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The Evaluation of Bioremediation Potential of a Yeast Collection Isolated from Composting

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Abstract

The influence of xenobiotic compounds on environment and on living organisms has been reported as an imminent public health problem. Among them we can list the contamination by Alkanes present in petroleum, hydrocarbons and organic contaminant substances from industrial effluents. Also, heavy metals are of particular interest because of their persistence in the environment contaminating the food webs. Among the innovative solutions for treatment of contaminated water and soil is the use of biological materials like living or dead microorganisms. Yeasts exhibit the ability to adapt to extreme condition such as temperature, pH and levels of organic and inorganic contaminants that make them a potential material to be used to remediate contaminated environment application. The goal of this work was to search for yeast isolates capable to use n-hexadecane (alkane hydrocarbon) as a primary carbon source and for those able to tolerate high concentration of lead (Pb) within a collection of 90 isolates obtained from the São Paulo Zoo composting system. The isolated yeast strains were identified by mass spectrometry (MALDI-TOF-MS) and by sequencing of the ribosomal DNA (18S and D1/D2) conserved regions. We found that the collection bares 23 isolates capable of utilizing n-hexadecane and one which is able to tolerate high concentration of lead (Pb) with a high biosorption index compared to the reference yeast strains (BY4742, PE-2, CAT-1 and BG-1). These results confirm the initial hypothesis that the São Paulo Zoo composting is the source for diverse yeasts species with biotechnological application potential.

Keywords

Biosorption, Lead, Yeast, *Trichosporon montevideense*, *Saccharomyces cerevisiae*

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1. Introduction

The influence of xenobiotic compounds on environment and on living organisms has been reported as an issue of concern and has been listed as one of 40 priorities for conservation policy and management [1]. Alkanes present in petroleum, hydrocarbons and organic contaminant substances from industrial effluents are increasingly being disposed into the soil and water leading to pollution [2]. Metals as contaminants are also of particular interest because of their liability, which make their persistence possible in the environment with the possibility of interconversion between species, favoring migration among physic compartments and food webs [3].

Lead is known by mankind for many years and it has been employed in several processes ever since. The anthropogenic Pb input in the environment decreased in the last decades after banishing its application as a gasoline additive in many countries. However, it is still widely applied in industries of paper, plastic, ceramic, automotive, semiconductors and pigments [4], and as a consequence Pb is still introduced in the environment nowadays [5].

Moreira *et al*. [6] reported several health problems related to Pb contamination, which may include neurological and hematological abnormalities, endocrine disruption, fetal development, and it may interfere with processes such as reproduction, renal, hepatics, gastrointestinal and cardiovascular functions, besides its carcinogenic effects. Therefore, it is urgency to develop new technologies for the treatment and recovery of Pb from industrial waste before its discard and disposal. Among the many biotechnology processes available to remediate the contamination by heavy metals, the use of microorganisms capable of accumulating these metals and metal species has gained prominence either by bioremediation through bioaccumulation or by the recovery of metals for a new application, converting an environmental liability into an economic asset, such as in biometallurgical process [7]-[9].

Different kinds of organisms have been successfully used for bioremediation, from plants to microbes (for reviews see [10]-[12]). The microbial metabolic diversity and versatility is part of the reason why they are suitable as agents for remediation among many living organisms. In general bacteria, fungi and yeast are able to assimilate complex and recalcitrant substrates as carbon and nitrogen sources, as well as to conduct biotransformation of recalcitrant substances that would accumulate in the environment. Metal bioaccumulation can also be achieved by the action of algae, plants, bacteria and fungi. Dursun *et al*. [13] obtained a maximum Pb uptake of 34.4 $mg \cdot g^{-1}$ by *Aspergillus niger*. Li *et al*. [14] evaluated Cd accumulation during the *Zygosaccharomyces rouxii* and *Saccharomyces cerevisiae* growth reaching 94% accumulation of Cd. Therefore, the prospection and evaluation of tolerant microorganisms capable of xenobiotic compound assimilation and metal accumulation have great interest for bioremediation.

