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FROM YEAST GENETICS TO BIOTECHNOLOGY*

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Roots of classical yeast genetics go back to the early work of Lindegreen in the 1930s, who studied thallism, sporulation and inheritance of wine yeast strains belonging to *S. cerevisiae*. Consequent mutation and hybridization of heterothallic *S. cerevisae* strains resulted in the discovery of life cycle and mating type system, as well as construction of the genetic map. Elaboration of induced mutation and controlled hybridization of yeast strains opened up new possibilities for the genetic analysis of technologically important properties and for the production of improved industrial strains, but a big drawback was the widely different genetic properties of laboratory and industrial yeast strains. Genetic analysis and mapping of industrial strains were generally hindered because of homothallism, poor sporulation and/or low spore viability of brewing and wine yeast strains [1, 2]. In spite of this, there are a few examples of the application of sexual hybridization in the study of genetic control of important technological properties, e.g. sugar utilization, flocculation and flavor production in brewing yeast strains [3] or in the improvement of ethanol producing *S. cerevisiae* strains [4]. Rare mating and application of karyogamy deficient (kar^-) mutants also proved useful in strain improvement [5].

Importance of yeasts in biotechnology is enormous. This includes food and beverage fermentation processes where a wide range of yeast species are playing role, *but S. cerevisiae* is undoubtedly the most important species among them. New biotechnology is aiming to improve these technologies, but besides this, a completely new area of yeast utilization has been emerged, especially in the pharmaceutical and medical areas. Without decreasing the importance of *S. cerevisiae*, numerous other yeast species, e.g. *Kluyveromyces lactis, Hansenula polymorpha, Pichia pastoris, Schizosaccharomyces pombe* and *Yarrowia lipolytica* have gained increasing potentialities in the modern fermentation biotechnology [6].

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Developments in yeast genetics, biochemistry, physiology and process engineering provided bases of rapid development in modern biotechnology, but elaboration of the recombinant DNA technique is far the most important milestone in this field. Other molecular genetic techniques, as molecular genotyping of yeast strains proved also very beneficial in yeast fermentation technologies, because dynamics of both the natural and inoculated yeast biota could be followed by these versatile DNA-based techniques.

Keywords: Yeast genetics, fermentation biotechnology

Hybridization of yeast cells by sexual and parasexual processes

Yeast genomes consist of chromosomes and mitochondrial DNA, moreover plasmids and viral nucleic acids are also found sometimes in the cells. Natural genetic transfer and recombination of all these elements can take place during sexual mating process, which is controlled by the mating type genes, the chromosome code for all. Teleomorphic (perfect) yeast species are considered heteiothallic, homothallic or both, which represent haplontic, diplontic or haplo-diplontic life cycles, respectively [7].

Laboratory ("academic") strains of *S. cerevisiae* are generally heterothallic, which can mate only after finding the opposite mating type cells. Genetics of mating type determination and control and that of mating type interconversion is one of the most studied area in yeast genetics and molecular biology, which elucidated the molecular mechanisms of gene regulation, recombination and signal transduction in this simple eukaryotic organism [8]. Cells of the anamorphic yeast species, or those of belonging to the same mating type is unable, however, for sexual crossing. According to our present knowledge, natural parasexual hybridization does not exist in yeasts.

Research groups at the Department of Microbiology, University of Szeged elaborated the methodology of induced protoplast fusion for many yeast species and applied it for hybridization and studying the genetic consequences of the fusion. In *S. cerevisiae*, stable diploid hybrids were obtained, when protoplasts belonging to the same mating type were fused [9, 10]. Mitochondrial genes were also transmitted during fusion of protoplasts, having identical (alpha) mating type [9]. Detailed genetic analysis of the fusion products with different parental nuclear and mitochondrial genetic background was performed by Maráz and Šubik [11] who demonstrated that the transmission and recombination of mitochondrial genes are governed by the same rules, irrespective of the mating type and the mode of cell fusion (i.e. by induced protoplast fusion or by sexual mating).

