BIODEGRADATION OF 1,2-DIBROMOETHANE BY *BRADYRHIZOBIUM JAPONICUM 273* STRAIN BY FREE AND IMMOBILIZED CELLS STIMULATED BY CONSTANT ELECTRIC FIELD

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Abstract

Halogenated compounds are ubiquitous in the environment. This work examines the potential of Bradyrhizobium japonicum 273 strain for degrading 1,2-dibromoethane. This study compares biodegradation with free and immobilized cells onto a granular polymeric support. Processes of dehalogenation were carried out with different initial substrate concentrations in shaken flasks and in a laboratory bioreactor in presence and absence of constant electric field. There was significant positive effect of electric field on the biodegradation rate. Complete mineralization was verified by measuring stoichiometric release of the biodegradation products.

Keywords: *1,2-Dibromoethane (DBE), Bradyrhizobium japonicum 273 strain, immobilized cells onto a granular polymeric support, processes in presence and absence of constant electric field*

1. INTRODUCTION

The lead scavenger 1,2-Dibromoethane (DBE) is a common additive to leaded gasoline, which is used to form volatile dihalides with lead deposits and reduce engine fouling [1].DBE is still used in aviation gasoline (AvGas) and other fuels used for high performance applications, such as automobile racing [2].The current widespread presence of DBE in the subsurface is mainly from historic releases, such as past underground storage tank and pipeline leaks[3]. DBE is highly toxic, a probable carcinogen, and causes both acute and chronic health effects. Acute effects can include damage to the liver, stomach, and reproductive system, while chronic health effects include damage to the respiratory system, nervous system, liver, heart, and kidneys [4]. In the past, DBE has also been used in agriculture as an insecticide, pesticide, and soil fumigant for various crops The United States Environmental Protection Agency poses maximum contaminant level (MCL) for DBE, of 0.05 µg/L, which is the second lowest for all drinking water contaminants [5]. The Massachusetts Department of Environmental Protection has set an even more stringent MCL of 0.02 µg/L [6]. DBE is one of the most commonly detected contaminants in public drinking water systems reliant on groundwater [7]. DBE is especially problematic in groundwater systems due to its mobility and persistence under certain conditions. The physical properties of DBE, including a water solubility of 4300 mg/L [8] and a low gasoline-water partition coefficient, indicate that DBE can rapidly dissolve out of free-phase gasoline. The apparent persistence of DBE in soils and groundwater is studied by Hatzingera et al. 2018 [9]. This persistence likely has multiple causes including (1) limited bioavailability in soil matrices [10], (2) groundwater concentrations that may be below thresholds for microbial growth [11], [12], and (3) absence of co-metabolic substrates such as propane, pentane, and ammonia in most aerobic groundwater environments.

Analysis of microbiota present in the soil obtained from across the world indicates that organisms from the *Bradyrhizobium* genus are the most ubiquitous of all microorganisms. Playing a critical role in nitrogen fixation and soil fertility, organisms from his genus should be used for studying the toxicity of pollutants. Bacterial strain *Bradyrhizobium japonicum sp*. degrades DBE successfully. It has been widely studied because of its symbiotic association with legumes and its biological nitrogen fixation capacity [13]. Symbiotic rhizobacteria belonging to the genus *Bradyrhizobium* have been extensively studied from the perspective of their association with legumes and the biological nitrogen fixation process [14]. Some members of this genus have also shown capacity to colonize certain non-legumes, survive in their rhizosphere and promote their growth in a free-living plantemicrobe

interaction model [15]. These are considered plant growth-promoting rhizobacteria (PGPR) [16]. The plant growth-promoting activity was primarily correlated with nitrogen fixation but the presence of other stimulatory actions was also assumed. They include the effects on (a) phosphorus availability [17], (b),siderophore production [18], and (c) phytohormone metabolism. Haloalkane dehalogenases (DHAs) are very promising biocatalytic tools for the bioremediation of environmental pollutants which consists of haloalkanes. These dehalogenases catalyze the hydrolytic cleavage of carbon–halogen bonds in a broad range of environmental pollutants such as aliphatic mono-, di-, and polyhalogenated alkanes. In the study of Skopelitoua et al. 2013 [19], different *Rhizobium* strains (*Sinorhizobiummeliloti 1021, Rhizobium leguminosarum bv. trifolii, Mesorhizobium loti MAFF, Bradyrhizobium japonicumusda 110*) were screened for their ability to produce stable and active 1,2-Dibromoethane degrading dehalogenase. The results showed that *B. japonicum* produces the most potent dehalogenase and the most stable enzyme.