Many microorganisms isolated from contaminated soil, waste waters, compost and extreme environments have been proven useful for bioremediation [15]-[18]. Their success to survive in such a harsh environment can be attributed to metabolic possibilities allowing microbes to explore, detoxify and survive in exotic and complex substrates. In this work, organic composting waste from São Paulo Zoo Park in Brazil, which is known as a rich source of microbial diversity [19]-[22] was used as the starting material for isolation of yeast strains capable to use hydrocarbon as main carbon source and tolerate and accumulate heavy metals. Two yeast collections containing 90 isolates were assembled and identified by MALDI-TOF biotyping and ribosomal DNA sequence. Among these, several Ascomycetes and Basidiomycetes able to use n-hexadecane as sole carbon source were found. Also, a yeast strain was identified which is highly tolerant to Pb and capable of uptaking and accumulating high concentration of this hazardous heavy metal. In this paper, the biotechnological potential of yeast isolates from São Paulo Zoo Park Compost will be revealed and emphasis will be given to the Pb bioaccumulation capabilities.

2. Material and Methods

2.1. Strains and Growth Conditions

General yeast medium was YPD (Yeast extract 5 g/L, Peptone 10 g/L, Dextrose 20 g/L, HCl 1 N 5 mL/L, agar 20 g/L). *S. cerevisiae* reference strain (S288c background) used in this work is BY4742 acquired from Genetics Research, industrial strains (PE-2, CAT-1 and BG-1) were a gift from Dr. Luis Carlos Basso [23].

2.2. Yeast Isolation

Samples from São Paulo Zoo Organic Compost Unit (UPCO) were collected from different composting cells

according to the protocol published before [19] [20] [22]. In brief, samples were diluted in 0.9% NaCl, homogenized and let settle at room temperature for 2 hours. Serial dilutions of the supernatant (10^{-1} to 10^{-8}) were platted on the following fungal enrichment media: Potato-Dextrose Agar, Czapek Dox, Malt Extract, ISP2, Sabouraud-Dextrose Agar and YEPD, all supplemented with 25 µg/mL chloramphenicol. Growth was observed every 24 hours during 15 days. Yeast-like and filamentous colonies were inspected under the stereomicroscope, isolates were purified to single colonies and analyzed for yeast-like micromorphology and stored in 15% glycerol at −80˚C in ultra freezer.

2.3. Yeast Identification by MALDI-TOF

MALDI-TOF spectrometry and Biotyper software were used for microbial identification [24]. Ethanol/formic acid extraction method was applied for each isolate as suggested by Bruker Daltonics. In brief, plates containing fresh colonies were used as source of cells which were scraped, transferred to microtubes and diluted in 300 µL of sterile water. Absolute ethanol (900 µL) was added, mixed, and cells were centrifuged at 10,000 g for 2 minutes at room temperature (25˚C). The supernatant was discarded and the pellet air dried. The dry pellets were mixed thoroughly with 50 µL 70% formic acid (Merck, USA) and 50 µL acetonitrile (Merck, USA). The suspension was centrifuged at 10,000 g for 2 minutes, and 1 µL of the supernatant was placed on Micro Scout Plate (MSP) 96 polished steel target (Bruker Daltonics GmbH, Germany) and dried at room temperature (25˚C). Each sample was overlaid with 1 µL of the matrix solution which consisted of a saturated solution of a-cyano-4-hydroxy-cinnamic acid (Sigma, USA) in 50% acetonitrile—2.5% trifluoroacetic (Sigma, USA), and the matrix/sample was co-crystallized by air drying at room temperature (25˚C). Measurements were performed with Microflex LT mass spectrometer (Bruker Daltonics) using FlexControl software (version 3.0, Bruker Daltonics). Positive linear mode was used to record Spectra (laser frequency, 20 Hz; ion source 1 voltage, 20 kV; ion source 2 voltage, 18.6 kV; lens voltage, 7.5 kV; mass range, 2000 to 20,000 Da). For each spectrum, 240 shots in 50-shot steps from different positions of the target spot (automatic mode) were collected and analyzed. *Escherichia coli* ribosomal proteins were used as internal calibration. The raw spectra were imported into the Biotyper software (version 2.0, Bruker Daltonics), processed by standard pattern matching with default settings, and the results reported in a ranking table. Outcomes of the pattern-matching process were expressed as proposed by MALDI-TOF Biotyper (MT) manufacturer with ID scores ranging from 0 to 3. Scores < 1.70 were considered not to have a generated reliable ID; a score of $1.7 \leq ID \geq 1.9$ was considered ID to genus, and a score > 1.9 was used for reliable species ID according to the software manufacturer (Bruker Daltonics).

