Research Paper

Influence of the metabolic state on the tolerance of Pichia kudriavzevii to heavy metals

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This work aims to examine the influence of the metabolic state of the yeast *Pichia kudriavzevii* on the susceptibility to a metals mixture (5 mg L^{-1} Cd, 10 mg L^{-1} Pb, and 5 mg L^{-1} Zn). Cells exposed to the metals mixture in the presence of 25 mmol L^{-1} glucose displayed a higher loss of membrane integrity and proliferation capacity, compared to cells incubated in the absence of glucose. The analysis of the effect of individual metals revealed that glucose increased the toxic effect of Cd marginally, and of Pb significantly. The increased susceptibility to heavy metals due to glucose was attenuated in the simultaneous presence of a mitochondrial respiration inhibitor such as sodium azide (NaN$_3$). ATP-depleted yeast cells, resulting from treatment with the non-metabolizable glucose analogue 2-deoxy-D-glucose, showed an increased susceptibility to heavy metals mixture. Pre-incubation of yeast cells with 1 or 1.5 mmol L^{-1} Ca^{2+} reduced significantly ($P < 0.05$) the loss of membrane integrity induced by the metals mixture. These findings contribute to the understanding of metals mechanisms of toxicity in the non-conventional yeast *P. kudriavzevii*.

Introduction

Heavy metal pollution is a worldwide problem. Industrial activities, such as mining, metal processing and electroplating, produce solid wastes, and/or wastewaters streams containing various metals [1, 2]. The release of these solid wastes or effluents into the environment causes metal pollution of soil, and of surface and ground waters.

In recent times, considerable effort has been expended in a search for bioremediation processes to replace physicochemical processes for treating heavy-metal pollution. In this context, it has been suggested that yeast cell biomass may be applied in bioremediation of metals pollution at low-cost [3]. Recently, the yeast *Pichia kudriavzevii* was described as a new alternative for heavy metals bioremediation because of its high metal-removing capacity [4]. Metal removal by yeast cells can occur by two main mechanisms: surface-accumulation (called biosorption) and metabolism-dependent process (designated as bioaccumulation). The latter process involves the passage of the metals through cell membrane of viable cells, by different mechanisms, and is influenced by the metabolic state of the cell [5].

Zinc is an essential metal since is required for the function of many proteins [6]. Uptake of Zn in the yeast *Saccharomyces cerevisiae* is mediated, mainly, by two specific transporters: Zrt1p (the high affinity transporter active in Zn deficient cells) and Zrt2p (the low affinity transporter active in Zn replete cells) [7, 8]. Zinc transport by Zrt1p is energy dependent [8]. Two other plasma membrane Zn transporters (Fet4p and Pho84p) are involved in Zn uptake by the yeast

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S. cerevisiae. The transporter Fet4p is involved in the low affinity uptake of iron, copper, as well as zinc [9]. Jensen et al. [10] suggested that the high affinity phosphate transporter Pho84p was also involved in Zn uptake.

Non-essential elements, like Cd and Pb, have no known biological functions being toxic at relatively low concentrations. It is assumed that for non-essential elements, there would be no specific uptake mechanisms. Alternatively, these metal ions might enter in yeast cells via uptake systems for essential cations. This is the case of the divalent metal transporter (DMT1), which belongs to the family of natural resistance-associated macrophage proteins (NRAMP) [11, 12]. In mammalian cells, DMT1 (also called DCT1 or Nramp2) has been associated with the transport of a variety of divalent metal ions, including Fe^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, and also with Cd^{2+} and Pb^{2+} uptake [12, 13]. DMT1 represents a large family of orthologous metal ion transporters that are highly conserved from bacteria to humans. In cell, DMT1 is mainly localized at the membrane level [11]. At tissue level, DMT1 is ubiquitously expressed; however, its levels of expression are particularly high in the proximal intestine when compared to areas such as kidney or brain [12]. Yeast cells overexpressing DMT1 displayed an increased Pb uptake [14]. Yeast cells of *S. cerevisiae* expressed three known DMT1 orthologues: Smf1p, Smf2p, and Smf3p, encoded by the *SMF1*, *SMF2*, and *SMF3* genes, respectively [11]. In *S. cerevisiae*, Smf1p and Smf2p transport Mn, Cu, Co, and Cd ions [15].

