



# Effect of different coating-forming agents on the efficacy of the biocontrol agent *Candida sake* CPA-1 for control of *Botrytis cinerea* on grapes



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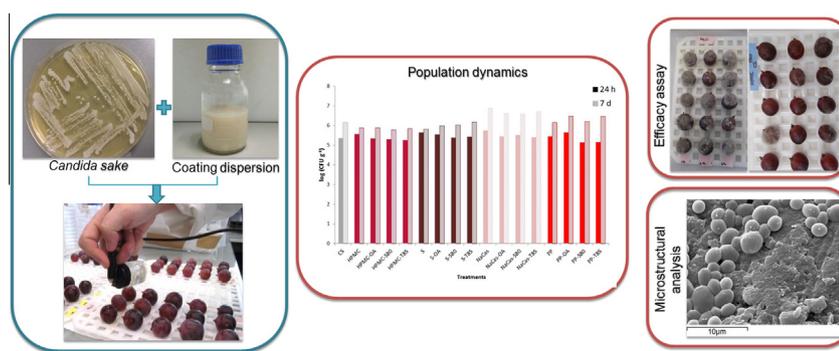
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## HIGHLIGHTS

- Polymer type influenced *Candida sake* survival and efficacy against *Botrytis cinerea*.
- High ratios solids:CFU promoted better biocontrol activity of *C. sake*.
- Surfactants allowed a better cell dispersion on grapes but not additional positive effect.
- Caseinate or starch are the most suitable coatings to obtain formulated biocontrol *C. sake* products.

## GRAPHICAL ABSTRACT



## ARTICLE INFO

### Article history:

Received 10 December 2015

Revised 16 February 2016

Accepted 17 February 2016

Available online 18 February 2016

### Keywords:

Biological control

*Candida sake*

Grapes

Biopolymer

Edible coating

Microstructural analysis

## 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.

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## 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 environ-

mental 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

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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 and 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 conditions associated with the fluctuation of environmental factors in the 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; Perdonés 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 they 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 and 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) (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 and Dimitroglou, 1999; McGuire, 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 and 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 gray mold, different yeasts have also exhibited antagonistic activity against *B. cinerea* (Elmer and 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

**Table 1**  
Treatments based on different edible coatings and *Candida sake* at  $5 \cdot 10^7$  CFU mL<sup>-1</sup> applied on grapes.

Treatment	Treatment description
CS	<i>Candida sake</i> 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)

(NYDA) medium (nutrient broth, 8 g L<sup>-1</sup>; dextrose 10 g L<sup>-1</sup>; agar 15 g L<sup>-1</sup>) 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<sub>2</sub>PO<sub>4</sub> 0.2 M, 70 mL; K<sub>2</sub>HPO<sub>4</sub> 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<sup>-1</sup>; urea 1.2 g L<sup>-1</sup>; water activity  $a_w = 0.996$ ), with adjustment of the initial concentration to  $1 \times 10^6$  CFU mL<sup>-1</sup>. Cell pellets were obtained by centrifugation at 6831g 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<sup>-1</sup> 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, 2013). CFDs were homogenized using a rotor-stator homogenizer (Ultraturrax T25, Janke and Kunkel, Germany) at 13,600 rpm for 4 min and sterilized at 121 °C for 15 min.

After cooling, *C. sake* was incorporated in CFD to a final yeast concentration of  $5 \times 10^7$  CFU mL<sup>-1</sup> (Calvo-Garrido et al., 2013a; Cañamás et al., 2011). The dispersions obtained were shaken for 15 min 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  $5 \times 10^7$  CFU mL<sup>-1</sup>. The seventeen considered treatments are summarized in Table 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  $1 \times 10^4$  conidia mL<sup>-1</sup> (Cañamás et al., 2011).

