Biokemijska i fizikalna analiza stanične stijenke kvasca

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Biochemical and biophysical studies of cell wall of industrial yeasts

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Sažetak: Cilj ovog rada je bio odrediti udio komponenti stanične stijenke kvasca *Saccharomyces cerevisiae*: manoproteina, β-1,3-glukana, β-1,6 glukana i hitina, koristeći novo razvijenu metodu koja se temelji na enzimskim i kemijskim metodama. Ova metoda je testirana na različitim sojevima kvasca i došlo se do zaključka da uvjeti rasta imaju utjecaj na udio staničnih komponenti. Metoda je korištena za procjenu sastava stanične stijenke industrijskih sojeva (autolizirani i osušeni sojevi) koji su bili pod utjecajem različitih fermentacijskih i drugih procesa. Korišteni su i fenotipski testovi da se uoči i potvrdi da rast različitih sojeva ovisi o sastavu hranjive podloge. U drugom dijelu rada je promatran utjecaj etanol stresa na rast te na udio komponenti stanične stijenke različitih laboratorijskih sojeva kvasca.

Ključne riječi: etanol, hitin, *Saccharomyces cerevisae*, stanična stijenka, β-glukan

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Biochemical and biophysics studies of cell wall of industrial yeasts

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Abstract: The aim of this study was to quantify the amount of specific components in the cell wall of yeast *Saccharomyces cerevisiae*, namely mannoproteins, β-1,3-glucans and β-1,6-glucans and chitin using newly developed enzymatic and chemical method. These new methods were validated on various cell wall mutants and it was observed that growth conditions have strong effects on the amount of cell wall components. This method was used to evaluate cell wall composition of industrial yeasts (autolysed and dried yeast) that were subjected to different fermentation and treatment processes. The phenotypic assay was used to confirm and to notice how the growth of various strains depends of composition of medium. The second part of the project was to examine the effect of ethanol stress on cell wall composition and to analyze cell wall composition in industrial yeast cells, to determine whether culture/process conditions have an impact on cell wall composition.

Keywords: chitin, ethanol, *Saccharomyces cerevisae*, cell wall, β-glucan
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1. INTRODUCTION

Yeasts are single-cell organisms that belong to the group of organisms called "fungi", which exist almost everywhere in nature. Yeasts are very helpful, especially with respect to baking, wine making, and brewing. Yeasts reproduce rapidly and grow especially well in substances containing sugar. Yeast has graduated from a position as the premier model for eukaryotic cell biology to become the pioneer organism that facilitated the establishment of entirely new fields of study called “functional genomics” and “systems biology“ (Botstein and Fink, 2011).

Yeast cells are surrounded by a thick, mechanically strong cell wall which serves several key physiological functions, namely maintaining cell shape and cell integrity, and protecting cell interior from harmful compounds from the environment. The cell wall also harbors several proteins that are implicated in molecular recognition and adhesion (Chaffin, 2008). The yeast cell wall consists of a microfibrillar network of β-glucans (β-1,3 and β-1,6-glucans) and highly glycosylated proteins decorated by long chains of mannose residues. Chitin, a linear polysaccharide of β-linked *N*-acetylglucosamine, is the third component of the yeast cell wall and represents 1 to 3 % of the cell wall mass (Lipke and Ovalle, 1998).

In this work amount of cell wall components will be determinate (β-1,3 and β-1,6-glucans, chitin and mannan) with newly developed method to quantify in a more accurate manner the amount of each component in the yeast cell wall.

The etanol effect on different laboratory and industrial strains of yeast *Saccharomyces cerevisae* will be investigated.

The effect of different dyes on the cell wall composition will be investigated on laboratory and industrial strains of *Saccharomyces cerevisae*.

2. THEORETICAL PART

2.1 The yeast *Saccharomyces cerevisiae*

Saccharomyces cerevisiae constitute a group of single-celled (unicellular) fungi, a few species which are commonly used to leaven bread, ferment alcoholic beverages, and even drive experimental fuel cells.

Physiologically, yeast can exist stably in either haploid or diploid states, with the haploid cell being either of two mating types called a and α. Under conditions of carbon and nitrogen starvation, the diploid cell will undergo meiosis to produce four haploid spores. It is possible to recover all four haploid products of the meiosis individually, which may facilitate many types of studies.

Saccharomyces cerevisiae is a species of the yeast. It has been used in winemaking, baking and brewing since ancient times. *Saccharomyces cerevisiae* has been referred to as the *Escherichia coli* of the eukaryotic world (Bergman, 2001). Yeast has been extensively characterized genetically and a complete physical map is now available. The complete genome sequence of *Saccharomyces cerevisiae* was published in April 1996 by Goffeau *et al*. Studying the biology of this yeast has enabled scientists to work out the connections between genes and proteins, and the functions they carry out in human and other higher eukaryotic cells.

Yeast can be grown in either liquid medium or on the surface of a solid agar plate. Yeast cells will grow on a minimal medium containing dextrose (glucose) as a carbon source and salts that supply nitrogen, phosphorus, and trace metals. Yeast cells grow much more rapidly in the presence of rich medium such as yeast extract and bactopeptone. These provide many of the metabolites that the cells would synthesize when growing under minimal growth conditions. During log-phase growth in rich medium, yeast cells divide once approximately every 90 min. The rich medium yeast extract, peptone, dextrose (YPD) is most commonly used for growing yeast under nonselective conditions (e.g., when maintaining plasmid slection is not required).

Wild-type yeast grows well at 30°C with good aeration and glucose as a carbon source. Erlenmeyer flasks work well for growing liquid cultures, and baffled-bottom flasks are good but not necessary. Although small volumes of cultures may be grown in culture tubes, in many cases the cells will settle out from suspension.

For optimal aeration and growth, the medium should constitute no more than 20% of the total volume of the flask, and growth should be carried out in a shaking incubator at 250–300 rpm.

On solid YPD medium at 30°C, single colonies may be seen after 24 h, but generally growth for at least 48 h is required prior to picking of colonies or replica plating. Dropout medium is medium to test the growth requirements of strains in which each of the commonly encountered auxotrophs is supplemented except the one of interest. Growth on dropout medium is approx 50% slower than that observed in YPD.

The approximate number of cells in a culture can be determined with a spectrophotometer by measuring the optical density (OD) at 600 nm. Cultures should be diluted so that the observed reading (OD600) is <1.0.

Yeast strains can be stored at –70°C in 15% glycerol and are viable for more than 3 years. Alternatively, they can be stored at 4°C on slants of rich medium for 6 months to 1 year.
Cells from yeast colonies grown on one medium can be tested for their ability to grow on another medium by replica plating. There are now several commercial sources for the purchase of both a replica-plating block and velveteen squares (Bergman, 2001).

The cell wall is a dynamic structure that can adapt to physiological (i.e. from logarithmic to stationary phase) and morphological changes (conjugation, sporulation or pseudohyphal growth) (Orlean, 1998). Moreover, a cell wall compensatory mechanism is activated in response to cell wall perturbing agents or cell wall mutations, which allows remodelling of the cell wall to combat cell lysis (Orlean, 1998; Klis et al. 2002). One of the major outcomes of this mechanism is a strong increase of chitin that can reach up to 20% of the cell wall dry mass (Popolo et al., 1997; Dallies et al., 1998; Lagorce et al., 2002). The cell walls of yeasts contain (Figure 1) chitin, chitosan, β-1,3-glucan, β-1,6-glucan, mixed β-1,3-/β-1,4-glucan, α-1,3-glucan, melanin, and glycoproteins as major constituents (Free et al., 2013).

The cell wall of yeast and other fungi determines the cell shape and integrity of the organism during growth and cell division. The cell wall of *Saccharomyces cerevisiae* is a strong and elastic structure that gives mechanical protection to the cell. The inner layer of the yeast cell wall is composed of β-glucan polymers cross linked to chitin, while the outer layer is composed of mannoproteins. In the process of autolysis, the medium is enriched by the compounds released as a result of the degradation of intracellular constituents. The two important aspects of yeast autolysis are degradation and solubilization of cellular components, and degradation of the cell wall (Klis et al., 2002).

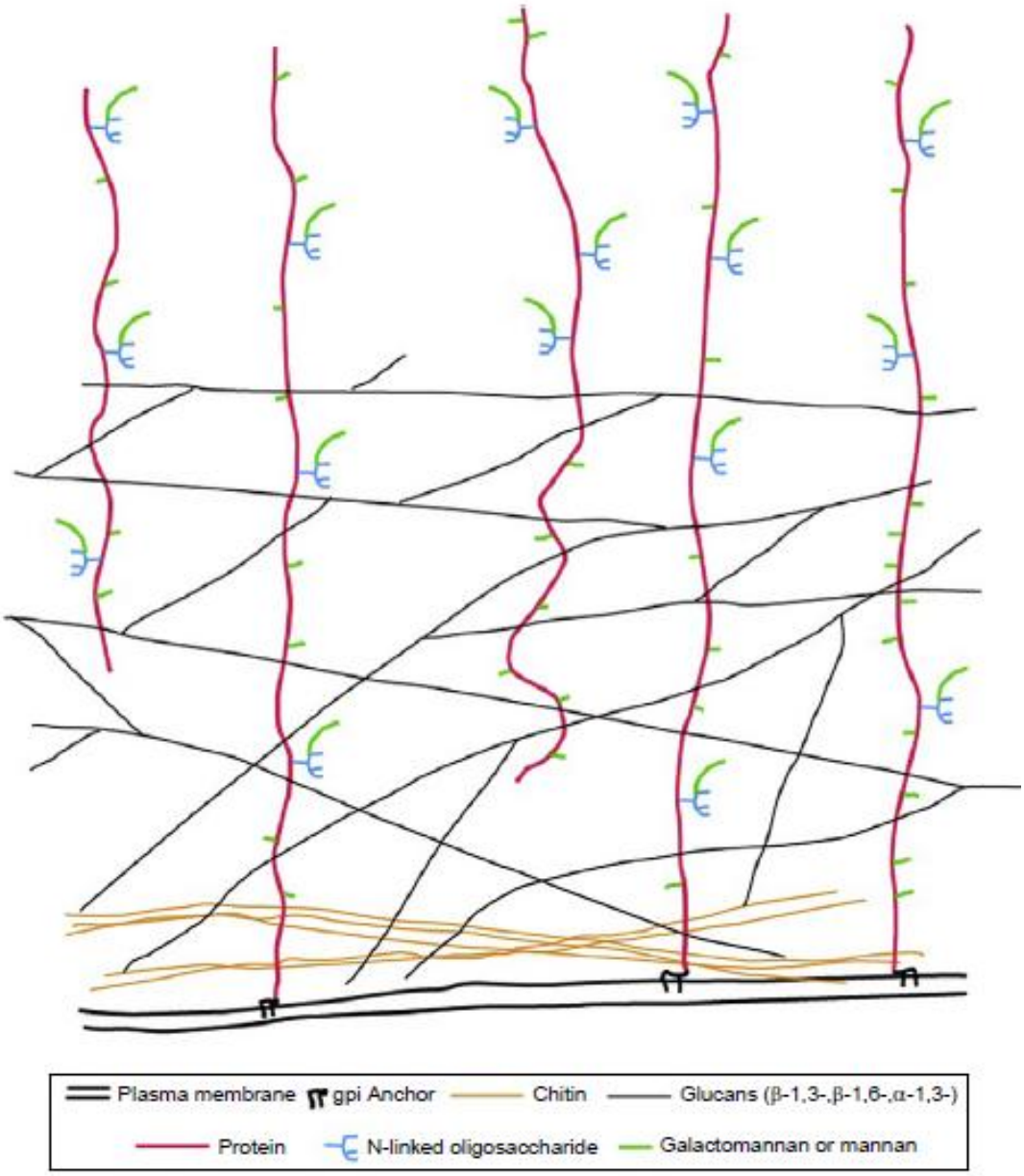


Figure 1. Basic fungal cell wall structure. Generalized representation of a fungal cell wall. The chitin and glucan matrix are cross-linked together to form a glucan/chitin matrix. The glucan fraction contains β-1,3-glucan as a major element and can include other types of glucans, such as β-1,6- glucan and α-1,3-glucan. GPI- anchored glycoproteins and non GPI-anchored glycoproteins are covalently attached to the cell wall. These proteins contain N-linked oligosaccharides that have been modified by addition of either galactomannan or mannans. Attached mannans can contain up to 200 mannose resuides, while attached galactomannans are much smaller. The glycoproteins also contain O- linked galactomannans or mannans (Free et al., 2013).

2.2 Composition and properities of the cell wall of *Saccharomyces cerevisiae*

S. cerevisiae spends a considerable amount of metabolic energy in cell wall construction. Depending on growth conditions, its mass in terms of dry weight may account for about 10–25% of the total cell mass. The molecular architecture of the cell wall is constantly remodelled according to growth conditions, morphological development or in response to cell surface stresses (Aguilar Uscanga *et al.*, 2003).

