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MINI-REVIEW

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The potential of genetic engineering for improving brewing, wine-making and baking yeasts

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Abstract The end of the twentieth century was marked by major advances in life technology, particularly in areas related to genetics and more recently genomics. Considerable progress was made in the development of genetically improved yeast strains for the wine, brewing and baking industries. In the last decade, recombinant DNA technology widened the possibilities for introducing new properties. The most remarkable advances, which are discussed in this Mini-Review, are improved process performance, off-flavor elimination, increased formation of by-products, improved hygienic properties or extension of substrate utilization. Although the introduction of this technology into traditional industries is currently limited by public perception, the number of potential applications of genetically modified industrial yeast is likely to increase in the coming years, as our knowledge derived from genomic analyses increases.

Introduction

Yeasts have been used to produce food and beverages since the Neolithic age. Their use in fermentation was recognized in 1836–1838; and Louis Pasteur demonstrated their unequivocal role in the conversion of sugar to ethanol and carbon dioxide in 1861. Originally, fermentation was spontaneous. The first pure yeast culture was obtained by Emil Christian Hansen from the Carlsberg Brewery in 1883. A pure culture of wine yeast was subsequently obtained by Müller-Thurgau from Geisenheim (Germany) in 1890.

The genetic improvement of industrial strains traditionally relied on classical genetic techniques (mutagenesis, hybridization, protoplast fusion, cytoduction), followed by selection for broad traits such as fermentation capacity, ethanol tolerance, absence of off-flavors (e.g. H_2S for wine strains), fast dough fermentation, osmotolerance, rehydration tolerance, organic acid resistance (baker's strains), flocculation and carbohydrate utilization (brewer's strains). Despite considerable work, a major limitation of these classical genetic techniques was the difficulties of adding or removing features from a strain without altering its performance. One major advantage of gene technology over classical genetic techniques is that just one characteristic can be precisely modified, without affecting other desirable properties. In addition, molecular biology approaches have introduced a new dimension. The expression of heterologous genes has substantially increased the possibilities. In the past 20 years, impressive progress has been made in the development of molecular techniques for *Saccharomyces cerevisiae*. These advances have been successfully applied to industrial strains in the past decade, allowing the development of a new generation of specialized industrial yeast strains. The principal targets for strain development fall into two broad categories: (1) improvement of fermentation performance and simplification of the process and (2) improvement of product quality, e.g. organoleptic and hygienic characteristics. The purpose of this paper is to review selected examples of the most advanced applications of yeast genetic engineering in the fields of winemaking, brewing and baking. Many targets for yeast improvement are relevant to several of these fields and will be presented in a sole section. Conversely, each of thesefields has specific demands, which will be reviewed in separate sections.

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History and genetics of industrial yeast

The use of pure culture strains in brewing has been widespread since the end of the nineteenth century. Top-fermenting (ale-brewing) yeasts form a diverse group of polyploid yeasts that are closely related to the laboratory strains of *S. cerevisiae* (Hansen and Kielland-Brandt 1997). Bottom-fermenting (lager-brewing) yeasts were

initially classified as *S. carlsbergensis*, later included in *S. cerevisiae* (Yarrow 1984) and then renamed *S. pastorianus* (Vaughan-Martini and Martini 1987) These strains are allo-tetraploid and exhibit poor sporulation and low spore viability. Extensive studies, mainly by Carlsberg, indicate that this species contains part of two divergent genomes, probably derived from *S. cerevisiae* and *S. monacencis* (reviewed by Hansen and Kielland-Brandt 1997). Conversely, other studies favor the formation of hybrids between *S. cerevisiae* and *S. bayanus* (Vaughan-Martini and Kurtzman 1985; Yamagashi and Ogata 1999). Despite this complex genetic constitution, brewers took an active interest in yeast genetics as early as the beginning of the 1980s, leading to the production of hybridized and genetically engineered yeasts with improved characteristics (Benitez et al. 1996; Hammond 1995; Hansen and Kielland-Brandt 1997; Hinchliffe 1992; Stewart and Russel 1986).

Compared to brewer's yeast, the study of wine yeast genetics is relatively recent. The majority of commercial wine yeasts are strains of *S. cerevisiae*, including those described by enologists as *S. bayanus*, which are in fact *S. cerevisiae* (Masneuf et al. 1996). These strains are predominantly homothallic, diploid/aneuploid and display low sporulation ability. Compared to laboratory strains, they exhibit chromosomal-length polymorphisms and possess rearranged chromosomes with multiple translocations (Bidenne et al. 1992; Rachidi et al. 2000). Classical genetic approaches were first applied to wine yeast strains in the middle of the 1980s, in response to increasing demand for new characteristics resulting from the development of pure culture strains. Strains with new properties, e.g. with mitochondrial markers, flocculation properties or expressing the killer toxin, have been generated by mutagenesis, hybridization or cytoduction (Barre et al. 1993). Only a few of these strains have been commercialized,including mitochondrial mutants, which have been very useful for implantation studies. This is mainly due to the lack of specificity of the methods, making it difficult to modify a characteristic precisely without altering other properties. In the past 10 years, the demand for more specialized wine yeasts has been growing and the number of commercialized, selected wine yeast strains has increased from about 20 to over 100. Substantial amounts of work took place during the 1990s to develop new strains, which mainly involved recombinant DNA approaches (Barre et al. 1993; Blondin and Dequin 1998; Butzke and Bisson 1996; Henschke 1997; Pretorius 2000; Pretorius and van der Westhuizen 1991; Querol and Ramon 1996).

