

Effect of Iron Nanoparticles Synthesized by a Sol-Gel Process on *Rhodococcus erythropolis* T902.1 for Biphenyl Degradation

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Abstract

Nanoparticles (NP_s) are considered as a new generation of compounds to improve environmental remediation and biological processes. The aim of this study is to investigate the effect of iron NP_s encapsulated in porous silica (SiO₂) on the biphenyl biodegradation by *Rhodococcus erythropolis* T902.1 (RT902.1). The iron NP_s (major iron oxide Fe_xO_y form) were dispersed in the porosity of a SiO₂ support synthesized by sol-gel process. These Fe/SiO₂ NP_s offer a stimulating effect on the biodegradation rate of biphenyl, an organic pollutant that is very stable and water-insoluble. This positive impact of NP_s on the microbial biodegradation was found to be dependent on the NP_s concentration ranging from 10⁻⁶ M to 10⁻⁴ M. After 18 days of incubation the cultures containing NP_s at a concentration of 10⁻⁴ M of iron improved RT902.1 growth and degraded 35% more biphenyl than those without NP_s (positive control) or with the sole SiO₂ particles. Though the microorganism could not interact directly with the insoluble iron NP_s, the results show that about 10% and 35% of the initial 10⁻⁴ M iron NP_s encapsulated in the SiO₂ matrix would be incorporated inside or adsorbed on the cell surface respectively and 35% would be released in the supernatant. These results suggest that RT902.1 would produce siderophore-like molecules to attract iron from the porous silica matrix.

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Keywords

Biodegradation, Biphenyl, *Rhodococcus erythropolis*, Encapsulated Nanoparticles, Sol-Gel, Iron

1. Introduction

Bioremediation of polluted soils is considered as a more effective and sustainable method to remove pollutants than physico-chemical technologies. It is also preferred to direct disposal of toxic and hazardous wastes [1] [2]. However, the bioremediation kinetics are very limited. Indeed, long time periods of a few months are needed for microbial degradation of highly complex or hydrophobic substances such as polyaromatic and polycyclic hydrocarbons.

Some studies showed that using certain metal ions at low concentrations accelerates the biodegradation rate [3]-[7]. Similarly, Chun-Wei and Barbara [8] demonstrated that the addition of cadmium, copper and chrome at low concentration of 0.01 ppm (*i.e.* about 10^{-7} M) enabled to achieve a 2-fold higher biodegradation rate of phenol, benzoate or 2-chlorophenol. By contrast, when comparing to the kinetics achieved at 2.5×10^{-5} M of metal, Kotresha and Vidyasagar [9] reported a 2-fold decrease of phenol biodegradation rate by *P. aeruginosa* MTCC 4996 in presence of 2×10^{-4} M of cobalt or nickel and up to 1.5×10^{-3} M of copper, cadmium or zinc. Furthermore these elements are heavy metals not suitable for environmental applications.

On the contrary, metallic iron may be accepted for environmental applications since it is less expensive and has a lower toxicity compared with the other metals [3] [9]-[11]. Bunesco *et al.* [3] reported that the presence of iron up to 10^{-3} M did not inhibit the biodegradation of 2-aminobenzothiazol by *Rhodococcus rhodochrous*. In contrast, Lin *et al.* [10] reported that the presence of copper at 1 mg/L and 10 mg/L (*i.e.* 7×10^{-6} M - 7×10^{-5} M) significantly reduced the degradation rate of methyl tert-butyl ether by *P. aeruginosa*.

Iron addition in the culture medium was shown to be effective for reductive degradation of a variety of toxic substances such as aromatic compounds [12] [13]. Santos *et al.* [12] showed that the presence of different forms of soluble iron at the concentrations of 10^{-4} M (FeCl_3 , $\text{Fe}(\text{NO}_3)_3$, Fe_2O_3 , FeSO_4) had a positive impact on cell growth and biodegradation of anthracene by *Pseudomonas* sp. with up to 25% higher biodegradation yield than in the control test with iron nitrate, the most soluble iron form. By contrast, concentrations higher than 2×10^{-4} M of this salt showed to inhibit the microorganisms. Choroa [13] emphasized that the use of a Fe (III)-nitrilotriacetic acid complex (FeNTA) up to 10^{-3} M stimulated the biodegradation of 2-aminobenzothiazol by *Rhodococcus rhodochrous*.

These recent studies used different forms of iron: iron salts and iron complexes for the optimization of pollutant biodegradation. By comparison, the iron NP_s are preferred as they have a large surface area and high reactivity [14]. Moreover, when compared to many other metallic NP_s, the iron NP_s are stable, less expensive and less toxic for use with the microorganisms. They are therefore suitable for large scale industrial applications such as bioreactors for water treatment [15].

Previous studies demonstrated that using iron NP_s (individual or joint with other metal) accelerates the biodegradation of pollutants [14] [16]. Murugesan *et al.* [16] reported that the presence of palladium-iron bimetallic NP_s (nFe-Pd) up to 0.1 g/L (estimated at 2×10^{-3} M Fe and 2×10^{-6} M Pd) in minimal salt medium is effective on the complete dechlorination of triclosan (2, 4, 40-trichloro 20-hydroxydiphenyl ether; up to 27 ppm initial concentration) and biodegradation of the intermediates metabolites by a strain of *Sphingomonas*. Zhang [14] showed that using iron NP_s is very effective for the transformation of a wide variety of common environmental contaminants such as chlorinated organic solvents.

Currently, the nanomaterials open up a window for a range of applications in numerous fields as well as usual products and processes [17]. For example, the NP_s of titanium dioxide (TiO_2) are used for self-cleaning surfaces, for treatment of polluted water and in sun creams [18]. SiO_2 NP_s are used as an anti-clumping-agent in common salt [18]. In the biological field, the NP_s have a wide range of applications. On the one hand, the nanomaterials are used as antibacterial agents (*i.e.* NP_s of silver) [19] [20]. On the other hand, some microorganisms may take advantages of nanoparticles to improve their potential in biological processes [21]. These applications have attracted much attention of researchers to develop new methods for the synthesis of the nanomaterials with specific properties such as thermal stability, high porosity and large surface area [22]-[25].

