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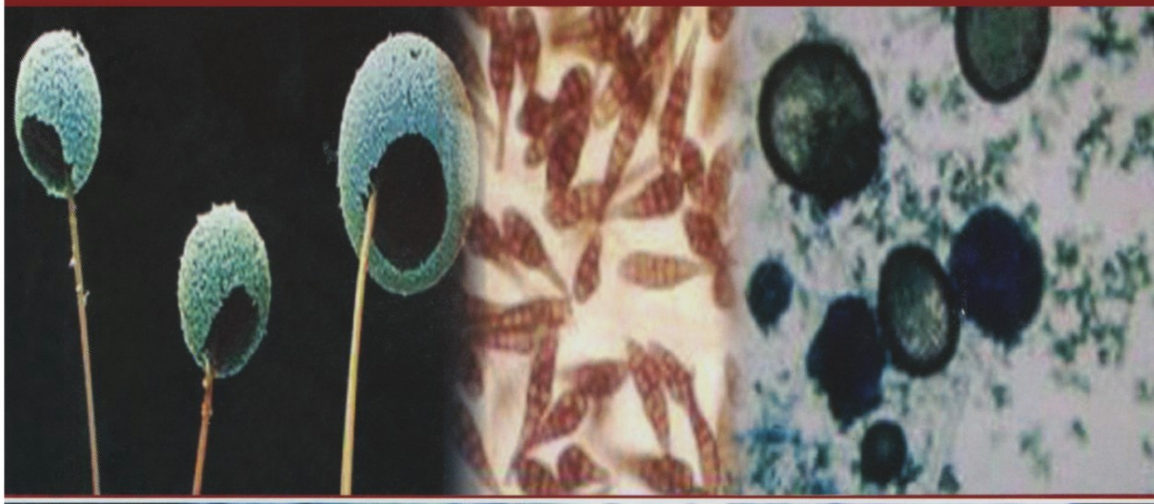
Progress in Mycology

Mahendra Rai George Kövics
Editors



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Preface

The people have been using fungi since ancient times. On one hand fungi are responsible for causing plant and human diseases, while on the other hand they are beneficial to human kind. There have been tremendous biotechnological advancement in the field of fungi in the last two decades. Various applications of the fungi include drugs, dyes, single-cell protein and growth promoters. Advancement in the field of molecular biology, proteomics and genomics have unravelled various doubts and provided new insights in the field of genetic improvement, transformations and phyllogenetic relationship of different genera and species. Fungi are not only involved in production of single-cell protein, wine and beer production and antioxidants but also used for bioremediation, growth promotion, as biosensors and fabrication of eco-friendly silver and gold nanoparticles. Some of these issues have been addressed in the present book.

The present book is aimed to provide the readers with current trends in the field of Mycology in general and fungal biotechnology in particular.

The book would be of utmost importance to students, researchers and teachers of botany, mycology, microbiology, medical microbiology, fungal biotechnology and nanotechnology. The readers should find the book full of information and reader friendly.

We are thankful to all the contributors for submission of their valuable manuscripts. MKR wishes to thank his students- Ravindra Ade, Avinash Ingle, Dnyaneshwar Rathod, Alka Yadav, Vaibhav Tiwari, Jayendra Kesharwani and Swapnil Gaikwad for help in editing and typesetting.

Mahendra Rai
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CHAPTER 1

BETTER YEAST FOR BETTER WINE - GENETIC IMPROVEMENT OF *SACCHAROMYCES CEREVISIAE* WINE STRAINS

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Introduction

The yeast species *Saccharomyces cerevisiae*, commonly called 'wine yeast', 'bakers yeast', 'brewers yeast' or 'distillers yeast' is the main yeast responsible for alcoholic fermentation and has been used for centuries in wine making, baking, brewing and distilling. With the emergence of molecular genetics and genomics, the industrial importance of *S. cerevisiae* continuously extended, providing a tremendous future potential for the development of genetically modified yeast strains (GMY) for the biofuel, bakery and beverage industries or for the production of enzymes and pharmaceutical products.

At present, most of the European wine production relies on the use of selected pure yeast cultures as an oenological practice to produce wine with desirable organoleptical characteristics and to guarantee the homogeneity of successive vintages. These yeast strains were selected from the fermentative flora of a given viticultural region mainly due to their good fermentation performance. There is considerable genetic variation within this species, since different strains of *S. cerevisiae* can vary significantly in their fermentative behavior and the production of compounds that benefit the sensory quality of wine. The accumulated knowledge of the *S. cerevisiae* cellular biology, physiology, biochemistry and genetics, in combination with intensive genomics and proteomics research, will illuminate phenotypic variation in natural populations.

Classical strain improvement approaches have a long-standing history of successful application and rely on repeated cycles of genetic diversity creation

through mutagenesis and/or genetic recombination followed by selection or screening of the desired phenotypes. Targeted genetic manipulation were undertaken even long before the publication of the *S. cerevisiae* genome sequence (Goffeau *et al.*, 1996). More recently, classical methods of strain selection became blended with the latest whole cell engineering approaches such as genome shuffling or evolutionary engineering, that mimic the principles of natural whole genome evolution in a laboratory setting. These procedures provide a promising means for the design of multiple complex, polygenic phenotypes in industrial yeasts, when coupled to high throughput screening and analytical technologies such as robotic miniaturization of assays. In parallel, unlocking the transcriptome, proteome and metabolome complexities in the post-"omics" era, decisively contributes to the knowledge about the genetic make-up of commercial yeast and will both allow to evaluate the consequences of the introduced changes on a genomic scale and speed up the development of novel strains.

Wine yeast strains obtained by genetic engineering using recombinant methods are still perceived in a very controversial manner by consumers, are not likely to become commercially feasible and probably will not receive approval in the European Union within the next future. Further obstacles are complex legal and regulatory issues that require a detailed safety and environmental impact evaluation. Non-recombinant modification and optimization of industrial strains by whole cell engineering approaches or by "self-cloning", based on the use of host-derived genetic material are most likely to receive approval by both authorities and consumers.

The present chapter gives a global overview of recent advances regarding the importance and implications of the use of engineered *S. cerevisiae* strains in the wine industry, considering a variety of aspects such as the genetic constitution, ecology and population genetics of indigenous *S. cerevisiae* strains, phenotypes of interest in wine-emaking, strategies and targets used for the construction of the strains, taking also into account data derived from genomic and proteomic studies. The final part focuses on current legislation requirements and environmental risk evaluations concerning the deliberate release of GMY strains and includes an analysis of the reasons responsible for critical consumer's attitudes toward their application in winemaking.

The ecology and population genetics of *Saccharomyces cerevisiae*

Winemaking is a human activity for several millennia and the species *S. cerevisiae* can be considered as mankind's oldest domesticated organism (Pretorius, 2000). Molecular evidence of the historical presence of *S. cerevisiae* in wine fermentation has been obtained from identification of this species in pottery jars found in the tomb of one of the first Egyptian kings, which dates back to 3150 bc (Cavalieri *et al.*, 2003).

