Lead (Pb) toxicity in *Saccharomyces cerevisiae:* the role of cell wall

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Resumo

O chumbo é utilizado em muitos produtos, tais como baterias, gasolina, tintas e corantes, resultando na sua libertação no meio ambiente. Neste trabalho, foi examinado o papel da parede celular da levedura *Saccharomyces cerevisiae* como uma barreira ou como alvo da toxicidade do chumbo.

A biodisponibilidade do Pb é muito reduzida pelos componentes do meio de cultura YEPD, o que dificulta a avaliação da toxicidade deste elemento em concentrações ambientalmente realistas. Para avaliar a toxicidade de Pb em *S. cerevisiae*, em condições de crescimento, foram efetuadas diferentes diluições (10-100 vezes) do meio YEPD, as quais foram misturadas com várias concentrações de Pb (0,1-1,0 mmol/l). Observou-se que o YEPD diluído 25 vezes constituía a melhor condição de compromisso entre o crescimento celular e a precipitação de Pb.

Os genes *CWP1* e *CWP2* codificam para duas grandes manoproteínas da parede celular da levedura *S. cerevisiae*; a deleção destes genes *CWP* aumenta a permeabilidade da parede celular. A suscetibilidade de células de levedura interrompidas no gene *CWP1* (estirpe *cwp1*Δ) ou *CWP2* (estirpe *cwp2*Δ) foi comparada com a da estirpe, isogénica, selvagem (WT). Verificou-se que o crescimento das estirpes *cwp1*Δ e *cwp2*Δ, no meio de cultura YEPD 25 vezes diluído, na presença de Pb, não diferiu do crescimento da estirpe WT. Este resultado sugere que a alteração da permeabilidade da parede celular não altera a sensibilidade de células de levedura ao Pb.

Foi investigada o impacto do Pb na parede celular de levedura. Para este efeito, comparou-se a suscetibilidade ao dodecil sulfato de sódio (SDS), ao calcofluor (CFW) e a uma enzima que degrada a parede da célula (liticase), em células da estirpe WT não expostas ou expostas a Pb durante 4, 8 ou 24 h. Além disso, o conteúdo de quitina da parede celular de levedura foi investigada por coloração das células com CFW. Os resultados não mostraram uma alteração da suscetibilidade ao SDS e ao CFW, nas células tratadas com Pb; contudo, nas células tratadas durante 24 h com Pb, observou-se um aumento da sensibilidade à liticase e um aumento da coloração com CFW. Estes resultados sugerem que o chumbo interage com a parede celular da levedura e influencia a sua composição. Deve ser levado a cabo trabalho adicional a fim de confirmar estes resultados.

Palavras-chave: chumbo (Pb); *CWP1*; *CWP2*; parede celular de levedura; *Saccharomyces cerevisiae*; toxicidade de metais.

Abstract

Lead is used in many products like batteries, gasoline, paints and dyes, resulting in leakage in the environment. In this work, the role of the cell wall of *Saccharomyces cerevisiae* as a barrier or as a target of Pb toxicity was examined.

Pb bioavailability is greatly reduced by YEPD medium components, which difficult the evaluation of Pb toxicity under environmentally realistic concentrations. To assess Pb toxicity to *S. cerevisiae,* in growing conditions, different dilutions (10-100 times) of the YEPD medium were made and mixed with several Pb concentrations (0.1-1.0 mmol/l). It was observed that YEPD 25 times diluted was the best compromising condition between cell growth and Pb precipitation.

CWP1 and *CWP2* genes encode two major mannoproteins of yeast cell wall of *S. cerevisiae*; deletion of these *CWP* genes enhances cell wall permeability. The susceptibility of yeast cells deleted on *CWP1* (*cwp1*Δ strain) or *CWP2* genes (*cwp2*Δ strain) was compared with the isogenic wild-type (WT) strain. It was found that the growth of *cwp1*Δ and *cwp2*Δ strains, in 25 times diluted YEPD medium, in presence of Pb did not differ from the growth of the WT strain of *S. cerevisiae*. This result suggests that the modification of cell wall permeability does not affect the sensitivity of yeast cells to Pb.

The impact of Pb on yeast cell wall was investigated. For this purpose the susceptibility to sodium dodecyl sulphate (SDS), calcofluor white (CFW) and cell wall-degrading enzyme (lyticase) were compared in cells of the WT strain not exposed or exposed to Pb for 4, 8 and 24 h. In addition, the chitin content of yeast cell wall was investigated by staining the cells with CFW. The results showed no modification of the susceptibility to SDS and CFW, an increased sensitivity to lyticase and an increase of cell staining with CFW, in Pb treated cells for 24 h. These results suggest that Pb interacts with yeast cell wall and influences its composition. A further work should be carried out in order to confirm these results.

Keywords: yeast cell wall; lead (Pb); *CWP1; CWP2*; metal toxicity; *Saccharomyces cerevisiae*

Abstract

Lood wordt gebruikt in vele producten zoals batterijen, brandstof, verf en kleurstoffen, waardoor het ook in de natuur terecht komt. In dit werk werd de rol van de celwand van *Saccharomyces cerevisiae* als barrière en doelwit voor Pb toxiciteit onderzocht.

De bio-beschikbaarheid van Pb verlaagd door componenten van YEPD medium, waardoor onderzoek van de toxiciteit in biologisch realistische concentraties bemoeilijkt wordt. Om de Pb toxiciteit bij *S. cerevisiae* in groeiomstandigheden te onderzoeken, werden verschillende verdunningen (10-100 keer) van het YEPD medium gemaakt, waaraan Pb in verschillende concentraties (0,1-1,0 mmol/l) werd toegevoegd. Uit waarnemingen bleek dat 25 keer verdund YEPD medium de beste compromis is tussen celgroei en Pb precipitatie/ complexvorming.

De genen *CWP1* en *CWP2* coderen voor twee belangrijke mannoproteinen van de celwand van *S. cerevisiae*; verwijderen van deze *CWP* genen verhoogt de permeabiliteit van de celwand. De gevoeligheid van gistcellen zonder het *CWP1* gen (*cwp1*Δ stam) of zonder het *CWP2* gen (*cwp2*Δ stam) werd vergeleken met de gevoeligheid van het isogene wilde type (WT). De groei van de *cwp1*Δ en de *cwp2*Δ stam, in 25 keer verdund YEPD medium, in aanwezigheid van Pb, verschilde niet van de groei van WT *S. cerevisiae*. Dit veronderstelt dat modificatie van de permeabiliteit van de celwand de sensitiviteit van gistcellen voor Pb niet beïnvloedt.

De impact van Pb op de celwand van gistcellen werd onderzocht. Hiervoor werd de gevoeligheid voor natrium dodecyl sulfaat (SDS), calcofluor white (CFW) en het celwand afbrekend enzym lyticase bij gistcellen van het wilde type onderzocht. De gevoeligheid werd vergeleken tussen cellen die niet blootgesteld werden aan Pb en blootgesteld werden aan Pb op de tijdstippen 4, 8 en 24 uur. Ook werd de hoeveelheid chitine in de gistcelwand onderzocht met behulp van kleuring met CFW. De resultaten tonen aan dat de gevoeligheid voor SDS en CFW, bij cellen 24 u blootgesteld aan Pb, niet verandert en de gevoeligheid voor lyticase toeneemt na 24 uur. Dit veronderstelt dat lood interactie aangaat met de celwand van gist en een invloed heeft op de samenstelling van de celwand. Verder onderzoek moet uitgevoerd worden om deze resultaten te bevestigen.

Kernwoorden: gistcelwand; lood (Pb), *CWP1; CWP2*; metaal toxiciteit; *Saccharomyces cerevisiae*

Work objectives

The first objective of this work was to find an appropriate medium to assess the Pb toxicity in yeast cells in growing conditions. The main objectives were to determine if the yeast cell wall is a barrier for Pb toxicity, by using gene-specific disruption mutants, and if the yeast cell wall is a target for Pb toxicity in growing conditions, by evaluating the effect of Pb on the level of chitin in the cell wall and the sensitivity to lyticase, CFW and SDS.

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1 Introduction

1.1 Use of Pb

Lead is a natural element of the crust of the earth. The most common forms of lead are lead acetate, lead chromate, lead chloride, lead oxide and lead nitrate (Steunpunt beleidsrelevant onderzoek 2014).

The worldwide production and consumption of lead has increased over the past 5 years (table 1). Lead is used in mine production and in the metal industry. The production of refined lead metal decreased in the past 5 years in the United States, but the production in Asia increased resulting in a total rise in production (International Zinc and Lead study group 2015).

Table 1: Worldwide use of Pb*

World Refined Lead Supply and Usage 2009 - 2014											
000 tonnes	**2009**	**2010**	**2011**	**2012**	**2013**	**2013 Jan-Nov**	**2014 Jan-Nov**	**2014 Aug**	**Sep**	**Oct**	**Nov**
Mine Production	3816	4168	4644	5035	5435	4977	4836	470.3	440.5	475.9	512.9
Metal Production	9247	9850	10606	10550	11122	10174	10300	979.5	979.5	993.3	1027.2
Metal Usage	9242	9812	10444	10484	11120	10194	10299	963.8	968.0	1007.1	1031.3

*Source: International Zinc and Lead study group (2015)

Batteries of cars, shots, ammunition, pipes, weights, cable covers, radiation shields, paints, dyes, ceramic glazes, caulk and stained glass can contain lead. Cheap jewellery, hair dyes and cosmetics can contain lead acetate (Abadin et al. 2007).

Lead is added to gasoline to improve the quality and increase the octane ratio. In large parts of the world, including Europe and the United States of America, it is forbidden to add lead to gasoline. In developing countries, lead containing gasoline is still used and the main source of lead in the air (Fig 1) (Fewtrell et al. 2003).

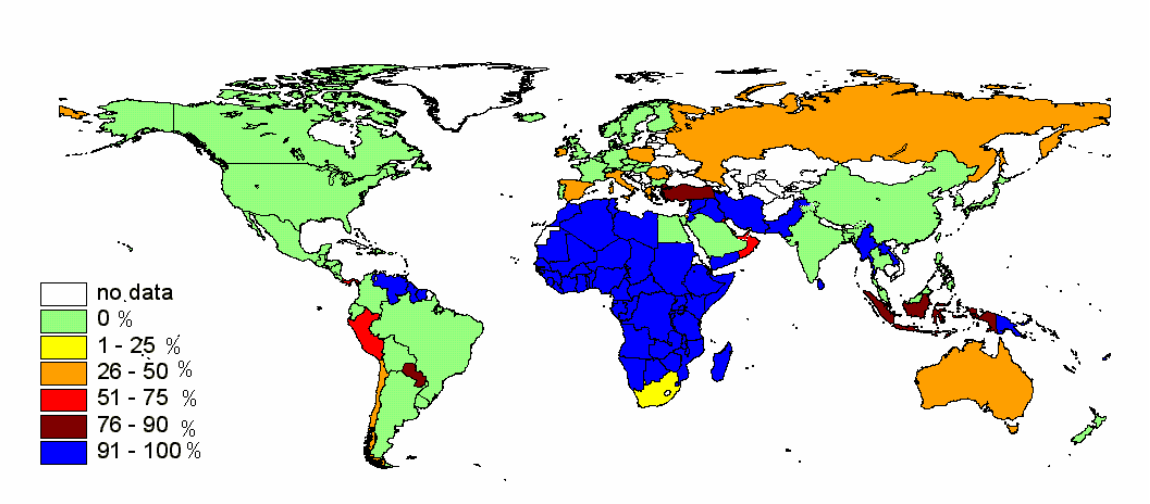

Figure 1: Sales of lead containing gasoline as a percentage of the total gasoline sales. Source: Fewtrell et al. (2003)

As shown in Fig 1, in Africa, lead containing gasoline is still sold more than gasoline without lead. This results in higher levels of Pb in the air. In North-America and Western Europe, lead containing gasoline is not sold anymore (Fewtrell et al. 2003).

1.2 Lead in the environment

The main sources of lead in the environment are storage batteries of cars and shots and ammunition. The industrial sources of lead are mined ores, recycled metal, oil, coal and waste products (Abadin et al. 2007).

The exhaust of vehicles using lead containing gasoline is the main source of lead in the air. Nowadays, as it was reported above, leaded gasoline is forbidden in many countries, resulting in a significantly decrease of levels of lead in the air and in blood of the population (Meyer et al. 2008). The industry related Pb in the air mainly comes from burning oil, waste and coal. Earlier, pesticides for orchards also contained lead. These pesticides aren't used anymore (Abadin et al. 2007).

