Bioremediation potential of Cd by transgenic yeast expressing a metallothionein gene from Populus trichocarpa

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Abstract

Cadmium (Cd) is an extremely toxic environmental pollutant with high mobility in soils, which can contaminate groundwater, increasing its risk of entering the food chain. Yeast biosorption can be a low-cost and effective method for removing Cd from contaminated aqueous solutions. We transformed wild-type *Saccharomyces cerevisiae* (WT) with two versions of a *Populus trichocarpa* gene (*PtMT2b*) coding for a metallothionein: one with the original sequence (*PtMT2b* 'C') and the other with a mutated sequence, with an amino acid substitution (C3Y, named here: *PtMT2b* 'Y'). WT and both transformed yeasts were grown under Cd stress, in agar (0; 10; 20; 50 µM Cd) and liquid medium (0; 10; 20 µM Cd). Yeast growth was assessed visually and by spectrometry OD_{600}. Cd removal from contaminated media and intracellular accumulation were also quantified. *PtMT2b* 'Y' was also inserted into mutant strains: *fet3fet4*, *zrt1zrt2* and *smf1*, and grown under Fe-, Zn- and Mn-deficient media, respectively. Yeast strains had similar growth under 0 µM, but differed under 20 µM Cd, the order of tolerance was: WT < *PtMT2b* 'C' < *PtMT2b* 'Y', the latter presenting 37% higher growth than the strain with *PtMT2b* 'C'. It also extracted ~80% of the Cd in solution, and had higher intracellular Cd than WT. Mutant yeasts carrying *PtMT2b* 'Y' had slightly higher growth in Mn- and Fe-deficient media than their non-transgenic counterparts, suggesting the transgenic protein may chelate these metals. *S. cerevisiae* carrying the altered poplar gene offers potential for bioremediation of Cd from wastewaters or other contaminated liquids.

Keywords: biosorption; environmental biotechnology; functional expression; heavy metals; transgenic yeast; waste treatment

1. Introduction

Cadmium (Cd) is an element that lacks a known biological function. It is one of the most hazardous metals in the environment, because it can affect animals, plants and microorganisms at relatively low concentrations (Alloway, 2013). Several anthropogenic activities are responsible for Cd addition into the environment, such as: atmospheric deposition, industrial and municipal wastes, mining activities, smelting and metal ore processing, battery production, soil fertilisation and sewage sludge application (Mirlean and Roisenberg, 2006; Smolders and Mertens, 2013; He et al., 2015; Khan et al., 2017). Sewage sludge is an inevitable by-product from industrial or domestic wastewater processing, and is commonly used as an organic amendment in soils; however if wastewater is not pre-treated for metal removal, it can lead to high metal contents being added into agricultural soils and crops (Chen and Wang, 2008; Jamali et al., 2009).

Cd is also highly mobile in soils (Lei et al., 2010) with a potential risk of contaminating the groundwater. Estimated leaching of Cd from European soils is between 100 to 5,700 mg Cd ha^{-1} $year^{-1}$ (Smolders and Mertens, 2013). Cd is readily taken up by plant roots and poses a risk when entering the food chain, possibly causing biomagnification, in which a low Cd concentration can increase and become even more toxic through different trophic levels (Janssen et al., 1993).

A low-cost and effective method of removing heavy metals from wastewater or aqueous solutions is by using natural materials of biological origin (algae, fungi, bacteria, yeast) in a process known as biosorption (Goksungur et al., 2005; Bulgariu and Bulgariu, 2016; Beni and Esmaeili, 2020). This process has many advantages, such as low operating costs, decreased volume of the sludge generated and high efficiency in detoxifying very dilute effluents (Marques et al., 2000). The yeast *Saccharomyces cerevisiae* has been frequently studied as a biosorbent for several heavy metals, such as Pb, Cr, Zn, Cu and Cd (Oliveira et al., 2012;

Vijayaraghavan and Balasubramanian, 2015). Although biosorption is a term commonly used for non-living biomaterials that bind and concentrate contaminants, this process occurs in both living and dead organisms (Amirnia et al., 2015).

Employing living microorganisms for metal biosorption has an advantage of simultaneously exploiting their inherent ability of absorbing and accumulating heavy metals intracellularly, a process known as bioaccumulation (Pankiewicz et al., 2015). Recently, a system of continuous growth of *S. cerevisiae* was demonstrated to be an efficient method of removing copper and lead ions from water (Amirnia et al., 2015). *S. cerevisiae* is a promising candidate for bioremediation of metal-contaminated waters or other liquids for several reasons, such as: (1) its reproduction by budding (asexual) or spore formation (sexual) (Wang and Chen, 2009); (2) it is easy to cultivate and available from various food and beverage industries (Wang and Chen, 2006); (3) it has high adsorbent capacity even in dead cells (Goksungur et al., 2005), (4) it can accumulate high intracellular amounts of heavy metals (Brady and Duncan, 1994; Joutey et al., 2013), (5) it can flocculate easily in metal solutions and sediment, which facilitates the separation process after remediation (Machado et al., 2008, Soares, 2011), and, finally, (6) *S. cerevisiae* is a model system in biology and can be easily manipulated genetically and morphologically for numerous purposes (Karathia et al., 2011; Farcasanu and Ruta, 2017).

Genetically engineered microorganisms appear to be the next frontier in terms of bioremediation and biodegradation of contaminants, in which remediation pathways are enhanced by inserting foreign genes of specific interest (Joutey et al., 2013; Kulshreshtha, 2013). Genes coding for phytochelatins (PCs) and metallothioneins (MT) are frequently the focus for engineering microorganisms for heavy metal remediation (Sriprang et al., 2003; Singh et al., 2008; Ruiz et al., 2011).

Metallothioneins are low-molecular weight proteins rich in Cys (usually 9-16 Cys residues), which are able to bind metals in metal-thiolate clusters (Cobbett and Goldsbrough,

2002; Sheoran et al., 2011), such as Zn^{2+} and Cu^{2+} (Bulgarelli et al., 2016). Most MT proteins belong to the sub-family MT2 of plants, which is known for binding divalent cations, such as Cd^{2+} (Cobbett and Goldsbrough, 2002), or some nutrients like Fe^{2+}, Zn^{2+} and Cu^{2+} (Jin et al., 2014). The MT2 sub-family has already been demonstrated to increase Cd tolerance through heterologous expression in yeast (Kohler et al., 2004) and *Arabidopsis thaliana* (Gu et al., 2012).

Similarly, we have recently demonstrated that the gene *PtMT2b* from tree species *Populus trichocarpa* cv 'Trichobel' was able to reduce Cd toxicity when expressed in *S. cerevisiae* (De Oliveira et al., 2020). This poplar clone is particularly tolerant to elevated Cd concentrations (De Oliveira and Tibbett, 2018), whose high expression of MT2b in roots was shown to be correlated to enhanced Cd sequestration (De Oliveira et al., 2020). Moreover, those yeasts expressing poplar MT2b may effectively remove Cd from contaminated water by preventing the excretion of metals back to the medium through chelation (Ruta et al., 2017).

Considering the role of MTs in binding divalent cations and micronutrients (e.g. Zn, Cu) (Jin et al., 2014), it is possible that MT2b in involved in binding other metals besides Cd, such as Fe, Mn and Zn. In this sense, the use of mutant *S. cerevisiae* strains, lacking a particular metal transporter, can help unveil these roles. For instance, DEY1453 is defective for low and high-affinity Fe^{2+} uptake systems, while ZHY3 lacks two Zn^{2+} transporters (ZRT1 and ZRT2) and SMF1 strain lacks a high affinity Mn^{2+} uptake gene (SMF1) (Ullah et al., 2018). If under nutrient deficiency these mutants (carrying MT2b) display growth improvement, it could mean that MT2b is also involved in binding those nutrients.

Therefore, our main objectives were to verify the effect of two versions of the poplar gene *PtMT2b* (the original gene and a mutated version) in Cd tolerance, accumulation and bioremediation potential of *S. cerevisiae*. We hypothesised that: i) *PtMT2b* increases Cd tolerance in transformed yeast; ii) a mutated version of the gene *PtMT2b* 'Y' (encoding a C3Y

substitution) is not as efficient in conferring Cd tolerance in yeast due to the lack of one cysteine in the peptide sequence; and iii) if transformed yeasts are more tolerant to Cd, they can also effectively bioremediate Cd from aqueous solutions (by surface biosorption or intracellular accumulation). Considering the role of MTs in binding divalent cations, it was also hypothesised that this metallothionein could improve the growth of mutant *S. cerevisiae* strains in nutrient depleted media (Fe, Mn or Zn), possibly by containing a larger internal metal storage than the non-transformed yeast.

2. Materials and Methods

2.1 Cloning of poplar's *PtMT2b*

2.1.1 RNA extraction and cDNA synthesis

DNA was extracted from *Populus trichocarpa* cv. 'Trichobel' roots and leaves with DNeasy Plant Mini Kit (Qiagen, UK), following the manufacturer's instructions. Total RNA was extracted from approximately 100 g of fresh weight material (roots) macerated in liquid nitrogen via TissueLyser II (Qiagen®). Extraction was performed by a modified version of the CTAB method (Jaakola et al., 2001): macerated samples were incubated with CTAB buffer (hexadecyltrimethylammonium bromide) for 25 min at 65^{o}C (instead of 10 min), while LiCl addition was 1/3 of total extract volume (instead of 1/4). After overnight precipitation at 4^{o}C, extract was centrifuged for 60 min (instead of 20 min); supernatant was then discarded and RNA pellets were purified with the RNeasy Plant Mini kit (Qiagen, UK), including a DNAse treatment (Qiagen, UK) for 20 min.

