Biocontrol products based on *Bacillus amyloliquefaciens* CPA-8 using fluid-bed spray-drying process to control postharvest brown rot in stone fruit

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ABSTRACT

Two products based on the biocontrol agent *Bacillus amyloliquefaciens* CPA-8 have been developed as an effective alternative to chemical applications to control postharvest brown rot in stone fruit. As part of the production and formulation processes, the effects of three different nitrogen sources on growth media and the effects of different carrier materials and protectants on fluid-bed spray-drying were studied. CPA-8 populations achieved $10^9$ CFU mL$^{-1}$ after 72 h of culture. However, the protein PROSTAR 510A at 20 g L$^{-1}$ provided better growth curves compared to the boiled extract from Defatted Soy Flour and protein PROSTAR 510A at 10 g L$^{-1}$. Furthermore, culture ages of 72 h were needed to obtain high endospore production and therefore, suitable heat tolerance of CPA-8. The use of the protectants 20 % sucrose plus 10 % skimmed milk resulted in the best formulations when either carrier material, maltodextrin or potato starch, was used. These two products were then selected for assays of shelf-life and efficacy. CAP-8 viability was unchanged after 15 months of storage at 4 and 22 ºC, maintaining concentrations between $7.8 \cdot 10^9$ and $1.2 \cdot 10^{10}$ CFU g$^{-1}$. Finally, the efficacy of the CPA-8 products against *Monilinia* spp. was confirmed (> 44.4 % disease reduction) on peaches, nectarines, flat peaches, cherries, apricots and plums.

**Keywords**: *Bacillus* spp.; protectants; shelf-life; *Monilinia* spp.; biocontrol efficacy.
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**INTRODUCTION**

Brown rot caused by *Monilinia* spp. is specially responsible for substantial postharvest losses of stone fruit, reaching even as high as 80% of the production in years when the conditions of temperature and wetness are favorable for the development of the disease, especially in late-ripening varieties (Mari et al., 2014; Usall et al., 2015). Traditionally, synthetic fungicides have been used to control postharvest decays; however, the appearance of fungicide-resistant population of pathogens and the concerns of the consumers about the possible toxicological risks of the residues have resulted in the need of developing other methods that involve a reduction in the number of field chemical applications (Droby et al., 2016; Usall et al., 2016a). For this reason, the biological control of postharvest pathogens using microbial antagonists has become in the last decades an effective alternative to reduce or replace the chemicals applied for controlling postharvest diseases of fruit and vegetables (Wisniewski et al., 2016). The efficacy of the biocontrol agent (BCA) *Bacillus amyloliquefaciens* CPA-8, formerly identified as *Bacillus subtilis* (Gotor-Vila et al., 2016), has been previously described against postharvest diseases caused by *Monilinia* spp. based on its capability of production of fengycin-like lipopeptides and emission of volatile organic compounds (Casals et al., 2012; Gotor-Vila et al., 2017a; Yánez-Mendizábal et al., 2012d).

While an abundance of reports exists describing beneficial microorganisms with effective antagonistic activity against postharvest diseases, little success rate of postharvest biocontrol products has been realised (Usall et al., 2016b). For the development of a commercial microorganism-based product, two fundamental steps should be considered: the economical production of large quantities of the microorganism and the development of a formulation strategy that ensures reasonable shelf-life (stored preferentially for 12-24 months at room temperature) and maintains efficacy on a wide range of hosts compared to fresh cells (Droby et al., 2016; Teixidó et al., 2011).

CPA-8 formulated products have been successfully obtained in a liquid state requiring refrigeration (Gotor-Vila et al., 2017b) and dried by different dehydration processes such as spray-drying (Yánez-Mendizábal et al., 2012b), freeze-drying and fluid-bed spray-drying (Gotor-Vila et al., 2017b). The feasibility of the fluid-bed spray-
drying, which operates with a large air volume and lower temperatures than spray-drying, makes this technology an attractive alternative to the traditional drying systems (Srivastava & Mishra, 2010).

Although drying methods are generally more suitable due to the storage capability and transportation, desiccation frequently produces cell damage (Fu & Chen, 2011; Morgan et al., 2006). Nevertheless, to provide a good matrix that allows stability and cell rehydration, substances such as polymers, sugars, milk, and polyols have been tested for their protective effect during drying (Navarta et al., 2011; Strasser et al., 2009). In many cases, such additives were found to be effective toward protection of dried bacteria because they replace structural water in cell membrane after rehydration and prevent unfolding and aggregation of proteins (Stephan et al., 2016).

The genus Bacillus is considered very amenable to drying methods because of its spore-forming ability that provides tolerance against diverse environmental stresses including heat, desiccation, and ionic strength (Nguyen Thi Minh et al., 2011). Thereby, the endospore production is a crucial factor that needs to be considered during the production process of BCAs. These structures confer exceptional ecological advantages and allow long-term storage and relatively easy development of Bacillus-based products (Collins & Jacobsen, 2003).

