INVESTIGACIONES Y AVANCES EN BIOTECNOLOGÍA DE LOS ALIMENTOS

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PRÓLOGO

Biotecnología es un término que se empezó a utilizar en los ’60 para describir procesos de naturaleza biológica, desarrollados industrialmente en esa época pero conocidos desde hacía 3000 a 6000 años a.C.. Inicialmente la Biotecnología se circunscribía a la microbiología industrial y la tecnología enzimática. Hoy podemos decir que la biotecnología es una actividad (tecnología) multidisciplinaria. Integra la bioquímica, la microbiología, la nanotecnología y la biología molecular con la química, la matemática, la informática y las tecnologías de proceso. En el área de alimentos, la Biotecnología abarca tecnologías biológicas para la producción, transformación y/o preservación de alimentos, o bien para la producción de materias primas, aditivos y coadyuvantes en la industria alimentaria.

Aquí se pone a consideración de los lectores, trabajos en el campo de la Biotecnología de Alimentos que fueron originalmente presentados de manera resumida durante el V Congreso Internacional de Ciencia y Tecnología de los Alimentos (2014).

En esta oportunidad se han abordado, entre otros, varios temas relacionados con revalorización de subproductos y el uso de enzimas para objetivos tan variados como favorecer la coagulación de la leche bovina, intentar eliminar el oxígeno en alimentos ricos en grasas para prevenir oxidaciones y aumentar la eficiencia en la extracción de pectinas de la cáscara de limón hasta obtener nanopartículas de plata usando extractos de cedrón.

Este libro es una contribución a la difusión del conocimiento científico-tecnológico en el Área de los alimentos que se desarrolla en Argentina y Latinoamérica y esperamos que resulte de interés y utilidad a los lectores.

Los Editores.

A NEW POTENTIAL MILK-CLOTTING ENZYME PRODUCED BY *BACILLUS* SP. P45

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Resumen: El objetivo de este trabajo fue investigar la aplicación de la enzima queratinasa obtenida del *Bacillus* sp. P45, sin purificación previa y purificada como una alternativa para la coagulación de la leche bovina. La enzima queratinasa se purificó a partir de dos procesos secuenciales de sistemas acuosos de dos fases integrados a una etapa de diafiltración. La actividad coagulante se realizó en la leche bovina a pH 6,5, 30°C utilizando diferentes concentraciones de la enzima sin purificación, purificada y comercial (quimosina). La enzima queratinasa fue altamente activa en la hidrólisis de las proteínas de la leche. Además, teniendoen cuenta la actividad coagulante de la enzima comercial, la enzima del *Bacillus* demostró ser adecuada como alternativa a coagulación de la leche en la concentración de 30 mg/ml. Los resultados indicaron que el tiempo de coagulación de la leche es dependiente de la concentración de la solución coagulante y disminuye al aumentar la concentración de la enzima. La aplicación de la enzima purificada en la coagulación de la leche puede ser utilizada en el desarrollo de procesos biotecnológicos innovadores, en particular para el desarrollo de nuevos productos lácteos.

Palabras clave: leche, proteasa, actividad de coagulación de la leche, coagulante alternativo, productos lácteos.

Abstract: The aim of this study was to investigate the application of the crude and purified keratinase enzyme obtained from *Bacillus* sp. P45 as a new potential milk-clotting. The keratinase was purified from a sequence of two aqueous two-phase systems integrated into the diafiltration process. The coagulant activity was performed on bovine milk at pH 6.5 at 30°C using different concentrations of purified, crude and commercial enzyme (chymosin). The enzyme from *Bacillus* sp. P45, both in crude and purified form, was highly active and efficiently applied in the hydrolyze of milk proteins considering the coagulant activity when compared to the commercial rennet, showing similar strength milk-clotting that the chymosin in the concentration of 30 mg/mL and suitability as an alternative coagulating for milk. The results indicated that the milk clotting time is dependent on the concentration of the coagulant solution and decreases with increasing of the enzyme concentration. The enzyme application in the milk coagulation showed that the purified enzyme could be used as interesting alternative in developing of innovative biotechnological processes, particularly for the development of new dairy products.

Keywords: milk, protease, milk-clotting activity, alternative coagulant, dairy products.

INTRODUCTION

Obtaining natural coagulants is a challenge for the dairy industry because rennet availability is limited compared with the growing demand. Natural coagulants of animal origin are often expensive, and their consumption has been restricted due to religious or dietary reasons, and even through bans on foods produced from genetically modified organisms (GMO). This scenario has led to a growing demand for novel rennet substitutes, promoting a search for new sources of proteases with coagulant properties (Roseiro *et al.* 2003, Jacob *et al.* 2010, Ahmed *et al.* 2012, Mazorra-Manzano *et al.* 2013). The milk market is important, since approximately a third of the world's milk production is used for cheese manufacture and the use of cheese for direct consumption and as an ingredient has increased tremendously (Farkye 2004). For example, there was a 17% worldwide increase in cheese production from 2000 to 2008, and 43% in Brazil (Embrapa 2010). Therefore the dairy industry still has the challenge of overcoming the shortage of rennet with coagulants from other sources (Merheb-Dini *et al.* 2012).

Milk coagulation, in most cheese-making processes, is caused by the action of proteases on milk proteins. The specific hydrolysis of the peptide bond between $phenylalanine_{105}$ and $methionine_{106}$ in *k*-casein provokes casein micelle destabilization and subsequent aggregation, resulting in the transformation of the milk into curd (Kumar *et al.* 2010). Proteases constitute one of the most important groups of industrial enzymes, accounting for about 60% of the total enzyme market (Kumari *et al.* 2012). They have been routinely used for various purposes such as, cheese making, baking, meat tenderization and preparation of hydrolysates (Tomar *et al.* 2008). The major application of proteases in dairy industry is in the manufacture of cheese. Different types proteases have been used for this purpose, including those extracted from vegetable (Corrons *et al.* 2012, Galán *et al.* 2012, Pontual *et al.* 2012, Mazorra-Manzano *et al.* 2013) and animals (Trujillo *et al.* 2000, Rolet-Repecaud *et al.* 2013, Shamtsyana *et al.* 2013), but their application may be limited by several factors such as cultivation and climatic variations, which can affect the production and supply.

Enzymes from microorganisms are attractive because they can be grown in large quantities over a relatively short period of time with lower production costs (Headon and Walsh 1994). Several proteases of microbial origin have been tested in milk coagulation, but they often cause extensive hydrolysis, reducing curd yields (Ustunol and Hicks 1990). *Bacillus* sp. P45, which was isolated from the intestine of the Jaraqui fish (*Piaractus mesopotamicus*) originating from the Amazon basin (Sirtori et al. 2006), produces an enzyme that presents great potential for protein hydrolysis (Daroit *et al.* 2009). Hydrolysates of ovine caseinate produced with this enzyme presented antioxidant and antimicrobial activities, and showed gelation and aggregation abilities (Daroit *et al.* 2012). Therefore, this protease may be useful in the milk clotting processes for the development of dairy products. The obtaining new proteases with high milk-clotting ability could be an interesting alternative to use in the cheese industry for development of dairy products, as well as other food and biotechnological industries. Thus, the aim of this study was to investigate the application of the crude and purified keratinase enzyme obtained from *Bacillus* sp. P45 as a new potential milk-clotting and interesting alternative in developing of innovative biotechnological processes, particularly for the development of new dairy products.

MATERIALS AND METHODS

Microorganism, inoculum and cultivation

Bacillus sp. P45 (GenBank accession number AY962474), maintained on brain-heart agar medium (BHA) at 4 ºC, was used to produce the enzyme. For inoculum preparation, this strain was grown on BHA at 30 ºC for 24 h. The cultures were scraped from the agar surface, added to a sterile 0.85% (w/v) NaCl solution, and mixed until a homogeneous suspension with $O.D._{600}$=0.5 was obtained. The enzyme was produced by submerged cultivation using feather meal as substrate as described by Daroit *et al.* (2011). After cultivation, the culture was then clarified by centrifugation (5000 × g for 20 min) and the supernatant containing the enzyme was used in the purification steps.

Enzyme purification

The enzyme extract obtained from the cultivation was purified from a sequence of two aqueous two-phase systems integrated into the diafiltration process to remove the polyethylene glycol (Sala *et al.* 2014). The first aqueous two-phase systems was composed (w/w) by 3 % polyethylene glycol 1500 Da, 23 % potassium phosphate pH 7.0, 8 % NaCl, and 20 % enzyme extract. The second aqueous two-phase systems was composed (w/w) by 36 % top phase (1st aqueous two-phase systems), 36 % tris-HCl buffer (100 mmol/L, pH 7.0), and 28 % $(NH_4)_2SO_4$. The polyethylene glycol removal was accomplished by diafiltration using a 10 kDa membrane (Millipore, regenerated cellulose), 5 diafiltration cycles, and pressure of 1.5 kgf/cm^2 at 15 ºC. The purified and the crude enzyme was lyophilized and stored at 4 ºC for use in later steps.

