

Original Research Paper

Production of Selenium-Enriched Yeast (*Kluyveromyces marxianus*) Biomass in a whey-based Culture Medium

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Abstract: Two important aspects of agriculture intensification are the reduction in the concentration of specific soil minerals that affects livestock production and the increase of agricultural by-products, which produce environmental pollution. In this regard, whey - a cheese by-product-often is considered a wasted-product. Due to its lactose concentration, (4.5%), when whey is discarded without treatment generates a high Biological Oxygen Demand (BOD) and a high Chemical Oxygen Demand (COD). Taking into account these two issues, we developed a whey-based culture medium to produce selenium-enriched *Kluyveromyces* biomass. Then, we evaluated the effect of its supplementation on calves blood selenium concentration. *Kluyveromyces marxianus* DSM 11954 and *Kluyveromyces lactis* DSM 3795 strains were used in this study. Different culture media were prepared using whey as a main component and supplemented with peptone, yeast extract, (NH₄)₂SO₄ and K₂HPO₄ as appropriate. In the selected whey culture medium, three sodium selenite concentrations between 10-30 µg/mL were tested to produce selenium-enriched biomass. After that, a scaled up to 5 L stirred-tank bioreactor was carried out to increase final yeast biomass levels. Finally, dietary supplementation experiments with selenium-enriched yeast were conducted to increase selenium content in calves. *K. marxianus* DSM 11954 showed a better growth performance than *K. lactis* DSM 3795 in a medium composed by whey, (NH₄)₂SO₄ 5 g/L, K₂HPO₄ 1 g/L (pH 6.5) so, this strain was chosen to continue the experiments. The results showed that sodium selenite addition at 20 µg/mL was adequate to generate selenium-enriched biomass. Our study demonstrated that whey is an optimal and economical culture medium to produce selenium-enriched- yeast biomass. Also, we proved that 10 days of yeast-biomass supplementation raised blood-selenium level in calves.

Keywords: Whey, Selenium, *Kluyveromyces Marxianus*, Supplementation, Calves

Introduction

Population growth led to an intensification of agricultural production systems. As a consequence of agriculture management, some soils present a reduction of specific minerals concentration that affects livestock. For instance, some geographic areas, especially in semi-arid regions, are lacking selenium (Gil *et al.*, 2004; Mirlean *et al.*, 2018). Even though selenium is required in low amounts (300 µg/dry matter intake), it is an

essential microelement for livestock (National Research Council, 2001). Many enzymes require selenium as a cofactor, being one of particular interest the glutathione peroxidase, which catalyzes lipid hydroperoxides to hydroxy acids and water (Smith *et al.*, 1974). Moreover, selenium is crucial for the correct function of the thyroid gland (Beckett *et al.*, 1993). Cows with selenium deficiency are associated with immunological alteration, increasing placental retention and decreased milk production (Julien *et al.*, 1976; Moeini *et al.*, 2009). In

calves, selenium deficiency causes a myodegenerative disorder called white muscle disease (Andrews *et al.*, 1968) and retards growth rate (Gleed *et al.*, 1983). Selenium is biologically active as selenium-containing amino acids, such as selenocysteine and selenomethionine, the latter being most commonly found in animals diet (Fairweather-Tait *et al.*, 2010). Livestock can be supplemented with inorganic or organic selenium. It has been reported that organic selenium has a higher bioavailability and lower toxicity than the inorganic form in cows (Slavik *et al.*, 2008; Brennan *et al.*, 2011; Salman *et al.*, 2013) and ewes (Hall *et al.*, 2012).

Another aspect of intensification is the increase of agricultural by-products that contribute to environmental contamination. In this regard, whey - a derivative of the cheese industry - represents 80-90% of the total volume of milk entering the cheese process. Due to its high lactose concentration (4.5%), whey is highly polluting (World Bank Group, 1998). Even when different alternatives have been proposed (e.g. animal feeding with no further process or dried whey production) whey is still discarded, causing a high environmental impact especially in developing countries where the drying process is not profitable. Yet, far from being a waste, whey is an economical culture medium due to its composition (Amado *et al.*, 2016). It would provide an alternative use, if its lactose were utilized as a carbon source for the development of microorganisms. Commercial organic selenium is based on *Saccharomyces cerevisiae*, however, this yeast does not express lactase, an essential enzyme for lactose energy source. Therefore, in this study, we produced selenium-enriched *Kluyveromyces* biomass as an organic selenium for calves' supplementation in a whey-based culture media. Through this process, the reuse of an agro-industrial byproduct is achieved, avoiding in this way environmental contamination.

