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Starch Derivatives in Coating Formulations as Carriers of a Biocontrol Yeast, *Candida sake*, to Control Fungal Decay on Grapes

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The biocontrol agent (BCA) *Candida sake* CPA-1 has proven to be effective against *Botrytis cinerea*, the fungus causing grey mold in many fruits. The performance of this yeast could be improved if applied in combination with edible coatings. Several combinations of starch derivatives and cell protectants were tested to obtain water-dispersible granular biocontrol products (BCPs) of *C. sake*. Those with better shelf life and stability were: BCP1 based on potato starch and pre-gelatinized potato starch, and BCP2 based on maltodextrins, sucrose, and skim milk powder. BCP1 and BCP2 were applied under controlled conditions on table grapes (Moscatel variety) and the study of the population dynamics of *C. sake* was carried out. Fruit visual appearance and weight loss were also examined. In each case, a progressive increase in the *C. sake* population took place after 1, 2 and 4 days of application, with a significantly greater increase in BCP1 formulations after 4 days. Grapes with both BCP formulations exhibited a slightly higher gloss than the uncoated control, although no marked differences in the fruit appearance were observed. After 7 days, considerable changes were observed in fruit appearance, but these changes were less marked in the case of the coated grapes. The lowest weight loss was observed with BCP2 although few differences were detected between formulations.

The increasing demand for a reduction in the use of potentially harmful agrochemicals in fruit by consumers, together with the development of stricter regulatory policies by authorities, have triggered an increasing interest in the search for alternatives to synthetic pesticides, which includes microbial antagonists (Marín et al., 2017a; Usall et al., 2016). The yeast *Candida sake* CPA-1 has been widely reported as an effective biocontrol agent (BCA) against the fungal pathogen *Botrytis cinerea*, causal agent of grey mold in grapes causing significant crop losses in many temperate regions (Calvo-Garrido et al., 2013; Cañamás et al., 2011; Elmer and Reglinski, 2006). Recently, Marín et al. (2016) demonstrated that edible coatings could be used as carriers of *C. sake* and improved its survival and efficacy against the decay of grapes caused by *B. cinerea*. Specifically, coating formulations based on starch showed remarkable results.

On the other hand, to achieve a successful effectiveness in practical conditions and to be useable by producers, microbial antagonists require a formulation process. The success of formulated biocontrol agent BCA-based products, however, has been limited for diverse reasons such as 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 (Usall et al., 2009). Therefore, an intensive research program has been devoted optimizing formulation processes.

Among the BCA-based formulations, solid biocontrol products (BCP) offer several advantages compared to liquid formulations such as easier storage, transport and quality control, longer shelf-life and lower contamination risks (Cañamás et al., 2008; Fravel, 2005; Fu and Chen, 2011; Usall et al, 2009).

Considering the aforementioned points, water-dispersible granular BCPs of the BCA *C. sake* were developed using different starch derivatives as cell carriers, and made using a fluidized-bed spray-drying technique (Carbó et al., 2017b). After rehydration, these granules can be sprayed on fruit surface and develop a coating, which may act as a support for the cells thanks to the coating-forming ability of starch and its derivatives (Shah et al., 2016). The preservation and shelf life in terms of cell viability and

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antifungal activity against *B. cinerea* of some of the developed formulations were studied, and optimal storage conditions were defined (Carbó et al., 2017a, 2017b; Marín et al., 2017c). Those BCPs showing the most promising properties were selected to be tested in field and controlled conditions.

This research was undertaken to assess the performance of dry formulations based on starch derivatives and containing the yeast *C. sake*, in terms of adherence and survival over time of the BCA on grapes surface during storage under controlled conditions. The effect of the applications on fruit weight and visual appearance was also examined.

Material and Methods

Formulations. Pre-gelatinized potato starch (PG) and native potato starch (PS), mixed in different proportions, or maltodextrins (MD, dextrose equivalent, DE: 12) were used as carriers of the yeast and purchased from Quimidroga. S.A. (Barcelona, Spain). Sucrose and skim milk powder were food grade products used as cells protectants during the drying process. The CPA-1 strain of *C. sake* was isolated from the surface of apples, belongs to the collection of Postharvest Pathology group of IRTA (Lleida, Catalonia, Spain). It was deposited in the Colección Española de Cultivos Tipo, Burjassot, Valencia, Spain (CECT-10817).