Although the formulated products could be stored and kept viable after long incubation periods, this does not guarantee that the biocontrol potential of the microorganism would be maintained. The narrow range of activity of many BCAs is a serious limitation to their commercial success. Many postharvest antagonists target a single major pest in a particular host, which generally limits their potential market size. To make development and commercialisation more successful, it would be beneficial to broaden the spectrum of action of these products to different hosts and pathogens (Droby et al., 2016; Glare et al., 2012).

The aim of this study was to develop stable and effective CPA-8-based products. In order to meet the requirements of product quality, we studied the following: (i) different nitrogen sources to improve the low cost medium for CPA-8 production, (ii) the endospore production of CPA-8 under different culture ages, (iii) the effect of
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different protectants and carriers on CPA-8 fluid-bed spray-drying and (iv) the efficacy of CPA-8 products against *Monilinia laxa* and *Monilinia fructicola* on a wide range of stone fruit.

**MATERIALS AND METHODS**

Microorganisms

*B. amyloliquefaciens* CPA-8 was isolated from a nectarine surface and belongs to the Postharvest Pathology Group Collection of IRTA (Lleida, Catalonia, Spain). Bacteria were subcultured on nutrient yeast dextrose agar (NYDA: 8 g L\(^{-1}\) nutrient broth, 5 g L\(^{-1}\) yeast extract, 10 g L\(^{-1}\) dextrose and 20 g L\(^{-1}\) agar) at 30 °C for 24 h when required.

*M. laxa* (CPML2) and *M. fructicola* (CPMC2) were obtained from decayed stone fruit, identified by the Department of Plant Protection of INIA (Madrid, Spain) and belong to the Postharvest Pathology Group Collection of IRTA (Lleida, Catalonia, Spain). Cultures were maintained on potato dextrose agar plates (39 g L\(^{-1}\) PDA, Biokar Diagnostics, France) at 25 ºC for 15 days.

**CPA-8 production**

Culture media optimisation

Low cost media with a different nitrogen source each were used to evaluate the CPA-8 growth in 2 L bioreactors (BioFlo/CelliGen 115, Eppendorf, New Brunswick, Canada). CPA-8 growth was previously described by setting defatted soy flour (DSF) as nitrogen source (Yáñez-Mendizábal *et al.*, 2012c). In the present study, the DSF medium was slightly modified using the soy flour extracted by boiling for 10 min: 100 g L\(^{-1}\) extracted defatted soy flour, 5 g L\(^{-1}\) molasses, 1.9 g L\(^{-1}\) KH\(_2\)PO\(_4\), 0.001 mg L\(^{-1}\) CuSO\(_4\), 0.005 mg L\(^{-1}\) FeCl\(_3\), 6H\(_2\)O, 0.004 mg L\(^{-1}\) Na\(_2\)MoO\(_4\), 0.002 mg L\(^{-1}\) KI, 3.6 mg L\(^{-1}\) MnSO\(_4\) H\(_2\)O, 0.92 g L\(^{-1}\) MgSO\(_4\) 7H\(_2\)O, 0.14 mg L\(^{-1}\) ZnSO\(_4\) 7H\(_2\)O, 0.01 mg L\(^{-1}\) H\(_3\)BO\(_3\) and 10 mg L\(^{-1}\) C\(_6\)H\(_8\)O\(_7\). Two more media were prepared replacing the soy flour by PROSTAR 510A (Brenntag Química, S.A.U., Barcelona, Catalonia, Spain), an isolated soy protein (90 % protein content) prepared at two concentrations (10 and 20 g L\(^{-1}\)). For every growth medium, subsamples were taken periodically and cell concentrations were recorded and presented.
in a growth curve. CFU mL$^{-1}$ were estimated by ten-fold dilutions and plated on NYDA. Each medium was tested in triplicate.

Culture conditions with the optimised medium

Fresh bacteria cultured overnight at 30 °C in NYDA plates and suspended in potassium phosphate buffer (PB, 70 mL KH$_2$PO$_4$ 0.2 mol L$^{-1}$; 30 mL K$_2$HPO$_4$ 0.2 mol L$^{-1}$ and 300 mL deionized water v/v/v pH 6.5) were used to prepare an appropriate volume of inoculum to inoculate 5 L laboratory scale bioreactors (BIOSTAT-A modular fermenters, Braun Biotech International, Melsungen, Germany) or 2 L bioreactors containing the culture medium. The initial concentration was adjusted at 2 $\cdot$ 10$^5$ CFU mL$^{-1}$. CPA-8 cells were grown for 68-72 h at 30 °C. Agitation was set at 200 rev min$^{-1}$ and the air feeding rate was 0.33vvm. Antifoam (30 % Simethicone emulsion USP, Dow Corning®, USA) was added as needed. This procedure was further used in sections 2.3, 2.4, and 2.5 for CPA-8 production.

