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# Biosorption of heavy metals by lactic acid bacteria and identification of mercury binding protein

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**Abstract**

Heavy metals cause various health hazards. Using lactic acid bacteria (LAB), we tested the biosorption of heavy metals e.g. cadmium (Cd) (II), lead (Pb) (II), arsenic (As) (III), and mercury (Hg) (II). Cd (II) sorption was tested in 103 strains using atomic absorption spectrophotometery (AAS). *Weissella viridescens* MYU 205 ($1 \times 10^8$ cells/ml) decreased Cd (II) levels in citrate buffer (pH 6.0) from one ppm to $0.459 \pm 0.016$ ppm, corresponding to 10.46 μg of Cd (II). After screening, 11 LAB strains were tested using various pH (pH 4.0, 5.0, 6.0, 7.0) showing the sorption was acid sensitive; and was cell concentration dependent, where the Cd (II) concentration decreased from one ppm to 0.042 (max)/0.255 (min) ppm at $1 \times 10^{10}$ cells/ml. Additionally, the biosorption of Pb (II), As (III), and Hg (II) were tested using an inductively coupled plasma mass spectrometer (ICP-MS). The Hg (II) concentration was reduced the most followed by Pb (II) and As (III). Many of the bacterial cell surface proteins of *W. viridescens* MYU 205 showed binding to Hg (II) using the Hg (II) column assay. Having a CXXC motif, a ~14 kDa protein may be one of the Hg (II) binding proteins. LAB biosorption may aid the detoxification of people exposed to heavy metals.


*Keywords:* Heavy metal; Biosorption; Lactic acid bacteria; Detoxification; Cadmium; Mercury

## 1. Introduction

Heavy metals cause various health hazards. Food contaminated with heavy metals may have detrimental effects on human and animal health even at low concentrations because of gradual accumulation. The four major pollution-caused illnesses in Japan are Itai–itai disease, Minamata disease, Niigata Minamata disease, and Yokkaichi asthma. Itai–itai disease and the two Minamata diseases are caused by the heavy metals, Cd and methylmercury, respectively. Presently, heavy metal contamination is especially serious in developing countries where accumulation in the human is a concern. Therefore, Cd, Pb, As, and Hg are specified by the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan as required harm factors for risk management.

LAB are common microbes used as probiotics. Probiotics show many beneficial effects, e.g. managing lactose intolerance (Savaiano and Kotz, 1989), lowering cholesterol (Danielson et al., 1989), improving immune function (Perdigón et al., 2002), preventing colon cancer (Lim et al., 2002), and the inhibiting the adherence of some pathogens (Chen et al., 2007; Mack et al., 1999; Varma et al., 2010). Probiotics such as *Lactobacillus* and *Bifidobacterium* can bind cholesterol on their cell surface, allowing efficient fecal discharge outside of the body (Gilliland et al., 1985; Tahri et al., 1996; Usman and Hosono, 1999).

Many reports show the biosorption of heavy metals by bacteria and fungi. For example, Rehman et al. (2010) reported *Candida tropicalis* CBL-1 removed Cd (II) from wastewater. Sochor et al. (2011) reported *Staphylococcus aureus* bound Cd

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(II) and may be used for the sensing of Cd (II). Lin et al. reported the biosorption of Au (III) (Lin et al., 2011) and Pt (IV) (Lin et al., 2009) in *Bacillus megatherium* D01. Li et al. (2011) reported *Bacillus cereus* showed resistance and biosorption of Ag (I). However, these species are unsuitable for food use. Conversely, people eat many LAB strains with food, especially fermented products, in which LAB are generally recognized as safe (GRAS) (Feord, 2002; Stiles and Holzapfel, 1997). We believe LAB can prevent the absorption of heavy metals into the body and oral ingestion of LAB can remove heavy metals from the body efficiently during defecation. Some reports show heavy metal biosorption by LAB (Bhakta et al., 2012; Ibrahim et al., 2006; Lin et al., 2005; Schut et al., 2011). However, further research is needed concerning the heavy metal binding capability of LAB as a probiotic for human health. Therefore, we examined the Cd (II) biosorption ability of bacteria derived from food using an *in vitro* mass-screening; and characterized other properties of selected LAB, e.g., pH effect, dose-dependence, and biosorption abilities for Pb (II), As (III), and Hg (II). Finally, we examined one of the mechanisms for Hg (II) biosorption and detoxification.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

One hundred three bacteria isolates from various foods, bovine and porcine intestines (called Horumon in Japan), Japanese pickles, Japanese Amazake, kimchee, and yogurt were used for bacterial samples including 50 *Lactobacillus* strains, 19 unidentified LAB derived from yogurt, eight *Weissella*, four strains of *Pediococcus*, two of *Streptococcus,* two *Enterococcus*, and 18 strains of unidentified bacteria (may be including non-LAB strains) to apply to fermented food in the future. Bacterial strains were propagated twice at 37 °C for 18 h in MRS broth with 2% (v/v) inoculum before the experiments.

### 2.2. Cd (II) biosorption assay

Cd (II) standard 1000 ppm solution (Cd $(NO_3)_2$ in 0.1 M $HNO_3$) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was diluted with 10 mM sodium citrate buffer (pH 6.0) and used as a one ppm Cd (II) solution.

