

Progress in Metabolic Engineering of *Saccharomyces cerevisiae*

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INTRODUCTION

Due to its importance in traditional biotechnology such as baking, brewing, and wine making, research activities historically have focused on the yeast *Saccharomyces cerevisiae* (21).

Thus, in-depth knowledge concerning genetics, physiology, and biochemistry as well as genetic engineering and fermentation technologies has accumulated over the time. The availability of highly efficient transformation methods (97) has aided *S. cerevisiae* genetic engineering. Furthermore, many specialized expression vectors, including episomal ones (267), and numerous other useful tools such as reporter genes, immunotags, and genetically selectable markers (107, 108, 135, 312) have been available. In addition, the extraordinarily high efficiency of homologous recombination in this species has facilitated targeted manipulations within chromosomes (172). The popular-

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ity of *S. cerevisiae* in basic and applied research is undoubtedly also influenced by its classification as GRAS (generally regarded as safe) by the U.S. Food and Drug Administration (FDA).

Baker's yeast *S. cerevisiae* was the first eukaryotic organism whose complete genomic sequence was determined (100). Several databases such as the *Saccharomyces* Genome Database (<http://www.yeastgenome.org/>) and the Comprehensive Yeast Genome Database (<http://mips.gsf.de/genre/proj/yeast/>) contain an enormous amount of information concerning *S. cerevisiae* genes, open reading frames, and gene products. In addition, databases provide access to results from genomewide microarray studies (<http://transcriptome.ens.fr/ymgv/index.php>) and networks of protein interactors (General Repository of Interaction Datasets [<http://www.thebiogrid.org/>]). The European *S. cerevisiae* Archive for Functional Analysis (<http://web.uni-frankfurt.de/fb15/mikro/euroscarf/>) as well as the Japanese Yeast Genetic Resource Center (http://yeast.lab.nig.ac.jp/nig/index_en.html) collect and store many useful tools for the yeast scientific community, e.g., strains and plasmids generated during various projects. The *Saccharomyces* Genome Deletion Project (391) has developed a collection of knockout strains covering 96% of the yeast genome. This collection of over 6,000 gene disruption mutants provides a unique tool for the functional analysis of the yeast genome. The complete strain collection or single deletion mutants are available for purchase at the European *S. cerevisiae* Archive for Functional Analysis or Open Biosystems (<http://www.openbiosystems.com/GeneExpression/Yeast/YKO/>). Due to its cutting-edge role, it is not surprising that the yeast *S. cerevisiae* has become a well-established eukaryotic model organism to study fundamental biological processes such as aging (29), mRNA transport (231), the cell cycle (35), and many more. *S. cerevisiae* also serves as a model organism for studying human diseases such as cancer (326, 396) and has been used as a tool for drug research (222), studying prions (55), basic and applied virus research (91), and ecotoxicology (346).

S. cerevisiae also plays a major role in applied research due to its outstanding capacity to produce ethanol and carbon dioxide from sugars with high productivity, titer, and yield. Baking, wine making, brewing, and production of bioethanol constitute the majority of the *S. cerevisiae* biotechnological industry. Moreover, this yeast has been used as a host organism for pharmaceutical protein production in the past (279, 280). *S. cerevisiae* is relatively tolerant to low pH values and high sugar and ethanol concentrations, i.e., properties which lower the risk of contamination in industrial fermentation. Moreover, this yeast is fairly resistant to inhibitors present in biomass hydrolysates and is able to grow anaerobically. These have been the major reasons for increasing *S. cerevisiae* exploration in industrial ("white") biotechnology, focusing on the fermentative production of industrially relevant biochemicals, e.g., glycerol, propanediol, organic acids, sugar alcohols, L-glycerol-3-phosphate (L-G3P), steroids, and isoprenoids. As discussed below, these substances are either directly utilized in the pharmaceutical or chemical industry or represent building blocks or precursors for further chemical or enzymatic syntheses.

Metabolic engineering, i.e., the intentional redirection of metabolic fluxes, has played an exceptional role in improving yeast strains for all industrial applications mentioned above. In

contrast to classical methods of genetic strain improvement such as selection, mutagenesis, mating, and hybridization (15, 266), metabolic engineering has conferred two major advantages: (i) the directed modification of strains without the accumulation of unfavorable mutations and (ii) the introduction of genes from foreign organisms to equip *S. cerevisiae* with novel traits. The latter is particularly crucial for industrial biotechnology to provide pathways that extend the spectrum of usable industrial media (e.g., lignocellulosic biomass) and/or to produce compounds not naturally formed by *S. cerevisiae*. Since the first introduction of metabolic engineering (16), there have been tremendous enhancements of its toolbox (245, 354), and several related disciplines have emerged, such as inverse metabolic engineering (18) and evolutionary engineering (305). These developments have strongly influenced yeast strain improvement programs in the past few years and have greatly enhanced the potential for using yeast in biotechnological production processes.

The current overview examines the concept of metabolic engineering in yeast strain improvement and discusses related engineering strategies. It then presents tools for targeted tuning and regulation of protein activities crucial for metabolic engineering. Examples of recently constructed engineered yeast strains follow. The major focus is on fermentative production processes used in food and industrial biotechnology. There have been numerous impressive successes since the previous comprehensive review published by Ostergaard et al. in 2000 (259).

ENGINEERING STRATEGIES FOR YEAST STRAIN IMPROVEMENT

The goal of metabolic engineering is the directed modification of metabolic fluxes. It encompasses approaches which improve the production of a metabolite naturally formed by an organism or provide the organism with the ability to utilize atypical substrates or form metabolites not naturally produced, either by this organism or any others. Metabolic engineering was introduced by Bailey in 1991 as a subdiscipline of engineering and pertains to "the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technology" (16, 17). A number of reviews of metabolic engineering have been published (16, 17, 175, 244, 285, 327–329).

Metabolic engineering is always based on genetic engineering, i.e., the targeted manipulation of a cell's genetic information. These two disciplines, of course, overlap, provided that "enzymatic, transport, and regulatory functions" are the target of directed genetic modifications and the "improvement of cellular activities" is the goal. If Bailey's definition was scrupulously applied, there might be cases where it is unclear whether or not a genetic engineering approach can also be considered metabolic engineering. It is, for example, questionable whether metabolic engineering encompasses stress tolerance improvement via modification of any of the above-mentioned cellular functions (i.e., is stress tolerance a cellular activity?). In fact, stress tolerance is crucial for yeast's performance in virtually all industrial fermentation processes. Thus, engineering approaches to improve such traits fall within the rubric of metabolic engineering. Indeed, the term "cellular

TABLE 1. Engineering approaches for microbial strain improvement

Strategy	Description	Advantage(s)
Rational metabolic engineering	Engineering metabolic pathways (mainly enzymes, transporters or regulatory proteins) based on available information	Known genetic modifications leading to strain improvement can be transferred to other strains
Inverse metabolic engineering	Starts from the phenotype; analysis of the genetic basis for the phenotype; transfer of the genetic basis to the production strain	Independent of preliminary knowledge about pathways, enzymes, and their kinetics; can reveal novel, unknown target genes for improvement
Random engineering strategies (support inverse metabolic engineering)		
Evolutionary engineering	Includes all random genetic modifications and perturbations; relies primarily on random mutagenesis of the entire genome or its parts; identification of clones with improved phenotypes via appropriate screening methods; identification of genetic modifications is difficult (except when a limited DNA sequence is mutated)	Independent of preliminary knowledge about pathways, enzymes, and their kinetics; multigenic traits can be addressed
Transposon mutagenesis and gene overexpression libraries ^a	Random deletion or overexpression of single genes; identification of clones with improved phenotypes by appropriate screening methods	Independent of preliminary knowledge about pathways, enzymes, and their kinetics; can reveal novel, unknown target genes for improvement; genetic modifications can be easily identified
Global transcription machinery engineering ^a	Mutagenesis of basal transcription factors leading to a global reprogramming of gene transcription; identification of clones with improved phenotype by appropriate screening methods	Independent of preliminary knowledge about pathways, enzymes, and their kinetics; multigenic traits can be addressed; the genetic modification that triggers the optimal global reprogramming can be identified and transferred to another strain

^a These strategies can, in principal, also be defined as evolutionary engineering, but they represent special cases (see text).

activities” was replaced by “product formation or cellular properties” in another definition of metabolic engineering (330).

The discipline of metabolic engineering has been subject to continuous enhancements and, as such, the current view on it is much more sophisticated than the definition. First, there have been dynamic developments of available tools for modifying, analyzing, and modeling metabolic fluxes (354). It is also increasingly obvious that improving cellular activities requires a holistic view of the entire cell rather than isolated metabolic pathways (330). Metabolic engineering nowadays is seen as an iterative process including several rounds of engineering, analysis, and modeling of metabolic fluxes (244, 285). In addition, other engineering disciplines such as inverse metabolic engineering or evolutionary engineering have facilitated rational metabolic engineering. The next three sections and Table 1 discuss the peculiarities, commonalities, and synergies of and between these engineering disciplines.

Rational Metabolic Engineering

Traditional metabolic engineering is rational and deductive and has been referred to as “rational, constructive” (18) or “reductionistic” (37). Rational metabolic engineering refers to the engineering of enzymes, transporters, or regulatory proteins based on available information about the pathways, enzymes, and their regulation. Based on this

knowledge, typically, a strategy is designed to optimize these protein activities in order to achieve the desired metabolic flux or phenotypic trait. Rational metabolic engineering has been fairly successful in a number of applications; however, many attempts failed, were less successful than predicted, or led to unwanted side effects. Moreover, there have been cases where a metabolic engineering approach worked well under laboratory conditions but not when transferred to industrial constraints. Only with a complete understanding of the complex global metabolic network and its responses to changing environmental conditions would one be able to predict all the secondary responses of a certain metabolic engineering approach.

The need to understand and model the cell as a whole has led to the discipline of systems biology. Although we are still far from this ambitious and challenging goal, it is generally accepted that models are critical to handle the growing information generated by global analysis methods that study the cell as a whole (245). Nonetheless, it is important to remember that models are based on experimental data and are only as good as the data used to construct the models. Often, models must rely on assumptions in the absence of sufficient experimental information, and any model refinement requires further experimental data. For example, a sophisticated metabolic model has already been helpful in predicting metabolic engineering strategies in yeast; however, the performance of the accordingly engineered strains

was less superior than predicted (38). The successful application of systems biology to strain improvement will require a very close collaboration between mathematical modelers, biologists, and bioengineers.

Evolutionary Engineering

The term evolutionary engineering encompasses all methods for empirical strain improvement, e.g., mutagenesis (natural or induced) and recombination or shuffling of genes, pathways, and even whole cells, followed by selection of cells with the desired phenotype (305). Often, multiple cycles of random genetic perturbation and selection are sequentially performed, resulting in various genetic alterations, including those which are phenotypically advantageous. These methods exploit “natural design principles” (306) and can be very effective in strain improvement. There have been many recent examples in which satisfactory performance of rationally designed strains was achieved only via subsequent evolutionary engineering (25, 185, 261, 325). Moreover, evolutionary engineering has greatly improved *S. cerevisiae* tolerance to multiple stressors (39, 218).

All methods of evolutionary engineering have the same limitation: their dependence on suitable screening methods for the improved trait. Therefore, the applications are restricted to only a few phenotypic improvements, such as better growth or substrate spectrum extension. It is a challenging task to further develop efficient selection strategies for other phenotypes, such as better product formation. High-throughput methods such as robotic microtiter plate screening assays or fluorescence-associated cell sorting can greatly facilitate such developments. An exciting development in this area is molecular detectors such as riboregulators whose activity can be controlled by the molecule of interest and connected to the expression of a reporter such as green fluorescent protein (24).

Evolutionary engineering per se is distinct from metabolic engineering as it relies on random methods; i.e., the genetic modifications are not directed. It can be very difficult to determine which genetic modifications are responsible for improved traits in an evolutionarily evolved strain, particularly if random mutations are spread over the entire genome. In such cases, evolutionary engineering does not lead directly to an improved understanding of the metabolic network. In addition, it is impossible to transfer the crucial genetic information to other strains. Nevertheless, evolutionarily evolved strains can provide useful starting points for inverse metabolic engineering approaches (see below) as long as attempts are made to identify the genetic modifications and link them to the phenotypic trait of interest. In this regard, evolutionary engineering methods contribute powerful tools to the field of metabolic engineering (98, 305).

Special evolutionary engineering approaches have facilitated identification of the actual genetic modification and its transfer to other strains. For example, to overcome the frequent difficulties in rationally identifying optimal targets for pathway engineering, it may be helpful to use random methods for overexpression or deletion of genes, i.e., overexpression of DNA libraries or transposon mutagenesis (285). In this way, vast numbers of genetic variants can be screened for the desired phenotype and the relevant genetic modification easily identified. As an example of random gene overexpression for

yeast strain improvement, Jin et al. (145) improved the growth of *S. cerevisiae* on xylose (see “Utilization of lignocellulosic biomass. (ii) Utilization of pentoses and other lignocellulosic carbon sources” below). Basically, a heterologous genomic fragment library was introduced into a recombinant *S. cerevisiae* strain which was able to slowly consume xylose. Subculturing in xylose medium led to the enrichment of clones which expressed *Pichia stipitis* genes (whose overexpression is advantageous for xylose utilization). The plasmids from these clones have been subsequently isolated, and sequencing of the inserts revealed the genes whose overexpression facilitated xylose consumption.

Another important approach is “global transcription machinery engineering” (gTME). It introduces random mutations in one or more protein factors (i.e., transcription factors) of the basal transcription machinery and induces perturbations in global gene expression. After selection of clones with improved traits, pertinent gene mutations can be identified. In contrast to other evolutionary engineering approaches, which accumulate random mutations in the entire genome, the gTME approach allows for genotype-phenotype correlations traceable to a single mutant protein. Therefore, the genetic modification leading to the desired phenotype is easily transferable to other relevant host strains. Although the multigenic changes induced by the mutated transcription factor are primarily unknown, appropriate methods such as transcriptome analysis can be applied to identify the relevant changes in gene expression. This approach is an interesting strategy to address polygenic traits such as tolerance toward different types of stress. Indeed, gTME has been demonstrated to successfully improve yeast tolerance to high sugar and ethanol concentrations (9).

Inverse Metabolic Engineering

Bailey et al. also introduced the approach of “inverse metabolic engineering” as a subdiscipline of metabolic engineering (18). They described “the elucidation of a metabolic engineering strategy by: first, identifying, constructing, or calculating a desired phenotype; second, determining the genetic or the particular environmental factors conferring that phenotype; and third, endowing that phenotype on another strain or organism by directed genetic or environmental manipulation” (18).

Primarily, inverse metabolic engineering takes advantage of phenotypic differences. Therefore, the starting point is a system with two or more different manifestations of the desired phenotype. This can, for example, result from (i) exposing the host strain to different environmental conditions, (ii) investigating a heterologous organism which possesses the desired trait and is more or less related to the host organism, or (iii) selecting a variant of the host strain which has evolutionarily evolved (see “Evolutionary Engineering” above). The next step is to identify the genetic basis for the differing trait values; this is the biggest challenge in inverse metabolic engineering. However, global methods of gene expression analysis such as transcriptomics, proteomics, metabolomics, and even fluxomics have strongly facilitated the identification of differences at various molecular levels and revealed useful targets for metabolic engineering (37, 98). The analytical possibilities conferred by such techniques has increased impressively in recent

years (245). Nevertheless, all levels of gene expression analysis have advantages and disadvantages for target gene identification. Using sequencing to conduct a global genome analysis will certainly reveal a huge number of differences between strains or conditions but will not pinpoint which of the differences are truly relevant for the phenotype under investigation. The analysis of transcriptome and proteome analysis will reduce the number of differences but at the cost of important information such as small mutations inside a coding sequence; this may alter protein-specific activity or regulatory features. The levels of metabolome and fluxome are highly informative since they are closely related to the phenotype. However, based solely on this information, it is difficult to identify the actual genetic players involved. Thus, it always remains challenging to link phenotypes with genotypes.

The last and crucial step in inverse metabolic engineering is to verify potential target genes identified by “omics” technologies. This is performed by genetic engineering of the production strain, most often by deletion or overexpression of the identified target genes. Afterwards the modified production strain is assessed to determine whether the genetic modification resulted in the desired phenotype. Inverse metabolic engineering has been successfully applied for yeast strain improvement in an increasing number of studies (36, 103, 145, 272), as discussed later in this review.

The concept of inverse metabolic engineering has several obvious advantages over rational approaches: (i) there is no need for preliminary knowledge regarding the proteins/enzymes of a pathway and their regulation, (ii) one can directly utilize industrial strains and real production conditions to identify crucial genetic players, (iii) the final strain improvement strategy is then based on homologous genes (i.e., the modified strain can be considered “self-cloned,” which is an important issue for consumer perception especially in the food area), and (iv) there is a good chance of discovering novel genetic targets for strain improvement which would have never been found by a rational method.

Notwithstanding the advantages of inverse metabolic engineering, it cannot, of course, replace rational approaches, in particular if the introduction of heterologous genes is required. It is the combination of all engineering disciplines which is likely to be most successful for strain development.

CONTROLLING PROTEIN ACTIVITY IN YEAST FOR METABOLIC ENGINEERING

The metabolism and other relevant phenotypic traits of a cell are controlled primarily by proteins. For example, the flux through a given pathway is determined by the abundance and biochemical properties of the pertinent enzymes, transporters, and regulatory proteins, as well as their interactions with each other and the metabolites generated. Each protein may exert a different degree of control over a pathway, and it is most often necessary to modify several or even all enzymes of a pathway for improved metabolic fluxes. Flux control is a topic in itself that is not addressed in the current review. The reader is referred to several relevant publications in this field (2, 243, 254, 330, 353). I focus here on how protein abundance and biochemical properties can be modified at the genetic level. Opportunities include engineering the (i) level of gene expres-

sion, (ii) regulation of gene expression, (iii) in vivo protein/enzyme activity (including V_{max} , K_m with regard to substrates and cofactors, and allosteric regulation), and (iv) protein sub-cellular location. These possibilities are discussed in the following sections together with yeast metabolic engineering examples.

