Rhizosphere-induced Selenium Precipitation for Possible Applications in Phytoremediation of Se Polluted Effluents

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Two bacterial isolates were obtained in axenic culture from the rhizosphere soil of *Astragalus bisulcatus*, a legume able to hyperaccumulate selenium. Both strains resulted of particular interest for their high resistance to the toxic oxyanion SeO_3^{2-} (selenite, Se^{IV}). On the basis of molecular and biochemical analyses, these two isolates were attributed to the species *Bacillus mycoides* and *Stenotrophomonas maltophilia*, respectively. Their capability in axenic culture to precipitate the soluble, bioavailable and highly toxic selenium form selenite to insoluble and relatively non-toxic Se^0 (elemental selenium) was evaluated in defined medium added with 0.2 or 0.5 mM Se^{IV}. Both strains showed to completely reduce 0.2 mM selenite in 120 h, while 0.5 mM Se^{IV} was reduced up to 67% of the initial concentration by *B. mycoides* and to about 50% by *S. maltophilia* in 48 h. Together in a dual consortium, *B. mycoides* and *S. maltophilia* increased the kinetics of selenite reduction, thus improving the efficiency of the process. A model system for selenium rhizofiltration based on plant-rhizobacteria interactions has been proposed.

Key words: Bacterial Reduction, Selenite, Wastewater Rhizofiltration

Introduction

Selenium can be considered as an essential trace element for many living organisms from bacteria to mammals (Shamberger, 1983). Nevertheless, the behaviour of selenium with particular respect to the health of humans and animals is dichotomous. While, as an essential trace nutrient, selenium has a recommended dietary requirement of 70 μg per day, conversely, at intake doses above 350 μg per day, it starts to exert toxic (Rayman, 2000) or even mutagenic effects (Shamberger, 1985).

In oxic environments, selenium is predominantly occurring as Se^{IV} in selenites (SeO_3^{2-}) and as Se^{VI} in selenates (SeO_4^{2-}). These inorganic oxidized forms can be found in high concentrations in some habitats as a consequence of agricultural practices or industrial discharges (Losi and Frankenberger, 1997). Severe Se pollution in agriculture is restricted to seleniferous soils where repeated irrigation leads to the accumulation of this metalloid into the drainage water, eventually entering the food chain. This phenomenon is apparently widespread in the western United States (Weres *et al.*, 1989; Stephens and Waddell, 1998). However, cases of elevated Se concentrations in soils have been reported in several countries world-wide, including Australia, Canada, China, Columbia, India, Ireland, Israel and Mexico (Dhillon and Dhillon, 2001). On the other hand, selenium-laden effluents and wastes are principally associated to oil refining and mining activities, to the production of pigments, metallurgical additives and pharmaceutical preparates or to the use in electronics and glass manufacturing (Bocovay, 1995).

The toxicity of selenium is related to its chemical form. Although the oxyanions selenite and selenate are both soluble and bioavailable, selenium in the form of Se^{IV} is more toxic to most organisms than selenium in the form of Se^{VI}. Contrarily, elemental selenium (Se^0) is insoluble; that is, it can not be absorbed by the biological systems (Barceloux, 1999)

Selenium undergoes various redox reactions within its biogeochemical cycle and microbes play a pivotal role in this context. A number of bacteria have been described as specifically able to reduce Se oxyanions to elemental selenium. *Pseudomonas fluorescens* K27 is a selenium-resistant, facultative anaerobe, that reduces selenite and selenate to Se^0 and volatile organo-selenium methylated com-