The main goal of this study is to investigate biodegradation of 1,2-dibromoethane with *Bradyrhizobium japonicum 273* cells. We compare an aerobic biodegradation in shaking flasks with various initial concentrations of substrate of 0,05, 0,1 and 0,3 g\l DBE. We analyzed also biodegradation with free and immobilized cells onto a granular polymeric support. Different processes of dehalogenation were carried out in a laboratory bioreactor in presence and absence of constant electric field and influence of electric field was investigated. Complete biodegradation was observed corresponding to the stoichiometric concentration of bromide ions in the medium.

2. MATERIALS AND METHODS

2.1. Bacterial strain, media and experimental conditions

Bradyrhizobium japonicum 273 was obtained from the National Bank for Industrial Microorganisms and Cell Cultures, Bulgaria (NBIMCC). The strain was grown in mineral medium (MMY) containing (per liter): 1 g of yeast extract, 0,2 g of NaCl, 0,2 g of $MgSO_4$, 0,5 g of K_2HPO_4, 10 g of Glucose, 1 g of tap water. The medium pH was adjusted to 7.2 before being autoclaved.

First experiments have been carried out in flasks of 250 ml in a rotary shaker at 28^0C and agitation speed of 100 rpm. In each flask the quantity of cultivated inoculums was 30 ml. Processes of dehalogenation have been carried out with different initial substrate concentrations of DBE of 0,05; 0,1 and 0,3 g/l.

The second group of experiments focused on biodegradation of DBE with free and immobilized cells onto a granular polymeric support. *Bradyrhizobium japonicum 273* produces haloalkane dehalogenase, which promotes the biodegradation halogenated aliphatic pollutants. In our study the inhibitory effect of substrate was reduced by immobilization. Processes of dehalogenation have been carried out in shaken flasks of 200 ml in a rotary shaker at 28^0C and agitation speed of 100 rpm. Experiments have been carried out with different initial substrate concentrations of DBE 0,1 and 0,3 g/l. Complete biodegradation was observed corresponding to stoichiometric concentration of bromide ions in the medium.

Immobilization has been carried out using the method described by Lalov et al., 2001 [20]. The supporting material was a copolymer of acrylonitrile and acrylamide formed as porous granules with an average diameter of 2 mm. The polymer granules were activated for 4 h, using a 12.5% solution of formaldehyde in 0.1M phosphate buffer at pH=7.5. The microbial cells were harvested from the inoculum culture by centrifuging at 5000 *x g* for 20 min, washed in phosphate buffer at pH=7.5, and re-suspended in the same buffer to achieve a biomass content of 10 mg/ml. Then the microbial suspension was mixed with the activated polymer beads for 20 min under careful stirring. Thereafter the granules were thoroughly washed with distilled water until no free cells were detected in the flush. The overall volume of the beads was 55 cm^3 with a specific area about 30 cm^{-1}. Processes of dehalogenation were carried out in shaken flasks.

The last group of experiments described the influence of the electric field on the degradation of -DBE. The experiments have been carried out under batch conditions in a 0,5 l Bioflo fermentor (New

Brunswick Scientific, Edison, NJ) at 30°C and agitation speed of 400 rpm. Processes of dehalogenation have been carried out with different initial substrate concentrations (0,05, 0,1 and 0,15 g/l) DBE in presence and absence of constant electric field. When applying electric field, one of the stainless steel baffles of the fermentor was used as a cathode (with surface of 13 cm^2). The cathode potential was maintained constant by a potentiostat at the value 0,1 V vs. quinhydrone electrode at 30 ^{0}C. At this temperature the operating voltage was + 0,6029 V.

The anode was attached to the system by an agar salt bridge, outside the fermentor, in order to avoid undesired anodic processes in the broth.

2.2. Analytical methods

Samples from the broth were taken periodically and analyzed for biomass, bromoethanol and bromide ions. The biomass growth was monitored by the optical density of the broth. The light absorption was measured at wavelength 600 nm using a spectrophotometer (VWR UV-1600 PC).

The quantity of bromoethanol was monitored by the optical density of the centrifuged broth at wavelength 216 nm using a spectrophotometer (VWR UV-1600 PC). The concentrations of bromoethanol were calculated from the optical density, using a calibration curve with correlation coefficient R^2=0,9901.

Bromides were analyzed by the colorimetric method of Bergman & Sanik, 1957 [21]. An aliquot of centrifuged sample (2.5 ml) was mixed with 1 ml of Fe (III) solution (8 g $(NH_4)Fe(SO_4).12H_2O$ in 100 ml of 6 M HNO_3) and 3 ml saturated solution of 1.5 g $Hg(SCN)_2$ in 500 ml of 98% ethanol. The mixture was shaken and after 10 min was filtered through 0.2 μm filter. The light absorption was measured at wavelength 460 nm using a spectrophotometer VWR UV-1600 PC [22]. The concentrations of bromide ions were calculated from the optical density, using a calibration curve with correlation coefficient R^2=0,9996.

