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A combination of absorption and enzymatic biodegradation: phenol elimination from aqueous and organic phase.

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Abstract
Peroxidase from *Brassica rapa* (BRP) was immobilized as cross-linked enzyme aggregates (CLEAs) and used to treat air containing phenol as a model molecule of volatile organic compounds (VOCs). Prior to enzymatic treatment, phenol was absorbed into aqueous or organic phase (silicone oil, Rhodorsil® 47V20) to reach concentrations ranging from 20 to 160 mg/L. The process of enzymatic degradation was carried out by introducing a desired weighing of BRP-CLEAs into preparations and reaction was started by injecting $\text{H}_2\text{O}_2$ solution to medium.

Optimization of the reaction conditions in the organic solvent revealed an optimal contact time of 60 min, 60 mg/L initial phenol concentration and 3 mM $\text{H}_2\text{O}_2$, leading to a maximum phenol removal yield of 70% for 3.4 UI/mL of BRP-CLEAs. These results were compared to those obtained in aqueous medium that showed 90% of VOC degradation yield after 40 min in the following conditions, 90 mg/L initial phenol amount, 2 mM of $\text{H}_2\text{O}_2$ and an enzyme activity of 2.5 UI/mL. Parameters of the Michaelis-Menten model, $K_m$ and $V_{max}$, were also determined for the reaction of phenol degradation by BRP-CLEAs in both silicone oil and water. Phenol removal by *Brassica rapa* cross-linked enzyme in silicone oil succeeded with 70% of conversion yield. It is promising regarding the transposition of such enzymatic process to hydrophobic VOCs.

**Keywords:** BRP-CLEAs; Silicone oil; absorption; aqueous medium; biodegradation.

Introduction
The increasing development of industrial processes generate a variety of molecules that may pollute air and waters due to negative impacts for ecosystems and humans (toxicity, carcinogenic and mutagenic properties) [1]. Among these pollutants, volatile organic compounds (VOCs) and odorous compounds discharged into the environment represent a potential source of danger [2].

Phenol is considered as a volatile organic compound with a vapour pressure of 42.93 Pa at 25°C [3,4]. Due to its toxicity, it has been classified as a priority pollutant by environmental...
institutions [5–7]. The US National Institute of Occupational Safety and Health (NIOSH) fixed the concentration immediately dangerous to life or health at 250 ppm [8], while the US Environmental Protection Agency has established a provisional Reference Concentration for phenol of 0.006 milligrams per cubic meter in air [7]. As other VOCs, phenol is released into the air through a variety of industrial activities, such as synthetic resins, petrochemical, petroleum refineries, steel mills, coke oven plants, coal gas, pharmaceuticals, paints, plywood industries and mine discharge [1,9].

