International Biodeterioration & Biodegradation 91 (2014) 29–36

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Impact of selected anions and metals on the growth and in vitro removal of methylmercury by *Pseudomonas putida* V1

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ARTICLE INFO

Article history:
Received 21 August 2012
Received in revised form
15 December 2013
Accepted 7 January 2014
Available online 1 April 2014

Keywords:
Plant nutrient
NaCl
Heavy metals
Synergistic effect
Bioremediation

ABSTRACT

The aim of this study was to evaluate the influence of plant nutrients (nitrate, phosphate, sulfate and molybdate), NaCl and heavy metals (lead, copper, chromium and nickel) on the growth and methylmercury removal by *Pseudomonas putida* V1 in vitro. The growth and methylmercury removal capacity of *P. putida* V1 were evaluated in Luria Bertani (LB) broth containing different doses of plant nutrients (50–300 mmol L^{-1}); NaCl (0.5–20%) and heavy metals (100 and 600 μmol L^{-1}). The analyses of the residual mercury were carried out in an atomic absorption/cold steam spectrophotometer. The results indicated a significant decline in the methylmercury removal from LB broth ($p < 0.05$) in the presence of the plant nutrients concentrations tested, however the growth of this bacteria was affected only in few conditions. *P. putida* V1 was capable of removing methylmercury in the presence of 10% NaCl. The concentrations of lead or copper tested and the addition of 2.3 μmol L^{-1} of methylmercury was highly inhibitory to growth of *P. putida* V1. In presence of 0.008 μmol L^{-1} of methylmercury and lead, this bacteria was able to remove 89% of methylmercury, whereas in the presence of copper it only removed between 45% and 12%. The resistance of *P. putida* V1 to NaCl and to some metals suggests the potential use of this microorganism in bioremediation processes of areas contaminated with these substances.

1. Introduction

The enrichment with salts and presence of heavy metals can interfere in the activity of the microbial flora present in ecosystems. Such contamination represents a great environmental problem in various parts of the world. The enrichment with nutrients is described as one of the main causes of eutrophisation (degradation of bodies and water reservoirs causing an excessive increase in algae, which limits biological activity). On the other hand, enrichment with salts can result, for example in the case of soils, in great degradation phenomena, such as dispersion, structural loss and reduction in agricultural productivity. Both, the enrichment with salt and nutrient affect the activity of the soil microorganisms (FAO, 1988; Metternicht and Zinck, 2003; Mavi et al., 2012).

Fertilizers are nutrient sources used for plant growth, but excessive application can lead to pollution not only of the soil but also of the water (Ray, 2001; Bustamante et al., 2011; Kros et al., 2011). The principal nutrients used in agriculture are phosphate, nitrate, sulfate and molybdate. The presence of phosphate and nitrate is frequently associated with environmental problems in lakes and streams, since they cause the eutrophisation of water bodies (Zhang et al., 2003). Sulfate and molybdate can be soluble in soils under both normal and inundated conditions, and can thus percolate to the water bodies (Kaiser et al., 2005; Bustamante et al., 2011).

The contamination of soils and water bodies by heavy metals constitutes another great environmental challenge. These contaminants originate from sewage sludge, industrial, animal residues and from the application of fertilizers that usually contain small amounts of these substances (Kabata-Pendias and Pendias, 2001; Li et al., 2008; Bertol et al., 2010). Heavy metals can present toxicity leading to the degradation and loss of the biological

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http://dx.doi.org/10.1016/j.ibiod.2014.01.021
0964-8305/

diversity of the ecosystems, especially due to their deleterious effect on microorganisms, plants, animals and man. In most cases, soil contamination by metals is associated with more than one element (Klauberg Filho et al., 2005), but interactions between metal contaminants and microorganisms are frequent and intense in the soil (Mcbride, 1994).

Mercury has been introduced into the environment as a result of industrial activity, especially petroleum extraction and coal mining. Among the various forms of mercury, methylmercury is the greatest cause of concern, since it is highly toxic and easily accumulates in live organisms. Methylmercury is mainly formed in lakes or anaerobic sediments by the action of sulfate-reducing bacteria, recognized as the principal methylators found in these locations (Compeau and Bartha, 1985; Gilmour and Henry, 1991). The most studied mechanism of resistance to this metal is that of enzymatic reduction, related to the *mer* operon system, which is governed by two main enzymes coded by the genes *mer*B and *mer*A, namely organomercurial lyase and mercury reductase, respectively (Osborn et al., 1997; Barkay et al., 2003; Wang et al., 2011). However, mercury methylation by an abiotic process can be indirectly related to biological activity in soil, due to the presence of dissolved organic matter (Weber, 1993; Barkay and Poulain, 2007). Due to this fact, few studies have reported the isolation of methylmercury-resistant soil bacteria (Olson et al., 1991). To the best of the authors' knowledge, there are no studies on the influence of plant nutrients and salts used in agriculture and of heavy metals on the methylmercury degradation potential by bacteria with potential for use in bioremediation. However, this information constitutes a primordial step in the bioprospecting of bacteria with potential for application in bioremediation processes.

Given the above, the aim of this study was to determine the influence of different concentrations of plant nutrients (nitrate, phosphate, sulfate and molybdate), heavy metals (lead, copper, chromium and nickel) and of salt (NaCl) on the growth and methylmercury removal capacity by *Pseudomonas putida* V1 under in vitro conditions.

