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The Effect of Carbon Source Loading on Selenite Reduction by *Enterococcus* spp

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This study investigated the bioremediation capabilities of *Enterococcus* spp. for mitigating selenite (SeO_3^{2-}) contamination, focusing on the impact of varied carbon source loadings. The investigation not only explored the bioremediation potential but also endeavoured to elucidate the underlying mechanisms governing selenite bioremediation. Carbon source selection emerged as a critical determinant, significantly influencing SeO_3^{2-} reduction, elemental selenium (Se^0) formation, and protein synthesis. Aerobic batch experiments were conducted to reduce an initial SeO_3^{2-} concentration of 1 mM. In each case, the carbon source was either glucose, cooking oil or crude oil. Glucose and cooking oil were identified as the most efficient carbon sources, facilitating rapid SeO_3^{2-} reduction. Of the initial SeO_3^{2-} concentration (1 mM) ,59 % and 56 % reduction was observed in the presence of glucose and crude oil respectively, after 50 h. In contrast, crude oil, only resulted in a 39 % reduction. Findings from this study not only underscored the efficiency of *Enterococcus* spp. in selenite reduction but also highlighted the influence of carbon sources on the overall bioremediation process. The identification of glucose and cooking oil as optimal carbon sources opens avenues for further research into tailoring bioremediation strategies based on specific environmental conditions.

1. Introduction

Selenium (Se), a vital micronutrient for both plants and humans, requires strict environmental monitoring and regulation due to its narrow safety range (Brozmanova *et al*, 2010; Huang *et al*, 2019). Anthropogenic activities like mining, coal burning, and agriculture (Mehdi *et al*, 2013) have elevated Se levels, contaminating surface waters with selenate and selenite oxyanions. The recommended maximum allowable concentration of Se oxyanions in surface water is 40 $\mu g.L^{-1}$ (Brink *et al*, 2018). Similar issues exist with other heavy metal ions such as lead which also exceeds recommended concentrations of between 10-15$\mu g.L^{-1}$ in drinking water (Chowdhury *et al*, 2022) due to human activities.

Conventional methods for remediating metal and non-metal ion contamination, such as physical and chemical techniques, are effective but often costly, environmentally unfriendly, and require extensive downstream processing. Biological methods offer promising solutions, particularly bacterial bioremediation, which mitigates these challenges (Lampis *et al*, 2014; Van Khanh Nguyen *et al*, 2016). By embracing the circular economy concept, the aim is to not only remediate but also to derive economic value from by-products (Wang *et al*, 2022). Some metals and non-metals can be transformed into valuable nanomaterials during reduction from ionic to elemental forms.

In this study, we investigated the bioremediation capabilities of selenite (SeO_3^{2-}) by *Enterococcus* spp. across various carbon sources, i.e. glucose, cooking oil, and crude oil. The choice of carbon sources is crucial, as it can significantly impact the microorganism's metabolic activity and subsequent production of other metabolites like biosurfactants. Microbes compete for carbon, which is a limited resource and serves as an energy source for them. The availability and choice of carbon source can influence the growth and competition of microorganisms, thereby affecting the overall efficiency of the bioremediation process (Ayilara and Babalola, 2023).

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Understanding how *Enterococcus* spp. responds to these diverse carbon substrates sheds light on its adaptability and potential for bioremediation and environmental applications. By exploring these different carbon sources, this can assist in future work on their potential role in the bioremediation of other heavy metals.

2. Materials and methods

2.1 Bacterial storage and cultivation

The bacterial cultures utilised in this study were *Enterococcus* spp., which were isolated from a Se-laden medium obtained from a laboratory at the University of Pretoria, South Africa. The characterisation and identification of the bacteria were performed using TEM, SEM, and 16s rRNA sequencing, with the latter outsourced to Inqaba Biotechnical Industries (Pty) Ltd (Tendenedzai *et al*, 2021). For cultivation and storage, the *Enterococcus* spp. were cultured in Tryptone Soy Broth (TSB) for 24 h at 28 °C on a rotary shaker operating at 120 rpm (FSIM-SPO8, Labcon, Johannesburg, South Africa). After 24 h, 0.8 mL of the TSB containing the bacteria was transferred to sterilised 2 mL vials, followed by the addition of 0.2 mL of a 50% glycerol solution. These vials were then placed in a freezer at -70 °C. To revive the bacterial strain, the frozen vials were removed from the freezer, and loops were used to streak the contents of the vials onto agar plates.

