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Use of *Saccharomyces cerevisiae* for Cu^{2+} removal from solution: the advantages of using a flocculent strain

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Abstract

A flocculent strain of *Saccharomyces cerevisiae* S646-1B accumulated more Cu^{2+} (81 nmol mg^{-1} dry wt) than the isogenic (except for the marker genes *ade1* and *trp1* and the gene *FLO1*) non-flocculent strain S646-8D (30 nmol mg^{-1} dry wt), in the first 10 min of contact of the cells with Cu^{2+}. Additionally, this strain flocculated in solutions of 0.2 mM Cu^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+}. The potential of using flocculent strains in the bioremediation of heavy metals contaminated waste waters is discussed.

Introduction

The bioremediation of waters contaminated with heavy metal ions has received a great deal of attention in recent years as an alternative technology to classical physico-chemical methods (Gadd 1990, Volesky & Holan 1995). Different groups of microorganisms (algae, fungi and bacteria) have been used to remove many heavy metal ions (Volesky & Holan 1995).

One limitation in the industrial application of microbial biomass for remediation technologies is linked with their small size and low density, which can limit the choice of suitable reactors and make it difficult to separate biomass from treated effluent. To overcome this problem, different immobilization techniques have been proposed: gel immobilization using polyacrylamide, calcium alginate, agar or cellulose-acetate have been used (Cassidy *et al.* 1996). However, immobilization techniques have some disadvantages: unsuitable at high pH and the cell viability and metal accumulation properties of the biomass decrease with time (Cassidy *et al.* 1996). Gel immobilization on a large scale can, also, be prohibitively expensive and resistance to diffusion within the gel is a major problem; thus, problems of immobilization have rendered large-scale engineering of bioaccumulation systems impractical.

A practical alternative may be to use flocculating yeasts. Several brewing yeasts have the ability to interact between them and flocculate (Stratford 1992, Soares & Seynaeve 2000); this property is under genetic control and several *FLO* genes and their suppressors have been described (Stratford 1992). Although, several researcher have studied the use of brewer's yeast (Volesky *et al.* 1993, Volesky & May-Philips 1995, Simmons & Singleton 1996, Blackwell & Tobin 1999) including flocculent ones (Avery & Tobin 1992, Ferraz & Teixeira 1999, Marques *et al.* 1999) in metals removing, the potentialities of flocculating characteristics have only been partially considered. Furthermore, different strains, grown in different cultural conditions, have been used which hinders a true comparison.

In this work, the ability of a flocculent and a non-flocculent yeast strain to accumulate Cu^{2+} was compared; the ability of the flocculent strain to settle in the presence of different heavy metals was also evaluated. The advantages of using flocculent strains in the bioremediation of heavy metals are discussed.

Material and methods

Strains, medium and culture conditions

Two isogenic strains of *Saccharomyces cerevisiae* were used in this work. They were S646-8D *(MAT* **a**/α, *HO/HO, trp1/trp1)* and S646-1B *(MAT* **a**/α, *HO/HO*, *FLO1/FLO1*, *ade1/ade1*), a non-flocculent and a flocculent strain, respectively (Miki *et al.* 1982). Strains were gifts from Dr Malcolm Stratford of Unilever Research, Colworth Laboratory, UK.

The pre-cultures were prepared in 40 ml YEPD broth (10 g yeast extract l^{-1}, 20 g peptone l^{-1}, 20 g glucose l^{-1}) in 100 ml Erlenmeyer flasks. The cells were incubated at 28 °C and shaken at 150 rpm for 24 h. This culture was inoculated at 4% (v/v) into YEPD broth, in 1-l Erlenmeyer flasks. Cultures were incubated in the same conditions of the pre-culture for 19 h (flocculent strain) or 22 h (non-flocculent strain), which corresponds to the end of the arithmetic respiro-fermentative growth phase/beginning of diauxic lag phase (data not shown).

Preparation of cell suspensions

After growth, cells were removed by centrifugation (2000 × *g*, for 5 min), washed twice with 30 mM EDTA, once with deionised water and then with MES pH buffer, 10 mM, at pH 6. The concentration of cell suspensions was adjusted by dilution with MES buffer. MES is a suitable pH buffer for heavy metal uptake studies, since it does not complex several metal ions (Soares *et al.* 1999a,b), and does not provoke physiological disturbs in yeast cells (Soares *et al.* 2000). Cell concentration was determined spectrophotometrically, at 600 nm, after dilution of the samples in 100 mM EDTA. Calibration curves (absorbance versus number of cells or versus dry wt), were previously performed.

