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Construction of copper biosensor using *Saccharomyces cerevisiae*

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ABSTRACT

The yeast *Saccharomyces cerevisiae* is widely used in the food industry to produce bread, beer, and wine, but also represents a good model organism for scientific studies, as well for biotechnology research. In collaboration with 'Italian Biotechnology,' a company actively involved in the field of oenology, we conducted some experiments resulting in the development of new fluorescent biosensors for copper, derived from *S.cerevisiae* natural strains selected from the company's collection. Employing the CRISPR/Cas9 technique, we successfully integrated into the genomes of each yeast strain a specific DNA fragment (reporter) encoding a green fluorescent protein (GFP), whose expression is directly dependent by the presence of copper in the environment. By subjecting the newly generated biosensors to both *in vivo* (e.g., fluorescence microscopy) and *in vitro* (Western blot, fluorimetry) assays, we characterized their properties as copper biosensors regarding both specificity and selectivity, providing evidence that the yeast biosensor characterized by highest sensitivity to copper was able to detect sub-nanomolar levels of the metal, representing an increase of 1000 times compared to the yeast-based biosensors previously reported.

RIASSUNTO

Il lievito *Saccharomyces cerevisiae* è ampiamente utilizzato nell'industria alimentare per produrre pane, birra e vino, ma rappresenta anche un ottimo organismo modello per gli studi scientifici, nonché per la ricerca biotecnologica. In collaborazione con "Italiana Biotecnologie", azienda attivamente impegnata nel campo dell'enologia, abbiamo condotto alcuni esperimenti che hanno portato allo sviluppo di nuovi biosensori fluorescenti per il rame, derivati da ceppi naturali di *S.cerevisiae* selezionati dalla collezione dell'azienda. Utilizzando la tecnica CRISPR/Cas9, abbiamo integrato con successo nei genomi di ciascun ceppo di lievito uno specifico frammento di DNA (reporter) che codifica per una proteina fluorescente verde (GFP), la cui espressione è direttamente dipendente dalla presenza di rame nell'ambiente. Sottoponendo i biosensori appena generati a test sia *in vivo* (ad esempio, microscopia a fluorescenza) che *in vitro* (Western blot, fluorimetria), abbiamo caratterizzato le loro proprietà come biosensori del rame per quanto riguarda sia la specificità che la selettività, fornendo la prova che il biosensore di lievito caratterizzato dalla massima sensibilità al rame è stato in grado di rilevare livelli sub-nanomolari del metallo, che rappresentano un aumento di 1000 volte rispetto ai biosensori a base di lievito precedentemente riportati.

KEYWORDS

Saccharomyces cerevisiae

Biosensor

CRISPR/Cas9

Copper

Green Fluorescent Protein (GFP)

TABLE OF CONTENTS

1.INTRODUCTION

1.1 *Saccharomyces cerevisiae*.

Saccharomyces cerevisiae is a single-shaped facultative anaerobic eukaryotic microorganism. The size of *S.cerevisiae* may vary depending on the strain, environmental conditions, and cell growth, but morphologically the yeast cells are normally spheroidal and measure 4-5 μm in diameter. It belongs to the phylum Ascomycota within the fungi kingdom.

The most common way for *Saccharomyces cerevisiae* to reproduce is budding, which is an asexual reproduction that occurs when a smaller daughter cell forms on the surface of a larger (mother) cell. The daughter cell eventually separates from the mother and becomes an independent cell as it grows. When the condition is favorable this process is rapid (approximately 90 minutes), and the yeast population expands quickly, while under stress or starving conditions, the budding will stop (Hartwell et al., 1974).

S. cerevisiae can be found in diploid or haploid form. Haploid cells tend to be smaller and rounder than diploid cells, which are generated when two haploid cells of opposite mating type (a and α) mate. Genetically, mating types are determined by the specific configuration of the *MAT* locus. Cells of the same mating type cannot mate while cells with opposite mating types can. MATa cells produce and secrete the pheromone a-factor, while MATα cells produce and secrete the pheromone α-factor. When a cell of one mating type binds to the pheromone of the opposite mating type, it triggers a series of events that lead to mating, resulting in a diploid cell which has one chromosome form “a” parent and one chromosome from “α” parent. Under severe stress conditions (especially lack of nitrogen), yeast diploid cells undergo sporulation. During this process, the genome the diploid cell is processed with the meiotic division and the genome rearrangement, finally resulting in four haploid spores (two MATa and two MATα) with a unique combination of chromosomes (M.mackenzie et al.,2020).

As all eukaryotic cells, yeast is delimited by the plasmatic membrane, a double phospholipid membrane that is selectively permeable and controls the movement of molecules into and out of the cell. Internally, yeast contains all the cellular organelles typically present in eukaryotic cells such as a nucleus, mitochondria, ER, Golgi apparatus, and peroxisomes, but also displays specific compartments as the vacuole (defined as the yeast lysosome) (Klis et al., 2002). Moreover, yeast cells are peculiarly surrounded by a robust cell wall (100-200 nm thickness), mainly composed by chitin and glucans, providing mechanical protection from environmental stress, and promoting the stability of the cell architecture.

Saccharomyces cerevisiae is among the best-studied experimental organisms, and its DNA was the first eukaryotic genome completely sequenced (Goffeau et al.1996). It is composed of 12,068 kilobases organized into 16 linear chromosomes, containing approximately 6200 genes, of which 5885 are potential protein-encoding genes and approximately 140 genes specifying ribosomal RNA. Approximately 30% of *S.cerevsiae* genes are conserved throughout evolution and homologous genes have been identified in human genome (Botstein et al.1997). These characteristics made *S.cerevisiae* a widely used yeast in cell biology as a model organism for the studies on fundamental processes such as cell cycle, cell division, or DNA replication, that have provided information for better understanding of cellular biology in more complicated organisms such as human (Cazzanelli et al.2018, Karathia et al.,2011). Furthermore, since *Saccharomyces cerevisiae* is easy and low-cost to grow and manipulate in laboratory, it has been successfully employed in the field of biotechnology (Wang et al.2023).

Finally, *Saccharomyces cerevisiae* had been so far used by human in both bakery and production of alcoholic beverages, such as beer and wine, because of its fermentative properties. Indeed, it was one of the first microorganisms that have been classified as GRAS (Generally Recognized As Safe) in the US by the FDA in 1973, and also classified as QPS (Qualified Presumption of Safety) by EFSA in 2007.

1.2 Yeast and wine production.

S. cerevisiae is a facultative anaerobic organism, it manages to obtain energy through aerobic or anaerobic processes, depending on the growth conditions. The two main factors on which the choice of metabolic pathway depends are the presence or absence of oxygen and the concentration of glucose in the medium. In aerobic conditions, the fermentation process is inhibited in the *S. cerevisiae* cell in favor of respiration (Pasteur effect), which is energetically more effective. However, this effect only occurs in the presence of low sugar concentrations. In a substrate with a sugar concentration higher than 9 g/L*, S. cerevisiae* will use alcoholic fermentation and respiration will be inhibited (Crabtree effect). In winemaking, using grape must as a substrate (>150 g/L sugars), the metabolic pathway will always be fermentative, with the production of ethanol and carbon dioxide (Barnett J. A., 2005). However, there is a rich diversity of yeast strains with distinct roles at different stages of fermentation. In the first fermentative phase, in the must there is the prevalence of some non-*Saccharomyces* yeast species (e.g., *Kloeckera spp, Candida spp*, and *Pichia spp*.), typically found on the grape skins

and in the winery environment, which may promote fermentation start and also contribute to the wine aroma with fruity and floral flavors (G.H.Fleet, 1993, Wine microbiology and Biotechnology). Since most of these yeast species have a relatively low tolerance to high levels of alcohol compared to *Saccharomyces cerevisiae*, after a while when fermentation goes on and the alcohol content reaches 4-6 degrees, *S.cerevisiae* outcompetes the other yeasts and becomes the dominant yeast in the must which completes the fermentation process.

Compared to other yeast types, *Saccharomyces cerevisiae* is further capable of producing more alcohol and has a higher ability to adapt and develop more rapidly in acidic environments like in the must. Additionally, *S.cerevisiae* yeast cells produce a series of secondary compounds that have a significant impact on the organoleptic properties of the wine and are crucial to determine its aromatic character (Graeme M Walker et al.2016). Several factors may interfere with the fermentation process, by reducing or blocking the activity of yeast cells, of either physical (e.g., temperature), or chemical (e.g., acidity) origin, including the excessive levels of heavy metals (Bauer et al.,2000). Among these, an issue that has recently been developing in wineries more frequently is an excess of copper in the must. Indeed, copper is normally used as a fungicide, since it is effective against a variety of fungi including the ones that cause grape diseases such as powdery mildew. Being relatively inexpensive in addition to its ease of use, has made the wine makers convinced to use copper and copper-based products in wine fields. However, besides all its benefits, copper concentration above 12-15mg/L can slow down or stop wine fermentation and copper levels higher than 20mg/L can stops the growth of *S.cerevisiae*, consequently resulting in the reduction of alcohol production (Sun Y et al.2015, Sun et al.2016). In addition to its impacts on fermentation process, it can also affect the health of consumers, if wine has a high copper content, posing a health risk to the consumers. In wine, high levels of copper can also combine with other heavy metals such as iron and lead and create harmful compounds (Araya et al.2003). Moreover, copper can be harmful for both plants and animals by building up in the soil over time or by reaching the groundwater and contaminating drinking water supplies (Garcia-Esparza et al.2006).

