The use of genetically modified *Saccharomyces cerevisiae* strains in the wine industry

Dorit Schuller [(*)] and Margarida Casal

Centro de Biologia, Universidade do Minho, 4710-057 Braga, Portugal

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*Corresponding author:

Dorit Schuller

Departamento de Biologia, Universidade do Minho

Campus de Gualtar

4710-057 Braga, Portugal

Phone: +351 253 604310

Fax: +351 253 678980

email: dschuller@bio.uminho.pt

Abstract

During the last decades, science and food technology have contributed at an accelerated rate to the introduction of new products to satisfy nutritional, socio-economic and quality requirements. With the emergence of modern molecular genetics, the industrial importance of *Saccharomyces cerevisiae,* continuously extended. The demand for suitable genetically modified (GM) *S. cerevisiae* strains for the biofuel, bakery and beverage industries or for the production of biotechnological products (e.g. enzymes, pharmaceutical products) will be continuously growing in the future.

Numerous specialized *S. cerevisiae* wine strains were obtained in the last years, possessing a wide range of optimized or novel oenological properties, capable to satisfy the demanding nature of modern winemaking practice. Unlocking the transcriptome, proteome and metabolome complexities contributes decisively to the knowledge about the genetic make-up of commercial yeast strains and will influence wine strain improvement by genetic engineering.

The most relevant advances regarding the importance and implications of the use of genetically modified yeast strains in the wine industry are discussed in this Mini-Review, considering a variety of aspects such as the strategies used for the construction of the strains with respect to current legislation requirements, environmental risk evaluations concerning the deliberate release of genetically modified yeast strains, methods for the detection of recombinant DNA and protein that are currently under evaluation, and the reasons for the critical public perception towards the application of such strains.

Introduction

The inoculation of selected pure yeast cultures into must is an oenological practice established since the seventies, in order to produce wine with desirable organoleptical characteristics and to guarantee the homogeneity of successive vintages. Nowadays, most of the European wine production relies on the use of such commercial starter yeasts that were selected mainly due to their good fermentation performance. Extensive biogeographical surveys over years and the evaluation of the fermentative flora of a given viticultural region of were the point of departure for further strain selection and improvement programs. However, the natural availability of yeast strains possessing an ideal combination of oenological characteristics is improbable. In the years following the publication of the *S. cerevisiae* genome sequence (Goffeau et al. 1996), new genetic tools turned the construction of genetically modified wine yeast (GMY) strains a great challenge. Currently, numerous research laboratories worldwide have obtained engineered strains, capable of improving for example processing efficiency, fermentation performance and wine's sensory quality. Their performance under oenological conditions has also been extensively evaluated. A future introduction of genetically modified wine yeast (GMY) also requires, in agreement with current legislation, a detailed safety and environmental impact evaluation and strains obtained by self-cloning, based on the use of host-derived genetic material, are most likely to receive approval. However, the critical attitudes of consumers towards the use of genetically modified yeasts for wine production has not changed significantly during the last 10 years, and are the most relevant reason for the absence of recombinant strains in the wine industry.

The present paper makes a global analysis of recent advances regarding the importance and implications of the use of genetically modified yeast strains in the wine industry, considering a variety of aspects such as the strategies used for the construction of the strains with respect

to current legislation requirements, environmental risk evaluations concerning the deliberate release of GMY strains, most relevant and sensitive methods for the detection of recombinant DNA and protein, and the reasons for the critical attitudes of consumers towards the application of such strains.

Selection of commercial wine yeast strains

Recent findings showed that residues inside one of the earliest known wine jars from Egypt contained ribosomal DNA from *S. cerevisiae*, indicating that this yeast was responsible for wine fermentation by at least 3150 B.C. (Cavalieri et al. 2003). 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. Selected yeast starters are nowadays widely used since they possess very good fermentative and oenological capabilities, contributing to both standardization of fermentation process and wine quality. Currently, about 150 different wine yeast strains, mainly *S. cerevisiae*, are commercially available. Considering the current trend towards the production of high quality wines with distinctive and very characteristic properties, the wine-makers demand “special yeasts for special traits” still remains to be satisfied (Mannazzu et al. 2002, Pretorius 2000, 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. The numerous compounds synthesized can vary greatly between *S. cerevisiae* strains, in particular within

different yeast species. As summarized in Table 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.

Finding wine yeast strains possessing an ideal combination of oenological characteristics is highly improbable and therefore strain selection was extended to non-*Saccharomyces* yeasts, e.g. *Candida, Kloeckera*, *Debaryomyces, 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 towards 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, Clemente-Jimenez et al. 2004, Granchi et al. 2002, Mingorance-Cazorla et al. 2003, Plata et al. 2003, Romano et al. 2003c), and considerable differences regarding these compounds were also found among commercial or autochthonous *S. cerevisiae* strains (Patel and Shibamoto 2003, Romano et al. 2003a, Steger and Lambrechts 2000).

