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Research Article

Viability of *Hanseniaspora uvarum* yeast preserved by lyophilization and cryopreservation

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Abstract

***Hanseniaspora* yeasts are known to produce volatile compounds that give fruity aromas in wine and fermented fruit. This study aimed to verify the feasibility of the *Hanseniaspora uvarum* strain that had been isolated and identified during a previous study and preserved by lyophilization and freezing at −80 °C (cryopreservation). This strain was assessed in relation to its macroscopic and microscopic morphology and for its ability to ferment apple must. After having been subjected to lyophilization and cryopreservation, viability was assessed in relation to these characteristics during 12 months of storage. The strain showed stable colonial features and its microscopic appearance was unchanged during all recoveries. The plate count results showed consistency in both processes. Regarding the fermentative capacity, the kinetic results showed 100% viability for the strain subjected to lyophilization, as well as for those preserved at −80 °C. These results demonstrate that the preservation methods used are compatible with the maintenance of the relevant characteristics of the strain for the period of evaluation of this study (12 months).**

Keywords: **cryopreservation; lyophilization; *Hanseniaspora uvarum***

Introduction

The genus *Hanseniaspora* is recognized for its aromatic contribution to ciders, fermented fruits and wines, due to the production of esters that provide these beverages with 'fruity' and/or 'floral' sensory notes (Viana *et al.,* 2009; Moreira *et al.,* 2005, 2008; Xu *et al.,* 2006; Xufre *et al.,* 2006).

According to Girão *et al.* (2004), the proper preservation of yeast cultures is essential for future studies because it is necessary to maintain the same morphological and physiological properties of the strains, regardless of the storage method that is chosen.

According to Baker and Jefries (2006) and Lastra *et al.* (2001), the maintenance of strains by the processes of lyophilization, cryopreservation or in sterile water are the best methods of preservation, because they permit the reduction of cell metabolism to a basal level, they the phenotypic and genotypic characteristics of the micro-organisms and also contribute to reducing the risk of contamination. Cavalcanti (2010) tested the methods of lyophilization and freezing at −80 °C in three genera of yeast (*Candida* spp., *Cryptococcus* spp. and *Trichosporon* spp.) and found 100% viability of the strains kept for 6 months or more under these conditions.

During the freezing process, the species to be frozen, in either liquid nitrogen or an ultra-freezer, should undergo multiplication in 3 ml liquid medium for 24 h, followed by stirring with the addition of 1 ml 60% glycerol solution; it should then be stored in containers that are appropriate for freezing.

The cultures are frozen at −30 °C for 30–60 min and taken to the ultra-freezer at −80 °C or in liquid nitrogen tanks (Kurtzman *et al.,* 2003; Yarrow, 1998). This process, widely used for the preservation of culture collections to ensure stability and reliability, is not intended for commercial purposes (Suárez Lepe, 1997).

The lyophilization process consists of the dehydration of the material through the sublimation of frozen water at low temperatures under vacuum. It is a process of simultaneously transferring mass and heat, in which heat is supplied to the frozen product and the water vapour is continuously removed. The initial freezing stage should be performed with the minimum possible formation of ice crystals. In the second stage (primary drying) the free water is removed from the product, and in the third stage (secondary drying) the water that is strongly chemically linked increases in temperature and leaves the product. After lyophilization, the dehydrated micro-organisms (with greatly reduced metabolic activity) are transferred into ampoules that are sealed and stored at room temperature, which facilitates transport and marketing (Pitombo, 2005; Robert *et al.,* 2006). The tolerance of residual moisture content for fungi is 1.0–2.5%. More severe drying will result in irreversible damage to the cells, due to the denaturation of proteins and lipids, while a higher moisture content reduces the useful life of the material (Melin *et al.,* 2011; Robert *et al.,* 2006).

However, there is no universally efficient method for all genera and species of fungi, due to differences in their metabolic and physiological characteristics. Thus, the behaviour of each micro-organism in relation to different conservation processes is the result of different biochemical and physiological factors (Paoli, 2005; Deshmuhk, 2003; Figueiredo, 2001; Silva *et al.,* 2008).