The yeast cell wall is endowed with remarkable biochemical properties; in winemaking, cell wall mannoproteins are used to reduce astringency caused by tannins and to capture some wine aromatic contaminants that give a mouldy taste to wine, in animal nutrition, cell wall β-glucan serves as a valuable microbiological binder of mycotoxins. Biological functions of the yeast cell wall appeal for a precise quantitative determination of its composition, owing to the fact that cell wall mass and the proportion of each of its component may dramatically vary according to growth conditions and process methods (Aguilar Uscanga et al., 2003).

The outer layer of cell wall, which consists of heavily glycosylated mannoproteins (Cappellaro et al., 1994; Baba and Osumi, 1987) is involved among others in cell-cell recognition events. It also limits the accessibility of the inner part of the wall and the plasma membrane to foreign enzymes such as cell wall-degrading enzymes in plant tissue (De Nobel et al., 1990, De Nobel et al., 2000, Zlotnik et al., 1984). The carbohydrate side chains of the cell surface proteins contain multiple phosphodiester bridges, resulting in numerous negative charges at the cell surface at physiological pH values (Jigami et al., 1999). These side chains are responsible for the hydrophilic properties of the wall, and may be involved in water retention and drought protection (Klis et al., 2002).

The inner layer is composed of β-1,3-glucan branched through β-1,6 linkages (80-90%), polymers of β-1,6-glucan chain and chitin (1-2%).

The mechanical strength of the wall is not due to a specific cell wall component (Table 1) but to linkage between cell wall components (Free et al., 2013). The chitin and glucans are synthesized and extruded into the cell wall space by plasma membrane-associated chitin synthases and glucan synthases. The glycoproteins are synthesized by ER-associated

ribosomes and pass through the canonical secretory pathway. Over half of the major cell wall proteins are modified by the addition of a glycosylphosphatidylinositol anchor. The cell wall glycoproteins are also modified by the addition of O-linked or N-linked oligosaccharides which are extensively modified during their passage through the secretory pathway. These cell wall glycoprotein posttranslational modifications are essential for cross-linking the proteins into the cell wall matrix (Ruiz- Herrera et al., 2006). Cross-linking the cell wall components together is essential for cell wall integrity. Properties of the wall may be involved in water retention and drought protection (Free et al., 2013).

Polysaccharides

β –1,3-Glucans

β-linked glucans compose 30–60% (Table 1) of the dry weight of the wall (Orlean, 2012). β-1,3-glucan is a major constituent of all of the characterized fungal cell walls, making up between 30% and 80% of the mass of the wall. In the cell wall, β-1,3-glucan is found as a branched polymer, with the branches being attached to the core polymer by β-1,6-branches. The glucan is synthesized by β-1,3-glucan synthase (Free et al., 2013). In stationary phase cells β-1,3-glucan molecules consist about 1500 glucose monomers (Klis et al., 2002).

β -1,6-Glucans

β-1,6-glucan has been shown to be an important component of the *S. cerevisiae*, it forms cross-links with β-1,3-glucan, chitin, and with the GPI anchor mannoproteins. The β-1,6-glucan plays an important role in the formation of the *S. cerevisiae* cell wall matrix (Klis et al., 2006; Lesage and Bussey, 2006). It is in mature form a highly branched, water-soluble polymer consisting on average of about 130 glucose monomers (Manners et al., 1973). Various ER-resident proteins, Golgi-resident proteins, and cell surface proteins strongly afect β-1,6-glucan levels in the cell wall (Klis et al., 2002).

Chitin

Chitin isolated from bud scars of *Saccharomyces cerevisiae* consists of about 190 N-acetylglucosamine monomers. The chitin polymer is elongated by the addition of N-acetylglucosamine to the nonreducing end of the polymer and is extruded into the cell wall space, reducing end first, during synthesis (Ruiz- Herrera et al., 2006). Chitin synthesis in *S. cerevisiae* involves three chitin synthases and is tightly regulated (Klis et al., 2002). *S. cerevisiae* has two sporulation-specific chitin deacetylase genes, and mutational analysis shows that the spore cell wall from the double mutant has an increased sensitivity to lysis, caused by digestive enzyme treatment, suggesting that the chitosan provides some protection to the spore (Christodoulidou et al., 1996).

Cell wall mannoproteins

The mannoproteins that form the outer cell wall layer are highly glycosylated with a carbohydrate fraction that often amounts to over 90%. The outer layer of mannoproteins is much less permeable to macromolecules than the internal fibrillar layer. In cell wall some glycoproteins are covalently linked into the cell wall matrix and might be called "integral cell wall proteins" but there is large number of "nonintegral" proteins that have important functions. There are two main classes of proteins covalently coupled to cell wall polysaccharides: GPI-dependent cell wall proteins (GPI-CWPs) which are generally indirectly linked to β-1,3-glucan through a β-1,6-glucan moiety and Pir proteins (Pir-CWPs) which are presumably directly linked to β-1,3-glucan through an alkali-sensitive linkage (Orlean, 1997).

Table 1. Cell wall macromolecules in *S.cerevisiae* (Klis et al., 2002).

Cell wall macromolecules in *S. cerevisiae*

Macromolecule	Wall dry wt. (%)	Site of synthesis	Mature form	
			DP	Branching
Mannoproteins	35–40	Secretory pathway	200[a]	Highly branched
β1,6-Glucan	5–10	(PM)	140	Highly branched
β1,3-Glucan	50–55	PM	1500	Moderately branched
Chitin	1–2	PM	190[b]	Linear

2.3 Methods to study the cell wall

S. cerevisiae was adopted as a model system for laboratory study in the 1930s, as investigators developed genetic tools to understand its life cycle and differentiation. It provided an important tool to understand recombination and the transmission of genetic material, and launched into greater prominence with the molecular era in the 70s, when it became identified as a sort of eukaryotic *E. coli*. With potent genetic tools and a typical eukaryotic cell organization, budding yeast became a favorite system to tackle cell biology questions (Hall and Linder, 1993).

Chemical and enzymatic methods to determine cell walls composition

Methods available for cell wall architecture analysis are based on the separation of structural components by either chemical or enzymatic methods. Most of them make use of chemical treatments of the cell wall with strong acids which can break down the cell wall polymers into their monomers of glucose, mannose and glucosamine. But some of these acidic methods are unable to make distinction between β-1,3 and β-1,6-glucan, some of them are harsh and may destroy the monomers and underestimate the content of the corresponding polysaccharide in the wall and some of them are not efficient enough to hydrolyse all chitin leading to an underestimation of this polymer. There is chemical fractionation method for estimate the proportion of β-1,3 and β-1,6-glucan that consisted in the separation of the cell wall into an alkali-soluble and alkali-insoluble fraction, and the latter was again separated into an alkali-insoluble acid-insoluble and alkali-insoluble acid-soluble fractions that contain the bulk of β-1,6-glucan, whereas β-1,3-glucan is essentially present in the alkali-soluble and alkali-insoluble acid-insoluble fraction. There is one method that is based on an enzymatic digestion after this fractionation procedure to determine β-1,3 and β-1,6-glucan using recombinant endo-β-1,3-glucanase from *Thermotoga neopolitana* and endo-β-1,6 glucanase from *Trichoderma harzianum*. This fractionation procedure is time consuming and imperfect as it leaves some 10–20% of β-1,6-glucan in the alkali-insoluble acid-soluble fraction. This method is developed to be more accurate on these polysaccharides quantification, based on the chemical and enzymatic fractionation of the cell wall obtained from yeast cells cultivated with radiolabeled glucose. But this method couldn't be applied for regular cell wall analysis because they require cultivating yeast cells with a radiolabeled sugar prior to hydrolysis.

New, simple and reliable method that is based on the sequential treatment of cell walls with specific hydrolytic enzymes, namely chitinase, endo and exo-β-1,3-glucanases followed by a mixture of endo-β-1,6-glucanase and β-glucosidase to determine more precisely chitin levels and to discern β-1,6 from β-1,3-glucan in cell wall β-glucan is developed (Schiavone et al., 2014).

Combined with the chemical treatment that hydrolyses mannans into mannoses units, this new method allowed faithfully quantifying each of the different polymers that compose the cell wall. This method has been applied to determine the cell wall composition of mutants defective in genes involved in the cell wall synthesis and remodelling, as well as to assess effects of ethanol stress, heat shock and the antifungal drug caspofungin on the cell wall composition of yeasts (Schiavone et al., 2014).

Phenotypic assays- effects of Calcoflour White and Congo Red on industrial strains of *Saccharomyces cerevisiae*

Calcofluor white (CFW) and Congo red (CR) are two related anionic dyes that both interfere with the construction and stress response of the cell wall. Both compounds contain two sulfonic-acid groups. CFW and CR exert their antifungal activities only when they are solubilized, when their sulfonic-acid groups are negatively charged.

CFW-based and CR-based susceptibility assays are simple and powerful methods for the identification of fungal cell wall mutants. Small differences in the susceptibility of the wild-type and mutant strains might require the use of a series of CFW or CR concentrations to identify which gives the largest growth difference (Ram et al., 2006). In the case of *Saccharomyces cerevisiae*, Calcofluor induces abnormal septa which apparently fail to develop abscission zones between mother and daughter cells, probably by inhibiting the action of chitinases. A similar effect was produced in *S. cerevisiae* by Congo red (Cabib et al., 1982). The abnormally thick septa may be the result of a massive deposition of polymers, mainly chitin. This assumption corresponds with the proposed morphogenic role of chitin in the formation of the yeast primary septum. The rate of chitin polymerization was specifically enhanced *in vivo* by either Calcofluor or Congo Red. The fact that *S. pombe*, whose cells do not contain chitin, is not affected by Calcofluor would also seem to suggest that chitin is the target polysaccharide. It is feasible to assume that the binding of Calcofluor to nascent, β-1,3-

glucan fibers or chitin fibers or both may interfere with the action of the hydrolytic activities presumably implicated in cell wall morphogenesis (Roncero et al., 1985). As CFW and CR are believed to bind to chitin, these compounds also directly counteract the cell wall stress response itself (Ram et al., 2006). Some mutants with lowered chitin levels in their walls become more resistant to CFW and CR. Most cell wall mutants, however, have more chitin in their walls than wild-type cells, because of activation of the cell wall stress response, and become more sensitive to CFW and CR (Ram et al., 1994 ; Imai et al., 2005).

2.4 The effect of ethanol on the cell wall

When yeast cells are exposed to environmental stresses, the changes usually will be compensated for by transient adaptation of the cells, through transcriptional, translational, or other types of regulations (Ding et al., 2009). Among environmental stresses that yeasts can experience, ethanol toxicity is a major stress, especially encountered in fermentation processes (Canneta et al., 2005). The effects of ethanol toxicity on yeast physiology are diverse, though cellular membranes appear to be the main sites of ethanol damage. Specific effects include growth inhibition, reduced cell size, reduced viability, reduced respiration and glucose uptake, reduced fermentation, enzyme inactivation, lipid modification, loss of proton motive force across the plasma membrane and increased membrane permeability and lowering of cytoplasmic pH (Gibson et al., 2007). The synthesis of heat shock proteins is increased, lipids become less unsaturated and sterol levels are reduced. Heat and ethanol stress consequently have a synergistic effect and the toxic effects of ethanol are influenced by temperature. Aside from making the plasma membrane less stable and more fluid, ethanol can damage the cell membrane functional proteins. Thus, factors that help stabilize or refold denatured proteins can contribute to ethanol tolerance, among which heat shock proteins and trehalose have been well-reported (Singer and Lindquist, 1998). Stress response is mediated by the transcription factors Msn2p and its homologous Msn4p (also called Msn2/4p) in *S. cerevisiae*. The *MSN2* and *MSN4* genes encode homologous and functionally redundant Cys2His2 zinc finger proteins. Both of them play central roles in response to a range of stresses by activating gene transcription *via* stress response elements (STRE) (Martinez-Pastor et al., 1996). Thus, when there is a high concentration of ethanol in the environment, Msn2p and Msn4p will bind specifically to STRE-containing oligonucleotides to stimulate the

expression of the downstream genes. Consistent with the importance of these two genes in yeast response to adverse conditions, when both *MSN2* and *MSN4* were deleted, the double mutants were shown hypersensitive to severe stress conditions, including a high concentration of ethanol, carbohydrate starvation, heat shock, severe osmotic stress, and so on (Moskvina et al., 1998).

3. EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Preparation of strains and growth conditions

First the different laboratory strains (BY4741 and BY4743) were precultured and prepared for acid and enzymatic hydrolysis.

Growing of the wt strains in YPD medium (protocol from Dallies et al., 1998): YPD medium: 2% glucose, 1% bactopeptone, 1% yeast extract. Strains were precultured in 5 ml of YPD- and put on mixing (200 rpm/ 30°C / 1 day- triplicate) in test tubes. After 1 day, OD_{600} was checked and strains were inoculated with 0.1 cell density. Cells were cultivated in 200 ml of medium under 200 rpm until OD_{600}=1 (OD_{600} is checked every 2 hours, because generation time of yeast is 2 hours) in Erlenmeyer's flasks of 500 ml. Medium was divided in 4 Falcon tubes (total 12 tubes, because triplication). Cells were harvested by centrifugation- 10 min at 4000 rpm, 21°C. The cells were washed 2 times with 5 ml sterilized cold water until pellets are pure (after washing centrifuge - 4000rpm/5min/4°C). The pellets were stored at -20°C.

