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Biodegradation of monochloroacetic acid used as a sole carbon and energy source by *Xanthobacter autotrophicus* GJ10 strain in batch and continuous culture

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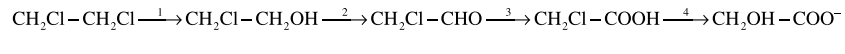
Abstract

Batch and continuous mode degradation of monochloroacetic acid used as a sole carbon and energy source in the concentration range of 0.9–48.4 mM by pure culture of *Xanthobacter autotrophicus* GJ10 was investigated. The substrate was completely degraded in each flask in batch system. Partial substrate inhibition occurred at the concentrations exceeding 25.4 mM. Temporary accumulation of glycolic acid in the medium indicated that dehalogenation was undergoing faster than further utilization of glycolate. Three different carbon substrates were used for inoculum preparation – 1,2-dichloroethane, tri-sodium citrate and a nutrient broth. The fastest growth on monochloroacetate occurred for 1,2- dichloroethane-grown inoculum. The assays of haloacid dehalogenase in crude extract indicated that the bacteria grown on 1,2-dichloroethane possessed higher level of the enzyme. The response of the GJ10 culture towards spikes of 20 mM monochloroacetate was tested in 2.5-l continuously stirred tank fermentor. The substrate was readily utilized within 7–8 h. Continuous degradation of monochloroacetate in the fermentor was demonstrated for monochloroacetate concentration of 20 mM and dilution rate 0.016 h^{-1}. Quantitative agreement between the amount of monochloroacetate introduced and chloride released was found. The results demonstrated that the strain *X. autotrophicus* GJ10 might be suitable for biodegradation of monochloroacetate contaminated media.

Introduction

Monochloroacetic acid (MCA) has been produced in amounts exceeding 300,000 t annually (Reimann et al. 1996). The main destinations of the compound include thioglycolic acid (intermediate in the manufacture of organotin stabilizers for PVC), herbicides (metolachlor, alachlor, propachlor and metazachlor), carboxymethylcellulose, preservatives for beverages and other chemicals.

The scale of MCA production and its versatility have resulted in a broad scientific interest aimed at detecting MCA in the environment and characterizing its toxicity and fate. The pollutant has been found in rainwater (Reimann et al. 1996; Römpp et al. 2001), surface waters (Hashimoto et al. 1998; Scott et al. 2000, 2002), drinking water (Pervova et al. 2002) and in snow (von Sydow et al. 1999). Typical concentrations of MCA in the aquatic systems have not exceeded μg/liter but in treated wastewaters levels of mg/l have been found (McRae et al. 2004). Numerous studies have demonstrated that monochloroacetate is toxic to aquatic life, in particular to algae (Kühn & Pattard 1990); MCA was recognized as the most toxic of all haloacetates toward several aquatic macrophytes, although toxicity was observed for concentrations higher than currently met in the



1 - haloalkane dehalogenase
2 - pyrrolo-quinoline quinone-containing alcohol dehydrogenase
3 - NAD-dependent aldehyde dehydrogenase
4 - haloacetate dehalogenase

Figure 1. 1,2-dichloroethane metabolic pathway in *Xanthobacter autotrophicus* GJ10 strain (Janssen et al. 1985).

environment (Hanson & Solomon 2004). Chloroacetic acid has relatively long lifetime in natural environment (Ellis et al. 2001) which may result from its resistance to photodegradation (Lifongo et al. 2004). On the other hand, spontaneous microbial dehalogenation of MCA in surface waters has been reported (Hashimoto et al. 1998; Ellis et al. 2001).

Although the existence of natural sources of MCA have been postulated (Woolard et al. 1979), severe risk of soil and water contamination with chloroacetate arises mainly from insufficient wastewater treatment, accidental spills as well as water disinfection via chlorination (Uden & Miller 1983) and partial degradation of herbicides (Wilson & Mabury 2000). Therefore, much scientific interest focused on biodegradation of MCA as a potential remedy. Many bacterial strains possessing inducible dehalogenases capable of cleaving off carbon-halogen bond in chloroacetate were isolated from soil (Hardman & Slater 1981) and sewage oxidation ponds (Olaniran et al. 2001). Since these bacteria tend to loose dehalogenating activity after growing on non-halogenated carbon sources, the authors concluded that the enzymes catalyzing dehalogenation were inducible. McRae et al. (2004) recently reported biodegradation of MCA in low concentrations by bacterial enrichment cultures. After prolonged incubation in 1-l batch fermentor the enrichment cultures were able to degrade completely the periodic spikes of 5.7μg/l up to 148 mg/l chloroacetate within 1–3 days. A strong inhibition was observed when the MCA concentration was equal to 2.04 mM (193 mg/l). The authors suggested that the consortia could be promising for biotreatment of surface waters and treated drinking water, in which MCA concentrations are much lower than 1 mg/l. However, the observed substrate inhibition makes the cultures unsuitable for the treatment of industrial wastewaters and neutralizing spills of MCA.

