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ORIGINAL PAPER

Optimisation of the operational conditions of trichloroethylene degradation using *Trametes versicolor* under quinone redox cycling conditions using central composite design methodology

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Abstract Extracellular radicals produced by *Trametes versicolor* under quinone redox cycling conditions can degrade a large variety of pollutant compounds, including trichloroethylene (TCE). This study investigated the effect of the agitation speed and the gas–liquid phase volume ratio on TCE degradation using central composite design (CCD) methodology for a future scale-up to a reactor system. The agitation speed ranged from 90 to 200 rpm, and the volume ratio ranged from 0.5 to 4.4. The results demonstrated the important and positive effect of the agitation speed and an interaction between the two factors on TCE degradation. Although the volume ratio did not have a significant effect if the agitation speed value was between 160 and 200 rpm, at lower speed values, the specific pollutant degradation was clearly more extensive at low volume ratios than at high volume ratios. The fitted response surface was validated by performing an experiment using the parameter combination in the model that maximised TCE degradation. The results of the experiments carried out using different biomass concentrations demonstrated that the biomass concentration had a positive effect on pollutant degradation if the amount of biomass present was lower than 1.6 g dry weight l^{-1}. The results show that the maximum TCE degradation was obtained at the highest speed (200 rpm), gas–liquid phase volume ratio (4.4), and a biomass concentration of 1.6 g dry weight l^{-1}.

Keywords White-rot fungi · Hydroxyl radicals · Surface response methodology · Scale-up

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Introduction

Trichloroethylene (TCE) is a chlorinated hydrocarbon classified as an organopollutant. This compound is found in the environment (soil, groundwater, river sediments) due to the improper disposal practices used during the twentieth century (ATSDR 2007; NRC 2006). This compound is resistant to degradation by microorganisms present in the environment due to its chemical structure, and as a consequence, TCE is highly persistent (Pant and Pant 2010). TCE is considered a toxic compound, although there is no

clear evidence of any carcinogenic effect of TCE in humans (Tabrez and Ahmad 2009).

It has been demonstrated that TCE and other chlorinated solvents are degradable by white-rot fungi (WRF) under aerobic conditions (Khindaria et al. 1995; Yadav et al. 2000; Marco-Urrea et al. 2008a, b). WRF can degrade a large variety of otherwise degradation-resistant pollutants as the result of the production of extracellular enzymes, principally laccase and peroxidases (D'Annibale et al. 2005; Baborova et al. 2006; Michniewicz et al. 2008; Acevedo et al. 2010), and intracellular enzymes, which are highly reactive. In the case of TCE, the intracellular cytochrome P450 system is responsible for the degradation (Marco-Urrea et al. 2008a).

Advanced oxidation processes (AOPs) are physico-chemical treatments that produce hydroxyl radicals ($^{\bullet}$OH), which are responsible for pollutant oxidation. Different AOP treatments have been applied successfully to degrade TCE and other chlorinated ethylenes (Hirvonen et al. 1996; Weeks et al. 2000; Kan et al. 2007; Young et al. 2008). This article proposes an alternative treatment using a biological process to produce hydroxyl radicals to degrade pollutants. This process, which is described in detail elsewhere (Gomez-Toribio et al. 2009a, b), consists of the induction in ligninolytic fungi of extracellular $^{\bullet}$OH production, based on a quinone redox cycling mechanism activated by the incubation of the fungi with a lignin-derived quinone (i.e., 2,6-dimethoxy-1,4-benzoquinone, DBQ) and chelated ferric ion (Fe^{3+}-oxalic acid). Because the hydroxyl radical is the strongest oxidant produced in white-rot fungi cultures (Backa et al. 1993) and because it is a non-selective oxidant, this process allows the degradation of pollutants that are usually very difficult to degrade by biological treatments.

Previous results have demonstrated that the induction of $^{\bullet}$OH production in *Trametes versicolor* via quinone redox cycling leads to the degradation of approximately 30% of the TCE present in closed test tubes (Marco-Urrea et al. 2009). The free chloride ion balance and the increase in the δ^{13} in the isotopic relationship analysis of the $^{13}C/^{12}C$ ratio of the CO_2 produced during the degradation process demonstrate that part of the total TCE degraded was mineralised. However, after a certain time, quinone redox cycling and TCE degradation stopped. A recent publication showed that the degradation of BTEX (benzene, toluene, ethylbenzene and xylene isomers) by *T. versicolor*, incubated under quinone redox cycling conditions, stopped when DBQ and oxygen were exhausted (Aranda et al. 2010).

