

Improvement of *Saccharomyces* Yeast Strains Used in Brewing, Wine Making and Baking

Ute E. B. Donalies¹ · Huyen T. T. Nguyen¹ · Ulf Stahl¹ · Elke Nevoigt^{1,2,3} (✉)

¹Department of Microbiology and Genetics, Berlin University of Technology,
 Seestr. 13, 13353 Berlin, Germany

²Laboratory of Molecular Cell Biology, Institute of Botany and Microbiology,
 Katholieke Universiteit Leuven, Kasteelpark Arenberg 31 – bus 2438,
 3001 Heverlee, Flanders, Belgium

³Department of Molecular Microbiology, VIB, Kasteelpark Arenberg 31 – bus 2438,
 3001 Heverlee, Flanders, Belgium
elke.nevoigt@bio.kuleuven.be

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Abstract Yeast was the first microorganism domesticated by mankind. Indeed, the production of bread and alcoholic beverages such as beer and wine dates from antiquity, even though the fact that the origin of alcoholic fermentation is a microorganism was not known until the nineteenth century. The use of starter cultures in yeast industries became a common practice after methods for the isolation of pure yeast strains were developed. Moreover, effort has been undertaken to improve these strains, first by classical genetic methods and later by genetic engineering. In general, yeast strain development has aimed at improving the velocity and efficiency of the respective production process and the quality of the final products. This review highlights the achievements in genetic engineering of *Saccharomyces* yeast strains applied in food and beverage industry.

Keywords Baker's yeast · Brewer's yeast · Genetic engineering · Metabolic engineering · *Saccharomyces cerevisiae* · Strain improvement · Wine yeast

1

Introduction

Beer, wine and bread have been produced with the help of microorganisms for thousands of years. Yeast has been found to be responsible for the occurrence of alcoholic fermentation, i.e., the production of ethanol and carbon dioxide from fermentable sugars during brewing, wine making and dough leavening. Apart from these major products, there are a huge number of other minor byproducts of yeast metabolism that determine the flavor of the different products. Indeed, yeast strains used in brewing, wine making and baking differ from each other in terms of their typical characteristic fermentation properties and byproduct formation, even though virtually all belong to *Saccharomyces cerevisiae* or are closely related to this species.

It is strongly assumed that yeast strains which are currently used in these industries are domesticated due to their long-term association with human-induced fermentation. Nevertheless, it is still controversial whether *S. cerevisiae* strains occasionally occurring in nature are derived from domesticated strains or vice versa [1, 2]. Indeed, there is some thought that all *S. cerevisiae* strains originally derive from close relatives occurring in nature. However, several recent genomic studies seem to invalidate this hypothesis by identifying natural *S. cerevisiae* populations not associated with human alcoholic beverage production [3].

As humans have carried out alcoholic fermentation over such a long period of time, it is not surprising that yeast was one of the first microbes to be

studied scientifically. A very comprehensive series of reviews regarding the history of yeast research has been provided by Barnett and colleagues [4–15]. The most important milestones are summarized as follows. The first investigations that suggested that yeast causes fermentation and is a microorganism were published between 1836 and 1838. At this time Schwann [16] identified yeast as a fungus (“Zuckerpilz”). Julius Meyen defined the genus *Saccharomyces* (Latin form of Schwann’s *Zuckerpilz*) in 1838 comprising three species, *S. cerevisiae*, *S. pomorum* and *S. vini*, their names simply indicating where they were found: in beer, fermenting apple juice and wine. The “microbial theory” of alcoholic fermentation evoked a remarkable attack by some leading chemists for a long period of time [17]. It took about 20 years until Pasteur’s study led, once and for all, to the general acceptance of the fact that alcoholic fermentation was a process performed by living organisms [18]. Another milestone was the work of Emil Christian Hansen, who developed techniques for obtaining pure yeast cultures in the early 1880s. He named a number of yeast species and made yeast taxonomy a practical proposition. Since that time, the use of isolated yeast strains as starters in different industrial processes has become possible, a practice which is nowadays the rule rather than the exception. Several companies have even established and evolved their own strains in order to guarantee and improve their products quality.

Although it has not often been obvious to consumers, there has been always a demand to improve yeast strains used in brewing, wine making and baking. However, due to the complex genetic structure of industrial yeast strains (see below), mating of industrial yeast strains has been a challenge and often simply not possible. In such cases, a direct analysis of tetrads and the isolation of recessive mutations are not possible. A number of alternative methods of genetic improvement such as rare mating, protoplast fusion, cytoduction and mutagenesis followed by laborious strain selection have been therefore applied for improvements of industrial yeast strains [19].

Owing to the rapid development in recombinant DNA technology (genetic engineering), directed improvements of distinct traits of brewer’s, wine and baker’s yeast strains have become possible. Recombinant DNA technology enables the targeted manipulation of a cell’s genetic information without the accumulation of disadvantageous mutations. The possibility of introducing or deleting genes within yeast chromosomes has even been facilitated the determination of the complete genome sequence of *S. cerevisiae* in 1996 [20].

When genetic engineering for strain improvement targets the modification of metabolic pathways (i.e., “enzymatic, transport and regulatory functions of the cell”), it is considered to be metabolic engineering [21, 22]. Most improvements of brewer’s, wine and baker’s yeast strains covered in this review are metabolic engineering approaches; however, there are a few cases such as the modification of flocculation or improved stress tolerances where it can be-

come tricky to decide whether the genetic modification can be assigned to metabolic engineering. We have used the term genetic engineering throughout this review for simplicity.

In order to improve relevant traits of yeast strains, a strategy for genetic engineering has often been conducted and first applied in laboratory strains. Indeed, it has been much easier to genetically modify well-characterized haploid laboratory strains than complex industrial yeast strains. Laboratory strains have been derived from naturally occurring *S. cerevisiae* strains and carry mutations or genetic modifications that make them superior for classical and molecular genetics, e.g., they prevent mating type switching (heterothallic strains) or result in a strain's auxotrophy for certain nutrients [23, 24]. Moreover, they are mostly haploid or diploid. This is in contrast to many industrial *Saccharomyces* strains used in baking, brewing and wine making. The latter strains are often homothallic¹, polyploid, aneuploid or even allopolyploid, and sporulate poorly or not at all [25–27]. Compared to laboratory yeast strains of *S. cerevisiae*, it has been much less efficient to transform industrial yeast strains and establish genomic integrations by homologous recombination. Moreover, such strains are usually not auxotrophic for certain nutrients, and require the use of dominant genetic markers, such as genes conferring resistance to antibiotics or toxic agents. Another possibility is the use of genes which enable the utilization of a substrate such as starch or melibiose that does not belong to those naturally used by *S. cerevisiae* (see Sects. 4.1.2 and 4.1.3).

When a genetic engineering approach has been shown to work well in laboratory yeast strains, it does not necessarily mean that the strategies will also work in industrially relevant conditions. As mentioned above, the genetic background of industrial yeast strains is different to those of laboratory strains; in addition, the relevant practical conditions also strongly differ from laboratory conditions (e.g., brewer's wort or grape must is used instead of the mineral or complex media usually used in the lab). In fact, several cases are known in which a strategy worked well in the laboratory but not in industrial yeast strains [28, 29].

This review gives an overview of the genetic and physiological properties of baker's, wine-maker's and brewer's yeast strains, and highlights the strategies used to improve them by means of genetic engineering. The improvement of both process performance and product quality has been the major goal in industrial yeast strain improvement. Although there are application-specific targets that allow for the specific nature of the product, there has also been a certain amount of overlap between efforts to improve baker's, brewer's and wine yeast strains.

¹ Haploid *S. cerevisiae* cells exist in two different mating types, a or α . The yeast can be either genetically stable (heterothallic yeast) or they can change their mating type (homothallic yeast).

