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Original paper

In vitro biosynthesis of organic selenium by *Lactobacillus casei* from inorganic selenium forms

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Abstract

The bioconversion of selenium in the form of sodium selenite (Na_2SeO_3) (SeIV) or sodium selenate (Na_2SeO_4) (SeVI) to organic form by *Lactobacillus casei* (*L. casei*) was investigated *in vitro*. MRS media was supplemented with 1, 2, 5, 10 or 20 ppm of Se, inoculated with 2% starter culture with about *105 cfu/ml* of *L. casei* and then incubated up to 24 hrs at 37°C. Increasing of selenite "Se(IV)" concentrations in the media markedly reduced the bacterial growth compared to selenate "Se(VI)" indicating cytotoxic effect of selenite. The media supplemented with 5 ppm or more of Se(IV) became reddish after 24 hr of incubation as a result of the formation of 100-200 nanometer particles of selenium (SeNPs). Se speciation of the cultured media supernatants and its corresponding cell fractions was carried out by HPLC-ICP-MS technique. The bioconversion rate of Se to organic form by *L. casei* was extremely higher in Se(IV) than Se(VI) in both fractions, however the media supernatant contained the highest content. Increasing the media Se content resulted in gradual increase of organic Se concentration in both cells and supernatant fractions. The medium supplemented with 1 ppm Se(IV) was completely depleted from the inorganic Se as it completely converted to organic form. Although the cell fractions from all Se(VI) supplemented media contained only organic Se, the media supernatant contained significant residual amount of the inorganic form. Our results demonstrate the ability of *L. casei* to convert Se(IV) or Se(VI) up to 20 ppm to organic form(s) either in the cultured media or inside the bacterial cells. However, Se(IV) but not Se(VI), at a limit concentration of 1 ppm, was completely converted and accumulated in an organic form in the cell fraction and the cultured medium.

Keywords *Lactobacillus casei*, sodium selenite, sodium selenate, organic selenium, selenium nanoparticles.

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Introduction

The importance of selenium as an essential micronutrient for animals and human nutrition was demonstrated and proved several decades ago. Selenium deficiency in the diet was reported as the main cause of several malnutrition disorders including cancer, cardiovascular disorders, poor immune response, white muscle disease and the osteochondropathy Kashin-Beck disease. Selenium exerts its biological functions through selenoproteins namely selenocysteine. There are twenty-five selenoproteins are encoded in the human genome **Kurokawa and Berry (2013)**. Selenium supplementation is tricky, as high selenium admissions can be dangerous, causing sickness, heaving, muscle fits and even demise, especially if the source is inorganic. Protein-bound selenium is more bioactive and less poisonous than inorganic one and there is enthusiasm for conveying selenium in natural structures in nourishment items requested by customers. Awareness of public health trends and recent scientific evidence of desirable features beyond nutrition stimulates consumer interest and demand for such food. Among the developed selenium enriched products, tea (**Xu *et al*., 2003**), table salt (**Cheng and Qian, 1990**), yoghurt (**Achanta *et al*., 2007**), sprout (**Lintschinger *et al*., 2000**), infant formula (**Tyrala *et al*., 1996**), mushroom (**Ogra *et al*., 2004**) onions (**Ip and Lisk, 1994**), garlic (**Ip and Lisk, 1995**) and prickly pear fruit (**Banuelos *et al*., 2011**). Milk and dairy products, when intended to satisfy particular human dietary demands, can become more appealing and useful. Therefore, production of selenium enriched functional dairy products was the aim of several researches. Enrichment of dairy cows fed with organic selenium is one of the approaches used to increase milk content of organic selenium; therefore the dairy products from such milk could be used to meet the recommended daily intakes of the consumers (**Liu *et al*., 2015).** Many Lactic acid bacteria were found to transform and accumulate inorganic Se to organic form in their cultured media and biomass (**Prokisch *et al*., 2008; Pophaly *et al*., 2014; Zommara and Prokisch, 2015; Kurek, *et al*., 2016; Pescuma *et al*., 2017**). In our previous study we have found that yoghurt culture (*S. thermophiles* and *L. bulgaricus*) cultivated in milk supplemented with sodium selenite Se(IV) was able to accumulate organic Se in the product during the fermentation process at a maximum level of 1 ppm (**Zommara and Prokisch, 2015**). The aim of the present study is to investigate the ability of *L. casei* to convert the inorganic selenium in the form of selenite Se(IV) or selenite Se(VI) to organic form in the cultured media supplemented with different concentrations of Se. The bacterial growth rate was followed up to 24 hr of incubation period and speciation of Se forms was carried out in the media supernatant and the lysozyme hydrolyzed cell pellets using HPLC-ICP-MS system.