To improve TCE degradation and extend the reaction time, we investigated the induction in *T. versicolor* of $^{\bullet}$OH production through quinone redox cycling to degrade TCE in higher-volume reactors, which has not been carried out previously. This experimental volume change allowed an increase in the amount of oxygen available and the addition of DBQ during the process due to an increase of the gas–liquid phase volume ratio relative to closed test tubes. As a result of this, TCE degradation was significantly improved in relation to that observed in previous experiments. Moreover, an extensive study of the effect of three parameters, the agitation speed, the gas–liquid phase volume ratio and the biomass concentration, in TCE degradation by *T. versicolor* under quinone redox cycling conditions is reported for the first time. The main objective was to study the effects of these parameters and their interaction on the degradation process to optimise this process for future scale-up to a reactor system.

Materials and methods

Chemicals

TCE (≥99.5%), DBQ (97%), 2-deoxyribose (≥98%) and 2-thiobarbituric acid (TBA, ≥98%) were obtained from Sigma-Aldrich (Barcelona, Spain). All other chemicals used were of analytical grade.

Fungus and culture conditions

Trametes versicolor (ATCC#42530) was maintained by subculturing on 2% malt extract and 1.5% agar slants (pH 4.5) at 25°C. Subcultures were started every 30 days. Malt extract was obtained from Scharlau Co. (Barcelona, Spain).

T. versicolor pellets were produced by inoculating 250 ml of malt extract medium in a 1 l Erlenmeyer flask with 1 ml of mycelial suspension, prepared as described previously (Vilaplana et al. 2008). This mixture was shaken (135 rpm $r = 25$ mm) at 25°C for 5 days. Subsequent pellets formed by this process

were transferred to 1 l Erlenmeyer flasks containing 250 ml of a defined medium described elsewhere (Marco-Urrea et al. 2008a) and were incubated under the same shaking conditions for 2 days.

TCE degradation experiments

TCE degradation experiments were carried out in 125 ml serum bottles (161 ml total volume) with Teflon-coated grey butyl rubber stoppers (Wheaton, Millville, USA) and aluminium crimps (Baxter Scientific Products, Deerfield, USA). Each bottle contained the same amount of 2-day-old washed mycelium pellets, 500 μM DBQ, 100 μM Fe^{3+}-300 μM oxalate, and 100 μM Mn^{2+} as $MnSO_4 \cdot H_2O$ in 20 mM phosphate buffer, pH 5, based on a previous $^{\bullet}OH$ production optimisation study using quinone redox cycling (Gomez-Toribio et al. 2009b). The stock solution of Fe^{3+}-oxalate was prepared by mixing equal volumes of 20 mM $Fe(NO_3)_3 \cdot 9H_2O$ and 60 mM oxalic acid. This solution was freshly prepared for each experiment. Before pollutant addition, each bottle was oxygenated for 1 min and sealed immediately. After that, the bottles were incubated horizontally at 25°C and the desired agitation speed (three samples for each point of analysis). All experiments, including non-inoculated and control bottles (without Fe^{3+}-ox and Mn^{2+}), were carried out in triplicate.

The desired concentration of TCE, dissolved in acetonitrile, was added to each bottle through the septum by means of a pressure-lock gas tight syringe (VICI Precision Sampling, Baton Rouge, USA). The percentage of TCE that has been degraded at each specified time was calculated by comparing the pollutant concentration in the control bottles and the concentration in the experimental bottles. Cultures were discarded after sampling.

The DBQ solution added during experiments was introduced through the septum using a plastic syringe.

TCE analysis

The concentration of TCE was determined by static headspace gas chromatography. All samples were equilibrated at 25°C before analysis. In the case of serum bottles, the appropriate volume of liquid sample from each experimental bottle was transferred to a 10 ml vial with the appropriate volume of 5% sodium azide, which was added to stop the reaction in the sample (Johannes and Majcherczyk 2000), to obtain a final liquid volume of 5 ml.

After sample addition, the vial was sealed immediately with a Teflon-coated stopper. The detailed GC operating conditions are described elsewhere (Vilaplana et al. 2008; Marco-Urrea et al. 2008a).

