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Biotransformation of halogenated compounds by lyophilized cells of *Rhodococcus erythropolis* in a continuous solid–gas biofilter

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Abstract

The irreversible hydrolysis of 1-chlorobutane to 1-butanol and HCl by lyophilized cells of *Rhodococcus erythropolis* NCIMB 13064, using a solid–gas biofilter, is described as a model reaction. 1-Chlorobutane is hydrolyzed by the haloalkane dehalogenase from *R. erythropolis*. A critical water thermodynamic activity (a_w) of 0.4 is necessary for the enzyme to become active and optimal dehalogenase activity for the lyophilized cells is obtained for a a_w of 0.9. A temperature of reaction of 40 °C represents the best compromise between stability and activity. The activation energy of the reaction was determined and found equal to 59.5 kJ/mol. The absence of internal diffusional limitation of substrates in the biofilter was observed. The apparent Michaelis–Menten constants K_m and V_{max} for the lyophilized cells of *R. erythropolis* were 0.011 (1-chlorobutane thermodynamic activity, a_{ClBut}) and 3.22 $\mu\text{moles}/\text{min g}$ of cell, respectively. The activity and stability of lyophilized cells were dependent on the quantity of HCl produced. Since possible modifications of local pH by the HCl product, pH control by the addition of volatile Lewis base (triethylamine) in the gaseous phase was employed. Triethylamine plays the role of a volatile buffer that controls the local pH and the ionization state of the dehalogenase and prevents inhibition by Cl^- . Finally, cells broken by the action of the lysozyme, were more stable than intact cells and more active. An initial reaction rate equal to 4.5 $\mu\text{moles}/\text{min g}$ of cell was observed.

Keywords: Haloalkane dehalogenase; *Rhodococcus erythropolis*; Solid–gas; Biofilter; Bioremediation

1. Introduction

Volatile organic compounds (VOCs) are widely used in (and produced by) both industrial and domestic activities. Their extensive use results in their occurrence in aquatic, soil and atmospheric environments. Many VOCs are toxic, and some are considered to be carcinogenic, mutagenic, or teratogenic. Emissions of VOCs also contributes to localized pollution problems of toxicity and odor [1,2].

Among the VOCs, halogenated organic compounds constitute one of the largest groups of environmental pollutants as a result of their widespread use as herbicides, insecticides, fungicides, solvents, plasticizers, intermediates for chemical syntheses [3]. Because of their toxicity, the biodegradation of these compounds has been widely studied. These compounds are quoted by the US Environmental Protec-

tion Agency as priority environmental toxic pollutants [4,5]. Bioremediation via microbial decontamination, is one natural solution to this environmental problem. The biotechnological approach to polluted air bioremediation is now a promising field of research which can supply reliable, simple and cheap technologies for preventing air contamination [6].

The carbon–halogen bond can be cleaved by enzymic dehalogenation, catalyzed by specific dehalogenases. Six mechanisms of dehalogenation are known so far: reductive dehalogenation, oxygenolytic dehalogenation, thiolytic dehalogenation, intramolecular substitution, dehydrohalogenation and hydrolytic dehalogenation. In the course of hydrolytic dehalogenation reactions, catalyzed by halidohydrolases, the halogen substituent is replaced by an hydroxyl group [7,8].

Rhodococcus sp. are known to play a significant role in the biodegradation of VOCs in the environment and are believed to be important for future bioremediation processes. The bacterium *R. erythropolis* can utilize a wide range of 1-haloalkanes as sole carbon source. Short chained

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1-chloroalkanes (C₂ to C₈) appear to be metabolized by the initial action of an hydrolytic dehalogenase (E.C. 3.8.1.5) to form the corresponding alcohol [9,10]. Haloalkane dehalogenases are the only enzymes known to be capable of direct hydrolytic dehalogenation of halogenated compounds, without the requirement of coenzymes or oxygen and are thus attractive catalysts for the biotransformation of volatile halogenated organic compounds.