Materials and methods

Cultivation of Lactobacillus casei with selenium sources

Pure lyophilized culture of *Lactobacillus casei* NCAIM B 1147 (*L. casei*) strain was obtained from the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary. The bacterial culture (2%) with about 10^5 cfu/ml was cultivated in MRS broth medium as described by **De-Man *et al*., (1960)** amended with 0, 1, 2, 5, 10 and 20 ppm of filter sterilized (Sartorius AG, Germany) sodium selenite, Na_2SeO_3. $5H_2O$ [Se(IV)], or sodium selenate, Na_2SeO_4. $10\ H_2O$ [Se(VI)] Sigma-Aldrich, Switzerland) and incubated at 37°C up to 24 h.

Determination of bacterial growth

The bacterial growth was monitored at 2 hr intervals for 12 hr and then after 24 hr of incubation the cultured media. The bacterial growth was estimated by measuring the absorbance at 650 nm (**Ayad *et al*., 2004**) and determination the pH value (Radelkis Electrochemical Instruments, Hungary) of the media.

Analysis of selenium species in medium supernatant and cell fraction

Ten ml aliquots of media were removed after 24 hr of incubation. The media were centrifuged at 4500g (7000 rpm) for 20 min at 10°C to spin down the bacterial cells. The supernatant was carefully collected and kept under freezing for Se speciation analysis. The cell pellets were washed 2 times by Tris-HCl buffer (50 mM, pH 7.5) and finally with ultra-pure water. To the cell pellet 1 ml Tris-HCl buffer (10 mM, pH 8.0) was added followed by 100 µl of 10% lysozyme solution (Sigma-Aldrish, 100.000 U/ mg) and incubated overnight at room temperature. The hydrolyzed cell pellet was centrifuged at 5000g for 20 min and the supernatant was collected (cell fraction) for Se speciation analysis. Media supernatant and the cell fractions were analyzed for Se species by inductively coupled plasma mass spectrometer (ICP-MS) (X series, Thermo Fisher Scientific, Germany) coupled to HPLC (Merk-Hitachi L06200A, Germany) equipped with an anion exchange chromatography column (Polyspher, IC-ANI, Merck, Germany) as previously described (**Zommara *et al.*, 2007**). Se (10 ppb) standards mixture namely; seleno-L-methionine (SeMet, Fluka Chemie, Switzerland), Se(IV) and Se(IV) were prepared in Milli-Q water.

Scanning electron microscopy (SEM), transmission electron microscopy (TEM) and SeNPs size determination

The SEM and TEM of the bacterial media were carried out according to **Nagy *et al*., (2016)** using Hitachi S 4300 SEM (Schaumburg, IL, USA) and TESLA BS 540 TEM (Brno, Czechoslovakia). Size of the SeNPs was determined by particle size analyzer (Malvern, Mastersizer 2000) Malvern Instruments Ltd, UK.

Results and discussion

Data illustrated in Fig. 1 (A & B) show the growth profile of *L. casei* strain incubated in MRS media with different concentration of Se(IV). The bacterial growth rate was monitored during 24 hr of incubation using the progress of medium acidity and absorbance as indicators. The pH data indicated no inhibition effect of Se(IV) on bacterial growth

up to 5 ppm in the medium. However, addition of 10 and 20 ppm to the medium had a slight negative effect on bacterial growth. The media absorbance confirmed the pH data although the media amended with 10 and 20 ppm Se(IV) resulted in high absorbance after 4 hr of incubation compared to the media with less Se(IV) content. This increase may be explained by the accumulation of selenium nanoparticles (SeNPs) in the cells and media (Fig. 2 A & B). The transmission electron microscope (TEM) photo of a bacterial cell (Fig. 2 A) and The scanning electron microscope (SEM) photo of the medium amended with 20 ppm Se(IV) (Fig. 2 B) showed the accumulation of SeNPs inside the bacterial cells and in the cultured medium, respectively. In this respect, **Alzate *et al*., (2010)** stated that supplementation of fermented milk with Se(IV) below 2 ppm had no negative effect in the growth of a mixed cultures of *S. thermophilus*, *L. bulgaricus*, *L. casei*, *L. paracasei* and *B. Lactis* until the 4th week of cold storage. They noticed segregated selenium as SeNPs in the fermented milk supplemented with 6, 10 and 20 ppm Se(IV). Our previous studies showed that different LAB including the traditional yoghurt culture (*S. thermophilus* and *L. bulgaricus*) and bifidobacteria were able to accumulate SeNPs when cultivated in suitable media amended with different concentrations of Se(IV) up to 20 ppm. (**Prokisch *et al*., 2008; Prokisch and Zommara, 2010; Zommara and Prokisch, 2015**). The ability of lactic acid bacteria to biotransform selenite Se(IV) to organic form(s) have been reported in plenty of research papers including different strains of lactobacilli and the traditional yoghurt starter culture (**Pophaly *et al*., 2014; Kurek, *et al*., 2016; Pescuma *et al*., 2017**).