This study aimed to evaluate the viability of *Hanseniaspora uvarum* in relation to two methods of preservation, lyophilization and freezing at −80 °C, during 12 months of storage.

Materials and methods

The *Hanseniaspora uvarum* strain that was used was part of the collection of strains at the Federal Technological University of Paraná, Ponta Grossa Campus (Pietrowski *et al.,* 2012). The culture media and reagents used were of analytical grade.

Conservation of *H. uvarum* by lyophilization

In order to increase the biomass to be lyophilized, a colony of a fresh strain of *H. uvarum* isolated in yeast–malt agar (YMA) was inoculated in 5 ml glucose peptone yeast extract broth (GPYB), incubated at 25 °C in an incubator and stirred (Marconi NA 832/1) for 24 h. This culture was transferred to an Erlenmeyer flask containing 100 ml of the same medium and incubated again under the same conditions for 48 h at 25 °C (Schmidell *et al.,* 2001), reaching a population of approximately 10^{12} cfu/ml.

The culture obtained was centrifuged (CELM, Combate) at 3000 rpm for 15 min, followed by two washes with sterile distilled water. This process reduced the population of yeasts to approximately 10^{9} cfu/ml. The clean biomass was placed into a sterile container and then the homogenized content was distributed into penicillin-type flasks (vials with 5 ml capacity), with previously sterilized rubber stoppers, for lyophilization (Terroni LD 1500).

The vials were frozen in a freezer (Consul 530) at −20 °C for 24 h and sent to the lyophilizer, which had an initial temperature of −56 °C and an initial vacuum of 1335 Vca and 66 μHg. After 24 h, when the vacuum reached zero, the process was completed. The vials were sealed, still under vacuum, by the equipment when the lyophilization process had ended. Subsequently, metal seals were fitted to the vials to ensure perfect sealing. The vials containing the lyophilized strain were kept in locked cabinets under light, and at room temperature, until the date of each recovery.

Conservation of *H. uvarum* at −80 °C (cryopreservation)

A fresh colony of the strain of *H. uvarum* was inoculated into glucose peptone yeast and malt extract broth (GYMP), sterilized in glass tubes with cotton plugs and incubated at 25 °C for 24 h or until growth of the yeast in the broth was observed by its turbidity. This culture was added to 50 ml GYMP and incubated at 25 °C for 24 h.

Aliquots (1 ml) of the culture obtained (population of approximately 10^{12} cfu/ml) were transferred to a sterile CryoTubes (Alfa), properly identified,

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and then four drops of glycerol, which had previously been sterilized for 2 h at 100 °C in a Pasteur oven (Fanem 320 SE), were added. The tubes were properly cooled, as a cryoprotectant. After homogenization in a vortex (Vision Scientific, KMC-1300v) for 30 s, the tubes were placed in the freezer at −20 °C for 2 h and were then transferred to a freezer at −80 °C (Nuaire NU9668GC, Series 8050105), where they were stored until the date of each recovery (Kurtzman *et al.,* 2003).

Recovery of *H. uvarum* preserved by lyophilization and cryopreservation

The recovery of the conserved *H. uvarum* occurred soon after the aforementioned processes (considered as 0 time of preservation) and after 3, 6, 9 and 12 months of storage. From the lyophilized material, 7 mg of the contents of the flasks was aseptically weighed and transferred to test tubes with 9 ml 0.1% peptone water and 1 ml glucose for rehydration. Of the material frozen at −80 °C, 0.5 ml samples were aseptically collected from the CryoTubes, once thawed, and recovered in tubes with 8.5 ml 0.1% peptone water and 1 ml glucose. The tubes were subjected to stirring with the help of a vortex (Vision Scientific KMC1300v) and then remained at rest for 20 min.

The initial population of 10^6 cfu/ml was used as an inoculum for checking the fermentative capacity of the conserved strain, for a plate count and also to verify the morphological characteristics.