The extracted RNA was converted into cDNA using the SensiFAST cDNA synthesis kit (Bioline, UK) following the manufacturer's instructions. The full coding sequence was then amplified with a *PtMT2b* primer set containing attB overhang (annealing temperature: 58^{o}C), with sequences (5' – 3'), according to De Oliveira et al., (2020):

147 F - GGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCTTGCTGTGGAGGAAA;

148 R - GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATTTGCAGGAGCATGGAT.

149 *PtMT2b* sequence has been deposited in GenBank (accession number: MN974475).

150

151 **2.1.2 Amino acid substitution (*PtMT2b* 'Y')**

152 During the cloning process, two different *PtMT2b* sequences were obtained due to a
153 probable error during DNA amplification (Fig. 1). This was later confirmed by sequencing the
154 *MT2b* gene directly from the genomic DNA extracted. One codon had a single nonsynonymous
155 nucleotide substitution, from the original 'TGC' to 'TAC', which consequently changed the
156 correspondent amino acid from a cysteine (C) to a tyrosine (Y) at the third position (C3Y).
157 Considering that cysteine is responsible for the divalent cation binding ability in MTs, it was
158 possible that the C3Y substitution would lead to a different Cd tolerance phenotype in yeast.
159 Therefore, these two versions of the same gene were used in yeast transformation, the original
160 (*PtMT2b* 'C') and the mutated (*PtMT2b* 'Y').

161

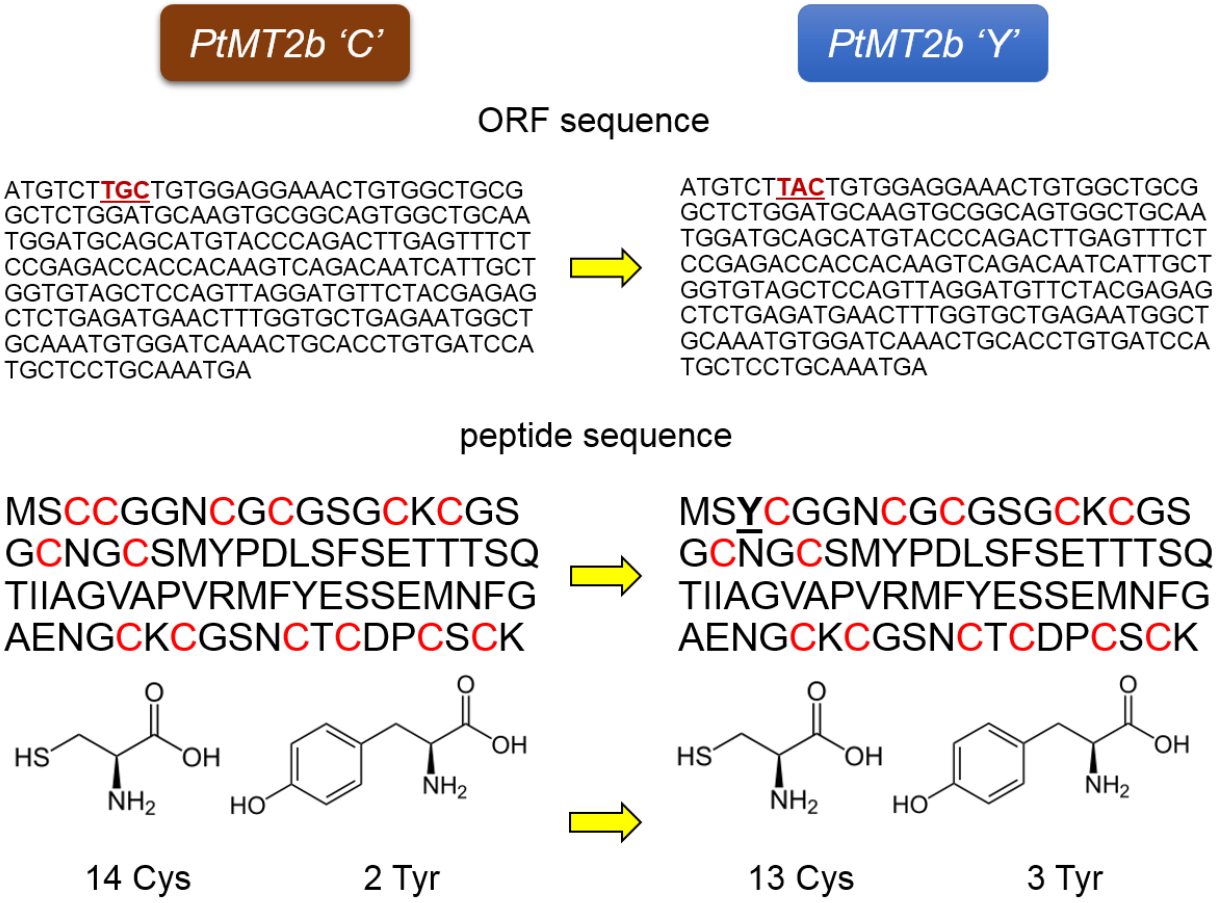

Fig. 1. Substitution of one single nucleotide in *Populus trichocarpa* MT2b gene during amplification (from TGC to TAC), leading to the cysteine in the third position being replaced by a tyrosine (C3Y). Left: original open reading frame (ORF) and peptide sequences. Right: altered sequences.

2.2 Yeast transformation

The wild-type *S. cerevisiae* strain DY1457 (WT) was used for transformations. The genes were introduced into a Gateway® donor vector pDONR221 (containing the kanamycin resistance gene – Fig. S1; Sup. Files) using Gateway® BP Clonase® II enzyme mix. Chemically competent *Escherichia coli* cells (TOP10) were transformed with the entry clones and grown overnight in LB agar + Kanamycin medium at 37°C. Plasmids were isolated from transformed *E. coli* and introduced into destination vector pDR195 (Fig. S2; Sup. Files) using

the Gateway® LR Clonase® II enzyme mix. *E. coli* cells were transformed with the expression vectors and grown in LB agar + Ampicillin, same parameters as before. WT yeast was transformed with either the expression vector containing *PtMT2b* 'C', *PtMT2b* 'Y' or an empty vector (pDR195) as control, the latter strain will be referred to as simply "WT" throughout the manuscript. The transformants were selected on synthetic complete (SC) drop-out medium without uracil [1 g/L drop out medium Y1501 Sigma® + 6.7 g/L yeast nitrogen base Invitrogen™] + 2% dextrose (v/v). Plasmids were restricted (entry vector: *Sac*I and *Ssp*I; expression vector: *Sac*I and *Hin*dIII) and sequenced at every stage to confirm ORF integrity and direction.

2.3 Expression of PtMT2b (C and Y) in yeast under Cd stress

Yeast cells were grown overnight at 30°C (250 rpm) in SC liquid media (5 mL; pH: 5.5). Cells were then pelleted by centrifugation, and re-suspended in 5 mL of sterile water. Optical density at 600 nm of wavelength (OD_{600}) was recorded using SpectraMax i3x (Molecular Devices) microplate reader. Cultures were diluted in sterile water to reach OD_{600} of 0.1, which were used for serial dilutions (1:10 v/v). All dilutions of transformed (*PtMT2b* 'C' and 'Y') and empty vector yeast ('WT') were spotted (5 µL) into SC agar plates at 0; 10; 20; and 50 µM Cd (in the form of $CdCl_2$), then grown at 30°C for 72 hours in the dark (three replicates). In order to quantify yeast growth under Cd stress, all strains were grown in liquid SC media (initial OD_{600}: 0.01), containing either 0; 10 or 20 µM Cd (three replicates) for 48 hours (30°C, 250 rpm; dark), after which the OD_{600} was recorded. The concentration of 50 µM Cd was not used in any liquid media assays due to high toxicity.

2.4 Cd accumulation and extraction in yeast containing PtMT2b 'Y'

The *PtMT2b* 'Y' and WT (empty vector) yeasts were grown in 5 mL of SC liquid media + 2% dextrose, containing 0; 10 and 30 µM Cd at 30°C in the dark with constant shaking (initial OD_{600}: 0.01; four replicates). After 72 h, OD_{600} was recorded and cells were pelleted by centrifugation (10 min, 4000 rpm). All contaminated media were transferred to new tubes without disturbing the pellet, these were denominated Left Over (LO) and were later analysed by ICP-MS to determine the remaining Cd concentration after yeast growth. Pelleted cells were re-suspended in 10 mL of EDTA (20 mM) and washed for 10 minutes (by inverting tubes) in order to remove adhering Cd ions from yeast surface (Ullah et al., 2018). Cells were pelleted again and washed twice with 10 mL of deionised water. Yeasts were oven-dried at 80°C for 48 hours. Dried cells were digested in 5 mL of 69% nitric acid (TraceSELECT™ grade) in closed glass vessels for 8 h at 110°C (in duplicates). Pure acid was used as blank and 0.05 g of reference material (IAEA-359 cabbage leaves) was digested in the same manner for quality control. Cd accumulation in cells and the remaining Cd in Left Over media were determined via ICP-MS (Thermo Scientific™ iCAP™ Q). Cd extraction potential was calculated by the following equation:

$$(1) \quad Cd\ extracted\ (\%) = 100 - \frac{LO\ Cd \times 100}{Initial\ Cd}$$

In which *"LO Cd"* is the Cd concentration determined in the Left Over media solution after yeast growth (mg L^{-1}); and "*Initial Cd"* the concentration of Cd added in the growth media before yeast inoculation, also determined via ICP-MS (mg L^{-1}).