Heat resistance test of CPA-8 for endospore production

As a crucial factor during the production of BCAs, heat resistance and consequently endospore production of 24-, 48- and 72-h-old CPA-8 cultures was tested. Triplicate samples of 50 mL each of CPA-8 cultures were taken from the bioreactor, centrifuged at 9820 g for 12 min at 10 °C in an Avanti J-20 XP centrifuge (Beckman Coulter, CA, USA) and concentrated 10 times in PB. Then, CPA-8 solutions were incubated at 80 °C for 12 min to kill the vegetative cells as reported Yánez-Mendizábal et al. (2011). The number of viable cells (vegetative cells and endospores) and the surviving cells corresponding to the number of viable CPA-8 endospores were determined by 10-fold dilutions plated on NYDA and incubated at 30 °C for 24 h (CFU mL$^{-1}$). The experiment was repeated twice.

CPA-8 formulation

Fluid-bed spray-drying optimisation process

To obtain fluid-bed spray-dried CPA-8 products, a fluid-bed spray-dryer (HüttlinGmbH, Bosch Packaging Technology Company, Schopfheim, Germany) was used. The bacterial solution was atomised by a 0.8 mm nozzle in bottom-spray position using a peristaltic pump, applying a spraying air pressure of 80 kPa. Each trial was sprayed onto 300 g of powdered carrier material previously loaded into the drying
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camera of the pilot scale fluid-bed dryer. To facilitate the grain formation, 3.5 g of pregelatinised potato starch were added to the cells solution due to its agglomerating function. Inlet air temperature was set to 65 °C which resulted in a maximal product temperature of 42 °C depending on the spraying rate, which ranged between 4 and 4.5 g min\(^{-1}\). The formulation process was performed with cells grown for 68-72 h and harvested by centrifugation at 9820 g for 12 min at 10 °C. The resulting pellet was resuspended approximately at 10\(^{10}\) CFU mL\(^{-1}\) in the same CPA-8 supernatant medium to include the antifungal lipopeptides synthesised by the bacterium during the production process (Yáñez-Mendizábal et al., 2012d). For every trial, 160 g of the suspension were supplemented with different protective substances in order to improve the CPA-8 survival during the drying process and storage. The CPA-8 suspensions with each protective substances were homogenised in a rotary shaker at 150 rev min\(^{-1}\) for 60 min at room temperature before drying. CPA-8 suspensions without additives served as control. Two different carrier materials, maltodextrin and potato starch, were used to obtain different formulations.

In a first approach, maltodextrin was tested with the following protectants: 10 % sucrose, 20 % sucrose, 10 % skimmed milk (SM), 10 % sucrose plus 10 % SM and 20 % sucrose plus 10 % SM. Subsequently, the best protectants obtained were tested using potato starch instead. Additionally, the protectants 5 % and 10 % maltodextrin and 10 % glucose syrup were included.

**CPA-8 survival after formulation**

Three replicate samples (0.5 g) of each dried product were rehydrated with 5 mL of distilled water, shaken vigorously for 1 min and then allowed to rehydrate for other 9 min in static. Ten-fold dilutions of each suspension were made and plated on NYDA to determine the CFU g\(^{-1}\).

The relative cell viability was calculated for each preparation by the difference between the cell concentration after drying and the initial cell concentration. The rate of surviving cells was calculated as follows:

\[
\text{Relative cell viability} = \log_{10} \left( \frac{N_f}{N_i} \right)
\]
Where \( N_i \) represents the CFU in the suspension of CPA-8 before being formulated: population of the suspension (CFU mL\(^{-1}\)) x amount of solution (mL), and \( N_i \) is the CFU of CPA-8 obtained after drying: population of the powder (CFU g\(^{-1}\)) x amount of powder (g).

Moisture content, \( a_w \), and shelf-life of CPA-8 formulations

To calculate the moisture content of the CPA-8 dried products, duplicate samples of 0.5 g each were placed in aluminium-weighing boats and dried in a convection oven at 100 °C for 24 h. The dry matter was calculated based on the weight loss after drying and expressed as relative humidity percentage (% RH). The water availability (\( a_w \)) of each formulation was checked with an Aqualab (Decagon Devices Inc, Pullman, WA, USA) \( a_w \)-meter to an accuracy of ± 0.003. Finally, CPA-8 formulations were stored in 250 mL plastic flasks (leak-resistant wide mouth translucent HDPE bottle and cap, Fisherbrand, Fisher Scientific S.L, Madrid, Spain) at 4 and 22 °C and the shelf-life was determined monthly for 15 months. Three replicate samples (0.5 g) of each dried formulation and temperature were sampled, rehydrated in 5 mL of distilled water and plated on NYDA (CFU g\(^{-1}\)).

