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review

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## Designer Yeasts for the Fermentation Industry of the 21<sup>st</sup> Century

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### Summary

The budding yeast, *Saccharomyces cerevisiae*, has enjoyed a long and distinguished history in the fermentation industry. Owing to its efficiency in producing alcohol, *S. cerevisiae* is, without doubt, the most important commercial microorganism with GRAS (Generally Regarded As Safe) status. By brewing beer and sparkling wine, mankind's oldest domesticated organism made possible the world's first biotechnological processes. With the emergence of modern molecular genetics, *S. cerevisiae* has again been harnessed to shift the frontiers of mankind's newest revolution, genetic engineering. *S. cerevisiae* is at the forefront of many of these developments in modern biotechnology. Consequently, the industrial importance of *S. cerevisiae* has extended beyond traditional fermentation. Today, the products of yeast biotechnologies impinge on many commercially important sectors, including food, beverages, biofuels, chemicals, industrial enzymes, pharmaceuticals, agriculture and the environment. Nevertheless, since ethyl alcohol produced by yeast fermentation is likely to remain the foremost worldwide biotechnological commodity for the foreseeable future, this review focuses on advances made with respect to the development of tailor-made yeast strains for the fermented beverage and biofuel industries.

*Key words:* *Saccharomyces cerevisiae*, tailor-made yeast strains, fermented beverages, biofuel industry

### Introduction

The power with which microorganisms have caused irreversible revolutions in the existence and activities of humankind during the course of history and with which these mostly invisible forms of life still influence the weal and woe of this planet can best be illustrated on the basis of the phenomenal list of achievements of the well known yeast, *Saccharomyces cerevisiae*. This simple, unicellular fungus boasts an unequalled track record, making it a true pioneer among microorganisms. As a matter of fact, since the beginning of recorded civilisation, this versatile trailblazer has been instrumental in planting the most important milestones on the road of

the development of modern humankind. *S. cerevisiae* therefore can be regarded as the key to the most hidden secrets of life, because, of all the microorganisms, it was the *first* to be (i) domesticated by people for the production of food (e.g. bread in ancient Rome in 100 BC) and beverages (e.g. beer and wine in Assyria, Caucasia, Mesopotamia and Sumer in 7000 BC), (ii) observed microscopically (by Antonie van Leeuwenhoek), (iii) described as a living biochemical agent of transformation (by Louis Pasteur), (iv) used as a host for the production of the first recombinant vaccine (against hepatitis B) and the first food enzyme (the milk coagulation enzyme,

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chymosin, for cheese making), and (*v*) used to reveal the entire nucleotide sequence of a eukaryotic genome. On the basis of the last-mentioned frontier-shifting milestone of modern science, there already are strong indications that *S. cerevisiae* will probably also become the first eukaryotic cell of which the complexities of the transcriptomes, proteomes and metabolomes will be unlocked. In this way, it will once again be the forerunner when the two strongest forces in modern day science, namely biotechnology and information technology, are combined in a breathtaking new discipline, bioinformatics. This will lead to the third millennium finally bidding farewell to the industrial economy and entering the exciting era of the information economy and the bio-economy.

There is no doubt that the pace of scientific and technological innovation will be much faster in the Bio-economy and that *S. cerevisiae*, as one of the most popular scientific models and commercial microorganisms, will continue to play a leading role in the future. It is expected that the traditional production of ethyl alcohol (for the beer, wine, distilled beverage and fuel industries) and yeast biomass (for the food and animal feed industries) will continue to provide the largest quantity of the world's fermentation products in the foreseeable future. This assumption is based on the fact that *S. cerevisiae* currently remains responsible for the production of all four of the leading commodity products of fermentation (in terms of worldwide tonnage per year), namely 60 million tons of beer, 30 million tons of wine, 800 000 tons of single cell protein and 600 000 tons of baker's yeast. However, it is an accomplished fact that, in the future, *S. cerevisiae* will become increasingly important as a host organism for the production of *low volume, high value* biotechnological products (*e.g.* commercially important enzymes, chemicals, therapeutic proteins and other pharmaceutical products).

