Evaluation of harmful heavy metal (Hg, Pb and Cd) reduction using *Halomonas elongata* and *Tetragenococcus halophilus* for protein hydrolysate product

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ABSTRACT

Background: Many health claims surrounding antioxidative, antihypertensive and anti-inflammatory properties have been addressed in natural protein hydrolysates, including fermented fish. Besides being sold as animal feed, tuna viscera is used for for the production of fermented products like fish sauce and Tai pla, fermented viscera. However, toxic heavy metals including Hg, Pb and Cd have been found in various food items, particularly within the internal organs of tuna. Therefore, the consumption of fermented tuna viscera containing heavy metal involves health risks. Consequently, the detoxification and reduction of these toxic elements are relevant and important issues, particularly with the use of their bacterial cells and metabolic products. *Halomonas elongata* is a moderately halophilic bacterium which has the ability to remove heavy metal, and is normally found in hypersaline environments. *Tetragenococcus halophilus* is a moderately halophilic lactic acid bacterium and probiotic which is found in fermented food products, such as fish sauce, shrimp paste, and fermented fish. Some scientific studies have reported using *T. halophilus* improves amino acid profiles and desirable volatile compounds, in addition to reducing biogenic amine content in fish sauce product. Therefore, it was hypothesized that using *H. elongata* and *T. halophilus* could reduce heavy metal content and improve the organoleptic quality of fermented fish viscera product (Tai pla).

Objective: This present work attempted to determine the growth characteristic of *H. elongata* and *T. halophilus* reared at various NaCl concentrations: 10, 15, 20 and 25%. Consequently, heavy metal reduction using these microorganisms reared at optimum NaCl concentration was evaluated.

Methods: *H. elongata* and *T. halophilus* were reared in saline nutrient broth (SNB) and de Man, Rogosa and Sharpe (MRS-broth) added with NaCl at concentration 10, 15, 20 and 25%, respectively. Cultures at each NaCl content were added with mercury (Hg), lead (Pb) and

cadmium (Cd) at concentration, 0.5, 1, and 3 mg/L, respectively. Subsequently, the supernatant of each condition was incubated at 48h and taken for heavy metal analysis at 96 h.

Results: The results showed that higher NaCl content resulted in slower late log and stationary phases, particularly in *T. halophilus*. This may due to *T. halophilus* not producing special metabolite such as exopolysaccharide, which was found in *H. elongata*. Regardless of heavy metal concentration, the results revealed that Cd at 3 mg/kg caused more cell death of *H. elongate,* but not that of *T. halophilus*. Furthermore, removal of Hg, Pb and Cd was 12.70, 84.78 and 75.83% respectively, by rearing with *H. elongata* for 48 h and by rearing with *T. halophilus* for 96 h was 12.68, 91.27 and 95.12%, respectively.

Conclusion: *H. elongata and T. halophilus* prefered SNB containing NaCl concentration between 10-20%. At higher NaCl concentration, 20-25%, the log phase was extended. Both *H. elongata and T. halophilus* were able to remove all test heavy metals. However, *T. halophilus* appeared to have higher Pb and Cd removal capability compared with *H. elongata*. Therefore, using *H. elongata* and *T. halophilus* for fermented tuna viscera is possible.

Keywords: *Halomonas elongata, Tetragenococcus halophilus*, Heavy metal, NaCl

INTRODUCTION:

Some heavy metals such as zinc, cobalt, iron copper, manganese and molybdenum are necessary to support life in very small doses. However, in large amounts these heavy metals are toxic and potentially hazardous [1, 2]. Moreover, other heavy metals like Pb, Cd and Hg are not essential for any living cell. An accumulation of these toxic elements over time can cause serious body illness once the accumulated level increases over the threshold. The releasing of heavy metals into the environment occurs through various process plants [3]. Expansion of many industries, including smelting, mining, refining, metallurgical, electroplating, petrochemical, and the discharge of industrial wastes has led to environment toxic metal contamination.

Environments contaminated with heavy metals pose a significant problem, mainly due to the toxic effects of these metals throughout the food chain. The toxic effects of these metals can cause serious ecological damage due to their solubility and mobility. Among the toxic heavy metals, Pb, Cd and Hg cause serious health problems when they accumulate in the living tissues [4], particularly in internal organs such as the spleen, liver, pancreas and stomach [5, 6, 7]. Since these elements are harmful chemicals for many human organs, ways to reduce and or eliminate these metals are of great interest and importance [8].

