CHEMICAL ENGINEERING TRANSACTIONS
VOL. 79, 2020
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Enrico Bardone, Antonio Marzocchella, Marco Bravi

ISBN 978-88-95608-77-8; **ISSN** 2283-9216
DOI: 10.3303/CET2079071

Microbial Pb(II) Precipitation: Yeast Extract Autolyzed from *Saccharomyces Cerevisiae* as a Sustainable Growth Substrate

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This study aimed to quantify the effectiveness of yeast extract, autolyzed from commercial baker's yeast (*Saccharomyces cerevisiae*), as a more economical growth medium for the Pb(II) removing consortium. Three parameters were tested, i.e. the amount Pb(II) removed, metabolic activity, and a measurement of precipitate formed (optical density). The experiments were conducted over a 48 h period, under anaerobic conditions, spiked with 80 ppm Pb(II) and with the produced yeast extract as sole carbon- and nitrogen source.
Most of the Pb(II) was removed (50 %) within the first 7 minutes of experimentation indicating rapid Pb(II) removal. From there it was observed that 100% of the Pb(II) was removed from solution within 24 h. Metabolic activity initially increased slowly to 5.36 ± 0.0692 absorbance units from 0 h to 8 h, followed by a growth spurt from 8 h to 48 h with a measured metabolic activity of 18.9 ± 0.0604 absorbance units. The optical density exhibited a slow increase to 3.60 ± 0.0453 absorbance unit up to 16 h, with a dramatic increase to 6.37 ± 0.0782 absorbance units after 24 h. A dark precipitate was observed in all the batch reactors as seen in previous studies conducted by this team. The initial rapid drop of Pb(II) concentration within the first few minutes before sampling may indicate a biosorption mechanism due to the high amount of yeast biomass present.
The remaining Pb(II) was subsequently removed during the period of slow metabolic activity and optical density increase, likely as a result of an initial detoxification mechanism (biosorption). The large metabolic activity and optical density increase appear to correspond to low Pb(II) concentrations, most probably due to a Pb(II) inhibitory effect or slow access to more complex components in the yeast hydrolysate.
It can be concluded that yeast extract, autolyzed from commercial baker's yeast can successfully be used as substrate in the bioremediation of Pb(II) from contaminated wastewater. However, more research is required to determine the inhibitory effect of Pb(II) and/or the effect of the complex substrate on the microbial consortium.

1. Introduction

Lead is a widely distributed and mobilized environmentally persistent non-essential element, that accumulates in the food chain (Zhang et al., 2015). It is a neurotoxic heavy metal found rarely in pure form in nature and commonly mined in the form of PbS (Rosenberg et al., 1979). High levels of human exposure can cause damage to various organs, such as the central nervous system, blood and kidneys and even death in some cases (Tong et al., 2000). The estimated maximum concentration of lead in drinking water is 0.01 ppm and the threshold for causing serious harm to aquatic life is about 0.0058 ppm (Duruibe et al., 2007). The bioremediation and biorecovery of lead from wastewater could provide a feasible mechanism to achieve economic and environmentally-friendly removal of lead from the biosphere.
The other reason for lead recovery is the continuing demand for lead and a limited supply of raw reserves. It is projected that there is about 17 years supply of lead available worldwide with an annual global consumption rate of about 5 million tonnes per annum (International Lead Association, 2019) and only a total of 83.3 million tonnes of reserve remaining (Statista, 2019).

