Chapter IV

Formulation of the biocontrol agent Bacillus amyloliquefaciens CPA-8 using different approaches: liquid, freeze-drying and fluid- bed spray-drying

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ABSTRACT

The present work focuses on the assessment and comparison of three different formulation technologies and the effect of protectants on cell viability, storage stability, and antagonistic activity of the biocontrol agent *Bacillus amyloliquefaciens* CPA-8. Cultures were concentrated with different protective substances such as $MgSO_4$, sucrose, and skimmed milk (SM) and subjected to liquid formulation, freeze-drying, and fluid-bed spray-drying. Results showed that CPA-8 freeze-dried cells without protectants or amended with SM suffered the highest losses in cell viability (0.41-0.48 log). Moreover, the cell viability of the tested freeze-dried products decreased after four months of storage at both tested temperatures (4 and 22 °C). Otherwise, liquid and fluid-bed spray-dried products were stable for four months at 4 °C and for twelve months at 22, 4 and -20 °C, respectively, and no effect of the protectants was observed. The most suitable CPA-8 products were then tested against *Monilinia laxa* and *Monilinia fructicola* on artificially wounded nectarines and in all cases, the antagonistic activity was maintained similar to fresh cells. The efficacy results revealed that the formulation process did not affect the biocontrol potential of CPA-8. This work led us to conclude that effective formulations with final concentrations ranging from $1.93 \cdot 10^9$ to $2.98 \cdot 10^9$ CFU mL^{-1} and from $4.76 \cdot 10^9$ to $1.03 \cdot 10^{10}$ CFU g^{-1} were obtained for liquid and dried products, respectively. Additionally, the suitability of the fluid-bed spray-drying technology should be taken into account to develop a stable and effective CPA-8 product for practical applications to control brown rot in stone fruit.

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

INTRODUCTION

Postharvest decays of fruit have been traditionally controlled by synthetic pesticides. However, the appearance of pathogen resistance to these compounds and the public concerns about environmental contamination and human health have revealed the need to develop other methods that would help reduce field chemical applications (Droby *et al.*, 2009; Janisiewicz & Korsten, 2002; Sharma *et al.*, 2009).

Postharvest losses can reach high values, reaching up to 50 % of the total fruit production (Nunes, 2012). Brown rot caused by the wound-invading fungus *Monilinia* spp. is one of the most important postharvest diseases affecting stone fruit (Larena *et al.*, 2005; Mari *et al.*, 2007). The application of environment-friendly approaches such as those using biological control agents (BCAs), either alone or in combination with physico-chemical treatments, has been strongly considered in recent years (Casals *et al.*, 2012; Casals *et al.*, 2010; Mari *et al.*, 2007; Usall *et al.*, 2015).

The spore-forming ability of *Bacillus* species provides high resistance to extreme environmental conditions, making this genus a good candidate for developing stable and efficient biocontrol products (Mari et al. 2014). The efficacy of the BCA *Bacillus amyloliquefaciens* CPA-8, formerly known as *B. subtilis* (Gotor-Vila *et al.*, 2016), has been previously described against postharvest diseases caused by *Monilinia* spp. based on its ability to produce fengycin-like lipopeptides (Yánez-Mendizábal *et al.*, 2012a; Yánez-Mendizábal *et al.*, 2012d)

One of the major bottle necks in the commercialisation of biocontrol products is the development of shelf-stable formulations (Droby *et al.*, 2009; Navarta *et al.*, 2011). A useful microbial formulation should be economical to produce, easy to distribute to the intended environment, contain enough colony forming units (CFU), and provide a long shelf-life (preferentially stored at room temperatures and maintained for at least six months) (Melin *et al.*, 2007; Teixidó *et al.*, 2011). Finally, biological formulations should maintain the efficacy against plant pathogens similar to fresh cells.

Formulations can be accomplished by different methods including liquid and dry preparations. Compared to liquid forms, dried products obtained by spray-drying,

drying with fluidised-bed dryers or freeze and vacuum-drying are more feasible due to the storage capability, transportation, and their ability to produce large amounts of dried products at low cost. However, dried products frequently show low viability rates because of the thermal and dehydration stress suffered during the drying process (Abadias *et al.*, 2005; Melin *et al.*, 2007). Nevertheless, the microbial cell viability and efficacy resulting after the formulation process can be improved by the addition of certain stabilising substances (e.g., polymers, sugars, albumin, milk, salts, honey, polyols or aminoacids) to the formulation medium. Components of the formulation media have two main functions in preserving the viability of formulated cells: to provide a dry residue and thus acting as a receptor in the rehydration process and also to protect biochemically the cells against damage during the drying process (Abadias *et al.*, 2001; Melin *et al.*, 2011; Sabuquillo *et al.*, 2010).

