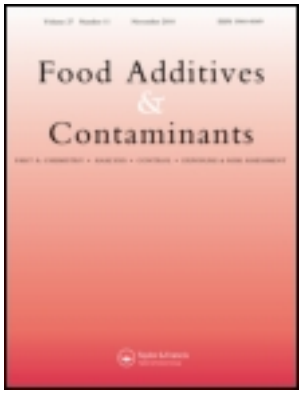

Food Additives & Contaminants: Part A: Chemistry, Analysis, Control, Exposure & Risk Assessment
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tfac20

Production of Fusarium verticillioides biocontrol agents, Bacillus amyloliquefaciens and Microbacterium oleovorans, using different growth media: evaluation of biomass and viability after freeze-drying

M. Sartori [a] , A. Nesci [b] & M. Etcheverry [b]

[a] Agencia Nacional de Promoción Científica y Tecnológica (FONCYT), Laboratorio de Ecología Microbiana, Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas, Físico Químicas y Naturales, Universidad Nacional de Río Cuarto, 5800 Córdoba, Agentina

[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Laboratorio de Ecología Microbiana, Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas, Físico Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta nacional 36, Km 601, 5800 Córdoba, Agentina

Available online: 24 Jan 2012

To cite this article: M. Sartori, A. Nesci & M. Etcheverry (2012): Production of Fusarium verticillioides biocontrol agents, Bacillus amyloliquefaciens and Microbacterium oleovorans, using different growth media: evaluation of biomass and viability after freeze-drying, Food Additives & Contaminants: Part A: Chemistry, Analysis, Control, Exposure & Risk Assessment, 29:2, 287-292

To link to this article: http://dx.doi.org/10.1080/19440049.2011.563369

Food Additives and Contaminants
Vol. 29, No. 2, February 2012, 287–292

Taylor & Francis
Taylor & Francis Group

Production of *Fusarium verticillioides* biocontrol agents, *Bacillus amyloliquefaciens* and *Microbacterium oleovorans*, using different growth media: evaluation of biomass and viability after freeze-drying

M. Sartori[a], A. Nesci[b] and M. Etcheverry[b]*

[a]*Agencia Nacional de Promoción Científica y Tecnológica (FONCYT), Laboratorio de Ecología Microbiana, Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas, Físico Químicas y Naturales, Universidad Nacional de Río Cuarto, 5800 Córdoba, Agentina;* [b]*Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Laboratorio de Ecología Microbiana, Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas, Físico Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta nacional 36, Km 601, 5800 Córdoba, Agentina*

(*Received 15 November 2010; final version received 10 February 2011*)

The aims of this study were to compare the viability and biomass production of *B. amyloliquefaciens* and *M. oleovorans* in different growth media, and the efficiency of a freeze-drying method as a possible formulation process. *B. amyloliquefaciens* and *M. oleovorans* were grown in 100 ml of four different media. Media water activity was modified at 0.99, 0.98, 0.97 and 0.96. Nutrient yeast dextrose broth (NYDB) and molasses soy powder (MSB) media were selected and survival levels of cells were determined before and after the freeze-drying process. *B. amyloliquefaciens* showed the highest survival after freeze-drying when grown in NYDB medium at 0.99 a_w, whereas, at 0.98, 0.97 and 0.96 a_w, the highest survival was obtained in MSB medium. *M. oleovorans* showed the highest survival in MSB medium at 0.99 a_w. MSB medium was select for biomass production due to high growth and survival after freeze-drying.

Keywords: microbiology; fumonisins; animal feed; animal feedingstuffs; cereals

Introduction

The use of biological strains with fungistatic or fungicide characteristics in controlling plant fungal diseases is one of the most promising alternatives to chemical fungicides (Butt 2000). In crops such as maize, seed treatment with biocontrol agents is the most suitable method for controlling pathogens at the rhizosphere level (Lewis 1991; Harman 1992; Hebbar et al. 1992). Previous studies showed that *Bacillus amyloliquefaciens* and *Microbacterium oleovorans* significantly reduced *Fusarium verticillioides* count and fumonisin B_1 and B_2 levels in maize grains (Pereira et al. 2007, 2009). In physiological adaptation assays, these strains were more tolerant to ionic and non-ionic potential stress than matric potential stress (Sartori et al. 2010). Therefore, cells of both biocontrol agents, adapted to osmotic and heat stress, could be more resistant to desiccation and pressure during freeze-drying in an appropriate growth medium.