The conventional technique for metal remediation includes common physic-chemical precipitation, such as inducing electrochemical treatment, chemical coagulation, reverse osmosis, ion exchange and ultrafiltration. However, these processes also have other disadvantages, including the need to find suitable places. Moreover, a majority of these process are outside industrial scale applications. Furthermore, the high capital and operational costs involved, high energy consumption, and generation of large amounts of sludge containing toxic compounds are not eco-friendly [9]. Therefore, biotechnological approaches which are less expensive, eco-

friendlier and have high efficiency for toxic metals remediation are more important to invest in [10]. Various microorganisms such as algae, bacteria, yeasts and mold have the capacity for metal removal through their functional groups and have been tested for their purposes [4, 11, 12]. For example, the moderate halophilic bacterium, *Halomonas elongata* ATCC 33315, resulted in the reduction of toxicity within Ag, As, Cd, Co, Cr, Cu, Hg, Ni, Pb and Zn [13]. An unclassified genus of halophilic bacteria isolated from the Dead Sea shore of Jordan, could absorb Pb and Cd added at 500 ppm in nutrient media to the extent of 83.39% and 90% within 2 and 3 weeks [14].

Fermented foods mainly involve lactic acid bacterial action. Thailand is famous for many fermented fishery products such as fish sauce or nam-pla, shrimp paste (kapi) [15] and budu [16], as well as fermented fish viscera called Tai-pla. Udomsil *et al.* [17] reported that *Tetragenococcus halophilus*, a halophilic lactic acid bacterium, played an important role in the aroma of fish sauce. Therefore, in order to produce good fermented tuna viscera and reduce the risk of heavy metal contamination, the specific microorganism requires carefully consideration. The present work attempted to determine growth characteristics, cell survival and metal reduction ability of *H. elongata* and *T. halophilus* when cultured in various salt concentrations and heavy metal contents in model media systems.

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 the media for microorganism culture were purchased from Becton, Dickinson and Company, France.

Bacterial strains: The halophilic and lactic acid bacteria strain used in this study were *Halomonas elongata* (ATCC 33173) and *Tetragenococcus halophilus* (ATCC 33315), respectively.

Microorganism preparation: *H. elongata* (ATCC 33173) and *T. halophilus* (ATCC 33315) were cultured in saline nutrient broth (SNB) and de Man, Rogosa and Sharpe broth (MRS-broth) under aerobic and anaerobic conditions respectively for 48 h at 37°C, with a final 10% salt concentration. Two times of subculture were made and utilized further [18, 19].

Optimal culture condition for growth measurements: 0.2 ml of the culture of the individual activated test organisms, *H. elongata* and *T. halophilus*, were separately transferred to sterilized Erlenmeyer flasks containing 20 ml of SNB or MRS-broth added with NaCl at concentrations of 10, 15, 20 and 25%, [18, 20]. The suspension of each test organisms was incubated under either aerobic and anaerobic conditions at 37°C for 180 h. At 0, 12, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132, 144, 156, 168 and 180 h, samples of the suspension of each organism were used to monitor the bacterial growth. Bacterial growth was followed by measuring absorbance at 600 nm using a spectrophotometer and viable plate count of each organisms using SNA and MRS-agar containing 10% NaCl and incubated at 37°C for 2 d under aerobic and 7 d anaerobic condition for *H. elongata* and *T. halophilus*, respectively. The growth constructed by plotting biomass or OD is presented on y-axis against time on line x-axis.

Effect of heavy metals on growth characteristics and removal capacities by test organism: SNB and MRS-broth were prepared and adjusted the pH to 7 by using 0.1 N NaOH and autoclaved at 121°C 15 min. Meanwhile, $HgCl_2$, ($Pb(NO_3)_2$) and $CdCl_2$ stock solutions at concentration 50, 100 and 300 mg/l were prepared [18]. Subsequently, each stock solution was diluted and filtered with sterile membrane pore size 0.22 µm and added into the sterilized SNB and MRS-broth to obtain Hg, Pb and Cd at concentrations of 0.5, 1 and 3 mg/L, respectively. Each SNB and MRS-broth containing heavy metal at the mentioned concentration was inoculated with *H. elongata* and *T. halophilus*, before being incubated under either aerobic and anaerobic conditions at 37°C for 192 h. At 24, 48, 72, 96, 120, 144, 168 and 192 h, the cultured broth was taken to analysis OD and cell viability, then centrifuged at 3000 x g for 15 min. Afterwards, the supernatant of each condition was taken for heavy metal analysis.

