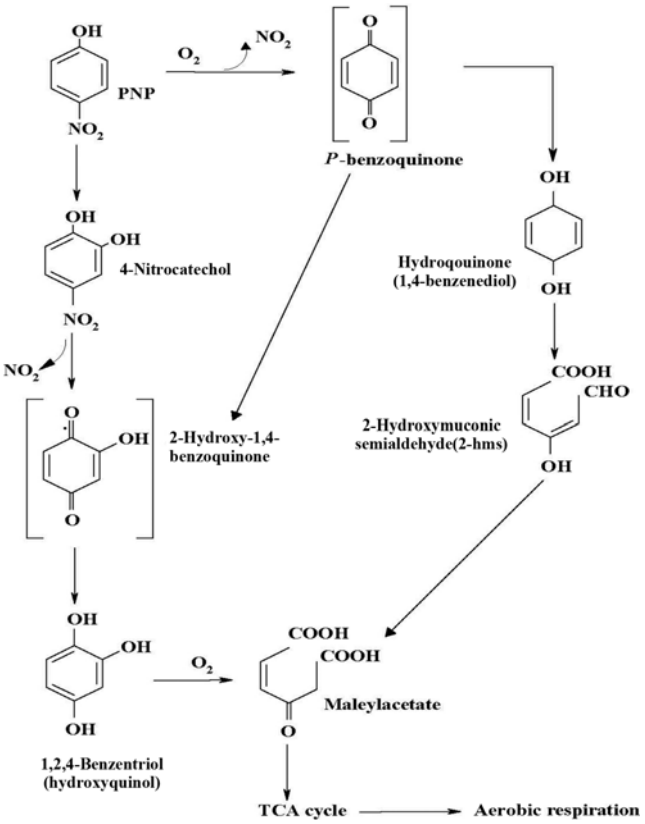


Fig. 4. A brief scheme for PNP degradation pathways via oxidative routes operative in microorganisms according to the literature.

Hydroquinone in the present study was the dominant intermediate (Fig. 5), among pathways of microbial degradation of PNP shown in Fig. 4. And this is in agreement with the literature [11-14,16].

2. Kissiris-immobilized Cells and PNP Cometabolic Degradation on Phenol in an [ICB]

The biodegradative performance of the Kissiris-immobilized *R. eutropha* in the ICB was improved considerably as compared with that of the free cells in the flask experiments. The time for complete degradation of the phenol (200 mg l^{-1}) in the ICB was 7 h, showing four-fold decrease in the time needed by the suspended cells in the flask (Tables 1 and 2). Resistance to the diffusion of substrate and release of product through the immobilized matrices are weak points in cell immobilization areas, and it is possible to reduce the cell demand for substrate by reducing cells loading in the supports, so that mass transfer has a better chance of supplying substrate at a proper rate [36]. SEM images presented in Fig. 6 show good accumulation of biofilm throughout the Kissiris matrix. Decrease of the cell demand for the phenol substrate in the present work and in the presence of PNP (15 mg l^{-1}) was through phenol feeding regime

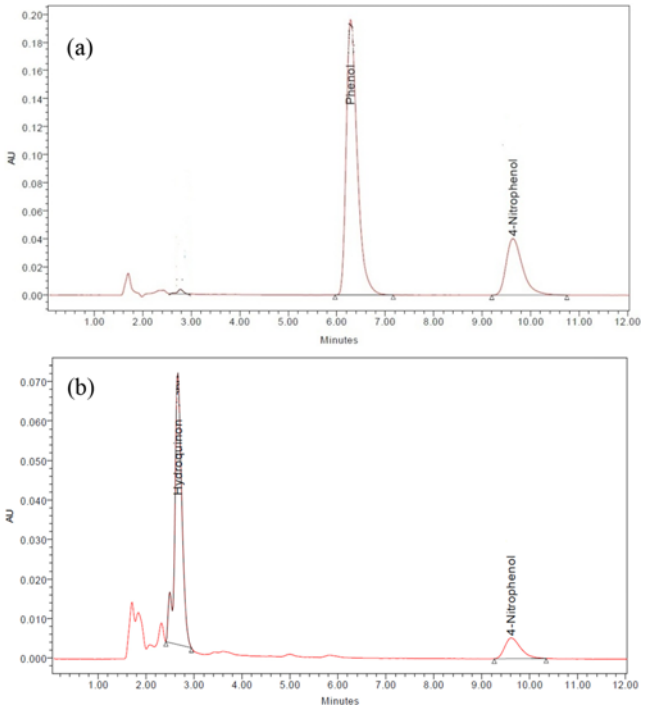


Fig. 5. HPLC chromatograms of the supernatant obtained from the culture of *R. eutropha* grown on (phenol 200 mg *l* and PNP 15 mg *l*) at time 0 h (a) and more than 70 h (b).

(200 mg l^{-1} per cycle). Lower activity of the Kissiris-immobilized *R. eutropha* towards phenol increased the chance of PNP utilization by the test bacterium (r_{PNP}=0.36 and 0.82 mg l^{-1} h^{-1}) (Table 2). Apparently, the cell demand in the [ICB] was provided by the cooperative actions of both phenol and PNP substrates (Fig. 7). Thus, the probability of simultaneous, and at the same time sequential, utilization of phenol and PNP increased through the phenol feeding regime used in the present study. Fig. 6 also shows the presence of void spaces within Kissiris pieces in the ICB. These may have had an enhancing effect on the substrate flux from the bulk liquid to the cells fixed onto the support, and this facilitated accumulation of the cells within porous structure of Kissiris pieces [37].

