Dry formulations of the biocontrol agent *Candida sake* CPA-1 using fluidised bed drying to control the main postharvest diseases on fruits

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Abbreviated running headline: Fluidised bed drying for *Candida sake*

ABSTRACT

BACKGROUND: The biocontrol agent *Candida sake* CPA-1 has been demonstrated to be effective against several diseases. Consequently, the optimisation of a dry formulation of *C. sake* to improve its shelf life and manipulability is essential to increase its potential for future commercial applications. The aim of this research was to optimise the conditions to make a dry formulation of *C. sake* using a fluidised bed drying system and then to determine the shelf life of the optimised formulation and its efficacy against *Penicillium expansum* on apples.

RESULTS: The optimal conditions for the drying process were found to be 40 °C for 45 minutes, and potato starch used as the carrier significantly enhanced the viability. However, none of the protective compounds tested increased the viability of the dried cells. A temperature of 25 °C for 10 min in phosphate buffer was considered the optimum condition to recover the dried formulations. The dried formulations should be stored at 4 °C and air-packaged; moreover, shelf life assays revealed good results after 12 months of storage. The formulated products maintained their biocontrol efficacy.

CONCLUSION: A fluidised bed drying system is a suitable process for dehydrating *C. sake* cells; moreover, the *C. sake* formulation is easy to pack, store and transport, and it is a cost-effective process.

Keywords: biocontrol, *Candida sake*, dehydration, fluidised bed, formulation, shelf life.

INTRODUCTION

Scientific knowledge related to biocontrol agents (BCAs) has been extensively developed during previous decades.[1] The efficacy of BCAs has been demonstrated against several postharvest diseases responsible for significant damage of fresh fruit and vegetables. Commercial use of BCAs would allow the reduced application of synthetic fungicides to treat fungal pathogens.[2] These facts, as well as an increased concern for human health, demonstrate the need to protect the environment for future generations, and the development of resistance to many fungicides by many postharvest pathogens is one of the most important reasons for the advancement of biological control research.

In spite of research advancements, the acceptance and widespread use of postharvest biocontrol products is still limited, probably because of their shortcomings when commercially applied.[1,3–6] Therefore, an improvement of handling of BCAs under commercial conditions is necessary, and one way to achieve this goal is to produce a formulation that is easy to package, store and transport, that has a long shelf life and retains its efficacy. All of these factors are the keys for the commercial success of BCAs.[7,8]

Candida sake strain CPA-1 is a well-known BCA. It was isolated from apples and demonstrated good efficacy against major postharvest diseases of pome fruits, such as blue mould (*Penicillium expansum*), grey mould (*Botrytis cinerea*) and Rhizopus rot (*Rhizopus stolonifer*).[9] Moreover, *C. sake* effectively reduced *B. cinerea* in a lab-based

assay with detached grape berries[10] and in field applications, different biologically based strategies significantly reduced the incidence and severity of botrytis rot under Mediterranean conditions[11] and under Atlantic climatology.[12] Sour rot of grapes was also reduced with some treatments including *C. sake.*[13]

A formulation process must principally achieve four basic objectives: It must i) stabilise the organism during production, distribution and storage; ii) aid in the handling and application of the product; iii) protect the agent from harmful environmental factors; and finally iv) enhance the activity of the organism at the site of use.[14] The maintenance of cell viability is fundamental for commercial BCA formulations but suggested minimum shelf life changes depending on the authors from six[15] to 12 months[16] or 18 months[2]. The efficacy of BCAs is dependent on the degree of success of the formulation, so it is necessary to carefully evaluate every detail of the formulation process to obtain high survival rates of viable microorganisms and to achieve an equal or even better efficacy as with fresh cells.

Different formulations have been developed for *C. sake* in previous years. Abadias *et at.* studied a freeze-dried *C. sake* formulation but obtained an efficacy significantly lower than that of fresh cells in all treatments.[17] The stability of the formulation decreased 10-fold after two months storage at 4 °C. Liquid formulation of BCA by modifying the water activity or adding protectants was also investigated, and the results were more satisfactory because the efficacy of the formulation was similar to that of fresh cells for *P. expansum* on apples, and the addition of sugars such as lactose and trehalose as protectants improved cell viability, which was greater than 70% after four months at 4 °C.[18] Furthermore, a liquid formulation in an isotonic solution based on trehalose (0.96 M) was developed and stored for seven months at 4 °C, and it retained its viability and efficacy against blue mould rot in apples.[19] A spray-drying system was evaluated as a dehydration method for CPA-1, but it was not satisfactory because of low cell survival, poor product recovery and low efficacy.[20] The induction of

thermotolerance in *C. sake* cells by mild heat treatments to improve survival by using spray-drying was also studied, but although these treatments improved the survival of the cells, they could not be considered as suitable formulation methods for this BCA.[21]

After all of this work, the liquid *C. sake* formulation in an isotonic solution of trehalose was found to be most suitable because it retained viability and efficacy for one year (unpublished data), and this formulation was commercialised by demand for a period of time under the name of Candifruit™ [3]. However, some shortcomings of this formulation must be noted: it should be stored and distributed under refrigeration, and large volumes of liquid have to be managed and transported. Therefore, CPA-1 can be considered an effective BCA, but it is still not a competitive product or a real alternative to fungicides.

