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Heavy metal removal ability of *Halomonas elongata* and *Tetragenococcus halophilus* in a media model system as affected by pH and incubation time

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Abstract

A serious public problem in many countries relate to toxic heavy metal contamination throughout the eco-system and food chain particularly in tuna viscera which is a raw material used for human consumption in some areas. *Halomonas elongata* a halophilic bacteria have been reported to remove Hg, Pb and Cd from waste water. In this work, 2 specific bacteria namely *H. elongata* (halophilic bacteria) and *Tetragenococcus halophilus* (halophilic lactic acid bacteria) were selected and studied for heavy metal removal in the media model system including saline nutrient broth (SNB) and de Man, Rogosa and Sharpe broth (MRS-broth). Initial concentration of Hg, Pb and Cd was set up 0.5, 1 and 3 mg/L, respectively. In addition, effects of pH and incubation time on metal removal of both organisms were monitored. The result revealed that potential of metal absorption was in the order as Pb>Cd>Hg in both organisms though not in the same level. *H. elongata* highly removed Hg, Pb and Cd at 69.52-81.11%, 97.82 - 98.47% and 74.17 - 89.16%, respectively when cultured pH was 5. Percentage removal of Hg and Cd were highest at 8.64 – 12.69% and 93.50 – 95.12%, respectively, when *T. halophilus* was cultured in media pH 7 while Pb removal was highest at 91.60 – 96.54% when the organism was cultured in media pH 6. In addition, the metal removal generally increased as increased incubation time up to 96 h. Therefore, it is possible that addition of *H. elongata* and *T. halophilus* as well as optimum condition of bacterial growth would be applied in tuna viscera for heavy metal absorption in next experiment.

Introduction

Though in a small dose of some metals including zinc, cobalt, iron, copper, manganese and, molybdenum are essential elements, in large portions are toxic, harmful and influenced to other living cells (Ogundiran *et al*., 2012; Serbaji *et al*., 2012). Other heavy metals such as Hg, Pb and Cd have never addressed as an essential element for any living cell (Florian *et al*., 2011). These toxic heavy metals have now become an international health problem because they can spread or enter into eco-system and food chain leading to serious illness. Increasing of industrialize plants such as smelting, mining, refining, metallurgical, electroplating, and petrochemical leads to more heavy metal contamination (Volesky and Holan, 1995). All Hg, Pb and Cd are classified as most dangerous heavy metals and normally found in internal organ of animals such as spleen, liver, pencrease and stomach (Sivalingam and Sani, 1980; Agusa *et al*., 2007; Jeenmhun, 2009). From preliminary test, it was found that raw internal tuna organ contaminated with Cd was 2 times higher than standars regulation of FDA which is set up as 1mg/L. In addition, the fermented tuna or Tai-pla was heavily contaminated with toxic heavy metals including Hg, Pb and Cd as 1.278, 1.719 and 2.653, respectively. In fact, reduction and/ or elimination of toxic heavy metals in both in vitro and in vivo have been focused for many eras (Levi, 2000).

The conventional technique for metal remediation includes common physicochemical precipitation inducing electrochemical treatment, chemical coagulation, reverse osmosis, ion exchange and ultrafiltration, however these processes also cause other disadvantages including consideration of suitability places, large portion of them outside of industrial scale applications due to the high capital and operational costs involved, high energy consumption, generation of large amount of sludge containing toxic compounds and which are not eco-friendly (Iyer *et al*., 2004). Therefore, biotechnological approaches because of inexpensive, eco-friendly and high efficiency for toxic metals remediation are more interesting (Chen *et al*., 2005). Various microorganisms such as algae, bacteria, yeasts and mold possess capacities for metal removal

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through their functional groups have been attempted to utilize (Volesky and Holan, 1995; Gadd, 1988; Brierley, 1990). For example, moderate halophilic bacteria *H. elongata* ATCC 33315 showed reduction toxicity of Silver (Ag), Arsenic (As), Cadmium (Cd), Cobalt (Co), Chromium (Cr), Copper (Cu), Mercury (Hg), Nickel (Ni), Lead (Pb) and Zinc (Zn) (Nieto *et al.*, 1989). Non classified genus of halophilic bacteria isolated from the Dead Sea shore, Jordan, could absorb added Pb and Cd (500 ppm) in nutrient media as 83.39% and 90% within 2 and 3 weeks, respectively (Massadeh et al., 2005).

