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Predicted ecological niches and environmental resilience of different formulations of the biocontrol yeast *Candida sake* CPA-1 using the Bioscreen C

Carbó, A.[1], Torres, R. [1], Teixidó, N. [1], Usall, J.[1], Magan, N. [2] and Medina, A.[2]

[1]IRTA, XaRTA-Postharvest, Edifici Fruitcentre, Parc Científic i Tecnològic Agroalimentari de Lleida, 25003 Lleida, Catalonia, Spain. [2]Applied Mycology Group, Cranfield Soil and AgriFood Institute, Cranfield University, Cranfield, Bedford MK43 0AL, United Kingdom.

Corresponding author:

Name: Neus Teixidó

Full address: IRTA, XaRTA-Postharvest, Edifici Fruitcentre, Parc Científic i Tecnològic Agroalimentari de Lleida, 25003 Lleida, Catalonia, Spain.

Telephone: +34 973003429

Fax: +34 973238301

Email: neus.teixido@irta.cat

Anna Carbó is a PhD student, currently based at IRTA-Fruitcentre, working on development of microorganism-based products to control pre- and postharvest diseases.

Dr. Rosario Torres is a researcher in postharvest pathology, mainly focused on fruit-pathogen interactions and on development of molecular tools to identify and quantify fungal pathogens and biocontrol agents.

Dr. Neus Teixidó is the head of the Postharvest program at IRTA, with a wide expertise in postharvest plant pathology and in production, formulation and stabilization of biocontrol agents (domestication).

Dr. Josep Usall is actually the IRTA CEO and before that the head of the Postharvest program, with 25+ years of expertise in postharvest plant pathology and alternative strategies to synthetic fungicides.

Prof. Naresh Magan is a Plant Pathologist and Head of the Applied Mycology Group, Cranfield University. He has been working on ecophysiology, improvement and formulation of biocontrol agents for 30+ years.

Dr. Angel Medina is a Senior Lecturer in Food Mycology at Cranfield University who has been working on molecular ecology and ecophysiology of fungi for 10+ years. He has developed rapid methods for quantifying ecological impacts on both beneficial and harmful fungi.

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Abstract

Environmental resilience of biocontrol microorganisms has been a major bottleneck in the development of effective formulations. *Candida sake* is an effective biocontrol agent (BCA) against *Penicillium expansum, Botrytis cinerea or Rhizopus stolonifer*, and different

formulations of the BCA have been optimised recently. The objective of this study was to compare the relative tolerance of different dry and liquid formulations of the biocontrol yeast *Candida sake* CPA-1 to interacting environmental conditions using the Bioscreen C. Initially, the use of this automated turbidimetric method was optimised for use with different formulations of the biocontrol yeast. The best growth curves were obtained for the *C. sake* CPA-1 strain when grown in a synthetic grape juice medium under continuous shaking and with an initial concentration of 10^5 CFUs ml^{-1}. All the formulations showed a direct relationship between optical density values and yeast concentrations. Temperature (15-30°C) and water activity (a_w; 0.94-0.99) influenced the yeast resilience most profoundly, whereas the effect of pH (3-7) was minimal. In general, the liquid formulation grew faster in more interacting environmental conditions but only the yeast cells in the dry potato starch formulation could grow in some stress conditions. This rapid screening method can be used for effective identification of the resilience of different biocontrol formulations under interacting ecological abiotic conditions.

Keywords: dehydrated yeast; temperature; stress; ecological niche, interacting environmental conditions; biocontrol; survival; *Candida sake*

1. Introduction

The development of biocontrol agents (BCAs) has received significant impetus as many crop protection chemical groups have been removed from the EU list of approved compounds for use in agriculture/horticultural crops (European Parliament 2009). This has been partially because of the need to protect the environment and minimise consumer exposure to such chemicals (Droby et al. 2009). Because BCAs are living organisms, their usage and efficacy are strongly dependent on environmental conditions, whereas for chemical products this effect is less significant. The latter products can be applied effectively over a wide range of climatic conditions. A key bottleneck for the use of BCAs in the field has been the narrow relative

humidity (RH) and sometimes temperature range for establishment and colonisation of foliar plant parts. Moreover, other unfavourable factors such as UV radiation or rainfall events could limit the development of BCAs under field conditions. Indeed, this has limited the commercial development of BCAs for control of many economically important pre-harvest fungal pathogens (Droby et al. 2016).

In contrast, post-harvest BCAs have been more successful because of the ability to control relative humidity and temperature and the application methods, which include dipping of fruit into formulations of the BCA (Usall et al. 2016). Conditions are conducive for colonisation of the fruit surface by the BCA, as has been demonstrated for *C. sake* CPA-1 which effectively controlled fungal diseases on pome fruits (Viñas et al. 1998). Indeed, such formulations have also been effective against *Botrytis cinerea* (Cañamás et al. 2011; Calvo-Garrido et al. 2013a; Calvo-Garrido et al. 2014b) and sour rot in grapes post-harvest (Calvo-Garrido et al. 2013b). Previously, *C. sake* CPA-1 formulations, both liquid (Abadias et al. 2003; Torres et al. 2003) and solid (Abadias et al. 2001; Abadias et al. 2005; Carbó et al. 2017a), have been optimised, especially for post-harvest application, where most of the applications are based on liquid formulations. The environmental stress tolerance of *C. sake* CPA-1 fresh cells has been studied. For example, Teixidó et al. (1998) studied the ecology of *C. sake* CPA-1 cells in response to water, temperature and pH stress which helped to identify the boundaries for growth in relation to these factors. However, the behaviour of fresh cells does not represent the real situation when exposed to fluxes in temperature and RH in the field, especially on phyllosphere surfaces. Some recent studies by Calvo-Garrido et al. (2014b) examined the effect of temperature and RH, and simulated rainfall on the establishment of liquid formulations *in vivo* on grapes. Survival and efficacy of liquid formulations of CPA-1 was improved by adding a commercial fatty acid-based product called Fungicover® (Cañamás et al. 2011; Calvo-Garrido et al. 2013a; Calvo-Garrido et al. 2014b; Calvo-Garrido et al. 2014a). While studies have previously focused on liquid formulations of this yeast biocontrol agent, very little information is available on dry formulations and their resilience under different interacting environmental conditions.

