Effect of culture media and pH on the biomass production and biocontrol efficacy of a Metschnikowia pulcherrima strain to be used as a biofungicide for postharvest disease control

Effect of culture media and pH on the biomass production and biocontrol efficacy of a *Metschnikowia pulcherrima* strain to be used as a biofungicide for postharvest disease control

Spadaro D.[12], Ciavorella A.[2], Dianpeng Z.[2], Garibaldi A.[2], Gullino M.L.[2]

[1]DiVaPRA – Plant Pathology, University of Turin, Grugliasco (TO), Italy. [2]Centre of Competence for the Innovation in the Agro-environmental Sector (AGROINNOVA), University of Turin, Grugliasco (TO), Italy.

Correspondence

Davide Spadaro, Di.Va.P.R.A. – Plant Pathology and AGROINNOVA - Centre of Competence for the Innovation in the Agro-environmental Sector, University of Turin, Via L. da Vinci 44, I-10095 Grugliasco (TO), Italy.

E-mail: davide.spadaro@unito.it

Telephone: +39-011-6708942

Fax: +39-011-6709307

Abstract

Few strains of *Metschnikowia pulcherrima* are under development for control of postharvest pathogens on fruit. A substrate was developed to optimize the biomass production *M. pulcherrima* strain BIO126. Different complex nutrient sources, with or without pH control, were tested. Growth in Yeast Extract provided at concentrations equal to or higher than 30 g l^{-1} resulted in the highest biomass. The addition of two carbon sources, D-Mannitol and L-Sorbose at 5 g l^{-1} each, significantly improved the yeast growth. Initial pH values of the medium ranging from 5.0 to 7.5 permitted the highest growth of the yeast. A combination of Yeast Extract, D-Mannitol and L-Sorbose (YEMS), probably with diauxic utilization, showed synergistic effect, widening the exponential phase (the maximum specific growth rate was 0.45 h^{-1}), and increasing the final cell number (1.5 x 10^9 cells ml^{-1}) and dry biomass (6.0 g l^{-1}) in well controlled batch fermentation. In efficacy trials on 'Golden Delicious' apples, the microorganism grown in YEMS effectively reduced incidence and severity of *B. cinerea* (51.1% and 70.8%) and *P. expansum* (41.7% and 14.0%). Also on 'Gala' apples, the best reduction of grey and blue mould incidence was obtained with cells grown in YEMS (58.1% and 50.5%, respectively).

Keywords: batch fermentation, biological control, pH effect, postharvest, yeast.

1. Introduction

Several microorganisms have been evaluated to obtain microbial based products useful in agriculture, such as biofertilisers or biopesticides. Among the biofungicides, during the last twenty years, several yeast have been widely investigated against postharvest fungal pathogens of different host species (Janisiewicz and Korsten 2002; Spadaro and Gullino 2004; Wilson and Wisnieswki 1994).

Among the microorganisms under development, there are few strains of *Metschnikowia pulcherrima* antagonistic against fungi causing postharvest decay of fruit. Some strains are effective against *Botrytis cinerea, Penicillium expansum*, or *Alternaria alternata* of apples (Janisiewicz et al. 2001; Piano et al. 1997; Spadaro et al. 2002; 2008), other strains were selected against *Penicillium digitatum* on grapefruit, *B. cinerea, Rhizopus stolonifer*, and *Aspergillus niger* on table grape, or *B. cinerea* and *R. stolonifer* on cherry tomato (Schena et al. 2000). Epiphytic isolates reduced *A. carbonarius* and *A. niger* colonization on grapes (Bleve et al. 2006). Moreover, a strain of *M. pulcherrima* proved effective in preventing the growth or survival of food-borne human pathogens, such as *Listeria monocytogenes* or *Salmonella enterica*, on fresh-cut apple tissue (Leverentz et al. 2006). *M. pulcherrima* could act through competition for iron (Saravanakumar et al. 2008) or production of hydrolases, such as chitinases and glucanases (Saravanakumar et al. 2009).

The efficacy of many antagonists of wound pathogens is directly related to the number of antagonist propagules applied (Hofstein et al. 1994). Sinigaglia et al. (1998) found that the antagonistic effects of isolates of four yeast species, including *M. pulcherrima*, against *Penicillium glabrum* were more pronounced at high cell concentrations. Moreover, an increasing efficacy in reducing the germination of *B. cinerea* was

demonstrated by increasing the number of yeast cells applied (Spadaro et al. 2002). A simple way to increase the effectiveness of such biofungicides is the application of a higher number of cells. Mass production of yeast cells is an essential step in the commercialization of a biocontrol agent. A rapid, efficient and cheap mass production of yeast antagonists, generally by liquid fermentation, is one of the key issues to achieve the commercial use of the biofungicide (Wraight et al. 2001).

Due to the recent interest over the use of the yeast species *M. pulcherrima* as biocontrol agent, efforts must be intensified to produce *M. pulcherrima* in a laboratory scale fermenter to provide relevant information for the scale-up production. Operating conditions (aeration, agitation, pH and temperature) as well as medium constituents may affect the quality and quantity of the tested microorganisms. To increase the biomass production of an antagonistic yeast on a laboratory scale, the optimization of the growth conditions, using different complex nutrient sources, is essential. The culture media can greatly influence the efficacy of the biocontrol agents (Wraight et al. 2001). The aim of developing a substrate for laboratory purposes is to optimize the biomass production, to find optimal conditions for stabilization and formulation, and to develop a quality control system. To scale-up a laboratory fermentation process to an industrial level, it is fundamental to find cheap nutrient sources, generally industrial by-products, with nutritional values similar to the laboratory standardized media.

In this work, the influence of different complex nutrient media on the growth of *M. pulcherrima* strain BIO126 in 5 L batch fermentation was considered. The aim of the research was to find which sources provided the highest biomass production (as number of living cells and as dry biomass) of the antagonistic yeast and what was the optimal concentration for the identified sources. The experiments were carried out first in shake

flasks as a preliminary screening, and then in a 5-liter fermenter, optimizing aeration, temperature and pH. Finally, biological control assays were used to test the efficacy of the yeast cells produced through well controlled batch fermentation.

2. Materials and Methods

2.1. Microorganism

Metschnikowia pulcherrima (Pitt) M.W. Miller strain BIO126 was isolated from the carposphere of a 'Golden Delicious' apple harvested from an unsprayed orchard located in Piedmont, Northern Italy (Spadaro et al. 2002). The strain was stored as a cell suspension in 20% V/V glycerol at –80°C at the Microorganism Culture Collection of the Centre of Competence for the Innovation in the Agro-environmental Sector of the University of Torino (Italy). The strain was deposited within the American Type Culture Collection on June 19, 2007 with deposit designation PTA-8486.

2.2. Inoculum preparation

The yeast inoculum was prepared by subculturing in Yeast extract-Peptone-Dextrose (YPD) [10 g l^{-1} granulated yeast extract (Merck, Darmstadt, Germany); 20 g l^{-1} triptone-peptone of casein (Difco, Detroit, MI, USA); 20 g l^{-1} D(+)-glucose monohydrate (Merck)] on a rotary shaker (100 rpm) at 25°C for 48 h. Yeast cells were collected by centrifugation at 2500 x *g* for 7 minutes, washed, resuspended in sterilized Ringer solution (pH 6.9±0.1; Merck) and used as inoculum for the different liquid substrates evaluated in shake flask and batch fermentation experiments.

2.3. Shake flask experiments

Cell suspensions of *M. pulcherrima* strain BIO126 (3 ml; 5 x 10^8 cfu ml^{-1}) were inoculated in 1 litre Erlenmeyer flasks containing 300 ml of liquid media and grown on a rotary shaker (150 rpm) at 25°C for 48 h. Three flasks were prepared per each medium and two samples were collected from each flask. The final number of viable cells (cfu ml^{-1}) was determined by plating on NYDA (Nutrient broth-Yeast extract-Dextrose-Agar as in Droby et al., 1989): 10-fold dilutions of each suspension were prepared in sterilized Ringer solution (pH 6.9±0.1; Merck) and spread-plated in order to calculate the cell number. Plates were incubated at 25°C for 48 h and the number of colony forming units per millilitre (cfu ml^{-1}) was determined. The shake flask experiments were repeated two times.

