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Microbial transformation of Se oxyanions in cultures of *Delftia lacustris* grown under aerobic conditions[§]

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Delftia lacustris **is reported for the first time as a selenate and selenite reducing bacterium, capable of tolerating and growing in the presence of ≥ 100 mM selenate and 25 mM selenite. The selenate reduction profiles of *D. lacustris* were investigated by varying selenate concentration, inoculum size, concentration and source of organic electron donor in minimal salt medium. Interestingly, the bacterium was able to reduce both selenate and selenite under aerobic conditions. Although considerable removal of selenate was observed at all concentrations investigated, *D. lacustris* was able to completely reduce 0.1 mM selenate within 96 h using lactate as the carbon source. Around 62.2% unaccounted selenium (unidentified organo-selenium compounds), 10.9% elemental selenium and 26.9% selenite were determined in the medium after complete reduction of selenate. Studies of the enzymatic activity of the cell fractions show that the selenite/selenate reducing enzymes were intracellular and independent of NADPH availability. *D. lacustris* shows an unique metabolism of selenium oxyanions to form elemental selenium and possibly also selenium ester compounds, thus a potential candidate for the remediation of selenium-contaminated wastewaters in aerobic environments. This novel finding will advance the field of bioremediation of selenium-contaminated sites and selenium bio-recovery and the production of potentially beneficial organic and inorganic reactive selenium species.**

Keywords: bioremediation, *Delftia lacustris*, aerobic selenate reduction, organo-selenium compounds, selenium speciation

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Introduction

Selenium (Se) is an essential trace element in living organisms. It is an important constituent of about 25 known selenoproteins including glutathione peroxidase, thioredoxin reductase, iodothyronine deiodonase, and plays an important role in intracellular signaling and redox homeostasis in higher organisms (Huawei, 2009). In spite of its essential requirement and beneficial health effects at low dietary uptake (40 μg/day), selenium has been listed as a priority pollutant (US EPA) because of its potential bioaccumulation (even at concentrations as low as 5 μg/L) and associated toxicity (Tan *et al.*, 2016). Selenium toxicity is associated with hair and nail loss and disruption of the nervous and digestive systems in humans and animals (Tinggi, 2008; Rayman, 2012).

Contamination of aquatic bodies with selenium is a serious concern because it can pass through trophic levels, bioaccumulate in living organisms to toxic concentrations and exert toxicity on cellular metabolism (Wu, 2004). Therefore, selenium release through anthropogenic sources such as agricultural drainage water and industrial effluents is tightly regulated. At present, the discharge limit to aquatic bodies has been set at 5 μg/L Se by the US EPA (Frankenberger *et al.*, 2004; Zhang *et al.*, 2008). Different methods such as ion exchange, reverse osmosis, adsorption, and chemical reduction using zero valent iron (ZVI) are available for treating selenium containing waters (Frankenberger *et al.*, 2004; Sasaki *et al.*, 2008). Among the variety of methods, microbial reduction of soluble selenium oxyanions (selenate and selenite) to insoluble and comparatively stable elemental selenium is the best available option because of its cost-effectiveness and eco-friendly nature for the treatment of selenium containing wastewaters (Nancharaiah and Lens, 2015a, 2015b; Tan *et al.*, 2016).

Reduction of selenium oxyanions to elemental selenium nanospheres has been found in phylogenetically diverse microorganisms isolated from pristine and contaminated environments (Nancharaiah and Lens, 2015b; Eswayah *et al.*, 2016). Selenite reduction has been found in several microorganisms under both aerobic and anaerobic conditions. For example, rhizospheric, nitrogen-fixing bacteria *Azospirullum brasilense* (Tugarova *et al.*, 2014) and *Rhizobium selenireducens* (Hunter *et al.*, 2007) have been reported to reduce selenite to selenium nanoparticles (SeNPs). The Gram-positive bacterium *Bacillus megaterium* isolated from mangrove soil (Mishra *et al.*, 2011) and *Bacillus mycoides* isolated from a

Table 1. Overview of bacteria reducing selenate under aerobic conditions

Organism	Optimum growth conditions			Se source	Product	Respiration	Maximum toxicity concentration for SeO_4^{2-} and SeO_3^{2-}	References
	pH	Temp (°C)	Medium					
Pseudomonas stutzeri NT-I	7.0	28°C	Tryptic soy broth	SeO_4^{2-}, SeO_3^{2-}, Se^0	DMSe, DMDSe	Aerobic	-	Kagami *et al.* (2013)
	7–9	20-50°C	Tryptic soy broth	SeO_4^{2-}, SeO_3^{2-}	Se^0	Aerobic	122 mM SeO_4^{2-}, 94 mM SeO_3^{2-}	Kuroda *et al.* (2011)
Enterobacter cloacae SLD1a-1	7.2	22°C	Tryptic soy broth	SeO_4^{2-}	Se^0	Aerobic	1.2 mM SeO_4^{2-}	Losi and Frankenberger (1997)
Clostridium sp. BXM	7	30°C for aerobic 37°C for anaerobic	Basal medium with 10% yeast extract	SeO_4^{2-}, SeO_3^{2-}	Se^0	Aerobic & anaerobic	1 mM SeO_4^{2-}, 1 mM SeO_3^{2-}	Bao *et al.* (2012)
Pseudomonas fluorescens	-	Room temperature	Tryptic soy broth	SeO_4^{2-}, SeO_3^{2-}	Se^0	Aerobic & anaerobic	10 mM SeO_4^{2-}, 10 mM SeO_3^{2-}	Hapuarachchi *et al.* (2004)
Stenotrophomonas maltophilia	7.3	Room temperature	Tryptic soy broth	SeO_4^{2-}, SeO_3^{2-}	Se^0, DMSe, DMDSe, DMSeS	Aerobic	1 mg/L SeO_4^{2-}, 1 mg/L SeO_3^{2-}	Dungan *et al.* (2003)
Delftia lacustris	7.0	Room temperature	MSM with 10 mM lactate	SeO_4^{2-}, SeO_3^{2-}	Se^0, Organic Se	Aerobic		This study

selenium hyper-accumulating plant (Lampis *et al.*, 2014) are able to reduce selenite to elemental selenium. A *Rhodopseudomonas palustris* strain (Li *et al.*, 2014) isolated from a sewage treatment plant and the novel *Paenibacillus selenitireducens* sp. (Yao *et al.*, 2014) isolated from selenium mineral soil collected from a mine in China reduce selenite to elemental selenium anaerobically.