Compared to the huge amount of work performed to engineer brewer's yeast and wine-making yeast, the improvement of baker's yeast has received less attention. Attempts to construct baker's yeast with improved broad traits, such as fermentative performance, osmo- and acid-tolerance and tolerance to rehydration (Evans 1990), were performed in the 1980s, using breeding procedures. These approaches were successful. For example, they generated new strains with rapid fermentative properties. Other traits, especially tolerance to stresses (drying, freezing, osmotic stress), which involves complex multigenic responses, are more difficult to improve by classical breeding programs. Our limited knowledge of the genetic basis of these important commercial properties has also limited their manipulation by recombinant DNA techniques. Therefore, much of the effort in genetic engineering of baker's yeast has gone into improving sugar degradation and fermentation performance (Randez-Gil et al. 1999).

Relevant targets for brewer's and wine-making yeasts

Flocculation

Yeast flocculation, the asexual aggregation of cells into flocs and their subsequent removal from the fermentation medium by sedimentation, is of major interest for some fermentation processes. In brewing, especially bottom fermentation, good flocculation towards the end of primary fermentation is essential for a bright beer with sufficient aroma. The onset of cell sedimentation is critical. If it occurs too late, the beer will be cloudy and hard to filter; and if it occurs too early, maturation will be inefficient.Flocculation is also of interest for the elaboration of sparkling wines. In the classic Champagne method, a secondary fermentation (the so-called "prise de mousse") is conducted in the bottle to develop specific organoleptic characteristics. After 1 year, the yeast cells are removed from the bottle by the procedure of "remuage", which is time-consuming and expensive. This process could be advantageously simplified by the sedimentation of flocculent yeast cells.

Yeast flocculation is an asexual, calcium-dependent, reversible aggregation of cells into flocs, involving a protein–sugar interaction between a specific cell-surface lectin and cell-wall mannan (Stratford 1992). Several attempts have been made to construct flocculent industrial strains by classical genetic approaches. Electrofusion was used to convert a non-flocculent brewer's yeast into a flocculent one with adequate brewing performance (Urano et al. 1993a, b). Flocculation was also introduced into wine yeast strains by hybridization or cytoduction (reviewed in Barre et al. 1993). Extensive work in the past decade has led to a better understanding of the molecular and biochemical basis of flocculation. Several dominant flocculation genes, *FLO1*, *FLO5* and *FLO8*, are involved in flocculation. The dominant *FLO1* gene has been isolated, characterized and shown to encode a cell-wall protein containing a lectin domain (Bidard et al. 1994; Bony et al. 1997a; Kobayachi et al. 1998; Watari et al. 1994). Transfer of the flocculation characteristics into a wine yeast strain was achieved by transforming the strains with a multicopy vector containing *FLO1*, isolated from a flocculent *S. cerevisiae* strain (Bidard et al. 1994). A strong flocculation phenotype was achieved in a non-flocculent brewer's yeast by inte-

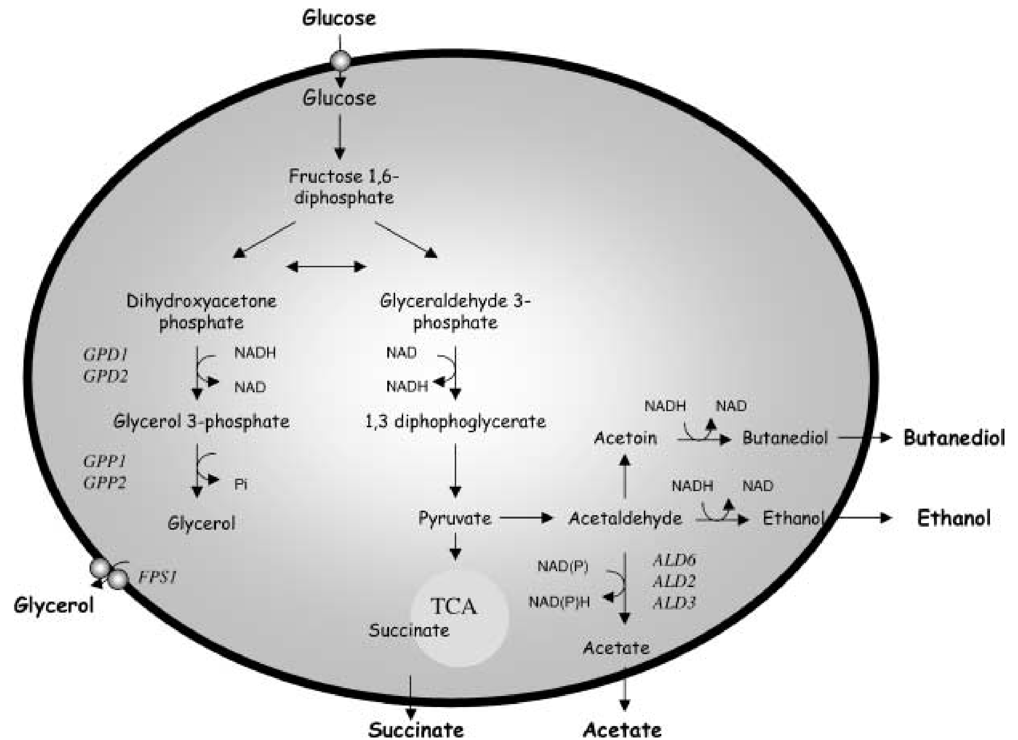

Fig. 1 Pathways for glycerol and acetate production during fermentation in *Saccharomyces cerevisiae*. *ALD2*, *ALD3*, *ALD6* cytosolic acetaldehyde dehydrogenases, *FPS1* glycerol facilitator, *GPD1*, *GPD2* glycerol-3-phosphate dehydrogenases, *GPP1*, *GPP2* glycerol-3-phosphatases

gration of the *ADH1*-regulated *FLO1* gene into the *ADH1* locus (Watari et al. 1994). However, the onset of flocculation still cannot be precisely controlled: flocculation of the recombinant strain occurred too early in brewing trials. A major difficulty in the development of precise control of gene expression is the lack of regulated promoters that can be used under industrial conditions. The most well known inducible promoters for yeasts cannot be used, due to the composition of the industrial medium and to regulatoryconstraints preventing major modifications to it. An example of a promoter that is induced by environment signals is *HSP30* (heat-shock protein 30). This gene is induced by factors that occur late or towards the end of fermentation (high ethanol concentration, depletion of sugars and nitrogen). When the native *FLO1* promoter was replaced by the *HSP30* promoter, flocculation occurred towards the end of fermentation, under laboratory conditions (Verstrepen et al. 2000). However, it is necessary to confirm that this behavior is also obtained during brewing fermentation.