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pounds in microaerophilic/anaerobic conditions (Zhang and Chasteen, 1994). Also *Escherichia coli* can transform both oxyanions to Se^0 (Turner *et al.*, 1998). Selenate-reducing bacteria were isolated from extreme environments such as hypersaline ponds (de Souza *et al.*, 2001). On the other hand, certain species, namely *Rhodospirillum rubrum* (Kessi *et al.*, 1999) and *Ralstonia metallidurans* (Roux *et al.*, 2001), have been shown to reduce only selenite. *Rhodobacter sphaeroides*, which reduces mostly selenite, can reduce SeO_4^{2-} to some extent (Van Fleet-Stalder *et al.*, 2000). Selenite is also respired anaerobically by other bacterial species including *Bacillus arsenicoselenatis*, *B. selenitireducens* (Switzer-Blum *et al.*, 1998), *Sulfurospirillum barnesii* (Stolz *et al.*, 1999), and *Thauera selenatis* (Macy *et al.*, 1993), while reduction of selenite in aerobic conditions has been reported in several strains of *Pseudoalteromonas* sp. (Rathgeber *et al.*, 2002) as well as in *Stenotrophomonas maltophilia* (Di Gregorio *et al.*, 2005). This latter was found able to reduce aerobically even selenate (Dungan *et al.*, 2003).

Microbial reduction of bioavailable selenium oxyanions into elemental selenium or to relatively non-toxic gaseous forms is of great interest for bioremediation, expecially for the treatment of Se-laden effluents and industrial outlets. At present, physico-chemical technologies such as chemical reduction and precipitation, adsorption, ion exchange, and membrane processes are mainly applied for remediating wastewater (Twidwell *et al.*, 2000). However, these methods are commonly very costly. Thus, an interesting alternative for a cost-effective abatement of Se oxyanions may be represented by biological treatments relying on the exploitation of either microbes or plants capable to reduce, volatilise or accumulate toxic selenium forms (Cantafio *et al.*,1996; Fujita *et al.*, 2002; Azaizeh *et al.*, 2003).

In the present work, two bacterial isolates from the rhizosphere of the Se hyperaccumulator legume *Astragalus bisulcatus* have been compared for their efficiency in reducing selenite to red amorphous metallic selenium (Se^0) *in vitro*. The main objective of this study was to test such a dual consortium of bacteria in terms of both Se tolerance and SeO_3^{2-} reduction rate in the perspective of possible application in hydroponic rhizofiltration reactors with bacteria entrapped in the root system of *A. bisulcatus*.

Materials and Methods

Chemicals

Chemicals purchased from Sigma-Aldrich were all analytical grade.

Culture media

Nutrient Broth (rich medium) and Bacteriological Agar were provided by Oxoid. Defined medium (DM) was prepared according to Di Gregorio *et al.* (2005), with minor modifications (addition of 0.1% glucose wt/vol. and 0.1% yeast extract wt/vol.).

Isolation of bacterial strains

Enrichment cultures for bacterial isolation were inoculated with rhizosphere soil from plants of *Astragalus bisulcatus* grown for six months in greenhouse conditions at 21 ± 1 °C on seleniferous soil collected at a mine site in Sardinia, Italy (Campostrini *et al.*, 1999). *A. bisulcatus* seeds were obtained from the Western Regional PI Station, Washington State University, Pullman, USA. Enrichment cultures were carried out in 250 ml Erlenmeyer flasks containing 100 ml Nutrient Broth added with 0.2 mM Se^{IV} sodium salt. Flasks were incubated in the dark at 28 °C on an orbital shaker (250 rev/min). After one week of incubation, serial dilutions of the culture medium were plated on agarised Nutrient Broth containing 0.2 mM Se^{IV} sodium salt. Plates were incubated in the dark at 28 °C for 5 d. Appearance of red colored colonies was interpreted as an indication for selenite reduction to elemental selenium (Se^0) (Sabaty *et al.*, 2001). Red colonies were isolated and streaked on fresh agarised Nutrient Broth plates containing Se^{IV} sodium salt. Pure cultures of three morphologically different bacteria were obtained. Minimum inhibitory concentration (MIC) for Se^{IV} was determined on agarised plates prepared with either DM or Nutrient Broth containing increasing concentrations of selenite. Microbial cells were checked for viability after 5 d of incubation. The strains, successively referred to as SeITE01 and SeITE02, revealed the highest value of MIC for Se^{IV} and were further characterized.