In the light of these 21st century developments and forecasts, the demand for suitable *designer* yeasts for a wide variety of existing and new products becomes increasingly greater and more urgent. Since *S. cerevisiae* and ethanol are likely to remain, respectively, the world's premier commercial microorganism and biotechnological commodity for many years to come, this overview is aimed at highlighting the most important targets for the development of tailored *S. cerevisiae* strains for the fermented beverage and biofuel industries and providing a vision for the use of such yeasts.

### Tailoring of Yeast Strains for the Fermented Beverage Industry

The healthful benefits of fermented alcoholic beverages, now largely attributed to the antimicrobial activity of yeast-derived ethanol, predate recorded history. Beer, wine and distilled spirits were some of the first commodities to be bartered by early civilisations engaged in international trade. Then, as now, the most successful producers of beer, wine, brandy and whisky were those who grasped the market forces of supply and demand, and whose products met the prevailing definition of quality (1). Today's fierce competition for market share has led to increased diversity and innovation within the

fermented beverage industry, much to the benefit of the consumer. This, in turn, has created an urgent quest for customised brewing, wine, brandy and whisky yeast strains. The knowledge of the physiology of genetic reference strains of *S. cerevisiae* that is now available is a great help in directing the modification of industrial yeasts toward practical goals, even though the industrial strains and species are less characterised and their genetic make-up is more complex (2).

### Strain fingerprinting

In the fermented beverage industry it is very important that the microorganisms associated with the various industrial processes have food grade status. Therefore, with the prominence of *S. cerevisiae* as starter culture in alcoholic beverages, it is important to ensure the identity and safety of each of the newly developed or selected strains. This quest for novel yeast strains that are designed to perform specific tasks set by the beverage industries has led to the development of rapid and reliable methods for yeast strain differentiation. Molecular genetic techniques can be used to discriminate between yeast strains that have similar physiological characteristics. The methods most frequently used today are electrophoretic karyotyping, restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), polymerase chain reaction (PCR), PCR with specific primers, temperature gradient gel electrophoresis (TGGE) or denaturing gradient gel electrophoresis (DGGE) of PCR-amplified products, amplified fragment length polymorphisms (AFLP) and Fourier-transform infrared microspectroscopy (3–6).

### Improvement of fermentation performance

The primary selection criteria applied to most strain development programmes in the fermented beverage industry relate to the overall objective of achieving a better than 98 % conversion of sugar to alcohol and carbon dioxide, at a controlled rate and without the development of off-flavours. The growth and fermentation properties of yeast starter cultures have yet, however, to be genetically defined. What makes the genetic definition of these attributes even more complex is the fact that lag phase, rate and efficiency of sugar conversion, resistance to inhibitory substances and total time of fermentation are affected strongly by the physiological condition of the yeast, as well as by the physicochemical and nutrient properties of the grape must.

This complexity explains why it is so difficult to define all the key genetic determinants of a yeast's fermentation performance that may be candidates for genetic engineering. However, general targets include increased stress tolerance and resilience; improved polysaccharide degradation (*e.g.* starch, pectin, cellulose and hemicellulose), sugar uptake and assimilation; increased ethanol tolerance; improved nitrogen assimilation; enhanced resistance to microbial metabolites and toxins; resistance to heavy metals and agrochemical residues; tolerance to chemical preservatives; and reduced foam formation (2,4,7,8).

As sterols, trehalose, glycogen and aquaporins play multiple roles in increasing the survival of *S. cerevisiae*

cells exposed to several physical and chemical stresses, they have important implications for the general stress tolerance, resilience, fitness and vigour of active dried yeast starter cultures upon reactivation. As a result, there is a strong incentive to develop yeast strains with a superior ability to accumulate these compounds. However, due to the complex stress response mechanisms in yeast, it is not yet clear whether the deletion of the *ATH1* trehalase gene and the modification of the expression levels of the genes involved in the metabolism of trehalose (*TPS1*, *TPS2*, *ATH1*), glycogen (*GSY1*, *GSY2*) and sterols (*SUT1* and/or *SUT2*), and in the synthesis of aquaporins (*AQY1*, *AQY2*) will result in an improvement in yeast viability and vitality (2,4).

