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Using High Performance Liquid Chromatography to Analyse Indoline Degradation during Lead Bioremoval

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Analysis was conducted on the degradation of indoline present in LB (Luria Bertani broth) broth used as rich growth medium during the bioremoval of Pb(II) using an industrially obtained microbial consortium. Indoline is a saturated analogue of indole, which is a well-known environmental pollutant and health concern found in industrial and agricultural wastewater. A method for Indoline analysis was developed using reverse phase high performance liquid chromatography (HPLC) run isocratically with methanol and 0.1% TFA (Trifluoroacetic acid). Experiments were conducted anaerobically in the presence of both 80 ppm and 500 ppm Pb(II) and each presented complete degradation of indoline within 33 hours. The study focuses on the novel method developed for HPLC analysis of indoline found in LB broth and its degradation in the presence of Pb(II).

1. Introduction

Indole is a typical nitrogen heterocyclic aromatic compound, of which indoline is a saturated analogue. Indole is widely available in everyday products and the environment with the indole ring found in proteins, phytohormones and multiple alkaloids. It is popular in the pharmaceutical industry as the nucleus of indole possess various biological capabilities for example for anticancer, antimicrobial and antiviral uses. Indole also adds to the unpleasant odour in mammal and bird faeces as it forms part of the tryptophan metabolism in the colon and rumen of monogastric animals (Tesso et al., 2019). Indole is regarded as a toxic *N*-heterocyclic aromatic with potential mutagenetic properties. The compound can cause animal hemoglobinuric nephrosis, hemolysis and even tumour formation. Indole can also induce bacterial membrane and oxidant toxicity, which prevents cell division as well as reparable DNA damage. The interest of the biodegradation of indole has thus spiked over recent years (Ma et al., 2018).

Due to its stable chemical structure conventional wastewater treatment methods are insufficient for the removal of indole, therefore more advanced treatment methods are sought. In literature ionizing radiation has been shown as an effective treatment method (He et al., 2022)(He et al., 2022), however ionizing radiation treatment is expensive and complicated limiting its scaling potential. Bioremoval poses a more cost-effective and environmentally friendly option for the removal of indole from wastewater. Various studies have demonstrated the removal of indole using, under both aerobic and anaerobic conditions. These include a study in which the oxidation of indole was followed by heterocyclic cleavage under aerobic conditions with *Acinetobacter* spp. One of the subspecies achieved 96.0 % removal after 2 days (Tesso et al., 2019). Various possible mechanisms exist under anaerobic conditions as well, such as the use of sulfate-reducing bacteria utilising indole as sole carbon source (Bak et al., 1986). Other studies have also shown that anaerobic denitrifying bacteria are capable of indole degradation (Berry et al., 1987).

The current study is founded on the work conducted by this team on Pb(II) bioremoval. The same microbial consortium used in previous studies for lead removal proved effective in the biodegradation of indoline during the lead removal. LB broth was continuously used as rich growth medium, containing tryptone and yeast extract (Hörstmann et al., 2020). The importance of lead removal and recovery from wastewater is well known as a toxic non-biodegradable heavy metal, which poses the threat of permanent damage to not only humans but also the environment. Lead affects almost all functions of the human body negatively and could lead to detrimental effects, such as anaemia, increase in blood pressure and damage to the nervous system (Wani et al., 2015).

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Many conventional Pb(II) removal techniques exist such as coagulation, chemical precipitation, ion exchange, and cementation (Arbabi et al., 2015). A comparison between the efficiency of a few conventional Pb removal techniques and with the current study is shown in Table 1 below, the last example refers specifically to a study conducted by this team with this microbial consortium. The study achieved 99.9 % removal of 2000 ppm Pb(II) with the use of a continuous reactor setup. The removal by this microbial consortium outshines conventional methods in its robust system, which is able to remove extreme amounts of lead in a cost effective environmentally friendly manner. Conventional methods pose the threat of not being very environmentally friendly or cost-effective, with many of the by-products being as harmful to the environment as the initial pollutant itself. Using new bioremoval and biorecovery methods instead of traditional physicochemical methods has shown to be the safer, more cost-effective alternative (Pan et al., 2012).

Table 1: Comparison between examples of conventional Pb removal techniques and the current study.

