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Characterization and Identification of Organic Selenium-enriched Bacteria Isolated from Rumen Fluid and Hot Spring Water

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In the present study, the isolation of selenium (Se)-enriched bacteria from rumen fluid and hot spring water was carried out. Rumen fluid samples were taken from cannulated goats fed a basal diet and the water samples were collected from Selayang hot spring, Selangor- Malaysia. A total number of 140 Se-tolerant isolates were obtained aerobically using an Se-enriched medium and spread plate technique. All the isolates were initially screened for the ability to transform the Se-containing medium to a red-orange culture using a spectrophotometer. Twenty isolates of dark red-orange medium were selected for a screening of the highest Se-containing protein accumulating strains using the dialysis technique and icp.ms to measure the Se content. Four isolates, identified as *Enterobacter cloacae* (ADS1, ADS7, and ADS11), and *Klebsiella pneumoniae* (ADS2) from rumen fluid origin, as well as, one isolate from hot spring water (*Stenotrophomonas maltophilia* (ADS18)), were associated with the highest biomass organic Se-containing protein when grown in a medium enriched with 10 μg/ml sodium selenite. In addition, around 50 μg/100 μg of the absorbed inorganic Se was accumulated as an organic form. Organic Se-containing protein in all the selected strains showed antioxidant properties in the range of 0.306 to 0.353 Trolox equivalent antioxidant capacity (TEAC) mg/ml. Therefore, these strains may offer a potential source of organic Se due to their Se-tolerant nature and higher biomass organic to inorganic Se ratio.

Keywords: **Accumulation, antioxidant, bacteria, isolation, organic selenium**

Introduction

Selenium (Se) is a micronutrient of vital environmental importance, it is essential for animals and humans, with a relatively narrow gap between toxic and essential values [1, 2]. Both oxyanions of Se, selenite ($SeO3^{2-}$) and selenate ($SeO4^{2-}$), are water soluble and acutely toxic, especially in high concentrations [3]. Selenite can damage the cellular antioxidant system, affect cellular respiration, and block DNA repair [4, 5]. However, selenium in its elemental form is insoluble with less toxicity and less availability [6]. The preferred form of Se is its organic form (selenoproteins), which is common in plants like garlic, onion, Brazil nuts, and Se-enriched yeast. This form is considered to be an efficient Se source with nutritional bioavailability, which can be absorbed and accumulated in animals and humans more easily than inorganic selenium [7]. Recently, different studies have suggested that finding a proper source of selenium supplementation is important, especially in Se-deficient regions; therefore, organic selenium might be a potential alternative source of Se.

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Organic Se can be produced biologically through selenate or selenite microbial reduction. Selenium resistance microorganisms can challenge selenite and selenate when grown in an Se-enriched medium; this resistance action is achieved through two different processes: reduction to red elemental Se form [8], or metabolic conversion to organic Se, such as selenocysteine and selenomethionine [9]. Bacterial selenite reduction results in a red-orange culture in liquid media due to the accumulation of intracellular deposits of red elemental Se [10]. Recently, a number of microorganisms, such as *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterococcus*, have been reported to take up and accumulate Se in their cells and can be used as Se-enriched probiotics [11–13]. The bacterial strain, *Lactobacillus retire* Lb2 BMDSM 16143, can uptake inorganic Se from the medium and metabolize it into an organic Se form and incorporate it into proteins as SeCys; however, this is associated with a bacterial biomass reduction [14]. The same result has been found for *L. bulgaricus*; the biomass is ($p < 0.05$) affected when the selenite concentration is greater than 0.46 mM [15]. Therefore, the poor selenite tolerance of lactic acids bacteria limits the application of Se-enriched *Lactobacillus* spp. in the food industry. In contrast, Gram-negative bacteria isolated from soil and metalloid water shows ($p < 0.05$) selenite resistance associated with less Se effect on their biomass [16]. However, although most of these Gram-negative strains are resistant to very high concentrations of the toxic Se and reduce it to a less toxic elemental Se, this process may be associated with organic Se accumulation as an intermediate step. Therefore, organic Se produced by un-probiotic bacteria can be extracted and used to deliver dietary levels of Se to livestock through feed supplementation.

Evidence has been accumulated that most of the microbial selenite reduction was in aerobic conditions however, some studies reported the ability of anaerobic conditions in selenite reduction [17]. Previous studies clearly demonstrated that natural ecosystem such as hot spring water contained a variety of microorganisms which they useful in metals bioremediation process [18]. As well as, rumen microorganisms which contain facultative anaerobic bacteria were capable of reduction inorganic Se and incorporating it into the microbial protein [19]. Therefore, once no more bacterial strains have been characterized and obtained in laboratory culture as an organic Se source, it is interesting to identify some strains as organic Se-enriched bacteria since it is able to absorb medium selenite and accumulate it as selenoproteins. Thus, the objective of this study is to isolate, enumerate, and to characterize several bacterial strains from rumen fluid and hot spring water that resist and reduce medium selenite and accumulate it as high biomass organic selenium.

