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Microbial Pb(II) Removal by Precipitation and Adsorption Mechanisms with *Klebsiella Pneumoniae* Isolated from an Industrially Obtained Consortium

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The study was focused on investigating the contribution of a single bacterial strain to lead removal from wastewater through microbial precipitation and adsorption. Previous studies on lead bioremediation have been conducted using a microbial consortium obtained from wastewater at a lead battery recycling plant in Gauteng, South Africa. The successful isolation of an active strain in the consortium was achieved and the strain was identified as a pure culture of *Klebsiella pneumoniae*. The rate of lead removal and the metabolic activity of the culture was determined over a period of 4 d.
The results indicated that microbial precipitation of Pb(II) by *K. pneumoniae* is possible with significant Pb(II) removal occurring in under 20 h. The amount of lead in solution was decreased to a value undetectable by the analysis equipment in approximately 63 h. No lead removal by adsorption was observed. It is suspected that a metabolically dependent mechanism was responsible for the precipitation of lead as the metabolic activity increased with increasing lead precipitation, but further studies would be required to confirm this.

1. Introduction

Lead can be introduced to wastewater from sources such as battery waste, fertiliser, industrial effluents, and mining waste among others. The occurrence of lead in wastewater is a cause for concern due to the highly toxic nature of the heavy metal which will result in numerous health risks for the population (Baysal et al., 2013). The conventional methods of lead removal include but are not limited to ion exchange, electro-winning, electro-coagulation, cementation, adsorption, and chemical precipitation (Ida and Eva, 2021). These methods have been shown to have high operation costs (Arbabi et al., 2015). The use of bioremediation for lead removal has lowered costs, higher efficiencies, and higher selectivity and is therefore a more desirable option.
Previous studies have been completed on the bioremediation of lead from wastewater using an industrially obtained consortium (Hörstmann et al., 2020b). These studies focused on the effect of various factors such as initial lead concentration and nutrient conditions on the bioprecipitation of lead and the biosorption of lead by the consortium. The results indicated that the consortium was able to remove approximately 50 % of lead in the first 3 h of the study while approximately 96 % of all lead in solution was removed in 15 d. This removal was attributed by Hörstmann et al. (2020a) to a faster biosorption mechanism alongside a slower bioprecipitation mechanism. Subsequent research has demonstrated the biosorption phase by the consortium by inhibiting metabolic processes with NaN_3 (van Veenhuyzen et al., 2021).
Studies by Hörstmann et al. (2020b) were conducted under anaerobic conditions and the precipitate formed was identified as primarily PbS. A BLAST analysis using 16S rDNA sequencing showed that *Klebsiella pneumoniae* and *Paraclostridium bifermentans* were the active species present in the Pb-bioremoval mechanism. To improve the understanding of the mechanisms employed by each active species, the strains were isolated to be investigated individually.

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This study aimed to isolate the *K. pneumoniae* strain, determine its lead removal capabilities, and investigate what types of Pb(II) removal mechanisms (biosorption, bioprecipitation, or both) were present. The isolation of *K. pneumoniae* was achieved with the use of the selective agar eosin methylene blue agar. This research is the first step to understanding the roles of the individual strains in the bioremoval of lead from wastewater using the above-mentioned industrial consortium as well as the Pb-removal mechanisms utilised by the isolated strain.

2. Materials and methods

2.1 Materials

Batch reactors for all experiments were prepared using stimulated LB broth containing yeast extract (Merck, Modderfontein, South Africa), tryptone (Sigma Aldrich, St. Louis, MO, USA) and NaCl (Glassworld, South Africa). The isolation of *K. pneumoniae* was achieved with the use of eosin methylene blue agar (EMB) (Oxoid, Hants, UK). Metabolic activity was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Aldrich, St. Louis, MO, USA) and dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO, USA).

