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ORIGINAL ARTICLE

Dehalogenation of a gaseous effluent by dehydrated whole cells in a solid/gas reactor: A study of catalyst stability

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Abstract
Dehydrated cells of recombinant *Escherichia coli* producing a halidohydrolase have been used as catalyst for the continuous hydrolysis of gaseous 1-chlorobutane. Viability was not required for catalysis but a low stability was observed. At 40°C and water activity of 0.7, this behavior could be explained by two main phenomena. Thermal denaturation induced the irreversible inactivation of 55% of the catalyst during the first 16 h of reaction, whereas accumulation of HCl acidified the medium and led to a reversible decrease in enzyme activity. Damage caused by the release of proteases during catalyst preparation did not seem to be a major factor. Since neither 1-chlorobutane nor 1-butanol accumulated in the bioreactor, the decrease in activity was not due to the accumulation of these compounds.

Keywords: *Dehalogenation, halogenated volatile organic compounds, solid/gas bioreactor, stability, gaseous effluent*

Introduction

Halogenated volatile organic compounds (VOCs) constitute one of the largest groups of environmental pollutants due to their widespread use, their toxicity and their persistence (Mohamed et al. 2002). These compounds are known to cause direct and indirect effects on the environment and human health (Le Cloirec 1998). Several chemical and physical processes have already been developed to reduce their emission. Among them, incineration and active coal fixation are the most common. Both of these techniques can be efficient for a large range of flow and pollutant concentrations but have some limitations, particularly for applications with dilute effluents (Hasnaa & Heitz 1999).

Bioprocesses have been proposed to overcome these limitations. They are particularly well suited for the removal of odorous, biodegradable compounds. Nevertheless, biofilters and biotrickling filters suffer from the low solubility of VOCs in water, and these processes can be limited by the toxicity of xenobiotics toward bacteria (Hasnaa & Heitz 1999). Selection of microorganisms with extended substrate ranges could help to solve this problem, and there have been some examples of application of biofilters and trickling filters for the degradation of compounds which were previously considered non-biodegradable (Diks & Ottengraph 1991a,b; Lackey et al. 2002). An alternative approach is offered by a new generation of biofilters using dry whole cells (Erable et al. 2004) for the hydrolysis of gaseous halogenated pollutants. This does not require the solubilization of VOCs in an aqueous phase and allows the direct treatment of gaseous effluents. Several bacteria producing a haloalkane dehalogenase have been tested and appear able to dehalogenate a range of pollutants in this reactor (Erable et al. 2004, 2005). *Xanthobacter autotrophicus* GJ10 and *Rhodococcus erythropolis* NCIMB 13064, which synthesize the haloalkane dehalogenases DhlA and DhaA respectively, have been used. These enzymes are well-characterized monomeric halidohydrolases that do not require a cofactor for catalysis. Their structure is similar, but they differ in the size and geometry of their active site cavities. They therefore have distinct substrate ranges, DhlA being more suitable for small haloalkanes. An association of strains producing both of these enzymes could be of interest for the treatment

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of mixtures of gaseous pollutants. Recombinant *Escherichia coli* strains producing either DhlA or DhaA have also been tested in the solid/gas reactor and proved to able to hydrolyze the same range of pollutants as *X. autotrophicus* and *R. erythropolis*. However, all lyophilized microorganisms used as catalyst exhibit poor stability (Erable et al. 2004).

Little information is available on the use of whole cells in solid/gas reactors and even less on the stability of such catalysts, with the exception of Grizon et al. (2004) and Erable et al. (2004, 2005). The decrease in activity observed by Grizon et al. (2004) with sonicated *Saccharomyces cerevisiae* cells used for oxido-reductive reactions was attributed to the existence of two enzyme fractions, one stable and retained in the cell matrix and the other released in the outer medium. The instability reported by Erable et al. (2004, 2005) with *R. erythropolis* and *X. autotrophicus* cells was mainly explained by an accumulation of HCl but no data were available on the amount of acid retained by the biofilter.

The aim of the present work was to understand and quantify the impact of several parameters on the dehalogenase activity of whole cells in a solid/gas reactor. For this purpose, the hydrolysis of 1-chlorobutane into 1-butanol and HCl was taken as a model reaction since it has already been used in several previous dehalogenation studies (Stafford, 1993; Dravis et al. 2000; Erable et al. 2004, 2005). A recombinant *E. coli* BL21 (DE3) strain producing the DhaA dehalogenase, and retaining the ability to dehalogenate 1-chlorobutane when dehydrated, was chosen as catalyst. We specifically studied the effect of bacterial viability, the influence of both temperature and water activity (a_w), and the effect of substrates and products on the stability of the catalyst.

Materials and methods

Strain and growth conditions

E. coli BL21 (DE3) expressing the DhaA dehalogenase was provided by Professor D.B. Janssen (Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, The Netherlands). This strain was grown in 1-L flasks containing 200 mL Luria Bertani (LB) medium adjusted to pH 7.0 and supplemented with ampicillin 100 $\mu g\ mL^{-1}$. The LB medium had the following composition ($g\ L^{-1}$): 10.0 tryptone; 5.0 yeast extract and 5.0 NaCl.