Changing Protein Cellular Levels

There are various ways to modify the cellular abundance of a particular protein. Primary targets are (i) DNA/gene content (by adjusting the copy number of homologous or heterologous genes), (ii) transcription efficiency (by using promoters of the appropriate strength), (iii) mRNA abundance and translation efficiency (e.g., by use of antisense RNA, ribozymes, riboregulators, RNA interference, secondary structures contributing to mRNA stability, and codon usage adaptation for heterologous genes), and protein abundance (e.g., via degradation control).

Modifications of gene copy number and transcription efficiency have been very common in yeast metabolic engineering. In contrast, RNA technologies and protein degradation control have not yet been extensively used (see below).

DNA/gene content. The copy number of a given homologous or heterologous gene can be changed by introducing single- or multicopy plasmid vectors. Alternatively, genomic insertions or deletions via homologous recombination can be carried out. The use of $2\mu\text{m}$ -based multicopy plasmids can greatly increase cellular gene copy number, i.e., to roughly 10 to 30 copies, as for the pRS series of $2\mu\text{m}$ -plasmids (56). This approach usually results in significant gene overexpression but has several drawbacks. First, the plasmid's high copy number can be maintained only under selective cultivation conditions, which clearly is not an option for industrial applications. Other problems with this technique are the metabolic burden of maintaining high-copy plasmids (see below) and cell-cell expression heterogeneity caused by copy number variance between individual cells of a culture. In addition, it is virtually impossible to reliably adjust the copy number of multicopy plasmids in yeasts to fine-tune gene expression (see “Fine-Tuning Gene Expression” below).

Alternatively, the copy number of a gene can be increased by stepwise integration of several copies into the yeast chromosome. Although laborious, this represents the only feasible strategy for genetically stable engineered yeast strains in industrial applications. There have been several attempts to introduce multiple copies of a gene within a single transformation experiment. In such cases, the gene integration was targeted into e.g., rRNA genes (88, 205) and sigma (181) or delta (80, 195, 253, 301) sequences. Copies of such sequences occur in multiple places throughout the yeast genome, and, after homologous recombination, clones with multiple copies of the inserted gene can be generated.

Transcription efficiency. The second target for modifying the level of gene expression is the regulatory sequence (promoter) upstream of a gene. For example, gene overexpression can be achieved by fusing a strong promoter upstream of the gene of interest. When combined with multicopy expression (see previous section), this approach maximizes the expression level (233). In order to engineer a full range of gene expres-

sion, one can take advantage of native *S. cerevisiae* promoters possessing different inherent activities. A collection of four yeast promoters (*CYC1p*, *ADH1p*, *TEFp*, and *GPDp*, in ascending order by strength) was introduced into either low-copy (CEN/ARS-based) or multicopy (2 μ m-based) yeast expression vectors (233). Using these plasmids, a crude adjustment of gene expression is possible.

Homologous *S. cerevisiae* gene expression can also be modified in the absence of autonomously replicating vectors, simply by replacing its native promoter with another *S. cerevisiae* promoter of the desired strength (378). However, the ratio between the strengths of the original and substitute promoters must be carefully considered. Indeed, it is virtually impossible to increase the level of a housekeeping protein or glycolytic enzyme by promoter replacement without simultaneously increasing the respective gene's copy number. In contrast, overexpression solely by promoter replacement works well for genes whose wild-type expression in cells is low or moderate. A good example has been the overexpression of the nonoxidative pentose phosphate pathway (PPP) genes by Kuyper et al. (184). Here, 1.7- to 8.5-fold increases in mRNA levels were achieved for the five genes relative to the reference strain, simply by replacing the native promoters of the respective genes with strong ones. This resulted in increased PPP flux.

The native *S. cerevisiae* promoters mentioned so far are generally considered to be constitutively expressed in yeast. Nevertheless, their inherent regulation modes and fluctuations in expression must be taken into account when choosing a promoter for a given application. For example, the high activity of the original *ADH1* promoter depends on the presence of a fermentable carbon source (352). Indeed, there have been multiple attempts to optimize the *ADH1* promoter for either brewing fermentation or heterologous protein production (257, 299).

In addition to modifying the regulatory sequence of a gene (i.e., the promoter), the deletion or mutation of specific transcription factors which regulate the expression of one or more discrete genes is another method to alter gene transcription. Prominent examples are the deletion of genes such as *MIG1*, *MIG2*, or *GAL80*, which encode transcriptional regulators of glucose repression. Deletion of these transcriptional regulators alleviated glucose repression and resulted in constitutive expression of genes involved in sucrose, maltose, and galactose utilization (244). Another example is the deletion of *PDC2* to reduce the activity of pyruvate decarboxylase (PDC), a key enzyme in alcoholic fermentation (241, 242). *Pdc2p* positively regulates transcription of the three structural PDC genes (*PDC1*, *PDC5*, and *PDC6*). *PDC2* deletion did not completely eliminate but strongly reduced PDC activity, to 19% residual activity.

mRNA stability and translation efficiency. RNA technologies are powerful tools for tuning gene expression and have been successfully used in other organisms such as plants (216). Their use in budding yeast, however, is still quite limited. To my knowledge, there are no examples to date of successfully utilizing ribozymes in *S. cerevisiae* metabolic engineering, although they appear to be useful engineering tools in filamentous fungi (230). The same holds for RNA interference. Recent attempts to control gene expression with antisense RNA were made in yeast metabolic engineering (31, 153). Bonoli et al. (31) stated that it is difficult to effectively and reliably

silence target gene expression via antisense RNA techniques in *S. cerevisiae*. Still, there have been promising reports that involved the use of antisense-based riboswitches (23, 24) and ribozyme-based riboswitches (388) in *S. cerevisiae*. Such riboswitches have not yet been applied in metabolic engineering but demonstrate the feasibility to control mRNA abundance by small-molecule concentration (389). The importance of RNA techniques for yeast metabolic engineering will undoubtedly increase in the future.

Well-established tools for adjusting translation efficiency in *S. cerevisiae* are rare, even though fundamental research is focusing on this issue (358, 380). The translation efficiency of heterologous genes depends on codon usage in yeast. In fact, it is highly recommended to adapt the codons of bacterial genes to those of highly expressed *S. cerevisiae* genes, thus ensuring maximal expression (387).

Protein degradation. Regulated degradation of a specific protein is an elegant way to adjust its concentration. In contrast to manipulating mRNA levels, this targets the protein directly. One example of reduced protein degradation for yeast metabolic engineering is given by Omura et al. (256). The reduction of lysine residues in Put4p (amino acid permease) led to its delayed ubiquitination. Thus, cellular abundance of this permease increased, along with improved proline assimilation. An interesting development in protein degradation control, albeit not yet seen in metabolic engineering, is the fusion of a protein to a "heat-inducible-degron cassette" that targets the protein for ubiquitin-dependent proteolysis at 37°C (74).

Fine-Tuning Gene Expression

Strong overexpression of a gene is not always the best approach. It can cause a "metabolic burden" on the cell, as detailed by Gorgens et al. (102) and confirmed in their own work. Explanations for this effect could be increased energy demand, dilution of molecular factors required for transcription and translation (e.g., proteins, RNAs, cofactors, and precursors), or simply limited cellular space. When enzyme-encoding genes are overexpressed, this can trigger complex metabolic changes detrimental for cells (e.g., cofactor depletion). Indeed, strong enzyme overproduction may considerably exceed the level that is optimal for the metabolic engineering goal. There are several examples of target phenotype optimization achieved by moderate rather than strong multicopy gene expression in *S. cerevisiae* (147, 150, 196, 345). Moreover, one example even demonstrates that very low enzyme activity can be optimal. This strongly reduced activity of an originally overexpressed enzyme was selected after evolutionary engineering as most favorable for the desired phenotype (25).

It is increasingly obvious that metabolic engineering requires highly flexible tools for fine-adjusting gene expression. As previously discussed, native *S. cerevisiae* promoters of different strengths are useful to crudely adjust protein levels; however, finer adjustments of expression level over a wider dynamic range may be required. This would be useful for identifying a given application's optimal gene expression level and for flux analysis studies. The best strategy is to create promoter libraries spanning a wide range of activities, as implemented first in bacteria (8, 141, 323) and subsequently in the yeast *S. cerevi-*

siae. One example of a synthetic promoter library for yeast is given by Jeppsson et al. (143). Three promoters from this library were chosen to tune the expression of the *ZWF1* gene, encoding glucose-6-phosphate dehydrogenase in yeast. Specific enzyme activities of 0.11, 0.77, and 1.82 U/mg protein were obtained, with the latter most appropriate for the metabolic engineering application. Recently, a full range of promoter activities was obtained by random mutagenesis of the strong constitutive *S. cerevisiae* *TEF1* promoter (8, 87). Out of this library, a collection of 12 promoters possessing a wide range of activities was selected based on the specific fluorescence of yeast clones expressing a fluorescent reporter gene downstream of the promoter mutants. The dynamic range of the promoter activities was comparable to that obtained by Jeppsson et al. (143). Subsequently, selectable genetic markers were cloned upstream of the promoter sequences. This generated promoter replacement cassettes which can be used to replace any given promoter in the yeast chromosomes (238). The utility of the promoter replacement cassettes was demonstrated by fine-tuning the specific activity of G3P dehydrogenase (GPD), the key enzyme of the glycerol production pathway in yeast. The results showed that optimal expression of *GPD1* (one of the isogenes encoding GPD) for high glycerol yields was significantly lower than multicopy overexpression from a 2 μ m plasmid (238).

Although more useful in yeast basic research than in industrial applications, the activity of an inducible promoter can be fine-tuned by modifying its regulatory properties or accurately titrating its inducer (143, 295). For example, gene expression driven by the *GALI* promoter has been modulated by several promoter deletion variants (232) or by finely controlling the concentration of the repressor/inducer molecule galactose (150). The latter method was also applied to adjust the transcription activity of the inducible promoters of *CUPI* (187), *CTR1/3* (187), and *MET25* (232). Recently the bacterial *tet* promoter system has been used for inducer-triggered repression of all essential genes in yeast (225, 402). It has been shown that doxycycline, the inducer of the *tet* promoter, had virtually no pleiotrophic effects on *S. cerevisiae* (392). Nevertheless, the use of inducers for fine-tuning gene expression is prohibitively expensive on an industrial scale. In addition, cell-cell heterogeneity is problematic with this strategy, because it is not clear if the level of induction is homogeneous across all cells.

Controlling Gene Expression Regulation

Metabolic engineering also requires tools which switch gene expression on or off. Such tools are useful for approaches that lead to a metabolic burden or produce toxic metabolites (279). In such cases, product formation must be uncoupled from growth. Promoters triggered by external inducers are mentioned in the previous section. An ideal inducible promoter for industrial processes employing yeast as a biocatalyst must (i) be tightly regulated, (ii) be inexpensive to induce, (iii) express at high levels after induction, and (iv) be easy to handle. None of the systems presently available for inducing gene expression in *Saccharomyces cerevisiae* satisfies all these requirements.

The traditional systems for inducing *S. cerevisiae* gene expression require either expensive or toxic inducers, e.g., galactose (*GALI-10* promoter) (331) or copper (*CUPI* promoter)

(187), and leaky expression may occur in the absence of the inducer. There have also been efforts to make the traditional *GALI* promoter system independent of its expensive inducer galactose (268). This has been achieved by isolating artificial transcription factors that functionally activate the *GALI* promoter. However, it is questionable whether these artificial transcription factors can be cost-effective. The *PHO5* promoter, which requires phosphate depletion for induction, is tightly regulated, but its expression level even after full induction is relatively low (383). Shimizu-Sato et al. (317) developed a sophisticated "light-switchable" promoter system in yeast that is, however, far from practical application and would require photofermentor systems that remain to be designed. Promoters induced by temperature shifts have also been tested in *S. cerevisiae* (321), but uniform heat transfer for large-scale fermentations would likely be challenging.

Recently, we proposed that a promoter induced by simply down-regulating the cultivation medium oxygen concentration would represent an inexpensive and manageable tool for conditional gene expression (237). This idea has already been pursued for *Escherichia coli* heterologous protein production using the *Vitreoscilla vgb* promoter, which is induced by low oxygen (201). The use of oxygen tension as a trigger for gene expression is attractive because (i) oxygen supply is limited in industrial fermentations, particularly at high biomass concentrations; (ii) ATP supply could be optimized for yeast biomass formation during the aerobic phase (360); and (iii) fermentative production of small molecules could be carried out in the anaerobic phase to ensure optimal yields (211).

A promising candidate for an oxygen-responsive system is the *S. cerevisiae* *DANI* gene promoter; it is one of several yeast promoters which strongly up-regulate transcription under anaerobic conditions. The anaerobic mRNA expression level was up to 140-fold higher than the aerobic level (186, 274, 348). The regulation of the *DANI* promoter is tightly controlled; i.e., there is virtually no basal level of expression under aerobic conditions. For efficient induction, the *DANI* promoter requires fastidious anaerobiosis, which was achieved in previous studies by bubbling nitrogen through cultures (60, 311). Given the attenuated mass transfer dynamics at large scales, this step could be time-consuming or its implementation unfeasible under industrial conditions. We therefore attempted to evolve the oxygen sensitivity of the *DANI* promoter (237). The evolved promoters enabled induction of reporter gene expression in yeast fermentation simply by depleting oxygen during cell growth. The maximal level of reporter expression achieved by a *DANI* promoter mutant was comparable to that with the fully induced *GALI* promoter (237); the latter is the most highly inducible promoter among those commonly used in yeast. Additional advantages, that the inducer incurs no costs and the modified *DANI* promoters do not suffer from leaky expression in the noninduced state, make these evolved promoters promising alternatives for metabolic engineering in yeast. The biggest challenge for applying them in industrial applications would be to guarantee an oxygen tension which is uniform and sufficiently depressed throughout a large fermentor.

Other examples of biotechnological processes that require switches of gene expression are wine or beer fermentations where a certain phenotypic trait, e.g., yeast flocculation, is not desired until the final stage of fermentation. Promoters of several *S.*

cerevisiae genes encoding heat shock proteins have been proposed for delayed gene expression during the stationary phase of fermentation (76, 293; K. Verstrepen, F. Bauer, C. Michiels, G. Derdelinckx, F. Delvaux, and I. Pretorius, presented at the Symposium on Yeast Physiology—a New Era of Opportunity, 1999).

In Vivo Protein Activity

In addition to its cellular abundance, the in vivo activity of an enzyme depends on protein specific traits such as V_{max} , K_m , allosteric regulation, catabolite inactivation, and cofactor specificity. These properties can, in principle, be modified by genetic engineering. Tyo et al. (354) emphasized the demand for enzymes optimized for high catalytic rates to circumvent strong overexpression of slow enzymes. Branduardi et al. (34) provide a recent example of yeast metabolic engineering utilizing site-directed mutagenesis to improve enzyme-specific activity and K_m . Using a rational approach to overproduce lactate, they generated a lactate dehydrogenase (LDH) that is catalytically more efficient. Moreover, it becomes increasingly obvious that several *S. cerevisiae* enzymes are regulated metabolically rather than by expression (40, 66, 188, 338), a fact which future metabolic engineering strategies should take into account. A recent report suggests that even transcriptional regulators are subject to metabolic regulation. Although the rationale behind this regulation is not yet fully understood, it seems that the cellular NADP level plays a key role in the *S. cerevisiae* GAL induction system (182).

Single point mutations can alter in vivo protein properties. For example, research on yeast phosphofructokinase identified mutations that modified allosteric regulation (116, 167, 296). Nonetheless, there are only a few recent examples of yeast metabolic engineering that take advantage of site-specific enzyme mutagenesis to modify regulatory properties. In such cases, useful mutations leading to higher protein/enzyme activities have usually been identified by earlier random approaches. For example, a mutant form of *PUTI* encoding an enzyme from the proline biosynthetic pathway was isolated from yeast mutants with increased proline levels (228). In contrast to the wild-type enzyme, the mutated version of Put1p was less sensitive to feedback regulation by proline, and its overexpression led to intracellular accumulation of proline (310, 347).

In contrast to targeting enzyme regulatory properties, the modification of cofactor specificity is relatively frequent in yeast metabolic engineering. Redox-factor imbalance can be an important issue in metabolically engineered cells; thus, altering an enzyme's preferred cofactor can be of great value. One example is *S. cerevisiae* engineering for xylose utilization (see "Utilization of lignocellulosic biomass. (ii) Utilization of pentoses and other lignocellulosic carbon sources" below and Fig. 1). To rescue cells from redox factor imbalance, several attempts have been made to identify xylose reductase mutants that prefer NADH over NADPH (142, 386).

Moreira dos Santos et al. (227) altered the subcellular localization of a yeast enzyme for metabolic engineering. Seeking an additional source of cytosolic NADPH, they removed the mitochondrial target sequence of yeast malic enzyme and indirectly demonstrated its functionality in the cytosol.

ENGINEERING *SACCHAROMYCES CEREVISIAE* FOR FERMENTATIVE PRODUCTION PROCESSES

Food and Beverage Industry

The metabolic engineering of yeast strains used in the beverage and food industry, including baker's, wine, brewer's, sake, and distillers' yeasts, raises particular challenges. First of all, the sensory quality of alcoholic beverages or bread is determined by many different compounds which must be present in well-balanced quantities. It is challenging to optimize certain metabolic pathways or phenotypic traits without affecting this overall balance.

In addition, genetic engineers in the food field are confronted with industrial yeast strains lacking the advantageous traits of laboratory yeast strains. Industrial *Saccharomyces* yeast strains are genetically diverse, prototroph, homothallic, and often polyploid, aneuploid, or even allopolyploid (72a). Knowledge of the genetics of industrial yeast strains lags behind that of laboratory strains of *S. cerevisiae*; however, there is an increasing interest in the functional genomics of these strains (72a).