Rhodes (1993) suggested that dehydration is one of the best ways to formulate microbial agents so they can be handled by normal distribution and storage channels.[22] Moreover, a successful dry formulation is easy to use and convenient to transport, and it has a long shelf-life and high consumer acceptance.[14] Nevertheless, when a dry formulation is optimised, some issues must be considered, such as the maintenance of viability when the BCA is dried or an optimal moisture content to avoid rapid deterioration.[14]

In this study, a fluidised bed drying system was evaluated to avoid the deficiencies of freeze-drying and spray-drying dehydration systems. The freeze-drying method usually maintains cell viability, but it is comparatively expensive. On the other hand, the main disadvantage of spray-drying is the destruction of microbial cells caused by the high drying temperature.[8] In contrast, during fluidised bed drying, the air to dry the cells is injected at the bottom so that the granules fluidise and the product dries relatively rapidly without a requirement for high temperatures, and the cost is lower than that of other drying methods. Fluidised bed drying is widely used to dry heat sensitive biologic materials, and some BCAs have been formulated using this system.[23–25]

The aim of this research was to optimise the conditions of fluidising-bed dehydration to produce a dry formulation of *C. sake* by studying several factors that could affect cell viability, shelf life, and efficacy against *P. expansum* on apples.

The specific objectives were: (i) the optimisation of drying conditions such as temperature and drying time, (ii) the selection of a carrier to mix with *C. sake* cell paste to facilitate extrusion, (iii) the determination of the effect of protectants on cell survival, (iv) the evaluation of the effect of the rehydration media and conditions on viable cell recovery from CPA-1 formulations; (v) the evaluation of the product shelf life under various storage conditions, and (vi) the evaluation of the biocontrol efficacy against *P. expansum* on apples.

MATERIAL AND METHODS

Microorganisms

Yeast isolate

The *C. sake* strain CPA-1 used in this investigation belongs to the collection of the University of Lleida-IRTA, Catalonia, Spain and it was deposited in the Colección Española de Cultivos Tipo (CECT-10817) at the University of Valencia, Burjassot, Spain. It was isolated from the surface of apples.

C. sake stock cultures were stored at 4 °C on nutrient yeast dextrose agar plates (NYDA: nutrient broth, 8 g l^{-1}; yeast extract, 5 g l^{-1}; dextrose, 10 g l^{-1}; and agar, 15 g l^{-1}) and were sub-cultured on NYDA plates at 25 °C for 48 h before use. Sub-cultured cells were transferred to potassium phosphate buffer (pH 6.5; KH_2PO_4 0.2 mol l^{-1}, 70 ml; K_2HPO_4 0.2 mol l^{-1}, 30 ml and deionised water, 300 ml) to obtain the suspension used as the starter inoculum for biomass production in liquid bioreactors and for the *in vivo* efficacy assays.

Cells were produced in a liquid production system with a 5 l working volume (BIOSTAT-A modular bioreactor, Braun Biotech International, Melsungen, Germany) at

25 °C, an agitation speed of 400 rpm and an aeration level of 150 l h^{-1} for 40 h. The starter inoculum of *C. sake* was added to 5 l of molasses-based medium (MB: cane molasses 40 g l^{-1} (Loiret & Haëntjens España S.A., Barcelona, Spain), urea 1.2 g l^{-1} (Panreac Química S.A., Barcelona, Spain), water activity (a_w) 0.996) at a final concentration of 10^6 colony forming units per ml (CFU ml^{-1}).

For biomass production in an Erlenmeyer flask for *in vivo* efficacy trials, the starter *C. sake* inoculum was added to 50 ml of molasses-based medium to a final concentration of 10^7 CFU ml^{-1}.

Pathogen isolate

The pathogen used was *P. expansum* CMP-1 belonging to the collection of University of Lleida-IRTA, Catalonia, Spain, and it was deposited in the Colección Española de Cultivos Tipo (CECT-20906) at the University of Valencia, Burjassot, Spain. It was a strain isolated from a rotten apple that had been in cold storage for several months. It was maintained on potato dextrose agar (PDA: 200 ml of extract from boiled potatoes, 20 g dextrose, 20 g agar and 800 ml water) at 25 °C from 7 to 15 days and was periodically sub-cultured and transferred through apple tissues to maintain virulence.

Fluidised bed drying

CPA-1 produced cells were centrifuged (Avanti™, Beckman) at 8631 g and 10 °C for 10 min to harvest the cells from the medium. The centrifuge was programmed at 10 ºC to avoid heating of yeast cells. The pellet was weighed, and the cell paste was homogenised in a hand mixer with the appropriate proportion of the carrier until the dough lost adherence. The yeast dough was extruded into particles of 0.2 to 0.5 mm diameter and 0.5 mm length using a cookie press from an icing set (LACOR) and perforated metallic plate. The initial concentration of the extruded yeast dough was determined on NYDA by dilution plating. Afterwards, the various optimisation studies were conducted by introducing 2 - 3 g of wet extruded dough into the tubes of a

fluidised bed dryer 350S (Burkard Manufacturing Co. Ltd, Hertfordshire, UK). After the fluidised bed drying was complete, 0.05 g of dry extruded particles were weighed and rehydrated with 5 ml of phosphate buffer, then shaken for 1 min and allowed to rehydrate for 9 min. The final concentration of dehydrated cells of *C. sake* was determined by dilution plating on NYDA. Viability after the fluidised bed drying process was expressed as log (N/N_0), where N_0 represents CFU per gram of extruded dough before drying and N is the CFU per gram of the same quantity of wet extruded dough after fluidised bed drying.