When lead based paint weathers off, it can enter the soil or the surface water. Landfills with waste from mining, ammunition, industrial activities are also sources of Pb in the soil. Because Pb sticks to the soil, it remains in the upper levels. Lead from pipes can be released into the water when the water is acidic and enter the soil (Abadin et al. 2007).

In Europe and the United States, there are guidelines and regulations to reduce the levels of lead in the environment and limit the exposure to lead. Nowadays, it is not allowed to add lead to gasoline or use lead in water pipes and paints. These regulations are in order to protect the population from the negative effects of lead on health (Abadin et al. 2007).

1.3 Toxic effect of Pb on humans

Lead can enter the human body by breathing in dust or chemicals containing lead. From the lungs, Pb enters the blood. When larger particles are breathed in, they are coughed up and swallowed. Lead can also enter the body with food. Only very small amounts of lead can enter the body through intact skin. When the skin is damaged, Pb can enter easily. The blood passes lead through the body to the organs. After a few weeks, the lead is stored in the teeth and bone. The amount that is not stored, is excreted in urine and faeces (Abadin et al. 2007). The poisoning caused by the presence of lead or lead salts in the body is known as plumbism or saturnism.

The nerve system is the main target for lead toxicity. Long-term exposure can lead to weakness in joints, an elevated blood pressure, anaemia and eventually organ failure. In small children, the consequences are even worse. A long-time exposure to high levels of lead can result in organ failure and eventually death (Abadin et al. 2007; Meyer et al. 2008). Fig 2 summarizes and correlates the levels of Pb in blood with the toxic effects in children and adults.

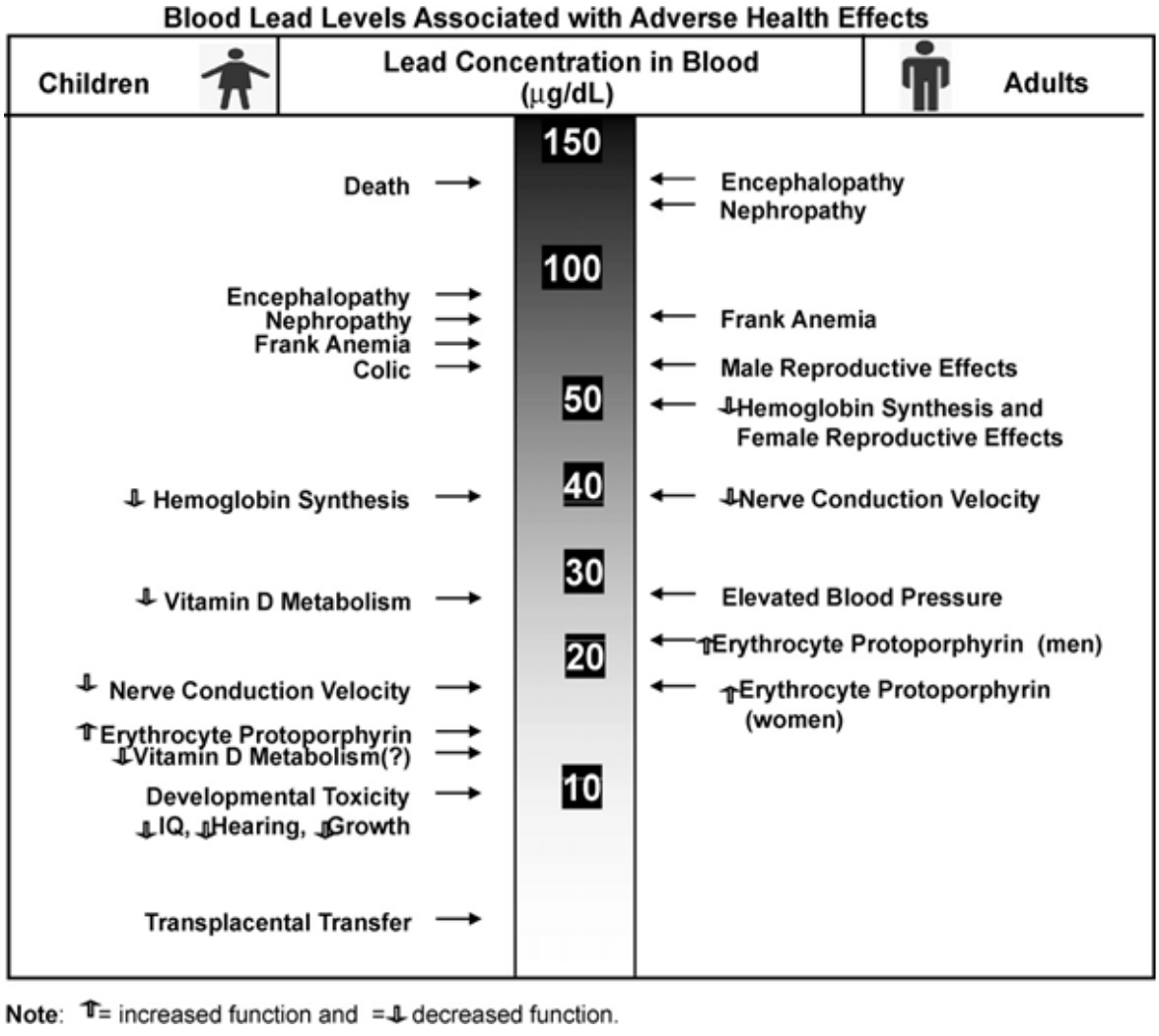

Figure 2: Effects of Pb on humans and adults. Source: Meyer et al. (2008)

Since only small amounts of lead reach the blood, most of the lead is stored or excreted and exposure levels are relatively small. These effects are not frequent in developed countries. People who live near waste sites, live in old houses, work in or nearby lead using companies and children are risk groups. In developing countries, especially due to poor work conditions and less strict safety regulations, lead poisoning is still an issue (Abadin et al. 2007; Meyer et al. 2008).

1.4 Toxic effects of Pb on *Saccharomyces cerevisiae*

On contrary to Zn or Cu, Pb is not a necessary growth factor for yeasts and it has no biological function. When *Saccharomyces cerevisiae* cells were exposed to Pb a loss of viability was observed (Heggen et al. 2010). Yeast cells exposed to lead, accumulated reactive oxygen species (ROS) intracellularly. DNA, lipids and proteins are very sensitive to ROS and are affected. Carbohydrates are less sensitive. Accumulation of ROS also leads to cell apoptosis. Apoptosis is a regulatory mechanism to remove death and damaged cells and for homeostasis (Bussche and Soares 2011). Pb also impaired ammonium assimilation and decreased the DNA/RNA ratio. Cells in lead containing medium also had an extended lag-phase (Chen and Wang 2007).

When the yeast cells were exposed to 1 mmol/l Pb for 6 hours, the cell membrane remained intact but the proliferation capacity was lost on solid YEPD medium. This loss of proliferation capacity was induced by the larger production of ROS (Bussche and Soares 2011). ROS are formed during the normal cell metabolism. When exposed to Pb, mitochondria are the main sources of ROS. ROS are by-products of the respiratory chain. Mitochondria are also the main target of ROS toxicity induced by Pb (Sousa and Soares 2014).

There are several mechanisms how ROS can be formed. These reactions (Fig 3) are catalysed by metals (e.g. Cu, Cr, Fe) and radicals are formed (Avery 2001; Wysocki and Tamás 2010). UV-radiation, ionizing radiation, ozone, heath and pesticides also induce forming of ROS (Dimova et al. 2008). Exposure of yeast cells to Pb resulted in an intracellular accumulation of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Sousa and Soares 2014). ROS formed can be partially reduced by the anti-oxidative redox system (AORS) to limit damage (Hosiner et al. 2014).

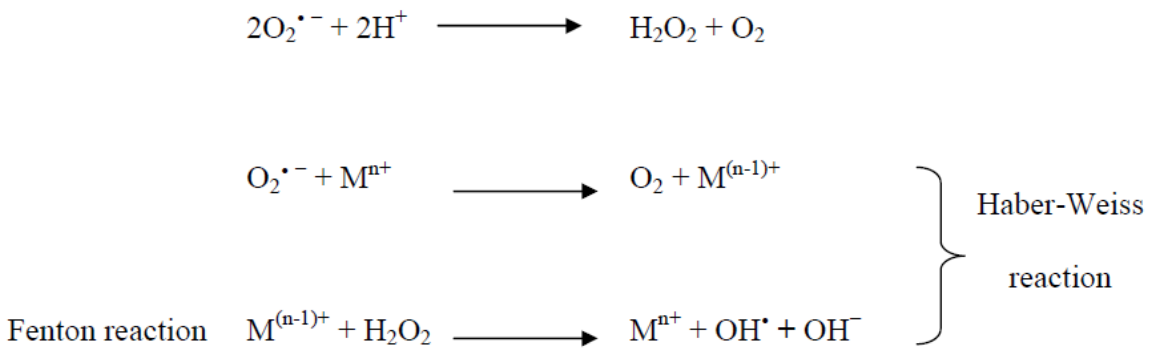

Figure 3: Reactions forming ROS. Source: Avery (2001).

Redox-inactive metals like Pb induce the forming of ROS indirectly. For example, Pb can inhibit enzymes with the function of antioxidants and stimulates a depletion of glutathione. Recently, it was described that Pb decreased the intracellular level of reduced glutathione in yeast cells (Perez et al. 2013). Pb also disturbs the lipids of the membrane and sensitizes these lipids for lipid oxidation. An elevated level of ROS results in protein, lipid and DNA oxidation (Avery 2001; Wysocki and Tamás 2010).

1.4.1 Oxidation of lipids

Lipid peroxidation can be enzymatic or non-enzymatic. In the presence of metals and oxygen, no enzymes are needed to catalyse the reaction. Unsaturated lipids of the cell membrane are highly susceptible for lipid peroxidation (Avery 2001). Polyunsaturated fatty acids contribute to membrane structure, fluidity, permeability and flexibility (Repetto et al. 2012). Polar lipid hydroperoxides, formed during lipid peroxidation, can cause an increase in membrane fluidity and thereby change the characteristics and functions of the membrane. The membrane permeability also increases. Even membrane disintegration and cell death can occur in case of high levels of peroxidation. By-products of lipid peroxidation may induce protein oxidation and DNA damage (Avery 2001). The effects of lipid peroxidation of the cell membrane are shown in Fig 4. When Pb or other positively charged elements binds to the negatively charged heads of the phospholipids on the membrane, the membrane becomes more sensitive to oxidative damage (Repetto et al. 2012).

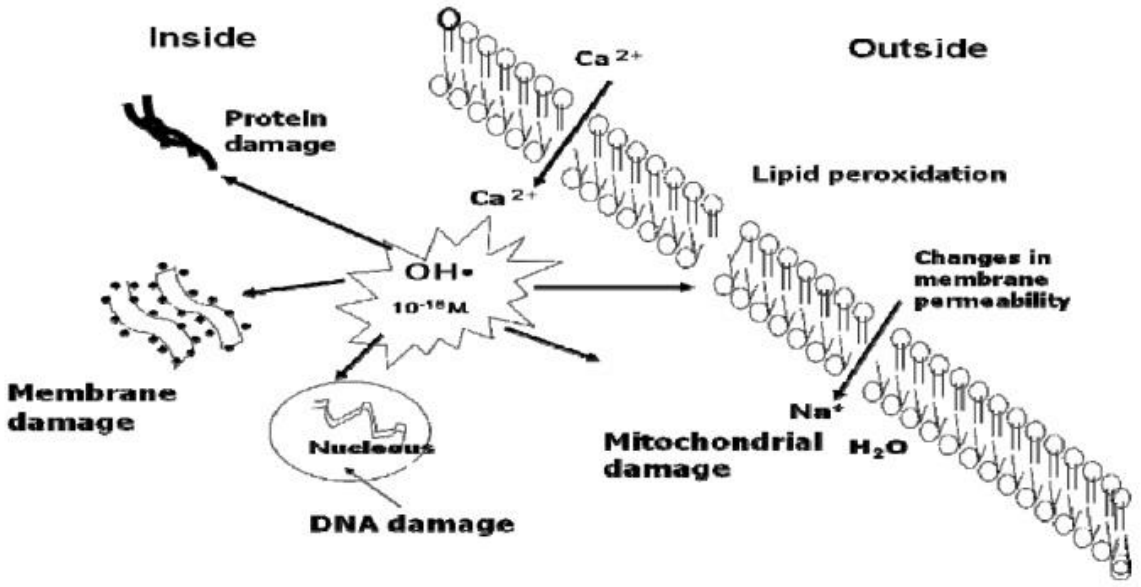

Figure 4: Mechanism and effects of lipid peroxidation. Source: Repetto et al., 2012.

