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PHISIOLOGICAL, BIOCHEMICAL AND MOLECULAR CHARACTERISATION OF FILM-FORMING WINE YEAST STRAINS

Theses of the PhD dissertation of

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1. INTRODUCTION

In Hungary the traditional wine technology still gives preference to the spontaneous fermentation, although the application of starter cultures gains greater and greater attention. Beside selection of yeasts with favourable characteristics for performing the main fermentation, further selection of yeast strains is going on for obtaining strains that are possessing special technological features and effects. The film-forming wine yeast strains belong to one of this special yeast groups.

Numerous famous wine specialities are known, like the Spanish Sherry and the French Vin Jaune, which are characterised by typical aroma evolving during aging for several years. The above mentioned film- or velum-forming ("flor") yeasts are responsible for completion of biological aging of wines. The flor yeasts of sherry and sherry-type wines were widely and extensively studied in particular by the Spanish researchers and the results were published in several international journals.

Film-formation takes place on the surface of high ethanol content dry white wines after fermentation of the must has been finished. During film formation cells of film-forming yeast strains attach to each other and a floating biofilm develops. During film formation cells undergo different alterations, e.g. metabolism, size and shape of the cells will be changed. The cells in the film will utilise the ethanol, glycerol and organic acids via oxidative metabolism during aging. In the course of their metabolism characteristic aroma compounds are produced, which will determine the organoleptic properties of the wine in a great extent. One of the main compounds produced during biological aging is the acetaldehyde. Several environmental factors influence film-formation, like the available nutritive compounds in the wine, the temperature of the environment and its fluctuation, as well other stress factors. Also we might suppose that cell surface hydrophobicity plays an important role in film-formation, similarly as in the case of flocculation and adhesion to solid surfaces, namely the hydrophobic cell surface could help the interconnection of cells and the formation of aggregates. In this manner they could retain the CO_2 bubbles produced during fermentation, which could promote the rising of cells to the wine surface. The exact mechanism how the yeast cells rise to the surface, is not well understood, especially the molecular characterisation of the participating cell components is missing. It could be supposed that the cell surface proteins of

Saccharomyces cerevisiae play an important role in the interconnection of cells, similarly to the flocculation, in which the Flo11 protein of the cell wall assists in aggregation of cell.
In Tokaj District of Hungary during the aging process of Tokaji Szamorodni a yeast film develops occasionally on the surface of wine, similarly to the Spanish Sherry and other sherry-type wines. In the fifties several technological studies started in connection with the biological aging of Tokaj wine specialities. At the Oenological Department, University of Horticulture wine aging under yeast film has been studied by Gyula Kádár and his group in the years of 1970. As the result Gyöngyös-Domoszló Ltd. started production and distribution of a sherry-like wine speciality named 'HELIOS' in 1975. Later on experiments were done to produce new wine specialities with the use of alcohol fortification and short heat treatment of wine at the beginning of aging, respectively. Further investigations were made concerning the chemical process of aging and possibilities for its regulation.
The molecular analysis of film-forming yeasts participating in aging of Tokaji Szamorodni is entirely missing, as well as the overall description of potential cell surface proteins playing role in attachment of film-forming yeast cells.

2. OBJECTIVES

Main objectives of my research work were the physiological, biochemical and molecular biological characterisation of film-forming wine yeasts presented in the film during the aging of Tokaji Szamorodni and comparing them with film-forming strains originated from Sherry and sherry-type wines.

To reach the aims the following steps were planed:

1. Isolation of the dominant film forming yeast strains involved in aging of Tokaji Szamorodni.
2. Comparison of the film-forming yeasts of Tokaji Szamorodni and Sherry wines at molecular level. Investigation of the rDNA of film-forming yeast by using PCR-RFLP and heteroduplex analysis. Further typing and comparison of the film-forming strains with the help of RAPD analysis.
3. Characterisation of the environmental factors influencing the growth and film-formation of film-forming wine yeasts and determination the optimal values. Focusing

on the factors characteristic to wines, namely on the effect of fermentable (glucose) and non-fermentable (ethanol) carbon sources and the pH values.

