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Enrichment of selenium and its selenium species analysis in *Bifidobacterium bifidum* BGN4

Bifidobacterium bifidum BGN4의
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Abstract

Enrichment of selenium and its selenium species analysis in *Bifidobacterium bifidum* BGN4

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Selenium is a trace element essential for human health that has received considerable attention given its nutritional value. In this research, to solve the poor selenium tolerance of lactic acid bacteria (LAB) and to select a strain with both high selenium uptake efficiency and the ability to transform inorganic selenium into an organic form, five strains of LAB were subjected to an experiment. The total amount of selenium was quantified using an inductively coupled plasma-atomic emission spectrometer (ICP-AES). Selenium species

were separated by anion-exchange chromatography and analyzed by inductively coupled plasma-mass spectrometry (ICP-MS). The reduction of bacterial cell mass was solved with an adjustment of the selenium addition time (12 h after incubation), and *Bifidobacterium bifidum* BGN4 was found to be the most potent selenium-enriched strain. The results showed that selenomethionine was the main organic selenium in selenium-enriched *B. bifidum* BGN4. Considering that *B. bifidum* BGN4 is a well-known probiotic strain with clinically proven beneficial effects, selenium-enriched *B. bifidum* BGN4 may provide dual healthy functions as a daily supplement of selenium and for the regulation of intestinal bacteria.

Keywords: Selenium, biotransformation, lactic acid bacteria, BGN4, HPLC, ICP-MS

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List of abbreviations

SS: Sodium selenite

ICP-AES: Inductively coupled plasma-atomic emission spectrometer

ICP-MS: Inductively coupled plasma-mass spectrometry

LAB: Lactic acid bacteria

L. bulgaricus KCTC 3188: *Lactobacillus bulgaricus* KCTC 3188

L. acidophilus KCTC 3142: *Lactobacillus acidophilus* KCTC 3142

L. casei KFRI 704: *Lactobacillus casei* KFRI 704

L. brevis 353: *Lactobacillus brevis* 353

B. bifidum BGN4: *Bifidobacterium bifidum* BGN4

1. Introduction

Selenium is a micronutrient essential for the maintenance of human health and also plays an important role in human diseases, such as cancer and cardiovascular diseases [1-7]. The recommended daily allowance (RDA) of selenium is 55 μg. Selenium deficiency can cause Keshan disease and Kashin-Beck disease, with high fatality rates [8, 9]. Wei et al. [10] demonstrated that the dietary selenium intake was useful in the suppression of the development of type 2 diabetes.

The bioactivity and toxicity of selenium are closely related to the selenium species, and several studies have suggested that the organic form of selenium is more bioavailable and less toxic than its inorganic form, although accurate knowledge of the pertinent mechanism remains unknown [11]. Selenium in elemental form is regarded as non-bioavailable when taken orally but less toxic than organic and inorganic form. The LD50 for red elemental selenium is 6,700 mg/kg while LD50 is 7 mg/kg for sodium selenite [12] and 25.6 mg for selenomethionine [13].

According to recent studies, some microorganisms, such as *Saccharomyces cerevisiae* and *Lactobacillus*, showed the ability to uptake selenium and transform the chemical form of inorganic selenium to the organic form [14, 15]. Yin et al. [16]

suggested that selenium-enriched *Bifidobacterium longum* displays tumor inhibitory activity. Various strains of microorganisms were screened to select strains with high selenium uptake efficiencies and the abilities to transform the inorganic selenium to the organic form. By applying these selected microorganisms, certain selenium-enriched foods, such as selenium-enriched yeast, wine, fermented milk, mushrooms were developed [14, 16-21].

However, due to the poor selenium tolerance, the growth of selenium-enriched bacteria was inhibited considerably simply by adding a small quantity of sodium selenite [17]. Accordingly, the harvested cell mass and selenium concentrations in selenium-enriched bacteria were not satisfactory.

Probiotics, especially lactic acid bacteria and *Bifidobacterium*, have received increasing attention in recent years owing to their function of balancing intestinal microbial balance and some medical benefits, such as anti-inflammatory effects, alleviation of lactose intolerance, relief of constipation, anticholesterolaemic effects, anticancer activity, anti-inflammatory and tolerogenic immune responses [22, 23].

