Desalination and Water Treatment
www.deswater.com
doi: 10.1080/19443994.2014.987177

57 (2016) 3957–3963
February

Taylor & Francis
Taylor & Francis Group

Significance of age, temperature, and aeration of yeast cell culture for the biosorption of europium from aquatic systems

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Received 18 May 2014; Accepted 7 November 2014

ABSTRACT

The Eu(III) uptake from aqueous solutions by *Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, and *Debaromyces hansenii* was studied as a function of the growth temperature, supply of air flow during the cultivation process, and the age of cells. Our results revealed that exponential phase cells and the optimum temperature of growth resulted in a higher metal uptake, while aeration did not have any significant effect on the uptake. These traits make the proposed bisorbents desirable for clean-up processes due to easy access and low cost, as well as low time- and low energy-consuming optimal conditions as specified in this study. The reason why younger cells grown close to the optimal temperature show higher metal removal capacity is related to qualitative and quantitative alterations of their membranes' content in fatty acids and carboxyl groups mainly.

Keywords: Biosorption; Europium; *Saccharomyces cerevisiae; Kluyveromyces marxianus; Debaromyces hansenii;* Growth conditions

1. Introduction

The aquatic waste produced by nuclear industry contains a wide range of toxic pollutants, including long-lived radiotoxic trivalent actinides such as Am^{3+} [1], and therefore their treatment and consequent safe disposal are of major importance. Europium and neodymium have been used as homologs of trivalent actinide elements, due to their similar physicochemical properties, in studies concerning the environmental behavior of the trivalent actinides [2]. Furthermore, considering the high cost of rare earth elements and the rapid growth of their applications such as in automotive catalytic converters, glass polishing and ceramics, petroleum refining, and electronics, studies of Eu (III) retention also aim at metal recovery for financial reasons [3,4].

Conventional physicochemical treatment methods (e.g. oxidation, precipitation, ion-exchange, reverse osmosis, etc.) exhibit serious drawbacks such as high operation cost, toxic sludge formation, and high requirement of reagents, just to name a few [5]. Biotechnological approaches can cover those niches. Biosorption is a collective term used for the uptake of pollutants by materials of biological origin through a wide array of mechanisms that act synergistically. Microbial masses can influence the mobility of dissolved metal ions by various microbiological processes, which result in reduction/oxidation of multivalent ions, passive sequestration (binding or precipitation) of metal ions on their surfaces. In biomass cell walls, a wide array of functional groups,

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such as hydroxyl, carboxylates, phosphates, and amino-groups are responsible for binding metal ions [6]. Exploitation of this phenomenon suggests the development of microbe-based remediation strategies to clean up metal-bearing waste streams and groundwaters and a potential use of micro-organisms, either in the treatment of radionuclides for reasons related to radioactive waste management or in the recovery of precious entities.

In most of the studies dealing with the interaction of micro-organisms with heavy metals or radionuclides, the parameters usually investigated were the uptake dependence on the metal concentration and the temperature, the pH of the solution and thermodynamic and kinetic data were obtained in order to elucidate the mechanism of metal uptake. But microorganisms are living organisms and the concentrations of their cell wall functional groups are not fixed depending on their growth physiology and metabolic state, as well as the growth state [7,8]. Hence, their surface characteristics can vary with their age as well as the temperature and the air inlet during cultivation. Up to now, such biotic or environmental variables, affecting metal uptake by yeast cells, have not been studied adequately. The objective of this study was to explore the possible dependence of Eu(III) uptake by common yeasts on biotic and environmental parameters. *Saccharomyces cerevisiae*, *Kluyveromyces marxianus,* and *Debaromyces hansenii* are yeasts involved in various food industries, such as breweries, wineries, bakeries, and industries of dairy products [9,10]. The use of waste biomass in remediation processes could increase economic competitiveness, since these microorganisms are cheap and easily accessible in large quantities. It is noted that despite *S. cerevisiae* being mentioned in literature for its retentive capacities, on the other hand, little is known about the biosorptive capacities of *K. marxianus* and *D. hansenii*.