The genus *Bacillus* is considered very amenable to drying because of its capability of spore production, which provides heat tolerance (Nicholson *et al.*, 2000; Yánez-Mendizábal *et al.*, 2012a). The study of spray-drying effect on *Bacillus* strain CPA-8 has been recently reported and spray-dried CPA-8 formulations have been obtained with final concentrations around 1.6–3.3 $\cdot 10^9$ CFU g^{-1} and 28-38 % of powder recovery using a pilot spray-dryer without preconditioning (Yánez-Mendizábal *et al.*, 2012b). The study of alternative formulation techniques, liquid or solid, amended or not with protecting agents can contribute to recover higher rates of the product with final concentrations considered acceptable for practical applications. Regarding drying methods, a lot of work is done by freeze-drying or fluidised-bed drying microorganisms. However, little is known about the innovative combination of spray-drying and conventional fluidised-bed drying techniques (fluid-bed spray-drying).

This work aimed to evaluate and compare different technologies to formulate CPA- 8: liquid, freeze-drying, and fluid-bed spray-drying. Specifically, we studied the following: (i) the effect of different protectants on cell viability and residual moisture content of CPA-8 formulations; (ii) the shelf-life and the effect of the storage temperature on CPA-8 formulations, and finally (iii) the efficacy of CPA-8 formulations against *Monilinia laxa* and *Monilinia fructicola* on nectarines.

MATERIALS AND METHODS

Antagonist strain and culture conditions

CPA-8 was originally isolated from a nectarine surface, identified by the Netherlands Culture Collection of Bacteria and belongs to the Postharvest Pathology Group Collection of IRTA (Lleida, Catalonia, Spain). Stock cultures were stored at 4 °C and 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. CRIOBILLES AEB 400100 (AES laboratory, Combourg, France) were used for long term storage at -80 °C.

Fresh bacteria suspended in potassium phosphate buffer (PB, 70 mL KH_2PO_4 0.2 mol L^{-1}; 30 mL K_2HPO_4 0.2 mol L^{-1} and 300 mL deionised water v/v/v pH 6.5) were used to inoculate a 5 L (BIOSTAT-A modular fermenters, Braun Biotech International, Melsungen, Germany) or 2 L (BioFlo/CelliGen 115, Eppendorf, New Brunswick, Canada) bioreactors containing growth medium as previously described Yánez-Mendizábal *et al.* (2012c) and slightly modified in the nitrogen source manipulation process: 100 g L^{-1} extracted defatted soy flour, 5 g L^{-1} molasses, 1.9 g L^{-1} KH_2PO_4, 0.001 mg L^{-1} $CuSO_4$, 0.005 mg L^{-1} $FeCl_3 \cdot 6H_2O$, 0.004 mg L^{-1} Na_2MoO_4, 0.002 mg L^{-1} KI, 3.6 mg L^{-1} $MnSO_4 \cdot H_2O$, 0.92 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.14 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.01 mg L^{-1} H_3BO_3 and 10 mg L^{-1} $C_6H_8O_7$. The initial inoculum was adjusted at $2 \cdot 10^5$ CFU mL^{-1} with an spectrophotometer (Yánez-Mendizábal et al., 2012c) and cells were grown for 68-72 h at 30 °C to obtain high endospore concentration (Yánez-Mendizábal *et al.*, 2012a). Agitation was set to 200 rev min^{-1} and the air feeding rate was 0.33 vvm. Antifoam (1 ml per litre) was added if needed (30 % Simethicone emulsion USP, Dow Corning®, USA).

Pathogens

M. laxa (CPML2) and *M. fructicola* (CPMC1) were isolated from decayed stone fruit, identified by the Department of Plant Protection of INIA (Madrid, Spain) and belongs to the Postharvest Pathology Group Collection of IRTA (Lleida, Catalonia, Spain). They were maintained on potato dextrose agar plates (PDA, Biokar Diagnostics, 39 g L^{-1}) at 25 °C for a maximum of 15 days. To induce conidial production, healthy fruit (peaches or

nectarines) were artificially infected with the pathogens. Infected fruit were maintained at 20 °C and 85 % relative humidity (RH) in the dark for 7 days.

Preparation of CPA-8 formulations

CPA-8 liquid formulation

For liquid formulation (LQF), cells were grown as described above and harvested by centrifugation at 9820 g for 12 min at 10 °C in an Avanti J-20 XP centrifuge (Beckman Coulter, CA, USA). The resulting pellet was resuspended in the same CPA-8 supernatant medium to include the antifungal lipopeptides synthesised by the bacterium during the production process. Samples of 50 mL of CPA-8 prepared approximately at $5 \cdot 10^9$ CFU mL^{-1} were mixed with 10 % sucrose, 10 % powder skimmed milk (SM), 10 % MgSO$_4$, 10 % sucrose plus 10 % SM or 10 % MgSO$_4$ plus 10 % SM and homogenised at 150 rev min^{-1} for 20 min at room temperature. One sample was non-supplemented with protectants and used as control (without protectant, WP). For each LQF product, the initial cell concentration per millilitre (CFU mL^{-1}) was determined by plating ten-fold dilutions on NYDA medium. The experiment was conducted twice. Subsequently, all LQF products were kept at 4 °C.