Materials and Methods

Chemicals

All the chemicals and microbiological media used in this study were of analytical grade. Nutrient agar and nutrient broth were purchased from Merck (Darmstadt, Germany), sodium selenite, Na_2SeO_3, ≥99%, were purchased from Sigma-Aldrich.

Sample collection

Rumen fluid samples were taken from cannulated goats fed a basal diet (Field 2, Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia), and the water samples were collected from Selayang hot spring located in the Gombak, Selangor, Malaysia (N.03°15.542' and E.101°38.766'). Both samples sources were collected in triplicates at different points from the sampling sites and were transported to the laboratory, in sterile capped bottles in a proper temperature and directly diluted serially for inoculation.

Culture media and bacterial growth

The selective medium of selenium-tolerant bacteria was prepared using nutrient agar media enriched with 10, 20, 30, 40, 50, and 100 μg/ml of Sodium selenite, as described by Shahverdi *et al.* [20]. A sodium selenite stock solution (2.19 g/l corresponding to 1 g/l of Se) was prepared and sterilized by filtration (single use syringe filter, 0.20 mm, Sartorius Stedim Biotech). The collected samples were serially diluted in sterile deionized water and spread onto the nutrient agar plates. Inoculated plates of rumen fluid and hot spring water samples were incubated aerobically for 48 h at 39℃ and 30℃, respectively. Each plate holding between 30 and 300 colonies was selected to be counted as colony-forming units (CFU) per ml of sample.

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Isolation of selenium-enriched bacteria. A total of 140 red single colonies of different morphological appearance were selected and re-streaked on new nutrient agar media supplemented with 10 μg/ml Na_2SeO_3 to obtain a pure bacterial culture of the isolates. The pure agar cultures were sub-cultured by the transfer of a single colony to a nutrient broth medium enriched with the same Na_2SeO_3 concentration. Among these cultures, 20 isolates were selected for this study according to their higher capability to reduce selenite to red elemental Se, which was chosen, based on the red color intensity using a spectrophotometer (624 nm). The pure cultures of the isolates were kept at -20℃ using 30% glycerol [21].

Screening of organic selenium accumulated strains. The screening was carried out by determination of organic selenium according to the method described by [12]. Aliquots of fresh culture (24 h) containing 1×10^6 of isolated bacterial cells were used to ensure that all the cultures were inoculated by the same amount of cells [22]. The culture was centrifuged at 3,220 ×*g* for 15 min to harvest the bacterial pellets and then washed two times using deionized water to remove inorganic selenium which might adsorb to the bacterial cells. The selenium-enriched bacterial cells were lyophilized at -20ºC for further use.

To determine the organic selenium, all measurements of the samples were made in triplicate; one gram of bacterial cells from each strain was dialyzed using dialysis sacks of flat width 25 mm, 12,000 Da, (Sigma-Aldrich). The dialysis process was performed against deionized water, which was changed every 12 h for a total of 96 hours to separate inorganic Se from its organic form [12]. The content in the dialysis tube was lyophilized and then used to determine the Se concentration using a Perkin Elmer ICP.MS by the same protocol as for the determination of the total selenium concentration [23]. The accumulating rate of Se in the bacterial biomass was calculated according to the following equation:

$$\text{Accumulation rate (\%)} = \frac{(\text{organic Se content in bacteria} \times \text{biomass in 10 ml medium})}{\text{Se content in 10 ml medium}} \times 100$$

Determination of Selenium Concentration

Selenium was determined in the supernatant and pellet (cell) fractions according to Garbisu *et al.* [24]. The supernatant fraction was analyzed directly; the cell fraction was subjected to an acid digestion procedure (30% H_2O_2 in 16 M HNO_3, 100℃, overnight) followed by reduction of any selenite generated with 6 N HC1 (100℃, 1 h). The samples were analyzed by inductively coupled plasma mass spectrometry.