Apart from bacteria producing inducible enzymes (haloacid dehalogenases) several microbial strains possessing constitutive haloacid dehalogenase were reported (van den Wijngaard et al. 1992). *Xanthobacter autotrophicus* GJ10 strain capable of complete mineralization of 1,2-dichloroethane (DCE) via metabolic pathway involving haloacetate dehalogenase activity was described by Janssen et al. (1985) (Figure 1). The authors found dehalogenating activity toward MCA in crude extract prepared from cells grown on 1,2- dichloroethane, citrate or mixture of H_2, CO_2 and O_2 (autotrophic growth). However, no growth of *X. autotrophicus* GJ10 cells was observed in liquid mineral medium supplemented with 5 mM MCA. van der Ploeg et al. (1995) observed a poor growth of the GJ10 strain on 5 mM MCA with the growth rate equal to 0.05 h^{-1}. When the strain was grown on agar containing 5 mM citrate and 10 mM bromoacetate a mutant designated as GJ10M50 that overproduced haloacid dehalogenase was selected (van der Ploeg et al. 1995). Due to possessing higher level of the enzyme the mutant cells grew on 5 mM MCA with the growth rate 0.087 h^{-1}.

While Janssen et al. (1985) reported on constitutive haloacid dehalogenase in *X. autotrophicus* GJ10, Meusel & Rehm (1993) observed significant loss of the specific activity of haloacid dehalogenase in the strain toward dichloroacetic acid (DCA) when succinate was a carbon source for cell growth. The phenomenon of the enzyme level variation was further addressed by van der Ploeg and Janssen (1995) who determined the DNA sequence upstream of the *dhlB* gene encoding the dehalogenase in GJ10. On the basis of the analysis the authors concluded that the enzyme is overexpressed in stationary phase grown cells and under maintained conditions with poor growth substrate, like methanol.

The promising results reported on biodegradation of DCA (Heinze & Rehm 1993; Meusel & Rehm 1993) allowed us to suppose that the *X. autotrophicus* GJ10 strain could be successfully used also for biodegradation of MCA. Since the

data published up to now in the scientific literature did not bring decisive conclusions on the possibility of MCA utilization by this strain, we decided to verify it experimentally. The aim of the work was to check if growth of the strain on monochloroacetate used as a sole carbon substrate in a broad range of concentrations was possible and whether the strain could be potentially used for large-scale biodegradation of MCA. Another objective was to verify whether the reported variations of the haloacid dehalogenase level in the cell (Meusel & Rehm 1993; van der Ploeg & Janssen 1995) could be used to optimize the strain for MCA biodegradation.

Materials and methods

Microorganism

Pure *X. autotrophicus* GJ10 strain was obtained from Department of Biochemistry, University of Groningen (Groningen, The Netherlands) via National Bank for Industrial Microbial Cell Cultures (NBIMCC, Sofia, Bulgaria). Throughout the experiments the purity of the culture was routinely tested by plating on agar (with nutrient broth or 5 mM MCA as the carbon source) or by microscopic observation of medium samples.

Medium

In all batch mode experiments the strain was grown in MMY medium containing (g/l): 5.37 $Na_2HPO_4{\cdot}12H_2O$, 1.36 KH_2PO_4, 0.5 $(NH_4)_2SO_4$, 0.2 $MgSO_4{\cdot}7H_2O$. When the fermentor was applied lower concentration of the phosphate buffer was used (g/l): 0.46 $Na_2HPO_4{\cdot}12H_2O$ and 0.16 KH_2PO_4. The media were supplemented with trace metal solution (5 ml/l) containing (g/l): 0.53 $CaCl_2$, 0.2 $FeSO_4{\cdot}7\ H_2O$, 0.01 $ZnSO_4{\cdot}7H_2O$, 0.01 H_3BO_3, 0.01 $CoCl_2{\cdot}6$ H2O, 0.004 $MnSO_4{\cdot}5\ H_2O$, 0.003 $Na_2MoO_4{\cdot}2\ H_2O$, 0.002 $NiCl_2{\cdot}6\ H_2O$. To satisfy the strains' need for vitamins 10 mg/l yeast extract (Difco, 30 mg/l in case of continuous culture) was added to the medium. MCA (pure, Fluka, Switzerland or 99%, Aldrich, Germany) was introduced as a concentrated aqueous solution. The solution was either filter-sterilized (0.2 μm, Schleicher & Schuell) or used without sterilization; in the latter case the sterility was confirmed in reference experiments with non-inoculated flasks. All other growth substrates were of analytical grade or of $>99\%$ purity. Prior to inoculation pH was always adjusted with sterile concentrated NaOH solution to 6.5–7.0.

Fermentation conditions

Batch mode experiments were carried out in 500-ml flasks closed with cotton plugs. About 3–5 ml of inoculum was added to 100 ml sterile medium supplemented with MCA. Flasks were incubated in darkness on Model G25 Incubator Shaker (New Brunswick Scientific Co., New Jersey) at 150 rpm in 30 °C. Samples of 2.5 ml were taken regularly from the media. Several experiments were done in duplicate or triplicate (including experiments with highest MCA concentrations). The additional flasks were not sampled at the same regular time intervals, but their content was analyzed after the experiments were finished. Neither monochloroacetate nor glycolate was detected in any of these flasks confirming that biodegradation was complete.

Continuous mode and spiking experiments were carried out in a fermentor described in details elsewhere (van den Wijngaard et al. 1993). Only important differences in the experimental setup are given here. The volume of the reactor was 2.5 l. The substrate was supplied together with culture medium via viton rubber tubing or spiked directly to the fermentor. The stirring rate was 400 rpm. The temperature was kept at 30 ± 0.1 °C by a temperature sensor and controller connected to electrical heating device. The value of pH was maintained at 6.90–7.15 by automatic addition of sterile 2 M KOH or 1 M H_2SO_4. Continuous aeration rate was proportional to the actual concentration of oxygen in the medium (oxygen meter/controller was used); maximum air flowrate (100 ml/min) was applied when the oxygen concentration dropped below 1% of saturation. Samples of 5 ml were taken either directly from the fermentor or from the outlet.

