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Oleuropein hydrolysis by lactic acid bacteria in natural green olives

Eva Ramírez, Manuel Brenes, Antonio de Castro, Concepción Romero, Eduardo Medina*

Food Biotechnology Department, Instituto de la Grasa (IG-CSIC), Campus Universitario Pablo de Olavide, Building 46. Ctra. Utrera, Km 1, 41013 Seville, Spain

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ABSTRACT

The presence of phenolic compounds in raw olives, particularly the bitter glucoside oleuropein, requires transforming this substance into other non-bitter to make the fruit palatable. An alkali treatment is currently carried out to hydrolyze the oleuropein but a high volume of wastewater is generated. The aim of this study was to develop a natural product without an alkali treatment and with appropriate organoleptic characteristics similar to those of Spanish-style green olives. Mild heat treatments (60 °C for 10 min) were sufficient to inactivate the β-glucosidase activity which prevented the formation of antimicrobial compounds and thereby the growth of lactic acid bacteria was promoted. By contrast, heating released a high concentration of oleuropein in fruits that remained at a very high level even after 6 months of fermentation. The inoculation of the brines of Manzanilla olives with selected lactic acid bacteria with oleuropeinolytic activity was insufficient for reducing the high concentration of the bitter glucoside. However, favorable results were obtained with varieties such as Gordal and Aloreña, which have lower oleuropein contents. The product obtained showed a very attractive color, similar to that of Spanish-style green olives and lighter than natural green olives, which is a positive aspect for consumer acceptability.

1. Introduction

Table olives have been a component of the Mediterranean diet for centuries and their consumption is increasing worldwide because of their nutritional and palatable characteristics. Among the different types of commercial table olives, Spanish-style green olives are the most popular. The presence of phenolic compounds in the raw fruit, particularly the bitter glucoside oleuropein, requires a step to transform this compound into other non-bitter. In alkaline conditions, the ester bond of the oleuropein is broken with the consequent formation of hydroxytyrosol and elenolic acid glucoside, both non-bitter compounds (Brenes & de Castro, 1998). Sodium hydroxide treatment allows for the sweetening of Spanish-style green olives in a short period of time and favors a product with a characteristic color, flavor and aroma due to spontaneous fermentation in brines. Precisely, lactic acid fermentation is a critical step in the process of Spanish-style green olives because it is responsible for the organoleptic properties of fruits and guarantees their preservation.

On the other hand, natural green olives, which are not treated with alkali, lose their bitterness slowly for months or even a year. The main microbiota in the brine of these olives is formed by yeasts and sometimes lactic acid bacteria when conditions are favorable (Garrido-Fernández, Fernandez-Díaz, & Adams, 1997). Some of these microorganisms possess β-glucosidase and esterase activity in their metabolism and many researchers have studied the possibility of using them to hydrolyze the oleuropein molecule and thus to accelerate the sweetening of the fruits. Ciafardini, Marsilio, Lanza, and Pozzi (1994) inoculated model solutions rich in oleuropein with 3 different strains of *Lactobacillus plantarum* isolated from natural black olives. These strains possessed β-glucosidase activity and hydrolyzed the phenolic glucoside *in vitro*. Similar tests were carried out later and confirmed that some *Lactobacillus plantarum* strains were able to hydrolyze oleuropein through the action of β-glucosidase forming its aglycone, and subsequently esterase activity released the final products of hydrolysis, elenolic acid and hydroxytyrosol (Marsilio, Lanza, & Pozzi, 1996; Ghabbour et al., 2011; Zago et al., 2013; Tofalo et al., 2014.). Some of these strains were able to hydrolyze 90% of the oleuropein in the model solution, obtaining hydroxytyrosol as the final hydrolysis product, while other researchers only detected the formation of an intermediate metabolite, oleuropein aglycone (Santos, Piccirillo, Castro, Kalogerakis, & Pintado, 2012). Enzymatic transformations of oleuropein have also been studied *in vitro* by different yeast strains

* Corresponding author.
E-mail address: emedina@ig.csic.es (E. Medina).

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isolated from natural green olives (Bautista-Gallego et al., 2011; Restuccia et al., 2011; Tofalo, Perpetuini, Schirone, Suzzi, & Corsetti, 2013) and black olives (Bonatsou, Benítez, Rodríguez-Gómez, Panagou, & Arroyo-López, 2015), with *Wicheramomyces anomalus* being the microorganism with the highest activity.

However, there are few studies on a pilot plant scale. Servili et al. (2006) selected 5 strains isolated from Italian natural black olive brines and found that a strain of *Lactobacillus pentosus* (1MO) was able to sweeten olives in only 8 days. Kaltsa, Papaliaga, Papaioannou, and Kotzekidou (2015) isolated five strains of *Lactobacillus plantarum* from natural green and black olives and found that *Lactobacillus* LP15 had a high β-glucosidase activity, whereas *Lactobacillus* LP20 had esterase activity in low salt fermentations. Nevertheless, no industrial application of these starters is known.

The production and consumption of organic and/or natural foods has increased in recent years, but not those of ecological and/or natural olives. There are several reasons for this phenomenon and among them is the need to remove the bitterness of the olives without the use of a NaOH solution and to provide a desirable product for consumers. The aim of this work was to develop a new natural green olive process to obtain a final product with appropriate organoleptic characteristics (color, firmness and flavor), and if possible, similar to those of the type of table olives universally accepted, such as Spanish-style green olives.

