SOUTH AFRICA, APRIL 30, 2006

NEW TRENDS IN THE USE OF SPECIALTY INACTIVATED YEAST DURING WINEMAKING

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LALLEMAND

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PROCEEDINGS
OF

LES XVIII^{es} ENTRETIENS SCIENTIFIQUES LALLEMAND

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FOREWORD

The world of wine is in constant evolution, and new research is being conducted on a variety of innovative tools, such as specialty inactivated yeast (SIY) products and their contributions to wine. SIY products were the focus of the *XVIII*^{es} *Entretiens Scientifiques Lallemand* held April 30, 2006, at the Kwa Maritane Lodge in South Africa. The program for these technical meetings included presentations reporting on current knowledge regarding the composition of yeast and its derivatives and their commercial equivalent – SIY products.

From current knowledge on the cell walls of yeast, which vary in composition according to the conditions in their environment (the cell walls redesign themselves to adapt to their environment), to the research on yeast hulls and their utilization in winemaking, the presentations were both applied and scientific. The importance of mannoproteins and peptides in the cell walls was clarified. The mannoproteins and peptides are released during aging, subsequent to autolysis, influencing the structure and sensory properties of the wine. This is a long process. To obtain wines with a richer sensory profile, the winemaker can accelerate the process by using selected yeast strains that produce large quantities of mannoproteins or yeast extracts high in mannoproteins or glutathione (to prevent oxidation). The practical experience on the subject of mannoproteins and oenological products high in *Saccharomyces cerevisiae* cell walls was also discussed. Aging on fine lees and the different factors that can help the winemaker, e.g., the quality of the grapes, the yeast strain, enzymes, maceration and the management of malolactic fermentation, were presented.

The results of the presenters' research show that the claims for SIY products are scientifically based. The oenological world is in the early stages of research into these new techniques that are already being used in wineries. By understanding the various impacts of SIY products based on the cell walls of different yeast strains, Lallemand hopes to have new products and better inform winemakers, thereby contributing to the making of quality wines.

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THE CELL WALL OF THE YEAST *SACCHAROMYCES CEREVISIAE*: AN INVESTIGATION OF ITS DYNAMIC COMPLEXITY FOR THE BETTER UTILIZATION OF ITS BIOTECHNOLOGICAL VALUES

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Abstract

Yeast cells are surrounded by a thick wall that ensures their shape and integrity, and constitutes the first interface with the environment. The cell wall is composed of three different types of polymers: ß-glucan (ß-1,3 + ß-1,6-glucan), mannan (mannose residues linked in α1➞2, α1➞3, and α1➞6) bound to proteins (making the mannoproteins) and chitin (N-acetyl glucosamine residues linked in ß1➞3). Each of these polysaccharides are endowed with interesting biotechnological properties. In particular, the ß-glucan matrix has been show to be the main binder of mycotoxins and a structural model of the interaction of mycotoxins with ß-glucans has been proposed for the first time. The practicability of this model for the better utilization of cell walls in mycotoxin adsorption is hampered by the difficulty of manipulating cell wall formation, to increase levels of ß-1,3-glucan for example, and to reduce chitin, which has a negative effect on mycotoxin adsorption. These difficulties are due mainly to the fact that the three polysaccharides that compose the cell wall are interconnected and form a three-dimensional structure subject to dynamic remodelling in response to environmental and developmental conditions. Accordingly, a major effort has been carried out over the past 10 years to identify the regulatory mechanisms that account for the remodelling of the yeast cell wall. In our laboratory, two lines of research have been taken to study the remodelling mechanism. The first was to develop a simple and quantitative method for cell wall composition, and use this method to investigate the effects of culture conditions on levels of ß-glucan, mannan and chitin. Overall, these studies have shown that the dry mass and polysaccharide content of the cell wall can vary by more than 50%, according to the nature of the carbon source, nitrogen limitation, pH, temperature and mode of cell cultivation. The other line of research was to investigate the molecular mechanism of cell wall remodelling using genomic and post-genomic technologies. This work, which confirmed a role of the Pkc1 signalling pathway in the integrity of the cell wall, also unravelled the complex interaction of this signalling pathway with the global stress response dependent on Msn2/Mns4 and the Ca^{2+}/calcineurin signalling systems. Further work is underway to identify the metabolic and regulatory networks that connect the cell wall to cell growth, with the ultimate goal of modelling the metabolic and regulatory pathways of the cell wall. This modelling could be used for biotechnological purposes, including a willingness to reshape cell wall composition for agro-food applications, and to identify new cell wall targets for antifungal agents.

Introduction

The yeast cell wall is the main determinant of cellular strength, and plays an important role in cell morphogenesis and cell growth.1 It is the first cellular structure in direct contact with the surroundings. Under laboratory culture conditions, the cell wall amounts to 18-22% of the cell dry mass.[2, 3] The yeast cells have a very high turgor pressure, and a minor chink in the cell wall can lead to bursting and death. Moreover, the cell wall is not a rigid structure, as it endures all the changes that the yeast cell undergoes during division, morphogenesis and dif-

ferentiation. To ensure the continuous integrity of the wall in accordance with its plasticity, elaborate mechanisms are operating, and they are strictly coordinated with those governing the cell cycle progression. In this presentation, I will focus on our recent work dealing with cell wall composition, biogenesis and remodelling in response to environmental stress and in relation to cell growth. The reasons for studying the yeast cell wall will be illustrated through an example of biotechnological application as a microbiological method for capturing mycotoxins in animal feed. This example will then justify the willingness to further investigate cell wall engineering. However, due to the complexity of yeast cell wall biogenesis, the difficulties of such engineering appear to be insurmountable. A possible solution to this problem could be through a System Biology approach, as described in the paper in reference [4].

Part 1: Cell Wall Composition and the Biogenesis and Biotechnological Use of ß-glucans

The cell wall of *Saccharomyces cerevisiae* is organized into two layers that are made up of four classes of covalent cross-linked macromolecules: ß-1,3-glucan, ß-1,6-glucan, chitin and cell wall mannoproteins (CWPs). The crystalline character of ß-glucan and its partial insolubility might be explained by its association with chitin through covalent bonds.[5] Electron microscopic studies have also shown that the mannoproteins form a fibrillar outer layer extending radially from the inner skeletal layer, which is formed by the polysaccharide fraction of the cell wall.[6] The architecture of the cell wall from the yeast *S. cerevisiae* is outlined in Figure 1.

1.1 Structure, enzymology and regulation of cell wall components

1.1.1 ß-1,3- and ß-1,6-glucans

Among the ß-glucosyl-polymers, ß-1,3-glucan is the most prominent carbohydrate in the cell wall, accounting for about 40-50% of the cell wall dry mass. They form a fibrillar structure composed of three helically entwined linear chains of about 1500 ß(1➞3)-linked glucose units. This structure provides the rigidity and integrity of the cell wall, and determines the cell shape. The ß-1,3-glucans are produced from UDP-glucose by the membrane-bound ß-1,3-glucan synthase (GS) encoded by two homologue genes, *FKS1*and *FKS2*.[7] However, the GS activity requires the presence of a 26 kDa GTP-binding protein encoded by *RH01*. This small G-protein is loosely attached to the plasma membrane through prenylation at the C-terminus. Rho1 acts as a switch for the GS activity, due to the conversion between an inactive GDP-bound protein to an active GTP-bound form catalyzed by the guanine exchange factors Rom1 and Rom2. Conversely, GTPase-activating proteins, encoded by *BEM2* and *SAC7*, switch off the GS activity by converting GTP-bound active Rho1 to the inactive GDP-bound form (reviewed in reference [8]).

The minor component of ß-glucan is ß-1,6-glucan, which is a polymer of about 100-350 ß(1➞6)-linked glucose units. A genetic approach was used to elucidate a great part of the biosynthesis of this polymer, taking advantage of the fact that cells devoid of ß-1,6-glucan are resistant to the K1 Killer toxin, called *kre* mutants for Killer REsistant. To summarize more than 10 years of work carried out mainly by the Bussey group (see reference[9] for an extensive review), ß-1,6-glucan biosynthesis is initiated in the endoplasmic reticulum, continues in the Golgi apparatus and ends up at the cell surface. After several trials, a UDP-glucose-dependent ß-1,6-glucan synthase activity was identified in the crude membrane of yeast cells, using an immunoassay.[10] Like ß-1,3-glucan synthase, the putative ß-1,6-glucan synthase activity seems be stimulated by GTP. The gene encoding this enzyme remains to be discovered. (See Figure 1 on next page)

1.1.2 Chitin

Chitin is an essential component of the yeast cell wall, although it is a minor component (<2% in wild-type cells in laboratory conditions).[2] Chitin is a linear homopolymer composed of about 120 N-acetylglucosamine residues that are linked by ß(1➞4) bonds. Chitin structure consists of antiparallel hydrogen-bonded chains called microfibrils that can retain the dye calcofluor white.[11] In *S. cerevisiae*, and most likely in other non pathogenic and pathogenic fungi, chitin is attached covalently to ß-1,3 and ß-1,6-glucans.[12, 13, 14] While in *S. cerevisiae*, the formation of chitin is taken over by three distinct chitin synthases, encoded by *CHS1*, *CHS2* and *CHS3*, this biosynthesis seems to be even more complex in other fungi, due to the presence of five to eight isoforms.[15] Chitin synthases are integral membrane proteins with the catalytic domain located at the cytosolic face. Except for CHS3, measurement of its activity requires prior *in vitro* activation by mild treatment of the crude extract with trypsin. Although the physiological meaning of this activation is still enigmatic, it may somehow be linked to the fact that chitin synthases have also been reported to be located in vesicles named chitosomes.[16, 17] The existence of three distinct chitin synthases raises questions about their specific roles and regulation mechanisms in yeast. These questions have been addressed at the genetic and biochemical levels. As

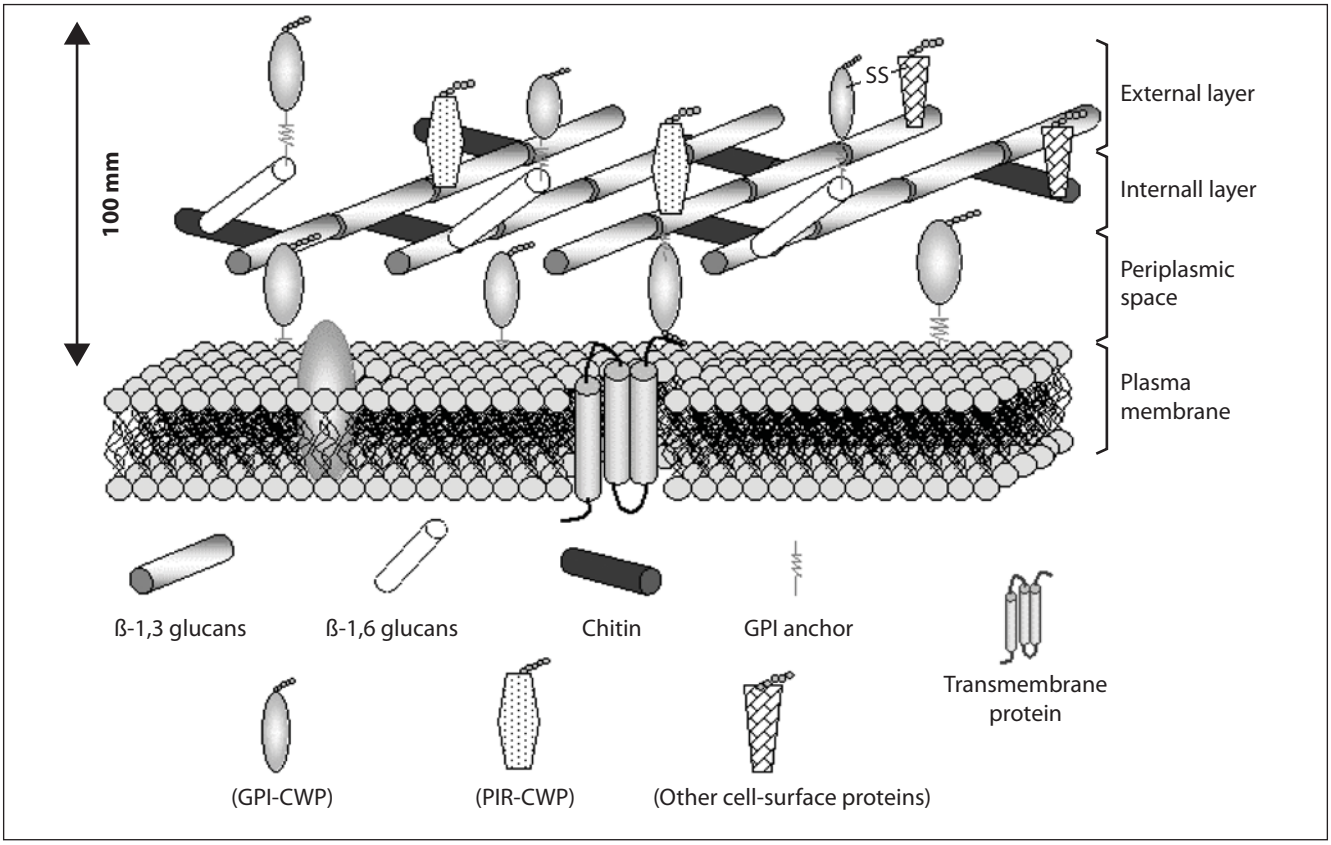
it was shown that chitin synthesis is a cell cycle regulated process, the action of each of the chitin synthases can be assigned to specific steps during this process. Chs1 is a repair enzyme that synthesizes chitin following separation of mother and daughter cells, while Chs2 is localized to the mother-bud septum and is responsible for primary septum formation between mother and daughter cells.[18] Chs3 is the most important enzyme-catalyzing about 90% of the chitin content of the cells. This enzyme is spatially and temporarily controlled by a number of regulatory proteins encoded by *CHS4*, *CHS5*, *CHS6* and *CHS7*. (For a review, see [5].)