Milk-clotting enzyme activity

The coagulant activity of the enzyme on milk was performed by Berridge methodology (1952) using bovine milk at pH 6.4 and prepared from milk powder (12 %) in calcium chloride ($CaCl_2$) solution (1.11 g/L of distilled water). Milk aliquots were incubated at 30 ºC and 1 mL of the enzyme solution (concentration ranging from 10 to 50 mg/mL) was added. The clotting time was determinated from the clots formation in the tube wall. The milk-clotting activity was performed using crude enzyme (without purification), purified enzyme, and commercial chymosin (Chymax®) as standard. Milk-clotting activity was determinated in relation to chymosin unit (Eq. 1). One unit of milk-clotting activity (MCA) is defined as the amount of enzyme which would clot 10 mL of reconstituted skim milk in 100 seconds at 30 ºC.

$$MCA = \frac{10.V}{t.v} \quad (1)$$

Where: *V* = volume of milk; *t* = clotting time (s); *v* = coagulant volume.

Preparation of coagulant solution

The coagulant solution was prepared from the dissolution of the lyophilized protease enzyme obtained from *Bacillus* sp. P45 in distilled water and stored for 24 h at 4 ºC to complete rehydration. After rehydration, the enzyme solution was used in the clotting activity determination.

Statistical analysis

Data were subjected to analysis of variance to detect significant differences between treatments by Tukey's test. Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Milk-clotting activity

Both crude and purified enzyme were highly active in relation to milk-clotting capability when compared to the commercial coagulant (Table 1). The enzyme, used in the concentration of 30 mg/mL, showed a similar milk-clotting activity to that observed with commercial chymosin and, therefore the potential to be used as an alternative

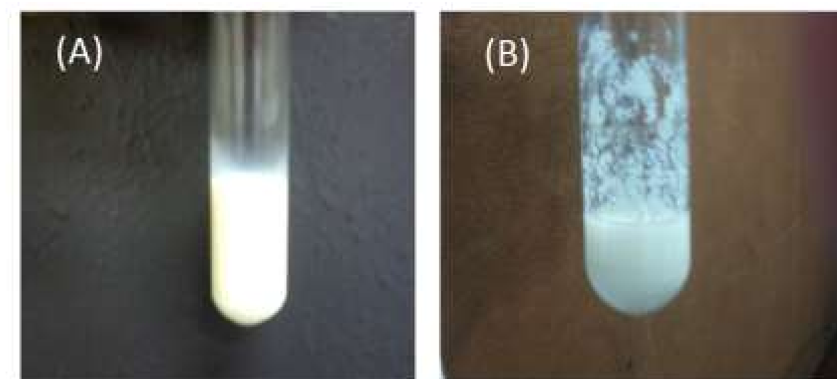
coagulant. The results indicated that milk-clotting activity is also dependent on the concentration of enzyme, as the milk-clotting time decreased as the enzyme concentration increased. This result was similar to that observed for milk-clotting enzymes from vegetables (Chazarra *et al.* 2007, Ahmed *et al.* 2010, Beka *et al.* 2014). A clear linear correlation between the inverse of the enzyme concentration and the milk-clotting activity is verified for the crude and the purified enzyme (Fig. 1), thus making possible the time prediction of coagulation and the coagulant activity of the enzyme (Najéra *et al.* 2003).

Table 1. Milk-clotting activity of crude enzyme extract, purified enzyme and commercial coagulant agent.

Enzyme	(mg/mL)	Clotting time (s)	Chymosin unit
Crude	10	239 ± 7.2 [e]	0.42 ± 1.10^{-2} [e]
	20	140 ± 2.0 [d]	0.71 ± 1.10^{-2} [d]
	30	105 ± 1.7 [c]	0.95 ± 2.10^{-2} [c]
	40	85 ± 1.5 [b]	1.17 ± 2.10^{-2} [b]
	50	76 ± 2.6 [a]	1.32 ± 5.10^{-2} [a]
Purified	10	264 ± 4 [e]	0.38 ± 5.10^{-3} [e]
	20	141 ± 3 [d]	0.71 ± 0.01 [d]
	30	105 ± 2 [c]	0.96 ± 0.02 [c]
	40	82 ± 2 [b]	1.22 ± 0.02 [b]
	50	74 ± 2 [a]	1.36 ± 0.03 [a]
Commercial	1 mL	106 ± 1 [c]	0.96 ± 0.01 [c]

The statistical analysis of the averages used the Tukey test, whereas same letters on the columns represents no significant differences at the significance level of 5 %.

Clots formed by the three enzymes showed the same characteristics: stable and with transparent whey exudate. Figure 2 shows bovine milk coagulated by the action of purified enzyme compared with the control (without enzyme). The application in milk coagulation showed that both the enzyme purified as the crude enzyme could be used as interesting alternative coagulant substitute for animal, vegetable and microbial (genetically modified) sources, since these may be restricted due to religious reasons, and even by regulatory limitations, such as in the case of GMOs.



The choice of optimal coagulant during the dairy products development is crucial, since it can influence the technological properties of the product, including the texture parameters (Borsting *et al.* 2015) and the sensory attributes.

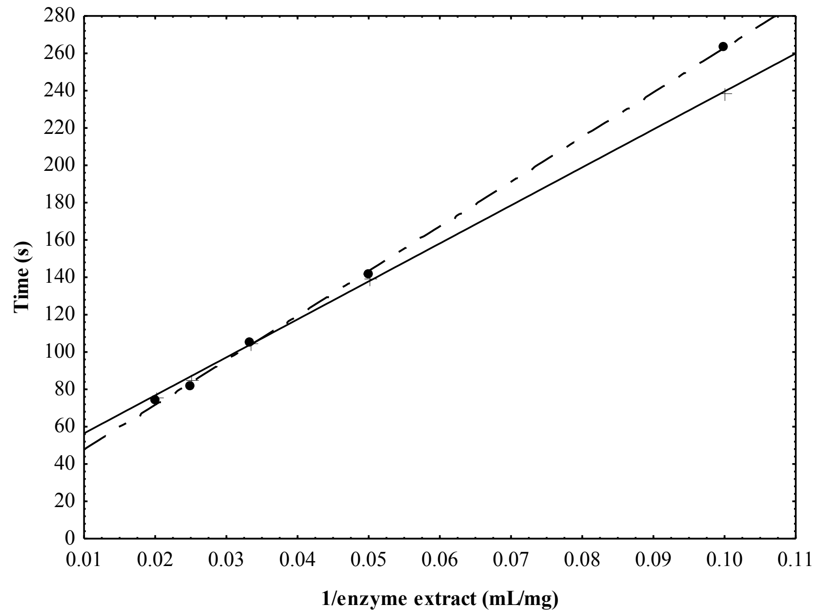
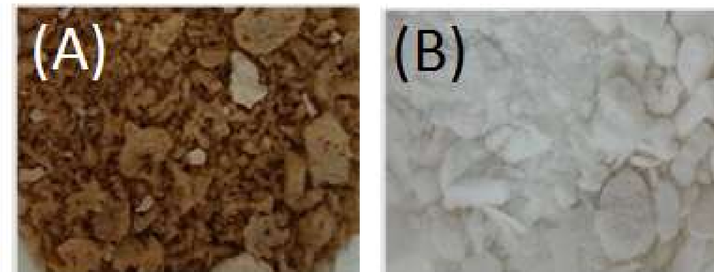


Figure 1. Milk-clotting activity of crude enzyme (+) and purified enzyme (●) obtained from *Bacillus* sp. P45 relative the enzyme extract concentration (mL/mg). Crude enzyme = 36.1 + 2034.9 * x (r^2 = 0.9994); Purified enzyme = 23.9 + 2390.6 *x (r^2 = 0.9995).

Figure 2. Milk-clotting test using the purified enzyme obtained from *Bacillus* sp. P45. (A) Control test (without enzyme) and (B) with purified enzyme addition.

Despite the similarity in the milk-clotting activity, the use of purified enzyme in the dairy product development could be advantageous. Preliminary sensory tests conducted by trained evaluators indicated that crude enzyme showed intense acetic odor and brown coloration (Fig. 3), affecting the color and aroma of the final product, and therefore the consumers acceptance. Thus, the purified enzyme can be chose and applied in the further milk-clotting experiments and in the development of dairy products.

Figure 3. Freeze-dried samples of crude enzyme (A) and purified enzyme (B) obtained from *Bacillus* sp. P45.



CONCLUSION

A novel protease from *Bacillus* sp. P45 was efficiently applied in the hydrolyze of milk proteins and showed a high coagulating activity. The enzyme application in the milk coagulation showed that the purified enzyme could be used as interesting alternative in developing of innovative biotechnological processes, particularly for the development of new dairy products.

