Biodegradation of phenol at low temperature using two-phase partitioning bioreactors

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Abstract Two-phase partitioning bioreactors offer many advantages for the removal of toxic pollutants. In particular, such systems can be loaded with very large quantities of pollutants without risks of microbial inhibition, they are self-regulated and they prevent the risks of hazardous pollutant volatilisation during aerobic treatment. However, their potential has never been tested at low temperatures. Phenol biodegradation by a cold adapted Pseudomonas strain was therefore tested at 14 or 4°C using 2-undecanone, diethyl sebacate or 2-decanone as organic phases in a two-phase partitioning bioreactor. The three solvents were biocompatible at 14°C but evidence was found that diethyl sebacate was biodegraded by the bacteria and this solvent was not tested further. Although only 2-decanone was suitable at 4°C, phenol biodegradation was more efficient in 2-undecanone at 14°C, reaching a maximum volumetric rate (based on the volume of aqueous phase) of approximately 1.94 g/L·day after 47 h of cultivation. In 2-decanone at 14°C, evidence was found that phenol degradation was limited by the release of biosurfactants, which increased the solubility and toxicity of the solvent in the aqueous phase inhibiting microbial activity. This study therefore shows that pollutant removal at low temperature is feasible but that the production of biosurfactants can have a negative impact on the process and must be taken into consideration when selecting the organic solvent. Future work should therefore focus on the selection of solvents suitable for use at temperatures below 14°C.

Keywords Biodegradation; biphasic; phenol; VOCs; wastewater; xenobiotics

Introduction
Two-phase partitioning bioreactors (TPPBs), also named biphasic liquid–liquid bioreactors, were originally developed to improve the fermentation of inhibitory biological products (Collins and Daugulis, 1997). Their principle is based on the use of a non-miscible and non-toxic organic solvent together with the aqueous phase in the reactor. Thus, an inhibitory compound can be extracted in situ in the bioreactor as soon as it is produced by the microorganisms and the biological reaction can be performed at much higher yields.

This concept was later extended to the field of environmental biotechnology and it was demonstrated that the organic phase could advantageously serve to deliver a toxic pollutant at a sub-inhibitory level in the aqueous phase (Daugulis, 2001; Déziel et al., 1999). Biphasic reactors are also advantageous by limiting the risks of pollutant volatilization during aerobic treatment and by being self-regulated because the pollutant delivery to the aqueous phase is controlled only by its removal rate (or the microbial activity) and its partitioning ratio between the organic and aqueous phases (Daugulis, 1997). Successful degradations were thus reported for phenol (Collins and Daugulis, 1997), BTEXs (Collins and Daugulis, 1999), styrene (El Aalam et al., 1993), chlorinated compounds (Ascon-Cabrera and Lebeault, 1993) and PAHs (Guieysse et al., 2001a). In the field of soil remediation, biphasic systems could be used for the treatment of any kind of concentrated mixture of contaminants that would originate, for instance, from soil extraction (Villemur et al., 2000; Guieysse et al., 2001b; Janikowski et al., 2002).

However, although a large part of the Earth’s biosphere temperature is below 5°C, TPPBs have always been tested at temperatures between 20 and 35°C (Collins and Daugulis, 1997). Temperature has a strong effect on the microbial activity and low...
temperatures are known severely to limit groundwater treatment in bioreactors as heating is not economically feasible (Langwaldt and Puhakka, 2000). In addition, the properties of the aqueous and organic phases (such as viscosity, toxicity, pollutant partitioning ratio, etc.) are influenced by temperature and it is necessary to develop processes suitable under cold climatic conditions.

This study was conducted to evaluate the potential of TPPBs at low temperature for the biodegradation of phenol by a cold-adapted Pseudomonas strain. Phenol was chosen as the model contaminant as being representative of toxic and semi-volatile contaminants found in many industrial wastes.

Material and methods

2-Undecanone (99%), diethyl sebacate (98%) and 2-decanone (98%), tested as organic phases, were obtained from Sigma-Aldrich.