In contrast, only a limited number of selenate reducing bacterial cultures have been isolated and characterized (Table 1). In the well-studied selenate reducing bacteria *Thauera selenatis* and *Bacillus selenatarsenatis*, the reduction of selenate to selenite is linked to anaerobic respiration and catalysed by selenate reductase (Yamamura *et al.*, 2007; Butler *et al.*, 2012). Very few microorganisms such as, *Pseudomonas stutzeri* NT-I, *Enterobacter cloacae* SLD1a-1, and *Stenotrophomonas maltophilia*, have been identified to reduce selenate under aerobic conditions (Table 1), but the understanding of the biochemical mechanism of bacterial selenate reduction under aerobic conditions is rather incomplete. *Pseudomonas stutzeri* NT-I is able to convert selenate, selenite and biogenic elemental selenium (Se^0) to volatile selenium compounds (i.e. dimethyldiselenide and dimethylselenide) under aerobic conditions (Kagami *et al.*, 2013). The selenate and selenite reducing bacterium, *Enterobacter cloacae* SLD1a-1 isolated by Losi and Frankenberger (1997) was hypothesized to reduce selenium oxyanions via membrane-associated reductases, followed by expulsion of the Se^0 precipitate outside the cells. Dungan *et al.* (2003) considered detoxification mechanisms for the reduction of both selenate and selenite to elemental Se and volatile Se compounds.

Detoxification mechanisms are survival strategies developed by microorganisms which convert toxic compounds to non-toxic forms (Nancharaiah and Lens, 2015b). In the present study, the reduction mechanism of selenium oxyanions (at toxic concentrations) to comparatively non-toxic and insoluble elemental Se of the pure culture is studied. A bacterium frequently encountered in a 100 mM selenate stock solution was isolated and identified based on 16S rRNA gene sequencing. The bacterium was selected for this study because it possesses excellent selenate resistance, survival and growth in a mineral medium containing more than 100 mM selenate. Another reason was that it allows studying biotransformation of Se oxyanions under aerobic conditions. The selenate reduction capabilities of this strain were determined by varying the initial selenate concentration, inoculum size, carbon sources and carbon source concentration in minimal salt medium (MSM).

Materials and Methods

Chemicals

Sodium selenate (purity > 98%) was procured from Sigma Aldrich. Nutrient broth and agar for isolation and culturing of bacteria was obtained from Oxoid Ltd. All other analytical-grade chemicals were purchased from Merck. A 100 mM selenate stock was prepared by dissolving 1.89 g of sodium selenate in 100 ml ultrapure water. A sodium lactate (1 M) stock solution was prepared by diluting 13.79 ml of 61.1% sodium lactate to 100 ml with ultrapure water. The stock solutions were stored at 4°C.

Isolation and growth conditions

The bacterium was isolated serendipitously as a contaminant on nutrient agar plates supplemented with 1 mM selenate from the 100 mM selenate stock solution. Red coloured colonies were re-streaked on MSM supplemented with 1.2%

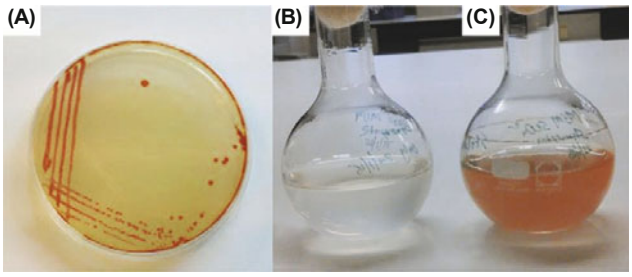

Fig. 1. *D. lacustris* cultured at 30°C on MSM supplemented with 1.2% (w/v) agar plate containing selenate (A) and grown in liquid MSM without (B) and with (C) selenate.

(w/v) agar and 1 mM selenate (Fig. 1). The composition of the MSM was based on the composition of a synthetic wastewater (Stams *et al.*, 1992) (g/L): 0.053 $Na_2HPO_4 \cdot 2H_2O$, 0.041 KH_2PO_4, 0.3 NH_4Cl, 0.01 $CaCl_2 \cdot 2H_2O$, 0.01 $MgCl_2 \cdot 6H_2O$, and 0.04 $NaHCO_3$ along with 0.1 ml/L acid and alkaline trace element solutions. The acid trace element stock solution contained (in mM): 7.5 $FeCl_2$, 1 H_2BO_4, 0.5 $ZnCl_2$, 0.1 $CuCl_2$, 0.5 $MnCl_2$, 0.5 $CoCl_2$, 0.1 $NiCl_2$, and 50 HCl. The alkaline trace metal solution was composed of (in mM) 0.1 Na_2WO_4, 0.1 Na_2MoO_4, and 10 NaOH.

MSM agar plates were incubated at 30°C overnight for the growth of selenate reducing colonies. Individual colonies were transferred to MSM broth containing 1 mM sodium selenate and 10 mM sodium lactate and incubated overnight at 30°C with constant shaking at 150 rpm. The isolate was sub-cultured in MSM broth and stored in a 50% (v/v) glycerol stock at -20°C to be used for further experiments for analysing growth and selenate reduction profiles under varying conditions.

Growth and selenate reduction by *D. lacustris*

Growth and selenate reduction under aerobic and anaerobic conditions : Sterile MSM containing 10 mM lactate and 0.1 mM selenate was used to study growth and selenate reduction by the isolate. For anaerobic conditions, the serum bottles were closed with butyl rubber stoppers, crimp sealed and purged with N_2 for 5 min. The bottles containing 100 ml MSM liquid with 0.1 mM selenate were inoculated with an overnight grown bacterial culture (3.25×10^{12} CFU/ml) under anaerobic conditions. For aerobic growth, the culture flasks plugged with cotton containing 100 ml MSM with 0.1 mM selenate were inoculated with an overnight grown culture and incubated on an orbital shaker set at 150 rpm. Aerobic culture flasks and anaerobic serum bottles were incubated at 30°C. Liquid samples of 5 ml medium were collected at regular time intervals under aseptic conditions for monitoring growth and concentrations of selenate, selenite, lactate, and total selenium.