Glycerol overproduction

Glycerol is the most abundant by-product of alcoholic fermentation, after ethanol and carbon dioxide. This polyol is thought to impart some sensory quality to beverages. In particular, high levels of glycerol may contribute to the perceived sweetness of wine. Therefore, many attempts have been made to increase the glycerol yield during fermentation. Another interest for re-routing the carbon flux towards glycerol, is the expected decrease in ethanol yield. This may be an alternative approach to the current methods for removing ethanol from beverages (e.g. dialysis), which are expensive and detrimental for aroma compounds.

In *S. cerevisiae*, glycerol is produced by reduction of dihydroxyacetone phosphate, catalyzed by the glycerol-3-phosphate dehydrogenase (GPDH), followed by a subsequent dephosphorylation realized by a glycerol-3-phosphatase. Glycerol, as a non-ionized molecule, can cross the plasma membrane by passive diffusion or be transported by facilitated diffusion through the major intrinsic protein channel Fps1p (Fig. 1). Glycerol export and glycerol-3-phosphate dehydrogenase, but not glycerol phosphatase, are rate-limiting for glycerol production during fermentation in *S. cerevisiae* (Remize et al. 2001). The most direct approach for overproducing glycerol is to overproduce the GPDH. Overexpression of *GPD1* in laboratory strains of wine and brewer's yeasts resulted in a 2- to 3-fold increase in glycerol production and a lower ethanol yield (Michnick et al. 1997; Nevoigt and Stahl 1996, 1998; Remize et al. 1999). Wine yeast strains transformed with multicopy plasmids carrying *GPD1* under the control of *ADH1* promoter produced 12–18 g of glycerol/l and about 1% (v/v) less ethanol (Remize et al. 1999). Interestingly, the fermentation rate of wine yeast strains overproducing glycerol was increased during the stationary phase under enological conditions, suggesting that the availability of NADH might be limiting for glycolysis. As a result of carbon re-routing and altered NADH metabolism, these strains exhibited increased production of by-products, mainly acetate, 2,3-butanediol and succinate. The high production of acetate is a major disadvantage; and this problem was solved by deleting *ALD6*, which encodes the $NADP^+$-dependent,

Mg^{2+}-activated cytosolic acetaldehyde dehydrogenase isoform (Fig. 1) in the strains overexpressing *GPD1*. High amounts of glycerol were obtained without increasing acetate formation and were accompanied by the reorientation of carbon flux towards the formation of succinate and 2,3-butanediol (Remize and Dequin 1998). Attention must be paid to acetoin, the production of which was shown to increasein strains producing very high amount of glycerol.

The same approach was applied to brewer's yeast. Overexpression of *GPD1* in brewer's yeast resulted in a lower ethanol yield (80% compared to the reference strain) and a 4-fold increase in glycerol yield (Nevoigt and Stahl 1998). Although the concentration of higher alcohols and esters was not strongly affected, there was a marked increase in the production of acetaldehyde, diacetyl and acetoin (Nevoigt and Stahl 1998).

Increased production of acetate esters

Isoamyl acetate (banana-like aroma) is an important determinant for beer, sake and young wines. This ester is produced by yeast from isoamyl alcohol, which is itself a by-product of leucine synthesis. Overexpression of one of the genes responsible for leucine synthesis (*LEU4* encoding α-isopropylmalate synthase) in a sake yeast strain resulted in a very slight increase in isoamyl alcohol concentrations and the corresponding ester (Hirata et al. 1992). Another strategy developed in a brewing strain was to increase the formation of acetate esters by overexpression of the *ATF1* gene, which encodes the alcohol acetyltransferase catalyzing the formation of esters from acetyl CoA and the relevant alcohols. This led to a 27-fold increase in the production of isoamyl-acetate during fermentation on a laboratory scale (Fuji et al. 1994). The strategy has been also successfully applied to wine yeast strains (Lilly et al. 2000). However, a major disadvantage of this approach is that it generates a simultaneous increase in the formation of esters, e.g. ethyl acetate, which can be undesirable in wine above a critical amount. Another approach developed on sake yeast strains was to disrupt the *EST2* gene, which codes for the major esterase hydrolyzing isoamyl acetate (Fukuda et al. 1998). However, this only resulted in a limited increase (approximately 2-fold) in isoamyl acetate production.

Elimination of ethylcarbamate

Ethylcarbamate is a suspected carcinogen found in many fermented foods and beverages, e.g. wine, sherry, brandy and sake (Ough 1976). It is mainly formed by the spontaneous chemical reaction of ethanol and urea at elevated temperatures in acidic media. Urea is produced mainly from the cleavage of arginine by arginase. To reduce the formation of urea in sake, the two copies of the *CAR1* gene, coding for arginase, were disrupted in an industrial sake yeast. This resulted in the elimination of urea and ethylcarbamate formation during sake brewing (Kitamoto et al. 1991). It may be also of interest to reduce the formation of ethylcarbamate in wines. Arginine is one of the most abundant amino acids in grape must and is quickly assimilatedby yeast. Therefore, wines made from arginine-rich grape musts may sometimes contain amounts of ethylcarbamate that exceed the authorized concentrations. Although the approach described above might be conceivable for wine yeast, it has the major drawbackof reducing the amount of nitrogen available during fermentation. Another possibility is to express an acidic urease that degrades urea into ammonia and carbon dioxide in yeast (Ough and Trioli 1988). The acidic urease from *Lactobacillus fermentum* was recently expressed in *S. cerevisiae*, with the aim of degrading urea during wine fermentation. However, this approach was ineffective, because *S. cerevisiae* lacks several auxiliary proteins and appropriate cofactors required for the correct folding of urease (Visser et al. 1999).

