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The Growth Potential and Bioaccumulation Ability of Probiotics under the Exposure of Different Heavy Metals

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ABSTRACT

The presence of heavy metals in aquaculture is a major concern due to possible toxicity effects to the organisms. Bioaccumulation with bacteria is an effective and economical way to remove heavy metals from the water. The objectives of this research were to measure the growth rate of probiotics (*Bacillus* sp. BpChIAY [BpChIAY] and *Bacillus thruingiensis*, [Bt]) under different concentrations of selected heavy metals, and to determine the ability of the probiotics to bioaccumulate selected metals. Bacterial strains were grown in nutrient broth with the addition of heavy metals (Cu, Cr, Cd, Zn, Ni) at 37°C to determine the growth under exposure to heavy metals. The bioaccumulation experiment was conducted by exposing the strains' pellets to heavy metals solutions. The concentrations of heavy metals were measured using Atomic Absorption Spectroscopy (AAS). The bacterial growth percentages when grown under 2 ppm Zn, Ni and Cd for BpChIAY were 128%, 103% and 67%, and for BT, the growth rates were 97.7%, 98.8% and 36.2%, respectively. The findings showed the order of the toxicity in the order from the most toxic: Cu>Cr>Cd>Zn>Ni. Both strains were able to bioaccumulate the heavy metals, and

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BpChIAY was a better metal absorber than BT at the rate of 0.0539 mg/g for Zn, 0.0781 mg/g for Ni and 0.0256 mg/g for Cd.

Keywords: *Bacillus* spp., *Chlorella* sp., heavy metals, probiotics, *Tor tambroides*

INTRODUCTION

The manufacturing industries, which consist of metal finishing processes such as electroplating, etching and preparations of metal components, are major contributors to heavy metals pollution in Malaysia. Among heavy metals released are nickel (Ni), zinc (Zn), aluminium (Al), cadmium (Cd), copper (Cu), iron (Fe) and chromium (Cr) (Yeoh, 1993). The metals concentrations in rivers of Malaysia have been extensively studied, which show that rivers sediments are mostly polluted with Pb, Zn and Cu due to industry discharges, sewage and runoff. Juru River in Penang, for example, contained 117 µg/g of Pb, 144 µg/g of Cu and 483 µg/g of Pb (Lim & Kiu, 1995). The Langat River of Negeri Sembilan contained 71-374 µg/g of Zn and 3.0-37.9 µg/g of Cd, which exceeded the natural average global shale values (Sarmani, 1989).

Heavy metals can have various toxicological effects on living organisms by disrupting the biochemical roles in metabolic processes such as the dysfunction of the endocrine system, reproductive system, growth, immune system as well as metabolism (Jakimska et al., 2011). This is an alarming issue because they can cause toxicity to aquatic organisms, being in close and prolonged contact with the soluble metals (Kaoud, 2015). Aquatic organisms may adsorb heavy metals from surrounding water and food, which may accumulate in various tissues and causing toxicological effects (Kaoud, 2015, Mazon et al., 2002).

Probiotics are known as living organisms that when applied in appropriate amounts will grant the hosts health benefits (Joint, 2002). They are able to provide benefits in fish where they can produce inhibitory responses against pathogens, provide essential nutrients as well as developing important immune responses (Verschuere et al., 2000). Some bacteria can reduce metal toxicity because they possess resistance mechanisms and are able to bind and sequester heavy metals onto the cell surfaces to remove heavy metals. *Lactobacillus* sp., for example, can reduce oxidative stress by heavy metals and detoxify them as well. For arsenic and mercury, *Lactobacillus* sp. resists by actively expulsing toxic metals from cytosol, govern by *mer* and *ars* operons in the DNA, respectively (Monachese et al., 2012). *Bacillus* spp., are Gram-positive, rod-shaped, and spore-forming bacteria. They are suitable to be consumed by living organisms due to their capability to withstand highly acidic conditions (Bader et al., 2012).