Uptake of Cu^{2+}

Cell suspensions (200 ml) with a concentration near 0.4 mg (dry wt) ml^{-1} were shaken in 500 ml plastic flasks at 150 rpm, at 28 °C and an appropriate volume of a metal salt solution (from a stock solution of 1000 mg metal ion l^{-1}) was added. Before and, at defined intervals of time, after adding the Cu^{2+}, samples were taken and filtered through a 0.45 μm-pore size filter. Filters were washed immediately with MES buffer and dried at 60 °C. At each filter, 65% HNO_3 (analytical grade) was added and gently boiled until digestion was completed. Samples were diluted with ultra-pure water and filtered through 0.45 μm pore filters. The filtrates were analysed for metal content by Atomic Absorption Spectroscopy with flame atomisation.

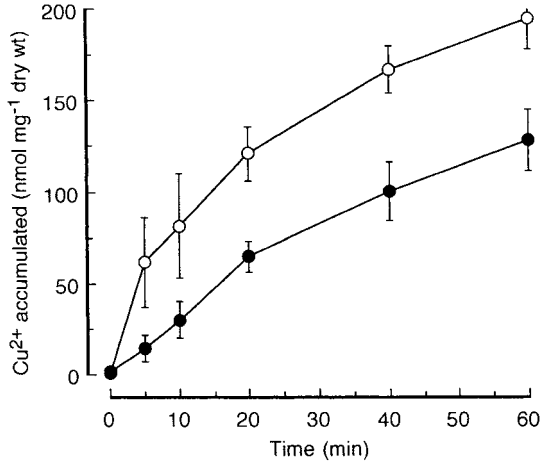

Fig. 1. Accumulation of Cu^{2+} by *S. cerevisiae* flocculent strain S646-1B (○) and non-flocculent strain S646-8D (●). Cells were suspended in 10 mM MES pH buffer, at pH 6, in a final concentration near 0.4 mg ml^{-1}. The initial metal concentration was 0.2 mM. Each point represents the mean of two independent experiments performed in duplicate; standard deviation are presented ($n = 4$).

Measurement of flocculation

Cell suspensions were placed in 100 ml Erlenmeyer flasks, in MES pH buffer (10 mM, pH 6), containing 0.2 mM of each cations tested. The final volume of the suspension was 40 ml with 1×10^8 cells ml^{-1}. Flocculation was initiated by the agitation of the flasks at 120 rpm. After agitation, the flasks were allowed to stand undisturbed during 30 sec, after which, samples were taken from just below the meniscus, dispersed in 100 mM EDTA solution and free-cell concentrations were determined spectrophotometrically, as described above.

Results and discussion

The kinetics of Cu^{2+} accumulation by the flocculent and non-flocculent strains are depicted in Figure 1. After 60 min of contact of the yeast with the metal, the flocculent strain accumulated about 195 nmol mg^{-1} (dry wt), while the non-flocculent strain only 128 nmol mg^{-1} (dry wt). Typically, the yeast metal uptake occurs in two steps: the first-step (initial biosorption), is rapid (in the first 10 min of contact with metal), as can be seen in Figure 1, is metabolism independent (Blackwell *et al.* 1995) and attributed to the interactions of yeast cell surface with the metal. This phenomenon can be due to one or more of the following mechanisms: adsorption, complexation, precipitation and crystallization with the multilaminate, microfibrillar of yeast cell wall structure (Gadd 1990). The second step, designated bioaccumulation, is usually considered metabolism-dependent and classically attributed to the intracellular metal uptake across the cell membrane (Gadd 1990, Blackwell *et al.* 1995). However, the increase in metal accumulated observed in both strains after 10 min of contact of cells with the metal, is unlikely that can be due to a energy-dependent uptake, since it was observed a severe lethality and increase in yeast cell membrane permeability, probably as a consequence of lesions of yeast cell membrane, after 10 min of contact of the yeast cells with 0.2 mM Cu^{2+} (EV Soares, K Hebbelinck & HMVM Soares, unpublished results); the increase in yeast Cu^{2+} accumulation is probably due to the exposition of further metal-binding sites inside the cell, as it was reported in other works where, similarly, the integrity of plasma membrane was destroyed by the action of detergents and an enhance in yeast metal uptake was observed (Gadd 1990).