A cold-adapted Pseudomonas mandelii (GenBank accession number AY179326) capable of using phenol as the sole carbon and energy source was used as inoculum (Soares et al., 2003). The organism was maintained on the following mineral salt medium (MSM, in mg/L): K₂HPO₄ 4,000, Na₂HPO₄ 4,000, KNO₃ 3,000, CaCl₂·7H₂O 10, MgSO₄·7H₂O 500, FeSO₄·7H₂O 10, MnCl₂·4H₂O 5.5, ZnCl₂ 0.68, CoCl₂·6H₂O 1.2, NiCl₂·6H₂O 1.2, CuCl₂·2H₂O 0.85, H₃BO₃ 0.0031, NaMoO₄·2H₂O 0.012, NaSeO₃·5H₂O 0.013, NaWO₄·2H₂O 0.0165. The pH was adjusted to 7.0 with sulphuric acid.

To provide inoculum for the subsequent tests, Pseudomonas sp. was cultivated in a 500 mL Erlenmeyer flask containing 200 mL of medium supplied with 200 mg phenol/L at 14°C and agitated at 100 rpm during 2 days. The inoculum was obtained by centrifuging the culture broth and removing approximately 170 mL of supernatant.

Measurement of partition coefficient

2-Undecanone, 2-decanone or diethyl sebacate, 0.5 mL of each, supplied with 10 g phenol/L was contacted with 5 mL of MSM in a 15 mL glass test tube and manually shaken during 30 s. The tubes were then left for equilibration for 1 h before being centrifuged at 1000 rpm for 5 min. The organic phase was removed using a Pasteur pipette and samples of the aqueous phases were withdrawn for phenol analysis. The phenol partitioning coefficient was then calculated by mass balance. The experiment was done in triplicate at room temperature (23 ± 2°C), 14°C and 4°C.

Solvent biocompatibility

Cultivation flasks of 125 mL were filled with 20 mL of MSM and 5 mL of organic solvent and inoculated with 1 mL of Pseudomonas culture. Yeast extract was provided at 5 g/L as an easily biodegradable carbon source. The flasks were then sealed with rubber septum to allow gas sampling from the headspace and incubated in the dark at 14°C and 100 rpm. Gas samples were taken every day to follow the cell activity by measuring the O₂ and CO₂ concentrations in closed bottles. When necessary, the flasks were open to replenish atmospheric O₂. All experiments were done in duplicate.

Phenol biodegradation and solvent biodegradability

A first series of experiment was done in 125 mL cultivation flasks filled with 2 mL of 2-undecanone, diethyl sebacate or 2-decanone supplied with phenol at 5 g/L, 10 mL of MSM supplied with 50 mg yeast extract/L and inoculated with 0.5 mL of Pseudomonas sp. culture. Control flasks that contained phenol at 200 mg/L in 50 mL MSM without adding any organic solvent were inoculated with 1 mL of Pseudomonas culture. Hence, all flasks were provided with the same total amount of phenol (10 mg) to allow comparison.
An additional set of experiments was done using the same procedure but with no phenol supplied in order to check whether the solvents were biodegradable by *P. mandelii*. All the flasks were incubated in the dark at 14°C and agitated at 100 rpm. Gas samples were taken every day to follow microbial respiration by measuring the O₂ and CO₂ concentrations in the flasks, headspaces. All experiments were done in duplicate.

A second series of experiments was conducted in 15 mL glass test tubes. Each tube was filled with 2.5 mL of MSM supplied with 50 mg yeast extract/L and 0.5 mL of solvent. The tubes were then inoculated with 0.2 mL of *P. mandelii* culture, closed with Teflon coated screw caps and incubated for 5 days in the dark at 14°C. Microbial activity in the tube was then stopped by heating the tubes at 80°C for 10 min. After cooling at room temperature, phenol concentration in the aqueous phase was determined by centrifuging the tubes at 1500 rpm for 10 min, removing the organic phases and measuring the phenol concentration in the aqueous phase by HPLC-UV. Phenol concentration in the organic phase was then calculated by mass balance using the experimental partition coefficient of each solvent. All experiments were done in duplicate.

**Bioreactor**

Phenol biodegradation was tested at 14°C in a 800 mL jacketed glass reactor filled with 100 mL of 2-undecanone or decanone containing 5 g phenol/L, 300 mL of MSM supplied with 0.5 g yeast extract/L and inoculated with 10 mL of *Pseudomonas* culture. The reactor was magnetically agitated at 300 rpm and mechanically aerated at 60 mL/min by bubbling moisturized air. The reactor temperature was maintained at 14°C by recirculating cooled water from a thermostated bath into the reactor jacket. Samples of 1 mL were periodically taken from the organic phase and contacted with 4 mL of purified water during 30 s in a separating funnel at room temperature. After 40 min, the two phases were separated and the phenol aqueous concentration was analysed by HPLC. The phenol concentration in the organic phase was then calculated using the partitioning coefficient at room temperature.