Formulation development involves a multidisciplinary approach, including microbial–plant ecology and pathology, to clarify the interactions between the biopesticide, pest and environment (Deacon 1991; Whipps 1997; Butt and Copping 2000). Formulation is necessary to optimize the efficacy, stability, safety and ease of application of the product (Rhodes 1993). In addition to biological considerations in choosing liquid or solid fermentation and in the selection of growing media and other system manipulations, the cost of these materials, the amount of time the fermentation system will be tied up and the labour involved must also be considered. The use of cheap substrates from food industries as nitrogen or carbon sources could be an alternative to attain high density and low cost (Costa et al. 2001). To reduce costs, inexpensive waste products, such as molasses, peanut hulls, maize cobs, fish meal, various chitin sources, yeast extracts, soy bean hulls and others, have been used for the production of biocontrol agents (Fravel et al. 1999). Once an optimized defined medium has been developed, a production medium can be formulated by replacing the nutritional components of the defined medium with low-cost, complex substrates (Wraight et al. 2001). Microbial physiology to optimize the fermentation, formulation variables and chemistry were study to select compatible adjuvants that sustain and promote optimum activity of the active ingredient (Hynes and Boyetchko 2006).

*Corresponding author. Email: metcheverry@exa.unrc.edu.ar

ISSN 1944–0049 print/ISSN 1944–0057 online

http://dx.doi.org/10.1080/19440049.2011.563369
http://www.tandfonline.com

Teixidó et al. (2005) suggested that it is possible to significantly improve environmental stress tolerance of bacterial biocontrol agents by physiologically growing them under conditions which facilitate synthesis of useful compatible solutes. This improvement can also give cross-protection during formulation against heat stress. Significant improvements in water and heat stress tolerance were observed when both biocontrol agents, *B. amyloliquefaciens and M. oleovorans*, were developed in media modified with glycerol. Cells of *B. amyloliquefaciens* not only had better tolerance at low a_w but also showed better survival under heat stress (Sartori et al. 2010).

Mass production is an important aspect in the commercial development of a biocontrol product. Commercial production requires low cost and high cells densities, and optimization of nutritional and environmental conditions (Lumsden and Lewis 1989). An appropriate selection of growing medium is essential to optimise cell viability (Abadías et al. 2001).

Freeze-drying is the most convenient and successful method of preserving bacteria, yeasts and sporulating fungi (Berny and Hennebert 1991). Bacterial cell survival during the freeze-drying process is dependent on many factors, such as cell growth conditions, protective suspension medium, initial cell concentration, freezing temperature and rehydratation conditions (Zhao and Zhang 2005). Therefore, the hypothesis is that cells of both biocontrol agents, adapted to osmotic and heat stress, would be more resistant to desiccation and pressure during freeze-drying in an appropriate growth medium. The aims of this study were to compare the viability and biomass production of *B. amyloliquefaciens* and *M. oleovorans* in different growth media plus the efficiency of the freeze-drying method to produce a low cost, highly effective and enhanced performance formulation.

Materials and methods

Bacterial strains

The strains used in this study were *Bacillus amyloliquefaciens* (GenBank accession # EU164542) and *Microbacterium oleovorans* (GenBank accession # EU164543) (Pereira et al. 2009). These strains were isolated from a commercial maize field and identified on the basis of 16S DNA gene sequence similarity and additionally identified based on their physiological profiling according to Bergey's Manual of Systematic Bacteriology (Holt 1993).

Growth media and culture conditions

B. amyloliquefaciens and *M. oleovorans* were grown in 200 ml of four different media: (1) nutrient yeast dextrose broth (NYDB) [nutrient broth ($8\,g\,l^{-1}$) + dextrose ($10\,g\,l^{-1}$) + yeast ($5\,g\,l^{-1}$]; (2) Molasses soy powder (MSB) [molasses ($20\,g\,l^{-1}$) + soy powder ($10\,g\,l^{-1}$)]; (3) Sucrose yeast broth (SYB) [sucrose ($10\,g\,l^{-1}$) + yeast extract ($5\,g\,l^{-1}$)] (Costa et al. 2001); (4) Lactose tapioca peptone ammonium sulphate broth (LTPAB) [lactose ($12.7\,g\,l^{-1}$) + tapioca ($16.7\,g\,l^{-1}$) + peptone ($8\,g\,l^{-1}$) +ammonium sulphate ($1.8\,g\,l^{-1}$)] (Rao et al. 2007).

