The potential for Probiotic Bacteria from milkfish intestine in reducing mercury metals in skimmed milk media

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Abstract. Mercury (Hg) is one of the heavy metals that is harmful to humans. The accumulation of mercury in the body is generally derived from food. Several types of bacteria from intestine of milkfish are known to reduce mercury concentration. People can take advantage of this bacterial ability by eating it through probiotic foods. This research conducted to figure out the potential for probiotic bacteria from milkfish intestine in reducing mercury. Isolation from probiotic bacteria from milkfish intestine conducted with grown the isolates in MRSA medium with addition of 1% CaCO$_3$. Twelve isolate were obtained from milkfish intestine. Mercury resistance tested was performed by measuring cell density using a spectrophotometer at concentrations of 10, 15 and 20 ppm respectively in skim milk media. Probiotic tests (gastric acid, bile salts and antimicrobial activity) for MRSB media was also conducted. Results showed that seven isolate were resistant to mercury in all concentrations and potential as probiotics. All resistant isolate then tested for skim milk media with addition of 5, 10, 20 ppm mercury acetate respectively. Result showed that only one isolated was able to reduce the concentration of mercury (Hg) in all variations on concentration and potential as mercury reducer probiotic bacteria.

1. Introduction
One of the most dangerous heavy metal pollutant in the level of toxicity is mercury (Hg). Although all forms into mercury are toxic chemicals, mostly concerns focus on methyl mercury. The main routes of exposure to these toxic elements are through fish or biota from contaminated waters. Mercury mostly present in methyl form as result of bioaccumulation and biomagnification of CH$_3$Hg in aquatic food chain [1].

An important step in tackling heavy metal pollution, especially mercury is by using potential microbes that can transform mercury (Hg) in the environment (bioconversion). Intestinal microbial from Milkfish used in heavy metal detoxification because of its ability in heavy metal binding. Fish intestinal bacteria are expected to be potent as probiotics and also resistant to heavy metals. It is important to note that isolates from mercury-resistant bacteria especially from probiotic groups have ability to detoxify mercury. Heavy metals from aquatic environment were accumulated mainly in the cell walls of bacteria when marine biota i.e. milkfish directly feeds contaminated food mercury in a pond.
2. Material and Methods
Preparation and isolation of probiotics candidate bacteria protocols were conducted base on Djide and Wahyuddin [2]. The milkfish intestines are cleaned and crushed with mortar. The scraped banding of milkfish intestine is then weighed as much as 1 g and fed into a sterile physiologic NaCl solution and diluted with diluted dilution ($10^{-1} - 10^{-6}$). A total of 1 ml of a solution of $10^{-6}$ dilution was inoculated on MRSA medium (Man Ragosa Sharpe Agar) which added CaCO$_3$ 1%, then incubated for 24-48 hours at 37ºC.

Selection of probiotics resistant bacterial candidates (Hg) and resistance test of probiotics candidate bacteria in mercury concentration (Hg) were conducted according to Badjoeri and Hafidh (2010). A total of 0.5 ml of suspension of each isolate of probiotic bacteria were inoculated on an MRSB (Man Ragosa Sharpe Broth) medium added with Mercury Acetate Hg(CH$_3$COO)$_2$, as much as 5 ppm (5 μg ml$^{-1}$). The isolate of probiotic candidate bacteria was inoculated on MRSB medium which added some concentration of mercury acetate Hg(CH$_3$COO)$_2$, i.e 10 ppm (10 μg ml$^{-1}$), 15 ppm (15 μg ml$^{-1}$) and 20 ppm (20 μg ml$^{-1}$). Then incubated for 72 hours. Growth was observed by looking at the value of bacterial cell density grown in culture using a uv-vis spectrophotometer with a wavelength of 580 nm. The highest optical density values are considered to be the most resistant.

3.1 Characterization of Probiotic Mercury-resistant Bacterial Isolates

3.1.1. Observation of Cell Morphology. On morphological observations every single colony formed after purification was then observed. Observations include colony shape, colony color, colony edge, and colony surface. Observation of cell morphology was done by gram staining technique.

