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Use of alginate for extending shelf life in a lyophilized yeast-based formulate in controlling green mould disease on citrus fruit under postharvest condition

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ABSTRACT

Tentative to extend shelf-life of lyophilized yeast-based formulations arouses great interest in the phytosanitary drug market. The challenge of this work was to set an simple, innovative and inexpensive system that use alginate for making gummosis gel beads able to preserve for longer time vitality, morphological stability and biological activity of lyophilized-yeast used as biocontrol mean against fruit commodity losses caused by the phytopatogenic fungus *Penicillium digitatum*, the main causal agent of green mould disease on citrus fruit under postharvest condition. Two lyophilized-yeast strains belonging to *Pichia guilliermondii* (5A) and *Candida oleophila* (13L), three Na-alginate doses (1, 2 and 3%), and three storage temperatures (0, 4 and 15 °C) were considered for assessing shelf life of immobilized-yeast into a sodium-alginate matrix. The results show that shelf life of this innovative biological product suitable for controlling citrus fruit green mould can be elongated up to fourteen months under storage temperature of 4 °C using 3% Na-alginate. Gel beads containing both yeast strains showed more than 60% citrus fruit decay inhibition if employed after storage at 4 °C for fourteen months. A positive correlation highly significant between sodium-alginate dose and yeast cells vitality with the citrus fruit decay inhibition was found.

1. Introduction

Losses due to postharvest diseases of citrus fruit can reach proportions of 30% contributing to a severe reduction of market value of these perishable commodities. Their control is traditionally performed with synthetic fungicides (as tiabendazole and imazalil), however their intense use has aroused important issues relating to environmental and human health prompting to search safer control means. In order to find valid alternatives for a sustainable management of the citrus fruit postharvest losses caused by phytopathogenic fungi, the use of antagonistic yeast as biocontrol mean is considered promising (Arras, Nicolussi, & Ligios, 1999; Lima, Arru, De Curtis, & Arras, 1999; Wilson & Wisniewski, 1989). Strains (or isolates) of *Pichia guilliermondii* and *Candida oleophila* selected against green mould disease caused by *Penicillium digitatum* have been considered among the most efficient (Arras, Demontis, & Sussarellu, 1996; Arras et al., 1999) because they possesses: 1) ability to control of a broad range of pathogens under different storage conditions of the fruit, 2) availability of inexpensive systems of multiplication on large scale, 3) wide compatibility with chemical fungicides, and 4) easiness of distribution. Nevertheless, for a viable development of yeast-based formulations in the biocontrol field into a more highly evolving phytosanitary industry, must be required a longer shelf life because the available systems based on yeast formulates are still not satisfaction for the phytosanitary drug market (Spadaro & Gullino, 2004). However, several techniques of cryopreservation and lyophilisation are nowadays considered as the best shelf life systems for cell preservation in filamentous fungi and yeast (Homolka, 2013). For example, several yeast-based formulations (liquid and dry) for controlling mould growth on moist cereal grains during airtight storage were improved using dried cells (Melin, Håkansson, & Schnürer, 2007).

Although some works regarding to use of eco-friendly biomaterials for controlling plant pathogenic fungi growth in food industry were reported (Kulkarni, Soppimath, Aminabhavi, Dave, & Mehta, 2000; Pola et al., 2016), nevertheless advanced researches for finding biomaterials capable to extend shelf life of lyophilized-yeast formulates are still poorly investigated. A suitable biomaterial is sodium alginate, the alginic acid sodium salt, that is an anionic polysaccharide comprising of