Media water activities were modified to 0.99; 0.98; 0.97 and 0.96 a_w using NaCl and glycerol (Dallyl and Fox 1980). Each treatment was inoculated with 1% fresh cultures of each strain of *B. amyloliquefaciens* and *M. oleovorans* that was grown initially in tryptic soy broth (TSB). Conical flasks were incubated on a rotary shaker (140 rpm) at 30°C. The growth in each treatment was determined spectrometrically to 620 nm and cell viability was estimated using the surface-plated method. Sample dilutions were made in TSB and spread-plated onto tryptic soy agar (TSA). Plates were incubated at 30°C for 24 h and the viable count was expressed as colony forming units per ml ($cfu\,ml^{-1}$). The experiments were conducted in three replicates for each treatment, and two independent assays. The growth parameters g (generation time) and k (constant growth rate) were calculated by linear regression of the exponential growth phase.

Inoculum production

NYDB and MSB media were selected. The water activity of these media was modified to 0.99; 0.98; 0.97 and 0.96 a_w by adding different amount of NaCl (Dallyl and Fox 1980).

To build up inocula, 1000-ml conical flasks containing 200 ml of medium were inoculated with 1% (v/v) inocula of *B. amyloliquefaciens* and *M. oleovorans*. Separately, the inocula were added aseptically to each flask containing the medium at different a_w values The inoculated flasks were incubated in a orbital shaker at 140 rpm and 30°C for 24 h. Inoculated cultures were serially diluted in nutrient broth and spread-plated (100 µl) onto the surface of TSA plates to estimate the inocula level. Plates were incubated at 30°C for 24 h and the initial number of colony forming units per millilitre ($cfu\,ml^{-1}$) was calculated (survival levels before freeze-drying).

The cells were protected by the addition of 10% sucrose solution before freeze-drying (Abadías et al. 2001). The conical flasks containing the inocula were frozen directly at −20°C for 4 h, then at −80°C for 24 h; these were then connected to a freeze-drier operating at a chamber pressure <0.05 mbar and −45°C for 72 h. The experiments were conducted in three replicates and repeated twice.

Table 1. Influence of growth media and water activities on growth parameters of *B. amyloliquefaciens*.

Growth media	0.99 a_w	0.98 a_w	0.97 a_w	0.96 a_w
NYDB *b*	*g*: 0.25 h *c* *k*: 2.76 h^{-1} *a*	*g*: 0.32 h *c* *k*: 2.15 h^{-1} *b*	*g*: 0.35 h *b* *k*: 1.97 h^{-1} *c*	*g*: 0.48 h *a* *k*: 1.43 h^{-1} *d*
MSB *b*	*g*: 0.20 h *c* *k*: 3.45 h^{-1} *a*	*g*: 0.26 h *c* *k*: 2.65 h^{-1} *b*	*g*: 0.35 h *b* *k*: 1.97 h^{-1} *c*	*g*: 0.54 h *a* *k*: 1.27 h^{-1} *d*
SYB *c*	*g*: 0.60 h *c* *k*: 1.15 h^{-1} *a*	*g*: 0.52 h *c* *k*: 1.32 h^{-1} *b*	*g*: 0.67 h *b* *k*: 1.03 h^{-1} *c*	*g*: 1.52 h *a* *k*: 0.45 h^{-1} *d*
LTPAB *a*	*g*: 0.38 h *c* *k*: 1.82 h^{-1} *a*	*g*: 0.37 h *c* *k*: 1.84 h^{-1} *b*	*g*: 0.39 h *b* *k*: 1.76 h^{-1} *c*	*g*: 0.49 h *a* *k*: 1.40 h^{-1} *d*

Notes: Data with the same letter are not significantly different according to Duncan's multiple range test ($P < 0.05$).
g: generation time.
k: constant growth rate.

Determination of viability

From the freeze-dried samples, 0.5 g of each treatment were rehydrated with 9 ml of nutrient broth, incubated for 1 h at room temperature, and homogenized with a vortex mixer. Serial dilutions were spread-plated on the surface of TSA plates. These plates were incubated at 30°C for 24 h and viable populations counted. Survival levels were expressed as the number of colony forming units per millilitre (cfu ml^{-1}).