Materials and Methods

Whey Composition

Sweet whey was obtained from a local cheese maker factory in La Pampa, Argentina and stored at -20°C until use. The characteristics of this whey are indicated in Table 1. The high lactose concentration in this medium increases the chances of biomass formation.

Table 1: Characteristics of the whey used for culture medium preparation

Parameter	Value
Lactose (g/L)	45.0
Proteins (g/L)	9.0
Fat (g/L)	6.6
Total solids (g/L)	68.0
pH	6.5
Colour	Yellow

Depending on the experiment, medium components and salts were diluted directly in the sweet whey and then autoclaved at 121°C for 20 min.

Biological Material

The type strains *Kluyveromyces marxianus* DSM 11954 and *Kluyveromyces lactis* DSM 3795 were purchased from the Leibniz-Institute DSMZ (German collection of microorganisms and cell culture). Competent yeast cells were prepared in YPD (1% yeast, 2% peptone, 2% dextrose and 2% agar, w/v) for 48 h and then were transferred into YPD liquid medium for another 24 h. Incubations were performed at 28°C.

Determination of Kinetic and Stoichiometric Parameters in Rich Culture Media

Kluyveromyces marxianus and *K. lactis* were cultured in Erlenmeyer flasks containing YPD and YPL (1% yeast, 2% peptone and 2% dextrose, w/v). The pH was adjusted to 5.5 with HCl 1N. The purpose was to compare kinetic (specific growth rate, μ) and stoichiometric (biomass yield coefficient based on substrate consumption, $Y_{x/s}$) parameters of *Kluyveromyces* strains in both media. Cultures were performed in an orbital shaker at 28°C and 250 rpm and were sampled periodically during incubation for biomass level measurement. The optical density of culture samples was measured at 600 nm using a spectrophotometer and converted to dry cell weights (DCW, in g/L) with a previously calculated calibration curve in accordance to the formula: $OD_{600\text{ nm}} = 2.14 \times DCW$, $R^2 = 0.990$. Kinetic and stoichiometric parameters were obtained from the growth curves of both *Kluyveromyces* strains.

Study of Kinetic Growth of *Kluyveromyces marxianus* in a Whey-based Culture Medium

Four different culture media were prepared from sweet whey: 1.- whey without adding any nutrients (pH 6.5); 2.- whey, peptone 2% and yeast extract 1%, w/v (pH 6.5); 3.- milk whey, peptone 0.5%, yeast extract 0.25% and K_2HPO_4 0.1%, w/v (pH 6.5); 4.- whey, $(NH_4)_2SO_4$ 0.5% and K_2PO_4 0.1%, w/v (pH 6.5). *K. marxianus* DSM 11954 was cultured in Erlenmeyer flasks containing such whey-based culture media at 28°C and 150 rpm. Kinetic (specific growth rate, μ) and stoichiometric (biomass yield, $Y_{x/s}$) parameters were determined from growth curves of *K. marxianus* in each culture medium. The pH of the culture media was adjusted to 6.5 because whey was obtained at this value from the cheese maker factory.

Production of *Kluyveromyces* Biomass in a whey-based Medium Supplemented with Selenium

To study the correlation between selenium concentration in culture medium and the yeast growth

rate, *K. marxianus* DSM 11954 was grown in a medium composed of whey, $(\text{NH}_4)_2\text{SO}_4$ 0.5% and K_2PO_4 0.1%, w/v (pH 6.5) with different Na_2SeO_3 concentration: 0 $\mu\text{g/mL}$; 10 $\mu\text{g/mL}$; 20 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$. Incubations of the cultures were performed at 28°C and 150 rpm, withdrawing samples periodically during incubation for biomass quantification. Specific growth rate (μ) and biomass yield ($Y_{x/s}$) were determined from the growth curves of *K. marxianus* in the whey-based medium supplemented with selenium.

