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

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Abstract

Nanoparticles (NPs) 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 NPs encapsulated in porous silica (SiO2) on the biphenyl biodegradation by *Rhodococcus erythropolis* T902.1 (RT902.1). The iron NPs (major iron oxide Fe3O4 form) were dispersed in the porosity of a SiO2 support synthesized by sol-gel process. These Fe/SiO2 NPs offer a stimulating effect on the biodegradation rate of biphenyl, an organic pollutant that is very stable and water-insoluble. This positive impact of NPs on the microbial biodegradation was found to be dependent on the NPs concentration ranging from 10^-6 M to 10^-4 M. After 18 days of incubation the cultures containing NPs at a concentration of 10^-4 M of iron improved RT902.1 growth and degraded 35% more biphenyl than those without NPs (positive control) or with the sole SiO2 particles. Though the microorganism could not interact directly with the insoluble iron NPs, the results show that about 10% and 35% of the initial 10^-4 M iron NPs 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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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 $2.5 \times 10^{-5}$ M of metal, Kotresha and Vidyasagar [9] reported a 2-fold decrease of phenol biodegradation rate by *P. aeruginosa* MTCC 4996 in presence of $2 \times 10^{-4}$ M of cobalt or nickel and up to $1.5 \times 10^{-3}$ 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]. Bunescu 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. $7 \times 10^{-6}$ M - $7 \times 10^{-5}$ 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$_3$, Fe(NO$_3$)$_3$, Fe$_2$O$_3$, 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 $2 \times 10^{-4}$ M of this salt showed to inhibit the microorganisms. Chorao [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 NPs are preferred as they have a large surface area and high reactivity [14]. Moreover, when compared to many other metallic NPs, the iron NPs 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 NPs (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 NPs (nFe-Pd) up to 0.1 g/L (estimated at $2 \times 10^{-3}$ M Fe and $2 \times 10^{-6}$ 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 NPs 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 NPs of titanium dioxide (TiO$_2$) are used for self-cleaning surfaces, for treatment of polluted water and in sun creams [18]. SiO$_2$ NPs are used as an anti-clumping-agent in common salt [18]. In the biological field, the NPs have a wide range of applications. On the one hand, the nanomaterials are used as antibacterial agents (i.e. NPs 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 NPS encapsulated in porous silica SiO$_2$ 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 alkoxysilanes: an SiO$_2$ network-forming reagent such as tetraethoxysilane (TEOS or Si(OC$_2$H$_5$)$_4$) and an alkoxysilane-functionalized ligand of the type (RO)$_3$Si-X-A, in which a functional organic group A, able to form a chelate with a metal such as iron, palladium or copper is linked to the hydrolyzable silyl group (RO)$_3$Si—via an inert and hydrolytically stable spacer X [22]-[24]. The iron NPS are encapsulated in the form of metal oxide (Fe$_x$O$_y$) in an inert matrix of silica. There is no chemical bond between SiO$_2$ and (Fe$_x$O$_y$). This is a physical blocking of metal oxides NPS of 2 to 3 nm size in microporous SiO$_2$ 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 NPS was investigated via the mass balance of iron liberated from the capsule of SiO$_2$ 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 NPS and SiO$_2$

The Fe/SiO$_2$ NPS 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[CH$_3$COCHCOCH$_3$]$_3$) in the initial homogenous solution of silica gel precursor tetraethoxysilane (Si(OCH$_2$)$_4$ or TEOS). This complex dried at 80°C after the gelation (Fe/SiO$_2$ dried). It is further calcined at high temperature (450°C to 550°C) to remove organic waste material and achieve the SiO$_2$ porous matrix encapsulating the iron oxide crystallites of Fe$_x$O$_y$. Moreover, the porous silica matrix without NPS, denoted SiO$_2$, was also synthesized by the sol-gel process to study the effect of the sole matrix of SiO$_2$ 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/SiO$_2$ calcined contained 1.53% (w/w) of active metal iron encapsulated in porous silica [27]. A $10^{-2}$ M Fe/SiO$_2$ suspension was prepared using 1.86 g of Fe/SiO$_2$ catalyst (agglomerates of microporous silica particles encapsulating iron NPS 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/SiO$_2$ 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 $10^6$ 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 NPS 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 NPS nor SiO$_2$ (positive control), with the sole SiO$_2$ matrix (positive control + SiO$_2$) or without inoculum (negative control with or without Fe/SiO$_2$ NPS). The negative controls were carried out in triplicates to examine the abiotic removal/evaporation of biphenyl. The optical density was measured at 600 nm (Ultrspec...
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 SorvallR RCSB+ 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 NPs

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 NPs and also for the control culture (without NPs).