To determine the dry matter of the initial wet dough, 1 g of the product was placed in duplicate aluminium weighing boats and dried in a convection oven at 105 ± 1 °C for 24 hours. The same process was carried out with the dry extruded product but only 0.5 g were weighed.

During the fluidised bed drying process, the room temperature was controlled at 20 ºC and relative humidity at 40% to achieve the same conditions for all assays. All fluidised bed drying experiments were completely random designs.

Optimisation of time and temperature of drying

A range of drying temperatures from 30 °C to 50 °C were used to study the effect of inlet air temperatures of 30, 40, 45 and 50 °C. Drying times of: 5, 10, 15, 20, 30, 45 and 60 min were also evaluated. Potato starch was used as a carrier in studies to optimise the drying time and temperature. Three replicates of each formulate were weighted for each time and temperature, and the viability was the mean of them. The experiment was repeated twice.

Optimisation of the carrier

Four substances were tested to identify an optimal carrier for a dry formulation of *C. sake* using fluidised bed drying, natural silicate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), potato starch (Panreac Química S.A., Barcelona, Spain), corn

starch (Fluka, Sigma-Aldrich Chemie GmbH) and rice starch (Sigma-Aldrich Chemie GmbH). The survival of the BCA was the mean of three replicates. The fluidised bed drying conditions for this assay were 40 °C for 45 min.

Optimisation of the protective compounds

Various concentrations of several protective compounds were used as protectants in the fluidised bed drying process, 100 g l^{-1} sucrose (SUC10), 10 g l^{-1} glucose (GLU1), 100 g l^{-1} glucose (GLU10), 100 g l^{-1} sorbitol (SOR10), 100 g l^{-1} dextran (DEX10), 10 g l^{-1} trehalose (TRE1), 50 g l^{-1} trehalose (TRE5), 100 g l^{-1} trehalose (TRE10), 100 g l^{-1} sorbitan monostearate (MON10), 200 g l^{-1} sorbitan monostearate (MON20), 100 g l^{-1} skimmed milk (MILK10), 10 g l^{-1} carboxymethyl cellulose (CMC1), 50 g l^{-1} carboxymethyl cellulose (CMC5), 100 g l^{-1} carboxymethyl cellulose (CMC10), 100 g l^{-1} glycerol (GLY10) and 100 g l^{-1} polyethylene glycol (PEG10). All the formulation products were dried using previously determined conditions.

This optimisation was carried out with different assays on different days. Three formulations with different protective compounds and a formulation without a protective substance (the control) were tested in each assay. The effect of protective compounds on the survival of the *C. sake* yeast cells after fluidised bed drying was expressed as: [log ($N_{f \text{ with protectant}}/N_{0 \text{ with protectant}}$) − log ($N_{f \text{ control}}/N_{0 \text{ control}}$)], where N_0 is the CFU per gram of the extruded dough before drying and N_f is the CFU per gram of the same quantity of wet extruded dough after fluidised bed drying. Each protective compound was tested at least in two drying processes. Two replicates of each formulate were weighted to evaluate the survival.

Optimisation of the rehydration media and the rehydration conditions

The rehydration process is an important factor because it can improve the viability of the final product. Two aspects of the rehydration process were considered: i) the composition of the rehydration media because it could facilitate the repair of cell

damage and the restoration of physiological function, and ii) the rehydration temperature and time, which could affect the cell viability after rehydration.

Phosphate buffer and skimmed milk (100 g l^{-1}) were tested as rehydration media for dried formulations. Rehydration times of 10 min, 2 h, 5 h and 24 h and rehydration temperatures of 6, 15, 25, 30 and 35 °C were studied with the previously optimised formulation. Three replicates of each formulate were weighted for each condition of rehydration and the viability was the mean of them. The experiment was repeated at least twice.

Shelf life of formulated products

To study the stability of the optimised formulation, 72 glass vials containing approximately 0.05 g of the formulation were sealed with laboratory film (Parafilm "M"). Then 36 vials were kept in an airtight container filled with silica gel to avoid sample humidification. The other 36 vials were stored under vacuum. The vials were stored at 4 °C and 25 °C, and 3 vials were removed at different times to determine survival by dilution plating on NYDA with phosphate buffer at room temperature for 10 min as the rehydration media. The resulted survival for each condition was the mean of the three vials. Shelf life assays were conducted for twelve months.

Formulated product efficacy

The efficacy of two optimised dry formulations was tested against *P. expansum* on apples. Both formulations were stored at 4 °C and air-packaged, one for one month and a half and another for six months. Both formulations were rehydrated with phosphate buffer at room temperature for 10 min, and their efficacy was compared to the efficacy of fresh cells. Fresh cells were obtained following growth in 100 ml conical flasks containing 50 ml of the same medium of formulated cells. Flasks were incubated for 48 hours at 25 °C and 150 rpm, and then the cells were centrifuged (*as section 2.2.*)

and resuspended in 50 ml of phosphate buffer. The cell density of *C. sake* was adjusted with phosphate buffer to 10^7 CFU ml^{-1}.