3. RESULTS AND DISCUSSION

3.1. Biodegradation with flasks in a rotary shaker

The experimental results as time evolution of biomass, bromoethanol and bromide release are presented in Fig. 1-2

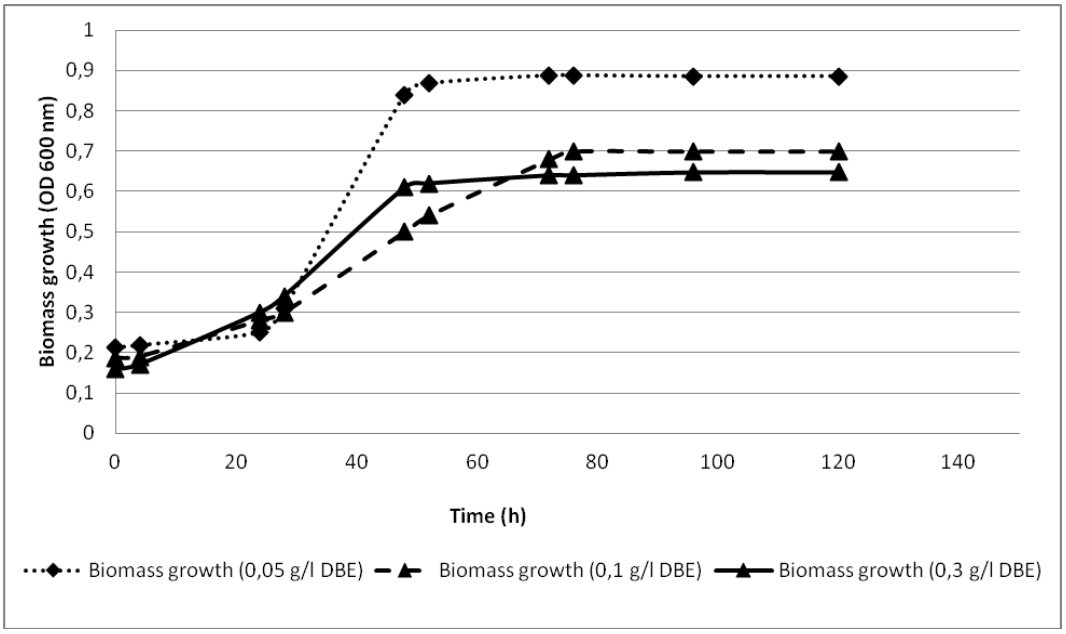

Fig. 1. Curves of biomass growth with 0,05 g/l, 0,1 g/l (dashed lines) and 0,3 g/l(solid line) initial DBE concentration

The curve of biomass growth with 0,05 g/l DBE concentration shows a clear lag-phase which continues to the 24th hour. After that fast biomass growth until 50th hour follows. It helps to accelerate biodegradation of substrate and reaches to the stationary phase of bacterial growth. The curve of biomass growth with 0,1 g/l DBE concentration shows a similar lag-phase. The exponential phase of bacterial growth continues until the 76th hour which is longer than in the previous experiment. The concentration of 0,3 g/l DBE was the highest one for our experiments. The curve of biomass growth is similar to the ones of the previous experiment. Biomass quantity increase to the 50th hour and after that a stationary phase of bacterial growth follows.

The curves for bromoethanol concentration and bromide profile at initial DBE concentration of 0,05, 0,1 and 0,3 g / l are presented in Fig. 2

