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MANIPULATION OF NI^{2+} TOLERANCE OF *SACCHROMYCES CEREVISIAE* CELLS: A PRIMARY STEP TO BIOREMEDIATION BY REMOVAL AND RECOVERY OF NI^{2+} FROM CONTAMINATED WATERS

Ileana C. FĂRCĂŞANU*, Codruţa PARASCHIVESCU, Lavinia RUŢĂ, Eliza OPREA and Sorin AVRAMESCU

Department of Organic Chemistry, Faculty of Chemistry, University of Bucharest, Sos. Panduri 90-92, Bucharest, Roumania

Received September 19, 2007

Metal remediation through common physico-chemical techniques is expensive and unsuitable in case of voluminous effluents containing complex organic matter and low metal contamination. Alternative biotechnological approaches received great deal of attention in the recent years. Engineering cell lines that would hyperaccumulate heavy metals can be an invaluable tool in removing such ions from aqueous environments. In this study we obtained *Saccharomyces cerevisiae* mutants that were tolerant to high concentrations of Ni^{2+}. Out of the five tolerant lines selected, two were shown to hyperaccumulate the Ni^{2+} ions in the vacuole. These mutants represent the first step in designing a system suitable for bioremediation of Ni^{2+}-contaminated waters.

INTRODUCTION

Heavy metals represent an important environmental problem due to their potential toxic effects, and their accumulation throughout the food chain leads to serious ecological and health problems. The increase of environment pollution by heavy metals generated the interest in the problem of live organisms resistance to these metals. Biosorption is one of the mechanisms of microorganisms resistance to heavy metals and yeasts as biosorbents are of special interest.[1-4] However, the low binding capacity of biomass for certain metals such as Ni^{2+} and failure to effectively remove metals from real industrial effluents due to presence of organic or inorganic ligands limit this approach. When pure biosorptive metal removal is not feasible, growing metal-resistant cells can ensure better removal through a combination of bioprecipitation, biosorption and continuous metabolic uptake of metals after physical adsorption. Such approach may lead to removal of toxic metals, as well as allow optimization through development of resistant species.

Nickel ion is a heavy metal ubiquitously distributed in nature and constitutes a trace element in most living cells.[5] It is reported that this metal acts as a cofactor of several enzymes, such as NiFe hydrogenase, methyl coenzyme M reductase, CO dehydrogenase, and urease, in various organisms.[6] At higher concentrations, Ni^{2+} can be very toxic to both eukaryotic and prokaryotic cells. Ni^{2+} potentially inhibits synthesis of macromolecules such as RNA and proteins.[7] Toxicity is also exhibited as an alteration in the metabolism of carbohydrates and organic ions by interfering with the role of other trace elements, such as Mg^{2+} and Fe^{3+}, and excretion of pyruvate or potassium ions from the cell probably because of damage to membrane integrity.[8]

The baker's yeast *Saccharomyces cerevisiae* is an excellent model system to study the uptake and accumulation of heavy metals by living cells. This simple organism can grown on low-cost media, can be manipulated easily, and is not pathogenic. Its genome has been entirely sequenced and many cellular processes have been identified at molecular level. Recently, we found that exogenous histidine augmented the ability of *Saccharomyces cerevisiae* cells to decrease the Ni^{2+} concentration in their environment.[9] Nevertheless, routine supplement of L-His to the environments contaminated with Ni^{2+} may be of little practical interest.

* Corresponding autor: farcasanu.ileana@unibuc.ro

Alternatively, it would be interesting to engineer yeast mutants that would hypperaccumulate Ni^{2+}.

In this work we focused on obtaining *Saccharomyces cerevisiae* mutants that are resistant to high concentrations of Ni^{2+} in their environment and we present the characterization of these tolerant mutants in terms of Ni^{2+} uptake, accumulation and cellular compartmentalization, aiming to select for cell lines that hyperaccumulate Ni^{2+} as a primary tool for bioremediation of wastewaters contaminated with Ni^{2+}.