2

Genetic Characteristics of Baker's, Wine and Brewer's Yeast Strains

According to recent taxonomic nomenclature, virtually all industrially used baker's, wine and brewer's yeast strains belong to the *Saccharomyces sensu stricto* species [30,31]. Baker's yeast, many wine starter yeast strains and top-fermenting brewer's yeast (ale yeast) have been assigned to the species *Saccharomyces cerevisiae* [32–35]. Thus, their genetic information should have a high degree of similarity to laboratory strains and the published genomic sequence of *S. cerevisiae* (obtained from a laboratory strain) [20]. This means that commercial *S. cerevisiae* microarrays can be used for global expression studies, and gene/protein sequences published in public databases such as *Saccharomyces* Genome Database (SGD) [36] and Comprehensive Yeast Genome Database (CYGD) [37] are useful tools for analysis (e.g., transcriptomics and proteomics) and engineering these strains. The situation is different for some wine yeast strains and lager brewer's yeast. Several industrially used wine yeast strains belong to *S. bayanus* or *S. uvarum* [38]. Bottom-fermenting (lager) brewer's yeast originally known as *S. carlsbergensis* according to Hansen [39] were later recognized as part of the *S. pastorianus* species [40].

In terms of their genetic constitution, bottom-fermenting (lager) brewer's yeast are most striking among industrial yeast strains. Several genetic studies have revealed lager brewer's yeast as being a natural allopolyploid interspecies hybrid between *S. cerevisiae* and a non-*S. cerevisiae* yeast. This is in contrast to top-fermenting (ale) yeast which is a diverse group of polyploid yeast strains but closely related to laboratory strains of *S. cerevisiae* [41]. Interestingly, the results of the different studies, which aimed at identifying the second "parent" of lager brewer's yeast, seemed to be contradictory at first glance. For instance, investigations of the restriction fragment polymorphism as well as analysis of the sequence of distinct non-*Saccharomyces* genes pointed to *S. monacensis* being the origin of the second genome of lager brewer's yeast [41–43]. The measurement of the DNA homology between *S. cerevisiae* and other yeast strains suggested that the second genome originates from *S. bayanus* [44, 45]. Two-dimensional (2-D) gel electrophoresis of the proteomes of lager brewer's yeast and type-strains of *S. bayanus*, *S. uvarum*, *S. carlsbergensis*, *S. monacensis* and *S. pastorianus* highlighted the fact that *S. carlsbergensis*, *S. monacensis* and *S. pastorianus* are hybrid yeast strains themselves, also containing *S. cerevisiae*-like proteins [46]. This at least explained the partial homology of lager brewer's yeast with *S. monacensis*. Based on these data, it was even trickier to explain the previously found partial homology to *S. bayanus*. Later, it was found that obviously different strains of *S. bayanus* exist, a few being hybrids containing genes homologous to *S. cerevisiae* and others that do not contain parts of the *S. cerevisiae* genome. The latter strains' (*S. bayanus* isolates IFO539 and IFO1948) might constitute "pure" genetic lines and could probably rep-

resent relatives of the second ancestor of lager brewer's yeast [47]. However, the origin of lager brewer's yeast will certainly continue to be a matter of discussion in the future.

There have been several studies of lager brewer's yeast using *S. cerevisiae* DNA microarrays [48, 49]. When discussing these data, one should however always bear in mind that this method has been a compromise due to the lack of non-*S. cerevisiae* gene sequences. A breakthrough in the molecular analysis of lager brewer's yeast has been the identification of the whole genomic sequence of a commonly used lager brewer's yeast strain, i.e., Weihenstephan No. 34 (34/70) [50]. As announced by the authors, this laid the basis for the development of DNA microarrays that are specifically designed for lager brewer's yeast, allowing reliable gene expression analyses and detailed studies of the genomic constitution of other lager brewer's yeast strains. Although the sequence has not yet been made publicly available, the exact chromosome structure of Weihenstephan No. 34 (34/70) has been published [50, 51]. These data confirmed nicely once and for all the hybrid character of lager brewer's yeast and the coexistence of three types of chromosomes: (1) pure *S. cerevisiae*-type, (2) pure non-*S. cerevisiae*-type and (3) mosaic-type chromosomes, the latter containing parts of both origins.

3

Physiological Properties of Industrial Yeast Strains

3.1

Baker's Yeast

Baker's yeast is responsible for dough leavening by CO₂ production from fermentable sugars, but also influences the taste and texture of bread and other bakery products. Wheat flour is composed mainly of starch, but also of maltose, sucrose, glucose, fructose and glucofructans. Maltose is continuously released from starch during the baking process due to the activity of crop amylases [35, 52]. Efficient fermentation of dough sugars, in particular maltose, by yeast cells is crucial for dough leavening. Moreover, it has been regarded as favorable if baker's yeast could be engineered to directly use starch, the major carbohydrate in dough. With regard to the desired traits of baker's yeast it is, however, not sufficient to solely look at its performance during dough leavening. The production of baker's yeast itself is also an important industrial branch. In this case, the fast propagation of baker's yeast is desired with high biomass yields related to the carbon sources available in the growth medium, which has mainly been molasses. Moreover, a high tolerance to stresses caused by high sugar concentration (sweet dough), drying and freezing (production of dried yeast and frozen dough, respectively) is necessary.

3.2

Wine Yeast

Starter strains in wine making are usually distributed in dried form. Therefore, fast and efficient growth during propagation and high tolerance to drying are important traits that have been already mentioned in the context of baker's yeast. For the wine-making process itself, the pattern of fermentation products determining wine flavor and body is most important to obtain good and reproducible product quality. Moreover, other traits are desirable, such as efficient use of the nutrients in grape must, high ethanol tolerance, optimal flocculation and prevention of contamination. Reduction of wine acidity is another target of yeast engineering. Often, grapes from colder regions have a high concentration of malic acid.

3.3

Brewer's Yeast

Brewer's wort is composed of 25% nonfermentable carbohydrates, mainly dextrans of varying length. Apart from dextrans, wort consists of fermentable monosaccharides (fructose, glucose), disaccharides (maltose, sucrose) and oligosaccharides (maltotriose). Maltose is the major fermentable sugar followed by maltotriose. Efficient utilization of maltose and maltotriose is therefore as important as it is in baking (see above).

In brewing, freshly propagated yeast is usually used for fermentation. A crucial factor for product quality is to inoculate beer fermentation with cells that are well adapted to the conditions used [53, 54]. Cells propagated up to the late logarithmical growth phase in wort, under conditions similar to brewing, are best at fulfilling this requirement. When cells from the stationary phase or even dried yeast were used, the product of the first batch of beer would be much less tasty due to unusual byproduct patterns. Indeed, this metabolic pattern strongly depends on yeast growth conditions and a well-balanced pattern of yeast metabolic products, such as esters and higher alcohols, is extremely important for beer flavor [54]. Brewing fermentations, in particular lager brewing fermentations, are usually carried out at low temperatures of about 8–15 °C. This is important for a well-balanced beer flavor. Higher growth would result in greater production of byproducts of yeast anabolism accompanied by off-flavors.

The production of lager beer requires a long period (up to 3 weeks) for maturation. Green beer contains buttery flavored diacetyl in a concentration above the accepted taste threshold. Diacetyl is a byproduct of yeast anabolism. Reducing yeast's diacetyl formation and shortening the maturation period is a major requirement in order to maximize a brewery's capacity in beer production.

4

Approaches to Optimize Industrial Yeast Strains Relevant in Baking, Wine Making and Brewing

4.1

Improving and Extending the Utilization of Available Substrates in Industrial Media

4.1.1

Improvement of Sucrose, Maltose and Galactose Utilization: Alleviation of Glucose Repression

Improvements in yeast growth in industrial media such as molasses have been important for both baker's and wine yeast propagation. Molasses is a mixture of sugars such as glucose, fructose, sucrose, raffinose, melibiose, and galactose. Although it is not the major sugar, glucose is always present in complex industrial media and, even at a very low concentrations, prevents the simultaneous utilization of other sugars such as maltose and galactose. This effect has been referred to as "glucose repression" and results in lengthened process times. Apart from being important during yeast propagation in molasses, glucose repression of maltose utilization also occurs during dough leavening and brewing fermentation, as maltose is the most important fermentable sugar in both dough and brewer's wort.