2. Experimental part

2.1. Biomass preparation

Commercial strains of *K. marxianus* and *D. hansenii* were purchased from Chr. Hansen A/S, Denmark, under the code names LAF-4 and LAF-3, respectively, while *S. cerevisiae* cells were grown from a commercial product of baker's yeast ("L' hirondelle") purchased from a local market. All yeasts were cultivated, under gentle stirring, in the same sterilized nutrient medium (temperature = 121°C, pressure = 1.1 atm for 15 min in autoclave), consisting of 40 g L^{-1} glucose, 4 g L^{-1} yeast extract (Merck), 1 g L^{-1} KH_2PO_4, 1 g L^{-1} $(NH_4)_2SO_4$, and 10 g L^{-1} $MgSO_4{\cdot}7H_2O$, with or without bubbling sterile air (aerobic or anaerobic growth, respectively) at 30 and 15°C. All the reagents used were of analytical grade, while the sterile air was produced by passing first through a filter (Millex-FG, 0.2 μm, PTFE, Millipore).

The growth of each micro-organism was monitored by measuring periodically the optical density of small volume samples withdrawn from the cell culture at 700 nm, against sterilized nutrient medium, which served as reagent blank. The values of optical density measured were correlated with the concentrations of cells, in terms of dry weight of cells per liter of suspension (g L^{-1}) by appropriate calibration curves. These curves had been obtained by measuring the optical density of lyophilized cell suspensions of known concentrations, which had just been prepared and homogenized in the sterile nutrient medium. It is known that during the progress of a cultivation process, just after the initial lag phase, the exponential or logarithmic phase of growth follows, which is characterized by an abrupt growth rate, followed by the stationary phase, which is characterized by a constant cell number. Through the plot of biomass concentration against time for each one of the yeasts studied, the exponential and the beginning of stationary phase were determined accurately. Based on these kinetic data of incubation, independent cell populations for each micro-organism were isolated from the nutrient medium by centrifugation (4,000 rpm, 15–20 min), at three different time intervals:

(1) approximately, in the middle of the exponential phase, denoted as exponential phase;
(2) at the beginning of the stationary phase, denoted as early stationary phase, and;
(3) after a time interval, about 2–4 times longer than that determined as early stationary phase, which was denoted as late stationary phase.

After the planned period of growth, the isolated biomass was washed twice with deionized–sterilized water (18 MΩ) via sequential resuspensions–centrifugations, to remove the excess of nutrient medium. Then, the biomass was lyophilized and finally was stored at −20°C.

2.2. Europium solutions

A stock europium solution was prepared by dissolving the appropriate amount of $Eu(NO_3)_3{\cdot}6H_2O$ (Riedel-deHaën, catalog number 14873) in acidified triply distilled water. Three samples of this solution were titrated with a standard EDTA solution (Merck,

Titrisol), using xylenol orange as an indicator, after the addition of few hundred milligrams of hexamine. The end point of the titration was determined by the sharp change in color from intensive red to lemon-yellow. The metal concentration of the stock solution was determined as the mean concentration estimated from the titrations. Working solutions were prepared fresh every day, by diluting appropriate portions of the stock solution with triply distilled water.

2.3. Sorption experiments

Batch sorption experiments were carried out in polypropylene vials, by suspending, 0.05 g of lyophilized cells, in 10 mL of europium solution with total concentration 100 mg/L at pH 4. Experiments carried out in higher pH values (e.g. pH 5) would yield higher metal removal, as the metal binding increases with pH [3]. The solution pH to be studied should reflect the nature of aqueous streams that biosorption is applied upon; e.g. the pH of mining effluents is usually 4 or lower [11]. Generally, experiments at pH values higher than 5.5 should be avoided due to metal precipitation as europium hydroxide, as predicted by Hydra-Medusa speciation software (Fig. 1).

The pH of the solutions was adjusted using dilute HNO_3 and NaOH solutions. The biomass and the sorbate were agitated for 24 h, at room temperature and then the supernatant was isolated by centrifugation at 4,000 rpm for 15 min. At the end of agitation time, the suspension was centrifuged and the residual (final) metal concentration in the supernatant was determined spectrophotometrically, by the Arsenazo III method [12]. The results were expressed, as percentage of metal uptake, using the following equation:

$$\text{Metal uptake } (\%) = \frac{(C_i - C_f)}{C_i} \times 100$$

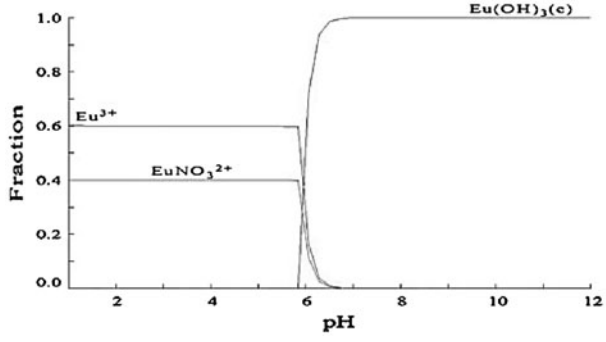

Fig. 1. Europium speciation obtained by means of Hydra-Medusa speciation software for the conditions studied.

where C_i and C_f stand for the initial and final europium concentrations, respectively. For each experiment, the mean value of uptake was calculated from a set of three vials, which were treated simultaneously, under identical conditions.