Because many halogenated compounds have low solubility in water, processing in non-conventional media insuring the transformation of halogenated compounds has a potential for waste treatment purposes. Biocatalysis in non-conventional media, especially in a solid–gas system, presents numerous advantages over the conventional liquid–solid system: (i) problems of solubility of substrates and products do not exist and the addition of solvents can be avoided, (ii) the recovery of the biocatalyst is simplified either for batch or continuous reactors, (iii) diffusion in the gaseous phase is more efficient than in solution, thus mass transfers are more efficient) [11,12]. Recently, we have reported the possibility of using lyophilized cells in a solid–gas bioreactor to transform volatile organic compounds and especially alcohols, aldehydes and ketones using lyophilized baker's yeast (*Saccharomyces cerevisiae*) [13,14].

The aim of this study is to investigate the ability of lyophilized cells of *R. erythropolis* to transform halogenated compounds, especially 1-chlorobutane, in a continuous solid–gas biofilter.

2. Materials and methods

2.1. Microorganism, culture conditions and chemical material

R. erythropolis NCIMB 13064, was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

The organism was grown in 1000 ml flasks containing 200 ml of minimal medium described by Radwan and Al-Muteirie [15] at pH 7 and 200 μ l of 1-chlorobutane as sole carbon source. Cultures were incubated at 28 °C on an orbital shaker (160 rpm). Cell growth was monitored by measuring the optical density at 690 nm. After 48 h of growth, cells were harvested by centrifugation at 7000 rpm for 10 min. The cell paste was washed by resuspension in Tris–HCl buffer 50 mM pH 9 and lyophilized prior their use in the solid–gas biofilter. All substrates were purchased from Sigma Chemical Co. or Aldrich (USA). Pure water was obtained via a Milli Q system (Millipore, France).

2.2. Treatment of cells with lysozyme

In order to break down cell walls, part of cells were treated with lysozyme: 200 mg of lysozyme were added to 600 mg of harvested cells suspended in 30 ml of Tris–HCl buffer,

50 mM pH 9. After 15 min incubation at 22 °C, this preparation was then frozen at –20 °C and lyophilized. Lysozyme was obtained from Sigma Chemical Co. (USA).

2.3. Solid–gas biofilter

The solid–gas biofilter used in this study has been previously described by Lamare and Legoy [16]. It is composed of a 9 cm long glass tube in which the lyophilized cells of *R. erythropolis* are packed between two layers of glass wool. Substrate feeding was obtained by flowing nitrogen, as carrier gas, through the substrate saturation flasks. Substrates were continuously passed through the biofilter and reacted with the lyophilized cells of *Rhodococcus erythropolis*. The gas leaving the biofilter was analyzed by gas chromatography. Acquisition and control of operating parameters (substrate thermodynamic activity, water thermodynamic activity (a_w), temperature and pressure) were monitored on line.

A typical experiment was run at 40 °C, with 100 mg of lyophilized cell. The total flow passing into the biofilter was 500 μ moles/min. 1-Chlorobutane thermodynamic activity (a_{ClBut}) was fixed at 0.06 and the a_w at 0.8. Under these conditions, less than 5% substrate were converted allowing the assumption that initial rates (expressed in μ moles of 1-butanol produced per minute and per gram of cell) were measured.

2.4. Chromatographic analysis

For these analyses, the vapor phase leaving the biofilter was sampled using a 250 μ l loop on a 6-way valve (Valco) maintained at 190 °C. Samples were automatically injected in the split injector of a gas chromatograph (Agilent model 5890 A) equipped with a Flame Ionization Detector (FID) for detection. The column used was a OV 1701 fused silica capillary column (25 m \times 0.25 mm i.d. \times 0.25 μ m film thickness; Chrompack, France). The split ratio was 43.2/2.7. The injector was kept at 200 °C and the detector at 250 °C. The column temperature was held at 40 °C for 2.5 min, then programmed to increase at 15 °C/min to 110 °C and kept for 1 min at this temperature. The carrier gas was nitrogen and the flow rate in the column was 2.7 ml/min. Hydrogen and air, were supplied to the FID at 38 and 398 ml/min, respectively. Quantitative data were obtained after integration on an HP 3396A integrator. An external standard method was used for the calculations.