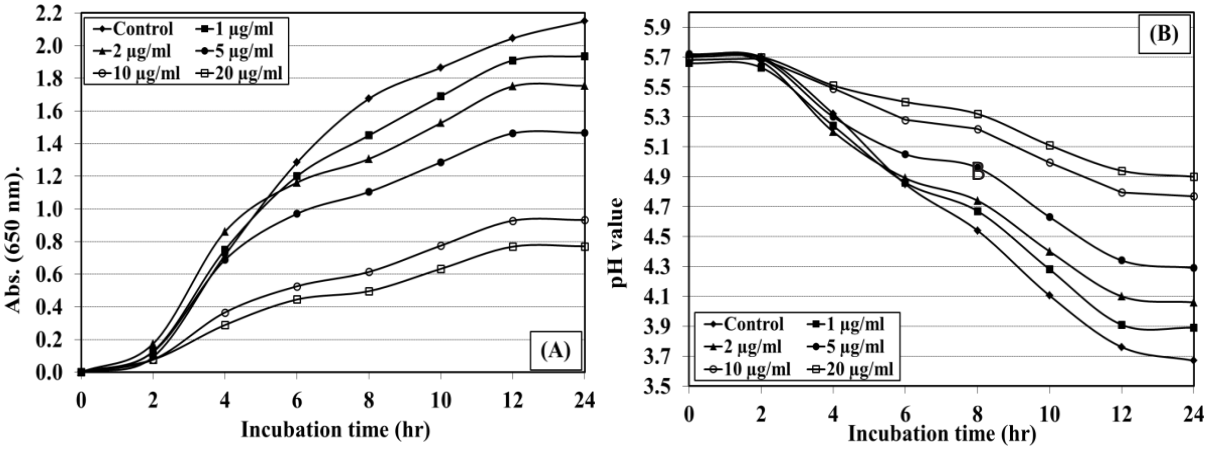

Figure 1. Effect of selenium (IV) concentration on growth of *L. casei* cultured in MRS broth media during 24 hr. of incubation at 37°C as indicated by cultured media absorbance (A) and pH value (B).

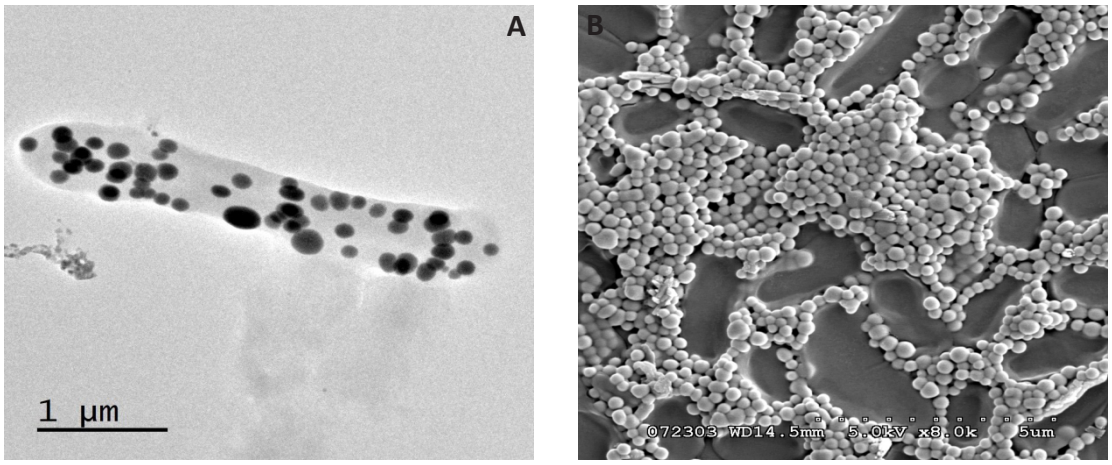

Figure 2. TEM of a single cell (A) and SEM of the cultured media (B) of *L. casei* cultivated in MRS media with 20 ppm of Se(IV) and incubated at 37°C for 24hr showing SeNPs inside (A) and outside (B) the cells.

