UNIVERSITY OF ECONOMIC SCIENCES AND PUBLIC ADMINISTRATION

CHARACTERIZATION OF YEAST AND MOULD BIOTA OF NOBLE ROTTED BERRIES IN TOKAJ WINE REGION

Thesis

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1. INTRODUCTION AND AIMS

Climatic conditions, soil circumstances and grape-varieties of Tokaj-region offer favourable parameters to the formation of noble rot caused by *Botrytis cinerea* on the grapes. The noble rotted aszú-berries represent the most important raw material of the worldwide known Tokaji Aszú wine. Due to the enzymatic and physical degradation of the grape skin, *Botrytis* opens free way for the growth of numerous saprophytic species (other moulds, yeasts, and bacteria). There have been only few studies on the composition of the microbiota colonising the aszú grapes, although this composition may have strong impact on the aszú technology, with special regard to the fermentation. Today the most wineries relay on spontaneous fermentation, which is initiated together by the natural yeast species on the surface of the grape and yeasts of the base wines used for soaking the aszú-berries.

After the transport to winery, aszú-berries generally are stored for more weeks before processing. The storage are carried out in opened vats, tanks or containers and, especially in the case of big wineries, treatment of sulphur-dioxide is widely used against acetic acid bacteria and wine-flies. During the storage, the quality and chemical composition of aszú-berries can be influenced by the yeast and mould biota harbouring the digested grape skin and in this way the sensory parameters of aszú wines can be modified, too. It is very important to have knowledge on the changes taking place during the storage.

The unique feature of Tokaj wine specialities is determined by the „aszú-character" derived from noble rot and the „Tokaj-character" coming from ageing. The formation of „aszú-character" is the result of the special metabolic activity of *Botrytis cinerea* infecting matured or overmatured grapes, which process is accompanied with activity of other mould strains (*Penicillium, Aspergillus*). There is a possibility of the formation of penicillin-derivates by the fact of being possible occurrence of *P. chrysogenum* and *P. notatum*. We have not found data on the penicillin-content of noble rotted berries and botrytized wines in the oenological literature, although the presence or detection of this compound at a certain concentration may serve as quality determining criterion to identify noble rot.

Out of the several research directions of this complex topic, I have selected the following questions:

1. Study on the microbiological features of aszú-berries from Tokaj-region: determination of the quantitative composition of yeast- and mould biota on the surface of noble rotted berries. These results may contribute to the survey on the botrytized grapes performed in other wine-districts of the world.
2. Examination of the share of fermentative and non-fermentative yeast species in the microflora.
3. Determination of the taxonomic composition of natural yeast biota.
4. Evaluation of the effect of year on the composition of yeast- and mould biota.
5. Analysis of microbiological changes during the storage.
6. Determination of the indigenous yeast species which can survive and effect the inoculated fermentation of aszú wines, too.
7. Scanning electron microscopic study of the botrytization process.
8. Survey for the presence of penicillin-V in the aszú grapes. Determination of the penicillin-content as secondary metabolite of *Penicillium* strains from precursors.
9. Analysis of aszú wines for penicillin-V (is this compound stable during vinification and ageing?)

From the results of my research work I intended to give also practical suggestions concerning the possible aszú-qualifying characteristic features according to theoretical consideration and our examinations.

2. MATERIALS AND METHODS

2.1. Study on the microflora of botrytized grapes

One part of the aszú berry samples was taken aseptically from the vineyards, directly from the vine stocks. Another part of the samples was collected from the storage places of various winemaking companies, after the manual picking, sorting, transporting and storage. Cells attached to the surface of aszú-berries were washed into sterile water with shaking, and then after diluting they were spread-plated on different differential media. The total yeast and mould count was determined in complex nutritive medium YEPD, pH 3.5 in 1998 and 1999. In 2000 and 2001 Dichloran-Rose-Bengal-Chloramphenicol DRBC agar (Merck, Germany) and Dichloran-glycerine DG18 agar (Merck, Germany) were used. The dichloran-content restricts the colony size of several moulds.

In 1998 I examined the share of fermentative and non-fermentative yeast-species. I put the Petri-dishes spread-plated on agars mentioned above in carbon-dioxide circumstances.