Antimicrobial properties

In brewing, sake-brewing and wine-making, there is always a risk that wild yeast becomes predominant. Many indigenous yeasts secrete zymocin, a toxin that kills sensitive strains. Zymocin production and immunity to this toxin are determined by a cytosolic, double-stranded RNA. There are three major types of killer toxins (K1–K3). As most of the yeast strains found in grape musts produce the K2 zymocin, the K1 dsRNA has been integrated into the genome of a K2 wine yeast (Boone et al. 1990). A brewing strain with increased resistance to contamination was also constructed by expressing the genes coding for zymocin productionand immunity. The modified yeast was resistant to the zymocin which can be produced by contaminating yeast and can kill yeast flora that is sensitive to the toxin (Hammond and Eckerlsey 1984). Although the idea of achieving a better control of yeast implantation is relevant, the utilization of starter strains with a selective advantage is questionable. Industrial yeasts, for example wine yeasts, are classically used without containment and are released into the environment. Therefore, great attention must be paid to the potential impact of yeast strains possessing a competitive advantage over the natural microflora.

The fermentation media are also susceptible to bacterial contamination. A brewer's strain carrying both the killer factor and antibacterial properties has been constructed (Sasaki et al. 1984). This strain was obtained by mating a respiratory-deficient strain with antimicrobial properties and a killer strain, followed by fusion of the resulting hybrid with a brewer's strain. Attempts to develop bactericidal wine yeast strains have been recently described. Two bacteriocin genes, encoding a pediocin and a leucocin gene from *Pediococcus acidilactici* and *Leuconostoc carnosum* respectively, have been expressed in *S. cerevisiae* (Schoeman et al. 1999). As these

bacteriocins have a rather narrow spectrum, this approach should be extended to other toxins to warrant a satisfying stability of wines. The use of bacteriocidal yeasts would be useful for the production of wine with reduced levels of sulfur dioxide and other chemical preservatives. However, as sulfur dioxide has other interesting properties as an antioxidant, its addition to wine will probably remain necessary.

Specific targets for brewer's yeast

Fermentation of dextrins

Brewer's yeast strains cannot utilize dextrins, which represent about 25% of malt wort sugars and contribute significantly to the calorific content of beer. Crude commercial preparations containing glucoamylases (usually from *Aspergillus*) are used to produce light beers. An early target for yeast genetic improvement was the development of amylolytic yeast strains that hydrolyze residual starch or dextrin in wort, producing low carbohydrate beers. Although classical genetic approaches have succeeded in transferring the amyloglucosidase activity of *S. diastaticus* (which hydrolyzes the α-1,4 linkages of dextrins) to brewer's strains, a major problem was the co-transfer of the *POF1* gene,causing phenolic off-flavor (reviewed by Hansen and Kielland-Brandt 1997). This side-effect was overcome by the expression of the *S. diastaticus STA2* gene encoding amyloglucosidase (Perry and Meaden 1988). To overcome plasmid instability problems, an all-yeast, multicopy plasmid carrying *STA2* under the control of the *PGK* promoter, was used to transform a commercial lager-brewing yeast strain (Vakeria and Hinchliffe 1989). The recombinant brewer's yeast secreting amyloglucosidase exhibited improved carbohydrate degradation and produced approximately 1% more ethanol, without altering the fermentation performance or the quality of the beer.

The degree of dextrin degradation was further increased by expressing amyloglucosidases able to hydrolyze α-1,6 links. The corresponding genes from *A. niger* (Yocum 1986) or *A. awamori* (Cole et al. 1988) have been integrated into the genome of brewer's yeast strains, to give recombinant strains with high levels of secreted enzymes and dextrin degradation. Good quality, superattenuated beers have been produced on a pilot scale by brewer's yeast secreting the *A. niger* enzyme (Gopal and Hammond 1992). Increased dextrin degradation has also been obtained with a brewer's yeast expressing the gene coding for the amyloglucosidase of *Schwanniomyces occidentalis*. This enzyme has both α-1,4 and α-1,6 activities and has the additional advantage of being thermolabile, thus being inactivated by pasteurization (Lancashire et al. 1989). This ensures that the beer does not become sweet during storage.

To increase starch degradation further, various *Saccharomyces cerevisae* yeasts coexpressing the *STA2* gene from *S. distaticus*, coding for a glucoamylase, and the *AMY* gene from *Bacillus amyloliquefaciens* were constructed. These strains were very efficient at starch degradation in laboratory conditions (Steyn and Pretorius 1991).