The genome of *S. cerevisiae* contains two genes, *CTS1*and *CTS2*, encoding endochitinases that cleave chitin into N-acetylglucosamine units. Cts1 is present in vegetative cells as a protein non-covalently bound to the cell wall. The main function of this hydrolase is to dissolve the chitinous primary septum that is synthesized by Chs2 during cell separation. Since excessive activity can be lethal for the cell, Cts1 is supposed to be tightly controlled by both transcriptional and post-translational mechanisms.[19] The *Cts2* gene is induced during sporulation, but the role of *Cts2* during this process remains unknown.

1.1.3 Mannoproteins

The outer layer of the yeast wall is made up of proteins that are bound via a serine, threonine or asparagine residue to a polysaccharide complex of 150 or more *D*-mannose units (the so-called mannan). Chemical studies have shown that mannans are composed of an α(1➞6) linked backbone of mannoses to which are attached short side chains of mannoses linked by α(1➞2) and α(1➞3) bonds. The polysaccharide structure can reach up to 50% of the cell wall dry mass. The biosynthetic pathway of mannans starts in the cytosol by the isomerization of fructose-6*P* into mannose-6*P* catalyzed by the phosphomannose isomerase (Pmi1), which is then epimerized into mannose-1*P*. The GDP-mannose is formed from mannose-1*P* and GTP by a GDP-mannose pyrophosporylase encoded by the essential gene *PSA1*.[20] A dolichol-phosphate synthase, localized at the cytosolic face of the ER and encoded by the essential gene *DPM1*, transfers the mannose from GDP-mannose to

Figure 1. Outline of the *Saccharomyces cerevisiae* cell wall.
The yeast cell wall consists of a 100 nm thick extracellular matrix. The internal skeletal layer is made of ß-1,3-glucans that form a three-dimensional network surrounding the entire cell and strengthened by chitin fibres. The ß-1,3-glucans are branched with ß-1,6-glucan side chains that also interact with chitin and function as a flexible tether for GPI-anchored mannoproteins (GPI-CWPs). A second class of mannoproteins (PIR-CWPs) are directly linked to ß-1,3-glucans. Other cell-surface proteins are attached to the cell wall either by disulphide bridges to other CWPs, or by non specific binding.



dolicholphosphate to form Dol-*P*-mannose. This molecule is a key intermediate in three protein glycosylation processes, namely the *N*-glycosylation, *0*-glycosylation and glycosylphosphatidyl-inositol (GPI) membrane anchoring.[21] *N*-glycosylation involves a set of glycosyltransferases encoded by *ALG1* to *ALG10* genes which produce the oligosaccharide precursor $GlcNac_2Man_9Glc_3$ on the polyisoprenoid carrier lipid Dol-*PP*. The last step in the ER is the transfer of the oligosaccharide to the amide group of an asparagine residue of the protein in the tripeptide "sequon" Asn-X-Ser/Thr, where X is any amino acid except proline. This reaction is catalyzed by the oligosaccharide transferase (OTase) complex composed of eight proteins, all of which are encoded by essential genes.[22] Maturation of glycolysated protein occurs in the Golgi apparatus by successive addition of mannose units from GDP-mannose catalyzed by specific α-mannosyltransferases. The *0*-mannosylation is initiated in the ER by transferring a mannose from Dol-*P*-mannose to a Ser or Thr residue of the protein. This reaction is carried out by a Dol-*P* Man::protein *O*-mannosyltransferase encoded by one of the five *PMT* genes.[23] Only the deletion of three of them (*PMT1*, *2* and *4* or *PMT2*, *3* and *4*) leads to inviability, leaving the other four with no clear function yet.[24]

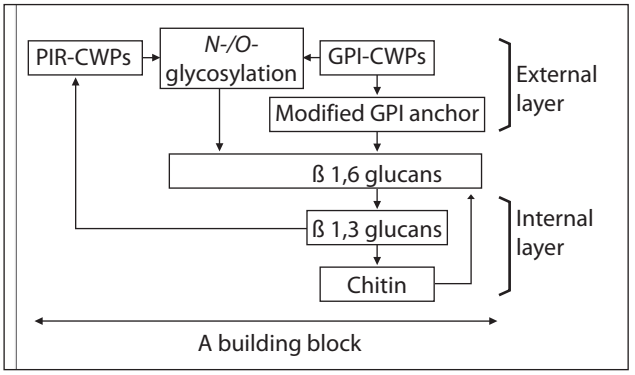
Yeast cells have evolved three different ways of attaching proteins to the polysaccharide moiety. Some proteins are bound to the ß-1,3-glucans non-covalently. In the so-called SCWs family, some are attached covalently through a remnant of the GPI anchor to ß-1,6-glucans (the so-called GPI-CWPs), and lack of this GPI anchor results in secretion of these CWPs into the medium.[25] A third class includes cell wall mannoproteins characterized by Protein Internal Repeat regions (PIR-CWPs or the CCWs family) that are directly linked to ß-1,3-glucan by a mechanism that is not completely understood.[26] A last category encompasses the so-called non-conventional secreted proteins that are loosely bound at the cell surface, as is surprisingly found for many glycolytic enzymes.[27] It is important to emphasize the redundancy of these cell wall proteins, suggesting an important function in cell wall integrity.

1.2 Cell wall composition and molecular architecture

1.2.1 Modular structure of the cell wall

The structural components described above are not simply juxtaposed. Rather, they are assembled to each other by covalent linkages, which probably generate a modular architecture of the yeast cell wall, as proposed by Klis1 and Lipke[28] (see Figure 2). These cell wall modules, *i.e.*, CWP→ß-1,6-glucan ← chitin and CWP→ß-1,6-glucan →ß-1,3-glucan ← chitin, involve glycosidic attachments between ß-glucans and chitin. GPI-CWPs mannoproteins are linked to ß-1,6-polysaccharides via a processed form of GPI anchors.

Figure 2. Typical building blocks of the yeast cell wall. Relationships among components of a cell wall module (or building block) are schematically depicted (adapted from reference [1]).



The different modules are interconnected by noncovalent interactions in the ß-glucan-chitin layer, and by covalent cross-links in the mannoprotein layer, including disulphide bonds between mannoproteins, and perhaps also through other mannoprotein-glucan links not yet characterized.[12]

Although the chemical bonds between the different cell wall components have been described in detail, the enzymes involved in the generation of these links are still largely unknown. These are the enzymes involved in the cleavage of the GPI-anchor of the GPI-proteins and the subsequent attachment to ß-1,6-glucans, as well as the enzymes involved in the coupling between ß-glucan and chitin. Yeast possesses several exo-glucanases that catalyze *in vitro* the hydrolysis of linkages at the non-reducing ends of ß-1,3-glucans, and endo-glucanase activities that cleave within the chains.[29] However, some of the identified ß-glucanases may also carry out glucosyl transferase activity, such as Bgl2, which was originally characterized as an endo-ß-1,3-glucanase[30], and Gas1, a ß-1,3-glucanosyltransferase[31]. Many other genes, such as the *SCW* gene family, *CHR1* and *CHR2*, encode proteins with putative glucosyl transferase activity, but so far several trials to demonstrate that these proteins may bear out such activity have failed.

1.2.2 No correlation between variation of cell wall composition and molecular architecture

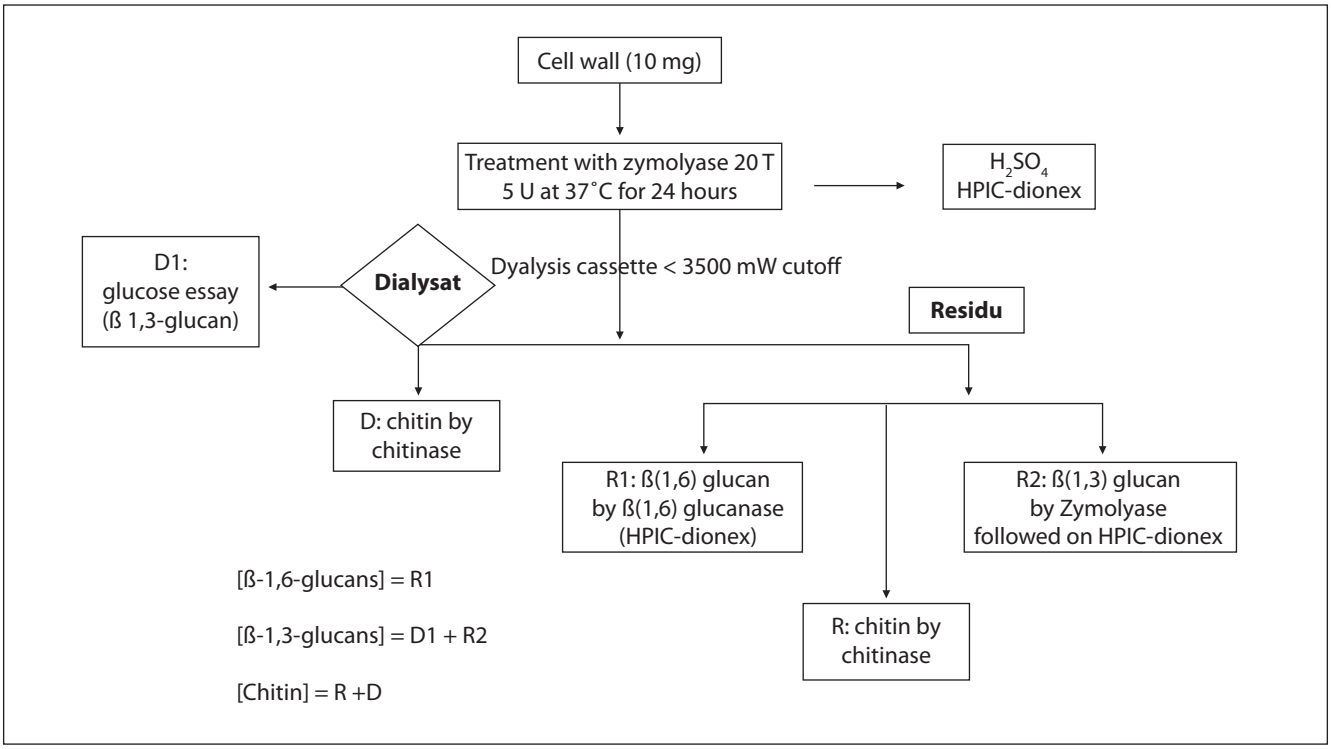
Methods available for cell wall composition and the characterization of molecular architecture analysis are based on the separation of structural components by either chemical or enzymatic methods. A method still currently used for quantification of cell wall composition is based on chemical fractionation of the cell wall polysaccharides. This results in three main fractions: an alkali-soluble

fraction consisting mainly of ß-1,3-glucan, mannan and some ß-1,6-glucan; an alkali-insoluble, acid-insoluble fraction containing ß-1,3-glucan linked to chitin; and an acid-soluble, alkali-insoluble fraction composed of ß-1,6-glucan.[2] An alternative method is based on the complete hydrolysis of complex cell wall polymers under acidic conditions, and determination of the monomers by high-performance anion-exchange chromatography (HPAEC) using pellicular resins and pulsed-amperometric detection (PAD). Several procedures, differing in the nature and concentration of acids, have been used to achieve to a complete hydrolysis of cell wall polymers from various organisms. However, in the case of yeasts, we developed a reliable and fast acid hydrolysis of purified *Saccharomyces cerevisiae* cell walls using sulphuric acid. This resulted in an almost complete release of glucose and mannose from cell wall polysaccharides, whereas the hydrolysis of chitin into its monomer glucosamine (the *N*-acetyl form is unstable under acidic conditions) was not reproducible. After the complete removal of sulphate ions by precipitation with barium hydroxide, the liberated monosaccharides are separated and quantified by HPAEC with PAD. The superiority of this method over hydrolysis in either trifluoroacetic (TFA) or hydrochloric acid (HCl) resides in its higher efficiency regarding the release of glucose from ß-1,6-glucan and of glucosamine from chitin. The simplicity and reliability of this procedure makes it the method of choice for the characterization of various *S. cerevisiae* strains and mutants.[3]

However, these chemical methods provide only the global content of cell wall composition, often estimated as a percentage of dry mass. In order to correlate cell wall composition to cell wall structure, we devised an enzymatic assay that enables us to determine the proportion of each component, particularly chitin and ß-1,6-glucans whose levels can be decisive with respect to cell wall structure/remodelling.

