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Pseudomonas Stutzeri NT-I: Optimal Conditions for Growth and Selenate Reduction

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In this study, Pseudomonas stutzeri NT-I growth and selenate reduction were examined using aerobic batch experiments. Optimal growth conditions were determined in a mineral salt medium in the presence of background selenium. Optimal conditions for the reduction of selenate to selenite and elemental selenium was identified using harvested cells in a mineral salt medium. The reduction profiles of selenium were monitored using selenite as indicator species. A glucose and nitrogen independent maximum biomass concentration of 0.64 g/L dry cell weight was measured for all glucose concentrations above 2 g/L, signifying the presence of a population density control mechanism. Optimal growth conditions for the culture were obtained at a pH of 7, temperature of 37 °C, a salinity of 10 – 20 g/L NaCl, and a background selenium concentration of 5 mM. Optimal selenium reduction rates were observed at a temperature of 37 °C, pH 7 – 8 and salinity less than 5 g/L NaCl. The similarity of conditions for maximum growth and selenium reduction rates provide evidence that optimal operation can be achieved for both parameters simultaneously, a requirement for continuous operation. The microbe was capable of practically complete reduction of up to 4 mM selenate in less than 3 h of operation, translating to a volumetric reduction rate of between 0.2 mM/h (for 0.5 mM selenate) and 1.33 mM/h (for 4 mM selenate). The increasing mass-based reduction rates of between 0.006 mmol/g.h (for 0.5 mM selenate) and 0.1 mmol/g.h (for 4 mM selenate) indicate that the increased reduction rate was a result of both increased biomass and increased biomass activity with increased selenate concentration. Results from the study demonstrate the potential of the organism Pseudomonas stutzeri NT-I for the biological remediation of selenate and subsequent removal from the environment.

1. Introduction

Selenium is a metalloid and chalcogen with several oxidation states: selenate (SeO_4^{2-}, Se(VI)), selenite (SeO_3^{2-}, Se(IV)), elemental selenium (Se^0, Se(0)), and selenide (Se^{2+}). An estimated 38 % – 41 % of environmental selenium can be attributed to anthropogenic sources (Lenz and Lens, 2009). These include acid mine drainage (Lenz et al., 2008), wastewater from oil refineries (Lawson and Macy, 1995), selenium refinery wastewater (Satoshi et al., 2012), and the extensive use of coal and other fossil fuels for energy (Wu, 2004).
Selenium causes birth defects and reproductive problems among fish and waterfowl, while selenium overexposure in humans leads to respiratory difficulty, gastrointestinal distress, liver damage, and impairment of the central nervous system (Kenward et al., 2006). The maximum allowable concentrations of total selenium are regulated internationally; the National Primary Drinking Water Standard and the National Fresh Water Quality Standard in the USA prescribe limits of 50 µg/L and 5 µg/L respectively (USEPA, 1999). The World Health Organization proposes a value of 40 µg/L for countries without a specified legislative framework in place (WHO, 2011).
Due to the regulation of selenium effluent concentrations, effective remediation strategies for seleniferous industrial wastewaters are required prior to discharge. Technologies for selenium removal from wastewaters include physical, chemical (Frankenberger et al., 2004), and biological methods (Staicu et al., 2015a).
Although the reduction of metal species is thermodynamically feasible, large activation energies are usually required to initiate these reactions. The introduction of enzymes through biological species/material allow these reactions to proceed under ambient conditions (Karp, 2009). Several studies have utilised this technique for the

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reduction of metals: Cr(VI) to Cr(III) (Molokwane and Chirwa, 2009), U(VI) to U(IV) (Chabalala and Chirwa, 2012), and Pb(II) to Pb(0) (Brink et al., 2017).
While most identified selenium oxyanions-reducing organisms function under anaerobic, low selenium concentration conditions, several bacterial strains capable of reducing selenium oxyanions under aerobic conditions have been isolated. The benefits of aerobic reduction include increased biological activity, as well as resilience to aerobic industrial conditions. Examples of aerobic selenium oxyanions reducers most frequently include Pseudomonas species, e.g. P. fluorescens (Belzile, 2006), P. aeruginosa (SNT-SG1) (Gupta et al., 2010), P. stutzeri NT-I (Kuroda et al., 2011), P. seleniipraecipitatus (Hunter and Manter, 2011), and P. moraviensis stanleyae (Staicu et al., 2015b).
The microbial strain P. stutzeri NT-I has recently gained significant interest due to its ability to serially reduce selenate to selenite and subsequently elemental selenium for elevated selenate concentrations of up to 122 mM. Further, the organism has been shown to fully reduce 5 mM selenate and 4 mM selenite in under 20 h of incubation (Kuroda et al. 2011). In comparison, P. fluorescens reduced 95 % of a 0.2 mM selenite solution after 45 h (Belzile et al., 2006), Desulvibrio desulfuricans required 7-25 h to reduce 86 % of 1 mM of selenate (Tucker et al., 1998), and Escherichia coli EWB32213 reduced 3.1 mM selenate in 48 h of incubation (Ji and Wang, 2017).
The current study investigates the optimal conditions for growth and selenium oxyanion reduction for the microorganism P. stutzeri NT-I in a mineral salt medium.