All these drawbacks resulted in the establishment of some regulations by government agencies and agricultural authorities regarding the maximum amount of copper that can be used in vineyards. The use of copper-based products in viticulture is regulated by the European community (EC) by the Law (Reg UE 2018/848 and EU Reg. 2018/1584), limiting their annual use to 6 kg per hectare and the maximum residue limits (MRLs) in vineyard soil, grapes and

wine of 140 mg/kg, 20mg/L,1mg/L respectively (EC regulation No. 1410 of 7/8/2003), however, copper in must can reduce the speed of fermentation or completely block it when the concentration reaches 12-15mg/L. At the end of the fermentation the copper can be found either as an insoluble precipitate (as sulfide) or attached to negatively charged cell wall of yeast cells.

1.3 Yeast and copper.

Copper is an essential microelement to many biological activities, e.g., it is the catalytic cofactor for various proteins or enzymes of oxidative pathways (Linder et al.1998) since it can be present in two different redox states (Cu^{2+} and Cu^{+}). However, excessive levels can have detrimental effects on yeasts, possibly leading also to cell death. Among copper-dependent damages, oxidative stress and enzymatic inhibition have to be mentioned: the redox reaction of free copper especially with molecular oxygen results in the formation of reactive oxygen species (ROS), directly causing oxidative stress and cellular damage; the excessive copper levels may interfere with the function of metal-dependent enzymes by displacing other metals from their active sites, resulting in impaired metabolic pathways. Therefore, copper homeostasis is highly controlled process in the cell, occurring by high and low-affinity membrane transporters such as the proteins encoded by the *CTR1* and *FET4* genes, respectively, which may finely regulate the import and export fluxes. Accordingly, the expression rate of copper transporters mainly depends on the metal availability in the medium (Puig et al.2002, Dancis et al.1994).

Importantly, yeast cells also possess specific mechanisms to defend themselves when the external level of copper exceeds the safe zone and starts to become dangerous, mainly by producing protective molecules, such as the antioxidant tripeptide glutathione (GSH) (Berterame et al.2018), or expressing specific proteins called metallothioneins. Since the presence of multiple cysteine residues, carrying thiol groups (-SH) with significant affinity for metal cations, such as copper, metallothioneins are able to efficiently bind and sequester free copper ions, decreasing their excessive levels, and thus acting as primary detoxification agents for yeast cells (Kumari et al.1998, Xie et al.2015).

In *S.cerevisiae*, the metallothionein protein is encoded by the *CUP1* gene, that can also be present in multiple copies in yeast genomes. Its expression is mainly regulated at transcriptional level by the Acel activator protein (Thiele et al.1988). Notably, the Acel factor directly binds

copper ions, leading to the formation of a metal-protein complex which is transcriptionally active and induces the transcription of the *CUP1* gene (Dameron et al.1991,1993). Relevantly, observations made in different strains of *Saccharomyces cerevisiae* indicated the direct link between the high copper tolerance and the high number of copies of the *CUP1* gene present in the strain genome, supporting the importance of metallothioneins in copper resistance (Adamo et al.2014).

1.4 Yeast as a Biosensor.

Biosensors are defined as analytical devices employed to specifically detect chemical substances, that combines a biological element with a physico-chemical detector system. Two components are strictly required to proper functionality: the sensor, responsible for receiving the signal (i.e., the analyte), and the transducer (reporter), necessary for signal translation, amplification, and conversion into an observable and measurable phenomenon, which is proportional to the concentration of the analyte in the sample. Interestingly, either enzymes, antibodies, or specific proteins can be efficiently used as reporter systems. In the case of microorganisms such as yeast, the two components are further combined in the same cell, which simultaneously acts as a biological receptor, signal transducer, and reporter system, finally resulting in more advantageous device to identify specific molecules in different complex mixtures (Liang Su et al.2011). Notably, yeast-based biosensors may provide more accurate measurements regarding the bioavailable quantity of a specific analyte, often consisting of a fraction of its total amount in the solution, unlike to traditional analytical techniques that normally provide information about the total quantity of the analyte present in the sample.

Microorganism-based biosensors have been (and are) used for a wide range of purposes, such as medical diagnostics, to detect molecules associated to several diseases including cancer, for environmental purposes, to monitor the environmental pollutants such as heavy metals, for food safety, to detect foodborne pathogens as salmonella, as well for agriculture, to monitor crop help and soil quality (M. Fraga-Corral et al.2020). Since *S.cerevisiae* has high ability to adopt to wide range of environmental conditions, such as high temperature, low oxygen levels and acidic environment, and is able to perceive and respond to different types of stimuli, it can represent a suitable eukaryotic cell model to create new biosensors, in order to detect and

evaluate multiple environmental contaminants, toxins, and in general chemical or organic elements that can be potentially dangerous for human health.

To date, several copper biosensors have been generated in *S.cerevisiae* by genetic engineering, using common laboratory strains equipped with different reporter systems, expressing various types of probes, detected by either amperometry (Lehmann et al.2000, Tag et al.2006), luminometry (Roda et al., 2011), colorimetry (Vopalenska, 2015, Fan et al.2021), or fluorescence analysis (Shetty et al.2004). All these biosensors showed to be specific to copper with different levels of copper sensitivity (up to 5μM). Notably, in all these studies, the expression of the reporter gene was controlled by the *CUP1* promoter and driven by the Acel factor which regulates transcription of *CUP1* promoter, as mentioned above.

Recently, the Laboratory of Yeast Genetics at the University of Padova, where I performed the Thesis stage, has been involved in a research project (in collaboration with the private company "Italiana Biotecnologie"), aimed to create by genetic engineering new copper biosensors, using natural wild-type strains of *Saccharomyces cerevisiae*, isolated and collected by the company. As first, the yeast strain named '330' was chosen to develop a biosensor based on the expression of the GFP protein as fluorescent reporter, driven by the copper-dependent activation of the *CUP1* promoter. Collectively, experimental evidence demonstrated that the chromosome-integrated copy of the GFP reporter was efficiently expressed in the engineered strain (330-BS) when copper was present in the medium, further indicating very high specificity for copper ions. However, the sensitivity of the biosensor (i.e., the minimal copper concentration detectable by 330-BS yeast cells) yet remained to be established.

2. AIM OF THE THESIS.

In the present study, besides a deeper characterization of the yeast 330-BS properties, I aim to generate additional copper biosensors endowed with higher sensitivity, by the use of natural yeast strains belonging to the Company's collection, and featured by very low copper resistance, which is indicative of extreme sensitivity. After selecting the most promising strain on copper-containing medium, yeast cells will be genetically modified by CRISPR/Cas9 technique, and the recombinant strains will be checked to evaluate the efficiency of the reporter (GFP) expression, as well both selectivity and sensitivity of new yeast biosensors to copper ions, by performing multiple assays such as Western blot, fluorescence microscopy, and fluorimetry analysis.

3. MATERIALS AND METHODS.

All the experiments carried out throughout this project were performed in the Laboratory of Yeast Genetics at the Department of Biomedical Sciences, University of Padua.

3.1 Yeast strains.

The strains of the yeast *S.cerevisiae* used in this work are listed in Table 1.

Strain	Genotype
CESPLG05	Diploid (2n); WT
330	Diploid (2n); WT
CENPK	Haploid (n); MATa , ura3-52, trp1-289, leu2-3,112, his3 Δ1
CESPLG05-BS	as CESPLG05, but *LEU2::pCUP1-GFP-URA3TT*
330-BS	as 330, but *LEU2::pCUP1-GFP-URA3TT*
CENPK-BS	as CENPK, but *leu2::pCUP1-GFP-URA3TT*

Table1: Genotype of the yeast strains used in this study.

3.2 Yeast media.

Yeast cells were grown in different culture media depending on the experiments, and media compositions are indicated in Table 2.