Although much progress has been made in understanding the molecular basis of glucose repression in yeast, it has not yet been completely resolved. It is generally accepted that glucose repression exerts its regulation at the level of transcription by binding regulatory proteins to the promoter region of the regulated genes. Transcriptional repression is caused by a protein complex consisting of Mig1p, Ssn6p, and Tup1p. Ssn6p and Tup1p are responsible for the actual inhibition, whereas Mig1p recruits the SSN6-TUP1 complex to glucose-repressed promoters [55]. Mig1p is a zinc finger protein, whose binding site contains a GC-box with the consensus sequence $G/C \text{ } ^C/TGGGG$. Binding sites for Mig1p were found in the promoter region of numerous glucose-regulated genes, including those encoding enzymes involved in utilization of sucrose (*SUC2*), maltose (*MALR*, *MALS*, *MALT*) and galactose (*GAL1/3/4/5*) [56]. Hence, *MIG1* has been a promising target to alleviate glucose repression in industrial yeast strains [52].

Sucrose is mainly found in molasses. It is converted to glucose and fructose by the enzyme invertase which is encoded by *SUC2* and secreted into the extracellular medium. The disruption of *MIG1* led to alleviation of glucose repression of *SUC2* in both laboratory and industrial strains. Hence, the lag phase of sucrose consumption in the presence of glucose was significantly reduced [56]. In addition to Mig1p, another zinc finger protein (Mig2p) has been shown to contribute to glucose control of *SUC2* [57]. Compared to

a *mig1* disruptant, an isogenic laboratory *S. cerevisiae* strain deleted in *MIG1* and *MIG2* showed an even greater ability to consume sucrose in the presence of high glucose concentrations [58].

Maltose is the major fermentable sugar in molasses, brewer's wort and dough. This disaccharide is transported into the cell by maltose permease, an active transporter. It is subsequently hydrolyzed into two glucose units and further metabolized by the central carbon catabolism. The *MAL* genes are centralized within the so-called *MAL* loci. One *MAL* locus contains three genes: *MALR*, *MALT* and *MALS*, encoding a transcriptional regulatory protein, maltose permease and maltase, respectively. The expression of these genes is repressed by glucose via *MIG1* and induced by maltose. Glucose influences gene expression regulation both at transcriptional level, and the turnover rate of mRNA [59]. Five *MAL* gene loci exist in industrial strains of *S. cerevisiae*, whereas haploid laboratory yeast only contain one *MAL* locus, which is obviously sufficient for maltose utilization [60].

Expression of *MAL* genes is under dual control: repression by glucose and induction by maltose. Induction is mediated by *MALR*, and Mig1p is involved in glucose repression and also controls *MALR* expression. The deletion of *MIG1* was able to alleviate glucose repression of *MAL* genes, although only in a haploid laboratory yeast strain. Several polyploid industrial strains were also deleted in *MIG1* and an alleviation of glucose repression was found only for sucrose and not for maltose [28]. One of the main differences between genes involved in sucrose and maltose utilization is that the first does not need induction by sucrose, whereas all *MAL* genes require the presence of maltose as an inducer [60]. It has been clearly shown that the polyploid *mig1* disruptants showed increased levels of *MALS* and *MALT*; hence, the stricter glucose control of maltose utilization must be caused by some other factor. There has been some discussion that the inactivation of maltose permease by glucose is the limiting factor for maltose utilization by *mig1* deletion strains in the presence of glucose [28, 56]. There is no plausible explanation for the different effects of *MIG1* deletion in haploid and polyploid strains, except that industrial yeasts are polyploid, i.e., they possess different *MAL* loci, whereas laboratory strains possess only one. One has to mention in this context that there have been other strategies to alleviate the lag in maltose consumption in media containing glucose [35]. For example, *MALS* and *MALT* can be placed under the control of constitutive promoters [61].

Galactose is present in molasses, cheese whey, and lignocellulose. The latter industrial media came into focus as an alternative for molasses in yeast propagation [28, 52, 58, 62]. Galactose is taken up by galactose permease encoded by *GAL2* and is then converted to glucose-6-phosphate by the enzymes of the Leloir pathway encoded by *GAL1*, *GAL7*, *GAL10* and *GAL5*. All of these *GAL* genes are subject to complex regulation involving the regulatory proteins Gal4p, Gal80p and Gal3p [56]. Similarly to maltose, utilization of galactose is under dual control, i.e., repression by glucose and induction by galactose.

Several *GAL* genes (*GAL1*, 3, 4 and 5) have been shown to be regulated by Mig1p at transcriptional level. Gal4p is responsible for the transcriptional activation of the *GAL* genes by galactose and Gal80p prevents the action of Gal4p as long as galactose is absent. The use of galactose by *S. cerevisiae* in industrial media encounters two problems: (1) glucose repression of *GAL* genes and (2) a low rate of galactose metabolization, significantly lower than in glucose (even when glucose is absent). Deletion of *MIG1* led only to a slight alleviation of glucose repression on galactose utilization. To improve galactose utilization, *GAL80* was disrupted in addition to *MIG1*. The disruption of *GAL80* led to a constitutive expression of the *GAL* genes [63].

As mentioned above, galactose is utilized very slowly even in the absence of glucose. This problem has recently been solved to a large extent. By means of inverse metabolic engineering, it has been shown that simply an increased level of *PMG2*, encoding the major isoenzyme of phosphoglucumutase, led to a 74% increase in flux through the Leloir pathway [59].

Recently, it has been suggested that glucose repression is mediated by inositol and phosphatidylinositol (PI) [64]. An increase in PI content may cause phosphorylation of Mig1 by a PI-type signaling pathway and its translocation to cytoplasm where it exerts glucose repression. Even though the mechanism of this regulation has not been completely clarified, it opens up a new avenue for the manipulation of glucose repression in yeast.

4.1.2

Utilization of Oligosaccharides such as Raffinose, Melibiose and Maltotriose

Apart from glucose repression, another major problem occurs during yeast propagation in molasses: the oligosaccharides raffinose and melibiose cannot be used by either baker's or wine yeast. Thus, waste streams still contain valuable carbon sources.

Raffinose, a component of molasses (8% of total sugar), is a trisaccharide that is cleaved by the enzyme invertase encoded by *SUC2* (see Sect. 4.1.1) into melibiose and fructose. Melibiose can be cleaved into glucose and galactose by the enzyme melibiase. However, the expression of melibiase encoded by *MEL1* is a specific feature of lager brewer's yeast (*S. pastorianus*) and most baker's yeast strains (*S. cerevisiae*) do not have this gene. Furthermore, *MEL1* expression is also controlled by glucose repression [56]. Therefore, efforts have been aimed at both expressing melibiase in *S. cerevisiae* industrial yeast strains and deregulating the glucose repression of *MEL1*. Both targets have been pursued at the same time by integrating the *MEL1* gene into the genomic loci of *MIG1* and *GAL80* [65]. This strategy resulted in a *S. cerevisiae* strain able to efficiently hydrolyze melibiose.

Another oligosaccharide which is important in brewing fermentation is maltotriose. This trisaccharide accounts for 15–20% of the total sugars in

brewer's wort and is the second most abundant sugar, apart from maltose (50–60%). Maltotriose is only utilized in the later stages of wort fermentation, a fact which is even more manifest in ale yeast. Several transporters are known that account for maltotriose uptake in *S. cerevisiae* [66, 67]. Recently, the *MTTI* gene has been determined to encode another maltotriose transporter, and its overexpression in a lager brewing strain resulted in a significant increase of maltotriose uptake [68].

4.1.3

Utilization of Polysaccharides such as Starch and Dextrins for Improved Dough Leavening and Low-Calorie Beer

Starch is the storage protein in plants and it is made up of glucose molecules joined by α -1,4 linkages (amylose) and additionally joined at branch points by α -1,6 linkages (amylopectin). Starch is the major carbohydrate in dough. Dextrins represent degradation products of starch, i.e., a mixture of large fragments. These starch degradation products result from partial hydrolysis. This hydrolysis is facilitated by the mashing step during brewer's wort production. As mentioned above, nonfermentable dextrins make up about 25% of the carbohydrates in wort. The dextrins in brewer's wort are important for fullness (body) of beer. Nonetheless, utilization of dextrins by brewer's yeast is required when it comes to the production of low-calorie beer.

