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Comparison of the Performance of Chlorococcum Ellipsoideum and Tetradesmus Obliquus as a Carbon Source for Reduction of Cr(VI) with Bacteria

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Chromium (Cr) is used in a variety of metallurgical, refractory and chemical processes, as well as in industrial activities such as the refining of ore, the production of steel and alloys, metal plating, and tanneries. Due to its widespread use in anthropogenic processes Cr(VI) is commonly released into the environment. Cr(VI) is known to be carcinogenic and mutagenic to living organisms, however some bacteria species have evolved a detoxification mechanism through which they reduce Cr(VI) to Cr(III), which is 100 times less toxic than Cr(VI). In previous studies of the bioreduction of Cr(VI), glucose and Luria-Bertani broth was used as the primary carbon source. In this study, an indigenous mixed culture of bacteria (Escherichia coli, Bacillus thermoamylovorans and Citrobacter sedlakii) is utilised to reduce Cr(VI) while consuming carbon sources produced by various algae species (Chlorococcum ellipsoideum and Tetradesmus obliquus). The different algae species were compared as possible carbon sources for the bioreduction process. Batch studies show that locally isolated bacteria co-cultured with algae achieved 100 % removal of Cr(VI) within 24 h. The performance of the different algae was very similar however bacteria utilizing Tetradesmus obliquus algae as a carbon source achieved the 100 % Cr(VI) reduction the fastest. Algae is sensitive to Cr(VI) toxicity and the algae growth is inhibited, therefore the algae must be cultured beforehand. SEM results indicate that the algae cells were adversely affected by the Cr(VI). The Cr(VI) destroyed the algae cell walls, allowing the bacteria to utilise the internal metabolites. Utilizing carbon sources produced by algae would be more practical to implement in the real world than adding glucose. This study demonstrates the potential of combining locally isolated Cr(VI) reducing bacteria and green algae to decontaminate Cr(VI) polluted sites in South Africa.

1. Introduction

In the environment Cr typically occurs either in its trivalent oxidation state as Cr(III), or in its hexavalent oxidation state as Cr(VI) (Dermatas et al., 2012). Cr(VI) is acutely toxic at high concentration and is classified as carcinogenic to humans (Concha et al., 2017). Cr(VI) is extremely mobile and therefore poses a high risk for groundwater contamination (Di Palma et al., 2012). Cr can accumulate in living organisms, which is why the World Health Organization (WHO) recommends that the maximum acceptable concentration level of Cr in drinking water is 0.05 mg/L (Seolatto et al., 2012). In trace amounts Cr(III) is considered to be essential for the proper functioning of living organisms, but Cr(III) is less bioavailable than Cr(VI) (Gomes et al., 2012). This has caused increased interest in technologies that are able to reduce Cr(VI) to Cr(III) through the use of bacteria.
Microorganisms, such as bacteria, exposed to toxic compounds such as Cr(VI) ions developed diverse resistance mechanisms to tolerate the toxicity. The resistance mechanisms involve specific biochemical pathways that can alter chemical properties of toxic metal (Igboamalu and Chirwa, 2016). Bacteria species that can tolerate Cr(VI) toxicity, and can reduce Cr(VI) to Cr(III), are called chromium reducing bacteria (CRB).
In most cases an organic carbon source is required, as either an energy source or as an electron donor, in order for a microorganism to reduce Cr(VI) (Zhiguo et al., 2009). In the past the carbon source was provided in forms similar to glucose and Luria-Bertani (LB) (Molokwane and Chirwa, 2008). Glucose is a carbon source of high cost. The cost of the carbon source is one of the most discussed drawbacks that presents the limitation of the

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commercial application of the bioremediation pathway (Vidotti et al., 2014). Recently Molokwane and Chirwa (2013) used saw dust as a natural alternative carbon source for Cr(VI) reduction.
The possibility of using algae and algae metabolites is yet still unexplored. Microalgae are photoautotrophic microorganisms that can produce biomass and energy by using sunlight and CO_2, which are renewable resources, for photosynthesis (phototrophic metabolism) (Visca et al., 2017). Thus, algae use the CO_2 already in the surrounding water, and CO_2 from the atmosphere, as its main carbon source. Algae produce extracellular and intracellular compounds. These cellular compounds, as well as the physical algae cell, can be used as a substrate for the bacteria (Dvoretsky et al., 2017). Chromium ruptures algae cell walls, causing cells to leak lipids, proteins, carbohydrates, and primary metabolites such as sugars, sugar alcohols, amino acids, and organic acids. These ruptured cell walls produce nutrients that surrounding bacteria can use (Cicci et al., 2017). A consortia of algae and bacteria can work in a synergistic manner to detoxify pollutants. Algal photosynthesis produces O_2 which the aerobic bacteria requires, and the bacteria provides CO_2 and other stimulatory means to support the photoautotrophic algae. The bioremediation processes, by means of algae-bacteria consortia, have the potential to be a self-sustaining system, which is cheaper compared to conventional remediation technologies, which have several disadvantages, such as high costs and the production of secondary pollutants (Fu and Secundo, 2016). The aim of this study is to determine if locally isolated CRB can utilise carbon sources produced by algae and further to compare how different algae species behave as carbon source. The effect of Cr(VI) toxicity on the algal cells is also investigated.

