Study on the yeast and mould biota of the botrytized grapes in Tokaj region in two years[1]

Bene, Zs. and **Magyar, I.**

Szent István University, Faculty of Food Sciences, Department of Oenology, Budapest

Summary: The famous wine Tokaji Aszú is produced from "noble rotted" grapes infected and modified by *Botrytis cinerea* under special condition. The objective of this study was quantitative and qualitative characterisation of saprophytic fungi present on the surface of aszú-berries, with special regard to yeasts. There were considerable differences in these populations depending on the origin of noble rotted berries, notably between berries taken from the vine or the ones taken from the winery. Beside *Botrytis*, other mould species like *Penicillium*, *Aspergillus* were commonly found, in widely varying population. Yeast counts were detected between 10^4 and 10^7 cfu/g berry. In the samples taken from the vineyard *Candida pulcherrima* predominated followed by some aerobic basidiomycetous species, but *Hanseniaspora* species were also present in relatively high population. After transport and storage of the grape in the wineries, population of aerobic yeasts and *C. pulcherrima* quickly declined and *Candida stellata* followed by other sugar tolerant species became dominant. This autoselection process directs attention to the importance of storage conditions.

Key words: Botrytis, noble rot, Tokaji, yeast

Introduction

Produced from "noble rotted" grapes, Tokaji Aszú is known as one of the oldest botrytized wines all over the world. Climatic conditions, soil circumstances and grape-varieties of Tokaj-region offer exceptionally favourable parameters to the formation of noble rot caused by *Botrytis cinerea*. Noble rotted grapes – locally called "aszú" berries – are the result of the special metabolic activity of *Botrytis* which can occur when there is an alternation of short damp periods and longer sunny days after the time when berries reached the whole maturity state. Under these conditions the contamination of mould takes plece by penetration of the germinating conidia and hyphae of *Botrytis* into the berry through microfissures of the visually intact skin. After infection the numerous destructive enzymes of mould start to cause chemical changes. Firstly the degradation of cell walls is realised, which causes cell death and the colour of skin turns into chocolate or greyish blue. In sunny days berries lose much water through the penetration cracks. Since it cannot be replaced from the vine through stem at the whole maturity state, the juice can be strongly concentrated by drying. As a common results of the enzymatic activity of *Botrytis* and the physical process of concentration the grapes undergo complex chemical modifications, which have been well studied (reviewed by *Dittrich*, 1987, *Doneche*, 1993). Also the aromatic compounds like free terpenoids go through considerable changes and new volatile compounds are produced *(Kerényi*, 1976).

Infection of *Botrytis* opens free way for other microorganisms such as other mould species (like *Penicillium, Aspergillus*) and yeasts. These microorganisms cannot penetrate into the berries unless hurt of the berry-skin takes place. This kind of injuries can be caused by *Botrytis* giving possibility to the concomitant microbiota for infection. Presence of other mould strains can cause formation of unpleasant flavour and smell. Yeast species invading the disrupted berry skin may, however, have either positive or negative impact on the quality of aszú wine during the special vinification process which includes storage of the aszú grapes, maceration of the berries with determined quantity of must or wine, re-fermentation of the mixture (mainly spontaneously) and long ageing of wine in small oak barrels. Among these steps, fermentation is the most critical one because of the extreme composition of wine and possibly because of the special composition of the indigenous yeast biota.

Microbiota of botrytized grapes and wines from different countries have been studied by several authors *(Domerque*, 1957, *Minarik*, 1963, *Le Roux* et al., 1973,) but there are few studies published relating to the Hungarian Tokaj wine district *(Magyar*, 1996, *Kalmár* et al., 1999).

In this work we have studied the microbiological features of aszú-berries from Tokaj-region, and we have made steps to identify the representative yeast species present on the surface of botrytized berries. The results from two vintages are presented here, of which the drier year 1999 provided better conditions for the noble rot than the year 1998.

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To establish a better control of Aszú fermentation, deeper knowledge is inevitable concerning the storage circumstances of noble rotted berries, too. We examined surface of berries taken from the vine-stock directly and also from the winery, after several weeks of storage. We paid attention to the change of yeast and mould populations as a function of origin and years.

Material and method

Samples of aszú–berries were taken aseptically partly from the vineyards, partly from the storage place of various winemaking companies. Cells attached to the surface of aszú-berries were washed into sterile water with shaking, then after diluting they were spread-plated on different selective media. The total yeast and mould count was determined in complex nutritive medium YEPD (yeast extract 1%, peptone 0.5%, glucose 2%), pH 3.5, and in many cases also in Dichloran-Rose-Bengal-Chloramphenicol agar (Merck) which restricts the colony size of several moulds. The lower detection limit of the method was about 10^2 cfu/g berry.