## 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 s 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 0.5 g L<sup>-1</sup> 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  $1 \times 10^4$  conidia mL<sup>-1</sup> 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 the 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 gray 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  $5 \times 10^7$  CFU mL<sup>-1</sup> 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 solids:BCA on *Candida sake* viability and efficacy

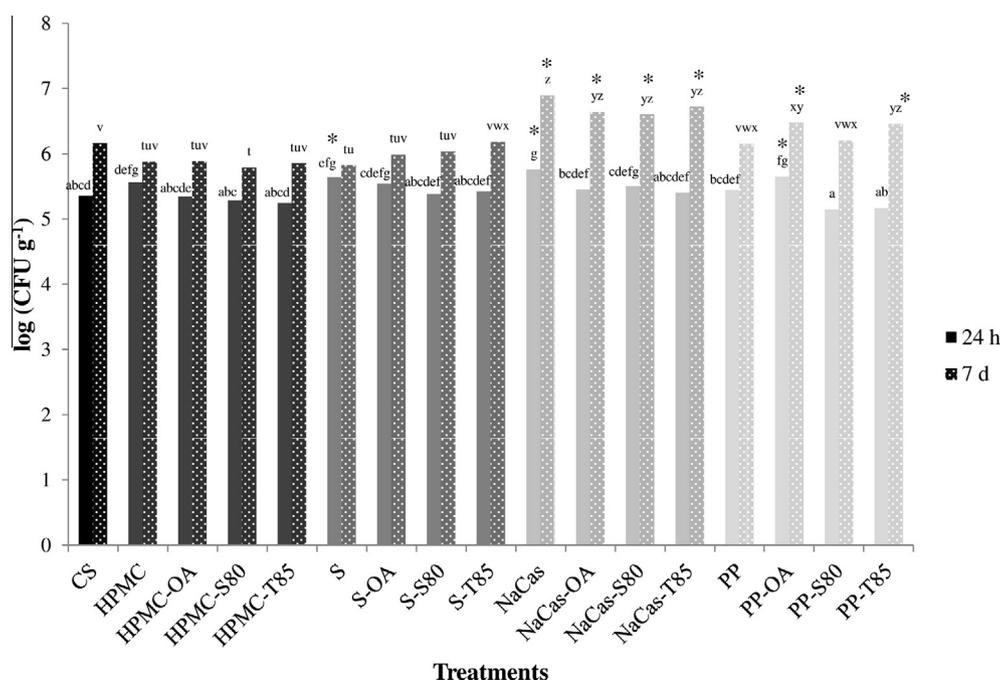
The influence of the ratio of the coating-forming solids with respect to the *C. sake* concentration in the CFDs on the antagonist efficacy was also analyzed for some selected formulations, in order to know if there is a critical ratio for promoting its viability and

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).

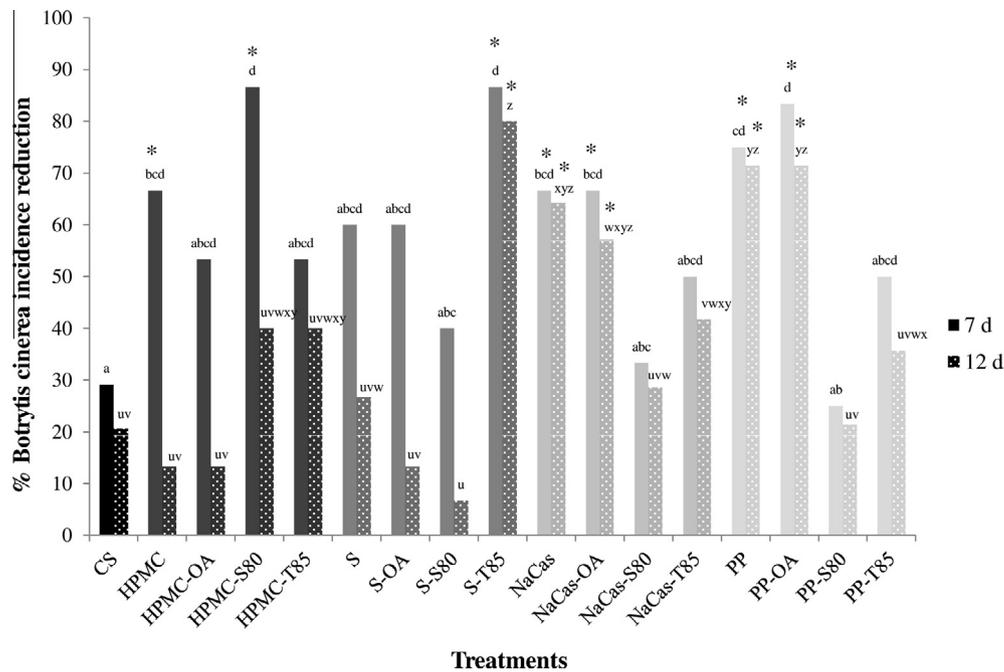
CFDs based on NaCas and S were prepared with different ratios of coating-forming solids maintaining a *C. sake* concentration of  $5 \times 10^7$  CFU mL<sup>-1</sup>. The concentrations of coating solids used were: 25, 12.5, 16.25, 5, 3.75, 2.5 or 1.25 mg mL<sup>-1</sup>. The mass of the hydrocolloid required for each treatment was dispersed in 50 mL deionized water and after its complete dispersion, the CFD was 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 ( $p < 0.05$ ).