**Analysis**

Gas samples of 100 μL were withdrawn from the closed bottles with a syringe and analysed with a GC (VARIAN 3350) equipped with a thermal conductivity detector (TCD). The carrier gas was helium at a flow rate of 12 mL/min and the column used was a Haysep Q, 80–100 mesh, 2 m × 1/8” × 2 mm. The column, injector and detector temperatures were 70, 90 and 150°C, respectively.

HPLC analyses were performed using a Lachrom L-7100 liquid chromatograph equipped with a Lachrom L-7250 autosampler and Lachrom L-7400 variable wavelength monitor. Samples were injected on a Supelcosil LC-8 column and eluted with a mixture of 60:39:1 methanol:H₂O:acetic acid. UV detection was performed at 280 nm and external standards were used to enable quantitative determination. The phenol concentration in the organic phase was then determined by mass balance using the experimental aqueous-organic partition coefficient of phenol in each solvent.

**Results and discussion**

2-Undecanone, diethyl sebacate and 2-decanone were selected according to the screening made by Collins and Daugulis (1997) when studying the biodegradation of phenol in biphasic systems by a *Pseudomonas putida* strain. There are indeed many criteria relevant to the selection of an organic solvent for use in TPPBs (Bruce and Daugulis 1991): the solvent must not inhibit microbial activity, it should have good partitioning properties in regard to the target pollutant in order significantly to reduce its toxicity in the aqueous
phase. Finally, the solvent must not be biodegradable and non-miscible in water, it should not form emulsions and should be available in bulk quantities at a reasonable price. Only 2-undecanone was used by Collins and Daugulis (1997) but diethyl sebacate and 2-undecanone were kept here for being suitable for use at temperatures lower than 13°C (Table 1).

Microbial activity (characterized by O2 consumption and CO2 release in the flasks) occurred at comparable rate in all flasks supplied with organic solvent (but in the absence of phenol) when yeast extract was added as carbon source, showing that the three solvents were biocompatible at 14°C. The phenol partitioning coefficients in 2-undecanone and 2-decanone were similar to the values of 47.6 and 49.7, respectively, experimentally measured by Collins and Daugulis (1997). However, the partitioning ratio in diethyl sebacate was significantly lower than the 69 predicted by the same authors. The partitioning coefficients logically increased when temperature decreased (since the aqueous solubility of organic compounds decreases with temperature). This also shows that phenol transfer to the aqueous phase is likely to decrease with the temperature. Additional tests (data not shown) showed that at 14°C Pseudomonas veronii was only capable of biodegrading phenol provided at an initial concentration of 400 mg/L, but was inhibited at 500 mg phenol/L. Hence, 2-undecanone and 2-decanone can be loaded up to 20 g/L of phenol compared with only 6 g/L for diethyl sebacate, in order to reach an aqueous phenol concentration below 400 mg/L.

A high microbial activity was recorded in the control flasks supplied with diethyl sebacate, indicating that this solvent was probably biodegraded by the Pseudomonas sp. (Fig. 1). Microbial activity was even initially higher when phenol was not provided, which suggests an inhibition effect caused by this compound. The P. mandelii strain used was isolated for its capacity to biodegrade the recalcitrant and poorly soluble endocrine disruptor nonylphenol (Soares et al., 2003) and might therefore be capable of also biodegrading other poorly soluble substrate. In these tests, microbial activity stopped at the end of cultivation due to oxygen limitation, and opening of the flasks to replenish atmospheric O2 was immediately followed by a strong recovery of microbial activity (data not shown). Finally, phenol degradation tests showed that the competitive degradation of the diethyl sebacate severely limited the removal of phenol (Table 1) and this solvent was not tested further.