3. Results and discussion

3.1. Continuous hydrolysis of 1-chlorobutane by lyophilized cells of *R. erythropolis* in solid–gas biofilter

Recently, Dravis et al. [17], have reported the possibility of using free lyophilized haloalkane dehalogenase in a solid–gas system to transform 1-chlorobutane. In the study,

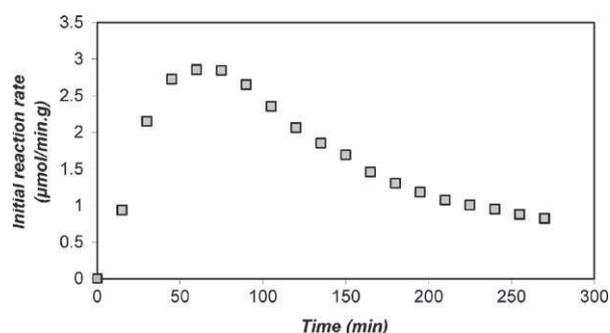


Fig. 1. Initial reaction rate of the hydrolysis of 1-chlorobutane catalyzed by *R. erythropolis*. The reaction was carried out at 40 °C with 100 mg of cells. The total flow passing into the biofilter was 500 µmoles/min. 1-Chlorobutane activity was fixed at 0.06 and a_w at 0.8.

lyophilized cells of *R. erythropolis* were used to carry out the irreversible hydrolysis of 1-chlorobutane to 1-butanol and HCl. In a first reaction, 100 mg of lyophilized cells of *R. erythropolis* were placed at 40 °C in the solid–gas biofilter. The total flow passing into the biofilter was fixed at 500 µmoles/min. a_{ClBut} was fixed at 0.06 (corresponding to 7.8 µmoles/min at 40 °C) and a_w at 0.8 (corresponding to 33.8 µmoles/min at 40 °C). The thermodynamic activity of each compound X in the reactor is calculated as follows:

$$a_X = \frac{Pp_X}{Pp_X^{\text{Sat}}}$$

with Pp_X is the partial pressure of compound X in the gas entering the biofilter, Pp_X^{Sat} the saturation vapor pressure of compound X (atm).

As shown in Fig. 1, the initial rate of 1-butanol formation corresponding to dehalogenation of 1-chlorobutane, increases up to a maximum equal to 3 µmoles/min g of cell corresponding to 38% of 1-chlorobutane conversion. Then, the initial rate of 1-butanol formation decreases to reach a steady state close to 0.6 µmoles/min g of cell. This experiment shows for the first time, that lyophilized cells of *R. erythropolis* can be used in solid–gas biofilter to transform halogenated compounds such as 1-chlorobutane. The ability of *R. erythropolis* cells to convert a wider range of chlorinated and brominated substrates into their corresponding alcohol was then examined.

3.2. Biotransformation of other halogenated substrates

Since *R. erythropolis* haloalkane dehalogenase is known to be able to convert numerous halogenated compounds into alcohols [18], the ability of dehydrated cells of this bacteria to transform several halogenated compounds was tested. The reactor, packed with 100 mg of cells, was fed with 1-chlorobutane, 1-chloropentane, 1-chlorohexane, 1-bromobutane or 1-bromohexane at a 0.06 thermodynamic activity. The reactor was maintained at 40 °C and a_w was fixed at 0.8 and the total flow passing in the solid–gas biofilter was fixed at 500 µmoles/min for all experiments.

Table 1
Haloalkane dehalogenase activity in cell measured on various halogenated substrates^a

Substrate	Relative activity (%)
1-Chlorobutane	100
1-Chloropentane	186
1-Chlorohexane	194
1-Bromobutane	98
1-Bromohexane	301

^a Dehalogenase activity was standardized with respect to the 1-chlorobutane dehalogenation rate, which was 3 µmoles/min g. The total flow passing into the biofilter was 500 µmoles/min. The halogenated substrate activity was fixed at 0.06 and the a_w at 0.8. The reaction was carried out at 40 °C with 100 mg of cells.

Maximal initial rates observed are reported in Table 1. Results are expressed as percentages of conversion of 1-chlorobutane conversion rate. Dehalogenation was found to be effective, whatever the halogenated compound tested. Moreover, the relative dehalogenase activity of *R. erythropolis* increased as the size of the halogenated compound increased. This could be interesting for depollution since long chain substrates have low solubility in water and are often difficult to remove by classical biofiltration. These experiments confirm the actual possibility of biologically removing halogenated substrates from air streams through a solid–gas biofilter.

The effects of various parameters on the initial rate of dehalogenation was next examined in order to define optimal conditions.