The concentration of heavy metals in the environment can be reduced by utilizing microorganisms through biosorption and bioaccumulation. The biosorption process refers to the binding of metals onto the cell wall's surface and it is a simple physicochemical process similar to conventional adsorption or ion exchange. Whereas bioaccumulation process refers to

the intracellular accumulation of metals that occur in two stages, biosorption and active transport system. This process is a much more complex process compared to biosorption in which it requires the metabolic activity of cells. In other words, the metals are required to go through biosorption first and only then the metals go through the next step, which is bioaccumulation by transporting them within the cells (Chojnacka, 2010).

Bioaccumulation works by transporting the metals across the cell wall and membrane. When the metals are within the cells, the metals will be bounded to intracellular structures. Intracellular accumulation and oxidation or reduction reactions are the mechanisms contributing to bioaccumulation in cells. There are several factors that impact the mechanisms which are the composition of growth medium, pH temperature, presence of other metals and inhibitors. The process of bioaccumulation relies on the synthesis of low molecular weight proteins metallothioneins that are rich with thiol groups that bind to the metals (Chojnacka, 2010).

Due to the hazardous effects of heavy metals to the environment, it is important to find appropriate ways to curb the issue of heavy metals pollution. Utilizing probiotics is an ideal method due to its metal bioaccumulation capabilities. Therefore, the objectives of this research are to measure the growth rate of the probiotics obtained from marine microalgae (BpChIAY) and freshwater fish (Bt) to grow under different concentrations of selected heavy metals, and to determine the ability of the probiotics to bioaccumulate the metals.

MATERIALS AND METHODS

Growth of Probiotics under Selected Heavy Metals

In this study, the following probiotics were used: *Bacillus* sp. BpChlAY isolated from marine microalgae, *Chlorella* sp., and *Bacillus thuringiensis* (Bt), isolated from freshwater fish, *Tor tambroides*. These strains were obtained from subculture from Faculty of Agriculture, UPM.

The probiotics were inoculated in 10 mL of nutrient broth in universal bottle for homogenizing purposes. The inoculated nutrient broths were placed on a rotary shaker at 160 rpm, for 24 hours at 37°C. After 24 hours, 50 µL of the homogenized probiotics were taken and placed in 5 mL of nutrient broth in HACH test tubes. The probiotics were grown for 8 hours on a rotary shaker at 160 rpm at 37°C. Absorbance readings for the probiotics were taken once every 2 hours until the final 8th hour. Experiments were done in triplicates. The readings obtained were then used to graph out growth curve graphs of the probiotics.

Probiotics Growth under Heavy Metals Analysis. The probiotics were homogenized in 10 mL of nutrient broth grown on a rotary shaker at 160 rpm, for 24 hours at 37°C. The selected heavy metals, Cd, Cr, Cu, Zn and Ni were added in 5 mL of nutrient broth in HACH test tubes and the probiotics were then inoculated in heavy metal containing nutrient broth. The absorbance readings for the probiotics were taken once every 3 hours until the final 6th hour. This experiment

was done only until the 6th hour because the bacteria's exponential phase started between the 2nd hour and the 6th hour. Experiments were done in duplicates. Two different concentrations of heavy metals (2 ppm and 10 ppm) were tested. The readings were then used to graph out the growth curve of the probiotics with heavy metals.

Bioaccumulation of Heavy Metals by Probiotics. 40 mL of bacterial culture, which were grown in nutrient broth, were centrifuged at 6000 rpm for 5 minutes. The pellets obtained were then washed with buffer and subsequently centrifuged. The supernatant was removed and the pallets in the falcon tubes were weighed. The pellets obtained were resolubilized and exposed to 50 mL of 2 ppm heavy metals solution. After two hours, the heavy metals solutions were extracted and sent to be analyzed under Atomic Absorption Spectroscopy (AAS) to identify the final concentration.