4. Investigation of the cell wall structure and function of film-forming wine yeasts. Investigation of the adhesive growth, pseudohypha formation and cell surface hydrophobicity of the strains. Determination of the effect of influencing factors, like pH and growth phase. Determination of the electron donor/electron acceptor characters of the yeast cells.
5. Investigation and comparison of the cell surface proteins of film-forming and non-film-forming wine yeast strains. Isolation of cell wall proteins and separation with PAGE. Identification of the cell wall proteins using immunoblotting technique and comparison of the genes coding for the potential cell wall proteins of film-forming and non-film-forming strains with the application of molecular methods.

3. MATERIALS AND METHODS

Film-forming wine yeasts were isolated from the surface-film of szamorodni wines aging for different years in oak barrels at Bene Cellar in Bodrogkeresztúr. Within the frame of molecular assays identification was performed using restriction analysis of the rDNA region amplified with NS3-ITS4 primer pair. Performing RFLP analysis of rDNA ITS-5.8S region comparison was made between film-forming yeasts of Tokaj District and flor yeasts of sherry and sherry-type wines. To determine the rDNA polymorphism of film-forming wine yeasts the ITS1 region of the strains was investigated using heteroduplex analysis. Typing of film-forming wine yeast strains was performed with the application of random and microsatellite primers. To determine the physiological race of film-forming strains sugar fermentation profiles of yeast strains were established.

The effect of environmental factors (glucose, ethanol and pH) on growth was investigated using microtiter plate cultures in MULTISKAN-ASCENT (Thermo, Electron Corporation) apparatus. Parallel to growth investigation of the film-formation was followed in tube cultures. To assess the cell surface hydrophobicity and electron donor/electron acceptor characters of film-forming yeasts distribution of cells between polar and non-polar solvents was measured. The effects of pH and growth phase on cell surface hydrophobicity were also assessed. Furthermore adhesion of film-forming strains to glass, polystyrene and agar was also determined.

For investigation of cell surface molecules, proteins of film-forming strains were labelled with biotin and the covalently and non-covalently linked proteins were isolated. Separation was made with PAGE followed by blotting to nitrocellulose membrane. After conjugation with avidin the cell wall proteins were detected on the membrane. The N-terminal sequence of the cell wall protein that was different in the case of laboratory and film-forming *S. cerevisiae* strains was determined. The gene coding for the protein was identified using the *S. cerevisiae* gene databank (http://db.yeastgenome.org/cgi-bin/seqTools). A specific primer pair was designed for amplification of the encoding gene (*HSP150*) by using http://seq.yeastgenome.org/cgi-bin/web-primer website. With the application of the designed primer pair PCR-RFLP analysis of film-forming and non-film-forming wine yeasts was performed. To investigate the polymorphic nature of the gene numerous *HSP150* genes of film-forming and non-film-forming wine yeasts were sequenced and the deduced amino-acid sequences of the strains were compared.

4. RESULTS

4.1. Isolation and molecular characterisation of film-forming yeasts

Fourteen film-forming yeast strains have been isolated from the surfaces of aging dry and sweet szamorodni wines of ten barrels at Bene Cellar in Bodrogkeresztúr. The isolates belonged to *Saccharomyces cerevisiae* according to the rDNA analysis. Majority of the 'Tokaj Szamorodni' film-forming yeasts possessed dimorphic ITS1 region. The genomes of these strains contained two different alleles of rDNA: one with a 24 bp deletion and the other without this deletion. Out of the investigated three foreign flor yeasts two also had dimorphic rDNA, while the third possessed only the rDNA with the 24 bp deletion. The existence of 'Szamorodni' film-forming strains without this deletion indicates that the evolution of film-forming yeasts followed different routes; this is why their genomes could differ in some regions. Investigation of the meiotic segregants of a diploid strain having dimorphic rDNA established that these two types of rDNA alleles were present in combination on each chromosome. The appearance of polymorphic ITS1 region and the lack of the typical deletion in some strains indicate genome rearrangement or hybridization of diverse strains in which the genes/alleles necessary for film-formation remained unaltered. All of the film-forming

strains showed high similarity to the *S. cerevisiae* type strain and completely consistent pattern with each other using RAPD-PCR analysis. This consistency indicates the isogenic nature of film-forming strains.