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DEVELOPMENT OF CREAMY CHEESE ENRICHED WITH CHIA AND QUINOA

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Resumen: El objetivo de este trabajo fue investigar el desarrollo de un queso untable cremoso enriquecido con chía y quínoa. El queso untable fue producido con la enzima queratinasa purificada de *Bacillus* sp. P45 como agente coagulante. El pH, la acidez, perfil de textura, retención de agua, la actividad de agua y la evaluación microbiológica del producto fueron determinados. Durante la preparación y el almacenamiento del producto se encontró una disminución del pH debido a un aumento en la concentración de ácido láctico.Esto es ocasionado por los microorganismos fermentativos que utilizan e hidrolizan la lactosa. La sinéresis fue menos de 5%, lo que indica la formación de un coágulo con alta estabilidad. Los resultados indicaron que el queso untable producido con la enzima queratinasa purificada y enriquecido con harina de chía y quínoa mostró condiciones sanitarias satisfactorias, compatibles con la legislación brasileña. Las características tecnológicas encontradas durante el desarrollo del queso untable demostraron que el producto es muy estable y viable, cumpliendo con las expectativas del mercado para este tipo de producto.

Palabras clave: leche, queso untable, chia, quínoa.

Abstract: The aim of this study was to investigate the development of a creamy cheese enriched with chia and quinoa. The cream cheese was made using the purified keratinase enzyme from *Bacillus* sp. P45 as coagulant agent. The pH, acidity, texture profile, water retention, water activity and microbiological evaluation of the product was analyzed. During the product preparation and storage, it was possible to verify the reduction of pH caused by lactic increase, due to the presence of fermentative microorganisms that use and hydrolyze lactose. The syneresis was practically nil, less than 5%, indicating the formation of a clot with high stability. These results indicate that the creamy cheeses made using the purified keratinase and enriched with chia and quinoa flour showed satisfactory sanitary conditions, compatible with current Brazilian laws. The technological characteristics found during the development of cream cheese enriched with chia and quinoa flour showed that the product is highly stable and viable, given market expectations regarding this type of product.

Keywords: milk,cream cheese, chia, quinoa.

INTRODUCTION

Milk coagulation, in most cheese-making processes, is caused by the action of proteases on milk proteins. The specific hydrolysis of the peptide bond between $phenylalanine_{105}$ and $methionine_{106}$ in *k*-casein provokes casein micelle destabilization and subsequent aggregation, resulting in the transformation of the milk into curd (Kumar *et al.* 2010).
The development of dairy products enriched with bioactive compounds that bring benefits to the human body are an interesting alternative, since it has increased the interest of the consumers for such products. Quinoa (*Chenopodium quinoa* Willd.) is a seed traditionally grown in the Andes region that is mostly used in flour development and animal feed (Stikic *et al.* 2012). It is recognized for the high content of essential amino acids, protein, fiber and minerals (Zhun *et al.* 2002, Madl *et al.* 2006, Nsimba *et al.* 2008). Moreover, quinoa presents a diversity of phenolic compounds, which have been associated with a high antioxidant activity, protection against lipid oxidation and prevention of inflammatory processes, cancer and other diseases related to oxidative stress (Gawlik-Dziki *et al.* 2013). On the other hand, chia (*Salvia hispanica* L.) presents high content of protein, fiber (Capitani *et al.* 2012, Marineli *et al.* 2014) and the higher content of α-linolenic acids among vegetable sources, up to 60% (Ayerza y Coates 2011). Also, it has high antioxidant activity, associated with tocopherols and polyphenolic compounds (Capitani *et al.* 2012), suggesting that the seeds can be used as functional ingredients in food formulations (Marineli *et al.* 2014).
To the best of our knowledge, no previous reports have described the development of cream cheese enriched with chia and quinoa flour, especially using an alternative coagulant. Furthermore, with the increase consumer demands for healthy and alternative products, the aim of this work was to investigate the application of a new milk-clotting enzyme to replace the conventional coagulants
for use in the development of an innovative dairy product enriched with chia and quinoa flour.

MATERIALS AND METHODS

Microorganism, inoculum, cultivation and purification

Bacillus sp. P45 (GenBank accession number AY962474), maintained on brain-heart agar (BHA) at 4ºC, was used to produce the enzyme. For inoculum preparation, this strain was grown on BHA at 30ºC for 24 h. The cultures were scraped from the agar surface, added to a sterile 0.85% (w/v) NaCl solution, and mixed until a homogeneous suspension with $O.D._{600}$ of 0.5 was obtained. The enzyme was produced by submerged cultivation using feather meal as substrate as described by Daroit *et al.* (2011). After cultivation, the culture was then clarified by centrifugation (5000 ×g for 20 min). The supernatant containing the enzyme was purified from a sequence of two aqueous two-phase systems integrated into the diafiltration process to remove the polyethylene glycol (PEG) (Sala *et al.* 2014). The purified enzyme was lyophilized and stored at 4ºC for use in the cream cheese development.

Cream cheese development using purified enzyme

The five cream cheese formulations enriched with chia and quinoa flour was produced by the process illustrated in Figure 1 using the starter culture and the coagulant agent (purified enzyme). The cream cheese formulations were different in the relative concentrations of cream, chia and quinoa flour added (Table 1).The product was evaluated by the following parameters in triplicate: pH and acidity (every 5 days); texture profile and water retention (days 1, 13 and 25 of storage); water activity (13th day of storage). In addition, microbiological analyses (coagulase-positive *Staphylococcus*, *Salmonella*, *Listeria monocytogenes*, total and fecal coliforms, aerobic mesophilic, molds and yeasts) were performed.

Table 1. Cream cheeses formulations made from the purified keratinase enzyme from *Bacillus* sp. P45 and enriched with chia and quinoa.

Ingredients	Formulations (%)				
	1	2	3	4	5
Cream	6.0	10.0	6.0	10.0	8.0
Chia flour	4.0	4.0	1.0	1.0	2.5
Quinoa flour	1.0	1.0	4.0	4.0	2.5

pH and acidity determination during cream cheese storage

The pH was measured potentiometrically and the acidity by direct titration with NaOH (0.1 mol L^{-1}) using phenolphthalein as indicator (AOAC 2005). The results were determined in Dornic degree and converted to lactic acid concentration (mol L^{-1}).

Water retention and syneresis

The water retention (%WR) analyses of the cream cheese enriched with chia and quinoa flour were performed during the storage by centrifugation and gravimetry. The %WR was determined by centrifugation of the samples (1000 x g, 20 min, at 4 ºC) stored at 4ºC in suitable containers containing 50 g of sample. After centrifugation the supernatant was drained and weighed to determine the water retention value (Eq. 2) and syneresis (Eq. 3).

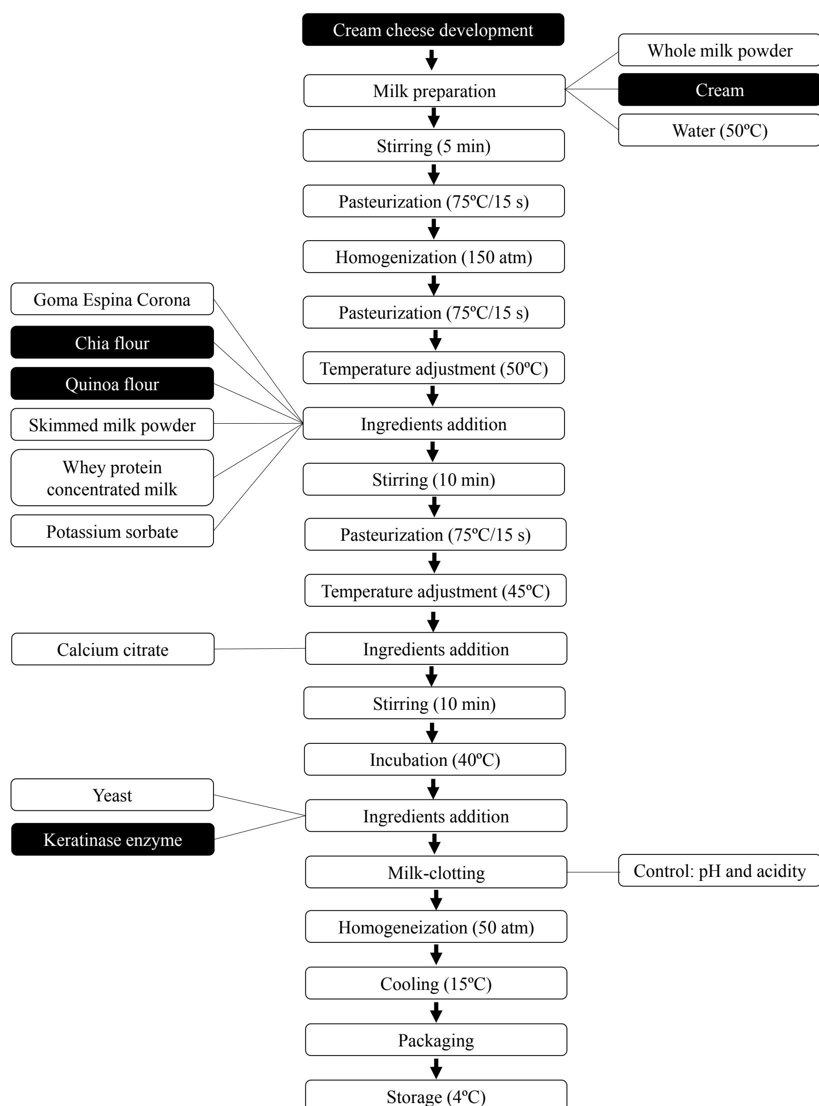
$$\%\mathrm{WR} = 100 - \left((\text{Initial weight} - \text{final weight})/(\text{inital weight})\right) \mathrm{x}\, 100$$

(2)

$$\%\,Syneresis = 100 - \%\,WR \qquad \textbf{(3)}$$

The water retention of cheeses by gravity was determined by weighing the tubes containing the samples stored in suitable containers at 4 ºC and containing 50 g of sample. The supernatant fluid present on the surface were drained and weighed to determine the parameters.