2.4. Yeast Identification by Ribosomal DNA Sequencing

Yeast genomic DNA was extracted from 3 mL overnight liquid cultures according to the protocol described by Hoffman and Winston [25]. Small Subunit RNA (18S) and intergenic region D1-D2 were amplified by PCR (polymerase chain reaction), using 1U Taq Polymerase (New England Biolabs, USA), 1× Buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 2 mM $MgCl_2$, 200 µM dNTP's (Fermentas, Finland), 0.2 µM of each primer (IDT, USA). **Table 1** describes the primers used. Amplification condition: 1 cycle 94˚C for 5 minutes, 35 cycles: denaturing at 94˚C for 30 seconds, primers annealing at 54˚C for 30 seconds, and extension at 72˚C for 1 minute, a final extension of 72˚C for 7 minutes. Single-band amplicons were purified with PCR Clean up Kit (Axigen Scientific, USA) and double stranded sequenced with BigDye Terminator (Applied Biosystems, USA) according to the manufacturer's instructions. The sequences were aligned using SeqMan module of software Lasergene

Table 1. Primers used for 18S and D1-D2 ribosomal DNA sequencing.

Primer	Sequence	Region	Amplicon	Annealing temperature	Reference
PRCP007	TTAGCATGGAATAATRRAATAGGA	18S	760 bp	60˚C	Valente *et al.* [53]
PRCP008	ATTGCAATGCYCTATCCCCA	18S	760 bp	58˚C	
PRCP039	GCATATCAATAAGCGGAGGAAAAG	D1-D2 (25S)	600 bp	68˚C	
PRCP040	GGTCCGTGTTTCAAGACGG	D1-D2 (25S)	600 bp	60˚C	

(DNAStar, USA) and blasted against Genebank. Identification at species level was considered for sequence similarities above 97%.

2.5. Yeast Assimilation of N-Hexadecane as Carbon Source

N-hexadecane assay was performed in 96 wells plates containing YNB with ammonium sulphate and amino acids supplemented with 1% of n-hexadecane (Sigma) as the main carbon source, in a final volume of 100 µL. Each well was inoculated with 10 µL of fresh culture grown overnight in YPD (30˚C, 150 rpm rotation). The wells were stained with 10 µg/mL MTT (Methylthiazolyldiphenyl-tetrazolium bromide, Sigma) and incubated with agitation (100 rpm) for 2 hours at 30˚C. Negative control was considered medium without inoculum or the lack of *E. coli* growth and the positive control was USM 537, which is a bacterial isolate that assimilates n-hexadecane as sole carbon source [20]. Yeast isolates considered positive for n-hexadecane assimilation are those that turned purple after incubation period of 2 hours as described above. All assays were made in triplicates.

2.6. Disk Diffusion Assay

Disk diffusion method was employed as a sensitivity screening test to find isolates tolerant to Pb [26]. Briefly: each isolate was grown overnight in 5 mL of YEPD, at 30˚C with agitation (150 rpm). Each culture was diluted in sterile 0.9% NaCl (1:10) and 100 µL aliquots were spread evenly onto YPD plates. Filter paper disks (0.5 cm) were previously soaked with 0.5, 1.0, 2.5 and 5.0 mg of Pb^{2+}. Positive control was performed with the same type of disks impregnated with 200 µg of fluconazole and negative control with water. The disks were laid on inoculated plates, which were incubated at 30˚C for 48 hours and plates were inspected every 12 hours. All experiments were made in triplicates.

The contact of Pb with the solid YPD formed a precipitate observed between the filter paper discs and the growing colony. Inhibitory concentration was considered when a clear zone (no growth) was observed around the edge of the disk. Tolerance was identified by no clear zone around the paper disk, there is, colony growth met edge of the paper disk soaked with different concentrations of Pb.

