Marín-Gozalbo, A.; Atarés Huerta, LM.; Cháfer Nácher, MT.; Chiralt, A. (2017). Stability of biocontrol products carrying Candida sake CPA-1 in starch derivatives as a function of water activity. Biocontrol Science and Technology. 27(2):268-287. https://doi.org/10.1080/09583157.2017.1279587

Stability of biocontrol products carrying *Candida sake* CPA-1 in starch derivatives as a function of water activity

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Acknowledgements

The authors are grateful to the Spanish Government for the financial support from the national project RTA2012-00067-C02 (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Spain and FEDER funds) and to the Conselleria d'Educació of the Generalitat Valenciana, (Spain) for A. Marín's PhD grant. The authors are also thankful to IRTA group, headed by Dra. Neus Teixidó, for the optimization and development of the different formulations.

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ABSTRACT

The preservation and shelf-life of formulations of the biocontrol agent *Candida sake* CPA-1 and starch derivatives as a function of water activity (a_W) was studied in terms of the physical stability of the products and cell viability. Formulations of biocontrol products (BCPs), based on combinations of potato starch and pre-gelatinized potato starch (F1 and F2), or maltodextrins (F3) containing cell protectants, were obtained by fluidized-bed drying. The carriers and the formulated products were stored at 20°C under different a_W conditions. The water sorption and water plasticization behavior of the different products were analyzed through the water sorption isotherms and glass transition temperatures (T_g). Likewise, the viability of *C. sake* over time was determined as a function of the a_W. The solubility of the products was also assessed. Although formulations stored at 20°C and low a_W ($\leq$ 0.33) exhibited a better shelf-life, a significant decrease in cell survival ratio after 180 storage days was observed. Cold storage (5°C) was required to better maintain the cell viability, thus prolonging the shelf-life of BCPs. Formulations containing maltodextrins were the most effective at preserving cell viability and also exhibited the highest water solubility. All the formulations were physically stable at ambient temperature; therefore, the cell stability is the critical point at which to establish both the a_w levels and temperature during storage. Packaging the product using high water vapor barrier material and under cold storage would be necessary to ensure a high number of viable cells and an effective and competitive biocontrol product.

Keywords: biocontrol products, *Candida sake*, cell carriers, maltodextrin, starch, water plasticization, water sorption, cell viability.

1. Introduction

In recent years, the biological control of plant diseases using microbial antagonists as an alternative to chemical products has attracted considerable interest and many potential biocontrol agents (BCAs) have been isolated and tested to ascertain their disease suppression capability (Chumthong, Kanjanamaneesathian, Pengnoo, & Wiwattanapatapee, 2008; Torres *et al.*, 2014). In order to use BCAs practically, it is key to formulate them effectively if they are to be used successfully as biocontrol products (BCPs) (Melin, Schnürer, & Håkansson, 2011).

The primary obstacle in the commercialization of BCPs is the development of shelf-stable products that preserve a high degree of cell viability over time, preferably at ambient temperatures for the purposes of avoiding cold storage. In this sense, solid formulations are preferable to liquid formulations since they allow for easier storage, transport and quality control (Cañamás *et al*., 2008; Fu & Chen, 2011). Drying is the main technique with which to formulate solid BCPs that remain physically and microbiologically stable in long-term storage. Thus, the general object of the drying of BCAs is to enable storage over extended periods of time whilst preserving the cell's viability and its effectiveness against pathogens and also to ensure the retrieval of its metabolic activity and biological properties upon rehydration (Fu & Chen, 2011; Melin, Håkansson & Schnürer, 2007; Morgan, Herman, White & Vesey, 2006). In many cases, it becomes necessary to incorporate adjuvants and protective agents to the formulations so as to preserve the viability of dried cells. Of the protective agents, skim milk and sugars, used either alone or in combination, have been widely used because of their relatively low prices and chemically innocuous nature (Costa, Usall, Teixidó, Torres & Viñas, 2002; ~~Khem, Woo, Small, Chen & May, 2015; Santivarangkna, Higl & Foerst, 2008~~).

Microorganisms can be dehydrated by employing several techniques, such as spray drying or freeze drying, which have commonly been used for the purposes of drying probiotics, starter cultures in the food industry and BCAs (Aponte, Troianiello, Di Capua, Romano & Blaiotta, 2016; ~~Corcoran, Ross, Fitzgerald & Stanton, 2004~~; Costa *et al.*, 2002; ~~Yánez-Mendizabal, Viñas, Usall, Cañamás & Teixidó, 2012~~). Nevertheless, they present some shortcomings related to the loss of cell viability, due to the damaging conditions to which they are subjected during the process. With regards to these drawbacks, fluidized-bed technology might represent a promising alternative method since it presents some advantages over more traditional methods, such as lower temperature gradients and operating times and less extreme water loss (Guijarro, Larena, Melgarejo & De Cal, 2006; Larena, Melgarejo, De Cal, 2003; Morgan *et al.*, 2006). Several authors have studied this technique for the drying of BCAs, obtaining interesting results (Larena *et al.*, 2003; Melin *et al.*, 2007; Mounir *et al.*, 2007). Fluidized-bed drying (FBD) allows granulated solids to be dried while spraying a coating material onto the granulated product. ~~The principle of this technique is the fluidization of solid particles by maintaining them in suspension by blowing hot air through the powder bed (Teunou & Poncelet, 2002). The bed of particles assumes the characteristics of a boiling liquid, hence the term fluidization (Andrade, Skurtys & Osorio, 2012). The coating material is sprayed through a nozzle onto the particles, in the form of a solution or suspension, and its moisture evaporates due to the heat of the air. After a succession of wetting and drying stages, the final dried product is obtained (Jacquot & Pernetti, 2004).~~

