Impact of multi-metals (Cd, Pb and Zn) exposure on the physiology of the yeast *Pichia kudriavzevii*

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Abstract Metal contamination of the environment is frequently associated to the presence of two or more metals. This work aimed to study the impact of a mixture of metals (Cd, Pb and Zn) on the physiology of the non-conventional yeast *Pichia kudriavzevii*. The incubation of yeast cells with 5 mg/l Cd, 10 mg/l Pb and 5 mg/l Zn, for 6 h, induced a loss of metabolic activity (assessed by FUN-1 staining) and proliferation capacity (evaluated by a clonogenic assay), with a small loss of membrane integrity (measured by trypan blue exclusion assay). The staining of yeast cells with calcofluor white revealed that no modification of chitin deposition pattern occurred during the exposure to metal mixture. Extending for 24 h, the exposure of yeast cells to metal mixture provoked a loss of membrane integrity, which was accompanied by the leakage of intracellular components. A marked loss of the metabolic activity and the loss of proliferation capacity were also observed. The analysis of the impact of a single metal has shown that, under the conditions studied, Pb was the metal responsible for the toxic effect observed in the metal mixture. Intracellular accumulation of Pb seems to be correlated with the metals' toxic effects observed.

Keywords Cell dead · Heavy metals · Membrane integrity · Metabolic activity inhibition · Metals toxicity · Yeast survival

Introduction

Heavy metal pollution is a worldwide problem mainly due the toxic effects of the metals on the biological systems. On the contrary of organic pollutants, metals are not degraded or destroyed over the time. In addition, they can be accumulated through the food chain originating serious ecological and health problems.

Cadmium is found in nature as ores, associated with other elements such as zinc, lead and copper. Cadmium is primarily used in batteries (silver-cadmium and nickel-cadmium), painting pigments, electroplating and stabilizers of plastics (ATSDR 2012; Naja and Volesky 2010). Cadmium can be found in air, water or soil due to non-ferrous metal mining and refining activities or due to the production and use of phosphate fertilizers; the combustion of fossil fuel as well as waste incinerations contributes to the release of cadmium in the environment (ATSDR 2012). Cd is considered to be of major concern for human health according to the World Health Organization and is classified as a human carcinogen by the International Agency for Research on Cancer (Nair et al. 2013). Although the mechanisms of Cd toxicity are not completely elucidated, it is believed that the major effects include oxidative stress, which might result in the damage of different organs such as the kidney, liver and lungs (Nair et al. 2013). In yeast cells, the intracellular accumulation of reactive oxygen species (ROS) originates oxidative damage, such as lipid peroxidation (Howlett and Avery 1997) and DNA damage (Avery 2001) and cell death by apoptosis (Nargund et al. 2008). Cd induces endoplasmic reticulum stress (ER) as consequence of Cd accumulation in the ER (Gardarin et al. 2010). It was also described that high concentration of Cd induces a

sudden boost in cytosolic Ca, which can initiate defence responses and increase Cd tolerance (Ruta et al. 2014).

Although lead occurs naturally, the high levels that can be found in the environment come mainly from human activities. In fact, lead, lead compounds and lead alloys have many applications and can be found, for example, in pipes and storage batteries in cars or in pigments in paints, dyes and ceramic glazes (ATSDR 2007; Naja and Volesky 2010). Lead found in soils arises from lead that falls to the ground from air (in the countries where lead is used in gasoline), or due to chipping of lead-based paint from houses and bridges, or from pesticides (ATSDR 2007; Rossi 2008). Like Cd, Pb is a non-essential element since it has no known biological roles. Pb is classified as a priority pollutant by the US Environmental Protection Agency (US-EPA 2006) and can cause, in humans, blood and brain disorders (ATSDR 2007). In the yeast *Saccharomyces cerevisiae*, Pb inhibits assimilation of nutrients (Chen and Wang 2007) and metabolic activity (Van der Heggen et al. 2010), decreases the intracellular level of reduced glutathione (Perez et al. 2013) and induces oxidative stress (Bussche and Soares 2011; Sousa and Soares 2014), DNA damage (Cui and Tang 2000; Yu et al. 2009; Yuan and Tang 1999), loss of proliferation capacity (Soares et al. 2003; Suh et al. 1999) and cell death displaying phenotypic markers of apoptosis (Bussche and Soares 2011). Pb is compartmentalized in the vacuole by two parallel pathways: one dependent of H^+ gradient generated by the V-ATPAse (Sousa et al. 2014) and another mediated by ABCC transporters (Ycf1p, Vmr1p, Ybt1p and Bpt1p), after conjugation with glutathione, in a process catalysed by glutathione transferases 1 and 2 (Gtt1p and Gtt2p) (Sousa et al. 2015). The compartmentalization of Pb in the vacuole can help the yeast cells to survive in the presence of this toxicant.