Recently Carbó et al. (2017b) examined the potential for enhancing the survival and environmental stress tolerance of dry formulations of biocontrol agents for application pre-harvest. These were fluidised-bed spray-drying based formulations including biodegradable coatings. This approach may also provide formulations with a wider range of activity against fungal pathogens on different crops. Some authors have suggested that the drying process may result in damage to the cells due to the combined effect of heat and dehydration (Fu and Chen 2011) and perhaps decrease viability and efficacy of dried BCA formulations under environmental stress fluxes, especially pre-harvest. Thus, tolerance to environmental stress conditions is critical in developing formulations which can maintain their competitiveness and occupation of the desired ecological niche (Magan and Aldred 2008) and exclude the pathogen (Samsudin et al. 2016).

There is thus interest in developing an environmental screening system to facilitate the comparison and identification of formulations of BCAs, which may have the desired ecological resilience, especially for pre-harvest applications. One such approach is the use of the Bioscreen C system which allows automated turbidimetric measurements of viability and resilience under different interacting abiotic and indeed nutritional factors (Medina et al. 2012; Mohale et al. 2013; Samsudin et al. 2016).

The objectives of the present study were (a) to optimise the Bioscreen C system to evaluate the growth of the BCA yeast *C. sake* CPA-1, (b) to compare the impact of interacting environmental factors of temperature (15-30°C), pH (3-7) and water activity (a_w; 0.94-0.99) on the viability and growth of the liquid (Candifruit) commercial formulation and two dry formulations of the BCA *C. sake* CPA-1 using the optimised turbidimetric method, and (c) to determine the Time To Detection (TTD) for growth of the *C. sake* CPA-1 formulations under interacting environmental conditions to compare their resilience. All the experiments were carried out with synthetic grape juice as a basal growth medium to simulate CPA-1 growth on grapes.

2. Methods

2.1. Microbial antagonist

Strain CPA-1 of *C. sake* used in the present study was obtained originally from University of Lleida-IRTA, Catalonia, Spain, and it was deposited at the Colección Española de Cultivos Tipo (CECT-10817) at the University of Valencia, Burjassot. *C. sake* CPA-1 stock cultures were stored in Criobilles tubes (Criobilles AEB 400100, AES Laboratory, Comburg, France) at -80 ºC for long term storage. When required, CPA-1 cells were sub-cultured 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}) at 25 ºC for 48 h. After growth, yeast cells were sub-cultured to use or to store at 4 ºC on NYDA plates for a short time. All assays were carried out with three different formulations of the BCA: (i) a liquid formulation registered in Spain under de name Candifruit™; (ii) a dry formulation based on potato starch; and (iii) a dry formulation based on maltodextrin. Both solid formulations were dried using fluidised-bed spray-drying system by adding biodegradable coatings to enhance the survival under stress conditions. The formulation process was done as optimised previously (Carbó et al. 2017b).

Concentration of *C. sake* (CFU ml^{-1}) was determined by plating 100 µl of serial dilutions on NYDA and incubating at 25 ºC for 48 h. Prior to starting the experiments, the initial viability of formulations was checked by serial dilutions as described previously to calculate the required amount of product to achieve the inoculum size. To carry out the assays, 50 ml of each formulation was prepared at 10^7 CFU ml^{-1} with sterile water, then the required dilution was made to achieve 15 ml of the target concentration of cells. Also 500 mg l^{-1} of ampicillin were added to each treatment to inhibit any bacterial growth.

2.2. Turbidimetric assay

Three Bioscreen C Microbiological Growth Analysers (Labsystems, Helsinky, Findland) were used for the analysis of the turbidity. For all the assays, 200 µl of inoculated media were loaded in each well of the non-standard, 100-well microtitre plates. The optical density (OD) was recorded periodically using the 600 nm filter over the required time for each experiment. The software Easy Bioscreen Experiment (EZExperiment) provided by the manufacturer was used to

record all the obtained data. Then, data was exported to a Microsoft® Excel® Professional 2013 (Microsoft Corporation, Redmond, Washington, USA) sheet for further analysis.

2.3. Culture medium preparation and optimisation

Synthetic grape juice (SGJ) (Mitchell et al. 2004; Tassou et al. 2009) was used as the basal growth medium for *C. sake* CPA-1 in turbidimetric assays. The medium had the following composition: D(+) glucose, 70 g; D(–) fructose, 30 g; L(–) tartaric acid, 7 g; L(–) malic acid, 10 g; $(NH_4)_2HPO_4$, 0.67 g; KH_2PO_4, 0.67 g; $MgSO_4.7H_2O$, 1.5 g; NaCl, 0.15 g; $CuCl_2$, 0.0015 g; $FeSO_4.7H_2O$, 0.021 g; $ZnSO_4 \cdot 7H2O$, 0.0075 g; (+) Catechin hydrate, 0.05 g; agar, 25 g; distilled water, 1000 ml. The pH of SGJ was adjusted to 4 with KOH (2 mol l^{-1}).

Three options were tested to optimise the growth media conditions: (i) semi-solid SGJ by adding 0.125% of agar to liquid SGJ without shaking; (ii) liquid SGJ shaken at medium intensity for 20 sec before every automatic reading; and (iii) liquid media with continuous shaking at medium intensity. The optimisation of the media was carried out with three concentrations of CPA-1: (i) 10^1 CFU ml^{-1}; (ii) 10^3 CFU ml^{-1}; and (iii) 10^5 CFU ml^{-1}. The Bioscreen C was run at 25 ºC for 5 days and the $OD_{600}$was recorded every 20 min.