3. Results and discussion

Typical growth curves for *K. marxianus* under different conditions of temperature and air flow are shown in Fig. 1. The growth curves of *S. cerevisiae* and *D. hansenii* at 30 and 15°C, under aerobic and anaerobic conditions (not shown), revealed similar behavior with those in Fig. 2. When the growth temperature of a micro-organism is reduced, the initial lag phase extends and the growth rate decreases.

The amounts of Eu(III) uptake by cells isolated from different growth phases and different growth conditions for the three yeasts studied, were compiled in Table 1.

From Table 1, it is clear that:

(1) The growth phase of the biomass affects the Eu(III) uptake quantitatively, as there was a statistically significant difference ($p < 0.05$; Student's t test) between metal uptake of exponential and stationary phase cells.
(2) The maximum uptake was obtained when the biomass had been harvested during the exponential phase, either at 30°C, that is the optimum growth temperature of yeasts [13] or even at 15°C.
(3) Equivalent values were obtained from aerobic and anaerobic conditions at the same temperature.
(4) The mean values differed significantly when the biomass was cultivated at 30 and 15°C (except the case of *S. cerevisiae* late stationary phase cells).

The ability of younger cells to sequester larger amount of metal, such as heavy metal uptake by *Zoogloea ramigera* [14], by bacterium *Thiothrix* Strain A1 [15], and by *S. cerevisiae* [16] has been reported by few research studies. Nevertheless, the authors of the above-mentioned reports were not able to document the effect of growth phase, due to the lack of kinetics of growth. The effect of growth phase is likely related to changes in the composition of the cell wall. This composition, in turn, depends on the composition of the nutrient medium, which apparently, does not remain constant. As the population grows up, the ingredients of the growth medium are consumed, their concentrations are reduced and the exponential

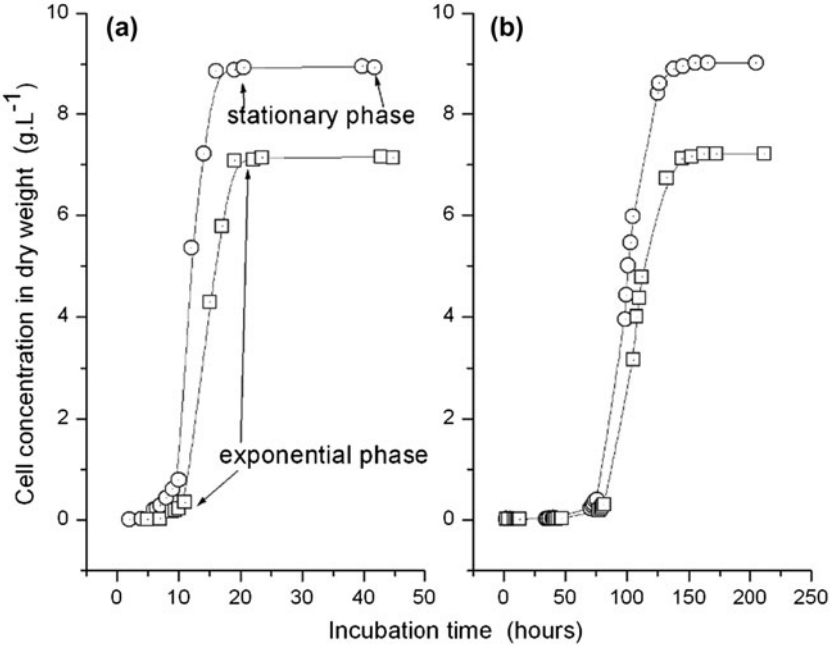

Fig. 2. The growth curves of *K. marxianus* in terms of cell concentration vs. incubation time under aerobic (circles) and anaerobic conditions (boxes) at 30°C (a) and at 15°C (b). The values shown on each growth curve are averaged from two independent cultures grown simultaneously, under identical conditions.