2. Materials and methods

The optimisation experiments were limited to 1 g/L – 6 g/L glucose, temperature range 20 °C – 45 °C, pH 6 – 9, salinities 5 g/L – 20 g/L NaCl, and background selenium concentrations of 0 – 5 mM. The reduction rate experiments of selenate to elemental selenium in a concentrated biomass reaction system were limited to initial selenate concentrations of 0.5 mM – 4 mM.

2.1 Media

The study utilised Tryptone Soy Broth/Agar (TSB, TSA) and a glucose supplemented mineral salt medium (glucose-MSM at pH 7: 30 mM NH_4Cl, 0.8 mM Na_2SO_4, and 0.2 mM $MgSO_4$, 50 nM $CaCl_2$ (Sigma-Aldrich, St. Louis, MO), 25 nM $FeSO_4$, 0.1 nM $ZnCl_2$, 0.2 nM $CuCl_2$, 0.1 nM NaBr, 0.05 nM Na_2MoO_2, 0.1 nM $MnCl_2$, 0.1 nM KI, 0.2 nM H_3BO_3, 0.1 nM $CoCl_2$, and 0.1 nM $NiCl_2$). All chemicals were sourced from Merck (Merck, Darmstadt, Germany) unless otherwise specified. For optimisation experiments, the pH was varied according to ratios of K_2HPO_4:KH_2PO_4 (10 mM K_2HPO_4, 20 mM KH_2PO_4 for pH 7), the salinities were varied according to NaCl addition, the background selenium concentration was varied by addition of Na_2SeO_4 (Sigma-Aldrich, St. Louis, MO) to the medium, and substrate was varied by initial glucose concentrations. For reduction rate tests, selenate as Na_2SeO_4 (0.5 mM, 1 mM, 2 mM and 4 mM) was initally added to the glucose-MSM medium.

2.2 Bacterial strain and growth rate cultivation conditions

P. stutzeri NT-I (JCM 5965), originally isolated and characterised by Kuroda et al. (2011), was obtained from the NITE Patent Microorganisms Depository (NMPD) in Chiba Ken, Japan. To determine the exponential growth rate, the bacteria was pre-cultured by taking a loop of bacterial cells from a TSA plate and inoculating into 20 mL of glucose-MSM (2 g/L glucose at pH 7) in a 100 mL serum bottle and incubating aerobically for 24 h at 37 °C. Subsequently 5 mL was transferred to three 250 mL Erlenmeyer flasks each containing 100 mL glucose-MSM with a specified background selenium concentration and aerobically incubated. 3 mL samples were taken every 1-2 h for optical density measurements at 600 nm (OD_{600}) (Biochrom WPA Lightwave II, Harvard Bioscience, Inc., Holliston, MA), for 24 h. The dry cell weight (DCW) evidences the following relation with OD_{600}: an OD_{600} of 1.0 represents 416 mg DCW/L. These experiments were repeated for the conditions specified in Table 1.

2.3 Selenate reduction experiments: Condition optimisation and rate profiles

Biomass harvesting and concentration

The biomass harvesting and amplification procedure were performed by inoculating 20 mL of TSB in a 100 mL serum bottle and incubating for 24 h at 37 °C. From this pre-culture, 10 mL was transferred to 100 mL fresh TSB in a 250 mL Erlenmeyer flask and cultivated for 8 h at 37 °C. Subsequently the cells were harvested by centrifugation (6,000 rpm, 15 min, room temperature, Eppendorf® Minispin Z606235, Hamburg, Germany) and washed with physiological saline.