2.4. Selection of an appropriate inoculum size

The composition of the formulations could cause some turbidity to the medium after inoculation, so it was preferable that the three formulations had a similar initial OD_{600} value for the turbidimetric assays. For this reason, despite an approximation of the initial cells concentrations was examined previously, a wide range of concentrations from 10^1 CFU ml^{-1} to 10^7 CFU ml^{-1} of CPA-1 was tested to select the appropriate inoculum size. Specifically, 10^1, 10^2, 10^3, 5×10^3, 10^4, 5×10^4, 10^5, 5×10^5, 10^6, 5×10^6 and 10^7 CFU ml^{-1} were tested.

Three wells of a microtitre plate were used for each inoculum size and in addition 200 µl of non-inoculated SGJ were loaded into ten wells as a control. The OD_{600} was recorded every 2 min using the 600 nm filter for 10 min, and then the average of the recorded values was calculated as the initial turbidity to be compared between the different concentrations. This experiment was conducted at 15 ºC to avoid rapid growth of the yeast and the previously optimised conditions for the medium were used.

2.5. Correlation between OD_{600} values and CPA-1 concentrations

Three CPA-1 solutions containing 10^5 CFU ml^{-1} were prepared, one with each formulation. Non-modified SGJ medium was used and each solution was loaded in a 100-well microtitre plate. Ten replicates for each condition were used. The OD_{600} was recorded after inoculation and then after 24 h, 45 h, 48 h and 72 h at 25 ºC. At the same times, CFU ml^{-1} was calculated by plating 100 µl of serial dilutions on NYDA and incubating at 25 ºC for 48 h. Afterwards, the CFU ml^{-1} were correlated with the OD_{600} obtained for that well at the tested time.

2.6. Evaluation of *C. sake* growth under different interacting parameters

Several interacting conditions of temperature, water activity (a_w) and pH were tested for each formulation with the turbidimetric assays. Specifically, the assays were carried out at four temperatures: (i) 15 ºC; (ii) 20 ºC; (iii) 25 ºC; and (iv) 30 ºC; four a_w: (i) 0.99; (ii) 0.98; (iii) 0.96; and (iv) 0.94; and finally, at three different pH values; (i) pH 3; (ii) pH 5; and (iii) pH 7.

The pH of the medium was adjusted with KOH (2 mol l^{-1}) and the a_w modified by adding different amounts of glycerol (a_w 0.99: 2.8% w/v; a_w 0.98: 9.2% w/v; a_w 0.96: 18.4% w/v; and a_w 0.94: 32.2% w/v). Vials containing 15 ml of the adjusted pH and a_w modified media were sterilised for 20 min at 121 ºC. Vials were then shaken and left at room temperature to cool. Afterwards, they were inoculated with the required amount of the concentrated solution of each *C. sake* formulation and then decanted into the microtitre plates using a multichannel pipette. Ten replicates were used for each treatment, with uninoculated sterile media used as a control. Means of the replicates were only considered if at least 8 of the 10 inoculated wells developed the growth curves. Although, the percentage of wells where *C. sake* CPA-1 achieved to grow was also represented to examine the resilience of the different formulations under the different interacting environmental conditions.

The OD_{600} was recorded every 20 min using the 600 nm filter for at least 7 days, with the total length of the assays depending on the applied stress to the yeast formulation. Before further analyses, the raw datasets were corrected to remove the background signal. The average of the measurements for each well during the first 60 min was calculated and automatically subtracted from all subsequent measurements in order to remove the different signal backgrounds. The

time to detection (TTD) for an OD_{600} of 0.5 was obtained for all environmental conditions using a Microsoft® Excel® template (kindly provided by Dr. R. Lambert; Samsudin et al. 2016), which used linear interpolation between successive OD_{600} readings to predict the TTD at $OD_{600} = 0.5$.

2.7. Statistical analysis

The ability to growth of *C. sake* formulations was analysed using a Generalised Linear Model based on a binomial distribution and a logit link function. Prior the analysis, the response variable TTD 0.5 (days) was transformed to binary data; where 0 values indicated no growth for an OD_{600} of 0.5, whereas 1 values indicated that *C. sake* achieved to growth at least until an OD_{600} of 0.5. Percentage of LogWorth was plotted to graphically show the weight of each factor in the whole model. LogWorth is defined as $-\log_{10}(p\text{-value})$, this transformation adjusts *p*-values to provide an appropriate scale for graphical display. Effects of different environmental stress factors were compared by orthogonal contrasts. All analysis was performed using JMP Pro12 software (SAS Instiute Inc., NC, U.S.A).

3. Results

3.1. Optimisation of the culture medium conditions for subsequent experiments with the Bioscreen

The growth curves obtained for *C. sake* CPA-1 on the synthetic grape juice (SGJ) medium are compared in Figure 1. This shows that growth of the yeast over time was most consistent with continuous shaking (Fig. 1c). The initial concentration of yeast cells also showed that the reproducibility was better at 10^5 CFUs ml^{-1} (Fig. 1d). Other semi-solid media or liquid media with intermittent shaking (every 20 sec before absorbance measurement) did not give well defined and distinct phases for the lag, exponential and stationary phases (Figs. 1a and 1b).

Differences between media conditions were observed at the end of the growth period when the 100-well microtitre plates were visually examined. The yeast cells appeared to be uniformly distributed in the wells containing liquid medium which was continuously shaken. In other conditions, the cells appeared to have been deposited at the bottom of the wells (semi-solid or non-agitated liquid media). The highest tested concentration for which no OD_{600} differences

amongst the formulations was obtained was 10^5 CFUs ml^{-1}. This coincided with the best results obtained due to the faster cell growth and the good inter-well reproducibility (Fig. 1d).

3.2. Optimisation of inoculum size

No differences were observed among formulations when low concentrations of *C. sake* CPA-1 cells were tested. However, the initial turbidity among the formulations varied from 5×10^5 to 5×10^6 CFUs ml^{-1} based on their natural formulated turbidity (Fig. 2).