'Golden Delicious' apples were surface-sterilised and wounded with a nail to produce an injury 2 mm in diameter and 2 mm deep at the stem (top) and another at the calyx (bottom). For each treatment, 15 µl of cell suspension of formulated *C. sake* was applied to the wounds and the fruits were allowed to dry at room temperature. Then the wounds were inoculated with 15 µl of an aqueous suspension of *P. expansum* at 10^4 conidia ml^{-1}. Each treatment was replicated four times with five apples per replicate.

The treated apples were incubated at 20 °C and 85% relative humidity (RH) for seven days, and then the rot lesion diameter (severity) and the number of infected wounds (incidence) were determined.

Statistical analysis

An analysis of variance was performed using the JMP8 software (SAS Institute Inc., Cary, NC, USA) on all data sets. Prior to the analyses, the incidence percentage and the CFU data were transformed by the square root of the arcsine and the logarithm, respectively, in order to improve the homogeneity of the variance. If a result was statistically significant ($P<0.05$), the Least Significant Difference (LSD) procedure was used for mean separation.

RESULTS

Optimisation of time and temperature of drying process

Several combinations of drying temperatures and times were tested and the best temperatures were 40 and 45 °C (Fig. 1). However, only drying at 40 °C for 45 min produced an optimal formulation moisture below 10% (8.5%), although the cell viability decreased to approximately 25%. In contrast, the highest temperature tested (50 °C) was too high, and the cell survival rapidly decreased to near 35% after 20 min, whereas survival was approximately 60% when the cells were dried at 40 °C for 20 min

(data not shown). A low temperature (30 °C) was insufficient to dry the product, so the final formulation had a high moisture content of approximately of 12.5% (data not shown). Drying the cells at a lower temperature or for less time to improve cell viability was considered, but both options increased the risk of an important decline in the shelf life of the final formulation due to excess available water in the dried product. Consequently, these temperatures were rejected, and 40 °C for 45 min was chosen to continue the study.

Optimisation of the carrier

A carrier substance was necessary to reduce the adhesiveness of the cell paste and achieve a malleable dough that could be extruded to obtain small particles for fluidised bed drying. In addition, it was important to choose a carrier that did not damage the cells or reduce their survival.

Two of the four substances tested as carriers proved to be not useful for this BCA. Specifically, natural silicate caused problems with the formulation process because the dough could not be extruded, and the corn starch produced non-soluble formulations during the rehydration process. Only potato starch and rice starch were useable as carriers in further experiments.

The viability of *C. sake* after drying the cells at 40 °C for 45 min was significantly higher with potato starch (31.3% viability) than with rice starch (18.5% viability) as the carrier. Likewise, the reduction of *C. sake* cells after drying was 0.5 log units when potato starch was used as carrier whereas the reduction was 0.73 log units with rice starch. The moisture content of the dried product was also better with potato starch (8.6%) because the dried product with rice starch had a moisture content above 10% that could compromise the shelf life of the formulated product.

Optimisation of protective compounds

Different protectant substances at different concentrations were tested to improve cell survival after the fluidised bed drying process (Fig. 2). Most of the compounds tested resulted in lower survival than the control formulation (without a protectant substance). Improved survival was achieved with all tested percentages of carboxymethyl cellulose (10, 50 and 100 g l^{-1}), 200 g l^{-1} sorbitan monostearate, 10 g l^{-1} glucose and 10 g l^{-1} trehalose, but no significant differences were observed. Moreover, despite of the improvement in *C. sake* survival, these formulations had too high moisture content for practical use (Table 1). The worst survival was observed when 100 g l^{-1} sucrose and 100 g l^{-1} polyethylene glycol were used.

Optimisation of the rehydration media and the rehydration conditions

Laboratory experiments showed that differences among phosphate buffer and skimmed milk were rarely significant at low rehydration temperatures (6 °C and 15 °C), and no significant differences were evident among the different rehydration times at these temperatures (Fig. 3). Phosphate buffer was a significantly better medium only when rehydration was carried out at 6 °C for two hours. At room temperature (25 °C), both rehydration media showed the same tendency with time in the experiment. Two hours of rehydration yielded the highest *C. sake* survival, although no significant differences were observed between 10 minutes and two hours. At higher temperatures (30 °C and 35 °C), significant differences were observed among rehydration times but not between the rehydration media.

In general, the cell recovery did not decrease with time at low temperatures (6 and 15 °C), whereas at high temperatures (25, 30 and 35 °C), a notable decrease of the cell survival with time was observed.

The highest survival was obtained with skimmed milk at 35 °C for 10 minutes (70.0%) although this was not significantly different from cells rehydrated with phosphate buffer.

Moreover, for both media, survival was not significantly different after 10 min of rehydration at 25 °C and 35 °C (data not shown).