Beer viscosity and filtration

The β-1,4 and β-1,6 linkages of β-glucans, a polysaccharide found in barley cell walls, are cleaved by a specific endo-β-glucanase during malting. This thermolabile enzyme is only present in small amounts in the wort, due to the elevated temperature during malt drying. Consequently, β-glucan degradation is often insufficient, which leads to increased viscosity, reduced filterability and the formation of gels and hazes in the beer. The filtration performance of beer can be significantly improved by the additionof a commercial preparation of bacterial β-glucanase to the fermenter. A cheaper solution is the use of yeast strains secreting glucanase. The secretion of *B. subtillis* β-glucanase in brewer's yeast strains was obtained by expressing the appropriate gene fused to the promoter and signal sequence of α-factor, in a multicopy plasmid (Lancashire and Wilde 1987). More efficient β-glucan degradation was subsequently achieved by expressing a β-glucanase of *Trichoderma reesei* with a lower optimum pH, between 4 and 5, instead of 6.7 for the *B. subtillis* enzyme (Enari et al. 1987; Penttilä et al. 1987a, b). Brewer's strains carrying one copy of the *T. reesei EG1* gene integrated into the chromosome (*ADH1* or*PGK1* locus) and devoid of bacterial sequences have been constructed and tested on a pilot scale. Almost all β-glucans were digested by some of the integrants during fermentation, resulting in a significant reduction of beer viscosity. The growth and brewing properties of the strains were unaltered (Suihko et al. 1991).

Diacetyl elimination

Brewer's yeasts positively or negatively influence the flavor of the beer by producing aroma compounds during primary fermentation. Vicinal diketones, in particular pentanedione and diacetyl, are considered unpleasant. The main reason for lagering (secondary fermentation) is to decrease the concentration of diacetyl, which can be tasted at concentrations of below 0.02–0.10 mg/l. Diacetyl is formed outside the cell by the chemical oxidation of α-acetolactate, an intermediate in the biosynthesis of valine, which diffuses into the fermenting wort (Fig. 2). Once diacetyl has been formed, it is enzymatically reduced inside the yeast cell to acetoin and finally to 2,3-butanediol, which has no impact on the flavor of the beer. The complete removal of diacetyl sometimes requires lengthy maturation (1–3 weeks). Various approaches based on the engineering of enzymes of the valine biosynthetic pathway have been tested to reduce yeast diacetyl formation (summarized by Hansen and Kiellandt-Brandt 1997). Diacetyl formation was reduced

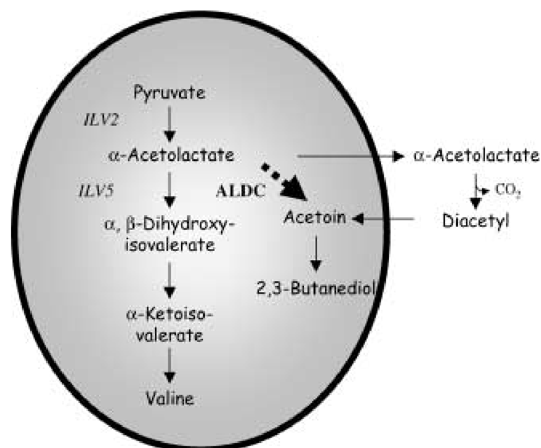

Fig. 2 Formation of diacetyl and strategies developed to reduce its production by *S. cerevisiae*. *ALDC* Heterologous acetolactate decarboxylase, *ILV2* acetohydroxyacid synthetase, *ILV5* reductoisomerase

in *ILV2* mutants and in strains in which the expression of *ILV5*, which codes for acetohydroxy acid reductoisomerase, was increased (Gjermansen et al. 1988; Mithieux and Weiss 1995; Villanueba et al. 1990). Another strategy, which has the advantage of not interfering with the biosynthesis of valine or isoleucine, consisted of expressing the acetolactate decarboxylase gene from *Enterobacter aerogenes* in brewer's yeast. Brewer's strains with the *ADHI*-controlled *ALDC* gene integrated into the chromosome produced considerably less diacetyl than the parent yeast in fermentation tests (Fuji et al. 1990; Sone et al. 1988).

Reduction of hydrogen sulfide production

Low levels of hydrogen sulfide are produced by *S. cerevisiae*, by the reduction of sulfate during methionine synthesis (Fig. 3). Due to its very low sensory threshold, trace amounts of hydrogen sulfide can alter the organoleptic characteristics of beer. This compound must be eliminated during beer maturation. An alternative strategyis to develop brewing yeasts with reduced hydrogen sulfide formation. Increased expression of *NHS5*, encoding the cystathionine β-synthase in brewing yeast, has been shown to suppress the formation of hydrogen sulfide in beer produced on the laboratory scale, without affecting other fermentation characteristics (Tezuka et al. 1992). Another approach, based on the overexpression of *MET25*, which encodes *o*-acetylhomoserine and *o*-acetylserine sulfhydrylase, resulted in a 10-fold decrease in hydrogen sulfide production in beer, in pilot-scale experiments (Omura et al. 1995). This can be explained by increased consumption of the substrate by the overproduced enzyme and by a decrease in sulfate uptake. An alternative approach was to partially or fully eliminate *MET10*, which encodes a putative sulfite reductase subunit. This led to a substantial reduction in hydrogen sulfide and the accumulation of sulfite. The beer produced showed increased flavor stability (Hansen and Kielland-Brandt 1996b).

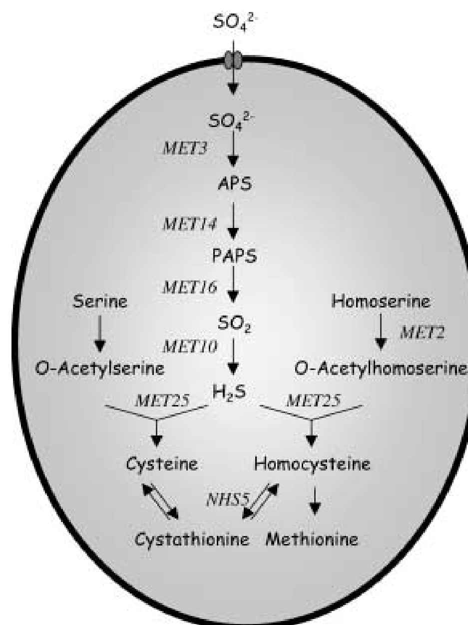

Fig. 3 Pathways for hydrogen sulfide and sulfite production in *S. cerevisiae*. *APS* Adenosine 5′-phosphosulfate, *MET2* serine acetyltransferase, *MET3* ATP sulphurylase, *MET10* sulfite reductase subunit, *MET14* APS kinase, *MET16* 3′-phospho adenosine 5′-phosphosulfate (*PAPS*) reductase, *MET25* *o*-acetylhomoserine or *o*-acetylserine sulfhydrylase, *NHS5* cystathionine β-synthase

