Journal of Environmental Management 222 (2018) 396–401

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Research article

Biosorption of multi-heavy metals by coral associated phosphate solubilising bacteria *Cronobacter muytjensii* KSCAS2

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ARTICLE INFO

Keywords:
Biosorption
Cronobacter muytjensii
Heavy metals
PSB

ABSTRACT

This paper examines the potential detoxification efficiency of heavy metals by phosphate solubilising bacteria (PSB) that were isolated from coral, sea grass and mangrove environment. Initially, four potential bacterial isolates were selected based on their phosphate solubilisation index from 42 strains and were used for the metal tolerance test. Among the four isolates, KSCAS2 exhibited maximum tolerance to heavy metals and the phenotype indicated the production of extra polymeric substances. In a multi-heavy metal experimental setup at two concentrations (100 and 200 mg L^{-1}), it has been demonstrated that the bacteria have extracellularly sequestered metal ions in amorphous deposits and this has been confirmed by scanning electron microscopy. In experiments with a 100 mg L^{-1} initial metal concentration, the percentages of metal removal by bacteria were 55.23% of Cd, 72.45% of Cr, 76.51% of Cu and 61.51% of Zn, respectively. In subsequent experiments, when the metal concentration was increased up to 200 mg L^{-1}, the metal removal capacity decreased as follows: 44.62%, 63.1%, 67% and 52.80% for Cd, Cr, Cu and Zn, respectively. In addition, the biosorption of heavy metals was confirmed by the Fourier transform infrared Spectroscopy (FT-IR) and scanning electron microscopy (SEM) analysis. The heavy metal concentrations in a broth culture were analysed by inductively coupled plasma-optical emission spectroscopy (ICP-OES). The study suggests that PSB *Cronobacter muytjensii* KSCAS2 can efficiently remove the heavy metals and these bacteria could be used for the metal removal from the agricultural soils.

1. Introduction

Soil is one the most important non-renewable natural resources. It forms the basis of food and fuel and serves as a critical component of many ecosystems. Soil is contaminated by heavy metals due to the increase in geologic and anthropogenic activities. Heavy metals which pollute the agricultural lands cause decrease in microbial activity and productivity of the crops (Jiang et al., 2008). The aggregations of heavy metals penetrate the crops through absorption and are highly hazardous to the top consumers in the food-chain including humans through bioaccumulation (McLaughlin and Singh, 1999).

Metals are essential to microorganisms at minimum concentration for various metabolic events, however, they are toxic at higher concentrations (Adriano, 2001). Some heavy metals exhibits toxicity even at low concentration (1.0–10 mg/L) for example Hg and Cd metal ions were found to be toxic even at a concentration of 0.001–0.1 mg^{-1} (Wang and Chen, 2006). This necessitates the elimination and control of the accumulation of heavy metals from agricultural lands.

Various conventional methods such as adsorption on activated carbon, reverse osmosis, ion exchange, chemical precipitation and membrane filtration have been used for the elimination of heavy metals from the soil (Rajasulochana and Preethy, 2016). Though these methods are effective, they are very expensive and detrimental to the environment. Bioremediation is an effective tool to overcome these difficulties. Bioremediation techniques are low-cost and eco-friendly and are carried out using renewable resources such as plants and microbes (Nies, 1999). Among the diverse bioremediation methodologies, phytoremediation is more prevalent because of the cost effectiveness. The main disadvantage of this technique is the time consumption and the disability of plants to tolerate high concentration of metals (Ali et al., 2013). These difficulties can be overcome by microbial bioremediation, which are carried out using bacteria (Iyer et al., 2005), yeast (Goksungur et al., 2005), fungi (Anayurt et al., 2009) and algae (Gupta and Rastogi, 2008).

Among all the microbes, bacteria have been identified to be one of the most important biosorbents used for metal detoxification. Bacterial cell walls comprise a variety of surface organic functional groups, which offer high affinity to binding of metals (Daughney et al., 2002).

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https://doi.org/10.1016/j.jenvman.2018.05.083
Received 19 September 2017; Received in revised form 23 April 2018; Accepted 26 May 2018

Hence, biosorption of heavy metals by bacterial cell wall has been in practise for a long time (Feng et al., 2012).

Phosphate solubilising bacteria is a group of bacteria which can solubilise the phosphate. It has received substantial attention in recent years due to its potential applications in heavy metal detoxification and diverse plant growth promoting traits, such as, production of Indole acetic acid (IAA), production of organic acids, ACC (1- aminocyclopropane-1-carboxylate) deaminase activity and phosphatases production (He et al., 2013; Oves et al., 2013; Ahmad and Kibert, 2013).

Heavy metal contamination of agricultural land might cause phytotoxicity and reduction in land usability for agricultural production ultimately causing food insecurity. This also poses a high risk to human health as a result of direct contact through ingestion and contact with contaminated soil as well as bioaccumulation. In addition to heavy metal contamination, insoluble phosphate is also considered as a major environmental problem in the agricultural field as they cannot be absorbed by plants (Rengel and Marschner, 2005). Considering the excess phosphate content and metal threat to agricultural soils in relation to human health, PSB can be involved in dual role of phosphate solubilising and heavy metal detoxification. The present investigation was carried out to characterise the detoxification efficiency of heavy metals by PSB bacterial strain *Cronobacter muytjensii* (KSCAS2) isolated from coral associated sediment, which could be potentially used for the process of bioremediation.