Culture media for yeast	
YPGA	● MiliQ H2O by volume ● Glucose 2% ● Yeast extract 1% ● Bacto peptone 1% ● Adenine 0.01% ● Agar 2% (in case of solid medium)
YPGA+G418	● MiliQ H2O by volume ● Adenine 0.01% ● Yeast extract 1% ● Bacto peptone 1% ● Glucose2% ● G418 200µg/mL ● Agar 2% (in case of solid medium)

Table2: composition of culture medias used in the project.

In general, to prepare the culture media, reagent powders were combined, dissolved in proper volume of MilliQ water, and then sterilized by autoclaving at 121 °C for 15min.

For solid medium, agar was added (2%) and after autoclaving the medium was poured into Petri plates (~25mL). Once the medium was solid, plates were stored at 4°C.

When required, media (liquid or solid) were added with the antibiotic G418 (200 µg/mL), or with specific heavy metals, diluted from stock solutions (1 M) prepared dissolving in milliQ appropriate amounts of the following saline compounds: copper sulphate, nickel sulphate, and zinc sulphate (Sigma Aldrich).

3.3 Yeast plasmids.

In this study, two plasmids were used to perform the genetic modification of yeast cells by the CRISPR/Cas9 system.

1) gLEU2-pMEL-Cas9, derived from the vector pMEL13 (Mans et al.2015), consisting of 11000 bp and composed by the following DNA elements:

- ori: replication origins of *E.coli* (vector propagation in bacteria).
- 2μ ori: replication origins (multicopy) (vector propagation in yeast)
- AmpR: Ampicillin resistance cassette (bacterial marker selection)
- KanMX: Gentamicin (G418) resistance cassette (yeast marker selection)
- Cas9MX: Cas9 endonuclease expression cassette
- *LEU2*gRNA: expression cassette for the guide RNA molecule, targeting the coding region of the *LEU2* gene of *S.cerevisiae.*

The plasmid, already available in the Laboratory, was used to perform the genetic modification of the *LEU2* locus in the yeast cells by CRISPR/Cas9 strategy.

2) pILGFP17, described in (Peng et al.2015), containing the following elements:

- ori: replication origins of *E.coli* (vector propagation in bacteria).
- 2μ ori: replication origins (multicopy) (vector propagation in yeast)
- AmpR: Ampicillin resistance cassette (bacterial marker selection)
- KIURA3MX: expression cassette for URA3 gene of *Kluiveromyces lactis* (yeast nutritional marker selection)
- Yeast expression cassette consisting of:
    1. Promoter of the *S.cerevisiae CUP1* gene, carrying specific consensus sites for the Acel transcriptional factor, whose DNA binding requires copper ions (Dameron et al. 1991, 1993)
    2. Coding sequence of the green fluorescent protein GFP
    3. Transcription terminator sequence of the yeast *URA3* gene

The plasmid, already available in the Laboratory, was used as DNA template to amplify by PCR the pCUP1-GFP-URA3TT expression cassette, generating the Donor DNA molecule required to perform the CRISPR/Cas9-assisted genome editing of yeast cells.

3.4 Polymerase Chain Reaction (PCR).

The Polymerase Chain Reaction (PCR) is a popular technique used to amplify in vitro a specific DNA template with known upstream and downstream ends. This procedure is composed of 3 main steps repeated from 30 to 40 times using a thermal cycler: 1) denaturation: which separates the two complementary strands of DNA (template DNA), this step happens by

heating the DNA up to about 95°C to break the hydrogen bonds. 2) Annealing: which specifies the region of the DNA that will be copied with the help of primers, the right value of temperature used in this step depends on the pair of primers that have been used in terms of length and sequence of primers (between 50-60°C). 3) Extension: which copies the DNA. In this step, we heat the reaction back up again (70-75 °C) to extend the new double-stranded areas where the primer and DNA template have been annealed using DNA polymerase which finds the short, double-stranded regions of DNA where the primers have bound and then moves along the DNA and adds the correct complementary DNA nucleotides. In this work, I used Taq polymerase, a thermostable enzyme found in thermophilic bacteria that is capable of remaining stable in reaction even when facing multiple rounds of heating and cooling.

To perform PCR, the components were mixed in sterile tubes with the following concentrations:

Component	**Final Concentration**
DNA template	1ng/µL
Taq mastermix	12.5 µL
Primer FOR	0.75 µL
Primer REV	0.75 µL
H2O	10 µL
Total	25µL

Table 3: components for PCR reactions used in this project.

In this study, I employed PCR in two different experimental assays, first, to amplify the GFP cassette using pILGFP plasmid as template and two specific primers. Secondly, to perform diagnostic PCR on the genomic DNA extracted from either wild-type or genetically modified yeast cells. The sequences of the primers used in this project are reported in Table 4.

PRIMERS	SEQUENCE	LENGHT
DGLEU2-F	GTGGGTGGTCCTAAATGGGG	20
DGLEU2-R	CTTTTTGTGTGGTGCCCTCC	20
LEU2-IR CUP F	GATGGTGTCGCTTGGGATAGTGAACAATACACCGTTCCAGAAGTGC AAAGAATCACAAGACTAGTTAGAAAAAGACATTTTTGCTGTCAGTC ACTG	96
LEU2-IR CUP R	AAGTTCAATGACAATTTCAACATCATTGCAGCAGACAAGATAGTTG GCGATAGGGTTGACCTTTAAATCATTACGACCGAGATTCCCGGG	89

Table 4: sequence of the primes used in this project.

3.5 Agarose gel electrophoresis.

Gel electrophoresis is a technique used to separate DNA molecules according to their size, and to visualize the DNA fragments thanks to the staining with fluorescent probes, as ethidium bromide. This method is based on an electric field and a gel matrix. The gel contains small pores in which the negatively charged molecules migrate to the positive electrode due to the electric field. This movement is affected by the shape, charge, and size of molecules. Due to the lower molecular weight, smaller molecules migrate faster and consequently further while the large ones move more slowly. The Gel is made by dissolving an appropriate amount of agarose (EuroClone) in 0.5X TBE buffer (45mM Tris-borate, 1mM EDTA) with the addition of ethidium bromide with the final concentration of 0.5µg/ml. The agarose is dissolved by heating, the solution is subsequently poured into special containers adding a particular comb that allows the formation of the wells in which the DNA samples must be loaded. Once the gel becomes solid, it should be placed in an electrophoretic tank filled with 0.5% TBE. The DNA samples should be loaded in the wells by adding the gel loading Dye Purple 6X (New England BioLabs), which increases the viscosity of the samples so that they can be deposited in the bottom of the wells. The gel is then subjected to a voltage of 90-100V for 30-60 minutes to make the DNA molecules migrate. At the end of the run, the gel is visualized by ultraviolet light. To compare the size of the DNA fragments, a molecular weight marker (DNA Ladder, New England BioLabs) has been used.

3.6 Yeast genome editing by CRISPR/Cas9.

CRISPR/Cas9 is a genome-editing technology that uses a specific endonuclease (Cas9) and a guide RNA. Cas9 is guided to a specific DNA target site with the help of guide causing a cut at the target site blocking the cell cycle until the damage is repaired. In this case, in the present of a DNA fragment (donor DNA) with a sequence identical to the regions flanking the break site, the recombination process is activated resulting in restoring cell proliferation. In the laboratory, the CRISPR/Cas9 is a well-established genome editing system with standardized protocols that is commonly used to modify both laboratory and natural (oenological) yeast strains.

In this study, I used the gLEU2-pMEL13-Cas9 plasmid to express in yeast cells the active gRNA-Cas9 complex targeted to *LEU2* gene. The Cas9-induced double-strand breaks can be efficiently repaired by homology recombination in presence of the donor DNA molecule, which consists of the GFP expression cassette surrounded by homology sequences flanking the *LEU2* locus, finally replacing the LEU2 gene with the GFP cassette.

3.7 Yeast transformation.

The transformation allows the entry of exogenous DNA molecules into the yeast cells by perturbing temporarily their permeability. In this project, I performed the chemical transformation based on the use of lithium acetate (Liac), which is a crucial component that facilitates the formation of a stable complex between foreign DNA and cell wall of yeast. Standard protocols, reported in (Chen et al.1992), were followed as described below.

The yeast strains were incubated in a rich medium (liquid YPGA) and left to grow at 28°C until they reached OD600=0.8 (late exponential phase which is ideal to apply the transformation).

5ml of each culture was taken and centrifuged for each transformation, following by discarding the supernatant and resuspending the pallets in 1ml of Liac 0.1M and subsequently incubating at 30°C for 15 minutes. After aliquoting 200µl of the solution into new tubes, the components were added in the following order:

1) 480µl of PEG 4000 50%

2) 72µl of Liac 1M

3) 5µg plasmid DNA

4) Repair, donor DNA (in this case reporter cassette) (based on the number of samples)

5) ddH2o

In the next step, the cells were resuspended by vortex and were then incubated at 30°C for 30 minutes and subsequently at 42°C for 20 minutes. The cells were then sedimented, transformation mixture was removed, and the pellets were resuspended in a rich medium (YPGA) and allowed to grow at 27 °C.

