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Bioremediation of halogenated compounds: Comparison of dehalogenating bacteria and improvement of catalyst stability

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Abstract

Five bacterial strains were compared for halogenated compounds conversion in aqueous media. Depending on the strain, the optimal temperature for dehalogenase activity of resting cells varied from 30 to 45 °C, while optimal pH raised from 8.4 to 9.0. The most effective dehalogenase activity for 1-chlorobutane conversion was detected with *Rhodococcus erythropolis* NCIMB13064 and *Escherichia coli* BL21 (DE3) (DhaA). The presence of 2-chlorobutane or propanal in the aqueous media could inhibit the 1-chlorobutane transformation.

Keywords: Haloalkane dehalogenase; *Rhodococcus erythropolis* NCIMB13064; *Sphingomonas paucimobilis* UT26; *Xanthobacter autotrophicus* GJ10

1. Introduction

Halogenated compounds are an important class of environmental pollutants (Mohamed et al., 2002). Because of their toxicity, the bioremediation of these compounds has been widely studied and the biotechnological approach is now a promising field which could in the future supply reliable, simple and cheap technologies to treat halogenated pollutants contamination (Hardman, 1991).

Several articles have dealt with strains capable of hydrolytic dehalogenation of halohaliphatic compounds. The physiology, biochemistry and genetics of dehalogenating bacteria and substrates specificity of their enzymes are currently being studied (Janssen et al., 1985; Keuning et al., 1985; Hardman, 1991; Nagata et al., 1997; Janssen et al., 2001; Damborsky et al., 2001; Janssen, 2004).

Xanthobacter autotrophicus GJ10 was one of the first isolated. This bacterium is able to use a number of halogenated short chain hydrocarbons as sole carbon source for growth (Janssen et al., 1985). Indeed it constitutively produces a haloalkane dehalogenase (DhlA) which catalyzes the hydrolytic dehalogenation of halogenated C_2–C_4 alkanes, including chlorinated, brominated and iodinated compounds (Keuning et al., 1985; Schanstra et al., 1996).

Other strains producing hydrolytic haloalkane dehalogenases belonging to the same family have been identified. *R. erythropolis* NCIMB13064 and *Sphingomonas paucimobilis* UT26 express respectively the DhaA (dehalogenase from *R. erythropolis*) and LinB (dehalogenase from *S. paucimobilis*) (Nagata et al., 1993; Curragh et al., 1994). The structure of these enzymes are very similar to the one of DhlA with some differences in the geometry and size of the active site cavity (Janssen, 2004). Nevertheless these three haloalkane dehalogenases differ by their substrate specificity and the bacteria producing these enzymes also differ in their range of growth

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substrate. Short chain 1-haloalkanes (C_3–C_{10}) appear to be metabolized by *R. erythropolis* NCIMB13064 (Curragh et al., 1994) while Nagata et al. (1993) showed that not only short haloalkanes but also 1-chlorodecane and 2-chlorobutane, which are poor substrates for DhlA and DhaA, were good substrates for resting *E. coli* cells overproducing LinB dehalogenase. These strains could also be used for complementary degradation of mixture of halogenated pollutants.

The aim of this article is to compare the ability of these different strains and two recombinant *E. coli* strains to convert halogenated pollutants in a continuous gas/liquid reactor. Moreover, gaseous effluents can sometimes contain several halogenated compounds and very few studies have been dedicated to the study of competition of these different substrates for the catalyst. We also studied the influence of a secondary halogenated pollutant on the depollution efficiency of such reactor.

2. Materials and methods

2.1. Microorganisms, culture conditions and chemicals

R. erythropolis NCIMB13064, *S. paucimobilis* UT26, *X. autotrophicus* GJ10 and two recombinant strains of *E. coli* BL21 (DE3) the first containing the gene coding for the dehalogenase from *R. erythropolis* NCIMB13064 (*E. coli* BL21 (DE3) (DhaA)) and the second containing the gene coding for the dehalogenase from *X. autotrophicus* GJ10 (*E. coli* BL21 (DE3) (DhlA)) were used for this study.