Growing of the wt strains in YNB medium : YNB medium: 2% glucose, 0.17% yeast nitrogen base without amino acid and ammonium, 0.5 % $(NH_4)_2SO_4$, required aminoacid according to strain auxotrophy. The rest of protocol is the same as for a YPD medium.

3.1.2. Isolation of cell wall

Cell wall needs to be isolated and analysed from yeast cell to get the informations about cell wall compounds.

The pellets of laboratory yeast strains were resuspended in 500 µl of 10 mM TrisCl + 1mM EDTA buffer pH 7.5 and transferred in tubes containing beads (MPBio, 6960).

50 mg of the industrial yeast strains (Table 2) (yeast cultures autolysed and dried according to industrial process of Lallemand company (confidential)) were resuspend in 5 ml of sterile water. Medium was centrifuged at 4800 rpm/25°C /10 min, cells were washed 2 times with 5 ml sterilized cold water until pellets are pure. After washing, pellets were centrifuged - 4000rpm/5min/4°C. The pellets were resuspended in 2.5 ml of 10 mM TrisCl + 1mM EDTA buffer pH 7.5 and divided in 10 tubes with beads (MPBio, 6960).

They were broken with a FastPrep system by several cycles of 20 sec at max speed (6.5 ms^{-1}) and left 1 min on ice during each cycle (protocol from Dallies et al., 1998).

Table 2. The names of the strains and their genotype and source of reference.

Strain	**Genotype**	**Source of Reference**
BY4743	MATa/MATα his3Δ 1/his3Δ 1; leu2Δ0 /leu2Δ 0; met15Δ 0/MET15; LYS2/lys2Δ 0; ura3Δ 0/ura3Δ 0	EUROSCARF
BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	EUROSCARF
*msn2*Δ/*msn4* Δ	BY4741 *msn2*::Kan *msn4*::Kan	Openbiosystem
yap1Δ	BY4741 *yap1*::Kan	Openbiosystem
A[1]		Lallemand collection (Blagnac, France)
B		Lallemand collection (Blagnac, France)
C		Lallemand collection (Blagnac, France)
D1		Lallemand collection (Blagnac, France)
D2		Lallemand collection (Blagnac, France)
D3		Lallemand collection (Blagnac, France)

To check the breakage of cells, pellets were observed by microscope by methylene blue test. The cell suspension was transferred in a Falcon tube of 15 ml, without taking the beads. The suspension was washed 4 times with 1 ml of cold water and each time the supernatant was collected. The Falcon tubes were centrifuged for 10 minutes at 4000 rpm in 4 °C. The

[1] Strains A, B, C, D1, D2 and D3 are industrial products which are yeast cultures autolysed and dried according to industrial process of Lallemand company (confidential).

supernatant was removed. At the end of this part, the cell walls were washed in Falcon tubes with 1 ml of cold water, divide it in 2 parts, and centrifuge again (4000rpm/10 min/4°C). The supernatant was removed. The cell walls were lyophilized with the freeze dry system (in liquid N_2).

3.1.3. Determination of cell wall composition

For determination of cell wall composition in this study acid (H_2SO_4) hydrolysis and newly developed enzymatic hydrolysis were used to prepare samples for High-Performance Anion-Exchange Chromatography (HPAEC- PAD).

Quantitative determination cell wall components after acid hydrolysis and HPAEC-PAD on different yeast strains

The 10 mg of lyophilized cell wall (dry mass) and 75 µl H_2SO_4 72% (under the hood with filtering tips) were added in a glass tube, left at room temperature for 3 hours (every half hour were vortexed). After 3 hours, 925 µl of water was added in order to dilute the acid to a final concentration at 2N and the tubes were placed in a dry bath set at 100 °C /4h.

Samples were diluted by 5 ml of water and transferred in 50 ml Falcon tubes. The 100 µl of bromophenol blue 1% was added (mixture was changed the color, from yellow to blue). The neutralization is done by adding the $Ba(OH)_2$ solution (5-8 ml) and mixed to form $BaSO_4$ precipitat. Adding of the $Ba(OH)_2$ was stopped when the color of the mixture became blue and the pH reaches 7. The samples were left over the night at 4 °C. The next day, while samples were in ice, the water was added until a final volume = 25 ml and the pH was checked. The precipitate was collected by centrifuge for 10 min/4000rpm/21°C. The 1 ml of supernatant was transferred in the Eppendorf tubes. It was centrifuged again 10 min x 15 000 rpm/min at 4°C and transferred in vials (using 0.45 µm microfilters) for the HPAEC-PAD analysis. That analysis provide information about concentration of glucose, glucosamin and mannose (protocol from Dallies et al., 1998).

Quantitative determination cell wall components after enzymatic hydrolysis and HPAEC- PAD on different yeast strain

Enzymatic hydrolysis: the 10 mg of purified cell walls and 200 µl of 50 Mm potassium acetate buffer pH 5 were added in Eppendorf tube and vortexed. That mixture was set in water bass (60 °C / 5 min) and after 5 min vortexed. The 25 µL of chitinase (4 U/ml) + 40 µL endo-β-1,3-glucanase (50 U/ mL) + 20 µl exo-β-1,3-glucanase (100 U/mL) were added in that mixture. That mixture was incubated in mixing machine at 37 °C / 24 hours.

After one day, the 50 µl of diluted aliquot (10x) was put in the vials and analysed (first step of measuring). In the rest of sample 25 µl β-glucosidase + 40 µl endo-1,6-glucanase were added and also incubated in mixing machine at 37°C/24 hours. After that, the diluted aliquot (10x) for measuring the step 2 of enzymatic hydrolysis was analysed. Rest of samples were stored at -20 ºC.

The samples were analysed by HPAEC-PAD on an ICS 3000 system (Thermofisher Scientific, Courtaboeuf, France). Separation of the released monosaccharides (glucose, mannose, glucosamine) and oligosaccharides was performed on a CarboPac PA10 analytical column (250 x 4 mm) with a guard column CarboPac PA10 using NaOH 200 mM (A), water (B) and NaOAc/NaOH (300 mM /200 mM) (Table 3). Sugar residues were detected on a pulsed amperometric system equipped with a gold electrode.

Table 3. Gradient of added NaOH 200 mM (A), water, NaOAc/NaOH and A as solvent (respectively) for HPAEC- PAD.

Time (min)	**% A solvent**	**%B water**	**% NaOAc/NaOH**
0	8	91	0
0	8	91	0

20	8	91	0
21	100	0	0
26	0	0	100
27	0	0	100
37	100	0	0
38	8	91	0
58	8	91	0

Reissig's method was done in the end. It is a colorimetric method to determine chitine. First solutions need to be prepared: standard of N-acetyl-D-glucosamine at 10 mM concentration (the 44.2 mg of N-acetyl-D-glucosamine was dissolved in 20 ml of water); solution of tetraborate potassium 0.8 M pH 9,0 (the 6.11 g was dissolved in 25 ml of water, pH was adjusted with KOH 3M) and Reissig's reagent 10X (the 10 g of 4-dimethylaminobenzaydedyde (Fluka) was dissolved in 12.5 ml of HCl 10 N and 87.5 ml of glacial acetic acid. Reissig's reagent should be stored in fridge (+4ºC), in dark for maximum 2 months. For the dosage the reagent is diluted 10 times in acetic acid.

The 125 µl of Reissig's reagent or standard was transferred in Eppendorf tubes and 25 µl of the potassium tetraborate 0.8 M pH 9.0 was added. It was heated at 100 °C for 8 min in dry bass. It was cooled at room temperature. In tubes was added 750 µl of the Reissig Reagent 1X (from the 10X solution, diluted with acetic acid). It was incubated immediately at 37 °C for 40 min. After that, samples were put in microplate, the OD was read at 550 nm. Standard curve of N-acetyl-D-glucosamine is made from 0 to 0.5 mM from the 10 mM stock solution.

In the end, this analysis provide data about:

- Mannan= concentration of mannans, acyd hydrolysis
- β-1,3-glucan (µg mg^{-1})= concentration of Glc, enzymatic hydrolysis, step 1
- β-1,6-glucan (µg mg^{-1})= concentration of Glc, enzymatic hydrolysis step 2 - concentration of Glc, enzymatic hydrolysis, step 1

- Chitin (µg mg^{-1})= concentration of N-acetylglucosamine; Reissig's method

3.1.4. Determination of trehalose and glycogen in whole cell
(protocol from Parrou and François, 1997)

Reagent: reactive glox (Glucose Oxidase from Aspergillus niger-Type VII, lyophilized powder, ≥100,000 units/g solid (without added oxygen). Synonym: β-D-Glucose:oxygen 1-oxidoreductase, G.Od., GOx, *SIGMA*).

The capsule of glox was dissolved in 39,2 ml of water; the 10 mg of o-dianisidine was dissolved in 1 ml of water (under hood). 400 µL of the o-dianisidine was added in glox solution. It was stored at 4 °C / 1 week.
The 10 mg of sample (whole yeast of industrial strains) was added to 250 µl of 0.25 M Na_2CO_3 and incubated in a water bath at 95 °C for 2-4h (the tubes were inverted from time to time). The 150 µl of 1 M acetic acid and 600 µl of 0.2 M sodium acetate pH 5.2 were added. It was splitted in 2 parts of 500 µl. In first part, the 10 µl of trehalose or 10 µl of H_2O for a blank were added. In another 500 µl, 10 µl of α-aminoglucosidase or 10 µl of water for a blank were added. Eppendorf tubes were incubated with trehalose at 37 °C over night, and α-amyloglucosidase at 56 °C over night. The day after, tubes were centrifuged 1 min/ 15000 rpm/ 4 °C. The 20 µl of sample + 200 µl of reagent (reactive glox) were added in microplate, incubated for 30 min/ 37 °C. The absorbance was measured at 490 nm.

3.1.5. Ethanol stress

3.1.5.1. Conditions of ethanol shock

In the second part of this study the effect of different ethanol concentration on a yeast cell growth, cell wall polysaccharide content and on release of glucose from laminarin (*in vitro*

assay) was investigated. Verified effects of ethanol on protein in cell walls of laboratory yeast strains are presented in the following chapter. This study was carried out on a wild type strain BY4741, and its isogenioc mutant *msn2/msn4Δ*. This later is defective in the transcription factor required for general stress response and thus, it was suspected that the lost of this function could show impact on the ethanol response and indirectly on the cell wall composition.

In this study the effect of different concentration of ethanol on a concentration of liberated glucose from laminarin, using exo-β-1,3-glucanase and endo-β-1,3-glucanase was investigated. Laminarin (Figure 2) from *Laminaria digitata (*polysaccharide substrate for laminarinase) is a storage glucan. It is created by photosynthesis and is made up of β-1-3-glucan with β-1-6-branched with a β-1-3:β-1-6 ratio of 3:1. Its hydrolysis is catalyzed by enzymes such as laminarinase that breaks the β-1-3 bonds. Laminarinase has a same activities as exo-β-1,3-glucanase and endo-β-1,3-glucanase.

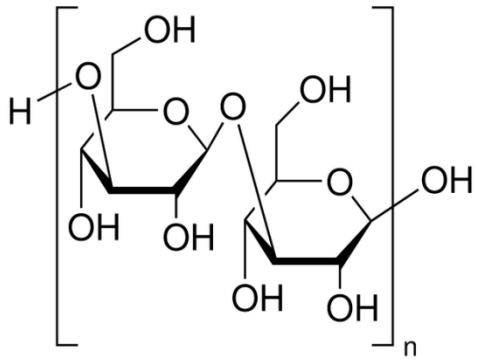


Figure 2. Structure of laminarin

3.1.5.2. Analysis of sugar released, measurement of glucose (Glox) and oligosaccharides **(HPAEC- PAD)**

Protocol for the degradation of laminarin using exo-β-1,3-glucanase: laminarin (10 mg ml^{-1}) was added in required volume of 50mM Na acetate buffer pH 5.5 and exo-β-1,3- glucanase

0.1 U ml^{-1} was added to the final volume of 1 ml (enzyme was diluted in buffer 10 x) (Table 4).

Table 4. The ratio of the volumes of the exo-β-1,3-glucanase, ethanol, laminarin, and buffer for degradation of laminarin using exo-β-1,3- glucanase.

Final volume (µl)	Volume of exo-β-1,3-glucanase (µl)	Volume of ethanol (µl)	Volume of laminarin (µl)	Volume of buffer (µl)
1000	10	0	500	490
1000	10	50	500	440
1000	10	90	500	400
1000	10	120	500	370

There were two blanks: without laminarin and without exo-β-1,3-glucanase. Incubation was at 37 °C (mixing). Reaction was stopped every 10 minutes (in 30 minutes) by taking 100 µl of mixture which was incubated in dry bath (80ºC/ 5 min), centrifuged on 3min/1500 rpm and diluted 2 times (the last one 4 times) with sterile water.