As indicated in Figure 3, this procedure involves a 24-hour hydrolysis with a 5U zymolyase, which is a crude preparation containing ß-1,3-glucanase from *Arthrobacter luteu*. This amount of zymolyase and the time of incubation were found to be optimal. After dialysis, the filtrated solution essentially contained zymolyase-released glucose, which was from ß-1,3-glucans and mannans. Chitin can be measured in this soluble fraction after chitinase treatment. Chitin and the major part of ß-glucans (both ß-1,3- and ß-1,6-glucans) were found in the non dialyzable solution. To distinguish ß-1,3- from ß-1,6-glucan, we incubated the two fractions with purified recombinant endo-ß-1,6-glucanase from *Trichoderma harzinium* expressed in *Pichia pastoris*.[32] In our experience, the enzyme was highly specific to pustulan (used as a template for the ß-1,6-glucan assay), and yielded mainly glucose and small amounts of gentiobiose and gentiotriose. This method was then utilized to evaluate the polysaccharide composition of the *Saccharomyces cerevisiae* cell wall under various growth conditions. It was found that the dry mass and polysaccha-

Figure 3. Sketch of the enzymatic digestion of the yeast cell wall.



rides content of the cell wall could vary by more than 50% with the nature of the carbon sources, nitrogen limitation, pH, temperature and aeration, and with the mode of cell cultivation (shake flasks versus controlled fermentors). We then tentatively compared the variation of cell wall composition to the cell wall architecture. This structure can be assessed by the formation rate of yeast spheroplasts. Lysis caused by digestion with zymolyase can be measured spectrophotometrically at 600 nm in a hypotonic solution. The lysis rate is somehow an indirect estimation of cross-linking between cell wall components.[33] While there was no obvious correlation between absolute levels of ß-glucans or mannans and the resistance of whole yeast cells to zymolyase treatment, the increase of ß-1,6-glucan levels, albeit modest in respect to the growth conditions investigated, was associated with a decrease in the rate of cell lysis (Table 1). This indicated lower sensitivity of yeast cells to lytic action by zymolyase. Therefore, we concluded that the cell wall structure is merely determined by cross-linking between cell wall polymers, and pointed to the role of ß-1,6-glucan in this process. Hence, this study reinforces the idea that enzymes involved in these cross-linking reactions are very important in structuring the modular architecture of the cell wall, indicating that they could be potential targets for antifungal drugs.

Table 1. Effects of growth conditions on cell wall mass, ß-1,6-glucan and the sensitivity of yeast cells to zymolyase (adapted from reference[34]).

Growth condition	Cell wall* (% dry mass)	Total ß-glucans (µg/mg cell mass)	ß1,6 glucans /total glucans (%)	Zymolyase sensitivity (MLR) x 10^{-2} min
YPD	24.5 ± 2.5	127.4 ± 3.2	18 ± 3.0	0.28
YNB	21.2 ± 2.4	72.6 ± 4.1	18 ± 2.5	0.32
SM	20.4 ± 2.8	71.4 ± 3.3	15 ± 2.0	0.68
glucose	18.3 ± 2.0	62.5 ± 2.9	14 ± 2.5	0.75
mannose	14.2 ± 1.8	64.3 ± 1.7	14 ± 2.2	0.66
sucrose	15.2 ± 1.8	62.7 ± 3.1	13 ± 3.6	0.68
maltose	14.5 ± 2.0	50.6 ± 1.8	16 ± 2.7	0.35
galactose	16.4 ± 2.0	78.2 ± 2.3	19 ± 3.2	0.15
ethanol	10.8 ± 1.5	38.7 ± 3.4	21 ± 2.4	0.03
pH 3	17.9 ± 3.0	60.4 ± 2.6	15 ± 2.0	0.49
pH 4	18.9 ± 2.0	80.8 ± 3.6	17 ± 3.0	0.62
pH 5	20.5 ± 2.0	66.5 ± 2.9	14 ± 2.0	0.75
pH 6	14.1 ± 1.6	58.4 ± 3.2	12 ± 1.5	0.86
T22°C	12.4 ± 2.1	60.1 ± 3.1	10 ± 2.0	0.85
T30°C	18.3 ± 2.6	68.5 ± 4.5	14 ± 2.0	0.75
T37°C	15.5 ± 2.0	88.9 ± 4.9	20 ± 2.0	0.59

Yeast cells were cultivated in a controlled batch fermentor. The growth medium was YPD (10 g/L yeast extract, 20 g/L bactopeptone and 20 g/L glucose), SD (1.7 g/L yeast nitrogen without amino acid or ammonium, 5 g/L ammonium sulphate and 20 g/L glucose) and SM (synthetic medium). Unless otherwise stated (see column 1), the pH was kept constant at 5.0, temperature at 30°C, and aeration at pO_2 ~50% saturation in the medium. The zymolyase sensitivity was carried out as described in reference[33]. The values reported are the mean ± SEM from four independent experiments, and for each experiment three samples were taken for cell wall analysis.

*Cell wall content was determined by the phenol/sulphuric methods.[3]

MLR: Maximal lysis rate.

nd: Not determined.

1.2.3 ß-glucans as a binder of mycotoxins: A molecular and structural approach.

Yeast cells or a preparation of yeast walls are currently added to animal diets in order to facilitate digestion and to protect the animals from pathogens. One of the apparent benefits of this additive in animal feed seems to be the protection of the animals against toxic effects resulting from mycotoxins. These toxins are secondary metabolites secreted by moulds, with most belonging to one of three genera: *Aspergillus*, *Penicillium* or *Fusarium*. They are produced in cereal grains and forage before and during harvest, or even during the processing and storage of forage. The presence of mycotoxins in feed may decrease feed intake, and affects animal performance, due to liver damage or the induction of tumour formation and immunosuppression.[35] Detoxification by metabolic activity in the host is relatively inefficient for most of the toxins. There is therefore a strong recommendation from food safety authorities to develop prevention programs and to encourage new methods for reducing mycotoxins in food and feeds. One of the most popular methods for controlling mycotoxins in animal feeds is based on the use of specific materials that can absorb mycotoxins, thus limiting their bioavailability in the body. The carbohydrates in yeast cell walls have been reported to express this capacity when administrated to chickens.[36] However, neither the nature of the component nor the mode of binding was determined. This was the focus of a research project under Dr. Alexandros Yiannikouris at INRA-Theix (France) in collaboration with Alltech and my laboratory. First, it was shown that the adsorption of toxins on cell walls follows a sigmoid curve that could be mathematically described by the Hill's equation. This model allows us to estimate cooperativity (or the number of accessible sites), which is an indication of the flexibility of the cell wall components to adsorb the toxin.[37] Identification of the major components in the cell wall that are responsible for mycotoxin retention was mapped by comparing several yeast strains differing in amount of ß-glucans, mannans and chitin (Table 2). Cells with higher levels of ß-glucans had higher maximum amounts of bound toxins (T^{max}_{bound}) and higher associations of constant and saturation points (K_D and K_{sat}). In contrast, high levels of chitin result in reduced flexibility of the ß-glucans and hence in a lower adsorption of the toxin. This finding is important, taking into ac-

count that manipulating cell wall composition to increase ß-glucans through a genetic approach leads to the induction of a cell wall compensatory mechanism culminating in a strong increase of chitin (see below).

Table 2. Adsorption parameters calculated with the Hill model with n sites equation (adapted from reference[38]).

Parameters	Glucan /mannan /chitin 45/53/2	Glucan /mannan /chitin 75/17/8	Glucan /mannan /chitin 35/62/3	Glucan /mannan /chitin 30/63/7
n	1.3 ± 0.2	1.9 ± 0.4	2.8 ± 0.4	2.1 ± 0.8
T^{max}_{bound}	15.2 ± 7.1	9.3 ± 2.5	3.8 ± 0.2	3.3 ± 0.7
K_D (µg/ml)	25.6	15.7	8.10	6.4
K_{sat} (µg/ml)	65.3	32	14	12.6

Since ß-glucans can be separated into alkali soluble and insoluble fractions, further work on zearalenone adsorption on these two fractions was investigated. Interestingly, it was found that the alkali-insoluble fraction exhibited a higher affinity rate for ZEN adsorption than the alkali-soluble fraction, and this difference was suggested to be due to the complex 3-D structure of ß-glucans.[39] It is noteworthy that the alkali-insolubility of ß-glucan is dependent on its ß-1,6-glucan content. These results suggest that the adsorption process is greatly dependent on the three-dimensional organization of the ß-glucans.

In general, polysaccharides can exist in two conformational forms: random coils and ordered structures. In solution, the polysaccharides adopt random coil conformation as the many flexible glycosidic linkages between the units in the polysaccharide chain allow rotation to occur around the glycosidic bonds. Using computer-assisted methods to calculate the energy maps for different polysaccharide conformations, it was shown that the most favourable conformation for short ß-1,3-glucans is an open single helix with six residues per turn. However, branched fungal ß-1,3-glucans having side chains of ß-1,6-linked glucosyl units can adopt triple-helical aggregates.[40, 41] (see Figure 4)

The molecular mechanism, and in particular the spatial conformation, of ZEN complexation to ß-glucans was investigated by means of NMR and X-ray diffraction techniques using model ß-glucans (laminarin). Using computer-assisted modelling, it was proposed that the ß-1,3-glucans form the backbone of the complexation process, which is stabilized and further strengthened by ß-1,6-glucans. Stable intermolecular hydrogen bonds are created which involved the hydroxyl and ketone groups of the ZEN molecule, plus the hydroxyl groups located at C(4), C(6) and, to a lesser extent, at the C(2) hydroxyl group of the ß-1,3-glucan molecule. In addition, the increasing length of the ß-1,6-glucan side chains appeared to exhibit stabilizing effects by enhancing the van der Waals interactions with ZEN. These theoretical findings are in full agreement with the structural parameters found for Zen and ß-glucans *in vivo*.

As a conclusion, this first study, which is the first to elucidate how ß-glucan chains have a specific and quantitative affinity for a toxin, could contribute significantly to proving the reliability of these adsorbents in fighting mycotoxins.[42, 43] However, *in vivo* application of ß-glucans as feed additives would require the use of insoluble yeast cell wall ß-glucans, which are not adsorbed in the digestive tract. This requires further work in both process engineering for the optimization of cell wall production and extraction preparation and genetic engineering for the intrinsic modification of the cell wall composition.

Figure 4. Computer-generated modelling of single ß1,3-glucan chains branched with three units of ß-1,6 glucans (adapted from reference[42]).

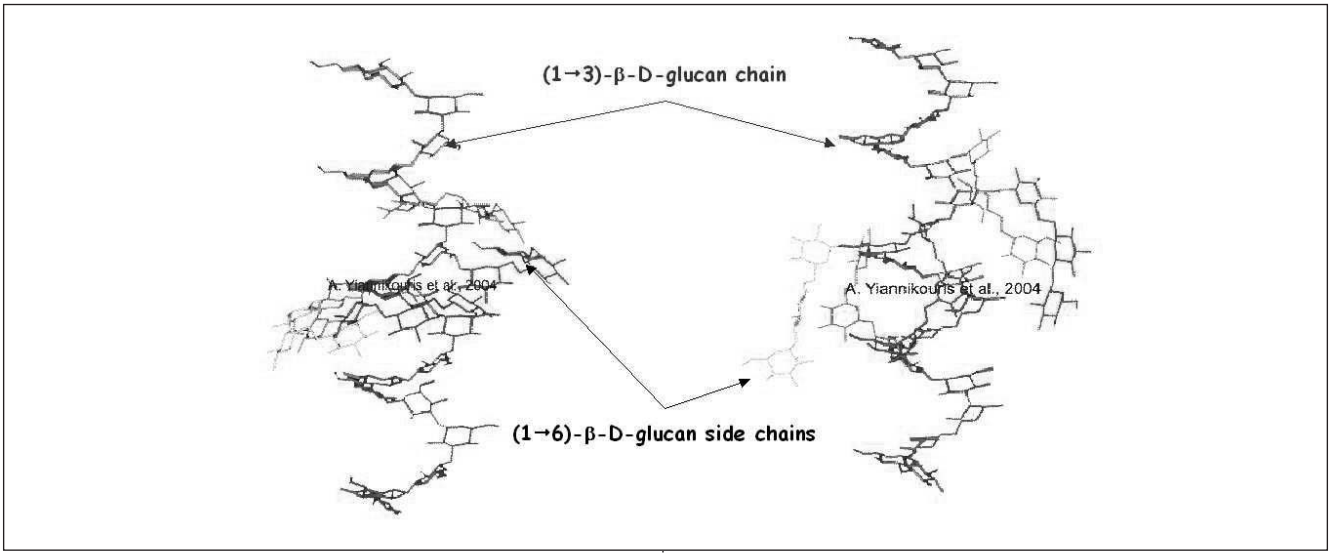
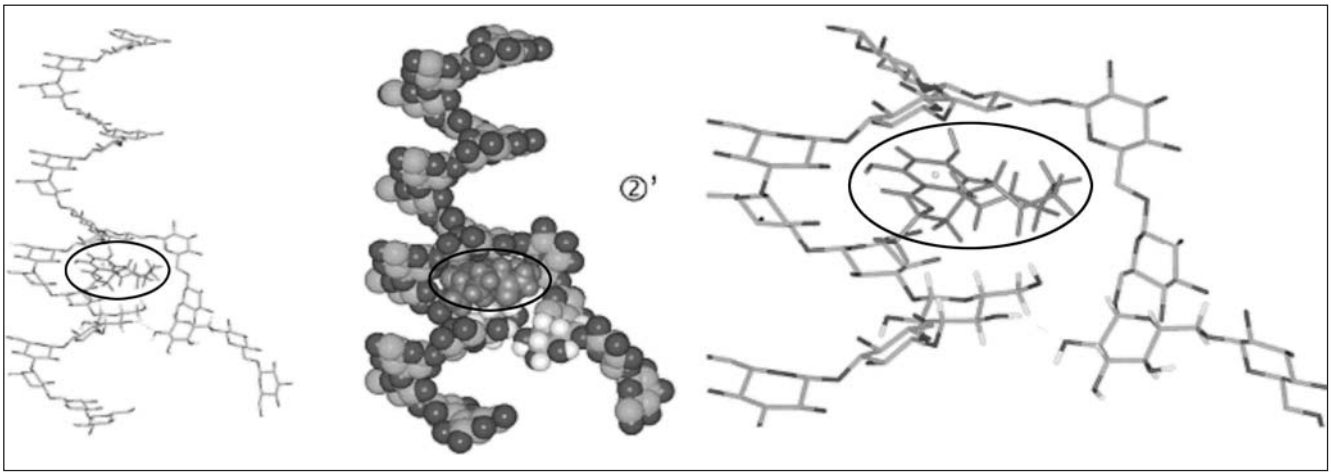


