UNIVERSITAT POLITÈCNICA DE VALENCIA

INSTITUTO UNIVERSITARIO DE INGENIERÍA DE ALIMENTOS PARA EL DESARROLLO

Coating forming agents as carriers of the biocontrol agent *Candida sake* with antifungal effect against *Botrytis cinerea* on grapes

DOCTORAL THESIS

Presented by:

Anna Marín Gozalbo

Supervisors:

Amparo Chiralt Boix

Lorena Atarés Huerta

Maite Cháfer Nácher

Valencia, julio 2016

Dra. Amparo Chiralt Boix, Catedrática de Universidad, perteneciente al Departamento de Tecnología de Alimentos de la Universitat Politècnica de València.

Dra. Lorena Atarés Huerta, Profesora Titular de Universidad, perteneciente al Departamento de Tecnología de Alimentos de la Universitat Politècnica de València.

Dra. Maite Cháfer Nácher, Catedrática de Universidad, perteneciente al Departamento de Tecnología de Alimentos de la Universitat Politècnica de València.

Hacen constar que:

La memoria titulada "Coating forming agents as carriers of the biocontrol agent *Candida sake* with antifungal effect against *Botrytis cinerea* on grapes" que presenta Dª Anna Marín Gozalbo para optar al grado de Doctor por la Universitat Politècnica de València, ha sido realizada en el Instituto de Ingeniería de Alimentos para el Desarrollo (IuIAD – UPV) bajo su dirección y que reúne las condiciones para ser defendida por su autora.

Valencia, 15 de junio 2016

Fdo. Amparo Chiralt Boix Fdo. Lorena Atarés Huerta Fdo. Maite Cháfer Nácher

This work has been funded by VALi+d 2013 grant of Generalitat Valenciana and by the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria through the subprojects RTA2012-00067-C01 and RTA2012-00067-C02 jointly coordinated by the Postharvest Pathology Laboratory of IRTA (Institut de Recerca i Tecnologia Agroalimentàries) in Lleida and the Instituto Universitario de Ingeniería de Alimentos para el Desarrollo of the Universitat Politècnica de Valencia.

No importa tanto la meta como en quien te conviertes para conseguirla

- *Anónimo*

AKNOWLEDGEMENTS

Después de tres años, y muchos kilómetros recorridos, es complicado plasmar en unas pocas líneas todas las experiencias vividas y todo aquello que me gustaría agradecer a las personas que han estado junto a mí durante este camino.

A Lorena Atarés por apostar por mí y darme la oportunidad de empezar el doctorado, aunque en su momento no lo tenía demasiado claro. Siempre es de agradecer saber que tienes una persona a la que recurrir cuando te encuentras baches en el camino y tú has sido esa persona.

A Maite Cháfer por esa energía positiva que desprendes. Gracias por esa cercanía y confianza que transmites y por siempre estar dispuesta ayudar y a aportar tu granito de arena.

A Amparo Chiralt por su dedicación y esfuerzo y por ser capaz de sacar tiempo de donde no lo hay. Trabajar contigo me ha dado la oportunidad de disfrutar de las explicaciones y razonamientos de un referente en el mundo de la investigación.

A Neus Teixidó y a todo el grupo de Patología de la Postcosecha del IRTA de Lleida. Gracias por acogerme como una más y por introducirme en el mundo del control biológico. Ha sido un placer poder trabajar con vosotros, tanto a nivel personal como a nivel profesional.

A parte de todos los conocimientos adquiridos y el trabajo realizado, sin duda, una de las cosas más importantes me llevo después de estos años sin duda sois vosotras, mis compañeras de laboratorio: las Ángelas, Amalia, Cristina, Emma, Justine, Olga y Raquel. Me llevo un poquito de todas y cada una de vosotras. Gracias por las risas y los buenos momentos pero también por los consejos y el apoyo en los momentos complicados.

No me puedo olvidar de dar las gracias a Susana y Mariola, compañeras de laboratorios vecinos y grandes amigas. ¿Qué hubiera sido de nosotras sin las terapias en el tren y nuestras reflexiones matutinas? Habéis sido un gran apoyo durante todo este tiempo.

A mis padres, porque sin ellos esta Tesis no hubiera sido posible. Gracias por enseñarme que el esfuerzo y la constancia siempre acaban teniendo recompensa. Gracias por vuestros consejos, por darme tranquilidad en los momentos complicados, por animarme siempre a mejorar, por respetar mis decisiones pero, sobre todo, gracias por demostrarme que estáis orgullosos de mí.

A Álex, porque una trocito de esta Tesis también es suyo. Gracias por tu apoyo incondicional, por hacerme olvidar un mal día con unas simples palabras (o incluso a veces con una canción) y por creerme capaz de todo. Después de todo este tiempo poco te puedo decir que no sepas. Gracias por haber estado ahí en todo momento y por todos los.

ABSTRACT

The biocontrol agent (BCA) *Candida sake* CPA-1, has proven to be effective against the pathogenic fungus *Botrytis cinerea*, causing agent of grey mold in many fruits. The aim of this Thesis was to develop biocontrol products (BCP), based on this BCA and coating forming agents (CFAs) with good stability and efficacy against fungus infection. Several formulations of CFAs, based on biopolymers (hydroxypropylmethylcellulose (HPMC), corn starch (S), sodium caseinate (NaCas) and pea protein (PP)), combined with surfactants (oleic acid (OA), Span 80 (S80) and Tween 85 (T85)), were obtained and analyzed as to their ability to improve the adherence, survival and efficacy of *C. sake* on grapes. The functionality of these formulations as coatings was also analyzed with and without yeast cells. Likewise, dry formulations based on low cost CFAs (starch derivatives) and *C. sake* were obtained by means of fluidized-bed drying and their physical and microbiological stability were studied as a function of product moisture content.

The application of *C. sake* in combination with CFAs permitted an improvement in the initial adherence of the yeast on the surface of grapes and also higher survival rates. The protein-based coatings (NaCas and PP), both with and without surfactants showed the best results, suggesting that these matrices are more adequate supports for the BCA. CFAs also enhanced the efficacy of the BCA efficacy at controlling grey mold with respect to *C. sake* applied in water. NaCas and PP, and also some of the formulations based on S, exhibited the highest reduction values as regards to the incidence and severity of the infection.

When the main properties of the coating forming dispersions and films were analyzed, it could be observed that the type of polymer, more than the presence or type of surfactant, greatly influenced the obtained values. The viability of *C. sake* on the different matrices was greatly reduced during storage at 25°C. However, protein-based coatings showed slightly higher counts. The coatings formed were not estimated to be tick and so they did not represent a relevant barrier to the gas exchanges of the fruit, although they were sufficient to improve the performance of *C. sake* as BCA.

The physical stability of the different BCPs based on *C. sake* and starch derivatives (potato starch, pre-gelatinized potato starch and maltodextrins) was ensured below a water activity (a_W) of 0.75 at room temperature, since BCPs were in a glassy state. However, the viability of *C. sake* at 20°C over time was greatly affected by the a_W. Thus, whereas an $a_W \geq 0.43$ caused fast reductions in the viability of the BCA in all of the formulations, an $a_W \leq 0.33$ better preserved the viability of the yeast. This is a key factor since 0.33 is the a_W value of a newly dried product and its moisturizing must be avoided in order to maintain its effectiveness in terms of cell viability. Nevertheless, 20°C was considered a non-adequate temperature since, even at low a_W, a remarkable decline in viable cells was observed. However, cold storage of BCPs at 5°C allowed for a very good preservation of viable cells even after 6 months. A BCP based on maltodextrins as the main carrier was the formulation that showed the best potential to formulate *C. sake* in terms of the cell viability preservation and feasibility of in-field application due to its faster water solubilization.

RESUMEN

El agente de biocontrol (ABC) *Candida sake* CPA-1 ha demostrado ser efectivo frente al hongo patogénico *Botrytis cinerea*, el agente causante de la podredumbre gris en muchas frutas. El objetivo de esta Tesis fue el desarrollo de productos de biocontrol (PBC) basados en este ABC y agentes formadores de recubrimiento (AFRs), con una buena estabilidad y efectividad frente a la infección fúngica. Se obtuvieron diversas formulaciones de AFRs, basadas en biopolímeros (hidroxipropilmetilcelulosa (HPMC), almidón de maíz (AM), caseinato sódico (NaCas) y proteína de guisantes (PG)), combinados con tensoactivos (ácido oleico (AO), Span 80 (S80) y Tween 85 (T85)). Todas ellas se analizaron en su capacidad para mejorar la adherencia, supervivencia y eficacia de *C. sake* en uvas. Se estudio también la funcionalidad de estas formulaciones como recubrimientos con y sin la incorporación de células de la levadura. Asimismo, se obtuvieron formulados secos basados en AFRs de bajo coste (derivados de almidón) y *C. sake* por secado en lecho fluido y su estabilidad física y microbiológica fue estudiada en función de su contenido en humedad.

La aplicación de *C. sake* en combinación con AFRs permitió una mejora de la adherencia inicial de la levadura en la superficie de uvas y también una mayor supervivencia. Los recubrimientos basados en proteínas (NaCas y PG) con y sin tensoactivos mostraron los mejores resultados, sugiriendo que estas matrices son soportes más adecuados para el ABC. Los AFRs también mejoraron la efectividad del ABC en el control de la podredumbre gris en comparación con *C. sake* aplicada con agua. NaCas y PG, así como algunas de las formulaciones basadas en AM, dieron lugar a los valores más elevados de reducción de la incidencia y severidad de la infección.

Cuando se analizaron las principales propiedades de las dispersiones formadoras de recubrimiento y las películas, se pudo observar que el tipo de polímero, más que la presencia o tipo de tensoactivo, afectó considerablemente los valores obtenidos. La viabilidad de *C. sake* en las distintas matrices se vio muy afectada durante su almacenamiento a 25°C. Sin embargo, los recubrimientos a base de proteínas mostraron recuentos ligeramente superiores. El espesor estimado de los

recubrimientos formados en las uvas fue muy bajo, por lo que éstos no supusieron un efecto barrera relevante para los intercambios de gases de la fruta, aunque fueron suficientes para mejorar la función de *C. sake* como ABC.

La estabilidad física de los diferentes PBCs basados en *C. sake* y derivados de almidón (almidón de patata, almidón de patata pregelatinizado y maltodextrinas) quedó asegurada a actividades de agua (a_W) por debajo de 0.75 a temperatura ambiente, ya que los PBCs se encontraban en estado vítreo. Sin embargo, la viabilidad de *C. sake* a 25°C durante el tiempo se vio altamente disminuida. Mientras valores de $a_W \geq 0.43$ causaron rápidas reducciones en la viabilidad del ABC en todas las formulaciones, valores de $a_W \leq 0.33$ preservaron mejor la viabilidad de la levadura. Esto es un factor clave ya que 0.33 es el valor de a_W de los productos recién obtenidos, por lo que su hidratación debe evitarse para mantener su efectividad en términos de viabilidad celular. No obstante, 20°C no fue considerada una temperatura de conservación adecuada ya que, incluso a valores bajos de a_W, se observó un marcado descenso en el número de células viables. Por su parte, el almacenamiento en frío a 5°C permitió un muy buen mantenimiento de células viables, incluso tras 6 meses de almacenamiento. El PBC basado en maltodextrinas como soporte principal fue el formulado que mostró el mejor potencial para formular *C. sake* en términos de mantenimiento de la viabilidad celular y practicidad para su aplicación en campo, debido a su rápida solubilidad en agua.

RESUM

L'agent de biocontrol (ABC) *Candida sake* CPA-1 ha demostrat s'efectiu enfront del fong patogènic *Botrytis cinerea*, l'agent causal de la podridura grisa en moltes fruites. L'objectiu d'aquesta Tesi va ser el desenvolupament de productes de biocontrol (PBC) basats en aquest ABC i agents formadors de recobriment (AFRs), amb una bona estabilitat i efectivitat enfront de la infecció fúngica. Es van obtindre diverses formulacions d'AFRs, basades en biopolímers (hidroxipropilmetilcelulosa (HPMC), midó de dacsa (M), caseinat sòdic (NaCas) i proteïna de pésol (PP)), combinats amb tensoactius (acid oleic (AO), Span 80 (S80) i Tween 85 (T85)). Totes elles van ser analitzades en la seua capacitat per a millorar l'adherència, supervivència i eficàcia de *C. sake* en raïm. La funcionalitat com a recobriments d'aquestes formulacions també va ser estudiada amb i sense la incorporació de cèl·lules del llevat. Tanmateix, es van obtindre formulats secs basats en AFRs de baix cost (derivats de midó) i *C. sake* per assecat en llit fluiditzat i la seua estabilitat física i microbiològica va ser estudiada en funció del seu contingut en humitat.

L'aplicació de *C. sake* en combinació amb AFRs va permetre una millora de l'adherència inicial del llevat sobre la superfície de raïm i també una major supervivència. Els recobriments basats en proteïnes (NaCas i PP) amb i sense tensoactius van mostrar els millors resultats, suggerint que aquestes matrius són suports més adequats per a l'ABC. Els AFRs també van millorar l'efectivitat de l'ABC en el control de la podridura grisa en comparació amb *C. sake* aplicada amb aigua. NaCas i PP, així com algunes de les formulacions basades en M, van donar lloc als valors més elevats de reducció de la incidència i severitat de la infecció.

Quan les principals propietats de les dispersions formadores de recobriment i pel·lícules van ser analitzades, es va poder observar que el tipus de polímer, més que la presència o tipus de tensoactiu, va afectar considerablement els valors obtinguts. La viabilitat de *C. sake* en les diferents matrius es va veure molt afectada durant el seu emmagatzemament a 25°C. No obstant això, els recobriments a base de proteïnes van mostrar recomptes lleugerament superiors. La grossària estimada dels recobriments

formats en el raïm va ser molt baixa, per la qual cosa aquests no van suposar un efecte barrera rellevant per als intercanvis de gasos de la fruita, encara que van ser suficients per a millorar la funció de *C. sake* com ABC.

L'estabilitat física dels diferents PBCs basats en *C. sake* i derivats de midó (midó de creïlla, midó de creïlla pregelatinitzat i maltodextrines) va quedar assegurada a activitats d'aigua (a_W) per davall de 0.75 a temperatura ambient, ja que els PBCs es trobaven en estat vitri. No obstant això, la viabilitat de *C. sake* a 25°C durant el temps es va veure altament afectada. Mentre que valors d'$a_W \geq 0.43$ van causar ràpides reduccions en la viabilitat de l'ABC en totes les formulacions, valors d'$a_W \leq 0.33$ an preservar millor la viabilitat del llevat. Açò és un factor clau ja que 0.33 és el valor d'a_W dels productes acabats d'obtindre, per la qual cosa la seua hidratació ha d'evitar-se per a mantindre la seua efectivitat en termes de viabilitat cel·lular. No obstant això, 20°C va ser considerada una temperatura no adequada ja que, fins i tot a valors d'a_W baixos, un marcat descens en el nombre de cèl·lules viables va ser observat. Per la seua banda, l'emmagatzemament en fred a 5°C va permetre un molt bon manteniment de cèl·lules viables, fins i tot després de 6 mesos. El PBC basat en maltodextrines com a suport principal va ser el formulat que va mostrar el millor potencial per a formular *C. sake* en termes de manteniment de la viabilitat cel·lular i practicitat per a la seua aplicació en camp, degut a la seua ràpida solubilitat en aigua.

PREFACE

DISSERTATION OUTLINE

This Doctoral Thesis is structured in five sections: Introduction, Objectives, Chapters, General Discussion and Conclusions. The INTRODUCTION section focuses on the use of biocontrol agents and the potential benefits of their incorporation in edible coatings. The application of edible coatings on fruits is also discussed. Both the general and the specific objectives of the Thesis are compiled in the OBJECTIVES section. The obtained results are divided into three CHAPTERS, which are presented as a collection of different scientific publications, including the usual parts: introduction, materials and methods and results and discussion. In the GENERAL DISCUSSION section, a global analysis of the main results obtained is carried out. Finally, the most important CONCLUSIONS of the Thesis are presented in the corresponding section.

The Thesis analyses the formulation of *Candida sake* as a BCA, based on coating-forming agents, using different approaches: a) the ability of different biopolymers to enhance cell viability and efficacy so as to control *Botrytis cinerea*, b) the coating-forming capacity of these formulations and their functional properties as films and c) the physical stability and cell viability of selected formulations as dried powders, obtained by fluidized-bed drying, analyzing the effect of a_W and temperature. For the purposes of selecting the most adequate formulation, the economic aspects and feasibility for field applications have been taken into account. The obtained results are organized as follows:

Chapter 1, entitled "Effect of different coating-forming agents on the efficacy of the biocontrol agent *Candida sake* CPA-1 for control of *Botrytis cinerea* on grapes" studies different formulations of edible coatings as a support to this biocontrol agent. Different coating-forming dispersions based on biopolymers (hydroxypropylmethycellulose, corn starch, sodium caseinate and pea protein) and surfactants (oleic acid, Span 80® and Tween 85®) were obtained and *Candida sake* was incorporated into them. In this work, both the adherence and survival of *Candida sake* applied with the edible coatings on

grapes and its efficacy against the pathogen *Botrytis cinerea* were tested. The positive results propitiated further studies for the purposes of finding out whether there was a critical ratio of coating solids which would enhance the viability and efficacy of the antagonist. In order to analyze the distribution of cells on the grape surface as well as their possible morphological changes throughout time, scanning electron microscopy observations were also carried out. After this study, information about the most adequate composition of the edible coatings to be used as carriers of *Candida sake* was obtained. Of the biopolymers tested as the main compounds in edible coatings, starch, and especially proteins, enhanced both the adherence and survival of the BCA on the grapes' surface and also its fungicidal action. The incorporation of surfactants did not improve either survival or disease control and, although they promoted a better cell dispersion on the fruit surface, their use for further studies was discarded.

The formulations studied in Chapter 1 were characterized as fruit coatings in their most relevant properties and the results are included in Chapter 2, entitled "Properties of biopolymer dispersions and films used as carriers of the biocontrol agent *Candida sake*". *Candida sake* was incorporated into the dispersions and films were obtained by casting. The influence of the incorporation of the biocontrol agent, the type of matrix and the type of surfactant on the optical and barrier properties of the films and their coating capacity on grapes was analyzed. Furthermore, the viability and survival of *Candida sake* in the films after the drying process and stored under different relative humidity conditions, was evaluated. This study provided insight into the characteristics of each formulation and its ability to act as an adequate carrier of the biocontrol agent. The properties of the dispersions and films were more clearly affected by the type of polymer than by the incorporation of surfactants. In general terms, the viability of the antagonist in the dry films was reduced during storage, although *Candida sake* exhibited a slightly higher survival rate in protein-based films.

Taking into account the results of the previous studies, the obtaining of a formulation based on edible-coating agents acting as carriers of *Candida sake* was considered. In this sense, starch derivatives were selected based on their availability and low cost and on the ability of starch to maintain cell viability and biocontrol efficacy. Chapter 3, entitled "Stability of biocontrol products carrying *Candida sake* CPA-1 in starch

derivatives as a function of water activity", studies the physical and microbiological stability of different dried biocontrol products obtained by fluidized-bed drying. The different compounds employed as cell protectants were also introduced into the formulations. The physical stability of the powder formulations was determined through the study of the relationships between water activity, moisture content and glass transition. As regards the shelf-life of the formulations, the viability of *Candida sake* in the different formulations was studied during storage both at room temperature (20°C) and at 5°C under several relative humidity conditions. The biocontrol products studied were physically stable under practical application conditions, while the cell viability in the powder formulations was the critical point. At room temperature, the viability of *Candida sake* decreased in long-term storage; the higher the relative humidity, the higher the death rate of the cells. A lower storage temperature enhanced cell survival during storage.

DISSEMINATION OF RESULTS

International Journals JCR

Published:

Marín, A., Cháfer, M., Atarés, L., Chiralt, A., Torres, R., Usall, J. & Teixidó, N. (2016). Effect of different coating-forming agents on the efficacy of the biocontrol agent *Candida sake* CPA-1 for control of *Botrytis cinerea* on grapes. Biological Control, 96, 108-119.

Submitted:

Marín, A., Atarés, L., Cháfer, M. & Chiralt, A. Properties of biopolymer dispersions and films used as carriers of the biocontrol agent *Candida sake*. Food and Bioprocess Technology

Pending submission:

Marín, A., Atarés, L., Cháfer, M. & Chiralt, A. Stability of biocontrol products carrying *Candida sake* CPA-1 in starch derivatives as a function of water activity.

The results have been also presented in national and international conferences:

Poster – Marín, A., Atarés, L., Chiralt, A. & Cháfer, M. (2014). Influencia de la incorporación de tensoactivos en las propiedades reológicas de dispersiones formadoras de recubrimientos. 9 Congreso Iberoamericano de Ingeniería de Alimentos, CIBIA, January, 13-16. Valencia, Spain

Poster – Marín, A., Atarés, L., Chiralt, A. & Cháfer, M. (2014). Evaluación de la retención de biopolímeros sobre superficie de uvas en aplicaciones por pulverización. II Congreso Internacional de Investigación e Innovación en Ingeniería, Ciencia y Tecnología de los alimentos (IICTA), May, 27-30. Medellín, Colombia.

Poster – Marín, A., Atarés, L., Chiralt, A. & Cháfer, M. (2014). Propiedades barrera al vapor de agua de películas biodegradables. Influencia del tipo de polímero y tensoactivo. III International Conference of Food Innovation, October, 20-23. Concordia, Entre Ríos, Argentina.

Oral – Marín, A., Cháfer, M., Atarés, L., Chiralt, A., Torres, R., Usall, J. & Teixidó, N (2014). Efecto de diferentes agentes formadores de recubrimiento en la efectividad de *Candida sake* para el biocontrol de *Botrytis cinerea* en uvas. XI Simposio Nacional y VIII Ibérico Sobre Maduración y Postcosecha, October 21-23. Valencia, Spain.

Poster – Marín, A., Atarés, L., Chiralt, A., Teixidó, N. & Cháfer, M. (2015). Efectividad de *Candida sake* incorporada a recubrimientos biodegradables en el control de *Botrytis cinerea* en uva. VIII Congreso CYTA/CESIA, April, 8-10. Badajoz, Spain.

Poster – Marín, A., Cháfer, M., Atarés, L., Chiralt, A., Torres, R., Usall, J. & Teixidó, N (2015). Effect of different film forming agents on the efficacy of *Candida sake* for biocontrol of *Botrytis cinerea* in grapes. II International Symposium on Postharvest Pathology, June, 7-11. Bari, Italia.

Poster – Marín, A., Atarés, L., Cháfer, M., Teixidó, N. & Chiralt, A. (2016). Influence of water activity on viability of *Candida sake* formulated in different polymer matrices. 4th International ISEKI_Food Conference, July, 6-8. Vienna, Austria.

TABLE OF CONTENTS

LIST OF ACRONYMS

a_W – water activity

BC – biological control

BCA – biocontrol agent

BCP – biocontrol product

CFDs – coating-forming dispersions

CFAs – coating forming agents

CFU – colony forming units

CMC - carboxymethylcellulose

CMC – critical moisture content

CWA – critical water activity

ECs – edible coatings

EOs – Essential oils

FBD – fluidized-bed drying

FFDs – film-forming dispersions

GRAS – generally recognized as safe

HLB - hydrophilic/lipophilic balance

HPMC – hydroxypropylmethylcellulose

MD – maltodextrins

MC – methylcellulose

MC – moisture content

NaCas – sodium caseinate

OA – oleic acid

OP – Oxygen permeability

OTR – oxygen transmission rate

PP – pea protein

PG – pregelatinized starch

PS – potato starch

RH – relative humidity

S – starch

S80 – Span 80®

SEM – scanning electron microscopy

T85 – Tween 85®

T_g – glass transition temperature

WTR – water transmission rate

WVP – water vapor permeability

1. INTRODUCTION

Fruit losses caused by fungal diseases both in the field, during storage and under commercial conditions can reach more than 25% of the total production in industrialized countries, and over 50% in developing countries (Nunes, 2012; Spadaro & Gullino, 2004). The losses in developing countries, especially in those located in tropical and subtropical regions, are more severe both due to environmental conditions in the field that are particularly conducive to fruit infection and also to inadequate post-harvest storage and transportation facilities (Janisiewicz & Korsten, 2002; Sharma *et al*., 2009). Several factors make the fruit especially susceptible to fungal decay. The composition of the fruit, characterized by a large amount of water and nutrients and low pH, is one of the main causes of its sensitivity to the attack of fungal pathogens (Harvey, 1978). The presence of wounds, often originated during harvest, transport, packing and storage processes, facilitates the access of the pathogens (Barkai-Goland, 2001; Spadaro & Gullino, 2004). Moreover, after harvest, the fruit loses the protection offered by the intrinsic decay resistance that it possesses while attached to the plant (Droby *et al*., 1992). The main pathogens causing the deterioration of fruit belong to the genera *Alternaria*, *Aspergillus*, *Botrytis*, *Fusarium*, *Geotrichum*, *Gloeosporium*, *Mucor*, *Monilinia*, *Penicillium* and *Rhizopus* (Barkai-Goland, 2001). Apart from economic considerations, some species of these genera may represent a potential risk since they are able to produce mycotoxins (Liu *et al*., 2013).

Fungal diseases can be somewhat controlled by using non-chemical methods or non-selective fungicides, such as sodium carbonate, sodium bicarbonate, active chlorine and sorbic acid. Moreover, the mechanical injuries can be minimized and proper sanitation procedures can be used (Shweleft, 1986; Tripathi & Dubey, 2004). However, all these strategies are insufficient and synthetic fungicides, applied both in orchard and post-harvest, represent the most widely-used method with which to control fungal diseases.

Nevertheless, chemical control presents several shortcomings. Firstly, synthetic pesticides are a source of environmental contamination and have a long degradation period (Tripathi & Dubey, 2004). Secondly, the use of these chemicals may lead to the presence of residues in food, which represent a toxicological hazard to human health. This is of particular importance in the case of fruit, since nowadays there is a rising consumer awareness of the need to follow a healthier diet, in which the role of fruit is

essential. In fact, The World Health Organization (WHO), Food and Agriculture Organization (FAO), United States Department of Agriculture (USDA) and European Food Safety Authority (EFSA) recommended an increase in fruit and vegetable consumption so as to decrease the risk of cardiovascular diseases, cancer and ageing (Allende *et al*., 2006). Ultimately, the continued use of chemical fungicides has generated resistance in the pathogen populations and, consequently, some of them have become inefficient against such strains (Brent & Hollomon, 2007; Panebianco *et al*., 2015; Tripathi & Dubey, 2004). Consumer awareness of in this regard has motivated an increasing demand for a reduction in the use of potentially harmful chemicals in order to obtain fruit free of pesticide residues (Liu *et al*., 2013). Additionally, the authorities have developed stricter regulatory policies that require the search for eco-friendly strategies as an alternative to the chemical control of fungal decay.

1.1 Biological control

In the past thirty years, biological control (BC) has been considered as one of the approaches with the greatest potential against fungal pathogens, either alone or as part of integrated systems for pest management (Spadaro & Gullino, 2004). Consequently, extensive research has been devoted to exploring and developing this field. BC consists of biologically-based processes to lower pathogen inoculum density and reduce crop and post-harvest losses (Cañamás *et al*., 2011).

One of the main advantages of BC is that it exploits mostly natural cycles with a reduced environmental impact (Spadaro & Gullino, 2004). Moreover, it is a good option for application on organic crops (Fravel, 2005). Since BC offers different modes of action from physical treatments and chemical pesticides, it may be applied as part of an integrated system, consisting of the combination of BC and physical and/or chemical methods. Many examples of the combination of BC with pesticides and physical treatments (hot water, controlled atmosphere, ultraviolet-C radiation or microwaves) have been reported in literature (Droby *et al*., 1998; Lima *et al*., 2011; Moretto *et al*., 2014; Yu *et al*., 2013; Torres *et al*., 2007; Zhang *et al*., 2013; Zhou *et al*., 2014). The combination of different treatments against fungal pathogens allows the amount of applied chemicals to be reduced.

Despite the benefits of BC, there are some potential hazards related to the use of living microorganisms which should be taken into account. Alabouvette & Cordier (2011) widely reported the main drawbacks of the use of microorganisms as biocontrol agents (BCAs). Since some microorganism species may include both pathogenic and beneficial strains, it is necessary to precisely identify the species and strains of the potential BCAs. However, in many cases, identification at the strain level is difficult and only molecular techniques enable a clear classification. Moreover, whenever the mechanism of action of the BCA consists of the production of secondary metabolites, it might be necessary to verify the non-toxicity of such products. Nevertheless, it is known that secondary metabolites are only locally produced in very limited quantities, and their production depends heavily on many factors, such as the age of the culture, the growth medium or the plant organ on which the BCA is applied.

As regards the effects of BCAs on human health, it is worth noting that most of them are isolated from natural sources, so it is extremely unlikely that humans have never been exposed to them. Often the antagonist exists in the plant, but at a density too low to be effective at controlling the disease. Alabouvette & Steinberg (1998) stated that microorganisms introduced from the natural environment will not become dominant when reintroduced into the same environment.

BCAs can be applied throughout most of the fruit development in order to reduce latent infections causing post-harvest decay caused by the loss of natural resistance mechanisms (Janisiewicz & Korsten, 2002). They can also be applied immediately before harvest (pre-harvest); therefore, they are able to pre-colonize the fruit's surface so that wounds inflicted during harvesting are protected from pathogen colonization (Ippolito & Nigro, 2000; Janisiewicz & Korsten, 2002; Sharma *et al*., 2009). The success of pre-harvest applications greatly depends on the tolerance of microorganisms to environmental stresses, such as high temperatures, UV irradiation or scarce nutrient availability (Nunes, 2012; Romanazzi *et al*., 2016). For that reason, due to the poor survival rate of antagonists in field conditions, the advantages of pre-harvest application are often lost. However, several examples of the success of this approach can be found in previous studies (Benbow & Sugar, 1999; Cañamás *et al*., 2008a; 2008b; Karabulut *et al*., 2004; Teixidó *et al*., 1999). In post-harvest applications, BCAs are applied after

harvesting as sprays or as dips in solutions containing them. This approach appears to be more effective than the pre-harvest one for the purposes of controlling post-harvest diseases of fruit and vegetables (Sharma *et al*., 2009).

1.2 Biocontrol agents: sources and mechanisms of action

Fungi, yeasts and bacteria are potential microorganisms to be used as antagonists for controlling the post-harvest diseases of fruits and vegetables. They either may be naturally occurring microbes or artificially introduced (Sharma *et al*., 2009). Natural microbial antagonists already exist on the surface of fruits and vegetables and, after isolation, can be promoted and managed in order to be used against fungal pathogens. In the case of artificially introduced BCAs, although they are initially isolated from natural sources, their target application is different from their source. The artificial introduction of microorganisms has been reported as being more effective at controlling organisms which cause post-harvest rots than other means of BC (Sharma *et al*., 2009).

Regardless of the source, an ideal BCA should meet a number of requirements, as reported by several authors (Abano & Sam-Amoah 2012; Droby *et al*., 2009; Sharma *et al*., 2009). The characteristics of an ideal antagonist are that it must be : genetically stable, effective at low concentrations, undemanding in terms of its nutrient requirements, capable of surviving under adverse environmental conditions, effective against a wide range of pathogens on different commodities, amenable to production on inexpensive growth media, amenable to formulation with a long shelf-life, easy to dispense, resistant to chemicals used in the post-harvest environment, not detrimental to human health, compatible with other chemical and physical treatments and not detrimental to the quality of the fruits and vegetables it preserves. In addition, a BCA should have an adaptive advantage over specific pathogens (Sharma *et al*., 2009; Wilson & Wisniewski, 1989). For instance, if the pathogen to be suppressed is tolerant to low temperatures, the antagonist should have the ability to survive under cold conditions.

1.2.1 Sources of biocontrol agents

The fruit surface has been reported as an excellent source of naturally occurring BCAs (Janisiewicz & Korsten, 2002) and a large number of examples of antagonists isolated

from fruit can be found (Bonaterra *et al*., 2003; El-Shanshoury *et al*., 2013; Lima *et al*., 2013; Metz *et al*., 2001; Spadaro *et al*., 2010; Teixidó *et al*., 2001). In this context, unmanaged orchards, where natural populations have not been disturbed by chemicals and the reserve of potential antagonists is greater than in chemically treated orchards, are preferable (Janisiewicz & Korsten, 2002). Wilson *et al*., (1993) utilized fruit wounds as source of potential yeast antagonists against post-harvest diseases. This methodology allows for a rapid selection of a number of possible antagonists at minimal cost and has been employed in many post-harvest programs (Droby *et al*., 2009), although it does have some drawbacks that limit the variety of potential BCAs to be isolated. It favors the selection of antagonists with a high growth capacity, which mainly exhibit protective rather than curative activity, and appear to have little effect on latent infections (Droby *et al*., 1989; Droby *et al*., 2009; El-Ghaouth *et al*., 2000). Moreover, this screening method also favors the selection of microorganisms whose primary mechanism of action is nutrient competition (Droby *et al*., 1989; Janisiewicz & Korsten, 2002; Wisniewski *et al*., 2007), despite the fact that a wide range of other mechanisms has been identified. The use of other methodologies would greatly increase the range of microbial species with BC potential.

BCAs may also be isolated from other sources, both related and unrelated to fruit (Table 1.1). The surface of leaves or phylloplane is another good source of microorganisms (Blakeman & Fokkeman, 1982; Janisiewicz & Korsten, 2002) and several studies aiming at the isolation and testing of BCAs from different plants and crops have been developed. Other food sources, and even starter cultures, used in the food industry may also yield effective antagonists. Likewise, the isolation of microbial antagonists has expanded to soils and sea water.

1.2.2 Mechanisms of action of biocontrol agents

An extensive body of research has been devoted to the understanding of the mechanisms by which BCAs exert their action against pathogens. Nonetheless, in many cases, the suggested modes of action whereby antagonists wield their biocontrol effect are not totally elucidated, especially due to the fact that several mechanisms frequently take place at the same time (Rivera-Ávalos *et al*., 2012; Janisiewicz and Korsten, 2002).

Figure 1.1 shows the complex interactions between the host, the pathogen, the antagonist, the epiphytic microflora of the fruit surface and their environment that hinder the total understanding of the different modes of action (Jamalizadeh *et al*. 2011; Nunes, 2012). Despite the difficulties, insight into the action modes involved will permit an improvement in both the biocontrol performance and the development of appropriate formulations and methods of application. Tables 1.2, 1.3 and 1.4 show representative fungi, bacteria and yeasts used as BCAs and their suggested mechanisms of action.

Table 1.1. Biocontrol agents isolated from different sources.

Source	Biocontrol agent	Reference
Plants and crops	*Aureobasidium pullulans*	Sperandio *et al*., 2015
	Aureobasidium pullulans, Epicoccum purpurascens and *Trichoderma polysporum*	Falconi & Mendgen, 1994
	Candida pelliculosa and *Rhodotorula rubra*	Dal Bello *et al*., 2008
	Pseudomonas syringae	Cirvilleri *et al*., 2005
	Pseudomonas syringae	Habibi *et al*., 2012
Food	*Aureobasidium pullulans, Metschnikowia Pulcherrima* and *Wickerhamomyces anomalus*	Parafati *et al*., 2015
	Bacillus spp.	Zhou *et al*., 2008
	Pichia anomala	Laitila *et al*., 2007
	Saccharomyces cerevisiae and *Wickerhamomyces anomalus*	Platania *et al*., 2012
Soil	*Streptomyces violascens*	Choudhary *et al*., 2015
	Saccharomyces cerevisiae	Nally *et al*., 2012
	Leucosporidium scottii	Vero *et al*., 2013
	Bacillus amyloliquefaciens	Zhao *et al*., 2013
Sea water	*Debaryomyces hansenii*	Medina-Córdova *et al*., 2016
	Pseudomonas aeruginosa	Manwar *et al.,* 2004
	Rhodosporidium paludigenum	Wang *et al*., 2010

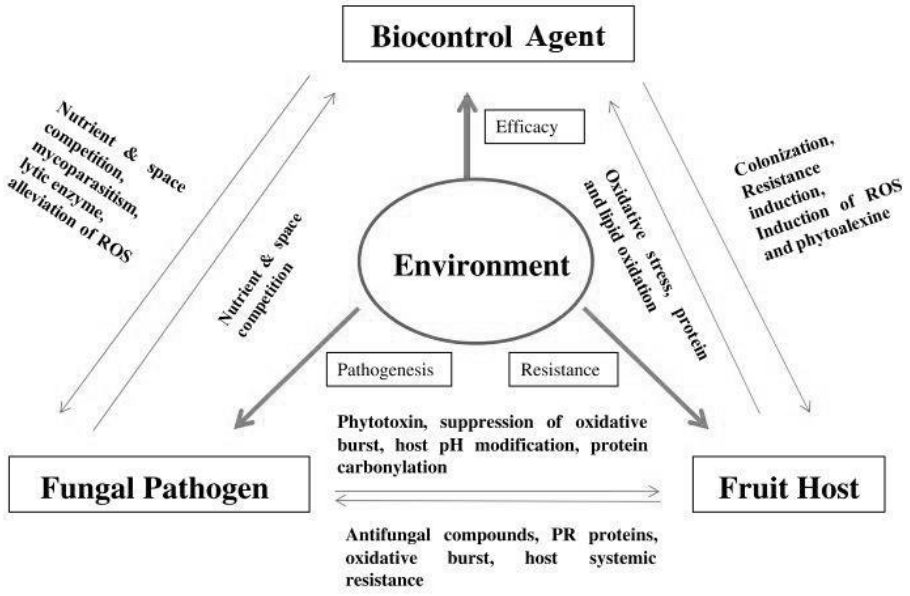


Figure 1.1. Possible interactions between host, pathogen and antagonist and their environment (adapted from Liu *et al*. 2013). ROS: reactive oxygen species; PR proteins: pathogenesis-related proteins.

Successful BCAs are generally equipped with several attributes which often work in concert and may be crucial for controlling disease development (Droby *et al*. 2009; Jamalizadeh *et al.* 2011). Competition for nutrients and space between the pathogen and the antagonist is considered to be the major mode of action, but other mechanisms such as parasitism, the production of secondary metabolites or the induction of host defenses, have also been reported. As described below, most of the modes of action are closely related.

Competition for nutrients and space

Competition for nutrients and space consists of a niche overlap resulting from a situation where there is a simultaneous demand for the same resource and space by two or more populations (Droby & Chalutz, 1994). To effectively compete, BCAs should grow more rapidly than the pathogen, have similar nutritional requirements, use low concentrations of nutrients and be able to survive under conditions that are unfavorable for the pathogen (Barkai-Goland, 2001; Jamalizadeh *et al*. 2011; Sharma *et al.* 2009). Under these conditions, the antagonist will deplete the available nutrients,

preventing the pathogen from using them, and physically occupy the space, thereby causing an inhibition of the germination of fungal spores (Liu *et al*. 2013; Nunes, 2012). In fact, it has been demonstrated through *in vitro* studies that antagonists consume the available nutrients and establish themselves more rapidly than pathogens (Droby and Chalutz, 1994; Droby *et al*. 1998). This mode of action is closely related to the initial amount of BCA since, to exert successful competition, there has to be a sufficient quantity at the correct time and location to multiply and colonize the fruit surface. In general, concentrations of 10^7 – 10^8 CFU/ml tend to be effective and higher concentrations are rarely required (El-Ghaouth *et al*. 2004; Sharma *et al*. 2009). Competition for space and nutrients is preferable to the use of antibiotic-producing microorganisms because of the potential issues related to human toxicity and the development of antibiotic resistance within pathogen populations (Jamalizadeh *et al*. 2011).

Direct attachment, secretion of enzymes and parasitism

Direct attachment to fungi, a mode of action intimately associated to parasitism that consists of the ability of BCAs to adhere to pathogen hyphae, is mainly observed in yeasts and bacteria (Wisniewski *et al*., 1991). It has been suggested that the attachment might facilitate a more efficient depletion of nutrients from the area subjacent to the mycelium and serves as a mechanical barrier to nutrient uptake by the target fungi, debilitating growth and inhibiting spore germination (Droby *et al*., 1989; 1992; Wilson & Wisniewski, 1989). Moreover, some authors have reported that the efficacy of the attachment seems to be dependent on the secretion of lytic enzymes, such as glucanases, chitinases and proteinases (Table 1.5). By means of these cell-wall-degrading enzymes and direct attachment, BCAs are able to parasitize fungal hosts. When parasitism takes place, the antagonists feed on or within the pathogen, and finally a destruction or lysis of its structures occurs (Jamalizadeh *et al*., 2011; Spadaro & Droby, 2016).

Based on the composition of the fungal cell wall, β-1,3-glucanase and chitinases are lytic enzymes related to the inhibition of pathogens by microbial antagonists. β-1,3-glucan is the filling material situated between the layers comprised of chitin which, together with

glucan, is one of the main components of the fungal cell wall. Given that glycoproteins are constituents of the cell walls of fungi, proteases also play an important role in biocontrol activity (Spadaro & Droby, 2016).

Production of secondary metabolites or antibiosis

Antibiosis is defined as the inhibition or destruction of pathogens caused by metabolic products of the antagonists (Heungens & Parke, 2001; Jamalizadeh *et al*., 2011). Against decay-causing fungi, the different substances that some BCAs are able to produce are killer toxins, antibiotics or volatile organic compounds.

Killer toxins are extracellular proteins produced by those yeasts with the denominated *killer phenotype*. These toxins have been described as lethal to sensitive microbial cells belonging to either the same or different species. Producers of this kind of toxins are able to kill each other but are immune to killer toxins of their own class (Spadaro & Droby, 2016). Many examples of killer yeasts can be found in literature (Breinig *et al*., 2002; Platania *et al*., 2012; Santos *et al*., 2009).

On the other hand, certain microorganisms possess the ability to produce antibiotics. This production may occur not only when there is a substantial quantity of substrate, mainly carbon, available but also when this availability decreases. It has been suggested that this last strategy could prevent other microorganisms from using the remaining substrates (Jamalizadeh *et al*., 2011).

Table 1.2. Summary of representative antagonistic fungi used as biocontrol agents and suggested mechanisms of action.

Biocontrol agent	Mechanism of action	Source	Pathogen	Application	Reference
Epiococcum nigrum	Production of secondary metabolites	Peach	*Monilinia laxa*	Peach	Larena *et al*., 2007
Gliocladium roseum	Competition for nutrients and space, parasitism	Strawberry	*Botrytis cinerea*	Strawberry	Peng & Sutton 1991
Penicillium frequentans	Production of secondary metabolites	Peach	*Monilinia laxa*	Peach	Guijarro *et al*., 2007
Penicillium oxalicum	Induction of host defense	Soil	*Fusarium oxysporum*	Tomato	Larena *et al*., 2003
Pestalotiopsis neglecta	Production of secondary metabolites	Avocado	*Colletotrichum gloeosporioides*	Avocado	Adikaram & Karunaratne, 1998
Trichoderma spp.	Competition for nutrients and space, induction of host defense, production of secondary metabolites, parasitism	Grape and kiwi	*Monilinia fructicola*	Stone fruits	Hong *et al*., 1998
		Soil	*Fusarium oxysporum*	Melon	Gava & Pinto, 2016
Trichoderma harzianum	Competition for nutrients and space, induction of host defense, production of secondary metabolites, parasitism	Soil	*Botrytis cinerea*	Apple	Batta, 2004
			Colletotrichum musae	Banana	Devi & Arumugam, 2005
			Colletotrichum gloeosporioides	Rambutan	Sivakumar *et al*., 2001
Trichoderma viride	Competition for nutrients and space, induction of host defense, production of secondary metabolites, parasitism	Soil	*Botryodiplodia theobromae*	Mango	Kota *et al*., 2006
Ulocladium atrum	Competition for nutrients and space	-	*Botrytis cinerea*	Grape	Metz *et al*., 2001

Table 1.3. Summary of representative antagonistic bacteria used as biocontrol agents and suggested mechanisms of action.

Biocontrol agent	Mechanism of action	Source	Pathogen	Application	Reference
Bacillus amyloliquefaciens	Competition for nutrients and space, production of secondary metabolites	Apple	*Botryosphaeria dothidea*	Apple	Li *et al*., 2013
		Citrus	*Penicillium digitatum*	Mandarin	Hao *et al*., 2011
Bacillus subtilis	Competition for nutrients and space, production of secondary metabolites	Soil and stone fruit	*Botrytis cinerea* *Colletotrichum acutatum* *Penicillium expansum*	Apple and stone fruit	Lee *et al*., 2012
			Monilinia fructicola	Stone fruit	Yánez-Mendizábal *et al*., 2012b
			Monilinia fructicola	Stone fruit	Pusey & Wilson, 1984
			Peronophythora litchi	Litchi	Jiang *et al*., 2001
Bacillus licheniformis	Competition for nutrients and space, production of secondary metabolites	Soil	*Botryosphaeria spp.* *Colletotrichum gloeosporioides*	Mango	Govender *et al*., 2005
Pantoea agglomerans	Competition for space and nutrients, attachment to pathogen, parasitism	Pear	*Monilinia laxa* *Rhizopues stolonifer*	Stone fruit	Bonaterra *et al*., 2003
		Plum	*Monilinia fructicola*	Plum	Janisiewicz *et al*., 2013
		Pear and apple	*Botrytis cinerea* *Penicillium expansum*	Pear	Nunes *et al*., 2001
		Apple	*Penicillium digitatum Penicillium italicum*	Orange	Teixidó *et al*., 2001 Torres *et al*., 2011
Paenibacillus brasiliensis	Competition for nutrients, secretion of enzymes	Kumquat	*Penicillium italicum*	Mandarin	Tu *et al*., 2013
Pseudomonas aeruginosa	Induction of host defense	Grape	*Aspergillus* spp.	Grape	El-Shanshoury *et al*., 2013
Pseudomonas cepacia	Production of secondary metabolites	Apple	*Botrytis cinerea* *Penicillium expansum*	Apple and pear	Janisiewicz *et al*., 1991 Janisiewicz & Roitman, 1988
Pseudomonas fluorescens	Production of secondary metabolites	Melon	*Botrytis mali*	Apple	Mikani *et al*., 2007
Rahnella aquatilis	Production of secondary metabolites	Apple	*Botrytis cinerea* *Penicillium expansum*	Apple	Calvo *et al*. ,2007

Table 1.4. Summary of representative antagonistic yeasts used as biocontrol agents and suggested mechanisms of action.

Biocontrol agent	Mechanism of action	Source	Pathogen	Application	Reference
Aureobasidium pullulans	Competition for nutrients, secretion of enzymes, induction of host defense	Apple	*Botrytis cinerea* *Penicillium expansum*	Apple	Ippolito *et al*., 2000
		Apple	*Botrytis cinerea*	Apple	Vero *et al*., 2009
		Cherry	*Botrytis cinerea* *Monilinia laxa*	Cherry and grape	Schena *et al*., 2003
		Pear	*Penicillium expansum*	Pear	Robiglio *et al*., 2011
		Plum	*Botrytis cinerea* *Monilinia laxa* *Penicillium expansum*	Apple peach and plum	Zhang *et al*., 2010a
Candida ciferri *Cryptococcus laurentii*	Competition for nutrients	Apple	*Penicillium expansum*	Apple	Vero *et al*., 2002
Candida oleophila (Aspire, commercial biocontrol product)	Competition for nutrients and space, parasitism, induction of host defense	Tomato	*Botrytis cinerea* *Penicillium expansum* *Monilinia fructicola* *Rhizopus stolonifer*	Apple and peach	Droby *et al*., 2003
			Penicillium expansum	Grapefruit	Droby *et al.,* 2002
			Colletotrichum gloeosporioides	Papaya	Gamagae *et al*., 2003
Candida saitoiana	Induction of host defense	Orange	*Botrytis cinerea*	Apple	El-Ghaouth *et al*., 1998
Candida sake	Competition for nutrients and space	Apple	*Botryticis cinerea* *Penicillium expansum* *Rhizopus nigricans*	Apple	Viñas *et al*., 1998
			Penicillium expansum	Apple	Teixidó *et al*., 1998
			Penicillium expansum	Apple	Usall *et al*., 2000
Cryptococcus albidus	Competition for nutrients	Peach	*Botrytis cinerea* *Penicillium expansum*	Apple	Qin & Tian, 2001

Table 1.4. (Continued)

		Apple	*Botrytis cinerea*	Apple	Roberts, 1990
Cryptococcus laurentii	Competition for nutrients	Pear	*Botrytis cinerea* *Penicillium expansum*	Pear	Yu *et al*., 2012
			Penicillium expansum	Pear	Zeng *et al*., 2015
			Rhizopus stolonifer	Strawberry	Zhang *et al*., 2007
			Botrytis cinerea *Penicillium expansum*	Pear	Yu *et al*., 2012
			Penicillium expansum	Pear	Zeng *et al*., 2015
			Rhizopus stolonifer	Strawberry	Zhang *et al*., 2007
Hanseniaspora uvarum	Competition for nutrients and space, induction of host defense	Strawberry	*Botrytis cinerea*	Grape	Qin *et al*., 2015
			Botrytis cinerea *Rhizopus stolonifer*	Strawberry	Cai *et al*., 2015
Kloeckera apiculata	Production of secondary metabolites, biofilm formation	Citrus	*Penicillium digitatum Penicillium italicum*	Citrus	Pu *et al*., 2014
Metschnikowia pulcherrima	Competition for nutrients, parasitism	Apple	*Botrytis cinerea*	Apple	Piano *et al*., 1997
		Apple	*Botrytis cinerea* *Penicillium expansum*	Apple	Spadaro *et al*., 2010
		Fig	*Botrytis cinerea* *Cladosporium cladosporioides* *Monilia laxa* *Penicillium expansum*	Apple and nectarine	Ruiz-Moyano *et al*., 2016
Meyerozyma caribbica	Competition for nutrients, secretion of enzymes, biofilm formation	Mango	*Colletotrichum gloeosporioides*	Mango	Bautista-Rosales *et al*., 2013
Pichia guilliermondii	Attachment to pathogen, secretion of enzymes, parasitism, induction of host defense	Fruit	*Botrytis cinerea* *Penicillium expansum*	Apple	Wisniewski *et al*., 1991
			Penicllium italicum	Citrus	Arras *et al*., 1998
Pichia membranaefaciens	Competition for nutrients and space, attachment to pathogen, induction of host defense	-	*Colletotrichum gloeosporioides*	Citrus	Zhou *et al*., 2016
			Penicillium digitatum	Citrus	Zhou *et al*., 2014

Table 1.5. Lytic enzymes with antifungal effect produced by biocontrol agents.

Enzyme	Biocontrol Agent	Reference
β-1,3-glucanase	*Aureobasidium pullulans*	Castoria *et al*., 2001 Vero *et al*., 2009
	Metschnikowia pulcherrima	Saravanakumar *et al*., 2008
	Pichia anomala	Jijakli & Lepoivre, 1998
	Pichia membranaefaciens	Masih & Paul, 2002
Chitinases	*Aureobasidium pullulans*	Castoria *et al*., 2001
	Cryptococcus albidus	Chan & Tian, 2005
	Metschnikowia pulcherrima	Banani *et al*., 2015 Saravanakumar *et al*., 2009
Proteases	*Aureobasidium pullulans*	Banani *et al*., 2014 Zhang *et al*., 2012
	Metschnikowia pulcherrima	Zhang *et al*., 2010b

To be effective, antibiotics should be produced *in situ*, in sufficient quantities and at precisely the right time in order to interact with the pathogens (El-Ghaouth *et al*., 2002). *Pseudomonas* sp. and *Bacillus* sp. are examples of bacteria genera producers of antibiotics that have been widely studied as BCA (Li *et al*., 2013; Janisiewicz & Roitman, 1988) and also other microorganisms, such as yeasts (Liu *et al*., 2007).

Volatile organic compounds produced by microorganisms are chemicals with low molecular weight and water solubility and high vapor pressure. These characteristics make them especially suitable for biofumigation, which is considered a better alternative because it does not imply a direct contact with food and involves less manipulation of commodities (Jamalizadeh *et al*., 2011; Nunes, 2012). Examples of studies in which volatile compounds produced by BCAs were responsible for fungal inhibition have been described by Di Francesco *et al*., (2015), Fredlund *et al*., (2004), Parafati *et al*., (2015) and Kwasiborski *et al*., (2014).

Induction of host defense

Some antagonists are able to induce defense responses on fruit by means of several mechanisms, including the production of inhibitors of cell-wall degrading enzymes of the pathogen and antifungal substances, such as phenolic compounds or phytoalexins (Droby *et al*., 2002; Spadaro & Gullino, 2004; Wisniewski *et al*., 1991). The induction of host defense has also been related to active oxygen species (Torres *et al*., 2011; Xu *et al*., 2008), changes in gene expression (Jiang *et al*., 2009; Tian *et al*., 2007) and the reinforcement of the host, for instance, through an increase in the cicatrization processes in wounds (Droby & Chalutz, 1994; Spadaro & Droby, 2016).

Biofilm formation

Biofilm formation is a process in which microorganisms form morphologically distinct structures and exhibit altered gene expressions and an enhanced resistance to stresses (Droby *et al*., 2009; Nobile & Mitchell, 2005). In a biofilm, colonies are enclosed in a hydrated matrix of microbe-produced proteins, nucleic acids and polysaccharides (Annous *et al*., 2009). Parsek & Greenberg (2005) illustrated that biofilm formation is closely connected to quorum-sensing regulation, which refers to the phenomenon whereby the accumulation of signaling molecules enables a single cell to sense the number of bacteria and enables the bacteria to coordinate their behavior. There is little information about the role of biofilms in the biocontrol activity of antagonists to manage post-harvest rots, but this has been proposed as an effective mechanism in some antagonistic yeasts (Giobbe *et al*., 2007; Pu *et al*., 2014). It is thought that the presence of biofilm would reduce the amount of physical space available for pathogen development and interfere with the flow of nutrients and/or germination signals from the host to the pathogen (Liu *et al*., 2013).

1.3 Yeasts as biocontrol agents

The role of yeasts as BCAs has gained importance to the detriment of bacteria and fungi, because many yeast species display most of the desirable traits of an ideal antagonist. Yeasts are relatively easy to produce and maintain and have several characteristics that can be modified in order to improve their use and efficiency (Robiglio *et al*., 2011).

Yeasts have simple nutritional requirements, since they can satisfactorily use a wide range of carbohydrates, which include disaccharides and monosaccharides, and nitrogen. They are able to colonize dry surfaces for long periods and resist the extreme environmental conditions that prevail both before and after harvesting (low and high temperatures, desiccation, wide range of relative humidity, low oxygen levels, pH fluctuations and UV radiation). They have low sensitivity to pesticides. Moreover, they can easily grow rapidly on inexpensive substrates in bioreactors and, therefore, can be produced in large quantities. Unlike fungi, they do not produce allergenic spores or mycotoxins. Being a major component of the epiphytic microbial communities on the surfaces of fruit and vegetables, yeasts are phenotypically adapted to fruit. They are able to produce extracellular polysaccharides that can promote adhesion to fruit, enhance their survivability and restrict the growth of pathogen propagules. For all these reasons, yeasts are useful and highly efficient for control purposes (Droby & Chalutz, 1994; Elmer & Reglinski, 2006; Parafati *et al*., 2015; Sharma *et al*., 2009; Spadaro & Droby, 2016).

As illustrated in **Table 1.4**, a number of recent studies have addressed the use of antagonistic yeasts against fungal pathogens in fruit, showing themselves to be highly effective at controlling fungal species, such as *Aspergillus*, *Botrytis*, *Monilinia*, *Penicillium* and *Rhizopus*.

1.3.1 Control of *Botrytis cinerea* with antagonistic yeasts

Botrytis spp. is a filamentous fungus of the *Sclerotiniaceae* family that is able to infect a broad range of hosts, including horticultural and fruit crops of great economic importance (Holz *et al*., 2004). Major post-harvest losses caused by this fungus occur in fresh fruit, such as apple, blackberry, blueberry grape, grape, kaki, kiwi, pear, raspberry and strawberry, among many others. Although *Botrytis* is not the main pathogen, it is also capable of causing considerable post-harvest losses in other fruits, such as apricot, lemon, orange, peach, plum or sweet cherry (Romanazzi *et al*., 2016).

The specie *Botrytis cinerea* is ranked second in the world's Top 10 fungal plant pathogens list, based on scientific and economic importance (Dean *et al*., 2012; Romanazzi *et al*., 2016). The annual economic losses caused by this pathogen in the

viniculture and fruit industries are considerable (Calvo-Garrido, 2013). *Botrytis cinerea*, the causal agent of gray mold or botrytis bunch rot on grapes, is responsible for significant losses in the vineyards of temperate regions worldwide. Depending on the environmental temperature and relative humidity (RH), it could cause the total loss of the crop or affect the sensory attributes of wine when it is produced with 5% of affected grapes (Ky *et al*., 2012; Parafati *et al*., 2015).

It is difficult to control this disease on vines because *B. cinerea* is able to survive saprophytically during the winter in the plant tissues (not only in the green tissue, but also if it is necrotic and senescent) from which, when the environmental conditions are favorable, it can release fresh conidia that provide an abundant inoculum for infection (Calvo-Garrido, 2013; Elmer and Reglinski, 2006). Given that prolonged wet periods during the late season period encourage the development of the pathogen, a common cultural practice has been to reduce moisture in the vineyards. Another more traditional method of managing grey mold on grapes has relied heavily upon the use of synthetic chemicals. This approach has triggered the emergence of fungicide-resistant strains of *B. cinerea* within vineyard populations (Elmer & Reglinski, 2006; Latorre *et al*., 2002; Leroux *et al*., 2002). Therefore, its use is more and more restrictively legislated in order to reduce the presence of residues in wine and table grapes (EC Regulation No. 396/2005).

Among the different alternative strategies developed for the purposes of controlling post-harvest grey mold in grapes and other fruit, the use of antagonistic yeasts has been extensively studied, with *B. cinerea* being one of the main target pathogens of these types of BCAs. Elad & Stewart (2007) reported that the most effective mechanism against this pathogen is competition for nutrients. *B. cinerea* is extremely susceptible to competition for nutrients, the main mode of action of yeasts, because of its dependence of an exogenous supply of nutrients for germination, germ tube growth and infection. On the plant surface, conidia or germ tubes are sensitive to the effect of antibiotics and lytic enzymes that may inhibit germination and lyse germ tubes. Induced resistance may also be effective against infection. Some examples of the control of *B. cinerea* using yeast are listed in Table 1.4. The studies developed by Masih & Paul (2002), Qin *et al*., (2015), Raspor *et al*., (2010), Vargas *et al*., (2012) and Zahavi *et al*.,

(2000) are specific examples of yeasts applied on grapes for the purposes of controlling grey mold.

