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New biosensor for detection of copper ions in water based on immobilized genetically modified yeast cells

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ABSTRACT

Contamination of water by heavy metals represents a potential risk for both aquatic and terrestrial organisms, including humans. Heavy metals in water resources can come from various industrial activities, and drinking water can be ex-post contaminated by heavy metals such as Cu^{2+} from house fittings (e.g., water reservoirs) and pipes. Here, we present a new copper biosensor capable of detecting copper ions at concentrations of 1–100 μM. This biosensor is based on cells of a specifically modified *Saccharomyces cerevisiae* strain immobilized in alginate beads. Depending on the concentration of copper, the biosensor beads change color from white, when copper is present in concentrations below the detection limit, to pink or red based on the increase in copper concentration. The biosensor was successfully tested in the determination of copper concentrations in real samples of water contaminated with copper ions. In contrast to analytical methods or other biosensors based on fluorescent proteins, the newly designed biosensor does not require specific equipment and allows the quick detection of copper in many parallel samples.

1. Introduction

Due to both natural sources and certain industrial enterprises, the environment, including water resources, becomes contaminated by various pollutants including heavy metals. Such contamination poses serious problems for terrestrial and aquatic organisms and to human health. The heavy metals accumulated in the environment usually cannot be degraded naturally. Considerable amounts of heavy metals also penetrate the surface water system and accumulate in sediments. Among the heavy metals, copper is both an essential nutrient, as a constituent of some enzymes, and a drinking-water contaminant. The value recommended by the World Health Organization (WHO) guidelines is 2 mg of Cu^{2+} per liter of water. This is based on the presumption that adults consume 2 or 3 l of water per day and ingest additional copper from food. Copper consumption should not exceed an upper limit of 10 mg per day. However, copper concentrations in drinking water can range from ≤ 0.005 to > 30 mg/L, primarily as a result of the interior corrosion of copper plumbing (WHO, 2008).

Heavy metals can be detected using many analytical methods such as atomic absorption spectrometry (AAS), inductively coupled plasma optical emission spectrometry (ICP/OES) and inductively coupled plasma mass spectrometry (ICP/MS) (WHO, 2008), which are costly and time-consuming and require expensive specialized equipment and highly qualified staff. The limits of copper detection are 0.02 μg/L by ICP/MS (Zhu et al., 2009), 0.3 μg/L by ICP/OES (WHO, 2008) and 0.5 μg/L by flame atomic absorption spectrometry (FAAS) (Pourreza and Hoveizavi, 2005). These methods can reliably determine the total concentration of ions of heavy metals, including their insoluble forms, but they do not provide any information about the bioavailable concentrations of these metals, which is the amount that could be dangerous for living organisms. One way to address this problem is to use biosensors.

A biosensor is an analytical device that combines a biological component with a physicochemical detector, and it can be used for a detection of a specific analyte (Su et al., 2011). For example, one biosensor group is based on the ability of protein or DNA to bind heavy metals. In proteins, the binding of heavy metals predominantly to Cys residues can cause inhibition or activation of protein/enzyme activity.

Several important biosensors are based on intact cells (e.g., Belkin, 2003). Some of these biosensors can use the same enzymatic reactions as those based on isolated enzymes, but they are cheaper and can provide data on the bioavailability of pollutants and/or their effects on living systems. Intact cells provide ideal conditions for enzyme function. Cell-based biosensors include

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either unmodified or genetically modified microorganisms, along with detection based on either the measurement of changes in the intensity of bioluminescence or fluorescence or the measurement of electrical parameters.

Biosensors using unmodified microorganisms are often based on the inhibitory effect of heavy metals on microbial cells. Thus, the decrease in luminescence intensity of *Photobacterium phosphoreum* was used to detect chromium reaching I_{50} at 0.85 nM Cr^{6+} (Lee et al., 1992). A conductometric biosensor detects the decrease in alkaline phosphatase activity of immobilized blue algae *Arthrospira platensis* caused by the presence of heavy metals (Tekaya et al., 2013). The I_{50} values were 10^{-19} M for cadmium and 10^{-17} M for mercury. In addition, lyophilized biomass of yeast *Rhodotorula mucilaginosa* (Yuce et al., 2010) or dead biomass of algae *Tetraselmis chuii* (Alpat et al., 2007) have been used to construct a voltammetric biosensor used to detect Cu^{2+}. These biosensors were based on the ability of the biomass to adsorb heavy metals from aqueous solutions, and it reached detection limits of 10^{-7}–10^{-5} M and 4.6×10^{-10} M, respectively.

Biosensors that use genetically modified microorganisms are usually based on the production of specific reporter protein (s) controlled by a promoter induced by heavy metal(s). The genetically modified bacillus *Alcaligenes eutrophus* increases its bioluminescence based on the heavy metal concentration. That detection is based on the reporter operon *luxCDABE* from *Vibrio fischeri* in combination with bacterial σ-54 promoter regions that are inducible by heavy metals. Thus, in combination with the *chrA* promoter, *A. eutrophus* predominantly detects chromium Cr^{6+} (K_2CrO_4); the presence of the *copSRA* promoter region, the mer regulatory region (*merR* and *mer* promoter) or the *pbrR* promoter enable the detection of copper, mercury or lead ions, respectively, with detection limits of 1.0 μM for Cu^{2+} and Cr^{6+} and 0.5 μM for Pb^{2+}, respectively (Corbisier et al., 1999). A similar biosensor but combined with optical fibers was used later to detect copper ions (Leth et al., 2002). Copper ions can also be detected by biosensors using recombinant yeast *Saccharomyces cerevisiae*. This copper sensing is based on the presence of a plasmid containing the lacZ gene (from *Escherichia coli*) or a GFP gene controlled by the *CUP1* promoter, which is inducible by copper ions. The *CUP1* promoter is inducible specifically by Cu^{2+} or by silver ions (Dameron et al., 1991; Shetty et al., 2004). In the lacZ-based sensor, the enzyme β-galactosidase (encoded by the lacZ gene) was produced only in the presence of Cu^{2+} and enabled the yeast to utilize lactose. This sensor detected Cu^{2+} concentrations of 0.5–2 mM $CuSO_4$ (Lehmann et al., 2000). In the GFP-based biosensor, the production of GFP protein in a Cu^{2+}-dependent manner can be detected by monitoring the GFP fluorescence (Shetty et al., 2004). This system can detect Cu^{2+} with a lower limit of 0.5 μM.