2.2 Isolation of *Klebsiella pneumoniae* from the consortium

K. pneumoniae was isolated from the microbial consortium with several rounds of streak plating and batch reactors using both simulated LB broth and EMB agar. Streak plates were initially prepared using EMB agar with no added lead, this was done to inhibit the growth of gram-negative strains. The colonies from these plates were streaked onto LB broth agar containing 80 mg L^{-1} Pb(II). Batch reactors were prepared containing 10 g L^{-1} yeast extract, 20 g L^{-1} tryptone, 1 g L^{-1} NaCl and 80 mg L^{-1} Pb(II) prepared from $Pb(NO_3)_2$ in 100 mL serum bottles. These batches were inoculated using desirable colonies from the LB agar plates and were grown aerobically to further inhibit the growth of obligate anaerobe *Paraclostridium bifermentans*. The batch reactors were used to streak LB agar plates containing 80 mg L^{-1} Pb(II). The final plates were analysed using 16S rDNA sequencing with amplification of the sequence achieved with the use of the universal primers 27F and 1492R.

2.3 Lead precipitation

Batch reactors were prepared using 10 g L^{-1} yeast extract, 20 g L^{-1} tryptone, 1 g L^{-1} NaCl and 80 mg L^{-1} Pb(II) prepared from $Pb(NO_3)_2$ in 100 mL serum bottles in triplicate. The reactors were inoculated with 0.2 mL of *K. pnuemoniae* preculture previously stored in 20 % glycerol at -77 °C. The reactors were purged with N_2 gas for 3 min to ensure anaerobic conditions and were then sealed. Samples were taken every 24 h for 7 d using a sterile hypodermic needle and syringe and stored in sterile vials at - 40°C.

2.4 Lead biosorption experiments

Batch reactors were set up in two stages, with the first stage being used to grow *K. pneumoniae* to a certain concentration and the second stage being used for lead biosorption. The first stage was set up with identical nutrient and preculture concentrations to those used for lead precipitation experiments, except for Pb(II) not being added before inoculation to prevent microbial precipitation during the growth phase. Inoculated batch reactors were incubated aerobically at 35°C and 120 rpm for 24 h. The optical density of the culture at 600 nm (OD_{600}) was measured before the culture was centrifuged at 5000 × *g* and washed with 1.45 g L^{-1} $NaNO_3$ solution twice. To ensure the inhibition of metabolic activity, washed bacteria was exposed to 5 M NaN_3 for 24 h before being centrifuged at 5000 × *g* and washed with 1.45 g L^{-1} three times. Thereafter, 1 mL of uninhibited and NaN_3 inhibited cultures at OD_{600} = 5.32 (equivalent to 4.32 g L^{-1} dry mass) were spiked in triplicate into separate reactors with 100 mg L^{-1} Pb(II) prepared from $Pb(NO_3)_2$. Reactors were adjusted to a pH of 5 using HNO_3 to prevent the formation of insoluble Pb-hydroxyl complexes (Wang and Chen, 2009). Control reactors without bacteria were also used. Reactors housing uninhibited bacteria were dosed with nutrients identical to reactors used in microbial culture preparation to ensure growth, whereas reactors housing the control and inhibited bacteria were dosed with 1.45 g L^{-1} $NaNO_3$ alone to solution to ensure identical ionic strength without the opportunity for nutrient uptake. Samples were taken at various intervals over a 3 h period.

2.5 Metabolic activity

The metabolic activity of the culture was determined at each time interval of the lead precipitation experiment. A solution of 5 g L^{-1} 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was prepared using ultrapure water and filtered using a 25 mm nylon syringe filter with 0.45 µm pores. This solution was stored in a dark container at -40 °C.

The samples obtained were centrifuged at 7711× *g* and 21 °C for 10 min (Hettich, Universal 320 R, Germany) and 0.5 mL of this sample was filtered using a 25 mm nylon syringe filter with 0.45 µm pores; this sample is a

representative of a sample without biomass. An amount of 0.5 mL of sample was not filtered and the metabolic activity of these two samples are subtracted to represent the reading without noise.
The samples are both diluted in 1.5 mL ultrapure water and 0.9 mL of the diluted sample is added to a 2 mL vial. The MTT is added to this vial (0.1 mL) and the vials are incubated at 35 °C for 1 h.
After incubation the samples are diluted with 1 mL of dimethyl sulfoxide (DMSO) and the absorbance is measured at 550 nm using a UV/Visible spectrometer (WPA-Lightwave II, Labotech, South Africa).