Assessment of the viability of strains

Plate counts of strains

To perform the plate count of *H. uvarum* preserved by both processes, aliquots of 1.0 ml suspension obtained in the recovery were withdrawn. After serial dilutions were completed, the plate surfaces were sown using a Drigalski handle in YMA. The plates were incubated at 25 °C for 48 h (Crous *et al.,* 2009).

Verification of morphology

The macroscopic appearance of the colonies of obtained *H. uvarum* was observed prior to the count. After recording the results of both the count and the macroscopic appearance, the colonies were used for the microscopic preparations of staining to observe the microscopic characteristics of the preserved strains.

Verification of fermentative capacity in apple must

The clarified, filtered and pasteurized apple must, obtained in accordance with Pietrowski *et al.* (2012), was packaged in 250 ml Erlenmeyer flasks, with a working volume of 200 ml, sterilized by autoclaving (Phoenix - AV75) at 121 °C for 15 min, and equipped with a bung. Chloramphenicol antibiotic was added (0.01%:99%; Henrifarma, São Paulo) to control bacterial growth. *H. uvarum*, preserved by both lyophilization and freezing at −80 °C, was inoculated into the Erlenmeyer flasks (study of standardization of the inoculum, data not shown; an initial count of 10^6 and 10^4 cfu/ml to strains cryopreserved and lyophilized, respectively) and identified and incubated in a bacteriological incubator (Quimis 316 B24) at 25 °C for 10 days. The fermentation kinetics were calculated by monitoring the mass loss of the fermentations (200 ml) through the release of carbon dioxide (CO_2) through the bung. Weighing occurred every 2 h in an analytical balance (0.001 g sensitivity; Bell Engineering 1300) until constant mass was attained (Roger *et al.,* 2002; Bely *et al.,* 1990). The fermentation rate was calculated by the variation in the loss of CO_2 vs time (equation 1):

$$V = \Delta CO_2/\Delta t \quad (1)$$

where V is the rate of fermentation (g CO_2/l/day), ΔCO_2 is the variation in production of CO_2 (g/l) and Δt is the variation in time (days).

Results and discussion

The results of the *H. uvarum* plate counts in Table 1 show the population stability (4.9×10^6–9.4×10^6 cfu/ml) in the samples preserved at −80 °C. However, even though they remained in the same logarithmic cycle at all times during the recovery, there was a significant difference between the samples, with the 6 months sample having the highest score. These differences may have occurred due to inadequate homogenization of the sample before transfer to the CryoTubes.

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Table 1. Cellular viability of *H. uvarum* preserved at −80 °C after different periods of storage

Preservation time (months)	Population (cfu/ml)	SD ± (cfu/ml)	Coefficient of variation (%)
0	5.85×10^{6c}	50 000	0.8
3	4.93×10^{6d}	152 752	3.1
6	9.41×10^{6a}	76 376	0.8
9	7.81×10^{6b}	175 594	2.2
12	6.42×10^{6c}	80 829	1.2

Average of three replicates: different letters indicate significant differences ($p < 0.05$) in the populations.

In the recovery of the strain preserved by lyophilization, the counts showed values in the range 1.17×10^4–1.68×10^4 cfu/ml (Table 2). It is noteworthy that the population was not significantly different from time 0 to 12 months of storage. However, significant differences were found in the samples recovered after 3 and 6 months. It seems that the cellular injuries were caused by the process without the use of cryoprotectants or to readjust its internal volume internal pressure (Goward *et al.,* 1986), apart from the regulation of other affected cell functions (Groleau *et al.,* 1995), which can occur after 3–6 months storage.

The differences in the scores of the strains of lyophilized (10^4 cfu/ml) and cryopreserved (10^6 cfu/ml) *H. uvarum* were due to different preparation for the preservation process. However, lyophilization results in more injuries to micro-organisms that cryopreservation because, in addition to freezing at −20 °C (part of both processes), it involves dehydration of the material due to the sublimation of frozen water at low temperatures under vacuum (Robert *et al.,* 2006). Some authors report morphological changes after lyophilization, or even the non-viability of micro-organisms in the freezing phase prior to lyophilization (Morgan *et al.,* 2006; Voyron *et al.,* 2009; Paoli, 2005; Figueiredo, 2001).