Scaling up the production of Selenium-Enriched *Kluyveromyces* Biomass

The scaling up of the production of selenium-enriched yeast was performed in a stirred-tank bioreactor applying a repetitive batch cultures. In order to obtain the inoculum for bioreactor fermentation, *K. marxianus* DSM 11954 cells grown on YPD agar plates were first cultured overnight in 20 mL YPD medium at 28°C and 250 rpm. The day after, 200 mL of YPD in a 1 L-Erlenmeyer flask were inoculated with the overnight culture and incubated at 28°C and 250 rpm until an OD_{600nm} of ~8. Finally, this culture was used to inoculate 5 L of whey-based culture medium contained in a 6 L-BioFlo 110 bioreactor (New Brunswick Scientific; Edison, NJ). The stirred bioreactor was used in interface with the Biocommand Bioprocessing software (New Brunswick Scientific) for parameter control and data acquisition. Temperature was maintained at 28°C through the fermentation process. Furthermore, pH was hold at a value of 5 by adding H_3PO_4 (42% v/v) and NH_4OH (26% v/v) which also served as nitrogen source. Dissolved Oxygen (DO) was controlled through agitation (maximum of 1200 rpm) and filter-sterilized (0.22 μm) air supply. The pH was determined using a pH electrode (Mettler-Toledo GmbH, Germany) and the oxygen concentration was measured with a polarographic probe (InPro6110/320, Mettler-Toledo GmbH). Foam formation was avoided by the addition of 0.3% (v/v) antifoam 289 (Sigma-Aldrich; St. Louis, MO). Fermentation samples were withdrawn throughout batch cultures with the purpose of evaluating biomass concentration and quantifying the yeast organic selenium.

Chemical Determinations of the Yeast Biomass

Organic selenium concentration was determined by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) in *Kluyveromyces* biomass obtained from bioreactor fermentations. Furthermore, total protein concentration and amino acid composition were evaluated in *Kluyveromyces* biomass obtained from fermentation. The analysis of total protein was performed by Kjeldahl method; and the amino acid composition by HPLC (Hewlett Packard Serie 1100)

according to the procedures described in the AOAC Standards Development Animal assay.

Effect of Dietary Supplementation with Selenium-Enriched Yeast in Calves

The effect of dietary supplementation with selenium-enriched yeast on selenium blood level was studied in 6 calves. Animal management was performed under the approval of School of Agriculture, University of La Pampa. Animals were 90 day-old and weighed approximately 100 kg. Selenium-enriched biomass was supplemented daily to the grain ration (0.60 mg Se/animal) during 10 days. Blood samples were taken from the jugular of each calf before the dietary supplementation and the day after the treatment had finished. Blood selenium was measured by hydride generation atomic absorption spectrometry at EEA "Ing. Agr. Domingo R. Pasquale" Veterinary Diagnostics Service, at the National Institute of Agricultural Technology (INTA), Balcarce.

Statistical Analysis

A two-way ANOVA was used to compare the effect of time and medium conditions. The effect of selenium supplementation in calves was analysed by paired samples t-test. These analyses were done with the R Project for Statistical Computing.

Results

Kluyveromyces marxianus and *Kluyveromyces lactis* Growth in Rich Culture Media

In order to study the ability of two strains of *Kluyveromyces* to grow in lactose medium, we first evaluated the kinetic growth of *K. marxianus* DSM 11954 and *K. lactis* DSM 3795 in two rich culture media (YPD and YPL). As shown in Fig. 1, *K. marxianus* strain presented a similar growth pattern independently of the two culture media evaluated reaching a biomass level of 13.8 and 13.5 g DCW/L for YPD and YPL medium respectively. In this sense, in the presence of both carbon sources, the lag phase lasted 2 h and the exponential stage was extended by 9 h and this was followed by the deceleration phase that lasted until the incubation finished. In contrast, the growth kinetic of *K. lactis* DSM 3795 depended on the carbon source employed in the culture medium. In the control YPD medium, *K. lactis* exhibited a growth pattern similar to that observed with *K. marxianus*, but with a slightly extended lag phase. However, *K. lactis* biomass levels in YPL (3.2 g DCW/L) was significantly lower than the one obtained in YPD medium (13.1 g DCW/L). Moreover, the specific growth rate (μ) was different between *Kluyveromyces* species.

