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Optimisation of Selenite Reduction using *Enterococcus* spp. under Anaerobic Conditions

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With the drastic increase in selenium-releasing anthropogenic activities, the bioaccumulation of toxic selenium oxyanion in the environment has increased significantly, posing deleterious effects to living organisms. The bio-removal of selenite by *Enterococcus* spp. under both aerobic and anaerobic batch conditions was examined using batch reactors containing Tryptone Soy Broth (TSB) laced with sodium selenite. The optimal conditions for bacterial growth and selenite reduction were determined under anoxic conditions. *Enterococcus* spp. reduced selenite more effectively under anaerobic conditions, with 77 % selenite reduction after 20 h. The bacteria also reduced selenite under aerobic conditions, but the process was much slower, with approximately 68 % reduction after 72 h. This correlated with the more rapid bacterial growth seen in the anaerobic batch reactor. Optimal growth conditions for *Enterococcus* spp. were obtained at a temperature of 25°C, pH of 6 and initial selenite concentration of 30 mg/L. Optimal selenite removal was at temperature of 35°C, pH of 7, and initial selenite concentrations of 30 mg/L. A red precipitate was formed during the selenite removal experiments, a probable sign that the selenite was being reduced to form elemental selenium. The results of this study demonstrated the capability of *Enterococcus* spp. to bio-transform toxic selenite oxyanions under both oxic and anoxic conditions.

1. Introduction

Selenium is a non-metallic chalcogen that naturally exists in the environment in four oxidation states: selenate (SeO_4^{2-}), selenite (SeO_3^{2-}), elemental selenium (Se^0) and selenide (Se^{2-}) (Lenz and Lens, 2009). It is an essential micronutrient for living organisms, and it makes up at least 25 proteins within the human body (Papp et al., 2007). Selenium deficiency in humans has dire consequences such as foetal growth restriction, reduced immune response and increased mortality (Ligowe et al., 2020). The recommended selenium intake in men ranges between 30 and 85 µg/d and 30 and 70 µg/d in women, depending on the geographic location (Kieliszek, 2019). Apart from its nutritional benefits, selenium has various applications in fields such as the glass, agriculture, medicinal and electronics industries (Tan et al., 2016, Shiri, 2020).

While selenium naturally exists in the environment and is beneficial, human activities have caused a drastic increase in its environmental concentrations (Lenz and Lens, 2009). A study done in 1989, estimated that anthropogenic activities make up approximately 41 % of the total selenium emissions (Nriagu, 1989). Since then, there has been a rise in industrialisation and urbanisation, consequently resulting in increased selenium emitting human activities. Coal combustion and the use of selenium-containing agricultural products are amongst the highest-ranking anthropogenic activities (Lemly, 2004). Selenium enters the aquatic environment through various sources including agriculture drainage water and residue from coal combustion (Lenz and Lens, 2009). Moreover, selenium predominantly exists as selenite (Garousi, 2015), which is toxic, soluble in water and bioaccumulate in the food chain, even at low concentrations.

Over the years, several treatment avenues for seleniferous wastewater have been explored including, physical, chemical, biological and hybrid treatment methods (Lenz and Lens, 2009). The biological treatment route has gained interest as it is more environmentally friendly and cost-effective (Tendenedzai et al., 2020).

Numerous microorganisms capable of transforming the toxic selenium oxyanions into less toxic elemental selenium and organic selenium compounds have been identified. Very few studies have been conducted to investigate the selenium reducing capabilities of lactic acid bacteria (LAB). *Enterococcus*, amongst other LAB,

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is a facultative anaerobe that is considered generally safe (GRAS), has probiotic properties, and is used in the food fermentation industry. *Enterococcus durans, Enterococcus faecalis* and *Enterococcus faecium* are amongst the previously discovered selenium-reducing *Enterococcus* species (Pieniz et al., 2011). A 2017 study illustrated that *Enterococcus faecalis* can aerobically reduce sodium selenite to elemental selenium nanoparticles (Shoeibi and Mashreghi, 2017). *Enterococcus faecium* 37 CCDM 922A assimilated approximately 98 % sodium selenite into selenium proteins (Krausova et al., 2020). Recent studies have also shown aerobic experiments in which *Enterococcus* spp. have reduced selenite both biotically (Tendenedzai et al., 2021) and abiotically (Tendenedzai et al., 2022).
In this study, the bio-removal of selenite by *Enterococcus* spp. under both aerobic and anaerobic conditions was investigated. The optimal bacterial growth and selenite reduction conditions were determined, in terms of temperature, pH and selenite concentrations, under anaerobic conditions.