The bacterial cultures were incubated at 30°C on an orbital shaker (160 rev min^{-1}). Growth was followed by measuring the optical density at 690 nm (OD_{690}). Cultures were started using an initial OD_{690} of 0.05 units. Preliminary experiments were conduced to select the culture conditions that gave the highest expression of the halidohydrolase, and 0.4 mM isopropyl-β-thiogalactopyranoside was added at an OD_{690} of 1.8.

Chemicals

All substrates were purchased from Sigma Co. (St. Quentin Fallavier, France) except NaCl, tryptone and yeast extract, which were obtained from Fluka (St. Quentin Fallavier, France). The broad specificity protease inhibitor cocktail was from Sigma and contained 4-(2-aminoethyl)benzene sulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin and sodium EDTA.

Cell harvesting

Cells were harvested at the end of exponential phase at an OD_{690} of 3 by centrifugation at 8000 rev min^{-1}, 8°C, for 7 min. The cell paste obtained from 1.8 L of culture was washed twice with 200 mL, and then resuspended in 10 mL of 50 mM borate buffer pH 9.0. This concentrated cell suspension was used for lyophilization or sonication.

Lyophilization

Ten milliliters of the concentrated cell suspension were diluted 15 times with borate buffer (150 mM, pH 9.0) before freezing at –24°C and lyophilizing using a Heto Lyopro 3000 freeze dryer. The dehydrated cells were then stored hermetically in a dessicator over phosphorous pentoxide at 4°C before use. For each lyophilizate the dry bacterial mass and the dry buffer salt mass were determined. Except where stated otherwise, the lyophilizates used for solid/gas catalysis contained 50% of borate salt and 50% of dry cells by weight.

Sonication and preparation of cell extracts

Ten milliliters of the concentrated cell suspension (OD_{690} of 300) were sonicated four times for 1 min at 50 W and 20 kHz (Vibra-cell 72434; Bioblock Scientific, France) while cooling on ice. After treatment, half of the lysate was diluted (1:15 in 150 mM borate buffer pH 9.0) with or without the addition of protease inhibitors (3 mg/250 mg dry cells), lyophilized and stored as described before. The other half was centrifuged for 30 min at 17 000***g*** and 4°C. The supernatant was then diluted (1:15 in 150 mM borate buffer pH 9.0), lyophilized and stored as

described before. It was used as a crude cell extract to check thermostability of the DhaA.

Dehalogenase activity in aqueous solution

The dehalogenase activity of whole or sonicated cells and the activities of cell extracts were measured by quantifying the initial rate of HCl production in 50 mM borate buffer, pH 9.0, at 40°C. Five reactors, consisting of 4 mL bacterial suspension at an OD_{690} of 1, or the corresponding amount of sonicated cells or extracts, introduced in a 5-mL glass tube closed hermetically with PTFE Mininerts™ caps, supplied by VWR International, Strasbourg, France, (to avoid 1-chorobutane evaporation) and magnetically stirred at 1100 rev min^{-1}, were prepared. They were started simultaneously by the addition of 10 mM 1-chlorobutane and single reactions were stopped after 0, 5, 10, 15 and 20 min of reaction by the addition of 700 μL of 20% HNO_3. Each suspension was then centrifuged (2 min at 14 000 rev min^{-1}), and the HCl produced was quantified in the supernatant with an Aquanal-plus chloride kit™ from Riedel-de Haën (Sigma). This method is based on the reaction of mercury(II) ions with chloride ions and consequent release of thiocyanate ions from mercury(II) thiocyanate added as reactant. Thiocyanate ions then react with the second reactant, iron(III) ions, and form a red complex; the intensity of color increases with the concentration of chloride ions. The product was measured spectrophotometrically at 460 nm. All initial rate determinations are the means of a triplicate measurement.

Reaction in the solid/gas reactor

The solid/gas biofilter used has been described previously by Lamare & Legoy (1995a,b). Briefly, the reactor was composed of a 9-cm long glass tube in which dehydrated *E. coli* cells were packed between two layers of glass wool. Gaseous substrates were continuously fed through the biofilter by flowing nitrogen, as carrier gas, through flasks containing the liquid substrates. Proper mixing of saturated gas (with 1-chlorobutane and water) and of an additional nitrogen flow allowed the desired thermodynamic activity to be obtained. The thermodynamic activity of each compound X in the reactor was: $a_X = PP_X/PP_X^{sat}$, where PP_X is the partial pressure (Pa) of X in the gas entering the biofilter and PP_X^{sat} is the saturation vapor pressure (Pa) of pure X at the working temperature.

The vapor phase leaving the biofilter was sampled using a 250 μL loop in a six-way valve (Valco™, Les Ulis, France) maintained at 190°C. Samples were automatically injected into the split injector of a gas chromatograph. Calibration of 1-butanol and 1-chlorobutane was carried out by programming a range of their partial pressures in the bioreactor packed with glass wool and by analyzing the vapors leaving the reactor. Operating parameters (temperature, pressure, substrates and water thermodynamic activities) were monitored on-line.