1.3.2 *Candida sake* CPA-1

Of the different genera of yeasts employed in the BC of *B. cinerea*, *Candida* spp., in particular the strain *Candida sake* CPA-1 has been proved to be effective on grapes and also other fruit and against other pathogens. The potential of *C. sake* CPA-1 as a BCA has been reported by numerous authors, both in laboratory assays and also in field applications (Cañamás *et al*., 2011; Calvo-Garrido *et al*., 2013a; 2013b; 2014a; Teixidó *et al*., 1998a; 1999; Usall *et al*., 2000; 2001; Viñas *et al*., 1998). *C. sake* is a ubiquitous natural organism and a component of the epiphytic community on mature fruits (Usall *et al*., 2000). The CPA-1 strain was isolated from the surface of "Golden Delicious" apples by the Postharvest Pathology Group from the UdL-IRTA Centre in 1990 and deposited in the "Colección Española de Cultivos Tipo" (CECT-10817) in the Universitat de València. Its colonies are creamy white, round in shape and with a slight central elevation. The morphology of a *C. sake* cell is elliptic and its reproduction occurs by multilateral germination.

This strain is able to grow in aerobic conditions and over a wide range of temperatures. For instance, it is very well adapted to cold storage (1°C) but it is capable of multiplying at higher temperatures (34°C) (Calvo-Garrido, 2013). Moreover, it has a very wide tolerance of water activity (a_W) and pH (Teixidó *et al*., 1998b). *C. sake* presents no toxicological risk since it has never been associated with warm-blooded animals (Hurley *et al*., 1987). Additionally, it has been demonstrated that its application in grapes used for vinification did not adversely affect the quality parameters of the obtained wine (Calvo-Garrido *et al*., 2013a).

As it has great potential, *C. sake* has been used in a great number of studies which have aimed to (a) increase its tolerance to adverse conditions - which would increase its applicability – and (b) optimize the formulation and processes by which it is obtained. Both types of studies will be described in more detail in the subsequent sections.

1.4 Enhancement of biocontrol agents

The potential BCAs often show some significant limitations, such as their sensitivity to both adverse environmental conditions and their fluctuations, and their narrow range of activity because BCAs act on specific hosts against well-defined pathogens (Spadaro & Gullino, 2004). For these reasons, the performance of biological-based control strategies in the field is subject to significant variability which constitutes a significant constraint to their practical implementation (Droby *et al*., 2009; Elmer & Reglinski, 2006; Wisniewski et al., 2007). Therefore, a principal goal in the development and implementation of biocontrol products (BCPs) is to improve the ability of antagonists to successfully control post-harvest diseases under a wider array of conditions and with minimal variability (Droby *et al*., 2003). The following approaches may be used to make the BCAs both more efficient and more consistent.

1.4.1 Use of mixed cultures

One of the possible ways of enhancing the efficacy of BCAs is by means of the combination of compatible antagonists, whose joint application may offer improved results. Nunes (2012) and Sharma *et al*., (2009) identified some advantages of this approach: (a) a widening of the spectrum of microbial activity resulting in the control of two or more post-harvest diseases; (b) an increase in the effectiveness under different situations, such as cultivars, maturity stages and locations; (c) an enhancement of the efficiency and reliability of BC as the components of the mixture act through different mechanisms of action; (d) a reduction of the cost of the treatments by reducing the biomass of the antagonist required to achieve an effective control.

The fact that the BC is enhanced by the application of mixtures of antagonists might be due to: a better utilization of the substrate, resulting in an acceleration of the growth rate; the removal of substances inhibitory to one organism by the other microbial agent; the production of nutrients by one microbe that may be used by another; and the formation of a more stable microbial community that may exclude other microbes, including pathogens (Janisiewicz, 1998; Sharma *et al*., 2009).

There are several reports of the successful use of mixtures of bacteria and yeasts, such as *Bacillus mycoides* with *P. guilermondii* (Guetsky *et al*., 2002) and *P. agglomerans* with *C. sake* (Nunes *et al*., 2002a), and different species of yeasts, such as *C. laurentii* with *Rhodotorula glutinis* (Calvo *et al*., 2003) and *C. laurentii* with *M. pulcherrima* (Conway *et al*., 2007).

1.4.2 Physiological and genetic manipulation

It is possible to increase the ecological competence and the capacity to control pathogens of antagonists by genetic and physiological manipulation. The physiological manipulation of BCAs has mainly focused on improving the ecological fitness of antagonists, which is particularly important in field applications where environmental conditions fluctuate widely (Janisiewicz & Korsten, 2002). Some microorganisms possess an inherent ability to adapt to unfavorable environments by the induction of various general and specific stress responses (Usall *et al*., 2009). These stress responses are characterized by the induction of different proteins and by physiological changes that generally enhance the ability of the microbe to withstand more adverse environmental conditions (Ang *et al*., 1991). Subjecting them to mild stress makes cells resistant to a lethal challenge with the same stress condition but can also render cells resistant to other stress-imposing conditions, a phenomenon which is referred to as *cross protection* (Sanders *et al*., 1999; Usall *et al*., 2009).

There are many studies dealing with the physiological manipulation of BCAs, mainly yeasts, by subjecting cells to mild stress levels. This has several purposes, such as the acquisition of tolerance to osmotic stress, high temperatures, low pH, oxidative stress, UV radiation or even low oxygen levels. Teixidó *et al*., (1998a) reported that populations of low a_W tolerant cells of *C. sake* CPA-1 grown on modified media with low $a_{W,}$ and applied to apples 2 days prior to harvest, increased until harvest time, whereas those from non-modified media remained relatively unchanged. Similarly, Abadias *et al*., (2001b) demonstrated that the growth of this BCA in a medium with low a_W led to higher viable counts in liquid media, compared to cells grown in low water activity (0.95) solid medium. Likewise, while working with *C. sake* CPA-1*,* Abadias *et al*., (2003) described that the using isotonic solutions to harvest cells resulted in an increased

retention of compatible solutes, such as trehalose and sugar alcohols. Compatible solutes permit the equilibration of the cytoplasmatic a_W with the surrounding environment, thereby retaining water in the cells and, thus, maintaining turgor pressure and helping to preserve protein function (Usall *et al*., 2009). Similar results have been obtained with other BCAs grown in media with modified a_W, such as *P. anomala* (Mokiou & Magan, 2008) and *P. agglomerans* (Teixidó *et al*., 2005; 2006).

A tolerance to high temperature is also a desirable trait of microbial antagonists, especially for the purposes of obtaining formulations for commercial use, a process which requires the application of in whose high temperatures. For instance, Cañamás *et al*., (2008c) induced thermotolerance in *C. sake* CPA-1 cells by mild heat treatments (30 or 33°C) applied at different phases of growth of the yeast. Liu *et al*., (2011) also induced thermotolerance and tolerance to oxidative stress on *Metschnikowia fructicola* cells by a pre-treatment at 40°C.

Oxidative stress resistance could be an important component of the fitness and suitability of microorganisms for their use as post-harvest BCAs (Sui *et al*., 2015). Liu *et al*., (2012) applied an oxidative stress in form of 5 mM H_2O_2 to *C. olephila*, which increased yeast tolerance to subsequently lethal levels of oxidative stress (50 mM H_2O_2), high temperature (40 °C), and low pH (pH 4).

Adaptation to other stressing environmental conditions, such as UV radiation, low oxygen levels and low pH, have also been described by several authors (Lahlali *et al*., 2011; Li & Tian, 2006; Wang *et al*., 2014).

Current efforts are focused on developing efficient and rapid procedures for tracking antagonists, both pre and post-harvest (Larena & Melgarejo, 2009; Nunes *et al*., 2008; Soto-Muñoz *et al*., 2014). Although fewer in number, several reports may be found dealing with the introduction of genes responsible for either biocontrol activity or for an increase in ecological competence (Janisiewicz *et al*., 2008; Ren *et al*., 2012). However, the use of genetically modified organisms for treating fruit is subject to regulation restrictions that should be taken into account since, in all likelihood, this will be an impediment for the registration of genetically modified BCA (Nunes, 2012).

1.4.3 Combination with additives and natural substances

Different substances, when applied in combination with some microbial antagonists, have demonstrated to improve their performance at controlling fungal pathogens. In this sense, it should be noted that this improvement will depend on the concentration of the antagonist, the concentration of the substance, their mutual compatibility and also for how long and at what time they are applied (Sharma *et al*., 2009).

Of these substances, salt additives, some of them used in the food industry and considered as GRAS (Generally Recognized as Safe by the Food and Drug Administration), have generated great interest. Some examples of these salts are: calcium chloride (Ippolito *et al*., 2005; Yu *et al*., 2012), calcium propionate (Droby *et al*., 2003), potassium sorbate (Karabulut *et al*., 2001) and sodium bicarbonate (Gamagae *et al*., 2003; Qin *et al*., 2015). As regards *C. sake*, Nunes *et al*., (2002b) reported that its combination with ammonium molybdate resulted both in an improvement in its antagonistic activity and in a significant reduction in the amount of the yeast biomass required to achieve post-harvest disease control of pears, with the consequent reduction in costs.

Another substance which has yielded interesting results is 2-deoxy-D-glucose. This glucose analog possesses antifungal properties and, in combination with different antagonists, has showed an enhancement of its biocontrol activity (El-Ghaouth *et al*., 2000b; 2001; Janisiewicz *et al*., 1994). Similarly, salicylic acid, a natural phenolic compound, provided a better control of *B. cinerea* on grape and peach when combined with the BCAs, *H. uvarum* and *R. glutinis*, respectively (Qin *et al*., 2015; Zhang *et al*., 2008); thus, it is a natural substance of great potential in the BC field.

Essential oils (EOs) and their components are also gaining attention due to their volatility and relatively safe status (Di Francesco & Mari, 2014). It has been described that the monoterpene components of EOs can increase the biofilm formation in gram-positive bacteria and, as described above, this mode of action is important for the bacteria's ability to act as BCA (Arrebola *et al*., 2010).

Additionally, numerous studies have reported that the combined application of microbial antagonists and chitosan (CH), an N-acetylated derivative of the polysaccharide chitin, or its derivatives, enhanced the biocontrol activity. There are descriptions of several approaches that have been used to achieve this enhancement. For instance, Yu *et al*., (2007) and Zhou *et al*., (2016) inoculated previously wounded fruits with CH at different concentrations and cell suspensions, separately. Another possible strategy is the incubation of the BCA in a medium amended with CH, as reported by Zhang *et al*., (2014), using *R. mucilaginosa* for the purposes of controlling *Rhizopus* decay and grey mold decay of strawberries. Finally, due to the CH coating-forming ability it is possible to apply it as a layer on fruit and then treat the product with the antagonist, a strategy which will be discussed in the following sections. Regardless of the method employed, since CH is a molecule with antimicrobial properties owing to its unique physiological and biological characteristics, prior to its combination with BCAs, it would be necessary to verify the absence of any toxic effect on the antagonists.

Other natural substances have been proved to be effective at improving the performance of microbial antagonists: gibberellic acid belonging to a family of plant growth regulators (Yu *et al*., 2006; Yu & Zheng, 2007); methyl jasmonate, a volatile compound present in plants (Ebrahimi *et al*., 2012; Guo *et al*., 2014); tea saponin, a tea-seed derived natural surfactant (Hao *et al*., 2011); and nitrogenous compounds, such as L-asparagine and L-proline (Janisiewicz *et al*., 1992).

1.4.4 Combination with coatings

The use of edible coatings (ECs) to protect fruits from fungal decay cannot be considered as a new approach anymore. In fact, there are a great number of studies dealing this topic, in which different matrices and active compounds are used, such as EOs and food preservatives. However, the use of ECs to carry antagonistic microorganisms is an area that has been less widely explored. Since the focus of the present Doctoral Thesis is the incorporation of a BCA into different ECs, this subject will be examined in depth in the following sections.

1.5 Edible coatings

1.5.1 Generalities about edible coatings and films

Although the terms *edible coatings* (EC) and *edible films* may be used synonymously, they refer to different concepts depending on the final purpose and use. González-Martínez *et al*., (2011) and Palou *et al*., (2015) defined films as stand-alone thin layers of biodegradable materials that are applied to the product after their formation. On the other hand, coatings imply the formation of films directly on the surface of the product to which they are to be applied, so that they form part of the final product. Those coatings and films obtained with food-grade ingredients can be eaten as part of the whole product and may be called edible. Therefore, the composition of ECs and films must conform to the regulation that applies to the food product concerned (Guilbert *et al*., 1995).

Obtaining ECs is possible due to the filmogenic capacity of natural biopolymers (Campos *et al*., 2011). Their basic components are typically hydrocolloids (polysaccharides and proteins) and lipids, and these can either be used individually or in combination, in order to obtain composite or blend coatings. The composite coatings take advantage of the specific functional characteristics of each group, reducing their drawbacks (González-Martínez *et al*., 2011; Greener & Fennema, 1994). Other components, such as plasticizers and emulsifiers (or surfactants), may be added to the matrices as a means of improving the flexibility, extensibility and/or the stability of the structure (Palou *et al*., 2015). Moreover, formulations can act as carriers of a very wide range of other minor compounds, such as antioxidants, antimicrobials, certain nutrients like vitamins, volatile precursors, flavors, firming agents and colorants (González-Martínez *et al*., 2011).

Polysaccharides

These hydrocolloids have the ability to form a continuous and cohesive matrix that is related to their chemical structure, which permits the association through hydrogen bonding of their polymeric chains (Campos *et al*., 2011). Vargas *et al*., (2008b) reported that polysaccharides vary according to their molecular weight, degree of branching,

conformation, electrical charge and hydrophobicity. Variations in these characteristics lead to variations in the ability of the different polysaccharides to form coatings, as well as to variations in the physicochemical properties and performance of the coatings formed. Almost all of them contain highly polar monomers with hydroxyl groups and exhibit good oxygen barrier properties (low oxygen permeability, OP), but low moisture barrier properties (high water vapor permeability, WVP) and a high degree of solubility due to their hydrophilic properties (Ortega-Toro *et al*., 2014a). This shortcoming is often solved by blending them with different types of biopolymers (Cano *et al*., 2015; Jiménez *et al*., 2012; Xu *et al*., 2005), adding hydrophobic compounds (Jiménez *et al*., 2010; Ortega-Toro *et al*., 2014a; Rodríguez *et al*., 2006) or the by chemically modifying the polymer structure (Seligra *et al*., 2016; Ortega-Toro *et al*., 2014b).

Starch (S) is the most commonly used natural polysaccharide in the formulation of ECs because it is inexpensive, abundant, biodegradable and easy to use (Vargas *et al*., 2008b). S granules contain two types of polymeric molecules: amylose, a linear chain of (1→4)-α-D-glucopyranosyl units, and amylopectin, a larger molecule which has a backbone of amylose and is highly branched with side units of D-glucopyranosyl linked by α-1,6-glycosidic bonds (Campos *et al*., 2011).

Cellulose derivatives have usually been used as polysaccharide EC components. They are obtained by the chemical substitution of some hydroxyl groups along the cellulose chain, which leads to the formation of ionic (carboxylmethylcellulose, CMC) and non-ionic (methylcellulose, MC; hydroxyprophylmethylcellulose, HPMC) cellulose ethers (Campos *et al*., 2011).

Chitosan (CH) is another interesting polysaccharide for the purposes of EC formulation. It is obtained from the deacetylation of chitin (poly-β-(1→4)-N-acetyl-D-glucosamine), which is a major component of the shells of crustaceans (Campos *et al*., 2011; Vargas *et al*., 2008b). CH shows antifungal and antibacterial properties, which are believed to originate from its polycationic nature (Cuero, 1999; Tharanathan & Kittur, 2003).

Other polysaccharide materials typically used to formulate ECs and films also include alginates, pectins and various gums. Additionally, coatings and films based on

polysaccharides produced by microorganisms, such as pullulan and polyhydroxyalkanoates, have also been developed.

Proteins

The ability of proteins to form ECs or films is highly dependent on their molecular characteristics, namely their molecular weight, conformation, electrical properties, flexibility and thermal stability (Vargas *et al*., 2008b). Protein-based ECs usually exhibit good gas barrier characteristics but, like polysaccharide-based ECs, due to their predominantly hydrophilic nature, they present poor water barrier characteristics (Lacroix & Vu, 2014; Palou *et al*., 2015). Nevertheless, proteins have a unique structure which confers a range of functional properties, especially a high intermolecular binding potential (Campos *et al*., 2011). The different approaches used to improve the barrier properties of ECs and films based on proteins have been reviewed by Bourtoom (2009). These methods include the modification of the properties of protein by chemical and enzymatic methods (Audic & Chaufer, 2005; Insaward *et al*., 2015; Masamba *et al*., 2016; Yildirim *et al*., 1996), the combination with hydrophobic materials (Fabra *et al*., 2009; 2011; Pérez-Gago *et al*., 2001; Rezvani *et al*., 2013) or some other polymers (Yoo & Krochta, 2011) and the use of physical methods in order to modify their structure (Benbettaïeb *et al*., 2016; Ouattara *et al*., 2002).

Milk is a habitual source of proteins, with interest in the area of ECs, given that, apart from their film-forming ability, milk proteins can provide a high nutritional added value (Campos *et al*., 2011). For instance, sodium caseinate (NaCas), obtained by the acid precipitation of casein (Audic & Chaufer, 2005), has been widely studied as a film-forming protein (Arrieta *et al*., 2013; Atarés *et al*., 2010; Fabra *et al*., 2009; 2011). Many studies have also studied the properties of films based on whey protein (Pérez *et al*., 2016a; Pérez-Gago *et al*., 2001; Yoo & Krochta, 2011).

Fewer studies deal with the use of proteins of vegetal origin, even though legume seeds are a cheap source of proteins with a high nutritional value and, hence, an interesting raw material for the purposes of producing protein isolates (Sánchez-González *et al*., 2013). Of the vegetal proteins from legumes, some studies can be found with pea

protein (PP) (Choi & Han, 2001; Kowalczyk *et al*., 2011, Pérez *et al*., 2016b, Fabra *et al*., 2014).

Other examples of proteins used to obtain ECs and films are soy protein, proteins from other legumes, such as lentils or kidney beans, wheat gluten, corn zein, rice bran protein and gelatin (Fakhouri *et al*., 2015; Ma *et al*., 2013; Masamba *et al*., 2016; Monedero *et al*., 2009; Rocca-Smith *et al*., 2016).

It should be noted that some proteins of potential use as major compounds of ECs may be responsible for allergies and intolerances in some people, a serious disadvantage that could restrict the use of protein-based ECs.

Composites

Since lipids are not polymers, they form films and coatings with poor mechanical properties and opaque characteristics (Palou *et al*., 2015; Rhim & Shellhammer, 2005). However, due to their low affinity for water, lipids exhibit low WVP (Vargas *et al*., 2008b). For these reasons, considerable research efforts have focused on the design of ECs and films based on the combination of lipids and hydrocolloids, which take advantage of the most desirable properties of each compound. Generally, lipids contribute to the improvement of the water vapor resistance, whereas hydrocolloids confer selective permeability to O_2 and CO_2, durability and structural cohesion and integrity (Krochta 1997, Vargas *et al*., 2008a). In these types of coatings, the efficiency of the lipid material depends on several attributes, including the lipid structure, its chemical arrangement, hydrophobicity, physical state and interaction with other components of the coating (Rhim & Shellhammer, 2005).

Composite coatings can be obtained as both bilayer composites or as stable emulsions. In bilayer composites, the lipid forms a layer over the previously formed hydrocolloid layer, whereas in emulsion coatings, the lipid is dispersed in a supporting hydrophilic phase of polysaccharides or proteins (Palou *et al*., 2015; Pérez-Gago & Rhim, 2014; Vargas *et al*., 2008b). Although emulsified coatings are less effective as a result of the non-homogeneous distribution of the lipids, they have received more attention since they need only one application step (Vargas *et al*., 2008b).

Reviewing the literature, it can be seen that many lipids have been used to formulate composite coatings, such as beeswax, candelilla wax, carnauba wax, triglycerides, acetylated monoglycerides, fatty acids, fatty alcohols, sucrose fatty acid esters and other surfactants (Fabra *et al*., 2009; 2011; Jiménez *et al*., 2010; Masamba *et al*., 2016; Navarro-Tarazaga *et al*., 2008a; 2008b; Ortega-Toro *et al*., 2014a; Vargas *et al*., 2009; Villalobos-Carvajal *et al*., 2009).

Plasticizers

Plasticizers are low molecular weight compounds of high polarity due to the fact they have a large number of well spaced-out polar groups (Palou *et al*., 2015). They are incorporated into ECs formulations in order to decrease the intermolecular forces between the polymer chains, which results in greater flexibility, elongation, and toughness but also in significant changes in the barrier properties (García *et al*., 2000; Navarro-Tarazaga *et al*., 2008a; Palou *et al*., 2015; Rodríguez *et al*., 2006). Therefore, plasticizers are particularly suited to form stable emulsions and improve mechanical properties when hydrocolloids are combined. Glycerol, polyethylene glycol, propylene glycol, sorbitol and sucrose are plasticizers commonly used in the formulation of ECs.

Emulsifiers or surfactants

Emulsifiers or surfactants are amphiphilic substances, having a lipophilic part, which tends to be in a non-polar environment, and a hydrophilic part, with a preference for a polar (aqueous) environment (Stauffer, 1999; Rodríguez *et al*., 2006). This property endows surfactants with the ability to interact at the water-lipid interface and reduce surface tension between the dispersed and continuous phases (Han & Gennadios, 2005; Palou *et al*., 2015). The balance among the two parts of these molecules, called hydrophilic/lipophilic balance (HLB), governs its functionality and influence on the properties of the resulting coatings (Rodríguez *et al*., 2006).

When incorporated into ECs, these ingredients promote good surface wetting, spreading and adhesion to the food surface (Hiemenz & Rajagopalan, 1997; Palou *et al*., 2015; Rodríguez *et al*., 2006). Some typical emulsifiers or surfactants used in ECs are

esters of fatty acids, ethylene glycol monostearate, fatty acids, glycerol monostearate, lecithin, polysorbates, sorbitan monostearate and sucrose esters.

1.5.2 Application of edible coatings on fruit

In nature, fruits and vegetables are coated by a natural waxy coating called cuticle. It consists of a layer of fatty acid-related substances, such as waxes and resins, of low water permeability, which help to prevent moisture loss, especially in the dry humid season (Baldwin, 1994; Tharanathan, 2003; Vargas *et al*., 2008a). The application of commercial coatings is a common practice for many fruits. These coatings are generically known as waxes, since their composition is based on paraffin wax or a combination of various other waxes, such as beeswax or carnauba. They are anionic microemulsions that may also contain synthetic components, such as polyethylene and petroleum waxes, ammonia or morpholine. They are applied to reduce fruit weight loss and shrinkage while improving their appearance and physical resistance. Furthermore, commercial waxes are often amended with synthetic fungicides in order to control post-harvest diseases (Palou *et al*., 2015).

Due to the potential hazards of synthetic coatings, such as the presence of potentially toxic substances on the fruit surface, the use of ECs as a replacement for these currently-used commercial waxes has been widely studied. When applied to fruit, ECs extend their shelf-life by acting as a barrier to water and gases related with fruit ripening. ECs also prevent physical damage, improve the product appearance and contribute to a reduction in the amount of synthetic packaging wasted as, in some cases, they can substitute the conventional plastics (González-Martínez *et al*., 2011).

Additionally, ECs may be used as carrier matrices of bioactive compounds to enhance the safety and the quality of fruit (Quirós-Sauceda *et al*., 2014). Bioactive compounds are non-nutritional constituents that typically occur in small quantities in foods and can be obtained by extraction from plants, animals, marine organisms and microorganisms and by biotechnological methods (Kris-Etherton *et al*., 2002; Quirós-Sauceda *et al*., 2014). Bioactive compounds can be transported from the ECs to the fruit skin by diffusion release, which is controlled by their solubility and permeability in the

structural matrix (Figure 1.2). It is possible to control this release as a function of time by means of encapsulation techniques (González-Martínez *et al*., 2011)

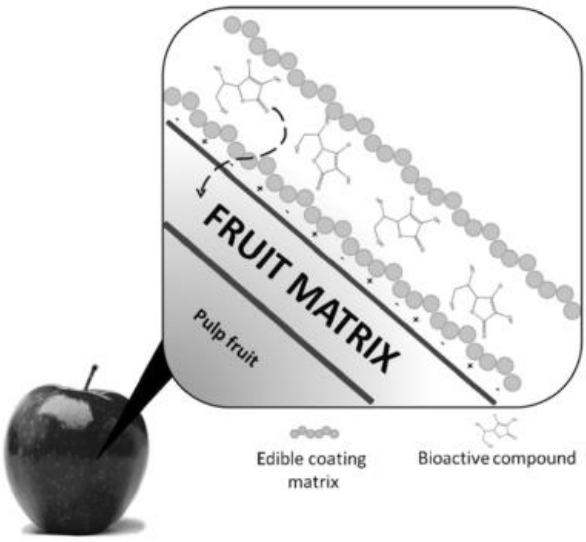


Figure 1.2. Release of bioactive compounds from edible coatings to the fruit (Quirós-Sauceda *et al*., 2014)

Antioxidants, antimicrobials, probiotics, flavors and nutraceutical substances are the most widely used bioactive compounds (Quirós-Sauceda *et al*., 2014).

Polysaccharides are the most commonly used components in fruit ECs, probably due to their better microbial and physical stability over time in comparison with protein-based coatings, especially in high RH environments (González-Martínez *et al*., 2011). Other compounds which are commonly used in fruit ECs are lipids, which have low WVP and are very useful for controlling their desiccation (Morillon *et al*., 2002; Vargas *et al*., 2008a). In fact, TAL-Prolong and Semperfresh are two commercially available composite coating formulations based on CMC, sucrose fatty acid ester, sodium salt and an emulsifier, used for the shelf-life extension of banana and other fruit (Nisperos-Carriedo *et al*., 1992; Tharanathan, 2003).

Intensive research has been devoted to the application of ECs as a means of improving the quality and shelf-life of fruit. For instance, Fakhouri *et al*., (2015) studied the effect of ECs based on starch and gelatin on the quality of grapes; Nadim *et al*., (2015) applied MC-based coatings to strawberries for the purposes of studying their quality throughout storage; and Muangdech (2016) developed ECs based on aloe vera gel,

chitosan and carnauba wax to study the post-harvest storage life of mango. These are only some recent examples of the numerous studies published on this topic

1.5.3 Antifungal edible coatings applied to fruit

As far as the prevention of microbial decay is concerned, especially that caused by fungi in fruit, ECs and films based on the biopolymer components described above (with the exception of CH) are not capable of accomplishing this task. Hence, in order to obtain ECs with antifungal properties, food-grade antimicrobial agents have to be incorporated into the formulations (Liu, 2009; Palou *et al.*, 2015). In this sense, the use of ECs containing antimicrobial substances may be more efficient than the direct addition of antimicrobial agents to the food, given that active compounds may selectively and gradually migrate from the coating onto the surface of the fruit, helping to maintain a high concentration of bioactive compounds where needed (Elsabee & Abdou, 2013; Perdones, 2015; Quirós-Sauceda *et al.*, 2014).

According to Palou *et al.*, (2015), the antifungal compounds that can be incorporated into ECs might be classified in the following categories: (a) synthetic food preservatives or GRAS compounds with antimicrobial activity, which include some organic and inorganic acids and their salts (benzoates, carbonates, propionates or sorbates) and parabens (ethyl and methyl parabens) and their salts, among others; (b) natural compounds, such as EOs or other natural plant extracts (capsicum, carvacrol, cinnamon, cinnamaldehyde, citral, eugenol, grape seed extracts, lemongrass, propolis extract, oregano, rosemary, thyme oil, vanilla, vanillin, etc.); (c) antimicrobial antagonists, such as BCA. Table 1.6 summarizes several examples of the application of ECs based on different matrices and antifungal components as well as the main effects they have on the quality parameters of fruit and the controlled microorganisms, when available.

1.5.4 Edible coatings containing biocontrol agents

In comparison with the large number of studies dealing with the incorporation of antifungal compounds into ECs for fruit applications, there is little information about coatings including antifungal microbial antagonists for the purposes of controlling fruit pathogens. Some studies were published in the 1990s, but there has been little recent

research. This approach, consisting of the combination of two methods as a means of preserving fruit from fungal decay has proven to be effective. This effectiveness is attributed to the advantages of both methodologies. On the one hand, the ability of the BCA to inhibit fungal development endows the EC with antimicrobial properties. On the other hand, the joint application with the EC improves the adherence and survival of the BCA on the fruit surface, and therefore, of its antagonistic activity. This strategy has been studied and developed in the present Doctoral Thesis.

More concretely, the potential benefits for the BCAs that can be derived from their combined application with ECs may be their performance as binding elements on the fruit surface, sources of nutrients and protector against ultraviolet (UV) radiation, desiccation, and rain and temperature variations in the field (Marín *et al*., 2016; Potjewijd *et al*., 1995). It has also been reported that coating-forming agents can improve the stability and dispersability of cell suspensions, which could allow for a more homogeneous spatial distribution on the fruit surface, and may slow down the microbial desiccation when applied, thereby extending the time available for the BCA to multiply and become established (Cañamás *et al*., 2011; Wang *et al*., 2011). Moreover, ECs can also exert a direct effect against a pathogen both via their intrinsic antifungal properties, as in the case of CH, or by acting as a mechanical barrier to protect fruit from pathogens (Chien *et al*., 2007; Meng *et al*., 2010).

Two possibilities might be distinguished as regards the technique whereby the combined application of ECs and BCAs takes place. Some authors, such as Meng *et al*. *al*., (2010) and Rahman *et al*., (2009) have reported the separate application of the EC and the BCA suspension. In these cases, the microbial antagonists might be applied before or after coating. Another option is the incorporation of the BCA directly into the coating forming dispersion (CFD) and then their combined application. This strategy simplifies the process because it permits the application of the treatment in only one step (McGuire & Baldwin, 1994).

Some examples of published studies that have developed the combined application of edible and commercial coatings containing antagonistic yeasts are summarized and shown in Table 1.7. In every case, it was possible to observe an enhancement of the cell

Table 1.6. Summary of edible coatings with antifungal effects applied to fruit. CH: chitosan, CMC: carboxymethylcellulose; EOs: essential oils; MC: methylcellulose; PS: potassium sorbate; SB: sodium benzoate; SP: sodium propionate; SEP: sodium ethyl paraben; SMP: sodium methyl paraben.

Matrix	Antimicrobial agent	Application	Pathogen	Main beneficial effects	Reference
CH	-	Apple	*Penicillium expansum*	-	Li *et al*., 2015
CH	-	Orange	*Penicillium digitatum* *Penicillium italicum*	Preservation of firmness, titratable acidity, ascorbic acidity and water retention	Chien *et al*., 2007
CH	Bergamot, tea tree, thyme EOs	Orange	*Penicillium italicum*	Preservation of firmness and slowing down of weight loss	Cháfer *et al*., 2012
CH	Citral, lemongrass EO	Lime and orange	*Penicillium digitatum* *Penicillium italicum*	-	El-Mohamedy *et al*., 2015
CH	Clove EO	Mandarin	*Penicillium digitatum*	Preservation of water retention, pH	Shao *et al*., 2015
CH	Lemon EO	Strawberry	*Botrytis cinerea*	Slowing down of respiration rate	Perdones *et al*., 2012
CH	Thyme or cinnamon EOs	Strawberry	*Botrytis cinerea*	-	Mohammadi *et al*., 2015
CH, aloe vera	-	Blueberry	*Botrytis cinerea*		Vieira *et al*., 2016
CH, aloe vera, arabic gum	Thyme EO	Avocado	*Colletotrichum gloeosporioides*	Slowing down of weight loss and preservation of firmness and flesh color	Bill *et al*., 2014
CH, HPMC	Bergamot EO	Grape	Molds, yeasts, aerobic mesophilic	Preservation of firmness and water content and slowing down of respiration rate	Sánchez-González *et al*., 2011
CH, HPMC	PS	Strawberry	*Cladosporium* sp. *Rhizopus* sp.	Slowing down of weight loss	Park *et al*., 2005

CH + locust bean gum	Citrus EOs	Date	*Aspergillus flavus*	-	Aloui *et al*., 2014b
CH + oleic acid + Tween 80	-	Strawberry	Molds	Preservation of firmness and slowing down of respiration rate	Vargas *et al*., 2006
CMC + CH, MC + CH	-	Strawberry	-	Slowing down of weight loss and preservation of total soluble solids, pH, titratable acidity, ascorbic acid content, phenolics and anthocyanins	Gol *et al*., 2013
HPMC	Propolis extract	Grape	Molds, yeasts, aerobic mesophilic	Slowing down of respiration rate and weight loss	Pastor *et al*., 2011
HPMC + beeswax	$(NH_4)_2CO_3$, NH_4HCO_3, $NaHCO_3$	Plum	*Monilinia fructicola*	-	Karaca *et al*., 2014
HPMC + beeswax	SMP, SEP, SB	Cherry tomato	*Alternaria alternata*	Preservation of firmness and slowing down of respiration rate and weight loss	Fagundes *et al*., 2015

Table 1.6. Continued.

HPMC + beeswax, shellac	SB, SP, PS	Mandarin and orange	*Penicillium digitatum* *Penicillium italicum*	-	Valencia-Chamorro *et al*., 2009
Sodium alginate	Carvacrol, methyl cinnamate	Strawberry	*Botrytis cinerea*	-	Peretto *et al*., 2014
Sodium alginate	Grapefruit EO or seed extract	Grape	*Penicillium digitatum*	Preservation of firmness and water content and slowing down of weight loss	Aloui *et al*. 2014a
Sodium alginate	Lemongrass EO	Fresh cut pineapple	Molds, yeasts, aerobic mesophilic	Preservation of firmness and sensory attributes and slowing down of respiration rate and weight loss	Azarakhsh *et al*., 2014

Sodium alginate, pectin	Citral, eugenol	Strawberry	Molds, yeasts, aerobic mesophilic and psychrophilic	Preservation of firmness and color and slowing down of weight loss	Guerreiro *et al.*, 2015
Starch	-	Strawberry	-	Preservation of firmness and slowing down of weight loss	García *et al*., 1998
Starch + CH	EOs mixture	Guava			de Aquino *et al*., 2015
Starch + gum	Ascorbic acid, $CaCl_2$, cinnamon oil	Fresh-cut apple	Molds, yeasts, aerobic mesophilic and psychrophilic	Preservation of firmness, delay of browning, respiration rate and CO_2 and ethylene production	Pan *et al*., 2013
Starch, guar gum	PS	Apple	*Cladosporium herbarum*	-	Mehyar *et al*., 2011
Pectin	Cinnamon leaf EO	Grape	*Botrytis cinerea*	-	Melgarejo-Flores *et al*., 2013
Pectin	PS, SB	Fresh-cut persimmon	Molds, yeasts, aerobic mesophilic and psychrophilic	Delay of browning	Sanchís *et al*., 2016
Pectin + beeswax	-	Avocado	*Lasiodiplodia theobromae*	Slowing down of changes in color and texture	Maftoonazad *et al*., 2007
Pullulan	-	Strawberry	Molds	Preservation of firmness, ascorbic acid and carotenoids and slowing down of weight loss	Eroglu *et al*., 2014
Pullulan	Sweet basil extract	Apple	*Rhizopus arrhizus*	Slowing down of weight loss and changes in color and soluble solids	Synowiec *et al*., 2014
Quinoa protein + CH + sunflower oil	-	Blueberry	Molds and yeasts	Preservation of firmness	Abugoch *et al*., 2016
Xanthan gum	Cinnamic acid	Fresh cut pear	Molds, yeasts, aerobic mesophilic and psychrophilic	Delay of browning	Sharma & Rao, 2015

Table 1.7. Edible coatings containing biocontrol agents, dose of application, fruit applied to and target pathogens inhibited. CMC: carboxymethylcellulose; HPC: hydroxypropylcellulose; MC: methylcellulose

Coating	Coating %	Biocontrol agent	CFU/ml	Fruit	Pathogen	Reference
CMC, HPC, MC	2	*Candida guilliermondii* *Debaromyces* spp.	$2.3 \cdot 10^8$ $1.7 \cdot 10^8$	Orange	*Geotrichum candidum* *Penicillium digitatum* *Penicillium italicum*	Potjewijd *et al*., 1995
CMC-sodium	0,3	*Rhodosporidium paludigenum*	10^8	Jujube	*Alternaria alternata*	Wang *et al*. ,2011
Cellulose, shellac, sucrose ester	-	*Candida oleophila* *Pseudomonas* spp.	$4 \cdot 10^8$ $8 \cdot 10^9$	Grapefruit	-	McGuire, 2000
Commercial EC (Fungicover®)	5	*Candida sake*	$5 \cdot 10^7$	Grape	*Botrytis cinerea*	Cañamás *et al*., 2011* Calvo-Garrido *et al*., 2013a*
	5	*Pantoea agglomerans*	$2 \cdot 10^8$	Orange	*Penicillium digitatum*	Cañamás *et al*., 2008b *
Commercial fruit waxes	20	*Pichia guilliermondii*	10^8	Orange	*Penicillium italicum*	Lahlali *et al*., 2014
Glycolchitosan	0,2	*Candida saitoana*	10^8	Apple, citrus	*Diplodia natalensis* *Penicillium digitatum* *Penicillium italicum* *Phomopsis citri*	El-Ghaouth *et al*., 2000a; 2000b

HPC, MC	4, 2	*Candida oleophila* *Cryptococcus albidus* *Rhodotorula mucilaginosa*	$4 \cdot 10^8$	Grapefruit	-	McGuire & Baldwin, 1994
HPMC, starch, sodium caseinate, pea protein	2	*Candida sake*	$5 \cdot 10^7$	Grape	*Botrytis cinerea*	Marín *et al*., 2016
Locust bean gum	0.5, 1	*Aureobasidium pullulans* *Metschnikowia pulcherrima* *Wickerhamomyces anomalus*	10^9	Mandarin	*Penicillium digitatum* *Penicillium italicum*	Parafati *et al*., 2016
Shellac	-	*Candida oleophila*	$4 \cdot 10^8$	Grapefruit	*Penicillium italicum*	McGuire & Dimitriglou, 1999
Sodium alginate	2	*Cryptococcus laurentii*	10^9	Strawberry	Non specified	Fan *et al*., 2009
Sodium alginate, locust bean gum	2, 1	*Wickerhamomyces anomalus*	10^7	Orange	*Penicillium digitatum*	Aloui *et al*., 2015

* Field application

yeast viability on the fruit surface and an increased control of the pathogens, in some cases even in applications carried out in the field.

1.6 Formulation of biocontrol agents

The formulation of BCAs into ECs can enhance their efficacy, extend the range of conditions over which are effective, increase their ability to withstand drastic changes in the phyllosphere and improve their survival under unfavorable microclimatic conditions (Cañamás *et al*., 2011). In this sense, the formulation process is decisive if these improvements are to be made and also has a significant influence on the successful delivery of the antagonists, the shelf-life and storage requirements of the BCP and on its cost (Janisiewicz & Korsten, 2002; Spadaro & Gullino, 2004; Yánez-Mendizábal *et al*., 2012c).

In comparison with the large number of effective antagonists under laboratory conditions, the success of formulated BCA-based products has been limited and just a few products have reached advanced stages of development and commercialization. Generally, information on the specific composition and production of formulations of commercial BCA is largely proprietary. Table 1.8 summarizes examples of different developed BCP, although some of them are not currently in the market.

Some of the reasons for the limited success of BCA-based commercial products are the inconsistency and variability of the efficacy under commercial conditions, the narrow tolerance to fluctuating environmental conditions of the BCAs and the difficulties in developing shelf-stable formulated products that retain a biocontrol activity similar to that of the fresh cells (Janisiewicz & Jeffers, 1997; Usall *et al*., 2009). Another drawback is the difficulty involved in the market penetration and perception of the customers/industry and small-sized companies whose available resources are too low to maintain development and commercialization (Spadaro & Droby, 2016).

The first step of the formulation process is the mass production of the BCA. Mass production aims to obtain the greatest quantity of efficacious cells in the shortest time, at a low price and maintaining biocontrol efficacy (Teixidó *et al*., 2011; Yánez-Mendizábal *et al*., 2012b). The nutrient for the growth medium must satisfy the

requirements for cell biomass and metabolite production by providing an adequate supply of energy for biosynthesis and cell maintenance (Stanbury *et al*., 1995).

Table 1.8. Commercial biocontrol products.

Product	Biocontrol agent	Application
AQ-10-biofungicide™	*Ampelomyces quisqualis*	Apple, curcubits, grape, strawberry,
Aspire™	*Candida oleophila*	Apple, citrus, pear
Binab™	*Trichoderma harzianum, T. polysporum*	Strawberry
Bio-Save 10LP, 11LP, 110™	*Pseudomonas syringae*	Apple, citrus, cherry, pear, potato
BlightBan A506™	*Pseudomonas fluorescens*	Apple, pear, potato, strawberry
BoniProtect™	*Aerobasidium pullulans*	Apple
Botry-Zen™	*Ulocladium oudemansii*	Grape
Candifruit™	*Candida sake*	Apple
Mangogreen™	*Bacillus licheniformis*	Mango
Nexy™	*Candida oleophila*	Banana, citrus, pome fruit
Serenade™	*Bacillus subtilis*	Apple, grape, pear, vegetables
Shemer™	*Metschnikowia fructicola*	Apricots, citrus, grape, peach, strawberry
TRICHODEX™	*Trichoderma harzianum*	Grape
Yieldplus™	*Cryptococcus albidus*	Pome fruit

In this sense, the use of agricultural waste and by-products from the food industry, such as nitrogen and carbon sources, has attracted considerable interest. These substrates meet most of the above criteria for production media and, in addition, are cheap. In general, yeasts and bacteria are produced by liquid fermentation, whilst fungi are produced by solid fermentation (Droby *et al*., 2016).

A biocontrol product (BCP) could be defined as a mixture of the active ingredient (BCA), a carrier providing physical support of the microorganism, and different adjuvants or protectants. Depending on the physical state of the carriers, BCPs may be liquid or dry. It is common practice to incorporate adjuvants and/or protectants as a means of achieving several purposes. These additives can be incorporated at different points, such as in the mass production, formulation and storage steps or just before the application in the mixing tanks (Burges, 1998: Cañamás *et al*., 2008a). Additives can be used as stickers, diluents, suppressants, dispersants, emulsifiers, wetters, gelants, humectants, brighteners, spreaders, stabilizers, sunscreens, synergists, thickeners, nutrients, binders, or protectants, depending on their function in the formulation (Burges & Jones, 1998; Cañamás *et al*., 2008a). As previously described, some of these functions can be accomplished by ECs.

According to Melin *et al*., (2007) an ideal BCP should satisfy a set of criteria. It should: be inexpensive to produce, be easy to distribute to the intended environment, have a long shelf-life, preferentially also upon storage at ambient temperature and be easily rehydrated (in the case of dry formulations). Finally, the biocontrol activity must be maintained through all the formulation steps, long-term storage and rehydration.

1.6.1 Liquid formulations of biocontrol agents

Liquid formulations are also known as flowable or aqueous suspensions and consist of biomass suspensions in water or oils, or combinations of both (emulsions) (Schisler *et al*., 2004). They are the simplest way to stabilize the viability of microbial cells, but require refrigeration (Droby *et al*., 2016).

In formulations based on water acting as the carrier of the BCA cells, different substances may be incorporated to adjust the a_W and obtain the same water chemical potential of the cells (isotonic solutions). Examples of the liquid formulation of *C. sake* have been reported by Abadias *et al*., 2003 and Torres et al., (2003). These authors observed that, under storage at 4°C, the liquid formulation of this yeast in isotonic solutions retained its viability and efficacy for 7 months and that the addition of protective substances had a beneficial effect on its viability. The best formulations were phosphate buffer supplemented with either 5% trehalose or 10% lactose. Furthermore,

Cañamás *et al.,* (2011) reported that the formulation of *C. sake* in isotonic solutions combined with an EC resulted in a *Botrytis* spp. control similar to that obtained by fungicide treatment. *C. sake* was developed in the form of a liquid BCP called Candifruit™ for use on pome fruit and grape. After being commercialized for some years, it was discontinued due to business and marketing-related shortcomings (Droby *et al*., 2016).

Similarly, other published studies have aimed to obtain liquid formulations of other BCAs. For instance, Melin *et al*., (2006; 2011) developed liquid formulations of *P. anomala* with a long shelf-life using trehalose as protectant, and Liu *et al*., (2009) reported an enhanced viability of *C. laurentii* and *P. membranaefaciens* in liquid formulations containing trehalose once again, and galactose.

Oils, or combinations of water and oils (emulsions), can also be used as support for the antagonistic cells. For instance, Batta (2004; 2007) developed an inverted emulsion (water-in-oil formulation), based on coconut and soybean oils, aiming to obtain a suitable formulation of *T. harzianum* conidia that was found to be effective in managing the pathogens *R. stolonifer*, *B. cinerea* and *P. expansum* on a variety of fresh fruits. Kim *et al*., (2005) developed a water-in-oil-in-water multiple emulsion to improve the storage stability of the antagonistic microorganism *Burkholderia gladioli*, and stability tests revealed that the viability of the formulated cells was greater than that of the unformulated ones. More recently, Peeran *et al*., (2014) obtained a formulation of the antagonist *P. fluorescens* based on an emulsion containing soybean and castor oils, among other ingredients, which allowed for an extended shelf-life.

1.6.2 Dry formulation of biocontrol agents

In general, dry formulations weigh less, have a longer shelf life and exhibit a lower risk of contamination than liquid ones, and allow for easier transport, distribution, storage and manipulation (Costa, 2012; Fravel, 2005). However, they also present some shortcomings, such as a marked loss of viability in the cells not only during dehydration and storage but also during the subsequent rehydration process (Melin *et al*., 2006).
Dry formulations of BCAs take several forms. Wettable powders consist of dry inactive and active ingredients (BCA cells) intended to be applied as a suspension in liquid. Dusts

are powder-like and consist of dry inactive and active ingredients to be applied dry, generally to seeds or foliage. Granules are described as free-flowing aggregated products composed of dry inactive and active ingredients (Schisler *et al*., 2004). Dry formulations can be applied directly to the target plant or, in the case of wettable powders and water dispersible granules, mixed into water where the suspension of biomass and inactive ingredients are applied as a spray.

Carriers of biocontrol agents in dry formulations

The inactive ingredients of dry formulations act as carriers of the antagonist and may be organic (grain flours, powders from plants, starches and their derivatives, etc.) or mineral (peats, talc, diatomaceous earths, kaolin, clay, etc.) (Brar *et al*., 2006; Lee *et al*., 2006; Mokhtarnejada *et al*., 2011; Schisler *et al*., 2016).

It is possible to use EC-forming agents as carriers in the formulation of dry BCP. In this approach, the EC compound would firstly act as support for the BCA cells during the drying and storage steps and, when applied, the EC would provide the BCA with the previously described benefits, such as an improved adherence and survival on the fruit surface or a source of nutrients. Working along these lines, wettable powders and water dispersible granules would be the most adequate presentations, since water is necessary to form the ECs.

There are few publications on the use of EC-forming agents as support for microbial antagonist based formulations and, in most cases, different kinds of starch and the derivatives are the ingredients used. This is because production cost is a key factor that must be borne in mind and kept to a minimum (Melin e*t al*., 2011), and starch is low cost and readily available (Vargas *et al*., 2008b). Lewis *et al*., (1995) obtained granular formulations using pre-gelatinized starch (PG) and the biocontrol fungus *Gliocladium virens*, whose viability was maintained for 6 months at 5°C. Similarly, Mounir *et al*., (2007) used maize starch to produce a formulation of *A. pullulans* by means of fluidized-bed drying (FBD) and observed that a drop in viability was observed in the first 30 days but, after that period, the viability remained constant for 7 months at 4°C. Soto-Muñoz *et al*., (2015) studied different dry formulations of *P. agglomerans*, one of which was obtained by means of FBD using potato starch as carrier.

Dehydration as a means of obtaining dry formulations

One of the best ways to formulate dry BCAs is by dehydrating them and subsequently keeping them in a dry environment, since it allows the products to be handled using the normal distribution and storage channels (Rhodes, 1993; Usall *et al*., 2009). Nevertheless, not all microorganisms are amenable to drying and many tend to lose viability during both the drying process and subsequent storage. The reduction in the survival time is related to the damage that is done to a large number of cellular components, including DNA, RNA, cytoplasmic membrane and cell wall, during the drying processes (Santivarangkna *et al*., 2008a; Silva *et al*., 2011; Soto-Muñoz *et al*., 2015). For that reason, drying is often a very critical step and many approaches have been developed in order to reduce the losses in viability, such as adding protectants to growth media or directly to cells (Abadias *et al*., 2001a; Pusey & Wend, 2012; Schisler *et al*., 2016; Yánez-Mendizábal *et al*., 2012b). Of the protectant agents, skim milk and sugars, used either alone or in combination, have been widely used because of their relatively low prices and chemically innocuous nature (Costa *et al*., 2002a; Khem *et al*., 2015; Santivarangkna *et al*., 2008b). Sugars, especially disaccharides, are able to protect the cell membranes from dehydration (Leslie *et al*., 1995). The proteins present in milk provide a protective coat for the cells and seem to restore injured cells during dehydration, avoiding osmotic shock, disruption and the death of cells (Champagne *et al*., 1991; Guijarro *et al*., 2006).

Subjecting cells to mild heat treatments or osmotically unfavorable conditions during production in liquid culture is another approach that has been proven to be effective at improving cell survival throughout dehydration processes (Cañamás *et al*., 2008c; Torres *et al*., 2014).

The general objective of drying BCAs is to enable storage over extended periods of time whilst preserving the viability of the cell and its effectiveness against pathogens, and also to ensure the retrieval of its metabolic activity and biological properties upon rehydration (Fu & Chen, 2011; Melin *et al*., 2007; Morgan *et al*., 2006). The classical dehydration processes are freeze-drying, spray-drying and fluidized-bed drying. Although these methods present several differences, there are certain phases generic

to all drying methodologies, as outlined in **Figure 1.3**. These techniques change the physical state of water by varying the temperature or pressure or by the combined effect of both parameters. Hence, cells dehydrated in these ways are subjected to simultaneous temperature and a_W stress, which can enhance their tolerance to unfavorable situations (Teixidó *et al*., 2006).

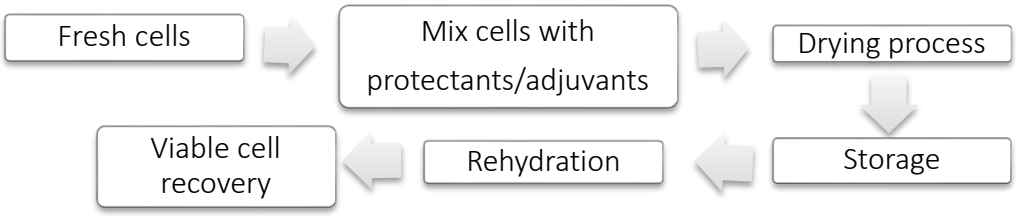


Figure 1.3. Generic drying process layout (adapted from Morgan *et al*. 2006)

Spray-drying consists of the removal of almost all the liquid, by vaporization, from a solution of a non-volatile solid (Santivarangkna *et al*., 2007; Silva *et al*., 2011). Generally, heat is applied as a heated atmosphere and evaporation is promoted by spraying the liquid feed into this atmosphere. This technique offers high production rates and low operating costs (Corcoran *et al*., 2004). Spray-drying has been widely studied as an alternative industrial process to freeze-drying for the purposes of preserving lactic acid starter cultures (Silva *et al*., 2011; Ying *et al.,* 2012). As regards the spray-drying of BCAs, Abadias *et al*., (2005) reported that spray-dried cells of *C. sake* were less effective than fresh ones at controlling *P.expansum* rot in apples. Moreover, spray-drying was not a good dehydration method, as it provided a low cell survival rate and poor product recovery. However, Yánez-Mendizábal *et al*., (2012b) used this dehydration method to develop shelf-stable formulations of *B. subtilis* which are effective at controlling brown rot on peach. With the high temperatures involved in this process, the species submitted to this technique require a certain level of thermotolerance (Morgan *et al*., 2006). In fact, it would be more suitable to apply this method to BCAs with endospore-forming ability (Yánez-Mendizábal *et al*., 2012a; 2012b. The results of a study carried out by Cañamás *et al*., (2008c) indicated that mild heat-adapted *C. sake* cells at 33°C had a longer survival time after spray-drying than non-adapted cells.

Freeze-drying or liophilization is one of the most commonly-used methods to obtain dry preparations of microorganisms. The process takes place in three phases: an initial freezing step, which should be performed so that the amounts and sizes of the ice crystals are kept to a minimum; a second step of primary drying in which free water is removed from the product; and a secondary drying that implies the application of a higher temperature that causes strongly bound water to leave the product (Melin *et al*., 2011). The main disadvantage of this drying method is the effects of freezing on cell viability (Morgan *et al*., 2006; Sui *et al*., 2015). Moreover, this technique implies high costs and energy consumption (Peighambardoust *et al*., 2011; Yánez-Mendizábal *et al*., 2012c). Nevertheless, it presents interesting advantages, such as protection from contamination or infestation during storage, long-term viability of microorganisms and ease of distribution (Abadias *et al*., 2001; Smith & Onions, 1983). Abadias *et al*., (2001a) reported that freeze-drying using 10% skim milk as a protectant was the best method to preserve the viability of yeast *C. sake* cells. In a complementary study, Abadias *et al*., (2001c) observed that the stability of freeze-dried samples decreased during storage and was influenced by temperature. Working with other BCAs, Torres *et al*., (2014) studied different storage strategies of a freeze-dried formulation of *P. agglomerans*, which resulted in a good shelf life and efficacy against the major post-harvest diseases of apples and citrus fruit. Spadaro *et al*., (2010) optimized the freeze-drying conditions for *M. pulcherrima*, whose cells maintained a high viability after 6 months of storage at 4°C.

Fluidized-bed drying (FBD) is a drying method in which the biomass is desiccated by being suspended or fluidized on a warm air stream inside a chamber (Melin *et al.,* 2011). It can incorporate a spray within the fluidized-bed dryer which can coat material onto a granulated product (Morgan *et al*., 2006). It is an expensive method but it is generally considered the least stressful of the dehydration methods, since it involves less extreme water loss and lower temperature gradients (Dunlap & Schisler, 2010; Larena *et al*., 2003; Morgan *et al*., 2006). There are several examples of the application of FBD for the purposes of dehydrating antagonists. For instance, Guijarro *et al*., (2006) demonstrated that *P. frequentans* conidia dried by fluidized bed-drying were as effective as fresh conidia at controlling brown rot of peaches; and Larena *et al*. ,(2003)

found that conidia of *P. oxalicum* dried by this technique maintained 100% viability after the process. As far as the application of FBD to the dehydration of yeasts is concerned, Mokiou & Magan (2008) developed formulations of *P. anomala* with different additives which were able to inhibit the growth of *Penicillium verrucosum* and exhibited good viability. Similarly, Mounir *et al*., (2007) reported that the formulation of *A. pullulans* in a fluidized-bed did not affect its antagonistic activity against blue mold on apples.

1.6.3 Stability of biocontrol formulations

The preservation of the cell viability during fermentation, drying, storage and rehydration is one of the main goals of the formulation process (Schisler *et al*., 2004). After the drying process, storage conditions have a great influence on the shelf-life of the dry BCP. The final moisture content or, preferably, a_W, of the products and temperature and %RH conditions during storage can profoundly affect the survival of BCA in the formulations (Connick *et al*., 1996; Fravel, 2005). Therefore, all of these parameters deserve careful research in order to maintain the formulation in optimal conditions for its further commercial applications (Torres *et al*., 2014).

Water activity and moisture content

Low moisture content after the drying process and maintaining it at the same level during storage has been reported as being critical to the conservation of viability (Guijarro *et al*., 2006; Mokiu & Magan, 2008). Dunlap & Schisler (2010) obtained dried granules based on *Cryptococcus flavescens* in an inert support with different moisture contents using a fluidized-bed dryer and evaluated its storage stability at 4°C for up to a year. These authors reported that 4% moisture content, the lowest tested, had the best long-term survival (1 year). Mokiou & Magan (2008) found that a moisture content of >10% was best for the viability of *P. anomala* formulations obtained by FBD.

As regards storage conditions, Dunlap & Schisler (2010) stated that sorption/desorption isotherms are particularly helpful in selecting storage water activity conditions. Interpreting the storage stability results in the context of the sorption/desorption isotherm provides a rational basis for guiding decisions to be made on storage condition for similar organisms. For most reported organisms, the optimum storage a_W lies in the

middle of the plateau region of the isotherm, in the 0.2 – 0.6 range. At high humidity conditions, water begins to act as a plasticizer and increases the molecular mobility of the components of the dry formulations (Poddar *et al*., 2014; Thomsen *et al*., 2005). This increase could result in the crystallization of the amorphous structures and in an instantaneous loss of microbial viability during storage, since the glassy (non-crystalline) state has been shown to enhance the storage life (Ananta *et al*., 2005; Crowe *et al*., 1998; Miao *et al*., 2008; Poddar *et al*., 2014; Sun & Leopold, 1997)

There are few reports dealing with the influence of a_W during storage on BCA-formulations and the majority of the published studies have been carried out using probiotics. For example, Poddar *et al*., (2014) studied the viability of dried *Lactobacillus paracasei* during storage at 25°C under different a_W. These authors reported that, at a_W of 0.11, cell viability loss was minimal, while viability was lost in all powders within 22 days at a_W of 0.52. Likewise, Miao *et al*., (2008) observed that the retention of the cell viability of *L. paracasei* and *Lactobacillus rhamnosus* was greatest for cells stored at a_W of 0.11 and compromised at higher a_W.

Temperature

The shelf-life of dry products containing microorganisms is highly dependent on the storage temperature. In general terms, as the storage temperature increases, mortality also increases and storage time is reduced (Costa *et al*., 2002). This could be due to the fact that temperatures of between 4 and 10°C cause a slowing down of both cell division and metabolic rate in microorganisms and, in this situation, cells are capable of withstanding the depletion of nutrients and the accumulation of toxic metabolites (Mejri *et al*., 2013; Trivedi *et al*., 2005).

Several studies have previously reported the effect of the storage temperature on the viability of different biological formulations. Abadias *et al*., (2001c) reported that storage at 4°C was required to maintain the viability of *C. sake* cells obtained by freeze-drying. Likewise, Torres *et al*., (2014) studied the viability of freeze-dried *P. agglomerans* cells, which was significantly higher at -20 and 5°C, as opposed to at 25°C. Other authors have described similar results (Kinay & Yilniz, 2008; Melin *et al*., 2011; Spadaro *et al*., 2010).

In this context, the present doctoral Thesis analyze different variables which affect the viability and efficacy of using *Candida sake* as BCA against *Botrytis cinerea* in formulated products based on coating forming agents, obtained by fluidized-bed drying; both the economic aspects and the feasibility of commercialization and field applications are also taken into account. The biopolymeric agents were selected in terms of their ability to promote cell viability and the physical stability of the formulated products and the cell viability *of C. sake* as a function of a_W and temperature was studied.

