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Biotechnology of Wine Yeasts

Fabienne Remize

1. Introduction

The story of biotechnology of wine yeasts started a long time ago. The selection of yeasts from wineries for commercial purposes was developed from the 1970s. The aim was to display satisfactory fermentation profiles and to ensure product quality. From the 1980s, classical breeding, interspecific breeding and mutagenesis were applied for the improvement of existing strains. This resulted in many developments: for example, better nitrogen assimilation and fermentation kinetics were obtained by random mutagenesis (Salmon and Barre 1998). These classical approaches are still used; EMS (ethyl methyl sulfonate) mutagenesis resulted in the selection of commercial wine strain variants which produce less reduced hydrogen sulfide due to mutations into genes encoding sulfide reductase subunits (Cordente et al. 2009). But genomic features of wine yeasts were rapidly identified as strong limitations for these approaches. Indeed, wine strains are mainly diploid, polyploid or aneuploid. They exhibit a chromosomal polymorphism (Bidenne et al. 1992). Chromosomal trisomies or tetrasomies result in impaired sporulation ability and in highly variable spore viability (Johnston et al. 2000). In addition, wine strains are generally homothallic, i.e., able to switch mating type when haploid, and highly heterozygous. All these features confer to wine yeast, high genome plasticity and limit the stability of lineage of variants.

The 1990s have been marked by considerable efforts to solve wine making process issues and to improve wine quality by metabolic engineering of wine yeasts. This was made possible by the incredible step beyond genetic improvement by the development of recombination methods. Genetic modification tools have the benefit of research in gene function understanding. Indeed, the first yeast sequenced genome was released in 1997 and revisited in 2000 (Blandin et al. 2000). Proteomic and

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transcriptomic studies resulted in a lot of useful data which was used to complete genome structure and to understand gene function. In this view, the considerable work realized on the understanding of osmotic stress response by *Saccharomyces cerevisiae* was exemplary. The genes involved in stress adaptation and the signaling pathways have been elucidated (Albertyn et al. 1994, Rep et al. 1999, Tamás et al. 2000, Pahlman et al. 2001, Rep et al. 2001). Consequently, the HOG (High Osmolarity Glycerol) pathway, a branched MAPK (Mitogen Activated Protein Kinase) pathway, has become a model to analyze systems level properties of signal transduction (Hohmann 2009).

Genetic engineering of wine yeasts was used to explore many issues. Among them, the examples which are hereby presented are representative of methods and are emblematic of research in the field.

2. Metabolic Engineering of Wine Yeasts

In the following section, the various aspects of genetic engineering of wine yeasts have been discussed.

2.1 Genetic Engineering of Malolactic Fermentation

One of the main focuses for wine making process improvement was to obtain a malolactic or a maloethanolic fermentation of wine from modified yeast. Malolactic fermentation is performed after alcoholic fermentation by a lactic acid bacterium, generally *Oenococcus oeni*. This bacterium converts the di-acid malic acid to the mono-acid lactic acid, thus leading to acidity decrease. Such a reaction is expected in most of white wines and in red wines from northern production areas of France. As a consequence of bacterial growth and metabolic activity, malolactic fermentation contributes also to wine stabilization (thanks to nutrient exhaust), and to flavor development. However, due to harsh conditions after alcoholic fermentation (ethanol level, low pH, low temperatures, sulfur dioxide and low nutrients level), bacterial implantation and growth in wine is difficult to control. As a consequence, many researches focused on the genetic engineering of yeast ability to use malic acid. A malolactic *S. cerevisiae* strain was obtained by expressing the genes encoding a malate transporter from *Schizosaccharomyces pombe* and of a malolactic enzyme from *Lactococcus lactis* (Bony et al. 1997, Volschenk et al. 1997). This strain efficiently converted malic acid to lactic acid. Similarly, an engineered *S. cerevisiae* strain, able to convert malic acid to ethanol, was obtained by the cloning of malate permease and malic enzyme genes from *Sc. Pombe* under the control of strong *S. cerevisiae* promoters (Volschenk et al. 2001). The Fig. 1 shows the two strategies, malolactic and maloethanolic, alternatively used in yeast.