Zinc is one of the most widespread elements in the Earth's crust. It is an essential element, being required in small amounts for normal cell growth. Zn has been associated at least 300 enzymatic reactions, as a co-factor. Consequently, this element plays an important role in a multitude of physiological processes. Examples of zinc proteins are as follows: metalloproteases, carboxypeptidases and zinc finger proteins, which include DNA- or RNA-binding proteins (Bleackley and MacGillivray 2011). Zinc has also been shown to act as a signalling molecule as Ca (Yamasaki et al. 2007). The uptake of Zn in *S. cerevisiae* is mainly mediated by two Zn transporters: Zrt1p, a high affinity transported, active in Zn-deficient cells (Zhao and Eide 1996a) and Zrt2p, a low affinity transported, active in Zn-replete cells (Zhao and Eide 1996b). In the presence of high Zn concentration, Zrt1p is removed from the cell surface to prevent the uptake of excess Zn (Gitan et al. 2003). In the presence of high levels of zinc, yeast cells store excess zinc in the vacuole in order to meet metabolic need, in case of zinc deficiency, and prevent zinc toxicity (Cyert and Philpott 2013). Zrc1p and Cot1p, located in vacuole membrane, are the transporters involved in Zn vacuole storage (MacDiarmid et al. 2000). When in excess, Zn affects membrane integrity and induces the release of K^+ (Mowll and Gadd 1983). Zinc is commonly used to prevent the corrosion of other metals (such as iron), in a process called galvanization, or combined with other metals to form alloys such as brass (copper and zinc). Zinc, combined with other elements, has many applications such as to make paints, wood preserving or in the drug industry as sun blocks or deodorants. Zinc can enter the environment through air, water and soil as a consequence of both natural processes and human activities. The levels of zinc in soils increase due to the disposal of zinc wastes from metal manufacturing industries and coal ash from electric utilities as well as from fertilizers (ATSDR 2005).

Yeast cells have been proposed, as alternative to conventional techniques, for the bioremediation of heavy metals (Soares and Soares 2012). This type of biomass has the ability to remove a wide range of metals and can be obtained at low cost as by-product of fermentation industry (Soares and Soares 2013; Wang and Chen 2009). Recently, it was described that the yeast *Pichia kudriavzevii* (previously named as *Issatchenkia orientalis*) seems to be a new alternative for heavy metal bioremediation due to the higher metal removal capacity (Ubeda et al. 2014).

Heavy metal removal by yeast cells can be carried out by two main mechanisms: a passive process, called biosorption, and a metabolism-dependent process, known as bioaccumulation. The last process only occurs in metabolic active (live) cells and is attributed to the passage of the metals through the cell membrane (Soares and Soares 2012). Bioaccumulation could be desirable for heavy metal removal (Mapolelo and Torto 2004). However, metal toxicity can affect the efficiency of metal removal by live biomass.

In most cases, the contamination of a given area is not limited to the presence of only one metal but, in a general away, occurs due to the presence of two or more metals. Thus, it is important to know not only the behaviour of individual heavy metals (e.g. Cd, Pb and Zn) but also possible interactions between them. In the present work, the toxic impact caused by multi-metals (Cd, Zn and Pb) on the non-conventional yeast *P. kudriavzevii* will be evaluated and discussed. Additionally, the toxic impact of single metal was also evaluated. As far as we know, this is the first time that the toxic impact of metals is evaluated in *P. kudriavzevii* yeast cells.

Material and methods

Yeast, media and growth conditions

A strain of *P. kudriavzevii* CCMA 0136 was used in this work. The strain belongs to collection of cultures of agricultural microbiology (CCMA), from Biology Department (DBI),

Federal University of Lavras (MG, Brazil). The gene sequence has been deposited in GenBank under the accession number KJ468031.1 (http://www.ncbi.nlm.nih.gov/nuccore/KJ468031). This strain was chosen from previously tolerated growth screening using Cd, Pb and Zn which took place in the Environmental and Industrial Microbiology Laboratory in DBI, Brazil.

The strain was maintained at 4 °C on YPD agar slants [10 g/l yeast extract (Difco-BD), 20 g/l peptone (Difco-BD), 20 g/l dextrose (Merck) and 20 g/l agar (Merck)].

Pre-cultures were prepared in 10 ml of YPD broth in 100-ml Erlenmeyer flasks. Cells were incubated at 25 °C on an orbital shaker at 150 rpm for 8–10 h. Cultures in the exponential growth phase were obtained by inoculating 40 or 100 ml of YPD broth in 100 or 250-ml Erlenmeyer flasks, respectively, with pre-cultures and then grown overnight (OD_{600} ~0.5) under the same conditions as the pre-culture.

Preparation of soil extract

Soil was suspended in water (1 kg/l) and heated at 100 °C for 2 h. Subsequently, the suspension was centrifuged, filtered through paper filter and then through a 0.45-μm-pore-size filter.

Exposure of yeast cells to metal stress

Cells in the exponential growth phase were harvested by centrifugation (2500×*g*, 5 min), washed twice and re-suspended in deionized water at ~1×10^8 cells/ml. Cells were suspended in soil extract or in 10 mmol/l [2-(N-morpholino) ethanesulfonic acid] MES pH buffer (Sigma-Aldrich), pH 6.8, at 1×10^7 cells/ml. Unless stated otherwise, individual metals or a mixture of metals were added at a final concentration: cadmium, 5 mg/l; lead, 10 mg/l and zinc, 5 mg/l. Cells suspensions were shaken in 100-ml Erlenmeyer flasks at 150 rpm at 25 °C. The following stock standard solutions (Merck) were used: 1000 mg/l $CdCl_2$, 2000 mg/l ($Pb(NO_3)_2$) and 2000 mg/l Zn Cl_2.