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 absorbed 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 $10^{-4} \text{ M}$ 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 of Fe$^{II}$N$_4$ 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 NPs at concentrations of 0 (positive control) or $10^{-5} \text{ M}$ 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$_2$HPO$_4$, KH$_2$PO$_4$, 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 L mol$^{-1}$ 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$_2$HPO$_4$, KH$_2$PO$_4$, 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$_2$HPO$_4$, KH$_2$PO$_4$, 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 NPs 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 NPs 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 NPs added in the culture medium at concentrations ranging from $10^{-6}$ to $10^{-4} \text{ M}$ the comparison of the impact of $10^{-4} \text{ M}$ of calcined iron NPs and dried iron NPs was assessed. In the third experiment, the recycling of calcined iron NPs 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/SiO$_2$ were carried out without microorganisms, control (+) without NPs nor SiO$_2$, control (+) + SiO$_2$ 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 NPS Concentration

Figure 1(a) and Figure 1(b) show that the presence of iron NPS without inoculum did not exhibit any effect on the degradation of biphenyl when compared to the negative control without Fe/SiO₂ NPS at all time points \((p > 0.05)\). It suggests that the presence of different iron NPS 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 NPS 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 NPS 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 NPS 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 NPS 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 NPS 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 NPS (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 Ninnekak [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 NPS.

<table>
<thead>
<tr>
<th>Metabolites concentration (ppm)</th>
<th>3 days</th>
<th>8 days</th>
<th>18 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Catechol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td>66.14 ± 0.09</td>
<td>33.00 ± 0.07</td>
<td>19.40 ± 0.08</td>
</tr>
<tr>
<td>Culture with Fe/SiO₂ 10⁻⁴ M</td>
<td>74.38 ± 0.06</td>
<td>43.36 ± 0.09</td>
<td>24.50 ± 0.03</td>
</tr>
<tr>
<td><strong>Benzoic acid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td>1.19 ± 0.10</td>
<td>3.09 ± 0.35</td>
<td>13.39 ± 0.96</td>
</tr>
<tr>
<td>Culture with Fe/SiO₂ 10⁻⁴ M</td>
<td>4.36 ± 0.14</td>
<td>10.30 ± 0.21</td>
<td>25.39 ± 0.92</td>
</tr>
</tbody>
</table>
3.3. Effect of Dried or Calcined NPS

The impact of $10^{-4}$ M Fe/SiO$_2$ calcined NPs on biphenyl biodegradation was compared with similar NPs that did not undergo the calcination step. Therefore the iron NPs were still complexed between the acetyl acetonate and tetraethoxysilane molecules [27] whereas the calcined iron NPs are in the form of metallic oxide (Fe$_x$O$_y$) [38].

Figure 2(a) and Figure 2(b) shows that dried or calcined iron NPs achieved similar improvement effect on biphenyl biodegradation (90% or 91%). These results confirm that iron NPs, 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

![Figure 2](image)

**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 $4 \times 10^{-4}$ M of magnetic Fe$_3$O$_4$ nanoparticles. They achieved a 56% higher desulfurization activity in basic salt medium than with the undecorated cells. Olle et al. [39] also reported that the presence of 1 M of colloidal dispersions of magnetite (Fe$_3$O$_4$) 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)-nitrilotriacetic acid complex (FeNTA) increased the degradation of 2-aminobenzothiazol by *Rhodococcus rhodochrous* without any inhibition up to $5 \times 10^{-4}$ M FeNTA.

### 3.4. Effect of NPS Recycling

Following the experiment with $10^{-4}$ M calcined Fe/SiO$_2$ NPS, two cycles of culture (fresh inoculum) were carried out with the initial $10^{-3}$ M NPS 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 NPS) but considering a potential additional concentration of soluble iron transferred from the NPS. As seen in Table 2, the biphenyl degradation yield in presence of Fe/SiO$_2$ NPS (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 $10^{-3}$ M NPS (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 $2 \times 10^{-5}$ M to $6 \times 10^{-5}$ M 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 NPS 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 NPS.