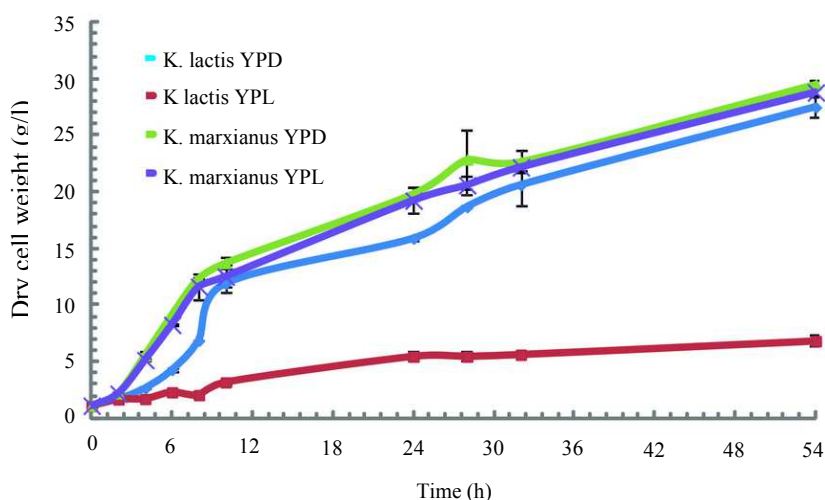


Fig. 1: Growth kinetics of *Kluyveromyces marxianus* DSM 11954 and *Kluyveromyces lactis* DSM 3795 in YPD medium (yeast extract 1%, peptone 2% and dextrose 2%) and YPL medium (yeast extract 1%, peptone 2% and lactose 2%).

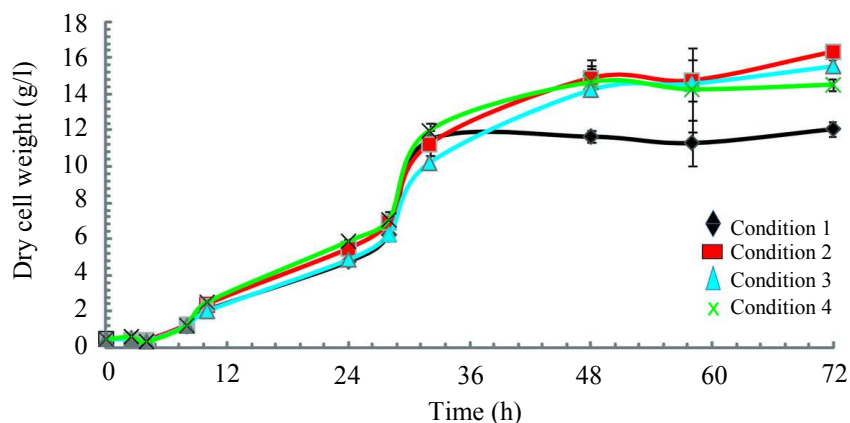


Fig. 2: Effect of whey-based culture media on *Kluyveromyces marxianus* DSM 11954 growth kinetics. Medium 1: Whey; Medium 2: whey, peptone 2%, yeast extract 1%; Medium 3: whey, peptone 0.5%, yeast extract 0.25%, K₂HPO₄ 0.1%; Medium 4: whey, (NH₄)₂SO₄ 0.5%, K₂PO₄ 0.1%.

As it is shown in Fig. 1, *Kluyveromyces marxianus* reached a specific rate of 0.36 ± 0.01 h⁻¹ in YPD and 0.34 ± 0.01 h⁻¹ in YPL. On the contrary, *Kluyveromyces lactis* specific rate was 0.23 ± 0.01 h⁻¹ when it grew in YPD medium and 0.05 ± 0.01 h⁻¹ in YPL. Due to these results, we chose *K. marxianus* strain to continue our studies.

Growth Kinetics of *Kluyveromyces marxianus* in Culture Media based on Milk Whey

The growth of *K. marxianus* DSM 11954 was evaluated in four different whey-based culture media: 1. Whey; 2. whey, peptone 2%, yeast extract 1%, w/v; 3. whey, peptone 0.5%, yeast extract 0.25%, K₂HPO₄ 0.1%, w/v; 4. whey, (NH₄)₂SO₄ 0.5% and K₂PO₄ 0.1%, w/v. As shown in Fig. 2, *K. marxianus* presented a prolonged lag phase in all whey-based culture media