The EC legislations and regulations on air quality and waste air treatment are being more and more serious regarding polluting industries [10], due to the high increase of risk and their consequences on human’s health. An effective process of waste air treatment efficient for all pollutants does not exist, every VOC has been the subject of a single study, but despite of its toxicity, very few data can be found in the literature concerning the treatment of phenol-containing gaseous streams [1]. Numerous remediation methods have been developed for the removal and decomposition of VOCs, taking the example of biodegradation by microorganisms [11], biofiltration [12–14], advanced oxidation processes [15,16], and finally absorption into liquids. The key variable of this last process is the choice of a suitable liquid absorbent. For instance, in the case of hydrophobic VOCs, water is not the most appropriate solvent and hence other absorbents are required [17]. Regarding phenol, according to its polarity and their partial reciprocal solubility, water can be chosen to remove phenol from gaseous streams by using 3 to 20% of sodium hydroxide solution as a scrubbing agent. Due to the weak acidity of phenol and the large solubility of sodium phenate in water, the process has revealed its weakness as important quantities of phenate-containing wastewater should be disposed or treated [1]. More recent studies revealed that silicone oils show high capacity to absorb VOCs from air or water [18]. Silicone oils also display certain characteristics which are selection criteria of choice for absorbents, such as low volatility, thermal and chemical stability and high absorption capacity [19–21]. In addition to the absorption step, VOC biodegradation was considered to allow solvent regeneration and hence the recycling of the VOC-free solvent owing to its cost. This biodegradation step was performed in a two partitioning phase bioreactor (TPPB) involving an aqueous phase containing microorganisms [22]. Nevertheless, the combined process coupling absorption and biodegradation presents some kind of complexity and side problems which could limit its applicability [23]. An alternative to the TPPB was examined in this study, which consisted to test an enzymatic process in place of the microbial degradation. In addition, the performances of the enzymes
for phenol degradation were tested in both aqueous and organic phases to examine if the presence of water can be totally avoided and hence if only one liquid phase can be used for both steps of the combined process, absorption and enzymatic biocatalysis. Phenol was considered in this study as a model VOC compound owing to its wide industrial use and since the enzymatic degradation of phenol by peroxidases in the presence of hydrogen peroxide is among the most studied subjects in the field of enzymatic processes in recent years [24–27]. The process presents many advantages, such as simplicity under mild conditions, high turn-over, selectivity, less drawbacks when compared to chemical processes and lower cost. Peroxidase (EC 1.11.1.7) can be extracted from several sources, but the most studied one is horseradish Peroxidase (HRP), while other sources such as Brassica rapa showed great capacities as shown for the degradation of recalcitrant compounds including phenol [28–31]. Peroxidases were also successfully considered after immobilization on an appropriate support to eliminate phenol from aqueous streams [32–34]. The purpose of its use under immobilized form is to enhance its stability, recovery, selectivity and to reduce the inhibition by the medium or products [35]. A novel, practical and inexpensive immobilization technique has been developed by Sheldon et al [36], which has been named, cross-linked enzyme aggregates (CLEAs). It combines two unit processes, purification and immobilization, into a single operation. CLEAs have attracted increasing attention, due to their simplicity, broad applicability, long conservation and high stability in organic solvents [36–39]. The last characteristic is the main issue which could be exploited in the design of an integrated process (Absorption/enzymatic biocatalysis) for phenol degradation as a proof of feasibility towards VOCs treatment. The main purpose of this work was therefore to study the degradation of phenol by BRP-CLEAs in an organic phase (silicone oil) and to compare to that observed in an aqueous phase. In this aim, the impact of phenol and hydrogen peroxide concentrations, as well as enzyme activity, on the overall degradation kinetic were examined, with the objective of finding the optimal conditions under which the maximum yield of phenol removal could be achieved.

Materials and Methods

Chemicals and Reagents
Glutaraldehyde (25% solution), Hydrogen peroxide aqueous solution (30% w/w), guaiacol (98%) were of analytical grade and obtained from Sigma–Aldrich (Germany). Phenol 99% and sodium phosphate used for the preparation of buffer solution were purchased from Merck (Germany). The analytical reagents like 4-aminoantipyrine (4-AAP) and potassium ferricyanide were obtained from Sigma Aldrich (Germany).

Silicone oil (polydimethylsiloxane), Rhodorsil® 47V20 was purchased from the Rhodia Company, France. Table 1 summarises its main chemical and physical properties [40].

**BRP-CLEAs Preparation**

BRP-CLEAs were prepared as described in our previous work [41]. When needed, peroxidase was freshly prepared by squeezing some *Brassica rapa* turnip fruits of about 15 cm; a specific dilution of 3/4 of the filtrate with phosphate buffer was prepared and then directly precipitated using acetone. The mixture was subsequently cross-linked using glutaraldehyde solution (25%) at a concentration of 2% (V/V). Aggregated enzymes (CLEAs) were finally separated using centrifugation; then thoroughly washed with distilled water and stored in phosphate buffer (50 mM) (pH= 7) at 4°C until use.