Most of the biosensors described above require specific devices for the detection of a pollutant. Here, we present a newly designed biosensor for detecting Cu^{2+} concentration visually by the evaluation of the red coloring of immobilized yeast *S. cerevisiae*; the evaluation of this sensor requires a comparison of the unknown sample (liquid or solid) with a parallel sample containing a known Cu^{2+} concentration. The biosensor uses a *S. cerevisiae* strain with the *ADE2* gene deleted from the genome and with the natural promoter regulating the expression of the *ADE5,7* gene replaced with the *CUP1* promoter (submitted Czech patent, Vopalenska et al., 2014). The resulting strain produces red pigment only in the presence of Cu^{2+} and in a quantity proportional to the copper concentration when in the range of 1–100 μM. The intensity of the red coloring and therefore of the Cu^{2+} detection are optimal when the strain is immobilized.

2. Materials and methods

2.1. Strains and media

Strain BY4742 (*MATα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*) was obtained from the EUROSCARF collection. Strains BY-*ade2* (*MATα; ade2Δ*) and BY-*ade2*-P_{CUP}-*ADE5,7* (*MATα; ade2Δ; Pcup1-ADE5,7*) were prepared in this study. The BY-*ade2* knockout strain was constructed using the kanMX replacement cassette amplified from plasmid pUG6 (obtained from the EUROSCARF collection) by PCR (see Table S1 for the primers). The BY-*ade2*-P_{CUP}-*ADE5,7* strain was derived from BY-*ade2* using the *natNT2*-P_{CUP1} cassette amplified from the plasmid pYM-N2 (EUROSCARF) (Table S1). The transformation was performed as described previously (Gietz and Woods, 2002).

Yeast cells were cultivated in liquid complete YPD (0.5% yeast extract, 1% peptone, 2% glucose), liquid YPAD (YPD, 40 mg/L adenine sulfate) or using agar media YPDA (YPD, 2% agar) and YPADA (YPAD, 2% agar). For selection of transformants, the media were supplemented with geneticin (400 mg/L) or nourseothricin (100 mg/L).

Red coloring of the BY-*ade2* and BY-*ade2*-P_{CUP}-*ADE5,7* strains was monitored in minimal SD medium (0.19% yeast nitrogen base without amino acids and without copper, 0.5% $(NH_4)_2SO_4$, 2% glucose) with concentrations of Cu^{2+} (ranging from 0 to 100 μM) with shaking. Amino acids supplementing strain auxotrophies were added at concentrations of 50 mg/L (L-histidine, L-leucine, L-lysine), 30 mg/L (uracil) and 4 mg/L (adenine sulfate).

Red coloring of immobilized BY-*ade2*-P_{CUP}-*ADE5,7* cells was monitored in SD medium with amino acid and uracil supplements and without adenine sulfate. Cu^{2+} was added in various concentrations (0–100 μM).

2.2. Preparation of alginate beads with immobilized yeast cells

Yeast cells were cultivated for 6 days in YPAD with shaking at 28 °C to reach the stationary phase. Then, 0.2 mL of the culture was mixed with 5 mL of 3% sodium alginate in distilled water (final cell concentration from 4 to 8×10^6/ml). Alginate beads with yeast cells were prepared by dropping the yeast cell-sodium alginate suspension into 5% $CaCl_2$ and incubating for 30 min at 4 °C. Yeast cells within the beads were propagated by cultivation in YPAD at 28 °C with shaking for 36 h and then stored in distilled water at 4 °C.

2.3. Visual documentation of the red coloring

Red coloring of the cell suspensions in liquid media was documented by photographing 10 mL cultures in Petri dishes or beakers in transmitted light. Alginate beads with immobilized yeast cells were placed on microscope slides, and images were captured in transmitted light. Image capture used a ProgRes® CT3 CMOS camera with a Cosmicar TV zoom objective, the Kaiser illumination and NIS Elements software (Laboratory Imaging).

2.4. Incubation of copper pipes

Individual pieces of new plumbing copper pipe fittings (T Cu 5130, 22 × 22 × 22 mm). were immersed into 90 ml of different buffers or to distilled water. After 13 h of the incubation, the buffers/water were used for detection of Cu^{2+} concentration.