The first step of lipid peroxidation is either hydrogen abstraction or addition of an oxygen radical. This is the initiation-step. In a next step, oxygen is added to the carbon-centred radical (R·) and a lipid peroxyl radical (ROO·) is formed. These radicals can react with other unsaturated lipid resulting in a new formed radical (propagation). Termination reactions can destroy the radicals and occur when two radicals react (Repetto et al. 2012). These reactions are shown in Fig 5.

$$R_1H + R\cdot \rightarrow R_1\cdot + RH \qquad \text{initiation}$$

$$R\cdot + O_2 \rightarrow ROO$$

$$R_1H + ROO\cdot \rightarrow R_1\cdot + ROOH$$

$$ROO\cdot + ROO\cdot \rightarrow ROH + RO\cdot + {}^1O_2 \qquad \text{termination}$$

Figure 5: Lipid oxidation reactions. Source: Repetto et al. (2012).

When lead or other metals are present, they can react with ROOH resulting in a raise of newly generated radicals (Fig 6) (Repetto et al. 2012).

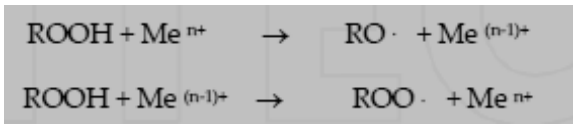

Figure 6: Lipid peroxidation reactions in the presence of metals. Source: Repetto et al. (2012).

1.4.2 Effects on proteins

Metals can effect proteins in different ways: by oxidation or by binding the proteins. When metals bind enzymes that protect cells from oxidative stress, ROS levels increase. Metals most commonly bind thiol groups of cysteine residues (Wysocki and Tamás 2010).

The susceptibility of proteins to oxidation differs and depends on the different side chains of the amino acids. When residues of amino acid oxidation accumulate, other proteins are damaged. This results in structural changes and inactivation. Some proteins are sensitive to both metals and oxidation (Avery 2001). Metals can bind the active site of proteins and inhibit the functions of the protein (Wysocki and Tamás 2010).

Fig 7 summarizes the mechanism and effects of protein-damage by ROS. An increased level of ROS due to the presence of Pb results in an accumulation of oxidized proteins. Some proteins can be repaired, but others are too damaged and are degraded by vacuolar proteases or by the 20S proteasome. Oxidized proteins can also be cross-linked resulting in oxidized protein aggregates. These aggregates effect the function of the mitochondria resulting in an increased ROS-production (Costa et al. 2007).

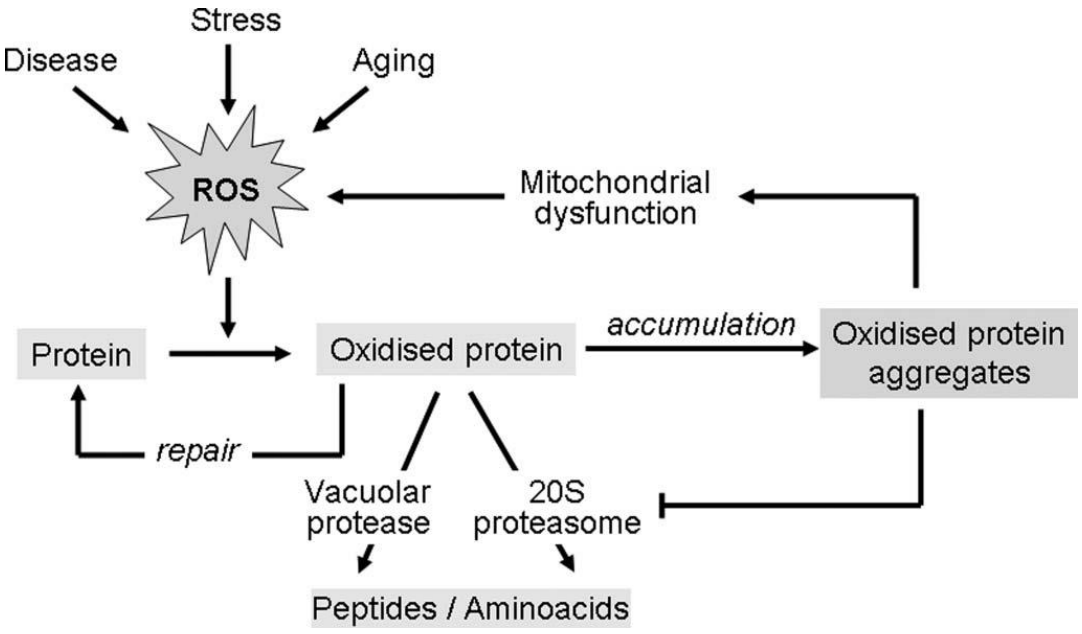

Figure 7: Effect of ROS on proteins. Source: Costa et al. (2007).

1.4.3 Effects on DNA

Metals do not directly influence DNA, but can interfere with the process of DNA repair. For example, Cd inhibits the DNA mismatch repair system resulting in higher levels of mutations and defects (Wysocki and Tamás 2010).

DNA oxidation, due to increased ROS levels, results in modification of bases, cross-linking between proteins, smaller chains and depurination. These modifications can result in mutagenicity or cell death when there is extensive damage (Avery 2001). Mitochondrial DNA is more sensitive to oxidative damage than nuclear DNA (Sousa and Soares 2014).

Certain metals e.g. Co, Cr(VI), Fe and Ni catalyse redox reactions, resulting in products that oxidize DNA. Cr(V) is reduced in cells by different factors, one of them being glutathione which is normally a protective factor against oxidative damage, resulting in reactive species containing Cr(VI) and Cr(V). During reduction of Cr-species, oxygen, carbon and sulphur containing radicals are formed. Other metals (Cu, Co, Fe and Ni) result in accumulation of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) which can damage DNA (Bal and Kasprzak 2002).

1.4.4 Other effects of metals

Apart from DNA, protein and lipid damage, metals can have all kinds of other effects. For instance, Cr interferes with mRNA translation; As(III) damages the actin and tubulin cytoskeleton and its syntheses. Metals can disrupt protein folding as well (Wysocki and Tamás 2010).

1.5 Metal uptake

Heavy metals can use the same pathways and transporters as crucial metals and other nutrients. The different pathways of metal uptake are shown in Fig 8. As(V) enters the cell through phosphate transporters (permeases) because of its similar structure to phosphate. As(III) is taken up through the Fps1p transporter. This transporter can also be used for Sb(III)-uptake and for efflux of As(III) and Sn(III). When three $As(OH)_3$ molecules form a ring structure, hexose transporters recognize the structure as hexose sugars and As(III) can enter the cell. Cd can also enter the cell through different pathways. Cd used transporters for the uptake of essential metals. Zrt1p is a transporter for Zn-uptake, Smf1p for Mn-uptake, Fet4p for Fe-uptake and Mid1p for Ca-uptake. Cr(VI), Mo(VI) and Se(VI) have similar structures as sulphate and can be imported through sulphate transporters (Wysocki and Tamás 2010).

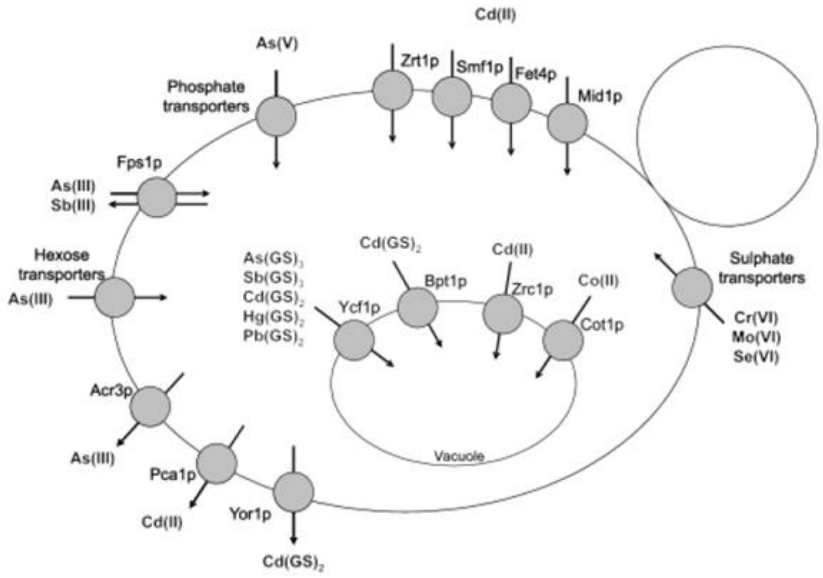

Figure 8: Metal uptake and detoxification (efflux) in yeast cells. Source: Wysocki and Tamás (2010).

Pb can enter cells via uptake systems for essential cations, such as divalent transporter (DMT1) (Au et al. 2008). Yeast cells overexpressing DMT1 displayed an increased Pb uptake (Bannon et al. 2002).

1.6 Metal detoxification

Metal can be removed from the cytosol by three different mechanism. Metals can be exported through different pathways: they can be chelated to proteins or peptides, transported across the cell membrane or they can be compartmentalized in the vacuole (Wysocki and Tamás 2010).

1.6.1 Efflux of metals

Transporters for the efflux of metals are highly specific (Fig 8) and tightly regulated by various mechanisms. The efflux of As(III) is catalysed by a prototype member of the arsenical resistance-3 family of transporters (Arc3p). Cells without the *ARC3* gene are highly sensitive to As(III). Arc3p is highly specific and normally does not transport Sb(III). However, yeast without the *ARC3* gene appeared also more sensitive to Sb(III) (Wysocki et al. 2001). When As(V) is present, it is converted to As(III) by Arc2p, an arsenate reductase, before export out of the cell. Contrary to Arc3p, the transporter Fps1p can export As(III) and Sb(III). This transporter can be used to export, but also to import the metals (Rosen 2002; Wysocki and Tamás 2010).

To export Cd, the Pca1p-transporter is used. This transporter belongs to the P_{1B}-type ATPase super-family. Yor1p, a transporter from the ATP-binding cassette (ABC) transporter family, can also export Cd (Wysocki and Tamás 2010).

1.6.2 Vacuolar sequestration

ABC-transporters catalyse transport of different substrates through the plasma and vacuolar membrane. ABC-transporters have a membrane spanning domain (MSD) and a nucleotide binding domain (NBD). These transporters are divided into subclasses. Subclass C (ABCC-transporters) contains vacuolar transporters (Paumi et al. 2009).

The main pathway for metal sequestration is the ABCC-transporter Ycf1p (Fig 8). Cells without this transporter are more sensitive to Pb and other metals (e.g. Cd, Hg, Cd). Ycf1p transports $As(GS)_2$, $Pb(GS)_2$, $Cd(GS)_2$, $Sn(GS)_2$ and $Hg(GS)_2$ actively into the vacuole (Wysocki and Tamás 2010). Vmr1p, Ybt1p and Bpt1p are other vacuolar transporters with a function similar as Ycf1p (Sousa et al. 2015).

Before the transport of metals can take place, the metals have to be bound to glutathione. Glutathione transferases (GSTs) are responsible for this action. Two GSTs catalyse the reaction with Pb: glutathione transferase 1 (GTT1) and glutathione transferase 2 (GTT2). The whole mechanism of detoxification by vacuolar sequestration mediated by ABCC transporters is shown in Fig 9 (Sousa et al. 2015).

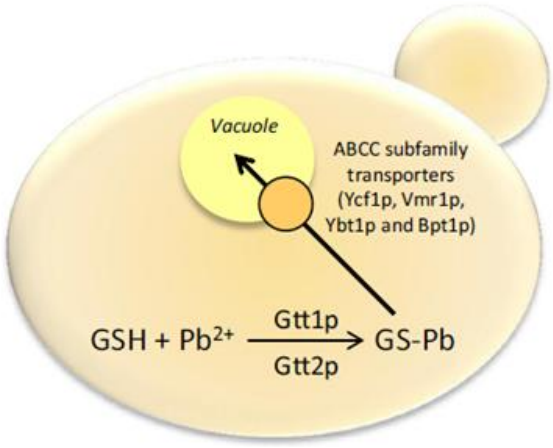

Figure 9: Vacuolar sequestration of Pb using ABCC subfamily transporters. Source: Sousa et al. (2015)

Vacuolar H^+-ATPase (V-ATPase) is also involved in vacuolar transport and sequestration of Pb. V-ATPase is responsible the acidic character of the vacuole. The H^+ gradient is necessary for the process of Pb-sequestration (Sousa et al. 2014).