Another sulfur compound that may cause organoleptic problems, especially for some lager beers, is dimethyl sulfide (DMS) which is produced during fermentation by reduction of dimethyl sulfoxide (DMSO). The *MXR1* gene was recently shown to encode a methionine sufoxide reductase (Hansen 1999). A *mxr1* disruption mutant was reported to be unable to reduce DMSO in laboratory conditions. This work opens up the construction of brewing strains that do not produce DMS.

Increased production of sulfur dioxide

Sulfite is an antioxidant and a key compound for the flavor stability of beer, because it combines with aldehydes. There has been extensive work to control the accumulation of sulfite by brewer's yeast. One approach, based on the overexpression of *MET3* and *MET14* in brewer's yeast, led to increased sulfite production (Korch et al. 1991; Fig. 3). Alternatively, brewer's yeast with several of the four copies of *MET2* inactivated was shown to produce higher amounts of sulfite (Fig. 3). However, hydrogen sulfide production was also increased (Hansen and Kiellandt-Brandt 1996a). As mentioned above, the inactivation of *MET10* was shown to be an efficient strategy for increasing sulfite formation and decreasing hydrogen sulfide production (Hansen and Kielland-Brandt 1996b).

Specific targets for wine yeast improvement

Malolactic fermentation

Alcoholic fermentation by pure-cultured wine yeast strains has become an increasingly well controlled process. In contrast, malolactic fermentation remains unreliable in numerous situations, due to the poordevelopment of lactic acid bacteria in wine. This secondary fermentation (decarboxylation of malate to lactate by the malolactic enzyme) has an essential role for the deacidification and stabilization of wine. Delayed or stuck malolactic fermentation leads to scheduling problems in cellars and increases the risks of wine alteration. To circumvent the problems of the unreliability of malolactic fermentation, *S. cerevisiae* strains able to degrade malic acid completely into lactic acid and carbon dioxide have been constructed. The malolactic gene from *Lactococcus lactis* has been cloned and efficiently expressed in *S. cerevisiae* (Ansanay et al. 1993a, b, 1996; Denayrolles et al. 1994, 1995). Due to the inability of *S. cerevisiae* to transport malate efficiently, the malate permease from *Schizosaccharomyces pombe* (Grobler et al. 1995) was coexpressed with the malolactic enzyme in *S. cerevisiae* (Bony et al. 1997b; Volschenk et al. 1997). The recombinant strains fully degraded up to 7 g of malate/l in 4 days, simultaneously with alcoholic fermentation and without affecting the growth properties and fermentation rate (Bony et al. 1997b). A careful comparison of wines, obtained on a pilot scale with engineered industrial strains and traditional malolactic fermentation, will be necessary.Attention will have to be paid to the impact on flavors, because bacterial malolactic fermentation has been described in some instances to improve the organoleptic complexity of wine. Similarly, it will be interesting to compare the advantages of both yeast and bacterial malolactic fermentation in terms of wine stabilization, because the composition of residual micronutrients might differ in the wines obtained by the two systems. Although the use of engineered yeast strains for malolactic fermentation to produce safer wines of high quality might compete with bacterial starter cultures in the future, the use of malolactic-engineered yeast would provide an additional advantage. Indeed, the elimination of secondary fermentation limits the risk of developing bacterial flora responsible for the alteration of wine and/or the production of undesirable metabolites (i.e. biogen amines).

Production of lactic acid

Achieving the correct balance between sugar and acidity is a major requirement for wine quality. In some hot regions, grape musts are often insufficiently acidic and the balance must be corrected. Wine acidification is usually accomplished by adding tartaric acid, which is authorized in certain specific situations and the efficiency of which is limited by the instability of the K-tartrate. Biological acidification using a lactic acid-producing *S. cerevisiae* strain is a promising alternative, due to the high stability and organoleptic properties of this organic acid. Moreover, lactic acid is naturally found in wines after malolactic fermentation. Therefore, *S. cerevisiae* strains performing a dual fermentation (ethanol and lactate) have been constructed by expressing a lactate dehydrogenase (*LDH*) gene from *Lactobacillus casei* (Dequin and Barre 1992, 1994). These strains can efficiently adjust the acidity of grape must: production of lactate at around 5 g/l increased total acidity by 0.2–0.3 pH units (Dequin et al. 1999). Consistent with the diversion of sugars towards lactate, the ethanol content of the wines obtained is slightly lower [approximately 0.25% (v/v) for 5 g lactate/l]. It is technically possible to construct a set of wine yeast strains producing different amountsof lactic acid across a range suitable to correct grape musts lacking in acidity. Lactate-producing yeasts are one of the most promising examples of wine-yeast improvement and may also be of interest in other fermentation fields (production of acidic doughs for baking, industrial lactate production).