According to Morgan *et al.* (2006), there are few studies that define the initial concentration of cells ideal for lyophilization; however, historically, quantities $> 10^8$ cells/ml ensure the recovery of viable cells in a lyophilized sample. Bozoglu *et al.* (1987) recommended cultures with concentrations $> 10^7$ cells/ml for lyophilization, in order to ensure sufficient cells remaining after lyophilization, long-term storage and successful reconstitution of the strain. Thus, considering that the initial concentration of yeasts in this study was approximately 10^{12} cfu/ml, exceeding the values mentioned in the literature, cryoprotectant substances were not used for the growth phase and freezing of *H. uvarum* prior to lyophilization.

Bozoglu *et al.* (1987) suggested that the survival of 0.1% of the original cell population is 'sufficient' to allow the continuation of a line after lyophilization. The results of our study showed counts in the range 1.17×10^4–1.68×10^4 cfu/ml. Therefore, counts < 0.1% of 10^{12} cfu/ml, the initial population of lyophilization, suggest that the lack of cryoprotectant during the cultivation of yeasts may have influenced their viability; however, this did not compromise the preservation of the *H. uvarum* strain over 12 months.

Morgan *et al.* (2006) point out that the reason for the preservation of cells at high concentrations is based on the premise that many cells die during storage for extended periods, but enough survive to allow the continuation of the lineage. However, in our study, the recovery of newly lyophilized yeasts and those recovered after 12 months showed no significant difference in count results, suggesting that the death of the yeasts occurred during lyophilization and not during storage.

Table 2. Cellular viability of *H. uvarum* preserved by lyophilization after different periods of storage

Preservation time (months)	Population (cfu/ml)	SD ± (cfu/ml)	Coefficient of variation (%)
0	1.20×10^{4c}	1732	14.4
3	1.68×10^{4a}	721	4.3
6	1.43×10^{4b}	608	4.2
12	1.17×10^{4c}	642	5.4

Average of three replicates: different letters indicate significant differences ($p < 0.05$) in the populations.

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Regarding the macro- and microscopic characteristics of colonies of *H. uvarum*, it can be noticed (Table 3) that in all the recoveries the samples remained unchanged, demonstrating the same characteristics observed in the natural strain, before being subjected to the studied processes. Thus, both in relation to the macroscopic appearance of the colonies and the cellular characteristics, this confirmed the results described for this yeast reported by Smith (1998). This shows that the preservation methods in the present study are compatible with these characteristics.

The results are in agreement with the reports of other authors, such as Mariano (2006), who, having examined five methods of serial conservation (transfer in solid medium, mineral oil, distilled water, freezing at −70 °C and lyophilization) of strains of clinical and industrial interest, found that all samples remained with their macro- and microscopic features stable and showed their colonial characteristics to be unchanged during all tests. Cavalcanti (2010) worked with three genera of yeasts, among other micro-organisms, and concluded that both lyophilization and freezing at −80 °C were suitable for the preservation of species of yeasts, since the viability of the isolated material kept under these conditions for 6 months was 100%.

The lyophilization process has been applied to yeast and filamentous fungi with good recovery results, preserving the phenotypic and genotypic characteristics of micro-organisms for prolonged periods (Cavalcante *et al.,* 2007; McGinnis *et al.,* 1974). However, some authors have reported morphological changes after lyophilization, or even the non-viability of the fungus in freezing prior to lyophilization (Voyron *et al.,* 2009; Paoli, 2005), a fact that was not observed in the *H. uvarum* strain preserved for 12 months in the present study.

In our experiments involving the fermentation of apple must with the strain of *H. uvarum* before being subjected to the processes of preservation tested in this study, we determined a maximum rate of 3.85 g CO_2/l/day in a time of 4.25 days.