Non-*Saccharomyces* strains produce and secrete several enzymes e.g. pectinase (increases juice extraction, improves clarification and facilitates wine filtration), ß-glycosidases (hydrolyse non-volatile glycosidic aromatic precursors from the grape) proteases (improve clarification process), esterases (contribute to aroma compound formation) or lipase (degrade lipids from grape or yeast autolytic reactions), interacting with grape-derived precursor compounds, contributing thus to reveal the varietal aroma and improve the winemaking process (Esteve-Zarzoso et al. 1998, Fernandez et al. 2000, Fleet and Heard 1993, Otero et al. 2003). *S. cerevisiae* is not a significant producer of enzymes with relevance in wine production, being mainly ß-glycosidase production reported for this species (Restuccia et al. 2002, Rodriguez et al. 2004). Non-*Saccharomyces* yeasts are commercially available, for example immobilized *Schizosaccharomyces pombe* cells (ProMalic, commercialized by PROENOL) for the deacidification of must by malic acid consumption (Silva et al. 2003).

Genetic engineering of *S. cerevisiae* wine yeast strains

Due to the demanding nature of modern winemaking practice, there is a continuously growing quest for specialized *S. cerevisiae* strains possessing a wide range of optimized or novel oenological properties. Genetic improvement of industrial strains by classical genetics (e.g. mutagenesis or protoplast fusion) was followed in the last 20 years by the use of recombinant DNA technologies. The publication of the complete *S. cerevisiae* genome (Goffeau et al. 1996), together with a growing arsenal of recombinant DNA technologies led to major advances in the fields of molecular genetics, physiology and biotechnology, and made the construction of specialized commercial strains possible, mainly by heterologous gene expression or by altered gene dosage (overexpression or deletion).

The most important targets for strain improvement relate to the improved production technology and quality, such as enhancement of fermentation performance, higher ethanol tolerance, better sugar utilization and nitrogen assimilation, enhanced organoleptical properties through altered sensorial characteristics as summarized by several reviewers (Blondin and Dequin 1998, Dequin 2001, Dequin et al. 2003, Pretorius 2000, Pretorius and Bauer 2002, Pretorius et al. 2003) and shown in table 2.

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, while the use of DNA sequences from species taxonomically closely related to pathogenic species should 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*), *Bacillus subtilis* (*padc*), *Pediococcus acidilactici* (*pedA*), *Schizosaccharomyces pombe* (*mae1* and *mae2*), hybrid poplar (*4CL216*), grapevine (*vst1*), *Aspergillus* sp. (*egl1*, *abfB*, *xlnA*, *rhaA*) or *Fusarium solani* (*pelA*), being others, such as *ATF1*, *GPD1* or *PGU1* derived from *S. cerevisiae* (Table 2).

In most cases strong promoters and terminators were used, derived from glycolytic enzymes that are constitutively expressed under fermentative conditions (*ADH1*, *ADH2*, *PGK*) but also from the actin gene (*ACT*). Industrial yeasts usually do not have auxotrophic markers (*LEU2*, *URA2*), therefore the yeast-derived cycloheximide resistance gene *CYH2* or heterologous drug-resistance markers were used such as *ble* (Tn5) or *G418* (Tn903), conferring resistance to phleomycine and geneticine, respectively. Engineering industrial strains with multi-copy shuttle vectors bearing *Escherichia coli* ampiciline resistance and yeast drug-resistance markers is not recommended, since the possibility of DNA transfer to gut microflora is

considered remote but existent. Nevertheless, for wine yeast strains this should not be relevant since cells are removed at the end of fermentation. Plasmid-encoded genes should be preferably integrated, since the elements inserted have to be stable in the newly constructed organism, but such approaches were used in few cases (Lilly et al. 2000, Malherbe et al. 2003, Volschenk et al. 2001). One-step gene disruption with auxotrophic markers as performed for the *GPD* gene (Michnick et al. 1997) results in a self-cloning strain, as previously defined (ILSI 1999), a much less problematic approach in terms of acceptability evaluation. Secretion of extracellular proteins, for example the *pedA* - encoding pediocin or *gox*-encoding glucose oxidase, was usually directed by the mating pheromone α factor's secretion signal ($MFa1_s$) (Malherbe et al. 2003, Schoeman et al. 1999).