3.3. Correlation between OD_{600} values and C. sake concentrations

The good correlation between C. sake concentrations and the OD_{600} readings, when adjusted to using the power trend method, confirmed that the OD_{600} readings were representative of cell concentrations (Fig. 3). This demonstrated that the three yeast formulations achieved a similar concentration for a specific OD_{600} value, so that they were comparable. In fact, when an OD_{600} value of around 1 was obtained, the three formulations achieved a similar concentration; the same occurred with an OD_{600} value >1.5 (Fig. 3). Nevertheless, after reaching an OD_{600} of 0.5 the power trend line slope decreased, and a higher increase in the OD_{600} value was required to show an increase in C. sake CPA-1 concentration.

3.4. Resilience of *C. sake* CPA-1 formulations under environmental stress

3.4.1.Comparison of liquid and dry formulations

There were significant differences in the ability of the yeast cells in the formulations to grow (df = 2; $\chi^2 = 26.45$; $P < 0.0001$), including between the two fluidised-bed dried formulations based on the percentage (%) LogWorth weightings for the different factors (Table 1; Fig. 4). Indeed, temperature, pH and a_w had differential effects on each of the three formulations (Fig. 4). In general, the pH appeared to have less impact on the viability of the formulations. Temperature and a_w were the main factors influencing the growth of *C. sake* CPA-1 formulations in terms of the relative influence based on the weighting of the factors for each formulation. Interestingly, the liquid formulation (Candifruit) was more sensitive to temperature (53.8%), whilst the dried potato starch formulation was predominantly affected by a_w (58.8%). In contrast, temperature (49.7%) and a_w (49.3%) affected the dried maltodextrin formulation with a similar weighting.

3.4.2.Effect of temperature

The viability and growth of the different yeast cell formulations was significantly affected by temperature (df = 3; χ^2 = 1116.37; P < 0.0001) (Table 2). Overall, 15 and 20ºC were the optimum temperatures for growth of the yeast formulations. For the liquid formulation (Candifruit) there was a significant influence of each temperature (df = 3; χ^2 = 445.02; P < 0.0001) with maximum growth at 20ºC (Fig. 5). For the dry potato starch-based formulation (df = 3; χ^2 = 317.44; P < 0.0001) there were no differences in the temperature range 15-25ºC. For the maltodextrin dry formulation (df = 3; χ^2 = 403.94; P < 0.0001) similar optimum growth were observed at 15 and 20ºC. However, at 25ºC the percentage of inoculated wells where CPA-1 grew was decreased significantly. At 30°C, yeasts cells in all three formulations had difficulty in becoming established, with only the dry potato starch formulation showing limited viability and growth.

3.4.3.Effect of water activity (a_w)

The effect of water stress on relative growth of the yeast cell formulations is shown in Figure 5. In all cases maximum and rapid growth occurred of all three formulations with relatively freely available water (0.99 a_w). Indeed, there was also no significant differences between viability and growth at 0.99 and 0.98 a_w for the liquid Candifruit and dried Maltodextrin formulations (Table 3). Differences among formulations were observed with relatively freely available water (0.99 $a_{w;}$ df = 2; χ^2 = 20.41; P < 0.0001) and under extreme water stress conditions (0.94 $a_{w;}$ df = 2; χ^2 = 15.48; P = 0.0004). Indeed, at 0.94 a_w only the liquid Candifruit formulation had the resilience for active growth.

3.4.4.Effect of pH

The maximum growth of the yeast formulations was in the synthetic grape juice medium adjusted to pH levels of 5 and 7 (Fig. 5). However, significant differences were observed between pH 5 and 7 for the liquid Candifruit formulation (Table 4), which appeared to be better adapted to pH 5. The poorest viability and growth was at pH 3 for all three tested formulations. No significant differences were observed among formulations at each individual pH level (pH 3:

df = 2; χ^2 = 1.59; P = 0.4520; pH 5: df = 2; χ^2 = 2.55; P = 0.2796; and pH 7: df = 2; χ^2 = 1.56; P = 0.4590).

3.5. Effect of interacting environmental stresses on the Time To Detection (TTD) for growth of the *C. sake* formulations

In general, the liquid Candifruit formulation grew faster in more interacting environmental conditions (Fig. 6). However, only the dry potato starch formulated yeast cells could grow in some temperature stress conditions whereas the liquid Candifruit formulation could not develop at all (Fig. 6).

Overall, all the formulations were able to grow in the interacting conditions of pH (3, 5 and 7), temperature (15 to 25ºC) and a_w levels of 0.99 to 0.96. The only exceptions were the liquid Candifruit and the dry maltodextrin formulations which were unable to grow at pH 3, 25ºC and 0.96 a_w. *C. sake* development at 30ºC or 0.94 a_w was significantly restricted due to the high temperature or water stress conditions. In fact, only the dry potato starch formulation could grow at 30ºC and pH 5 or 7 and 0.99 a_w. In contrast, the best resilience at extreme a_w levels for growth of this yeast (0.94 a_w) was restricted to the liquid Candifruit formulation at pH 5 and 20ºC.

All two and three-factor interactions among different environmental conditions (pH, temperature and a_w) were statistically significant ($P<0.0001$). As shown in Fig. 4, temperature and water activity had a high impact on the yeast growth and affected the TTD.

4. Discussion

Resilience of different formulations of a biocontrol agent in relation to environmental stress was examined to identify the relative range of conditions and boundaries for effective viability and establishment. This study has shown that there are significant differences between a liquid and two dry formulations in terms of growth and establishment, especially under interacting conditions of a_w, temperature and pH. This environmental screening approach may be beneficial in helping to identify those formulations which may have better viability in the field with conserved biocontrol efficacy. The use of the Bioscreen C as a rapid automated turbidimetric tool for screening and identifying resilience of different types of formulations could be very

beneficial in helping to address this key bottleneck in the development of effective BCA formulations for field use.