Figure 1. Flow chart of cream cheese development using the purified enzyme from *Bacillus* sp. P45 and enriched with chia and quinoa flour.



Water activity determination

The water activity was determined at 25 ºC using the Aqua Lab CX-2T model 1998 (Decagon) equipment.

Texture profile

The texture profile during the storage was determined by double penetration using Instron Bluehill® texturometer from the stress curve in Newton (N) versus time in seconds. The following mechanical properties were determined: hardness, adhesiveness, springiness, cohesiveness, gumminess, chewiness and resilience (Santini *et al.* 2007). The parameters used in the tests were: penetration of 30 mm, speed of 1 mm/s, bristle of 10 N, penetrometer diameter of 12 mm and cylinder diameter of 36 mm at 10 ºC.

Microbiological analyses

Microbiological analyses were performed for coagulase-positive *Staphylococcus*, *Salmonella*, *Listeria monocytogenes*, total and fecal coliforms, aerobic mesophilic, molds and yeasts, following standard protocols (Doores *et al.* 2013).

Statistical analysis

Data were subjected to analysis of variance to detect significant differences between treatments by the Tukey test. Differences were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

pH and acidity

Similar behavior, pH decline and proportional increase on lactic acid production, is observed in all samples (Figure 2). The pH during fermentation ranged from 6.6 to 6.3 at the beginning to 5.3 at the end of fermentation process. The lactic acid production ranged from 0.02 to 0.03 at the initial phase, reaching from 0.06 to 0.1 $molL^{-1}$ at the end of fermentation process, lasting about 5 h.

The pH reduction and increase in acidity occurs because once the yeast is added, the lactic fermentation is started. The microorganisms performed the hydrolysis of lactose into glucose and galactose, and the glucose is subsequently transformed into lactic acid. The lactic acid production causes the drop of the pH, even though most of the enzymes remain active and microorganisms remain viable. This effect however, is less marked during the storage due to the fact that lower temperatures are used (Rojas-Castro *et al.* 2007).

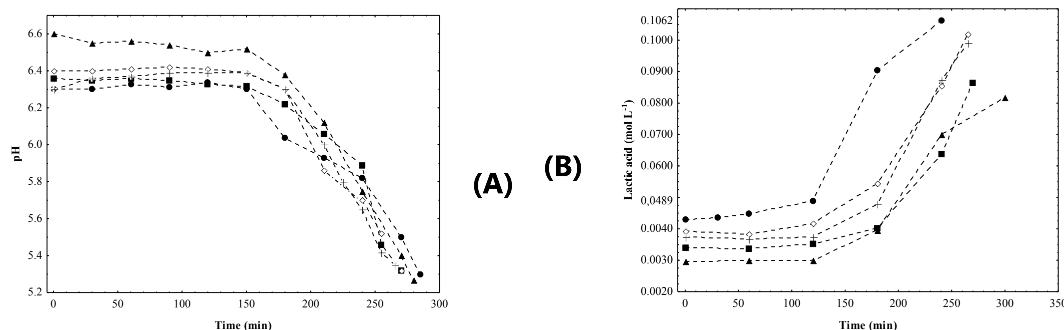


Figure 2. pH (A) and acidity (B) of the formulations prepared from purified enzyme during the fermentation process. (●) formulation 1: 6% cream, 4% chia, 1% quinoa; (■) formulation 2: 10% cream, 4% chia, 1% quinoa; (◇) formulation 3 - Cream 6%; Chia 1%; Quinoa 4%; (▲) formulation 4 - Cream 10%; Chia 1%; Quinoa 4%; (+) formulation 5 - Cream 8%; Chia 2.5%; Quinoa 2.5%.

The pH reduction (Fig. 3A) and acidity increase (Fig. 3B) was observed in all cream cheese formulations during the 25 days storage period. During the first ten days of storage the pH decrease and lactic acid production is more pronounced than in subsequent periods, with a tendency to stabilize at the end of storage.

This could be due to **(i)** inhibition of the enzymatic activity present in the lactic culture, **(ii)** viability of microbial load and **(iii)** lactose depletion combined with the low temperature (Tamime y Robinson 1985, Rojas-Castro *et al.* 2007).

The pH stabilizing effect and lower lactic acid production at the end of the storage is a natural and desirable phenomenon during the development of fermented dairy products and is described by several authors. Also, the pH values and acidity observed in this work are comparable with those reported by other authors (Olmedo *et al.* 2013, Brighenti *et al.* 2008, Deegan *et al.* 2014). The results obtained here for cream cheeses made using the purified enzyme and enriched with chia and quinoa flour, it can be assumed that the pH and acidity values are consistent with those usually expected for this product.

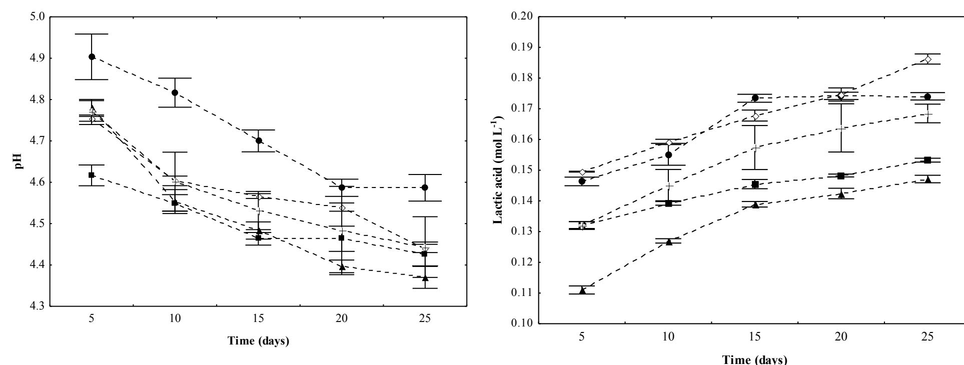


Figure 3 – pH **(A)** and acidity **(B)** of the formulations prepared from purified keratinase enzyme during the storage by 25 days. (●) formulation 1: 6% cream, 4% chia, 1% quinoa; (■) formulation 2: 10% cream, 4% chia, 1% quinoa; (◇) formulation 3 - Cream 6%; Chia 1%; Quinoa 4%; (▲) formulation 4 - Cream 10%; Chia 1%; Quinoa 4%; (+) formulation 5 - Cream 8%; Chia 2.5%; Quinoa 2.5%.

(B)

Water activity of cream cheese

The water activity (a_w) was similar for all samples, independently of the amount of cream, chia or quinoa flour used. Pinto *et al.* (2011) also noted the similar water activity during the storage of cheese during 60 days. The determination of water activity is important since it is related to the water availability for deleterious reactions or microbial metabolism (Ostrowska–Ligeza y Lenart 2014**)**. The a_w values around 0.96 indicate that the product would be susceptible to most bacteria and fungi, but the acidic pH of the cream cheese protects against several pathogenic bacteria.

Water retention and syneresis

In relation to water retention and syneresis, no important differences were observed among the formulations for the initial period of storage. The values were identical when evaluated by gravity (Fig. 4) and very similar by centrifugation (Fig. 5). However, a decrease in the water retention and respective increased syneresis was observed in the 13th day of storage, with a significant elevation (p < 0.05) during storage for 25 days. This occurrence was more evident when the samples were evaluated by centrifugation (Fig. 5), indicating a greater water release

from cheese matrix. Despite the water release increased during storage for 25 days, the syneresis values obtained in the gravity analysis that simulates the product exposure under normal storage conditions is practically null, indicating a low risk that the defect occurs. This allows us to assume that the enzyme application in combination with the components used in the cheese produces a clot with high stability and water retention ≥ 99.0%. The stability may be also favored by the addition of chia and quinoa flour, since they present a high water retention capacity (Bhargava *et al.* 2006, Capitani *et al.* 2012), especially due the high protein content. In addition, stabilizers used in the cream cheese samples, such as *espina corona* gum tends to "bind" water in the gel structure, generally improving the hydration of proteins.