The introduced modifications should not change essential characteristics of the host in the fermentation process. For most genetic modifications it could be shown that apart from the introduced metabolic change, no significant differences were found between wines produced with commercial strain and the corresponding modified strain regarding their oenological characteristics. Contrarily, enhanced glycerol production due to modulated *GPD* expression led to a decreased ethanol yield (1%, v/v) and by-product accumulation such as pyruvate, acetate, acetoin and 2,3-butanediol in consequence of carbon flux redirection (Michnick et al. 1997). Deletion of *ALD6* led to reduced acetic acid production (-40-70%) and re-routed the carbon flux towards glycerol, succinate and butanediol (Remize et al. 2000). It was also shown that grape must acidification due to enhanced *LDH* expression and consequent L(+) lactic acid production depends on the *S. cerevisiae* genetic background and also on the grape variety used for must preparation (Dequin et al. 1999). Wines containing 1.8-2.0% less alcohol were obtained from glucose-oxidase overexpressing strains, since this enzyme produced also D-glucono-δ-lactone and gluconic acid from glucose (Malherbe et al. 2003).

Recently, a sake yeast strain was approved as self-cloning yeast by the Japanese Government and does not need to be treated as GMY (Akada 2002). A two-step gene replacement was used for the construction of a strain free of bacterial and drug-resistant marker sequences. A point mutation (Gly1250Ser) in the yeast fatty acid synthetase *FAS2* confers cerulenin resistance and is associated with a higher production of the apple-like flavor component ethyl caproate in Japanese sake. A novel counter-selection marker was used, that consisted of a galactose-inducible overexpression promoter and the *GIN11* growth inhibitory sequence (*GALp-GIN11*). Cells that retain the marker do not grow on galactose because of the growth inhibitory effect mediated by *GIN11* overexpression. A plasmid containing the mutated *FAS2* gene, a drug resistance marker and the counter-selectable marker was integrated into the wild-type *FAS2* locus, and the loss of plasmid sequences from the integrants was done by growth on galactose, which is permissive for the loss of *GAL*p-*GIN11*. Counter-selected strains contained either the wild type or the mutated *FAS2* allele, but not the plasmid sequences, and the resulting difference between the described mutant and the corresponding wild type strain is a single base (Akada et al. 1999, Aritomi et al. 2004). The mentioned type of counter-selections can also be used for multiple chromosomal gene introductions, as required for engineering of metabolic pathways. Other strategies, for example site-directed mutagenesis of the sulfite-reductase *MET10* gene were used to develop wine yeast with lowered ability to produce hydrogen sufide (Sutherland et al. 2003). The allele *LEU4-1* confers resistance to 5,5,5-trifluoro-DL-leucine and the corresponding strains produce twice the amount of isoamyl-alcohol in laboratory-scale fermentations as the respective parental strains (Bendoni et al. 1999).

S. cerevisiae was the first eukaryotic genome sequenced, and will probably become the first organism whose transcriptome, proteome and metabolome complexities will be unlocked.

Since many physiological traits are consequences of complicated multigene regulation, understanding the way genes are expressed during wine fermentation will contribute to the knowledge about the genetic make-up of commercial yeast strains and influence wine strain improvement by genetic engineering. The same approaches are the most appropriate to show that the introduced changes are not associated with adverse or unexpected side-effects such as the production of toxic substances.

Specific strains may serve in future as a natural gene pool for yeast improvement programs, since linking observed phenotypes with global-expression analysis provides further information that might be useful for the construction of self-cloning yeast strains. Genes could be uncoupled from their regulatory controls and induced only under fermentation-specific conditions. Such *S. cerevisiae* strains could be for example strains possessing ß-glycosidase activity (Rodriguez et al. 2004) or the capability to reduce copper content in the must by excessive intracellular accumulation (Brandolini et al. 2002), strains with absent sulphite reductase activity (Mendes-Ferreira et al. 2002, Spiropoulos et al. 2000), or strains producing low amounts of acetic acid (Romano et al. 2003a).

Regulations concerning genetically modified organisms for food use

In May 1997 the European Regulation EC258/97 on novel foods and novel food ingredients (EC 1997) came into force and includes within its scope foods and food ingredients containing or consisting of genetically modified organisms (GMO) or produced by genetically modified organisms, whereas these are not present in the food. The safety of a food derived from a genetically modified organism had to be evaluated by comparing it with the most similar food which has a history of safe use. This means that, if a food derived from a GMO is substantially equivalent, it is “as safe as” the corresponding conventional food item and

should be treated as such, whereas identified differences are the subject for further toxicological, analytical and nutritional investigations. Detailed knowledge of both the overall characteristics and genetic background of the organisms, the source of the transferred gene(s) and the function of the modified genes is essential for this evaluation. Considering that the final outcome of a genetic modification is based on processes that are controlled by numerous different genes, whereas the function of many genes is still poorly understood, powerful methods for the identification and characterization of unintended effects on a genomic, proteomic and metabolomic scale are currently evaluated for their routine use (Corpillo et al. 2004, Kuiper and Kleter 2003, Kuiper et al. 2002).