Effect of selenate and selenite concentration : Selenate and selenite reduction at different initial selenate and selenite concentrations was determined by inoculating 2% (v/v) overnight grown culture (6.5×10^{12} CFU/ml) in 100 ml sterile MSM containing 20 mM sodium lactate as carbon source. Growth, selenate and selenite concentrations were determined at different initial selenate and selenite concentrations of 0.1, 0.5, 1, and 2 mM each. Culture flasks were incubated at 30°C with constant shaking at 150 rpm.

Effect of initial cell density : Initial cell density was measured in terms of colony forming units (CFU) on nutrient agar. The cell density was varied by inoculating 1%, 2% or a cell pellet of an overnight grown culture (5.4×10^{12} CFU/ml) in sterile MSM containing 10 mM sodium lactate and 0.1 mM sodium selenate, incubated at 30°C with constant shaking at 150 rpm.

Effect of carbon source and concentration : Growth and selenate reduction was determined in the presence of different carbon sources such as lactate, acetate and glucose. Tests were carried out by inoculating 2% (v/v) overnight grown culture in sterile MSM containing 0.1 mM sodium selenate and 20 mM sodium lactate, 20 mM sodium acetate, 20 mM sodium citrate, 20 mM mannitol or 55 mM D-glucose, incubated at 30°C with constant shaking at 150 rpm. Growth and selenate reduction were also determined at different initial lactate concentrations of 10 mM and 20 mM. A fixed selenate concentration of 0.1 mM sodium selenate was used. Culture flasks were incubated at 30°C with constant shaking at 150 rpm.

Effect of tungstate on selenate and selenite reduction

Molybdenum has been identified as the constituent of selenate reductase and act as the co-factor for SeO_4^{2-} reduction in selenium reducing microorganisms such as *Enterobacter cloacae* SLD1a-1, *Escherichia coli* and *Thauera selenatis* (Schröder *et al.*, 1997; Bébien *et al.*, 2002; Watts *et al.*, 2003). The selenate reductase activity in these microorganisms was significantly decreased in the presence of tungstate in the medium due to competitive inhibition. Therefore, the presence of tungstate in the medium results in significant decrease in selenate reduction by microorganisms containing molybdenum-dependant selenate reductase. The effect of tungstate on selenate and selenite reduction was determined by inoculating 2% of fully grown culture in sterile MSM containing 0.1 mM sodium selenate, 10 mM sodium tungstate and 20 mM lactate. MSM containing 0.1 mM sodium selenate and 20 mM lactate inoculated with bacterial culture served as control. For determining the impact of tungstate on selenite reduction, a similar experiment was performed by replacing selenate with selenite.

Minimum inhibitory concentration of selenate

Nutrient broth and MSM containing a final concentration of 0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 mM of sodium selenate and 10 mM lactate as carbon source with a final volume of 100 ml was sterilized by autoclaving. The flasks were inoculated with 1% bacterial culture (3.25×10^{12} CFU/ml) so that the initial absorbance at 600 nm (indicating cell density) was around 0.02 and incubated at 30°C with constant shaking at 150 rpm overnight. Growth was monitored by measuring absorbance at 600 nm. The concentration of selenate which caused complete inhibition in bacterial growth was considered as the minimum inhibitory concentration.

Selenate reduction by spent medium, cell lysate, and resting cells

The bacterial culture was grown in nutrient broth overnight. This culture was centrifuged at 5,000 × *g* for 15 min to pellet the bacterial cells. The supernatant was filter-sterilized with 0.22 µm sterile filter disks. Control samples were obtained after heat inactivation by autoclaving at 121°C for 15 min. Sodium selenate was added to filtered spent medium at a final concentration of 0.1 mM and incubated at 30°C. Selenate and selenite concentrations were analysed at 0, 2, 4, 8, 24, 48, and 72 h of incubation. The pellet was re-suspended and washed twice with 10 mM Tris HCl, pH 7.5. Bacterial cells were lysed by pulse sonication at 4°C. Sodium selenate or sodium selenite were added separately to adjust the final concentration to 0.1 mM and incubated at 30°C. Selenate and selenite were analysed at 0 and 4 h of incubation. The influence of NADPH addition was analysed by adding 2

mM NADPH to the lysed cells and supernatant along with sodium selenate and selenite separately.

To obtain the resting cells, the strain was grown at 30°C in nutrient broth for 48 h to ensure complete utilization of the energy source in the medium and microbial cells were in the stationary phase. The cells were then harvested by centrifugation at 6,000 × *g* for 10 min, followed by washing and inoculation in sterile MSM. Sterile sodium selenate stock was added to MSM containing resting cells to adjust the final concentration of 0.1 mM and incubated at 30°C with constant shaking at 150 rpm under aerobic or anaerobic conditions. Selenate, selenite and total selenium were analysed at regular time intervals.

Extraction of organic selenium compound from spent culture medium

Culture was grown in selenate or selenite containing MSM as described above. After 5 days of incubation, the culture was centrifuged at 6,000 × *g* for 10 min to separate cells from the spent MSM and the supernatants were extracted by 2-phase liquid-liquid extraction using ethyl acetate. The aqueous and ethyl acetate phases were analysed by TLC as described by Bottura and Pavesi (1987). The solvent system used was ethyl acetate: methanol (4:1) and after the run on the silica gel TLC plates (Sigma Aldrich), the spots were observed under UV light. The ethyl acetate phase was further concentrated in a rotary evaporator. A white precipitate formed at the bottom of the flask was then dissolved in dimethyl sulfoxide (DMSO) for ^{77}Se NMR analysis on a Bruker Avance 500 MHz. The NMR spectra were recorded at the frequency of 95.41 MHz using deuterated DMSO as a solvent.

