ELSEVIER

Food Research International 36 (2003) 747–751

FOOD RESEARCH INTERNATIONAL

www.elsevier.com/locate/foodres

Short communication

Autochthonous microbial population in a Niagara Peninsula icewine must

R.E. Subden[a,*], J.I. Husnik[a], R. van Twest[b], G. van der Merwe[c], H.J.J. van Vuuren[c]

[a] *Department of Food Science, University of Guelph, Guelph, ON, Canada N1G 2W1*
[b] *Department of Microbiology, University of Guelph, Guelph, ON, Canada N1G 2W1*
[c] *Wine Research Center, The University of British Columbia, Vancouver BC, Canada V6T 1Z4*

Received 18 July 2000; accepted 31 January 2003

Abstract

We have characterized the microbial population in icewine musts from three locations in an Ontario vineyard. More than 3×10^3 colony forming units (cfus) were isolated from icewine must from the 1999 vintage. The cfus were enumerated and representative colonies were selected and identified using classical morphological, auxanographic and physiological tests. Species distribution and frequency of microbes in icewine musts were compared with those found in an earlier study. In icewine must, bacteria accounted for 2% of the total cfus enumerated. *Acetobacter* and *Gluconobacter* were not found. The only fermentative yeast found was *Hanseniaspora*. The rest of the yeasts were non-fermentative Basidiomycetous yeasts that would have little effect on fermentations. Ninety percent of the mold cfus were the Basidiomycetous non-fermentative *Aureobasidium pullulans*. We conclude that in this sample, the autochthonous micro flora may possibly have some effect on the grapes in the vineyard but have only a minor role in the fermenting must.

1. Introduction

We have previously enumerated the naturally occurring or autochthonous yeasts and bacterial species in the table-wine must of a vineyard in the Niagara Peninsula (Holloway, Subden, & LaChance, 1990). In a succeeding work we characterized the common wild yeasts by analyzing some of the key metabolites that affect the organoleptic quality of wines (Holloway & Subden, 1991).

Since the early 1980s, Canadian wineries have developed a successful icewine industry. The production parameters for Icewine in Canada are set out in the Canadian Vintners Quality Assurance (VQA) regulations which state that the harvest temperature of the grapes must be lower than −8 °C and that the must Brix be >35 (VQA, 1997). There is a correlation between the harvest temperatures and quality so many of the better quality icewine producers wait to harvest when the temperature drops to −20 °C or even lower with a resultant must density of >45B. The freezing removes water through crystallization (Fennema & Powrie, 1964) accompanied by; changes in pH; titratable acidity; ionic strength; viscosity; osmotic pressure; vapor pressure; freezing point; surface and interfacial tensions; as well as the oxidation–reduction potential (Fennema, Powrie, & Marth, 1973). The change of interest to the winemaker is primary the intensification of flavors.

It is not known what effect the freezing and thawing prior to harvest has on the indigenous microbial (especially the dominant yeasts) population. In contrast to the abundant amount of literature on the microbiology of table wines (Benda, 1964; Davenport, 1974; Fleet, Lafon-Laforcade, & Ribereau-Gayon, 1984; Minarik, 1964; Rosini, Frederici, & Martini, 1982) there is comparatively little information for icewines especially the wild yeast population in the bloom. The wild yeasts and other indigenous micro flora are significant as it can affect the flavor characteristics of the wine (Gil, Mateo, Jiménez, Pastor, & Huerta, 1996; Holloway & Subden, 1991; Soles, Ough, & Kunkee, 1982; Suomalainen & Lehtonen, 1979). The principle metabolites of both wild

* Corresponding author. Tel.: +1-319-824-4120x3818; fax: +1-319-824-6631.
E-mail address: rsubden@uoguelph.ca (R.E. Subden).

0963-9969/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0963-9969(03)00034-6

and selected commercial yeasts affecting flavors include: higher alcohols, acetic acid, aldehydes, ketones, esters and sulfur compounds (Wagener & Wagener, 1968).

Holloway et al., (1990) have identified and enumerated the yeasts and yeast-like molds in the must from Riesling grapes grown in the Niagara grape-producing area of Ontario. Although the number and diversity of these yeasts species are similar to those found in various wine districts if the world (Fleet et al., 1984; Moore, Johnson, & Morris, 1988), Holloway, vanTwest, Subden, and LaChance (1992) did find a strain of *Candida stellata* which was atypical in carbon source assimilation tests and which produced 240 mg of 2-methyl-1-propanol per litter which was 20% higher than the published concentration range (Amerine, Chunk, Ough, & Web, 1980; Amerine and Ough, 1980; Schreier, 1979) and was well over the sensory threshold (Rankine, 1967). This flavor compound is perceived as being "vinous" or "heady" and could be responsible for "off flavors" in wine. It is the desire of many wineries to control these off flavors.