2.7. Pb Uptake by Yeast Cells

Pb uptake experiments were performed with fresh yeast cells prepared as follow: 500 mL of YPD was inoculated with 5 mL of fresh culture grown overnight in YPD and incubated 30˚C, 16 hours with agitation (150 rpm). The cells were washed three times in sterile 0.9% NaCl solution by centrifugation (4000 rpm, 10 minutes at 10˚C). After the last wash, cells were brought to suspension in NaCl (0.9%) and divided in aliquots of the same volume for further Pb uptake experiments.

2.8. Yeast Inactivation by UV Irradiation

Aliquots of fresh yeast cells prepared as described above were placed in Petri dishes (150 × 15 mm) and exposed to UV irradiation (30 W lamp) at 20 cm distance for 0, 10, 20, 30, and 50 minutes under constant shaking (150 rpm). Samples from each irradiation exposure time were serial diluted and spotted on YPD (5 µL of the dilutions: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) and incubated at 30˚C for 48 hours. Colonies counts were conducted to evaluate the cell viability and identify the time point suitable to complete inactivation. Cells were maintained in the dark during and after irradiation in order to avoid DNA repair mechanism activation.

2.9. Pb Quantification

Viable and UV inactivated cells were suspended in 5 mL of a solution containing Pb (5000 $mg \cdot L^{-1}$) and placed in an orbital shaker (150 rpm) at room temperature for 1, 5 and 10 minutes. Cells were harvested by centrifugation (2 minutes at 4000 rpm) and the supernatants were discharged. The pellets were frozen in dry ice, lyophilized for 24 hours and stored at room temperature. In order to quantify Pb adsorbed 0.1 g of lyophilized cells exposed and not exposed (blanks) to Pb were digested in 1 mL of HNO_3 sub-boiled added with 2 mL H_2O_2 3% v/v in a microwave oven (2 min at 750 W, 2 min 0 W, 18 min at 800 W, 6 min at 0 W and 30 min of cooling). A SpectrAA-800 GFAAS (Varian, Mulgrave Victoria, Australia) equipped with deuterium lamp as background

corrector and a GTA 96 auto sampler was used for Pb determination performed at 2833 nm, with a slit-width 0.2 nm. Pyrolytically coated graphite tubes without chemical modifier and L'vov platform (Varian, Mulgrave Victoria, Australia) were used. A volume of 20 µL of sample was injected in the GFAAS by the auto sampler after appropriated dilutions. The results were obtained in peak areas and the quantification was performed using analytical curves. The Pb pyrolysis and atomization temperatures were set as 700˚C and 1000˚C, respectively [27]. The ANOVA statistical analysis followed by Student's t-test was applied to verify statistical difference between averages of the treatments.