Fermented foods mainly involve with lactic acid bacteria action. Thailand is famous for many fermented fishery products such as fish sauce or nam-pla, shrimp paste (kapi) (Thongsant *et al*., 2002) and budu (Rosma *et al*., 2009) as well as fermented viscera of fish called Tai-pla. Udomsil *et al*. (2010) reported that *T. halophilus* (one of the halophilic lactic acid bacteria) played an important role in fish sauce aroma. To produce a good fermented tuna viscera and reduce the risk of heavy metal contamination in the next experiment, specific microorganism are to be studied. Therefore, this present work is aimed to determine the growth character, cell survival and metal reduction of *H. elongata* and *T. halophilus* when cultured in media model systems having various pH and heavy metal contents.

Materials and Methods

Chemicals and culturing media

Lead nitrate ($Pb(NO_3)_2$), cadmium chloride ($CdCl_2$) and mercury chloride ($HgCl_2$), were obtained from Ajax Finechem Pty Ltd, Australia. All media for microorganism culture were purchased from Becton, Dickinson and Company, France.

Bacterial strains

The halophilic and lactic acid bacteria strains used in this study were *Halomonas elongata* ATCC 33173 and *Tetragenococcus halophilus* ATCC 33315.

Microorganism preparation

Lyophilized *H. elongata* ATCC 33173 and *T. halophilus* ATCC 33315 were individually transferred into sterilized saline nutrient broth (SNB) and de Man, Rogosa and Sharpe broth (MRS-broth) containing 10% NaCl and cultured under aerobic and anaerobic conditions, respectively for 48 h at 37°C. Twice subcultures were made before use (Amoozegar *et al*., 2012). In addition, to storage the cell for further study,

H. elongata and *T. halophilus* cultured in the SNB and MRS-broth containg 10% NaCl were taken to put into glycerol at ratio 1: 1 and kept at -20°C.

Efficacy of heavy metal removal by test organism

SNB and MRS-broth were prepared and adjusted pH to 5, 6 and 7 to a cover pH range of fermented fish (5.5-7) by using 0.1 N NaOH and 0.1 N HCl autoclaved at 121°C for 15 min. Thereafter, $HgCl_2$, ($Pb(NO_3)_2$) and $CdCl_2$ were prepared for a stock solution with 0.1 M HNO_3 at concentration 50, 100 and 300 mg/L, respectively. Stock solution of each heavy metal element was calculated, diluted and filtered with sterile membrane pore size 0.22 µm then added into the sterilized SNB and MRS-broth to obtained Hg, Pb and Cd at concentration, 0.5, 1, and 3 mg/L, respectively based on the heavy metal level of contamination in the fermented viscera fish product as mentioned earlier. Each organism cultured in each pH without addition of any heavy metal and adjusted the pH was used as control. Thereafter, the solution of Hg, Pb and Cd was taken to analysis for initial heavy metal (Ci). 0.5 ml of the *H. elongata* and *T. halophilus* were aseptically transferred to a 125 ml Erlenmeyer flask containing 50 ml of the above specified sterile basal medium to obtain the cell counts approximately 3-3.5 log CFU/ml. The cultured media were incubated at 37°C for 192 h. At 0, 24, 48, 72, 96, 120, 144, 168 and 192 h before taken to analysis for pH value and cell viability. In addition, at 48 and 96 h the samples were taken to centrifuge at 3000 x g for 15 min. Thereafter, supernatant of each condition was subjected to analysis for final heavy metal content (Cf). Pb and Cd concentration were determined with Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) and Hg was determined with atomic absorption spectroscopy (CVAAS) (AOAC, 2000). The percentage of each metal removal was calculated based on its initial concentration following method of Gourdon *et al.* (1990) as followed equation:

$$\% \text{ Biosorption removal} = \frac{Ci - Cf}{Cf} x100$$

Ci = Initial concentration of metal in the solution before cultured with tested organism (mg/L)

Cf = Final concentration of metal in the solution after cultured with tested organism (mg/L)

Statistical analysis

Completely randomized design (CRD) was applied though out the experiment. Results were analyzed using one way analysis of variants (ANOVA) and mean comparisons were performed using the Duncan's new multiple range test (DMRT).