The most relevant data concerned with yeast surviving freezing is from workers interested in producing frozen bread dough for in-store bakeries (Hino, Takano, & Takana, 1987; Hino et al., 1992; Murakami, Hahn, Yokigawa, Endo, & Kawi, 1994) and involves the analysis of intracellular concentration of carbohydrates (Continho, Bernardes, Felix, & Panek, 1987; Hino, Mihara, Nakashima, & Takano, 1990), fatty acid concentration of membranes (Sajbidor, Breierova, & Kockova-Kratochvilova, 1989) and free water distribution (Ratner, Kamkova, Loseva, & Fikhte, 1987). The sensitivity of wild yeasts found in the musts from Niagara grapes to freezing has been reported (Chamberlain, Husnik, & Subden, 1998). There are no reports of the micro flora of icewine musts.

2. Materials and methods

2.1. Isolation procedure

Samples of Riesling icewine must were taken from three locations in a vineyard in the Niagara Peninsula Viticultural Area in Ontario, Canada. Media, procedures for the isolation and enumeration (except for the acetic acid producing bacteria, AAB) are similar to those used in a previous work (Holloway et al., 1990).

A total of four 100 ml must samples were taken at 2-h intervals during the second day of the icewine harvest in the winters of 2000, 2001 and 2002 (corresponding to the 1999, 2000 and 2001 vintages). Samples were batched, then serial diluted to 10^{-0}, 10^{-2} and 10^{-4} in physiological saline. Saline was selected for a dilution medium after a study comparing survival rates of the species associated with grape blooms in phosphate, 1% peptone and physiological saline. Aliquots of 0.1 mL from the initial sample and each dilution were spread on 100 plates of MEA (Malt Extract Medium, Difco Laboratories, Detroit, MI) medium. MEA is commonly used for the selection of microbes of interest to the fermentation industry (Heard & Fleet, 1986; Holloway et al., 1990). Using only the plates that produced colony counts $>25<250$ per plate, morphologically distinct colony types were defined, enumerated and representative colonies were and streaked onto YPDA (Yeast Extract, Peptone, Dextrose Agar, Difco Laboratories, Detroit, MI) plates, re-examined for purity, then stored at 5 °C until needed for further examination. Complete analyses were made only on the samples from the 1999 vintage. Samplings of the 2000 and 2001 vintage were for the detection of AAB only.

Both Frauter and Carr media (Sharpe, 1996), were used to isolate acetic acid bacteria during the examination of the 2001 and 2002 icewine vintages.

Cultures were examined microscopically and grouped on the basis of their cellular morphology into: bacteria, yeast or mold cultures.

2.2. Strains used

Authentic strains of: *Candida stellata*, *Hanseniaspora uvarum, Aureobasidium pullulans, Candida diversa, Pichia kluyveri, Rhodotorula glutinis, Cryptococcus laurentii, Issatchenkia terricola, Metschnikowia pulcherrima, Saccharomycopsis (Endomycopsella) crataegensis, Sporobolomyces roseus*, the most commonly encountered strains in the bloom of grapes harvested in the Niagara region in early October, were donated by Paul Holloway of the Dept. of Biology, University Winnipeg, Manitoba. Type strains of bacterial cultures were obtained from the University of Guelph culture collection. *Acetobacter pasteurianus* (ATCC9432) and *Gluconobacter oxydans* (ATCC621) strains were obtained from the American Type Culture Collection (Atlanta GA)

2.3. Identification

Bacterial isolates were transferred to Tryptic Soy Broth or agar (Difco Laboratories, Detroit, MI) and cultured for a week at ambient room temperature. Isolates were then examined for colony morphology, cellular morphology and a battery of primary tests including Gram stain, oxidase activity, catalase activity, Hugh–Liefson oxidation fermentative test, glucose fermentation tests, nitrate reduction and motility tests. Secondary tests included: gelatin liquifaction, starch, Tween 80 sensitivity and casein hydrolysis (Cowan, 1974). Other physiological tests for carbohydrate assimilation tests for gram positive bacteria and gram

negative bacteria were performed on GP Micro plates™ and GN Micro plates™ (Biolog, 3938 Trust Way, CA) respectively. All results were compared with profiles contained in Bergey's *Manual of Determinative Bacteriology* (9th ed). (Soc. Amer. Bacteriol., 1994).