2.4. Culture media

Complex nutrient media selection. The complex sources selected for the experiments were rich organic sources (yeast extract, nutrient broth, malt extract, meat peptone, casein peptone, bacto-peptone and casein hydrolyzed). Every source was tested at 10 g l^{-1}. The pH values were registered at the beginning of the experiment, after 24 and 48 h of culture. In a second experiment, the initial pH of the seven complex nutrient sources was adjusted to 7.00 ± 0.05, using a 1.0 M phosphate buffer (Na_2HPO_4 and NaH_2PO_4) solution, in order to evaluate the pH effect on the yeast growth. Every source was tested at 10 g l^{-1}. The pH values were registered after growth for 24 and 48 h.

Concentration of the complex nutrient source. Yeast extract medium was tested at different concentrations (5, 10, 15, 20, 30, 40, 50 and 60 g l^{-1}). The pH values were registered after growth for 48 h.

154

155 *Initial pH.* Yeast extract (30 g l^{-1}) was put in all the flasks. The pH of the media
156 prepared was adjusted using either 0.1 N HCl or NaOH to obtain initial pH ranging
157 from 1.0 to 11.0. The pH values were registered after growth for 24 and 48h.

158

159 *Carbon source addition.* In a first assay, the carbon sources were tested in a medium
160 containing 30 g l^{-1} of yeast extract. Three monosaccharide sugars (D-Glucose, D-
161 Fructose and L-Sorbose), two disaccharide sugars (Maltose and Sucrose, Sigma
162 Chemical Co.) and two sugar alcohols (L-Sorbitol and D-Mannitol) were added to the
163 yeast extract, at 10 and 20 g l^{-1}. The pH values were registered after growth for 48h.
164 In a second assay, D-Mannitol and L-Sorbose were tested either individually at different
165 concentrations (from 2.5 g l^{-1} to 20 g l^{-1}) or mixed at concentrations of 5+5 g l^{-1} or 6+6 g
166 l^{-1} in a medium containing 30 g l^{-1} of yeast extract. The pH values were registered after
167 growth for 48h. The results of the two experiments were similar, so they could be
168 analyzed together and combined (Table 3).

169

170 *2.5. Fermentation experiments*

171 Well-controlled fermentations of 4.0 L working volume (nominal volume, 5 L) were
172 carried out in Applikon BioConsole ADI 1025 glass stirred tank vessels (Applicon^{TM}
173 Biotechnology, Schiedam, The Netherlands), integrated with the software Bioexpert
174 Lite for data acquisition. Operating conditions included temperature controlled at 25±
175 0.2° C, dissolved oxygen permitted to float and monitored using a polarographic probe,
176 agitation with two equally spaced Rushton impellers controlled at a constant speed of
177 450 rpm, and air sparging through a submerged ring sparger controlled at 4.0L/min or 1

178 vvm (volume of air per volume of medium).

179 The tested substrates were YE (Yeast Extract 30 g l^{-1}), YEM (Yeast Extract 30 g l^{-1}; D-
180 Mannitol 10 g l^{-1}), YES (Yeast Extract 30 g l^{-1}; L-Sorbose 10 gl^{-1}) and YEMS (Yeast
181 Extract 30 g l^{-1}; D-Mannitol 5 g l^{-1}; L-Sorbose 5 g l^{-1}). 0.05 ml/L of silicone antifoam
182 (Sigma antifoam 204) were added. Every 2 h, starting from the inoculation of the
183 fermenter to the end of the experiment, a 5 ml sample was harvested in order to measure
184 the microorganism cell concentration. The concentration of viable cells (cfu ml^{-1}) was
185 determined by serial dilutions and plating, as indicated in the shake flask experiments.
186 The fermenter experiments were carried out twice.

187 In order to know the dry biomass produced by fermentation, the dry weight was
188 determined. After 36 h, the liquid cultures were collected and centrifuged (7500 rpm) at
189 4°C for 10 min (Beckman J21-2 centrifuge, Palo Alto, CA, USA) and the supernatant
190 was discarded. The cell pellet was dried at 105°C for 30h and the dry mass was
191 weighed.

192

193 *2.6. Biocontrol assay*

194 To evaluate the effect of the growth of *M. pulcherrima* strain BIO126 in four substrates
195 (YE, YEM, YES and YEMS) on the biocontrol efficacy, four trials were carried out
196 against *Botrytis cinerea* and *Penicillium expansum* on 'Golden Delicious' and 'Gala'
197 apples. Yeast cells were grown in the four substrates for 36h, centrifuged and suspended
198 to 10^7 cfu ml^{-1}in 100 l tanks. Five strains per each pathogen were isolated from rotted
199 apples and selected for their virulence. Each strain was stored in slant on Potato
200 Dextrose Agar (PDA; Merck) with 50 mg l^{-1} streptomycin Merck at 4°C. Spore
201 suspensions were prepared by growing the fungal pathogens on Petri dishes for two

202 weeks on PDA with 50 mg l^{-1} of streptomycin. Spores from the five strains were
203 collected, suspended in sterile Ringer's solution, filtered through 8 layers of sterile
204 cheese-cloth and brought to a final concentration of 10^5 spores ml^{-1} per strain. Apples
205 were artificially wounded at the equatorial region (3 mm diameter; 6 mm depth; 3
206 wounds per fruit). The fruits were artificially inoculated by dipping for 60 seconds in a
207 100 l tank containing a conidial suspension (10^5 spores ml^{-1} per pathogen) of *B. cinerea*
208 or *P. expansum*. After 3 hours, biocontrol isolates were applied at 10^7 cells ml^{-1} by
209 completely dipping the boxes of fruits for 60 sec in a 100 l tank containing the yeast cell
210 suspensions prepared as described. Fifty apples per replicate and three replicates per
211 treatment were used. After incubation at 1°C for 28 days, the incidence of rotten fruits
212 and the lesion diameters were measured. A chemical control treatment consisted of
213 fruits treated with thiabendazole (Tecto 20 S, Elf Atochem Agri Italy, 19.7 % a.i., 20 g
214 a.i. 100 l^{-1}). The experiment was carried out twice.
215
216 *2.7. Statistical analysis*
217 The fermentation experiments in bioreactor were performed twice, while the growth
218 experiments in shake flasks and the biocontrol experiments were repeated twice. No
219 significant differences were found among corresponding experiments so that the trials
220 were pooled and statistical analysis was performed by using the SPSS software (SPSS
221 Inc., version 13.0, Chicago, IL, USA). Statistical significance was generally judged at
222 the level of $P<0.05$ for the shake flasks growth and biocontrol experiments, but at
223 $P<0.01$ for the assay of concentration of the complex nutrient source. When the analysis
224 of variance was statistically significant either in the shake flask growth or in the
225 biocontrol experiments, Duncan's multiple range test was used for the separation of

means.

3. Results

3.1. Growth in complex nutrient media

Maximum exponential growth rate and biomass production of the strain BIO126 of *M. pulcherrima* varied with the complex nutrient source (Table 1a). The total nitrogen content of the tested media ranged from 8.0% to 15.4%, but it was very low for Malt Extract (1.1%). The highest yield was obtained with Yeast Extract (1.2×10^8 cfu ml^{-1}) and Nutrient Broth (8.5×10^7 cfu ml^{-1}), followed by Malt Extract and Meat Peptone. The Ringer solution, used as control, permitted to keep alive the initial inoculum.