Characterization and identification of isolated bacteria

The best five organic Se producing bacteria ADS1, ADS2, ADS7, ADS11, and ADS18 were identified using phenotypic characterization and genetic characterization:

Phenotypic characterization. All isolates were subjected to Gram staining according to standard microbiological protocol. The colonies were distinguished through visual observation of the colony morphology. Individual colonies were characterized by a specific biochemical test using commercially available biochemical Kits (Api-20E) API® bioMérieuxs. API 20E data were compared to those in the bioMérieux's database (bioMérieux's 1990). All tests were performed in duplicate, and negative controls were obtained using a fresh medium. Their characteristics are summarized in Table 4.

Genetic characterization. Genomic DNA was extracted using PureLink® Genomic DNA Kits (Invitrogen). The DNA of each bacterial isolate was PCR amplified directly with primer pair 27F/1492R (27F: 5'-AGA GTT TGA TCC TGG CTC AG-3'; 1492R: 5'-TAC CTT GTT ACG ACT T-3'). The reaction product was analyzed using agarose gel electrophoresis. The PCR products were sent to a private laboratory (First Base, Malaysia) for purification and sequencing. The sequences obtained were analyzed using the National Centre for Biotechnology International (NCBI) BLAST, which is available on the Internet at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The sequences in the FASTA form were aligned in this software. BLAST was used to search for a similar sequence in the GenBank and compared to the query sequence.

Antioxidant capacity using $ABTS^{\cdot+}$ method

The $ABTS^{\cdot+}$ radical cation decolorization assay was determined according to Chan *et al.* [25]. ABTS was produced by reacting 7 mM ABTS aqueous solution with

2.4 mM potassium persulfate in the dark for 12–16 h at room temperature. After the addition of 1 ml of diluted ABTS solution to 10 µl of test sample in ethanol, the spectrophotometric absorbance of the sample mixture was recorded at 734 nm after 10 min. The level of inhibition of the samples was calculated as:

ABTS Inhibition = (1 – AS/A0) (TEAC) mg/ml

where A0 is the absorbance of the blank and AS is the absorbance of the sample mixture. Trolox was used as a positive reference. TEAC is the Trolox equivalent antioxidant capacity.

Statistical analysis. The data were analyzed using the Statistical Analysis System (SAS 1998) package software for the analysis of variance (ANOVA), Duncan's test. All experiments were carried out in triplicate. The significance was established at $p < 0.05$. Statistical model used for all estimated parameters was;

$$Y_{ij} = \mu + T_j + \varepsilon_{ij}$$

where, µ was overall mean,

T_j was the effect of different examined strains, and

ε_{ij} was difference within examined strain means (error term).

Results

Isolation and characteristics of selenite-reducing bacteria

Selenium-enriched bacteria was isolated from rumen fluid and hot spring water using a spread plate procedure with nutrient agar medium containing 10 µg/ml sodium selenite. Out of more than 350 colonies in the control media, 140 isolates had the ability to tolerate sodium selenite in the medium. Therefore, sixty red colonies with different morphological shapes were selected and isolated as selenium-enriched bacteria from both samples (Fig. 1). As shown in the Table 1 isolated strains were variable according to their Gram staining and morphological shape. However, most of the isolated strains were Gram negative-rod shape.

Table 1. Origin of isolation and morphology of isolated selenium-enriched bacteria.

Origin of isolation and morphology				
	Rumen fluid		Hot spring water	
	Rod	Cocci	Rod	Cocci
Gram Positive	1	6	5	9
Gram Negative	20	3	14	2

Effect of sodium selenite on the viability of selenium bacteria

In order to isolate a broad range of selenium reducing bacterial strains, bacteria were isolated in an agar medium containing different amounts of sodium selenite ranging from 0 to 100 µg/ml to determine the optimum concentration that leads to a significant variation in bacterial viability. As shown in Fig. 2, although a concentration of 10 µg/ml sodium selenite was associated with the highest bacterial viability compared to the other sodium selenite concentrations, it reduced the viability of the bacteria compared to the control group by around 40 and 48 CFU/100 CFU in 1 ml of rumen fluid and hot spring water samples, respectively. Increasing the sodium sele-

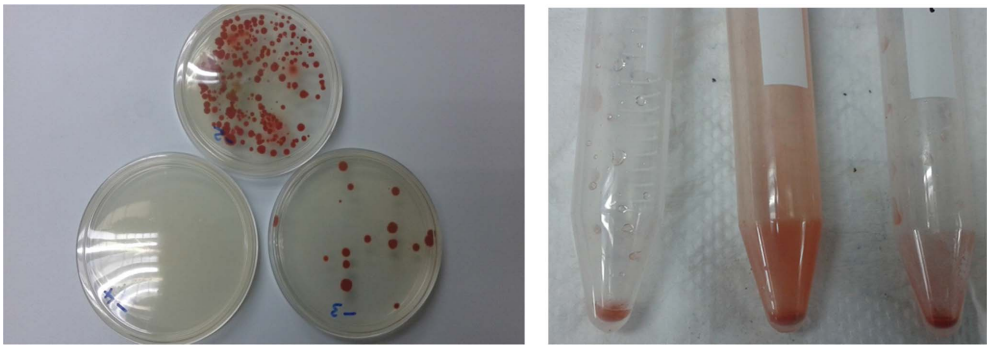

Fig. 1. Photograph of bacterial growth in the selenium-enriched medium. Se-enriched bacteria isolated as red colonies in solid media or red biomass in broth medium.