**The activity assay of BRP-CLEAs**

The guaiacol colorimetric assay was used for measuring BRP-CLEAs activity [42]. The reaction mixture consisted of 4.05 µL of 98% guaiacol, 2 mg of CLEAs, 3.9 ml of phosphate buffer (50 mM, pH= 7). The reaction was started by the addition of 5 µl of H₂O₂ (0.8 M), ending up with a volume of 4 mL. The initial rate of colour formation at a wavelength of 470 nm was monitored using a SHIMADZU UV–VIS spectrophotometer model UV mini-1240 and a molar extinction coefficient of 4279 M⁻¹.cm⁻¹. The BRP-CLEAs activity was checked regularly before use in phenol biodegradation assays.

**Phenol absorption into silicone oil (Rhodorsil® 47V20)**

In order to generate the contaminated phase of silicone oil by the waste air stream of phenol, a laboratory model was adopted by dissolving a specific amount of phenol in Rhodorsil® 47V20, to achieve a final concentration of 1000 mg/L, the solution was kept under magnetic agitation for 2 hours at 50°C until all phenol crystals were dissolved. This preparation was considered as a stock solution for preparing further diluted solutions. It was conserved in a dark bottle at 4°C. In order to check its conservation, a calibration curve of phenol
concentration was daily established using phenol UV absorption method as described below ('Analytical methods' sub-section). The extinction coefficient was also determined.

**Phenol removal with the BRP-CLEAs**

Batch experiments were conducted to remove phenol from silicone oil HV20 as follows: reactions were carried out in 20 mL volumetric vials equipped with a magnetic stirrer and closed with PTFE-coated silicone rubber septa (PerkinElmer, France) sealed with aluminium caps. The mixtures consisted of 5 mL of silicone oil containing a specific concentration of phenol which was added from the stock solution. BRP-CLEAs (5 mg) were added to each vial. The reaction was initiated by adding hydrogen peroxide (5 µL) using a micro-syringe of 25 µL through the rubber septa. The vials were maintained at room temperature (25±3°C) under 600 rpm magnetic stirring to keep CLEAs permanently dispersed in the silicone oil phase during the reaction time.

Similarly, phenol degradation in aqueous environment was studied using a phosphate buffer solution (pH=7, 10 mM) as reaction medium. Other conditions were kept the same as described above using silicone oil as the liquid phase. At the end of the reaction, the solutions were centrifuged at 5000 rpm and the supernatant was assessed for residual phenol amount.

Aiming to maximize phenol degradation yield in both silicone oil and aqueous medium, the kinetics of phenol degradation was followed under different experimental conditions, namely various initial phenol concentrations in the range 20 to 160 mg/L, hydrogen peroxide from 0.5 to 20 mM and BRP-CLEAs amounts from 2.5 to 20 mg corresponding to an activity of 0.4 to 5 UI/mL.

Two parameters were considered to assess for the efficiency of phenol removal in both phases, silicone oil and aqueous phase, the conversion yield and the initial rate (V₀), which was determined by extrapolating the slope of the time course of the substrate concentration at initial time.

Experiments were performed in triplicate. Control experiments were done under the same conditions in organic or aqueous phases free from BRP-CLEAs then free from H₂O₂ to estimate the abiotic degradation of phenol.

**Analytical methods**

Residual phenol concentration in silicone oil was determined by direct analysis at the maximum absorbance wavelength of phenol (270 nm) using a UV-Vis spectrophotometer,
against a blank of silicone oil. Residual concentration of phenol was determined by using an appropriate calibration curve previously established with an extinction coefficient of \( \varepsilon_1 = 0.016 \text{ L/mg} \).

