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Preservation of the antibacterial activity of enzymes against *Alicyclobacillus* spp. through microencapsulation

Márcia M. Anjos [a], Eliana H. Endo [a], Fernanda V. Leimann [b], Odinei H. Gonçalves [b], Benedito P. Dias-Filho [a], Benício Alves de Abreu Filho [a, *]

[a] State University of Maringá, Brazil
[b] Federal Technological University of Paraná, Brazil

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ABSTRACT

The genus *Alicyclobacillus* spp. can deteriorate foods with low pH, such as citrus juices, changing their odor and taste. Different studies seek an alternative for the control of this microorganism in orange juice, and the antibacterial effect of papain and bromelain proteases has already been demonstrated under these conditions. However, one of the limitations of the application of these compounds is the drastic decrease of the antibacterial action when the product is subjected to high temperatures, such as pasteurization. The objective of the present study was to microencapsulate these enzymes with alginate and chitosan and evaluate their action following thermal processes. Microencapsulation was performed by spray drying and the compounds were subjected to high temperatures. Their inhibitory and bactericidal activity against five different species of *Alicyclobacillus* was then evaluated. The microcapsules were characterized by Scanning Electron Microscopy (SEM), Differential Scanning Calorimetry (DSC) and Infrared Spectroscopy (FT-IR). The microencapsulation of the particles was evidenced. The results showed that papain microencapsulated with chitosan or alginate maintained low minimum inhibitory concentration values after submission to heat treatment, demonstrating its effectiveness and potential application as a biopreservative.

1. Introduction

Alicyclobacillus spp. are bacilli Gram-positive, thermophilic, acidophilic, non-pathogenic with the ability to form spores. They are present in the soil and are often related to the deterioration of acidic products such as citrus drinks and juices (Silva, Gibbs, Vieira, & Silva, 1999; Silva & Gibbs, 2001; Goto et al., 2002; Matsubara et al., 2002; Goto, 2003).

This bacterium survive at high temperatures and low pH due to two main factors: the composition of its membrane, where cyclic fatty acids are found and its ability to form spores. Although it is not pathogenic, its ability to produce undesirable odors and flavors in products such as citrus juices, isotonic drinks, iced teas and tomato extracts represents a major economic problem for the food industry (Chang & Kang, 2004; Walker & Phillips, 2005).

Alicyclobacillus acidoterrestris is the most important species associated with this type of deterioration. It is capable of producing compounds such as 2-methoxyphenol (guaiacol) and 2,6-dibromophenol, which are the main compounds responsible for changes in the odor and taste of juice. These changes are described as a medicinal or antiseptic odor and taste (Chang & Kang, 2004; Durak, Churey, Danyluk, & Worobo, 2010; Goto et al., 2002; Matsubara et al., 2002).

Thermal treatments are employed in the concentrated orange juice industry to deactivate many pathogenic and deteriorating microorganisms. *A. acidoterrestris* spores, however, can survive these thermal treatments, germinate, grow and deteriorate juice after reconstitution (Savaş Bahçeci et al., 2004).

Preservation methods other than thermal treatments, such as the application of natural antimicrobial agents in food, are therefore desirable. These provide a potential strategy for inhibiting a wide variety of microorganisms without risk to the health of consumers (Bevilacqua, Sinigaglia, & Corbo, 2008; Burt, 2004).

As described by Anjos et al. (2016), the enzymes papain and bromelain have demonstrated efficacy as antibacterial agents against strains of *A. acidoterrestris* inoculated in orange juice, and

* Corresponding author.
E-mail address: baafilho@uem.br (B.A. Abreu Filho).

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are therefore potential preservative agents for this product. The addition of these enzymes to juice after reconstitution and pasteurization eliminated four logs of the microorganism after 48 h of contact with the product.

Papain is an important peptidase extracted from papaya (*Carica papaya*). It has a high proteolytic capacity and hydrolyzes large proteins in small peptides and amino acids. Studies have demonstrated the antibacterial activity of papain and other papaya extracts against *Bacillus subtilis, Enterobacter cloacae, Escherichia coli, Listeria monocytogenes, Salmonella* Typhimurium, *Staphylococcus aureus* and *Proteus vulgaris* (Eshamah, Han, Naas, Acton, & Dawson, 2014; Osato, Santiago, Remo, Cuadra, & Mori, 1993).

Bromelain, which is also a proteolytic enzyme, is derived from pineapple (*Ananas comosus*), which is also a member of the Bromeliaceae family. Studies have demonstrated its antimicrobial effect, as well as its anthelmintic activity against gastrointestinal nematodes and its anti-*Candida* activity (Rowan, Buttle, & Barrett, 1990).

Papain and bromelain are widely used in the food, medicine and pharmaceutical industries and in clinical-laboratory analysis technical procedures (Dutta & Bhattacharyya, 2013; Hale, Greer, Trinh, & James, 2005). Although the antibacterial action of papain and bromelain against *A. acidoterrestris* in orange juice is effective, the addition of these compounds as preservatives must occur after the pasteurization of the juice, as the heat treatment interferes with the structure of the enzyme and decreases its antibacterial effect.