Eventually, a genetically-stable malolactic yeast strain was constructed (Husnik et al. 2006). Fermentation kinetics, growth kinetics, analytical and sensorial profile of wine obtained with this strain, called ML01, were investigated. The resulting wines were compared to wines obtained by the traditional process, i.e., by successive yeast and lactic bacteria fermentations (Husnik et al. 2007). ML01 received

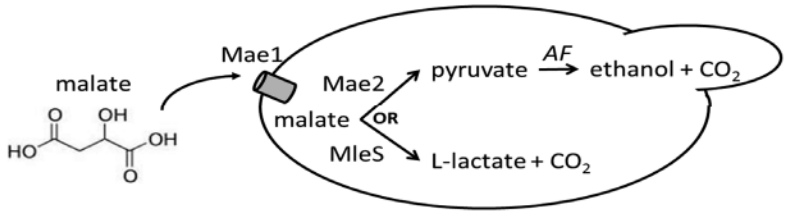

Figure 1. Malolactic and maloethanolic pathways, which were introduced in *S. cerevisiae* yeast. Mae1: *Schizosaccharomyces pombe* malic acid transporter; Mae2: *Sc. pombe* malic enzyme; MleS: *Lactococcus lactis* malolactic enzyme; AF: alcoholic fermentation.

approval from the Food and Drug Administration (USA) and the Health Canada and Environment Canada agencies. It is commercialized since 2004.

In parallel, alternative non-GM solutions were researched and co-inoculation of musts with yeast and lactic acid bacteria has attracted attention. Recently, another approach, that does not employ genetically modified microorganisms either, was investigated. It was observed that *Lactobacillus plantarum* was able to cope with wine conditions and exhibited interesting enzymatic activities to enhance wine aroma formation. *Lb. plantarum* starter cultures were selected to perform malolactic fermentation (du Toit et al. 2011, Lerm et al. 2011).

2.1.1 Engineering of resveratrol level

Health effects of a moderate consumption of wine are recognized as positive for the reduction of coronary diseases. Several wine molecules are detrimental to health, for instance ethyl carbamate, a carcinogenic molecule, biogenic amines or sulfites. Some others, however, exert beneficial effects, like resveratrol involved in the so-called 'French paradox'. Resveratrol (3, 5,4'-*trans*-trihydroxystilbene) is a phenolic compound with a demonstrated bioactivity and its main source in the diet is wine (Fernández-Mar et al. 2012). This compound is naturally present in grape, especially in the skin and seeds. As a consequence of skin maceration process, its level is higher in red wines than in white or rosé wines. Its concentration depends on many factors, among them biotic or abiotic stress exposure of vine plants, as resveratrol is a plant defense compound (phytoalexin). Its level in wine can be increased either by an increase of its release from grapes (González-Candelas et al. 2000) or by its formation by yeast from free aminoacids or derivatives. The resveratrol formation pathway is not present in yeast but was described in several plants (Fig. 2). The first studies that managed to produce resveratrol in yeast relied on over-expression of heterologous CoenzymeA ligase (C4L) gene from a hybrid poplar and resveratrol synthase (VST) gene from grapevine in a medium containing p-coumaric acid (Becker et al. 2003).

Other gene origins, other promoters, a synthetic scaffold strategy and fusion proteins were successively investigated and resulted in a further increase of resveratrol production by yeast (Shin et al. 2011, Wang and Yu 2012). The main increase in production was obtained by the adaptation of codon usage to yeast (Wang et al. 2011). The same author showed that the expression of the poorly characterized

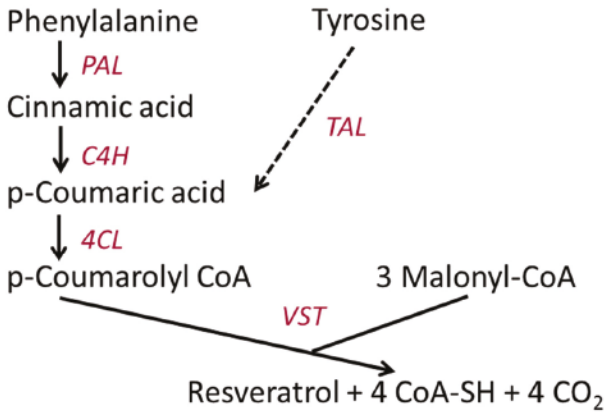

Figure 2. Resveratrol formation pathway. PAL: Phenylalanine ammonia-lyase; C4H: Cinnamate 4-hydroxylase; 4CL: CoA-ligase; VST: Resveratrol synthase; TAL: Tyrosine ammonia lyase.