Traditional wine fermentation occurs in a spontaneous way when yeast, part of the indigenous microbial flora of the grape's surface, are brought in contact with the sugar-rich (20-30%) grape must, that is obtained from pressed crushed grapes. The composition of the grape's yeast flora depends on a large variety of factors such as climatic conditions including temperature and rainfalls, geographic localization of the vineyard (Parish and Carroll, 1985; Longo *et al.*, 1991), antifungal applications (Monteil *et al.*, 1986), soil type (Farris *et al.*, 1990), grape variety and the vineyard's age (Martini *et al.*, 1980; Rosini, 1982; Pretorius *et al.*, 1999). Predominant species on healthy grapes are apiculate yeasts like *Hanseniaspora uvarum* (and its anamorph form *Kloeckera apiculata*) and oxidative species such as *Candida*, *Pichia*, *Kluyveromyces* and *Rhodotorula* (Fleet and Heard, 1993). Fermentative species of the genus *Saccharomyces*, predominantly *S. cerevisiae*, occur in extremely low number on healthy undamaged grape berries (<0.1%) or in soils (Parish and Carroll, 1985; Frezier and Dubourdieu, 1992; Martini *et al.*, 1996), while damaged grapes are believed to be an important source, providing inocula of 10^2–10^3cells/ml of must (Mortimer and Polsinelli, 1999). Insects (e.g. *Drosophila* spp., honey bees and wasps), birds and wind are important agents for the dispersal of yeasts in habitats related to winemaking environments. Several ecological surveys, using molecular methods of identification, report a large diversity of genetic patterns among the enological fermentative flora. *S. cerevisiae* strains seem to be widely distributed in a given viticultural region, can be found in consecutive years and there are also predominant strains in the fermenting flora, hypothesizing the occurrence of specific native strains that can be associated with a *terroir* (Frezier and Dubourdieu, 1992; Vezinhet *et al.*, 1992; Versavaud *et al.*, 1995; Sabate *et al.*, 1998; van der Westhuizen *et al.*, 2000; Torija *et al.*, 2001; Lopes *et al.*, 2002; Schuller *et al.*, 2005; Valero *et al.*, 2007).

Independent studies report the prevalence of *S. cerevisiae* strains among the wineries resident flora (Longo *et al.*, 1991; Vaughan-Martini and Martini, 1995;Constanti *et al.*, 1997; Beltran *et al.*, 2002; Sabate *et al.*, 2002). This lead to the discussion whether the vineyard is a natural environment of *S. cerevisiae*, or just provides a source of "domesticated" isolates that passed through consecutive series of must fermentations and survived in the vineyard/winery until the following harvest and fermentation. The isolation of *S. cerevisiae* far from vineyards, for example from soils associated with oak trees in the north-eastern United States (Naumov *et al.*, 1998), oak exudates and other broad-leafed trees (Sniegowski *et al.*, 2002), but also from the Danube River (Slavikova and Vadkertiova, 1997) and the gut of insects supports a natural occurrence of this species in very diverse habitats.

When the yeast genome was fully sequenced, the community of yeast researchers has developed a keen interest in genetic variation of natural populations and its functional and evolutionary implications. One of the first population-genetic variation studies was undertaken by sequencing the four loci *CDC19*, *PHD1*, *FZF1* and *SSU1* in 27 *S. cerevisiae* strains. Sequence analysis of

each gene distinguished strains collected from a Pennsylvanian oak forest and strains collected from vineyards, perhaps due to ecological rather than geographic factors (Aa *et al.*, 2006). However, the finding of *S. cerevisiae* isolates in other sources still cannot exclude the prevailing idea that they simply represent migrants from fermentations and derived from a domesticated species, specialized for the fermentation of alcoholic beverages. A recent study showed that the species as a whole is not domesticated and consists of both ''wild'' and ''domesticated'' populations. In this study, genealogical relationships from DNA sequence diversity at five loci in 81 strains of *S. cerevisiae* isolated from fermentations, tree exudates and immuno-compromised patients were established. At least two independent domestication events lead to specialized breeds of *S. cerevisiae*, one for the production of grape wine and one for the production of sake wine. The oldest lineages and most of variation were found in strains from sources that are not related to wine production, suggesting that strains of *S. cerevisiae* specialized for the production of alcoholic beverages derived from natural populations unassociated with alcoholic beverage production, rather than the opposite (Fay and Benavides, 2005).

Parallel to the selection and development of new *S. cerevisiae* strains for enological applications, molecular methods were developed and validated to study the evolution of yeast flora in spontaneous, but also in inoculated fermentations. The most widely used typing methods are based on chromosome separation by pulsed field electrophoresis (Carle and Olson, 1985; Blondin and Vezinhet, 1988), restriction fragment length polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) (Dubourdieu *et al.*, 1984; Vezinhet *et al.*, 1990; Querol *et al.*, 1992; Lopez *et al.*, 2001), randomly amplified polymorphic DNA, PCR fingerprinting followed by enzymatic restriction of amplified DNA (Baleiras Couto *et al.*, 1996), PCR-amplification of inter-delta sequences (Ness *et al.*, 1993, Legras and Karst, 2003) and multi locus sequence typing (MLST) (Ayoub *et al.*, 2006). In the last few years, fingerprinting of microsatellite or SSR (Simple Sequence Repeats) *loci*, short (1-10 nucleotides) DNA tandem repeats dispersed throughout the genome and with a high degree of variability, revealed to be very useful to discriminate *S. cerevisiae* strains (Gallego *et al.*, 1998; Hennequin *et al.*, 2001; Pérez *et al.*, 2001; Techera *et al.*, 2001; Schuller *et al.*, 2004). These loci exhibit a substantial level of polymorphism and have been used in humans for paternity tests, forensic medicine and population structure studies. Despite the higher equipment investment and need for skilled human resources, PCR-based microsatellite amplification and detection by capillary electrophoresis should be considered the method of choice, because of the easy high-throughput data generation, the absence of errors resulting from local experimental conditions and the possibility of sharing data by different laboratories. Besides the high level of discrimination and unequivocal results, expressed as base pair number (or as repeat number), the generated data are suitable to complete computational population genetic analysis. Twelve highly polymorphic micr-osatellite loci were used to assess the genetic diversity among 651 *S. cerevisiae* strains from 56 worldwide geographical origins. The genotypes clustered in

subgroups, according to the technological use (i.e. bread, beer, wine, sake). Bread strains displayed a combination of alleles intermediate between beer and wine strains, and strains used for rice wine and sake were most closely related to beer and bread strains. Macrogeographical differentiation of strains from Asia, Europe and Africa accounted for 28% of the observed genetic variation, which suggests clonal reproduction and local domestication of natural strains originating from the same geographic area. The data also indicated a Mesopotamia-based origin of most wine strains, and a migration route along the Danube Valley and around the Mediterranean Sea. The close association between vine migration and wine yeast favors the hypothesis that yeast may have followed man and vine as a commensal member of grapevine flora (Legras *et al.*, 2007). Similar phylogenetic relationships related to technological applications were observed when clustering of *S. cerevisiae* strain was based on 32 single-nucleotide polymorphism markers (Ben-Ari *et al.*, 2005) or amplified fragment length polymorphism (AFLP) analysis (Azumi and Goto-Yamamoto, 2001). Microsatellite revealed as informative markers for distinguishing populations from vineyards in very close geographical locations (50–100 km). Genetic differences among *S. cerevisiae* populations were rather apparent from gradations in allele frequencies than from distinctive "diagnostic" genotypes, and the accumulation of small allele-frequency differences across six loci allowed the identification of population structures. Within a vineyard, the genetic differentiation increased with the distance between sampling points suggesting a pattern of isolation by distance (Schuller and Casal, 2007).

