The Influence of Different Factors on Manganese Incorporation into *Saccharomyces cerevisiae*

Vesna STEHLIK-TOMAS
Jasna MRVČIĆ (✉)
Damir STANZER
Slobodan GRBA

Summary

Yeast biomass as well as biomass enriched with trace minerals have been demonstrated to be useful in improving animal health and growth performance. In this work, process for the production of *Saccharomyces cerevisiae* biomass enriched with manganese, a microelement with antioxidant properties in the form of high bioavailable Mn-protein complex, has been studied. The influences of media composition, Mn^{2+} concentration and Mn salt were investigated in shaken cultures. The change of biomass and ethanol yields was not observed in molasses media with addition of Mn, while in sucrose media the decrease was observed at Mn^{2+} concentrations higher than 0.8 mM (added as sulphate) and 0.2 mM (added as chloride). It was established that aeration mode (anaerobic, shaken flask or aerated culture) influences amount and dynamic of Mn incorporation into the yeast biomass, and that this incorporation was *S. cerevisiae* strain dependent. The Fourier transform infrared (FTIR) spectrophotometry on blank and Mn loaded biomass suggested that carboxyl groups, N-H groups of secondary amide, and sulfonate groups are involved in mechanism of manganese binding.

Key words

manganese, *Saccharomyces cerevisiae*, feed supplement

University of Zagreb, Faculty of Food Technology and Biotechnology,
Pierottijeva 6, HR-10000 Zagreb, Croatia
✉ e-mail: jmrvcic@pbf.hr

Received: May 6, 2009 | Accepted: July 29, 2009

Introduction

The role of trace elements in the metabolism of microorganisms and higher organisms has been well documented. As structural and/or functional constituents of numerous metalloproteins, enzymes, hormones or vitamins, trace elements are involved in many metabolic processes (Simić and Budić, 2003). Certain ions also neutralize electrostatic forces present in various cellular anionic units. Because of that, microelements have been recognised as an essential dietary supplement in human and animal nutrition, providing beneficial effects on the health.

Manganese is a cofactor important for several key enzymes, especially for one of the body's superoxide dismutases (Mn-SOD). Mn deficiency causes damage to mitochondrial membrane in mammals, due to decrease of Mn-SOD activity that results in increased lipid peroxidation and reduced antioxidant potential. On the other hand, Mn supplementation resulted in significant increase of human lymphocyte Mn-SOD activity and also in Mn-SOD activity in selected brain regions (Husain, 1997). Manganese is rather poorly absorbed; in human body it is only 1-4 % of dietary intake. Absorption is facilitated by chelating agents and antagonised by diets high in calcium, iron and phosphorus (Underwood, 1962). The dietary deficiencies in animals and poultry can also result in retarded growth, impaired lipid metabolism, reproduction and glucose tolerance (Freeland-Graves and Llanes, 1994), thus suggesting that the consumption of recommended daily manganese intake is highly important.

Pharmacological preparations of trace elements now available on the market contain non-organic and organic salts in the form of chlorides, oxides, carbonates and lactates. Still, they are characterised by a low availability for human and animal organism; whereas microelements bounded in the form of protein complexes (metalloproteins or bioplexes) are highly bioavailable and have a better resumption from the digestive tract of humans and animals (Dobrzanski and Jamroz, 2003). *Saccharomyces cerevisiae* represents a valuable supplement to human diet because of high content of proteins and vitamins. It is known for its ability to accumulate metal ions from aqueous solutions and to form organically bounded microelements (Mrvčić et al., 2007; Stehlik-Tomas et al., 2003). The efficiency of metal uptake by biomass depends on the chemistry of the metal ions, the specific surface properties of the organisms, cell physiology and physicochemical influences from the environment (Godlewska-Zlkiewicz, 2006). Yeast cells bind double valence metals on their outer surface (biosorption) due to the phosphomannan content in their cell walls and the presence of free carboxyl, hydroxyl, amine, phosphate and hydrosulphide groups in the surface proteins (Blackwell et al., 1995). The highest biosorption of microelements from the medium usually takes place in the first period of biomass cultivation. This stage involves the accumulation of metal ions on the outer surface of a cell and their binding with intramicrofibrillar structures of the cell wall by adsorption, complexing, ion exchange and precipitation. The process is fast and temperature and energy independent. After that, a slower binding of microelements with intracellular structures takes place (bioaccumulation) (Blackwell et al., 1995).

