World Academy of Science, Engineering and Technology
International Journal of Bioengineering and Life Sciences
Vol:4, No:11, 2010

Isolation and Probiotic Characterization of Arsenic-Resistant Lactic Acid Bacteria for Uptaking Arsenic

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***Abstract*—**The growing health hazardous impact of arsenic (As) contamination in environment is the impetus of the present investigation. Application of lactic acid bacteria (LAB) for the removal of toxic and heavy metals from water has been reported. This study was performed in order to isolate and characterize the As-resistant LAB from mud and sludge samples for using as efficient As uptaking probiotic. Isolation of As-resistant LAB colonies was performed by spread plate technique using bromocresol purple impregnated-MRS (BP-MRS) agar media provided with As @ 50 µg/ml. Isolated LAB were employed for probiotic characterization process, acid and bile tolerance, lactic acid production, antibacterial activity and antibiotic tolerance assays. After As-resistant and removal characterizations, the LAB were identified using 16S rDNA sequencing. A total of 103 isolates were identified as As-resistant strains of LAB. The survival of 6 strains (As99-1, As100-2, As101-3, As102-4, As105-7, and As112-9) was found after passing through the sequential probiotic characterizations. Resistant pattern pronounced hollow zones at As concentration >2000 µg/ml in As99-1, As100-2, and As101-3 LAB strains, whereas it was found at ~1000 µg/ml in rest 3 strains. Among 6 strains, the As uptake efficiency of As102-4 (0.006 µg/h/mg wet weight of cell) was higher (17 – 209%) compared to remaining LAB. 16S rDNA sequencing data of 3 (As99-1, As100-2, and As101-3) and 3 (As102-4, As105-7, and As112-9) LAB strains clearly showed 97 to 99% (340 bp) homology to *Pediococcus dextrinicus* and *Pediococcus acidilactici*, respectively. Though, there was no correlation between the metal resistant and removal efficiency of LAB examined but identified elevated As removing LAB would probably be a potential As uptaking probiotic agent. Since present experiment concerned with only As removal from pure water, As removal and removal mechanism in natural condition of intestinal milieu should be assessed in future studies.

***Keywords*—**Lactic acid bacteria, As-resistant, characterization, *Pediococcus* sp., As removal probiotic.

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I. Introduction

ARSENIC (As) is the most common toxic metal widely occurring in the environment which poses serious hazardous impacts not only from human health perspective but also from broader ecosystem viewpoint during last few decades. It is already identified as a common cause of acute heavy metal poisoning with severe health risks reported by a number of scientists [1], [2]. Drinking of As contaminated water is responsible for the development of hyperpigmentation, skin cancer, liver cancer, circulatory disorders, and other ailments [3], [4]. The United States Environmental Protection Agency (USEPA) has identified arsenic as a group A "known" carcinogen.

Generally, various geogenic and anthropogenic factors are responsible for As contamination in the environmental and in turn water and food are the primary sources of As contamination in any organism. Robertson stated that priority natural sources of As element in soils are arsenic bearing rocks and minerals [5]. Inspite of it, mining, burning of arsenic containing fossil fuels, various industrial activities, volcanic eruptions and weathering processes are recognized as major origins to introduce substantial amounts of arsenic into the environment. Geochemical reactions and industrial waste discharges or agricultural uses of pesticides are greatly responsible for arsenic contamination in the aquatic environment [6]. Moreover, application of metal contaminated wastewater in aquaculture leads to a silence metal poisoning in freshwater organism especially in fish [7]–[9] and mollusks by bioaccumulation.

To prevent various adverse impacts of As, the USEPA promulgated the new arsenic rule that lowered the maximum contaminant level (MCL) in drinking water to 10 µg/l (10 ppb) for both community and non-transient, non-community water systems [10]. Therefore, it is necessary to remove As from the contaminated environment to achieve the above MCL of As in water.