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mannuronic acid and guluronic acid residues. It was mainly extracted from the cellular walls of brown seaweeds, but it is also produced from the bacterial sources being extracted from the main component of biofilms (Donati & Paoletti, 2009). Through binding with water, it forms a viscous gum by water absorbing and quickly solidifying. Its colour ranges from white to yellowish-brown, and it is sold in granular or powdered forms. Alginates from different seaweed species often have variations in their chemical structure and physical properties: some of them may be yielded as alginate that gives a stronger gel, another a weaker gel, some may readily give a cream/white alginate, and another difficultly forming gel. Owing to its gelling, thickening, stabilizing and viscose properties, alginate is a prominent component for food (Holdt & Kraan, 2011), textile and paper industries (Pallerla & Chambers, 1997), as well as in pharmaceutical and medical fields (Augst, Kong, & Mooney, 2006; Gombotz & Wee, 2012). Alginate is a well-known biomaterial that is widely used for drug delivery (Tønnesen & Karlsen, 2002) and in tissue or cell engineering (Kuo & Ma, 2001; Machida-Sano, Matsuda, & Namiki, 2010; Quraishi et al., 2015) due to its high biocompatibility, low toxicity, low cost, and simple gelation mechanism (Lee & Mooney, 2001). For example, 3% Na-alginate with 0.1 M calcium chloride has been used for producing encapsulated nodal segments for plant regeneration by non-embryogenic synthetic seeds in the micro-propagation field of plant tissue (Verma, Khosla, Choudhary, & Lal, 2015).

The aim of the present work was to bring out common techniques of yeast cell preservation, as lyophilisation, with inexpensive and eco-friendly new biomaterials, as Na-alginate, for extending shelf life of a lyophilized yeast-based formulate able to reduce the postharvest losses caused by the biotic stress factors on citrus fruit without use of synthetic fungicides. The goal idea of this interdisciplinary approach was therefore to set an simple, innovative and inexpensive system that uses Na-alginate as a gummosis matrix able to protect for longer time (beyond one year) morphological stability, vitality and overall biological activity of two yeast strains belonging to *P. guilliermondii* and *C. oleophila* for controlling green mould on citrus fruit under postharvest condition using immobilized-yeast into an alginate matrix rather than free-lyophilized and/or fresh cells.

2. Materials and methods

2.1. Starting materials

2.1.1. Lyophilized-yeast production

Colonies of *P. guilliermondii* (strain 5A, isolated from fig, inducing 96–100% decay inhibition on citrus fruit) and *C. oleophila* (strain 13L, isolated from mandarin, causing 92–98% inhibition) grown into a 100 mm Petri plates for 48 h at 25 ± 1 °C on Nutrient-Yeast-Dextrose-Agar (NYDA, Difco Laboratories, Detroit, MI, USA) medium, were provided by the BIO-PLANTA Consortium (Modugno, Bari, Italy) and used as inoculum starters for multiplication on large scale. Colonies of each yeast strain were transferred into a 500 mL Erlenmayer flasks on sterile Nutrient-Yeast-Dextrose-Broth (NYDB, Difco) medium and grown for 7 d at 25 °C ± 1 °C under rotary shaking. Inoculum were employed for multiplication in a bench-top bioreactor of 5 L, and successively transferred into an industrial unit composed of two bioreactors of 75 and 500 L using a sterile minimal medium broth in deionized water composed of 10 g L^{-1} Sucrose, 2 g L^{-1} $(NH_4)_2SO_4$, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.8 g L^{-1} KH_2PO_4 and 1.5 g L^{-1} Yeast Extract adding a commercial antifoam provided by the Braun Biotech International Company (Milan, Italy). As micronutrients were added 1 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 5 mg L^{-1} $FeSO_4 \cdot 7H_2O$, 60 mg L^{-1} $CaCl_2 \cdot 2H_2O$, 5 mg L^{-1} $CuSO_4 \cdot 7H_2O$ and 5 mg L^{-1} $MnSO_4 \cdot H_2O$ (Di Bonito et al., 2006). A batch culture of 5 L from the 1st-bioreactor (Mod. Braun Biotech International "BIOSTAT B", Milan, Italy) was used to inoculate a volume of 50 L on the 2nd-bioreactor (Mod. BBI "BIOSTAT D-75"), and 50 L of culture from the 2nd-bioreactor served to inoculate a volume of 350 L on the 3th-bioreactor (Mod. BBI "BIOSTAT D-500"). The microbiological biomass produced after scaling-up containing no pooled yeast cells of each strain was separated from the exhausted broth by centrifugation (5,000*g* × 2 min, 4 °C) under sterile condition, and washed twice with sterile deionied water without antifoam use.