The objective of this work was to improve biphenyl biodegradation by RT902.1 using iron NP_s encapsulated in porous silica SiO₂ synthesized by sol-gel methods. Indeed, several authors used the sol-gel method to obtain monometallic catalyst particles finely dispersed on a mineral support [26]. The process is based on the simultaneous hydrolysis and condensation of two alkoxy silanes: an SiO₂ network-forming reagent such as tetraethoxysilane (TEOS or Si(OC₂H₅)₄) and an alkoxy silane-functionalized ligand of the type (RO)₃Si-X-A, in which a functional organic group A, able to form a chelate with a cation of a metal such as iron, palladium or copper is linked to the hydrolyzable silyl group (RO)₃Si—via an inert and hydrolytically stable spacer X [22]-[24]. The iron NP_s are encapsulated in the form of metal oxide (Fe_xO_y) in an inert matrix of silica. There is no chemical bond between SiO₂ and (Fe_xO_y). This is a physical blocking of metal oxides NP_s of 2 to 3 nm size in microporous SiO₂ crystallite (10 to 20 nm diameter) whose internal porosity is less than 1 nm.

The mechanism involved in the improvement of biphenyl biodegradation by RT902.1 and mediated by NP_s was investigated via the mass balance of iron liberated from the capsule of SiO₂ and the enzymes involved in the two stages of biphenyl biodegradation (hydroxylation and cleavage of aromatic ring).

2. Materials and Methods

2.1. Preparation and Characterization of Iron NP_s and SiO₂

The Fe/SiO₂ NP_s were prepared by the sol-gel method, as described by Heinrichs *et al.* [27]. This method consists of dissolving the iron (III) acetyl acetonate (Fe[CH₃COCHCOCH₃]₃) in the initial homogenous solution of silica gel precursor tetraethoxysilane (Si(OC₂H₅)₄ or TEOS). This complex dried at 80°C after the gelation (Fe/SiO₂ dried). It is further calcined at high temperature (450°C to 550°C) to remove organic waste material and achieve the SiO₂ porous matrix encapsulating the iron oxide crystallites of Fe_xO_y (Fe/SiO₂ calcined). Moreover, the porous silica matrix without NP_s, denoted SiO₂, was also synthesized by the sol-gel process to study the effect of the sole matrix of SiO₂ on biphenyl biodegradation [24]. The samples were characterized (textural analysis, electron microscopy, X-ray diffraction) by using the methods described by Lambert *et al.* [24] and Heinrichs *et al.* [27]. They showed that the catalyst Fe/SiO₂ calcined contained 1.53% (w/w) of active metal iron encapsulated in porous silica [27]. A 10⁻² M Fe/SiO₂ suspension was prepared using 1.86 g of Fe/SiO₂ catalyst (agglomerates of microporous silica particles encapsulating iron NP_s produced by sol-gel process and finely pounded at micrometersize for accurate weighing) in 50 mL ultrapure water (Milli-Q water, 18.2 MΩ cm). According to the experimental conditions, a defined volume of the homogenized 10⁻² M Fe/SiO₂ suspension was transferred rapidly, due to fast settlement, in the culture medium prior to sterilization in order to reach a final iron concentration ranging from 10⁻⁴ to 10⁻⁶ M.

2.2. Cultures

Precultures were prepared in 250 mL baffled flasks containing 50 mL of M284 minimal medium [28], complemented by 5 g/L of glucose. The medium was sterilized for 20 minutes at 121°C and cooled down to room temperature before inoculation at an initial cell density of about 10⁹ cell/mL by transfer of colonies of RT902.1 developed on M284 + agar medium. The microbial suspension was incubated for 3 days at 30°C and 150 rpm orbital agitation. The glucose was totally consumed after 3 days of incubation (assayed by the Kit RTU glucose BioMerieux, F). For the biphenyl biodegradation experiments, the culture medium was prepared similarly in 100 mL flasks (in triplicates) with 20 mL M284 minimal medium supplemented with calcined or dried iron NP_s at concentrations ranging from 0 (positive control) to 10⁻⁴ M of metal element. After sterilization, 1 mL of a 10 g/L biphenyl solution in *n*-hexane was added on the culture medium to achieve a biphenyl initial concentration of 500 ppm as source of carbon and energy. The evaporation of *n*-hexane was allowed overnight under a ventilated hood leaving biphenyl crystals suspended in the M284 minimal medium. A relatively homogenous suspension of biphenyl crystals in aqueous phase was achieved by vigorous mixing for 2 minutes using a mixer (POLYTRON® PT 1200 E, KINEMATICA AG, CH) sterilized by immersion in three successive solutions: SDS 5%, sodium hypochlorite 4% and norvanol 9%. The resulting suspension was inoculated with 3 mL of preculture and incubated at 30°C (150 rpm orbital agitation). Test controls were also prepared in the same conditions but without added NP_s nor SiO₂ (positive control), with the sole SiO₂ matrix (positive control + SiO₂) or without inoculum (negative control with or without Fe/SiO₂ NP_s). The negative controls were carried out in triplicates to examine the abiotic removal/evaporation of biphenyl. The optical density was measured at 600 nm (Ultrospec

III, Pharmacia LKB) at different time points of incubation (after 3, 8 and 18 days). The interference of NP_s on absorbance was corrected with the control samples that contained only NP_s to confirm the bacterial growth.