Previous studies have highlighted the need to optimise the methodology prior to the development of ecophysiological experiments using the Bioscreen C with the main parameters including media consistency, shaking and inoculum size (Weiss et al. 2004; Medina et al. 2012). Medina *et al.* (2012) optimised the semi-solid medium to analyses filamentous fungal growth using the Bioscreen C, whereas Xuan et al. (2017) used liquid media shaken for 20 sec before each measurement for studies with *Listeria monocytogenes*. Thus, our methodology was optimised to obtain the best results in terms of relative yeast growth: the liquid SGJ medium, which was continuously shaken, with an initial concentration of 10^5 CFU ml^{-1} which gave the best growth over time for this biocontrol yeast. Moreover, a direct relationship between OD_{600} values and yeast concentrations was observed when the three formulations were analysed together. An OD_{600} value of 0.5 was assigned to obtain the TTD in different environmental conditions because changes in *C sake* CPA-1 concentrations were clear at this value. Previously, an OD_{600} value of 0.2 was assigned for screening antifungal compounds (Medina et al. 2012) or an OD_{600} = 0.1 to calculate the temporal carbon utilisation sequences of *Aspergillus flavus* strains (Mohale et al. 2013).

Despite the differences in formulation resilience, temperature and a_w were the environmental factors that had a major effect on viability and growth of the biocontrol yeast, whereas the impact of pH was minor. In fact, temperature and a_w are the two most important stress factors influencing yeast activity, although the host surface pH may also reduce the growth of the biocontrol agent (Sui et al. 2015). Exposure to heat stress is expected when *C. sake* CPA-1 is applied pre-harvest to grapes; therefore high temperatures can reduce the viability of the BCA. The a_w of grapes is about 0.977±0.001 (Fernández-Salguero et al. 1993) and their pH varies from 3.0 to 4.5 (Worobo and Splittstoesser 2005). Assuming that the a_w after the BCA application depends on the environmental conditions within the fruit (Sui et al. 2015), the biocontrol yeast cells applied to the surface of grapes will be exposed to microclimatic conditions representing a_w values >0.97 a_w, at least during the ripening stage. When *C. sake*

formulations were subjected to these stress conditions (pH 3 to 4.5 and a_w >0.97), both liquid and dry formulations had viable yeast cells which could effectively grow at 15 to 25 ºC.

The yeast cells in the liquid formulation (Candifruit) usually developed faster than the dry formulations but differences were less pronounced under environmental stress conditions. For example, at 25ºC and 0.96 a_w at pH 3 and 7. The vital functions of microorganisms can often reach a dormant state during the drying process (Berninger et al. 2018) and they only become active again during rehydration (García 2011). This may give liquid formulations an advantage allowing slightly faster recovery and growth when favourable environmental conditions occur. When comparing the TTD (OD of 0.5) of both fluidised-bed spray-dried formulations, generally the TTDs for the potato starch formulation was shorter than the TTDs for the the maltodextrin formulation. This suggests that skimmed milk and sucrose incorporated into the maltodextrin formulation improved the viability of cells after the drying process (Carbó et al. 2017b) but these protective compounds do not favour the development of the yeast after rehydration. It may be possible that carbohydrate composition of pregelatinised potato starch could be more favourable than maltodextrin carbohydrate composition for *C. sake* CPA-1 viability and development, although maltodextrin molecular chains are shorter. Previous studies with nutritional utilisation patterns have shown that depending on the environmental conditions the TTD and the rates of utilisation of C-sources may vary for biocontrol agents and fungal pathogens (Mohale et al., 2013; Samsudin et al., 2016). The TTD could be used effectively as a rapid tool for comparing and identifying promising formulations for targetted applications pre-harvest.

In general, none of the three studied *C. sake* formulations could develop in extreme environmental conditions at 0.94 a_w, or high temperatures (30-35ºC). The yeast cells in the liquid formulation were able to grow under extreme a_w conditions at 20ºC and pH 5. However, under temperature stress, the potato starch formulation was more resilient than the other two tested. Only yeast cells in this fluidised-bed spray-dried formulation which included biodegradable coatings was able to grow at 30ºC + freely available water (0.99 a_w) and pH 5 or 7. These results support recent studies by Carbó et al. (2017b) where field applications of these

three formulations showed that the dry potato starch formulation showed better establishment with the best resilience on grapes surfaces. Interestingly, the efficacy and survival of *C. sake* CPA-1 fresh cells was previously shown to be improved by the addition of coatings in the formulation (Marín et al. 2016). Indeed, these types of coatings have been demonstrated to improve efficacy of other yeast and bacterial BCAs (Aloui et al. 2015; Parafati et al. 2016).

In summary, the optimised environmental screen using the Bioscreen C enables the rapid comparison of the resilience of different formulations in the target ecological niche. For the three formulations evaluated in the present study, it was demonstrated that the *C. sake* CPA-1 BCA is effective over a wide range of environmental conditions, with some limitations under extreme environmental stress conditions. Although fluidised-bed spray-dried formulations resulted in some cell damage, they appear to show effective environmental resilience after desiccation. The potato starch formulation was able to extend the ecological niche range, especially tolerance of high temperature, possibly due to the incorporation of biodegradable coatings during the drying process.

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Compliance and Ethical Standards

Conflict of interest: The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1 Growth curves obtained during the optimisation of the synthetic grape juice used as media for *C. sake* growing in the Bioscreen C assays: (a) average growth curves (ten individual replicate) obtained with semi-solid media without shaking; (b) average growth curves (ten individual replicates) obtained with liquid media shaken 20 seconds before the automatic reading; (c) average growth curves (ten individual replicates) obtained with liquid media with continuous shaking; and (d) individual curves obtained with liquid media shaken for 20 s before the automatic reading. Different concentrations of each formulation are represented: Candifruit at 10^1 CFU ml^{-1} (CAN01); Candifruit at 10^3 CFU ml^{-1} (CAN03); Candifruit at 10^5 CFU ml^{-1} (CAN05); potato starch formulation at 10^1 CFU ml^{-1} (PS01); potato starch formulation at 10^3 CFU ml^{-1} (PS03); potato starch formulation at 10^5 CFU ml^{-1} (PS05); maltodextrin formulation at 10^1 CFU ml^{-1} (MAL01); maltodextrin formulation at 10^3 CFU ml^{-1} (MAL03); and maltodextrin formulation at 10^5 CFU ml^{-1} (MAL05).