CPA-8 freeze-drying formulation

For each suspension prepared in LQF (including WP), samples of 50 mL each were distributed in autoclaved flasks and frozen at -20 °C overnight. Thereafter, the flasks were connected to a freeze-dryer (Cryodos, Telstar, S.A, Terrasa, Catalonia, Spain) operating at 1 Pa and -50 °C for 24 h. The experiment was conducted twice and the dried products were stored at 4 and 22 °C.

To determine the CPA-8 viability after freeze-drying (FD), three replicate samples (0.5 g) of each FD product were rehydrated with 5 mL of PB. Samples were shaken vigorously for 1 min and then allowed to rehydrate for other 9 min in static. Ten-fold dilutions of each suspension were plated on NYDA to determine the cell concentration per gram (CFU g^{-1}). The relative cell viability was calculated for each FD product by the difference between the CPA-8 concentration after drying and the initial concentration. The rate of surviving cells was calculated as follows:

$$\text{Relative cell viability} = \text{Log} (N_f / N_i)$$

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_f is the CFU of CPA-8 obtained after drying: population of the powder (CFU g^{-1}) x amount of powder (g).

To calculate the moisture content of the CPA-8 dried products, duplicate samples of 0.5 g 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 % RH.

CPA-8 fluid-bed spray-drying formulation

Fluid-bed spray-drying (FBSD) of CPA-8 was performed with cells cultured for 68-72 h, harvested by centrifugation and resuspended approximately at 10^{10} CFU mL^{-1} as described before. Suspensions of 500 mL each were prepared as control (WP) or supplemented with 10 % MgSO$_4$ as protectant. A fluid-bed spray-dryer (HüttlinGmbH, Bosch Packaging Technology Company, Schopfheim, Germany) was used. Each suspension was sprayed 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 camera of the pilot scale fluid-bed spray-dryer. To facilitate the grain formation, 3.5 g of binder were added to the cells solution. 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} to avoid the agglomeration of particles. The experiment was repeated and the FBSD products were stored at 22, 4, and -20 °C.

To optimise the final powder recovery, potato soluble starch, pregelatinised potato starch and pea protein were tested as carrier materials and two different binders were evaluated: pregelatinised potato starch and hydroxymethylpropylcellulose (HPMC).

The viability of CPA-8 cells after FBSD was calculated for both, the control and the suspension with 10 % MgSO$_4$ by the difference between the CFU after the drying process

(rehydrated with PB) and before drying, as it has been explained for FD products. The moisture content of each formulation was also estimated.

Shelf-life and effect of storage temperature on viability of CPA-8 formulations

Polypropylene tubes containing solid and liquid products were sealed with laboratory film (Parafilm 'M'; Picgenet Plastic Packaging, Chicago, IL, USA) and then kept in an airtight container filled with silica gel in order to avoid product humidification. CPA-8 LQF products were maintained under refrigeration conditions (4 ºC). Solid formulations were stored at 22 and 4 °C (FD products) and at 22, 4 and -20 °C (FBSD products). To determinate the shelf-life of each product, cell viability was determined monthly for four months for LQF and FD products and for a year in the case of FBSD products. Three replicates of each product and temperature were sampled and ten-fold dilutions were plated on NYDA to determine the CFU mL^{-1} or CFU g^{-1}. In case of dried formulations, 0.5 g were rehydrated in 5 mL of PB.

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

Antagonistic activity of CPA-8 to control brown rot caused by *M. laxa* and *M. fructicola* was tested on nectarines cv. 'Early Sungrand'. Treatments were prepared from those liquid and solid CPA-8 products that showed the best cell viability after formulation and their efficacy was compared to fresh cells and water as the treatment without CPA-8 (control CK).

Fruit selected without visible injuries and rots and homogeneous in maturity and size was stored at 0 °C and 85 % RH until required for experimentation (not exceeding 15 days). Fruit was wounded in the equator with a sterile nail (3 mm wide and 3 mm deep) and inoculated with 15 µL of *M. laxa* or *M. fructicola* conidial suspension. Conidia of each pathogen were scraped from infected fruit using a sterile loop and transferred to 5 mL of sterile distilled water amended with Tween-80 (one drop per litre). Conidia concentrations were adjusted at 10^3 conidia mL^{-1} by hemocytometer.

