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Original article

Studies Regarding Cytotoxicity of Copper, Lead and Zinc Ions Solutions on Living *Saccharomices Cerevisae* Cells

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Abstract

Brewer's yeast is a type of fungus formally known as *Saccharomyces cerevisae*. Along with other Saccharomyces species, brewer's yeast is used to brew beer and can be also used as a nutritional supplement in active form. To evaluate the cytotoxicity of some heavy metal ions on living *Saccharomyces cerevisae* cells it were used copper, lead and zinc solutions at different concentrations ranged between 10^{-3}M to 10^{-5}M. The end points for cytotoxicity evaluation are: proliferation rate, lactate dehydrogenase quantification and lipid peroxidation. Regarding the proliferation rate all the metal ions inhibit the growth of the cells, especially copper ions and the optimal pH was established to be 5.5. Concentrations of 10^{-3}M of Cu^{2+}, Pb^{2+} and Zn^{2+} inhibit the proliferation rate about 18% for lead, 24.5% for zinc and 27% for copper after 24h at pH=5.5 and 31.8%, 35% and 43.5% at pH=8. Concerning the cytotoxicity test, if the level of lactate dehydrogenase released is under 0.15 nmoli NADH/min/10.000 cells it is considered that there is no cytotoxic activity in the cell. The results obtained demonstrated that only lead and zinc ions at the concentration of 10^{-5}M could be considered non-cytotoxic on *Saccharomyces cerevisae* cells. The peroxidation level measured by MDA-TBA complex formed was higher for Cu^{2+} ions at all concentrations; for Pb^{2+} and Zn^{2+} the higher prooxidant activity was determined for the concentration of 10^{-3}M and 10^{-4}M, so the maximum level admitted for this study is 10^{-5}M.

Keywords: grape; brewer's yeast, heavy metals, cells proliferation

1. Introduction

The development of various industries in the past century has remarkably increased the amount and complexity of toxic waste effluents, which may be bioremediated by appropriate plants and microbes, either natural occurring or tailor-made for the specific purpose.

This technology is termed as bioremediation. Bioremediation can be defined as any process that uses microorganisms or their enzymes to return the environment altered by contaminants to its original condition. Biosorption can be defined as the selective sequestering of metal soluble species that result in the immobilization of the metals by microbial cells. Metal sequestering by different parts of the cell can occur via various processes: complexation, chelation, coordination, on exchange, precipitation, reduction. Biosorption is a process with some unique characteristics.

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It can effectively sequester dissolved metals from very dilute complex solutions with high efficiency. This makes biosorption an ideal candidate for the treatment of high volume low concentration complex waste-waters [1, 2, 3]. Intensification of agriculture and manufacturing industries has resulted in increased release of a wide range of xenobiotic compounds to the environment. Excess loading of hazardous waste has led to scarcity of clean water and disturbances of soil thus limiting crop production [4]. Compared to other methods, bioremediation is a more promising and less expensive way for cleaning up contaminated soil and water. Bioremediation uses biological agents, mainly microorganisms, yeast, fungi or bacteria to clean up contaminated soil and water [3]. In bioremediation processes, microorganisms use the contaminants as nutrient or energy sources [5].

Metals play an integral role in the life processes of living organisms. Heavy metals are metals with densities higher than 5 g cm^{-3}. Some metals (Ca, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni and Zn) are essential, serve as micronutrients and are used for redox-processes, to stabilize molecules through electrostatic interactions; as components of various enzymes; and regulation of osmotic pressure. Heavy metals in wastewater come from industries and municipal sewage, and they are one of the main causes of water and soil pollution [6]. Accumulation of these metals in wastewater depends on many local factors such as type of industries in region, people's way of life and awareness of the impacts done to the environment by careless disposal of wastes. Therefore the presence of heavy metals in wastewater is not only of great environmental concern but also strongly reduces microbial activity, as a result adversely affecting biological wastewater treatment processes.

Biosorption, using biomaterials such as bacteria, fungi, yeast and algae, is regarded as a cost-effective biotechnology for treatment of high volume and low concentration complex wastewaters containing heavy metal(s) 1 to 100 mg L^{-1}. Among the microorganisms used for biosorption, *Saccharomyces cerevisiae* is an inexpensive, readily available source of biomass for heavy metal removal from wastewater. Two types of *Saccharomyces cerevisiae*: lab culture and brewery waste cells were used as biosorbent. The recycling of waste yeast from brewery can be solved by using it as biosorbent material.