A typical experiment was run at 40°C, with 100 or 200 mg lyophilized cells. The total flow through the biofilter was 500 μmol min^{-1} (12.3 mL min^{-1}), a_w was fixed at 0.7 (9.64 mL min^{-1}) and 1-chlorobutane activity ($a_{1\text{-chlorobutane}}$) was fixed at 0.06 (0.66 mL min^{-1}). 1 IU corresponds to 1 μmole of 1-butanol formed per minute.

The rate of dehalogenation was not measured by the disappearance of 1-chlorobutane because only a small fraction (1%) of the substrate was converted and the concentration of this compound in the gas phase was obtained with a lower experimental precision than that of the 1-butanol.

GC analysis

Samples were analyzed by GC using a Hewlett-Packard 5890 gas chromatograph with a flame ionization detector connected to a 3396 integrator. The column used was an OV 1701 fused silica capillary column (25 m × 0.25 mm i.d. × 0.25 μm film thickness; Chrompack™, France). The injector and detector were kept at 220°C and 250°C, respectively. The column temperature was held at 60°C for 5 min, then programmed to increase at 10°C min^{-1} to 150°C and kept for 1 min at this temperature.

Extraction and measurement of 1-chlorobutane and 1-butanol

After catalysis, the contents of the solid/gas reactor were extracted for 2 min with 10 mL decane or resuspended and stirred for 2 min in 10 mL water at ambient temperature. These suspensions were then centrifuged at 11 000 rev min^{-1} for 2 min and the supernatant analyzed by GC.

Measurement of HCl accumulation on the biocatalyst

After catalysis the content of the reactor was dissolved in 150 mL water; 4 mL of this suspension were then acidified with 700 μL HNO_3 (20%) to precipitate proteins and cell debris. The suspension was then centrifuged (2 min at 11 000 rev min^{-1}), and the HCl produced was quantified in the supernatant with the Aquanal-plus chloride kit™.

Bacterial cell counting

Viable cell count was determined by plating suitably diluted aliquots of the lyophilized powder or of cell suspension on Petri dishes containing nutrient agar. Dilutions were made with peptone water (peptone 1 g L^{-1}; NaCl 5 g L^{-1}). Plates were incubated at 30°C for 72 h. For each dilution series, the number of colony-forming units (CFU) per milliliter was based on the average of at least triplicate plate counts. Viability was expressed as the number of viable cells per gram of lyophilizate or per gram of dried cells.

Measurement of the water content of the biocatalyst

The water content of the biocatalyst was calculated on the basis of the mass difference measured before and after drying aliquots of lyophilizate at 105°C for 24 h.

Residual dehalogenase activity after reaction at the solid/gas interface

When lyophilized whole cells were recovered from the solid/gas reactor, HCl could have accumulated on the biocatalyst. This acid could change the pH and hinder quantification of residual activity measured by the rate of production of HCl during the hydrolysis of 1-chlorobutane in aqueous batch. It was therefore necessary to remove HCl before measuring the residual activity.

To this end, the content of the bioreactor was resuspended in 10 mL deionized water, and HCl was eliminated by dialysis (6–8000 Da) against 2 L ultra pure water at 4°C for 24 h. The ultra pure water was changed after 12 h. This HCl-free bacterial suspension was diluted with 50 mM borate buffer pH 9.0 to correspond to an $OD_{690} = 1$ suspension and its activity was measured spectrophotometrically (at 460 nm) with the Aquanal-plus chloride kit™ as described above for determination of the activity in aqueous solution.

In order to compare residual activity of biocatalysts exposed to 1-chlorobutane or not, and also HCl-free, this treatment was applied whatever the operating conditions. All residual activity measurements were obtained from three independent experiments.

Microscopy

Lyophilized cells were gently suspended in 50 mM sodium cacodylate buffer (pH 7). The fixation step was performed using solutions of glutaraldehyde 2% and osmic acid 2% v/v. Successive baths of acetone (50, 70, 90 and 100% v/v) and hexamethyldisilazane (50% with acetone and 100% v/v) were used for the dehydration steps. The preparations were examined using a Philips Quanta 200 ESEM/FEG microscope with an Everhart–Thornley BSE detector.

Results and discussion

A preliminary experiment was conduced with 222 mg lyophilized recombinant *E. coli* maintained at 40°C and fed by 500 μmol min^{-1} flow of gaseous effluent containing 1-chlorobutane and water at 0.06 and 0.7 thermodynamic activities, respectively. In order to quantify the amounts of 1-chlorobutane and 1-butanol accumulated on the catalyst, these compounds were extracted with either water or decane as solvent. No trace of 1-chlorobutane or 1-butanol could be found, even after 48 h of hydrolysis. The dehalogenation reaction rate could thus be evaluated by the amount of 1-butanol carried out of the reactor by the nitrogen.