Yeasts isolates on YPDA and Yeast Morphology Agar (YMA, Difco Laboratories, Detroit Michigan) were examined for colony morphology. Corn Meal Agar (CMA, Difco Laboratories, Detroit Michigan) plus Tween 80 medium was used for Dalmau plates to ascertain the ability to form hyphae or pseudohyphae. Vegetative reproduction and asexual spore formation were studied using phase contrast microscopy using overnight cultures in YPD broth. Ascopore formation was determined by periodically examining cultures on sporulation medium using the Schaeffer Fulton's Spore Stain (Barnett, Payne, & Yarrow, 1990). Carbohydrate assimilation test were conducted using API 20C strips (Bio-Merieux-Vitec, Hazelwood, MO) and glucose fermentation was determined using Glucose Fermentation Broth (Barnett et al., 1990).

Mold isolate colony morphology was determined by culture on PDA, CMA, and PCA agar. Microscopic examination of mycelia, spores, and fruiting bodies was performed using phase contrast at ×400. The Hyphomycetes were identified according to the keys of G. L. Barron (1968).

3. Results and discussion

3.1. Bacteria

The identification and frequency of bacterial cfus is given in Table 1. The low frequency of bacterial colonies (2%) of total cfus enumerated, is similar to other studies (including a previous study from the same vineyard (Holloway et al., 1990) using must samples collected during the normal October harvest.

We were surprised to note that there was no *Acetobacter* or *Gluconobacter* in icewine musts as acetic acid is often attributed to these species growing in juice from damaged berries. We repeated the sampling for acetic acid bacteria in the winters of 2001 and 2002 using Frauter's and Carr's selective medium and again no AAB were found. Acetic acid production is a major concern for icewine producers (Monk & Cowley, 1984; Shimazo & Wantanabe, 1981).

None of the bacteria isolated, proliferate rapidly in environments like icewine must with high osmotic stress and a low pH (3.1–3.6).

3.2. Yeasts

In most parts of the world, including Ontario, *Hanseniaspora* (or its anamorph *Kloeckera*) is one of the most commonly encountered yeast genera in musts collected during the normal October harvest (Fleet et al., 1984; Rosini et al., 1982). In icewine musts it was the only Ascomycetous yeast isolated and it was the only fermentative yeast isolated. No *Kloeckera* was found.

In the normal harvest period, Candida is the most commonly found wild yeast genus in Ontario musts (Holloway & Subden, 1991). None was found in the icewine musts used in this survey (Table 2).

The remaining cfus were all identified as non-fermentative Basidiomycetous yeasts routinely associated with plant material.

3.3. Molds

Ninety percent of the molds isolated were the Basidiomycetous *Aureobasidum pullulans* (Table 3). *A. pullulans* is a phenotypically diverse dimorphic yeast and many isolates are partially filamentous on the isolation medium. It is the most commonly found mold on both wine grapes and brewer's barley. Under some conditions *A. pullulans* may produce significant amounts of fermentation-stable polysaccharides such as pullulan (Simon, Bouchet, Bremond, Gallant, & Bouchonneau, 1998). It rarely, if ever, presents a problems in the wine industry as it is an obligate aerobe and is not active during fermentation. *A. pullulans* cannot be retrieved from fermentations after a drop of 1% in the sugar concentration (Holloway & Subden 1991). A search of the literature has revealed that there is no evidence for pullulan synthesis in wines of any kind. It may be that the carbon source concentration in the grape skin

Table 1
Bacterial isolates from icewine musts

Isolate code	Species	Total cfu×10^4/ml	% Of total bacterial population	% Of total microbiological population
N	*Pantoea agglomerans*	20	44.44	0.97
H	*Curtobacterium flaccumfaciens*	13	28.89	0.63
I	*Pseudomonas corrgata*	9	20.00	0.43
Q	*Curtobacterium pusillum*	3	6.67	0.14
		45[a]		2.17

[a] Total bacterial cfu×10^4/ml isolated.

Table 2
Yeasts isolated from icewine must

Isolate codes	Species	Total cfu$\times 10^4$/ml	% Of total yeast population	% Of total microbial population
C,DD,P	*Cryptococcus albidus*	543	47.93	26.14
W,E	*Rhodotorula* sp.	230	20.3	11.07
D	*Sporobolomyces* sp.	143	12.62	6.89
K,O,J	*Hansenisapora* uvarum	119	10.5	5.73
HH,F,G	*Sporobolomyces roseus*	92	8.12	4.43
M	*Sporidiobolus* sp-.	3	0.26	0.14
R	*Rhodotorula* sp *glutinis*	1	0.088	0.048
Ub	*Rhodotorula* sp. (black colonies) {Chromatorula}	1	0.088	0.048
Uw	*Cryptococcus laurentii*	1	0.088	0.048
		1133[a]		54.55

[a] Total yeast cfu$\times 10^4$/ml isolated.