Table 1: Experimental conditions for determination of optimal growth rate conditions

Experiment	Glucose concentration (g/L)	Temperature (°C)	pH	Selenium concentration (mM)	Salinities (g/L)
1	0, 1, 2, 4, 6	37	7	1	5
2	2	20, 30, 37, 45	7	1	5
3	2	37	6, 7, 8	1	5
4	2	37	7	0, 1, 2, 5, 10	5
5	2	37	7	1	0, 5, 10, 20

Optimisation of selenate reduction

After the cells were harvested and washed, the biomass was resuspended in three 100 mL serum bottles each containing 20 mL glucose-MSM (1 mM selenate, 5 g/L glucose). The experimental conditions for the optimisation experiments are summarised in Table 2.

Table 2: Experimental conditions for determination of optimal selenium reduction conditions

Experiment	Temperature (°C)	pH	Salinities (g/L)
6	30, 37, 45	7	5
7	37	6, 7, 8, 9	5
8	37	7	0, 5, 10, 20

The average selenium reduction rate was quantified from the amount of selenium in the solid phase at termination of the experiment (16 h). This experimental timespan was chosen to ensure maximal selenium reduction with minimal selenium volatilisation (Kagami et al. 2013). At termination of the experiments, the reactor volumes were centrifuged and the selenium present in the plug quantified by ICP-OES. To confirm the selenium mass balance, the selenium present in the supernatant from the plug was analysed by ICP-OES.

Reduction experiments: Cultivation, sampling, and analysis

The selenate/selenite reduction profile experiments (experiments 9 – 12) were performed by concentrating and washing the biomass as described previously, and by resuspending the biomass in three 100 mL flasks each containing 30 mL of MSM (0.5, 1, 2, 4 mM selenate, 10 g/L glucose, pH 8). The flasks were incubated at 37 °C on a rotary shaker operated at 120 rpm. 1 mL samples were taken at specified time intervals and centrifuged (6,000 rpm, 15 min, room temperature, Eppendorf® Minispin Z606235, Hamburg, Germany). The supernatant was analysed for selenite concentration; selenite concentrations were used as an indicator for selenate reduction as it has been shown previously (Kuroda et al., 2011) that P. stutzeri NT-I irreversibly reduces selenate to selenite and subsequently elemental selenium.
The selenite concentration was quantified by the colourimetric method (3500-Se C Colorimetric method) in the Standard Methods for the Examination of Water and Waste (APHA et al., 2012). The method involves the reaction of selenite with 2,3-diaminonaphthalene (DAN); this produces a brightly coloured, fluorescent piazsenol which is extracted in cyclohexane and measured colourometrically at 480 nm. All the reagents for this method were obtained from Merck (Merck, Darmstadt, Germany), except Sodium Ethylenediaminetetraacetate (Na-EDTA), DAN, sodium selenate, and sodium selenite which were obtained from Sigma Aldrich (Sigma-Aldrich, St. Louis, MO).

3. Results

Experiment 1 showed that the biomass concentration reached a plateau at a DCW concentration of 0.64 g/L after about 10 h of fermentation (profile not shown) for all glucose concentrations over 2 g/L. This finding points to an independence of the maximum DCW on the glucose concentration. Using the standard biomass formula, $C_nH_{1.8n}O_{0.5n}N_{0.2n}$ (Villadsen et al., 2011), approximately 0.76 g/L glucose was required for the production of the observed biomass, suggesting that the observed maximum biomass was not a result of glucose depletion. An alternative cause for the discontinuation of biomass growth could have been due to nitrogen limitation in the medium. The only source of nitrogen in the MSM was NH_4Cl with an initial concentration of 30 mM. Using the standard biomass formula, the supplied nitrogen would theoretically yield a final biomass concentration of 3.69 g/L. According to literature, the ratio of nitrogen to carbon ranges from 0.26:1 for *Aerobacter aerogenes* to 0.16:1 for *Penicillium chrysogenum* and *Saccharomyces cerevisiae* (Villadsen et al., 2011), clearly demonstrating that a limited variation in this ratio is observed in nature. These results indicate that a population-dependent regulating mechanism such as quorum sensing (March and Bentley, 2004) is present which actively controls

the population density within the reaction volume. Therefore, irrespective of fermentation time, the biomass concentration of the organism would not exceed a maximum population density. Hence the cellular density would have to be artificially increased if higher reaction rates are sought.

The results from the growth rate optimisation experiments (experiments 2-5), the selenium reduction experiments (experiments 6-8), and comparative results from experiments performed by Kuroda et al. (2011) in the absence of a background selenium, are shown in Figure 1.