As previously observed for *R. erythropolis* NCIMB 13064, *X. autotrophicus* GJ10 and recombinant *E. coli* strains (Erable et al. 2004, 2005; Erable 2005), after a couple of hours the dehalogenase activity decreased (Figure 1). Since no sorption of the organic substrate and product occurs in the reactor, the lack of stability cannot be explained by inhibition due to these compounds. At this stage, four hypotheses could be proposed: (i) a correlation between cell viability and catalytic activity; (ii) inhibition/denaturation by the accumulation of the second product, HCl; (iii) denaturation of the catalyst by the combined action of temperature and

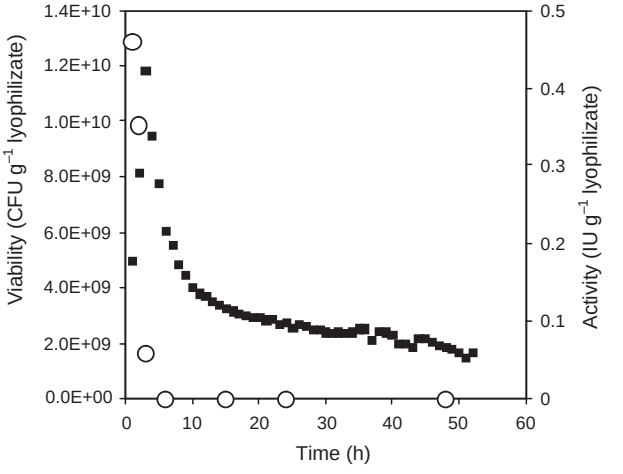

Figure 1. *E. coli* BL21 (DE3) DhaA viability (○) and dehalogenase activity, measured by the rate of 1-butanol formation (■) in the solid/gas reactor. The reaction was carried at 40°C with 222 mg lyophilized biocatalyst containing 50% cells and 50% borate buffer salts per gram of dry mater, $a_w = 0.7$ and $a_{1\text{-chlorobutane}} = 0.06$. The total flow into the reactor was 500 μmol min^{-1}.

water; and (iv) inactivation by proteases released by damaged cells.

Relationship between cell viability and the dehalogenase activity in the solid/gas reactor

Preparation of the catalyst requires washing and resuspension of bacteria in borate buffer, freezing and lyophilization. The viability of cells was measured at each step and their morphology observed by electron microscopy (Figure 2). Moreover, dehydrated cells were resuspended in borate buffer and the dehalogenase activity was quantified both in the supernatant and in the pellet obtained after centrifugation of this suspension (Table I).

The first step of preparation, involving two washings with borate buffer, does not alter cell viability. Conversely, freezing decreases the numbers of culturable bacteria by 39%. This can be explained by the slow rate of freezing and the lack of cryoprotectant. Lyophilization also had an important effect on viability, as only 0.8% of bacteria remained culturable after this step. These measurements correlated with the changes in cells' morphology. After freezing and even more after dehydration, cell walls appeared damaged (Figure 2). After rehydration of lyophilized cells and the centrifugation of this suspension, 78% of the activity was found in the supernatant and 22% in the pellet whereas the entire activity was found in the pellet for fresh cells (Table I). These results agree with the two previous observations and indicate that cell damage occurs during catalyst preparation. Moreover, they indicate that cells could release part of their contents upon rehydration. Pembrey et al. (1999) observed that resuspension and washing (in either 100 mM phosphate buffer or 2.4% NaCl-buffered solution) did not change the viability of *E. coli* significantly but that freeze drying without any cryoprotectant led to the alteration of cell walls and the loss of 83–98% of viability.

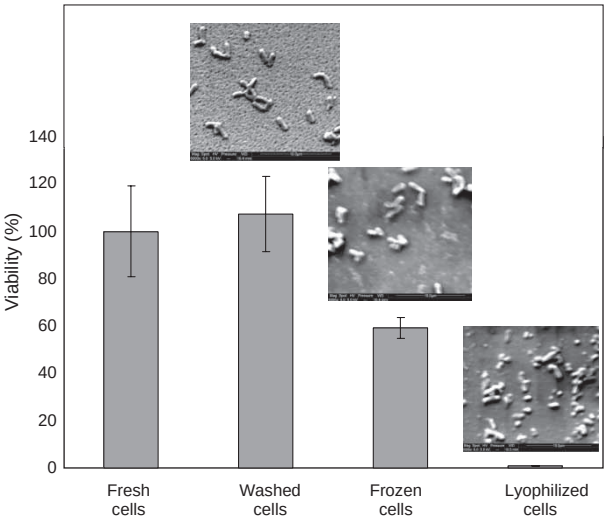

Figure 2. Viability and morphology of *E. coli* BL21 (DE3) DhaA at different steps of preparation for solid/gas catalysis. Viability of the fresh cells was taken as 100% for reference (1.9×10^{12} CFU g^{-1} dry mass of cells). Values represent means ± SD.

Table I. Dehalogenase activity of *E. coli* BL21 (DE3) measured in the supernatant and the pellet of centrifuged fresh cells and lyophilized cells (after rehydration)[a]. The dehalogenase activity is expressed as the percentage of the total activity[b].

	% of activity in the pellet	% of activity in the supernatant
Fresh cells	100	0
Lyophilized cells	22	78

[a]Fresh cells were diluted to obtain an $OD_{690} = 1$ suspension and lyophilized cells were resuspended to obtain an equivalent concentration of cells. The dehalogenase activity was measured at 40°C in borate buffer (50 mM, pH 9.0) (see Materials and methods). [b]The total activity of fresh cells corresponded to 15 IU g^{-1} dry bacteria and 100% of the activity of resuspended lyophilized cells was equal to 10.8 IU g^{-1} dry bacteria.