3. Results and Discussion

3.1. Yeast Collection

São Paulo Zoo Compost Unit has proven to be a source of relevant microorganisms for biotechnological applications [19]-[22]. This compost is the result of microbial transformation of excreta from animals coming from all around the world and the waste of Atlantic forest vegetation into fertilizer [19]. The zoo compost gathers great unexplored microbiology diversity. Our previous reports used metagenomic approach to show the bacterial community structure present in this substrate [21] also, several bacterial strains from this exotic environment have been appointed as potential biotechnology tools [20] [22]. However, the diversity of yeast and filamentous fungi has never been described in São Paulo Zoo Park Compost. The yeast cells are known as metabolic versatile [28] [29] also yeast cell wall is well known for its ability to bind metals [9] [30]-[33], therefore, they may have several potential applications [34]. In this study we assembled a yeast collection isolated from compost aiming to explore its potential as bioremediation agents. A total of 90 strains were isolated and protein profiles were generated by MALDI-TOF spectrum. The Biotyper software was used to compare these profiles with those deposited at the Brucker Daltonics database. As positive controls *E. coli*, USM 470 and a *C. neoformans* serotype A (*var. grubbii*) laboratory strain H99 were added to each MALDI-TOF plate. Scores higher than 1.9 were used to attribute genus and species to each isolate. Out of 90 isolates, 35 generated novel profiles, for which similarities were not found at Brucker Daltonics database. These isolates were submitted to ribosomal DNA sequence at 18 or 25S regions. **Table 2** describes the consolidated results of yeast identification by MALDI-TOF and DNA sequence. The majority of yeast strains isolated belongs to the genus *Candida* (57%), followed by *Pichia* (17%) and *Trichosporon sp*. (6.5%). The genus *Candida* was the most diverse (9 different species), followed by *Pichia* (5 species) as described in **Table 2**. Also, the Ascomycete (*Candida*, *Pichia*, *Debaryomyces*, *Hanseniaspora*, *Meyerozyma* and *Stephanoascus spp.*) predominated over the Basidiomycete (*Trichosporon*, *Rhodotorula* and *Cryptococcus*) which is consistent with the literature considering that the *Ascomycota phylum* is larger than *Basidiomycota* [35] [36]. Fungi are known as saprophytic microbes that decompose a large range of organic matter, even the most complex and recalcitrant as lignocelluloses, especially the Basidiomycete are well known for this ability (for review see [37]). The diverse metabolic arsenal present among the yeasts and their easy and simple manipulation reflect on their applicability as bioremediation agents [34]. In this work we inquired whether our yeast collection from São Paulo Zoo Compost has isolates suitable to bioremediation applications. To address this question we searched the collection for two important phenotypes associated to bioremeditation: ability to assimilate n-hexadecane, a model xenobiotic considered a recalcitrant Carbon source [38] and tolerance to high levels of Pb, an important heavy metal that contaminates the environment.

3.2. n-Hexadecane Assimilation by Yeasts

Our previous results demonstrated that several bacteria from compost are able to metabolize n-hexadecane and therefore, have potential for bioremediation [20]. We used the same method (broth micro assay with n-hexadecane as main carbon source) to screen 90 isolates and identified 23 yeast capable of growth in 1% n-hexadecane (**Table 3**). Among these isolates, 18% and 62.5% belong to *phylum* Ascomycota and Basidiomycota, respectively, suggesting this phenotype is more often found in the later *phylum*. This observation is in accordance with the literature, since Basidiomycetes are known to consume complex carbon sources efficiently [37] [38].

The literature report several uses of Ascomycetes in bioremeditation. *C. catenulata* is able to assimilate oil from contaminated soil [15]. In this report 84% of hydrocarbon was removed from oil by composting it with *C. catenulata*. Other reports, mention the use *C. krusei* and *C. rugosa*, *Pichia gilliermondii* and *Meyerozyma guil liermondii* in biotransformation of pigments, lipase sources, degradation of polycyclic aromatic compounds and

Table 2. Taxonomic identification of yeasts isolated from São Paulo Zoo Compost.

Code	Taxonomic identification	MALDI-TOF-MS score	DNA sequence similarity
USM 384	*Candida tropicalis*	2.298	-
ULP 385	*Pichia kluyveri*	2.050	100%
ULP 387	*Pichia kluyveri*	2.423	100%
ULP 388	*Candida krusei*	2.388	-
ULP 389	*Candida tropicalis*	2.177	-
ULP 390	*Candida tropicalis*	2.232	-
ULP 391	*Candida krusei*	2.162	-
ULP 392	*Candida krusei*	2.334	-
ULP 393	*Pichia kluyveri*	2.547	100%
ULP 394	*Pichia kluyveri*	-	100%
ULP 395	*Candida krusei*	2.204	-
ULP 398	*Pichia guilliermondii*	-	99%
USM 399	*Debaryomyces hansenii*	-	100%
USM 400	*Pichia guilliermondii*	2.720	99%
USM 402	*Candida tropicalis*	2.225	-
USM 404	*Debaryomyces hansenii*	-	100%
USM 405	*Debaryomyces hansenii*	-	98%
USM 406	*Debaryomyces hansenii*	2.155	100%
USM 407	*Candida catenulata*	2.252	-
ULP 412	*Candida krusei*	2.506	-
USM 417	*Pichia guilliermondii*	2.652	99%
USM 418	*Candida olivae*	-	100%
ULP 432	*Candida orthopsilosis*	-	99%
USM 433	*Candida catenulata*	2.289	-
USM 434	*Candida catenulata*	2.172	-
USM 436	*Candida catenulata*	2.479	-
USM 440	*Trichosporon asahii*	-	100%
ULP 469	*Candida catenulata*	2.070	-
ULP 470	*Candida catenulata*	2.028	-
ULP 471	*Candida catenulata*	2.283	-
ULP 473	*Candida catenulata*	2.385	-
ULP 475	*Pichia heedii*	-	100%
ULP 476	*Candida krusei*	2.306	-
UED 493	*Candida catenulata*	2.286	-
UED 494	*Rhodotorula mucilaginosa*	2.273	-
UED 495	*Rhodotorula mucilaginosa*	2.267	-
UED 496	*Candida catenulata*	2.190	-
UED 497	*Candida catenulata*	2.165	-
UED 498	*Rhodotorula glutinis*	-	99%
UED 499	*Candida catenulata*	2.377	-
UED 500	*Candida catenulata*	2.378	-
UED 501	*Candida catenulata*	2.240	-
UED 502	*Candida catenulata*	2.323	-
UED 503	*Candida catenulata*	2.337	-