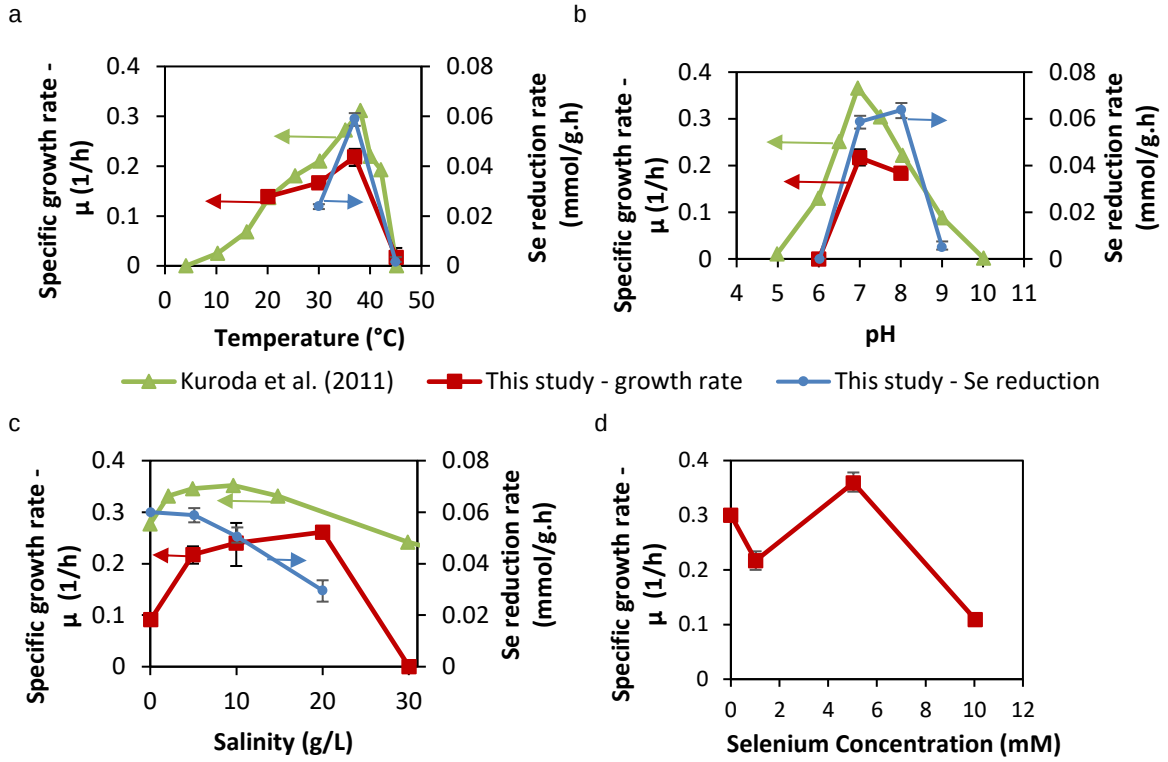

Figure 1: Results from the growth and selenate/selenite reduction optimisation experiments show the effect of (a) temperature, (b) pH, (c) salinity (concentration of NaCl) and (d) the background selenium concentration on the specific growth rate of P. stutzeri NT-I and the specific selenium reduction rates by P. stutzeri NT-I. Data points represent the average, and error bars indicate one standard deviation measured in three separate experiments.

Results from Figure 1 evidence that optimal growth conditions in the presence of background selenium were observed at a temperature of 37°C, pH 7, salinity between 10 g/L and 20 g/L NaCl, and a background selenium concentration of 5 mM. The optimal temperature, pH, and salinity results were similar to those observed by Kuroda et al. (2011) in the absence of selenium, with the difference being that the current study measured a slightly lower growth rate than previously reported. This corresponds with the decreased growth rate between 0 mM and 1 mM selenium (Figure 2d). It is interesting to note the increased growth rate at 5 mM as compared to 1 mM, which suggests a limited co-metabolic function of selenium as electron acceptor. It has been shown previously that P. stutzeri is capable of reducing selenate to selenite anaerobically (Kuroda, 2011), indicative of an anaerobic respiration mechanism with selenate as terminal electron acceptor. The decrease in growth rate at selenium concentrations above 5 mM is likely a result of the toxicity of selenium at elevated concentrations.

The optimal selenium reduction rate conditions were observed at a temperature of 37 °C, pH 7 – 8, and salinity of 0 – 5 g/L NaCl. These values approximate the optimal growth conditions of the culture, implying that the optimal reduction of selenium and biomass growth could be achieved simultaneously. This means that the biomass could by kept viable while reduction takes place, a requirement for continuous operation of the remediation process.