After 2 h at room temperature, a 15 µL suspension (10^7 CFU mL^{-1}) of each CPA-8 treatment was applied. The treatments were prepared as follows: CPA-8 fresh suspension from 72 h-old culture in NYDB medium (NYDA without agar) and formulated CPA-8

treatments from stored powders or liquid suspensions based on CPA-8 concentrations determined on the storage stability assay. Five nectarines constituted a single replicate and each treatment was replicated four times. The percentage of fruit infected (disease incidence) and the mean lesion diameter of brown rot (disease severity) were determined after five days of storage at 20 ºC and 85 % RH.

Data analysis

Relative cell viability of CPA-8 was estimated on the basis of CFU counted before (fresh cells) and after being formulated (dried cells). Brown rot incidence and severity data were also analysed and expressed as percentage of rotten fruit and rot diameter over the fruit surface (cm), respectively. Incidence percentage was transformed to the arcsine of the square root to normalise the data. Differences in relative cell viability as well as the brown rot incidence and severity data were evaluated using analysis of variance (ANOVA) with the JMP®8 statistical software (SAS Institute, Cary, NC, USA). Statistical significance was judged at the level P<0.05. When the analysis was statistically significant, the least significance difference (LSD) test was used for mean separation. Cell viability (CFU mL^{-1} and CFU g^{-1}) from the shelf-life study were log-transformed to achieve a normal distribution of the data and plotted in figures where the error was represented by the standard deviation (±SD) of three replications of each sampling data.

RESULTS

Effect of protectants on shelf-life of CPA-8 after liquid formulation

In order to test whether addition of a specific additive can protect CPA-8, either 10 % sucrose, 10 % SM, 10 % MgSO$_4$, 10 % sucrose plus 10 % SM or 10 % MgSO$_4$ plus 10 % SM was mixed with CPA-8 cells and the cell viability of each product was compared to cells that were resuspended in a formulation medium without protectants. The initial concentrations of CPA-8 LQF products ranged from 2.92 to 4.47 $\cdot$10^9 CFU mL^{-1}. After four months of storage (Fig. 1), no appreciable alterations in cell viability were observed (1.93-2.98 $\cdot$10^9 CFU mL^{-1}). Furthermore, no effects were detected in the addition of protectants to the formulation medium. According to these results, all liquid preparations showed

acceptable shelf-life and the CPA-8 LQF product with no protectants (WP) was selected for efficacy evaluation.
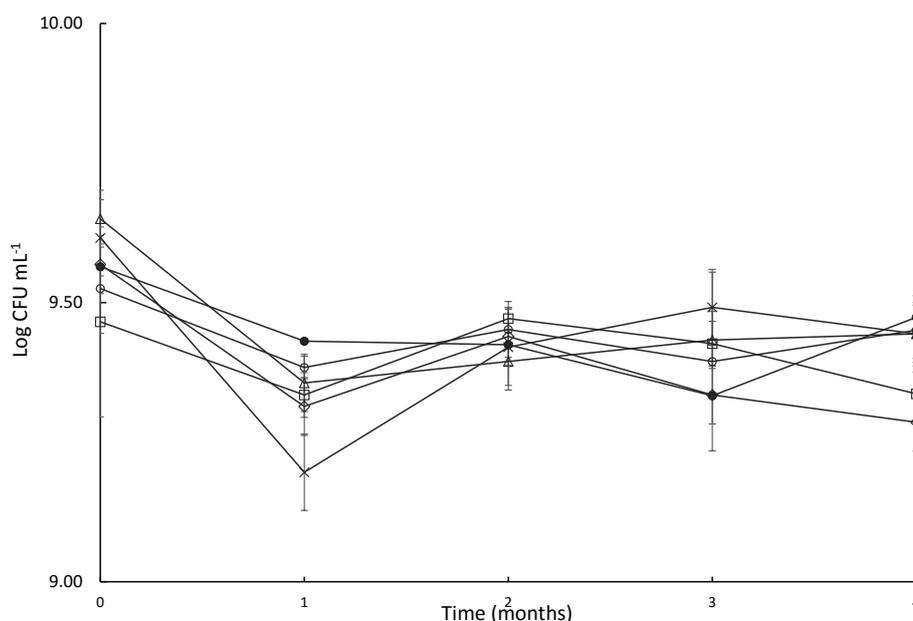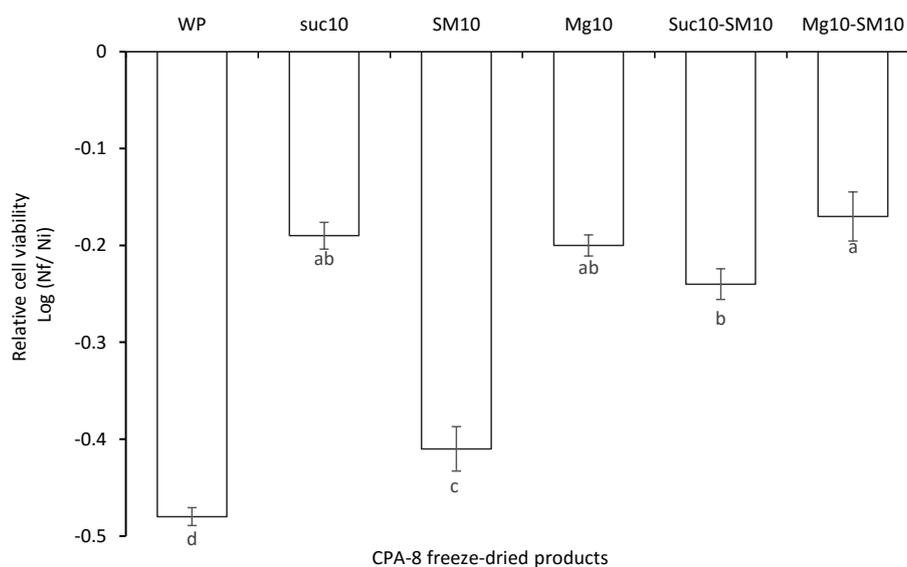