2.5 Cell Dry Weight vs OD_{600}

In order to estimate Cd concentration in terms of cell dry weight (CDW), transformed (*PtMT2b* 'Y') yeast was grown in conical flasks (three replicates), containing 60 mL of uncontaminated SC media, with OD_{600} starting at 0.01. Every 3 h an aliquot of 10 mL from

each flask had its OD_{600} determined, cells were pelleted and washed with deionised water and dried in previously weighed glass vials at 80°C. After 72 h, dry weight was recorded. The relationship between CDW and OD_{600} was determined by linear regression model ($\alpha = 0.05$; 15 samples).

2.6 PtMT2b 'Y' expression in mutant yeast under nutrient deficiency

In order to verify the specificity of this gene, transgenic mutant yeast were subjected to nutrient deficient conditions (Fe, Mn and Zn). If MT2b 'Y' proteins also bind these nutrients, these yeast strains would be able to grow under deficiency due to a higher nutrient storage capacity in their cells. Strains used for transformation were the single mutant SMF1 (*smf1*), and the double mutants DEY1453 (*fet3fet4*) and ZHY3 (*zrt1zrt2*), as well as the corresponding parental wild type strain DY1457. All strains were transformed either with *PtMT2b* 'Y' or an empty vector (e.v.) as control. Mutant yeasts were also transformed with *TcNramp5,* a metal transporter gene from cocoa trees known to increase Cd^{2+} and Zn^{2+} uptake in yeast (Ullah et al., 2018), and were used as a positive control. Transformations were carried out as described previously.

Primary cultures were stablished from a single colony, and grown in 10 mL SC media supplemented with either 0.4% (v/v) Fe, 0.2% Mn or 0.4% Zn; for DEY1453, SMF1 and ZHY3 strains, respectively (30°C, 72h, 250 rpm, dark). Initial growth in a rich media was carried out to promote a nutrient stock in yeast cells before being transferred to deficient media (pre-growth stage). Afterwards, cultures were serial diluted and spotted (5 µL) into SC + agar plates, with or without chelating agents to decrease nutrient availability: 10 µM BPS (Bathophenanthrolinedisulfonic acid) for creating iron deficient plates (- Fe); 12.5 mM EGTA (Ethylene glycol-bis(2-aminoethylether)-N,N,N′) for Mn deficiency (- Mn); and 100 µM EDTA (Ethylenediamine tetraacetic acid) for Zn deficiency (- Zn).

2.7 Statistical analyses

ANOVA and Tukey test were performed for all datasets that met ANOVA's assumptions. Some variables needed transformation (x^2) to attain normality and homoscedasticity, i.e. OD_{600} values for *SMF1* strains (with and without MT2b) grown under Mn deficiency. After being unable to transform the data for Cd content (µg g^{-1}) to attain normality, the non-parametric Kruskal-Wallis test was performed. Linear regression analysis was used for obtaining the CDW (mg mL^{-1}) and OD_{600} relationship, in which the Min/Max accuracy and MAPE (mean absolute percent error) were used to assess the model accuracy. All statistical analyses were performed using R software.

3. Results

3.1 Amino acid substitution in MT2b increased Cd tolerance in yeast

The spot assay clearly showed that the strains transformed with both versions of the *PtMT2b* gene were able to cope with higher Cd concentrations than the strain transformed with the empty vector only, especially at 50 µM, in which its growth was completely suppressed (Fig. 2).

In liquid media contaminated by Cd, yeast strains had similar growth under 0 µM, but differed under 10 and 20 µM Cd (ANOVA: $p < 0.001$). Under the highest Cd concentration the order of tolerance was WT < *PtMT2b* 'C' < *PtMT2b* 'Y'; determined after Tukey test (variation coefficient = 6.5%), in which the growth of yeasts carrying the tyrosine-replaced MT2b was around 37% higher than strain expressing the original gene sequence (*PtMT2b* 'C') (Table 1).

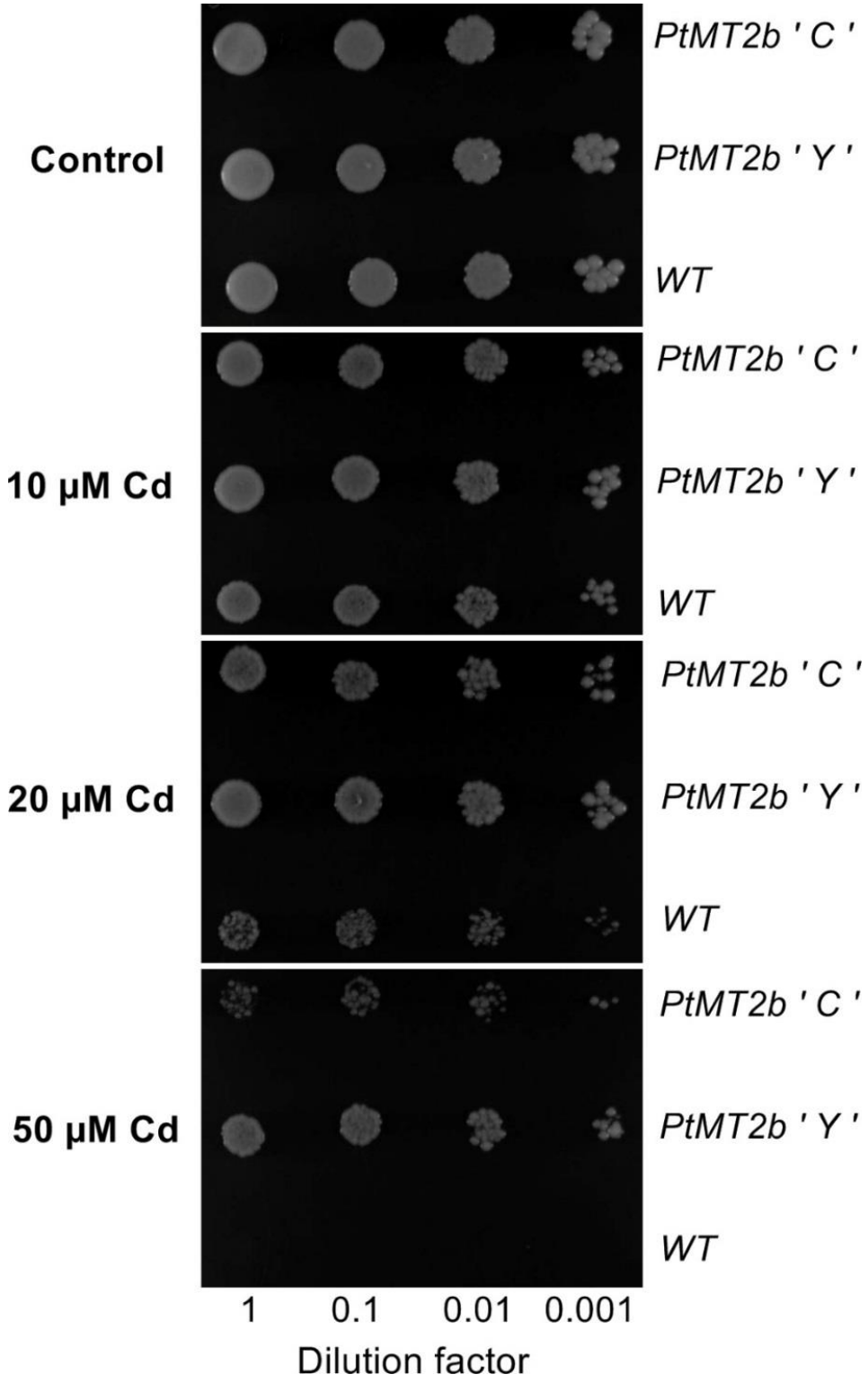

Fig. 2. Heterologous expression of *PtMT2b* in *S. cerevisiae* growing under increasing Cd concentrations. WT: Wild type (DY1457) strain with empty vector; PtMT2b 'C': wild type yeast transformed with the original *PtMT2b*; PtMT2b 'Y': transformed yeast expressing the modified *PtMT2b* gene, with cysteine to tyrosine replacement (C3Y).

266

Table 1 – Growth of transformed *S. cerevisiae* strains under Cd stress, determined by OD_{600} after 48 hours (mean ± st. error)[1]. WT: wild type with empty vector; *PtMT2b* 'C': gene with original sequence; and *PtMT2b* 'Y': gene with cysteine to tyrosine replacement (C3Y).