An imbalance in the high levels of carbon and low levels of nitrogen in some of the raw materials used in the fermented beverage industry (e.g. grape must) is another cause of poor fermentative performance. Sluggish or stuck fermentations occur because nitrogen depletion irreversibly arrests hexose transport. The main focus to ensure the efficient utilisation of sugars under conditions of nitrogen limitation is to increase the rate of glycolytic flux by replacing any non-functional mutant alleles of genes encoding the key glycolytic enzymes, to enhance the efficiency of hexose (especially fructose) uptake, and to alleviate the assimilation of proline and arginine (accounting for 30 to 65 % of the total amino acid content of grape juice) from nitrogen catabolite repression. In the case of incomplete fermentations, the preference of, for example, wine yeasts for glucose over fructose can lead to excessive residual fructose levels that compromise the quality of the wine. It is hypothesised that the rate of alcohol production by yeast is limited primarily by the rate of sugar uptake, especially the uptake of fructose, in the presence of high sugar levels during the early phase of fermentation and during the final stages of nitrogen depletion coupled with nutrient limitation. Therefore, several laboratories focus on phosphorylation by the *HXK1*- and *HXK2*-encoded hexokinases and the *GLK1*-encoded glucokinase, as well as on hexose transporters encoded by *HXT1*-*HXT18* and *SNF3*. There is anecdotal evidence that the overexpression of the *S. pastorianus* *FSY1*-encoded fructose/H<sup>+</sup> symporter, together with some of the other *HXT1*-*HXT18* and *SNF3*-encoded hexose transporters and the *HXK1*-encoded hexokinase (with the highest affinity for fructose, but still a significantly lower affinity than for glucose), result in improved glucose and fructose uptake during wine fermentations. Furthermore, the deletion of the *URE2*-encoded repressor of the *PUT1*-encoded proline oxidase and *PUT2*-encoded pyrroline-5-carboxylate dehydrogenase represents the first step towards the development of wine yeasts that can efficiently assimilate the abundant supply of proline and arginine in grape juice under fermentative conditions (2,4,7,8).

Since *S. cerevisiae* is only able to metabolise mono-, di- and tri-hexoses, while some raw materials used for the production of potable ethanol (e.g. beer wort and whisky grain mash) contain substantial amounts of polysaccharides such as starch, there is considerable interest in improving the fermentation performance by developing yeast strains that are able to use a wider range of carbohydrates. For example, in the whisky and brew-

ing industries, modified *S. cerevisiae* strains that are able to hydrolyse and ferment polysaccharides would convert these raw materials to ethanol much more effectively than the current starter culture strains. In an attempt to avoid the use of expensive enzyme preparations (often containing unwanted, contaminating or side activities) in the production of grain whisky, low carbohydrate diabetic beers and low calorie »light« beers, several heterologous amylase-encoding genes have been expressed in *S. cerevisiae* (9). In some instances, these amylolytic strains have also been improved with respect to their effectiveness to ferment hexoses other than glucose (e.g. maltose and melibiose).

Another thrust to improve the fermentation performance of *S. cerevisiae* starter culture strains is to increase their resistance to toxic microbial metabolites (e.g. ethanol, acetic acid, medium chain fatty acids, etc.), zymocins (yeast-derived killer toxins or zymocins), chemical preservatives (e.g. sulphite) and agrochemicals containing heavy metals (e.g. copper). For example, the modification of the expression of the *SUT1*, *SUT2*, *PMA1* and *PMA2* genes results in increased sterol accumulation and cell membrane ATPase activity, thereby increasing the resistance to ethanol (2). In addition, the genetic, mycoviral determinants and other genes encoding killer toxins (zymocidal peptides) and immunity factors can be incorporated into *S. cerevisiae* starter culture strains to make them insensitive to the zymocins of contaminating wild yeasts. With respect to resistance to agrochemicals, an increase in the copy number of the *CUP1* copper chelatin gene enables yeast to tolerate higher levels of copper residues in the fermentation medium (4).