The Novel Food Regulation has been recently amended by three new regulations concerning genetically modified organisms including derived foods and feeds: EC1829/2003 (EC 2003a), 1830/2003 (EC 2003b) and 65/2004 (EC 2004), which define the procedures for authorization, labeling and traceability. Regulation 1829/2003 describes the information to be provided by an applicant seeking authorization to place a product on the market. The applicant has to show that the referred food must not (i) have adverse effects on human and animal health and the environment, (ii) mislead the consumer and (iii) differ from the food which it is intended to replace to such an extent that its normal consumption would be nutritionally disadvantageous for the consumer. Such products must undergo a safety assessment before being placed on the market, including a technical dossier with detailed information concerning results obtained from research and developmental releases in order to evaluate the GMOs impact on human health and environment. This is defined in Annex III of Directive 2001/18/EC (EC 2001) on the deliberate release into the environment of genetically modified organisms for placing on the market or for any other purpose, that repealed the former Council Directive 90/220/EC (EC 1990). Since placing on the market includes

deliberate release into the environment, an environmental risk assessment in accordance with Annex II of Directive 2001/18/EC has to be carried out (EC 2002). The product then goes through the approval procedure between the European Food Safety Agency (EFSA) in Brussels, the European Commission and member states. Labeling is mandatory, even if the recombinant DNA or the corresponding protein cannot be detected in the final product. Foods containing GMOs have to be labeled “genetically modified” or “produced from genetically modified (name of the ingredient)”. Labeling is not required for foods containing traces of GMOs, which are adventitious and technically unavoidable, in a proportion lower than the threshold of 0.9% of the food ingredients (relation between recombinant and non-recombinant ingredient). Whereas the Novel Food Regulation was based on the principle of evidence, in the sense of mandatory labeling for food products containing more than 1% GMOs, Regulation EC1829/2003 is supported by the principle of application, making the declaration of GMO use during the production of food compulsory, but declaration does not rely on the detection of recombinant DNA or protein in the final product. According to Regulations Nº 1830/2003 (EC 2003b) and 65/2004 (EC 2004), GMOs and products derived from GMOs must be traceable during all stages of their placing on the market through the production and distribution chain, in order to facilitate withdrawal of products when necessary and to facilitate the implementation of risk management measures.

USA regulations do not require mandatory labeling and segregation of genetically modified products. No special labeling is required for “bioengineered foods” the term used by FDA for those derived by GM technology, “as they are not considered to differ from other foods in any meaningful or uniform way or, as a class, to present any different or greater safety concern than foods developed by traditional plant breeding” (Federal Register of May 29, 1992 57 FR 22984). Evaluation and approval before marketing is only required when the introduced gene

encodes a product that had never been a component of any other food, such as a new sweetening agent for example. The labeling requirements that apply to foods in general therefore also apply to foods using biotechnology. A label must “reveal all material facts” about a food, for example if a bioengineered food is significantly different from its traditional counterpart, has a significantly different nutritional property or if a potential allergen is present.

Wines produced by GMY should be, in general, considered as substantially equivalent to “traditional” wines. Compounds like glycerol, acetate ester, malic or lactic acid are natural wine substances, and their content would be merely adjusted or optimized in the sense of enhanced organoleptical characteristics. The expected concentration is very likely to lie within the range that can be found in different wine styles. Besides, facilitated and more economic technological process such as the use of a *S. cerevisiae* strain expressing pectolytic enzymes will have no impact on the composition or properties of the final product since the addition of commercial enzymes is a habitual oenological practice. Anyway, a careful evaluation based on a case-by-case study is indispensable.

Assessing environmental risks associated with the use of genetically modified yeasts

The future use of genetically modified yeasts will be dependent on the ability to assess potential or theoretical risks associated with their introduction into natural ecosystems.

Tracking the spreading of industrial yeast strains in vineyards close to the wineries where these strains were used during the last 5-10 years was used as an experimental model to assess the fate of genetically modified yeast strains in natural environments. These large-scale studies, carried out over a 3-years period in vineyards located in North Portugal and South

France, revealed that dissemination of commercial yeast in the vineyard is limited to short distances and periods of times and is largely favoured by the presence of water runoff. In samples taken at distances from wineries higher than 100 m, less than 2% of the fermentative microflora had a genetic profile identical to that of commercial yeast. In samples taken at very close proximity to the winery and to water rills, the proportion of commercial yeasts increased to 10-43%. The vast majority (94%) of commercial yeasts were found at a distance of between 10 and 200 m from the winery. Commercial strains, despite their intensive annual utilization, do not seem to implant in vineyards, and do not predominate over the indigenous flora, being their presence characterized by natural fluctuations of periodical appearance/dissappearance as autochthonous strains (Valero, personal communication)