Hybridization by protoplast fusion is circumventing most of the difficulties arising from polyploidy, homothallism and non-mating of the majority of industrial strains. Novel genetic properties can be effectively transferred to industrial yeasts from strains belonging to the same or closely related species (e.g. *S. cerevisiae* and *S. bayanus*). New wine yeast strains were constructed by fusion of genetically different wine yeast strains, when natural genetic markers and mitochondrial mutations were used in the selection of hybrids [12]. Another example is the construction of dextrin fermenting brewing yeast strain when the glucoamylase (*STA*) gene was transferred from an auxotrophic mutant of *S. cerevisiae* var. *diastaticus* into a lager yeast strain [13]. Karyotype analysis of hybrids and mitotic segregants obtained, proved very useful in the selection of those descendants, which harbored most of the chromosomes of the original brewing yeast strain. A major drawback of the method is, however, that undesired properties are generally also transferred, therefore a laborious, time-consuming post-selection work is needed.

Yeast *STA* genes and gene dosage effect

There are three unlinked *STA* genes (*STA1*, *STA2*, and *STA3*) in *S. cerevisiae* var. *diastaticus*, which are coding for the extracellular glucoamylase isoenzymes GAI, GAII and GAIII, respectively [14, 15]. Every *S. cerevisiae* strain has the sporulation-specific *SGA1* (intracellular) glucoamylase gene [16]. The *STA1*, *STA2* and *STA3* genes are located on chromosomes IV, II and XIV, respectively, while the *SGA1* gene on the chromosome IX [17].

In our research work we were looking for the answers to two questions:

- How much is the glucoamylase enzyme activity influenced by increasing the chromosomal copy number of *STA* genes in different combinations under normal physiological conditions?
- Do changes in enzyme activity have any effect on the growth rate?

To find the answers to these questions, we constructed hybrid yeast strains using haploid *S. cerevisiae* and *S. cerevisiae* var. *diastaticus* strains by sexual crossing. Haploid meiotic segregants were further isolated, which had more than one copy of *STA1*, *STA2* and *STA3* genes on their chromosomes. Number and chromosomal location of *STA* and *SGA1* genes were determined by electrophoretic karyotyping followed by Southern blotting with a 1.7 kb gene probe, specific for all the four genes. Glucoamylase enzyme production of nearly isogenic haploids with one, two or three copies of *STA* genes on their chromosomes increased significantly in starch containing medium in accordance with the copy number of genes. Although the increase of glucoamylase production was not directly proportional to the copy number, we could

still conclude that the enzyme production was not under significant glucose repression. Enhancement of *STA* gene copy number, however, did not influence the growth of strains, μ_{max} values were essentially the same, independently of the *STA* copy number [18].

Brewing yeasts are not able to hydrolyze starch, they are considered to have STA^0 genotype. In spite of this, we could detect *STA* or *SGA1* homologue gene sequence on chromosome XIV in addition to the *SGA1* gene on chromosome IX of four bottom fermenting (lager) yeast strains [19].

Killer characters in yeasts

Killer yeast strains produce proteinaceous extracellular protein, named killer toxin or zymocin. Zymocin has a lethal effect to the sensitive cells, which belong generally to the same or a closely related species. The toxin producer itself is resistant (immune) to its own toxin but can have cross sensitivity for an other type of toxin [20].

Killer yeasts are typed either by the characteristics of the toxin (e.g. molecular mass, pH optimum, sensitivity for temperature inactivation) and by the size of the genetic determinant of the toxin (M dsRNA). Less is known, however, about the cause of resistance, but differences in the cell wall structure can be crucial from this respect.