About 2.0 L of fresh semi-liquid biomass were transferred into a sterile stainless steel hawk (40 × 60 × 5 cm) and dried using an industrial lyophilisation unit (Mod Terruzzi & Fercalx "TLC 150", Milan, Italy). About 10–15 L of yeast culture were processed throughout one cycle of freezing at −30 °C for 90 min, followed by 11 cycles of vacuum (from 47 to 67 Pa)–heating (at 30 °C), and a final maintenance phase at 30 °C for 3 h at 40 Pa. Lyophilised cells were then collected from each hawk by a sterile spatula, admitted into a sterile bottle, and stored at 4 °C until use.

2.1.2. Chemicals

Three Na-alginate solutions were prepared at different concentrations (1, 2 and 3% w/v) by overnight stirring of Na-alginate with deionized water and brought at pH 4.5 for producing gel beads. Sodium alginate for immobilization microorganisms grade was purchased in the finest white powdered form from Sigma Life Science (Germany), catalogue n. 71238, for further use. After preparation, all the solutions were bottled and stored at 5 °C until used. Calcium chloride (light, precipitated powder, particle size ca. 1 μm) was purchased from Sigma Life Science, and a solution of 0.2 M $CaCl_2$ in deionized water was prepared for producing alginate gel beads. Deionized water at pH 5.8 was used throughout the following studies.

2.2. Alginate gel bead production with yeast strains

The production of porous beads with controlled pore size on their surface with an adequate distribution into gummosis matrix for avoiding anoxic conditions to yeast cells has been patented by ENEA (IT Patent n. 87659/ENEA, Issue of 24th March 2013). Samples of sterile lyophilized-yeast (200 g L^{-1} dry weight, w/v) were collected under sterilized condition, suspended for 30 s into each Na-alginate solution by mixing. The final suspensions of 1, 2 and 3% Na-alginate containing yeast were pumped by a peristaltic pump into a calcium chloride solution through a sterile capillary for making gel beads (Ø = 3–5 mm). Sodium ions were exchanged with calcium ions into alginate matrix during pumping for achieving a better solidity to the beads. Alginate gel beads were collected after 30 min, washed twice with sterile water, and stored into a sterile tank till used. Four different alginate gel beads were prepared: 1) without yeast (control), 2) with *P. guilliermondii* (isolate 5A), 3) with *C. oleophila* (isolate 13 L) and 4) with pooled yeast strains.

2.3. Alginate bead characterization

Characteristics of the gel bead were determined during a monitoring program long more one year for each used dose of Na-alginate. This program started from the alginate gel bead production (time = 0) and continued every two months until fourteen months after production (times = 2, 4, 6, 8, 10, 12 and 14) determining the following features of the beads under three different storage temperatures (0, 4 and 15 °C): 1) water uptake, 2) yeast cells inclusion, 3) cells vitality, 4) detection of microbial contaminants, and 5) identification of the two strains.

2.3.1. Water uptake

Water uptake study was performed using alginate gel bead suspended in Tris–HCl (pH 7.4) as buffer solution. Samples of alginate gel bead containing *P. guilliermondii* (5A), *C. oleophila* (13L) and pooled yeast strains collected at the zero time were placed into test tubes, filled with adequate amount of buffer, and then placed into water bath at 37 °C for 5 min under rotary shaking. Tris–HCl buffer was chosen in place of PBS due to its lower affinity to calcium ions because phosphate

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