RESULTS

Selection of Ni^{2+}-tolerant mutants

The parental *Saccharomyces cerevisiae* strain used (also called by us "wild type" strain) can grow on YPD plates containing $NiCl_2$ up to 5-6 mM. To obtain Ni^{2+}-resistant mutants, we exposed the parental strain to the chemical mutagen ethyl methanesulfonate (EMS). The EMS-mutagenized cells were spread onto YPD plates that contained lethal concentrations of $NiCl_2$ (10 mM) at approximate density 10^6 cells/plate. After 6-8 days incubation at 28ºC, Ni^{2+}-resistant colonies appeared (3-4 colonies/plate). We selected 200 resistant colonies and we passed them five times on fresh YPD plates, checking each time for the Ni^{2+}-tolerant phenotype, to exclude the false-positive candidates. Out of the 200 initial colonies, only 11 retained the phenotype of interest. These cells were back-crossed with the wild type cells, the diploids were sporulated and tetrads were dissected. Out of the 11 mutants, only 5 exhibited 2:2 segregation of the Ni^{2+}-resistant phenotype, suggesting that this was probably the result of a mutation in a single gene. These mutants were designated as *nir1*, *nir2*, *nir3*, *nir4*, and *nir5* (for **Ni-R**esistant) and selected for further analysis.

Growth characteristics of the Ni^{2+}-tolerant lines

The five mutants were tested for growth abilities in the presence of high concentrations of Ni^{2+} in their environment. Thus, it was shown that all five mutants could grow on YPD plates containing up to 12-15 mM $NiCl_2$, conditions under which the parental line no longer survives (data not shown). The dynamics of mutant cells growth in liquid YPD containing Ni^{2+} was also determined. All five mutants exhibited improved growth properties in the presence of Ni^{2+}, when compared to the parental type (Fig. 1).

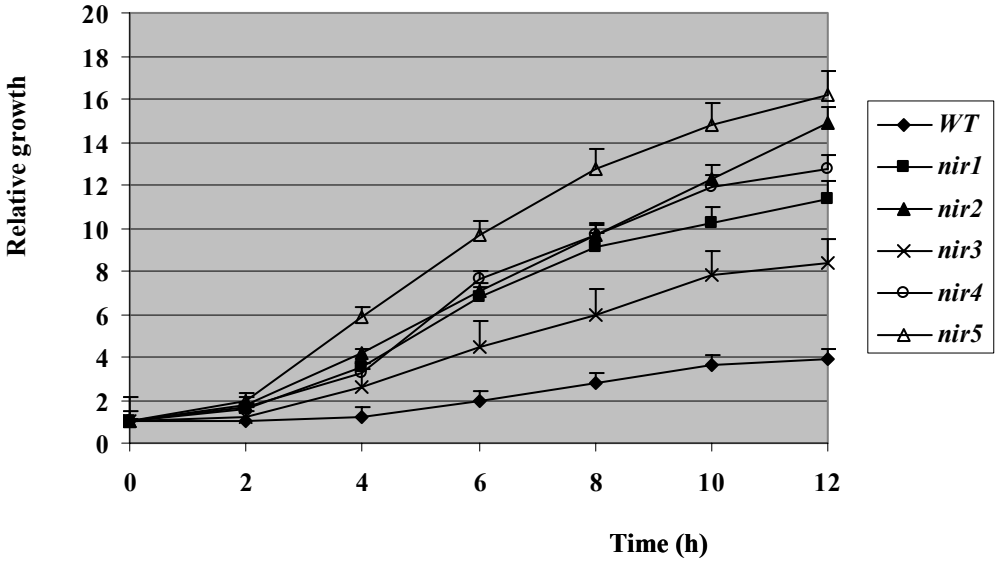

Fig. 1 – Growth of the Ni^{2+}-tolerant mutants in Ni^{2+}-containing media. Cells were inoculated from an overnight culture into liquid YPD to density 10^6 cells/mL and incubated with shaking at 28°C for two hours before $NiCl_2$ was added (final concentration 2 mM). Growth of cells was assessed at various times by measuring the absorbance of the cell suspension at 600 nm (OD_{600}). The growth was calculated relatively to the absorbance measured at the moment when $NiCl_2$ was added (considered time 0). Data represent triplicate determinations and are expressed as means standard deviations (for clarity reasons, error bars are shown only in one direction). *WT*, wild type (parental strain); *nir*, isogen Ni^{2+}-resistant strain.