In a previous work, it was found that *P. kudriavzevii* exposed for 6 h to a metals mixture (Cd, Pb, and Zn) lost the metabolic activity and proliferation capacity with a small loss of membrane integrity [16]. In a subsequent work, it was found that disruption of membrane integrity can be attributed to the intracellular accumulation of reactive oxygen species [17]. These previous observations prompted us to examine the influence of the metabolic state of the non-conventional yeast *P. kudriavzevii* on the susceptibility to the action of heavy metals (Cd, Pb, and Zn). Thus, in the present work, the influence of glucose, sodium azide (NaN_3) (mitochondrial respiratory inhibitor), and ATP depletion on metals-induced toxicity was studied. In addition, the influence of Ca^{2+} on the toxicity response of the yeast to metals mixture was tested. The information obtained in the present study constitutes a step forward in the knowledge of toxic impact of heavy metals on *P. kudriavzevii*, and can be useful for further use of this yeast in the bioremediation of heavy metals.

Materials and methods

Yeast, media, and growth conditions

A strain of *P. kudriavzevii* CCMA 0136 was used in this work. The strain belongs to the Collection of Agricultural Microbial Cultures (CCMA) in the Biology Department (DBI) of the Federal University of Lavras (MG, Brazil). The gene sequence of the yeast strain used has been deposited in GenBank under the accession number KJ468031.1 (http://www.ncbi.nlm.nih.gov/nuccore/KJ468031).

The strain was maintained at 4 °C on YPD agar slants [10 g L^{-1} yeast extract (Difco-BD), 20 g L^{-1} peptone (Difco-BD), 20 g L^{-1} glucose (Merck), and 20 g L^{-1} agar (Merck)]. Yeast pre-cultures were prepared in YPD broth. Cells were incubated at 25 °C on an orbital shaker at 150 rpm for 8–10 h. *P. kudriavzevii* cultures in their exponential growth phase were obtained by inoculating YPD broth with pre-cultures, and then incubating overnight to an OD_{600} of $\sim$0.5 under the same conditions as the pre-cultures.

Exposure of yeast cells to metal stress

Exponential-growth-phase cells were harvested by centrifugation (2500×g, 5 min), washed twice, and re-suspended in deionised water at $\sim$1 × 10^8 cells ml^{-1}. Cells were placed at 1 × 10^7 cells ml^{-1} in 10 mmol L^{-1} (2-(N-morpholino) ethanesulfonic acid) MES buffer (Sigma–Aldrich), pH 6.8. MES is an appropriate pH buffer for toxicity studies because it does not complex the metals under study [18–20].

In the assessment of the influence of glucose on metals toxicity, cells were suspended in 10 mmol L^{-1} MES with 25 mmol L^{-1} glucose. Glucose addition to yeast cells leads to the acidification of the medium, mainly because of the action of the plasma-membrane H^+-ATPase [21]. However, previous control experiments, with cells plus 25 mmol L^{-1} glucose (with or without metals) have shown a modification of pH values by less than 0.05 units (data not shown), which means that MES pH buffer, at a concentration of 10 mmol L^{-1}, is effective in buffering the solution.

Metals mixture or individual metals were added at a final concentration: cadmium, 5 mg L^{-1}; lead, 10 mg L^{-1}; zinc, 5 mg L^{-1}. The following stock standard solutions (Merck) were used: 1000 mg L^{-1} $CdCl_2$; 2000 mg L^{-1} $Pb(NO_3)_2$; 2000 mg L^{-1} $ZnCl_2$. The metal mixture and the concentration of each metal were chosen according to previous screening experiments, performed to select yeast strains for bioremediation purposes, which were carried out in the Environmental and Industrial Microbiology Laboratory in UFLA/DBI, Brazil. Cell suspensions (final volume of 20 ml) were shaken in 100-ml Erlenmeyer flasks at 150 rpm at 25 °C for 6 h.

This incubation time (a chronic exposure of yeast cells to heavy metals) corresponds to more than five times the doubling time of *P. kudriavzevii* in YPD broth, under the growth conditions described above.