3.1.2. Probiotic Test. Probiotic test conducted following Djide and Wahyuddin [2].

3.1.3. Test of Resilience to Gastric acidity (pH). The acid resistance test was performed using MRSB medium added with 0.1 N HCl to obtain a pH of 2.5-3 (according to gastric pH). One ose of each bacterial isolate was taken from culture stock and then inoculated on MRSB-HCl medium. Then incubated for 2x24 hours at 370C. Positive result indicated if bacterial growth occurs on MRSB-HCl medium and negative result showed if no bacterial growth occurs on MRSB-HCl medium.

3.1.4. Resistance Test against Gall bladder. The MRSB medium is added with synthetic bile salts (ox bite), with a concentration of 5%. One ose, each bacterial isolate extracted from culture stock was inoculated on MRSB-bile salt, then incubated for 2-3 x 24 hours at 37ºC.

3.2. Fermentation of Probiotic Bacteria IPB 9 on Scaly Milk Media with Addition of Mercury
A total of 10% starter was introduced into 1200 ml of skimmed milk medium then homogenized. Skimmed milk media was then put into a sterile glass container each of 100 ml then treated with the addition of Mercury Acetate (Hg(CH$_3$COO)$_2$) as follows:
- P1: Milk Media Skim + mercury concentration 5 ppm
- P2: Milk Media Skim + mercury concentration 10 ppm
- P3: Milk Media Skim + mercury concentration 20 ppm
- P4: Skimmed Milk Media without mercury addition (control)

All subsequent media at close the meeting then incubated at room temperature for 3 days. In incubation day 1, day 2 and day 3, mercury, acidity (pH) and calculation of bacterial count were measured.

3.3. Calculation of Number of Bacteria
Calculation of bacterial count is done by Standard Plate Count (SPC) method. A total of 1 ml of sample, diluted using physiological NaCl (0.9%) with diluted dilution, to dilution $10^6$. A total of 1 ml of dilution result was grown on MRSA + CaCO$_3$ 1% medium by pouring method. Then incubated for 1x24 hours. Furthermore, the colonies number of bacteria that grow in the plate were calculated.
3.4. Mercury Analysis on Skim Milk Media

After fermentation, each sample of milk media that has been added with mercury acetate (Hg(CH₃COO)₂) was tested to analyze mercury levels contained in the media after fermentation process. Mercury analysis was conducted at the Center of Makassar Health Laboratory using AAS (Atomic Absorption Spectrophotometry) method. At first, the sample was filtered with filter paper 0.045 m. The pH is set to about 3.5-4 by adding concentrated HNO₃ and then added 1 ml of concentrated HNO₃ solution and 5 ml of acetate buffer abrasive mixture. Then 5 ml of Ammonium Pyrrolidine Diphosphocarbonate (APDC) was added and shaken for about 5 min. An organic solvent, 10 ml Methyl Isobutyl Ketone (MIBK), were added to the solution, shake about 3 minutes and leave it to two separate phases.

3.5. Accommodated water phase

This aqueous phase is used for the preparation of laboratory and standard blank solutions. Addition of 10 ml distilled water in solution is required. The solution then will shake about 5 seconds until both phases apart.

3.6. Discard the water phase

Addition of 5 ml of HCl concentration dropped to the solution. The solution then shaken briefly and left for about 15 minutes. Addition of 10 ml of CI₃La7H₂O then gave to the solution that will shake about 2 minutes. After this process, sample are ready to be measured with AAS using dry ice method with wavelength 358 nm.

3.7. Data Analysis

Data obtained from mercury level analysis, acidity change (pH), number of microorganisms, discussed descriptively, and processed in the form of tables and graphs.

4. Results and Discussion

An incubation of 2 x 24 h from 10⁻⁴ dilution was obtained by 12 isolates showing clear zone as probiotic bacterial candidate. Medium MRSA is a medium often used to grow lactic acid bacteria. The result of bacterial selection of probiotic candidate on mercury showed from 12 isolates, only 7 isolates showed growth in MRSB media added mercury i.e: IPB 1, IPB 2, IPB 3, IPB 8, IPB 9, IPB 11, and IPB 12.