Genetic constitution of *Saccharomyces cerevisiae* wine strains

When the *S. cerevisiae* genome sequencing project was completed, it became clear that this yeast has a genome of around 13000 kb, containing ca. 6000 protein-encoding genes that are distributed on 16 linear chromosomes, varying in length from 200 to 2200 kb, with a very low number of introns and little repetitive DNA (Goffeau *et al.*, 1996). Wild strains of *S. cerevisiae*, isolated from wine, cellars or vineyards are predominantly diploid, homothallic and mostly homozygous (65%), with low (Bakalinsky and Snow, 1990; Barre *et al.*, 1992; Guijo *et al.*, 1997) to high (>85%) sporulation capacity (Mortimer, 2000). Aneuploid strains, with approximately diploid DNA contents, have been described (Codon *et al.*, 1997; Nadal *et al.*, 1999; Puig *et al.*, 2000) and meiosis seems not to be a common occurrence in their life-cycle (Bakalinsky and Snow, 1990; Barre *et al.*, 1992). Such wine yeast strains present essentially an asexual life cycle and are characterized by high karyotype instability, which is believed to be a potential source of genetic variability (Bidenne *et al.*, 1992; Longo and Vezinhet 1993; Nadal *et al.*, 1999; Carro *et al.*, 2003). Haploid laboratory strains do not undergo by far such extensive changes (Longo and Vezinhet, 1993).

Gross mitotic chromosomal rearrangements, such as large regions fusion between homologous and non-homologous chromosomes occur in wine yeast with frequencies around 10^{-5} (Puig *et al.*, 2000). In chromosome I, several

membrane-associated genes are located in subtelomeric regions, and it was hypothesized that subtelomeric plasticity may allow rapid adaptive changes of the yeast strain to specific substrates (Carro *et al.*, 2003). The *SSU1-R* allele, generated by reciprocal translocation between chromosomes VIII and XVI, confers sulfite resistance to yeast cells and was described as the first case of adaptive evolution, occurring probably because sulfite was used as a preservative in wine production (Goto-Yamamoto *et al.*, 1998; Pérez-Ortin *et al.*, 2002). Retrotransposons may also be involved in chromosomal recombinations. *S. cerevisiae* strains contain between two and 30 copies of at least five retrotransposons (Ty1-Ty5), being the copy number of each highly variable, depending on the strain examined. Multiple Ty elements mediated reciprocal recombinations (chromosome I/III or III/VII) were shown by fine-mapping of the junctions, demonstrating their crucial involvement in karyotype alterations in natural and industrial strains (Rachidi *et al.*, 1999; Umezu *et al.*, 2002; Carro *et al.*, 2003), together with insertions/ transpositions of Y′elements (Neuvéglise *et al.*, 2000).

Among *Saccharomyces* yeasts used in wine, beer and cider production, genetically stable interspecies hybrids, that possesses nuclear DNA from two or three species are quite common. The strain CID1, which was isolated from a home fabricated apple cider (Masneuf *et al.*, 1998) is a triple hybrid between *S. cerevisiae*, *S. kudriavzevii*, and *S. bayanus* var. *uvarum*, as was shown by analysis of the partial sequence of the *ACT1* gene, flow cytometry analysis (Naumova *et al.*, 2005a) and by amplified fragment length polymorphism analysis (de Barros Lopes *et al.*, 2002). *S. cerevisiae* x *S. bayanus* var. *uvarum* hybrids were also identified among baker's yeast and hybrids were also obtained from the surface of black-currant berries (Naumova *et al.*, 2005b). *S. cerevisiae* x *S. bayanus* var. *uvarum* diploid hybrids were isolated from spontaneous fermentations and microsatellite DNA analysis identified strains isolated in the same cellar as potential parents belonging to *S. bayanus* var. *uvarum* and *S. cerevisiae*. Such genetic mixes may be useful from a technological standpoint because they lead to the emergence of more vigorous, competitive strains, combining the specific properties of the parental strains (le Jeune *et al.*, 2007). *S. cerevisiae* x *S. kudriavzevii* hybrids can also be involved in wine fermentation, as was shown by sequence analysis of the mitochondrial gene *COX2* and restriction analysis of nuclear and ribosomal genes (5.8S rRNA) (Gonzalez *et al.*, 2006).

The advent of DNA microarray technology has enabled the analysis of global patterns of gene expression and diverse networks of coordinated function. However, the genetic differences examined have been primarily differences between growth conditions or between mutant strains and this knowledge has accumulated on a narrow range of laboratory yeast genetic backgrounds, selected due to their suitability to laboratory conditions. In the last few years, genetic variation among laboratory, but also natural isolates, became unravelled on a genomic scale. The studies, summarized as follows, revealed considerable genetic divergence among *S. cerevisiae* strains.

Natural vineyard populations of *S. cerevisiae* harbor alleles that cause massive alterations in gene expression as was shown by combining classical Mendelian segregation analysis with microarray-based genomics. The four progeny of a natural isolate (M28) from Tuscany segregated 2:2 for filagree and smooth colony phenotypes. In cultures derived from middle-logarithmic phase in YPD medium (yeast extract 1% w/v, peptone 2% w/v, and glucose 2% w/v), almost 400 genes, mostly associated with amino acid biosynthesis and transport, sulphur or nitrogen assimilation were differentially expressed between the two phenotypes. The filagree progeny poorly express genes for amino acid transport and instead abundantly express genes for the synthesis of amino acids. Differentially expressed genes segregated as a suite of traits, due to variation in a few regulatory loci that either act on hundreds of loci or initiate cascades of transcriptional control. These studies showed that natural vineyard populations of *S. cerevisiae* can harbor alleles that cause massive alterations in the global patterns of gene expression (Cavalieri *et al.*, 2000). Under the same experimental conditions, another study examined gene-expression variation of the M28 strain to three other isolates from the same set of vineyards around Montalcino, Italy. Among the four isolates, 433 genes were expressed at significantly different levels between at least two isolates, and most variation was found in genes associated with amino acid metabolism, protein synthesis and degradation, metal ion transport and transposable element activity (Townsend *et al.*, 2003). The commercial wine yeast strain T73 and the laboratory strain S288C showed significant differential expression patterns in 40 genes during logarithmic growth in YPD medium. These genes were mainly associated with small changes in promoter regions or variations in gene copy number (Hauser *et al.*, 2001).

DNA-array-based hybridisation is an emerging and powerful method for scanning genomes that allows for genome-wide genotyping. By commercial high-density oligonucleotide arrays that contain up to 200 000 different 25 mers features from the yeast genomic sequence genome-wide diversity between strains can be determined with a level of detail previously impossible. Single-base pair changes between two 25 bp sequences, especially in the central zone, can disrupt hybridization. They are used to determine the genetic variation (locations of allelic differences) existing between two strains and whether functional classes of genes or particular genome regions show higher rates of variability. One of the first large-scale studies to discover variable genes within *S. cerevisiae* populations was published by Winzeler *et al.* (2003). Using 14 yeast strains, common laboratory strains and natural isolates, it was shown that intra-species genome variability is biased toward subtelomeric regions at the ends of chromosomes, where genes related to fermentation and transport are located (Winzeler *et al.*, 2003). This approach will be fundamental for future genome evolution and population genetic studies in yeast, but has also great potential for the rapid identification of loci that are responsible for imparting positive attributes.

Unexpectedly wide differences exist even when comparing laboratory strains. The popular laboratory strains S288C and CEN.PK113-7D showed significant physiological differences in protein expression and lipid metabolism. Comparison by high-density oligonucleotide arrays revealed divergent hybridization patterns in 288 genes, due to differential amplification, gene absence or sequence polymorphisms. Seventeen genes were absent in CEN.PK113-7D and eight genes did not show hybridization signals due to significant differences at the DNA level compared to S288C (Daran-Lapujade *et al.*, 2003).

A global view of genetic variation among commercial wine strains both at intra- and inter- strain level has been obtained by microarray karyotyping, also known as "arraycomparative genomic hybridization" ("aCGH"), giving information on whole or partial chromosome aneuploidies, non-reciprocal translocations and isolated gene deletions or amplifications by the examination of copy number changes for every gene. The analysis of three independent isolates of each of four commonly used commercial *S. cerevisiae* wine strains relative to each other and to the sequenced *S. cerevisiae* strain S288C, showed that a major group of shared genomic differences, found among all wine strains, is associated with genes coding for membrane transporters or genes involved in drug resistance pathways (Dunn *et al.*, 2005). The low level of inter-strain variability suggests that it can be relatively easy to discover whether the observed differences do indeed confer different sensory properties in the finished wine, but differences in the fermentation and organoleptic properties may also arise from single nucleotide changes, of which there may be many, and that are not detected by microarray karyotyping.