Another difficulty particular to the food and beverage industry is that containment of engineered yeast within the industrial plant cannot be guaranteed. Engineered yeast strains used in food and beverage production could be consumed by humans and released to the environment. Thus, such products are regulated as novel food using genetically modified organisms (GMOs), and engineered yeast strains must pass safety evaluations and follow labeling regulations. On the international level, the Codex Alimentarius Commission has established principles for the assessment of GMO food-related safety (http://www.who.int/foodsafety/biotech/codex_taskforce/en/), and the Cartagena Protocol on Bio-safety outlines principles for an environmental assessment of living modified organisms (<http://www.biodiv.org/biosafety/default.aspx>). However, there are tremendous differences between countries in how GMOs are approved, classified, and labeled. The European legislation is particularly strict; for example, the risk assessment procedure for genetically modified nonpathogenic microorganisms used in fermentation is the same as that for GMOs from plant and animal origin (333).

Official approval of GMOs does not guarantee successful commercialization. Indeed, there is a very low acceptance by consumers of GMOs in the food and beverage industry. Although many advantageous industrial yeast strains have been engineered and two have obtained approval from the British government for commercial use several years ago (4), no genetically modified yeast has entered commercial use until recently (126). This problem, influenced by complex cultural, social, ethical, environmental, and technical factors, has been intensively addressed by Pretorius and Bauer (284), with wine yeasts as an example.

Within the context of GMO legislation and consumer acceptance, the term "self-cloning" enters the discussion. Self-cloning refers to targeted genetic modifications generating an organism which contains DNA exclusively from species phylogenetically closely related to the host organism. That is, any foreign DNA temporarily used as a cloning tool, e.g., vector DNA and genetically selectable markers, has been removed. For the exact definition of the European Legislature, the reader is referred to the Council Directive 98/81/EC (<http://rod>

.eionet.europa.eu/show.jsv?id=569&mode=S). It is debatable whether, under the risk assessment procedure, self-cloned organisms should be equated with uncontrolled genetically altered organisms, e.g., those generated by induced mutagenesis (333). When containment is not ensured, the European legislation does not differentiate self-cloned strains from those obtained by the expression of heterologous genes (transgenic organisms). In contrast, the Japanese government does not consider a self-cloned yeast to fall under the GMO regulations (122).

In general, it has been assumed that self-cloned yeasts have a greater potential for commercialization because of better consumer acceptance compared to transgenic organisms. In fact, yeast genetic engineers have expended significant effort on reinforcing self-cloning strategies (4, 14, 123, 339, 385). It is also obvious that the focus on self-cloning in food-related yeast metabolic engineering will, in the future, direct more attention to inverse metabolic engineering approaches, i.e., exploiting the natural diversity of yeast. Nonetheless, more than merely sophisticated engineering strategies will be needed to facilitate consumer acceptance of genetically modified yeasts for novel food production. It has been suggested that product labeling emphasize the benefits of the novel food, the nature of the GMO, the risk assessment procedure, and the nature of the product's conventional counterpart. This transparency together with improved education of the public on these matters may help overcome the refusal to use GMOs (333).

Baker's yeast. Engineering of baker's yeast has been previously reviewed by Randez-Gil et al. (287, 288). In addition, further reviews address approaches to improving baker's and other yeast strains (71, 259). Most genetic engineering approaches to optimize baker's yeast relate to either (i) yeast propagation or (ii) dough performance and bread quality.

Molasses is the medium classically used for yeast propagation. It is a mixture of sugars such as glucose, fructose, sucrose, raffinose, melibiose, and galactose. Although it is not the primary sugar, glucose is always present and prevents the simultaneous utilization of other sugars such as maltose, sucrose, and galactose. This effect has been referred to as "glucose repression" and results in lengthened process times. Apart from its importance during yeast propagation in molasses, glucose repression of maltose utilization also occurs during dough leavening (see below) and brewing fermentation (see "Brewer's yeast" below), as maltose is the most important fermentable sugar in both dough and brewer's wort.

Other low-cost media such as whey, starch, or lignocellulose have also been considered as alternatives to molasses for yeast propagation. There have been many approaches aimed at extending the substrate spectrum of *S. cerevisiae* to enable it to hydrolyze/utilize starch, cellulose, hemicelluloses, melibiose, raffinose, lactose, xylose, or arabinose (71, 259, 288). The extension of yeast's substrate spectrum and the simultaneous utilization of different sugars during fermentation are of general importance in yeast biotechnology, particularly when using lignocellulosic biomass as a renewable substrate. This will therefore be discussed in detail later in this review (see "Bioethanol Production" below).

Another challenge for effective yeast biomass propagation using sugar-containing media is the Crabtree effect present in *S. cerevisiae*. This leads to a predominant ethanol fermenta-

tion, even under aerobic conditions, as soon as the glucose concentration exceeds 0.1 g/liter (86, 375). Hence, optimal biomass yields in industrial growth media such as molasses can be obtained only if *S. cerevisiae* is grown under glucose limitation with high aeration; this can be achieved in either well-controlled continuous or fed-batch fermentations. The absence of ethanol formation is particularly critical when *S. cerevisiae* is used for the production of heterologous proteins. A promising approach to obtaining yeast with a fully respiratory phenotype in sugar-containing industrial media such as molasses has been published by Elbing et al. (82). They generated a yeast strain which is defective in the most important hexose transporter genes (*HXT1* to -7) and expresses a chimeric hexose transporter. This fusion between *HXT1* and *HXT7* abolished the Crabtree effect in *S. cerevisiae*. Another approach to prevent overflow metabolism (i.e., ethanol formation) in the presence of a high sugar concentration has been the expression of a water-forming NADH oxidase from *Lactococcus lactis* in *S. cerevisiae* (120). The lower cytosolic NADH level in the strain reduced ethanol formation in batch fermentation (121). Parallel work performed by Vemuri et al. (374) demonstrated that reduction of ethanol yield was even stronger when a *Histoplasma capsulatum* alternative oxidase (*AOX1*) was introduced instead of the water-forming *Streptococcus pneumoniae* NADH oxidase. Although these approaches could optimize yeast propagation, one has to consider that if used for subsequent dough or beverage fermentation, cells must regain ethanolic fermentation. Nevertheless, yeast strains free of the Crabtree effect could be of interest in reducing ethanol in alcoholic beverages (121) (see "Wine yeast" below).

Typically, yeast cells must cope with various known stresses during industrial propagation in molasses. Expression studies of suitable marker genes revealed osmotic and oxidative stress as the major causes of the stress response under such conditions (271). Indeed, there have been numerous attempts to improve yeast's tolerance toward these stressors, most of them dating back to the 1990s (287). Oxidative stress tolerance has also been the focus of a recent study by Chen et al. (54). Their approach, aimed at increasing the intracellular proline concentration, was based on their previous finding that supplemental proline protected yeast cells from lethal levels of reactive oxygen species generated by paraquat (53). Moreover, deletion of *PUT1*-encoded proline dehydrogenase conferred increased tolerance to hydrogen peroxide (H_2O_2) (347), whereas *PUT1* overexpression caused a strongly reduced intracellular proline concentration and a hypersensitivity to oxidants such as H_2O_2 and paraquat (54). The same authors used the *PUT1*-overexpressing strain background to perform a conditional life/death screen for suppressors of reactive oxygen species 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. For example, 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 , it was 1.8 times and 7 times higher, respectively, than that the corresponding strains lacking tQM expression (54).

The QM protein is a small basic protein which was first identified as a putative tumor suppressor from the Wilms' tumor cell line (77). It is highly conserved in mammals, plants,

worms, insects, and yeasts (83). Phenotypic analysis of an *S. cerevisiae* mutant deficient in *GCR5*, a yeast homologue of the QM gene, suggested that QM is involved in multiple cellular functions, including growth control and proliferation, cytoskeletal function, and energy metabolism (246). Although a two-hybrid analysis showed a physical interaction between tQM and Put1p, it remains to be clarified how tQM increases the intracellular proline concentration and protects the *PUT1*-overexpressing strain from oxidative stress (54). It is worth noting that high intracellular proline also positively affects freeze tolerance (347), desiccation (340), and ethanol tolerance (339, 341) in yeast (see below and "Improving tolerance to ethanol" below). If present in the medium, proline may also serve as an osmoprotectant in yeast (349).

Baker's yeast improvements which fall into the second category (dough performance and bread quality) equip the yeast with supplemental enzymes, such as amylases, hemicellulases, proteases, and lipases. These enzymes alter the physicochemical properties of the dough and/or the quality of the bread itself (e.g., flavor, dough rheology, or shelf life) (287, 288). The second category also includes the enhancement of dough fermentation and CO₂ production. Wheat flour is composed mainly of starch, as well as maltose, sucrose, glucose, fructose, and glucofructans. Maltose is continuously released from starch during the baking process due to the activity of crop amylases (255, 287). Efficient fermentation of dough sugars, in particular maltose, by yeast cells is crucial for dough leavening. A major limiting factor for fermentation rate in baker's yeast is the carbon catabolite repression of maltose-utilizing enzymes and the inactivation of maltase enzyme by glucose. This results in a maltose consumption lag phase which can be avoided by derepressing maltose-utilizing enzymes, i.e., replacing the native, glucose-controlled promoters of maltase and maltose permease with constitutive ones. This engineered baker's yeast which produces carbon dioxide more quickly than conventional baker's yeast was the first one cleared for food use in the United Kingdom, in March 1990 (6). For more details concerning these previous examples of engineering baker's yeast, the reader is referred to the reviews mentioned at the beginning of this section. There have been also attempts to alleviate glucose repression of maltose-utilizing enzymes by deleting proteins which act as transcriptional regulators, such as Mig1p (168, 169).

Approaches that facilitate baker's yeast dough performance also improve cryoresistance in frozen dough as well as osmotolerance in sweet frozen dough. There have been several recent attempts to improve freeze-thaw stress tolerance in *S. cerevisiae*. Izawa et al. (131, 132) have reported two engineering approaches to address intracellular glycerol accumulation in baker's yeast. Here, the most promising genetic modification is the deletion of *FPS1*, encoding a glycerol channel. The engineered cells showed a roughly threefold increase in intracellular glycerol accumulation. Survival after 7 days at -20°C (measured as the portion of the number of viable cells prior to freezing) was roughly 80% in the *fps1* Δ mutant but only about 15% in the wild type. Moreover, in contrast to wild-type cells, the engineered cells retained their high leavening ability even after the dough was frozen.

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 (Asp154Asn) of *PRO1*, encoding γ -glutamyl kinase, plus wild-type *PRO2*, encoding γ -glutamyl phosphate reductase, increased the engineered strain's tolerance for freezing (347). It was subsequently demonstrated that the selected mutant allele of *PRO1* was less sensitive to feedback inhibition by proline. The researchers were able to isolate other mutant enzymes which performed even better than Asp154Asn with respect to proline accumulation (310).

Furthermore, the *car1* Δ arginase mutant accumulated higher levels of arginine or glutamate (depending on the cultivation conditions), which increased leavening ability during the frozen-dough baking process; i.e., the number of viable cells after three freeze-thaw cycles was 1,000 times higher in the *car1* Δ mutant than in the control (316). Yeast strains overexpressing heterologous aquaporin had been believed to provide new perspectives on the development of freeze-resistant strains (344). Later studies, however, have shown that they have less potential for use in frozen dough than originally thought (343).

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*) demonstrated improved viability during freezing; the number of viable cells after 13 days at -20°C was 32-fold higher than that of the reference strain. There was also a 30% increase in CO₂ production (ml CO₂ per mg [dry weight] yeast) after storage at -20°C for 40 days and inoculation of model liquid dough, compared to the control (265). A recent approach focused on the impact of unsaturated fatty acids on freezing tolerance. The multicopy overexpression in *S. cerevisiae* of either *FAD2-1* or *FAD2-3*, encoding two different desaturases from sunflower, increased the dienoic fatty acid content, particularly 18:2 Δ (9, 12), plus the unsaturation index, yeast membrane fluidity, and freezing tolerance. For example, about 90% of the *FAD2-3*-overproducing cells survived for 35 days when frozen at -20°C , in contrast to roughly 50% wild-type cell survival (297). Given the numerous examples of cryoresistance improvements in baker's yeast, it will be interesting to see whether combined approaches might result in additive effects.

Wine yeast. There are numerous examples of genetically modified wine yeast strains, and previous approaches have been well described in other, including quite recent, reviews (48, 71, 99, 259, 282–284, 307). Targets for wine yeast genetic improvements are divided into fermentation performance, wine processing efficiency, growth prevention of wine-spoiling microorganisms, sensory quality, and wholesomeness. The latter includes, for example, the reduction of compounds deleterious to human health such as ethyl carbamate, biogenic amines, or even ethanol and the increase of beneficial compounds such as resveratrol (284). Wine yeast propagation is performed in aerated molasses and does not differ significantly from baker's yeast production. Therefore, all targets for the genetic improvement of baker's yeast propagation mentioned in the previous section can be generalized to wine yeast.

With regard to wine fermentation itself, one major focus specific to yeast improvement has been the efficient use of nitrogen sources, given the imbalance between high carbon levels and limited nitrogen sources in grape must (33). Other

wine-specific targets are, for example, ethanol tolerance, increased glycerol production for body and fullness, the degradation of malic acid for acidity control, and several other properties. For details, the reader is referred to the reviews mentioned above. I will focus here only on a few recent reports on wine yeast improvement.

First, a genetically stable industrial yeast strain was constructed to fully decarboxylate 5.5 g/liter of malate in Chardonnay grape must during the 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*) into the *URA3* locus. This malolactic yeast strain enjoys GRAS status from the FDA and was the first genetically enhanced wine yeast to be commercialized (126).

Glycerol overproduction has also been a target of wine yeast engineering for two reasons: (i) glycerol improves the body of the wine, and (ii) reduced ethanol formation is a valuable strategy to produce low-ethanol beverages. Ethanol reduction in alcoholic beverages is currently of great commercial interest. Consumer demand for these beverages continues to increase due to both increased health awareness and stricter laws regarding drinking and driving. Glycerol formation is an alternative pathway for reoxidation of glycolytic NADH (Fig. 2) and could partially replace ethanolic fermentation. Rerouting carbon flux toward glycerol formation was tried first in laboratory yeasts (241) and subsequently in industrial strains such as brewer's (239) and wine (41, 289, 290) yeasts. Cambon et al. (41) combined (i) the overproduction of GPD (by overexpressing *GPD1*; see Fig. 2), the rate-controlling step in glycerol biosynthesis, with (ii) the deletion of *ALD6*, which was previously shown to result in lower acetate production in a laboratory yeast strain (79). In general, a major drawback of glycerol overproduction is the concomitant increase in other by-products of yeast metabolisms, particularly acetoin and acetaldehyde; these are undesirable in both beer and wine (223, 239, 290).

Another approach for ethanol reduction in wine has been the expression of *Aspergillus niger* glucose oxidase in *S. cerevisiae* (214). When excreted into the grape must, the enzyme converts a certain percentage of glucose into gluconic acid, thereby reducing the amount of sugar which can be metabolized into ethanol. Using this approach, the ethanol content of Chardonnay wine was reduced from 11.9% (vol/vol) to 10.1% (vol/vol). It was mentioned in the previous section that recent attempts to abolish the Crabtree effect in yeast could be a novel avenue to reducing ethanol in beverages. Based on the approach of Elbing et al. (82), a non-ethanol-producing wine yeast strain was developed by modifying hexose transporters (117). Moreover, introducing heterologous enzymes to increase NADH oxidation has been shown to reduce overflow metabolism, i.e., ethanol formation, in a wine yeast strain (121). As such approaches rely on oxygen availability or, at least, microaeration, their use for low-alcohol beverage production will require sophisticated fermentation strategies. Traditional beer and wine production is typically performed under oxygen limitation, and full aeration would definitely affect yeast's metabolism, by-product formation, and, therefore, flavor and taste.

Controlled and targeted modification of wine flavor is desirable

to winemakers. Lilly et al. (199), by altering the activities of the branched-chain amino acid transaminases involved in the formation of higher alcohols, investigated the impact on general yeast physiology, aroma, flavor, and sensory characteristics of wines and distillates. The formation of many aroma compounds, particularly higher alcohols, was significantly affected. In addition to higher alcohols, esters have been shown to form an important group of aromatic compounds in beer and wine (377). Another study by Lilly et al. (198) examined the overexpression of four genes encoding ester-synthesizing and ester-degrading enzymes in wine yeast. The genetic modifications resulted in specific ester profiles. Thus, the latter two approaches could increase the variability of wine flavors without affecting other fermentation behaviors. Approaches to modulating the formation of volatile sulfur compounds by wine yeast have been recently reviewed by Schwiegers and Pretorius (332).

Wine yeast transformants expressing heterologous enzymes able to degrade various polysaccharides, such as xylan, glucan, and pectin, have been shown several positive effects on wine filterability as well as on color and flavor (206, 367).

Autolysis products of yeasts strongly contribute to the sensory quality of sparkling wines produced by the traditional "méthode champenoise." Genetic engineering approaches have been applied to accelerate autolysis of *S. cerevisiae* in order to enhance sparkling wine production (49, 335).

Brewer's yeast. Previous approaches for engineering brewer's yeasts were thoroughly reviewed by Hammond (113), Hansen and Kielland-Brandt (115), and other authors who describe improvements to brewer's yeast strains, among others (71, 259). In addition, one review covers approaches exclusively related to beer flavor (359).