Treatment of yeast cells with metabolic inhibitors and calcium ions

Cells were ATP depleted as previously described in Ref. [22]. Briefly, 1×10^7 cells ml^{-1} were resuspended in filter-sterilized (0.45 μm pore size) 0.5% (w/v) 2-deoxy-D-glucose (2-DOG) (Sigma–Aldrich) in 10 mmol L^{-1} MES, pH 6.8. Cell suspensions were shaken at 150 rpm for 16 h at 25 °C. Then, cells were pelleted by centrifugation, washed two times with deionized water, resuspended in 10 mmol L^{-1} MES, pH 6.8, and subsequently placed in the absence (control) or presence of metals mixture.

Mitochondrial function was impaired using NaN$_3$ [23]. Yeast cells were treated with NaN$_3$ as previously described in Ref. [24], with the required modifications. Thus, cells were suspended at 1×10^7 cells ml^{-1} in 10 mmol L^{-1} MES, pH 6.8, containing 25 mmol L^{-1} D-glucose and 20 mmol L^{-1} NaN$_3$ (Sigma–Aldrich), and were preincubated for 10 min before adding the metals mixture.

To study the protector effect of calcium ions against the loss of membrane integrity induced by heavy metals mixture, 1×10^7 cells ml^{-1} were suspended in 10 mmol L^{-1} MES, pH 6.8. The cells were preincubated without or with different calcium concentrations (0.5–1.5 mmol L^{-1} CaCl$_2$), for 1 min, before adding the metals mixture, as previously described in Ref. [25]. Cells exposed to the highest Ca^{2+} concentration (1.5 mmol L^{-1}), in the absence of heavy metals, did not loss membrane integrity.

Assessment of membrane integrity

Membrane integrity was evaluated by staining yeast cells with trypan blue (TB), as previously described in Ref. [16]. Briefly, cells were washed twice with deionised water and suspended in 10 mmol L^{-1} MES, pH 6.8, at 1×10^7 cells ml^{-1}. Yeast suspensions were incubated with TB solution (Aldrich), at a final concentration of 0.2% (w/v), at room temperature, for 20 min. Cells were examined by light microscopy, at the times reported in the figures. For each sample, at least three replicates of 200 cells (>600 cells) were scored in randomly selected fields. Unstained cells were scored as having retained plasma membrane integrity (TB negative cells), whereas, cells with disrupted membrane appeared blue (TB positive cells).

Measurement of cell viability by CFU count

At time intervals indicated in the figures, samples (1.0 ml) were taken (two replicates), serially diluted with sterile deionised 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. No further colonies appeared after that incubation time. The percentage viability was calculated using the number of colony-forming units (CFU) ml^{-1} at zero time (before the addition of metals) as reference (100%).

Reproducibility of the results and statistical analysis

All experiments were repeated, independently, at least three times. The data reported are the mean values ± standard deviation (SD), presented with 95% confidence. Statistical differences between cells exposed to metals mixture in the absence or the presence of Ca^{2+} were tested using unpaired t test.

Results

Glucose enhances metal toxicity

In the present study, the influence of glucose on the toxic impact of a heavy metals mixture comprising of 5 mg L^{-1} Cd, 10 mg L^{-1} Pb, and 5 mg L^{-1} Zn was evaluated. To this end, yeast cells of *P. kudriavzevii* were exposed to metals mixture in the absence or the presence of 25 mmol L^{-1} glucose. The percentage of cells with intact membrane (TB negative cells) dropped to ∼40% in the yeast population incubated for 6 h in the simultaneous presence of glucose and heavy metals (Fig. 1a). A similar pattern was observed in yeast cells growing in colonies on the surface of agar medium. After 6 h of metals exposure, in the presence of glucose, only ∼8% of the yeast cells remained able to proliferate (Fig. 1b). A much small toxic effect was observed in *P. kudriavzevii* yeast cells exposed to metal mixture in the absence of glucose (Fig. 1). *P. kudriavzevii* cells incubated for 6 h in MES buffer at pH 6.8, in the presence of glucose, but in the absence of metals (control), did not lose membrane integrity or proliferation capacity (Fig. 1).