Results and Discussion

Effect of pH and heavy metal on growth organisms

The effect of pH (5, 6 and 7) and heavy metals (Hg, Pb and Cd) on growth of *H. elongata* is depicted in Figure 1(A, B and C). It was found that the H. elongata reared in Hg at pH 5, 6 and 7 provided the cell peak as approximately 6.7, 6.5 and 6.5 log CFU/ml, respectively. It pointed out that pH around 5-7 did not strongly affect for growth rate of *H. elongata* cultured in Hg. In addition, both cell counts and metabolizing of *H. elongata* depended mainly on toxic of Hg. The similar trend was also found in

H. elongata cultured in Pb. However, it was found that cell counts of *H. elongata* reared with Cd at pH 5 was significantly different from other pH. Surprisingly, *H. elongata* reared with Cd at pH 5 had a more acidic condition when compared with pH 6 and 7 did not have a lowest cells growth. This phenomenon was not in agreement in hurdle effect as weak acid and toxic agent, which provided growth inhibition (Montville and Matthews, 2013). A higher cell counts and OD at pH 5 of H. elongata cultured with Cd for 72 and 168 h, respectively may be explained by 2 mechanisms; (1) increasing of amine compounds, as a result of H. elongata bacterial growth leading to pH raised up and closed up to its optimum pH (2) initial acidity and Cd induced cell adaptation/mutation. It was noticed that *H. elongata* cultured at pH 5 produced less viscous exopolysaccharide (EPS) determined by OD and compared with other treatments.

Cox (1995) reported that pH of media for culturing Pedomicrobium sp increased as incubation time increased because of amine products from decarboxylation of amino acids. Llamas *et al.* (2012) mentioned that a decrease of EPS of halophilic species occurred when the organisms were incubated with high salt content, long incubation time and temperature as well as excess glucose concentration condition. In addition, Pham *et al.* (2000) stated production of exopolysaccharide by *Lactobacillus rhamnosus* R that was highest after incubation for 120 h, thereafter declined, probably due to enzymatic degradation.

The effect of pH on growth character of *T. halophilus* reared with different test of heavy metals is represented in Figure 2 (A, B and C). In general, it was found that both pH and heavy metals influenced

T. halophilus growth particulary at pH 5. As known that *T.halophilus* is lactic acid bacteria at lower pH (5) caused high cell death particularly when the cells are treated with Cd (Figure 2C). It

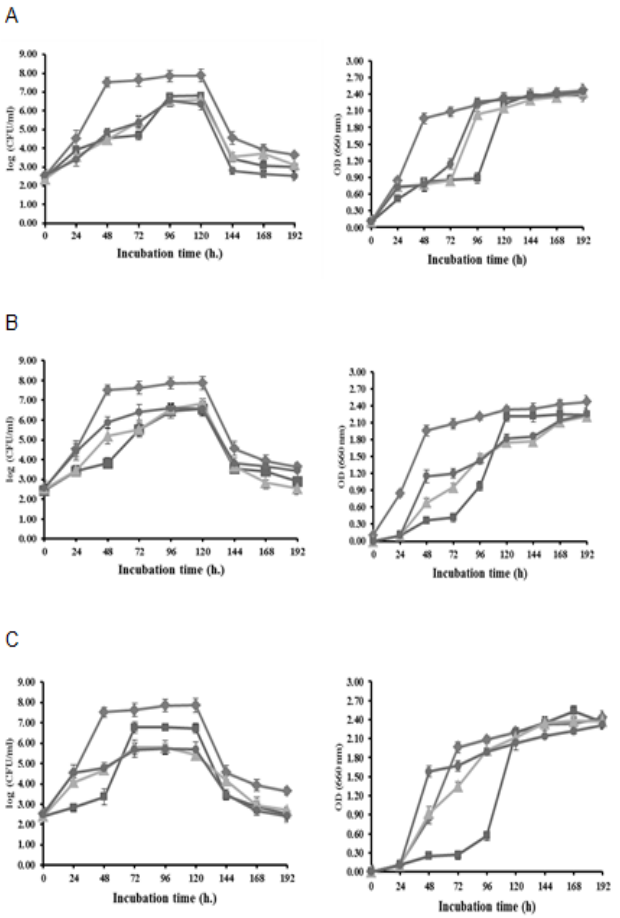

Figure 1. Effect of pH (Control; pH 7 (◆), 5(■), 6(▲) and 7(●)) on growth of *H. elongata* reared in heavy metals Hg (A), Pb (B) and Cd (C), 10% NaCl SNB and incubation at 37°C.