Despite the increasing of syneresis during the storage ($p < 0.05$), the highest value obtained with respect to gravity (0.72%) is insignificant and does not affects this quality attribute. Rozycki *et al.* (2010) also observed a high water retention (≥ 99,0%) for a cholesterol-free probiotic cream cheese developed with reduced fat, showing high stability of the clot throughout the storage, without whey separation, which is a common defect in fermented dairy products. Zulkurnain *et al.* (2008) observed that creamy cheeses made from soy proteins also showed high stability against syneresis over storage of 20 days. The high protein and fat content contribute with a stable structure retaining water inside during the storage.

The findings of Zulkarnain *et al.* (2008) agree with the current study where the formulations 2 and 4, with the highest cream concentration (10%), showed the highest water retention (%WR) when measured by centrifugation, and consequently the lower syneresis during the storage. In the other hand, the opposite is also observed: samples with less cream addition (6%) showed the greatest water release from the cheese matrix. The same phenomenon was verified by Mateo *et al.* (2009) that observed a decreased in the whey release with increasing fat concentration during the cheese development, mainly due to the emulsifying properties.

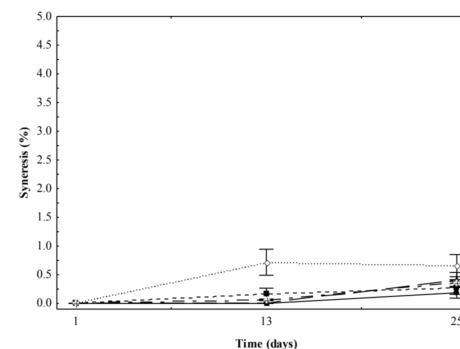
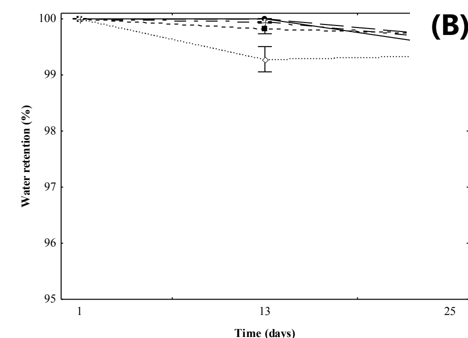


Figure 4. (A) Syneresis and (B) water retention (%WR) determined by gravity. (●) formulation 1: 6% cream, 4% chia, 1% quinoa; (■) formulation 2: 10% cream, 4% chia, 1% quinoa; (◇) formulation 3 - Cream 6%; Chia 1%; Quinoa 4%; (▲) formulation 4 - Cream 10%; Chia 1%; Quinoa 4%; (+) formulation 5 - Cream 8%; Chia 2.5%; Quinoa 2.5%.



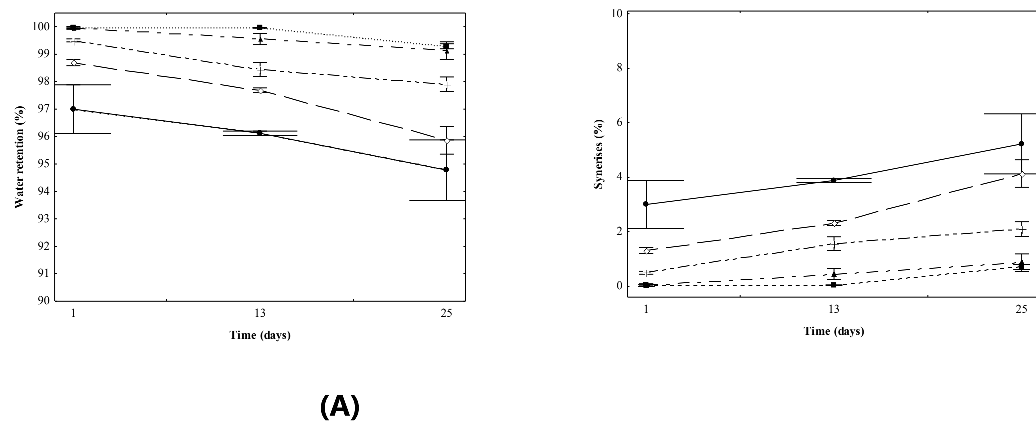


Figure 5 - (A) Syneresis and (B) water retention (%WR) of five cream cheese formulation determinated by centrifugation. (●) formulation 1: 6% cream, 4% chia, 1% quinoa; (■) formulation 2: 10% cream, 4% chia, 1% quinoa; (◇) formulation 3 - Cream 6%; Chia 1%; Quinoa 4%; (▲) formulation 4 - Cream 10%; Chia 1%; Quinoa 4%; (+) formulation 5 - Cream 8%; Chia 2.5%; Quinoa 2.5%.

Texture profile

The texture profile of cream cheese enriched with chia and quinoa flour was evaluated by the following parameters: hardness, adhesiveness, springiness, cohesiveness, gumminess and chewiness. The parameters were compared among the different samples for the same period of storage and the same samples for the different storage time.

In general, all parameters showed significant differences ($p < 0.05$) as a function of time and the formulations studied. The elasticity was the only parameter that showed no significant difference, suggesting no change in the recovery of initial cheese dimensions after the removal of deforming force (Santini *et al.* 2007).

The formulation 3, with the highest quinoa and less cream content, had the highest hardness. This suggests that the high capacity of quinoa to absorb water, combined with lower fat concentration, which is responsible for creaminess and smoothness of the cream cheese, can contribute to a greater resistance to deformation (Santini *et al.* 2007). The formulations 1, 2 and 5 showed an increase in hardness on the 13th day of storage, while formulations 3 and 4 showed increased hardness on the 25th day of storage.

The adhesiveness was higher for samples with higher quinoa content, indicating the formation of a structure more resistant that requires greater force for removing the cheese from the contact surface of the mouth, usually the palate or the tongue (Bryant *et al.* 1995, Karamana y Akalın 2013). The formulations 1, 2 and 3 showed no significant variation in adhesiveness during the storage of 25 days. However, the formulations 4 and 5 showed an increase in the adhesiveness on the 13th day of storage.

The cohesiveness was significantly higher for formulation 1, where a higher chia and lower cream content is present. These conditions favor the production of a more elastic gel and consequently, require greater effort to reach the break point of the product. During the storage time there was no change in cohesiveness parameter for the formulations 1 and 5; however, it was significantly altered in formulations 2, 3 and 4, mainly for the 25 days of storage.

The gumminess and chewiness (secondary attributes) showed the same behavior. Highest values were found for samples with higher quinoa content, demonstrating their influence on the cheese texture profile. Both gumminess and chewiness remain equal for the formulation 3 during the 25 days of storage. However, there was significant difference for formulations 1, 2 and 5 on the 13th day and significant change in these attributes only for the formulation 4 on the 25th day of storage.

The changes in the cream cheese texture profile, in some cases, during the storage time can be triggered by proteolysis processes, glycolysis, lipolysis, and pH changes. These alterations, in addition with the full solubility of the cheese constituents and the enzymatic activities continuous cause a change in texture of the cream cheese (Lucey *et al.* 2003). Furthermore, the acidity increase during the cream cheese storage may also cause changes in the characteristics of protein aggregates and in the texture profile (Buriti *et al.* 2005, Queiroga *et al.* 2013).

In general the different formulations of cream cheese showed no significant variations during the development and storage, thus suggesting the use of formulations made with lower cream contents, since consumers seek products with healthier and functional properties. Furthermore, high cream content may not be desirable for contributing to increased health risk, since cardiovascular diseases are linked to 17 million deaths annually, mainly due to dietary and behavioral problems (Andrade *et al.* 2013).

Thus, taking into account the desirable final characteristics of the product, especially acceptability by consumers, two products could be indicated for marketing: (a) with high chia content (formulation 1) and (b) with high quinoa content (formulation 3); both with reduced cream content. The properties of chia and quinoa seeds, such as amino acid composition, protection against lipid oxidation, and potential in the prevention of inflammatory processes, cancer and other diseases related to oxidative stress such as atherosclerosis, suggest their use as functional ingredients with a high potential for application in this type of product (Zhun *et al.* 2002, Madl *et al.* 2006, Nsimba *et al.* 2008, Ayerza y Coates 2011, Stikic *et al.* 2012, Gawlik-Dziki *et al.* 2013, Marineli *et al.* 2014).

Microbiological analyses

The cream cheese made using the purified enzyme and enriched with chia and quinoa flour showed satisfactory sanitary conditions during the storage, compatible with current Brazilian regulations (MAPA, 1996; ANVISA, 2001). Coagulase-positive *Staphylococcus* was absent in 0.01 g of cream cheese samples. Absence of *Salmonella* spp. and *Listeria monocytogenes* was found in 25 g samples. The results for total and fecal coliform were < 0.3 MPN/g (Most Probable Number). Total counts of aerobic mesophilic were around 1.0 x 10^4 CFU/g (Colony Forming Units), and yeasts and molds around 3.6 x 10^3 CFU/g. Thus, the preservatives used and the appropriate manufacturing process were sufficient to ensure satisfactory sanitary conditions and microbial stability to the cheese samples over the 25 days of storage.