Table 1
Uptake of Eu(III) as a function of growth conditions for the three yeasts studied ± standard deviation of triplicate experiments

		Uptake of Eu^{3+} (%)			
		Incubation at 30°C (optimum temperature)		Incubation at 15°C (below optimum temperature)	
Type of yeast	Growth phase	Aerobic	Anaerobic	Aerobic	Anaerobic
D. hansenii	Exponential	37 ± 2^a	35 ± 2^a	28 ± 2^b	28 ± 1^b
	Early stationary	25 ± 3^b	26 ± 3^b	15 ± 4^c	14 ± 4^c
	Late stationary	23 ± 2^b	22 ± 2^b	17 ± 4^c	18 ± 3^c
K. marxianus	Exponential	36 ± 1^a	37 ± 3^a	28 ± 3^b	27 ± 3^b
	Early stationary	30 ± 2^b	29 ± 2^b	19 ± 3^c	16 ± 3^c
	Late stationary	25 ± 2^b	25 ± 1^b	14 ± 3^c	15 ± 2^c
S. cerevisiae	Exponential	33 ± 3^a	34 ± 4^a	25 ± 2^b	24 ± 2^b
	Early stationary	25 ± 1^b	24 ± 2^b	16 ± 1^c	15 ± 2^c
	Late stationary	16 ± 1^c	14 ± 2^c	12 ± 2^c	11 ± 2^c

The same superscripts (a, b, c) denote equivalent values statistically (significance level 0.05).

phase cells enter stationary phase. The abundance of nutrients available to cells in the exponential phase, allows them to develop cell walls with high concentration of suitable binding sites. However, as the concentration of nutrients decreases and the population moves to the stationary phase, cell walls with lower densities of favorable binding sites are formed [7]. Macaskie and Dean [17], Chang et al. [18], and Daughney et al. [7] report higher heavy metal uptake by *Citrobacter* sp., *Pseudomonas aeruginosa*, and *Bacillus subtilis*, respectively, when cells were harvested at exponential phase. In contrast, Friis and Myers-Keith [19] and Andrès et al. [8] have found that early stationary phase cells of *Streptomyces longwoodensis*

and *P. aeruginosa* respectively, remove higher amounts of U(VI). Therefore, it seems that the effect of growth phase/age of cells seems to be metal- and speciesspecific and this is a factor that needs to be taken into consideration, especially when designing recovery systems for high-value metals and radionuclides.

On the other hand, the reduced metal capacity of micro-organisms grown at temperature below optimal is associated with possible changes of their membrane, mainly fatty acids. Beales [20] and Russell et al. [21] have reported that the cell membrane of both bacteria and yeasts contain higher proportion of unsaturated fatty acids as the growth temperature decreases. Furthermore, a number of less reported alterations can occur such as, shortening in the mean fatty acid chain length, an increase in the amount of branch fatty acids, and a reduction in the proportion of cyclic fatty acids [20–22], all related to fatty acids. The decrease in the carbon chain of membrane acids may result in a slight decrease in the deprotonation constant (pK_a) of the corresponding functional group (carboxyl group) as this is the general trend comparing the pK_a values of short-chain aliphatic carboxyl acids, e.g. ethanoic–methanoic acid and propionic–propenoic acid. Carboxyl groups, associated with cell wall surface components, are considered to be among the main groups of cell membranes responsible for metal binding [23]. In metal uptake studies, slightly acidic or neutral solutions are generally used and therefore a decrease in p*K* of some surface functional groups will increase the membrane's negative charge density, because of the deprotonation of some binding sites and consequently, sorption of cationic species is favored.

Our results from Table 1 indicate clearly that yeast cells grown under aerobic or anaerobic conditions exhibited approximately the same metal capacities and consequently, the air (O_2+N_2) and the additional agitation provided by the air stream, do not seem to have any significant effect on Eu(III) uptake by the studied yeasts.

Another important trait of micro-organisms' surface is the presence of phosphates. Phosphate moieties are present on the membrane of micro-organisms in the form of phospholipids [24] and are able to bind with metals. Several studies have shown the high affinity of phosphate groups present in micro-organisms with various metallic cations, such as trivalent lanthanides [3], uranium [25], Pb [26], and Cd [27]. Despite the establishment of phosphate groups as a major contributor in metal sequestration, it is not clear if phosphate concentration is affected by the morphological alterations that take place due to cell age or growth temperature.

