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Effect of culture age, protectants and initial cell concentration on viability of freeze-dried cells of *Metschnikowia pulcherrima*

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Running title

Freeze-drying of *Metschnikowia pulcherrima*

ABSTRACT

The effect of freeze-drying using different lyoprotectants at different concentrations on the viability and biocontrol efficacy of *Metschnikowia pulcherrima* was evaluated. The effects of initial yeast cell concentration and culture age on the viability were also considered. Yeast cells grown for 36 h were more resistant to freeze-drying than 48 h-old cells. An initial concentration of 10^8 cells mL^{-1} favoured the highest survival after freeze-drying. When maltose (25% w v^{-1}) was used as protectant, a high cell viability was obtained (64.2%). Cells kept a high viability after 6 months of storage at 4°C. The biocontrol efficacy of freeze-dried cells was similar to the activity of fresh cells on 'Gala' apples, and slightly lower on 'Golden Delicious' apples. After optimizing the freeze-drying conditions, the viability of *M. pulcherrima* cells were similar to that obtained in other studies. The results constitute a first step towards the commercial development of *M. pulcherrima* as biocontrol agent.

Key words

Apple fruit, biological control, freeze-drying, postharvest, yeast.

INTRODUCTION

Several microorganisms, particularly yeast naturally occurring on the surface of fruit and vegetables, have been widely studied for the control of postharvest diseases (Spadaro and Gullino 2004). The acceptance of biocontrol agents (BCAs) depends on their effectiveness and compatibility with current handling and storage practices used in the agrochemical marketplace (Wraight et al. 2001). Formulation is necessary in order to present the product in a usable form and to optimize its efficacy, stability, safety and ease of application. The formulation must therefore provide conditions which retain viability during preparation, storage and application, and favour survival of the agent, in the environment (Rhodes 1993).

Among the microorganisms under development, there are some antagonistic yeast strains belonging to the species *Metschnikowia pulcherrima* (Pitt) M.W. Miller effective against postharvest decay of apple, table grape, grapefruit, cherry tomato, and peach (Schena et al. 2000; Janisiewicz et al. 2001; Spadaro et al. 2002, 2008; Zhang et al. 2010). *M. pulcherrima* normally acts as competitor for scarce nutrients, such as iron (Saravanakumar et al. 2008), or by producing hydrolases, such as chitinases, able to degrade the cell wall of pathogenic fungi (Saravanakumar et al. 2009).

Biocontrol of postharvest pathogens using competitive yeast is only effective when applying high (at least 10^7-10^8 cells mL^{-1}) concentrations of microorganisms (Hofstein et al. 1994), and its effectiveness increases with the concentration. The need of storing and preserving the microbial pesticides requires an increase in the shelf-life, obtained by stabilizing the viability of the microorganisms (Burges 1998). This can be achieved i) by refrigeration in liquid state; ii) by freezing in presence of cryoprotectant substances; iii) by dehydrating the product (Montesinos, 2003).

Freeze-drying is the most convenient and successful method of preserving bacteria, sporulating fungi and yeast (Berny and Hennebert 1991). Freeze-drying, compared to other dehydration techniques (To and Etzel 1997), has the advantages of protecting from contamination during storage, long viability and ease of distribution (Miyamoto-Shinohara et al. 2006). Formation of a maximally freeze-concentrated matrix with entrapped microbial cells is essential in freezing prior to freeze-drying. Freeze-drying must retain a solid amorphous state of protectant matrices. Freeze-dried matrices contain cells entrapped in the protective matrices in the freezing process. The retention of viability during storage seems to be controlled by water plasticization of the protectant matrix and possibly interactions of water with the dehydrated cells. Highest cell viability has been obtained in glassy protective media (Pehkonen et al. 2008). Some investigators have already studied the effect of freeze-drying on survival and efficacy of yeast biocontrol agents, such as *Candida sake* (Abadias et al. 2001), *Cryptococcus laurentii* (Li and Tian 2007) and *Pichia anomala* (Melin et al. 2007). The aim of the present research was to evaluate the effect of freeze-drying on the survival of *M. pulcherrima*. Furthermore, the effect of different lyoprotectants used at different concentrations was studied on the viability of yeast cells after freeze-drying. Moreover, the initial yeast cell concentration and culture age on the viability of freeze-dried yeast cells were taken into consideration. Finally, the biocontrol efficacy of frozen, freeze-dried and fresh cells were compared.

MATERIALS AND METHODS

Microorganisms

Metschnikowia pulcherrima (Pitt) M.W. Miller strain BIO126 was isolated from the carposphere of an apple ‘Golden Delicious’ harvested in an unsprayed orchard located in

Piedmont, Northern Italy (Spadaro et al. 2002). The strain was stored at -80°C in a cell suspension with 20% v v^{-1} of glycerol.