arabinose transporter *araE* was able to enhance resveratrol permeability through lipid membranes in yeast, and then, to further increase resveratrol production. Resveratrol was also produced from tyrosine thanks to the additional over-expression of the gene encoding tyrosine ammonia lyase that converts the amino acid into p-coumaric acid (Shin et al. 2011, Jeandet et al. 2012). The different strategies used to produce resveratrol from yeast do not currently result in wine application. However, the progress led to issues for the industrial production of the stilbene for cosmetic or nutraceutical applications (Jeandet et al. 2012, Liu et al. 2013).

2.1.2 Low-Alcohol wines

Another topic widely investigated was to produce wines with lower ethanol content. The consumer's expectation towards wines with reduced ethanol level has been reinforced by health considerations. In addition, climatic changes result in higher grape maturity at the harvest stage, and thus, in higher sugar contents that, in turn, lead to higher ethanol levels in wines. Besides, physical and chemical approaches to decrease wine ethanol content generally result in color or/and aromatic reduction. Ethanol is produced by yeast *via* the glycolytic pathway followed by alcoholic fermentation of pyruvate. The complete pathway of ethanol formation is redox balanced and produces two molecules of ATP per C6-sugar consumed. In parallel during wine fermentation, biomass is produced from a part of carbon available in sugars. Biomass formation generates reduced cofactors. Redox homeostasis under anaerobiosis is ensured by the formation of reduced by-products, thereby re-oxidizing co-factors. The main by-product is glycerol, whose content in wine generally ranges between 6 and 9 g/L (Remize et al. 2000b). Glycerol formation during wine fermentation is not only driven by redox imbalance but mainly by the osmotic stress response due to high sugar content at the beginning of the process, and further, by the increasing ethanol level (Albertyn et al. 1994, Pahlman et al. 2001, Remize et al. 2003).

Glycerol does not exhibit aromatic properties by itself, but contributes to wine sensorial characteristics by providing sweetness and fullness. Its formation at the expense of carbon flux towards ethanol synthesis has been investigated (Remize et al. 1999, Remize et al. 2001). It was established that the first enzymatic step after triose-phosphate formation was rate-limiting. The overexpression of one of the genes encoding this enzyme, glycerol-3-phosphate dehydrogenase, under the control of a strong promoter, resulted in a 1.5–2.5 fold increase in glycerol content (Remize et al. 1999). As a consequence, ethanol content was reduced by 5–10 g/L. From that first genetic engineering modification, secondary effects were deeply examined (Fig. 3). Among those, an increase in acetic acid production was demonstrated. This effect was linked to the dynamic requirement of redox equivalents during wine fermentation. The PDH by-pass was then engineered to combine the high glycerol formation with a limited increase in oxidized by-products (Remize et al. 2000a, Cambon et al. 2006). The modified yeast exhibited fermentative properties comparable to control strains but with an efficient diversion of carbons from sugar towards glycerol instead of ethanol. The ethanol content was decreased by 15–20%. The formation of another by-product, acetoin, was increased by lowering ethanol content. *BDH1*, coding for native NADH-dependent butanediol dehydrogenase, was over-expressed in a yeast strain, overexpressing glycerol-3-phosphate dehydrogenase, and inactivated