Figure 1. Cell viability of CPA-8 liquid products stored at 4 ºC in the absence "WP" (●—) and in the presence of the protectants 10 % sucrose (□—), 10 % SM (△—), 10 % $MgSO_4$ (×—); 10 % sucrose-10% SM (○—) and 10 % $MgSO_4$-10 % SM (◇—). Values are the averages of three determinations and bars indicate the standard deviations.

Effect of protectants on viability of CPA-8 after freeze-drying formulation

The protective ability of sucrose, SM, and $MgSO_4$ on CPA-8 during the FD process was evaluated against non-amended (WP) CPA-8 cells (Fig. 2). The ANOVA analysis revealed significant differences between formulations ($F_{5,29}$=46.33 $P<0.0001$). After means separation, the results indicated a positive effect in the addition of protectants. The use of 10 % $MgSO_4$ plus 10 % SM showed the best results in CPA-8 viability after the drying process (0.17-log cell viability reduction) but not significant differences were observed when it was compared to 10 % sucrose and 10 % $MgSO_4$. However, when CPA-8 was amended with only SM, the CPA-8 viability was reduced 0.41-log. For FD products without protectants (WP), the cell viability was also decreased almost five times (0.48-

log). For shelf-life and efficacy evaluation, CPA-8 FD products supplemented with MgSO$_4$ alone and combined with SM, were selected.

Figure 2. Relative cell viability of CPA-8 after freeze-drying in the absence "WP" and in the presence of the protectants 10 % sucrose (suc10), 10 % SM (SM10), 10 % MgSO4 (Mg10); 10 % sucrose-10 % SM (Suc10-SM10) and 10 % MgSO4-10 % SM (Mg10-SM10). Different letters indicate significant differences ($P<0.05$) according to LSD test. Values are the averages of six determinations and bars indicate the standard error.

## Effect of protectants on viability of CPA-8 after fluid-bed spray-drying formulation

Two CPA-8 solutions were tested, one without protectants (WP) and another one amended with 10 % MgSO$_4$. No significant differences were found when comparing both FBSD products ($F_{1,9}$=3.87 P=0.0806) and minor reductions in cell viability (0.06 and 0.14-log for WP and 10 % MgSO$_4$, respectively) were observed. Consequently, both FBSD products were selected for shelf-life and efficacy trials.

Shelf-life and effect of storage temperature on viability of CPA-8 dried formulations: freeze-drying and fluid-bed spray-drying

The cell viability of each solid CPA-8 product was tested at different shelf-life periods and storage temperatures (Fig. 3). In general, the viability was stable

