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Detection of some chloroorganic compounds by a microbial sensor system

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Cells of an isolated *Pseudomonas* sp. MB58 strain containing DL-2-haloacid dehalogenase were used as a biological component in microbial bioassay for the detection of 2,2-dichloropropionic and D-2-chloropropionic acids. The cells were entrapped in various matrices. The highest specific activity and stability have been obtained in calcium alginate. Temperature between 293 and 313 K did not affect the enzymatic activity of the cells, and pH between 6.0 and 8.0 had a little influence. Several organic substances did not influence the conversion, whereas some heavy metal ions inhibited the dehalogenase activity.

Key words: *detection; microorganism; whole cell biosensor; dehalogenation; chloroorganic compounds*

1. Introduction

Despite innumerable benefits, industrialization and new technologies create various environmental problems. Heavy metals originating from industrial production bioaccumulate in the aquatic food chain [1]. Organic compounds originating from widespread use of petroleum products are highly toxic and pose a threat for soil and drinking water quality [2].

Conventional chromatographic methods for evaluating contaminated sites are both expensive and technically complicated. In recent years, bacterial whole cell biosensors have been developed as tools to detect and quantify the toxicity of samples from various environments. Many studies used bacteria containing constitutively expressed *luxCDABE* operon from *Vibrio fischeri* to evaluate the presence of toxic compounds. The *lux* genes are usually highly expressed in cells that are not exposed to toxic effects in the medium. Measurements of the decrease in light production from these cells will therefore reflect an inhibitory effect of the compounds added to bacteria. This has led

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to a development of commercial toxicity tests such as Microtox [3], using the naturally luminescent *Vibrio fischeri.* As a consequence, similar constructs have been made in bacteria such as *Pseudomonas fluorescens* [4, 5] and *Rhizobium leguminosarum* [6]. Such measurements are nonspecific because many conditions decreasing the metabolic activity of a biosensor strain may reduce light production. Conditions such as pH, metals, xenobiotics and many other compounds are potential inhibitors of light production. These nonspecific sensors have been used to determine the toxicity level of such diverse substances as nitric oxide [7], xenobiotics [8–11], metals [5, 12, 13] and tetracyclines [14, 15]. Recently a novel type of bacterial biosensor has been developed using a recombinant DNA technology [16].

The application of biosensors for the specific estimation of various organic compounds has been investigated by several authors [17–21]. Rawson et al. [22] used the electron transfer chain of the photosynthetic microorganism *Synechococcus sp.* to detect chlorotoluron. A biosensor using *Escherichia coli* cells as a biological compound was introduced in the detection of pentachlorophenol tasted on its effect on respiration [23]. A bioassay for the determination of halogenated hydrocarbons was described by Hutter et al. [24, 25]. It is based on the liberation of halogen ions by the action of the alkyl-halidohydrolase enzyme (EC 3.8.1.1) present in the cells of *Rhodococcus* sp.

The aim of the experiments described in this article was to establish a microbial system for the detection of 2-chloroalkanoic acid in water samples. Immobilized cells of *Pseudomonas* sp. MB58 and potentiometric ion selective electrodes (ISE) were used as sensing elements. The MB58 strain produces DL-2-haloacid dehalogenase (data not shown). This enzyme dehalogenates both D- and L-enantiomers of 2-haloalkanoic acids and the corresponding L- and D-2-hydroxyalkanoic acids are produced. The performance of the system and the influence of physical parameters and the number of heavy metals and salts on the system response were studied in detail.

2. Experimental

Microorganism and growth. Pseudomonas sp. strain MB58, grown on 2,2-dichloropropionate was isolated from soil in this laboratory, and was used for the immobilization and biodechlorination experiments. The MB58 strain was cultivated in a mineral medium containing per dm^3: $Na_2HPO_4 \times 12H_2O$ (5 g), NaH_2PO_4 (2 g), $(NH_4)_2SO_4$ (0.5 g), $MgSO_4 \times 12\ H_2O$ (0.1g), yeast extract (50 mg). Chloroorganic compound was filter-sterilized to prevent thermal dechlorination and added as the sole carbon and energy source. Bacteria were grown aerobically at room temperature under conditions of rotary shaking (120 rpm). After 72 h of incubation the cells were collected by centrifugation (3200 rpm, 15 min, 277 K). Then the packed cells were resuspended in a 10 mM phosphate buffer (pH = 7.6) and used for immobilization.