2.6 Analysis

Samples were centrifuged at 7711 × *g* for 10 min (Hettich, Universal 320 R, Germany) and diluted in ultra pure water before the residual lead concentration was determined using an atomic absorption spectrometer with a Pb lumina hollow cathode lamp (PerkinElmer AAnalyst 400, Waltham, Massachusetts). Zeta potentials were determined with a Malvern Zetasizer Nano ZS for pH values between 2 and 12 in intervals of 2. The 16S rDNA sequencing and generation of the phylogenetic tree was outsourced to Inqaba Biotech (Pty) Ltd. (Pretoria, South Africa).

3. Results and discussion

3.1 Isolation of *Klebsiella pneumoniae* from the consortium

After several rounds of streak plating on both LB agar and EMB agar, it was possible to obtain an isolated culture, the EMG agar plate from the first round of isolation as well as the LB agar plate from the final round of isolation can be seen in Figure 1. The isolated strain was identified using 16S rDNA sequencing and the phylogenetic tree of the strain can be seen in Figure 2. The sample was identified as a pure culture of *Klebsiella pnuemoniae* (BKP21E).

(a) (b)

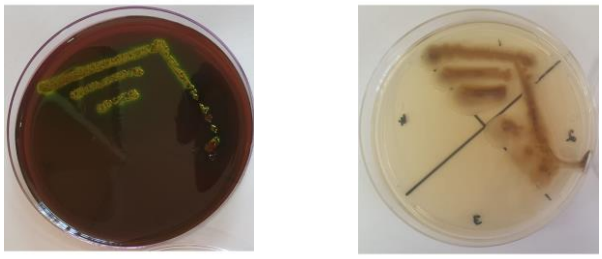

Figure 1: The agar plates used in the isolation experiment with (a) EMB agar in the initial isolation round and (b) LB broth agar in the final isolation round.

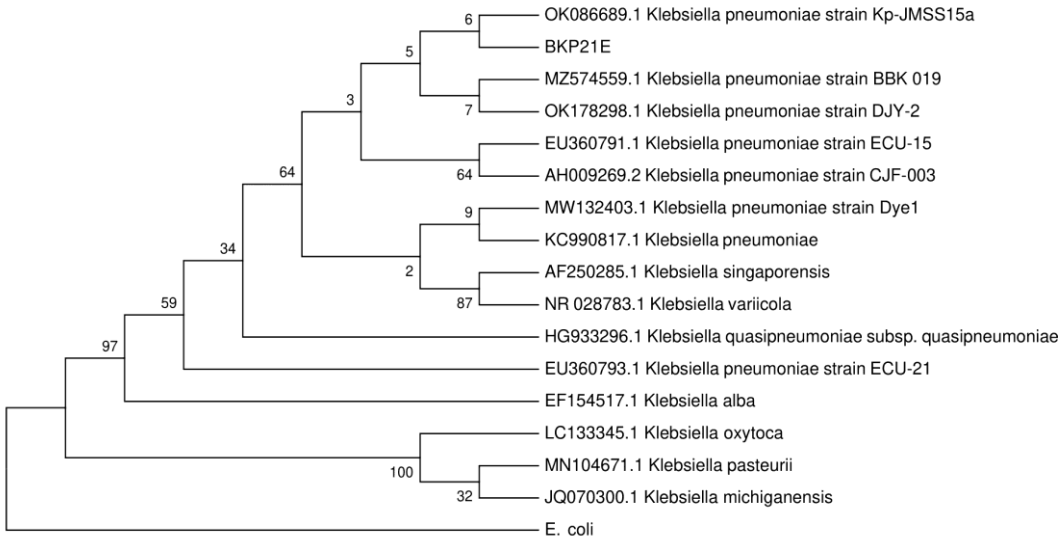

Figure 2: Phylogenetic tree indicating the identity of the isolated Klebsiella pneumoniae strain (BKP21E).