2.3. Biphenyl and Major Metabolites Analysis

Biphenyl residual concentration in the culture medium was measured by HPLC at different time points of incubation (after 3, 8 and 18 days) after liquid-liquid extraction in organic solvent. Glass tubes of a total volume of 10 mL (culture tube 16 × 100 SVL SCRE, PyrexR, UK) with Teflon seal and containing 2 mL sample and 4 mL *n*-hexane were mixed for 24 h at 30°C. After centrifugation at 7000 rpm (SAL-1500 rotor in Sorvall RC5B+ centrifuge) for 15 min the organic phase was transferred in glass tubes for overnight evaporation of solvent under a constant ventilated hood. Crystals of biphenyl were resuspended in 10 mL methanol before analysis by HPLC. The major metabolites from biphenyl biodegradation *i.e.* catechol and benzoic acid were analyzed in the aqueous phase recovered after liquid-liquid extraction. It was centrifuged at 13,000 rpm for 2 min and the supernatant was filtered through a 0.2 μm cellulose acetate filter before HPLC analysis. HPLC analyses were performed using an Agilent 1100 Series equipment and a C18 column (LiChroCART® 250-4.6 HPLC-cartridge Purospher® STAR RP-18 endcapped 5 μm, Merck, D) maintained at 30°C. The mobile phase contained acetonitrile and Milli-Q water in the ratio 70/30 or 35/65 in order to determine the biphenyl concentration or the metabolites respectively. Its pH was adjusted to 2.75 with 1.5 M phosphoric acid. The flow rate was 0.8 mL/min and 10 μL of the sample were injected. Biphenyl, benzoic acid and catechol were detected at 254 nm and their concentration in samples was calculated from a standard graph determined using pure compounds.

2.4. Recycling of Calcined Iron NP_s

After 18 days of incubation, the culture was centrifuged at 7000 rpm for 15 min. The pellet was calcined for 4 hours in porcelain crucible directly on the flame in order to completely oxidize the organic compounds. After calcination, the inorganic matrix was added for a first cycle in 100 mL flasks, with 20 mL of M284 minimal medium and 3 mL of fresh inoculum. The supernatant volume of the culture (about 14 mL after 3 samples collection of 2 mL) was transferred in another 100 mL flask supplemented to 20 mL with M284 minimal medium before inoculation with 3 mL of fresh inoculum. A second and a third cycles were carried out using the same procedure applied to the flasks containing the NP_s and also for the control culture (without NP_s).

2.5. Iron Titration

The total iron concentration was determined using the method slightly modified from Bunescu [29]. The total iron concentration was determined after the reduction of Fe³⁺ with a solution of ascorbic acid (3%) and reaction with orthophenanthroline forming a strong-colored complex with Fe²⁺. In a 10 mL flask, 300 μL of sample were mixed with 4200 μL of ascorbic acid (3% w/v in ultrapure water) and stirred. After, 2 min wait and addition of 500 μL of a 1% orthophenanthroline solution (w/v in ethanol for analysis), the optical density was measured at 510 nm. This procedure was used to determine the iron concentration in the different compartments of the RT902.1 biomass (extracellular, intracellular, and at the cell surface) at the end of the experiment (after 18 days of incubation) with calcined 10⁻⁴ M Fe/SiO₂ and 500 ppm biphenyl. According to Bezkorovainy *et al.* [30] 4 mL of the culture medium (in triplicates) was sampled after rapid settlement of Fe/SiO₂ catalyst and separated into two fractions. The first 2 mL fraction (way A) was centrifuged at 13,000 rpm for 20 min before measurement of the soluble iron in the supernatant (A₀). The pellet was washed with 2 mL of ultrapure water (Milli-Q water, 18.2 MΩ cm). After centrifugation, the iron concentration in the supernatant (A₁) was determined. This step with ultrapure water was repeated three times more. The sum (A₁ + A₂ + A₃ + A₄) gave the concentration of the iron adsorbed at the surface of the cells. The pellet was then resuspended with 2 mL of ultrapure water, and the cells were disrupted by sonication at a frequency of 10 kHz for 3 min. After centrifugation at 13,000 rpm for 20 min, iron concentration was measured in the supernatant A₅ and in the pellet after resuspension in 2 mL of ultrapure water A₆. The sum (A₅ + A₆) corresponds to the intracellular iron concentration. All the steps described previously were carried out at 4°C. The second 2 mL fraction (way B) was disrupted by sonication at a frequency of 10 kHz for 3 min and centrifuged at 13,000 rpm for 20 min. The total iron concentration (B₁ + B₂) was determined as the sum of the soluble iron concentration in the supernatant (B₁) and the iron adsorbed on the cell membrane determined after analysis of the pellet resuspended in 2 mL ultrapure water (B₂). The concentration

of the total iron measured in both fractions A and B (*i.e.* $9.8 \times 10^{-5} \text{ M} \pm 0.01$ and $11 \times 10^{-5} \text{ M} \pm 0.01$ respectively) were similar to the theoretical value of 10^{-4} M confirming the suitability and accuracy of the procedure. The procedure applied to the positive control ($1.9 \times 10^{-5} \text{ M}$ theoretical iron concentration due to the presence $\text{Fe}^{\text{III}}\text{NH}_4$ citrate added in the culture medium at a higher level than in the first experiment of **Figure 1** for better reproducibility) lead to a total iron concentration measured in the first fraction A (*i.e.* $2 \times 10^{-5} \text{ M} \pm 0.01$) that was close to that of the second fraction B (*i.e.* $9 \times 10^{-6} \text{ M} \pm 0.01$).

2.6. Enzyme Assays

For the enzyme assays needing larger biomass material, the cultures were carried out in 500 mL flasks (in triplicates) with 100 mL of M284 minimal medium complemented by iron NP_s at concentrations of 0 (positive control) or 10^{-4} M of metal element. The cultures were sterilized for 20 minute at 121°C . After sterilization, 5 mL of 10 g/L biphenyl solution in *n*-hexane were added as the sole carbon and energy source. The other steps were as described above (Section "Cultures"). After 18 days of incubation, the cells were centrifuged at 13,000 rpm for 20 min and washed twice with a potassium phosphate buffer 0.05 M (K_2HPO_4 , KH_2PO_4 , pH 7.5). The pellets were resuspended in a sufficient volume of the same buffer to obtain a cellular concentration of 0.05 g of wet cells per mL. The cell suspension was then sonicated at a frequency of 10 kHz for 2 min and centrifugated at 13,000 rpm for 40 min. The supernatant containing crude cell free extracts was used to determine the biphenyl dioxygenase activity and catechol1, 2 dioxygenase activity. During all these operations, the extracts were maintained at 4°C .