Five out of seven complex nutrient sources, with an initial pH almost neutral, favoured the growth of *M. pulcherrima*, and the two substrates providing the highest biomass resulted in an increased pH value after 24 and 48 h from the inoculum. In the case of Malt Extract and Casein Hydrolyzed, the pH, initially acidic, decreased further at the end of the microorganism growth. For this reason, the experiment was repeated, adjusting the initial pH to 7.00±0.05 with a phosphate buffer (Table 1b). Yeast Extract and Nutrient Broth confirmed the highest viable biomass. The pH values after 24 and 48 h were higher and slightly acidic when the microorganism was grown in buffered Malt Extract, but the final biomass obtained was lower. On the opposite, the viable cells obtained in buffered Casein Hydrolyzed were higher compared to the not buffered substrate, and the pH value after 48 h was basic (8.04).

An increase in initial Yeast Extract concentration from 5 to 30 g l^{-1} gave a proportional increase in the biomass produced at the stationary phase (Fig.1). No significant increase

250 in biomass was observed from 40 to 60 g l^{-1} of Yeast Extract, which is likely due to the
251 Crabtree effect (Crabtree 1928; Boulton et al. 1998). At the stationary phase, pH values
252 of the culture substrate ranged from 8.07 to 8.70.
253 To assess the effect of the initial pH value on the final biomass produced, *M.*
254 *pulcherrima* was grown in Yeast Extract (30 g l^{-1}) whose initial pH was adjusted at
255 values ranging from 1.0 to 11.0 (Table 2). The pH values lower than 3.0 and higher than
256 10.0 did not permit the growth of *M pulcherrima.* A growth of at least 10^8 cfu ml^{-1} was
257 possible at initial pH values ranging from 4.0 to 8.5. When the initial pH ranged
258 between 5.0 and 7.5, a viable population higher than 3.0 x 10^8 cfu ml^{-1} was achieved
259 and the final pH ranged between 8.05 and 8.34.
260
261 *3.2. Effect of carbon addition*
262 The effect of different carbon sources on the growth of *M. pulcherrima* was assessed in
263 presence of Yeast Extract at two different concentrations: 10 and 20 g l^{-1} (Table 3). All
264 the carbon sources tested increased the yeast biomass production when used at 10 g l^{-1}.
265 At 20 g l^{-1} of carbon source, only D-Fructose did not provide a statistically significant
266 increase in the biomass of *M. pulcherrima*. In general, 20 g l^{-1} of carbon source did not
267 improve the yeast biomass compared to 10 g l^{-1} suggesting that high external carbon
268 source concentration are not beneficial to growth of this yeast. Only D-Glucose, applied
269 at 20 g l^{-1}, provided a *M. pulcherrima* biomass higher than at 10 g l^{-1}.
270 D-Mannitol and L-Sorbose at the concentration of 10 g l^{-1} provided the highest biomass,
271 1.5 x 10^9 cfu ml^{-1} and 8.0 x 10^8 cfu ml^{-1}, respectively. Also Sucrose, either at 10 or 20 g
272 l^{-1}, was a good carbon source, resulting in 6.7 and 6.9 x 10^8 cfu ml^{-1} of *M. pulcherrima*,
273 respectively.

The addition of different carbon sources resulted in a lower pH after 48 h. For D-Fructose, D-Mannitol, D-Sorbitol, and Sucrose, the pH was below 7.0 even after 48 h.

D-Mannitol and L-Sorbose were used alone or combined at different concentrations in a second assay to evaluate the effect on the final growth of the yeast strain (Table 3). The maximum growth was obtained using 5.0 g l^{-1} of D- Mannitol + 5.0 g l^{-1} of L-Sorbose, that caused increase in the final number of cells to 1.7 x 10^9 cfu ml^{-1}. At equal concentrations, D-Mannitol provided more yeast growth than L-Sorbose. In particular, the addition of 7.5 and 10.0 g l^{-1} of D-Mannitol were the most effective concentrations resulting in a final cell number of 1.5 x 10^9 cfu ml^{-1}. The highest growth with L-Sorbose was achieved at concentration of 12.5 g l^{-1} (1.2 x 10^9 cfu ml^{-1}). Addition of D-Mannitol and the mixture of D-Mannitol and L-Sorbose reduced pH after 48h growth. Increasing concentrations of D-Mannitol contributed to increase in the final pH value, while increasing concentrations of L-Sorbose had the opposite effect.

3.3. Fermentation experiments

The biomass production process was scaled-up from shaking flasks to a 5-l fermenter. The yeast biomass resulted significantly higher (1.6 x 10^9 cfu ml^{-1}) in YEMS medium compared to simple YE, YE with L-Sorbose, or YE with D-Mannitol (Fig.2*a*). At 5 l min^{-1} of aeration and 450 rpm, the stationary phase was achieved after 32 h of batch culture in YEMS medium, while in the other substrates it was reached from 2 to 4 h later. The maximum specific growth rate during the exponential phase was 0.45 h^{-1} in YEMS, while it was 0.33 h^{-1} in YES and 0.34 h^{-1} in YE and YEM.

Initial pH was 6.9 in YE and in YEM (Fig.2*b*). The presence of L-Sorbose contributed to lower the initial pH to 6.5 in YES and YEMS. As the growth approached the

298 stationary phase, pH tended to increase. The metabolism of D-Mannitol contributed to
299 lower the pH of 0.4 units: the minimum value achieved was 6.40 for YEM and 6.03 for
300 YEMS.
301 The consumption of oxygen is an indication of exponential growth (Fig.2*c*). The
302 reduction in the dissolved oxygen became visible when the viable population reached
303 around 10^7 cfu ml^{-1}. In YE, the length of the exponential phase and the final number of
304 cells obtained were reduced. In YES and YEM, the dissolved oxygen started to decrease
305 earlier than in YE. In YE, moreover, even during the exponential phase, the dissolved
306 oxygen was never reduced to 0%, but in YEMS it declined to 0% from the hour 24 to
307 the hour 31, for the longest period, indicating a long exponential phase. The evolution
308 of the dissolved oxygen indicated the sequential metabolism of D-Mannitol, followed
309 by L-Sorbose and finally by the amino acids and proteins contained in Yeast Extract.
310 At the end of the fermentation experiments, 1000 ml of cultural broth were harvested,
311 centrifuged and dried to measure the wet and the dry biomass produced. Using YE,
312 YES, YEM and YEMS, the wet biomass was 11.1, 12.0, 19.8, and 26.4 g l^{-1},
313 respectively, and the dry biomass was 1.4, 2.1, 4.2, and 6.0 g l^{-1}, respectively. These
314 results confirmed the values obtained by plate counting.
315
316 *3.4. Efficacy trials*
317 In the efficacy trials carried out on apples by treating with *M. pulcherrima* strain
318 BIO126 grown on four substrates, yeast cells grown in YEMS were more effective.
319 Generally, yeast concentrations being equal, the efficacy of the antagonistic cell
320 suspension was influenced by the growth culture substrate (Fig. 3). On 'Golden
321 Delicious' apples (Fig.3*a*), grey mould incidence and severity on the fruits treated with

322 BIO126 grown on YEMS were 48.9% and 29.2% compared to the inoculated control
323 (whose incidence and lesion diameter were 91.3% and 51.9 mm, respectively). Among
324 the fruits treated with the four yeast cell suspensions, grey mould incidence was
325 significantly lower when fruit were treated with cells grown in YEMS, but the mean
326 lesion diameters observed were not significantly different. Considering the efficacy
327 against *P. expansum* on 'Golden Delicious' apples, the cell suspension of *M.*
328 *pulcherrima* BIO126 grown in YEMS reduced the incidence from 86.3% (inoculated
329 control) to 50.3% and the mean lesion diameter from 37.9 mm (inoculated control) to
330 32.6 mm. On the opposite, when the yeast was grown in YE, it was ineffective in
331 reducing blue mould severity compared to the control.
332 On 'Gala' apples (Fig. 3*b*), the grey mould incidence was significantly reduced by
333 BIO126 grown on YEMS, YES, or YEM, but not when the yeast was grown on YE.
334 The lesion diameter of grey rots was not significantly reduced by any yeast application.
335 Similarly, against *Penicillium expansum* on 'Gala' apples, none of the biological
336 treatments could significantly reduce the lesion diameter, but cell suspensions of the
337 yeast grown in YES or YEMS significantly reduced blue mould incidence.
338 The fungicide thiabendazole reduced the incidence of grey mould and blue mould on
339 both apple cultivars, but its efficacy in reducing the lesion diameter was higher against
340 *B. cinerea* on 'Golden Delicious' apples, lower, although significant, against *P.*
341 *expansum* on 'Golden Delicious' apples, and not significant against both pathogens on
342 'Gala' apples.
343
344 **4. Discussion**
345 The cell production is an essential step in the commercialization of a yeast with