The behavior of genetically modified yeast strains (GMY) within microbial populations of a confined wine cellar and greenhouse vineyard has also been evaluated. From the commercial strain VIN13 different genetically modified strains were constructed, containing heterologous genes expressing α-amylase (*LKA1*), endo-β-1,4-glucanase (*end1*), xylanase (*XYN4*) or pectate lyase (*peh1*) under the control of strong promoters and terminators and using the *kanMX* or *SMR*-410 resistance markers. After initial characterization of the autochthonous yeast flora of a newly established greenhouse vineyard, the vines of four blocks (each consisting of 20 vines) were sprayed with yeast suspensions containing 2.5 x 10^6 CFU/ml according to a previously defined scheme. Despite of the high initial cellular concentrations, only few *S. cerevisiae* strains were isolated during the weekly monitoring of yeast populations on grapes, leaves, stems and soil. Results showed that (i) no significant difference between the occurrence of the modified strains compared to the parental commercial strains was evident, even for GM strains that were supposed to have a selective advantage over the parental strains (secreting glucanases and pectinases) showing that the mentioned

modifications did not confer any fitness advantage (ii) the overall yeast populations on the sprayed blocks were very similar to the untreated control vines, leading to the conclusion that neither commercial strains nor GMY affect the ecological balance of vineyard-associated flora in a confined system, (iii) no significant differences among the strains were detected concerning their fermentation performance during spontaneous micro-vinifications (Bauer et al. 2003).

Horizontal DNA transfer can occur between yeast species belonging to the *sensu stricto* complex, generating viable hybrids with both parental chromosomal sets (Marinoni et al. 1999). Natural transformation of baker's yeast with plasmid DNA was observed under non-artificial starvation conditions when non-growing cells metabolize sugars without additional nutrients. This was proposed to be an evolutionary mechanism contributing to genetic diversity, being a plausible scenario in natural environments (Nevoigt et al. 2000). At present, studies are underway to evaluate the likelihood of both horizontal and vertical gene transfer among modified commercial wine yeast strains under wine production conditions (Bauer et al. 2003).

Another issue, equally important for the safety assessment of GMY use in wine production, is the evaluation of the potential release and stability of recombinant DNA and the corresponding protein(s) during alcoholic fermentation and wine aging on yeast lees. Autolysis of yeast cells is characterized by a loss of membrane permeability, hydrolysis of cellular macromolecules such as DNA and proteins, followed by leakage of the breakdown products in the extracellular environment and occurs after yeast cells have completed their life cycle and entered the death phase. Autolysis experiments were performed in laboratory culture media and showed that incubation at 40ºC during 10-14 days at pH 4.0-7.0 led to

degradation of 55% of total DNA, associated with leakage of mainly deoxyriboncleotides and a fewer amount of polynucleotides into the extracellular environment (Zhao and Fleet 2003).

Methods for the detection of genetically modified DNA or protein

In "experimental" wines produced by genetically modified yeast (GMY), no data are so far available about the occurrence and concentration of recombinant cells, DNA and protein. It can be estimated that the number of recombinant cells per bottle would be rather low (1-10 cells), since they are removed by filtration or inactivated by thermal treatment. This implies the use of highly sensitive techniques for tracing recombinant DNA during the wine production chain and in final products. Taking into account the recent European Regulations Nº 1829/2003 and 1830/2003, it is clear that reliable and accurate analytical methods are necessary for food containing GMO or produced from GMO. During the past years, both protein- and DNA-based methods have been developed and applied mostly for detection of transgenic soy and maize and their derivatives.

For protein-based detection, specific monoclonal and polyclonal antibodies have been developed mainly for immunochemical detection, Western blot analysis and ELISA (enzyme-linked immunosorbent assays). The immunochromatographic assays, also known as lateral flow strip tests, Reveal®CP4 and Reveal®Cry9C detect EPSPS (5-enol-pyruvyl-shikimate-3-phosphate synthase) derived from *Agrobacterium* sp. strain CP4 which confers resistance to the herbicide glyphosate in soybeans and corn, and *Bacillus thuringiensis* Cry proteins that confers protection against insects in corn plants, seeds and grains, respectively. Both kits are commercialized by Neogen (www.neogen.com) and detect GMO presence in 5-20 minutes at a low price, with high sensitivity (< 0.125% mass fraction of GMO) being a reliable field test

for controlling the distribution of biotechnology-derived products (Ahmed 2002, Auer 2003, Brett et al. 1999, Rogan et al. 1999, Stave 1999, van Duijn et al. 1999, van Duijn et al. 2002).