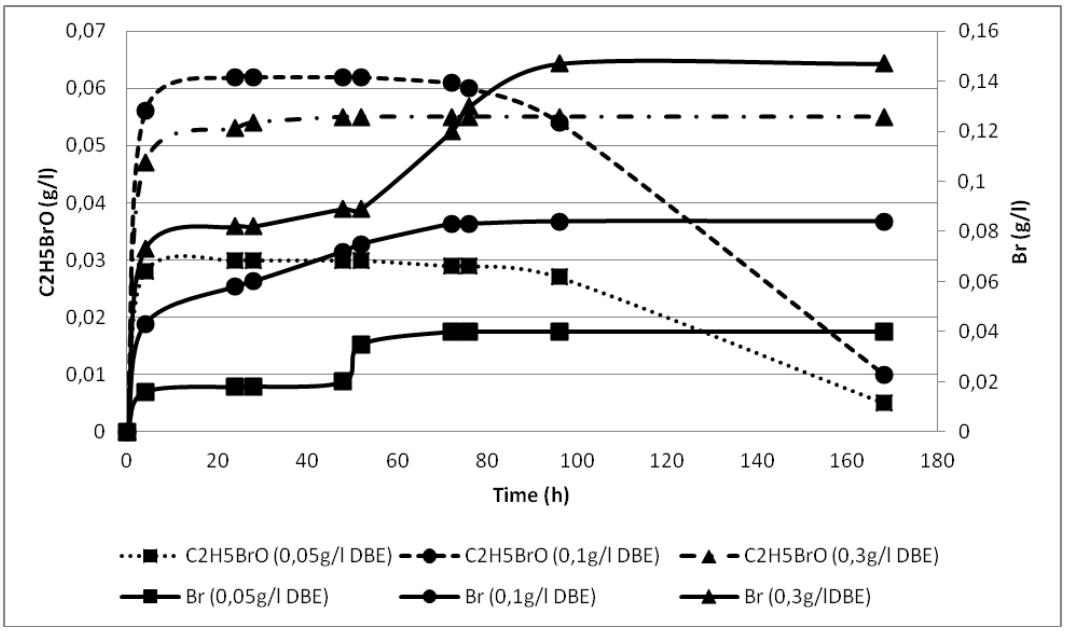

Fig. 2. Curves of bromoethanol concentration(dashed lines) and bromide profile(solid lines) with 0,05 g/l, 0,1 g/l and 0,3 g/l initial DBE concentration

The experimental results with 0,05 g/l initial DBE concentration are shown in Fig.2 Fast increase of bromoethanol quantity until reaching its stoichiometric concentration 0,033 g/l was observed. During a period from the start and the 50th hour only a half of bromide ions were released completely. After 50th hour a decrease of bromoethanol concentration with the corresponding increase of concentration of bromide ions was observed. At the 168th hour complete degradation of DBE was obtained with final accumulation of bromide ions with a concentration being comparable to the stoichiometric one (0,0425 g/l).

The experimental results with 0,1 g/l initial DBE concentration are shown in the same Fig.2 Similarly to the previous experiment, the results for bromoethanol and bromide ions concentration show that in the beginning fast increase of bromoethanol concentration took place. In this experiment the concentration of bromoethanol also reaches its stoichiometric value: 0,066 g/l. Dehalogenation is more active during the exponential phase of bacterial growth. After 76th hour decrease of bromoethanol concentration was observed until 168th hour, when it was completely removed. In contrast to the previous experiment bromide concentration increases slightly and after 76th hour reach stoichiometric concentration of 0,0851 g/l.

The experimental results with 0,3 g/l initial DBE concentration are presented in the same figure. This substrate concentration is already much higher. It is visible that until 50th hour stagnation in accumulation of bromoethanol and bromide ions is observed. Biodegradation was inhibited at this

initial substrate concentration. The concentration of bromoethanol is 0,055 g/l compared to the stoichiometric one 0,199 g/l. After 50th hour the process of bromide ions release continued, but partially only, as it was at the previous substrate concentration. The final concentration of bromide was 0,132 g/l which is approximately a half of stoichiometric 0,255 g/l and corresponds to complete release of only one bromine atom from the DBE molecule.

3.2. Biodegradation with free and immobilized cells

The experimental results of bromide release are presented in Fig. 3

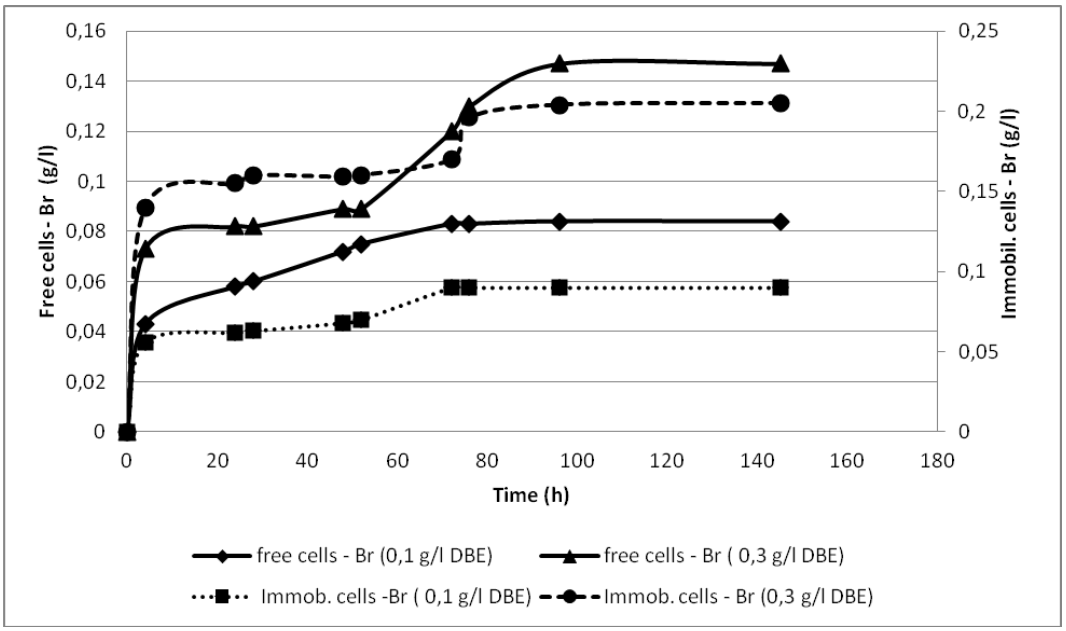

Fig. 3. Curves of bromide profiles with free (solid lines) and immobilized cells (dashed lines) with 0,1 g/l and 0,3 g/l initial DBE concentration