The aim of the present study was to evaluate the optimal growth conditions and suitable source of manganese enrichment of the yeast biomass (with manganese) as a promising mineral food and feed supplement. Additionally, the mechanism of Mn-biomass interaction was investigated by FTIR method.

Materials and methods

Inoculum preparation

Saccharomyces cerevisiae strains TVG4 and SM2 used in this work were taken from the culture collection of the Faculty of Food Technology and Biotechnology, University of Zagreb. The yeasts were maintained on slant agar yeast medium (YM) containing (in g/L): glucose - 20; bactopeptone - 10; yeast extract, 5 and agar - 20. For the inocula preparation the yeasts strains were reinoculated from agar slants into test tubes each containing 10 mL of sterile liquid YM and incubated in a thermostat at 30 ^{0}C for 24 h. After that 200 mL of sterile liquid YM in 500 mL Erlenmeyer flasks were inoculated with 5 % of the obtained inocula. Flasks were shaken on a rotary shaker at 150 rpm and 30 ^{0}C for 12 h. Such prepared inocula were used in fermentation experiments.

Batch Process

The composition of the basal medium (BM) for yeast cultivations was (in g/L): sucrose – 50; bactopeptone – 10; yeast extract – 5. The composition of the molasses medium (MM) was (in g/L): molasses – 90 (corresponding to 50 g/L sucrose); $(NH_4)_2HPO_4$ – 10; $(NH_4)_2SO_4$ – 5. The medium was sterilised at 121 ^{0}C for 10 min. After cooling to 30 ^{0}C, it was inoculated with about 1 g yeast dry matter /L. The batch processes were performed in 500 mL Erlenmeyer flasks with 200 mL of BM or MM. Cultivations were performed in BM or MM with and without addition of Mn^{2+} (added as $MnSO_4$ or $MnCl_2$), in static conditions (thermostat), shaken cultures (shaker, at 150 rpm) or aerated cultures (bioreactor, at 400 rpm and 3 l air/Lmin) and at 30^{0}C during 12 hours. There were three runs referring to various Mn^{2+} concentrations (0–2.4 mM). After fermentation, yeast biomass was washed twice with deionised water. Samples were analysed three times in parallel for yeast biomass (expressed as biomass dry matter) and ethanol concentrations.

Analysis

The yeast dry matter was determined by drying the yeast biomass at 105 ^{0}C to a constant weight after centrifuging 5 mL of samples at 2325 g for 10 min on a portable centrifuge. Ethanol concentration in the medium was determined by alcohol dehydrogenase method (Bergmeyer, 1974). Manganese ion concentrations in yeast cells were analysed using a "Varian" Spectra AA 300 Atomic Absorption Spectrophotometer (Varian Techton Pty. Limited, Mulgrave, Australia). Manganese concentrations were determined by reference to an appropriate standard metal solution. As qual-

ity control samples whey powder (IAEA-155) was used as commercial reference material. The analysis of variance was carried out with StatSoft, Inc. STATISTICA (data analysis software system), version 7.1 (Tulsa, USA).

FT-IR spectroscopy

The FT-IR spectra of two samples: dried blank biomass and biomass enriched with manganese were recorded. After incubation times, samples were taken and centrifuged at 2325 g, for 20 min. Yeast cells were washed twice with ultra pure water and dried in vacuum. The samples for FT-IR analysis were prepared by pressing powdered KBr pellets mixed with 1 % of ground powder of each samples and determined on a Bomem MB 100 Mid FT spectrophotometer (ABB Bomem, Quebec, Canada). The spectra were recorded in the region of 2000 – 500 cm^{-1} at a resolution of 4 cm^{-1}.

Results and discussion

The influence of manganese addition and media composition on yeast growth and ethanol production

The aim of the experiments was to illustrate the influence of manganese addition to different fermentation media on yield of biomass and ethanol. The rates of yeast growth and ethanol production influenced with Mn addition in sucrose and molasses media are presented in Fig. 1 and Fig. 2.

It has been reported that the addition of microelements in fermentation medium can increase the biomass yield (Bayer, 1983). Our results show (Fig. 1. and Fig. 2.) that the addition of Mn^{2+} to both media did not increase the growth of *Saccharomyces cerevisiae* SM2. The yeast growth was inhibited after Mn addition at higher concentrations into sucrose medium (Fig. 1). In the molasses medium the inhibitory effect of manganese ions has not been observed due to numerous organic ligands present in molasses that can bind metal ions (Azenha et al., 1995). Statistically significant ($p<0.05$) difference in inhibitory effect between different types of added manganese salts was observed in sucrose medium. The growth was significantly inhibited over 0.2 mM when Mn^{2+} was added as chloride or 0.8 mM when Mn^{2+} was added as a sulpfate.