The 103 bacterial strains were cultured at 37 °C for 18 h in MRS broth (Difco Laboratories, Detroit, MI) using 2% (v/v) inoculum. Bacterial cells after culture were washed three times with sterile distilled water. After washing, the pellets were suspended in 20 ml of one ppm Cd (II) solution (pH 6.0) adjusted to $1.0 \times 10^8$ cells/ml and incubated at 37 °C for 1 h. After incubation, the bacterial cells were removed using a 0.2 μm Minisart syringe filter (Sartorius Stedim Biotech GmbH, Germany); and the Cd (II) concentration of the filtrate was measured using an AAS SpectrAA-55 (Agilent Technologies, Santa Clara, CA). Buffer was used instead of bacteria in the negative control (NC).

For Cd (II) biosorption assays using various pH conditions, 10 ml of 10 mM citrate-phosphate buffer was used at pH 4.0, 5.0, 6.0, and 7.0 instead of sodium citrate buffer.

The heat-treatment test was performed at pH 5.0 and 7.0 with *Weissella viridescens* MYU 205 and *Lactobacillus mucosae* MYU 224. The bacteria were washed three times with sterile distilled water, adjusted to $1.0 \times 10^8$ cells/ml, and suspended in 10 ml of one ppm Cd (II) solution (pH 5.0 or 7.0). The bacterial suspensions were treated at 100 °C or room temperature for 1 h, and then incubated at 37 °C for 1 h. After incubation, the bacterial cells were removed using a 0.2 μm Minisart syringe filter (Sartorius Stedim Biotech GmbH); and the Cd (II) concentration of the filtrate was measured using AAS.

For Cd (II) biosorption assays at various bacterial concentrations, $1 \times 10^7$, $1 \times 10^8$, $1 \times 10^9$, and $1 \times 10^{10}$ bacteria cells/ml in one ppm Cd (II) solution with 10 ml of 10 mM sodium citrate buffer (pH 6.0) was used.

### 2.3. Pb (II), As (III), and Hg (II) biosorption assay

Each 1000 ppm standard solution: Pb (II) (Pb $(NO_3)_2$ in 0.1 M $HNO_3$) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), As (III) ($As_2O_3$ and sodium chloride (0.05%) in 1M HCl solution) (Kanto Chemical Co., Inc., Saitama, Japan), and Hg (II) ($HgCl_2$ in 0.1 M $HNO_3$) (Kanto Chemical Co., Inc., Saitama, Japan) was diluted with 10 mM sodium citrate buffer (pH 6.0) and used as one ppm Pb (II), As (III), or Hg (II) solution, respectively.

Pb (II), As (III), and Hg (II) biosorption assays in 10 ml of one ppm solution were performed using the same methods as the Cd (II) biosorption assays. The filtrate after biosorption was diluted 20 times with 2% nitric acid ($HNO_3$) solution and the concentration of the diluted solution was measured using ICP-MS ELAN DRC-e (Perkin Elmer SCIEX, Boston, MA). The concentration of the undiluted solution was evaluated 20 times compared to the concentration of a diluted solution. Buffer was used instead of bacteria in the NC.

### 2.4. Examination of the influence of the heavy metals on cell growth

The influence of the heavy metals on cell growth of *W. viridescens* MYU 205, *Lactobacillus sakei* MYU 10, and *L. mucosae* MYU 224 was investigated. The strains were propagated in MRS broth containing one ppm Cd (II), Pd (II), As (III), or Hg (II) with 2% (v/v) inoculum, and the pH and optical density at 600 nm ($OD_{600}$) of these culture media were measured after 0, 4, 8, 12, 16, 24, 36, and 48 h. MRS broth containing no heavy metal was used as a control.

### 2.5. Identification of Hg (II) binding proteins of W. viridescens *MYU 205*

To prepare cell wall surface proteins from *W. viridescens* MYU 205 cultured in MRS broth, the cells were washed three times with sterilized distilled water; and extracted using 2 M guanidine hydrochloride (GHCl, pH 6.0) solution at 37 °C for

2 h with gentle shaking. After dialysis of the extract against distilled water for 2 days at 4 °C, the dialysate was lyophilized.

To identify Hg (II) binding proteins, we used a HiTrap Chelating HP column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's instructions. The chelating column was washed with 5 ml distilled water and 0.5 ml of 0.1 M Hg (II) in distilled water was added and washed 5 ml of distilled water. After column preparation, the column was equilibrated with 10 ml binding buffer (0.02 M sodium phosphate, pH 7.2), and 5 ml of 10 mg/ml of the lyophilized cell surface protein in the binding buffer was applied. To ensure binding of the proteins to the column, the eluate was recovered and re-applied to the column. The column was washed with 10 ml of the binding buffer, and the proteins bound to the column were eluted with 10 ml of the elution buffer (0.02 M sodium phosphate, 0.5 M NaCl, 0.05 M EDTA, pH 7.2). After dialysis against distilled water for 2 days at 4 °C, the dialysate was lyophilized. A column containing no Hg (II) was used as a negative control using the same protocol. The lyophilized proteins binding to the Hg (II) column and the non-specific binding proteins in the negative control column were dissolved in SDS buffer (60 mM Tris, 25% glycerol, 2% SDS, 2% β-mercaptoethanol and 0.1% bromophenol blue; pH 6.8) and used for SDS-PAGE. The Hg (II) binding protein was identified using the N-terminal amino acid sequence with the Edman degradation.