2. Materials and method

2.1 Algae cultivation

Chlorococcum ellipsoideum were collected from Hartbeespoort dam in South Africa and isolated using streak plating. Tetradesmus obliquus were sent from China. The algae strain was cultured axenically in the modified recipe of 3-fold Nitrogen, Bold Basal Media with Vitamins (3N-BBM+V). Cultures were grown in continuously stirred 1,000 mL Erlenmeyer flasks under the required algal light conditions (Osram L 36W/77 Floura) at 20 - 23 °C (Birungi and Chirwa, 2017). After 14 days the algae cells were harvested, and then centrifuged for 10 minutes at 6,000 rpm at 4 °C.

2.2 Bacteria cultivation

The CRB were isolated from dried sludge samples from the Brits Wastewater Treatment Plant in South Africa. These sludge samples were used as inoculum to culture the CRB. The bacteria strain identification was based on the ± 700 bp partial sequence of the 16S rRNA gene of the organisms. The sequences were compared against the GenBank of the National Centre for Biotechnology in the United States of America using a basic BLAST search. The isolated bacteria were identified as Escherichia coli, Bacillus thermoamylovorans and Citrobacter sedlakii. The different species of bacteria were used as a reconstituted consortium in experiments. The bacteria were stored as soon as possible after isolation in a -70 °C freezer to preserve the bacteria's chromium reducing capabilities. The bacteria that will be used in the batch studies were grown aerobically for 24 h in a 1,000 mL Erlenmeyer flask containing 400 mL LB broth. Cells were collected, and centrifuged for 10 minutes at 6,000 rpm at 4 °C. The supernatant was decanted, and the remaining pellet was washed three times in a sterile saline solution (0.85 % NaCl) (Igboamalu and Chirwa, 2016).

2.3 Analytical method

Cr(VI) was measured using the UV/Vis spectrophotometer (WPA, light wave II, Labotech, South Africa) at a wavelength of λ = 540 nm (10 mm light path). The appearance of a purple colour after acidification with 1N N_2SO_4 and adding 1,5-diphenyl carbazide indicates the presence of Cr(VI) in the sample. The intensity of the purple colour is related to the Cr(VI) concentration. Total Cr was determined in the Varian AA–1275 Series Flame Atomic Adsorption Spectrophotometer (AAS) at 359.9 nm wavelength equipped with a 3 mA and chromium hollow cathode lamp.

2.4 Cr(VI) reduction in batch setup

First the algae and bacteria were cultivated separately as described in Section 2.1 and 2.2. After the bacteria and algae cells were harvested and re-suspended in 50 mL Erlenmeyer flasks containing different concentrations of Cr(VI) and 3N-BBM+V media (pH of 7), the flasks were placed in an orbital shaker (Labotec, Gauteng, South Africa) at 30 ±2 °C. Samples taken at certain intervals were centrifuged at 6,000 rpm for 10 min in a Minispin® Microcentrifuge (Eppendorf, Hambury, Germany) and the supernatant was used for Cr(VI) analysis. In all the experiments the same biomass concentration for bacteria and algae were used, i.e. 6,100 mg/L bacteria and 1,000 mg/L algae.

3. Results and discussion

3.1 Algae as potential carbon source comparison

All the algae species used in this study proved to be suitable carbon sources for the bacterial reduction of Cr(VI). Figure 1 compares the percentage of Cr(VI) reduction, after 14 h, while utilizing different algae species and their metabolites as carbon sources. At lower initial Cr(VI) concentrations, 30 mg/L, all the algae species allowed for the reduction of more than 90 % of the Cr(VI). However at higher initial Cr(VI) concentrations a small inhibitory effect is observed; as Chlorococcum ellipsoideum carbon sources could only allow for 80 % Cr(VI) reduction. Figure 1 indicates that Tetradesmus obliquus performed the best as a potential carbon source. This could be attributed to the fact that Tetradesmus obliquus produced more accessible intracellular and extracellular compounds for the CRB to consume. A drawback of Tetradesmus obliquus algae is that it has a low specific growth rate compared to Chlorococcum ellipsoideum which leads to longer cultivation periods to attain the same dry biomass weight.