PCR-based methods are also applied for detection of GMOs by amplification of genetic elements present in most currently available GMOs in Europe. Detection limits range between 20 pg and 10 ng target DNA, which can correspond to 0.0001 – 1% mass fraction of GMO. (Ahmed 2002, Auer 2003, ILSI 1998, ILSI 2001, Meyer 1999, van Duijn et al. 1999, van Duijn et al. 2002). Quantitative-competitive PCR (QC-PCR) relies on parallel amplification of the transgene and of an endogenous reference gene that provides a control for both the lack of inhibition and amplificability of the target DNA in the sample. Quantification is possible by comparing PCR product concentrations from amplifications with varying proportions of target DNA:standard DNA. This approach was successfully tested in collaborative studies involving 12 European control laboratories, and allowed the detection of 0.1% GMO DNA (Hübner et al. 1999, Lüthy 1999). A hybrid method consisting of multiplex quantitative PCR coupled to subsequent DNA array technology (MQDA-PCR) was able to test a variety of food and feed products for seven different maize constructs simultaneously at levels as low as 0.1% GM (Rudi et al. 2003). Real-time PCR technologies are highly sensitive and suitable for precise DNA quantification at low thresholds, measuring the production of DNA amplicons during the log-linear phase of PCR amplification. (Ronning et al. 2003, Vaitilingom et al. 1999). PCR products quantitation by means of enzyme linked immunoabsorbent assays (PCR-ELISA) were recently described as a highly sensitive and cheap alternative to real-time PCR (Liu et al. 2004, Petit et al. 2003).

While raw foods can readily be identified as GMOs, detection is more difficult when they are processed: complex processed foodstuffs contain degraded DNA and substances that interfere even with the PCR reaction. Inter-laboratory assessment of procedures was essential and gave

rise to international standards development (e.g. DIN, ISO, EN) concerning sampling (DIN 2003), DNA extraction (DIN 2002b), DNA-based GMO detection (DIN 2002a) and protein-based GMO detection (DIN 2002c).

Technological evolution in GMO design, modifications of government regulations and adoption of risk-assessment guidelines will continue to drive the development of analytical techniques that will be in the future applied to genetically modified organisms. New profiling methods using transcriptomics, proteomics and metabolomics were proposed as the most adequate non-targeted approaches to detect secondary effects (Kuiper and Kleter 2003) and proteome analysis demonstrated “substantial equivalence” between a genetically modified virus-resistant tomato and the unmodified hybrids (Corpillo et al. 2004).

Consumer’s perceptions and attitudes

In 1988, Gist-Brocade obtained a baker’s strain where the genes coding for maltose permease and maltase were substituted with a more efficient set of genes from another strain. Since no non-*Saccharomyces* DNA was present, the UK authorities granted consent in 1989. A few years later, a recombinant brewer’s strain, obtained in 1993 by Brewing Research International was equally approved. This *S. cerevisiae* strain contained an amylase gene from *Saccharomyces diastaticus* together with a gene for copper resistance. Because of the unwillingness of the industries to face a negative consumer reaction none of the strains has gone into commercial production (Moseley 1999). For the same reasons, no application for the industrial use of genetically modified wine strains has been submitted in the last few years, although many strains were developed, as previously shown in Table 2, in consequence of the increased demand for diversity and innovation within the fermented beverage industry.

One of the most extensive (in terms of the number of people surveyed) public opinion analysis conducted in Europe is the Eurobarometer survey, that has been monitoring changes in attitude towards biotechnology in different European member states since the early 1990s. The last survey conducted in 2001 (Anonymous 2001) questioning 16 000 Europeans showed a generalized positive view of science and technology, but scientific advance is not regarded as an universal panacea for all problems. Almost all (95%) respondents indicated the consumer's lack of choice about consuming genetically modified food (GMF) as main reason for their negative attitude and 60% expressed the view that GMOs had the potential to have negative effects on the environment. In view of the fact that many scientific concepts are unknown to the public, the consumer's risk perception and attitudes to risk differ significantly from those defended by scientific risk experts, turning discussions about transgenic technologies complex, increasing at the same time distrust and negativity towards biotechnology in general, and GMO in particular. The fears by the critics of GM technology include alterations in nutritional quality of foods, potential toxicity, possible antibiotic resistance, potential allergenicity and carcinogenicity from consuming GM foods, environmental pollution, unintentional gene transfer, possible creation of new viruses and toxins, religious, cultural and ethical concerns, as well as fear from the unknown (Uzogara 2000).