The biphenyl dioxygenase activity was determined as the initial rate of indigo formation monitored spectrophotometrically at 610 nm. The molar extinction coefficient for indigo at 610 nm was calculated at $15.900 \text{ L mol}^{-1}\cdot\text{cm}^{-1}$ [31]. For the determination of specific rates of indigo formation, a 100 μL volume of concentrated cell free extracts was added in a 1.5 mL tube preincubated for 5 min at 30°C to 390 μL of 0.05 M phosphate buffer (K_2HPO_4 , KH_2PO_4 , pH 7.5) and 10 μL of a 10 M indole solution in N, N dimethyl-formamide. The amount of indigo formed in the samples was calculated from a standard graph determined using pure compound of indigo. One unit of enzyme activity was expressed in mg of indigo liberated/minute/mg protein. For the determination of catechol1,2dioxygenase specific activity, a 100 μL volume of concentrated cell-free extracts was added to 900 μL of 0.05 M phosphate buffer (K_2HPO_4 , KH_2PO_4 , pH 7.5) and 20 μL of 10 mM catechol. The specific activity was monitored at 260 nm ($\epsilon = 16.8 \text{ mM}$) [32]. The amount of *cis, cis*-muconic acid formed in the samples was calculated from a standard graph determined using pure compound of *cis, cis*-muconic acid. One unit of enzyme activity was defined as the amount of enzyme catalyzing the production of 1 μmol *cis, cis*-muconic acid per minute at 30°C .

2.7. Protein Concentration

Protein concentration in culture samples was measured by the Bradford method using Bovine Serum Albumin as a standard [33].

2.8. Statistical Analysis

The SAS software (SAS Institute 2001) was used for all statistical analyses. The general linear model (GLM) was used to determine whether the effect of metallic NP_s was significant on the biodegradation potential of RT902.1. Least square means and standard errors were calculated. A *p*-value of <0.05 was chosen as the threshold for significance of all statistical comparisons.

3. Results and Discussion

3.1. Biodegradation of Biphenyl in the Presence of Iron NP_s Fe/ SiO_2 Synthesized by Sol-Gel Process

Three biodegradation experiments were carried out successively in 100 mL flasks containing 20 mL of M284 culture medium with 500 ppm biphenyl and inoculated by 3 mL of preculture. First we investigated the effect of calcined iron NP_s added in the culture medium at concentrations ranging from 10^{-6} to 10^{-4} M the comparison of the impact of 10^{-4} M of calcined iron NP_s and dried iron NP_s was assessed. In the third experiment, the recycling of calcined iron NP_s was carried out for 2 further cultures in order to test their effect on the long term.

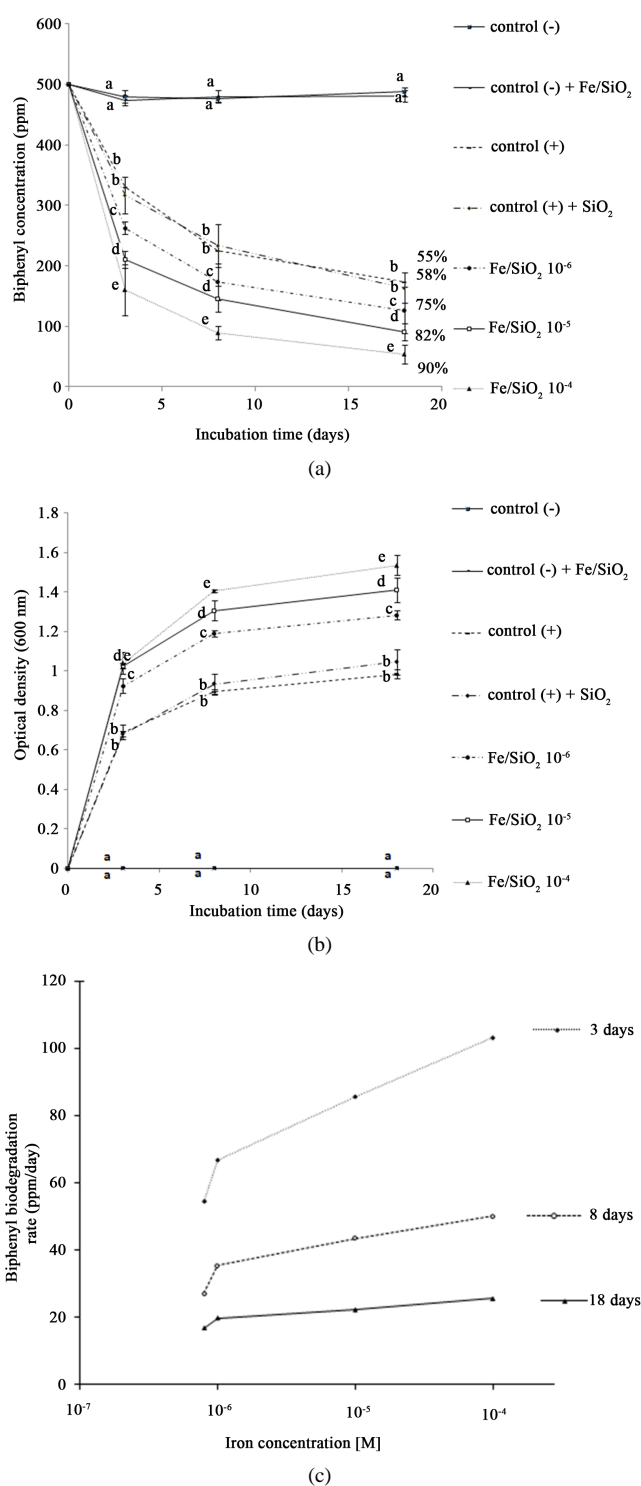


Figure 1. Evolution of biphenyl biodegradation by *Rhodococcus erythropolis* T902.1 in 100 mL flasks containing 20 mL of M284 minimal medium, 500 ppm biphenyl and different concentrations of calcined iron nanoparticles from 10^{-6} to 10^{-4} M: (a) biphenyl concentration; (b) growth measured by optical density at 600 nm; (c) biphenyl biodegradation rate after 3, 8 and 18 days of culture. Control (-) and control (-) + Fe/SiO₂ were carried out without microorganisms, control (+) without NPs nor SiO₂, control (+) +SiO₂ without NPs. The similar letters (aa, bb, cc, dd and ee) indicate that no significant statistical differences were observed (p -value > 0.05) at different time points of biphenyl biodegradation by RT902.1.