Slight bromide release was registered at initial DBE concentration of 0,1 g/l. After 72 hours bromide concentration reaches 0,08 g/l. This value, being comparable to stoichiometric one (0,085 g/l), is an evidence that the biodegradation of DBE was almost completed.

One plateau was observed in the bromide curve at initial DBE concentration of 0,3 g/l. During a period of 50 hours (from 50 th to 100 th) bromide concentration reaches 0,14 g/l. For this substrate concentration the stoichiometric bromide value is 0,255 g/l. This result shows that a half of the bromide ions were released completely, i.e. only one of the bromide atoms is released from the substrate molecule. This is might be due to accumulation of intermediates (e.g. bromoethanol and bromo-acetaldehyde) acting as inhibitors of substrate biodegradation.

The bromide profiles for experiments with cells immobilized onto a granular polymeric support are presented in the same figure. The results are different. At experiments with initial 0,1 g/l DBE concentration bromide release after 80 hours and the finally bromide concentration was 0,084 g/l. This value is practically the same as the stoichiometric one (0,085 g/l). The bromide profile for experiments with initial DBE concentration of 0,3 g/l have similar trend. A plateau is observed until 80 hours. Probably there are intermediate compounds in the broth for this period preventing the full bromine release. After 80th hour complete dehalogenation is observed. The bromide concentration reaches 0,205 g/l which is lower but comparable with the stoichiometric value 0,255 g/l.

Curves for bromoethanol concentration at the experiments with immobilized cells are shown in Fig.4.

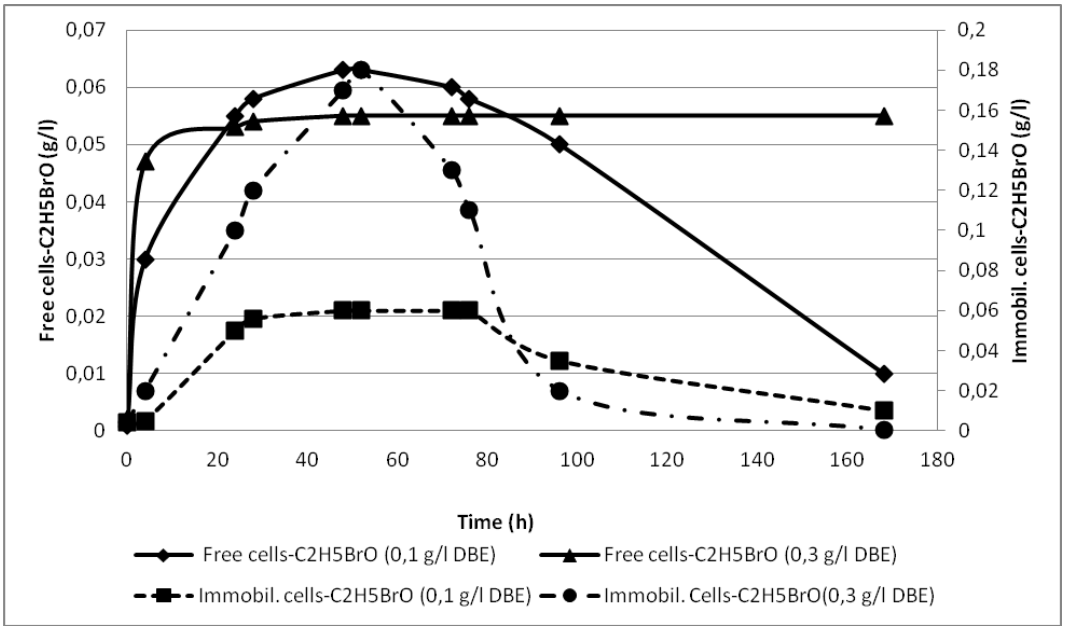

Fig. 4. Curves of bromoethanol concentration with free (solid lines) and immobilized cells (dached lines) with 0,1 g/l and 0,3 g/l initial DBE concentration

Experimental results with free cells with 0,1 g/l initial DBE concentration show slowly increase of bromoethanol quantity. During a period of 20th hours the concentration of bromoethanol reaches its stoichiometric value 0,066 g/l. After 80th hour decrease of bromoethanol concentration was observed until 168th hour, when it was completely removed. Experimental result with free cells with 0,3 g/l initial DBE concentration are different. At the beginning for 5 hours concentration of bromoethanol reaches only 0,05 g/l which is a half of stoichiometric value 0,199 g/l. Until the end of the process this concentration doesn`t change. Compared with bromide profile this result shows that a half of the bromide ions were released completely, i.e. only one of the bromide atoms is released from the substrate molecule. This is evidence that bromoethanol acted as inhibitors of substrate biodegradation.