16S rRNA gene sequencing

The genomic DNA of the bacterial isolate was extracted using the PowerSoil DNA isolation kit (MO BIO Laboratories Inc.). For this, the strain was grown overnight in nutrient broth and centrifuged at 6,000 × *g* for 10 min. The genomic DNA was isolated from the cell pellet by following the manufacturer's instructions. The 16S rRNA gene was PCR amplified from genomic DNA using universal primers and analysed by DNA sequencing by Eurofins Genomics. The sequence was analysed using Basic Local Alignment Search Tool (BLAST) from the nucleotide database of the US *National Center for Biotechnology Information* (NCBI, www.ncbi.nlm.nih.gov) to obtain the phylogenetic identity of the isolate.

Electron microscopic imaging

The morphology of the selenate reducing bacterial cells was analysed using a Jeol JSM-6010LA scanning electron microscopy (SEM). The culture was washed with phosphate buffered saline, pH 7, followed by fixation with 2.5% glutaraladehyde and dehydration using a graded ethanol series (Kaláb *et al.*, 2008). Bacterial cells grown in nutrient broth, and MSM with and without 0.1 mM selenate were fixed in glutaradehyde and dehydrated as described by Kaláb *et al.* (2008) and subsequently observed using SEM.

Analysis

Growth was routinely monitored by measuring absorbance at 600 nm using UV-Vis spectrophotometry (Shimadzu UV-2501 PC). The samples were filtered through a 0.22 µm syringe filter and analysed for selenate, selenite and lactate. Selenite was analysed by UV-Vis spectrophotometry using ascorbic acid reagent (Mal *et al.*, 2016). Selenate was measured by an ion chromatograph equipped with AG14A 3 mm guard column (Dionex ICS series AS-DV) using 8 mM sodium carbonate and 1 mM sodium bicarbonate as eluant with a flow rate of 0.5 ml/min (Dessì *et al.*, 2016). The retention time for selenate was 8.5 min.

After acidification of samples using 65% nitric acid, total selenium was analysed by a graphite furnace atomic absorption spectrophotometer (GF-AAS) (Solaar AA Spectrometer GF95, Thermo Electrical, Unity lab services) as described by Dessì *et al.* (2016). For quantification of elemental Se the samples were centrifuged at 37,000 × *g* for 15 min to separate Se(0) particles and bacterial cells from dissolved Se (Mal *et al.*, 2016). Pellet and supernatant samples were then digested with 65% nitric acid, diluted and analysed by GF-AAS as intracellular and extracellular elemental Se particles and total dissolved Se, respectively. One-way analysis of variance ANOVA (at 95% confidence levels) was applied to evaluate statistical significance for Se reduction for each condition studied.

Unaccounted selenium was calculated by subtracting the selenate and selenite concentration from the total dissolved selenium:

$$\text{Unaccounted Se} = \text{Total dissolved Se} - (\text{Selenate} + \text{Selenite}) \quad \text{(Eq. 1)}$$

Where total dissolved Se, selenate and selenite were measured using GF-AAS, ion chromatography and UV-Vis spectrophotometry, respectively, as described above.

Results

Characterisation of the isolate

The 16 rRNA gene sequence of the selenate reducing isolate showed 100% identity with *Delftia lacustris* (Jørgensen *et al.*, 2009). The sequence was submitted to the NCBI GenBank (Accession number: MH158542). Selenate reduction by *D. lacustris* under aerobic growth conditions was intriguing (Fig. 1) with respect to Se speciation, hence the reduction process was investigated in detail.

Scanning electron microscopy (SEM) images showed distinct variation in the cell morphology of the *D. lacustris* cells, with rod shaped cells in chains when cultured in nutrient broth medium and rod shaped bacterial cells in the form of aggregates when cultured in minimal medium (Supplementary data Fig. S1). No significant difference was, however, observed in the morphology of bacterial cultures grown in MSM with or without 0.1 mM selenate.

Growth of *D. lacustris* and selenate reduction profiles

Lactate and selenate removal under aerobic and anaerobic conditions : Removal profiles of lactate and selenate under aerobic and anaerobic conditions are shown in Fig. 2. In

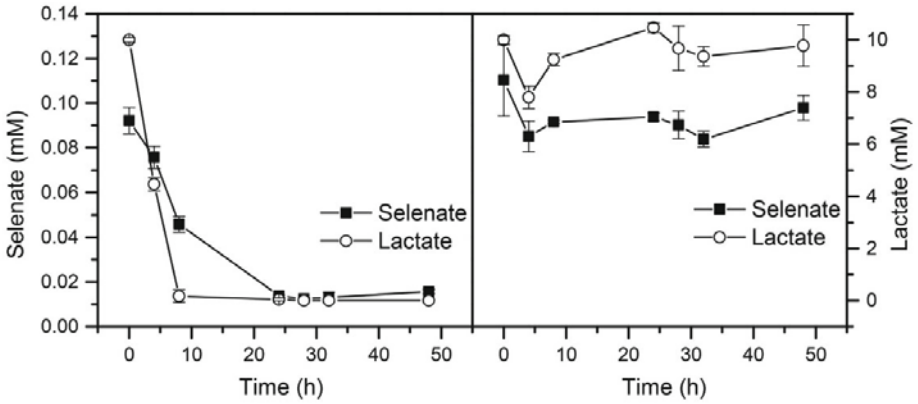

Fig. 2. **Selenate and lactate removal profiles by *D. lacustris* in mineral salts medium under aerobic (A) and anaerobic (B) growth conditions.**

aerobic conditions, lactate was completely utilized within the first 10 h (Fig. 2A). Selenate removal was around 50% within 10 h after inoculation. Conversely, no major change in either lactate or selenate concentration was observed under anaerobic conditions (Fig. 2B). In fact, no change in the turbidity was observed, suggesting that *D. lacustris* is incapable of growing in the presence of lactate, and reducing selenate (Fig. 2B) under anaerobic conditions.