Technique	More information	Relevant Pb(II) concentration	P(II) removal	Reference
Coagulation and flocculation	Using Hydroxide precipitation	Range between 1 ppm and 14 ppm	Maximum removal of 99.04 %	(Pang et al., 2009)
Chemical precipitation	Sulfur-containing precipitation agents	50 ppm	Maximum removal of 99.90 %	(Pohl, 2020)
Ion exchange	Polyacrylic acid capped Fe_3O_4-Cu	500 ppm	Maximum removal of 93 %	(Goyal et al., 2021)
Pb(II) bioremediation (Current research team)	Microbial consortium	2000 ppm	99.9 %	(Chimhundi et al., 2021)

The discovery of indoline in the samples was first reported by this team in 2021 (Cilliers et al., 2021). The project focused on giving insight into the metabolism of lead resistant microbes and indoline was found to be one of the main metabolites consumed during lead bioremoval. The focus of the study, however, was on the metabolism responsible for effective lead removal and various aspects were discussed such as metabolic activity and nitrate depletion. The current study focuses on the method developed to obtain quantitative measurements for indoline as no other study to the authors' knowledge have investigated the possibility of HPLC indoline analysis and microbial indoline degradation, coupled with Pb(II) removal.

2. Materials and Methods

2.1 Materials

100 mL Anaerobic batch reactors were prepared in serum bottles. The reactors were spiked with a Pb(II) stock solution of $Pb(NO_3)_2$ (Merck, Kenilworth, NJ, USA) at 80 ppm and 500 ppm Pb(II). LB broth (Sigma Aldrich, St. Louis, MO, USA) was prepared as growth medium for 80 ppm batches and simulated LB for 500 ppm. Simulated LB broth contains double the amount of nutrients compared to standard LB broth with a reduced amount of NaCl to avoid precipitation of PbCl.

Indoline (HPLC grade, ReagentPlus®, 99%) standard was used for HPLC measurements (Sigma-Aldrich, Bryanston, Sandton, South Africa). TFA (trifluoroacetic acid, HPLC grade, ≥ 99.0%, Sigma-Aldrich) was prepared with Ultrapure water and methanol (HPLC grade, gradient grade, ≥ 99.9%, Sigma-Aldrich) HPLC solvents.

2.2 Microbial culture

The microbial consortium used in this study as well as various previous studies were collected in South Africa, in the province of Gauteng in a borehole at an automotive battery recycling plant. The original inoculant was prepared from 1 g of lead-contaminated soil added to a 100 mL LB broth anaerobically, spiked with 80 ppm Pb(II). The batch was incubated overnight at 32ºC and 120 rpm. The inoculant was stored cryogenically at -77˚C in glycerol (20% v/v ratio). Precultures were prepared at 80 ppm and 500 ppm Pb(II) from the stored inoculum. The precultures were incubated at 35ºC and 120 rpm until a dark precipitate was formed and stored cryogenically in the same manner as the original inoculant and used directly after thawing for each experiment.

2.3 Experimental and Sampling

The LB broth (standard and simulated) in 100 mL serum bottles and Pb(II) stock solution was prepared separately and autoclaved. The media were cooled down and prepared in a sterile environment to 80 ppm and 500 ppm respectively. The batches containing LB broth and Pb(II) were subsequently inoculated with 0.2 mL of

preculture and purged with nitrogen gas for 3 minutes and sealed with a rubber stopper and metal cap to obtain anaerobiosis. The batches were incubated at 35ºC and 120 rpm for experimentation.
Samples were taken in 3 h intervals for a period of 33 h (3, 6, 9, 24, 27, 30 and 33 h). The batch reactors were shaken thoroughly and sampled using a sterile hypodermic needle and syringe. The samples were stored for later analysis.

2.4 Analysis and Measurements

The amount of Pb(II) removed from solution was obtained using an atomic absorption spectrometer (Perkin Elmer AAnalyst 400, Waltham, Massachusetts), coupled with a Pb Lumina hollow cathode lamp. The samples were centrifuged to split the precipitate from the supernatant. This remaining supernatant was subsequently diluted to the required amount and measured as the amount of Pb(II) remaining in solution.
High-performance liquid chromatography was used to establish the initial amount of Indoline present in rich growth medium LB broth. A Waters 2695 Separations module coupled with a 2489 UV/Vis detector at 280 nm was used, as well as a Waters PAH C_{18} 5 µm 4.6 mm × 250 mm column. The samples as well as indoline standard was dissolved with methanol and prepared directly before analysis. The system was washed between each sample with ultra-pure water to ensure minimal carry-over.

3. Results and discussion

3.1 HPLC method development

A method was developed for the measurement of indoline in LB broth using high-performance liquid chromatography.
Three reference methods were combined to establish the current indoline HPLC analysis method. The current study used the same separations module and a C_{18} column as in the study by Yong et al., (2017). The method by Yong et al. (2017) was used for measuring various indole compounds in sugar cane juice. Ten indole compounds were measured, including indole-3-acetic acid, but not indoline.
The selection of mobile phase was more complicated. Yong et al. (2017) used a methanol-formic acid buffer eluted isocratically at 20:80, v/v. Their solvent (A) consisted of 100% methanol and solvent (B) 0.1 % formic acid (w/v) (Yong et al., 2017). In contrast, multiple methods were found where TFA (Trifluoroacetic acid) was used instead of formic acid (Cohen et al., 1995 and Verma et al., 2012). A guide by Sigma-Aldrich (Brandes et al., 2010) for separation of peptides indicated that formic acid tends to generally produce poorer peak shape and lower peak capacity than TFA. Hussain et al. (2019) quantified various proteins using 0.1 % TFA in water and 100 % methanol with an injection size of 20 µL.
The injection size used by Yong et al. was 5 µL and 10 µL, 5 µL was however chosen. Yong et al. used a flow rate of 0.3 mL/min, however it was found that better peak results were obtained at double the amount of flow of 0.6 mL/min.
The final method used for this study was as follow:

- Isocratic
- Methanol as solvent A
- 0.1 % TFA as solvent B
- 20:80 ratio of A:B
- Constant flow rate of 0.6 mL/min
- Injection size of 5 µL at 30ºC

Indoline was tested on its own as standard and the spectra acquired can be seen in Figure 1 below. The R^2 value obtained was 0.9993 and shown in Figure 2 below, followed by one of the samples containing LB broth and lead (80 ppm) in Figure 3.

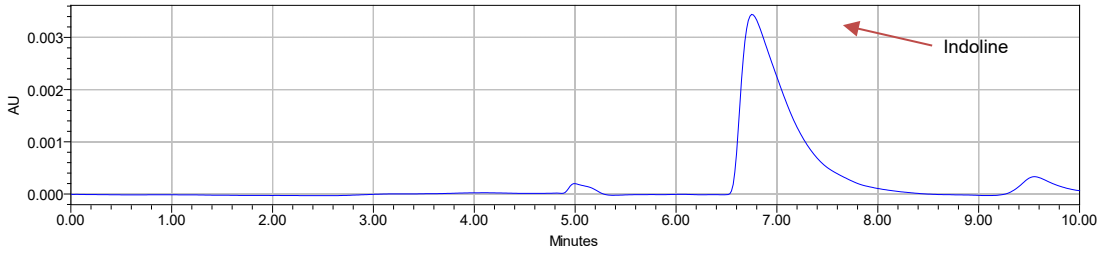

Figure 1: HPLC spectra Indoline standard with the final method

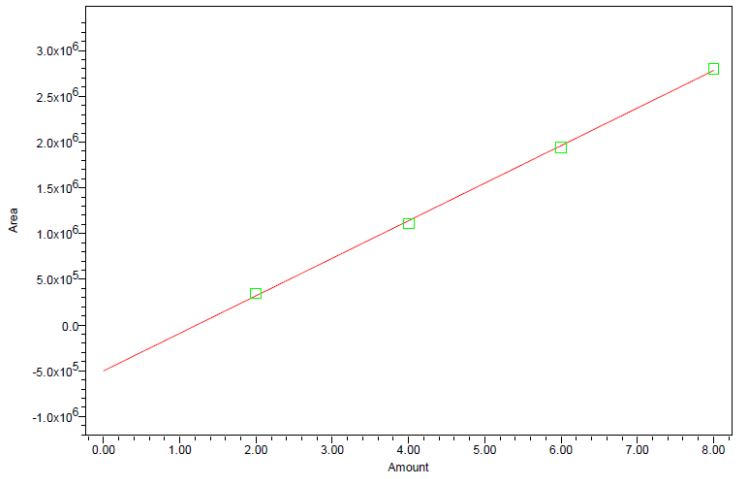

Figure 2: Calibration results for Indoline

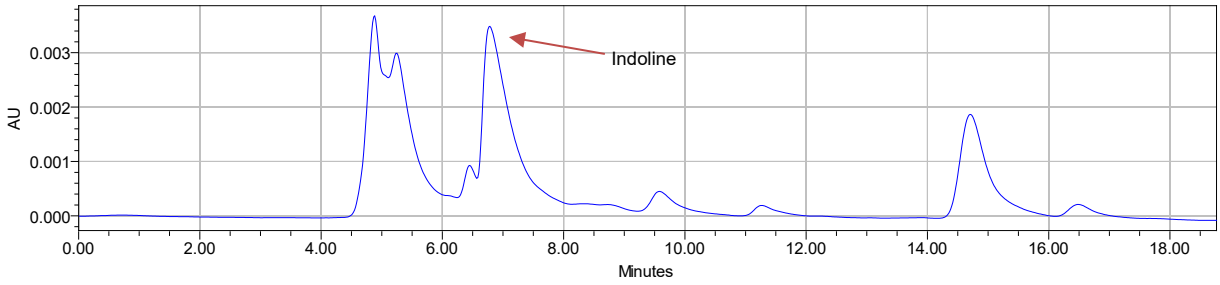

Figure 3: B80 sample HPLC spectra with the final method

The method developed produced a clear peak for indoline in a pure indoline mixture and a relatively clear peak on LB broth. The peak also eluted quite early at just before 7 minutes, making it effective and quick.