Neither baker's nor brewer's yeasts are able to hydrolyze starch or dextrins, as they do not excrete starch-decomposing enzymes such as α -amylase (which cleaves α -1,4 glycosidic bonds) and isoamylases (debranching enzymes that hydrolyze α -1,6 glycosidic bonds) [69]. Therefore, α -amylases are often added to dough in the baking industry. In order to produce low-calorie beer, enzymes such as amyloglucosidase from *Aspergillus niger* were added [70]. These practices could be omitted if baker's and brewer's yeasts were provided with starch/dextrin-decomposing enzymes.

S. diastaticus has amylolytic activity, i.e., it is able to excrete amyloglucosidase into the surrounding medium. Amylolytic hybrids between brewer's yeast and dextrin-fermenting yeast have been generated by classical genetic methods. However, some undesirable characters of *S. diastaticus* have also been transferred into the brewing yeast. Thus, for example, the *POF1* gene was cotransferred, leading to the ability to decarboxylate ferulic acid causing a phenolic off-flavor [70]. This effect could be avoided by directly transferring the *DEX1* (= *STA2*) gene of *S. diastaticus*, encoding the glucoamylase into brewer's yeast using recombinant DNA technology. The transformants produced extracellular glucoamylase and showed an improved degradation of carbohydrates [71, 72]. The *S. diastaticus* glucoamylase encoded by *STA2* does not have α -1,6-debranching activity. To obtain the complete degradation of dextrins, an enzyme able to hydrolyze the α -1,4 as well as the

α -1,6 linkages (amyloglucosidase) has therefore been regarded as advantageous. Such an enzyme was identified in *A. niger*. The gene was cloned and integrated into the genome of brewer's yeast [73]. Gopal and Hammond [74] carried out brewing experiments with brewer's yeast secreting the *A. niger* enzyme and demonstrated half of wort dextrins being degraded. One crucial disadvantage of the amyloglucosidase from *A. niger* is its very high thermostability. Thus, it is still active after pasteurization and the beer may become sweet after storage. The amyloglucosidase from *Schwanniomyces occidentalis* is less heat stable. Lancashire et al. [75] isolated this amyloglucosidase gene (*GAM1*) and transferred it into brewer's yeast under the control of the *ADH1* promoter. The resulting transformants were able to degrade dextrins very well and the enzyme was inactivated after pasteurization.

Amylolytic baker's yeast that is able to degrade starch was generated by a similar strategy. For example, Hollenberg and Strasser [76] have transformed industrial *S. cerevisiae* strains with the amyloglucosidase gene (*GAM1*) in combination with the α -amylase gene (*AMY1*) from *Schwanniomyces occidentalis*.

Some other amylolytic brewer's yeast strains have also been constructed. However, such recombinant industrial yeast strains often contained antibiotic (and/or drug) resistance markers from bacteria or yeast that have pathogenic relatives and thus have not been acceptable for applications in the food industry. There has indeed been a general low consumer acceptance of genetically modified organisms, as reviewed in detail using wine yeast as an example [77]. This has always posed a challenge to scientists who work on the genetic modification of industrial yeast strains. Recently, Liu et al. [78] constructed an amylolytic brewer's yeast, *S. pastorianus*, that was free of vector sequence and drug-resistant genes, bearing α -amylase of *Saccharomycopsis fibuligera* [79, 80]. This brewer's yeast was able to utilize starch as its sole carbon source.

4.1.4

Degradation of β -Glucan, Pectin and Xylan for Better Filterability of Beer and Wine

Barley cell walls contain β -glucan, polysaccharides containing β -1,4- and β -1,3-linked glucose molecules. These linkages are cleaved by specific endo- β -glucanases from barley during the malting process. Depending on the conditions used, β -glucan degradation is often incomplete. The enzyme from barley is extremely thermolabile and, due to high temperatures during the drying of malt and mashing, little active β -glucanases are present in wort. Thus, β -glucan is often found in beer, where it reduces filterability by the for-

mation of gels and blocks the filter. Moreover, β -glucan is found in the final product and causes hazes [70, 81].

One can add isolated microbial β -glucanases to mash and wort, respectively, to improve the filterability of beer. Meanwhile, the genes encoding β -glucanases have been cloned from bacteria and fungi, characterized and expressed in brewer's yeast. Cantwell et al. [82] expressed a β -glucanase-encoding gene from *Bacillus subtilis* under the control of the *ADH1* promoter in yeast. However, very little enzyme was secreted by the engineered yeast. Lancashire and Wilde [83] fused the *B. subtilis* gene to the promoter and signal sequence of the *S. cerevisiae* α -factor, a naturally secreted enzyme. Brewer's yeast transformed with this expression cassette secreted functional β -glucanase into the medium, which reduced the β -glucan content of the wort during fermentation.

Enari et al. [84] expressed the *Trichoderma reesei* β -glucanase gene under control of the *PGK1* promoter. Specific yeast-secretion signal sequences were not required, since fungal extracellular enzymes are efficiently excreted by the yeast [85]. Wort β -glucan was effectively degraded and beer filterability was remarkably improved in brewing experiments using the transformed yeast strain [84, 86]. The heat-labile β -glucanase from barley, which is responsible for β -glucan degradation during malting and mashing was fused downstream of a mouse α -amylase-secretion signal sequence and expressed in *S. cerevisiae* under the control of the *ADH1* and *PGK1* promoters, respectively [87, 88]. Brewer's yeast, transformed with this construct, produced beer with reduced β -glucan content in brewing experiments [81]. Recently, the β -glucanase of *Rhizopus microsporus* var. *microsporus* was characterized [89]. This enzyme was shown to be able to hydrolyze barley β -glucan and its maximum activity was detected at temperatures of 50–60 °C. This enzyme was also able to reduce both the viscosity of the brewing mash and the filtration time, and is thus a promising candidate for industrial application.

Glucan, pectin and xylan are polysaccharides that also hamper wine clarification. These polysaccharides are derived from grapes or formed by other microorganisms, respectively. They are responsible for turbidity, viscosity and filter stoppages. Pectinases and glucanases have very often been added to the grape must during wine making in order to improve filterability [25]. Some recombinant yeast strains have been developed that are able to express and secrete these enzymes. For example, a glucanolytic wine yeast was developed expressing fungal or bacterial β -1,4-glucanases [90, 91]. Recently, recombinant wine yeast strains have been constructed that were able to degrade the problem-causing grape polysaccharides, glucan and xylan, by separately integrating the *Trichoderma reesei* XYN2 xylanase gene and the *Butyrivibrio fibrisolvens* *END1* glucanase into the genome of a commercial wine yeast strain of *S. cerevisiae* [92].

4.1.5

Improved Utilization of Nitrogen Sources

Apart from the improvement of carbon assimilation, nitrogen uptake has also been challenged. Proline is the most abundant amino acid in brewer's wort and is, oddly enough, the only amino acid that is virtually unassimilated during brewing fermentation. A lager brewer's yeast expressing a modified version of the *PUT4* gene, encoding a highly specific proline permease, was able to efficiently assimilate proline from brewer's wort without any negative impact on beer quality [93]. The site-directed mutagenesis of the Put4 protein resulted in a less degradable form of the permease.

The efficient use of nitrogen sources has also been a major focus for wine yeast improvement, since there is an imbalance between the high level of carbon and low level of nitrogen sources in grape must [94]. Again, proline (together with arginine) accounts for 30–65% of grape amino acids and is also barely utilized during wine fermentation [33]. Salmon and Barre [95] deleted the *URE2* gene encoding a repressor of the *PUT1*-encoded proline permease and the *PUT2*-encoded pyrroline-5-carboxylate dehydrogenase. The mutant strains produced more biomass and exhibited a higher maximum CO₂ production rate than the wild type. An additional effect of nitrogen starvation is the limitation of sugar uptake. Thus, sugar transporters have been assumed to be interesting targets for strain optimization; however, a breakthrough has not yet been achieved in this field [33].