The ethanol yield decreased with the addition of Mn^{2+} in sucrose medium. The ethanol synthesis was significantly inhibited in media with Mn^{2+} concentration higher than 0.8 mM when Mn^{2+} was added as chloride or higher then 1.2 mM when Mn^{2+} was added as sulphate. The results for the *Saccharomyces cerevisiae* TVG4 were almost the same (data not shown).

Incorporation of Manganese Ions into Yeast Biomass During Alcoholic Fermentation

The influences of the added manganese salt type (0.6 mM, inhibitory concentration of chloride but non-inhibitory concentration of sulphate), yeast strain, and cultivation conditions on the Mn^{2+} accumulation in the yeast biomass grown on sucrose medium are presented in Fig. 3. and Fig. 4. Metal ions are taken up by actively growing yeast cells through specific membrane transporters. Mn^{2+} uptake is regulated by the posttranslational control of Smf1p and Smf2p trafficking and degradation. In order to be transported intracellularly metals should be in free ionic forms in yeast growth media (Godlewska-Zlkiewicz, 2006). So, with an equal concentration of manganese ions added to both media, there was a very low mass of ions incorporated into yeast biomass grown on

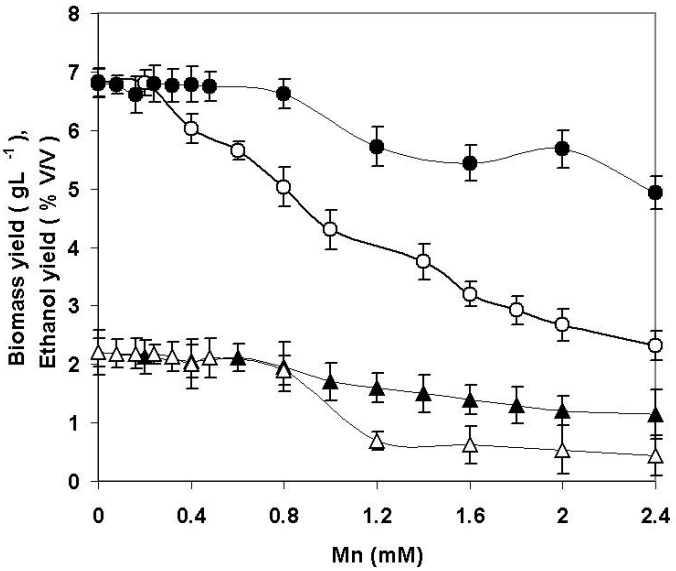

Figure 1. Yields of yeast biomass *S. cerevisiae* SM2 (o) and ethanol (Δ) obtained after 12 h of fermentation, with the addition of 0–2.4 mM Mn^{2+}; as sulphate (full symbol) or chloride (open symbol) to sucrose medium under shaken culture conditions.

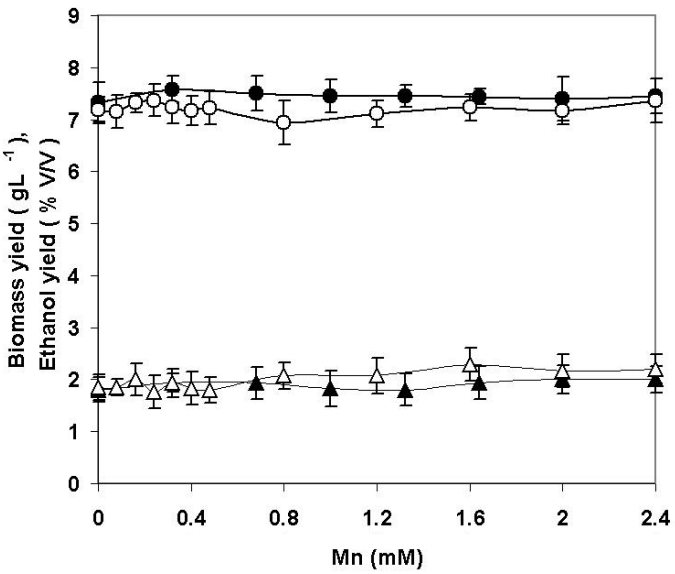

Figure 2. Yields of yeast biomass yeast *S. cerevisiae* SM2 (o) and ethanol (Δ) obtained after 12 h of fermentation, with the addition of 0–2.4 mM Mn^{2+}; as sulphate (full symbol) or chloride (open symbol) to molasses medium under shaken culture conditions.