2.2 Batch reduction experiments

All chemicals used were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. The experiment was conducted to investigate the bioreduction of SeO_3^{2-} (as Na_2SeO_3) using *Enterococcus* spp. in batch reactors. Initially, agar plates were prepared, and the bacteria were streaked onto the plates. The plates were then incubated overnight at 35 °C. After 24 h, colonies that had grown on the plates were selected and inoculated into a broth culture medium. The broth culture was further incubated for an additional 24 h to facilitate bacterial growth. Three batches of Mineral Salt Medium (MSM) described by Tendenedzai *et al* (2021) were prepared in triplicate with a SeO_3^{2-} concentration of 1 mM (173 ppm), and each batch was supplemented with a different carbon source: glucose, cooking oil, or crude oil. The carbon sources were added at a concentration of 1% w/v. The MSM batches were sterilized and distributed into separate batch reactors. Each reactor was inoculated with 10 ml of the broth culture prepared earlier before the reduction reactions (35±2 °C, 120 rpm, pH≈8, 50 h). At various time intervals, samples were collected from each batch reactor. The experiments were carried out in triplicate and with appropriate controls, including control samples without bacteria or carbon sources, to account for any abiotic changes in selenite concentration or other parameters.

2.3 Analytical methods

The SeO_3^{2-} concentrations in the samples were determined using a 940 Professional IC Vario ion chromatograph (Metrohm, Herisau, Switzerland) with separation column Metrosep C 6-250/4.0 (Metrohm, Switzerland) and C 6 eluent 8 mM oxalic acid (Metrohm, Herisau, Switzerland). The protein concentration in the samples was determined using the Bradford assay (Bradford, 1976; He, 2011). The Bradford protein assay is a widely used method for protein quantification, employing the binding of protein molecules to Coomassie dye under acidic conditions, leading to a noticeable colour transition from brown to blue. This colour change occurs due to the dye's absorption maximum shifting from 465 nm to 595 nm upon binding to proteins, allowing for monitoring of the increased absorption at 595 nm (Bradford, 1976). Elemental selenium was quantified as total selenium using a Varian AA–1275 Series Flame AAS (Perkin Elmar, Varian, Palo Alto, CA, USA) at 196.03 nm wavelength equipped with a 290 mA selenium lamp. The pellet was first digested in 0.1 mL 55% nitric acid (Glassworld, Johannesburg, South Africa) and 0.1 mL distilled water. Data analysis involved plotting the SeO_3^{2-} or Se^0 concentrations over time for each carbon source (glucose, cooking oil, and crude oil) to observe the reduction kinetics. Additionally, the protein concentration at different time points was determined to evaluate biomass growth.

3. Results and interpretation

3.1 SeO_3^{2-} reduction and Se^0 formation

Figure 1 depicts the different colours scheme after 50 h, using different carbon sources for SeO_3^{2-} reduction. Glucose had the most intense red colouration followed by cooking oil and lastly crude oil. This is indicative of the level of Se^0 formation indicating a precipitation mechanism used by the bacteria (Stabnikova *et al*, 2023).

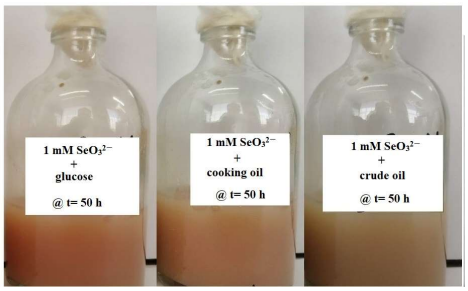
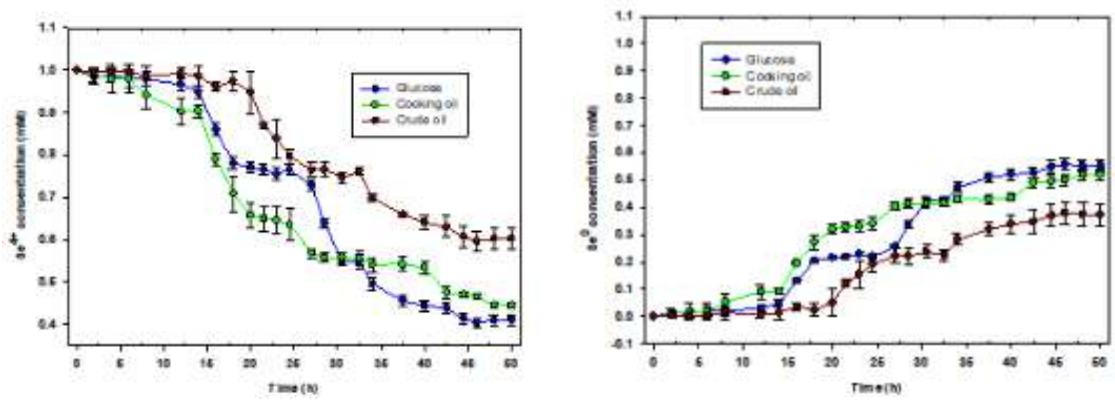