Statistical analysis

Analysis of variance (ANOVA) was made for growth parameters and viable counts using a SAS program (SAS System for Windows 6.11; SAS Institute, Cary, NC, USA). To establish significant differences, Duncan's multiple range test ($p < 0.05$) was performed.

Results

Effect of growth media and osmotic potential on bacterial growth parameters

B. amyloliquefaciens and *M. oleovorans* growth parameters in all media plus a_w assay results are shown in Tables 1 and 2. The constant growth rate (k) and generation time (g) of *B. amyloliquefaciens* and *M. oleovorans* at different a_w levels for each growth medium tested was statistically significant ($p < 0.05$) for the medium and four water activities (ANOVA test). The k values decreased as the water activity level decreased in all media, with the lowest number of generations per unit of time at 0.97 and 0.96 a_w. Changes in a_w alone reduced the growth rate by more than 60% between 0.99 and 0.96 a_w for *B. amyloliquefaciens* and 90% for *M. oleovorans*. Both bacteria showed the highest reduction in k values at 0.96 a_w in MSB medium. The highest constant growth rate for *B. amyloliquefaciens* and *M. oleovorans* was observed in MSB medium at 0.99 a_w ($k = 3.45$ and 1.97 h^{-1}, respectively). Both bacteria achieved the lowest duplication time ($g = 0.20$ and 0.35 h, respectively) under these conditions. In NYDB medium, *B. amyloliquefaciens* showed acceptable growth ($k = 2.76\ h^{-1}$) and low generation time ($g = 0.25$ h). Similarly, *M. oleovorans* showed a growth rate of $k = 1.30\ h^{-1}$ and a generation time of $g = 0.53$ h. *B. amyloliquefaciens* and *M. oleovorans* showed a growth rate reduction of more than 60% to 40% and 38% to 30% in SYB and LTPAB media, respectively, at 0.99 a_w compared to MSB medium. The biomass obtained in SYB and LTPAB media at 0.97 and 0.96 a_w was insufficient to carry out the freeze-drying process. Based on the growth parameters in MSB and NYDB media, both were selected to obtain biomass for the freeze-drying process.

Resistance of freeze-drying

The cells of *B. amyloliquefaciens* and *M. oleovorans* were grown in MSB and NYDB media. NaCl was selected to modify media water activities as media modified with glycerol did not dry completely during the freeze-drying process. The addition of 10% sucrose to growth media increased cell viability (data not shown) and, therefore, sucrose was added to all treatments. The viable count of *B. amyloliquefaciens* and *M. oleovorans* before and after the freeze-drying process are shown in Tables 3 and 4. Significant differences in viable counts were observed before and after freeze-drying. *B. amyloliquefaciens* showed a better tolerance to freeze-drying when grown in MSB medium at 0.98, 0.97 and 0.96 a_w. *M. oleovorans* showed better survival after freeze-drying in NYDB medium at 0.99, 0.98 and 0.97 a_w. No survival was observed in NYDB medium at 0.96 a_w.

The greatest difference in cell viability for *B. amyloliquefaciens* was obtained at 0.99 a_w in NYDB

Table 2. Influence of growth media and water activities on growth parameters of *M. oleovorans*.

Growth media	0.99 a_w	0.98 a_w	0.97 a_w	0.96 a_w
NYDB *a*	*g*: 0.53 h *c* *k*: 1.30 h^{-1} *a*	*g*: 0.54 h *c* *k*: 1.27 h^{-1} *b*	*g*: 1.14 h *b* *k*: 0.60 h^{-1} *c*	*g*: 1.26 h *a* *k*: 0.55 h^{-1} *d*
MSB *a*	*g*: 0.35 h *c* *k*: 1.97 h^{-1} *a*	*g*: 0.73 h *c* *k*: 0.94 h^{-1} *b*	*g*: 1.07 h *b* *k*: 0.64 h^{-1} *c*	*g*: 5.55 h *a* *k*: 0.12 h^{-1} *d*
SYB *c*	*g*: 0.57 h *c* *k*: 1.21 h^{-1} *a*	*g*: 0.88 h *c* *k*: 0.78 h^{-1} *b*	*g*: 2.31 h *b* *k*: 0.29 h^{-1} *c*	*g*: 2.44 h *a* *k*: 0.28 h^{-1} *d*
LTPAB *b*	*g*: 0.50 h *c* *k*: 1.38 h^{-1} *a*	*g*: 0.60 h *c* *k*: 1.15 h^{-1} *b*	*g*: 0.93 h *b* *k*: 0.74 h^{-1} *c*	*g*: 0.98 h *a* *k*: 0.70 h^{-1} *d*

Notes: Data with the same letter are not significantly different according to Duncan's multiple range test ($p < 0.05$).