#### Improvement of processing efficiency

The main objectives of product processing, fining (e.g. addition of adsorptive compounds followed by settling or precipitation) and clarification (e.g. sedimentation, racking, centrifugation, filtration, etc.) include the removal of excess amounts of certain components and microbial cells to achieve clarity and to ensure the physicochemical stability of the end product. The fining and clarification of fermented beverages often include expensive and laborious practices that generate large volumes of lees for disposal, thereby causing a loss of product and the removal of important aroma and flavour compounds from the remaining product. In order to minimise the disadvantages of these harsh fining and clarification practices, an increasing spectrum of relatively expensive commercial enzyme preparations (e.g. proteases, pectinases, glucoamylases, xylanases, arabinofuranosidases, etc.) are often added to some fermentation media (e.g. grape must and wine). As an alternative strategy to the addition of costly enzyme preparations that often contain unwanted contaminating or side activities, starter culture yeasts are being developed to secrete proteolytic and polysaccharolytic enzymes that would remove haze-forming proteins and filter-clogging polysaccharides, respectively. To this end, the overexpression of several bacterial, fungal and yeast genes resulted in the development of proteolytic, pectinolytic, glucoamylolytic and xylanolytic yeasts (9,10).

A second target for the improvement of clarification and filtration is to efficiently remove all yeast cells from

the liquid phase of the fermentor, tank or barrel (11). The regulated expression of the flocculation genes is important to guarantee a high suspended yeast count for a rapid fermentation rate during the fermentation process, while efficient settling is needed to minimise problems with wine clarification at the end of sugar conversion. Yeast flocculation is particularly important for the production of bottle-fermented sparkling wine, and the controlled onset of yeast flocculation at the appropriate time during sparkling wine production can simplify this costly process. The expression of the *FLO1* flocculin gene, linked to the late-fermentation *HSP30* promoter, can be induced by a heat-shock treatment, confirming that controlled flocculation is indeed possible during fermentation (12). Cell aggregation also plays a key role in the production of flor sherry, during which a related cellular process results in the flotation of the yeast cells, thereby forming a velum (biofilm) on the surface of the wine. By placing the *MUC1* (also known as *FLO11*) mucin gene under the control of the *HSP30* promoter, the formation of the biofilm can be promoted at the end of fermentation, thereby simplifying the development of the flor.

#### Improvement of sensory quality

The single most important factor in the fermented beverage industry is the organoleptic quality (appearance, aroma and flavour) of the final product. The endless variety of flavours stems from a complex, completely non-linear system of interactions among many hundreds of compounds. The bouquet of a beer, wine, brandy or whisky is determined by the presence of a well-balanced ratio of desirable flavour compounds and metabolites, and the absence of undesirable ones. The perceived flavour of these products is the result of absolute amounts and specific ratios of many of these interactive compounds, rather than being attributable to a single »impact« compound. Subtle combinations of trace components derived from the raw material usually elicit the characteristic flavour and aroma notes, whereas the products of yeast fermentation (*e.g.* esters, alcohols, *etc.*) contribute to the generic background flavour and aroma, as well as to the complexity and intensity of the aroma and taste of the final product (13). However, the flavour of the product immediately after fermentation or distillation only approximates that of the finished product. After the sudden and dramatic changes in composition during fermentation and distillation, chemical constituents generally react slowly during ageing to move to their equilibria, resulting in gradual changes in flavour. The harmonious complexity of some products, such as wine, brandy and whisky, subsequently can be increased further by volatile extraction during oak barrel ageing.

There is an obvious need for the development of *S. cerevisiae* starter culture strains that could impart specific desirable characteristics to beer, wine, brandy and whisky. To this end, significant progress has been made in the construction of yeasts producing colour- and aroma-liberating enzymes (*e.g.* pectinases, glycosidases, glucanases, arabinofuranosidases, *etc.*) and ester-modifying enzymes (*e.g.* alcohol acetyl transferases, esterases, isoamyl acetate hydrolysing enzyme, *etc.*) (11). Further-

more, yeasts have been developed that produce optimal levels of glycerol (the overexpression of *GPD1*, *GPD2* and *FPS1*, together with the deletion of the *ALD6* acetaldehyde dehydrogenase gene), fusel oils (*e.g.* isobutyl alcohol, isoamyl alcohol, *etc.*), and phenolic acids (modified expression of the yeast *PAD1* phenyl acrylic acid decarboxylase gene, as well as the expression of bacterial *pdc* *p*-coumaric acid decarboxylase and *padc* phenolic acid decarboxylase genes).