346 industrial application as a biopesticide. To our knowledge, there are not studies that
347 have addressed the production process of *M. pulcherrima*, considering the biomass
348 viable count and the biocontrol efficacy as an objective function of the process.
349 The growth parameters were initially optimized in flask experiments: the complex
350 nutrient source that provided the maximum biomass of the antagonistic yeast *M.*
351 *pulcherrima* was Yeast Extract. Although other sources tested (nutrient broth and the
352 three peptones) contained higher nitrogen content, probably Yeast Extract possesses a
353 more balanced equilibrium of amino acids and peptides (it contained 11.4% nitrogen),
354 together with vitamins and carbohydrates, able to promote and sustain a rapid growth of
355 a yeast microorganism (Peppler 1982; Perez et al. 1992). Moreover Yeast Extract shows
356 a buffer ability (Gaudreau et al. 1997) and this could contribute to reach the highest
357 viable cells concentration. The concentrations of Yeast Extract providing the highest
358 growth were 30 g l^{-1} or more.
359 The trial carried out using the complex source media adjusted to pH 7.00 ± 0.05 allowed
360 to determine the effect of the pH control on the biomass production by the strain
361 BIO126. The buffering permitted to significantly improve the final yeast biomass
362 obtained using Casein Hydrolyzed and Nutrient Broth, but it did not affect the result
363 with the other complex source media. For this reason, the trials continued using Yeast
364 Extract without phosphate buffer.
365 A wide range of pH permitted the growth of *M. pulcherrima* (from 3.0 to 10.0),
366 although initial pH values ranging from 5.0 to 7.5 provided the highest culture growth.
367 Thus the subsequent trials were carried out measuring the initial pH values.
368 The results obtained in flask experiments were confirmed in well-controlled batch
369 fermentations. The higher viable cells count obtained in the top bench fermenter could

370 be linked to a better oxygenation as compared to the shake flasks. Maximum
371 exponential growth rate, maximum culture density and maximum wet and dry
372 biomasses were reached using YEMS medium. Moreover, the stationary phase was
373 reached in YEMS from 2 to 4 h earlier than in the other substrates tested. Our results on
374 *M. pulcherrima* are in accordance or higher than previously obtained results. Abadias et
375 al. (2003) obtained 8 x 10^8 cfu ml^{-1} of *Candida sake* after 30 h growth in a 5 l lab-scale
376 fermenter. By growing *Rhodotorula minuta* in a shake flasks for 48 h, the best results in
377 terms of viable microorganisms (over 10^9 cells ml^{-1}) were obtained with a PYD, a
378 medium containing soluble potato starch, dextrose and yeast extract (Patiño-Vera et al.
379 2005).
380 In the fermentation experiments performed, L-Sorbose lowered the initial pH when it
381 was added to YES and YEMS, and the metabolism of D-Mannitol contributed to lower
382 the pH of the medium during the exponential growth phase. For this reason the
383 combination of the two carbon sources, probably with diauxic utilization, showed a
384 synergistic effect, improving the final yeast biomass over those obtained with the single
385 carbohydrates, and lowering the pH up to 6.08 during the exponential phase. The
386 sequential metabolism of D-Mannitol, followed by L-Sorbose, and finally by the amino
387 acids and proteins contained in Yeast Extract, suggests that the growth response is
388 diauxic, reflecting a sequential rather than simultaneous utilization of the carbon sources
389 (Collier et al. 1996).
390 Actively growing yeast acidifies the growth medium through a differential ion uptake
391 and direct secretion of organic acids and carbon dioxide (Walker 1998). An increase of
392 the pH was related to the beginning of the stationary phase. During the experiments,
393 when the microorganism was approaching the stationary phase of growth, the

394 respiration growth decreased, carbon dioxide production decreased and, consequently,
395 pH tended to increase. An experiment was carried out in the top bench fermenter using
396 YEMS as a substrate and with pH control kept at 6.00 (data not shown). The final cell
397 concentration obtained was not significantly different from the experiment without pH
398 buffering, as already shown in the shake flask experiments, so that pH control was not
399 considered as an essential factor to increase the yeast biomass.
400 Oxygen consumption could be considered an indicator of exponential growth (Abadias
401 et al. 2003). The dissolved oxygen rapidly decreased during the exponential phase
402 because of cell respiration and, on the opposite, it started back to increase because of a
403 decrease in the respiration rate of the cells (Meesters et al. 2003).
404 Cells were grown for 48 h in shake flask experiments and 40 h in well-controlled batch
405 experiments, periods largely sufficient for the microorganism to reach the stationary
406 phase of growth. Harvesting stationary phase cells is desirable to enhance cell survival
407 under stress conditions such as low water potential or drying (Abadias et al. 2001). This
408 could be an advantage as, after production, *M. pulcherrima* cells have to be formulated,
409 probably, through a drying process, such as freeze drying (Melin et al. 2007) or fluid
410 bed drying (Bayrock and Ingledew 1997). In both cases, yeast cells harvested at the
411 stationary phase are more resistant to the osmotic stress caused by the drying process
412 (Wraight et al. 2001).The efficacy trials conducted on two commercial varieties of apple
413 permitted to test the influence of the growing media on the biocontrol capability of *M.*
414 *pulcherrima* BIO126. The growth of both pathogens and the biocontrol efficacy of
415 BIO126 were affected by the host cultivar. The two different cultivars chosen for the
416 test, “Gala” and “Golden Delicious”, have different pH and titrable acidity, able to
417 modify the growth and fitness of postharvest pathogens (Morales et al. 2008). On ‘Gala,

418 the incidence of fruits with grey or blue mould was lower than on 'Golden Delicious'
419 apples. On 'Golden Delicious' apples, the microorganism cells grown in YEMS
420 provided a higher reduction of incidence and severity of *B. cinerea* and *P. expansum*.
421 Results were more promising against grey mould than blue mould, probably because *P.*
422 *expansum* is growing at a faster rate. On 'Gala' apples, the best results on the reduction
423 of the disease incidence were obtained with cells grown on YEMS or YES. Previous
424 studies, carried out to test the influence of the growing media on the biocontrol efficacy
425 of other antagonists, such as *Pantoea agglomerans*, did not show any statistically
426 significant effect of the substrates (Costa et al. 2001).
427 As a chemical control, thiabendazole was used. Benzimidazoles can effectively control
428 grey mould on apples, but they are almost ineffective against *P. expansum*, due to the
429 high level of resistance developed by most of the Italian strains of this pathogen
430 (Bertetti et al. 2003). To represent conditions more similar to the postharvest
431 environment, both pathogen mixtures used in the efficacy trials were formed by four
432 benzimidazole-sensitive isolates and one benzimidazole-resistant isolate. *Penicillium*
433 *expansum* can cause economical losses due to blue mould, but also produces patulin, a
434 mycotoxin often found in apple juices (Spadaro et al. 2007). For this reason, effective
435 control strategies against blue mould are necessary, and strain BIO126 could constitute
436 an effective alternative for blue mold control after harvest.
437 The results obtained were encouraging and a good substrate was developed for
438 laboratory purposes (i.e. to find optimal conditions for biomass production,
439 stabilization, formulation, and to develop a quality control system), but the following
440 step will be the scaling up of the production process to the level of pilot plant and the
441 subsequent stabilization and formulation. To scale-up a laboratory fermentation process

