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Metallomic study of selenium biomolecules metabolized by the microalgae *Chlorella sorkiniana* in the biotechnological production of functional foods enriched in selenium*

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Abstract: The optimization of culture conditions for selenium (Se) bioaccumulation in the microalgae *Chlorella sorokiniana* growth in a Na_2SeO_4-enriched medium was explored, in order to obtain a suitable approach for the biotechnological production of a Se-enriched food. Se concentration (as Na_2SeO_4) in the culture medium until 100 μg ml^{-1} allows the growth of algae colonies during long periods of time, until 300 h, but higher concentrations cause the collapse of the colony. The bioaccumulation process causes a concentration of Se in algae in the order of 3 μg g^{-1} in about 100 h. A metallomic analytical approach based in the coupling high-performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS), which uses two chromatographic switched columns (C-18 and chiral columns) with ICP-MS detection, has been applied to characterize the different Se metabolites, including chiral forms, in both the algae and culture medium. The results reveal that selenate present in the culture is biotransformed in selenocystine ($SeCys_2$), selenomethylselenocysteine (SeMeSeCys), and mainly selenomethionine (SeMet) by the algae, although appreciable concentration of Se(VI) is also present in cells. When algae are cultured under sulfur (S) deficiency conditions, the accumulation of Se in the cell is enhanced owing to the great chemical similarity between S and Se that promotes the substitution of S by Se in the cell metabolism, therefore, SeMet concentration in the algae increases from about 7 to 15 μg g^{-1} and Se(VI) from 15 to 25 μg g^{-1}, after 500 h of exposure.

Keywords: chemical speciation; functional food; high-performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS); liquid chromatography; metallomics; selenium.

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INTRODUCTION

Selenium (Se) is an essential element with important biological functions associated with its antioxidative action, regulation of thyroid hormone metabolism, and cell growth [1]. Se is a key element for important biomolecules such as the enzyme glutathione peroxidase [2] and other selenoenzymes or selenoproteins [3], which are involved in the removal of hydrogen peroxide and lipid peroxides produced during oxidative process in cells. In mammals, Se deficiency has been associated with muscular, neurological, and immune disorders, and also with an increase in cancer incidence [4,5]. In addition, Se deficiency in humans may be related to several diseases such as ischemic heart disease [6], Keshan disease [7], rheumatoid arthritis [8], and others. For this reason, many Se supplements and selenized foods have been proposed to avoid the problems related with the deficiency of this element. One important question is the chemical forms in which Se is ingested, which affects their toxicity, nutritional value, and metabolic fate [9]. The chemical forms of Se depend on the food or supplement considered [10], thus, selenized vegetables such as broccoli, tomato leaves, and cucumbers, accumulate Se as selenite, whereas wheat grain, corn, rice, soybeans, and yeast, predominantly accumulate selenomethionine (SeMet), similarly to some edible mushrooms [11]. However, garlic, onions, and sprouts mainly accumulate Se-methylselenocysteine (SeMeSeCys) and γ-glutamyl-Se-methylselenocysteine [12–14]. Lobinski et al. [15] claimed for a deeper analysis of Se-containing molecules in Se-supplemented foods, using yeast as a model, since only a few molecules of low molecular weight were usually identified but no selenocompounds with molecular masses exceeding 1000 Da, including Se-containing proteins. Nevertheless, the knowledge of virtually all forms of Se present in the supplemented food candidate is also important to fulfill with regulations of governmental agencies issuing sales authorizations [16]. For this reason the development of methods allowing the qualitative and quantitative characterization of the set of Se metabolites is necessary [17] and at the same time is a precious tracer of the origin of the product and the biotechnological process involved [18].

