Copper, cobalt and cadmium binded by *Debaryomyces occidentalis* cell wall polymers

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Fijación de cobre, cobalto y cadmio a los polímeros de la pared celular de *Debaryomyces occidentalis*

Resumen. Se estudió la capacidad quelante de los polímeros aislados de la pared celular de *Debaryomyces occidentalis* involucrados en la bioasorción de cadmio, cobre y cobalto, con la finalidad de evaluar la capacidad de las levaduras medioambientales para detoxificar áreas contaminadas. Los componentes de la pared celular aislados acumularon mayores porcentaje de metales pesados que las paredes intactas, lo que muestra que la extracción expuso sitios de fijación iónica. La cantidad de metal secuestrado se decrementó en el orden cobre, cadmio y cobalto, excepto en las muestras con manosa que fijaron mayor proporción de Co. La adsorción de cationes en las levaduras tratadas enzimáticamente mostraron una correlación significativa con la relación proteína: carbohidrato y el rendimiento de pared celular remanente de la degradación enzimática. Un 15% de incremento en la fijación iónica después de la digestión de manosa se debió a al exposición de puntos adicionales de quelación en las proteínas de la fracción manosa-proteína. En conclusión, se observó que la ligación de los metales pesados fue más efectiva en el orden: proteína>manosa>quitina> glucano.

Palabras clave: *Debaryomyces occidentalis*, Adsorción de iones, biorremediación, polímeros pared celular metales pesados.

Abstract. A study was made of *Debaryomyces occidentalis* cell wall polymers involved in the biosorption of cadmium, copper and cobalt in order to evaluate the environmental yeasts ability to detoxify polluted habitats. The cell wall components accumulated more heavy metals than the intact cell wall, indicating that many binding sites were exposed during the extraction procedures. The decreasing order of the metal chelating was copper, cadmium and cobalt, except in the mannan samples where more cobalt than cadmium was fixed. The metal accumulation by enzymatically modified yeast showed a significant correlation with the protein:carbohydrate ratio and the yield of cell wall material remained after enzymatic degradation. A 15% increase in metal fixing after mannan digestion suggested that additional metal binding sites were exposed on the proteins of the mannan-protein fraction. In conclusion, it was observed that the cell wall components binded the metals in the order: protein>mannose>chitin> glucan.

Key words: *Debaryomyces occidentalis*, ion biosorption bioremediation, cell wall components, heavy metals.

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ORIGINAL

Introduction

A major group of toxic elements in ecosystems are the heavy metals and they are usual components of human polluted areas, hence organisms have adopted different strategies to protect against toxicity, such as avoidance and sequestration [16]. Although it is known that heavy metals bind to yeast cell wall, the knowledge of the behaviour of the cell wall components in fixing the ions need further researches [2, 7]. Some biotechnological aspects of the interactions of fungi with metals have been reviewed, being the immobilized systems with filamentous fungi and nonliving biomass recently suggested [14, 17]. However, the use of native yeasts as heavy metal bioaccumulators for wastewater and soil remediation is a significant approach as metal detoxification.

Debaryomyces occidentalis cell wall include polymers such as glucan (28 %, w/w), mannan (31 %), proteins (13 %), lipids (8.5 %) and chitin and chitosan (2.5 %) [1]. The aim of this study was to determine which of the components of *D. occidentalis* cell wall were involved in the sequestration of cadmium, copper and cobalt, and to evaluate the yeast cell ability to detoxify polluted habitats.

Materials and methods

Preparation of isolated cell walls

Yeasts studied were isolated from soil samples of an agricultural area near La Plata, Argentina, and was selected because it was dominant in the sampled soils. The cells were grown aerobically in a molasses wort adjusted with phosphoric acid and harvested in the stationary phase. Yeast cells were disrupted by an homogenizer with 0.5 mm diam. glass beads at 4 ºC, using ice and liquid nitrogen to minimize heat buildup and enzymatic autolysis.

Freeze-dried cell walls were resuspended in 5 mmol 1,4-piperazinediethanesulfonic acid (PIPES) / l buffer and centrifuged at 4 ºC, 3000 g for 10 min, then the supernatant was discarded. The pellet was freeze-dried and stored under dessicated conditions until used.

Cell wall component extraction

Mannan and glucan were extracted from 150 mg freeze-dried cell walls under nitrogen, the extracts were dialyzed twice at 4 ºC against 1 l distilled water, and freeze-dried again. Chitin and chitosan were obtained from 100 mg freeze-dried yeast walls [10]; the obtained pellet was dialyzed and freeze-dried.

Two 1.2 ml chambers clamped together bisected by a semipermeable membrane (Spectrapor dialysis tubing, 6 to 8 kDa cutoff) made up the dialysis appparatus. Comparative metal binding studies of the cell wall components were realized at 22 ºC, in a shaking water-bath with 2 perpex balls in each chamber to agitate the solution.