442 to an industrial level, it is fundamental to find nitrogen and carbon sources that provide
443 maximum biomass production and minimum cost of media, whilst maintaining
444 biocontrol efficacy (Mousdale et al. 1999). Yeast extract was the best complex
445 nutritional source for BIO126 but it is expensive for an industrial process. The use of
446 commercial by-products with the same nutritional qualities can result in a cheap
447 alternative for the yeast biomass production (Ghribi et al. 2006). Anyway, often by-
448 products are not standardized as purified products and they may contain impurities that
449 need to be removed before fermentation (Stanbury et al., 1995). Moreover, their
450 composition may vary according to season and origin. For these reasons, appropriate
451 procedures should be employed to standardize the industrial growth media (Thomsen
452 2005). Commercial dry beer yeast could replace synthetic yeast extract, but it should be
453 filtered before autoclaving, to discard the insoluble fraction (Reed and Nagodawithana
454 1991).
455 The knowledge gained about the addition of D-Mannitol and L-Sorbose will be useful
456 to develop a cheap substrate containing a complex nutritional source similar to yeast
457 extract together to the sugars selected in this study. After producing the biomass of the
458 antagonistic yeast, the next step will be the cell stabilization by freeze-drying or fluid
459 bed drying (Brian and Etzel 1997).

460 **Acknowledgements**

461 This research was carried out with a grant from the Piedmont Region for the project
462 "Selection, study of the efficacy and of the mechanism of action, characterization and
463 development of antagonistic yeast against post-harvest fruit pathogens". The authors
464 gratefully acknowledge Dr. Pilar Bustos, Dr. Piergiuseppe Nestola and Ms. Incoronata
465 Luongo for their help in the realization of the trials and Dr. Jeanne Griffin for her

linguistic advice. A special thank to the anonymous reviewers of the manuscript.

References

Abadias, M., Teixidó, N., Usall, J., and Viñas, I. 2003. Optimization of growth conditions of the postharvest biocontrol agent *Candida sake* CPA-1 in a lab-scale fermenter. J. Appl. Microbiol. **95**: 301-309.

Abadias, M., Teixidó, N., Usall, J., Viñas, I., and Magan, N. 2001. Improving water stress tolerance of the biocontrol yeast *Candida sake* grown in molasses-based media by physiological manipulation. Can. J. Microbiol. **4**: 23-129.

Bayrock, D. and Ingledew, W.M. 1997. Fluidized bed drying of baker's yeast: moisture levels, drying rates, and viability changes during drying. Food Res. Int. **30**: 407-415.

Bertetti, D., Kejji, S., Garibaldi, A., and Gullino, M.L., 2003. Valutazione della sensibilità di alcuni agenti di marciume su pomacee in post-raccolta nei confronti di diversi fungicidi. Inf.tore Fitopatol. **53** (6): 57-60.

Bleve, G., Grieco, F., Cozzi, G., Logrieco, A., and Visconti, A. 2006. Isolation of epiphytic yeasts with potential for biocontrol of *Aspergillus carbonarius* and *A. niger* on grape. Int. J. Food Microbiol. **108**: 204-209.

Boulton, R.B., Singleton, V.L., Bisson, L.F., and Kunkee, R.E. 1998. Principles and Practices of Winemaking. Aspen Publishers, New York.

Brian, C.S. and Etzel, M.R. 1997. Spray drying, freeze drying, or freezing of three different lactic acid bacteria species. J. Food Sci. **62:** 576-578.

Collier, D.N., Hager, P.W., and Phibbs, P.V. 1996. Catabolite repression control in the Pseudomonads. Res. Microbiol. **147:** 551–561.

Costa, E., Teixidó, N., Usall, J., Atarés, E., and Viñas, I. 2001. Production of the

490 biocontrol agent *Pantoea agglomerans* strain CPA-2 using commercial products and
491 by-products. Appl. Microbiol. Biotechnol. **56**: 367-371.
492 Crabtree, H.G. 1928. The carbohydrate metabolism of certain pathological overgrowths.
493 Biochem. J. **22** (5): 1289-1298.
494 Droby, S., Chalutz, E., Wilson, C.L., and Wisniewski, M.E. 1989. Characterization of
495 the biocontrol activity of *Debaryomices hansenii* in the control of *Penicillium digitatum*
496 on grapefruit. Can. J. Microbiol. **35**: 794-800.
497 Gaudreau, H., Champagne, C.P., Goulet, J., and Conway, J. 1997. Lactic fermentation
498 of media containing high concentrations of yeast extract. J. Food Sci. **62**: 1072-1075.
499 Ghribi, D., Zouari, N., Trigui, W., Jaoua, S. 2006. Use of sea water as salts source in
500 starch- and soya bean-based media, for the production of *Bacillus thuringiensis*
501 bioinsecticides. Process Biochem. **42**: 374–378.
502 Hofstein, R., Friedlender, B., Chalutz, E., and Droby, S. 1994. Large scale production
503 and pilot testing of biocontrol agents of postharvest diseases. *In* Biological Control of
504 Postharvest Diseases – Theory and Practice. *Edited by* C.L. Wilson and M. Wisniewski.
505 CRC Press Inc, Boca Raton, pp. 89-100.
506 Janisiewicz, W.J., and Korsten, L. 2002. Biological control of postharvest diseases of
507 fruits. Annu. Rev. Phytopathol. **40**: 411-441.
508 Janisiewicz, W.J., Tworkoski, T.J., and Kurtzman, C.P. 2001. Biocontrol potential of
509 *Metschnikowia pulcherrima* strains against blue mold of apple. Phytopathology **91**:
510 1098-1108.
511 Leverentz, B., Conway, W.S., Janisiewicz, W., Abadias, M., Kurtzman, C.P., and
512 Camp, M.J. 2006. Biocontrol of the food-borne pathogens *Listeria monocytogenes* and

513 *Salmonella enterica* serovar Poona on fresh-cut apples with naturally occurring bacterial
514 and yeast antagonists. Appl. Environ. Microbiol. **72**:1135–40.
515 Meesters, P.A.E.P., Huijberts, G.N.M., and Eggink, G. 1996. High-cell density
516 cultivation of the lipid accumulating yeast *Cryptococcus curvatus* using glycerol as
517 carbon source. Appl. Microbiol. Biotechnol. **45:** 575-579.
518 Miller, M.W., and Phaff, H.J. 1998. *Metschnikowia* Kamienski. *In* The Yeasts. *Edited*
519 *by* C.P. Kurtzmann and J.W. Fell. Elsevier, Amsterdam, pp. 256-257.
520 Morales, H., Barros, G., Marín, S.,Chulze, S., , Ramos, A.J., and Sanchis, V. 2008.
521 Effects of apple and pear varieties and pH on patulin accumulation by *Penicillium*
522 *expansum*. J. Sci. Food Agric. **88**: 2738-2743.
523 Mousdale, D.M., Melville, J.C., and Fischer, M. 1999. Optimization of fermentation
524 process by quantitative analysis: from analytical biochemistry to chemical engineering.
525 *In* Fermentation Microbiology and Biotechnology. *Edited by* E.M.T. El-Mansi and
526 C.F.A. Bryce. Taylor and Francis, London, pp. 147-178.
527 Patiño-Vera, M., Jiménez, B., Balderas, K., Ortiz, M., Allende, R., Carrillo, A. and
528 Galindo, E., 2005. Pilot-scale production and liquid formulation of *Rhodotorula minuta*,
529 a potential biocontrol agent of mango anthracnose. J. Appl. Microbiol. **99**: 540-550.
530 Peppler, H.J. 1982. Yeast extracts. *In* Fermented Foods. *Edited by* A.H. Rose. Academic
531 Press, London, pp. 293-312.
532 Perez, F., Riba, J.P., and Strehaiano, P. 1992. Effect of yeast extract concentration on
533 growth of *Schizoccharomyces pombe*. Biotechnol. Lett. **14**: 123-126.
534 Piano, S., Neyrotti, V., Migheli, Q., and Gullino, M.L. 1997. Characterization of the
535 biocontrol capability of *Metschnikowia pulcherrima* against Botrytis postharvest rot of
536 apple. Postharvest Biol. Technol. **11**: 131-140.