3.3. Effect of *R. erythropolis* amount

The effect of *R. erythropolis* on the initial rate of the biotransformation was studied. Between 0 and 200 mg of lyophilized cells of *R. erythropolis* were placed at 40 °C in the solid–gas biofilter. The total flow passing into the biofilter was fixed at 500 µmoles/min, a_{ClBut} was fixed at 0.06 and a_w at 0.8. The initial reaction rate of 1-chlorobutane transformation increased almost linearly over the range investigated (Fig. 2) indicating that internal diffusion of substrates was higher than hydrolytic reaction rates.

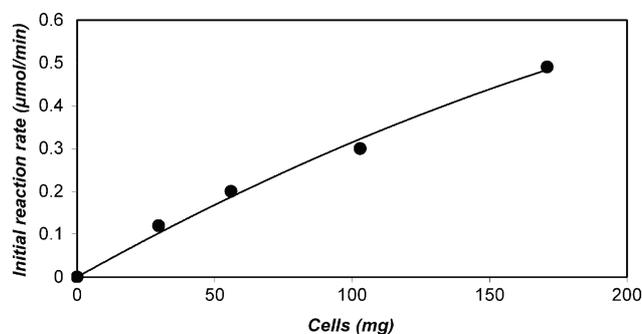


Fig. 2. Initial reaction rate of the hydrolysis of 1-chlorobutane as a function of amount of cells of *R. erythropolis*. The reaction was carried out at 40 °C. The total flow passing into the biofilter was 500 µmoles/min. a_{ClBut} was fixed at 0.06 and a_w at 0.8.

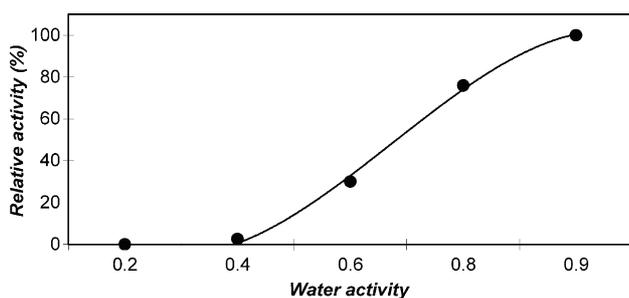


Fig. 3. Effect of a_w on relative activity of *R. erythropolis*. The reaction was carried out at 40 °C with 100 mg of cells. The total flow passing into the biofilter was 500 $\mu\text{moles}/\text{min}$. The a_{ClBut} was fixed at 0.06. The maximum initial reaction rate of hydrolysis of 1-chlorobutane was taken as 100% and was observed for a 0.9 a_w value.

Moreover, tests were performed using 100 mg of dehydrated cells grown on nutritive broth without addition of 1-chlorobutane thus presenting a lack in dehalogenase activity (checked by degradation tests). Calibrations curves obtained when increasing 1-chlorobutane activities were identical to those obtained with an empty reactor, meaning that *R. erythropolis* cells do not accumulate 1-chlorobutane. Thus, 1-chlorobutane conversion observed with cells grown on minimal medium supplemented with 1-chlorobutane cannot be attributed to sorption and accumulation of substrates on the biofilter.

3.4. Water effects

Free enzyme activity (lipase, cutinase) and the activity of lyophilized cells (baker's yeast) in the gas phase, have been reported to be related to a_w [13,14,19]. Reactions were therefore carried out under different a_w conditions. Hundred milligrams of lyophilized cells of *R. erythropolis* were placed at 40 °C in the solid–gas biofilter. The total flow passing into the biofilter was 500 $\mu\text{moles}/\text{min}$. a_{ClBut} was fixed at 0.06.

In all experiments, the initial rate of 1-butanol formation increases and reaches a maximum. Then, the initial rate of 1-butanol formation decreases to reach a steady state near to 0.6 $\mu\text{moles}/\text{min}$ of cell. This maximal rate of 1-butanol formation is strongly influenced by the a_w (Fig. 3). Contrary to the reports of Dravis et al. [17] in which free lyophilized haloalkane dehalogenase was used, the haloalkane dehalogenase in the cells was not able to catalyze the hydrolysis of 1-chlorobutane at low water activities. Indeed, it was observed that a critical a_w of 0.4 was necessary for the dehalogenase of *R. erythropolis* to become active. Over the range of water activities tested, optimal dehalogenase activity for the lyophilized cells was obtained for a a_w of 0.9. Since water is also a substrate of the reaction, an increase of a_w enhances the rate of biotransformation. Moreover high a_w supports molecular mobility, resulting in a higher reaction rate.