3.2 Batch reactors, residual lead, and metabolic activity

The Pb(II) removal and the metabolic activity of the experiments can be seen in Figure 3. A significant increase in Pb(II) removal is evident in the first 20 h which coincides with an increase in metabolic growth, possibly indicating a metabolic mechanism of lead precipitation at least within the first 20 h. As the amount of Pb(II) removed from solution tends towards a maximum value, the metabolic activity depicts a sharp increase. This can be due to environmental conditions improving after the removal of Pb(II). The lead in solution was reduced to amounts undetectable by the atomic absorption spectrometer after approximately 63 h.

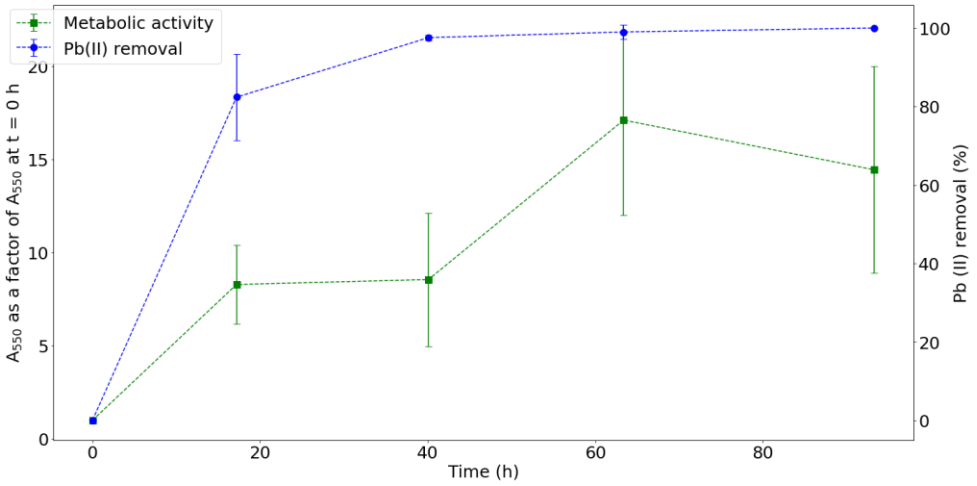

Figure 3: The comparison between metabolic activity reported as a fraction of A_{550} measured at t = 0 and the Pb(II) percent removal over 5 d.

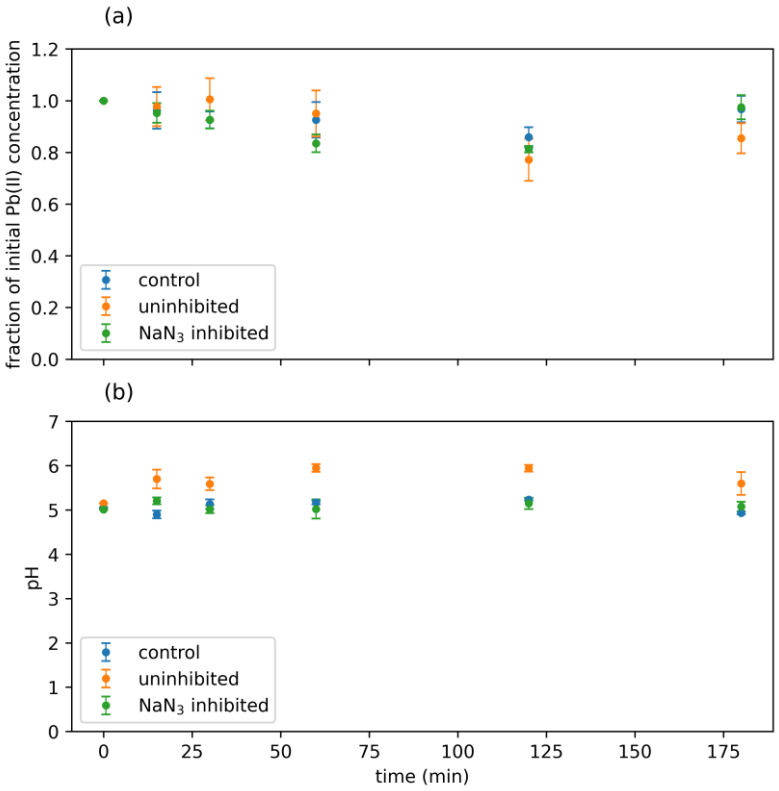

Figure 4: Fraction of original Pb(II) concentration (a) and changes in pH (b) over a 3 h period in reactors without bacteria, with metabolically active bacteria, and with bacteria metabolically inhibited with NaN_3 (a).