2.5. Quantification of the data

For quantification of copper concentrations, the pictures of beads incubated in different Cu^{2+} concentrations were evaluated by image analysis using UltraQuant 6.0 software. The maximum OD of particular evaluated area was used; at least three parallel series and 2–3 areas of each sample were evaluated. To estimate calibration curve parameters, a computer-assisted non-linear

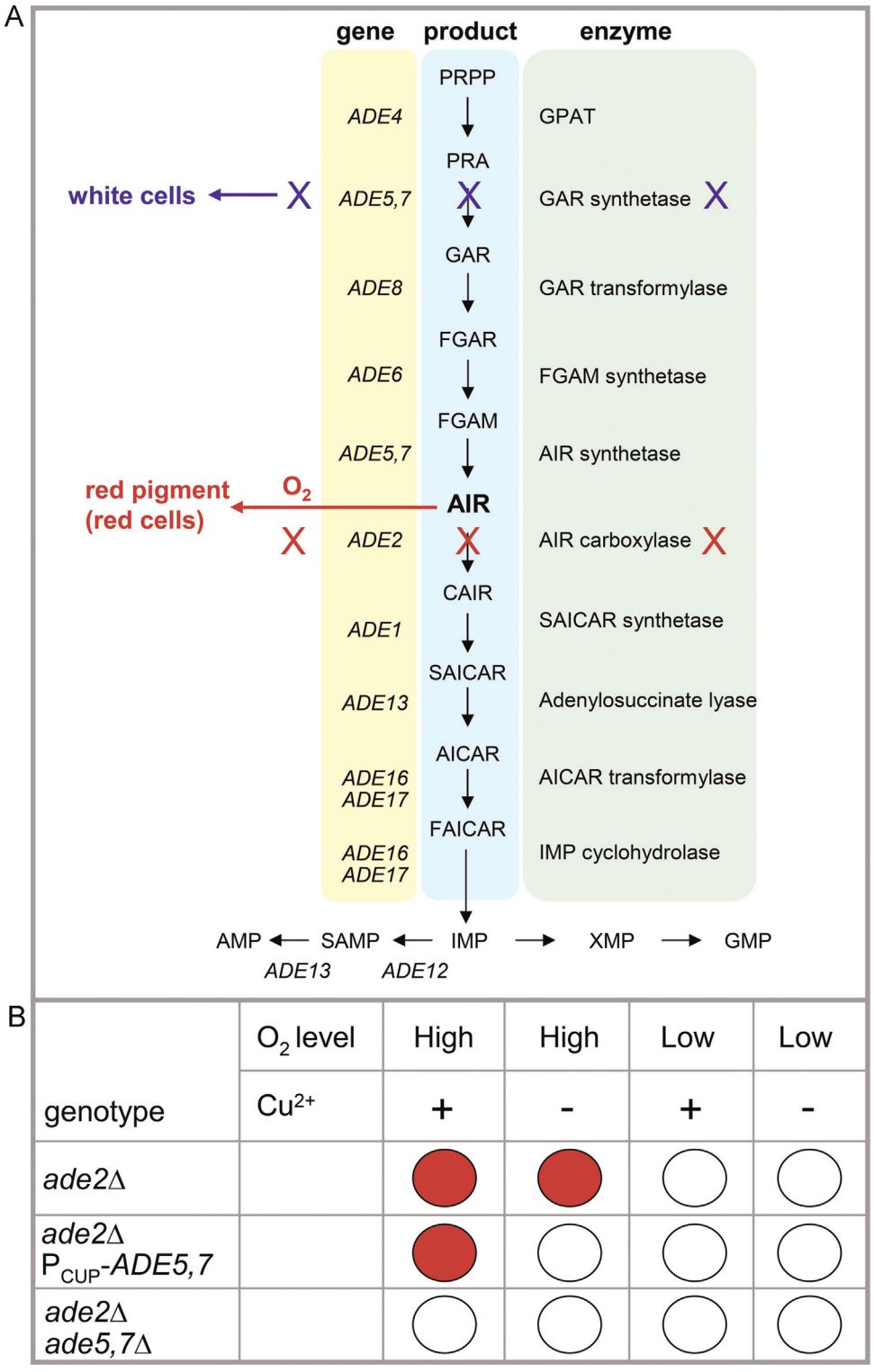

Fig. 1. Principle of the new Cu^{2+} detection system using the AMP pathway. (A) Scheme of the AMP pathway, depicting synthesis intermediates, genes and their products. The enzymatic activities whose disruption leads to changes in cell coloring are marked by blue ("white" phenotype) and red ("red" phenotype). For a detailed description, see the results section. AIR, 5′-phosphoribosylaminoimidazole; CAIR, 5′-phosphoribosylaminoimidazole carboxylate; FAICAR, 5′-phosphoribosyl 4-carboxamide-5-formaminoimidazole; FGAM, 5′-phosphoribosyl-N-formylglycinamidine; FGAR, 5′-phosphoribosyl-N-formylglycinamide; GAR, 5′-phosphoribosylglycinamide; IMP, inosine 5′-monophosphate; PRA, 5-phosphoribosylamine; PRPP, 5-phosphoribosyl-1-pyrophosphate; SAMP, adenylosuccinate; XMP, xanthosine 5′-monophosphate. (B) Predicted phenotypes of various yeast knockout strains under various growth conditions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

regression analysis was used (using the GraphPAD software). The parameters were used to calculate Cu^{2+} concentrations in unknown samples.