Paper Received: 26 July 2019; Revised: 27 December 2019; Accepted: 1 March 2020
Please cite this article as: Horstmann C., Naidoo S., Brink H., Chirwa E.M., 2020, Microbial Pb(ii) Precipitation: Yeast Extract Autolyzed from Saccharomyces Cerevisiae as a Sustainable Growth Substrate, Chemical Engineering Transactions, 79, 421-426 DOI:10.3303/CET2079071

The experiments were conducted under strict anaerobic conditions, it is thus important to consider anaerobic respiration for the following study. Anaerobic respiration is a metabolic process, during which bacteria transfer electrons to terminal electron acceptors, such as oxidized chemicals or metal ions. It is used in processes where the microbe requires the removal of residual electrons to manage the internal redox balance in the cell. Compounds such as nitrate and fumarate have been used in the past as electron acceptors for oxidising NAD(P)H to NAD(P)+ (Kim et al, 2016).
The following research team have conducted countless studies on Pb(II) removal using the same industrially obtained microbial consortia under anaerobic conditions. The team has effectively proven that bioremoval of Pb(II) is possible with this consortium, producing a dark grey precipitate with the use of Luria Bertani broth as growth substrate (Brink et al., 2017a). It is known that LB broth contains rich nutrients, yeast extract and tryptone. The precipitate was identified in following studies as mostly PbS and a fractional amount of Pb0 (Brink et al., 2019b). LB broth, however, is known to be an expensive growth medium and would not be ideal for continuous industrial application, a substitute was sought after. Various other options were investigated such as glucose and xylose but were proven invaluable as Pb(II) removal was not as effective (Brink et al., 2018c).
Yeast Extract from *Saccharomyces cerevisiae* has been used in other studies in the past for a cost-effective microbial culture media (Zareia et al., 2016). Viable *Saccharomyces cerevisiae* biomass has effectively been implemented in the biosorption and subsequent removal of heavy metal manganese from solution, this, however, did not result in precipitation (Fadel et al., 2019). It was also seen in a study conducted with non-viable yeast cells that biosorption of Pb(II) was highly effective and that 50 % of Pb(II) was removed within the first 5 minutes (Farhan & Khadom, 2015). *Saccharomyces cerevisiae* was however not initially used as substrate, as the key growth and Pb(II) precipitation ingredient in LB broth were initially unknown and to be identified first (Brink et al., 2018c).
The possibility for using autolyzed yeast extract from *Saccharomyces cerevisiae* as substrate and biosorpent in combination with Pb(II) removing microorganisms was investigated in the following study.

2. Materials and methods

2.1 Materials

Anaerobic batch reactors were set up with 100 mL serum bottles. The lead stock solution was prepared using $Pb(NO_3)_2$ (Merck, Kennelworth, NJ). The yeast extract was autolyzed from commercial baker's yeast. Optical density and metabolic activity measurements were conducted with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) (Sigma Aldrich, St Louis, MO).

2.2 Microbial culture

A lead resistant microbial consortium was collected from a borehole at an automotive-battery recycling plant in Gauteng, South Africa. The inoculum was prepared by adding 1 g of Pb(II) contaminated soil to a mixture of LB (Luria Bertani) broth and 80 ppm Pb(II) in a 100 mL serum bottle, which was then incubated anaerobically for 24 hours at 32 °C at 120 rpm. Glycerol was added to a final ratio of 20% v/v and cryogenically stored at -77 °C. The preculture was subsequently prepared from the stored stock cultures. It was prepared by inoculating one loop of stock culture to 100 mL anaerobic serum bottles containing LB broth spiked with 80 ppm Pb(II). The serum bottles were purged with nitrogen gas for roughly 3 minutes and sealed to obtain anaerobic conditions, then incubated at 30°C and 120 rpm for three days before inoculation of the experiments took place.

2.3 Yeast Extract preparation

The methods of procedure used were outlined by Zarei et al. (2016). An aqueous suspension of yeast cells was prepared by adding 250 g/L baker's yeast (*Saccharomyces cerevisiae*) to ultra-purified water. The suspension was then autoclaved for 10 min at 115 °C, followed by rapid cooling on ice. Thereafter, the cell debris was separated by centrifugation at 5 430 rpm for 10 min. The supernatant was collected, centrifuged a second time to ensure that all the debris was removed and autoclaved and again under the same conditions to ensure that the yeast extract was sterile.