3.3 Biosorption

The inhibition of metabolic activity was seen by A_{550} readings that differed as 14.2 and 2.42 for uninhibited and NaN_3 inhibited bacteria respectively. As seen in Figure 4(a), both inhibited and uninhibited bacteria demonstrated similar behaviour to the control where negligible Pb(II) removal was observed. This suggests that the rapid, abiotic Pb(II) removal mechanism observed in the consortium from which *K. pneumoniae* was isolated is not present in the pure culture. This observation is similar to Johnson et al. (2007), where no significant difference in heavy metal adsorption between inhibited and uninhibited Gram-negative bacteria was observed. It is therefore likely that biosorption from the original consortium was brought about by other strains.
Additionally, Figure 4(b) does not indicate the release of H^+ ions commonly observed in biosorption that arises from binding site competition between cations and protons (Hammaini et al., 2007).
While metabolic inhibition of other Gram-negative species has been found to make surface charge more negative (Martinez et al., 2008), and while more negative surface charges ease binding with positive cations (Hammaini et al., 2007), Figure 4 and Figure 5 respectively show that metabolic inhibition caused neither in *K. pneumoniae*. This further demonstrates how the species does not support biosorption as a removal mechanism.

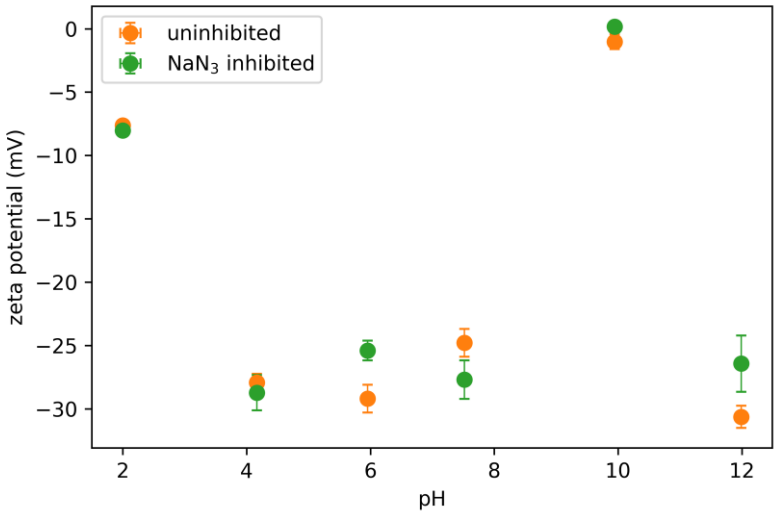

Figure 5: Zeta potential of metabolically active bacteria and bacteria metabolically inhibited with NaN_3 across a range of initial pH values.

4. Conclusions

The Pb(II) removal results indicate that the isolated strain of *K. pneumoniae* is highly capable of lead removal in solution. The contribution of biosorption to lead removal was determined to be negligible, and no evidence for the enhancement of biosorption by metabolic inhibition was observed. The exact mechanism of lead bioprecipitation and the identity of the precipitate are unknown. These results are, however, favourable for further studies on the microbial precipitation of Pb(II) by *K. pnuemoniae*. The lead removal capabilities of *K. pneumoniae* are slightly superior to the removal capabilities of the industrial consortium bioprecipitation value of approximately 96 % over 15 d (Hörstmann et al., 2020b). The results indicate a metabolically dependent mechanism of lead removal since the metabolic activity increases as lead precipitation occurs, a total organic carbon (TOC) analysis can be done in further studies to determine whether a drop in organic carbon occurs translating to a drop in substrate, this will then be an indication of whether lead precipitation is dependent on microbial growth.
Further studies can be conducted which focus on the precipitation mechanism and the identification of the precipitate identity. The effect of nitrates on bioprecipitation of lead by the strain can be investigated.

Acknowledgements

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