After 3 hours of growth (to allow the expression of antibiotic resistance cassette), the solutions were centrifuged, and the pallets were resuspended in MiliQ water. The final mixture was then plated in a rich solid medium with the addition of the G418 antibiotic (YPG+G418) and incubated for 2-3 days at 30 °C.

3.8 Extraction of genomic DNA from yeast cells.

The genomic DNA extraction was carried out following the method described in (Lõoke et al.2011). This procedure is based on the chemical lysis of cells using repeated freeze-thaw cycles with the help of a buffer containing Triton X-100 and SDS which results in the precipitation of DNA in alcohol. The procedure starts with resuspending the yeast in a lysis solution (Liac 0.2M and SDS 1%), incubating the samples at 75 °C for 10 minutes and washing them using alcohol (ethanol 100% and 75%) causing the DNA to become less soluble and subsequently precipitated. After centrifugation, the genomic DNA is resuspended in sterile water.

3.9 Growth analysis of yeast strain on solid medium (spot assay).

Growth assay is a biological experiment that measures the viability of cells by constantly monitoring their growth rate. It is a practical method for comparing the behavior of different cells placed in the same environmental condition. In this project, the ability of yeast cells to propagate and grow in the presence of different concentrations of copper has been evaluated. For this purpose, the yeast strains were left to grow to the late-exponential phase (OD600=1.0). Five serial dilutions (1:10) have been prepared from each strain and an aliquot (5µl) from each dilution has been deposited on YPG solid mediums prepared with different concentrations of

copper (0 mM $CuSO_4$, 1 mM $CuSO_4$, 5 mM $CuSO_4$, and 10 mM $CuSO_4$). The plates were then incubated at 30 °C and the growth rate was constantly observed for 48 to 72 hours.

3.10 Fluorescence microscopy analysis.

Fluorescence microscopy was performed to evaluate *in vivo* the expression of the green fluorescent protein GFP, i.e., in yeast living cells. The procedure started with the incubation (12 hours) of either wild-type, or genetically modified yeast cells in a medium containing copper at a concentration of 5 mM. The samples have been then collected, deposited on the special slides, and visualized by microscope detecting both fluorescence and differential interference contrast (DIC) signals. In this study, the LEICA DMI 6000 microscope with 63x Dry lens, equipped with the GFP-FLUO filter to detect green fluorescent signal was used.

3.11 Protein extraction from yeast cells.

To evaluate the expression of GFP protein in the yeast cells, which have been incubated (6 hours) in either copper-free (as control), or in media supplemented with copper at different concentrations (1mM and 10mM), the total yeast protein content was extracted by the method described in (Wright et al. 1989). Briefly, 10 mL of liquid culture (OD600=1) for each yeast strain was centrifuged and resuspended with 100 µL of TCA (20%). In the next step the glass beads (about 0.2 mg) were added to each solution and the mechanical lysis of the yeasts was applied by repeated breaking cycles with the help of specific instrument (MagNA Lyser, Roche). 500 µL of TCA (5%) was then added to each lysate and the solutions were centrifuged at least for two times and the supernatant was discarded. The pallets obtained from centrifugation were resuspended in 100 µL of 1x LSB buffer (Laemmli sample buffer: SDS 2%, glycerol 10%, bromophenol blue 0.02%, DTT 50mM, Tris-HCI at a PH of 6.8) and the samples were denatured by incubation at 95 °C for 10 minutes.

Once the protein extractions of yeast cells were obtained, samples have been quantified by Bradford's assay, based on the spectroscopic properties of the protein dye Coomassie Brilliant Blue G-250. For this assay, a solution with 1-2µl of the sample that should be quantified (in this case the extracted protein), 800 µl of miliQ water, and 200 µl of Bradford (Sigma-Aldrich) should be prepared and the absorbance should be measured at the wavelength of 595 nm. To calculate the amount of protein, a standard curve with known concentrations of a protein

standard (i.e., purified BSA protein) was used and protein concentration was determined by measuring its absorbance and comparing it to the standard curve.

3.12 SDS-PAGE and Western blot.

SDS-PAGE (Sodium Dodecyl/Sulfate-polyacrylamide Gel Electrophoresis) is a method that gives the possibility of detecting a target protein from a complex mixture of proteins. By this method we can separate proteins based on their size and molecular weight. SDS is a detergent that denatures proteins, meaning that it breaks down their three-dimensional structure and unfolds them. This converts all proteins into linear chains of amino acids with a uniform negative charge which causes the migration of proteins towards the positive electrode.

The polyacrylamide gel is made using the components mentioned in the Table 3 as described in Sambrook et al. (1989)

	Stacking Gel	**Running Gel**
H2O MilliQ	70%	22%
Acrilammide 30%	17%	50%
Tris 1.5M	12.5% Ph 6.8	26% pH 8.8
APS 10%	0.1%	1%
SDS 10%	0.1%	1%
TEMED	0.01%	0.1%

This gel is made of 2 different parts: 1) the upper part (stacking gel), where the extracted proteins will be loaded in the wells based on their protein concentration, well size, and gel thickness. The stacking part determines the same starting point for all the proteins. 2) The lower part of the gel (Running gel) is the actual part of the gel in which the protein migration occurs. The gel should be polymerized between two support glasses. When the gel is ready, it should be placed in an electrophoresis unit, and filled with SDS-PAGE 1x (Tris-HCI 25mM PH8.3, Glycine 192mM , and SDS 0.1%), then the extracted proteins(solved in LSB buffer 1X and incubated at 95°C for 5minutes) should be loaded into the wells and at the end, the molecular weight marker should be loaded to assess the molecular weight of the protein of interest, once the samples and marker are loaded, a constant electric field at the appropriate voltage (16-24mM) for 90 minutes should be applied.

After electrophoresis, the protein sample is transferred from the gel to a nitrocellulose membrane. This is done by placing the gel and membrane in a special instrument (Trans-blot turbo, Biorad), in the presence of the blotting buffer (glycine 192mM, methanol 20%, SDS 0.03%, Tris HCI 25 mM pH 8.4) and subjected to an electric current (350mM) for 30 minutes. The electric current causes the proteins to move from the gel to the membrane.

Once all the proteins are transferred to the membrane, the membrane should be incubated with a Ponceau red solution. This step allows us to verify that equal amounts of protein extracts were loaded onto the membrane from all the samples. After washing membrane with TBS-T swab (TBS - NaCI 150 mM, Tris 20 mM pH 7.4, Tween20 0.1%) to remove all the dye, the membrane should be then saturated through incubation for 60 minutes at room temperature with a solution of powdered milk (5% in TBS-T) which prevents non-specific binding of the antibodies. The membrane should be then incubated for 60 minutes at the room temperature with the primary antibody diluted in TBS-T (1:1000), in this project, we employed GFP (D5.1) Rabbit mAb (Cell Signaling) which is specific to the GFP protein thus binds to the GFP protein on the membrane. After repeated washes with TBS-T to remove any unbound primary antibody, the membrane is incubated for 60 minutes at the room temperature in a solution of secondary antibody diluted in TBS-T (1:10000) The secondary antibody binds to the primary antibody. In this project, we used Anti-Rabbit IgG (Sigma) which is directed against rabbit IgG. After washing the membrane with TBS to remove any unbound secondary antibody, the membrane should be incubated for a couple of minutes in an appropriate solution (Immobilion Western Chemiluminescent HRP substrate, Millipore) to produce a chemiluminescent signal. In this work, to visualize the immunoreactive bands, we used UVITEC (Eppendorf) instrument.

3.13 Fluorimetry assay.

Fluorometer is a specific instrument capable of measuring the intensity of fluorescence emitted by a sample. The fluorescence occurs when a substance absorbs light of a certain wavelength and emits it at a different wavelength. The sample which contains fluorescent molecules should be placed in a microplate well, they should be exposed to a specific wavelength of light, which causes the molecules within the sample to absorb energy and become excited to a higher energy state. Once the excited molecules return to their original lower energy state, they release the additional energy in the form of light (fluorescence emission) which can be measured by fluorometer.

In this study, genetically modified yeast cells were incubated in a nutrient-rich liquid medium (YPG) overnight at 28°C. The following day, when the OD600 reaches 1, each culture has been centrifuged and the resulting pellet of yeast cells were resuspended in 200 µL of YPG supplemented with different concentrations of copper solution, and then transferred to microtiter (multiwells) plate, which was incubated at 28°C for 2 hours while shaking to prevent yeast sedimentation. Plates were finally placed in the fluorometer apparatus (TECAN, infinite 200Pro), and the fluorescence emitted by the GFP protein was directly measured under the instrument with a manual gain of 120.