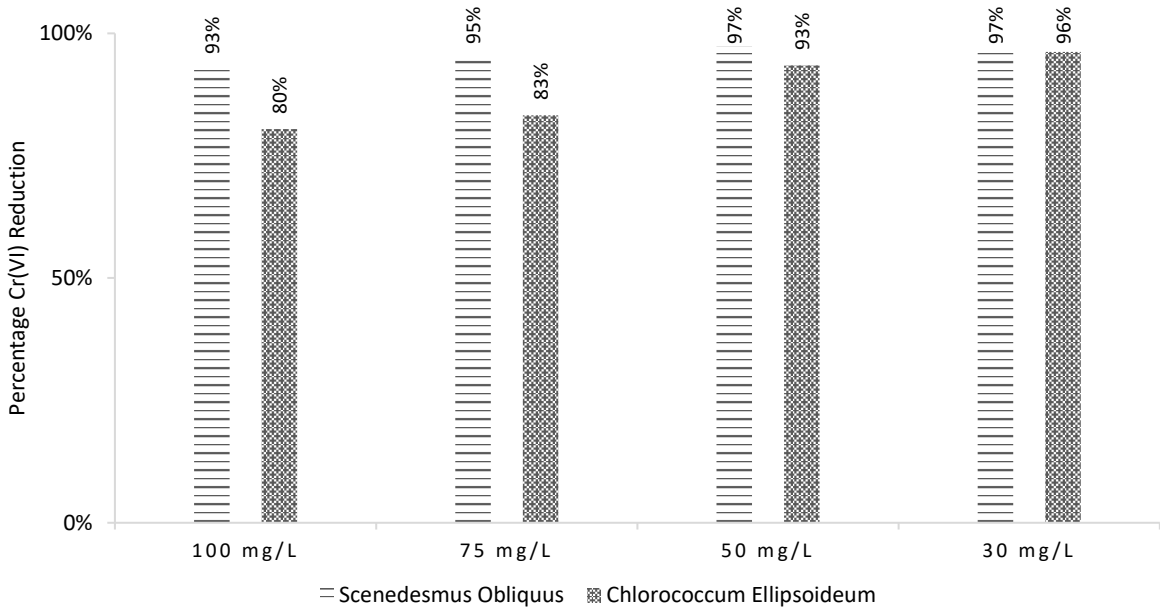

Figure 1: A comparison of the different algae species utilise as carbon sources for bacterial Cr(VI) reduction at different initial Cr(VI) concentrations after 14 h.

3.2 Cr(VI) reduction

In the control experiments, where no carbon source was used, only 25 % Cr(VI) was reduced by the mixed culture of bacteria. This can be attributed to either some of the Cr(VI) being adsorbed into the algae cells, but a very small amount was reduced to Cr(III), or that the bacteria were able to use their own metabolites to some extent as a carbon source. Another control experiment only contained algae cells and Cr(VI) and there was very little change in the Cr(VI) concentration, thus the possibility of using the algae as a biosorbent at a pH of 7 is not viable. As the CRB require a neutral pH for Cr(VI) reduction. The Cr(VI) however have adverse effects on the algae. The Cr(VI) inhibit the algae growth significantly and reduced the chlorophyll content in the algae cells which is necessary for photosynthesis. Bacterial Cr(VI) reduction using glucose as a carbon source were done in previous studies (Roestorff and Chirwa, 2018). Consuming glucose CRB were able to remove 100 % of the Cr(VI) within 7 h.

Figure 2 and 3 shows the results for the experiments in which Tetradesmus obliquus and Chlorococcum ellipsoideum were used in cooperation with CRB to remove Cr(VI). Out of the two algae species, Tetradesmus obliquus allowed for the highest overall Cr(VI) reduction. Complete Cr(VI) reduction of a solution with 100 mg/L initial Cr(VI) concentration were achieved while utilizing Tetradesmus obliquus as carbon source within 24 h as shown in Figure 2. Chlorococcum ellipsoideum, under the same conditions, allowed for just 92 % Cr(VI) reduction within 24 h.