The effect of glucose on the toxicity of individual metals of the mixture was also examined. Yeast population exposed for 6 h to Pb in the presence of glucose also displayed a decreased percentage of cells with intact membrane and proliferation capacity, comparatively to the cells exposed to Pb in the absence of glucose (Fig. 2). Cells treated with Cd, in the presence of glucose, did not lose membrane integrity and displayed a small (<20%) loss of proliferation capacity (Fig. 2). Cells exposed to Zn, in the presence of glucose, did not loss membrane integrity or proliferation capacity (Fig. 2). Yeast cells incubated for 6 h in MES buffer, in the presence or absence of glucose and
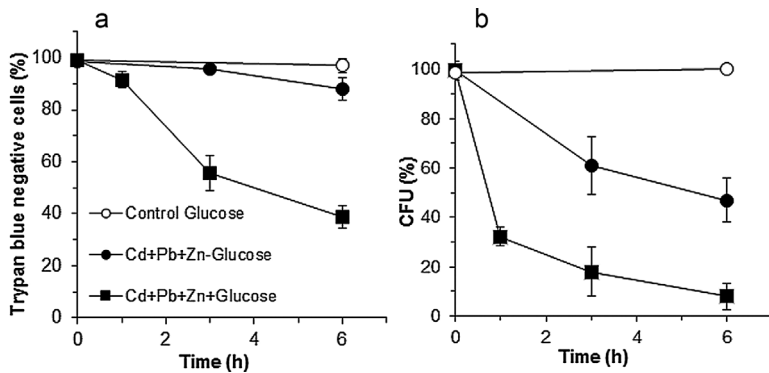
Figure 1. Effect of glucose on the toxicity caused by the heavy metals mixture in the yeast *P. kudriavzevii* CCMA 0136. Cells were exposed to a metals mixture in the absence (Cd + Pb + Zn-glucose) or in the presence of 25 mmol L^{-1} glucose (Cd + Pb + Zn + glucose). As a control (control glucose), cells were incubated in 25 mmol L^{-1} glucose, in the absence of metals. (a) Assessment of membrane integrity by microscopic determination of cells excluding trypan blue (TB) (TB negative cells). (b) Quantification of yeast survival using a clonogenic assay (colony-forming units, cfu, on YPD agar). The data represent the mean (±SD) of three independent experiments for (a) and (b).

metals (control), did not lose membrane integrity or proliferation capacity (data not shown for pictorial clarity).

Together, these results show that at the metal concentrations used glucose increased, particularly, Pb toxicity (Fig. 2).

Sodium azide (NaN$_3$) alleviates metal toxicity

The effect of sodium azide (NaN$_3$, an inhibitor of mitochondrial electron transport chain), on the susceptibility of the cells of *P. kudriavzevii* to metals was examined. Yeast cells incubated in the presence of glucose, NaN$_3$ and the metals mixture had approximately twice as many cells with an intact plasma membrane compared to the cell population exposed to the metals in the absence of NaN$_3$ (Fig. 3a). In the same way, cells incubated with glucose, NaN$_3$, and metals mixture for 6 h, displayed a higher proliferation capacity compared to cells exposed to metals in the absence of

NaN$_3$ (Fig. 3b). These results show that NaN$_3$ alleviate heavy metals toxic effect. Yeast cells were also incubated with glucose and NaN$_3$, in the absence of metals mixture (control); in these conditions, cells did not lose membrane integrity (Fig. 3a) or proliferation capacity (Fig. 3b), which means that NaN$_3$ was not toxic to *P. kudriavzevii*.

A similar behavior was observed in the presence of Pb. The cell population simultaneously exposed to glucose, NaN$_3$, and Pb displayed a higher percentage of cells with intact membrane and proliferation capacity, compared to cells incubated in the absence of NaN$_3$ (Fig. 4).