**Fig. 1.** Population of *Candida sake* applied with different coating-forming dispersions on grape surface at 24 h and 7 days after application: CS: *C. sake* in water; hydroxypropylmethylcellulose (HPMC), corn starch (S), sodium caseinate (NaCas), pea protein (PP), oleic acid (OA), Span 80 (S80) and Tween 85 (T85). 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.



**Fig. 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. CS: *C. sake* in water; hydroxypropylmethylcellulose (HPMC), corn starch (S), sodium caseinate (NaCas), pea protein (PP), oleic acid (OA), Span 80 (S80) and Tween 85 (T85). 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.

### 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 Fig. 1. 24 h after application, *C. sake* populations on berry surface were between 5 and 6 log CFU g<sup>-1</sup> in every case, as previously reported by Cañamás et al. (2011) in field experiments using *C. sake* 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 and Dimitroglou, 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 and Dimitroglou (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 guilliermondii* and *Debaryomyces* spp.

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<sup>-1</sup>, 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 and 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 Fig. 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 saitoana* with glycol chitosan was more effective

controlling gray mold on apple caused by *B. cinerea* than the independent applications of *C. sakeoana*.

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.

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.

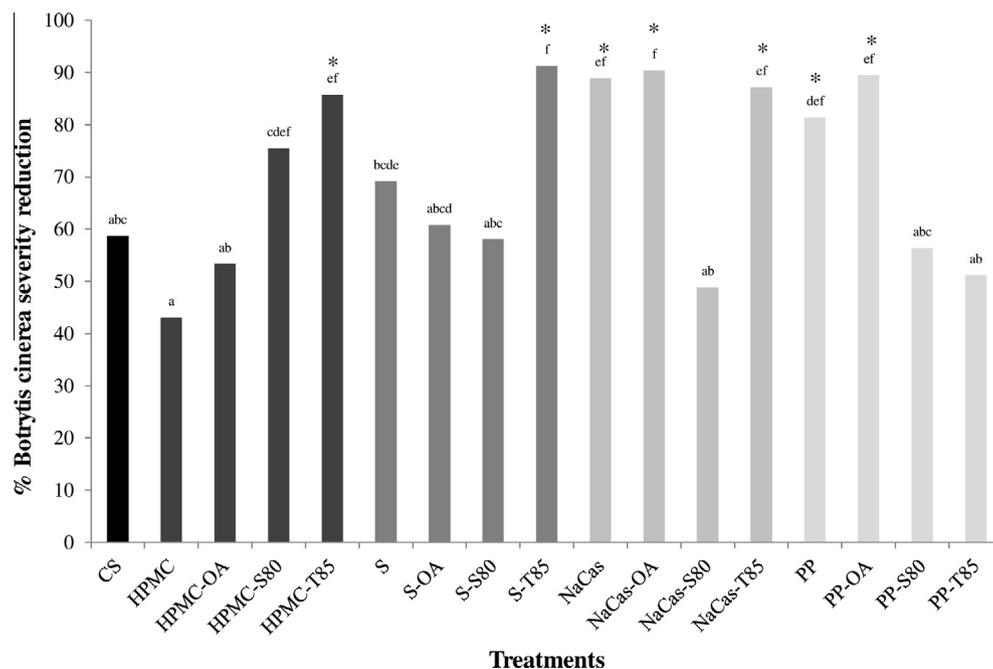
Nevertheless, several authors have found effectiveness against some pathogens of some coatings. For example, Calvo-Garrido et al. (2014a) demonstrated the efficacy of a fatty acid-based product with coating-forming ability against *B. cinerea* by a multiple mode of action. Other studies have reported the use of fatty acid-based products in other fruit crops to act against *B. cinerea* and other fungal pathogens (Hou and 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; Perdonés 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 and Hagenmaier (1996) reported a presumable effect of some compounds of commercial coatings, such as surfactants, on microbial survival, including that of pathogens (see Fig. 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 (24 h 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. Fig. 4 shows representative images of the surface of grapes



**Fig. 3.** Percentage of reduction of *Botrytis cinerea* severity on grape berries by applications of *Candida sake* incorporated in different coating-forming dispersions after 12 days of incubation. CS: *C. sake* in water; hydroxypropylmethylcellulose (HPMC), corn starch (S), sodium caseinate (NaCas), pea protein (PP), oleic acid (OA), Span 80 (S80) and Tween 85 (T85). Different letters in the bars indicate significant differences determined using LSD test ( $p < 0.05$ ) between treatments. \* indicate the treatments that significantly improved the results of CS treatment.