Phylogenetic analysis of SeITE01 and SeITE02 strains

Bacterial genomic DNAs of strains SeITE01 and SeITE02 were extracted with the NucleoSpin Tis-

sue Kit (Clontech) following the manufacturer's instructions. Amplification of the genes encoding for the 16S rRNAs (rDNAs) was performed with the primers F8 and R11 (Weisburg *et al.*, 1991).

The products of amplification were directly double-strand sequenced, aligned to the database sequences using BLASTN (Altshul *et al.*, 1997), and analysed by means of ARB (Strunk and Ludwig, 1993–2002).

Se^{IV} reducing activity induction

Stationary phase bacterial cultures grown in presence of 0.2 mM Se^{IV} sodium salt were used to inoculate either 100 ml DM or 100 ml DM added with, respectively, 0.2 and 0.5 mM Se^{IV} sodium salt (initial turbidity: 0.01).

Microbial growth

Microbial growth was evaluated in DM amended with 0.2 or 0.5 mM Se^{IV} by quantifying the turbidity of cell suspensions with a Helios β spectrophotometer, Unicam. All analyses were performed in triplicate. Microbial growth in presence of Se^{IV} was compared to control cultures incubated in DM added with no Se^{IV} sodium salt.

Se^{IV} content determination

Se^{IV} concentration in cultures amended with 0.2 or 0.5 mM Se^{IV} was determined by reading absorbance at 377 nm of the selenium-2,3-diaminonaphthalene complex in cyclohexane as described by Kessi *et al.* (1999). Sterile cultures were also tested for Se^{IV} concentration as negative controls.

Results

Three different bacterial strains (SeITE01, SeITE02 and SeITE03) were isolated from the rhizosphere of the selenium hyperaccumulator legume *Astragalus bisulcatus* (Neuhierl and Boek, 1996). Values of MIC for Se^{IV} were determined. The strains SeITE01 and SeITE02, showing the highest resistance to Se^{IV} (up to 15 and 50 mM, respectively), were further characterized. The corresponding 16S rRNA genes were amplified by PCR. The 1.5 kb fragments obtained were double-strand sequenced and successively analyzed by using BLASTN (Altshul *et al.*, 1997) and ARB (Strunk and Ludwig, 1993–2002). Strain SeITE02 was previously characterized (Di Gregorio *et al.*, 2005) and recognized as belonging to the genus *Stenotrophomonas*, species *maltophilia* (99% identity with *Stenotrophomonas maltophilia* VUN10-075, AF100734). On the other hand, strain SeITE01 resulted very close to the *Bacillus cereus* group (99% identity with *Bacillus cereus* G9667). Nevertheless, further biochemical characterization suggested the possible attribution of SeITE01 to the species *B. mycoides* (Table I). Both strains formed

Table I. Morphological, biochemical, and growth characteristics of *Bacillus* sp. strain SeITE01 isolated from the rhizosphere of the selenium hyperaccumulator *Astragalus bisulcatus.*

Cell morphology (size [μm])	Short, ovoid rods (0.7×1.5)
Colour and aspect of colonies:	
without Se^{IV} addition	Opaque, creamy, rhizoid growth
with Se^{IV} addition	Bright red, rhizoid growth
Fluorescent pigment	–
Growth at 4 °C	–
Growth at 18 °C	–
Growth at 28 °C	+
Growth at 37 °C	+
Growth at 42 °C	–
Growth at pH 4.5	–
Growth at pH 7.2	+
Growth at pH 9	–
Oxidase activity	–
Catalase activity	+
Lipase activity	+
Urease activity	–
Methyl red test	–
V/P test	+
Nitrate reduction	+
Utilisation of:	
Acetate	–
Caprylate	–
Citrate	–
Gluconate	+
Malate	+
Oxalate	–
Succinate	+
Arabinose	–
Fructose	+
Galactose	+
Glucose	+
Lactose	+
Maltose	+
Mannitol	+
Mannose	+
N-Acetyl-glucosamine	–
Raffinose	+
Rhamnose	+
Saccharose	+
D-Xylose	+
Hydrolysis of:	
Starch	+
Gelatin	+
Esculin	+