Means of the logarithmic cell counts obtained from different sampling places were statistically compared by *t-probe* (unequal number of observations with homogenous variances).

The characteristic yeast colonies were isolated from several dilution levels, purified by serial streaking, investigated microscopically then identified according to *Kurtzmann & Fell* (1998). The classic morphological (colour in different media, type of vegetative reproduction, sporulation and conjugation on McClary-acetate agar, malt extract agar, pseudomycelium formation on corn meal agar) and physiological (fermentation, multiplication at 37 °C, urease–test, assimilation of galactose, sucrose, maltose, xylose, raffinose, mannitol, erithritol, lactose, cellobiose, melibiose, ethyl-amine, lysine, nitrate) examinations served as basis of the identification. All strains were identified to the level of genera and most of them to the level of species.

Moulds were identified to the level of genera according to the morphological stamps.

Results

Quantitative composition of microorganisms on the surface of aszú – berries

Figure 1 displays the main results of our studies in 1998, *Figure 2* shows the composition of examined aszú-berries in 1999. Mean values of the measured populations with some statistical parameters are summarised in *Table 1*. In the quantitative composition of microbiota considerable differences were found depending on the place of sampling-, notably between berries taken from the vine directly and those taken from the cellars *(Table 1)*.

Moulds

In the vineyard as expected, the order of magnitude of mould-population was significantly higher, than those commonly found in sound grapes. It has to be noted that, as a consequence of surface washing method, the published mould-counts actually mean conidia–counts on the surface of examined berries, which is not necessarily proportional with the mass of mycelium spread through skin.

Botrytis conidia were present in comparable density in both years in the samples from the vineyards (mean logarithmic values 5.9 and 5.7 cfu/g), although the variances of the samples were very high *(Table 1)*. Other mould species like *Penicillium, Aspergillus* were measured at much lower average population (mean value: 3.7), with individual data from the detection limit to 10^6 cfu/g counts.

After 2–3 weeks of storage no significant changes were detected in the conidia count of either *Botrytis* or other moulds in 1998, but large reduction was found in both populations in the drier year 1999 that produced higher quality aszú grapes. This must be a consequence of higher sugar content of the grapes.

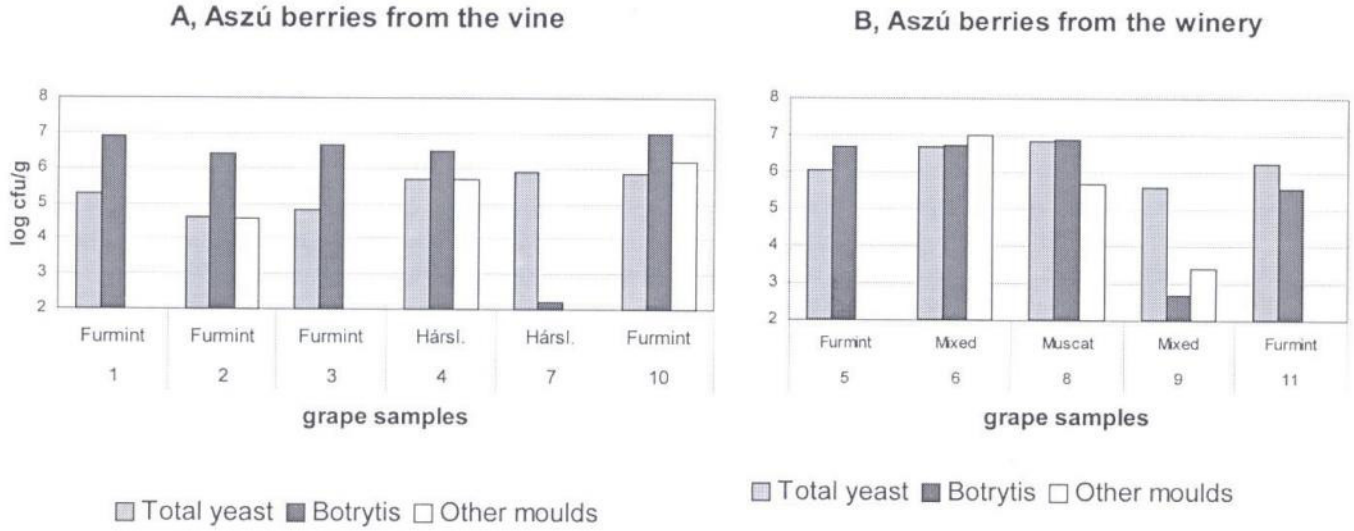

Figure 1 Yeast and mould counts on the surface of noble rotted grapes taken from the vine before harvest (A) and from the winery after storage (B) in the year 1998.