R. erythropolis NCIMB13064 was obtained from the National Collection of Industrial and Marine Bacteria LTD, Aberdeen, Scotland. The organism was grown in 1 l Erlenmeyer flasks sealed with Teflon line screw caps; the flasks contained 200 ml of a minimal medium described by Sorkhoh et al. (1990) adjusted at pH 7.0. The medium was sterilized by autoclaving for 15 min at 121 °C. After cooling, 100 μl of filtered sterilized 1-chlorobutane (0.22 μm Minisart sartorius filters) was added twice as sole carbon source at 24 h interval. This method was chosen since it has been shown that adding twice 100 μl of 1-chlorobutane at 24 h interval, provide both a good growth and an interesting specific dehalogenase activity (Erable et al., 2004).

S. paucimobilis UT26 and *X. autotrophicus* GJ10 were grown in 1 l aerobic flasks containing 200 ml of Luria Bertani (LB) medium at pH 7.0. The LB medium had the following composition (gl^{-1}): 10.0 Tryptone, 5.0 Yeast extract, and 5.0 NaCl. The medium was sterilized by autoclaving for 15 min at 121 °C.

E. coli BL21 (DE3) (DhlA) and *E. coli* BL21(DE3)(DhaA) were grown in 1 l aerobic flasks containing 200 ml of LB medium adjusted at pH 7.0 and supplemented with 100 $\mu g\,ml^{-1}$ ampicillin. The culture was incubated at 30 °C until it reached an OD_{690} of 0.7 after which it was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

All the bacterial cultures were incubated at 30 °C on an orbital shaker (160 rpm). Cell growth was monitored by measuring the optical density at 690 nm.

All substrates for culture media were purchased from Sigma Company (USA) except Tryptone and Yeast extract, which were obtained from Fluka (USA). All halogenated compounds: 1-chloropropane, 1-chlorobutane, 1-chloropentane, 1-chlorohexane, 1-chloroheptane, 1-chlorohexane, 1-chloroheptane, 1-chlorooctane, 1.2-dichloroethane, 1.2-dibromoethane, 1-bromobutane, 1-bromopentane, 1-bromohexane, 1-iodobutane, 2-chlorobutane, 2-bromobutane and 1-propanol, propanal and propionic acid were purchased from Flucka (USA). Their purity was 99%. Deionised water was obtained via a Milli-Q system (Millipore, France).

2.2. Preparation of cells

Cells grown for 24 h (*E. coli* BL21) or 48 h (*R. erythropolis*, *S. paucimobilis*, *X. autotrophicus*) were harvested by centrifugation (8000 rpm for 10 min), washed with 50 mM Tris/HCl buffer at pH 8.4 (*S. paucimobilis*) pH 8.5 (*X. autotrophicus* and the corresponding *E. coli* BL21 (DE3) (DhlA)) or at pH 9.0 (*R. erythropolis* and the corresponding *E. coli* BL21 (DE3) (DhaA)) and resuspended in 50 mM Tris/HCl buffer at the same pH and at an approximate OD_{690} of 15.

2.3. Dehalogenation in a batch system

The haloalkane dehalogenase activity of resting cells was assayed at 30 °C in 50 mM Tris HCl buffer (pH 8.4, 8.5 or 9.0). Tests were performed with 50 ml of diluted cell suspension (approximate OD_{690} of 3) (in Tris/HCl buffer) in 100 ml bottles. Kinetics were started by the addition of 100 μl of 1-chlorobutane ($1.8\ g\,l^{-1}$). 2 ml was periodically sampled and cells were removed by centrifugation at 1000 rpm for 2 min. 1 ml of the supernatant was assayed for butan-1-ol concentration by gas chromatography analysis using 1-hexanol as an internal standard and the same analytical conditions as for the analysis of the gas phase exiting the reactor.