Protocol for glucose-oxidase method to measure the concentration of glucose released from laminarin required: reagent (the capsule of glox was dissolved in 39.2 ml of water, the 10 mg of o- dianisidine was dissolved in 1 ml of water (under hood) and 400 µl of o-danisidine was added in glox solution) and standards (glucose solution).

The 20 µl sample and standards were put in microtiter plate and 200 µl of reagent was added in each wells. It was incubated for 30 min at 30 ºC and the absorbance was measured at 490 nm.

Protocol for the degradation of laminarin using endo-β-1,3-glucanase: laminarin (10 mg ml^{-1}) was added in required volume of 50mM Na acetate buffer pH 5.5 and endo-β-1,3- glucanase 0.1 U ml^{-1} was added to final volume of 1 ml (enzyme was diluted in buffer 10 x) (Table 5).

Table 5. The ratio of the volumes of the endo-β-1,3-glucanase, ethanol, laminarin, and buffer for degradation of laminarin using endo-β-1,3- glucanase.

Final volume (µl)	Volume of endo-β-1,3-glucanase	Volume of ethanol (µl)	Volume of laminarin (µl)	Volume of buffer (µl)

	(µl)			
1000	20	0	500	480
1000	20	50	500	430
1000	20	90	500	390
1000	20	120	500	360

There were two blanks: without laminarin and without exo-β-1,3-glucanase. Incubation was at 37 °C (mixing). Reaction was stopped every 10 minutes (in 30 minutes) by taking 100 µl of mixture which was incubated in dry bath (80ºC/ 5 min), centrifuged on 3min/1500 rpm and diluted 2 times with sterile water.

STANDARDS:

Glucose- 25 µmol ml^{-1} and 12.5 µmol ml^{-1};

L2 (laminarin composed of two glucose molecules)- 25 µmol ml^{-1} and 12.5 µmol ml^{-1};

L3 (laminarin composed of three glucose molecules) - 50 µmol ml^{-1} and 12,5 µmol ml^{-1};

L5 (laminarin composed of five glucose molecules)- 50 µmol ml^{-1} and 12,5 µmol ml^{-1};

L6 (laminarin composed of six glucose molecules)- 50 µmol ml^{-1} and 12,5 µmol ml^{-1}

The samples were analysed by HPAEC-PAD on an ICS 3000 system (Thermofisher Scientific, Courtaboeuf, France). Separation of the released monosaccharides (glucose) and oligosaccharides was performed on a CarboPac PA100 analytical column (250 x 4 mm) with a guard column CarboPac PA100 using A- NaOH 100 mM, water and C- NaOAc/NaOH (400 mM /200 mM) (Table 6).

Table 6. Gradient of solvents A (NaOH 100 mM) and C (NaOAc/ NaOH) for HPAEC- PAD.

Time	**%A**	**%C**
0	91	2

0	91	2
2	91	2
15	65	35
22	57	43
23	0	100
28	0	100
30	91	2
35	91	2

3.1.5.3. Effect of ethanol stress on a laboratory strains

This protocols were used for comparation and better understanding how ethanol effect on wild type and its mutants (*msn2/msn4* and *yap1*).

In this protocol YPD with different concentrations of EtOH was used as medium (YPD, YPD + 5% EtOH, YPD + 9% EtOH and YPD + 12% EtOH).

Wild type and mutants were precultured in YPD medium (200 rpm/ 30°C) in test tubes. Cells were cultivated in 100 ml of YPD medium under shaking 200 rpm/ 30 ºC until OD_{600}=1 (OD_{600} is checked every 2 hours) in Erlenmeyer's flasks of 500 ml. Cells were harvested when OD_{600} reached 1 by centrifugation at 300 rpm/3 min. Pellets were divided in 4 fractions (4 Erlenmeyer's flasks of 500 ml) with different concentrations of ethanol in YPD medium. Erlenmeyer's flasks were incubated at 30 ºC under shaking (200 rpm) for 5 hours. Strains were affected with different concentrations of ethanol. OD_{600} were checked every hour.

In the next protocol the quantity of cell wall components of mutant *msn2/msn4* was determinated in two medium, YPD and YPD + 9% EtOH.

The preculture of mutant was grown in YPD medium 30 °C/200 rpm/24h. Yeast strain was cultivated in 200 ml of YPD medium under shaking at 200 rpm/30 °C in Erlenmeyer's flasks of 1 l. The cells were harvested when OD_{600} was 1 by centrifugation at 3000rpm/3 min. The

cell suspensions were divided in 2 fractions. In the first fraction the 200 ml of YPD medium were added. In the second fraction were added 200 ml YPD + 9% EtOH. Erlenmeyer's flasks of 1l were incubated at 30 °C /200 rpm. The samples were analysed after 5 hours of incubation time (the cell walls were isolated and analyzed).

3.1.6. Phenotypic tests on plates based on Calcoflour White, Congo Red and ethanol

Phenotypic tests on plates with included ethanol, Calcoflour White and Congo Red were used to investigated ethanol effect on growth and on the level of chitin in a different strains.

To prepare different concentration of CFW, CR and ethanol appropriate volume of CFW, CR and ethanol (previously calculated) were added in tube and then YPD agar was added till 20 ml in Falcon tubes and pour out in Petri dish. When it was dry, the Petri dishes were closed covered with paraffin and stored at +4 °C.

Calcoflour White

Preparation of Calcofluor White solution 5 mg ml^{-1}: the 50 mg of the CFW was added to 10 ml of water. The 10N NaOH (20 µl) was added until the solution became translucent. The volume was made up to 10ml with water. CFW was added to the cooled medium just before solidification in the form of a freshly prepared solution sterilazed by filtration. Plates were made with different concentrations of CFW, from 0.01 to 0.1 mg ml^{-1}.

Congo Red

The Congo Red is added to the cooled medium just before solidification in the form of a freshly prepared solution of 1 mg ml^{-1} and sterilized by filtration with 0.45 microns. Plates were made with different concentrations of CR from 0.01 to 0.1 mg ml^{-1}.

Ethanol

YPD medium were composed of 2% glucose, 1% bactopeptone, 1% yeast extract and of different concentrations of ethanol- 5, 9 and 12 percentage.

The strains were cultivated till OD_{600} was 1. The 1 ml of culture was added in Eppendorf tubes, centrifuged 4000 rpm/5 min/ 4 °C. Pellets were resuspend in 100 µl of sterile water. In microplate the 20 µl of sample + 180 µl water were added. The cells from the microplate were deposited in drops at different dilutions (1/10, 1/100 and 1/1000) in the medium containing the Calcofluor White, Congo Red, Ethanol and YPD. The Petri dishes were incubated at 30 °C for 2 days.

4. RESULTS AND DISCUSSION

The yeast cell wall is a strong, but elastic, structure that is essential not only for the maintenance of cell shape and integrity, but also for progression through the cell cycle. Cell wall construction is tightly controlled. Polysaccharide composition, structure and thickness of the cell wall vary considerably, depending on environmental conditions.

Yeast cells divide mitotically by forming a bud, which is subsequently pinched off to form a daughter cell. Yeast can also be grown on a completely defined medium, which has allowed the isolation of numerous nutritional auxotrophs. In this work it was confirmed that growth rate depended on the medium in which the yeast was cultivated.

4.1. Cell wall analysis of laboratory and industrial strains

The *S. cerevisiae* cell wall represents 30% of the dry weight of the cell and is composed largely of polysaccharides (85%) and proteins (15%). Acid and enzymatic analyses confirmed composition of the total carbohydrates of cell walls: glucose (80-90%), mannose (10-20%) and *N*-acetylglucosamine (GlcNAc) (1-2%). Previous methods based on acid hydrolysis were not effective enough to release all chitin that was bound to β-(1,3) and β-(1,6)-glucan, but the newely developed method that was a combination of acid and enzymatic metods, can quantify all chitine. This method can quantify the content of components that have biotechnological values. Glucose residues are linked to other glucose molecules through β-1,3 and β-1,6 linkages and to GlcNAc via β-1,4 bonds. Mannoproteins can be linked to β-1,6-glucose chains through a processed glycosylphosphatidylinositol (GPI) anchor or to β-1,3-glucan through an alkali-labile bond. These are simple methods and they don't require any complex manipulations.

4.1.1. Growth curve of laboratory strains

Figures 3 and 4 show a growth curve of laboratory strain BY4743 in YPD medium and in YNB. The growth of BY4743 is faster in YPD because yeast cells grow much more rapidly in the presence of rich medium that contains reagents such as yeast extract and bactopeptone. These provide many of the metabolites that the cells would synthesize when growing under minimal growth conditions.

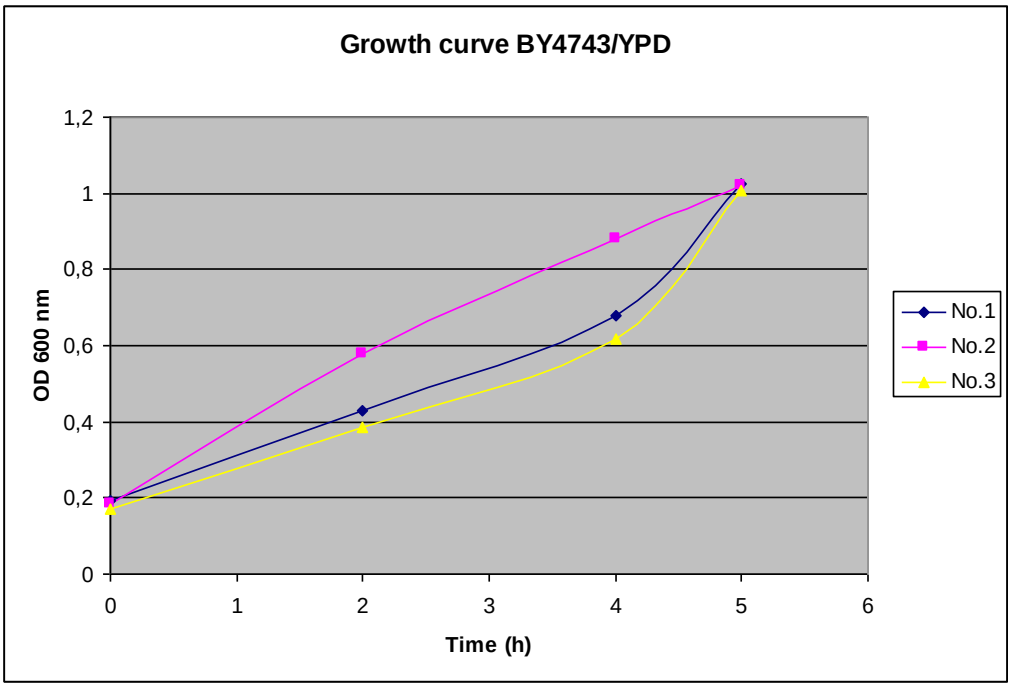


Figure 3. The growth curve in the first 6 hours of growth of yeast strain BY4743 in YPD medium obtained in three independent experiments as indicated in the legend at right.

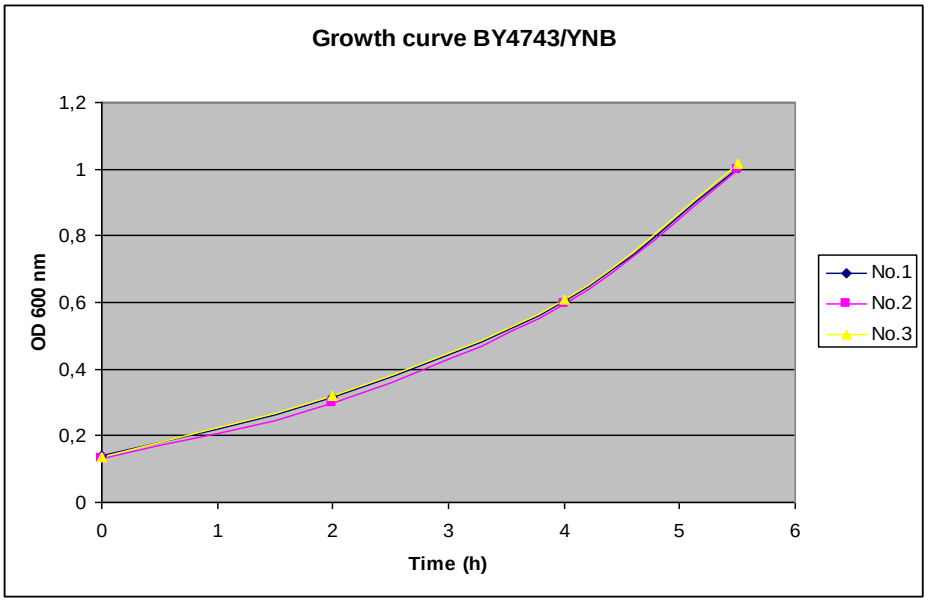


Figure 4. The growth curve in the first 6 hours of growth of yeast strain BY4743 in YNB medium obtained in three independent experiments as indicated in the legend at right.