Strain	Media Cd concentration	
	10 µM	20 µM
WT	0.20 ± 0.009 a	0.15 ± 0.009 a
WT + *PtMT2b* 'C'	0.31 ± 0.005 b	0.25 ± 0.003 b
WT + *PtMT2b* 'Y'	0.29 ± 0.005 b	0.35 ± 0.012 c

[1]- Different letters correspond to significant differences among strains within columns (same Cd concentrations), as determined by Tukey test after ANOVA ($p < 0.001$).

267

268 **3.2 Mutated PtMT2b gene increased Cd accumulation and removal by yeast**

269 Since yeast carrying the mutated gene sequence (*PtMT2b* 'Y') were more tolerant than
270 the strains expressing the original gene, they were ultimately selected for Cd bioremediation
271 trials. Results showed that WT strain (empty vector) was significantly affected by Cd toxicity,
272 while growth of transformed strain was unaffected by Cd additions (Fig. 3).

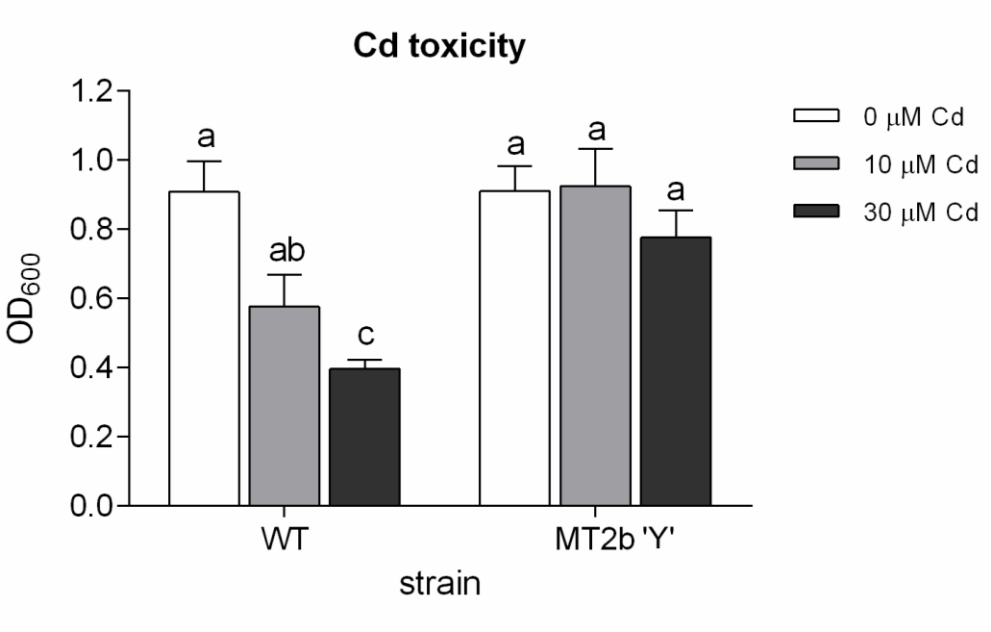

Fig. 3. *S. cerevisiae* growth under three Cd concentrations, as determined by OD_{600} in liquid SC media after 72h. WT: Wild type (DY1457) with empty vector; MT2b 'Y': transformed yeast carrying mutated *PtMT2b* gene, with an amino acid substitution (C3Y). Different letters represent significant differences among treatments by Tukey test (ANOVA; $p = 0.00085$)

273

274 Transformed yeast accumulated high contents of Cd within cells, with concentrations
275 at least 30 times higher than the strains carrying empty vectors only (Fig. 4a). In the WT yeast,
276 internal Cd uptake was similar regardless of media concentration, but in transformed yeast,
277 accumulation significantly increased under the highest Cd dose (30 μM).

278

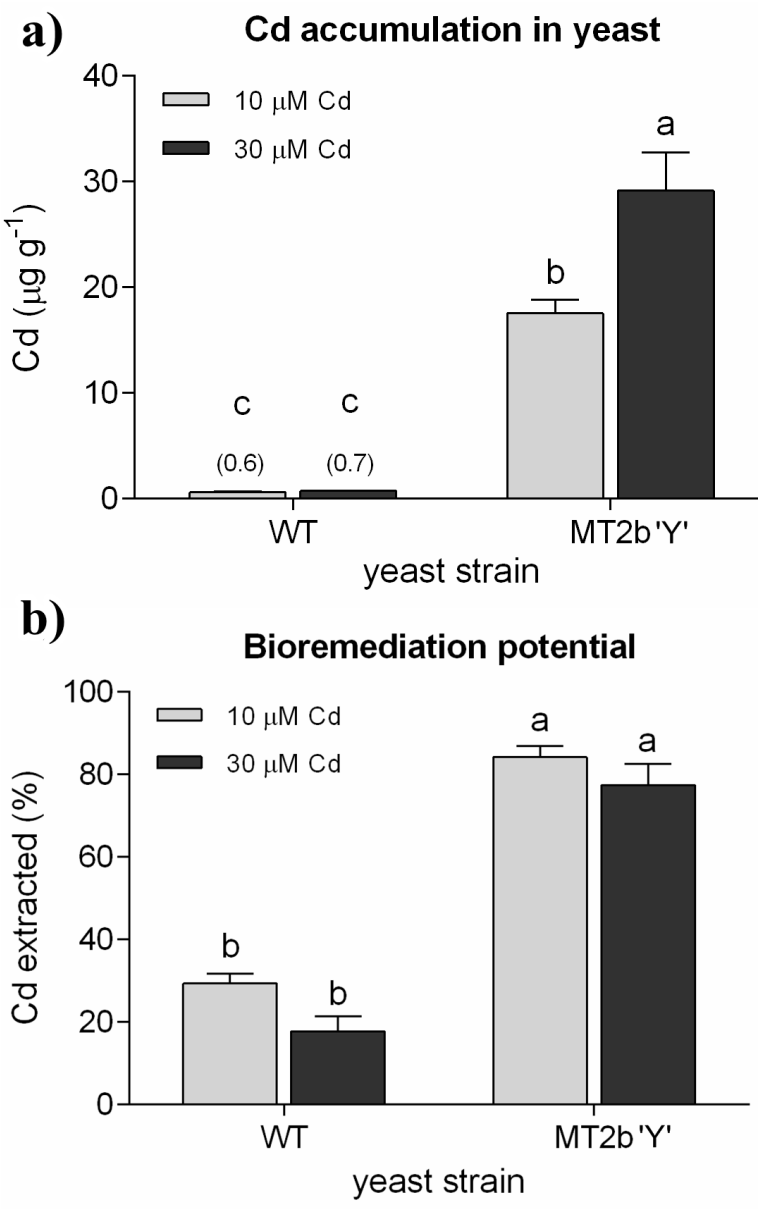

Fig. 4. Cd accumulation in *S. cerevisiae* strains after 72 hours of growth. WT: Wild type + empty vector (DY1457); MT2b 'Y': transformed yeast carrying mutated *PtMT2b* gene, with an amino acid substitution (C3Y). a) Amount of Cd in dried yeast cells (μg g^{-1}) after EDTA washing and acid digestion. b) Percentage of Cd removal from liquid media after yeast growth (72 h). Different letters represent significant differences among treatments by Kruskal-Wallis and Dunn test ($p = 0.004$) in a); and by Tukey test (ANOVA; $p < 0.001$) in b)

279

In order to convert the OD_{600} values into CDW (cell dry weight) and express the results in µg of Cd per g CDW, the following equation was used:

$$CDW_{(mg/ml)} = 2.496 \times OD_{600} + 0.0303$$

This equation was obtained by a linear regression analysis between CDW (mg mL^{-1}) and OD_{600} values of 15 samples at different growth stages ($R^2 = 0.974$; $p < 0.001$); with 94.8% of Min/Max accuracy and 5.6% of MAPE (mean absolute percent error) (Fig. S3; Sup. Files).

In terms of Cd removal from the media (%), which includes internal Cd accumulation, cell wall binding and sorption processes; the transformed yeast removed around 84% and 77% of the total Cd concentration initially added (10 µM and 30 µM, respectively) in a 72 h period, while in WT strain those values were on average under 30% (Fig. 4b).

3.3 Mutated PtMT2b gene slightly increases yeast growth under Fe and Mn deficiency

Spot assay of transformed mutant yeasts under deficient conditions showed that *PtMT2b* 'Y' could not recover the growth of double mutant *zrt1zrt2* under Zn deficiency, but slightly promoted growth in *SMF1* and *fet3fet4* strains in Mn and Fe deficient plates, respectively (Fig. 5). From those strains, the *PtMT2b* 'Y' effect appeared to be more pronounced only in *SMF1* (Fig. 5b). For quantification purposes, this mutant strain was cultivated in liquid media under Mn deficiency, under which conditions *SMF1* + *PtMT2b* 'Y' had on average 71% higher growth (OD_{600}: 0.90 ± 0.01) than when carrying an empty vector (OD_{600}: 0.52 ± 0.13) (ANOVA; $p = 0.008$).

