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Enriched Selenium and Its Effects on Growth and Biochemical Composition in *Lactobacillus bulgaricus*

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Se-enriched *Lactobacillus bulgaricus* (*L. bulgaricus*) was generated by administration of sodium selenite (0, 1, 4, 8, 16, 32, and 64 mg/L, respectively) in MRS medium and enriched selenium manifestation in *L. bulgaricus* was investigated using transmission electron microscopy and energy-dispersive X-ray spectrometry and alterations of essential elements and amino acids in the organism were evaluated. We demonstrate that administration of sodium selenite in the dosage of 1−16 mg/L is suitable for selenium enrichment in *L. bulgaricus* and can enhance nutritive value in the organism by elevating the contents of essential elements including P, Mg, Mn, Zn, Ca, and total amino acids as well as reducing selenite to insoluble elemental selenium, an electron-dense and amorphous Se (0) granule, thereby depositing it both in the cytoplasm and in the extracellular space of *L. bulgaricus*. Thus, Se-enriched *Lactobacillus* can provide a potential dietary source of nontoxic selenium and functional regulator used for food and medical industry.

KEYWORDS: Selenium; *Lactobacillus bulgaricus*; grow; amino acids

INTRODUCTION

Selenium (Se) has received considerable attention as an essential micronutrient for animals and humans. It functions in the active site of a large number of selenium-dependent enzymes such as iodothrine 5′-deiodinase and glutathione peroxidase, which participate in the antioxidant protection of cells (*1*, *2*). The current Recommended Dietary Allowance (RDA) for both men and women is 55 μg (0.7μmol) of Se per day (*3*). Se deficiency in humans results in some endemic diseases such as Keshan and Kashin-Beck diseases (*4*). In addition, a low concentration of selenium in plasma has been identified as a risk factor for several diseases, including certain types of cancer, cardiovascular disease, osteoarthritis, and AIDS (*5*−*7*). Therefore, selenium supplement is now becoming popular for nutritional demand and chemoprevention. Since it is generally believed that organic Se compounds are better and safer than inorganic Se as a dietary supplement, a variety of Se-enriched biological products have been developed including yeast, wheat, fruits, and vegetables such as tomato, broccoli, onions, and garlic cultured in selenite-enriched soil or medium (*8*−*12*). The Se supplementation using microorganisms has received much attention in the past decade (*13*).

Lactic acid bacteria are GRAS (generally recognized as safe) microorganisms and have long been used in the food industry to produce fermented foods and beverages, including dairy products (yogurt and cheese), fermented vegetables (olives, pickles, and sauerkraut), and fermented meats (salami), etc. (*14*). Furthermore, many reports show the usefulness of lactic acid bacteria as probiotics for human and animals which have extensive physiological effects such as antimicrobial, immunomodulatory, anticarcinogenic, antidiarrheal, antiallergenic, and antioxidant activities (*15*). Calomme et al. found that various lactobacillus species could concentrate Se intracellular as selenocysteine in biomass and then provided a potential dietary source of organic selenium (*16*). Our previous observations reveal that concomitant Se-enriched *Lactobacillus* administration to mice and rats subjected to CCl_4-induced liver injury results in a reliable hepatoprotection against hepatic damage by elevating antioxidant enzyme activities and reducing lipid peroxidation reaction in the liver, as well as enhancing peripheral blood lymphocyte proliferation and red blood cell (RBC) immune function to keep normal and beneficial effects (*17*−*19*). However, the detailed information about enriched selenium characteristics in lactobacillus and its effects on *Lactobacillus* was not completely clear so far. Thus, the present study was aimed to investigate enriched selenium characteristics in *Lactobacillus bulgaricus* (*L. bulgaricus*) using transmission electron microscopy and energy-dispersive X-ray spectrometry and to evaluate alterations of essential elements and amino acids in the organism to further understand biological effect and significance in Se-enriched *Lactobacillus*.

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Table 1. Total Selenium Content (mg/(g of Dry Weight)) and Mean Biomass (g of Dry Weight/L) of *Lactobacillus bulgaricus* Grown under Different Selenite Concentrations[a]

	0 mg/L Na_2SeO_3	1 mg/L Na_2SeO_3	4 mg/L Na_2SeO_3	8 mg/L Na_2SeO_3	16 mg/L Na_2SeO_3	32 mg/L Na_2SeO_3	64 mg/L Na_2SeO_3
total Se	nd[b]	0.28 ± 0.05	1.15 ± 0.15	2.38 ± 0.33	5.37 ± 0.51	8.87 ± 0.78	12.45 ± 1.04
biomass	0.73 ± 0.03	0.82 ± 0.02[c]	0.77 ± 0.02[c]	0.74 ± 0.04	0.72 ± 0.03	0.66 ± 0.02	0.63 ± 0.01

[a] Data are presented as means ± SEM ($n = 3$). [b] Nondetectable. [c] $P < 0.05$ compared to Na_2SeO_3-untreated group.