The detection limit of the method was about 10^2 CFU g^{-1} berry.

Botrytis cinerea and other moulds were separated according to the morphological stamps.

The characteristic yeast colonies were isolated from several dilution levels, purified by serial streaking, investigated under microscope then identified according to KURTZMANN and FELL (1998). All isolates were identified to the level of genera and most of them to the level of species.

The morphological stamps of *Botrytis cinerea* and the mechanism of noble rot were also investigated by scanning electron microscopy typed TESLA BS-300.

The random sampling in wineries having different storage conditions and wine-making technology is not suitable for exploring the exact effects of storage circumstances on the quantitative composition of microbiota on the surface of aszú-berries. To clarify some of these effects, I performed an experimental storage with homogeneous noble rotted berries stored under controlled circumstances (temperature, SO_2) at the Department of Oenology, BKÁE University, Budapest.

2.2. Determination of penicillin-content

The sample taking was realised partly from aszú-berries taken from the storage places of several winemaking-companies in Tokaj Wine Region. Another part of the samples was collected from different raw-aszú wines, aszú wines and nature “eszencia”(essence). I determined the penicillin-content in case of 7 noble rotted berries and 13 aszú wines.

Wine-sample was extracted with dichloride-methane in a bolting-funnel. After splitting up of phases, lower phase involving penicillin-V was collected and evaporated in 60˚C under vacuum in a rotator. The residue was dissolved in 1-ml ethanol of HPLC-quality and used for the measuring of penicillin-V content.

In the case of aszú-berries, firstly noble rotted berries were measured and diluted with sterile water. Then there was a revealing method with a mixer without hurting the grape-seed. The juice was steeped in 1 hour, then strained twice and I continued sample preparation in the same way as described above in the case of wines.

The prepared samples were measured with HPLC-technique.

The calibration-curve was taken up with penicillin-V (Sigma) chromatographic standard.

3. RESULTS

The main results of my research work are as follows:

- The microbiota of noble rotted berries is *quantitatively* and *qualitatively* different from that of non-botrytized grapes. The activity of *B. cinerea* causes changes in the composition of associated saprophytic species.

- 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.

  - In the vineyard as expected, the order of magnitude of mould-population was significantly higher than those commonly found in sound grapes. I measured 10^4-10^7 cfu/g for *Botrytis*, and 10^2-10^5 for other moulds. The order of magnitude of yeast populations in the samples from vine was also somewhat higher (10^3-10^6) than that of the sound grapes.
  - The quantity of cells on the surface of aszú-berries is significantly influenced by years, although the differences were not as high as I expected from the extremity of years.
  - After transport and storage of the aszú grapes in the wineries, the mould count generally decreased, the yeast count typically increased or sometimes decreased. The differences between years enhanced during storage, which directs our attention to the significance of the storage conditions.

- Also the *qualitative* composition of the yeast biota was different from the typical yeast composition of sound grapes, and significant differences were found depending on the place of sampling, too.

  - On the aszú-berries taken from the *vineyards* directly, the most frequently found yeast species was the *Candida pulcherrima* beside *Hanseniaspora* species, followed by basidiomycetous-related aerobic species. In the dry, high quality vintages (1999, 2000) among the yeast species, *C. pulcherrima* and sugar-tolerant species like *C.stellata*, *Zygosaccharomyces* species were found in high quantity. The rainy autumn in 1998 and 2001 provides an intensive growth of *Botrytis*, producing low quantity and lower quality

noble rotted berries leaded to the prevalence of aerobic yeasts and *Hanseniaspora* species as that of sound grape.

- The taxonomic composition of yeast-flora on the samples taken from *wineries* is different from the pattern described above. Aerobic yeast, *Hanseniaspora* species and the *C. pulcherrima* generally disappeared or declined, while the sugar-tolerant *C. stellata* was found in high population. Other sugar tolerant yeasts like *Zygosaccharomyces* species with good fermentative skill were enriched, too.

- From these results I concluded that the effect of postharvest treatments on yeast-biota of aszú-grapes before vinification is highly significant. During picking, sorting, transport and storage, the yeast biota of noble rotted berries undergoes a considerable autoselection process due to the special microecological conditions. From that reason, the high diversity of the yeast-flora detected in the vineyard gradually decreases in the winery. Elaborating a control of Aszú-fermentation, we have to consider the species of indigenous yeast-biota especially *C. stellata* and *Zygosaccharomyces sp.* because they can have either positive or negative impact on the quality of aszú-wines.