Cell concentration was determined spectrophotometrically at 600 nm after appropriate dilution of the samples. A calibration curve (absorbance versus number of cells) was previously made.

Measurement of cell proliferation capacity by colony-forming unit count

Samples were taken (two replicates) at different intervals of time, serially diluted with sterile deionized water and plated on YPD agar (two replicates of the convenient dilutions). The colonies were counted after 1–2 days of incubation at 25 °C. The viability (CFU %) was calculated using the number of colony-forming units (CFU)/millilitre at zero time as reference (100 %).

Leakage of UV_{260}-absorbing compounds

For the measurement of the leakage of cell content (UV_{260}-absorbing materials), cell suspensions were centrifuged (2500×*g*, 10 min), the supernatants were carefully removed and the optical density at 260 nm determined using a quartz cuvette. Buffer solutions with the same metal concentrations were used as blank.

Microscopy

Membrane integrity was evaluated by staining yeast cells with trypan blue (TB). Cells were washed twice with deionized water and suspended in 10 mmol/l MES buffer, pH 6.8, at 1×10^7 cells/ml. Yeast suspensions were incubated with TB solution (Aldrich), at final concentration of 0.2 % (*w*/*v*), at room temperature, for 20 min. Cells were analysed by light microscopy. For each sample, at least three replicates of 200 cells (>600 cells) were scored in randomly selected fields. Unstained cells were scored as retained plasma membrane integrity (trypan blue negative cells), whereas cells with disrupted membrane appeared blue (trypan blue-positive cells).

Metabolic activity was assessed using the probe [2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinolinium iodide] FUN-1. Thus, cells were washed with deionized water and then with 10 mmol/l 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (Sigma-Aldrich), pH 7.2, containing 2 % (*w*/*v*) glucose and resuspended in the same buffer at 1×10^7 cells/ml. Subsequently, cells were stained with FUN-1 (Molecular Probes, Invitrogen) as previously described (Van der Heggen et al. 2010). Cells were examined using an epifluorescence microscope equipped with a HBO-100 mercury lamp and a filter set I3 (excitation filter BP 450–490, dichromatic mirror 510 and suppression filter LP 515) from Leica. For each sample, at least 600 cells were scored in randomly selected microscope fields.

Yeast cell wall was stained using calcofluor white M2R. Therefore, cells were washed twice and resuspended in deionized water. Calcofluor white M2R (Sigma) was added at a final concentration of 100 μmol/l, and the cells were incubated in the dark at room temperature for 30 min. After staining, cells were washed twice, resuspended in deionized water and examined using an epifluorescence microscope equipped with a filter set A (excitation filter BP 340–380, dichromatic mirror 400 and suppression filter LP 425), from Leica.

All images were acquired with a Leica DC 300F camera, using a N plan ×100 objective, and were processed using Leica IM 50-Image manager software.

Uptake of heavy metals

Cell suspensions (200 ml) containing 1×10^7 cells/ml, in MES buffer at pH 6.8, were shaken in 500-ml plastic flasks at 150 rpm, at 25 °C. An appropriate volume of a metal salt solution [$CdCl_2$, $Pb(NO_3)_2$ and $ZnCl_2$] was added, as described above, at a final concentration: cadmium, 5 mg/l; lead, 10 mg/l and zinc, 5 mg/l. Before and after adding the metal, samples (30 ml) were taken at defined intervals of time and filtered through a 0.45-μm-pore-size filter. The filters containing yeast cells were washed, two times, with 15 ml of 10 mmol/l EDTA and then washed, two times, with 15 ml MES buffer. Subsequently, filters were transferred to 50-ml Falcon tubes and dried at 60 °C. Each filter was transferred to a beaker and 2 ml of 65 % HNO_3 (analytical grade) was added. The filters were then slowly boiled on a hot plate until digestion was completed, transferred to 25-ml volumetric flasks, diluted with ultra-pure water and filtered through 0.45-μm-pore-size filters to remove any undigested particulate matter. The metal content was analysed by atomic absorption spectroscopy (AAS) with flame atomization in a Perkin Elmer AAnalyst 400 spectrometer (in the case of Zn) or by AAS with electrothermal atomization in a Perkin Elmer AAnalyst 600 spectrometer (in the case of Cd and Pb), after appropriate dilution of the samples.

Reproducibility of the results

All experiments were repeated, independently, three to eight times. The data reported are the mean±standard deviation (SD), presented with 95 % confidence.

Results

Multiple metals disturbs membrane integrity in a time-dependent manner

The effect of the simultaneous presence of Cd, Pb and Zn on the membrane integrity of the yeast *P. kudriavzevii* CCMA 0136 was evaluated by two methodologies: dye-exclusion assay with trypan blue (TB) and assessment of the leakage of UV_{260}-absorbing materials.

When exposed for 3 h to the action of metal mixture, the majority (>97 %) of *P. kudriavzevii* yeast cells were able to exclude TB and thus remained unstained (TB-negative cells), which suggests that membrane integrity was not affected. Yeast cell population exposed to metal mixture for 6 h displayed ~88 % of TB-negative cells. These results suggested that the loss of membrane integrity of cell population started after 3 h of contact of yeast cells with metal mixture. Increasing the exposure time to 24 h, the permeabilization (disruption) of plasma membrane occurred in the majority of yeast population. At this time, only ~35 % of cell population remained as TB-negative cells. Yeast cells incubated in MES pH buffer, in the absence of metals (control), remained with intact membrane (>99 % TB-negative cells) (Fig. 1a).