Carbon source type and concentration and initial cell density : *D. lacustris* was able to grow by utilizing different carbon sources such as acetate, citrate, lactate, and mannitol under aerobic condition (Fig. 3A). It was, however, unable to use glucose as the carbon source. Moreover, selenate was removed from the MSM when supplemented with acetate, citrate, lactate, and mannitol as the carbon source. Reduction of selenate by *D. lacustris* was more efficient when lactate, acetate or mannitol were the carbon sources (Fig. 3A). Se speciation showed formation of selenite, Se(0) and unaccounted dissolved Se after 7 days of incubation (Fig. 3B).

A major portion of the dissolved Se could not be detected either as selenate or selenite and thus is hereafter referred to as unaccounted dissolved Se (Fig. 3B). Profiles of progressive selenate disappearance typically followed an initial lag phase, a phase of fast selenate removal and stationary phase with almost no selenate transformation (Fig. 4). Both selenate reduction and bacterial growth increased with increasing initial lactate concentration. In MSM containing 20 mM lactate, complete reduction of 0.1 mM selenate was observed after 96 h (Fig. 4A and B).

Selenate reduction by *D. lacustris* was monitored at two inoculum densities. Cell pellets harvested from *D. lacustris* culture grown overnight were resuspended and added to medium at 1 and 2% (v/v). The number of cells corresponding to 1 and 2% inoculum are 3.25×10^{10} CFU/ml and 6.50×10^{10} CFU/ml, respectively. The cell density of the overnight grown cultures determined by serial dilution and plating on nutrient agar was found to be 3.25×10^{12} CFU/ml. Fast reduction of selenate occurred when the initial cell density of *D. lacustris* inoculated into the culture medium was higher (2% inoculum) (Fig. 4B and D) compared to cultures containing 1% inoculum (Fig. 4A and C). Complete reduction of 0.1 mM selenate was observed after 96 h when the 2% inoculum was added to the culture (Fig. 4B). The selenate reduction rate was unchanged with increase in inoculum concentration greater than 2% of its initial concentration (Fig. 4E).

Organic selenium analysis : Thin layer chromatography (TLC)

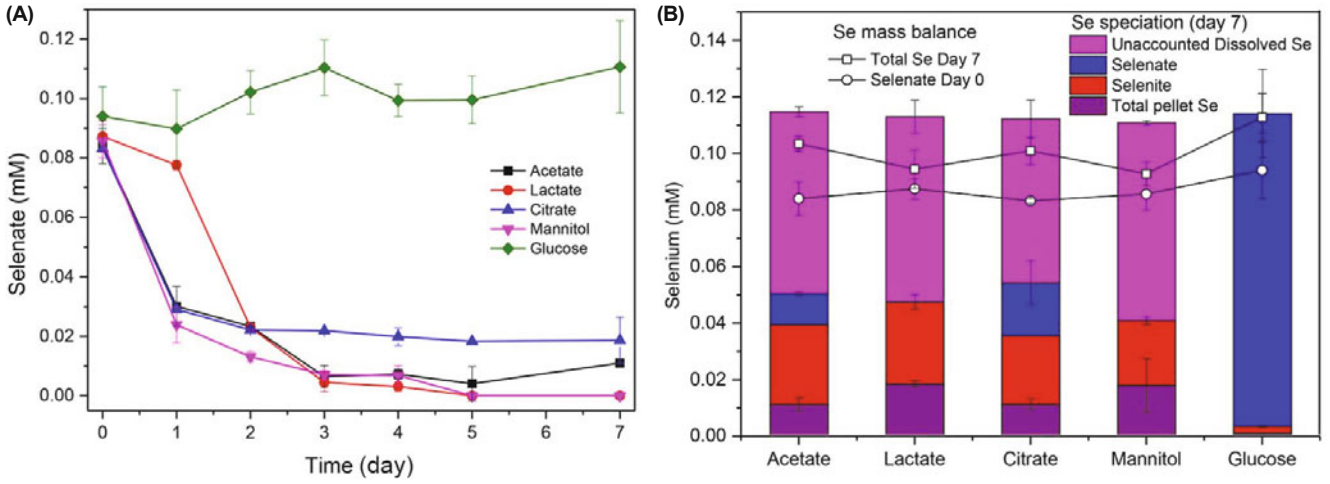

Fig. 3. **Selenate reduction (A) by *D. lacustris* in mineral salts medium supplemented with different carbon sources and selenium mass balance and speciation after 7 days (B) by *D. lacustris* in the presence of different carbon sources.**