Continued

UED 504	*Candida catenulata*	2.203	-
UED 505	*Candida catenulata*	2.243	-
UJF 805	*Candida krusei*	2.375	-
UJF 809	*Candida rugosa*	2.175	-
UJF 812	*Pichia barkeri*	2.388	-
UJF 814	*Stephanoascus ciferrii*	-	100%
UJF 815	*Pichia fermentans*	-	99%
UJF 817	*Cryptococcus neoformans var. grubii*	-	100%
UJF 820	*Candida humilis*	-	100%
UJF 821	*Pichia fermentans*	2.144	-
UJF 822	*Debaryomyces hansenii*	-	100%
UJF 823	*Candida ethanolica*	-	100%
UJF 824	*Candida humilis*	2.173	-
UJF 825	*Pichia kluyveri*	2.045	-
UJF 826	*Candida sorbosa*[*]	1.947	-
UJF 827	*Candida sorbosa*[*]	2.297	-
UJF 829	*Candida ethanolica*	-	99%
UJF 830	*Pichia barkeri*	-	98%
UJF 831	*Rhodotorula mucilaginosa*	-	100%
UJF 832	*Candida krusei*	2.430	-
UJF 833	*Candida humilis*	2.265	-
UJF 834	*Candida ethanolica*	2.122	-
UJF 840	*Pichia kluyveri*	-	99%
UJF 842	*Hanseniaspora guilliermondii*	-	99%
UJF 844	*Candida krusei*	2.542	-
UJF 845	*Candida krusei*	2.474	-
UJF 847	*Candida rugosa*	2.057	-
UJF 848	*Candida krusei*	2.445	-
UJF 849	*Pichia barkeri*	2.080	-
UJF 850	*Candida krusei*	2.278	-
UJF 853	*Candida krusei*	2.081	-
UJF 1428	*Candida catenulata*	2.088	-
UJF 1430	*Meyerozyma guilliermondii*	-	100%
UJF 1432	*Candida catenulata*	1.990	-
UJF 1433	*Trichosporon jirovecii*	-	100%
UJF 1435	*Candida catenulata*	2.136	-
UJF 1438	*Trichosporon jirovecii*	2.553	-
UJF 1439	*Trichosporon jirovecii*	2.764	-
UJF 1441	*Trichosporon montevideense*	-	100%
UJF 1444	*Candida catenulata*	1.925	-
UJF 1445	*Cryptococcus neoformans var. grubii*	-	100%
UJF 1454	*Trichosporon asahii*	2.545	-
UJF 1469	*Cryptococcus neoformans var. grubii*	-	100%
UJF 1470	*Cryptococcus neoformans var. grubii*	-	100%
UJF 1471	*Cryptococcus neoformans var. grubii*	-	100%
UJF 3006	*Rhodotorula mucilaginosa*	-	100%

[**] *Issatchenkia occidentalis.*

Table 3. Isolates that use n-hexadecane as the sole carbon source.