2.4. Dehalogenation in a continuous system

The gas/liquid bioreactor consisted of a 1 l flask Erlenmeyer containing 200 ml of bacterial suspension at $5\ g\,l^{-1}$. Substrate feeding was obtained by passing nitrogen, used as a carrier gas, through substrate saturation flasks. Substrates were continuously passed through the bioreactor and reacted with the suspended resting cells. The gas leaving the bioreactor was analysed by gas chromatography. The acquisition and control of the operating parameters (substrate concentration, temperature, and pressure) were monitored on line as shown in Fig. 1. The vapour phase leaving the reactor was sampled using a 250 μl loop on a six-way valve (Valco) maintained at 190 °C. Samples

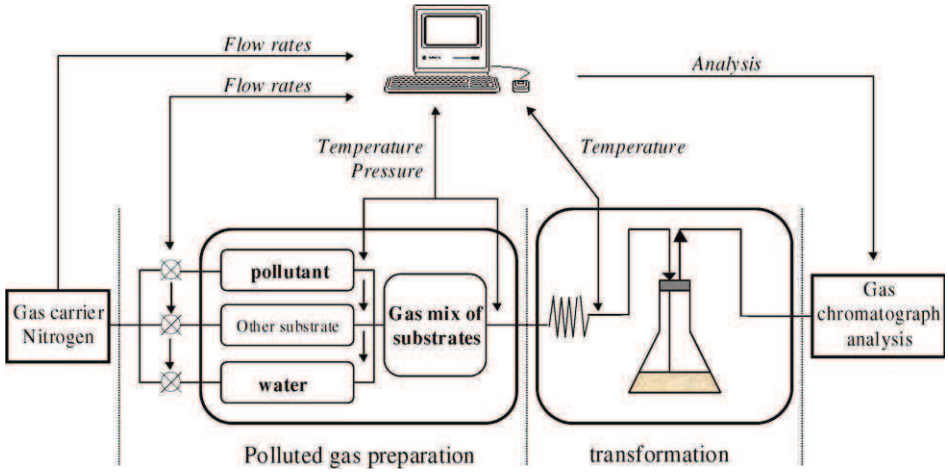

Fig. 1. Representation of the continuous gas/liquid bioreactor.

were automatically injected into the split injector of a gas chromatograph. Calibration for 1-chlorobutane and 1-butanol leaving the reactor were performed by sampling vapours generated with one of the saturation flask filled with 1-chlorobutane or 1-butanol and by programming increasing the vapour concentration of this substrate through an empty reactor.

A typical test was carried out at 30 °C, with 200 ml of bacterial suspension at 5 g l^{-1}. The total flow passing into the Erlenmeyer flask was 500 μmol min^{-1} and the 1-chlorobutane flow was fixed at 17 μmol min^{-1} (1.5 mg min^{-1}). The dehalogenase activity was expressed in μmol of alcohol per min and per *g* of cells.

2.5. Chromatographic analysis

Analysis were performed on a gas chromatograph (Hewlett Packard model 5890 A), equipped with a Flame Ionisation Detector (FID). 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 split ratio was 43.2/2.7. The injector was kept at 200 °C, and the detector was kept at 250 °C. The column temperature was held at 60 °C for 2 min, then programmed to increase at 10 °C min^{-1} to 140 °C. Nitrogen was used as a carrier gas and the flow rate in the column was 2.1 ml min^{-1}. Hydrogen and air were supplied to the FID at 40 and 400 ml min^{-1} respectively. Quantitative data were obtained after integration on a HP 3396A integrator.

3. Results and discussion

3.1. Comparison of dehalogenating bacteria

DhlA and LinB were constitutively produced by *X. autotrophicus* GJ10 and *S. paucimobilis* UT26 respectively. The expression of DhaA was induced by 1-chlorobutane addition for *R. erythropolis* NCIMB13064 cells. DhlA and DhaA produced by the recombinant *E. coli* BL21 were induced by IPTG (Table 1).

The generation time depended on the strain considered (Table 1). *R. erythropolis* NCIMB13064 possessed the highest generation time (310 min). However, this was also the only strain cultivated on mineral medium supplemented with 1-chlorobutane. After growth, the cells were recovered and tested for the degradation of 1-chlorobutane. The effects of several parameters such as temperature, pH or substrate concentration were first observed on the dehalogenase activities of resting cells (Table 2).