Table 3. Results of macro- and microscopic characteristics of strains of *H. uvarum* preserved by freezing and lyophilization after different periods of storage

	Preservation method			
	Lyophilization		**Cryopreservation at −80 °C**	
Preservation time (months)	**Aspects of colonies**	**Microscopic aspect**	**Aspects of colonies**	**Microscopic aspect**
0	Whitish, smooth and shiny edges	Peaked cells	Whitish, smooth and shiny edges	Peaked cells
3	Whitish, smooth and shiny edges	Elongated cells	Whitish, smooth and shiny edges	Peaked cells
6	Whitish, smooth and shiny edges	Elongated cells	Whitish, smooth and shiny edges	Elongated cells
12	Whitish, smooth and shiny edges	Elongated cells	Whitish, smooth and shiny edges	Peaked cells

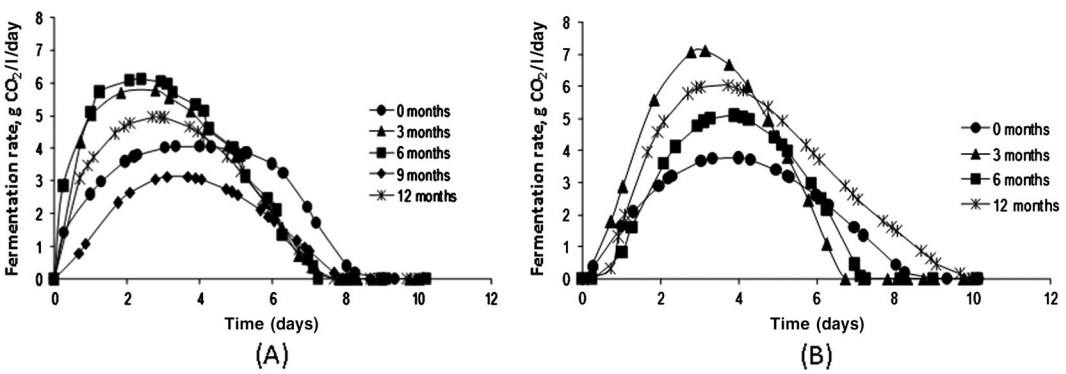

Figure 1. Fermentation rates of *H. uvarum* during apple must fermentation after being preserved (A) at −80 °C and (B) by lyophilization for different periods of storage

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These values were used as the default behaviour for the studied yeast strain, given that until then it had been stored in inclined tubes containing YMA (yeast malt agar) at a temperature of 7–10 °C, with periodical sampling at 3 months, a process that does not subject the micro-organism to the damage suffered in the investigated preservations.

The results of the fermentative capacity of *H. uvarum* in apple must are shown in Figure 1(A) and demonstrate that the yeast maintained its high fermentative activity after being preserved at −80 °C for 12 months. However, the maximum fermentation rate was variable (3.0–6.0 g CO_2/l/day) and occurred between 2.0 and 3.5 days. This difference in fermentation rate may be related to the problem of biomass homogenization in cryopreservation procedures, as observed in Table 1.

Figure 1B shows the fermentative activity of *H. uvarum* in apple must after different periods of preservation by lyophilization. Due to a lower initial cell population (10^4 cfu/ml), the maximum rate of fermentation took longer to achieve (3.0 g CO_2/l/day to 4.0 days), in comparison to the cryopreserved strain, which had a higher initial inoculum (10^6 cfu/ml). The maximum rates of fermentation were variable, due the differences of population observed in Table 2. However, the values were similar to those found in cryopreserved strains.

At time zero (Figure 1B) the lower maximum speed was observed in yeast subjected to preservation by lyophilization. This may have been due to the short time between the processes of freezing, dehydration and rehydration (lyophilization), causing the water bound to the polar heads of the phospholipids of the cells being lost, to a greater extent, modifying the membrane fluidity and integrity and thus compromising cellular metabolism (Crowe *et al.,* 1984); this was not observed in the sample preserved at −80 °C (Figure 1A).