The results presented above strongly suggest that the toxicity caused by the metals mixture, and especially Pb, is an energy-dependent process, since the abolishment of oxidative phosphorylation enhanced the cell viability in the presence of the metals mixture (Fig. 3) or Pb (Fig. 4).
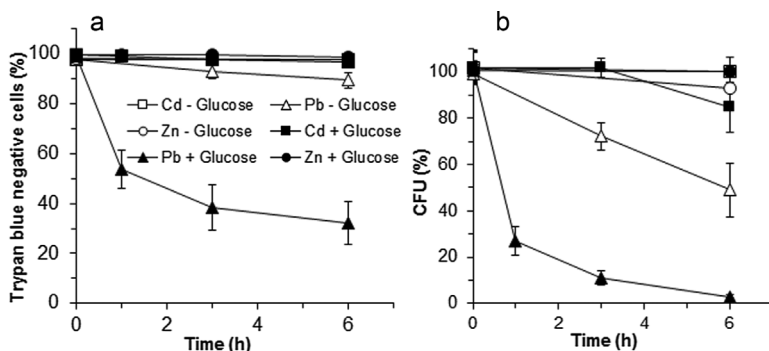
Figure 2. Effect of glucose on the toxicity caused by a single metal in *P. kudriavzevii* CCMA 0136. Cells were exposed to individual metals in the presence of 25 mmol L^{-1} glucose (closed symbols). For comparative purposes, cells were also exposed to metals in the absence of glucose (open symbols). (a) Assessment of membrane integrity by microscopic determination of TB negative cells. (b) Quantification of yeast survival using a clonogenic assay. The data represent the mean (±SD) of four independent experiments for (a) and (b).
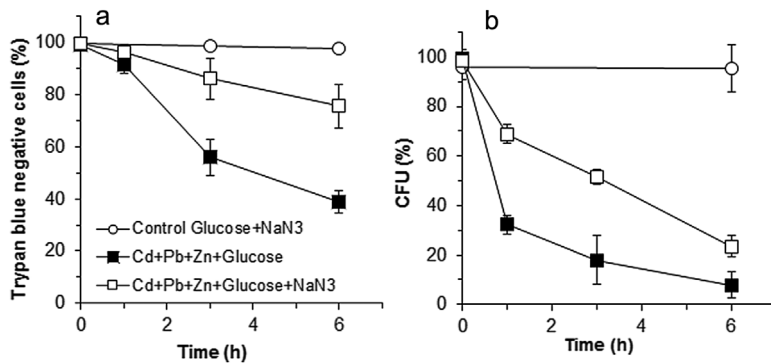
Figure 3. Influence of sodium azide (NaN_3) on the toxicity caused by heavy metals mixture in *P. kudriavzevii* CCMA 0136. Cells were exposed to a metals mixture in the presence of 25 mmol L^{-1} glucose without (Cd + Pb + Zn + glucose) or with 20 mmol L^{-1} NaN_3 (Cd + Pb + Zn + glucose + NaN_3). As a control, cells were incubated in 25 mmol L^{-1} glucose and 20 mmol L^{-1} sodium azide, in the absence of metals (control glucose + NaN_3). (a) Assessment of membrane integrity by microscopic determination of TB negative cells. (b) Quantification of yeast survival using a clonogenic assay. The data represent the mean (±SD) of four independent experiments for (a) and (b).

**ATP depleted cells are more susceptible to the metals**

In natural environments, yeast cells are exposed to extreme variations in nutrient availability, specifically, their carbon, and energy source [26]. In order to test the effect of energy depletion (ATP) on the susceptibility of the yeast to heavy metal, cells of *P. kudriavzevii* were treated overnight with 2-deoxy-D-glucose (2-DOG). 2-DOG is a nonmetabolisable glucose analogue which causes ATP depletion [22].

Yeast cells treated with 2-DOG and subsequently exposed to meals mixture for 6 h, displayed a greater loss of membrane integrity (Fig. 5), and proliferation capacity (data not shown), than cells not exposed to 2-DOG. As control, yeast cells were treated with 2-DOG and subsequently suspended in MES buffer, in the absence of metals; these cells retained membrane integrity (control) (Fig. 5) and proliferation capacity (data not shown), which means that 2-DOG was not toxic to *P. kudriavzevii*.