Fresh yeast cells and biomass were obtained as described by Carbó et al. (2017b) and the final *C. sake* formulations were obtained with a bottom fluidized-bed spray-dryer (Hüttlin Solidlab 1, Bosch GmbH, Stuttgart, Germany). A cell dispersion was pumped and sprayed on the fluidized carriers at an approximate flow rate of 4 mL/min and 55 °C.

Table 1 shows the composition of the dry formulations and their final yeast concentration, expressed as colony-forming units (CFU)/g dry product. In previous studies, these formulations were physically stable at ambient temperature, showed a shelf-life of up to 12 months when stored at 4 ºC and maintained their biocontrol efficacy (Carbó et al., 2017b; Marín et al., 2017c)

Dispersion of BCPs. Each BCP was weighted and dispersed in deionized sterile water for 1 min, using a vortex shaker, to achieve a final yeast concentration of 2.5×10^7 CFU/mL (Carbó et al., 2017b). As a control, a dispersion of *C. sake* fresh cells in sterilized deionized water (CS) was prepared at the same concentration.

Application of BCPs on grapes. Five-berry clusters of table grapes (*Vitis vinifera* L., Moscatel variety) homogeneous in size and shape were selected on the basis of their maturity stage and without signs of mechanical damage or fungal decay. Three replicates consisting of five clusters of table grapes per treatment and storage time were used for yeast population dynamics and weight loss assessment.

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 sealed plastic boxes for incubation at 25 ºC and 68% relative humidity (RH) for either 0, 1, 2, 4, or 7 d. These conditions were selected to simulate the possible ambient conditions in the field.

Table 1. Composition of the different BCP based on *Candida sake* CPA-1 and starch derivatives as well as yeast concentration expressed as colony-forming units (CFU)/g of dry product.

Formulation	Carrier	Protectant[z]	CFU/g
BCP1	PG:PS[y] (2:1)	—	$1.07 \cdot 10^9$
BCP2	MD	Su:SMP (2:1)	$1.47 \cdot 10^9$

[z]20g·100 g^{-1} carrier.
[y]PG = pre-gelatinized potato starch, PS = native potato starch, MD = maltodextrins, Su = sucrose, SMP = skim milk powder.

Population dynamics of *C. sake* on grapes. To study the population dynamics of *C. sake* over time, each sample was weighed and transferred to an Erlenmeyer flask containing 100 mL of sterile deionized water with 0.01% (w/v) Tween 85. 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 wash water were performed in duplicate and plated onto trypticase soy agar medium with streptomycin sulphate (0.5 g·L^{-1}) (Sigma-Aldrich, Madrid, Spain) to prevent bacterial growth. Plates were incubated for 48 h at 25 ºC and typical *C. sake* colonies were then counted based on their morphological characteristics. Results were expressed as log CFU per gram of treated grape.

Grapes weight loss. Samples were weighted before and after the application of the formulations and control, and after the corresponding storage times. Weight loss was determined following a gravimetric method and expressed as the percentage loss of the initial weigh. Uncoated grapes were used as control.

Visual appearance. Images of the control and treated samples were taken after 1, 2, 4, and 7 d of storage at 25 ºC and 68% RH in order to assess the visual appearance of the fruit.

Statistical analysis. The statistical analyses of the population counts of *C. sake* and the grapes weight loss were performed through an analysis of variance (ANOVA) using Statgraphics Centurion XVI version 16.1.17 (Manugistics Corp., Rockville, MD). The CFU data were log-transformed prior to ANOVA to improve the homogeneity of variances. Significant differences were determined using the least significant difference (LSD) test ($P \leq 0.05$).