Few studies consider the use of algae as Se bioaccumulator, the microalgae *Spirulina platensis* and its processing products have been employed as feed and food additives in agriculture, food industry, pharmaceutics, and recently as food supplement [19,20], although it has been constated that Se from Se-rich spirulina was less effective and less bioavailable than Se from sodium selenite and SeMet in repleting GSHPx activity and Se presence in most organs and tissues of Se-depleted rats [20]. Exposure experiments to selenite have been carried out with unicellular marine algae, namely, *Dunaliella primolecta*, *Porhyridium cruetum*, and *Chlorella* sp. [21] in which Se was accumulated in several biochemical components, including proteins, amino acids (*D. primolecta* and *Chlorella* sp.), and lipids (*P. cruetum*), as well as soluble carbohydrates (*D. primolecta* and *Chlorella* sp.). Another study considers the biotransformation of arsenic in the microalgae *Chlorella vulgaris* reveals that major species in a methanol/water (1:1) extract, after exposure to arsenate, is also arsenate (87–100 %), with small amounts of arsenite, dimethylarsinic acid, and three different arsenosugars [22]. The microalgae *C. vulgaris* has been studied by Burianova et al. [23] for iodine (I) accumulation with the purpose to be used as food supplement and a preliminary study for the characterization of I species accumulated by this microalgae has been performed [24]. The algae *C. vulgaris* has been used as model eucaryotic organism to study the bioconcentration and metabolism of Se and other metals; this algae induces the synthesis of phytochelatins in response to Se exposure [25], which has a potential significance if it can increase the storage of dietary Se when the algae is used as dietary supplement. On the other hand, the antagonistic interactions between sulfate and Se in *C. vulgaris* was observed by Shrift [26] in 1954; sulfate and selenate compete for uptake at the sulfate ion transporter(s) [26–28] and also may share the same assimilation pathways [27,29,30]. Under S starvation, algae are more susceptible to Se uptake [31], altough the toxic action of Se can also be enhanced.

A study from Stadtman [32] about Se in *C. sorokiniana* reveals that three major amino acids were found in protein hydrolysates: selenocysteic acid, SeMeSeCys, and Se-methylselenocysteine selenoxide. On the other hand, the prevailing fraction of Se analogues in free amino acids and proteins of

C. sorokiniana is a mixture containing selenocysteine (SeCys), selenocystine ($SeCys_2$), and SeMet. The results suggest that the seleno-aminoacid pattern of algae exposed to sublethal Se concentrations depends on the stage of the algae growth. It has also been established that when exposed to sublethal, but above trace levels of Se, the algal cells tend to substitute Se for sulfur (S). Thus, under overloading conditions, Se appears to use the S enzymatic system, while under normal levels, Se specific enzyme systems seem to be in operation.

The present work considers the optimization of Se bioaccumulation in *C. sorokiniana* from enriched Se media. The characterization of Se species in the algae and culture medium, including organic and inorganic species as well as chiral SeMet forms was performed using a metallomic approach based on high-performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS). Experiments under deficiency of S conditions in the culture medium and studies about change of Se speciation during algae assimilation process were also performed in order to get a biotechnological approach suitable for the production of functional food enriched in Se.

MATERIALS AND METHODS

Instrumentation

Chromatographic separation and detection of Se species has been performed with a HPLC system Agilent 1100 series (Agilent Technologies, Waldbronn, Germany). Two columns were connected using a column-switching system comprising two Rheodyne valves model 7725i (Bellefonte, PA, USA): Phenomenex Luna C18 (250 × 4.60 mm, 5 µm, 100 Å pore size) and Astec Chirobiotic T columns (250 × 4.6 mm, 5 µm). The injection volume was 50 µL. The column outlet was directly connected to the nebulizer of the ICP-MS system by PEEK tubing. Elemental detection was performed using an ICP-MS Agilent 7500ce (USA) equipped with a Micromist nebulizer.

An ultrasonic probe (HD 2200, Bandelin electronic, GmbH & Co. Kg, Berli) with a MS 72 tip was used for the extraction of the species. A centrifuge model Sigma Laborzentrifugen 4-10 (Osterode, Germany) was used to accelerate the phase separation process in the extraction of the compounds.

The total chlorophyll and carotenoids was calculated from the absorbance data obtained with a UV–vis spectrometer (Ultrospec 3100 pro, Biochromm Ltd.). The photosynthetic activity was determined by using a Clark electrode (Hansatech, UK). A Neubauer camera and an optical microscope Olympus model CX41 was used to measure the number of cells.

Mineralization of algae samples to determine the total content of Se was performed by using a microwave-accelerated reaction system model MARS (CEM Corporation, Mattews, NC, USA) and MARSXpress vessels.

Standard solutions and reagents

All reagents used were of highest purity. The following reagents were purchased from Sigma-Aldrich (Steinheim, Germany): KH_2PO_4, $Na_2HPO_4{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, KNO_3, EDTA, $Na_2EDTA{\cdot}2H_2O$, H_3BO_3, $MnCl_2{\cdot}4H_2O$, $ZnSO_4{\cdot}7H_2O$, $CuSO_4{\cdot}5H_2O$, $NaHCO_3$, DL-selenomethionine (DL-SeMet), L-selenomethionine (L-SeMet), SeMeSeCys, $SeCys_2$, and Na_2SeO_3 were purchased from Sigma-Aldrich (Steinheim, Germany). Na_2SeO_4 and tetraethylammonium chloride (TEAC) were obtained from Fluka (Buchs, Switzerland).