Table 3. Viable count (log cfu ml^{-1}) of *Bacillus amyloliquefaciens* before and after freeze-drying in NYDB and MSB growth media.

	NYDB		MSB	
a_w	Before freeze-drying	After freeze-drying	Before freeze-drying	After freeze-drying
0.99	Stationary phase	7.92 ± 0.46	Stationary phase	6.77 ± 0.74
0.98	Stationary phase	4.30 ± 0.99	Stationary phase	7.54 ± 1.31
0.97	11.82 ± 0.18	3.38 ± 0.12	Stationary phase	5.08 ± 0.87
0.96	11.27 ± 0.62	3.34 ± 0.49	Stationary phase	4.23 ± 0.00

Table 4. Viable count (log cfu ml^{-1}) of *Microbacterium oleovorans* before and after freeze-drying process in NYDB and MSB growth media.

	NYDB		MSB	
a_w	Before freeze-drying	After freeze-drying	Before freeze-drying	After freeze-drying
0.99	Stationary phase	8.59 ± 1.84	Stationary phase	8.81 ± 0.91
0.98	Stationary phase	10.85 ± 0.63	Stationary phase	8.82 ± 0.09
0.97	Stationary phase	9.75 ± 2.41	Stationary phase	7.69 ± 0.55
0.96	7.42 ± 0.09	–	Stationary phase	7.24 ± 0.10

medium (7.92 ± 0.46 log cfu ml^{-1}); at 0.98 a_w in MSB medium, the count was 7.54 ± 1.31 log cfu ml^{-1} after freeze-drying. For *M. oleovorans,* cell viability after freeze-drying was highest at 0.98 a_w in NYDB and MSB media, with counts of 10.85 ± 0.63 and 8.82 ± 0.09 log cfu ml^{-1}, respectively.

Discussion

The results obtained in this study show that the use of MSB medium with molasses (20 g l^{-1}) + soy powder (10 g l^{-1}), supported a rapid growth rate and high biomass production. Molasses used in our experiments contained ~50% sucrose (Imrie 1969) and, in agreement with Costa et al. (2001), this high sucrose concentration may explain the high biomass obtained. The low cost of molasses could be an advantage for production of the biocontrol agents, *B. amyloliquefaciens* and *M. oleovorans,* and it has been widely used in the production of microorganisms (Reed and Nagodawithana 1991). Soy powder contains more than 40% protein, 56–60% carbohydrate and 2% fat (Wickramanayake 1987) and, hence, is a good source of nitrogen and carbon (Navaratnam et al. 1996). In addition, its unsaturated fat can enhance microbial enzyme production and secretion. Unsaturated fatty acids increase the fluidity of the membrane (Nicholas et al. 1985; Mishra 1989).

The survival of biocontrol agents under field conditions can be improved by the use of physiological strategies (Cañamas et al. 2008). In previous studies, the physiological adaptation of *B. amyloliquefaciens* and *M. oleovorans* showed that both strains were more tolerant to ionic and non-ionic than matric stress (Sartori et al. 2010). Many studies have reported that modifying water potential in the culture medium can increased cells' tolerance to desiccation (Aleshina et al. 1986; Harman et al. 1991; Teixidó et al. 1998). Cells of *B. amyloliquefaciens* grown in liquid media modified with glycerol not only had better tolerance of low a_w but also showed better survival under heat stress (Sartori et al. 2010). Teixidó et al. (2006) suggested that it is possible to improve stress tolerance in microorganisms during the formulation process without affecting its biocontrol ability and, thus, improve its behaviour with uncontrolled environmental conditions. In this study, NaCl and glycerol were added to modify the osmotic potential of growth media, with the objective of obtaining cells osmotically adapted and with improved viability during freeze-drying. This adaptation also provided better cell performance under field conditions. Freeze-drying is a three-step process that ideally will result in an end-product with a maintained structure that is easily reconstituted upon rehydration (Tang and Pikal 2004). Good results were not obtained by freeze-drying when the water activity was modified with glycerol, due to its high point of evaporation and its hygroscopicity. The freeze-dried product obtained was very viscous and, therefore, based on previous studies (Sartori et al. 2010), NaCl was chosen as the osmotic solute to modify growth media water activity.