In this study, FBD has been employed to obtain biocontrol water-dispersible granular formulations based on the BCA, *Candida sake* CPA-1, in combination with different polymeric carriers in order to ensure a good drying performance and the product's

101 physical stability. *C. sake* has previously been formulated in both liquid and dry forms
102 (Abadias, Usall, Teixidó & Viñas, 2003; Abadias, Teixidó, Usall, Solsona & Viñas,
103 2005; Cañamás *et al*., 2008; Torres, Usall, Teixidó, Abadias & Viñas, 2003) but it has
104 not so far been formulated by means of FBD and in combination with compounds
105 which, in addition to supplying drying feasibility and stability to the cells, permit
106 coating formation when the BCP is applied, thus better supporting the BCA. In previous
107 studies, the combination of *C. sake* with different coating-forming agents has been
108 demonstrated to be effective against the pathogen *Botrytis cinerea* on grapes when
109 applied in liquid form (Marín *et al*., 2016). Edible coatings were able to improve the
110 adherence of *C. sake* to grapes and its survival time and also its efficacy against *B.*
111 *cinerea*, when compared to the application of the antagonist without any support.
112 The correct selection of the components that comprise the final product is essential,
113 since the successful delivery of the BCA, the shelf-life, the stability and effectiveness
114 under the application conditions are all greatly dependent on the formulation (Kinay &
115 Yildiz, 2008). In the FBD formulation, the carrier is the primary material that acts as
116 support for the BCA and allows the bioproduct to be dispersed effectively (Kinay &
117 Yildiz, 2008). From an economic point of view, the production cost is another key
118 factor to be considered and kept to a minimum (Melin *et al*., 2011). For this reason,
119 using starch derivatives as carriers for BCAs is a good option~~, not only~~ because they are
120 both low cost and also readily available (Lafargue, Lourdin & Doublier, 2007).
121 Moreover, starch derivatives offer different advantages: a) they present high critical
122 moisture content values for water plasticization, which is essential if both the drying
123 feasibility and physical stability of the BCP in the glassy state during storage must be
124 ensured (Roos, 1995) b) starch-*C. sake* formulations exhibit high degree of cell
125 viability and are highly effective against *Botrytis cinerea* (Marín *et al*., 2016), c) they

have the ability to form coatings on the treated product, which help to protect the antagonist during the application phase (Cañamás *et al.*, 2011). Several studies have reported the use of starch derivatives as carriers of BCA-based formulations (Lewis, Fravel, Lumsden & Shasha, 1995; Lee *et al.*, 2006) ~~obtained granular formulations with pre-gelatinized starch and the biocontrol fungus *Gliocladium virens*, whose viability was maintained for 6 months at 5°C. Lee *et al*., (2006)~~; some of these obtaining BCA formulations by using FBD (Mounir *et al*., 2007; Soto-Muñoz *et al.*, 2015) ~~developed different wettable powder formulations of *Bacillus lincheniformis*; corn starch was the carrier material that delivered the biocontrol bacteria on tomato most efficiently. Similarly, Mounir *et al*., (2007) used maize starch to produce a formulation of the yeast *Aerobasidium pullulans* by means of FBD, observing a drop in cell viability in the first 30 days; after that period, however, the cell viability remained constant for 7 months at 4°C. Soto-Muñoz *et al*., (2015) studied different dry formulations of *Pantoea agglomerans*, one of which was obtained by means of FBD, using potato starch as carrie~~r. Soluble starch and maltodextrines, both obtained by starch hydrolysis, have also been employed as cell protectants during the freeze drying and FBD of BCAs and probiotics (Stephan, Matos Silva & Bisutti, 2016; ~~Strasser, Neureiter, Geppl, Braun & Danner, 2009~~; Stummer *et al*., 2012).

In terms of the stability of BCP after drying and during storage, water sorption and water plasticization are key features in the physical stability of dry products (Roos, 1995; Rahman, 2009). Water sorption relates water content and water activity while plasticization relates water content and glass transition temperature (T_g) (Nurhadi, Roos & Maidannyk, 2016). Moreover, for a specific dry formulation of BCAs, preserving the viability of the antagonists is of vital importance. Thus, it is necessary to discern how the viability of the BCA is affected by the water activity of the product, which defines

151 the water availability for cells. Likewise, the good dispersion of the dry BCPs in water
152 under practical conditions is another fundamental point, since their application in the
153 field requires a quick solubilization and a simple preparation. If all these characteristics
154 are known, it will permit us to establish the most adequate formulation, and its water
155 activity, in terms of the best physical and microbial stability of the dry BCP and the
156 feasibility of its application.
157 The aim of the present study was to analyse both preservation and shelf-life as a
158 function of the water activity of dry formulations based on starch derivatives used as
159 carriers of the BCA *Candida sake* CPA-1, in terms of the physical stability of the
160 formulations and the cell viability. The solubility in water of the granular formulations
161 was also studied.

162 **2. Materials and methods**

163 **2.1 Materials**

164 Potato starch (PS), pre-gelatinized potato starch (PG) and maltodextrines (MD)
165 (dextrose equivalent, DE: 12) were purchased from Quimidroga. S.A. (Barcelona,
166 Spain). The salts, P_2O_5, LiCl, $MgCl_2$, K_2CO_3, $Mg(NO_3)_2$, KI, NaCl, KCl and K_2SO_4,
167 were supplied by Panreac Química, S.L.U (Barcelona, Spain). Trypticase soy agar and
168 streptomycin sulphate were obtained from Scharlab (Barcelona, Spain) and Sigma –
169 Aldrich (Madrid, Spain), respectively. Sucrose and skim milk powder were food grade
170 products.