Aqueous stock standard solutions of 1000 mg Se l^{-1} were prepared from DL-SeMet and L-SeMet in ultrapure water, Se-MeSeCys, and $SeCys_2$ in 3 % (v/v) HCl solution, sodium selenate (Na_2SeO_4) in 2 % (v/v) HNO_3 solution, and sodium selenite (Na_2SeO_3) in 2 % (v/v) HCl solution.

Working solutions were prepared daily by stepwise dilution of their stock solutions with high-purity deionized water. Ultrapure water (18 MΩ cm) was obtained from a Milli-Q water-purification

system and was used throughout (Millipore, UK). All the standard solutions were stored at 4 °C in darkness until the analysis.

The chromatographic mobile phase was prepared daily as follows: 0.075 % TEAC aqueous solution at pH 4.5. The pH of mobile phase was adjusted with HCl and NaOH solutions (Merck, Darmstadt, Germany). Protease type XIV was supplied by Sigma-Aldrich (Steinheim, Germany).

A tuning solution containing Li, Y, Tl, and Ce (1 mg l^{-1} each) was purchased from Agilent Technologies (USA).

Determination of total selenium

For the determination of total Se in the culture medium, the supernatant obtained from the centrifugation of the cultures was filtrated through a 0.45-µm filter and an aliquot of 5 ml was digested with 6 ml of HCl (37 % w/v) and 2 ml of HNO_3 (65 % w/v). The samples were digested from room temperature to 165 °C in 10 min and hold for 25 min at this last temperature.

For the determination of Se in *Chlorella*, the pellet obtained from the centrifugation of the culture was lyophilized and an amount of 0.040 g was accurately weighted and submitted to acid digestion with 8 ml of HNO_3 (65 % w/v) and 2 ml of H_2O_2 (30 % w/v). The samples were digested from room temperature to 175 °C in 15 min and hold for 40 min at this last temperature.

The digested solutions were filtered through 0.45 µm PVDF filters and the elements measured by ICP-MS using the operational conditions shown in Table 1.

Table 1 Operating conditions for ICP-MS.

Forward power	1500 W
Sampling depth	7–8 mm*
Auxiliary gas flow rate	0.10–0.15 ml/min*
Extract 1	0–3 V*
Extract 2	–137.5 V*
Omega Bias-ce	–20 V
Omega Lens-ce	–1.6 V
Cell entrance	–40 V
QP Focus	–15 V
Cell Exit	–44 V
OctP RF	190 V
OctP Bias	–18 V
H_2 flow	3.8 ml/min
QP Bias	–16 V
Discriminator	8 m V
Analog HV	1840 V

Extraction of selenium species

The culture of *Chlorella* was centrifuged to separate the pellet from the medium. The medium was filtrated using 0.45-µm filters (PVDF) and then was directly injected into the HPLC-ICP-MS analysis. Liquid nitrogen was applied to the pellet to break the cells wall and an amount of 0.020 g was weighted in a centrifuge tube, then 0.02 g of protease XIV was added. The extraction was performed with the assistance of an ultrasonic probe at 25 % power during 2 min. After the extraction, the sample was centrifuged for 5 min at 6000 rpm and the supernatant collected. Finally, the supernatant was filtered through 0.45 µm and injected into HPLC-ICP-MS for analysis.

Determination of selenium species

The instrumental approach for the determination of Se species is based on reversed-phase chromatographic separation coupled to ICP-MS detection. The optimal operation conditions for ICP-MS detection are similar to those for the determination of total Se (Table 1). The isotopes ^{77}Se, ^{80}Se, and ^{82}Se were monitored for analysis, but only ^{80}Se was used for quantification. A solution containing Li, Y, Tl, and Ce (1 μg l^{-1} each) prepared in the mobile phase was used to tune the ICP-MS for sensitivity, resolution, percentage of oxides, and doubly charged ions.