1.6.3 Chelation

Different proteins and peptides can be used to chelate metals. Proteins and peptides with a low molecular weight and which contain a lot of cysteine take part in the detoxification process. For example, glutathione (GSH) and metallothioneins (MTs) have an important role in the process. By chelation, the reactivity and toxicity of the metals is reduced (Wysocki and Tamás 2010).

S. cerevisiae contains two types of MTs: Cup1p and Crs5p. The main function of Cup1p is Cu detoxification, but Cup1p can also bind Cd and Zn. Crs5p contains more cysteine than Cup1 and its main role is the homeostasis of Zn. Crs5p can also bind Cu and Cd (Wysocki and Tamás 2010).

GSH or l-γ-glutamyl-l-cysteinyl-glycine is an electron donor for enzymes, the most important redox buffer of the cell and a fundamental factor in metal detoxification and protection against oxidative stress. As reported above, in some cases, GSH has to bind metals before they can be transported to the vacuole, where metals are stored. GSH also offers protection against oxidation induced by metals and can bind reactive sulfhydryl groups on proteins. By binding these groups, metals cannot bind and the protein is protected to oxidation. A recently discovered function of GSH is extracellular chelation of metals. When yeast cells are in a broth with As(III), the cells export GSH that binds extracellular to As(III). The GSH-As(III)-complex cannot be taken in by the yeast cell (Wysocki and Tamás 2010). When lead was present in the medium, intracellular GSH levels decreased, indicating GSH has a role in Pb detoxification (Perez et al. 2013).

The methods of detoxification by chelation and vacuolar sequestration are summarized in Fig 10.

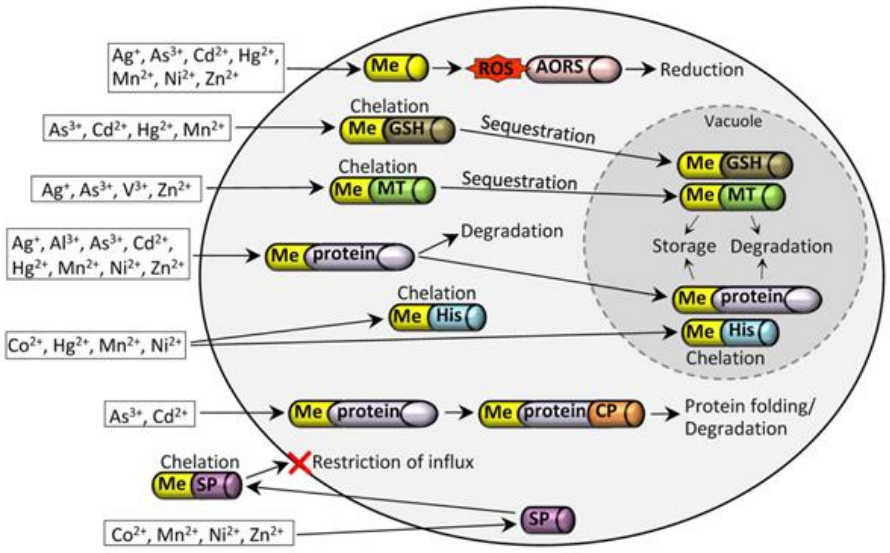

Figure 10: Detoxification by chelation and vacuolar sequestration. Source: Hosiner et al. (2014).

1.7 Structure of the *S. cerevisiae* cell wall

The cell wall is a ridged structure for osmotic support and physical protection of the cell (Klis et al. 2002). The cell wall of *S. cerevisiae* is a porous matrix which has a width of 110-200 nm. It mainly consists of β-1,6-linked glucans, mannoproteins and chitin. These components can vary in amount, depending of the growth stage and conditions of the medium. Because of this, the characteristics of the cell wall can differ (Orlean 2012).

The cell wall consists of two layers: an electron-dense outer layer that consists of glycosylated mannoproteins and an inner layer consisting of glucans and chitin providing mechanical strength. The outer layer is involved in cell-cell recognition processes and protects the inner layer against cell-wall damaging enzymes. This layer is negatively charged due to phosphodiester bridges on the carbohydrate side chains of the protein layer. The side chains are accountable for the hydrophilic characteristics of the outer layer and its protection to dehydration. The outer layer is less permeable to macromolecules than the inner layer (Klis et al. 2002).

1.7.1 Chitin

Chitin consists of linear chains of β-1,4-linked N-acetylglucosamine, with an average length of 110 residues. Only 1-2 % of the dry matter of the cell wall is chitin. Chitin is present in the neck between mother and daughter cell, in scars from buddying and in the division septa. In the cell wall, chitin can be free or bound to glucans. In the neck between the buddying cells, chitin is mainly bound to β-1,3-glucans. In other zones, chitin is bound to β-1,6-glucans, who are linked to mannans and β-1,3-glucans (Orlean 2012).

1.7.2 β-Glucans

At about 30-60 % of the dry matter of the cell wall are glucans. β–glucan is constituted by the fractions of β(1→3) and β(1→6)-linked glucose residues. β(1→3) glucans have a hollow helix conformation, responsible for the flexibility of the cell wall (Klis et al. 2002). The β-glucans can be separated into fractions. The first fraction, an acid- and alkali-insoluble fraction, of approximately 1500 β-1,3-linked glucan units and β-1,3-linked glucan side chains at branching β-1,6-glucose units. Chitin can be linked at the non-reducing ends of the chains. This makes the fraction insoluble. The second fraction has a similar composition as the first fraction, but it is alkali-soluble because there is no crosslinking with chitin. A third fraction originates from the action of acids or endo-β-1,3-glucanase on the first fraction. This action results in β-1,6-glucan chains of approximately 140 units, some with β-1,3 glucose side chains (Orlean 2012).

1.7.3 Mannoproteins

The cell wall proteins of *S. cerevisiae* can carry O-linked manno-oligosaccharides, asparagine-N-linked-glycans and glycosylphosphatidylinositol (GPI). Proteins are important for the construction of the cell wall (hydrolases or transglycosidases), for flocculation and the detection of cell wall stress. Proteins covalently bound to glucans of the cell wall are called CWP (Orlean 2012). Proteins with internal repeat, containing high levels of threonine or serine, are proteins extensively linked to manno-oligosaccharides (Lesage and Bussey 2006). Due to linkage of highly branched carbohydrates to proteins and disulphide bridges, the outer layer is less permeable to macromolecules than the inner layer (Klis et al. 2002).

1.7.4 Role of CWP1 and CWP2

CWP1 and *CWP2* are genes that encode for the cell wall mannoproteins cwp1p and cwp2p. Cwp2p can be found in mother cells and daughter cells and has a function stabilizing the cell wall. Cwp1p is mainly found in bud scars (Smits et al. 2006).

Both genes are located on chromosome XI (van Der Vaart et al. 1995) and are suppressed in anaerobic conditions. When the *CWP1* and *CWP2* genes are deleted, the cells show an increased sensitivity to calcofluor white and Congo Red. Without *CWP2*, the outer layer of the cell wall is also thinner (Lesage and Bussey 2006). Since mannoproteins are key factors in the permeability of the cell wall, the permeability also increases when the *CWP1* and *CWP2* genes are deleted (Zhang et al. 2008).

1.7.5 Linkage between cell wall components

Fig 11 shows the global structure of the lateral cell wall of *S. cerevisiae* (Levin 2011). CWP can be bound to β-1,3-glucans by alkali sensitive bounds. In *S. cerevisiae* a family of covalent bounded cell wall proteins are called Pir proteins (Ecker et al. 2006). Pir proteins are linked to CWP by disulphide bounds. GPI-CWP-complexes are linked to β-1-6 glucans by GPI remnant anchors (Lesage and Bussey 2006).

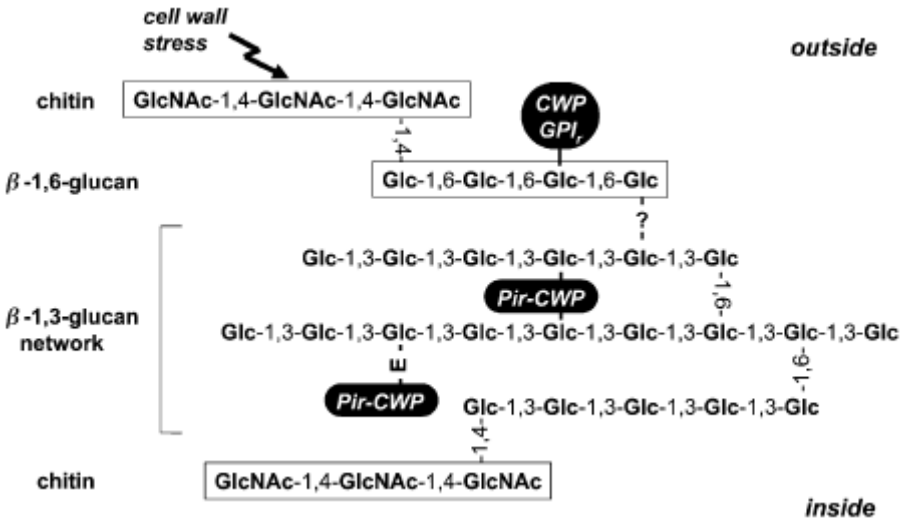

Figure 11: Cell wall structure of S. cerevisiae. Source: Levin (2011).

1.7.6 Influence of toxics on the cell wall

PbS-nanoparticles (80 nm) added to media increase the cell wall chitin content. The damage of the particles to the cell wall results in activation of the Cell Wall Integrity (CWI) pathway. Activation of this pathway results in chitin synthesis and an increased chitin content (Sun et al. 2014).

2 Materials and methods

2.1 Yeast strains

Saccharomyces cerevisiae strains used in the present work are listed in table 2. Wild-type (BY4741) and single gene deletion strains were obtained from EUROSCARF collection (Frankfurt, Germany).

The strains were routinely maintained at 4 °C on yeast peptone dextrose (YEPD) agar slants (please see below YEPD composition – section 2.2).

Table 2: *Saccharomyces cerevisiae* yeast strains used in this work

Reference	Strain	Genotype	Description
BY4741	Wild type (WT)	MAT*a*; *his*3Δ1; *leu*2Δ0; *met*15Δ0; *ura*3Δ0	Control strain
Y04945	*cwp1*Δ	BY4741; *YKL096w*::*kanMX4*	Without cell wall mannoprotein
Y07026	*cwp2*Δ	BY4741; *YKL096w-a*::*kanMX*4	Without cell wall mannoprotein

2.2 Media composition

The medium used for preparation of pre-cultures, cultures and growth was YEPD broth. Using as a base YEPD medium, different dilutions were carried out; the composition of these media can be found in table 3.

Table 3: Media composition

Medium	Yeast Extract (g/l)	Peptone (g/l)	Glucose (g/l)
YEPD	10	20	20
YEPD 10x dil	1.0	2.0	20
YEPD 25x dil	0.4	0.8	20
YEPD 50x dil	0.2	0.4	20
YEPD 100x dil	0.1	0.2	20

Diluted YEPD media was buffered with 20 mmol/l 2-(N-morpholino) ethanesulphonic acid (MES) at pH 5.5.

2.3 Pre-culture and culture

Pre-cultures were obtained by inoculating 10 ml YEPD broth in 100 ml Erlenmeyer flasks with one loop of the stock culture. The cells were incubated during 8-9 h at 25 °C, on an orbital shaker (150 rpm).

Cultures, in exponential phase of growth, were obtained by inoculating 40 ml of YEPD broth, in 100 ml Erlenmeyer flasks, with pre-cultures and grown 15-16 h (OD_{600} ~1.0) under the same conditions described for the pre-cultures.

2.4 Measuring cell concentrations

Cell concentrations were measured with a spectrophotometer at a wavelength of 600 nm ($OD_{600\,nm}$), after appropriate dilution of the samples with deionised water. Deionised water was used as blank. The following calibration curve was used:

$$Number\ of\ cells\ \times 10^6/ml = 20.237 \times OD_{600\,nm} - 0.1348$$

2.5 Growth curves

Because of the problem of Pb precipitating with medium components, different media were tested. Thus, different dilutions of the medium were carried out and buffered with 20 mmol/l MES buffer (pH 5.5) to avoid growth limitations by the acidification of the medium. The growth without and with Pb in these different media were tested to define an optimal condition for tests. The conditions should result in a low level of precipitate, but still a good growth of yeast.

The different media were inoculated with culture in exponential phase of growth as previously described (section 2.3) starting with an initial OD_{600nm} of 0.050. At defined times the OD_{600nm}, the pH and the glucose content were measured.

2.6 Determination of 24h-EC_{50}

To determine the Pb concentration for the Pb sensitivity tests, an assay in test tubes was executed. The lead concentration at final of 1000, 560, 420, 320, 240, 210, 180 and 130 µmol/l was added to a 25 times diluted medium. The medium was inoculated with 10^6 cells/ml (initial OD_{600nm}=0.050).