Several improved and innovative technologies including several bioremedial methods have been evolved to reclaim the As contaminated environment. Recently, application of favourable microorganisms as probiotic is a potentially emerging field to the scientists of aquaculture industry for the welfare of aquatic animals as well as conservation of aquatic

environment. *Escherichia coli*, *Bacillus subtilis,* and *Saccharomyces* sp. have been employed to remove heavy and toxic metals from aquatic environment [11], [12]. Generally, bacteria of lactobacilli and bifidobacteria are commonly isolated from the environmental samples and gut content for using as probiotic in respect to nutritional, growth, disease controlling [13], [14] and immunological [15]–[18] respects. Besides these, application of lactic acid bacteria (LAB) in removing the toxic metals from the water has been studied by Halttunen *et al*. [19]–[21]. Lactic acid bacteria have also been reported to remove mycotoxins [22] and cyanotoxins [23], [24] from food and water, respectively. A combination of two probiotic strains has also been reported to reduce the gastro-intestinal absorption of aflatoxin B1 in young Chinese men [25]. In this context, it is also apparent that heavy metal contamination induces the development of resistant ability of microbial community in the environment. Soil with heavy metals affects the structure (qualitative and quantitative) of microbial communities, resulting in decreased metabolic activity and diversity [26]. It has been reported many soil bacteria are tolerant to heavy metals and play important roles in mobilization of heavy metals [27], [28].

From the above understanding of probiotic applications as well as metal resistant properties of bacteria, it is obvious that no such study has been performed so far regarding the development of metal removing probiotic from metal resistant bacterial community of the environment. Therefore, the present study has been focused for isolation and probiotic characterization of As-resistant LAB to develop the As uptaking probiotic.

II. Materials and Methods

A. Collection and Processing of Sample

The present study used 53 mud and sludge samples collected from few costal aqua-farming area and effluents flowing canals in India (10 samples) and Viet Nam (40 samples), whereas only sludge samples were procured from a wastewater treatment plants in Japan (3 samples).

Equal parts of all samples of each place were blended properly to get a homogenous sample for each station. Thus, three samples were prepared, leveled (as S1, S2, and S3 for the samples of India, Japan, and Viet Nam, respectively) and preserved in refrigerator at -20°C as parent stock samples for the isolation of As-resistant LAB.

B. Isolation and Morphological Characterization of As-resistant LAB

Preserved samples were thawed and 1 g of each sample was suspended in 9 ml 0.85% physiological saline (PS) by vortex. One milliliter aliquot of each sample suspension was inoculated in 9 ml MRS (De Man, Rogosa, and Sharpe, Difco) broth media and incubated for 7 d at 37°C anaerobically using the Anaero-pack Rectangular jar with an Anaeropack-Anaero sachet (Mitsubishi Gas Chemical Company, Tokyo) statically to enrich the population of LAB. Aliquot of broth culture was serially diluted (10^{-1} – 10^{-8}) with PS, 100 µl aliquot of each broth was then inoculated over the 0.017% bromocresol purple (for isolating yellow colony) impregnated-MRS (BP-MRS) agar media plates supplemented with As @ 50 µg/ml and incubated at 37°C for 24 h anaerobically. The As stock solution (2000 mg/l) were prepared from As_2O_3 (Cica-Reagent, Kanto Chemical Co., Inc., Tokyo, Japan) and sterilized for using in different experiments.

Colonies (25 – 50) with distinct yellow zones were randomly picked from the plates of higher dilution by tooth pick to represent the metal resistant bacterial isolates and re-streaked two times in 50 µg/ml As containing MRS agar plates for purification. The pure culture of each isolates was applied for catalase reaction using one drop of 3% hydrogen peroxide solution on each isolates of the re-streaked plates. Immediate formation of bubbles indicated the presence of catalase in the bacterial cells. Only catalase negative isolates were selected and used for morphological study. All isolates were maintained in MRS broth containing 20% glycerol at -85°C for subsequent studies.

C. Probiotic Characterization

Screening of potential probiotic LAB was done by the following sequential probiotic characterization process:

1. Acid pH Tolerance

The acid tolerant LAB was selected following the modified method of Erkkila and Petaja [29]. Each LAB isolate was grown in MRS broth at 37°C for 24 h incubation. The cells were harvested and washed twice with PS by centrifugation at 13000 rpm for 5 min. The cell pallets were suspended (~10^7 CFU/ml) in sterile phosphate-buffered saline (PBS; NaCl, 9 g/l, $Na_2HPO_4.2H_2O$, 9 g/l, and KH_2PO_4, 1.5 g/l) adjusted with a pH 2.5 using 5 M HCl and incubated at 37°C. After 2 h, the bacterial suspension was used for platting in MRS agar media and tolerant LAB was assessed in terms of colony growth in plate after 48 h anaerobic incubation.