Fig. 2 Initial turbidity values obtained during the optimisation of the inoculum size used in the Bioscreen assays. Three formulations are represented: Candifruit (■); potato starch formulation (▲); and maltodextrin formulation (●). Linear regression for each formulation are represented with the dotted lines. Liquid SGJ media with continuous shaking treatment was used. Values are means of 5 readings recorded every 2 min during the first 10 min after inoculation. Three wells were used for

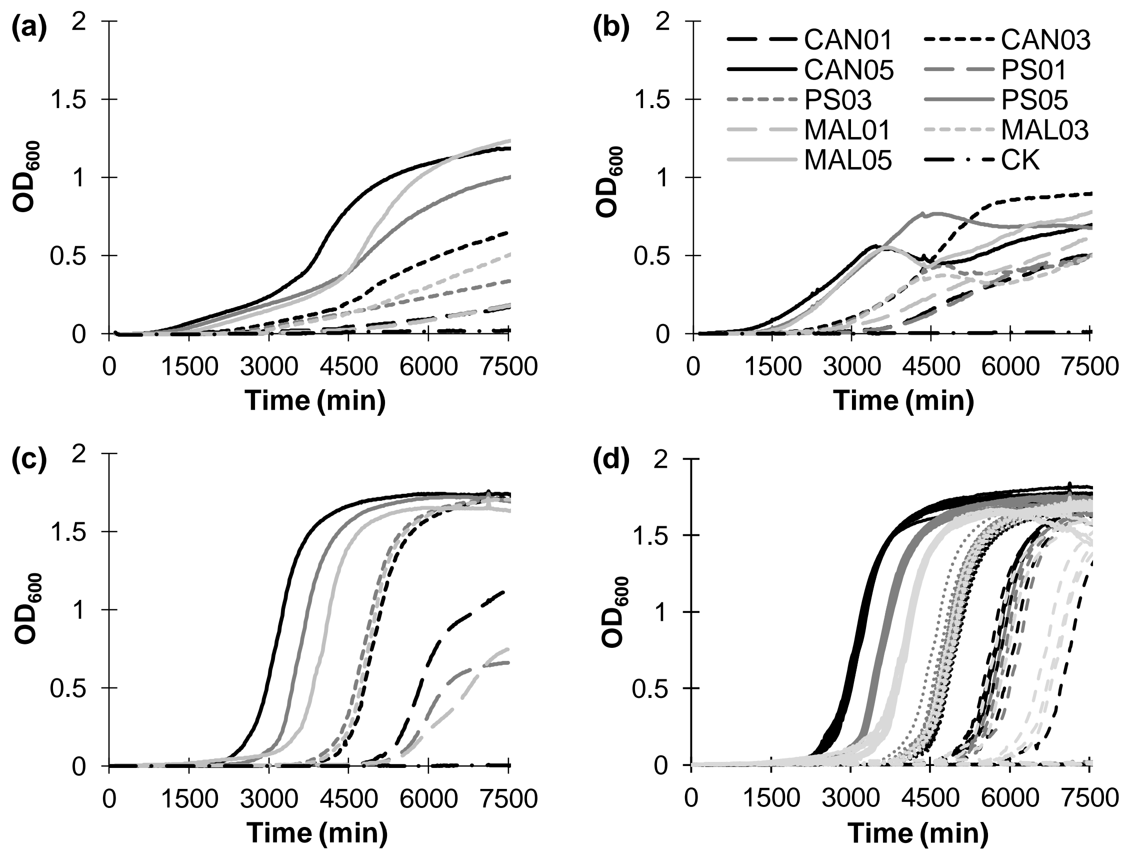
each formulation. Error bars indicate standard errors of the means. When bars are not visible, they are smaller than symbol size.

Fig. 3 Correlation between OD_{600} and *C. sake* (CFU ml^{-1}) at different growing times with an initial inoculum size of 10^5 CFU ml^{-1}. Three formulations are represented: Candifruit (■); potato starch formulation (▲); and maltodextrin formulation (●). The power trend line is represented with the dotted line. Liquid SGJ media with continuous shaking was used.

Fig. 4 Summary effect of each tested formulation: Candifruit, potato starch, and maltodextrin. The three studied formulations are represented as LogWorth percentage values to compare the effects in the model: temperature (□); water activity (■); and pH (■).

Fig. 5 Graphical representation of the percentage of inoculated wells where *C. sake* CPA-1 achieved growth for each evaluated condition (temperature, water activity and pH). Three formulations are represented in each graph: Candifruit (□); potato starch formulation (■), and maltodextrin formulation (■). For each treatment and source, percentage values linked by the same letter are not significantly different ($p < 0.05$) according to orthogonal contrasts analysis. Different uppercase letters indicate significant differences between CPA-1 formulations within the same condition (temperature, water activity or pH); different lowercase letters indicate significant differences between different evaluated temperature, water activity or pH conditions within the same formulation. Error bars indicate standard errors of the means.

Fig. 6 Graphical representation of the TTD (0.5) against temperature and water activity at three different pH: (a) pH 3; (b) pH 5; and (c) pH 7. Three formulations are represented in each graph: Candifruit (□); potato starch formulation (■), and maltodextrin formulation (■). Results are the mean of growth curves for ten replicate wells. NG means no growth under those conditions. Error bars indicate standard errors of the means. When bars are not visible, they are smaller than 0.1 days.



