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Biogenic production of selenocysteine by *Enterococcus faecium* ABMC-05: an indigenous lactic acid bacterium from fermented Mexican beverage

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Abstract

Lactic acid bacteria have been studied for their ability to accumulate inorganic selenium, which is reduced to its elemental form or could be integrated into selenoproteins in the form of selenocysteine. This ability to produce bioavailable organic selenium for humans is an advantage that could be exploited in the production of functional foods. In this work, *Enterococcus faecium* ABMC-05 isolated from a traditional Mexican beverage (tepache) and with NCBI entry number OL413240 was used. Microorganism selenization was performed after the determination of minimal inhibitory concentration of Na_2SeO_3 (184 mg/L), which was calculated using the graphical method of Talmadge and Fitch. The selenium concentration of accumulated selenium was calculated by inductively coupled plasma optical emission spectroscopy (ICP-OES). The concentration of selenium at 48 h of fermentation was 39.92 mg/L, which represented 23.8% of selenium at medium. This value was consistent with those reported for other lactic acid bacteria. Finally, it was determined the selenocysteine presence in biomass recovery after fermentation using RP-HPLC technique. With these results it was confirmed the biogenic production of selenocysteine by *Enterococcus faecium* ABMC-05 using an inorganic source.

Keywords: selenium; slenocysteine; lactic acid bacteria; *Enterococcus*; selenization.

Practical Application: Biogenic production of selenocysteine by lactic acid bacteria isolated from traditional Mexican fermented beverage is an opportunity to confront challenges in the new starters field, making these kinds of bacteria an important ingredient in fermented food manufacture.

1. Introduction

Selenium (Se) is an essential trace element that has important biological functions for human health. Unlike the other elements, Se is incorporated into proteins through a selenocysteine (SeCys) using a co-translation mechanism (Sumner et al., 2019). This amino acid is considered number 21 and is part of the synthesis of selenoproteins. So far, 25 genes that code for selenoproteins have been identified, which are mainly enzymes (Gladyshev et al., 2016; Kryukov et al., 2003; Schmidt & Simonović, 2012). Thus, the biological effects of SeCys are related to its structural integration in functional selenoproteins, which are involved in physiological processes such as antioxidant defense (glutathione peroxidase), and redox regulation of cellular processes (thioredoxin reductase). In addition, some selenoproteins are known to perform more specific essential functions, such as iodothyronine deiodinases (IODs), which participate in the metabolism of thyroid hormones. Likewise, GPx4 is essential for spermatogenesis, and selenophosphate synthetases 2 (SPS2) participate in the selenoproteins biosynthesis (Winther et al., 2020; Qazi et al., 2019; Santos et al., 2018).

On the contrary, a deficiency of Se is closely associated with hypothyroidism, cardiovascular diseases, cancer development, diabetes mellitus, and diseases of the immune system (Kawai et al., 2018). Likewise, the intake of Se worldwide depends on its concentration in the soil and the ability of edible plants to accumulate it (Sullivan et al., 2013). Due to the interest in consumption of organic Se as SeCys, which is more efficiently retained in organisms and tissues than inorganic Se (selenate and selenite), research has focused on its potential use as a functional ingredient. In this sense, Se-enriched yeasts and lactic acid bacteria (LAB) represent a possibility because they have been considered as a source of organic Se (Adadi et al., 2019; Martínez et al., 2019; Medina Cruz et al., 2018). Therefore, Se supplementation using microorganisms has received attention in the last decade.