B. amyloliquefaciens showed the highest viability after freeze-drying in MSB medium, while *M. oleovorans* showed good viability in MSB medium under all water activities assayed. The advantages of freeze-drying are protection from contamination or infestation during storage, long viability and ease of strain distribution (Smith and Onions 1983). This study has demonstrated that molasses ($20\,g\,l^{-1}$) + soy powder ($10\,g\,l^{-1}$) medium gave the best biomass production to *B. amyloliquefaciens* and *M. oleovorans*, and showed high viability and enhanced performance after freeze-drying. The results have important implications in optimizing the formulation process of proven biocontrol agents against *F. verticillioides,* in addition to enhancing survival under environmental stress conditions.

Acknowledgements

This work was carried out through grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT) FONCYT-PICT START-UP 1521/06. SECYT-UNRC 2009–2010. Res. 807/09.

References

Abadías M, Teixidó N, Usall J, Viñas I, Magan N. 2001. Improving water stress tolerance of the biocontrol yeast *Candida sake* grown in molasses-based media by physiological manipulation. Can J Microbiol. 47:123–129.

Aleshina IM, Evpak AN, Lomovskays TF. 1986. Preparation of bacterial entomopathogenic preparate by cultivation of *Bacillus thuringiensis* adding specific salt compounds to increase yield and improve properties. Patent SU1510813 A890930.

Berny JF, Hennebert GL. 1991. Viability and stability of yeast cells and filamentous fungus spores during freeze drying: effects of protectants and cooling rates. Mycologia. 83:805–815.

Butt TM, Copping LG. 2000. Fungal biological control agents. Pestic Outlook. 11:186–191.

Butt TM, Jackson C, Magan N. 2001. Fungi as biocontrol agents: progress, problems and potential. 1st ed. London: CAB International.

Cañamas TP, Viñas I, Usall J, Casals C, Solsona C, Teixidó N. 2008. Control of postharvest diseases on citrus fruits by preharvest application of biocontrol agent *Pantoea agglomerans* CPA-2. Part I. Study of different formulation strategies to improve survival of cells in unfavourable environmental conditions. Postharvest Biol Technol. 49:86–95.

Costa E, Teixidó N, Usall J, Atarés E, Viñas I. 2001. Production of the biocontrol agent *Pantoea agglomerans* strain CPA-2 using commercial products and by-products. Appl Microbiol Biotechnol. 56:367–371.

Dallyl H, Fox A. 1980. Spoilage of material of reduced water activity by xerophilic fungi. Technical Series 15. London: Academic Press. p. 219–239.

Deacon J. 1991. Significance of ecology in the development of biocontrol agents against soil-borne plant pathogens. Biocontrol Sci Technol. 1:5–20.

Fravel D, Rhodes D, Larkin R. 1999. Production and commercialization of biocontrol products. In: Albajes R, Gullino ML, Van Lenteren JC, Elad Y, editors. Integrated pest and disease management in greenhouse crops. Dordrecht: Kluwer Academic. Chapter 26. p. 365–376.

Harman GE. 1992. Development and benefits of rhizosphere competent fungi for biological control of plant pathogens. J Plant Nutr. 15:835–843.

Harman GE, Jin X, Stasz TE. 1991. Production of conidial biomass of *Trichoderma harzianum*. Biol Control. 1:23–28.

Hebbar KP, Davey AG, Dart PJ. 1992. Rhizobacteria of maize antagonistic to *F. moniliforme*, a soil-borne fungal pathogen: isolation and identification. Soil Biol Biochem. 24:979–987.

Holt JG. 1993. Bergey's manual of systematic bacteriology. 9th ed. Baltimore (MA): Williams & Wilkins.

Hynes RK, Boyetchko SM. 2006. Research initiatives in the art and science of biopesticide formulations. Soil Biol Biochem. 38(4):845–849.

Imrie FKE. 1969. Fermentation media. Sugar and molasses. Process Biochem. 1:34–35.