2. Bile Salt Tolerance

Bile tolerance test was performed only for the LAB, those were successful in acid tolerance test following the modified method of Arihara *et al*. [30]. Acid tolerant LAB strains were grown at 37°C for 24 h in MRS broth without bile salt and 1 ml aliquot of broth was employed in MRS agar with bile salt (Sigma-Aldrich) concentrations @ 1000, 2000, and 4000 mg/l. After 48 h anaerobic incubation at 37°C, the growth of bacteria was evaluated to select the bile tolerant LAB strains.

3. Antimicrobial Activity, Lactic Acid, and pH Measurement

The bile tolerant LAB was cultured in 4 ml MRS broth at 37°C for 24 h. The supernatant of each cultured LAB was separated by centrifugation at 13000 rpm for 5 min and sterilized by passage through 0.2 µm Millipore membrane (Millipore, USA). Antimicrobial activity was measured by agar disk-diffusion assay described by Balcazar *et al*. [31] with some modifications using the non-neutralized and

neutralized (pH 6.8) filter sterilized supernatant. The indicator strains (*E. coli* and *Salmonella* sp.) were subcultured in tryptic soy broth and 100 µl of culture (~10^7 CFU/ml) were flooded over the Mueller–Hinton agar (MHA; Difco Laboratories, Detroit, MI) plates and air dried for 30 min. The discs of 8 mm (Advantec, Tokyo Roshi Kaisha Ltd.) were then overlaid onto the bacterium seeded agar plates and impregnated with 50 µl sterilized supernatant of test LAB culture. The agar plates were incubated at 37°C for 24 h and diameter of the clear zone around each disk was measured.

Likewise antimicrobial activity, one part of the supernatant from 24 h MRS broth cultured of LAB was collected after centrifugation, filtered, and used for the determination of lactic acid production using HPLC and another part employed for measuring the pH of the broth changed by producing the lactic acid.

4. Antibiotic Resistant Profile

LAB seeded (~10^7 CFU/ml) MHA plates were prepared for antibiotic susceptibility assay using the disc-diffusion method as described previously. The discs (8 mm) were placed onto the bacterium seeded agar plate and impregnated with 50 µl solutions with four different concentrations of antibiotics (trimethoprim and streptomycin @ 50, 100, 300, and 500 µg/ml, chloramphenicol and oxytetracycline @ 10, 50, 100, and 300 µg/ml). Antibiotic resistant ability was assessed based on measuring the diameter (mm) of the clear zone around the disc after 24 h incubation at 37 °C.

D. Metal Resistant Pattern

Previously described disc-diffusion assay was followed to determine the minimum inhibitory concentrations (MICs) of identified LAB against As, Cd, and Pb but MRS agar was used to prepared the bacteria seeded plate instead of MHA. Different concentrations of As (50 – 2000 µg/ml), Cd (10 – 1000 µg/ml), and Pb (50 – 1000 µg/ml) were used for the impregnation of discs overlaid on LAB seeded MRS plates. MIC was assessed determining the lowest concentration of bacterial growth inhibition.

E. Metal Removal by Resistant Isolate

The metal removal ability of LAB was determined by measuring the metals (As, Cd, and Pb) uptake of the resting LAB cells following the method described by Pazirandeh *et al.* [32] with some modifications. Freshly cultured cells were harvested in 2 ml centrifuge tubes, centrifuged at high speed to pellet the cells and washed twice by sterilized MQ water. Cells of LAB resuspended (3 mg/ml [wet weight]) in sterilized As (@ 100 µg/l) Cd (@ 1000 µg/l), and Pb (@ 6000 µg/l) solutions and incubated at 37°C. Samples were collected at 2 h, centrifuged to pellet bacterial cells and metals content in water was determined using the ICP-AES (ICPS-1000IV; Shimadzu, Japan) for As and AAS (AA-6800; Shimadzu, Japan) for Cd and Pb.

F. Identification of LAB

Extraction of DNA was executed following the chloroform/isoamyl alcohol (24:1) method described by Ruiz-Barba *et al*. [33]. Fragments of bacterial 16S rDNA were amplified by PCR using the universal primers FProR (5′-AGAGTTTGATCCTGGCTCAG-3′) and R534 (3′-GGTCGTCGGCGCCATTA-5′) (Invitrogen) with the thermocycler PC818 (ASTEC programme temperature control system). The PCR reaction mixture (20 µl) consisting of 10 µl AmpliTaq Gold® 360 Master Mix with 0.5 µl 360 GC Enhancer (Applied Biosystems), 1 µl of each primer, 2.5 µl nuclease free water, and 5 µl template DNA. The thermocycle program was as follows: 95°C for 10 min; 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min; and a final extension step at 72°C for 7 min. The PCR products were analyzed by electrophoresis on a 1.2% agarose gel.