3. Results

3.1. Construction of yeast strains able to change color depending on the level of extracellular copper

The AMP pathway of purine synthesis (Rebora et al., 2001) (Fig. 1A) was modified in *S. cerevisiae* BY4742 in two steps. In the first step, the gene *ADE2* (coding for AIR carboxylase) was deleted in the genome by the insertion of the kanMX cassette. In the resulting strain, BY-*ade2*, the AMP pathway is interrupted, leading to the accumulation of the Ade2p substrate AIR (P-ribosylaminoimidazole) (Fig. 1). Under conditions of oxidative metabolism, AIR is oxidized to a red pigment (Smirnov et al., 1967) that accumulates in cellular vacuoles (Weisman et al., 1987). Cells of the strain BY-*ade2* thus become red when growing under respiratory conditions in medium with limited adenine. With excess adenine, sufficient quantities of ADP and ATP are formed to inhibit Ade4p (first enzyme of AMP pathway) and thus also the rest of the AMP pathway. Consequently, AIR is not accumulated, and the cells remain white. In the second step, the BY-*ade2* strain was modified by introducing the promoter P_{CUP1} (*CUP1* promoter, inducible by Cu^{2+}) in front of the gene *ADE5,7*, which encodes an enzyme that catalyzes the initial step(s) of AMP pathway (synthesis of GAR and AIR intermediates, Fig. 1A). In the absence of copper ions, *ADE5,7* is not expressed in BY-*ade2*-P_{CUP}-*ADE5,7*, and the cells behave like any *ade2*Δ *ade5,7*Δ strain, without forming the red pigment and remaining white (Fig. 1B). However the addition of Cu^{2+} to the medium should induce *ADE5,7* gene expression, Ade5,7p production and the formation of AIR, forming the red pigment (red cells under respiratory conditions). As shown in Fig. 2, according to this prediction, the strain BY-*ade2*-P_{CUP}-*ADE5,7* changes color from white to red upon the addition of Cu^{2+} and depending on the level of oxygen. This strain was therefore used as a "tester" strain in subsequent experiments.

3.2. Detection of copper ions using the strain BY-ade2-P_{CUP}-ADE5,7

With the aim of optimizing the detection of copper ions using the tester strain BY-*ade2*-P_{CUP}-*ADE5,7*, various cultivation conditions were tested, including different media (YPD and SD with different supplements) and growth conditions (solid or liquid media). Both the tester strain and the parental control strain BY-*ade2* were used. In these experiments, we monitored the following: i) efficiency of red coloring of the BY-*ade2* cells independent of the Cu^{2+} level, indicating the ability of the strain to accumulate oxidized AIR red pigment under particular conditions; ii) preservation of the white color of the tester strain when growing under conditions without Cu^{2+} (documenting that P_{CUP} is not artificially induced) and iii) colorization of the tester strain after the addition of copper ions to the medium. In addition, we monitored possible differences in properties of the strains that could be caused by either an absence or an excess of Cu^{2+} as well as the stability of the cell coloring during prolonged incubation.

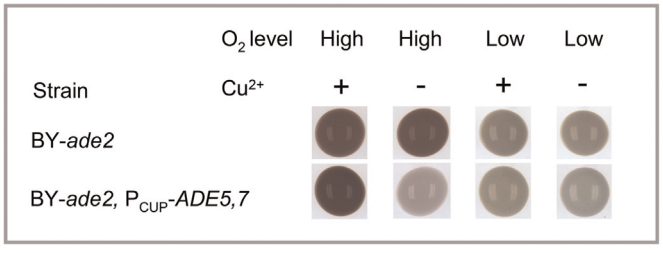

Fig. 2. Coloring of newly prepared strains under different cultivation conditions. Cells were grown for 2 days in liquid complete YPAD with shaking and then stored in distilled water at 4 °C. Then, they were cultivated 22 h in liquid minimal SD with 100 μM Cu^{2+} or without Cu^{2+} in combination with normoxic or hypoxic conditions. After cultivation, the cells were collected by centrifugation, and drops (30 μL) of the cell suspension were photographed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Comparing the YPD and SD media showed that SD medium is more suitable for Cu^{2+} detection. The complete YPD medium is less defined and usually contains traces of copper ions, which induce weak background expression from P_{CUP} and thus interfere with the Cu^{2+} to be detected. In addition, the coloring of cells by the red pigment is partially obscured by the intrinsic color of the YPD medium. Therefore, we used SD medium for testing various concentrations of nutrient and adenine sulfate additives. Although differences in glucose concentration in the range of 2–8% in SD did not affect the red coloring of cells during 3 days of cultivation (data not shown), the level of adenine sulfate was crucial because both high and very low adenine sulfate levels decreased the efficiency of red coloring (Fig. S1A). The optimal medium for the visualization of red coloring of the BY-*ade2* strain was SD medium with 4 mg/L of adenine sulfate (SD-A4). Importantly, strain BY-*ade2*-P_{CUP}-*ADE5,7* develops only a very light pinkish color in this medium during 2 days of cultivation in the absence of Cu^{2+}. SD-A4 medium was then used for monitoring the effects of different Cu^{2+} concentrations on the coloration of both strains.

Fig. S1B shows that differences in color of the BY-*ade2*-P_{CUP}-*ADE5,7* cultures after the addition of Cu^{2+} are clearly visible when the strain is cultivated with shaking in SD-A4 medium for 2 days. However, even under these optimized cultivation conditions, some inconsistencies in the detection persist. First, partial decolorization of the cell suspension of both the tester strain, BY-*ade2*-P_{CUP}-*ADE5,7*, and the control "red" strain, BY-*ade2*, occurs after prolonged cultivation times when using higher copper concentrations (50–100 μm) (Fig. S1B, blue arrows). Second, in the absence of copper ions or in the presence of very low concentrations of copper ions (0.16 μM), the speed of the red colorization is slow even in the control BY-*ade2* strain (Fig. S1B, red arrow), which decreases the range of the color difference compared with the BY-*ade2*-P_{CUP}-*ADE5,7* "tester" strain. Both of these problems were solved by the immobilization of the yeast strains in alginate beads (see below).