Preparation of crude extract and haloacid dehalogenase assay

About 50–100 ml of medium sample were centrifuged (15 min at 9000 rpm), the pellet was resuspended in TEMAG H_2SO_4 (pH 7.5) buffer containing Tris (25 mM), EDTA (1 mM),

2-mercaptoethanol (1 mM), sodium azyde (0.02%) and glycerol (10% v/v) and centrifuged again (15 min at 9000 rpm, 15 °C). The pellet was transferred to 2-ml Eppendorf tubes and the cells were disrupted by ultrasonication. A crude extract was obtained by centrifugation (15 min at 13,000 rpm, 4 °C). Haloacid dehalogenase was assayed by adding 50–100 μl of crude extract to 3 ml of 50 mM glycine NaOH buffer containing 5 mM MCA or DCA followed by incubation of the mixture at 30 °C. Samples of 0.5 ml were taken at different time intervals and chloride concentration was determined.

Protein concentration in the crude extract was estimated by the method of Bradford (Bradford 1976) using bovine serum albumin as a standard. The specific dehalogenase activity was expressed as micromoles of chloride produced per minute per milligram of protein (units per milligram, U/mg).

Analytical methods

The biomass was monitored by optical density measurements at 500 nm. Chlorides were determined by a spectrophotometric method described in literature (Bergmann & Sanik 1957). A sample of centrifuged incubation mixture (2.5 ml) was mixed with 1 ml of Fe(III) solution (36.2 g $(NH4)Fe(SO_4)\cdot 12H_2O$ in 250 ml of 6M HNO_3) and 3 ml of saturated solution of $Hg(SCN)_2$ in 98% ethanol. The mixture was shaken and after 10 min filtered through 0.2 μm filter; the absorption was measured on Heliosβ Unicam UV/Vis spectrophotometer at 460 nm. Chloride concentration was calculated from a standard calibration curve. The concentrations of MCA and GLC were measured on a Perkin–Elmer Series 10 HPLC equipment with Bio-Rad column for organic acid analysis (Aminex Ion Exclusion HPX-87H) and spectrophotometric Knauer variable wavelength detector at 210 nm, coupled with an integrator Shimadzu C-R6A Chromatopac. As a mobile phase a 0.01 N sulfuric acid was used at an elution flow rate of 0.6 ml/min. About 2-ml medium samples were centrifuged (15 min at 5000 rpm) and 20 μl aliquots from the supernatant were directly introduced to the chromatograph. If the concentration was not determined immediately, the samples were kept in a freezer at −20 °C. A standard calibration curve was prepared to calculate the concentrations from the peak area. The detection limits were ca. 0.25 mM for MCA and GLC.

In all cases only the linear parts of the calibration curves were used. The linear ranges and line coefficients (A and B in the general equation $y = Ax + B$, where y was the respective concentration in moles/l and x was the peak area or absorbance measured) were as follows:

– for chlorides 0−1 mM, $A = 1.873.10^{-3}$ and $B = 1.026{\cdot}10\text{-}5$;

– for MCA 0.25 – 10 mM, $A = 1.045.10^{-8}$ and $B = \text{-}6.353.10^{-4}$;

– for glycolate 0.25 – 10 mM, $A = 1.015.10^{-8}$ and $B = 5.917.10^{-4}$.

Results and discussion

Growth of Xanthobacter autotrophicus *GJ10 on MCA in batch mode*

The bacterium *X. autotrophicus* GJ10 produces haloacid dehalogenase active towards MCA and chloroacetate is degraded as an intermediate in 1,2-dichloroethane metabolic pathway (Janssen et al. 1985). It could be expected that MCA should support the strain's growth as sole carbon substrate. In order to verify this experimentally three series of flasks containing ca. 1–20 mM of MCA in mineral medium were inoculated with *X. autotrophicus* GJ10. Three separate inocula were prepared: a citrate-grown (1% of trisodium citrate), a nutrient broth-grown (0.8% NB in mineral medium), and 1,2-dichloroethane-grown (5 mM) one. The non-halogenated substrates (NB and citrate) were much richer carbon sources for the strain. Therefore, the initial optical densities in the flasks inoculated with the NB- and citrate grown cultures were of one order of magnitude higher than for DCE-grown inocula. Interestingly, the exponential phase of growth first started in the media with 1,2-dichloroethane pre-grown bacteria (Figure 2c). The length of the lag-phase in the flasks inoculated with DCE was approximately 25 h. When the strain was pre-grown on citrate the lag-phase lasted c.a. 50–80 h (Figure 2a), while in case of the richest inoculum (grown on NB) the exponential phase started after 100 h (Figure 2b). Thus, the type of the carbon source used for inoculum growth influenced the strain growth on MCA more than did the total number of bacteria intro-