4.2

Optimal Flocculation Behavior of Brewer's and Wine Yeasts for Biomass Separation

Flocculation is an asexual, reversible aggregation of yeast cells to form flocs when lectins on cell walls bind to sugar residues on the walls of neighboring cells [96, 97]. This phenomenon is advantageous in brewing and wine making, since flocculated cells can be easily and efficiently separated after sedimentation. However, the flocculation behavior of many industrial yeast strains is far from optimal; some yeast strains are even unable to flocculate.

It is very important that flocculation starts at the correct time during the fermentation of beer and wine [25, 26, 70]. When the onset of flocculation is too early, fermentation is incomplete which results in an abnormal aroma. When flocculation is too late, yeast cells cannot be separated completely. In general, the flocculation of brewing yeast is associated with the onset of the stationary phase, but the factors which control the precise timing and degree of flocculation are not completely understood. At least it is known that the onset of flocculation depends on many external factors, e.g., ethanol concentration, temperature, osmotic stress and nutrient availability [98–101]. Other

factors involved are pH, calcium and zinc ions, and oxygen, as well as growth phase and cell density [102].

In addition, flocculation is dependent on genetic factors [70, 97, 103]. Three different flocculation phenotypes have been identified, based on the lectins on their cell walls, their sensitivities to sugar inhibition and proteolytic enzymes, and their dependency on growth conditions and pH. These three phenotypes are Flo1, NewFlo [104, 105] and Mannose-Insensitive (MI) [106, 107]. The majority of top-fermenting brewing yeasts belong to the NewFlo phenotype, whereas bottom-fermenting brewing yeast strains are assigned to the Flo1 phenotype [96, 104]. The MI phenotype is rare and occurs only in some top-fermenting yeast strains. The Flo1 phenotype is only inhibited by mannose [102, 108] whereas the NewFlo phenotype is also inhibited by glucose and maltose [107]. Different genes have been identified to be responsible for the flocculation behavior in yeast cells. *FLO* genes encode glycoproteins (lectins), so-called flocculins, which are located at the cell surface and bind specifically to mannose residues at the wall of neighboring cells [109]. The dominant genes *FLO1* and *FLO5* were isolated and characterized. Both genes encode an integral membrane protein that could be the postulated lectin [110, 111]. Nonflocculating brewer's, wine or laboratory yeast strains can attain the ability to flocculate after transformation with *FLO1* and *FLO5*, respectively [26, 110–112]. The Flo1 phenotype is associated with the *FLO1* gene [109, 113] whereas the lectin of NewFlo phenotype is encoded by the *FLO10* gene [114], which is not expressed until the onset of the stationary phase.

There have been some achievements in improving the flocculation of yeast by genetic engineering. The constitutive expression of *FLO1* led to premature flocculation [26]. Thus, it is necessary to regulate the gene expression in such a way that flocculation occurs during the stationary phase at the end of fermentation. The main problem here has been the lack of promoters that can be used for stationary-phase expression under industrial conditions. Therefore, Riou et al. [115] examined gene expression during the stationary phase during wine fermentation and showed that the *HSP30* promoter could be suitable for delayed gene expression during the stationary phase under wine fermenting conditions. Verstrepen et al. [116] replaced the native *FLO1* promoter by the *HSP30* promoter in a laboratory yeast strain and showed that flocculation occurred towards the end of fermentation under laboratory conditions. It still remains to be confirmed whether this strategy can cause flocculation specifically during the stationary phase under industrial conditions. Donalies and Stahl [117] examined the promoters of several genes encoding heat shock proteins and proposed that the *HSP26* and *HSP30* promoters could be suitable candidates for phase-specific gene expression under brewing conditions, since the authors used maltose as a carbon source in their experiments.

4.3

Modification of Byproduct Formation

4.3.1

Reduction of Diacetyl Formation in Brewer's Yeast to Facilitate Maturation of Lager Beer

Vicinal diketones, in particular diacetyl, impart the unwanted butter-like flavor to green beer and cause an off-flavor if present in finished beer. Diacetyl is the product of a nonenzymatic oxidative decarboxylation of α -acetolactate, the latter being an intermediate of the valine biosynthetic pathway as shown in Fig. 1 [70]. The taste threshold of diacetyl in beer is very low, and its presence in green beer is the main reason for the time-consuming secondary fermentation (lagering) required in lager brewing. Diacetyl is metabolized to acetoin and 2,3-butanediol during lagering. There is a high demand to reduce yeast's diacetyl production in order to be able to accelerate lager beer production.

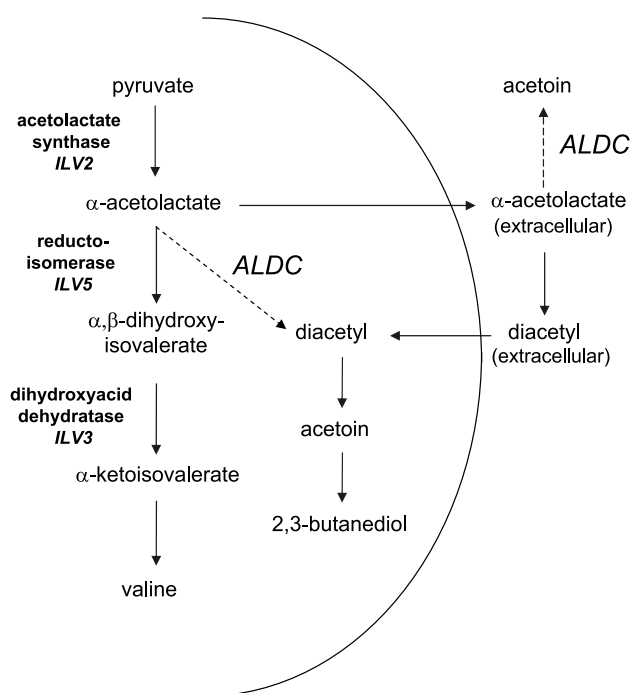


Fig. 1 Diacetyl formation in *Saccharomyces cerevisiae* fermentation originates from α -acetolactate which is an intermediate of the valine biosynthetic pathway. Diacetyl formation can be prevented by heterologous expression of bacterial *ALDC* genes, encoding acetolactate decarboxylase

One strategy has been to introduce a bacterial gene encoding the enzyme acetolactate decarboxylase (ALDC). This enzyme catalyzes the decarboxylation of α -acetolactate to acetoin, and thus prevents oxidative decarboxylation to diacetyl (Fig. 1). Originally, preparations of bacterial ALDC were added to wort; however, this procedure is expensive and not compatible with the German beer-brewing purity laws ("Reinheitsgebot"). Later, the ALDC gene from *Enterobacter aerogenes* [118, 119], *Klebsiella terrigena* [120, 121], *Lactococcus lactis* [122] and *Acetobacter aceti* var. *xylinum* [123] were cloned and expressed in brewer's yeast. Diacetyl formation was reduced significantly during fermentation with episomal as well as integrative transformants. Thus, the lagering period became obsolete [120, 124, 125]. The disadvantage of ALDC expression is that yeast is rendered auxotrophic for valine, leucine and isoleucine because of a deficiency of intracellular α -acetolactate. Even though brewer's wort contains amino acids, transformed strains showed reduced growth because of a very slow uptake of these amino acids in brewer's yeast [126]. To solve this problem, the ALDC gene was fused to the prepro-sequence of the α -factor leader. Thereby, the expressed ALDC was secreted into the medium and the extracellular α -acetolactate was decarboxylated to acetoin. The transformants showed a reduced diacetyl formation and the yeast was not auxotrophic for the amino acids valine, leucine and isoleucine [126]. It has been suggested that the ALDC genes from *L. lactis* or *Acetobacter* sp. would be preferable for food use as these organisms are already used in food production [122]. It has been assumed that engineered yeast strains without foreign genes ("self-cloned") are more readily accepted by consumers [127–131].