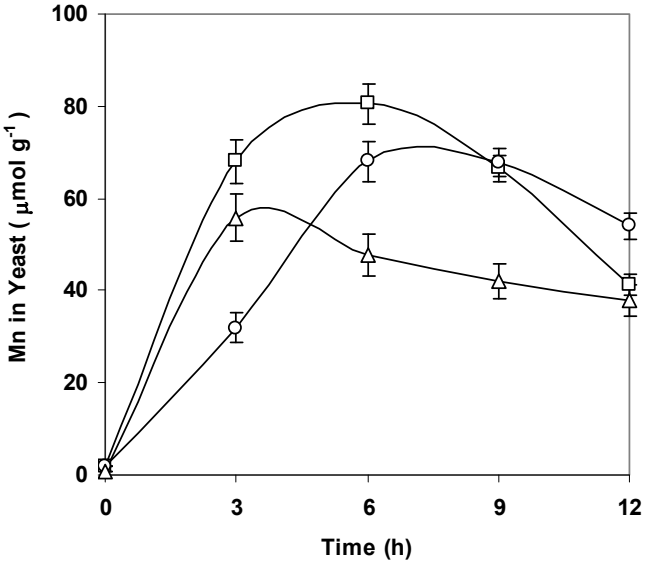

Figure 3. Accumulation of manganese in biomass of yeast *S. cerevisiae* SM2 with the addition of 0.6 mM Mn^{2+}, added as chloride into sucrose medium: static conditions (-□-), shaken culture (-o-) and aerated culture (-Δ-).

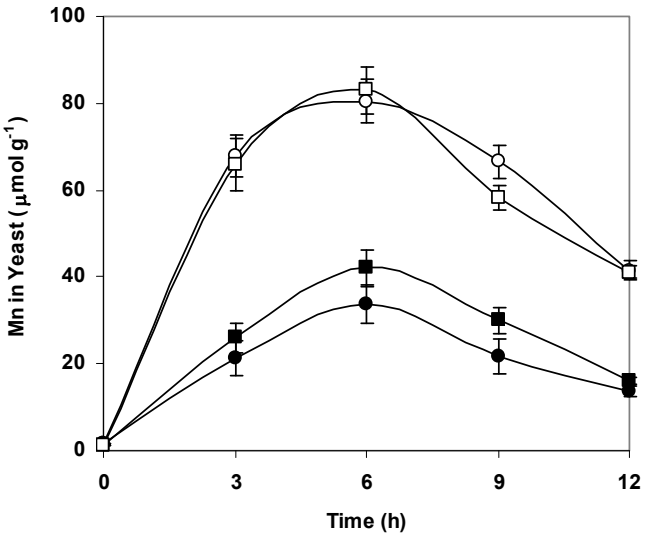

Figure 4. Accumulation of manganese in biomass of yeast *S. cerevisiae* SM2 (-o-) and *S. cerevisiae* TVG_4 (-□-) with the addition of 0.6 mM Mn^{2+}; as chloride (open symbol) or sulphate (full symbol) into sucrose medium.

molasses medium when compared with the sucrose media (data not shown). This is why the kinetics of manganese incorporation into yeast biomass has been studied only in the sucrose media.

The kinetics presented in Fig. 3. show a substantial difference ($p<0.05$) in the rate and mass of the incorporated manganese under different aeration conditions. A higher amount of manganese was incorporated into the yeast biomass under a lower aeration. The highest value of incorporated manganese was obtained during the first six hours of fermentation in static conditions, while in aerated cultures the highest value was obtained during three hours of fermentation. Apparently, the rate of manganese ions incorporation in aerated cultures is highest during the first few hours when the vitality of the yeast cells is at maximum and when cell division is taking place. The results concur with findings that the highest uptake rates of various metal ions by yeast biomass occur in various growth phases. The maximum rate of iron incorporation into the yeast cells of *S. cerevisiae* in the molasses medium occurs in the first four hours of fermentation (Stehlik-Tomas et al., 2003), while the highest uptake of zinc occurs in the first hour of fermentation (Walker, 2004). Blackwell et al. (1995) claimed that the manganese uptake and toxicity is strongly dependent on the intracellular and extracellular magnesium concentrations. The results also show that the mass of the incorporated manganese ions was reduced towards the end of fermentation. It was reported that cells release metal ions as a consequence of the aging process and the reduction in the cell charge as well (Walker, 2004). Blazejak et al. (2002) explained the release of Mg^{2+} at the stationary growth stage of brewer's yeasts by activation of cell mechanisms to prevent the cell from magnesium supersaturation. Mixing during fermentation can be assumed to cause the release of those Mn^{2+} ions that are bound to extracellular groups in the cell wall.