MATERIALS AND METHODS

Chemicals. Sodium selenite (Na_2SeO_3) was from Shanghai Xingta Chemical Engineering Factory, Shanghai, China. 2,3-Diaminonaphthalene (DAN) was purchased from Fluka Co., USA. Other chemicals were purchased from local commercial sources and were of analytical grade quality.

The MRS medium contained the following (L): K_2HPO_4, 2 g; CH_3-COONa, 5 g; $(NH_4)_3$citrate, 1.45 g; $MgSO_4 \cdot 7H_2O$, 0.32 g; $MnSO_4 \cdot H_2O$, 0.19 g; Tween 80, 1 g; glucose, 20 g; yeast extract, 5 g; beef extract, 10 g; peptone, 10 g (pH 6.4).

Preparation of Se-Enriched *Lactobacillus*. *Lactobacillus bulgaricus* was obtained from the Institute of Microbiology, Chinese Academy of Sciences, Beijing, China, and was batch-cultured in 250 mL Erlenmeyer flasks containing MRS medium at 42 °C. After initial growth for 24 h, the cultures were exposed to Se at final concentrations of 0, 1, 4, 8, 16, 32, and 64 mg/L Na_2SeO_3 for an additional 24 h, respectively. At the end of cultivation, bacteria cells were harvested by centrifugation at 4500 rpm (Allegra 21R, Beckman Inc., USA) for 15 min and washed three times with deionized water. The washed pellets were then frozen, lyophilized, and weighed.

Assay for Total Selenium and Essential Elements in *Lactobacillus bulgaricus*. For the determination of P, K, Mg, Mn, Zn, Cu, Fe, and Ca concentrations, a 0.4 g lyophilized sample was mixed with 5 mL of nitric acid (HNO_3) and 0.5 mL of perchloric acid ($HClO_4$) and then digested on an electric hot plate (model EH-35A, Labtech Inc., Holliston, MA). The resulting solution was cooled to room temperature and adjusted to a final volume of 20 mL with deionized water. Elemental concentrations were detected by inductively coupled argon plasma atomic emission spectrometry (ICP-AES; Model Prodigy, Leeman Labs Inc., Hudson, NH). The accuracy of the method was evaluated by analyzing a standard reference material of bovine liver (GBW 080193). The values of P, K, Mg, Mn, Zn, Cu, Fe, and Ca obtained with the present method showed good agreement with the certified values as judged from the relative standard deviation.

Selenium contents were assayed using a modification diaminonaphthalene (DAN) fluorometric method as described by Olson et al. (*20*). In brief, lyophilized samples in 100 mL flasks were digested on an electric hot plate with 10 mL of nitric and perchloric acids (V_{HNO_3}: $V_{HClO_4} = 2{:}1$) followed by 5 mL of 15% hydrochloric acid used to reduce Se(VI) to Se(IV). The digested solution was allowed to cool to room temperature and adjusted pH to 1.5−2.0. Then 3 mL of 0.1% 2,3-diaminonaphthalene in 0.1 M HCl was added. After the contents were mixed, the flasks were incubated in boiling water for 5 min and then cooled to room temperature immediately. The selenium−2,3-diaminonaphthalene complex was extracted with 5 mL of cyclohexane by shaking the flasks vigorously for about 5 min. The fluorescence intensity of the organic phase was determined with a Shimazu RF-540 fluorescent spectrophotometer where $\lambda_{ex} = 376$ nm, $\lambda_{em} = 520$ nm. The detection limit for Se was 5 ng, and RSD was below 5%. The method has been verified with the national certified reference material (GBW 080193) with satisfactory results.