Antagonistic activity of CPA-8 formulations against *M. laxa* and *M. fructicola* in stone fruit

Antagonistic activity of CPA-8 formulations to control brown rot caused by *M. laxa* and *M. fructicola* was evaluated in a wide range of stone fruit. Treatments prepared from no stored formulations and from those kept for 10 months at either, 4 or 22 °C were tested in ‘Ruby Rich’ peaches, ‘Noracila’ nectarines, ‘UFO-4’ flat peaches and ‘Early Bigi’ cherries. ‘Fropria’ apricots and ‘Saphire’ plums were used to test formulations stored for 6 months at both temperatures (4 and 22 °C). Efficacy was compared to 72 h-old fresh cells and water as the control treatment (CK).

Fruit with no visible injuries and similar in size and maturity was selected, wounded in the equator with a sterile nail (3 mm wide and 3 mm deep) and then inoculated with 15 μL of a pathogen conidial suspension adjusted at \(10^5\) conidia mL\(^{-1}\). Conidia of each pathogen were transferred to 5 mL of sterile distilled water amended with Tween-80 (one drop per litre). After air-drying, 15 μL of each CPA-8 formulation suspended in distilled water (\(10^7\) CFU mL\(^{-1}\)) were applied. Five fruits constituted a single replicate and each
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treatment was replicated four times. With cherry fruit, there were three replicates with 10
fruits each. The percentage of fruit decayed (disease incidence) and the mean lesion
diameter (cm) of brown rot (disease severity) were determined after 5 or 7 days of storage
at 20 °C and 85 % RH when *M. fructicola* or *M. laxa* were used, respectively. In the case of
cherry fruit, only the disease incidence was evaluated after 7 days of storage.

**Statistical analysis**

Data from CPA-8 grown in different culture conditions and the shelf-life assays of CPA-8
formulated products were log-transformed (log$_{10}$ CFU mL$^{-1}$ and log$_{10}$ CFU g$^{-1}$, respectively)
and plotted in figures where the error was represented by the mean standard deviation (±SD)
of three replications of each sampling data. CFU mL$^{-1}$ of CPA-8 heat resistance tests were also
log-transformed. After formulation trials, the relative cell viability of CPA-8 was estimated on
the basis of CFU counted before (fresh cells) and after being dried (formulated cells). Finally,
for the efficacy evaluation of the CPA-8 formulated products, brown rot incidence and brown
rot severity were analysed. The formulation and efficacy trials were evaluated using analysis
of variance (ANOVA) with the JMP®8 statistical software (SAS Institute, Cary, NC, USA). In
case of no homogeneity of variances, the Wilcoxon test was applied. Statistical significance was
judged at the level *P*<0.05. When the analysis was statistically significant, the Tukey’s HSD
test was used for separation of means.

**RESULTS**

**CPA-8 culture media optimisation**

Growth curves for CPA-8 in low cost media with three different nitrogen sources
are shown in Figure 1. After 6 h, the medium with protein PROSTAR 510A at 20 g L$^{-1}$
provided higher CPA-8 growth (6.3 log CFU mL$^{-1}$) compared to protein PROSTAR 510A
at 10 g L$^{-1}$ and the modified DSF medium (5.7 and 5.6 log CFU mL$^{-1}$, respectively),
indicating a faster exponential growth phase. A decrease was observed after 22-30 h
(time in which maximum CPA-8 growth was observed) when protein PROSTAR 510A
was used at low concentration and it was even more pronounced in the modified DSF
medium curve. However, this effect was not detected when protein PROSTAR 510A
was used at 20 g L$^{-1}$. Although all media provided good CPA-8 growth after 72 h of
culture, achieving stable CPA-8 concentrations around 10$^{9}$ CFU mL$^{-1}$ (8.9-9.0 log
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CFU mL\(^{-1}\)), the medium including protein PROSTAR 510A at 20 g L\(^{-1}\) was selected for CPA-8 production in later trials.

![Figure 1](image.png)

**Figure 1.** CPA-8 growth curves in low cost media using different nitrogen sources: Defatted soy flour (DSF) extract (---), protein PROSTAR 510A at 10 g L\(^{-1}\) (---) and protein PROSTAR 510A at 20 g L\(^{-1}\) (---). Values are the averages of three determinations and bars indicate the standard deviation.