The shape of the Cr(VI) reduction curves in Figure 2 and 3 suggest that there is more than one carbon source present. Which means that the intracellular and extracellular compounds, and the algal metabolites produced

by any one algae species are different and are utilize in a dissimilar manner by the CRB. The curves does not correspond with the standard Monod model which is normally used to describe Cr(VI) reduction when only glucose is present. In the Monod model, shown in Eq(1), the maximum reduction rate, k_m, and the half velocity constant, K_c, describes an unique enzyme-substrate relationship. Therefore, multiple carbon sources will have multiple k_m and K_c values, which is the present case.

$$-\frac{\mathrm{dCr(VI)}}{\mathrm{dt}} = \frac{\mathrm{k_m Cr(VI)}}{\mathrm{Cr(VI) + K_c}} \cdot \mathrm{X} \tag{1}$$

In the first section of the curves, shown in Figure 2 to 4, there is rapid Cr(VI) reduction, which leads to the conclusion that one of the carbon sources is readily accessible to the CRB and is completely consumed. The carbon source that is available in the second section of the curves only allows for slow reduction of Cr(VI). The carbon sources are consumed in a sequential manner. It could also be that the CRB has consumed all the extracellular compounds and the Cr(VI) toxicity is slowly breaking down the algal cell walls, which will cause the intracellular compounds to become available. The reduction of Cr(VI) can be expressed with Eq(2) where the first term represents the fast Cr(VI) reduction, the second term represents the slow Cr(VI) reduction and α plus β are a corresponding fraction.

$$\mathrm{Cr(VI)_{total}} = \alpha \mathrm{Cr(VI)_{fast}} + \beta \mathrm{Cr(VI)_{slow}} \tag{2}$$

Before combining the Monod model, Eq(1), with Eq(2) two assumptions are made; Firstly, the biomass concentration remains relative constant throughout the batch experiment so that X is X_0, the initial biomass concentration. Secondly, the K_c value is much larger than the Cr(VI) concentration such that the whole denominator term is dominated by K_c and the Monod model becomes first order. With the above assumptions the Monod model integrated form is shown in Eq(3)

$$\mathrm{Cr(VI)} = \mathrm{Cr(VI)_0} e^{-\frac{X_0 k_m}{K_c} t} \tag{3}$$

The, $\frac{X_0 k_m}{K_c}$, term can be simplified and represented by either K_{fast} or K_{slow}. Eq(3) can be substituted into Eq(2) as shown in Eq(4).

$$\mathrm{Cr(VI)} = \alpha \mathrm{Cr(VI)_0} \mathrm{e^{-K_{fast} t}} + \beta \mathrm{Cr(VI)_0} \mathrm{e^{-K_{slow} t}} \tag{4}$$

The kinetic parameters for the different algae species are given in Table 1. Each algae species produce different carbon sources and therefore have unique substrate-enzyme relationships with the CRB and unique parameters.

Table 1: Kinetic parameters for the derived Cr(VI) bioreduction model, Eq(4), of the different algae species.

Parameter	α	β	K_{fast} (min^{-1})	K_{slow} (min^{-1})	R^2
Tetradesmus obliquus	0.26	0.74	0.113	0.0026	0.98
Chlorococcum ellipsoideum	0.39	0.61	0.033	0.0018	0.96

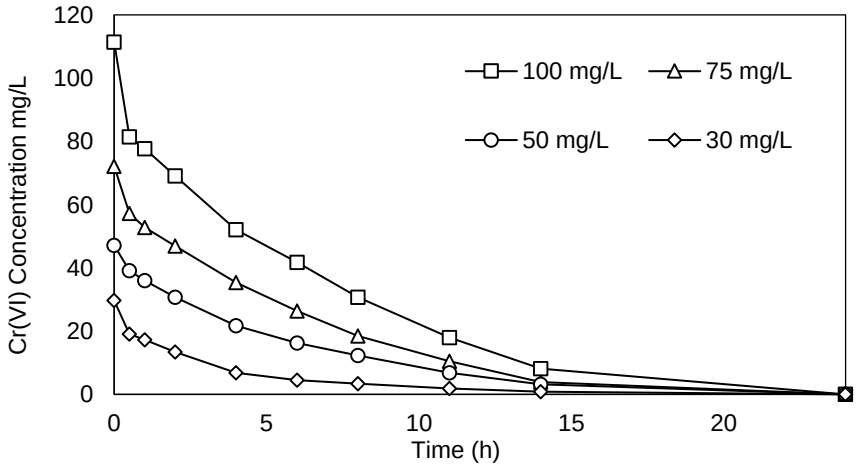

Figure 2: Cr(VI) concentration in batch experiments in which CRB utilise Tetradesmus obliquus as a carbon source.