### 2.6. SDS-PAGE analysis

The cell surface proteins prepared from *W. viridescens* MYU 205 were analyzed using SDS-PAGE according to the method of Laemmli (1970). Samples were denatured in SDS buffer; followed by heating at 95 °C for 5 min. Electrophoresis was performed using polyacrylamide gels with a discontinuous buffer system with a 4.5% stacking gel and a 12.5% separation gel (10 × 12 cm). The electrophoresis was performed at a constant voltage of 125 V in running buffer (pH 8.5): 0.025 M Tris, 0.2 M glycine and 0.1% SDS. Protein bands were visualized staining the gels with Coomassie brilliant blue (CBB) (EsStain AQua; Atto Co., Ltd., Tokyo, Japan). Molecular weight markers from 14.3 to 97.2 kDa (AE-1440 EzStandard, Atto Co., Ltd., Tokyo, Japan) were used.

### 2.7. Statistical analysis

The assays were performed three or four times. All data were reported as the mean ± SD. The data was assessed using analysis of variance (ANOVA). When ANOVA was significant, the significance of differences was determined by the two-tailed *t*-test with Bonferroni adjustments ($p < 0.01$).

## 3. Results

### 3.1. Cd (II) biosorption assay

The Cd (II) biosorption assay was performed three times for 103 strains. These bacteria showed various biosorptions from 0.459 ± 0.016 ppm (max) to 0.951 ± 0.007 ppm (min) and all samples showed a significant difference against NC ($p < 0.01$). The NC containing no bacterial cells was 0.982 ± 0.005 ppm. *W. viridescens* MYU 205 showed the highest biosorption of the 103 strains where the Cd (II) concentration decreased from one ppm to 0.459 ± 0.016 ppm, corresponding to 10.46 μg of Cd (II). The amount of Cd (II) biosorption with MYU 205 was 5.23 fg per bacterial cell. Table 1 shows Cd (II) biosorption of each bacterial species. The genus *Weissella* showed the highest average value of Cd (II) biosorption of the 103 strains, at 0.577 ± 0.089 ppm ($n = 8$), where all seven strains of *W. viridescens* were in the top 11 in biosorption. The average of the Cd (II) biosorption value in 50 identified *Lactobacillus* was 0.845 ± 0.087 ppm. Ten strains of *L. sakei* showed the highest biosortion in *Lacobacillus* species at 0.723 ± 0.089 ppm (max: 0. 577 ppm in MYU 10, min: 0.906 ppm in MYU 92). Four strains of *L. mucosae/L. fermentum* showed 0.781 ± 0.074 ppm. Two strains of *Streptococcus alactolyticus* and two strains of *Enterococcus faecalis* showed comparatively high biosorption at 0.651 and 0.633 ppm, respectively; whereas four strains of *Pediococcus pentosaceus* showed comparatively low biosorption at 0.874 ± 0.037 ppm. Average biosorption values in 19 strains of an unidentified LAB derived from yogurt and 18 strains of unidentified bacteria were 0.776 ± 0.070 ppm and 0.759 ± 0.111 ppm, respectively.

We selected 11 different LAB strains: MYU 205, MYU 10, MYU 217, MYU 73, MYU 213, MYU 224, MYU 96, MYU 49, MYU 135, MYU 89, and MYU 221, representing different species and different biosorption abilities in the Cd (II) biosorption assay (Table 2). The selected 11 LAB strains were tested for Cd (II) biosorption at pH 4.0, 5.0, 6.0, and 7.0 (Fig. 1A). All selected LAB showed a significantly lower concentration of Cd (II) under the different pH condition compared with NC ($p < 0.01$). The results showed pH affected the Cd (II) biosorption. Significant difference was observed between each different pH group ($p < 0.01$). The amount of Cd (II) biosorption tended to be high when the pH was high in many strains such as MYU 205, MYU 217, MYU 73, MYU 213, MYU 49, MYU 89, and MYU 221. No pH effect was observed in the MYU 96 and MYU 135 strains. The biosorptions in MYU 10 and MYU 224 were the highest at pH 5.0 and lowest at pH 7.0. MYU 205 also showed high biosorption at different pHs, although MYU 224 was the highest at pH 4.0 and 5.0, showing reduction from one ppm to 0.486 ± 0.001 ppm and 0.312 ± 0.003 ppm, respectively. Changes in the amount of biosorption after heat-treatment were examined at pH 5.0 and pH 7.0 in MYU 205 and MYU 224, respectively ($n = 4$). No influence of heat-treatment was observed in MYU 205 although significant difference was observed in MYU 224 ($p < 0.01$). In MYU 224, at pH 5.0, the Cd (II) biosorption value after heat-treatment was lower (0.800 ± 0.026 ppm) than non-treatment (0.518 ± 0.070 ppm). However, at pH 7.0, the Cd (II) biosorption value after heat-treatment was higher (0.518 ± 0.047 ppm) than non-treatment (0.785 ± 0.051 ppm).