The yeast cell growth was measured after 24 h. As control, the medium was inoculated with yeast cells in the absence of Pb.

The Pb concentration that results in 50 % reduction of the growth in 24 h (24h-EC_{50}), compared to a positive control (medium without Pb), was determined. 24h-EC_{50} was calculated using the linear interpolation method (TOXCALC version 5.0.32, Tidepool Scientific Software).

2.7 Determining of total reducing sugars content

The glucose concentration in culture medium was determined as total reducing sugars by 3,5-dinitrosalicyle acid (DNS) method (Chaplin and Kennedy 1986).

Samples of the culture were centrifuged for 5 min, 2500xg and the supernatant (0.5 ml) was recovered and stored at -20°C, until sugar determination. This supernatant was, if necessary, diluted several times with water. This diluted sample was mixed with DNS and boiled for exactly 5 min. The samples were cooled rapidly in water and deionised water was added to each sample. The blank was made by adding 0.5 ml of deionised water to 0.5 ml DNS reagent. The OD at 540 nm of the samples was measured against the blank. The total reducing sugars were determined using a calibration curve, previously performed (see appendix 1).

2.8 Precipitation assays

Lead precipitates with medium components, resulting in a higher OD_{600nm} measured in samples with Pb. Precipitation assays had the purpose to define the amount of Pb that precipitates and the amount of Pb that remains soluble. Different dilutions of the YEPD medium were combined with different Pb concentrations and were placed for 2 h at 25 °C. Then, the OD_{600nm} was measured to determine the amount of precipitate. Subsequently, the media were centrifuged (3220xg, 10 min) and the soluble Pb was determined by atomic absorption spectroscopy with flame atomization, after appropriate dilution of the samples.

2.9 Lyticase assay

The cell wall sensitivity was assessed with the lyticase assay. For this purpose, 320 µmol/l Pb was added to YEPD medium 25 times diluted and subsequently centrifuged (2500xg, 10 min), resulting in a culture medium with 13 µmol/l of soluble Pb. The yeast cells were inoculated at $1x10^6$ cells/ml and incubated for different time in the absence (control) or in the presence of Pb. Then, the cells were centrifuged (2500xg, 10 min), washed with 40 mmol/l PBS buffer (pH 6.8) and resuspended in PBS buffer obtaining a sample (1.5 ml) with an initial OD_{600nm} of ~0.600. Subsequently, lyticase was added

to a final concentration of 4 U/ml. At defined times, the OD_{600nm} was measured and the percentage of lysis was determined. The percentage of OD_{600nm} reduction, in percentage, was calculated with a formula.

$$OD_{600\,nm}reduction\ (\%) = 100 - (\frac{OD_t}{OD_i}) \times 100$$

where, OD_i and OD_t are the OD_{600nm} at initial and at a given time, respectively.

2.10 Cell sensitivity to SDS and calcofluor white

The sensitivity to sodium dodecyl sulphate (SDS) and calcofluor white (CFW) was examined by the growth on YEPD medium containing different concentrations of SDS or CFW (see below). Samples of growing yeast in absence and presence of Pb (in the same conditions described above for lyticase assay) were taken at defined times. Cell concentrations of the samples were adjusted to $1.0x10^6$ cells/ml and the samples were serially diluted 3 times (10 times diluted each time), the first time with 3 mmol/l EDTA and the other times with deionized water. 5 µl from the original sample and the 3 dilutions were plated on YEPD agar (control), YEPD agar with 0.01, 0.05 and 0.1 % (w/v) SDS and with 50 and 100 µg/ml CFW.

2.11 Evaluation of plasma membrane integrity

The integrity of the plasma membrane was examined by the trypan blue (TB) staining. A sample with an OD_{600nm} of 0.050 was centrifuged (2500xg, 5 min), washed with PBS buffer (10 mmol/l) and centrifuged again (2500xg, 5 min). The cells were resuspended in PBS buffer. 15 µl TB 0.4% (w/v) (final concentration 0.2 %) was added to 15 µl of the cell suspension and mixed. After an incubation time of 20 min, at least 200 cells were counted in random fields, 3 times. Cells with an intact plasma membrane were unstained (TB negative cells); cells with a disrupted plasma membrane were stained (blue; TB positive cells).

2.12 Cell wall staining with calcofluor white (CFW)

Chitin in the cell wall was stained with CFW. The cell suspension (10^6 cells/ml) was centrifuged (2500xg, 5 min) and washed two times and resuspended in 0.5 ml water. CFW 5 mmol/l was added to obtain a final CFW concentration of 50 µmol/l. The cells were incubated 30 min in the dark at room temperature. The cells were observed using an epifluorescence microscope with filter A (excitation filter BP 340–380, dichromatic mirror 400 and suppression filter LP 425). Photos of representative

cells were acquired with a Leica DC 300 F camera (Leica Microsystems, Switzerland) using a 100× oil immersion N plan objective and processed using Leica IM 50- Image manager software.

2.13 Reproducibility of the results and statistical treatment

All experiments were repeated, independently, three to six times. The date reported are the means ± standard deviation (SD). In Fig 19, the statistical difference between control and Pb treated cells were tested using unpaired *t* test.

3 Results and discussion

3.1 Evaluation of Pb toxicity in YEPD medium

The influence of Pb on the growth of *S. cerevisiae* was examined. The growth of the yeast in YEPD was compared to the growth in YEPD with 1.0 mmol/l Pb. There was no observed difference between the growth in presence or absence of Pb. The change in pH of the culture medium was the same for both conditions as well as the glucose consumption (Fig 12).

It is described in the literature that the incubation of *S. cerevisiae* yeast cells with 1.0 mmol/l Pb, in the absence of complexation/precipitation (MES pH buffer), induced a loss of proliferation capacity and intracellular accumulation of ROS (Sousa and Soares 2014). Thus, it can be hypothesized that the lack of Pb toxicity in YEPD medium can probably be due to the abiotic complexation/precipitation of Pb by medium components. In order to test this possibility, a precipitation assay was executed. For this purpose, YEPD medium was diluted 10-100 times and Pb was added (0.1-1.0 mmol/l) at each diluted medium. As it can be seen in Fig 13b, for a Pb concentration of 1 mmol/l, the level of soluble Pb was very low (6 µmol/l). More than 99% Pb was precipitated with media components (Fig 13a). These results explain why there was no observed difference between the growth without and with 1.0 mmol/l Pb. These results evidence that the medium YEPD cannot be used for to study the impact of Pb on yeast cells in growing conditions.

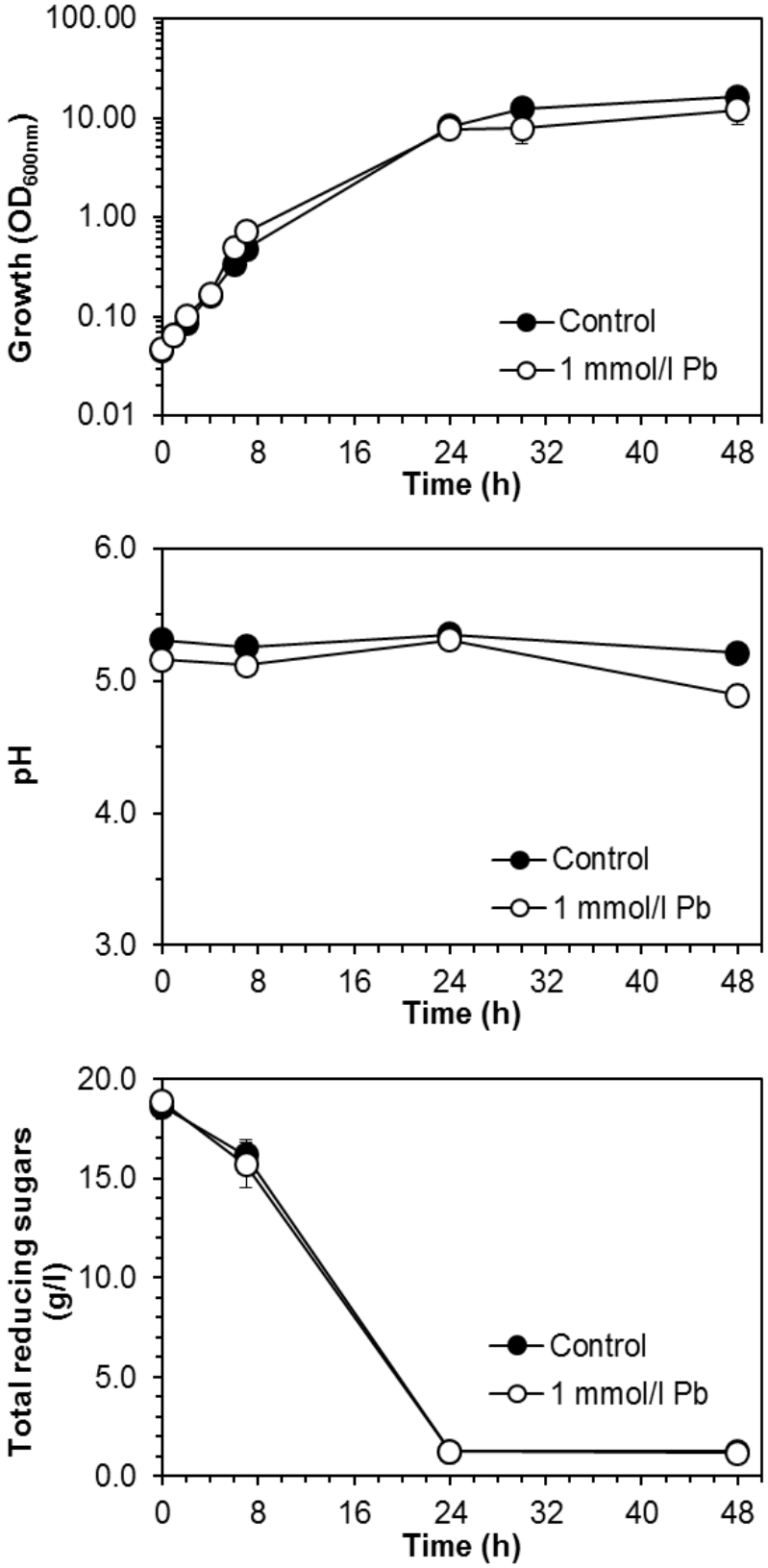

Figure 12: Exposure of *S. cerevisiae* yeast cells to Pb in YEPD medium. Yeast cells (1x10^6 cells/ml) were inoculated in the absence (control) or in the presence of 1.0 mmol/l Pb. Evolution of growth (a), pH of culture medium (b) and total reducing sugar in the culture medium (c). Each point represents the mean (±SD) of three independent experiments.

3.2 Defining a suitable growth medium

In order to be possible the study of the impact of Pb on yeast cells in growing conditions, the design of an appropriate medium was attempted. Thus, as reported above, different dilutions (10-100x) of the YEPD medium were made and a precipitation assay was executed on these media. Pb concentrations of 0.1-1.0 mmol/l were added to growth media.

As can be seen in Fig 13a, in 100 times diluted medium, different Pb concentration lead to the same amount of precipitate. The soluble Pb concentration increased with an increasing Pb concentration of the growth medium (Fig 13b). Most likely, the proteins in the medium were the limiting factor because all proteins precipitated with Pb. In 50 times diluted medium, 0.25-1.0 mmol/l Pb resulted in the same amount of precipitate (Fig 13a). Thus, a Pb concentration of 0.25-1.0 mmol results in precipitation of protein components. When the Pb concentration was 0.25 mmol/l or higher, there was detectable soluble Pb (Fig 13b). In 25 times diluted medium, the amount of precipitate is the approximately the same for a Pb concentration of 0.5-1.0 mmol/l (Fig 13a). There was soluble Pb present if the Pb concentration was higher than 0.25 mmol/l. In 10 times diluted medium and undiluted medium, the amount of precipitate increased with increasing Pb concentrations (Fig 13a). Fig 13b indicates that there was only detectable Pb present with a Pb concentration of the medium of 1.0 mmol/l. In undiluted medium, there was a very low Pb detectable.