Shelf life of formulated products

In general, storage temperature had a strong influence on the shelf-life viability of fluidised bed dried *C. sake* cells, and better results were obtained at 4 °C than at 25 °C (Fig. 4). At 25 °C, the survival of the formulated cells stored decreased sharply under both air conditions, and after two months, the air-packaged formulation showed a survival of less than 10%, a decrease of 0.69 log units. At 4 °C, the viability remained stable for both storage conditions after 12 months, and the differences between an air-vacuum and air-packaging were only significant at the first and the twelfth month of storage.

Formulated product efficacy

The optimised dry formulation process using fluidised bed drying was used to determine the efficacy of formulated *C. sake* cells stored at 4 °C against *P. expansum* in Golden Delicious apples. The efficacy experiments showed that both *C. sake* formulations significantly inhibited development of blue mould (Fig. 5). Two different storage times (one and a half and six months) were tested to demonstrate that the efficacy did not decrease during storage. Both the incidence and severity of *P. expansum* on apples treated with *C. sake* cells were reduced up to 52 and 72%, respectively. No significant differences were found between both storage formulations and fresh cells.

DISCUSSION AND CONCLUSIONS

The present study demonstrated that a dry formulation of the biocontrol agent *Candida sake* CPA-1 using fluidised bed drying is appropriate for cell survival and efficacy. The optimised formulation was dried at 40 ºC for 45 min using potato starch as carrier and without protectant compounds. The chosen rehydration media was phosphate buffer at

25 °C for 10 min and it can be stored air-packaged at 4 °C at least one year. Moreover, compared with freeze-drying, vacuum-drying or spray-drying systems, fluidised bed drying has the lowest fixed and manufacturing costs.[26] Likewise, the optimised formulation presented more benefits compared to previously developed formulations of *C. sake*. On one hand, compared with liquid formulation, fluidised bed dried formulation was a lot less bulky, so it would be easy to manage and transport. On the other hand, compared with the other processes used previously to dry *C. sake*, the optimised process encompassed three important benefits: it was cheaper, it was faster and it maintained cell viability and efficacy.

The optimum *C. sake* formulation using fluidised bed drying was achieved by drying for 40 min at 45 °C. Similar conditions have been used for other microorganisms such as *Saccharomyces cerevisiae*, which was also optimised for 40 min but at 30 °C[27] or *Pichia anomala* at 40 °C for approximately 1 h[28]. The best carrier for *C. sake* was native potato starch. Potato starch mixed with carbohydrates was also used as a carrier to dry *Lactobacillus plantarum* in a fluidised bed drier to minimise the inactivation of the cells due to dehydration.[29]

Experiments with protective compounds demonstrated that the best choice is to avoid the addition of protective substances to achieve an optimal water content for a long shelf life of the formulated product. The results obtained in this study demonstrated that most protectant substances tested did not improve the viability of dried cells, which could be explained by two reasons: (1) the use of potato starch as carrier could have had a protective effect on the yeast because it is a polysaccharide[30]; and (2) direct mixing of a solid protectant with wet cell paste could decrease the water activity of the final dough prior to drying and affect the stress tolerance of the microorganisms[29]. A final moisture content of <8% is recommended for dried *C. sake* cells[20], but in this study with fluidised bed drying, the moisture content of the control formulation (10.3%) was deemed acceptable, and its shelf life was evaluated to ensure that the moisture

content was satisfactory. According to our goal, a high survival rate immediately after drying is less important than low inactivation during storage, an aspect which is crucial for commercial exploitation[31].

The optimum rehydration medium and rehydration conditions are phosphate buffer at 25 °C for 10 min. Nevertheless, a combination that produced a higher survival rate, skimmed milk media at 35 °C after 10 minutes, was not significantly different from phosphate buffer at 25 °C for 10 min. For this reason, heated skimmed milk was not chosen as the rehydration medium because heating the rehydration medium is an expensive and unsuitable option for fruit applications. In contrast, phosphate buffer at 25 °C is cheaper. Yánez-Mendizábal *et al.* obtained lower viabilities by rehydrating spray-dried *Bacillus subtilis* CPA-8 cells with skimmed milk than with other rehydration media.[32] Moreover, at 35 °C, the viability decreased drastically after 10 min, so the CPA-1 would have to be applied immediately after recovery, which could be difficult. However, at 25 °C, the cell viability begins to decrease after 2 h of rehydration. A minor loss of viability is offset by greater convenience and a lower cost.

Muller *et al.* reported that the reconstitution technique had a significant effect on the bacterial recovery and described the rehydration process as a vital step for the achievement of maximal viability.[33] A critical parameter of the rehydration process is the temperature of the rehydration medium[29] which is usually from 20 °C to 30 °C for fluidised bed drying[34,35]. Nevertheless, cell survival during rehydration is affected by many factors such as the strain, the temperature, the rehydration kinetics or the pH of the medium[29].