Considering the ease of sample preparation, the method of preservation at −80 °C may be regarded as the most viable form of preservation of the yeasts studied. However, the cost of electric power for maintenance at this temperature, as well as possible risks of power failure, with the resultant potential loss of material, should be considered. Thus, the method of lyophilization, although requiring a little more work in terms of sample preparation, ensures the preservation of the samples at room temperature, eliminating the costs and risks associated with using an electrical supply.

Conclusion

Analysis of the results showed that both studied methods of conservation provided proper maintenance of the strain of *H. uvarum*, ensuring stability in macro- and microscopic characteristics as well as the counts and fermentative capacity. Thus, our study attests to the viability of yeast studied after up to 12 months of preservation by lyophilization stored at room temperature, and also frozen at −80 °C in an ultra-freezer.

References

Baker M, Jefries P. 2006. Use of commercially available cryogenic vials for long-term preservation of dermatophyte fungi. *J Clin Microbiol* **44**: 617–618.

Bely M, Sablayrolles JM, Barre P. 1990. Description of alcoholic fermentation kinetics: its variability and significance. *Am J Enol Viticult* **41**: 319–324.

Bozoglu TF, Ozilgen M, Bakir U. 1987. Survival kinetics of lactic acid starter cultures during and after freeze drying. *Enzyme Microb Tech* **9**: 531–537.

Cavalcante SC, Freitas RS, Vidal MSM, *et al.* 2007. Evaluation of phenotypic and genotypic alterations induced by long periods of subculturing of *Cryptococcus neoformans* strains. *Mem I Oswaldo Cruz* **102**: 41–47.

Cavalcanti SDB. 2010. Aplicação de metodologias de preservação e caracterização de fungos na coleção de culturas do Instituto de Medicina Tropical de São Paulo. MA Dissertation, Department of Medicine, University of São Paulo; pp 61.

Crous PW, Verkley GJM, Groenewald JZ, Samson RA (eds). 2009. Fungal Biodiversity. CBS–KNAW Fungal Biodiversity Centre: Utrecht, The Netherlands; 189–197.

Crowe LM, Mouradian R, Crowe JH, *et al.* 1984. Effect of carbohydrates on membrane stability at low water activities. *Biochim Biophys Acta* **769**: 141–150.

Deshmuhk SK. 2003. The maintenance and preservation of keratinophilic fungi and related dermatophytes. *Mycoses* **46**: 203–207.

Figueiredo MB. 2001. Métodos de preservação de fungos patogênicos. *Biológico* **63**: 73–82.

Girão MD, Prado MR, Brilhante RSN, *et al.* 2004. Viabilidade de cepas de *Malassezia pachydermatis* mantidas em diferentes métodos de conservação. *Rev Soc Bras Med Trop* **37**: 229–233.

Goward CR, Atkinson T, Scawen MD. 1986. Rapid purification of glucokinase and glycerokinase from *Bacillus stearothermophilus* by hydrophobic interaction chromatography. *J Chromatogr* **369**: 235–239.

Groleau D, Chevalier P, Tse Hing Yeun TLS. 1995. Production of polyols and ethanol by the osmophylic yeast *Zygosaccharomyces rouxii*. *Biotechnol Lett* **17**: 315–320.

Kurtzman C, Boekhout T, Robert V, *et al.* 2003. Methods to identify yeast. In Yeast in Food: Beneficial and Detrimental Aspects, Boekhout T, Robert V (eds). CRC Press: Cambridge; 69–121.

Lastra CLL, Hajek AE, Humber RA. 2001. Effects of two cryopreservation techniques on viability and pathogenicity of entomophthoralean fungi. *Can J Bot* **79**: 861–864.

Mariano PDLSA. 2006. Diferentes processos de armazenamento de leveduras; estudos sobre a variabilidade fenotípica e genotípica. PhD thesis, Department of Dentistry, State University of Campinas, Piracicaba; pp 118.