Figure 1 SeO_3^{2-} reduction with different carbon sources



(a) (b)

Figure 2: (a) SeO_3^{2-} reduction and (b) Se^0 formation with various carbon sources

Figure 2 (a) shows the trends in selenite reduction. For glucose as the carbon source, *Enterococcus* spp. demonstrated a dual response. Initially, there was rapid SeO_3^{2-} reduction, with its concentration decreasing sharply from 1 mM at time 0 h to 0.411 mM at time 50 h (59 % reduction). This indicated that glucose strongly supports selenite reduction.Cooking oil as a carbon source exhibited a trend similar to that of glucose but with some differences. SeO_3^{2-} reduction started at 8 h and continued gradually, resulting in a final concentration of 0.445 mM at 50 h, indicating effective reduction (56 %), although slightly slower than with glucose.
Crude oil on the other hand displayed a distinct trend. Initially, there was no significant SeO_3^{2-} reduction or protein production for the first 16 h. However, SeO_3^{2-} reduction and protein formation were notable thereafter. The SeO_3^{2-} concentration decreased to 0.603 mM at 50 h (39 % reduction), indicating a slower reduction rate compared to glucose and cooking oil.Figure 2 (b) depicts elemental selenium formation. In the presence of glucose, there was no measurable formation at time 0 h. However, as time progressed, Se^0 began to accumulate steadily. The rate of Se^0 formation increased notably at 16 h and its final measured concentration was 0.552 mM at 50 h. Glucose appears to support the gradual formation of Se^0, with a more significant increase observed in the later stages of the experiment.Similar to glucose, with cooking oil there was no initial Se^0 formation at time 0 h and it only became apparent after 16 h, and its concentration increased steadily over time. The highest measured accumulation of Se^0 was 0.519 mM at 50 h. The trend is consistent with the gradual accumulation of Se^0, with a slight lag compared to glucose.Crude oil as a carbon source showed a slow but still significant support for Se^0 formation. Se^0 concentration reached a peak of 0.379 mM at 46 h and remained relatively stable thereafter.

Table 1: Selenium balance across all the carbon sources

Carbon source	SeO_3^{2-} measured (mM)	Se^0 measured (mM)	Total Selenium (mM)
Glucose	0.411	0.552	0.963
Cooking oil	0.445	0.519	0.964
Crude oil	0.603	0.379	0.982

Table 1 shows the total selenium balance done across the different carbon sources. Each row represents a specific carbon source, and the columns display the measured concentrations of SeO_3^{2-}, Se^0 and the calculated total selenium in mM.The selenium accountability for glucose, cooking oil, and crude oil was 96 %, 96 %, and 98 %, respectively. These high recovery rates indicate reliability in both SeO_3^{2-} measurement and Se^0 recovery methods. Marginal differences observed suggest minor experimental errors during measurement and digestion. It was concluded that almost all of the reduced SeO_3^{2-} was successfully recovered as Se^0 across all carbon sources, eliminating assumptions of volatilisation. This finding is significant as it mitigates concerns regarding volatilization, which has been observed with certain bacterial species (Kagami *et al*, 2013).

3.2 Protein synthesis

Protein production remained low during the first 6 h but then increased significantly, reaching a peak of 2.243×10^{-3} g.L^{-1} at 50 h with glucose as the carbon source. This is shown in Figure 3. The protein content was not used only as an indicator of the likely metabolites produced by the bacteria but also as a proxy indicator for the growth and viability of the bacteria throughout the reduction of selenite (Zheng *et al*, 2014). The substantial increase in protein production suggests that glucose also promotes robust protein synthesis. Overall, glucose serves as an efficient carbon source for both selenite reduction, elemental selenium formation and protein production by *Enterococcus* spp.