In another approach, membrane integrity was evaluated by the release of UV_{260}-absorbing compounds. These compounds are principally constituted by nitrogen compounds derived from intracellular nucleotides and related molecules (Delisle and Phaff 1961). The amount of UV_{260}-absorbing materials released by *P. kudriavzevii* cells, exposed to the metals mixture, for 6 h, was similar to the control cells (Fig. 1b). Yeast cells exposed for 24 h to multi-metals released UV_{260}-absorbing materials (Fig. 1b). This leakage of UV_{260}-absorbing compounds was only observed when the majority of cell population displayed a disrupted cell membrane (Fig. 1). As a positive control, *P. kudriavzevii* cells were treated with Cu, an ion known by its rapid effect on yeast

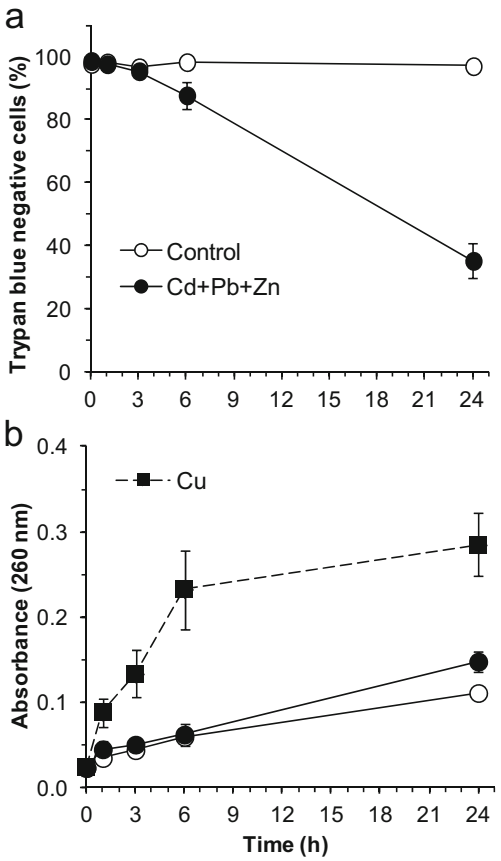

Fig. 1 Effect of multi metals on the membrane integrity of *P. kudriavzevii*. Yeast cells were suspended in 10 mmol/l MES pH buffer (pH 6.8) in the absence (control) or in the presence of a mixture of metals comprised by 5 mg/l cadmium, 10 mg/l lead and 5 mg/l zinc. **a** Assessment of membrane integrity by microscopic determination of cells excluding trypan blue (TB) (TB-negative cells). The data represent the mean (±SD) of three independent experiments. **b** Leakage of UV_{260}-absorbing cellular components. Copper (13 mg/l) was used as positive control. The data represent the mean (±SD) of four independent experiments

membrane integrity (Ohsumi et al. 1988; Soares et al. 2003). As expected, a fast and pronounced leakage of UV_{260}-absorbing compounds was observed as consequence of plasma-membrane permeabilization (Fig. 1b).

The results obtained suggest that the mixture of Cd, Pb and Zn induces the disruption of membrane integrity in a time-dependent manner. However, contrary to what happens with copper, for the metal mixture, plasma membrane does not seem to be the immediate target. These results prompted us to evaluate other physiological effects of the mixture of the heavy metals on the yeast *P. kudriavzevii*.

The loss of proliferation capacity and metabolic activity in yeast cells exposed to multiple metals is an early event

The ability of a single cell to divide and form a colony (colony-forming unit, CFU) on the surface of rich-medium plates, such as YPD (clonogenic assay), is typically used to evaluate toxic effects (Mirisola et al. 2014). The exposure of yeast cells to a mixture of Cd, Pb and Zn induced a progressive loss of cell proliferation (Fig. 2a). The incubation of yeast cells with the metal mixture, for 1 h, induced a loss of ~30 % of the proliferation capacity of cell population. The exposure to metal stress for 6 h provoked a loss of ~50 % of cell proliferation capacity. When incubated with metals for 24 h, <20 % of cell population retained the proliferation capacity (Fig. 2a).

The fluorescent probe FUN-1 has been used to distinguish between metabolically active and inactive yeast cells (Millard et al. 1997). In this context, FUN-1 has been used in the assessment of the toxic impact of organic and inorganic compounds on yeast metabolic activity (Fiolka et al. 2012; Parisi-Duchene et al. 2006; Van der Heggen et al. 2010). Before metal treatment, >97 % yeast cells were metabolically active and thus able to process and concentrate FUN-1 in the vacuole, as orange-red cylindrical intravacuolar structures (CIVS, FUN-1-positive cells) (Fig. 2c). Yeast cells metabolically inactive (FUN-1-negative cells) showed a green cytoplasmatic fluorescence without CIVS (Fig. 2c). The exposure of *P. kudriavzevii* cells to the metal mixture originated a loss of metabolic activity, since cell population, progressively, becomes unable to process FUN-1 (Fig. 2b). About 70 % of yeast cells incubated with the metals mixture, for 1 h, were able to process FUN-1. After 6 h of metal exposure, ~40 % of