Figure 5. Computer-generated views of the energy-minimized structure of the docking of the most favourable conformation of ZEN (circled) into a single-helix ß-1,3-glucan chain branched with five ß-1,6-glucosyl linked residues (adapted from reference[42]).



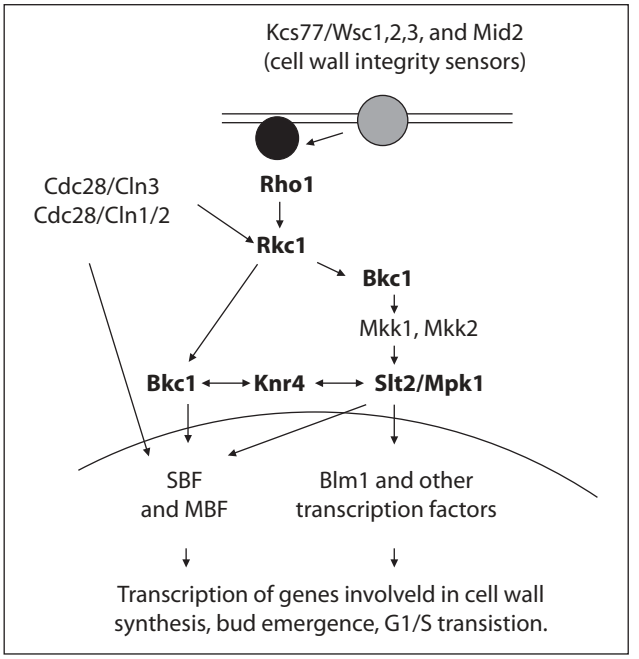
Part 2
Coordination of Cell Wall Integrity and Cell Growth

2.1 The Slt2-dependent cell wall integrity pathway

Cells sense and respond to environmental constraints via signalling pathways. Five signalling pathways based on a module of three protein kinases, highly conserved among eukaryotes and which culminate in the activation of a mitogen-activated protein kinase (MAPK), have been analyzed in great detail in the yeast *S. cerevisiae*.[44, 45] The MAPK cascade dependent on the protein kinase C encoded by *PKC1* is considered the main pathway controlling cell wall integrity (reviewed in reference[8] (Fig. 6). This signalling pathway consists of two branches that diverge downstream from the Pkc1. One of them is a linear pathway consisting of the sequentially activated protein kinases Bck1, the redundant Mkk1/Mkk2 and the Slt2/Mpk1 MAPK, which ultimately activates (by phosphorylation) transcription factors, including Rlm1 and SBF complex. The evidence that there is another branch arose from the finding that phenotypes of a *pkc1* null mutant are stronger than those of mutants downstream from the cascade. However, the components of this second branch have not been completely worked out (Figure 6).

The Pkc1-MAP kinase pathway is activated by cell wall stress, such as heat, hypo-osmotic conditions, mutations of cell wall structural genes, drugs that perturb cell walls (e.g., calcofluor white, Congo red, caffeine, SDS and zymolyase). This activation is transmitted through plasma membrane sensors, including Hcs77/Slg1/Wsc1, Wsc2, Wsc3, Wsc4 and Mid2. *MID2* and *WSC1* genes have the major in vivo role, as deletion of each of them results in *pkc1Δ*-like phenotypes, such as sorbitol-remediable cell lysis at high temperature, and high sensitivity to drugs that affect cell walls, while deletion of their homologues causes only minor phenotypes. Mid2 is believed to transmit a calcium signal of the pheromone response, as well as signals from cell wall perturbations, whereas the Wsc1 protein probably sends signals to the Pkc1 kinase resulting from membrane perturbations in response to hypo-osmotic and temperature shock. These sensors relay the signal to Rho1, an essential and highly conserved small GTP-binding protein that directly activates Pkc1. Meanwhile, Rho1 also controls cell wall synthesis by activating the. ß-1,3-glucan synthase, and by taking part in the actin

Figure 6. A model proposed for the function of Knr4 protein in the Slt2 MAP kinase pathway of *Saccharomyces cerevisiae*. The dual interaction of Knr4 with Bck2 and Slt2 is one of the elements by which the Pkc1 pathway coordinates cell wall integrity with cell growth. Solid arrows indicate activation, double arrowheads indicates protein interaction, and the dashed arrow is suggested interaction.



cytoskeleton organization during polarized growth.[46] The activation of the Pkc1-MAP kinase pathway by environmental stress, α-factor treatment, heat shock or cell wall perturbing molecules leads to the dual phosphorylation of the downstream Slt2 MAP kinase on Thr202 and Tyr204 residues. It is supposed that this dual phosphorylation results in an active Slt2 kinase.[47] This activated kinase in turn activates transcription factors, two of which are well known: Rlm1, which plays a key role in the activation of cell wall related genes, and SBF, which causes activation of G1-S regulated genes.

2.2. Knr4, a potential scaffold protein implicated in coordinating Slt2-dependent cell wall integrity and cellular growth

Cell proliferation and cell wall biogenesis must be coordinated to allow budding. The process of bud emergence and bud expansion involves the remodelling of the mother cell wall, as well as the synthesis and assembly of new cell wall components. Coherent with this picture, a periodic transcription has been reported for many cell wall genes with a maximum expression in the late G1/early S phase of the cell cycle, coincident with the early stages of budding. Cytokinesis, in turn, requires the expression of genes encoding specific enzymes related to the synthesis and degradation of cell wall components. Recent work from the Ohya group identified the existence of a checkpoint control that ensures coupling of cell wall synthesis with mitosis.[48]

Our laboratory has been involved for several years in studying the connection between cell wall assembly and cellular growth. The initial insertion in this field came from the molecular cloning of a gene originally called *SMI1* (for *s*uppressor of *m*atrix-association region *i*nhibition), and later *KNR4* (*k*iller *n*ine *r*esistant 4) as a suppressor of several calcofluor white hypersensitive mutants.[49] This gene was originally cloned by the complementation of mutants hyper resistant to the killer toxin HM-1 from *Hansenula mrakii*.[50] Interestingly, many of the phenotypes of a *knr4* null mutant were identical to those harboured by mutants of the Pkc1 pathway, which includes a hypersensitivity to caffeine, SDS, Congo red, calcofluor white, caspofungin and cercosporamide, and an arrest of growth with a small bud at temperatures above 37°C.[49, 51] This important result pointed to Knr4 as a potential component of the Pkc1-Slt2 pathway. However, this gene cannot be located in the linear signalling pathway as all mutations in *PKC1* to *SLT2* were synthetically lethal with deletion of *KNR*. As indicated in Figure 6, a first clue about the function of *KNR4* came from the finding that the Knr4 protein physically interacts with the MAP kinase Slt2, and is required for the strong increase of Slt2 kinase activity induced by heat shock. Remarkably, the signalling through the *PKC1* pathway that leads to the dual phosphorylation of Slt2 protein is not impaired by the loss of KNR4 function, but the lack of Knr4 prevents Slt2 kinase from efficiently phosphorylating some of its substrates. Moreover, in cells defective for *KNR4*, the transcriptional activity of Rlm1 is dramatically reduced, whereas the transcriptional activity of SBF is strongly increased, and the phosphorylated, and probably activated, form of Swi6 is more abundant. Thus, during vegetative growth, Knr4 seems to monitor the fine tuning of the output signals of the Pkc1-MAP kinase, acting as a switch to favour Rlm1, and hence the cell wall synthesis genes, versus SBF and cell cycle progression[52] (see Figure 6).

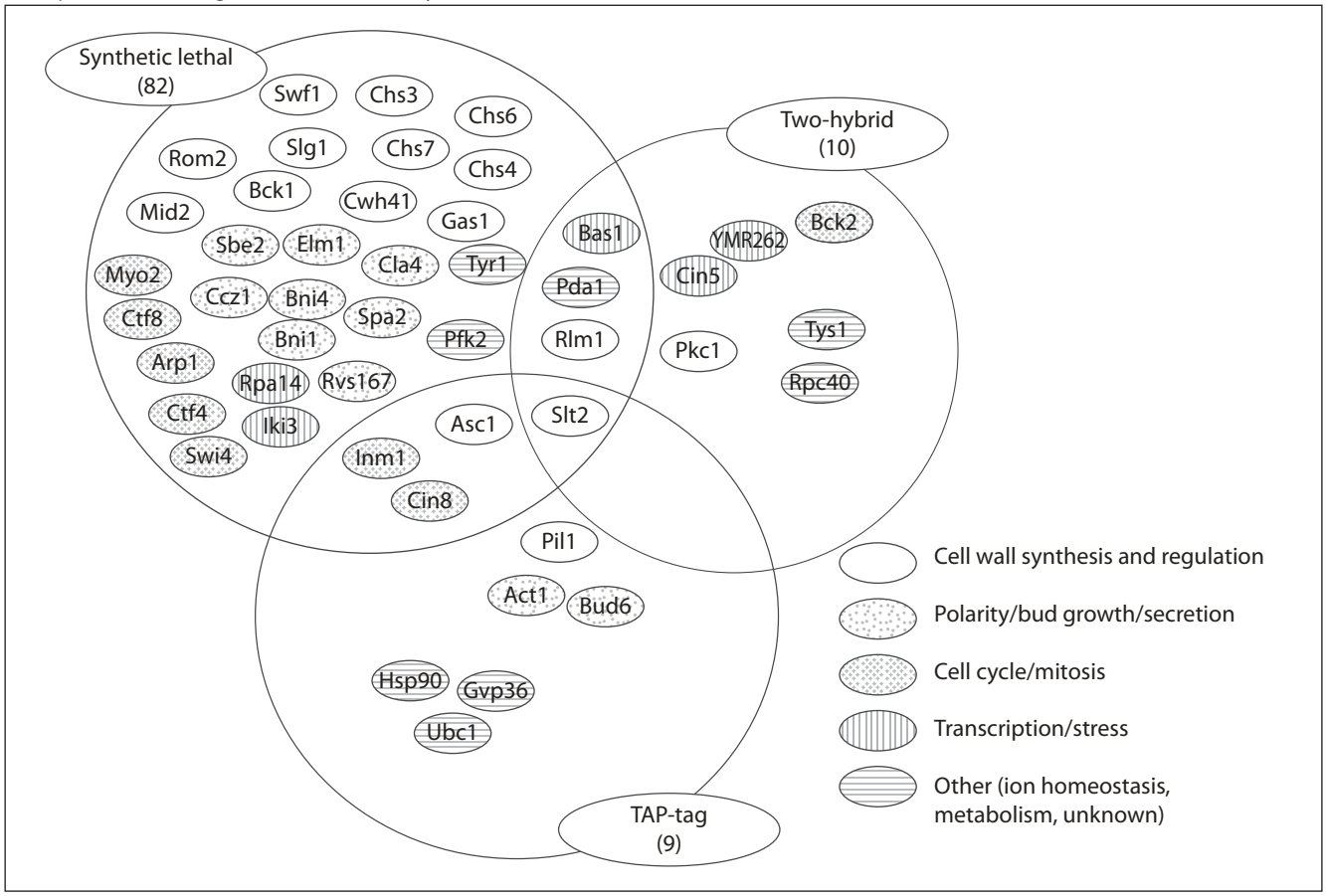
To further consolidate the role of Knr4 in controlling cell wall assembly with cell growth, we found a genetic interaction between *KNR4* and *BCK2* (Figure 6). *BCK2* was isolated in a genetic screen for suppressors of *pck1Δ* cell lysis.[53] Wijnen and Futcher[54] gave additional clues about the function of Bck2 protein in yeast. They reported that activation of SBF and MBF, two transcription factors required for regulation of G1 to S transition depends on both Bck2 and Cln3. However, unlike Cln3, Bck2 is capable of inducing its transcriptional targets in the absence of a functional Cdc28, and this activation is partially dependent on SBF- and MBF-binding sites. A synthetic lethality between *BCK2* and *CLN3* was shown, and in addition, the *bck2 pck1Δ* double mutant displays an extremely severe growth defect. These findings highlighted the idea that the Bck2 protein is in a parallel pathway to Pkc1 and led to the suggestion that Knr4 may be the linkage between these two pathways. Consistent with this hypothesis, we found that a double *knr4cln3* mutant was also a synthetic lethality, but this lethality could be rescued by the over-expression of *BCK2*, while the converse was not true (*i.e., bck2cln3* was not suppressed by high copy numbers of *KNR4*) and that Knr4 interacts weakly with Bck2 in a two-hybrid system. Nevertheless, a genome-wide analysis of cells over expressing *BCK2* or *KNR4* indicates that Bck2, when it is over-produced, can act independently of Knr4. These independent effects can account for the fact that over-expression of this gene, but not of *KNR4*, can rescue cell lysis of a *pck1Δ*.[55]