3.2. Effect of NP_s Concentration

Figure 1(a) and **Figure 1(b)** show that the presence of iron NP_s without inoculum did not exhibit any effect on the degradation of biphenyl when compared to the negative control without Fe/SiO₂ NP_s at all time points ($p > 0.05$). It suggests that the presence of different iron NP_s concentrations in the absence of bacteria did not contribute to an abiotic mechanism of biphenyl degradation. By comparison, Lipczynska-Kochany *et al.* [34] reported that the addition of iron (Fe⁰) without inoculum exhibits abiotic effect on the degradation of carbon tetrachloride. This effect was increased in the presence of sulphur compounds such as sodium sulphate, sodium sulphide, and ferrous sulphide. However, such abiotic effect on partial degradation and/or bioavailability improvement of complex molecules for further biodegradation was mainly reported for halogenated compounds [16] [34]. Moreover the abiotic effect would not be effective in our experimentations because the biphenyl is insoluble in the culture medium and iron NP_s are encapsulated inside the porous silica matrix [27] preventing a direct contact with each other. By contrast, the present study shows that biphenyl concentration decreases with RT902.1 growth (measured by OD at 610 nm) and that the biodegradation rate increases with increasing the Fe/SiO₂ concentration (**Figure 1**). Iron NP_s at the concentration of 10⁻⁴ M significantly influenced the biphenyl biodegradation efficiency when compared to the concentration of 10⁻⁵ M and 10⁻⁶ M ($p < 0.05$) since biphenyl biodegradation yields up to 90%, 82% and 75% respectively after 18 days of incubation (**Figure 1(a)**).

Figure 1(c) also shows that the biphenyl degradation rate increases significantly (*i.e.* by about 30%) at day 3 when adding 10⁻⁶ M of iron NP_s to the culture medium already containing 7×10^{-7} M iron ammonium citrate and, during the exponential growth phase (*i.e.* results recorded at 3 days of incubation), this rate increases logarithmically with increasing NP_s concentration. As a consequence, since the presence of 10⁻⁴ M Fe/SiO₂ lead to biphenyl degradation rate about 2 fold higher than in the positive control it was therefore unfavorable for further industrial application to test higher NP_s concentrations. Rapid tests up to 2×10^{-3} M Fe/SiO₂ did not achieved higher biodegradation yields after 18 days of incubation (results not shown).

The decrease of biphenyl biodegradation rate after three days of culture, regardless of the concentration of NP_s (**Figure 1(c)**) should be related with the release of soluble metabolites, which gives a yellow color to the medium [35]. HPLC analysis of the aqueous phase of the culture medium revealed the presence of two compounds identified as catechol and benzoic acid (**Table 1**) (retention time of 4.3 min and 7.9 min respectively). Benzoic acid concentration increased regularly whereas catechol appeared in the culture during the growth phase and disappeared during the stationary phase. These compounds were already identified with the *Micrococcus* species and *Rhodococcus* species in the presence of biphenyl as the sole source of carbon [36] [37]. Since the yellow color appeared with RT902.1 growth on biphenyl and disappeared after about 12 days of incubation, the analysis of the carbon mass balance considering this substrate and the major metabolites (**Table 1**) indicates that biphenyl is partially metabolized by RT902.1. This result is in agreement with Bevinakatti and Ninnekar [36] who showed that the culture medium became yellow colored during growth of *Micrococcus*, indicating that the biphenyl was metabolized by this strain. Additionally, Kuony [35] also reported the production of yellow compounds without increasing the bacterial density, indicating that the aromatic hydrocarbons (fluorene and fluoranthene) were partially metabolized by *Mycobacterium* sp. 6py1.

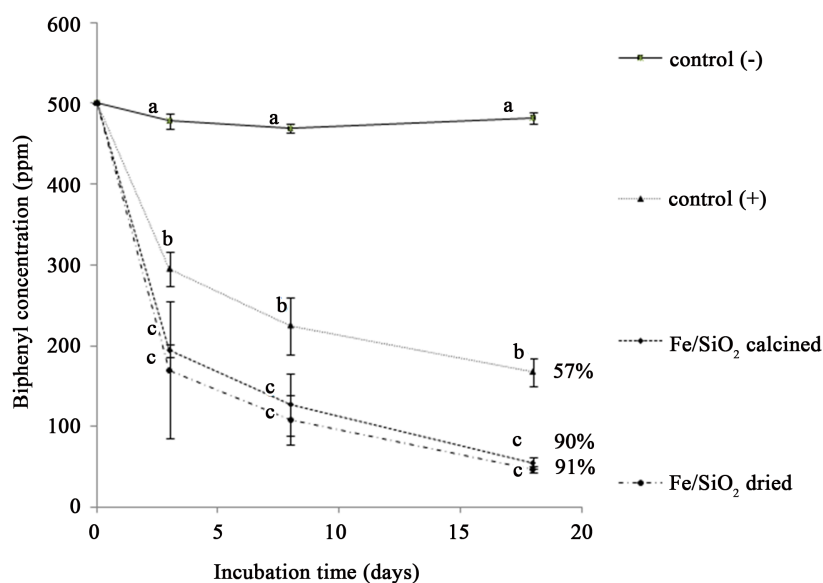
Table 1. Evolution of benzoic acid and catechol production from biphenyl biodegradation (500 ppm initial concentration) by *Rhodococcus erythropolis* T902.1 cultured for 18 days in 100 mL flasks containing 20 mL of M284 minimal medium with or without 10⁻⁴ M of calcined iron NP_s.