According to the lectin-like mechanism, flocculation occurs as the result of the interaction between specific proteins (lectins), present only on flocculent cells, with the carbohydrate residues (receptors) of the walls of neighbouring cells; in this process Ca^{2+} seems to ensure the correct conformation of the lectins (Miki *et al.* 1982). Cell walls of flocculent strains are able to fix more Ca^{2+} than non-flocculent strains (Stewart & Russell 1981); it is likely that heavy metals can also occupy lectin Ca^{2+} binding sites with a consequent increase in heavy metals biosorption, since the cell walls of flocculent cells may provide additional metal-binding sites than the non-flocculent cells. In fact, a significant increase in the metal accumulation ability of flocculent cells was observed in the first

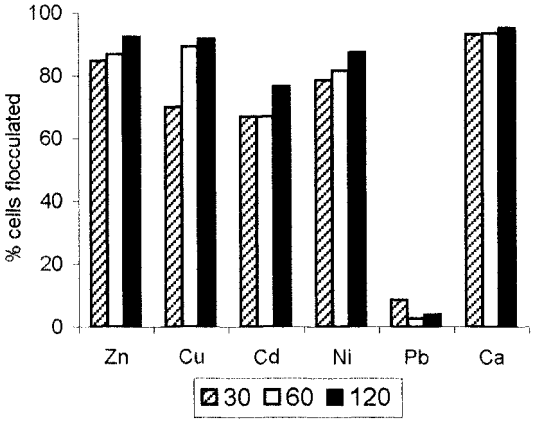

Fig. 2. Time course of flocculation of *S. cerevisiae* S646-1B in the presence of different cations. 1×10^8 cells ml^{-1} were suspended in MES buffer (10 mM, pH 6), containing 0.2 mM of different cations. Suspensions were agitated at 120 rpm, before flocculation was measured at a given time represented (30, 60 and 120 min). Bars are means of duplicate readings. This is a typical example of an experiment performed two times.

10 min of contact of the cells with the Cu^{2+} (Figure 1). The difference in Cu^{2+} binding capacity between the flocculent (81 nmol mg^{-1} dry wt) and non-flocculent cell walls (30 nmol mg^{-1} dry wt) can probably be due to the presence of the characteristics surface proteins of flocculating yeasts. In this work, differences between the flocculent and non-flocculent strains, as well as differences in the physiological state of the cells (namely different cultural age) (Volesky & May-Philips 1995, Simmons & Singleton 1996), which can be responsible for the differences of yeast metal uptake, was excluded since the strains are isogenic [except for the marker genes *ade1* and *trp1* and the gene *FLO1*, which controls flocculation (Miki *et al.* 1982)] and the growth of both strains was stopped in the same physiological phase.

The *S. cerevisiae* S646-1B strain has the ability to flocculate in the presence of Zn^{2+}, Cu^{2+}, Cd^{2+} and Ni^{2+}, while remained dispersed in the presence of Pb^{2+} (Figure 2); in the case of Zn^{2+}, Cu^{2+} and Ni^{2}, the strain flocculated practically with the same percentage than in the presence of Ca^{2+} (control ion). The kinetics of flocculation is rapid, being more than 80% of cells aggregated after 5 min of contact with Ca^{2+} (data not shown).

It is a debatable question if flocculation is exclusively promoted by Ca^{2+} ions (Stratford 1992) or if other cations are able to induce the correct conformation of the flocculation lectins (Sousa *et al.* 1992); however, for practical purposes, yeast cells flocculate

in solutions with different metal ions (Figure 2) and most likely in wastewaters loaded with heavy metals, being a natural and inexpensive process of cell separation after the effluent has been treated. It means that flocculent cells can be used in several configurations of suspended biomass reactors, without the risk of washout of the biomass; additionally, the exploitation of sedimentation characteristics of flocculating yeasts presents several advantages such as design simplicity and energy coasts (Teixeira *et al.* 1990). It is also important to point out that flocculent yeasts retain their sedimentation properties after a heat killed process, which means that they can be used as living or dead cells in the removal of heavy metals.

From the results obtained in this work, it can be concluded that flocculent cells can be used with advantages in the bioremediation of effluents loaded with heavy metals, since they can be used without the necessity of cell immobilization and, additionally, the walls of flocculent cells have a higher ability to remove Cu^{2+} from solution than non-flocculent cells. Using isogenic and genetically defined strains of yeast (flocculent and non-flocculent), we begin a comprehensive examination of using flocculent cells in the removal of heavy metals. Future work should be carried out to confirm the higher ability of flocculent cells to remove other heavy metals.

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