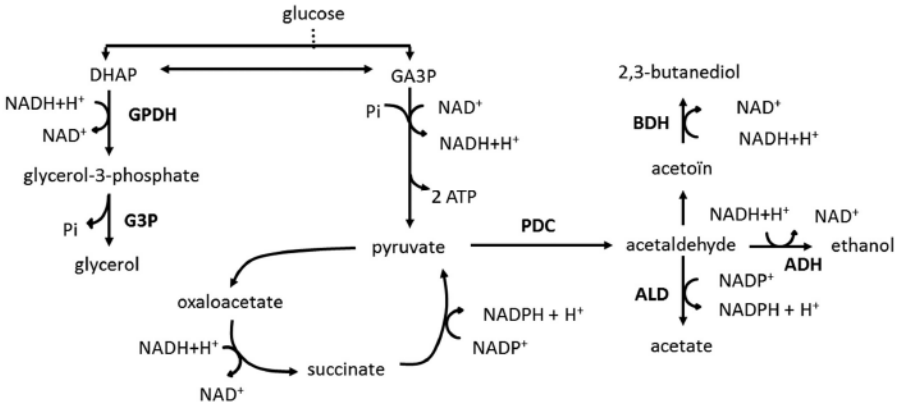

Figure 3. Glycerol formation pathway and formation of other by-products. ADH: alcohol dehydrogenase; ALD: acetaldehyde dehydrogenase; BDH: butanediol dehydrogenase; GPDH: glycerol-3-phosphate dehydrogenase; G3P: glycerol-3-phophatase; PDC: pyruvate decarboxylase; DHAP: dihydroxyacetone phosphate; GA3P: glyceraldehyde-3-phosphate.

for the *ALD6* gene encoding acetaldehyde dehydrogenase (Ehsani et al. 2009). This additional genetic modification was one step beyond for low-alcohol producing yeasts with acceptable organoleptic profiles.

2.1.3 Quantitative Trait Loci (QTL) approaches

The increase of availability of genomic sequences and the development of genomic tools over the last decade increased the global understanding of wine yeast response

to its changing environment. The QTL (Quantitative Trait Loci) approach has been applied to link phenotypes with genomic loci. For instance, the QTL approach has been explored in *S. cerevisiae* to increase ethanol formation yield at the expense of glycerol (Hubmann et al. 2013). A strain producing unusually low glycerol levels and a commercial strain were compared. To achieve that, haploid segregants with phenotypes similar to the parental strains were obtained and further crossed for genetic mapping of the diploid resulting strains. With that approach, a recessive allele of *SSK1*, expressing a truncated and partially mistranslated protein, was identified and used for reverse genetic engineering. While the glycerol/ethanol ratio reduction was stronger in the strain which carried the identified allele than in the deletion mutant, side effects on growth, on ethanol productivity and on osmosensitivity were low. *SSK1* encodes a protein of the osmolarity two-component signal transduction system which controls the activity of the HOG pathway.

Wine applications of QTL are still few. In a study, acetic acid production in wine was investigated by QTL mapping (Marullo et al. 2007). Acetic acid concentration is tightly related to volatile acidity and it has to be kept low. With haploïd derivatives of commercial wine strains producing very different levels of acetic acid, a major QTL was mapped. It corresponded to a variant of the *APS1* gene encoding, type 1 asparaginase, which carried a mutation in the catalytic motif of the enzyme. More recently, Ambroset et al. (2011) examined the relationships between genetic polymorphism, expression QTL and phenotypic traits associated with wine fermentation by using a laboratory yeast strain and a haploid derivative of a commercial wine strain. Expression QTL aim to evidence relationships between genetic polymorphism and transcript abundance. In this study, fermentation of must by the two parental strains exhibited differences in fermentation parameters, in particular carbon dioxide production rates depending on the fermentation stage (Ambroset et al. 2011). By the original approach of combining expression QTL, genetic QTL and phenotypic traits, new associations were evidenced and an allelic variant of *ABZ1* gene was mapped from overlapping results. It was shown that this gene is involved in the control of fermentation rate through nitrogen utilization modulation.

QTL approaches have now demonstrated their interest for biotechnology of wine yeasts: they allow identifying allelic variants not directly involved in the pathway of a metabolite formation and thus define new targets for metabolic reengineering.

2.1.4 Evolutionary engineering for wine yeast improvement

From the 2000s, European Union positioning towards GMO was clarified. In several other countries, regulations are more permissive for GMOs. Whatever the case may be, consumers' acceptance for GMO suffers from a bad image and alternative strategies were investigated, more and more frequently. In addition, metabolic engineering failed to define genetic targets related either to phenotypes resulting from pleiotropic regulations or to phenotypes resulting from multiple cellular properties that do not share a defined molecular basis. In that perspective, evolutionary engineering was developed as alternative strategy (Çakar et al. 2005, Çakar et al. 2012). This approach is based on the generation of high clonal diversity, by classical mutagenesis, and the progressive selection of phenotype. The challenging step is phenotype selection

which, contrarily to classical approaches, is performed by taking into consideration multiple characteristics.