The optimal temperature for the dehalogenase activity of *R. erythropolis* NCIMB13064 resting cells was 45 °C. Stafford (1993) determined an optimal temperature of 45 °C for the free DhaA. The optimal temperature for dehalogenating activity of these resting cells corresponds to the optimal temperature of the free enzyme rather than to the optimal temperature for the growth of this microorganism (28 °C). The dehalogenase activity from *E. coli* BL21 (DE3) (DhaA) is more sensitive to the temperature than that observed in the strain of origin since 40 °C was

Table 1
List of selected bacteria and media used to produce the different dehalogenases

Bacterial strain	Dehalogenase	Growth medium	Added substrate	Mean generation time (min)
R. erythropolis NCIMB13064	DhaA	Mineral	1-Chlorobutane	310
E. coli BL21 (DE3) (DhaA)	DhaA	Luria Bertani	IPTG	40
X. autotrophicus GJ10	DhlA	Luria Bertani	/	48
E. coli BL21 (DE3) (DhaA)	DhlA	Luria Bertani	IPTG	38
S. paucimobilis UT26	LinB	Luria Bertani	/	39

Table 2
Optimal physicochemical parameters for the dehalogenase activity of resting cells

Bacterial strain	Optimal temperature for dehalogenase activity (°C)	Optimal pH for dehalogenase activity	K_m (mM)	Maximal rate of 1-chlorobutane transformation (μmol min^{-1} g^{-1})
R. erythropolis NCIMB13064	45	9.0	3	16
E. coli BL21 (DE3) (DhaA)	40	9.0	5	23
X. autotrophicus GJ10	30	8.5	15	6
E. coli BL21 (DE3) (DhlA)	30	8.5	19	9
S. paucimobilis UT26	35	8.4	23	23

The tests of transformation of the 1-chlorobutane were carried out in Tris/HCl 50 mM buffer at the optimal pH for each strain. The concentration of 1-chlorobutane varied from 0 to 20 mM. The production of butan-1-ol was measured by GC.

its optimal temperature. In the case of *X. autotrophicus* GJ10, *E. coli* BL21 (DE3) (DhlA) and *S. paucimobilis* UT26, optimal temperatures of whole cells dehalogenase activities corresponded to those generally published for the purified enzyme (Janssen et al., 1985; Nagata et al., 1997). It should be noted that an increase of 5 °C above the optimal temperature involved a 50% loss of the activity for the resting cells.

The optimum pH varied from 8.4 to 9.0 depending on the strain (Table 2). Various studies with free dehalogenases report similar data (Keuning et al., 1985; Nagata et al., 1997; Dravis et al., 2000). Under these optimal conditions (temperature and pH), all the studied strains could transform 1-chlorobutane but maximal rates of conversion were different. Indeed, the more efficient dehalogenating bacteria were *E. coli* BL21 (DE3) (DhaA) and *S. paucimobilis* UT26 with a maximal transformation velocity of 23 μmol min^{-1} g^{-1} (Table 2).

However, *E. coli* BL21 (DE3) (DhaA) had higher affinity for 1-chlorobutane than *S. paucimobilis* UT26 (Km = 5 and 23 mM respectively). In contrast, *X. autotrophicus* GJ10 was less effective with a 6 μmol min^{-1} g^{-1} rate of 1-chlorobutane conversion. The 1-chlorobutane is not the best substrate for all the studied strains but it is often used as a reference substrate. DhlA from *X. autotrophicus* GJ10 is better known for its affinity for short dihalogenated substrates such as 1.2-dichloroethane or 1.2-dibromoethane whereas LinB from *S. paucimobilis* is known for its affinity for mono-halogenated substrates (C_4–C_{10}) (Damborsky and Koca, 1999).

Since the behavior of the free dehalogenases varies with respect to the substrates, the dehalogenating activity of resting cells was also tested with different substrates. All strains tested were active on the brominated, chlorinated and iodinated compounds selected. Except for 1-chloropropane, the short chain compounds were better transformed in aqueous medium than those with long carbon chain. For almost all strain, 1-chlorobutane was better transformed than 1-chloropentane which was better degraded than 1-chlorohexane. The same trend was noticed for brominated compounds. This observation can be directly connected to the aqueous solubility of the compounds. However *S. paucimobilis* UT26 is more active than the other strains for the long chain compounds (C_6–C_{10}). Except for *S. paucimobilis* resting cells, rate of conversion of 1-bromohexane was always higher than that of 1-chlorohexane. These data observed with whole cells are in agreement with those reported for the purified enzymes (Janssen et al., 1985; Stafford, 1993; Damborsky, 1996). But the reverse trend can be observed when comparing rates of conversion of 1-bromobutane and 1-chlorobutane or 1-bromopentane and 1-chloropentane. Only *S. paucimobilis* UT26 was able to transform secondary mono-halogenated compounds such as 2-chlorobutane with rates comparable to those of 1-chlorobutane degradation. *X. autotrophicus* GJ10 clearly showed a specificity for short dihalogenated compounds such as 1.2-dichloroethane or 1.2-dibromoethane.