On the other hand, there was no inhibition effect of Se(VI) on the growth of *L. casei* as indicated by following the media absorbance and pH (Fig. 3 A & B), respectiveley although, the bacterial growth rate was slightly reduced in the medium supplemented with 20 ppm Se(VI). Unlike Se(IV), use of Se(VI) did not led to accumulate SeNPs inside the cells or outside in the cultured media (data not shown).

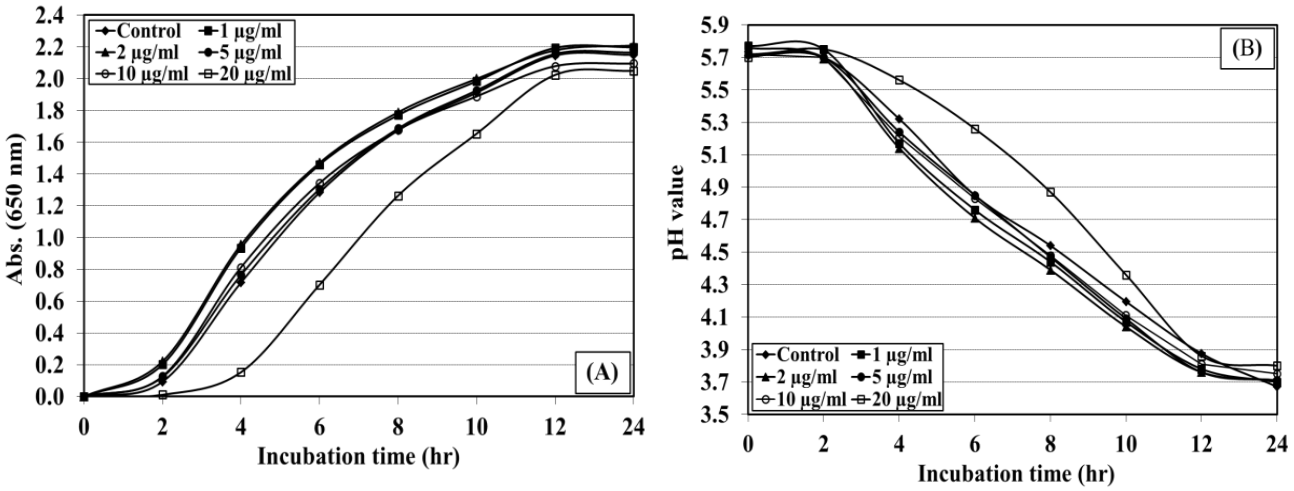

Figure 3. Effect of selenium (VI) concentration on growth of *L. casei* cultured in MRS broth media during 24 hr of incubation at 37°C as indicated by cultured media absorbance (A) and pH value (B).

The ability of *L. casei* to convert different concentrations of Se(IV) to organic form in MRS media supernatant and cell biomass are shown in Fig. (4) and Fig. (5). The data illustrated in Fig. (4) clearly show that most of Se(IV) in the cultured media was converted to an organic form during the incubation period (24hr.). However, The medium supplemented with 1 ppm almost depleted from the inorganic Se(IV). On the other side, the bacterial cell fractions had higher concentration of organic Se (Fig. 5) compared to its corresponding cultured medium supernatant (Fig. 4). Except for the medium supplemented with 20 ppm Se(IV), selenium was almost detected in an organic form inside the bacterial cell.

The formation of organic selenium takes place by replacing sulfur with Se in the sulfied amino acids in the proteins namely cysteine and methionine to form selenocysteine(Se-cys) and selenomethionine (Se-met) as the main organic selenium species found in plant and animal tissue (**Alzate *et al*., 2007; Alzate *et al*., 2008; Galano *et al*., 2013; Palomo *et al*., 2014; Pescuma *et al*., 2017**). Also, the production of SeNPs by *L. casei* cultivated in suitable medium supplemented with Se(IV) was repeatedly confirmed by many researchers (**Nagy *et al*., 2016; Rajasree and Gayathri, 2015; Eszenyi *et al.,* 2011**). Our results demonstrated that *L. casei* produce SeNPs with 100-200 nm dimeter when cultivated with MRS media amended with Se(IV). **Diowksza *et al*., (1999)** also observed a red color in *L. plantarum*, *L. brevis*, *L. sanfrancisco* biomass grown in MRS medium amended with Se(IV) exceeding 10 ppm. The formation of SeNPs by the bacterial cultures may be attributed to a detoxification mechanism (**Prokisch *et al*., 2008**).