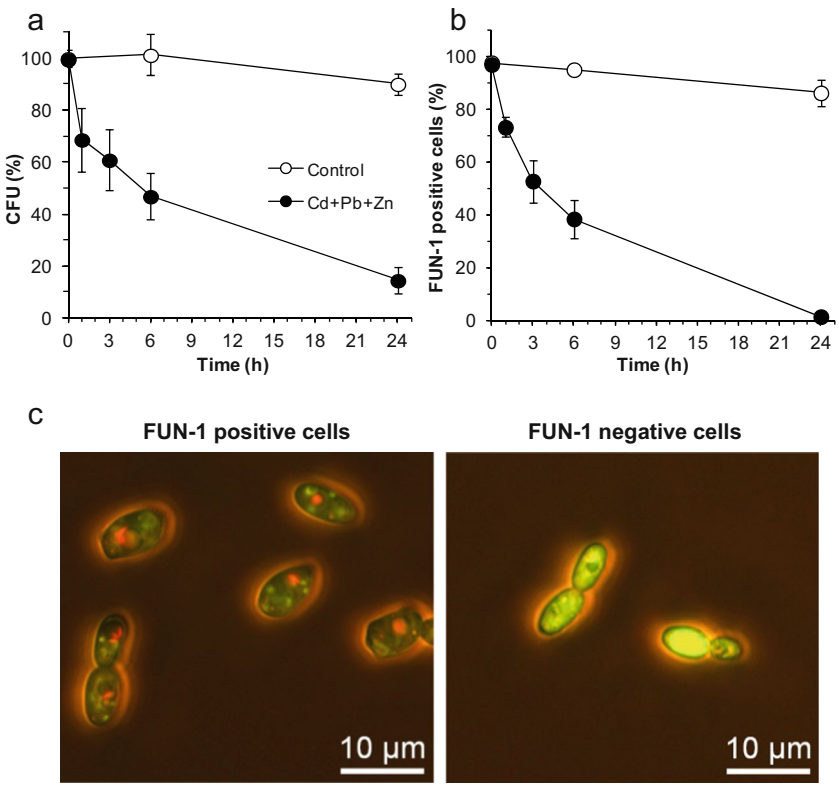

Fig. 2 Influence of multi metals on the proliferative capacity and metabolic activity of *P. kudriavzevii*. Yeast cells were suspended in 10 mmol/l MES pH buffer (pH 6.8) in the absence (control) or in the presence of metal mixture, reported in Fig. 1. **a** Quantification of yeast survival using a clonogenic assay (colony-forming units, CFU, on YPD agar). The data represent the mean (±SD) of eight independent experiments. **b** Evaluation of the metabolic activity of yeast cells, by fluorescence microscopy, after staining with FUN-1; the data represent the mean (±SD) of five independent experiments. **c** Microphotographs illustrative of FUN-1-stained cells. FUN-1-positive cells: cells metabolically active with cylindrical intravacuolar structures, CIVS (orange-red structures); FUN-1-negative cells: metabolically inactive cells, green stained, without CIVS

cells were FUN-1 positive. After 24 h of metal exposure, only ~1 % of cells were FUN-1 positive. The loss of metabolic activity (assessed by the staining with FUN-1) accompanied the loss of proliferation capacity evaluated through the clonogenic assay (Fig. 2a, b). After 24 h of incubation in buffer, in the absence of metals, a loss <10 % of FUN-1-positive cells were observed in the control. Probably, the starvation induced by the long (24 h) incubation in buffer medium can explain this result. In fact, FUN-1 processing is a metabolic-dependent process (Millard et al. 1997).

Cell wall was not modified by the exposure to multi-metals

The impact of the exposure of multi-metals on the cell wall of *P. kudriavzevii* was assessed using calcofluor white (CFW), a specific chitin dye (Costa-de-Oliveira et al. 2013; Pringle 1991). Cell walls of untreated cells (control) of *P. kudriavzevii*, stained with CFW, displayed a weak fluorescence, while the septa (junction between the mother cell and the buds-daughter cells) displayed a bright fluorescence (Fig. 3; arrow). These results suggest that septa are chitin-rich regions. Chitin seems to be almost exclusively located in the scar formed between the daughter and mother cell. Replicative aged yeast cells (i.e. cells that undergone several divisions) (Longo et al. 2012), which can be assessed by counting the number of budding scars, also displayed a faint surface fluorescence (Fig. 3). Cells incubated with a mixture of metals for 6 h retained the normal morphology. In addition, these cells displayed a typical faint fluorescence on their surface and presented visible bud scars (photos not shown). These observations suggest that the exposure to metal mixture up to 6 h did not induce cell morphology modification nor redistribution of chitin through the cell wall in *P. kudriavzevii*.