Previous studies have shown limitations on the preparation of selenium-enriched lactic acid bacteria attributing to the low yield of selenium enrichment and lethal effect of selenium on the growth of bacterial cells. Thus, the objectives of this study were to solve the reduction of cell mass due to the poor selenium tolerance of

lactic acid bacteria (LAB) and by quantifying the selenium uptake efficiencies and the biotransformation abilities to select a promising strain capable of providing dual healthy functions both as a supplement of selenium and as a regulator of intestinal bacterial.

Materials & Methods

2.1. Materials

2.1.1. Chemicals

Sodium selenite (Na_2O_3Se), sodium selenate decahydrate ($Na_2O_4Se \cdot 10H_2O$), seleno-DL-methionine ($C_5H_{11}NO_2Se$) and se-(methyl) selenocysteine hydrochloride ($C_4H_9NO_2Se \cdot HCl$) used as standards and the enzyme pronase E (protease XIV type) were purchased from Sigma-Aldrich (St. Louis., MO, USA). Phosphate buffered saline (PBS) was prepared by dissolving 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 7.4 g KH_2PO_4 in 1 L of de-ionized water (DW) with a final pH of 7.4. The buffer was autoclaved at 121°C for 15 min before use.

2.1.2. Microorganisms

The experimental bacterial strains were obtained from the Food Microbiology Laboratory of Seoul National University (Seoul, Korea).

2.2. Methods

2.2.1. Preparation of a selenium-enriched LAB samples

Five strains of *Lactobacillus* were used to prepare the selenium-enriched probiotics. All of the stocks were stored at -80°C in cryogenic vials with 30% (v/v) sterile glycerol (50% w/v) before use. First, they were cultured in MRS broth (Becton, Dickson and Company, USA) with 0.05% (w/v) L-cysteine·HCl at 37°C for 18 h. The culture media (15 mL) containing 1 mM of sodium selenite (added at 0 time or after being cultured for 12 h) were incubated in a water bath (37°C, 150 rpm) for 2 days. The selenium-enriched LAB samples were harvested by centrifugation (139 × g, 20 min) and washed twice with DW. Finally, the samples were freeze-dried.

2.2.2. Preparation of a 0.1 M sodium selenite (SS) solution

The sodium selenite (SS) stock solution (172.9 mg dissolved in 10 ml of DW) was prepared and filtered by a 0.2 μm syringe filter. The 15 μL stock solution was added to the culture medium (15 mL). The final selenium concentration in the culture medium was 1 mM.

2.2.3. Quantification of total selenium, organic selenium and analysis of selenium species

For the quantification of total selenium, dried cells harvested from 15 mL of culture were dissolved with 8 mL of nitric acid overnight and then digested by a microwave digestion system (Ethos 1, Milestone, Bergamo, Italy). The volume of the dissolved samples was adjusted with DW to 50 mL. The total selenium was quantified using an inductively coupled plasma-atomic emission spectrometer (ICP-AES, Optima-4300 DV, Perkin Elmer, Norwalk, CT, USA).

For the extraction of the total selenium organic compounds and an assessment of the selenium species, the selenium-enriched LAB powder was dissolved in 5 mL of phosphate buffer containing 20 mg of pronase E and then incubated at 37°C for 16 h [24, 25]. The samples were then filtered through a sterile filter (0.45 μm, Pall Corporation, Port Washington, NY). The determination of selenium species was performed using an inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7700, Agilent Technologies, Tokyo, Japan) equipped with an octopole collision cell. Chromatographic separations were performed using a Model 1260 HPLC pump (Agilent, Wilmington, DE, USA) as the delivery system. The selenium species separations were carried out using a Hamilton (Reno, NV) PRP X-100 anion exchange column (250 mm X 4.1 mm id, 10μm particles) at 40°C. The flow rate was kept at 1 mL/min, and the injection volume was 20 μL. The detailed

HPLC analytic conditions are presented in Table 1 and ICP-MS operating conditions were summarized in Table 2.

Table 1. Anion Exchange Chromatographic Parameters

Parameters	
Column	PRP X-100 (250 X 4.1 mm, 10 μm), Hamilton
Mobile phase	A, 2 mM Ammonium Citrate in 2% Methanol
	B, 10 mM Ammonium Citrate in 2% Methanol
Flow rate	1 mL/min
Injection Vol.	20 μL
Gradient	0-2 min 20% B
	2-10 min 20-100% B
	10-18 min 100% B

Table 2. ICP-MS parameters

Parameters	
Forward Power	1550 W
Plasma Gas Flow Rate	10.5 L/mL
Carrier Gas Flow Rate	0.8 L/mL
Makeup Gas Flow Rate	0.30 L/min
Sample Depth	8.0 mm
Collision Gas (He Gas) Flow	3.5 mL/min
Quadrupole Bias	-16 V
Octopole Bias	-18 V
Isotopes Detection Type	TRA
Isotopes monitored (m/z)	77Se, 78Se

3. Statistical analysis

Experiments were performed in triplicate, and the data was shown as mean ± standard deviation. Unpaired t-test and one-way analysis of variance (ANOVA) with Duncan post-hoc test ($p < 0.01$) were performed with the statistics package GNU R, version 3.1.2 [26].