4.1.2. Quantitative determination of cell wall components after acid hydrolysis and HPAEC-PAD of the different yeast strains

Acid hydrolysis is a part of the metod for quantitative determination of the proportion of cell wall components, such as glucosamine from chitin, glucose from β-glucan and mannose from mannans. The total amount of component that was analyzed by acid hydrolysis is shown in Table 7.

Table 7. Amount of cell walls components after acid hydrolysis of BY4743 and YCW[2].

Sample	**Chitin (µg mg^{-1})**	**β-glucan (µg mg^{-1})**	**Mannan (µg mg^{-1})**
YCW A	15,9 +/- 1,9	456,5 +/- 31,7	587,9 +/- 545
YCW C	10,4 +/- 1,9	347,0 +/- 78,1	318,1 +/- 81,1
YCW D1	12,2 +/- 2,59	408,3 +/- 116,7	338,3 +/- 114,2
YCW D2	12,6 +/- 5,7	397,3 +/- 1,3	322,8 +/- 9
YCW D3	17,0 +/- 5,8	692,4 +/- 25,7	227,4 +/- 18,1
BY4743/YPD	14,1 +/- 0,9	565,2 +/- 30,6	377,5 +/- 20,5
BY4743/YNB	23,5 +/- 2,1	616,2 +/- 15,3	384,4 +/- 15,2
YCW B	4,2 +/- 0,8	409,5 +/- 9	489,2 +/- 38,9

4.1.3. Quantitative determination of cell wall components after enzymatic hydrolysis and HPAEC- PAD of the different yeast strains

Enzymatic hydrolysis is a part of the metod for quantitative determination of the proportion of cell walls components, such as chitin, β-1,3-glucans and β-1,6-glucan. The total amount of component that was analyzed by enzymatic hydrolysis is shown in Table 8.

Table 8. Amount of cell walls components after enzymatic hydrolysis.

Sample	**Chitin (µg mg^{-1})**	**β-1,3-glucan (µg mg^{-1})**	**β-1,6-glucan (µg mg^{-1})**
YCW A	38,9 +/- 1,1	387,1 +/- 48,3	251,6 +/- 32,4
YCW C	37 +/- 20,3	425,6 +/- 228,2	490 +/- 27,3
YCW D1	26,8 +/- 20,7	571,1 +/- 241,3	306,6 +/- 51,1
YCW D2	43,9 +/- 19,4	728,3 +/- 185,4	165,1 +/- 237,9
YCW D3	30,2 +/- 0,7	930,8 +/- 414,1	122,8 +/- 230

[2] Fraction YCW are industrial products which are yeast cultures autolysed and dried according to industrial process of Lallemand company (confidential).

BY4743/YPD	61,1 +/- 1,2	637 +/- 16,5	226,9 +/- 128,9
BY4743/YNB	36,8 +/- 4,7	441,8 +/- 19,2	223,2 +/- 18,3
YCW B	18,5 +/-0,7	326,2 +/- 32,9	148,3 +/- 24,6

Table 7 and 8 show amount of chitine. In acid hyrolysis, hydrolytic yield of chitin is not very high because there are cross-linkages between chitin and β-glucans.

Total content of components (Table 9) in the laboratory strain BY4743 inoculated in YPD medium is higher than in YNB, because yeast cells grow much more rapidly in the rich medium that contains reagents such as yeast extract and bactopeptone. As it is shown, in the cell walls the most representing compound is β-1,3-glucan, then mannan and β-1,6- glucan and in the end is chitin.

Table 9. Content of total carbohydrates after acid and enzymatic hydrolysis shown in percentage.

Sample	Total carbohydrates	% chitin	% β-1,3 glucan	% β-1,6 glucan	% mannan
BY4743/YPD	1302,6	4,7	48,9	17,4	28,9
BY4743/YNB	1090,2	3,3	40,5	20,8	35,2
YCW C	1270,7	2,9	33,4	38,5	25
YCW D1	1242,9	2,1	45,9	24,6	27,2
YCW A	1265,6	3	30,5	19,8	46,4
YCW B	982,3	1,8	33,2	15	49,8
YCW D2	1260,2	3,4	57,7	13,1	25,6
YCW D3	1311,2	2,3	70,9	9,3	17,3

4.3. Determination of trehalose and glycogen in whole cells

(industrial strains, Lallemand)

Glycogen and trehalose are two important glucose stores of the yeast *Saccharomyces cerevisiae*. The ratio of glycogen and trehalose (Table 10) is strongly and rapidly changeful, depending of the environmental conditions. Amount of glycogen is higher than trehalose in cell walls.

Table 10. Content of glycogen and trehalose in whole cells of industrial strains of yeast.

	YCW A	***YCW B***	***YCW C***	***YCW D1***	***YCW D2***	***YCW D3***
Glycogen (µg mg^{-1})	186,3 +/- 13,7	59,6 +/- 18,4	79,6 +/- 16,4	165,4 +/- 1,3	148,3 +/- 5,6	132,9 +/- 12,1
Trehalose (µg mg^{-1})	4,3 +/- 0,9	23,3 +/- 4	11,2 +/- 4,1	17,4 +/- 1,4	13,6 +/- 0,8	8,4 +/- 1,2

4.4. Ethanol effect on cell wall composition

4.3.1. Growth curve

BY4741 (Figure 6) is a laboratory strain of yeast *Saccharomyces cerevisiae*. In this work growth curve of BY4741 and its mutants *msn2/msn4Δ* and *yap1Δ* (Figure 5) are compared. The *MSN2* and *MSN4* genes encode homologous and functionally redundant Cys2His2 zinc finger proteins. Both of them play central roles in response to a different kinds of stresses by activating gene transcription via STRE. Thus, when there is a high concentration of ethanol in the environment, Msn2p and Msn4p (Figure 7) will bind specifically to STRE-containing oligonucleotides to stimulate the expression of the downstream genes. Consistent with the importance of these two genes in yeast response to adverse conditions, when both msn2 and msn4 were deleted, the double mutants were shown hypersensitive to severe stress conditions, including a high concentration of ethanol, carbohydrate starvation, heat shock, severe osmotic stress. Strains lacking the transcriptional activators Msn2p and Msn4p are unable to accumulate trehalose in response to stress. The activation of the STRE element of inducible genes is dependent upon two zinc finger transcriptional activators (Msn2p and Msn4p). An increase in the transcription of genes encoding enzymes involved in trehalose biodegradation,

as well as synthesis, on exposure to stress, indicates that rapid trehalose turnover rather than accumulation may be the factor governing cellular protection against oxidative stress in yeast. This stress response is mediated by the transcription factors Msn2p and its homologous Msn4p in *S. cerevisiae*. Oxidative stress is indirectly generated under ethanol stress. Yap1p is a basic leucine zipper required for oxidative stress tolerance. Many ethanol-induced genes contain a Yap1p binding motif. The *YAP1* gene was obtained as a suppressor, deletion of *YAP1* gene exacerbates Ca^{2+} sensitivity. It encodes a bZIP-containing transcription factor that is essential for the normal response of cells to oxidative stress.

The grown rate of laboratory strain BY4741 in YPD is slightly higher than its mutants *msn2/msn4Δ* and *yap1Δ* (Figure 5). There is no big divergence in growth curve of laboratory strain and its mutants in rich medium.

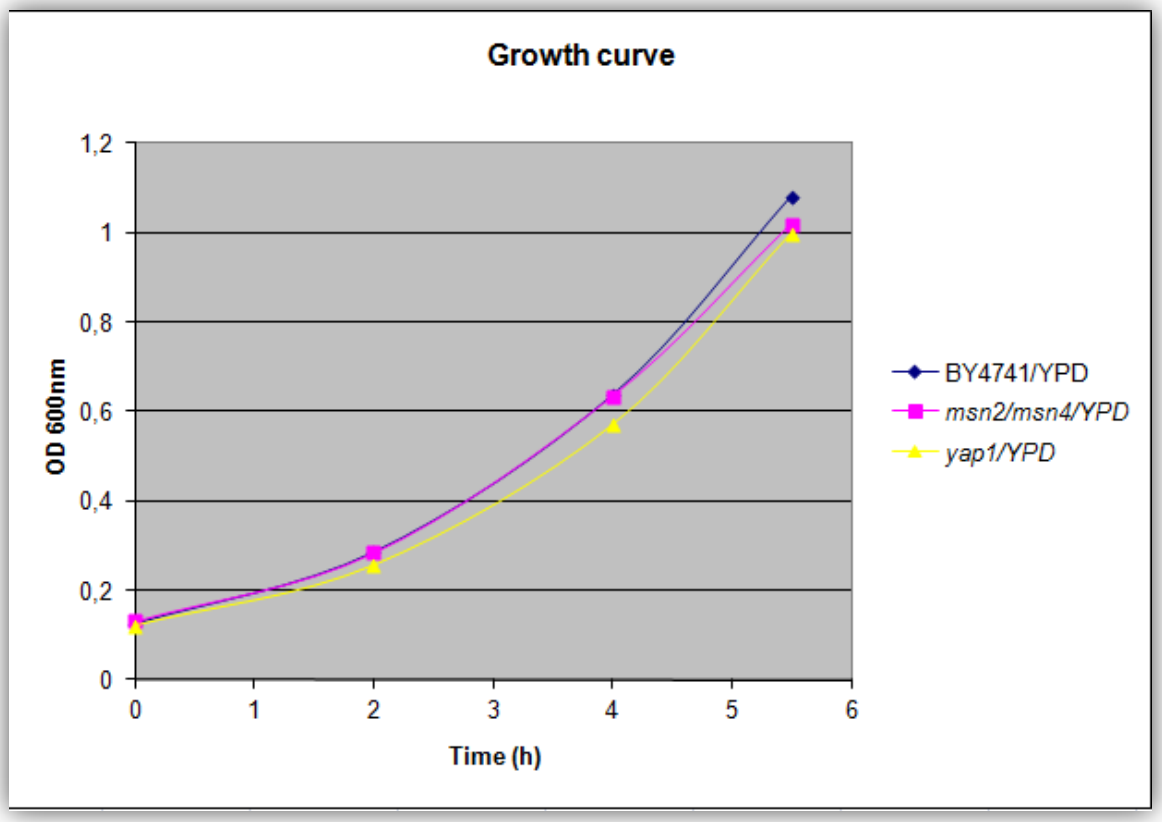


Figure 5. The growth curve in the first 6 hours of growth of yeast strains BY4741 and its mutants *msn2/mns4Δ* and *yap1*Δ in YPD

When the wild type (BY4741) and its mutants were exposed to a different concentrations of ethanol, the growth of wild type was depended on a concentration of ethanol; higer concentration, growth was slower. Four samples were cultivated till OD_{600} was 1. After that, pellets were incubated for 5 hours with differents concentrations of EtOH. OD_{600} was checked every hour. The growth curve of wild type was slower at higher concentration of ethanol (Figure 6).