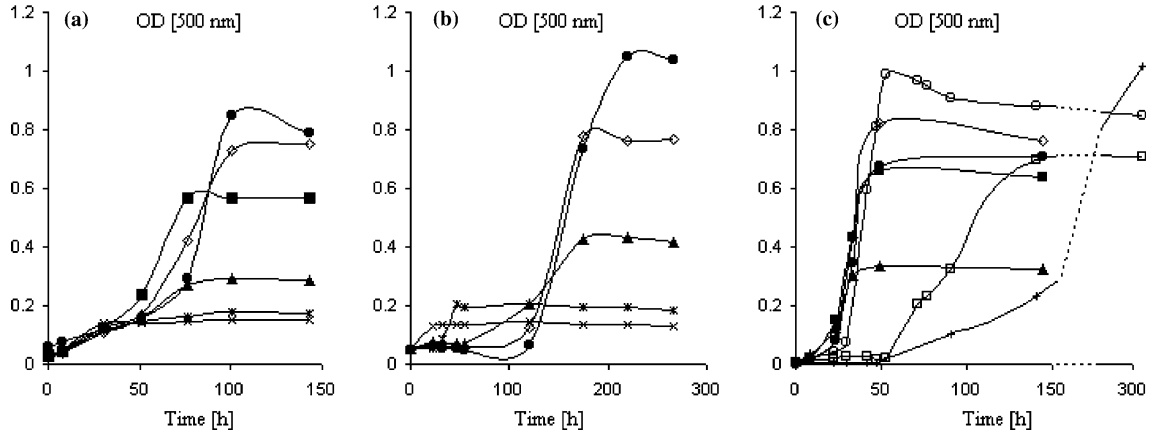


Figure 2. Growth of *Xanthobacter autotrophicus* GJ10 on MCA used as a sole carbon and energy source; (a) citrate-grown inoculum (MCA concentration: × 0.9 mM, * 1.8 mM, ▲ 5.5 mM, ■ 11.0 mM, ◇ 15.6 mM, ● 19.6 mM), (b) NB-grow inoculum (MCA concentration: × 1.1 mM,) * 2.5 mM, 6.9 mM, ◇ 12.7 mM, ● 18.6 mM), (c) DCE-grown inoculum (MCA concentration: 4.3 mM, ■ 9.1 mM, ◇ 14.0 mM, ● 19.0 mM, ○ 24.9 mM, □ 36.9 mM, +48.4 mM).

duced. This phenomenon is discussed in next sections.

In general, an extension of the lag-phase's duration with the increase of initial substrate concentration was observed in all series of experiments, especially for citrate and NB-grown inocula (Figure 2a, b). Such phenomenon is not uncommon in microbiological processes and could be explained by time needed for developing adaptation mechanisms enabling selection of cells which are more tolerant to higher substrate concentrations (Velizarov & Beschkov 1998).

Regardless of the length of the lag-phase a good growth was observed for monochloroacetic acid concentrations between 4.3 and 19 mM (Figure 2). Since no substrate inhibition was found in this range, three additional flasks containing 24.9, 36.9 and 48.4 mM of MCA, respectively, were inoculated with DCE-grown inoculum and the growth kinetics was followed. The flasks were incubated for an extended period of time (310 h). For the lowest concentration of the three ones the lag-phase was not significantly changed compared to the growth on MCA in the range previously tested. For 36.9 mM and especially for 48.4 mM MCA a significant delay in the growth was observed (Figure 2c). The lag-phase was approximately doubled for 36.9 mM MCA and the exponential phase of growth was slower. In case of the highest concentration tested no clearly distinguished exponential phase could be found. The final optical densities after this time were 0.71 and 1.015 for 36.9 mM and 48.4 mM of initial MCA concentration, respectively (Figure 2c).

On the basis of the biomass profiles (Figure 2) specific growth rate μ of the strain on monochloroacetate in batch mode was estimated by solving the equation:

$$\frac{dX}{dt} = \mu \cdot X,$$

where X was the biomass concentration and dX/dt was the slope of best linear fit through the points at the exponential growth. In case of DCE-grown inoculum no dependence of the μ value on the initial monochloroacetate amount was found within the range of 4.3–19 mM MCA. The average μ value for the culture was equal to 0.094 h^{-1}. The value found for citrate-grown culture was 0.058 h^{-1} and was similar to the value reported for the wild-type strain GJ10 (0.05 h^{-1}) (van der Ploeg et al. 1995). In case of NB-grown culture the highest μ value was found for the highest MCA concentration tested and was equal to 0.019 h^{-1}. Although the values are only rough estimates, they clearly indicate that the cells pre-grown on the poorest of the carbon sources (DCE) were best able to utilize MCA as a sole growth substrate.

Utilization of MCA by Xanthobacter autotrophicus GJ10 in batch mode

The experimental profiles of MCA in the flasks versus time of incubation were presented in