171 **2.2 Obtaining of formulations by fluidized-bed drying**

172 BCA formulations containing *C. sake* and carrier, binder and protective agents were
173 obtained with a bottom fluidized-bed dryer (Hüttlin Solidlab 1, Bosch GmbH, Stuttgart,
174 Germany). PG and PS, mixed in different proportions, or MD, were used as carriers of

175 the yeast; were fluidized in powdered form by the air current in the drying chamber of
176 the equipment.

177 The CPA-1 strain of *C. sake* (Colección Española de Cultivos Tipo, Spain, CECT-
178 10817), with proven bioactivity against *B. cinerea* (Cañamás *et al*., 2011) was used in
179 this study. Fresh *C. sake* cells were obtained by liquid fermentation in a BIOSTAT-A
180 modular bioreactor (Braun Biotech 140 International, Melsungen, Germany), as
181 described by Cañamás *et al*., (2011). Then, cell pellets were obtained by centrifugation
182 and suspended in potassium phosphate buffer solution (pH 6.5; KH_2PO_4 0.2 mol/L, 70
183 ml; K_2HPO_4 0.2 mol/L, 30ml and deionized water, 300 ml). A binder agent and
184 protectants were added to the cell suspension and the blend was homogenized using a
185 rotor-stator homogenizer (Ultraturrax T25, Janke and Kunkel, Germany). Then, the cell
186 dispersion was pumped and sprayed on the fluidized carriers as droplets through the
187 nozzle at an approximate flow rate of 4 ml/min. The composition of the different
188 formulations was optimized in a previous study to obtain a target cell count in the
189 powder of 10^9 CFU/g, by applying an inlet air temperature of 55°C for 60 min, which
190 did not to affect *Candida sake* survival (unpublished data). Table 1 shows the
191 composition of the three considered dry formulations, as well as the product's moisture
192 content and final yeast concentration in the products, expressed as CFU/g dry product.

193 **2.3. Water sorption and water plasticization methods**

194 ***2.2.1 Water sorption***

195 Water sorption isotherms of both the different formulations and the carrier materials
196 were obtained via a static gravimetric method (Spiess & Wolf, 1983) at 20°C. Three
197 replicates of each product were accurately weighed using an analytical balance
198 (ME235P-SD, SARTORIUS AG, Germany) and placed in hermetic recipients

containing oversaturated solutions of different salts, which provided the known equilibrium relative humidity (RH). The different salts used were LiCl, $MgCl_2$, K_2CO_3, $Mg(NO_3)_2$, KI, NaCl, KCl and K_2SO_4 and provided an a_W range of 0.11 to 0.98 (Greenspan, 1977). The samples were periodically weighed until constant weight when equilibrium was assumed. The equilibrium moisture content of the samples was determined from their initial moisture content and the corresponding weight gain at equilibrium. Initial moisture content of four samples per product was determined by drying for 24 h at 60°C in a vacuum oven and subsequent conditioning in a desiccator containing P_2O_5.

The experimental data were fitted to the Guggenheim–Anderson-de Boer (GAB) model (Equation 1) over the entire a_W range.

$$W_e = \frac{W_o \cdot C \cdot K \cdot a_w}{(1 - K \cdot a_w) \cdot (1 + (C - 1) \cdot K \cdot a_w)} \quad (1)$$

where W_e is the equilibrium moisture content on dry basis; W_0, the monolayer moisture content; and C constant related to the heat sorption of multilayer and K factor correcting properties of the multilayer molecules (Bizot, 1983)

2.2.2 Water plasticization

The glass transition temperature (T_g) of the different products and carriers was determined as a function of their a_w by means of differential scanning calorimetry (DSC) using a DSC TA Instruments, model DSC1 STAR System, Mettler Toledo, Switzerland. The measurements for the different formulations and carrier materials conditioned at the different a_w were taken in duplicate. For that purpose, samples of approximately 9 mg were weighed and sealed in aluminium pans. An empty pan was used as reference. Three cycles of scanning (heating-cooling-heating) at 10°C/min were performed using a 20 mL/min nitrogen flow. The temperature range of each

measurement was fitted according to the sample moisture content at between 0 and 160ºC. T_g was determined as the midpoint temperature of the glass transition in the second heating scan.

The relationship between T_g and water content at various water activities were modelled by using the Gordon & Taylor equation (Equation 2).

$$T_g = \frac{(1 - x_w) \times T_{g(s)} + k \times x_w \times T_{g(w)}}{(1 - x_w) + k \times x_w} \quad (2)$$

were x_w is the moisture content; $T_{g(s)}$ is the T_g value of the anhydrous solids; $T_{g(w)}$ is the T_g value of the amorphous water; and k is a model parameter.

The goodness of fit for both water sorption and water plasticization was analysed using the value of relative percent root mean square (Equation 3), whose value of under 10 indicates the very good fit of the model (Rizvi, 2005).

$$\%\, RMS = \left[\sqrt{\frac{\sum \left[\frac{M^{exp} - M^{calc}}{M^{exp}} \right]^2}{N}} \right] \times 100 \quad (3)$$

2.3 Viability of *Candida sake* during storage

In order to analyse the influence of the product's water activity and storage time on the yeast cells, the viability of *C. sake* in the different formulations stored at 20°C at different RH% was determined after 2, 7, 14, 30, 60 and 180 days of storage. Samples of the different formulations were placed in desiccators over different relative humidities (11, 33, 43, 54 and 69%), which were achieved with oversaturated solutions of different salts as explained in section 2.2.1. Likewise, the viability of *C. sake* in the different formulations was also evaluated in the products with their original a_w, stored in hermetic jars at 5°C after 90 and 180 days.