CONCLUSION

A new cream cheese enriched with chia and quinoa flour was successfully developed. The cream cheeses were highly stable and viable, meeting the market expectations for this type of product. The product was very stable in relation to syneresis, and shows high level of water retention (≥ 99.0%) during 25 days storage. The cream cheese made using the purified enzyme showed suitable sanitary conditions during the storage, compatible with current Brazilian regulations. Moreover, the results indicate the feasibility of the purified enzyme as an alternative coagulant to the development of innovative biotechnological processes, such as the development of new dairy products enriched with functional ingredients.

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UTILIZACIÓN DEL ALPERUJO COMO SUSTRATO PARA EL CRECIMIENTO MICROBIANO

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Resumen: El proceso de producción de aceite de oliva mediante el sistema centrífugo de dos fases produce un residuo denominado alperujo, el cual exhibe un alto porcentaje de agua (~65 %) y la presencia de biofenoles. Estos compuestos son tóxicos y altamente contaminantes para el suelo y cursos de agua. En este trabajo evaluamos la capacidad de un extracto acuoso de alperujo (EAA) para sostener el crecimiento microbiano, en un intento para valorizar este residuo. Mediante la técnica de enriquecimiento de muestras de alperujo se aislaron 1 hongo, 6 levaduras y 5 cepas bacterianas (3 bacilos Gram-, 1 coco Gram+ y un coco Gram-). Entre las levaduras se aisló la cepa productora de carotenos *Rhodotorula spp.*, la que se utilizó como organismo modelo para evaluar la capacidad del EAA para sostener su crecimiento y la producción de carotenos. Se observó una relación inversa entre el crecimiento de *Rhodotorula spp.* y la producción de carotenos con la concentración de EAA. El crecimiento de *Rhodotorula spp.* no decoloró el EAA, ni redujo el contenido total de biofenoles. Estos resultados demuestran que el EAA, puede ser utilizado como sustrato para el crecimiento microbiano siendo una alternativa para valorizar el alperujo.

Palabras clave: alperujo, biofenoles, crecimiento microbiano, valorización de residuos

Abstract: The production process of olive oil by the two-phase centrifugal system leaves a residue named “alperujo” which has a high percentage of water (65%) and contains biophenols. These compounds are toxic and pollute the soil and waterways. The aim of this work is to evaluate the ability of an aqueous extract from “alperujo” (EAA) to sustain microorganism growth in an attempt to valorize this waste. By sample enrichment technique, 1 fungus, 6 yeast and 5 bacteria (3 bacilli Gram-, 1 coco Gram+ and 1 coco Gram-) were isolated. Among the yeast isolated, *Rhodotorula spp.* -a carotene producing yeast- was found. *Rhodotorula spp.* was used as a model organism to assess the ability of EAA to sustain its growth and carotenoid production. An inverse relationship between *Rhodotorula spp.* growth and carotenoid production with EAA concentration was observed. *Rhodotorula spp.* growth neither decolorized nor reduced total biophenol content. These results demonstrate that EAA can be used as a substrate for microbial growth, being this strategy an alternative for “alperujo” valorization.

Keywords: alperujo, polyphenol, microbial growth, waste valorization

INTRODUCCIÓN

La producción de aceites de oliva vírgenes extra en Argentina, se ha incrementado notablemente durante los últimos años, sumándose a las provincias productoras tradicionales nuevas áreas productivas a menor escala, entre ellas el sudoeste bonaerense (COI 2012). La extracción del aceite de oliva puede realizarse por el método tradicional, también llamado discontinuo o por prensado, y por los sistemas continuos que utilizan centrífugas horizontales de dos o tres fases. Actualmente están más difundidos los sistemas continuos siendo el sistema centrífugo de dos fases el más utilizado en nuestro país. En este sistema de extracción se obtiene un residuo semisólido denominado alperujo en una proporción de hasta 80 toneladas por cada 100 de aceitunas procesadas. Tiene un alto contenido de humedad (~65%), un pH ligeramente ácido y casi un 90% de su peso seco es materia orgánica, en su mayoría lignina, celulosa y hemicelulosa así como grasas, proteínas y carbohidratos. Además, es altamente contaminante para el suelo y los cursos de agua debido a su contenido en biofenoles (Morillo *et al.* 2009, Mekki *et al.* 2006, Nasini *et al.* 2013, Ouzounidou *et al.* 2010).

Pese a su contenido en biofenoles, los cuales inhiben el crecimiento microbiano, diversas especies de bacterias, hongos y levaduras han sido aisladas del alperujo (Ntougias *et al.* 2013). Estos hallazgos indican que el alperujo tiene algún potencial para ser utilizado como sustrato para el crecimiento de diversos microorganismos, lo que valorizaría este residuo (D'Annibale *et al.* 2006). Entre las especies aisladas, se encuentran varias con potencial biotecnológico, como por ejemplo las levaduras oleaginosas del genero *Rhodotorula spp.* (Gonçalves *et al.* 2009). Este género de levaduras se destaca por su eficiencia en la producción de lípidos y por la capacidad de producir carotenoides (Frengova y Beshkova 2009). El objetivo de este trabajo es evaluar la capacidad del extracto acuoso de alperujo proveniente del sudoeste bonaerense de sostener el crecimiento de la levadura *Rhodotorula spp.* y la producción de carotenos.

MATERIALES Y MÉTODOS

Muestras de alperujo

Se tomaron muestras de alperujo de las variedades *Frantoio*, *Arbequina* (cosecha 2014) y *Nevadillo* (cosecha 2013) en una planta ubicada en Coronel Dorrego (provincia de Bs. As.) que utiliza la separación centrífuga de dos fases. Las muestras correspondieron a frutos de la propia finca. Los alperujos fueron guardados a -20 °C hasta su procesamiento. El contenido de humedad de las muestras se determinó en estufa de vacío (50 ºC) por gravimetría a partir de aproximadamente 5 g de alperujo.

Extractos acuosos de alperujo (EAA)

Una cantidad previamente pesada de alperujo fue extraída con agua destilada en relación alperujo:agua 1:3 (m:V) durante 2 h a 4 ºC con agitación continua. El extracto acuoso (EAA) obtenido se centrifugó durante 15 min a 2500 rpm, se filtró y se esterilizó en autoclave. A partir de este EAA al 25 % (m/V) se realizaron diluciones al medio (12,5 %) y al cuarto (6,25 %) con agua destilada estéril.

Enriquecimiento y aislamiento de microorganismos

Aproximadamente 2 g de alperujo de *Frantoio* fresco se incubaron con 50 ml de medio mínimo mineral (KH_2PO_4 0,25 %, $NaHPO_4$ 0,25 %, NH_4NO_3 0,1 %) durante 7 días a 30 ºC. Luego de la incubación, una alícuota diluida del sobrenadante fue sembrada en placas de Agar Tripticasa Soya (ATS, Britania, C.A.B.A., Argentina) y de Extracto de

levadura, Glucosa y Cloramfenicol (YGC, Britania, C.A.B.A., Argentina) para aislar y cuantificar los microorganismos presentes. Las colonias fueron estudiadas en cuanto a su morfología pasándose cada colonia diferente a tubos y placas hasta obtener un cultivo uniforme. Se caracterizaron los microorganismos mediante la tinción de Gram. Los recuentos (UFC) se expresaron en base seca de alperujo. La cuantificación del número de microorganismos se realizó por triplicado mediante el recuento en placa.

Determinación de fenoles totales

Se aplicó el método espectrofotométrico de Folin-Ciocalteu sobre el EAA antes y después del crecimiento de microorganismos, previa centrifugación de las muestras. Los resultados fueron expresados en mg de ácido cafeico por g de alperujo (b. s.) (Vázquez-Roncero *et al*. 1973).

Extracción de azúcares del alperujo

El alperujo fue desengrasado mediante extracción con n-hexano durante 20 min a temperatura ambiente con agitación. Luego, aproximadamente 2 g de alperujo fueron sometidos a la extracción de los azúcares solubles utilizando 40 ml de etanol 80 % con agitación durante 4 h a 80ºC. El líquido se concentró en un evaporador rotatorio para eliminar el etanol y se transfirió a un tubo de centrífuga en donde se procedió a precipitar las proteínas con acetato de plomo neutro. La muestra se centrifugó y se dejó reposar durante 15 min, el sobrenadante se diluyó con agua destilada, se filtró y se añadió Na_2CO_3 anhidro hasta precipitar el plomo. Nuevamente se filtró y se llevó a volumen en matraz de 100 ml obteniéndose un extracto de azúcares (EAZ).

Determinación del contenido total de azúcares

Se realizó por espectrofotometría de acuerdo al método de metahidroxidifenilo (Kintner y van Buren 1982). El ensayo fue aplicado sobre el EAZ y sobre el EAA antes y después del crecimiento de microorganismos, previa centrifugación de las muestras.