The bacterial cell fraction of the medium fortified with 1 ppm Se(IV) contained no inorganic selenium which indicate a complete conversation of Se(IV) to organic form at that concentration or less. In this respect, **Prokisch *et al*., (2008)** and **Zommara and Prokisch (2015)** stated that whey media cultivated with yoghurt culture (*S. thermophilus* and *L. bulgaricus*) and different concentrations of Se(IV) markedly increased the organic form of selenium in the supernatant. On contrast, *L. casei* showed weak ability to convert Se(VI) to an organic form compared to Se(IV). The data illustrated in Fig. (6) and Fig. (7) obviously show the remaining of high residual content of Se(VI) in the cultured medium supernatant (Fig. 6); however, all the Se detected in the cell fraction was in the organic form (Fig. 7).

Conclusion

In conclusion, The obtained results indicate that *L. casei* could be used as Se enriched probiotic culture using Se(IV) or Se(VI) up to 20 ppm with superior to Se(IV), or as a starter culture for producing Se rich fermented dairy foods using Se(IV) at a limit concentration of 1 ppm. Also, in higher Se(IV) concentration *L. casei* produces SeNPs which may be applied in different applications, however more adequate research still in need in this regard.

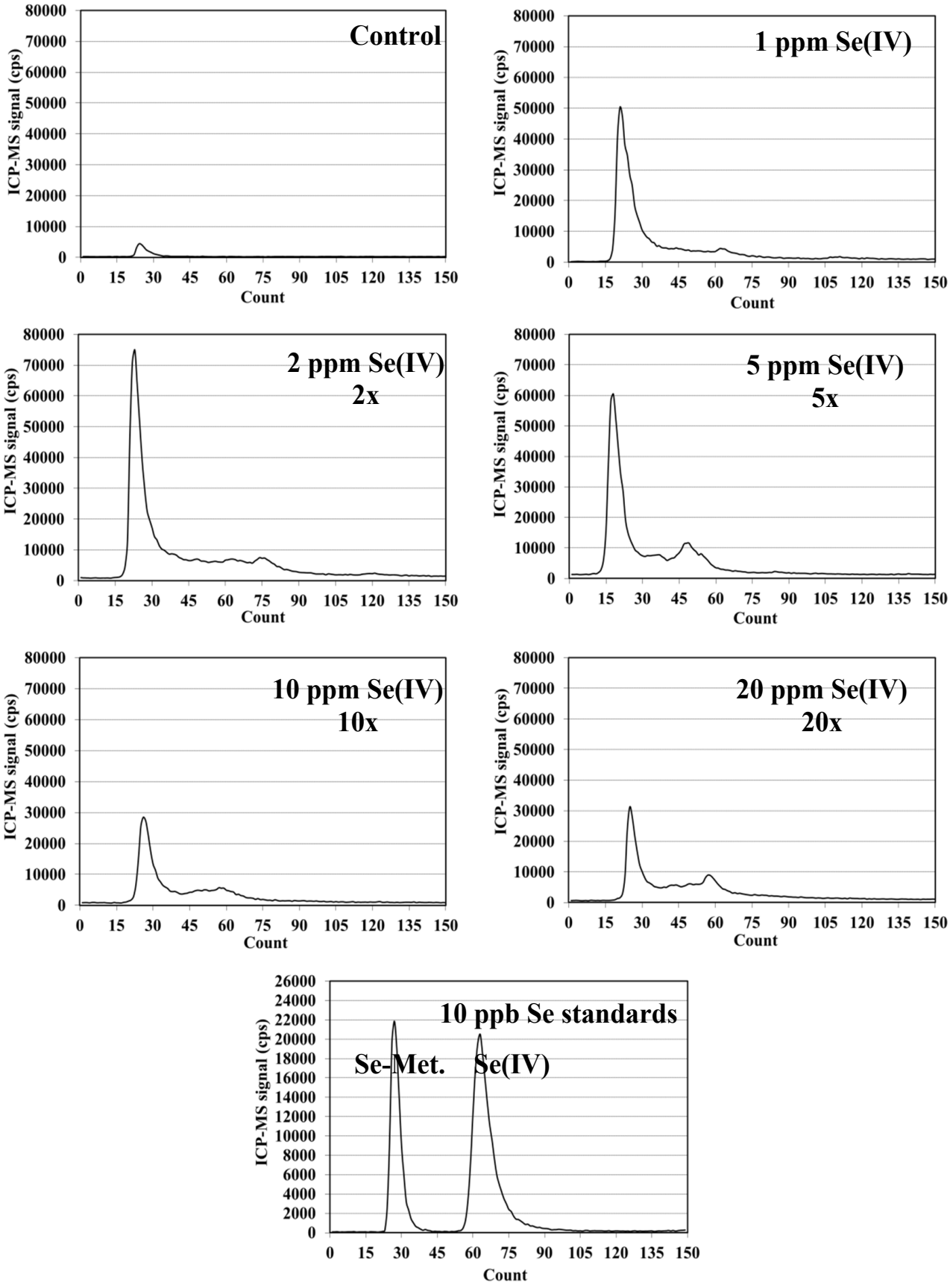

Figure 4. Selenium content in MRS media supernatant of *L. casei* incubated with different concentrations of Se(IV) at 37°C for 24hr measured as count per second (cps).