Most LAB are considered GRAS (generally recognized as safe) microorganisms and have been used in the food industry to produce fermented foods and beverages (Mathur et al., 2020; Mora-Villalobos et al., 2020). In addition, several scientific studies have shown the usefulness of certain LAB as probiotics for humans and animals, which have been associated with beneficial effects such as antimicrobial, immunomodulatory, anticancer, antidiarrheal, anti-allergic, and antioxidant activities

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(Reuben et al., 2020). In recent years, it has been reported that LAB strains bioaccumulate and biotransform Se from the growth medium through a detoxification mechanism, which reduces inorganic Se to its elemental form (Se^0) as a nanoparticle or produce organic species such as selenocysteine, selenomethionine, and methylselenocysteine (Tugarova et al., 2020; Rehan et al., 2019; Debieux et al., 2011; Turner et al., 1998). In this way, it has been possible to develop probiotic LAB with Se and thus increase the health benefits and nutritional value of foods containing these microorganisms. Therefore, in this study, the objective was to isolate a lactic acid bacterium from a traditional Mexican fermented beverage to develop it as a Se-enriched microorganism and evaluate its ability to accumulate and biotransform it to SeCys.

2 Materials and methods

2.1 Microorganism

The microorganism used was previously isolated from "tepache", which is a typical Mexican fermented beverage (Escobar-Ramírez et al., 2020) and was identified as *Enterococcus faecium* ABMC-05 with the NCBI entry number (OL413240). The frozen-preserved strain (-20 °C in glycerol 1:1) was activated by subculturing three times in Man Rogosa Sharpe broth (MRS, BD Difco Laboratories, Thermo Fisher Scientific Inc.). The incubation conditions were 37 °C for 12 h in anaerobiosis. With the streak-plate procedure, a mixture of cells was spread on the surface of solid MRS-agar (BD Difco) in a Petri dish (BD Difco), and after 24 h of incubation at 37 °C, a pure colony was placed in 10 mL of MRS broth, incubating again at 37 °C for 12 h. Subsequently, the culture was plated with 15% (v/v) glycerol in eppendorf tubes and stored at -20 °C to be used as a working strain. To verify the morphology of the microorganism, a Gram stain was performed.

2.2 Determination of the minimum inhibitory concentration of Na_2SeO_3

The determination of the minimum inhibitory concentration of Se in *Enterococcus faecium* was carried out according to Castañeda-Ovando et al. (2019), with some modifications.

Preparation of the medium with selenite. It was inoculated 0.1 mL (1 x 10^9 CFU/mL) of *Enterococcus faecium* in 5 mL of MRS broth supplemented with Na_2SeO_3 (Sigma Aldrich) at different concentrations: 0, 100, 200, 300, 400 and 500 mg/L. A stock solution of 1000 mg/L Na_2SeO_3 in deionized water was prepared to obtain the desired concentrations. This solution was sterilized through a 0.22 µm sterile syringe filter (Millipore) and immediately supplemented with MRS medium into the tubes.

Determination of growth. The experiment was carried out at 37 °C for 36 and 48 h of incubation under anaerobic conditions. At the end of fermentation, pH (Conductronic pH120) and growth of cells by optical density at 600 nm (OD_{600}) (UV-1240, Shimadzu, Kyoto, Japan) were measured. The control consisted of MRS supplemented with sodium selenite without bacteria. The entire experiment was carried out in separate tubes.

Talmadge and Fitch graphical method. The minimum inhibitory concentration (MIC) was calculated according to the Talmadge and Fitch graphical method modified by González-Olivares et al. (2016). With the OD_{600} data obtained, a graph was made and it was adjusted to a polynomial trend line of third order. The graph of the polynomial function was obtained and the following protocol was carried out: a) The minimum point of the graph was determined by means of the second derivative of the function and with this value a tangent line (t) was drawn to the curve; b) A slope of the upper region of the curve (y=ax+b) was plotted; c) The slope line and the tangent line were extended to their intersection; d) Once the intersection point was obtained, a bisector was drawn until intercepting the polynomial curve, from which a tangent was obtained and e) This tangent was extended until it intersected the tangent of part a, this intersection was called CMI. The graphs and equations were developed with the Geogebra software (5.0.536.0-d, 2019).

2.3 Enterococcus faecium ABMC-05 growth to MIC of Na_2SeO_3

The bacteria were inoculated (100 µL; 1 x 10^9 CFU/mL) in tubes with 5 mL of MRS-broth and Na_2SeO_3 at the calculated minimum inhibitory concentration. Fermentation was carried out at 37 °C for 48 h under anaerobic conditions. As a control, a culture inoculated in MRS broth without Na_2SeO_3 was used. Samples were taken from separate tubes every 2 h. The pH and biomass concentration from each sampling by OD_{600} to build the growth curve, were measured.