RESULTS:

Optimal culture conditions for growth: The effects of salinity on growth of *H. elongata* and *T. halophilus* are shown in Figures 1 and 2. Comparing the OD of the medium and viable plate count of *H. elongata* and *T. halophilus* organisms during incubation revealed that higher NaCl content was proportional to slower late log and stationary phases of *H. elongata* cultured in 10, 15, 20; 25 % NaCl reached the OD approximately 1.98, 0.94, 0.62 and 0.22 and cell counts of approximately 7.8, 6.8, 5.4 and 4.4 log CFU/ml. Figure 2 demonstrates the fast growth of the *T. halophilus*, which reached a peak at OD 0.78, 0.27, 0.17 and 0.09 and cell counts of 9.7, 8.74, 6.64 and 4.83 log CFU/ml at concentration 10, 15, 20 and 25%.

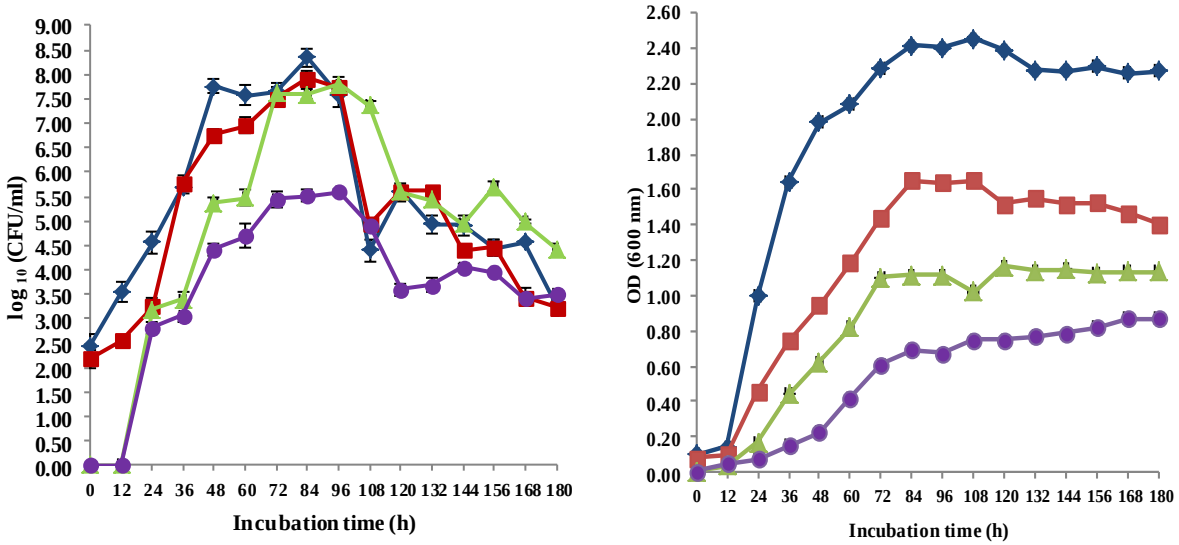

Figure 1. Effects of salinity on the growth of *H. elongata* (ATCC 33173) reared in 10(◆), 15 (■), 20(▲) and 25 (●) % NaCl SNB and incubated at 37°C. All data was obtained using triplicate determined every 12 h during incubation.