2. Methods

2.1. Sample collection, isolation and screening of phosphate solubilising bacteria (PSB)

Twelve sediment samples were collected from three distinct ecosystems such as coral reef, sea grass beds and mangroves. Sediments samples from coral reef (Lat. 9º18′20.14″N, Long. 79° 20′32.33″) and seagrass beds (Lat. 9º19′03.65″N, Long. 79° 20′21.52″) were collected at depths ranging from 1.5 to 3 m by well-trained scuba divers. Samples from mangrove region (Lat. 11º29′24.77″N, Long. 79º45′57.87″) were collected during low tide. The top layer (5 cm) of sediments were collected and transferred into an insulated box preserved at 4 °C. They were immediately transported to laboratory and processed under aseptic conditions. One gram of wet soil was serially diluted by using 25 mM of phosphate buffer and spread over Pikovskaya's (PKV) agar plates containing tricalcium phosphate 2.5 g, glucose 13 g, $(NH_4)_2SO_4$ 0.5 g, NaCl 0.2 g, MgSO4·7H2O 0.1 g, KCl 0.2 g, yeast extract 0.5 g, MnSO4 trace, FeSO4·7H2O trace, agar 15 g, pH adjusted to 7.2 and dissolved in 1000 ml distilled water. The plates were incubated at 30 °C for 48–96 h and colonies with clear halo zone were marked for positive phosphorous solubilisation (Freitas et al., 1997). These were sub cultured 10–15 times in freshly prepared PKV agar plates to obtain pure strains. The high phosphate solubilising strains including KSCAS1, KSCAS2, KSCAS3 and KSCAS4 were selected from the isolates for further study. Phosphate solubilising activity was measured by plate screening method; the bacteria were inoculated in the centre of PKV agar plates and were incubated for five days at 30 ± 2 °C. Clear zones around the bacterial culture denote solubilisation and the phosphate solubilisation index (SI) was calculated using the formula (Edi-Premono et al., 1996) given below:

$$\text{Solubilization Index (SI)} = (\text{Colony diameter} + \text{Halo zone diameter}) / \text{Colony diameter.}$$

2.2. Enrichment and assessment of heavy metal resistant efficacy by PSB

The selected bacterial cultures were amended with heavy metals prior to the assessment of multi-element tolerance capacity. 0.1 mM of Cd, Cr, Cu and Zn were mixed in 100 ml of Zobell marine broth (ZMB) and incubated at 30 °C for 48 h in a shaking incubator rotating at a speed of 200 rpm/min (Kato et al., 2016).

The metal resistance capability of PSB was confirmed using well diffusion method (Hassen et al., 1998). The bacterial strains were spread over the solid agar medium containing different concentrations of Cd, Cu, Cr and Zn ranging from 100 to 500 mg^{-1}. The plates were incubated at 30 °C for a period of 24 h. The maximum concentration tolerance of individual metal by each strain was determined based on the zone of inhibition test.

2.3. Characterisation of bacteria

Among the four PSB strains, KSCAS2 exhibited the highest resistance capacity for all the metals and hence was selected and characterised by morphological, physiological and biochemical tests. The biochemical properties of the strain KSCAS2 including gram reaction, indole production, methyl red test, citrate utilization test and Voges-Proskauer test were determined by standard methods given in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994).

The strain KSCAS2 was subjected to 16S rRNA gene sequence using the universal eubacterial primers 5′ AGAGTTTGATCCTGGCTCAG 3′ and 5′ ACGGCTACCTTGTTACGACTT 3′ (Weisburg et al., 1991). Attained sequence was submitted in the gene-bank database. The taxonomic classification of the strain (KSCAS2) was done using BLASTn online programme in the NCBI database. Phylogenetic tree was constructed using MEGA6.1 software.

2.4. Metal tolerance capacity

The multi-metal tolerance capacity was observed at different metal concentrations. Individual metals were added to the nutrient broth at concentrations of 100 and 200 mg^{-1}. The bacterial culture was inoculated and incubated at 30 °C for 48 h. The growth pattern of the selected strain was observed by measuring the optical density at regular intervals using UV-VIS spectroscope (SHIMADZU, 1800) (Oves et al., 2013).

2.4.1. Metal sorption by bacterial strains

Selected bacterial cells were grown in Zobell marine broth for a period of 24 h and the measured density was 10^9 CFU/ml. The biomass was harvested by centrifugation and washed with 1 ml saline solution (0.8%). The harvested pellets were inoculated into Zobell marine broth containing a mixture of heavy metals. The heavy metals mixture was prepared at two different concentration 100 and 200 mg of Cd, Cr, Cu and Zn. The method described in this experiment was slightly modified (3 mM Cd, 1.1 mM Cu, 1 mM Pb and 1.1 mM Zn) from the method used by Mergeay et al. (1985). The bacterial growth was measured at different time intervals. The cell free supernatants were subjected to inductively coupled plasma optical emission spectroscopy (ICP-OES) (Model 5100, Agilent, UK) for the determination of residual metal contents.

Biosorption isotherms were calculated using the Langmuir equation:

$$q_{ex} = [(C_0 - C)_x V]/M$$

where q_{ex} is the quantity of biosorption onto the adsorbent (mg g^{-1}), C_0 is initial concentration of the metal ions (mg L^{-1}), C is the residual concentration of metal ions (mg L^{-1}) present in medium, V is the volume of the experiment and M is the amount of adsorbent (g).

The heavy metal removal potential was calculated using the formula:

$$\% \text{ removal} = \frac{\text{Heavy metal ions removed (mg)} \times 100}{\text{Heavy metal ions available (mg)}}$$