2.4 Experimental

The Pb(II) stock solution and yeast extract medium were prepared and then autoclaved separately. When cooled to room temperature, the Pb(II) stock solution was added to the yeast extract in a biological safety cabinet, in the presence of an open flame to ensure sterile conditions. The serum bottles were then inoculated with a loop from the prepared preculture. The batch reactors were purged with N_2 gas for 3 min, sealed with a

rubber stopper and clamped with a metal cap to ensure anaerobic conditions. The batch reactors were placed in a shaker incubator at 120 rpm and 35 °C for a period of 48 h. The experiments were conducted in triplicate.

2.5 Sampling

Samples were taken from hour zero followed by 7 min, 2 h, 4 h, 8 h, 16 h, 24 h and 48 h under strict sterile conditions. The sealed serum bottles were shaken thoroughly before sampling. A hypodermic needle and sterile syringe were used to pierce the rubber stopper. A sample volume of 5 mL was removed from each reactor, from which 1 mL was stored at 5°C. At a later stage, the 1 mL sample was centrifuged for 10 minutes at 9 000 rpm and 20°C, after which the supernatant was decanted from the solid precipitate (pellet) for the purpose of Pb(II) analysis. The remaining 4 mL was immediately used for metabolic activity and optical density measurements.

2.6 Analysis

The metabolic activity measurements are performed immediately after sampling. All the dilutions made, were considered when calculating final data. The metabolic activity of bacterial cells was quantified using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Wang et al., 2010). MTT is a water-soluble yellow dye that can be reduced to water-insoluble purple formazan crystals by the dehydrogenase system of active cells. The formazan concentration can be measured spectrophotometrically after dissolution in an organic solvent dimethyl sulfoxide (DMSO). It is assumed that the formazan concentration is directly related to the number of metabolically active cells in the sample. The MTT solution contained 5 g/L MTT in ultra-purified water, which was filtered with sterile filters and stored at -40 ºC. Two sets of analyses were performed, one with biomass and the other without. One of the 1 mL samples were filtered with 25 mm nylon syringe filters with 0.45 µm pores (Anatech) before analysis was done on it, to represent the sample without biomass. The analysis was conducted by diluting the 1 mL sample (with or without biomass) to a total of 4 mL with ultra-purified water. The MTT solution (0.2 mL) was then added to 1.8 mL of the diluted sample and incubated for one hour at 35 °C. The samples were then dissolved in 2 ml DMSO after incubation. The absorbance at 550 nm on a V spectrophotometer was measured and recorded as an indication of metabolic activity.
The optical density measurements were also measured with 1 mL of sample directly after sampling on a UV spectrophotometer at 600 nm. The measurements were also done on a sample containing biomass and one without as mentioned above.
The residual aqueous Pb(II) was measured using an atomic absorption spectrometer (Perkin Elmer AAnalyst 400, Waltham, Massachusetts), with a Pb Lumina hollow cathode lamp. The supernatants of the 1 mL samples stored at 5 °C were used, as to avoid any solids and so avoid causing blockages on the instrument.

3. Results and discussion

The visual results are presented in Figure 1 below, they clearly indicate strong growth coupled with dark grey precipitation only at 16 h.

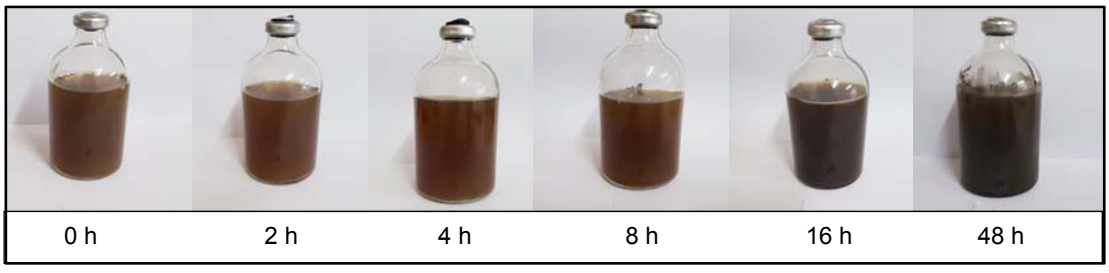

Figure 1: Visual changes in the batch reactors over 48 h.