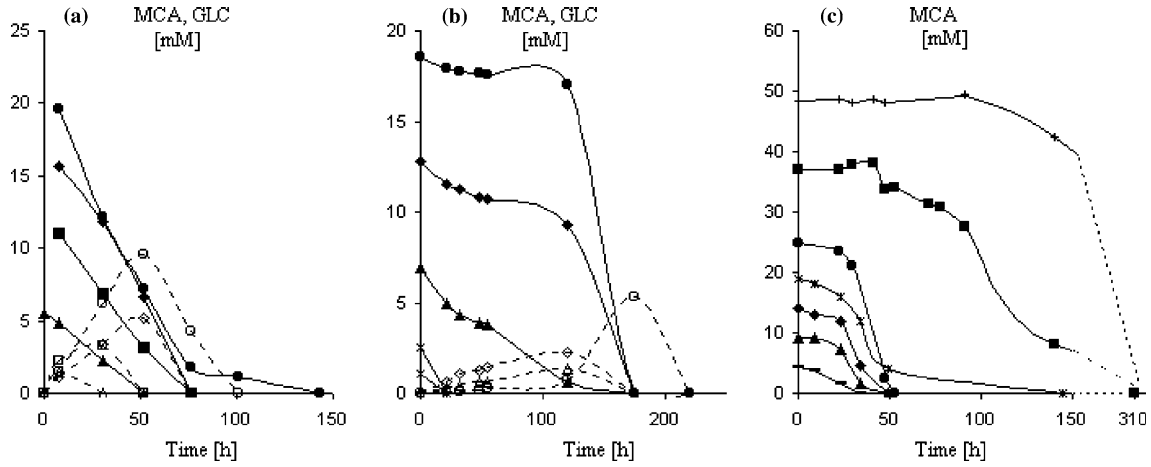


Figure 3. Utilization of MCA and GLC formation by *Xanthobacter autotrophics* GJ10; (a) citrate-grown inoculum, (b) NB-grown inoculum, (c) DCE-grown inoculum (here only MCA concentration shown). Full symbols and continuous lines – MCA concentrations, open symbols and dotted lines – respective GLC concentrations.

Figure 3. In all experiments the concentration of monochloroacetate dropped below the limit of detection. Abiotic processes of MCA degradation were excluded since neither chloride production nor MCA disappearance was observed in non-inoculated flasks (data not shown).

Degradation of MCA was accompanied by the release of chloride (Figure 4). In most cases the agreement between the amount of chloride released and MCA degraded was quantitative (the highest differences did not exceed 20% and were likely to be caused by analytical errors) confirming that no accumulation of chloroacetate in the cells can be assumed.

For lower initial MCA concentrations a correlation was observed between the amount of MCA utilized and biomass produced. The logarithmic phase of growth was accompanied by the fastest rates of substrate disappearance (compare Figures 2 and 3) and chloride production. For higher initial concentrations of monochloroacetate, especially in case of citrate- and nutrient broth-grown inocula, the exponential phase of growth started when some amount of MCA had already been degraded (Figure 3). To explain this phenomenon the broth was analyzed with HPLC for the presence of intermediate products. The chromatograms showed that a temporary accumulation of glycolate in the medium occurred. The accumulation was lowest for DCE-grown inoculum (data not plotted for the reason of better readability of Figure 3c), the concentration of glycolate did not exceed 2 mM with the exception of the three highest concentrations of MCA (up to 5 mM was then observed). In cases of NB and citrate-grown inocula the accumulation was significant and the logarithmic phase of growth coincided with the utilization of glycolate rather than monochloroacetate (Figure 4).

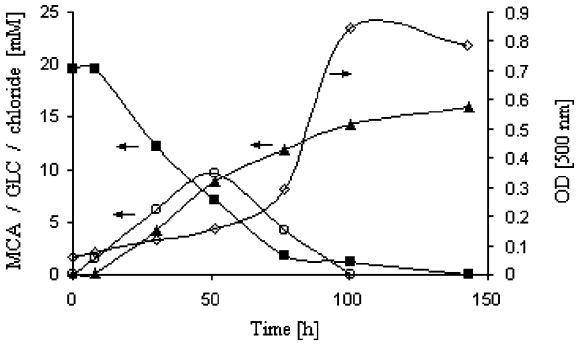


Figure 4. Biomass (optical density, ◇), MCA (■), GLC (○) and chloride (▲) profiles for *Xanthobacter autotrophicus* GJ10 cultivation on MCA in batch mode (citrate-grown inoculum, initial MCA concentration 19.6 mM).

The temporary presence of glycolate in the medium suggests that the activity of haloacetate dehalogenase was higher than the activity of enzymes responsible for glycolate degradation.

pH changes after MCA biodegradation in batch mode

The transformation of monochloroacetate to glycolate by haloacid dehalogenase from

X. autotrophicus GJ10 is a hydrolysis reaction (Janssen et al. 1985). Due to the reaction HCl molecule is released to the surrounding medium and pH drop may occur. This drop, in turn, may stop further cell growth and haloacid degradation, as shown by Meusel & Rehm (1993). These authors reported strong inhibition due to pH drop for GJ10 growth on dichloroacetic acid in concentrations exceeding 20 mM. In this work a partial inhibition was observed for the concentration of MCA equal to 36.9 mM. A stronger inhibition was found for 48.4 mM MCA but complete biodegradation after 310 h was found.

In order to verify whether pH drop may play a role in the batch biodegradation of MCA additional experiments with pH control were carried out. Final pH values measured after complete biodegradation of MCA (initial concentrations 10 and 20 mM) were compared to the values of pH obtained after addition of 10 and 20 mM HCl, respectively, to MMY medium in the absence of cells. The results show that the pH drop in the inoculated flasks was negligible and the final values were comparable to the initial pH, before biodegradation started (6.9 and 6.6, respectively). On the other hand, strong pH drop was observed when 20 mM HCl was added to the medium (pH equal to 6.2 and 3.7, for 10 and 20 mM HCl, respectively). Hence, it can be concluded that the H^+ cations released during the dehalogenation of MCA were utilized during further biodegradation of glycolate. This conclusion was supported by observations of pH changes in the fermentor (see next paragraph).