In general, there are two types of brewer's yeast: (i) lager (bottom-fermenting) and (ii) ale (top-fermenting) strains. Ale yeasts are subdivided into *S. cerevisiae* and lager yeast strains originally known as *Saccharomyces carlsbergensis* (114) but later recognized as part of the *Saccharomyces pastorianus* species (22, 220). Bottom-fermenting (lager) brewer's yeast is most striking among industrial *Saccharomyces* strains due to its genetic constitution. In contrast to baker's, wine, and ale brewer's yeast strains, which contain a pure *S. cerevisiae* genome, lager brewer's yeast results from genetic hybridization of at least two different species of the *Saccharomyces sensu stricto* species complex. One genetic ancestor is clearly *S. cerevisiae*, whereas the origin of the heterologous fraction of the lager yeast genome has been a matter of controversy (151). A recent in-depth genetic study of a large number of industrial lager brewing yeast strains confirmed that the non-*S. cerevisiae* portions might be derived from *Saccharomyces bayanus* and/or *Saccharomyces uvarum* (286). The genome of the current lager strains may have resulted from chromosomal loss, replacement, or rearrangement within the hybrid genetic lines. The hybrid nature of lager brewer's yeast strains is reflected by their allopolyploid genomes, extremely low degree of sporulation, and spore viability. Indeed, the complex genetics of lager brewer's yeast has complicated engineering of those strains. Moreover, reliable analyses of global gene expression in lager brewer's yeasts are not possible. The commercially available microarrays for *S. cerevisiae* do not contain probes for the genes representing the non-*S. cerevisiae* ancestor of lager brewer's yeasts. Still, there have been several studies of lager brewer's yeast using *S. cerevisiae* DNA microarrays (134, 252). When discussing

these data, one should always bear in mind that this method represents a compromise due to the lack of non-*S. cerevisiae* gene sequences. Proteomics has also been hampered by the lack of sequences encoding the non-*Saccharomyces* genes/proteins in lager brewer's yeast. Researchers have put much effort into identifying non-*Saccharomyces* protein spots by sequence homologies (152).

The identification of the entire genomic sequence of a commonly used lager brewer's yeast strain, i.e., Weihenstephan Nr. 34 (34/70), represents a breakthrough in the molecular analysis of lager brewer's yeast (174). According to the researchers, this laid the basis for developing DNA microarrays specific to lager brewer's yeast, thus allowing reliable gene expression analysis and detailed genomic constitution studies of other lager brewer's yeast strains. Although the sequence has not yet been made publicly available, the exact chromosomal structure of Weihenstephan Nr. 34 (34/70) has been published (174; Y. Nakao, Y. Kodama, N. Nakamura, T. Ito, M. Hattori, T. Shiba, and T. Ashikari, presented at the 29th Congress of the European Brewery Convention, Dublin, Ireland, 2003). These data confirm the hybrid character of lager brewer's yeast and the coexistence of three chromosomal types: (i) pure *S. cerevisiae* type, (ii) pure non-*S. cerevisiae* type, and (iii) mosaic-type chromosomes, i.e., hybrids of both.

Brewer's yeast strain development principally aims to improve (i) the production process for time and cost savings and (ii) product quality. Some targets for genetic improvement of brewer's yeasts resemble those for wine yeasts, e.g., flocculation, by-product formation, ethanol reduction, glucanase expression for polysaccharide clarification, and microbial spoilage prevention. Other targets are specific to the nature of beer and its production.

As mentioned previously, maltose is the major fermentable sugar in brewer's wort. Thus, approaches to improve maltose utilization of *Saccharomyces* yeast strains are of interest in brewing. The trisaccharide maltotriose is the second most abundant fermentable sugar in brewer's wort. Several known transporters carry out maltotriose uptake in *S. cerevisiae* (67, 68). The recently identified *MTTI* gene encodes another maltotriose transporter, and its overexpression in a lager brewing strain resulted in significantly increased maltotriose uptake (73). Dextrins make up about 25% of the carbohydrates in wort and are usually nonfermentable by industrial *Saccharomyces* strains. Dextrins represent degradation products of starch, i.e., mixtures of large fragments, and result from partial hydrolysis facilitated by the mashing step during brewer's wort production. The dextrins in brewer's wort are important for the fullness and body of beer. Still, dextrin utilization by brewer's yeast is necessary for the production of low-calorie beer. A dextrin-assimilating brewer's yeast is the second genetically engineered strain to receive official approval for commercial use from the British Government (4).

In addition to improving carbon assimilation, nitrogen uptake has also presented a challenge with brewer's yeast. Proline is the most abundant amino acid in brewer's wort and is, oddly, the only amino acid that is virtually unassimilated during brewing fermentation. A lager brewer's yeast expressing a modified version of the *PUT4* gene to encode a highly specific proline permease efficiently assimilated proline from brewer's wort

without negatively impacting beer quality (256). The modifications within the Put4 protein resulted in a less degradable form of the permease.

Diacetyl production, another target for improving brewer's yeast, has been an important issue. The formation of this butyry-flavored substance, a by-product of yeast's amino acid metabolism, entails a "second fermentation" of lager beer (2 to 3 weeks). During this maturation period, diacetyl is enzymatically converted to acetoin and then to 2,3-butanediol, a substance which has a much higher taste threshold in beer than diacetyl or acetoin. Accelerated lager beer production is of great interest for the brewing industry, and there have been numerous attempts to decrease diacetyl formation in brewer's yeast. These have been, in part, quite successful, particularly when a bacterial α -acetolactate decarboxylase was expressed in yeast. These studies mostly date to the 1980s; the reader is referred to several prior reviews (71, 113, 217, 259, 359). Interestingly, diacetyl production has never presented a problem in wine production. In fact, wine yeast possesses much higher 2,3-butanediol dehydrogenase activity (E. Nevoigt et al., unpublished results). This difference in specific 2,3-butanediol dehydrogenase activity may contribute to the varying diacetyl production capacities of wine and beer yeasts.

Another beer-specific target is the increased production of sulfite, an antioxidant that forms complexes with aldehydes. The latter may cause the so-called "cardboard flavor" in bottled beer as a result of oxidative reactions. Thus, sulfite is a key compound for the flavor stability of beer. The highest sulfite production in laboratory yeast was obtained by the simultaneous overexpression of *MET14*, encoding adenylylsulfate kinase, and *SSU1*, encoding a sulfite pump (75). Similar results were recently obtained with lager brewer's yeast strains (G. Kristan, personal communication).

Yeast osmotolerance has not been a major limitation when using wort of 12 degrees Plato. However, the situation changes when breweries switch to high-gravity brewing in which highly concentrated wort is fermented. In this case, it is worthwhile to engineer yeast strains which better resist high osmolarity at the beginning and a high ethanol concentration at the end of the fermentation. By subjecting a pool of UV-induced variants to consecutive rounds of fermentation in very-high-gravity wort, Blicek et al. (30) isolated variants of an industrial lager brewer's yeast strain with improved performance in terms of fermentation velocity and final wort attenuation under brewing-relevant conditions in highly concentrated wort (>22 degrees Plato). A number of differentially expressed genes have been identified in the variants by comparative transcriptome analysis. Unfortunately, the complex genetic structure of lager brewer's yeast hampered the verification of the potential target genes by genetic engineering.

Other yeast biomass-derived products. Apart from yeast biomass used in baking, there are several other industrial uses of yeast biomass (e.g., fodder yeast) and biomass extracted cell products (e.g., yeast B-complex vitamins) which have been addressed in detail by Walker (383). One final recent example of genetic engineering in this field is the increase of iron-carrying yeast capacity by expression of heterologous ferritin (52, 164, 318). The iron stored in the ferritin is bioavailable; such recombinant yeast therefore could have the potential to help address the global problem of iron deficiency.

Bioethanol Production

Burning fossil fuels result in atmospheric CO₂ accumulation and climate change. Per the 2006 final report of the European Union Biofuels Research Advisory Council, the vehicular transportation sector accounts for more than 30% of total energy consumption in the European Community (<http://www.biofuelstp.eu/overview.html#visirept>). Over the next 25 years, demand for petroleum and other liquid fuels is expected to increase more rapidly in the transportation sector than in any other end use sector. The United States is the largest consumer of transportation energy among the nations in the Organization for Economic Cooperation and Development and is projected to consume 54 percent of the region's total for that sector in 2030 (<http://www.eia.doe.gov/oiaf/ieo/enduse.html>). Moreover, many countries are attempting to decrease their dependency on imported oil. In the medium to long term, the limitations on fossil fuels as a world resource will become increasingly important. This explains the increasing interest in biofuels generated from renewable sources.

Ethanol can serve as a liquid fuel (84), and microbial fermentation from renewable resources is one technical pathway for its production. Bioethanol make a significant contribution as a transportation fuel in the short to medium term. Bioethanol production from sugar- and starch-containing biomass is state of the art in Brazil and the United States. In contrast to sugarcane in Brazil, corn or grain (i.e., starch) has been the main substrate for bioethanol production in the United States and many European countries. Whereas bioethanol production in Brazil successfully competes with fossil fuel, bioethanol production in other countries is not yet economically viable. Governments have subsidized bioethanol production in different ways (213).

It is generally accepted that large-scale use of ethanol for fuel will require lignocellulose utilization (84). The sole use of sugar- and starch-containing biomass (i) generates significant competition between bioethanol and food production and (ii) is not cost efficient. Instead, the entire biomass of a plant should be used. In fact, lignocellulose represents the most abundant global biomass source (90%). Therefore, designated energy crops or agricultural remains, such as corn stover, sugarcane waste, straw, forestry and paper mill discards, and the paper portion of municipal waste, represent attractive low-cost substrates for fuel production.

The yeast *S. cerevisiae* traditionally has been used in the bioethanol process (32, 398). Approaches for improving yeast bioethanol production comprise (i) the classical process using starch or sugar as the starting material, (ii) those which utilize lignocellulosic biomass substrate, and (iii) those independent of the starting material that increase sugar-to-ethanol conversion efficiency or yeast ethanol tolerance.

Supplemental starch-degrading enzymes. Biochemically, starch is a combination of two polymeric polysaccharides, amylose and amylopectin. Amylose consists of glucose monomer units joined via head-to-tail alpha-1,4 linkages. Amylopectin differs from amylose in that branching occurs with an alpha-1,6 linkage every 24 to 30 glucose monomer units. *S. cerevisiae* cannot directly utilize starchy materials, so large amounts of amylolytic enzymes must be added to convert starch into glucose. Efficient starch-degrading *S. cerevisiae* strains will lay the

foundation for consolidated bioprocessing, i.e., the simultaneous production of enzymes for saccharification of polysaccharides, hydrolysis, and fermentation by a single microorganism (209, 370). This will greatly improve the economic viability of the bioethanol industry (92, 208) and also play a role in beer and whisky production.

Of the numerous attempts to resolve this problem with recombinant yeasts, the examples given here are far from complete. Engineers expressed glucoamylase either alone (177, 303) or in combination with α -amylase (81, 163, 315) or even with pullulanase (136). Many approaches have relied on anchoring starch-degrading enzymes on the yeast cell surface using the Flo1 protein (163, 177, 303, 315). The sources of heterologous genes encoding amylolytic enzymes are many, including the amylolytic yeast *Saccharomyces diastaticus*, several bacteria, and filamentous fungi. Efforts have been also undertaken to improve the starch-hydrolyzing properties of amylolytic enzymes. For example, a hybrid glucoamylase-encoding gene has been recently constructed by in-frame fusion of the *S. diastaticus* *STAI* gene and the DNA fragment that encodes the starch binding domain of *A. niger* glucoamylase (193).

Utilization of lignocellulosic biomass. Lignocellulosic biomass shows significant potential as a future substrate for bioethanol production. Lignocellulose is composed of cellulose, hemicelluloses, and lignin. Cellulose, a long linear polymer of 1,4- β -linked D-glucose, is the major component of plant cell walls and the most abundant carbohydrate in plant biomass. Hemicelluloses (polyoses) have an intermediate degree of complexity and are made up of different pentose and hexose residues, which are often acetylated, and generally form branched chains. They also vary greatly in amount and complexity with cell type and over different plant species. Typically, hemicelluloses in softwood are glucomanans, whereas those in hardwood are mainly xylans with variable percentages of galactose, arabinose, rhamnose and methylglucuronic acid units, and acetyl groups.

Lignin is a three-dimensional network built up of dimethoxylated, monomethoxylated, and nonmethoxylated phenylpropanoid units, derived from the corresponding *p*-hydroxycinnamyl alcohols, which form a variety of subunits via various ether and carbon bonds. Lignin is highly resistant to chemical and biological degradation and confers mechanical resistance to wood (219). Unlike cellulose or hemicelluloses, the lignin fraction of lignocellulose cannot be converted into fuels or chemicals by fermentation. However, this portion can be burned for efficient heating and electrical production and, in the longer term, specialty chemical production (92). The negative impacts of lignin on lignocellulose-based fermentative processes are (i) inhibition of cellulases (50) and (ii) formation during lignocellulose pretreatment of phenolic degradation products which inhibit microbial growth (11, 192).

An optimal microorganism for lignocellulose-based bioethanol production must be able to hydrolyze sugar polymers, utilize all fermentable compounds, and convert them into ethanol at a high rate, yield, and productivity. No single microorganism is known to possess all of these characteristics. For example the traditional ethanol producer, *S. cerevisiae*, cannot hydrolyze cellulose and hemicelluloses. Moreover, it cannot utilize many compounds which are generated during hydrolysis of lignocel-

lulosic biomass, such as xylose, arabinose, glucuronic acids, and cello-oligosaccharides. Another problem that arises during biomass pretreatment in a lignocellulosic process is the formation of several substances which inhibit yeast growth, such as furans, organic acids, phenols, and inorganic salts. These challenges of using lignocellulose in fuel ethanol production have already been addressed through numerous metabolic engineering approaches in yeast. Previous achievements in this field have been discussed in detail by several authors (13, 112, 140, 397). Recent progress with respect to metabolic engineering of *S. cerevisiae* in this field is reviewed below.

(i) Supplemental cellulose- and xylan-degrading enzymes.

Typically, lignocellulose hydrolysis is achieved through various chemical and physical pretreatments followed by the addition of appropriate enzymes (58, 165, 166). However, yeast which can directly utilize cellulose or hemicelluloses, in particular xylan, for ethanol production would greatly reduce the costs of ethanol production from cellulosic biomass. In fact, consolidated bioprocessing, combining cellulase/xylanase production, cellulose/xylan hydrolysis, and fermentation in one step, shows outstanding potential for the bioethanol industry (208, 209).

As cellulose is the most abundant biopolymer in nature, it is of major interest as a renewable carbon source for industrial biotechnology, particularly biofuel production. However, the enzymatic hydrolysis of cellulose is complex. For example, the cellulolytic filamentous fungus *Trichoderma reesei* (*Hypocrea jecorina*) is equipped with different types of endoglucanases, cellobiohydrolases (exoglucanases), and β -glucosidases (cellobiases) which act synergistically. Endoglucanases (EC 3.2.1.4) attack one of the cellulosic chains within the molecule (preferably in the amorphous cellulose fiber regions), creating new chain termini. Then exoglucanases attach to loose ends, pull the cellulose chains from the crystalline structure, and cleave off cellobiose (glucose dimers) or glucose. Exoglucanases include cellobiohydrolases (EC 3.2.1.91) and cellodextrinases (EC 3.2.1.74); the latter act specifically on cello-oligosaccharides. Of the two types of cellobiohydrolases, cellobiohydrolase I attaches to the "reducing" end and cellobiohydrolase II attaches to the "nonreducing" end. Finally, after β -glucosidases (EC 3.2.1.21) split cellobiose into two glucose molecules, these become available for processing into chemicals or fuels. For an excellent review of microbial cellulose degradation, the reader is referred to reference 210.

Numerous attempts to transfer single cellulolytic enzymes to *S. cerevisiae* have been reviewed elsewhere (210, 370). Recent examples of the heterologous expression of β -glucosidases, enabling yeast to utilize cellobiose and cello-oligosaccharides, are given by van Rooyen et al. (369) and Katahira et al. (161). Promising results for endoglucanase expression, allowing yeast to attack amorphous cellulosic substrates, have been presented by Toda et al. (350). Cellulases (exoglucanases and endoglucanases) possess cellulose binding domains that are crucial for these enzymes to interact with their substrate cellulose (45). To improve the activity of single cellulolytic enzymes expressed and secreted by *S. cerevisiae*, domain engineering of cellulose binding domains has been a frequent target in the recent past (109, 130, 229).

The most promising strategy for converting cellulose to ethanol in yeast is certainly the concerted heterologous expression of all types of cellulolytic enzymes to maximize their synergies (19, 403). In fact, such a cocktail of enzymes (isolated from cellulolytic

filamentous fungi) was responsible for increased efficiency in industrial cellulases and greatly reduced costs for cellulose hydrolysis in past years (http://www1.eere.energy.gov/biomass/cellulase_enzyme.html). The following examples of yeast metabolic engineering address the challenges of cellulose as a solid substrate whose degradation requires the concerted action of multiple cellulolytic enzymes (70). The first report of an *S. cerevisiae* strain engineered to secrete four cellulolytic enzymes was given by van Rensburg et al. (368). Here, an endo- β -1,4-glucanase from *Butyrivibrio fibrisolvens* (*END1*), a cellobiohydrolase from *Phanerochaete chrysosporium* (*CBH1*), and a cellobiose from *Endomyces fibuligera* (*BGL1*) were cosecreted with a cellodextrinase from *Ruminococcus flavefaciens* (*CEL1*). Compared to the wild type, the engineered yeast released higher quantities of reducing sugars from several cellulosic substrates. However, there are no published data concerning the performance of this yeast during ethanol production.

A whole-cell yeast biocatalyst that directly produced ethanol from amorphous cellulose was constructed through simultaneous expression of *Trichoderma reesei* endoglucanase II, cellobiohydrolase II, and *Aspergillus aculeatus* β -glucosidase I (89). All three cellulolytic enzymes were displayed on the yeast's cell surface. Although amorphous cellulose was not completely consumed ($3 \text{ g} \cdot \text{liter}^{-1}$ residual total sugar, compared to about $9.5 \text{ g} \cdot \text{liter}^{-1}$ at the beginning of the fermentation), a final ethanol concentration of $3 \text{ g} \cdot \text{liter}^{-1}$ was achieved after 40 h, and the ethanol yield was $0.45 \text{ g} \cdot (\text{g sugar consumed})^{-1}$, i.e., 88.5% of the theoretical yield. In this study, the engineered strain was pregrown in medium containing glucose as a carbon source, and subsequently amorphous cellulose fermentation was inoculated with a high optical density (optical density at 600 nm of 50).