4. Results and Discussion

4.1. The effect of the selenium addition time on bacterial cell mass

Though the preliminary screening process, the strains *L. bulgaricus* KCTC 3188, *L. acidophilus* KCTC 3142, *L. casei* KFRI 704, *L. brevis* 353, and *B. bifidum* BGN4 were selected and assessed experimentally to quantify the total selenium content and the selenium species. Table 1 shows the dry weights of the selenium-enriched LAB samples depending on the addition time of SS into the growth medium. The addition of SS at 0 h inhibited the growth of *L. bulgaricus* KCTC 3188, *L. acidophilus* KCTC 3142, *L. casei* KFRI 704, *L. brevis* 353, and *B. bifidum* BGN4 by 40, 26.9, 41.9, 31.1 and 75.9%, respectively, compared to the control group without SS (Table 3, $p < 0.01$). On the other hand, the addition of SS at 12 h did not lead to a reduction of the cell mass ($p > 0.01$).

Selenium compounds can inhibit the growth of *Staphylococcus aureus* [27, 28] but also can inhibit the growth of *Lactobacillus* and *Bifidobacterium*. Deng et al. [17] reported that a concentration of SS which exceeds 10 μg/mg can inhibit the growth of bacteria, and Yin et al. [16] showed that the biomass of *L. brevis* was reduced by 59.3% when grown in a MRS medium containing 1 mM of SS. Since

the addition of selenium at 12 h after incubation did not lead to a reduction of final cell mass of LAB, the present method would be a good solution to overcome the reduction of bacterial cell mass caused by poor selenium tolerance of LAB.

Table 3. Dry weight of selenium-enriched LAB grown in MRS containing 1 mM (172.9 mg/L) of sodium selenite (SS)

Strain	Dry weight of selenium-enriched LAB (mg)		
	SS added at 0 h	SS added at 12 h	Control (no SS added)
L. bulgaricus KCTC 3188	15 ± 0.62*	24.3 ± 0.55	25 ± 0.38
L. acidophilus KCTC 3142	16.3 ± 0.40*	22.8 ± 0.42	22.3 ± 0.26
L. casei KFRI 704	15 ± 0.49*	25.6 ± 0.31	25.8 ± 0.56
L. brevis 353	21 ± 0.49*	31.1 ± 0.55	30.5 ± 0.81
B. bifidum BGN4	5.3 ± 0.50*	20.9 ± 0.53	22 ± 0.35

Data is shown as the mean ± SD from triplicate experiments.

*Values are significantly different compared to control group ($p < 0.01$).

4.2. Total selenium and organic selenium contents in selenium-enriched LAB quantified by ICP-AES

Total selenium quantification by nitric acid extraction using microwave oven digestion could achieve a high recovery rate (96.6%) [29]. Meanwhile, the oxidation states of selenium were altered by nitric acid [30]. Therefore, the method in which nitric acid is applied is not appropriate for identifying the chemical form of selenium. Enzymatic hydrolysis with proteinase K (pronase E) has been proven as the most effective method for the quantification of organic selenium species without altering their oxidation states [31]. Therefore, nitric acid digestion (Table 4) and a proteolytic treatment were applied for the quantification of the total selenium and the total organic selenium, respectively, in this study. Previously, microorganisms such as *Saccharomyces* and LAB showed the ability to transform the chemical form of tetravalent inorganic selenium in a culture medium into elemental selenium and organic selenium [14, 32-34]. Based on Tables 4 and 5, *L. casei* KFRI 704 showed relatively low levels of total and organic selenium concentrations of 7.3 mg/g and 35 μg/g, respectively, while *B. bifidum* BGN4 showed the highest concentrations of total and organic selenium, amounting to 33.3 mg/g and 207.5 μg/g, respectively. Yan et al. [16] prepared selenium-enriched *Bifidobacterium longum* with an organic selenium concentration of 81.6 μg/g. Yeast is considered to be an effective selenium-enriching microorganism, and commercial products typically contain from 1,000 to 2,000 micrograms of