The total amount of TCE in the experimental bottles and its concentration in the liquid media were determined by comparing the peak areas with those of external standards and by using Henry's law constants chosen from among the bibliographic values found (Gossett 1987) and then verified in our laboratory.

Other analyses

TBA-reactive substances (TBARS) production from deoxyribose (Halliwell and Gutteridge 1981) was used to estimate $^{\bullet}OH$ production. The TBARS concentration was determined as follows: 0.5 ml of 2.8% (w/v) trichloroacetic acid and 0.5 ml of 1% (w/v) TBA in 50 mM NaOH were added to 1 ml samples, which were then heated for 10 min at 100°C. After cooling the samples, the absorbance was read at 532 nm against appropriate blanks (Gutteridge 1984).

The pellet dry weight was determined by vacuum filtering the cultures with glass filters of known weight (Whatman GF/C, 47-mm-diameter, Maidstone, England). The filters containing the biomass were placed in glass dishes and dried at 105°C to constant weight.

Experimental design methodology and statistical analysis of TCE degradation experiments using a quinone redox cycling process

Central composite design (CCD) methodology with two factors ($k = 2$) was applied to study the effect of the agitation speed (x_1) and the gas–liquid phase volume ratio (x_2) on TCE degradation by *T. versicolor* under quinone redox cycling conditions. This methodology is commonly used in process optimisation because it requires fewer experiments than a full factorial design. CCD allows the fitting of a full quadratic polynomial model, and this method permits the statistical differentiation of the roles of the different factors and the random error associated with the experiments. The factor levels were normalised and coded in the range $(-\alpha, +\alpha)$.

Table 1 Design matrix and response values of TCE degradation

Experiments	Actual levels		Coded levels		Response
	Agitation speed (rpm)	V_G/V_L	Agitation speed	V_G/V_L	TCE degradation (mg g^{-1} dry weight)
1	90	2.4	−1.414	0	9.32
2	90	2.4	−1.414	0	8.68
3	90	2.4	−1.414	0	8.09
4	106	1.0	−1	−1	20.73
5	106	1.0	−1	−1	19.62
6	106	3.8	−1	1	11.15
7	106	3.8	−1	1	14.56
8	145	4.4	0	1.414	12.92
9	145	4.4	0	1.414	7.75
10	145	4.4	0	1.414	10.49
11	145	2.4	0	0	10.59
12	145	2.4	0	0	9.40
13	145	0.5	0	−1.414	14.72
14	145	0.5	0	−1.414	16.03
15	145	0.5	0	−1.414	17.75
16	184	1.0	1	−1	21.32
17	184	1.0	1	−1	23.58
18	184	1.0	1	−1	23.39
19	184	3.8	1	1	25.14
20	184	3.8	1	1	24.77
21	184	3.8	1	1	25.76
22	200	2.4	1.414	0	23.39
23	200	2.4	1.414	0	18.90
24	200	2.4	1.414	0	17.82

CCD consists of 2^k factorial points representing all combinations of the codified values (± 1), 2^k axial points at a distance $\pm\alpha$ from the origin, and at least three central points with an encoded value of zero. The α value is 1.414 ($\alpha = F^{1/4}$, where $F = 2^k$), which represents the extreme value of each factor involved in the design. Typically, three replicates at the central point are sufficient to evaluate the experimental uncertainty. However, in this experimental design, each combination was tested in triplicate to increase the confidence of the fitted model. A detailed explanation of the method and its possible applications can be found in the literature (Deming and Morgan 1987).

Nine combinations of the independent variables were evaluated, thus leading to an experimental design that included 27 runs. Table 1 shows the experimental design matrix with the coded and actual values obtained from the combination of five levels for each factor. The agitation speed ranged from 90 up to 200 rpm. The range of the gas-to-liquid volume ratio was chosen as a compromise between the need to treat adequate volumes and the need to increase oxygen availability. The response of the TCE degradation rate (y) to each combination of parameters is also shown.

Each response function can be fitted to a second-order polynomial model (Eq. 1) considering the factors level.

$$y = b_0 + b_1 \times x_1 + b_2 \times x_2 + b_{11} \times x_1^2 + b_{22} \times x_2^2 + b_{12} \times x_1 \times x_2 \quad (1)$$

The model parameters (b_i) were estimated and the statistical analysis was performed based on the experimental values using the Sigmaplot© 11.0 software package (Systat Software Inc., San Jose, USA).