Another recent example of the coexpression in *S. cerevisiae* of two cellulolytic enzymes, including an endoglucanase, is the work of Den Haan et al. (70). These authors expressed an endoglucanase of *Trichoderma reesei* (*EGI*) plus the β -glucosidase of *Saccharomycopsis fibuligera* (*BGL1*), which enabled the yeast to convert amorphous cellulose to ethanol in one step. The maximal titer was $1 \text{ g} \cdot \text{liter}^{-1}$, and the yield was 73% of the maximal theoretical yield. In contrast to the report of Fujita et al. (89), Den Haan et al. (70) demonstrated anaerobic growth of the engineered strain on phosphoric acid-swollen cellulose. Based on the yeast biomass yield, these authors calculated that 27% of the phosphoric acid-swollen cellulose was enzymatically converted to fermentable glucose.

There have also been many attempts to engineer *S. cerevisiae* for xylan degradation. Hemicellulases enter the picture when the pretreatment method (for lignocellulosic biomass) fails to completely hydrolyze hemicelluloses (210). Two heterologous enzymes are required to hydrolyze xylan into D-xylose: (i) endo- β -xylanase and (ii) β -D-xylosidase. Xylan-degrading *Trichoderma* and *Aspergillus* spp. supply genes that encode the required enzymes. For example, Katahira et al. (160) codisplayed *xynII*-encoded xylanase II from *T. reesei* and *xylA*-encoded β -D-xylosidase from *A. oryzae* on the *S. cerevisiae* cell surface. This engineered yeast was simultaneously equipped with the three initial enzymes required for consumption of xylose, the xylan hydrolysis product (see the following section). Fermentation of birch wood xylan (100 g/liter) yielded $7.1 \text{ g} \cdot \text{liter}^{-1}$ ethanol after 62 h. The residual total sugar concen-

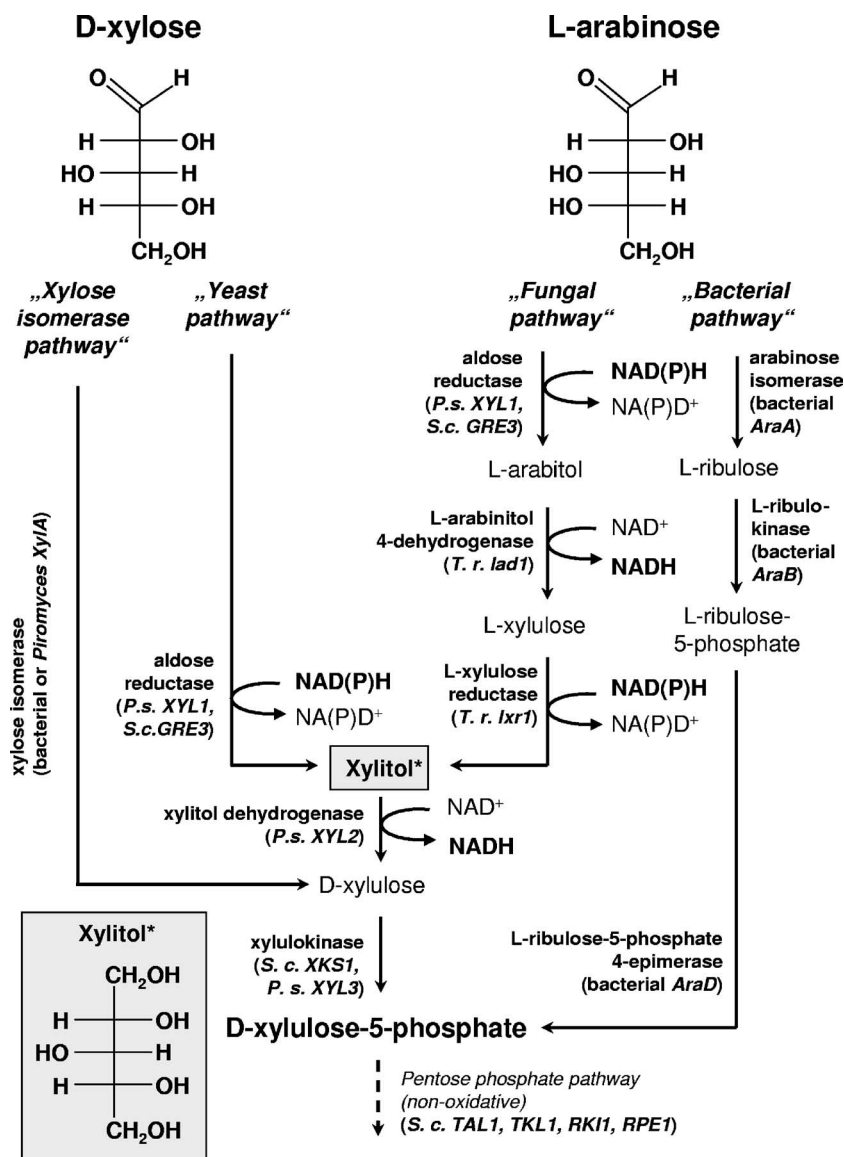


FIG. 1. Microbial utilization pathways for D-xylose and L-arabinose transferred to *S. cerevisiae*. *S.c.*, *S. cerevisiae*; *P.s.*, *Pichia stipitis*; *T.r.*, *Trichoderma reesei* (*Hypocrea jecorina*).

tration (xylan) at the end of fermentation was about $75 \text{ g} \cdot \text{liter}^{-1}$. Taking into account only the consumed sugar, the ethanol yield was $0.3 \text{ g} \cdot \text{g}^{-1}$ in this study. The ethanol production rate during the sugar-consuming phase was $0.13 \text{ g} \cdot \text{liter}^{-1} \cdot \text{h}^{-1}$. Although the processes of cellulosic and hemicellulosic ethanol production require further optimization, these examples demonstrate that consolidated bioprocessing with the yeast *S. cerevisiae* is becoming a reality.

(ii) **Utilization of pentoses and other lignocellulosic carbon sources.** Hemicellulosic hydrolysis yields several monosaccharides which cannot be used by *S. cerevisiae*. In particular, the pentose sugars xylose and arabinose can amount to a substantial fraction of the fermentable sugars in lignocellulose hydrolysates (93). To reduce the ethanol production cost, it is necessary to achieve high ethanol yields. Therefore, the complete conversion of all available substrates, including pentoses, is crucial (92).

This is an important reason why microorganisms other than

S. cerevisiae came to the fore in bioethanol production from lignocellulosic biomass. Indeed, many microorganisms are able to efficiently utilize pentose sugars but cannot naturally produce ethanol with sufficient yield and productivity. Nevertheless, there have been substantial successes in engineering those microorganisms for ethanol production (137, 138, 140). The future will reveal which is more successful: (i) establishing efficient ethanol production in microbes which naturally utilize pentoses, such as the bacteria *E. coli* and *Klebsiella oxytoca* (137) or the yeast *P. stipitis* (138, 140), or (ii) supplying ethanologenic microorganisms, such as the conventional yeast *S. cerevisiae* (57, 110, 111, 138, 362) or the ethanologenic bacterium *Zymomonas mobilis* (298), with pentose utilization pathways.

Two enzymatic pathways for xylose consumption are known to exist in nature (Fig. 1), and both have been independently transferred to *S. cerevisiae*. In one pathway the conversion of D-xylose to D-xylulose is performed by a xylose isomerase. This

pathway had been previously found only in bacteria, until a xylose isomerase in the anaerobic rumen fungus *Piromyces* was reported (183). Although this has often been referred to as a “bacterial pathway” in previous publications, I refer to it as the “xylose isomerase pathway,” to account for this novel discovery. The second natural pathway found primarily in certain fungi and yeast species consists of two enzymatic steps: aldose (xylose) reductase and xylitol dehydrogenase. Approaches which have used this “yeast pathway” for engineering *S. cerevisiae* rely on the corresponding genes (*XYL1* and *XYL2*) from the xylose-fermenting yeast *Pichia stipitis*. The introduction of either pathway enables *S. cerevisiae* to consume xylose; however, the rates are very low (110, 364).

One clear limitation for xylose utilization by the engineered strains is the nonoxidative PPP. In fact, the overexpression of PPP enzymes (starting from D-xylulose) led to improved growth on xylose; this result is independent of the initial heterologous pathway that converts xylose into D-xylulose (145, 157, 184). Significant improvements were also achieved by applying evolutionary engineering to the strains previously equipped with one of the two heterologous pathways (185, 382). Potential limitations for efficient ethanol production from xylose by engineered *S. cerevisiae* are, for example, the redox imbalance of the “yeast pathway” (138, 302), a low xylose uptake rate (185, 308), an insufficient ATP production rate (324), and the fact that xylose does not, unlike glucose, induce fermentative catabolism in yeast (146, 302).

Most investigations using engineered xylose-consuming *S. cerevisiae* strains have been carried out under laboratory conditions (111). However, industry is very interested in studying the performance of engineered *S. cerevisiae* strains under practical conditions, i.e., in lignocellulose hydrolysates. Hahn-Hagerdal et al. (110) focused on this issue and showed that several industrial *S. cerevisiae* strains with the “yeast pathway” for xylose utilization successfully cofermented xylose and glucose. The highest ethanol yield reported in that paper was $0.44 \text{ g} \cdot (\text{g carbohydrate consumed})^{-1}$, based on batch fermentation of corn stover hydrolysate with a strain constructed at the Laboratory of Renewable Resources Engineering (309). Keeping in mind the final goal of consolidated bioprocessing, the “yeast pathway” in *S. cerevisiae* strains engineered for xylose utilization was combined with arabinose utilization (see below), xylan hydrolysis (160), and cello-oligosaccharide metabolism (161).

As recently announced, the “xylose isomerase pathway” is also well on its way to industrial application (364). An engineered (rational metabolic engineering plus evolutionary engineering) *S. cerevisiae* laboratory strain efficiently fermented wheat-straw hydrolysates in fed-batch fermentation followed by a batch phase. Simultaneous glucose and xylose consumption and an ethanol yield of $0.51 \text{ g} \cdot (\text{g carbohydrate consumed})^{-1}$ were achieved after 55 h of fermentation. The often-emphasized advantage of the “xylose isomerase pathway” in comparison with the “yeast pathway” is its redox neutrality. This is expected to enable higher sugar conversion and ethanol production rates, low concomitant xylitol production, and independence of aeration. In fact, a laboratory strain based on the “xylose isomerase pathway” has the highest specific ethanol production rate ($0.49 \text{ g} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$) reported to date from xylose under laboratory conditions (362). This strain, RWB 218, was obtained by introducing *Piromyces xylA*; overexpression of *XKSI*, *TALI*, *TKLI*, *RPE1*, and *RKII*;

deletion of *GRE3* (Fig. 1); and then evolutionary engineering (for growth rate improvement on xylose). To better dissect the impacts of rational engineering and evolutionary engineering in RWB 218, Karhumaa et al. (158) overexpressed *XKSI*, *TALI*, *TKLI*, *RPE1*, and *RKII* in a laboratory strain and deleted the *GRE3* gene. They subsequently introduced the “yeast pathway” and the “xylose isomerase pathway” to compare their respective impacts on ethanol yield and production rate. The strain carrying the “xylose isomerase pathway” showed a higher ethanol yield of 0.43 versus $0.33 \text{ g} \cdot (\text{g carbohydrate consumed})^{-1}$ but a slightly lower specific ethanol production rate (0.02 versus $0.04 \text{ g} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$). The results indirectly demonstrate that the much higher ethanol production rate of strain RWB 218 was based primarily on mutations accumulated during evolutionary engineering.

There are also two different arabinose utilization pathways in nature, the bacterial and the fungal. The redox-neutral bacterial pathway is comprised of three enzymes, whereas the fungal pathway consists of five enzymes, including four oxidoreductases (Fig. 1). Both pathways have been independently expressed in yeast (25, 291). The expression of the bacterial pathway together with the overexpression of the endogenous galactose transporter gene (*GAL2*) resulted in an ethanol production rate of $0.08 \text{ g} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$, over one order of magnitude higher than that with the fungal pathway. Gal2p has been previously shown to transport arabinose to a certain extent (180), and the ethanol production rate was achieved only after subsequent evolutionary engineering of the rationally constructed strain (25). The genetic background of the evolved strain was combined with the “yeast pathway” for xylose utilization (159). The results obtained with the recombinant *S. cerevisiae* strain showed that arabinose and xylose can be co-consumed, but efficient ethanol production from L-arabinose requires further optimization. Recently, a laboratory yeast strain which anaerobically converts arabinose to ethanol in batch fermentation was reported (393). This strain was obtained by introducing the “bacterial pathway” for arabinose utilization (Fig. 1) from *Lactobacillus plantarum*, overexpressing *S. cerevisiae* genes encoding the nonoxidative PPP enzymes, and subsequent evolutionary engineering. An ethanol yield of $0.43 \text{ g} \cdot (\text{g carbohydrate consumed})^{-1}$ and a specific ethanol production rate of $0.29 \text{ g} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ from arabinose as the sole carbon source were achieved. The strain also efficiently produced ethanol from sugar mixtures containing glucose and arabinose, at $0.43 \text{ g} \cdot (\text{g carbohydrate consumed})^{-1}$. Recently, another engineered *S. cerevisiae* strain (BWY1-4S) was reported to grow on arabinose. The bacterial genes (*B. licheniformis araA* and *E. coli araB* and *araD*) were codon optimized for *S. cerevisiae* glycolytic enzymes. The strain produced ethanol from arabinose with a yield of $0.39 \text{ g} \cdot \text{g} \cdot (\text{carbohydrate consumed})^{-1}$ and a production rate of $0.036 \text{ g} \cdot \text{g}^{-1} \cdot \text{h}^{-1}$ (387). It is expected that further evolutionary engineering of this strain will greatly enhance its ethanol production rate.

To overcome the critical failure to efficiently transport pentoses into the *S. cerevisiae* cells, genes encoding pentose transporters have been cloned from pentose-utilizing yeast species for expression in *S. cerevisiae*. The first active heterologous expression of a glucose/xylose facilitator (*GXF1*) and a glucose/xylose symporter (*GXS1*) from *Candida intermedia* was reported by Leandro et al. (194). Moreover, the first transporter

specific for arabinose from *P. stipitis* was functionally expressed in *S. cerevisiae* (162a). When introduced into *S. cerevisiae* strains previously engineered for xylose and arabinose fermentation, these transporters potentially could further increase pentose consumption rates. Based on the achievements in engineering *S. cerevisiae* for pentose fermentation, the simultaneous conversion of different sugars in lignocellulose hydrolysates by yeast appears to be feasible. Yeast strains capable of simultaneous hexoses, xylose, and arabinose fermentation are expected to emerge in the near future.

Another sugar which is present in several industrial media, such as molasses, cheese whey, and lignocellulose, is galactose. *S. cerevisiae* can naturally utilize this monosaccharide via the Leloir pathway, but at a significantly lower rate than glucose. In addition, galactose utilization is repressed if glucose is present in the medium. Therefore, it has been proposed to deregulate the glucose repression of galactose utilization by genetic engineering. The overexpression of *GAL4* or the simultaneous deletion of *GAL6*, *GAL80*, and *MIG1* resulted in an increase in the specific galactose uptake rate of 26% or 41%, respectively (258). By using an inverse metabolic engineering strategy, it was recently found that an increased level of *PMG2*, encoding the major isoenzyme of phosphoglucosyltransferase, increases the flux through the Leloir pathway by 74%, even without modifying transcriptional regulators involved in glucose repression (36). Other carbohydrates such as galacturonic acid and rhamnose, present in lignocellulose hydrolysates, also have potential as *S. cerevisiae* carbon sources, and appropriate metabolic engineering strategies have recently been suggested (362).

(iii) Tolerance to inhibitory substances present in lignocellulose hydrolysates. Lignocellulosic plant biomass requires chemical pretreatment, exposing the polysaccharides (cellulose and hemicelluloses) to enzymatic hydrolysis and fermentation. Current pretreatment processes, which usually rely on high temperatures, acid hydrolysis, and/or high pressure (50, 93, 200), form several degradation products with various inhibitory effects on yeast fermentation (170, 171). These substances fall into the following classes: carboxylic acids, furans, phenolic compounds, and inorganic salts (397).

Furfural and 5-hydroxymethylfurfural (HMF) are sugar degradation products (50, 93). Both substances greatly inhibit yeast growth and fermentation (226, 264, 336, 337). Engineered yeasts, which better tolerate furfural and HMF, more efficiently convert lignocellulose to ethanol. Strains of *S. cerevisiae* exhibit significant differences in inhibitor tolerance, and yeast is able to adapt to furfural and HMF to a certain extent (162, 202–204). Improved tolerance is associated with an increased capability for NADH- and/or NADPH-dependent transformation of furfural and HMF into their respective lower-toxicity alcohols (247). These studies have increased the potential for in situ detoxification and suggest that high reductase activity may provide the basis for tolerance.

To identify genes involved in furfural tolerance, one strategy screened a *Saccharomyces cerevisiae* gene disruption library for mutants which exhibit growth deficiencies in the presence of furfural (103). Surprisingly, several mutants defective in the PPP were found to be inefficient at reducing furfural to furfuryl alcohol. The researchers suggested that these data result from an overall decreased abundance of reducing equivalents in the

presence of furfural or from NADPH's role in stress tolerance. In fact, overexpression of *ZWF1*, encoding glucose-6-phosphate dehydrogenase, in *S. cerevisiae* allowed growth at furfural concentrations that are normally toxic.

Another inverse metabolic engineering approach was launched to identify the enzyme(s) responsible for HMF conversion in *S. cerevisiae*. Here, microarray analysis compared the expression profiles of two strains that exhibit differing HMF detoxification abilities. *ADH6*, encoding a strictly NADPH-dependent alcohol dehydrogenase, was among the genes up-regulated in the more tolerant strain and identified as a potential target for improved HMF tolerance. Strains overexpressing *ADH6* demonstrated (i) increased HMF conversion activity in cell-free crude extracts with NADPH as the cofactor, as well as (ii) a substantially higher in vivo HMF conversion rate in both aerobic and anaerobic cultures. Obviously, this genetic modification did indeed increase the desired reduction capacity (272).