selenium per gram [35]. Thus, although the organic selenium contents were less than 1% in total selenium, *B. bifidum* BGN4 was better at enriching selenium than other microorganisms reported earlier, where the selenium source were added at the beginning of the growth. By solving the poor selenium tolerance of LAB, a higher concentration of selenium accumulated in LAB was achieved. The culture medium turned into red color during the incubation with the addition of SS. According to Xia et al. [36], the red color of the medium was attributed to the formation of non-toxic elemental selenium after the experimental cells were treated with a high selenium concentration (higher than 4 mg/L). The notable difference in the selenium contents between the results with the nitric acid method and the proteolytic treatment was mainly due to the formation of elemental selenium. Considering both the harvested cell mass and the uptake efficiency, *B. bifidum* BGN4 is the most desirable for use as selenium-enriched probiotics.

Table 4. Total selenium and organic selenium contents in selenium-enriched LAB quantified by ICP-AES

Strain	Total selenium contents in LAB (mg/g)	Total organic selenium contents in LAB (μg/g)
L. bulgaricus KCTC 3188	28.1 ± 0.01[a]	111.7 ± 0.16[a]
L. acidophilus KCTC 3142	31.4 ± 0.00[b]	134.4 ± 1.18[b]
L. casei KFRI 704	7.3 ± 0.00[c]	35 ± 0.20[c]
L. brevis 353	23.1 ± 0.01[d]	111.8 ± 0.86[a]
B. bifidum BGN4	33.3 ± 0.01[e]	207.5 ± 1.25[d]

Data is shown as the mean ± SD from triplicate experiments.

[a,b,c,d,e] Values with different superscripts within the same columns indicate significant differences.

4.3. Comparison of the effect of different SS addition time on the bacterial cell mass and uptake efficiency

The total and organic selenium contents in selenium-enriched *B. bifidum* BGN4 with SS added at 6 h (33.3 mg/g, 224.1 μg/g) and 12 h (33.3 mg/g, 207.5 μg/g) showed no significant differences ($p > 0.01$). However, the bacterial cell mass was significantly decreased when selenium was added at 6 h (Table 5, $p < 0.01$). When selenium was added at the beginning of the growth of *B. bifidum* BGN4, the growth was considerably retarded (data not shown). The decrease of cell mass were 75.9% and 62.7% when SS was added at 0 h and 6 h after incubation. As expected, *B. bifidum* BGN4 was very sensitive to selenium. In the previous studies, a comparison of different selenium addition times was not performed.

Table 5. Comparison of the dry weights and the uptake efficiency of *B. bifidum* BGN4 (BGN4) with SS added after 6 h and 12 h incubation

Groups	Dry weight of selenium-enriched BGN4 (mg)	Total selenium contents in selenium-enriched BGN4 after acid treatment (mg/g)	Total organic selenium contents in selenium-enriched BGN4 after enzymatic hydrolysis (μg/g)
SS added at 6 h	8.3 ± 0.61	33.3 ± 0.02	224.1 ± 0.78
SS added at 12 h	20.9 ± 0.53	33.3 ± 0.01	207.5 ± 1.25

Data is shown as the mean ± SD from triplicate experiments.

4.4. Selenium species analysis by HPLC-ICP-MS

For the selenium species analysis, enzymatic hydrolysis using pronase E was done prior to the species analysis step. Pronase E is a non-specific protease which is used regularly to break down the peptide bonds of selenium-containing proteins. It is also considered as the most effective protease [37]. As shown in Figure 1, the major species in selenium-enriched *B. bifidum* BGN4 was selenomethionine, at 169.6 μg/g.

The previous study suggested that the relative bioavalibility value of selenomethionine compared to SS was 147% for liver glutathione peroxidase activity and 336% for weight gain in channel catfish which indicated that selenium allowance in diets of channel catfish can be reduced when using selenomethionine instead of inorganic selenium [38]. Claire et al. [39] suggested that selenomethionine inhibited the growth of the human tumor cell lines (MCF-7/S breast carcinoma, DU-145 prostate cancer cells and UACC-375 melanoma) in the range from 45 to 130 μM while growth inhibition of normal diploid fibroblasts required 1 mM selenomethionine, much higher than for the cancer cell lines. Considering bioavalibility and toxicity, selenomethionine is appropriate supplemental form of selenium for humans and animals [40].