Fig. 1B shows the data from the Cd (II) biosorption assays at various concentrations of LAB cells: $1 \times 10^7$, $1 \times 10^8$, $1 \times 10^9$, and

Table 1
Cd (II) biosorption of each bacterial species.

| Bacterial species | Number of bacterial strains | Average of the Cd (II) concentration (ppm) of the filtrate after biosorption (SD) | Average of total amount of Cd (II) sorbed by bacteria (μg) | Average of amount of Cd (II) sorbed per a bacterial cell (fg/cell) |
|---|---|---|---|---|
| Negative control (without bacterial cell) | – | 0.982 (0.005) | – | – |
| Total *Lactobacillus* species | 50 | 0.845 (0.087)[b] | 2.74 | 1.37 |
| (Detail in *Lactobacillus* species) | | | | |
| *L. sakei* | 10 | 0.723 (0.089)[b] | 5.19 | 2.59 |
| *L. plantarum/L. pentosus*[a] | 8 | 0.906 (0.014)[b] | 1.52 | 0.76 |
| *L. casei/L. paracasei*[a] | 6 | 0.848 (0.049)[b] | 2.69 | 1.35 |
| *L. rhamnosus* | 5 | 0.916 (0.020)[b] | 1.33 | 0.66 |
| *L. mucosae/L. fermentum*[a] | 4 | 0.781 (0.074)[b] | 4.02 | 2.01 |
| *L. reuteri* | 3 | 0.890 (0.070)[b] | 1.83 | 0.92 |
| *L. gasseri* | 3 | 0.915 (0.011)[b] | 1.34 | 0.67 |
| *L. coryniformis* | 2 | 0.870 (–)[b] | 2.25 | 1.12 |
| *L. alimentarius* | 2 | 0.886 (–)[b] | 1.92 | 0.96 |
| *L. curvatus* | 1 | 0.828 (–)[b] | 3.09 | 1.54 |
| *L. ruminis* | 1 | 0.833 (–)[b] | 2.98 | 1.49 |
| *L. gallinarum* | 1 | 0.858 (–)[b] | 2.48 | 1.24 |
| *L. johnsonii* | 1 | 0.868 (–)[b] | 2.28 | 1.14 |
| *L. agilis* | 1 | 0.872 (–)[b] | 2.19 | 1.10 |
| *L. rossiae* | 1 | 0.876 (–)[b] | 2.12 | 1.06 |
| *L. brevis* | 1 | 0.927 (–)[b] | 1.10 | 0.55 |
| *W. viridescens & W. paramesenteroides* | 8 | 0.577 (0.089)[b] | 8.10 | 4.05 |
| *P. pentosaceus* | 4 | 0.874 (0.037)[b] | 2.15 | 1.08 |
| *S. alactolyticus* | 2 | 0.651 (–)[b] | 6.62 | 3.31 |
| *E. faecalis* | 2 | 0.633 (–)[b] | 6.97 | 3.49 |
| Unidentified LAB derived from yoghurt | 19 | 0.776 (0.070)[b] | 4.13 | 2.06 |
| Unidentified bacteria | 18 | 0.759 (0.111)[b] | 4.46 | 2.23 |

The biosorption assay was performed in triplicate by incubation for 1 h at 37 °C in 20 ml of one ppm Cd (II) solution containing $1.0 \times 10^8$ cells/ml of bacteria cells. The average in triplicate data was used as one data of the strain, and the standard deviation (SD) in the bacterial species was calculated.

[a] Two species couldn't be distinguished in the identification assay of bacterial species.

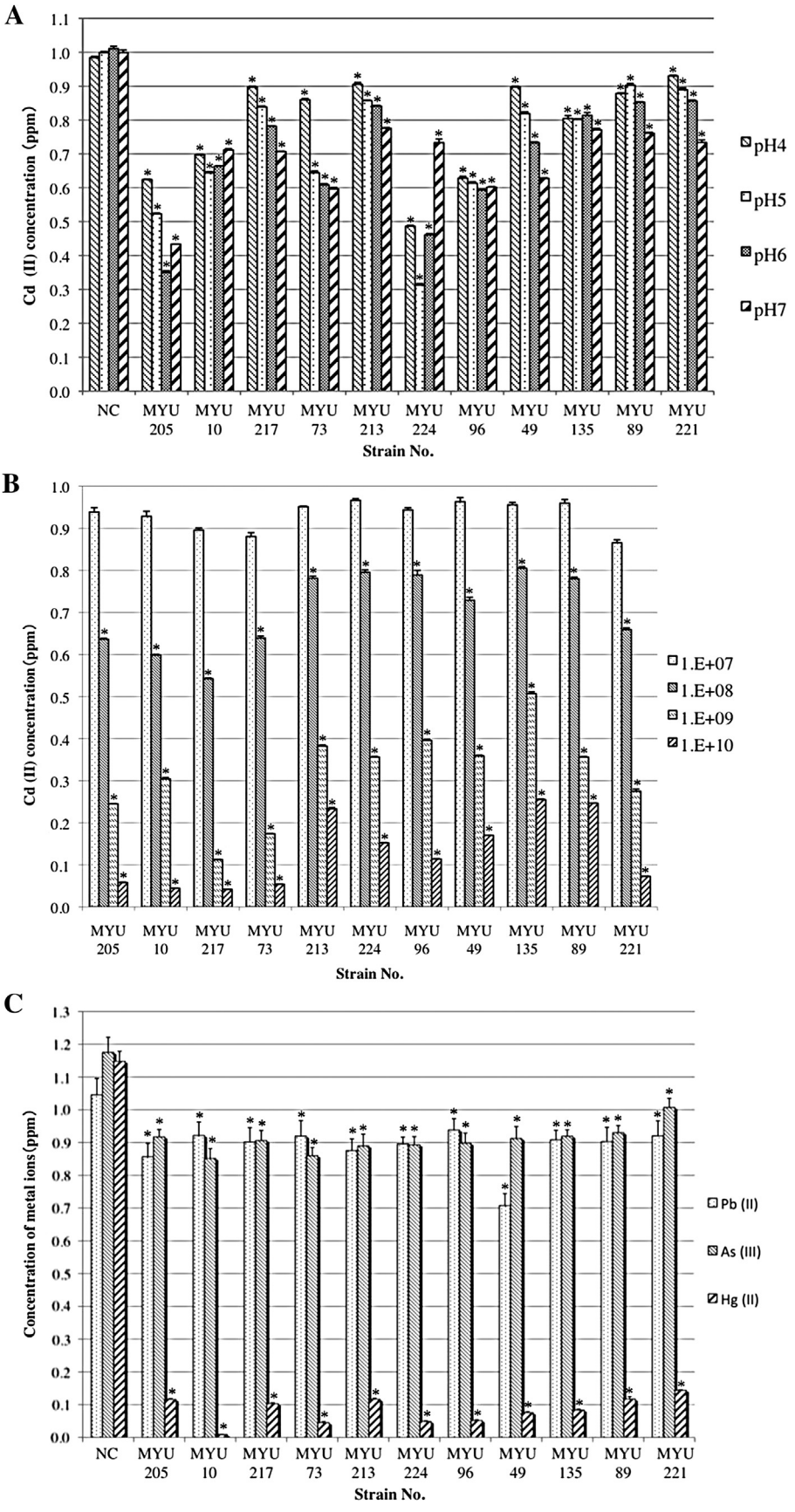
[b] Significant difference against the negative control ($p < 0.01$).