3.3. Detection of copper ions using immobilized yeast strain

Cells of both the tester BY-*ade2*-P_{CUP}-*ADE5,7* and control BY-*ade2* strains were immobilized in alginate beads and pre-cultivated for 36 h in liquid YPAD medium with shaking. During this cultivation, the cells grown within the beads remained white because of the high concentration of adenine sulfate in YPAD medium. Pre-cultivation for 36 h was proved as the best; pre-cultivation shorter than 30 h resulted in less intense red coloring of the beads in the presence of Cu^{2+}, pre-cultivation longer than 45 h caused slight red coloring of the beads without presence of any copper. The alginate beads filled with the grown yeast cell biomass were stored in distilled water at 4 °C and then used for copper ion detection. The beads with pregrown yeast can be stored for at least 50 days before the cells within the beads lose their ability to respond to the presence of Cu^{2+} (see below).

The ability of immobilized BY-*ade2*-P_{CUP}-*ADE5,7* to detect copper ions was analyzed as follows: alginate beads with this strain pregrown as described above were incubated with shaking in liquid SD medium without adenine but with various concentrations of Cu^{2+} (0–100 μM). Beads with the control strain BY-*ade2* were subjected

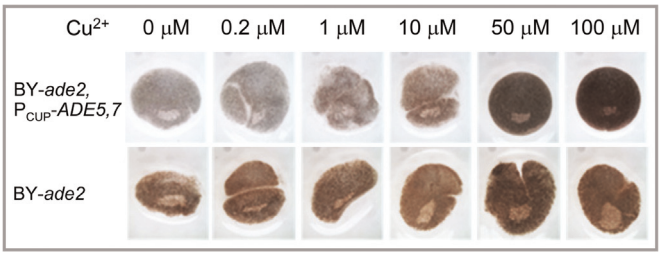

Fig. 3. Effect of Cu^{2+} concentrations on coloring of cells within alginate beads. Cells of both strains were immobilized in sodium alginate. After pre-cultivation in YPAD, alginate beads were cultivated for 18 h with shaking in liquid SD without adenine sulfate and supplemented with Cu^{2+} at various concentrations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to the same treatment in parallel. The biomass of cells of both strains in the beads remains constant because the cells cannot grow and divide when incubated in SD medium without adenine. This arrangement prevents the release of cells from the alginate beads during the incubation. The intensity of the red coloring of the beads was evaluated after 18 h of incubation (Fig. 3). Whereas the intensity of red color of the BY-*ade2*-P_{CUP}-*ADE5,7* beads corresponded to the concentration of copper in the incubation medium, the red color of beads with the BY-*ade2* strain was approximately the same in every medium tested, independent of the Cu^{2+} concentration. Thus, as shown in Fig. 3, using cells pre-grown in alginate beads with an excess of adenine followed by incubation in SD medium without adenine eliminated both the slow colorization at low concentrations and the de-colorization in higher Cu^{2+} concentrations observed in liquid cultures of non-immobilized cells. In other words, alginate beads allowed the separation of cell growth from the detection of copper.

3.4. Induction of coloring of immobilized yeast strains using increased oxygen levels

As mentioned above, the coloration of *ade2*Δ cells is caused by oxidation of the intermediate AIR to red pigment (Smirnov et al., 1967). Therefore, the alginate beads with pre-grown yeast cells were incubated in medium with or without copper ions with