The chromatographic separation was performed on the basis of a previously described instrumental coupling [33]. Briefly, (a) when chiral species are not separated the sample is loaded in the first Rheoyne valve at the inject position, which allows mobile phase flowing through the C18 column and then entering the second valve that is maintained in the load position, therefore the mobile phase get the ICP-MS detector; (b) when chiral species are separated, the chiral column is activated after $SeCys_2$ and Se-MeSeCys elution (5 min), changing the valve 2 to the inject position, which allows both columns to work in series, therefore L- and D-SeMet enter the chiral column where they are retained. After 6.7 min, valve 2 is moved again to the load position and Se(VI) elutes from the C18 column. Finally, from 9 to 16 min, the chiral column is again activated allowing L- and D-SeMet separation. The retention times for Se species when the chiral column is not activated are: 3.0 min ($SeCys_2$), 4.3 min (SeMeSeCys), 7.2 min (SeMet), and 8.3 min [Se(VI)], these values changes when two columns work in series and chiral forms are separated, as follows: 3.0 min ($SeCys_2$), 4.3 min (SeMeSeCys), 8.1 min [Se(VI)], 13.8 (L-SeMet), 14.8 min (D-SeMet). Se(VI) is allowed to elute before SeMet in the second chromatographic approach to avoid peak broadening. Chromatographic performance was checked regularly by measuring control standards to ensure a suitable separation between species and sensitivity of the method after a considerable number of samples

Preparation of *C. sorokiniana* cultures

Microalgae *C. sorokiniana* CCAP 211/8K was obtained from the UTEX culture collection. It was maintained in modified M-8 medium [34] with 160 μmol of photons m^{-2} s^{-1} (continuous illumination) at 30 °C. The culture medium was prepared as follows (composition expressed in mol l^{-1}): KH_2PO_4, 5.4×10^{-3}; $Na_2HPO_4 \cdot 2H_2O$, 1.5×10^{-3}; $MgSO_4 \cdot 7\ H_2O$, 1.6×10^{-3}; $CaCl_2 \cdot 2H_2O$, 0.9×10^{-4}; KNO_3, 30×10^{-3}; EDTA, 0.3×10^{-3}; $Na_2EDTA \cdot 2H_2O$, 0.1×10^{-3}; H_3BO_3, 1.0×10^{-6}; $MnCl_2 \cdot 4H_2O$, 0.7×10^{-4}; $ZnSO_4 \cdot 7H_2O$, 0.1×10^{-4}; $CuSO_4 \cdot 5H_2O$, 0.7×10^{-5}; $NaHCO_3$, 5×10^{-3}. The pH was adjusted to 6.7 using NaOH. The sterilization of the medium was carried out during 20 min at 120 °C. The medium was constantly bubbled with air containing 5 % (v/v) CO_2 for culture homogenization. Cells growth in Roux flasks (5 L) of borosilicate glass Pyrex® supervised with the optical microscope to check the culture viability and asepsis. From these cultures, samples were taken for subsequent analysis.

Bioaccumulation experiments on *C. sorokiniana*

Exposure experiments were performed with increasing concentrations of SeO_4^{2-} in the media, from 20 to 500 μg ml^{-1}. The concentration of Se in the culture medium was evaluated every day.

Measurement of the biochemical parameters

Algae dry mass was calculated using 0.45-μm filters (cellulose acetate). The filters were cleaned with Milli-Q water and dried at 80 °C during 24 h in an oven. Then, 5 ml of the culture was filtered and dried. The dry mass was calculated by weight difference.

The total content of chlorophyll and carotenoids was determined using the Arnon method. 1 ml of the culture was centrifuged during 5 min at 4400 rpm. The supernatant was discarded and 4 ml of

methanol was added to the residue for chlorophyll extraction by shaking during 20 s. The sample was incubated during 30 min at 60 °C and then during 10 min at 0 °C. After that, the sample was centrifuged during 5 min at 4400 rpm obtaining a white pellet. The UV–vis absorbance was measured at 665, 653, and 470 nm. Total content of chlorophyll and carotenoids was calculated using the equations proposed by H. Lichtenthaler [35].

RESULTS AND CONCLUSIONS

Bioaccumulation of selenium in *Chlorella*

Suitable conditions for the accumulation of Se in *Chlorella* are strongly depending on Se concentration in the culture medium. Increasing concentrations of inorganic Se (SeO_4^{2-}) from 20 to 500 μg ml^{-1} were tested in a culture medium containing nutrients such as phosphate, nitrate, and sulfate, and trace elements such as Mn, Zn, Cu, and B. It has been considered the highest concentration of Se (as selenate) in the culture medium that allows longer times of exposure and therefore a high intake of this element. The concentration of chlorophyll is a good marker to evaluate the metabolic status of algae culture and rate of growth. How it can be seen in Fig. 1, algae culture grows acceptably during 100 h for Se concentrations in the medium ranging from 20 to 75 μg ml^{-1} (Fig. 1a). For high concentrations of Se, until 200 μg ml^{-1}, the culture only grows in a suitable manner during 75 h (Fig. 1b) but higher concentrations cause colony collapse. Figure 2 shows the behavior of algae culture by longer periods of time, until 12 days, using concentrations of selenate of 50 and 100 μg ml^{-1}. Both levels of Se in the medium allow successful algae growth during long periods, and a final compromise was adopted for 50 μg ml^{-1} that was used in further experiments.