Knr4 is a protein 505 amino acids long that contains five putative PEST domains and several potential sites for protein phosphorylation. This protein is localized near the presumptive bud site in unbudded cells, and at the mother-bud neck in budded cells, which provided further evidence for a role of Knr4 in cell wall synthesis and probably in bud formation.[49] However, the burning question that remains is how this protein can exert its regulatory

function, some of which involve transcriptional effects, while residing in the cytosol. To answer this question, we searched for protein partners by setting up three different approaches.[56, 57] The first approach was to use the two-hybrid method in which Knr4 was the bait. Our work together with two other large-scale two-hybrid studies allowed us to isolate about 10 partners (Figure 7). Interestingly, the most potent interaction was found between the Knr4 and tyrosyl-tRNA synthetase encoded by TYS1. We suggested that this physical interaction could be required for dityrosine formation during the sporulation process. The other major interaction found in this screen was with Slt2/Mpk1. The second strategy was to search for synthetic lethality using single yeast deletants from the Euroscarf collection. However, while establishing the precept of this technique, another group published a large-scale synthetic genetic array (SGA) analysis for interactions with the *knr4* null mutation, as well as for many mutations of cell wall encoding genes. This large-scale genomic analysis expanded the network of *KNR4* interactions, implicating this gene in more than 80 synthetic interactions that were distributed in several cellular functions, including cell wall synthesis (the highest number of partners), chromosome segregation and polarized growth, signal transduction, transcription and spore formation[58, 61, 62] (see Figure 7). The third strategy was to identify the physical partner of Knr4 using an affinity chromatography technique called the TAP-tag strategy.[63] This method led to the identification of nine potential partners. As indicated in Figure 7, the overlap of these three strategies was rather poor, giving rise to only one common component identified by the three techniques. However, the sole common component was Slt2, which definitively ascertained the main function of Knr4 in the control of cell wall integrity. Although the three methods did not yield the same partner, they were convergent in highlighting the three major biological processes in which Knr4 is directly involved, namely cell wall maintenance, polarity/bud emergence and cell cycle/mitosis.[57]

Knr4 is one of the cell wall-related proteins exhibiting the largest numbers of partners. So how can this protein physically interact with so many proteins? For structural reasons, it is obvious that these interactions cannot occur at once, but they are distributed in space and time. In addition, we could not isolate a specific domain of interac-

Figure 7. Overlapping of data sets from synthetic lethal, two-hybrid and TAP-tag interaction screens. Out of the 82 synthetic lethal genes with *KNR4* deletion reported by reference [58], only those with known functions related to cell wall, morphogenesis, transcription and metabolism are reported in the diagram. Data from two-hybrid interaction were from references[55, 56, 59, 60].



tion, although some of the partners harbour a preferential interaction with the N-terminal domain of Knr4. Another peculiarity of Knr4 is to exhibit several features that characterize unfolded or intrinsically disordered proteins. This was not only predicted by *in silico* analysis of the protein sequence, but was supported by the aberrant mobility of this protein on gel electrophoresis under denaturating conditions. These results are interesting to place in a more general perspective because, contrary to general belief, the function of a protein is not necessarily dictated by its three-dimensional structure. There are an increasing number of proteins that lack intrinsic secondary structure, and they acquire a specific 3-D structure upon binding to partners.[64, 65] How exactly Knr4 operates in coordinating wall assembly with cell grow has not yet been elucidated, as the same role seems to be taken over by other proteins[66], which can explain why the loss of *KNR4* is not lethal. However, structure-function studies are underway to further characterize the mechanism by which Knr4 exerts its regulatory function. Moreover, the possibility of using this protein as a drug target is an open question, since the *KNR4* gene is present only in yeasts and moulds.

2.3. Molecular analysis of the cell wall compensatory mechanism

As yeast cells are living in environmental conditions that can weaken their walls, it is not surprising that they have developed some mechanisms for cell wall rearrangement to combat cell lysis. Consistent with this idea, cell wall damage induced by wall-perturbing drugs such as calcofluor white, caffeine, SDS or zymolyase, or by mutations in cell wall-related genes, are accompanied by dramatic changes in the composition and molecular structure of the cell wall.[3, 45, 67] Three major responses characterize the so-called cell wall salvage/compensatory system. First, the balance among the cell wall polysaccharides components is modified, with chitin content being the most affected as it can reach up to 20% of the cell wall mass. Secondly, the type of association among components is changed. For instance, lowering the amount of ß-1,6-glucan leads to a larger fraction of the cell wall proteins to become linked directly to ß1,3-glucan and chitin, consistent with increased levels of PIR proteins. A third response that ensures the strengthening of the cell wall is a transient redistribution of the cell wall synthesis and repair machinery that is normally focused on active growth regions all over the cell periphery.

As a major consequence of cell wall damage is a considerable increase in chitin, we investigated the molecular mechanism that accounted for this pronounced increase. We demonstrated that *GFA1*-encoding glutamine-fructose-6-phosphate amidotransferase (Gfa1), the first committed enzyme of the chitin biosynthesis pathway, plays a major role in this process. Using the terminology of the Metabolic Control Analysis[68], we showed that the reaction catalyzed by Gfa1 has a flux-coefficient control in the range of 0.90, indicating that the major control of the chitin metabolic pathway takes place at the level of this reaction. Moreover, our data established that the control of the chitin metabolic pathway is mainly hierarchical, i.e., dominated by a transcriptional control of *GFA1*.[69]

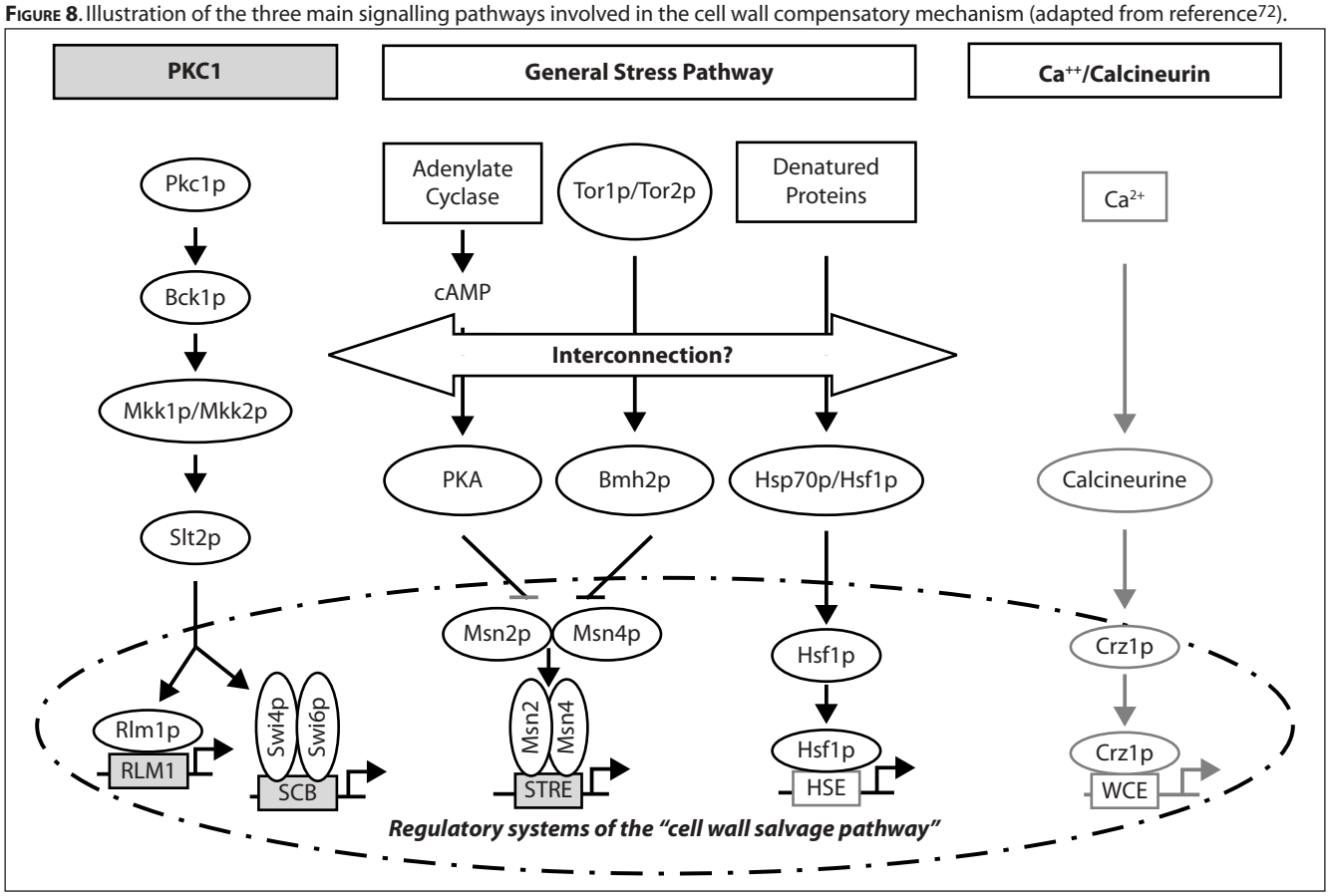
We then considered the use of DNA microarrays as the advised technology to decipher the molecular mechanisms underlying the cell wall compensatory mechanism. A first attempt was previously made by Jung and Levin[70] who created a permanent activation of the linear Pkc1-Slt2 MAP kinase cascade using a gain-of-function allele of MKK1 (MKKS^{368P}) that was placed under the galactose-inducible promoter. This work led to the identification of a collection of about 25 genes whose up-regulation was totally dependent on Rlm1. Another genome-wide study was carried out by comparing expression profiles of a *fks1Δ* mutant with its isogenic wild type.[71] This work also led to the identification of genes whose transcriptional changes were clearly dependent on the Pkc1-Slt2 kinase pathway. Interestingly, *SLT2* transcript was up-regulated, indicating a positive feedback loop of the phosphorylated Slt2 on its own expression. Both of these genome-wide analyses illustrate the role of the Pkc1-MAP kinase cascade in the cell wall repair mechanism, and show that the transcriptional response implicates Rlm1 as well as other transcriptional factors. Therefore, to enlarge this analysis, we conducted a genome-wide survey of gene-expression changes caused by five independent cell wall mutations, namely mutations in genes implicated in cell wall structure (*mnn9*, *fks1* and *kre6*) in the interconnection of cell wall components (*gas1*) and in the regulation of cell wall biosynthesis (*knr4*). Overall, roughly 300 genes were responsive, with transcriptional changes ranging from 1.4 to more than tenfold. The repartition of the differentially expressed genes into functional categories revealed an enrichment of genes that belong to energy metabolism and cell defence. A two-dimensional hierarchical clustering method identified a major group of about 80 genes that were up-regulated in the five cell wall mutants, among which roughly 30% have no annotated function, and less than 10% were known to be controlled by the *PCK1*-dependent cell integrity pathway. Using available software for promoter analysis, the over-represented DNA sequences were identified in the upstream non-coding regions of these genes. They correspond to binding sites of known transcriptional factors involved in the activation of cell wall genes (Rlm1) and in stress and heat shock

responses (Msn2/4 and Hsf1), in the cell cycle (Sok2), as well as in a novel putative 6-bp regulatory motif for 30% of the co-regulated genes. This motif turned out to be the binding site for the calcium-calcineurin-regulated Crz1 transcription factor. Taken together, our data led to the proposal that the cell wall compensatory mechanism, as triggered by cell wall mutations, integrates three major regulatory systems, namely the *PKC1-SLT2* MAP kinase-signalling module, the "global stress" response mediated by Msn2/4p, and the Ca^{++}/calcineurin-dependent pathway[72] (see Figure 8).