Code	Species
UJF 1428	*Candida catenulata*
UJF 1432	
UJF 1435	
UJF 1444	
UJF 832	*Candida krusei*
USM 418	*Candida olivae*
UJF 847	*Candida rugosa*
UJF 817	*Cryptococcus neoformans var. grubii*
UJF 1445	
USM 399	*Debaryomyces hansenii*
USM 404	
USM 405	
USM 406	
UJF 1430	*Meyerozyma guilliermondii*
ULP 398	*Pichia guilliermondii*
USM 400	
UED 498	*Rhodotorula glutinis*
UJF 831	*Rhodotorula mucilaginosa*
USM 440	*Trichosporon asahii*
UJF 1454	
UJF 1433	*Trichosporon jirovecii*
UJF 1438	
UJF 1439	
UJF 1441	*Trichosporon montevideense*

glycerol [39]-[43]. However, other species as *D. hansenii* are well known as food spoilage agents due to its salt and cold tolerance and no reports on its ability to degrade xenobiotic compounds have been reported.

Regarding Basidiomycetes found in this work, in spite of the small number compared to Ascomycetes, most of them (62.5%) are able to use n-hexadecane as main carbon source. *Rhodotorula spp*. has been associated to the production of beta-carotene, torulene and enzymes as phenylalanine ammonia lyase. Also, some species were reported as able to convert recalcitrant substrates, as lignin, tannins and pectin [34] [44]-[48]. The genus *Cryptococcus* and *Trichosporon* have also been reported as capable of biotransformation of a wide range of substrates as well as xenobiotics substances [34].

3.3. Selection of Pb Tolerant Yeasts

The yeast *S. cerevisiae* has been used to remediate heavy metals due to the fact that its cell wall has the ability to serve as biosorbent [3]. In this work we searched our yeast collection for isolates capable to tolerate Pb levels higher than those observed by *S. cerevisiae* strains. We expected that higher tolerance would reflect higher uptake. The disk diffusion method was employed in this experiment in order to overcome Pb precipitation in liquid culture medium. Disks containing 0.5, 1.0, 2.5 and 5 mg of Pb were placed in triplicates on plates inoculated with each test isolate and reference strains. **Table 4** shows that *S. cerevisiae* BY4742 and BG-1 were inhibited

Table 4. Pb tolerance of the yeast reference strains and selected isolates from São Paulo Zoo collection.

Yeast isolate	Pb tolerance level (mg)
UJF 502 (*C. catenulata*)	2.5
UJF 1441 (*T. montevideense*)	5.0
BY4742 (*S. cerevisiae* S288c)	1.0
BG-1 (Industrial strain)	1.0
PE-2 (Industrial strain)	2.5
CAT-1 (Industrial strain)	2.5

by 1.0 mg, whereas PE-2 and CAT-1 were inhibited by 2.5 mg of Pb. *C. catenulata* isolate UJF 502 was no more tolerant than the industrial strains (PE-2 and CAT-1). However, UJF 1441 (*T. montevideense*) was not inhibited by 5 mg Pb. Therefore, UJF 1441 was considered the most tolerant isolate in the collection and it was selected for further studies. All the other isolate in the collection were inhibited by 1.0 mg and were not pursued further. This result is in accordance to previous reports in the literature, Muñoz *et al.* [17] reported several yeasts isolated from waste waters, among them a *T. montevideense* and *Trichosporon sp.* which are very tolerant to different heavy metals (Pb, Zn and Ag), however those strains were not assayed for heavy metal cell uptake.

3.4. Pb Uptake by Selected Yeasts

Isolate UJF 1441 (*T. montevideense*) and the reference strain CAT-1 were compared regarding their ability to uptake Pb. In many instances the cell wall is responsible for binding the heavy metal but, also, the uptake may dependent upon the microbial metabolism and causes the biotransformation of the metal [49]. To address this question we submitted both strains to UV inactivation and quantified the uptake by Graphite Furnace Atomic Absorption Spectroscopy (GF-AAS) in active and inactive cells. The UV inactivation was chosen since it induces cell death by nucleic acid damage with reduced chance of cell wall damage, as it would if a chemical inducer such as heat was to be used [50]. A survival curve (0, 10, 20, 30, and 50 minutes) was performed to define the time of UV irradiation necessary to completely inactivate cells. Although our results showed that 10 minutes of UV-irradiation was enough to inactivate the yeast cells (CAT-1 and UJF 1441) we irradiated the cells for 30 min to assure complete cell inactivation.