red cell suspensions, in aerated DM liquid medium added with Se^{IV}, revealing the capacity to reduce Se^{IV} to Se^0 (Sabaty *et al.*, 2001). Conversely, Se^{IV} was totally recovered in sterile control cultures suggesting for a biologically mediated Se^{IV} reduction.

As reported elsewhere (Di Gregorio *et al.*, 2005), the incubation of *S. maltophilia* strain SeITE02 with 0.2 mM Se^{IV} resulted in an increase of either the capability to reduce Se^{IV} to Se^0 or the cell viability in presence of increasing concentration of selenite. Thus, in the present study, both *B. mycoides* SeITE01 and *S. maltophilia* SeITE02 were pre-induced with 0.2 mM Se^{IV} before evaluating selenite resistance and capability to reduce Se^{IV} to Se^0 in both strains.

Se^{IV} toxicity towards strains SeITE01 and SeITE02 was evaluated in liquid culture by checking for bacterial cell growth in presence of increasing concentrations of Se^{IV} (Fig. 1). At the two concentrations tested (0.2, 0.5 mM), Se^{IV} slightly affected the microbial growth of *B. mycoides* strain SeITE01, determining a modest decrease in turbidity compared to the control. On the other hand, neither significant effects by Se^{IV} on cell growth of *S. maltophilia* SeITE02 were revealed nor Se^{IV} negatively influenced the growth of the dual consortium consisting in strain SeITE01 and strain SeITE02 together.

With reference to the capacity of both bacterial strains to reduce Se^{IV} to Se^0, this activity was evaluated for either each strain and for the dual consortium of them (Fig. 2). At 0.2 mM Se^{IV}, *S. maltophilia* strain SeITE02 completely reduced the oxyanion within 120 h. Se^{IV} reduction started at the early exponential phase of growth, lasting throughout the stationary phase (Fig. 1). At 0.5 mM Se^{IV}, strain SeITE02 could reduce only 50% of the initial oxyanion concentration, in 120 h (Fig. 2). Actually, this Se^{IV} reduction was recorded

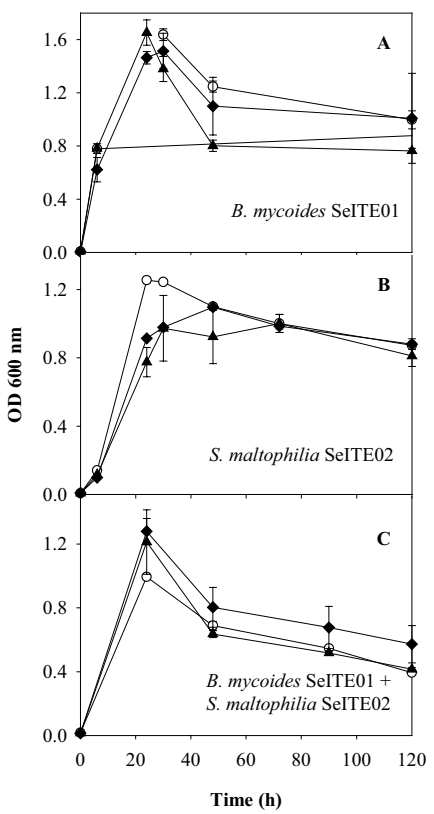

Fig. 1. Time courses of turbidity in presence of 0.2 mM (–▲–), 0.5 mM Se^{IV} (–◆–), or with no selenium (control) (–○–) corresponding to cell suspensions of: (A) *Bacillus mycoides* strain SeITE01, (B) *Stenotrophomonas maltophilia* strain SeITE02, and (C) the dual bacterial consortium consisting of strains SeITE01 and SeITE02 together. Each curve is the mean of three separate experiments.