As shown in Figure 1, consumer's concern about genetic modification depended on many factors, being minor modifications to food products associated with minor concern, whereas the need for them and the advantages they offer were also rated low. For GM applications in food, benefits were perceived to be marginal, abstract or only on the producer's side. This was verified especially for genetically modified beer, followed by tomatoes, strawberries and salmon. Being beer a traditional lifestyle and convenience beverage like wine, it can be

estimated that wine produced by gene technology use would share a comparable consumer opinion. Any modification involving humans and animals was associated with high levels of ethical concern, whereas medical applications such as pharmaceuticals and applications relevant to hereditary disease were perceived to be the most important and necessary (Frewer 2003, Frewer et al. 1997).

In conclusion, the recent availability of clear legal regulations defining requirements for construction and safety evaluation of genetically modified organisms as well as the labeling of products obtained by their use can be considered as a crucial step to assist the consumer in making an informed choice, and the next future will show whether this strategy was appropriate to contribute towards a less negative consumer attitude. The construction of genetically modified wine yeast strains should be obtained by strategies based on self-cloning. In this context, the exploration of specific strains in winemaking environments, harboring desirable oenological traits, may serve in future as a natural gene pool for the construction of such strains, conferring the exploration of strain diversity a new dimension.

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463 Table 1

464 Oenological characteristics considered in the selection of *Saccharomyces cerevisiae* wine
465 strains (Brandolini et al. 2002, Caridi et al. 2002, Esteve-Zarzoso et al. 2000, Guerra et al.
466 1999, Maifreni et al. 1999, Mannazzu et al. 2002, Martinez-Rodriguez et al. 2001, Mendes-
467 Ferreira et al. 2002, Perez-Coello et al. 1999, Rainieri and Pretorius 2000, Regodon et al.
468 1997, Romano et al. 1998, Steger and Lambrechts 2000).

469

470 Table 2

471 Targets for *S. cerevisiae* strain improvement (adapted from Pretorius 2000, Pretorius et al.
472 2003) , indicating, whenever possible, examples of the strategies used for genetic
473 modifications.

474

475 Figure 1

476 Public perceptions of risk versus benefit of genetically modified foods (adapted from Frewer
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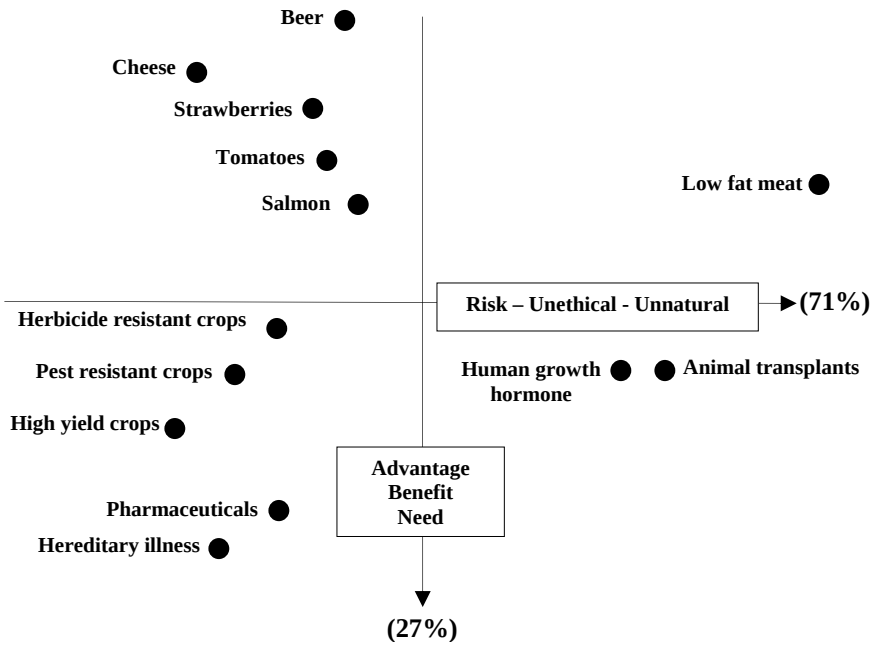

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784 Figure 1

Table 1

Oenological characteristics	**Comment**
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 28ºC

Table 1 (cont.)

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

Table 1 (cont.)