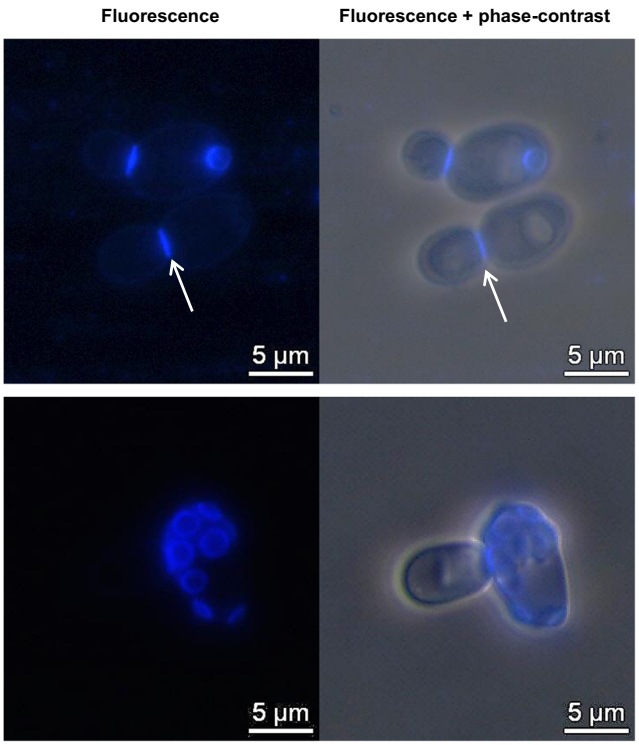

Fig. 3 Visualization of the cell wall of *P. kudriavzevii*. Microphotographs illustrative of calcofluor white-stained cells. *Arrow* indicates the chitin-enriched bud neck

Pb is the metal responsible for the toxic effects

In order to identify what was (were) the metal(s) responsible for the toxic effect, yeast cells were exposed to a single metal in the same concentration used in the mixture. As it can be seen in Fig. 4, Cd and Zn, under the concentration used, were not toxic to *P. kudriavzevii* cells, since no loss of membrane integrity or proliferation capacity occurred over a period of 24 h. Pb induced a loss of membrane integrity and cell proliferation capacity (Fig. 4) in a similar

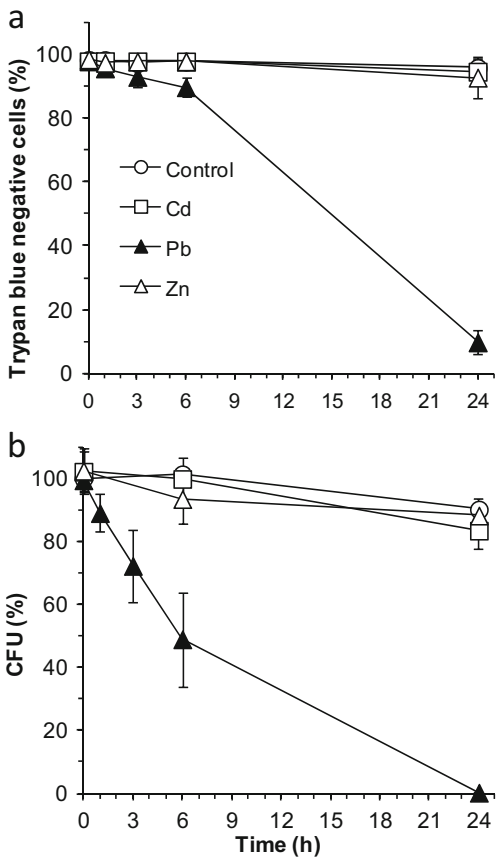

Fig. 4 Impact of single metals on the physiology of *P. kudriavzevii*. Yeast cells were suspended in 10 mmol/L MES pH buffer (pH 6.8) in the absence (control) or in the presence of 5 mg/l cadmium or 10 mg/l lead or 5 mg/l zinc. **a** Assessment of membrane integrity using the trypan blue assay; the data represent the mean (±SD) of three independent experiments. **b** Quantification of yeast viability using a clonogenicity assay; the data represent the mean (±SD) of four independent experiments

pattern to the one induced by the metals mixture (Figs. 1a and 2a). Together, these results suggest that, under the concentrations studied, Pb was the metal responsible for the toxic effect of the mixture.

Metal accumulation

The kinetics of metal accumulation inside the yeast cells were determined in order to verify if metal accumulation can be correlated with the toxic symptoms. As expected, yeast cells not exposed to Cd or Pb did not present these metals (Fig. 5). However, yeast cells contained Zn, which is in agreement with the abundance of this element in nature and their physiological functions. Pb was rapidly accumulated in the first hour. The intracellular level of this metal remained approximately constant up to 3 h. After this time, an increase of the intracellular content of the three metals occurred (Fig. 5).