REFERENCES

Abadias, M., Benabarre, A., Teixidó, N., Usall, J., & Viñas, I. (2001a). Effect of freeze drying and protectants on viability of the biocontrol yeast *Candida sake*. *International Journal of Food Microbiology*, *65*(3), 173–182.

Abadias, M., Teixidó, N., Usall, J., Viñas, I., & Magan, N. (2001b). Improving water stress tolerance of the biocontrol yeast *Candida sake* grown in molasses-based media by physiological manipulation. *Canadian Journal of Microbiology*, *47*(2), 123–129.

Abadias, M., Teixidó, N., Usall, J., Benabarre, A., & Viñas, I. (2001c). Viability, efficacy, and storage stability of freeze-dried biocontrol agent *Candida sake* using different protective and rehydration media. *Journal of Food Protection*, *64*, 856–861.

Abadias, M., Usall, J., Teixidó, N., & Viñas, I. (2003). Liquid formulation of the postharvest biocontrol agent *Candida sake* CPA-1 in isotonic solutions. *Phytopathology*, *93*(4), 436–442.

Abadias, M., Teixidó, N., Usall, J., Solsona, C., & Viñas, I. (2005). Survival of the postharvest biocontrol yeast *Candida sake* CPA-1 after dehydration by spray-drying. *Biocontrol Science and Technology*, *15*(8), 835–845.

Abano, E. E., & Sam-Amoah, L. K. (2012). Application of antagonistic microorganisms for the control of postharvest decays in fruits and vegetables. *International Journal of Advanced Biological Research*, *2*(1), 1–8.

Adikaram, N. K. B., & Karunaratne, A. (1998). Suppression of avocado anthracnose and stem-end rot pathogens by endogenous antifungal substances and a surface inhabiting *Pestalotiopsis* sp. *ACIAR Proceedings Series*, *80*, 72–77.

Alabouvette, C., & Cordier, C. (2011). Regulation of Biological Control Agents. In R.-U. Ehlers (Ed.), *Regulation of Biological Control Agents* (pp. 157–173). Stockholm.

Alabouvette C. & Steinberg C. (1998) Fate of microorganisms introduced into soils, effects on autochthonous communities and activities. Workshop on microbiological plant protection products: scientific basis for risk assessment, KEMI, Stockholm, 26–28/10/1998.

Allende, A., Tomás-Barberán, F., & Gil, M. (2006). Minimal processing for healthy traditional foods. *Trends in Food Science & Technology*, *17*(9), 513–519.

Aloui, H., Khwaldia, K., Sánchez-González, L., Muneret, L., Jeandel, C., Hamdi, M., & Desobry, S. (2014a). Alginate coatings containing grapefruit essential oil or grapefruit seed extract for grapes preservation. *International Journal of Food Science and Technology*, *49*(4), 952–959.

Aloui, H., Khwaldia, K., Licciardello, F., Mazzaglia, A., Muratore, G., Hamdi, M., & Restuccia, C. (2014b). Efficacy of the combined application of chitosan and locust bean gum with different citrus essential oils to control postharvest spoilage caused by *Aspergillus flavus* in dates. *International Journal of Food Microbiology*, *170*, 21–28.

Aloui, H., Licciardello, F., Khwaldia, K., Hamdi, M., & Restuccia, C. (2015). 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*. *International Journal of Food Microbiology*, *200*, 22–30.

Ananta, E., Volkert, M., & Knorr, D. (2005). Cellular injuries and storage stability of spray-dried *Lactobacillus rhamnosus* GG. *International Dairy Journal*, *15*(4), 399–409.

Ang, D., Liberek, K., Skowyra, D., Zylicz, M., & Georgopoulos, C. (1991). Biological role and regulation of the universally conserved heat-shock proteins. *Journal of Biological Chemistry*, *266*, 24233–24236.

Annous, B. A., Fratamico, P. M., & Smith, J. L. (2009). Quorum sensing in biofilms : Why bacteria behave the way they do. *Journal of Food Science*, *74*(1), R24–R37.

Arras, G., De Cicco, V., Arru, S., & Lima, G. (1998). Biocontrol by yeasts of blue mould of citrus fruits and the mode of action of an isolate of *Pichia guilliermondii*. *The Journal of Horticultural Science and Biotechnology*, *73*(3), 413–418.

Arrebola, E., Sivakumar, D., Bacigalupo, R., & Korsten, L. (2010). Combined application of antagonist *Bacillus amyloliquefaciens* and essential oils for the control of peach postharvest diseases. *Crop Protection*, *29*(4), 369–377.

Arrieta, M. P., Peltzer, M. A., Garrigós, M. D. C., & Jiménez, A. (2013). Structure and mechanical properties of sodium and calcium caseinate edible active films with carvacrol. *Journal of Food Engineering*, *114*(4), 486–494.

Atarés, L., Bonilla, J., & Chiralt, A. (2010). Characterization of sodium caseinate-based edible films incorporated with cinnamon or ginger essential oils. *Journal of Food Engineering*, *100*(4), 678–687.

Audic, J.-L., & Chaufer, B. (2005). Influence of plasticizers and crosslinking on the properties of biodegradable films made from sodium caseinate. *European Polymer Journal*, *41*(8), 1934–1942.

Baldwin, E. A. (1994). Edible coatings for fresh fruits and vegetables: Past, present, and future. In J. M. Krochta, E. A. Baldwin, & M. O. Nisperos-Carriedo (Eds.), *Edible Coatings and Films to Improve Food Quality* (pp. 25–65). Lancaster, Pennsylvania (USA): Technomic Publishing Company, Inc.

Banani, H., Spadaro, D., Zhang, D., Matic, S., Garibaldi, A., & Gullino, M. L. (2015). Postharvest application of a novel chitinase cloned from *Metschnikowia fructicola* and overexpressed in *Pichia pastoris* to control brown rot of peaches. *International Journal of Food Microbiology*, *199*, 54–61.

Barkai-Goland, R. (2001). *Postharvest diseases of fruit and vegetables: development and control*. (Elsevier, Ed.). Amsterdam (The Netherlands): Elsevier.

Batta, Y. A. (2004). Postharvest biological control of apple gray mold by *Trichoderma harzianum* Rifai formulated in an invert emulsion. *Crop Protection*, *23*, 19–26.

Batta, Y. A. (2007). Control of postharvest diseases of fruit with an invert emulsion formulation of *Trichoderma harzianum* Rifai. *Postharvest Biology and Technology*, *43*(1), 143–150.

Bautista-Rosales, P. U., Calderón-Santoyo, M., Servín-Villegas, R., Ochoa-Álvarez, N. A., & Ragazzo-Sánchez, J. A. (2013). Action mechanisms of the yeast *Meyerozyma caribbica* for the control of the phytopathogen *Colletotrichum gloeosporioides* in mangoes. *Biological Control*, *65*(3), 293–301.

Benbettaïeb, N., Karbowiak, T., Brachais, C.-H., & Debeaufort, F. (2016). Impact of electron beam irradiation on fish gelatin film properties. *Food Chemistry*, *195*, 11–8.

Benbow, J. M., & Sugar, D. (1999). Fruit surface colonization and biological control of postharvest diseases of pear by preharvest yeast applications. *Plant Disease*, *83*(9), 839–844.

Bill, M., Sivakumar, D., Korsten, L., & Thompson, A. K. (2014). The efficacy of combined application of edible coatings and thyme oil in inducing resistance components in avocado (*Persea americana* Mill.) against anthracnose during post-harvest storage. *Crop Protection*, *64*, 159–167.

Blakeman, J. P., & Fokkema, N. J. (1982). Potential for biological control of plant diseases on the phylloplane. *Annual Review of Phytopathology*, *20*, 167–190.

Bleve, G., Grieco, F., Cozzi, G., Logrieco, A., & Visconti, A. (2006). Isolation of epiphytic yeasts with potential for biocontrol of *Aspergillus carbonarius* and *A. niger* on grape. *International Journal of Food Microbiology*, *108*(2), 204–209.

Bonaterra, A., Mari, M., Casalini, L., & Montesinos, E. (2003). Biological control of *Monilinia laxa* and *Rhizopus stolonifer* in postharvest of stone fruit by *Pantoea agglomerans* EPS125 and putative mechanisms of antagonism. *International Journal of Food Microbiology*, *84*, 93–104.

Brar, S. K., Verma, M., Tyagi, R. D., & Valéro, J. R. (2006). Recent advances in downstream processing and formulations of *Bacillus thuringiensis* based biopesticides. *Process Biochemistry*, *41*(2), 323–342.

Breinig, F., Tipper, D. J., & Schmitt, M. J. (2002). Kre1p, the plasma membrane receptor for the yeast K1 Viral Toxin. *Cell*, *108*(3), 395–405.

Brent, K. J., & Hollomon, D. W. (2007). *Fungicide resistance: the assessment of the risk*. *Fungicide resistance in crop pathogens: how can it be managed? FRAC Monograph No. 1 (second, revised edition).* Brussels: Global Crop Protection Federation. Burges, H. D. (1998). Formulation of mycoinsecticides. In H. D. Burges (Ed.), *Formulation of Microbial Biopesticides* (pp. 131–186). London (UK): Kluwer Academic Publishers.

Burges, H. D., & Jones, K. A. (1998). Formulation of bacteria, viruses and Protozoa to control insects. In H. D. Burges (Ed.), *Formulation of Microbial Biopesticides* (pp. 31–128). London (UK): Kluwer Academic Publishers.

Cai, Z., Yang, R., Xiao, H., Qin, X., & Si, L. (2015). Effect of preharvest application of *Hanseniaspora uvarum* on postharvest diseases in strawberries. *Postharvest Biology and Technology*, *100*, 52–58.

Calvo, J., Calvente, V., De Orellano, M. E., Benuzzi, D., & De Tosetti, M. I. S. (2003). Improvement in the biocontrol of postharvest diseases of apples with the use of yeast mixtures. *BioControl*, *48*(5), 579–593.

Calvo, J., Calvente, V., de Orellano, M. E., Benuzzi, D., Isabel, M., & Sanz de Tosetti, M. I. (2007). Biological control of postharvest spoilage caused by *Penicillium expansum* and *Botrytis cinerea* in apple by using the bacterium *Rahnella aquatilis*. *International Journal of Food Microbiology*, *113*, 251–257.

Calvo-Garrido, C. (2013). Control de la podredumbre por *Botrytis cinerea* mediante la aplicación de *Candida sake* CPA-1 y otras estrategias alternativas a los fungicidas químicos en uva de vinificación. Lleida: Universitat de Lleida, PhD Thesis.

Calvo-Garrido, C., Elmer, P. a G., Viñas, I., Usall, J., Bartra, E., & Teixidó, N. (2013a). Biological control of botrytis bunch rot in organic wine grapes with the yeast antagonist *Candida sake* CPA-1. *Plant Pathology*, *62*(3), 510–519.

Calvo-Garrido, C., Viñas, I., Elmer, P., Usall, J., & Teixidó, N. (2013b). *Candida sake* CPA-1 and other biologically based products as potential control strategies to reduce sour rot of grapes. *Letters in Applied Microbiology*, *57*(4), 356–361.

Calvo-Garrido, C., Viñas, I., Elmer, P. A., Usall, J., & Teixidó, N. (2014a). Suppression of *Botrytis cinerea* on necrotic grapevine tissues by early-season applications of natural products and biological control agents. *Pest Management Science*, *70*(4), 595–602.

Calvo-Garrido, C., Viñas, I., Usall, J., Rodríguez-Romera, M., Ramos, M. C., & Teixidó, N. (2014b). Survival of the biological control agent *Candida sake* CPA-1 on grapes under the influence of abiotic factors. *Journal of Applied Microbiology*, *117*(3), 800–811.

Campos, C. A., Gerschenson, L. N., & Flores, S. K. (2011). Development of edible films and coatings with antimicrobial activity. *Food and Bioprocess Technology*, *4*(6), 849–875.

Cano, A., Fortunati, E., Cháfer, M., Kenny, J. M., Chiralt, A., & González-Martínez, C. (2015). Properties and ageing behaviour of pea starch films as affected by blend with poly(vinyl alcohol). *Food Hydrocolloids*, *48*, 84–93.

Cañamás, T. P., Viñas, I., Usall, J., Casals, C., Solsona, C., & Teixidó, N. (2008). Control of postharvest diseases on citrus fruit by preharvest application of the biocontrol agent *Pantoea agglomerans* CPA-2. Part I. Study of different formulation strategies to improve survival of cells in unfavourable environmental conditions. *Postharvest Biology and Technology*, *49*(1), 86–95.

Cañamás, T. P., Viñas, I., Usall, J., Torres, R., Anguera, M., & Teixidó, N. (2008b). Control of postharvest diseases on citrus fruit by preharvest applications of biocontrol agent *Pantoea agglomerans* CPA-2. Part II. Effectiveness of different cell formulations. *Postharvest Biology and Technology*, *49*(1), 96–106.

Cañamás, T. P., Viñas, I., Usall, J., Magan, N., Solsona, C., & Teixidó, N. (2008c). Impact of mild heat treatments on induction of thermotolerance in the biocontrol yeast *Candida sake* CPA-1 and viability after spray-drying. *Journal of Applied Microbiology*, *104*(3), 767-775.

Cañamás, T. P., Viñas, I., Torres, R., Usall, J., Solsona, C., & Teixidó, N. (2011). Field applications of improved formulations of *Candida sake* CPA-1 for control of *Botrytis cinerea* in grapes. *Biological Control*, *56*(2), 150–158.

Castoria, R., De Curtis, F., Lima, G., Caputo, L., Pacifico, S., & De Cicco, V. (2001). *Aureobasidium pullulans* (LS-30) an antagonist of postharvest pathogens of fruits: study on its modes of action. *Postharvest Biology and Technology*, *22*(1), 7–17.

Cháfer, M., Sánchez-González, L., González-Martínez, C., & Chiralt, A. (2012). Fungal decay and shelf life of oranges coated with chitosan and bergamot, thyme and tea tree essential oils. *Journal of Food Science*, *77*(8), 182–187.

Champagne, C. P., Gardner, N., Brochu, E., & Beaulieu, Y. (1991). The freeze drying of lactic acid bacteria: A review. *Canadian Institute of Science and Technology*, *24*, 118–128.

Chan, Z., & Tian, S. (2005). Interaction of antagonistic yeasts against postharvest pathogens of apple fruit and possible mode of action. *Postharvest Biology and Technology*, *36*(2), 215–223.

Chien, P.-J., Sheu, F., & Lin, H.-R. (2007). Coating citrus (*Murcott tangor*) fruit with low molecular weight chitosan increases postharvest quality and shelf life. *Food Chemistry*, *100*(3), 1160–1164.

Choi, W., & Han, J. H. (2001). Physical and mechanical properties of pea-protein-based edible films. *Journal of Food Science*, *66*(2), 319–322.

Choudhary, B., Nagpure, A., & Gupta, R. K. (2015). Biological control of toxigenic citrus and papaya-rotting fungi by *Streptomyces violascens* MT7 and its extracellular metabolites. *Journal of Basic Microbiology*, *55*(12), 1343–56.

Cirvilleri, G., Bonaccorsi, A., Scuderi, G., & Scortichini, M. (2005). Potential biological control activity and genetic diversity of *Pseudomonas syringae* pv. *syringae* strains. *Journal of Phytopathology*, *153*, 654–666.

Connick, W. J., Daigle, D. J., Boyette, C. D., Williams, K. S., Vinyard, B. T., & Quimby, P. C. (1996). Water activity and other factors that affect the viability of *Colletotrichum truncatum* conidia in wheat flour-kaolin granules (’Pesta'). *Biocontrol Science and Technology*, *6*(2), 277–284.

Conway, W. S., Janisiewicz, W. J., Leverentz, B., Saftner, R. A., & Camp, M. J. (2007). Control of blue mold of apple by combining controlled atmosphere, an antagonist mixture and sodium bicarbonate. *Postharvest Biology and Technology*, *45*(3), 326–332.

Corcoran, B. M., Ross, R. P., Fitzgerald, G. F., & Stanton, C. (2004). Comparative survival of probiotic lactobacilli spray-dried in the presence of prebiotic substances. *Journal of Applied Microbiology*, *96*, 1024–1039.

Costa, E., Usall, J., Teixidó, N., Torres, R., & Viñas, I. (2002). Effect of package and storage conditions on viability and efficacy of the freeze-dried biocontrol agent *Pantoea agglomerans* strain CPA-2. *Journal of Applied Microbiology*, *92*(5), 873–878.

Côté, J.-C., Vincent, C., Son, K.-H., & Bok, S. H. (2001). Persistence of insecticidal activity of novel bioencapsulated formulations of *Bacillus thuringiensis* var. *kurstaki* against *Choristoneura rosaceana* [Lepidoptera: Tortricidae]. *Phytoprotection*, *82*, 73–82.

Crowe, J. H., Carpenter, J. F., & Crowe, L. M. (1998). The role of vitrification in anhydrobiosis. *Annual Review of Physiology*, *60*, 73–103.

Cuero, R. G. (1999). Antimicrobial action of exogenous chitosan. In P. Jollés & R. A. A. Muzzarelli (Eds.), *Chitin and Chitinases*. Birkhäuser Verlag, pp. 315–333.

Dal Bello, G., Mónaco, C., Rollan, M. C., Lampugnani, G., Arteta, N., Abramoff, C., Ronco, L., & Stocco, M. (2008). Biocontrol of postharvest grey mould on tomato by yeasts. *Journal of Phytopathology*, *156*, 257–263.

Dean, R., Van Kan, J. A. L., Pretorius, Z. A., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., Rudd, J. J, Dickman, M., Kahmann, R., Ellis, J., Foster, G. D. (2012). The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology*, *13*(4), 414–430.

Devi, A. N., & Arumugam, T. (2005). Studies on the shelf life and quality of Rasthali banana as affected by postharvest treatments. *Orissa Journal of HorticutIture*, *33*(2), 3–6.

Di Francesco, A., & Mari, M. (2014). Use of BCAs in combination with physical and chemical treatments. *Stewart Postharvest Review*, *1*.

Di Francesco, A., Ugolini, L., Lazzeri, L., & Mari, M. (2015). Production of volatile organic compounds by *Aureobasidium pullulans* as a potential mechanism of action against postharvest fruit pathogens. *Biological Control*, *81*, 8–14.

Droby, S., Chalutz, E., Wilson, C. L., & Wisniewski, M. E. (1989). Characterization of the biocontrol activity of *Debaryomyces hansenii* in the control of *Penicillium digitatum* on grapefruit. *Canadian Journal of Microbiology*, *35*, 794–800.

Droby, S., Chalutz, E., Wilson, C. L., & Wisniewski, M. E. (1992). Biological control of postharvest diseases: a promising alternative to the use of synthetic fungicides. *Phytoparasitica*, *20*, 149–153.

Droby, S., & Chalutz, E. (1994). Mode of action of biocontrol agents of postharvest diseases. In C. L. Wilson & M. E. Wisniewski (Eds.), *Biological Control of Postharvest Diseases: Theory and Practice* (pp. 63–76). Boca Raton (US): CRC Press Inc.

Droby, S., Cohen, L., Daus, A., Weiss, B., Horev, B., Chalutz, E., Katz, H., Keren-Tzur, M., Shachnai, A. (1998). Commercial testing of Aspire: a yeast preparation for the biological control of postharvest decay of citrus. *Biological Control*, *12*(2), 97–101.

Droby, S., Vinokur, V., Weiss, B., Cohen, L., Daus, A., Goldschmidt, E. E., & Porat, R. (2002). Induction of resistance to *Penicillium digitatum* in grapefruit by the yeast biocontrol agent *Candida oleophila*. *Phytopathology*, *92*(4), 393–399.

Droby, S., Wisniewski, M., El Ghaouth, A., & Wilson, C. (2003). Influence of food additives on the control of postharvest rots of apple and peach and efficacy of the yeast-based biocontrol product Aspire. *Postharvest Biology and Technology*, *27*(2), 127–135.

Droby, S., Wisniewski, M. E., Macarisin, D., & Wilson, C. L. (2009). Twenty years of postharvest biocontrol research: Is it time for a new paradigm? *Postharvest Biology and Technology*, *52*(2), 137–145.

Droby, S., Wisniewski, M., Teixidó, N., Spadaro, D., & Jijakli, M. H. (2016). The science, development, and commercialization of postharvest biocontrol products. *Postharvest Biology and Technology*, (April).

Dunlap, C. A., & Schisler, D. A. (2010). Fluidized-bed drying and storage stability of *Cryptococcus flavescens* OH 182.9, a biocontrol agent of *Fusarium* head blight. *Biocontrol Science and Technology*, *20*(5), 465–474.

Ebrahimi, L., Etebarian, H. R., Aminian, H., & Sahebani, N. (2012). Enhancement of biocontrol activity of *Torulaspora delbrueckii* with methyl jasmonate against apple blue mould disease. *Archives Of Phytopathology And Plant Protection*, *45*(19), 2355–2363.

EC Regulation No. 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin.

Elad, Y., & Stewart, A. (2007). Microbial control of *Botrytis* spp. In Y. et al. Elad (Ed.), *Botrytis: Biology, Pathology and Control* (pp. 223–241). New York (US): Springer.

El-Ghaouth, A., Wilson, C. L., & Wisniewski, M. E. (1998). Ultrastructural and cytochemical aspects of the biological control of *Botrytis cinerea* by *Candida saitoana* in apple fruit. *Phytopathology*, *88*, 282–291.

El-Ghaouth, A., Smilanick, J. L., Brown, G. E., Ippolito, A., Wisniewski, M., & Wilson, C. L. (2000a). Application of *Candida saitoana* and glycolchitosan for the control of

postharvest diseases of apple and citrus fruit under semi-commercial conditions. *Plant Disease*, *84*(3), 243–248.

El-Ghaouth, A., Smilanick, J. L., & Wilson, C. L. (2000b). Enhancement of the performance of *Candida saitoana* by the addition of glycolchitosan for the control of postharvest decay of apple and citrus fruit. *Postharvest Biology and Technology*, *19*(1), 103–110.

El-Ghaouth, A., Smilanick, J. L., Wisniewski, M., & Wilson, C. L. (2000c). Improved control of apple and citrus fruit decay with a combination of Candida saitoana and 2-deoxy-D-glucose. *Plant Disease*, *84*, 249–253.

El-Ghaouth, A., Smilanick, J., Brown, G., Ippolito, A., & Wilson, C. (2001). Control of Decay of apple and citrus fruits in semi-commercial tests with *Candida saitoana* and 2-deoxy--glucose. *Biological Control*, *20*, 96–101.

El-Ghaouth, A., Wilson, C. L., Wisniewski, M., Droby, S., Smilanick, J. L., & Korsten, L. (2002). Biological control of post-harvest diseases of citrus fruit. In S. S. Gnanamanickam (Ed.), *Biological Control of Crop Diseases* (pp. 289–312). New York (US): Dekker Inc.

El-Ghaouth, A., Wilson, C. L., & Wisniewski, M. E. (2004). Biologically based alternatives to synthetic fungicides for the postharvest diseases of fruit and vegetables. In S. A. M. H. Naqvi (Ed.), *Diseases of Fruit and Vegetables, vol. 2.* (pp. 511–535). The Netherlands: Kluwer Academic Publishers.

Elmer, P. A. G., & Reglinski, T. (2006). Biosuppression of *Botrytis cinerea* in grapes. *Plant Pathology*, *55*(2), 155–177.

El-Mohamedy, R. S. R., El-Gamal, N. G., & Bakeer, A. R. T. (2015). Application of chitosan and essential oils as alternatives fungicides to control green and blue moulds of citrus fruits. *International Journal of Current Microbiology and Applied Sciences*, *4*(6), 629–643.

Elsabee, M. Z., & Abdou, E. S. (2013). Chitosan based edible films and coatings: a review. *Materials Science & Engineering. C, Materials for Biological Applications*, *33*(4), 1819–41.

El-Shanshoury, A. E. R. R., Bazaid, S. A., El-Halmouch, Y., & Ghafar, M. W. E. (2013). Control the post-harvest infection by *Aspergillus* spp. to Taify table grape using grape epiphytic bacteria. *Life Science Journal*, *10*(1), 1821–1636.

Eroglu, E., Torun, M., Dincer, C., & Topuz, A. (2014). Influence of pullulan-based edible coating on some quality properties of strawberry during cold storage. *Packaging and Technology and Science*, *27*, 831–838.

Fabra, M. J., Talens, P., & Chiralt, A. (2009). Microstructure and optical properties of sodium caseinate films containing oleic acid-beeswax mixtures. *Food Hydrocolloids*, *23*(3), 676–683.

Fabra, M. J., Pérez-Masiá, R., Talens, P., & Chiralt, A. (2011). Influence of the homogenization conditions and lipid self-association on properties of sodium caseinate based films containing oleic and stearic acids. *Food Hydrocolloids*, *25*(5), 1112–1121.

Fabra, M. J., López-Rubio, A., & Lagaron, J. M. (2014). Biopolymers for food packaging applications. In: Aguilar, M. R. & J.S. Román (Eds.) *Smart Polymers and their Applications* (pp. 476-509). Cambridge (UK): Woodhead Publishing

Fabra, M. J., Jiménez, A., Talens, P., & Chiralt, A. (2014). Influence ohf Homogenization conditions on physical properties and antioxidant activity of fully biodegradable pea protein-alpha-tocopherol films. *Food and Bioprocess Technology*, 7(12), 3569–3578.

Fagundes, C., Palou, L., Monteiro, A. R., & Pérez-Gago, M. B. (2015). Hydroxypropyl methylcellulose-beeswax edible coatings formulated with antifungal food additives to reduce alternaria black spot and maintain postharvest quality of cold-stored cherry tomatoes. *Scientia Horticulturae*, *193*, 249–257.

Fakhouri, F. M., Martelli, S. M., Caon, T., Velasco, J. I., & Mei, L. H. I. (2015). Edible films and coatings based on starch/gelatin: Film properties and effect of coatings on quality of refrigerated Red Crimson grapes. *Postharvest Biology and Technology*, *109*, 57–64.

Falconi, C. J., & Mendgen, K. (1994). Epiphytic fungi on apple leaves and their value for control of the postharvest pathogens *Botrytis cinerea*, *Monilinia fructigena* and *Penicillium expansum*. *Zeitschrift Für Pflanzenkrankheiten Und Pflanzenschutz*, *101*, 38–47.

Fan, Y., Xu, Y., Wang, D., Zhang, L., Sun, J., Sun, L., & Zhang, B. (2009). Effect of alginate coating combined with yeast antagonist on strawberry (*Fragaria* × *ananassa*) preservation quality. *Postharvest Biology and Technology*, *53*(1-2), 84–90.

Fravel, D. R. (2005). Commercialization and implementation of biocontrol. *Annual Review of Phytopathology*, *43*, 337–359.

Fredlund, E., Druvefors, U. Ä., Olstorpe, M. N., Passoth, V., & Schnürer, J. (2004). Influence of ethyl acetate production and ploidy on the anti-mould activity of *Pichia anomala*. *FEMS Microbiology Letters*, *238*(1), 133–137.

Fu, N., & Chen, X. D. (2011). Towards a maximal cell survival in convective thermal drying processes. *Food Research International*, *44*, 1127–1149.

Gamagae, S. U., Sivakumar, D., Wijeratnam, R. . W., & Wijesundera, R. L. C. (2003). Use of sodium bicarbonate and *Candida oleophila* to control anthracnose in papaya during storage. *Crop Protection*, *22*(5), 775–779.

García, M. A., Martino, M. N., & Zaritzk, N. E. (1998). Starch-based coatings : effect on refrigerated strawberry (*Fragaria ananassa*) quality. *Journal of Science Food and Agriculture*, *76*(3), 411–420.

García, M. A., Martino, M. N., & Zaritzky, N. E. (2000). Microstructural characterization of plasticized starch-based films. *Starch/Staerke*, *52*(4), 118–124.

Gava, C. A. T., & Pinto, J. M. (2016). Biocontrol of melon wilt caused by *Fusarium oxysporum* Schlect f. sp. *melonis* using seed treatment with *Trichoderma* spp. and liquid compost. *Biological Control*, *97*, 13–20.

Giobbe, S., Marceddu, S., Scherm, B., Zara, G., Mazzarello, V. L., Budroni, M., & Migheli, Q. (2007). The strange case of a biofilm-forming strain of *Pichia fermentans*, which controls *Monilinia* brown rot on apple but is pathogenic on peach fruit. *FEMS Yeast Research*, *7*(8), 1389–1398.

Gol, N. B., Patel, P. R., & Rao, T. V. R. (2013). Improvement of quality and shelf-life of strawberries with edible coatings enriched with chitosan. *Postharvest Biology and Technology*, *85*, 185–195.

González-Martínez, C., Cháfer, M., Vargas, M., Sánchez-González, L., & Chiralt, A. (2011). Edible coatings for fresh and minimally processed fruits and vegetables. In *Advances in Postharvest Treatments and Fruit Quality and Safety* (pp. 59–84). New York (US): Nova Science Publishers.

Greener, I., & Fennema, O. (1994). Edible films and coatings: Characteristics, formation, definitions, and testing methods. In J. M. Krochta, E. A. Baldwin, & M. O. Nisperos-Carriedo (Eds.), *Edible coatings and films to improve food quality.* (pp. 1–24). Lancaster, Pennsylvania (US): Technomic Publishing Company, Inc.

Guerreiro, A. C., Gago, C. M. L., Faleiro, M. L., Miguel, M. G. C., & Antunes, M. D. C. (2015). The use of polysaccharide-based edible coatings enriched with essential oils to improve shelf-life of strawberries. *Postharvest Biology and Technology*, *110*, 51–60.

Guetsky, R., Shtienberg, D., Elad, Y., Fischer, E., & Dinoor, A. (2002). Improving biological control by combining biocontrol agents each with several mechanisms of disease suppression. *Phytopathology*, *92*(9), 976–985.

Guijarro, B., Larena, I., Melgarejo, P., & De Cal, A. (2006). Effect of drying on conidial viability of *Penicillium frequentans*, a biological control agent against peach brown rot disease caused by *Monilinia* spp. *Biocontrol Science and Technology*, *16*(3), 257–269.

Guilbert, S., Gontard, N., & Cuq, B. (1995). Technology and applications of edible protective films. *Packaging Technology and Science*, *8*(6), 339–346.

Guo, D., Yang, B., Ren, X., & Zhu, L. (2016). Effect of an antagonistic yeast in combination with microwave treatment on postharvest blue mould rot of jujube fruit. *Journal of Phytopathology*, *164*(1), 11–17.

Guo, J., Fang, W., Lu, H., Zhu, R., Lu, L., Zheng, X., & Yu, T. (2014). Inhibition of green mold disease in mandarins by preventive applications of methyl jasmonate and antagonistic yeast *Cryptococcus laurentii*. *Postharvest Biology and Technology*, *88*, 72–78.

Habibi, R., Kazempor, M. N., Elahinia, A., Rabiei, B., & Ebadi, A. A. (2012). Differentiation by simplified AFLP of *Pseudomonas syringe* pv. *syringae* isolates from fields, panicles and nurseries of the Guilan province – Iran. *Journal of plant protection research*, *52*(2), 268–274.

Han, J. H., & Gennadios, A. (2005). Edible films and coatings: A review. In J. H. Han (Ed.), *Innovations in Food Packaging: First Edition* (pp. 239–262). Amsterdam (The Netherlands): Elsevier.

Hao, W., Li, H., Hu, M., Yang, L., & Rizwan-ul-Haq, M. (2011). Integrated control of citrus green and blue mold and sour rot by *Bacillus amyloliquefaciens* in combination with tea saponin. *Postharvest Biology and Technology*, *59*(3), 316–323.

Harvey, J. M. (1978). Reduction of losses in fresh market fruits and vegetables. *Annual Review of Phytopathology*, *16*, 321–341.

Hernández-Montiel, L. G., Holguín-Peña, R. J., Larralde-Corona, C. P., Zulueta-Rodríguez, R., Rueda-Puente, E., & Moreno-Legorreta, M. (2012). Effect of inoculum size of yeast *Debaryomyces hansenii* to control *Penicillium italicum* on Mexican lime (*Citrus aurantiifolia*) during storage. *CyTA -Journal of Food*, *10*(3), 235–242.

Heungens, K., & Parke, J. L. (2001). Postinfection biological control of oomycete pathogens of pea by Burkholderia cepacia AMMD. *Phytopathology*, *91*, 383–391.

Hiemenz, P. C., & Rajagopalan, R. (1997). Surface tension and contact angle. In P. C. Hiemenz (Ed.), *Principles of colloid and surface chemistry* (pp. 248–255). New York (US): Marcel Dekker.

Holz, G., Coertze, S., & Williamson, B. (2004). *The Ecology of Botrytis on Plant Surfaces*. Dordrecht (The Netherlands): Springer.

Hong, C., Michailides, T. J., & Holtz, B. A. (1998). Effects of wounding, inoculum density, and biological control agents on post-harvest brown rot of stone fruits. *Plant Disease*, *82*(11), 1210–1216.

Hu, H., Yan, F., Wilson, C., Shen, Q., & Zheng, X. (2015). The ability of a cold-adapted *Rhodotorula mucilaginosa* strain from Tibet to control blue mold in pear fruit. *Antonie van Leeuwenhoek*, *108*(6), 1391–404.

Hurley, R., de Louvois, J., & Mulhall, A. (1987). Yeast and human and animal pathogens. In A. H. Rose & J. S. Harrison (Eds.), *The Yeasts, Vol. 1* (pp. 207–281). New York (US): Academic Press.

Insaward, A., Duangmal, K., & Mahawanich, T. (2015). Mechanical, optical and barrier properties of soy protein film as affected by phenolic acid addition. *Journal of Agricultural and Food Chemistry*, *63*(43), 9421–9426.

Ippolito, A., El-Ghaouth, A., Wilson, C. L., & Wisniewski, M. E. (2000). Control of postharvest decay of apple fruit with *Candida saitoana* and induction of defense responses. *Postharvest Biology and Technology*, *19*, 265–272.

Ippolito, A., & Nigro, F. (2000). Impact of preharvest application of biological control agents on postharvest diseases of fresh fruits and vegetables. *Crop Protection*, 19, 715–723.

Ippolito, A., Schena, L., Pentimone, I., & Nigro, F. (2005). Control of postharvest rots of sweet cherries by pre- and postharvest applications of *Aureobasidium pullulans* in combination with calcium chloride or sodium bicarbonate. *Postharvest Biology and Technology*, *36*(3), 245–252.

Jamalizadeh, M., Etebarian, H. R., Aminian, H., & Alizadeh, A. (2011). A review of mechanisms of action of biological control organisms against post-harvest fruit spoilage. *EPPO Bulletin*, *41*(1), 65–71.

Janisiewicz, W. J. (1994). Enhancement of biocontrol of blue mold with the nutrient analog 2-deoxy-D-glucose on apples and pears. *Applied and Environmental Microbiology*, *60*(8), 2671–2676.

Janisiewicz, W. J. (1998). Biocontrol of postharvest diseases of temperate fruit: challenges and opportunities. In J. Boland & L. D. Kuykendall (Eds.), *Plant Microbe Interactions and Biological Control,* (pp. 171–198). New York (US): Marcel Dekker.

Janisiewicz, W. J., & Roitman, J. (1988). Biological Control of Blue Mold and Gray Mold on Apple and Pear with Pseudomonas cepacia. *Phytopathology*, *78*(12), 1697–1700.

Janisiewicz, W., Yourman, L., Roitman, J., & Mahoney, N. (1991). Postharvest control of blue mold and gray mold of apples and pears by dip treatment with pyrrolnitrin, a metabolite of Pseudomonas cepacia. *Plant Disease, 75*, 490–494

Janisiewicz, W. J., Usall, J., & Bors, B. (1992). Nutritional enhancement of biocontrol of blue mold on apples. *Phytopathology*, *8*, 1364–1370.

Janisiewicz, W. J., & Jeffers, S. N. (1997). Efficacy of commercial formulation of two biofungicides for control of blue mold and gray mold of apples in cold storage. *Crop Protection*, *16*(7), 629–633.

Janisiewicz, W. J., & Korsten, L. (2002). Biological control of postharvest diseases of fruits. *Annual Review of Phytopathology*, *40*, 411–41.

Janisiewicz, W. J., Pereira, I. B., Almeida, M. S., Roberts, D. P., Wisniewski, M., & Kurtenbach, E. (2008). Improved biocontrol of fruit decay fungi with *Pichia pastoris* recombinant strains expressing Psd1 antifungal peptide. *Postharvest Biology and Technology*, *47*(2), 218–225.

Janisiewicz, W. J., Jurick, W. M., Vico, I., Peter, K. A., & Buyer, J. S. (2013). Culturable bacteria from plum fruit surfaces and their potential for controlling brown rot after harvest. *Postharvest Biology and Technology*, *76*, 145–151.

Jiang, F., Zheng, X., & Chen, J. (2009). Microarray analysis of gene expression profile induced by the biocontrol yeast *Cryptococcus laurentii* in cherry tomato fruit. *Gene*, *430*(1-2), 12–6.

Jiang, Y. M., Zhu, X. R., & Li, Y. B. (2001). Postharvest control of litchi fruit rot by *Bacillus subtilis*. *LWT - Food Science and Technology*, *34*(7), 430–436.

Jijakli, M. H., & Lepoivre, L. (1998). Characterization of an exo-□-1,3-glucanase produced by *Pichia anomala* strain K, antagonist of *Botrytis cinerea* on apples. *Phytopathology*, *88*, 335–343.

Jiménez, A., Fabra, M. J., Talens, P., & Chiralt, A. (2010). Effect of lipid self-association on the microstructure and physical properties of hydroxypropyl-methylcellulose edible films containing fatty acids. *Carbohydrate Polymers*, *82*(3), 585–593.

Jiménez, A., Fabra, M. J., Talens, P., & Chiralt, A. (2012). Effect of sodium caseinate on properties and ageing behaviour of corn starch based films. *Food Hydrocolloids*, *29*(2), 265–271.

Karabulut, O. A., Lurie, S., & Droby, S. (2001). Evaluation of the use of sodium bicarbonate, potassium sorbate and yeast antagonists for decreasing postharvest decay of sweet cherries. *Postharvest Biology and Technology*, *23*(3), 233–236.

Karabulut, O. A., Tezcan, H., Daus, A., Cohen, L., Wiess, B., & Droby, S. (2004). Control of preharvest and postharvest fruit rot in strawberry by *Metschnikowia fructicola*. *Biocontrol Science and Technology*, *14*(5), 513–521.

Karaca, H., Pérez-Gago, M. B., Taberner, V., & Palou, L. (2014). Evaluating food additives as antifungal agents against *Monilinia fructicola* in vitro and in hydroxypropyl methylcellulose-lipid composite edible coatings for plums. *International Journal of Food Microbiology*, *179*, 72–9.

Khem, S., Woo, M. W., Small, D. M., Chen, X. D., & May, B. K. (2015). Agent selection and protective effects during single droplet drying of bacteria. *Food Chemistry*, *166*, 206–214.

Kim, H. J., Cho, Y. H., Bae, E. K., Shin, T. S., Choi, S. W., Choi, K. H., & Park, J. (2005). Development of W/O/W multiple emulsion formulation containing *Burkholderia gladioli*. *Journal of Microbiology and Biotechnology*, *15*(1), 29–34.

Kinay, P., & Yildiz, M. (2008). The shelf life and effectiveness of granular formulations of *Metschnikowia pulcherrima* and *Pichia guilliermondii* yeast isolates that control postharvest decay of citrus fruit. *Biological Control*, *45*(3), 433–440.

Kota, V. R., Kulkarni, S., & Hegde, Y. R. (2006). Postharvest diseases of mango and their biological management. *Journal of Plant Disease Science*, *1*(2), 186–188.

Kowalczyk, D., & Baraniak, B. (2011). Effects of plasticizers, pH and heating of film-forming solution on the properties of pea protein isolate films. *Journal of Food Engineering*, *105*(2), 295–305.

Kris-Etherton, P. M., Hecker, K. D., Bonanome, A., Coval, S. M., Binkoski, A. E., Hilpert, K. F., Griel, A. E., & Etherton, T. D. (2002). Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *The American Journal of Medicine*, *113*(9), 71–88.

Krochta, J. M. (1997). Edible composite moisture-barrier films. In B. Blakistone (Ed.), *Packaging Yearbook: 1996* (pp. 38–54). Washington DC (USA).

Kwasiborski, A., Bajji, M., Renaut, J., Delaplace, P., & Jijakli, M. H. (2014). Identification of metabolic pathways expressed by *Pichia anomala* Kh6 in the presence of the pathogen *Botrytis cinerea* on apple: New possible targets for biocontrol improvement. *Plos One*, *9*(3), 1-12.

Ky, I., Lorrain, B., Jourdes, M., Pasquier, G., Fermaud, M., Geny, L., Rey, P., Doneche, B., & Teissedre, P. L. (2012). Assessment of grey mould (*Botrytis cinerea*) impact on

phenolic and sensory quality of Bordeaux grapes, musts and wines for two consecutive vintages. *Australian Journal of Grape and Wine Research*, *18*(215-226).

Lacroix, M., & Vu, K. D. (2014). *Edible Coating and Film Materials: Proteins*. (J. H. Han, Ed.)*Innovations in Food Packaging: Second Edition*. London (UK): Elsevier.

Lahlali, R., Brostaux, Y., & Jijakli, M. H. (2011). Control of apple blue mold by the antagonistic yeast *Pichia anomala* strain K: screening of UV protectants for preharvest application. *Plant Disease*, *95*(March), 311–316.

Lahlali, R., Hamadi, Y., Drider, R., Misson, C., El Guilli, M., & Jijakli, M. H. (2014). Control of citrus blue mold by the antagonist yeast *Pichia guilliermondii* Z1: Compatibility with commercial fruit waxes and putative mechanisms of action. *Food Control*, *45*, 1–7.

Laitila, A., Sarlin, T., Kotaviita, E., Huttunen, T., Home, S., & Wilhelmson, A. (2007). Yeasts isolated from industrial maltings can suppress *Fusarium* growth and formation of gushing factors. *Journal of Industrial Microbiology and Biotechnology*, *34*, 701–713.

Larena, I., De Cal, A., & Melgarejo, P. (2007). Effects of stabilizers on shelf-life of *Epicoccum nigrum* formulations and their relationship with biocontrol of postharvest brown rot by Monilinia of peaches. *Journal of Applied Microbiology*, *102*, 570–582.

Larena, I., Melgarejo, P., & De Cal, A. (2003). Drying of conidia of *Penicillium oxalicum*, a biological control agent against Fusarium wilt of tomato. *Journal of Phytopathology*, *151*(11-12), 600–606.

Larena, I., & Melgarejo, P. (2009). Development of a new strategy for monitoring *Epicoccum nigrum* 282, a biological control agent used against brown rot caused by *Monilinia* spp. in peaches. *Postharvest Biology and Technology*, *54*(2), 63–71.

Latorre, B., Spadaro, I., & Rioja, M. (2002). Occurrence of resistant strains of *Botrytis cinerea* to anilinopyrimidine fungicides in table grapes in Chile. *Crop Protection*, *21*(10), 957–961.

Lee, G.-W., Ko, J.-A., Oh, B.-T., Choi, J.-R., Lee, K.-J., Chae, J.-C., & Kamala-Kannan, S. (2012). Biological control of postharvest diseases of apples, peaches and nectarines by *Bacillus subtilis* S16 isolated from halophytes rhizosphere. *Biocontrol Science and Technology*, *22*(3), 351–361.

Lee, J. P., Lee, S. W., Kim, C. S., Son, J. H., Song, J. H., Lee, K. Y., Kim, H. J., Jung, S. J., & Moon, B. J. (2006). Evaluation of formulations of *Bacillus licheniformis* for the biological control of tomato gray mold caused by *Botrytis cinerea*. *Biological Control*, *37*(3), 329–337.

Leroux, P., Fritz, R., Debieu, D., Albertini, C., Lanen, C., Bach, J., Gredt, M., & Chapeland, F. (2002). Mechanisms of resistance to fungicides in field strains of *Botrytis cinerea*. *Pest Management Science*, *58*(9), 876–888.

Leslie, S. B., Israeli, E., Lighthart, B., Crowe, J. H., & Crowe, L. M. (1995). Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying, *61*(10), 3592–3597. *Applied and environmental microbiology*, *61*(10), 3592–3597.

Lewis, J. A., Fravel, D. R., Lumsden, R. D., & Shasha, B. S. (1995). Application of biocontrol fungi in granular formulation of pregelatinized starch-flour to control damping off diseases caused by *Rhizoctonia solani*. *Biological Control*, *5*, 397-404.

Li, B. Q., & Tian, S. P. (2006). Effects of trehalose on stress tolerance and biocontrol efficacy of *Cryptococcus laurentii*. *Journal of Applied Microbiology*, *100*(4), 854–861.

Li, H., Wang, Y., Liu, F., Yang, Y., Wu, Z., Cai, H., Zhang, Q., Wang, Y., & Li, P. (2015). Effects of chitosan on control of postharvest blue mold decay of apple fruit and the possible mechanisms involved. *Scientia Horticulturae*, *186*, 77–83.

Li, Y., Han, L. R., Zhang, Y., Fu, X., Chen, X., Zhang, L., Mei, R., & Wang, Q. (2013). Biological control of apple ring rot on fruit by *Bacillus amyloliquefaciens* 9001. *Plant Pathology Journal*, *29*(2), 168–173.

Lima, G., Castoria, R., De Curtis, F., Raiola, A., Ritieni, A., & De Cicco, V. (2011). Postharvest biology and technology integrated control of blue mould using new

fungicides and biocontrol yeasts lowers levels of fungicide residues and patulin contamination in apples. *Postharvest Biology and Technology*, *60*(2), 164–172.

Lima, J. R., Gondim, D. M. F., Oliveira, J. T. a, Oliveira, F. S. a, Gonçalves, L. R. B., & Viana, F. M. P. (2013). Use of killer yeast in the management of postharvest papaya anthracnose. *Postharvest Biology and Technology*, *83*, 58–64.

Liu, J., Tian, S. P., Li, B. Q., & Qin, G. Z. (2009). Enhancing viability of two biocontrol yeasts in liquid formulation by applying sugar protectant combined with antioxidant. *BioControl*, *54*(6), 817–824.

Liu, J., Wisniewski, M., Droby, S., Norelli, J., Hershkovitz, V., Tian, S., & Farrell, R. (2012). Increase in antioxidant gene transcripts, stress tolerance and biocontrol efficacy of *Candida oleophila* following sublethal oxidative stress exposure. *FEMS Microbiology Ecology*, *80*(3), 578–590.

Liu, J., Sui, Y., Wisniewski, M., Droby, S., & Liu, Y. (2013). Review: Utilization of antagonistic yeasts to manage postharvest fungal diseases of fruit. *International Journal of Food Microbiology*, *167*(2), 153–160.

Liu, J., Wisniewski, M., Droby, S., Tian, S., Hershkovitz, V., & Tworkoski, T. (2011). Effect of heat shock treatment on stress tolerance and biocontrol efficacy of *Metschnikowia fructicola*. *FEMS Microbiology Ecology*, *76*(1), 145–155.

Liu, S. X. (2009). Antimicrobial coatings for ensuring safety of fresh produces. *Fresh Produce*, *3*(1), 73–79.

Liu, X., Wang, J., Gou, P., Mao, C., Zhu, Z.-R., & Li, H. (2007). In vitro inhibition of postharvest pathogens of fruit and control of gray mold of strawberry and green mold of citrus by aureobasidin A. *International Journal of Food Microbiology*, *119*(3), 223–9.

Ma, W., Tang, C.-H., Yang, X.-Q., & Yin, S.-W. (2013). Fabrication and characterization of kidney bean (*Phaseolus vulgaris L.*) protein isolate–chitosan composite films at acidic pH. *Food Hydrocolloids*, *31*(2), 237–247.

Manwar, A. V., Khandelwal, S. R., Chaudhari, B. L., Meyer, J. M., & Chincholkar, S. B. (2004). Siderophore production by a marine *Pseudomonas aeruginosa* and its antagonistic action against phytopathogenic fungi. *Applied Biochemistry and Biotechnology*, *118*(1), 243–251.

Marín, A., Cháfer, M., Atarés, L., Chiralt, A., Torres, R., Usall, J., & Teixidó, N. (2016). Effect of different coating-forming agents on the efficacy of the biocontrol agent *Candida sake* CPA-1 for control of *Botrytis cinerea* on grapes. *Biological Control*, *96*, 108–119.

Masamba, K., Li, Y., Hategekimana, J., Ma, J., & Zhong, F. (2016). Effect of drying temperature and pH alteration on mechanical and water barrier properties of transglutaminase cross linked zein–oleic acid composite films. *LWT - Food Science and Technology*, *65*, 518–531.

Masih, E. I., & Paul, B. (2002). Secretion of β-1,3-glucanases by the yeast *Pichia membranaefaciens* and its possible role in the biocontrol of *Botrytis cinerea* causing grey mold disease of the grapevine. *Current Microbiology*, *44*(6), 391–395.

McGuire, R. G. (2000). Population dynamics of postharvest decay antagonists growing epiphytically and within wounds on grapefruit. *Phytopathology*, *90*(11), 1217–1223.

Mcguire, R. G., & Baldwin, E. A. (1994). Compositions of cellulose coatings affect populations of yeasts in the liquid formulation and on coated grapefruits. *Proceedings of the Florida State Horticultural Society*, *107*, 293–297.

McGuire, R. G., & Dimitroglou, D. A. (1999). Evaluation of shellac and sucrose ester fruit coating formulations that support biological control of post-harvest grapefruit decay. *Biocontrol Science and Technology*, *9*(1), 53–65.

Mehyar, G. F., Al-Qadiri, H. M., Abu-Blan, H. A., & Swanson, B. G. (2011). Antifungal effectiveness of potassium sorbate incorporated in edible coatings against spoilage molds of apples, cucumbers, and tomatoes during refrigerated storage. *Journal of Food Science*, *76*(3), 210–217.

Mejri, D., Gamalero, E., & Souissi, T. (2013). Formulation development of the deleterious rhizobacterium *Pseudomonas trivialis* X33d for biocontrol of brome (*Bromus diandrus*) in durum wheat. *Journal of Applied Microbiology*, *114*(1), 219–28.

Melgarejo-Flores, B. G., Ortega-Ramírez, L. A., Silva-Espinoza, B. A., González-Aguilar, G. A., Miranda, M. R. A., & Ayala-Zavala, J. F. (2013). Antifungal protection and antioxidant enhancement of table grapes treated with emulsions, vapors, and coatings of cinnamon leaf oil. *Postharvest Biology and Technology*, *86*, 321–328.

Melin, P., Hakansson, S., Eberhard, T. H., & Schnürer, J. (2006). Survival of the biocontrol yeast *Pichia anomala* after long-term storage in liquid formulations at different temperatures, assessed by flow cytometry. *Journal of Applied Microbiology*, *100*(2), 264–271.

Melin, P., Håkansson, S., & Schnürer, J. (2007). Optimisation and comparison of liquid and dry formulations of the biocontrol yeast *Pichia anomala* J121. *Applied Microbiology and Biotechnology*, *73*(5), 1008–1016.

Melin, P., Schnürer, J., & Håkansson, S. (2011). Formulation and stabilisation of the biocontrol yeast *Pichia anomala*. *Antonie van Leeuwenhoek*, *99*(1), 107–12.

Meng, X.-H., Qin, G.-Z., & Tian, S.-P. (2010). Influences of preharvest spraying *Cryptococcus laurentii* combined with postharvest chitosan coating on postharvest diseases and quality of table grapes in storage. *LWT - Food Science and Technology*, *43*(4), 596–601.

Metz, C., Oerke, E. C., & Dehne, H. W. (2001). Biological control of grey mould (*Botrytis cinerea*) with the antagonist *Ulocladium atrum*. *Mededelingen (Rijksuniversiteit Te Gent. Fakulteit van de Landbouwkundige En Toegepaste Biologische Wetenschappen)*, *67*(2), 353–359.

Miao, S., Mills, S., Stanton, C., Fitzgerald, G. F., Roos, Y., & Ross, R. P. (2008). Effect of disaccharides on survival during storage of freeze dried probiotics. *Dairy Science and Technology*, *88*(1), 19–30.

Mikani, A., Etebarian, H. R., Aminian, H., & Alizadeh, A. (2007). Interaction between *Pseudomonas fluorescens* isolates and thiabendazole in the control of gray mold of apple. *Pakistan Journal of Biological Sciences*, *10*(13), 2172–2177.

Mohammadi, A., Hashemi, M., & Hosseini, S. M. (2015). The control of Botrytis fruit rot in strawberry using combined treatments of chitosan with *Zataria multiflora* or *Cinnamomum zeylanicum* essential oil. *Journal of Food Science and Technology*, *52*(11), 7441–7448.

Mokhtarnejada, L., Etebariana, H. R., Fazelib, M. R., & Jamalifarb, H. (2011). Evaluation of different formulations of potential biocontrol yeast isolates efficacy on apple blue mold at storage condition. *Archives of Phytopathology and Plant Protection*, *44*(10), 970–980.

Mokiou, S., & Magan, N. (2008). Physiological manipulation and formulation of the biocontrol yeast *Pichia anomala* for control of *Penicillium verrucosum* and ochratoxin A contamination of moist grain. *Biocontrol Science and Technology*, *18*(10), 1063–1073.

Monedero, F. M., Fabra, M. J., Talens, P., & Chiralt, A. (2009). Effect of oleic acid-beeswax mixtures on mechanical, optical and water barrier properties of soy protein isolate based films. *Journal of Food Engineering*, *91*(4), 509–515.

Moretto, C., Lima Cervantes, A. L., Filho, A. ., & Kupper, K. C. (2014). Integrated control of green mold to reduce chemical treatment in post-harvest citrus fruits. *Scientia Horticulturae*, *165*, 433–438.

Morgan, C. A., Herman, N., White, P. A., & Vesey, G. (2006). Preservation of micro-organisms by drying: A review. *Journal of Microbiological Methods*, *66*(2), 183–193.

Morillon, V., Debeafort, F., Blond, G., Capelle, M., & Voilley, A. (2002). Factors affecting the moisture permeability of lipid-based edible films: A review. *Critical Reviews in Food Science and Nutrition*, *42*, 67–89.

Mounir, R., Durieux, A., Bodo, E., Allard, C., Simon, J.-P., Achbani, E.-H., El-Jaafari, S., Douira, A., & Jijakli, M.-H. (2007). Production, formulation and antagonistic activity of the biocontrol like-yeast *Aureobasidium pullulans* against *Penicillium expansum*.

Muangdech, A. (2016). Research on using natural coating materials on the storage life of mango fruit cv. Nam Dok Mai and technology dissemination. *Walailak Journal of Science and Technology*, *13*(3), 205–220.

Nadim, Z., Ahmadi, E., Sarikhani, H., & Amiri Chayjan, R. (2015). Effect of methylcellulose-based Edible coating on strawberry fruit's quality maintenance during storage. *Journal of Food Processing and Preservation*, *39*(1), 80–90.

Nally, M. C., Pesce, V. M., Maturano, Y. P., Noz, C. J. M., Combina, M., Toro, M. E., Castellanos De Figueroa, L. I. & Vazquez, F. (2012). Biocontrol of *Botrytis cinerea* in table grapes by non-pathogenic indigenous *Saccharomyces cerevisiae* yeasts isolated from viticultural environments in Argentina. *Postharvest Biology and Technology*, *64*, 40–48.

Navarro-Tarazaga, M. L., Sothornvit, R., & Pérez-Gago, M. B. (2008a). Effect of plasticizer type and amount on hydroxypropyl methylcellulose- beeswax edible film properties and postharvest quality of coated plums (Cv. Angeleno). *Journal of Agricultural and Food Chemistry*, *56*(20), 9502–9509.

Navarro-Tarazaga, M. L. L., Del Río, M. A., Krochta, J. M., & Pérez-Gago, M. B. (2008b). Fatty acid effect on hydroxypropyl methylcellulose-beeswax edible film properties and postharvest quality of coated "ortanique" mandarins. *Journal of Agricultural and Food Chemistry*, *56*(22), 10689–10696.

Nisperos-Carriedo, M. O., Baldwin, E. A., & Shaw, P. E. (1992). Development of edible coating for extending post-harvest life of selected fruits and vegetables. *Proceedings of Florida State Horticultural Science*, *104*, 122–125.

Nobile, C. J., & Mitchell, A. P. (2005). Regulation of cell-surface genes and biofilm formation by the *C. albicans* transcription factor Bcr1p. *Current Biology : CB*, *15*(12), 1150–5.

Nunes, C. A. (2012). Biological control of postharvest diseases of fruit. *European Journal of Plant Pathology*, *133*(1), 181–196.

Nunes, C., Usall, J., Teixidó, N., & Vias, I. (2001). Biological control of postharvest pear diseases using a bacterium, *Pantoea agglomerans* CPA-2. *International Journal of Food Microbiology*, *70*, 53–61.

Nunes, C. A., Usall, J., Teixidó, N., Torres, R., & Viñas, I. (2002a). Control of *Penicillium expansum* and *Botrytis cinerea* on apples and pears with the combination of *Candida sake* and *Pantoea agglomerans*. *Journal of Food Protection*, *65*(1), 178–184.

Nunes, Usall, J., Teixidó, N., Abadias, M., & Viñas, I. (2002b). Improved Control of Postharvest Decay of Pears by the Combination of *Candida sake* (CPA-1) and Ammonium Molybdate. *Phytopathology*, *92*(3), 281–7.

Nunes, C. A., Bajji, M., Stepien, V., Manso, T., Torres, R., Usall, J., & Jijakli, M. H. (2008). Development and application of a SCAR marker to monitor and quantify populations of the postharvest biocontrol agent *Pantoea agglomerans* CPA-2. *Postharvest Biology and Technology*, *47*(3), 422–428.

Ortega-Toro, R., Jiménez, A., Talens, P., & Chiralt, A. (2014a). Effect of the incorporation of surfactants on the physical properties of corn starch films. *Food Hydrocolloids*, *38*, 66–75.

Ortega-Toro, R., Jiménez, A., Talens, P., & Chiralt, A. (2014b). Properties of starch-hydroxypropyl methylcellulose based films obtained by compression molding. *Carbohydrate Polymers*, *109*, 155–165.

Ouattara, B., Canh, L. ., Vachon, C., Mateescu, M. ., & Lacroix, M. (2002). Use of γ-irradiation cross-linking to improve the water vapor permeability and the chemical stability of milk protein films. *Radiation Physics and Chemistry*, *63*(3-6), 821–825.

Palou, L., Valencia-Chamorro, S., & Pérez-Gago, M. (2015). Antifungal Edible Coatings for Fresh Citrus Fruit: A Review. *Coatings*, *5*(4), 962–986.

Pan, S. Y., Chen, C. H., & Lai, L. S. (2013). Effect of tapioca starch/decolorized Hsian-tsao keaf gum-based active coatings on the qualities of fresh-cut apples. *Food and Bioprocess Technology*, *6*(8), 2059–2069.