3.2. Continuous dehalogenation in a gas/liquid bioreactor

We observed in the preceding study that the cells of *E. coli* BL21 (DE3) (DhaA) could be useful in the bioremediation of the 1-chlorobutane. Indeed, the kinetic parameters obtained in batch reaction for resting cells (dehalogenating activity = 23 μmol min^{-1} g^{-1} and Km = 5 mM) and the low generation time of this strain (40 min) could be interesting for the transformation of 1-chlorobutane. Consequently, fresh harvested *E. coli* BL21 (DE3) (DhaA) cells were tested in a continuous gas/liquid bioreactor for the transformation of 1-chlorobutane (Fig. 1). We observed that it is not possible to preserve a stable dehalogenase activity when resting cells were placed in a standard Tris/HCl 50 mM, pH 9.0 buffer (Fig. 2). Maximal dehalogenase activity was detected after only 50 h of reaction. Rate of conversion then decreased and no activity was detected after 400 h of reaction. The rate of deactivation was found to be 0.3% maximal activity per hour.

This low stability of the dehalogenase activity was already observed in batch system with free dehalogenases. The hydrolysis of 1-chlorobutane produces both butan-1-ol and HCl. This acid is known to have two effects: acidifying the medium (by H^+) and inhibiting dehalogenase (by Cl^-) (Oakley et al., 2002). The presence of an alcohol produced by the hydrolysis of halogenated compounds or the products of its metabolism (alcohol → aldehyde → carboxylic acid) could also have an effect on the dehalogenase activity of resting cells.

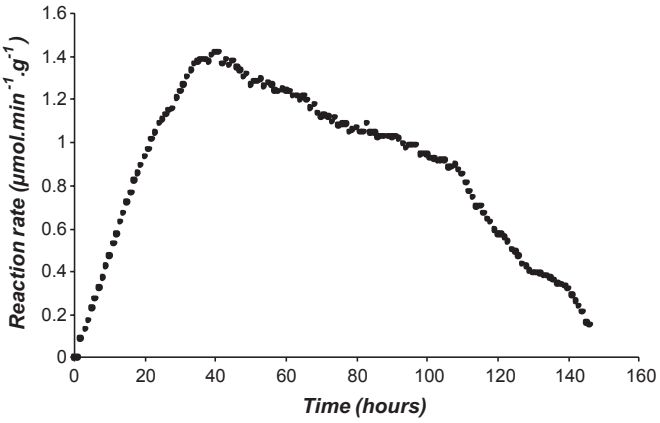

Fig. 2. Continuous 1-chlorobutane transformation by resting cells of *E. coli* BL21 (DE3) (DhaA) at 40 °C. Dehalogenase activity is expressed as the 1-chlorobutane transformation (µmol min^{-1} g^{-1} of bacteria).

3.3. Effect of the addition of an alcohol, an aldehyde or an acid on conversion of 1-chlorobutane by resting cells

Consequently, we examined the effect of secondary products resulting from the hydrolysis of 1-chloropropane (propanol, propanal, propionic acid) on the dehalogenase activity of whole cells (Fig. 3). Propanol, propanal and propionic acid were used as effectors for reasons of separation and solubility. Propanol, propanal or propionic acid were added in the reaction medium at a concentration of 2 ml l^{-1} and butan-1-ol production was determined, using 1-chlorobutane as a substrate.

First of all, the presence of propanol in the reaction medium had no significant effect on the velocity of 1-chlorobutane transformation in butan-1-ol. The propanal had a more important effect on the dehalogenase activity. This could be due the toxicity of the aldehyde. Cells of *R. erythropolis* NCIMB13064 were the most sensitive to the presence of propanal in the medium. Indeed 95% of the dehalogenase activity of *R. erythropolis* was inhibited in the presence of propanal at a concentration of 2 ml l^{-1}. A similar effect was noticed in the presence of propanoic acid except in the case of *R. erythropolis* cells.