537 Reed, G. and Nagodawithana, T.W. 1991. Yeast Technology, 2nd edition, Van Nostrand
538 Rheinold, New York.
539 Saravanakumar, D., Ciavorella, A., Spadaro, D., Garibaldi, A., and Gullino, M.L. 2008.
540 *Metschnikowia pulcherrima* strain MACH1 outcompetes *Botrytis cinerea*, *Alternaria*
541 *alternata* and *Penicillium expansum* in apples through iron depletion. Postharvest Biol.
542 Technol. **49**: 121-128.
543 Saravanakumar D., Spadaro D., Garibaldi A., and Gullino M.L. 2009. Detection of
544 enzymatic activity and partial sequence of a chitinase in *Metschnikowia pulcherrima*
545 strain MACH1 used as postharvest biocontrol agent. Eur. J. Plant Pathol. **123**: 183-193.
546 Schena, L., Ippolito, A., Zahavi, T., Cohen, L., and Droby, S. 2000. Molecular
547 approaches to assist the screening and monitoring of postharvest biological yeasts. Eur.
548 J. Plant Pathol. **106**: 681-691.
549 Sinigaglia, M., Corbo, M.R., and Ciccarone, C. 1998. Influence of temperature, pH and
550 water activity on "in vitro" inhibition of *Penicillium glabrum* (Wehmer) Westling by
551 yeasts. Microbiol Res. **153**:137-43.
552 Spadaro, D., and Gullino, M.L. 2004. State of art and future perspectives of biological
553 control of postharvest fruit diseases. Int. J. Food Microbiol. **91**: 185-194.
554 Spadaro, D., Sabetta, W., Acquadro, A., Portis, E., Garibaldi, A., and Gullino, M.L.
555 2008. Efficacy and genetic diversity of *Metschnikowia pulcherrima* strains isolated
556 from different food matrices against postharvest diseases in apple. Microbiol. Res. **163**:
557 523-530.
558 Spadaro, D., Vola, R., Piano, S., and Gullino, M.L. 2002. Mechanisms of action,
559 efficacy and possibility of integration with chemicals of four isolates of the yeast
560 *Metschnikowia pulcherrima* active against postharvest pathogens on apples. Postharvest

561 Biol. Technol. **24**: 123-134.

562 Spadaro, D., Ciavorella, A., Frati, S., Garibaldi, A., and Gullino, M.L. 2007. Incidence
563 and level of patulin contamination in pure and mixed apple juices marketed in Italy.
564 Food Cont. **18**: 1098-1102.

565 Stanbury, P.F., Whitaker, A., and Hall, S.J. 1995. Principles of Fermentation
566 Technology. Pergamon Press, Oxford.

567 Thomsen, M.H. 2005. Complex media from processing of agricultural crops for
568 microbial fermentation. Appl. Microbiol. Biotechnol. **68**: 598-606.

569 Walker, G.M. 1998. Yeast Physiology and Biotechnology. John Wiley and Sons,
570 Chichester.

571 Wilson, C.L., and Wisniewski, M.E. 1994. Biological Control of Postharvest Diseases.
572 Theory and Practice. CRC Press, Boca Raton.

573 Wraight, S.P., Jackson, M.A., and de Kock, S.L. 2001. Production, Stabilization and
574 Formulation of Fungal Biocontrol Agents. *In* Fungi as Biocontrol Agents. *Edited by*
575 T.M. Butts, C. Jackson and N. Magan. CAB International, Wallingford, Great Britain,
576 pp. 253-287.

577

Table 1 Growth of *Metschnikowia pulcherrima* strain BIO126 (cfu ml^{-1}) in different complex nutrient media (10 g l^{-1} each), without (a, left) or with (b, right) adjustment of initial pH_0 7.00±0.05 with a phosphate buffer. Values of pH (±0.05) at initial time (pH_0), after 24h (pH_{24}) and 48h culture (pH_{48}) are shown. Cultures (5 x 10^5 cfu ml^{-1}) were inoculated in 300 ml of liquid media and grown on a rotary shaker (150 rpm) at 25°C for 48 h.

	Total nitrogen content	Complex nutrient media (a)					Buffered complex nutrient media (b)			
		pH_0	pH_{24}	pH_{48}	Mean (cfu ml^{-1})		pH_{24}	pH_{48}	Mean (cfu ml^{-1})	
Yeast Extract	11.4	7.13	7.91	8.56	1.2 x 10^8	a*	8.05	8.61	1.1 x 10^8	a*
Nutrient Broth	13.0	7.11	7.72	8.57	8.5 x 10^7	a-b	8.19	8.73	1.2 x 10^8	a
Malt Extract	1.1	5.17	4.94	3.84	5.3 x 10^7	b	6.77	6.70	1.8 x 10^7	c
Meat Peptone	13.9	7.36	7.30	7.20	2.3 x 10^7	b-c	7.10	7.34	1.8 x 10^7	c
Casein Peptone	13.5	7.04	7.35	7.50	1.5 x 10^7	c	7.14	7.22	2.7 x 10^7	c
Bacto-peptone	15.4	7.09	7.56	7.53	1.3 x 10^7	c	7.26	7.35	1.4 x 10^7	c
Casein Hydrolyzed	8.0	5.42	5.53	5.67	1.3 x 10^7	c	7.45	8.04	8.0 x 10^7	b
Ringer solution	--	6.35	6.30	6.54	4.9 x 10^5	d	7.42	7.32	5.6 x 10^5	d

* Values in the same column followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P<0.05$).

589 **Table 2** Effect of the initial pH value on the growth of *Metschnikowia pulcherrima*
590 strain BIO126 (inoculum: 5 x 10^5 cfu ml^{-1}) produced in liquid medium containing yeast
591 extract (30 g l^{-1}) at 25°C for 48 h. Values of pH (±0.05) at initial time (pH_0), after 24
592 (pH_{24}) and 48h culture (pH_{48}) are shown.
593

pH_0	pH $_{24h}$	Mean $_{24h}$ (cfu ml^{-1})	pH $_{48h}$	Mean $_{48h}$ (cfu ml^{-1})
1.0	1.06	no living cells	0.94	no living cells
1.5	1.58	no living cells	1.42	no living cells
2.0	2.08	no living cells	1.95	no living cells
2.5	2.53	no living cells	2.45	no living cells
3.0	3.22	5.2 x 10^5	7.35	9.5 x 10^6 b*
3.5	6.03	1.2 x 10^6	7.67	1.6 x 10^7 b-c
4.0	7.72	1.2 x 10^8	7.32	2.6 x 10^8 d-f
4.5	7.70	1.1 x 10^8	6.59	2.1 x 10^8 d-e
5.0	8.02	1.3 x 10^8	8.12	4.9 x 10^8 g
5.5	7.02	1.6 x 10^8	8.27	3.8 x 10^8 f-g
6.0	6.80	1.5 x 10^8	8.16	3.7 x 10^8 f-g
6.5	8.23	1.4 x 10^8	8.33	3.2 x 10^8 e-g
7.0	7.98	7.3 x 10^7	8.34	3.1 x 10^8 e-g
7.5	7.68	5.8 x 10^7	8.05	3.4 x 10^8 e-g
8.0	7.91	5.5 x 10^7	6.70	2.2 x 10^8 d-f
8.5	8.30	6.3 x 10^7	8.28	1.6 x 10^8 d
9.0	7.05	3.3 x 10^7	7.62	3.6 x 10^7 c
9.5	8.53	1.4 x 10^7	8.23	2.3 x 10^7 b-c
10.0	8.86	3.9 x 10^5	8.90	1.5 x 10^5 a
11.0	9.30	3.9 x 10^5	9.43	no living cells

594
595 * See Table 1.
596

Table 3 Growth of *Metschnikowia pulcherrima* strain BIO126 (cfu ml^{-1}) in media containing yeast extract (30 g l^{-1}) and different carbon sources. Cultures (5 x 10^5 cfu ml^{-1}) were inoculated in 300 ml of liquid media and grown on a rotary shaker (150 rpm) at 25°C for 48 h. Values of pH (±0.05) at initial time (pH_0) and 48h culture (pH_{48}) are shown.