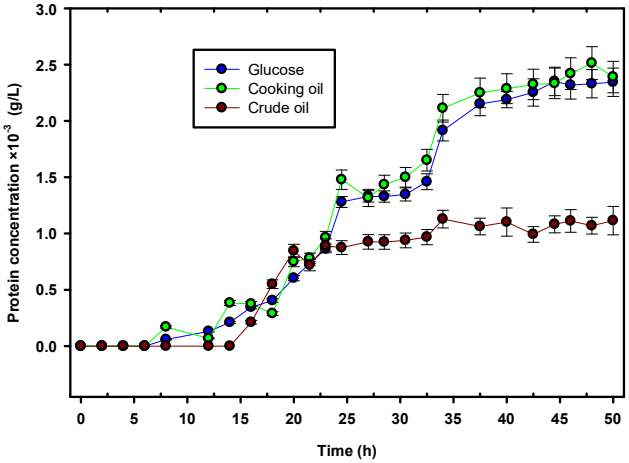


Figure 3: Protein concentration with different carbon sources during SeO_3^{2-} reduction

In the presence of cooking oil, protein production also began around the 8 h mark with a maximum protein concentration of 2.513×10^{-3} g.L^{-1} at end of the run. While the timing of protein production was similar to SeO_3^{2-} reduction, the protein yield was notably high with cooking oil.

Crude oil appeared to delay its support for selenite reduction and protein production, suggesting that the bacteria may require a longer adaptation period for metabolization. Protein production increased gradually, reaching a peak of 1.115×10^{-3} g.L^{-1} at 50 h. Overall, crude oil can ultimately serve as a carbon source for both selenite reduction and protein synthesis, but the response is delayed compared to glucose and cooking oil and less effective due to lower selenium reduction degree and protein production.

Crude oil is a complex mixture of hydrocarbons and other organic compounds, which makes it a difficult carbon source for bacteria to metabolise compared to simpler substances like glucose or cooking oil (Nikolova and Gutierrez, 2021). In contrast, glucose is easier to metabolise providing a readily available source of energy and carbon for metabolic processes. Similarly, cooking oil, although composed of a mixture of fatty acids and lipids, is easier to metabolise relative to crude oil (Kachienga, 2020; Patowary *et al*, 2017).

The recalcitrance of oil-based substrates for *Enterococcus* spp. in selenite reduction and thus their availability to the microorganism is also related to their solubility in water. This affects the accessibility of the substrate to the microorganisms and can affect the reduction process (Melati *et al*, 2019).

Being highly soluble in water, glucose can serve as a readily available carbon source for *Enterococcus* spp., promoting their growth and metabolic activities (Tendenedzai *et al*, 2023). On the other hand, due to their poor solubility in water, crude oil and cooking oil are not readily accessible carbon sources for *Enterococcus* spp.

The hydrophobic nature of these oils makes them less available for uptake by the bacteria, potentially limiting their growth and metabolic processes (García-Solache and Rice, 2019). This has an impact on the uptake mechanisms by the bacteria. However, *Enterococcus* spp., can utilise hydrocarbons from cooking oil and crude oil as carbon sources through mechanisms such as biosurfactant production and emulsification, which enhance the bioavailability of these substrates (Sharma *et al*, 2015).
In summary, the choice of carbon source has a significant impact on the selenite reduction, elemental selenium formation and protein synthesis by *Enterococcus* spp. Glucose and cooking oil are efficient carbon sources, with glucose leading to rapid and robust responses in all processes. Crude oil, although initially slower in this regard, can still support selenite reduction, elemental selenium formation and protein production, but with a delayed onset.

4. Conclusion

The study offers valuable insights into the bioremediation potential of *Enterococcus* spp. in the context of selenite reduction. The choice of carbon sources significantly influences the bacteria's capacity for selenite reduction, elemental selenium formation, and protein synthesis (viability). Glucose and cooking oil emerged as efficient carbon sources, while crude oil exhibited delayed but substantial support for these processes. The bacteria employed bioprecipitation for selenite reduction as indicated by red elemental selenium formation. These findings hold great promise for the development of sustainable bioremediation strategies, particularly for selenium and other contaminant ions.

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