Results and discussion

Quinone redox cycling process scale-up to degrade trichloroethylene

Satisfactory TCE degradation results using the quinone redox cycling process were obtained in 8 ml closed tubes with a gas–liquid phase volume ratio of 1 (Marco-Urrea et al. 2009). To date, the oxygen availability during the process and the low DBQ concentration have been the main limitations of TCE degradation by quinone redox cycling. TCE is highly volatile, and consequently it must be kept in a hermetically closed system, thus limiting the addition of reagents such as oxygen during the process. In addition, DBQ has a low solubility, making it impossible to use a high initial DBQ concentration.

To improve the TCE degradation yield, an experiment was performed using higher-volume reactors (161 mL) that allow an increase in the gas–liquid phase volume ratio in the experimental system. Therefore, a higher level of oxygen was available for the fungus, and it was possible to add DBQ, thus prolonging the process.

In this experiment, the gas–liquid phase volume ratio was increased to 1.7, and a noticeable increase in pollutant degradation was observed at 6 h. A level of TCE degradation of 13.7 ± 1.5 mg g^{-1} dry weight was obtained for the amount to TCE that had been degraded, in comparison with a degradation level of 10.0 ± 0.3 mg g^{-1} dry weight obtained in the test tube experiments. This result suggests that oxygen could be a limiting factor in this process because the TCE degradation yield was improved by increasing the gas phase volume.

The amount of $^{\bullet}$OH produced during the quinone redox cycling process can be calculated by estimating the production of TBARS from 2-deoxyribose, as the level of TBARS formed has been shown to be quantitatively correlated to the formation of Fenton's reagent during the process in *Pleurotus eryngii* (Gomez-Toribio et al. 2009a). Figure 1 shows the formation of hydroxyl radicals in serum bottles under the same experimental conditions as the TCE degradation experiments. An increase of TBARS production was observed over time, indicating that $^{\bullet}$OH radicals were produced during the entire process. The changes observed in the parameters over time in this figure correspond to the typical production behaviour of

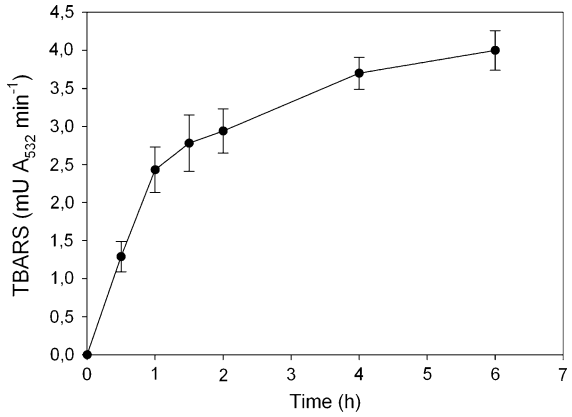

Fig. 1 $^{\bullet}$OH production, estimated as TBARS production by *Trametes versicolor* under quinone redox cycling conditions in serum bottles

hydroxyl radicals by the fungus under quinone redox cycling conditions, which usually stops after 6 h due to DBQ and oxygen depletion (Aranda et al. 2010).

During the experiments in the serum bottles, DBQ was added at 6 h, at which time the original DBQ supply had been depleted, to determine which compound is depleted first, causing the quinone redox cycling to stop. Six hours after addition, the TCE degradation had increased from 13.7 ± 1.5 mg g^{-1} dry weight to 23.4 ± 4.2 mg g^{-1} dry weight. This result implies that the oxygen supply had not been exhausted at this time because the degradation continued.

This result indicates that the first limiting factor is DBQ. The addition of DBQ is expected to cause a marked increase in the liquid phase volume due to the low solubility of DBQ. As a result of this volume increase, the concentration of TCE is reduced in the liquid phase, provoking a decrease in the degradation rate compared with that achieved before the addition of DBQ, as it is reported that TCE degradation by hydroxyl radicals can be adjusted to a pseudo-first-order degradation reaction, which depends directly on the TCE concentration in the liquid phase (Weeks et al. 2000; Chen et al. 2001). Future research should concentrate on the search for a quinone, such as anthrahydroquinone-2,6-disulfonate, juglone, lawsone or purified humic acids, that is as efficient as DBQ in terms of $^{\bullet}$OH production and is more soluble than DBQ.