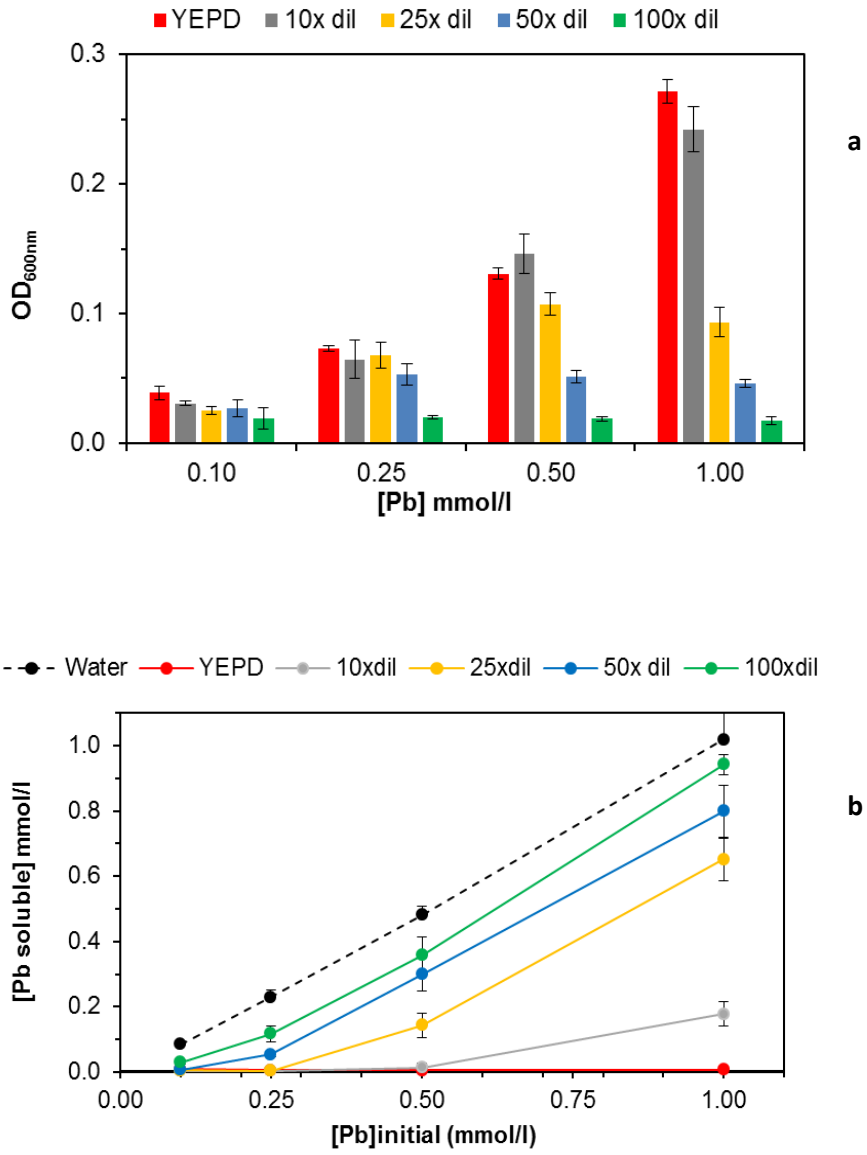

Figure 13: Interaction of Pb with the YEPD components. A-Level of precipitation of the media components, evaluated by the measurement of $OD_{600\,nm}$. B-Amount of Pb soluble after removal of the precipitate. Each point represents the mean (±SD) of three independent experiments.

To study the impact of Pb on growing yeast cells, soluble Pb should be present. Therefore, the medium had to be diluted or the Pb concentration had to be increased. To minimize the interference of the precipitate with OD measurement, the medium was diluted. By diluting, the level nutrients for yeast cells were reduced, influencing the growth of yeast cells. Thus, the growth of *S. cerevisiae* was observed in 10-100 times diluted media without (control) and with 1.0 mmol/l Pb. The pH was measured to exclude growth inhibition due to a low pH. The glucose concentration was measured to exclude the deficiency of glucose as a growth limiting factor.

In case of the controls (cells not exposed to Pb), growth was observed in all diluted media (Fig 14a). The glucose consumption reduced with the increase of the dilution of the medium (Fig 14c); for the

media 25-100 times diluted, glucose was not the limiting nutrient. For 10 and 25 times YEPD diluted media, a higher decrease of the pH was observed (Fig 14e). In the presence of 1.0 mmol/l Pb, in 25-100 times diluted media, the yeast growth was arrested (Fig 14b). Consequently, the glucose consumption was low (Fig 14d) and the decrease in pH was small (Fig 14 f).

Taking into account the compromising between Pb precipitation (with the consequent impact on the concentration of Pb soluble) and the level of cell growth, the YEPD 25 times diluted medium was selected to be used in further experiments. In this medium, the number of cells increased more than 20 times, in 24h, using an initial OD_{600nm}=0.05.

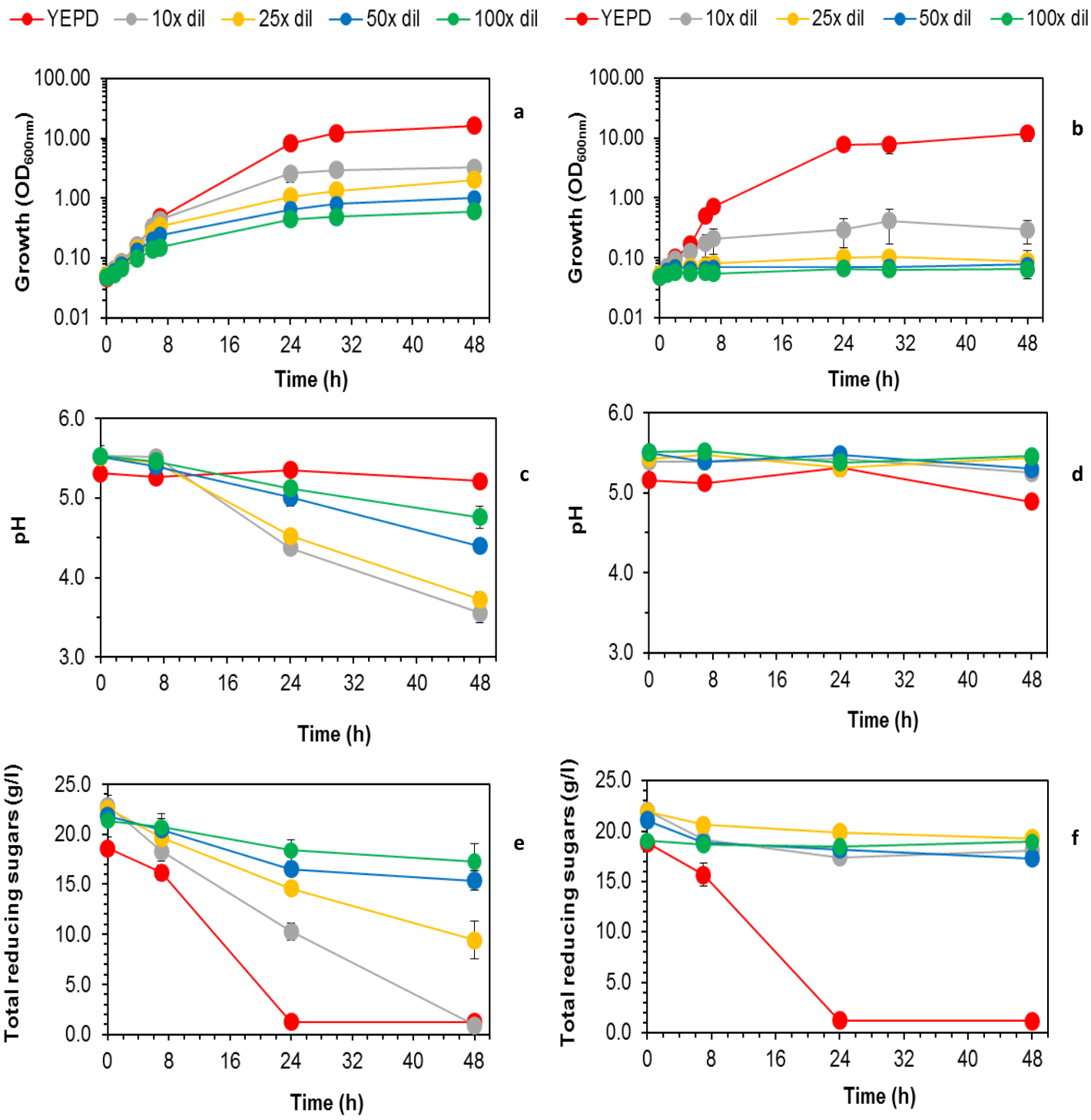

Figure 14: Exposure of *S. cerevisiae* yeast cells to Pb in undiluted YEPD medium and 10-100 times diluted medium. Yeast cells (1x10^6 cells/ml) were inoculated in the absence (control) or in the presence of 1 mmol/l Pb. Evolution of growth in absence (a) and presence (b) of 1 mmol/Pb. pH of culture medium in absence (c) and presence (d) of 1 mmol/l Pb. Total reducing sugar in the culture medium in absence (e) and presence (f) of 1 mmol/l Pb. Each point represents the mean (±SD) of three independent experiments.

In order to validate the suitability of the use of YEPD medium 25 times diluted in the evaluation of Pb toxicity, cells of *S. cerevisiae*, in exponential phase of growth, were exposed to Pb in a concentration range of 130-1000 µmol/l, for 24 h. A dose-response was obtained by plotting the growth (OD_{600nm}) after 24 h against the concentration range of Pb (Fig 15). It was possible to determine the 24h-EC_{50} value, which represent the concentration of Pb that inhibited 50% of the growth (214 µmol/l Pb; 95% confidence limits 205 – 223).

Remarkably, there was already an inhibitory effect measured from a Pb concentration of 180 µM, although the results of the precipitation assay (section3.2) showed a very low amount of soluble Pb (4 µmol/l) in 25 times diluted media with 250 µmol/l Pb. This can be explained by a shift in the equilibrium between soluble Pb and Pb bound to proteins (precipitate). When yeast accumulates Pb, the very small concentration of soluble Pb that is present decreases, resulting in resolubilisation of precipitated Pb.

Additionally, it was determined the impact of Pb on membrane integrity of *S. cerevisiae* in growing conditions. As it can be seen in Fig 16, a progressive loss of membrane integrity was observed with the increase of Pb in the culture medium.

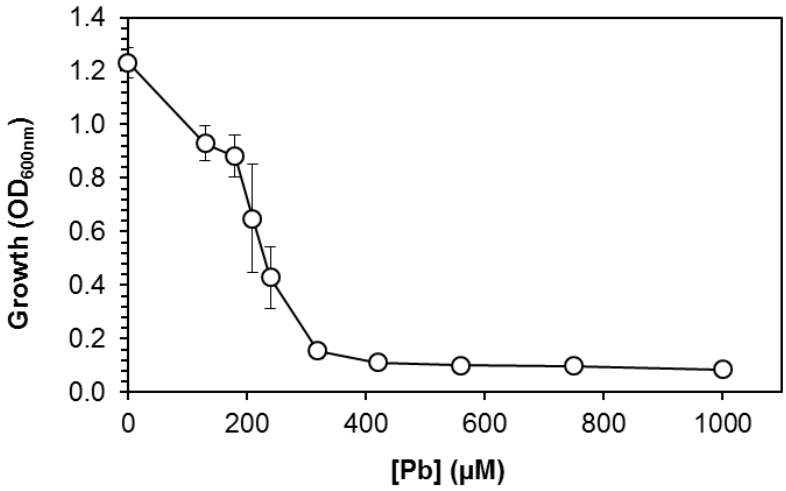

Figure 15: Dose–response plot of cells of *S. cerevisiae* exposed to Pb. Cells in the exponential phase of growth ($1.0x10^6$ cells/ml) were inoculated in YEPD medium 25 times diluted, containing different concentrations of Pb. Cell quantification ($OD_{600\ nm}$) was carried out after 24 h. Each point represents the mean (±SD) of six independent experiments.

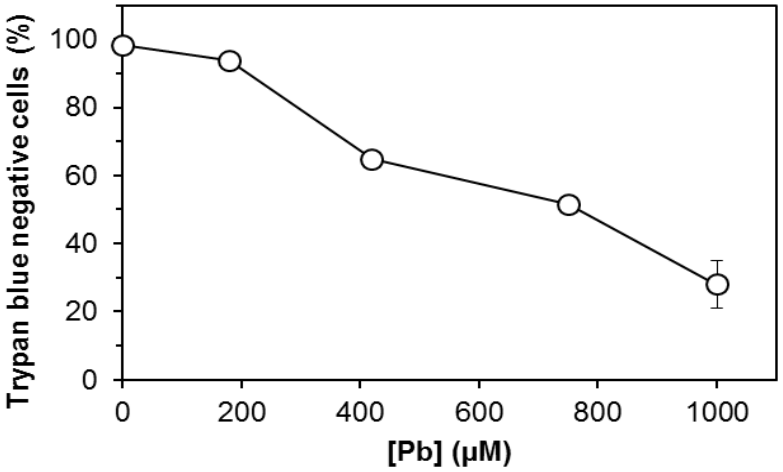

Figure 16: Effect of Pb on the membrane integrity of *S. cerevisiae*. Cells in the exponential phase of growth (1.0x10^6 cells/ml) were inoculated in YEPD medium 25 times diluted, containing different concentrations of Pb. Membrane integrity was assessed after 24 h by microscopic determination of cells excluding trypan blue (TB) (TB negative cells). Each point represents the mean (±SD) of three independent experiments.