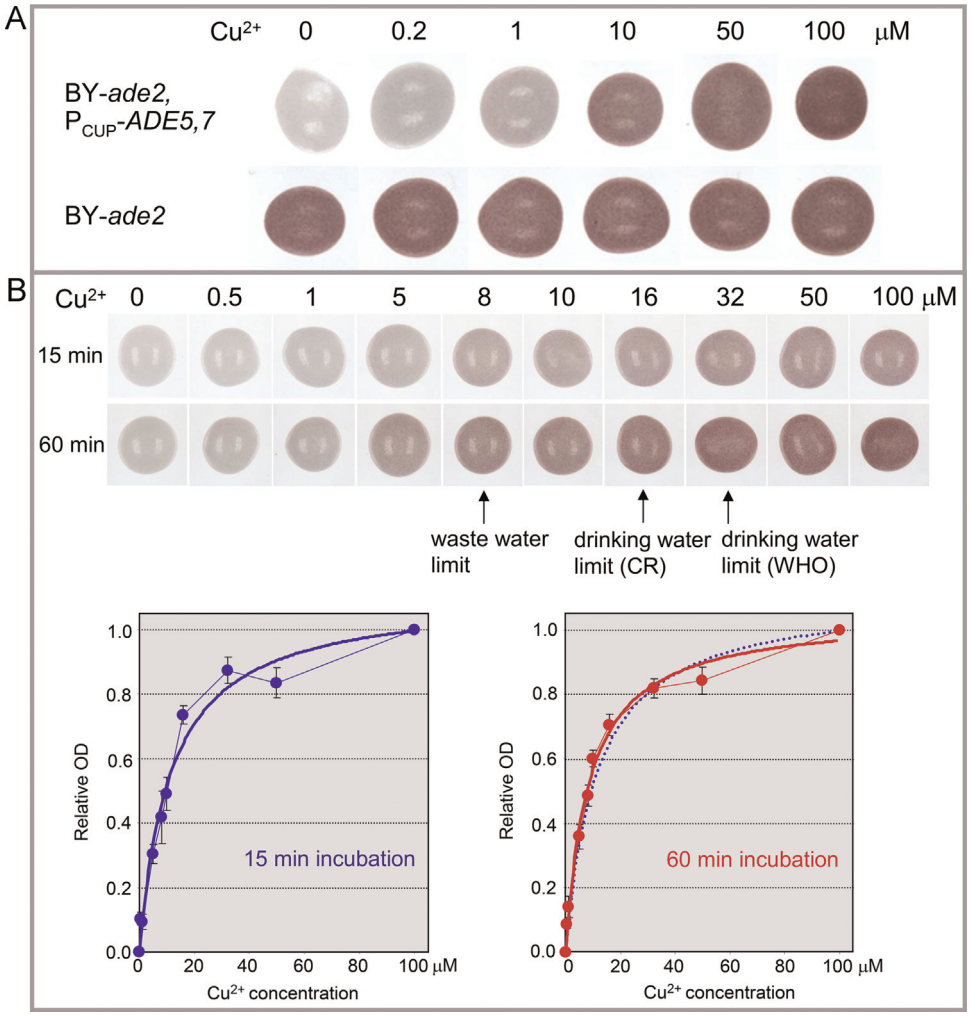

Fig. 4. Sensitivity of the new alginate-bead Cu^{2+} detection system (optimized protocol). (A) Comparison of the coloring of the control strain BY-*ade2* and the tester strain BY-*ade2*-P_{CUP}-*ADE5,7* in various Cu^{2+} concentrations (0–100 μM). After pre-cultivation in YPAD, alginate beads were cultivated for 20 h without shaking in liquid SD without adenine sulfate and then incubated for 1 h while being exposed to air. (B) Calibration curves showing sensitivity of the alginate beads with BY-*ade2*-P_{CUP}-*ADE5,7* after 15 and 60 min of incubation in air. Alginate beads were incubated for 17 h without shaking in liquid SD medium without adenine sulfate. Cu^{2+} concentration limits in wastewater (0.5 mg Cu^{2+}/L=7.87 μM Cu^{2+}) and drinking water are indicated by arrows. CR, limit in Czech Republic (1 mg Cu^{2+}/L=15.74 μM Cu^{2+}), WHO, limit recommended by the WHO (2 mg Cu^{2+}/L=31.47 μM Cu^{2+}). Bottom panels: calibration curves determined by the image analysis. Individual average values of experimental data (circles) with SEM bars and curves (thick lines) obtained by non-linear regression analysis of the experimental data are shown for both 15 min (blue) and 60 min (red) incubation of alginate beads on air. Dotted blue line in the right panel shows non-linear regression curve for 15 min incubation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

shaking. This incubation, however, sometimes resulted in the rupture of alginate beads (Fig. 3). The efficiency of coloring could also be affected by the intensity of the shaking, which can affect O_2 availability for AIR oxidation. We therefore further modified the procedure to optimize the detection of Cu^{2+}.

The beads with immobilized cells pre-grown in YPAD medium were incubated statically (in an incubator) in SD without adenine and with various copper concentrations. Because of the low oxygen accessibility, the cells within the beads of both tester and control strains remained white even after 19 h of incubation, independent of the copper concentration. However, when the beads of either BY-*ade2*-P_{CUP}-*ADE5,7* or BY-*ade2* were taken from the medium and exposed to O_2, e.g., by deposition on a glass slide (transfer to conditions with sufficient oxygen diffusion from the air), the beads with cells producing AIR began to change color within a few minutes (Fig. 4), becoming first pink and then red. After 10–20 min of incubation on the glass slide, BY-*ade2*-P_{CUP}-*ADE5,7* beads were colored according to the concentration of copper ions (1–100 μM) present in the original incubation medium. After 1 h of incubation, the differences were even more prominent (Fig. 4B). The intensity of the coloring of the beads was quantified by image analysis. To be able to compare parallel independent calibration series, the value obtained with 100 μM Cu^{2+} was set as 1. The results obtained after 1 h incubation of the beads on air provided better calibration data and Cu^{2+} determination was thus more precise as shown in Fig. 4B. The profiles of the calibration curves obtained by non-linear regression, however, did not significantly differ when comparing 15 min and 1 h incubation times. The calibration curves were similar regardless of the age of the beads, if the beads were stored from 2 to 53 days at 4 °C before they were used for the calibration test (Fig. S2). The range of the detection (calibration curves) was reproducible with beads of different batches when using standard pre-cultivation of the beads for 36 h, followed by 17–24 h of cultivation with copper and 1 h exposition on air. Concentrations higher than 100 μM Cu^{2+} caused only slight increase of the bead coloring (because the saturation of the color is almost reached), but only concentrations above 2000 μM Cu^{2+} started to inhibit the red coloring of the beads, because of copper toxicity to cells. As predicted, all beads with the BY-*ade2* strain turned red independent of the copper ion concentration. In contrast to shaken cultivation, the beads were not disrupted, and their coloring was faster and more efficient.

3.5. Detection of copper ions in samples of water from different sources

To evaluate our new Cu^{2+} detection system in practice, we analyzed well water from a village near Prague (sample WW) as well as the same water after heating in water heaters with copper coils (boiler) by two village residents (samples WW-HA and WW-HB) (Fig. 5). As shown in Fig. 5A, low concentration of copper ions (~0.2 μM) was determined in the water from the well using calibration curves (no difference between this sample and the negative control was visible by naked eye), whereas significant amounts of Cu^{2+} (7–10 μM Cu^{2+} according to the calibration scale) appeared in the same water after heating with copper coil (boiler). Analysis of the same water samples by the VIS laboratory, accredited by CAI (Water Engineering Services, Inc.), confirmed the absence of Cu^{2+} in the well water (WW < 0.079 μM Cu^{2+}) and the presence of Cu^{2+} in the heated samples (WW-HA = 15.1 μM Cu^{2+}; WW-HB = 17.31 μM Cu^{2+}). The slightly higher amount of Cu^{2+} detected by the VIS laboratory is likely caused by the fact that some of the Cu^{2+} in the water occurs as tiny insoluble particles (visible to eye).