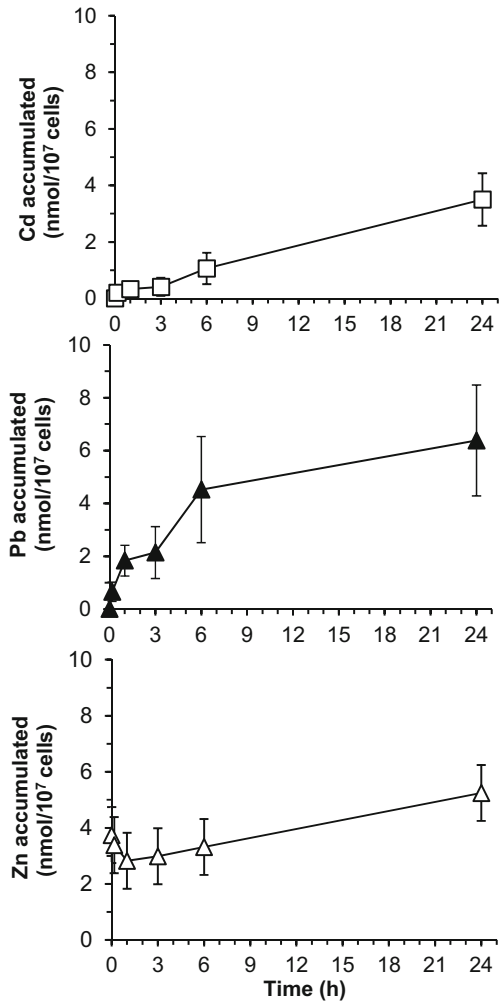

Fig. 5 Intracellular accumulation of metals by *P. kudriavzevii*. Cells were suspended in 10 mmol/l MES buffer (pH 6.8), in a final concentration of 1×10^7 cells/ml in the presence of 5 mg/l cadmium, 10 mg/l lead and 5 mg/l zinc. The data represent the mean (±SD) of three independent experiments performed in duplicate

Survival and metabolic activity of yeast cells exposed to multi-metals in soil extract is similar to the one observed in buffer

In order to test, in the future, the feasibility of using biomass of *P. kudriavzevii* in the bioremediation of soil contaminated with heavy metals, the toxic impact of the mixture of heavy metals was evaluated in soil extract. A similar trend of loss of proliferation capacity was observed in soil extract (Fig. 6a) and in buffer solution (Fig. 2a). The decrease, over time, of FUN-1-positive cells (Fig. 6b) accompanied the decline of proliferation capacity (Fig. 6a) and is consistent with the decrease observed in buffer solution (Fig. 2b). These results show that even in soil extract, metals exert a toxic impact.

Discussion

The yeast *P. kudriavzevii* has been isolated from different food fruit sources, such as cocoa beans, as the strain used in the present work (Pereira et al. 2012). This yeast has a biotechnological potential for bio-ethanol (Dhaliwal et al. 2011) production, in agriculture practices as bio fertilizer (Chan et al. 2012) or in the bioremediation of heavy metals (Ubeda et al. 2014).

The efficiency of heavy metal bioremediation by a metabolism-dependent process is influenced by the impact of the heavy metals on yeast cells. It is described that for several metals, such as Cu, plasma membrane is the first and one of the main targets of toxicity in the yeast *S. cerevisiae*. This metal originated the disruption of membrane integrity with the consequent release of UV_{260}-absorbing compounds (Ohsumi et al. 1988; Soares et al. 2003). In the present work, it was observed that the exposure of *P. kudriavzevii* CCMA 0136 up to 6 h, to a mixture of 5 mg/l cadmium, 10 mg/l lead and 5 mg/l zinc, originated a small loss of membrane integrity, without an important leakage of UV_{260} compounds, comparatively to the control cells (Fig. 1). The majority of cell population exposed for 6 h to metals mixture lost the proliferation capacity and the ability to process the FUN-1 (Fig. 2). This means that the exposure for 6 h to the simultaneous action of Cd, Pb and Zn induced in *P. kudriavzevii* cells a physiological state usually defined as "viable but not culturable" (Davey and Hexley 2011; Davey and Kell 1996): cells retained membrane integrity but were not able to form colonies on solid media. In fact, ~88 % of cell population retained the membrane integrity, but only ~50 % was able to proliferate and 40 % of cells were FUN-1 positive (Figs. 1a and 2). The exposure for 24 h to the action of multi-metals induced a loss of membrane integrity in the majority of cell population with the leakage of intracellular components (Fig. 1); this resembles a necrotic scenario (Eisenberg et al. 2010). At this time (24 h), a dramatic loss of proliferation capacity and the ability of yeast cells to process FUN-1 occurred (Fig. 2). Together, these results

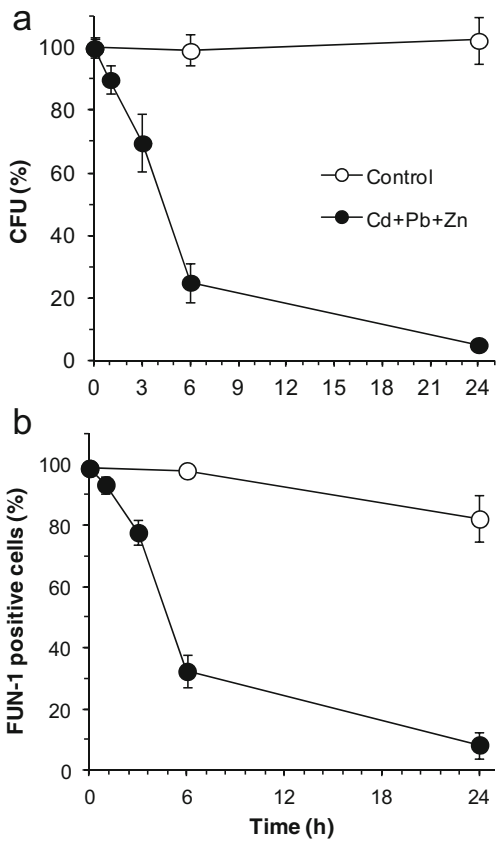

Fig. 6 Impact of multi metals, present in soil extract, on the physiology of *P. kudriavzevii*. Yeast cells were suspended in soil extract in the absence (control) or in the presence of a mixture of metals, reported in Fig. 1. **a** Quantification of yeast cells viability using a clonogenic assay; the data represent the mean (±SD) of six independent experiments. **b** Evaluation of metabolic activity by cell staining with FUN-1; the data represent the mean (±SD) of three independent experiments

indicate that target(s) of metal mixture, in *P. kudriavzevii*, had an intracellular localization. The disruption of membrane integrity, observed after 24 h of metals exposure, was most likely the consequence of the intracellular disorder.