2. Material and methods

2.1. Raw material

Fruits of the Manzanilla, Hojiblanca, Gordal and Aloreña cultivars (*Olea europaea* L.) in the ripening stage corresponding to the green–yellow color on the surface were supplied by local farmers.

2.2. Effect of temperature on the β-glucosidase activity and phenolic compound concentration in olives

The olive fruits (Manzanilla cv.) were dipped in a water bath at different temperatures (50, 60 and 70 °C) for 3, 5, 10, 15 and 40 min. Then, the fruits were rapidly chilled in cool water. Ten grams of olive pulp were put in 10 mL of distilled water and homogenized in an Ultra-turrax (IKA-T25, S25N-18G). The mixture was centrifuged at 12,000 × g for 5 min at 4 °C. The aqueous phase was filtered through a 0.22 μm pore size nylon filter and phenolic compounds were analyzed as described below.

Simultaneously, raw olives (Manzanilla and Hojiblanca cv.) were treated at the following heating conditions before brining: i) No heat treatment (control); ii) 60 °C for 10 min; iii) 70 °C for 5 min; and iv) 80 °C for 5 min. After treatments, the olive fruits were placed in containers of 3 L capacity with 1.8 kg of fruit and 1.2 L of brine (50 g/L NaCl and 5 g/L of acetic acid). After 24 h of brining, the vessels were inoculated with a cocktail of 4 selected strains of lactic acid bacteria (*Lactobacillus brevis* Z17BP, *Lactobacillus pentosus* ATCC 8041, *Lactobacillus plantarum* ATCC 14917 and *Leuconostoc mesenteroides* LM51) to reach an initial population of 5×10^7 CFU/mL. These strains were chosen and tested as described in sections 2.3 and 2.4. The vessels were left at ambient temperature for six months and were periodically analyzed to know their microbiological and chemical evolution.

In another experiment, Manzanilla and Hojiblanca olives from two different seasons (2010/2011 and 2011/2012) were heat-treated at 60 °C for 15 min. Subsequently, the oleuropein concentration in the pulp of the fruit was analyzed as described below.

2.3. Strain sources and maintenance

A total of 105 lactic acid bacteria (LAB) strains from different origins were used in this work. Some strains were purchased from the American Type Culture Collection (ATCC strains) and the Spanish Type Culture Collection (CECT strains), although most of them were isolated by the authors from olive brines belonging to diverse cultivars, locations and manufacturing methods. All the strains were routinely cultured in MRS broth (Oxoid, Basingstoke, UK) under anaerobic conditions (AnaeroGen, Oxoid), and kept frozen at −80 °C in broth with glycerol (200 g/L). Catalase-negative bacteria that were able to grow on MRS agar with sodium azide (0.2 g/L) were considered LAB.

2.4. Depletion of oleuropein by the different strains in synthetic broth and brine model solution

A minimum medium mimicking the conditions prevailing in the brines of natural olives was formulated in order to favor the utilization of oleuropein by the isolates. The composition was: glucose (Panreac, Barcelona, Spain) 1 g/L; neutralized bacteriological peptone (Oxoid) 10 g/L; yeast extract (Oxoid) 4 g/L; sodium chloride (Panreac) 50 g/L; and oleuropein (Sigma, Aldrich, St. Louis, MO) 5 g/L in an acetic-acetate buffer pH 4. The medium was sterilized by filtration and 250 μL were dispensed into sterilized vials. Cultures of the strains were carried out in MRS broth, firstly without NaCl, and a second culture in MRS with 50 g/L of NaCl to adapt the isolates. After overnight incubation at 30 °C, 1 mL of each culture was centrifuged. The pelletized cells were washed with 1 ml of saline solution (9 g NaCl/L) and centrifuged again. Finally the pellets were re-suspended with 0.5 mL of saline solution and 10 μL of suspension were used to inoculate the vials. With this method, the expected initial inocula were between 10^7-10^8 cfu/mL. The duplicate vials for each strain were incubated at 30 °C in anaerobic conditions for 10 days and then analyzed to know the remaining concentration of oleuropein.

In another experiment, brine from heat-treated olives aseptically stored for two months containing a concentration of 11.36 mM oleuropein was used as a brine model. The brine was sterilized by filtration and distributed in 250 μl volumes into sterile vials. Inoculation with the selected strains, incubation and analysis were performed as described above for the minimum medium. Uninoculated brines were used as control.

2.5. Depletion of oleuropein by selected strains during olive fermentations

Twelve fermentation vessels were filled with raw olives (Manzanilla cv.) and covered with acidulated brine (100 g NaCl/L and acetic acid 3 g/L). Except for control A, the rest of the fruits suffered a heat treatment at 60 °C for 15 min with the aim of inactivating the endogenous enzymatic activity. The specific treatments were: no heat treatment and no inoculation (Control A); heat-treated but no inoculation (Control B); inoculated with *Lactobacillus plantarum* CECT 748 (treatment C); inoculated with *Lactobacillus pentosus* LAB 80 (treatment D); inoculated with *L. pentosus* LAB 64 (treatment E); and inoculated with *L. plantarum* LAB 46 (treatment F). LAB 80, LAB 64, and LAB 46 were classified according to Torriani, Felis, and Dellaglio (2001). Inoculation was carried out at day 7 and the experiment was performed in duplicate. Cultures of the different strains in MRS broth plus 50 g NaCl/L were incubated at 30 °C for 24 h, then centrifuged and the pellets resuspended in saline for washing the cells, next they were centrifuged again, and finally the pellets were re-suspended with brine from the corresponding vessels to be inoculated, and injected