2.4 Determination of total Se concentration by inductively coupled plasma optical emission spectroscopy (ICP-OES)

The methodology of Castañeda-Ovando et al. (2019) with some modifications was used. The biomass produced was collected in eppendorf tubes at 0, 24 and 48 h. In order to separate biomass from the medium, 1 mL of the sample was centrifuged at 10,000 x *g* for 15 minutes at 4 °C. Cells were washed in 100 µL of 0.3% (w/v) di-thiothreitol (DTT) and centrifuged (10,000 x *g* for 15 minutes at 4 °C) to release any possible Se bonds with amino groups of membrane proteins. The supernatant from the first and second centrifugation were mixed to determine residual Se. Biomass was dried at 60 °C for 24 h. Finally, the dry biomass was weighed in tubes at constant weight and stored in a desiccator for SeCys determination.

The supernatant collected were used to determine residual Se in medium. Mixture of 1 mL of supernatant and 10 mL of concentrate HNO_3 was digested in a microwave accelerated reaction system (MARS 5 microwave, CEM Corporation, Matthews, NC). A temperature ramp (175 °C for 5.5 min and 175 to 180 °C for 4.5 min) with a pressure limit of 110 psi (7.0307 Kg/cm^2) was used. After digestion, the resulting solution was completed to a final volume of 25 mL with deionized water. A calibration curve of Se from 0.2 to 4 ppm was made. To construct the curve, a 50 ppm solution of Se standard (Sigma-Aldrich) in 5% HNO_3 was used. Samples were analyzed by ICP-OES (Optima 8300 ICP-OES, PerkinElmer; Waltham, MA) at a wavelength of 166 nm. Percentage of Se absorbed by microorganism was calculated

by difference of Se in medium at time 0 and Se determined at 24 and 48 h.

2.5 Selenocysteine determination

The determination was made according to the methodology of carboxymethylation reaction proposed by Castañeda-Ovando et al. (2019) without modifications. Reaction products of the standard used were checked by proton nuclear magnetic resonance (^{1}H NMR).

2.6 Statistical analysis

Data were analyzed using the one-way ANOVA test. Significant differences between mean values were determined by Tukey's test at a confidence level of 95% ($P \leq 0.05$) using the program Minitab 18.

3 Results

3.1 Minimum inhibitory concentration

To determine the MIC, the effect of selenite concentration in the culture medium on the viability of *Enterococcus faecium* ABMC-05 was determined. Results of the determination of viability by OD_{600} at different concentrations of sodium selenite are shown in Table 1.

Growth at 36 and 48 h of incubation showed a significant reduction ($P > 0.05$) from 100 mg/L of sodium selenite. In general, as the concentrations increased from 100 to 500 mg/L, a decrease in growth was observed in both incubation times. These results differ from those reported by other researchers who tested lower concentrations. Pieniz et al. (2011) evaluated 36 strains of *Enterococcus* sp. isolated from a Minas Frescal cheese, of which 11 showed microbial growth with OD_{600} close to 1 after 24 h in a medium containing 10 mg/L Se. Recently, Krausova et al. (2020) reported that the OD_{660} parameters were not affected in any of the tested concentrations (0, 1, 5, 10, 30 and 50 mg/L of sodium selenite) for *Enterococcus faecium* CCDM 922A and *Streptococcus thermophilus* CCDM144. In other lactic acid bacteria such as *Lactobacillus* sp. the presence of low concentrations of selenite (0, 30 and 60 mg/L of sodium selenite) did not affect the growth of these strains after 28 and 32 h of incubation. However, when testing concentrations of 200 mg/L a significant decrease in *Lactobacillus paracasei* is observed (Mörschbächer et al., 2018).