Panebianco, A., Castello, I., Cirvilleri, G., Perrone, G., Epifani, F., Ferrara, M., Polizzi, G., Walters, D.R, & Vitale, A. (2015). Detection of Botrytis cinerea field isolates with multiple fungicide resistance from table grape in Sicily. *Crop Protection*, *77*, 65–73.

Parafati, L., Vitale, A., Restuccia, C., & Cirvilleri, G. (2015). Biocontrol ability and action mechanism of food-isolated yeast strains against *Botrytis cinerea* causing post-harvest bunch rot of table grape. *Food Microbiology*, *47*, 85–92.

Parafati, L., Vitale, A., Restuccia, C., & Cirvilleri, G. (2016). The effect of locust bean gum (LBG)-based edible coatings carrying biocontrol yeasts against *Penicillium digitatum* and *Penicillium italicum* causal agents of postharvest decay of mandarin fruit. *Food Microbiology*, *58*, 87–94.

Park, S. I., Stan, S. D., Daeschel, M. A., & Zhao, Y. (2005). Antifungal coatings on fresh strawberries (*Fragaria* x *ananassa*) to control mold growth during cold storage. *Journal of Food Science*, *70*(4).

Parsek, M. R., & Greenberg, E. P. (2005). Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends in Microbiology*, *13*(1), 27–33.

Pastor, C., Sánchez-González, L., Marcilla, A., Chiralt, A., Cháfer, M., & González-Martínez, C. (2011). Quality and safety of table grapes coated with hydroxypropylmethylcellulose edible coatings containing propolis extract. *Postharvest Biology and Technology*, *60*(1), 64–70.

Peeran, M. F., Nagendran, K., Gandhi, K., Raguchander, T., & Prabakar, K. (2014). Water in oil based PGPR formulation of *Pseudomonas fluorescens* (FP7) showed enhanced resistance against *Colletotrichum musae*. *Crop Protection*, *65*, 186–193.

Peighambardoust, S. H., Golshan Tafti, A., & Hesari, J. (2011). Application of spray drying for preservation of lactic acid starter cultures: A review. *Trends in Food Science and Technology*, *22*(5), 215–224.

Peng, G., & Sutton, J. C. (1991). Evaluation of microorganisms for biocontrol of *Botrytis cinerea* in strawberry. *Canadian Journal of Plant Pathology*, *13*(3), 247–257.

Perdones, A. (2015). Antifungal chitosan-based films and coatings containing essential oils for fruit applications. Valencia: Universitat Politècnica de València, PhD Thesis.

Perdones, A., Sánchez-González, L., Chiralt, A., & Vargas, M. (2012). Effect of chitosan-lemon essential oil coatings on storage-keeping quality of strawberry. *Postharvest Biology and Technology*, *70*, 32–41.

Peretto, G., Du, W. X., Avena-Bustillos, R. J., Berrios, J. D. J., Sambo, P., & McHugh, T. H. (2014). Optimization of antimicrobial and physical properties of alginate coatings containing carvacrol and methyl cinnamate for strawberry application. *Journal of Agricultural and Food Chemistry*, *62*(4), 984–990.

Pérez, L. M., Piccirilli, G. N., Delorenzi, N. J., & Verdini, R. A. (2016a). Effect of different combinations of glycerol and/or trehalose on physical and structural properties of whey protein concentrate-based edible films. *Food Hydrocolloids*, *56*, 352–359.

Perez, V., Felix, M., Romero, A., & Guerrero, A. (2016b). Characterization of pea protein-based bioplastics processed by injection moulding. *Food and Bioproducts Processing*, *97*, 100–108.

Pérez-Gago, M. B., & Rhim, J. W. (2014). Edible coating and film materials: lipid bilayers and lipid emulsions. In J. H. Han (Ed.), *Innovations in Food Packaging* (2nd ed., pp. 325–350). London (UK): Elsevier.

Pérez-Gago, M. B., & Krochta, J. M. (2001). Lipid particle size effect on water vapor permeability and mechanical properties of whey protein/beeswax emulsion films. *Journal of Agricultural and Food Chemistry*, *49*(2), 996–1002.

Piano, S., Neyrotti, V., Migheli, Q., & Gullino, M. L. (1997). Biocontrol capability of *Metschnikowia pulcherrima* against *Botrytis* postharvest rot of apple. *Postharvest Biology and Technology*, *11*(3), 131–140.

Platania, C., Restuccia, C., Muccilli, S., & Cirvilleri, G. (2012). Efficacy of killer yeasts in the biological control of *Penicillium digitatum* on Tarocco orange fruits (*Citrus sinensis*). *Food Microbiology*, *30*(1), 219–25.

Poddar, D., Das, S., Jones, G., Palmer, J., Jameson, G. B., Haverkamp, R. G., & Singh, H. (2014). Stability of probiotic *Lactobacillus paracasei* during storage as affected by the drying method. *International Dairy Journal*, *39*(1), 1–7.

Potjewijd, R., Nisperos, M. O., Burns, J. K., Parish, M., & Baldwin, E. A. (1995). Cellulose-based coatings as carriers for *Candida guillermondii* and *Debaryomyces* sp. in reducing decay of oranges. *HortScience*, *30*(7), 1417–1421.

Pu, L., Jingfan, F., Kai, C., Chao-an, L., & Yunjiang, C. (2014). Phenylethanol promotes adhesion and biofilm formation of the antagonistic yeast *Kloeckera apiculata* for the control of blue mold on citrus. *FEMS Yeast Research*, *14*(4), 536–546.

Pusey, P. L., & Wend, C. (2012). Potential of osmoadaptation for improving *Pantoea agglomerans* E325 as biocontrol agent for fire blight of apple and pear. *Biological Control*, *62*(1), 29–37.

Pusey, P. L., & Wilson, C. L. (1984). Postharvest biological control of stone fruit brown rot by *Bacillus subtilis*. *Plant Disease*, *68*, 753–756.

Qin, X., Xiao, H., Xue, C., Yu, Z., Yang, R., Cai, Z., & Si, L. (2015). Biocontrol of gray mold in grapes with the yeast *Hanseniaspora uvarum* alone and in combination with salicylic acid or sodium bicarbonate. *Postharvest Biology and Technology*, *100*, 160–167.

Qing, F., & Tian, S. P. (2001). Postharvest biological control of grey mold and blue mold on apple by *Cryptococcus albidus* (Saito) Skinner. *Postharvest Biology and Technology*, *21*(3), 341–350.

Quirós-Sauceda, A. E., Ayala-Zavala, J. F., Olivas, G. I., & González-Aguilar, G. A. (2014). Edible coatings as encapsulating matrices for bioactive compounds: a review. *Journal of Food Science and Technology*, *51*(September), 1–12.

Rahman, M. A., Mahmud, T. M. M., Kadir, J., Abdul Rahman, R., & Begum, M. M. (2009). Enhancing the efficacy of *Burkholderia cepacia* B23 with calcium chloride and chitosan to control anthracnose of papaya during storage. *The Plant Pathology Journal*, *25*(4), 361–368.

Raspor, P., Miklič-Milek, D., Avbelj, M., & Čadež, N. (2010). Biocontrol of grey mould disease on grape caused by *Botrytis cinerea* with autochthonous wine yeasts. *Food Technology and Biotechnology*, *48*(3), 336–343.

Ren, X., Kong, Q., Wang, H., Yu, T., Tang, Y. J., Zhou, W. W., & Zheng, X. (2012). Control of apple blue mold by *Pichia pastoris* recombinant strains expressing cecropin A. *Bioprocess and Biosystems Engineering*, *35*(5), 761–767.

Rezvani, E., Schleining, G., Sümen, G., & Taherian, A. R. (2013). Assessment of physical and mechanical properties of sodium caseinate and stearic acid based film-forming emulsions and edible films. *Journal of Food Engineering*, *116*(2), 598–605.

Rhim, J. W., & Shellhammer, T. (2005). Lipid-based edible films and coatings. In J. H. Han (Ed.), *Innovations in Food Packaging* (1st ed., pp. 362–383). Amsterdam (The Netherlands): Elsevier.

Rhodes, D. J. (1993). Formulation of biological control agents. In D. G. Jones (Ed.), *Exploitation of microorganisms* (pp. 411–439). London (UK): Chapman & Hall.

Rivera-Ávalos, S., Martínez-Peniche, R. Á., Soto-Muñoz, L., & Chávaro-Ortiz, M. D. S. (2012). Modos de acción de cuatro cepas de levaduras antagónicas contra *Penicillium expansum* link en manzana. *Revista Chapingo Serie Horticultura*, *XVIII*(2), 227–238.

Roberts, R. G. (1990). Postharvest biological control of grey mould of apple by Cryptococcus laurentii.pdf. *Phytopathology*, *80*, 526–530.

Robiglio, A., Sosa, M. C., Lutz, M. C., Lopes, C. A., & Sangorrín, M. P. (2011). Yeast biocontrol of fungal spoilage of pears stored at low temperature. *International Journal of Food Microbiology*, *147*(3), 211–6.

Rocca-Smith, J. R., Marcuzzo, E., Karbowiak, T., Centa, J., Giacometti, M., Scapin, F., Venir, E., Sensidoni, A., & Debeaufort, F. (2016). Effect of lipid incorporation on functional properties of wheat gluten based edible films. *Journal of Cereal Science*, *69*, 275–282.

Rodríguez, M., Osés, J., Ziani, K., & Maté, J. I. (2006). Combined effect of plasticizers and surfactants on the physical properties of starch based edible films. *Food Research International*, *39*(8), 840–846.

Romanazzi, G., Smilanick, J. L., Feliziani, E., & Droby, S. (2016). Integrated management of postharvest gray mold on fruit crops. *Postharvest Biology and Technology*, *113*, 69–76.

Ruiz-Moyano, S., Martín, A., Villalobos, M. C., Calle, A., Serradilla, M. J., Córdoba, M. G., & Hernández, A. (2016). Yeasts isolated from figs (*Ficus carica* L.) as biocontrol agents of postharvest fruit diseases. *Food Microbiology*, *57*, 45–53.

Sánchez-González, L., Pastor, C., Vargas, M., Chiralt, A., González-Martínez, C., & Cháfer, M. (2011). Effect of hydroxypropylmethylcellulose and chitosan coatings with and without bergamot essential oil on quality and safety of cold-stored grapes. *Postharvest Biology and Technology*, *60*(1), 57–63.

Sánchez-González, L., Quintero Saavedra, J. I., & Chiralt, A. (2013). Physical properties and antilisterial activity of bioactive edible films containing *Lactobacillus plantarum*. *Food Hydrocolloids*, *33*(1), 92–98.

Sanchís, E., González, S., Ghidelli, C., Sheth, C. C., Mateos, M., Palou, L., & Pérez-Gago, M. B. (2016). Browning inhibition and microbial control in fresh-cut persimmon (*Diospyros kaki* Thunb. cv. Rojo Brillante) by apple pectin-based edible coatings. *Postharvest Biology and Technology*, *112*, 186–193.

Santivarangkna, C., Kulozik, U., & Foerst, P. (2007). Alternative drying processes for the industrial preservation of lactic acid starter cultures. *Biotechnology Progress*, *23*, 302–315.

Santivarangkna, C., Kulozik, U., & Foerst, P. (2008a). Inactivation mechanisms of lactic acid starter cultures preserved by drying processes. *Journal of Applied Microbiology*, *105*(1), 1–13.

Santivarangkna, C., Higl, B., & Foerst, P. (2008b). Protection mechanisms of sugars during different stages of preparation process of dried lactic acid starter cultures. *Food Microbiology*, *25*(3), 429–441.

Santos, A., San Mauro, M., Bravo, E., & Marquina, D. (2009). PMKT2, a new killer toxin from *Pichia membranifaciens*, and its promising biotechnological properties for control of the spoilage yeast *Brettanomyces bruxellensis*. *Microbiology (Reading, England)*, *155*(Pt 2), 624–34.

Saravanakumar, D., Ciavorella, A., Spadaro, D., Garibaldi, A., & Gullino, M. L. (2008). *Metschnikowia pulcherrima* strain MACH1 outcompetes *Botrytis cinerea*, *Alternaria alternata* and *Penicillium expansum* in apples through iron depletion. *Postharvest Biology and Technology*, *49*(1), 121–128.

Saravanakumar, D., Spadaro, D., Garibaldi, A., & Gullino, M. L. (2009). Detection of enzymatic activity and partial sequence of a chitinase gene in *Metschnikowia pulcherrima* strain MACH1 used as post-harvest biocontrol agent. *European Journal of Plant Pathology*, *123*(2), 183–193.

Schena, L., Nigro, F., Pentimone, I., Ligorio, A., & Ippolito, A. (2003). Control of postharvest rots of sweet cherries and table grapes with endophytic isolates of *Aureobasidium pullulans*. *Postharvest Biology and Technology*, *30*(3), 209–220.

Schisler, D. A., Slininger, P. J., Behle, R. W., & Jackson, M. A. (2004). Formulation of *Bacillus* spp. for biological control of plant diseases. *Phytopathology*, *94*(11), 1267–1271.

Schisler, D. A., Slininger, P. J., & Olsen, N. L. (2016). Appraisal of selected osmoprotectants and carriers for formulating Gram-negative biocontrol agents active against *Fusarium* dry rot on potatoes in storage. *Biological Control*, *98*, 1–10.

Seligra, P. G., Medina Jaramillo, C., Famá, L., & Goyanes, S. (2016). Biodegradable and non-retrogradable eco-films based on starch-glycerol with citric acid as crosslinking agent. *Carbohydrate Polymers*, *138*, 66–74.

Shao, X., Cao, B., Xu, F., Xie, S., Yu, D., & Wang, H. (2015). Effect of postharvest application of chitosan combined with clove oil against citrus green mold. *Postharvest Biology and Technology*, *99*, 37–43.

Sharma, R. R., Singh, D., & Singh, R. (2009). Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: A review. *Biological Control*, *50*(3), 205–221.

Sharma, S., & Rao, T. V. R. (2015). Xanthan gum based edible coating enriched with cinnamic acid prevents browning and extends the shelf-life of fresh-cut pears. *LWT - Food Science and Technology*, *62*(1), 791–800.

Sivakumar, D., Wijeratnam, R. S. W., Marikar, F. M. M. T., Abeyesekere, M., & Wijesundera, R. L. C. (2001). Antagonistic effect of *Trichoderma harzianum* on postharvest pathogens of rambutan (*Nephelium lappaceum*). *Acta Horticulturae, 553*, 389–392.

Smith, D., & Onions, D. H. S. (1983). *The Preservation and Maintenance of Living Fungi*. Kew (UK): Commonwealth Mycological Institute.

Soto-Muñoz, L., Teixidó, N., Usall, J., Viñas, I., Abadias, M., & Torres, R. (2014). Technology Molecular tools applied to identify and quantify the biocontrol agent *Pantoea agglomerans* CPA-2 in postharvest treatments on oranges. *Postharvest Biology and Technology*, *100*, 151–159.

Soto-Muñoz, L., Torres, R., Usall, J., Viñas, I., Solsona, C., & Teixidó, N. (2015). DNA-based methodologies for the quantification of live and dead cells in formulated

biocontrol products based on *Pantoea agglomerans* CPA-2. *International Journal of Food Microbiology*, *210*, 79–83.

Spadaro, D., & Gullino, M. L. (2004). State of the art and future prospects of the biological control of postharvest fruit diseases. *International Journal of Food Microbiology*, *91*, 184–194.

Spadaro, D., Ciavorella, A. A., Lopez-Reyes, J. G., Garibaldi, A., & Gullino, M. L. (2010). Effect of culture age, protectants, and initial cell concentration on viability of freeze-dried cells of *Metschnikowia pulcherrima*. *Canadian Journal of Microbiology*, *56*(10), 809–815.

Spadaro, D., & Droby, S. (2016). Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists. *Trends in Food Science & Technology*, *47*, 39–49.

Sperandio, E. M., do Vale, H. M. M., & Moreira, G. A. M. (2015). Yeasts from native Brazilian Cerrado plants: Occurrence, diversity and use in the biocontrol of citrus green mould. *Fungal Biology*, *119*(11), 984–93.

Stanbury, P. F., Whitaker, A., & Hall, S. J. (1995). Media for industrial fermentations. In *Principles of fermentation technology* (pp. 93–121). Oxford (UK): Pergamon Press.

Stauffer, C. E. (1999). *Emulsifiers. Practical guides for the food industry*. Minnesota (US): American Association of Cereal Chemists.

Sui, Y., Wisniewski, M., Droby, S., & Liu, J. (2015). Responses of yeast biocontrol agents to environmental stress. *Applied and Environmental Microbiology*, *81*(9), 2968–2975.

Sun, W. Q., & Leopold, A. C. (1997). Cytoplasmic vitrification and survival of anhydrobiotic organisms. *Comparative Biochemistry and Physiology*, *117*(3), 327–333.

Shweleft, R. L. (1986). Postharvest treatment for extending the shelf-life of fruit and vegetables. *Food Technology*, *40*,70– 80

Synowiec, A., Gniewosz, M., Kraśniewska, K., Przybył, J. L., Bączek, K., & Węglarz, Z. (2014). Antimicrobial and antioxidant properties of pullulan film containing sweet basil extract and an evaluation of coating effectiveness in the prolongation of the shelf life of apples stored in refrigeration conditions. *Innovative Food Science and Emerging Technologies*, *23*, 171–181.

Teixidó, N., Viñas, I., Usall, J., & Magan, N. (1998a). Control of blue mold of apples by preharvest application of *Candida sake* grown in media with different water activity. Phytopathology, 88(9), 960–964.

Teixidó, N., Vinas, I., Usall, J., Sanchis, V., & Magan, N. (1998b). Ecophysiological responses of the biocontrol yeast *Candida sake* to water, temperature and pH stress. *Journal of Applied Microbiology*, *84*(2), 192–200.

Teixidó, N., Usall, J., & Viñas, I. (1999). Efficacy of preharvest and postharvest *Candida sake* biocontrol treatments to prevent blue mould on apples during cold storage. *International Journal of Food Microbiology*, *50*(3), 203–210.

Teixidó, N., Usall, J., Palou, L., Asensio, A., Nunes, C., & Viñas, I. (2001). Improving control of green and blue molds of oranges by combining *Pantoea agglomerans* (CPA-2) and sodium bicarbonate. European Journal of Plant Pathology.

Teixidó, N., Cañamás, T. P., Usall, J., Torres, R., Magan, N., & Viñas, I. (2005). Accumulation of the compatible solutes, glycine-betaine and ectoine, in osmotic stress adaptation and heat shock cross-protection in the biocontrol agent *Pantoea agglomerans* CPA-2. Letters in Applied Microbiology, 41(3), 248–252.

Teixidó, N., Cañamás, T. P., Abadias, M., Usall, J., Solsona, C., Casals, C., & Viñas, I. (2006). Improving low water activity and desiccation tolerance of the biocontrol agent *Pantoea agglomerans* CPA-2 by osmotic treatments. Journal of Applied Microbiology, 101(4), 927–937.

Teixidó, N., Torres, R., Viñas, I., Abadias, M., & Usall, J. (2011). Biological control of postharvest diseases in fruit and vegetables. In: Lacroix, C. (ed.), *Protective Cultures,*

Antimicrobial Metabolites and Bacteriophages for Food and Beverage Biopreservation (pp. 364-402). Sawston, Cambridge (UK): Woodhead Publishing Limited.

Tharanathan, R. N. (2003). Biodegradable films and composite coatings: Past, present and future. *Trends in Food Science and Technology*, 14(3), 71–78.

Tharanathan, R. N., & Kittur, F. (2003). Chitin — The undisputed biomolecule of great potential. *Critical Reviews in Food Science and Nutrition*, *43*(1), 61–87.

Thomsen, M. K., Jespersen, L., Sjøstrøm, K., Risbo, J., & Skibsted, L. H. (2005). Water activity - Temperature state diagram of amorphous lactose. *Journal of Agricultural and Food Chemistry*, *53*(23), 9182–9185.

Tian, S. P., Yao, H. J., Deng, X., Xu, X. B., Qin, G. Z., & Chan, Z. L. (2007). Characterization and expression of β-1,3-glucanase genes in jujube fruit induced by the microbial biocontrol agent *Cryptococcus laurentii*. *Phytopathology*, *97*(3), 260–268.

Torres, R., Usall, J., Teixidó, N., Abadias, M., & Viñas, I. (2003). Liquid formulation of the biocontrol agent *Candida sake* by modifying water activity or adding protectants. Journal of Applied Microbiology.

Torres, R., Nunes, C., García, J. M., Abadias, M., Viñas, I., Manso, T., Olmo, M., & Usall, J. (2007). Application of *Pantoea agglomerans* CPA-2 in combination with heated sodium bicarbonate solutions to control the major postharvest diseases affecting citrus fruit at several Mediterranean locations. *European Journal of Plant Pathology*, *118*, 73–83.

Torres, R., Teixidó, N., Usall, J., Abadias, M., Mir, N., Larrigaudiere, C., & Viñas, I. (2011). Anti-oxidant activity of oranges after infection with the pathogen *Penicillium digitatum* or treatment with the biocontrol agent *Pantoea agglomerans* CPA-2. *Biological Control*, *57*(2), 103–109.

Torres, R., Solsona, C., Viñas, I., Usall, J., Plaza, P., & Teixidó, N. (2014). Optimization of packaging and storage conditions of a freeze-dried *Pantoea agglomerans* formulation for controlling postharvest diseases in fruit. *Journal of Applied Microbiology*, *117*(1), 173–184.

Tripathi, P., & Dubey, N. (2004). Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. *Postharvest Biology and Technology*, *32*(3), 235–245.

Trivedi, P., Pandey, A., & Palni, L. M. S. (2005). Carrier-based preparations of plant growth-promoting bacterial inoculants suitable for use in cooler regions. *World Journal of Microbiology and Biotechnolog*y, 21, 941-945.

Tu, Q., Chen, J., & Guo, J. (2013). Control of postharvest blue mold of Nanfeng Mandarin by Application of Strain YS-1 *Paenibacillus brasilensis*. *Journal of Food Science*, *78*(6), 868–873.

Usall, J., Teixidó, N., Fons, E., & Viñas, I. (2000). Biological control of blue mould on apple by a strain of *Candida sake* under several controlled atmosphere conditions. *International Journal of Food Microbiology*, *58*(1-2), 83–92.

Usall, J., Teixidó, N., Torres, R., Ochoa de Eribe, X., & Vias, I. (2001). Pilot tests of *Candida sake* (CPA-1) applications to control postharvest blue mold on apple fruit. *Postharvest Biology and Technology*, *21*(2), 147–156.

Usall, J., Teixidó, N., Abadias, M., Torres, R., Cañamás, T. P., & Viñas, I. (2009). Improving formulation of biocontrol agents manipulating production process. In D. Prusky & M. L. Gullino (Eds.), *Post-harvest Pathology* (pp. 149–169). Springer.

Valencia-Chamorro, S. A., Pérez-Gago, M. B., Del Río, M. A., & Palou, L. (2009). Curative and preventive activity of hydroxypropyl methylcellulose-lipid edible composite coatings containing antifungal food additives to control citrus postharvest green and blue molds. *Journal of Agricultural and Food Chemistry*, *57*(7), 2770–2777.

Vargas, M., Albors, A., Chiralt, A., & González-Martínez, C. (2006). Quality of cold-stored strawberries as affected by chitosan-oleic acid edible coatings. *Postharvest Biology and Technology*, *41*(2), 164–171.

Vargas, M., Pastor, C., Albors, A., & Chiralt, A. (2008a). Development of edible coatings for fresh fruits and vegetables : possibilities and limitations. *Fresh Produce*, *2*(2), 32–40.

Vargas, M., Pastor, C., Chiralt, A., McClements, D. J., & González-Martínez, C. (2008b). Recent advances in edible coatings for fresh and minimally processed fruits. *Critical Reviews in Food Science and Nutrition*, *48*(6), 496–511.

Vargas, M., Albors, A., Chiralt, A., & González-Martínez, C. (2009). Characterization of chitosan-oleic acid composite films. *Food Hydrocolloids*, *23*(2), 536–547.

Vargas, M., Garrido, F., Zapata, N., & Tapia, M. (2012). Isolation and selection of epiphytic yeasts for biocontrol of *Botrytis cinerea* on table trapes. *Chilean Journal of Agricultural Research*, *72*(3), 332–337.

Vero, S., Mondino, P., Burgueño, J., Soubes, M., & Wisniewski, M. (2002). Characterization of biocontrol activity of two yeast strains from Uruguay against blue mold of apple. *Postharvest Biology and Technology*, *26*(1), 91–98.

Vero, S., Garmendia, G., González, M. B., Bentancur, O., & Wisniewski, M. (2013). Evaluation of yeasts obtained from Antarctic soil samples as biocontrol agents for the management of postharvest diseases of apple (*Malus* × *domestica*). *FEMS Yeast Research*. ht

Vero, S., Garmendia, G., González, M. B., Garat, M. F., & Wisniewski, M. (2009). *Aureobasidium pullulans* as a biocontrol agent of postharvest pathogens of apples in Uruguay. *Biocontrol Science and Technology*, *19*(10), 1033–1049. http://doi.org/10.1080/09583150903277738

Vieira, J. M., Flores-López, M. L., de Rodríguez, D. J., Sousa, M. C., Vicente, A. A., & Martins, J. T. (2016). Effect of chitosan–Aloe vera coating on postharvest quality of blueberry (*Vaccinium corymbosum*) fruit. *Postharvest Biology and Technology*, *116*, 88–97.

Villalobos-Carvajal, R., Hernández-Muñoz, P., Albors, A., & Chiralt, A. (2009). Barrier and optical properties of edible hydroxypropyl methylcellulose coatings containing surfactants applied to fresh cut carrot slices. *Food Hydrocolloids*, *23*(2), 526–535.

Viñas, I., Usall, J., Teixidó, N., & Sanchis, V. (1998). Biological control of major postharvest pathogens on apple with *Candida sake*. *International Journal of Food Microbiology*, *40*(1-2), 9–16.

Wang, Y., He, S., Xia, J., Yu, T., & Zheng, X. (2014). Acid adaptation and biocontrol efficacy of antagonistic marine yeast Rhodosporidium paludigenum. Annals of Microbiology, 64(2), 503–508. http://doi.org/10.1007/s13213-013-0681-2

Wang, Y., Yu, T., Xia, J., Yu, D., Wang, J., & Zheng, X. (2010). Biocontrol of postharvest gray mold of cherry tomatoes with the marine yeast *Rhodosporidium paludigenum*. *Biological Control*, *53*(2), 178–182.

Wang, Y., Tang, F., Xia, J., Yu, T., Wang, J., Azhati, R., & Zheng, X. D. (2011). A combination of marine yeast and food additive enhances preventive effects on postharvest decay of jujubes (*Zizyphus jujuba*). *Food Chemistry*, *125*(3), 835–840.

Wilson, C. L., & Wisniewski, M. E. (1989). Biological control of postharvest diseases of fruits and vegetables: an emerging technology. *Annual Review of Phytopathology*, *27*, 425–441.

Wilson, C. L., Wisniewski, M. E., Droby, S., & Chalutz, E. (1993). A selection strategy for microbial antagonists to control postharvest diseases of fruits and vegetables. *Scientia Horticulturae*, *53*(3), 183–189.

Wisniewski, M. E., Biles, C., Droby, S., McLaughlin, R., Wilson, C. L., & Chalutz, E. (1991). Mode of action of the postharvest biocontrol yeast, *Pichia guilliermondii*: I. Characterization of attachment to *Botrytis cinerea*. *Physiological and Molecular Plant Pathology*, *39*, 245–258.

Wisniewski, M. E., Bassett, C., Artlip, T., Janisiewicz, W. J., Norelli, J., Goldway, M., & Droby, S. (2005). Overexpression of a peach defensin gene can enhance the activity of postharvest biocontrol agents. In: *Acta Horticulturae* (pp. 1999–2006).

Wisniewski, M. E., Wilson, C. L., Droby, S., Chalutz, E., El-Ghaouth, A., & Stevens, C. (2007). Postharvest biocontrol: new concepts and applications. In C. Vincent, M. S.

Goettel, & G. Lazarovits (Eds.), *Biological Control A Global Perspective* (pp. 262–273). CABI, Cambridge, MA, USA.

Xu, X., Qin, G., & Tian, S. (2008). Effect of microbial biocontrol agents on alleviating oxidative damage of peach fruit subjected to fungal pathogen. *International Journal of Food Microbiology*, *126*(1-2), 153–8.

Xu, Y. X., Kim, K. M., Hanna, M. A., & Nag, D. (2005). Chitosan–starch composite film: preparation and characterization. *Industrial Crops and Products*, *21*(2), 185–192.

Yánez-Mendizabal, V., Viñas, I., Usall, J., Cañamás, T., & Teixidó, N. (2012a). Endospore production allows using spray-drying as a possible formulation system of the biocontrol agent *Bacillus subtilis* CPA-8. *Biotechnology Letters*, *34*(4), 729–735.

Yánez-Mendizábal, V., Viñas, I., Usall, J., Torres, R., Solsona, C., Abadias, M., & Teixidó, N. (2012b). Formulation development of the biocontrol agent *Bacillus subtilis* strain CPA-8 by spray-drying. *Journal of Applied Microbiology*, *112*(5), 954–965.

Yánez-Mendizábal, V., Viñas, I., Usall, J., Torres, R., Solsona, C., & Teixidó, N. (2012c). Production of the postharvest biocontrol agent *Bacillus subtilis* CPA-8 using low cost commercial products and by-products. *Biological Control*, *60*(3), 280–289.

Yildirim, M., Hettiarachchy, N. S., & Kalapathy, U. (1996). Properties of biopolymers from cross-linking whey protein isolate and soybean 11s globulin. *Journal of Food Science*, *61*, 1129–1131.

Ying, D., Sun, J., Sanguansri, L., Weerakkody, R., & Augustin, M. A. (2012). Enhanced survival of spray-dried microencapsulated *Lactobacillus rhamnosus* GG in the presence of glucose. *Journal of Food Engineering*, *109*, 597–602.

Yoo, S., & Krochta, J. M. (2011). Whey protein-polysaccharide blended edible film formation and barrier, tensile, thermal and transparency properties. *Journal of the Science of Food and Agriculture*, *91*(14), 2628–2636.

Yu, C., Zhou, T., Sheng, K., Zeng, L., Ye, C., Yu, T., & Zheng, X. (2013). Effect of pyrimethanil on *Cryptococcus laurentii*, *Rhodosporidium paludigenum* and *Rhodotorula glutinis* biocontrol of *Penicillium expansum* infection in pear fruit. *International Journal of Food Microbiology*, *164*(2-3), 155–160.

Yu, T., Wu, P. G., Qi, J. J., Zheng, X. D., Jiang, F., & Zhan, X. (2006). Improved control of postharvest blue mold rot in pear fruit by a combination of *Cryptococcus laurentii* and gibberellic acid. *Biological Control*, *39*(2), 128–134.

Yu, T., Li, H. Y., & Zheng, X. D. (2007). Synergistic effect of chitosan and *Cryptococcus laurentii* on inhibition of *Penicillium expansum* infections. *International Journal of Food Microbiology*, *114*(3), 261–6.

Yu, T., & Zheng, X. D. (2007). An integrated strategy to control postharvest blue and grey mould rots of apple fruit by combining biocontrol yeast with gibberellic acid. *International Journal of Food Science & Technology*, *42*(8), 977–984. http://doi.org/10.1111/j.1365-2621.2006.01321.x

Yu, T., Yu, C., Lu, H., Zunun, M., Chen, F., Zhou, T., Sheng, K., & Zheng, X. (2012). Effect of *Cryptococcus laurentii* and calcium chloride on control of *Penicillium expansum* and *Botrytis cinerea* infections in pear fruit. *Biological Control*, *61*(2), 169–175.

Zahavi, T., Cohen, L., Weiss, B., Schena, L., Daus, A., Kaplunov, T., Zutkhi, J., Ben-Arie, R., & Droby, S. (2000). Biological control of *Botrytis*, *Aspergillus* and *Rhizopus* rots on table and wine grapes in Israel. *Postharvest Biology and Technology*, *20*, 115–124.

Zeng, L., Yu, C., Fu, D., Lu, H., Zhu, R., Lu, L., Zheng, X., & Yu, T. (2015). Improvement in the effectiveness of *Cryptococcus laurentii* to control postharvest blue mold of pear by its culture in β-glucan amended nutrient broth. *Postharvest Biology and Technology*, *104*, 26–32.

Zhang, C., Chen, K., & Wang, G. (2013). Combination of the biocontrol yeast *Cryptococcus laurentii* with UV-C treatment for control of postharvest diseases of tomato fruit. *Biocontrol*, *58*, 269–281.

Zhang, D., Spadaro, D., Garibaldi, A., & Gullino, M. L. (2010a). Efficacy of the antagonist *Aureobasidium pullulans* PL5 against postharvest pathogens of peach, apple and plum and its modes of action. *Biological Control*, *54*(3), 172–180.

Zhang, D., Spadaro, D., Garibaldi, A., & Gullino, M. L. (2010b). Selection and evaluation of new antagonists for their efficacy against postharvest brown rot of peaches. *Postharvest Biology and Technology*, *55*(3), 174–181.

Zhang, D., Spadaro, D., Valente, S., Garibaldi, A., & Gullino, M. L. (2012). Cloning, characterization, expression and antifungal activity of an alkaline serine protease of *Aureobasidium pullulans* PL5 involved in the biological control of postharvest pathogens. *International Journal of Food Microbiology*, *153*(3), 453–64.

Zhang, H., Zheng, X., Wang, L., Li, S., & Liu, R. (2007). Effect of yeast antagonist in combination with hot water dips on postharvest *Rhizopus* rot of strawberries. *Journal of Food Engineering*, *78*, 281–287.

Zhang, H., Ma, L., Wang, L., Jiang, S., Dong, Y., & Zheng, X. (2008). Biocontrol of gray mold decay in peach fruit by integration of antagonistic yeast with salicylic acid and their effects on postharvest quality parameters. *Biological Control*, *47*(1), 60–65.

Zhang, H., Ge, L., Chen, K., Zhao, L., & Zhang, X. (2014). Enhanced biocontrol activity of *Rhodotorula mucilaginosa* cultured in media containing chitosan against postharvest diseases in strawberries: Possible mechanisms underlying the effect. *Journal of Agricultural and Food Chemistry*, *62*, 4214–4224.

Zhao, Y., Li, P., Huang, K., Wang, Y., Hu, H., & Sun, Y. (2013). Control of postharvest soft rot caused by *Erwinia carotovora* of vegetables by a strain of *Bacillus amyloliquefaciens* and its potential modes of action. *World Journal of Microbiology and Biotechnology*, *29*, 411-420.

Zhou, T., Schneider, K. E., & Li, X.-Z. (2008). Development of biocontrol agents from food microbial isolates for controlling post-harvest peach brown rot caused by *Monilinia fructicola*. *International Journal of Food Microbiology*, *126*(1-2), 180–5.

Zhou, Y., Deng, L., & Zeng, K. (2014). Enhancement of biocontrol efficacy of *Pichia membranaefaciens* by hot water treatment in postharvest diseases of citrus fruit. *Crop Protection*, *63*, 89–96.

Zhou, Y., Zhang, L., & Zeng, K. (2016). Efficacy of *Pichia membranaefaciens* combined with chitosan against *Colletotrichum gloeosporioides* in citrus fruits and possible modes of action. *Biological Control*, *96*, 39–47.

2. OBJECTIVES

The general objective of this Doctoral Thesis is to **design formulations of the biocontrol agent *Candida sake* CPA-1 with coating-forming ability, which preserve cell viability and efficacy against the pathogen *Botrytis cinerea* on grapes**, by means of studying the coating properties of different biopolymer-based matrices and their ability to act as a support to the biocontrol agent, thus enhancing its viability and efficacy.

In order to achieve this purpose, the following specific objectives were defined:

1. The study of the adherence, survival and efficacy of *Candida sake* against *Botrytis cinerea* when applied on grapes in combination with the different coating-forming agents. .

2. The characterization of different coating-forming formulations to be used as carriers of the biocontrol agent. The most relevant properties of the coating-forming dispersions and films made from four selected biopolymers, with and without surfactants, were characterized as well as their ability to preserve the viability of *Candida sake*.

3. The obtaining of dry formulations based on *Candida sake* and coating-forming agents and the study of their shelf-life in terms of their physical stability and ability to maintain cell viability throughout storage time, as a function of temperature and relative humidity.

3. CHAPTERS

CHAPTER 1

EFFECT OF DIFFERENT COATING-FORMING AGENTS ON THE EFFICACY OF THE BIOCONTROL AGENT *Candida sake* CPA-1 FOR CONTROL OF *Botrytis cinerea* ON GRAPES

Anna Marín[1], Maite Cháfer[1], Lorena Atarés[1], Amparo Chiralt[1], Rosario Torres[2], Josep Usall[2] & Neus Teixidó[2]

Biological Control, *96* (2016), 108–119.

[1]Instituto de Ingeniería de Alimentos para el Desarrollo, Departamento de Tecnología de Alimentos. Universitat Politècnica de València, 46022, Valencia, Spain

[2] IRTA, XaRTA-Postharvest, Edifici Fruitcentre, Parc Científic i Tecnològic Agroalimentari de Lleida, Parc de Gardeny, 25003 Lleida, Catalonia, Spain

ABSTRACT

Multiple formulations of known biocontrol agent (BCA) *Candida sake*, containing different coating-forming polymers and surfactants were tested at different polymer:BCA ratios, in order to improve control of *Botrytis cinerea* on grapes. The BCA cell viability on the grape surface was analyzed and reduction in disease incidence and severity was determined. Coating-forming solids improved the survival and efficacy of *C. sake* as a BCA against *B. cinerea*, depending on the polymer type and ratio. The incorporation of surfactants did not improve survival or disease control, although they promoted a better cell dispersion on the grape surface. Cell growth of the antagonist during incubation led to the formation of aggregates, even when surfactants were present. Sodium caseinate and starch were the most suitable polymers to formulate *C. sake* preparations to obtain coating-forming systems with this BCA and to increase its survival and efficacy at the minimum economic cost of the ingredients.

Keywords: biological control, *Candida sake*, grapes, biopolymer, edible coating, microstructural analysis

1. INTRODUCTION

The fact that major post-harvest pathogens have developed resistance to many fungicides and the public demand for a reduction in pesticide use, stimulated by a greater awareness of environmental and health issues, have generated an increasing interest in alternative methods to fungicidal treatments in the control of fruit diseases (Teixidó *et al.,* 2011; Zahavi *et al*., 2000). Biological control, which consists of biologically-based processes to lower pathogen inoculum density and reduce crop loss, is one of the most effective and practical alternatives to chemical fungicides (Cañamás *et al*., 2011). Biocontrol has been extensively studied during the last twenty years; however, it is difficult to observe the successful results obtained under laboratory or controlled condition in pre-harvest conditions. Its commercial application has been greatly limited due to the narrow range of environmental conditions in which biocontrol agents (BCAs) are able to survive and effectively control pests and diseases. Hence, a main aim in the development and implementation of biological control products is to improve the ability of the antagonists to survive and successfully control postharvest diseases under a wider array of conditions and with minimal variability (Droby *et al*., 2003).

Several strategies have been employed to improve the behavior of BCAs in practical conditions. Physiological manipulation has been one of the strategies used to enhance the tolerance of BCAs to environmental stress conditions obtaining interesting results (Abadias *et al*., 2001; Liu *et al*., 2012; Mokiou & Magan, 2008; Teixidó *et al.,* 1998). Furthermore, diverse additives, such as coatings, can act as protectors during the preparation, conservation and application phases of antagonist-based products (Droby *et al*., 2009). These additives might maintain the viability of BCAs more effectively and promote their biocontrol efficacy. Moreover, additives could not only improve the spray deposition, droplet size and spreadability of the products but also enhance survival and persistence of the BCAs under the stressing of conditions associated with the environmental fluxes in field. Cañamás *et al*., (2008a; 2008b) observed that the application of an edible coating improved the effectiveness of *Pantoea agglomerans* at controlling postharvest pathogens in orange fruit. Likewise, Cañamás *et al*., (2011) and

Calvo-Garrido *et al*., (2013a; 2014b) demonstrated similar effect on *Candida sake* applied on grapes. This was attributed to the improvement in the environmental stress tolerance and ecological competence of this BCA. Other functions of coatings have been described so as to aid and enhance BCA survival, including protection from ultraviolet (UV) radiation, desiccation, rain and temperature variations and by acting as a source of nutrients. In addition, coatings may also slow the microbial desiccation, thereby extending the time available for the BCA to multiply and become established and improve their homogeneity and distribution on the plant surface (Cañamás *et al*., 2011). Therefore the combined application of BCAs and edible coatings offers many possibilities, both because of the wide variety of matrices which can be used and their potential benefits for the survival and retention of the antagonists.

Edible coatings, produced from biopolymers and food-grade additives, are thin layers of material that cover the surface of the food and can be consumed as a part of the whole product (Vargas *et al*., 2008). They have been widely studied for the purposes of maintaining the quality of coated products, mainly in post-harvest treatments of fruits and vegetables (Hernández-Muñoz *et al*., 2008; Pastor *et al*., 2011; Perdones *et al*., 2012). Thus, the pre-harvest application of edible coatings that incorporate a BCA could be a good strategy for the preservation of crops since them might enhance the activity of the antagonist and also provide benefits to fruit.

There is a wide spectrum of biopolymers (polysaccharides and proteins) that can be used as the main compounds in the obtaining of edible coatings. Among polysaccharides, hydroxypropylmethylcellulose (HPMC), is a remarkable coating-forming compounds and corn starch (S) is extensively used due to its low cost and high availability (Rodríguez *et al*., 2006). Likewise, dairy and plant proteins, such as sodium caseinate (NaCas) and pea protein (PP), are also coating-forming compounds of interest (Choi & Han, 2001; Sánchez-González *et al*., 2013). In order to enhance the wettability on the plant tissue and the adhesion of the coatings, it is good practice to incorporate surfactants to the biopolymer matrices as a means of decreasing the surface tension of the coating-forming dispersions (CFDs) (Krochta, 2002; Ortega-Toro *et al*., 2014). The balance between the polar and non-polar groups of the surfactant molecules

determines their hydrophilic-lipophilic balance (HLB), which has a great influence on their surface activity depending on the blend components.

The enhancement of the efficacy of BCAs applied in combination with edible coating forming compounds has been demonstrated by several authors (Aloui *et al*., 2015; El Ghaouth *et al*., 2000; Fan *et al*., 2009; McGuire & Dimitriglou, 1999; McGuire *et al*., 2000; Potjewijd *et al*., 1995). However, there are, as yet, few studies aimed at the joint formulation of edible coatings and BCAs. Some isolated studies do exist, although a general overview is needed in order to acquire information about which is the most adequate design of edible coating in order to optimize the viability and effectiveness of the antagonists under practical conditions. The different physicochemical nature of the coating components can affect not only the viability and survival of the BCA but also their activity against the pathogen. It is important to consider the establishment of specific interactions between the polymeric matrix and the BCA and their influence on its biocontrol activity (Sánchez-González *et al*., 2013). Moreover, the type and concentration of coating-forming solids in relation to the incorporated BCAs could change the environmental conditions and, consequently, affect their activity. Furthermore, these aspects could influence other features of key importance for practical application, such as the BCA adherence, or the thickness of the coating layer on the fruit surface.

The filamentous fungus *Botrytis cinerea* is the dominant bunch rot-causing pathogen of gray mold in grapes in many temperate regions of the world, producing significant crop losses (Elmer & Reglinski, 2006; Zahavi *et al*., 2000). Some studies have revealed that it is possible to protect grapes from gray mold disease using postharvest antifungal coatings (Romanazzi *et al*., 2007; Sánchez-González *et al*., 2011; Xu *et al*., 2007). Against grey mold, different yeasts have also exhibited antagonistic activity against *B. cinerea* (Elmer & Reglinski, 2006; Zahavi *et al*., 2000). Several studies have reported the efficacy of *Candida sake* CPA-1 yeast in controlling both gray mold in grapes (Calvo-Garrido *et al*., 2013a; 2013b; 2014b; Cañamás *et al*., 2011). In addition, it has been demonstrated that the use of the edible coating Fungicover® based on fatty acids allows to improve the efficacy of CPA-1 (Calvo-Garrido *et al.,* 2013a; 2014a).

Combining biocontrol and CFD agents in joint formulations to obtain active coatings could represent a good strategy to improve biocontrol efficacy. Hence, the aim of this work was to evaluate the effect of different CFDs containing *C. sake* CPA-1, based on different biopolymers (HPMC, S, NaCas or PP) with and without the addition of surfactants (oleic acid, OA, HLB: 1; Span 80, S80, HLB: 4.3; Tween 85, T85, HLB: 11), on the adherence, viability and survival of *C. sake* cells, as well as to test its biocontrol efficacy against *B. cinerea* infections of coated grapes. The effect of coating-forming solids concentration respect to the BCA on these aspects was also analyzed for selected formulations. Likewise, scanning electron microscopy (SEM) was used to analyze the distribution of *C. sake* on the surface of coated grapes for some formulations and times post application.

2. MATERIALS AND METHODS

2.1 *Candida sake* inoculum production

Strain CPA-1 of *Candida sake* (Viñas *et al.,* 1998) was originally isolated from the surface of apples by UdL-IRTA group (Lleida, Catalonia, Spain), and was deposited at the "Colección Española de Cultivos Tipo" (CECT-10817) in the "Universidad de Valencia" (Burjassot, Valencia, Spain). Cell production and formulation were carried out following methods described by Cañamás *et al*., (2011). Briefly, stock cultures were stored on nutrient yeast dextrose agar (NYDA) medium (nutrient broth, 8 g/L; dextrose 10 g/L; agar 15 g/L) at 4°C. When required, *C. sake* CPA-1 was sub-cultured onto NYDA plates at 25°C. Then, sub-cultured cells were suspended on potassium phosphate buffer (KH_2PO_4 0.2 M, 70 mL; K_2HPO_4 0.2 M, 30 mL; deionized water 300 mL) were added as inoculum starter to 5 L of molasses-based medium (cane molasses 40 g/L; urea 1.2 g/L; water activity a_w = 0.996), with adjustment of the initial concentration to $1 \cdot 10^6$ CFU/mL. Cell pellets were obtained by centrifugation at 6831 g for 10 min at 10°C after 40 h of liquid fermentation in a BIOSTAT-A modular bioreactor (Braun Biotech International, Melsungen, Germany) at 25°C, 400 rpm agitation speed and 150 L/h aeration level. Re-suspended pellets were then formulated in an isotonic solution, with adjustment of the water potential with trehalose as described by Abadias *et al*., (2003).

2.2 Preparation of the coating-forming dispersions with *Candida sake*

HPMC and NaCas were supplied by Sigma-Aldrich (Madrid, Spain). S and PP were purchased from Roquette Laisa España, S.A. (Valencia, Spain). CFDs, with and without surfactants, were prepared by dispersing the biopolymers (2% w/v) in deionized water. HPMC was heated to 80°C for 10 min and maintained under magnetic stirring at 25°C overnight. S was maintained under stirring at 95°C for 30 min to induce starch gelatinization. NaCas and PP were dispersed at 25°C for 2 h. After polymer dispersion, glycerol (Panreac Química, S.L.U, Barcelona, Spain) was incorporated as plasticizer in S, NaCas and PP CFDs at a hydrocolloid:glycerol mass ratio of 1:0.25. Surfactants (all supplied by Sigma-Aldrich, Madrid, Spain) were added at a hydrocolloid:surfactant mass ratio of 1:0.1. The hydrocolloid:glycerol and hydrocolloid:surfactant ratios were selected on the basis of previous studies (Jiménez *et al*., 2012; Sánchez-González *et al*., 2009; Sánchez-González *et al*., 2013). CFDs were homogenized using a rotor-stator homogenizer (Ultraturrax T25, Janke and Kunkel, Germany) at 13,600 rpm for 4 minutes and sterilized at 121°C for 15 min.

After cooling, *C. sake* was incorporated in CFD to a final yeast concentration of $5 \cdot 10^7$ CFU/mL (Calvo-Garrido *et al*., 2013a; Cañamás *et al*., 2011). The dispersions obtained were shaken for 15 minutes at 150 rpm in a rotatory shaker (Selecta, Abrera, Barcelona, Spain) to achieve a homogeneous distribution of the microorganisms. As a control, a dispersion of *C. sake* in sterilized deionized water (CS) was prepared at $5 \cdot 10^7$ CFU/mL. The seventeen considered treatments are summarized in Table 1.1.

2.3 *Botrytis cinerea* inoculum

An isolate of *Botrytis cinerea* obtained from infected grapes collected in a local vineyard in Lleida was used in this study because it was the most virulent isolate from IRTA collection. The isolate was grown on potato dextrose agar (PDA) for 15 days at 20°C with a daily 14 h photoperiod of near ultraviolet light and 10 h dark to induce sporulation. Conidial suspensions were prepared by adding 10 mL of sterile distilled water containing 0.01% (w/v) Tween 80 to *B. cinerea* cultures. Conidia were scraped from the agar using a sterile loop, sonicated for 5 min to facilitate conidial dispersion, and then adjusted to $1 \cdot 10^4$ conidia/mL (Cañamás *et al*., 2011).

Table 1.1. Treatments based on different edible coatings and *Candida sake* at $5 \cdot 10^7$ CFU mL^{-1} applied on grapes.

Treatment	Treatment description
CS	*Candida sake* in sterilized deionized water
HPMC HPMC OA HPMC S80 HPMC T85	Coating forming dispersions based on hydroxypropylmethylcellulose (HPMC) without surfactants or with oleic acid (OA), Span 80 (S80) or Tween 85 (T85).
S S OA S S80 S T85	Coating forming dispersions based on corn starch (S) without surfactants or with oleic acid (OA), Span 80 (S80) or Tween 85 (T85).
NaCas NaCas OA NaCas S80 NaCas T85	Coating forming dispersions based on sodium caseinate (NaCas) without surfactants or with oleic acid (OA), Span 80 (S80) or Tween 85 (T85).
PP PP OA PP S80 PP T85	Coating forming dispersions based on pea protein (PP) without surfactants or with oleic acid (OA), Span 80 (S80) or Tween 85 (T85).

2.4 Population dynamics of *Candida sake* on grapes

Six replicates, consisting of five berries of table grapes (*Vitis vinifera* L., Red Globe variety) homogeneous in size and shape, were used for the application of each treatment. The berries were selected on the basis of their maturity stage and without signs of mechanical damage or fungal decay. Each sample was placed separately on a plastic grid and sprayed for 5 seconds with its corresponding treatment, including CS control, using an air brush. The samples were left to dry at room temperature and then placed in a sealed plastic box for incubation at 20°C and 85% RH for either 24 h or 7 days. To study the population dynamics, each sample was weighed and transferred to

Erlenmeyer flasks containing 100 mL of sterile deionized water with 0.01% (w/v) Tween 80. They were shaken in a rotatory shaker at 150 rpm for 20 min and sonicated for 10 min in an ultrasound bath (Selecta, Abrera, Barcelona, Spain) to achieve the maximum detachment of the yeast from the grape surface. Serial dilutions of the washings were performed in duplicate and plated onto NYDA agar medium with streptomycin sulphate (Sigma-Aldrich, Madrid, Spain) at a concentration of 0.5g/L to prevent bacterial growth. Plates were incubated for 48 h at 25°C in the dark and typical *C. sake* colonies were then counted based on their morphological characteristics. Results were expressed as log CFU per gram of treated grape.

2.5 Efficacy of *Candida sake* against *Botrytis cinerea* on grapes

Three replicates of five berries each per formulation were used to study the effectiveness of the antagonist in the biocontrol against *B. cinerea*. Samples were washed with water, left to dry and placed separately on plastic grids. Sandpaper was used to induce the rupture of the grape cuticle and favour pathogen infection. The different CFDs with *C. sake* were applied as described in section 2.4, as well as the CS control and an additional deionized water control without the antagonist (W). When the berries were dried, a conidial suspension of *B. cinerea* at $1 \cdot 10^4$ conidia mL^{-1} was applied with an air brush and left to dry again at room temperature. Samples were incubated at 20°C and 85% RH for either 7 or 12 days. Likewise, CFDs without the incorporation of the BCA were applied in order to ascertain if they exert any antifungal effect against de pathogen.

The incidence of the pathogen rot was visually evaluated by counting the number of berries with the typical *B. cinerea* conidia. The severity of the pathogen infection was visually estimated and expressed as the percentage of berry surface affected by grey mold (Cañamás *et al*., 2011). The results were expressed as the percentage reduction of the incidence and severity as referred to W.

2.6 Microstructural analysis of coatings containing *Candida sake* on grape surface

The microstructural analysis of grape surfaces coated with the formulations S, S-OA, S-T85, NaCas, NaCas-OA and NaCas-T85 containing $5 \cdot 10^7$ CFU mL^{-1} of *C. sake* was carried out after 24 h and 7 days post application by cryoSEM using a Scanning Electron

Microscope (JEOL JSM-5410, Japan). Samples were cryofixed in slush nitrogen and observed, after gold coating, using an accelerating voltage of 10 kV. Images of the coated grape surface were obtained to analyze the distribution of *C. sake* on the grape surface with different coating formulations.

2.7 Influence of the ratio of coating-forming solid :BCA on *Candida sake* viability and efficacy

The influence of the ratio of the coating-forming solids with respect to the *C. sake* concentration in the CFDs on the antagonist efficacy was also analyzed for some selected formulations, in order to know if there is a critical ratio for promoting its viability and efficacy as BCA. In practical terms, it would be preferable a minimum amount of solids in order to limit the quantity of non-active material and to obtain a final product with a competitive price. To this end, two of the initially tested biopolymers were selected: NaCas, for its positive results and S due to its low cost. Some modifications of the initial formulations were introduced: native corn starch was replaced by pre-gelatinized corn starch to avoid the necessary gelatinization; and the use of glycerol as plasticizer was discarded since its incorporation into the CFDs did not have a positive effect on the activity of the antagonist, as deduced from a previous study (data not shown).

CFDs based on NaCas and S were prepared with different ratios of coating-forming solids maintaining a *C. sake* concentration of $5{\cdot}10^7$ CFU/mL. The concentrations of coating solids used were: 25, 12.5, 16.25, 5, 3.75, 2.5 or 1.25 mg/mL. The mass of the hydrocolloid required for each treatment was dispersed in 50 mL deionized water and after its complete dispersion, the CFD were sterilized at 121°C for 15 min. *C. sake* was incorporated into CFDs at the yeast concentration required as in section 2.2. The adherence and survival of *C. sake* on grapes and its efficacy against *B. cinerea* for the different CFDs were analyzed as described in sections 2.4 and 2.5. The results of the population dynamics were expressed as the difference of log CFU per gram of treated grape with respect to the CS treatment without coating solids. For the efficacy assays, the percentage of incidence and severity reduction referred to the W was also reported.

2.8 Statistical analysis

The statistical analysis of the population dynamics of *C. sake* and the incidence and severity of *B. cinerea* infection was performed through an analysis of variance (ANOVA) using Statgraphics Centurion XVI version 16.1.17 (Manugistics Corp., Rockville, Md.). CFU data were log-transformed prior to ANOVA to improve the homogeneity of variances. Significant differences were determined using LSD test ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1 Population dynamics of *Candida sake* on grapes

The influence of the different CFD formulations on the population data of *C. sake* on grapes can be observed in **Figure 1.1**. 24 h after application, *C. sake* populations on berry surface were between 5 and 6 log CFU/g in every case, as previously reported by Cañamás *et al*. (2011) in field experiments using CPA-1. Significantly ($p < 0.05$) higher values, compared to the control treatment (CS), were observed for S, NaCas and PP-OA formulations. In this sense, a high rate of yeast survival after the application step is important to ensure that there is a high number of CFU available to colonize the fruit surface (McGuire & Dimitriglou, 1999). After 7 days of incubation, an increase in the *C. sake* population was observed for all treatments, including the one without coating-forming agents. All NaCas-based coatings, with and without surfactants, PP-OA and PP-T85 showed a significantly higher ($p < 0.05$) population on berries than the rest of treatments, including the control. These results indicate that all of the coating formulations used were suitable carriers for the microorganism and allowed *C. sake* cells to effectively establish on the fruit surface. It is likely that the layer created by these coatings on the berry surface could generate a beneficial environment for the BCA that would stimulate its survival (Cañamás *et al*., 2011). Other authors have reported beneficial effects of some components on the survival of some BCAs. McGuire and Baldwin (1994) and McGuire & Dimitriglou (1999) reported that coatings based on cellulose or sucrose esters supported high numbers of the yeast *Candida oleophila* when applied on grapefruits. Similarly, Potjewijd *et al*., (1995) observed that a methylcellulose-based coating applied on oranges was the best carrier for the pathogen antagonists *Candida guillermondii* and *Debaromyces spp*.

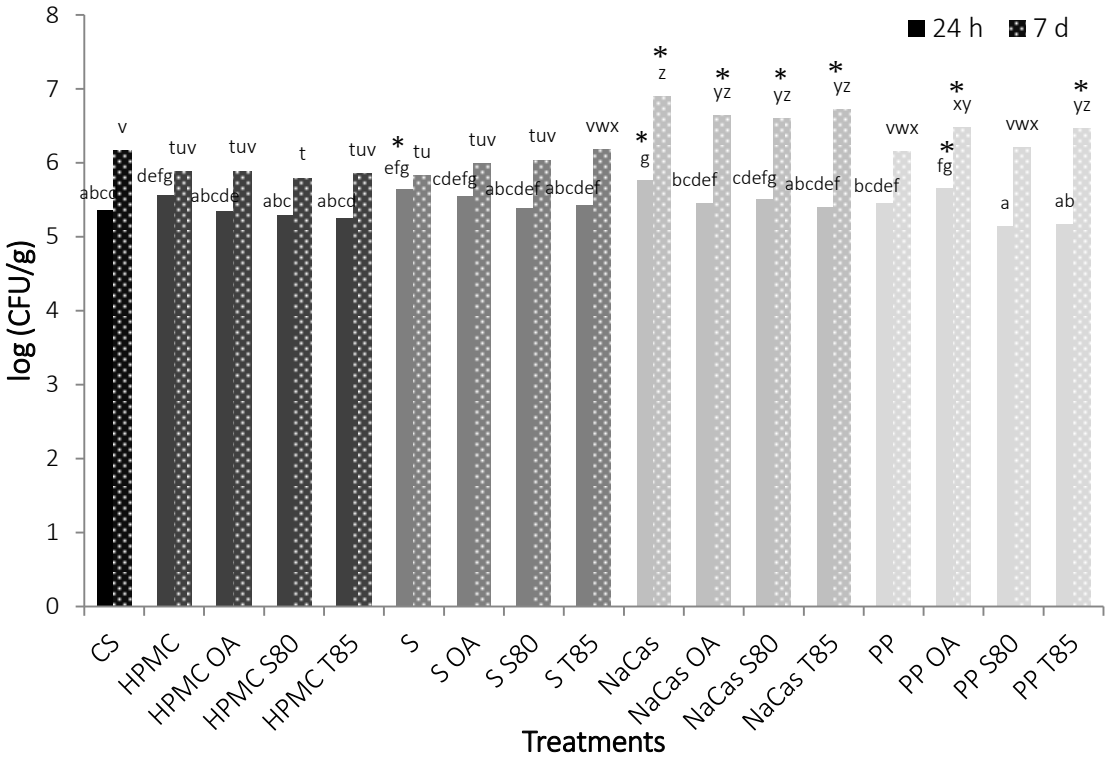


Figure 1.1. Population of *Candida sake* applied with different coating-forming dispersions on grape surface at 24 h and 7 days after application. Different letters in the bars indicate significant differences determined using LSD test ($p < 0.05$) for each time. * indicate the treatments that significantly improved the population with respect to CS.