independently of the temperature. For CPA-8 FD products, a minor reduction in cell viability was detected when the storage temperature was either, 4 or 22 °C. In every case, logarithm of CFU g^{-1} decreased between 0.24-0.34. CPA-8 cells amended with 10 % MgSO$_4$ and stored at 22 °C (most pronounced decrease) meant a reduction from $1.79 \cdot 10^{10}$ to $8.09 \cdot 10^9$ CFU g^{-1}. The CFU g^{-1} of FBSD formulations were practically unchanged during one year of storage; after this period, viability values between 4.76 and $8.16 \cdot 10^9$ CFU g^{-1} were obtained.
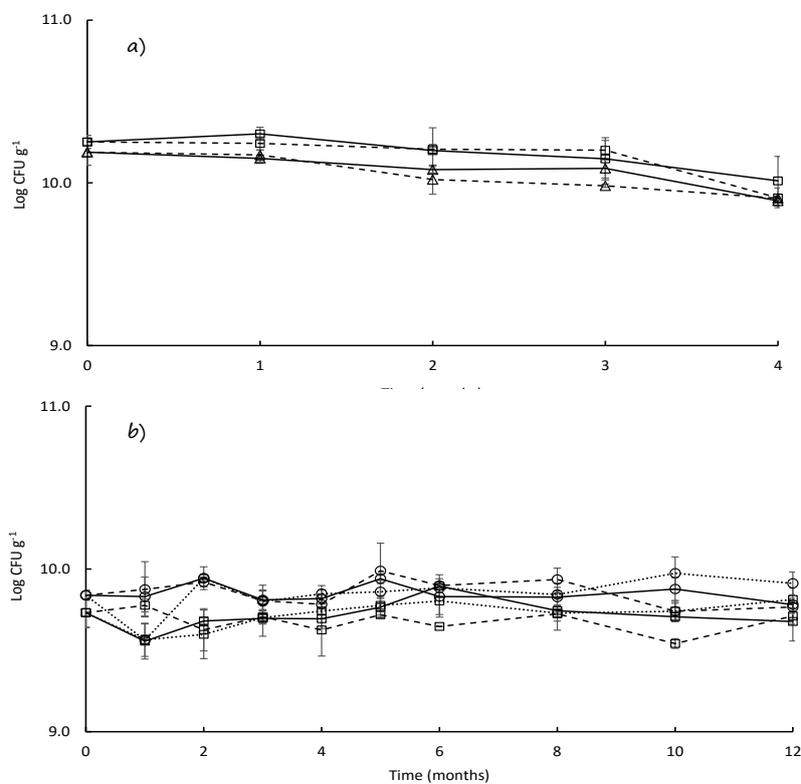
Figure 3. Cell viability of CPA-8 dried products after long-term storage at different temperatures. In the figure, only the most successful products are shown. Values are the averages of three determinations and bars indicate the standard deviations. (a): four months shelf-life of CPA-8 freeze-dried products previously amended with 10 % MgSO$_4$ and stored at 4 (⎯□⎯) and at 22 °C (--□--); and four months shelf-life of CPA-8 freeze-dried products previously amended with 10 % MgSO$_4$-10 % SM and stored at 4 (⎯△⎯) and at 22 °C (--△--); (b): one year shelf life of CPA-8 fluid-bed spray-dried products without protecting agents "WP" stored at 4 (⎯○⎯), at 22 (--○--) and at -20 °C (····○····) and one year shelf-life of CPA-8 fluid-bed spray-dried products previously amended with 10% MgSO$_4$ stored at 4 (⎯□⎯), at 22 (--□--) and at -20 °C (····□····).

Moisture contents of CPA-8 resulting powders

Residual moisture contents of FD products laid in the range between 2.3 and 8.8 %. In contrast, FBSD products varied from 7.0 to 11.4 %. The results indicated that FD yielded drier products than BFSD and that the introduction of SM into the formulation medium before drying significantly reduced the moisture content of the final products. When SM was added, the products obtained contained the lowest % RH values (from 2.3 to 4.9 %). For both techniques, acceptable final moistures were obtained.

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

Figure 4 shows the effect of formulated CPA-8 products on the reduction of brown rot decay caused by *M. laxa* and *M. fructicola* compared to untreated fruit and fruit treated with CPA-8 fresh cells. The most successful formulations stored for 2 months at 4 °C were tested and taking into account the feasibility of the FBSD technology, FBSD products stored for 9 months at 4 and 22 °C were also evaluated.

After 5 days at 20 °C and 85 % RH, all formulated products maintained similar antagonistic activity than fruit treated with fresh cells. The ANOVA analysis revealed that the treatments applied significantly affected brown rot decay (*M. fructicola* incidence: $F_{8,27}$=21.99 P<0.0001; *M. fructicola* severity: $F_{8,27}$=64.55 P<0.0001; *M. laxa* incidence: $F_{8,27}$=8.04 P<0.0001; *M. laxa* severity: $F_{8,27}$=21.51 P<0.0001). The percentage of disease incidence in untreated fruit inoculated with *M. fructicola* was 70 %, with a mean lesion diameter of 3.9 cm. In contrast, fresh cells showed decay incidence of 5 % with 0.1 cm of mean lesion diameter and no statistical differences were observed compared to the formulated products.