$1 \times 10^{10}$ cells/ml. The Cd (II) concentration decreased dose-dependently and a significant difference was observed between different concentrations ($p < 0.01$). When bacterial counts were $1 \times 10^8$, $1 \times 10^9$, and $1 \times 10^{10}$ cells/ml, all selected LAB showed a significantly lower concentration of Cd (II) compared with $1 \times 10^7$ cells/ml ($p < 0.01$). When bacterial counts were $1 \times 10^7$ cells/ml, little difference between bacterial strains was observed comparing $1 \times 10^8$, $1 \times 10^9$, and $1 \times 10^{10}$ cells/ml. When bacterial counts were $1 \times 10^9$ cells/ml, the highest biosorption observed was $0.112 \pm 0.001$ ppm in MYU 217, and the lowest

Table 2
The selected lactic acid bacteria by Cd (II) biosorption assay.

| Selected LAB strain | Isolated source | Average of the Cd (II) concentration (ppm) of the filtrate after biosorption (SD) | Average of total amount of Cd (II) sorbed by bacteria (μg) | Average of amount of Cd (II) sorbed per a bacterial cell (fg/cell) |
|---|---|---|---|---|
| *W. viridescens* MYU 205 | Bovine intestine | 0.459 (0.016) | 10.46 | 5.23 |
| *L. sakei* MYU 10 | Japanese Takuan pickle | 0.577 (0.003) | 8.11 | 4.05 |
| *S. alactolyticus* MYU 217 | Porcine intestine | 0.644 (0.004) | 6.76 | 3.38 |
| *L. sakei* MYU 73 | Japanese Amazake | 0.656 (0.007) | 6.52 | 3.26 |
| *E. faecalis* MYU 213 | Porcine intestine | 0.684 (0.005) | 5.96 | 2.98 |
| *L. mucosae* MYU 224 | Porcine intestine | 0.691 (0.005) | 5.82 | 2.91 |
| *W. paramesenteroides* MYU 96 | Japanese Nukazuke pickle | 0.758 (0.002) | 4.49 | 2.24 |
| *L. casei* MYU 49 | Pickle | 0.763 (0.004) | 4.39 | 2.19 |
| *L. ruminis* MYU 135 | Porcine intestine | 0.833 (0.004) | 2.98 | 1.49 |
| *P. pentosaceus* MYU 89 | Celery's pickle | 0.835 (0.005) | 2.93 | 1.47 |
| *L. reuteri* MYU 221 | Porcine intestine | 0.940 (0.015) | 0.84 | 0.42 |

The data shows Cd (II) concentration of filtrate after biosorption. The assay was performed in 20 ml of one ppm Cd (II) solution adjusted $1.0 \times 10^8$ cells/ml of bacteria cells ($n = 3$).


A
Cd (II) concentration (ppm)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
pH4
pH5
pH6
pH7
NC
MYU 205
MYU 10
MYU 217
MYU 73
MYU 213
MYU 224
MYU 96
MYU 49
MYU 135
MYU 89
MYU 221
Strain No.
B
Cd (II) concentration (ppm)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1.E+07
1.E+08
1.E+09
1.E+10
MYU 205
MYU 10
MYU 217
MYU 73
MYU 213
MYU 224
MYU 96
MYU 49
MYU 135
MYU 89
MYU 221
Strain No.
C
Concentration of metal ions (ppm)
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Pb (II)
As (III)
Hg (II)
NC
MYU 205
MYU 10
MYU 217
MYU 73
MYU 213
MYU 224
MYU 96
MYU 49
MYU 135
MYU 89
MYU 221
Strain No.