The bioadjustment of acidity in wine can be achieved by recombinant wine yeasts containing combinations of genes cloned from *Schizosaccharomyces pombe* and lactic acid bacteria (14,15). A wine yeast that contains the *S. pombe mae1* malate permease gene and the *mae2* malic enzyme gene converts malic acid to ethanol (maloethanolic fermentation), whereas a transformant carrying the *mae1* gene, together with the *Oenococcus oeni* (*mleA*), *Lactococcus lactis* (*mleS*) or *Lactobacillus delbrueckii* (*mleS*) malolactic enzyme gene, converts malic acid into lactic acid (malolactic fermentation). The maloethanolic wine yeast would be preferred for low pH wines from the cooler wine-producing regions, while the malolactic wine yeast would provide the best solution for high pH wines from the warmer regions. In the case of high pH wines, the production of additional lactic acid during fermentation can be achieved by incorporating the *Lactobacillus casei* *LDH1* lactic dehydrogenase gene into the malolactic wine yeast strain (2,4). These yeasts also preclude the requirement for the use of bioamine-forming malolactic bacteria in red wine and certain styles of white wine that need to undergo malolactic fermentation.

Since yeast can also be responsible for the production of unwanted byproducts, such as hydrogen sulphide, dimethyl sulphide, sulphite, diacetyl and phenolic off-flavours, there is an incentive to delete some endogenous genes from commercial strains of *S. cerevisiae*. For example, yeasts carrying disrupted alleles of the *MET14* adenosylphosphosulphate kinase, *MET10* sulphite reductase, *MRX1* methionine sulphoxide reductase and/or *MET2* homoserine *O*-acetyl transferase have been constructed so that the production of sulphite and sulphide can be controlled during fermentation. Some of these modified yeasts have been shown to produce reduced levels of sulphide and optimal levels of sulphite, which facilitate increased flavour stability (2,4,16). Furthermore, two different strategies have been used in attempts to reduce the levels of diacetyl in beer. In the first, diacetyl formation was reduced in *ILV2* mutants and in strains in which the expression of *ILV5* was increased. The second strategy consisted of expressing the *ALDC* acetolactate decarboxylase gene from *Enterobacter aerogenes* in *S. cerevisiae*. This transformant produced considerably less diacetyl during fermentation than the parent yeast (16).

#### Biopreservation of spoilage microorganisms

Uncontrolled microbial growth before, during or after fermentation can alter the chemical composition of the end product, thereby detracting from its sensory properties of appearance, aroma and flavour. Unspoiled raw material and sound, hygienic fermentation practices are the cornerstones of the industry's strategy to prevent

the uncontrolled proliferation of spoilage microbes. Added safety is provided by the addition of chemical preservatives, such as sulphur dioxide, dimethyl dicarbonate, benzoic acid, fumaric acid and sorbic acid, which control the growth of unwanted microbial contaminants. However, the excessive use of these chemical preservatives is harmful to the quality of the end product and is confronted by mounting consumer resistance. Consumer preferences have shifted to products that are less heavily preserved with chemicals, less processed, of higher quality, more natural and healthier. Therefore, biopreservation with yeast-derived metabolites (*e.g.* formation of SO<sub>2</sub> or hydrogen peroxide during wine fermentations), antimicrobial enzymes (*e.g.* lysozyme, chitinases, endoglucanases, *etc.*) and peptides (zymocins and bacteriocins) is currently being considered as an alternative strategy to chemical preservation. However, the use of purified antimicrobial enzymes and bacteriocins is expensive, resulting in an increase in retail costs. This problem might be circumvented by expressing effective antimicrobial enzymes and peptides in wine yeast starter culture strains, thereby addressing the wine industry's call for wines of higher quality and purity. To this end, the hen egg white lysozyme gene (*HEL1*), the *Pediococcus acidilactici* pediocin gene (*PED1*) and the *Leuconostoc carnosum* leucocin gene (*LCA1*) have been used to engineer bactericidal yeasts (4,16–18). The antifungal yeast *CTS1*-encoded chitinase and *EXG1*-encoded exoglucanase have also been expressed in *S. cerevisiae* (2,19). The main approach in the construction of zymocidal strains entails the inclusion of a combination of mycoviral killer toxin determinants of *S. cerevisiae* (*e.g.* a K<sub>1</sub>/K<sub>2</sub> double killer) and zymocin-encoding genes from other yeasts (*e.g.* *Hanseniaspora*, *Kluyveromyces*, *Pichia*, *etc.*) into *S. cerevisiae* starter culture strains. The ideal would be to incorporate all of these antimicrobial activities into a single starter culture strain, thereby counteracting all contaminating spoilage bacteria, yeasts and moulds (2,16,20,21).