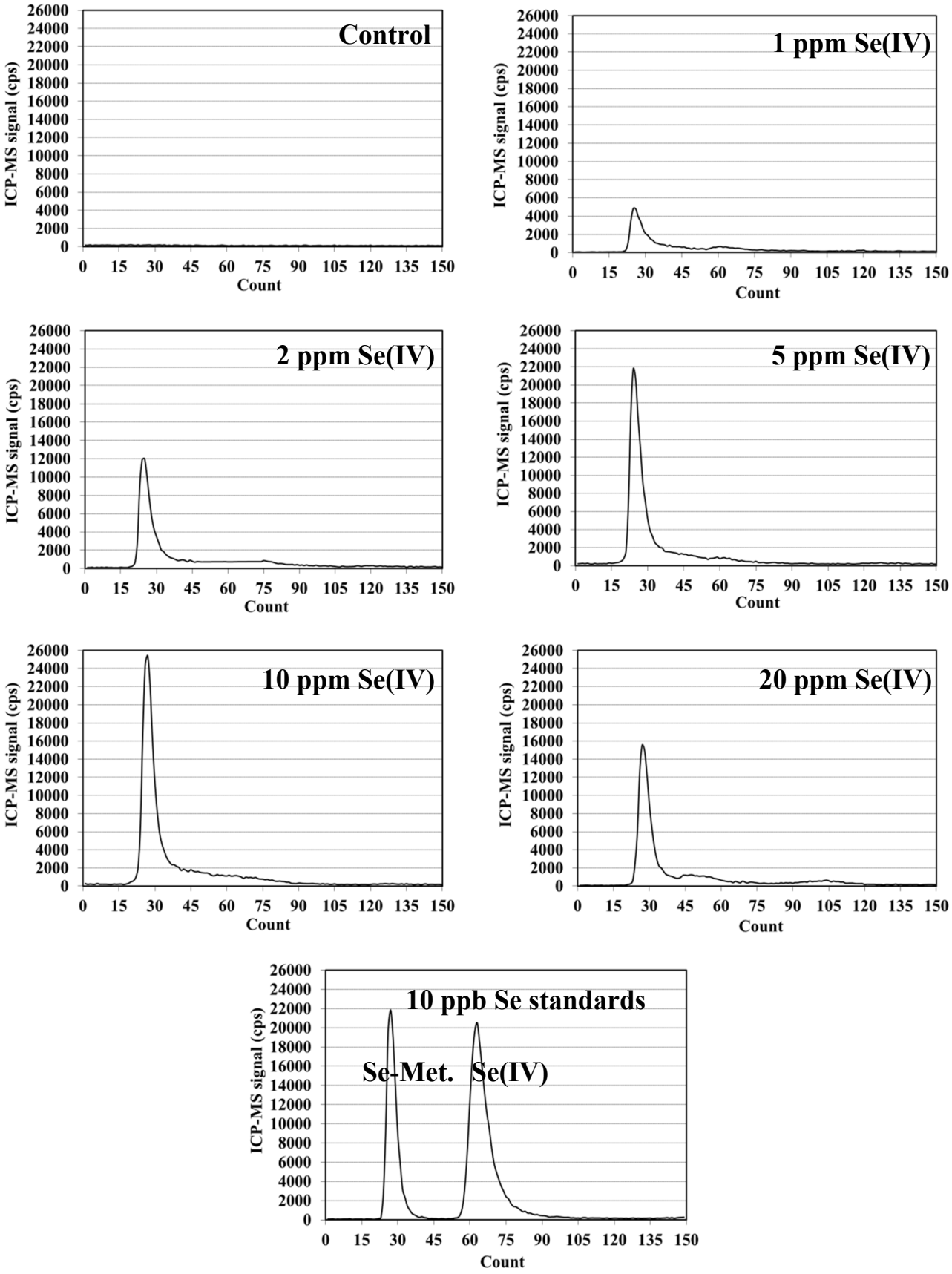

Figure 5. Selenium content in cell fraction of L. casei incubated in MRS media with different concentrations of Se(IV) at 37°C for 24hr measured as count per second (cps).