It was noticed that the total activity of resuspended lyophilized bacteria, i.e. 10.8 IU g^{-1} dry bacteria, corresponded to only 72% of the initial activity measured with fresh cells (15 IU g^{-1} dry bacteria). Thus, a significant fraction of the dehalogenase was also denatured upon preparation of the catalyst. Nevertheless, the dehalogenase activity in the liquid phase was more than one order of magnitude higher than that observed in the solid/gas reactor. A similar difference has been observed with whole dehydrated cells of *R. erythropolis* used for oxido-reductive reactions in an aqueous and in the gas phase (Marchand et al. 2008). This could partly be explained by differences in substrate activities. Indeed, water activity is higher in the aqueous medium (1.0) than in the gas phase (0.7). Diffusional limitations could also be involved.

The remaining culturable bacteria (1.3×10^{10} CFU g^{-1} lyophilizate) were not able to withstand the solid/gas reactor conditions for more than a couple of hours (Figure 1). This high mortality is logical since cells were exposed to a low water activity (0.7), a 1-chlorobutane flow and a rather high temperature (40°C) compared with their optimal growth temperature. After 6 h of catalysis no colonies could be observed from plating the reactor contents on nutrient agar, whereas the dehalogenation continued for more than 48 h (Figure 1). Some of the activity therefore clearly does not correlate with viability of cells.

However, cell death can lead to protease release and as a consequence affect enzyme integrity, contributing to the loss of activity.

Search for protease activity

E. coli has numerous cytoplasmic, membrane-bound and periplasmic proteases that can be released upon cell disruption (Cook 1988; Maurizi 1992). To investigate the possible involvement of proteases in the decrease of activity, cells, sonicated or not, and cell extracts, with or without addition of a bacterial protease inhibitor cocktail, were lyophilized. They were then incubated in borate buffer pH 9.0 at 40°C for 2 h and their dehalogenase activity measured (Figure 3). Similar dehalogenase activity was observed with whole, sonicated and also damaged cells (Figure 3A) and the addition of protease inhibitors did not affect the retention of dehalogenase activity after 2 h.

Accumulation of HCl on the catalytic bed

1-Butanol and HCl are produced stoichiometrically during the dehalogenation of 1-chlorobutane. It has already been shown that 1-butanol did not accumulate on the catalyst. Since the amount of 1-butanol formed could be quantified by GC, the amount of HCl produced can be estimated. In order to determine if some of the HCl condensed on the catalyst we compared the HCl produced with the amount of HCl retained by the catalyst (Figure 4) and found them to be virtually the same. It was noticed that there was a systematic and increasing difference between the HCl concentration estimated by the on-line 1-butanol analysis and the HCl directly measured on the biofilter. This could be due to a slight underestimate of the 1-butanol produced as a function of reaction time, resulting in an appreciable error after 30 h of catalysis.

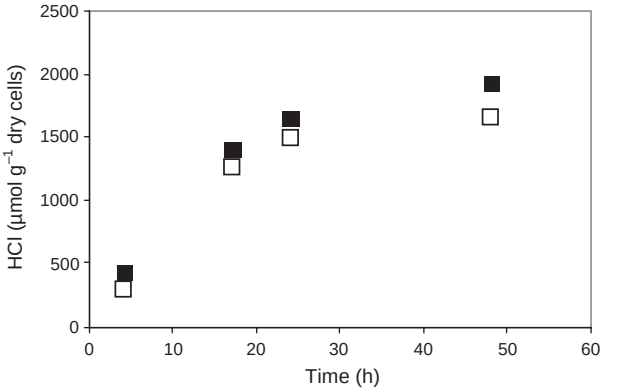

Figure 4. Comparison of the amount of HCl produced during the dehalogenation reaction in the solid/gas reactor (estimated by quantification of 1-butanol produced) (□) and the amount of HCl accumulated on the catalyst (■). The reaction was carried at 40°C with 100 mg lyophilizate, $a_w = 0.7$ and $a_{1\text{-chlorobutane}} = 0.06$. The total flow into the reactor was 500 μmol min^{-1}.

In an additional experiment, vapors coming from the reactor were bubbled into 10 mL water for 100 h and the amount of HCl measured. No HCl could be found, confirming that all the HCl produced accumulated on the catalyst. This would decrease the local pH around the enzyme, explaining some of the decrease of activity. This effect has already been proposed by Erable et al. (2004) to explain the decrease of dehalogenase activity observed with *R. erythropolis* NCIMB 13064 during the hydrolysis of 1-chlorobutane, and was supported by the restoration of the activity upon addition of a volatile base (triethylamine) in the carrier gas. Nevertheless, previous research did not quantify the extent of HCl retention on the biofilter, or the proportion of the catalyst which was reversibly inhibited as a consequence and that denatured either by the HCl or by