#### *Improvement of wholesomeness*

It is generally accepted that moderate drinking of alcoholic beverages (especially wine) can be socially beneficial and effective in the management of stress and the reduction of coronary heart disease. The principal protective compounds found in products such as wine include the phenolic compounds, resveratrol, salicylic acid and alcohol (4,22). However, prudent consumers of these products are increasingly fastidious about the presence of undesirable compounds in fermented beverages. These unwanted compounds include suspected carcinogens, such as ethyl carbamate, neurotoxins, such as biogenic amines, and asthmatic chemical preservatives, such as sulphites. The most finicky among these fussy consumers are even concerned about high levels of alcohol in fermented beverages. When yeast starter strains are developed, it therefore is of the utmost importance to focus on these health aspects and to develop yeasts that may enhance the benefits (*e.g.* production of resveratrol, carnitine, *etc.*) and reduce the risks (*e.g.* eliminating ethyl carbamate and biogenic amines, and reducing the levels of alcohol) associated with moderate alcohol consumption (2,16).

With regard to the production of resveratrol during fermentation, progress has already been made by constructing a wine yeast that expresses the *4CL9/216* co-enzyme A ligase, *VST1* stilbene synthase and *BGL1*  $\beta$ -glucosidase genes (23). The development of a bactericidal yeast that is deleted for the *CAR1* arginase gene (blocking the secretion of urea, the precursor for the formation of ethyl carbamate) or that is transformed with heterologous urease genes (enabling the degradation of urease) would reduce the levels of added sulphite, yeast-derived ethyl carbamate and bioamines formed by bacterial contaminants. The bio-reduction of the levels of alcohol in fermented beverages can be achieved by redirecting the carbon flux away from ethanol formation and towards the production of glycerol and gluconic acid. A significant increase in the level of extracellularly accumulated glycerol and concomitant decreases in ethanol concentrations have been achieved by the overexpression of the endogenous *GPD1* and *GPD2*-encoded glycerol-3-phosphate dehydrogenase isozymes of *S. cerevisiae*, together with the constitutive expression of its *FPS1*-encoded glycerol transport facilitator (2,4,24). Similar decreases in ethanol levels have been achieved by the expression of the *Aspergillus niger* *GOX1* glucose oxidase gene in *S. cerevisiae* (25).

#### **Tailoring of Yeast Strains for the Biofuel Industry**

Fuel ethanol, which has a higher octane rating than gasoline, accounts for approximately two-thirds of the world's total annual ethanol production of more than 31 billion litres (26). The primary reasons for considering the expanded use of biofuel ethanol concern sustainable resource supply, enhanced security and the realisation of macroeconomic benefits for rural communities and the economy at large (27,28). All indications are that dramatic changes in energy supply will occur in the 21<sup>st</sup> century, particularly relating to oil. It is estimated that world oil production will peak within the next 25 years and then follow a permanent decline. The time required to develop and deploy new technologies that allow a smooth transition from oil to plant biomass as the only foreseeable sustainable source of biofuels is running out. Furthermore, more than two-thirds of the world's remaining oil reserves lie in the Middle East (including the Caspian Sea), which is an increasingly prominent concern in the context of post-Cold War considerations relating to security of nations (27,28). If a transition from fossil fuels to biofuels becomes affordable, this unwelcome dependence on the Middle Eastern cartels controlling the production, manufacturing and marketing of oil could diminish and the world's security could be improved in many ways. A transition to biofuel ethanol would increasingly limit the ability of oil-exporting countries and regimes to shape world events and would democratise the world's energy markets. Since biomass feedstocks are available as both residues and dedicated crops at a lower price on a per energy basis than oil, it is foreseen that the agricultural sector and the economic health of rural communities could be promoted, with huge benefits for the economy at large. In addition to these political and socioeconomic considerations, a tran-