**Heat resistance of CPA-8 by endospore production**

The heat resistance of CPA-8 cells grown in the medium including protein PROSTAR 510A at 20 g L\(^{-1}\) and incubated at 80 °C for 12 min is shown in Figure 2. Although a significant interaction was observed between the two repetitions of the experiment, a clear tendency in CPA-8 endospore production was detected. While the number of viable cells (vegetative cells and endospores) remained practically unchanged after 24, 48, and 72 h of culture (3.9-6.2 \(10^6\) CFU mL\(^{-1}\) and 1.9-3.6 \(10^9\) CFU mL\(^{-1}\) in the first and second repetition, respectively), the number of viable cells previously exposed to heat at 80 °C was higher as the age of the culture increased. Therefore, CPA-8 endospores after 72 h of culture (4.3 \(10^8\) and 4.0 \(10^7\) CFU mL\(^{-1}\) in the first and second repetition, respectively) were higher than earlier times (48 (5.2 \(10^7\) and 7.9 \(10^6\) CFU mL\(^{-1}\)) and 24 h (2.7 \(10^5\) and 1.1 \(10^6\) CFU mL\(^{-1}\)). In the first repetition, the differences between samples...
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exposed or not to heat incubation ranged from 4.3 to 2.0 and 1.0 log units reduction, whereas in the second repetition populations decreased 3.3, 2.7 and 1.8 log units compared to the initial concentration after being cultured 24, 48, and 72 h, respectively.

**Figure 2.** Endospore production of CPA-8 from 24-, 48- and 72-h-old cultures grown in low cost medium using protein PROSTAR 510A at 20 g L\(^{-1}\) as nitrogen source. The figure shows CPA-8 cells (■) and CPA-8 endospores after incubation at 80 °C for 12 min (□). (a) First repetition of the experiment and (b) second repetition of the experiment. Within the same figure, different letters in the same column pattern indicate significant differences (\(P<0.05\)) according to Tukey’s HSD test.

**Fluid-bed spray-drying CPA-8 formulations**

Maltodextrin as carrier material

To obtain good formulated products, the protective ability of sucrose and SM was evaluated and compared against cells formulated without protectants (control) (Fig. 3a). After drying, good survival of CPA-8 was generally observed, obtaining reductions in cell viability lower than 0.4 log unit. Compared to the control, the best formulations obtained included 20 % sucrose plus 10 % SM and 10 % sucrose plus 10 % SM as protectants. Moreover, the formulation with 20 % sucrose plus 10 % SM allowed to obtain products with the highest final concentration (3.4·10\(^9\) CFU g\(^{-1}\)) and large quantity of powder recovered from the dryer (341.1 g) (Table 1).

Potato starch as carrier material

A second formulation approach was conducted using 300 g of potato starch as carrier material instead of maltodextrin. In this case, non-amended CPA-8 cells (control)
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and cells mixed with 20% sucrose plus 10% SM and with 10% sucrose plus 10% SM were formulated. Moreover, two more formulations were obtained using 5% maltodextrin and 10% glucose syrup as protectants. When the concentration of maltodextrin was increased at 10%, the poor solubility triggered clogging the spray nozzle and it was not found appropriate for CPA-8 formulation (data not shown). Regarding the relative cell viability ratio, all protectants provided good CPA-8 survival after drying except 5% maltodextrin, which could not improve the ratio obtained in the control (Fig. 3b). Once more, the yield of the process was considerably improved when the combination 20% sucrose plus 10% SM was used as protectant. In this case, 257.7 g of product with $8.6 \times 10^9$ CFU g$^{-1}$ were obtained (Table 2).

Consequently, CPA-8 formulated products with 20% sucrose plus 10% SM using either, maltodextrin or potato starch as carrier material, were analysed for residual moisture content and $a_w$ and then selected for subsequent assays of shelf-life and biocontrol efficacy.

Figure 3. Survival of CPA-8 cells after fluid-bed spray-drying with different protective substances: non-amended cells (CK, control), 10% sucrose (suc 10), 20% sucrose (suc 20), 10% skimmed milk (SM 10), 10% sucrose plus 10% skimmed milk (suc 10-SM 10), 20% sucrose plus 10% skimmed milk (suc 20-SM 10), 5% maltodextrin (MD 5), and 10% glucose syrup (syrup 10). (a) CPA-8 formulations by using maltodextrin as carrier material and (b) CPA-8 formulations by using potato starch as carrier material. For each figure, different letters indicate significant differences ($P<0.05$) according to Tukey’s HSD test.
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Table 1. Powder recovery (g of dried product) and final concentration (CFU g⁻¹ and total CFU) of the resulting formulations of CPA-8 after fluid-bed spray-drying using maltodextrin as carrier material.