In this investigation, previous studies were carried out testing concentrations of less than 100 mg/L of Na_2SeO_3 and it was observed that from 20 mg/L a decrease in cell concentration was generated without reaching total inhibition (data not shown), for this reason, the experiment was carried out at concentrations greater than 100 mg/L of Na_2SeO_3. Using these concentrations, one was found in which the accumulation of Se by the microorganism was ensured without a total inhibition of its growth being observed. So, the selenite concentration calculated as the growth inhibition point was 184 mg/L Na_2SeO_3 (Figure 1).

Table 1. Optical density (OD_{600}) of *Enterococcus faecium* ABMC-05 growth in enriched media fermented during 36 and 48 h at different Se concentrations.

Se concentration (mg/L)	36 h of fermentation	48 h of fermentation
0	0.627 ± 0.000	0.654 ± 0.042
100	0.218 ± 0.037	0.443 ± 0.005
200	0.025 ± 0.018	0.337 ± 0.013
300	0.041 ± 0.013	0.171 ± 0.052
400	0.066 ± 0.011	0.248 ± 0.019

Similar data have been reported for other lactic acid bacteria. Castañeda-Ovando et al. (2019) found a critical growth inhibition concentration of 140 mg/L of sodium selenite for *Streptococcus thermophilus*, using the Talmadge and Fitch method. In contrast, Yang et al. (2018) found that *Lactobacillus delbruekii* and *Streptococcus thermophilus* reached a maximum of 8.82 and 8.87 log cfu/mL live cells, respectively, when the selenite concentration was 80 µg/mL. Therefore, they considered this concentration of sodium selenite as the concentration necessary for the selenization of the microorganisms.

3.2 Growth from LAB to MIC of Na_2SeO_3

Microbial growth of *Enterococcus faecium* ABMC-05 at an MIC of 184 mg/L is shown in Figure 2.

The growth of the microorganism was affected by the addition of Na_2SeO_3 compared to the control. Both growth curves showed different logarithmic and stationary phases. The growth curve with Na_2SeO_3 presented a logarithmic phase from 2 to 10 hours of incubation. After 10 h, the bacteria reached the stationary phase, but there is a process of deceleration in growth, which decreases the replication capacity of the microorganism since a gradual increase in cell concentration is subsequently observed. The stationary phase was observed at 26 h (0.744 absorbance unit (AU)). In the case of the control, this phase was reached at 14 h (0.81 AU) and it was observed until 48 h (0.707 AU).

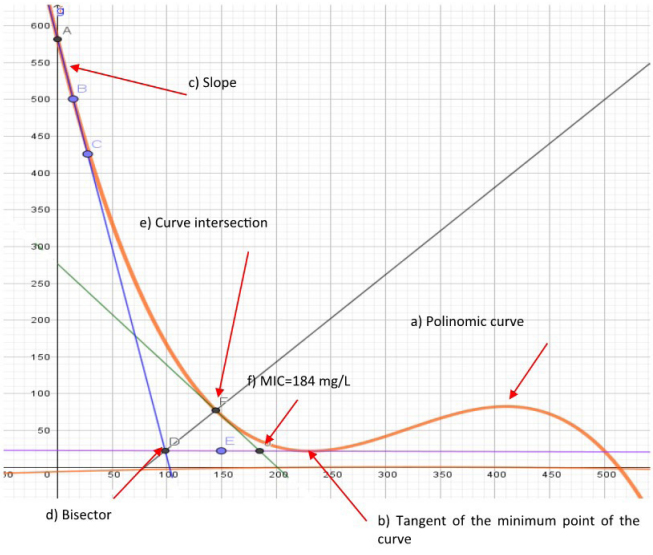

Figure 1. Calculation of the minimum inhibitory concentration of Na_2SeO_3 on the growth of *E. faecium* ABMC-05 using the Talmadge and Fitch method. Graph obtained from Geogebra software (5.0.536.0-d, 2019).