- During an experimental storage I found, that the sulphite addition and the temperature had strong effect on the yeast and mould populations present on the aszú grapes. I consider beneficial a relatively high (20-22 ^{o}C) temperature and 300 mg/kg SO_2 treatment combined with a slight mechanic compression of the grapes.

- The mechanism of botrytization can be well followed by scanning electronmicroscope, which provides a better understanding of the interaction among the berry, *Botrytis cinerea* and the saprophytic microorganisms.

- One of the *Penicillium* strains on the surface of aszú-berries produces penicillin as secondary metabolite from precursors such as aminoacids, volatile phenols and aldehydes. In the aszú grapes penicillin-V was present in measurable quantity, although the small number of samples does not allow us to draw conclusions about the effect of years. In Tokaji wine specialities we also could detect penicillin-V concentration, which means that it is stable during vinification and ageing.

4. NEW SCIENTIFIC ACHIEVEMENTS

1. I characterised *quantitatively* the microbiota presented on the surface of noble rotted berries in Tokaj Wine Region as follows:
   Botrytis conidia were present in 10^4-10^7 cfu/g quantity, other mould species like *Penicillium, Aspergillus* were measured in the range of 10^2 – 10^5 cfu/g. In this way, the order of magnitude of mould-population was significantly higher, than those commonly found in sound grapes. The total yeast count was measured in the range of 10^3 – 10^6 cfu/g, which is somewhat higher than values in the case of sound grapes. The *quantitative* composition of yeast- and mould biota of aszú-berries first of all depends on the transport and storage circumstances after picking, secondly on the effect of years.

2. The *qualitative* composition of yeast biota of botrytized grapes is different from that of non-botrytized grapes and it is highly influenced by the years. Beside the effect of years, the storage of the grapes in the wineries before vinification is a very important factor on the quality of aszú-berries and aszú wines.

   - 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 *Sporidiobolus, Trichosporon.* In the rainy years *Hanseniaspora* species can be found in higher frequency similarly to the surface of non-botrytized grapes. *Saccharomyces cerevisiae* was not found in the surface of aszú-berries.
   - There is a change in the taxonomic composition of yeast biota during the transport and storage. Aerobic yeasts, *Hanseniaspora* species generally declined or disappeared, while the sugar-tolerant *Candida stellata* (and/or *Candida zemplinina* described as new species) was found in high population. Other sugar-tolerant yeasts like *Zygosaccharomyces, Kluyveromyces* species were enriched, too.

3. During my research work I isolated 158 yeast-strains, the biggest part of that yeast-strains was identified to the level of species, too. Most of the strains be found in the strain collection in the Department of Oenology and may serve as genetic basis for further scientific research works.

4. The yeast- and mould count on the surface of aszú-berries is highly influenced by the storage circumstances, especially by the treatment with sulphur-dioxide, temperature and storage period.

    - The yeast species on the surface of botrytized grapes are not sensitive to the sulphiting during the storage. The temperature and the storage period are the most important factors to their multiplication. The total population is growing at higher temperature and there is an autoselection process among species due to the longer storage period.
    - The mould species respond more sensitively to the sulphiting than yeast species: with the increasing dose of SO2 the mould population decreases. The rise of temperature has inhibitory effect on growth of moulds and the physical compression of the grapes implies further impediment to the mould growth.

5. According to our findings, one of the *Penicillium* strains on the surface of aszú-berries produces penicillin as secondary metabolite from precursors present inside noble rotted berries. Therefore the concomitant saprophytic fungi of botrytized berries may lead to appearance of further biologically active compounds. The Penicillin-content can be measured in the range of 0 – 74 mg/kg.

    We pointed out that the penicillin-V content measured in aszú-berries does not brake down completely during the different phase of aszú wine-making method and it can be also measured in the case of Tokaji wine specialities in the range of 0.4 – 26 mg/l. However the measured values are not high enough to lead to antibiotic-resistance in human body. It can not be considered therapeutic dose either, because 0.5 – 4 % of the medically suggested quantity is found in one litre Tokaji wine speciality, which consumption is typically occasional.