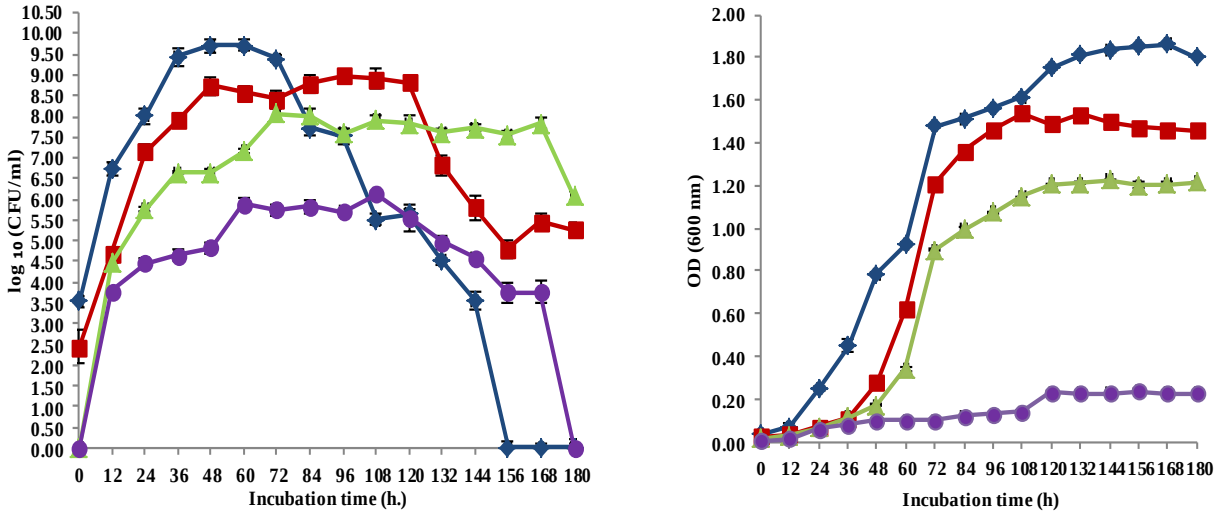

Figure 2 Effects of heavy metals on the growth of *T. halophilus* (ATCC 33315) reared in 10 (◆), 15(■), 20(▲) and 25 (●) % NaCl MRS-broth and incubated at 37°C. All data was obtained using triplicate determined every 12 h during incubation.

Effect of heavy metals on growth characteristics: The effect of heavy metals (control, Hg, Pb and Cd) on the growth of *H. elongata* is represented in Figure 3. It was discovered that the organism reached cell peaks of approximately 7.5, 6.5, 6.3 and 5.6 log CFU/ml, respectively. Additionally, it was revealed that *T. halophilus* obtained from control, Hg, Pb and Cd condition reached peak counts of approximately 9.7, 6.6, 6.3 and 7.0 log CFU/ml (Figure 4). *H. elongata* was sensitive to Cd > Pb > Hg, while *T. halophilus* was responsive to Pb > Hg > Cd.

Efficacy of heavy metal removal by test organism: The effect of heavy metal removal by the biomass of *H. elongata* is shown in Figure 5. The maximum removal of Hg, Pb and Cd was 12.70%, 84.78% and 75.83% respectively in the cultured cell at pH 7 for 48 h. Percentages of the test heavy metal removal by *T. halophilus* after incubation for 48 and 96 h are shown in Figure 6. The removal of heavy metals by *T. halophilus* increased slightly after 96 h of incubation time. The maximum removal of Hg, Pb and Cd was 12.68%, 91.27% and 95.12%, when the cells were cultured at pH 7 for 96 h. It revealed that Hg removal by both organisms was the lowest when compared with other metals.

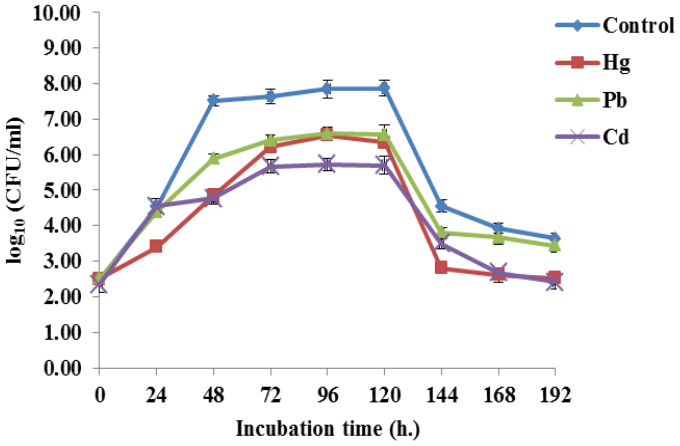

Figure 3 Effect of heavy metals on the growth of *H. elongata* (ATCC 33173) reared in 10% NaCl SNB and incubated at 37°C. All data was obtained using triplicate determined every 24 h during incubation.