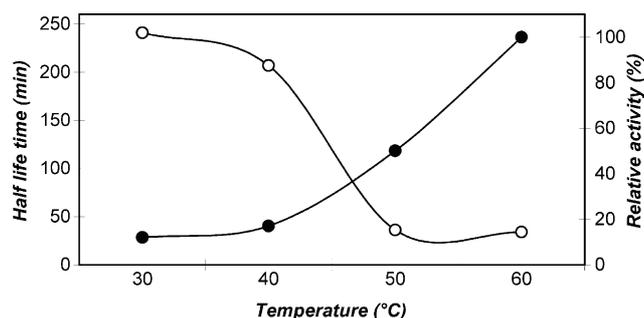


Fig. 4. Effect of temperature on stability (○) and relative activity (●) of *R. erythropolis*. The reaction was carried out with 100 mg of cells. The total flow passing into the biofilter was 500 $\mu\text{moles}/\text{min}$. a_{ClBut} was fixed at 0.06 and a_w at 0.8. Half-life is defined as reaction time at which conversion is half of that at the maximum initial reaction rate.

3.5. Temperature effect

In order to evaluate the effect of temperature on the thermal stability and dehalogenase activity of lyophilized cells of *R. erythropolis* in the gas phase, a series of reactions was performed at various temperatures. Reactions were carried out with 100 mg of cells. The total flow passing into the biofilter was 500 $\mu\text{moles}/\text{min}$. Water and 1-chlorobutane activities were fixed at 0.8 and 0.06, respectively.

The effect of temperature on the dehalogenase activity of cells was observed over the range 30–60 °C. As shown in Fig. 4, the initial rate of chlorobutane hydrolysis increases with temperature. The optimal temperature to reach the highest initial reaction rate under the selected conditions was 60 °C. Nevertheless, with an increase in activity, the half-life of the lyophilized cells decreases. Forty degree Celsius seems to be the best compromise between the loss of stability and activity since the half-life is about 90% of that obtained at 30 °C, while activity doubles between 30 and 40 °C.

The activation energy of the reaction as determined by an Arrhenius plot was 59.5 kJ mol^{-1} which is similar to values already reported for haloalkane dehalogenases [18,20].

3.6. Chlorobutane effects

The effect of a_{ClBut} on the dehalogenase activity of *R. erythropolis* was studied. Hundred milligrams of bacteria was placed at 40 °C in the solid–gas biofilter. The total flow passing into the biofilter was 500 $\mu\text{moles}/\text{min}$. The chlorobutane thermodynamic activity was variable while a_w was fixed at 0.8. As shown in Fig. 5A, the maximal reaction rate measured for 1-butanol increases with chlorobutane thermodynamic activity. In the range tested, no inhibitory effect of chlorobutane was detected and the apparent Michaelis–Menten constants K_m and V_{max} for the lyophilized cells of *R. erythropolis* were determined. In the present case, the $1/V_{\text{max}}$ axis is not the usual y -axis but a parallel axis corresponding to $1/a_{1\text{-chlorobutane}}$ values of 1,

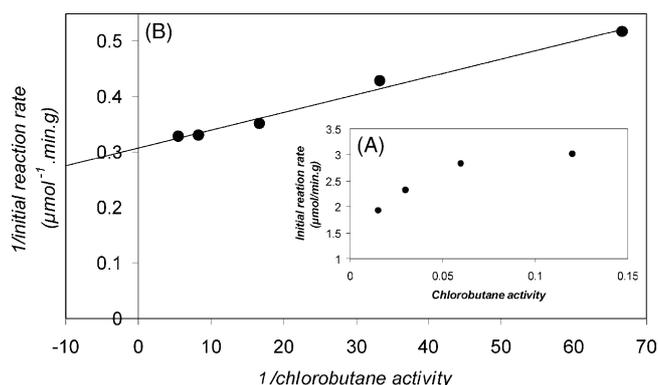


Fig. 5. Initial reaction rate of the hydrolysis of 1-chlorobutane as a function of a_{ClBut} (A) and reciprocal initial reaction rate of the hydrolysis of 1-chlorobutane vs. reciprocal a_{ClBut} (B). The reaction was carried out at 40°C with 100 mg of cells. The total flow passing into the biofilter was fixed at $500 \mu\text{moles}/\text{min}$ and the a_w at 0.8.