In the other tests, microbial activity in the flasks supplied with phenol was higher than in the control flasks not supplied with phenol, showing that this compound was mineralised (Fig. 1). Microbial activity in the control flasks containing 2-undecanone and especially 2-decanone was more likely due to the biodegradation of impurities present in the solvents rather than the solvent partial biodegradation since the O2 consumption and CO2 released dropped after 1–2 days of cultivation. In the presence of phenol, the maximum microbial activity during the exponential phase was faster in the flasks not supplied

<table>
<thead>
<tr>
<th>Solvent</th>
<th>K (g/g)</th>
<th>Melting point (°C)</th>
<th>Log Pow</th>
<th>Toxic</th>
<th>Phenol removal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>23°C</td>
<td>14°C</td>
<td>4°C</td>
<td>P_des</td>
<td></td>
</tr>
<tr>
<td>2-Undecanone</td>
<td>48.5</td>
<td>53.7</td>
<td>–</td>
<td>3.91</td>
<td>No</td>
</tr>
<tr>
<td>Diethyl sebacate</td>
<td>15.2</td>
<td>17.1</td>
<td>18.2</td>
<td>4.35</td>
<td>No</td>
</tr>
<tr>
<td>2-Decanone</td>
<td>52.9</td>
<td>60.0</td>
<td>65.6</td>
<td>3.39</td>
<td>No</td>
</tr>
</tbody>
</table>

1 The partition coefficient (K) is calculated as the ratio of phenol concentration in the organic phase by phenol aqueous concentration.
2 Results from the tests conducted in 15 mL test tubes supplied with 0.5 mL of solvent.
with an organic phase than in the flasks supplied with 2-undecanone or 2-decanone. This could have been caused by toxicity effects due to the solvent and/or by poor phenol transfer into the aqueous phase. First, according to the phenol partitioning coefficients, the initial phenol aqueous concentrations were significantly lower when these solvents were provided (approximately 70 mg/L) than in the controls (200 mg/L). Then, since the aqueous–organic mixtures were not intensely homogenised, phenol biodegradation could have become limited by its transfer to the aqueous phase. These tests were, however, not conducted to compare the two systems but to evaluate the feasibility of TPPBs for various solvents. Phenol removal in the biphasic systems was confirmed for both solvents in test tubes but phenol removal was significantly better in 2-undecanone than 2-decanone (Table 1).

At 4°C, O₂ consumption and CO₂ release occurred at slower maximal rates (during the exponential phases) than at 14°C (Fig. 2), which is logical knowing that the P. mandelli used had an optimum temperature around 10°C (Soares et al., 2003). This effect was much more pronounced in the flasks supplied with 2-decanone as this time, initial microbial activity was even higher in the controls (without phenol). However, after 5 days of incubation, microbial activity became higher in the flasks supplied with phenol. Microbial activity was much faster in the tests not supplied with organic solvent than in the test supplied with 2-decanone, showing a negative effect of temperature on toxicity and/or phenol mass transfer. However, these tests showed that phenol removal in TPPBs is feasible at temperature as low as 4°C.

According to these results, 2-undecanone was the most suitable solvent for the bioremediation of phenol at 14°C but only 2-decanone was suitable at lower temperatures.
As the tests above were conducted under conditions of poor aeration and homogenisation, the efficiency of TPPBs constructed with 2-undecanone and 2-decanone was therefore compared at 14°C in a well-mixed and well-aerated reactor.

When 2-undecanone was used as the organic phase (Fig. 3), phenol removal started after an initial lag phase of 24 h and reached a maximum volumetric rate (based on the volume of aqueous phase) of approximately 1.94 g/L·day after 47 h of cultivation. In 2-decanone, phenol was only removed by approximately 15% during the first 40 h of incubation after which phenol biodegradation apparently stopped (data not shown). Analyses of the aqueous phase showed that after 60 h of incubation, the phenol aqueous concentration was approximately 87 mg/L, which indicates that phenol removal was limited by low microbial activity rather than poor mass transfer. This aqueous

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**Figure 2** O2 consumption (squares) and CO2 release (circles) in flasks supplied with 2-decanone (A) or without organic solvent (B) and supplied with phenol (filled symbols) or not (open symbols). All flasks were inoculated with *P. mandelii* and incubated at 4°C at 100 rpm. Vertical bars represent the standard deviation on duplicates. The amount of O2 and CO2 was calculated using the ideal gas law at 4°C and subtracting the amount of O2 and CO2 consumed and released in the control flasks not supplied with organic solvent or phenol (the amount of gases dissolved was neglected).
concentration is significantly higher than the 54 mg/L predicted from the coefficient partition for 2-decanone at 14°C and the phenol remaining organic concentration in the organic phase at the time of sampling. This suggests that \textit{P. mandelii} released biosurfactants that increased phenol solubility biosurfactant production by the same bacterial strain has indeed been reported (Soares \textit{et al.}, 2003). HPLC analysis confirmed the release of an unknown metabolite in the aqueous phase and solvent emulsification was observed in all the biphasic tests. This substance was also detected when 2-undecanone and diethylsebacate were used (both in the absence or presence of phenol) but not during phenol biodegradation in the absence of an organic phase. Hence, since additional analysis confirmed that microbial inhibition was not due to pH effects or nutrient depletion, it is believed that the biosurfactants increased the solubility and toxicity of 2-decanone, which caused microbial inhibition (according to its lowest Pow coefficient, 2-decanone was also the most soluble and toxic solvent, \textit{Table 1}). More research is therefore needed to find a solvent suitable at temperatures lower than 14°C.