3.4. Effect of 2-chlorobutane on conversion of 1-chlorobutane by resting cells

Gaseous effluents can contain a mixture of pollutants and the treatment of several halogenated compounds by biological method is even more difficult than the degradation of a single halogenated pollutant. 2-chlorobutane is a recalcitrant substrate for the majority of the strains stud-

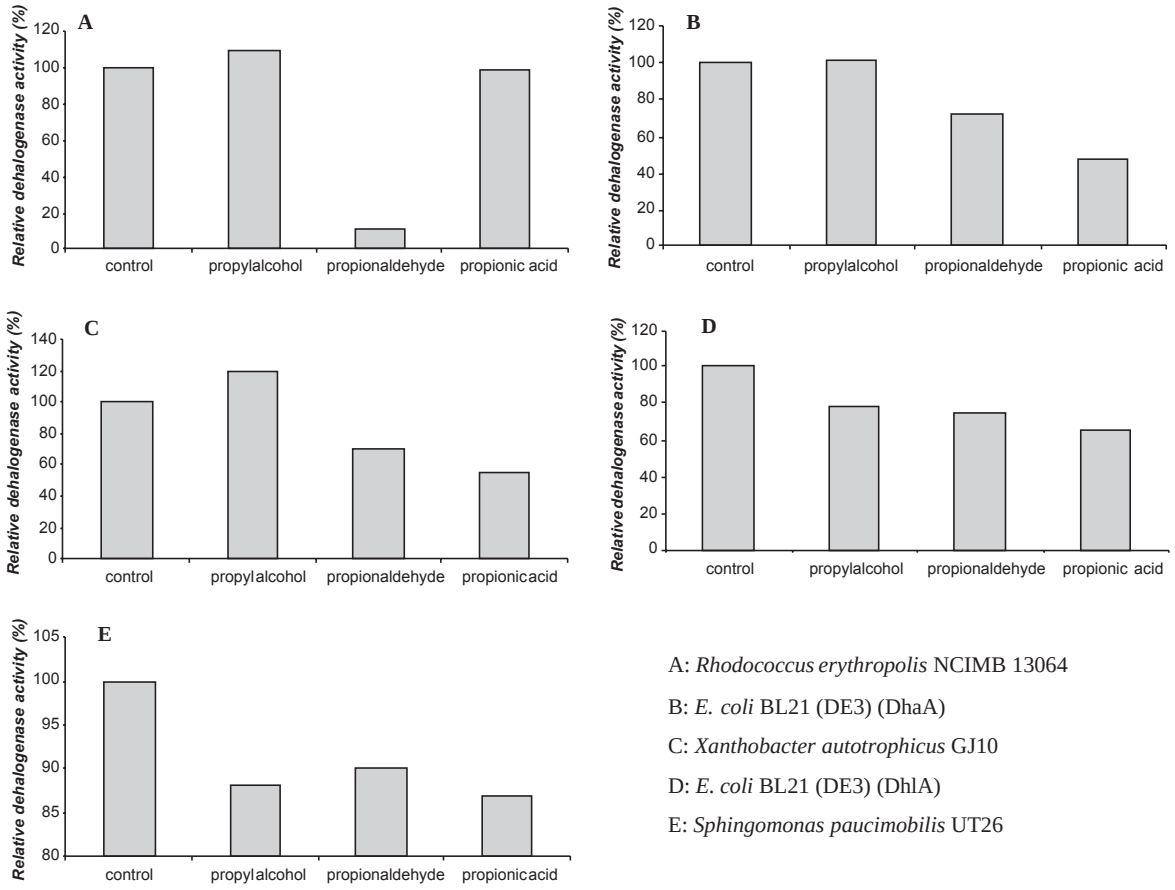

Fig. 3. The effect of propanol, propanal and propionic acid on resting cells dehalogenase activity. The tests of transformation of the 1-chlorobutane were carried out in Tris/HCl 50 mM buffer. The temperature and pH were those determined as optimal for each strain. The concentration of 1-chlorobutane was 14 mM and the concentration of secondary product was 2/1000 (v/v). The production of butan-1-ol was measured by GC.