In aqueous solution, residual phenol was determined using a colorimetric assay in which the phenolic compounds within a sample reacts with 4-AAP in the presence of potassium ferricyanide reagent under alkaline conditions [43–45]. The procedure was as follows: a sample of 2.4 mL from the treated solution was withdrawn using a syringe and diluted with phosphate buffer (pH7) (phenol concentration up to 0.2 mM). The mixture was then added to 0.3 mL of potassium ferricyanide (83.4 mM) and 0.3 mL of 4-AAP (20.8 mM), and the mixture absorbance was measured after few minutes at 510 nm using an extinction coefficient of \( \varepsilon_2 = 9.8 \times 10^{-3} \text{ L/mg} \).

Results and Discussion

The BRP-CLEAs were prepared according to a method previously described [41]; and it was proved that they were quite stable in 47HV20 silicone oil which was encouraged for use in the considered absorption/enzymatic degradation process. The prepared CLEAs had a relative activity of 0.82 UI/mg.

Effect of the phenol concentration

The kinetics of any enzymatic reaction are affected by substrate concentration. Figure 1(a) and 1(b) show the evolution of phenol concentration with time. At constant peroxide and enzyme concentration, the effect of the initial phenol concentration \( C_0 \) on the initial rate \( V_0 \) and conversion yield (%), is reported in Figure 1(c) and 1(d). It is clear that \( V_0 \), as well as the removal yield, depend on the initial substrate concentration \( C_0 \). Similar remarks could be drawn for both aqueous and organic media. The curves of initial phenol concentration versus first reaction rate \( V_0 \) show that the rate increased rapidly and linearly at low phenol concentrations, but it gradually tended towards a limiting value at high concentrations of the substrate. A slight decrease in \( V_0 \) for phenol concentrations higher than 120 mg/L in silicone oil was found. In this region, all the enzyme molecules are bound to the substrate molecules, and the rate becomes zero order in substrate concentration. The optimal values of concentration of phenol were defined as 90 mg/L and 60 mg/L in aqueous phase and silicone oil, respectively.
At fixed hydrogen peroxide concentration and activity, the reaction proceeded faster in aqueous phase (40 min) than in silicone oil (60 min) (Figure 1(a) and 1(b)). Although phenol degradation in the organic solvent, silicone oil (47V20), did occur with BRP-CLEAs as a catalyst, only 15% of removal was achieved after one hour when phenol concentration was 60 mg/L. Another phenomenon which altered negatively the degradation in the organic solvent was the accumulation of product degradation on the surface of BRP-CLEAs, which limited the access of the substrate to the active sites and the overall reaction rate [46,47].

It was an expected result due to mass transfer limitations in the organic phase lowering the total activity of the enzyme [38,48,49]. Another explanation could be added which is based on solvent-substrate interactions which could lower the free energy of the substrate and thereby its reactivity. Rhodorsil® 47V20, a nonpolar solvent, dissolves phenol very efficiently lowering the free energy, decreasing consequently the rate of the reaction. The solvent also influences the equilibrium position of reactions, and hence the solvation of both substrates and products must be considered [50].

Kinetic parameters

To evaluate the effect of the silicone oil on the kinetics of phenol degradation by BRP-CLEAs, the Michaelis-Menten model was adopted and values ($K_m, V_{max}$) were determined using the Lineweaver–Burk double reciprocal plot (Equation (1)) in each phase (Figures. 2(a), 2(b)).

$$\frac{1}{\tilde{v}_0} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \times \frac{1}{[\text{Phenol}]_0}$$  

(1)

The apparent half-saturation constant ($K_m$) value reflects the affinity of the BRP-CLEAs for phenol. An enzyme with a high $K_m$ has a low affinity for its substrate [51], whereas $V_{max}$ is considered as the maximal rate at which a reaction could proceed. It is evident from table 2 that $K_m$ is affected by the nature of the solvent. The lower $K_m$ value obtained in silicone oil indicated a better affinity of BRP-CLEAs to phenol. However, the ratio $V_{max}/K_m$, which characterizes the specificity of the enzyme to the substrate (the larger the ratio, the more effective is the substrate utilization by the enzyme) [52], was in favor of the aqueous medium. Nevertheless, this could lead to false conclusions since these parameters are only apparent values regarding mass transfer limitations occurring with immobilized enzyme.