To detoxify phenols, laccase from the white rot fungus *Trametes versicolor* has been expressed in *S. cerevisiae*. The laccase-producing transformant was able to ferment a dilute-acid hydrolysate from spruce at a higher rate than the reference strain (190). Moreover, the overexpression of *S. cerevisiae* *PADI*, encoding phenolic acid decarboxylase, improved ethanol productivity in spruce hydrolysates; this was attributed to the engineered yeast's increased biotransformation of several phenolic compounds (191). To date, metabolic engineering strategies which have addressed yeast's tolerance to organic acids present in lignocellulose hydrolysates are rare. Nevertheless, one interesting target for improving weak acid tolerance is Pdr12p (a plasma membrane H⁺-ATPase), which has been identified in research regarding yeast's resistance to weak acid food preservatives (368a). Enhanced basic research under way in this field is expected to identify further target genes for acid tolerance improvement (see, e.g., reference 66a). With regard to inorganic salt tolerance, fundamental research is addressing osmotolerance in yeast. In these studies, unusually high concentrations of NaCl (up to 1.4 M) have often been used to induce hyperosmotic stress (124, 125). Proteins that were identified as important in yeast osmoregulation are potential targets for improved tolerance to inorganic salts present in lignocellulose hydrolysates.

Although there has been considerable progress in addressing individual inhibitory substances, it bears mention that different inhibitory compounds in lignocellulose hydrolysates have additive or synergistic effects (171). Moreover, conditions relevant in industrial-scale fermentations, such as elevated temperatures, may further increase deleterious inhibitor effects (106). This, along with the fact that the composition of a hydrolysate strongly depends on the source material and pretreatment method, will certainly be a challenge for genetic engineers. Multiple tolerances are multigenic, and rational strategies will therefore fail in this context. Alternative approaches such as evolutionary engineering or gTME will be necessary to address these challenges. Martin et al. (218) have recently pursued this idea via improving the multiple-inhibitor tolerance of a xylose-fermenting *S. cerevisiae* strain through evolutionary engineering. Ongoing research is also directed at establishing alternative pretreatment strategies which produce lower levels of inhibitors (165, 166).

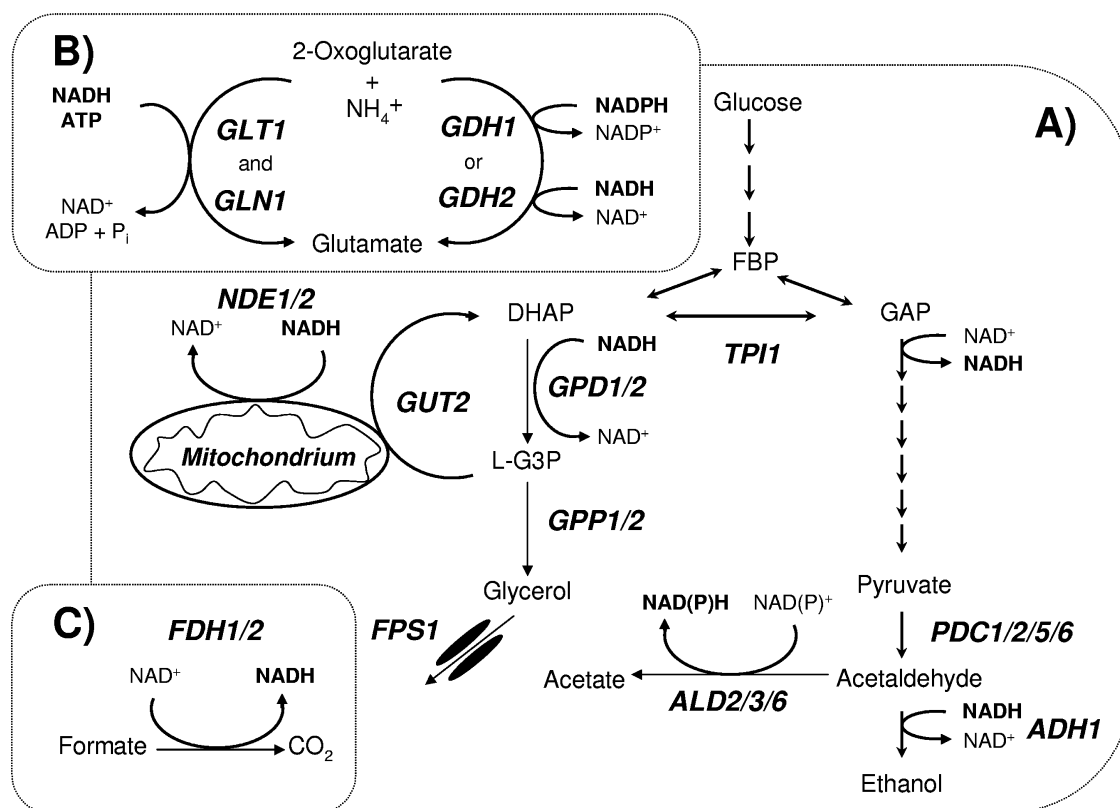


FIG. 2. Glycerol biosynthesis and other major pathways contributing to cytosolic NAD⁺/NADH balance in *S. cerevisiae*. All genes with roles in either increasing or abolishing glycerol formation as well as accumulating L-G3P are included. (A) Glycolytic pathway linked to the formation of glycerol, ethanol, and acetate. (B) Redox factor utilization during glutamate formation from 2-oxoglutarate modified per data from Nissen et al. (250) to reduce cytosolic NADH generation and glycerol formation. (C) Glycerol overproduction in *S. cerevisiae*, per data from Geertman et al. (95), with formate dehydrogenase (*FDH1*) overexpression and formate feeding to generate an additional cytosolic NADH source. Gene names: *ADH1*, alcohol dehydrogenase; *ALD4/5/6*, cytosolic and mitochondrial acetaldehyde dehydrogenases; *GPD1/2*, cytosolic L-G3P dehydrogenase; *GPP1/2*, L-glycerol 3-phosphatase; *GUT2*, mitochondrial FAD⁺-dependent G3P dehydrogenase; *FDH1/2*, formate dehydrogenase; *NDE1/2*, external mitochondrial NADH dehydrogenase; *PDC1/2/5/6*, PDC; *TPI1*, triosephosphate isomerase; *GLT1*, glutamate synthase (NADH); *GLN1*, glutamine synthetase; *GDH1*, glutamate dehydrogenase (NADP⁺); *GDH2*, glutamate dehydrogenase (NAD⁺); *FPS1*, glycerol facilitator (glycerol export). FBP, fructose-1,6-bisphosphate; GAP, glyceraldehyde phosphate; P_i, inorganic phosphate; NH₄⁺, ammonium ions.

Reduction of fermentation by-products. Another important aspect of improving the bioethanol production process is to ensure complete conversion of sugars into valuable products. However, there are several by-products of yeast fermentation, such as carbon dioxide, yeast biomass, glycerol, and other, minor products, which lower the total ethanol yield. Glycerol production in *S. cerevisiae* can be quite substantial, i.e., 2.0 to 3.6% of glucose consumed when calculated as g · g⁻¹ (269). This depends on the yeast strain, fermentation conditions (94), and medium composition, particularly the type of nitrogen source (5). Elimination of glycerol formation and redirection of carbon flux toward ethanol formation could increase the ethanol yield by at least 10% (249). Glycerol reduction in the fermentation broth would also decrease the ethanol extraction costs and waste volumes.

Glycerol production in *S. cerevisiae* (Fig. 2) under nonstress conditions results primarily from maintenance of the cytosolic redox balance (NADH), particularly under anaerobic conditions (12, 20, 292, 356). The cellular NADH concentration is low, and continuous reoxidation is crucial to maintain glycolytic flux. In principle, ethanol formation from glucose is redox

neutral. However, some cytosolic NADH generated during glycolysis (via glyceraldehyde-3-phosphate dehydrogenase) cannot be reoxidized. This is due to the fact that a certain portion of carbon compounds such as pyruvate is lost to synthetic pathways for biomass formation. This in turn reduces the amount of NADH acceptor molecules (acetaldehyde) available for ethanol formation. Moreover, several biosynthetic pathways result in net NADH formation. Another source of excess cytosolic NADH is the excretion of partially oxidized products such as acetate. Glycerol biosynthesis serves as a mechanism to reoxidize this surplus NADH, particularly under anaerobic conditions. Under aerobic conditions, NADH is assumed to be partly reoxidized via the external NADH dehydrogenases Nde1p and Nde2p (207), which channel the electrons to the mitochondrial electron transport chain. This might be the main reason that glycerol formation was considerably reduced when microaeration instead of anaerobiosis was applied during ethanol fermentation (7).

Previous metabolic engineering approaches to reduce glycerol have primarily attempted to lower flux into the glycerol

biosynthetic pathway, reduce biomass formation, or decrease NADH formation during biomass synthesis. The first metabolic engineering approach to reduce glycerol was reported several years ago (249). Deleting *GPD1* and *GPD2* completely abolished yeast's glycerol formation and increased ethanol yield in aerobic batch fermentation by 12%. However, the resulting volumetric ethanol productivity did not approach industrial relevance, since the growth of the *gpd1Δ gpd2Δ* double mutant was severely affected. The growth defect under anaerobic conditions is not surprising, since glycerol formation is the only outlet for NADH reoxidation under such conditions. However, growth of the mutant was also strongly impaired under aerobic conditions. This has been attributed to a limited capacity of respiratory NADH reoxidation by the external NADH dehydrogenases Nde1p and Nde2p and the mitochondrial L-G3P/dihydroxyacetone phosphate (DHAP) shuttle (249).

Other attempts to reduce glycerol formation relied on the introduction of bacterial transhydrogenases into yeast to convert excess NADH into NADPH. These approaches failed since on one hand, the *Azotobacter vinelandii* transhydrogenase produced the opposite of the expected effect (248), and on the other hand, the membrane-bound *Escherichia coli* transhydrogenase was not properly targeted in yeast (10).

The ammonium assimilation of *S. cerevisiae* has been modified (250) to reduce NADH formation in biomass production (amino acid biosynthesis). A yeast strain was constructed in which *GLN1*, encoding glutamine synthetase (Fig. 2), and *GLT1*, encoding glutamate synthase (Fig. 2), were overexpressed and *GDH1*, encoding NADPH-dependent glutamate dehydrogenase (Fig. 2), was deleted. The glycerol yield per glucose consumed was reduced by 38%, and the ethanol yield was increased by 10%. It was stated in a later publication by the same authors (38) that, to properly function, the latter approach requires that the yeast utilizes ammonium as a nitrogen source. Therefore, any use of complex nitrogen sources containing amino acids, e.g., corn steep liquor, grain and corn mash, or sugar cane juice, particularly when proteases are added (133), might reduce the success of this approach. Surprisingly, Kong et al. (179), who overexpressed homologous *GLT1*, encoding NADH-dependent glutamate synthetase, in a *gpd2Δ* mutant of *S. cerevisiae*, obtained similar results (for increased glycerol and ethanol yields compared to wild type) as Nissen et al. (250) even though they used complex medium (YPD).

To evaluate further metabolic engineering strategies for improved ethanol yield in *S. cerevisiae*, an in silico study was carried out using a genome-scale *S. cerevisiae* metabolic model (38). These approaches have been designed to prevent excess NADH production through biomass synthesis and thus reduce the need to produce glycerol. Based on the authors' predictions, several approaches should achieve increased ethanol yields of up to 10.4%. One of the predicted strategies, the expression of a nonphosphorylating, NADP⁺-dependent glyceraldehyde-3-phosphate dehydrogenase encoded by the *gapN* gene of *Streptococcus mutants*, was tested in vivo and resulted in a 40% reduction of glycerol yield but only a 3% increase in ethanol yield. The latter result exemplarily demonstrates that predictive modeling is a helpful tool in metabolic engineering; however, the models will need to be further refined.

Reduction of glycerol production has also been attempted

by deleting *FPS1*, encoding a glycerol facilitator in yeast (Fig. 2). In fact, it was previously shown that glycerol efflux is one of the rate-controlling steps in glycerol production (289). Glycerol yield (per glucose consumed) was decreased by 18.8% while ethanol yield was improved by 10% in an *fps1Δ* mutant (400). When *FPS1* deletion was combined with *GLT1* overexpression, the glycerol yield per glucose consumed was 30% lower than that in the parental strain, and the ethanol yield was 14% higher (178). According to the authors, there was no further increase of ethanol yield by additional deletion of *GPD1* or *GPD2* in this genetic background (43).

To date, all studies aimed at reducing glycerol formation have been carried out under laboratory condition; no industrial yeast strains have been engineered for low glycerol production and tested in industrial-scale fermentations. The industrial constraints occurring during bioethanol production, such as anaerobiosis, high ethanol concentration, and high temperature and osmolarity, aggravate metabolic engineering attempts to reduce glycerol formation. In fact, the glycerol biosynthetic pathway in yeast has various important biological functions, particularly under stress conditions. Intracellular glycerol has been shown to be involved in osmoadaptation (240), oxidative stress protection (263), and response to heat shock (320). Thus, reducing glycerol formation without negatively affecting cell fitness under industrial conditions is an important challenge. The level to which glycerol production can be reduced without deleterious effects on cell viability remains to be seen.

Improving tolerance to ethanol. Ethanol inhibition of yeast growth begins at concentrations of less than 5%, depending on the yeast strain and growth conditions (273). Increasing ethanol concentrations also strongly depress fermentation rate and yeast viability. For detailed information on the toxic effects of ethanol, the reader is referred to previous comprehensive reviews (47, 65, 139, 275). The extent of yeast's tolerance to ethanol determines its ethanol productivity and final titer, which is especially relevant in very-high-gravity fermentation (72).

The mechanisms underlying yeast tolerance to ethanol are very complex and not fully understood (139). Many studies investigating ethanol tolerance have focused on sake yeasts (also classified as *S. cerevisiae*), since they have an extraordinarily high ethanol tolerance compared to wine and brewing yeasts. In fact, the final ethanol concentration reaches about 20% in sake mash (173). Genetic engineering has identified several factors which may contribute to the higher ethanol tolerance of sake yeast, such as ergosterol (127), unsaturated fatty acids (154, 155), palmitoyl coenzyme A (palmitoyl-CoA) (251), trehalose (153), inositol (90), and L-proline (341). However, in many cases researchers merely showed that the deletion of a certain gene causes a decrease in ethanol tolerance (90, 127). Examples of real improvements in ethanol tolerance for sake or other industrial yeasts via rational metabolic engineering are rare. Successful improvements were achieved by decreasing intracellular trehalose degradation or increasing L-proline accumulation (153, 339, 341). A diploid sake yeast engineered for proline accumulation showed a slight reduction in fermentation time (19 versus 22 days) compared to the wild type (339). Recently, screening of the homozygous diploid yeast deletion pool of nonessential genes identified two null mutants (*ura7Δ* and *gal6Δ*) that grew and consumed glucose

TABLE 2. Attempts to generate novel products from simple carbon sources by engineered *S. cerevisiae*^a

Chemical	Use	Genetic modification(s)	Carbon source and essential cosubstrates	Yield or concn ^b	Reference
p-Hydroxycinnamic acid	Starting material for different applications (e.g., flavors, pharmaceuticals, cosmetics)	<i>Rhodotorula glutinis</i> phenylalanine ammonia lyase/tyrosine ammonia lyase, <i>Helianthus tuberosus</i> C4H cytochrome P-450 and P-450 reductase	2% raffinose, 5% galactose (for promoter induction)	202.6 μM	366
Poly[(R)-3-hydroxybutyrate]	Biopolymer	Coexpression of <i>Cupriavidus necator</i> β-ketothiolase, poly[(R)-3-hydroxybutyrate] reductase and synthase	1% D-glucose, 1% D-galactose (for promoter induction)	Roughly 9% of cell dry wt (avg)	44
Polyhydroxyalkanoates	Biopolymer	Medium-chain-length polyhydroxyalkanoates, expression of <i>Pseudomonas oleovorans</i> mcl polymerase (<i>phaCI</i>); short-chain-length polyhydroxyalkanoates, targeting the <i>Ralstonia eutropha</i> scl synthase (<i>phbC</i>) to the peroxisome	1% glycerol, 0.4% Tween 80, appropriate fatty acids	Up to 7% of cell dry wt	401
Naringenin, pinocembrin	Flavonoids/isoflavonoids (plant pigments, flavors; some are used as pharmaceuticals)	Expression of <i>Rhodospiridium toruloides</i> phenylalanine ammonia lyase (<i>PAL</i>), <i>Arabidopsis thaliana</i> 4-coumarate: coenzyme A ligase (<i>4CL</i>), <i>Hypericum androsaemum</i> chalcone synthetase (<i>CHS</i>)	2% D-galactose (for promoter induction)	0.35 mg naringenin · (mg L-galactose) ⁻¹ , 0.04 mg pinocembrin · (mg L-galactose) ⁻¹	144
Nicotianamine	Metal chelator (antihypertensive effects)	<i>Arabidopsis thaliana</i> nicotinamide synthase (<i>NAS</i>)	2% D-glucose	Roughly 800 μg · (g wet wt) ⁻¹	381
Methylmalonyl-CoA	Polyketide precursor (various therapeutic applications)	(i) Expression of <i>Streptomyces coelicolor</i> PCC pathway; (ii) expression of <i>Streptomyces coelicolor</i> Mat pathway	(i) 2% D-glucose, propionate; (ii) 2% D-glucose, methylmalonate	Detectable amounts	234
S-Adenosylmethionine	Chemotherapeutic agent for various diseases	Deletion of <i>S. cerevisiae</i> C-24(28) sterol reductase (<i>erg4Δ</i>)	5% D-glucose, 0.15% L-methionine	16.3 mg · (g dry wt) ⁻¹	319
Vitamin C (L-ascorbic acid) ^c	Vitamin	Overexpression of <i>Arabidopsis thaliana</i> L-galactose dehydrogenase (<i>LGDH</i>), overexpression of <i>S. cerevisiae</i> D-arabinono-1,4-lactone oxidase (<i>ALOI</i>)	250 mg/liter L-galactose	0.4 mg of L-ascorbic acid · (mg L-galactose) ⁻¹	304

^a Attempts that are not discussed in the text.