The minimum requirement for the prevention of Keshan disease is 20 μg/day. The physiological requirement for maximal glutathione peroxidase (GPx) and selenoprotein P was estimated to be 45–50 μg/day and the requirement for iodothyronine 5 deiodinases

(IDIs) is 30 μg/day. In addition, protection against some cancers, such as lung cancer and prostate cancer, requires 120 μg/day [41]. *B. bifidum* BGN4 is considered as one of the most promising probiotics given its clinically proven beneficial effects [42]. Research has suggested that the oral feeding of *B. bifidum* BGN4 can prevent T cell-mediated inflammatory bowel disease by the inhibition of disordered T cell activation processes [43].

Since the low toxicity of elemental selenium, high bioactivity of selenomethionine and the high selenium uptake efficiency of *B. bifidum* BGN4 shown in the current study, selenium-enriched *B. bifidum* BGN4 may provide sufficient supplementation of dietary selenium and regulation of intestinal bacteria.

Fig. 1. Chromatographic profiles of mixture of selenium standards obtained by HPLC-ICP-MS

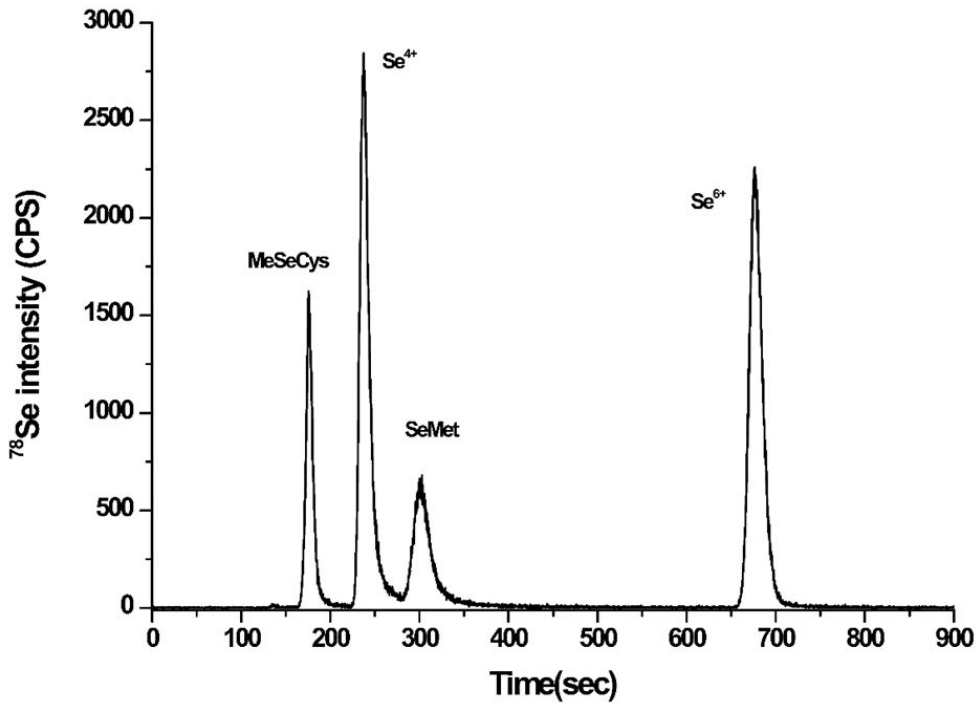
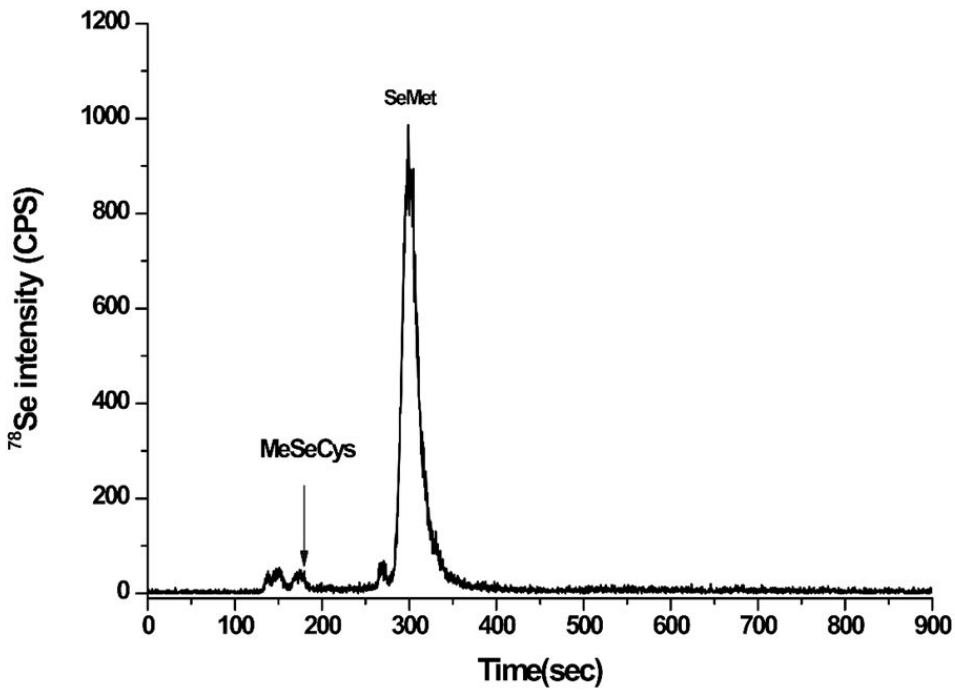