The latest researches to engineer the ethanol levels in wines investigated adaptative evolution (Cadière et al. 2011, Kutyna et al. 2012). To achieve that, a poor carbon source, δ-gluconolactone, was used (Cadière et al. 2011). This carbon source is consumed *via* the pentose-phosphate pathway that generates reducing equivalents and thus is interconnected, *via* the cellular redox level, to glycerol formation. A long-term serial transfer procedure on δ-gluconolactone resulted in the isolation of variants with a better growth on this substrate while the growth rate on glucose was maintained. The accurate characterization of strain properties with ^{13}C flux analysis highlighted a re-routing of metabolic flux from the glycolytic pathway towards the pentose phosphate pathway. As a consequence, acetate production from the evolved strain was reduced, whereas ethanol level reduction was hardly detectable. Kutyna et al. (2012) selected a haploid variant from successive generations on a medium containing high sulfite concentration at alkaline pH. Sulfite stress was previously shown to result in increased glycerol formation. The heterozygous diploid obtained after backcrossing did not exhibit an increased glycerol production as the genetic modification was recessive. Other applications of adaptative evolution for winemaking were investigated, particularly in the enhancement of desirable aromatic properties (Cadière et al. 2012) and improvement of fermentation kinetics (Novo et al. 2014). Promising results were obtained but require further studies to result in a usable commercial starter.

3. Wine Ecosystems and Terroir

Whereas wine alcoholic fermentation is essentially performed by *S. cerevisiae* thanks to its peculiar resistance to ethanol, non-*Saccharomyces* yeasts are dominant during the first stages of sugar assimilation, and their contribution to wine complexity has been recognized for decades. It was established that yeasts in must originate from grapes and cellar equipment (Renouf et al. 2007). Yeast population on grapes averages 10^3 cfu/g. In must, its population rapidly reaches 10^8 cfu/mL and then gradually decreases to stabilize thereafter (Torija et al. 2001).

The diversity of the species present on grape berries has been described (Zott et al. 2008, Barata et al. 2012). Recent molecular approaches have allowed understanding the ecological dynamics during wine making (Esteve-Zarzoso et al. 1998, Pramateftaki et al. 2000, Schuller et al. 2004, Hierro et al. 2006). The most recent studies highlight a possible geographic effect on the yeast selection so much so that the most adapted to the ecological niche constitute an ecosystem fingerprint (Pramateftaki et al. 2000, Raspor et al. 2006, Dequin and Casaregola 2011, Gayevskiy and Goddard 2012, Schuller et al. 2012). Domestication of yeasts from millenniums, not only for winemaking but also for baking and brewing, has impacted genetic structures (Legras et al. 2007) and results in specific phenotypic traits shared by isolates from the same ecological niche (For a comprehensive review, see Dequin and Casaregola 2011). Starter strains, which are well-adapted to their ecosystem, could disseminate in the cellar and around, to the vines. This might then result in a diversity loss in the particular environment. A study has shown that yeast dissemination was restricted to close environments (Valero et al. 2005, Schuller et al. 2012). This evidence is

strengthened by genomic characterization of yeasts that shows a relative geographic diversity within domestic wine yeasts (Martínez et al. 2007). From this point of view, the ecological adaptation of yeasts could partly explain the terroir of wines. Commercial starters which claim to respect wine terroir typicity are now developed.

Conversely, several tentative to develop non-*Saccharomyces* starters are reported (Hong and Park 2013). Starters, combining non-*Saccharomyces* and *Saccharomyces* strains, are commercialized in order to improve the aromatic complexity (See lallemandwine.com). Eventually, the use of non-*Saccharomyces* yeasts able to consume sugar by respiration in the harsh must conditions without producing off-flavors was investigated (Gonzalez et al. 2013, Quirós et al. 2014) in order to produce less ethanol in wines. Research in that direction is required to propose in the future, yeast cocktails that warranty wine quality without any loss of terroir influence.

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