Ni^{2+}-accumulation by the mutant cells

The tolerance to high concentrations of Ni^{2+} can be acquired by at least two different mechanisms: 1) Exclusion of the ions from the cells by stimulating the export process. 2) Intracellular accumulation of ions in non-toxic forms, usually by compartmentalization in the organelles (mainly vacuole). We therefore tested our mutant lines for the ability to accumulate Ni^{2+} from their culture media, and we found that only mutants *nir4* and *nir5* accumulated more Ni^{2+} than the parental strain (Fig. 2). The *nir1* and *nir2* clearly gained their tolerance to Ni^{2+} by acquiring a low level of Ni^{2+} accumulation, while mutant *nir*3 was no different from the parental line (Fig. 2). For bioremediation purposes, only the mutant lines that (hyper)accumulate the ions from the environment would be useful, therefore mutants *nir4* and *nir5* were selected for further investigation.

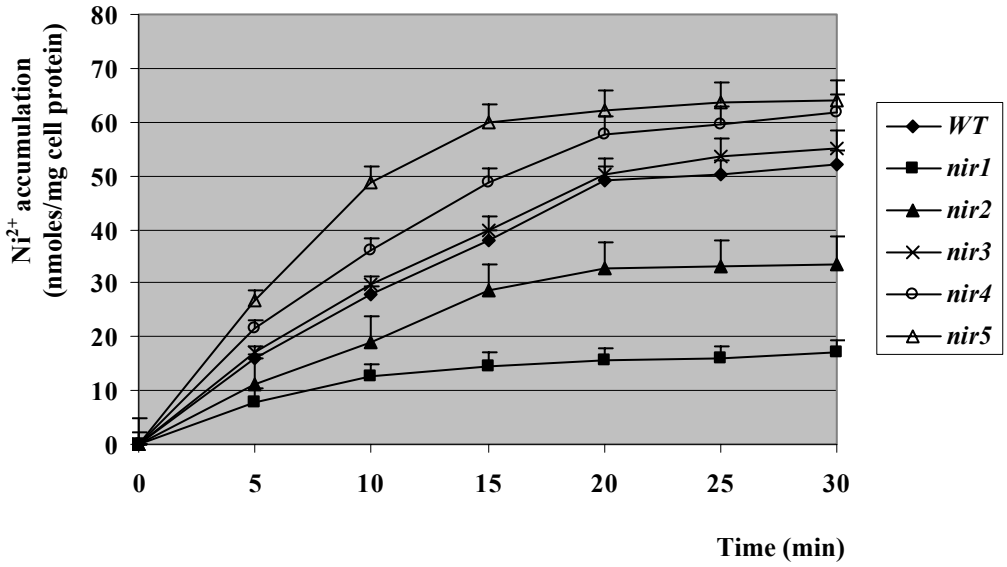

Fig. 2 – Ni^{2+} accumulation by Ni^{2+}-resistant mutants. Cells were inoculated from an overnight culture into liquid YPD to density 10^6 cells/mL and incubated with shaking at 28°C for two hours before $NiCl_2$ was added (final concentration 2 mM). Aliquots of cells were harvested and used to determine cellular Ni^{2+} as described in the *Experimental* section. Data represent triplicate determinations and are expressed as means standard deviations (for clarity reasons, error bars are shown only in one direction). *WT*, wild type (parental strain); *nir*, isogen Ni^{2+}-resistant strain.

Intracellular distribution of Ni^{2+} ions in mutants *nir4* and *nir5*

For bioremediation use, the tolerant mutants that accumulate the ions in organelles are more promising, as compartmentalized ions are not so easily mobilized for export and thus they can be retained within the cell for times long enough to allow various technologic processes. We therefore determined the intracellular distribution of Ni^{2+} ions in mutants *nir4* and *nir5* following the uptake across cell membrane. While both *nir4* and *nir5* showed comparable level of cytosolic Ni^{2+}, both mutant *nir4* and *nir5* exhibited higher vacuolar content than the parental strain (Fig. 3), in good agreement with the overall cellular accumulation (Fig. 2). The absence of cross-contamination between the cytosolic and vacuolar extracts was monitored by assaying the activities of cytosolic enzyme glucose-6-phosphate dehydrogenase and vacuolar enzyme carboxypeptidase Y (data not shown).

Mutants *nir4* and *nir5* can decrease the Ni^{2+} content of their environment

We further wanted to determine whether the mutant cells are capable of taking up enough Ni^{2+} to cause a detectable decrease of its concentration in the growth environment. We found that when adding non-toxic concentrations of Ni^{2+} to a log-phase culture (10^7 cells/mL), an approximate 18% and 21% of the total medium Ni^{2+} was removed by the *nir4* and *nir5* cells respectively. This was detected over a 24 hours incubation time, with a maximum activity in the first 6 hours (Fig. 4). Further increase of culture time did not have any effect upon decreasing the Ni^{2+} concentration in the culture medium (data not shown).