The Pb(II) concentration results are presented in Figure 2 below with error bars. The first Pb(II) measurement taken after 7 minutes was measured at 39.9 ± 7.35 ppm, which is a decrease in Pb(II) concentration of 50 % from 80 ppm Pb(II). These results strongly indicate biosorption of Pb(II) onto biomass and correspond to evidence found in literature (Farhan & Khadom et al., 2015). The remainder of the Pb(II) was mostly removed within the first 2 hours, with a drop in Pb(II) from a measurement of 39.9 ± 7.35 ppm to 15.3 ± 3.14 ppm. The change, however, was not coupled with any colour change, still indicating the presence of a biosorption mechanism. 100 % of the Pb(II) was removed in all the reactors at 24 h. The results indicate a biosorption mechanism followed by bioprecipitation (mineralization) of Pb(II).

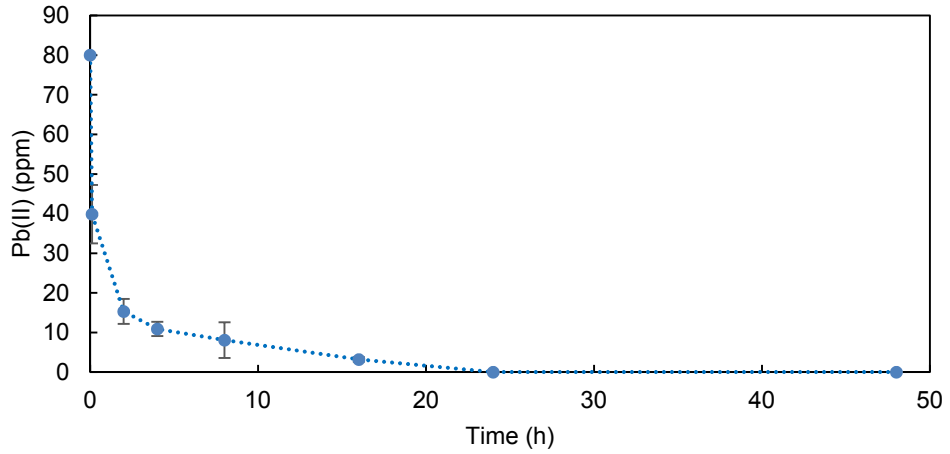

Figure 2: Pb(II) concentrations over time.

The results for optical density (OD_{600}) and metabolic activity (MA) are presented in Figure 3 and 4 below with error bars. The maximum increase in OD_{600} measurements was observed from 16 h to 24 h, this was coupled with the sudden change in appearance and the formation of precipitate. The maximum OD_{600} measurement was at 24 h and measured 6.37 ± 0.0782 absorbance units.
The most significant increase in growth was however observed between 8 h and 16 h, this proceeds the change in colour and optical density. The lag in OD_{600}, as opposed to metabolic activity, coincides with initial growth followed by precipitation. The sudden increase in metabolic activity is consistent with a decrease observed in the Pb(II) concentration, indicating an inhibitory effect of Pb(II) on the microbial culture. The maximum MA reading for the experiments was measured at a reading of 18.9 ± 0.0604 absorbance units at termination of the experiment. It was clear that growth had not yet stopped, but a decrease in growth rate was apparent, with a plateau forming from 24 h and onwards. The decrease in growth might be due to a depletion in substrate and available nutrients and/or a lack of available electron acceptors. It is known that anaerobic respiration is the use of alternative electrons acceptors to produce cellular energy. The specifics for this alternative electron sink should be investigated.