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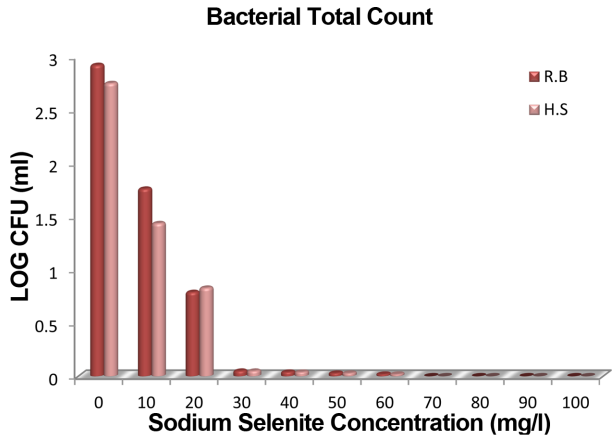

Fig. 2. Bacterial count of Se-enriched bacteria in rumen fluid and hot spring water under of different selenium concentrations. R.B-rumen fluid source, H.S-hot spring water source. CFU/ml showed the optimum level of Se that leads to a significant variation in the bacterial viability.

nite in the medium gradually reduced the bacterial CFU/ml until 50 mg/l selenite.

The capability of organic Se accumulation by Se-enriched isolates

The total organic Se was measured in twenty isolates associated with dark red color in the culture. The biomass of selected strains was extracted (proteomics extraction) and dialyzed against deionized water to separate inorganic Se. Then, the organic Se concentration in the bacterial biomass was measured by ICP.MS. Organic selenium was found to have accumulated in all the selected strains when sodium selenite was added to the culture medium (Fig. 3). The highest organic selenium was observed in the ADS2 strain, which accumulated around 8.36 μg/g Se in an organic form. Other strains that accumulated ($p < 0.05$) high organic selenium compared to the rest of the isolated strains were ADS1, ADS7, ADS11, and ADS18.

Distribution of selenium in Se-enriched isolated culture

The distribution of selenium in the culture of the isolated strains was determined after growth in the medium amended with 10 μg/ml of Se (IV) for 24 h (Table 2). The soluble Se, which indicated unabsorbed Se, was determined in the culture supernatant. The ADS18 strain showed the highest soluble Se with ($p < 0.05$) differences compared to the other strains. However, the same range of soluble Se and bacterial Se absorption rate was observed in the other isolates. The biomass Se content in the isolates was determined before dialysis as total Se, and after dialysis as an organic Se, and then the accumulation rate was calculated. The results indicated that ADS2 accumulated the highest biomass Se, and, subsequently, accumulated the largest organic Se among the isolated strains. Moreover,