2. Material and methods

2.1 Growth media

Tryptone Soy Agar (TSA) (sourced from Oxoid Ltd., Basingstoke, Hants, UK), used for colony isolation and growth, was prepared by dissolving 40 g of the TSA powder in 1 L distilled water. Tryptone Soy Broth (TSB) (sourced from Oxoid Ltd., Basingstoke, Hants, UK), the growth media for all experiments, was prepared by dissolving 30 g in 1 L of distilled water. Both media were sterilised by autoclaving at 121 °C and 4 MPa for 15 min.

2.2 Selenite stock solution

A selenite stock solution of 100 mM (12 697 mg/L = ppm) was prepared by dissolving 17.293 g of 99 % Na_2SeO_3 (Sigma-Aldrich, St. Louis, MO, USA) in 1 L of distilled water and autoclaved at 121 °C for 15 min. This stock solution was used for all the experiments conducted.

2.3 Bacteria isolation, cultivation, and storage

The *Enterococcus* spp. used in this study was the product of an accidental bacteria contamination in the laboratory, and it was previously analysed and identified at the Inqaba Biotechnical Industries (Pty) Ltd. (Pretoria, South Africa) in a study by (Tendenedzai et al., 2021). The *Enterococcus* spp. was cultivated by transferring the culture into 50 mL TSB in a serum bottle capped with aluminium foil and inoculating for 24 h at 120 rpm and 35 ˚C on a rotary shaker (FSIM-SPO8, Labcon, Johannesburg, South Africa). After 24 h, a 0.2 mL aliquot was transferred into pre-sterilised vials containing 0.8 mL of 50 % glycerol solution. The vials were stored in a -70 °C storage chamber until their usage. The strain in the frozen vials was revived by being naturally thawed and streaked on agar plates using a wired loop. The bacteria culture was left to grow for 24 h in a 35 °C incubator before being stored at 0 °C for future use.

2.4 Batch reduction experiments

Batch experiments were conducted under aerobic and anaerobic conditions to determine the effect of aeration on bacterial growth and selenite removal. A loop of bacteria cells from the agar plates was inoculated in 50 mL of TSB, placed in a 100 mL serum bottle covered with aluminium foil, on a rotary shaker (120 rpm, 35±2 °C, pH ≥7) for 24 h. After 24 h, 200 µL of the aliquot was transferred to 100 mL serum bottles containing fresh TSB laced with 150 mg/L selenite. For the aerobic reactors, the serum bottles were covered by aluminium foil while the anaerobic reactors were firstly purged with nitrogen for 5 min to displace the oxygen and then sealed with rubber caps. Samples were taken at predetermined time intervals to measure the bacterial growth and selenite concentration over the 72-h experimental time. A syringe was used to take samples from the anaerobic reactors and a pipette for the aerobic ones. All experiments were conducted in triplicate.

2.5 Optimisation of anoxic selenite reduction

The optimum conditions for the selenite bio-removal under anaerobic conditions were determined in terms of pH, temperature, and initial selenite concentration. For all the parameters, the bacterial growth and selenite concentrations were measured, and all experiments were conducted in triplicate.

Temperature

The effect of temperature on bacterial growth and selenite reduction was determined by incubating the cultures in temperatures ranging from 25 to 45 °C for 24 h. Firstly, a loop of the bacteria strains in 50 mL TSB under aerobic conditions, and thereafter, 200 µL (OD_{600} = 0.667) of the inoculum was transferred into 50 mL fresh TSB medium amended with 140 mg/L Na_2SeO_3. The mixture was purged with nitrogen for approximately

5 min before being sealed with a rubber cap. The initial pH in reactors was approximately 7, and measurements were taken at the start of the experiment and 24 h later.

pH

Aluminum foil for 24 h. Then, 200 µL (OD600 = 1.505) inoculum was placed into 50 mL fresh broth laced with Na2SeO3 (140 mg/L), the mixture was purged with nitrogen to displace the oxygen and inoculated for 24 h. The pH in the reactors was varied from 5 to 9, and 300 mM HCl and 300 mM NaOH were used to alter the pH to the desired valuesSimilar to temperature, a loop of culture was inoculated in 50 mL of TSB in 100 mL serum bottles covered with.