The results obtained in this study showed that an easy to recover dry product with a high viability had been achieved, but knowledge about the time that it could be stored without undergoing a significant decrease in survival was necessary. This study demonstrated that the survival of the fluidised bed dried *C. sake* cells is closely related to the storage temperature, and is better at 4 °C than at 25 °C. Fu and Chen

associated the loss of cell viability at elevated temperature with the degradation of life-essential macromolecules during storage.[29] Previous studies with this BCA showed that a cold storage temperature for *C. sake* was better to keep metabolic activity low and maintains stability with time[17–19]. In this study, a significant difference between vacuum conditions and air-packaging was only seen after 12 months of storage at 4 °C; but during this time, the dried cell survival stored with air-packaging decreased by 0.3 log units. Therefore, the storage of air-packaged formulations at 4 °C is the best and most economical for CPA-1. In contrast, Yánez-Mendizábal *et al.* did not observe significant differences when *Bacillus subtilis* CPA-8 was stored at 4 °C or 20 °C.[32]

The storage of air-packaged formulations at 4 °C demonstrated the longest shelf life for a *C. sake* formulation obtained to date. Previous shelf life studies showed that the survival of freeze-dried *C. sake* cells after storage was not satisfactory, decreasing significantly after 15 days[17], and the longest published shelf life for a liquid *C. sake* cell formulation was seven months in an isotonic solution based on trehalose (0.96 M)[19]. Nevertheless, this liquid formulation can be stored for one year (unpublished data).

Additionally, we have proven that this dry formulation retains its efficacy against *P. expansum* on Golden Delicious apples after six months of storage and is similar to fresh *C. sake* cells. Both the incidence and severity were reduced by 42 and 64%, respectively. Previous studies of freeze-dried *C. sake* cells with *P. expansum* on apples showed that this formulation was not as effective as fresh cells.[17] Furthermore, spray-dried cells were not as effective as fresh cells against blue mould on pome fruit.[20] Therefore, this is the first dry formulation of *C. sake* CPA-1 that retained its efficacy against blue mould on apples. Other fluidised bed dried BCAs, such as *Penicillium frequentans*[36] or *P. anomala*[28], retained an efficacy similar to fresh cells. Also efficacy of freeze-dried *Pseudomonas spp.* was nearly identical to that of the fresh cells.[37]

In summary, we have demonstrated that a fluidised bed drying system is suitable for dehydrating *C. sake* cells because the dry formulation had satisfactory solubility,

retained its viability for 12 months and was as effective as fresh cells against *P. expansum* on apples. In addition, it is easy to package, store and transport and is cost effective. The cell concentration after 12 months is very satisfactory because just over one kilogram of formulation can treat a 1000 litre drencher, which is a reasonable amount of product to use.

Based on these findings, we assume that this dry formulation overcomes most of the shortcomings hindering the commercialisation of this biocontrol product. However, the extrusion of the yeast dough is an involved process that must be performed manually, so further research should focus on the development a similar formulation using an automatic process. Optimisation of a fluid-bed spray-drying process for *C. sake* that takes advantage of the benefits of this method could be the next step.

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Table 1. Moisture of the formulations with the best protective compounds after fluidised bed drying.

Protective compound	Formulation moisture[a] (%)
Control	10.3
5% carboxymethyl cellulose	12.3
1% carboxymethyl cellulose	13.3
10% carboxymethyl cellulose	15.2
20% sorbitan monostearate	11.3
1% glucose	12.6
1% trehalose	11.3