McGinnis MR, Padhye AA, Ajello L. 1974. Storage of stock cultures of filamentous fungi, yeasts, and some aerobic actinomycetes in sterile distilled water. *Appl Microbiol* **28**: 218–222.

Melin P, Schnürer J, Hakansson S. 2011. Formulation and stabilisation of the biocontrol yeast *Pichia anomala*. *Antonie Van Leeuwenhoek* **99**: 107–112.

Moreira N, Mendes F, Pinho PG, *et al*. 2008. Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* grown as pure and mixed cultures in grape must. *Int J Food Microbiol* **124**: 231–238.

Moreira N, Mendes F, Pinho PG, *et al*. 2005. Alcohols, esters and heavy sulphur compounds production by pure and mixed cultures of apiculate wine yeasts. *Int J Food Microbiol* **103**: 285–294.

Morgan CA, Herman N, White PA, Vesey G. 2006. Preservation of micro-organisms by drying: a review. *J Microbiol Meth* **66**: 183–193.

Paoli P. 2005. Biobanking in microbiology: from sample collection to epidemiology, diagnostics and research. *FEMS Microbiol* **29**: 817–910.

Pietrowski GAM, Dos Santos CME, Sauer E, *et al*. 2012. Influence of fermentation with *Hanseniaspora* sp. yeast on the volatile profile of fermented apple. *J Agr Food Chem* **60**: 9815–9821.

Pitombo RNM. 2005. Liofilização. In Purificação de Produtos Biotecnológicos, Pessoa A Jr, Kilikian BV (eds). Manole: Barueri (SP); 332–348.

Robert V, Stalpers J, Boekhout T, Tan S. 2006. Yeast biodiversity and culture collections. In Biodiversity and Ecophysiology of Yeasts, Rosa CA, Péter G (eds). Springer-Verlag: Berlin, Heidelberg; 31–44.

Roger JM, Sablayrolles JM, Steyer JP, Bellon-Maurel V. 2002. Pattern analysis techniques to process fermentation curves: application to discrimination of enological alcoholic fermentations. *Biotechnol Bioeng* **79**: 804–815.

Schmidell W, Lima UA, Aquarone E, Borzani W (eds). 2001. Biotecnologia Industrial: Engenharia Bioquímica. Edgard Blücher: São Paulo.

Silva JO, Costa PP, Reche SHC. 2008. Yeasts maintenance for freezing at −20 °C. *Rev Bras Anal Clin* **40**: 73–74.

Smith MT. 1998. *Hanseniaspora* zikes. In The Yeasts: A Taxonomic Study, 4th edn, Kurtzman C, Fell JW (eds). Elsevier Science: Florida, FL; 214–220.

Suárez Lepe JA. 1997. Levaduras Vínicas: Funcionalidad y uso en Bodega. Mundi-Prensa: Madrid; 221–241.

Viana F, Gila JV, Vallésa S, Manzanares AP. 2009. Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae*. *Int J Food Microbiol* **135**: 68–74.

Voyron S, Roussel S, Manaut F, *et al*. 2009. Vitality and genetic fidelity of white-rot fungi mycelia following different methods of preservation. *Mycol Res* **113**: 1027–1038.

Xu Y, Zhao GA, Wang LP. 2006. Controlled formation of volatile components in cider making using a combination of *Saccharomyces cerevisiae* and *Hanseniaspora valbyensis* yeast species. *J Ind Microbiol Biot* **33**: 192–196.

Xufre A, Albergaria H, Inácio J, *et al*. 2006. Application of fluorescence *in situ* hybridisation (FISH) to the analysis of yeast population dynamics in winery and laboratory grape must fermentations. *Int J Food Microbiol* **108**: 376–384.

Yarrow D. 1998. Methods for the isolation, maintenance and identification of yeasts. In The Yeasts: A Taxonomic Study, 4th edn, Kurtzman C, Fell JW (eds). Elsevier Science: Florida, FL; 77–100.