Initial concentration

The effects of different initial selenite concentrations were determined using TSB medium amended with 15, 30, 60 and 120 mg/L of selenite. The selenite laced TSB media were inoculated with 200 µL aliquots of *Enterococcus* spp. and incubated at 35 °C for 24 h. Inoculum preparation was similar to temperature optimisation.

2.6 Analytic methods

Parameters were measured at the sampling extraction time intervals namely: bacterial growth and selenite concentrations. Bacteria growth was quantified using optical density (OD_{600}), with TSB containing selenite in the absence of the bacterial cells as the blank. The cell growth was determined by placing 2 mL of the sample into cuvettes and measuring the OD_{600} on a spectrophotometer (WPA, Light Wave II, Labotech, South Africa). To determine the unreduced selenite concentration in the reactors, 2 mL of the samples were placed into small centrifuge tubes and centrifuged at 6 000 rpm and 25 °C for 10 min. From the supernatant, 1 mL was taken a placed into a small plastic bottle, and 9 mL of NaOH (300 mM) was added to stabilise the solution. Measurements were taken using the Varian AA–1275 Series Flame atomic absorption spectrometer (AAS) (manufactured by Perkin Elmar, Varian, Palo Alto, CA (USA)).

3. Results and discussions

Since *Enterococcus* is a facultative anaerobe, its ability to grow and reduce selenite under aerobic and anaerobic conditions was tested. Figure 1a compares the bacterial growth of *Enterococcus* spp in the presence of selenite under oxic and anoxic conditions over 72 h. Despite the stationary phase being reached after 20 h for both setups, the biomass production under anaerobic conditions was higher. A 2-h lag occurred under both conditions. At 28 h, an unexpected rise in bacterial growth was observed for the aerobic setup, but at 72 h, the growth declined drastically.

Despite most of the previous studies on selenite removal using *Enterococcus* species being conducted under aerobic conditions, the results in Figure 1b show more rapid selenite removal under anaerobic conditions. A control experiment was done in the presence of selenite but in the absence of biomass to show that the bacterial cells were responsible for selenite reduction. At 20 h, approximately 77 % selenite removal had occurred under anaerobic conditions, and beyond this, no more selenite removal occurred. For the aerobic batch reactors, only 22 % selenite reduction had occurred after 20 h and 68 % removal after 72 h. The reduction of selenite occurred during the exponential phase for the anaerobic setup, but for the aerobic one, selenite removal only began once the exponential phase was reached. The variation in the growth phase at which reduction occurs may be because of the bacteria using different mechanisms for selenite reduction under oxic and anoxic conditions.

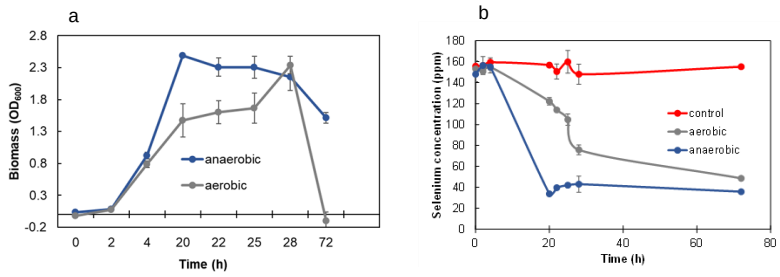

Figure 1: Time course of a) biomass production b) selenite reduction under aerobic and anaerobic conditions.

The optimum temperature for bacteria growth was studied, and as seen in Figure 2a, the optimum temperature was 25 °C with an OD_{600} of 2.411. At 35 °C, there was a slight decrease in bacteria growth. The effect of temperature on the cell growth of the *Enterococcus* species has previously been reported. A study by Pieniz et al. (2011) on *Enterococcus faecalis* and *Enterococcus faecium* found the optimum biomass production was at 25 °C for both isolates, but contrary to our study, there was a significant decrease in growth seen at 45 °C. For *Enterococcus durans*, a decline in bacterial growth was seen at 40 °C, and the maximal bacterial growth was observed at 35 °C (Pieniz et al., 2013).
The amount of selenite bio-removed over various temperatures was measured within a 24-h reaction period. A control experiment, without any cells to show that biomass was required for selenite reduction was conducted and the results are shown in Figure 2b corroborate this hypothesis. Contrary to the observation for bacterial growth, 35 °C displayed the optimum selenite removal, with approximately 75 % selenite removal. A significant decline in the selenite removed by the bacteria was observed at 25 °C. For *Enterococcus* faecalis and *Enterococcus* f*aecium*, the selenite removed was relatively constant from 25 to 45 °C (Pieniz et al., 2011). For *Enterococcus durans*, the selenium removal results were consistent with the bacterial growth results; the optimum selenite removal temperature was 30 °C (Pieniz et al., 2013).