[a] The results are the means of at least four measurements.
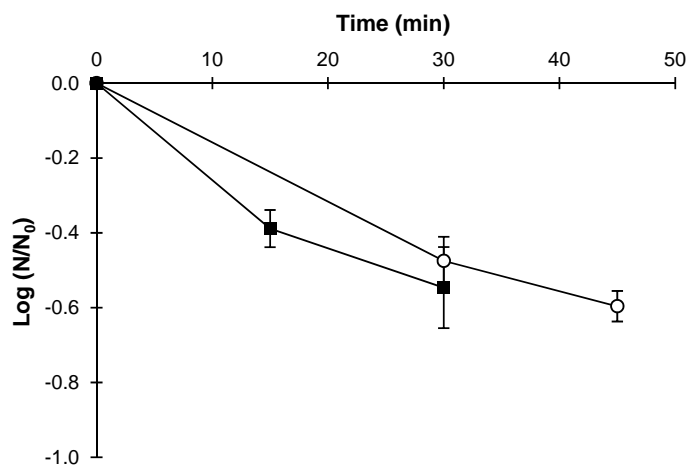
Fig. 1. Survival of *C. sake* yeast cells after fluidised-bed drying at 40 °C (○) and 45 °C (■) for various times. The mean value of six determinations and the standard deviation of the mean are shown.
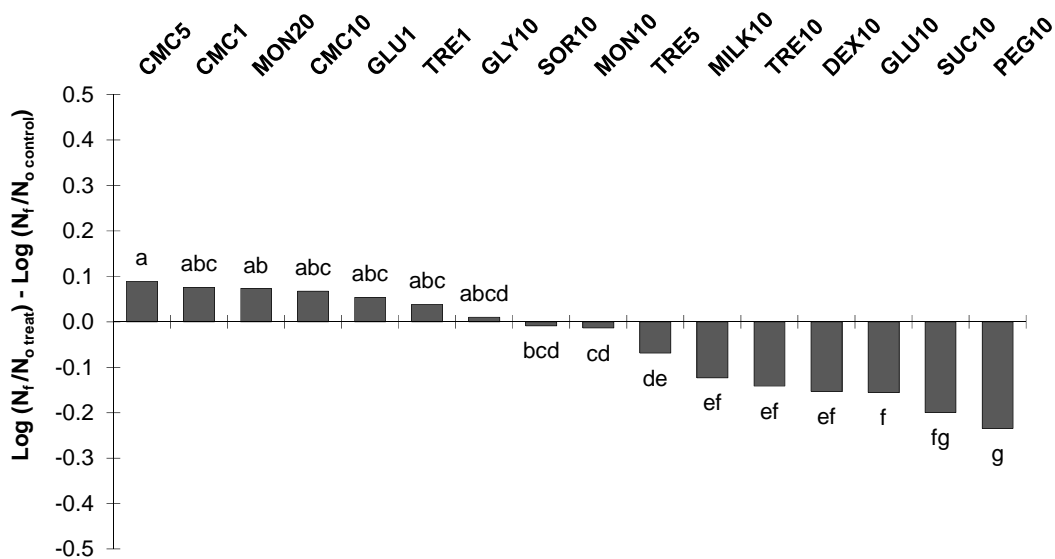
Fig. 2. Effect of protective compounds on the survival of *C. sake* yeast cells after fluidised-bed drying at 40 °C for 45 minutes with potato starch as the carrier. The protective substances used were: 50 g l^{-1} carboxymethyl cellulose (CMC5), 10 g l^{-1} carboxymethyl cellulose (CMC1), 100 g l^{-1} carboxymethyl cellulose (CMC10), 200 g l^{-1} sorbitan monostearate (MON20), 10 g l^{-1} glucose (GLU1), 10 g l^{-1} trehalose (TRE1), 100 g l^{-1} glycerol (GLY10), 100 g l^{-1} sorbitan monostearate (MON10), 100 g l^{-1} sorbitol (SOR10), 50 g l^{-1} trehalose (TRE5), 100 g l^{-1} skimmed milk (MILK10), 100 g l^{-1} trehalose (TRE10), 100 g l^{-1} dextran (DEX10), 100 g l^{-1} glucose (GLU10), 100 g l^{-1} sucrose (SUC10) and 100 g l^{-1} polyethylene glycol (PEG10). Columns with different letters indicate significant differences (*P*<0.05) according to the LSD test.
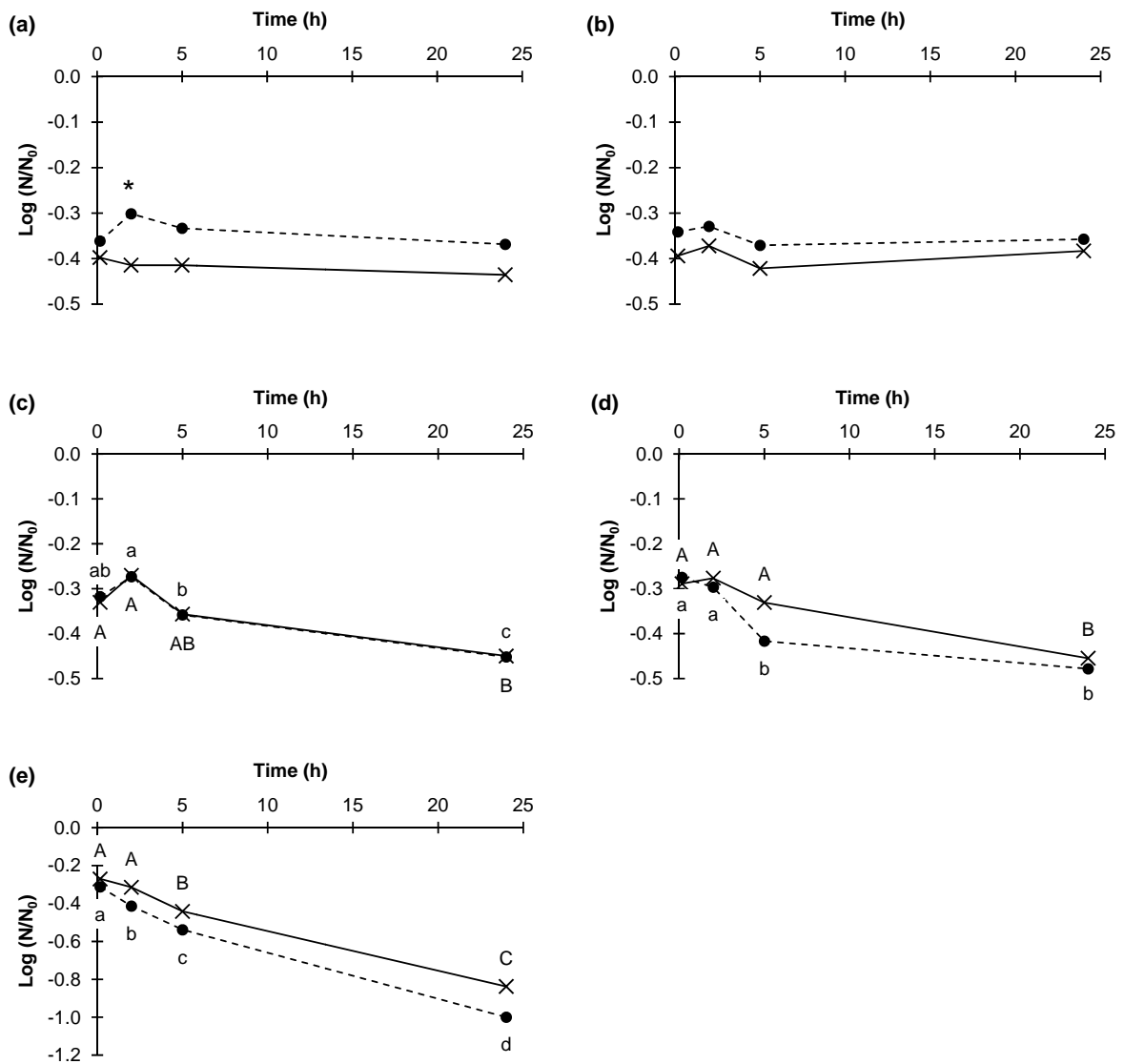