In general, all protein-based coatings (NaCas and PP) led to the highest initial adherence and survival rate of the yeast, promoting their growth during incubation time, which was especially notable for formulations with NaCas. In these formulations, the highest value of log CFU/g, 6.89, was observed with no significant effect of surfactants. In general, surfactants did not significantly affect ($p > 0.05$) the BCA survival, except for PP formulations, where the addition of OA and T85 had a marked positive effect after 7 incubation days. The positive effect of proteins could be attributed to a better availability of adequate nutrients for *C. sake*. In the case of polysaccharides, S showed a significantly higher population of cells with respect to CS, but only after 24 h of incubation.

The presence of a high number of CFU available to rapidly germinate and grow on the fruit surface before the arrival of the pathogen is a key factor in the prevention or

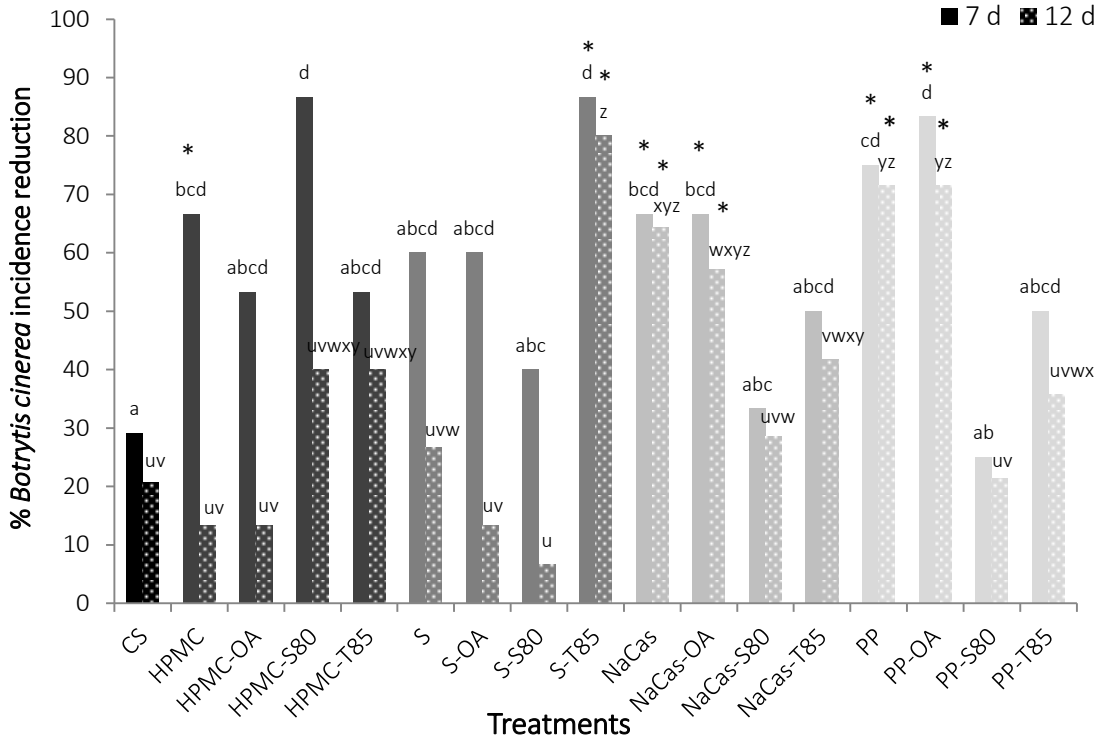
reduction of the disease development, especially when the mechanism of action is based on the competition for space and nutrients, such as it is described for *C. sake* (Fokkema, 1996; Ippolito & Nigro, 2000). In this sense, formulations with NaCas and S would be suitable to improve the biocontrol effect of the yeast.

3.2 Efficacy of *Candida sake* against *Botrytis cinerea* on grapes

The effect of the different formulations on the effectiveness of *C. sake* in the biocontrol of *B. cinerea* is shown in **Figure 1.2**, as the percentage of incidence reduction with respect to the control sample W, after 7 and 12 days of inoculation. The untreated controls showed an incidence infection of 86% and 96.5% after 7 and 12 days of infection, respectively, and a value of infection severity of 70.5%. In general, all treatments exhibited a similar or higher reduction than the CS control after 7 days of incubation. Several treatments showed a significantly ($p < 0.05$) higher reduction of the infection with respect to the solid-free formulation of *C. sake*. The highest reduction was obtained for the S-T85, HPMC-S80 and PP-OA treatments, with reduction values higher than 80%. PP, HPMC, NaCas and NaCas-OA treatments also reached good levels of biocontrol but the reductions were slightly lower. In agreement with our results El Ghaouth *et al*. (2000) reported that the combination of the yeast *Candida satoiana* with glycolchitosan was more effective controlling gray mold on apple caused by *B. cinerea* than the independent applications of *C. saitoana*.

After 12 days of incubation, the reduction of the incidence decreased in every case because of the progression of the existing infection. Nevertheless, some of the applied treatments (S-T85, NaCas, NaCas-OA, PP and PP-OA) still maintained a significantly higher reduction of the *Botrytis* incidence than CS control. Among the treatments which better controlled the pathogen growth at 7 days, those containing HPMC were not effective after 12 days of incubation, which could be associated with the lack of yeast viability after long times in this substrate. The infection control with both proteins and S coatings was coherent with the greatest viability of *C. sake* in these supports, as previously mentioned.

Figure 1.2. Percentage of reduction of *Botrytis cinerea* incidence on grape berries by applications of *Candida sake* incorporated in different coating-forming dispersions after 7 and 12 days of incubation. Different letters in the bars indicate significant differences determined using LSD test ($p < 0.05$) for each time. * indicate the treatments that significantly improved the results of CS treatment.



The effectiveness of the CFDs without BCA was also evaluated (data not shown) and no significant effect could be observed in the control of the infection, since all formulations showed low or no effect. This indicates that components used in the CFDs did not themselves exhibit antifungal effects, although they could enhance the BCA action through different mechanisms, such as supplying adequate nutrients or water retention contribution.

Nevertheless, several authors have found effectiveness against some pathogens of some coatings. For example, Calvo-Garrido *et al*. (2014a) demonstrated the efficacy of a fatty acid-based product with coating-forming ability against *B. cinerea* by a multiple mode of action. Other studies have reported the use of fatty acid-based products in other fruit crops to act against *B. cinerea* and other fungal pathogens (Hou & Forman 2000; Montealegre *et al*., 2010; Řiháková *et al*., 2001). Likewise, chitosan-based

coatings have been widely studied due to its antimicrobial properties (Reglinski *et al*., 2010; Romanazzi *et al*., 2009) which can be promoted by the incorporation of other bioactive compounds (Sánchez-González *et al*., 2011; Perdones *et al*., 2012). Aloui *et al*., (2014; 2015) also reported that sodium alginate and locust bean gum based coatings had a slight indirect effect on the fungal decay of oranges and grapes.

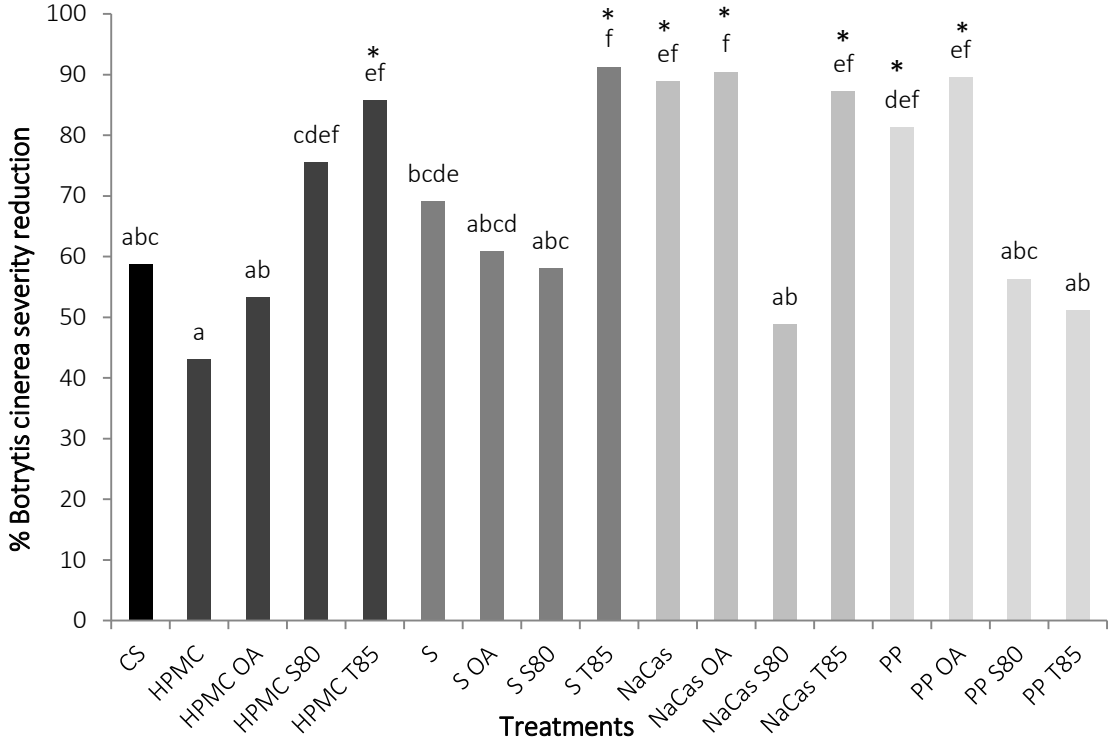
The effect of surfactants on the infection control was not related to their effect observed on the viability of yeast cells. For NaCas, the same cell viability was obtained for treatments with and without surfactants, whereas the incidence reduction by formulations was only notable in surfactant-free samples or those with OA. In the case of PP treatments, T85 enhanced yeast viability but did not improve the infection control. On the contrary, T85, which was not effective at promoting *C. sake* viability in S formulations, significantly improved its biocontrol efficacy. This suggests that the interactions of the support components, not only with the BCA but also with the infectious agent, play an important role in biocontrol.

All protein-based coatings showed a better control of the severity of the infection. NaCas, NaCas-OA, NaCas-T85, PP and PP-OA significantly improved ($p < 0.05$) the reduction of infection severity with respect to CS treatment. S-T85 treatments achieved levels of control similar to those of the mentioned CFDs based on proteins. These results suggest that the overall balance of interactions among molecular components of CFD and the antagonist cells affected the final action of the BCA against the pathogen. In fact, McGuire & Hagenmaier (1996) reported a presumable effect of some compounds of commercial coatings, such as surfactants, on microbial survival, including that of pathogens (Figure 1.3).

3.3 Microstructural analysis of coatings containing *Candida sake* on grape surface

In order to analyze the distribution of cells on the grape surface as well as their possible morphological changes throughout time, SEM observations were carried out on newly NaCas and S coated samples (24h after coating treatment) and on those stored for 7 days at 20°C and 85% RH . The same samples, with the addition of OA and T85 surfactants, were also observed in order to analyze the effect of these surfactants on the cell distribution on the grape surface. Figure 1.4 shows representative images of the

Figure 1.3. Percentage of reduction of *Botrytis cinerea* severity on grape berries by applications of *Candida sake* incorporated in different coating-forming dispersions after 7 and 12 days of incubation. Different letters in the bars indicate significant differences determined using LSD test ($p < 0.05$) for each time. * indicate the treatments that significantly improved the results of CS treatment.



surface of grapes coated with *C. sake* dispersions in water (CS) and in the CFDs based on NaCas and S. The grape surfaces were partially covered by the coatings and the BCA cells were surrounded by a biofilm (**Figure 1.4b**), which was probably excreted for their protection. In grapes with CS, the typical crystalline formations of the epicuticular natural surface wax were observed (Fava *et al.*, 2011). This waxy structure appeared coated with a polymer layer when bioactive coatings incorporating *C. sake* were applied on grape surface. Grape surface appeared smoother, more homogeneous, and more uniform as a result of coatings application. In general, the coating distribution was uneven since coated and uncoated areas were observed in the samples. The surface coated with NaCas formulations (**Figures 1.4c** and **1.4d**) exhibited a more granular appearance due to the globular structure of the protein. In contrast, S based coatings (**Figures 1.4e** and **1.4f**) led to a smoother and more homogeneous surface.

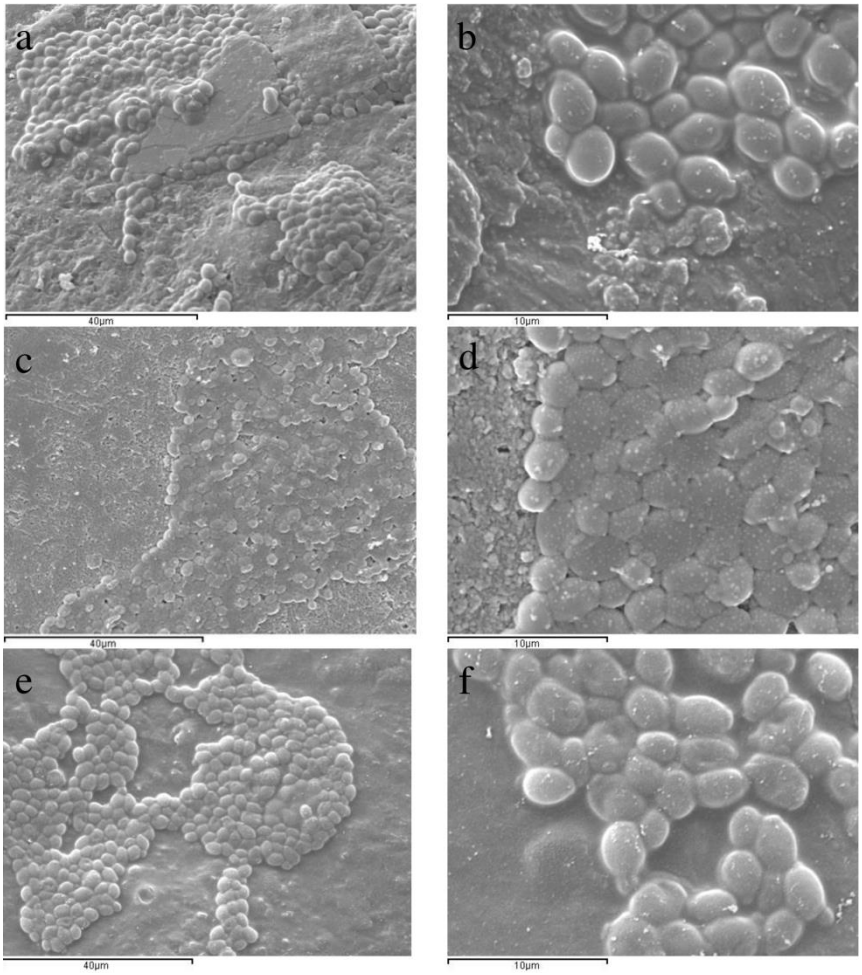


Figure 1.4. SEM images of coated grape surface with *Candida sake* formulations: water (a, b); sodium caseinate (NaCas) (c, d); corn starch (S) (e, f).

The formation of cell aggregates was observed, probably as result of the natural tendency of microorganisms to attach onto solid surfaces thereby forming biofilms (Domínguez-Manzano *et al.,* 2012), while it could be also promoted by the loss of water during the coating drying. Biofilm formation includes the bonding of the cells to a solid surface and the presence of an extracellular matrix (Nobile & Mitchell, 2007). Cell aggregates were more extensive and multilayered in water-coated grapes, whereas the presence of coatings resulted in monolayer accumulations.

In grapes treated with NaCas coatings (**Figures 1.4c** and **1.4d**), the cells appeared more irregularly coated and small globular protein particles were observed on their surface. On the contrary, a greater coverage was observed in grapes treated with S (**Figures 1.4e**

and 1.4f). This could be explained by the ability of the polysaccharide chains to coat the cells and fill the gaps between them.

In Figures 1.5 and 1.6, the effect of the incorporation of the surfactant on the formulation of NaCas and S coatings can be observed after 1 and 7 days of application. Surfactants induced a greater disaggregation of cells, which appeared much more dispersed and isolated on the surface as compared to grapes coated with both polymers without surfactants. So, the incorporation of surfactants reduced the formation of aggregates. Likewise, the appearance of the coatings was more heterogeneous due to the lack of miscibility of the surfactants with the polymers, which gave rise to lipid dispersed particles inside the polymer matrix, depending on the polymer-surfactant interactions. The observed heterogeneity of the coatings with surfactants has been previously described also from SEM micrographs of starch-surfactant based films by Jiménez *et al*., (2012) and Ortega-Toro *et al*., (2014). After 7 days of incubation, clusters of cells were again observed on the grape surface treated with S and NaCas with surfactants, which might be attributed to the yeast growth from the initial isolated cells with the subsequent increase in their population, as previously commented on. In the case of grapes coated with S and surfactants, some *C. sake* cells exhibited a more elongated appearance probably associated to their division process (Figures 1.6b and 1.6d). In grapes coated with NaCas and surfactants the aggregates showed different layers (Figures 1.5b and 1.5d). The cells in the layers below presented a dehydrated aspect as compared to cells in the upper part. The appearance observed for the new cells in NaCas and S films was different. Cells in S treated samples became more dehydrated and were less vital in appearance than those coated with NaCas. Thus, SEM images revealed an apparently better preservation and vitality of *C. sake* when NaCas was used in BCA formulation. This agrees with the higher counts obtained for NaCas treated samples after 7 incubation days.

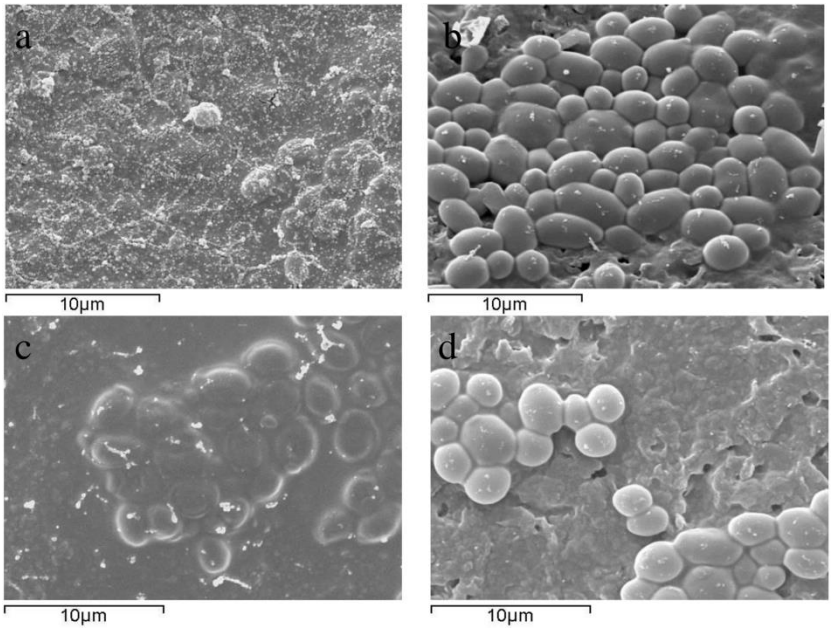


Figure 1.5. SEM images of coated grape surface *with Candida sake* formulations: sodium caseinate (NaCas) with oleic acid (OA) at 24 h and 7 days (a, b); NaCas with Tween 85 (T85) at 24 h and 7 days (c, d).

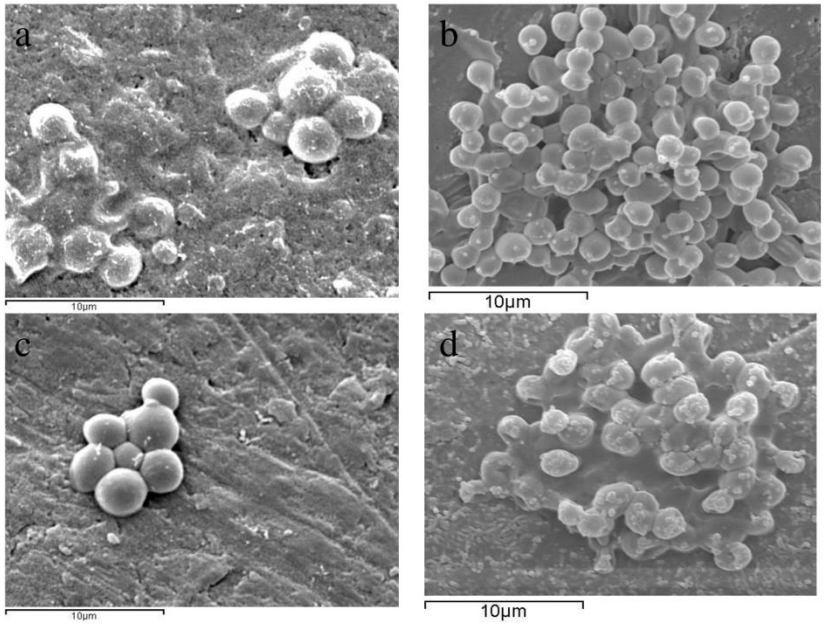


Figure 1.6. SEM images of coated grape surface with *Candida sake* formulations: corn starch (S) with oleic acid (OA) at 24 h and 7 days (a, b); S with Tween 85 (T85) at 24 h and 7 days (c, d).

3.4 Influence of the ratio of coating-forming solids:BCA on *Candida sake* viability and efficacy

The effect of the proportion of coating-forming solids with respect to the concentration of BCA was analyzed in order to establish the minimum amount of solids that improve the antagonistic activity. For this purpose, coatings based on NaCas and S were selected, as explained above. Data were analyzed in terms of the relative increase in the BCA population (Δlog CFU) with respect to the corresponding control (CS) after the different incubation times (24 h and 7 days; Figure 1.7). It is remarkable that the coatings had positive (+Δlog CFU) and negative effects (-Δlog CFU) on the population of *C. sake,* depending on the incubation time, solid ratios and polymer type. In general, after 24 h, coatings based on S had a positive effect over the whole range of solid ratios, while those based on NaCas only had a positive effect when applied at low concentrations (2.5, 3.75 and 5 mg/mL). The behavior of NaCas-based coatings with low solid concentrations was contrary to the observed tendencies (lines in the plot), whereby the higher the solid ratio the higher the Δlog increase. Concerning the incubation time, a negative effect was always observed in coatings based on S, whereas for NaCas-based coatings a positive effect was found but only for the highest concentrations (upper 5 mg mL^{-1}) where lower counts were obtained after 24 h of incubation. For a high ratio of solids:BCA, these results agreed with those observed in the first experimental series carried out with a solid concentration of 20 mg mL^{-1}. As previously commented on, a population increase in *C. sake* was observed for NaCas coatings, whereas no significant cell growth occurred in S coated samples during the 7 incubation days. Likewise, the SEM micrographs also showed the *C. sake* growth in NaCas coated grapes during 7 incubation days, whereas although cells in S coated grapes seemed to grow, they appeared altered in shape in the micrographs.

This behavior suggests that in order to ensure the better survival of *C. sake* during the coating drying and incubation time, a minimum concentration of coating solids is required, although this value is dependent on the kind of solids. NaCas better preserved the viability of *C. sake* during incubation time, promoting its growth; and the greater the solid ratio was, the higher the cell count difference with respect to the control after 7

incubation days. Although this same tendency was observed for S coatings, the colony number significantly decreased after 7 incubation days.

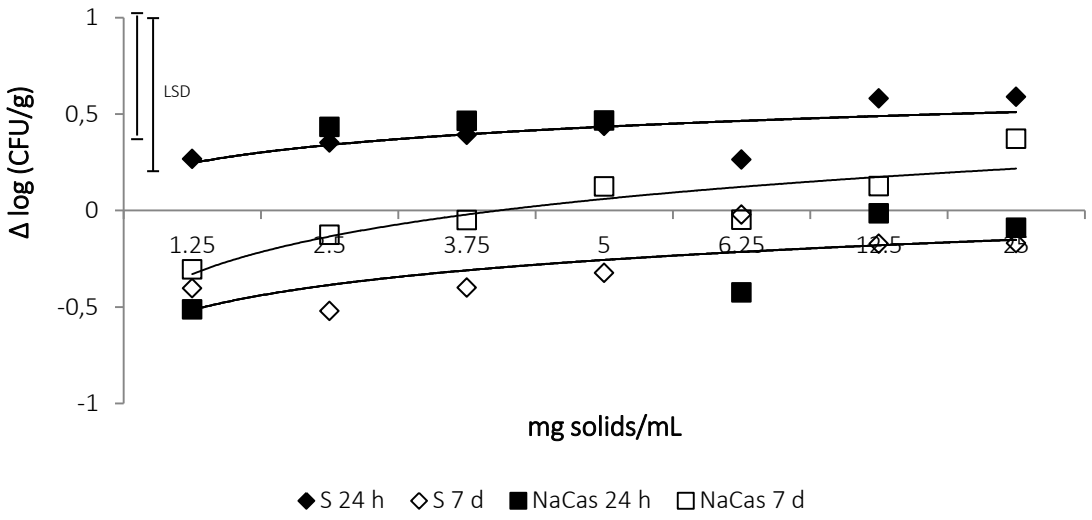


Figure 1.7. Relative population increase of *Candida sake* with respect to the corresponding control as a function of the amount (mg/mL) of coating-forming solids with respect to the BCA colonies ($5 \cdot 10^7$ CFU/mL), for corn starch (S) and sodium caseinate (NaCas) coatings applied on grapes surface after 24 h and 7 days of application. Lines: tendencies of Δlog vs. solid concentration). LSD intervals of the controls at 24 h and 7 days.

The effect of the coating solid ratio on the reduction of the incidence and severity of the *B. cinerea* infection was also analyzed after 6 days of incubation (Figures 1.8 and 1.9). A significantly greater incidence reduction was observed for high solid ratios. The treatments with a significant reduction in the incidence with respect to the CS treatment were those containing more than 5 mg mL^{-1} of NaCas or more than 2.5 mg mL^{-1} of S (except intermediate values, 5 and 6.5 mg mL^{-1} for S, where no significant differences were found). Similarly, the reduction in the severity of the infection (Figure 9) was significantly higher than that of the CS treatment when the NaCas concentration was higher than 5 mg mL^{-1} and when the S concentration was 2.5, 3.75 or 25 mg mL^{-1}. Therefore, the amount of coating solids in relation to the CFU had an effect on the efficacy of *C. sake* against *B. cinerea*, which was also dependent on the kind of polymer. The use of NaCas gave rise to a good efficacy of the BCA at a higher solid ratio than S, in line with its better support for the growth of the *C. sake* during incubation time. The

improvement in the efficacy of *C. sake* at controlling *B. cinerea* agreed with the increase in the population of the BCA throughout time and the vital appearance of the cells in SEM micrographs, which guarantees their biocontrol action. This was confirmed in the second experimental series with different ratios of coating-forming solids with respect to the BCA CFUs. After 7 incubation days, greater cell counts could be observed for NaCas than for S coatings, both of which were higher when the coating-forming solids increased in the formulation. The greater nutrient availability for cells on the grape surface and the better limitation of cell drying throughout time, when a high ratio of coating-forming solids covered the grapes, could explain this finding.

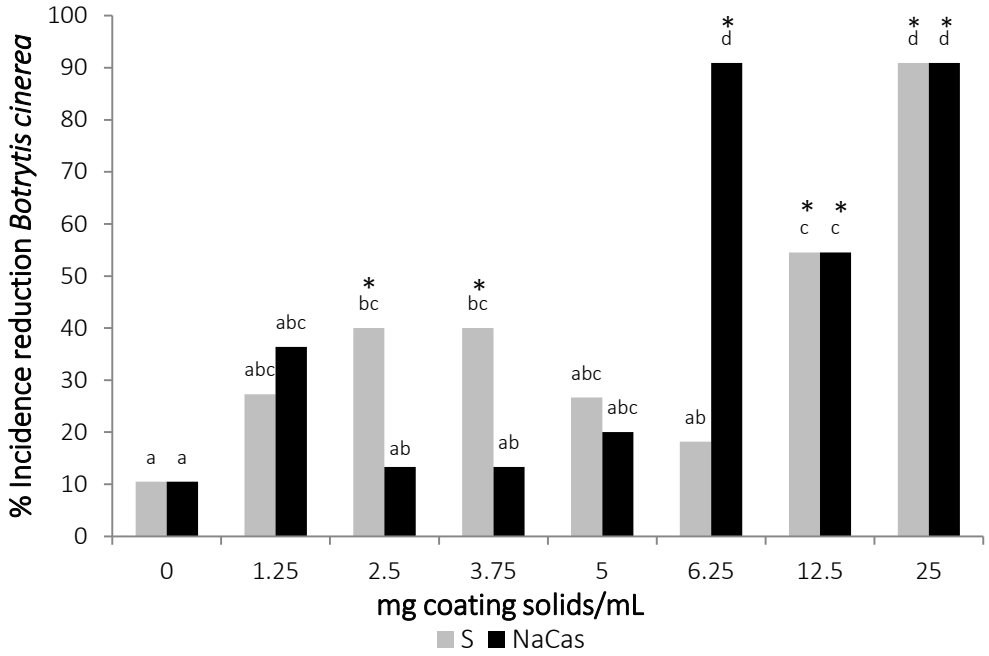


Figure 1.8. Percentage of reduction of *Botrytis cinerea* incidence on grape berries by applications of *Candida sake* as a function of the amount (mg mL^{-1}) of coating-forming solids with respect to the BCA colonies ($5 \cdot 10^7$ CFU/mL) after 6 days of incubation. S: corn starch, NaCas: sodium caseinate. Different letters in the bars indicate significant differences determined using LSD test ($p < 0.05$). * indicate the treatments that significantly improved the results of CS treatment.

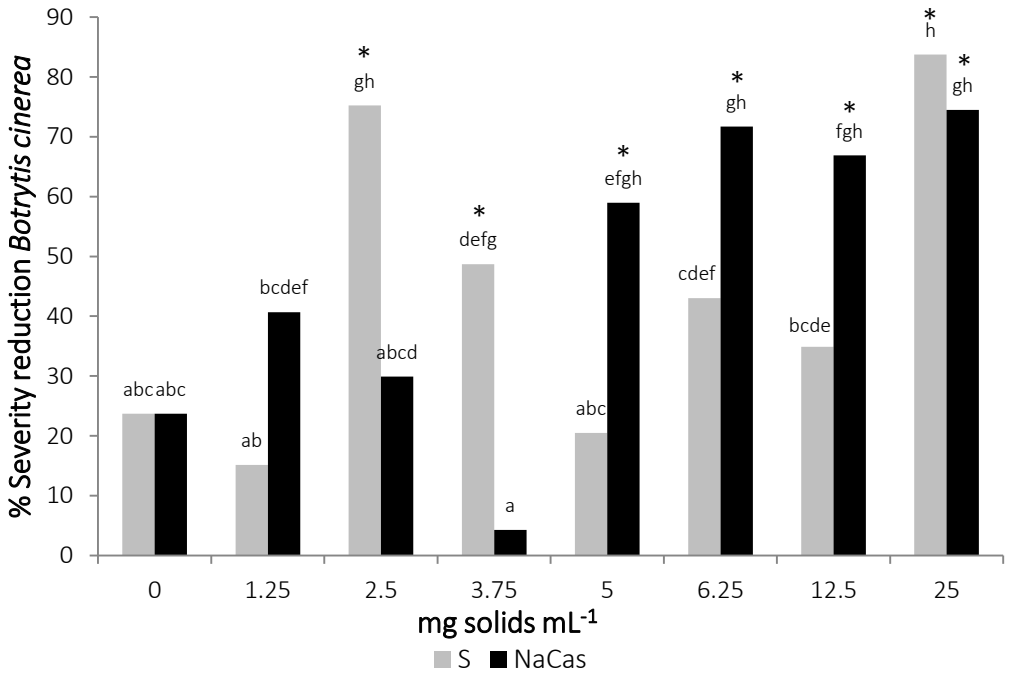


Figure 1.9. Percentage of reduction of *Botrytis cinerea* severity on grape berries by applications of *Candida sake* as a function of the amount (mg/mL) of coating-forming solids with respect to the BCA colonies ($5 \cdot 10^7$ CFU/mL) after 10 days of incubation. S: corn starch, NaCas: sodium caseinate. Different letters in the bars indicate significant differences determined using LSD test ($p < 0.05$). * indicate the treatments that significantly improved the results of CS treatment.

4. CONCLUSIONS

In conclusion, coating-forming solids improved the survival and efficacy of *C. sake* as BCA of *B. cinerea*, depending on the polymer type and ratio of coating solids. The addition of surfactants did not imply additional positive effects, although they promoted a better cell dispersion onto the grape surface. Nevertheless, cell growth during the incubation time led to the formation of cell aggregates, even when surfactants were added to the formulations. Taking into account the relative increase in the survival and efficacy of *C. sake,* and the cost of ingredients, NaCas or S are recommended to formulate preparations in order to obtain coating-forming systems with this BCA against *B. cinerea* in grapes. The highest polymer:CFU ratios in the formulation exhibited better biocontrol properties and so, this is also recommended. For NaCas, at least 6 mg for $5 \cdot 10^7$ CFU/mL was required to ensure the effective

biocontrol of *B. cinerea*. In the case of S, 2.5 mg for $\cdot\times10^{7}$ mL^{-1} CFU also led to an improved effective biocontrol.

AKNOWLEDGEMENTS

The authors are grateful to the Spanish Government for the financial support from the national project RTA2012-00067-C02 (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Spain and FEDER funds) and to the Conselleria d'Educació of the Generalitat Valenciana, (Spain) for A. Marín's PhD grant.

REFERENCES

Abadias, M., Teixidó, N., Usall, J., Viñas, I., & Magan, N. (2001). Improving water stress tolerance of the biocontrol yeast *Candida sake* grown in molasses-based media by physiological manipulation. *Canadian Journal of Microbiology*, 47, 123–129.

Abadias, M., Usall, J., Teixidó, N., & Viñas, I. (2003). Liquid formulation of the postharvest biocontrol agent *Candida sake* CPA-1 in isotonic solutions. *Phytopathology 93*, 436–442.

Aloui, H., Khwaldia, K., Sánchez-González, L., Muneret, L., Jeandel, C., Hamdi, M., & Desobry, S. (2014). Alginate coatings containing grapefruit essential oil or grapefruit seed extract for grapes preservation. *International Journal of Food Science and Technology*, *49*, 952–959.

Aloui, H., Licciardello, F., Khwaldia, K., Hamdi, M., Restuccia, C. (2015). 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. *International Journal of Food Microbiology*, *200*, 22–30.

Calvo-Garrido, C., Elmer, P. a G., Viñas, I., Usall, J., Bartra, E., Teixidó, N. (2013a). Biological control of botrytis bunch rot in organic wine grapes with the yeast antagonist *Candida sake* CPA-1. *Plant Pathology*, *62*, 510–519.

Calvo-Garrido, C., Viñas, I., Elmer, P., Usall, J., & Teixidó, N. (2013b). *Candida sake* CPA-1 and other biologically based products as potential control strategies to reduce sour rot of grapes. *Letters of Applied Microbiology*, *57*, 356–361.

Calvo-Garrido, C., Elmer, P. a G., Parry, F.J., Viñas, I., Usall, J., Torres, R., Agnew, R.H., & Teixidó, N. (2014a). Mode of action of a fatty acid-based natural product to control *Botrytis cinerea* in grapes. *Journal of Applied Microbiology*, *116*, 967–979.

Calvo-Garrido, C., Viñas, I., Usall, J., Rodríguez-Romera, M., Ramos, M.C., & Teixidó, N. (2014b). Survival of the biological control agent *Candida sake* CPA-1 on grapes under the influence of abiotic factors. *Journal of Applied Microbiology*, *117*, 800–811.

Cañamás, T.P., Viñas, I., Usall, J., Casals, C., Solsona, C., & Teixidó, N. (2008a). Control of postharvest diseases on citrus fruit by preharvest application of the biocontrol agent *Pantoea agglomerans* CPA-2. Part I. Study of different formulation strategies to improve survival of cells in unfavourable environmental conditions. *Postharvest Biology and Technology*, *49*, 86–95.

Cañamás, T.P., Viñas, I., Usall, J., Torres, R., Anguera, M., & Teixidó, N. (2008b). Control of postharvest diseases on citrus fruit by preharvest applications of biocontrol agent *Pantoea agglomerans* CPA-2. Part II. Effectiveness of different cell formulations. *Postharvest Biology and Technology*, *49*, 96–106.

Cañamás, T.P., Viñas, I., Torres, R., Usall, J., Solsona, C., & Teixidó, N. (2011). Field applications of improved formulations of *Candida sake* CPA-1 for control of *Botrytis cinerea* in grapes. *Biological Control*, *56*, 150–158.

Choi, W., Han, J.H., 2001. Physical and mechanical properties of pea-protein-based edible films. *Journal of Food Science*, *66*, 319–322.

Domínguez-Manzano, J., Olmo-Ruiz, C., Bautista-Gallego, J., Arroyo-López, F.N., Garrido-Fernández, A., & Jiménez-Díaz, R. (2012). Biofilm formation on abiotic and biotic surfaces during Spanish style green table olive fermentation. *International Journal of Food Microbiology*, *157*, 230–238.

Droby, S., Wisniewski, M., El Ghaouth, A., & Wilson, C. (2003). Influence of food additives on the control of postharvest rots of apple and peach and efficacy of the yeast-based biocontrol product Aspire. *Postharvest Biology and Technology*, *27*, 127–135.

Droby, S., Wisniewski, M., Macarisin, D., Wilson, & C. (2009). Twenty years of postharvest biocontrol research: Is it time for a new paradigm? *Postharvest Biology and Technology*, *52*, 137–145.

El-Ghaouth, A., Smilanick, J.L., & Wilson, C.L. (2000). Enhancement of the performance of *Candida saitoana* by the addition of glycolchitosan for the control of postharvest decay of apple and citrus fruit. *Postharvest Biology and Technology*, *19*, 103–110.

Elmer, P.A.G., & Reglinski, T. (2006). Biosuppression of *Botrytis cinerea* in grapes. *Plant Pathology*, *55*, 155–177.

Fan, Y., Xu, Y., Wang, D., Zhang, L., Sun, J., Sun, L., & Zhang, B. (2009). Effect of alginate coating combined with yeast antagonist on strawberry (*Fragaria* × *ananassa*) preservation quality. *Postharvest Biology and Technology*, *53*, 84–90.

Fava, J., Hodara, K., Nieto, A., Guerrero, S., Alzamora, S.M., & Castro, M.A. (2011). Structure (micro, ultra, nano), color and mechanical properties of *Vitis labrusca* L. (grape berry) fruits treated by hydrogen peroxide, UV-C irradiation and ultrasound. *Food Research International*, *44*, 2938–2948.

Fokkema, N.J. (1996). Biological control of fungal plant diseases. *Entomophaga*, *41*, 333–342.

Hernández-Muñoz, P., Almenar, E., Valle, V., Velez, D., & Gavara, R. (2008). Effect of chitosan coating combined with postharvest calcium treatment on strawberry (*Fragaria* × *ananassa*) quality during refrigerated storage. *Food Chemistry*, *110*, 428–435.

Hou, C.T., & Forman III, R.J. (2000). Growth inhibition of plant pathogenic fungi by hydroxy fatty acids. *Journal of Industrial Microbiology and Biotechnology*, *24*, 275–276.

Ippolito, A., & Nigro, F. (2000). Impact of preharvest application of biological control agents on postharvest diseases of fresh fruits and vegetables. *Crop Protection*, *19*, 715–723.

Jiménez, A., Fabra, M.J., Talens, P., & Chiralt, A. (2012). Effect of re-crystallization on tensile, optical and water vapour barrier properties of corn starch films containing fatty acids. *Food Hydrocolloids*, *26*, 302–310.

Liu, J., Wisniewski, M., Droby, S., Norelli, J., Hershkovitz, V., Tian, S., & Farrell, R. (2012). Increase in antioxidant gene transcripts, stress tolerance and biocontrol efficacy of *Candida oleophila* following sublethal oxidative stress exposure. *FEMS Microbiology Ecology*, *80*, 578–590.

McGuire, R.G. (2000). Population dynamics of postharvest decay antagonists growing epiphytically and within wounds on grapefruit. *Phytopathology*, *90*, 1217–1223.

Mcguire, R.G., & Baldwin, E.A. (1994). Compositions of cellulose coatings affect populations of yeasts in the liquid formulation and on coated grapefruits. *Proceedings of the Florida State Horticultural Society*, *107*, 293–297.

McGuire, R.G., & Dimitroglou, D.A. (1999). Evaluation of shellac and sucrose ester fruit coating formulations that support biological control of post-harvest grapefruit decay. *Biocontrol Science and Technology*, *9*, 53–65.

McGuire, R.G., & Hagenmaier, R.D., 1996. Shellac coatings for grapefruits that favor biological control of *Penicillium digitatum* by *Candida oleophila*. *Biological Control*, *7*, 100–106.

Mokiou, S., & Magan, N. (2008). Physiological manipulation and formulation of the biocontrol yeast *Pichia anomala* for control of *Penicillium verrucosum* and ochratoxin A contamination of moist grain. *Biocontrol Science and Technology*, *18*, 1063–1073.

Montealegre, J.R., López, C., Stadnik, M.J., Henríquez, J.L., Herrera, R., Polanco, R., Di Piero, R.. M., & Pérez, L.M. (2010). Control of grey rot of apple fruits by biologically active natural products. *Tropical Plant Pathology*, *35*, 271–276.

Nobile, C.J., & Mitchell, A.P. (2007). Microbial biofilms: e pluribus unum. *Current Biology*, *17*, 349–353.

Ortega-Toro, R., Jiménez, A., Talens, P., & Chiralt, A. (2014). Effect of the incorporation of surfactants on the physical properties of corn starch films. *Food Hydrocolloids*, *38*, 66–75.

Pastor, C., Sánchez-González, L., Marcilla, A., Chiralt, A., Cháfer, M., & González-Martínez, C. (2011). Quality and safety of table grapes coated with hydroxypropylmethylcellulose edible coatings containing propolis extract. *Postharvest Biology and Technology*, *60*, 64–70.

Perdones, A., Sánchez-González, L., Chiralt, A., & Vargas, M. (2012). Effect of chitosan-lemon essential oil coatings on storage-keeping quality of strawberry. *Postharvest Biology and Technology*, *70*, 32–41

Potjewijd, R., Nisperos, M.O., Burns, J.K., Parish, M., & Baldwin, E.A. (1995). Cellulose-based coatings as carriers for *Candida guillermondii* and *Debaryomyces sp*. in reducing decay of oranges. *HortScience*, *30*, 1417–1421.

Reglinski, T., Elmer, P. a G., Taylor, J.T., Wood, P.N., & Hoyte, S.M. (2010). Inhibition of *Botrytis cinerea* growth and suppression of botrytis bunch rot in grapes using chitosan. *Plant Pathology*, *59*, 882–890.

Řiháková, Z., Plocková, M., Filip, V., & Šmidrkal, J. (2001). Antifungal activity of lauric acid derivatives against *Aspergillus niger*. *European Food Research and Technology*, *213*, 488–490.

Rodríguez, M., Osés, J., Ziani, K., & Maté, J.I. (2006). Combined effect of plasticizers and surfactants on the physical properties of starch based edible films. *Food Research International*, *39*, 840–846.

Romanazzi, G., Karabulut, O.A., & Smilanick, J.L. (2007). Combination of chitosan and ethanol to control postharvest gray mold of table grapes. *Postharvest Biology and Technolog*y, *45*, 134–140.

Romanazzi, G., Gabler, F.M., Margosan, D., Mackey, B.E., & Smilanick, J.L. (2009). Effect of chitosan dissolved in different acids on its ability to control postharvest gray mold of table grape. *Phytopathology*, *99*, 1028–1036.

Sánchez-González, L., Vargas, M., González-Martínez, C., Chiralt, A., & Cháfer, M. (2009). Characterization of edible films based on hydroxypropylmethylcellulose and tea tree essential oil. *Food Hydrocolloids*, *23*, 2102–2109.

Sánchez-González, L., Pastor, C., Vargas, M., Chiralt, A., González-Martínez, C., & Cháfer, M. (2011). Effect of hydroxypropylmethylcellulose and chitosan coatings with and without bergamot essential oil on quality and safety of cold-stored grapes. *Postharvest Biology and Technolog*y, *60*, 57–63.

Sánchez-González, L., Quintero Saavedra, J.I., & Chiralt, A. (2013). Physical properties and antilisterial activity of bioactive edible films containing *Lactobacillus plantarum*. *Food Hydrocolloids*, *33*, 92–98.

Teixidó, N., Viñas, I., Usall, J., & Magan, N. (1998). Control of blue mold of apples by preharvest application of *Candida sake* grown in media with different water activity. *Phytopathology*, *88*, 960–964.

Teixidó, N., Torres, R., Viñas, I., Abadias, M., & Usall, J. (2011). Biological control of postharvest diseases in fruit and vegetables. In: Lacroix, C. (ed)., *Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage biopreservation* (pp. 364-402. Sawston, Cambridge (UK): Woodhead Publishing Limited.

Vargas, M., Pastor, C., Chiralt, A., McClements, D.J., & González-Martínez, C. (2008). Recent advances in edible coatings for fresh and minimally processed fruits. *Critical Reviews in Food Science and Nutrition, 48*, 496–511.

Viñas, I., Usall, J., Teixidó, N., & Sanchis, V. (1998). Biological control of major postharvest pathogens on apple with *Candida sake*. *International Journal of Food Microbiology*, *40*, 9–16.

Xu, W.T., Huang, K.L., Guo, F., Qu, W., Yang, J.J., Liang, Z.H., & Luo, Y.B. (2007). Postharvest grapefruit seed extract and chitosan treatments of table grapes to control *Botrytis cinerea*. *Postharvest Biology and Technolog*y, *46*, 86–94.

Zahavi, T., Cohen, L., Weiss, B., Schena, L., Daus, a., Kaplunov, T., Zutkhi, J., Ben-Arie, R., & Droby, S. (2000). Biological control of *Botrytis*, *Aspergillus* and *Rhizopus* rots on table and wine grapes in Israel. *Postharvest Biology and Technolog*y, *20*, 115–124.

CHAPTER 2

PROPERTIES OF COATING-FORMING BIOPOLYMER DISPERSIONS AND FILMS USED AS CARRIERS OF THE BIOCONTROL AGENT *Candida sake* CPA-1

Anna Marín, Lorena Atarés, Maite Cháfer & Amparo Chiralt

Submitted to *Food and Bioprocess Technology*

Instituto de Ingeniería de Alimentos para el Desarrollo, Departamento de Tecnología de Alimentos. Universitat Politècnica de València, 46022, Valencia, Spain

ABSTRACT

Films and coatings formulated from different biopolymers (Hydroxypropylmethylcellulose: HPMC, corn starch: S, sodium caseinate: NaCas and pea protein: PP) with and without surfactants (oleic acid: OA, Span 80: S80 and Tween 85: T85) have been characterized as to their barrier and optical properties in order to analyze their impact on fruit when applied as carriers of *Candida sake* CPA-1, as biocontrol agent. The properties of the film-forming dispersions (FFDs) that are relevant as regards their stability and fruit application were also analyzed. The properties of the FFDs were more clearly affected by the type of polymer than by the incorporation of a surfactant and the influence of this compound was heavily dependent on the type of polymer. NaCas dispersions formed the thickest coatings, but these were very thin in every case, which led to there being no predicted relevant effect on the gas exchanges of the fruit. The cell viability in the films without fruit support was good during film drying in every case, especially in the case of protein films; however, it decreased after 7 storage days at 68% relative humidity, particularly in polysaccharide films. Comparison of this behavior with that reported in coated grapes led to the conclusion that cell survival increased when there are a fruit support.

Keywords: edible coating, edible film, biocontrol agent, *Candida sake*, cell viability.

1. INTRODUCTION

Edible films or coatings contribute to food preservation, acting as a barrier for gases or water transport and also contributing to an improvement in the appearance of the product (e.g. gloss) (González-Martínez *et al*., 2001; Vargas *et al*., 2008). They can also be used as carriers of antioxidant or antimicrobial agents to enhance the product stability. Antimicrobial edible films can be formulated via the incorporation of different compounds such as essential oils, organic acids and their salts, bacteriocins or enzymes in the formulation of film-forming dispersions (FFDs) (Suppakul *et al*., 2003). These films may be applied in food products to prolong the shelf-life, but could also be used in the field of crop protection when applied pre-harvest.

In the formulation of antimicrobial films, some microorganisms, such as lactic acid bacteria (LAB), have been used due to their ability to produce antibiotics and other antimicrobial metabolites effective against some foodborne pathogenic bacteria (López De Lacey *et al*., 2012; Romano *et al*., 2014; Sánchez-González *et al*., 2013; 2014; Soukoulis *et al*., 2016). The main applications of films and coatings with LAB have been described for foodstuffs of animal origin, which are susceptible to the growth of *L. monocytogenes*, one of the pathogens which are of greatest concern to the food industry (Concha-Meyer *et al*., 2011; Gialamas *et al*., 2010). Other microorganisms which can act as microbial antagonists are yeasts, which are naturally present in fruit. These have received considerable attention as controlling agents of diseases caused by molds in fruits and vegetables, both at pre and post-harvest (Liu *et al*., 2013; Teixidó *et al*., 1998). The so-called biological control, i. e. the use of living agents to control pests or plant pathogens, is considered as a reliable alternative to pesticide use both in the field and post-harvest (Droby *et al*., 2009, Teixidó *et al*., 2011). The formulation of coatings/films, containing living agents, for biological control purposes (biocontrol agents: BCA) represents an interesting alternative means of applying this kind of preservation method in crops. The film constituents can help to keep the microorganisms alive by acting as a source of nutrients, at the same time as they protect them from environmental damage (rain, sun radiation or temperature

variations), favoring their adhesion to the plant (Cañamás et al., 2008; Marín *et al*., 2016).

There are few published studies dealing with edible films or coatings used as carriers of antagonistic yeasts, aiming to control plant diseases. González-Estrada *et al*., (2015) obtained arabinoxylan films with *Debaryomyces hansenii* as BCA and studied its influence on the physical properties of the films. The incorporation of this yeast into the films led to an increase in yellowness and influenced the mechanical properties, reducing tensile strength, elongation at break and Young's modulus. Aloui *et al*., (2015) developed films based on sodium alginate and locust bean gum, which were able to maintain more than 85% of the initial *Wickerhamomyces anomalus* yeast population for 21 days. Fan *et al*., (2009) incorporated the BCA *Cryptococcus laurentii* in alginate coatings to be applied on strawberries. In this study, cell viability was maintained at 50% after 20 days of storage. Further studies about yeast BCA application in edible coatings on fruits have been reported by Calvo-Garrido *et al*., (2013) and Cañamás *et al*., (2011), El-Ghaouth *et al*., (2000), McGuire (2000) and Potjewijd *et al*., (1995). In every case, it was possible to observe an enhancement of the cell yeast viability on the fruit surface and an increased control of the pathogens when the BCAs were applied in combination with the different coatings, which, in some cases, was also proven in crop applications.

Of the wide variety of antagonistic yeasts which are of potential use as BCA, particular attention has been paid to *Candida sake* CPA-1, due to its ability to control some fungal diseases, such as grey mold caused by *Botrytis cinerea* (Calvo-Garrido *et al*., 2013; Cañamás *et al*., 2011). This fungus causes heavy losses in table and wine grapes worldwide (Masih *et al*., 2001). Competition for nutrients and space is the proposed mechanism whereby CPA-1 is able to inhibit fungal diseases (Calvo-Garrido *et al*., 2013). This mode of action requires the presence of a high number of cells on the fruit surface to ensure their antifungal efficiency. In this sense, their application in edible coatings could improve the cell viability and, in turn, the antifungal action, as has been reported in a recent study (Marín *et al*., 2016). These authors observed that coatings based on different hydrocolloids improved the survival and efficacy of *C. sake* as a BCA against *B. cinerea*.

Accordingly, the selection of coating components is necessary both to ensure their ability to be carriers of BCAs, in terms of compatibility and viability enhancement, as well as to confer suitable functional properties to the coating. Therefore, both cell viability and coating functional properties must be taken into account in BCA formulations with coating forming agents. In this sense, barrier or optical properties, which could affect the exchanges of water and respiration gases of the plant or its appearance, should be analyzed to identify suitable formulations. Biopolymers, such as polysaccharides and proteins, have been widely studied as edible film or coating forming agents (Arrieta *et al*., 2013; Choi & Hoy, 2001; Jiménez *et al*., 2014; Sánchez-González *et al*., 2009). Hydroxypropylmethylcellulose (HPMC), corn starch (S), sodium caseinate (NaCas) and pea protein (PP) are examples of these compounds, which have shown good compatibility with *C. sake* when applied on grapes, with or without surfactants in the coating formulation (Marín *et al*., 2016). Cell viability increased to a different extent with respect to the coating-free cell applications, as did the antifungal efficacy. The use of surfactants in the active coating formulations could improve the adherence on the plant surface at the same time as they could modulate the film barrier properties due to their more hydrophobic nature, as compared with hydrocolloid matrices (Ortega-Toro *et al*., 2014; Santacruz *et al*., 2015; Villalobos-Carvajal *et al*., 2009). For surfactants, the balance of the size and strength of the hydrophilic and lipophilic groups of the molecules, known as hydrophilic-lipophilic balance (HLB), is a determining factor of their potential influence on the film properties (Pasquali *et al*., 2008).

This work was undertaken to determine the functional properties as films or coatings of different formulations, of proven compatibility with the biocontrol agent *C. sake* CPA-1, in order to predict their effects when applied on the fruit. The hydrocolloid FFD and films were based on HPMC, S, NaCas or PP with or without surfactants with different HLB (oleic acid: OA, Span 80: S80 and Tween 85: T85). The properties of film forming dispersions relevant to their spray application on the plant and the barrier and optical properties of the films, with and without cells, were analyzed to predict coating functionality. Likewise, the viability of the antagonist BCA in the films without fruit support, conditioned at different relative humidity, was studied.

2. MATERIALS AND METHODS

2.1 Materials

Hydroxypropylmethylcellulose (HPMC), sodium caseinate (NaCas), surfactants (oleic acid: OA, Span 80®: S80 and Tween 85®: T85) and streptomycin sulphate were supplied by Sigma – Aldrich (Madrid, Spain). Corn starch (S) and pea protein (PP) were purchased from Roquette Laisa España, S.A., (Valencia, Spain) and glycerol, magnesium nitrate-6-hydrate, phosphorus pentoxide and potassium iodide from Panreac Química, S.L.U (Barcelona, Spain). Trypticase soy agar was purchased from Scharlab (Barcelona, Spain).

2.2 Preparation of the film forming dispersions (FFDs)

FFDs with and without surfactants were prepared by dispersing the biopolymers in deionized water. The concentration of polymers was chosen in order to obtain viscosities that allow the solutions to be sprayed (3–15 mPa·s), taking previous studies into account (Jiménez *et al*., 2012; Sánchez-González *et al*., 2009; 2013). HPMC (2% wt.) was heated until 80°C for 10 min and maintained under magnetic stirring at 25°C overnight. S (2% wt.) was stirred at 95°C for 30 min to induce starch gelatinization. NaCas and PP (4% wt.) were dispersed at 25°C for 2 h. After polymer dispersion, glycerol was incorporated as plasticizer in S, NaCas and PP FFDs at a hydrocolloid:glycerol mass ratio of 1:0.25. Surfactants were added at a hydrocolloid:surfactant mass ratio of 1:0.1. Prior to film formation, FFDs were homogenized using a rotor-stator homogenizer (Ultraturrax T25, Janke and Kunkel, Germany) at 13,600 rpm for 4 minutes and sterilized at 121°C for 15 min to eliminate possible microbial contamination.

2.3 Characterization of the film forming dispersions

2.3.1 Density, pH, particle size and ζ-potential

Film forming dispersions were characterized as to density, pH, particle size and ζ-potential. Density (ρ) was measured at 25°C by means of a pycnometer, using water as the reference liquid. A pH-meter (GLP +21 Crison Instruments SA, Hospital del Llobregat,

Barcelona, Spain) was used to determine the pH at 25°C. Both tests were performed in triplicate.

The droplet size distribution, volume-length mean diameter ($D_{4.3}$) and volume-surface mean diameter ($D_{3.2}$) of the FFD were measured by using a laser diffractometer (Mastersizer 2000, Malvern Instruments, Worcestershire, UK). The samples were diluted in deionized water at 2000 rpm until an obscuration rate of 10% was obtained. Three samples of each FFD were measured in quintuplicate.

ζ-potential was measured in triplicate using a Zetasizer nano-Z (Malvern Instruments, Worcestershire, UK). The electrophoretic mobility of the droplets was transformed into ζ-potential values using the Smoluchowsky model. The samples were diluted with water to a droplet concentration of 0.02%.

2.3.2 Rheological behavior

The rheological behavior of FFDs was analyzed in duplicate at 25°C by means of a rotational rheometer (HAAKE Rheostress 1, Thermo Electric Corporation, Karlsruhe, Germany) with a Z34DIN Ti type sensor system of coaxial cylinders. Up and down curves of shear stress (σ) vs. shear rate ($\dot{\gamma}$) from 0 to 800 s^{-1} were obtained: 6 minutes to reach the maximum shear rate and 6 minutes to attain zero shear rate. Either the Ostwald de Waele model or the Herschel-Bulkey model (Eqs. 1 and 2 respectively) was fitted to the experimental up-curve data depending on whether the curves show yield shear stress (σ_y) or not. The consistency index (K) and the flow behavior index (*n*) were determined in all samples. Additionally, the apparent viscosities at 100 s^{-1} were given for each formulation.

$$\sigma = K \cdot \dot{\gamma}^n \quad (1)$$

$$\sigma = \sigma_y + K \cdot \dot{\gamma}^n \quad (2)$$

2.3.3 Coating capacity of film forming dispersions on the grape surface

The coating capacity of the different formulations on the fruit surface was studied following a gravimetric method. Bunches of table grapes were selected on the basis of their similar weight and lack of mechanical damage and four replicates were coated with the FFDs by spraying them (three applications) using an Elite E4182 sprayer.