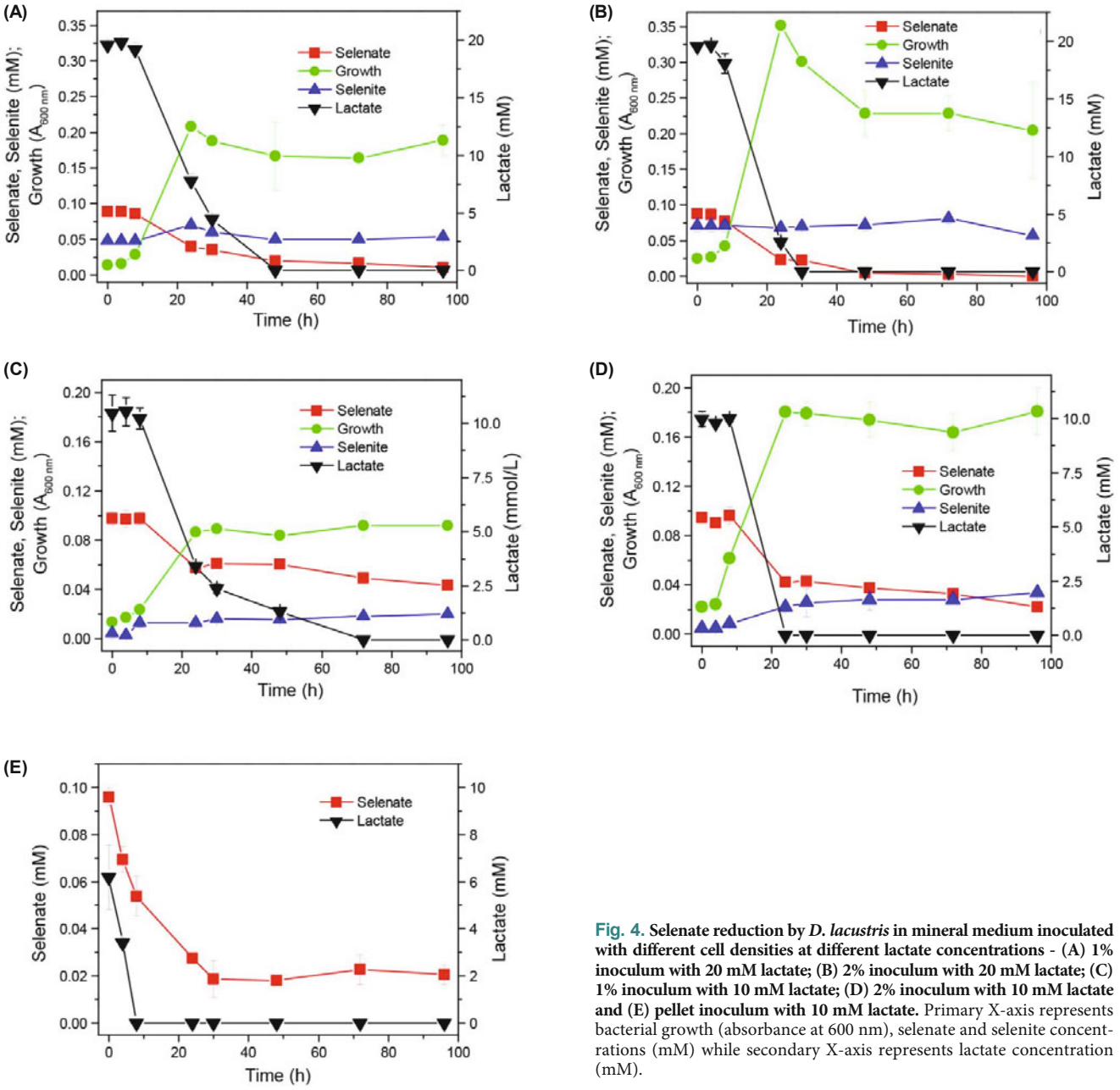

Fig. 4. Selenate reduction by *D. lacustris* in mineral medium inoculated with different cell densities at different lactate concentrations - (A) 1% inoculum with 20 mM lactate; (B) 2% inoculum with 20 mM lactate; (C) 1% inoculum with 10 mM lactate; (D) 2% inoculum with 10 mM lactate and (E) pellet inoculum with 10 mM lactate. Primary X-axis represents bacterial growth (absorbance at 600 nm), selenate and selenite concentrations (mM) while secondary X-axis represents lactate concentration (mM).

was performed to determine the components present in aqueous extract as well as ethyl acetate extracts obtained from both selenate and selenite-reducing culture medium. Supplementary data Table S1 presents each spot and the R_f values ob-

Table 2. Removal efficiencies and concentrations of elemental selenium and unaccounted selenium produced by *D. lacustris* after 96 h incubation at 30°C with varying initial concentration of selenate and selenite

Se source	Selenate			Selenite		
Concentration (mM)	Removal efficiency (%)	Elemental Se (mM)	Unaccounted Se* (mM)	Removal efficiency (%)	Elemental Se (mM)	Unaccounted Se* (mM)
0.1	100	0.009 ± 0.002	0.051 ± 0.009	69.5 ± 0.8	0.022 ± 0.002	0.040 ± 0.002
0.2	71.6 ± 3.3	0.003 ± 0.0003	0.095 ± 0.007	70.9 ± 2.6	0.031 ± 0.009	0.085 ± 0.009
0.3	54.1 ± 9.9	0.006 ± 0.001	0.102 ± 0.015	71.9 ± 0.3	0.038 ± 0.003	0.148 ± 0.002
0.4	49.1 ± 0.7	0.007 ± 0.002	0.120 ± 0.005	69.8 ± 0.5	0.045 ± 0.001	0.168 ± 0.001
0.5	42.9 ± 2.7	0.008 ± 0.001	0.113 ± 0.003	60.2 ± 2.4	0.043 ± 0.006	0.215 ± 0.001

* Unaccounted Se was determined by subtracting dissolved selenate and selenite from total dissolved selenium after 96 h.

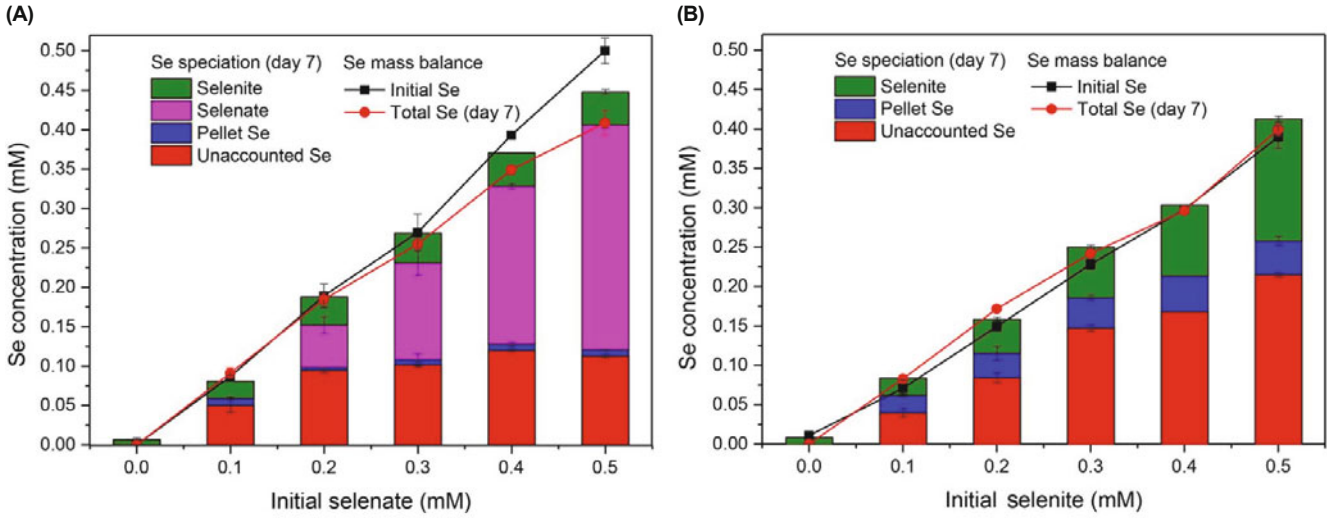

Fig. 5. Selenate (A) and selenite (B) reduction at varying concentrations from 0.1–0.5 mM showing reduction of selenate and selenite to elemental Se, and an unidentified soluble organo-Se compound. Pellet Se includes elemental selenium as well as intracellular organic selenium.