sition from fossil fuel to biofuel ethanol would also contribute to a cleaner environment (26). The incomplete combustion of fossil fuels emits smog-forming gases, while their extraction, processing and combustion result in the pollution of the air, water and soil, and are thus hazardous to the environment and to public health. By using biomass-derived ethanol, the net reduction in the levels of carbon dioxide (the main polluting greenhouse gas) could range between 60 and 90 % relative to gasoline-consuming vehicles (26).

In the light of the above-mentioned aspects relating to oil exhaustion, a possible interruption in oil supply caused by political meddling, a superior net performance of biofuel ethanol in comparison with gasoline, and a significant reduction in pollution levels when fossil fuel is replaced by bioethanol as a sustainable energy source for fuel transportation, it can be expected that the demand for cheap, renewable substrates and cost-effective ethanol production processes will become ever more urgent. In this regard, plant biomass is the only foreseeable sustainable source of fuel bioethanol because of its relatively low cost and plentiful supply (26–28). The central technological impediment to more widespread utilisation of this important resource is the general absence of low-cost technology for overcoming the recalcitrance of plant biomass (28). A promising strategy to overcome this obstruction involves the metabolic engineering of *S. cerevisiae* strains for use in an integrated process, known as direct microbial conversion (DMC) or consolidated bioprocessing (CBP) (26,28). Such an integrated process differs from the earlier SHF (separate hydrolysis and fermentation) and SSF (simultaneous saccharification and fermentation in which enzymes from external sources are used) strategies in that the production of polysaccharide-degrading enzymes, the hydrolysis of biomass and the fermentation of the resulting sugars to ethanol takes place in a single process via a polysaccharide-fermenting yeast strain (9). The CBP strategy offers a very large cost reduction if *S. cerevisiae* strains that possess the required combination of substrate utilisation and product formation properties can be developed. The desired substrate utilisation properties include the production of a hydrolytic enzyme system allowing high rates of hydrolysis and the utilisation of the resulting hydrolysis products under anaerobic conditions. The desired product formation properties include high product selectivity and high concentrations. An efficient polysaccharide-degrading yeast with this combination of properties has not been described to date. The often-underestimated diversity of yeast species encompasses organisms with a broad range of properties that differ from *S. cerevisiae* and could be useful for CBP. However, *S. cerevisiae* has received the most attention with regard to the heterologous expression of amylases, pectinases, cellulases and hemicellulases, as well as the production of ethanol.

While industrial strains of *S. cerevisiae* are unable to ferment polysaccharides, certain strains produce saccharolytic enzymes with limited activity on polysaccharides. These endogenous genes include the glucoamylase genes (*STA1*, *STA2*, *STA3*, and *SGA1*), the pectinase genes (*PGU1* and *PGL1*) and the glucanase genes (*EXG1*, *EXG2*, *BGL2* and *SSG1*) (9). These genes are of interest because their encoded enzymes may augment the effec-

tiveness of heterologous polysaccharolytic components and because the expression of natural *S. cerevisiae* saccharases may provide clues to the effective expression of recombinant enzymes.

A large number and wide variety of genes encoding amylases, pectinases, cellulases and hemicellulases have been cloned from various bacteria, yeasts, filamentous fungi, plants and animals and have successfully been expressed in *S. cerevisiae* (9,28). However, limited growth on non-native polysaccharide substrates has been reported for only a small number of these recombinant strains of *S. cerevisiae*. Furthermore, this research is at present more advanced with respect to  $\alpha$ -linked substrates (starch) than  $\beta$ -linked substrates (cellulose and its derivatives). The growth of *S. cerevisiae* on starch, under aerobic conditions in most, if not all, cases, has been demonstrated in several studies. A significant breakthrough study has achieved the utilisation of 100 g/L starch with the production of 44 g/L ethanol and 8 g/L cells. Although the ethanol and cell yields on starch were similar to those on glucose, the specific growth rate was nearly 10-fold lower on starch (28).