Saccharomyces cerevisiae can remove toxic metals, recover precious metals and clean radio-nuclides from aqueous solutions to various extents. *S. cerevisiae* is a product of many single cell and alcohol fermentations, it can be procured in large quantity at low cost. Saccharomyces has the ability to differentiate between different metals such as selenium, antimony and mercury based on their toxicity. This property makes *S. cerevisiae* useful in analytical measurements [7]. Brewer's yeast is made from a one-celled fungus called *Saccharomyces cerevisiae* and is used to make beer. It also can be grown to make nutritional supplements. Brewer's yeast is a rich source of minerals, especially chromium, an essential trace mineral that helps the body maintain normal blood sugar levels, selenium, amino acids, protein and the B-complex vitamins [8].

2. Material and Methods

Saccharomyces cerevisae biomass was supplied as a by-product from industrial ethanol production. Prior use as a biosorbent, the biomass was pretreated in order to remove fine particles and to displace any metals already bound to the sorption sites. The waste biomass as washed with deionized water by stirring followed by centrifugation at 3000 rpm for 20 minutes. The supernatant was discarded and the pellet was reslurried in deionized water. The procedure was repeated for three times until the supernatant was clear.

Metals solution were prepared using the mixture of their salts $CuSO_4 \cdot 5H_2O$, $PbNO_3 \cdot 2H_2O$, $ZnSO_4$ of analytical reagent grade. The concentrations of metals ions established were 1 mg L^{-1} for Cu^{2+}, Zn^{2+}, Pb^{2+} and were obtained by dissolving the appropriate salts in deionized water.

Experimental procedure:

Metal ion binding experiments were performed by incubation of 25 ml biomass with 25 ml mixture of metals ions-containing solution in 125-ml Erlenmeyer flask on an orbital rotary shaker at 120 rpm for 120 minutes. The experiment was conducted at two pH values: 5.5 and 8 and were established by adjusting it with HCl 0.1M or NaOH 0.1M solutions.

For metal ions biosorption the pH is one of the most important environmental factor. Experiments could not be performed at higher pH value due to the possibility of the lead ions precipitation.

a) *Evaluation of yeasts growth rate*

The proliferation rate of the yeast may be regard as an overall indicator of the physiology status of the cells. Therefore, the effect of metal ions on different cells functions will be reflected by changes in the rate of proliferation. The extinction of the yeast suspension is measured at 525 nm, using a calibration curve for correlation the absorbance with cells number. Proliferation rate was

measured spectrophotometrically using a calibration curve for yeast cells number at 24h after incubation at two pH values: 5.5 and 8.

Brewer yeast was growth on complete medium contain: 2% glucose, 1% yeast-extract, 0.5% peptone at 28-31^{o}C. Yeast are co-cultivated in the presence of three different metal ions solutions (copper, zinc, lead), at three concentrations: 10^{-3}M, 10^{-4}M and 10^{-5}M.

b*) Lactate dehydrogenase activity*

Lactate dehydrogenase can be used in cytotoxicity studies as a marker of cell damage. The normal plasma membrane is impermeable to LDH, but damage if the cell membrane results in change in the membrane permeability and subsequent leakage of LDH into the extracellular fluid. In vitro release of LDH from cells provide an accurate measure of cell membrane integrity and cell viability. LDH activity was determinate by measuring the speed of NADH oxidation which is direct proportional with enzymatic activity of LDH.

LDH activity was determined spectrophotometrically at 340 nm using the reaction: Pyruvate + NADH + H^+ → L-lactate + NAD^+. Speed of NADH oxidation is directly proportional with enzymatic activity of the LDH.

c) *Peroxidation level using MDA-TBA test*

Malondialdehyde is an indicator of lipid peroxidation. MDA occurs in biological materials in the free state and in various covalently bound forms. The most widely employed method for the determination of MDA in biological materials is based on the reaction with thiobarbituric acid to form a pink complex MDA-TBA with an absorption maximum at 532-535 nm. For lipid peroxidation it was used 1 ml yeast treated with metal ions solutions mixed with 2 ml working solution containing 15% (w/v) thiobarbituric acid, 0.25 N HCl and heated for 15 minute in boiling water. After cooling, the precipitate was removed by centrifugation at 1000 *g* for 10 min. The results are calculated using a standard curve for MDA-TBA.