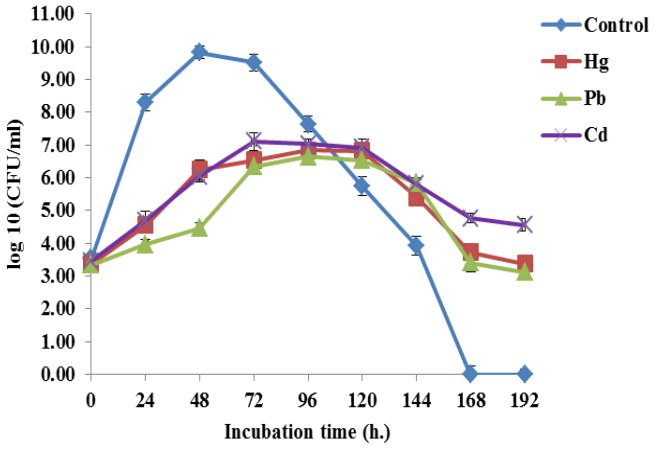

Figure 4 Effect of heavy metal on the growth of *T. halophilu* (ATCC 33315) reared in 10% NaCl MRS-broth and incubated at 37°C. All data was obtained using triplicate which determined every 24 h during incubation.

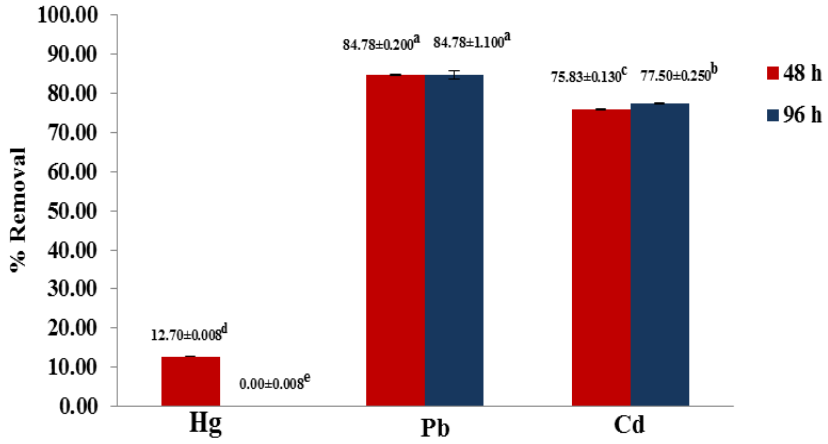

Figure 5 Heavy metal (Hg, Pb and Cd) removal by biomass of the *H. elongata* (ATCC 33173) at 48 and 96 h. and pH 7.

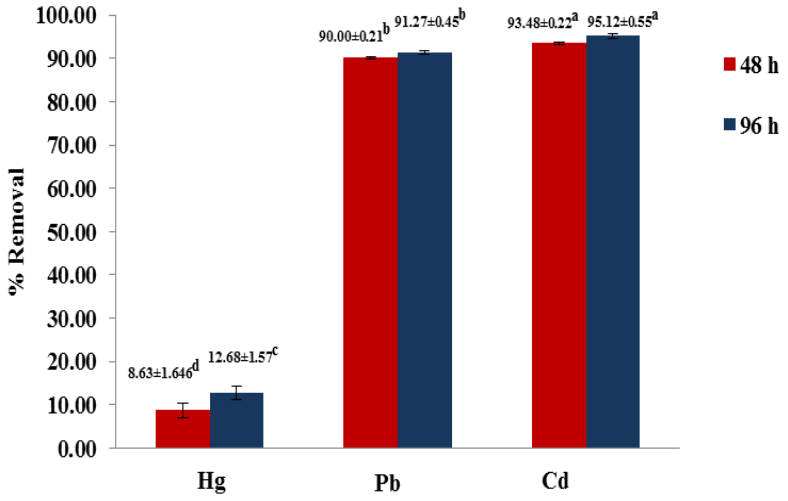

Figure 6 Heavy metal (Hg, Pb and Cd) removal by biomass of the *T. halophilus* (ATCC 33315) at 48 and 96 h. and pH 7.

DISCUSSION:

Optimal culture conditions for growth: *H. elongata* cultured in 10-20 % NaCl reached the OD and cell count approximately 2.3-2.5 and 8.2-8.3 log CFU/ml. In contrast, this phenomenon did

not occur when the organism was reared in 25% NaCl. Therefore, our results suggested that *H. elongata* preferred NaCl content at lower levels of 10-20%, compared to that of 25%. These results support the findings of Vreeland *et al.* [20], who reported that 10% NaCl was optimum condition for *H. elongata* growth. Additionally, OD of *H. elongata* at the peak was signficiantly high, measuring over 2. This may be due to the bacterial cell size of about 0.6–1.0 μm in width and 2.0–3.2 μm in length [21] and its metabolite as exopolysaccharide producing during growth [18].