With respect to  $\beta$ -linked substrates, the growth of recombinant *S. cerevisiae* has been demonstrated on cellobiose and cellodextrins (28). Anaerobic SSF with Avicel as the substrate has also been investigated, using an *S. cerevisiae* strain expressing endo/exoglucanase and  $\beta$ -glucosidase originating from a species of *Bacillus*. This strain produced filter paper activity under both aerobic and anaerobic conditions, but was not shown to grow or to produce ethanol in the absence of added cellulase (28).

Despite only a relatively small number of studies, significant progress has been made in the area of utilisation of non-native substrates by virtue of the heterologous expression of saccharolytic enzymes. In the case of starch, some results are encouraging, not only with regard to the feasibility of starch utilisation, but also in respect of the overall feasibility of enabling the utilisation of non-native substrates. Increased rates of growth and hydrolysis, as well as the use of insoluble substrates, would appear to be logical objectives for starch utilisation (28). Work aimed at the microbial utilisation of  $\beta$ -linked substrates was undertaken much later than the work aimed at starch, with the first such studies appearing in 1998, and is less advanced. Growth on cellobiose and cellodextrins provides a point of departure that can be built upon in future work. A logical next step would appear to be anaerobic growth in the absence of added cellulase enzymes, perhaps first on non-crystalline cellulose and then on crystalline cellulose (28).

## Concluding Remarks and Future Prospects

Genetic engineering has rapidly revolutionised several areas of basic and applied yeast biology. The past decade has seen marked advances in the depth and breadth of scientific understanding of the fields of molecular yeast genetics, physiology and biotechnology. However, no genetically engineered yeast is currently used in the commercial production of fermented beverages and fuel bioethanol. And, in the case of fermented beverages, this will continue to be the case unless both

the consumers and the industry are satisfied that the products derived from genetically engineered yeasts are safe, of high quality and beneficial. The successful commercialisation of transgenic yeasts for the fermentation industry will depend on a multitude of scientific, technical, economic, marketing, safety, regulatory, legal and ethical issues. However, we are optimistic that, in the long run, these hurdles will be overcome and that genetically tailored yeast strains will be of immense importance to the fermentation industry of the 21<sup>st</sup> century.

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## Konstruirani tipovi kvasaca za fermentativnu industriju u 21. stoljeću

#### Sažetak

Klijajući kvasac, *Saccharomyces cerevisiae*, ima dugu i bitnu ulogu u fermentacijskoj industriji. Zbog svoje sposobnosti da proizvede alkohol, *Saccharomyces cerevisiae* je nedvojbeno najvažniji komercijalni mikroorganizam sa statusom GRAS (generally regarded as safe). Budući da je najstariji udomaćeni mikroorganizam čovječanstva omogućio proizvodnju piva i pjenušavog vina, pridonio je razvoju i prvih svjetskih biotehnoških procesa. Pojavom moderne molekularne genetike, *Saccharomyces cerevisiae* je ponovno pridonio pomicanju granica najnovije ljudske revolucije, genetičkog inženjerstva. *Saccharomyces cerevi-*

*siae* je najistaknutiji u razvoju novih biotehnoških grana pa se njegova industrijska važnost proteže izvan granica tradicionalne fermentacije. Danas proizvodi biotehnologije kvasca zadiru u mnoga komercijalno važna područja, uključujući hranu, pića, biološka goriva, kemikalije, industrijske enzime, farmaceutske proizvode, agrikulturu i okoliš. Nedvojbeno, budući da se etilni alkohol proizvodi fermentacijom kvasca, vjerojatno će ostati vodeći svjetski biotehnoški proizvod u predvidivoj budućnosti. Ovaj je rad usmjeren na napredak postignut razvojem sojeva kvasca posebno konstruiranih za fermentirana pića i industriju bioloških goriva.