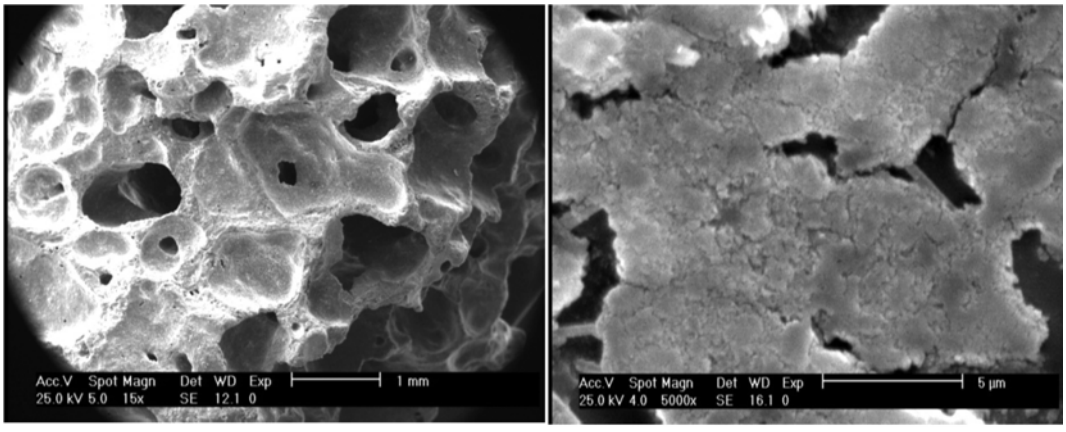
Table 2. Cometabolic degradation of PNP by the Kissiris- immobilized *R. eutropha* on phenol (200 mg l^{-1}) in the ICB operated in a batch recycling mode. The data for the system with phenol feeding regime (200 mg l^{-1}) are also shown

Substrate concentration (mg l^{-1})	r_{phenol} (mg l^{-1} h^{-1})	r_{PNP} (mg l^{-1} h^{-1})
Phenol only	28.3 (7$^{\alpha}$)	-
+PNP (5)	23.7 (8$^{\alpha}$)	0.44
+PNP (10)	13.4 (14$^{\alpha}$)	0.37
+PNP (15)	13.3 (14^{v})	0.36
Number of phenol feeding cycle		
1	14.3 (13$^{\alpha}$)	0.38
2	20.5 (9$^{\alpha}$)	0.23
3	31.7 (6$^{\alpha}$)	0.25
4	32.2 (6$^{\alpha}$)	0.34
5	48.75 (4$^{\alpha}$)	0.82

$^{\alpha}$=The number in the parentheses show the time of complete removal of phenol

CONCLUSIONS

The point in cometabolic studies is the simultaneous utilization of two substrates, where the degradation dependency of the cometabolic substrate on the primary substrate is advantageous, considering removal of more than one pollutant in waste biotreatment processes. Degradation of PNP by *R. eutropha* using phenol as the primary (growth substrate) has been reported in the present work. Phenol degradation at 200 mg l^{-1} initial concentration was almost complete within 30 hr, but in the presence of PNP (15 mg l^{-1}) only 20% of the phenol was degraded in this time. Performance of the cells improved considerably using Kissiris as the cells support in an ICB operated in a repeated batch recycling mode, time for complete consumption of phenol was 7 hr. By applying phenol feeding regime, the cells demand was cooperatively provided by the both of the sub-



(a) (b)

Fig. 6. SEM microphotograph of *R. eutropha* grown on the kissiris pieces in the ICB: (a) general view of the kissiris pieces (magnification: 15×), (b) image of the grown cells (magnification 5000×).

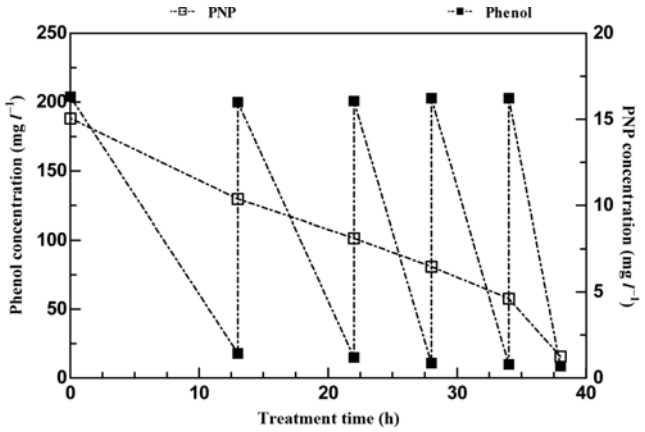


Fig. 7. Time course of PNP (15 mg l^{-1}) cometabolite degradation with phenol (15 mg l^{-1}) by Kissiris-immobilized *R. eutropha* cells in the ICB operated in a repeated batch recycling mood (phenol at 200 g l^{-1} was feed to the system in the five cycles, the feeding regime are performed when the level of phenol decreased to below 10 mg l^{-1}.

strates and the lower activity of the cells towards phenol in the presence of PNP, increased chance of PNP utilization by the Kissiris-immobilized cells. The ICB was found to be an efficient bioreactor configuration for this cometabolism studies. Mechanical rigidity in structure of Kissiris, inert nature of this cell support, and its availability at low price are some advantages which cannot be ignored for large scale applications of the mineral Kissiris in waste biotreatment processes.

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