The *T. halophilus* grew quickly and reached a peak at OD and cell count at 1.8 and 9.5 log CFU/ml when reared in 10% NaCl. However, this organism appeared to be sensitive to high NaCl content. Therefore, at concentrations of NaCl > 15%, the OD and cell counts did not reach the same peak as in 10% NaCl. The OD and cell counts of *T. halophilus* reared in 25% NaCl at the peak were lowest, with only 1.1 and 1.5 log CFU/ml, respectively. These results confirmed that this organism is a moderately halophilic bacterium, for which the optimum NaCl concentration for growth is about 5-10% [22].

Moreover, it was revealed that *T. halophilus* could not resist higher NaCl concentrations when compared with *H. elongata.* This may be the result of *T. halophilus* not producing special metabolites like exopolysaccharide, which is found in *H. elongata.* For example, Amoozegar *et al.* [18] reported that the moderately and extremely halotolerant bacteria produce more glycine-betaine to protect the cell at higher levels of NaCl in the habitat, though they also need certain amounts of NaCl for their growth metabolism. These results revealed a difference between OD and cell counts of the two organisms. It was also revealed that using only OD value to predict cell viability count is not appropriate or reliable when different growth stages and organism type and not identified. Collins *et al.* [22] reported that cell size of *T. halophilus* was smaller, (0.5-0.8 μm) compared with cell size of *H. elongata*, slightly curved 0.7-1.0 μm in width and 2.0-3.0 μm in length [21]. Additionally, there are still no records of *T. halophilus* producing the exopolysaccharide found in *H. elongate*, which makes suspension opaque and viscous and also leads to high OD value.

Effect of heavy metals on growth characteristics: The effect of heavy metals on growth of *H. elongata* is demonstrated in Figure 3. The results demonstrated that the organism reached cell peaks of approximately 7.5, 6.5, 6.3 and 5.6 log CFU/ml. Regardless of heavy metal concentration, the results revealed Cd caused more cell death of *H. elongata.* Maier *et al*. [23] reported that metal toxicity can occur in a number of ways including the displacement of essential metals from their normal binding sites on biological molecules. For example, cadmium normally competes with zinc, which acts as a co-enzyme in many metabolic pathways of the living cell. Additionally, the test elements Hg, Pb and Cd did not only reduce cell viability and OD but also shifted the organisms' log phase. Consequently, it was revealed that these poisonous elements react or bind with active sites, like ionic interaction in various pathways such as proteins disruption through binding to sulfhydryl group, and nucleic acid interference by binding to phosphate or hydroxyl groups. As a result, protein and DNA conformations are changed and the function is imbalanced. Additionally, Hughes and Pool [24] reported that heavy metal modified DNA synthesis process involved in binary fission and cell expansion is then inhibited.

These results supported the finding of Majid *et al.* [25], who reported that the heavy metals shifted the pattern of microbial populations, reduced their diversity, and changed species composition, reproduction and activity of indigenous microorganisms. For example, waste water with high heavy metals contamination demonstrated a significant decrease in the numbers of bacteria in biological system. Maier *et al.* [23] stated heavy metals reduced growth, increased abnormal morphology, and inhibited biochemical process in individual bacterial cells. *T. halophilus* obtained from control, Hg, Pb and Cd condition reached peak counts of approximately 9.7, 6.6, 6.3 and 7.0 log CFU/ml, respectively. The results demonstrated that Hg and Pb caused more cell death. Interestingly, *T. halophilus* reared in the media with added heavy metals exhibited slower growth, longer stationary phase and death time, while the control samples reached the peak point within 48 h and could not be detected at 168 h. It was hypothesized that heavy metal contamination may be a key role for increasing of living cell mutation. Toxicity effect of each heavy metal on *H. elongata* and *T. halophilus* did not in show similar trends. It has been revealed that different mechanism in the responses of each organism still need to be further investigated.