Nitrogen source	Carbon source		$pH_{0\,h}$	pH_{48h}	Mean $_{48h}$ (cfu ml^{-1})	
Yeast extract (30 g l^{-1})	D-Glucose	10 g l^{-1}	6.88	7.38	3.7 x 10^8	b-c*
		20 g l^{-1}	6.80	8.18	5.2 x 10^8	b-c
	D-Fructose	10 g l^{-1}	6.89	8.54	3.8 x 10^8	b-c
		20 g l^{-1}	6.90	6.78	3.0 x 10^8	a-b
	Sucrose	10 g l^{-1}	6.90	7.64	6.7 x 10^8	c-e
		20 g l^{-1}	6.89	6.85	6.9 x 10^8	c-e
	Maltose	10 g l^{-1}	6.89	8.20	6.2 x 10^8	b-d
		20 g l^{-1}	6.87	7.70	5.7 x 10^8	b-d
	D-Sorbitol	10 g l^{-1}	6.92	7.18	5.9 x 10^8	b-d
		20 g l^{-1}	6.90	6.94	5.6 x 10^8	b-d
	D-Mannitol	2.5 g l^{-1}	6.90	6.40	2.4 x10^8	a-b
		5.0 g l^{-1}	6.92	6.40	6.5 x 10^8	c-e
		7.5 g l^{-1}	6.92	6.50	1.5 x 10^9	g
		10.0 g l^{-1}	6.90	6.60	1.5 x 10^9	g
		12.5 g l^{-1}	6.90	7.00	1.2 x 10^9	f-g
		15.0 g l^{-1}	6.90	7.20	1.3 x 10^9	f-g
		17.5 g l^{-1}	6.91	7.20	8.2 x 10^8	d-e
		20.0 g l^{-1}	6.91	7.50	6.6 x 10^8	c-e
	L-Sorbose	2.5 g l^{-1}	6.90	8.40	1.5 x 10^8	a
		5.0 g l^{-1}	6.90	8.40	2.4 x 10^8	a-b
		7.5 g l^{-1}	6.91	8.30	7.3 x 10^8	d-e
		10.0 g l^{-1}	6.89	8.20	8.0 x 10^8	d-e
		12.5 g l^{-1}	6.90	8.20	1.2 x 10^9	f-g
		15.0 g l^{-1}	6.89	8.00	9.0 x 10^8	e-f
		17.5 g l^{-1}	6.90	7.95	7.0 x 10^8	c-e
		20.0 g l^{-1}	6.90	7.95	7.0 x 10^8	c-e
	D-Mannitol + L-Sorbose	5.0 g l^{-1} + 5.0 g l^{-1}	6.97	6.69	1.7 x 10^9	g
		6.0 g l^{-1} + 6.0 g l^{-1}	6.93	7.23	1.3 x 10^9	f-g
	None		6.91	8.40	1.5 x 10^8	a

* See Table 1.

Fig. 1 Effect of the Yeast Extract concentration on the growth of *Metschnikowia pulcherrima* strain BIO126 (cfu ml^{-1}) produced in shake flasks at 25°C for 48 h. Values of pH after 48h culture are shown between parentheses.

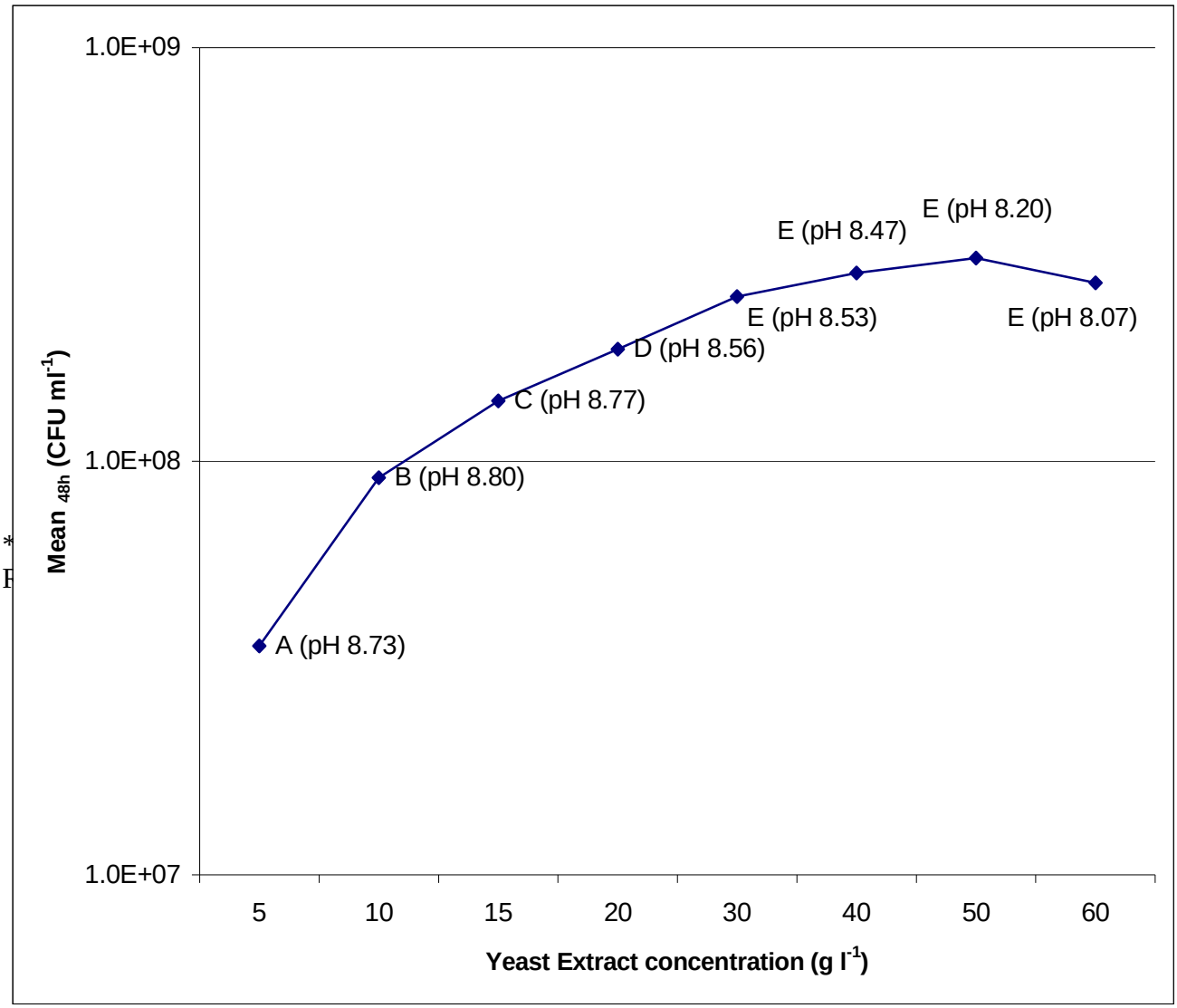
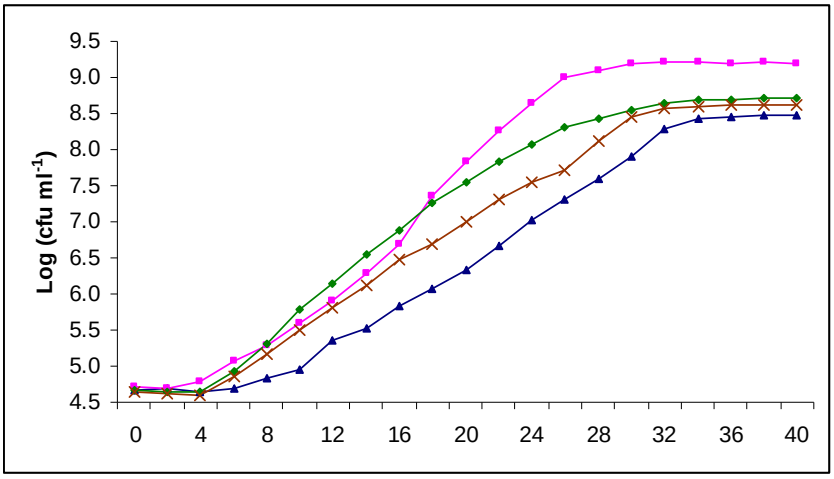
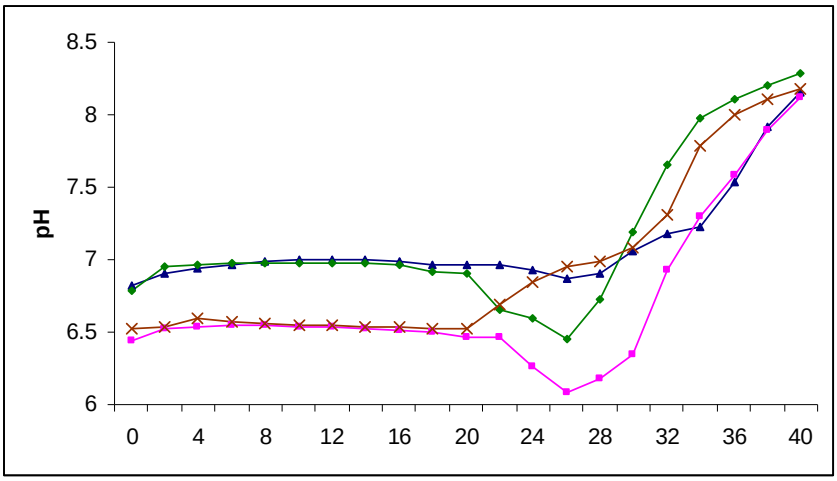


