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Physical properties and antifungal activity of bioactive films containing *Wickerhamomyces anomalus* killer yeast and their application for preservation of oranges and control of postharvest green mold caused by *Penicillium digitatum*

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ABSTRACT

This study assessed the ability of two bio-based films, obtained from sodium alginate (NaAlg) and locust bean gum (LBG), to protect the viability of *Wickerhamomyces anomalus* cells and control the growth of *Penicillium digitatum*. The effect of microbial cell incorporation on physical properties of the developed films was evaluated in terms of barrier, mechanical and optical properties. Furthermore, the application of these two matrices as bioactive coatings was investigated in order to evaluate their efficacy in preserving the postharvest quality of 'Valencia' oranges and inhibiting the growth of *P. digitatum* on artificially inoculated fruits. Results showed that NaAlg and LBG films were able to maintain more than 85% of the initial *W. anomalus* yeast population and that the developed films incorporating the killer yeast completely inhibited the growth of *P. digitatum* in synthetic medium. Likewise, NaAlg and LBG coatings enriched with *W. anomalus* yeast were effective at reducing weight loss and maintaining firmness of 'Valencia' oranges during storage, and reduced green mold in inoculated fruits by more than 73% after 13 days.

1. Introduction

Citrus fruits are among the most consumed fruits in the world, mainly because of their high content of vitamin C and other bioactive compounds including flavonoids and phenolic acids (Widmer and Montanari, 1996), known for their beneficial effect in reducing risk of cancer and cardiac diseases incidence (Attaway, 1994). However, during harvesting, postharvest handling, transportation and storage, citrus fruits are susceptible to injury and colonization by various fungi. *Penicillium digitatum* (Pers.) Sacc., the causal agent of green mold, has been reported to be one of the most devastating postharvest fungal pathogens of citrus fruits. This mold may cause 60–80% decay under ambient conditions (Moscoso-Ramírez et al., 2013) which results in severe economic losses, especially for exporting countries. Over the last few decades biological control, using antagonistic microorganisms including yeasts, yeast-like fungi and bacteria, has emerged as one of the most promising treatments for controlling wound-invading postharvest pathogens (Janisiewicz and Korsten, 2002). Among microbial antagonists, yeasts have received particular attention as potential biocontrol agents against postharvest diseases in fruits, mainly because of their high inhibitory capacity, rapid colonization of fruit wounds (Rosa-Magri et al., 2011) and simple nutritional requirements enabling them to colonize dry surfaces for long periods of time (El-Tarabily and Sivasithamparam, 2006). Moreover, in contrast to filamentous fungi, yeasts do not produce allergenic spores or mycotoxins, reinforcing their safe use for human consumption purposes (Fan and Tian, 2000). In the last decade, several scientific studies have demonstrated the efficacy of antagonistic yeasts as biocontrol agents against many phytopathogenic fungi including species of *Penicillium* (Bautista-Rosales et al., 2013; Manso and Nunes, 2011; Platania et al., 2012; Restuccia et al., 2006; Wang et al., 2010).

Among a wide variety of antagonistic yeasts particular attention has been directed toward the use of yeasts that exhibit a killer phenotype (K+) for controlling postharvest decay in fruits, due to their ability to secrete extra cellular protein toxins designated as killer proteins or killer toxins. These proteins have the potential to kill other species of yeasts, molds and pathogenic bacteria through different mechanisms, including the hydrolysis of the major cell wall component β-1,3-glucans (Izgü and Altinbay, 2004; Muccilli et al., 2013), the ion leakage by ion channel formation on the cytoplasmic membrane of the target cell, and the inhibition of DNA synthesis (Schmitt and Breinig, 2002). Among the killer species, *Wickerhamomyces anomalus* (previously named *Pichia anomala*) has

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been reported to produce high levels of killer toxins with a wide spectrum of killing activity and a relatively high stability, compared with toxins of other killer yeasts (Wang et al., 2008). Furthermore, *W. anomalus* has been granted Qualified Presumption of Safety (QPS) status by European Food Safety Authority (EFSA), which may authorize its use as a novel microorganism in food preservation (Sundh and Melin, 2011).

In the last decade, several studies have demonstrated the efficacy of *W. anomalus* as a biological control agent against different postharvest phytopathogenic fungi. In this sense, Lima et al. (2013) reported a significant reduction in the incidence of infection, for up to 6 days after inoculation, when assessing the efficiency of *W. anomalus* (strain 422) for the biocontrol of the anthracnose disease caused by *Colletotrichum gloeosporioides* in papaya. Similar effects were obtained by Lassois et al. (2008) and Platania et al. (2012), when evaluating the antagonistic activity of different strains of *W. anomalus* against the fungi causing crown rot disease in banana fruit and *P. digitatum* on Tarocco oranges, respectively.

Recently, Sánchez-González et al. (2013, 2014) developed bioactive polymeric films through the incorporation of different strains of bacteriocin-producing lactic acid bacteria into polysaccharide and protein matrices. These authors demonstrated the efficacy of the different developed films at maintaining the viability of incorporated cells and controlling the growth of *Listeria innocua* on an artificially contaminated synthetic medium.

Among many polysaccharides, sodium alginate (NaAlg) and locust bean gum (LBG) have been reported as potential coating components not only because of their excellent film forming properties and selective permeabilities to O_2 and CO_2 (Mikkonen et al., 2007; Oms-Oliu et al., 2008), but also for their ability to act as effective matrices for the entrapment of bioactive compounds (Aloui et al., 2014a,b).