Fig. 2. Chromatographic profiles of the enzymatically extracted organic selenium from selenium-enriched *B. bifidum* BGN4 analyzed by HPLC-ICP-MS



5. Conclusion

The adjustment of selenium addition time (12 h after incubation) enhanced the cell mass of the selenium-enriched LAB by overcoming the poor selenium tolerance of LAB. The comparison of the selenium uptake efficiency of the various experimental LAB showed that selenium-enriched *B. bifidum* BGN4 was the most potent selenium-enriched probiotics in this study. However, further researches are need to improve the biotransformation performance of *B. bifidum* BGN4 and prove the medical beneficial of the selenium-enriched *B. bifidum* BGN4.

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KOREAN ABSTRACT

셀레늄은 우리 몸에 필수적인 미량 영양소로서 자유 라디칼의 생성을 억제할 수 있는 글루타티온 과산화효소 (glutathione peroxidase)의 중요한 구성성분으로 세포를 산화적 손상으로부터 보호하는 역할을 한다. 이 외에도 셀레늄의 항암, 심혈관 질병 예방에 대한 연구 결과도 보고되는 등 영양소로서 역할이 주목받고 있다. 셀레늄은 구조에 따라 독성과 생물학적 이용도가 다른데 유기 셀레늄이 무기 셀레늄보다 낮은 독성과 높은 생물학적 이용도를 가지고 있다고 보고되었다. 여러 미생물들이 셀레늄을 uptake 하고 무기 셀레늄을 유기 셀레늄으로 생물전환 할 수 있다는 능력이 보고되면서 많은 효모와 유산균의 셀레늄 생물전환능력을 평가·탐색하여 고효율의 균주를 선별하고, 이를 이용하여 식품산업에 적용할 수 있는 셀레늄 강화 효모, 유산균을 제조한 사례가 보고되었다. 하지만 대부분의 유산균이 셀레늄 내성이 약하여 배지에 적은 농도의 셀레늄을 첨가하여도 균의 성장에 억제가 일어나는 약점이 있었다. 따라서, 본 연구의 목적은 약한 셀레늄 내성에 의한 유산균의 성장 억제를 해결하고 보다 더 효율적으로 셀레늄 강화 유산균 제조를 도모하며, 5 균주의 유산균 (*L.bulgaricus* KCTC 3188, *L.acidophilus* KCTC 3142, *L. casei* KFRI 704, *L.brevis* 353, *B.bifidum* BGN4) 을 이용하여 총 셀레늄과 총 유기 셀레늄의 함량을 분석하여 셀레늄 uptake 효율이 높은 균주를 선택하고 종 분석을 하는것이다. 연구결과, 유산균 배양 12 시간 후 셀레늄을 첨가하는 방식으로 셀레늄 첨가에 의한 유산균이 정상적인 생장 양상을 보였으며, 따라서 더욱 효

율적으로 셀레늄 강화 유산균을 제조할 수 있었다. *B. bifidum* BGN4 의 높은 셀레늄 uptake 효율과 무기 셀레늄을 유기 셀레늄으로 생물전환 하는 능력 역시 다른 균주들에 비해 우수하다는 것을 알 수 있었다.

주요어: 셀레늄, 생물전환, 유산균, BGN4, HPLC, ICP-MS

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