3. Results and Discussions

a) *Evaluation of yeasts growth rate*

After the yeast treatment with different metal ions concentrations the proliferation rate was measured spectrophotometrically using the calibration curve at two different pH and the results are presented in the next table:

Table 1. The effect of different metal ions concentrations on cells viability

Samples	Cells density after 24h Cells/ml x 10000 (pH=5.5)	Cells density after 24h Cells/ml x 10000 (pH=8)
Control	58	56
Yeast +Pb 10^{-5}M	46	46
Yeast + Pb 10^{-4}M	41	40
Yeast + Pb 10^{-3}M	32	31
Yeast + Zn 10^{-5}M	51	50
Yeast + Zn 10^{-4}M	47	44
Yeast + Zn 10^{-3}M	42	39
Yeast + Cu10^{-5}M	43	38
Yeast + Cu 10^{-4}M	34	32
Yeast + Cu 10^{-3}M	28	24

The potential of yeasts for accumulating a range of metal cations from aqueous solutions is well known. Therefore, heavy metals inhibitory effects on S. cerevisiae were to be studied. Low concentrations of certain heavy metal ions are necessary for the vitality of all microbial cells. Low concentrations of Cu2+ and Zn2+ even stimulate the growth and the activity of the metabolic process.

At high heavy metal concentration, the growth may be restrained.

Non-essential metals such as Pb2+ can interact with fungal cells and be accumulated by physico-chemical mechanisms [8].

Yeast biosorption largely depends on parameters such as pH, the ratio of the initial metal ion and initial biomass concentration, culture conditions, presence of various ligands and competitive metal ions in solution and to a limited extent on temperature. Soares et al. (2003) [9] stated that the decreasing order of toxicity of select heavy

metals on the S. cerevisiae, pH buffer at pH 6.0, was found to be Cu2+, Pb2+ and Ni2+.

Heavy metal at 10-3M induced a decrease in the number of viable cells by about 62% in the first 24h for Cu2+ and 45% for Pb2+ and 28% for Zn2+ at the pH=5.5.

Regarding the other metal ions concentrations 10-4M and 10-5M it can be concluded that the cytotoxic effects ranged between 21% and 30% for Pb, 12% and 19% for Zn and 38% and 42% for copper.

Chen et al. (2007) [10] indicated that the toxicity of heavy metal ions correlated with their inhibiting effect on transmembrane potential or parameters related to the operation of the generators of transmembrane potential of microbial cells.

LDH activity. Lactate dehydrogenase (LDH) can be used in cytotoxicity studies, as a marker of cell damage. The normal plasma membrane is impermeable to LDH, but damage of the cell membrane results in a change in the membrane permeability and subsequent leakage of LDH into the extracellular fluid. In vitro release of LDH from cells provide an accurate measure of cell membrane integrity and cell viability. LDH activity is the most used test and reliable for cytotoxicity [11].

Level of LDH from extracellular medium is expressed in nm NADH/min/10000 cells and calculated with the next formulae:

$$\frac{DO/\min \times 3ml \times 1000 « l}{3.3 \times 100 « l}$$

The results are presented in the next table:

Table 2. LDH levels for yeast cells treated with different concentrations of metal ions at pH=5.5 and pH=8

Samples	LDH level (nmols NADH/10.000 cells) (pH=5.5)	LDH level (nmols NADH/10.000 cells) (pH=8)
Control	0.101	0.112
Yeast +Pb 10^{-5}M	0.179	0.188
Yeast + Pb 10^{-4}M	0.166	0.179
Yeast + Pb 10^{-3}M	0.133	0.146
Yeast + Zn 10^{-5}M	0.183	0.197
Yeast + Zn 10^{-4}M	0.163	0.180
Yeast + Zn 10^{-3}M	0.139	0.148
Yeast + Cu10^{-5}M	0.201	0.216
Yeast + Cu 10^{-4}M	0.187	0.195
Yeast + Cu 10^{-3}M	0.174	0.186