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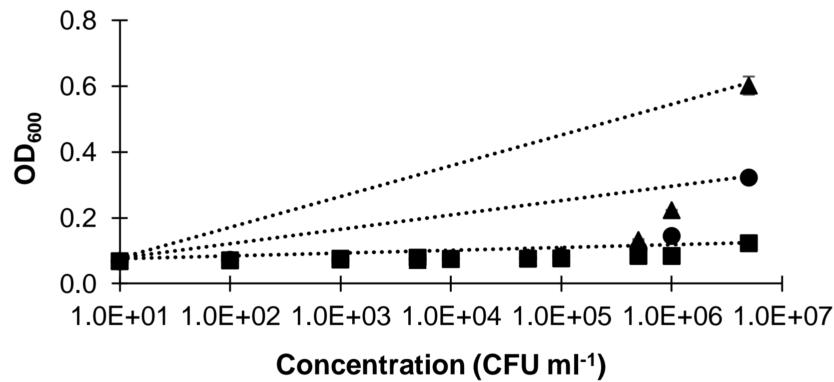

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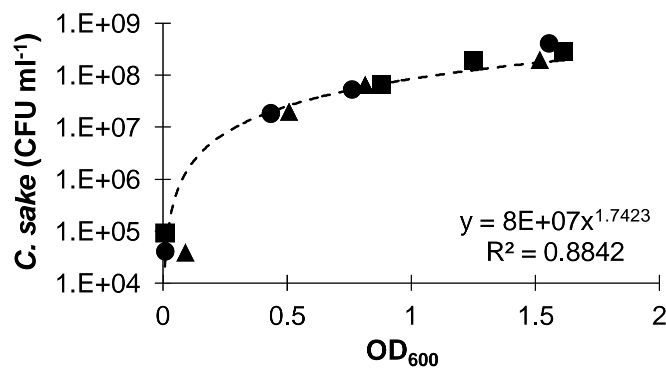

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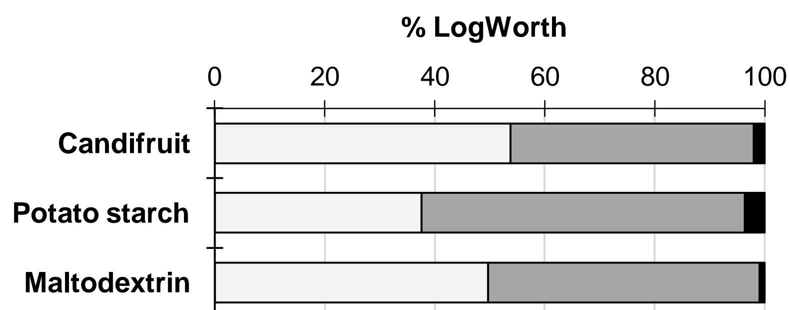

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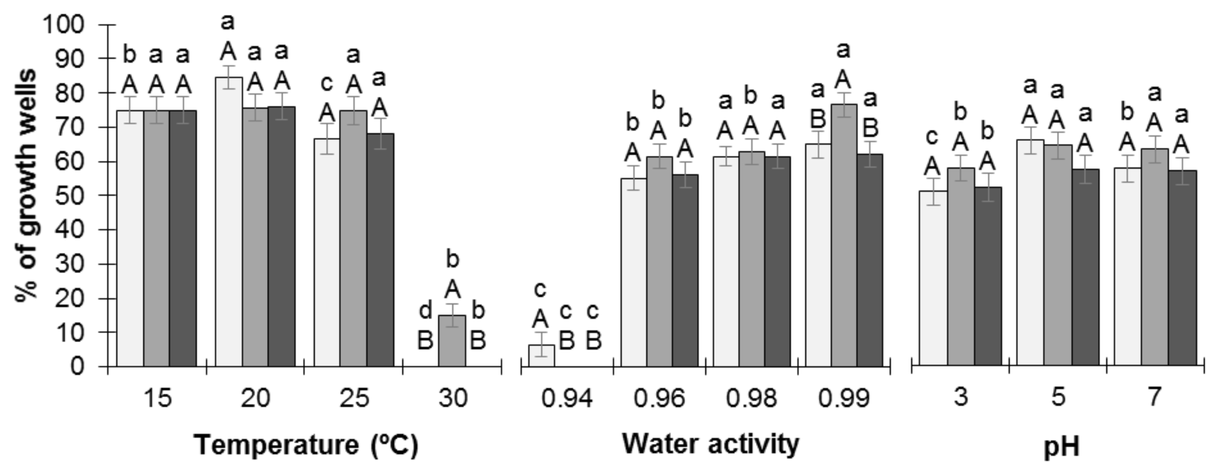

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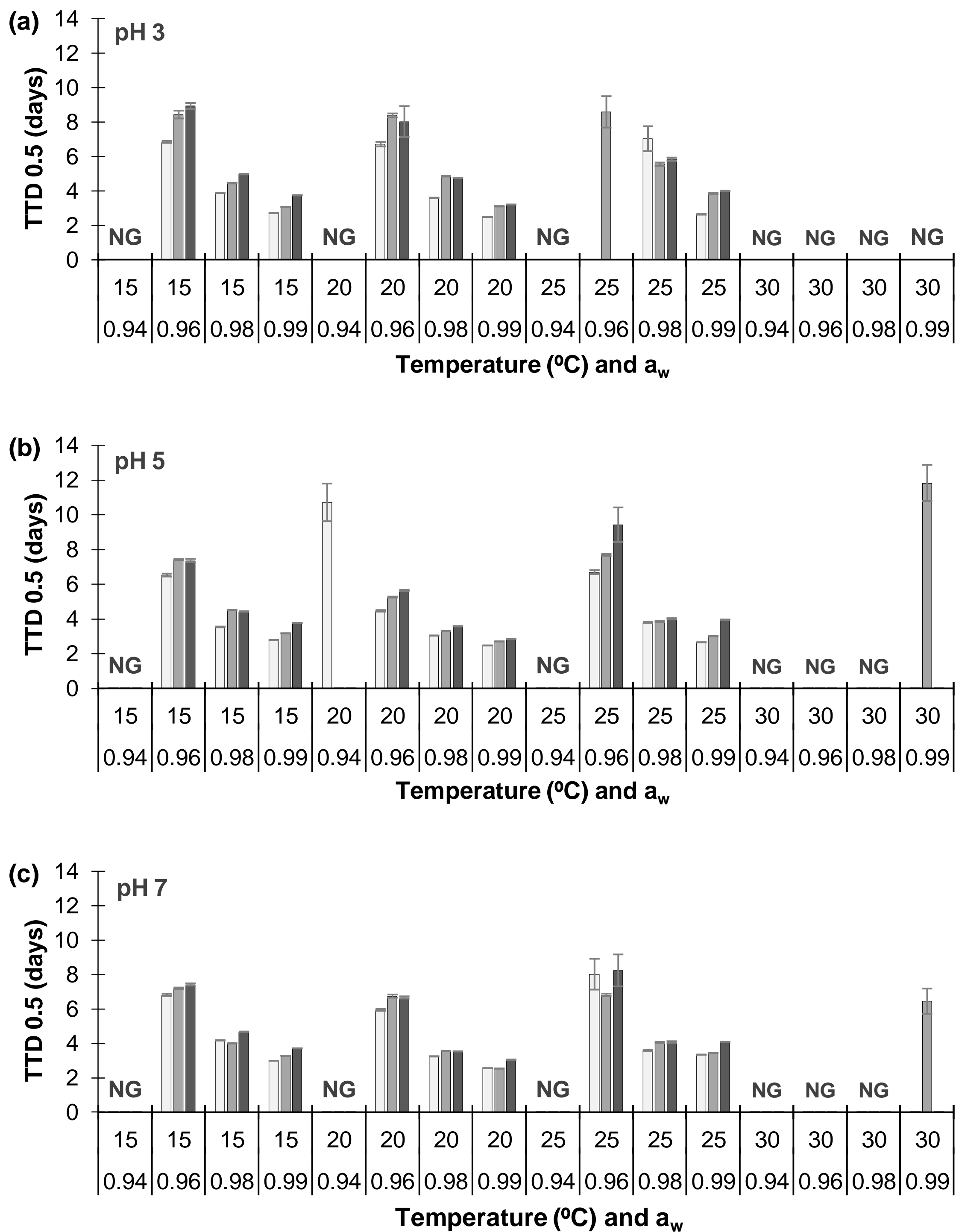