Figure 2a shows the normalised selenite concentrations for the reduction experiments at various initial selenate concentrations. Note that practically complete removal of selenium oxyanions was achieved for all initial concentrations of selenate after 3 h. This is almost an order of magnitude faster than comparative reduction experiments from literature, the fastest known being 5 mM selenate in 20 h (Kuroda, 2011). The rates of selenite reduction increased with elevated selenate feed. The fractional amount of selenite reached a maximum of 40 %

of the feed amount for the 0.5 mM, 1 mM, and 2 mM selenate feed, i.e. 0.2 mM, 0.4 mM and 0.8 mM selenite respectively. The fractional amounts of selenite in the 4 mM selenate experiment only reached 10 % (0.4 mM selenite).

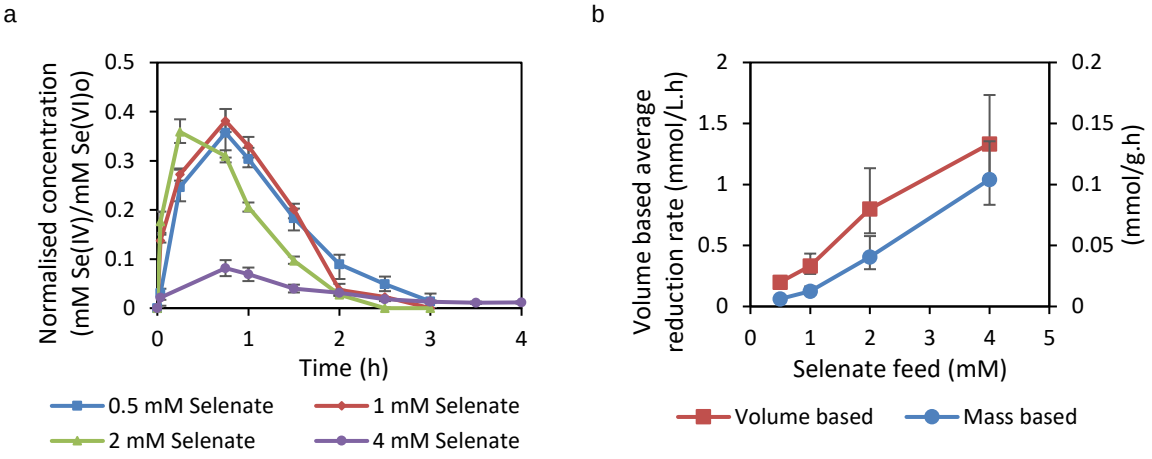

Figure 2: a) The normalised selenite concentrations measured in the reduction rate profile experiments (experiment 9 – 12). b) The volumetric and mass-based average selenate reduction rates observed in experiments 9 – 12.

Figure 2b shows the volume- and mass-based average selenate reduction rates for the various initial concentrations of selenate. Interestingly, the both the volumetric and mass-based rates of selenate reduction increased with rising selenate feed concentrations. This implies that the increased volumetric reaction rates were not only a result of increased biomass concentrations, but also of an intrinsic increase in the reducing ability of the biomass as demonstrated by increases in the mass-based reaction rate. It can be hypothesised that the organism responds to an increase in selenate concentration with the production of various enzymes or equivalent electron carriers to compensate for the increased selenate concentrations. Zawadska et al. (2006) found that the strain P. stutzeri KC excretes a siderophore (pdtc) that is capable of reducing selenium and tellurium extracellularly and appears to act as an external defense against elevated concentrations of these metals.

4. Conclusions

In conclusion, the organism P. stutzeri NT-I exhibited maximum growth and selenium reduction rates at similar mesophilic conditions. Because of this, optimal growth and reduction can be achieved simultaneously, a requirement for continuous selenium removal processes. The microorganism was capable of practically complete reduction of up to 4 mM selenate in less than 3 h in an artificially concentrated biomass reaction system. As such, even though a maximum cellular density of 0.64 g/L was observed in batch operation, it is possible to increase the volumetric reduction rates of selenate/selenite in the system by artificially concentrating the biomass in the reactor.

The increasing volumetric and mass based reduction rates for increasing selenate loading implies that the observed reduction rates were not only a result of increased biomass concentrations, but also an intrinsic escalation in reduction activity of the biomass in response to the elevated selenate concentrations. This suggests that the organism responds to the background concentration of selenium in the medium by increasing the reduction activity. Consequently, the exposure time of the microbe to dissolved selenium in the medium is reduced. The study highlights the potential of P. stutzeri NT-I for use as a biological reduction catalyst for the immobilisation and removal of selenate and selenite from contaminated water sources.

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