16S rDNA gene amplicons were purified by DNA gel extraction kit (Wizard® SV Gel and PCR Clean-Up System, Promega) according to the manufacturer's instructions. Purified DNA suspension employed for sequencing PCR using BigDye with the R534 primer. Nucleotide sequencing was performed with an automated DNA sequencer (Applied Biosystems, 3100-Avant Genetic Analyzer). Bacterial identification was done searching the homology in the Genbank DNA database using BLAST.

G. Statistical Analysis

All mean data of at least two independent experiments were considered for statistical analysis using SPSS 10. Correlation studies were significant at the 0.01 level.

III. Results and Discussion

A. Isolation and Morphological Characterization of As-resistant LAB

To isolate As-resistant LAB, clear yellow colonies were selected from the As containing BP-MRS agar plates as a preliminary screening. One hundred and twenty colonies with yellow colour were picked up from the plates of three sampling stations. Generally, impregnated BP in the MRS media turns the colonies colour into yellow due to acidic condition developed by lactic acid of LAB in anaerobic condition. Therefore, BP was used as indicator in the preliminary LAB screening process. However, only 103 resistant LAB (S1 - 50, S2 - 28, and S3 - 25) were identified as catalase negative strains (Table I). Phase contrast microscopic observation of 103 LAB revealed the cocci shaped morphological characteristics.

B. Probiotic Characterization

1. Acid pH and Bile Salt Tolerance

According to general phenomena of application and establishment, probiotic bacteria challenge to successive high acidic and bile salty environments in stomach and intestine, respectively. High acidity in the stomach and the high concentration of bile components in the proximal intestine of the host influence probiotic strain selection [34]. Due to this fact, higher acid and bile salt tolerant strains of LAB would be

TABLE I
ACID AND BILE SALT TOLERANT LAB ISOLATES IN THREE SAMPLING STATIONS

Types of sample	As-resistant isolates				
	Catalase negative isolates	Acid pH tolerant isolates	Bile tolerant isolates		
			1000 mg/l	2000 mg/l	4000 mg/l
S1	50	22	19	13	5
S2	28	15	5	5	3
S3	25	18	12	12	2
Total	103	55	36	30	10

a potential probiotic. Accordingly, all isolated catalase negative LAB strains were employed for testing their abilities to grow at pH 2.5 for 2 h to select the acid tolerant strains, 55 isolates were selected as acid tolerant strains from 103 As-resistant isolates (Table I). Pennacchia *et al*. selected LAB in PBS buffer pH 2.5 for 3 hour at 37°C [35]. Succi *et al*. reported the survival of *Lactobacillus rhamnosus* strain at pH 3.0 after 2 h period [36]. Prasad *et al*. identified four acid tolerant strains from 200 LAB isolates at pH 3 for 3 h culture [37]. The results of the present study are in agreement with the above results obtained by various probiotic selections using different acidic pH.

Strompfova´ and Laukova´ proposed the resistance against bile salt is the second important criterion for the colonization and metabolic activity of probiotic bacteria in the small intestine of the host [38]. After the passage through acid tolerance test, all As-resistant acid tolerant strains were employed for bile salt survival study. Out of 55 acid tolerant LAB, 36, 30, and 10 strains were successfully passed in the bile salt tolerance test at the concentration level 1000, 2000, and 4000 mg/l, respectively (Table I). Ten LAB with highest bile salt (4000 mg/l) tolerant strains were selected to carry out next step of probiotic characterization of the present study. In support of bile salt tolerance test, Pennacchia *et al*. [35] and Erkkila and Petaja [29] were able to grow the Lactobacillus strains in MRS agar supplemented with 3000 mg/l bile salt. The mean bile salt concentration of human GI-tract is about 3000 mg/l, which is considered as critical and high enough to screen for resistant strains [39], [40].