Spray drying is an important and widely applied technique in the pharmaceutical and food industries. It is a viable method for the preparation of microparticles, being a single step process, and is an alternative to microencapsulation methods that use various steps and organic solvents. Atomization occurs through compressed air, which breaks up the liquid fed to the nozzle into small droplets, and the solvent in these droplets is rapidly evaporated by the high temperatures. The technique can be applied to heat sensitive substances and hydrophilic and hydrophobic polymers can be used. It can also be easily adapted to industrial scale production (Giunchedi et al., 2002).

Natural biopolymers are potentially effective encapsulating agents due to their biodegradability and biocompatibility. Chitosan is a natural cationic polysaccharide obtained from the N-deacetylation of chitin, while alginate is a natural anionic polysaccharide of the linear copolymers α-L-guluronate and β-D-mannuronate. Both are suitable for use in food applications (Coppi, Iannuccelli, Bernabei, & Cameroni, 2002).

The objective of the present study was to microencapsulate papain and bromelain enzymes with alginate and chitosan and to verify the effectiveness of this technique in maintaining antibacterial activity against *Alicyclobacillus* spp. after heat treatment.

2. Materials and methods

2.1. Microbial lineages, enzymes and polymers

The lineages of the species of *Alicyclobacillus* spp. used were obtained from the Coleção Brasileira de Micro-organismos de Ambiente e Indústria (the Brazilian Environmental and Industrial Microorganisms Collection) (CBMAI), situated in the Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas (the Pluridisciplinary Center for Chemical, Biological and Agricultural Research) (CPQBA/UNICAMP), São Paulo, Brazil. The following reference species were studied: *A. acidoterrestris* CBMAI 0244^T; *A. hesperidum* CBMAI 0298^T; *A. acidophilus* CBMAI 0247^T; *A. cycloheptanicus* CBMAI 0297^T; and *A. acidocaldarius* CBMAI 0299^T.

The papain and bromelain enzymes, as well as the alginate and chitosan polymers, were obtained commercially from Sigma-Aldrich®, the papain was produced in Switzerland and the bromelain was produced in Indonesia.

2.2. Microencapsulation of enzymes

The alginate and chitosan microparticles containing the papain and bromelain enzymes were produced by the spray-drying technique. First, alginate (Sigma-Aldrich, viscosity of 250 cps at 2%) was dissolved in distilled water under constant stirring at a concentration of 2% w/v. The enzyme (papain or bromelain) was dispersed in an aqueous alginate solution to give an enzyme-polymer ratio of 1:2. The final suspension was spray-dried in a LM-MSD 1.0 Mini model spray dryer with a 0.7 mm nozzle, under the following conditions: air inlet temperature 100 °C, air outlet temperature 50–60 °C and a spray flow rate of 1.0 L/h. The microparticles of the enzymes prepared with chitosan (Sigma-Aldrich, low molar mass, viscosity 20,000 cps) were obtained from the dissolution of chitosan in 1% acetic acid at a concentration of 2% w/v, under stirring. The enzymes were dispersed in chitosan acetic acid solution, resulting in an enzyme-polymer ratio of 1:2. The atomization conditions were the same as those described for alginate.

The encapsulation efficiency was calculated by dosing the proteins of the particles before and after the microencapsulation in a UV-Vis spectrophotometer at 280 nm, and the microparticles obtained were dissolved in sterile distilled water at pH 4.0 and filtered in 0.45 μm membrane before analysis.

2.3. Characterization of microparticles

2.3.1. Scanning electron microscopy (SEM)

For morphological analysis, the microparticles were fixed on stubs, coating with gold and observed in a Shimadzu SS-550 scanning electron microscope. The particles were sized using the SizeMeter 1.1 (2001) software package and the polydispersion index (PDI) was calculated using the following equation:

$$PDI = \frac{SD^2}{D^2}$$

where: PDI = polydispersion index; D = mean diameter of the particles and SD = standard deviation of the mean diameter of the particles.

2.3.2. Differential scanning calorimetry (DSC)

Thermal analysis of the microparticles was performed using Differential Scanning Calorimetry (DSC) (Shimadzu DSC50). Prior to analysis, the samples were kept in a desiccator for three days. Approximately 5 mg of the powder was used for analysis, with the sample sealed in an aluminum vessel, and an empty vessel used as a reference. The heating rate used was 20 °C/min under a nitrogen atmosphere.

2.3.3. Infrared Spectroscopy (FT-IR)

Fourier Transform Infrared Spectroscopy - FT-IR, Shimadzu IR AFFINITY-1, from 400 to 4000 (1/cm), 1 (1/cm) resolution - was used to investigate the interactions between the polymers (alginate and chitosan) and the enzymes. The particles were dispersed in spectroscopic grade potassium bromide (KBr). The pellet was then formed by compressing the sample at 150 MPa and the FT-IR spectra were collected in transmission mode.