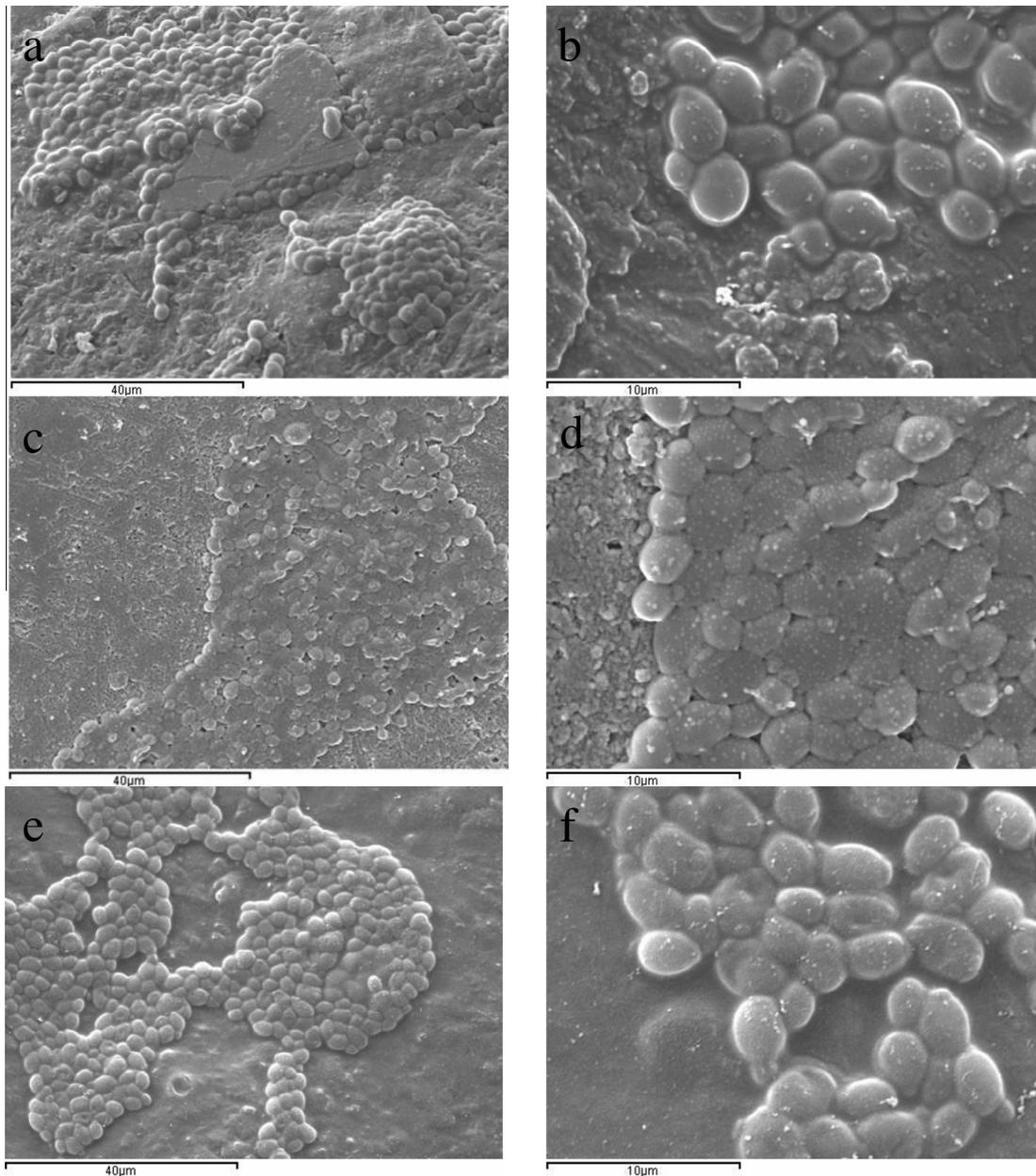


Fig. 4. SEM images of coated grape surface with *Candida sake* formulations: water (a and b); sodium caseinate (NaCas) (c and d); corn starch (S) (e and f).