evaluated. The first 24 h of incubation, growth kinetics and biomass production of *K. marxianus* were similar in all culture media tested (4.76 g DCW/L, 5.46 g DCW/L, 4.87 g DCW/L and 5.87 g DCW/L from medium 1 to 4). However, in the presence of whey alone, *K. marxianus* DSM 11954 reached the stationary phase earlier. Interestingly, when adding (NH₄)₂SO₄ 0.5% and K₂PO₄ 0.1%, w/v (medium 4, Fig. 2), growth showed a similar rate compared with any of the two other enriched media (medium 2 and 3, Fig. 2). Maximum biomass was achieved at 72 h of incubation with culture media 1 (11 g DCW/L), 2 (16 g DCW/L) and 3 (15 g DCW/L). On the other hand, medium 4 reached maximum biomass (14 g DCW/L) at 48 h of incubation (Fig. 2). When specific growth rates were calculated, a similar performance in all medium (0.08 ± 0.01 h⁻¹) was obtained.

Production of Selenium-Enriched *Kluyveromyces* Biomass in a Whey-based Medium

Since medium 4 is an economical culture medium and *K. marxianus* showed to grow without restriction and similarly to conventional medium containing yeast extract and peptone, we used this media to scale the process. First, to obtain enriched-selenium-*Kluyveromyces*- biomass, we studied the toxicity effect of sodium selenite concentration on the yeast growth rate. To do this, *K. marxianus* DSM 11954 was grown in medium 4 supplemented with four different Na_2SeO_3 concentrations: 0 $\mu\text{g/mL}$; 10 $\mu\text{g/mL}$; 20 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$. In order to shorten the delay phase, *K. marxianus* was grown in YPD overnight and then inoculated into the different selenium concentration media. As it is shown in Fig. 3, the presence of selenium in the media

did not inhibit *K. marxianus* growth at any concentration. However, adding 30 $\mu\text{g/mL}$ of Na_2SeO_3 to the medium resulted in a decrease of the maximum level of biomass compared to the other three media tested. When supplementing the whey-based culture medium with 10 and 20 $\mu\text{g/mL}$ of Na_2SeO_3 , the maximum level of biomass reached was 9.6 g DCW/L and 9.9 g DCW/L respectively after 36 h of incubation. Specific growth rates varied lightly between culture media starting with a rate of $0.26 \pm 0.01 \text{ h}^{-1}$ (in control condition, 0 $\mu\text{g/mL}$) following by 0.25, 0.24 and $0.23 \pm 0.01 \text{ h}^{-1}$ (10, 20 and 30 $\mu\text{g/mL}$ of Na_2SeO_3 respectively). Therefore, the whey-based culture medium supplemented with 20 $\mu\text{g/mL}$ of Na_2SeO_3 was chosen for the scaling up of *Kluyveromyces* biomass production in stirred-tank bioreactor since it was the maximum selenium concentration that did not affect yeast growth.

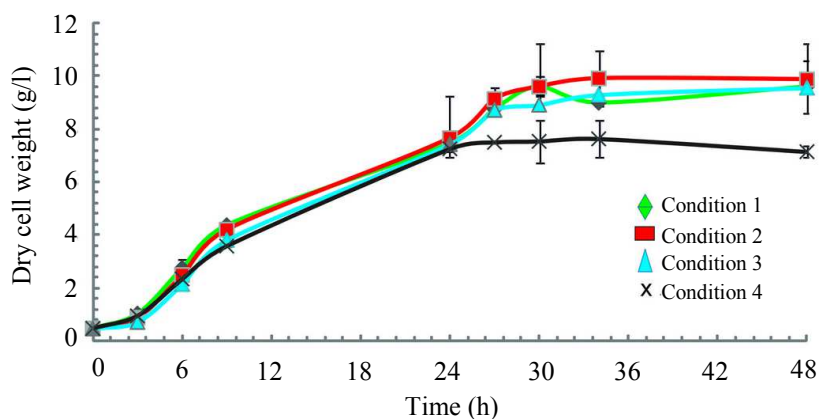


Fig. 3: Effect of sodium selenite concentration on *Kluyveromyces marxianus* DSM 11954 growth kinetics in a whey-based culture medium (whey, $(\text{NH}_4)_2\text{SO}_4$ and K_2PO_4) supplemented with four different Na_2SeO_3 concentrations: 0 $\mu\text{g/mL}$ (Medium 1); 10 $\mu\text{g/mL}$ (Medium 2); 20 $\mu\text{g/mL}$ (Medium 3) and 30 $\mu\text{g/mL}$ (Medium 4)