Infante *et al.* (2003) performed a detailed microarray karyotyping study of the genomic differences between two *S. cerevisiae* "flor" yeasts, obtained from the velum, a unique biofilm, which develops on the surface of the wine during the sherry wine making. The strains differed from one another in genomic regions that are flanked by repeated sequences or other recombination hotspots and that could mediate the observed chromosomal rearrangements by nonallelic interactions. However, the authors only compared the two "flor" yeasts to each other, and not to the sequenced S288C laboratory strain (Infante *et al.*, 2003).

Selection of *Saccharomyces cerevisiae* strains with desirable characteristics

S. cerevisiae populations harbor genetic variation that is associated with geographical ecological factors and that is the basis of the well-described phenotypic variability that has been explored for decades in strain selection programs. It is consensual among winemakers that the choice of wine yeast strain has a major impact on the sensory characteristics of both still and sparkling wines. Selection for millennia of wine-making may have created unique and interesting oenological traits, but they are not widely distributed, nor can be found in combination in one strain. Clonal selection of wild *Saccharomyces* strains isolated from natural environments belonging to the viticultural areas of

interest is always the starting point for a wine yeast selection program. It is desirable to evaluate the phenotypic diversity for as much as possible traits, such as glycerol production (Remize *et al.*, 2000b), hydrogen sulphite formation (Mendes-Ferreira *et al.*, 2002) or the modulation of grape-derived volatile thiols such as 4-mercapto-4-methylpentan-2-one (4MMP) during wine fermentation (Howell *et al.*, 2004). Currently, about 150 different wine yeast strains, mainly *S. cerevisiae*, are commercially available as active dry yeast, and are widely used due to their superior oenological properties, contributing to both standardization of fermentative processes and wine quality. Contrarily, spontaneous fermentations are usually used by small boutique wineries that wish to emphasize vintage variability, reflecting the specificity of a particular region, and that rely merely on indigenous yeasts present on the grape skin, which are thought to produce wines with a fuller palate structure. The first commercialized wine yeast strains were simply expected to ensure complete fermentation with rapid kinetics, but the criteria have evolved over the years, since the particular strain used should be most suitable for each type of wine to be produced. The current trend toward the production of high quality wines with distinctive and very characteristic properties requires the use of "special yeasts for special traits" (Pretorius, 2000; Mannazzu *et al.*, 2002; Romano *et al.*, 2003b).

Definition of the appropriate selection strategy should always depend on the traits that a wine strain is supposed to harbor and the number of strains to be screened. As summarized in Table 1.1, numerous oenological characteristics were proposed to be evaluated. Technologically relevant data can be obtained by monitoring the fermentation progress, and quantitative traits are determined by chemical analysis at the end of fermentation.

Table 1.1. Oenological characteristics considered in the selection of *S. cerevisiae* wine strains (Regodon *et al.*, 1997; Romano *et al.*, 1998; Guerra *et al.*, 1999; Maifreni *et al.*, 1999; Perez-Coello *et al.*, 1999; Esteve-Zarzoso *et al.*, 2000; Rainieri and Pretorius 2000; Steger and Lambrechts, 2000; Martinez-Rodriguez *et al.*, 2001; Brandolini *et al.*, 2002; Caridi *et al.*, 2002; Mannazzu *et al.*, 2002; Mendes-Ferreira *et al.*, 2002).

Oenological characteristics	Comments
Fermentation vigor	Maximum amount of ethanol (%, v/v) produced at the end of the fermentation; Desirable: good ethanol production
Fermentation rate	Grams of CO_2 produced during the first 48 hours of fermentation Desirable: prompt fermentation initiation
Mode of growth in liquid medium	Dispersed or flocculent growth, sedimentation speed Desirable: dispersed yeast growth during, but sedimentation at the end of fermentation
Foam production	Height of foam produced during fermentation Undesirable: increased foam production

Optimum fermentation temperature	Thermotolerance and cryotolerance is related to oenological properties Optimum fermentation temperature ranges between 18 and 28°C
Volatile acidity, acetic acid production	Selected strains should not release more than 100 – 400 mg l^{-1} during fermentation Undesirable: increased volatile acidity/acetic acid production
Malic acid degradation or production	Whether degradation of production is desirable depends on the characteristics of the must. Malic acid degradation varies between 0-20% depending on the *S. cerevisiae* strain
Glycerol production	Desirable major fermentation by-product (5-8 g l^{-1}) contributing to wine sweetness, body and fullness
Acetaldehyde production	Desirable metabolite in sherry, dessert and port wines being an important character for selection of strains to be applied in wine ageing
Esters, higher alcohols and volatile compounds	Desirable metabolites, markedly influence wine flavor and depend on the presence of precursors related to both grape cultivar and grape maturity. Limited amounts contribute positively to global sensorial characteristics
SO_2 tolerance and production	Antioxidant and antimicrobial agent Desirable: high fermentation vigor and rate in the presence of SO_2 concentrations usually applied in winemaking; Undesirable: excessive SO_2 production
H_2S production	Determined as the strains colony color on a bismuth containing indicator medium, e.g. BIGGY Agar; H_2S is detrimental to wine quality, considered as off-flavor with very low threshold value (50-80 µg/l)
Stress resistance	Tolerance to combined acid/osmotic stress
Copper resistance	High copper concentrations may cause stuck fermentations Desirable: high copper resistance and the ability to reduce the copper content

As mentioned in the previous section, recent research has provided interesting findings of naturally occurring *Saccharomyces* hybrid strains, for example triple hybrids S. *cerevisiae*, x *S. bayanus* x *S. kudriavzevii* (Gonzalez *et al.*, 2006). *S. cerevisiae* × *S. kudriavzevii* hybrids were found to have a promising enological potential, since they were better adapted to alcoholic fermentations carried out at lower temperatures (14-22°C), produced less acetic acid and intermediate amounts of glycerol in combination with increased amounts of higher alcohols (Gonzalez *et al.*, 2007). Strains with improved technological properties were also obtained from hybrids between cryotolerant *S. bayanus* and thermotolerant *S. cerevisiae* strains (Rainieri *et al.*, 1998; Masneuf *et al.*, 2002; Coloretti *et al.*, 2006).

Finding wine yeast strains possessing an ideal combination of oenological characteristics is highly improbable and therefore selection programs were extended to non-*Saccharomyces* species, e.g. *Candida*, *Kloeckera*, *Debaryo-*

myces, *Hanseniaspora*, *Hansenula*, *Pichia*, *Metschnikowia*, *Schizosaccharomyces*, *Saccharomycodes* or *Rhodotorula*. Although non-*Saccharomyces* species lack competitiveness in oenological conditions mainly because they are not vigorously fermenting and display a lower stress resistance when compared to *S. cerevisiae*, the use of mixed starter cultures or sequential fermentation (e.g. *C. cantarellii*/ *S. cerevisiae*) for directing fermentations toward enhanced glycerol and reduced acetic acid production has been successfully used (Toro and Vazquez, 2002). The yeasts *Torulaspora delbrueckii* and *Candida stellata* are considered to be positive contributors to the overall organoleptic wine characteristics, while apiculate yeasts such as *Kloeckera apiculata* have a negative influence on wine quality due to pronounced acetic acid and ethyl acetate formation associated with low ethanol production (Ciani and Maccarelli, 1998).