Killer yeasts are closely associated with the winemaking process. A number of wild killer yeasts from various genera have been isolated from grape must so far. Wild killer yeast strains may interfere with wine fermentation and quality. They can suppress the inoculated starter cultures and cause stuck fermentations and elevate the level of undesirable flavor and aroma compounds. Starter cultures, however, which harbor killer property, can have several advantages over conventional wine yeasts when employed for fermentation. They have grater chance to dominate from the very beginning of the fermentation and suppress certain wild yeasts, which can cause some problems such as delay of fermentation and production of off-flavors.

Killer property and sensitivity are very important in the wine fermenting yeasts, the incidence of killers and the type of toxin can vary, however, significantly in the different geographical areas, even in different wineries of the same district. Occurrence of killer activity has been observed also among strains, which are employed in the commercial fermentation of wine (see the review by Shimizu [21]).

Several strain improvement techniques can be used to construct wine yeast starter cultures with stable and desired killer property. The most important are the transfection of isolated killer virions to non-killer yeasts or transferring the genetic determinants of killer by sexual and parasexual hybridization.

Killer dsRNA molecules can be introduced into industrial strains by induced fusion of protoplasts without transferring chromosomal genes. In our experiments, protoplast fusion was applied for transferring killer dsRNA molecules of a K1-type *S. cerevisiae* strain to brewing yeast [13]. We developed a new technique of killer fusion product selection when no mutagenic treatment or any genetic modification of the brewing strain was necessary before the fusion. Killer toxin production of the resulted fusion products provided the only selective advantage of them. Mitochondrial DNA of the killer plasmid donor partner was eliminated by ethidium bromide, because the transfer of foreign mitochondria to brewer's yeasts occasionally decreases the maltose fermentation rate and alters the aroma production. Killer clones carrying brewing yeast genome mainly were elected on the basis of their inability to sporulate and lack of inducible segregation of the *S. cerevisiae* parental auxotrophic marker. One of the improved killer brewing strain was tested for beer fermentation in semipilot scale. The killer strain showed good fermentation ability, comparable or even better than the brewing parent. Moreover, the beer produced by the killer strain, had a diacetyl content significantly lower than that of the control.

Industrial importance of *Schizosaccharomyces pombe*

It has long been known that some species of the fission yeasts belonging to the *Schizosaccharomyces* genus are able to metabolize high amount of L-malic acid under anaerobic growth condition, via the maloalcoholic fermentation pathway. Although this mechanism exists in many other yeasts including *S cerevisiae*, only *Schiz. pombe* and *Schiz. malidevorans* were reported to be capable for complete degradation of malic acid in must. The high maloalcoholic activity of *Schiz. pombe* is assigned to the high substrate affinity of its malic enzyme and to its active transport system for malate [22].

Maloalcoholic fermentation by *Schiz. pombe* could serve as a technological means to reduce the malic acid content in musts or wines. Numerous studies were performed on *Schiz. pombe* from technological aspects, but its applicability to vinification has been found controversial. The main drawback for the process is that many of the tested strains were found to produce off-flavors, including H_2S.

We screened nineteen *Schiz. pombe* and three *Schiz. octosporus* strains for growth rate, L-malic acid fermentation rate and hydrogen sulphide production at low pH (pH 3.5) under semianaerobic condition. Each of these characters was found highly variable among the strains studied. The ethanol tolerance was found again strain dependent. Most strains had a decreased growth rate even at 5% (v/v) ethanol, while all were inhibited by 10% (v/v) ethanol. Low pH was beneficial for the growth rate, either without ethanol or with increasing ethanol concentration. Biomass yield was severely

reduced by 10% (v/v) ethanol both at pH 3.5 and 6.0, while the growth was totally repressed by 15% (v/v) ethanol. Malic acid fermentation rate was also strongly influenced by ethanol, but the degree of inhibition was not always proportional with the inhibition of growth, suggesting that ethanol had a specific way of action upon the maloalcoholic activity. Sulphide production of selected strains was considerably reduced by 10% (v/v) ethanol, even in higher extent than the growth. Based on these results, potential parental strains were selected to improve *Schiz. pombe* strains for enological purposes. Moreover, we selected a flocculating strain, RIVE 4-2-1, which presented a new type of flocculation but had a very poor malic acid fermenting activity.