Samples were weighed before and after pulverization, and FFD mass adhered on the grape surface was expressed as mg/g grape. In order to calculate the total adhered solids, the mass fraction of each FFD was considered. These values were used to estimate the thickness of applied coatings, by using a relationship between film thickness and solid surface density, which was obtained by preparing films with different amounts of solids per surface area in Petri plates and measuring their respective thicknesses.

To observe the formed coating on the fruit surface CryoSEM observations of fruit surface were carried out, using a Scanning Electron Microscope (JEOL JSM-5410, Japan). Samples were cryofixed in slush nitrogen and observed, after gold coating, using an accelerating voltage of 10 kV.

2.4 Film preparation

The mass of each FFD containing 1 g of solids was spread evenly over 15 cm diameter polytetrafluorethylene plates (PTFE) resting on a level surface. The final solid surface density was 5.6 mg/cm^2. Films were formed by drying for approximately 48 h at 45% RH and 25°C and peeled off the casting surface. Prior to film characterization, the films were stored for seven days in desiccators at 25°C and 53%RH using an oversaturated solution of magnesium nitrate-6-hydrate. Additionally, films of the different polymers without surfactants were also prepared by adding the cell culture suspensions as described in section 2.6.

2.5 Characterization of the films

2.5.1 Optical properties

The most important properties to assess the direct impact on the appreciation of color and appearance of a coated product are the gloss and the transparency (Hutchings, 1999). The gloss of the films was measured at an incidence angle of 60°, according to the ASTM standard D523 (ASTM, 1999) using a flat surface gloss meter (Multi-Gloss 268, Minolta, Germany). Measurements were taken in triplicate of each sample and three films of each formulation were considered. Results were expressed as gloss units, relative to a highly polished surface of black glass standard with a value close to 100.

The transparency of the films was determined through the surface reflectance spectra from 400 to 700 nm with a spectrocolorimeter CM-3600d (Minolta Co., Tokyo, Japan) on both a white and a black background. Measurements were taken in triplicate on the free film surface. The Kubelka-Munk theory for multiple scattering to the reflection spectra was applied in order to determine the transparency (Hutchings, 1999). Internal transmittance (T_i) was quantified using Eq. (3) in which R_0 is the reflectance of the film on an ideal black background. a and b parameters were calculated by Eqs. (4) and (5), where R is the reflectance of the sample layer backed by a known reflectance R_g. R_∞ (Eq. (6)) values (reflectance of an infinitely thick layer of the material) were used to determine L*, a* and b* values from the CIELab color space, using D65 illuminant and 10° observer. From these values, whiteness index (WI) was obtained (Eq. 7).

$$T_i = \sqrt{(a - R_0)^2 - b^2} \tag{3}$$

$$a = \frac{1}{2}\left(R + \frac{R_0 - R + R_g}{R_0 \cdot R_g}\right) \tag{4}$$

$$b = \sqrt{a^2 - 1} \tag{5}$$

$$R_\infty = a - b \tag{6}$$

$$WI = 100 - \sqrt{(100 - L^*)^2 + a^{*2} + b^{*2}} \tag{7}$$

2.5.2 Thickness, moisture content and barrier properties

A digital micrometer (Electronic Digital Micrometer, Comecta S.A., Barcelona, Spain) was used to measure film thickness to the nearest 0.0001 mm. Measurements were taken at six points of each film randomly.

Moisture content of the film specimens was determined gravimetrically. Four samples per formulation were dried for 24 h at 60°C in a vacuum oven. Afterwards they were placed in a desiccator containing phosphorus pentoxide (P_2O_5) at room temperature for 2 weeks, until constant weight was reached.

The water vapor permeability (WVP) of the films was measured according to a modification of the ASTM E-96-95 (McHugh *et al*., 1993) at 25°C and for a 53 – 100 % RH gradient, generated by using an oversaturated solution of magnesium nitrate-6-hydrate and distilled water respectively. Measurements were taken in triplicate in each formulation by placing them on Payne permeability cups (Elcometer SPRL, Hermelle/s Argenteau, Belgium). The side of the film in contact with the PTFE plate during drying was exposed to the highest RH value. The cups were weighed periodically (± 0.0001 g). After the steady state was reached, the slope obtained from the weight loss *vs.* time was used to calculate the water vapor transmission (WVTR). The vapor pressure on the film's inner surface (p_2) was obtained with Eq. (8), proposed by McHugh *et al*., (1993), to correct the effect of concentration gradients established in the stagnant air gap inside the cup:

$$WVTR = \frac{P \cdot D \cdot Ln\left[\frac{(P - p_2)}{(P - p_1)}\right]}{R \cdot T \cdot \Delta z} \quad (8)$$

where P, total pressure (atm); D, diffusivity of water through air at 10 and 25°C (m^2/s); R, gas law constant ($82.0 \cdot 10^{-3}$ $m^3 \cdot atm/kmol \cdot K$); T, absolute temperature (K); Δz, mean stagnant air gap height (m), considering the initial and final z value; p_1, water vapor pressure on the solution surface (atm); and p_2, corrected water vapor pressure on the film's inner surface (atm).

Using Eq. (9) permeance was calculated and the water vapor permeability was determined by multiplying the permeance by the average film thickness.

$$Permeance = \frac{WVTR}{(p_1 - p_2)} \quad (9)$$

The oxygen permeability (OP) of the films was determined at 53% RH and 25°C using an OX-TRAN model 2/21 ML Mocon (Lippke, Neuwied, Germany). Three samples were placed in the equipment for analysis and they were conditioned in the cells for 6 h. The transmission values were determined every 20 min until the equilibrium was reached. The exposure area during the tests was 50 cm^2.

2.6 *Candida sake* incorporation to the films and viability over film storage

Strain CPA-1 of *C. sake* was originally isolated from the surface of apples by UdL-IRTA Centre (Lleida, Spain) (Viñas *et al*., 1998) and was deposited at the "Colección Española de Cultivos Tipo" (CECT-10817) in the "Universidad de Valencia" (Burjassot, Spain). Cell production and formulation was carried out according to methods described by Cañamás *et al*., (2011).

C. sake was incorporated into each FFD, aiming for a final yeast population of $5 \cdot 10^7$ CFU per film (which corresponded to $2.8 \cdot 10^5$ CFU/cm^2 in the formed film). Suspensions obtained were shaken for 15 minutes at 150 rpm to achieve a homogeneous distribution of the microorganisms. The films with the BCA were obtained as described in section 2.3., and after drying these were stored in desiccators at 25°C and 53% or 68% RH, using oversaturated solutions of magnesium nitrate-6-hydrate or potassium iodide respectively. Films stored at 53% RH were characterized as to moisture content, barrier properties and gloss for comparisons with cell-free films.

Viability of *C. sake* was tested immediately after drying (2 days) and over storage time (7, 14 and 21 days) at 53 and 68 % RH. For the viability test, film specimens were placed in sterile plastic bags containing 100 mL sterile deionized water with 0.01% (w/v) Tween 85, and homogenized for 6 min in a Stomacher blender (Bag Mixer 400, Interscience). Serial dilutions were made by duplicate and plated onto trypticase soy agar (TSA) medium with streptomycin sulphate (0.5 g/L) to prevent bacterial growth. Plates were incubated for 48 h at 25°C in the dark and typical *C. sake* colonies were then counted based on their morphological characteristics.

2.7 Statistical analysis

Statistical Analyses were performed using Statgraphics Centurion XVI 16.1.17 (Manugistics Corp., Rockville, Md.) Homogeneous sample groups in ANOVA were obtained by using LSD test (95% significance level). Multifactor ANOVA was also used to analyze the effect of polymer and surfactants on the different properties. Principal Component Analysis (PCA) was used to compare all film forming dispersions and films using Unscrambler 10.X software.

3. RESULTS AND DISCUSSION

3.1 Properties of film forming dispersions

3.1.1 Density, pH, particle size and ζ-potential

Table 2.1 shows the values of density, pH, average diameters of the particles and their ζ-potential for the different FFDs. The density was always similar to that of water, given the low solid proportion (between 2 and 4 g per 100g FFD). The highest values were found for the proteins, which were incorporated into the highest mass ratio (4%wt.). The pH values were in the neutral range, between 6.5 and 7.8. NaCas FFDs were slightly more acid than the rest, probably due to aminoacid dissociation. Likewise, for a determined polymer, FFD with OA showed the lowest pH.

Table 2.1. Density (kg/m^3), pH, ζ-potential (mV) and mean particle size of the different film forming dispersions with and without surfactants (mean values and standard deviation). HPMC: hydroxypropylmethylcellulose, S: starch, NaCas: sodium caseinate, PP: pea protein, WS: without surfactant, OA: oleic acid, S80: Span 80, T85: Tween 85.

Property		Formulation			
		HPMC	S	NaCas	PP
Density (kg/m^3)	WS	1003.3 ± 0.7^{b}	1005.0 ± 0.6^{ab}	1011.0 ± 2.0^{a}	1010.1 ± 1.1^{a}
	OA	1002.3 ± 0.4^{ab}	1004.9 ± 1.1^{a}	1010.0 ± 0.9^{a}	1008.9 ± 1.4^{a}
	S80	1001.0 ± 2.0^{a}	1006.4 ± 0.8^{b}	1010.7 ± 1.1^{a}	1008.7 ± 1.7^{a}
	T85	1003.1 ± 0.5^{ab}	1005.3 ± 0.6^{ab}	1010.6 ± 0.8^{a}	1010.2 ± 0.1^{a}
pH	WS	6.61 ± 0.15^{a}	7.10 ± 0.20^{b}	6.96 ± 0.08^{b}	7.84 ± 0.05^{d}
	OA	6.90 ± 0.05^{b}	6.28 ± 0.09^{a}	6.48 ± 0.09^{a}	7.01 ± 0.02^{a}
	S80	7.32 ± 0.04^{d}	7.24 ± 0.10^{b}	6.93 ± 0.03^{b}	7.55 ± 0.01^{b}
	T85	7.14 ± 0.09^{c}	7.15 ± 0.03^{b}	6.92 ± 0.04^{b}	7.71 ± 0.03^{c}
ζ-potential (mV)	WS	-7.9 ± 1.4^{a}	-10.0 ± 0.5^{a}	-18.1 ± 1.7^{a}	-19.9 ± 1.1^{a}
	OA	-7.5 ± 0.8^{a}	-12.8 ± 1.3^{c}	-34.9 ± 3.0^{c}	-26.2 ± 0.5^{d}
	S80	-19.0 ± 3.0^{b}	-11.5 ± 0.7^{b}	-24.5 ± 1.8^{b}	-23.2 ± 0.6^{c}
	T85	-24.0 ± 2.0^{c}	-9.9 ± 0.7^{a4}	-38.0 ± 4.0^{d}	-22.1 ± 0.3^{b}
$D_{4,3}$	WS	-	26.0 ± 12.0^{c}	47.4 ± 6.3^{d}	14.6 ± 1.4^{a}
	OA	2.6 ± 0.2^{a}	10.4 ± 0.6^{a}	34.0 ± 3.0^{b}	17.2 ± 0.8^{c}
	S80	24.9 ± 0.7^{b}	9.6 ± 0.5^{a}	42.6 ± 8.0^{c}	15.6 ± 0.9^{b}
	T85	-	16.0 ± 6.0^{b}	20.8 ± 2.0^{a}	14.6 ± 1.6^{a}
$D_{3,2}$	WS	-	8.3 ± 0.9^{d}	7.7 ± 0.9^{d}	7.9 ± 0.5^{a}
	OA	1.5 ± 0.2^{a}	4.1 ± 0.1^{a}	4.9 ± 0.2^{b}	9.8 ± 0.4^{b}
	S80	4.9 ± 0.2^{b}	5.7 ± 0.1^{c}	6.5 ± 1.1^{c}	7.7 ± 0.4^{a}
	T85	-	5.2 ± 0.1^{b}	4.4 ± 0.1^{a}	7.7 ± 0.5^{a}

Different superscripts (a-d) within the same column indicate significant differences ($p < 0.05$) among formulations for the same polymer.

Figure 2.1 shows the particle size distribution curves of all FFDs, and the corresponding average diameters are shown in Table 1. Neither HPMC dispersions without surfactants nor those with T85 could be characterized in their size distribution, since they did not reach the required obscuration level for measurement, coherently with the good water solubility of the polymer and T85. In the other cases, polymer aggregates were formed in the aqueous media giving rise to measurable size particles. Most of the particle size distributions were monomodal, except for NaCas T85 and HPMC S80. Surfactants do not have a notable effect on the proteins (except NaCas T85 sample) or S. However, the surfactant greatly affected the size distributions of the HPMC dispersions, and S80 yielded greater aggregates as compared to the more hydrophobic OA. Likewise, T85 reduced the aggregation degree of NaCas particles, showing a greater population of smaller particles. This indicates particular interactions between the different surfactants and polymer chains, which affected the compound dispersion in water.

According to McClements (2005), if the droplet charge is sufficiently high, the system may be stable against aggregation due to electrostatic repulsion. The values of ζ–potential of all FFDs are shown in Table 1. All particles were negatively charged in agreement with both the adsorption of the negative ions on neutral polysaccharides and the negatively-charged protein chains at a pH above their isoelectric point. Protein FFDs showed higher values of ζ–potential due to their ionisable groups. Generally, surfactant addition resulted in changes of the surface charge of the particles, thus indicating the interactions/adsorption of these amphiphilic compounds with/on the polymer chains. This was remarkable for the HPMC S80 system and for all FFDs with proteins (NaCas, and PP) and surfactants. In all cases, a greater exposure of negative charges at the particle surface occurred due to the established interactions. Molecular interactions between amphiphilic molecules, surfactants and proteins, have been widely described, and will affect the properties and stability of the FFDs.

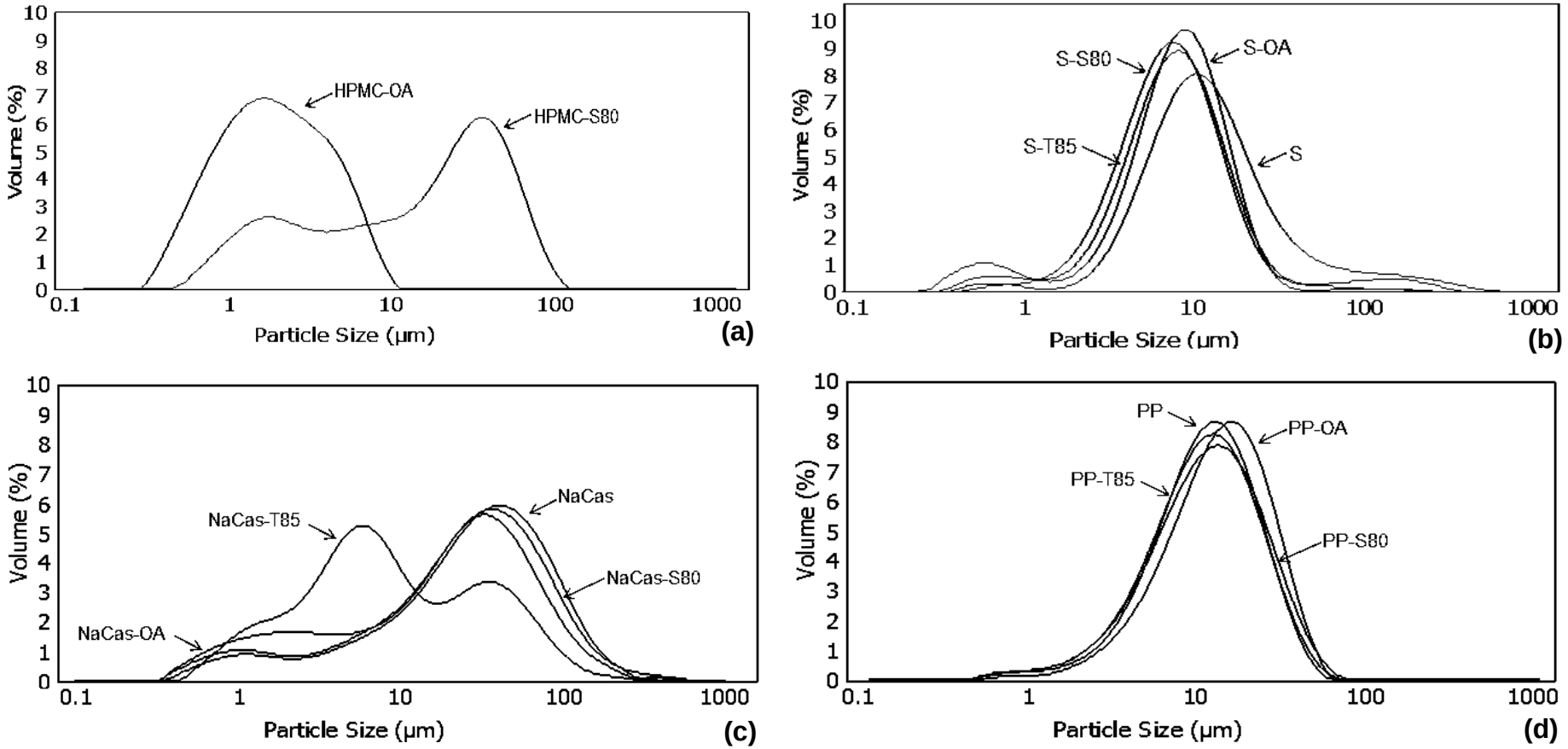


Figure 2.1. Particle size distributions of the different film forming dispersions based on HPMC: hydroxypropylmethylcellulose (a), S: starch (b), NaCas: sodium caseinate (c) and PP: pea protein (d) with and without surfactants. OA: oleic acid, S80: Span 80, T85: Tween 85.

3.1.2 Rheological behavior

All FFDs, except starch with surfactants, exhibited a non-time dependent behavior, practically Newtonian, below a limit shear rate ranging from 250 to 540 s^{-1}, where a change in the shear stress-shear rate relationship was observed. Figure 2.2 shows the flow curves of starch dispersions where those containing surfactants exhibited time-dependent behavior (different up and down curves) depending on the surfactant. The Ostwald de Waele model was fitted to the experimental data up to the limit shear rate values reported in Table 2.2. Table 2.2 shows the rheological parameters (flow and consistency indices) of the different FFDs with and without surfactants, including the apparent viscosities at 100 s^{-1} and the highest shear rate value up until which the model was fitted (limit $\dot{\gamma}$). Repeatability of rheological behavior was very high in all formulations, as deduced from the low values of the variation coefficients (VC) of rheological parameters obtained for the different replicates. These were lower than 3% in all cases for *n* values, (except in S where these were < 9 %) and lower than 10% for K values (except in S and NaCas with S80 and T85, where these were slightly higher).

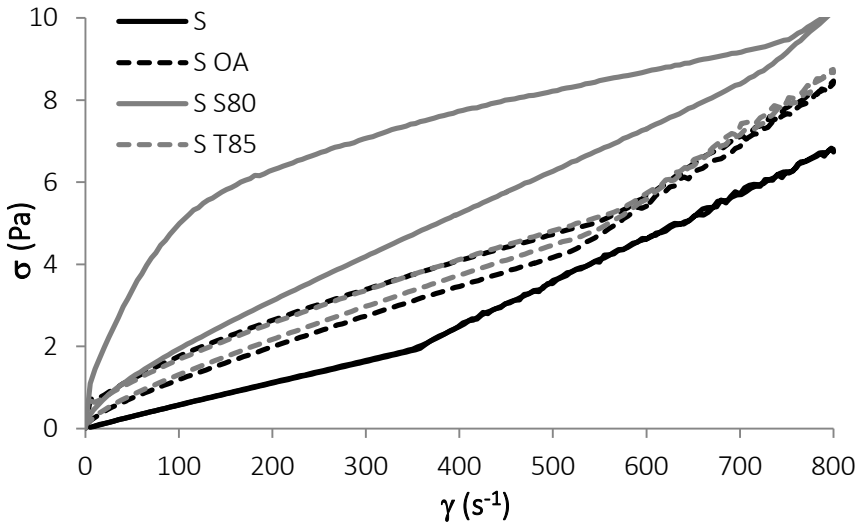


Figure 2.2. Flow curves at 25°C of the film forming dispersions based on starch (S) with and without surfactants. OA: oleic acid, S80: Span 80, T85: Tween 85.

Both for HPMC and NaCas FFDs, the flow index (close to 1) was similar to those reported by Sánchez-González *et al*., (2011) and Fabra *et al*., (2011) respectively.

Likewise, S dispersions without surfactants behaved similarly to that previously reported by Ortega-Toro *et al*., (2014).

Surfactant incorporation did not entail significant changes in the rheological behavior of HPMC, NaCas and PP FFDs, despite the interactions deduced from the ζ–potential values. The flow curves of all these polymer dispersions exhibited two different trends below and above the limit shear rate, and the apparent viscosity sharply increased at 350 s^{-1} (S), 300 s^{-1} (HPMC) or 250 s^{-1} (NaCas and PP). Nevertheless, an almost linear stress-strain relationship was observed in both periods. This sudden increase in apparent viscosity could be related to an increase in the hydrodynamic volume of the polymer chains due to the changes in their conformation (unfolding) and aggregation (shear flocculation) as a consequence of the shear flow.

Surfactant addition to S dispersions led to a remarkable increase in the apparent viscosity, promoting thixotropic behavior. The greatest hysteresis area in flow curves was found for S S80 (1622 Pa s^{-1}) (Figure 2.2), as compared to those of S OA (360 Pa·s^{-1}) and S T85 (255 Pa·s^{-1}). This effect of surfactants must be attributed to the aggregation of amylose–lipid complexes formed through the helical conformation of amylose, entrapping hydrophobic chains of surfactants (Wokadala *et al*., 2012). Due to their large size, these aggregates cause an increase in the stress-strain relationship at the same time as they can be disrupted during shear, thus causing thixotropic effects.

In starch-surfactant FFDs, the Ostwald de Waele model was fitted to the up flow curve up to a limit shear rate value ranging between 350 and 540 s^{-1} where flow behavior changed. S-S80 showed the lowest flow index (0.43) and the highest consistency index, as well as the greatest thixotropic effects, which suggests a higher degree of amylose complex formation. Furthermore, low values of yield stress were found for S OA (σ_y = 0.75 Pa) and S T85 (σ_y = 0.77 Pa), in line with the formation of the three-dimensional structure of aggregated amylose-lipid complexes. In these cases, the Herschel-Bulkley model was fitted up to 540 and 520 s^{-1}, respectively, in order to obtain yield stress values (Table 2.2).

Multifactorial ANOVA revealed that, the type of polymer and surfactant and the interaction of these two factors significantly ($p < 0.05$) affected the values of apparent

viscosity. Nevertheless, in practical terms, apparent viscosity of FFDs at 100 s^{-1} were similar (3-4 mPa·s) in all cases, except for S dispersions. In S samples, especially with surfactants, the apparent viscosity reached higher values.

Table 2.2. Rheological parameters (*n* and K), apparent viscosity (mPa·s), highest shear rate value up until which the model was fitted (limit $\dot{\gamma}$) and adherence on grapes surface of the different film forming dispersions with and without surfactants (mean values and standard deviation). HPMC: hydroxypropylmethylcellulose, S: starch, NaCas: sodium caseinate, PP: pea protein, WS: without surfactant, OA: oleic acid, S80: Span 80, T85: Tween 85.

Property		Formulation			
		HPMC	S	NaCas	PP
n	WS	1.06^{a}	0.95^{b}	1.05^{a}	1.02^{a}
	OA	1.06^{a}	1.08^{bc}	1.05^{a}	1.02^{a}
	S80	1.06^{a}	0.43^{a}	1.04^{a}	1.03^{a}
	T85	1.05^{a}	1.15^{c}	1.04^{a}	1.02^{a}
K (Pa·s^{n})	WS	3.20^{a}	7.11^{a}	3.03^{a}	3.42^{b}
	OA	3.33^{b}	5.53^{a}	3.02^{a}	2.94^{a}
	S80	3.29^{ab}	626.90^{b}	3.35^{a}	3.54^{b}
	T85	3.30^{ab}	4.06^{a}	3.22^{a}	3.26^{ab}
η_{ap} at 100 s^{-1} (mPa·s)	WS	4.16 ± 0.01^{a}	5.70 ± 0.04^{a}	3.78 ± 0.03^{a}	3.70 ± 0.30^{b}
	OA	4.29 ± 0.06^{b}	15.50 ± 0.30^{b}	3.84 ± 0.09^{a}	3.20 ± 0.05^{a}
	S80	4.30 ± 0.03^{b}	46.10 ± 0.90^{c}	4.00 ± 0.30^{a}	4.00 ± 0.17^{b}
	T85	4.24 ± 0.01^{ab}	14.90 ± 0.30^{b}	3.80 ± 0.20^{a}	3.63 ± 0.07^{ab}
Limit $\dot{\gamma}$ (s^{-1})	WS	300	350	250	250
	OA	300	540	250	250
	S80	300	350	250	250
	T85	300	520	250	250
Adherence of FFD (mg/ g grape)	WS	12.6 ± 1.1^{b}	7.7 ± 1.7^{a}	6.5 ± 0.2^{a}	4.0 ± 0.6^{b}
	OA	9.0 ± 2.0^{a}	5.0 ± 2.0^{a}	8.5 ± 1.6^{a}	4.1 ± 0.7^{b}
	S80	7.7 ± 1.4^{a}	7.0 ± 3.0^{a}	7.0 ± 3.0^{a}	4.0 ± 0.4^{b}
	T85	12.0 ± 3.0^{b}	7.3 ± 1.8^{a}	8.0 ± 3.0^{a}	2.7 ± 0.2^{a}
Adherence of solids (mg/g grape)	WS	0.25 ± 0.04^{bc}	0.19 ± 0.04^{a}	0.33 ± 0.01^{a}	0.20 ± 0.03^{b}
	OA	0.20 ± 0.05^{ab}	0.14 ± 0.06^{a}	0.45 ± 0.08^{a}	0.20 ± 0.05^{b}
	S80	0.17 ± 0.03^{a}	0.18 ± 0.08^{a}	0.36 ± 0.16^{a}	0.20 ± 0.03^{b}
	T85	0.27 ± 0.06^{c}	0.20 ± 0.05^{a}	0.41 ± 0.18^{a}	0.15 ± 0.01^{a}

Different superscripts (a-c) within the same column indicate significant differences ($p < 0.05$) among formulations for the same polymer.

3.1.3 Coating capacity of film forming dispersions on grape surface

Table 2.2 shows the mass of FFD adhered to the fruit surface during coating after the corresponding drainage (both in terms of mass of FFD per mass of grape and mass of

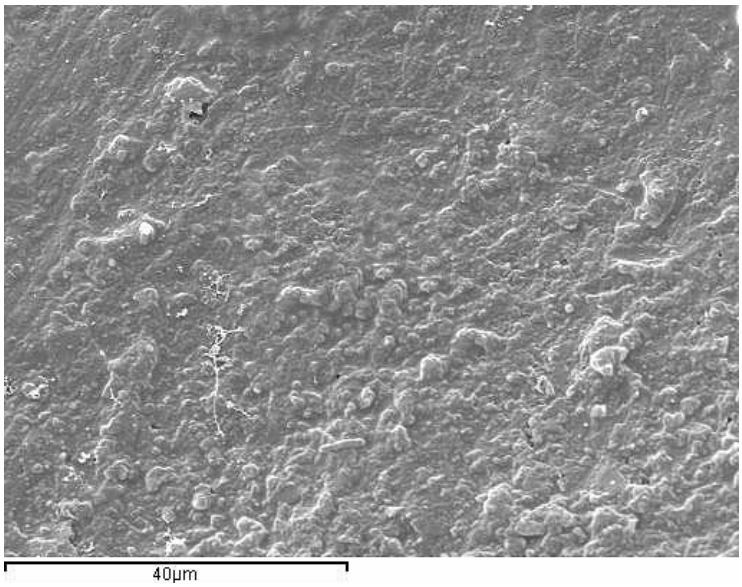
solids per mass of grape). These values will not only be affected by the adhesion forces of the film-forming dispersions on the surface of grapes (contact angle), but also by the coating gravitational drainage influenced by viscosity. S and NaCas dispersions exhibited a similar coating capacity, regardless of the presence of surfactants, this being about 5-8 mg FFD/g grapes, whereas PP showed less coating capacity (3-4) and HPMC FFDs were better spread and retained on the grape surface (8-12). No significant effect of surfactants was observed, except in HPMC, where OA and S80 reduced the coating capacity, and in PP where T85 produced the same effect. Therefore, despite the expected effect of surfactants on the contact angle and adhesion forces, no notable effect was observed in practical terms in most of the cases. Despite the higher viscosity of starch dispersions at low shear rates, no greater retention against gravitational drainage of the surface coating was observed.

The total solid surface density on the fruit determines the thickness of the formed coating. From the adhered mass of the different FFDs, the total solid mass coating of the fruit was estimated by considering their respective concentrations (Table 2.2). The NaCas FFDs provided the highest values of adhered solid mass, and hence, the formation of the thickest coatings is expected in this case.

Linear relationships between the total solids and the film thickness were obtained by correlating the solid surface density (g/cm^2) and film thickness of films prepared with different amounts of solids per surface unit. The slope of the fitted straight lines ($r^2 >$ 0,98) were 8.08, 6.52, 6.92 and 6.53, for coatings of HPMC, S, NaCas and PP, respectively. From these values and the obtained mass of solids adhered to the grape surface, the expected thicknesses of the coatings were estimated, which were 0.8, 0,5, 1.2 and 0.6 μm, respectively for HPMC, S, NaCas and PP. To this end, grapes of 2.5 cm diameter and 1.1 g/cm^3 were considered. The estimated values indicate that coatings represent a very thin layer on the fruit, which will have little impact on its water vapor and gas exchanges. Nevertheless, the appearance of the fruit at microscopic level changed as a result of the coating, as can be observed in Figure 2.3, where the SEM micrographs of the uncoated and coated fruit surface can be observed. For uncoated samples, the characteristic roughness of the waxy cuticle of fruit surface was observed

(Fava *et al*., 2011). Coatings confer a smoother surface to the fruit, reducing irregularities of the natural wax layer.

A PCA was carried out, taking all the properties into account, for the purpose of comparing FFDs. Figure 2.4 shows the typical plot of the two functions, PC1 and PC2, which explain 69 % of the variance (39% PC1), where the different FFDs are well grouped by the type of polymer. PC1 allows protein and polysaccharide FFDs to be differentiated and PC2 separates the FFDs of each polymer. In the case of polysaccharides, only HPMC S80 FFD overlaps in the group of S samples. The presence of surfactants particularly affected the HPMC samples, where the group was more dispersed in the plot. Therefore, the properties of the FFDs were more clearly affected by the type of polymer than by the incorporation of surfactants and the influence of this compound was heavily dependent on the type of polymer.



(a)

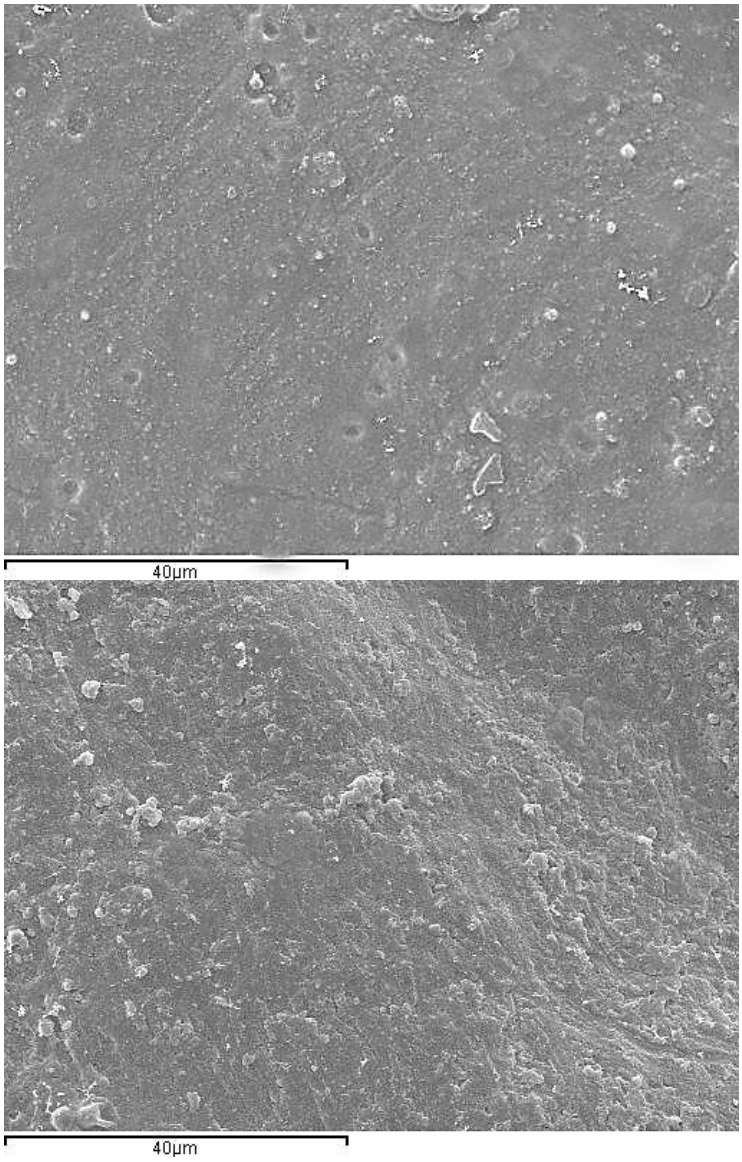


Figure 2.3. SEM images of uncoated grape surface (a) and coated with starch (b) and sodium caseinate (c) dispersions.

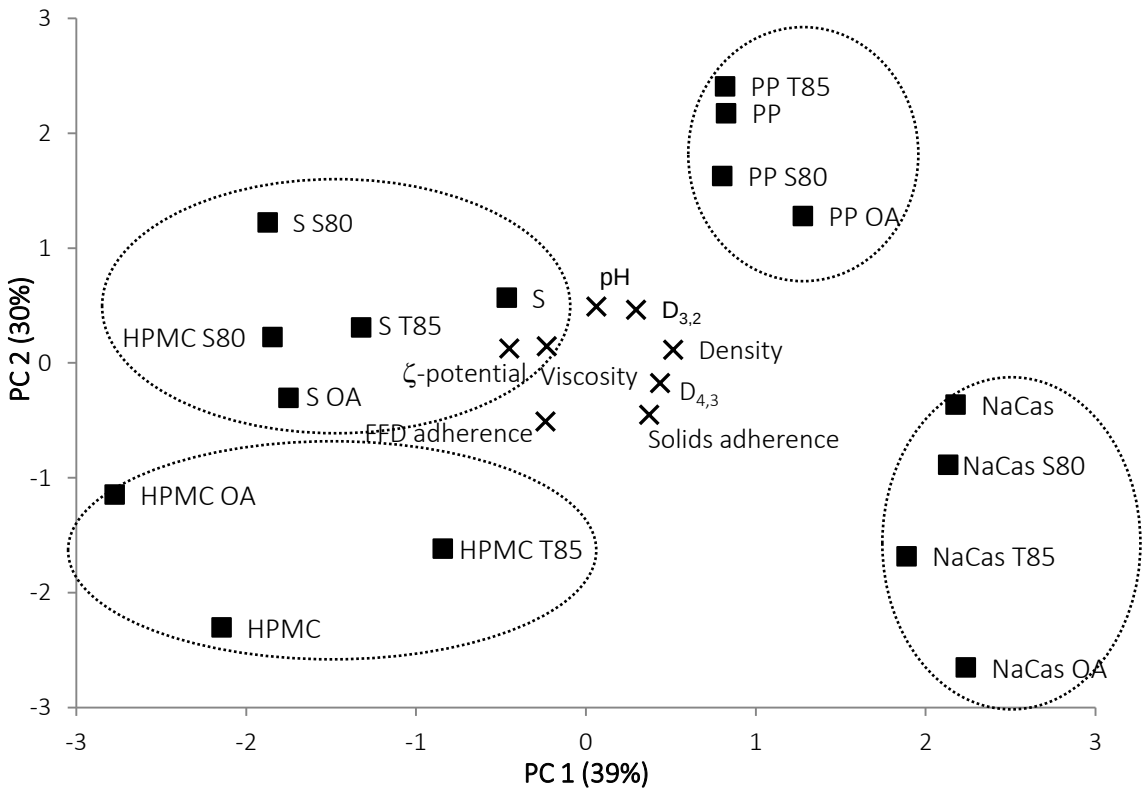


Figure 2.4. Principal component analysis for the properties of the film forming dispersions. HPMC: hydroxypropylmethylcellulose, S: starch, NaCas: sodium caseinate, PP: pea protein, WS: without surfactant, OA: oleic acid, S80: Span 80, T85: Tween 85.

3.2 Properties of the films

3.2.1 Optical properties

Gloss is related to the surface morphology of the films (Sánchez-González *et al.*, 2011) and generally, the smoother the film surface, the higher the film gloss. As all the films had gloss values lower than 70 (Table 2.3), they could be considered as matt (Trezza & Krochta, 2000). The kind of polymer significantly affected the film gloss ($p < 0.05$). PP films in general showed the highest gloss, which were comparable to those obtained by Sánchez-González *et al.*, (2013). On the other hand, HPMC films with surfactants showed the lowest gloss values. The incorporation of all surfactants into HPMC and S films resulted in a significant gloss reduction ($p < 0.05$), as reported in previous studies (Jiménez *et al.*, 2010; Sánchez-González *et al.*, 2009). Surfactant addition increased the heterogeneity and roughness of the film surface, thus reducing gloss (Jiménez *et al.*, 2012; Villalobos *et al.*, 2005). In protein matrices, OA incorporation resulted in a

significant gloss increase, which could be attributed to this lipid filling the gaps on the film surface, making it more even and glossier.

CIE L*a*b* color coordinates were calculated, and lightness, chrome, hue and whiteness index, shown in Table 2.3, were obtained. The kind of biopolymer greatly affected the color of the films. As compared to proteins, polysaccharides gave rise to lighter films with less saturated color, more yellow and less red in hue. Consequently, the whiteness index of protein films was lower. Surfactant incorporation led to a slight decrease in the lightness of HPMC films, which can be attributed to the changes in the film opacity due to the greater amount of dispersed phase.

Table 2.3 shows the values of internal transmittance at 400 nm where the greatest differences among films were observed. The internal transmittance (T_i) is related to the internal structure of the dried film, which, in turn, is affected by the initial microstructure of the formulation and its stability during film drying (Villalobos *et al*., 2005). The translucency of the films was evaluated by quantifying the internal transmittance, whose higher values are related to structural homogeneity and transparency. The highest T_i values correspond to HPMC and S with no surfactant, which is probably caused by the high packing of polysaccharide chains giving rise to homogeneous structures and increased transparency. The greatest opacity was found for PP films, as reported in previous studies (Sánchez-González *et al*., 2013).

The incorporation of surfactant caused slight T_i decreases both in HPMC and S films, especially S80 and T85 respectively. Ortega-Toro *et al*., (2014), studied the microstructure of corn starch films with S80, and observed the lipid separation in the starch matrix. The surfactant formed microdroplets which increased the heterogeneity of the film and its opacity. On the other hand, adding surfactants to NaCas films did not result in T_i modifications, other than a slight increase after OA was added. Particular interactions between NaCas and OA, which affect film properties, were described for these blend films (Fabra *et al*., 2009a; 2009b).

Table 2.3. Optical properties of the different films: gloss at 60°, color coordinates (lightness (L*), chrome (C^*_{ab}), hue (h^*_{ab}), whiteness index (WI) and internal transmittance (T_i) at 400 nm (mean values and standard deviation). HPMC: hydroxypropylmethylcellulose, S: starch, NaCas: sodium caseinate, PP: pea protein, WS: without surfactant, OA: oleic acid, S80: Span 80, T85: Tween 85.

Property		Formulation			
		HPMC	S	NaCas	PP
Gloss (60°)	WS	39 ± 20^{b}	46 ± 14^{c2}	19 ± 8^{a}	52 ± 15^{bc}
	OA	7 ± 2^{a}	21 ± 8^{a}	48 ± 20^{b}	55 ± 13^{c}
	S80	7 ± 4^{a}	22 ± 6^{a}	22 ± 4^{a}	45 ± 13^{b}
	T85	12 ± 3^{a}	31 ± 10^{b}	14 ± 3^{a}	31 ± 10^{a}
L*	WS	85.4 ± 0.2^{c}	85.5 ± 0.8^{b}	77.0 ± 0.8^{a}	68.0 ± 1.2^{a}
	OA	82.3 ± 0.6^{b}	83.2 ± 0.4^{a}	79.6 ± 1.0^{c}	70.5 ± 0.2^{a}
	S80	79.0 ± 3.0^{a}	85.0 ± 1.1^{b}	77.9 ± 0.7^{b}	67.3 ± 0.9^{a}
	T85	80.7 ± 0.4^{ab}	85.5 ± 0.4^{b}	77.0 ± 0.3^{a}	70.5 ± 5.0^{a}
C^*_{ab}	WS	4.0 ± 0.1^{a}	3.6 ± 0.1^{b}	15.2 ± 0.9^{a}	16.4 ± 0.2^{b}
	OA	4.8 ± 0.5^{a}	3.0 ± 0.3^{a}	15.3 ± 0.8^{a}	18.5 ± 0.7^{c}
	S80	4.4 ± 1.5^{a}	3.9 ± 0.6^{b}	15.1 ± 0.1^{a}	16.2 ± 0.1^{ab}
	T85	5.1 ± 0.8^{a}	4.0 ± 0.3^{b}	16.9 ± 0.3^{b}	15.7 ± 0.7^{a}
h^*_{ab}	WS	91.3 ± 1.3^{a}	100.1 ± 1.8^{b}	79.7 ± 1.3^{b}	80.7 ± 0.3^{a}
	OA	96.0 ± 1.3^{b}	97.7 ± 1.5^{a}	81.2 ± 0.6^{c}	79.7 ± 0.1^{a}
	S80	92.0 ± 3.0^{a}	98.8 ± 1.2^{ab}	78.0 ± 0.9^{a}	80.4 ± 0.5^{a}
	T85	90.2 ± 1.1^{a}	102.9 ± 0.8^{c}	78.8 ± 0.8^{ab}	83.0 ± 3.0^{b}
WI	WS	84.8 ± 0.2^{c}	85.3 ± 0.3^{c}	72.4 ± 1.1^{ab}	64.1 ± 1.2^{a}
	OA	81.7 ± 0.6^{b}	82.9 ± 0.3^{a}	74.5 ± 1.2^{c}	65.0 ± 1.1^{a}
	S80	79.0 ± 3.0^{a}	84.5 ± 0.9^{b}	73.3 ± 0.8^{b}	63.0 ± 0.1^{a}
	T85	79.8 ± 0.7^{ab}	84.9 ± 0.4^{bc}	71.8 ± 0.4^{a}	67.0 ± 5.0^{a}
T_i (400 nm)	WS	0.85 ± 0.01^{c}	0.84 ± 0.01^{c}	0.76 ± 0.01^{a}	0.66 ± 0.02^{ab}
	OA	0.84 ± 0.01^{b}	0.83 ± 0.01^{b}	0.77 ± 0.01^{b}	0.68 ± 0.01^{b}
	S80	0.83 ± 0.10^{a}	0.83 ± 0.01^{b}	0.76 ± 0.01^{a}	0.62 ± 0.02^{a}
	T85	0.84 ± 0.01^{ab}	0.82 ± 0.01^{a}	0.75 ± 0.01^{a}	0.67 ± 0.08^{ab}

Different superscripts (a-c) within the same column indicate significant differences ($p < 0.05$) among formulations for the same polymer.

3.2.2 Thickness, moisture and barrier properties

Table 2.4 shows the values of thickness, moisture content, water vapor permeability (WVP) and oxygen permeability (OP) of the different films. The film thickness ranged between 40 and 65 μm, despite the constant amount of solids used per unit of surface area. The protein films were slightly thicker than those of polysaccharide matrices, which indicates the tighter packing of S and HPMC chains, giving rise to thinner films. Likewise, the incorporation of surfactants led to thicker films, especially in the case of

polysaccharides, in line with the effects of their interruption on the polymer matrix. No significant effect was observed for PP and NaCas OA, in agreement with the better compatibility of amphiphilic molecules (surfactant and protein) which led to their more compact packing in the matrix.

HPMC films had a significantly lower equilibrium moisture content than the rest of the tested polymers, while S films exhibited the greatest water holding capacity, which coincides with the differences in the hydrophilic nature of the macromolecules. Regardless of the polymer, surfactant addition generally resulted in a significant ($p < 0.05$) decrease in the film's moisture content, coherent with the greater hydrophobic nature of these compounds, which limits the global water sorption capacity of the film solids, as reported in a previous study (Ortega-Toro *et al*., 2014). In HPMC, the effect of the surfactant was not significant due to the more hydrophobic nature of the chain. The establishment of hydrogen bonds between the hydrocolloid chains and the surfactant polar groups could also reduce the active sites for the adsorption of water molecules (Villalobos *et al*., 2006). There was no clear tendency found of the effect of the surfactant with a different HLB.

As shown in Table 2.4, HPMC films were the most efficient as water vapor barriers, coherent with their greater hydrophobicity, as commented on above, which limits the solubility of water molecules in the matrix. The WVP values obtained were similar to those found by Sánchez-González *et al.,* (2011). On the other hand, NaCas films showed the highest WVP, whereas S and PP exhibited intermediate values. The effect of incorporating surfactants on the WVP depended on both the surfactant and the polymer. Generally, OA addition led to a significant WVP decrease, which is probably due to its greater hydrophobicity (Fabra *et al*., 2008). The rest of the surfactants did not significantly affect WVP, except in starch matrices where a slight increase was observed. Similar effects were observed by Ortega-Toro *et al.,* (2014) for S matrices containing different surfactants.

Table 2.4 shows the values of oxygen permeability (OP) at 53% RH and 25°C, for the different films. The HPMC films were the most permeable and the OP values could not be quantified since they were above the threshold sensitivity of the equipment used.

The S films had better oxygen barrier properties than the protein films. For all three matrices, surfactant addition (especially OA addition) worsened the oxygen barrier properties of the films, which may be linked to the incorporation of hydrophobic agents where the oxygen solubility in the matrix is promoted

Table 2.4. Thickness (µm), equilibrium moisture content (g water/ 100 g dry film), water vapor permeability and oxygen permeability of the different films (mean values and standard deviation in brackets). HPMC: hydroxypropylmethylcellulose, S: starch, NaCas: sodium caseinate, PP: pea protein, WS: without surfactant, OA: oleic acid, S80: Span 80, T85: Tween 85.

Property		Formulation			
		HPMC	S	NaCas	PP
Thickness (µm)	WS	44 ± 1^{a}	42 ± 5^{a}	62 ± 6^{ab}	48 ± 3^{a}
	OA	48 ± 4^{ab}	47 ± 1^{b}	54 ± 6^{a}	51 ± 9^{a}
	S80	52 ± 5^{b}	59 ± 4^{c}	65 ± 2^{b}	54 ± 5^{a}
	T85	49 ± 4^{ab}	46 ± 2^{ab}	64 ± 7^{b}	56 ± 8^{a}
% moisture content (d.b.)	WS	5.0 ± 1.3^{a}	9.4 ± 0.8^{b}	8.3 ± 0.5^{b}	9.4 ± 0.5^{c}
	OA	3.6 ± 1.6^{a}	8.3 ± 0.5^{a}	8.0 ± 0.6^{b}	6.7 ± 0.4^{a}
	S80	3.6 ± 0.4^{a}	8.9 ± 0.7^{ab}	7.0 ± 0.6^{a}	6.9 ± 0.5^{a}
	T85	4.3 ± 1.5^{a}	8.4 ± 0.8^{ab}	6.2 ± 0.5^{a}	8.5 ± 0.4^{b}
WVP (g/Pa·s·m) × 10^{11}	WS	62 ± 17^{ab}	121 ± 7^{a}	196 ± 14^{b}	171 ± 5^{ab}
	OA	49 ± 3^{a}	152 ± 8^{b}	145 ± 17^{a}	130 ± 30^{a}
	S80	81 ± 13^{b}	178 ± 9^{c}	201 ± 14^{b}	156 ± 14^{ab}
	T85	68 ± 13^{ab}	160 ± 30^{bc}	211 ± 8^{b}	180 ± 50^{b}
OP (cm^3/Pa·s·m) × 10^{11}	WS	> L.D.*	16 ± 1^{a}	98 ± 2^{a}	150 ± 20^{a}
	OA	> L.D.*	132 ± 9^{c}	167 ± 2^{c}	244 ± 23^{b}
	S80	> L.D.*	106 ± 6^{b}	132 ± 12^{b}	156 ± 5^{a}
	T85	> L.D.*	114 ± 3^{b}	200 ± 14^{d}	173 ± 25^{a}

Different superscripts (a-c) within the same column indicate significant differences ($p < 0.05$) among formulations for the same polymer.

*> L.D. Above the detection limit (200 cm^3/m^2·day)

A PCA was used to compare all the analyzed properties of films from different matrices. **Figure 2.5** shows the PCA plot, where PC1 explains 54% of total variance and PC2 24%. Polysaccharide films were clearly differentiated from protein films in terms of PC1 while HPMC and S films were differentiated by PC2. The protein films, being closer, were separated by PC1.

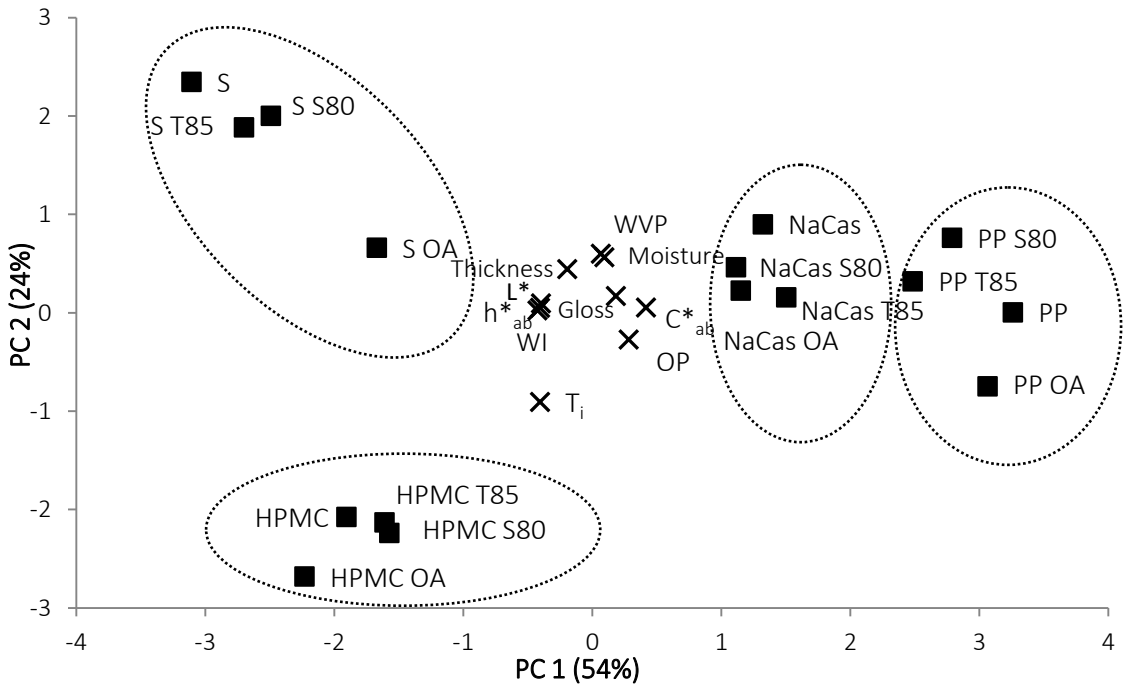


Figure 2.5. Principal component analysis for the properties of the films. HPMC: hydroxypropylmethylcellulose, S: starch, NaCas: sodium caseinate, PP: pea protein, WS: without surfactant, OA: oleic acid, S80: Span 80, T85: Tween 85.

Taking into account the data obtained, and considering their estimated thicknesses, the oxygen (OTR) and water (WTR) transmission rates of the coatings applied on grapes, were obtained and plotted in **Figure 2.6**. Due to the very thin coatings, very high values of WTR and OTR were obtained, which will not imply serious restrictions for the water vapor and oxygen exchanges of the coated fruit. Nevertheless, the location of the samples in the WTR-OTR map indicates that NaCas coatings will better limit water vapor and oxygen exchanges, especially NaCas and NaCas S80, mainly due to their higher coating capacity, whereas S without surfactants will be the most effective at limiting the exchange of oxygen.

From a comparison of the data obtained for the different matrices, it can be summarized that the S matrices were the best ones for the purposes of reducing gas (oxygen) exchanges in the coated product, whereas the HPMC matrices implied a better control of the water exchange, the proteins exhibiting intermediate barrier properties. The incorporation of surfactants did not imply that the matrices had a better coating capacity on grapes, while they did reduce the oxygen barrier properties with no notable

reduction of the WVP. They also reduced the film gloss and transparency. In terms of the thickness of the applied coatings, NaCas allows for the thickest coatings due to the possibility of preparing more concentrated- low viscosity solutions and their good adherence capacity on the grape surface. S coatings also exhibited good adherence capacity, but the high viscosity of its FFDs limits the starch concentration in the FFDs for practical applications.

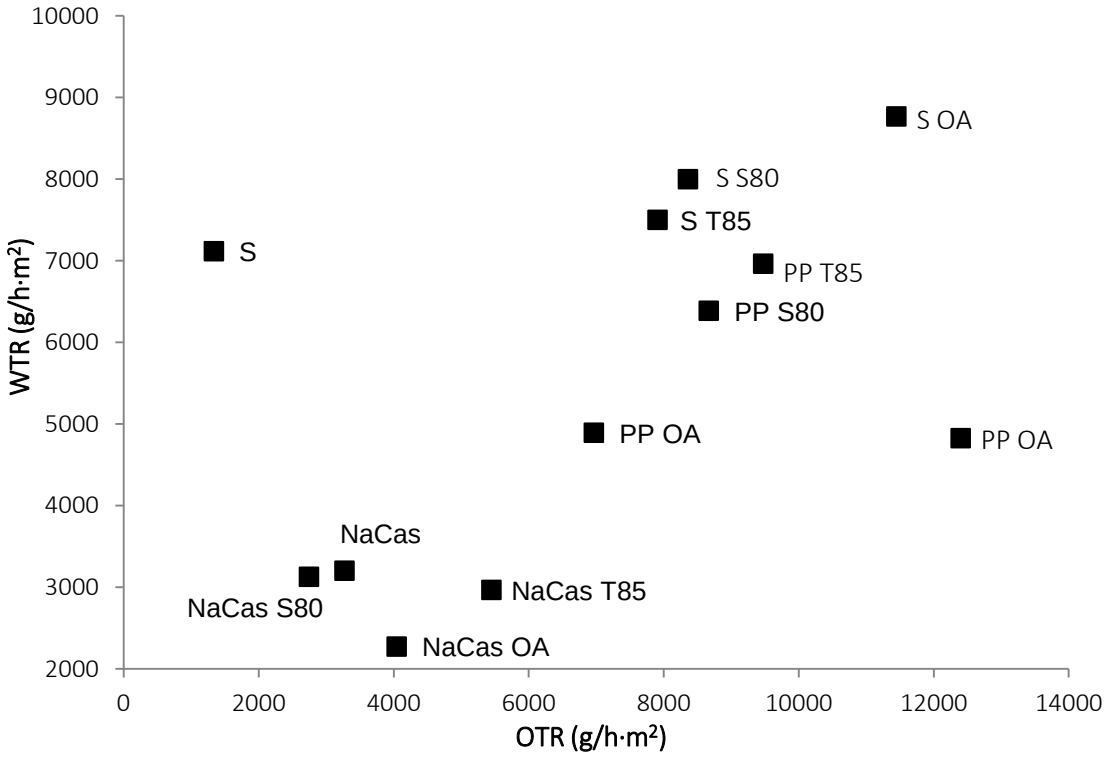


Figure 2.6. Water and oxygen transmission rates of the coatings applied on the grape surface. S: starch, NaCas: sodium caseinate, PP: pea protein, OA: oleic acid, S80: Span 80, T85: Tween 85.

3.3 Effect of the *Candida sake* incorporation on film properties

The effect of the BCA incorporation on the water sorption capacity and water and oxygen barrier properties of the films was analyzed in surfactant free films. **Figure 2.7** shows the values of these properties for both cell-free films and those containing cells.

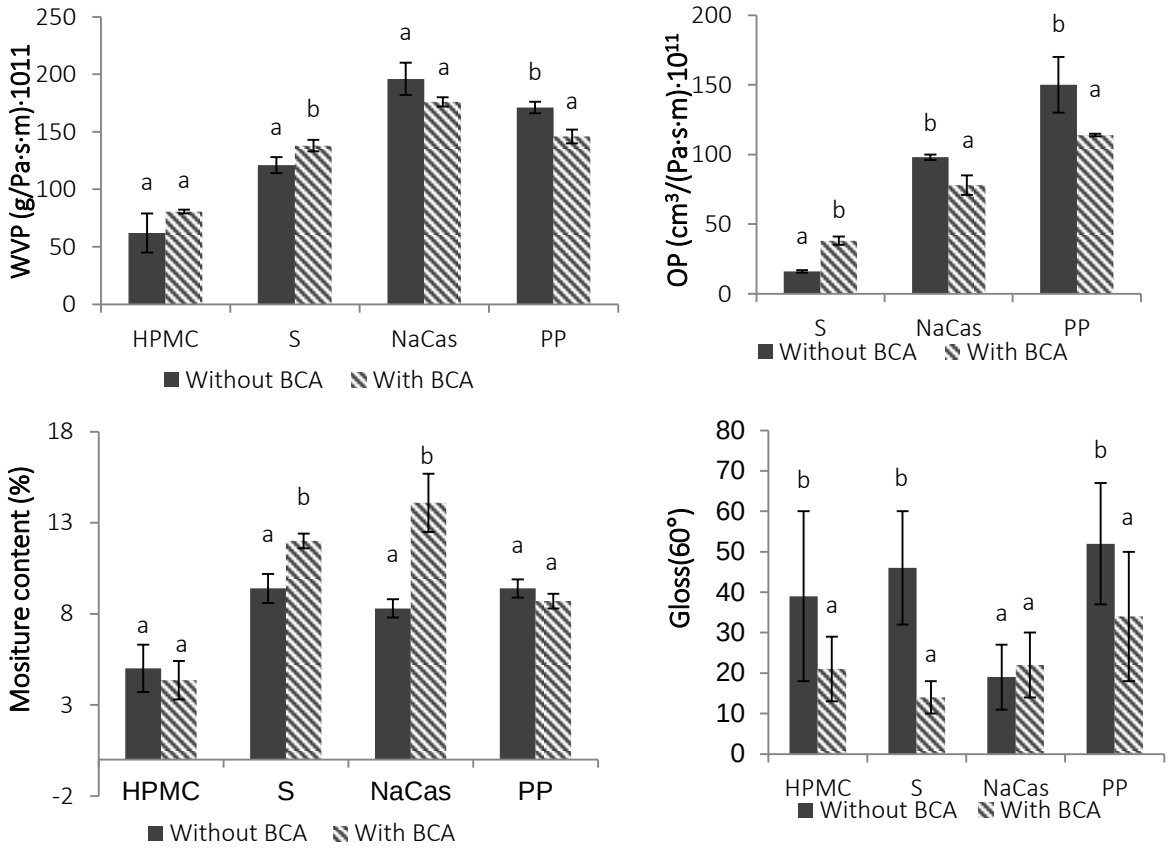


Figure 2.7. Water vapor permeability (WVP), oxygen permeability (OP), moisture content (g water/ 100 g dry film) and gloss of the surfactant free films without and with the BCA (mean values and standard deviation). HPMC: hydroxypropylmethylcellulose, S: starch, NaCas: sodium caseinate, PP: pea protein, BCA: biocontrol agent. Different superscripts (a-b) for the same polymer indicate significant differences ($p < 0.05$) due to the incorporation of *Candida sake*.