3.3 Is the yeast cell wall a barrier against Pb toxicity?

As it was reported above (section 1.7.4), *CWP1* and *CWP2* genes encode two major mannoproteins of *S. cerevisiae* cell wall; deletion of these *CWP* genes enhances cell wall permeability, increasing the susceptibility to genotoxic chemicals (Zhang et al. 2008). With the purpose to investigate if the yeast cell was acts as a barrier against Pb toxic effects, the susceptibility of yeast cells deleted on *CWP1* (*cwp1*Δ strain) or *CWP2* genes (*cwp2*Δ strain) was compared with the isogenic wild-type (WT) strain. As it can be seen in Fig 17, the growth of *cwp1*Δ and *cwp2*Δ strains, in 25 times diluted YEPD medium, in presence of different Pb concentrations (130-1000 µmol/l) did not differ from the growth of the WT strain of *S. cerevisiae*. Consistently, the 24h-EC_{50} values were similar: 214 $\pm$ 9 µmol/l (WT), 219 $\pm$ 9 µmol/l (*cwp1*Δ strain) and 231 $\pm$ 5 µmol/l (*cwp2*Δ strains). This result suggests that the modification of cell wall permeability did not affect the sensitivity of yeast cells to Pb.

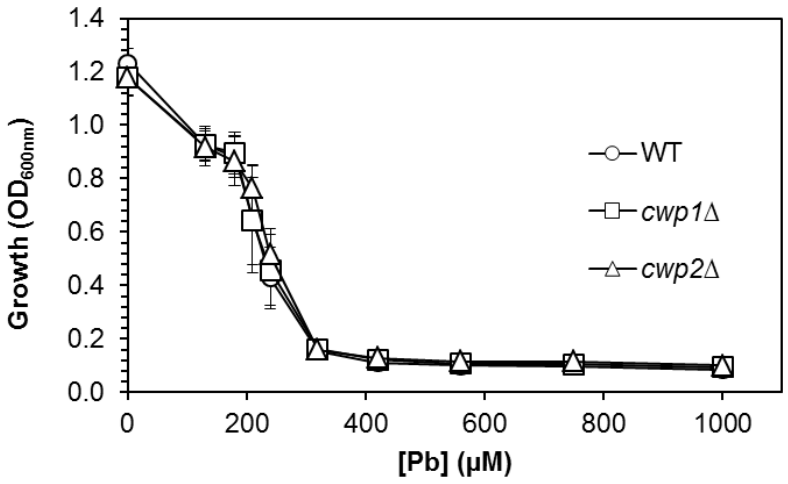

Figure 17: Dose-response curve of *S. cerevisiae* yeast cells exposed. Yeast cells (1x10^6 cells/ml) in exponential phase were inoculated in YEPD medium 25 times diluted in the absence (control) or in the presence of different Pb concentrations. Cell quantification ($OD_{600\ nm}$) was carried out after 24 h. Each point represents the mean (±SD) of six independent experiments.

3.4 Is the yeast cell wall a target of Pb toxicity?

In order to examine if the yeast cell wall is a target of Pb toxicity, the sensitivity of yeast cells to sodium dodecyl sulphate (SDS), calcofluor white (CFW) and the cell wall-degrading enzyme lyticase was assessed in growing conditions, in absence (control) and presence of soluble 13 µmol/l Pb, at defined times. To evaluate the impact of Pb on the growth, $OD_{600\ nm}$ was measured during 24 h (Fig 18). In Pb medium, a growth inhibition of ~20.0 % was observed after 24 h, compared to the control. Also, the impact of Pb on membrane integrity of *S. cerevisiae*, in growing conditions, was evaluated (Fig 19). The % of cells with intact membrane (TB negative cells) of Pb exposed cells were extremely significantly different from the control (Fig 18). Progressively, a small loss of membrane integrity was observed in Pb exposed cells. This suggests that Pb affects the membrane integrity.

The obtained results showed no difference in the rate of lysis between growing yeast cells in absence or in presence of Pb at time 0 h (Fig 20a) and at time 4 h (Fig 20b). After 24 h, a decrease of sensitivity to lyticase was observed in both control and Pb exposed cells (Fig 20c), probably, due to the stationary phase of the cells, instead of the exponential phase (times 0 and 4 h). Also, there was a difference observed between the control and the Pb exposed cells. At time 180 min, there was a significant difference (*P<0.05*) between the control and the Pb exposed cells. This suggests an increase of sensitivity to lyticase due to exposure to Pb. Further work, with a higher Pb concentration, should be carried out in order to confirm the interaction of Pb with the yeast cell wall.

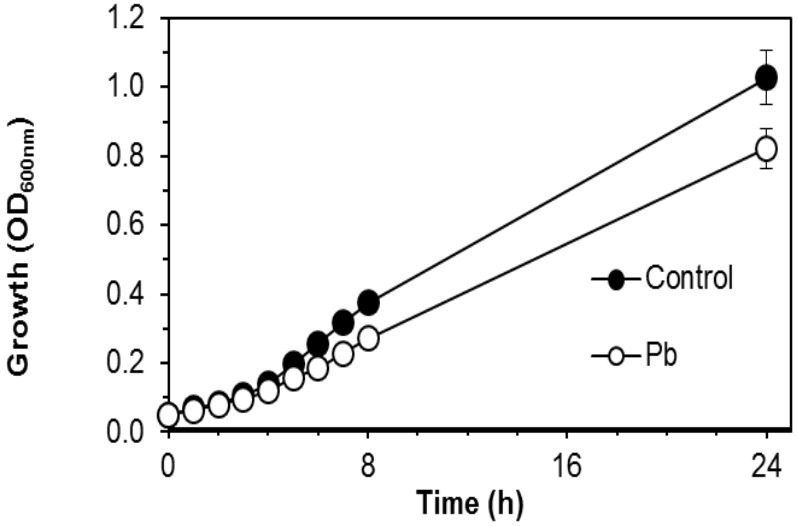

Figure 18: Growth of *S. cerevisiae* yeast cells exposed to Pb in 25 times diluted YEPD medium. Yeast cells ($1x10^6$ cells/ml) were inoculated in the absence (control) or in the presence of soluble 13 µmol/l Pb. Each point represents the mean (±SD) of three independent experiments.

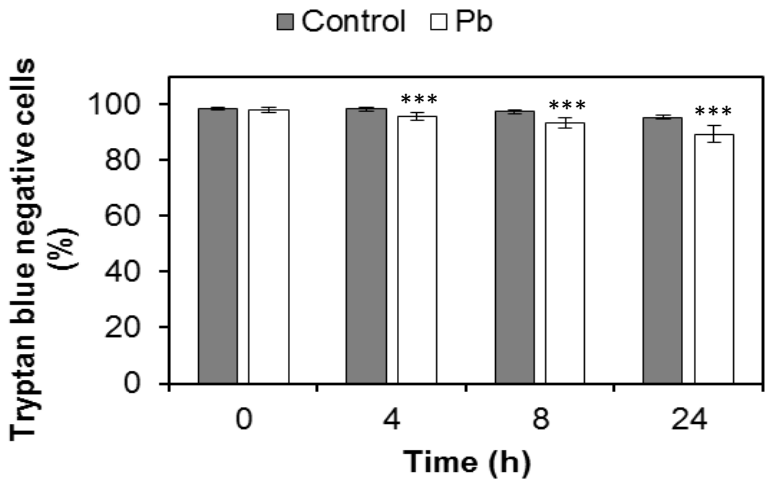

Figure 19: Effect of Pb on the membrane integrity of *S. cerevisiae*. Cells in the exponential phase of growth ($1.0x10^6$ cells/ml) were inoculated in YEPD medium 25 times diluted in absence (control) or in the presence of soluble 13 µmol/l Pb. Membrane integrity was assessed at defined times by microscopic determination of cells excluding Tryptan blue (TB) (TB negative cells). Each point represents the mean (±SD) of three independent experiments. For Pb, means with triple asterisks (***) are extremely significantly different (*P*<0.001*)* from the control.

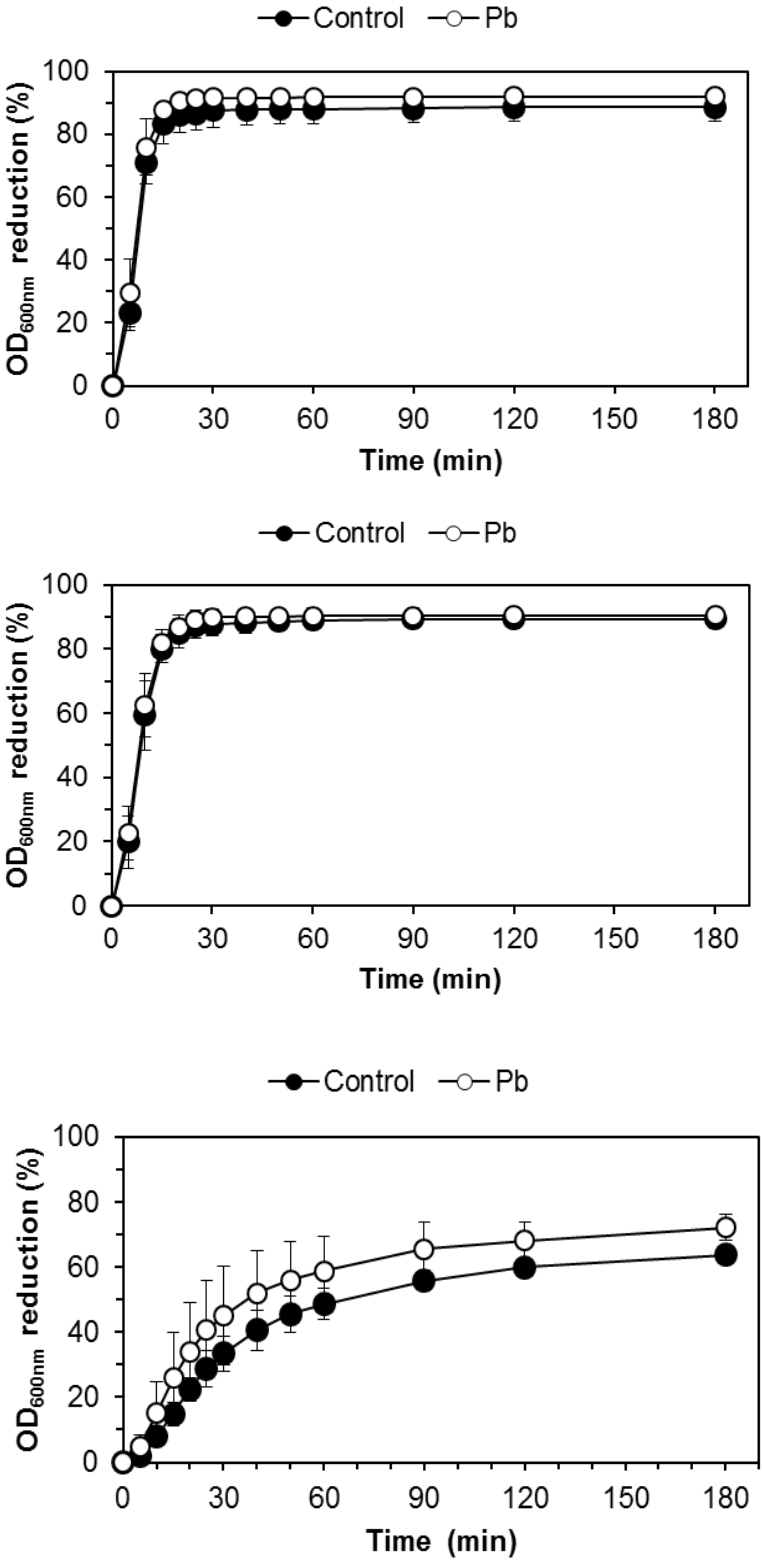

Figure 20: Effect of Pb on the sensitivity to lyticase of *S. cerevisiae*. Yeast cells (1x10^6 cells/ml) in exponential phase were inoculated in YEPD medium 25 times diluted in the absence (control) or in the presence of soluble 13 µmol/l Pb. Lyticase sensitivity defined as $OD_{600\,nm}$ reduction at time of Pb exposure: 0 h (a), 4 h (b) and 24 h (c). Each point represents the mean (±SD) of four independent experiments.