To decipher the relative importance and the connection between these regulatory systems in the cell wall compensatory mechanism, a complementary DNA microarray analysis was to investigate the response to transient cell wall damage as induced by the cell wall drugs Congo red and zymolyase. Treatment of the cells with both of these agents elicited the up-regulation of 132 genes and down-regulation of 101 genes respectively, with the main functional groups involved in cell wall construction and metabolism. Clustering these data with those from the response to constitutive cell wall damage revealed the existence of a cluster of co-regulated genes that was strongly induced under all conditions assayed. As reported in the previous study, the same regulatory systems were identified. However, with the exception of few genes, the transcriptional response to both of these cell wall drugs was totally eradicated upon deletion of *SLT2*, indicating that the cell wall damage compensatory response was most predominant under the dependence of the *PCK1-SLT2* MAP kinase-signalling module.[73]

However, other research indicated that the cell wall compensatory/repair mechanism cannot be restricted solely to these signalling pathways, because, for instance, the removal of the major GPI-anchored cell wall proteins (mutant ΔGPI) encoded by *CCW12*, *CCW13/DAN1*, *CCW14*, *TIP1* and *CPW1* does not trigger the activation of the Pkc1-dependent cell integrity pathway, although it does cause the strong up-regulation of two cell-surface proteins encoded by *SED1* and *SRL1* that seem to be necessary to reconstruct cell wall stability in the absence of multiple GPI-anchored mannoproteins.[74] Likewise, we recently found that yeast cells challenged with caffeine, a drug known to cause hyperphosphorylation of Slt2 kinase, can remodel cell walls in an Rlm1-independent pathway. This finding also highlighted, for the first time, a clear dissociation of the phosphorylation of Slt2 kinase and the activation of its downstream targets (Kuranda *et al.*, submitted to Molecular Microbiology).

Figure 8. Illustration of the three main signalling pathways involved in the cell wall compensatory mechanism (adapted from reference[72]).



To summarize, yeast cells are surrounded by a thin cell wall endowed with high biotechnological value. However, the wall is a highly dynamic organelle that constantly adapts to the environmental and developmental situation. Through genomic and transcriptomic approaches, we have just been able to identify the complex regulatory systems underlying this "dynamic flexibility." Whatever the regulatory mechanism, the most important consequence is a remodelling of the cell wall due to the activation of several enzymes that branch and cross link chitin with ß-glucan. At this time, the nature of the enzymes responsible for this structural remodelling is largely uncharacterized. Their identification will be a major research objectivein the near future.

3. Conclusions and Perspectives

A clear picture of the cell wall architecture is now emerging, thanks to recent large-scale genomic and transcriptomic analyses. This knowledge is very important as it will orient future research programs aimed at optimizing the biotechnological value of yeast cell walls (e.g., for pre- and probiotics) and identify new antifungal targets. However, the complex regulation of the synthesis of cell wall components and their assembly indicates that these objectives are extremely challenging. Coming back to the use of cell walls as a mycotoxin binder, this work has shown the prominent role of the ß-glucan matrix in this mechanism. However, the efficacy of the adsorption is dependent on a good compromise between linear ß-1,3-glucan chains with branched ß-1,6-glucosyl residues. Unfortunately, this ß-glucan structure represents a minor constituent of the yeast cell wall and it is relatively insoluble. Therefore, it will be an interesting challenge to increase the proportion of this structure in the yeast cell wall, while rendering it accessible to the toxins. Other biotechnological challenges could be in modifying content in mannoproteins for winemaking or reducing cell wall strength for easier breaking of the cells. To meet these challenges, we must strongly invest in the biochemical pathways and enzymatic reactions that take place at the cell surface and involve many yet uncharacterized cell wall remodelling enzymes harbouring glycosyl and glucanosyl transferase activity. Together with this enterprise, there will also be a need to identify the 3-D structure of the yeast cell wall, as has recently been accomplished for Gram-positive bacteria.[75]

Another more challenging strategy that may be successful for cell wall reshaping in accordance with biotechnological applications is through a Systems Biology approach. This new field of research aims at integrating omic data sets obtained from genomic and post-genomic technologies into biological networks to eventually model the cellular machinery *in silico*. Though there is still a long way to go to achieve this goal, it is now evident that molecular networks represent the backbone of molecular activity within the cell. However, the identification and analysis of these networks are still in their infancy, needing new strategies and theoretical frameworks to filter, interpret and organize the flood of information into a model of cellular function.[76] With respect to the cell wall network in yeast, there is already a huge body of interactomic data, rising from synthetic genetic array analysis (SGA) and large-scale two-hybrid systems.[77] While these interactional networks could be relevant for target identification in drug discovery programs, they are of limited use in terms of cell wall reshaping, because they are purely qualitative and not connected to the metabolic network. A stoichiometric model of the cell wall metabolic and regulatory network seems to be an urgent task to accomplish, although much kinetic and regulatory information is still missing. Use of metabolomic/ fluxomic analysis of cell wall synthesis in yeast under various growth conditions, and in cell wall mutants, could help in this metabolic network construction.

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OENOLOGICAL IMPACT OF MANNOPROTEINS AND PEPTIDES FROM YEAST: EXAMPLES OF INTERACTION WITH WINE POLYPHENOLS

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Introduction

Today, consumers have lost interest in some red wines which, without being of poor quality, are described as lacking in fruitiness and sweetness, with tannins that are too astringent and too bitter. Aging on lees appears to be a good technique to improve these wines, as it generally gives quite good results, with rounder and fleshier wines. It is well known that, during autolysis, the dead yeast cells in the lees release a high number of cellular constituents that have an influence on the organoleptic characters of wine.

In this presentation, we will focus on mannoproteins, and more specifically on their interaction with polyphenols, and on peptides, some of which bring sweetness while others, such as glutathion, lower the redox potential. We will also discuss the enrichment of wines with products originating from yeast that reinforce or accelerate the effects of autolysis.

Management, composition and evolution of lees

For white winemaking, the wine is kept on fresh lees that settle after the end of alcoholic fermentation. For red winemaking, the wine is left to decant for a period (from a few hours to a few days) after devatting, in order to keep mainly the fine lees, as the heavy lees are rich in vegetal fragments that might give herbaceous flavours (Table 1).

Table 1. Management of lees in winemaking.

- Destemming / Pressing
- Clarification (200-400 NTU)
- Alcoholic fermentation in tank / barrel
- Aging on total lees (stirring / batonnage)
- Racking after 6-8 months
- Back to the barrel for 4 to 12 months prior to bottling

or Back to the tank and bottling

- Destemming / Crushing
- Alcoholic fermentation / Maceration
- Decanting phase (hours to days)

Disposal of heavy lees

Wine in barrel + fine lees

Separation of fine lees

Wine in barrel+4% fine lees

- Aging on fine lees with/without batonnage
- Optional racking after 6 to 8 months

Of course, the composition of the lees varies according to the method of vinification. Table 2 (Renouil and Ferret 1988) gives the average composition of the dry residue obtained through the desiccation of fresh lees. Desiccated lees represent about 25% of the weight of fresh lees. The biomass is composed of yeast and bacteria from malolactic fermentation, but their weight is negligible.

Table 2. Composition of fresh lees. (Renouil and Ferret 1988)

- 25% to 35% tartaric crystals
- 35% to 45% microorganisms (yeast and bacteria)
- 30% to 40% minerals (iron, copper) and organic impurities (proteins, pectins)

The biomass is found essentially in the fine lees whose composition strongly depends on the time spent decanting the wine after devatting. The longer the decanting, the richer the lees are in nitrogen compounds and polysaccharides. For instance, after 72 hours of decanting, the total nitrogen content of lees rises from 15 to about 25 mg N/g of dry weight and the polysaccharide content goes from about 350 to 500 mg glucose/g of dry weight.

The dry weight of the lees decreases throughout aging. In Champagne, for example, with wine kept with the deposit of lees that ensured the bottle fermentation a continuous decrease of the dry weight of this deposit was observed by Leroy et al. (1990), reaching about 50% after 25 months of contact. This degradation of lees corresponds to yeast autolysis.

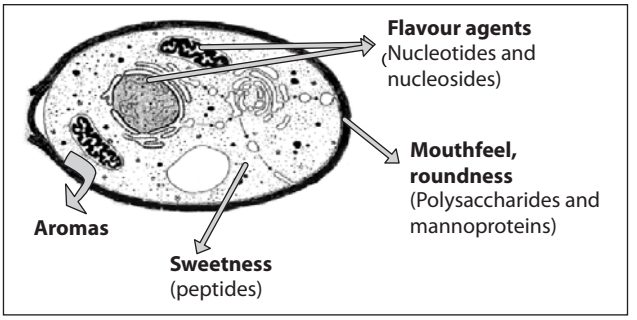
Yeast autolysis and oenological interest in the products released: a reminder

Yeast autolysis is an enzymatic self-degradation of cellular constituents beginning when the yeast cell has completed its life cycle and has entered the death phase. In winemaking, yeast autolysis occurs during the storage of wine on lees, as in the case of Burgundy wines, with or without periodic stirring, and sparkling wines. However, wines impose particular conditions on the autolytic process, namely low pH (3.0 to 3.5) and low temperature (often under 15°C). These conditions are very different from the optimal conditions of autolysis (pH 5.0, temperature 45°C), but the length of the aging time on lees (several months or years) compensates for the decrease in the autolytic process (Feuillat 1998).

Autolysis compounds are released into the exocellular environment. Figure 1 shows the main autolytic products released from different parts of the cell, including nucleotides, fatty acids, amino acids, peptides, polysaccharides and glycoproteins, as well as their sensory properties.

The oenological role of autolytic products, especially mannoproteins, is important for wine flavour and stability.

Figure 1. Origin and organoleptic properties of yeast compounds released during aging. (Charpentier and Feuillat)

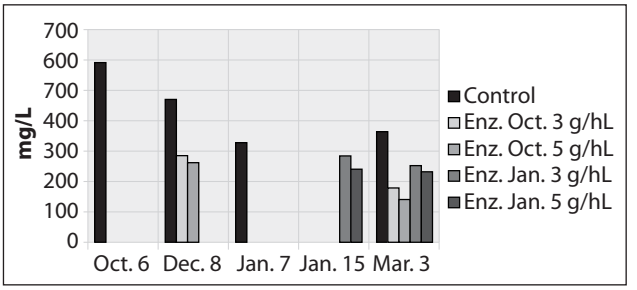


Yeast mannoproteins are produced by yeast during the fermentation and storage of wines. Their release is due to ß-1,3 glucanase activity. This activity present in biomass goes into supernatant and hydrolysis glucans. The glucose/mannose ratio in supernatant increases during cell wall autolysis.

Various parameters of wine aging on lees, such as stirring and the addition of exogenous ß-glucanase, can modify the quantities of mannoproteins. Stirring with or without oxygenation induces an increase in the quantity of mannoproteins released during the aging of wine on lees. This increase is approximately 30 mg/L of mannoproteins after six months (Feuillat, 1998). The effect of stirring is probably due to the increase of wine contact with lees, facilitating the diffusion of yeast cell wall mannoproteins into the wine.

Many trials of the exogenous ß-glucanase addition have shown that, after a few months of aging, wine systematically contains fewer total polysaccharides (Figure 2).

Figure 2 : Influence of glucanase addition on total polysaccharides content of wine



In spite of the higher release of glucans and mannoproteins caused by the addition of ß-glucanase, as shown in a synthetic medium by Vuchot (2001), one can suppose that the hydrolysis of glucans released in wine is itself more important. Moreover, industrial preparations of ß-glucanase may contain secondary activities of the pectinase type degrading the polysaccharides of the grape. Ex-

ogenous ß-glucanase can be associated with inactivated yeast, as shown in the following trials.

Interactions of yeast macromolecules with phenolic compounds

Wine polysaccharides are known as protective colloids of phenolic compounds. The colour is more stable and tannin aggregation is lower.

- **Influence of different additives on wine composition, colour and the quality of tannins**

Some trials with inactivated yeast associated with exogenous ß-glucanase were carried out and compared with other additives that could influence the colour and the quality of tannins. Table 3 shows the results of a trial with a control wine fermented by the BM 45 strain and compared to the same wine with the addition of:
- Inactivated yeast and glucanase (30 g/hL)
- Glucanase alone (5 g/hL)
- Tannins (10 g/hL).

Table 3. Influence of different additives on the composition, colour and the quality of tannins (4-5 months aging). (Feuillat et al.)