Results and Discussion

Fig. 1 shows the population of *C. sake* applied as BCP1 and BCP2, as compared with the water dispersion of fresh cells (CS

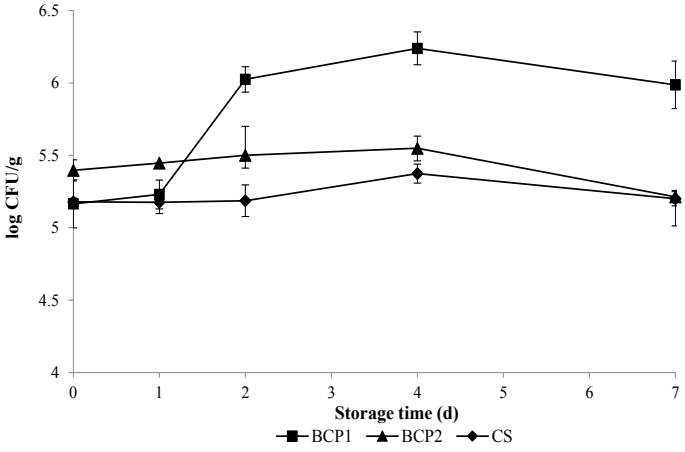

Fig. 1. Population of *Candida sake* applied on grapes in formulations based on starch derivatives (BCP1 and BCP2) and in water (CS) stored at 25 °C and 68% RH. Values are means (with standard deviation bars) of three replicates per treatment and storage time.

control). The BCA initial adherence to fruit surface is a key factor, especially in those antagonists whose mechanism of action is nutrient and space competition, such as *C. sake* (Viñas et al., 1998). A high rate of antagonist survival after the application step is necessary to ensure that there is a high number of CFU available to colonize the fruit surface and perform their antagonistic activity (McGuire and Dimitroglou, 1999). Initial yeast count right after the application of the formulations was about 5.2 log CFU/g for BCP1 and CS, in agreement with previously reported studies (Cañamás et al., 2011; Marín et al., 2016). However, with BCP2, significantly higher initial counts (5.4 log CFU/g) were achieved ($P \leq 0.05$). In all formulations, a progressive increase in the *C. sake* population took place after 1, 2 and 4 d of application when the yeast multiplied and established itself on the grape surface. This increase in the BCA count was especially pronounced in the case of BCP1 formulation, reaching values of about 6.2 log CFU/g (from the initial 5.2 log CFU/g) after 4 d of incubation. Similar results were observed by Carbó et al., 2017b, who reported that the yeast survival with the potato starch formulation was always better than for the maltodextrin based BCP under environmental climate conditions. These results could be due to the greater viscosity of BCP1 dispersion which allows better adherence during spraying.

Seven days after application, a decrease in the *C. sake* population was observed in every case, although with BCP1, the final population was 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, BCP1 could be considered a more nutritious formulation since it better maintained the development of the antagonist.

After 1 and 2 d of storage under controlled conditions, the percentage fruit weight loss varied between 1.1 and 2.5 (Fig. 2). No significant differences ($P > 0.05$) between BCPs and both controls were observed. Weight loss increased 4 d after the application and the differences between BCP formulations and controls were more marked. BCP2 led to significant lower weight loss when compared to BCP1 and the control. The same trend was detected after 7 d of storage, when BCP2 had a significantly lower weight loss (3.1% compared to an average of 4.7% for BCP1, CS, and control). Similar results were reported by Castelo Branco Melo et al. (2018), when antifungal chitosan coatings were applied on table grapes. In all cases, samples weight loss was below 5%, which is the normal acceptable limit for table grapes, according to Deng et al. (2006). However, more marked differences between coated and uncoated samples were expected, suggesting that the coatings formed on fruit surfaces were too thin to effectively control water loss. In a previous work, Marín et al. (2017b) determined the thicknesses of biopolymer-based coatings developed on grapes surface using an air brush and reported that coatings based on corn starch had an average thickness of 0.5 μm, which represented a very thin layer on the fruit surface. In that study, the oxygen and water vapor transmission rates of the coatings formed on fruit surface were also estimated and the high values obtained indicated that the coatings would not imply serious restrictions for the water vapor and oxygen exchanges.