The absence of growth in some isolates because it is suspected that mercury is toxic to bacterial cells. Mercury kill the bacterial cell while other isolates that grow on mercury-containing MRSB medium have their own way in overcoming the toxic environment. The bacteria adapted to this extreme condition through the mechanism of resistance either the mechanism of metal ion biosorption with the compilation of complex cell walls or induce mer operon systems in plasmids containing mercury-resistant genes. Mercury acetate Hg(CH₃COO)₂; that used in this study has the same characteristic with methyl mercury (CH₃Hg). Both compound is the most common form of organic mercury that accumulated in the body of aquatic organisms and has known for its very high toxicity.

In relation to mercury resistance levels, Zulaika [3] explained that isolates which is able to grow on synthetic media containing mercury (Hg) ≥ 5 mg/l categorized as high resistance to mercury. Whereas those isolates that only grow at 1 mg/l mercury has weak resistance.

The results of the isolate resistance test on some mercury concentrations can be seen from the value of OD (optical density). This value determine whether or not there is growth on mercury-added media. According to [4], this growth can be measured by cell density (dry weight of cells of cell cultures).
Turbidity level analysis showed that in 10 ppm of mercury concentration, IPB 2, IPB 3, IPB 8 and IPB 12 have much turbidity and cell precipitation compared with other isolates in accordance with the results of cell density measurements showing the highest value (figure 1). This is because the isolates are able to tolerate the presence of mercury in the media so that it is still experiencing growth. At concentrations of 15 ppm, IPB 2 and IPB 8 still showed high growth, while the low growth occurred in IPB 1, IPB 9 and IPB 11 ranged from 0.04 - 0.08 OD. At the concentration of 20 ppm only IPB 2 showed the highest growth (1.26 OD), while the other isolates ranged from 0.04 - 0.1 OD which indicates the growth is still low. Bacterial resistance to mercury can be through biosorption and bioaccumulation mechanisms. The biosorption is a passive mechanism, so the metal does not poisoned the bacterial cells. On the contrary, bioaccumulation mechanism is an active process in which heavy metals can poisoned the bacterial cells [5].

4.1. Characterization of Probiotic Bacterial Isolates
The result of pH test after incubation 2 x 24 hours showed that isolate IPB 2 had very high turbidity and sediment level, IPB 1 had moderate growth, while in other isolates had less precipitate but media did not have turbidity. Lactic acid bacteria are capable of maintaining an alkali-pH of intracellular rather than extracellular pH, but intracellular pH decrease persists with decreasing extracellular pH that supports tolerance to acid [6].

Table 1. Characterization of Probiotic Isolate from Milkfish Intestine

| Type of Characterization | IPB 1 coccus | IPB 2 bacil | IPB 3 bacil | IPB 8 coccus | IPB 9 bacil | IPB 11 coccus | IPB 12 coccus |
|--------------------------|--------------|-------------|-------------|--------------|-------------|--------------|--------------|
| Cell Morphology          |              |             |             |              |             |              |              |
| Bile Salt                | -            | +           | +           | +            | -           | -            |              |
| Probiotic Test:          |              |             |             |              |             |              |              |
| pH 2.5 Resistance        | +            | +           | +           | +            | +           | +            | +            |
| Bile Salt Resistance 5%  | +            | +           | +           | +            | +           | +            | +            |
The bile salt resistance test showed excellent growth for all isolates characterized by turbidity in the media and there were many deposits at the bottom of the tube, but the most common sediment was found in IPB isolate 2. The growth occurring in bile salts for all isolates indicated that all isolates of ALB (Acid Lactid Bacteria) were able to withstand high bile salt conditions. Bile salts affect the permeability of bacterial cells, Bacteria that are not resistant to bile salts are suspected of permeability of cell membranes resulting in leakage of large intracellular material and causing cell lysis [7]. The ALB group in general can withstand with high bile salt stress conditions.