5. PRACTICAL CONCLUSIONS AND PERSPECTIVES

- In the rainy years the formation of *Botrytis*-conidia have more noticeable degree, which has strong effect on the yeast- and other mould-count, too. Generally there is an increase both in yeast and other mould population during the storage of grapes in the wineries before vinification.

- In years when the circumstances are more favourable for the formation of noble rot, aszú-berries have higher dry-material content. These years support mycelial growth of *Botrytis*, while conidiophore and conidium formation is suppressed. There is a decrease in other mould count, too. In the case of high quality aszú-grapes, when the humidity is high during the formation of noble rotted berries, there is an increase in the originally low yeast-count during the storage. When the humidity is lower, the opposite tendency can be noticed.

- Within the total yeast-count, the non-fermentative yeast species generally prevailed in the samples taken from the vineyard directly. As opposed to that feature, in the samples taken from wineries, the ratio of fermentative yeast species is higher. Therefore yeast species with good fermentative skill are able to grow better during the transport and storage.

- As next step of the research I suggest the oenological examination of the yeast species of good fermentative skill taken from the wineries. Their effects on the chemical composition, aroma, bouquet and general quality of aszú wines have to be studied to explore the role of these yeasts in the aszú fermentation. We have to pay special regard to the importance of the sugar-tolerant *Candida stellata* and *Zygosaccharomyces* species, because they can enrich the aroma of aszú-wines, compared to the fermentations with *Saccharomyces* species only. In this case we should use storage circumstances that support the growth of indigenous yeast biota. I consider beneficial a relatively high (20-22 ^{o}C) temperature and 300 mg/kg SO_2 treatment combined with a slight mechanic compression of the grapes.

- I believe that isolation and selection of local *Saccharomyces* strains for aszú fermentation may be a good tool for the fermentation control without significant change of the traditions and the sensory quality of aszú. However, the isolation of these *Saccharomyces*-strains is not expected from the aszú-berry.

- The methods in practise for qualitative classification of aszú-berries such as sensory analyses (seed, texture) and analytical measurements (sugar, glycerol-content) are not sufficient themselves. There is an indispensable need for the microbiological assessments and determination of precise chemical composition of noble rotted grapes (sugar-gluconic acid, glycerol-gluconic acid correlations, polyphenol-, penicillin-, metal ions-, biogenic amines –content, Ochratoxin-A-concentration).

6. PUBLICATIONS IN THE TOPIC OF THESIS

6.1. Publications in scientific periodicals

1. Magyar Ildikó – **Bene Zsuzsanna** – Clara Kardos (2001)**:** Az élesztő- és penészflóra összetétele és változása Tokaji aszúbogyók felületén két évjáratban. Borászati Füzetek, Tudományos melléklet, **XI/4**: 7-9.

2. **Bene Zsuzsanna** – Magyar Ildikó (2002): Az élesztő- és penészflóra összetétele és változása Tokaji aszúbogyók felületén a 2000-es évjáratban. Borászati Füzetek, Tudományos melléklet, **XII/1**: 1-4.

3. Kállay Miklós – **Bene Zsuzsanna** (2003): Tokaji borkülönlegességek penicillintartalmának vizsgálata. Borászati Füzetek, Tudományos melléklet, **XIII/2**: 18-20.

4. **Bene Zsuzsanna** – Magyar Ildikó (2003): A tárolás hatása a tokaji aszúbogyók élesztőflórájára. Élelmezési ipar, **LVII/6**: 168-171.

5. **Bene, Zs.** – Magyar, I. (2002): Study on the yeast and mould biota of the botrytized grapes in Tokaj region in two years. International Journal of Horticultural Science, **8/3-4**: 61-65.

6. Kállay, M. – **Bene, Zs.** (2003): Study on the *Penicillin*-content of botrytized wines and noble rotted berries in Tokaj-region. International Journal of Horticultural Science, **9/1**: 29-33.

7. **Zs. Bene** – I. Magyar (2004): Characterization of yeast and mould biota of botrytized grapes in Tokaj Wine Region in the years 2000 and 2001. Acta alimentaria, **33/3**

6.2. Conference proceedings

1. **Bene Zs.** (2000): Tokaji aszúbogyók mikrobiotájának tanulmányozása. MÉTE XIII. Országos Tudományos Diákköri Konferencia, Mosonmagyaróvár. Összefoglalók: 52-55.