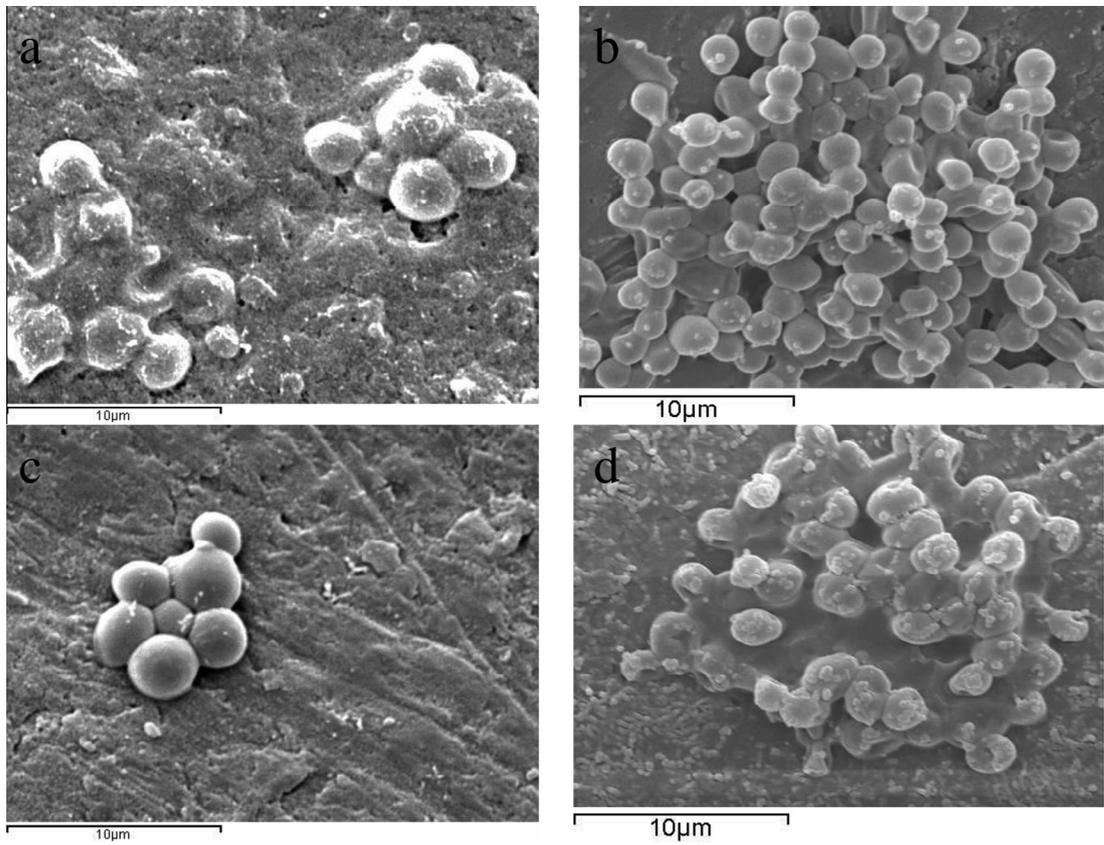
coated with *C. sake* dispersions in water (CS) and in the CFDs based on NaCas and S. The grape surfaces were partially covered by the coatings and the BCA cells were surrounded by a biofilm (Fig. 4b), which was probably excreted for their protection. In grapes with CS, the typical crystalline formations 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 (Fig. 4c and d) exhibited a more granular appearance due to the globular structure of the protein. In contrast, S based coatings (Fig. 4e and f) led to a smoother and more homogeneous surface.