For the biocontrol assays, five strains of *Botrytis cinerea* were isolated from rotten apples and selected for their high virulence. Each strain was stored on a slant of Potato Dextrose Agar (Merck, Darmstadt, Germany) with 50 mg L^{-1} streptomycin Merck at 4°C. Spore suspensions were prepared by growing the fungal strains on Petri dishes on PDA amended with 50 mg L^{-1} of streptomycin for 10 days. Spores from the five strains were collected, resuspended in sterile Ringer's solution (Merck, Darmstadt, Germany), filtered through 8 layers of sterile cheese-cloth and brought to a final concentration of 10^5 spores mL^{-1} per strain.

Yeast growth

Before use, the yeast was subcultured in Petri dishes with nutrient yeast dextrose agar (NYDA, Droby et al. 1989). The growth medium was YEMS (yeast extract 30 g L^{-1}, D-mannitol 5 g L^{-1} and L-sorbose 5 g L^{-1}), which favoured a high yeast biomass (Spadaro et al. 2010). Well-controlled fermentations of 4.0 L working volume (nominal volume, 5 L) were carried out in Applikon BioConsole ADI 1025 glass stirred tank vessels (Applikon™ Biotechnology, The Netherlands), integrated with the software Bioexpert Lite for data acquisition. Yeast cultures were inoculated to 5×10^5 cfu mL^{-1}. Operating conditions included temperature controlled at 25± 0.2°C, floating dissolved oxygen monitored using a polarographic probe, agitation with two equally spaced Rushton impellers controlled at a constant speed of 450 rpm, and air sparging through a submerged ring sparger controlled at 4.0 L min^{-1}.

Sample preparation

Cells were collected at the beginning of the stationary phase by centrifugation (7500 rpm for

10 min at 4°C) in a J21-2 centrifuge (Beckman, Brea, USA) and resuspended in Ringer solution. The concentration of the resuspended cells was determined by Bürker chamber and adjusted to the desired concentrations. An aliquote (500 µl) of the yeast cells suspensions were then deposited into vials.

The protective agents tested were two monosaccharide sugars (D-glucose and D-fructose), four disaccharide sugars (lactose, maltose, sucrose and trehalose), a sugar alcohol (L-sorbitol) and a complex matrix (yeast extract). Each protectant was dissolved in deionised water, autoclaved and added to the yeast cell suspension to obtain the final concentration (w v^{-1}). Throughout the experiments, different concentrations (1%, 5%, 10%, 15%, 20%) of protectants were tested and also the effect of the initial cell concentration was evaluated. 4.5 ml of each protectant suspension were added into each vial containing the yeast cell suspension (500 µl), to get the desired protectant concentration. For each protectant and/or initial concentration tested, tenfold dilutions of the suspensions were made in Ringer solution, and 100 µl aliquots of the suspensions were spread plated in triplicate on Petri dishes containing NYDA in order to determine the initial fresh cell concentration. Dishes were incubated at 25±1°C for 48 h, and the initial number of colony forming units per millilitre (cfu mL^{-1}) was determined.

Freeze-drying

The suspension was frozen at -20°C for 4 h and at -40°C for 12 h and subsequently lyophilized in a Heto FD 1.0 Freeze-Dryer (Waltham, Massachusetts, USA) operating at a chamber pressure of 1 Pa and at -45°C for 24 h. After freeze-drying, the vials were sealed under vacuum. Experiments were replicated three times for each protectant and concentration.

Reyhdration and yeast cell viability

For each protectant and/or initial concentration tested, three replicates of freeze-dried vials were resuspended in autoclaved Ringer solution to the original volume (5 mL) and left on a rotary shaker for 15 min. Tenfold dilutions of the suspensions were made in Ringer solution, and 100 µl aliquots of the suspensions were spread plated in triplicate on Petri dishes containing NYDA. Dishes were incubated at 25±1°C for 48 h, and the number of cfu mL^{-1} was determined. Viability percentage for each protectant was assessed as ratio between freeze-dried and fresh cells concentration. For the last viability assays about initial cell concentration with high protectant, the vials were stored at 4°C for 2, 4, and 6 months before rehydration and viability assessment. Experiments were repeated three times.