4.2. Physiological characterisation of film-forming yeasts

Majority of the film-forming yeasts belonged to the *beticus* race of *S. cerevisiae* according to the sugar fermentation profile. Only four strains belonged to the *cheresiensis* race and one strain to the *montuliensis* and *rouxii* races each. The effect of different environmental factors on the film-forming isolates originated from dry and sweet Szamorodni wines were compared. Application of different glucose concentrations had no effect on the growth of the investigated strains. Dry and sweet 'Szamorodni' film-forming isolates had probably similar sensitivity for glucose repression. Differences were found between the film-formation ability of dry and sweet wine isolates at different glucose concentrations. The beginning of film-formation of the sweet wine isolates was delayed by the increasing of glucose concentration. The ethanol had strong effect on the growth of the strains. About 12 % ethanol content of wines decreased the growth of the strains but the film-formation was not inhibited. The film-forming strains were able to survive even higher ethanol concentrations up to 18 % but in this case film-formation failed. The pH optimum of the growth and film-formation reflected the general pH values of the wines. In both cases the optimal pH ranges were between 3 and 5.
The cell surface of film-forming yeasts was highly hydrophobic at acidic pHs, while at neutral and alkalic pH values the surface changed to hydrophilic. In contrast to the film-forming yeasts the cell surface of non-film-forming yeasts was hydrophilic at all pH values. The growth phases of cells had no effect on the degree of hydrophobicity. Only a little increase could be observed at the beginning of the growth, after this period no change took place and the characteristic hydrophobicity values of the cell surfaces were measured. The hydrophobic nature was due to the presence of hydrophobic cell wall proteins and not to the cell surface oxilipin (oxidized fatty acids). As the consequence of hydrophobic cell surface of film-forming yeasts they were able to form hydrophobic interactions. They were capable to attach to polystyrene, glass and agar surfaces. In spite of adhesive growth they were unable to penetrate into the agar (having no invasive character) or to switch to pseudohyphal growth.

4.3. Characterisation of cell wall structure of film-forming yeasts

Comparing the cell wall protein patterns of film-forming and non-film-forming yeasts a characteristic ca. 117 kDa protein Ccw7p/Pir2p/Hsp150p belonging to PIR-proteins could not be detected. On the other hand all the film-forming yeasts had an extra, ca. 87 kDa protein, which proved to be a modified Ccw7p according to the sequencing results. Using molecular analysis it was established that the film-forming yeasts possessed a shorter gene than the *S. cerevisiae* S288c strain deposited in the databank. The size difference was the consequence of a deletion within the repetitive region of the *HSP150* gene. All the film-forming yeasts had the same sequence alteration, but heterozygotic strains could also be found. In contrast to the film-forming yeasts the *HSP150* gene of non-film-forming strains showed high sequence polymorphism. Some of the non-film-forming yeasts were also heterozygotic for the gene; it could be presumed that the chromosomes of these strains contained alleles of different lengths. Analysis of the heterozygotic TD04 diploid film-forming strain showed that the longer *HSP150* allele contained two extra repetitive parts within the repetitive region, which was the consequence of an intragenic duplication of a 135 bp sequence, which was probably the consequence of a replication slippage process. The altered alleles of the non-film-forming strains might have been generated by the same process. This variability could play a role in the change of cell surface properties and adaptation to the stressful condition in the wine (e.g. low pH, high ethanol content).