Measurement of Amino Acid Composition in *Lactobacillus bulgaricus*. Amino acids analysis was carried out on HP-1050 high-performance liquid chromatograph equipped with HP-1050 quadruple gradient pump, automatic pipet, HP-1046A flourodetector and LC-3D chromatographic workstation. Samples (~50 mg) were hydrolyzed under vacuum in 6 N HCl for 24 h at 110 °C in sealed tubes. After precolumn derivatization of the amino acids with *o*-phthalaldehyde and 9-fluorenylmethyl chloroformate (OPA-FMOC) in an online automatic pipet, the hydrolysate was separated on a Hypersil BDS C18 column (4.6 mm × 15 cm; Dalian Elite Analytical Instruments Co., China). Mobile phase A was 20 mmol/L sodium acetate buffer (pH = 7.2) containing 3 g/L tetrahydrofuran, and B was 100 mmol/L sodium acetate buffer (pH=7.2)−methanol−acetonitrile (20:45:35 by volume). The elution program was 100% A with 0% B at the start (0 min), and 42% A with 58% B at 25 min, and then 0% A with 100% B at the end (29.5 min) of the program. The 17 amino acids were separated satisfactorily in 29.5 min with $R > 1.5$ under above the conditions. Tryptophan was not analyzed. Sulfur amino acids were analyzed after oxidation, in the form of cysteic methionine sulfone and cysteic acid.

TEM and EDX Analysis of Enriched Se in *Lactobacillus bulgaricus*. The bacterial cells grown in MRS medium with or without 64 mg/L Na_2SeO_3 were collected by centrifugation and then washed three times in sterile water. Samples of 5 μL aliquots were directly added onto copper grids which were covered with 0.3% polyvinyl formal, and the copper grid was dehydrated at room temperature. Additionally, bacterial cells grown in MRS medium containing 64 mg/L Na_2SeO_3 were embedded in Epon-Araldite resin after fixing with 2.5% glutaraldehyde and 1% osmium tetroxide as described by Hess (*21*). Sections were prepared by means of a Reichert Ultracut S ultra microtome (Leica) equipped with a diamond knife. Observations were performed with a FEI company (Hillsboro, Oregon), model Tecnai G^2 20 S-TWIN transmission electron microscope (TEM) equipped with an energy-dispersive X-ray (EDX) microanalysis system. The dwelling time for the EDX analysis was 100 s.

Statistical Analysis. Data were presented as mean values ± standard error. Statistical analysis was performed by Student's *t*-test (STATISTICA, Stat soft Inc., Tulsa, USA) on a conventional personal computer. $P < 0.05$ was considered statistically significant. All experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Selenium Accumulation and Effect on the Growth of *Lactobacillus bulgaricus*. Addition of an increasing selenium in culture medium resulted in an increased selenium accumulation in *L. bulgaricus*. As demonstrated in **Table 1**, total selenium content on the dry weight basis rapidly increased from 0.28 ± 0.05 to 12.45 ± 1.04 mg/g with a dosage increase of Na_2SeO_3 administration (1, 4, 8, 16, 32, and 64 mg/L), revealing that *L. bulgaricus* could accumulate large amounts of selenium and incorporate them into living body. The finding is consistent with previous studies by Calomme et al. (*16*).

To understand growth and activity in Se-enriched *L. bulgaricus*, the biomass of administered different Na_2SeO_3 dosage in bacteria was evaluated. The results showed **(Table 1)** that treatment with low Na_2SeO_3 concentrations (≤4 mg/L) significantly facilitated the growth of *L. bulgaricus* ($P < 0.05$), while administration of high Na_2SeO_3 concentrations (>4 mg/L) in the culture medium inhibited the bacteria growth. Especially, a reduction of 13.7% in the biomass of *L. bulgaricus* exposed to 64 mg/L Na_2SeO_3 was observed compared to that of bacteria grown in the medium without Na_2SeO_3. With a selenite concentration as high as 80 mg/L Na_2SeO_3, the normal physiological condition of *L. bulgaricus* was disturbed and an unhealthy situation was noted (data not displayed). Similar results have also been observed with other microorganisms (*22*).