0.507 ± 0.005 ppm in MYU 135. When bacterial counts were $1 \times 10^{10}$ cells/ml, Cd (II) concentrations of five strains, MYU 205, MYU 10, MYU 217, MYU 73, and MYU 221, were lower than 0.1 ppm. Especially, the Cd (II) concentration of MYU 217 and MYU 10 decreased from one ppm to 0.042 and 0.044 ppm, corresponding to a sorption of 9.38 and 9.40 μg of Cd (II), respectively. Cd (II) concentration was decreased to 0.255 ± 0.002 ppm even though MYU 135 showed the least biosorption ability at $1 \times 10^{10}$ cells/ml. The average of the total amount of Cd (II) biosorption in 10 ml of Cd (II) solution with $1 \times 10^{7}$, $1 \times 10^{8}$, $1 \times 10^{9}$, and $1 \times 10^{10}$ cells/ml of selected strains were 0.50, 2.77, 6.67, and 8.51 μg, respectively; the average of the amount of Cd (II) biosorption per cell was 5.03, 2.77, 0.67, and 0.09 fg per cell, respectively.

### 3.2. Pb (II), As (III), and Hg (II) biosorption assays

The selected 11 LAB strains were tested for Pb (II), As (III), and Hg (II) biosorption (Fig. 1C). All selected LAB showed a significant lowering of concentration under the different metal ions compared with NC ($p < 0.01$). Especially, all strains significantly decreased Hg (II) concentration to under 0.15 ppm ($p < 0.01$). MYU 10 showed the highest biosorption at 0.009 ± 0.001 ppm, corresponding to a sorption of 19.82 μg of Hg (II); and indicated 0.198 pg of Hg (II) sorption per cell (99.1% sorption). Pb (II) concentration of MYU 49 was significantly decreased to 0.708 ± 0.036 ppm ($p < 0.01$); although the Pb (II) biosorption of other strains was not as high compared to Cd (II) and Hg (II). As (III) biosorption was less; MYU 10 strain showed the highest of 11 strains at 0.852 ± 0.030 ppm. No significant difference was observed between Pb (II) and As (III) although significant difference was observed in Hg (II) compared to Pb (II) and As (III) ($p < 0.01$).

Fig. 2 shows the results of growth in MRS broth containing the heavy metals. Hg (II) influenced the growth of the bacteria whereas Cd (II), Pb (II), and As (III) did not. Especially, the growth of MYU 205 after 48 h in Hg (II) was inhibited; the $OD_{600}$ was lower than the control and other metals. Delay of growth in the presence of Hg (II) was also observed in MYU 10 and MYU 224.

### 3.3. Identification of Cd (II) binding proteins of W. viridescens *MYU 205*

Fig. 3 shows the data using the chelating column for the identification of Hg (II) binding proteins of *W. viridescens* MYU 205. Many bands were detected from the Hg (II) column, although a few weak bands were detected in the negative control without Hg (II). Especially, the ~14 kDa band from the Hg (II) column (arrows in Fig. 3) was clear and thick. The N-terminal amino acids from the ~14 kDa band were sequenced after electroblotting, and nine amino acid residues were determined to be $NH_2$-MRVKGVDLV-. This partial sequence was compared to the NCBI database and was 78% identical to a conserved hypothetical protein from *Lactobacillus oris* PB013-T2-3 (Accession No. ZP_07728887). This hypothetical protein has a NAD(P) binding site and Cys-X-X-Cys (CXXC) motif although its function is unknown.