The yeast cell wall is dynamic structure. Environmental stress can lead to activation of the cell wall integrity pathway, resulting in a change of cell wall composition (Harrison et al. 2004). As reported in section 2.12, chitin in the cell wall and bud scar can be stained with CFW. When the growing yeast cells were stained with CFW, at times 0 h (Fig 21a) and 4 h, there was no difference observed between the yeast cells growing in the absence (control) or presence of Pb. The staining was mainly located in the bud scars and cell wall, visible as a small line around the cell. After 24 h, the majority of the cells growing in the presence of Pb (Fig 21c) were stained more than the control (Fig 21b). The staining was located more diffuse in the whole cell and the bud scars were brighter; although, few cells were stained as the control cells. This result suggests that the level of chitin in the cell wall increased due to the exposure to Pb.

The sensitivity to SDS and CFW was assessed during 24 h. It was observed that the growth of yeast cells in absence or presence of Pb on YEPD agar (Fig 22 a) did not differ to the growth on YEPD agar with 0.01 (w/v) SDS (Fig 22b) or YEPD agar with 50 or 100 µg/ml CFW (Fig 22c). The plates with 50 and 100 µg/l were similar, therefore, only photos of plates with 100 µg/ml CFW were included. Thus, in the described conditions, exposure to Pb concentration in the defined conditions do not increase the sensitivity to SDS or CFW. It was also observed that, in a period of 24 h, the sensitivity to SDS and CFW did not increase in time. On YEPD agar plates with 0.05 and 0.01 % SDS, there was no visible growth after 72 h of the control and the Pb treated cells (data not shown).

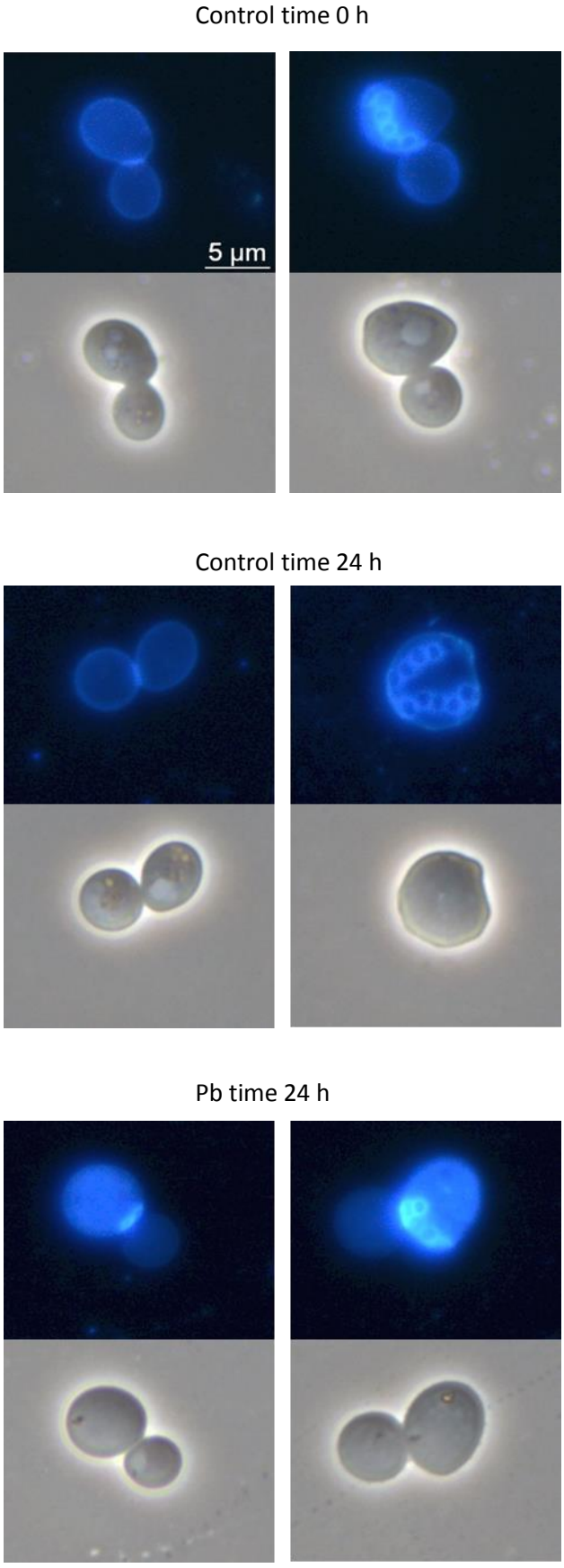

Figure 21: Impact of Pb on the chitin level in the cell wall of *S. cerevisiae*. Growing yeast cells (1x10^6 cells/ml) were stained with CFW (final concentration 50 µmol/l) and observed using an epifluorescence microscope. A- Control cells at time 0 h. B- Control cells at time 24 h. C- Pb exposed cells (13 µmol/l soluble Pb) at time 24 h. This is a typical result of an experiment performed four times .

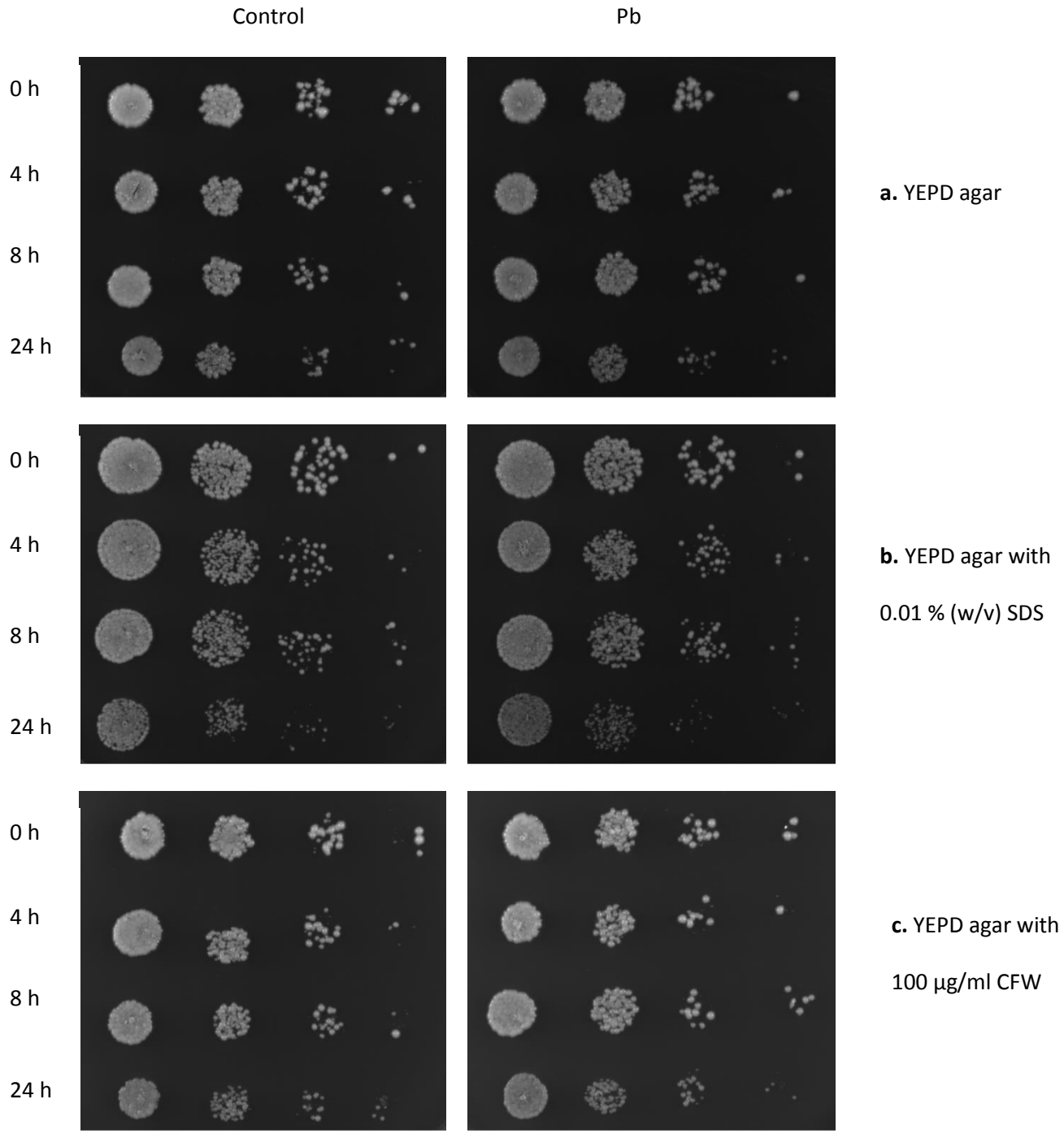

Figure 22: Sensitivity to dodecyl sulphate (SDS) or calcofluor white (CFW) of *S. cerevisiae cells* not exposed or exposed to 13 µmol/l soluble Pb. Yeast cells ($1x10^6$ cells/ml) in exponential phase were inoculated in YEPD medium 25 times diluted in the absence (control) or in the presence of Pb, for the times indicated in the figure. Samples of $1x10^6$ cells/ml and 3 dilutions (10 times diluted each time) were spotted on normal YEPD agar (a), YEPD agar with 0.01 % (w/v) SDS (b) or YEPD agar with 100 µg/ml CFW (c). This is a typical result of an experiment performed three times.

4 Conclusions

In undiluted YEPD medium, there was no observed difference in growth in absence or in presence of 1.0 mmol/l Pb, due to precipitation of Pb by medium components, resulting in a very low level of soluble Pb. YEPD medium could not be used to examine the influence of Pb on growing yeast cells, under environmentally realistic Pb concentrations. The obtained results permitted to define that YEPD 25 times diluted medium was the best compromise between precipitation and growth, allowing the assessment of Pb toxicity, in growing conditions, of *S. cerevisiae* yeast cells.

Comparing the susceptibility to Pb (130-1000 µmol/l) of yeast cells deleted on *CWP1* (*cwp1*Δ strain) or *CWP2* genes (*cwp2*Δ strain) with the isogenic wild-type (WT) strain, in 25 times diluted YEPD, showed no difference. This result suggests that the modification of permeability of cell wall of *S. cerevisiae* did not affect the sensitivity of yeast cells to Pb.

In 25 times diluted medium, with 13 µmol/l of soluble Pb, there was a decrease of ~20 % of the growth, compared to the control, after 24 h. A small loss of membrane integrity was observed in Pb exposed cells. The sensitivity to 0.01 % SDS and 50 or 100 µg/ml CFW did not modify due to Pb exposure. There was observed an increase in lyticase sensitivity and an increase of yeast cell staining with calcofluor white, after an exposure of 24 h to Pb. Together, these results suggest that Pb interacts with the yeast cell wall and influences the cell wall composition.

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Appendix

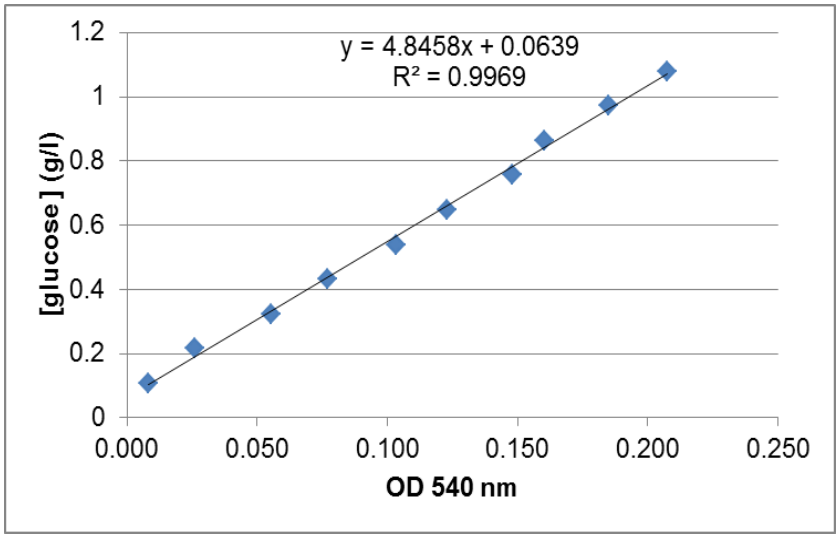

Figure 23: Calibration curve for determination of the total reducing sugar content with the DNS method.