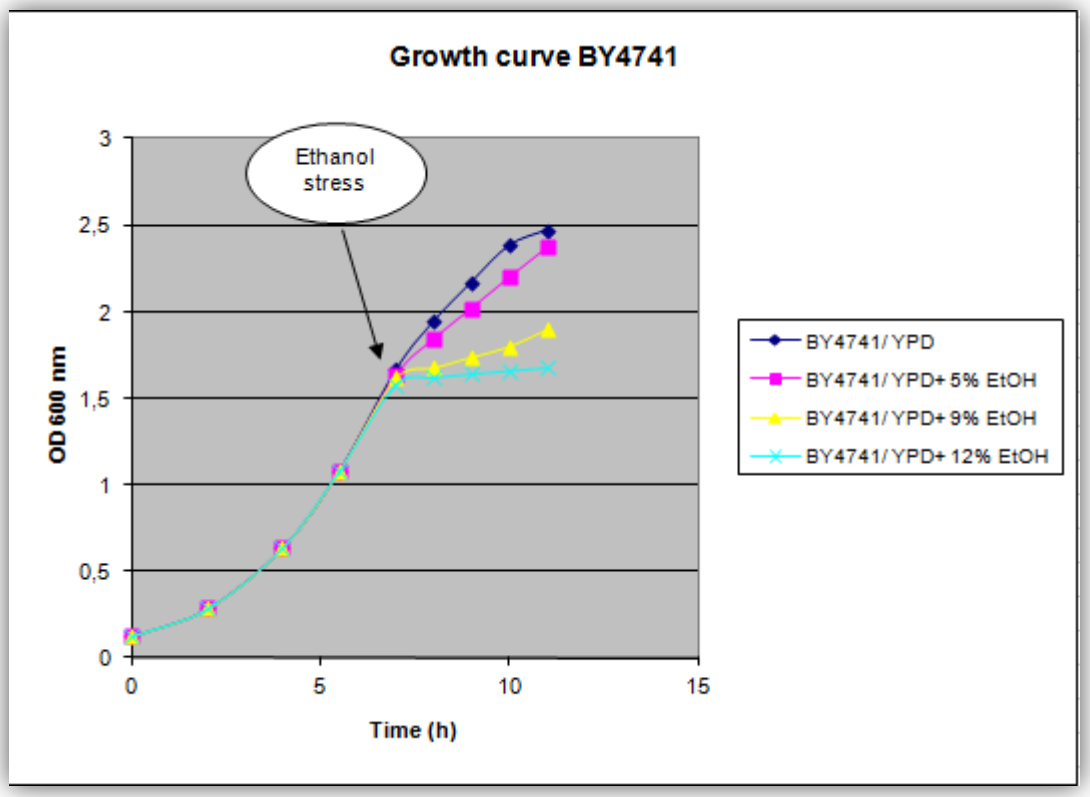
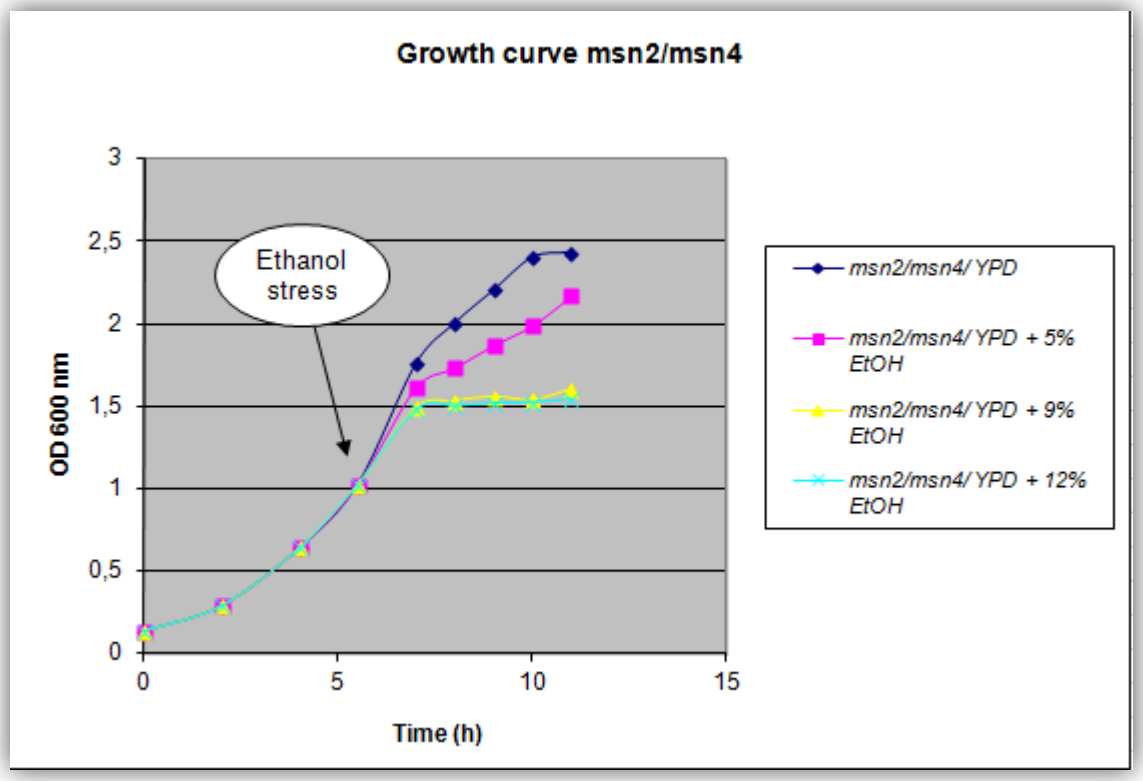


Figure 6. The growth curve of yeast strain BY4741 in YPD medium in first 5 hours till OD_{600} was 1 and after effected with different concentrations of ethanol

The growth curve in Figure 7 shows that different concentrations of EtOH have effects on the number of *msn2/msn4Δ* cells. In the medium without EtOH the number of *msn2/msn4Δ* cells

is higher than in medium with EtOH. Increasing concentration of EtOH, number of



msn2/msn4Δ cells is decreasing.

Figure 7. The growth curve of yeast strain *msn2/msn4* in YPD medium in first 6 hours, till OD_{600} was 1, and after effected with different concentrations of ethanol.

Like in the previous case, Figure 8 shows that different concentrations of EtOH also have effects on a number of *yap1Δ* cells. In medium without EtOH the concentration of *yap1Δ* is higher than in medium with EtOH. Increasing concentration of EtOH growth of *yap1Δ* is decreasing.

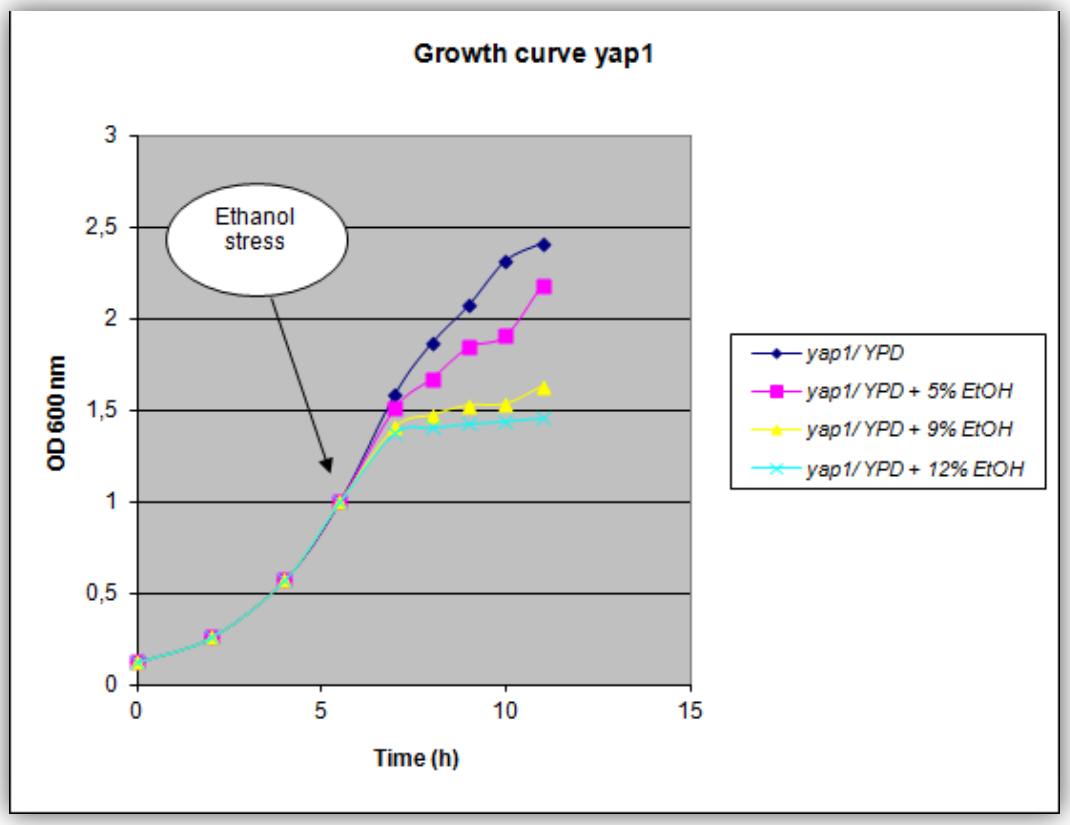


Figure 8. The growth curve of yeast strain *yap1* in YPD medium in first 6 hours, till OD_{600} was 1, and after effected with different concentrations of ethanol.

As shown in figures 6, 7 and 8 the growth of BY4741 is slightly faster than its mutants, *msn2/msn4Δ* and *yap1Δ.* When the suspension reached OD_{600}=1, it was affected with different concentrations of ethanol. At low concentrations of EtOH the growth is faster than at high concentrations. BY4741 is somewhat more resistant to EtOH than its mutants (Figures 6, 7 and 8). Ethanol affects the growth of BY4741 after 7 hours and the growth of its mutants (*msn2/msn4Δ and yap1Δ*) earlier, already after 6 hours. With this experiment it is confirmed that the lack of *MSN2* and *MSN4* led to hypersensitivity to severe stress conditions such as high ethanol concentration.

When cells are exposed to increased ethanol concentrations, they activate specific mechanisms to deal with ethanol stress. These mechanisms not only initiate the repair of macromolecular damages but also establish a state tolerant to certain adverse effects. In this study it was shown that the lost of some genes, as *MSN2*/*MSN4* and *YAP1* have a role in growth under ethanol stress.

4.3.2. Phenotypic test on plates containing ethanol

Cells were grown in YPD medium with and without EtOH in Petri dishes. Their cell density was observed (Figure 10). The percentage of ethanol in YPD medium influenced cell growth. Effect of EtOH on growth is rather similar in wild type and mutants, so the loss of *MSN2*, *MSN4* or *YAP1* did not impair sensitivity to ethanol stress. An example of order of spots and dilutions is shown in Figure 9.

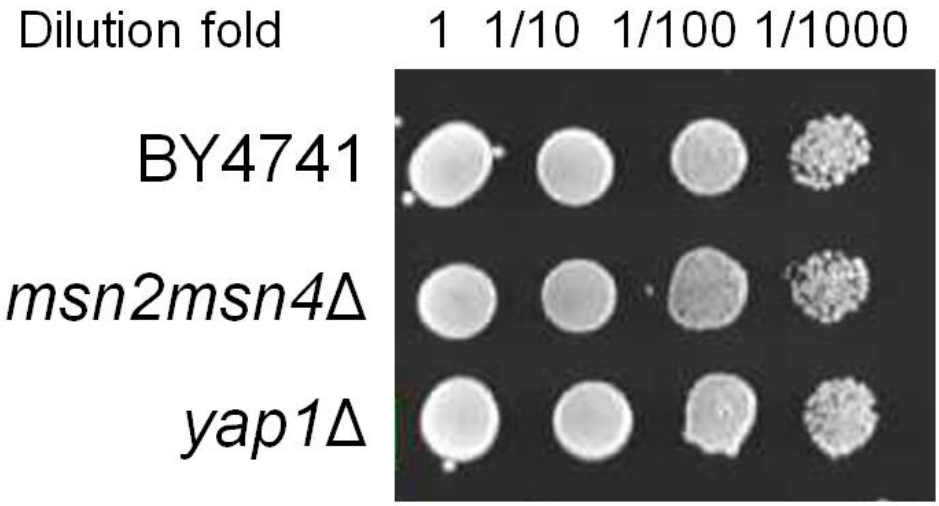


Figure 9. Order of spots of strains and their dilutions.

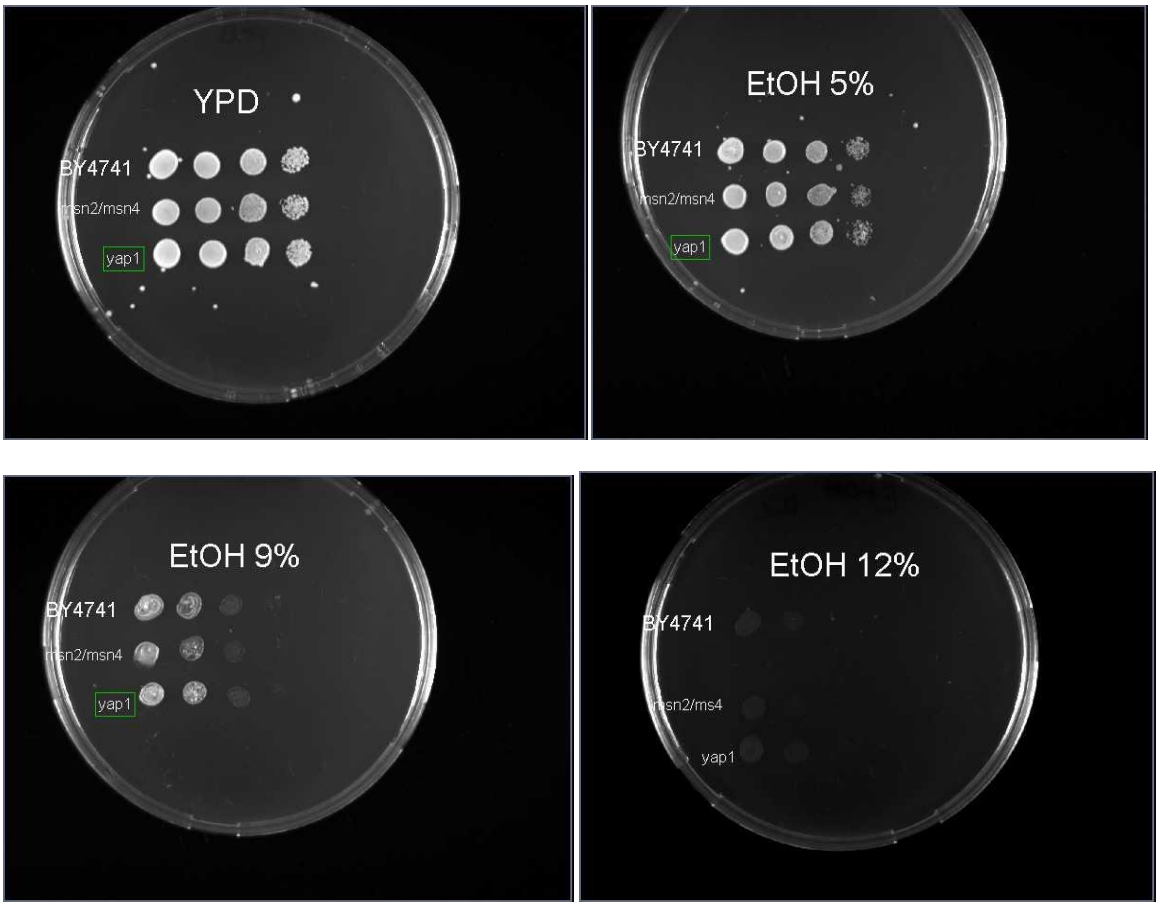


Figure 10. Different cell density due different composition of medium. EtOH has effect on wild type (BY4741) and its mutants, but there is no difference in cell density between wt and its mutants in the same medium.