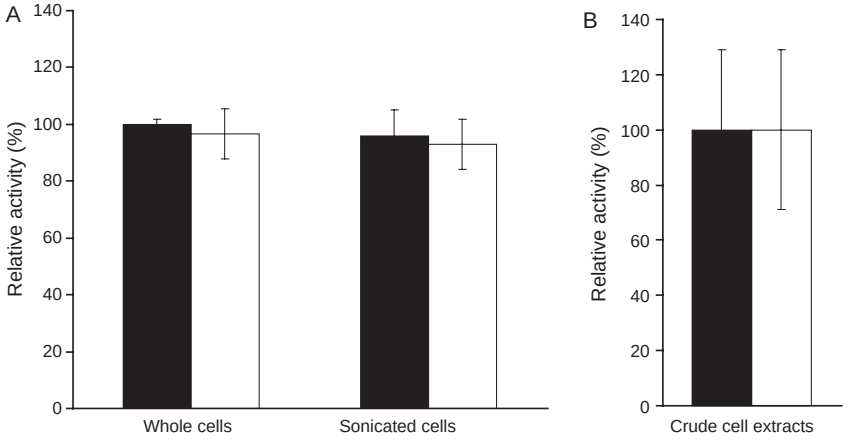

Figure 3. Relative dehalogenase activity in aqueous solution observed with (A) whole or sonicated cells of *E. coli* BL21 (DE3) DhaA and (B) cell extracts, with (□) or without (■) addition of a protease inhibitor cocktail (3 mg/250 mg dry cells), after 2 h of incubation at 40°C in 50 mM borate buffer pH 9.0. The dehalogenase activity of whole cells of recombinant *E. coli* without any addition of protease inhibitors after 2 h of incubation was taken as the 100% reference in (A). The dehalogenase activity of cell extracts without protease inhibitors after 2 h of incubation was taken as reference in (B). Values represent means ± SD.

the combined effects of temperature and water content.

Evaluation of the thermostability of the dehalogenase

It was possible that part of the DhaA was denatured either by HCl accumulation or thermal denaturation, but no data were available on the thermostability of the DhaA dehalogenase. Therefore, the thermostability of DhaA was evaluated by measuring the loss of dehalogenase activity in cell extracts of *E. coli* BL21 (DE3) incubated in borate buffer (50 mM, pH 9.0) at different temperatures (Figure 5A). In aqueous solution the dehalogenase activity of extracts was lost gradually at 24°C and 30°C and more drastically at 40°C and 50°C. At 40°C the entire activity was lost after 7 h of reaction. When whole lyophilized cells were rehydrated and incubated at 40°C in borate buffer (50 mM, pH 9.0) their dehalogenase was completely lost after 14 h of incubation (Figure 5B). Thus, the hydrolase seems to be sensitive to thermal denaturation, especially at 40°C and above, a phenomenon that warrants further investigation.

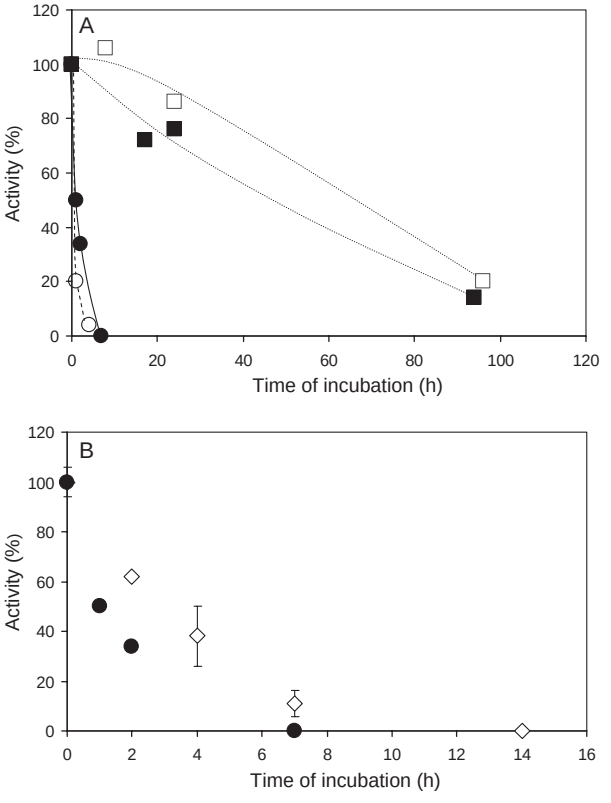

Figure 5. Dehalogenase activity of: (A) cell extracts of recombinant *E. coli* BL21 (DE3) DhaA (140 mg protein L^{-1}) after incubation in borate buffer (pH 9.0, 50 mM) at different temperatures (□, 24°C; ■, 30°C; ●, 40°C; ○, 50°C); and (B) whole lyophilized cells rehydrated (corresponding to an $OD_{690} = 1$ of fresh cells) and incubated at 40°C in borate buffer (50 mM, pH 9.0) (◇; values represent means ± SD). Dehalogenase activity of cell extract incubated in borate buffer (50 mM, pH 9.0) at 40°C has been reported from Figure 5A (●).

Residual activity of the catalyst

To quantify the extent to which the catalyst is reversibly inhibited and/or irreversibly denatured, the residual activity was measured at different temperatures and water activities, with or without exposure to 1-chlorobutane.

Since HCl accumulates during biocatalysis and could also decrease the pH and hinder further measurements, it was necessary to remove it prior to quantifying the activity. The reaction was stopped at different times and the contents of the reactor resuspended in deionized water and dialyzed. The activity of the bacterial suspension was then measured in a batch aqueous reactor, giving the residual activity of the cells. For strict comparability, this treatment was applied whatever the operating conditions.