Lanthanide, and more specifically europium, uptake reported in literature varies, depending on the biomass and the conditions sorption was studied. Diniz and Voleksy [3] report 0.8–0.9 mmol Eu g^{-1} of *Sargassum* biomass for pH range 3–5, while Cadogan et al. [28] report maximum monolayer sorption capacity of 114 and 3.2 mg g^{-1} by chitosan nanoparticles and crab shell powder, respectively. Maximum sorption capacity of Eu by crab shell particles at pH 6 was found to be 49.5 mg g^{-1} [29] and neodymium (an homolog of europium of same physicochemical properties) uptake lied between 10 and 12 mg g^{-1} by *Candida colliculosa*, *Debaryomyces hansenii*, and *Kluyveromyces marxianus* yeasts at pH 1.5 [2]. In the present study, under optimal conditions, Eu removal was found to be 37%, which corresponds to 8 mg Eu g^{-1}. Europium retention varies dramatically due to very different nature of sorbents, as well as different operating conditions, such as pH, temperature, sorbent dose, and solute volume. There is a very limited database concerning sorbents' performance for Eu sequestration. Taking into consideration that europium was declared by US Department of Energy as one of the most critical rare earth elements [28], the need for further studies and exploitation of new biosorbents, is imperative.

Biosorption is a complex process where multiple mechanisms, such as ion-exchange, surface complexation, and electrostatic interactions take place simultaneously. Different binding schemes have been proposed to describe the interaction of surface active groups with metal cations. An ion-exchange type of interaction between europium ions and hydrogen attached to sorbent binding site can be described by the following reaction:

$Eu^{3+} + 3HB \leftrightarrow EuB_3 + 3H^+$, where B is the monovalent carboxylic group [30].

On the other hand, surface complexation between europium and deprotonated carboxylic and phosphate groups can be described by the following reactions:

$$Eu^{3+} + R\text{-}COO^- \leftrightarrow R\text{-}COO\text{-}Eu^{2+}$$

$$Eu^{3+} + 2R\text{-}COO^- \leftrightarrow (R\text{-}COO)_2\text{-}Eu^+$$

$$Eu^{3+} + R\text{-}PO_4^- \leftrightarrow R\text{-}PO_4\text{-}Eu^{2+}$$

where R represents the micro-organism biomass that carboxylic and phosphate groups are attached [31]. The fact that all the reactions proposed, make several hypotheses such as ignoring that neighboring functional groups can affect each other, confirms the complex nature of biosorption process.

Biosorption studies for metal sequestration have used both living and non-living micro-organisms. Both living and dead cells are capable of metal accumulation, through different mechanisms though. Especially, in the case of living cells, metabolism might play an active role in metal sequestration; when dead cells are used, sequestered metals are believed to remain mainly on the surface and while when living cells are used, metals may be incorporated in the biomass through metabolic channels [32]. Consequently, living cells might show some metabolically derived resistance to heavy metal uptake. In addition, two other important factors that need to be taken into consideration when using live micro-organisms are the possible saturation, when toxicant concentration levels become too high, and the cell maintenance [33]. Hence, the use of dead cells might seem more appealing for reducing complexity. Nevertheless, the contribution of metabolic pathways is underappreciated, since living cells, apart from sorbing, can transport and transform metals and metalloids and possess both specific and non-specific transport systems [34]. Therefore, the application of living cells in both batch systems and large-scale operations need optimization in order to ensure maximum performance and cost-effectiveness.

4. Conclusions

The optimization of techniques for the treatment of metal-bearing effluents and the recovery of precious entities is of capital importance. Studying europium sorption serves double purposes: Eu(III) serves as an ideal homolog for the removal of highly radiotoxic trivalent actinides, whereas the recovery of europium itself is important due to its high industrial demand. Therefore, it is important to maximize cost-effectiveness when designing a system based on micro-organisms as sorbent material taking into consideration the effect of growth conditions of the biomass. In the present study, *S. cerevisiae*, *D. hansenii*, and *K. marxianus*, which are low-cost and easily accessible yeasts, showed significant Eu(III) retention under the conditions studied. Cells harvested from the exponential phase and grown at the optimum temperature (30˚C) showed higher sorption capacity, whereas air inlet was found to play no role. These traits render the above-mentioned yeasts more promising and cost-effective sorbent materials as there is no need for long cultivation time and air allowance and the optimum cultivation temperature is really close to room temperature.

Acknowledgments

The authors gratefully acknowledge Professors A.A. Koutinas and M. Kanellaki for providing the knowledge of the biomass preparation. Special thanks to Dr. L. Bosnea for useful discussions on the field of fermentation and yeast technology. We also thank the European Social Fund (ESF), Operational Program for Educational and Vocational Training II (EPEAEK II), and particularly the Program PYTHAGORAS II, for funding the above work.

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