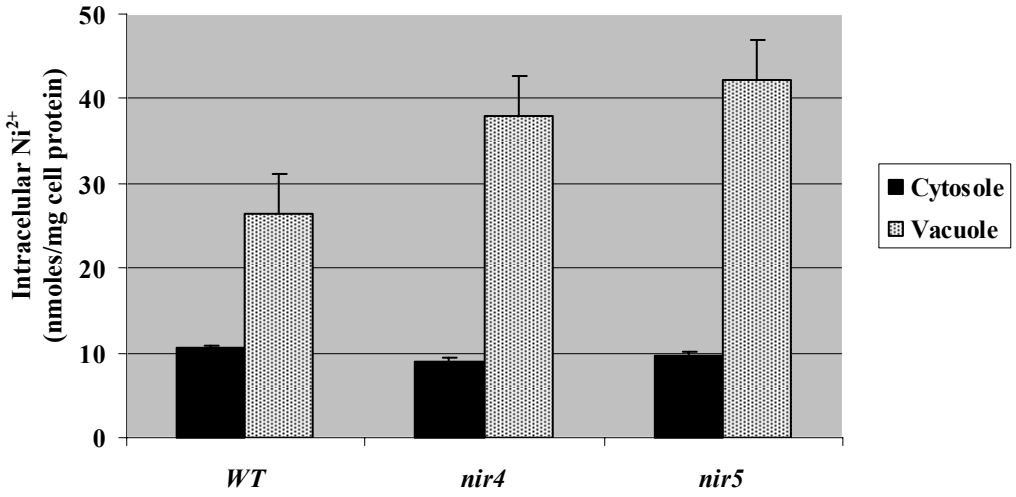

Fig. 3 – Intracellular distribution of Ni^{2+}. Cells were inoculated from an overnight culture into liquid YPD to density 10^6 cells/mL and incubated with shaking at 28°C for two hours before $NiCl_2$ was added (final concentration 2 mM). Aliquots of cells were harvested after 30 min; cytosolic and vacuolar fractions were prepared and Ni^{2+} was determined as described in the *Experimental* section. Data represent triplicate determinations and are expressed as means standard deviations. *WT*, wild type (parental strain); *nir*, isogen Ni^{2+}-resistant strain.

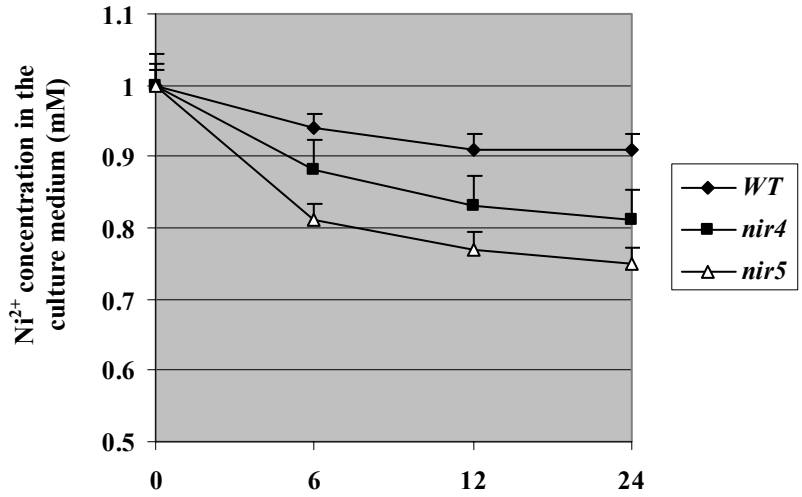

Fig. 4 – Ability of mutant yeast cells to decrease the Ni^{2+} concentration in their environment. Cells were incubated in YPD from a pre-culture and grown up to 10^7 cells/mL before $NiCl_2$ was added (1 mM final concentration). The decrease in Ni^{2+} concentration was monitored spectrophotometrically as described in the *Experimental* section. Data represent triplicate determinations and are expressed as means standard deviations (for clarity reasons, error bars are shown only in one direction). *WT*, wild type (parental strain); *nir*, isogen Ni^{2+}-resistant strain.