To analyze yeast viability, 0.5 g of each product was dispersed in deionized sterile water (15, 20 o 5 ml for F1, F2 and F3 respectively) for 1 min, using a vortex shaker to achieve the complete dispersion of the granular products. After 9 min of repose time to promote complete cell rehydration (Yánez-Mendizabal *et al.*, 2012), serial dilutions were performed in duplicate and plated in TSA agar medium plates with streptomycin sulphate at 0.5 g/L to prevent bacterial growth. Plates were incubated at 25°C for 48 h and *C. sake* colonies were then counted based on their morphological characteristics. Results were expressed as log CFU per gram of dry solids in the formulation. Each assay was carried out in triplicate.

The experimental data were fitted to the Weibull model (Equation 4) in order to describe survival curves (~~Albert & Mafart 2005~~; Coronel-Aguilera, Jiménez-Munguía & López-Malo, 2009).

$$\log N = \log N_0 - \left(\frac{t}{\delta}\right)^p \qquad (4)$$

where N is the number of microorganisms at time t; N_0 is the initial number of microorganisms; δ is the time that causes a one log reduction in the cell population; and p is a dimensionless shape parameter.

2.4. Solubility analysis

The water solubility of the dry formulations was determined at 5, 15 and 25°C for different contact times (between 5 and 50 minutes) to evaluate how temperature and time may influence the rehydration of the powders. The tests were carried out under mild agitation conditions (200 rpm) in order to simulate that might take place in agitation tanks in the case of in-field applications. The determination of the solubility was conducted following the method described by Cano-Chauca, Stringheta, Ramos & Cal-Vidal (2005), with some modifications. Specifically, 0.25 g of sample dispersed in

25 mL of deionized water and stirred with a magnetic stirrer at 200 rpm for each time and temperature. Afterwards, the samples were centrifuged at 3000×g for 5 min to separate the non-solubilized phase. Then, 6.25 mL of the supernatant were transferred into pre-weighed glass Petri dishes, which were oven-dried for 5h at 105°C to determine the mass of dissolved solids per ml. The solubility was expressed as % of dissolved solids with respect to the initial mass of dry powder. The assay was carried out in triplicate. Solubility data were fitted to Peleg model (Equation 5) (Peleg, 1988).

$$S = S_0 + \frac{t}{K_1 + K_2 \times t} \quad (5)$$

where S is the percentage of solubilized solids at time t; S_0 is the instantaneous solubility; t is time (min), K_1 and K_2 are the Peleg rate (min) and Peleg capacity constant ($\%^{-1}$), respectively

2.5 Statistical analysis

Statistical comparisons were made through an analysis of variance (ANOVA) using Statgraphics Centurion XVI version 16.1.17 (Manugistics Corp., Rockville, Md.). The differences were considered significant when $p < 0.05$. The viability data in CFU/g were log-transformed (log CFU/g) in order to improve the homogeneity of variances.

3. Results and discussion

3.1 Water sorption and water plasticization of the products

The water sorption and water plasticization behaviour of the different formulations was analysed, in comparison with that of the carriers used, PS, PG and MD, as the main components of the formulations. The moisture content and T_g values of each formulation, equilibrated at the different levels of a_w at 20 ºC, are shown in Table 2, which also shows the corresponding values for carriers. It is possible to observe the

298 greater water uptake of F1, in agreement with its greater proportion of PS, which
299 exhibited the greatest water sorption capacity (Figure 1). The isotherms obtained for the
300 BCPs and carriers were well fitted by the GAB model (Figure 1). The GAB parameters
301 for formulations and carriers are shown in Table 3, together with the %RMS, whose
302 value of under 10 indicated the very good fit of the model (Rizvi, 2005).
303 The initial moisture contents of the dry BCP formulations were 8.81 ± 0.05, 6.90 ± 0.50
304 and 6.75 ± 0.13 g/100 g product, respectively, for F1, F2 and F3; these were close to the
305 values of the respective monolayer moisture content (Table 3), which corresponded to
306 a_w values of nearly 0.33 after the drying step. The moisture content of dried powders is
307 usually within the range of the monolayer content if process conditions are adequately
308 optimized (Fabra, Márquez, Castro & Chiralt, 2011). In this sense, the isotherms
309 obtained corresponded to the adsorption curves for a $a_W \geq 0.33$ (the main part of the
310 curve) and to the desorption data for $a_w < 0.33$.
311 The carrier exhibiting the highest water binding capacity was PS, and the obtained
312 isotherm was similar to that previously reported for potato starch (Anzai, Hagiwara,
313 Watanabe, Komiyama & Suzuki, 2011; Bizot, 1983). Likewise, although Torres & Seijo
314 (2016) reported very similar values of GAB parameters in the case of water adsorption
315 of rice starch at 25ºC, the water binding capacity decreased in the desorption isotherms.
316 Of the three formulations, F1 had the greatest proportion of PS, and this formulation
317 also exhibited a more marked water binding capacity compared to F2 and F3, whose
318 water sorption behaviour was very similar. The high mean molecular weight of the
319 substrates resulted in a limited water gain at the highest a_w levels due to the low
320 incidence of solute-solvent effects, which produce great water gains with relatively
321 small increases in a_w when low molecular solutes are present in the matrices. These
322 effects were more marked MD due to the presence of free glucose molecules, which

interact with water molecules through solute-solvent mechanisms at high a_w. The obtained MD isotherm was similar to that previously reported by Nurhadi *et al*., (2016) for maltodextrine DE 10. Previously reported water sorption data for pre-gelatinized starch (PG) also showed a loss in the water binding capacity of the starch polymers as compared with that of the granules due to the loss in native structure where more water can be retained (Carvalho, 2008).