Effect of the hydrogen peroxide concentration
Figure 3 illustrates the effect of hydrogen peroxide concentration on the reduction of phenol concentration after 40 and 60 minutes of reaction time and for initial phenol concentration of 90 mg/L and 60 mg/L in aqueous phase and silicone oil, respectively. Hydrogen peroxide concentration in the reaction medium was varied by injecting four times volumes of 5 µL every 10 minutes in aqueous phase and every 15 minutes in silicone oil from freshly prepared peroxide solutions.

For the investigated range of hydrogen peroxide concentrations (0.5-20 mM), the rate and the percentage of phenol conversion increased with the peroxide concentration to reach an optimum between 2 to 3 mM of \( \text{H}_2\text{O}_2 \) in the case of aqueous phase (Figure 3(a)). Maximal conversion was about 60 to 65 % for an initial rate of 1.41 mg/L.min. While in the case of silicone oil, maximum conversion was about 40% for a lower initial rate 0.41 mg/L.min at a concentration of 3mM \( \text{H}_2\text{O}_2 \) (Figure 3(b)).

At peroxide concentrations higher than the optimal values detected in both phases, the rate and the yield of phenol conversion decreased dramatically. This decrease was most likely due to a deactivating effect of the hydrogen peroxide on peroxidase that irreversibly oxidize the enzyme ferriheme group essential for peroxidase activity [53] or to the fact that an excess of peroxide produces more intermediate products which inhibit the activity of the enzymes [32]. This result was an agreement with some studies dealing with phenol removal by peroxidase [54,55]. The above results show that the adequate molar ratio of hydrogen peroxide dose to the initial phenol concentration (\( \text{H}_2\text{O}_2 \)/[Phenol]) is 2:1 in aqueous phase and 4:1 in silicone oil (47V20); from this, BRP-CLEAs require a higher dose of hydrogen peroxide in organic medium compared to that needed in aqueous phase.

**Effect of the BRP-CLEAs activity**

The fourth parameter that could enhance the reaction of phenol degradation is the BRP-CLEAs concentration or activity in the reaction medium. All above experiments were carried out with a constant mass of enzyme 5 mg (≈0.86 UI/mL). To examine the impact of the BRP-CLEAs concentration, the molar ratio of phenol to hydrogen peroxide was kept constant at its optimal value, 1:2 in aqueous phase and 1:4 in silicone oil, and the enzyme activity was varied from 0.4 to 4.5 UI/mL by weighing the correspondent amount of BRP-CLEAs. Results obtained in both solvents are represented in (Figure 4). It is important to note that increasing the BRP-CLEAs amount in silicone oil was faced with the problem of their dispersion. In
order to avoid this problem and to ensure a good homogeneity of the system, the mixture was sonicated in ultrasound bath for 15 minutes at 25°C prior to peroxide injection. Data elucidate that enzyme concentration has a significant effect on phenol degradation in both solvents. The reaction rate increased rapidly when the concentration raised from 0.4 to 2.0 UI/mL in aqueous phase and from 0.5 to 2.8 UI/mL in silicone oil. This result was predictable since the availability of enzyme sites induce an attraction of substrate molecules and subsequently an increase in the number of molecules converted per unit of time.

Maximum performance of BRP-CLEAs was achieved at an optimal concentration of 2.5 UI/mL and 3.4 UI/mL in aqueous phase and organic solvent, respectively. While, increasing the enzyme concentration beyond these points had no noticeable effect because the substrate concentration became the limiting factor.

The conditions under which maximum phenol removal was achieved by BRP-CLEAs are summarized in Table 3.