3.2 Results

The results for Pb(II) removal are presented in Figure 4 a and b. These results were previously published by this team but adapted for this study as it carries weight in the conclusions (Cilliers et al., 2021). From these findings, it was determined that 70.77 ± 8.99 % of 80 ppm Pb(II) and 57.42 ± 4.92 % of 500 ppm Pb(II) was removed during 33 h.

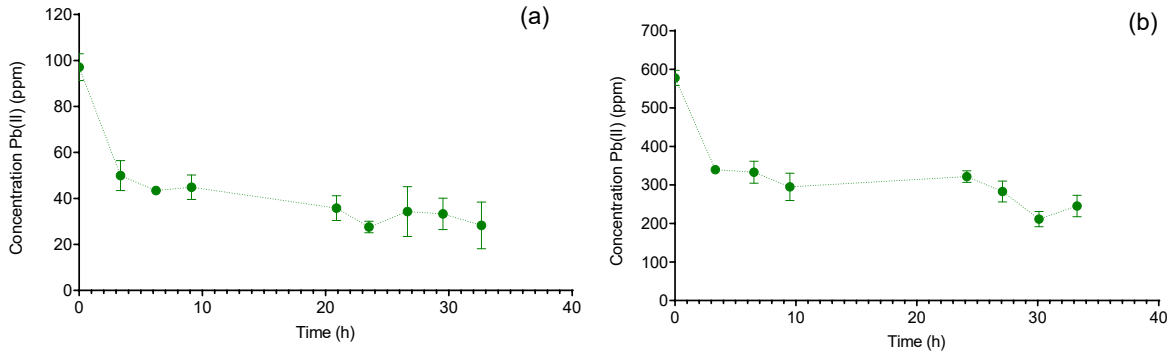

Figure 4: (a) Pb(II) removal for B80 samples and (b) Pb(II) removal for B500 samples

The HPLC method for indoline analysis was developed and used on samples containing 80 ppm and 500 ppm Pb(II) as well as LB broth. The initial indoline measurement was found to be 25.48 ppm for 80 ppm sample and 45.54 ppm for 500 ppm shown in Figure 5 a and b below. This made sense as the simulated LB broth had

approximately twice the amount of yeast extract as the standard commercial LB broth (see methods section). 100 % indoline degradation of indoline was achieved after 24 h at 80 ppm, containing standard LB broth and after 33 h at 500 ppm, containing simulated LB broth.

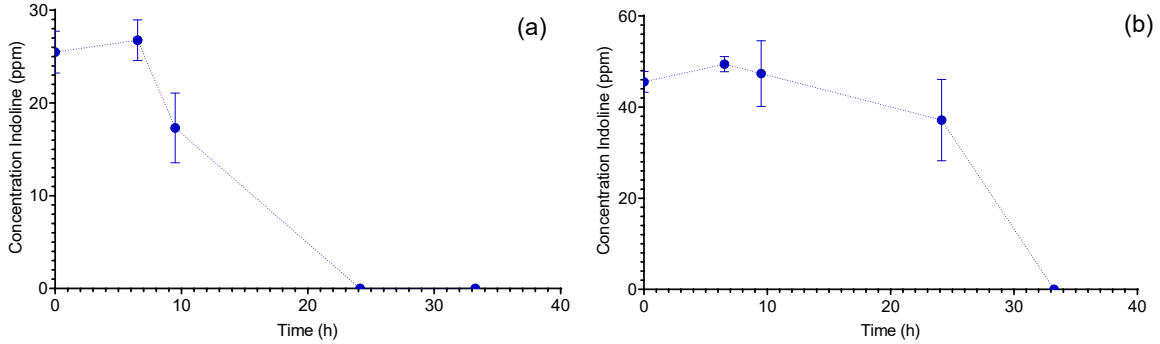

Figure 5: (a) B80 HPLC Indoline measurements and (b) B500 HPLC Indoline measurements

4. Conclusions

It can be concluded that LB broth does contain indoline and that complete indoline degradation is possible using a locally acquired microbial consortium under anaerobic conditions. This consortium has proven highly effective in lead removal in the past but has now proven as efficient in indoline degradation. A novel method was developed to measure the presence of indoline in a complex medium such as LB broth specifically using high-performance liquid chromatography, by combining various sources in literature to devise the best method. It was shown that total degradation of indoline was possible within 33 h as well as substantial Pb(II) removal. The study proves that the microbial consortium could remove indoline in the presence of lead spiked at 80 ppm and 500 ppm, substantiating the resilience of the culture and system in which it was grown to exert dual functionality. Future studies could be in the degradation of indole, a known organic pollutant, in the presence of Pb(II).

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