When the lyophilized whole cells were submitted only to a nitrogen flux at 20°C (without 1-chlorobutane or water), the residual activity was stable for 140 h, whereas 30% of the activity was lost at 40°C (Figure 6). In these experiments no water was provided in the gas phase but it may be presumed that the water content of the catalyst after lyophilization (2.5%) was sufficient to promote inactivation of the enzyme at 40°C.

At 40°C with a_w of 0.7 but without any 1-chlorobutane flow, hydration of the catalyst increased up to 15%. The residual activity decreased

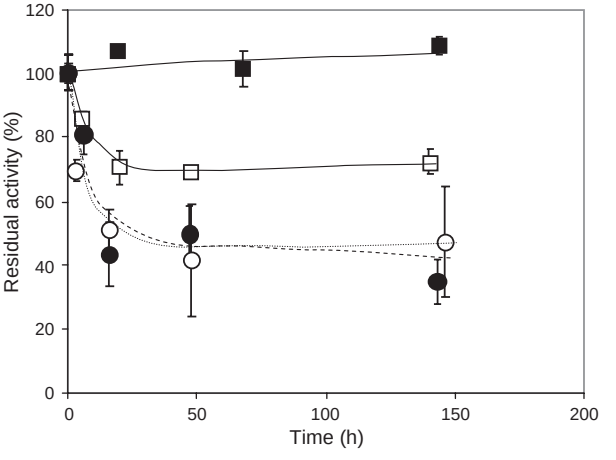

Figure 6. Residual activity of lyophilized *E. coli* BL21 (DE3) DhaA cells after catalysis in the solid/gas reactor performed at different temperatures, water activity, and with or without 1-chlorobutane flow: ■, at 20°C without water or 1-chlorobutane flow; □, at 40°C without water or 1-chlorobutane flow; ○, at 40°C, $a_w = 0.7$ and without 1-chlorobutane flow; ●, at 40°C, $a_w = 0.7$ and $a_{1\text{-chlorobutane}} = 0.06$. Values represent means ± SD. The residual activities were measured, after removing HCl by dialysis, in aqueous batch reactors with the following conditions: pH 9.0; 40°C; 10 mM 1-chlorobutane.

drastically during the first 16 h of reaction, then slowly to reach a stable value corresponding to 45% of the maximal activity (Figure 6). It is now well known that water content influences enzyme stability in non-conventional media (Zaks & Klibanov 1985; Adlercreutz & Mattiasson 1987; Klibanov 1989; Turner et al. 1995) and for catalysis at the solid/gas interface, it has been shown that the stability of enzymes is improved by reduced hydration (Pavaresh et al. 1992; Lamare & Legoy 1995b; Lamare et al, 2001). More recently it has been reported by Goubet et al. (2002) and Erable et al. (2004) that the half-life of whole dehydrated cells used as catalysts is decreased by exposure to increasing water activities. This is related to the sensitivity of enzymes to molecular motion caused by the combined action of water and temperature, leading to protein unfolding and incorrect refolding or aggregation, peptide hydrolysis, breaking of disulfide bonds, deamidation and Maillard reactions.

The dehalogenase is thermosensitive, especially above 40°C, and the cumulative effect of the temperature and a_w seems also to irreversibly denature this enzyme during the first 16 h of reaction. At 40°C and a_w of 0.7, a major part of the decrease of activity (up to 55%) is due to thermal denaturation. Nevertheless, it is interesting to note that the extent of inactivation is lower than in the aqueous phase, with 45% of the initial activity being retained for 150 h whereas the entire activity was lost after 7 h of incubation in aqueous solution (Figure 5B).

When dehydrated cells were submitted to a flow of both water (0.7 activity) and 1-chlorobutane (0.06 activity) at 40°C, the pattern of residual activity was similar to that observed at 40°C without any 1-chlorobutane (Figure 6), showing that the HCl produced during the hydrolysis of 1-chlorobutane did not denature the catalyst. Thus, the activity loss during the first 16 h of reaction can mainly be attributed to thermal denaturation of the enzyme, after which the further decrease occurs as HCl accumulates, lowering the pH but not denaturing the enzyme. Nevertheless, despite the reversibility of the latter, accumulation of chloride on the biofilter can constitute a serious limitation and has to be addressed for potential applications.

The decrease in activity and subsequent stabilization could be explained by different localizations of the enzyme after rehydration of the catalyst: one part could be intracellular and another part mixed with the borate buffer salts, with one of these fractions being protected against denaturation by its microenvironment. The intracellular enzymes could be protected by the bacterial wall and intracellular solutes. It has already been observed by Grizon et al. (2004) that the alcohol dehydrogenase retained by *S. cerevisiae* matrix was more stable than the dehydrated enzyme recovered from the supernatant of sonicated cells. Alternatively, the free dehalogenase fraction may be stabilized by the buffer salts after rehydration.

Conclusion

Lyophilized whole cells of a recombinant *E. coli* BL21 (DE3) DhaA can work as 'enzyme bags' in the solid/gas reactor for the degradation of halogenated substrates. The reactor operating temperature (40°C) and water activity (0.7) were found to irreversibly denature more than 50% of the catalyst during the first 16 h of reaction, but the residual activity was then stable. Unlike the 1-butanol, all of the HCl produced during the reaction accumulates on the biocatalyst causing reversible inhibition. This acidification caused a gradual, but reversible decline in the residual activity.