RESULTS AND DISCUSSION

As shown in Figure 1, BpChIAY underwent lag phase from 0th hour until the 2nd hour. The bacterium entered the exponential phase from the 2nd hour until the final 8th hour. The bacterium Bt showed a similar growth trend as BpChIAY in which the lag phase started from 0th hour to the 2nd hour, and entered the exponential phase from the 2nd hour until the 8th hour. There was no observable growth during the lag phase due to the bacteria synthesizing the necessary nutrients and molecules for growth, and to adapt to the new environment. In the exponential phase, the bacteria's population increased at the maximum rate. The exponential growth rates of Bt and BpChIAY were 0.09372hr^{-1} and 0.08292hr^{-1}, respectively.

Growth of the Probiotics under Selected Heavy Metals. It was observed that when grown under 10 ppm of heavy metals, none of the bacteria were able to grow (Table 1). When the concentration was reduced to 2 ppm, both bacteria showed positive growth (Table 1). For BpChlAY, the growth rate under Zn, Ni and Cd were 0.123 hr^{-1}, 0.123 hr^{-1} and 0.0848 hr^{-1}, respectively. Whereas for Bt, the growth rates were 0.106 hr^{-1} for Zn, 0.118 hr^{-1} for Ni and 0.0485 hr^{-1} for Cd.

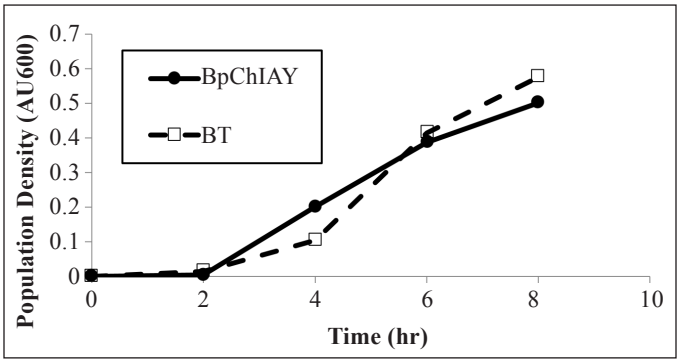

Figure 1. The growth of probiotics, BT and BpChIAY in nutrient broth

Table 1
The growth rates of BpChIAY *and* Bt *under different heavy metals*

	2 ppm		10ppm	
	BpChIAY growth rate (hr^{-1})	Bt growth rate (hr^{-1})	BpChIAY growth rate (hr^{-1})	Bt growth rate (hr^{-1})
Control	0.083	0.094	0.083	0.094
Cd	0.085	0.049	NA	NA
Cu	0.010	0.001	NA	NA
Cr	0.032	NA	NA	NA
Ni	0.123	0.118	NA	NA
Zn	0.123	0.106	NA	NA

NA = Not Available

BpChIAY growth rose higher when grown with Ni and Zn than the no-metal control at growth percentages of 128% and 103%, respectively (Figure 2). For Cd, Cu and Cr, BpChIAY's growth were inhibited where the percentages were only 67%, 5.02% and 22.2%, respectively (Figure 2). For Bt, only Cd, Ni and Zn did not inhibit growth whereas Cu and Cr inhibited it completely. Under Cd, Ni and Zn, the bacterium was able to grow at 36.2%, 98.8% and 97.7% and under Cu and Cr, the growth were completely inhibited (Figure 3).