Table 3
Relative dehalogenase activity of the different resting cells

	R. erythropolis NCIMB13064	*E. coli* BL21 (DE3) (DhaA)	*X. autotrophicus* GJ10	*E. coli* BL21 (DE3) (DhlA)	*S. paucimobilis* UT26
Experimental conditions	30 °C, pH 9.0	30 °C, pH 9.0	30 °C, pH 8.5	30 °C, pH 8.5	30 °C, pH 8.4
1-Chloropropane	50 (48)	44	75 (165)	116	30 (135)
1-Chlorobutane	100 (100)	100	100 (100)	100	100 (100)
1-Chloropentane	82 (78)	56	18 (16)	4	56 (120)
1-Chlorohexane	25 (75)	19	5 (10)	1	60 (145)
1-Chloroheptane	ND (59)	7	0 (0)	0	70 (161)
1-Chlorooctane	5 (33)	2	0 (0)	0	76 (139)
1-Chlorodécane	ND (10)	0	0 (ND)	0	8 (27)
1-Bromobutane	99 (106)	144	82 (87)	102	56 (234)
1-Bromopentane	58 (51)	35	67 (ND)	145	28 (355)
1-Bromohexane	45 (32)	20	37 (ND)	114	13 (ND)
1-Iodobutane	47 (58)	137	34 (ND)	30	67 (ND)
2-Chlorobutane	8 (8)	2	0 (ND)	0	73 (ND)
2-Bromobutane	34 (52)	47	0 (ND)	18	99 (ND)
1,2-Dichloroéthane	ND (ND)	66	159 (323)	223	10 (ND)
1,2-Dibromoéthane	ND (ND)	211	116 (303)	200	201 (ND)

The tests were carried out in Tris/HCl 50 mM buffer. The temperature and pH are those determined as optimal for each strain. The concentration of substrates in the reaction medium is 10 mM. The production of corresponding alcohol is evaluated in the course of time by GC. Rate of degradation of 1-chlorobutane is taken as the reference (100%).
The values between brackets are reported from the literature and represent values already observed with the purified dehalogenase (Janssen et al., 1985; Stafford, 1993; Damborsky, 1996).
ND: Not determined.

Table 4
The effect of inhibitor (2-chlorobutane) on resting cells dehalogenase activity for 1-chlorobutane

2-chlorobutane concentration (mM)	*R. erythropolis* NCIMB13064	*E. coli* BL21 (DE3) (DhaA)	*X. autotrophicus* GJ10	*E. coli* BL21 (DE3) (DhlA)
0	100	100	100	100
6	95	82	90	85
14	91	71	81	77
19	84	51	77	63

The tests of transformation of the 1-chlorobutane were carried out in Tris/HCl 50 mM buffer. The temperature and pH were those determined as optimal for each strain. The concentration of 1-chlorobutane was 14 mM. The production of butan-1-ol was measured by GC.

ied (Table 3), only *S. paucimobilis* UT26 has a good affinity for this compound. We studied the influence of 2-chlorobutane on the transformation of the 1-chlorobutane by resting cells (Table 4).

The dehalogenase activity of the various strains with exception of *S. paucimobilis* UT26 was measured for increasing concentrations of 2-chlorobutane. The dehalogenase activity of resting cells was inversely proportional to the concentration of 2-chlorobutane. *E. coli* BL21 was the most sensitive strain to 2-chlorobutane. Half of the initial dehalogenase activity of these cells was inhibited by the presence of 19 mM of 2-chlorobutane.

4. Conclusions

The study in aqueous phase made it possible to define the optimal temperature and pH conditions for dehalogenase activity of resting cells. For the transformation of the 1-chlorobutane, two strains have a strong effectiveness: *R. erythropolis* NCIMB13064 and *E. coli* BL21 (DE3) (DhaA). Production of the cells of *E. coli* BL21 (DE3) (DhaA) is eight times faster than that of *R. erythropolis* NCIMB13064 and should also be preferred for remediation. However the use of *E. coli* BL21 (DE3) (DhaA) for the continuous conversion of 1-chlorobutane is limited by the low stability of its dehalogenase activity. The studied strains have different specificities for halogenated substrates and thus the association of these strains could be interesting to treat a broad range of halogenated compounds. Since the optimal pH and temperature differ depending on the strain, they should be used in successive batch reactor rather than in association.

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