Countless references report the beneficial and detrimental influence of non-*Saccharomyces* yeasts on the volatile composition of musts from varying grape varieties (Ciani and Maccarelli, 1998; Granchi *et al.*, 2002; Mingorance-Cazorla *et al.*, 2003; Plata *et al.*, 2003; Romano *et al.*, 2003c; Clemente -Jimenez *et al.*, 2004), and considerable differences regarding these compounds were also found among commercial or autochthonous *S. cerevisiae* strains (Steger and Lambrechts, 2000; Patel and Shibamoto, 2003; Romano *et al.*, 2003a). Non-*Saccharomyces* yeasts, especially selected and commercialized for aroma and flavor enhancement in wine, for example as a blend of *S. cerevisiae* / *Kluyveromyces thermotolerans* / *Torulaspora delbrueckii* or *S. cerevisiae* / *Kluyveromyces thermotolerans*. Immobilized *Schizosaccharomyces pombe* cells are also commercially available for the biological reduction of wine acidity by malic acid consumption (Silva *et al.*, 2003).

Non-*Saccharomyces* strains metabolize grape-derived precursor compounds, contributing thus to reveal the varietal aroma and improve the winemaking process (Fleet and Heard, 1993; Esteve-Zarzoso *et al.*, 1998; Fernandez *et al.*, 2000; Otero *et al.*, 2003). Pectinases increase juice extraction, improve clarification and facilitate wine filtration, ß-glycosidases hydrolyse non-volatile glycosidic aromatic precursors from the grape, proteases improve the clarification process, esterases contribute to aroma compound formation and lipases degrade lipids from grape or yeast autolytic reactions. *S. cerevisiae* is not a significant producer of such enzymes with relevance in wine production, being mainly ß-glycosidase production reported for this species (Restuccia *et al.*, 2002; Rodriguez *et al.*, 2004).

Albeit the high amount of phenotypic variation that can be found among *S. cerevisiae* strains, and the inclusion of non-*Saccharomyces* and hybrid starter strains with a whole range of specialized properties that can add value to the final product, there is no doubt that significant progress of technological, fermentative and aromatic characteristics can only be achieved via targeted breeding and genetic engineering programs.

S. cerevisiae strain modification based on classical methods

Wine yeast strains exhibit a wide variability in their biotechnological properties, and the genetic diversity of native isolates has provided ample material from which to select wine yeasts expressing specific traits. In fact, the vast majority of *S. cerevisiae* strains currently on the market derived from isolation and screening of strains obtained from wineries or from vineyards. However, the natural availability of strains possessing an ideal combination of oenological characteristics is highly improbable because the most important enological traits, such as ethanol tolerance, low volatile acidity production or hydrogen sulphide production, are polygenic features, with complex interactions between alleles. A population of 50 progeny clones derived from four industrial wine strains of *S. cerevisiae* demonstrated that many clones presented better aptitudes than the parental strains in regard to ethanol tolerance, volatile acidity and hydrogen sulphide production, and that traits are in part inheritable and clearly polygenic (Marullo *et al.*, 2004).

Classical methods for strain modifications include mutagenesis or hybridization, where large genomic regions or entire genomes are recombined or rearranged. Elimination of undesirable characteristics and enhancement of favorable properties has been addressed through mutagenesis, using UV radiation or chemical agents such as ethylmethane sulfonate due to the low average spontaneous mutation frequency in yeast populations. A drawback of such methods is the effect of ploidy, which reduces efficiency in diploid or polyploid strains, and the presence of non-mutated alleles that cannot be easily detected. Therefore, haploid strains are preferred when inducing mutations, and mutagenesis is usually applied to isolate new variants of wine yeast strains before further genetic manipulation. A mutant wine strain was obtained by UV-mutagenesis, carrying a recessive allele of *ure2* that deregulated the proline utilization pathway, characterized by abolished nitrogen catabolite repression through ammonium ions. The strain showed an improved fermentation performance in media where proline and other poorly assimilated amino acids are the major potential nitrogen source, as is the case for most fruit juices and grape musts (Salmon and Barre, 1998). Mutants with an accelerated autolysis during second fermentation of sparkling wines were also obtained. This process is associated with the release of intracellular compounds that modify the chemical composition and sensory properties and usually lasts from a few months to years (Gonzalez *et al.*, 2003; Nunez *et al.*, 2005). UV mutagenesis was also used to obtain a thermosensitive autolytic mutant affected in cell wall integrity, with improved capability to release cell wall mannoproteins during alcoholic fermentation (Giovani and Rosi, 2007). Such polysaccharides confer greater body and smoothness to white and red wines (Vidal *et al.*, 2004).

Modulating a specific property can be easily achieved by intra-specific hybridization, based on the sexual cycle of *S. cerevisiae*, where a new heterozygous diploid cell is produced by sporulating parental diploids, recovering individual haploid ascospores and mating haploid progeny of opposite mating

types. Several hybrid strains are currently on the market, and this approach is still considered the most effective method for improving and combining traits under polygenic control, particularly if the molecular nature of the mechanisms involved has not been elucidated. Intra-specific hybridization was used, for example, to eliminate undesirable properties like SO_2 formation or excess foaming (Eschenbruch *et al.*, 1982). A flocculent *S. cerevisiae* strain to be used in the production of sparkling wines and not producing H_2S was obtained by hybridizing a flocculent strain with a H_2S non-producing strain (Romano *et al.*, 1985). Classical sexual reproduction has proven difficult for the case of homothallic strains, in which mating type reversals and cell fusion/diploid formation occurs in a spontaneous way. In this situation, particular forms of hybridization can be applied, such as spore-cell mating in which homothallic ascospores from the same ascus are placed into direct contact with heterothallic haploid cells. Spore-cell mating was used for the optimization of 11 relevant enological traits, by crossing two strains derived from commercial wine strains, a homozygous heterothallic strain carrying the *ho::KanMX*4 allele with the ascospores of a homothallic strain. In an additional targeted sexual cross, from the segregating progeny, all the optimal characters from both parents were combined in a single strain, showing the usefulness of this method for obtaining a wine strain with numerous fermentative qualities (Marullo *et al.*, 2006).

There are other forms of hybridization such as protoplast fusion, which is a direct, asexual technique that can be used to fuse non-sporulating yeast strains, surpassing the natural barriers of hybridization. Desirable (and undesirable) characteristics of both parental strains will recombine in the offspring. This approach can also be used to fuse cells with different levels of ploidy. Triploid strains can be obtained by fusion of a diploid to a haploid strain.

One of the limitations when hybridizing strains belonging to the same species is that traits to be exchanged or introduced in the hybrid culture and in its progeny are limited to the species-specific characteristics. Recent genetic analysis showed that there are no barriers to interspecific conjugation among *Saccharomyces sensu stricto* yeasts (Masneuf *et al.*, 1998;de Barros Lopes *et al.*, 2002;Naumova *et al.*, 2005a; Naumova *et al.*, 2005b;Gonzalez *et al.*, 2006), and that introgression may lead to the emergence of more vigorous, competitive strains, combining the specific properties of the parental strains (Coloretti *et al.*, 2006; le Jeune *et al.*, 2007). *S. bayanus*, for example, is a cryotolerant species and has a better fermentative profile at low temperatures compared to *S. cerevisiae* (Kishimoto and Goto, 1995). Wines fermented by *S. bayanus* are characterized by smaller amounts of acetic acid and ethanol, higher amounts of glycerol, succinic and malic acid (Kishimoto *et al.*, 1993; Zambonelli *et al.*, 1997). Besides, this species produces wines with higher amounts of flavor-active esters, such as β-phenylethyl alcohol and β-phenylethyl acetate (Masneuf *et al.*, 1998). Hybrids of *S. cerevisiae* x *S. bayanus* obtained in the laboratory exhibited such characteristics at midway the parental strains. This can present an advantage in wine making, especially for white wines, which are fermented at a low

temperature and for which intermediate amounts of β-phenylethyl alcohol and its acetate are desirable.