The increase of intracellular Pb level (Fig. 5) is compatible with the toxic symptoms displayed by yeast cells, namely, the loss of proliferation capacity and the ability to process the FUN-1 probe (Fig. 2). On the other hand, the loss of membrane integrity, which occurred after 3 h of cell exposure to the metals (Fig. 1), can explain the increase of intracellular metals content (particularly Cd and Pb) that occurred between 3 and 24 h (Fig. 5). Most likely, the loss of membrane integrity allowed the exposure of further metal binding sites present inside the cells. A similar observation was described in *S. cerevisiae* cells, which membrane was permeabilized by the action of detergents (Gadd 1990), HCHO (Strandberg et al. 1981), thermal-treatment or copper exposure (Machado et al. 2009).

Yeast cell wall is a dynamic structure. In order to ensure cell integrity, the cell wall is constantly remodelled and reorganized in response to growth signals or environmental stress (Harrison et al. 2004). It was described that *Candida krusei* (*P. kudriavzevii)* isolates from soil sediments, with different levels of environmental pollution, had different chitin content and chitin synthase activities. Isolates coming from the most polluted areas (receiving discharges from petroleum refinery or places contaminated with industrial and domestic effluents) displayed the highest chitin content (Romero et al. 2000). In this line, recently, the modification of the cell wall structure and composition of the fungi *Trichosporon asahii* upon exposure to Cd stress was described (Ilyas et al. 2014). It was also shown that the exposure to the antifungal caspofungin induced chitin accumulation in the cell wall of different *Candida* species, including *C. krusei*. It was proposed that the raise of chitin content and cell wall rearrangements are mechanisms of adaptation (tolerance), which resulted in the decrease of cell wall permeability and susceptibility to the drug (Rueda et al. 2014; Walker et al. 2013). Thus, in order to understand the physiological responses of *P. kudriavzevii* to heavy metals exposure, the impact of metals mixture on yeast cell wall was evaluated. In the present work, no abnormal pattern of chitin deposition or increase of chitin content on yeast cell wall of *P. kudriavzevii*, exposed to metals mixture, up to 6 h, comparing with non-exposed cells was observed. In both types of cells (metals treated and non-treated), chitin remained mainly in the scars. Together, these results suggest that the exposure to metal mixture (in the concentrations tested) did not induce alteration of yeast cell wall of *P. kudriavzevii*.

The evaluation of the individual impact of the three metals on membrane integrity and proliferation capacity of *P. kudriavzevii* yeast cells strongly indicates that Pb is the main element responsible for the toxic impact of the mixture. Cd and Zn, in the concentrations tested, were not toxic to *P. kudriavzevii* yeast cells (Fig. 4). The exposure of yeast cells to Pb for 6 h induced a loss of proliferation capacity with a small loss of membrane integrity, similarly to the one described in *S. cerevisiae* cells (Bussche and Soares 2011).

The media composition, namely, the complexation capacity, can affect the metal toxicity. In fact, metal toxicity is correlated with the available metal (i.e. the free and labile metal) concentration and not with the total amount of metal in solution (Huebert and Shay 1992; Sunda and Guillard 1976). Thus, the characteristics of the media where the cells are suspended, namely, the pH and the presence of organic or inorganic ligands (which can complex metals), can affect the amount of available metal and, thus, influence metal toxicity (Angle and Chaney 1989). The buffer here used (MES) does not complex Cd, Pb or Zn (Soares et al. 1999a; Soares et al. 1999b). This means that the total metal concentration is equal to the fraction of metal available, and consequently, all the toxic effects can be correlated with the total concentration of metal added. The loss of proliferation capacity and metabolic

activity induced by metal mixture displayed the same trend when cells were suspended in buffer (Fig. 2) or in soil extract (Fig. 6). Most likely, soil extract used presented low complexing properties, which can explain the similar toxic effects observed in both media (buffer and soil extract).

In conclusion, *P. kudriavzevii* yeast cells exposed to a metal mixture (Cd, Pb and Zn) up to 3 h retained membrane integrity, but presented a loss of metabolic activity and proliferation capacity. The exposure to metal mixture for 24 h induced a loss of membrane integrity with the leakage of intracellular components, which was accompanied by a deep disturbance of metabolic activity and proliferation capacity. The exposure of yeast cells to single metals revealed that, in the concentrations tested, Pb was the metal responsible by the toxic effects. Pb intracellular accumulation seems to be correlated with toxic effects observed. The information obtained about the impact of metal mixture on yeast physiology can be useful for further research about metal tolerance mechanisms in *P. kudriavzevii* and in the use of this yeast in the bioremediation of wastewaters or soils contaminated with heavy metals.

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