Biocontrol assays

To evaluate the efficacy of freeze-dried cells of *M. pulcherrima* strain BIO126 against *B. cinerea* on apple, freeze-dried cells (with 25% w v^{-1} maltose or 25% w v^{-1} fructose) were rehydrated as described above and compared with frozen and fresh yeast cells. Freeze-dried and frozen cell concentration was adjusted at 10^8 cfu mL^{-1} with Ringer solution. Fresh yeast cells were obtained by growing them in YEMS for 36 h, by centrifuging (7500 rpm for 10 min at 4°C) and resuspending them in Ringer solution to 10^8 cfu mL^{-1}. For a chemical control, fruit were treated with thiabendazole (Tecto 20S, Syngenta Crop Protection, Milan, Italy, 20% a.i., 200 µg mL^{-1}).

Two assays were carried out on two apple cultivars, ‘Golden Delicious’ and ‘Gala’. Apples were artificially wounded at the equatorial region (3 mm diameter; 6 mm depth; 3 wounds per fruit). A 30 µl yeast suspension was applied to each wound and, after 3 h, wounds were inoculated with a 30 µl of *B. cinerea* suspension (10^5 spores mL^{-1}). Five apples per replicate and three replicates per treatment were used. Treated apples were stored at 20±1°C for 7 days, after which the grey mould lesion diameters were measured. The assays were repeated twice.

Data analysis

Each experiment was repeated at least twice. No significant differences were found among corresponding experiments so that the trials were pooled and statistical analysis was performed by using the SPSS-WIN software. Statistical significance was judged at the level of $P<0.05$. When the analysis of variance was statistically significant, Duncan's multiple range test was used for the separation of means.

RESULTS AND DISCUSSION

Viability assays: culture age

In a first set of experiments, the cells of *M. pulcherrima* strain BIO126 grown for 36 and 48 h in YEMS were freeze-dried starting from an initial concentration of 1×10^9 cfu mL^{-1} and adding different protectant agents at 1% w v^{-1} (Table 1). The formulations after freeze-drying were porous cakes. Highest cell viability was obtained in glassy protective media, such as sugars. In general, the addition of any of the eight protectants tested provided a survival rate statistically higher than Ringer solution. No significant difference was found in the viability of yeast cells after freezing for the protectants tested. For 36 h old cultures, a statistically higher viability was obtained with maltose (8.81%) and fructose (9.94%). A noticeable result was obtained also by the addition of yeast extract (7.52%). For 48 h old cultures in YEMS, the viability was always lower than for 36 h old cultures, the protectant being equal. The highest viability was obtained by using maltose (3.21%), followed by trehalose (2.12%) and yeast extract (2.00%). Without protectants, viability after freeze-drying was extremely low: 0.11% for 36 h old cultures and 0.22% for 48 h old cultures. Cultures grown for 36 h in YEMS were chosen to continue the experiments.

In 5-liter fermenter experiments, the cultures of *M. pulcherrima* reached the stationary phase after 32 h growth in YEMS (Spadaro et al. 2010), so 36 h old cultures corresponded to the plain stationary phase, while 48 h old cultures corresponded to the beginning of the decline phase, when viable count started to turn down. Indeed, preliminary experiments showed that younger (grown for 24 or 30 h) or older cultures (grown for 42 or 48 h) had lower viability after freeze-drying (data not shown). Similarly, freeze-drying of other biocontrol agents was performed on cultures in plain stationary phase. In particular, the cultures of the bacterial antagonist *Pantoea agglomerans* and the yeast BCA *Candida sake* were harvested respectively after 24 h (Costa et al. 2000) and 38 h growth in liquid medium (Abadias et al. 2001).

Viability assays: initial cell concentration with low protectant

In a second set of experiments, the number of protectants tested was reduced to five, by keeping one monosaccharide sugar (D-fructose), one sugar alcohol (L-sorbitol), one complex matrix (yeast extract) and two disaccharide sugars (maltose and trehalose). The microorganism cultures were freeze-dried after 36 h growth at three initial cell concentrations (10^{11}, 10^{10} and 10^{9} cfu mL^{-1}) and in presence of 5% w v^{-1} of protectant (Figure 1). The lowest initial concentration of microorganism (10^{9} cfu mL^{-1}) provided the highest final viability. In particular, starting with 10^{11} cfu mL^{-1}, fructose (0.69%) and sorbitol (0.63%) were the protectants providing statistically higher viability. With an initial concentration of 10^{10} cfu mL^{-1}, yeast extract (1.50%) and maltose (1.44%) resulted the most effective protectants. Fructose (6.3%), followed by maltose (5.1%) and sorbitol (4.2%) provided the highest yeast viabilities, when freeze-drying was realized on an initial concentration of 10^{9} cfu mL^{-1}.