Operating conditions need to be adapted to minimize thermal denaturation and further research is needed to understand the role of different microenvironments in stability. Conditions that provide the most protective microenvironment for the dehalogenase could then be applied for preparation of a stable catalyst. Future work is also needed to remove chloride from the catalytic bed or to avoid its accumulation.

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References

Adlercreutz P, Mattiasson B. 1987. Aspects of biocatalyst stability in organic solvents. Biocatalysis 1:99–108.

Cook RA. 1988. Periplasmic proteases of *Escherichia coli*. Crit Rev Biotechnol 8:159–175.

Diks RMM, Ottengraph SPP. 1991a. Verification studies of a simplified model for the removal of dichloromethane from waste gases using a biological trickling filter. Part I. Bioprocess Eng 6:93–99.

Diks RMM, Ottengraph SPP. 1991b. Verification studies of a simplified model for the removal of dichloromethane from waste gases using a biological trickling filter. Part II. Bioprocess Eng 6:131–140.

Dravis BC, Lejeune KE, Hetro AD, Russell AJ. 2000. Enzymatic dehalogenation of gas phase substrates with haloalkane dehalogenase. Biotechnol Bioeng 69:235–241.

Erable B. 2005. Nouveau procédé de traitement de composés organiques volatils (COV) par biofiltration solide/gaz: application à la transformation des composés halogénés volatils par des microorganismes déshydratés. PhD Thesis. La Rochelle: La Rochelle University.

Erable B, Goubet I, Lamare S, Legoy M-D, Maugard T. 2004. Haloalkane hydrolysis by *Rhodococcus erythropolis* cells: comparison of conventional aqueous phase dehalogenation and non-conventional gas phase dehalogenation. Biotechnol Bioeng 86:47–54.

Erable B, Goubet I, Lamare S, Seltana A, Legoy MD, Maugard T. 2005. Nonconventional hydrolytic dehalogenation of 1-chlorobutane by dehydrated bacteria in a continuous solid-gas biofilter. Biotechnol Bioeng 91:304–313.

Goubet I, Maugard T, Lamare S, Legoy MD. 2002. Role of water activity and temperature on activity and stability of dried whole cells of *Saccharomyces cerevisiae* in a continuous solid-gas bioreactor. Enzyme Microb Technol 31:425–430.

Grizon V, Legoy M-D, Lamare S. 2004. Effect of yeast disruption on ADH activity for redox reactions with *in situ* cofactor regeneration in a continuous solid/gas bioreactor. Biocatal Biotransform 22:177–182.

Hasnaa J, Heitz M. 1999. Traitement de l'air par biofiltration. Can J Civ Eng 26:402–424.

Klibanov A. 1989. Enzymatic catalysis in anhydrous organic solvents. Trends Biochem Sci 14:413–418.

Lackey LW, Gamble JR, Boles JL. 2002. Bench-scale evaluation of a biofiltration system used to mitigate trichloroethylene contaminated air streams. Adv Environ Res 7:97–104.

Lamare S, Legoy M-D. 1995a. Solid/gas biocatalysis: how to fully define the system? Biotechnol Technol 9:127–132.

Lamare S, Legoy M-D. 1995b. Working at controlled water activity in a continuous process: the gas/solid system as a solution. Biotechnol Bioeng 45:387–397.

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Lamare S, Caillaud B, Roule K, Goubet I, Legoy M-D. 2001. Production of natural esters at the pre-industrial scale by solid/gas biocatalysis. Biocatal Biotransform 19:361–377.

Le Cloirec P. 1998. Les composés organiques volatils (COV) dans l'environnement. Paris: Tec & Doc, Lavoisier.

Marchand P, Rosenfeld E, Lamare S, Maugard T, Erable B, Goubet I. 2008. Coupled oxidation–reduction of butanol–hexanal by resting *Rhodococcus erythropolis* NCIMB 13064 cells in liquid and gas phases. Enzyme Microb Technol doi:10.1016/j.enzmictec.2008.07.004.

Maurizi MR. 1992. Proteases and protein degradation in *Escherichia coli*. Experientia 48:178–201.

Mohamed MF, Kang D, Aneja VP. 2002. Volatile organic compounds in some urban locations in United States. Chemosphere 47:863–882.

Pavaresh F, Robert H, Thomas D, Legoy M-D. 1992. Gas phase transesterification reactions catalyzed by lipolitic enzymes. Biotechnol Bioeng 39:467–473.

Pembrey RS, Marshall KC, Schneider RP. 1999. Cell surface analysis techniques: what do cell preparation protocols do to cell surface properties? Appl Environ Microbiol 65:2877–2894.

Stafford TM. 1993. The microbial degradation of chloroalkanes. PhD Thesis. Belfast: Queens University of Belfast.

Turner NA, Duchateau DB, Vulfson EN. 1995. Effect of hydration on thermostability. Biotechnol Lett 17:371–376.

Zaks A, Klibanov A. 1985. Enzyme-catalyzed processes in organic solvents. Proc Natl Acad Sci USA 82:3192–3196.