Lewis JA. 1991. Formulation and delivery systems of biocontrol agents with emphasis on fungi. In: Keister DL, and Cregan PB, editors. The rhizosphere and plant growth. Dordrecht: Kluwer Academic. Chapter 14. p. 279–287.

Lumsden RD, Lewis JA. 1989. Biotechnology of fungi for improved plant growth. Problems and progress in the selection, production, formulation and commercial use of plant disease control fungi. London: British Mycological Society. p. 171–190.

Mishra P. 1989. Relationship between ethanol and fatty acyl composition of *Saccharomyces cereviseae*. Appl Microbiol Biotechnol. 30:194–198.

Navaratnam P, Arasaratnam V, Mahendran S, Balasubramaniam K. 1996. Formulation of Medium and Recycling of Biomass for Glucoamylase production by *Botryodiplodia theobromae*. Process Biochem. 3:77–80.

Nicholas A, Jacques VL, Wolf AC, Wittenberger CL. 1985. Does an increase in membrane unsaturated fatty acids account from Tween 80 stimulation of glycosyl transferase secretion by *Streptococcus salivaricus*. J Gen Microbiol. 131:67–72.

Pereira P, Nesci A, Etcheverry M. 2007. Effects of biocontrol agents on *Fusarium verticillioides* count and fumonisin content in the maize agroecosystem: impact on rhizospheric bacterial and fungal groups. Biol Control. 42:281–287.

Pereira P, Nesci A, Etcheverry M. 2009. Efficacy of bacterial seed treatments for the control of *Fusarium verticillioides* in maize. BioControl. 54:103–111.

Rao YK, Tsay K, Wu W, Tzeng Y. 2007. Medium optimization of carbon and nitrogen sources for the production of spores from *Bacillus amyloliquefaciens* B128 using response surface methodology. Process Biochem. 42:535–541.

Reed G, Nagodawithana TW. 1991. Yeast technology. 2nd ed. New York: Van Nostrand Reinhold.

Rhodes DJ. 1993. Formulation of biological control agents. In: Jones DG, editor. Exploitation of Microorganisms. London: Chapman & Hall. p. 411–439.

Sartori M, Nesci A, Etcheverry M. 2010. Impact of osmotic/matric stress and heat shock on environmental tolerance induction of bacterial biocontrol agents against *Fusarium verticillioides*. Res Microbiol. 161:681–686.

Smith D, Onions AHS. 1983. The Preservation and Maintenance of Living Fungi. Kew (UK): Commonwealth Mycological Institute. p. 51–52.

Tang X, Pikal MJ. 2004. Design of freeze-drying processes for pharmaceuticals: practical advice. Pharm Res. 21(2):191–200.

Teixidó N, Viñas I, Usall J, Magan N. 1998. Improving ecological fitness and environmental stress tolerance of the biocontrol yeast *Candida sake* (strain CPA-1) by manipulation of intracellular sugar alcohol and sugar content. Mycol Res. 102:1409–1417.

Teixidó N, Cañamas TP, Abadías M, Usall J, Torres R, Magan N, Viñas I. 2005. Accumulation of compatible solutes, glycine-betaine and ectoine, in osmotic stress adaptation and heat shock cross-protection in the biocontrol agent *Pantoea agglomerans* CPA-2. Lett Appl Microbiol. 41:248–252.

Teixidó N, Cañamas TP, Abadías M, Usall J, Solsona C, Casals C, Viñas I. 2006. Improving low water activity and desiccation tolerance of the biocontrol agent *Pantoea agglomerans* CPA-2 by osmotic treatments. J Appl Microbiol. 101:927–937.

Wickramanayake TW. 1987. Food and nutrition. Sri Lanka: Trumpet Publishers. p. 80–160.

Whipps JM. 1997. Ecological considerations involved in commercial development of biological control agents for soil-borne diseases. In: Van Elsas JD, Trevors JT, Wellington EMH, editors. Modern soil microbiology. New York: Marcel Dekker. p. 525–545.

Wraight SP, Jackson MA, Kock SL. 2001. Production, stabilization and formulation of fungal biocontrol agents. In: Butt TM, Jackson C, Magan N, editors. Fungi as biocontrol agents: progress, problems and potential. 1st ed. London: CAB International. Chapter 10. p. 253–288.

Zhao G, Zhang G. 2005. Effect of protective agents, freezing temperature, rehydratation media and viability of malolactic bacteria subjected to freeze-drying. J Appl Microbiol. 99:333–338.