In particular, cultures were found to turn red due to the occurrence of Se(0) during the stationary phase under higher

Table 2. Mineral Compositions of *Lactobacillus bulgaricus* Grown under Different Selenite Concentrations[a]

Na_2SeO_3 (mg/L)	P (%)	K (%)	Mg (%)	Mn (μg/g)	Zn (μg/g)	Cu (μg/g)	Fe (μg/g)	Ca (μg/g)
0	2.09 ± 0.20	0.66 ± 0.05	0.55 ± 0.02	124.4 ± 69.6	18.7 ± 2.2	25.1 ± 7.6	8.2 ± 4.9	9.1 ± 4.6
1	2.13 ± 0.23	0.58 ± 0.02	0.56 ± 0.01	125.6 ± 40.7	19.0 ± 2.9	24.8 ± 8.8	10.6 ± 5.3	17.3 ± 5.5
4	2.15 ± 0.27	0.69 ± 0.05	0.61 ± 0.06	143.2 ± 35.5	23.4 ± 4.8	26.7 ± 7.4	8.2 ± 4.4	21.9 ± 8.4
16	1.97 ± 0.34	0.60 ± 0.03	0.57 ± 0.08	142.9 ± 57.4	18.5 ± 3.2	22.6 ± 6.9	3.8 ± 3.8	13.6 ± 3.8

[a] Data are presented as means ± SEM ($n = 3$).

Table 3. Total Amino Acid Compositions of *Lactobacillus bulgaricus* Samples Grown under Different Selenite Concentrations

	content for given samples, g/(100 g of DW)			
amino acid	0 mg/L Na_2SeO_3	1 mg/L Na_2SeO_3	4 mg/L Na_2SeO_3	16 mg/L Na_2SeO_3
Asp	5.476	5.847	6.345	6.388
Glu	6.913	7.250	7.209	6.353
Ser	1.528	1.527	1.501	1.338
His	0.716	0.692	0.647	0.637
Gly	2.463	2.656	2.622	2.349
Thr	1.431	1.491	1.692	1.615
Arg	1.756	1.615	1.476	1.653
Ala	4.824	5.069	5.225	4.865
Tyr	1.074	1.064	1.179	1.080
Cys-Cys	0	0	0	0
Val	1.635	1.721	1.870	2.072
Met	0.964	0.877	0.812	1.007
Phe	1.151	1.153	1.063	1.086
Ilu	1.402	1.486	1.587	1.578
Leu	2.700	2.884	2.888	2.667
Lys	4.285	4.540	2.490	1.762
Pro	1.233	1.883	2.698	2.443
total amino acids	39.551	41.755[a]	41.304[a]	38.893

[a] $P < 0.05$ compared to Na_2SeO_3-untreated group.

selenite stress (>4 mg/L Na_2SeO_3) and the color rose with selenite concentration increase, suggesting that *L. bulgaricus* is able to resist higher selenite by a detoxification process of reducing Se(IV) to nonsoluble Se(0).

Elemental Determinations. To evaluate the effect of enriched Se on the mineral composition of *L. bulgaricus*, concentrations of some essential elements including P, K, Mg, Mn, Zn, Cu, Fe, and Ca were detected by ICP-AES. As shown in **Table 2**, *L. bulgaricus* contained large amounts of P (2.09 ± 0.20%), K (0.66 ± 0.05%), and Mg (0.55 ± 0.02%). However, the Fe, Ca contents of *L. bulgaricus* were low (<10 μg/(g of dry weight)). The treatment of *L. bulgaricus* with Na_2SeO_3 (1 and 4 mg/L, respectively) could increase the contents of P, Mg, Mn, Zn, and Ca when compared to that of control *L. bulgaricus*, and administration of 4 mg/L Na_2SeO_3 in the medium of *L. bulgaricus* resulted in the higher values. But all detected elements of *L. bulgaricus* exposed to 16 mg/L Na_2SeO_3 decreased. Taken together, these findings suggest that the appropriate Na_2SeO_3 dosage in the medium for obtaining Se-enriched *Lactobacillus* without affecting essential element contents is within 4 mg/L.

Amino Acid Composition. Amino acids, a class of biologically active compounds present in food and beverages, are important for human nutrition. Using precolumn derivatization RP-HPLC, we determined the contents of 17 amino acids in *L. bulgaricus* treated with different concentrations of inorganic selenium. **Table 3** showed the results of these analyses (in order of elution). For the common *L. bulgaricus*, the four most abundant amino acids were glutamic acid (Glu), aspartic acid (Asp), alanine (Ala), and lysine (Lys), which accounted for roughly 54% of the total amino acid content. Other amino acids