Immobilization. The immobilized cell preparations were carried out as described in [26] with the following modification: agarose/ carrageenan 2 ml cell suspension (0.5 g of wet cells) was mixed with 20 ml of 6% agarose (Sigma, type VI) or 4% carrageenan (Sigma, type 1) and poured onto Petri dishes on ice in order to form gel. The gels were cut into 3×3×3 mm^3 pieces. Alginate 2 ml cell suspension (0.5 g of wet cells) was mixed with 20 ml of 4% Na-alginate and the mixture was added dropwise to 0.02 M $Ca(NO_3)_2$, thus forming beads with the diameter of about 3 mm. After hardening for 1 h or 6 h in the solution, the beads were washed several times with water and used in the experiments. Instead of $CaCl_2$ a $Ca(NO_3)_2$ was chosen to prevent the falsification of the measurement of the chloride concentrations.

Determination of the dehalogenating activity of cells. The activity of the immobilizate was assayed by the incubation (293 K, 120 min) of 2 g immobilizate (wet weight) in 20 ml phosphate buffer (10 mM, pH = 7.6) containing 20 mM of 2,2-dichloropropionate as a substrate. After removing the immobilizate by centrifugation (3200 rpm, 15 min, temp. 277 K), silver(I)-chromate (0.03 g) was added to 2 ml of the solution. The chloride content was determined as described by Isaacs [27]. One unit is defined as the activity catalyzing the formation of 1 μmol chloride/min under the conditions described above. The activity of resting cells was assayed in the same way using 20 mg cells (dry weight) and 20 mM substrate in 2 ml of phosphate buffer (10 mM, pH = 7.6).

Measuring procedure. The contents of organochlorides in aqueous solutions was determined as follows: 0.3 ml of $NaNO_3$ solution was added to 20 ml of the sample. The chloride selective electrode and an Ag/AgCl reference electrode were immersed into the solution. The electrodes were interfaced with a pH ionometer. The potential of the electrodes was allowed to stabilize for approximately 2 min, and 1 g of cell immobilizate was added to start the conversion. When monitoring the formation of chloride ions, 5 min proved to be a sufficiently long period to obtain a significant decrease in the electrode potential. The decrease in potential was recorded and used for the calculation of results. All experiments were carried out at room temperature.

Effect of physical and chemical parameters. The effect of several physical and chemical parameters on the dehalogenating activity of *Pseudomonas sp.* MB58 strain was investigated using 20 mM 2,2-dichloropropionate as a substrate with the incubation period of 30 min.

Cells of the tested strain were incubated at temperatures ranging between 293 K and 343 K at pH 8.0. The effect of pH on the dehalogenating cellular activity was determined in the range of pH between 2.0 and 10.0, at 293 K. The inhibition of the dehalogenating activity by several heavy metal ions and organic substances was studied at 293 K and pH = 8.0. For these experiments various salts ($CuSO_4$, $BaSO_4$, HgCl, $PbCl_2$) at the concentrations of 0.1 mM or 1mM were added at the beginning of incubation. Organic compounds such as glucose, urea, sodium citrate and sodium acetate at the concentration of 1 mM were used.

3. Results and discussion

Fresh cells of *Pseudomonas* sp. MB58 harvested from the cultivation broth by centrifugation showed the specific activity of 4.8 U/g dry biomass using 2,2-chloropropionate as a substrate. Catalytic active layers were formed by entrapment of bacterial cells in agarose, carrageenan and alginate. For estimating the efficiency in immobilization, specific activities of small pieces/beads which can be applied directly to the ion selective electrode were determined. A high specific activity of the immobilizate was achieved by using alginate as a matrix, resulting in the specific activity of 57 mU/g (100%) immobilizate using 2,2-chloropropionate as a substrate. Only a slightly reduced activity was found in cells immobilized in agarose (96%) and carrageenan (93%). Investigations into long-term stability were performed by storing pieces/beads at 277 K for six weeks in a solution of 2% sodium phosphate (agarose), ammonium sulphate (carrageenan) or calcium nitrate (alginate). The decrease of about 5% in the dehalogenating activity of cells immobilized in alginate beads was observed. The carrageenan and agarose immobilizate lost over 40% of the dehalogenating activity after six weeks at 277 K. Therefore, immobilized cells of *Pseudomonas* sp. MB58 in alginate were used as a component of a bioassay with chloride selective electrodes as sensors.

In our experiments, the response of this kind of biosensor was analysed by the addition of 2,2-dichloropropionate (final concentration 20 mM) to ionically stabilized water samples. Typical response curves from our experiments are shown in Fig. 1.