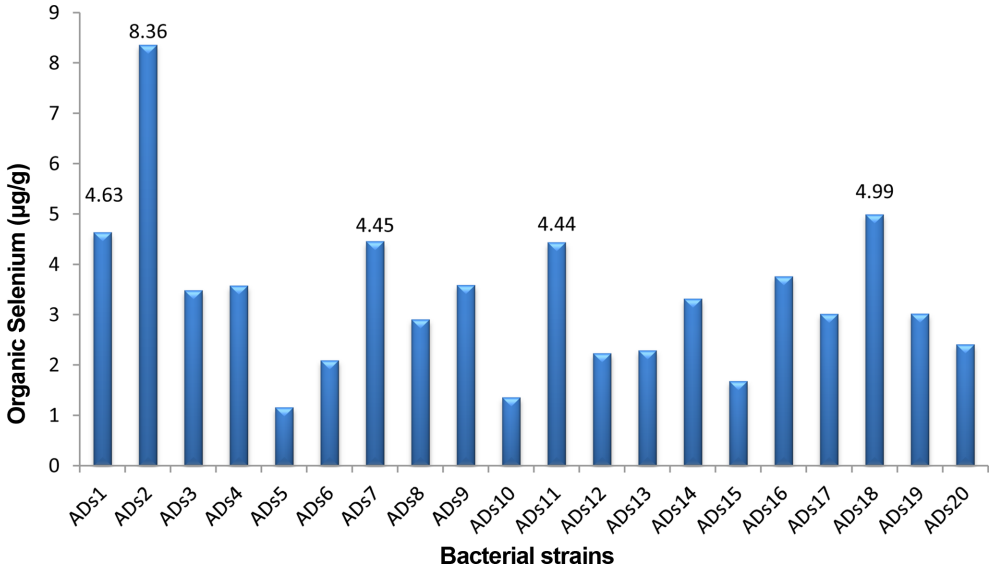

Fig. 3. Biomass organic selenium content (μg/g) of the isolated bacterial strains. Different isolated strains were associated with different levels of biomass Se-containing protein.

Table 2. Distribution of selenium in a liquid culture of isolated bacterial strains.

Parameters	Bacterial species						
	ADS1	ADS2	ADS7	ADS11	ADS18	P-value	SEM
Soluble Selenium (µg/ml)	39.67 ^{b}	40.33 ^{b}	39.98 ^{b}	44.26 ^{ab}	49.15 ^{a}	0.008	1.15
Total Selenium (µg/g)	42.22 ^{b}	64.99 ^{a}	48.11 ^{b}	43.92 ^{b}	45.79 ^{b}	<0.001	2.42
Organic Selenium (µg/g)	21.40 ^{b}	41.46 ^{a}	19.35 ^{b}	21.41 ^{b}	21.93 ^{b}	<0.001	2.24
Bacterial biomass (mg)	203 ^{a}	198 ^{a}	200 ^{a}	199 ^{a}	202 ^{a}	0.104	6.52
Accumulation Rate (%)	42.79 ^{b}	82.92 ^{a}	38.71 ^{b}	42.81 ^{b}	43.85 ^{b}	0.003	2.38

Values followed by different ^{a,b} letters in the same row are different by the Duncan test ($p < 0.05$). values are the means ($n = 3$). (ADS1, ADS7, and ADS11) *Enterobacter cloacae*, (ADS2) *Klebsiella pneumoniae* and (ADS18) *Stenotrophomonas maltophilia*.

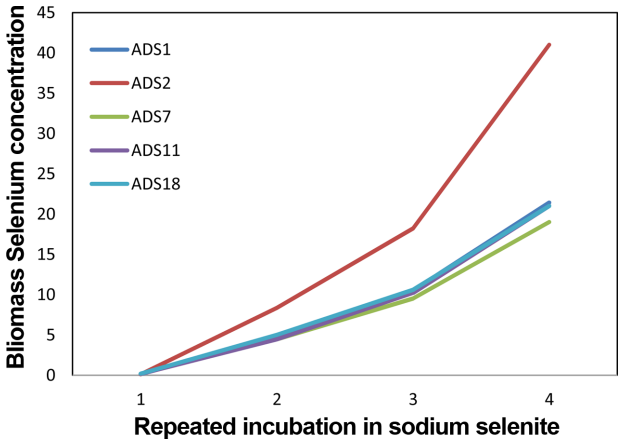

Fig. 4. Selenium accumulation in the bacterial biomass through repeated incubation in sodium selenite. (ADS1, ADS7, and ADS11) *Enterobacter cloacae*, (ADS2) *Klebsiella pneumoniae* and (ADS18) *Stenotrophomonas maltophilia*. Repeated incubation of the isolated bacterial strains in the Se-enriched medium increased their biomass Se concentration.

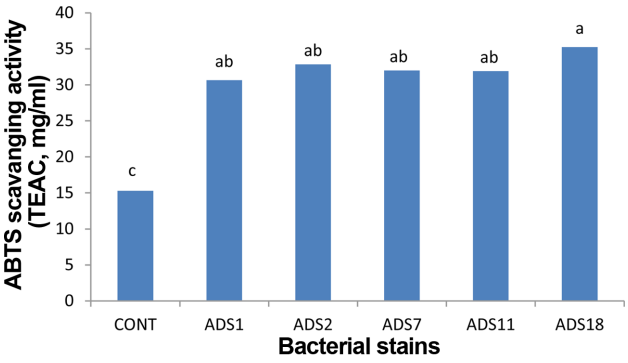

Fig. 5. ABTS radical scavenging activity of bacterial organic selenium extract. Values are means of three replicate determinations ($n = 3$). Different ^{a,b} letters are ($p < 0.05$) different.

accumulation of Se in the bacterial biomass showed a positive ($p < 0.05$) correlation with the subsequent incubation in sodium selenite (Fig. 4); this indicated the ability of the strain to preserve part of the absorbed Se in their cells.