As regards BCP formulations, it was remarkable that the up interval of isotherms was less pronounced in the formulated BCPs than in carriers, which could be due to the effects of the cells on the product moisture control at high a_w values. A reduction in the availability of low molecular sugars could be brought about by cell consumption, thus decreasing the solute-solvent effects that influence the water binding capacity at high a_w values. To the best of our knowledge, there are no published studies about the water sorption behavior of BCA-based dry formulations using starch derivatives as carriers.

As far as water plasticization is concerned, Figure 2 shows the typical DSC thermograms corresponding to the first heating scan of the products equilibrated at different a_W. As is commonly observed in starch derivatives, relaxation endotherms appeared near the glass transition and which disappeared in the second scan (Nurhadi *et al*., 2016). This has been attributed to the relaxation of a part of the amorphous phase, when molecular rearrangement occurs during aging. As observed by Nurhadi *et al*., (2016), relaxation endotherms were not present at very low a_w in the case of MD, due to the fact that relaxation times at lower water contents are longer, as expected. To avoid enthalpy relaxation effects, the second scan was considered to determine T_g. The midpoint glass transition values of the different formulations are shown in Table 2 and Figure 3 reflects the water plasticization effects in terms of a_w and moisture content. As expected, T_g decreased as the moisture content or a_w increased, since, in biological

materials, water plasticizes the amorphous structures (Roos & Karel 1991). On a kinetic level, the glassy state is considered to be more stable than the rubbery state (Cano-Chauca *et al*., 2005; Genin & René 1995) and so, in addition to the physical stability of the powders, cell viability could also be affected by the state of the carrier's solid matrix.

The plasticization behavior varied markedly from carrier to carrier, with MD being the most sensitive to water plasticization in line with the greatest proportion of low molecular compounds (hydrolysed sugars). Both PS and PG were less plasticized as previously observed by other authors (Perdomo *et al*., 2009), due to the lack of low molecular compounds which interact with water molecules through solute-solvent interactions. In this sense, the fitting of Gordon & Taylor equation gave values of k parameter higher for MD than for PS and PG, in agreement with its greater sensitivity to water plasticization. The obtained values of Gordon and Taylor parameters for the MD carrier were similar to those previously reported (Nurhadi *et al*., 2016) for DE 10 MD.

The water plasticization behavior of BCPs greatly differed from that of the carriers. An unexpected plasticizing effect of water was observed for the three cell-containing formulations, which, in turn, showed a very similar trend of T_g-a_w relationships. T_g varied within a narrow interval, between 45-60 ºC, in the 0.11-0.75 a_w range. At low a_w levels, BCPs showed lower T_g values than the carriers while the T_g of the BCPs decreased to a lesser extent when the water content increased. This behavior was not previously observed for other encapsulated microbial cells. For instance, for lactic acid bacteria encapsulated in MD and whey proteins, the T_g values did not significantly differ from those of the encapsulating carriers (Ying, Sun, Sanguansri, Weerakkody & Augustin, 2012). However, the presence of yeast cells in the formulations greatly affected the water plasticizing effects in the studied BCPs. This behaviour suggests that,

whereas the solid composition determines the water uptake capacity of the product at a given a_w, the cells could retain a determined amount of water molecules in their mechanisms for survival, making them more or less available to plasticize the solids as a function of the cell demand. The secretion of some low molecular metabolites by the yeasts could also contribute to the low T_g values at low a_w. The similar T_g-a_w relationship for the different BCPs seems to indicate that cell action carried more weight in water plasticization than the different solid composition of the BCP.

From the obtained data, it can be seen that in no case was the critical moisture exceeded for products stored at a relative humidity of below 75% at 20ºC. So, the glassy state can be assumed in all the BCPs stored at under 11, 33, 43, 54 and 69% RH, where the cell viability was analysed as a function of storage time.

3.2 Viability of *Candida sake* during storage

For the purposes of identifying the optimum moisture content of the BCPs in order to better maintain cell viability during storage at 20°C, samples were stored under different RH (11 to 69%). The cell counts of *C. sake* in F1, F2 and F3 throughout storage time under these conditions are shown in Figure 4.

The initial cell viability was 9.04, 9.02 and 9.16 log CFU/g dry product for F1, F2 and F3, respectively. When stored at a_W of up to 0.43, the formulations completely lost cell viability throughout the tested period. This loss was especially significant at 0.69, since none of the formulations showed any viable cells of *C. sake* after 14 days of storage. However, storage at low a_W levels (0.11 and 0.33) better preserved the cells of the antagonist in every case. These results agree with that reported by Dunlap & Schisler (2010) for a dry formulation of the yeast *Cryptococcus flavescens.* In their study, the yeast's storage stability varied significantly across the tested a_w range (0.22-0.57); products stored at 0.22 a_w exhibited the best long-term survival of the yeast, while those

stored at 0.57 showed the worst shelf-life. Other authors have reported similar results for the viability of probiotics in powder formulations, which exhibited a total loss of viability when stored at 0.52 a_W at 25°C within 22 days (Poddar *et al*., 2014). However, storage at a_w 0.11 gave rise to the slowest decline in the viable bacterial count. To explain this behavior, Moore, Langewald & Obogno (1996) pointed to the adverse effect of moisture gain in cell viability when the product rehydrates at high RH, due to the rapid water uptake by dry cells, which may cause membrane damage. On the other hand, under high moisture conditions the dormant state acquired by the yeast cells in the drying process is reverted and the available water and nutrients are insufficient to allow cells to perform their vital functions and, consequently, their death occurs.