Fig. 3. Survival of formulated *C. sake* cells after rehydration at different temperatures: (a) 6 °C; (b) 15 °C; (c) 25 °C; (d) 30 °C and (e) 35 °C. Phosphate buffer (- ● -) and skimmed milk 10% (—✗—) were used as the rehydration media. The means of six replicates and the separation of means with the LSD test are shown. Means not followed by the same letter are significantly different (*P*<0.05). Upper case letters refer to skimmed milk medium and lower case letters to phosphate buffer. Means marked with an asterisk are significantly different. The lack of a letter indicates that no significant difference was present.
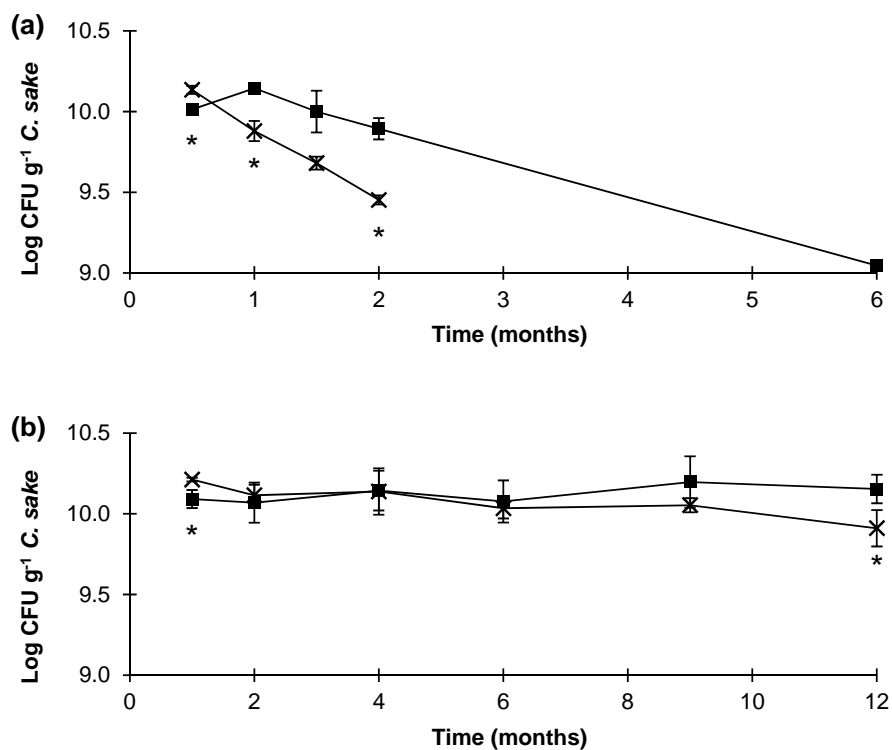
Fig. 4. Shelf life of the optimised *C. sake* formulation at different temperatures: (a) 25 °C and (b) 4 °C, and different storage conditions: vacuum (■) and air-packaged (✕). Mean values of three replicates are shown and the vertical bars indicate the standard deviation of the mean. Means marked with an asterisk are significantly different according to the LSD.
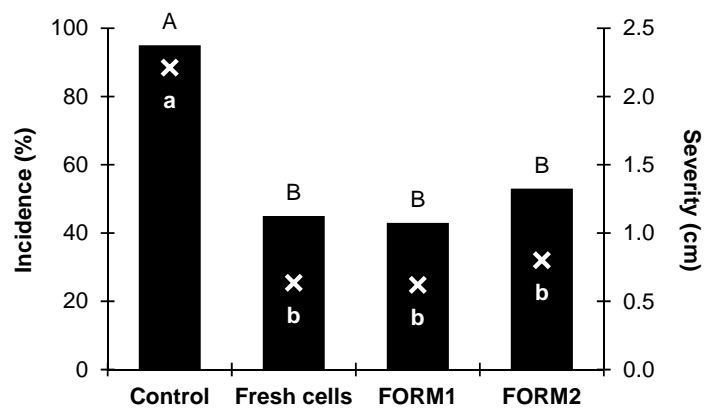
Fig. 5. Efficacy of fluidised-bed drying formulated *C. sake* cells stored a 4 °C for one month and a half (FORM1) and for six months (FORM2) against *P. expansum* on Golden Delicious apples compared with an untreated (control) and fresh cells. The percentage of incidence (■) and the severity (✕) are represented. The values are the means of four replicates. Different upper case letters indicate significant differences for the incidence, whereas different lower case letters indicate significant differences for the severity, both according to the LSD.