pointed out that toxicity of tested heavy metal, Hg, Pb and Cd may be more pronounced when solubility of metal is higher. It was surprised that though cell counts of *T. halophilus* in pH 5 and Cd did not detect since 48 h incubation, the OD significantly increased at 144 and 168 h and was not significantly different when compared with other pHs, (Figure 2C). This may be due to hydrolysis and/or solubilized cell by organic acids including lactic acid, acetic acid, formic acid and so on produced from lactic acid bacteria. Butler *et al*. (2013) reported that when cell is lysised it causes intracellular proteins leakage making a cloudy culture. In addition, Ramírez-Nuñez *et al.* (2011) studied the effect of pH and salt gradient on the autolysis of *Lactococcus lactis* strains and found that at low pH (5.4) and high salt (2.98%) resulted to high autolysis values. Toxicity of heavy metals depend on type of organism which relates to methylation process (Maier *et al*., 2001), type of heavy metal (Gourdon *et al*., 1990), pH of system (McLean and Beveridge, 1990), age of organisms (Odokuma, 2009), and salt concentration (Amoozegar *et al*., 2012). However, this experiment

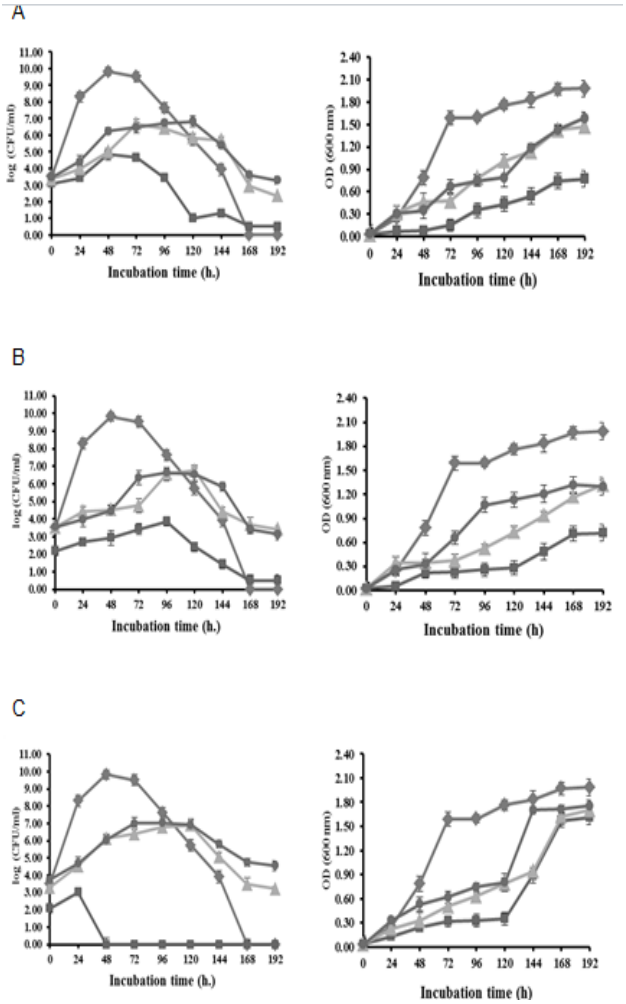

Figure 2. Effect of pH (Control; pH 7 (◇), 5(□), 6(△) and 7(○)) on growth of *T. halophilus* reared in heavy metals Hg (A), Pb (B) and Cd (C), 10% NaCl MRS-broth and incubation at 37°C.