No great differences in barrier properties were observed as a result of cell incorporation, despite the fact that an increase in the equilibrium moisture content occurred in some films (S and NaCas); however, it is remarkable that whereas cells enhanced the barrier capacity in protein films, they slightly reduced them in polysaccharide films. Similar effects were previously observed when lactic acid bacteria were added to biopolymer films (Sánchez-González *et al*., 2013). Gialamas *et al*., (2010) and Aloui *et al*., (2015) also observed no relevant changes in WVP of films based on NaCas and sodium alginate and locust bean gum films, respectively, when different microorganisms were incorporated into the respective matrices.

As shown in Figure 2.7, cell incorporation implied a decrease in film gloss, in the glossiest films (HPMC, S and PP), which could be attributed to the presence of cells on the film surface, introducing surface roughness and reducing the film gloss. In NaCas films, this effect could not be relevant due to the low gloss value of these films.

3.4 Viability of *Candida sake* in the films

The viability of cells in the different film matrices was also been studied in order to identify their different ability as carriers of BCA, regardless of the fruit support. Table 2.5 shows the viability of *C. sake* (log CFU/cm^2) in the films both after the drying period (48h) and storage (7 and 14 days) under 53 and 68 % RH and at 25°C.

In no case were any viable cells found after 21 days of storage. After the drying period, the cell viability was slightly higher in the protein films, which could be explained by the nutrition effect of the aminoacids present, favoring viability. In fact, the population of *C. sake* in protein films after the film drying is even higher than that inoculated (5,4 log CFU/cm^2), pointing to cell growth during the 48h drying step. This trend agrees with that found in previous studies. Sánchez-González *et al*., (2013) also found a greater viability of *Lactobacillus plantarum* in protein films (PP) than in polysaccharide films (HPMC) after film drying. The statistical analysis did not reveal a clear pattern as regards the effect of surfactants on the viability after drying, which was limited in any case. In HPMC, S80 and T85 seemed to favor cell survival, while in S they provoked a decrease in cell population. Likewise, T85 and OA reduced cell viability in NaCas and PP, respectively.

After 7 and 14 days of storage, although the viability was very much reduced in HPMC and S films, protein films better maintained the *C. sake* viability. This could also be explained by the nutritional effect of proteins through the free aminoacids. The ambient relative humidity (water activity in the film), greatly affected the yeast viability throughout storage. In S films, no cells were viable after 7 storage days either at 0.53 or 0.68 a_w. In HPMC and NaCas films, the yeast viability was maintained after 7 storage days at 53% RH, but drastically dropped at the highest a_w. However, for PP films, the greatest counts after 7 storage days were obtained at a_W 0,68 and they maintained cell

survival after 14 storage days at this a_W, although only in the case of PP and PP S80 samples.

Table 2.5. Viability of *Candida sake* in the films (log CFU/cm^2) after film drying and 7 and 14 days of storage at 25°C and 53% or 68% RH (mean values and standard deviation). HPMC: hydroxypropylmethylcellulose, S: starch, NaCas: sodium caseinate, PP: pea protein, OA: oleic acid, S80: Span 80, T85: Tween 85. – indicate absence of viable cells.

Formulation	log CFU/cm^2				
	After drying	7 days		14 days	
		53% RH	68% RH	53% RH	68% RH
HPMC	4.5 ± 0.5^{ab}	-	-	-	-
HPMC OA	4.2 ± 0.3^{a}	-	-	-	-
HPMC S80	5.6 ± 0.4^{c}	3.2 ± 0.1^{a}	-	-	-
HPMC T85	6.1 ± 0.1^{d}	4.6 ± 0.3^{b}	-	-	-
S	5.9 ± 0.1^{cd}	-	-	-	-
S OA	4.9 ± 0.5^{b}	-	-	-	-
S S80	4.9 ± 0.7^{b}	-	-	-	-
S T85	4.4 ± 0.3^{ab}	-	-	-	-
NaCas	7.1 ± 0.3^{ef}	7.1 ± 0.4^{c}	-	-	-
NaCas OA	6.7 ± 0.7^{e}	4.8 ± 0.5^{b}	-	-	-
NaCas S80	7.2 ± 0.5^{f}	6.3 ± 0.9^{c}	3.9 ± 0.1^{a}	-	-
NaCas T85	5.5 ± 0.2^{c}	-	-	-	-
PP	6.9 ± 0.1^{ef}	-	5.6 ± 0.5^{c}	-	3.8 ± 0.9^{a}
PP OA	5.9 ± 0.3^{cd}	-	4.3 ± 0.1^{ab}	-	-
PP S80	6.7 ± 0.3^{e}	-	5.1 ± 0.9^{bc}	-	3.4 ± 0.2^{a}
PP T85	8.8 ± 0.1^{ef}	4.7 ± 0.1^{b}	4.2 ± 0.5^{ab}	-	-

Different superscripts (a-f) within the same column indicate significant differences ($p < 0.05$) among formulations.

These results suggest that, at a lower aw (a_W 0.53), the yeast could be in a more latent state, due to the low water availability, prolonging its survival, whereas under more vital conditions (a_W 0.68), cells extenuate themselves fighting for survival in a water stressed medium without adequate nutrients. In S films, the greater availability of nutritive glucose could accelerate cell death due to the lack of water availability under both 0.53 and 0.68 a_w conditions. This trend was similar to that found by Romano *et al*., (2014), who studied the effect of a_w on the viability of lactic acid bacteria in methylcellulose films. At a low a_w, the microbial cells do not fight for survival, and remain viable in a latent state. On the contrary, with restricted, but greater, availability of water, vital cell activity occurs but the stress conditions result in cell death. The opposite effect observed in PP films points to specific survival mechanism for the cells in the chemical

context of this protein. No clear tendencies in the role of surfactants on cell survival during storage could be observed.

When the cell survival in net films was compared to that determined in coatings with a similar composition applied on grapes, stored at 20°C and 85% RH for 7 days (Marín *et al*., 2016), different results were obtained, which indicates that the fruit support affected cell viability. These authors reported that formulations based on proteins allowed for a better survival of this BCA when applied on the fruit surface. In fact, under storage an increase in the population of *C. sake* over time was observed. This could be explained by the fact that this yeast is naturally present in fruit surface. Therefore, when *C. sake* is present in its natural environment and supported in a thin coating is able to better survive and multiply. However, entrapped in a film matrix with thickness about 50 µm, its viability is more compromised.

4. CONCLUSIONS

Polysaccharides and proteins, with and without surfactants, can be used as carriers of the biocontrol agent *C. sake* to be applied on fruits, such as grapes, susceptible to attack by *B. cinerea*, in order to control the fungal infection, at the same time as coatings can modulate the exchange of gases and water vapor, without their great transparency introducing any negative effects on the product's appearance. NaCas permits a greater coating capacity, and so a thicker coating. The thickness and barrier properties of the matrices will determine the water and gas transmission rates on the coated product surface, although the very thin layers applied will not seriously affect the fruit's gas exchanges. Likewise, although the yeast's viability was better maintained in the PP films at higher a_W, in NaCas films this took place at lower a_W. Nevertheless, the fruit support affects the resulting cell viability. The formulation of PP-NaCas blend films could be a good strategy with which to prolong yeast viability regardless of the film's a_W, which will depend on the RH of the ambient/environment where the coatings are applied.

ACKNOWLEDGEMENTS

The authors are grateful to the Spanish Government for the financial support through the national project RTA2012-00067-C02 (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Spain and FEDER funds) and to the Conselleria d'Educació of the Generalitat Valenciana, (Spain) for A. Marín's PhD grant. The authors are also thankful to IRTA group, headed by Dra. Neus Teixidó, for their assistance in the microbiological study.

REFERENCES

Aloui, H., Licciardello, F., Khwaldia, K., Hamdi, M., & Restuccia, C. (2015). 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*. *International Journal of Food Microbiology*, *200*, 22–30.

Arrieta, M. P., Peltzer, M. a., Garrigós, M. D. C., & Jiménez, A. (2013). Structure and mechanical properties of sodium and calcium caseinate edible active films with carvacrol. *Journal of Food Engineering*, *114*(4), 486–494.

ASTM. (1995). Standard test methods for water vapor transmission of materials. Standards Designations: E96-95. In Annual book of ASTM standards. Philadelphia, PA: American Society for Testing and Materials., pp. 406–413.

ASTM. (1999). Standard test method for specular gloss. Designation (D523). Annual book of ASTM standards Philadelphia, PA: American Society for Testing and Materials.

Bleve, G., Grieco, F., Cozzi, G., Logrieco, A., & Visconti, A. (2006). Isolation of epiphytic yeasts with potential for biocontrol of *Aspergillus carbonarius* and *A. niger* on grape. *International Journal of Food Microbiology*, *108*(2), 204–209.

Calvo-Garrido, C., Elmer, P., Viñas, I., Usall, J., Bartra, E., Teixidó, N. (2013). Biological control of botrytis bunch rot in organic wine grapes with the yeast antagonist *Candida sake* CPA-1. Plant Pathology, 62(3), 510–519.

Cañamás, T. P., Viñas, I., Usall, J., Torres, R., Anguera, M., Teixidó, N. (2008). Control of postharvest diseases on citrus fruit by preharvest applications of biocontrol agent *Pantoea agglomerans* CPA-2. Part II. Effectiveness of different cell formulations. Postharvest Biology and Technology, 49(1), 96–106.

Cañamás, T. P., Viñas, I., Torres, R., Usall, J., Solsona, C., & Teixidó, N. (2011). Field applications of improved formulations of *Candida sake* CPA-1 for control of Botrytis cinerea in grapes. *Biological Control*, *56*(2), 150–158.

Choi, W., & Han, J. H. (2001). Physical and mechanical properties of pea-protein-based edible films. *Journal of Food Science*, *66*(2), 319–322.

Concha-Meyer, A., Schöbitz, R., Brito, C., & Fuentes, R. (2011). Lactic acid bacteria in an alginate film inhibit *Listeria monocytogenes* growth on smoked salmon. *Food Control*, *22*(3-4), 485–489.

Droby, S., Wisniewski, M. E., Macarisin, D., & Wilson, C. L. (2009). Twenty years of postharvest biocontrol research: Is it time for a new paradigm? *Postharvest Biology and Technology*, *52*(2), 137–145.

El-Ghaouth, A., Smilanick, J. L., & Wilson, C. L. (2000). Enhancement of the performance of *Candida saitoana* by the addition of glycolchitosan for the control of postharvest decay of apple and citrus fruit. *Postharvest Biology and Technology*, *19*(1), 103–110.

Fabra, M. J., Talens, P., & Chiralt, A. (2008). Tensile properties and water vapor permeability of sodium caseinate films containing oleic acid-beeswax mixtures. *Journal of Food Engineering*, *85*(3), 393–400.

Fabra, M. J., Jiménez, a., Atarés, L., Talens, P., & Chiralt, a. (2009). Effect of fatty acids and beeswax addition on properties of sodium caseinate dispersions and films. *Biomacromolecules*, *10*(6), 1500–1507.

Fabra, M. J., Talens, P., & Chiralt, A. (2009). Microstructure and optical properties of sodium caseinate films containing oleic acid-beeswax mixtures. *Food Hydrocolloids*, *23*(3), 676–683.

Fabra, M. J., Pérez-Masiá, R., Talens, P., & Chiralt, A. (2011). Influence of the homogenization conditions and lipid self-association on properties of sodium caseinate based films containing oleic and stearic acids. *Food Hydrocolloids*, *25*(5), 1112–1121.

Fan, Y., Xu, Y., Wang, D., Zhang, L., Sun, J., Sun, L., & Zhang, B. (2009). Effect of alginate coating combined with yeast antagonist on strawberry (Fragaria × ananassa) preservation quality. *Postharvest Biology and Technology*, *53*(1-2), 84–90.

Fava, J., Hodara, K., Nieto, A., Guerrero, S., Alzamora, S. M., & Castro, M. A. (2011). Structure (micro, ultra, nano), color and mechanical properties of Vitis labrusca L. (grape berry) fruits treated by hydrogen peroxide, UV-C irradiation and ultrasound. *Food Research International*, 44(9), 2938–2948.

Gialamas, H., Zinoviadou, K. G., Biliaderis, C. G., & Koutsoumanis, K. P. (2010). Development of a novel bioactive packaging based on the incorporation of *Lactobacillus sakei* into sodium-caseinate films for controlling *Listeria monocytogenes* in foods. *Food Research International*, *43*(10), 2402–2408.

González-Estrada, R., Calderón-Santoyo, Carvajal-Millan, E., Ascencio Valle, F. J., Ragazzo-Sánchez, J. A., Brown-Bojorquez, F., & Rascón-Chu, A. (2015). Covalently cross-Linked arabinoxylans films for *Debaryomyces hansenii* entrapment. *Molecules*, *20*(6), 11373–11386.

González-Martínez, C., Cháfer, M., Vargas, M., Sánchez-González, L., & Chiralt, A. (2011). Edible coatings for fresh and minimally processed fruits and vegetables. In *Advances in Postharvest Treatments and Fruit Quality and Safety* (pp. 59–84). New York: Nova Science Publishers.

Hutchings, J. B. (1999). *Food Color and Appearance, 2nd Edition*. Maryland: Aspen Publishers.

Jiménez, A., Fabra, M. J., Talens, P., & Chiralt, A. (2010). Effect of lipid self-association on the microstructure and physical properties of hydroxypropyl-methylcellulose edible films containing fatty acids. *Carbohydrate Polymers*, *82*(3), 585–593.

Jiménez, A., Fabra, M. J., Talens, P., & Chiralt, A. (2012). Effect of re-crystallization on tensile, optical and water vapor barrier properties of corn starch films containing fatty acids. *Food Hydrocolloids*, *26*(1), 302–310.

Jiménez, A., Sánchez-González, L., Desobry, S., Chiralt, A., & Tehrany, E. A. (2014). Influence of nanoliposomes incorporation on properties of film forming dispersions and films based on corn starch and sodium caseinate. *Food Hydrocolloids*, *35*, 159–169.

Liu, J., Sui, Y., Wisniewski, M., Droby, S., & Liu, Y. (2013). Review: Utilization of antagonistic yeasts to manage postharvest fungal diseases of fruit. *International Journal of Food Microbiology*, *167*(2), 153–160.

López De Lacey, a. M., López-Caballero, M. E., Gómez-Estaca, J., Gómez-Guillén, M. C., & Montero, P. (2012). Functionality of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* incorporated to edible coatings and films. *Innovative Food Science and Emerging Technologies*, *16*, 277–282.

Marín, A., Cháfer, M., Atarés, L., Chiralt, A., Torres, R., Usall, J., & Teixidó, N. (2016). Effect of different coating-forming agents on the efficacy of the biocontrol agent *Candida sake* CPA-1 for control of *Botrytis cinerea* on grapes. *Biological Control*, *96*, 108–119.

Masih, E. I., Slezack-Deschaumes, S., Marmaras, I., Barka, E. A., & Vernet, G. (2001). Characterisation of the yeast *Pichia membranaefaciens* and its possible use in the biological control of *Botrytis cinerea*, causing the grey mould disease of grapevine. *FEMS Microbiology Letters*, *202*, 227–232.

McClements, D. J. (2005). *Food emulsions. Principles, practices, and techniques*. Boca Raton (US): CRC Press Inc.

McGuire, R. G. (2000). Population dynamics of postharvest decay antagonists growing epiphytically and within wounds on grapefruit. *Phytopathology*, *90*(11), 1217–1223.

McHugh, T. H., Avena-Bustillos, R., & Krochta, J. M. (1993). Hydrophilic edible films: modified procedure for water vapor permeability and explanation of thickness effects. *Journal of Food Science.*

Ortega-Toro, R., Jiménez, A., Talens, P., & Chiralt, A. (2014). Effect of the incorporation of surfactants on the physical properties of corn starch films. *Food Hydrocolloids*, *38*, 66–75.

Pasquali, R. C., Taurozzi, M. P., & Bregni, C. (2008). Some considerations about the hydrophilic – lipophilic balance system. *International Journal of Pharmaceutics*, *356*, 44–51.

Potjewijd, R., Nisperos, M. O., Burns, J. K., Parish, M., & Baldwin, E. A. (1995). Cellulose-based coatings as carriers for *Candida guillermondii* and *Debaryomyces* sp. in reducing decay of oranges. *HortScience*, *30*(7), 1417–1421.

Romano, N., Tavera-Quiroz, M. J., Bertola, N., Mobili, P., Pinotti, A., & Gómez-Zavaglia, A. (2014). Edible methylcellulose-based films containing fructo-oligosaccharides as vehicles for lactic acid bacteria. *Food Research International*, *64*, 560–566.

Sánchez-González, L., Vargas, M., González-Martínez, C., Chiralt, A., & Cháfer, M. (2009). Characterization of edible films based on hydroxypropylmethylcellulose and tea tree essential oil. *Food Hydrocolloids*, *23*(8), 2102–2109.

Sánchez-González, L., Chiralt, A., González-Martínez, C., & Cháfer, M. (2011). Effect of essential oils on properties of film forming emulsions and films based on hydroxypropylmethylcellulose and chitosan. *Journal of Food Engineering*, *105*(2), 246–253.

Sánchez-González, L., Quintero Saavedra, J. I., & Chiralt, A. (2013). Physical properties and antilisterial activity of bioactive edible films containing *Lactobacillus plantarum*. *Food Hydrocolloids*, *33*(1), 92–98.

Sánchez-González, L., Quintero Saavedra, J. I., & Chiralt, A. (2014). Antilisterial and physical properties of biopolymer films containing lactic acid bacteria. *Food Control*, *35*(1), 200–206.

Santacruz, S., Rivadeneira, C., & Castro, M. (2015). Edible films based on starch and chitosan. Effect of starch source and concentration, plasticizer, surfactant's hydrophobic tail and mechanical treatment. *Food Hydrocolloids*, *49*, 89–94.

Soukoulis, C., Singh, P., Macnaughtan, W., Parmenter, C., & Fisk, I. D. (2016). Compositional and physicochemical factors governing the viability of *Lactobacillus rhamnosus* GG embedded in starch-protein based edible films. *Food Hydrocolloids*, *52*, 876–887.

Suppakul, P., Miltz, J., Sonneveld, K., & Bigger, S. W. (2003). Active packaging technologies with an emphasis on antimicrobial packaging and its applications. *Journal of Food Science*, *68*(2), 408–420.

Teixidó, N., Viñas, I., Usall, J., & Magan, N. (1998). Control of blue mold of apples by preharvest application of *Candida sake* grown in media with different water activity. *Phytopathology*, *88*(9), 960–964.

Teixidó, N., Torres, R., Abadias, M., Viñas, I., Usall, J., (2011) Biological control of postharvest diseases in fruit and vegetables. In: Lacroix, C. (ed). Protective cultures, antimicrobial metabolites and bacteriophages for food and beverage biopreservation. Woodhead Publishing Limited, 364-402.

Trezza, T. A., & Krochta, J. M. (2000). The gloss of edible coatings as affected by surfactants , lipids , relative humidity and time. *Journal of Food Science*, *65*(4), 658–662.

Vargas, M., Pastor, C., Chiralt, A., McClements, D. J., & González-Martínez, C. (2008). Recent advances in edible coatings for fresh and minimally processed fruits. *Critical Reviews in Food Science and Nutrition*, *48*(6), 496–511.

Villalobos, R., Chanona, J., Hernández, P., Gutiérrez, G., & Chiralt, A. (2005). Gloss and transparency of hydroxypropylmethylcellulose films containing surfactants as affected by their microstructure. *Food Hydrocolloids*, *19*(1), 53–61.

Villalobos, R., Hernández-Muñoz, P., & Chiralt, A. (2006). Effect of surfactants on water sorption and barrier properties of hydroxypropylmethylcellulose films. *Food Hydrocolloids*, *20*(4), 502–509.

Villalobos-Carvajal, R., Hernández-Muñoz, P., Albors, A., & Chiralt, A. (2009). Barrier and optical properties of edible hydroxypropyl methylcellulose coatings containing surfactants applied to fresh cut carrot slices. *Food Hydrocolloids*, *23*(2), 526–535.

Viñas, I., Usall, J., Teixidó, N., Sanchis, V. (1998). Biological control of major postharvest pathogens on apple with *Candida sake*. International Journal of Food Microbiology, 40(1-2), 9–16.

Wokadala, O. C., Ray, S. S., & Emmambux, M. N. (2012). Occurrence of amylose-lipid complexes in teff and maize starch biphasic pastes. *Carbohydrate Polymers*, *90*(1), 616–22.

CHAPTER 3

STABILITY OF BIOCONTROL PRODUCTS CARRYING *Candida sake* CPA-1 IN STARCH DERIVATIVES AS A FUNCTION OF WATER ACTIVITY

Anna Marín, Lorena Atarés, Maite Cháfer & Amparo Chiralt

Pending submission

ABSTRACT

The preservation and shelf-life of formulations of the biocontrol agent *Candida sake* CPA-1 and starch derivatives as a function of their water activity was studied in terms of the physical stability of the products and their cell viability. Formulations of biocontrol products (BCPs), based on combinations of potato starch and pre-gelatinized potato starch in different proportions (F1 and F2), or maltodextrins (F3) containing cell protectants, were obtained by fluidized-bed drying. The carriers and the formulated products were stored at 20°C under different water activity (a_W) conditions. The water sorption and water plasticization behavior of the different dry products were analyzed through the water sorption isotherm and glass transition temperature (T_g). Likewise, the viability of *C. sake* over time was determined as a function of the a_W. The solubility of the products was also assessed. Although formulations stored at 20°C and low a_W (≤ 0.33) exhibited a better shelf-life, a significant decrease in cell survival ratio after 180 storage days was observed in every case. Cold storage (5°C) was required to better maintain the cell viability, thus prolonging the shelf-life of BCPs. Formulations containing maltodextrins were the most effective at preserving cell viability and also exhibited the highest water solubility. All the formulations were physically stable at ambient temperature; therefore, the cell stability is the critical point at which to establish both the a_w levels and temperature during storage. In this sense, packaging the product using high water vapor barrier material and under cold storage would be necessary to ensure a high number of viable cells and, consequently, an effective and competitive biocontrol product.

Keywords: biocontrol products, *Candida sake*, cell carriers, maltodextrin, starch, water plasticization, water sorption, cell viability.

1. INTRODUCTION

In recent years, the biological control of plant diseases using microbial antagonists as an alternative to chemical products has attracted considerable interest and many potential biocontrol agents (BCAs) have been isolated and tested to ascertain their disease suppression capability (Chumthong *et al.,* 2008; Torres *et al*., 2014). In order to use BCAs practically, it is key to formulate them effectively if they are to be used successfully as biocontrol products (BCPs) (Melin *et al*., 2011).

The primary obstacle in the commercialization of BCPs is the development of shelf-stable products that preserve a high degree of cell viability over time, preferably at ambient temperatures for the purposes of avoiding cold storage. In this sense, solid formulations are preferable to liquid formulations since they allow for easier storage, transport and quality control (Cañamás *et al*., 2008; Fu & Chen, 2011). Drying is the main technique to formulate solid BCPs that remain physically and microbiologically stable in long-term storage. Thus, the general object of the drying of BCAs is to enable storage over extended periods of time whilst preserving the cell's viability and its effectiveness against pathogens and also to ensure the retrieval of its metabolic activity and biological properties upon rehydration (Fu & Chen, 2011; Melin *et al*., 2007; Morgan *et al*., 2006). In many cases, it becomes necessary to incorporate adjuvants and protective agents to the formulations so as to preserve the viability of dried cells. Of the protective agents, skim milk and sugars, used either alone or in combination, have been widely used because of their relatively low prices and chemically innocuous nature (Costa *et al*., 2002; Khem *et al*., 2015; Santivarangkna *et al*., 2008).

Microorganisms can be dehydrated by employing several techniques, such as spray drying or freeze drying, which have commonly been used for the purposes of drying probiotics, starter cultures in the food industry and BCAs (Aponte *et al*., 2016; Corcoran *et al*., 2004; Costa *et al*., 2002; Yánez-Mendizabal *et al*., 2012). Nevertheless, they present some shortcomings related to the loss of cell viability, due to the damaging conditions to which they are subjected during the process. With regards to these drawbacks, fluidized-bed technology might represent a promising alternative method since it presents some advantages over more traditional methods, such as lower

temperature gradients and operating times and less extreme water loss (Guijarro *et al*., 2006; Larena *et al*., 2003; Morgan *et al*., 2006). Several authors have studied this technique for the drying of BCAs, obtaining interesting results (Larena *et al*., 2003; Melin *et al*., 2007; Mounir *et al*., 2007). Fluidized-bed drying (FBD) allows granulated solids to be dried while spraying a coating material onto the granulated product. The principle of this technique is the fluidization of solid particles by maintaining them in suspension by blowing hot air through the powder bed (Teunou & Poncelet, 2002). The bed of particles assumes the characteristics of a boiling liquid, hence the term fluidization (Andrade *et al*., 2012). The coating material is sprayed through a nozzle onto the particles, in the form of a solution or suspension, and its moisture evaporates due to the heat of the air. After a succession of wetting and drying stages, the final dried product is obtained (Jacquot & Pernetti, 2004).

In this study, FBD has been employed to obtain biocontrol water-dispersible granular formulations based on the BCA, *Candida sake* CPA-1, in combination with different polymeric carriers in order to ensure a good drying performance and the product's physical stability. *C. sake* has previously been formulated in both liquid and dry forms (Abadias *et al*., 2003; Abadias *et al*., 2005 Cañamás *et al*., 2008; Torres *et al*., 2003) but it has not so far been formulated by means of FBD and in combination with compounds which, in addition to supplying drying feasibility and stability to the cells, permit coating formation when the BCP is applied, thus better supporting the BCA. In previous studies, the combination of *C. sake* with different coating-forming agents has been demonstrated to be effective against the pathogen *Botrytis cinerea* on grapes when applied in liquid form (Marín *et al*., 2016). Edible coatings were able to improve the adherence of *C. sake* to grapes and its survival time and also its efficacy against *B. cinerea*, when compared to the application of the antagonist without any support.

The correct selection of the components that comprise the final product is essential, since the successful delivery of the BCA, the shelf-life, the stability and effectiveness under the application conditions are all greatly dependent on the formulation (Kinay & Yildiz, 2008). In the FBD formulation, the carrier is the primary material that acts as support for the BCA and allows the bioproduct to be dispersed effectively (Kinay & Yildiz, 2008). From an economic point of view, the production cost is another key factor

to be considered and kept to a minimum (Melin *et al*., 2011). For this reason, using starch derivatives as carriers for BCAs is a good option, not only because they are both low cost and also readily available (Lafargue *et al*., 2007). Moreover, starch derivatives offer different advantages: a) they present high critical moisture content values for water plasticization, which is essential if both the drying feasibility and physical stability of the BCP in the glassy state during storage must be ensured (Roos, 1995) b) starch-*C. sake* formulations exhibit high degree of cell viability and are highly effective against *Botrytis cinerea* (Marin *et al*., 2016), c) they have the ability to form coatings on the treated product, which help to protect the antagonist during the application phase (Cañamás *et al*., 2011). Several studies report the use of starch derivatives as carriers of BCA-based formulations. Lewis *et al*., (1995) obtained granular formulations with pre-gelatinized starch and the biocontrol fungus *Gliocladium virens*, whose viability was maintained for 6 months at 5°C. Lee *et al*., (2006) developed different wettable powder formulations of *Bacillus lincheniformis*; corn starch was the carrier material that delivered the biocontrol bacteria on tomato most efficiently. Similarly, Mounir *et al*., (2007) used maize starch to produce a formulation of the yeast *Aerobasidium pullulans* by means of FBD, observing a drop in cell viability in the first 30 days; after that period, however, the cell viability remained constant for 7 months at 4°C. Soto-Muñoz *et al*., (2015) studied different dry formulations of *Pantoea agglomerans*, one of which was obtained by means of FBD, using potato starch as carrier. Soluble starch and maltodextrins, both obtained by starch hydrolysis, have also been employed as cell protectants during the freeze drying and FBD of BCAs and probiotics (Stephan *et al*., 2016; Stummer *et al*., 2012; Strasser *et al*., 2009).

In terms of the stability of BCP after drying and during storage, water sorption and water plasticization are key features in the physical stability of dry products (Roos, 1995; Rahman, 2009). Water sorption relates water content and water activity while plasticization relates water content and glass transition temperature (T_g) (Nurhadi *et al*., 2016). Moreover, for a specific dry formulation of BCAs, preserving the viability of the antagonists is of vital importance. Thus, it is necessary to discern how the viability of the BCA is affected by the water activity of the product, which defines the water availability for cells. Likewise, the good dispersion of the dry BCPs in water under practical

conditions is another fundamental point, since their application in the field requires a quick solubilization and a simple preparation. If all these characteristics are known, it will permit us to establish the most adequate formulation, and its water activity, in terms of the best physical and microbial stability of the dry BCP and the feasibility of its application.

The aim of the present study was to analyze both preservation and shelf-life as a function of the water activity of dry formulations based on starch derivatives used as carriers of the BCA *Candida sake* CPA-1, in terms of the physical stability of the formulations and the cell viability. The solubility in water of the granular formulations was also studied.

2. MATERIALS AND METHODS

2.1 Materials

Potato starch (PS), pre-gelatinized potato starch (PG) and maltodextrins (MD) (dextrose equivalent, DE: 12) were purchased from Quimidroga. S.A. (Barcelona, Spain). The salts, P_2O_5, LiCl, $MgCl_2$, K_2CO_3, $Mg(NO_3)_2$, KI, NaCl, KCl and K_2SO_4, were supplied by Panreac Química, S.L.U (Barcelona, Spain). Trypticase soy agar and streptomycin sulphate were obtained from Scharlab (Barcelona, Spain) and Sigma – Aldrich (Madrid, Spain), respectively. Sucrose and skim milk powder were food grade products.

2.2 Obtaining of formulations by fluidized-bed drying

BCA formulations containing *C. sake* and carrier, binder and protective agents were obtained with a bottom fluidized-bed spray dryer (Hüttlin Solidlab 1, Bosch GmbH, Stuttgart, Germany). PS and PG, mixed in different proportions, or MD, were used as carriers of the yeast; were fluidized in powdered form by the air current in the drying chamber of the equipment.

The CPA-1 strain of *C. sake* (Colección Española de Cultivos Tipo, Spain, CECT-10817), with proven bioactivity against *B. cinerea*, was used in this study. Fresh *C. sake* cells were obtained by liquid fermentation in a BIOSTAT-A modular bioreactor (Braun Biotech 140 International, Melsungen, Germany), as described by Cañamás *et al.*,

(2011). Then, cell pellets were obtained by centrifugation and suspended in potassium phosphate buffer solution (pH 6.5; KH_2PO_4 0.2 mol/L, 70 ml; K_2HPO_4 0.2 mol/L, 30 ml and deionized water, 300 ml). A binder agent and protectants were added to the cell suspension and the blend was homogenized using a rotor-stator homogenizer (Ultraturrax T25, Janke and Kunkel, Germany). Then, the cell dispersion was pumped and sprayed on the fluidized carriers as droplets through the nozzle at an approximate flow rate of 4 ml/min. The drying process was carried out at an inlet air temperature of 55°C for 60 min, which had previously been demonstrated not to affect *Candida sake* survival time. The composition of the different formulations was optimized in a previous study (unpublished data) to obtain a target cell count in the powder of 10^9 CFU/g. **Table 3.1** shows the composition of the three considered dry formulations, as well as the product's moisture content and final yeast concentration in the products, expressed as CFU/g dry product.

Table 3.1. Composition of the different BCP formulations of *Candida sake*. PS: potato starch; PG: pre-gelatinized potato starch; MD: maltodextrins, Su: sucrose, SMP: skim milk powder, MC: moisture content, d.s.: dry solids.

Formulation	Carrier	Binder (1)	Protectant(2)	MC (3)	CFU/g d.s.
F1	PG:PS (1:2)	PG	-	8.81 ± 0.05	$1.57 \cdot 10^9$
F2	PG:PS (2:1)	PG	-	6.85 ± 0.53	$1.07 \cdot 10^9$
F3	MD	MD	Su:SMP (2:1)	6.75 ± 0.13	$1.47 \cdot 10^9$

(1): 1,16 g/100 g carrier (2): 20 g/100 g carrier (3): g water/100 g product

2.3 Water sorption and water plasticization methods

2.3.1 Water sorption

Water sorption isotherms of both the different formulations and the carrier materials were obtained via a static gravimetric method at 20°C. Three replicates of each product were accurately weighed using an analytical balance (ME235P-SD, SARTORIUS AG, Germany) and placed in hermetic recipients containing oversaturated solutions of different salts, which provided the known equilibrium relative humidity (RH). The different salts used were LiCl, $MgCl_2$, K_2CO_3, $Mg(NO_3)_2$, KI, NaCl, KCl and K_2SO_4 and provided an a_W range of 0.11 to 0.98 (Greenspan, 1977). The samples were periodically

weighed until constant weight when equilibrium was assumed (Spiess & Wolf, 1983). The equilibrium moisture content of the samples was determined from their initial moisture content and the corresponding weight gain at equilibrium. For the purposes of moisture content analysis, four samples per product were dried for 24 h at 60°C in a vacuum oven and, afterwards, were placed in a desiccator containing P_2O_5 at room temperature until constant weight was reached.

The experimental data were fitted to the Guggenheim–Anderson-de Boer (GAB) model (Equation 1) over the entire a_W range.

$$W_e = \frac{W_o \cdot C \cdot a_w}{(1 - K \cdot a_w) \cdot (1 + (C - 1) \cdot K \cdot a_w)} \quad (1)$$

where W_e is the equilibrium moisture content on dry basis; W_0, the monolayer moisture content; and C constant related to the heat sorption of multilayer and K factor correcting properties of the multilayer molecules (Bizot, 1983)

2.3.2 Water plasticization

The glass transition temperature (T_g) of the different products and carriers was determined as a function of their a_w by means of differential scanning calorimetry (DSC) using a DSC TA Instruments, model DSC1 STAR System, Mettler Toledo, Switzerland. The measurements for the different formulations and carrier materials conditioned at the different a_w were taken in duplicate. For that purpose, samples of approximately 9 mg were weighed and sealed in aluminum pans. An empty pan was used as reference. Three cycles of scanning (heating-cooling-heating) at 10°C/min were performed using a 20 mL/min nitrogen flow. The temperature range of each measurement was fitted according to the sample moisture content at between 0 and 160ºC. T_g was determined as the midpoint temperature of the glass transition in the second heating scan.

The relationship between T_g and water content at various water activities were modeled by using the Gordon & Taylor equation (Equation 2).

$$T_g = \frac{(1 - x_w) \times T_{g(s)} + k \times x_w \times T_{g(w)}}{(1 - x_w) + k \times x_w} \quad (2)$$

were x_w is the moisture content; $T_{g(s)}$ is the T_g value of the anhydrous solids; $T_{g(w)}$ is the T_g value of the amorphous water; and k is a model parameter.

The goodness of fit for both water sorption and water plasticization was analyzed using the value of relative percent root mean square (Equation 3), whose value of under 10 indicates the very good fit of the model (Rizvi, 2005).

$$\% RMS = \left[\sqrt{\frac{\sum \left[\frac{M^{exp} - M^{calc}}{M^{exp}} \right]^2}{N}} \right] \times 100 \quad (3)$$

2.4 Viability of *Candida sake* during storage

In order to analyze the influence of the product's water activity and storage time on the yeast cells, the viability of *C. sake* in the different formulations stored at 20°C at different RH% was determined after 2, 7, 14, 30, 60 and 180 days of storage. Samples of the different formulations were placed in desiccators over different relative humidities (11, 33, 43, 54 and 69%), which were achieved with oversaturated solutions of different salts as explained in section 2.2.1. Likewise, the viability of *C. sake* in the different formulations was also evaluated in the products with their original a_w, stored in hermetic jars at 5°C after 90 and 180 days.

To analyze yeast viability, 0.5 g of each product was dispersed in deionized sterile water (15, 20 o 5 ml for F1, F2 and F3 respectively) for 1 min, using a vortex shaker to achieve the complete dispersion of the granular products. After 9 min of repose time to promote complete cell rehydration (Yánez-Mendizabal *et al.*, 2012), serial dilutions were performed in duplicate and plated in TSA agar medium plates with streptomycin sulphate at 0.5 g/L to prevent bacterial growth. Plates were incubated at 25°C for 48 h and *C. sake* colonies were then counted based on their morphological characteristics. Results were expressed as log CFU per gram of dry solids in the formulation. Each assay was carried out in triplicate.

The experimental data were fitted to the Weibull model (Equation 4) in order to describe survival curves (Albert & Mafart, 2005; Coronel-Aguilera *et al.*, 2009).

$$\log N = \log N_0 - (\frac{t}{\delta})^p \quad (4)$$

where N is the number of microorganisms at time t; N_0 is the initial number of microorganisms; δ is the time that causes a one log reduction in the cell population; and p is a dimensionless shape parameter.

2.5 Solubility analysis

The water solubility of the dry formulations was determined at 5, 15 and 25°C for different contact times (between 5 and 50 minutes) to evaluate how temperature and time may influence the rehydration of the powders. The tests were carried out under mild agitation conditions (200 rpm) in order to simulate that might take place in agitation tanks in the case of in-field applications. The determination of the solubility was conducted following the method described by Cano-Chauca *et al*., (2005), with some modifications. Specifically, 0.25 g of sample dispersed in 25 mL of deionized water and stirred with a magnetic stirrer at 200 rpm for each time and temperature. Afterwards, the samples were centrifuged at 3000×g for 5 minutes to separate the non-solubilized phase. Then, 6.25 mL of the supernatant were transferred into pre-weighed glass Petri dishes, which were oven-dried for 5 h at 105°C to determine the mass of dissolved solids per ml. The solubility was expressed as % of dissolved solids with respect to the initial mass of dry powder. The assay was carried out in triplicate. Solubility data were fitted to Peleg model (Equation 5) (Peleg, 1988).

$$S = S_0 + \frac{t}{K_1 + K_2 \times t} \quad (5)$$

where S is the percentage of solubilized solids at time t; S_0 is the instantaneous solubility; t is time (min), K_1 and K_2 are the Peleg rate (min) and Peleg capacity constant ($\%^{-1}$), respectively

2.6 Statistical analysis

Statistical comparisons were made through an analysis of variance (ANOVA) using Statgraphics Centurion XVI version 16.1.17 (Manugistics Corp., Rockville, Md.). The

differences were considered significant when $p < 0.05$. The viability data in CFU/g were log-transformed (log CFU/g) in order to improve the homogeneity of variances.

3. RESULTS AND DISCUSSION

3.1 Water sorption and water plasticization of the products

The water sorption and water plasticization behavior of the different formulations was analyzed, in comparison with that of the carriers used, PS, PG and MD, as the main components of the formulations. The moisture content and T_g values of each formulation, equilibrated at the different levels of a_w at 20°C, are shown in **Table 3.2**, which also shows the corresponding values for carriers. It is possible to observe the greater water uptake of F1, in agreement with its greater proportion of PS, which exhibited the greatest water sorption capacity (**Figure 3.1**). The isotherms obtained for the BCPs and carriers were well fitted by the GAB model (**Figure 3.1**). The GAB parameters for formulations and carriers are shown in **Table 3.3**, together with the %RMS, whose value of under 10 indicated the very good fit of the model (Rizvi, 2005).

Table 3.2. Values of glass transition temperature (T_g) and moisture content (MC: g water/100 g product) of the BCP formulations and of the carriers equilibrated at different a_W. PS: potato starch; PG: pre-gelatinized potato starch; MD: maltodextrins.

	F1		F2		F3	
a_W	T_g (°C)	MC (%)	T_g (°C)	MC (%)	T_g (°C)	MC (%)
0.11	53 ± 1^{e}	5.4 ± 0.2^{a}	55 ± 1^{c}	2.4 ± 0.1^{a}	62 ± 1^{c}	3.7 ± 0.1^{a}
0.33	50 ± 1^{d}	8.7 ± 0.1^{b}	51 ± 1^{bc}	6.4 ± 0.2^{b}	57 ± 1^{cd}	5.7 ± 0.1^{b}
0.43	48 ± 1^{c}	10.4 ± 0.1^{c}	49 ± 2^{ab}	7.3 ± 0.1^{c}	51 ± 2^{b}	7.3 ± 0.1^{c}
0.54	46 ± 2^{ab}	11.9 ± 0.7^{d}	46 ± 3^{a}	8.6 ± 0.2^{d}	51 ± 1^{b}	7.9 ± 0.3^{c}
0.69	44 ± 1^{a}	14.1 ± 0.1^{e}	46 ± 1^{a}	11.4 ± 0.4^{e}	45 ± 1^{a}	10.6 ± 0.4^{d}
0.75	44 ± 1^{a}	15.3 ± 0.1^{e}	45 ± 1^{a}	12.7 ± 0.1^{f}	45 ± 1^{a}	12.6 ± 0.3^{e}
	PS		PG		MD	
a_W	T_g (°C)	MC (%)	T_g (°C)	MC (%)	T_g (°C)	MC (%)
0.11	114 ± 3^{e}	8.0 ± 0.1^{a}	100 ± 2^{f}	4.2 ± 0.1^{a}	149 ± 2^{f}	4.5 ± 0.3^{a}
0.33	89 ± 1^{d}	13.7 ± 0.5^{b}	70 ± 1^{e}	6.8 ± 0.1^{b}	112 ± 2^{e}	6.3 ± 0.1^{b}
0.43	70 ± 1^{c}	15.6 ± 0.1^{c}	55 ± 1^{d}	7.9 ± 0.3^{c}	86 ± 1^{d}	9.4 ± 0.2^{c}
0.54	60 ± 2^{b}	17.4 ± 0.1^{d}	50 ± 2^{c}	8.6 ± 0.1^{d}	62 ± 1^{c}	13.1 ± 0.2^{d}
0.69	51 ± 1^{a}	19.6 ± 0.1^{e}	44 ± 1^{b}	10.9 ± 0.3^{e}	41 ± 1^{b}	13.3 ± 0.1^{d}
0.75	49 ± 1^{a}	20.0 ± 0.1^{e}	40 ± 1^{a}	11.9 ± 0.3^{f}	31 ± 1^{a}	15.0 ± 0.5^{e}

Different superscripts (a – f) in the same column indicate statistically significant differences ($p < 0.05$) for the same formulation or carrier.

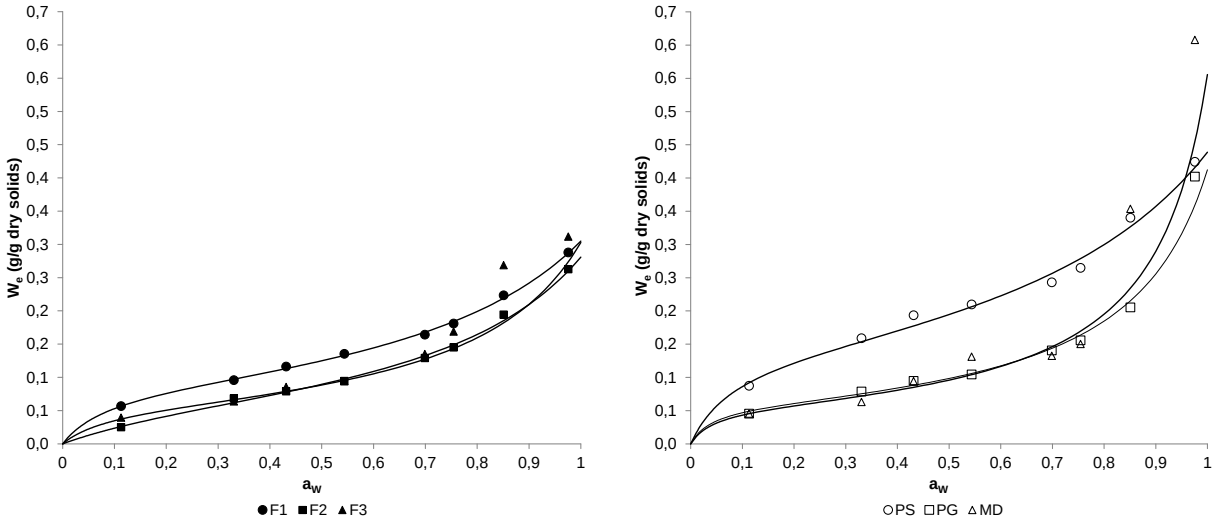


Figure 3.1. Moisture sorption isotherms of: (a) BCP formulations (F1, F2, F3) and (b) carriers (PS: potato starch, PG: pre-gelatinized potato starch, MD: maltodextrins). The solid lines represent the GAB model fitted curves.

The initial moisture contents of the dry BCP formulations were 8.81 ± 0.05, 6.90 ± 0.50 and 6.75 ± 0.13 g/100 g product, respectively, for F1, F2 and F3; these were close to the values of the respective monolayer moisture content (**Table 3.3**), which corresponded to a_w values of nearly 0.33 after the drying step. The moisture content of dried powders is usually within the range of the monolayer content if process conditions are adequately optimized (Fabra *et al.*, 2011). In this sense, the isotherms obtained corresponded to the adsorption curves for a $a_W \geq 0.33$ (the main part of the curve) and to the desorption data for $a_w < 0.33$.

The carrier exhibiting the highest water binding capacity was PS, and the obtained isotherm was similar to that previously reported for potato starch (Anzai *et al.*, 2011; Bizot, 1983). Likewise, although Torres & Seijo (2016) reported very similar values of GAB parameters in the case of water adsorption of rice starch at 25°C, the water binding capacity decreased in the desorption isotherms. Of the three formulations, F1 had the greatest proportion of PS, and this formulation also exhibited a more marked water binding capacity compared to F2 and F3, whose water sorption behavior was very

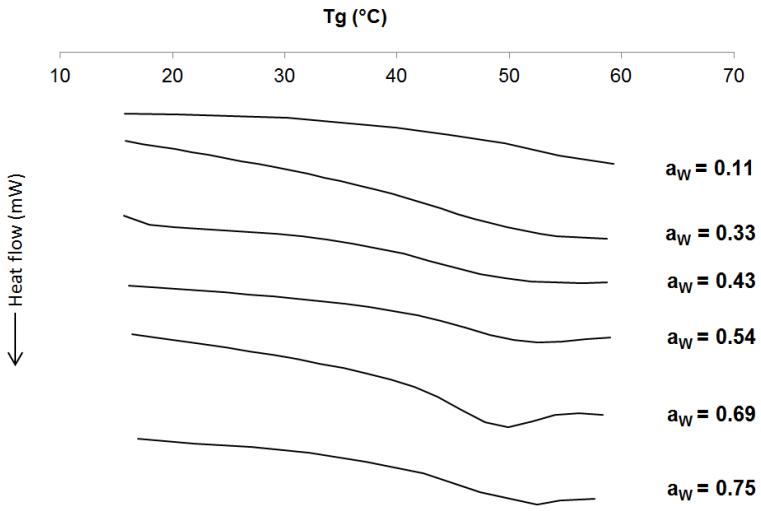
similar. The high mean molecular weight of the substrates resulted in a limited water gain at the highest a_w levels due to the low incidence of solute-solvent effects, which produce great water gains with relatively small increases in a_w when low molecular solutes are present in the matrices. These effects were more marked in MD due to the presence of free glucose molecules, which interact with water molecules through solute-solvent mechanisms at high a_w. The obtained MD isotherm was similar to that previously reported by Nurhadi *et al.*, (2016) for maltodextrin DE 10. Previously reported water sorption data for pre-gelatinized starch (PG) also showed a loss in the water binding capacity of the starch polymers as compared with that of the granules due to the loss in native structure where more water can be retained (Carvalho, 2008).

Table 3.3. GAB and Gordon & Taylor parameters of the different BCP and of the carriers. PS: potato starch, PG: pre-gelatinized potato starch, MD: maltodextrins, W_0: monolayer moisture content (g water / 100 g dry solid); C: constant related to the heat sorption of multilayer, K: factor correcting properties of the multilayer molecules, r^2: correlation coefficient, %RMS: relative percent root mean square.

GAB	F1	F2	F3	PS	PG	MD
W_0	8.94	7.38	5.82	14.17	5.88	5.53
C	15.89	5.12	13.72	17.75	28.45	24.74
K	0.71	0.75	0.81	0.68	0.85	0.90
r^2	0.99	0.99	0.85	0.99	0.97	0.88
% RMS	0.27	0.45	3.47	4.07	1.60	5.66
Gordon & Taylor	**F1**	**F2**	**F3**	**PS**	**PG**	**MD**
Tg(s)	-	-	-	172	143	212
K	-	-	-	2.6	4.8	5.8
r^2	-	-	-	0.99	0.99	0.99
% RMS	-	-	-	5.1	10	13

As regards BCP formulations, it was remarkable that the up interval of isotherms was less pronounced in the formulated BCPs than in carriers, which could be due to the effects of the cells on the product moisture control at high a_w values. A reduction in the availability of low molecular sugars could be brought about by cell consumption, thus decreasing the solute-solvent effects that influence the water binding capacity at high a_w values. To the best of our knowledge, there are no published studies about the water sorption behavior of BCA-based dry formulations using starch derivatives as carriers.

As far as water plasticization is concerned, Figure 3.2 shows the typical DSC thermograms corresponding to the first heating scan of the products equilibrated at different a_W. As is commonly observed in starch derivatives, relaxation endotherms appeared near the glass transition and which disappeared in the second scan (Nurhadi *et al*., 2016). This has been attributed to the relaxation of a part of the amorphous phase, when molecular rearrangement occurs during aging. As observed by Nurhadi *et al*., (2016), relaxation endotherms were not present at very low a_w in the case of MD, due to the fact that relaxation times at lower water contents are longer, as expected. To avoid enthalpy relaxation effects, the second scan was considered to determine T_g. The midpoint glass transition values of the different formulations are shown in Table 3.2 and Figure 3.3 reflects the water plasticization effects in terms of a_w and moisture content. As expected, T_g decreased as the moisture content or a_w increased, since, in biological materials, water plasticizes the amorphous structures (Roos & Karel, 1991). On a kinetic level, the glassy state is considered to be more stable than the rubbery state (Cano-Chauca *et al*., 2005; Genin & René, 1995) and so, in addition to the physical stability of the powders, cell viability could also be affected by the state of the carrier's solid matrix.



(F1)

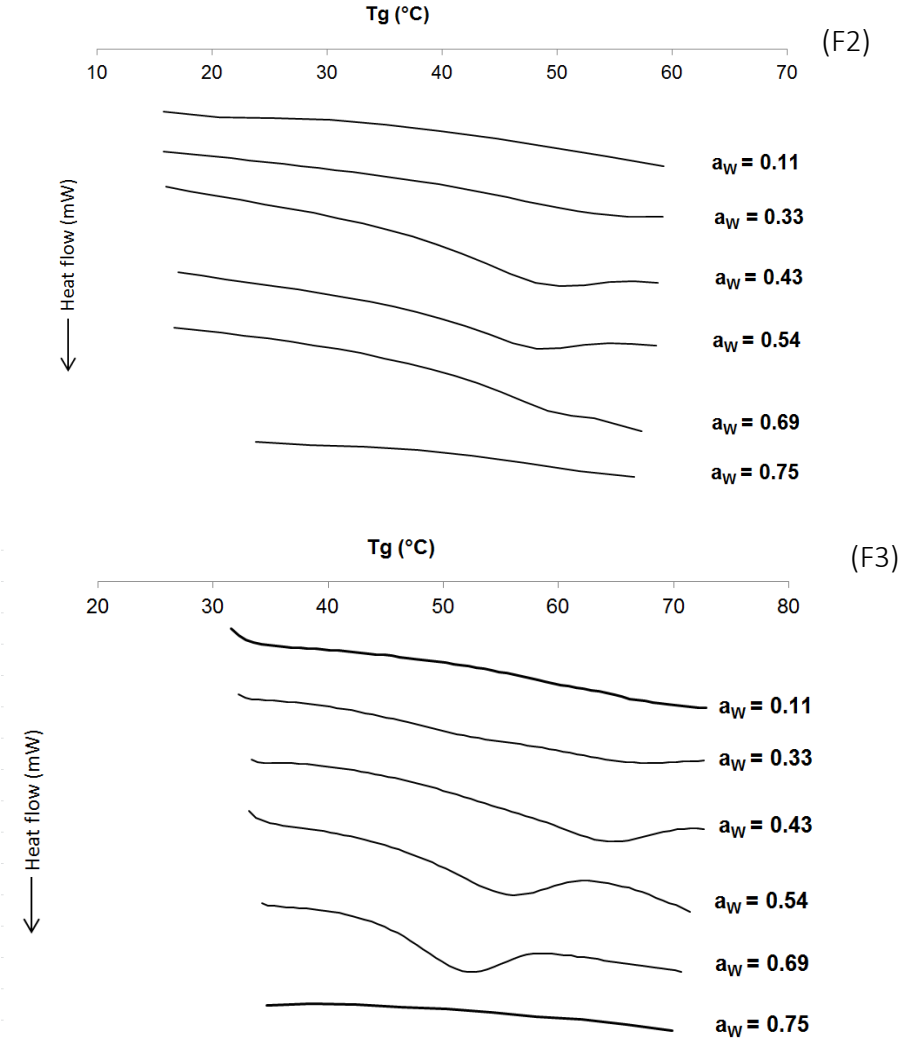


Figure 3.2. Typical DSC thermograms of the BCP formulations of *Candida sake* and starch derivatives.

The plasticization behavior varied markedly from carrier to carrier, with MD being the most sensitive to water plasticization in line with the greatest proportion of low molecular compounds (hydrolyzed sugars). Both PS and PG were less plasticized as previously observed by other authors (Perdomo *et al.*, 2009), due to the lack of low molecular compounds which interact with water molecules through solute-solvent interactions. In this sense, the fitting of Gordon & Taylor equation gave values of k parameter higher for MD than for PS and PG, in agreement with its greater sensitivity to water plasticization (Table 3.3). The obtained values of Gordon and Taylor parameters for the MD carrier were similar to those previously reported (Nurhadi *et al.*, 2016) for DE 10 MD.

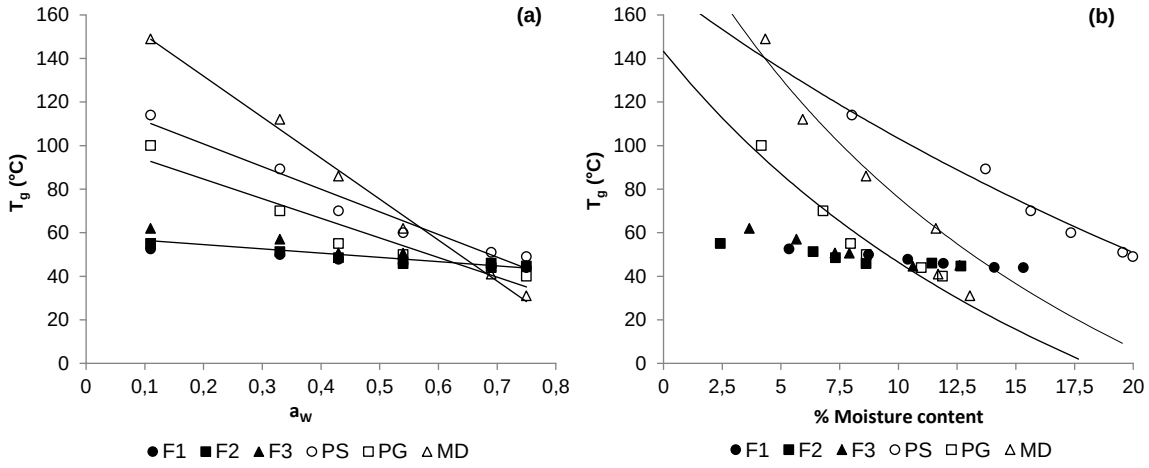


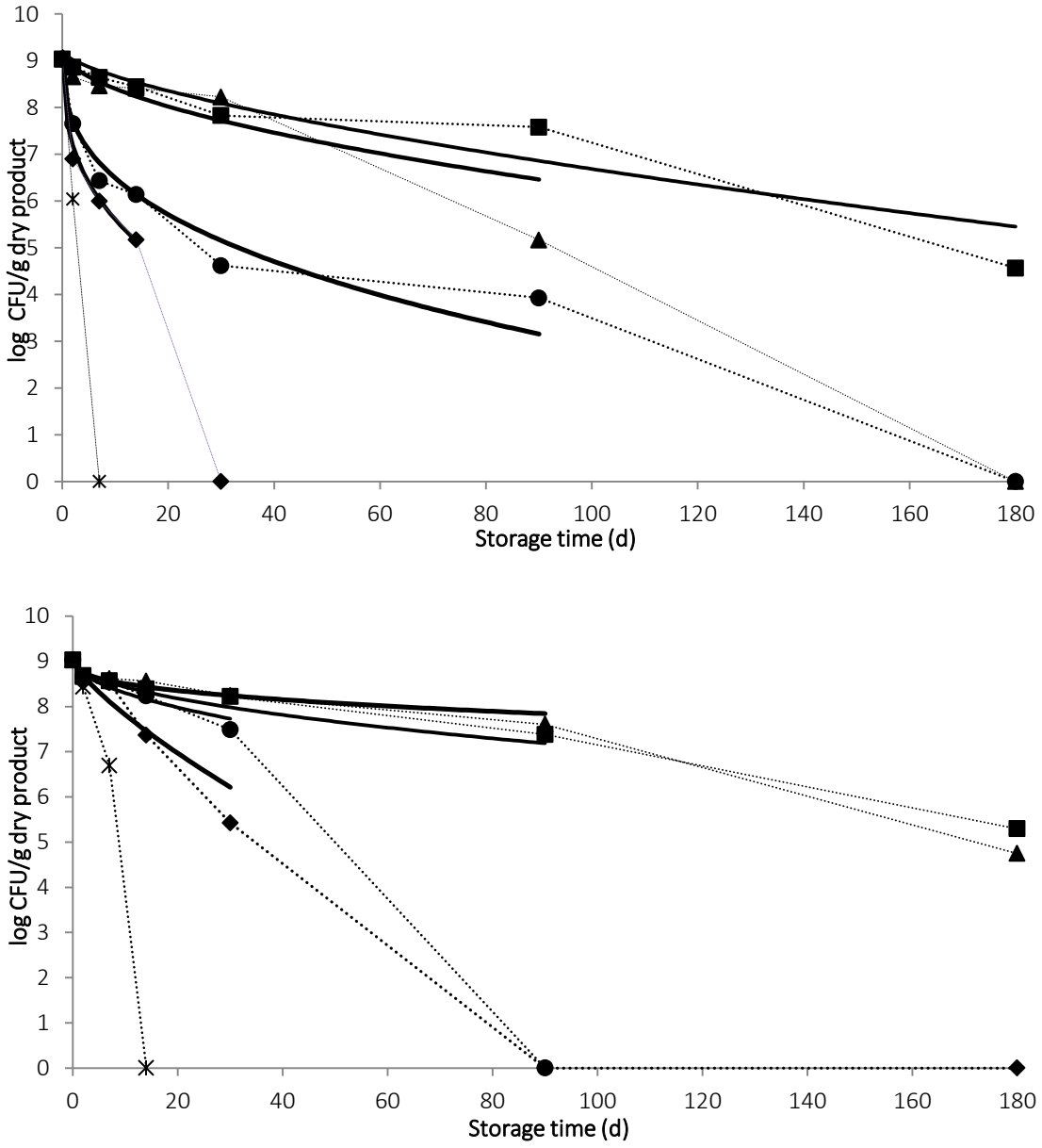
Figure 3.3. Glass transition temperatures of the BCP and carrier materials as a function of the a_W (a) and moisture content (g water/100 g product) (b). PS: potato starch, PG: pre-gelatinized potato starch, MD: maltodextrins. The solid lines in (b) represent the Gordon & Taylor model fitted curves.