^b As specified by the authors.

^c Resting rather than growing cells were used in this approach.

faster than the wild-type strain in ethanol-rich medium. Results point to modified cell wall integrity and membrane fluidity as the reasons for the disruptants' improved capability to cope with ethanol stress (394).

In addition, gTME offers a promising alternative approach to access polygenic traits such as multiple stress tolerance. Random mutagenesis of one or more general transcription factors elicits a global reprogramming of gene expression (9). For example, an *S. cerevisiae* mutant library of the TATA binding protein Spt15 was subjected to multiple stresses: high glucose and high ethanol concentrations. The authors were able to select one *spt15* mutant clone which contained three point mutations; its optical density was 13-fold after 20 h of growth in minimal medium supplemented with 100 g/liter glucose and 6% (vol/vol) ethanol. Interestingly, in addition to improved ethanol tolerance, the specific ethanol production rate of this strain also increased (by 41%) in comparison to that of the control strain (9). It remains to be seen whether transferring the *spt15* mutant to an industrial strain will similarly affect ethanol tolerance and productivity. Usually, industrial strains of *S. cerevisiae* have a higher inherent tolerance to ethanol than laboratory strains.

Aside from high ethanol concentrations, yeast cells also

must cope with several other types of stress during the process of bioethanol production, e.g., carbon dioxide (3) and pressure (85). Additionally osmotic stress is problematic, particularly in very-high-gravity fermentation (72), as is heat stress in areas/seasons of high ambient temperature where cooling is unavailable or energetically undesirable (1). Thus, a challenge for metabolic engineers will be the generation of multiple stress resistances in yeast (see "Utilization of lignocellulosic biomass. (i) "Tolerance to inhibitors present in lignocellulose hydrolysates" above). Evolutionary engineering has been recently shown to successfully isolate greatly improved multiple-stress (including ethanol stress)-resistant mutants of baker's yeast (39).

Production of Fine and Bulk Chemicals

This section highlights continuing attempts to produce in yeast various useful compounds in addition to ethanol, such as glycerol, propanediol, organic acids, sugar alcohols, L-G3P, isoprenoids, ergosterol, and other steroids. In addition to these compounds, which are individually discussed below, there are many further substances whose production in yeast has also been recently attempted (Table 2). In general, most of the

approaches mentioned here are still in the early phases and will require a great deal of effort to optimize yield and productivity. However, these examples demonstrate the potential of *S. cerevisiae* to produce many interesting chemical compounds from renewable sources, which undoubtedly will further increase in the future.

The reader might question what yield, titer, or productivity of a given production organism is necessary to make a fermentative production process economically viable. One way to assess process economics is to evaluate whether the production cost plus profits of the novel microbial process is lower than either the market price or the production cost plus profits of the petrochemical process (119). It is clear that process viability strongly depends on the price of the feedstock used, i.e., crude oil price versus sugar price, as well as on the market price of the product, which is determined by demand and supply. Therefore, the required yield, titer, and productivity of a microbial process may vary depending on whether the product is a bulk or niche chemical. Moreover, steadily rising raw oil prices will soon convert an increasing number of formerly nonviable microbial processes into economically viable ones (119). Process economics could also be significantly improved in the future by lignocellulosic biomass becoming fully available as a starting material.

Glycerol. Glycerol has been traditionally used for the production of nitroglycerin as well as in cosmetics, drugs, and toothpastes. Fermentative production of glycerol by *S. cerevisiae* has a long history dating back to Neuberg and Rheinfurth's "second form" of fermentation, which relied on the addition of steering agents such as sulfite or alkali to trigger glycerol overproduction (236). Different microorganisms, including algae, bacteria, and yeasts (especially several osmotolerant yeasts), which naturally form comparably large amounts of glycerol have been also explored for their potential in commercial glycerol production (384). Currently, there are several trends which influence the demand for microbial glycerol production. Large quantities of glycerol-rich streams are generated during the production of biodiesel (395), providing strong competition to microbial glycerol processes. In parallel, the chemical industry has shown renewed interest in the use of glycerol as a central raw material (262). For example, glycerol can be used as a precursor for the production of 1,3-propanediol (1,3-PD) (399) (see "Propanediol" below), which is highly in demand. Another emerging trend is the increasing use of plant biomass hydrolysates (sugars) as raw material in industrial biotechnology. When demand for the production of glycerol-derived compounds from such hydrolysates rises, metabolic engineering approaches for glycerol overproduction will regain favor. Demand will certainly be influenced by whether biodiesel production remains viable or not.

Previous metabolic engineering attempts to improve glycerol synthesis in *S. cerevisiae* (Fig. 2), including our approach of overexpressing *GPD1* in a *pdc2Δ* mutant (241), did not lead to industrially relevant glycerol yields until the triose phosphate isomerase gene (*TPI1*) was used as a target for deletion (62). The *tpi1Δ* mutant formed 0.46 g glycerol · (mol glucose)⁻¹ in shake flasks with glucose as a sole carbon source, albeit with a severe growth defect. This growth defect was later attributed to an accumulation of DHAP, which has been shown to inactivate *myo*-inositol-3 phosphate synthase and render the cells auxo-

trophic for inositol (313). Addition of inositol partly restored growth of the *tpi1Δ* mutant. The growth defect was also partly overcome by the additional deletion of the two isogenes *NDE1* and *NDE2*, encoding mitochondrial external NADH dehydrogenase, and *GUT2*, encoding the FAD⁺-dependent mitochondrial GPD (261). As these enzymes are primarily responsible for mitochondrial reoxidation of cytosolic NADH, their deletion increased the availability of cytosolic NADH, thereby reducing the accumulated DHAP pool. The *tpi1Δ nde1Δ nde2Δ gut2Δ* quadruple mutant did grow on glucose, but the specific growth rate was much lower than that of the wild type. Evolutionary engineering of the quadruple mutant resulted in a strain which yielded 0.99 mol glycerol · (mol glucose)⁻¹, which is very close to the theoretical maximum of 1 mol · (mol glucose)⁻¹ (261).

Another approach, which was also based on a *tpi1Δ* mutant and led to a glycerol yield of 0.91 mol glycerol · (mol glucose)⁻¹, was recently published by Cordier et al. (63). Here, the deletion of *TPI1* was combined with the overexpression of *GPD1* (encoding one isoenzyme of GPD), the deletion of *ADH1* (encoding the major NAD⁺-dependent alcohol dehydrogenase), and the overexpression of *ALD3* (encoding a cytosolic NAD⁺-dependent aldehyde dehydrogenase).

The theoretical maximum for glycerol production in *S. cerevisiae* is limited by the fact that only half of the glucose carbon can be used for NADH-consuming glycerol production, whereas the other half is required for NADH generation via glyceraldehyde 3-phosphate dehydrogenase in the "pyruvate branch" of glycolysis. This problem was recently addressed by Geertman et al. (95). They rerouted glycolytic flux using a *pdc1Δ pdc5Δ pdc6Δ nde1Δ nde2Δ gut2Δ* mutant strain combined with *GPD2* overexpression; however, their primary innovation was the simultaneous overexpression of *FDHI*, encoding a NAD⁺-linked formate dehydrogenase. Cells supplied with an additional source of cytosolic NADH achieved a glycerol yield of 1.08 mol glycerol · (mol glucose)⁻¹, exceeding the theoretical maximum. However, this strategy requires formate cofeeding to properly function (Fig. 2).

To overcome the fixed redox stoichiometry and significantly increase glycerol formation from glucose without cofeeding an additional substrate, a completely new strategy is needed. Therefore, we proposed to switch the redox factor specificity of glycerol formation in *S. cerevisiae* from NADH to NADPH by (i) introducing a NADPH-dependent DHAP reductase and (ii) redirecting the main carbon flux through the PPP by reducing phosphoglucose isomerase activity. Using this strategy, both the DHAP and reducing equivalents (NADPH) needed for glycerol formation are supplied by a direct, noncompetitive pathway (Fig. 2), and the theoretical maximum glycerol yield would be 1.66 mol glycerol · (mol glucose)⁻¹. To date, we have successfully introduced the *Bacillus subtilis gpaA* gene, encoding a NAD(P)H-dependent DHAP reductase, into an *S. cerevisiae pgi1* mutant. Unfortunately, this resulted in an increase of glycerol yield per glucose consumed of only 35% compared to that for the *pgi1* mutant without the heterologous gene (242a). One limitation of this approach which must be overcome in the future is the low specific activity of the bacterial *gpaA* gene product in the yeast transformant.

Propanediol. The structural isomers 1,2-PD and 1,3-PD have similar physical properties but different industrial appli-

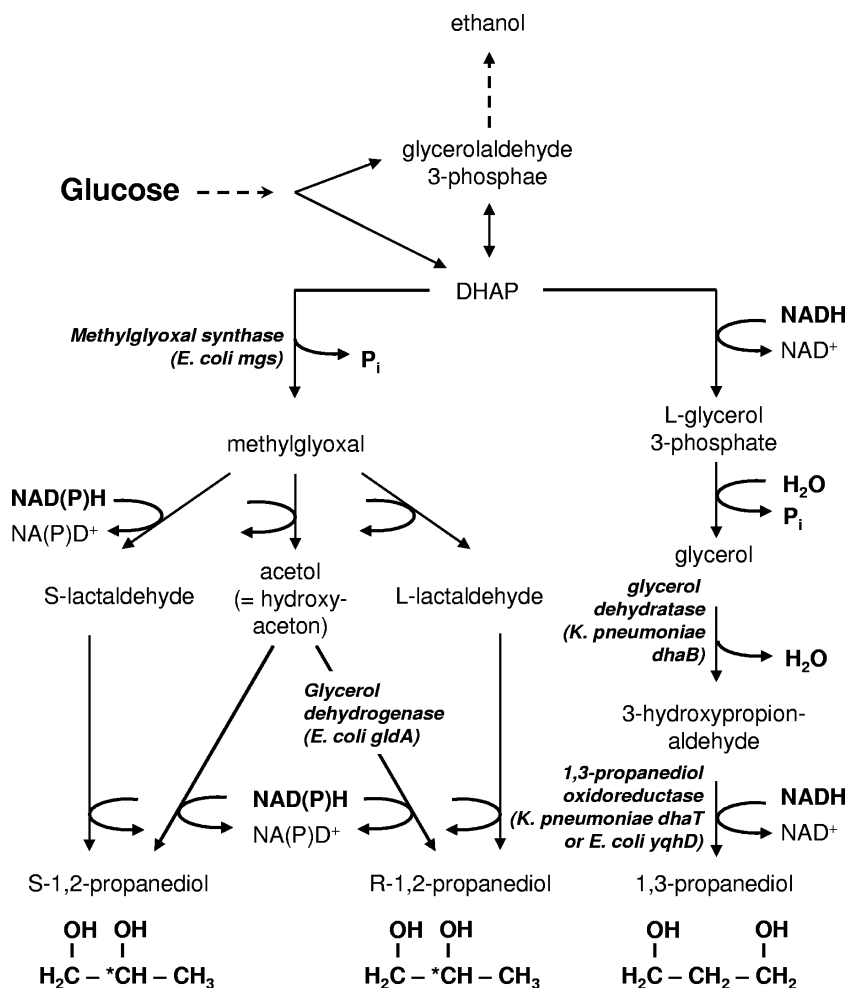


FIG. 3. Pathways for 1,2-PD and 1,3-PD formation from glycolytic DHAP. *E. coli* genes encoding methylglyoxal synthase (*mgs*) and glycerol dehydrogenase (*glaA*) were introduced into *S. cerevisiae* per Lee and DaSilva (196) to enhance 1,2-PD production. In attempts to produce 1,3-PD in *S. cerevisiae*, bacterial genes encoding glycerol dehydratase and 1,3-PD oxidoreductase were expressed (42, 212) (see "Propanediol" in text).

cations (42). Whereas 1,2-PD is already a commodity chemical with important applications in unsaturated polyester resins, liquid laundry detergents, pharmaceuticals, cosmetics, antifreeze, and deicers, 1,3-PD has long been considered a specialty chemical. However, the demand for the latter has greatly increased during recent years in light of the commercialization of the promising new polyester polypropylene terephthalate, which has unique properties for carpeting and textiles.

The most promising route for economical commercial microbial production of 1,2-PD and 1,3-PD is their total synthesis from sugars via a single organism. In fact, both substances can be synthesized from the glycolytic intermediate DHAP, via different pathways (Fig. 3). However, the ability to produce 1,2-PD by use of nonengineered microorganisms is highly limited (26, 28, 42), and no known natural microorganism is able to convert simple sugars to 1,3-PD.

It is still debated whether yeast is able to form 1,2-PD from glucose naturally (26). At a minimum, baker's yeast does contain the enzyme required to convert acetol into (*S*)-1,2-PD (176). Recently, two *E. coli* genes, *mgs* and *glaA*, which encode methylglyoxal synthase and glycerol dehydrogenase, respectively (Fig. 3),

were each integrated into the *S. cerevisiae* genome under the control of the *CUP1* promoter (196). Different copy numbers of both genes were tested in combination. The production of 1,2-PD did correlate with methylglyoxal synthase activity, but high glycerol dehydrogenase activity was found to be inhibitory. Moreover, the product was mainly found intracellularly; i.e., extracellular 1,2-PD was not detectable. The maximum concentration achieved in this study was fairly low, $0.15 \text{ g} \cdot (\text{g yeast dry weight})^{-1}$.

The following paragraphs deal with the microbial production of 1,3-PD, the monomer highly in demand for production of the polypropylene terephthalate polymer. Although 1,3-PD cannot be naturally formed from sugars via fermentation, a wide range of microorganisms (e.g., *Klebsiella pneumoniae*) are able to ferment glycerol to 1,3-PD. However, these organisms lack the enzymes required to form glycerol from glycolytic DHAP. In contrast, many organisms, including baker's yeast, are excellent glycerol producers but are unable to accomplish its subsequent conversion into 1,3-PD. Therefore, the challenge for metabolic engineering is to integrate both parts of the pathway into a single organism.

Most efforts to produce 1,3-PD via a microbial route have addressed genetic engineering in *E. coli*, which possesses nei-

ther of the two partial pathways. Genencor and Dupont's approach has been extraordinary successful (189). Nonetheless, researchers have also attempted to engineer *S. cerevisiae* for 1,3-PD production because of the various advantages of yeast as a biocatalyst in fermentative production processes originating from biomass hydrolysates. In addition, *S. cerevisiae* possesses a natural pathway to produce glycerol, the 1,3-PD precursor, from sugars. Moreover, glycerol overproduction up to theoretical maximum yields by metabolic engineering is state of the art (see above). In order to directly produce 1,3-PD from glucose in *S. cerevisiae*, heterologous enzymes which convert glycerol to 1,3-PD are required. Unfortunately, the coexpression of four *K. pneumoniae* 1,3-PD genes (*dhaB3*, *dhaB3a*, *dhaB4*, and *dhaB4a*) encoding glycerol dehydratase as well as the *K. pneumoniae* *dhaT* gene encoding 1,3-PD oxidoreductase in yeast (Fig. 3) did not yield detectable 1,3-PD in the fermentation broth (42). In fact, the activities of both enzymes were quite low when coexpressed. Still, even if all the genes could be successfully expressed, researchers may face another challenge, as coenzyme B₁₂ is needed for glycerol dehydratase activity and yeast can neither produce this vitamin nor actively transport it into the cell (42). The recently discovered B₁₂-independent glycerol dehydratase from a *Clostridium* strain might therefore be of great interest (101). Nonetheless, Ma et al. (212) reported the production of 1,3-PD in a recombinant *S. cerevisiae* strain expressing the *Klebsiella* *dhaB* gene and the *E. coli* *yqhD* gene (Fig. 3).

Organic acids. Recent efforts to produce organic acids in *S. cerevisiae* have concentrated on lactic, pyruvic, and succinic acids. One general advantage of using yeast rather than bacteria for the production of organic acids lies in its high tolerance to low pH values. This may greatly decrease the need for neutralizing agents during the production process; pH controls are unnecessary, obviating the formation of vast amounts of the by-product gypsum, which greatly complicates downstream processing (148). In addition, the risk of microbial contamination is reduced.

Lactate has versatile applications in the food, pharmaceuticals, textile, leather, cosmetics, and chemical industries. Lactic acid has recently received increasing attention in industrial biotechnology, since it can be used to produce polylactic acid, the biodegradable polymer with plastic properties (118). So far, lactate is produced primarily via biotechnology using various *Lactobacillus* species. Interest in *S. cerevisiae* as an alternative biocatalyst for lactic acid production from renewable biomass resources has recently increased. In addition to the advantages mentioned before, this yeast does not require complex nitrogen sources and vitamins.

As *S. cerevisiae* does not naturally produce lactate, heterologous *LDH* expression is necessary to introduce the pathway for NADH-dependent reduction of glycolytic pyruvate to lactate (128, 300, 322). A recent study revealed that lactate production in *LDH*-expressing *S. cerevisiae* strongly depends on the yeast strain background and the source of the heterologous *LDH* gene (34). Selection for *S. cerevisiae* mutants producing lactate at higher titers can be accomplished by screening for cells with higher intracellular pH (357). Such cells seem to better tolerate high lactate concentrations.

Additional genetic modifications for lactate production have suppressed competing pathways that consume pyruvate, in par-

ticular alcoholic fermentation (129, 322, 365). For example, ethanol production was completely abolished in a *pdc1Δ pdc5Δ pdc6Δ* mutant that lacked all structural genes encoding PDC and overexpressed a *Lactobacillus casei* LDH (365). Although the strain produced lactate as the main aerobic fermentation product, it was not able to grow anaerobically. The authors suggested that the export of lactate may be an ATP-consuming process such that there is no net ATP production under anaerobic conditions. To date, no lactate exporter but only a lactate uptake has been identified in yeast. The overexpression of the corresponding gene (*JEN1*) slightly increased the lactate yield of a yeast strain that overexpressed *Lactobacillus plantarum* LDH (34). However, at least one other lactate transporter must have been present, because the *JEN1* deletion did not affect lactate production. Other researchers have shown that nicotinic acid is a limiting factor for lactate production during fermentation with an LDH-expressing *S. cerevisiae* strain, but the underlying mechanisms are poorly understood (61). Thus, industrial application of a homofermentative lactate-producing *S. cerevisiae* strain would require aeration unless metabolic engineering succeeds in creating a strain which is independent of respiration. Nevertheless, van Maris was able to achieve a final lactate concentration of 110 g · liter⁻¹ using an evolved Pdc⁻ strain expressing bacterial LDH in aerated batch culture (361); for a laboratory yeast strain, this is a promising result compared to the titers achieved with lactic acid bacteria (149).