Fig. 3 shows representative images of uncoated grapes, used as a control, coated grapes with the BCP1 and BCP2 formulations and grapes with *C. sake* applied with water (CS). Compared to the control and CS samples, the grapes coated with both BCA formulations 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 significantly changed after 7 d of storage due to fruit ripening. These changes were less marked in the case of coated grapes whose texture remained the firmest (estimated by compressing the berry between index finger and thumb) and the color did not change as much as in the control fruit.

This study showed that starch based formulation coatings made a good support for the BCA *C. sake*, and slightly extended

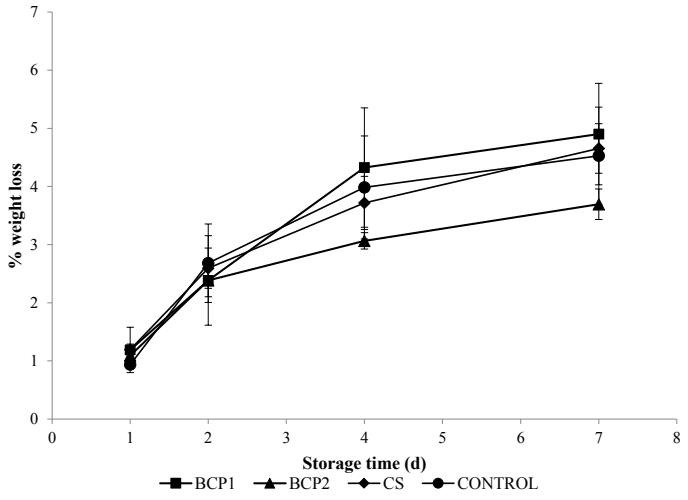

Fig. 2. Weight loss of grapes coated with BCP based on *Candida sake* and starch derivatives (BCP1 and BCP2), *C. sake* fresh cells in water (CS) and uncoated (CONTROL). Values are means (with standard deviation bars) of three replicates per treatment and storage time.

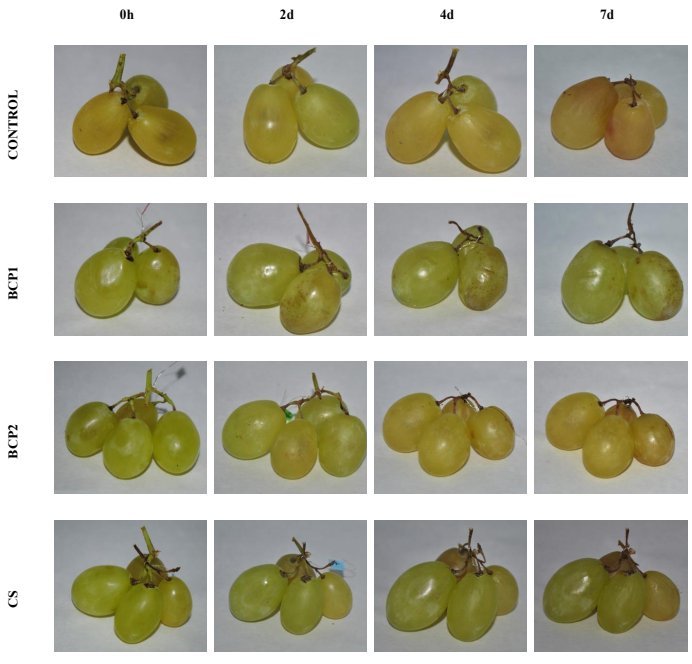

Fig. 3. Representative images of uncoated grapes (control), grapes coated with BCP1 and BCP2 and grapes with fresh *C. sake* in water (CS) throughout storage at 25 °C and 68% RH.

grape shelf-life. BCPs based on starch derivatives were able to improve the survival of *C. sake* on grape surface by providing a physical support and source of nutrients for the yeast. The formed coatings were also able to delay the deterioration of fruit appearance, although they did not effectively control their weight loss. From an economical point of view, the BCPs studied in this work are highly recommendable due to their high availability and the low price of the raw materials. Moreover, as has been previously mentioned, the products are easy to package and to transport thanks to their dry state.

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