Incubation time	Metabolites concentration (ppm)		
	3 days	8 days	18 days
Catechol			
Positive control	66.14 ± 0.09	33.00 ± 0.07	19.40 ± 0.08
Culture with Fe/SiO ₂ 10 ⁻⁴ M	74.38 ± 0.06	43.36 ± 0.09	24.50 ± 0.03
Benzoic acid			
Positive control	1.19 ± 0.10	3.09 ± 0.35	13.39 ± 0.96
Culture with Fe/SiO ₂ 10 ⁻⁴ M	4.36 ± 0.14	10.30 ± 0.21	25.39 ± 0.92

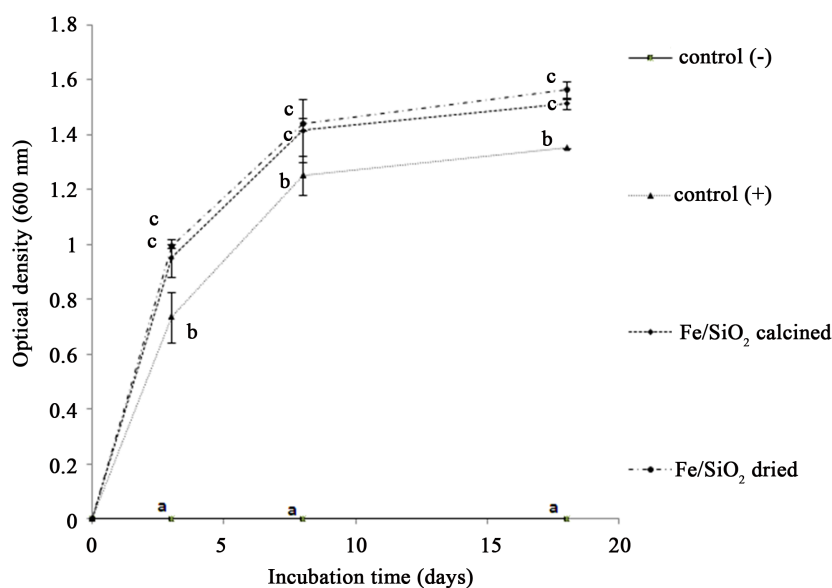
3.3. Effect of Dried or Calcined NP_s

The impact of 10^{-4} M Fe/SiO₂ calcined NP_s on biphenyl biodegradation was compared with similar NP_s that did not undergo the calcination step. Therefore the iron NP_s were still complexed between the acetyl acetonate and-tetraethoxysilane molecules [27] whereas the calcined iron NP_s are in the form of metallic oxide (Fe_xO_y) [38].

Figure 2(a) and **Figure 2(b)** shows that dried or calcined iron NP_s achieved similar improvement effect on biphenyl biodegradation (90% or 91%). These results confirm that iron NP_s, whatever reduced, complexed or oxidized have a positive impact on the potential of RT902.1. They are consistent with those reported by Ansari *et al.* [15] about dibenzothiophene biodesulfurization carried out by *Rhodococcus erythropolis* IGST8 decorated



(a)



(b)

Figure 2. Evolution of biphenyl biodegradation by *Rhodococcus erythropolis* T902.1 in 100 mL flasks containing 20 mL of M284 minimal medium, 500 ppm biphenyl and 10^{-4} M of calcined or dried iron nanoparticles: (a) biphenyl concentration and (b) growth measured by optical density at 600 nm. The similar letters (aa, bb, and cc) indicate that no significant statistical differences were observed (p -value > 0.05) in the presence of 10^{-4} M of calcined or dried iron nanoparticles.

with 4×10^{-4} M of magnetic Fe_3O_4 nanoparticles. They achieved a 56% higher desulfurization activity in basic salt medium than with the nondecorated cells. Olle *et al.* [39] also reported that the presence of 1 M of colloidal dispersions of magnetite (Fe_3O_4) improves cell density in bacterial cultures. Regarding the presence of iron complexed by potentially inhibiting-organic molecules such as acetyl acetone and tetraethoxysilane in dried Fe/SiO_2 , Bunescu *et al.* [3] reported that the presence of Fe(III)-nitriolotriacetic acid complex (FeNTA) increased the degradation of 2-aminobenzothiazol by *Rhodococcus rhodochrous* without any inhibition up to 5×10^{-4} M FeNTA.

3.4. Effect of NP_s Recycling

Following the experiment with 10^{-4} M calcined Fe/SiO_2 NP_s, two cycles of culture (fresh inoculum) were carried out with the initial 10^{-4} M NP_s recovered at the end of each culture by centrifugation and calcination to remove biomass. In parallel a similar procedure was applied to the supernatant of the cultures (without NP_s) but considering apotential additional concentration of soluble iron transferred from the NP_s. As seen in **Table 2**, the biphenyl degradation yield in presence of Fe/SiO_2 NP_s (pellet) decreased progressively with time (3 cycles during 64 days) from 90% to 84% and 82% respectively. Compared with **Figure 1(a)** this trend did not overtake the results with 10^{-5} M NP_s (82%). By contrast, the biphenyl degradation yield increased progressively in the different supernatants from the successive cultures with increase of available iron released from the capsule of SiO_2 . Indeed the concentration of iron in the supernatants increased from 2×10^{-5} M ± 0.01 to 6×10^{-5} M ± 0.01 respectively. The positive control (flasks with pellet and flasks with supernatant) showed similar biphenyl degradation yields and iron concentrations during the three cycles. Since, as measured by ICP-AES method [38], only 0.02% of the iron releases naturally from NP_s in culture medium without microorganisms (probably due to some unstable Fe/SiO_2 capsules in autoclave), these results indicate that interactions should exist between the bacteria and the metallic encapsulated elements. Therefore iron would be attracted progressively from the capsule of SiO_2 . One can imagine that this mechanism is initiated for consumption of iron by the bacteria cells.

Table 2. Evolution of total iron concentration, biphenyl biodegradation yield in % and growth measured by optical density of *Rhodococcus erythropolis* T902.1 after three successive cycles of 18 days of incubation in 100 mL flasks containing 20 mL of minimal medium and 500 ppm biphenyl with or without 10^{-4} M of calcined iron NP_s.