EXPERIMENTAL RESULTS:

3.1 Growth analysis of the selected strains.

As mentioned in the Introduction, in the Laboratory was recently generated the 330-BS yeast biosensor, carrying in the genome the GFP reporter under the control of copper-dependent *CUP1* promoter. The strain was created by CRISPR/Cas9-assisted genome modification of the 330 natural strain of *S.cerevisiae*, which was initially selected because of the general robustness, i.e., its elevated tolerance to environmental stress, as indicated by previous data obtained in the laboratories of the partner Company "Italiana Biotecnologie". Here, in order to generate copper biosensors possibly endowed with higher sensitivity, I explored the use of yeast strains displaying very low copper resistance, which would correspond to extreme sensitivity to the metal.

Therefore, several yeast strains of the Company collection have been preliminary checked for their ability to grow on copper-containing medium, finally leading to the selection of the CESPLG05 strain. Representative results of the growth assay on rich medium, added with increasing concentration of copper, are reported in Figure 1 for the three strains of *Saccharomyces cerevisiae* thereafter used in this research: the 330 strain, already adopted in previous study; CENPK, considered as reference of common laboratory strain; the CESPLG05 strain, so far judged as one of the most sensitive strains present in the Company collection.

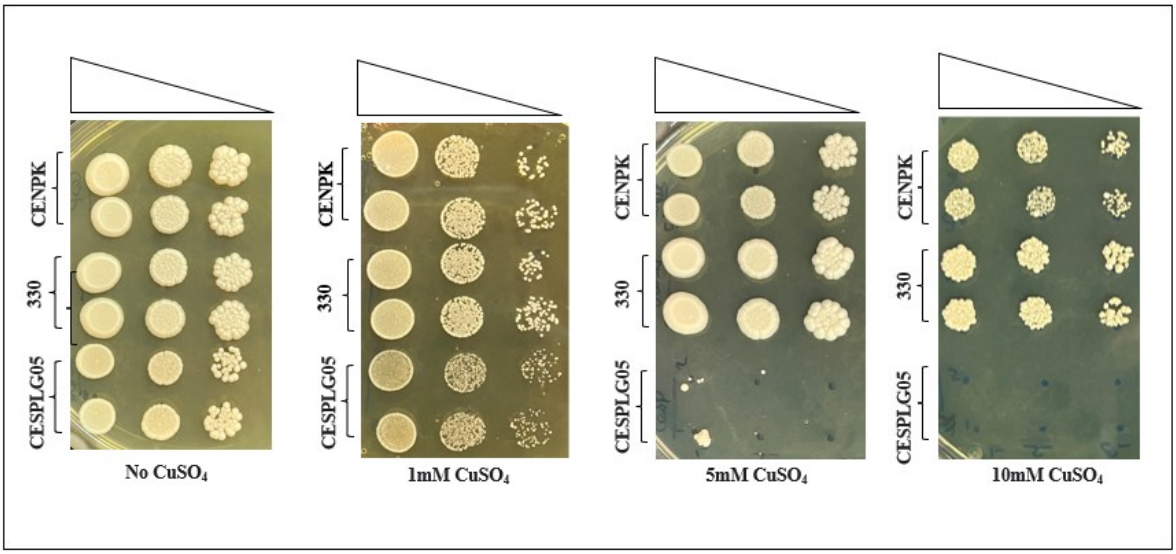

Figure 1: Growth analysis of the selected yeast strains (330, CENPK, and CESPLG05). Liquid cultures of each yeast strain were serially diluted, and equal amounts of cells were plated on either copper-free solid medium, or plates containing different concentration of $CuSO_4$ (1mM, 5mM, and 10mM). Plates were then incubated at 30°C for three days.

As shown in Figure 1, all yeast strains displayed similar growth in either absence, or at low concentration of copper (1 mM). However, at higher copper levels (5-10 mM), the growth of the CESPLG05 cells was clearly affected with respect to both 330 and CENPK yeast strains, thus demonstrating its higher sensitivity to copper. Notably, data also supported that the 330 strain was slightly more resistant than the laboratory CENPK strain, as indicated by the bigger colony size of the 330 strain in high copper concentration (10 mM).

4.2 Yeast genetic modification.

To originate the biosensors from both CENPK and CESPLG05 strains, I applied the CRISPR/Cas9-based strategy previously adopted to successfully modify the genome of the 330 strain. Similarly, in the *LEU2* locus it was stably integrated the specific DNA sequence to express in yeast cells the fluorescent reporter GFP, under the control of the copper-dependent *CUP1* promoter. The DNA fragment was obtained by PCR amplification using as template the pIL-GFP plasmid (already available in the laboratory), and the specific primers (reported in methodology section). The PCR products were then analyzed by agarose gel electrophoresis, confirming the amplification of the DNA fragment of the correct size (approximately 1500 bp) (Figure2). Once purified by column chromatography, the fragments were used as "Donor DNA" to perform the yeast cells transformation.

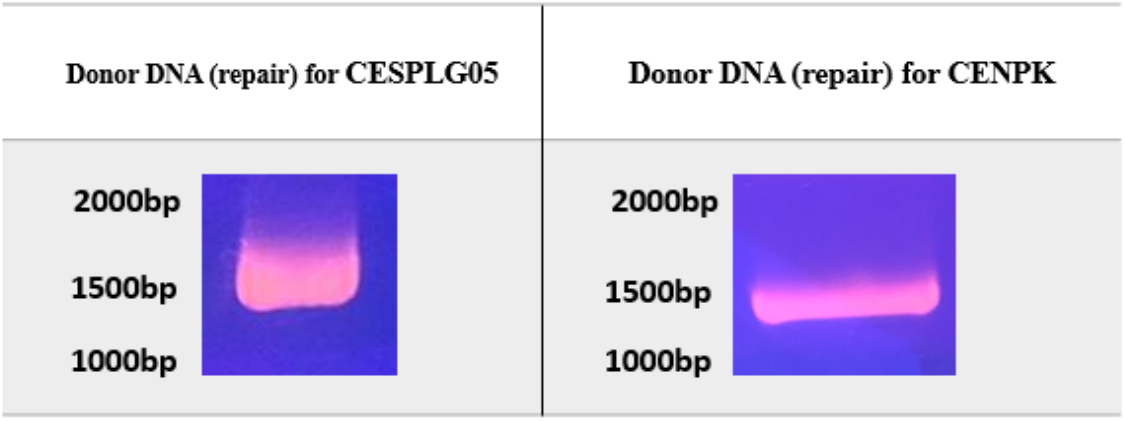

Figure 2: Analysis of PCR products. The DNA donor fragment of about 1.5kbp was generated by PCR with specific primers and plasmidic DNA as template (see Methods for details). Reaction products were then subjected to electrophoresis in agarose gel (1%), stained with ethidium bromide.

Yeast cells of either CESPLG05 or CENPK strains were transformed by the LiCl standard method (described in methods), using the plasmid gLEU2-pMEL13-Cas9 (expressing both Cas9 endonuclease and the *LEU2*-guide RNA molecule), with or without the "Donor DNA" previously purified. Transformation results are shown in Figure 3, where it can be observed that addition of Donor DNA (upper panels) determined the formation of new recombinant colonies for both CESPLG05 and CENPK strains, while in the absence of Donor DNA (bottom panels), no clones have been detected. Six different recombinant clones were then selected from each strain to be subjected for further studies.

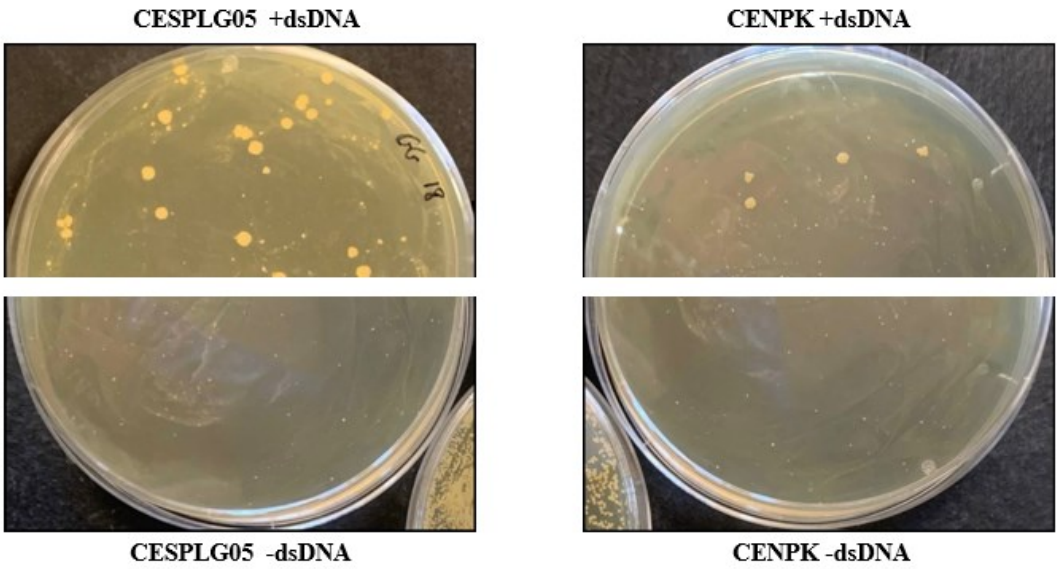

Figure 3: Yeast transformation. The CESPLG05 (left) and CENPK (right) strains were transformed with the guide plasmid (gLeu2-pMEL13-Cas9) and the Donor DNA repair (upper panel), or without repair as control (bottom panel), and then incubated in selective medium (YPG+G418) at 30 °C for 48-72 hours.