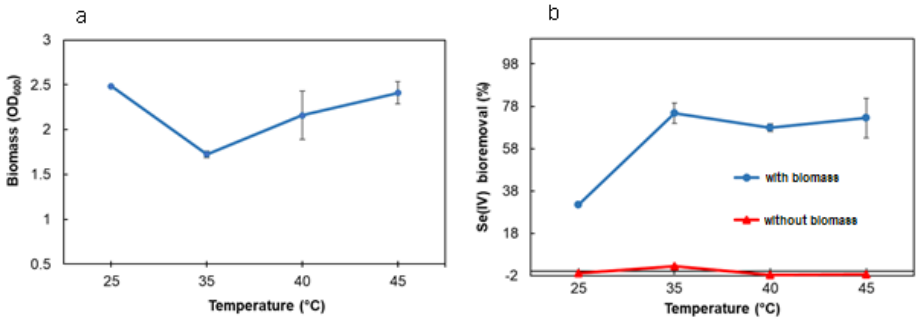

Figure 2: Effect of temperature (25 -45 °C) on a) bacterial growth b) selenite bio-removal after 24 h incubation.

Figure 3a illustrates the results obtained from experiments conducted to investigate the impact of pH on bacterial growth. Luxuriant growth was observed for initial pH ranges between 6 and 8; however, maximal bacterial growth was at 6 (OD_{600} = 2.613). There was a slight decline in growth observed at a pH of 5, and the growth reduced quite significantly at a pH of 9. (Wessels and Chirwa, 2017) compiled a table of optimum pH for selenium removing bacteria and concluded that the optimum bacterial growth rate was mainly between a pH of 7 and 8. For *Enterococcus faecalis* and *Enterococcus faecium*, optimal growth occurred at an initial pH range of 6 – 9 but poor growth at acidic conditions (pH of 4 and 5) (Pieniz et al., 2011). The trend observed for the effect of pH on *Enterococcus durans* bacteria growth was similar to the one observed in this study (Pieniz et al., 2013). In this study, the optimal pH for selenite removal was 7, which falls within the range of optimal bacterial growth. At pH 8, the selenium removal capacity was very close to the one observed at pH 7. Overall, pH of 7 and 8 was the optimum for growth and selenite removal. Despite a pH of 6 resulting in good bacterial growth, a significant decline in selenium removal was observed. As seen in Figure 3b, selenite removal was poor at pH 5. Selenite removal remained constant for the pH range 6 - 9 for *Enterococcus faecalis and Enterococcus faecium*. *Enterococcus durans* bioaccumulated more selenite ions for a pH range of 6 - 8, and selenite removal decreased for pH lower than 6.

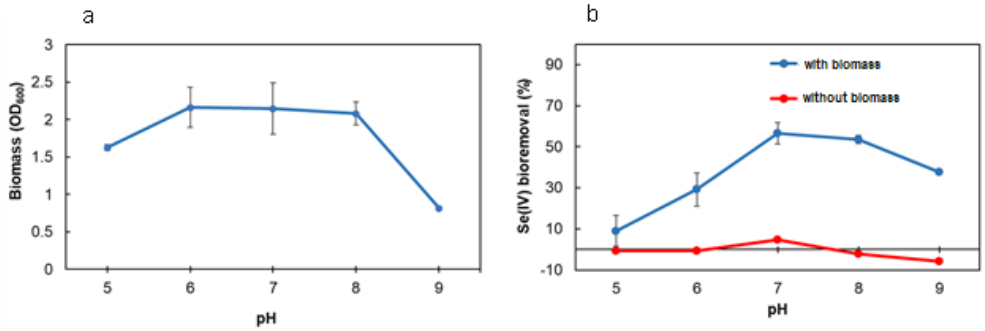

Figure 3: Effect of initial pH (5-9) on a) bacterial growth b) selenite bio-removal after 24 h incubation.