Table 3
Moulds isolated from icewine musts

Isolate(s) Code(s)	Species	Total cfu$\times 10^4$/ml	% Of total yeast population	% Of total microbiological population
A,GG,EE,S	*Aureobasidium pullulans*	814	90.55	39.19
B	*Trichosporon* sp.	41	45.61	1.97
T,V,BB,Y	*Penicillium* sp.	24	2.67	1.16
L	*Geotrichum* sp.	9	1.00	0.43
X	*Fusarium* sp.	5	0.56	0.24
Z	*Cladosporium* sp.	2	0.22	0.096
FF	*Paecilomyces* sp.	2	0.22	0.096
CC	*Trichoderma* sp	1	0.11	0.048
AA	*Alternaria* sp.	1	0.11	0.048
		899[a]		43.28

[a] Total mold cfu$\times 10^4$/ml isolated.

environment is too low to foster the production of pullulan or other fermentation stable polysaccharide.

4. Conclusions

No laboratory-controlled organoleptic evaluation of the icewines was performed. However, the wines from which the must samples were taken in the 1999 vintage went on to win three gold medals (including "Best of Show" at Challenge International du Vin, Bordeaux, France Expo) in 2000, and one gold in 2001. The most recent vintage has not been bottled yet. We conclude the icewine microflor described in this work did not have a detrimental effect on quality.

We conclude that because the autocthonous microflora of icewine is dominated by the presence of bacteria, yeasts and molds incapable of activity in icewine musts, they, with the possible exception of *Hanseniaspora* have only a minor role in the fermenting must. They may play a role in the vineyard where the temperature and condition of the grapes is variable but in this sample, their contribution to taint formation during fermentation was minimal.

Acknowledgements

Support from Klaus Reif of Reif Estate Winery, Karl Kaiser of Inniskillin Wines Inc and Ed Gurinskas of Lakeview Cellars Estate Winery Ltd. is gratefully acknowledged.

References

Amerine, M. A, Chunk, R. E., Ough, C. S., & Web, A. D. (1980). *The technology of winemaking* (4th ed). Westport, CT: AVI Pub.

Amerine, M. A., & Ough, C. A. (1980). *Methods for analysis of musts and wines*. New York: John Wiley Publishing Co.

Barron, G. L. (1968). *The genera of Hyphomycetes from soil*. Baltimore, MD, USA: The William & Wilkins Co.

Barnett, R. W., Payne, R. W., & Yarrow, D. (1990). *Yeasts: characteristics and identification* (2nd ed). Cambridge, UK: Cambridge Univ Press.

Benda, I. (1964). The yeast flors of the district of Fraconia. *Weinberg Keller*, *11*, 67–74.

Chamberlain, G., Husnik, J. I., & Subden, R. E. (1998). Freeze-desiccation survival in wild yeasts in the bloom of icewine grapes.

Coutinho, C., Bernardes, E., Felix, D., & Panek, A. (1987). Trehalose as cryoprotectant for preservation of yeast strains. *Journal of Biotechnology*, *7*, 23–32.

Davenport, R. R. (1974). Microecology of yeasts and yeast like-organisms associated with an English vineyard. *Vitis*, *13*, 123–130.

Fennema, O., & Powrie, W. (1964). Fundamentals of low-temperature food preservation. *Adv. Food. Res.*, *13*, 219–347.

Fennema, O. R., Powrie, W. D., & Marth, E. H. (1973). *Low temperature preservation of foods and living matter*. New York: Marcel Dekker.

Fleet, G. H., Lafon-Laforcade, S., & Ribereau-Gayon, P. (1984). Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. *Applied and Environmental Microbiology*, *48*, 1034–1038.

Gil, J. V., Mateo, J. J., Jiménez, M., Pastor, A., & Huerta, T. (1996). Aroma compounds in wine as influences by apiculate yeasts. *J. Food Sci.*, *61*, 1247–1249.

Heard, G. M., & Fleet, G. H. (1986). Evaluation of selective media for enumeration of yeast during fermentation. *Journal of Applied Bacteriology*, *60*, 67.

Hino, A., Takano, H., & Takana, Y. (1987). New freeze tolerant yeast for frozen dough preparations. *Cereal Chemistry*, *64*(4), 269–275.