4.2. Growth of Probiotic Bacteria IPB 9 on Skimmed Milk Media with Addition of Mercury

Probiotic bacterial fermentation is done by inserting a starter of 10% into a skim milk production medium. Then added mercury with various concentrations. The absorption of heavy metals by other probiotic bacteria has now been developed. Some members of the intestinal microbiota, such as Lactobacillus used in food applications, are potentially additive to reduce the toxicity of metals in humans. This is because they have an effective resistance mechanism in preventing damage to their cells and can bind and absorb heavy metals onto the cell surface, then later the cells will come out through the feces. Heavy metal resistance genes and antibiotics are often encoded together on the same plasmid [8].

4.3. Dynamics of Growth of Bacteria IPB 9 on Milk Media Skim with Various Concentrations of Mercury.

Calculation of the number of colonies of probiotic bacteria L. plantarum starts from calculating T0 treatment to T4 treatment with 1 day interval. The dilution results of each dilution were inoculated on the selective medium of lactic acid bacteria i.e., MRSA (Man Ragosa Sharpe Agar) which added 1% CaCO₃ using the casting method.

![Figure 2. Diagram of isolated probiotic bacteria IPB 9 grown on different mercury concentration.](image)

The addition of mercury concentration did not significantly affect the amount of bacteria produced. Even in the addition of high mercury concentrations, the bacteria are still able to grow and multiply because in addition to fermenting lactose in skim milk, the bacteria also bind and absorb heavy metals onto the surface of their cells.

4.4. Changes in pH in Skimmed Milk Media during Fermentation Process

Day 1 incubation (T1 treatment) pH control value was 6.0, pH value of mercury concentration 5 ppm was 6.2, pH value of 10 ppm concentration was 6.2 seta pH value of 20 ppm was 6.0. As for incubation day 2 (T2 treatment) pH control value is 5.9, pH value of 5 ppm is 5.9, pH value of 10 ppm is 6.0 and pH value of 20 ppm is 5.9. While for 3 days incubation (T3 treatment) the pH value of control was 5.9,
the pH value of 5 ppm was 5.9, the pH concentration of 10 ppm was 6.0 and the pH value of 20 ppm was 6.0.

![Figure 3](image)

**Figure 3.** Graph of pH change on skimmed milk medium during fermentation process.

The pH of skim milk ranges from 6.5 to 6.7. IPB 9 isolates can decrease the pH of milk during fermentation. The increase of pH along with the destruction of fermented milk is characterized by the rupture of solids in fermented milk, the texture turns roughly, the increase of the liquid separates from the solid and the incidence of unpleasant odors.

4.5. Analysis of Mercury Levels Using AAS (Atomic Absorption Spectrophotometry) with Dry Method (Dry Ice)

Mercury-resistant bacteria and a mechanism for dealing with mercury stress in the presence of mercury-resistant genes called mer-operon genes comprising the metaregulator (merR) genes, mercury transport genes (merT, merP, merC), mercury reductase (merA) and gene organomercuri liase (merB) (Chein, et al., 2010). The merB gene will catalyze the bonding of the Me-Hg yielding the organic compound and the Hg^{2+} ion, the Hg^{2+} ion will be bound to merC or merT. While Hg^{2+} outside the bacterial environment will enter the periplasm with cysteine merp residue pairs. Then merP transfer Hg^{2+} to cysteine residues merT or merC. Furthermore, Hg^{2+} ions cross the cytoplasmic membrane through a ligand exchange reaction process toward NADPH dependent on mercury reductase (the merA gene). Mercury reductase gives its 2 electrons to NADPH so that Hg^{2+} turns into a volatile Hg 0 without generating energy for the bacteria, then Hg0 is removed from the cell [9].