2. Antimicrobial Activity, Lactic Acid, and pH Measurement

Ten bile salt tolerant LAB were used to study the antimicrobial activity using non-neutralized and neutralized supernatant by agar diffusion method. Out of 10, the non-neutralized supernatant of 6 LAB strains (As99-1, As100-2, As101-3, As102-4, As105-7, and As112-9) showed the growth inhibition activity in *E. coli* and *Salmonella* sp, whereas no clear zones were pronounced in neutralized supernatant of 10 LAB. The diameter of growth inhibition zones varied from 0 to 11 mm and 0 to 10.5 in *E. coli* and *Salmonella* sp., respectively in all tested LAB. No inhibition effects were found by As103-5, As104-6, As106-8, and As113-10 strains against both *E. coli* and *Salmonella* sp. (Table II).

All 10 bile salt tolerant LAB strains were employed for determining the amount of lactic acid production and pH

TABLE II
ANTIMICROBIAL ACTIVITY OF ISOLATED LAB STRAINS PASSED BY ACID AND BILE SALT TOLERANCE TEST

Strains	Activity (mm)	
	E. coli	*Salmonella* sp.
As99-1	8.6	8.6
As100-2	8.6	8.6
As101-3	8.6	8.6
As102-4	11	9.8
As103-5	0	0
As104-6	0	0
As105-7	11	10.5
As106-8	0	0
As112-9	10	10
As113-10	0	0

developed in MRS broth culture within 24 h growth period. The concentration of lactic acid and pH of the MRS broth widely ranged from 246 to 478 mM and 4.00 to 5.21 in As-resistant LAB tested, respectively (Fig. 1). Rengpipat *et al*. reported that LAB *Weissella confusa* produced 730 mM lactic acid in 24 h culture period [41].

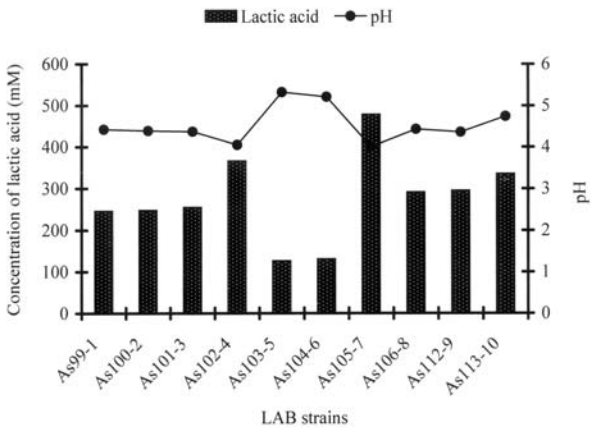

Fig. 1 Relationship between lactic acid concentration and pH of 24 h LAB cultured MRS broth media

Correlation studies clearly revealed a significant negative relationship (r = -0.843) between the lactic acid concentration

and pH of the cultured broth that signifying the pH of MRS broth is directly affected by lactic acid concentration. Levels and types of organic acids produced during the fermentation process depended on LAB species or strains, culture compositions, and growth conditions [42].

Excepting few strains, no antimicrobial activity pronouncing LAB showed higher pH (4.4 – 5.32) in the MRS broth compared to that of the antimicrobial activity exerting LAB (pH 4 – 4.4) (Fig. 1). On the other hand, pH level and antimicrobial activity of the respective LAB also exhibited a significant negative correlation (*E. coli* r = -0.815, *Salmonella* sp. r = -0.799) which strongly implied that antimicrobial activity of isolated LAB strains was supposed to be the function of lower pH level of supernatant developed by lactic acid but not for bacteriocin. Similar results were also proposed by Hwanhlem *et al*. [43]. It has been proposed that pH lower than 4.4 could inhibit the growth of *E. coli* [44] and *Salmonella* sp. [45]. From this proposition it can be mentioned the bacteriocin is not produced by the LAB strains isolated in the present investigation. The antibacterial activity of LAB may often be due to the production of organic acids, with a consequent reduction in pH, or to the production of hydrogen peroxide [46].