Experimental results with immobilized cells with initial DBE concentration of 0,1 g/l show increase until 20th hour and decrease after 70th hour of bromoethanol quantity. For 50 hours (from 20th until 70th hour) the bromoethanol quantity is 0,06 g/l. This value is comparable to stoichiometric one (0,066 g/l). The concentration of bromoethanol decreased from 70th hour to 160th hour. Probably in the broth for this period there are intermediate compounds preventing the full bromine release. Experimental results with immobilized cells with initial DBE concentration of 0,3 g/l showed fast increase until 20th hour and fast decrease after 80th hour of bromoethanol quantity. At 50th hour the curve its own peak reaches. The value is 0,18 g/l compared to the stoichiometric value 0,199 g/l. After this value the curve decreases faster. At 160th hour complete degradation of bromoethanol is obtained with final accumulation of bromide ions.

The main conclusion from this experiment is that the immobilized cells are more persistent to the inhibition of substrate and bromoethanol even at the highest studied initial concentration of 1,2-DBE.

3.3. Biodegradation in the presence and absence of constant electric field

The study was focused on biodegradation at constant anode potential. The anode potential was maintained constant by a potentiostat of value 0,1 V vs. quinhydrone electrode.

For experiments with electric field was used the following experimental installation (Fig.5).

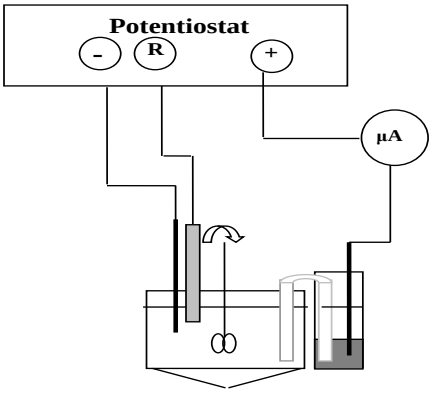

Fig. 5. Experimental installation with constant electric field

The experimental results as time evolution of bromoethanol and bromide release are presented in Fig 6-8.

Curves of bromoethanol concentration and bromide profiles with 0,05 g/l 1,2-DCA are shown in Fig.6.

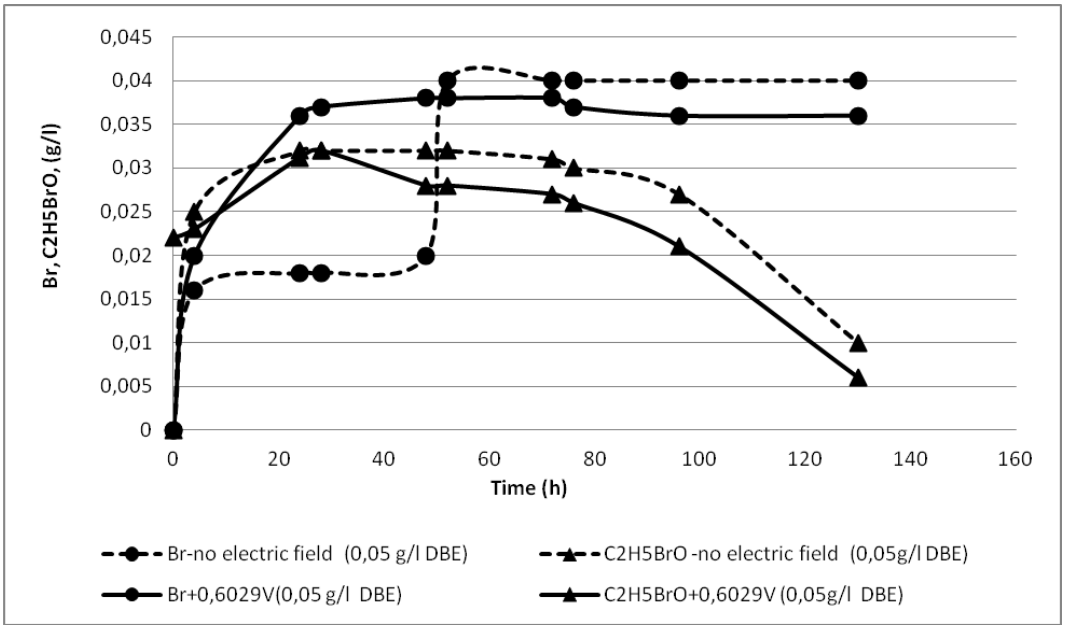

Fig. 6. Curves of bromoethanol concentration and bromide profiles with 0,05 g/l DBE in presence (solid lines) and absence of electric field (dashed lines)