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

Table 2

Improvement	Metabolism / protein(s)		Gene(s)	Source	Construction					Reference
					P	T	Pla	M	Chr	
Sensory quality	Aroma-liberating enzymes	Endoglucanase	*egl1*	*Trichoderma longibrachiatum*	*ACT*	-	2µ	*CYH2*	-	(Pérez-González et al. 1993)
		Arabinofuranosidase	*abfB*	*Aspergillus niger*	*ACT*	-	2µ	*CYH2*	-	(Sanchez-Torres et al. 1996)
		Endoxylanase	*xlnA*	*Aspergillus nidulans*	*ACT*	-	2µ	*CYH2*	-	(Ganga et al. 1999)
		Rhamnosidase	*rhaA*	*Aspergillus aculeatus*	*GPD*	*PGK*		*TRP*	-	(Manzanares et al. 2003)
Background flavor complexity and intensity	Acidity adjustment	Malate permease Malic enzyme	*mae1* *mae2*	*Schizosaccharomyces pombe*	*PGK1*	*PGK1*	2µ	*SMR1-140*	+	(Volschenk et al. 2001)
		Malolactic enzyme	*mleS*	*Lactococcus lactis*	*PGK1*	*PGK1*	2µ	*URA3*		(Volschenk et al. 1997)
		Acetaldehyde dehydrogenase	*ALD6* (deletion)	*Saccharomyces cerevisiae*				*kanMX4*		(Remize et al. 2000)
		Lactate dehydrogenase	*LDH*	*Lactobacillus casei*	*ADH1*	*ADH1*	2µ	*G418 (Tn903)*	-	(Dequin et al. 1999)
	Glycerol production	Glycerol-3-phosphate dehydrogenase	*GPD1*	*Saccharomyces cerevisiae*	*ADH1*	*ADH1*	2µ	*ble (Tn5)*	-	(Michnick et al. 1997, Remize et al. 1999)

Table 2 (cont.)

	Volatile phenol formation	Phenolic acid decarboxylase	*pdc* *padc*	*Lactobacillus plantarum* *Bacillus subtilis*	*PGK1*	*PGK1*	2µ	*URA3*		(Smit et al. 2003)
	Acetate ester production	Alcohol acetyltransferase	*ATF1*	*Saccharomyces cerevisiae*	*PGK1*	*PGK1*	2µ	*LEU2*	+	(Lilly et al. 2000)
	Hydrogen sulphide production	Sulphite reductase	*MET10*	*Saccharomyces cerevisiae*						(Sutherland et al. 2003)
Safety and health aspects	Resveratrol production	ß-glucosidase	*bglN*	*Candida 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*	-	
	Ethyl carbamate elimination	Blocking urea secretion	*CAR1* (deletion)	*Saccharomyces cerevisiae*						(Pretorius et al. 2003)
Spoilage microorganism control	Production of antimicrobial enzymes	Pediocin	*pedA*	*Pediococcus acidilactici*	*ADH1*	*ADH1*	*2µ*	*URA3*	-	(Schoeman et al. 1999)
		Chitinase	*CTS1-2*	*Saccharomyces cerevisiae*	*PGK1*	*PGK1*	*2µ*		-	(Carstens et al. 2003)
		Leucocin	*lcaB*	*Leuconostoc carnosum*	*ADH1*	*ADH1*	*2µ*	*URA3*	-	(du Toit and Pretorius 2000)
		Glucose oxidase	*gox*	*Aspergillus niger*	*PGH1*	*PGK1*		*URA3*	+	(Malherbe et al. 2003)

Table 2 (cont.)

Fermentation performance	Stress tolerance	Trehalose	*TPS1,TPS2, ATH1*	*Saccharomyces cerevisiae*	(Pretorius et al. 2003)
		Glycogen	*GSY1, GSY2*		
		Sterols	*SUT1, SUT2*		
	Sugar uptake and assimilation	Hexose transporters	*HXT1-18*	*Saccharomyces cerevisiae*	(Pretorius et al. 2003)
		Hexose kinases	*HXK1, HXK2*		
Achieving a complete conversion of sugar to alcohol and CO_2 without the development of off-flavors	Nitrogen assimilation	Proline oxidase	*PUT1*	*Saccharomyces cerevisiae*	(Pretorius et al. 2003)
		Pyrroline-5-carboxylate dehydrogenase	*PUT2*		
		PUT1 and *PUT2* repressor	*ure2*	*Saccharomyces cerevisiae*	(Salmon and Barre 1998)
	Ethanol tolerance	Sterol accumulation	*SUT1, SUT2,*	*Saccharomyces cerevisiae*	(Pretorius et al. 2003)
		Membrane ATPase activity	*PMA1, PMA2*		
	Agrochemicals resistance	Copper chelatin	*CUP1*	*Saccharomyces cerevisiae*	(Pretorius et al. 2003)

Table 2 (cont.)

Processing efficiency Fining and clarification	Removal of filter-clogging polysaccharides	Endopolygalacturonase	*PGU1*	*Saccharomyces cerevisiae*	*PGK1*	*PGK1*	*LEU2*		-	(Vilanova et al. 2000)
		Pectate Lyase	*pelA*	*Fusarium solani*	*ACT*	-	*CYH*	*2µ*	-	(Gonzalez-Candelas et al. 1995)
	Flocculation timing	Flocculin	*FLO1, FLO11*	*Saccharomyces cerevisiae*	*HSP30*					(Pretorius et al. 2003)

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