The previously mentioned methods of strain modification by mutagenesis or hybridization are used to improve and combine traits under polygenic control, but the introduced genetic changes remain hidden. Since they rely on classical breeding methods by which large genomic regions or entire genomes are recombined or rearranged, the resulting strains are not considered as GMOs according current legislative definitions.

Targets for genetic modifications by recombinant DNA technologies

Genetic improvement of industrial strains by classical genetics was followed in the last 20 years by the use of recombinant DNA technologies that made the construction of specialized commercial strains possible, mainly by heterologous gene expression or by manipulation of a specific metabolic pathway associated with altered gene dosage by modification of the gene promoter. Recombinant strain construction is easy and feasible, as far as the desired trait is encoded by one or few well-characterized genes.

The most important targets for wine strain improvement are related to higher ethanol tolerance, enhanced wholesomeness and organoleptical properties through altered sensorial characteristics, and improvements for processing efficiency (Blondin and Dequin, 1998; Pretorius, 2000; Dequin, 2001; Pretorius and Bauer, 2002; Dequin *et al.*, 2003; Pretorius *et al.*, 2003; Marullo *et al.*, 2004; Marullo *et al.*, 2006; Verstrepen *et al.*, 2006). Table 1.2 shows examples of the way in which *S. cerevisiae* wine yeast strains are currently being developed.

The worldwide growing demand for wines containing lower levels of alcohol has been addressed by engineering wine yeast strains that produce lower amounts of ethanol during alcoholic fermentation. This issue has been addressed by integration of the *Aspergillus niger* glucose oxidase *GOX1* gene into the *S. cerevisiae* genome. Wines produced with this yeast had 1.8–2.0% less alcohol, which was ascribed to production of d-glucono-δ-lactone and gluconic acid from glucose by *GOX* (Malherbe *et al.*, 2003). Efficient decrease (15–20%) of ethanol yield was succeeded through metabolic re-routing of glucose toward glycerol through overexpression of *GPD1*, encoding glycerol-3-phosphate dehydrogenase, combined with *ALD6* deletion, encoding acetaldehyde dehydrogenase to abolish excessive acetate production as a major side effect. However, this strain accumulated acetoin, which has a negative sensorial impact on wine (Cambon *et al.*, 2006). An alternative strategy was based on constitutive expression of an H_2O-NADH oxidase from *Lactoccocus lactis* in *S. cerevisiae*. However, the marked decrease in the intracellular NADH pool lead to reduced growth and fermentative performance (Heux *et al.*, 2006a), that could be improved when the anaerobic growth phase was followed by a microaeration phase with nongrowing cells. This strain reduced ethanol yield by 7%, but still showed a specific metabolite redistribution pattern, characterized by the presence of undesirable

oxidized metabolites such as acetaldehyde, acetate and acetoin (Heux *et al.*, 2006b).

The aromatic profile of a wine comprises hundreds of compounds that are interacting in a highly complex manner. Well-balanced wines must evidence characteristic flavor and aroma notes, whereas undesirable flavor compounds and metabolites should be absent. Metabolic yeast metabolites such as esters or alcohols contribute to the complexity and intensity of the final wine. Numerous approaches have been undertaken to develop *S. cerevisiae* starter strains that could impart specific desirable aromas and flavors by constitutive expression or overexpression of enzymes such as endoglucanase, arabinofuranosidase, endoxylanase or rhamnosidase for the cleavage of aroma components from their glycosylated precursors, producing wines with an increased fruity aroma (Pérez-González *et al.*, 1993; Sanchez-Torres *et al.*, 1996; Ganga *et al.*, 1999; Manzanares *et al.*, 2003). Starter strains have been constructed with optimized decarboxylation activity of phenolic acids, resulting in volatile phenols such as 4-vinyl and 4-ethyl derivatives that positively influence wine aroma (Smit *et al.*, 2003). Cysteinylated thiols are grape-derived non-volatile precursors of volatile thiols, that enhance the varietal characters and impart flavors of passionfruit, grapefruit, gooseberry, blackcurrant, lychee, guava and box hedge. A *S. cerevisiae* strain expressing tryptophanase with strong cysteine-beta-lyase activity released up to 25 times more volatile thiols and the produced wines displayed an intense passionfruit aroma (Swiegers *et al.*, 2007). Some of the most important yeast-derived aroma compounds produced are esters such as ethyl acetate and isoamyl acetate. Volatile esters represent the largest and most important group of flavor compounds produced during fermentation, and C_4–C_{10} fatty acid ethyl esters confer characteristic fruity odors such as apple-like (hexyl acetate, ethyl caproate and ethyl caprylate) or banana-like (isoamyl acetate) (Swiegers *et al.*, 2005). Recent approaches have been undertaken investigating the interactive roles of ester-synthesizing and ester-hydrolyzing enzymes in wine yeast to develop strains with differential ester-producing capabilities that could assist winemakers in their effort to consistently produce wines according to definable flavor specifications and styles. Overexpression of the *ATF1* gene encoding alcohol acetyltransferases significantly increased the concentrations of ethyl acetate, isoamyl acetate, 2-phenylethyl acetate and ethyl caproate, that were efficiently degraded by the the *IAH1*-encoded esterase. *EHT1*-encoded ethanol hexanoyl transferase overexpression resulted in a marked increase in ethyl caproate, ethyl caprylate and ethyl caprate (Lilly *et al.*, 2006a). Manipulation of the intracellular pool of acetyl-CoA was shown to play a role in the development of ester aromas such as ethyl acetate and isoamyl acetate (Cordente *et al.*, 2007). Branched chain amino acids are the precursors for the biosynthesis of higher alcohols, also known as fusel alcohols. They are quantitatively the largest group of aroma compounds in wines. Lilly *et al.* (2006b) showed that constitutive expression of the branched-chain amino acid transaminase *BAT1* and *BAT2* facilitates the production of optimized concentrations of higher alcohols during wine fermentations.

Glycerol has no aromatic characteristics but rather contributes to the sensory character of wine by its sweet taste, and is quantitatively the most important fermentation product after ethanol and carbon dioxide. A 1.5- to 2.5-fold increase in glycerol production and a slight decrease in ethanol formation under conditions simulating wine fermentation was achieved by overexpression of the *GPD1* gene, encoding a glycerol-3-phosphate dehydrogenase. However, the resultant change in redox balance caused excessive formation of secondary metabolites such as succinate, acetate, acetoin and 2,3-butanediol (Michnick *et al.*, 1997; Remize *et al.*, 1999).

L-tartaric and L-malic acid are the predominant organic acids in wine and represent 70–90% of total grape acidity. Flavor problems associated to insufficient or excessive acidity may occur in wines produced in climatic hot or colder regions, respectively. Efficient biological acidity correction is of biotechnological interest to produce a high-quality wine with a fine balance between the sugar and the acid content. *S. cerevisiae* strains degrade malic acid in must only partially (10-25%) during alcoholic fermentation by the mitochondrial malic enzyme (Riberéau-Gayon *et al.*, 2000), and the capacity to use malic acid varies among *S. cerevisiae* strains (Subden *et al.*, 1998). The commercial strain Lalvin71B is promoted as malate degrading strain, but may have a variable performance, depending on the must type (our unpublished results). Genetically modified *S. cerevisiae* strains have been constructed by coexpression of the malate permease from *Schizosaccharomyces pombe* and the *mleS* malolactic gene from *Lactococcus lactis* (Bony *et al.*, 1997; Volschenk *et al.*, 1997a; Volschenk *et al.*, 2001) or the *mleA* malolactic gene from *Oenococcus oeni* (Husnik *et al.*, 2006).