One mililiter of 4 mmol / l of each heavy metal solutions (cadmium chloride, copper chloride and cobalt chloride) was pipetted into one chamber, whereas 1 mg cell wall material suspended in 1 ml PIPES-tetramethylammonium hydroxide (TMAH) buffer was pipettted into the opposite ones. After 4 h, equilibrium was reached and a 0.1 ml subsample was removed from each compartment, to obtaine the free metal amounts using flame atomic absorption spectroscopy. The Cd^{2+}, Cu^{2+} and Co^{2+} bounded by the cell walls were then determined by subtraction the free cation amounts from the total ones.

Enzymatic modification of yeast cell walls

Enzymatic digested cell walls were centrifugated with distilled water (3000 g for 10 min) and assayed for cross-specificity. Protease activity was determined using the Folin-Lowry reaction to quantify free peptides and aromatic amino acids [9]. Chitinase activity was obtained using chitin dyed Remazol Brilliant 5R [6] and -mannosidase activity by Li (1967, [8]). The enzymes were obtained from Sigma Co. and dialyzed against distilled water, then the digested reactions were performed by shaking for 6 h.

Pronase (150 units), the multiple exo- and endopeptidases with a broad specificity for peptide sequences was used to digest 50 mg dry mass of freeze-dried cell walls at 37 ºC in 0.05 mol / l Tris buffer (pH 7.5). Dried cell walls (50 mg) were digested with 1 unit chitinase at 25 ºC in PIPES buffer (0.1 mol/l, pH 6.1 adjusted with TMAH); with 2.5 units laminarinase at 37 ºC, pH 5.0 in 0.1 mol/l acetate buffer, and with 50 units mannosidase at 25 ºC, pH 4.5 in 0.1 mol / l acetate buffer. -Glucuronidase, 13.9 x 10^3 units, digested 50 mg cell wall at 37 ºC in 0.1 mol / l sodium acetate buffer (pH 5).

Heavy metals binding assay

The metal binding capacity of the enzymatically digested cell walls was determined by atomic absorption spectrophotometry. PIPES (0.1 mol / l, pH 6.2 adjusted with TMAH), a hydrogen ion buffer that does not chelate cations was used for binding studies.

Cell wall protein and carbohydrate content

Samples of the cell wall components were hydrolyzed before chemical analysis. Chitin, 1 mg, was digested in 1 ml 4 N hydrochloric acid at 100 ºC for 4 h, whereas all other samples were hydrolyzed for 6 h at 100 ºC with 2 N hydrochloric acid. Mannan was dialyzed against 0.1 mol / l ethylenediaminetetraacetic acid (EDTA) in distilled water and then treated to remove complexed metal prior to acid hydrolysis. The Folin-Lowry assay was used to determine protein contents [9] and Nelson's test [3] for reduced sugars was employed to determine the carbohydrate amount in the digested cell wall.

Infrared analysis of the isolated cell walls, its components and enzymatically digested cell wall was conducted in a Perkin-Elmer model 180 infrared spectrophotometer, by mixing 1 mg freeze-dried sample with potassium bromide and then pressing into solid-state disks.

Statistical analysis

Data were subjected to analysis of variance and linear regression by ANOVA [18] and the significance levels were set at $P = 0.01$.

Results and discussion

The assays were performed with *Debaryomyces occidentalis* (van der Walt) Kurtzman & Robnett var. *persoonii*. The cell wall components were extracted by alkali and acid digestion or by enzymatic treatments to yield purified products and the efficiency of these procedures were obtained by their infrared analysis.

The cell wall components, glucan, mannan and chitin, accumulated greater quantities of copper, cobalt and cadmium than the intact cell wall, indicating that other unavailable binding sites were exposed during the extraction procedures. More copper than cadmium than cobalt was chelated by the polymers, except in the mannan samples where slightly more cobalt than cadmium was fixed. Of the three carbohydrates extracted, glucan and chitin accumulated twice and three-fold more ions, respectively, than the intact cell walls; moreover, mannans sequestrated the greatest quantity of cations, particularly copper (Fig. 1).

Infrared analysis of the *D. occidentalis* intact cell walls and its components yielded distinct patterns. The peaks at 1650 cm^{-1} (C = O, amide I) and 1550 cm^{-1} (N - H, amide II) represented the protein fraction, and at 1380 cm^{-1} the strong O-H stretch peak was detected. Between 1000 and 1100 cm^{-1}, the C-O stretch peak was observed, which represented the carbohydrate components. Compared to the isolated cell walls, the extracted components contained less amides showing a decrease in protein conten and therefore partial purification of the carbohydrate fractions.