The effect of various initial selenite concentrations (0, 15, 30, 60 and 120 mg/L) on *Enterococcus* spp. growth was investigated, and the results are in Figure 4a. The highest cell density occurred in the absence of

selenite, which shows the toxic effect of selenite ions on the bacteria. In the presence of selenite, the maximal bacteria growth was at 30 mg/L, and there was a decline in biomass production beyond this concentration. A study by Tendenedzai et al. (2021) and Shoeibi and Mashreghi (2017) on similar bacteria (*Enterococcus* spp.) and *Enterococcus faecalis*, respectively, also showed a decline in biomass production with an increase in initial selenite concentration. Prior studies were conducted on other *Enterococcus* strains with the same initial concentrations. Contrary to this study, biomass production increased with concentrations for these species such as *Enterococcus faecium*, *Enterococcus faecalis* and *Enterococcus durans* (Pieniz et al., 2011).
For all experiments conducted under both aerobic and anaerobic conditions, a time-dependent colour change was observed (not shown). The colour changed from a clear yellow/brown colour initially, to a red colour in the presence of selenite. For the experiments conducted with biomass in the absence of selenite, the colour changed into a cloudy yellow over the experimental time. Figure 5 shows the colour change over a 2-h incubation period for different initial selenite concentrations. From the visuals, the red colour got darker with an increase in initial selenite concentration. Although the red was darker at higher initial selenite concentrations, the highest quantified selenite removed was at 30 mg/L, and the lowest was at 120 mg/L. The trend was similar to the one seen for biomass production in Figure 4a. The colour change to red usually is attributed to the formation of elemental selenium as reported by Tendenedzai et al. (2022). Since elemental selenium is less toxic than selenite, this could be the reason for the higher biomass production corresponding to more rapid selenite removal.

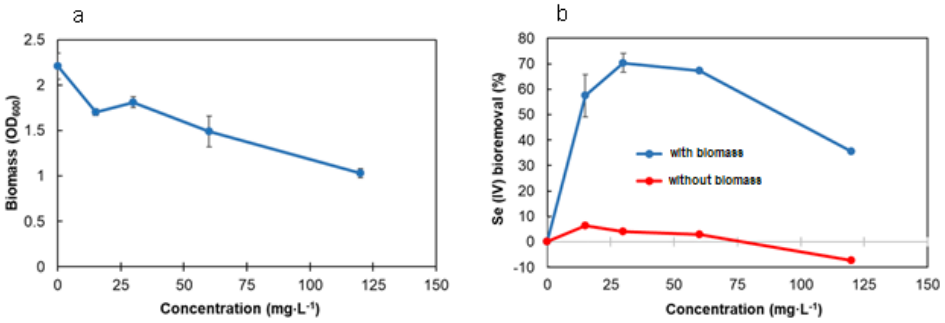

Figure 4: Effect of initial selenite concentration (0-150 mg/L) on a) bacterial growth b) selenite bio-removal after 24 h incubation.

Figure 5: Selenium suspensions for various concentrations 0 mg/L (A), 15 mg/L (B), 30 mg/L (C), 60 mg/L (D) and 120 mg/L (E)) of Na_2SeO_3 in 50 mL medium culture after 24 h.

4. Conclusion

The finding of this study illustrated a higher growth rate and selenite removal rate for *Enterococcus* spp. under anaerobic conditions. Approximately 77 % selenite was removed from the media by the bacteria after 20 h in the anaerobic batch setup. The maximum selenite removed from the aerobic batch reactors was 68 %, and this was after 72 h. The optimum pH, temperature, and initial selenite concentrations for bacterial growth and selenite reduction conditions were determined for anaerobic batch reactors. The optimum growth conditions were 25 °C, pH 6 and initial selenite concentration of 30 mg/L, while the optimum conditions for selenite removal were 35 °C, pH 7 and an initial selenite concentration of 30 mg/L. Given that *Enterococcus* spp. is a facultative anaerobe and has proved capable of reducing selenite under both anaerobic and aerobic conditions, it requires more attention as a selenite-reducing microbe. Further research will be required to increase the selenite percentage reduction and to also understand the mechanisms involved.

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