Different concentrations of ethanol have effect on cell density but comparing cell density of BY4741 with the mutants there is no significant difference (Figure 10). When is a high concentration of ethanol in the plate, Msn2p and Msn4p will bind specifically to STRE-containing oligonucleotides to stimulate the expression of the downstream genes. So when both msn2 and msn4 were deleted, the double mutants were shown hypersensitive to a high concentration of ethanol. In these results mutants are not sensitive than wild type.

4.3.3. Content of polysaccharides in strain *msn2/msn4* (mutant) in a YPD and YPD medium with 9% ethanol

Treatment with acyd hydrolysis and HPAEC- PAD

Components in cell wall of *msn2/msn4Δ* are more represented in YPD medium without EtOH what means that EtOH has effects on amount of cell wall components (Table 11).

Table 11. Concentrations of cell wall components of *msn2/msn4* after acid hydrolysis.

Sample	Chitin (µg mg^{-1})	β-glucans (µg mg^{-1})	Mannans (µg mg^{-1})
***msn2/msn4*Δ in YPD**	14,17 +/- 0,47	448,4 +/- 0,39	274,92 +/- 6,48
***msn2/msn4*Δ in YPD + 9% ethanol**	13,32 +/- 1,62	410,5 +/- 1,6	237,55 +/- 16,51

Treatment with enzymatic hydrolysis and HPAEC- PAD

As it is show in the Table 12, EtOH effects the amonut of chitin, β-1,3- and β-1,6-glucans.

Table 12. Concentrations of cell wall component of *msn2/msn4* after enzymatic hydrolysis.

Sample	Chitin(µg mg^{-1})	β-1,3-glucans (µg mg^{-1})	β-1,6-glucans (µg mg^{-1})
***msn2/msn4*Δ in YPD**	39,7 +//- 4,3	482,5 +/- 64,1	124,6 +/- 60,7
***msn2/msn4*Δ in YPD + 9% ethanol**	23,6 +/- 0,6	269,5 +/- 18,4	116,1 +/- 4,2

In table 13 content of compounds in cell wall of *msn2/msn4*Δ in YPD meduimu and YPD with 9% EtOH is shown. Ethanol reduces the quantity of total carbohydrates, and also has an effect on the growth and amount of compounds in cell walls, especially on the amount of β-1,3- glucans, and here it is confirmed that *MSN2/MSN4* are important genes of *S. cerevisiae*. It is confirmed that *S. cerevisiae* shows a tendency to decrease the amount of glucan under stress conditions such is ethanol stress.

Table 13. Total content of carbohydrates in cell wall of *msn2/msn4* in YPD medium and in YPD + 9% EtOH, shown in percent.

Sample	Total carbohydrates (μg mg^{-1})	% Chitin	% β-1,3 glucan	% β-1,6 glucan	% Mannan
***msn2/msn4Δ*/YPD**	921,7	4,3	52,3	13,5	29,8
***msn2/msn4Δ*/YPD + 9%EtOH**	716,7	3,2	37,6	11,9	26,1

4.3.4. Laminarin test- ethanol effect on cell wall hydrolytic enzymes

Laminarin from *Laminaria digitata (*polysaccharide substrate for laminarinase) is a storage glucan. Its hydrolysis is catalyzed by enzymes such as laminarinase that breaks the β-1-3 bonds. Laminarinase has the activity of exo-β-1,3-glucanase and endo-β-1,3-glucanase. In this experiment ethanol effect on a concentration of the liberated glucose from laminarin, using exo-β-1,3-glucanase and endo-β-1,3-glucanase was shown. In Figure 11 effect of EtOH on the concentration of glucose using exo-β-1,3-glucanase (catalys hydrolysis of glucose units from non-reducing ends of β-1-3-glucans, releasing glucose) is shown. Ethanol affects the concentration of liberated glucose. An increase in the concentration of ethanol leads to a decrease in the concentration of liberated glucose.

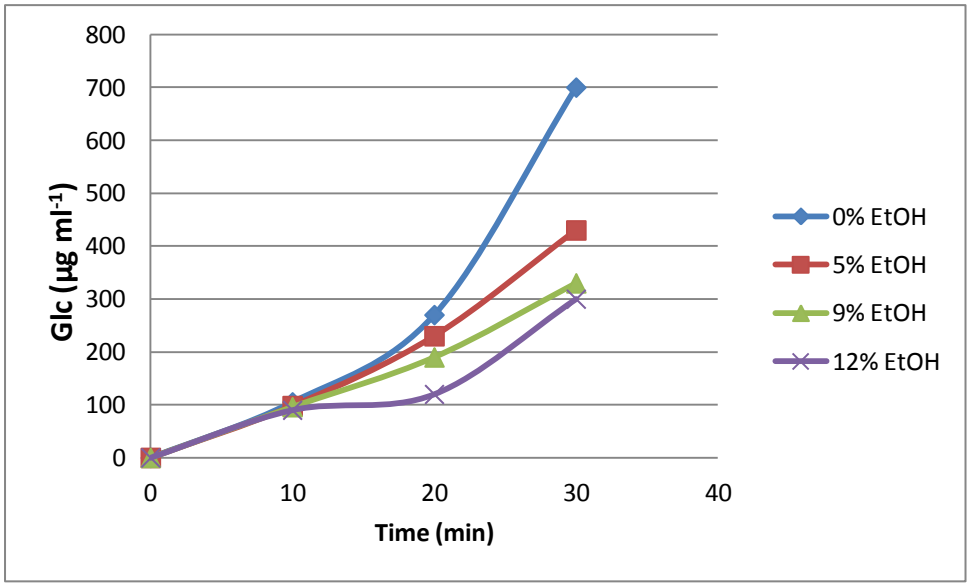


Figure 11. Concentration of liberated glucose from laminarin treated with exo-β-1,3-glucanase. Ethanol effect on a activity of exo-β-1,3-glucanase.

Figure 12 shows how different concentration of EtOH effect on activity of endo-β-1,3-glucanase which catalyses endohydrolysis of laminarin releasing α- glucose.

The concentration of liberated glucose is higher becase exo-β-1,3-glucanase catalyses laminarin hydrolysis from non-reducing ends.

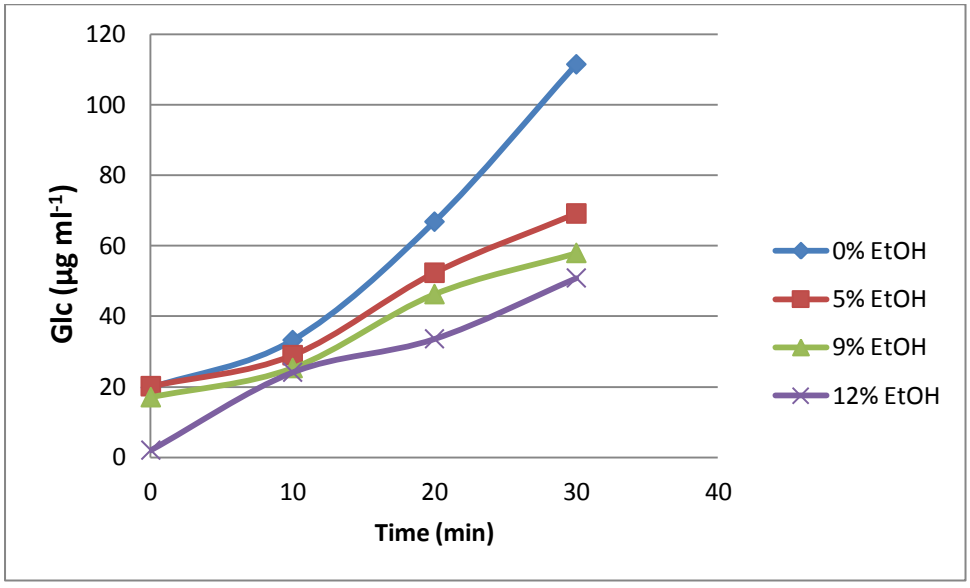
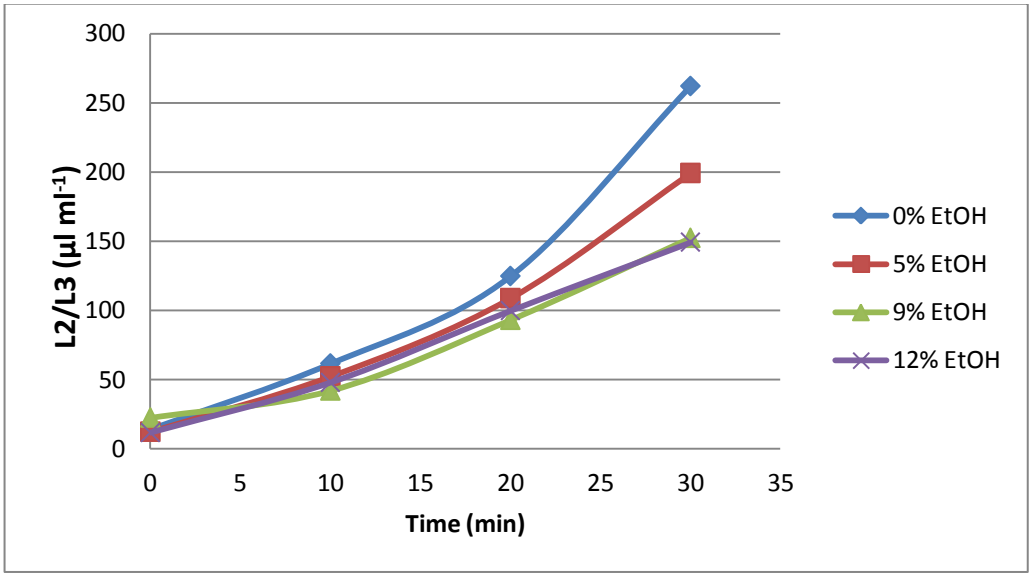


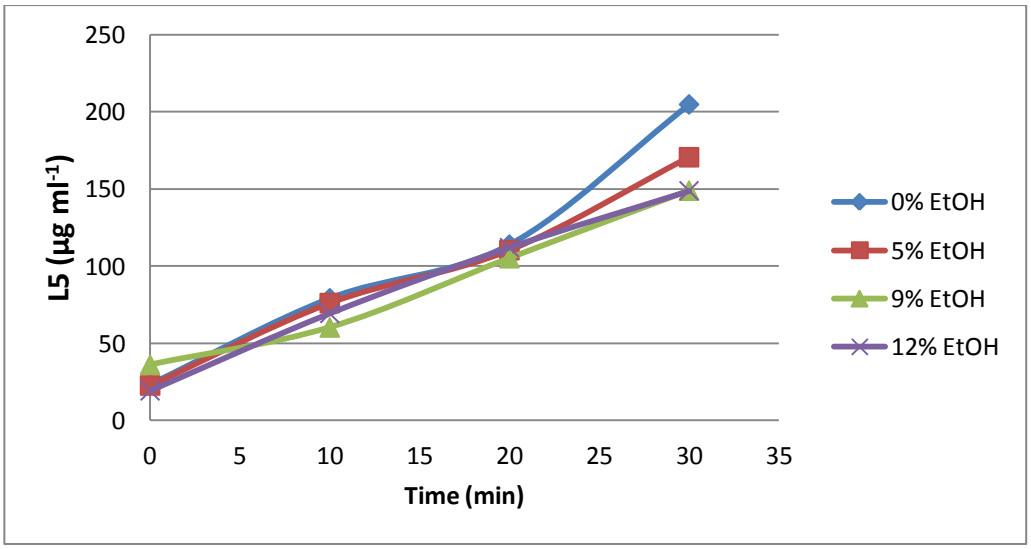
Figure 12. Concentration of liberated glucose from laminarin treated with endo-β-1,3-glucanase. Ethanol effect on a activity of endo-β-1,3-glucanase.