Decrease of volatile acidity

The level of acetic acid, the main component of volatile acidity, is critical for the quality of wines. The concentration of acetic acid in wines is usually approximately 0.5 g/l and must remain below 0.8 g/l. Yeasts sometimes produce excessive acetic acid, due either to the genetic background of the yeast or to the wine-making processes (e.g. excessive clarification). Despite the importance of this organic acid, thegenetic basis of acetate production during alcohol fermentation is still largely unknown. It has recently been shown that the level of acetate produced by *S. cerevisiae* can be effectively controlled by increasing or decreasing the level of expression of the cytosolic acetaldehyde dehydrogenase encoded by *ALD6* (Remize et al. 2000). Inactivation of the two alleles of this gene in a wine yeast leads to a 2-fold reduction in the amount of acetate produced during wine fermentation. As aconsequence of the resulting redox imbalance, glycerol, succinate and 2,3-butanedediol production is slightly increased.

Improved polysaccharide degradation

The use of pectinases and glucanases in winemaking has several advantages. These enzymes can be used to facilitate wine clarification and to improve liquefaction of grapes, thereby increasing the juice yield. Polysaccharide-degrading enzymes may also enhance the liberation of various compounds trapped in grape skins, thereby improving the bouquet and color of the wine. As these commercial enzyme preparations are expensive, much effort has been made to develop yeast strains that secrete heterologous pectinases, glucanases, xylanases or a com-

bination of these enzymes (Pretorius 2000). For example, polypectate degradation was increased by a wine yeast co-expressing the *Erwinia chrysanthemi* pectate lysate gene and the *E. carotovora* polygalacturonase gene (Laing and Pretorius 1993). Another approach was based on the expression of the *Fusarium solani* pectate lyase gene (Gonzalez-Candelas et al. 1995). Glucanolytic wine yeast strains have been constructed by the expression of fungal and bacterial endo-β-1,4-glucanases, (Perez-Gonzalez et al. 1993; Van Rensburg et al. 1994), or a combination of endo- and exo-glucanases (Van Rensburg et al. 1997).

Improved liberation of varietal aroma

The bouquet of a wine is determined by a combination of many compounds, the production of which is influenced by grape variety, *terroir*, viticulture practices, enological practices and yeast strain fermentation conditions. Due to this complexity, only a small number of suitable targets can be defined. A well identified target for aroma produced by yeast metabolism is isoamyl acetate (described above). In addition to producing aromatic compounds, yeast can increase the liberation of varietal aroma by producing enzymes that hydrolyze non-aromatic precursors in the grape must. For example, various grape monoterpenols of (e.g. linalol, geraniol) are linked to diglycosides, which can be converted to monoglucosides by cleaving of the 1,6-osidic linkage. The flavor compound is then liberated by the action of a β-glucosidase. However, grape and yeast β-glucosidases are inhibited by glucose, are unstable at low pH and therefore poorly hydrolyze monoterpenyl-glucosides during wine fermentation. The addition of commercial preparations of fungal β-glucosidases after fermentation has been shown to improve the hydrolysis of the glycoconjugated aroma compounds. Progress in this area relies on the isolation of new glucose-tolerant β-glucosidases that are stable at low pH. Ahighly glucose-tolerant β-glucosidase has been purified from *Candida peltata* (Saha and Bothast 1996). A β-glucosidase from *A. oryzae* that is highly resistant to inhibition by glucose and is stable at low pH was recently described (Günata et al. 1997) and the corresponding gene cloned (Riou and Günata 1998; Riou et al. 1998). This enzyme has a broad-specificity, because it can hydrolyze 1,3-, 1,4-, and 1,6-β-diglycosidase and can release flavor compounds such as geraniol, nerol and linalol from the corresponding monoglucosides in a rich-glucose medium at pH 2.9.

An alternative approach for increasing the flavor of wine involves the modification of existing *S. cerevisiae* metabolic pathways associated with the production of aromatic compounds. Interestingly, mutants of the ergosterol biosynthetic pathways have been shown to produce monoterpenes (geraniol, citronelol, linalool) similar to those of the floral grape cultivars (Chambon et al. 1990; Javelot et al. 1990).

Reduced sulfide

Hydrogen sulfide is a highly undesirable compound in wine and is a product of the yeast's sulfur metabolism (Fig. 3). Hydrogen sulfide is produced during wine fermentation, mainly in response to the depletion of nitrogen and possibly certain vitamins; and its production is influenced by many environmental factors and by the yeast strain. In conditions of nitrogen starvation, hydrogen sulfide may accumulate and diffuse out of the cells (Jiranek et al. 1995). Wine yeast strains with low hydrogen sulfide production have been obtained by hybridization (Romano et al. 1985). Alternatively, reduced sulfide production might be achieved by manipulating the sulfate metabolic pathway, as described above for brewer's yeast. However, strategies such as the elimination of *MET10* cannot be used for wine yeast, because they result in an increased sulfite production. The final amount of sulfur dioxide in wine is regulated; and sulfur dioxide is currently added to grape must and wine as a preservative. Moreover, due to the current unfavorable public perception of sulfites, the tendency is to reduce its content in wine.

Specific targets for baker's yeast

Melibiose-utilizing strains

Molasses, a commonly used raw material for the production of baker's yeast, contains up to 8% raffinose in addition to sucrose. This trisaccharide (fructose/glucose/galactose) is hydrolyzed by yeast invertase to fructose and the disaccharide melibiose. Bakers' yeast cannot utilize melibiose, because it does not have α-galactosidase (melibiase), the enzyme responsible for the hydrolysis of melibiose into the fermentable sugars, galactose and glucose. However, the α-galactosidase enzyme is found in bottom-fermenting brewers' yeast strains. The *MEL1* gene encoding this enzyme has been cloned and transferred into baking strains (Liljeström-Suominen et al. 1988). Strains expressing *MEL1* on multicopy plasmids or integrated into the *LEU2* locus have been reported to secrete significant amounts of α-galactosidase. All available melibiose was utilized in a beet molasses medium, resulting in higher yeast yields (Liljeström et al. 1991; Liljeström-Suominen et al. 1988). Recombinant *MEL*+ bakers' yeast strains devoid of plasmid sequences have been developed by Gasent-Ramirez et al. (1995). These strains exhibit an 8% increase in biomass yield without alteration of the growth rate.