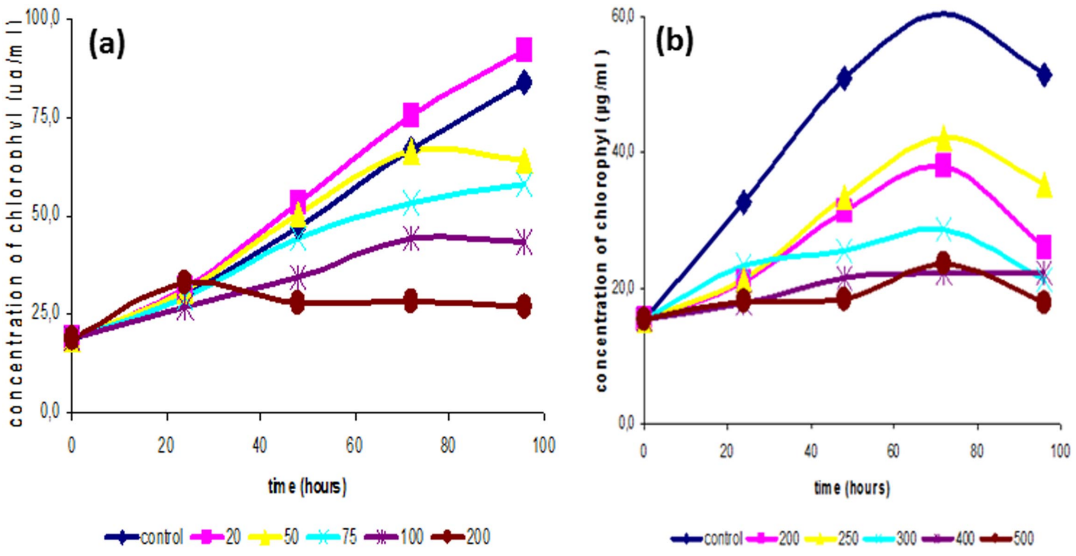

Fig. 1 Algae culture growth in presence of increasing concentrations of Se (SeO_4^{2-}), μg ml^{-1}.

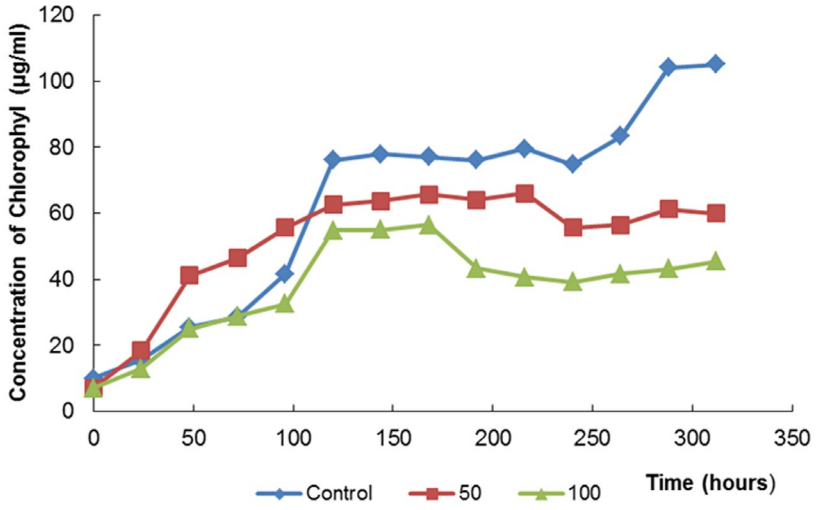

Fig. 2 Algae culture growth for long periods in presence of Se (SeO_4^{2-}), µg ml^{-1}.

It can be seen (Fig. 3) how the Se concentration in the culture medium decreases with the time of exposure, increasing correlatively the presence of Se in the cells. A concentration of about 3 µg g^{-1} of Se in the pellet was found after 100 h of exposure.