Operon Mer function is to give the ability of bacteria as absorbent agent molecule Hg (II). Combination of specific Mer genes as transport functions (MerTP) with metallothionein belonging to eukaryotes located in the cytoplasm of the Escherichia coli strain. This system is very specific. MerR has an affinity feature for reducing Hg (II) by sorbent by fusion with polypeptide (ELPs) [10].

Based on the results of mercury (Hg) analysis, results obtained that probiotic bacteria IPB 9 is able to reduce various mercury concentrations. At T1 the mercury concentration of 5 ppm was reduced to 3.56 ppm, the concentration of 10 ppm was reduced to 8.71 ppm, the concentration of 20 ppm was reduced to 11.93 ppm and on the undetectable control of mercury (Hg). While at T2 the mercury concentration of 5 ppm was reduced to 3.45 ppm, the concentration of 10 ppm was reduced to 7.41 ppm, the concentration of 20 ppm was reduced to 10.02 ppm and the control of undetectable mercury metal (Hg) was not detected. While at T3 the mercury concentration of 5 ppm was reduced to 3.02 ppm, the concentration of 10 ppm was reduced to 6.69 ppm, the concentration of 20 ppm was reduced to 9.00 ppm and on the undetectable control of mercury (Hg).
5. Conclusion
Isolation of milkfish intestine obtained 7 isolates of probiotic bacteria that had growth in MRSB media added mercury. Probiotics IPB 9 able to grow and can reduce mercury metal (Hg) at concentrations of 5 ppm, 10 ppm and 20 ppm on skim milk media. The number of Lactobacillus plantarum bacteria during the fermentation process from T0 treatment to T3 treatment increased.

References
[1] Barkay, T., and W. D. Irene, 2005. Microbial Transformations of Mercury: Potentials, Challenges, and Achievements in Controlling Mercury Toxicity in The EnvironmentDepartment of Biochemistry and Microbiology. New Jersey. Advances In Applied Microbiology. Vol. 57
[2] Djide, M. N., and E., Wahyudin, 2008. Isolation of Lactic Acid Bacteria from Mother’s Milk, and Its Potential in Lowering Cholesterol In Vitro. Pharmacy and Pharmacology Magazine. Vol. 12 (3)
[3] Zulaika, E., A. Widiyanti, and M. Shovitri, 2011. Endogenic Mercury Endogenous Bacteria Downstream Kalimas Surabaya. Journal. Biology Study Program, FMIPA ITS. Surabaya
[4] Khodijah, S., B. J. Tuasical, I. Sugoro and Yusneti, 2006. Growth of Streptococcus agalactiae As Bacteria Cause Subclinical Mastitis in Dairy Cattle. National Seminar on Livestock Technology. UIN Syarif. Jakarta
[5] Chojnacka, K., 2010. Biosorption and Bioaccumulation, The Prospects for Practical Applications. Environment International. 36: 299 – 307
[6] Siegumfeldt, H., B. K. Rechninger and M. Jacobsen, 2000. Dynamic Changes of Intracellular pH in Individual Lactic Acid Bacterium Cells in Response to a Rapid Drop in Extracellular pH. Appl. Environ Microbiol. 66: 2330 - 2335
[7] Kusumawati, N., L. J. Bettsyri, S. Siswa, Ratihdewanti and Hariadi, 2003. Selection of Lactic Acid Bacteria Indigenous as Probiotics Galur with Cholesterol Lowering Ability. Journal of Microbiology Indonesia. Vol. 8 (2): 39-43
[8] Monachese, M., J.P. Burton, and G. Reid, 2012. Bioremediation and Tolerance of Humans to Heavy Metals through Microbial Processes: a Potential Role for Probiotics?. Applied and Environmental Microbiology. 78 (18). P. 6397-6404
[9] Brown, N., Y. Shih, C. Leang, K. Glendinning, J. Hobman, and J. Wilson, 2002. Mercury Transport and Resistance. Biometals, International Biometals Symposium
[10] Kostal, J., A. Mulchandani, K. E. Gropp and W. Chen, 2003. A Temperature Responsive Biopolymer for Mercury Remediation. Environ. Sci. Technol. 37.4457 4462