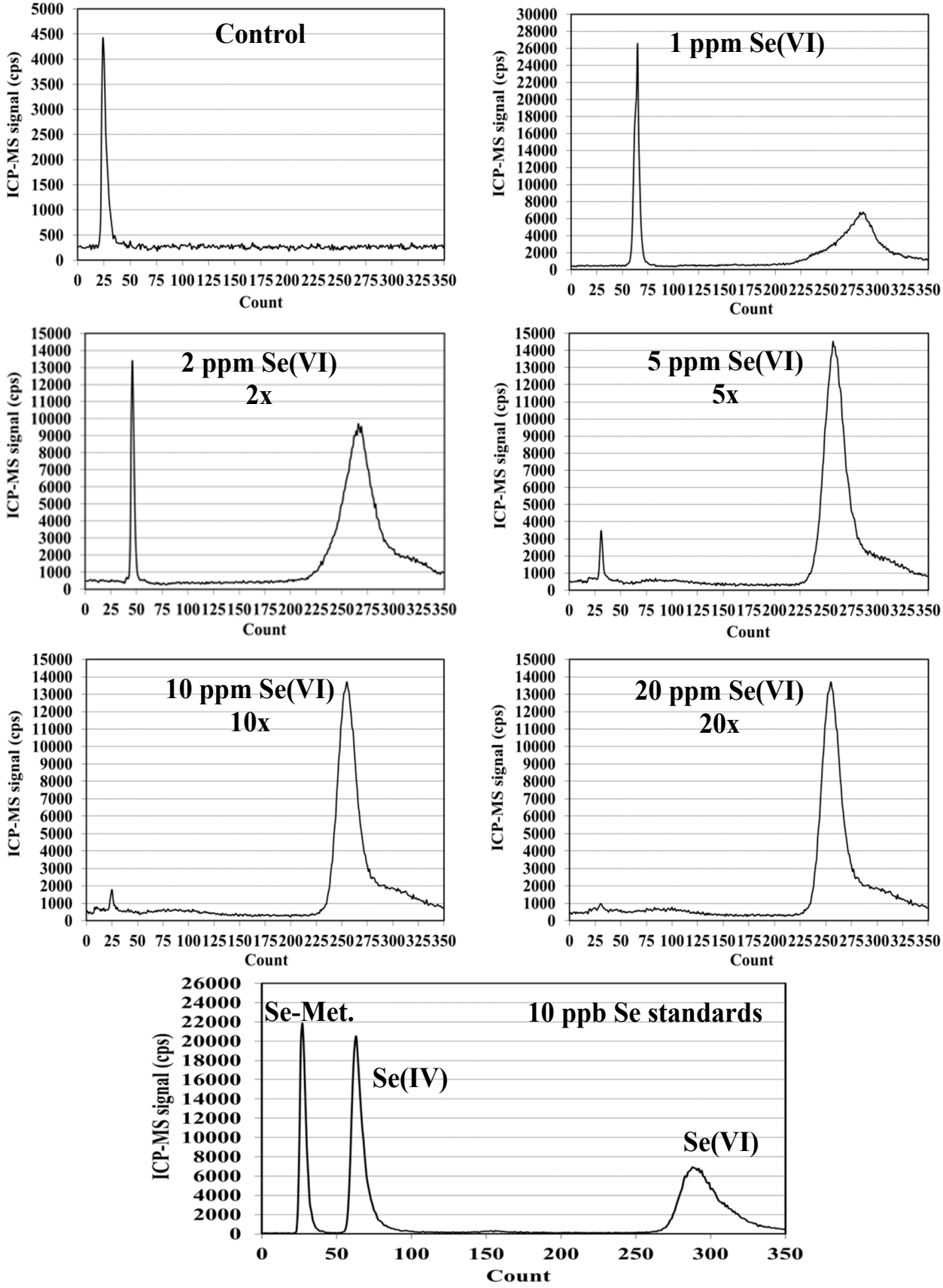

Figure 6. Selenium content in MRS media supernatant of *L. casei* incubated with different concentrations of Se(VI) at 37°C for 24hr measured as count per second (cps).