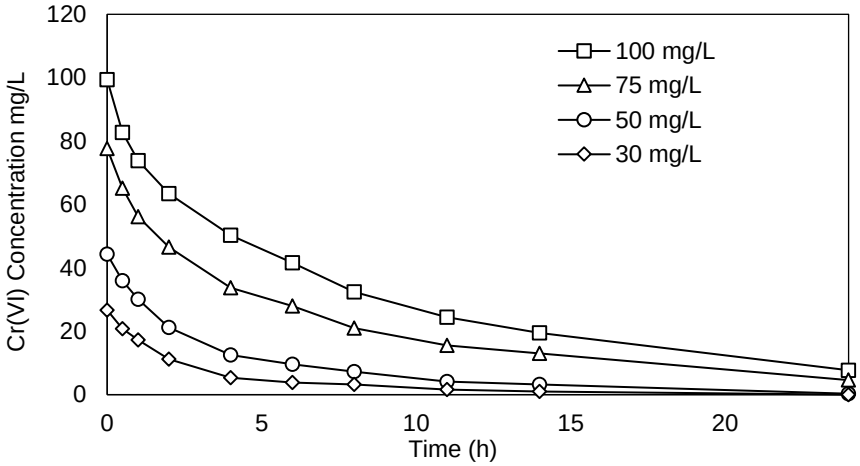

Figure 3: Cr(VI) concentration in batch experiments in which CRB utilise Chlorococcum ellipsoideum as a carbon source.

3.3 SEM results

In Figure 5 the SEM images of algae and bacteria cells are shown. The bacterial cells are the smaller cylindrical cells and the algae cell is the larger round cell. Figure 5a shows the cells before Cr(VI) was added and the algae cell wall is still intact. Figure 5b shows the cells after Cr(VI) exposure and the algae cell is shriveled up and has lost structural integrity. It is possible that a portion of the internal metabolites has leaked from the algae cell and were then utilized by CRB.

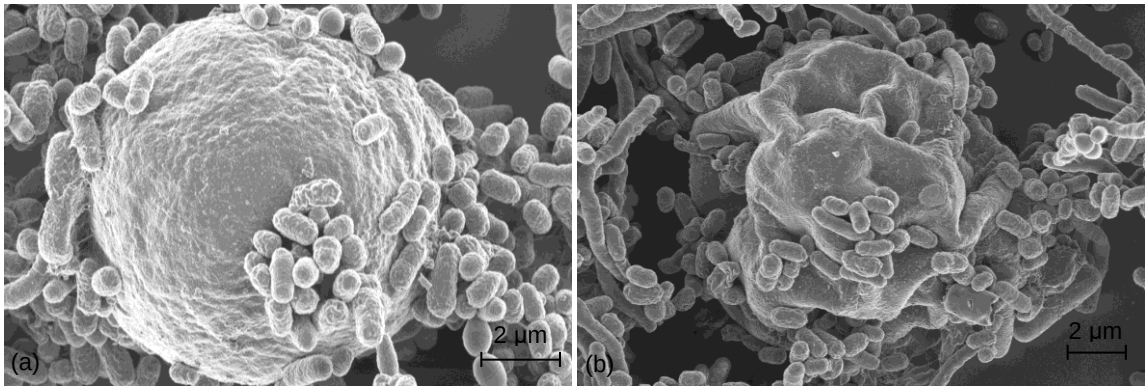

Figure 4: SEM images of the algae and bacteria cells. (a) Before the cells were exposed to Cr(VI). (b) After the cells were exposed to Cr(VI)

4. Conclusions

Successful Cr(VI) removal was achieved for all the different algae species that were used as a carbon source for CRB. Tetradesmus obliquus had the best performance as a carbon source, but due to the long cultivation period associated with Tetradesmus obliquus it would be better to use Chlorococcum ellipsoideum which was locally isolated. This research indicates that it is possible for the CRB to use various carbon sources to achieve Cr(VI) reduction including those provided by the algae. Some light was shed on the processes involved in the algae-bacteria system, but further research is warranted. The practicality and self-sustaining nature of using algae and CRB in a continuous system must be evaluated, and if possible pursued.

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