corresponding to the highest possible value of $a_{1\text{-chlorobutane}}$. The apparent Michaelis–Menten constants K_m and V_{max} for the lyophilized cells of *R. erythropolis* have been found to be 0.011 (1-chlorobutane thermodynamic activity) and $3.22 \mu\text{moles}/\text{min}\cdot\text{g}$ of cell, respectively (Fig. 5B). This apparent V_{max} observed is eight-fold higher than V_{max} observed for free lyophilized haloalkane dehalogenase in gas phase by Dravis et al. [17]. This difference can be explained by the experimental conditions which are different. Dravis et al. used a free lyophilized enzyme, in a batch system, at 39°C , with a_w near to 1 and with 1-chlorobutane-saturated vapor phase (e.g. a_{ClBut} near to 1). In this study, lyophilized whole cells were used, in a continuous system, at 40°C , with a_w fixed at 0.8 and with a maximal a_{ClBut} of 0.12.

3.7. Effect of HCl produced

In non-conventional media (gaseous system, supercritical fluids, organic solvents, etc.), as in aqueous media, the enzyme activity is dependent on the ionization state of the enzyme. It has been shown that the enzyme activity is dependent on the last aqueous pH to which the enzyme was exposed prior to drying and suspension in the organic solvent (pH memory) [21,22]. However, acid–base conditions may subsequently change due to acidic or basic reactants (e.g. [23,24]).

In order to control the ionization state of enzyme preparation in non-conventional media, buffers can be added to the reaction mixture. These buffers are composed of either an acid and its sodium salt or an amine and its hydrochloride [25–27]. For example, tridodecylamine has been shown to be useful as a basic extractant in several solvents (e.g. extraction of HCl from the aqueous phase into *n*-decanol).

In this study, the decrease of the dehalogenase activity and the stability of cells observed in all experiments could be attributed to the deactivation of haloalkane dehalogenase

by HCl produced. Indeed, HCl is considered as a very hydrophilic molecule, which diffuses very slowly through the membrane of cells in non-conventional media. The accumulation of HCl into the cell during the process could have several roles:

- (i) In this study, the cells were prepared at pH 9, which corresponds to optimum pH of the haloalkane dehalogenase [18]. If HCl accumulates, the pH of the microenvironment of the dehalogenase decreases and the dehalogenase activity also decreases.
- (ii) HCl can play the role of inhibitor of the dehalogenase. Indeed, in aqueous solutions, it has been shown that the halides are uncompetitive inhibitors of haloalkane dehalogenase from *Rhodococcus* sp., and the K_i for chloride and bromide was pH-dependent [28]. Consequently, in the reaction of dehalogenation by the haloalkane dehalogenase, the rate-limiting step is the release of the halogen ion from the active site.

In order to confirm the effect of HCl, a non-reactive hydrophobic base (triethylamine) which is not a substrate of the dehalogenase was added under steady state conditions. When the triethylamine is added (thermodynamic activity fixed at 0.05, corresponding to $3.9 \mu\text{moles}/\text{min}\cdot\text{g}$ at 40°C), the conversion of 1-chlorobutane is stimulated and the initial rate of 1-butanol formation increases (Fig. 6). This result confirms that the dehalogenase was not denatured but only inhibited by HCl. This stimulation is not only the result of the increase of the pH of microenvironment of the enzyme, but also, the result of the increase of the rate of release of the Cl^- from the active site, as a result of triethylamine action. The triethylamine would scavenge the HCl that is produced during reaction. This phenomenon has been already described for the free haloalkane dehalogenase in gas phase [17].