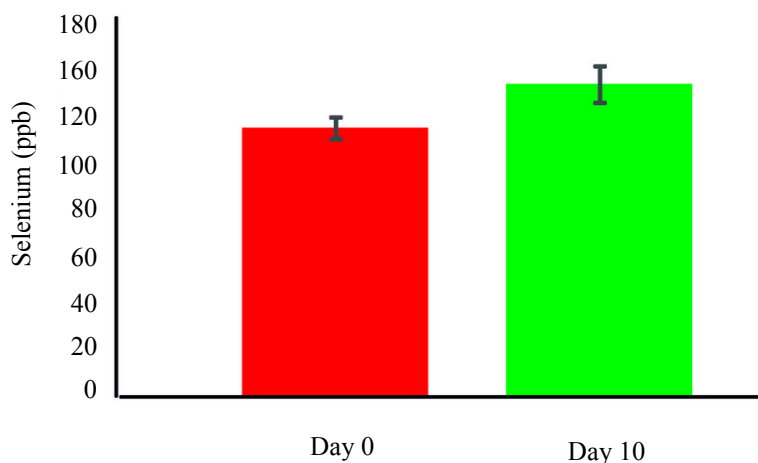


Fig. 4: Selenium blood levels (ppb) in calves before and the day after the selenium-enriched yeast supplementation

Scaling up the Selenium-Enriched *Kluyveromyces* Biomass Production

In order to scale up the selenium-enriched-*Kluyveromyces* biomass production, fermentation cultures were performed in a stirred-tank bioreactor by a process consisting of 2 repetitive batch cultures. A *K. marxianus* DSM 11954 culture was inoculated into 5 L of the selected whey-based culture medium (whey, (NH₄)₂SO₄ and K₂PO₄) supplemented with 20 µg/mL of Na₂SeO₃ in a 6 L BioFlo 110 bioreactor. Batch fermentation was carried out under the following conditions: 30°C, pH 6.0, constant agitation of 1000 rpm and air supply of 1 LLM. After 36 h of batch fermentation a spike of dissolved oxygen was generated since the carbon source was completely consumed. At that moment, the biomass achieved a maximum level of 19.5 g DCW/L and therefore, a second batch fermentation was initiated. Hence, a volume of 4 L of culture was removed from the bioreactor and centrifuged at 3500 g for 20 min to recover the biomass. Four fresh whey-based culture medium liters were added to the bioreactor, starting another batch culture with the same conditions as described below. At the end of the process, a total of 550 g Wet Cell Weight (WCW) was obtained. Selenium concentration was 85 mg/kg yeast.

Enriched Selenium Yeast Supplementation in Calves

To study the effect of selenium-enriched yeast supplement on selenium blood level, 6 calves received 7 g of wet selenium-enriched *K. marxianus* (corresponding to 0.60 mg selenium/animal/day) during 10 days. Blood selenium was measured before and after the supplementation treatments. The yeast biomass was mixed in a small amount of grains and given to the animals before they were fed. As expected, calves showed no signs of reluctance to food supplemented with yeast biomass. The level of selenium in blood, after ten days of supplementation with selenium-enriched *K. marxianus* biomass, experienced a statistically significant ($p = 0.0053$) increase of 19.2 mg/L. The mean at the endpoint was 144.8 mg/L (SD =11.5 mg/l) (Table 4). The statistical analysis showed that blood selenium after treatment was significantly higher than those at day 0.

Since we were interested in yeast biomass as feed supplementation, we furthermore analyzed the amino acid composition of the *K. marxianus* biomass obtained from culture grown in the selected medium (whey, (NH₄)₂SO₄ and K₂PO₄). As shown in Table 2, *K. marxianus* presents a relatively high methionine concentration (1.1 g/100 g protein).