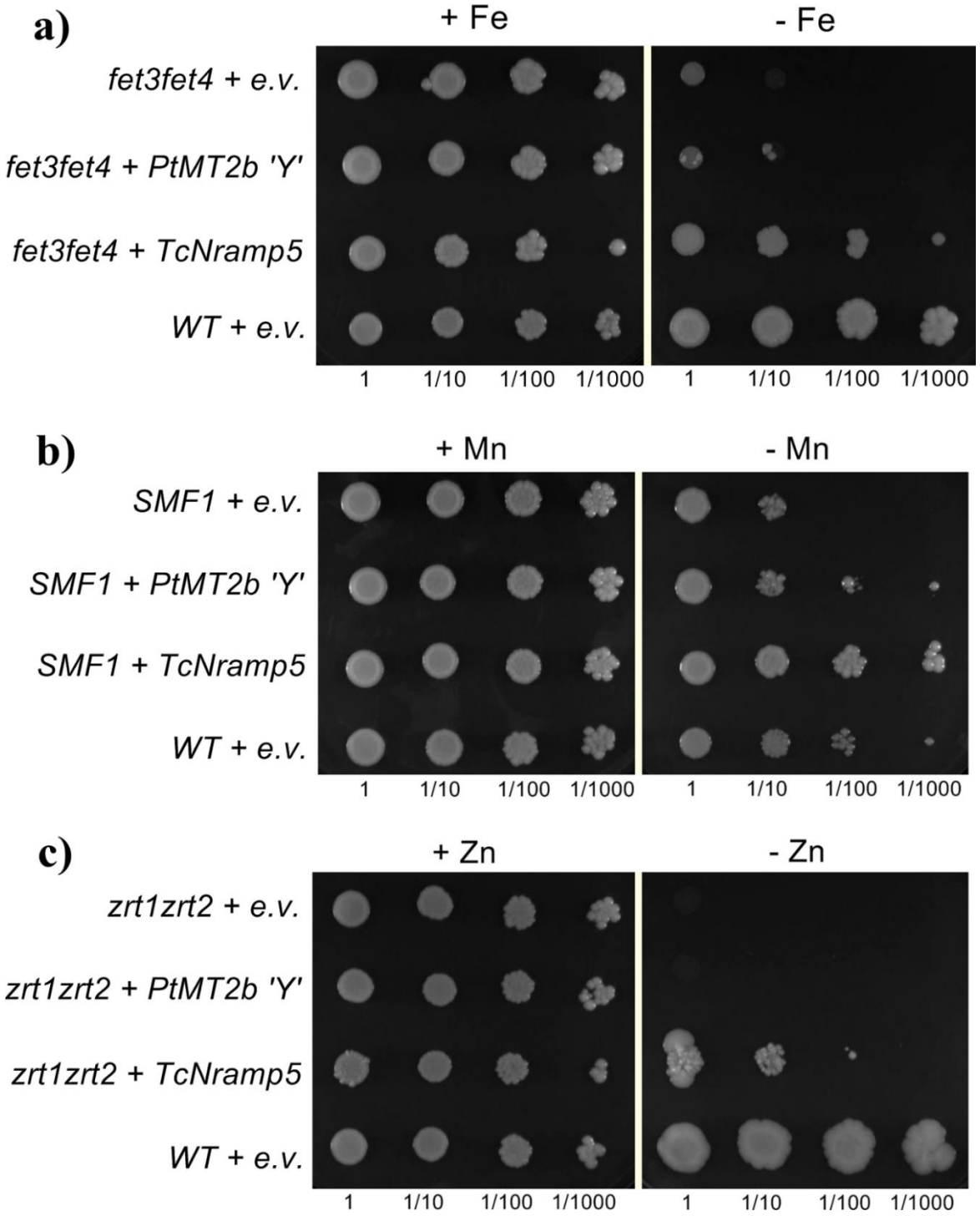

Fig. 5. Growth of mutant *S. cerevisiae* strains in nutrient sufficient (+ X) and nutrient deficient (- X) plates for 72 hours. Yeast strains were: *fet3fet4*, with double mutations for Fe uptake (a); *SMF1*, with single mutation for Mn uptake (b); and *zrt1zrt2*, with double mutations for Zn uptake (c). WT: wild type; e. v.: empty vector (DY1457); *PtMT2b* 'Y': poplar metallothionein with cysteine to tyrosine replacement (C3Y); and *TcNramp5*: cocoa tree metal transporter Nramp5. Dilution 1 = 0.1 OD_{600}

302

4. Discussion

4.1 Poplar's metallothionein confers Cd tolerance in yeast

As demonstrated previously (Fig. 2), *P. trichocarpa* metallothionein MT2b is indeed able to increase Cd tolerance in transformed *S. cerevisiae* (De Oliveira et al., 2020). Besides chelating and inactivating metals in their toxic forms, such as Cd^{2+}, MTs have a role in scavenging reactive oxygen species (ROS) from cells under stress (Wong et al., 2004; Ruttkay-Nedecky et al., 2013). Genes for ROS tolerance are highly expressed in wild type *S. cerevisiae* exposed to Cd (Thorsen et al., 2009); therefore it is clear that the addition of *PtMT2b* would enhance Cd tolerance by producing even more ROS-scavenging proteins than a WT strain.

Heterologous expression of other plant metallothionein genes in yeast have been assessed under heavy metal stress, with similar results, but mostly from herbaceous plant species (Zhou and Goldsbrough, 1994; Guo et al., 2008; Zhang et al., 2014a; Zhang et al., 2014b). For Cd, metallothioneins from sunflower, rice, *Arabidopsis*, *Noccaea caerulescens*, and even mycorrhizal fungi *Rhizophagus irregularis* and *Hebeloma cylindrosporum* were shown to complement Cd sensitivity in mutant yeast (Farcasanu and Ruta, 2017). In the present study, the non-transgenic strain (WT + empty vector) had a decrease in biomass of around 50% under 10 µM Cd in liquid media (Fig. 3), which is in accordance with the results from Hosiner et al., (2014), who reported an EC50 (half maximal effective concentration) of 10 µM $CdCl_2$ for *S. cerevisiae*. In the transgenic strain, however, growth was barely affected even at 30 µM Cd, confirming our initial hypothesis that *PtMT2b* increases Cd tolerance.

4.2 Amino acid substitution further enhanced Cd tolerance

Metallothioneins are characterised by their high content of Cys residues - generally 10 to 17 in plants – which are able to bind divalent metal cations in their sulfhydryl (R–SH) group, thus forming thiolate bonds (Hassinen et al., 2011; Nguyen et al., 2017) and, in the case of the

type II sub-family, their amino-terminal portion has a highly conserved domain, starting with Cys-Cys arrangement (Bulgarelli et al., 2016). Because of this obvious role of the cysteine content in providing metal binding sites in these proteins, it was interesting to observe that *PtMT2b* 'Y', a gene encoding a MT with one fewer Cys residue (replaced by one tyrosine - Tyr), not only did not lose its function as we hypothesised, but in fact enhanced Cd tolerance in transformed yeast. We could speculate two main reasons for this: 1) the tyrosine aromatic ring; 2) the position in which the substitution took place (C3Y).

Despite lacking the characteristic sulfhydryl group from Cys, Tyr has a phenolic aromatic ring that can also effectively bind divalent cations such as Cd^{2+} in their aromatic structure forming tyrosine-metal complexes (Hu et al., 1995), from which different conformations have been proposed (Fig. S4; Sup. Files). In this sense, Vandenbossche et al., (2015) developed a synthetic material enriched with tyrosine molecules that was able to efficiently remove copper from contaminated waters.

Another reason for increased Cd tolerance is also related to the aromatic group in Tyr, which can form a non-covalent bond with cationic metals, known as cation-π interactions. This interaction is essentially electrostatic, in which a cation is attracted to the negatively charged cloud of electrons from aromatic groups (π systems), and is considered one of the strongest noncovalent interactions (Ma and Dougherty, 1997; Mahadevi and Sastry, 2013). Although mostly reported for monovalent cations, cation-π can also happen with divalent metal ions, such as seen with Mg^{2+} (Stewart et al., 2013).

The position in which the substitution took place may possibly have influenced the results observed. Plant MTs have two short cysteine-rich terminal domains linked by a long spacer, devoid of Cys, and of around 40 amino acids (Domenech et al., 2006). These Cys domains in opposite ends can interact with each other and bind metals, forming a cluster, conferring the *hairpin* structure model typical of MT2 proteins (Hassinen et al., 2011). In the

353 present work, *PtMT2b* 'Y' had only the third amino acid of the peptide chain (Cys) replaced
354 by a Tyr (C3Y; Fig. 1), which means that it is unlikely for it to have affected the overall protein
355 folding, considering that this domain had another seven Cys residues to interact with the six
356 Cys from the opposite domain. Moreover, the domain in which this substitution occurred may
357 also explain why there was no loss of protein function. For instance, Cismowski et al. (1991)
358 observed that yeast carrying a mutated mammalian MT gene (Cys to Tyr substitution) had a
359 markedly lower resistance to Cd when it occurred in one domain (C50Y), but no effects when
360 this mutation was present in another domain (C13Y). Nevertheless, our results have shown for
361 the first time that the Cys to Tyr (C3Y) substitution in a plant metallothionein gene can in fact
362 increase Cd tolerance and accumulation in yeast. This suggests that metallothionein
363 manipulation and editing could be further explored to enhance bioremediation capacity in
364 microorganisms.
365
366 **4.3 Bioaccumulation and removal of Cd by transgenic yeast**
367 Yeast can remove metals from solutions by mainly two mechanisms, one is passive and
368 requires no energy expenditure (e.g. cell wall binding and metal diffusion) and the other active,
369 metabolism-dependent and being carried out only by living cells, involving
370 compartmentalisation in subcellular organelles such as vacuole or mitochondria (Vijver et al.,
371 2004; Wang and Chen, 2009). Metal binding by metallothioneins is one of the most important
372 strategies for metal accumulation (or toxicity avoidance) in living cells, a process seen in
373 almost all eukaryotic organisms, such as animals, plants, yeast and ectomycorrhizal fungi
374 (Vijver et al., 2004; Nguyen et al., 2017). Although in *S. cerevisiae* the induction of MT
375 production seems to occur mainly through exposure to Cu (Wang and Chen, 2006) or Ag
376 (Hosiner et al., 2014).