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

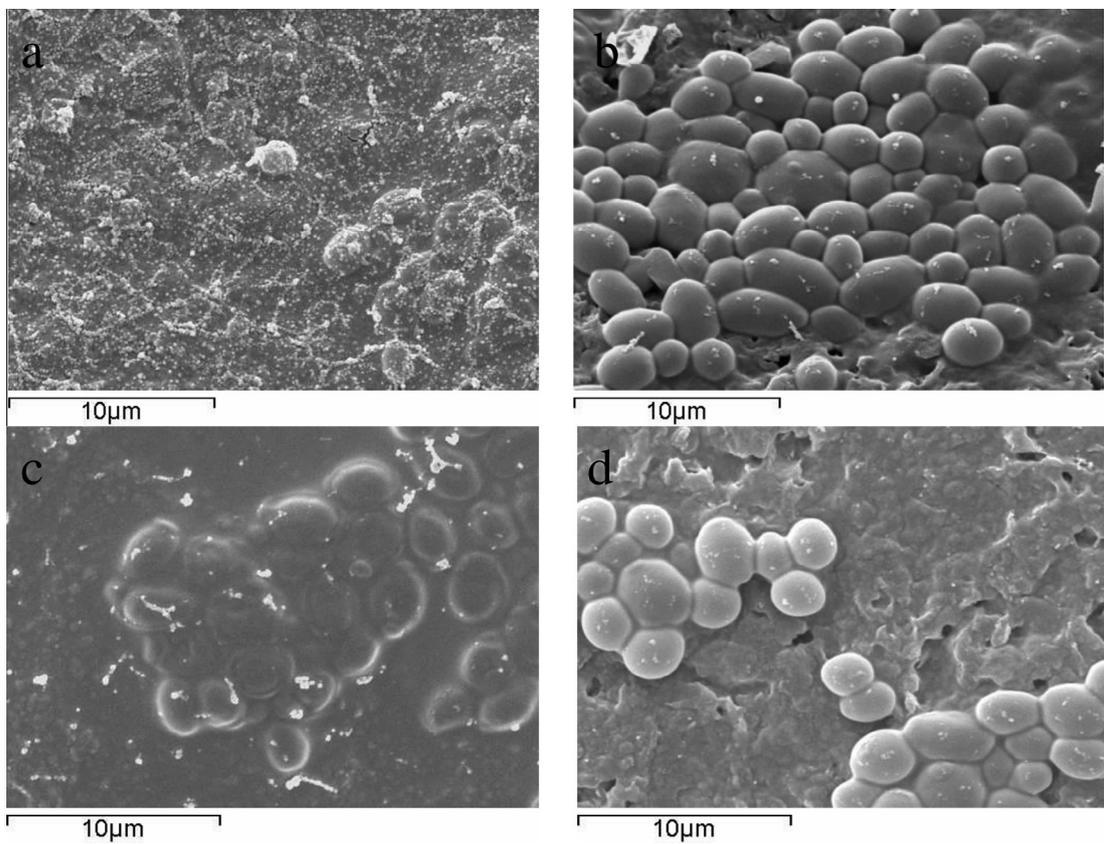
et al., 2012), while it could be also promoted by the loss of water during the coating drying. Biofilm formation includes the bonding of the cells to a solid surface and the presence of an extracellular matrix (Nobile and Mitchell, 2007). Cell aggregates were more extensive and multilayered in water-coated grapes, whereas the presence of coatings resulted in monolayer accumulations.

In grapes treated with NaCas coatings (Fig. 4c and d), 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 (Fig. 4e and f). This could be explained by the ability of the polysaccharide chains to coat the cells and fill the gaps between them.

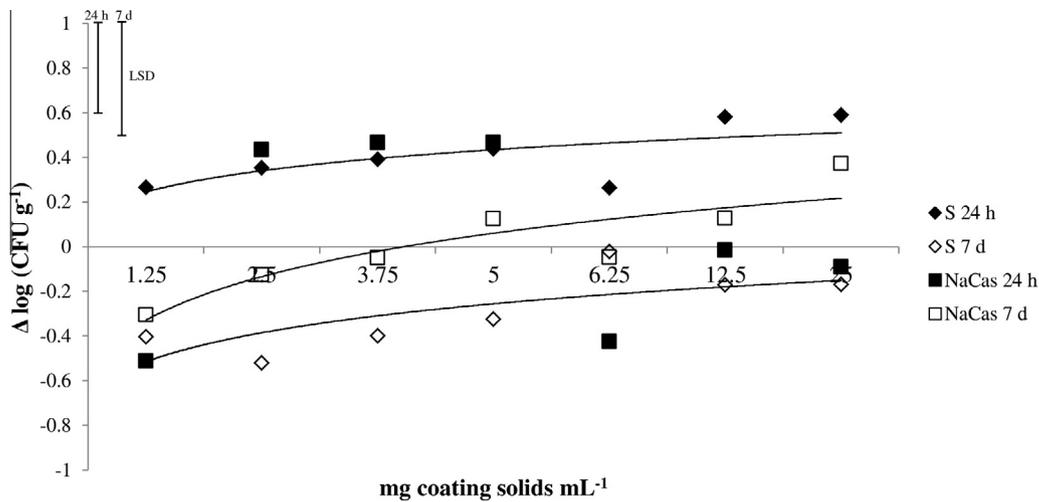
In Figs. 5 and 6, the effect of the incorporation of the surfactant on the formulation of NaCas and S coatings can be observed after 1 and 7 days of application. Surfactants induced a greater disaggregation of cells, which appeared much more dispersed and isolated on the surface as compared to grapes coated with both polymers without surfactants. So, the incorporation of surfactants reduced