DISCUSSION

In recent years, strict environmental regulations compel industries to shift to cleaner production methods, demanding the development of environmental friendly, low-cost and efficient treatment technique for metal rich effluents. Under such circumstances, biotechnological approaches to clean contaminated environments received great deal of attention in the recent years. Growing metal-resistant cells that accumulate heavy metals can ensure better removal through a combination of bioprecipitation, biosorption and continuous metabolic uptake of metals after physical adsorption.

The *Saccharomyces cerevisiae* cells develop an acidic environment (creating in their environment pH 5.5). Thus, the cell walls behave like polyanionic aggregates having a high capacity to reversibly bind metallic ions from their environment. This is

why when suspending *Saccharomyces cerevisiae* cells in a liquid medium that contains metallic ions, the latter will be initially sorbed onto the surface of the cells. Metabolically active cells will then transport the ions across the cell membrane into the cytosol, from where they are either compartmentalized (usually in the vacuole) or excluded back in the environment. Both processes are aimed to protect the cell from the harmful effect of the metallic ions. Engineering cell lines that would hyperaccumulate heavy metals can be an invaluable tool in removing such ions from aqueous environments. Subsequent removal of these cells (by centrifugation, decantation or filtration) would result in partial removal of contaminating ions. Repeating the process would decrease the metal concentration even more. The metallic ions taken up by the cells can be released through cell digestion, followed by metal separation.

In this study, we obtained two *Saccharomyces cerevisiae* lines that are both tolerant to high concentrations of Ni^{2+} and can also accumulate this metal within the vacuoles. Equally important, these lines had the ability to decrease the Ni^{2+} concentration in their growing medium in a single culture cycle, making them good candidates for bioremediation effectors. The possibility to use such cells for decontamination of Ni^{2+}-containing wastewaters is now under investigation.

EXPERIMENTAL

Strains, media, and growth conditions

The *Saccharomyces cerevisiae* strain W303 1A (*MAT***a** *trp1 leu2 ade2 ura3 his3 can1-100*) was used throughout our experiments. Cell growth, manipulation, genetic analysis were done as described.[10] Cells were grown in YPD (yeast extract-polypeptone-dextrose) supplemented with adenine (400 μg/mL) and uracil (200 μg/mL). For solid medium, 2% agar was used. For liquid cultures, overnight pre-cultures were used for inoculation, then cells were incubated with shaking for at least two hours at 28ºC before $NiCl_2$ was added from sterile stocks. The yeast cell manipulation was done on legally based decisions about principles of good laboratory practice.

Selection of Ni^{2+}-resistant mutant cells

The *Saccharomyces cerevisiae* cells were mutagenized by exposure for 10 minutes to non-lethal concentration of the chemical mutagen ethyl methanesulfonate (EMS).[10] Cells were subsequently washed, and spread onto YPD plates containing lethal concentrations of Ni^{2+} (approximately 10^6 cells/plate). Plates were incubated at 28ºC before the first resistant colonies appeared.

Ni^{2+} accumulation

Ni^{2+} loading of cells was done essentially as described by Farcasanu *et al.*[11] Cells grown in media containing various concentrations of $NiCl_2$, were harvested by centrifugation and were washed three times with 10 mM 2-(N-morpholino) ethanesulfonic acid (MES)-Tris buffer, pH6, at 0ºC. All centrifugation (1 min, 5000 rpm) was done at 4ºC. Cells were finally suspended (10^9 cells/mL) and used for Ni^{2+} assay. The Ni^{2+} cellular content was normalized to total cellular proteins.

Differential extraction of Ni^{2+} soluble pools from the cytosol and vacuoles

We used DEAE-dextran to obtain cytosolic extracts, and 60% methanol to obtain vacuolar extracts, as described earlier.[12,13] The absence of cross-contamination between the cytosolic and vacuolar extracts was monitored by assaying the activities of glucose-6-phosphate dehydrogenase (cytosolic marker)[14] and carboxypeptidase Y (vacuolar marker).[15]

Ni^{2+} assay

The amount of Ni^{2+} in biological material was determined using 1-(2-pyridylazo)-naphtol (PAN) assay[16] modified for aqueous solutions. 100 μL solubilized biologic material (whole or partial cell extract) was added to 400 μL PAN solution, and left for two minutes at room temperature for color development. The formation of Ni^{2+}-PAN complex was detected at 560 nm, using a Shimadzu UV-Vis spectrophotometer, model UV mini-1240.

Protein assay

Cellular total protein was assayed using the method described by Bradford.[17]

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