In general, there was higher incidence and bigger diameter of rot decay in fruit inoculated with *M. laxa* than with *M. fructicola*. The rot incidence of untreated fruit inoculated with *M. laxa* was 85 % and the mean diameter of lesion was 5.7 cm. No differences were observed between CPA-8 formulated products and fresh cells in disease severity. Regarding the percentage of disease incidence, the results showed 35 % reduction in case of fresh cells and over 62 % in all formulated treatments compared to untreated fruit. No statistical differences were obtained between formulated treatments

and all of them (except nine months-old CPA-8 FBSD product stored at 22 °C) showed better efficacy against *M. laxa* than fresh cells.
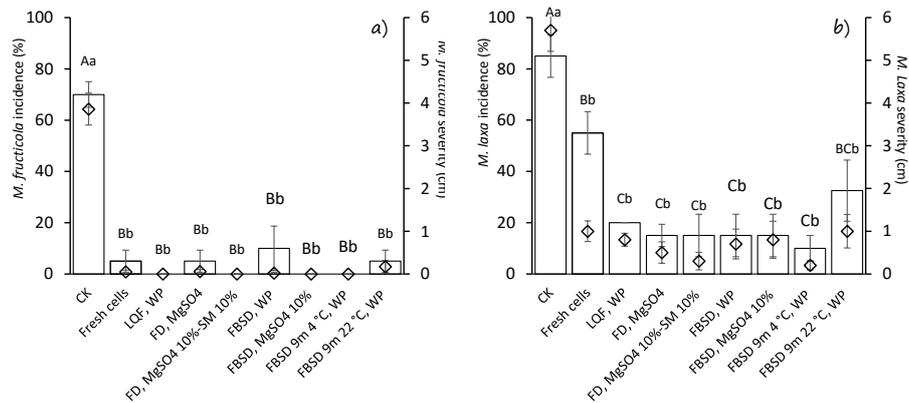
Figure 4 Antagonistic activity of CPA-8 fresh cells and formulations (10^7 CFU mL^{-1}) against artificial infection with *M. fructicola* (a) and *M. laxa* (b) on nectarines after 5 days of incubation at 20 °C and 85% RH. Within the same figure, different letters indicate significant differences ($P<0.05$) according to LSD test. Uppercases and bars refer to *Monilinia* incidence (%) and lowercases and diamonds refer to *Monilinia* severity (cm). Values are the averages of four determinations and bars indicate the standard error. The treatments tested were: negative control, without CPA-8 (CK); 72 h-old CPA-8 fresh cells (Fresh cells); two months (stored at 4 °C) liquid formulation of CPA-8 without protectants (LQF, WP), two months (stored at 4 °C) CPA-8 freeze-dried cells amended with 10 % $MgSO_4$ (FD, $MgSO_4$ 10 %) and with 10 % $MgSO_4$ plus 10 % SM (FD, $MgSO_4$ 10 %-SM 10 %), two months (stored at 4 °C) CPA-8 fluid-bed spray-dried cells without protectants (FBSD, WP) and amended with 10 % $MgSO_4$ (FBSD, $MgSO_4$ 10 %), nine months (stored at 4 °C) CPA-8 fluid-bed spray-dried cells without protectants (FBSD 9m 4 °C, WP) and nine months (stored at 22 °C) CPA-8 fluid-bed spray-dried cells without protectants (FBSD 9m 22 °C, WP).

DISCUSSION

This work focused on a direct comparison of LQF, FD, and the innovative FBSD technology in terms of viability of CPA-8 cells as alternative formulations to spray-drying, which was previously described (Yánez-Mendizábal *et al.*, 2012b). The effect of protective agents (sucrose, $MgSO_4$ and SM, alone or combined) was also investigated because the preconditioning of bacterial cells prior the drying process and during storage has been demonstrated to be of practical importance.

The decisive role of fengycin-like lipopeptides in the antagonism of CPA-8 against *Monilinia* spp. has been previously determined by Yánez-Mendizábal *et al.* (2012d). For

this reason, CPA-8 was not separate from the growth medium and cells were formulated with the supernatant containing synthesised lipopeptides.

In general, storage at 4 °C has given the highest degree of cell viability for many microorganisms formulated into a liquid form, while depending on species and strains, the shelf-life can be short at ambient or elevated temperatures (Abadias *et al.*, 2003; Melin *et al.*, 2007). According to our results, we have confirmed that CPA-8 supernatant medium with no protectants should be used as medium for preservation of CPA-8 aqueous cells because a suitable number of living cells were obtained after four months of storage. This suggests that the culture medium retains the nutrients needed for the metabolism of the microorganism and as no cells are died during formulation, the addition of protectants does not mean a beneficial effect in the bacteria's viability during shelf-life. It is noteworthy that LQF requires the manipulation of high volumes with no protection from contamination. For this reason and taking into account that the refrigerated conservation system has the disadvantage to involve high costs of storage, this was not the system chosen to formulate CPA-8 and no more than four months of shelf-life were studied.