**Fig. 5.** SEM images of coated grape surface with *Candida sake* formulations: corn starch (S) with oleic acid (OA) at 24 h and 7 days (a and b); S with Tween 85 (T85) at 24 h and 7 days (c and d).



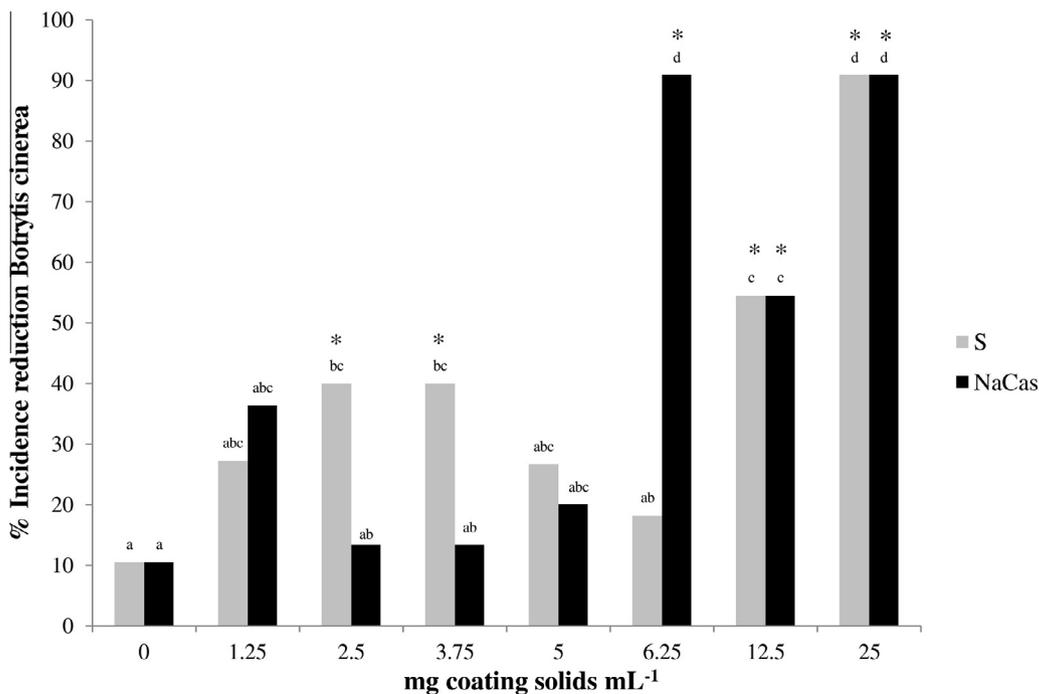
**Fig. 6.** SEM images of coated grape surface with *Candida sake* formulations: sodium caseinate (NaCas) with oleic acid (OA) at 24 h and 7 days (a and b); NaCas with Tween 85 (T85) at 24 h and 7 days (c and d).