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Table 1 Orthogonal contrasts testing formulation effects on *C. sake* CPA-1 cell growth on synthetic grape juice medium.

Effect test	**df**	χ^2	$P > \chi^2$
Candifruit vs Potato starch	1	11.1212 *	0.0008
Candifruit vs Maltodextrin	1	7.2497 *	0.0071
Potato starch vs Maltodextrin	1	23.2325 **	<0.0001

Note: * significant *P*<0.05; ** significant *P*<0.0001

Table 2 Orthogonal contrasts testing the temperature effect on *C. sake* CPA-1 cell growth on synthetic grape juice medium. The three tested formulations were: Candifruit liquid formulation, potato starch dry formulation and maltodextrin dry formulation. All temperatures were only contrasted when no significant differences were observed among successive temperatures.

Effect test		**df**	χ^2	$P > \chi^2$
Candifruit	15 ºC vs 20 ºC	1	7.6183 *	0.0058
	20 ºC vs 25 ºC	1	15.8247 **	<0.0001
	25 ºC vs 30 ºC	1	237.6401 **	<0.0001
Potato starch	15 ºC vs 20 ºC	1	$3.5074 \cdot 10^{-6}$ NS	0.9985
	20 ºC vs 25 ºC	1	$7.5777 \cdot 10^{-5}$ NS	0.9931
	25 ºC vs 30 ºC	1	201.9604 **	<0.0001
	15 ºC vs 25 ºC	1	$4.7221 \cdot 10^{-5}$ NS	0.9945
Maltodextrin	15 ºC vs 20 ºC	1	$1.7070 \cdot 10^{-4}$ NS	0.9896
	20 ºC vs 25 ºC	1	4.9110 *	0.0267
	25 ºC vs 30 ºC	1	226.8288 **	<0.0001

Note: * significant *P*<0.05; ** significant *P*<0.0001; NS, not significant

Table 3 Orthogonal contrasts testing water activity effect on *C. sake* CPA-1 growth on synthetic grape juice. Three tested formulations are itemised: Candifruit liquid formulation, potato starch dry formulation and maltodextrin dry formulation.

Effect test		**df**	χ^2	$P > \chi^2$
Candifruit	0.94 a_w vs 0.96 a_w	1	23.9165 **	<0.0001
	0.96 a_w vs 0.98 a_w	1	6.9798 *	0.0082
	0.98 a_w vs 0.99 a_w	1	0.0648 NS	0.7990
Potato starch	0.94 a_w vs 0.96 a_w	1	9.7335 *	0.0018
	0.96 a_w vs 0.98 a_w	1	$2.1172 \cdot 10^{-4}$ NS	0.9884
	0.98 a_w vs 0.99 a_w	1	4.7122 *	0.0299
Maltodextrin	0.94 a_w vs 0.96 a_w	1	13.9316 *	0.0002
	0.96 a_w vs 0.98 a_w	1	5.0854 *	0.0241
	0.98 a_w vs 0.99 a_w	1	$1.2641 \cdot 10^{-5}$ NS	0.9972

Note: * significant *P*<0.05; ** significant *P*<0.0001; NS, not significant

Table 4 Orthogonal contrasts testing pH effect on *C. sake* CPA-1 growth on synthetic grape juice. Three tested formulations are itemised: Candifruit liquid formulation, potato starch dry formulation and maltodextrin dry formulation.

Effect test		df	χ^2	$P > \chi^2$
Candifruit	pH 3 vs pH 5	1	15.9014 **	<0.0001
	pH 5 vs pH 7	1	7.5805 *	0.0059
	pH 3 vs pH 7	1	8.0868 *	0.0044
Potato starch	pH 3 vs pH 5	1	4.4223 *	0.0355
	pH 5 vs pH 7	1	$2.5959 \cdot 10^{-4}$ NS	0.9871
	pH 3 vs pH 7	1	4.5971 *	0.0320
Maltodextrin	pH 3 vs pH 5	1	5.5924 *	0.0180
	pH 5 vs pH 7	1	$8.8017 \cdot 10^{-5}$ NS	0.9925
	pH 3 vs pH 7	1	5.6231 *	0.0177

Note: * significant *P*<0.05; ** significant *P*<0.0001; NS, not significant