Table 2: Amino acid concentration (g /100 g protein) of *Kluyveromyces marxianus* DSM 11954 grown in whey-based culture medium (whey, (NH₄)₂SO₄ 0.5% and K₂PO₄ 0.1%)

Amino acid	(g /100 g protein)
Aspartic Acid + Asparagine	9.8
Glutamic + Glutamine	18.1
Glycine	3.8
Serine	5.6
Threonine	5.5
Histidine	2.3
Tirosine	2.6
Arginine	5.0
Alanine	6.7
Methionine	1.1
Valine	3.3
Tryptophan	0.2
Phenylalanine	0.2
Isoluecine	3.8
Leucine	3.3
Lysine	9.4
Proline	4.7
Hydroxyproline	0.0
Ornithine	0.8
Taurine	0.0
Cystine + Cysteine	4.3

Discussion

Although whey can be dried and used in different products, sometimes it is not a viable alternative since the drying process is expensive in terms of energy. Therefore, it is important to develop an alternative use that generates a new product on the one hand and reduces the pollution by its untreated waste on the other hand. Therefore, we proposed the selenium-enriched yeast (*Kluyveromyces marxianus*) production through a whey-based culture media. Yeast biomass produced as “Single Cell Protein (SCP)” is the final product of cell mass grown in large scale culture systems. When SCP is harvested, it can be used for protein supplementation or - as in our trial- as mineral organic supplementation in livestock. This system presents two advantages: first its high productivity and second its non-toxic nature as *K. marxianus* is recognized as a GRAS microorganism. This biotechnology application could be successfully applied when different kinds of industrial wastes are used as substrates, as has been reported using a poultry slaughterhouse wastewater to produce *Rhodocyclus gelatinosus* reducing the chemical oxygen demand (Ponsano *et al.*, 2003).

In the present work, first we studied the *K. Marxianus* and *K. lactis* growth rate in a whey-based culture. We observed a higher growth rate of *K. Marxianus* compared to *K. lactis*. This might be the consequence of a lower capacity of the latter to incorporate lactose from the culture. Since the purpose of this work was to produce *Kluyveromyces* biomass enriched with selenium using a

lactose-based culture medium, we continue our studies with *K. marxianus* strain.

It has been reported that yeasts are hypersensitive to sodium selenite; high concentrations of Se lead to DNA damage when the cells are growing in the exponential phase (Izquierdo *et al.*, 2010). Studying different sodium selenite concentration, we did not observe an inhibition of *K. marxianus* growth. However, a decrease of the maximum level of biomass was observed adding 30 µg/mL of Na₂SeO₃ to the medium compared to the other three concentrations tested. We scaled up the production and we obtained 61 g WCW/L with 85 mg selenium per kg yeast. Finally, the SCP was used to supplement six calves. *K. marxianus* presents a relatively high methionine (1.1 g/100 g protein) concentration which contributes to the selenium binding capacity. This concentration agrees with previous ones reported in *K. marxianus* (Páez *et al.*, 2008) and shows to be higher from those reported in *Saccharomyces Cerevisiae* (0.6g/100g protein) (Chiao and Peterson, 1953). Chemical and genetic modifications of yeast strain were reported to increase natural methionine concentration, e.g. mutant *Candida Tropicalis* increases from 0.42 to 0.49% methionine concentration (Halasz and Lasztity, 1991). In this context, *K. marxianus* shows a naturally high methionine concentration as well as lysine (9.4 g/100 g). Both amino acids are supplements in cows therefore, *K. marxianus* SCP might be used as a protein supplementation.

We conclude that our process was successful as the tested calves blood showed increased selenium after 10 days of supplementation.

Conclusion

Whey is a product of high biological value that is often discarded in cheese factories. In this study, whey was used to produce selenium-enriched *Kluyveromyces*. Even when organic selenium is commercially available in the form of *Saccharomyces cerevisiae*, there are several advantages in the use of *Kluyveromyces* as organic supplementation. Selenium replaces sulfur and binds to sulfur-containing amino acids such as methionine and cysteine. *Kluyveromyces* showed a 1.1% methionine and 4.3% cysteine concentration. These values are higher than those reported in *Saccharomyces cerevisiae* (Watson, 1976). Therefore, *Kluyveromyces* has an increased potential to bind selenium. In addition, *Saccharomyces* needs glucose as a carbon source since it does not express lactase. On the other side, *Kluyveromyces* species express lactase (Tovar-Castrom *et al.*, 2008) and, as we showed here, they can grow optimally in a whey-based culture medium. Here we showed that after supplementing calves with selenium-enriched *Kluyveromyces* biomass for 10 days, the selenium level in blood increased. In

conclusion, whey proves to be an efficient and economical media for the production of selenium-enriched yeast and *K. marxianus* represents a valuable feeding supplement for calves. It is important to find new alternatives for the reuse of agricultural by-products. In this sense, we believe that our research constitutes a contribution.

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Conflict Of Interest

The authors declare no conflict of interest.

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