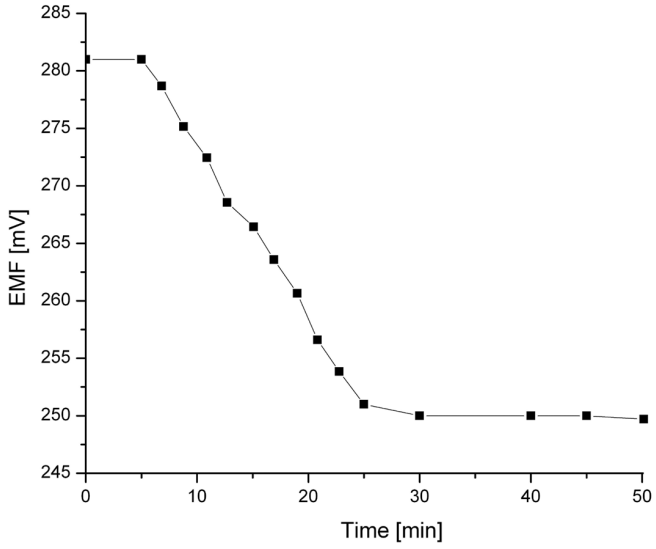

Fig. 1. Response of the microbial assay in a sample solution containing 2,2-dichloropropionate upon the addition of the chlorinated substrate at 0 min

After a dead time of 5 min, a first decrease in the electromotive force (EMF) was observed. A constant EMF was attained 30 min after sample application, thus repre-

senting the moment of attaining a steady state in the enzymatic conversion. In further experiments, the concentrations of 2,2-dichloropropionate and D-2-chloropropionate were varied with the aim to perform the calibration and to estimate the detection limit. The results are given in Fig. 2.

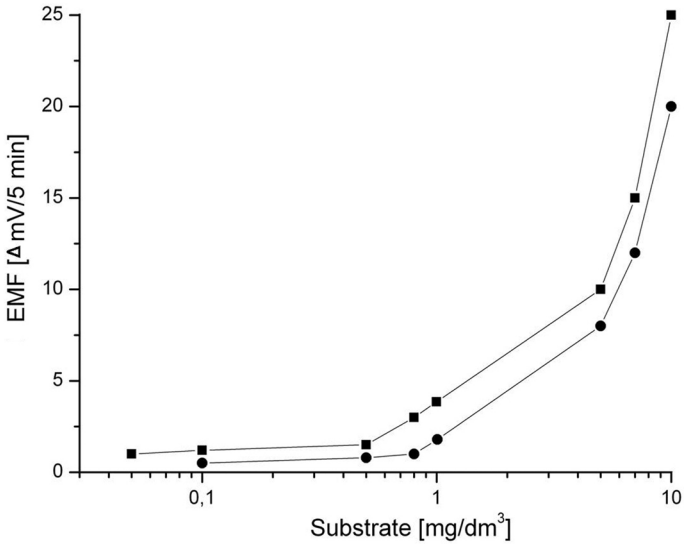

Fig. 2. Calibration graphs for the bioassay:
■ 2,2-dichloropropionic acid, ● D-2-chloropropionic acid

Table 1. Relative degradation of chlorinated compounds by immobilized cells of *Pseudomonas* sp. MB58

Substrate	Relative activity [%]
2,2-dichloropropionate	100
D-2-chloropropionate	148
L-2-chloropropionate	136
2-chlorobutyrate	95
chloroacetate	15
dichloroacetate	10
trichloroacetate	8
1-chlorobutane	0
1-chlorohexane	0

Non-linear calibration plots were obtained due to the non-linearity of the potential of a chloride electrode in the range close to the detection limit. The detection limits for 2,2-dichloropropionate and D-2-chloropropionate were 0.1 mg/dm^3 and 0.05 mg/dm^3, respectively. The relative standard deviation has been calculated at 6.8%.

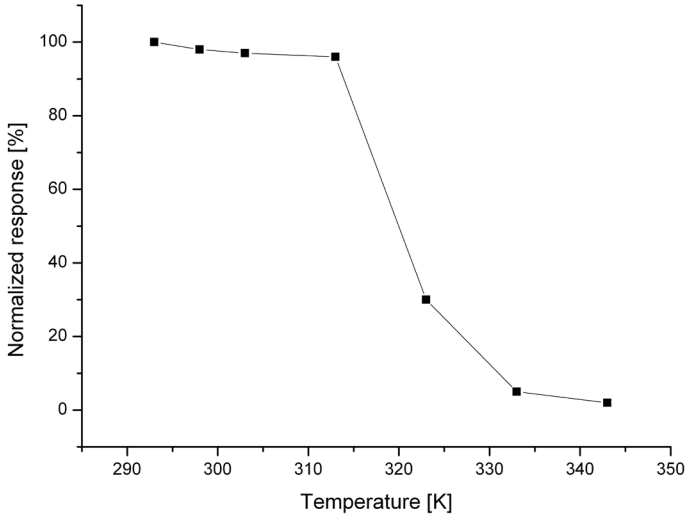

Fig. 3. Effect of temperature on dehalogenating cellular activity; the activities are expressed as percentages of the rate observed with 2,2-dichloropropionate at 293 K and pH = 8.0