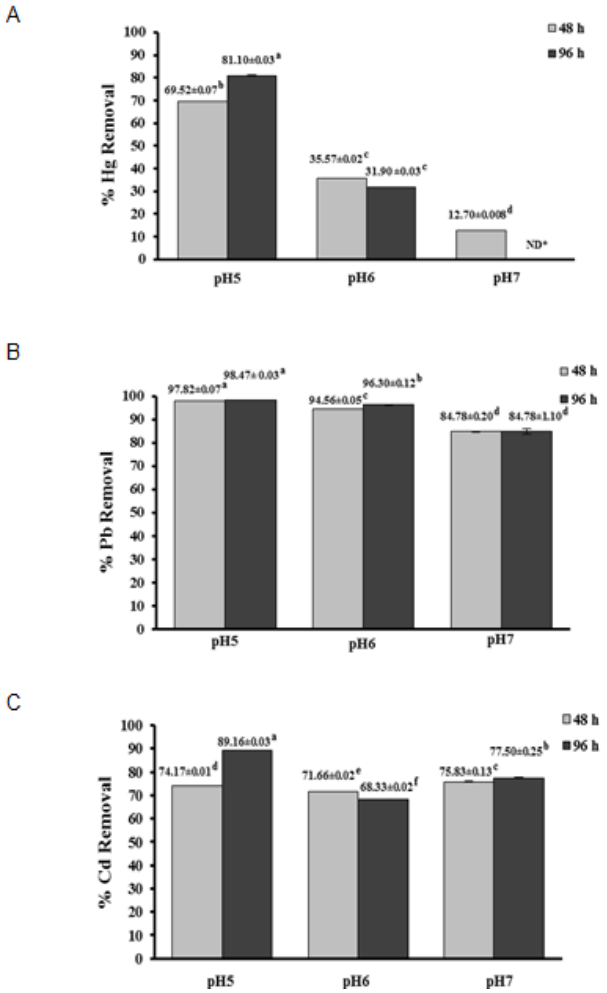

Figure 3. Effect of pH (5, 6 and 7) on heavy metals (Hg, Pb and Cd) removal by *H. elongata* incubated SNB at 37°C. All of the data were obtained after the 48 and 96 h. incubation.

confirmed that *T. halophilus* was more sensitive to various factors including pH, heavy metals and incubation time when compared with H. elongata. The results obtained from Figure 1-2 pointed out that using only OD as indicator for bacterial growth may lead to fault interpretation. Therefore, viable cell counts are necessary to determine particularly in longer incubation time. In addition, cell history or growth period of each organism in tested condition must be investigated and declared.

Heavy metals removal as affected of bacterial types, pH and incubation time

In general, it was found that percentage of heavy metal removal by *H. elongata* and *T. halophilus* were Pb>Cd>Hg (Figure 3-4). Amoozegar *et al.* (2012) stated that EPS of *H. elongata* ATCC 33173 could reduce more than 90% and 50% of Pb and Cd, respectively as a result of bigger molecular size. In addition, Halttunen *et al.* (2007) found that the maximum removal of Pb and Cd by Bifidobacterium longum 46 (LAB) were 175.7 mg/g and 54.7 mg/g dry biomass. Therefore, it pointed out that biosorption of each heavy metal may explain by molecular size and atom mass if bigger (Pb>Cd) then easier to bind with active size of EPS (Davis *et al.* 2003), in addition, ionic radius (Pb>Cd) also was a determinant factor for metal removal by *B. longum* 46 (Halttunen *et al.*, 2007). Omoike and Chorover (2004) addressed that biosorption of EPS depended on metal types, electric charges, molecular size, atom mass and deformability of metal ions. In comparison, *H. elongata* could absorb Hg and Pb higher when compared with T.halophilus. This may be due to *H. elongata* producing EPS and uronic acid at its cell wall (Iyer *et al.*, 2005). However, the highest Cd absorption was obtained from *T. halophilus* (95.13%) at pH 7, 96 h. Monachese *et al.* (2012) explained that Gram-positive bacteria exhibited heavy metal adsorption with anionic groups including carboxylate and phosphate in peptidoglycan and teichoic acid containing in cell wall. Gadd (1990) mentioned that