The apparent Michaelis–Menten constants K_m and V_{max} for the cells of *R. erythropolis* have been measured with several triethylamine activities. An increase of triethylamine

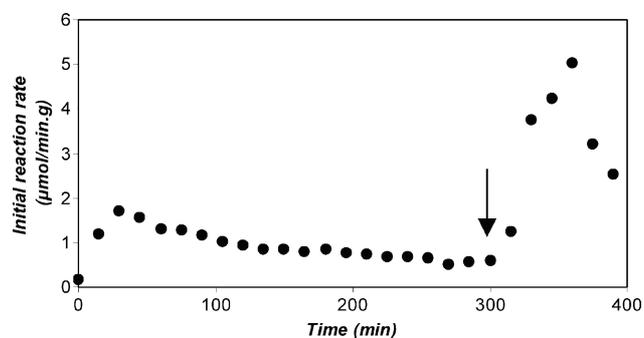


Fig. 6. Continuous initial rate of the hydrolysis of 1-chlorobutane catalyzed by *R. erythropolis* as a function of reaction time. Effect of the triethylamine addition. The total flow passing into the biofilter was $500 \mu\text{moles}/\text{min}$. a_{ClBut} was fixed at 0.06 and the a_w at 0.6. The reaction was carried out at 40°C with 100 mg of cells. Addition of triethylamine in the gas phase ($a_{\text{TEA}} = 0.05$) is indicated by the arrow.

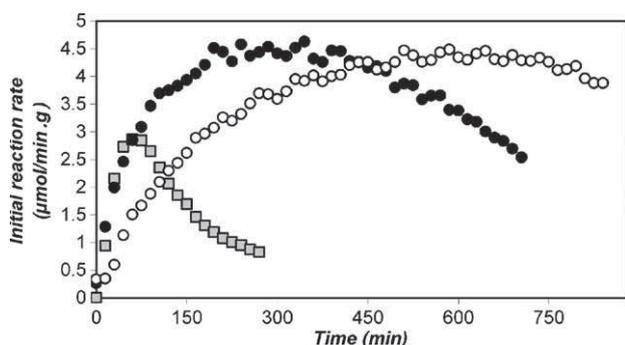


Fig. 7. Continuous initial rate of the hydrolysis of 1-chlorobutane catalyzed by *R. erythropolis* as a function of reaction time. The total flow passing into the biofilter was 500 $\mu\text{moles}/\text{min}$. a_{ClBut} was fixed at 0.06 and a_w at 0.8. The reaction was carried out at 40 °C with 100 mg of untreated cells (■), cells treated with lysozyme (●), or cells treated with lysozyme and with triethylamine in the gaseous phase ($a_{\text{TEA}} = 0.12$) (○).

thermodynamic activity increases the V_{max} and decreases the K_m . For example, when triethylamine thermodynamic activity was fixed at 0.1, the K_m and V_{max} were equal to 0.43 and 9.8 $\mu\text{moles}/\text{min g}$, respectively. This decrease of the affinity could be the result of a steric hindrance of the triethylamine, which prevents access to the catalytic site.

3.8. Lysozyme effect

To conclude this study, we carried out a reaction of dehalogenation of 1-chlorobutane with cells, which were first treated by the lysozyme, for breaking down the cell walls of bacteria. Cells treated by lysozyme, were more active and stable over the standard reaction (Fig. 7). The initial rate of 1-butanol formation increases to reach a maximum rate equal to 4.5 $\mu\text{moles}/\text{min g}$ of cell. When the membranes of the cells are broken, HCl diffuses freely and does not accumulate. The decrease of pH and the inhibition of the dehalogenase do not occur. Triethylamine and lysozyme combined action, confirms these assumptions. The activity of the enzyme remains equal to 4.5 $\mu\text{moles}/\text{min g}$.

4. Conclusion

The possibility of obtaining continuous hydrolysis of halogenated compounds using lyophilized whole cells of *R. erythropolis* in a continuous solid–gas biofilter was demonstrated. Hydrolysis of 1-chlorobutane was studied as a model reaction. The stability and the dehalogenase activity of lyophilized cells of *R. erythropolis* in the biofilter was strongly dependent of various parameters (temperature, substrate thermodynamic activity, water thermodynamic activity, HCl diffusion and pH of microenvironment). Dehalogenase activity and the stability of cells were also dependent on HCl produced. Thus, pH control by the addition of volatile Lewis base (triethylamine) can be highly

beneficial. Triethylamine plays the role of a volatile buffer which controls the ionization state of the dehalogenase and prevents Cl^- inhibition. We observed that cells broken by the action of the lysozyme, were more active and more stable than intact cells. In addition, in similar conditions, four other halogenated compounds were hydrolyzed with excellent yield.

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