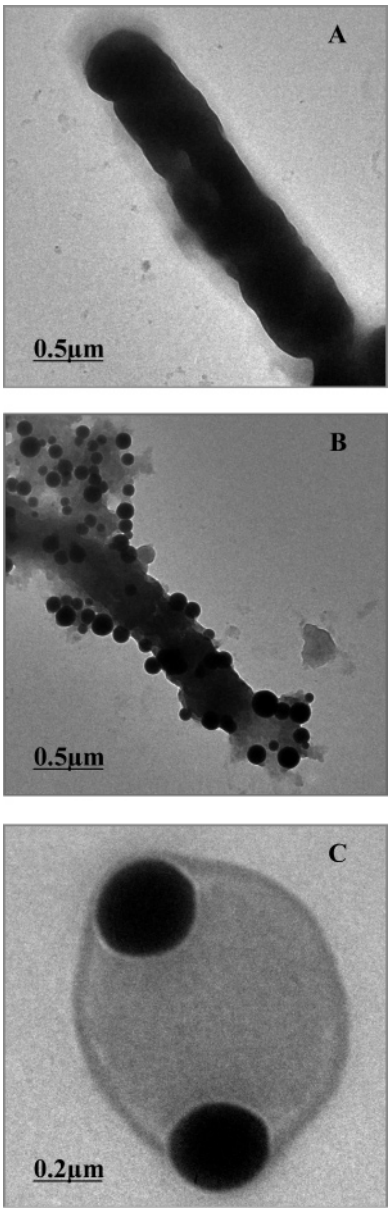

Figure 1. TEM photographs of *Lactobacillus bulgaricus* treated with or without 64 mg/L Na_2SeO_3. Control *L. bulgaricus* without supplement of Na_2SeO_3 (photograph **A**) showed no deposits. However, a lot of deposits near the periphery of the cell wall were observed in *L. bulgaricus* of exposure to Na_2SeO_3 (photograph **B**). Similar granules of deposits in the cytoplasm of Na_2SeO_3-treated *L. bulgaricus* (photograph **C**) were found using the ultrathin sectioning method.

such as methionine (Met), histidine (His), and cysteine (Cys-Cys) were always present in very low amounts. The administration of selenium increased the concentrations of Asp, Thr, Ala, Val, Ilu, and Pro and decreased that of Ser, His, and Arg in three treatment groups. Enriched Se in *L. bulgaricus* seemed to

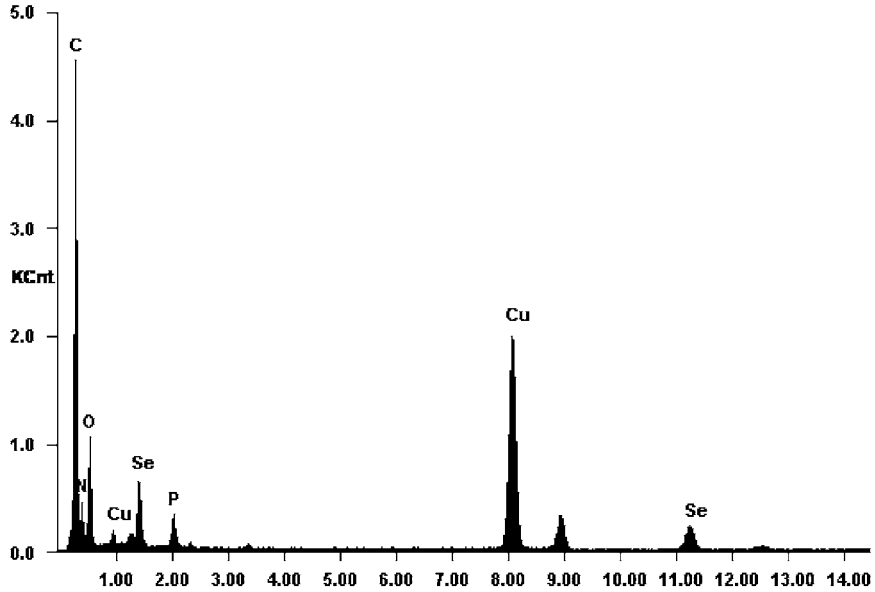

Figure 2. Representative EDXS spectrum of deposits found in cultures containing 64 mg/L Na_2SeO_3. The large Cu peak is from the copper electron microscopy grid used to support the cells. The energy range is from 0 to 14 keV. The dwelling time for the EDX analysis is 100 s.

have a biphasic effect on the production of its total amino acid. As compared to Na_2SeO_3-untreated *L. bulgaricus*, treatment of bacteria with low Na_2SeO_3 ($\leq$4 mg/L) significantly elevated the total amino acid levels ($P < 0.05$). However, an apparently inhibited effect existed after administration of high concentrations of Na_2SeO_3 (>4 mg/L) in the culture medium. Observations from the present study demonstrate that administration of suitable Se to *L. bulgaricus* in the culture medium may maintain and even increase the total amino acid levels, thereby elevating the nutritive value in the organisms, which is similar to a previous finding in *Ganoderma lucidum* (*23*).