First cycle				
Experience condition	Culture with Fe/SiO_2 10^{-4} M		Positive control	
	pellet	supernatant	pellet	supernatant
Biphenyl biodegradation yield (%)	90	64	55	56
Optical density	1.60 ± 0.07	0.85 ± 0.04	0.75 ± 0.06	0.73 ± 0.07
Total iron (M)	$9.2 \times 10^{-5} \pm 0.01$	$2.01 \times 10^{-5} \pm 0.01$	$6 \times 10^{-6} \pm 0.01$	$2 \times 10^{-6} \pm 0.01$
Second cycle				
Experience condition	Culture with Fe/SiO_2 10^{-4} M		Positive control	
	pellet	supernatant	pellet	supernatant
Biphenyl biodegradation yield (%)	84	65	58	57
Optical density	1.54 ± 0.09	1 ± 0.08	0.76 ± 0.06	0.74 ± 0.09
Total iron (M)	$8.6 \times 10^{-5} \pm 0.01$	$3.0 \times 10^{-5} \pm 0.03$	$4 \times 10^{-6} \pm 0.02$	$3 \times 10^{-6} \pm 0.01$
Third cycle				
Experience condition	Culture with Fe/SiO_2 10^{-4} M		Positive control	
	pellet	supernatant	pellet	supernatant
Biphenyl biodegradation yield (%)	82	69	57	56
Optical density	1.52 ± 0.06	1.00 ± 0.09	0.76 ± 0.06	0.75 ± 0.07
Total iron (M)	$8.1 \times 10^{-5} \pm 0.01$	$6.0 \times 10^{-5} \pm 0.01$	$5 \times 10^{-6} \pm 0.00$	$3 \times 10^{-6} \pm 0.02$

3.5. Iron Distribution in the Different Compartments of RT902.1

Iron concentration was measured in the different compartments of the biomass (extracellular, intracellular and at the cell surface) after 18 days of incubation (first cycle of the experiment of section “Effect of NP_S recycling”). **Table 3** shows that while the biomass was not totally similar in the cultures with NP_S and the positive control, about 35% of the iron initially present in the 10⁻⁴ M Fe/SiO₂ NP_S was found in solution (3.5 × 10⁻⁵ M ±0.01) a similar content was adsorbed at the cell surface (3.4 × 10⁻⁵ M ±0.01) and about 10% was considered intracellular (9 × 10⁻⁶ M ±0.01).

This distribution also enables to calculate that about 25% of the initial 10⁻⁴ M iron NP_S was still encapsulated after the first cycle and, assuming a similar distribution at each of the 3 cycles experimented in the section “Effect of NP_S recycling”, it can be estimated that about 35% of the iron was lost in each supernatant. Therefore the total iron content in the third cycle would reach 10⁻⁵ M with a low residual iron amount in the Fe/SiO₂ catalyst. This is in accordance with the decreasing trend of biphenyl degradation yields in the successive cycles. To our knowledge, the literature does not report any precise answer about the metabolism of iron NP_S during biphenyl biodegradation by *Rhodococcus* strains. These bacteria are not able to degrade SiO₂ and the iron NP_S, with sizes of about 2 - 3 nm, are embedded in inorganic microporous silica particles of around 10 to 20 nm diameter with pores not exceeding 1 nm diameter [24]. Therefore, *Rhodococcus erythropolis* cells cannot enter into the silicon dioxide matrix to directly catch the iron. As a consequence RT902.1 would produce specific microbial sensors such as siderophores in order to attract iron from the inside of the silicon dioxide microporous structure as suggested by some authors [40] [41]. This result is in agreement with Kasemets *et al.* [42] who proposed that since in the normal conditions NP_S cannot enter the yeast cells, these cells would produce specific microbial sensors, for the internalization of nanoparticles. Kraemer [41] also showed that siderophore stimulated the solubility and dissolution kinetics of iron oxides in environments.

3.6. Effect of NP on Major Enzymes Activity

Regarding the reduction of iron, there are two main ways reported in the literature. First extracellularly, the flavin reductase reduces Fe³⁺ to Fe²⁺, then Fe²⁺ is transported by a specific membrane transporter into the cell [29]. In the second way, the Fe³⁺ is chelated by a siderophore that is integrated into the cell, where Fe³⁺ is reduced [43].

Intracellularly, an enzyme complex simultaneously reduces Fe³⁺ to Fe²⁺ and activates the enzymes which are responsible of the main steps of biphenyl biodegradation. Indeed, the structure of purified dioxygenase confirms the presence of Rieske cluster {²F-²S} and Fe²⁺ on different sites. It could be inhibited when Fe²⁺ is oxidized in Fe³⁺ and reactivated by the addition of Fe²⁺ [44] [45]. Nadaf *et al.* [32] found that the enzymatic activity of catechol1,2dioxygenase was completely inhibited with addition of 10⁻³ M of Fe³⁺. This study also confirms the Fe²⁺ form of the iron inside the cells. The dioxygenase activity assayed by indigo production from indole added to culture samples [46]. **Figure 3(a)** showed that the presence of 10⁻⁴ M Fe/SiO₂ NP_S has a positive impact on the enzyme. Indeed, the enzyme activity achieved a specific rate of 0.016 mg indigo/mg protein/min after 48 hours of incubation compared to the 0.01 mg indigo/mg protein/min in the positive control, then it decreased dramatically until the end of the experiments (75 hours of incubation). Similarly **Figure 3(b)** suggests that Fe/

Table 3. Iron concentration in the different compartments of *Rhodococcus erythropolis* T902.1 cells (extracellular, intracellular and at the cell surface) after 18 days of incubation in 100 mL flasks containing 20 mL of M284 minimal medium, 500 ppm biphenyl and 10⁻⁴ M of calcined iron NP_S (first cycle).