The genomic DNA of the 6 clones selected for each strain (CESPLG05 and CENPK) was extracted following the protocol described in methods, and purified DNAs were then used as template to set up the diagnostic PCR, in order to verify the replacement of the *LEU2* locus with the reporter DNA sequence (pCUP1-GFP). PCR products were finally analyzed by agarose gel electrophoresis, whose results are presented in Figure 4.

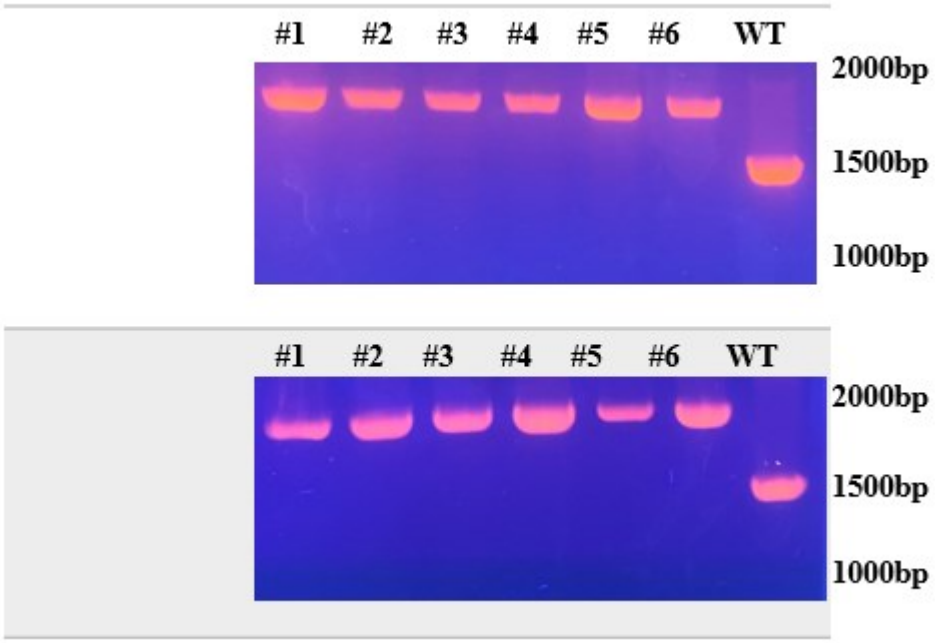

Figure 4: Analysis of diagnostic PCR products. Electrophoresis analysis in agarose gel (1%) of the product of PCR reactions (primers dgLEU2-F/dgLEU2-R) on the genomic DNA extracted from the indicated clones (#1-6), selected between the CESPLG05 (upper panel) and CENPK (lower panel) transformants. WT: parental (unmodified) genomic DNA from each strain was considered as control.

With respect to the parental (unmodified) strains (WT), considered as control, in all selected clones the PCR produced DNA fragments with increased size, demonstrating that the *LEU2* gene was successfully replaced by the reporter DNA fragment, definitely confirming that all clones were modified as expected, and thereafter renamed CESPLG05-BS and CENPK-BS strains, respectively.

4.3 GFP reporter expression analysis by fluorescence microscopy.

The expression of the GFP protein reporter was initially checked by fluorescence microscopy in both CESPLG05-BS and CENPK-BS strains, following the protocol described in methods. Resulting data, shown in Figure 5, supported that the presence of copper activated in the yeast cells the expression of the GFP protein, as clearly indicated by the green fluorescence emission, while no signal could be detected for the cells incubated in a metal-free medium.

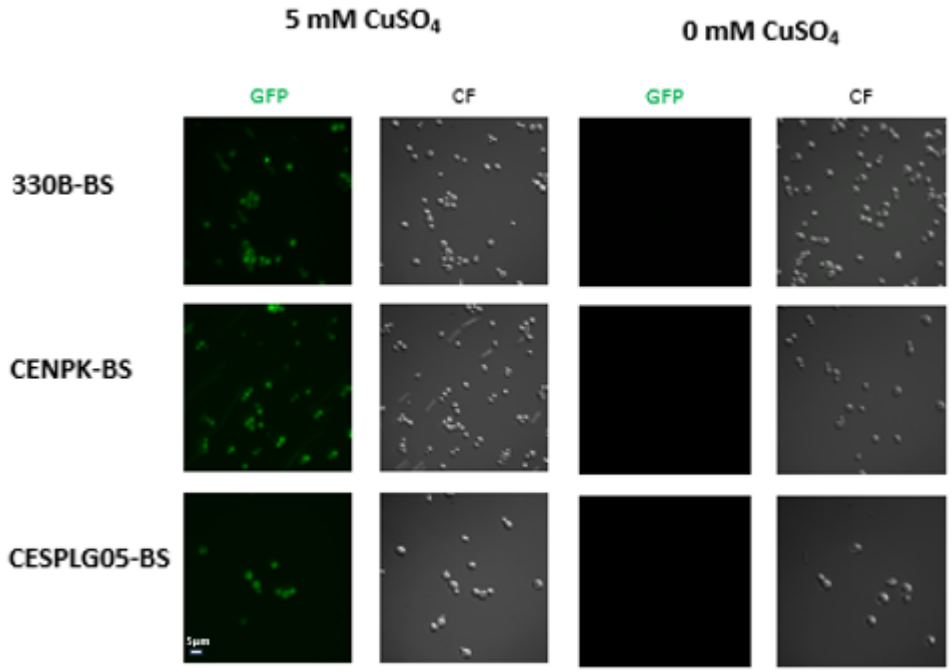

Figure 5: Analysis of GFP expression in the modified strains (i.e., 330-BS, CENPK-BS, and CESPLG05-BS) by fluorescence microscopy. Images were obtained by incubating (12 hours) the genetically modified cells with copper (5 mM) at 28°C (left panels), or in copper-free medium (right panels). Green fluorescence (GFP) and differential interference contrast (CF) fields are shown. Scale bar 5 µm.

Collectively, data demonstrated that the yeast strains integrated in their genome a functional copy of the DNA reporter sequence (pCUP1-GFP), as the expression was directly activated by copper and the protein was definitely able to produce the green fluorescence.

4.4 GFP reporter expression analysis by Western blot.

The newly generated biosensors have been further characterized by a series of biochemical assays (Western blot). Notably, the expression of the reporter GFP was monitored in each yeast strain (i.e., 330-BS, CESPLG05-BS, and CENPK-BS) upon exposure to different levels of copper. Therefore, cells were incubated for 6 hours with medium containing either 0 (as control), 1 or 10 mM copper (see methods for details), then total proteins were extracted and subsequently quantified by Bradford assay. Equal amounts of each protein extract (10 µg) were subsequently subjected to Western-Blot assay (described in methods), using the Anti-GFP specific antibody. Resulting data, shown in Figure 6, supported that the expression of the GFP protein occurred in dose-dependent fashion, as in each strain the protein levels increased with

increasing the concentration of copper in the medium (GFP panel). Although such analysis was not quantitative, some variability in GFP levels was observed between the three yeast strains, which was however not related to differences in protein loading (as indicated by the Ponceau staining of the membrane), and thus could possibly reflect strain-specific properties.