<table>
<thead>
<tr>
<th>Protectant</th>
<th>Powder recovered (g)</th>
<th>Concentration (CFU g⁻¹)</th>
<th>CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ck, control without protectants</td>
<td>314.1</td>
<td>1.4·10⁹</td>
<td>4.3·10¹¹</td>
</tr>
<tr>
<td>10 % sucrose</td>
<td>315.0</td>
<td>2.3·10⁹</td>
<td>7.3·10¹¹</td>
</tr>
<tr>
<td>20 % sucrose</td>
<td>331.0</td>
<td>1.6·10⁹</td>
<td>5.3·10¹¹</td>
</tr>
<tr>
<td>10 % skimmed milk (SM)</td>
<td>314.0</td>
<td>1.0·10⁹</td>
<td>4.8·10¹¹</td>
</tr>
<tr>
<td>10 % sucrose – 10 % SM</td>
<td>330.2</td>
<td>2.3·10⁹</td>
<td>7.6·10¹¹</td>
</tr>
<tr>
<td>20 % sucrose – 10 % SM</td>
<td>341.1</td>
<td>3.4·10⁹</td>
<td>1.2·10¹²</td>
</tr>
</tbody>
</table>

Table 2. Powder recovery (g of dried product) and final concentration (CFU g⁻¹ and total CFU) of the resulting formulations of CPA-8 after fluid-bed spray-drying using potato starch as carrier material.

<table>
<thead>
<tr>
<th>Protectant</th>
<th>Powder recovered (g)</th>
<th>Concentration (CFU g⁻¹)</th>
<th>CFU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ck, control without protectants</td>
<td>205.3</td>
<td>6.8·10⁹</td>
<td>1.4·10¹²</td>
</tr>
<tr>
<td>10 % sucrose – 10 % SM</td>
<td>236.3</td>
<td>8.7·10⁹</td>
<td>2.0·10¹²</td>
</tr>
<tr>
<td>20 % sucrose – 10 % SM</td>
<td>257.7</td>
<td>8.6·10⁹</td>
<td>2.2·10¹²</td>
</tr>
<tr>
<td>5 % maltodextrin</td>
<td>227.1</td>
<td>3.3·10⁹</td>
<td>7.4·10¹¹</td>
</tr>
<tr>
<td>10 % glucose syrup</td>
<td>235.8</td>
<td>3.1·10⁹</td>
<td>7.3·10¹¹</td>
</tr>
</tbody>
</table>

Residual moisture content, \(a_w\), and shelf-life evaluation

The residual moisture content and the \(a_w\) values for 20 % sucrose plus 10 % SM CPA-8-based products formulated with either, maltodextrin or potato starch, were in the range between 7.0-9.8 % and 0.33- 0.36, respectively (Table 3). The viability of CPA-8 in each formulation at different shelf-life periods and storage temperatures was also studied (Fig 4). In general, the viabilities were unchanged during 15 months of storage regardless of the temperature; after this period, cell concentrations between 7.8·10⁹ and 1.2·10¹⁰ CFU g⁻¹ were maintained.

Table 3. Average residual moisture contents (% RH) and water availability (water activity, \(a_w\)), of the most suitable CPA-8 formulations after fluid-bed spray-drying.

<table>
<thead>
<tr>
<th>Carrier</th>
<th>Protectant</th>
<th>% RH</th>
<th>(a_w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltodextrin</td>
<td>20 % sucrose – 10 % skimmed milk (SM)</td>
<td>7.0</td>
<td>0.33</td>
</tr>
<tr>
<td>Potato starch</td>
<td>20 % sucrose – 10 % SM</td>
<td>9.8</td>
<td>0.36</td>
</tr>
</tbody>
</table>
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Figure 4. Shelf life of the most suitable CPA-8 products after long-term storage at different temperatures: 20 % sucrose plus 10 % skimmed milk CPA-8 formulations with maltodextrin as carrier material stored at 4 °C (---) and 22 °C (-----) and 20 % sucrose plus 10 % skimmed milk CPA-8 formulations with potato starch as carrier material stored at 4 °C (-----) and 22 °C (-----). Values are the averages of three determinations and bars indicate the standard deviations.