Although formulations stored under low a_W conditions showed a better shelf-life, a significant ($p < 0.05$) decrease in the antagonist's survival time was observed for the three BCPs after 180 days. The decrease in viability was above 3 log units in every case. Specifically, the viability of *C. sake* was reduced to 50% (in log scale) in F1 stored at 0.11 a_W, whilst at 0.33 a_W, no viable cells were found at this time. 41 and 47% viability was lost in the case of F2 at a_w 0.11 and 0.33, respectively, and 33 and 38% in the case of F3. These results are not satisfactory from a commercial point of view, which requires the BCPs to have a shelf-life of at least 6 months and preferably 1-2 years, without a significant reduction in the initial number of viable cells (Pusey, 1994; Rhodes, 1993).

As regards the influence of carrier composition, F1 was the worst support in terms of *C. sake* cell viability, which may be related to its greater water binding capacity, possibly contributing to a faster cell death. Conversely, the lowest rate of yeast death was obtained with the formulation based on MD (F3) containing protectants (sucrose and milk powder), which may help to keep the cells alive. This observation is supported by

423 the fact that sucrose, lactose and other disaccharides, are known to extend microbial
424 survival via hydrogen bonding to the polar head group of the cell membrane
425 phospholipids, thus protecting them from the drying injuries (Corcoran *et al*., 2004;
426 Crowe, Crowe & Chapman, 1984; Stummer *et al*., 2012) Additionally, milk could
427 supply a variety of nutrients that favour the survival of *C. sake* (Costa *et al*., 2002).
428 The number of viable cells changed throughout storage time and these changes could be
429 fitted to the Weibull model (Figure 4) at a_W from 0.11 to 0.54, up to a critical time when
430 a sharper drop in viable cells occurred (limit time for the fitting). The obtained
431 parameters, related to the microbiological stability of the different formulations for each
432 a_W, can be seen in Table 4. The values of δ (time that causes a 1 log reduction in the cell
433 population) decreased as the a_W rose in every case. Likewise, F1 exhibited the lowest
434 values of δ, confirming that this support was the least adequate to carry *C. sake*,
435 whereas F3 allowed the highest δ values to be obtained, while showing a greater
436 number of viable cells in the second period with faster cell death.
437 Since temperature is a key factor for microbiological stability, BCPs with their initial
438 moisture content (a_W of about 0.33) were also stored at a low temperature (5ºC). Figure
439 5 shows the cell survival ratio for F2 and F3 products, which exhibited the best
440 microbial support, stored for 90 and 180 days at 5 and 20 ºC. The longer cell survival
441 time was observed at low temperatures for both cases. Similar results have been widely
442 reported with other BCAs (Kinay & Yildiz, 2008; Mejri, Gamalero & Souissi, 2013;
443 Torres *et al*., 2014). Temperatures of 4 – 10°C cause both the cell division and
444 metabolic rate of microorganisms to slow down. In this situation, cells are capable of
445 withstanding the depletion of nutrients and the accumulation of toxic metabolites (Mejri
446 *et al*., 2013; Trivedi, Pandey & Palni, 2005). Thus, storing the BCPs at a low
447 temperature maintains the microorganism in a state of low metabolic activity (Elzein,

448 Kroschel & Müller-Stöver, 2004) and this would be recommendable for the particular
449 case of the studied *C.sake* formulations. Although cold storage implies a higher product
450 cost, low temperatures greatly favour the cell viability in long-term storage.

451 **3.3 Solubility analysis**

452 The values of the percentage solubility of the BCPs as a function of stirring time at
453 different temperatures are plotted in Figure 6. Formulation type and temperature
454 affected the solubilization kinetics. As can be observed in the Figure, F3 exhibited the
455 fastest solubilization, reaching levels of nearly 100% after 20 min of stirring at 15 and
456 25°C. Likewise, similar behavior was observed for F1 and F2 at a given temperature,
457 both showing a more limited solubility. This behavior is coherent with the BCP
458 composition. Maltodextrines are reported as starch derivatives with the highest water
459 solubility (Cano-Chauca *et al.*, 2005) and so, the more soluble components of F3
460 contribute to its faster water dissolution. The presence of PS or PG hindered the
461 solubility of the powders, in line with the lower solubility of amylose and amylopectin
462 chains, especially in the starch granules where they are in a semi-crystalline structure
463 (Eliasson & Gudmundsson 1996; Mandala & Bayas 2004).
464 In every case, the solubility curves showed two steps; in the first step, a slower increase
465 in soluble solids was observed over time, while a fast dissolution of one part of the
466 powder occurred. In the second step, an slow, asymptotic increase in soluble solids was
467 observed. In most of the cases, the first step took about 10 minutes at 5 or 15ºC,
468 whereas it was shorter (7.5 min) at 25ºC, and 15ºC in F3. During this step, different
469 amounts of solids were instantaneously dissolved (S_0) depending on the formulation
470 and temperature, whereas another part rehydrated before their slower dissolution
471 (retarded solubility). These results agree with what was reported by Fang, Selomulya &
472 Chen (2008) for food powder rehydration, which takes place through different phases:

473 the wetting of particles overcoming the surface tension at the solid-liquid interface,
474 followed by its dissolution. Table 5 gives the S_0 values corresponding to the
475 instantaneous solubility of the products, which greatly increased as the temperature rose
476 and was markedly higher for the F3 product. The Arrhenius plot for S_o values allows
477 the activation energy to be determined for each product, these values being 24, 42 and
478 10 KJ for F1, F2 and F3, which indicates that more temperature requirements are
479 needed to dissolve the F2 product instantaneously. The increase in kinetic energy
480 generated by higher temperatures allows the solvent molecules to break apart the solute
481 molecules that are held together by intermolecular attractions more effectively. Data up
482 to S_0 (retarded solubility period) were fitted by Peleg equation (Equation 5) to predict
483 the total percentage solubility as a function of temperature and time for each product.
484 Table 5 also shows the constants of the model and the predicted asymptotic value of
485 solubility (S_∞). It can be observed that, whereas temperature notably affected S_0 values,
486 kinetic constant K_1 was less affected by temperature in the considered range. No
487 significant differences in the K_1 values of the different products between 15 and 25ºC
488 were found. The differences in the values at 5ºC can be attributed to the marked
489 difference in S_o values, which affected the driving force for retarded dissolving of the
490 different products. In this sense, F3, which had higher S_0 values, exhibited a slower
491 solubilization rate (the inverse of K_1) in the retarded period. Values of K_2 are related
492 with the asymptotic value S_∞ (Table 5). No significant differences in K_2 values were
493 obtained for F1 and F2 at the different temperatures, whereas greater values were
494 obtained for F3 which increased as the temperature rose. This indicates that similar
495 amounts of product (about 60%) were dissolved in the retarded period for F1 and F2,
496 regardless of the temperature, and that the differences in S_∞ were mainly determined by

497 S_0 values. In F3, total dissolution was obtained at every temperature, with about 30 and
498 45 % being the solubility in the retarded period at 5 ºC and 15-25, respectively.
499 From this analysis, the F3 formulation would be the best one for the purposes of the in-
500 field application of BCPs as water dispersion, since solubility is considered a key
501 quality characteristic for product reconstitution in order to avoid prolonging the process
502 (Selomulya & Fang 2013; Hla & Hogekamp, 1999). F3 exhibited the highest
503 instantaneous dissolution and total dissolution within the 5 to 25ºC range, which is a
504 common range for practical applications.
505
506 **4. Conclusions**
507 The viability of *C. sake* during storage at 20 ºC was highly dependent on the water
508 activity of the formulated BCP. The best preservation was obtained at a_w values below
509 0.33. This value corresponds to the monolayer moisture content of the products, when
510 water was strongly bonded to the solid matrix, and is their usual water content after
511 drying. MD was the starch derivative that best supported the yeast in terms of the
512 preservation of cell viability at low a_w. The incorporation of protectants (sucrose and
513 milk powder) could also contribute to the improvement in the functionality of the
514 carrier. This formulation also exhibited the best water solubility, which is a key factor
515 for BCP applications. Nevertheless, 20ºC is not low enough to maintain an adequate
516 cell count for prolonged storage times and cold storage would be required to ensure an
517 appropriate BCP shelf-life. Likewise, material with water vapour high barrier properties
518 must be used in the product packaging so as to avoid moisturizing, since water uptake
519 would lead to important significant losses in cell viability. The knowledge acquired in
520 this study provides a basis that might guide both the development of BCPs based on
521 similar antagonists and also the choice of the optimal storage conditions.
522

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722

723 **TABLES**

724 Table 1. Composition of the different BCP formulations of *Candida sake*. PS: potato
725 starch; PG: pregelatinized potato starch; MD: maltodextrines, Su: sucrose, SMP: skim
726 milk powder, MC: moisture content, d.s.: dry solids

Formulation	Carrier	Binder (1)	Protectant(2)	MC (3)	CFU/g d.s.
F1	PG:PS (1:2)	PG	-	8.81 ± 0.05	$1.57 \cdot 10^9$
F2	PG:PS (2:1)	PG	-	6.85 ± 0.53	$1.07 \cdot 10^9$
F3	MD	MD	Su:SMP (2:1)	6.75 ± 0.13	$1.47 \cdot 10^9$

727 (1): 1.16 g/100 g carrier (2): 20 g/100 g carrier (3): g water/100 g
728 product

729

730 Table 2. Values of glass transition temperature (T_g) and moisture content (MC: g
731 water/100 g product) of the BCP formulations and of the carriers equilibrated at
732 different a_W. PS: potato starch; PG: pregelatinized potato starch; MD: maltodextrines.

	F1		F2		F3	
a_W	**T_g (°C)**	**MC (%)**	**T_g (°C)**	**MC (%)**	**T_g (°C)**	**MC (%)**
0.11	53 ± 1^{e}	5.4 ± 0.2^{a}	55 ± 1^{c}	2.4 ± 0.1^{a}	62 ± 1^{c}	3.7 ± 0.1^{a}
0.33	50 ± 1^{d}	8.7 ± 0.1^{b}	51 ± 1^{bc}	6.4 ± 0.2^{b}	57 ± 1^{cd}	5.7 ± 0.1^{b}
0.43	48 ± 1^{c}	10.4 ± 0.1^{c}	49 ± 2^{ab}	7.3 ± 0.1^{c}	51 ± 2^{b}	7.3 ± 0.1^{c}
0.54	46 ± 2^{ab}	11.9 ± 0.7^{d}	46 ± 3^{a}	8.6 ± 0.2^{d}	51 ± 1^{b}	7.9 ± 0.3^{c}
0.69	44 ± 1^{a}	14.1 ± 0.1^{e}	46 ± 1^{a}	11.4 ± 0.4^{e}	45 ± 1^{a}	10.6 ± 0.4^{d}
0.75	44 ± 1^{a}	15.3 ± 0.1^{e}	45 ± 1^{a}	12.7 ± 0.1^{f}	45 ± 1^{a}	12.6 ± 0.3^{e}
	PS		**PG**		**MD**	
a_W	**T_g (°C)**	**MC (%)**	**T_g (°C)**	**MC (%)**	**T_g (°C)**	**MC (%)**
0.11	114 ± 3^{e}	8.0 ± 0.1^{a}	100 ± 2^{f}	4.2 ± 0.1^{a}	149 ± 2^{f}	4.5 ± 0.3^{a}
0.33	89 ± 1^{d}	13.7 ± 0.5^{b}	70 ± 1^{e}	6.8 ± 0.1^{b}	112 ± 2^{e}	6.3 ± 0.1^{b}
0.43	70 ± 1^{c}	15.6 ± 0.1^{c}	55 ± 1^{d}	7.9 ± 0.3^{c}	86 ± 1^{d}	9.4 ± 0.2^{c}
0.54	60 ± 2^{b}	17.4 ± 0.1^{d}	50 ± 2^{c}	8.6 ± 0.1^{d}	62 ± 1^{c}	13.1 ± 0.2^{d}
0.69	51 ± 1^{a}	19.6 ± 0.1^{e}	44 ± 1^{b}	10.9 ± 0.3^{e}	41 ± 1^{b}	13.3 ± 0.1^{d}
0.75	49 ± 1^{a}	20.0 ± 0.1^{e}	40 ± 1^{a}	11.9 ± 0.3^{f}	31 ± 1^{a}	15.0 ± 0.5^{e}