If the level of LDH was under 0.15 nmols NADH/min/10000 cells it can be concluded that the compounds are not cytotoxic [12]. From the above results it could be demonstrated that the lower concentrations of lead and zinc metal ions (10^{-5}M) are not cytotoxic for yeast cells at the both pH level and can be used for the recovery of biomass. Instead, the copper ions proved to be cytotoxic at all concentrations and pH experimented. The level of cytotoxicity could be proportional with the level of the metal ions uptake by the yeast. Microorganisms uptake metal either actively (bioaccumulation) and/or passively (biosorption) [13]. Feasibility studies for large-scale applications demonstrated that, biosorptive process are more applicable than the bioaccumulative processes, because living systems (active uptake) often require the addition of nutrients and hence increase biological oxygen demand (BOD) or chemical oxygen demand (COD) in the effluent. In addition, maintenance of healthy microbial population is difficult due to metal toxicity and other unsuitable environmental factors. In addition, potential for desorptive metal recovery is restricted since metal may be intracellulary bound, metabolic products may be form complexes with metals to retain them in solution and mathematical modeling of a non-defined system is difficult [14].

c) Lipid peroxidation

Lipid peroxidation is the oxidative deterioration of polyunsaturated fatty acids with the production of lipid hydroperoxides, conjugated diene, cyclic peroxides and finally fragmentation to ketones and aldehydes (including malondialdehyde – MDA).

The results are expressed as nanomols MDA/10^6 cells and are represented in the next table.

Table 3. MDA levels for yeast cells treated with different concentrations of metal ions at pH=5.5 and pH=8

Samples	MDA level (nmols MDA/10^6cells) (pH=5,5)	MDA level (nmols MDA/10^6cells) (pH=8)
Control	0.034	0.042
Yeast +Pb 10^{-5}M	0.759	0.774
Yeast + Pb 10^{-4}M	0.621	0.703
Yeast + Pb 10^{-3}M	0.513	0.625
Yeast + Zn 10^{-5}M	0.688	0.739
Yeast + Zn 10^{-4}M	0.563	0.606
Yeast + Zn 10^{-3}M	0.477	0.510
Yeast + Cu10^{-5}M	0.965	1.116
Yeast + Cu 10^{-4}M	0.883	0.979
Yeast + Cu 10^{-3}M	0.791	0.835

The degree of plasma membrane fatty acid unsaturation and the copper, zinc and lead ions sensitivity of *Saccharomyces cerevisiae* are closely correlated.

The level of lipids peroxidation depend based on the type of metal ion used and it concentration. According with Bayoumi et al. (2012) [15] the toxicity decreasing order of the investigated heavy metal salts on tested yeast strains was found to be Cu2+ > Pb2+ > Zn2+ and our results are in concordance with it.

Levels of thiobarbituric acid-reactive substances (lipid peroxidation products) were up to eightfold higher in copper 10^{-3}M concentration treated cells than in control cells following metal addition.

The copper ions solutions present the higher prooxidant effect comparing with zinc or lead ions. The results demonstrate heavy metal-induced lipid peroxidation in a microorganism and indicate that the metal sensitivity on *S. cerevisiae* may be attributable to elevated levels of lipid peroxidation in these cells.

Oxidative damage of lipid molecules generally involves lipid peroxidation, an autocatalytic process initiated by the oxidation of polyunsaturated fatty acids (PUFAs) into labile lipid hydroperoxides, by OH radicals.

Lipid hydroperoxides propagate the synthesis of further hydroperoxides and other reactive derivatives, all of which can inflict extensive oxidative damage to cell biomolecules [16, 17]. Yeast cells are incapable of synthesizing PUFAs, but will readily incorporate them into their membrane structures if cultured in PUFA-enriched growth medium, thus raising the risk of lipid peroxidation on exposure to oxidative stress [18].

4. Conclusions

The yeast play a vital role in the remediation of heavy metals and other pollutants. In the present study it was studied the effect of different metal ions concentrations at pH=5.5 and pH=8 on brewer's yeast living cells and quantified the cytotoxicity levels of these compounds. It could be concluded that the cytotoxicity is depended on the metal ions type, the concentrations of it and the pH level. The most harmful metal for brewer yeast is copper at all three concentrations experimented. Lead and zinc ions at lower concentration (10^{-5}M) have lower cytotoxicity level and could be used for bioremediation and for the biomass recovery. It may be concluded that brewer's yeasts can tolerate against the heavy metals and they are armed with various resistance and catabolic potentials. This catabolic potential of yeasts is enormous and is advantageous to mankind for a cleaner and healthier environment through bioremediation.

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