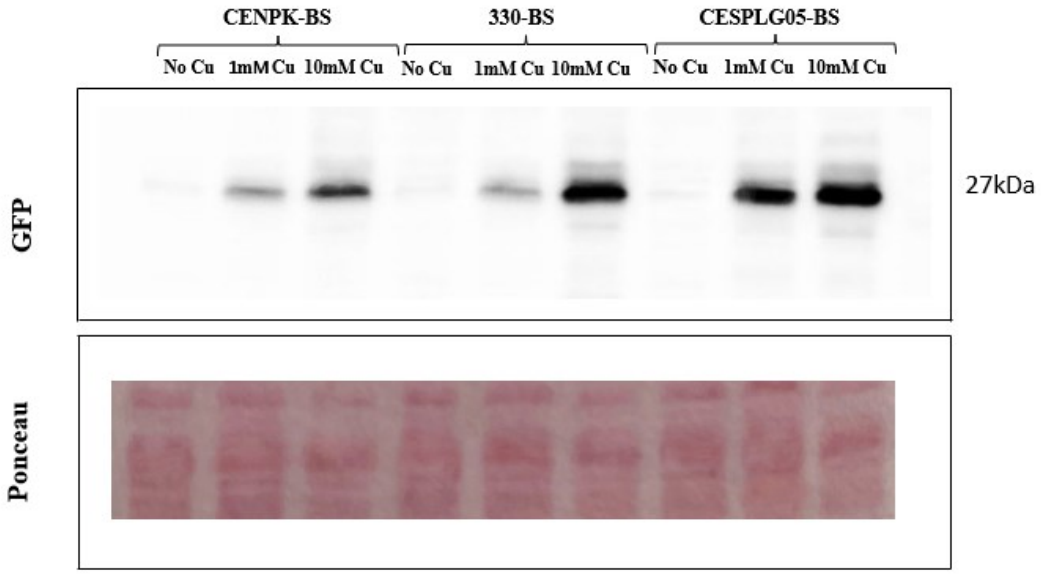

Figure 6: Analysis of GFP expression in the modified strains (330-BS, CENPK-BS, and CESPLG05-BS) by Western-blot assay. Equal amounts of protein extracts, obtained from each strain after incubation for 6 hours with different concentrations of copper (0mM,1mM and 10mM), were subjected to Western blot and the membrane probed with Anti-GFP antibody (upper panel), after the staining with Ponceau red, as gel loading control (lower panel).

Moreover, the same experimental procedure was performed to check the specificity of the yeast biosensors, by the incubation of each strain in medium added with other divalent metals, such as Nickel and Zinc, which could interfere with the association between copper and Ace1 protein. Similarly to previous experiments, yeast cells were incubated for 6 hours in presence of either Zn or Ni cations (10 mM), and total proteins were extracted to perform the Western blot assays. As shown in Figure 7, the band corresponding to the GFP protein was revealed for all yeast strains only in presence of copper, while no signal was detected in cells incubated with both Zn and Ni, therefore confirming that the new biosensors exhibit not only dose-dependent behavior but also high specificity towards copper.

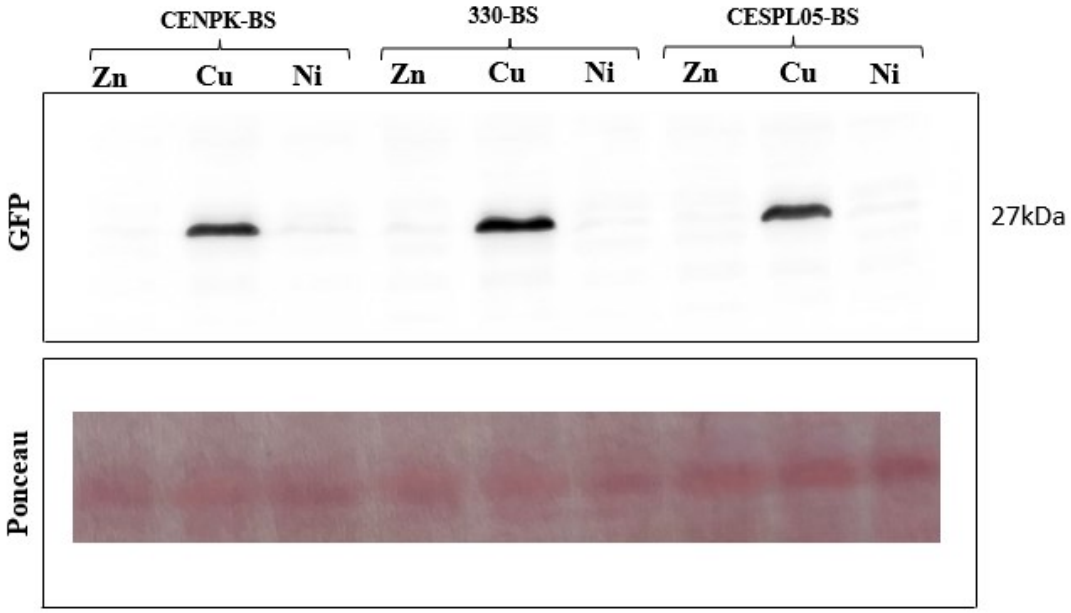

Figure 7: Analysis of GFP expression in the modified strains (330-BS, CENPK-BS, and CESPLG05-BS) by Western-blot assay. Equal amounts of protein extracts, obtained from each strain after incubation for 6 hours with 10 mM of either zinc-, nickel-, or copper-containing medium, were subjected to Western blot and the membrane probed with Anti-GFP antibody (upper panel), after the staining with Ponceau red, as gel loading control (lower panel).

4.5 Biosensors sensitivity evaluation by fluorimetry assay.

The copper sensitivity of the yeast biosensors, i.e., the minimal amount of the metal that can be detected by each strain, was determined by fluorimetric assays, as described in Methods, measuring the green fluorescence signal produced by the yeast cells incubated with medium containing different copper concentration, ranging from 10^{-12} to 10^{-3} M. As shown in Figure 8, the fluorescence emitted by all strains was comparable to background until the copper concentration was at picomolar levels (10^{-9} M), when the most sensitive CESPLG05-BS started to increase fluorescence production. Instead, both 330-BS and CENPK-BS remained to background levels, even when copper was in micromolar range (10^{-6} M). However, both 330-BS and CENPK-BS strains produced similar fluorescence signals upon exposure to millimolar levels of copper, consistently to their lower sensitivity to the metal, as indicated by previous data (see Figure 1).

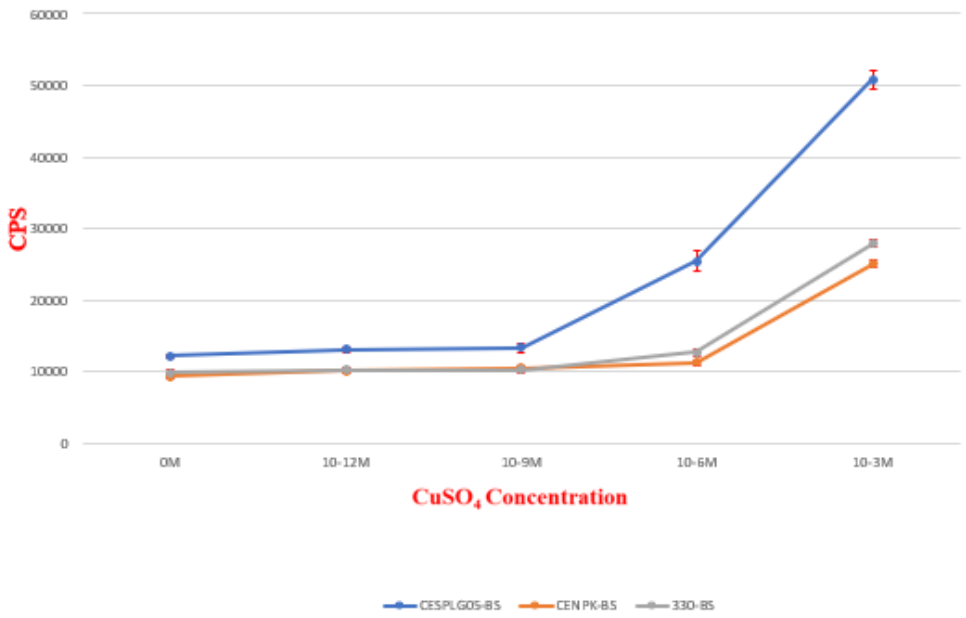

Figure 8. Analysis of fluorescence emission by Fluorometry. The modified strains (i.e., CESPL05-BS, 330-BS, and CENPK-BS) were incubated 2 hours at 25°C with medium containing different concentrations of copper (0, 10^{-12}, 10^{-9}, 10^{-6}, 10^{-3} M), and the fluorescence emission was measured. CPS values are the mean (±SD) of 4 independent experiments.

Additional assays were then performed to determine more precisely the minimal amount of copper able to activate the GFP reporter, by challenging each strain with different levels of metal, similarly to the experiments described above. In this case, however, copper concentration was increased by only 10 times, rather than 1000 times as previously. Resulting data, shown in Figure 9, further supported the highest sensitivity of the CESPLG05-BS strain, which was able to detect copper when the metal concentration was between 10^{-10} and 10^{-9} M. Data however indicated that in both 330-BS and CENPK-BS strains the reporter activation occurred when copper levels were between 10^{-7} and 10^{-6} M.