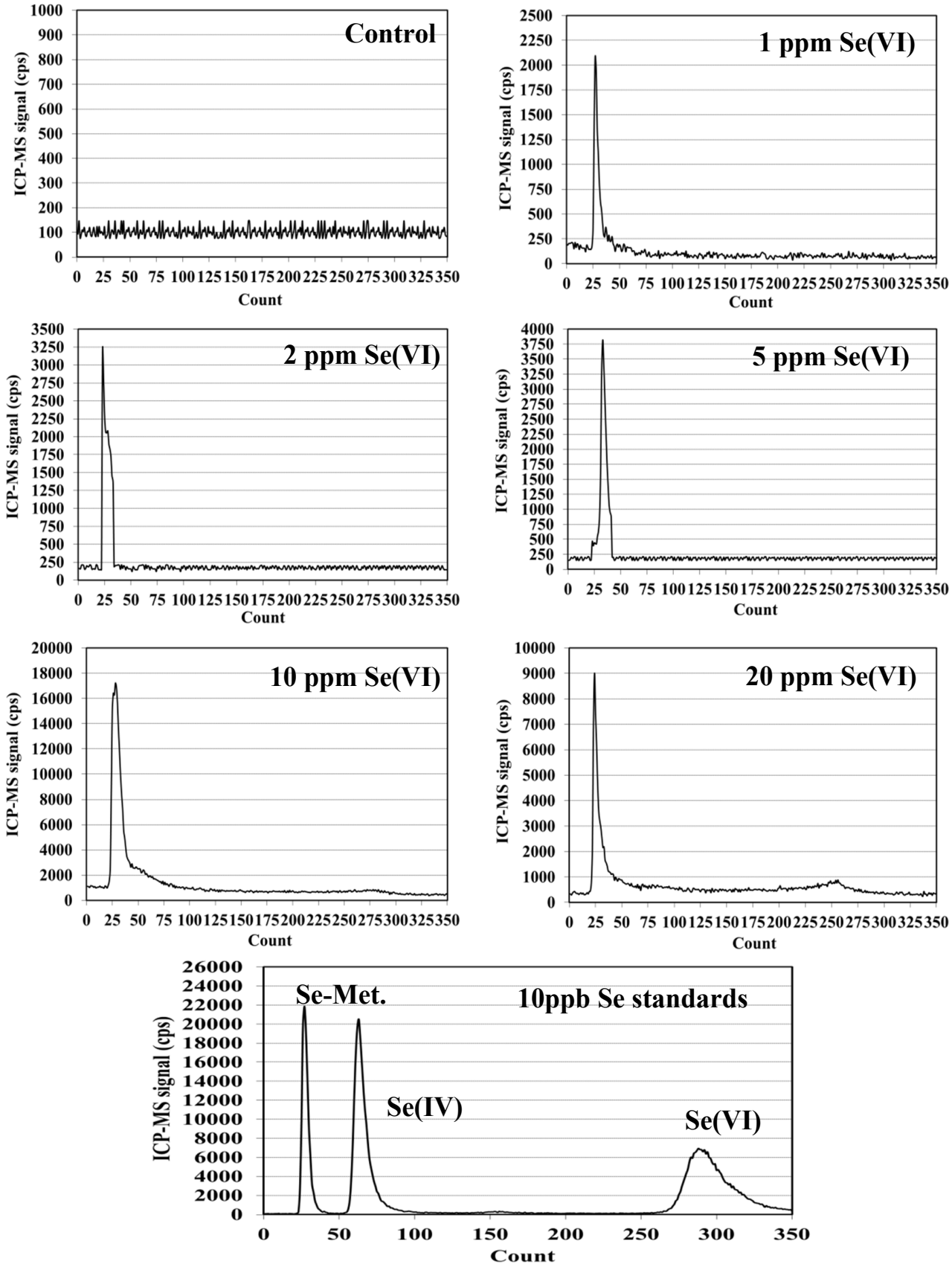

Figure 7. Selenium content in cell fraction of L. casei incubated in MRS media with different concentrations of Se(VI) at 37°C for 24hr measured as count per second (cps).

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