Identificación y cuantificación de azúcares

El contenido de azúcares fue determinado por HPLC-IR siguiendo el método utilizado por Martínez Ruiz (2005) con las modificaciones realizadas por Baümler *et al*. (2012). Brevemente una alícuota del EAZ se liofilizó, se resuspendió en agua, se filtró y se inyectó en un cromatógrafo Alliance e2695 (Waters, EEUU), equipado con una columna Rezex ROA Organic Acid (Phenomenex, EEUU) de 300mm x 7.8 mm y detector de índice de refracción modelo 2414 (Waters, EEUU). Como fase móvil de empleó una solución de $5*10^{-3}$ N de H_2SO_4 con un flujo de 0,3 mL/min. Los datos se procesaron con el programa Empower 2 (Shimadzu, Japón). Como estándares se utilizaron: Almidón, Rafinosa, Celobiosa, Maltosa, Sacarosa, Glucosa, Xilosa, Galactosa, Fructosa, Manitol, Ramnosa y Arabinosa (Anedra) en concentraciones de 1-10 mg/ml. Los resultados obtenidos se expresaron en mg de azúcares/ g de alperujo (b.s.).

Producción, extracción y cuantificación de carotenos

Un inóculo de la levadura productora de carotenos, *Rhodotorula spp.,* aislada del alperujo, fue sembrado en erlenmeyers conteniendo 50 ml de EAA (puro, diluido 1/2 y al 1/4, correspondientes a un concentración m/V de 25 %, 12,5 % y 6,25 %) e incubado durante 14 días a 30 ºC con agitación. Al final de la incubación una alícuota de las muestras fue separada para determinar el crecimiento microbiano mientras el resto fueron centrifugadas 15 min a 3000 rpm. El sobrenadante se reservó para determinar azúcares y biofenoles. Los

pellets obtenidos fueron congelados y descongelados 2 veces y luego incubados con HCl 1 M a 70 ºC durante 30 min. Al final de la incubación se centrifugaron y lavaron con agua destilada. Los carotenos fueron luego extraídos con 30 ml de una mezcla acetona:metanol (1:1) durante toda la noche. Luego de centrifugar 15 min a 3000 rpm, el sobrenadante fue separado, el solvente evaporado y los carotenos resuspendidos en 3 ml de acetona para su cuantificación (Schneider *et al.* 2012). La concentración total de carotenos fue determinada espectrofotométricamente midiendo la $A_{450\ nm}$ en un espectrofotómetro Shimadzu UV-160A, considerando un $A_1^{1\%}$ promedio de 2500 (Britton y Young 1993).

RESULTADOS Y DISCUSIÓN

Composición del alperujo

En la Tabla 1 se presenta los contenidos totales de azúcares determinados espectrofotométricamente y los resultados del análisis cuali y cuantitativo determinado por HPLC-IR del alperujo de las tres variedades estudiadas. La cantidad de azúcares totales varió de acuerdo a la variedad de aceituna, presentando *Arbequina* la mayor proporción. Los azúcares son la fuente de carbono más fácilmente metabolizable por la mayoría de los microorganismos, por lo que esta variedad presentaría en este aspecto mayor potencial como sustrato para el crecimiento de microorganismos. A partir del análisis cromatográfico se halló que el contenido y perfil de azúcares varió de acuerdo a la variedad considerada. Almidón, fructosa, glucosa, sacarosa y ramnosa constituyeron los azúcares mayoritarios presentes en todas las muestras.

No hay antecedentes de trabajos previos que describan en detalle la composición de azúcares del alperujo, sólo hay reportes en que se cuantifica sacarosa, glucosa y fructosa en el alpechín que es el residuo obtenido de la centrífuga de tres fases (Sassi *et al.* 2006).

Tabla 1- Contenido de azúcares totales y azúcares individuales del alperujo de distintas variedades.

Azúcares	**Arbequina**	**Frantoio**	**Nevadillo**
Totales [a]	323±10	171±8	243±11
Individuales [b]			
Almidón	89,22±3,68	366,09±10,54	104,14±2,17
Rafinosa	1,32	2,18±1.40	n.d.
Celobiosa	3,87±0,80	8,52±2.54	7,28±2.42
Maltosa	1,36±0,18	n.d.	n.d.
Sacarosa	0,84±0,27	5,16±1.49	2,16±1.28
Glucosa	7,09±0.31	35,45±1.26	0,61±0.31
Xilosa	0,60±0.53	n.d.	0,71±0.13
Galactosa	n.d.	n.d.	0,17
Fructosa	207±18	81,82±2.08	26,22±0.37
Ramnosa	7,24±1.15	14,98±0.92	8,27±0.37
Arabinosa	1,51±0.34	1,24±0.42	0,43±0.16

[a] Determinado espectrofotométricamente
[b] Determinados por HPLC
Los resultados se expresan mg/g alperujo (b.s.) y se muestran valores promedio ± desviación estándar (n=3)

Morillo *et al.* (2006) mostraron un comportamiento bifásico para el crecimiento de microorganismos en función de la concentración de un EAA, atribuido a que se debe lograr un balance entre los nutrientes presentes en el EAA (principalmente azúcares solubles) y la presencia de compuestos inhibitorios (principalmente biofenoles). Los biofenoles inhiben el crecimiento microbiano, por lo cual es importante determinar su cantidad (Capasso *et al.* 1995).

Tabla 2- Contenido de biofenoles totales del alperujo de distintas variedades.

Variedad	Biofenoles totales
Arbequina	6,9±0,5
Nevadillo	8,2±0,3
Frantoio	11,3±0,1

*valores promedio ± desviación estándar, (n=3). Resultados expresados en mg/g

La Tabla 2 presenta el contenido de biofenoles totales del EAA en las tres variedades estudiadas. Los resultados obtenidos concuerdan con trabajos anteriores (Soberon *et al.* 2012). Es de notar que de las tres variedades, el alperujo de *Frantoio* muestra las condiciones más desfavorables para el crecimiento de microorganismos, es decir menor concentración de azúcares y mayor concentración de biofenoles (Tabla 1 y 2). Por este motivo, este varietal fue seleccionado como sustrato para el crecimiento de *Rodhotorula spp*. con un EAA de *Frantoio*, al ser la situación más compleja.

Potencial biotecnológico del alperujo

Previo enriquecimiento y aislamiento de los microorganismos presentes en el alperujo, se procedió a evaluar el tipo y la cantidad de microorganismos que crecen naturalmente en este medio. Según las características morfológicas de las colonias se hallaron 1 hongo, 6 levaduras y 5 cepas bacterianas (3 bacilos Gram –, 1 coco Gram + y 1 coco Gram –). El recuento total en ATS fue de $4,6 \cdot 10^8$ UFC/g alperujo (b.s.) y en YGC fue de $9,0 \cdot 10^6$ UFC/g de alperujo (b.s.). Estos resultados muestran la variedad de microorganismos que pueden adaptarse al crecimiento en un EAA, indicando que es viable utilizarlo como sustrato. Diversas investigaciones estudiaron la microbiota asociado a los residuos olivícolas, y encontraron que las levaduras son las que mejor se adaptan ya que pueden tolerar la alta concentración de biofenoles y el pH ácido de estos residuos (Rincón *et al.* 2006, Sassi *et al.* 2006, 2008). Asimismo, entre las levaduras aisladas se encontró una cepa cuya macro y micro morfología era similar a la de la levadura *Rhodotorula spp.*, fuente de pigmentos carotenoides y de lípidos (Frengova y Beshkova, 2009).

Crecimiento de *Rhodotorula spp.* y producción de carotenos

Dada la aplicación biotecnológica de la levadura *Rhodotorula spp.*, evaluamos la capacidad de distintas diluciones de EAA de *Frantoio* de sostener el crecimiento de esta levadura. Además utilizamos dos concentraciones iniciales de inóculo de *Rhodotorula spp.* ($3,0 \cdot 10^5$ UFC/ml y $6,0 \cdot 10^5$ UFC/ml).

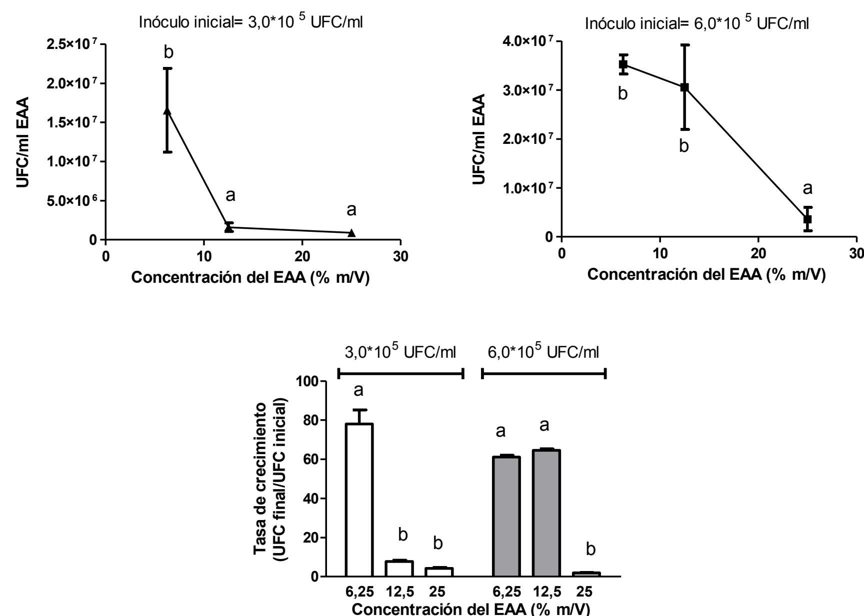

Figura 1- Crecimiento de la levadura *Rhodotorula spp* durante 10 días en distintas concentraciones de EAA y con distinto inóculo inicial. Media ± S.D. (n=3).