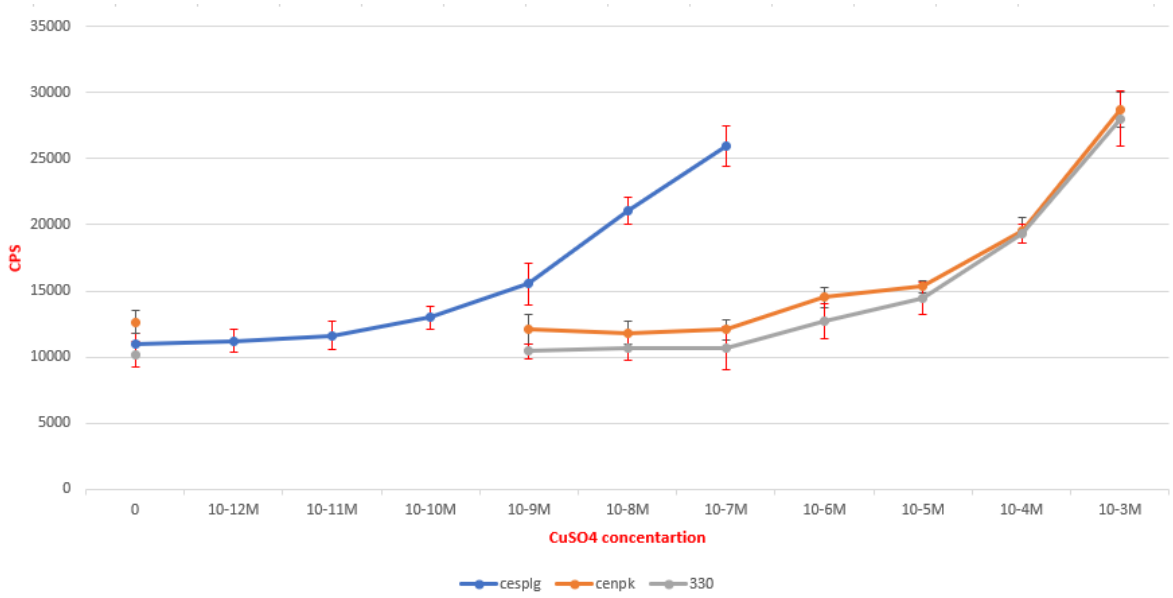

Figure 9. Analysis of fluorescence emission by Fluorometry. The modified strains (i.e., CESPL05-BS, 330-BS, and CENPK-BS) were incubated 2 hours at 25°C with medium containing the indicated concentrations of copper, and the fluorescence emission was measured. CPS values are the mean (±SD) of 4 independent experiments.

Collectively taken, experimental data indicated that copper sensitivity largely varied between the yeast strains considered in this project. Very similar performances were observed for the 330-BS and the CENPK-BS strains, both displaying the ability to detect copper at micromolar levels, which was consistent to the values previously reported for other laboratory strains. Data further supported that CESPLG05-BS was the most sensitive yeast biosensor generated in this Thesis, as it required significantly lower copper concentration to activate the GFP reporter and to produce detectable fluorescence signals (sub-nanomolar levels).

5. Discussion.

Heavy metals are a group of chemical elements potentially harmful for living organisms even in low quantities (Duruibe et al.,2007). They are released in the environment by natural processes (i.e., geochemical cycles), but human activities, such as mining, smelting, and manufacturing, have profoundly increased their dispersion into the environment, posing significant threats to human health, causing a range of adverse effects, including cancer, neurological damage, and respiratory issues (Mahurpawar et al., 2015). Additionally, heavy metal pollution disrupts ecosystems and degrades the environment, causing water, soil, and air pollution (Zaynab et al., 2022).

Among heavy metals, copper has been extensively employed as main component of pesticides in the wine industry for over a century, as its effectiveness against a variety of plant diseases, including downy mildew, one of the most destructive grapevine pathologies. Copper-based fungicides are relatively inexpensive and easy to apply, making them a popular choice for grape growers. However, the increasing use of such compounds, and consequently the increasing amounts in the must, may perturb or even block the fermentation process, finally becoming an emerging issue. Since traditional methods for measuring copper concentrations often require specialized technicians or complex equipments, the 'Italiana Biotecnologie' company (involved in oenology industry) started a research project in collaboration with the University of Padova, aimed to develop a yeast biosensor for copper, based on fluorescent reporter, generated by the genetic modification of natural strains belonging to the company's private collection. Initially, the '330' strain was selected because of its great adaptability and high resistance to environmental stress, and the preliminary characterization as copper biosensor of the modified strain (330-BS) demonstrated both full functionality and high specificity, i.e., the ability to express the reporter protein (GFP) only in presence of copper, but it remained still to be determined the sensitivity, i.e., the minimal copper concentration detectable by the yeast 330-BS biosensor.

The starting point of this thesis however consisted of a change in the logic behind the initial strain selection. Considering that lower resistance stands for higher sensitivity, the tolerance to copper of some natural strains of company's collection was checked, in order to identify the most sensitive strains, and finally focusing to the natural CESPLG05 strain to generate a novel biosensor. Moreover, as comparative control, it was considered the laboratory CENPK strain, which displayed similar copper resistance to the 330 strain.

Various methods have been reported in previous studies to create yeast-based copper biosensors, either based on the reporter integration as transgene into the yeast genome (Vopalenska 2015), or by the transient reporter expression by plasmidic vector (Lehmann et al.,2000; Tag et al.,2006), as for the biosensor described in (Shetty et al., 2004), which uniquely used green fluorescence as reporter. However, in this thesis, I applied the CRISPR/Cas9 technique to perform the stable integration of the fluorescence reporter cassette (pCUP1-GFP), by replacing the *LEU2* gene of both CESPLG05 and CENPK yeast strains, similarly to the previous modification performed for the 330 strain. Importantly, the use of CRISPR/Cas9 system generated recombinant strains carrying a single copy of the reporter cassette stably integrated in the genome, which diminished the variability of protein reporter expression among the cell population.

Specificity and sensitivity are two fundamental characteristics that are necessary to be evaluated when introducing a new biosensor. Collectively, the experimental results of this thesis demonstrated the specificity towards copper for all yeast biosensors, further excluding nonspecific activation by Zinc or Nickel cations, accordingly to (Shetty et al., 2004). However, future experiments are necessary to assess the behavior of the biosensors in presence of other metals such as Cadmium, Silver, or Cobalt, which were observed to compete/interfere with copper for the binding to the Ace1 transcription factor (Dameron et al.1993, Gokhale et al.,2006).

Regarding sensitivity, the determination of the minimal concentration of copper that can be detected by the biosensor was established by specific fluorimetry measurements, which gave the possibility to evaluate the behavior of each biosensor with a wide range of different copper concentrations at the same time, while fluorescence microscopy and biochemical (Western blot) assays allow to process and analyze only a small number of samples. Based on the results obtained from fluorimetry assays, the yeast biosensor CESPLG05-BS, derived from the most copper-sensitive *S.cerevisiae* strain of the company's collection, was able to detect copper at very low concentration (10^{-10} M), strongly supporting the potentiality of using natural strains to develop more sensitive biosensors. Notably, CESPLG05-BS biosensor sensitivity was considerably higher than previously reported yeast-based biosensors, such as those described by Shetty et al. (2004) ($5x10^{-7}$ M) and Fan et al. (2022) ($5x10^{-6}$ M). Moreover, data indicated similar sensitivity for 330-BS and CENPK-BS strains, according to their similar copper resistance, which allowed to detect copper at 10^{-7} M concentration. Importantly, it was consistent to the sensitivity reported in (Shetty et al. 2004) by the use of another laboratory

yeast strain (BY4742), which further corroborated the experimental evidence collectively provided by this work.

6.Conclusions.

In this project, I successfully generated two yeast-based biosensors by the genetic modification of two different strains of *Saccharomyces cerevisiae* (i.e., CESPLG05 and CENPK), which were both effectively able to express the fluorescent protein GFP upon exposure to environmental copper, specifically generating signals in dose-dependent fashion. The use of different strains of *S.cerevisiae* gave the possibility to perform comparative analyses of their copper sensing abilities, allowing to identify the most sensitive yeast biosensor among the strains generated during this project. Experimental evidence indicated that the biosensor originated from the CESPLG05 natural strain can be ranked among the most sensitive reported biosensors (10^{-10} M), and it could be considered as a valuable tool also for the wine industry, helping winemakers to detect even very low levels of copper in must, and thus to prevent problems during the fermentation. However, future experiments are yet required to assay the ability of the novel biosensors in natural must, i.e., the most relevant environment where biosensors have to be challenged.

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