**Conclusion**

Coupling absorption to enzyme conversion seems to be a promising new technique for the removal of hydrophobic compounds, which could be first absorbed in a suitable solvent containing the specific enzyme in the appropriate form. Results of the present study showed the feasibility of the process of phenol removal by *Brassica rapa* cross-linked enzyme into silicone oil. It was demonstrated that the process is significantly affected by substrate and enzyme concentrations which were optimized to obtain a maximal degree of phenol elimination. Mass transfer limitations and solvent-enzyme-substrate interactions are to be considered and well understood prior to any industrial process design calculations. These promising results also open new and promising perspectives towards the transposition of such enzymatic process to hydrophobic VOCs.
References


Figure legends

Figure 1. Effect of phenol concentration on the kinetics of enzymatic degradation by BRP-CLEAs in aqueous phase (a, c) and silicone oil (b, d). Reaction mixture: 5 mL of silicone oil or phosphate buffer (10 mM), 5µL of H₂O₂ (1M), 5mg of CLEAs (0.82UI/mL) at room temperature.

Figure 2. Lineweaver–Burk double reciprocal plot of phenol degradation by BRP-CLEAs in aqueous phase (a) and silicone oil (47V20) (b).

Figure 3. Effect of hydrogen peroxide concentration on phenol degradation using BRP-CLEAs in aqueous phase (a) and silicone oil (b). Reaction mixture: 5 ml of silicone oil or phosphate buffer (pH=7, 10 mM), 90 mg/L of phenol in aqueous phase and 60 mg/L in silicone oil (47 HV20), 5×4µL of H₂O₂, 5 mg of CLEAs (0.82 UI/mL) at room temperature.

Figure 4. Effect of BRP-CLEAs on phenol degradation in aqueous phase (a) and silicone oil (b). Reaction mixture: 5 ml of silicone oil or phosphate buffer (pH=7, 10 mM), 90 mg/L of phenol in aqueous phase and 60 mg/L in silicone oil (47HV20), 5×4µL of H₂O₂ (2 mM) in aqueous phase and (3 mM) in silicone oil at room temperature.
Tables legends

Table 1. Chemical and physical properties of Rhodorsil® 47V20.

Table 2. Kinetic constants of phenol degradation by BRP-CLEAs in aqueous phase and silicone oil (47V20)

Table 3. Optimal conditions for phenol removal by BRP-CLEAs in aqueous and organic media
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Table 1. Chemical and physical properties of Rhodorsil® 47V20.

<table>
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<tr>
<th>Property</th>
<th>Value</th>
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<tbody>
<tr>
<td>Molecular weight (g/mol)</td>
<td>2800 to 3200</td>
</tr>
<tr>
<td>Viscosity à 25 °C (mPa)</td>
<td>20</td>
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<tr>
<td>Density at 25°C</td>
<td>0.950</td>
</tr>
<tr>
<td>Dielectric constant (Debye)</td>
<td>2.68</td>
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</table>
Table 2. Kinetic constants of phenol degradation by BRP-CLEAs in aqueous phase and silicone oil (47V20)

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$K_m$ (mg/L)</th>
<th>$V_{max}$ (mg/L.min)</th>
<th>$V_{max}/K_m$ (1/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer (pH 7)</td>
<td>92.28</td>
<td>2.22</td>
<td>0.024</td>
</tr>
<tr>
<td>Silicone oil (47V20)</td>
<td>62.03</td>
<td>0.32</td>
<td>0.005</td>
</tr>
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</table>
**Table 3.** Optimal conditions for phenol removal by BRP-CLEAs in aqueous and organic media

<table>
<thead>
<tr>
<th></th>
<th>Aqueous phase</th>
<th>Silicone oil (47V20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol concentration (mg/L)</td>
<td>90</td>
<td>60</td>
</tr>
<tr>
<td>Hydrogen peroxide (mM)</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>CLEAs activity (UI/mL)</td>
<td>2.5</td>
<td>3.4</td>
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<tr>
<td>Reaction time (min)</td>
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<td>60</td>
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