Ca^{2+} reduces the loss of membrane integrity heavy metal-induced

Divalent ions can protect against heavy metals toxicity by various mechanics: (i) competition with heavy metals uptake, reducing their intracellular levels; (ii) by modification of microbial electrosurface properties making their surface less negative and increasing K_m for cation uptake; or (iii) stabilization of yeast plasma membrane [27, 28]. For this reason, it is of interest to examine the effect of Ca^{2+} on the alleviation of the loss of membrane integrity caused by
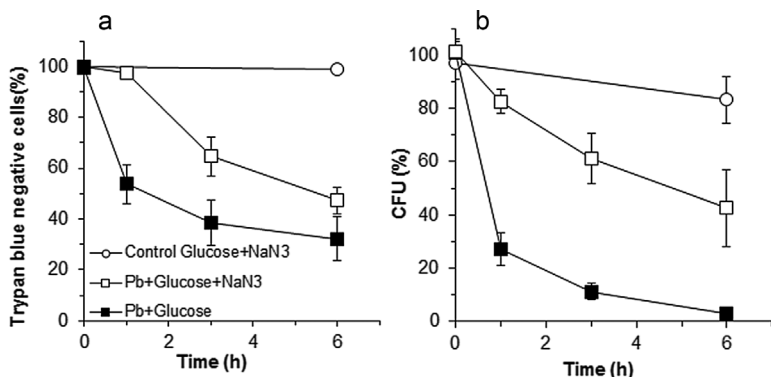
**Figure 4.** Influence of sodium azide (NaN_3) on the toxicity caused by lead (Pb) in *P. kudriavzevii* CCMA 0136. Cells were exposed to 10 mg L^{-1} Pb in the presence of 25 mmol L^{-1} glucose without (Pb + glucose) or with 20 mmol L^{-1} sodium azide (Pb + glucose + NaN_3). As a control, cells were incubated in 25 mmol L^{-1} glucose and 20 mmol L^{-1} sodium azide, in the absence of metals (control glucose + NaN_3). (a) Assessment of membrane integrity by microscopic determination of TB negative cells. The data represent the mean (±SD) of three independent experiments. (b) Quantification of yeast survival using a clonogenic assay. The data represent the mean (±SD) of four independent experiments.
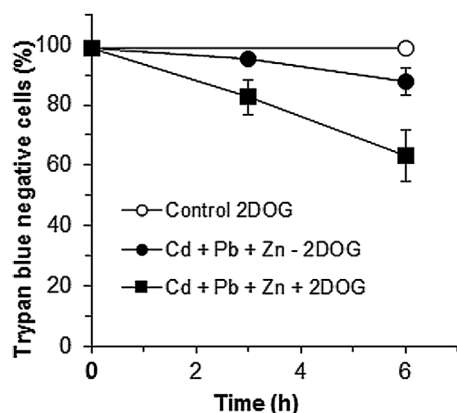
Figure 5. Loss of membrane integrity in *P. kudriavzevii* CCMA 0136 pre-treated with 2-DOG and then exposed to a mixture of heavy metals. Cells were incubated for 16 h with 0.5% (w/v) 2-DOG and subsequently washed and exposed to metals mixture in MES buffer (Cd + Pb + Zn + 2DOG). Control (control 2DOG): cells incubated for 16 h with 0.5% (w/v) 2-DOG and then washed and resuspended in MES buffer in the absence of heavy metals. For comparative purposes, cells non-treated with 2-DOG were also exposed to metals mixture (Cd + Pb + Zn-2DOG). Membrane integrity was assessed by microscopic determination of TB negative cells. The data represent the mean ($\pm$SD) of four independent experiments.

heavy metals in *P. kudriavzevii*. To determine if such an effect could be observed, cells of the yeast were preincubated in the presence of different calcium concentration (0.5–1.5 mmol L^{-1} Ca^{2+}) and subsequently exposed to the heavy metals mixture. The high Ca^{2+} concentration tested (1.5 mmol L^{-1}) corresponded to ~9 times the sum of the heavy metals and ~20–30 times the
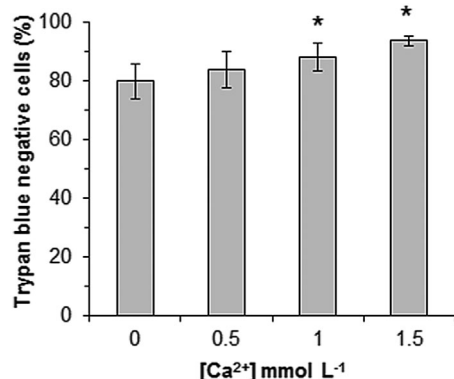
Figure 6. Influence of calcium ions on the loss of membrane integrity caused by heavy metals mixture in *P. kudriavzevii* CCMA 0136. Cells were exposed for 6 h to a metals mixture in the absence or the presence of different calcium concentrations. Membrane integrity was evaluated by microscopic determination of TB negative cells. The data represent the mean ($\pm$SD) of four independent experiments. The difference between cells not incubated with calcium or incubated with different calcium concentrations was tested using an unpaired *t* test. The means with asterisks are significantly different (*$P < 0.05$).

concentration of individual metals (Cd, Pb, or Zn). The presence of 1 or 1.5 mmol L^{-1} Ca^{2+}, reduced significantly ($P < 0.05$) the loss of membrane integrity induced by metals mixture (Fig. 6).