Fig 2 (*a*) Evolution of biomass production (cfu ml^{-1}) of *Metschnikowia pulcherrima* strain BIO126, (*b*) pH and (*c*) dissolved oxygen using YE (Yeast Extract; ▲), YES (Yeast Extract + D-Sorbitol; x), YEM (Yeast Extract + L-Mannitol; ♦) and YEMS (Yeast Extract + D-Sorbitol + L-Mannitol; ■) media in a 5 l fermenter, maintaining temperature at 25°C, stirring at 450 rpm and oxygen flow at 5 l min^{-1} for 40 h.

(*a*)



(*b*)



(*c*)

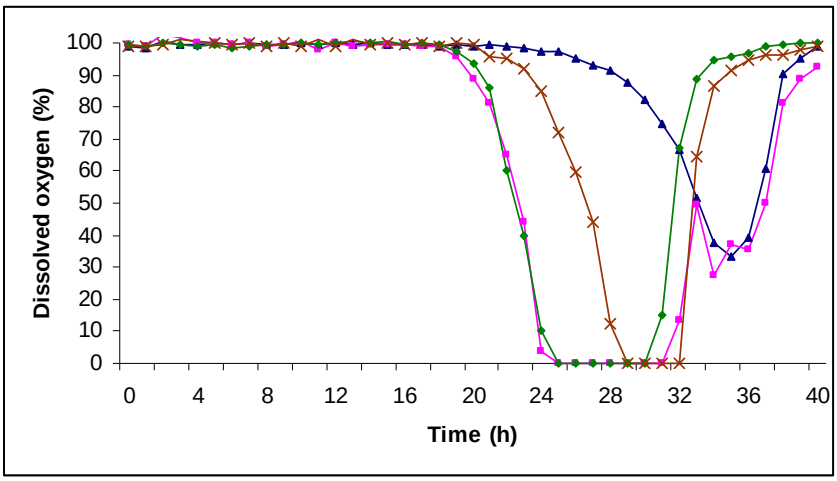
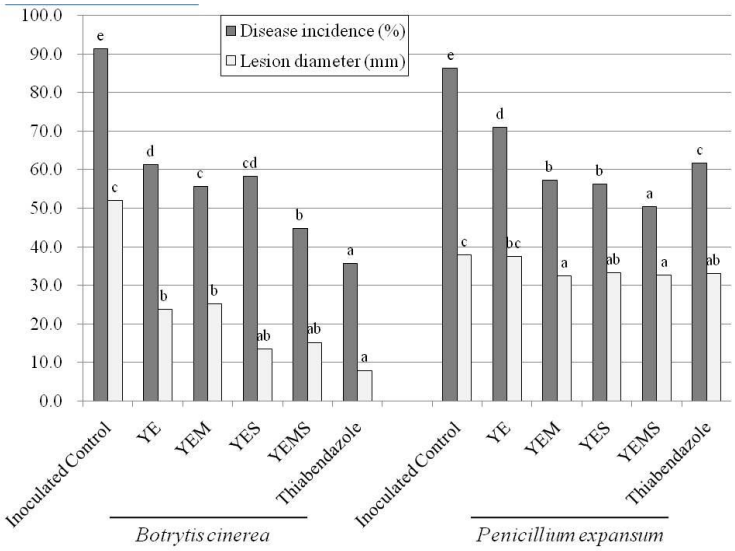
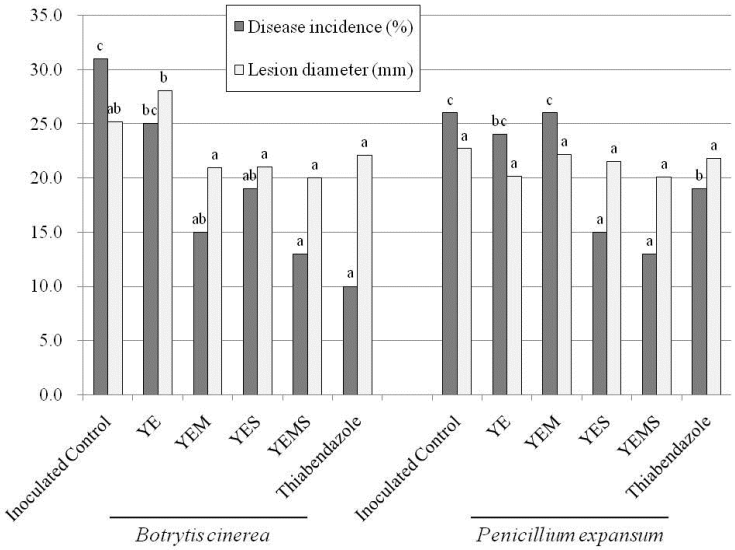


Fig 3 Influence of the cultivation substrate on the efficacy of *M. pulcherrima* strain BIO126 (10^7 cfu ml^{-1}) against *Botrytis cinerea* (left) and *Penicillium expansum* (right) on apples 'Golden Delicious' (***a***) and 'Gala' (***b***). Fruits were artificially inoculated with the pathogen (10^5 conidia ml^{-1}) 3 hours before treatment with the biocontrol agent, and then stored at 1°C for 28 days. Disease incidence was expressed as percentage of rotten fruits and disease severity was assessed by measuring the lesion diameter of the rots (mm). Thiabendazole was used as chemical control (Tecto 20 S, Elf Atochem Agri Italy, 19.7 % a.i., 20 g a.i. 100 l^{-1}).

a



b



Values in columns of the same colour followed by the same letter are not statistically different by Duncan's Multiple Range Test ($P < 0.05$).