	Control BM 45	Inactive yeats + glucanase (30 g/hL $^{-1}$)	Glucanase (5 g/hL $^{-1}$)	Tannins (10 g/hL $^{-1}$)
Total nitrogen (mg/L $^{-1}$)	301	322	280	280
Polysaccharides (mg/L $^{-1}$)	710	740	625	695
Colour intensity ($A_{420}+A_{520}+A_{620}$)	30.5	32.3	33.3	34.9
Tint ($A_{420}+A_{520}$)	0.75	0.76	0.74	0.76
Gelatine index (%)	32	25	38	40
mDP	13.1	8.8	10.7	11.3

Total polysaccharides are higher with inactivated yeast and glucanase, and lower with glucanase alone. The colour intensity is higher with the three additives. The tint was the same for all samples. The gelatine index and the degree of polymerization (mDP) are lower with inactivated yeast and glucanase than with glucanase alone. These last results concur with sensory analysis (Table 4).

Table 4. Sensory analysis of wines obtained with different yeast strains and different additives (triangle test). (Feuillat et al.)

Yeast	BM 45	QA 23	BRG
Control / inactive yeast	5%	1%	ns
Control / glucanase	1%	0.1%	ns
Control / tannins	ns	ns	
Inactive yeast / glucanase	5%	2%	

Samples with inactivated yeast or with glucanase are always identified as different from control. Glucanase sample is more astringent than control and inactive yeast sample.

The experiment was carried out with three different yeast strains: BM 45, QA 23 and BRG. A triangle test compared control/inactivated yeast and glucanase, control/glucanase and control/tannins. The results are significant for BM 45 and QA 23, but not for the BRG strain and tannins.

The comparison of inactivated yeast and glucanase, and glucanase alone is significant. Glucanase alone gives more astringent wines (Feuillat et al., to be published).

- **Tannin aggregation**

Astringency decreases thanks to less tannin aggregation. Riou et al. (2002) studied the ability of different wine polysaccharides to interfere with tannin aggregation. Aggregates were detected and sized by dynamic light scattering (DLS) measurement.

The main results are shown in Table 5. Rhamnogalacturonans (RG), and more precisely RGIId, could enhance the aggregation of seed tannins from 11.3 mDP. Arabinogalactans (AG) did not have any impact. Mannoproteins (MP) can prevent aggregation.

Table 5. Influence of grapes and yeast polysaccharides on wine tannin aggregation. This study was done by particle measurement using dynamic light scattering (DLS). (Riou et al. 2002)

RG: Could enhance aggregation
AG: Have no impact on aggregation, even in high concentration
MP: Can prevent aggregation

Escot (2003) and Charpentier et al. (2004) have shown by spectrometry (700 nm) that the influence of glycosylated yeast proteins on tannin aggregation varies according to the strain. In the presence of BM 45, released during alcoholic fermentation, tannin stabilization is twice as high as in the presence of glycosylated proteins from the yeast RC212. The polysaccharidic part of the glycosylated proteins could be important in tannin aggregation. The main composition difference between RC212 and BM 45 glycosylated proteins is the mannose/glucose ratio. This ratio is near 1 for BM 45 glycosylated proteins, and near 17 for RC212 glycosylated proteins. Polysaccharide adsorption of particles formed by tannins, preventing particle growth, is probably the best hypothesis.

Origin and roles of yeast peptides

At the end of alcoholic fermentation and during aging on lees, peptides are released as oligopeptides (Perrot et al. 2002). Wines are 10 to 15 times richer in peptides than the corresponding musts (Carnevillier et al. 1999). Two properties of peptides are important:
- The sensory properties
- The antioxidant properties (glutathione).

• **Sensory properties**

Several peptides that were characterized in Champagne (Desportes et al., 2001) and whose amino acid sequence was determined were tasted at 5 mg/L in water (Table 6).

Table 6. Flavour of some synthetic peptides characterized in wine. (Desportes et al. 2001)

Amino acid composition	Flavour in water (5 mg/L)
Ile-Arg	Tasteless
Tyr-Lys	Lightly sweet
Ile-Val, Val-Ile, Phe-Arg-Arg	Bitter
Ser-LyT-Ser-Pro-Tyr	Lightly bitter
Phe-Lys	Sour
Lys-Met-Asn	Umami

Four peptides were described as bitter: Ile-Val, Val-Ile, Phe-Arg-Arg, Ser-Lys-thi-Ser-Pro-Tyr. All the peptides contain hydrophobic amino acids, such as Val and Ile, or hydrophobic residues, such as Arg, Pro, Tyr.

The tripeptide Lys-Met-Asn was described as umami, probably because it contains the Asn residue. Only one peptide, Tyr-Lys was descibed as lightly sweet.

Peptide concentrations in wine are certainly low, lower than their detection thresholds. However, interactions of peptides with other wine compounds and the synergetic effects probably occur.

• **Glutathione**

Among wine peptides, glutathione was recently studied for its ability to protect wine from oxidation. Dubourdieu and Lavigne (2005) have shown that glutathione decreases in musts during most of the alcoholic fermentation, then increases at the end of this fermentation, and more and more at the beginning of autolysis, to reach about 10 to 12 mg/L.

Vivas et al. (2005) added glutathione (20 mg/L) to a red wine. After 60 days of storage, absorbance at 420 nm (yellow colour) is lower (Table 7). The colour tint (450/520) and the colour intensity (420+520+620) are lower. These characteristics of wine colour correspond to slower oxidation. The lower absorbance, at 620 nm (blue colour), would indicate smaller quantities of anthocyanin-tannin combinations by ethanal, which is an oxidation process.

Table 7. Influence of glutathione on wine colour. (Vivas et al. 2005)

	Control			With glutathione (20 mg/L)		
Days	0	30	60	0	30	60
Abs. 420 nm	0.251	0.316	0.388	0.251	0.260	0.274
Abs. 520 nm	0.315	0.367	0.372	0.315	0.320	0.352
Abs. 620 nm	0.093	0.142	0.182	0.093	0.101	0.126
Colour tint 420 nm/ 520 nm	0.79	0.86	1.04	0.79	0.79	0.77
Colour intensity	0.659	0.825	0.942	0.659	0.689	0.752
% abs. 620 nm	14.1	17.2	19.0	14.1	14.6	16.7

Conclusion

Autolysis plays an important role in winemaking. Released mannoproteins and peptides result in wines with more suppleness, more flavour and less oxidation. But the autolytic process is very slow in wine.

Alternative ways to increase mannoprotein and peptide concentrations more quickly could be considered, such as:

- The selection of yeast strains able to produce high quantities of mannoproteins during alcoholic fermentation or able to autolyse quickly at the end of it
- The shortening of aging on lees, either by adding exogenous glucanase or, preferably, a mixture of inactivated yeast with glucanase
- The peptide enrichment of wine by adding yeast autolysates to increase flavour and glutathione, or better still, by adding yeast with a high glutathione content to protect phenolic compounds.

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YEAST WALLS: A PROMISING FUTURE?

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Abstract

Yeast derivatives, such as yeast hulls (yeast ghosts), are largely employed in the wine industry. The usefulness of yeast hulls in oenology was first demonstrated by Lafon-Lafourcade et al. (1984). The action of the hulls was postulated to be due to removal of toxic fermentation by-products, i.e., the medium-chain fatty acids (hexanoic, octanoic and decanoic acids). Since then, it has been reported that yeast cell walls from either whole cells or yeast hulls are able to adsorb different kinds of constituents, such as esters, volatile phenols, fungicide, micotoxin, thiols, etc.

It is clear from these different studies that yeast cell walls are of considerable technological importance because of their role in the hygienic and organoleptic preservation of wines. The absorption capacity of yeast is dependent on interfacial processes and thus on cell surface physicochemical properties and chemical composition. The relationships between chemical composition and the physicochemical properties of yeast walls have received little attention. However, research carried out in our institute and reported by other groups has shown that the different polarities and the hydrophilic or hydrophobic nature of the cell wall define the capacity of yeast to retain or adsorb different wine molecules.

Yet the physicochemical properties of the yeast surface depend on a number of different parameters that will be presented. The surface properties of yeast cell walls are also strain-, species- and genus-dependent. Consequently, cell walls isolated from yeast belonging to other genus than *Saccharomyces* may be beneficial to improve absorption ability versus different compounds.

Introduction

Yeast cell walls possess different functions, including the stabilization of internal osmotic conditions, protection against physical stress, maintenance of the cell shape, and as a scaffold for proteins. These are considered to be collective functions, but such specific functions as flocculation, mating and biofilm formation also exist (Klis et al., 2006). These specific functions are often controlled by glycoproteins. The composition of the protein layer depends on growth conditions (Aguilar-Uscanga and François, 2003). In anaerobic conditions, when the oxygen level decreases, some cell wall proteins decrease, whereas the transcript level of others, like *DAN1*, *DAN2*, *DAN3*, *TIR1*, *TIR2* are strongly up-regulated (Abramova et al. 2001). Growth at low pH leads to various adaptations in cell wall composition. In such conditions, cell walls are more resistant to glucanases (Kapteyn et al., 2001). Changes in cell wall composition are expected to induce modification of the cell surface properties. Indeed, it has been shown for *Lactobacillus acidophilus* that, depending on medium composition, changes in cell wall composition correlate with changes in surface properties (Schär-Zammaretti et al., 2005).

Yet, the surface properties of yeast cells seem to have potential interest in oenology. We will discuss some examples of interest and look at future perspectives.

Yeast cell wall utilization in the wine industry

The presence of medium-chain fatty acids during alcoholic fermentation could result in stuck or sluggish fermentation. This phenomenon can be prevented by the addition of yeast ghosts or yeast hulls, which correspond to the co-product of yeast extract. The action of the hulls was postulated to be due to the physical adsorption of medium-chain fatty acids (Lafon-Lafourcade et al., 1984). Cell walls are able to adsorb up to 60% decanoic acid (Alexandre et al., 1997). In fact, yeast hulls also alleviate sluggish fermentation because they favour CO_2 release and enrich the medium with sterols and lipids (Munoz and Ingledew, 1989).

Other potential interests regarding yeast cell walls

The case of aroma compounds

The capacity of the yeast cell wall to bind volatile compounds was investigated in a model wine solution (Lubbers et al. 1994). This study demonstrated that the effect of cell walls on the volatility of aroma depended on the physicochemical nature of volatile compounds. Thus the level of binding increased with the hydrophobic nature of the aroma. Although lipid-free yeast walls bound fewer volatile compounds, the binding capacity of yeast walls is not due only to lipid matter.

The case of volatile phenols

The phenols 4-ethylphenol and 4-ethylguaiacol are volatile phenols produced by *Brettanomyces*. These compounds in wine are associated with animal, leather and horse sweat aromas. We have shown that red wine containing yeast lees has lower volatile phenol levels compared to the same wine aged without lees (Guilloux-Benatier et al., 2001). This could be explained by the ability of yeast cell walls to sorb volatile phenols (Chassagne et al., 2005).

The case of phenolic compounds

In the Champagne region there is a marginal but traditional practice based on the use of lees after alcoholic fermentation to discolour the must or wine. Vasserot et al. (1997) have shown that the reducing colour action is due to adsorption of anthocyanins on yeast walls. In this study, yeast ghosts showed a better adsorption capacity than yeast lees, which could be related to a larger adsorption surface.

A similar strategy has been proposed to decrease the colour of white wines (Razmkhab et al., 2002). The addition of dehydrated yeast to brown wines decreases the degree of browning. According to these authors, whole cells were more efficient than cell walls. The adsorption ability of yeast versus phenolic compounds has been confirmed in other studies. Morata et al. (2003) suggest that adsorption involves hydrophobic interactions. Indeed, acyl-derivatives are more strongly absorbed than non-acyl derivatives. On the other hand, adsorption of anthocyanins by yeast lees has been reported to be not related to their polarity (Mazauric and Salmon, 2005). These discrepancies might be due to differences in the physiological status of the yeast used.

What affects the adsorption phenomena of yeast walls?

Physicochemical parameters of the environment

Physical parameters could act either on the adsorption mechanism or by modifying the cell surface, or both. Classic oenological parameters such as temperature, ethanol, pH and SO_2 have been shown to alter adsorption of anthocyanins (Vasserot et al., 1997). Low temperature, for example, is more effective for anthocyanin adsorption, while the amount of anthocyanin adsorbed decreases with increasing ethanol concentration.

Treatment of yeast by heating or acidity enhances ochratoxin removal (Bejaoui et al., 2004). Heating might induce protein denaturation or Maillard reaction products. Acidic treatment could affect polysaccharides by releasing monomers. These released products could offer more adsorption sites.

Composition of the cell wall

Many studies suggest that the yeast cell wall composition determines adsorption capacity. In the concept of biosorption, several physical and/or chemical adsorption processes may be involved, such as physical and/or chemical adsorption, ion exchange, coordination, complexation, chelation and microprecipitation.

The nature of the process will depend in part on the available functional groups of the yeast cell wall. Among the functional groups we find carboxyl, hydroxyl, phosphate, sulphate and amino groups. All these groups allow different adsorption mechanisms, such as hydrogen bonding, ionic bonding and hydrophobic bonding. In addition, these functional groups often have ion exchange properties. Thus, the nature and the number of functional groups will affect the extent of adsorption capacity.

The nature and the number of functional groups depend in part on cell wall composition and porosity, which are both modified according to the growth medium, the yeast growth phase and the genus and species of yeast. Remodelling of the yeast cell wall has been observed during the

shift from logarithm phase to stationary phase, when the nature of the carbon source changes, during nitrogen limitation, during pH, temperature, aeration or ethanol concentration changes (Klis et al., 2006; Aguilar-Uscanga and François, 2003; Parascandola et al., 1997).