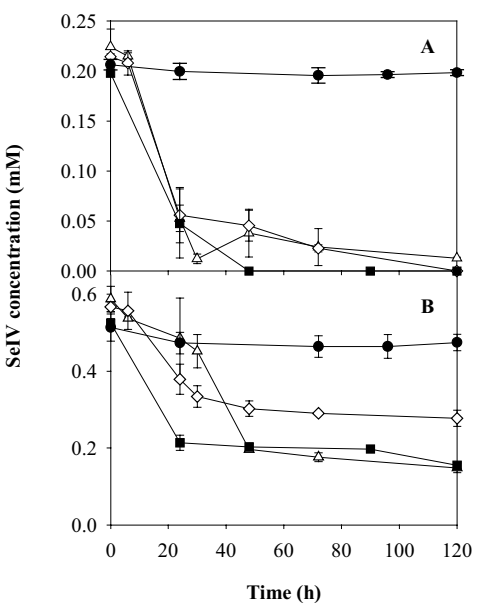

Fig. 2. Time courses of Se^{IV} reduction to Se^0 carried out by *Bacillus mycoides* strain SeITE01 (–△–), *Stenotrophomonas maltophilia* strain SeITE02 (–◇–), and the dual bacterial consortium consisting of strains SeITE01 and SeITE02 together (–■–), in presence of 0.2 mM (A) or 0.5 mM (B) Se^{IV}. Sterile cultures (not inoculated) were also tested for Se^{IV} concentration as negative control (–●–). Each curve is the mean of three separate experiments.

within the first 48 h of incubation. Afterwards, *S. maltophilia* was not capable to reduce the oxyanion any further.

At 0.2 mM Se^{IV}, also *B. mycoides* SeITE01 completely reduced selenite in 120 h (Fig. 2). Conversely, strain SeITE01 did not reduce completely selenite in presence of 0.5 mM Se^{IV}. In fact, *B. mycoides* reduced only 67% of the initial Se^{IV} concentration, mostly within the first 48 h of incubation.

With the dual bacterial consortium consisting of strain SeITE01 and strain SeITE02, the process of selenite reduction was accelerated in comparison with axenic cultures of each single strain. This result was observed at both Se^{IV} concentrations tested. Interestingly, at 0.2 mM Se^{IV}, complete oxyanion reduction occurred in only 48 h vs to 120 h needed with cultures of single strains (Fig. 2). Moreover, at 0.5 mM Se^{IV}, the higher percentage of reduction (67%) previously observed in *B. mycoides* SeITE01 was reached in only 24 h instead of 48 h (Fig. 2). Selenite reduction was recorded in correspondence to an increase of turbidity (*i.e.* cell growth) in cultures of the dual bacterial consortium (Fig. 1).

Discussion

Two bacterial species isolated from the rhizosphere of *Astragalus bisulcatus*, a leguminous plant able to hyperaccumulate selenium, resulted of particular interest for their high resistance to the toxic oxyanion SeO_3^{2-} associated to the capability of reducing selenite to non-bioavailable Se^0. Actually, while the strain SeITE01 assigned to the species *Bacillus mycoides* showed a MIC of 15 mM, either in rich or defined medium, on the other hand, *Stenotrophomonas maltophilia* strain SeITE02 resisted up to 50 mM Se^{IV}. These MIC values for SeO_3^{2-} are much higher than those reported for other naturally occurring, selenite-resistant bacteria such as *Bacillus subtilis* (Garbisu *et al.*, 1999), *Rhodospirillum rubrum* (Kessi *et al.*, 1999), *Rhodobacter spheroides* (Van Fleet-Stalder *et al.*, 2000), and *Ralstonia metallidurans* (Roux *et al.*, 2001). For these species, MIC values ranging from about 2.0 mM to 6.0 mM Se^{IV} have been ascertained in similar growth conditions. Some authors reported MIC values for Se^{IV} up to 40 mM in aerated bacterial cultures, however these isolates were not identified (Rathgeber *et al.*, 2002). The high resistance to selenite and the capability to detoxify SeO_3^{2-} through the reduction to metallic Se^0 make the bacterial isolates of this study attractive for possible application in the remediation of seleniferous effluents.