Hino, A., Mihara, K., Nakashima, K., & Takano, H. (1990). Trehalose levels and survival ratio of freeze-tolerant versus freeze sensitive yeasts. *Applied and Environmental Microbiology* 1386–1391.

Hino, A., Wongkhalaug, C., Kawi, S., Murao, S., Yano, K., Takano, H., & Takagi, M. (1992). Construction of a genetic transformation system for a freeze tolerant yeast Kluyveromyces thermotolerans. *Biosci. Biotech. Biochem.*, *56*(2), 228–232.

Holloway, P., Subden, R. E., & LaChance, M. A. (1990). The yeasts in a Riesling must from the Niagara grape-growing region of Ontario. *Can. Inst. Sci. Technol. J.*, *23*, 212–216.

Holloway, P., & Subden, R. E. (1991). Volatile metabolites produced in a Riesling must by wild yeast isolates. *Can. Inst. Sci. Technol. J.*, *24*, 212–216.

Holloway, P., vanTwest, R. A., Subden, R. E., & LaChance, M. A. (1992). A strain of *Candida stellata* of special interest to enologists. *Food Research International*, *25*, 147–149.

Minarik, E. A. (1964). Die Hefeflora von Jugenweinen in der Tschechoslowakei. *Mit. Rebe u Wein, Serie A (Klosterneuberg)*, *14*, 306–311.

Monk, P. R., & Cowley, R. J. (1984). Effect of nicotinic acid and sugar concentration of grape juice and temperature on accumulation of acetic acid during yeast yeast fermentation. *J. Ferment. Technol.*, *62*, 515–521.

Moore, K. J., Johnson, M. G., & Morris, J. R. (1988). Indigenous yeast microflora on Arkansas white Riesling (Vitis vinifera) grapes and in model must systems. *Journal of Food Science*, *53*, 1725–1728.

Murakami, Y., Hahn, Y., Yokoigawa, K., Endo, K., & Kawi, H. (1994). Induction of freeze-sensitive mutants from a freeze tolerant yeast, Torulaspora delbruckii. *Biosci. Biotec. Biochem.*, *58*(1), 206–207.

Rankine, B. C. (1967). Formation of higher alcohols by wine yeasts and relationship to taste thresholds. *Journal of Agricultural and Food Chemistry*, *21*, 50–59.

Ratner, E. N., Kamkova, S. P., Loseva, L. D., & Fikhte, B. A. (1987). Free water distribution in the cytoplasm of *Cryptococcus laurentii* cryotolerant cells. *Mikrobiol*, *55*, 851–854.

Rosini, G., Frederici, F., & Martini, A. (1982). Yeast flora of grape berries during ripening. *Microbiol. Ecol*, *8*, 83–91.

Sajbidor, J., Breierova, E., & Kockova-Kratochvilova, A. (1989). The relationship between freezing resistance and fatty acid composition of yeasts. *FEMS Microbiol. Lett.*, *58*, 195–198.

Schreier, P. (1979). Flavor composition of wines: a review. *CRC Critical Rev. Food Sci. Nutr.*, *12*, 59.

Sharpe, E. (1996). Laboratory procedures for the isolation of acetic acid bacteria. In B. M. Gibbs, & F. A. Skinner (Eds.), *Identification methods for microbiologists* (pp. 62–67). Academic Press.

Shimazo, Y., & Wantanabe, M. (1981). Effect of yeast strains and environmental conditions on formation of organic acids in must fermentation. *J. Ferment. Technol.*, *59*, 27–32.

Simon, L., Bouchet, B., Bremond, K., Gallant, D. J., Bouchonneau, M. R. (1998). Studies on pullulan extracellular production and glycogen intracellular content in *Aureobasidum pullulans*. *Can. J. Micro.*, 1182–1185.

Society of American Bacteriologists. (1994). *Bergey's manual of determinative bacteriology* (9th ed). Baltimore, MD, USA: The William & Wilkins Co.

Soles, R. M., Ough, C. S., & Kunkee, R. E. (1982). Ester concentration differences in wine fermented by various species and strains of yeasts. *Am. J. Enol. Vitic.*, *33*, 94–98.

Suomalainen, H., & Lehtonen, M. (1979). The production of aroma compounds by yeasts. *J. Inst. Brew.*, *85*, 149–156.

VQA Rules and Regulations, 1997. Government of Ontario Publications, Toronto, ON.

Wagener, W. W. D., & Wagener, G. W. W. (1968). The influence of ester and fusel alcohol content upon the quality of dry white wine. *S. Afr. Agric. Sci.*, *11*, 469–476.