The integrity of cell walls was verified by staining with Calcofluor followed by fluorescent microscopy observations. Comparing irradiated and non irradiated cells in both strains there was no difference in calcofluor staining pattern, suggesting the treatment did not change the microscopic characteristics of the yeast cell wall (data not shown).

The graph in **Figure 1** show the amount of Pb precipitated on the cell of active and inactive yeast at various time points (1, 5 and 10 minutes contact of cells and Pb). At all times active or inactive *T. montevideense* cells were capable of significant higher Pb sorption than *S. cerevisiae* CAT-1 strain. For both strains the inactive form was more efficient regarding biosorption. Machado *et al.* [51] reported similar behavior for *S. cerevisae* from beverage industry when they tested their sorption for Cu, Ni and Zn and concluded that the yeasts inactivated by heat (45˚C) were more efficient for biosorption than active cells.

Biosorption depends exclusively on the sorption sites present in the cell wall structure and in the plasma membrane of the inactive microorganisms; on the contrary bioaccumulation depends upon live cell metabolism [52]. Therefore, the increase sorption of Pb^{2+} observed in this work by the inactive cells suggests a biosorption mechanism rather than a bioaccumulation. In fact, the Student's t-test indicates that inactive cells have the highest sorption capacity average for both strains evaluated, *T. montevideense* and CAT-1 (Student's t-test, $p < 0.05$) when compared to the their active form. Moreover, considering that *T. montevideense* was more tolerant and retained more Pb, a relationship of dependence between tolerance and sorption capacity may established here, which is indicated by the Pearson correlation between these two variables ($r^2 = 1$; Student's t-test, $p < 0.05$).

It is important to mention that due the precipitation of Pb, the sorption studies were not executed in the culture media, which could favor the retention Pb^{2+} by the active isolates because of the ideal conditions of growth and development. However, the observed sorption capabilities of the inactive *T. montevideense* cells (**Figure 1**),

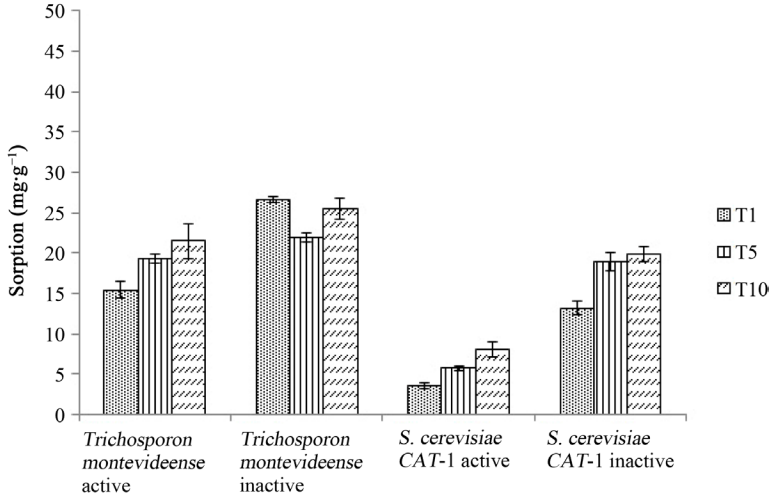

Figure 1. Sorption of Pb (5000 mg·L^{-1}) for *Trichosporon montevideense* and *S. cerevisiae* CAT-1 in different exposure times: 1 minute (T1), 5 minutes (T5) and 10 minutes (T10).

may indicate that this isolate can potentially be used in proccesses involving biosorption. Taken together these results show that 1) yeast collection isolated from São Paulo Zoo Park Compost has a large diversity of yeast species, 2) MALDI-TOF and Biotyper are reliable, easy, time and cost effective method for taxonomic identification, and finally 3) our collection has isolates with great potential for biotechnology applications, mainly related to bioremediation; 23 strains are able to consume a xenobiotic substance, therefore may be applied in polutant removal and inactive *T. montevideense* is a very robust biosorption agent regarding Pb uptake and further studies may allow its use in the near future for metal removal from the environment.

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