The *Bacillus cereus* group, which the isolate SeITE01 can be attributed to, includes Gram-positive bacteria belonging to four species: *Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus mycoides*. In particular, *B. mycoides* has recently been recognized as a plant growth-promoting bacterium associated to the roots of different tree species (Petersen *et al.*, 1995). The genus *Bacillus* is presently studied for a possible exploitation in bioremediation of heavy metals (Fujita *et al.*, 2002; Zaidi and Musarrat, 2004). On the other hand, the species *Stenotrophomonas maltophilia*, which the strain SeITE02 belongs to, is a Gram-negative, aerobic, non-fermentative bacterium widespread in different environmental niches. It has been isolated either from soils or aquatic environments (Berg *et al.*, 1999). Moreover, it occurs in the rhizosphere of a variety of plants such as wheat, oat, cucumber, maize, oilseed rape and potato (Lambert and Joos, 1989; Berg *et al.*, 1996). Even the genus *Stenotrophomonas* is nowadays considered for its potential application in bioremediation (Juhasz *et al.*, 2000; Dungan *et al.*, 2003).

As already reported for other Se^{IV} resistant microbes (Tomei *et al.*, 1992; Yamada *et al.*, 1997; Kessi *et al.*, 1999; Van Fleet-Stalder *et al.*, 2000; Roux *et al.*, 2001; Rathgeber *et al.*, 2002; Dungan *et al.*, 2003), selenite resistance of strains SeITE01 and SeITE02 depends on their capacity to reduce the highly toxic SeO_3^{2-} to the non-bioavailable and relatively non-toxic elemental selenium. Under experimental growth conditions, selenite reduction occurred mainly during the microbial exponential growth, lasting for only a part of the stationary phase. At the lower selenite concentration tested (0.2 mM Se^{IV} corresponding to 15 mg/l selenium), the efficiency of both bacterial isolates to reduce SeO_3^{2-} was similar. Meanwhile, *B. mycoides* strain SeITE01 showed a higher efficiency in Se^{IV} reduction at the higher content of selenite in the medium (0.5 mM Se^{IV} corresponding to 45 mg/l selenium), by reducing up to the 67% of the initial Se^{IV} compared to only the 50% reduced by *S. maltophilia* strain SeITE02. When the dual consortium of SeITE01 and SeITE02 was grown in presence of selenite, SeO_3^{2-} reduction proceeded faster. In fact, the maximum of selenite reduction

was reached in a shorter time, although the percent value of this reduction remained unchanged.

The concentrations of selenite tested in the study are comparable to those reported for soluble Se in agricultural drainage wastewaters (~ 0.65 mg/l selenium) (Cantafio *et al.*, 1996) or in industrial wastewater (4–60 mg/l selenium) (Fujita *et al.*, 2002). Consequently, the bacterial isolates SeITE01 and SeITE02 can be considered suitable for application, at either laboratory or pilot scale, in continuously fed wastewater treatment systems in order to optimise the abatement of selenite. Actually, it is interesting to note that, under the growth conditions adopted in this study, the combination of both bacterial isolates in a dual consortium resulted in a decrease of turbidity of the relative cell cultures, at least, when compared to the suspensions of *B. mycoides* strain SeITE01 alone. However, the dual consortium apparently accelerated selenite reduction with respect to what was observed in axenic cultures of each single bacterial isolate. Growth of the two strains within the dual consortium was likely sub-optimal because of a possible reciprocal inhibition. Nevertheless, strain SeITE02 has been shown to maintain its capability in reducing SeO_3^{2-} even under sub-optimal growth conditions (Di Gregorio *et al.*, 2005). Hence, although the mechanisms of selenite reduction by the strains SeITE01 and SeITE02 need to be further analyzed, the capacity to maintain Se^{IV} reducing activity in sub-optimal growth conditions encourages the application of these strains to treat seleniferous effluents.