Biodegradation of spikes of MCA in the fermentor

After the ability of the strain to biodegrade MCA in batch mode was confirmed, the response of the bacterium towards spiking of high concentrations of the substrate was tested in 2.5-l fermentor. Initially, the fermentor was inoculated with 250 ml of nutrient broth grown cells followed by continuous feeding with 20 mM MCA. Since slow washout was observed (data not shown), the flow was stopped and the fermentor was spiked with 10 mM sodium succinate in order to attain higher biomass concentrations. This substrate was consumed within ca. 15 h and resulted in significant growth of the optical density, up to 1.2 (data not shown). Afterwards, the fermentor was faced with 21-h starvation in order to stimulate the cells to increase the level of haloacid dehalogenase. Although real starvation conditions were not achieved (glycolate and other unidentified intermediates were still detected), the dehalogenase amount was almost doubled during the period (Table 1).

Despite that the level of the enzyme was still relatively low compared to most of the cultures

Table 1. Specific haloacid dehalogenase activities determined towards MCA and DCA in crude extract

Growth substrate	Age of the culture (h)	Specific dehalogenase activity[a] (mU mg^{-1}protein)		Age of the culture (h)	Specific dehalogenase activity[b] (mU mg^{-1}protein)	
		Towards MCA	Towards DCA	Towards MCA	Towards DCA	
1,2 dichloroethene (7.4 mM)	77	5140	7665	Not tested	–	–
1,2 Monochloroacetate (10 mM)	166.5	3207	5232	42.6	1609	2714
Monochloroacetate (20 mM)	196.5	1865	2950	44.3	2077	3178
Monochloroacetate (20 mM, second spike to fermentor)	26.7[c]	1199	1871	–	–	–
Dichloroacetate (10 mM)	78.5	883	2023	99.5	1258	2936
Citrate (10 mM)	76.5	676	992	77	408	580
Succinate (10 mM)	74.5	1057	1470	75	2002	2842
Nutrient broth (0.8%)	72.5	888	1473	Not tested	–	–
Succinate (10 mM, spike to fermentor)	40.5[c]	392	6663	–	–	–
Succinate (10 mM, spike to fermentor)	61.5[c]	742	1147	–	–	–

[a] Inoculum from NB agar plate (concerns batch cultures).
[b] Inoculum prepared on the same substrate as the growth one.
[c] Time between the spiking and crude extract preparation.

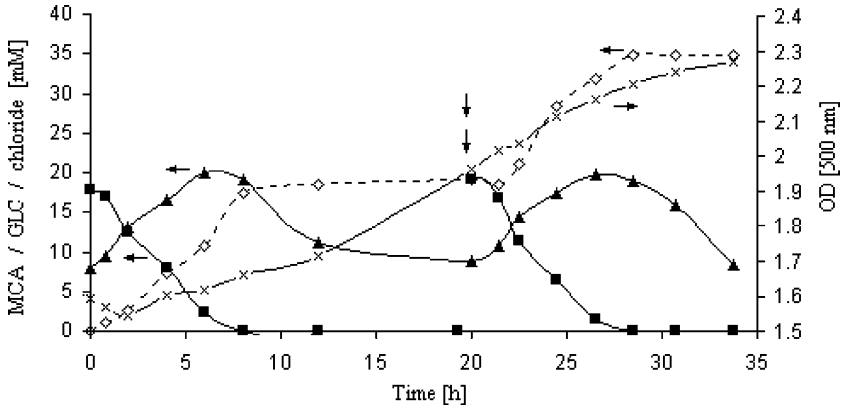


Figure 5. Degradation of MCA spikes in the fermentor; ■ MCA, ▲ GLC, ◇ chloride produced, × optical density at 500 nM. The first spike at time 0, the other at time 20 h (indicated by double arrow).

harvested from batch flask fermentors (Table 1), the first spike of 18 mM MCA to the bioreactor was rapidly consumed (Figure 5). After 8 h no more chloroacetate was found in the broth. The amount of chloride released coincided well with the decrease of MCA concentration, which proved that chloroacetate was dehalogenated and no accumulation in biomass occurred. Immediately after the spiking the concentration of oxygen in the medium dropped from 90% of saturation to ca. 1% (data not shown) indicating rapid biological action.

The profile of oxygen suggested that O_2 transport could be rate-limiting in the carbon source utilization. As a consequence glycolate accumulated in the broth reaching the concentration of 20 mM. The low level of oxygen did not change for the next 24 h probably due to the utilization of intermediate products.

The next spike of MCA, 19.2 mM, was also rapidly consumed within approximately 8 h (Figure 5). After MCA in the fermentor was completely degraded (Figure 5) the activity of haloacetate dehalogenase of the cells harvested from the fermentor was determined. Its value was 3 times higher than after the spiking of succinate (Table 1) but did not exceed values obtained in most batch mode experiments with other growth substrates.

The pH in the fermentor was regulated by automatic addition of acid (H_2SO_4) or base (KOH). Initially, only base was delivered to the bioreactor suggesting some acidification of the medium. When the concentration of MCA dropped below the detection limit, only the acid was introduced to the bioreactor, which suggests that H^+ ions were consumed during glycolate degradation.

Strong increase of optical density and a formation of thin biofilm in the fermentor as well as foam formation in the broth were observed after the spikings. The last phenomenon was probably caused by polysaccharide slime produced by *X. autotrophicus* GJ10 (Kern 1985).

Continuous degradation of MCA in the fermentor

The continuous mode was performed immediately after the experiments with spikings without exchanging the broth. The initial concentration of chloride accumulated in the fermentor was as high as 42.7 mM (Figure 6) and glycolate was still present in the amount of 7.4 mM. Low dilution rate (0.016 h^{-1}) and constant MCA inlet concentration (20 mM) were applied throughout the experiment lasting ca. 320 h.