6.3. Presentations on conferences

6.3.1. Conference abstracts

1. Magyar I. – Kardos, C. – Maráz A. – Pomázi A. – **Bene Zs.** (2000): A Tokaji Aszú erjesztésének mikrobiológiája. XIII. Élelmiszertudományi konferencia, Budapest. Összefoglalók:12.

2. Magyar I. - **Bene Zs.** – Kardos, C. (2000): A mikrobiota összetétele és változása Tokaji aszúbogyók felületén. Composition and evolution of the microbiota on the botrytized grapes in Tokaj. Lippay János – Vas Károly Tudományos Ülésszak, Budapest. Összefoglalók: 22-23.

3. **Bene Zs.** (2001): Tokaji aszúbogyók mikrobiotájának tanulmányozása. Study on the microorganisms of Aszú-berries. XXV. Országos Tudományos Diákköri Konferencia, Sopron. Összefoglalók:109, Abstracts: 97.

4. **Zs. Bene** - I. Magyar (2002): Composition and evolution of the microbiota on the surface of the botrytized grapes in Tokaj Wine District. II. Magyar Mikológiai Konferencia, Szeged. Acta Microbiologica et Immunologica Hungarica, 49 (2-3), pp 373-374.

5. **Bene Zs.** - Magyar I. (2002): A természetes élesztőflóra taxonómiai összetétele Tokaji aszúbogyók felületén az 1998, 1999, 2000 –es évjáratban. II. Mikológiai Nagygyűlés, Balatonfüred. Összefoglalók: 14.

6. **Zs. Bene** - J. Jarecsni (2002): Protection of origin and quality of the wine specialities of Tokaj-region, adaptibility of wine-categories to the EU-regulation. EFLA Kongresszus, Budapest. Abstracts: p13.

7. **Bene Zs.** (2003): A tárolás hatása a tokaji aszúbogyók élesztőflórájára. Tavaszi szél 2003, Sopron. Összefoglalók: 216.

8. **Zs. Bene** - I. Magyar (2003): Quantitative and qualitative characterisation of the yeast biota present on the botrytized aszú grapes in Tokaj wine district. ISSY-23, 23 rd International Specialised Symposium on Yeasts, „Interactions between Yeasts and other organisms", Budapest. Book of Abstracts: p 86.

9.**Zs. Bene** - I. Magyar (2003): Quantitative and qualitative change in the yeast biota of aszú-berries in Tokaj Wine Region during the storage. 14 th International Congress of the Hungarian Society for Microbiology, Balatonfüred. Book of Abstracts: p15.

10. Kállay M. – **Bene Zs.** (2003): Vizsgálatok különböző termőhelyű aszúszemek kémiai összetételére, különös tekintettel az élettani hatású vegyületekre. Examinations on the chemical composition of aszú-berries taken from different vineyards in special regard to the compounds with physiological effect. Lippay János – Ormos Imre – Vas Károly Tudományos Ülésszak, Budapest. Összefoglalók/Abstracts: 22/23.

11. **Zs. Bene** – M. Kállay (2004): Study on the compounds with physiological effect of noble rotted berries from Tokaj Wine-District. 2 nd Central European Congress on Food, Budapest. Book of Abstracts: p 177.

6.4. Posters and lectures on conferences

1. **Bene Zs.** (2002): Tokaji aszúbogyók felületi mikrobiotájának elektronmikroszkópos tanulmányozása. Fiatal magyar tudományos kutatók és doktoranduszok hatodik világtalálkozója, Gödöllő.

2. **Bene Zs.** (2002): Az aszúsodás folyamata. Szőlészeti-Borászati Napok, Bodrogkeresztúr.

3. **Bene Zs.** (2002): A tokaji aszú titkai – a botrítiszes nemesrothadás. VIII. Tokaji Bornapok, Tokaj.

4. **Bene Zs.** (2003): A *Botrytis cinerea* által bekövetkező kémiai változások. Szőlészeti-Borászati Napok, Bodrogkeresztúr.