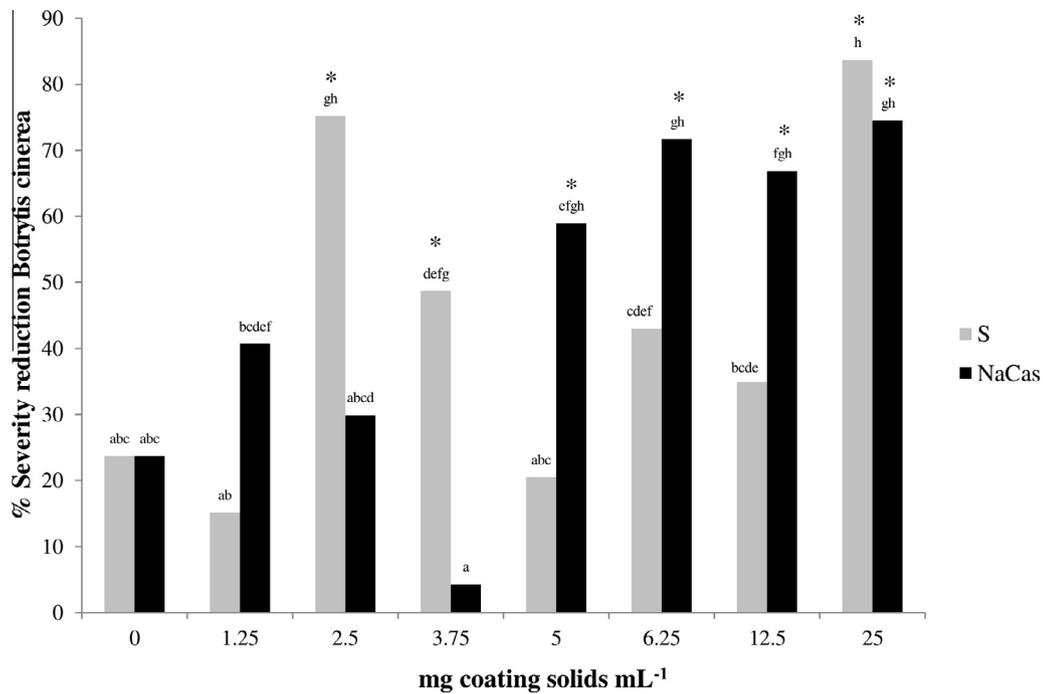
**Fig. 7.** Relative population increase of *Candida sake* with respect to the corresponding control as a function of the amount (mg mL<sup>-1</sup>) of coating-forming solids with respect to the BCA colonies ( $5 \times 10^7$  CFU mL<sup>-1</sup>), for corn starch (S) and sodium caseinate (NaCas) coatings applied on grapes surface after 24 h and 7 days of application (lines: tendencies of  $\Delta\log$  vs. solid concentration). LSD intervals of the controls at 24 h and 7 days.

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 (Fig. 6b and d). In grapes coated with NaCas and surfactants the aggregates showed different layers (Fig. 5b and d). 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.



**Fig. 8.** Percentage of reduction of *Botrytis cinerea* incidence on grape berries by applications of *Candida sake* as a function of the amount (mg mL<sup>-1</sup>) of coating-forming solids with respect to the BCA colonies ( $5 \times 10^7$  CFU mL<sup>-1</sup>) 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.



**Fig. 9.** Percentage of reduction of *Botrytis cinerea* severity on grape berries by applications of *Candida sake* as a function of the amount ( $\text{mg mL}^{-1}$ ) of coating-forming solids with respect to the BCA colonies ( $5 \times 10^7 \text{ CFU mL}^{-1}$ ) 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.

#### 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 ( $\Delta \log \text{ CFU}$ ) with respect to the corresponding control (CS) after the different incubation times (24 h and 7 days; Fig. 7). It is remarkable that the coatings had positive ( $+\Delta \log \text{ CFU}$ ) and negative effects ( $-\Delta \log \text{ CFU}$ ) 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  $5 \text{ mg mL}^{-1}$ ). 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  $\Delta \log$  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  $5 \text{ mg mL}^{-1}$ ) 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  $20 \text{ mg mL}^{-1}$ . 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.

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 (Figs. 8 and 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  $5 \text{ mg mL}^{-1}$  of NaCas or more than  $2.5 \text{ mg mL}^{-1}$  of S (except intermediate values, 5 and  $6.5 \text{ mg mL}^{-1}$  for S, where no significant differences were found). Similarly, the reduction in the severity of the infection (Fig. 9) was significantly higher than that of the CS treatment when the NaCas concentration was higher than  $5 \text{ mg mL}^{-1}$  and when the S concentration was 2.5, 3.75 or  $25 \text{ mg mL}^{-1}$ . 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.

#### 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  $5 \times 10^7$  CFU mL<sup>-1</sup> was required to ensure the effective biocontrol of *B. cinerea*. In the case of S, 2.5 mg for  $5 \times 10^7$  mL<sup>-1</sup> CFU also led to an improved effective biocontrol.

#### Acknowledgments

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.

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