A second test was performed with new copper pipes that we

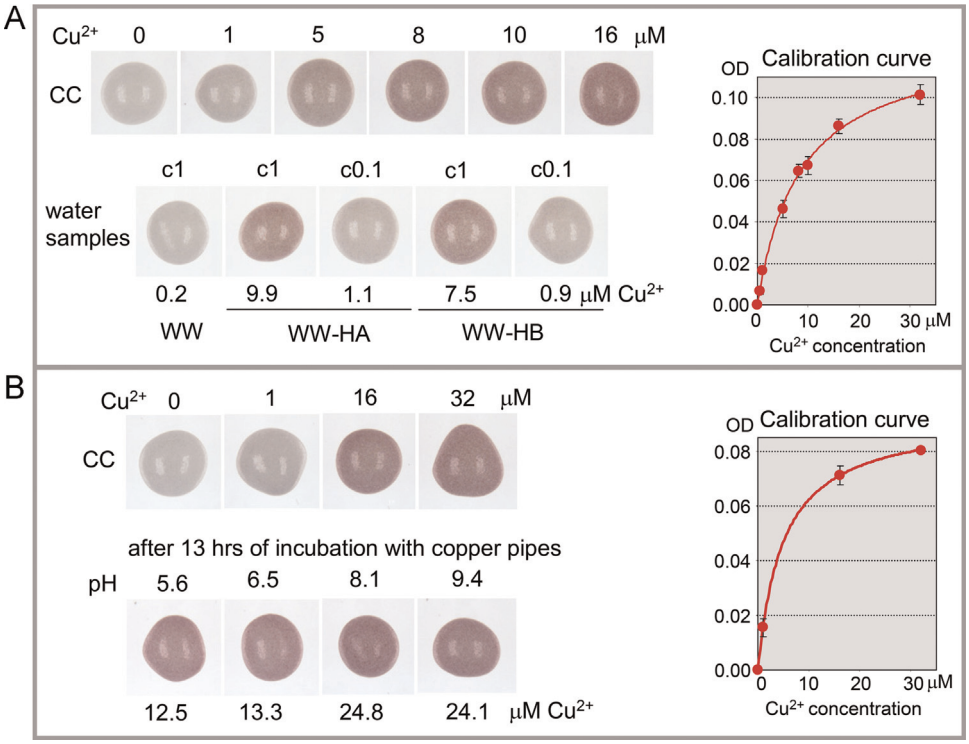

Fig. 5. Cu^{2+} detection in real water samples. (A) Cu^{2+} detection in samples of well water from a village near Prague before (WW) and after heating with a copper coil (in boilers) (WW-HA and WW-HB). A calibration curve of beads treated in 0–16 μM Cu^{2+} is shown (CC). Both sample and calibration beads were incubated for 1 h exposed to air after 17 h of static growth. Undiluted (c1) and 10x diluted (c0.1) samples were used. Right, a calibration curve determined by image analysis; bars, SEM. Parameters of the curve determined by Graph Pad software were used to estimate Cu^{2+} concentration of water samples (values specified below the particular bead). (B) Cu^{2+} detection in 10.5 mM KCl, with pH values as indicated, in which new copper pipe was incubated for 13 h. A calibration curve of beads treated in 0–32 μM Cu^{2+} is shown (CC). Both sample and calibration beads were incubated for 30 min with exposure to air after 23 h of static growth. Right, a calibration curve determined by image analysis; bars, SEM. Parameters of the curve determined by Graph Pad software were used to estimate Cu^{2+} concentration of water samples (values specified below the particular bead).

incubated in 10.5 mM KCl for 13 h. Because it has been reported (WHO, 2008) that Cu^{2+} release is more efficient at lower pH, we varied the pH of the 10.5 mM KCl in the range from 5.6 to 9.4. As shown in Fig. 5B, after 13 h, Cu^{2+} was released from the pipes at a concentration that at least in two samples exceeded the drinking water limit (16 μM Cu^{2+}), but we did not observed the reported pH effect. The efficiency of Cu^{2+} release thus could differ according to the quality of particular pipe fittings. Cu^{2+} in concentrations higher than 16 μM was also released in distilled water without KCl (not shown).

4. Discussion

Contamination of water by heavy metals coming from various industrial sources represents a serious problem for the health of both aquatic and terrestrial organisms, including human. Most of the heavy metals are toxic even in relatively low concentrations, and they cannot be naturally removed from the environment. Preventing the release of heavy metals to the environment requires the efficient detection of their presence in water produced as industrial waste.