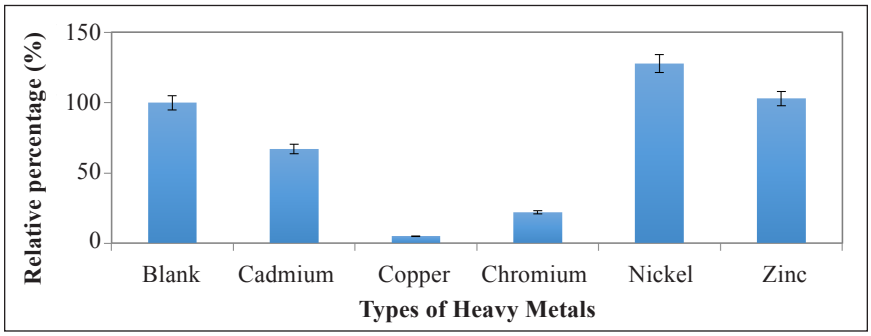

Figure 2. The percentage of growth of BpChIAY under different types of selected heavy metals

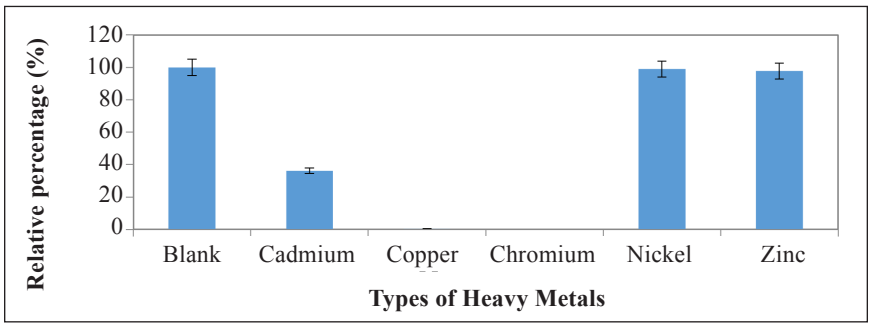

Figure 3. The percentage of growth of Bt under different types of selected heavy metals

The results showed that the bacteria were only able to grow when the concentration of heavy metals were reduced from 10 ppm to 2 ppm. This is expected because heavy metals at an increasing concentration can cause motility thus inhibiting the growth of the bacteria.

When bacteria are exposed to high concentrations of Zn, specifically Zn^{2+}, growth will be inhibited. This works by the reaction of Zn^{2+} with the mercapto group of the biological macromolecule which destroys the proteins and reject proliferation thus decreasing the rate of growth of cells. Besides that, Zn^{2+} can also cause the cell membrane permeability to change and disrupt the transportation of nutrients and waste across the membrane by deactivating the target sites in a cell (Yao et al., 2005).

For Cr, no growth were observed when the concentration was at 10 ppm or 2 ppm on both bacteria. This is because Cr, when presented in its toxic form, Cr^{6+}, may damage the cells' DNA. Cr^{6+} can easily be transported across the membranes due to non-specific phosphate or sulphate transporter activity. When Cr^{4+} is present within the cells, it will be reduced by reducing agents such as non-enzymatic Asc and thiol groups. The resulting Cr^{4+} intermediates will react and form complexes with the DNA, or proteins that will induce mutations, chromatid exchange and chromosomal instability (Younan et al., 2016).

Bacterial growth were also inhibited under the effect of Cu. Cu, when presented in high concentration interrupts the energy transport system, the enzyme active sites and the integrity of cell membranes similar to Zn. Cu can also cause the lipids, proteins and DNA within the bacteria to be damaged (Shenge et al., 2014). Similar to the other metals, the exposure of Cd interrupts the cell membrane functions and chemical reactions with cellular components. It can inhibit or compete with the cell's enzyme systems. This is caused by the interactions of the metals onto the cell surface receptors (Yamina et al., 2014).

Ni toxicity exhibits itself at 10 ppm to both of the bacteria. This is because there are four mechanisms of Ni toxicity on microorganisms which replaced essential metal of metalloproteins with Ni, the binding of Ni to catalytic residues of non-metalloenzymes, binding of Ni to the outside catalytic site of an enzyme to inhibit allosterically and lastly oxidative stress onto the microorganisms caused indirectly by nickel (Macomber & Hausinger, 2011).