Linear regression resulted in a good prediction model for converting OD_{600} measurements into cell dry weight (CDW) and allowed converting Cd concentrations in yeast to µg of Cd per gram of biomass. It should be noted, however, that those predictions should be applied only under the experimental conditions of the present work (strain type, growth period, temperature etc.), as well as the equipment use for OD_{600} determination, since it can vary according to the device used (Ude et al., 2014).

Transgenic strains carrying the mutated *PtMT2b* gene were not only highly tolerant but also effectively accumulated more Cd (in µg g^{-1}) than wild type yeast, with Cd contents at least 10 times higher, which supports our hypothesis that Cd tolerance can lead to enhanced Cd accumulation. Ruta et al., (2017) recently showed that *S. cerevisiae* transformed with *NctMT2a* and *NcMT2b* (from *Noccaea caerulescens*) had a 5-fold and a 4-fold increase in Cd accumulation, respectively, compared to the non-transformed strain. Yeast expressing *SaMT2* from hyperaccumulator *Sedum alfredii* also had a 50% increase in Cd accumulation in relation to the control (Zhang et al., 2014b). However enhanced Cd accumulation is not always observed, such as the case of the *S. cerevisiae* strains transformed with a range of MTs from *A. thaliana* (Guo et al., 2008). Bacteria may also display similar effects, such as the *E. coli* expressing a metallothionein from mice (*mt-1*), in which the gene promoted higher tolerance and accumulation of mercury from contaminated media (Ruiz et al., 2011), and the *CeMT2b* gene from tolerant weed species *Colocasia esculenta*, that doubled Cd accumulation in *E. coli* (Kim et al., 2011).

Due to their biosorption characteristics, yeast cell walls can remove heavy metals from aqueous wastes even if the cells are no longer alive. Machado et al., (2008) verified that after applying dead *S. cerevisiae* biomass (12 mg mL^{-1}) in nickel contaminated water, almost 80% of the Ni^{2+} in solution was removed after only 30 minutes. By using the OD_{600} to CDW (mg mL^{-1}) conversion equation previously determined, we were able to estimate that despite

402 removing around 80% of Cd^{2+} from the growth media, this amount would represent a
403 biosorption capacity of 1.5 mg g^{-1} of dried yeast. Even though this assay ran for only 72 hours
404 and did not reach saturation, the result is quite low compared to other biosorbent materials,
405 such as dried chestnut burr, which is able to remove 16.2 mg of Cd per gram, pinecones (4.3
406 mg g^{-1}) or the breakthrough biosorbent known as MMBB (a mix of tea wastes, mandarin peels
407 and maple leaves), which can absorb 31.7 mg g^{-1} of Cd from solution (Kim et al., 2015;
408 Abdolali et al., 2016). However, those are dead materials, and are not susceptible to metal
409 toxicity effects. Living yeasts provide a constant source of biosorbent material, which is also
410 able to actively accumulate metals within cells, removing metals continuously through internal
411 detoxification mechanisms (Wang and Chen, 2006). In this sense, Amirnia et al., (2015)
412 developed a continuous bioreactor-biosorption system, which is efficient for simultaneous
413 production of *S. cerevisiae* and removal of Cu^{2+} and Pb^{2+} from liquid waste without requiring
414 much nutritional input for yeast growth. The authors also suggested that this process is
415 facilitated by using flocculant strains that are easily able to decant and separate from the growth
416 solution (Soares, 2011), a feature that was observed in the WT strains in the present work.
417

418 **4.4 *PtMT2b* 'Y' possible role in binding Mn and Fe in mutant yeast**

419 Considering the evidence that metals such as Zn and Cu can affect the expression of
420 MT2b in white poplar (Cicatelli et al., 2010), and that both Cd and Zn concentrations were
421 verified to be highly correlated to *MT2b* expression in leaves of *P. tremula* x *P. tremuloides*
422 (Hassinen et al., 2009), we hypothesised that the double mutant strain *zrt1zrt2*, lacking two Zn
423 transporters, would have increased growth if carrying the *PtMT2b* gene. This was based on the
424 concept that prior to yeast inoculation into the Zn-depleted media, during pre-growth stage,
425 transgenic yeast would have built up a larger nutrient storage capacity within their cells by
426 forming MT-Metal chelates, which could then be accessed under nutrient deficiency. The same

was tested for Fe and Mn, using their respective mutant strains, for it is known that MTs are also able to bind these metals (Zhang et al., 2014a; Farcasanu and Ruta, 2017).

In our work, the spot assay showed that mutant strain *zrt1zrt2* had no effects from *PtMT2b* 'Y' transformation under Zn deficiency, showing virtually no growth. One reason could be that the double mutation did not allow enough Zn to penetrate the yeast cells during pre-growth. *S. cerevisiae* acquires Zn via mainly three transporters: Zrt1 (high affinity), Zrt2 (low affinity) and Fet4 (non-specific), therefore, this mutation severely hinders Zn acquisition pathways (Zhao and Eide, 1996; Schothorst et al., 2017). The *PtMT2b* gene was also shown to have slightly higher expression in poplars under high Zn concentrations (De Oliveira et al., 2020), so it is probably involved in Zn binding; however it is possible that the amino acid substitution (C3Y) in this gene could have led to a protein with lower Zn affinity, resulting in poor Zn storage.

However, yeasts carrying *PtMT2b* 'Y' were able to grow, to some extent, in Mn- and Fe- agar deficient media, confirming in part our initial hypothesis, although only by verifying metal contents intracellularly could we reach a more empirical conclusion. When grown in liquid media, the transgenic *SMF1* strain had a 71% increase in growth under Mn deficiency, suggesting that this gene is involved in Mn binding. The involvement of MTs in Mn homeostasis has not been thoroughly explored in plants thus far, except for a few studies with MTs from animals or plants (Kobayashi et al., 2007; Benatti et al., 2014).

5. Conclusions

Heterologous expression of the metallothionein gene (*PtMT2b*) from the Cd tolerant tree *Populus trichocarpa* is able to confer tolerance to *S. cerevisiae* under Cd concentrations up to 50 µM. Contrary to our hypothesis, replacement of Cys by Tyr (C3Y) in the amino acid sequence did not affect protein function, and, in fact, increased yeast growth under Cd stress.

The transgenic strains carrying the mutated gene were able to extract up to 80% of Cd from contaminated media solution, mostly due to continuous growth and constant metal biosorption. This specific strain offers great potential for bioremediation of Cd from waters or effluents, possibly in a bioreactor system, and further studies should be carried out to assess its potential use in a mixture of cationic metals, such as Zn, Mn or Cu, as well as tested on different bioreactors.

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7. Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

8. References

Abdolali A., Ngo H.H., Guo W., Lu S., Chen S.S., Nguyen N.C., Zhang X., Wang J., Wu Y., 2016. A breakthrough biosorbent in removing heavy metals: Equilibrium, kinetic, thermodynamic and mechanism analyses in a lab-scale study. Sci. Total. Environ. 542, 603–611. http://dx.doi.org/10.1016/j.scitotenv.2015.10.095

Alloway B.J., 2013. Heavy metals in soils, 3rd edn. Springer, Netherlands. 10.1007/978-94-007-4470-7

Amirnia S., Ray M.B., Margaritis A., 2015. Heavy metals removal from aqueous solutions using *Saccharomyces cerevisiae* in a novel continuous bioreactor–biosorption system. Chem. Eng. J. 264, 863–872. http://dx.doi.org/10.1016/j.cej.2014.12.016

Benatti M.R., Yookongkaew N., Meetam M., Guo W.J., Punyasuk N., AbuQamar S., Goldsbrough P., 2014. Metallothionein deficiency impacts copper accumulation and redistribution in leaves and seeds of Arabidopsis. New. Phytol. 202, 940–951. https://doi.org/10.1111/nph.12718

Beni A.A., Esmaeili A., 2020. Biosorption, an efficient method for removing heavy metals from industrial effluents: A Review. Environ. Technol. Innov. 17, 100503. https://doi.org/10.1016/j.eti.2019.100503

Brady D., Duncan JR., 1994. Bioaccumulation of metal cations by *Saccharomyces cerevisiae*. Appl. Mibrobiol. Biot. 41, 149–154. https://doi.org/10.1007/BF00166098

Bulgarelli R.G., Araujo P., Tezotto T., Mazzafera P., Andrade S.A.L., 2016. Expression of metallothionein genes in coffee leaves in response to the absence or excess of Cu and Zn. Theor Exp. Plant. Phys. 28, 371–383. https://doi.org/10.1007/s40626-016-0075-5

Bulgariu D., Bulgariu L., 2016. Potential use of alkaline treated algae waste biomass as sustainable biosorbent for clean recovery of cadmium(II) from aqueous media: batch and column studies. J. Clean. Prod. 112, 4525-4533. https://doi.org/10.1016/j.jclepro.2015.05.124

Chen C., Wang J., 2008. Removal of Pb^{2+}, Ag^{+}, Cs^{+} and Sr^{2+} from aqueous solution by brewery's waste biomass. J. Hazard. Mater. 151, 65–70. https://doi.org/10.1016/j.jhazmat.2007.05.046

Cismowski M.J., Narula S.S., Armitage I.M., Chernaik M.L., Huang PC., 1991. Mutation of invariant cysteines in mammalian metallothionein alters metal binding capacity, cadmium resistance and ^{113}Cd NMR spectrum. J. Biol. Chem. 266, 24390–24397.