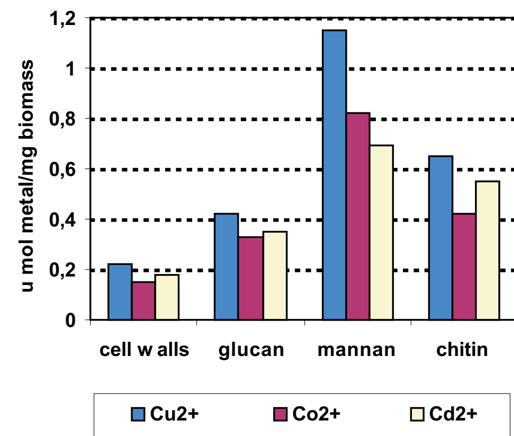

Figure 1. Heavy metal binding by *D. occidentalis* cell walls and by the extracted cell wall components

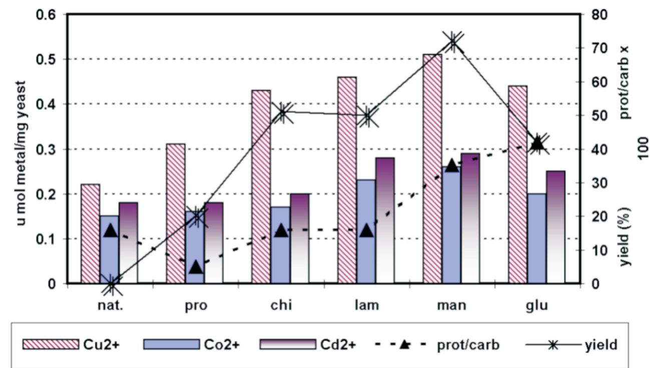

Figure 2. Yield (%, cell wall mass remained after enzymatic degradation), protein: carbohydrate ration (x 100) and metal binded amounts (u mol/mg yeast) by enzymatically modified cell walls (nat: without treatments; pro: pronase; chi: chitinase; lam: laminarinase; man: mannosidase; glu: glucuronidase treatment)

Infrared analysis of the enzymatically modified cell walls was different to the intact ones. Whereas protease treated walls diminished the amide peaks, as would be expected by the protein removal, mannosidase treatment decreased the C-O ones, in concordance with the -glucuronidase assay; and both showed different carbohydrate removal degrees.

The binded metal by cell walls partially degraded by enzymes showed a significant correlation with the protein: carbohydrate ratio ($P > 0.01$), although the preparations with the lower values decreased the copper, cadmium and cobalt adsorption. Whereas most of the proteins were removed from the cell wall, the least cation fix occured, and a 29.5 % decrease was obtained compared to the intact cell wall. This was also the cell wall preparation with the lowest yield of product after enzymatic degradation (Fig. 2).

A good correlation between metal accumulation and the yield of cell wall material remaining after enzymatic degradation was also observed ($P > 0.01$). A 15 % increase in copper fixing by the cell wall after mannan digestion, suggested that additional metal binding sites were exposed on the protein of the mannan-protein fraction. Glucan removal by laminarinase and -glucuronidase did not modify the adsorption capacity of cell walls for Cu^{2+}, although a slight increase was observed after laminarinase digestion.

Enzymatic degradation of *D. occidentalis* indicated a relationship between protein content and metal binding potential, whereas selective removal of mannan increased the three metal sequestration, due to the exposure fixing sites on the protein fraction. This data suggested that proteins were important binding components and basing in the amino acid composition, they were regarded as polyanionic ones. The *D. occidentalis* proteins had a high sulfur-to-protein ratio [13] probably due to cystine content or reduced residues that could be involved in the Cd^{2+}, Cu^{2+} and Co^{2+} biosorption. Bovine serum albumin accumulated 0.054 mol Cd^{2+}/ mg protein [11] and also a linear relationship between the absorbed metal within a cell and the cell protein content had been found [5].

Other researchers remarked the relevance of the outer layer in cation binding [4] and the removal of mannan-protein layer with its phosphodiester links by proteases decreased the ion fixing capacity by 50 % [17]. By complexing with copper, the mannan could be selectively precipitated out of the alkali solution [12], indicating that mannans were effective metal chelating polymers.In this study, mannans fixed up 0.046 mol / mg Cd^{2+} that was associated with protein rather than to carbohydrate content, because protease digestion greatly decreased cadmium chelation. Moreover, phosphate groups were associated with the extracted mannan, that explained the high level metal binding of this component.

Heavy metal chelating by yeasts were only realized with *Saccharomyces cerevisiae* strains [2], *Candida* spp. [19] and *Schizosaccharomyces pombe* [15], but in this study was assessed with a *D. occidentalis* strain isolated from agricultural soils without previous metal treatments.

Even more, this research confirmed that the yeast cell wall components binded cadmium, copper and cobalt, in the decreased order: protein mannose chitin glucan. Similar to the Co^{2+} adsorption by *Candida utilis* and *Torulopsis famata* [4], *D. occidentalis* binded the tested metals in relation to the cell wall components. Moreover, cation chelating analysis by cell wall polymers indicated that mannans were the most efficient accumulators of heavy metals compared to the other carbohydrates.

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