Here, we present a new, simple and efficient system allowing the detection of copper ions in water with high sensitivity. The system is based on a strain of the yeast *S. cerevisiae* with modifications to two steps of the purine biosynthesis pathway (tester strain). Cells of this strain increase the intensity of red color produced proportionally to the concentration of copper ions in incubation medium, allowing the detection of copper ions in the range of 1–100 μM. The system was optimized for the use of tester strain cells immobilized in alginate beads and static incubation in the presence of various copper ion concentrations under conditions of low aeration. The final coloration of the beads develops quickly (in 30–60 min) after the beads are exposed to aerial oxygen. This optimized protocol has numerous advantages over both non-immobilized cells and the beads with immobilized cells incubated with shaking. First, the biomass of cells in the beads is sufficiently high before the copper detection step, which avoids the negative effects of high or low copper ion concentrations on cell growth, as observed for free cells in liquid cultures. Moreover, the high cell density within the beads results in much more intense and thus more easily detectable coloration than the coloration observed in sparse cell populations in liquid cultivation. The use of immobilized sensors thus leads to more precise readings. Second, because the cells within the beads do not need to grow but only to synthesize specific enzymes (produced through the induction of the CUP promoter) and to accumulate the intermediate AIR, the detection of copper by immobilized sensors is quicker than in growing liquid cultures. Third, because the cells within the beads do not increase their biomass, they neither damage the beads nor leak out of the beads. Finally, static incubation of the beads with copper ions under hypoxic conditions allows sufficient accumulation of the intermediate AIR, which is subsequently quickly oxidized once the beads are exposed to the air. This allows better calibration of the system and detection of various copper ion concentrations. Static cultivation also helps to maintain the structure of the beads.

The system presented here takes advantage of a very easy detection that is readable by the naked eye with no need of either specialized equipment such as in the case of detection based on changes in the fluorescence intensity of GFP or other fluorescent protein(s) (Shetty et al., 2004), or changes in the amount of β-galactosidase, which is detected by changes in O_2 consumption by cells in the biosensor (Lehmann et al., 2000), or by a relatively laborious method using biosensor cells on filters and X-gal, a substrate of β-galactosidase (Freire-Picos and Lamas-Maceiras, 2006). In addition, all three cited systems are based on a gene or genes expressed from plasmids, which risks plasmid loss or changes in plasmid copy number (both affecting the measured data) during biosensor growth and the necessity of using antibiotics for selection pressure when preparing biosensor cells. Our detection system, however, is based on a stable biosensor strain with genetic modifications in its chromosomes. Our biosensor also allows rough Cu^{2+} quantification if the detection is performed in parallel with a calibration of coloring intensity using samples of known Cu^{2+} concentration. Either direct visual reading or more precise image analysis can be used for construction of the calibration curves. Additional detection controls can help to exclude false-negative readings. For example, toxic compounds in wastewater could decrease viability of the biosensor. Addition of known Cu^{2+} concentration to wastewater samples is therefore suitable control to prove biosensor functionality. The insufficient coloring of such control sample indicates presence of toxic compound (s) and necessity of lowering of the toxicity by the sample dilution. Testing of real samples of drinking or service water proved that the new biosensor system is valuable in practical usage. It can, for example, show the release of copper ions from copper plumbing including quantification of the amount of copper ions in washing water. The reported biosensor can also be easily modified for the detection of other chemical substances by replacing the *CUP1* promoter with another promoter that is induced specifically by the particular substance.

New biosensor enables detection of copper ion concentration as low as 60 μg/L (1 μM Cu^{2+}), approximately. Such sensitivity is comparable with the best colorimetric detection systems that usually range from 0.1–1 mg of Cu^{2+}/L (Abe et al., 1989; Rizk et al., 1992), but it is, of course, lower than highly sensitive FAAS requiring special expensive instrumentation (FAAS detection limit is 0.5 μg Cu^{2+}/L; (Pourreza and Hoveizavi, 2005)). The new biosensor is based on baker yeast *S. cerevisiae* modified in their AMP metabolic pathway and the detection assay does not require any hazardous/toxic compound(s) like in some other colorimetric assays that reach similar or even slightly higher sensitivity. For example, probably the most sensitive colorimetric assay with detection limit of 20 μg of Cu^{2+}/L uses toxic resorcinol (Mir, 2011). Similarly, chelating agent diethyldithiocarbamate (DDTC) forms a complex with Cu^{2+} that is insoluble in water and has to be extracted to toxic organic solvents (such as carbon tetrachloride or chloroform) for Cu^{2+} detection using DDTC and ultraviolet-visible spectrophotometry (Uddin et al., 2013). Pre-cultivated beads of the new biosensor can be stored ready to use with reproducible results for at least 50 days and the detection requires overnight incubation of the beads in tested water samples. Commercial Copper test strips EM Quant® (www.galladechem.com) allow to detect Cu^{2+} more quickly (in 1–10 min), however, they are designed to detect only relatively high copper concentrations (10–300 mg/L). The new biosensor uses *CUP1* promoter specifically responding to Cu^{2+} without cross-reacting with other metal cations (such as Zn^{2+}, Fe^{3+}, Cd^{2+}, Co^{2+} and Ni^{2+}) with exception of silver (Dameron et al., 1991; Shetty et al., 2004; and data not shown).

5. Conclusions

This study presents new biosensor allowing the detection of copper ions in the range of 1–100 μM. The biosensor thus fully covers the copper concentrations accepted as the limit of copper ions in waste water (7.87 μM Cu^{2+}) and drinking water (15.74 μM Cu^{2+} in Czech Republic and 31.47 μM Cu^{2+} according to WHO). The biosensor is based on *S. cerevisiae* cells immobilized in alginate beads and genetically modified in their genome (i.e., with no risk of bias caused by gene copy number changes, as in biosensors

based on plasmids) in a way leading to production of red pigment upon presence of extracellular copper ions. The biosensor takes advantage of an easy detection and rough quantification of copper concentration in many parallel samples of water that is readable by the naked eye with no need of any specialized equipment. The naked eye readings are confirmed by color intensity quantification using image analysis. The biosensor beads ready for use can be stored at least 50 days before the cells lose their ability to respond to the presence of copper. The biosensor was successfully used for the determination of copper concentrations in real samples of water contaminated with copper ions.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2015.05.006.

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