2. Material and methods

2.1. Microorganism and preparation of cell suspension

A methylmercury-resistant strain of *Pseudomonas* (*P. putida* V1), isolated from a landfarming soil sample from the petrochemical pole in Triunfo, RS, Brazil, was used in this study (Cabral et al., 2013). The pre-inoculum was prepared from a loopful of *P. putida* V1 inoculated into Luria Bertani (LB) broth containing 2.3 μmol L^{-1} of methylmercury (CH_3Hg^+), followed by incubation at 29 °C for 24 h with shaking at 100 rpm. This concentration of methylmercury was determined as stated in Cabral et al. (2013). An aliquot was removed after 24 h and added to LB broth so as to obtain a reading of 0.3 units $OD_{600\ nm}$ (approx. 10^7 CFU/mL) using a Spectronic-20, GENESYSTM spectrophotometer (Spectronic Analytical Instruments, Rochester, NY, USA).

2.1.1. Methylmercury concentration

The concentration was chosen based on preliminary experiments (Cabral et al., 2013). In that study, *P. putida* V1 exhibiting the highest removal percentage of methylmercury was inoculated in LB medium containing different concentrations of methylmercury: low concentration (2.3 μmol L^{-1}), minimal inhibitory concentration (11.5 μmol L^{-1}) and high concentration (12 μmol L^{-1}). When *P. putida* was inoculated in the culture medium containing 2.3 μmol L^{-1} methylmercury, its growth was very similar to when no methylmercury was present. Because of this, the methylmercury concentration of 2.3 μmol L^{-1} was used in the experiments.

2.2. Effect of the presence of plant nutrients (molybdate, phosphate, nitrate and sulfate) and NaCl on the in vitro methylmercury removal capacity by P. putida V1

The effects of the presence of molybdate (MoO_4^{2-} – $Na_2MoO_4 \times 2H_2O$), phosphate (PO_4^{3-} – K_2HPO_4), nitrate (NO_3^- – KNO_3) and sulfate (SO_4^{2-} – K_2SO_{4-}) on the methylmercury removal capacity of *P. putida* V1 were determined using LB broth with the following concentrations of each nutrient: 50, 100, 200 and 300 mmol L^{-1}. These concentrations were based on cell inhibition studies using molybdate and nitrate in concentrations varying from 5 to 75 mmol L^{-1}, carried out by Rincón et al. (2008). Since it was observed that *P. putida* V1 could grow in a culture medium containing this range of compounds, the concentrations used in the present study were raised, reaching 50, 100, 200 and 300 mmol L^{-1}. The methylmercury removal capacity of *P. putida* V1 was also evaluated in a mixture of the plant nutrients (molybdate, phosphate, nitrate and sulfate) at 1, 1/2 and 1/3 of the maximum concentration (300 mmol L^{-1}) for each of the 4 plant nutrients, that is, total plant nutrients concentrations of 1200 mmol L^{-1}, 600 mmol L^{-1} and 400 mmol L^{-1}. These concentrations were based on previous experiments and considering that the entire dose of the mixture of plant nutrients (molybdate, phosphate, nitrate and sulfate), with a total of 1200 mmol L^{-1}, could be inhibitory.

The effect of the NaCl content on the methylmercury removal capacity of *P. putida* V1 was evaluated using LB with the addition of various concentrations of NaCl (0.5%, 1%, 3%, 5%, 10% and 20%). These concentrations were chosen based on the study of Nakamura et al. (1999), but due to ability of *P. putida* V1 to grow in a culture medium containing up to 15% NaCl, higher concentrations were used in the current study. Non-inoculated LB broth containing different concentrations of plant nutrients or salts and metals constituted the negative controls. LB broth inoculated with *P. putida V1* and methylmercury (2.3 μmol L^{-1} of CH_3Hg^+), but with no plant nutrients or salts was used as positive control. The residual mercury was quantified by the generation of cold steam as described in Section 2.4.

2.3. The effect of the presence of heavy metals (copper, nickel, chromium and lead) on the growth and in vitro methylmercury removal by P. putida V1

A suspension of cells prepared as described in item 2.1 was used to assess the growth of *P. putida* V1 in the presence of copper ($CuCl_2 \times 2H_2O$), nickel ($NiCl_2 \times 6H_2O$), chromium (CrK_2O_4) and lead ($Pb[C_2H_3O_3]_2 \times 3H_2O$) after 48 h of incubation at 29 °C. After inoculation of LB broth, each metal was added to reach the following final concentrations: 100, 300 and 600 μmol L^{-1}. These concentrations were chosen based on preliminary experiments (Cabral et al., 2013) that showed the minimal inhibitory concentration (MIC) of *P putida* in LB medium against copper, lead, nickel, and chromium were 1000 μM, respectively. The concentration of methylmercury (2.3 μmol L^{-1}) was determined as stated in Cabral et al. (2013). As the aim of this step of the study was to evaluate the growth of *P. putida* V1 in LB, with and without methylmercury (2.3 μmol L^{-1}), concentrations above 600 μmol L^{-1} were not used because they were toxic this bacterium. In order to evaluate the combined influence of metals (copper, lead, nickel and chromium) and methylmercury on the growth of *P. putida* V1, 2.3 μmol L^{-1} of methylmercury was added to the LB medium. All the tubes were incubated at 29 °C, and cell growth was determined from the optical density ($OD_{600\ nm}$) in a Spectronic-20, GENESYSTM spectrophotometer (Spectronic Analytical Instruments, Rochester, NY, USA). No-inoculated LB containing different concentrations of heavy metals constituted the negative controls. LB broth inoculated