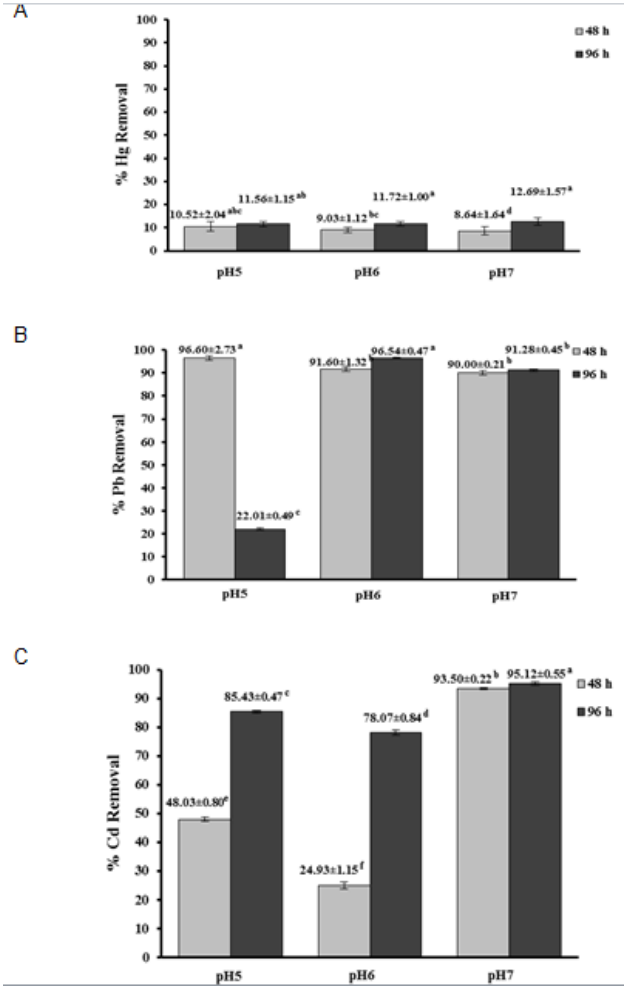

Figure 4. Effect of pH (5, 6 and 7) on heavy metals (Hg, Pb and Cd) removal by *T. halophilus* incubated MRs-broth at 37°C. All of the data were obtained after the 48 and 96 h. incubation

heavy metal removal depended on bacterial species. Daboor *et al*. (2014) also reported that active sites for metal binding were different according to bacterial species and metal types. However, from this present work, it pointed out that metal adsorption did not only depend on bacterial group as Gram- negative or Gram-positive but deeply information as lactic acid production, cultured pH or condition also are other determinant factors. Therefore, higher Cd removal with *T. halophilus* cultured at pH 7 may due to homeostatic phenomenon. As known that *T. halophilus* produced lactic acid during growth then pH of the culture reduced leading to facilitate Cd solubility then be trapped with cell wall of the bacteria. However, if *T. halophilus* was cultured in lower pH, 5 then excessive acid would destroy cell wall and membrane until homeostasis was destroyed.

Based on pH adjusting, it was found that an increase of all metal removals found in *H. elongata* was at lower pH (Figure 3 A, B and C). A decrease of pH significantly increased of heavy metal removals then at pH 5 the highest removal of Hg, Pb and Cd was 69.52 – 81.11, 97.82 – 98.47 and 74.17 – 89.16%, respectively. Many researchers reported that biosorption mechanism were strongly related to physicochemical interaction of metal in solution (Aksu *et al*., 2002; Lodeiro *et al*., 2006; Amini *et al*., 2008). The higher acidic pH (pH < 2.0) led to higher positive charge on the active site, then metal cations were difficult to compete with protons to bind with active site on cell wall which resulted in lower metal uptake (Lqbal and Edyvean, 2004). However, when pH of solution increased from 2 to 6, the negatively charged on biosorbent surface was exposed, and the functional group as carboxyl groups of the biomass were more deprotonated thus available for metal ions to bind (Sari and Tuzen, 2008). In addition, Farah *et al.* (2007) found that pH 3 to 6 was favorable for biosorption, due to the negatively charged carboxyl groups (pKa 3–5), which were responsible for the binding metal cations via ion exchange mechanism. Amini *et al.* (2008) stated that biosorption of dyes, depended or dye classes which required different pH ranges. A decrease of metal removal by organisms at higher pH 7 may be due to the metals being insoluble and transform into oxide or hydroxides complexes and precipitates at alkali pH, however, it could not be considered as a biosorption behavior of the all cell species (Amini *et al.*, 2008).

Incubation time is one of the important parameters of the biosorption process, because it relates to bacterial life cycle and metal exposure function. Figures 3-4 portray the effect of incubation time on the biosorption of Hg, Pb and Cd by *H. elongata* and *T. halophilus*. The result revealed that the percentage of removal of heavy metals increased considerably with increasing incubation time up to 96 h. H. elongata provided highest Hg (81.10%) at pH 5, 96 h and Pb (98.47%) pH 5, 96 h. and *T. halophilus* (95.13%) at pH 7, 96 h. Halttunen *et al.* (2007) explained that bacteria adsorbed heavy metals in 2 steps; (1) used cell wall with passive transport which has higher efficiency and (2) used active transport to push heavy metal into the inside cell which is lower in capacity.

Conclusion

The ability of removal by *H. elongata* and *T. halophilus* were Pb>Cd>Hg. *H. elongata* could absorb Hg and Pb higher when compared with *T.halophilus*. In contrast, higher Cd absorption was obtained from *T. halophilus*. Significantly increased of heavy metal removal by *H. elongata* was obtained at pH 5. The highest removal of Pb, Cd and Hg by *H. elongata* cultured at pH 5 was 97.82 – 98.47,

74.17 – 89.16 and 69.52 – 81.11%, respectively. *T. halophilus* exhibited highest Pb absorption at pH 5 (96.59%), while highest Cd and Hg absorption at pH 7 was 95.13% and 12.68%, respectively.

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