Se observó que luego de 10 días de incubación con EAA 25%, la levadura seleccionada era capaz de tolerar la concentración de biofenoles, aunque en esta situación no mostraba crecimiento, independientemente del inóculo inicial. En cambio, el EAA al 12,5%, permitió el crecimiento de la levadura únicamente en el caso del inóculo inicial mayor. Mientras el EAA al 6,25 % permitió el crecimiento de *Rhodotorula spp.* en todos los casos (Figura 1). La tasa de crecimiento, definida como las UFC finales/UFC iniciales aumentó a medida que disminuyó la concentración de EAA (Figura 1).

Se ha estudiado el crecimiento y la producción de carotenos de *Rhodotorula spp.* en distintos sustratos de origen agroindustrial, como residuos de la producción de azúcar y de quesos (Buzzini y Martini 2000). Si bien en estos trabajos el crecimiento de *Rhodotorula spp.* es más rápido que lo observado en este trabajo para EAA, esto puede deberse a que la mayoría de los residuos utilizados como sustrato son suplementados con fuentes de N e incluso en algunos casos se agrega sacarosa (Jarboui *et al.* 2012, Malisorn *et al.* 2008, Buzzini y Martini, 2000). Los tiempos de crecimiento observados en el presente trabajo (10 días) son similares a los de otras levaduras crecidas en alpechín diluido (Bleve *et al.* 2011).

El crecimiento de *Rhodotorula spp*. fue acompañado por un consumo de azúcares pero no de biofenoles. Es así que no se observa disminución en el contenido de biofenoles en el EAA en ninguna de las condiciones evaluadas (Figura 2). Tampoco se produjo una decoloración de la muestra, medida como la absorbancia a 395 nm. Este parámetro está relacionado con la fracción de biofenoles complejos y su disminución en residuos de la industria olivícola sólo ha sido reportada para el caso de tratamientos con microorganismos productores de lacasas y ligninasas, como los hongos filamentosos (Perez *et al.* 1998).

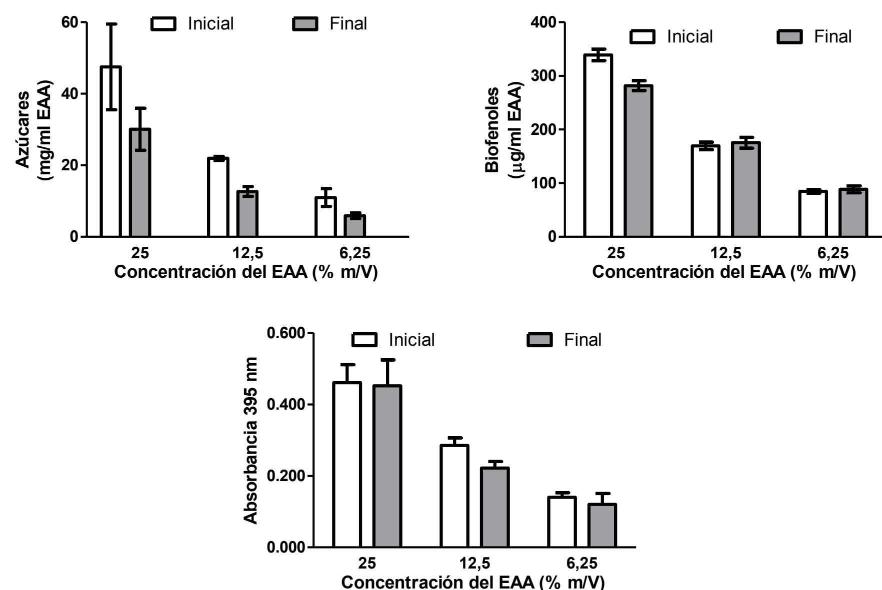

Figura 2- Concentración de azúcares, biofenoles y determinación del color de EAA antes y después del crecimiento de la levadura *Rhodotorula spp*. Media ± S.D. (n=3).

Al evaluar la producción de carotenos por parte de *Rhodotorula spp.* a las distintas concentraciones de EAA, la cantidad de carotenos totales fue mayor cuando disminuyó la concentración de EAA, acompañando el crecimiento de la levadura (Tabla 3). La misma tendencia fue hallada al relacionar la cantidad de carotenos con la biomasa producida (medida como UFC), Tabla 3. La producción de carotenos ocurre en forma más abundante en la fase de crecimiento tardío de la levadura, y como respuesta al stress (Marova *et al.*, 2010). Al haber más levaduras en el EAA (6,25%) es esperable que además las mismas produzcan más carotenos ya que se ven deprivadas de nutrientes.

Tabla 3- Producción de carotenos por *Rhodotorula spp.* a distintas concentraciones de EAA

Concentración de EAA (% m/V)	Carotenos totales (ng)	mg Carotenos/100 g de alperujo (b.s.)	ng Carotenos/10^6 UFC
25	5,74 ±0,01	0,317±0,001	8,7±0,2
12,5	16,06±0,06	3,55±0,01	13,16±0,05
6,25	878±2	97,2±0,2	292 ±1

Valores promedio ± D.S. (n=3)

CONCLUSIONES

Los resultados obtenidos en este trabajo demuestran que es viable la utilización del extracto acuoso de alperujo como sustrato para el crecimiento de microorganismos con interés biotecnológico. Asimismo mostramos que la levadura *Rhodotorula spp.* es capaz de crecer en el extracto acuoso de alperujo y producir carotenos, tolerando la alta concentración de biofenoles presentes.

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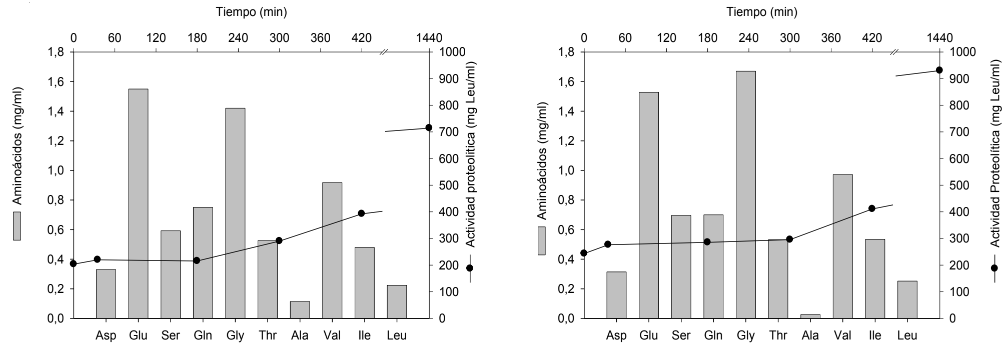
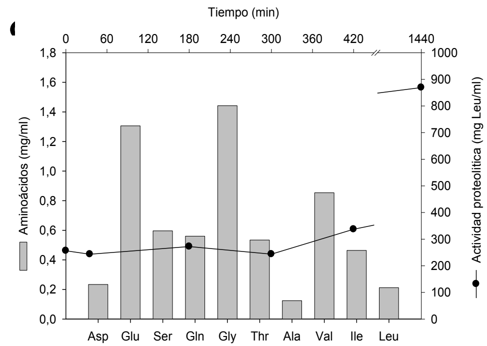
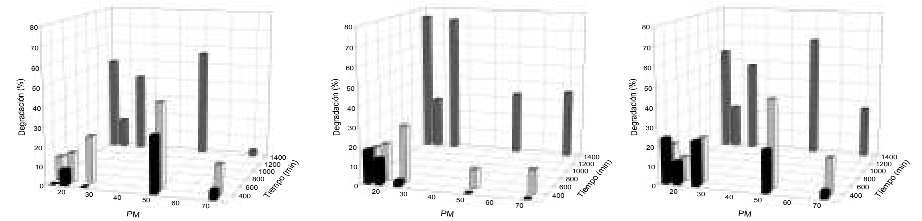


Tiempo (min)
Aminoácidos (mg/ml)
Actividad proteolítica (mg Leu/ml)
Asp Glu Ser Gln Gly Thr Ala Val Ile Leu



Tiempo (min)
Aminoácidos (mg/ml)
Actividad proteolítica (mg Leu/ml)
Asp Glu Ser Gln Gly Thr Ala Val Ile Leu



Degradación (%)
PM
Tiempo (min)