Despite the low temperature and the fact that the \textit{P. mandelii} used in this study were not specifically acclimatised to phenol, the maximum phenol removal rate achieved was in the range of the most of the values reported for phenol degradation at temperatures of 23 to 30°C (\textit{Table 2}). In addition, although very high removal capacities have be achieved using immobilised cells, various authors have reported inhibition effects due to high phenol concentrations (Santos \textit{et al.}, 2001; Santos and Linardi, 2004) and process failure under conditions of sudden pollutant load (González \textit{et al.}, 2001). Such effects would probably be avoided in TPPBs because the organic phase can act as a buffer to regulate the aqueous concentration of the pollutants. When 2-undecanone was used, it is not know if microbial growth was limited by \textit{O}₂ supply, phenol transfer to the aqueous phase or microbial activity. The system was operated at a low aeration rate (0.15 vvm based on the total liquid volume in the system) in order to prevent solvent emulsification during intensive aeration. This represents an intrinsic limitation to biphasic systems supplied with high loads of contaminants.

Conclusions
This study shows that TPPBs are also suitable for the treatment of high loads of toxic contaminants at low temperatures. Unfortunately, this study also shows that a new limitation of the use of TPPBs can occur as a consequence of the production of biosurfactants that lead to an increase in the solubility and toxicity of the organic phase. Such
Table 2 Comparison of phenol biodegradation in various systems

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Biodegradation tests</th>
<th>Load* (g/L)</th>
<th>Max removal rate (g/L day)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusarium sp.</td>
<td>Batch with suspended growth, 30°C</td>
<td>0.56–1.13</td>
<td>0.48</td>
<td>Santos and Linardi 2004</td>
</tr>
<tr>
<td>Trichosporon sp.</td>
<td>Batch with immobilized cell in 2% alginate gel beads, 30°C</td>
<td>1.88</td>
<td>1.46</td>
<td>Santos et al., 2001</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>Continuous with immobilised in polyacrylamide gel beads, 30°C</td>
<td>1.0–5.0</td>
<td>7.68</td>
<td>Chen et al., 2002</td>
</tr>
<tr>
<td>P. putida</td>
<td>Continuous in stirred tank and fluidised bed reactors with cell immobilised in calcium alginate gel beads, 30°C</td>
<td>1.0</td>
<td>3.2–4</td>
<td>González et al., 2001</td>
</tr>
<tr>
<td>P. putida</td>
<td>Continuous with immobilised in alginate gel beads, 25°C</td>
<td>0.1</td>
<td>1.4</td>
<td>Mordocco et al., 1999</td>
</tr>
<tr>
<td>Rhodococcus erythropolis</td>
<td>Continuous in a packed bed reactor with Biolite® carrier, 23 ± 3°C</td>
<td>0.20–0.50</td>
<td>7.2–14</td>
<td>Prieto et al., 2002</td>
</tr>
<tr>
<td>P. putida</td>
<td>Batch in a biphasic aqueous:2-undecanone, 30°C</td>
<td>0.60</td>
<td>0.432</td>
<td>Hamed et al., 2004</td>
</tr>
<tr>
<td>P. putida</td>
<td>Batch and fed-batch in an biphasic aqueous − 2-undecanone reactor, 30°C</td>
<td>2–10</td>
<td>3.24–4.2</td>
<td>Collins and Daugulis</td>
</tr>
<tr>
<td>P. mandelli</td>
<td>Batch in a biphasic aqueous − 2-undecanone reactor, 14°C</td>
<td>1.67</td>
<td>1.95</td>
<td>Present study</td>
</tr>
</tbody>
</table>

* Inlet concentration for continuous tests or initial concentration in batch and fed-batch tests.
phenomena must therefore be carefully studied during the screening suitability of organic phases. More research is therefore needed to find an organic phase suitable for applications at temperatures lower than 14 °C.

Acknowledgements
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References