There have been also attempts to reduce diacetyl formation without the use of bacterial genes. For this purpose, two different modifications of the valine biosynthetic pathway were attempted. Firstly, the formation of α -acetolactate was prevented by deleting the *ILV2* gene encoding acetolactate synthase (Fig. 1) This resulted in yeast strains unable to form α -acetolactate [132]. However, these strains were also auxotrophic for the amino acids and grew very badly [85]. Moreover, Falco and Dumas [133] have shown that some *S. cerevisiae* mutants resistant to the herbicide sulfometuron methyl carry an allele of *ILV2*, leading to a less active form of the enzyme acetolactate synthase. Mutant strains forming less α -acetolactate and showing good brewing properties were selected [134, 135]. A reduction of the acetolactate synthase activity was also achieved by employing *ILV2* antisense RNA; Vakeria et al. [136] reported the construction of brewer's yeast strains that formed less α -acetolactate and performed well in brewing trials. A rather recent attempt to reduce acetolactate synthase activity by disrupting *ILV2* in brewing yeast was performed by Liu et al. [78]. The authors employed the α -amylase gene as a reporter gene for detecting the deletion mutant (see Sect. 4.1.3). The obtained strain showed a 30% reduction in acetolactate synthase activity and a 70% reduction in diacetyl in comparison to the reference strain.

Another strategy to modify yeast's valine biosynthetic pathway was to enhance the conversion of α -acetolactate, preventing its accumulation and spontaneous diacetyl formation. The genes *ILV5*, encoding the enzyme reductoisomerase, and *ILV3*, encoding dihydroxy-acid dehydratase, were overexpressed for this purpose (Fig. 1). Brewer's yeast harboring the *ILV5* gene, either on a multicopy plasmid or integrated within the genome, showed increased reductoisomerase activity, resulting in decreased diacetyl formation [137–140]. In contrast, the overexpression of *ILV3* did not lead to a change in diacetyl content, although the encoding dihydroxy-acid dehydratase activity was enhanced [141]. Gjermansen et al. [132] integrated an additional *ILV5* gene into the *ILV2*-locus, leading to a strain with enhanced reductoisomerase activity linked with a reduced acetolactate synthase activity.

4.3.2

Optimization of Formation of Sulfur-Containing Compounds in Beer and Wine

There is a set of volatile sulfur-containing compounds in beer and wine, e.g., sulfite, hydrogen sulfite (H_2S), or dimethyl sulfide (DMS), which have high reactivity, low taste thresholds and different impacts on flavor.

4.3.2.1

Sulfite

S. cerevisiae produces sulfite as an intermediate product during the assimilatory reduction of sulfate to sulfide, which is important for the biosynthesis of the sulfur-containing amino acids methionine and cysteine (Fig. 2). Moderate amounts of sulfite in beer are important for flavor stability. During storage, an ageing aroma, the so-called “cardboard flavor”, can be formed in bottled beer as a result of oxidative reactions. This off-flavor is caused mainly by carbonyls such as *trans*-2-nonenal. Sulfite can act as an antioxidant, preventing oxidative reactions. It can also stabilize flavor by forming adducts with carbonyls. These sulfite–carbonyl complexes have a much higher flavor detection threshold than free carbonyls.

To improve the flavor stability of beer, different strategies to increase sulfite formation of the yeast were developed. Korch et al. [142] found that overexpression of *MET14*, encoding APS kinase (Fig. 2), increases sulfite formation in a *met5* mutant (inactive sulfite reductase), but not in a *MET5* prototrophic laboratory strain. In accordance with this, an increase in sulfite production was detected in a sulfite reductase-deficient strain after chromosomal integration of the *MET14* gene under the control of the constitutive *TPI* promoter (Johannesen, personal communication). Moreover, brewer's yeast with reduced *ILV2* gene function produced more than the usual amount of sul-

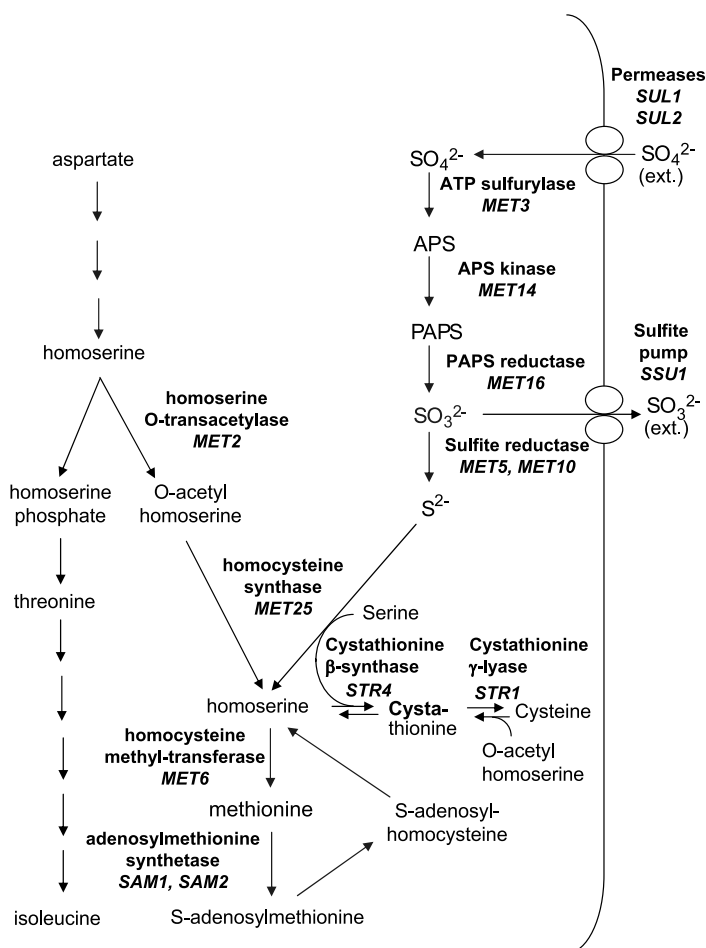


Fig. 2 The assimilatory reduction of sulfate to sulfite in *S. cerevisiae* during the biosynthesis of methionine and cysteine. APS Adenosyl phosphosulfate, PAPS phosphoadenosylphosphorsulfate ext. extracellular

fite [70]. This phenomenon could be explained by an increase in the threonine pool, leading to a derepression of the sulfur assimilation pathway.

Another possibility to increase sulfite accumulation is to prevent the reduction of sulfite to sulfide. A disruption of all the alleles of the *MET10* gene encoding a subunit of sulfite reductase resulted in enhanced sulfite accumulation in the medium compared to the isogenic wild-type strain [143, 144]. Moreover, the inactivation of all copies of the *MET2* gene encoding homoserine O-acetyl transferase led to an increase in sulfite formation [145]; *met2* mutants produced more H_2S , an undesirable byproduct (see Sect. 4.3.2.2).

One problem accompanied with the strategies described above is the early onset of sulfite accumulation. When sulfite accumulation has already commenced at the beginning of the fermentation process, it forms complexes with wort carbonyls and prevents the reduction of these carbonyls to the corresponding alcohol, thus negatively influencing beer flavor [146]. This is indeed the reason that some breweries prefer yeast strains which have low sulfite formation. Johannesen et al. [147] inactivated all copies of the *MET14* gene in brewer's yeast for this purpose. Beer produced by such engineered yeast showed accelerated ageing, which can be compensated for by sulfite supplementation before bottling. However, sulfite supplementation is expensive and is not permitted in Germany, because of the German "Reinheitsgebot" (beer purity laws). Hence, it was important to establish a strategy to control sulfite formation during fermentation, i.e., accumulation should not occur until the beginning of the stationary growth phase. Overexpression of *MET14* under the control of the *HSP26* promoter was able to solve this problem [143]. Furthermore, in contrast to the results discussed above, sulfite formation could also be enhanced in the *MET5* wild-type background when using this strong promoter for *MET14* expression.

Sulfite is usually added during wine making. Here, the main function of sulfite is to repress the growth of undesired microorganisms. One problem in this context is that different wine yeast strains differ significantly in their sulfite tolerances. Thus, one task for genetic optimization of wine yeast has been to increase sulfite tolerance [25]. Obviously, sulfite resistance in *S. cerevisiae* is determined by the expression level of the *SSU1* gene, encoding a putative sulfite pump [148–150]. Cells lacking the Ssu1p are sensitive to sulfite, while cells overexpressing this protein are resistant. Park and Bakalinsky [149] hypothesized that Ssu1p is responsible for sulfite resistance by transporting sulfite out of the cell.