In the absence of electric field, at the 5th hour concentration of bromoethanol reaches 0,025 g/l. After that at 60th hocurs (for 20th to 80th) the concentration is 0,03, This value is practically the same as the stoichiometric one (0,033 g/l). After 80th hour fast decreases begin and at 130th hour full biodegradation was observed. In the presence of electric field (0,1 V/QHE) the curve is similar. At this substrate concentration of DBE at 30 hour measured value is 0,03 g/l and at 130 hour full degradation was achieved.

In this figure at this substrate concentration of DBE, bromide profiles in the absence and in the presence of electric field also was presented. In the absence of electric field one plateau was observed. During a period of 45 hours (from 5 th to 50 th) bromide concentration did not change. At the same time the concentration of bromoethanol is the highest. After 50th hour complete degradation of DBE is

obtained with final accumulation of bromide ions with a concentration being comparable to the stoichiometric one (0,0425 g/l). In the presence of electric field there is a slowly increase and after 30th hour bromide concentration is 0,04 g/l. Constant electric field accelerates biodegradation. This results shown that in this concentration of DBE there was significant positive effect of electric field on the biodegradation rate. At 130th hour complete degradation of DBE is obtained with final accumulation of bromide ions

Curves of bromoethanol concentration and bromide profiles with 0,1 g/l 1,2-DCA are shown in Fig.7

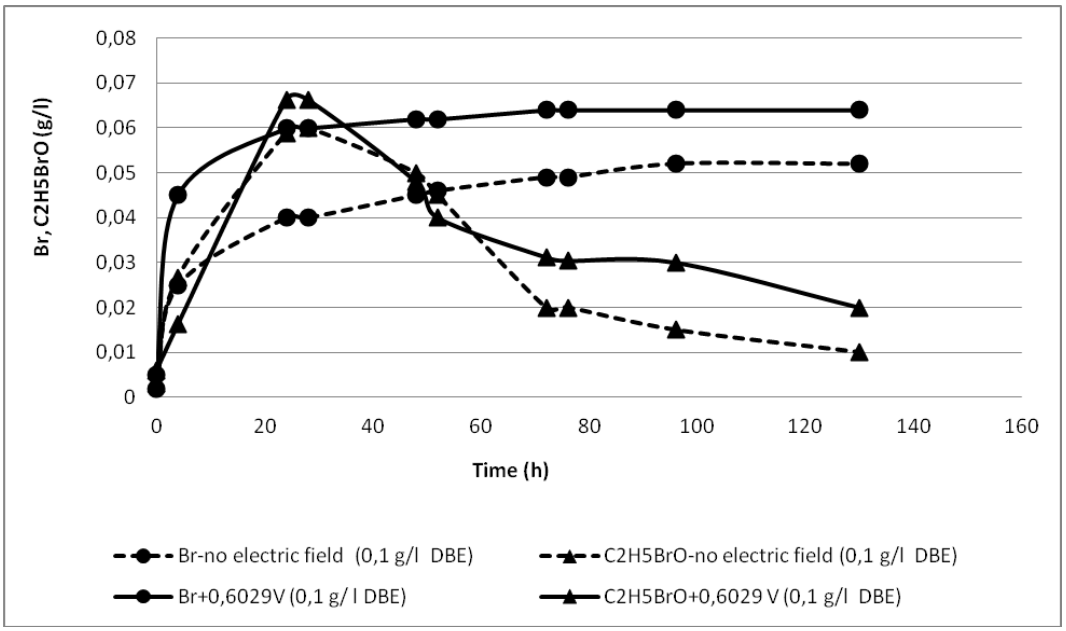

Fig. 7. Curves of bromoethanol concentration and bromide profiles with 0,1 g/l DBE in presence (solid lines) and absence of electric field (dashed lines)

With 0,1 g/l DBE curves of bromoethanol concentration in the absence and in the presence of const electric field have similar trend. At 30th hour concentration is the highest and reaches 0,06 g/l. This value corresponding to stoichiometric one 0,066 g/l.

It is visible that both curves of bromide profiles have similar trend but they are removed over time. In the absence of electric field the maximum value is 0,05 g/l. In the presence of electric field it is 0,065g/l.

Curves of bromoethanol concentration and bromide profiles with 0,15 g/l DBE are shown in Fig.8.