Cobbett C., Goldsbrough P., 2002. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Ann. R. Plant. Biol. 53, 159–182. https://doi.org/10.1146/annurev.arplant.53.100301.135154

De Oliveira V.H., Tibbett M., 2018. Tolerance, toxicity and transport of Cd and Zn in *Populus trichocarpa*. Environ. Exp. Bot. 155, 281–292. https://doi.org/10.1016/j.envexpbot.2018.07.011

De Oliveira V.H., Ullah I., Dunwell J.M., Tibbett M., 2020. Mycorrhizal symbiosis induces divergent patterns of transport and partitioning of Cd and Zn in *Populus trichocarpa*. Environ. Exp. Bot. 171, 103925. https://doi.org/10.1016/j.envexpbot.2019.103925

Domènech J., Mir G., Huguet G., Capdevila M., Molinas M., Atrian S., 2006. Plant metallothionein domains: functional insight into physiological metal binding and protein folding. Biochimie 88, 583–593. https://doi.org/10.1016/j.biochi.2005.11.002

Farcasanu I.C., Ruta L.L., 2017. Metallothioneins, *Saccharomyces cerevisiae*, and heavy

metals: a biotechnology triad? In: Lucas C., Pais C. (Eds) *Old Yeasts - New Questions*. InTech Open, pp 21–39. http://dx.doi.org/10.5772/intechopen.70340

Goksungur Y., Uren S., Guvenc U., 2005. Biosorption of cadmium and lead ions by ethanol treated waste baker's yeast biomass. Bioresource Technol. 96, 103–109. https://doi.org/10.1016/j.biortech.2003.04.002

Gu C.S., Liu L.Q., Zhao Y.H., Deng Y.M., Zhu X.D., Huang S.Z., 2014. Overexpression of *Iris lactea* var. *chinensis* metallothionein llMT2a enhances cadmium tolerance in *Arabidopsis thaliana*. Ecotox. Environ. Safe. 105, 22-28. http://dx.doi.org/10.1016/j.ecoenv.2014.04.002

Guo W.J., Meetam M., Goldsbrough P.B., 2008. Examining the specific contributions of individual *Arabidopsis* metallothioneins to copper distribution and metal tolerance. Plant Physiol. 146, 1697–1706. https://doi.org/10.1104/pp.108.115782

Hassinen V., Vallinkoski V.M., Issakainen S., Tervahauta A., Karenlampi S., Servomaa K., 2009. Correlation of foliar MT2b expression with Cd and Zn concentrations in hybrid aspen (*Populus tremula* x *tremuloides*) grown in contaminated soil. Environ. Pollut. 157, 922–930. https://doi.org/10.1016/j.envpol.2008.10.023

Hassinen V.H., Tervahauta A.I., Schat H., Karenlampi S.O., 2011. Plant metallothioneins – metal chelators with ROS scavenging activity? Plant Biol. 13, 225–232. https://doi.org/10.1111/j.1438-8677.2010.00398.x

He S., He Z., Yang X., Stoffella P.J., Baligar V.C., 2015. Soil biogeochemistry, plant physiology, and phytoremediation of cadmium-contaminated soils. In: Sparks D.L. (Ed.) Advances in Agronomy. Elsevier Inc, Oxford, pp 135–225. https://doi.org/10.1016/bs.agron.2015.06.005

Hosiner D., Gerber S., Lichtenberg-Frate H., Glaser W., Schuller C., Klipp E., 2014. Impact of acute metal stress in *Saccharomyces cerevisiae*. Plos One. 9:e83330. https://doi.org/10.1371/journal.pone.0083330

Hu P., Sorensen C., Gross M.L., 1995. Influences of peptide side chains on the metal ion binding site in metal ion-cationized peptides: participation of aromatic rings in metal chelation. J. Am. Soc. Mass. Spectr. 6, 1079–1085. https://doi.org/10.1016/1044-0305(95)00549-8

Jaakola L., Pirtilla M., Halonen M., Hohtola A., 2001. Isolation of high quality RNA from Bilberry (*Vaccinium myrtillus* L.) fruit. Mol. Biot. 19, 201–203. https://doi.org/10.1385/MB:19:2:201

Jamali M., Kazi T.G., Arain M.B., Afridi H.I., Jalbani N., Kandhro G.A., Shah A.Q., Baig J.A., 2009. Heavy metal accumulation in different varieties of wheat (*Triticum aestivum* L.) grown in soil amended with domestic sewage sludge. J. Hazard. Mater. 164, 1386–1391. https://doi.org/10.1016/j.jhazmat.2008.09.056

Janssen M.P.M., Ma W.C., Van Straalen N.M., 1993. Biomagnification of metals in terrestrial ecosystems, Sci. Total. Environ. 134, 511–524. https://doi.org/10.1016/S0048-9697(05)80053-1

Jin S., Sun D., Wang J., Li Y., Wang X., Liu S., 2014. Expression of *rgMT* gene, enconding for a rice metallothionein-like protein in *Saccharomyces cerevisiae* and *Arabidopsis thaliana*. J. Genet. 93, 709–718.

Joutey N.T., Bahafid W., Sayel H., Ghachtouli N.E., 2013. Biodegradation: involved microorganisms and genetically engineered microorganisms. In: Chamy R. (Ed.) Biodegradation - Life of Science. InTech, Rijeka, pp 289–320. http://dx.doi.org/10.5772/56194

Karathia H., Vilaprinyo E., Sorribas A., Alves R., 2011. *Saccharomyces cerevisiae* as a model organism: a comparative study. *Plos One* 6:e16015. https://doi.org/10.1371/journal.pone.0016015

Khan M.A., Khan S., Khan A., Alam M., 2017. Soil contamination with cadmium, consequences and remediation using organic amendments. Sci. Total Environ. 601–602, 1591–1605. http://dx.doi.org/10.1016/j.scitotenv.2017.06.030

Kim N., Park M., Park D., 2015. A new efficient forest biowaste as biosorbent for removal of cationic heavy metals. Bioresource Technol. 175, 629–632. http://dx.doi.org/10.1016/j.biortech.2014.10.092

Kim Y.O., Patel D.H., Lee D.S., Song Y., Bae H.J., 2011. High cadmium-binding ability of a novel *Colocasia esculenta* metallothionein increases cadmium tolerance in *Escherichia coli* and tobacco. Biosci. Biotech. Bioch. 75, 1912–1920. https://doi.org/10.1271/bbb.110289

600 Kobayashi K., Kuroda J., Shibata N., Hasegawa T., Seko Y., Satoh M., Tohyama C., Takano
601 H., Imura N., Sakabe K., Fujishiro H., Himeno S., 2007. Induction of metallothionein by
602 manganese is completely dependent on interleukin-6 production. J. Pharmacol. Exp. Ther. 320,
603 721–727. https://doi.org/10.1124/jpet.106.112912
604
605 Kohler A., Blaudez D., Chalot M., Martin F., 2004. Cloning and expression of multiple
606 metallothioneins from hybrid poplar. New Phytol. 164**,** 83–93. https://doi.org/10.1111/j.1469-
607 8137.2004.01168.x
608
609 Kulshreshtha S., 2013. Genetically engineered microorganisms: a problem solving approach
610 for bioremediation. J. Bioremediat. Biodegrad. 4:e133.7. doi:10.4172/2155-6199.1000e133
611
612 Lei M., Zhang Y., Khan S., Qin P., Liao B., 2010. Pollution, fractionation, and mobility of Pb,
613 Cd, Cu, and Zn in garden and paddy soils from a Pb/Zn mining area. Environ. Monit. Assess.
614 168, 215–222. https://doi.org/10.1007/s10661-009-1105-4
615
616 Ma J.C., Dougherty D., 1997. The Cation−π Interaction. Chem. Rev. 97, 1303–1324.
617 https://doi.org/10.1021/cr9603744
618
619 Machado M.D., Santos M.S.F., Gouveia C., Soares H.M.V.M., Soares E.V., 2008. Removal of
620 heavy metals using a brewer's yeast strain of *Saccharomyces cerevisiae*: the flocculation as a
621 separation process. Bioresource Technol. 99, 2107–2115.
622 https://doi.org/10.1016/j.biortech.2007.05.047
623
624 Mahadevi A.S., Sastry G.N., 2013. Cation−π interaction: its role and relevance in chemistry,

biology, and material science. Chem. Rev. 113, 2100–2138. https://doi.org/10.1021/cr300222d