Pyruvic acid is a central intermediate in carbohydrate and protein metabolism and the metabolic precursor for many biosynthetic pathways, including those of several amino acids. Besides being a precursor, pyruvic acid is also directly utilized. For example, there have been reports on its use as a dietary supplement for weight loss enhancements (156), positive effects in acne treatment (27), and a variety of cardioprotective outcomes (215). The PDC-negative *pdc1Δ pdc5Δ pdc6Δ* strain mentioned above represents an interesting platform for microbial production of pyruvate since it accumulates high levels of this compound. However, this strain shows several growth defects: (i) growth on synthetic medium in glucose-limited chemostates requires the addition of small amounts of ethanol or acetate for anabolic purposes, and (ii) even in the presence of a C₂ compound, these strains cannot grow in batch cultures on synthetic glucose medium. While the reason for the latter is unknown, evolutionary engineering has solved most of these problems. Although the exact molecular mechanisms underlying the altered phenotype were not additionally elucidated, the resulting strain produced pyruvate with an overall yield of 0.54 g of pyruvate · (g glucose consumed)⁻¹ (363). The final pyruvate concentration achieved (135 g · liter⁻¹) was two times higher than the maximal values of any microbial fermentation (197).

Recently, initial attempts to produce succinate in *S. cerevisiae* have been announced (270). Succinate is a desirable building block for a wide variety of added-value chemicals. Yields are still fairly low; the engineered strains produced only 0.03 (260a) and 0.07 (327a) g · (g glucose consumed)⁻¹, respectively.

Sugar alcohols. Several sugar alcohols, such as xylitol, can be used as sweeteners. The sweetening power of xylitol, a five-carbon sugar alcohol, is comparable to that of sucrose, but xylitol has several advantages over sucrose: (i) its energy value is lower; (ii) its metabolism in the human body is independent of insulin,

making xylitol ideal for diabetics; and (iii) it inhibits infections of the middle ear (355) and teeth (224). Due to these beneficial effects, xylitol has become a widely used sweetener. Industrially, xylitol is produced by chemical reduction of pure xylose, obtained from hardwood hydrolysates. Microbial production of xylitol from either glucose or xylose has been studied but has not yet been able to outcompete chemical synthesis (104, 105).

Xylitol has gained much attention as an unwanted by-product when *S. cerevisiae* is equipped with heterologous enzymes for xylose utilization (see above) (Fig. 1). In this context, engineering approaches have focused mainly on reducing xylitol production. Still, the intensive study of pentose metabolism and its regulation in yeast increased knowledge which also has been helpful for attempts to produce xylitol in *S. cerevisiae*. Granstrom et al. (104, 105) reviewed the achievements and challenges in producing xylitol from xylose in *S. cerevisiae*.

Recent efforts have been made to engineer *S. cerevisiae* xylitol production from glucose. Toivari et al. (351) deleted both transketolase isogenes (*TKL1* and *TKL2*) and the xylulokinase gene (*XKS1*) and overexpressed *Pichia stipitis* xylitol dehydrogenase (*XYL2*) and endogenous sugar phosphate dephosphorylase (*DOG1*) genes. Besides xylitol, another sugar alcohol, ribitol, was simultaneously formed in this strain. The final titers after 100 h of incubation in medium containing 2% glucose were 290 mg · liter⁻¹ xylitol and 440 mg · liter⁻¹ ribitol. If xylitol and ribitol were totaled, the yield would correspond to 0.036 g · (g glucose consumed)⁻¹. Even though this study demonstrates the feasibility of such a metabolic engineering approach, significant improvements would be necessary for commercial application. The yield was also much lower than those from other microbial production methods studied (105). For example, 23 g xylitol · (g glucose consumed)⁻¹ was achieved with metabolically engineered *Bacillus subtilis* (281).

L-G3P. The platform chemicals discussed above, such as ethanol, glycerol, propanediol, etc., are predominantly end products of microbial metabolism; i.e., all are nonphosphorylated and easily released into the fermentation broth. Phosphorylated intermediates of cellular metabolism are also potentially useful as starting materials in either chemical or enzymatic syntheses. However, microbial production of such substances faces multiple challenges: (i) cellular phosphate homeostasis effects and (ii) efficient product retention by the plasma membrane such that it is not released into the culture medium.

L-G3P is a promising precursor for the enzymatic synthesis of carbohydrates. We demonstrated that L-G3P can be accumulated in *S. cerevisiae* via pathway engineering (242). For example, a strain lacking both isogenes encoding glycerol 3-phosphatase, *GPP1* and *GPP2*, combined with the overexpression of *GPD1* (Fig. 2) could achieve intracellular L-G3P levels of about 200 mM. By up-scaling the process, a titer of up to 348 mg · liter⁻¹ L-G3P could be achieved, with about one-third of this amount located outside the cells at the end of the fermentation (278).

Ergosterol and other steroids. Steroids are a large group of diverse bioactive molecules that play an important role in the drug industry. Hundreds of distinct steroids have been identified in plants, animals, and fungi. Ergosterol is the predominant steroid alcohol (sterol) naturally formed in *S. cerevisiae*. Free ergosterol is found in membranes, whereas sterol esters, the storage form of sterols, are sequestered in cytosolic lipid

particles. Ergosterol, also known as provitamin D₂, is of great commercial interest. In addition, several intermediates of the ergosterol biosynthetic pathway are also useful high-value compounds. A compilation of these substances and a review of the progress in their production via *S. cerevisiae* biotechnology were given by Veen and Lang (372). Rational metabolic engineering of the ergosterol pathway led to increased production of early and late sterols. The total sterol cellular content was up to three times higher than that in wild-type cells; however, the end product, ergosterol, could be only marginally increased (276, 277, 373). Clearly, it is a challenge to understand and overcome the complex regulation of this pathway (371).

With the exception of ergosterol, wild-type yeast does not produce steroids. A major breakthrough in the microbial production of human steroid hormones has been the biosynthesis of hydrocortisone in yeast by introducing eight mammalian genes and engineering five *S. cerevisiae* genes (334). The achievements in producing such complex bioactive chemicals in yeast were recently reviewed by Dumas et al. (78).

Isoprenoids. Another group of interesting and diverse natural compounds whose production has been addressed by yeast metabolic engineering, isoprenoids, are used as flavors, fragrances, food colorants, or pharmaceuticals. Isoprenoids include mono-, sesqui-, di-, sester-, tri-, tetra-, and polyterpenes. The biosynthesis of isoprenoids starts from isopentenyl diphosphate (IPP), an intermediate of the ergosterol biosynthetic pathway (mevalonate pathway) in *S. cerevisiae*. Approaches to microbial isoprenoid production have been described in comprehensive reviews (51, 221). Here, I focus only on recent reports in the field of isoprenoid production in yeast.

One metabolic engineering approach is of general interest for the production of all isoprenoid substances in yeast, as it enhances the supply of acetyl-CoA. This is the starting point for the biosynthesis of different isoprenoid precursors, such as IPP, geranyl diphosphate (GPP), and farnesyl diphosphate (FPP) (Fig. 4). Increased acetyl-CoA availability was achieved by engineering the pyruvate dehydrogenase bypass in *S. cerevisiae*: the homologous *ALD6* gene, encoding an acetaldehyde dehydrogenase, was overproduced, and a *Salmonella enterica* acetyl-CoA synthetase variant was simultaneously expressed (314).

The precursor for the synthesis of monoterpenoids in *S. cerevisiae* is GPP. The expression of geraniol synthetase from *Ocimum basilicum* efficiently converted GPP to geraniol, a monoterpenoid (260). It has been proposed that an alternative pathway for the de novo synthesis of monoterpenes, the "MCC pathway," exists in *S. cerevisiae*, which might influence future engineering strategies (46).

The precursor for sesquiterpenes is FPP. To increase FPP, the *S. cerevisiae* mevalonate pathway (Fig. 4) was deregulated by overexpressing a homologous soluble form of 3-hydroxy-3-methyl-glutaryl-CoA reductase derived from *HMG1*, down-regulating *ERG9* expression, introducing the *upc2-1* mutation for sterol uptake under aerobic conditions, and overexpressing *ERG20*-encoded FPP synthase (294). These modifications combined with the introduction of the *ADS* gene from *Artemisia annua*, encoding amorphadiene synthetase, resulted in a strain that produced 153 mg · liter⁻¹ amorphadiene, a sesquiterpene. By simultaneously overexpressing the *Artemisia annua* cytochrome P450 monooxygenase and its native redox partner,

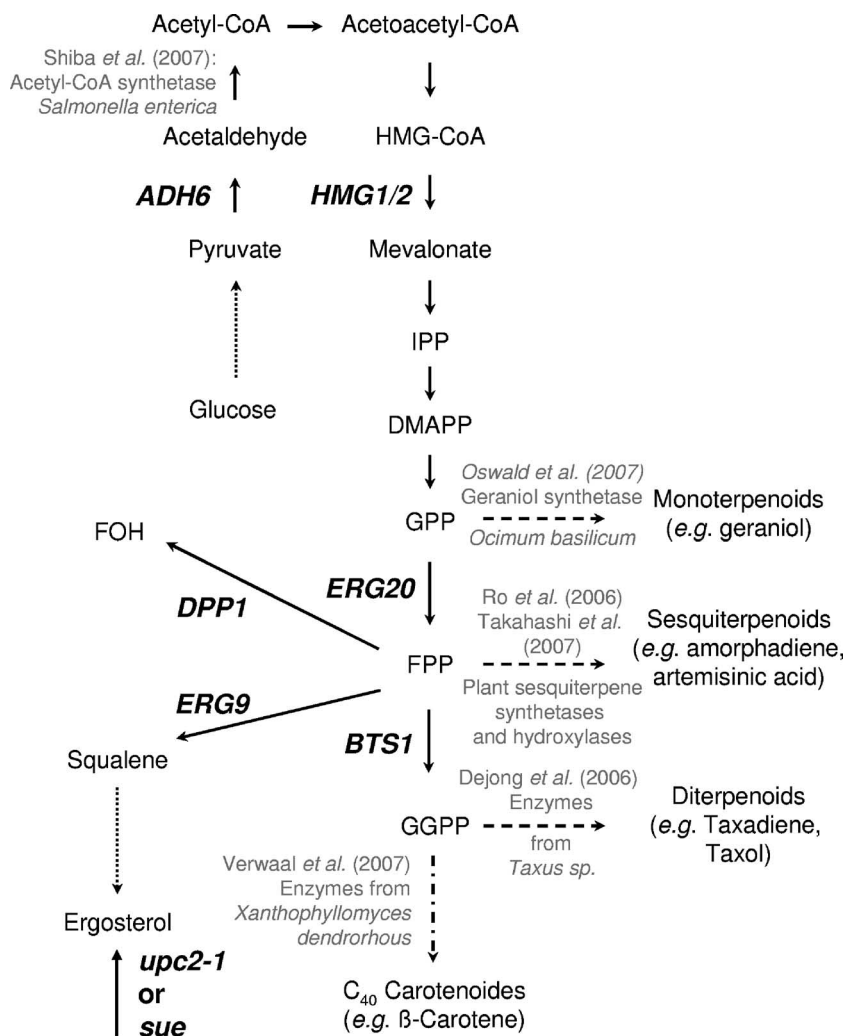


FIG. 4. Pathways, enzymes, and genes for engineering *S. cerevisiae* isoprenoid biosynthesis. Gene names and mutations: *ADH6*, alcohol dehydrogenase (NADP⁺); *BTS1*, GGPP synthase; *DPP1*, DGPP phosphatase; *ERG9*, squalene synthase; *ERG20*, farnesyl-diphosphate synthase; *HMG1/2*, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase; *upc2-1*, mutation in the transcriptional regulator of anoxic genes (Upc2p) responsible for the aerobic uptake of sterol (64); *sue*, unknown mutations selected for aerobic sterol uptake (342). Other abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FOH, farnesol.

NADPH:cytochrome P450 oxidoreductase, the same authors generated an artemisinic acid-producing yeast, which has attracted a lot of attention (294). Artemisinic acid can be used as a substrate for the cost-effective and environmentally friendly synthesis of artemesin, a sesquiterpene lactone endoperoxide that is highly effective against the malaria parasite *Plasmodium*. Artemisinic acid titers of up to 100 mg · (liter fermentation broth)⁻¹ were reported. It appears that artemisinic acid was efficiently transported out of yeast cells but remained bound to the cell surface when it was protonated under acidic culture conditions; more than 96% of the synthesized artemisinic acid was removed from the cell pellet by washing with alkaline buffer (pH 9 Tris-HCl buffer supplemented with 1.2 M sorbitol), with less than 2% remaining in the washed cell pellet or culture medium.

To increase FPP supply, Takahashi et al. (342) combined deregulated 3-hydroxy-3-methyl-glutaryl-CoA reductase activity with *ERG9* and *DPP1* deletion and evolutionary engineering for aerobic sterol uptake ("*sue*") (Fig. 4). The simultaneous

expression of plant terpene synthetases yielded more than 80 mg/liter sesquiterpene. By coexpression of terpene hydroxylase, the authors obtained 50 mg/liter hydrocarbon and hydroxylated products.

Geranyl-GPP (GGPP) is produced from FPP by *BTS1*-encoded GGPP synthetase in yeast (Fig. 4). GGPP serves as a precursor for the synthesis of diterpenoids in other organisms. For example, paclitaxel (Taxol) is a diterpenoid and a widely used anticancer isoprenoid produced by the secondary metabolism of yew (*Taxus* sp.) trees. However, only limited amounts of Taxol or related metabolites (taxoids) can be obtained from currently available sources. Recently, Dejong et al. (69) reported the functional expression of eight taxoid biosynthetic genes from *Taxus brevifolia* in yeast. Metabolite analysis of the engineered yeast grown in glucose demonstrated that yeast isoprenoid precursors could be utilized in the reconstituted pathway. However, only the first two steps of the engineered pathway appeared to be functional in the constructed strain, such that the intermediate taxadiene accumulated to mg/liter

levels.

β -carotene is another isoprenoid (tetraterpenoid) compound whose biosynthesis initiates from GGPP (Fig. 4). Its production has been recently attempted in *S. cerevisiae* (379). Apart from modifications of several homologous genes, carotenoid production was achieved by introducing heterologous genes from *Xanthophyllomyces dendrorhous*, and a β -carotene concentration of $5.9 \text{ mg} \cdot (\text{g yeast dry weight})^{-1}$ was obtained.

The mevalonate pathway in yeast is subject to complex native regulations, which generally limit pathway engineering successes. However, genes encoding the enzymes of an alternative bacterial pathway for IPP biosynthesis, the “MEP pathway” from *E. coli*, have been recently transferred into yeast (A. Clark, J. Maury, M. A. Asadollahi, K. Moeller, J. Nielsen, and M. Schalk, 23 August 2007, international patent application WO 2007/09396243). The authors cloned seven genes, verified the presence of the corresponding mRNAs, and demonstrated that the *E. coli* MEP pathway was able to sustain yeast growth in the presence of lovastatin, a specific inhibitor of the endogenous mevalonate pathway.

CONCLUDING REMARKS AND FUTURE PROSPECTS

As a model for understanding eukaryotic organisms and a cell factory in classical and modern biotechnological processes, the yeast *S. cerevisiae* is of great importance. Its broad use in industry is closely related to its role as a major platform for metabolic engineering, which aims to enhance yeast biotechnology. This underpins baking, brewing, wine making, and bioethanol production as well as the microbial production of a growing number of other interesting compounds with various applications.

In addition to the enormous body of knowledge on yeast physiology and genetics, the popularity and potential of baker's yeast is due to its process robustness, particularly its ability to grow anaerobically and its high tolerance to low pH, high osmotic pressure, and high ethanol concentrations. Consequently, yeast is unlikely to be easily replaced by other microorganisms in the bioethanol production field, and this organism is in use or undergoing tests in a constantly growing number of industrial (white) biotechnology applications. The food and beverage industry would also benefit from utilizing engineered yeasts. This would, however, require a greater acceptance by consumers. To achieve this, it will be necessary to provide more effective community education and to ensure greater transparency regarding the legal provisions governing approval of GMOs for food applications.

Former metabolic engineering approaches were primarily rational, i.e., based on available knowledge about the metabolic pathways and enzymes involved. A more recent alternative is inverse metabolic engineering, for which the phenotype is the starting point. The goal is to exploit natural variability and analyze the molecular basis of various manifestations of a trait, thereby elucidating novel targets for strain improvement. The methods of global gene expression analysis (“omics” technologies) have strongly facilitated inverse metabolic engineering, and several successful examples demonstrate the power of this approach. One challenge in using the “omics” technologies is the vast amount of data that they generate. Novel methods which are able to link the gene expression data with the geno-

type and the phenotype are required. Recent examples of yeast metabolic engineering have shown that a cell's evolutionary potential should not be underestimated in strain improvement. Evolutionarily evolved strains can form suitable starting points for inverse metabolic engineering approaches.

To develop an understanding of the cell as a whole, sophisticated computational methods capable of integrating copious amounts of information are required. This would be very advantageous for rational metabolic engineering, since optimized metabolic models are better able to predict successful engineering strategies. In addition, basic yeast research will remain fundamental to metabolic engineering to better understand cellular mechanisms and overcome current limitations. This will also support the development of novel tools for metabolic engineering, including those required for enhanced enzymes, fine-tuning of enzyme activities, and efficient up- or down-regulation of metabolic pathways.

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