FD (or lyophilisation), is a common industrial technique. However, it is a cost-sensitive application whose damaging factors in drying the microorganisms must be considered. For avoiding the undesirable effects, such as denaturation of proteins and decrease in cell viability of many cell types, some protecting agents have been added to the medium. In this work, sucrose, $MgSO_4$, and SM were tested. The SM at concentrations of 1-10 % has often been used for cryopreservation but even more frequently in mixtures with other cryoprotectants (Hubalek, 2003; Li & Tian, 2007). As described Navarta *et al.* (2011), the use of SM in the FD process of the BCA *Ranhella aquatilis* had lower protective effect than the SM used in combination with other additives such as sugars or polyols. This concords with the results obtained in this work. The lowest cell viability obtained for FD products, and even obtained when compared to all formulated products, was shown for FD products amended with 10 % SM and for those formulated without additives (relative cell viabilities of 0.41 and 0.48-log, respectively). The poor results in viability were probably caused because during the drying process it was too fast to let the internal water migrate outside the cell, and water frozen inside the cell resulted in lethal damage. In contrast, CPA-8 cells amended with sucrose or sucrose plus SM provided higher degree of cell protection during drying. Other authors have reported the beneficial role of

sugars (Costa *et al.*, 2000; Navarta *et al.*, 2011; Zhan *et al.*, 2011), which replace structural water in membrane after rehydration and prevent unfolding and aggregation of proteins (Champagne *et al.*, 1991). The results obtained when $MgSO_4$ was added to CPA-8 cells prior FD –alone or in mixture with SM- are in agreement with the work conducted by Yánez-Mendizábal *et al.* (2012b). It reported the importance to use $MgSO_4$ salts in spray-dried CPA-8 cells to thus obtaining formulations with acceptable shelf-life even at ambient temperatures.

In this work, satisfactory FD products were generally obtained. Although a minor reduction in cell viability after storage was observed, it was not considered decisive. However, this process is highly time-consuming and involves elevated costs in massive production. For that, the authors considered that FD is not the best alternative to formulate the BCA CPA-8.

FBSD is currently known as an attractive alternative to FD, spray-drying or traditional fluidised-bed drying systems. By operating with a large air volume, liquid can be spray-dried in the chamber at an inlet air temperature much lower than that used for traditional spray-drying. Srivastava and Mishra (2010) have also reported that FBSD products generally involve larger quantities, have better dispersibility and flowability, and exhibit a narrow particle-size distribution. Furthermore, comparing to conventional fluidised-bed drying, FBSD does not need previous extrusion and pelletization of the sample. This combined technology also offers lower operating costs and shorter process times compared to FD (Strasser *et al.*, 2009).

In this work, three different carriers and two binders were tested to obtain a suitable FBSD product. The poor solubility of pea protein and pregelatinised potato starch as carrier materials triggered clogging the spray nozzle and were not appropriated for CPA- 8 formulation. Moreover, the agglutinant power of the polymer HPMC also meant the formation of agglomerates. At the end, the combination of potato soluble starch as carrier and pregelatinised potato starch as binder were chosen for enhancing powder recovery with no agglomerates and also for being low-cost commercial products (data not shown).

In terms of CPA-8 viability, we have experimented with two cell suspensions: one without additives and other one amended with 10 % $MgSO_4$. $MgSO_4$ salt was chosen for

its good results in CPA-8 FD -reported in this work- and also for the results showed in the work conducted by Yánez-Mendizábal *et al.* (2012b) on CPA-8 spray-drying. Both formulations provided high bacterial viability after drying and were found shelf-stable in all storage temperatures tested (22, 4 and -20 ºC) extending the viability of CPA-8 for up to twelve months. This means that practically all the cells survived successfully the process. However, different findings were reported by Strasser *et al.* (2009) when lactic acid bacteria were dried using the same technique. In that case, without the addition of protectants, the viability of the cells was higher after FD than after FBSD. The results we obtained are important for subsequent CPA-8 distribution and application, considering that commercial products must have a long storage life and also should not require special cold conditions that increase their management cost.

Although CPA-8 can be stored and kept viable after long incubation periods, this does not guarantee that the biocontrol potential of the strain would be maintained. The biocontrol efficacy after the formulation process should be retained as one of the most important requisites for commercial purposes. Based on this, the antifungal activity of CPA-8 formulations against *M. laxa* and *M. fructicola* was tested on nectarines. CPA-8 formulated products have successfully controlled the pathogen compared to untreated fruit. These results supported that the formulating process did not have any negative effect on the biocontrol efficacy of CPA-8.

In conclusion, we could confirm that apart from the reported spray-drying method to formulate *B. amyloliquefaciens* CPA-8, this strain could be satisfactorily formulated with all the techniques evaluated in this work: LQF, FD and FBSD. In all cases, CPA-8 formulated products were obtained with satisfactory final concentrations and stable shelf-life. Taking into account the considerations mentioned, FBSD appeared to be the most suitable approach. Although the results presented are promising, CPA-8 FBSD needs to be improved in order to develop a commercially available product. Nevertheless, this work presented and discussed that FBSD technology ensures the efficacy, stability, and low-cost easily application of CPA-8 formulated products.

Chapter IV

Acknowledgments

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