BpChIAY and Bt can grow in Cd, Ni and Zn at low concentration due to their resistance mechanisms. There are three main mechanisms of action for bacteria against heavy metals which are ion exchange of the metals with peptidoglycan and teichoic acid, precipitation through nucleation reactions, and the complexation with oxygen and nitrogen ligands (Monachese et al., 2012).

The results also showed that the growth of BpChIAY grew significantly higher than the control when supplemented with 2 ppm of Zn by 28%. This is because Zn^{2+} in low concentrations, protect the integrated cells. This in return will cause the cells to have appropriate flow quality and is also helpful

in the synthesis of DNA and RNA (Yao et al., 2005). For Ni, trace concentrations of Ni can be utilized by microorganisms to assist in various cellular processes. One is by taking up Ni via nickel-specific permeases or ATP-binding binding systems after the microorganisms have sensed the Ni concentration. The metal will then be incorporated into enzymes that are nickel-dependent via complex assembly processes in which it will help the microorganisms to thrive in a Ni polluted environment (Mulrooney & Hausinger, 2003).

As obtained from the readings in Figures 2 and 3, the bacteria were able to thrive the best when supplemented with Ni and Zn, but growth was reduced by 33% for BpChIAY and 63.8% for Bt when grown under Cd. The order of toxicity of the metals to the bacteria was found to be Cu > Cr > Cd > Zn > Ni. This result was similar to a study done by Qing et al. (2007) that tested the growth of bacteria when grown under five different heavy metals. Qing et al. (2007) discovered that *Enterobacter clocae* and *Bacillus cereus* (*B. cereus*) are both tolerant to heavy metals particularly Cd and Zn. Malik et al. (2002) studied the metal tolerance of 70 industrial and agricultural soils bacterial isolates discovered that 88.8% of the bacteria isolates are tolerant to Ni, 82.8% to Zn and 71.4% to Cd.

Bioaccumulation of the Heavy Metals by Probiotics. BpChIAY metal absorbed per gram of bacteria for Zn was 0.0539 mg/g, 0.0781 mg/g for Ni and 0.0256 mg/g of Cd, whereas for Bt, the values for Zn, Ni and Cd were 0.0454 mg/g, 0.0309 mg/g and 0.0196 mg/g, respectively (Figure 4). When comparing the three metals, Zn and Ni have the higher removal rate compared to Cd for both bacterial strains. The ability of bacteria to adsorb heavy metals shows great usability in the open field like rivers in Malaysia. As an example, in Langat River in Selangor, the Zn concentration was observed to be at 71-374 ppm higher than the National Water Quality Standard of Malaysia, at 5 ppm (Sarmani, 1989).