Efficacy of heavy metal removal by test organism: *H. elongata* achieved maximum removal of Hg, Pb and Cd of 12.70%, 84.78% and 75.83%, when the cells were cultured at pH 7 for 48 h. Halttunen *et al.* [26] reported that resistance determinants of bacterial cell could be encoded on the chromosome or mobile genetic elements such as plasmids and transposons. The lowest mercury removal of *H. elongata* may due to conversion of Hg^{2+} to CH_3Hg^+, which is called methylation and exhibits higher toxicity and causes more death cell. CH_3Hg^+ is a volatile form having high lipophilicity leading to cell membrane permeability failure [27]. However, Maier *et al*. [23] reported that the methylation process of some heavy metals could facilitate the metal diffusion away from the cell leading to less toxicity. *H. elongata* was sensitive to Cd > Pb > Hg while *T. halophilus* was responsive to Pb > Hg > Cd. Nieto *et al*. [13] explained that metal tolerance pattern in moderately halophilic eubacteria was depended on type and dose of metal and bacterial species. Based on cell survival of control *T. halophilus*, it was found that this bacterium grew very fast and had short stationary phase time of only about 24h compared with the groups treated with heavy metals is which the stationary phase was about 72h. Therefore, this organism may have a specific mechanism to adapt itself to survive in pollutated environments.

Surprisingly, although Cd can cause the highest cell death in *H. elongata*, metal removal was not the lowest, while Hg removal by *H. elongata* was actually the lowest. Cell survival or growth and metal removal of this bacterium may not use the same pathway. Amoozegar *et al*. [18] reported that bioremoval for lead and cadmium by *Halomonas* sp occurred after cultured for 2 d. Additionally, percentage of removal of lead was higher than that of Cd. Malekzadeh *et al*. [14] explained that uptake of metal ions can be divided into two stages: the a stage and slow stage: in the rapid stage, the metal ions were rapidly adsorbed onto the surface of microorganisms, thereafter, the metal ions transported across the membrane into the cytoplasm with slow rate. Percentage of the test heavy metals removal by *T. halophilus* after incubation for 48 and 96 h was similar to bioremoval found in *H. elongata.* Additionally, percent removal of each metal by *T. halophilus* was not time dependent. Kaewchai and Prasertsan [28] reported that

Gram positive bacteria possessed higher metal adsorption ability compared with Gram negative bacteria due to chemical composition of the cell wall of each group. Gourdon *et al*. [29] stated that Gram positive bacteria exhibited approximately 20% more Cd bioabsorption at 30°C, pH 6.6 than Gram negative bacteria because of higher teichoic acid content in the glycoprotein on cell wall of Gram positive while Gram negative bacteria responded to heavy metal bioabsorption by using lipopysaccharide containg in outer membrane layer [29]. These results revealed that due to the percentage of metal removal, bacterial cell played a more important role when compared with its metabolite products, like EPS. Therefore, *T. halophilus* was a better organism for metal removal. However, *T. halophilus* is normally used in fermented fish product and has never been reported to exhibit metal removal ability. Consequently, this is the first report for heavy metal bioremoval material.

CONCLUSION:

H. elongata and T. halophilus prefer SNB containing NaCl concentration between 10-20%. The higher NaCl concentration, 20-25%, log phase was extended. *H. elongata* significantly removed $CdCl_2$ and ($Pb(NO_3)_2$) but not $HgCl_2$. Culturing *H. elongata* for 48 h was a suitable time for removal those heavy metals. The removal of Hg, Pb and Cd by biomass of *T. halophilus* was successful after being within the culture for 48-96 h. *T. halophilus* could remove more of the test metals than *H. elongate*.

Abbreviations: Saline nutrient broth (SNB); de Man, Rogosa and Sharpe (MRS-broth); mercury (Hg); lead (Pb); cadmium (Cd)

Competing interests: The authors declare that they have no competing interests.

Author's contributions: Ruttiya Asksonthong, BSc, MSc is a Food Technologist and performed all of the laboratory work for the study and provided statistical analysis and assisted in writing the manuscript.

Sunisa Siripongvutikorn, PhD is an Assistant Professor of Food Technology. She is principal investigator for this study providing oversight and contributing fundamental conceptualization for the research, writing the grant proposal and manuscript.

Worapong Usawakesmanee, PhD is a Food Technology. He coordinated the initiatives to accelerate the development and subsequent production of the intervention meal. He also contributed in the study design and assisted in writing the manuscript.

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