733

734 Different superscripts (a – f) in the same column indicate statistically significant
735 differences ($p < 0.05$) for the same formulation or carrier.

736

737 Table 3. GAB and Gordon & Taylor parameters of the different BCP and of the carriers.
738 PS: potato starch, PG: pregelatinized potato starch, MD: maltodextrines, W_0: monolayer
739 moisture content (g water / 100 g dry solid); C: constant related to the heat sorption of
740 multilayer, K: factor correcting properties of the multilayer molecules, r^2: correlation
741 coefficient, %RMS: relative percent root mean square.

GAB	**F1**	**F2**	**F3**	**PS**	**PG**	**MD**
W_0	8.94	7.38	5.82	14.17	5.88	5.53
C	15.89	5.12	13.72	17.75	28.45	24.74
K	0.71	0.75	0.81	0.68	0.85	0.90
r^2	0.99	0.99	0.85	0.99	0.97	0.88
% RMS	0.27	0.45	3.47	4.07	1.60	5.66
Gordon & Taylor	**F1**	**F2**	**F3**	**PS**	**PG**	**MD**
Tg(s)	-	-	-	172	143	212
K	-	-	-	2.6	4.8	5.8
r^2	-	-	-	0.99	0.99	0.99
% RMS	-	-	-	5.1	10	13

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743

744 Table 4. Weibull parameters from viability of *Candida sake* in the BCP formulations
745 based on starch derivatives. p: dimensionless shape parameter, δ: time, in days (d), that
746 causes a one log reduction in the cell population. The limit time (t_l) for time of the
747 model is also shown.

	F1			F2			F3		
a_W	**p**	**δ (d)**	**t_l (d)**	**p**	**δ (d)**	**t_l (d)**	**p**	**δ (d)**	**t_l (d)**
0	0.68	25	≤ 180	0.51	27	≤ 90	0.12	6137	≤ 90
0.33	0.56	15	≤ 90	0.37	57	≤ 90	0.31	51	≤ 90
0.43	0.37	1	≤ 90	0.52	18	≤ 30	0.26	43	≤ 14
0.54	0.36	0.3	≤ 14	0.76	8	≤ 30	0.70	4	≤ 14

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749

750 **Table 5**. Peleg parameters obtained from the fitting of solubility data for the BCP
751 formulations as a function of the temperature. S_0: instantaneous solubility, S_∞:
752 asymptotic value of solubility.

BCP	T (°C)	S_0 (%)	K_1 (min)	K_2 ($\%^{-1}$)	S_∞
	5	21	0,077	0,016	82
F1	**15**	37	0,141	0,015	102*
	25	42	0,155	0,014	112*
	5	12	0,062	0,016	74
F2	**15**	33	0,158	0,014	105*
	25	41	0,127	0,015	110*
	5	54	0,293	0,018	109*
F3	**15**	71	0,238	0,029	106*
	25	74	0,207	0,034	104*

753
754 *Values > 100 related to the mathematical fitting
755 Mean variation coefficient for S determinations: 6%
756 $S_\infty = S_0 + 1/K2$
757

FIGURE CAPTIONS

Figure 1. Moisture sorption isotherms of: (a) BCP formulations (F1, F2, F3) and (b) carriers (PS: potato starch, PG: pregelatinized potato starch, MD: maltodextrines). The solid lines represent the GAB model fitted curves

Figure 2. Glass transition temperatures of the BCP and carrier materials as a function of the a_W (a) and moisture content (g water/100 g product) (b). PS: potato starch, PG: pregelatinized potato starch, MD: maltodextrines. The solid lines in (b) represent the Gordon & Taylor model fitted curves

Figure 3. Typical DSC thermograms of the BCP formulations of *Candida sake* and starch derivatives

Figure 4. Viability of *Candida sake* in the BCP formulations during storage at 20°C under different a_W: ■ a_W = 0.11, ▲ a_W = 0.33, ● a_W = 0.43, ◆ a_W = 0.54, ✳ a_W = 0.69. The solid lines represent the Weibull model fitted curves

Figure 5. Viability of *Candida sake* referred to the initial counts in the BCP formulations (F2 and F3) stored at 20°C or 5°C

Figure 6. Percentage of solubility of the BCP formulations as a function of contact time at 5 (black symbols), 15 (grey symbols) or 25 °C (open symbols) for F1 (circles), F2 (squares) and F3 (triangles). Starting time for the retarded solubility period (t_0) is indicated.