3. Antibiotic Resistant Profile

TABLE III
ANTIBIOTIC RESISTANT PROFILE OF 6 ANTIMICROBIAL ACTIVITY EXERTING LAB STRAINS

Strains	Trimethoprim		Chloramphenicol		Streptomycin		Oxytetracycline	
	Activity (mm)	Conc.	Activity (mm)	Conc.	Activity (mm)	Conc.	Activity (mm)	Conc.
As99-1	10	500	15	50	0	>500	12	10
As100-2	0	>500	14	50	0	>500	18	10
As101-3	12.5	500	15	50	12	500	10	50
As102-4	9	300	13	50	10	500	16	10
As105-7	19	300	20	50	12	500	10	100
As112-9	12	300	13.5	50	10.5	500	11	300

Antibiotic resistant profile of 6 antimicrobial activity showing LAB strains pronounced an antibiotic dependant response. Table III revealed the antibiotic activity (mm) and growth inhibition zones showing concentrations of the antibiotics (trimethoprim, streptomycin, chloramphenicol, and oxytetracycline) in LAB strains. The clear growth inhibition zones of 4 tested antibiotics varied from 0 to 20 mm against 6 LAB. All LAB strains showed clear zones at ≥300 µg/ml in trimethoprim and streptomycin, whereas it was found at ≤50 µg/ml in chloramphenicol and oxytetracycline excepting 2 LAB strains (As105-7 and As112-9) in oxytetracycline. The results clearly apprehended all LAB strains were highly resistant to trimethoprim and streptomycin compared to that of the chloramphenicol and oxytetracycline. This antibiotic profile study clearly demonstrated not only metal resistant ability but also a broad spectrum of antibiotic resistance proficiency was acquired by the isolated LAB strains.

C. Metal Resistant Pattern

Six LAB pronounced a wide variations with significantly higher magnitude of metal resistant pattern. The MIC values of LAB for As and other heavy metals, Cd and Pb were shown in the Table IV. As it can be seen in the Table IV, the MICs of 6 LAB strains were greater in As (1000 – 2000 µg/ml) and Pb (>1000 µg/ml) than that of the Cd (50 – 250 µg/ml). In this context, it may be inferred that identified LAB supposed to be high resistant against As and other heavy metals also.

TABLE IV
MINIMUM INHIBITORY CONCENTRATION (MIC) PATTERN OF ISOLATED 6 LAB STRAINS AGAINST As, Cd, AND Pb

Strain	MIC (µg/ml)		
	As	Cd	Pb
As99-1	2000	50	>1000
As100-2	2000	110	>1000
As101-3	2000	110	>1000
As102-4	1000	50	>1000
As105-7	1000	50	>1000
As112-9	1000	250	>1000

Results of metal resistant ability also implied that tested LAB can easily survive with high As and Pb containing environment, whereas their upper survival limit for Cd contaminated environment would probably be lower than As and Pb. Though Cd-resistant *Bacillus cereus* and *Enterobacter cloacae* exhibited 1200 and 2000 mg/l MIC values, respectively but high resistant ability was also found against several other heavy metals compared to control bacterial species, *B. cereus* and *E. coli* showed 50 mg/l MIC value for Cd [47]. The ability of microorganisms to resist antibiotics and tolerate metals seems to be the result of exposure to metal-contaminated environments that cause coincidental selection of resistance factors for heavy metals and antibiotics [48], [49].

D. Metal Removal by Resistant Isolate

Since, most of the LAB strains were with higher metal resistant ability; therefore, all 6 LAB strains were employed

for As, Pb, and Cd removal study to determine the metal removal efficiency. Total removal of As, Cd, and Pb were varied from 25.47 to 41 µg/l, 692.53 to 804.73 µg/l, and 2598 to 4609 µg/l, respectively (Fig. 2). The Pb removal efficiency (0.43 – 0.76 µg/h/mg wet weight of cell) was remarkably higher compare to that of Cd (0.11 – 0.13 µg/h/mg wet weight of cell) and As (0.002 – 0.006 µg/h/mg wet weight of cell) in 6 tested LAB (Fig. 2). These results signifies that identified LAB can be used not only for the As removal but also for uptaking the Cd and Pb. Among 6 LAB the As uptake efficiency of As102-4 strain (0.006 µg/h/mg wet weight of cell) was higher (17 – 209%) over the remaining LAB, whereas As105-7 (0.13 µg/h/mg wet weight of cell) and As112-9 (0.76 µg/h/mg wet weight of LAB cell) pronounced 8 to 18% and 7 to 77% elevated Cd and Pb removal efficiency than the remaining LAB, respectively.