Results were not consistent with those reported for other LAB in which growth in selenized media is affected in the *lag* phase and in consequence times of logarithmic phase and the stationary are disturbed (Castañeda-Ovando et al., 2019; Fernández-Llamosas et al., 2017; Palomo-Siguero et al., 2016). Thus, the behavior of *Enterococcus faecium* ABMC-05 in the presence of selenite could be to the cell stress induced by Se, showing limited capacity to reduce high concentrations of selenite during the adaptation response (Pusztahelyi et al., 2015). This behavior has been presented in other LAB such as *L. paracasei* CH139. Mörschbächer et al. (2018) demonstrated a low adaptation response of this microorganism during the growth at different Se concentrations (100, 150 and 200 mg/L Na_2SeO_3). However, strains ML13 y CH135 did not show negative effect at concentrations of 100 and 150 mg/L, but at 200 mg/L the presence of Se inhibited growth of this strains

The metabolic deceleration due to the presence of Se in the medium is verified by both the time of the logarithmic phase and the changes in pH during fermentation. After 36 h of fermentation pH in enriched medium decrease to 5.23, while in the control the pH was 4.32. In contrast, it was reported that at low Se concentrations (0-50 mg/L) *Enterococcus faecium* 922A and *Streptococcus thermophilus* CCDM the pH of medium remains without significant changes (Krausova et al., 2020). Martínez et al. (2019) demonstrated that the growth of *L. brevis* CRL 2051 is not affected by pH changes in enriched medium with inorganic selenium. With these reports exist the possibility that Se concentration affects the metabolism of *E. faecium* ABMC-05 decelerating the log phase in which Se accumulation is presented.

3.3 Se accumulation

The concentration of Se bioaccumulated by *E. faecium* ABMC-05 was determined from samples collected at 24 and 48 h of fermentation in media enriched with the MIC (184 mg/L) of Na_2SeO_3. The results are shown in Table 2.

Results demonstrated the accumulation of Se by *E. faecium* ABMC-05. Even though at 48 h of fermentation there was higher accumulation than at 24 h (12.89 mg/L and 10.47 mg/L, respectively), the fermentation time did not show significant effect in these values.

Castañeda-Ovando et al. (2019) reported a Se accumulation in *Streptococcus thermophillus* of 10.55% (6.13 mg/L Se) at the end of the logarithmic phase (12 h) in the presence of 140 mg/L Na_2SeO_3. On the other hand, it is known that the accumulation of Se by *L. fermentum* and *L. plantarum* is between 2.53 to 3.28 mg/L of Se when adding 10 mg/L of Na_2SeO_3 (Saini & Tomar, 2017). In contrast, *Lactobacillus bulgaricus* in the presence of the same Se salt, at two exposure levels (1 and 10 µg/mL Na_2SeO_3), is capable of accumulating a higher concentration of Se (50-60% accumulation) when it is found in media enriched with 10 µg/mL and is also independent from the exposure time (24, 48 and 72 hours) (Palomo-Siguero et al., 2016).

3.4 Determination of SeCys

SeCys was determined in the recovered cells through RP-HPLC analysis. Figure 3 shows the chromatogram obtained.

The seleno-amino acid signal was located between 1.72 and 1.82 min. These results demonstrate the capacity of the microorganism under study, not only to accumulate Se but also to biotransform it into organic species such as SeCys from a medium enriched with an inorganic salt of Se, as has been reported for other LAB (Alzate et al., 2008, 2010).

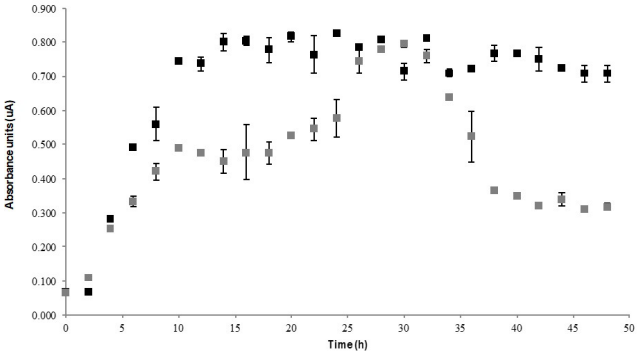

Figure 2. Growth curves of *Enterococcus faecium* ABMC-05 in media (■) with MIC of Na_2SeO_3 and (■) without Na_2SeO_3.