Discussion

In the present work, it was shown that the presence of energy source (glucose) enhanced the toxicity of Pb and of the metals mixture in the cells of *P. kudriavzevii* CCMA 0136 (Fig. 1). Probably, the presence of a metabolizable substrate (glucose) enhanced Cd and Pb bioaccumulation which caused an increase of toxic effects in yeast cells (Fig. 2). This possibility is consistent with the previous results, which showed that Pb toxic effects in *P. kudriavzevii* were related to intracellular Pb level [16]. It was considered the possibility of Cd and Pb could be transported, in yeast cells, by the divalent metal transporter (DMT1). DMT1 has been shown to mediate active transport of a broad range of divalent cations, including essential (for example, Fe and Mn) and non-essential (such as Cd and Pb) metals [12, 14]. However, glucose did not enhance Zn toxicity, even though intracellular Zn accumulation occurs by a metabolic-dependent process in yeasts [6, 29]. The absence of an enhancement of Zn toxicity, in the presence of glucose, can be attributed to a high tolerance of the yeast *P. kudriavzevii* to this essential metal.

The inhibition of cytochrome oxidase, and thus, mitochondrial function due to the presence of NaN$_3$ alleviated the toxicity caused by metal mixture (Fig. 3). A similar effect was observed in the case of the toxicity caused by Pb (Fig. 4). The attenuation of the toxicity caused by the metals mixture and by Pb, resulting from the decrease in energy production by the glucose metabolism, is consistent with the possibility raised above about the uptake of metals by an active transport. In fact, cells treated with NaN$_3$ are restricted to non-oxidative pathways for ATP synthesis [23].

Yeast cells pre-incubated with 2-DOG and subsequently exposed to metal mixture displayed an increased susceptibility to toxic effects of the metals (Fig. 5). 2-DOG is a glucose analogue that is actively taken up by hexose transport and phosphorylated, but not subsequently fully metabolized [30], resulting in ATP depletion of the cell. ATP-depleted cells can not actively accumulate Cd, Pb, or Zn. Thus, it would be expected that such cells would be less sensitive to the toxic effects of the metals mixture. However, the opposite effect (high susceptibility) was observed

(Fig. 5), indicating that ATP is required for some cellular functions associated with defence mechanisms, and cell survival under heavy metal stress.

It is known that Ca reduces the rates of intracellular Cd uptake [31] and protects the cell membrane from disruption by Cd [27], conferring protection against Cd toxicity in *S. cerevisiae* [32]. Similarly, Ca inhibits Zn uptake [33]. In the present work, for high Ca concentrations (1 and 1.5 mmol L^{-1}) a protective effect against the loss of membrane integrity caused by metals mixture was observed.

In conclusion, the present work showed that the metabolic state of *P. kudriavzevii* strongly influenced the susceptibility of the cells to heavy metals. The presence of glucose enhanced the metals mixture toxicity, evaluated through the loss of membrane integrity and proliferation capacity. Probably, the presence of energy source enhanced Cd and Pb bioaccumulation, by the divalent metal transporter (DMT1). Consistently, the impairment of energy production, caused by NaN$_3$, alleviated heavy metals toxicity. However, ATP-depleted yeast cells were more susceptible to heavy metals stress, which indicates that ATP is required for defence mechanisms under heavy metal stress. Ca ions, at high concentrations, protects the yeast cells of *P. kudriavzevii* against the toxicity caused by the metals mixture.

These findings on the influence of the metabolic state of the yeast cells on their sensitivity to metals contributes to the understanding of metals mechanisms of toxicity in the yeast *P. kudriavzevii*. In addition, these information may be useful in the application of *P. kudriavzevii* in bioremediation of different metal-polluted environments, for example, soils, sediments, and wastewaters. The metabolic state of the yeast cells is very important in determining its efficiency in the bioremediation of metal pollution, especially if the bioremediation process involves bioaccumulation [5].

Acknowledgments

Conflict of interest

Authors declare no conflict of interest.

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