Marques P.A.S.S., Rosa M.F., Pinheiro H.M., 2000. pH effects on the removal of Cu^{2+}, Cd^{2+} and Pb^{2+} from aqueous solution by waste brewery biomass. Bioprocess. Eng. 23, 135–141. https://doi.org/10.1007/PL00009118

Mirlean N., Roisenberg A., 2006. The effect of emissions of fertilizer production on the environment contamination by cadmium and arsenic in southern Brazil. Environ. Pollut. 143, 335–340. https://doi.org/10.1016/j.envpol.2005.11.022

Nguyen H., Rineau F., Vangronsveld J., Cuypers A., Colpaert J.V., Ruytinx J., 2017. A novel, highly conserved metallothionein family in basidiomycete fungi and characterization of two representative *SlMTa* and *SlMTb* genes in the ectomycorrhizal fungus *Suillus luteus*. Environ. Microbiol. 19, 2577–2587. https://doi.org/10.1111/1462-2920.13729

Oliveira R.P.S., Basso L.C., Junior A.P., Penna T.C.V., Borghi M.D., Converti A., 2012. Response of *Saccharomyces cerevisiae* to cadmium and nickel stress: the use of the sugar cane vinasse as a potential mitigator. Biol. Trace Elem. Res. 145, 71–80. https://doi.org/10.1007/s12011-011-9156-0

Pankiewicz U., Sujka M., Jamroz J., 2015. Bioaccumulation of the selected metal ions in *Saccharomyces cerevisiae* cells under treatment of the culture with Pulsed Electric Field (PEF). J. Membrane Biol. 248, 943–949. https://doi.org/10.1007/s00232-015-9844-3

Ruiz O.N., Alvarez D., Gonzalez-Ruiz G., Torres C., 2011. Characterization of mercury

650 bioremediation by transgenic bacteria expressing metallothionein and polyphosphate kinase.
651 BMC Biotechnol. 11, 1–8. https://doi.org/10.1186/1472-6750-11-82
652
653 Ruta .LL., Lin Y.F., Kissen R., Nicolau I., Neagoe A.D., Ghenea S., Bones A.M., Farcasanu
654 I.C., 2017. Anchoring plant metallothioneins to the inner face of the plasma membrane of
655 *Saccharomyces cerevisiae* cells leads to heavy metal accumulation. *Plos One* 12:e0178393.
656 https://doi.org/10.1371/journal.pone.0178393
657
658 Ruttkay-Nedecky B., Nejdl L., Gumulec J., Zitka O., Masarik M., Eckschlager T., Stiborova
659 M., Adam V., Kizek R., 2013. The role of metallothionein in oxidative stress. Int. J. Mol. Sci.
660 14, 6044–6066. https://doi.org/10.3390/ijms14036044
661
662 Schothorst J., Van Zeebroeck G., Thevelein J.M., 2017. Identification of Ftr1 and Zrt1 as iron
663 and zinc micronutrient transceptors for activation of the PKA pathway in *Saccharomyces*
664 *cerevisiae*. Microb. Cell. 4, 74–89. 10.15698/mic2017.03.561
665
666 Sheoran V., Sheoran A.S., Poonia P., 2011. Role of hyperaccumulators in phytoextraction of
667 metals from contaminated mining sites: a review. Environ. Sci. Technol. 41, 168–214.
668 https://doi.org/10.1080/10643380902718418
669
670 Singh S., Kang S.H., Mulchandani A., Chen W., 2008. Bioremediation: environmental clean-
671 up through pathway engineering. Curr. Opin. Biotech. 19, 437–444.
672 https://doi.org/10.1016/j.copbio.2008.07.012
673
674 Smolders E., Mertens J., 2013. Cadmium. In: Alloway B.J. (Ed.) Heavy metals in soils, 3rd

edn. Springer, Nehterlands, pp 283–311. 10.1007/978-94-007-4470-7

Soares E.V., 2011. Flocculation in *Saccharomyces cerevisiae*: a review. J Appl Microbiol 110, 1–18. https://doi.org/10.1111/j.1365-2672.2010.04897.x

Sriprang R., Hayashi M., Ono H., Takagi M., Hirata K., Murooka Y., 2003. Enhanced accumulation of Cd^{2+} by a *Mesorhizobium* sp. transformed with a gene from *Arabidopsis thaliana* coding for phytochelatin synthase. Appl. Environ. Microbiol. 69, 1791–1796. https://dx.doi.org/10.1128%2FAEM.69.3.1791-1796.2003

Stewart M., Dunlap T., Dourlain E., Grant B., McFail-Isom L., 2013. Cations form sequence selective motifs within DNA grooves via a combination of cation-pi and ion-dipole/hydrogen bond interactions. Plos One 8:e71420. https://doi.org/10.1371/journal.pone.0071420

Thorsen M., Perrone G.G., Kristiansson E., Traini M., Ye T., Dawes I.W., Nerman O., Tamas M.H., 2009. Genetic basis of arsenite and cadmium tolerance in *Saccharomyces cerevisiae*. BMC Genomics 10, 105. https://doi.org/10.1186/1471-2164-10-105

Ude C., Schmidt-Hager J., Findeis M., John GT., Scheper T., Beutel S., 2014. Application of an online-biomass sensor in an optical multisensory platform prototype for growth monitoring of biotechnical relevant microorganism and cell lines in single-use shake flasks. Sensors 14, 17390–17405. https://doi.org/10.3390/s140917390

Ullah I., Wang Y., Eide D.J., Dunwell J.M., 2018. Evolution, and functional analysis of Natural Resistance-Associated Macrophage Proteins (NRAMPs) from *Theobroma cacao* and their role

700 in cadmium accumulation. Sci. Rep. 8:14412. https://doi.org/10.1038/s41598-018-32819-y

701

702 Vandenbossche M., Dehaese A., Casetta M., Jimenez M., Traisnel M., 2015. Tyrosine: an
703 efficient natural molecule for copper remediation. Green Mater. 3, 1–9.
704 https://doi.org/10.1680/gmat.14.00006

705

706 Vijayaraghavan K., Balasubramanian R., 2015. Is biosorption suitable for decontamination of
707 metal-bearing wastewaters? A critical review on the state-of-the-art of biosorption processes
708 and future directions. J. Environ. Manage. 160, 283–296.
709 https://doi.org/10.1016/j.jenvman.2015.06.030

710

711 Vijver M.G., Gestel C.A.M., Lanno R.P., Van Straalen N.M., Peijnenburg W.J.G.M., 2004.
712 Internal metal sequestration and its ecotoxicological relevance: a review, Environ. Sci.
713 Technol. 38, 4705–4712. https://doi.org/10.1021/es040354g

714

715 Wang J., Chen C., 2006. Biosorption of heavy metals by *Saccharomyces cerevisiae*: a review.
716 Biotechnol. Adv. 24, 427–451. https://doi.org/10.1016/j.biotechadv.2006.03.001

717

718 Wang J., Chen C., 2009. Biosorbents for heavy metals removal and their future. Biotechnol.
719 Adv. 27, 195–226. https://doi.org/10.1016/j.biotechadv.2008.11.002

720

721 Wong H.L., Sakamoto T., Kawasaki T., Umemura K., Shimamoto K., 2004. Down-regulation
722 of metallothionein, a reactive oxygen scavenger, by the small GTPase *OsRac1* in rice. Plant
723 Physiol. 135, 1447–1456. https://doi.org/10.1104/pp.103.036384

724

725 Zhang M., Takano T., Liu S., Zhang X., 2014a. Abiotic stress response in yeast and metal-
726 binding ability of a type 2 metallothionein-like protein (*PutMT2*) from *Puccinellia tenuiflora*.
727 Mol. Biol. Rep. 41, 5839–5849. https://doi.org/10.1007/s11033-014-3458-1
728
729 Zhang J., Zhang M., Tian S., Lu L., Shohag M.J.I., Yang X., 2014b. Metallothionein 2 (*SaMT2*)
730 from *Sedum alfredii* Hance confers increased Cd tolerance and accumulation in yeast and
731 tobacco, Plos One 9:e102750. https://doi.org/10.1371/journal.pone.0102750
732
733 Zhao H., Eide D., 1996. The *ZRT2* gene encodes the low affinity zinc transporter in
734 *Saccharomyces cerevisiae*. J. Biol. Chem. 271, 23203–23210. 10.1074/jbc.271.38.23203
735
736 Zhou J., Goldsbrough P.B., 1994. Functional homologs of fungal metallothionein genes from
737 *Arabidopsis*. Plant Cell 6, 875–884. https://doi.org/10.1105/tpc.6.6.875