4.4. Practical aspects of the research results obtained

Earlier research results proved that the film-forming yeasts play an important role in the biological aging of Tokaji Szamorodni, since they contribute to the expected organoleptic properties of these wines by the production of different metabolites. According to my results the indigenous film-forming *S. cerevisiae* strains of Tokaj District showed beside the similarities significant differences in comparison with the flor strains originated from Sherry and sherry-type wines. One of the most important differences is that ‘sherry’ flor strains grow only on the surfaces of dry wines, while in the case of Szamorodni wines film-formation could be observed on the surface of sweet wines, too. This phenomenon is inconsistent with that physiological rule that fermentable carbon sources repress the utilization of non-fermentable carbon sources (e.g. glycerol and ethanol); this is why ‘sweet wine’ film-forming

isolates are considered as scientific novelties. Through they practical application (e.g. as starter cultures) an opportunity would be presented to control the biological aging of sweet wines. The similar yields of the film-forming and non-film-forming strains may indicate that the selected film-forming yeasts in Tokaj cellars could be applied as starter cultures to accomplish the main fermentation of Tokaji Szamorodni wines. An important question is whether the film-forming yeasts are already present during spontaneous fermentation of Tokaji Szamorodni and whether they are capable to become dominant during fermentation or they emerge only at the end of the fermentation when the ethanol content is rising. It would be interesting to examine the population dynamics of yeasts involved in fermentation in details. Further research task would be necessary to investigate whether the film-forming strains originated from different cellars could compete with each other if they are inoculated in Szamorodni wines of different parameters and if a selection could take place among them so that one of them becomes dominant. On the basis of these results starter film-forming strains having different technological properties could be applied for directed application.

5. NEW SCIENTIFIC ACHIEVEMENTS

(1) Film-forming yeasts involved in biological aging have been isolated from the velum developed on the surface of Tokaji Szamorodni. These isolates belonged to the ’flor’ race of *Saccharomyces cerevisiae* according to my results. It has been verified that the isolated *S. cerevisiae* film-forming wine yeast strains showed high similarity at genome structure level.

(2) It has been investigated whether the 24 bp deletion, as marker in rDNA ITS1 region of ‘sherry’ flor yeasts described by Fernandez-Espinar et al. (2000) is also characteristic to film-forming yeasts originated from Tokaj region. It has been established that most of the ‘Tokaj’ film-forming strains possessed dimorphic rDNA, they contained the rDNA both with deletion and without deletion. Assessing the haploid meiotic segregants of a diploid film-forming strain it has been verified that the dimorphism of rDNA was characteristic for either homologous chromosome.

(3) The optimal glucose and ethanol concentrations and pH values have been determined in terms of film-formation. It has been demonstrated that the cell surface of ‘Tokaj’ film-forming wine yeasts was highly hydrophobic while the cell surface of non-film-forming

strains was hydrophilic. The cell surface hydrophobicity had similar values during the growth phases. Only change in the pH had an effect on the hydrophobicity; increasing the pH from pH 5 to pH 7 decreased the hydrophobicity dramatically. The cells of film-forming strains possessed electron donor properties besides the hydrophobic character. Examining the invasiveness and adhesiveness of film-forming yeasts it was established that they were capable to adhere to glass, polystyrene and agar surfaces, which was the consequence of hydrophobic interactions and over-expression of *FLO11* gene.

(4) Cell wall proteins of laboratory and film-forming *S. cerevisiae* strains have been compared. It has been determined that the film-forming yeasts differed only in the size of Pir2 protein from the laboratory *S. cerevisiae* strain, which was not the consequence of the decreased glycosylation level of the protein.

(5) The *CCW7/PIR2/HSP150* gene have been analysed and it was found that the smaller size of the Pir2 protein in the case of film-forming yeasts was due to the shorter gene size. Analysing the sequence of the gene it was verified that it contained deletion of three repetitive parts within the repetitive region in comparison with the laboratory *S. cerevisiae* strain. Investigating the meiotic segregants of heterozygotic TD04 film-forming strain it has been established that the two different alleles of *PIR2* gene segregated during meiosis. The sequence analysis of the gene revealed that alleles with different size probably resulted from a replication slippage process characteristic to the genes having tandem repeats.

6. PUBLICATIONS

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