As described above, sulfite formation can be increased by overexpression of *MET14*, but sulfite accumulation is limited by an active sulfite reductase converting sulfite to sulfide (Fig. 2). Donalies and Stahl [143] investigated whether the overexpression of the putative sulfite pump could increase sulfite efflux, preventing the reduction to sulfide. The simultaneous overexpression of *SSU1* and *MET14* together did indeed lead to a significant increase in sulfite production. This result also confirmed the hypothesis that *SSU1* encodes a sulfite pump.

4.3.2.2

Sulfide

Sulfide is formed from sulfate, sulfite, sulfur, and cysteine during fermentation [151]. Hydrogen sulfide (H_2S) can negatively influence the organoleptic characteristics of beer and wine due to a very low flavor threshold. H_2S is produced mainly in response to the depletion of nitrogen and the vitamin

panthothenate, respectively [26]. The beer maturation process decreases H₂S concentration. Thus, yeast able to form less H₂S would shorten the beer production process [134]. Tezuka et al. [152] have overexpressed *NHS5* (=STR4), encoding the cystathionine β -lyase, resulting in a suppression of H₂S formation by enhancing conversion of homocysteine to cysteine via cystathionine in brewing yeast (Fig. 2). Omura and Shibano [153] overexpressed *MET25* encoding O-homocysteine synthase in brewing yeast, which resulted in a strong decrease in H₂S concentration in beer. A third method to reduce H₂S formation has been the disruption of the *MET10* gene, leading to an inactive sulfite reductase [144]. All strategies applied in brewer's yeast to decrease H₂S production could also be used for wine yeast strains [26].

4.3.2.3

Dimethyl Sulfide

DMS is another sulfur-containing compound. It negatively influences the organoleptic properties of beer, especially lager beer. DMS is produced by thermal degradation of S-methyl methionine during wort production or by enzymatic reduction of dimethyl sulfoxide (DMSO) during fermentation [154]. Hansen [155] showed that a *mrx1* disruption mutant is unable to reduce DMSO. The *MRX1* gene encodes the enzyme methionine sulfoxide reductase and its disruption in brewing yeast reduces the DMS content in beer [156].

4.3.3

Increasing Glycerol Formation for Improved Wine Viscosity and in Order to Reduce the Ethanol Content in Alcoholic Beverages

Glycerol affects the sensory quality of wine, due to its nonvolatile character and slightly sweet taste. Therefore, a moderate increase in glycerol content is desired; it improves viscosity, sweetness, consistency and the overall body of wine [25]. Glycerol overproduction has been also considered a valuable strategy to produce beverages with decreased ethanol content, as any increase in glycerol production will simultaneously lead to a decrease in ethanol yield. Consumer demand for low-ethanol beer and wine is indeed continuously increasing due to both increased awareness for health and stricter laws regarding drinking and driving.

Glycerol in *S. cerevisiae* is produced by the reduction of dihydroxyacetone phosphate, catalyzed by the glycerol-3-phosphate dehydrogenase (GPDH, Fig. 3). As glycerol overproduction has also been of interest in industrial biotechnology, there have been several approaches to overproduce glycerol in laboratory strains of *S. cerevisiae* [157]. Indeed, engineered *S. cerevisiae* laboratory strains that virtually reached the maximal theoretical yield of

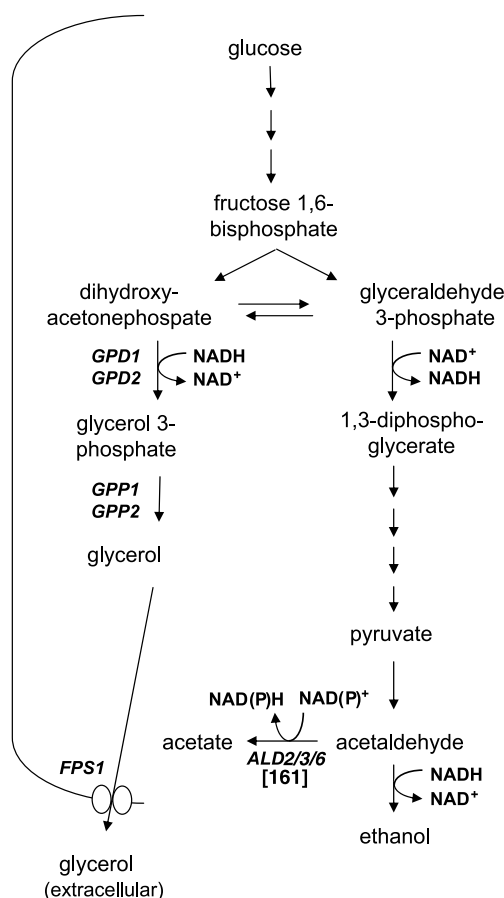


Fig. 3 Glycerol, ethanol and acetate production linked to the glycolytic pathway in *S. cerevisiae*. Genes and enzymes discussed in this review: *GPD1* and *GPD2* isogenes encoding glycerol-3-phosphate dehydrogenase, *GPP1* and *GPP2* isogenes encoding glycerol-3-phosphatase, *ALD2/3/6* genes encoding cytosolic acetaldehyde dehydrogenase, *FPS1* encoding a channel protein which facilitates glycerol transport through the plasma membrane

glycerol per glucose consumed (1 mol mol^{-1}) have been successfully constructed [158–160].

The first strategy to improve glycerol production in brewer's and wine yeasts was the overproduction of glycerol 3-phosphate dehydrogenase (GPD), which is the rate-controlling step in glycerol biosynthesis [161–163]. The increase in glycerol production by overexpression of *GPD1*, one of the isogenes encoding GPD, was first established in a laboratory yeast strain [164]. The overexpression of *GPD2*, encoding the other isoform of GPDH, had a similar effect. Extracellular glycerol accumulation could be further increased by

the additional constitutive expression of the gene *FPS1*, encoding a channel protein facilitating diffusion of glycerol through the cell membrane [161]. Overexpression of *GPP1*, encoding glycerol-3-phosphatase, did not enhance glycerol production [161]. A general major drawback of glycerol overproduction is the concomitant overproduction of unwanted byproducts. Owing to the altered metabolic flux, the formation of acetate, 2,3-butanediol, succinate, diacetyl and acetoin was increased [29, 162, 163]. The high production of acetate has been the main disadvantage in wine yeast. Cambon et al. [165] therefore combined the overexpression of *GPD1* with the deletion of *ALD6*, which was previously shown to result in lower acetate production in laboratory yeast [166].

A novel avenue to reduce ethanol in beverages could be the use of yeast strains with an abolished Crabtree effect. Based on the approach of Elbing et al. [167], a nonethanol-producing wine yeast strain was developed by modification of hexose transporters [168]. Moreover, introducing heterologous enzymes leading to increased NADH oxidation has been shown to reduce overflow metabolism, i.e., ethanol formation, in wine yeast [169]. As the functionality of such approaches relies on oxygen availability or, at least, microaeration, their use for production of low-alcohol beverages will require sophisticated fermentation strategies. Indeed, full aeration would strongly change yeast's metabolism and byproduct formation in comparison to traditional beer and wine production carried out under oxygen-limited conditions.

4.4

Reduction of Wine Acidity

Wine acidity is an important quality character. Here, the predominant organic acids are tartaric and malic acid. The malic acid content is often very high in must produced by grapes from colder regions. The so-called malolactic fermentation is one way to biologically deacidify wine. During malolactic fermentation, L-malic acid is decarboxylated to L(+)-lactic acid and carbon dioxide by lactic acid bacteria, in particular *Oenococcus oeni* [25]. Lactic acid has a much milder taste than malic acid. This secondary fermentation has been unreliable in numerous situations because wine, e.g. by high sulfite content growth of lactic acid bacteria is inhibited in.