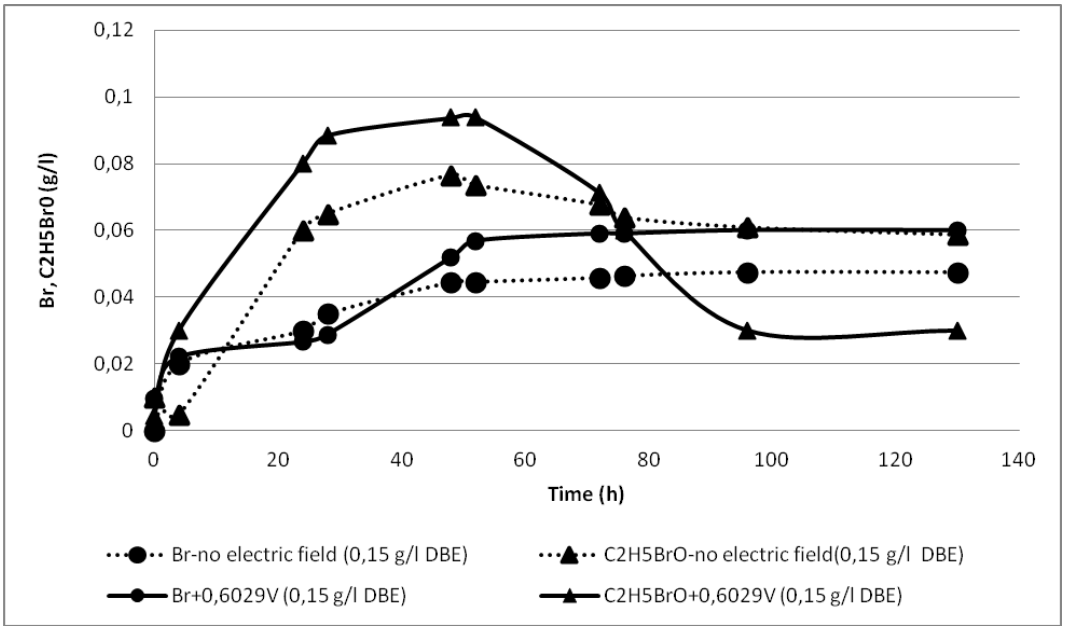

Fig. 8. Curves of bromoethanol concentration and bromide profiles with 0,15 g/l DBE in presence (solid lines) and absence of electric field (dashed lines)

With 0,15 g/l initial substrate concentration of DBE, curve of bromoethanol concentration (without electric field) increases slightly until 50th hour and reaches value of 0,08 g/l. After 80th hour biodegradation was impeded and the bromoethanol concentration was 0,06 g/l. It is lower than the stoichiometric value 0,099 g/l. Curve of bromoethanol concentration (with electric field) has the same trend. At the beginning until 25th hour the curves reach 0,09 g/l and after 80 hour decrease slowly to 130th hour. The quantity of bromoethanol reaches stoichiometric value and decrease up to 0,03 g/l.

On the same figure curves of bromide profiles are presented. At the beginning until 25 th hour the concentration in the both graphs is similar (0,03 g/l). Curve of experiment without electric field supports constant trend until the end of the process. In the absence of electric field the maximal quantity of bromide ions was 0,045 g/l. In the presence of electric field the final amount was 0, 06 g/l. The final amount of bromides (0,045 g/l and 0,06 g/l) (without and with electric field respectively) was much lower than the stoichiometric value 0,1276 g/l. Even in the case of electric field stimulation only one of the bromide atoms is released. These results might be due to accumulation of intermediates (e.g. 2-bromoethanol and bromoacetaldehyde) acting as inhibitors of growth and substrate biodegradation.

CONCLUSIONS

On the basis of the presented experimental results the following conclusions can be drawn:

1. Bacterial strain *Bradyrhizobium japonicum 273* biodegrades successfully 1,2-dibromoethane at low initial substrate concentrations up to 0,1 g/l in flasks in a rotary shaker.
2. At this concentration full dehalogenation with free and immobilized cells is observed too.
3. Using free cells at high initial substrate concentrations of 0,3 g/l intermediate bromoethanol inhibits dehalogenation. In these cases bromoethanol is not fully converted, and the process doesn't reach full degradation of DBE.
4. When no electric field was applied the biodegradation was impeded. It stopped before one bromide atom in the substrate molecule was released completely.

5. The constant electric field leads to complete degradation and the concentration of bromide ions in the medium reaches stoichiometric values.
6. Full biodegradation with immobilization onto a granular polymeric support was successful. Biodegradation with immobilized cells was complete at higher substrate concentration of 0,3 g/l DBE. Concentration of bromide ions in the medium almost reaches stoichiometric values.

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