Ethanol also has an impact on the concentration of liberated laminarin's subunits, increasing concentration of ethanol, the concentration of laminarin's subunits is decrasing (Figure 13). L2, L5 and L6 (the number refers to the number of glucoses) are composed of glucose linked by β-1,3 links. Subunits composed of more glucose units takes longer time to liberate.

With the increasing concentration of ethanol the kinetics of relase of longer laminarin units (for example L5 and L6) decreases. In other words it takes longer time to accumulate the same amount of shorter laminarinase units.



13 a)



13 b)

13 c)

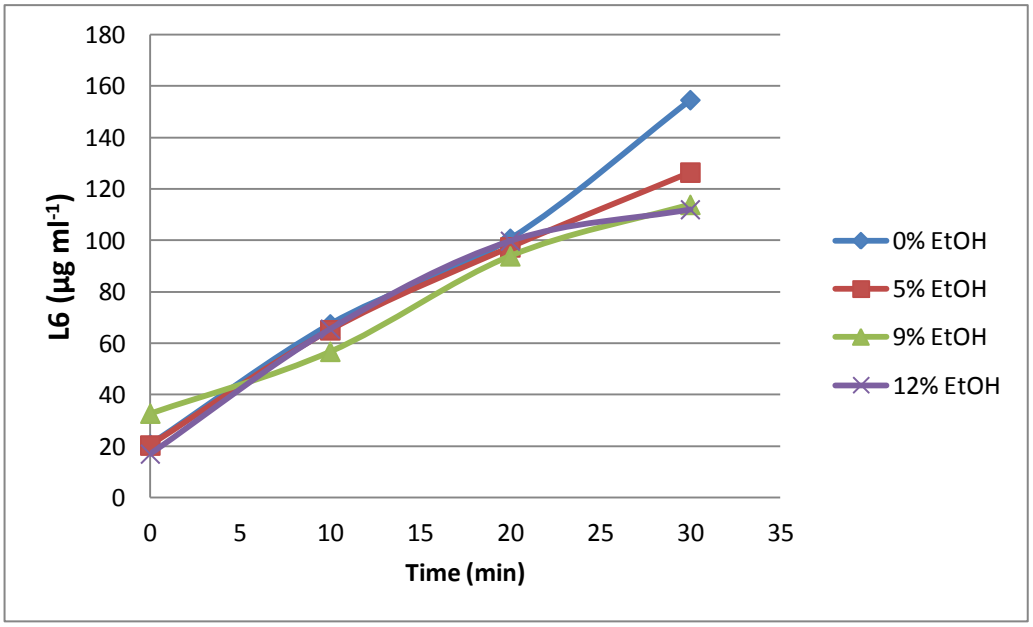


Figure 13. Concentration of liberated L2, L3, L5 and L6 units of laminarin treated with endo-β-1,3-glucanase. 13 a) shown concentration of liberated L2/L3 subunits of laminarin, composed of 2 and 3 glucose units, 13 b) shown concentration of liberated L5 subunit of laminarin, composed of 5 glucose units and 13 c) shown concentration of liberated L6 subunit of laminarin, composed of 6 glucose units.

4.5. Effect of Calcoflour White and Congo Red on wild types and industrial yeast strains

The exposure of *S. cerevisiae* to Calcofluor White and Congo Red induces formation of multicellular aggregates. Calcofluor induces abnormal septa which apparently fail to develop abscission zones between mother and daughter cells probably by inhibiting the action of chitinases. A similar effect was produced in *S. cerevisiae* by Congo red. CFW-based and CR-based susceptibility assays are simple and powerful methods for the identification of fungal cell wall mutants (Figures 14 and 15).

Sensitivity to CFW and CR depends on several parameters. Small differences in the susceptibility of the wild-type and mutant strains might require the use of a series of CFW or CR concentrations to identify the largest growth difference.

As CFW and CR are believed to bind to chitin, these compounds also directly counteract the cell wall stress response itself. Some mutants with lowered chitin levels in their walls become more resistant to CFW and CR.

Most cell wall mutants, however, have more chitin in their walls than wild-type cells, because of activation of the cell wall stress response, and become more sensitive to CFW and CR. Sensitivity of yeast cells to Congo Red (CR) or Calcofluor White(CFW) was tested on YPD agar plates using the different concentrations of the drug. CR and CFW have significant effeect on the growth of yeast strains.

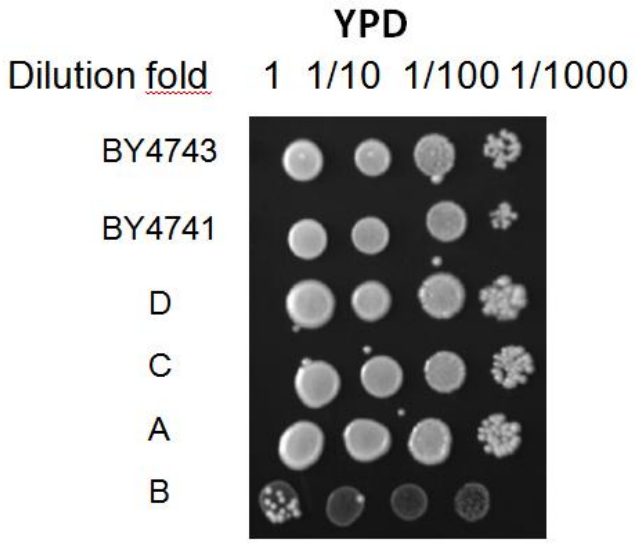
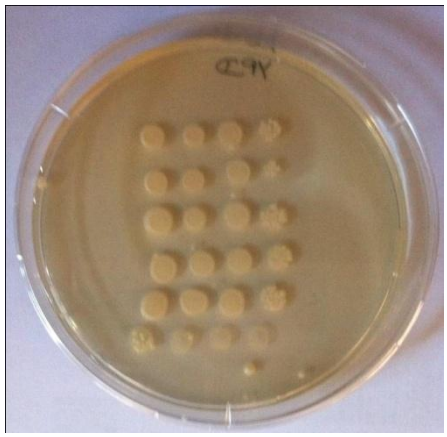


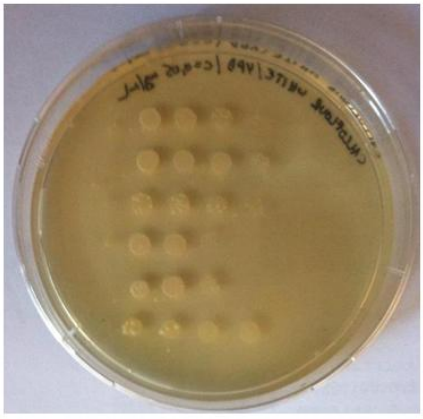
Figure 14. Order of spots of strains and their dilutions; A, B, C, D are industrial products which are yeast cultures autolysed and dried according to industrial process of Lallemand company (confidential).

Plate with YPD (control)

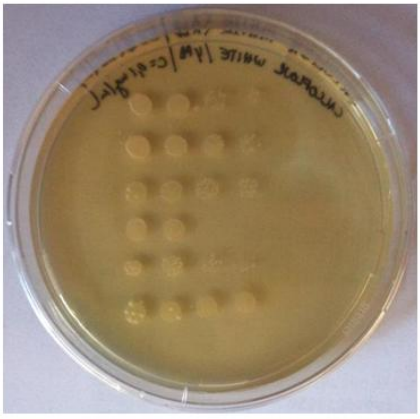


a) plate with CFW c=0.01 mg/ml

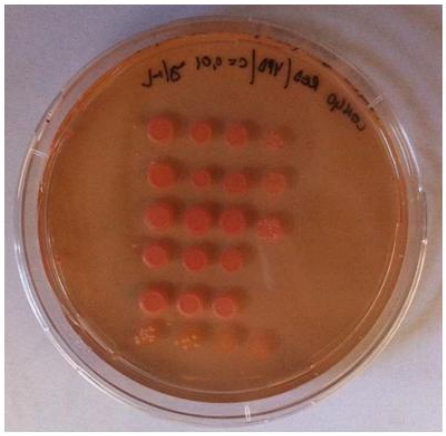
b) plate with CFW c=0.05 mg/ml



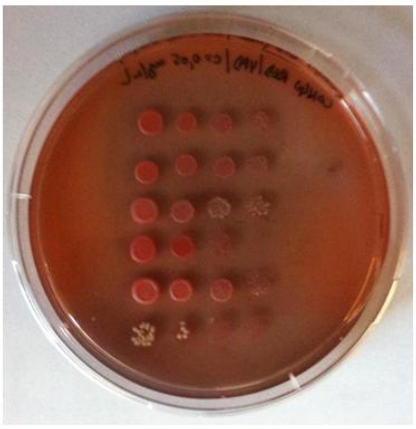
c) plate with CFW c=0,1 mg/ml



d) plate with CR c=0.01 mg/ml



e) plate with CR c=0.05 mg/ml



f) plate with CR c=0,1 mg/ml

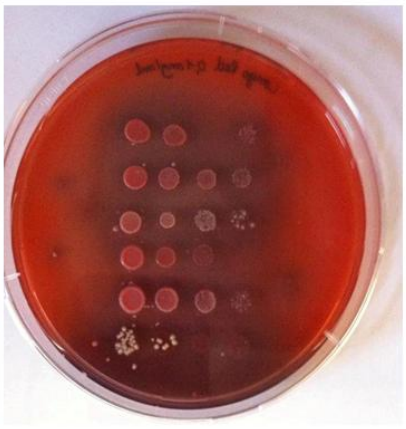


Figure 15. Sensitivity of wild type and mutants to Calcoflour White and Congo Red. Medium rich plate YPD is control plate; 15. a, b and c shows the cell density of samples in YPD medium with different concentration of CFW from 0.01, 0.05 to 0.1; 15. d, e and f shows the cell density of samples in YPD medium with different concentration of CR from 0.01, 0.05 to 0.1.

Plates in Figure 15 show various strains and their resistance to CFW and CR. BY4741 and BY4743 are more resistant to CWF and CR than some industial strains. In Table 9 is shown content of total carbohydrates after acid and enzymatic hydrolysis. It shows that industrial strain C has a very high concentration of chitine in cell wall what is also shown in Figure 15. Industrial strain C is more sensitive to CFW and CR than other industrial strains because strain C has the higher concentration of chitin. This suggest that chitin is target polysaccharide to CFW and CR. *S. Pombe* is not affected by CFW or CR because there is no present chitin in cell wall (Roncero et al., 1985).

5. CONCLUSION AND PERSPECTIVES

1. In this research a newly developed method using combined enzymatic and chemical treatments to quantify the amount of each yeast cell wall component (mannans, β-1,3 and β-1,6-glucans and chitin) in a more accurate manner was worked out. Various cell wall mutants were used. It was observed that growth conditions have a strong effect on the ratio of these components. While a similar approach was previously attempted by others to characterize cell wall structure and/or quantify cell wall polysaccharides composition here is provided additional inputs that allow conducting a simple protocol to achieve this goal, without requiring any fractionation, dialysis procedure or labelling of the yeast cells to trace the fate of the polysaccharides during their separation. The main inputs were to identify and select a very active chitinase to fully hydrolyse chitin into its monomer N-acetylglucosamine, which can be there after determined by a simple colorimetric method. Here is showed that maximal hydrolysis of chitin in the yeast cell wall required the incubation of this bacterial chitinase with endo/exo-β-glucanases, likely because the latter enzymes allowed better access of chitinase to chitin microfibrils, as proposed earlier. Previous methods based on acid hydrolysis were likely not effective enough to release all chitin that is bound to β-(1,3) and β-(1,6)-glucan, and which gives rise to a tightly knit mesh. This new evaluation of the chitin content in the cell wall may better fit with the crucial role of this polymer in yeast morphogenesis.

2. The addition of ethanol caused a delay in the cell cycle resulting in an increase in cell size and cell densitiy. The influence of ethanol stress on the physiology and morphology of yeasts is strain-dependent. Ethanol reduces the quantity of total carbohydrates, and also has an effect on the growth and amount of compounds in cell walls. With this experiment it is confirmed that the lack of *MSN2*, *MSN4* and *YAP1* led to hypersensitivity to severe stress conditions such as high ethanol concentration.

3. It is relevant to notice a good correlation between the sensitivity of the cell wall of wild type and industrial strains to Calcoflour White and Congo Red, two colloidal dyes that are known to bind preferentially to chitin microfibrils and chitin content, as higher this content, greater is the sensitivity to CFW and CR.

4. The results convincingly show that cell wall properties and composition, and especially protein composition, can vary considerably. This indicates that cell wall formation in *S. cerevisiae* is tightly controlled.

5. More work is required to take the yeast specific aspects of wall biogenesis to the next level of understanding. These efforts will involve charting new biochemical territory. With the identification of so many components involved in cell wall biogenesis, there are so many biochemical challenges. With improving knowledge of wall composition, we can look forward to deepening our comprehention of the complexities of yeast cell wall biogenesis.

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