Although oxygen is the second limiting factor in the process, the addition of oxygen is not

experimentally viable because the serum bottles must be hermetically closed to prevent TCE loss.

Although it has been demonstrated that TCE can be degraded by the quinone redox cycling process in serum bottles, it is important to study the different parameters that influence TCE degradation to optimise the process and to obtain the maximum TCE degradation yield in this experimental system.

Response surface and statistical analysis of TCE degradation by *T. versicolor* under quinone redox cycling conditions

The TCE degradation rate obtained at 6 h (Table 1) was used to fit the second-order polynomial model as a function of the agitation speed and the gas–liquid phase volume ratio. Out of the 27 experiments, 3 had to be rejected due to practical incidences. Table 1 shows appreciable differences in pollutant degradation between all of the experiments, with values ranging from 7.7 to 25.8 mg TCE g^{-1} dry weight.

Table 2 shows the b_i coefficient values of the fitted model. The results demonstrate that the full second-order model is suitable to fit the experimental values and that all of the parameters are significant. However, the correlation coefficient value (R^2) was 0.705, which indicates that there may be factors other than the studied factors that affect the degradation process. Nevertheless, for such a complex system, this correlation level is very common, and the results can be used to predict, with sufficient accuracy, the degradation levels in the studied range.

Table 2 Values of the b_i coefficients and statistical analysis of the fitted model

Coefficients	Coefficient values	Standard error	*t*-ratio	*P* value
b_0	9.964	2.750	3.624	0.002
b_1	4.209	0.832	5.056	<0.0001
b_2	−1.677	0.837	−2.004	0.060
b_{11}	3.643	1.542	2.362	0.030
b_{22}	3.098	1.542	2.009	0.059
b_{12}	2.536	1.241	2.044	0.055

Effect of the agitation speed and the gas–liquid phase volume ratio on TCE degradation by *T. versicolor* under quinone redox cycling conditions

The coefficients related to the agitation speed shown in Table 2 indicate that this parameter has a positive and greater effect on TCE degradation than the gas–liquid phase volume ratio. The fact that an increase in the agitation speed has a positive effect on pollutant degradation is not surprising because this increase improves the mass transfer rates of TCE and oxygen between the phases. Therefore, it is important to operate at the highest agitation speed that is experimentally possible to avoid limiting the mass transfer between phases and to obtain the maximum level of pollutant degradation.

On one hand, the b_2 coefficient value shows that the gas–liquid phase volume ratio has a negative effect on TCE degradation, but its positive quadratic effect is significant. On the other hand, statistical analysis showed that the interaction between the two factors has a positive and significant effect on TCE degradation. The effect of both factors can be better analysed by representing the surface response as shown in Fig. 2. According to this representation, the highest TCE degradation is obtained at the maximum agitation speed (200 rpm) and the maximum volume ratio (4.4), but this level of degradation was not

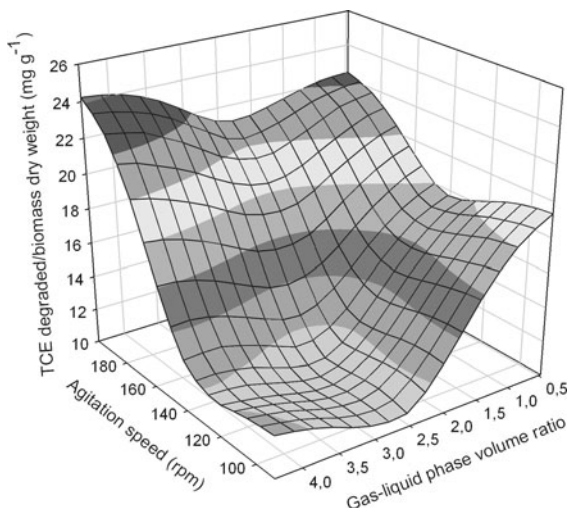

Fig. 2 Surface response of TCE degradation by *Trametes versicolor* under quinone redox cycling conditions carried out at different agitation speeds and gas–liquid phase volume ratios

significantly higher than the values obtained at all of the other volume ratios for the highest agitation speed range, between 180 and 200 rpm, as a maximum TCE degradation difference of approximately 13% was obtained in this range.