Acetate is the main component of volatile acidity and plays a significant role in the organoleptic balance of wine. In wine, acetic acid is a by-product of yeast alcoholic fermentation, and is highly undesirable above the threshold of 0.8 g/l. A substantial decrease (40-75%) in acetate yield was achieved by *ALD6* gene disruption, encoding acetaldehyde dehydrogenase (Remize *et al.*, 2000a). Engineered *S. cerevisiae* strains over-expressing a bacterial lacticodehydrogenase (*LDH*) have been described to perform mixed lactic acid-alcoholic fermentation under enological conditions and increased total acidity by 50% through production of 5 g/L of L(+) lactic acid (Dequin *et al.*, 1999).

Yeast can also be responsible for the production of unwanted byproducts, such as hydrogen sulphide, that is synthesized during alcoholic fermentation. A promising strategy was designed for reducing hydrogen sulfide production, based on site-directed mutagenesis to lower the activity of NADPH-dependent sulfite reductase, a key enzyme in the biosynthesis of sulfur-containing amino acids (Sutherland *et al.*, 2003).

Novel wine yeast strains have been developed that could contribute to improved health-protective effects by increased resveratol formation, a health-promoting stilbene that is mainly formed in the grape skin. This compound is possibly associated with the "French paradox", i.e. a lower heart disease

incidence among the French population, where a high-fat diet is combined with the regular consumption of wine. Resveratrol synthesis was engineered by co-expression of the grapevine stilbene ligase gene (*VST1*) and co-enzyme A ligase encoding gene (*4CL216*) from hybrid poplar. Resveratrol production occurred from the synthesized p-coumaroyl-CoA and the yeast-derived 3-malonyl-CoA by stilbene ligase (Becker, *et al.*, 2003). Resveratrol content of white wine has also been increased by expression of *Aspergillus niger abfB* gene encoding an alpha-L-arabinofuranosidase or *Candida molischiana bglN* gene encoding a beta-glucosidase to increase free resveratrol from its glycosylated precursors (Gonzalez-Candelas *et al.*, 2000).

Focusing on health aspects, yeasts were developed that could minimize the risks associated with moderate wine consumption by elimination of ethyl carbamate, a suspected carcinogen that is sometimes formed in wine through spontaneous reaction of ethanol with urea, which is secreted by yeast cells. Under fermentative conditions in the presence of nitrogen sources, urea catabolism to ammonia by urea amidolyase, the product of the *DUR1,2* gene, is transcriptionally repressed. When *DUR1,2* was constitutively expressed, ethyl carbamate could be reduced by 89.1% (Coulon *et al.*, 2006).

The physicochemical characteristics and sensory properties of wine can be altered by undesired bacterial growth before, during or after fermentation. In general, growth control of unwanted microbial contaminants is provided by the addition of chemical preservatives such as sulphur dioxide or other antibacterial compounds and enzymes. The expression of antimicrobial enzymes and peptides in starter strains has been achieved by distinct approaches. Bactericidal yeasts, engineered by expressing genes encoding *Pediococcus acidilactici* pediocin (*PED1*) (Schoeman *et al.*, 1999) and *Leuconostoc carnosum* leucocin (*LCA1*) (du Toit and Pretorius, 2000) have been used to obtain bactericidal yeasts. The antifungal *CTS1*-encoded chitinase has also been expressed in *S. cerevisiae* (Carstens *et al.*, 2003), as well as the *GOX1*-encoded exoglucanase to inhibit wine spoilage organisms, such as acetic acid bacteria and lactic acid bacteria during fermentation (Malherbe *et al.*, 2003).

Clarification and physicochemical stability of wines is usually achieved by an increasing spectrum of relatively expensive commercial polysaccharase enzyme preparations, due to the inability of indigenous *S. cerevisiae* strains to degrade grape-derived polysaccharides such as glucan and xylan. Recombinant strains were obtained, by integrating the *Trichoderma reesei XYN2* xylanase gene construct and the *Butyrivibrio fibrisolvens END1* glucanase gene cassette into the genome of a commercial wine yeast strain. Wines obtained with the polysaccharide-degrading strains resulted in significant improvements in juice extraction, colour intensity and stability, as well as alterations in the aromatic profiles (Louw *et al.*, 2006). Pectinolytic *S. cerevisiae* strains have been constructed by expressing enzymes of fungal origin (Gonzalez-Candelas *et al.*, 1995) or polygalacturonase encoded by *PGU1* as an alternative to commercial enzyme preparations (Vilanova *et al.*, 2000). Proficient clarification at the end of

fermentation can also be achieved by regulated expression of the flocculation genes to guarantee efficient settling at the end of fermentation (Verstrepen *et al.*, 2001; Verstrepen *et al.*, 2006).

Strategies for genetic modifications

In general, all genetic material used for the construction of microorganisms used for food fermentation should be derived from the host species (self-cloning) or GRAS (generally regarded as safe) organisms with a history of safe food use. The use of DNA sequences from species taxonomically closely related to pathogenic species has to be avoided. Heterologous gene expression was used in most cases, being the genes of interest isolated for example from *Lactobacillus casei* (*LDH*), *Lactobacillus plantarum* (*pdc*), *Lactobacillus lactis* (*noxE*, *mleS*), *Leuconostoc carnosum* (*LCA1*), *Oenococcus oeni* (*mleA*), *Bacillus subtilis* (*padc*), *Pediococcus acidilactici* (*pedA*), *Fusarium solani* (*pelA*), *Trichoderma reesei* (*XYN2*), *Butyrivibrio fibrisolvens (END1), Erwinia chrysanthemi (PEL5), Erwinia carotovora (PEH1), Candida molischiana* (*bglN*), *Schizosaccharomyces pombe* (*mae1* and *mae2*), hybrid poplar (*4CL216*), grapevine (*vst1*), *Aspergillus* sp. (*GOX egl1*, *abfB*, *xlnA*, *rhaA*), *E.coli (tnaA)* or *Fusarium solani* (*pelA*), being others, such as *ATF1*, *GPD1* or *PGU1* derived from *S. cerevisiae* (Table 1.2).

Table 1.2. Targets for *S. cerevisiae* strain improvement, indicating, whenever possible, examples of the strategies used for genetic modifications

Modification	Protein(s)	Gene(s)	Source	Construction					Reference
				P	T	Pla	M	Chr	
Reduce ethanol content	Glucose oxidase	*gox*	*A. niger*	*PGH1*	*PGK1*		*URA3*	+	(Malherbe *et al.*, 2003)
	Glycerol-3-phosphate dehydrogenase	*GPD1*	*S. cevevisiae*	*ADH1*	*ADH1*	2μ	Tn5 *ble*	-	(Cambon *et al.*, 2006)
	Acetaldehyde dehydrogenase	*ALD6* deletion	*S. cevevisiae*				*kanMX4*		
	NADH oxidase	*noxE*	*L. lactis*	*TDH3*			*URA3*	+	(Heux *et al.*, 2006a; Heux *et al.*, 2006b)
Cleavage of aroma components from their glycosylated precursors	Endoglucanase	*egl1*	*T. longibrachiatum*	*ACT*	-	2μ	*CYH2*	-	(Pérez-González *et al.*, 1993)
	Arabinofuranosidase	*abfB*	*A. niger*	*ACT*	-	2μ	*CYH2*	-	(Sanchez-Torres *et al.*, 1996)
	Endoxylanase	*xlnA*	*A. nidulans*	*ACT*	-	2μ	*CYH2*	-	(Ganga *et al.*, 1999)
	Rhamnosidase	*rhaA*	*A. aculeatus*	*GPD*	*PGK*		*TRP*	-	(Manzanares *et al.*, 2003)
Increase volatile phenol aromas	Phenolic acid decarboxylase	*pdc* *padc*	*L. plantarum* *B. subtilis*	*PGK1*	*PGK1*		*URA3*	+	(Smit *et al.*, 2003)