Maltose utilization

A high fermentation rate is a prerequisite for bakers' yeast strains. The free sugars present in the flour (sucrose, glucose, fructose, maltose) are sequentially consumed. Fermentation continues due to the action of amy-

lases present in the dough, which release maltose from starch. Maltose utilization by yeast requires a maltose permease and a maltase; and both are induced in the presence of maltose. A major factor limiting the dough fermentation rate is the repression of the synthesis of maltose-utilizing enzymes and the inactivation of the maltase enzyme by glucose (Needleman 1991). Low concentrations (1–2%) of free sugars (mainly glucose and fructose) in the dough repress maltose utilization, causing a lag phase in carbon dioxide production. To avoid this lag phase, maltose-utilizing enzymes have been derepressed by replacing the native promoters of the maltase and maltose permease with constitutive promoters (Osinga et al. 1988).

Baker's yeast strains display intrinsic differences in their rates of maltose utilization. Non-lagging bakers' strains are characterized by rapid maltose fermentation, unlike lagging strains, which exhibit a lag phase. It has been reported that the non-lagging phenotype depends on a high level of maltase expression in the presence of glucose (Oda and Ouchi 1990). More recently, it has been suggested that non-lagging strains display higher constitutive basal levels of expression of *MAL* genes than lagging strains, under non-inducing and non-repressing conditions (Hazell and Attfield 1999; Higgins et al. 1999). It has been proposed that these differences are due to divergences in the genetic structure of the *MALX3* gene, which codes for an activator of the other *MAL* genes. Therefore, expression of a *MALX3* gene isolated from a non-lagging strain in a lagging strain might improve maltose metabolism in unsweetened dough (Higgins et al. 1999).

Other approaches to increase the fermentative capacity

Several attempts have been made to increase the glycolytic flux. Various control steps have been suggested, including sugar uptake, the hexokinase, the phosphofructokinase and the pyruvate kinase. However, overexpression of a combination of these enzymes and other glycolytic enzymes failed to increase the glycolytic flux (Schaaff et al. 1989). An alternative strategy was based on the hypothesis that a decrease in the intracellular ATP concentration would result in an increased carbondioxide production rate. This was tested by derepression of the ATP-consuming gluconeogenic enzymes, fructose 1,6-diphosphatase and phosphoenolpyruvate carboxykinase (Navas et al. 1993). Although no evidence could be obtained of functional futile cycling, this resulted in a 25% increase in the rate of glucose consumption. This strain also displayed a significant reduction in biomass yield. Recently, recombinant strains, simultaneously overexpressing a set of seven enzymes involvedin the lower part of glycolysis, were constructed. The recombinant strain exhibited increased glycolytic flux, but only under conditions of increased ATP demand (Smits et al. 2000).

Conclusion

Considerable progress has been made during the past two decades in the development of industrial strains possessing optimized and new characteristics. For the most advanced examples, genetically modified industrial strains have been constructed according to the general requirements for genetically modified organisms (GMOs), particularly the absence of resistance markers and stability (usually obtained by chromosomal integration); and the new properties have been confirmed on a pilot-scale. However, despite the remarkable progress during the past 20 years, only two have so far received official approval (both from the British government) for commercial use, but they are not currently used commercially. The first is a baker's yeast derepressed for maltase and maltose permease (Aldhous 1990). The second is a brewer's yeast gene expressing the *STA2* gene and producing exocellular glucoamylase (Hammond 1995).

Public acceptance considerations remain the major obstacle to the commercialization of genetically modified industrial yeast strains. One of the major difficulties is that the benefits of genetically modified yeast strains are in most cases not perceptible to consumers, except for some nutritional or hygiene advantages.

Efforts to inform and to discuss with the general public have been limited and need to be developed to increase public awareness of the potential benefits (safe production, high quality/low cost) of recombinant DNA technology. The acceptability of GMOs in foodwill also depend on the presence (e.g. bread, wine) or absence (e.g. beer, filtered wines) of the GMO in the product. Detection methods will have to be developed to differentiate these two types of product. The debate should also include aspects concerningthe practical consequences of the introduction of this new technology for the industry. For example, the risks associated with GMO release. Each industrial field may have to be considered separately and specific approaches may have to be defined and implemented for each.

The end of the twentieth century has been marked by the explosion of life technologies, in particular those related to genomics. *S. cerevisiae* recently became the first eukaryotic microorganism whose entire genome has been sequenced (Goffeau et al. 1996). The challenge for the next years will be to expand the knowledge and data obtained for laboratory strains of *S. cerevisiae* in laboratory conditions to industrial strains and conditions. The genome of industrialyeast is more complex (e.g. alloploidy, polyploidy, aneuploidy, chromosomal rearrangements) than that of laboratory strains. Furthermore, these strains are exposed to many environmental stresses, e.g. alcohol concentration, desiccation, high osmolarity, freezing and nitrogen depletion, either simultaneously or sequentially. Their adaptation and response to these various stresses is largely unknown (Attfield 1997; Bauer and Pretorius 2000). Several commercialtraits (fermentation capacity, metabolite production, stress tolerance, etc.) are under multigenic control and their molecular

bases are largely unknown. New possibilities are now available for exploring the metabolic and genetic control of gene expressionon a genomic scale (DeRisi et al. 1997; Wodicka et al. 1997). The development of functional genomic approaches in relevant industrial conditions will obviously help us to identify genes that are regulated coordinately, transcription factors that control metabolism and relevant technological properties. Besides a huge increase in the knowledge of the adaptation mechanisms developed by industrial strains, new targets for genetic engineering can be expected.

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