The fission yeast *Schizosaccharomyces pombe* is able to effectively convert malate to ethanol during the so-called maloethanolic fermentation, but also produces off-flavors [170]. In principle, *S. cerevisiae* is also able to assimilate malate; however, conversion is very inefficient. In contrast to *S. pombe*, *S. cerevisiae* lacks an active malate transport system and L-malate enters wine yeast only by simple diffusion. The biochemical pathways for malate degradation in *S. cerevisiae* and *S. pombe* are the same: malate dehydrogenase (malic enzyme) decarboxylates malate to pyruvate which is further degraded

to ethanol and CO₂. The substrate specificity of the *S. cerevisiae* malic enzyme is about 15-fold lower than that of the *S. pombe* enzyme [171].

Using genetic engineering, wine yeast strains were constructed that are able to simultaneously conduct alcoholic fermentation and efficient malate degradation. The malolactic genes (*mleS*) from *Lactococcus lactis* [113, 172–174] and *Lactobacillus delbrueckii* [175] as well as the gene *mleA* from *O. oeni* [176] were cloned and expressed in *S. cerevisiae*. However, due to the absence of an active malate transport system in *S. cerevisiae*, the transformants could not metabolize malate efficiently [171]. Efficient malolactic fermentation was achieved only when the *L. lactis mleS* gene was coexpressed with the *S. pombe mae1* gene, encoding malate permease. Likewise, an efficient maloethanolic *S. cerevisiae* strain was constructed by coexpressing *S. pombe mae1* and *mae2* (the latter gene encoding malic enzyme). Here, the recombinant *S. cerevisiae* strain produced higher amounts of ethanol in comparison to the wild-type strain because the *S. pombe* enzymes converted L-malate to ethanol [171].

Recently, a genetically stable industrial yeast strain was constructed that was able to fully decarboxylate 5.5 g⁻¹ of malate in Chardonnay grape must during alcoholic fermentation. This was achieved by integrating a linear cassette containing the *Schizosaccharomyces pombe* malate permease gene (*mae1*) and the *Oenococcus oeni* malolactic gene (*mleA*) under control of the *S. cerevisiae* PGK1 promoter and terminator sequences into the *URA3* locus. This malolactic yeast strain enjoys “Generally Regarded As Safe” status from the FDA and is the first genetically enhanced wine yeast that has been commercialized [177].

4.5

Improving Tolerance to Several Types of Stress

Expression studies of suitable marker genes revealed osmotic and oxidative stress as the major causes of stress response under such conditions [178]. Indeed, there have been numerous attempts to improve yeast's tolerance towards these stressors, most of them dating back to the 1990s [35]. Oxidative stress tolerance has also been recently addressed by Chen et al. [179], based on their previous finding that externally added proline was able to protect yeast cells from lethal levels of reactive oxygen species (ROS) generated by paraquat [180]. Their approach, aimed at increasing intracellular proline concentration, is supported by the fact that the deletion of *PUT1* encoding proline dehydrogenase led to increased tolerance to hydrogen peroxide (H₂O₂) [181], whereas overexpression of *PUT1* caused a strongly reduced intracellular proline concentration and a hypersensitivity to oxidants such as H₂O₂ and paraquat [179]. The same authors used the *PUT1*-overexpressing strain background to perform a conditional life/death screen for suppressors of ROS hypersensitivity. Using a tomato cDNA library, they identified

a tomato QM-like protein (tQM) which seems to protect *S. cerevisiae* cells against oxidative stress by regulating intracellular proline levels. The QM protein is a small basic protein which was first identified as a putative tumor suppressor from the Wilms' tumor cell line [182]. It is highly conserved in mammals, plants, worms, insects and yeast [183]. When tQM was expressed in the wild type or in a strain overexpressing *PUT1* and the intracellular proline level was analyzed in the presence of 3 mM H_2O_2 , there was a 1.8 times and 7 times increase compared to the corresponding strains without tQM expression, respectively [179]. Phenotypic analysis of a *S. cerevisiae* mutant deficient in *GCR5*, a yeast homologue of QM, suggested that QM is involved in multiple cellular functions, including growth control and proliferation, cytoskeletal function, and energy metabolism [184]. However, it remains to be clarified how tQM increases intracellular proline concentration and protects the *PUT1*-overexpressing strain from oxidative stress, even though a two-hybrid analysis showed a physical interaction between tQM and Put1p [179].

Approaches targeted at stress tolerance of baker's yeast also comprise the improvement of cryoresistance in frozen dough, as well as osmotolerance in sweet frozen dough. There have been several recent studies to improve freeze-thaw stress tolerance in *S. cerevisiae*. Izawa et al. [185, 186] have reported two engineering approaches to accumulate intracellular glycerol in baker's yeast. Here, the most promising genetic modification is the deletion of *FPS1* encoding a glycerol channel. The engineered cells acquired tolerance to freeze stress and retained high leavening ability, even in dough after frozen storage.

Certain amino acids such as proline, arginine, and glutamate have also been shown to have cryoprotective activity in *S. cerevisiae*. For example, proline accumulation by simultaneous overexpression of a mutant allele of *PRO1* encoding γ -glutamyl kinase and wild-type *PRO2* encoding γ -glutamyl phosphate reductase resulted in a higher tolerance of the engineered strain for freezing [181]. It was later demonstrated that the selected mutant allele of *PRO1* was less sensitive to feedback inhibition by proline [187]. Furthermore, an arginase mutant *car1* Δ accumulated higher levels of arginine and/or glutamate and showed increased leavening ability during the frozen-dough baking process [188]. Yeast strains overexpressing heterologous aquaporin had been regarded as opening new perspectives for the development of freeze-resistant strains [189]. Later studies, however, have shown that they have less potential for use in frozen dough than originally thought [190]. Another recent approach to improve freezing tolerance has been the heterologous expression of antifreeze proteins. An industrial yeast strain expressing the recombinant antifreeze peptide GS-5 from the polar fish grubby sculpin (*Myoxocephalus aeneus*) showed improved viability during frozen storage and an increase in CO_2 production in model liquid dough compared to the control [191]. A very recent approach focused on the impact of unsaturated fatty acids on tolerance to freezing. The multicopy overexpression of either *FAD2-1* or *FAD2-3*, encoding two different desaturases from sunflower, in *S. cerevisiae* increased

the content of dienoic fatty acids, especially 18:2 Δ (9,12), the unsaturation index, the fluidity of the yeast membrane and the tolerance to freezing [192].

5

Concluding Remarks and Future Prospects

Important progress has been made in the field of recombinant DNA technology in the past 20 years. As the sequence of the complete *S. cerevisiae* genome has become available, targeted and stable genetic modifications can be easily performed in this yeast. Knowledge about the genetics of industrial yeast strains lags behind that of laboratory strains of *S. cerevisiae*. Nevertheless, there have been also recent achievements regarding the genomic structure of industrial yeast strains, in particular brewer's yeast. This will strongly facilitate future strain development programmes and help in understanding the specific nature and evolution of interspecies hybrid yeast strains.

As described in previous chapters, a multiplicity of recombinant yeast strains have been developed in order to improve production of beer, wine and bread. However, the majority of these strains have not been commercialized. The reason for this phenomenon is a lack of public acceptance of the use of genetically modified yeast for the production of food and beverage. The enlightenment of the general public about the advantages, limitations and dangers of genetic engineering, as well as simplified legal regulations, are the most important requirements for the commercial utilization of recombinant yeast strains.

Apart from sequencing industrial yeast strains, it will be crucial for future strain optimization to unravel the function and expression regulation of all genes. *S. cerevisiae* laboratory strains will certainly remain the cutting edge within this research field; however, the knowledge will also facilitate the understanding and improvement of industrial yeast. It has been generally accepted that the coordinated expression and/or inhibition of several genes is required for optimizing phenotypic traits rather than modifying a single or a few gene(s). Moreover, genetic/metabolic engineers have become aware of the necessity to consider the cell as a whole when modifying certain metabolic pathways. Indeed, the modern tools of global analysis (e.g., for the analysis of genomes, transcriptomes, proteomes, metabolomes and fluxomes) will have an important impact in yeast strain improvement [193, 194].

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