<table>
<thead>
<tr>
<th>Experience condition</th>
<th>Culture with Fe/SiO$_2$ $10^{-4}$ M</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pellet</td>
<td>supernatant</td>
</tr>
<tr>
<td><strong>Biphenyl biodegradation yield (%)</strong></td>
<td>90</td>
<td>64</td>
</tr>
<tr>
<td><strong>Optical density</strong></td>
<td>$1.60 \pm 0.07$</td>
<td>$0.85 \pm 0.04$</td>
</tr>
<tr>
<td><strong>Total iron (M)</strong></td>
<td>$9.2 \times 10^{-3} \pm 0.01$</td>
<td>$2.01 \times 10^{-3} \pm 0.01$</td>
</tr>
<tr>
<td><strong>Second cycle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Biphenyl biodegradation yield (%)</strong></td>
<td>84</td>
<td>65</td>
</tr>
<tr>
<td><strong>Optical density</strong></td>
<td>$1.54 \pm 0.09$</td>
<td>$1 \pm 0.08$</td>
</tr>
<tr>
<td><strong>Total iron (M)</strong></td>
<td>$8.6 \times 10^{-3} \pm 0.01$</td>
<td>$3.0 \times 10^{-3} \pm 0.03$</td>
</tr>
<tr>
<td><strong>Third cycle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Biphenyl biodegradation yield (%)</strong></td>
<td>82</td>
<td>69</td>
</tr>
<tr>
<td><strong>Optical density</strong></td>
<td>$1.52 \pm 0.06$</td>
<td>$1.00 \pm 0.09$</td>
</tr>
<tr>
<td><strong>Total iron (M)</strong></td>
<td>$8.1 \times 10^{-3} \pm 0.01$</td>
<td>$6.0 \times 10^{-4} \pm 0.01$</td>
</tr>
</tbody>
</table>
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/SiO$_2$ NPS was found in solution ($3.5 \times 10^{-5}$ M ± 0.01) a similar content was adsorbed at the cell surface ($3.4 \times 10^{-5}$ M ± 0.01) and about 10% was considered intracellular ($9 \times 10^{-6}$ M ± 0.01).

This distribution also enables to calculate that about 25% of the initial $10^{-4}$ M iron NPS 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/SiO$_2$ 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 SiO$_2$ 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 Fe$^{3+}$ to Fe$^{2+}$, then Fe$^{2+}$ is transported by a specific membrane transporter into the cell [29]. In the second way, the Fe$^{2+}$ is chelated by a siderophore that is integrated into the cell, where Fe$^{3+}$ is reduced [43].

Intracellularly, an enzyme complex simultaneously reduces Fe$^{3+}$ to Fe$^{2+}$ 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 ($2\text{Fe}-2\text{S}$) and Fe$^{2+}$ on different sites. It could be inhibited when Fe$^{2+}$ is oxidized in Fe$^{3+}$ and reactivated by the addition of Fe$^{2+}$ [44] [45]. Nadaf *et al.* [32] found that the enzymatic activity of catechol1,2dioxygenase was completely inhibited with addition of $10^{-3}$ M of Fe$^{3+}$. This study also confirms the Fe$^{3+}$ 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/SiO$_2$ 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/
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) 1,2 catechol dioxygenase specific activity versus time. The similar letters indicate that no significant statistical differences were observed between the two curves ($p$-value > 0.05).

SiO$_2$ stimulates the catechol1,2 dioxygenase 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 NPs 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 NPs encapsulated in porous silica. This improvement was found to be dependent on the Fe/SiO$_2$ 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 NPs than in the control conditions without NPs. The performance increase is mainly recorded in exponential growth phase with a logarithmic increase of biphenyl degradation rate with increase of iron NP$_3$ concentration from $10^{-5}$ to $10^{-4}$ M. It was also shown that the positive effect of $10^{-4}$ M Fe/SiO$_2$ NPs was related to a higher enzyme activity since the biphenyl dioxygenase and catechol1,2dioxygenase 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 NPs encapsulated in the SiO$_2$ 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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