Increase aroma-enhancing thiols from cysteinylated precursors	Tryptophanase with cysteinelyase activity	*tnaA*	*E. coli*	*PGK1*	*PGK1*		*SMR1-410*	+	(Swiegers *et al.*, 2007)
Modulate acetate ester aromas	Carnitine acetyltransferase	*CAT2*	*S. cerevisiae*	*PGK1*	*PGK1*	2μ	*URA3*	-	(Cordente *et al.*, 2007)
	Alcohol acetyltransferase	*ATF1, ATF2*	*S. cerevisiae*	*PGK1*	*PGK1*	2μ	*SMR1-410*	-	(Lilly *et al.*, 2000; Lilly *et al.*, 2006a)
	Ethanol hexanoyl transferase	*ETH1*	*S. cerevisiae*	*PGK1*	*PGK1*	2μ	*SMR1-410*	-	(Lilly *et al.*, 2006a)
	Esterase	*IAH1*	*S. cerevisiae*	*PGK1*	*PGK1*	2μ	*SMR1-410*	-	(Lilly *et al.*, 2006a)
Increase aromas associated with higher alcohols	Amino acid transaminase	*BAT1, BAT2*	*S. cerevisiae*	*PGK1*	*PGK1*	2μ	*SMR1-410*	-	(Lilly *et al.*, 2006b)
Increase glycerol formation	Glycerol-3-phosphate dehydrogenase	*GPD1*	*S. cerevisiae*	*ADH1*	*ADH1*	2μ	Tn5 *ble*	-	(Michnick *et al.*, 1997; Remize *et al.*, 1999)
Reduce malic acid concentration	Malolactic enzyme	*mleS*	*L. lactis*	*PGK1*	*PGK1*	2μ	*URA3*	-	(Volschenk *et al.*, 1997b)
	Malate permease	*mae1*	*S. pombe*				*SMR1-410*	+	(Volschenk *et al.*, 2001)
	Malic enzyme	*mae2*							
	Malate permease	*mae1*	*S. pombe*	*PGK1*	*PGK1*		*URA3*	+	(Husnik *et al.*, 2006)
	Malolactic enzyme	*mleA*	*O. oeni*	*PGK1*	*PGK1*		*URA3*	+	
Reduce acetic acid concentration	Acetaldehyde dehydrogenase	*ALD6* deletion	*S. cerevisiae*				*kan MX4*		(Remize *et al.*, 2000a)
Increase wine acidity by lactic acid production	Lactate dehydrogenase	*LDH*	*L. casei*	*ADH1*	*ADH1*	2μ	*G418*	-	(Dequin *et al.*, 1999)
Decrease hydrogen sulphide synthesis	Sulphite reductase	*MET10*	*S. cerevisiae*	Site – directed mutagenesis (lowering enzymatic activity)					(Sutherland *et al.*, 2003)
Increase production of the antioxidant resveratrol	ß-glucosidase	*bglN*	*C. molischiana*	*ACT*	*ACT*	2μ	*CYH2*	-	(Gonzalez-Candelas *et al.*, 2000)
	Resveratrol synthase	*4CL216*	*Hybrid poplar*	*ADH2*	*ADH2*	2μ	*URA3*	-	(Becker *et al.*, 2003)
	Coenzyme-A ligase	*vst1*	*Grapevine*	*ENO2*	*ENO2*	2μ	*LEU2*	-	
Reduce ethyl carbamate content	Urea amidolyase	*DUR1,2*	*S. cerevisiae*	*PGK1*	*PGK1*		*URA3*	+	(Coulon *et al.*, 2006)
Synthesis of antimicrobial enzymes or peptides	Pediocin	*PED1*	*P. acidilactici*	*ADH1*	*ADH1*	2μ	*URA3*	-	(Schoeman *et al.*, 1999)
	Leucocin	*LCA1*	*L. carnosum*	*ADH1*	*ADH1*	2μ	*URA3*	-	(du Toit and Pretorius, 2000)
	Chitinase	*CTS1*	*S. cerevisiae*	*PGK1*	*PGK1*	2μ	*URA3*	-	(Carstens *et al.* 2003)
	Glucose oxidase	*GOX*	*A. niger*	*PGH1*	*PGK1*		*URA3*	+	(Malherbe *et al.*2003)

Increase degradation of filter-clogging polysaccharides	Endopoly-galacturonase	*PGU1*	*S. cerevisiae*	*PGK1*	*PGK1*		*LEU2*	-	(Vilanova *et al.*2000)
	Pectatelyase	*pelA*	*F. solani*	*ACT*	-	2μ	*CYH*	-	(Gonzalez-Candelas *et al.*, 1995)
	Xylanase	*XYN2*	*T. reesei*	*ADH2, SSA1*	*ADH2*		*SMR*	+	(Louw *et al.*, 2006)
	Glucanase	*END1*	*B. fibris-olvens*	*ADH1*	*TRP5*		*SMR*	+	
	Pectate lyase	*PEL5*	*E. chrys-anthemi*	*ADH1*	*TRP5*		*SMR*	+	
	Polygalacturonase	*PEH1*	*E. caroto-vora*	*ADH1*	*TRP5*		*SMR*	+	

P: promoter; T: terminator; Pla: Plasmid; M: Marker; Chr: Chromosomal integration.

In most cases strong promoters and terminators were used, derived from glycolytic enzymes that are constitutively expressed under fermentative conditions (*ADH1*, *ADH2* and *PGK*), but also from the actin gene (*ACT*). A promoter collection comprising 11 mutants of the strong constitutive *S. cerevisiae TEF1* promoter has been recently constructed, that were used for fine-tuning of gene expression across a full continuum of possible expression levels. The activities of the mutant promoters range between about 8% and 120% of the activity of the unmutated *TEF1* promoter. In addition, promoter replacement cassettes were constructed that enable genomic integration of the mutant promoter collection upstream of any given yeast gene, allowing detailed genotype-phenotype characterizations (Nevoigt *et al.*, 2006).

Industrial yeasts usually do not have auxotrophic markers (e.g. *LEU2*, *URA2*), therefore the yeast-derived cycloheximide resistance gene *CYH2* or heterologous drug-resistance markers were used such as *ble* (from bacterial transposon Tn5, coding for a bleomycin binding protein) or *G418* (from bacterial transposon Tn903, coding for aminoglycoside phosphotransferase), conferring resistance to phleomycine and geneticine, respectively.

Plasmid-encoded genes should be preferably integrated, since the inserted elements have to be stable in the newly constructed organism, but such approaches were used in few cases (Volschenk *et al.*, 2001; Malherbe *et al.*, 2003; Smit *et al.*, 2003; Coulon *et al.*, 2006; Husnik *et al.*, 2006; Louw *et al.*, 2006). One-step gene disruption with auxotrophic markers as performed for the *GPD* gene (Michnick *et al.*, 1997) results in a self-cloning strain, a much less problematic approach in terms of acceptability evaluation according to the guidelines of the International Life Science Institute Europe (ILSI, 1999).

For heterologous expression of extracellular proteins, for example the *pedA*-encoding pediocin or *gox*-encoding glucose oxidase, secretion was usually directed by the mating pheromone α factor's secretion signal ($MFa1_S$) (Schoeman *et al.*, 1999; Malherbe *et al.*, 2003; Louw *et al.*, 2006).