Antioxidant activity of bacterial organic Se extract

The antioxidant activity of bacterial organic Se extract, which was evaluated using the $ABTS^{\bullet+}$ method, is presented in Fig. 5. The cellular extract of organic Se-enriched isolated strains exhibited an ability to scavenge the radical $ABTS^{\bullet+}$ with an inhibitory level of 0.306 to 0.352 TEAC (mg/ml). These results indicated that all the isolated strains could be considered to have bacterium antioxidant potential and may be useful to reduce the oxidative damage in food and feed. However, compared to the other isolates, ADS18 showed the highest antioxidant properties and ability to scavenge the free radicals.

Identification and characterization of organic Se-enriched isolates

Microscopic examination of isolates ADS1, ADS2, ADS7, ADS11, and ADS18 revealed that all were Gram-negative bacilli, motile and catalase positive (Table 3). The isolates grew luxuriantly in sealed tubes indicating that they were facultatively anaerobic. As shown in Table 4, all the isolates were identified by biochemical tests using API20E, and then confirmed by 16S rRNA gene sequencing. For genetic identification of the bacterial strains, DNA was isolated from all pure culture and quantified by spectrophotometer to ensure it was sufficient for the PCR reaction. The DNA was amplified using universal primers of bacteria, and the PCR product of required band size was obtained (Fig. 6). The

Table 3. Physiological characteristics of isolated selenium-enriched bacteria.

Bacterial strains	Gram stain test	Morphological shape	Growth in different temperature		Catalase Test	Oxidase test	Motility
			15 ℃	45 ℃			
ADS1	Negative	Short rod	+	+	+	-	+
ADS2	Negative	Rod	+	+	+	-	+
ADS7	Negative	Short Rod	+	+	+	-	+
ADS11	Negative	Short Rod	+	+	+	-	+
ADS18	Negative	Rod	+	+	+	+	+

(+) Indicate a positive test result, (-) Indicate a Negative test result. (ADS1, ADS7, and ADS11) *Enterobacter cloacae*, (ADS2) *Klebsiella pneumoniae* and (ADS18) *Stenotrophomonas maltophilia*.

Table 4. Biochemical characteristics of organic Se-enriched isolates.

TRAITS	ADS1	ADS2	ADS7	ADS11	ADS18
B-galactosidase production	+	+	+	+	-
Arginine dihydrolase	+	-	+	+	-
Lysine decarboxylase	-	+	-	-	+
Ornithine decarboxylase	+	-	+	+	-
Citrate utilization	+	+	+	+	+
Hydrogen sulfide	-	-	-	-	-
Urease	-	+	-	-	-
Tryptophan deaminase	-	-	-	-	-
Indole	-	-	-	-	-
Acetoin production	+	+	+	+	-
Gelatin liquefaction	-	-	-	-	+
Acid from glucose	+	+	+	+	-
Acid from mannitol	+	+	+	+	-
Acid from inositol	-	+	-	-	-
Acid from sorbitol	+	+	+	+	-
Acid from rhamnose	+	+	+	+	-
Acid from sucrose	+	+	+	+	-
Acid from melibiose	+	+	+	+	-
Acid from amygdalin	+	+	+	+	-
Acid from arabinose	+	+	+	+	-
Nitrate reduction	+	+	+	+	+
Identified Strain	*E. cloacae*	*K. pneumoniae*	*E. cloacae*	*E. cloacae*	*S. maltophilia*

(+) Indicate a positive test result, (-) Indicate a Negative test result. (ADS1, ADS7, and ADS11) *Enterobacter cloacae*, (ADS2) *Klebsiella pneumoniae* and (ADS18) *Stenotrophomonas maltophilia*.

bands were excised, and purified PCR products were sent for sequencing. GenBank Blast analysis of partial 16S rRNA gene sequence identified isolates ADS1, ADS7, and ADS11 as *Enterobacter cloacae* (97%, 96%, and 96% identity, respectively), ADS2 as *Klebsiella pneumoniae* (98%, identity) which they were rumen fluid origin, and ADS18 from hotspring water as *Stenotrophomonas maltophilia* (96%, identity).