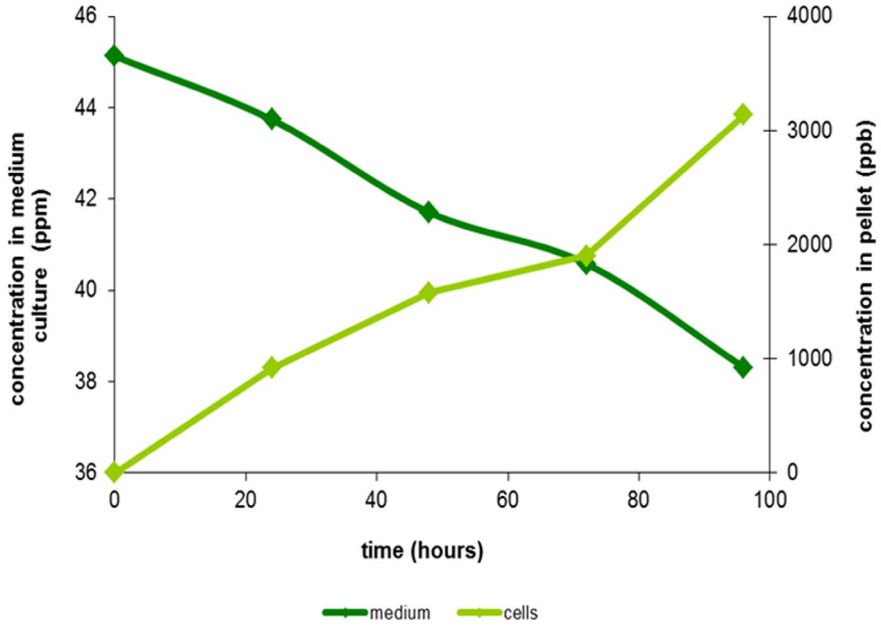

Fig. 3 Se concentration in culture medium and algae cell during a exposure experiment (starting levels of Se in the medium 50 µg ml^{-1}).

Presence of selenium metabolites in the algae

A study of Se species in the pellet was performed after extraction with Protease XIV, by using the coupling reversed-phase (RP) HPLC-ICP-MS. As it you can see in Fig. 4, selenate is biotransformed in $SeCys_2$, SeMeSeCys, and SeMet, mainly in this latter that rise a concentration of about 2.5 μg g^{-1} in the pellet over 200 h. However, inorganic Se [Se(VI)] is also accumulated in the pellet in a remarkable concentration (about 1.5 μg g^{-1}), which can impair the use of *Chlorella* as food due to the higher toxicity of this specie of Se. Other Se species such as $SeCys_2$ and SeMeSeCys are also present in the pellet but at lower concentrations (in the order of 500 ng g^{-1}).

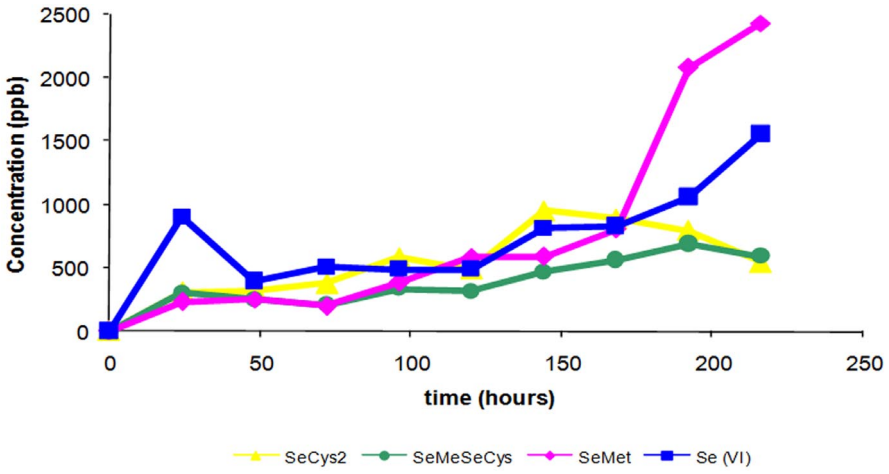

Fig. 4 Bioaccumulation of Se species in *C. sorokiniana* exposed to 50 μg ml^{-1} in the culture medium.

Algae culture under sulfur-deficiency conditions

The essential character of S is well known for a suitable growth of algae culture. The sulfate ions cross the cell wall to be reduced until sulfide following the typical pathway for sulfur assimilation in algae, which is incorporated into the carbon skeleton of *o*-acetylserine (OAS) to form the S amino acid L-cysteine. This process occurs under control of two enzymatic reactions addressed by serine acetyltransferase (SAT) and *o*-acetylserine(thiol)lyase (OASTL). The OASTL transforms the S in L-cysteine (Fig. 5), however, when Se (SeO_4^{2-}) crosses the cell wall it can also participate in the same process producing $SeCys_2$. Other S and Se compounds such as methionine, SeMet, and glutathione can also be synthetized from the previous species. It has been reported that uptake of selenate in *C. vulgaris* can be performed through a shared high-affinity sulfate ion transporter [27,28]. Expression of the high-affinity sulfate ion transporter genes is regulated by either the availability of sulfate, the demands for reduced S compounds (e.g., glutathione and cysteine), and the supply of precursors needed in the cysteine assimilatory pathway (e.g., OAS) [27,36,38]. Reduced-S compounds regulate this expression in the form of a negative-feedback loop.