We have shown that cell wall porosity is influenced by the physiological state of the yeast and the growth medium (Boivin et al., 1997). An increased surface area provided by intersticial space might favour adsorption capacity. Comparison of the cell surface hydrophobicity between forming flor yeast and non-forming flor yeast gives an example of the potential of different yeast species. Indeed, we have shown that the cell surface hydrophobicity of velum-forming yeast was much higher than the cell surface hydrophobicity of classic Saccharomyces (Alexandre et al., 1998). These differences could be explained by the cell wall composition, which is different between these types of yeast (Alexandre et al., 2000). High cell surface hydrophobicity is conferred by the presence of specific mannoproteins, such as Hsp12 (Zara et al., 2002), Flo11 (Zara et al., 2005) and Awa1p (Miyashita et al., 2004).

Conclusion

Most of the studies conducted on yeast cell walls and their adsorption capacity have been conducted either in synthetic mediums or with pure compounds. The aim of these studies was not to optimize the adsorption capacity. However, it is clear from all this research that adsorption capacity versus a specific compound could be improved in different ways. Medium composition, harvest growth phase, cell surface treatment and yeast or cell wall processing are potential steps by which cell wall composition could be altered and consequently might allow semi-specific biosorbents to be obtained for multiple purposes in winemaking.

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MANNOPROTEINS AND AROMATIC COMPOUNDS IN WINE

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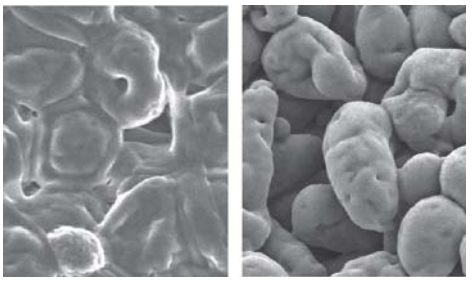
In 2003, the Institut Coopératif du Vin (ICV) conducted a study in collaboration with the INRA of Montpellier (T. Doco) and the Université de Montpellier (P.Chalier) to measure the interactions between the mannoproteins released by yeast and the aroma compounds produced or released during alcoholic fermentation and aging on lees.

Mannoproteins belong to a group of polysaccharides in the yeast and bacteria cell walls. They are cited in the literature as participating in the stabilization of wine and stimulating the bacterial flora. They are liberated into wine through yeast autolysis. The kinetics of yeast autolysis are dependent mainly on:

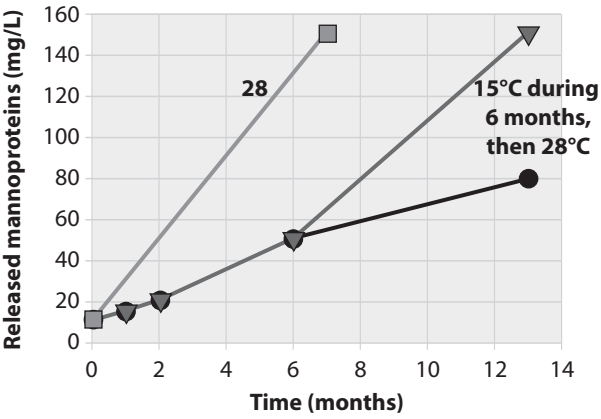
- The presence of polyphenols in wine (Figure 1)
- The stirring of lees
- Temperature (Figure 2).

Figure 1. Yeast cells after autolysis with and without polyphenols.



Autolysis without polyphenols | Autolysis with polyphenols

Figure 2. Mannoproteins released over time at different temperatures.



The study was aimed at harvesting the mannoproteins of two ICV yeast strains (ICV D21® and ICV D80®) that fermented on a synthetic must, and to measure their interactions with the classic aroma compounds found in wine via analytical and sensory methods.

The main results are the following:

- Analytical tools available today cannot measure significant chemical or structural differences between the mannoproteins produced by the two yeast strains.
- Nonetheless, the retention capacity of the aromatic compounds is different for the two yeast strains. The retention capacity is measured by a percentage. The higher the percentage, the more the mannoprotein fraction retains the molecule and its aromatic perception is more stable in time because the volatility is lower (Figure 3).

- Fractions of different molecular weights have different retention capacities for aromatic compounds, which is proof of the complex interactions between mannoproteins and aromatic compounds. Aromatic stabilization is therefore dependent on the quantity released during the autolysis but also on the quality of mannoproteins released. This conclusion leads to a second one: the choice of yeast for alcoholic fermentation also has an impact on the aromatic stabilization of the wine (Figure 4).
- The sensory analysis done by an expert trained panel confirms the phenomenon of retention by mannoproteins as shown on the graph below (Figure 5).

Figure 3. Retention of aroma compounds by two different yeast strains.

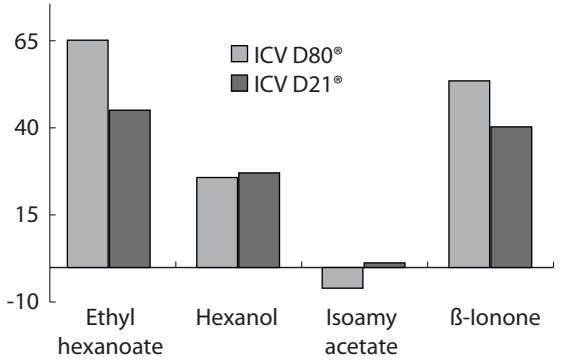


Figure 4. Retention capacity of the the different mannoprotein fractions of ICV D21®.

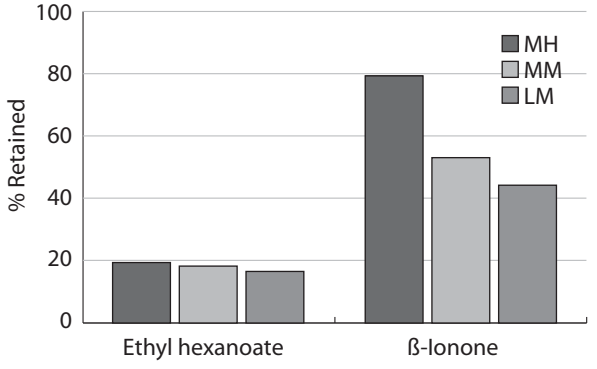
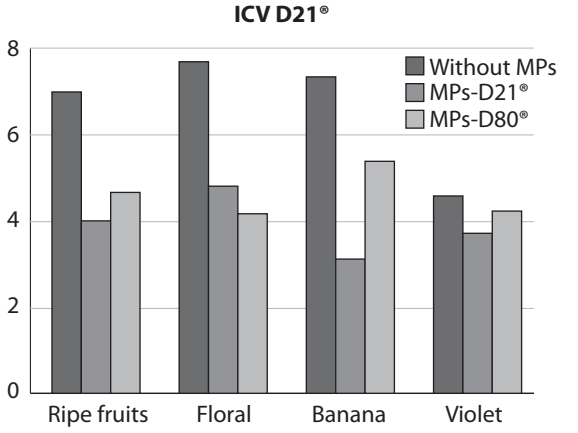


Figure 5. Sensory results of wine containing mannoproteins from different yeast strains compared to the control without mannoproteins.



THE MANAGEMENT OF LEES

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The Institut Coopératif du Vin has been working for the past 15 years to help winemakers manage the aging of wines, especially through good management of the lees.

An operational definition consists of calling "heavy lees" all the elements that settle in the container during the 24 hours following wine movement (stirring, pumping, bâtonnage, etc.). The "fine lees" are all the elements that remain in suspension. Heavy lees are composed mainly of tartaric acid crystals, colouring matter conglomerates, vegetal cellular fractions and dead yeast cells fixing the colour. Their presence will render the pH and the colour of the wine unstable, significantly increasing the risk of sulphur off-flavours and vegetal aromas. Good management consists of removing them as soon as they are formed, at a pace that decreases during aging or storage. The empirical practice was invented years before microscopic examination was used by oenologists.

Fine lees are composed principally of yeast. The autolysis of this yeast releases compounds that are interesting from a sensory point of view. The speed and level of autolysis are dependent on the temperature and wine movements. Those movements also avoid compaction of lees, which generates negative sulphur off-flavours. The facility with which the heavy lees are eliminated and the interest of the compounds liberated by the fine lees are dependent on the maturity and the sanitary state of the berries, the choice of maceration enzyme, the extraction technique used, the choice of yeast strain and the optimization of the yeast population by good alcoholic fermentation practices, and the choice of malolactic bacteria, in addition to the movement and temperature of the wine during aging.

Barrels are often where contaminating microorganisms will develop, and so alternatives to classic aging on lees were studied, using the addition of ADY or white wine lees. In the first instance, after five to six months the woody notes are more integrated, the original fruitiness more present and the balance has more foremouth volume with tannins that are less astringent. In the second instance, the dosage is particularly important: 1-2% appears to be the maximum. Soft sweet notes develop and the volume and mouthfeel are increased.

Aging techniques of fine lees separated from the wine are interesting if some rules are followed: tasting the lees to eliminate those with sulphur off-flavours, strong acidification and the addition of SO_2, early removal of the supernatant, bâtonnage and regular tastings, and testing before adding to the wine (use various dosages and measure the effect after four to six days of contact).

EFFECTS OF INACTIVATED DRY YEAST IN FERMENTATION

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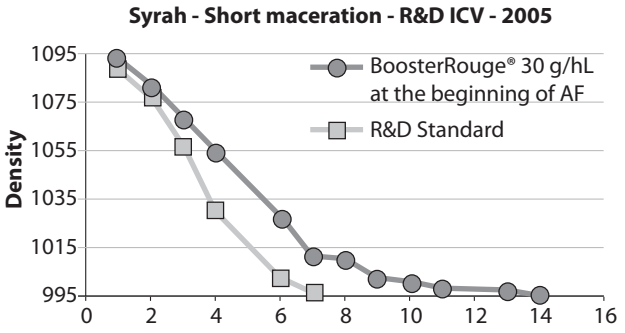
Products based on inactivated dry yeast (IDY) of the ICV range (Booster®) were tested in 2005 on several grapes or musts selected to produce premium wines, with or without FermaidE®. Chardonnay and Sauvignon varietals with direct pressing on musts clarified at less than 50 NTU, fermented with D47®; Grenache Noir in rosé (pre-fermentation identical to the whites), fermented with GRE®; and Merlot, Syrah and Grenache Noir in short maceration, fermented with GRE®. These six different trials were produced by the ICV R&D Department in our experimental winery.

Our first result (Figure 1) is clearly important for alcoholic fermentation management: Booster® additions alone have no effect on fermentation kinetics and are no alternative to reliable products such as FermaidE® in nitrogen deficiency situations.

Figure 1. Fermentation of Syrah with BoosterRouge®.



The dosage effect is clear: the sensory effect is perceivable only at above 30 g/hL, even with BoosterBlanc® in reductive-type processes. There is no technical or organoleptic interest in reducing the doses.

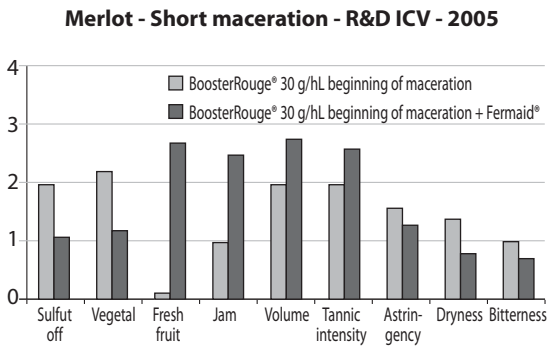
With the correct dose, the effects observed are:

- Increased fruity aromas
- Reduced vegetal notes
- Increased volume perception
- In reds, increased the tannic intensity in the middle palate
- Reduced bitterness
- Positive synergy with FermaidE®. In the absence of nitrogen compounds, sulphur off-flavours develop and flavours are more aggressive (astringency, dryness, bitterness).

In red wines, the fruity notes can be less intense when compared to the control, but the fruit is fresher and less vegetal, and the astringency is more pronounced, but with more volume and less bitterness.

The last interesting result (Figure 2) is illustrated with the organoleptic profile of a Merlot fermented with BoosterRouge®, with or without Fermaid®:

Figure 2. Organoleptic profile of Merlot with the addition of BoosterRouge® with and without Fermaid®



A deficit in yeast assimilable nitrogen results in a loss of most of the positive impacts of inactivated dry yeast additions, with more sulphur off-flavours and vegetal aromas, more astringency and dryness, less fruit and foremouth volume.

Booster® products do not transform average grapes into premium ones. They will however balance and stabilize the wine with aromatic and flavour notes better adapted to today's consumers of premium wines.

Cover Design: Bruno Loste – Layout and Printing: MODULI INC.

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NEW TRENDS IN THE USE OF SPECIALTY INACTIVATED YEAST DURING WINEMAKING

SOUTH AFRICA, APRIL 30, 2006

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