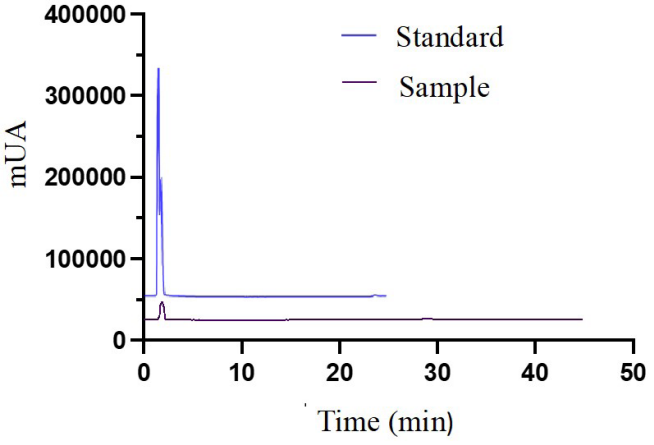

Figure 3. RP-HPLC of selenocysteine determined within cells of *Enterococcus faecium* ABMC-05 developed in enriched medium.

Table 2. Se consumption by *Enterococcus faecium* ABMC-05 at 184 mg/L of sodium selenite during 24 and 48 hours.

Time	[Se] mg/L in medium	[Se] mg/L in cell	Consumption %	log cfu/mL	µg Se/log ufc
0	52.39 ± 2.50	ND	ND	7.10 ± 0.04	0.00
24	41.50 ± 3.02	10.89 ± 3.02	20.79[a]	6.10 ± 0.14	1.79[a]
48	39.92 ± 1.32	12.47 ± 1.32	23.80[a]	5.49 ± 0.09	2.27[a]

ND = Selenium concentration not detected.

It is known that when a microorganism inserts Se within cell from the medium, it could be non-specifically incorporated into cysteine and methionine due to their analogous structure, with the consequent generation of the corresponding amino acids (Pieniz et al., 2017). It has been studied the ability to biotransform inorganic Se into selenoamino acids by multiple LAB, which produce mainly SeCys (Martínez et al., 2019, 2020; Kousha et al., 2017; Pophaly et al., 2014). In addition, reports such as those by Krausova et al. (2020) has reported the presence of MeSeCys and seleno-methionine (SeMet) and the report of Pieniz et al. (2017) demonstrated the Se accumulation in selenized *Enterococcus* species. However, it has been shown that the organic species with the highest presence in selenized *Enterococcus faecium* CCDM 922A is SeMet but, despite the fact that MeSeCys is of lower concentration inside the cell, it is absorbed more efficiently (Krausova et al., 2021). The SeCys produced by this LAB could be incorporated into proteins through specialized cellular machinery called "selenocysteine insertion assembly", which has an important role in all forms of life (Martínez et al., 2020; Kieliszek et al., 2017; Pieniz et al., 2017; Pophaly et al., 2014). With the results of this study, it has been verified that an indigenous lactic acid bacterium isolated from a traditional Mexican fermented beverage is capable of absorbing Se, accumulating it and producing selenocysteine, from an inorganic source of Se.

4 Conclusions

Selenium is concentrated in biomass by *Enterococcus faecium* ABMC-05 as organic selenium when the media in which it grows are enriched with sodium selenite. The results of this study demonstrate the capacity of this indigenous microorganism isolated from a traditional Mexican fermented beverage in the production of selenocysteine, which is an amino acid of great importance in the structural conformation of seleno-enzymes that have a direct incidence in metabolic cycles in humans. In addition, this study has revealed that traditional fermented beverages could be a source of beneficial bacteria for human health. This discovery adds to the information on the metabolism of *Enterococcus* species, which will increase the scientific knowledge of this group of microorganisms, which in many cases have probiotic characteristics and could be used as starter cultures in the transformation of functional foods as well as for the biogenic production of organic selenium particles.

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