served in the TLC plates of aqueous and ethyl acetate extracts. Two spots each were observed for samples A, B, and D (data not shown), with the R_f value of the first spot matching with that of the SeO_2 standard. When observed under UV light, the spot glowed indicating the presence, of conjugated double Se bonds of an aromatic compound, like those present in phenyl selenide (PhSe) compounds or selenate esters. The standard indicated (Supplementary data Table S1) the presence of Se compounds in the extracts (R_f 0.33). The spots with higher R_f values also exhibited fluorescence under UV indicating the presence of aromatic compounds.

Selenate and selenite concentration

Complete selenate reduction was observed only when the selenate concentration in the medium was 0.1 mM. The percentage reduction decreased with increased selenate concentration (0.1–0.5 mM) in the medium. In contrast, reduction of selenite was incomplete even at the lowest (0.1 mM) selenite concentration tested. Selenite was reduced by 60–72% in all the culture flasks, irrespective of the initial selenite concentration in the medium (Table 2). Se speciation analysis showed no major changes in the elemental Se concentration during transformation at different selenate concentrations, while the elemental Se concentration increased with increasing selenite concentration. Nevertheless, the fraction of unaccounted Se increased with increasing initial selenate and selenite concentration (Fig. 5).

Minimum inhibitory concentration of selenate and selenite

Bacterial growth was observed in all the concentrations tested up to 100 mM selenate in both nutrient broth as well as MSM containing lactate (Fig. 6). The growth of *D. lacustris* in nutrient broth in the presence of 50 and 100 mM selenate was lower compared to the control (nutrient medium without selenate), suggesting growth inhibition at higher selenate

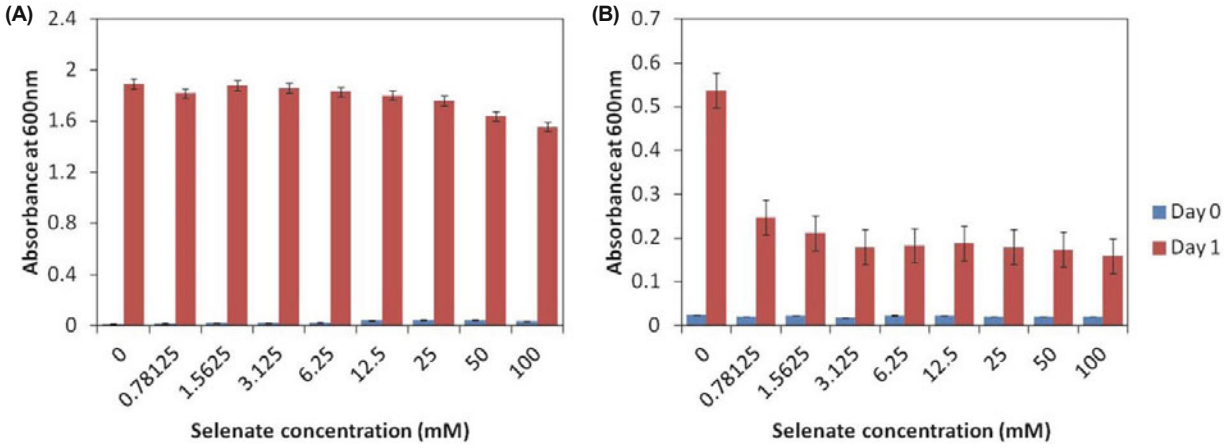

Fig. 6. *D. lacustris* growth in the presence of different concentrations of selenate in nutrient broth (A) and MSM (B). Presence of selenate in MSM negatively affects the growth of the bacterium.

concentrations. Furthermore, the bacterial growth in MSM was decreased in presence of all the selenate concentrations tested. The strain was, nevertheless, able to grow even in the presence of 100 mM selenate in this medium. Thus, the minimum inhibitory concentration of selenate was > 100 mM. Similarly, the bacterial growth was inhibited at a concentration of > 25 mM selenite in nutrient broth, suggesting that the MIC for selenite is 25 mM for *D. lacustris*.

Selenate reduction by spent growth medium, cell lysate, and resting cells

Selenate reduction did not occur in spent growth medium without *D. lacustris* cells. The selenate reduction ability was lost when *D. lacustris* cells were lysed or autoclaved (Supplementary data Fig. S2). No significant difference ($P < 0.05$) was observed in the selenate reduction profile between the untreated and sterile lysate sample and Tris-HCl (control).

About 60% of selenite was absorbed immediately by the cell lysate with or without autoclaving. The reduction continued by the cell lysate and removed up to 80% after 6 h (Supplementary data Fig. S2). Untreated cell lysate was shown to reduce selenite, whereas autoclaved lysate and Tris-HCl (control) did not induce selenite reduction. The autoclaved lysate did not show selenite reduction, suggesting the heat labile nature of the reducing agent or reductase enzyme.

No reduction or adsorption of selenate or selenite was observed in control setups with Tris-HCl. Resting cells also did not reduce selenate under both aerobic and anaerobic conditions. Addition of NADPH did also not increase the reduction rate of selenate or selenite (Supplementary data Fig. S3) by the lysed cells or the supernatant, indicating the selenate-reducing enzymes are NADPH independent.

Effect of tungstate on selenate and selenite reduction

In selenate reduction experiments, growth and selenate reduction were unaffected by the presence of sodium tungstate in the medium (Supplementary data Fig. S4). However, the selenite concentration in the medium could not be measured because sodium tungstate interfered with the selenite measurement by the UV-Vis spectrophotometric method. Visual inspection showed nevertheless that the characteristic red colour of elemental Se did not develop in the medium containing tungstate (data not shown). This suggests that reduction of selenite to elemental Se by *D. lacustris* was inhibited by the presence of sodium tungstate. Tungstate has been reported as a competitive inhibitor of molybdenum of the membrane-bound molybdo-enzyme active site, a putative selenate reductase in *E. cloacae* SLD1a-1 (Watts *et al.*, 2003).