As shown in Figure 6 no MCA was detected in the outlet (MCA efflux) of the fermentor during the whole experiment. The concentration of glycolate (GLC efflux) decreased below the limit of detection after 150 h, thus biodegradation was complete, without accumulation of the intermediate. The final concentration of chloride achieved approximately 20 mM, which was in quantitative agreement with the amount of MCA in the inlet. The optical density decreased during the first 150 h, finally reaching a constant value of approximately 1.0. The probable reason of the decrease was low amount of carbon source available to the cells due to low inlet flowrate. This was

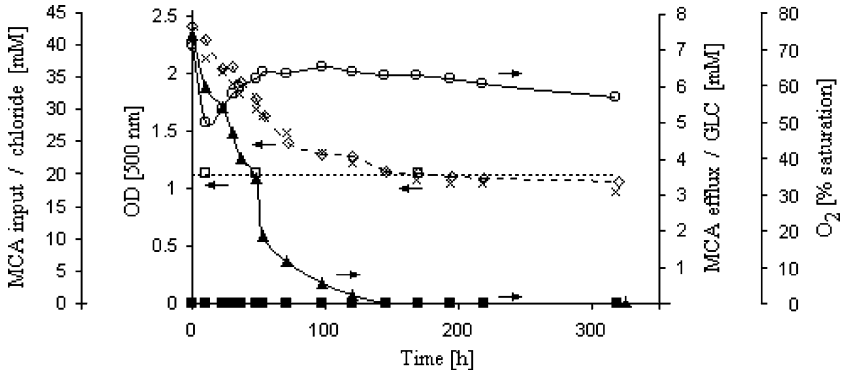


Figure 6. Biodegradation of MCA in the continuous culture of *Xanthobacter autotrophicus* GJ10: □ (dot line) MCA input, ■ MCA efflux, ▲ GLC efflux, ◇ (dash line) total concentration of chloride in the fermentor, × (not connected with line) optical density at 500 nm, ○ oxygen saturation in the broth.

also confirmed by the oxygen profile in the fermentor (Figure 6). The concentration of O_2 stabilized after approximately 50 h on the level of 56–64% of saturation (continuous aeration compensated O_2 consumption). Such high concentration of oxygen, compared to ca. 1% during the biodegradation of MCA spikes (data not shown), indicated low biological action. A short drop of oxygen concentration in the first 30 h might be caused by a transient adaptation of the culture to the continuous process conditions.

The profiles presented in Figure 6 suggested that a steady state process was achieved after 120–150 h.

Dehalogenase assay in crude extract

To verify whether the differences in specific growth rate for citrate- and 1,2-DCE-grown inocula could be caused by different haloacid dehalogenase level in the cells, crude extract activities towards MCA and DCA were determined. The latter substrate was more stable in the glycine NaOH buffer used therefore all assays were routinely performed on both acids. The results of the assays were collected in Table 1. In case of batch cultures cells were always harvested when high optical densities were obtained. The results (Table 1) indicated that there was a dependency between the activity of the enzyme and the growth substrate used for inoculum preparation. The difference in dehalogenase activities of citrate and DCE-grown cells towards MCA was one order of magnitude and was that most probable reason for different growth rates and lag-phase duration on MCA observed for DCE, citrate and nutrient broth-grown inocula. In general, the lowest activities were observed for the richest growth substrates (citrate, nutrient broth). The conclusion is in agreement with the data reported by van der Ploeg & Janssen (1995) on overexpression of haloacid dehalogenase by GJ10 in carbon-limitation conditions.

The reported loss of activity when succinate was the carbon source (Meusel & Rehm 1993) was not confirmed. The activity of the dehalogenase of succinate-grown cells towards MCA and DCA was comparable to the ones found for other substrates. On the other hand, high variations of the enzyme level for some substrates were observed. This observation, also reported in literature (van der Ploeg & Janssen 1995), suggests that haloacid dehalogenase level may depend on the phase of cell growth. Although the time of cultivation was similar for both assays prepared on succinate-grown cells, in the second incubation the substrate was apparently consumed faster and the cells were for some time faced with starvation, which could cause overexpression of dehalogenase.

Conclusions

The utilization of monochloroacetic acid by *X. autotrophicus* GJ10 strain in batch, spiking and continuous conditions was demonstrated. Within the whole of the investigated range of MCA concentrations (0.9–48.4 mM) growth of *X. autotrophicus* GJ10 was observed. For the concentrations of MCA above 25 mM the growth delayed due to

the extension of the lag-phase duration. The substrate inhibition phenomenon was, however, not complete, since the growth started after cells' accommodation to high chloroacetate concentrations.

The utilization of MCA in the concentration range tested was complete; the final products were chloride ions, biomass and carbon dioxide (not determined in the work). Temporarily, strong accumulation of glycolic acid in the medium was observed. On the basis of the oxygen concentration drop during rapid degradation of MCA spikes it can be concluded that glycolate accumulation in the fermentor might be caused by limited oxygen transport.

The results of haloacid dehalogenase assays in crude extract together with the lag-phase duration and specific growth rate values allow to conclude that a significant improvement in the biodegradation of MCA can be expected when a poor growth substrate is used for the strain precultivation in batch mode. The increase in the enzyme level occurs also under starvation conditions, which was demonstrated in the fermentor.