Biocontrol efficacy assay of CPA-8 formulations

Fluid-bed spray-dried CPA-8 products were effective in controlling brown rot caused by *M. laxa* and *M. fructicola* in artificially inoculated peaches, nectarines, flat peaches, and cherries regardless of the age of the formulation and temperature of storage (Fig. 5). The formulations were as effective as fresh cells, exhibiting disease incidence from 35 to 0 % and from 50 to 0 % for *M. laxa* and *M. fructicola*, respectively, compared to values generally higher than 80 % in the untreated controls (Fig. 5a-d). In flat peaches inoculated with *M. laxa*, the disease incidence in the control was low (53.3 %); however, it was noticeably different compared to the treatments (0-10 %) (Fig. 5c). Regarding the disease severity for both pathogens, *M. laxa* and *M. fructicola*, the decayed fruit in the untreated control showed mean lesion diameters from 4.4 to 2.1 cm, which were much bigger than those obtained in fruit treated with CPA-8 formulations (1.5-0 cm) (Fig. 5a-c). When compared to the control, all the formulations applied revealed disease percentage reductions ranging from 44.4 to 100 % in disease
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incidence and from 46.2 to 100 % in disease severity. In general, higher percentages of
decayed fruit and bigger lesion diameters were observed in fruit inoculated with
M. fructicola than with M. laxa. The efficacy of CPA-8 formulations stored for 6 months
against M. laxa and M. fructicola was also tested on artificially inoculated apricots and
plums. The percentage of disease reduction compared to the control ranged from 45.0 to
95.0 % and from 45.5 to 100 % regarding disease incidence and severity, respectively,
except in apricots treated with CPA-8 formulated with maltodextrin (in which no more
than 25 % reduction of disease incidence could be observed). These data indicate that
drying and storage conditions (at low or ambient temperature) did not have any negative
effect on the biocontrol efficacy of the CPA-8-based products.
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Figure 5. Antagonistic activity of CPA-8 formulations and fresh cells against artificial infection with *M. laxa* and *M. fructicola* on peaches (a), nectarines (b), flat peaches (c) and cherries (d). The percentage of fruit decayed (disease incidence) and the mean lesion diameter (cm) of brown rot (disease severity) were determined after 5 or 7 days of storage at 20 °C and 85 % RH when *M. fructicola* or *M. laxa* were used, respectively. In the case of cherry fruit, only the disease incidence was evaluated after 7 days of storage. Within the same figure, different letters indicate significant differences (*P*<0.05) according to Tukey’s HSD test. Uppercases and bars refer to disease incidence (%) and lowercases and diamonds refer to disease severity (cm). The treatments tested were: CK (control, without CPA-8); 72 h-old CPA-8 fresh cells; CPA-8 fluid-bed spray-dried products stored for 10 months (10 m) at 4 and 22 °C and non-stored CPA-8 fluid-bed spray dried products (0 h). The most suitable CPA-8 formulations were used: CPA-8 cells mixed with 20 % sucrose plus 10 % skimmed milk, using maltodextrin (MD suc20-SM10) or potato starch (Starch suc20-SM10) as carrier materials.
DISCUSSION

In this work, two optimised CPA-8 products with long-term shelf-life and effective against brown rot caused by *M. laxa* and *M. fructicola* on a wide range of stone fruit have been developed. These biocontrol products are particularly suitable for further applications in commercial trials under field conditions.

Economical production of microorganisms in a culture medium requires intensive investigation to determine the optimum conditions that ensure a large, stable, and effective microbial population prior to the formulation process (Hynes & Boyetchko, 2006). The work conducted by Yánez-Mendizábal et al. (2012c) showed that the use of commercial products and by-products such as DSF in combination with molasses and mineral trace supplements provided high CPA-8 growth while maintaining its biocontrol efficacy. However, the use of crude soy proved to be a source of contamination due to its high microbial load. Furthermore, its low solubility during the medium preparation was a major drawback in the production and scaling-up process. To solve these hurdles, three different nitrogen sources were tested. After 22-30 h of fermentation, a slight decrease in CPA-8 growth was observed when the BCA was grown in media using the boiled extract from DSF and protein PROSTAR 510A at 10 g L$^{-1}$. This effect could be due to the endospores production, which occurs in high cell density populations with stressful conditions (during the stationary growth phase when nutrients are exhausted). This stress probably mean the moment in which CPA-8 derives its metabolic energy to produce endospores rather than keep growing (Posada-Uribe et al., 2015). Nevertheless, the results revealed better CPA-8 growth curves when the isolated soy protein PROSTAR 510A was used at 20 g L$^{-1}$. Although maximum CPA-8 growth was obtained when the concentration of protein PROSTAR 510A was high (20 g L$^{-1}$), the low cost of this substrate allows its use without reducing its suitability for a commercial production. Moreover, the medium preparation is not time consuming as there is no need in boiling previously the flour.

Similarly to the work conducted by Yánez-Mendizábal et al. (2012a), high CPA-8 endospore production ($4.3 \times 10^8$ CFU mL$^{-1}$) was obtained with the new optimised medium based on protein PROSTAR 510A. After heat incubation, CPA-8 cells (and consequently, CPA-8 endospores) significantly increased from 24 to 72 h, suggesting
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that the ability of CPA-8 to survive the drying process greatly depends on the age of the culture. These results indicate that, similar to many other Bacillus species, CPA-8 produced endospores as a means of surviving temperatures up to 80 °C (Baril et al., 2012; Nguyen Thi Minh et al., 2011). This ability is important for the formulation of the BCAs, especially in drying systems that use high temperatures.

In previous works, CPA-8 has been successfully formulated by using both, liquid and dried forms (Gotor-Vila et al., 2017b; Yánez-Mendizábal et al. 2012b). Among the drying technologies used, fluid-bed spray-drying resulted the most suitable one (Gotor-Vila et al., 2017b), obtaining larger quantities of the product with lower operating costs and shorter process times. Once the best drying process was defined for CPA-8 formulation, different processing parameters to obtain high performance under commercial conditions need to be optimised. In the current work, we focussed our attention on the effect of protectants and carrier materials.