Discussion

Selenate reduction by *D. lacustris* is linked to growth and availability of carbon source

D. lacustris was first isolated from mesotrophic lake water in 2009 as a peptidoglycan degrading bacterium with extracellular lysozyme and chitinase activity (Jørgensen *et al.*, 2009). The bacterium is aerobic, Gram-negative, non-spore forming, motile, rod shaped and incapable of utilizing glucose as the carbon source. The strain was found to be oxidase and catalase positive and utilizes oxygen as terminal electron acceptor. *D. lacustris* has been reported to reduce nitrate to nitrite (Jørgensen *et al.*, 2009), but there are no reports on its ability to reduce either selenate or selenite. This is the first report showing the aerobic bacterium *D. lacustris* can reduce both selenate and selenite.

D. lacustris isolated from a selenate stock solution showed consistent reduction of selenate under aerobic growth conditions (Fig. 2). The selenate reduction ability depended on the density of inoculum (Fig. 4), carbon source type (Fig. 3), and concentration (Fig. 4). Rapid growth and selenate reduction was observed when grown in the presence of higher lactate concentrations (Fig. 4). Interestingly, selenate reduction was not observed in the resting cell experiments, suggesting that selenate reduction by this strain only occurs in the presence of external carbon source and is linked to growth.

Selenite reduction was inhibited, while selenate reduction was unaffected in the presence of tungstate, suggesting selenite and selenate reduction by *D. lacustris* is molybdenum dependent and independent, respectively (Supplementary data Fig. S4). Different enzymes or molecules may be involved in selenate and selenite reduction. Similarly, Zheng *et al.* (2014) inferred that different enzymes were involved in selenate and selenite reduction in *Comamonas testosterone*, where selenate reduction by the microorganism is a molybdenum dependent reduction inhibited by tungstate, while NADPH dependent enzymes are involved in selenite reduction. In this study, however, reduction of both selenate and selenite by *D. lacustris* was found to be NADPH independent. Further characterisation of the enzymology and intermediates of the selenate metabolism of *D. lacustris* is required. Nevertheless, attributes such as fewer growth requirements, ease of culturing and selenate reduction under aerobic conditions turn *D. lacustris* into a potential microorganism for bioremediation of selenate bearing wastewaters and soils.

Reduction of selenate to elemental Se and unaccounted soluble Se fraction

In the experiment with varying selenate concentrations, selenate removal accompanied by the appearance of selenite and elemental Se (Fig. 5) confirms reduction of selenate by *D. lacustris* under aerobic conditions. The negligible difference between the initial and final total selenium concentration (Fig. 5) suggested an insignificant role of volatilization in selenate or selenite removal by *D. lacustris*. Upon subtracting the selenate and selenite concentrations from total dissolved Se at the end of the experiment, a significant fraction of unaccounted form of Se was observed in dissolved form, which could be in the form of assimilatory Se compounds such as alkyl selenide or other organo-Se compounds. Se speciation analysis found that more than 60% of the selenate was directed towards organo-Se compound synthesis, irrespective of the initial selenate concentration (Table 2).

Similar experiments performed with variable initial selenite concentration showed that about 20% more organo-Se compound with increasing the initial selenite concentration (Table 2), while around 70% of selenite was reduced irrespective of its initial concentration. Lampis *et al.* (2014) reported that

the selenite reduction activity of *Bacillus mycoides* is linked to the initial selenite concentration and total number of bacterial cells, rather than the bacterial growth phase. The selenite reduction profile of *B. mycoides*, when carried out under aerobic conditions with increasing initial selenite concentration, shows that the selenite reduction rate increases with increased selenite concentration (Lampis *et al.*, 2014).

After 7 days of incubation, a low elemental Se concentration was observed with increasing selenate or selenite initial concentration. Thus, selenate and selenite reduction was mainly directed towards the formation of organo-Se compounds. Similar results were observed for a *Ralstonia metallidurans* strain, where selenite was reduced to alkyl selenide by an assimilatory pathway, followed by detoxification to form Se(0) (Sarret *et al.*, 2005). However, ^{77}Se NMR, ion trap mass spectrometry and FT-ICR MS analysis of the ethyl acetate extracts from the supernatant of the *D. lacustris* cultures grown in selenate and selenite could not unambigiously identify such alkyl selenium species as accumulated intermediates (data not shown).

Intracellular enzymes are involved in selenite and selenate reduction

Removal of selenate is possibly mediated by two parallel mechanisms which form elemental Se and an unidentified non-volatile Se compound, likely a selenite ester. Indeed, TLC to determine the components present in selenate and selenite-reducing culture medium hinted towards the present of phenyl selenide or selenate ester-like compounds, still not ruling out the presence of other organic or inorganic reactive Se species at this time (data not shown).

D. lacustris is known to produce extracellular enzymes, i.e. chitinase and lysozyme and has been the first microorganism to report for extracellular degradation of peptidoglycan (Jørgensen *et al.*, 2009). However, in the present study, experiments with microbial cell lysate suggest that the microorganism reduces selenite and selenate intracellularly to organo-Se compounds and red elemental Se. The enzyme involved in selenate and selenite reduction to red elemental-Se is an intracellular enzyme.

Extracellular reduction of selenite has been reported previously by Lampis *et al.* (2014). Selenite was reduced by cell lysate of *D. lacustris* under ambient conditions (Supplementary data Fig. S2), suggesting that selenite reducing enzymes or molecules are produced constitutively by the bacterium. In contrast, selenate reduction was not achieved using cell lysate (Supplementary data Fig. S2), probably because it is an energy-driven reaction necessitating reducing equivalents or other co-factors. Previous studies have shown that supplementation of NADPH and NADH to the cell lysate and supernatant has improved reduction of selenite under abiotic conditions (Zheng *et al.*, 2014).

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