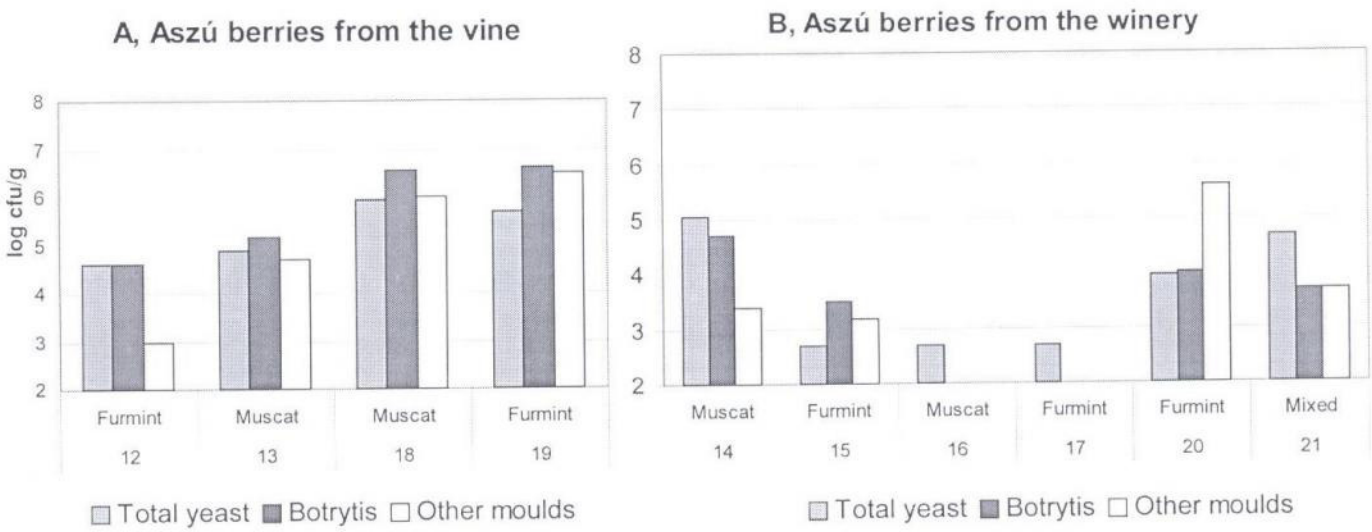

Figure 2 Yeast and mould counts on the surface of noble rotted grapes taken from the vine before harvest (A) and from the winery after storage (B) in the year 1999.

Table 1 Comparison of the mean values of cell counts (log cfu/g) detected on the surface of aszú grapes taken from different sampling places (vineyard or winery) in two years.

Lo g N/g		1998			1999		
		Place of sampling			Place of sampling		
		vineyard	winery	difference	vineyard	winery	difference
Total yeast	**mean**	**5.362**	**6.287**	**–0.925***	**5.281**	**3.630**	**1,650***
	variance	0.3138	0.2531		0.4025	1.1643	
	n	6	5		4	6	
	t	–2.85*		p= 0.0190	2.73*		p= 0.0260
	t5%	2.26			2.31		
Botrytis	**mean**	**5.903**	**5.709**	**0.195**	**5.730**	**3.216**	**2.515****
	variance	3.7125	3.1047		1.0130	1.5459	
	n	6	5		4	6	
	t	0.17		p= 0.8663	3.36**		p=0.0099
	t5%	2.26			2.31		
Other mould	**mean**	**3.737**	**4.019**	**–0.283**	**5.044**	**3.313**	**1.732**
	variance	3.9003	5.0618		2.4216	1.7725	
	n	6	5		4	6	
	t	–0.22		p= 0.8291	3.36		p= 0.0955
	t5%	2.26			2.31		

n = number of samples
t = calculated t-value
t5% = critical t-value at p=0.05
p = significance level of t-probe. Difference between the mean values is considered significant if p<0.05 (*) and highly significant if p<0.01 (**).

Yeasts

The order of magnitude of yeast populations in the samples from vine was similar in the two vintages (mean logarithmic values are 5.36 and 5.21, respectively) but the samples from the winery showed quite different patterns in the two years. While the conditions of the lower quality aszú grapes supported significant yeast growth on the surface of the aszú berries in 1998, considerable reduction (from 5.28 to 3.63 log cfu/g) could be experienced in 1999, similarly to the tendency of mould counts. The total yeast population decreased under 10^4 cfu /g in the majority of samples. From the results it is clear that circumstances on the surface of higher dry-material berries do not give favourable parameters to the multiplication of yeasts during the storage.

There was no noticeable correlation between yeast counts and grape varieties, although this question should be further investigated in larger number of samples.