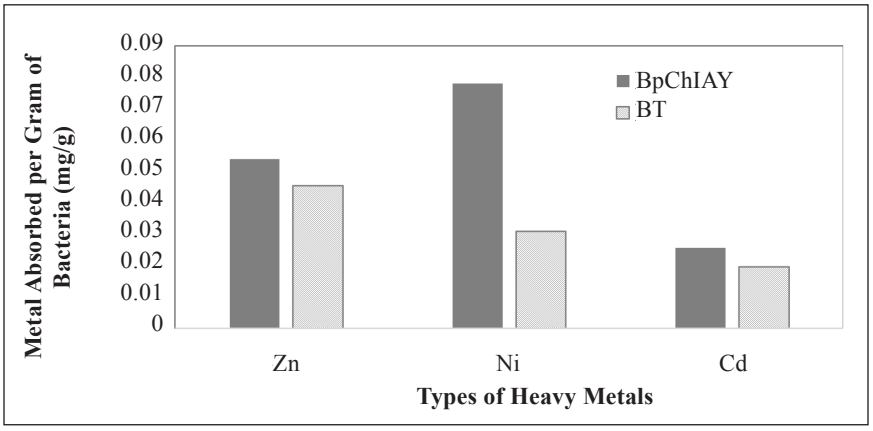

Figure 4. The average weight of heavy metal absorbed per gram of bacteria

The *Bacillu*s spp. are good metal adsorbers because they have higher adsorptive capacity from the high content of peptidoglycan and teichoic acid in the cell walls (Monachese et al., 2012). The carboxylic group in the glutamic acid of peptidoglycan of the bacteria's cell walls is a major site of metal deposition (Bhakta et al., 2012). For Cd-resistance bacteria, specifically gram-positive bacteria, its resistance depends on cadmium efflux mediated by the cadmium-exporting P-type ATPase CadA pump. An example would be in *Saccharomyces cerevisiae*, in which the Cd will be bounded by glutathione thus producing a Cd-bisglutathionato complex which will then be transported to the vacuole by an ABC transporter (Nies, 1999).

For the bioaccumulation of the heavy metals by the bacteria, only Cd, Ni and Zn at 2 ppm were selected to be tested with the bacteria as they did not inhibit growth entirely. It was observed that both bacteria are capable of accumulating Ni and Zn better than Cd. This confirmed previous results which showed that the bacteria grew better under Ni and Zn than Cd. When comparing the two bacteria's bioaccumulation ability, BpChIAY had a better rate of heavy metal removal from polluted environment compared to Bt. Ni was removed the most by BpChIAY followed by Zn and Cd. Bt removed Zn the most followed by Ni and Cd.

There are various ways of how microorganisms accumulate heavy metals through adsorptive interactions and metabolism mediated mechanisms. In metabolism mediated mechanisms, it is divided to metal transport within the cells for intracellular storage and intracellular detoxification (Juwarkar & Yadav, 2010). Intracellular detoxification is supported by the synthesis of metallothioneins (MT), proteins that are of low molecular weight. MT is produced by the cells when heavy metals are detected in the environment, and upon secretion, MT will bind to the heavy metals thus excluding it out of the cells' metabolical reactions (Chojnacka, 2010). The presence of MT in cells helps to increase the resistance against heavy metals and enhance the metal tolerance, sequestration or accumulation (Juwarkar & Yadav, 2010).

Costa & Duta (2001) studied the bioaccumulation capabilities of *Bacillus* spp., *B. cereus*, *Bacillus sphaericus* (*B. sphaericus)* and *Bacillus subtilis* (*B. subtilis*) which showed that the bacteria used were capable of bioaccumulating four types of heavy metals tested namely Cu, Zn, Cd and Pb with the best results shown by *B.subtilis* and *B.cereus*. Al-Taee (2015) studied the bioaccumulation ability of Bt isolated from soil, showed that the bacterium can grow in a Pb and Cd polluted environment. His results demonstrated that Bt was capable of accumulating Cd at 23.2 mg/g in the concentration of 100 mg/L. This is significant as the present study also tested the same bacterium capable of accumulating Cd.

CONCLUSION

The growth of both BpChIAY and Bt were observed under the effect of selected heavy metals at 2 ppm but no observable growth was obtained under 10 ppm. BpChIAY grew the best under Ni, Zn and Cd at growth percentages of 128%, 103% and 67% respectively. BT showed similar results as well by growing at 97.7% in Zn, 98.8% in Ni and 36.2% in Cd. However both bacteria showed little to no growth under Cu and Cr thus the order of toxicity of the heavy metals is in this order from the most toxic: Cu > Cr > Cd > Zn > Ni. This study also shows that the bacterial strains are capable of bioaccumulation where both bioaccumulated all three tested heavy metals, Cd, Ni and Zn. BpChIAY showed a higher metal absorbed per bacterial gram than Bt at 0.0539 mg/g for Zn, 0.0781 mg/g for Ni and 0.0256 mg/g for Cd**.** A recommendation in the future is to conduct more studies regarding on how to fully utilize these bacterial strains in the open field for the remediation of metals pollution in the environment. Other than the environment, the study on how we can use these strains in aquacultural species can be conducted to overcome heavy metal exposure to aquacultural species via ingestion as the *Bacillus* spp. are known to be able to grow under acidic environments.

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