The water plasticization behavior of BCPs greatly differed from that of the carriers. An unexpected plasticizing effect of water was observed for the three cell-containing formulations, which, in turn, showed a very similar trend of T_g-a_w relationships. T_g varied within a narrow interval, between 45-60°C, in the 0.11-0.75 a_w range. At low a_w levels, BCPs showed lower T_g values than the carriers while the T_g of the BCPs decreased to a lesser extent when the water content increased. This behavior was not previously observed for other encapsulated microbial cells. For instance, for lactic acid bacteria encapsulated in MD and whey proteins, the T_g values did not significantly differ from those of the encapsulating carriers (Ying *et al.*, 2012). However, the presence of yeast cells in the formulations greatly affected the water plasticizing effects in the studied BCPs. This behavior suggests that, whereas the solid composition determines the water uptake capacity of the product at a given a_w, the cells could retain a determined amount of water molecules in their mechanisms for survival, making them more or less available to plasticize the solids as a function of the cell demand. The secretion of some low molecular metabolites by the yeasts could also contribute to the low T_g values at low a_w. The similar T_g-a_w relationship for the different BCPs seems to indicate that cell action carried more weight in water plasticization than the different solid composition of the BCP.

From the obtained data, it can be seen that in no case was the critical moisture exceeded for products stored at a relative humidity of below 75% at 20°C. So, the glassy state can be assumed in all the BCPs stored at 11, 33, 43, 54 and 69% RH, where the cell viability was analyzed as a function of storage time.

3.2 Viability of *Candida sake* during storage

For the purposes of identifying the optimum moisture content of the BCPs in order to better maintain cell viability during storage at 20°C, samples were stored under different RH (11 to 69%). The cell counts of *C. sake* in F1, F2 and F3 throughout storage time under these conditions are shown in Figure 3.4.



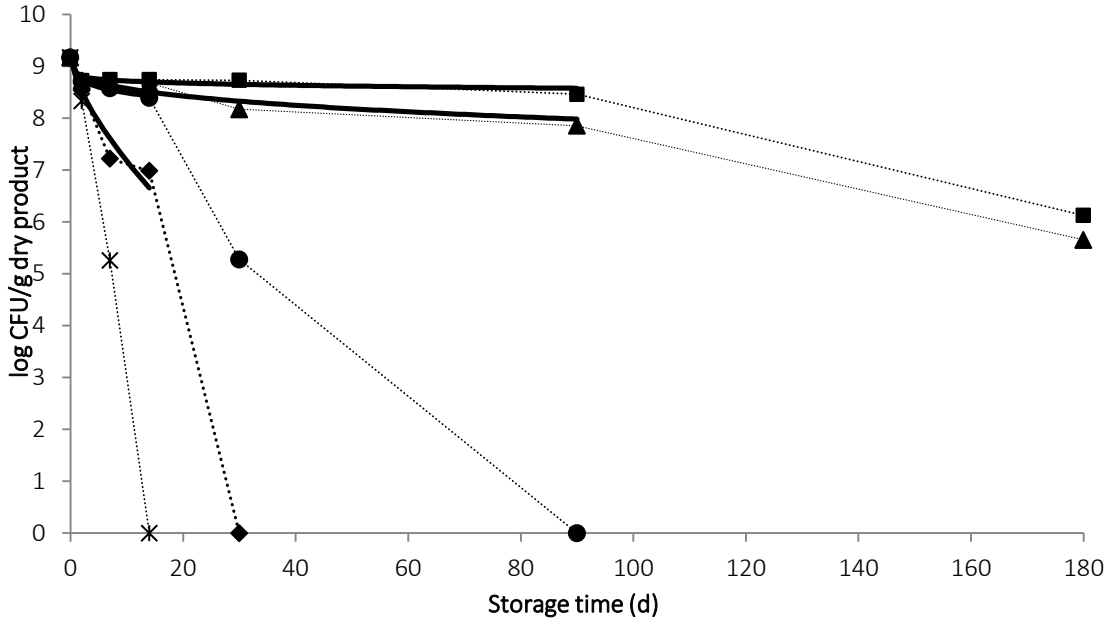


Figure 3.4. Viability of *Candida sake* in the BCP formulations during storage at 20°C under different a_W: a_W = 0.11, a_W = 0.33, a_W = 0.43, a_W = 0.54, a_W = 0.69. The solid lines represent the Weibull model fitted curves.

The initial cell viability was 9.04, 9.02 and 9.16 log CFU/g dry product for F1, F2 and F3, respectively. When stored at a_W of up to 0.43, the formulations completely lost cell viability throughout the tested period. This loss was especially significant at 0.69, since none of the formulations showed any viable cells of *C. sake* after 14 days of storage. However, storage at low a_W levels (0.11 and 0.33) better preserved the cells of the antagonist in every case. These results agree with that reported by Dunlap & Schisler (2010) for a dry formulation of the yeast *Cryptococcus flavescens.* In their study, the yeast's storage stability varied significantly across the tested a_w range (0.22-0.57); products stored at 0.22 a_w exhibited the best long-term survival of the yeast, while those stored at 0.57 showed the worst shelf-life. Other authors have reported similar results for the viability of probiotics in powder formulations, which exhibited a total loss of viability when stored at 0.52 a_W at 25°C within 22 days (Poddar *et al*., 2014). However, storage at a_w 0.11 gave rise to the slowest decline in the viable bacterial count. To explain this behavior, Moore *et al*. (1996) pointed to the adverse effect of moisture gain in cell viability when the product rehydrates at high RH, due to the rapid water uptake by dry cells, which may cause membrane damage. On the other hand, under high moisture conditions the dormant state acquired by the yeast cells in the

drying process is reverted and the available water and nutrients are insufficient to allow cells to perform their vital functions and, consequently, their death occurs.

Although formulations stored under low a_W conditions showed a better shelf-life, a significant ($p < 0.05$) decrease in the antagonist's survival time was observed for the three BCPs after 180 days. The decrease in viability was above 3 log units in every case. Specifically, the viability of *C. sake* was reduced to 50% (in log scale) in F1 stored at 0.11 a_W, whilst at 0.33 a_W, no viable cells were found at this time. 41 and 47% viability was lost in the case of F2 at a_w 0.11 and 0.33, respectively, and 33 and 38% in the case of F3. These results are not satisfactory from a commercial point of view, which requires the BCPs to have a shelf-life of at least 6 months and preferably 1-2 years, without a significant reduction in the initial number of viable cells (Pusey, 1994; Rhodes, 1993).

As regards the influence of carrier composition, F1 was the worst support in terms of *C. sake* cell viability, which may be related to its greater water binding capacity, possibly contributing to a faster cell death. Conversely, the lowest rate of yeast death was obtained with the formulation based on MD (F3) containing protectants (sucrose and milk powder), which may help to keep the cells alive. This observation is supported by the fact that sucrose, lactose and other disaccharides, are known to extend microbial survival via hydrogen bonding to the polar head group of the cell membrane phospholipids, thus protecting them from the drying injuries (Corcoran *et al*., 2004; Crowe *et al*., 1984; Stummer *et al*., 2012). Additionally, milk could supply a variety of nutrients that favor the survival of *C. sake* (Costa *et al*., 2002).

The number of viable cells changed throughout storage time and these changes could be fitted to the Weibull model (Figure 3.4) at a_W from 0.11 to 0.54, up to a critical time when a sharper drop in viable cells occurred (limit time for the fitting). The obtained parameters, related to the microbiological stability of the different formulations for each a_W, can be seen in Table 3.4. The values of δ (time that causes a 1 log reduction in the cell population) decreased as the a_W rose in every case. Likewise, F1 exhibited the lowest values of δ, confirming that this support was the least adequate to carry *C. sake*, whereas F3 allowed the highest δ values to be obtained, while showing a greater number of viable cells in the second period with faster cell death.

Table 3.4. Weibull parameters from viability of *Candida sake* in the BCP formulations based on starch derivatives. p: dimensionless shape parameter, δ: time, in days (d), that causes a one log reduction in the cell population. The limit time (t_l) for time of the model is also shown.

	F1			F2			F3		
a_W	p	δ (d)	t_l (d)	p	δ (d)	t_l (d)	p	δ (d)	t_l (d)
0	0.68	25	≤ 180	0.51	27	≤ 90	0.12	6137	≤ 90
0.33	0.56	15	≤ 90	0.37	57	≤ 90	0.31	51	≤ 90
0.43	0.37	1	≤ 90	0.52	18	≤ 30	0.26	43	≤ 14
0.54	0.36	0.3	≤ 14	0.76	8	≤ 30	0.70	4	≤ 14

Since temperature is a key factor for microbiological stability, BCPs with their initial moisture content (a_W of about 0.33) were also stored at a low temperature (5°C). **Figure 3.5** shows the cell survival ratio for F2 and F3 products, which exhibited the best microbial support, stored for 90 and 180 days at 5 and 20°C.

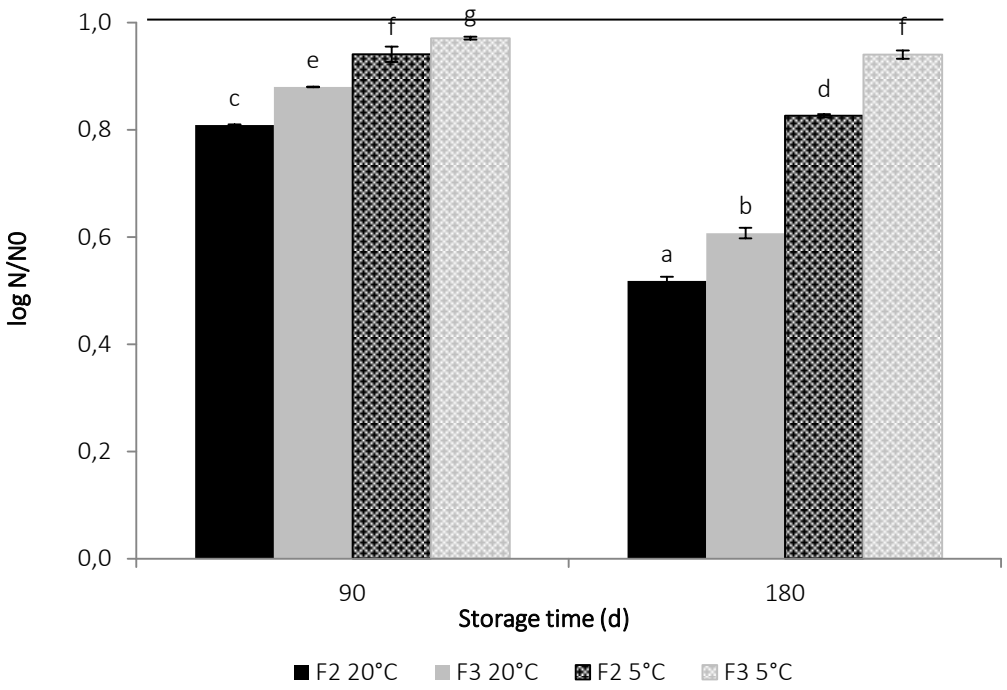


Figure 3.5. Reduction in population of *Candida sake* referred to the initial counts in the BCP formulations (F2 and F3) stored at 20°C or 5°C.

The longer cell survival time was observed at low temperatures for both cases, with F3 showing significantly ($p < 0.05$) higher values. Similar results have been widely reported with other BCAs (Kinay & Yilniz, 2008; Mejri *et al.*, 2013; Torres *et al.*, 2014).

Temperatures of 4 – 10°C cause both the cell division and metabolic rate of microorganisms to slow down. In this situation, cells are capable of withstanding the depletion of nutrients and the accumulation of toxic metabolites (Mejri *et al*., 2013; Trivedi *et al*., 2005). Thus, storing the BCPs at a low temperature maintains the microorganism in a state of low metabolic activity (Elzein *et al*., 2004) and this would be recommendable for the particular case of the studied *C.sake* formulations. Although cold storage implies a higher product cost, low temperatures greatly favor the cell viability in long-term storage.

3.3 Solubility analysis

The values of the percentage solubility of the BCPs as a function of stirring time at different temperatures are plotted in Figure 3.6.

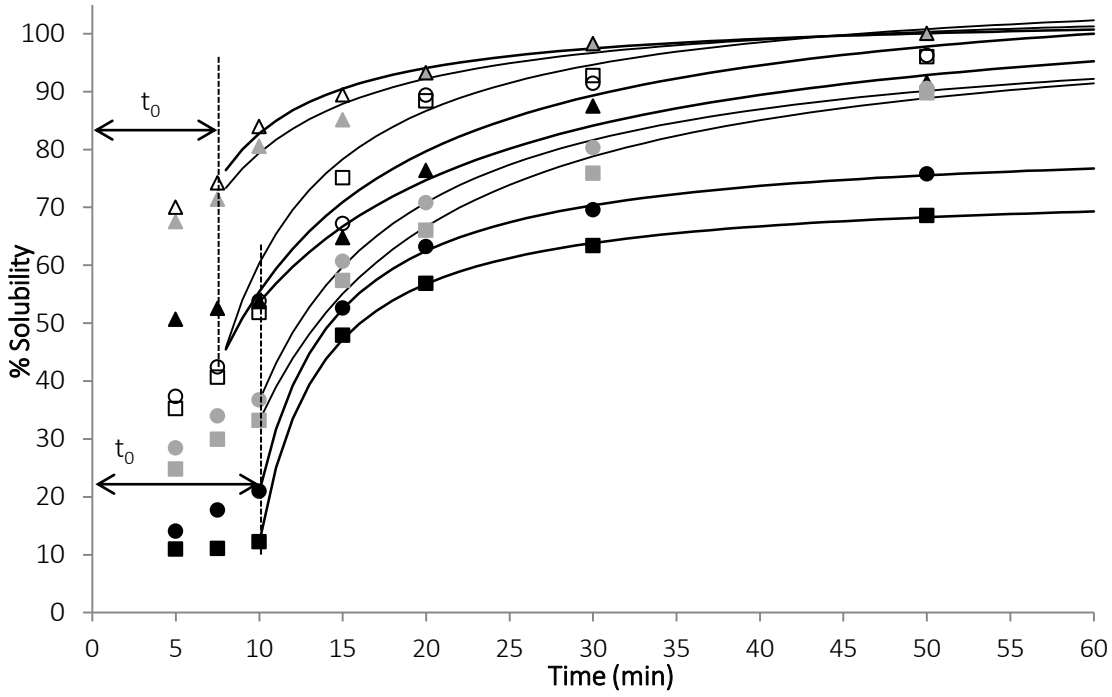


Figure 3.6. Percentage of solubility of the BCP formulations as a function of contact time at 5 (black symbols), 15 (grey symbols) or 25 °C (open symbols) for F1 (circles), F2 (squares) and F3 (triangles). Starting time for the retarded solubility period (t_0) is indicated. Mean variation coefficient for determinations: 6%.

Formulation type and temperature affected the solubilization kinetics. As can be observed in the figure, F3 exhibited a significantly fastest solubilization, reaching levels

of nearly 100% after 20 min of stirring at 15 and 25°C. Likewise, similar behavior was observed for F1 and F2 at a given temperature, both showing a more limited solubility with respect to F3. F2 showed a lower solubility than F1 according to a multifactorial analysis. This behavior was coherent with the BCP composition. Maltodextrins are reported as starch derivatives with the highest water solubility (Cano-Chauca *et al.*, 2005) and so, the more soluble components of F3 contribute to its faster water dissolution. The presence of PS or PG hindered the solubility of the powders, in line with the lower solubility of amylose and amylopectin chains, especially in the starch granules where they are in a semi-crystalline structure (Mandala & Bayas, 2004; Eliasson & Gudmundsson, 1996).

In every case, the solubility curves showed two steps; in the first step, a slower increase in soluble solids was observed over time, while a fast dissolution of one part of the powder occurred. In the second step, an slow, asymptotic increase in soluble solids was observed. In most of the cases, the first step took about 10 minutes at 5 or 15°C, whereas it was shorter (7.5 min) at 25°C, and 15°C in F3. During this step, different amounts of solids were instantaneously dissolved (S_0) depending on the formulation and temperature, whereas another part rehydrated before their slower dissolution (retarded solubility). These results agree with what was reported by Fang *et al.*, (2008) for food powder rehydration, which takes place through different phases: the wetting of particles overcoming the surface tension at the solid-liquid interface, followed by its dissolution. Table 3.5 gives the S_0 values corresponding to the instantaneous solubility of the products, which greatly increased as the temperature rose and was markedly higher for the F3 product. The Arrhenius plot for S_0 values allows the activation energy to be determined for each product, these values being 24, 42 and 10 kJ for F1, F2 and F3, which indicates that more temperature requirements are needed to dissolve the F2 product instantaneously. The increase in kinetic energy generated by higher temperatures allows the solvent molecules to break apart the solute molecules that are held together by intermolecular attractions more effectively. Data up to S_0 (retarded solubility period) were fitted by Peleg equation (Equation 5) to predict the total percentage solubility as a function of temperature and time for each product. Table 5 also shows the constants of the model and the predicted asymptotic value of solubility

(S_∞). It can be observed that, whereas temperature notably affected S_0 values, kinetic constant K_1 was less affected by temperature in the considered range. No significant differences in the K_1 values of the different products between 15 and 25°C were found. The differences in the values at 5°C can be attributed to the marked difference in S_o values, which affected the driving force for retarded dissolving of the different products. In this sense, F3, which had higher S_0 values, exhibited a slower solubilization rate (the inverse of K_1) in the retarded period. Values of K_2 are related with the asymptotic value S_∞ (Table 5). No significant differences in K_2 values were obtained for F1 and F2 at the different temperatures, whereas greater values were obtained for F3 which increased as the temperature rose. This indicates that similar amounts of product (about 60%) were dissolved in the retarded period for F1 and F2, regardless of the temperature, and that the differences in S_∞ were mainly determined by S_0 values. In F3, total dissolution was obtained at every temperature, with about 30 and 45 % being the solubility in the retarded period at 5°C and 15-25, respectively.

Table 3.5. Peleg parameters obtained from the fitting of solubility data for the BCP formulations as a function of the temperature.

BCP	T (°C)	S_0 (%)	K_1 (min)	K_2 ($\%^{-1}$)	S_∞
F1	5	21	0,077	0,016	82
	15	37	0,141	0,015	102*
	25	42	0,155	0,014	112*
F2	5	12	0,062	0,016	74
	15	33	0,158	0,014	105*
	25	41	0,127	0,015	110*
F3	5	54	0,293	0,018	109*
	15	71	0,238	0,029	106*
	25	74	0,207	0,034	104*

*Values > 100 related to the mathematical fitting

Mean variation coefficient for S determinations: 6%

$S_\infty = S_0 + 1/K2$

From this analysis, the F3 formulation would be the best one for the purposes of the in-field application of BCPs as water dispersion, since solubility is considered a key quality characteristic for product reconstitution in order to avoid prolonging the process (Selomulya & Fang, 2013; Hla & Hogekamp, 1999). F3 exhibited the highest instantaneous dissolution and total dissolution within the 5 to 25ºC range, which is a common range for practical applications.

4. CONCLUSIONS

The viability of *C. sake* during storage at 20°C was highly dependent on the water activity of the formulated BCP. The best preservation was obtained at a_w values below 0.33. This value corresponds to the monolayer moisture content of the products, when water was strongly bonded to the solid matrix, and is their usual water content after drying. MD was the starch derivative that best supported the yeast in terms of the preservation of cell viability at low a_w. The incorporation of protectants (sucrose and milk powder) could also contribute to the improvement in the functionality of the carrier. This formulation also exhibited the best water solubility, which is a key factor for BCP applications. Nevertheless, 20°C is not low enough to maintain an adequate cell count for prolonged storage times and cold storage would be required to ensure an appropriate BCP shelf-life. Likewise, material with water vapor high barrier properties must be used in the product packaging so as to avoid moisturizing, since water uptake would lead to important significant losses in cell viability. The knowledge acquired in this study provides a basis that might guide both the development of BCPs based on similar antagonists and also the choice of the optimal storage conditions.

ACKNOWLEDGEMENTS

The authors are grateful to the Spanish Government for the financial support from the national project RTA2012-00067-C02 (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Spain and FEDER funds) and to the Conselleria d'Educació of the Generalitat Valenciana, (Spain) for A. Marín's PhD grant.

REFERENCES

Abadias, M., Teixidó, N., Usall, J., Solsona, C., & Viñas, I. (2005). Survival of the postharvest biocontrol yeast *Andr* CPA-1 after dehydration by spray-drying. *Biocontrol Science and Technology*, *15*, 835–846.

Abadias, M., Usall, J., Teixidó, N., & Viñas, I. (2003). Liquid formulation of the postharvest biocontrol agent *Candida sake* CPA-1 in isotonic solutions. *Phytopathology*, *93*(4), 436–442.

Albert, I., & Mafart, P. (2005). A modified Weibull model for bacterial inactivation. *International Journal of Food Microbiology*, *100*(1-3), 197–211.

Andrade, R. D., Skurtys, O., & Osorio, F. A. (2012). Atomizing Spray Systems for Application of Edible Coatings. *Comprehensive Reviews in Food Science and Food Safety*, *11*(3), 323–337.

Anzai, M., Hagiwara, T., Watanabe, M., Komiyama, J., & Suzuki, T. (2011). Relationship between enthalpy relaxation and water sorption of ball-milled potato starch. *Journal of Food Engineering*, *104*(1), 43–48.

Aponte, M., Troianiello, G. D., Di Capua, M., Romano, R., & Blaiotta, G. (2016). Impact of different spray-drying conditions on the viability of wine Saccharomyces cerevisiae strains. *World Journal of Microbiology and Biotechnology*, *32*(1), 13.

Bizot, H. (1983). Using the GAB model to construct sorption isotherms. In R. Jowitt *et al*., (Ed.), *Physical properties of foods* (pp. 43–54). New York (USA): Applied Science Publishers.

Cano-Chauca, M., Stringheta, P. C., Ramos, a. M., & Cal-Vidal, J. (2005). Effect of the carriers on the microstructure of mango powder obtained by spray drying and its functional characterization. *Innovative Food Science and Emerging Technologies*, *6*(4), 420–428.

Cañamás, T. P., Viñas, I., Torres, R., Usall, J., Solsona, C., & Teixidó, N. (2011). Field applications of improved formulations of *Candida sake* CPA-1 for control of *Botrytis cinerea* in grapes. *Biological Control*, *56*(2), 150–158.

Cañamás, T. P., Viñas, I., Usall, J., Magan, N., Solsona, C., & Teixidó, N. (2008). Impact of mild heat treatments on induction of thermotolerance in the biocontrol yeast *Candida sake* CPA-1 and viability after spray-drying. *Journal of Applied Microbiology*, *104*(3), 767–775.

Carvalho, A. J. F. (2008). Chapter 15 – Starch: Major Sources, Properties and Applications as Thermoplastic Materials. In *Monomers, Polymers and Composites from Renewable Resources* (pp. 321–342).

Chumthong, A., Kanjanamaneesathian, M., Pengnoo, A., & Wiwattanapatapee, R. (2008). Water-soluble granules containing *Bacillus megaterium* for biological control of rice sheath blight: Formulation, bacterial viability and efficacy testing. *World Journal of Microbiology and Biotechnology*, *24*(11),

Corcoran, B. M., Ross, R. P., Fitzgerald, G. F., & Stanton, C. (2004). Comparative survival of probiotic lactobacilli spray-dried in the presence of prebiotic substances. *Journal of Applied Microbiology*, *96*, 1024–1039.

Coronel-Aguilera, C., Jiménez-Munguía, M., & López-Malo, A. (2009). Modelos para la descripción de curvas de supervivencia microbiana en alimentos. *Temas Selectos de Ingenieria de Alimentos*, *3*(1), 11–23.

Costa, E., Usall, J., Teixidó, N., Torres, R., & Viñas, I. (2002). Effect of package and storage conditions on viability and efficacy of the freeze-dried biocontrol agent Pantoea agglomerans strain CPA-2. *Journal of Applied Microbiology*, *92*(5), 873–878.

Crowe, J. H., Crowe, L. M., & Chapman, D. (1984). Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science*, *223*, 701–703.

Dunlap, C. A., & Schisler, D. a. (2010). Fluidized-bed drying and storage stability of *Cryptococcus flavescens* OH 182.9, a biocontrol agent of *Fusarium* head blight. *Biocontrol Science and Technology*, *20*(5), 465–474.

Eliasson, A. C., & Gudmundsson, M. (1996). Starch: Physico-chemical and functional aspects. In A. C. Eliasson (Ed.), *Carbohydrates in food* (pp. 431–503). New York (USA): Marcel Dekker.

Elzein, A., Kroschel, J. & Müller-Stöver, D. (2004). Optimization of storage conditions for adequate shelf-life of ‘‘pesta” formulation of Fusarium oxysporum ‘‘Foxy 2”, a potential mycoherbicide for Striga: effects of temperature, granule size and water activity. *Biocontrol Science and Technology*, *14*, 545-559.

Fabra, M. J., Márquez, E., Castro, D. & Chiralt A. (2011). Effect of maltodextrins in the water-content-water activity-glass transition relationships of noni (*Morinda citrifolia L*.) pulp powder. *Journal of Food Engineering*, *103* (1), 47-51

Fang, Y., Selomulya, C., & Chen, X. D. (2004). On measurement of food powder reconstitution properties. *Drying Technology*, *26*, 3–14.

Fu, N., & Chen, X. D. (2011). Towards a maximal cell survival in convective thermal drying processes. *Food Research International*, *44*, 1127–1149.

Genin, N., & René, F. (1995). Analyze du role de la transition vitreuse dans les procedés de conservation agro-alimentaires. *Journal of Food Engineering*, *26*, 391–408.

Greenspan, L. (1977). Humidity fixed points of binary saturated aqueous solutions. *Journal of Research of the National Bureau of Standards Section A: Physics and Chemistry*, *81A*(1), 89.

Guijarro, B., Larena, I., Melgarejo, P., & De Cal, A. (2006). Effect of drying on conidial viability of *Penicillium frequentans*, a biological control agent against peach brown rot disease caused by *Monilinia* spp. *Biocontrol Science and Technology*, *16*(3), 257–269.

Jacquot, M., & Pernetti, M. (2004). Spray coating and drying processes. In *Fundamentals of cell immobilisation biotechnology* (pp. 343–356). Amsterdam (The Netherlands): Springer.

Khem, S., Woo, M. W., Small, D. M., Chen, X. D., & May, B. K. (2015). Agent selection and protective effects during single droplet drying of bacteria. *Food Chemistry*, *166*, 206–214.

Kinay, P., & Yildiz, M. (2008). The shelf life and effectiveness of granular formulations of *Metschnikowia pulcherrima* and *Pichia guilliermondii* yeast isolates that control postharvest decay of citrus fruit. *Biological Control*, *45*(3), 433–440.

Kyaw Hla, P., & Hogekamp, S. (1999). Wetting behaviour of instantized cocoa beverage powders. *International Journal of Food Science and Technology*, *34*, 335–342.

Lafargue, D., Lourdin, D., & Doublier, J. L. (2007). Film-forming properties of a modified starch/-carrageenan mixture in relation to its rheological behaviour. *Carbohydrate Polymers*, *70*(1), 101–111.

Larena, I., Melgarejo, P., & De Cal, a. (2003). Drying of conidia of *Penicillium oxalicum*, a biological control agent against Fusarium wilt of tomato. *Journal of Phytopathology*, *151*(11-12), 600–606.

Lee, J. P., Lee, S. W., Kim, C. S., Son, J. H., Song, J. H., Lee, K. Y., Kim, H. J., Jung, S. J. & ¡Moon, B. J. (2006). Evaluation of formulations of *Bacillus licheniformis* for the biological control of tomato gray mold caused by *Botrytis cinerea*. *Biological Control*, *37*(3), 329–337.

Lewis, J. A., Fravel, D. R., Lumsden, R. D., & Shasha, B. S. (1995). Application of biocontrol fungi in granular formulation of pregelatinized starch-flour to control damping off diseases caused by *Rhizoctonia solani*. *Biological Control*.

Mandala, I. G., & Bayas, E. (2004). Xanthan effect on swelling, solubility and viscosity of wheat starch dispersions. *Food Hydrocolloids*, *14*, 191-201.

Marín, A., Cháfer, M., Atarés, L., Chiralt, A., Torres, R., Usall, J., & Teixidó, N. (2016). Effect of different coating-forming agents on the efficacy of the biocontrol agent *Candida sake* CPA-1 for control of *Botrytis cinerea* on grapes. *Biological Control*, *96*, 108–119.

Mejri, D., Gamalero, E., & Souissi, T. (2013). Formulation development of the deleterious rhizobacterium *Pseudomonas trivialis* X33d for biocontrol of brome (*Bromus diandrus*) in durum wheat. *Journal of Applied Microbiology*, *114*(1), 219–28.

Melin, P., Håkansson, S., & Schnürer, J. (2007). Optimisation and comparison of liquid and dry formulations of the biocontrol yeast *Pichia anomala* J121. *Applied Microbiology and Biotechnology*, *73*(5), 1008–1016.

Melin, P., Schnürer, J., & Håkansson, S. (2011). Formulation and stabilisation of the biocontrol yeast *Pichia anomala*. *Antonie van Leeuwenhoek*, *99*(1), 107–12.

Moore, D., Langewald, J., & Obognon, F. (1996). Effects of rehydratation on the conidial viability of *Metarhizium flavoviride* mycopesticide formulations. *Biocontrol Science and Technology*, *7*, 87–94.

Morgan, C. A., Herman, N., White, P. A., & Vesey, G. (2006). Preservation of micro-organisms by drying; A review. *Journal of Microbiological Methods*, *66*(2), 183–193.

Mounir, R., Durieux, a, Bodo, E., Allard, C., Simon, J.-P., Achbani, E.-H., El-Jaafari, S., Douira, A. & Jijakli, M.-H. (2007). Production, formulation and antagonistic activity of the biocontrol like-yeast *Aureobasidium pullulans* against *Penicillium expansum*. *Biotechnology Letters*, *29*(4), 553–9.

Nurhadi, B., Roos, Y. H., & Maidannyk, V. (2016). Physical properties of maltodextrin DE 10: water sorption, water plasticization and enthalpy relaxation. *Journal of Food Engineering*, *174*, 68–74.

Peleg, M. (1988). An Empirical Model for the Description of Moisture Sorption Curves. *Journal of Food Science*, *53*(14), 1216–1217.

Perdomo, J., Cova, A., Sandoval, A. J., García, L., Laredo, E., & Müller, A. J. (2009). Glass transition temperatures and water sorption isotherms of cassava starch. *Carbohydrate Polymers*, *76*(2), 305–313.

Pusey, P. L. (1994). Enhancement of biocontrol agents for postharvest diseases and their integration with other control strategies. In C. L. Wilson & M. E. Wisniewski (Eds.), *Biological Control of Postharvest Diseases: Theory and Practice* (pp. 77–78). Boca Raton (USA): CRC Press Inc.

Rahman, M. S. (2009). Glass transition data and models of foods. In M.S. Rahman (Ed.), *Food Properties Handbook, second ed.* Boca Raton (USA): CRC Press Inc.

Rhodes, D. J. (1993). Formulation of biological control agents. In D. G. Jones (Ed.), *In Exploitation of Microorganism* (pp. 411– 439). London (UK): Chapman & Hall.

Rizvi, S. (2005). Thermodynamic properties of foods in dehydration. In M. A. Rao, S. S. H. Rizvi, & A. K. Datta (Eds.), *Engineering Properties of Foods, third ed.* Boca Raton (USA): CRC Press Inc.

Roos, Y. H. (1995). *Phase transitions in foods*. San Diego, CA (USA): Academic Press.

Roos, Y. H., & Karel, M. (1991). Phase transitions of mixtures of amorphous polysaccharides and sugars. *Biotechnology Progress*, *7*(1), 49–53.

Santivarangkna, C., Higl, B., & Foerst, P. (2008). Protection mechanisms of sugars during different stages of preparation process of dried lactic acid starter cultures. *Food Microbiology*, *25*(3), 429–441.

Selomulya, C., & Fang, Y. (2013). 15 - Food powder rehydration. *Handbook of Food Powders : Processes and Properties*, 379–408.

Spiess, W. E. L., & Wolf, W. R. (1983). The results of the COST 90 Project on water activity. In R. Jowitt, F. Escher, B. Hallstrom, H. F. T. Meffert, W. E. L. Spiess, & G. Vos (Eds.), *Physical properties of foods* (pp. 65–91). London (UK), New York (USA): Applied Science Publishers.

Stephan, D., Matos, A.-P., Silva, D., & Bisutti, I. L. (2016). Optimization of a freeze-drying process for the biocontrol agent *Pseudomona*s spp. and its influence on viability, storability and efficacy. *Biological Control*, *94*, 74-81.

Strasser, S., Neureiter, M., Geppl, M., Braun, R., & Danner, H. (2009). Influence of lyophilization, fluidized bed drying, addition of protectants, and storage on the viability of lactic acid bacteria. *Journal of Applied Microbiology*, *107*, 167–177.

Stummer, S., Toegel, S., Rabenreither, M.-C., Unger, F. M., Wirth, M., Viernstein, H., & Salar-Behzadi, S. (2012). Fluidized-bed drying as a feasible method for dehydration of *Enterococcus faecium* M74. *Journal of Food Engineering*, *111*, 156-165.

Teunou, E., & Poncelet, D. (2002). Batch and continuous fluid bed coating – review and state of the art. *Journal of Applied Microbiology*, *53*, 325–340.

Torres, M. D., & Seijo, J. (2016). Water sorption behaviour of by-products from the rice industry. *Industrial Crops and Products*, *86*, 273–278.

Torres, R., Usall, J., Teixidó, N., Abadias, M., & Viñas, I. (2003). Liquid formulation of the biocontrol agent *Candida sake* by modifying water activity or adding protectants. *Journal of Applied Microbiology*, *94*(2), 330–339.

Torres, R., Solsona, C., Viñas, I., Usall, J., Plaza, P., & Teixidó, N. (2014). Optimization of packaging and storage conditions of a freeze-dried *Pantoea agglomerans* formulation for controlling postharvest diseases in fruit. *Journal of Applied Microbiology*, *117*(1), 173–184.

Yánez-Mendizabal, V., Viñas, I., Usall, J., Cañamás, T., & Teixidó, N. (2012). Endospore production allows using spray-drying as a possible formulation system of the biocontrol agent *Bacillus subtilis CPA-8. Biotechnology Letters, 34(4), 729–735.*

Ying, D., Sun, J., Sanguansri, L., Weerakkody, R., & Augustin, M. A. (2012). Enhanced survival of spray-dried microencapsulated *Lactobacillus rhamnosus* GG in the presence of glucose. *Journal of Food Engineering*, *109*, 597–602.

4. GENERAL DISCUSSION

In the present Doctoral Thesis, the improvement in the performance of a biocontrol agent (BCA) through its combined formulation with coating forming agents (CFAs) has been investigated in order to obtain stable and effective biocontrol products (BCP). A great number of strategies aimed at the improving of the resistance and efficacy of antagonists have been developed in area of the biological control, however, the joint formulation of BCAs and CFAs for the purposes of obtaining BCP, has been less widely explored.

CFAs based on different hydrocolloids were formulated in order to be used as carriers for the BCA *Candida sake* CPA-1, effective against the pathogenic fungus *Botrytis cinerea*, the causing agent of grey mold in many fruits, including grapes. Several matrices, two polysaccharides and two proteins (hydroxypropylmethylcellulose, HPMC; corn starch, S; sodium caseinate, NaCas; pea protein, PP) were selected and combined with surfactants with a different hydrophilic-lipophilic balance (oleic acid, OA; Span 80, S80; Tween 85, T85). The potential of the different formulations to improve the adherence and survival and, consequently, the efficacy of the antagonistic yeast was tested in *in vivo* studies. For that purpose, the CFAs were applied in combination with *C. sake* ($5 \cdot 10^7$ CFU/ml) on grapes. Analyses of population dynamics 24 h and 7 days after the application revealed that the major part of CFAs provided a better adherence after 24 h of the microorganism and improved its survival rate throughout time to a different extent, depending on their nature. Moreover, the combination *of C. sake* and the CFAs, applied on grapes inoculated with *B. cinerea,* led to a better control of the infection.

All the formulations improved the initial adherence of *C. sake* on grapes with respect to the application of the yeast in water, with values ranging from 5 to 6 log CFU/g. Furthermore, after 7 days of incubation under controlled conditions, the initial population of the antagonist increased in every case. This was especially true for the coatings based on proteins, which gave rise to a maximum value of 6.89 log CFU/g, suggesting that these matrices represented a better support for *C. sake*. As regards the biocontrol efficacy, CFAs also improved the control of gray mold exerted by the BCA. The coatings based on NaCas and PP also showed a more positive effect, although starch formulations exhibited comparable results. In these cases, reductions of over 80% were obtained in the incidence of disease caused by *B. cinerea*. A microstructural

analysis of the coatings containing *C. sake* on grapes surfaces allowed complementary information about cell distribution and development to be obtained after applying the BCP. This analysis revealed the natural tendency of the yeast cells to form aggregates which were reduced when surfactants were present in the formulations. Despite this effect of the surfactants, which might be positive for achieving a more homogeneous distribution of the yeast on the fruit surface, no additional positive effects were observed. Likewise, the increase in the *C. sake* population on the fruit was confirmed through the formation of cell aggregates, where, as a consequence of their growth, different layers of BCA cells were formed.

Bearing in mind that the ultimate objective of this study was to obtain a BCP to be applied in field, both the economic aspects and the product durability must be considered, in order to develop a highly effective competitive product. In this sense, different aspects were considered: the ratio between BCA and CFA in the formulation, the ability of formulations to form a coating on the fruit, and the physical and microbial stability of selected formulations obtained by fluidized-bed drying.

Two of the initial matrices were selected for the purposes of determining if there was a critical ratio of coating forming agents with respect to the concentration of *C. sake* for promoting its viability and efficacy: NaCas, for its especially positive results, and S, for its low price and ready availability. The results showed that the highest polymer:CFU ratios in the formulations exhibited better biocontrol properties, both in population dynamics and efficacy assays. Nevertheless, when the ratios for the two matrices were compared, starch permitted the use of a smaller amount of coating forming solids, which represented an advantage of the final BCP formulation, since a product with a higher cell concentration could be obtained.

Once the CFAs were confirmed to be effective at improving the adherence, survival and efficacy of *C. sake*, the most relevant properties of the initially tested coating forming dispersions (CFDs) were characterized, as well as their functional properties as coatings. In the area of CFAs, especially when they are intended for fruit application, the optical and barrier properties are of particular interest. In order to characterize the coatings in these terms, separated films had to be obtained. For that reason, films based on the

different combinations of hydrocolloids and surfactants were obtained and they were characterized in terms of gloss, water vapor permeability (WVP) and oxygen permeability (OP), among other properties. Films containing *C. sake* were also obtained for the purposes of determining its viability in the different matrices and comparing it with the viability on the grapes surface. Additionally, the films containing the BCA were also characterized and the results were compared with those of the free-cell films, in order to determine if the presence of *C. sake* significantly modified the film functionality. This study gave a general overview of the properties of the different coating dispersions and films, which were extrapolated to the estimated coating applied on grapes.

Firstly, the study of the influence of both the type of polymer type and the incorporation and type of surfactants on the properties of the CFDs and films without BCA incorporation was assessed. It was possible to conclude that the kind of matrix, more than the incorporation or type of surfactant, had a significant influence on the studied properties of both CFDs and films. As far as the most relevant properties of coatings when applied on fruit are concerned, HPMC films were the most effective as water vapor barriers, whereas the starch based coatings showed the lowest oxygen permeability. Protein-based films, had similar barrier properties and were the least efficient barriers water vapor barriers. In terms of gloss, although PP exhibited the highest values, the incorporation of surfactants reduced this attribute in every case.

The adherence of the different CFAs on the grapes was determined and used to predict the thickness of the coatings formed on the fruit surface. In every case, the coatings formed were of approximately 1 µm, with NaCas giving rise to the thickest coating. Taking into account both the films' barrier properties and the estimated thicknesses of the coatings', the oxygen (OTR) and water (WTR) transmission rates of the coatings applied on grapes were predicted. Due to the limited thickness of the coatings, very high values of WTR and OTR were obtained. Thus, it might be concluded that the coatings do not imply serious restrictions for the water vapor and oxygen exchanges of the coated fruit. For a pre-harvest application, which is the objective of this study, this is of interest since it would mean that the coatings would not interfere in the fruit development although they would exert their effect as support for the BCA.

The incorporation of *C. sake* into the film matrices led to different effects, depending on both the properties studied and the type of polymer. Nevertheless, in general terms, the incorporation of *C. sake* into the different films did not imply any notable change. Although in some cases the presence of the cells caused increases in the moisture content of the films, few differences in the barriers properties were observed. As regards the optical properties, the incorporation of the antagonist, in general, caused reductions in the gloss values of the films, consistent with the introduction of surface roughness.

The viability of *C. sake* in the films stored at 25°C and at under 53 or 68% of relative humidity (%RH) was also studied. These storage conditions were selected in order to simulate the possible temperature and RH that could take place in a potential field application. The viability assays were performed at different times: 48 h after preparing the films, in order to estimate whether the drying step of the films had an influence on the survival of the BCA; and after 7, 14 and 21 days of storage under the aforementioned conditions. In every case, *C. sake* was viable after the films were dried. In fact, the counts of viable cells in protein films were higher than what was inoculated (6.6 with respect to the initial 5.4 log CFU/cm^2). These results confirmed the greater suitability of these matrices to act as support of *C. sake*. After 7 and 14 days of storage, a marked reduction in viability of the antagonist was observed in every case, although this decrease was again less pronounced in protein-based films. On the other hand, no viable cells of *C. sake* were found in S films. A considerable influence of the %RH during storage was observed; whilst 53% RH favored the survival in HPMC and NaCas films, 69% RH better preserved the viability of the yeast in PP films. No clear pattern of the role of the surfactants with regard to cell viability was found.

The viability of *C. sake* in the different film formulations was compared to that determined in the coatings applied on the grape surface, obtaining different results. In *in vivo* assays, an increase in the *C. sake* population was observed after storage, suggesting that the fruit support enhances the survival of the yeast, while isolated films represent a worse support. This could be explained by the fact that this yeast is naturally present on the fruit surface. Therefore, when *C. sake* is present in its natural environment and supported in a thin coating, it is better able to survive and multiply.

However, entrapped in the film matrices with thicknesses of about 50 µm, its viability is compromised.

The final point of the study was to obtain a biocontrol product (BCP) based on *C. sake* and coating forming agents for field applications. Of the different kinds of formulations (liquid or solid) and different techniques used for their purposes of obtaining them, the dry formulation obtained by fluidized-bed drying was selected. The longer shelf-life and the easier transport, distribution, storage and manipulation of dry formulations were some of the reasons that influenced this decision. As regards the drying technique, fluidized-bed is considered the least stressful of the dehydration methods, since it involves less extreme water loss and lower temperature gradients.

Before obtaining the final dry formulations based on *C. sake* and coating-forming agents, the drying process was optimized in the Postharvest Pathology Laboratory of IRTA. For that purpose, different drying temperatures and times were tested as well as different combination of CFA-based carriers, binders and protectors. Despite the potential of NaCas, its use was discarded in favor of starch, mainly due to economic aspects. Working with starch, different combinations of starch and starch derivatives were tested and finally potato starch (PS), pre-gelatinized potato starch (PG) and maltodextrins (MD) were the carriers selected. The filmogenic capacity of the different starch derivatives was tested and, although the isolated films formed were brittle and stiff and their manipulation was difficult, their ability to coat was confirmed. Three final BCPs were obtained: F1, based on a combination of PS and PG in a proportion of 2:1; F2, based on the same carriers in a ratio of 1:2 and F3 based on MD. In the case of F3, protectors, such as sucrose and skim milk powder, were also incorporated in order to preserve the cell viability to a greater extent during drying. In all every case, the final *C. sake* concentration was about 10^9 CFU/g dry product.

The characterization of the different formulations was addressed in terms of the physical stability and viability of the BCA, as a function of the a_W (or moisture content) during storage. The BCPs were stored under different a_W conditions (0.11, 0.33, 0.43, 0.54, 0.69, 0.75) at 20°C and their water sorption and water plasticization (glass transition temperatures: T_g) behavior was analyzed in each case. Then, information

about the physical stability of the dry products, both formulations and carriers, was obtained. As concerns the microbiological stability of the BCPs, the viability of *C. sake* in the formulations stored under the a_W range of 0.11 – 0.69 at 20°C was determined for up to 6 storage months. At the same time, the formulations with their initial moisture content (a_w of nearly 0.33) were also stored at refrigeration temperature, in order to discern whether the temperature has an influence on the survival rate of the yeast. Bearing in mind practical considerations, the solubilization rate of the BCPs in water as a function of the temperature (5-25°C) was also studied so as to discover the feasibility of the BCP water dispersion for field applications.

The water sorption behavior of the three formulations was similar and it was observed that cells reduced the water uptake capacity with respect to that the carries at high relative humidities. Through the T_g values, the water plasticization of the different products was studied compared with the carriers. In the case of the carriers, a significant decrease in T_g was observed as the a_W or moisture content increased. However, the presence of *C. sake* cells introduced an unexpected plasticizing effect; at low moisture contents, the T_g was lower than expected while high water contents did not affect the product to the same extent as in the carriers. Thus, the variation range of the T_g values of the products was narrower (45 - 60°C). Nevertheless, in every case, even at the highest a_W (0.75), the obtained T_g values were above room temperature (20°C), hence, the glassy state could be assumed, which means that the physical stability was guaranteed in the tested a_W range.

However, the viability of *C. sake* during storage at 20°C was highly dependent on the water activity of the formulated BCP and the same pattern was observed in all of the formulations. While an $a_W \geq 0.43$ supposed high loses of viability, storage under low a_W conditions (≤ 0.33) better preserved the cell viability. The formulation based on MD at the lowest a_W was the best support for the yeast, since it showed the smallest decline in cell viability (from the initial $1.47 \cdot 10^9$ to $2.36 \cdot 10^6$ CFU/g dry product after 6 months of storage at 20°C). The incorporation of protectants probably contributed to the fact that F3 performed as support for *C. sake*.

The results of the viability study at 20°C revealed that this temperature was not useful to ensure an adequate shelf-life of the *C. sake* formulations, since the maintenance of higher levels of viable cells for longer periods is needed. For that reason, the viability of the yeast in the F2 and F3 formulations with their original moisture content (aw ≈ 0.33), stored at 5°C was determined. Their water activity is within the range which provides more stable products from the point of view of cell viability. Refrigeration temperature was proved as more appropriate for preserving *C. sake* viability in the starch derivative-based formulations. When F3 was stored at 5°C, a reduction in the yeast viability of less than 1 log unit was observed (from an initial $1.7 \cdot 10^9$ to $3.5 \cdot 10^8$ CFU/g dry product) after 6 months of storage.

The maltodextrin based formulation (F3) was also the most water soluble formulation, reaching levels of nearly 100% after 20 min of stirring at 15 and 25°C, whilst F1 and F2 presented a more limited solubilization.

Taking into account the results of the characterization of the different BCPs, their ideal conservation conditions could be defined. Since it has been proven that the water uptake by the products causes important losses in cell viability, the recommended packaging material should have high water vapor barrier properties, in order to prevent the product from moisturizing. Moreover, cold storage must be recommended to ensure a better maintenance of the *C. sake* survival.

Formulations F2 and F3, which showed the best results in terms of the maintenance of cell viability, were selected in order to be applied in practical conditions (field and laboratory applications). The field application was carried out by the IRTA group in a vineyard located in Lleida during the summer of 2015. In that study both the population dynamic of *C. sake* and its efficacy at controlling *B. cinerea* infection were studied.

As regards the application under controlled conditions, 25°C and 68% RH were the conditions selected for the purposes of studying the population dynamics of formulated *C. sake* on table grapes (Moscatel variety). The adherence and survival of the BCA was determined after different storage times (0, 1, 2, 4 and 7 days after application). The visual appearance of the fruit was also examined.

Figure 4.1 shows the population of *C. sake* applied in the form of F2 and F3 products, as compared with the water dispersion of fresh cells (CS control). The initial adherence of *C. sake* was very similar when applied as F3 and CS, whereas with the F2 product, higher initial counts were achieved. This could be due to the greater viscosity of the product which permits greater liquid adherence during spraying. In each case, a progressive increase in the *C. sake* population took place after 1, 2 and 4 days of application. During these periods, the yeast multiplied and established itself on the grape surface. This increase in the BCA count was especially accentuated in the case of F3 formulation, reaching values of about 6.25 log CFU/g (from the initial 5.15 log CFU/g) after 4 days of incubation. These results, suggest that this BCP favors the development and survival of the BCA to a greater extent, confirming the good potential of this support in in dry frormulations of *C. sake*.

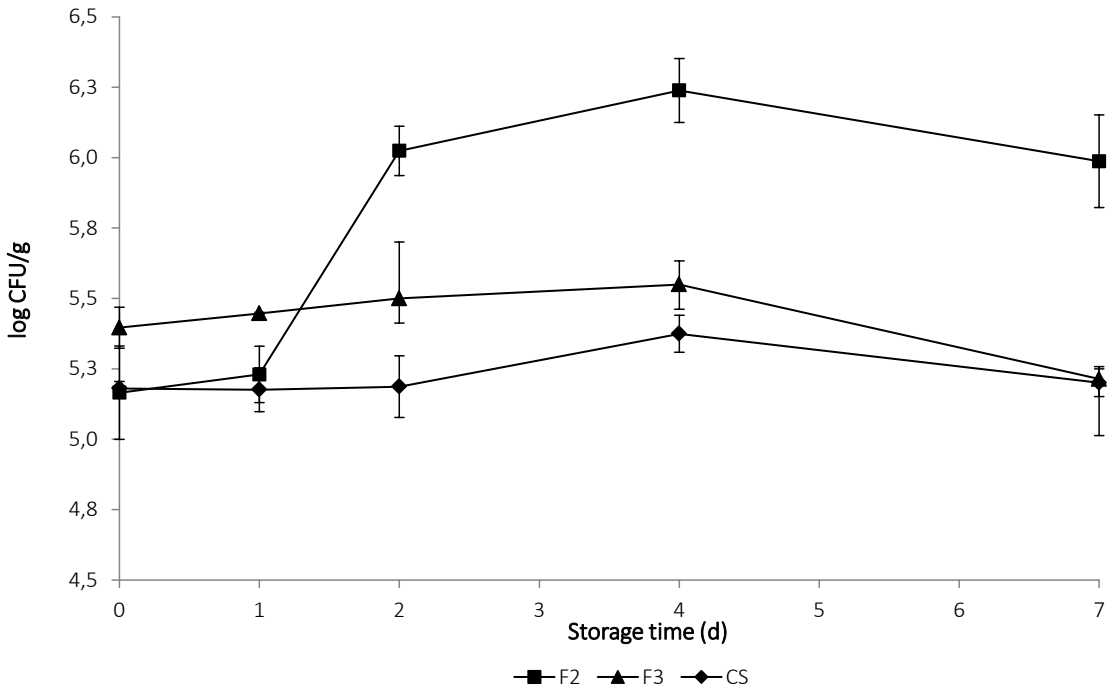


Figure 4.1. Population of *Candida sake* applied on grapes in formulations based on starch derivatives (F2 and F3) and in water (CS) stored at 25°C and 68% RH.

Seven days after application, a decrease in the *C. sake* population was observed in every case, although with F3, the final were still higher than the initial. This reduction in the viability could be attributed to a decrease in the availability of nutrients on the grapes

surface as the yeast multiplied. In this sense, F3 could be considered to be a more nutritious formulation since it better maintained the development of *C. sake*.

Figure 4.2 shows representative images of uncoated grapes, used as control, coated grapes with the F2 and F3 formulations and grapes with *C. sake* applied with water (CS). Compared to the control and CS, the grapes coated with F2 and F3 exhibited a slightly higher gloss, consistent with the coating action, although no marked differences in the fruit appearance were observed. As the incubation time progressed, considerable changes were observed in the control grapes, whose color and turgidity changed after 7 days of storage, depending on to the action of the ripening mechanisms of the fruit. These changes were less marked in the case of the coated grapes. In these cases, the grape texture remained the firmest and the color changed to a slightly lesser extent.

In conclusion, starch derivatives, especially maltodextrins, led to a good dry formulation of the BCA *Candida sake*, with adequate shelf life under cold storage conditions and packaged in water impermeable materials. These carriers favor the establishment and growth of the BCA on the fruit, conditions thus being adequate for preventing the attack by the *Botrytis*. The carrier also acts as a coating on the fruit, which could also help to maintain the fruit quality.

From a practical point of view, the obtaining of these BCPs offers many advantages both to the production sector and to consumers. For producers, the BCA-based products might be a way of reducing both the losses caused by fungal diseases and the presence of pesticide residues on their fruit, thus being able to respond to the increasing demand for chemical-free products by consumers and supply chains. The agrochemical companies could also benefit from the use of the BCPs in several ways. They might represent a viable alternative to the use of chemical products that would permit them to gain access to both the organic fruit and vegetable markets and to integrated production systems, which have shown huge potential in the last few years. Likewise, consumers, as the final recipients of the products treated with the BCPs, will have high-quality and healthy fruit at their disposal, without this implying an increase in price, since the carriers of the microorganism are safe, cheap and widely available.

In terms of legislation, the use of the coating forming agents would not imply any special regulation since all the carriers used are food grade ingredients. However, since Candida sake is not yet registered, it would be necessary to register BCP before commercial use as a required food safety procedure, according to the European Commission (EC) Regulation 1107/2009. This regulation deals, among other things, with the registration for the application of plant protection products and also defines time frames for the registration process.

Taking into account that the low success rate of postharvest biocontrol products has, in part, been attributed to the problems associated with the formulation of the antagonist, the results obtained represent an important advance for the commercial development of BCPs based on *Candida sake*, by considering their good expected stability and feasibility for storage and crop applications. This greatly improves the performance of the biocontrol product when used under commercial conditions.

Figure 4.2. Representative images of uncoated grapes (control), grapes coated with F2 and F3 and grapes with fresh *C. sake* with water (CS) during storage at 25°C and 68% RH.

5. CONCLUSIONS

1. Coating-forming solids improved the survival and efficacy of *C. sake* as BCA of *B. cinerea*, depending on the polymer type and ratio of coating solids. The addition of surfactants did not imply additional positive effects, although they promoted a better cell dispersion onto the grape surface. Nevertheless, cell growth during the incubation time led to the formation of cell aggregates, even when surfactants were added to the formulations. Taking into account the relative increase in the survival and efficacy of *C. sake,* and the cost of ingredients, NaCas or S are recommended to formulate preparations in order to obtain coating-forming systems with this BCA against *B. cinerea* in grapes. The highest polymer:CFU ratios in the formulation exhibited better biocontrol properties and so, this is also recommended. For NaCas, at least 6 mg for $5{\cdot}10^7$ CFU/mL was required to ensure the effective biocontrol of *B. cinerea.* In the case of S, 2.5 mg for 10^7 CFU/ml also led to an improved effective biocontrol.

2. Polysaccharides and proteins, with and without surfactants, can be used as carriers of the biocontrol agent *C. sake* to be applied on fruits, such as grapes, susceptible to attack by *B. cinerea*, in order to control the fungal infection, at the same time as coatings can modulate the exchange of gases and water vapor, without their great transparency introducing any negative effects on the product's appearance. NaCas permits a greater coating capacity, and so a thicker coating. The thickness and barrier properties of the matrices will determine the water and gas transmission rates on the coated product surface, although the very thin layers applied will not seriously affect the fruit's gas exchanges. Likewise, although the yeast's viability was better maintained in the PP films at higher a_W, in NaCas films this took place at lower a_W. Nevertheless, the fruit support affects the resulting cell viability. The formulation of PP-NaCas blend films could be a good strategy with which to prolong yeast viability regardless of the film's a_W, which will depend on the RH of the ambient/environment where the coatings are applied.

3. The viability of *C. sake* during storage at 20°C was highly dependent on the water activity of the formulated BCP. The best preservation was obtained at a_w values below 0.33. This value corresponds to the monolayer moisture content of the products, when water was strongly bonded to the solid matrix, and is their usual

water content after drying. MD was the starch derivative that best supported the yeast in terms of the preservation of cell viability at low a_w. The incorporation of protectants (sucrose and milk powder) could also contribute to the improvement in the functionality of the carrier. This formulation also exhibited the best water solubility, which is a key factor for BCP applications. Nevertheless, 20°C is not low enough to maintain an adequate cell count for prolonged storage times and cold storage would be required to ensure an appropriate BCP shelf-life. Likewise, material with water vapor high barrier properties must be used in the product packaging so as to avoid moisturizing, since water uptake would lead to important significant losses in cell viability. The knowledge acquired in this study provides a basis that might guide both the development of BCPs based on similar antagonists and also the choice of the optimal storage conditions.