The experimental results demonstrated here proved that the strain *X. autotrophicus* GJ10 utilizes well MCA as a sole carbon and energy source and could be potentially used for biodegradation of monochloroacetate contaminated media. The advantage of the strain over other MCA degraders results from its constitutive haloacid dehalogenase, which does not require MCA presence in order to be expressed. Another feature of the strain is complete mineralization of monochloroacetate, with only temporary intermediate excreted outside the cell.

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References

Bergmann JG & Sanik J (1957) Determination of trace amounts of chlorine in naphtha. Anal. Chem. 29: 241–243

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254

Ellis DA, Hanson ML, Sibley PK, Shadid T, Fineberg NA, Solomon KR, Muir DCG & Mabury SA (2001) The fate and persistence of trifluoroacetic and chloroacetic acids in pond waters. Chemosphere 42: 309–318

Hanson ML & Solomon KR (2004) Haloacetic acids in the aquatic environment. Part I: macrophyte toxicity. Environ. Pollut. 130: 371–383

Hardman DJ & Slater JH (1981) Dehalogenases in soil bacteria. J. Gen. Microbiol. 123: 117–128

Hashimoto S, Azuma T & Otsuki A (1998) Distribution, sources and stability of haloacetic acids in Tokyo Bay. Jn. Environ. Sci. Technol. 17: 798–805

Heinze U & Rehm H-J (1993) Biodegradation of dichloroacetic acid by entrapped and adsorptive immobilized *Xanthobacter autotrophicus* GJ10. Appl. Microbiol. Biotechnol. 40: 158–164

Janssen DB, Scheper A, Dijkhuizen L & Witholt B (1985) Degradation of halogenated aliphatic compounds by *Xanthobacter autotrophicus* GJ10. Appl. Environ. Microbiol. 49: 673–677

Kern R (1985) Production of drag reducing polymers by hydrogen bacteria. Proc. Am. Inst. Phys. Conf. 137: 135–142

Kühn R & Pattard M (1990) Results of the harmful effects of water pollutant to green algae (*Scenedesmus suspiciatus*) in the cell multiplication inhibition test. Water Res. 24: 31–38

Lifongo LL, Bowden DJ & Brimblecombe P (2004) Photodegradation of haloacetic acids in water. Chemosphere 55: 467–476

McRae BM, LaPara TM & Hozalski RM (2004) Biodegradation of haloacetic acids by bacterial enrichment cultures. Chemosphere 55: 915-925

Meusel M & Rehm H-J (1993) Biodegradation of dichloroacetic acid by freely suspended and adsorptive immobilized *Xanthobacter autotrophicus* GJ10 in soil. Appl. Microbiol. Biotechnol. 40: 165–171

Olaniran AO, Babalola GO & Okoh AI (2001) Aerobic dehalogenation potentials of four bacterial species isolated from soil and sewage sludge. Chemosphere 45: 45–50

Pervova MG, Kirichenko VE & Pashkevich KI (2002) Determination of chloroacetic acids in drinking water by reaction gas chromatography. J. Anal. Chem. 57: 326–330

Reimann S, Grob K & Frank H (1996) Chloroacetic acids in rainwater. Environ. Sci. Technol. 30: 2340–2344

Römpp A, Klemm O, Fricke W & Frank H (2001) Haloacetates in fog and rain. Environ. Sci. Technol. 35: 1294–1298

Scott BF, MacTavish D, Spencer C, Strachan WMJ & Muir DCG (2000) Haloacetic acids in Canadian lake waters and precipitation. Environ. Sci. Technol. 34: 4266–4272

Scott BF, Spencer C, Marvin CH, MacTavish D & Muir DGC (2002) Distribution of haloacetic acids in the water columns of the Laurentian Great Lakes and Lake Malawi. Environ. Sci. Technol. 36: 1893–1898

Uden PC & Miller JW (1983) Chlorinated acids and chloral in drinking water. J. Am. Water Works Assoc. 75: 524–527

van den Wijngaard AJ, van der Kamp KWHJ, van der Ploeg J, Pries F, Kazemier B & Janssen DB (1992) Degradation of 1,2-dichloroethane by *Ancylobacter aquaticus* and other facultative metylotrophs. Appl. Environ. Microbiol. 58: 976–983

van den Wijngaard AJ, Wind RD & Janssen DB (1993) Kinetics of bacterial growth on chlorinated aliphatic compounds. Appl. Environ. Microbiol. 59: 2041–2048

van der Ploeg J & Janssen DB (1995) Sequence analysis of the upstream region of *dhlB*, the gene encoding haloalkanoic dehalogenase of *Xanthobacter autotrophicus* GJ10. Biodegradation 6: 257–263

van der Ploeg J, Willemsen M, van Hall G & Janssen DB (1995) Adaptation *of Xanthobacter autotrophicus* GJ10 to bromoacetate due to activation and mobilization of the haloacetate dehalogenase gene by insertion element IS1247. J. Bacteriol. 177: 1348–1356

Velizarov S & Beschkov V (1998) Biotransformation of glucose to free gluconic acid by *Gluconobacter oxydants*: substrate and product inhibition situations. Proc. Biochem. 33: 527–534

von Sydow L, Borén H & Grimvall A (1999) Chloroacetates in snow, firn and glacier ice. Chemosphere 39: 2479–2488

Wilson RI & Mabury SA (2000) The photodegradation metolachlor: isolation, identification, and quantification of monochloroacetic acid. J. Agric. Food Chem. 48: 944–950

Woolard FX, Moore RE & Roller PP (1979) Halogenated acetic and acrylic acids from the Red alga *Asparagopsis taxiformis*. Phytochemistry 18: 617–620