Experience condition	Iron concentration (M)		
	Soluble extracellular	Absorbed at cell surface	Intracellular
Positive control	8.1 × 10 ⁻⁶ ± 0.01	5.4 × 10 ⁻⁶ ± 0.01	6.7 × 10 ⁻⁶ ± 0.01
Culture with Fe/SiO ₂ 10 ⁻⁴ M	4.3 × 10 ⁻⁵ ± 0.01	3.9 × 10 ⁻⁵ ± 0.01	1.6 × 10 ⁻⁵ ± 0.01
Iron released from the SiO ₂ capsule			
Molar unit	3.5 × 10 ⁻⁵ ± 0.01	3.4 × 10 ⁻⁵ ± 0.01	9 × 10 ⁻⁶ ± 0.01
%	35.6	34.6	9.5

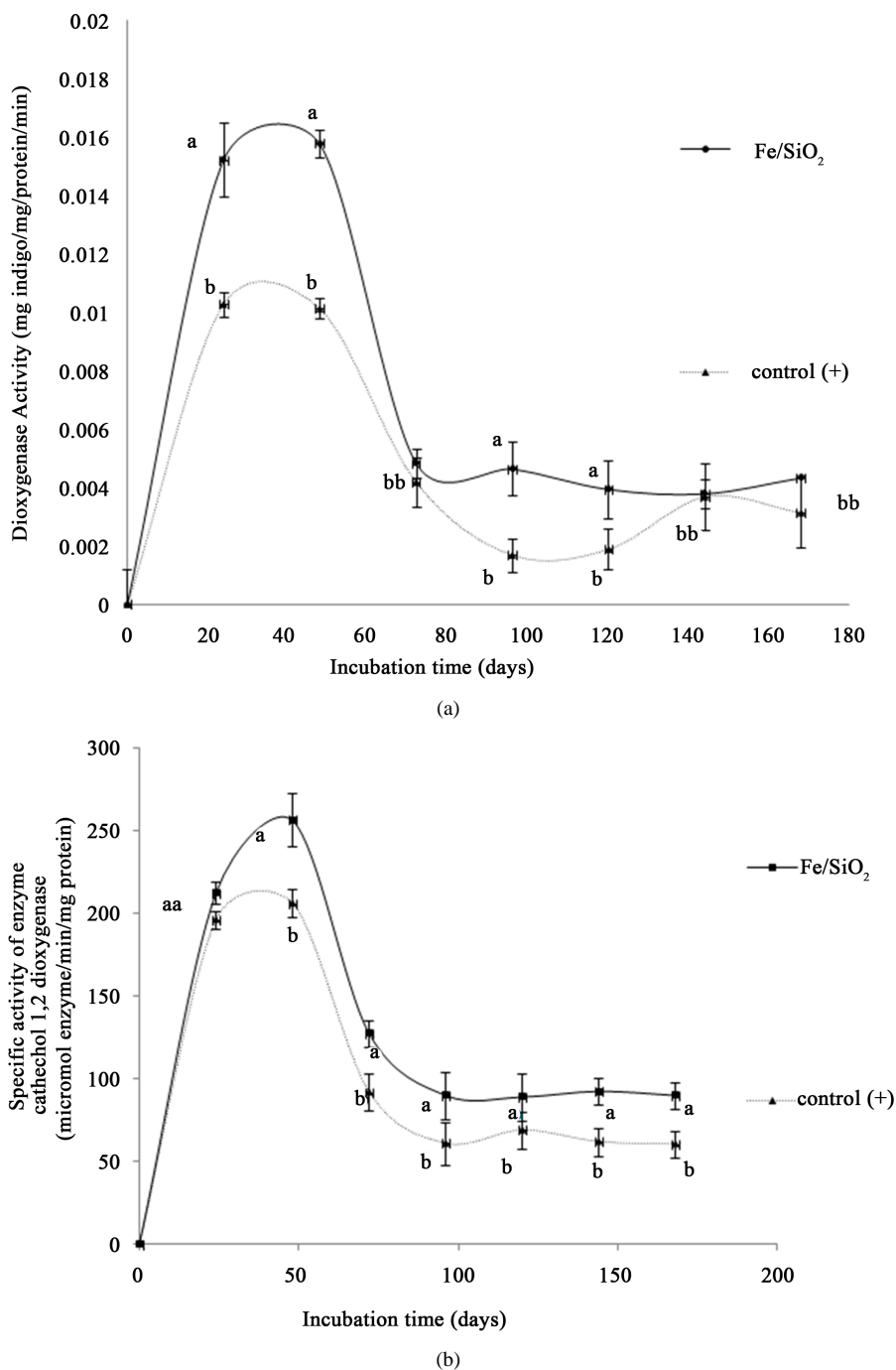


Figure 3. Evolution of biphenyl biodegradation by *Rhodococcus erythropolis* T902.1 in 100 mL flasks containing 20 mL of M284 minimal medium, 500 ppm biphenyl and 10^{-4} M of calcined iron nanoparticles: (a) Biphenyl dioxygenase activity defined as the rate of indigo formation per mg protein and (b) 1,2catechol dioxygenase specific activity versus time. The similar letters indicate that no significant statistical differences were observed between the two curves (p -value > 0.05).

SiO₂ stimulates the catechol1,2dioxygenase activity ($p < 0.05$). These results are consistent with those of Yeom and Yoo [7] and Dinkla *et al.* [47] who showed that the activity of the enzymes responsible of the biodegradation of pollutants increased after the addition of iron in the culture medium. It should also be mentioned that the

higher cell density achieved in presence of iron NP_s or the higher iron availability for the microorganisms could lead to higher biphenyl bioavailability through an improved production of biosurfactants that are often mentioned in the literature regarding hydrocarbons biodegradation by *Rhodococcus* strains [48].

4. Conclusion

In conclusion, the results of this study have shown that the biphenyl biodegradation by RT902.1 is improved in the presence of the iron NP_s encapsulated in porous silica. This improvement was found to be dependent on the Fe/SiO₂ concentration but not on the iron form whatever calcined as iron oxide or simply dried as iron complex with organic compounds. The highest biodegradation performance with a 35% higher biphenyl degradation yield was recorded with 10⁻⁴ M of iron NP_s than in the control conditions without NP_s. The performance increase is mainly recorded in exponential growth phase with a logarithmic increase of biphenyl degradation rate with increase of iron NP_s concentration from 10⁻⁶ to 10⁻⁴ M. It was also shown that the positive effect of 10⁻⁴ M Fe/SiO₂ NP_s was related to a higher enzyme activity since the biphenyl dioxygenase and catechol1,2-dioxygenase reached an about 50% and 25% higher maximum activity respectively. In addition, the analysis of the iron distribution showed that about 10% and 35% of the initial 10⁻⁴ M iron NP_s encapsulated in the SiO₂ matrix would be incorporated inside or adsorbed on the cell surface and 35% would be released in the supernatant. These results suggest that RT902.1 would produce siderophore-like molecules to attract iron from the porous silica matrix.

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