Although the efficacy of many antagonistic yeasts against a wide variety of phytopathogenic fungi has been well documented in literature, there are only few published data on their incorporation into coating formulations for fresh fruits (McGuire and Hagenmaier, 1996; McGuire and Dimitroglou, 1999; Fan et al., 2009). To our knowledge, no research has been reported on the use of edible coatings carrying *W. anomalus* killer yeast for controlling fungal decay and preserving postharvest quality of fruits.

The objectives of this work were to evaluate the effect of *W. anomalus* yeast incorporation on the functional and optical properties of NaAlg and LBG bio-based films, and to investigate the ability of these two polysaccharide matrices to maintain viability and antifungal potential of incorporated cells. Likewise, the application of these matrices as bioactive coatings was investigated to evaluate their effectiveness at preserving the postharvest quality of 'Valencia' oranges and controlling fungal spoilage caused by *P. digitatum*.

2. Materials and methods

2.1. Raw materials

'Valencia' oranges (*Citrus sinensis* L. Osbeck) were purchased from a wholesale distributor located in Catania (Italy) at commercial maturity and transported to the laboratory in polystyrene boxes to avoid mechanical damage, at ambient conditions of temperature and humidity (18 °C and 75% RH). Oranges were visually selected on the basis of uniform shape, size, color, firmness and absence of mechanical injuries or fungal infection. Before coating application, selected oranges were washed with a solution of sodium hypochlorite (0.01%) for 3 min, then drained and air-dried at room temperature. Coating experiments were carried out on the same day.

NaAlg (molecular weight ~ 80,000 Da, CAS Number 9005-38-3, Sigma Aldrich, Steinheim, Germany) and LBG (molecular weight ~ 310,000 Da, Sigma Aldrich, Steinheim, Germany) were used as coating materials. Glycerol (≥99% purity; Sigma-Aldrich) was used as a plasticizer and was purchased from Sigma Aldrich (Steinheim, Germany).

2.2. Microorganisms

W. anomalus BS 91 killer yeast strain used in this study, obtained from the DiGeSA collection (Department of Agri-Food and Environmental Management Systems, University of Catania), was isolated from naturally fermented olives and identified by sequencing the D1/D2 region of the 26S rRNA gene (Muccili et al., 2011). The selection of this strain was based on its high antifungal activity against *P. digitatum* according to our preliminary experiments (data not shown). The killing mechanism of *W. anomalus* BS 91 was reported to be based on β-glucanase production (Muccilli et al., 2013).

Freeze dried culture of *P. digitatum* DSM 2748 was obtained from DSMZ culture collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The fungus was rehydrated following the supplier's instructions, inoculated on potato dextrose agar (PDA) (Oxoid, Basingstoke, Hampshire, England) and incubated at 25 °C until sporulation.

2.3. Preparation of the bioactive film forming solutions

Pure NaAlg and LBG film forming solutions were prepared by dissolving either NaAlg (2%, w/v) or LBG (1%, w/v) powder in distilled water heated at 70 °C with constant agitation, until all particles were thoroughly dispersed. Glycerol (20%, w/w based on biopolymer content) was added as a plasticizer to enhance film flexibility, overcome brittleness and facilitate film detachment. The mixture was then stirred overnight at room temperature, before being degassed to remove entrapped air bubbles.

W. anomalus BS 91 killer yeast suspension used for the preparation of bioactive film forming solutions, was prepared from cells grown in YPD broth (1% yeast extract, 2% peptone, 2% glucose; Oxoid, Basingstoke, Hampshire, England) for 24 h at 25 °C. The yeast culture was centrifuged at 8000 ×*g* for 10 min and the pellet was washed twice with sterile water. *W. anomalus* BS 91 cells were then incorporated into NaAlg and LBG film forming solutions, cooled to 30 °C, at a concentration of 10^7 CFU/mL (~6 logs CFU/cm^2 in dry film). The concentration of antagonistic yeast in the stand alone films and film-forming solutions was chosen from results of preliminary laboratory tests (data not shown). This concentration was able to completely inhibit the growth of *P. digitatum* without affecting the physical properties of sodium alginate and LBG films.

The bioactive film forming solutions were subsequently placed under magnetic stirring for 5 min.

2.4. Preparation of the stand alone coatings

A casting method was used to produce NaAlg and LBG stand-alone coatings with and without yeast cells. In order to obtain films with a similar thickness, a constant amount that provided a solid surface density of 50 g/m^2 was poured into Petri plates, and dried at room temperature for approximately 48 h. Once formed, films were peeled from Petri plates and preconditioned in climatic chamber at 25 °C and 75% relative humidity (RH), before testing.

2.5. Characterization of the stand alone coatings

2.5.1. Mechanical properties

The mechanical properties of the films including tensile strength (TS) and elongation at break (%E) were determined using the Instron Universal Testing Machine (Model 3345, USA) according to a standard method of ISO 1924-2-1994. Samples were cut into 15 mm wide and 100 mm long strips and fixed with an initial clamp separation of 50 mm at a test speed of 100 mm/min. TS was calculated by dividing the maximum load on the film before failure by the cross-sectional area of the initial film specimen. %E was determined by dividing the extension at the moment of breakage by the initial gauge length of the