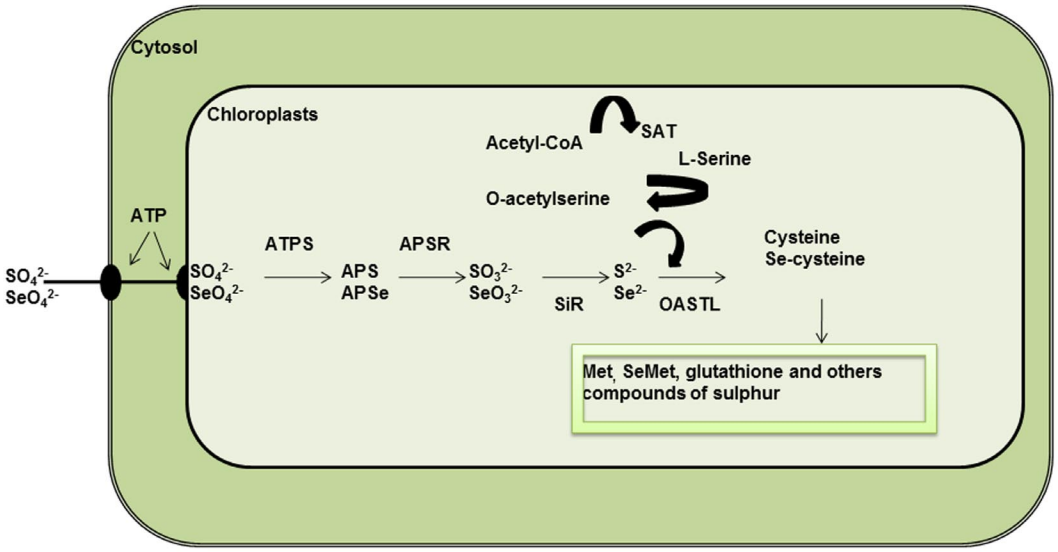

Fig. 5 S/Se metabolism pathway scheme in algae.

Therefore, algae cultured in a S-deficient medium could enhance the accumulation of Se in the cell [38]. In addition, the enzymatic activity of OASTL depends on the level of S or Se in the cell, which can be used as marker of S- or Se-compounds production along the algae metabolism. Figure 6 shows the results of different experiments performed with increasing deficiency of S in a culture medium that maintains a concentration of 50 μg ml^{-1} of Se. It can be seen that OASTL enzymatic activity is retained, although S level in the medium decreases, which assures cell function. However, the algae colony collapses in the total absence of this element.

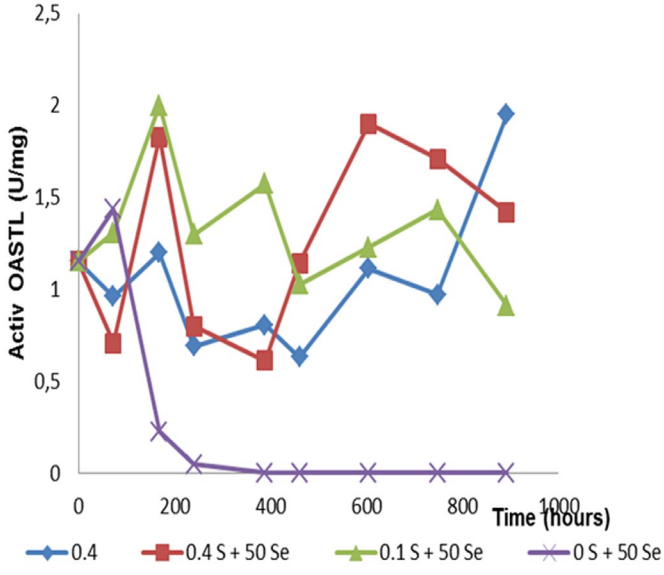

Fig. 6 Enzymatic activity of OASTL in deficiency of S: **0.4S**, culture containing 400 μg ml^{-1} of S; **0.4S + 50Se**, culture with 400 μg ml^{-1} of S and 50 μg ml^{-1} of Se; **0.1S + 50Se**, culture with 100 μg ml^{-1} of S and 50 μg ml^{-1} of Se; **0S + 50Se**, culture in absence of S with 50 μg ml^{-1} of Se.