The viability of microorganisms, particularly of *M. pulcherrima*, was very poor after freeze-drying without the use of any protective agent. Thus several protectants were tested at

different concentrations, throughout the experiments, without adding other chemical compounds, to evaluate its single effect on viability and to keep the formulation as simple as possible, as suggested by Nail et al. (2002). The experiments carried out showed that the type and concentration of the protective agent used had a strong effect on the viability of the cells of *M. pulcherrima*. Previously, Costa et al. (2000) found that maximum protection of cells of *P. agglomerans* during freeze-drying was achieved with sugars, and in particular with disaccharides. Sugars replace structural water in membranes after dehydration and prevent unfolding and aggregation of proteins by hydrogen bonding with polar groups of proteins (Patist and Zhoerb 2005).

Viability assays: protectant concentration

In the following series of experiments, cells with an initial concentration of 10^9 cfu mL^{-1} were freeze-dried in presence of four concentrations (5%, 10%, 15%, and 20% w v^{-1}) of four protective agents (Figure 2). At 5% w v^{-1} protectant, fructose and maltose provided a cell survival respectively of 9.2% and 6.2% after freeze-drying, significantly higher than the other two protectants. At 10% w v^{-1} protectant, fructose (13.3%), followed by maltose (7.6%), was the most effective protective agent tested. At 15% w v^{-1}, yet the most effective protectants were fructose and maltose, able to guarantee respectively 11.8% and 10.7% viability. The highest *M. pulcherrima* cell viability was achieved at high concentration (20%) of protective agents: in particular, maltose (20.6%), followed by fructose (14.6%) and sorbitol (12.8%) provided the best results.

Viability assays: initial cell concentration with high protectant

The last set of experiments related to the viability of *M. pulcherrima* was carried out by freeze-drying the yeast cells at three initial concentrations and in presence of two high

concentrations (20 and 25%) of maltose and fructose (Figure 3). At the highest initial concentration (10^9 cells mL^{-1}) 25% of maltose permitted the highest yeast viability (47.0%). When the initial cell concentration was 10^8 cells mL^{-1}, noticeable viabilities were obtained for both protectants and both concentrations tested. In particular, 20% and 25% of maltose provided a survival rate respectively of 62.5% and 64.2%. The viability in presence of 20% and 25% fructose were respectively 21.4% and 43.4%. The viability when the initial cell concentration was 10^7 cells mL^{-1} was lower compared to the other two initial concentrations, and the best results was provided by 25% maltose (35.4%) or 25% fructose (35.6%).

When increasing the protectant concentration, a lower microorganism concentration (10^8 cells mL^{-1}) guaranteed a higher viability. With 5% protectant, the highest viability was guaranteed with 10^9 cells mL^{-1} of antagonistic yeast (Figure 1), but with 20% or 25% protectant, the highest viability was obtained with 10^8 cells mL^{-1}(Figure 3). A high initial yeast concentration (higher than 10^{10} cells mL^{-1}) was related to a drastic decrease in the viable count. Costa et al. (2000) studied the effect of the initial cell concentration on the viability of *P. agglomerans*, finding that the effect of the initial cell concentration was related to the protective medium used. When sucrose was used as a protectant, the highest recovery was obtained at high concentration (10^{10} cfu mL^{-1}). On the opposite, when protein based protectants were used, the lowest viabilities were obtained at high bacterial concentration. Bozoglu et al. (1987) suggested that the death of microorganisms is proportional to their area of contact with the external medium. Pehkonen et al. (2008) showed that the differences in cell viabilities after dehydration were depending on the protective agents used, and in particular on the glass transition properties of the disaccharides used.

The protective effect provided by some disaccharides, such as maltose or trehalose, on living organisms under desiccation could be explained by several mechanisms. In the water-replacement hypothesis, the stabilization was attributed to the formation of hydrogen bonds

between sensitive components and disaccharide molecules when water is removed, maintaining the structural integrity of membranes and proteins (Crowe et al. 1987). A second hypothesis is related to the ability of disaccharides to form a glassy structure during drying under suitable conditions where the sensitive components are embedded (Crowe et al. 1993). Lodato et al. (1999) found that the presence of a certain amount of amorphous disaccharides (such as maltose) during freeze-drying was critical for ensuring the cell viability of *Saccharomyces cerevisiae.*

The shelf-life of a biological product refers to the period of time during which the antagonistic cells remain viable and effective (Elzein et al. 2004). In our study, no significant loss of viability on freeze-dried cells appeared after a storage at 4°C of the vials obtained in the last experiments for 2, 4, and 6 months. In particular, when 10^8 cells mL^{-1} of BIO126 were freeze-dried in presence of 20% or 25% maltose, the cell viabilities after 2, 4, and 6 months were 61.4%, 61.3%, and 59.7% with 20% maltose and 63.8%, 63.2% and 60.3% with 25% maltose. The low temperature helped to keep the metabolic activity at a low level, and contributed to increase the storage stability and shelf life. Freeze-drying is probably the most effective desiccation technique for microorganism long storage, and recent studies have showed that it is suitable to keep alive not only gram-negative or gram-positive bacteria, but also yeast for up to 20 years (Miyamoto-Shinohara et al. 2006).