Flocculation of this strain developed in the presence of ethanol only, both under aerobic and anaerobic growth conditions. Cell surface proteins which participated in cell-aggregation were characterized by susceptibility of flocculation to different proteolytic enzymes, heat treatment, denaturing and thiol-compounds and by inhibition of flocculation by sugars and derivatives. It was shown that a galactose-specific lectin was involed in this new type of flocculation.

In a breeding program, we created a good malic acid fermenting and flocculating strain, which could be the candidate for a continuous malic acid degradation in wine. The high sulphite production of this strain is still problematic, while we are working now on the study of sulphur metabolism of *Schiz. pombe*.

Application of molecular typing methods for identification and population studies of hiotechnologically important yeasts

Number and organization of chromosomal sets are typical for distinct species, while polymorphism of homologue chromosomes reflects difference or identity of strains belonging to the same species. Electrophoretic karyotyping is the most promising method in karyotype analysis, especially in industrial strains belonging to the *Saccharomyces* genus. Reproducible chromosomal fingerprints are obtained when intact chromosomes are separated under identical conditions of electrophoresis. Combination of electrophoretic karyotyping and Southern hybridization of special genes, Tamai *et al*. [23] showed that the bottom fermenting brewing yeast, *S. pastorianus* has two types of chromosomes, *S. cerevisiae*-type and *S. bayanus*-type, and that these chromosomes co-exist independently without undergoing reciprocal rearrangements. That is, *S. pastorianus* is a natural hybrid of *S. cerevisiae* and *S. bayanus* as was thought earlier by Vaughan-Martini and Kurtzman [24].

Development and changes of fermenting wine yeast populations are influenced by many factors, from the environmental conditions, via the viticultural and enological practices to the selection factors acting in wines. Molecular genetic techniques are very

powerful for following the population kinetical changes during the fermentation processes.

Production of "Tokaj aszu" wine is a traditional spontaneous fermentation process, which proceeds by the indigenous yeast biota of the base wine or must. Beside the yeast strains, belonging to *Saccharomyces sensu stricto* group, some other yeast species of good ethanol tolerance (*Candida stellata, Zygosaccharomyces rouxii, Z. bailii, Hanseniaspora* spp.), participate in the fermentation process, but their role and importance in the development of special "aszu" aroma have to be cleared.

The fermenting yeast biota of "Tokaj aszu" wines was studied by the electrophoretic karyiotype analysis and by RAPD–PCR method and the molecular fingerprints of yeast strains, isolated from different stages of wine fermentation were prepared. Spontaneous and experimental (starter inoculated) fermentations were studied.

Wine yeast populations were characterized by the frequency of the killer yeast strains, too. It showed a wide variety, ranging from 0 to 90%. Killer yeasts formed two separate groups on the basis of killer toxin produced: strains belonged to the first group produced an active toxin at pH 4, while the toxin produced by the strains of the second group was active at pH 5, too. The extracted nucleic acids of the killer strains were run on the agarose gel. We could detect dsRNA in all cases: the L plasmids were 4.7 kb, while the size of M plasmids was different from each other.

Comparison of the isolated strains by molecular typing methods showed higher heterogeneity of killer strain than the non-killer populations. This result was supported both by karyotyping and RAPD–PCR analysis.

Strains isolated from spontaneous fermentation belonged also to more than one cluster based on the RAPD–PCR analysis, while the RAPD patterns of the strains, originated from the induced fermentations were mostly identical with the starter cultures.

Molecular genetic studies proved that the populations responsible for the "Tokaj aszu" wine fermentation were genetically very variable, which became more homogenous to the last phase of fermentation. The reason could be that due to the presence of inhibiting substances in the botrytized wine and also to the increasing ethanol concentration, the more tolerant strains would be selected at the late phase of fermentation.

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