Discussion

A variety of microbial genera capable of tolerating and transforming selenium oxyanions have been isolated from contaminated water and soil over the past few

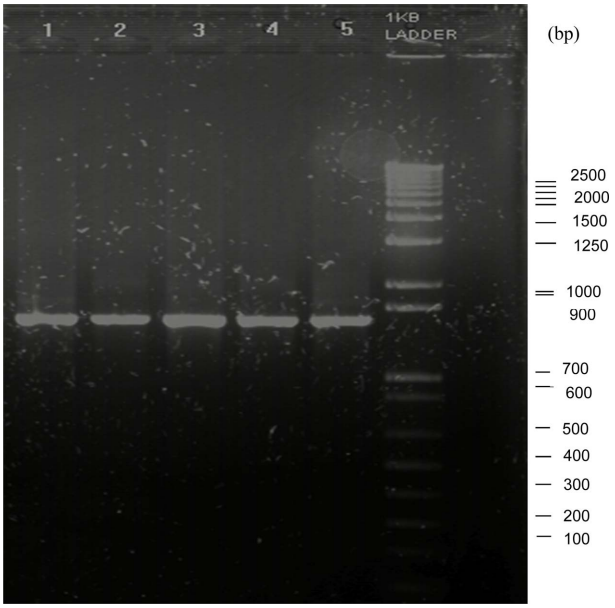

Fig. 6. Agarose gel electrophoresis of the partially amplified gene of ADS1, ADS2, ADS7, ADS11 and ADS18 PCR product in Lanes 1, 2, 3, 4, and 5 of the respectively.

years and studied for a better understanding of the mechanism enabling Se bioremediation purposes [26]. Selenium reducing microorganisms use various selenium conversion pathways, such as reducing toxic selenium oxyanions into non-toxic and insoluble Se^{o}, volatilization through hydrogen selenide (H_2Se) or dimethyl-selenide (Me_2Se) as well as conversion to methyl-selenocysteine [27]. In this study, Selenium-enriched bacteria were isolated aerobically from rumen fluid and hot spring water using different concentrations of sodium selenite in the medium. Selenium-enriched bacteria from both of samples appeared as red colonies with different morphological shapes; this agrees with the findings of Mainville *et al.* [28] and Huber *et al.* [18], who isolated Se-enriched bacteria from rumen fluid and hot spring water, respectively. Inorganic Se can be metabolized by rumen bacteria and incorporated into microbial protein as a component of the selenomethionine and selenocysteine amino acids. The inclusion of Se into protein occurs through a number of intermediate steps leading to the generation of hydrogen selenide (H_2Se), which serves as a precursor for the synthesis of essential selenoproteins [29]. Moreover, hot spring water bacteria use the same process of selenite reduction as the biological method for the remediation of toxic forms of selenium to an environmentally-friendly elemental Se, which is the end product of the reduction and is responsible for the red-orange color observed in the bacterial culture. Inclusions of inorganic Se (sodium selenite) in the bacterial medium reduced the bacterial CFU/ml and limited the bacterial viability. This agrees with the findings of Xia *et al.* [15] who found that low concentrations of sodium selenite (<4 μg/ml) in a medium of *Lactobacillus bulgaricus* strain did not affect the viability of the bacterial cells, while the administration of a concentration higher than 4 μg/ml in the culture medium inhibited the bacterial growth. The level of selenite resistance in our isolates was higher than the level observed in lactic acid bacteria but lower than some bacterial strains, such as *Azoarcus* spp. CIB [30]. *Pseudomonas* spp., and *Rhizobium leguminosarum bv.viceae* [5], which were able to grow in the presence of 8 mM to 200 mM selenite in the medium. Although the critical inhibitory concentration of selenite is specific for each microorganism, high selenite concentrations in a medium are toxic for most microbes, and there is a direct relation between the medium Se concentration and the viability of the bacteria. This explains our result of no bacterial cell viability above 50 mg/l of sodium selenite. Therefore, according to our experimental results, a sodium selenite concentration of 10 μg/ml was chosen to evaluate the growth curve of the strain and as a stimulus for the Se proteomic experiment.

Bacterial The bacterial screening of higher organic Se accumulation was done by measuring the biomass organic Se after dialysis against deionized water. Organic Se was found to be concentrated in all isolated strains when sodium selenite was added to the culture medium, but the concentration of organic Se varied among the isolates in the range of 1.2 to 8.3 μg/g. Other studies showed that organic Se could be produced biologically through some bacterial strains, such as *Bifidobacterium animalis* 01 accumulated 528, 641, 898 and 1017 μg/g dry weight organic Se, respectively, when it was grown in a medium containing 2.5, 5.0, 8.0 and 10.0 μg/ml Se. Most of the organic selenium in *Bifidobacterium animalis* 01 was found in the protein fraction, while the rest was distributed in the polysaccharide fraction, nucleic acid fraction, and may be other lipids and low molecular weight selenocompounds [12]. According to Calomme *et al.* [31], lactic acid bacteria can also concentrate selenium

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as selenocysteine as the only seleno-amino acid in the intracellular selenoproteins. Dialysis of the bacterial extract proved that at least 80% of the total selenium is associated with organic molecules. Accumulation of selenium inside the bacterial cells as organic or inorganic compounds can be due to two mechanisms: extracellular binding by the active groups of biopolymers present in the structure of the cell membrane conjunction and intracellular binding coupled with the ion transportation through the biological membrane into the cell [32]. Therefore, the ADS1, ADS2, ADS7, ADS11 and ADS18 isolated strains have the ability to transform inorganic Se to an organic form through binding with organic molecules and accumulating it in their cells, and, hence, can be considered as a potential source of organic Se.