Figure 7 shows the concentration of Se species in the algae under exposure to conventional (0.4S + 50Se) and S-deficient (0.1S + 50Se) conditions. It can be observed that concentration of SeMet in the algae, after 500 h of exposure, increases from about 7 to 15 μg g^{-1} when the concentration of sulfur decreases in the culture medium from 0.4 to 0.1 M, although the concentration of Se(VI) also increases and exceeds SeMet.

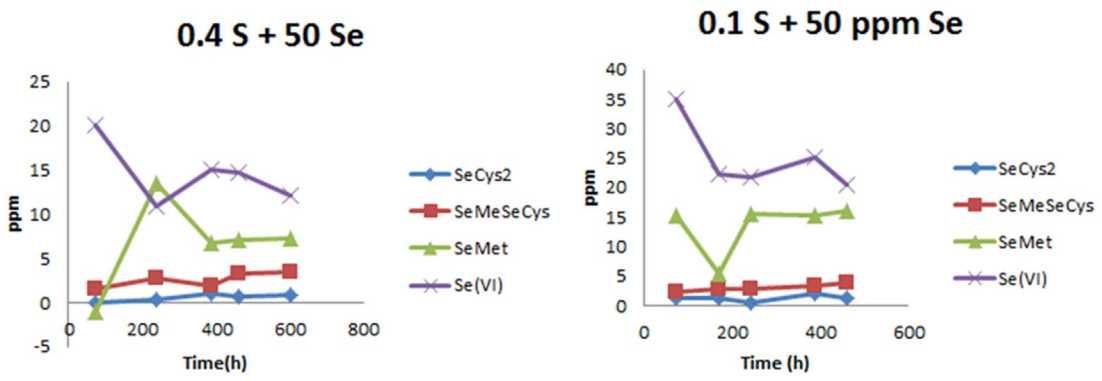

Fig. 7 Concentration of Se species along the time in the algae under Se exposure. **0.4S + 50Se**, culture with 400 μg ml^{-1} of S and 50 μg ml^{-1} of Se; **0.1S + 50Se**, culture with 100 μg ml^{-1} of S and 50 μg ml^{-1} of Se.

Changes of selenium speciation during the algae assimilation process

The transformations suffered by Se species were studied in both algae and culture medium during 100 h. The total concentration of Se in the culture solution decreased from 45 to 38 μg ml^{-1} in this period of time (Fig. 3), remaining as Se(VI), which denotes the absence of traffic of this element from the algae colony to the culture solution. However, both Se(VI) and SeMet were the predominant Se species into the algae, Fig. 4, in which Se is bioaccumulated up to 3 μg g^{-1}.

A series of experiments using chiral separation were performed to evaluate the chirality of SeMet in the algae. The resulting chromatogram revealed that SeMet was present as L-SeMet while D-SeMet was practically absent. Therefore, Se(VI) after passing the cell wall is transformed into L-SeMet, which is the active form of this element in *Chlorella*.

CONCLUSIONS

The microalgae *C. sorokiniana* can be used for the bioaccumulation of Se in the production of functional food enriched in this element. The optimization of algae growth conditions to promote Se bioavailable species accumulation from the culture medium has been combined with a metallomic analytical approach to identify and quantify the Se species in which the algae transforms the inorganic Se from the medium. The instrumental coupling is based on a bidimensional chromatographic separation by in series switching of a reversed-phase column (C-18) and a chiral column (Chirobiotic T), which allows the identification of $SeCys_2$, SeMeSeMet, Se(VI), and L-SeMet in the algae burden, however, no changes were observed in Se speciation in the culture medium that remains as Se(VI) along the study.

Suitable conditions for L-SeMet accumulation in the algae were obtained by exposure to 50 μg ml^{-1} of SeO_4^{2-} in the culture medium during 100 h, nevertheless, a remarkable concentration of the more toxic Se(VI) species was also observed in the algae.

Complementary experiments under S-deficient conditions in the culture revealed the enhancement of SeMet concentration in the algae, although the concentration of Se(VI) also increased.

Further studies have to be conducted to develop analytical approaches to identify Se incorporated into proteins (selenoproteoma) by coupling size exclusion chromatography with ICP-MS in combination to mass spectrometry. In addition, tests to assure the algae Se species bioavailability by mammals and finally by humans will be necessary.

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