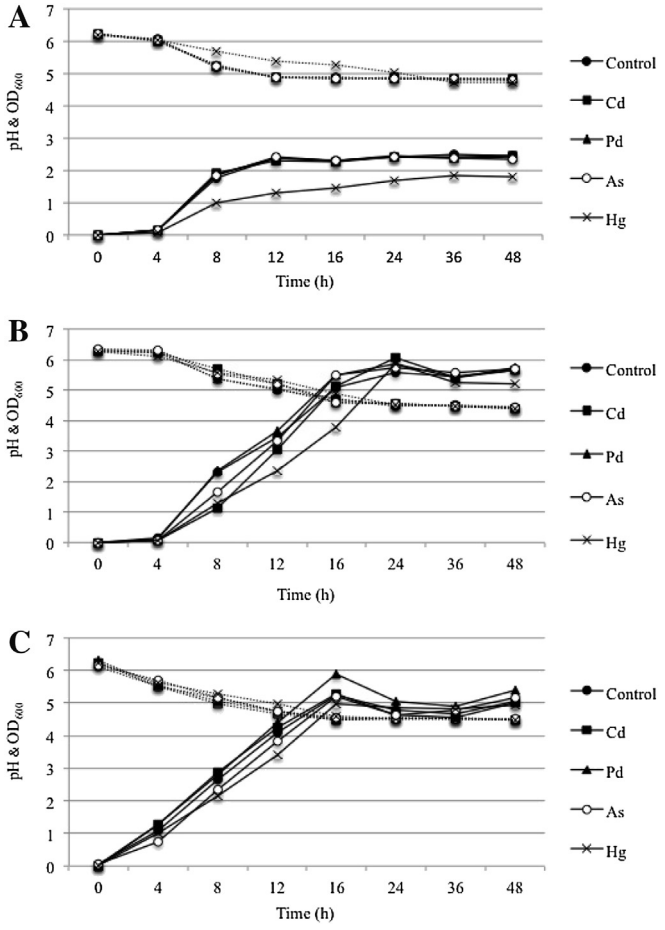



Fig. 2. Growth in MRS broth containing heavy metals. A: *W. viridescens* MYU 205, B: *L. sakei* MYU 10, C: *L. mucosae* MYU 224. These strains were propagated in MRS broth containing one ppm of Cd (II), Pd (II), As (III), and Hg (II), respectively with 2% (v/v) inoculum, and then the pH (dotted line) and the $OD_{600}$ (solid line) of these culture media were measured after 0, 4, 8, 12, 16, 24, 36, and 48 h ($n = 4$). MRS broth containing no heavy metal was used as a control.

Fig. 1. (A) Cd (II) biosorption assay at various pH conditions. The bacterial cells were suspended with one ppm Cd (II) solution (pH 4.0, 5.0, 6.0, and 7.0) adjusted to $1.0 \times 10^{8}$ cells/ml and incubated at 37 °C for 1 h. After incubation, the bacterial cells were removed and the Cd (II) concentration was measured using AAS ($n = 3$). NC: Negative control (without bacterial cells). *: Significant difference against negative control (NC) ($p < 0.01$). (B) Cd (II) biosorption assay at various bacterial concentrations. The bacterial cells were suspended in one ppm Cd (II) solution (pH 6.0) and adjusted to $1 \times 10^{7}$, $1 \times 10^{8}$, $1 \times 10^{9}$, and $1 \times 10^{10}$ cells/ml, respectively. After incubation, the Cd (II) concentration of the filtrate was measured using AAS ($n = 3$). *: Significant difference against the value at $1 \times 10^{7}$ cells/ml ($p < 0.01$). (C) Pb (II), As (III), and Hg (II) biosorption assays. The bacterial cells were suspended in one ppm Pb (II), As (III), and Hg (II) solution (pH 6.0) adjusted to $1.0 \times 10^{8}$ cells/ml, respectively. After incubation, the concentration of the filtrate was measured using ICP-MS ($n = 3$). NC: Negative control (without bacterial cells). *: Significant difference against negative control (NC) ($p < 0.01$).

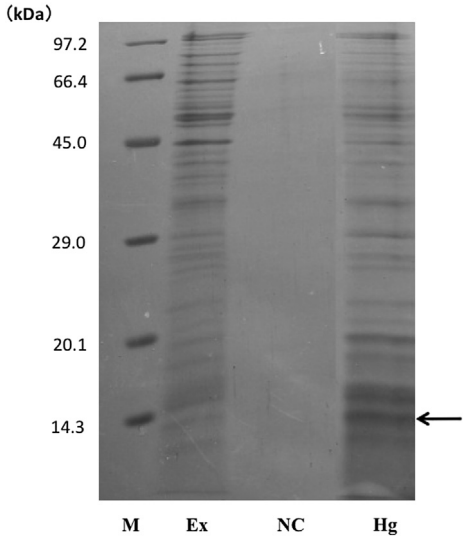



Fig. 3. SDS-PAGE of Hg (II) binding proteins after chelating on a column using *W. viridescens* MYU 205. The lyophilized cell surface proteins were applied to a chelating column containing Hg (II), and the Hg (II) binding proteins were eluted with elution buffer. Protein bands were visualized using CBB. M: molecular weight marker, Ex: the extract before chelating column, NC: the eluted proteins when using the column containing no Hg (II) (Negative control), Hg: the eluted proteins when using the Hg (II) column. The arrows indicate the clearest thick band at ~14 kDa.

## 4. Discussion

There is concern about the damage caused by heavy metals, where Cd (II), Pb (II), As (III), and Hg (II) are presently specified by the MAFF of Japan as required harm factors for risk management. Therefore, we examined the biosorption abilities of LAB for heavy metals as a potential means of detoxification.

All 103 strains showed Cd (II) biosorption, although the biosorption ability of each was different. This suggests all bacteria have potential for biosorption of heavy metals though there is a difference in their abilities. Cd (II) biosorption tended to be high at neutral pH (Fig. 1A). This suggests (i) the vital activity of LAB becomes weak in low pH and Cd (II) absorption is decreased and/or (ii) the cell surface proteins are positively charged below the isoelectric point and have difficulty adsorbing Cd (II) at low pH. Because the heat-treatment did not influence the amount of Cd (II) biosorption in MYU 205, the cell surface proteins may be related to Cd (II) biosorption. The data shown in Fig. 3 supports this. Kim et al. (2005) reported recombinant *Escherichia coli* JM109 harboring a manganese transport gene (*mntA*) and a metal-sequestering protein (metallothionein) gene that showed the most accumulation of Cd (II) at pH 7.0. Volesky et al. (1993) reported Cd (II) biosorption by *Saccharomyces cerevisiae* was inhibited by protons under acidic conditions. Conversely, an exception exists in some strains. MYU 10 and MYU 224 strains were the highest at pH 5.0 and lowest at pH 7.0. The heat-treatment test suggests more Cd (II) may be absorbed onto the cells at pH 5 than at pH 7 at room temperature (non-treatment). With heat-treatment, the bacterial cells may be disrupted using heating so that intracellular proteins may bind the Cd (II). Feng et al. (2012) reported the exopolysaccharide (EPS) of *Lactobacillus plantarum* showed maximum adsorption of Pd (II) at pH 5.0. This suggests the EPS may be related to biosorption of Cd (II) in MYU 10 and MYU 224, and the EPS may be broken or modified by heat-treatment. However, EPS may not bind Cd because Schut et al. (2011) report one of the *Lactobacillus* strains boiled at 100 °C for 1 h (the same condition as in this study) showed maximum biosorption of copper (Cu) (II) at pH 5.0. Binding may be different for each strain and heavy metal.