Biocontrol assays

To test the efficacy of the freeze-dried cells of *M. pulcherrima* strain BIO126 in controlling *B. cinerea* on apple, two trials were carried out on the cultivars ‘Golden Delicious’ and ‘Gala’ (Figure 4). The fruit were stored at 20°C for 7 days. The biocontrol trials showed a significant reduction of grey mould severity on both apple cultivars by fresh, frozen as well as freeze-dried yeast cells. The biocontrol efficacy of the freeze-dried cells was similar on ‘Golden

Delicious' and 'Gala' apples, with a disease severity reduction of 45.3% and 48.1% respectively, as compared to the control. On both cultivars, the antagonistic activity of fresh and frozen cells amended with protective agents was similar. On 'Golden Delicious' apples, freeze-dried yeast cells showed a slightly lower biocontrol efficacy, as compared to the fresh and frozen cells, but on 'Gala' apples the efficacy of freeze-dried cells was not statistically different from the biocontrol provided by the fresh cells.

The biocontrol efficacy trials confirmed that freeze-dried cells of *M. pulcherrima* after rehydration not only were viable, but still kept a biocontrol activity against *B. cinerea* similar to that shown by fresh cells of the same strain when applied at the same concentration. Previous, Abadias et al. (2001) showed a lower efficacy of freeze-dried *Candida sake* cells against *P. expansum* on apples compared to the fresh cells, partially due to the damage created to the cells, which could not totally recover their function after the dehydration-rehydration process. On the opposite, the biocontrol activity of freeze-dried cells of *Pichia anomala* against moulds on moist cereal grains remained intact (Melin et al. 2007).

The present work permitted to set up a freeze-drying protocol for the cells of *M. pulcherrima* characterized by a high viability and the preservation of the biocontrol efficacy against postharvest pathogens. Previous studies showed that the cell viability could significantly increase when a suitable rehydrating medium is used instead of Ringer solution. Future research will focus on the effect of the rehydrating media, of longer storage times and of storage temperatures on yeast cell viability.

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Figure captions

Figure 1 Viability of *Metschnikowia pulcherrima* cells (%) after 36h growth in YEMS and freeze-drying, using different sugar solutions (5% w v^{-1}) as protectants and Ringer solution as rehydration medium. Cells at three different initial concentrations were freeze-dried. Samples were analyzed as soon as the freeze-drying process finished. Each bar represents the mean value and standard deviation of 12 samples obtained from three independent experiments. Bars of the same colour followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P < 0.05$).

Figure 2 Viability of *Metschnikowia pulcherrima* cells (%) after 36h growth in YEMS and freeze-drying, using different sugar solutions at different concentrations (5%, 10%, 15%, 20% w v^{-1}) as protectants and Ringer solution as rehydration medium. Initial microorganism concentration was 10^9 cfu mL^{-1}. Samples were analyzed as soon as the freeze-drying process finished. Each bar represents the mean value and standard deviation of 12 samples obtained from three independent experiments. Bars followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P < 0.05$).

Figure 3 Viability of *Metschnikowia pulcherrima* cells (%) after 36h growth in YEMS and freeze-drying, using maltose and fructose, at two concentrations (20% and 25% w v^{-1}) as protectants and Ringer solution as rehydration medium. Three different initial concentrations were freeze-dried. Samples were analyzed as soon as the freeze-drying process finished. Each bar represents the mean value and standard deviation of 12 samples obtained from three independent experiments. Bars followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P < 0.05$).

Figure 4 Efficacy of fresh, frozen or freeze-dried cells of *Metschnikowia pulcherrima* strain BIO126 in reducing the development of *Botrytis cinerea* on apples 'Golden Delicious' and 'Gala'. Fruit were artificially wounded, treated with 5×10^8 antagonist cells mL^{-1} and inoculated after 3h with 5×10^5 conida mL^{-1} of *Botrytis cinerea*. Thiabendazole (200 µg mL^{-1}; p.a. 19.7%) was used as chemical control. Fruit were stored at 20±1°C for 7 days. Lesion diameters (cm) were measured. Bars of the same colour followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P < 0.05$).