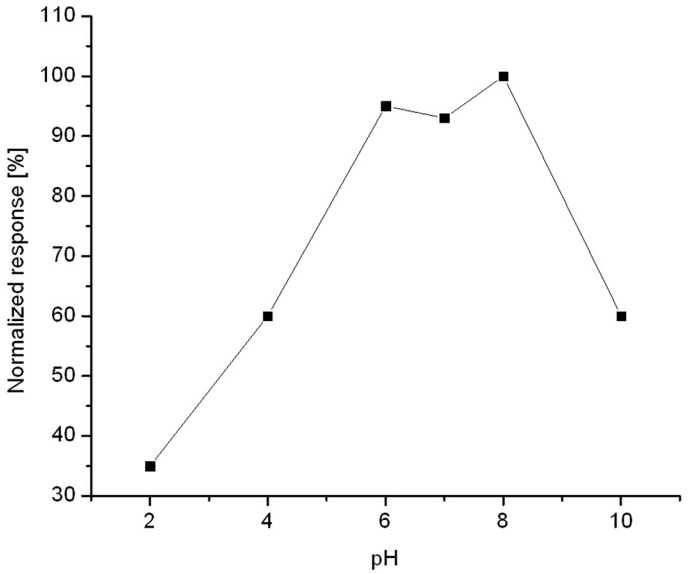

Fig. 4. Effect of pH on dehalogenating cellular activity; the activities are expressed as percentages of the rate observed with 2,2-dichloropropionate at pH = 8.0 and 293 K

The initial rates for the conversion of several chloroorganic compounds by immobilized cells were determined using 20 mM substrate. The measurements were carried out with alginate-immobilized cells. The results are given in Table 1, expressed relative to the conversion rate of 2,2-dichloropropionate. The data are averages of a triple

assay with the standard deviation of 3–5%. The activities are expressed as the percentage of the rate determined with 2,2-chloropropionic acid at 293K.

The studied enzyme acted towards both diastereoisomers D- and L-2-chloroalkanoic acids, however, an increased rate of dehalogenation of D-2-chloropropionic acid has been observed. Chloroacetate, di- and trichloroacetate were converted at a reduced rate. 1-chloroalkanes (1-chlorobutane and 1-chlorohexane) showed no detectable conversion. These results conform to data on substrate specificity obtained with the purified DL-2-halidohydrolase isolated from other bacterial strains [28].

The effect of several physical and chemical parameters on the dehalogenating activity of the studied MB58 strain was investigated using 20 mM 2,2-dichloropropionate as a substrate with the incubation period of 30 min. The results shown in Figs. 3 and 4 are mean values from a triple determination.

The temperature dependence of the dehalogenating activity of the studied MB58 strain has also been investigated in the temperature range 293–343 K (Fig. 3). There was no effect on dehalogenating activity at temperatures below 313 K, neither was there any significant deviation in the electric signal if measurements were performed within pH of 6.0–8.0 (Fig. 4). The influence of several heavy metals and organic compounds is summarized in Table 2.

Table 2. Inhibition of dehalogenating activity of *Pseudomonas* sp. MB58

Potential inhibitor	Concentration (mM)	Inhibition [%]
Glucose	1	0
Urea	1	4
Sodium citrate	1	0
Sodium acetate	1	5
Cu^{2+}	1	20
Ba^{2+}	1	45
Ba^{2+}	0.1	11
Hg^{2+}	1	89
Hg^{2+}	0.1	65
Pb^{2+}	1	74

Heavy metal ions inhibited the dehalogenating activity of the cells at concentrations of more than 0.1 mM. Several other substances like glucose, urea, citrate, acetate at the 1mM level caused only minor inhibition. These concentrations are far above the levels that can be expected to be present in water.

4. Conclusion

2-chloroalkanoic acids can be detected by the application of whole cell biosensors in a sensitive and inexpensive way. Cells of *Pseudomonas* sp. MB58 producing DL-2-haloacid dehalogenase can be easily immobilized as a very active catalytic layer. The intracellular dehalogenase of tested microorganism enables the dehalogenation of several 2-

chloroalkanoic acids. Ion selective electrodes for chloride are applicable as specific transducer element, thus forming a very compact biosensor for some chlorinated acids in water samples e.g. 2,2-dichloropropionic acid known as the herbicide DALAPON.

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