In this study, isolated Se-enriched bacteria showed a high correlation between the biomass total Se and organic Se. The level of organic Se in the isolated strains ADS1, ADS2, ADS7, ADS11, and ADS18 was 54.8, 63.8, 40.4, 48.7, and 48.09 (μg/100 μg of total Se), respectively, indicating that, in all strains, around 50 μg/100 μg of selenium was incorporated into the bacterial cells as an organic fraction. This is supported by previous findings of selenium uptake by *Lactobacillus reuteri* (Lb2 BM-DSM), which indicated that about half of the absorbed Se was covalently incorporated into proteins [33]. However, Se-enriched *B. animalis* 01 showed that around 16.7–39.6 μg/100 μg of inorganic selenium in the medium was absorbed and transformed into organic selenium, which accounted for 77.4 μg/100 μg of the biomass total Se when the strain was grown in 10 μg/ml sodium selenite [12]. Different research indicated that selenium has the ability to enhance the antioxidant properties of proteins, and the present study showed that organic Se-containing protein in all isolated bacteria has antioxidant properties in the range of 0.306 to 0.353 TEAC (mg/ml). according to $ABTS^{\cdot+}$ scavenger activity. This may be due to the release of selenium related antioxidant enzymes from the bacterial cells showing antioxidant activity in the protein content when the bacterial wall is broken. Also, it may be due to other bioactive compounds like se-exopolysaccharide and an organic acid that possesses potent antioxidant activity and can be produced by some bacterial strains, such as *Enterobacter cloacae* Z0206 when grown in a Se-enriched medium [34]. Another study of the production of Se-enriched yogurt by lactic acid bacteria revealed that two proteins involved in antioxidant functions (thioredoxin and glutaredoxin) were detected in the selenoprotein extract [35]. Therefore, in this study, we conclude that isolated organic Se-enriched strains showed *in-vitro* antioxidant properties, however, the particular mechanism needs to be elucidated in further research.

Genomic sequencing of the selected strains of higher organic Se, identified ADS1, ADS7, and ADS11 as *Enterobacter cloaca*, ADS2 as *Klebsiella pneumonia*, and ADS18 as *Stenotrophomonas maltophilia*. According to Losi and Frankenberger [36]. *Enterobacter cloaca* is capable of removing the selenium (Se) oxyanions from the medium and using it as an alternate electron acceptor. Furthermore, cell suspensions of *Enterobacter cloacae* SLD1a-1 produced dimethyl-selenide (DMSe) from inorganic selenium [37]. In addition, elemental selenium nanoparticles were produced when the strain of *Klebsiella pneumonia* and *Stenotrophomonas maltophilia* SeITE02 were grown in a selenium chloride and sodium selenite medium, respectively [38]. The previous studies proved that all the identified strains are selenium-tolerant bacteria and can produce different Se species, however, no study examined the accumulation of organic Se in these strains and the capability of extracting it as a source of Se in animal feed. As a conclusion, selenium-enriched bacteria from rumen fluid and hot spring water can be isolated with high cells availability using 10 μg/ml sodium selenite. Association of isolates with a dark orange-red culture results in a biomass organic Se accumulation in a range of 1.2 to 8.3 μg/g. Five strains ADS1, ADS2, ADS7, ADS11, and ADS18 accumulate high biomass organic Se (around 50 μg/100 μg of an absorbed Se), when grown in a Se-enriched medium. Moreover, organic Se-containing protein in all the strains has antioxidant properties in the range of 0.306 to 0.353 TEAC (mg/ml). $ABTS^{\cdot+}$ scavenging activity. Therefore, the selected five strains, *Enterobacter cloacae* (ADS1, ADS7, and ADS11), *Klebsiella pneumoniae* (ADS2), and *Stenotrophomonas maltophilia* (ADS18), have an ability to transform inorganic Se to organic form through binding with organic molecules and accumulating it in their cells, and, hence, can be considered as a potential source of organic Se.

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Conflict of Interest

The authors declare that they have no competing interest.

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