The degradation values at an agitation speed between 160 and 180 rpm were also very similar for all volume ratios, which indicate that this parameter had no significant effect on TCE degradation at high agitation speeds. Therefore, this result suggests that another factor, such as dissolved oxygen, could have a more important effect on TCE degradation than the gas–liquid phase volume ratio. The importance of another factor is also suggested by the significance level of the coefficients related to the volume ratio, which are higher than the accepted alpha level (Table 2). However, at agitation speed values below 160 rpm, the volume ratio has an important effect on pollutant degradation; the degradation process is clearly improved if it is carried out at a low gas–liquid phase volume ratio, and the volume ratio has a maximum effect at an agitation speed of 90 rpm. The fact that the maximum degradation difference between different volume ratios was obtained at the minimum agitation speed is not surprising because the pollutant mass transfer depends on the agitation speed.

To validate the fitted model, an experiment was carried out using a specific combination of parameters. The values chosen were an agitation speed of 200 rpm and a volume ratio of 4.4, which corresponds to the combination estimated to maximise TCE degradation. The pollutant degradation obtained for a time of 6 h was 22.9 ± 1.6 mg TCE g^{-1} dry weight, which is very similar to the value obtained from the response surface at the same parameter combination, approximately 24 mg TCE g^{-1} dry weight. Therefore, the results of the validation procedure confirm the results shown in Table 2, which show that the model has a suitable level of fit to the experimental values.

Effect of the *T. versicolor* concentration on TCE degradation under quinone redox cycling conditions

After the optimisation of the process in relation to the agitation speed and the gas–liquid phase volume ratio, it was concluded that the highest TCE degradation values are obtained for an agitation speed between 180 and 200 rpm and any volume ratio between 0.5 and 4.4. Because it was observed that the volume ratio is not an important parameter if the agitation speed is higher than 180 rpm, the maximum agitation speed (200 rpm) and the minimum volume ratio were chosen to determine the effect of different biomass concentrations on TCE degradation under quinone redox cycling conditions. High biomass levels may indicate a high degradation rate but also a high oxygen uptake rate. As a consequence, the oxygen mass transfer from the gas to the liquid phase can be even more critical and can therefore affect the degradation rate.

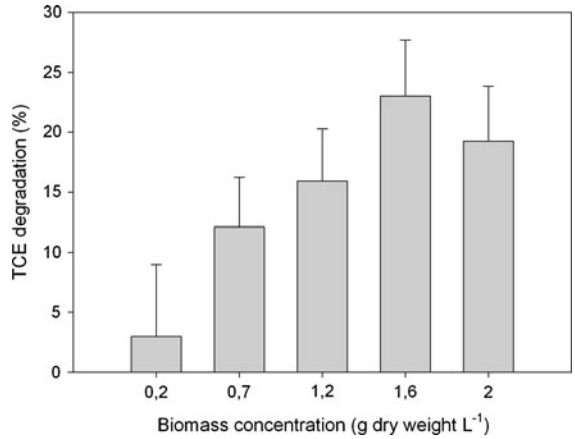

Fig. 3 Effect of the biomass concentration on TCE degradation by *Trametes versicolor* under quinone redox cycling conditions (an agitation speed of 200 rpm and a gas–liquid phase volume ratio of 0.5)

The TCE degradation percentages obtained with different biomass levels are shown in Fig. 3. This parameter has a positive effect on TCE degradation if its values are lower than 1.6 g dry weight l^{-1}, and the maximum degradation percentage (23%) was obtained at this biomass concentration.

Conclusion

The effects of the agitation speed, the gas–liquid phase volume ratio and the biomass concentration on TCE degradation by *T. versicolor* under quinone redox cycling conditions was studied. The results obtained show that the agitation speed has an important and positive effect on TCE degradation. With respect to the gas–liquid phase volume ratio, its

effect on pollutant degradation depends on the agitation speed, and the effect of this parameter is not significant in the highest agitation speed range. This fact might suggest that dissolved oxygen affects TCE degradation to a greater extent than the volume ratio does. Finally, the effect of the biomass concentration on TCE degradation is not negligible, and higher pollutant degradation percentages were obtained as the biomass concentration increased, except for the highest biomass assayed of 2.0 g dry weight l^{-1}.

In summary, the information obtained from the optimisation process can be very useful for future scale-up to a reactor system, in which oxygen and DBQ can be added during the degradation process. Additionally, the use of a quinone other than DBQ, which would not exhibit the experimental limitations observed for DBQ, could help to improve the process efficiency in terms of pollutant degradation.

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