Enriched Se Manifestation in *Lactobacillus bulgaricus*. To investigate the effect of Se(IV) administraion on cell morphology, whole amounts of cells grown either in Na_2SeO_3-free MRS medium or in 64 mg/L Na_2SeO_3-administered MRS medium were viewed by TEM. The electron micrographs showed that the cells of *L. bulgaricus* grown in Na_2SeO_3-free MRS medium were straight and rod-shaped with about 2.4 μm in length and 0.5 μm in diameter and contained no deposits (**Figure 1A**). When grown in the presence of Se(IV) the cells did not exhibit altered morphology, but a lot of deposits ($\leq$180 nm in diameter) were noticeably observed near the periphery of the cell wall (**Figure 1B**). Similar granules of deposits were also found in the cytoplasm under TEM using the ultrathin sectioning method (**Figure 1C**).

To further understand Se characteristics of enriched granules in *L. bulgaricus*, elemental analysis of deposits was performed using EDX spectroscopy (EDXS). Characteristic energy peaks for Se were found at 1.379, 11.207, and 12.492 keV (**Figure 2**). This evidence, in combination with the red color of the deposit, suggests that it is amorphous Se(0), not crystalline Se(0), which is gray in color, and clearly demonstrates that *L. bulgaricus* is able to reduce selenite to insoluble elemental selenium, an electron-dense Se(0) granule, thereby depositing it both in the cytoplasm and in the extracellular space.

Actually, Se(IV) reduction occurring as a common feature has been studied in many microorganisms, including *Rhodospirillum rubrum*, *Rhodobacter sphaeroides*, *Ralstonia metallidurans* CH34, and *Stenotrophomonas maltophilia* (*24*−*27*). However, mechanisms of Se(IV) reduction to Se(0) are not completely clear so far. According to Tomei et al. (*28*), the particles containing elemental selenium found outside cells are released by cell lysis, while Losi and Frankenberger (*29*) suggested that the reduction reaction occurs close to the membrane, possibly as a result of a membrane-associated reductase(s), and that the precipitate is rapidly expelled by a membrane efflux pump. With reference to *L. bulgaricus*, it is possible that the Se oxyanions are reduced via membrane-associated reductase(s) in the inner membrane surface, followed by expulsion of Se deposits from the cell. The actual enzymology mechanisms of Se(IV) reduction reaction in *L. bulgaricus* need to be further analyzed.

Additionally, our group found that most of selenium (~90%) accumulated in *L. bulgaricus* grown in the medium containing 64 mg/L of sodium selenite was present in the insoluble cell debris fractions with little of it (~5%) in soluble cell intercellular extract. Similar results have been observed with other Se-enriched microorganisms, including *Spirulina platensis* (*30*). These results further indicate that, consistent with previous studies by Anderoni et al. (*31*), elemental Se is probably the dominant form of selenium present in *L. bulgaricus*, while other selenium forms including organic (e.g., selenocysteine) and inorganic Se are only in minor. However, no elemental Se deposits were found under TEM in the bacteria grown under low concentrations of selenium (<4 mg/L Na_2SeO_3), suggesting most of the selenium is possibly in organic forms. To gain more information about the selenium species and distribution in *L. bulgaricus*, further efforts need to be done.

Conclusion. The main finding of this study is that administration of sodium selenite in the dosage of 1−16 mg/L is suitable for selenium enrichment in *L. bulgaricus* and can enhance nutritive value and biological effect in the organism by elevating the contents of essential elements including P, Mg, Mn, Zn, Ca, and total amino acids as well as reducing selenite to insoluble elemental selenium, an electron-dense and amorphous Se(0) granule, thereby depositing it both in the cytoplasm and in the extracellular space of *L. bulgaricus*. Our previous studies have demonstrated that Se-enriched *L. bulgaricus* is a safe and healthy resource when used as a supplement to the diet even up to concentrations of 4 ppm Se (data not displayed). Moreover, extensive effects such as elevating antioxidant enzyme activities,

enhancing immune function, and preventing liver injury and fibrosis, etc., in the body existed (*17*−*19*). Thus, Se-enriched *Lactobacillus* can be processed into capsules or directly added to foods such as yogurt, and then provide a potential dietary source of nontoxic selenium and functional regulator used for food and medical industries.

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