Many authors have reported the protective efficiency of certain substances in response to osmotic and ionic stress that desiccation causes on cells (Abadias et al., 2001; Costa et al., 2000). After fluid-bed spray-drying, the combined use of 20 % sucrose plus 10 % SM resulted in the best CPA-8 formulations when either, maltodextrin or potato starch, was used as carrier. The damage caused by the drying process may be reduced by incubating bacterial cells with some carbohydrates as their presence during desiccation resulted in higher survival rates than those of non-treated cells (Strasser et al., 2009). Several physical principles such as the ability of sucrose to stabilise cell membranes have been underlined as a mechanism of cells stabilisation in the dry state (Fu & Chen, 2011; Morgan et al., 2006). The use of SM at concentrations of 1-10 % has often been used for cell’s viability preservation during drying, but even more frequently in mixtures with other protectants (Navarta et al., 2011; Santivarangkna et al., 2007). Moreover, the protective effect of SM during the cell rehydration process has been considered very amenable (Abadias et al., 2001; Costa et al., 2000). The appropriate water binding capacities of the non-reducing disaccharide sucrose also enabled residual moisture and \( a_w \) contents that are particularly favourable for survival and storability of CPA-8. Moisture content in the dry product plays a key role in the maintenance of cell viability, suggesting that high levels tend to decrease the cell survival during the storage period (Fu & Chen, 2011). In this work, products with moisture contents lower than 10 % and \( a_w \) values between 0.33-
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0.36 enabled good final concentrations (7.8·10⁹-1.2·10¹⁰ CFU g⁻¹), extending the viability of CPA-8 for up to 15 months at both temperatures of storage studied, 4 and 22 ºC.

In the fluidised-bed process, liquid mixtures of cells and protectants are deposited on a solid substance and subsequently dried resulting in homogeneous powders with uniform thickness that exerts specific functions desired in the final products (Srivastava & Mishra, 2010). The spherical carrier material is loaded into the chamber of the dryer and then fluidised by the air flow applied. In this work, two different carriers were tested in order to obtain formulated products with different flowability and dispersability. The polysaccharides maltodextrin and potato starch were chosen for enhancing powder recovery with no agglomerates and also for being low-cost commercial products. These two materials differ in the Dextrose Equivalent (DE) measure, which indicates the degree of polymerisation from starch to sugars. Maltodextrin is typically composed of an amount of reducing sugars between 3-20 % (whereas starch is close to zero) resulting in products with higher solubility (Shamekh *et al.*, 2002). Moreover, maltodextrin also shows larger size of particles that avoid losses during drying and thus, major quantities of dried product were recovered improving the yield of the process.

Although these two formulations provide good quality requirements, it is noteworthy that biocontrol efficacy needs to be retained as one of the most important requisites for commercial purposes. Based on this, our results confirmed the biocontrol activity of CPA-8 formulations to reduce brown rot caused by *M. laxa* and *M. fructicola* in different stone fruit such as peaches, nectarines, flat peaches, cherries, apricots, and plums. These results provided a broad host range effect, suggesting an attractive opportunity for marketing biopesticides as products with an added value (Glare *et al.*, 2012; Usall *et al.*, 2016b). The biocontrol ability of this bacterium was previously reported by Casals *et al.* (2012) and by Yánez-Mendizábal *et al.* (2011) not only against *Monilinia* spp. in stone fruit (‘Baby Gold 9’ peaches, ‘Andros’ peaches and ‘Big Top’ nectarines) but also against *Botrytis cinerea* in ‘Golden Delicious’ apples, demonstrating the capability of this BCA to control different postharvest pathogens. Additionally, this study demonstrated the long-term storability (even at room temperature) of the two CPA-8-based products developed, which did not show any negative effect in the biocontrol efficacy of CPA-8, providing suitable product delivery.
In this study, the complete development of two effective biocontrol products has been optimised. An easily handling and low cost growth medium for CPA-8, which ensures an efficient endospore production and therefore heat tolerance during the bacteria drying process, has been described. This work also proved that the use of maltodextrin or potato starch as carriers combined with 20 % sucrose plus 10 % SM as protectants is a good tool for reaching an adequate stability and efficacy of CPA-8 formulated products. These results supported the suitability of the fluid-bed spray-drying technology as a promising way to preserve the BCA CPA-8 and consequently, other microorganism-based products. Further commercial trials with these two effective products are now the next research step. In conclusion, this work demonstrates that products based on biological formulations of \textit{B. amyloliquefaciens} CPA-8 could be a suitable approach to the management of postharvest brown rot control.

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