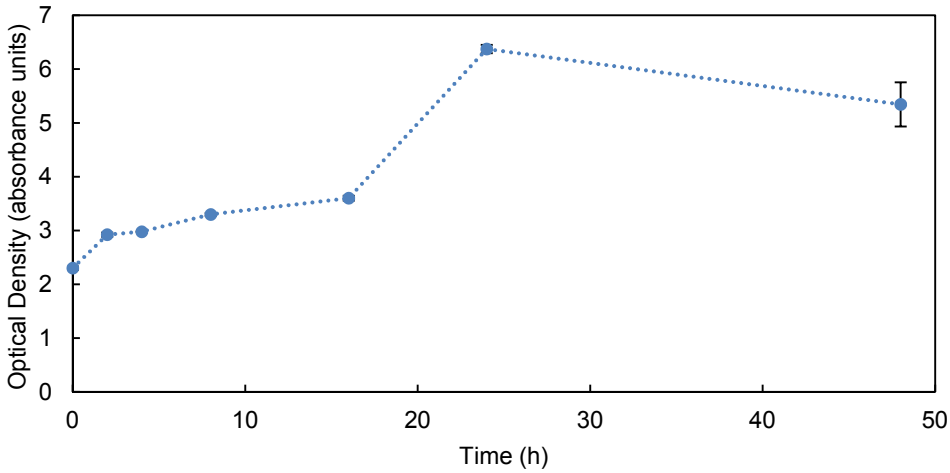

Figure 3: Optical density at 600 nm over time.

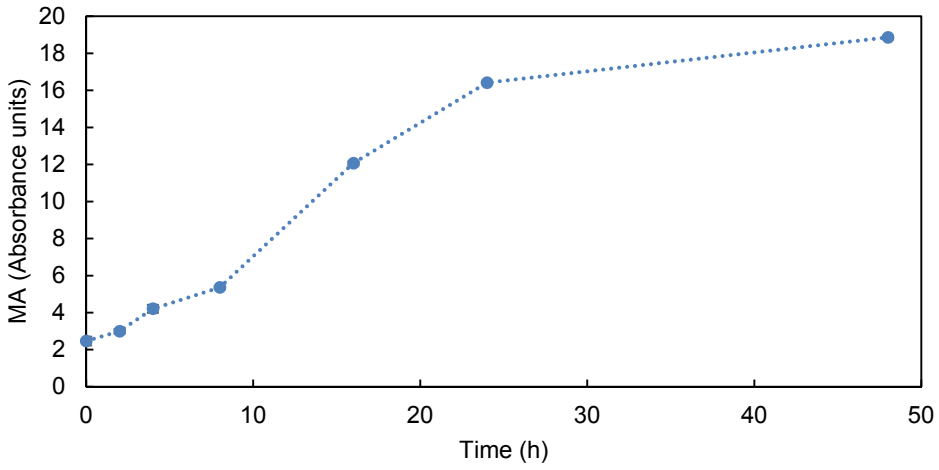

Figure 4: Metabolic activity at 550 nm over time.

4. Conclusions

It can be concluded that yeast extract autolyzed from *Saccharomyces cerevisiae* is an effect economically viable an environmentally friendly substitute for conventional LB broth when culturing Pb(II) removing bacteria. The autolyzed yeast extract is more effective in aiding Pb(II) removal as a biosorption mechanism is proposed to be present within the first few hours of Pb(II) removal. A Pb(II) precipitation (mineralization) mechanism follows biosorption of Pb(II) onto biomass to produce dark grey participation. Growth might be inhibited by the presence of Pb(II) and the biosorption mechanism acts as an initial detoxification mechanism. More research should thus be conducted into the specifics of the biosorption mechanism and the proposal of an anaerobic respiratory mechanism and the use of alternative electron acceptors to produce and maintain cellular energy. Research should also be conducted into the diluting of the produced yeast extract a growth medium of 100% yeast extract was used in this research project. The dilution of the food medium might increase the financial benefits of the already cost effect medium produced. Further studies could be conducted into the design and proposal of a continuous reactor setup, that could be used for future industrial application.

Acknowledgement

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed, and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

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