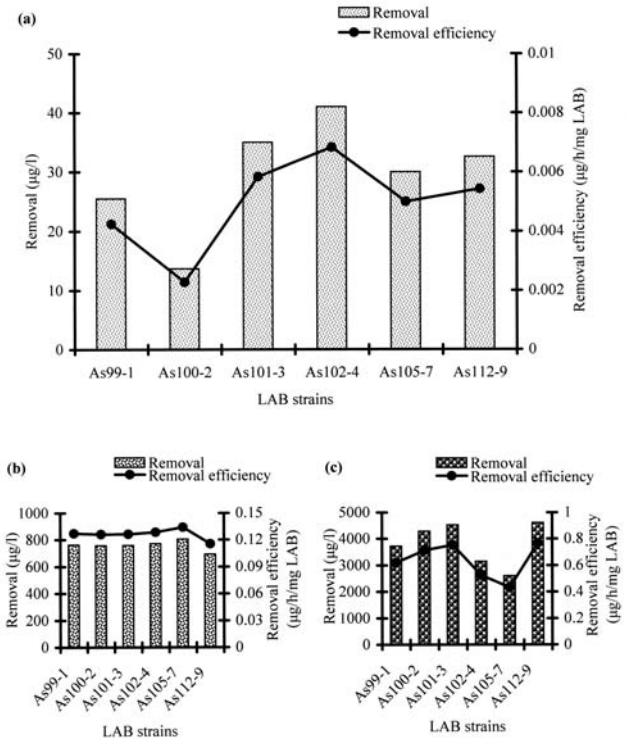

Fig. 2 Metal removal and removal efficiency characteristics of six isolated LAB strains: (a) As, (b) Cd, and (c) Pb

Irrespective of 3 tested metal species, As102-4, As105-7, and As112-9 LAB strains exerted highest As, Cd, and Pb removal efficiencies compared to rest of the LAB, respectively. Furthermore, in consideration of 3 metal species, each LAB showed the following order of variations in their metal removal efficiencies: As<Cd<Pb. In essence, it implied that each LAB has the highest Pb removal capacity followed by Cd and As. The capability of specific LAB to remove cadmium and lead from water has been reported [19], [20].

E. Identification of LAB

The PCR amplification of 16S rDNA of above As-resistant 6 LAB resulted in the synthesis of characteristic single band of about 500 bp using the primers FProR and R534. The sequencing data (340 bp) of purified 16S rDNA amplicons of all isolates were employed for bacterial identification. 16S rDNA sequencing data of 3 (As99-1, As100-2, and As101-3) and 3 (As102-4, As105-7, and As112-9) LAB strains clearly showed 97 to 99% homology to *Pediococcus dextrinicus* and *Pediococcus acidilactici*, respectively. Enterococcus such as *E. faecium* and Pediococcus such as *P. acidilactici* are mainly bacterial strains of gram-positive bacteria which were used in animal feed in the European Union (EU) [50] and as starters in the industrial fermentation of meat and vegetables.

IV. Conclusions and Suggestions

Summarily, it can be concluded that (1) Out of one hundred and three As-resistant and catalase negative LAB isolates, 3 (As99-1, As100-2, and As101-3) and 3 (As102-4, As105-7, and As112-9) LAB strains were selected based on the probiotic characterization and identified as *Pediococcus dextrinicus* and *Pediococcus acidilactici*, respectively, (2) Identified above 6 LAB showed a broad spectrum of As-resistant as well as good removal efficiency which indicating that identified LAB could be used as potential As removing probiotic agent within the animal system. Besides that, due to having elevated Cd and Pb removing capacities, selected LAB can also be utilize for removing the Cd and Pb, and (3) According to metal removal efficiency, particularly, As102-4, As105-7, and As112-9 LAB strains might be an effective As, Cd, and Pb removal probiotics which functions would be associated with the potential As, Cd, and Pb withdrawal mechanism from ambient environment, respectively.

Since, present experiments concerned with metal removal only from pure water, isolated LAB should be further studied in challenge experiments in natural condition of intestinal milieu for ascertaining the exact removal mechanism. Though, the present study is entirely in preliminary stage for developing such types of LAB, but a further great affords is needed to investigate more potential metal uptaking LAB strains considering the more metal polluting samples where chance factor would be more to get such relatively better LAB for developing efficient probiotic agent.

Acknowledgment

Authors are grateful to Govt. of Japan for sponsoring the Grant-in-Aid for Scientific Research fund to carry out the present study. Dr. Bhakta is also especially grateful to JSPS for providing the fellowship under the "FY2009 JSPS postdoctoral fellowship for foreign researcher".

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