The Cd (II) biosorption assay data in various concentrations of LAB strains show the Cd (II) concentration of the filtrate decreased dose-dependently. When bacterial counts were $1 \times 10^{10}$ cells/ml, even the lowest strain decreased Cd (II) to $0.255 \pm 0.002$ ppm (Fig. 1B). This suggests the possibility that many LAB commonly have Cd (II) biosorption abilities.

The Pb (II), As (III), and Hg (II) biosorption assay data show all selected strains decreased Hg (II) concentrations to under 0.15 ppm, where MYU 10 showed the highest biosorption at $0.009 \pm 0.001$ ppm corresponding to 99.1% reduction of Hg (II) contained in the one ppm Hg (II) solution (Fig. 1C). The affinity of metallothionein (MT) to the heavy metals is reported to be Hg (II) $\geqq$ Ag (I) $>$ Cu (I) $>$ Cd (II) $>$ Pb (II) $>$ Zn (II) (Kagi and Kojima, 1987; Ryvolova et al., 2011). This is similar to our data where the amount of biosorption of LAB was high in order of Hg (II) $>$ Cd (II) $>$ Pb (II) and As (III). Hg (II) influenced the growth of the bacteria although Cd (II), Pb (II), and As (III) did not (Fig. 2). This suggests the toxicity of Hg (II) is higher than other heavy metals because of the high affinity of Hg (II) to LAB. It is known that Hg (II) strongly binds to thiol groups of cysteine. In this study, part of the Hg (II) may be taken into the cell. The growth of LAB may be reduced or inhibited because intracellular thiol enzymes such as a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that is an essential house keeping enzyme, are inhibited by Hg (II).

We focused on the cell surface proteins to determine Hg (II) binding proteins. Many proteins were detected from the Hg (II) column, where a 14 kDa protein was identified as the major Hg (II) binding protein using the N-terminal amino acid sequencing. A hypothetical protein from *L. oris* PB013-T2-3 was the most similar candidate considering the bacterial species, molecular weight, and the location of sequences. This 14 kDa hypothetical protein has a CXXC motif (X is any amino acid) although the function is unknown. CXXC motif is known as a heavy metal binding motif binding to Cd (II), Co (II), Cu (II), and Zn (II) ions. CopA, CopZ and McsA in *S. aureus* have the heavy metal binding domain CXXC motifs and are capable of binding various types of heavy metals (Boonyodying et al., 2012; Sitthisak et al., 2007, 2012). This suggests the 14 kDa protein may bind to Hg (II) through the CXXC motif. However, it is necessary to prove whether the CXXC motif is in the 14 kDa protein using DNA cloning. Further, Roesijadi (1986) reported two low molecular weight Hg-binding proteins (HgBP) in marine mussels containing high levels of halfcystine (26%) and glycine (16%). MTs are well known as cysteine-rich heavy metal binding proteins

(Nordberg et al., 1972; Weser et al., 1973). MTs maintain intracellular ion homeostasis and contribute to the detoxification of heavy metal ions. Kim et al. (2011) reported heterologous expression of MT from *Colocasia esculenta* in *E. coli* greatly enhanced Cd (II) tolerance and accumulated Cd (II) content.

There are not many reports concerning heavy metal biosorption by LAB. Recently, Bhakta et al. (2012) reported seven and four LAB strains isolated from mud and sludge displayed relatively elevated Cd (II) and Pb (II) removal efficiencies from water. *Lactobacillus reuteri* Cd70-13 and Pb71-1 showed the highest Cd (II) (25%) and Pb (II) (59%) removal capacity from MRS culture medium. Chang et al. (2012) performed biological extraction of heavy metals from wood treated with chromated copper arsenate (CCA) using bacteria. The extraction rates of heavy metals using *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* were highest. Schut et al. (2011) investigated the biosorption of Cu (II) by eight wine-relevant *Lactobacillus* species to remove Cu (II) from must and wine. *Lactobacillus buchneri* DSM 20057 showed the highest biosorption at a maximum of 46.17 μg Cu (II) bound per mg cell in deionized water. Ibrahim et al. (2006) reported binding isotherms for Cd (II) and Pb (II) were characterized for *Lactobacillus rhamnosus* LC-705, *Propionibacterium freudenreichii* subsp. *shermanii* JS and a mix showed Cd (II) and Pb (II) sorption. This suggests LAB are potential biosorbents of heavy metals and have metal binding proteins or EPS on the cell surface.

In this study, many LAB showed biosorption abilities for heavy metals in a mass-screening. We believe oral ingestion of LAB showing high biosorption of heavy metals can prevent the absorption of heavy metals into the body, and can discharge them from the body efficiently. Thus, a detoxification using LAB such as strain MYU 205 may be possible.

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