Conclusions

Metal pollutants in industrial-process waters or in groundwaters are most commonly removed by precipitation or flocculation, followed by sedimentation and disposal of the resulting sludge. A promising alternative to this conventional clean-up method is rhizofiltration, a phytoremediative technique designed for the removal of metals in aquatic environments (Dushenkov *et al.*, 1995; Zhu *et al.*, 1999). On the basis of preliminary evidences from the present study, it appears worth of attention the hypothesis to apply selenite/selenate-resistant and reducing bacteria in hydroponic systems for treating selenium-laden water streams. In these continuous open bioreactors (Fig. 3), the synergic action of hyperaccumulating plants, such as *A. bisulcatus*, which absorb and concentrate the metalloid in their roots and shoots and rhizosphere colonising bacteria can be exploited. While the plants extract the contaminant in its soluble forms from the effluent flowing through sequencing hydroponic ponds, selenite/selenate-reducing bacteria may cause selenium to precipitate onto

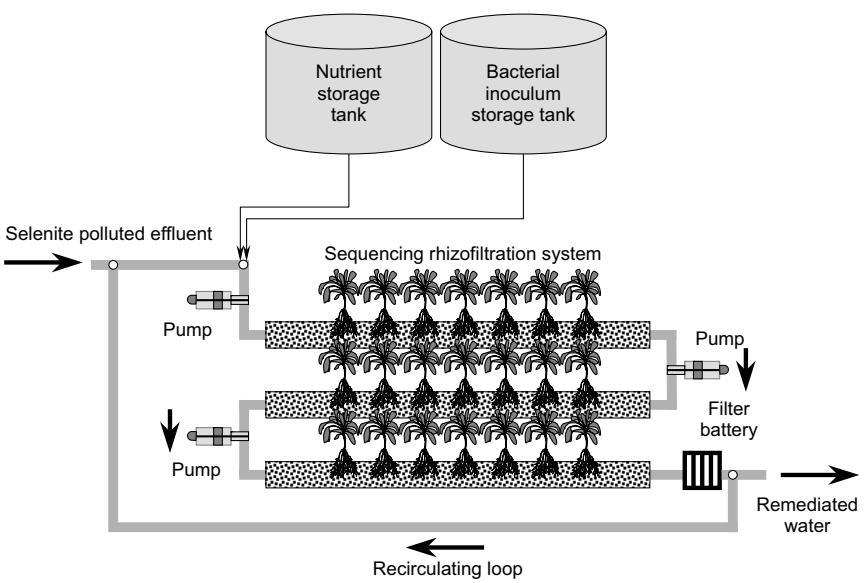

Fig. 3. Schematic representation of a hydroponic rhizofiltration system for the treatment of selenium-laden water streams by means of the synergic activity of a Se hyperaccumulator plant and selenite/selenate reducing bacteria.

the root surfaces or to settle to the bottom. As the plants become saturated with the contaminant, roots or whole plants are harvested for disposal. On the other hand, the remaining suspended Se^0 may be removed from the water by filtering the stream before its final release. Moreover, the possibility to rely on bacterial consortia, whose members exert different capabilities in terms of either selenite/selenate-resistance or reduction kinetics of Se oxyanions, makes the bioprocess more flexible and able to face pollutant fluctuations. For instance, the strain of *Stenotrophomonas* sp. isolated in this study might resist to possible peaking of wastewater selenite concentration, then allowing the *Bacillus* isolate to more efficiently reduce the contaminant in normal steady-state conditions.

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