Taxonomic composition of the yeast biota of aszú grapes

In these studies we isolated 75 yeast strains then they were identified according to *Kurtzmann & Fell* (1998). *Table* 2. displays qualitative composition of yeast–biota in both years. The identification was not successful in some cases

Table 2 Taxonomic composition of the yeast biota detected on the surface of aszú grapes taken from the vine or from the winery, in two years

Year	Place of sampling	Grape-variety	Code of samples	Cell count of the dominant yeast-strain (log cfu/g)	Dominant yeast genera or species	Other yeast-species isolated
1998	vine-stock	Furmint	1	5.30	*Hanseniaspora guillermondii*	*Rhodotorula sp.*
		Furmint	2	4.60	*Non-identified*	
		Furmint	3	4.60	*Rhodotorula sp.*	*C.pulcherrima, C.apicola*
		Furmint	10	5.70	*Candida pulcherrima*	*Hanseniaspora uvarum*
		Hárslevelű	4	5.48	*Candida stellata*	*C.pulcherrima, C.paludigena, H.uvarum*
		Hárslevelű	7	6.00	*Non-identified*	*Zygosacch. sp., C.cantarelli, C.multigemmis, Kluyv.lactis*
	winery	Furmint	5	5.78	*Candida stellata*	*P.angusta, Kluyv.thermotolerans*
		Mixed	6	6.00	*Hanseniaspora occidentalis*	*C.stellata, C.pulcherrima*
		Muscat	8	6.30	*Candida floricola*	*C.pulcherrima, Rhodotorula sp., H'spora uvarum, C. sake*
		Mixed	9	5.30	*Zygosaccharomyces rouxii*	*Rhodotorula sp., Br.bruxellensis, H'spora uvarum*
		Furmint	11	5.00	*Brettanomyces bruxellensis*	*Kluyveromyces lactis*
1999	vine-stock	Furmint	12	3.90	*Cryptococcus albidus*	*Cryptococcus macerans, Sporidiobolus pararoseus*
		Muscat	13	4.30	*Sporidiobolus pararoseus*	*C.pulcherrima, Lalaria sp., Arxula terrestris, Cryptococcus macerans*
		Mixed	18	5.78	*Candida pulcherrima*	*H'spora uvarum, C. stellata, Sympodiomycopsis sp.*
		Furmint	19	4.48	Candida pulcherrima	
	winery	Muscat	14	4.00	*Candida stellata*	
			15	2.85	*Candida stellata*	
		Furmint	17	2.70	*Non-identified*	*Zygosacch. sp.*
		Furmint	20	3.78	*Torulaspora sp.*	*C. stellata, C. pulcherrima*
		Mixed	21	6.30	*Candida stellata*	*Torulaspora delbrueckii, Zygosacch.rouxii*

because the strain died off, and one part of the isolated strains could be determined only to the level of genera.

On the aszú-berries taken from the vineyard directly, the most frequently recognised yeast species was the *Candida pulcherrima* (teleomorphic state: *Metschnikowia pulcherrima*) followed by basidiomycetes- related aerobic species like *Rhodotorula, Cryptococcus, Sporidiobolus*. In the year of 1998 when circumstances were not as favourable as in 1999, *Hanseniaspora* species could be found in higher frequency, similarly to the surface of non-botrytized grapes.

As opposed to that feature, the taxonomic composition of yeast–flora on the samples taken from wineries is very different. Aerobic yeasts, *Hanseniaspora* species and the *Candida pulcherrima* generally disappeared or declined, while the sugar-tolerant *Candida stellata* was found in high population. Other sugar-tolerant yeasts like *Zygosaccharomyces, Torulaspora* species with good fermentative skill were enriched, too.

Dominance of these species on noble rotted grapes – with special regard to *C. stellata*- had been reported long ago in the wine districts of Gironde *(Domerck,* 1957) and often associated with the *Botrytis* infection *(Le Roux* et al., 1973., *Gandini,* 1973, *Minarik* et al., 1978, *Heard & Fleet,* 1985). High presence of this species was reported also on the sound grapes in Mediterranean areas and in Australia *(Mora* et al., 1988, *Martínez* et al., 1989, *Fleet & Heard,* 1993) but not in cooler winegrowing regions.

In this study dominance of *C. stellata* was found only after the transport and storage of the aszú berries into the wineries. *Saccharomyces cerevisiae* was not found in the surface of aszú-berries, irrespective of the place of sampling or year.

Conclusions

According to our study the yeast biota of noble rotted berries is different from that of non-botrytized grapes and it is highly influenced by the storage of the grapes in the wineries before vinification. The effect of year on the yeast biota was also significant, although less pronounced. The results show that during picking, sorting, transport and storing, microbiota of the noble rotted berries undergoes a considerable autoselection process due to the special micro-ecological conditions.

Further research is in progress concerning the quality-determining role of the dominant yeasts of aszú grapes with special regard to the importance of *Candida stellata.* As next steps the optimisation of the storage conditions of Aszú – berries has to be elaborated, too.

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