Statistical analysis of the data presented in Fig. 4. has not pointed out any significant difference ($p>0.05$) in incorporated manganese ions between various strains of the yeast *S. cerevisiae*. On the other hand, type of added Mn^{2+} salts and cultivation conditions have a statistically significant impact ($p<0.05$) on the incorporation of manganese. It is evident that higher amounts of manganese are incorporated if manganese is added as chloride. The comparison of these results with the results presented in Fig. 1 showed out that the inhibition of yeast growth with chloride was higher then with sulphate. Most probably, the reason for that is the concentration of free Mn^{2+} in the medium that can interact with yeast cell. Mn^{2+} is classified among hard/transitive cations (Avery and Tobin, 1993). Considering its electronegativity, charge and ionic radius, it binds preferentially to hard ligands. Stability of Mn^{2+} complex with hard anion SO_4^{2-} is higher than stability with soft anion, such as Cl^- (Avery and Tobin, 1993). Thus, when Mn^{2+} is added in the form of chloride, its availability for interactions with groups on cell surface is higher, which correlates with prerequisite conditions of transporting metal into the yeast cell.

FT-IR spectrum of *S. cerevisiae*

The role of various functional groups in the mechanism of manganese binding can be defined by FT-IR spectroscopy. FT-IR spectra of microorganisms are usually divided into five regions (Naumann et al., 1991). These regions contain information from different cell components: 3000 – 2800 cm^{-1}: fatty acids in the bacterial cell membrane; 1800 – 1400 cm^{-1}: amide bands from proteins and peptides; 1400 – 1200 cm^{-1}: region of proteins and fatty acids; 1200 – 900 cm^{-1}: polysac-

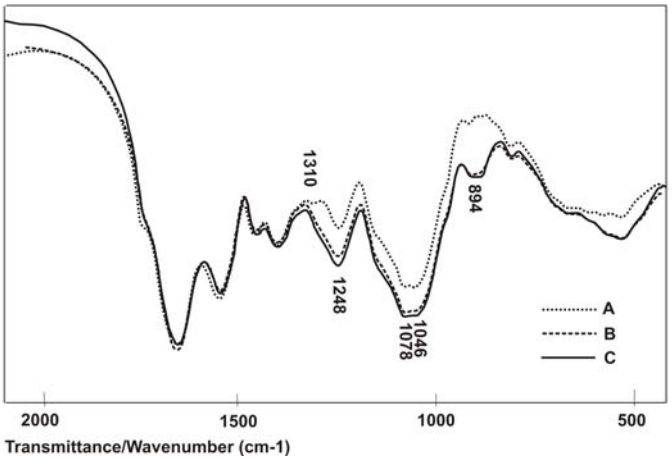

Figure 5. FT-IR spectra of *S. cerevisiae* SM2: blank biomass (A); biomass enriched with 0.6 mM Mn^{2+} in form of $MnSO_4$ (B) and biomass enriched with 0.6 mM Mn in form of $MnCl_2$ (C) after 12 h of fermentation in shaken culture conditions on sucrose medium.

charides within the cell wall and 900 – 500 cm^{-1} region containing bands which cannot be assigned to special functional groups. The FT-IR spectra of blank (a) and manganese loaded yeast biomass (b, c) are presented in Fig.5.

The major IR absorption bands of the *S. cerevisiae* are peptides and saccharides (Lin et al., 2005). Because of that specific region of proteins and peptides and polysaccharides within the cell wall (2000 – 500 cm^{-1}) was picking out. The spectrum of the blank biomass displays several typical functional groups; distinct and sharp adsorptions at 1653 cm^{-1} (N-H bending) and 1545 cm^{-1} (C-O stretching) indicating amide I and amide II band of amide bond in *N*-acetyl glucosamine polymer or of the protein peptide bond and 1456 cm^{-1} indicating CH_3 bending in proteins. Absorptions at 1399 cm^{-1} and 1242 cm^{-1} indicate O-H bending and C-O stretching bands of the carboxylate ion group (COO^-). Also, distinct absorption peaks can be seen at 1078 cm^{-1} and 1046 cm^{-1}, indicating saccharides hydroxyl groups. In comparison between blank (A) and manganese loaded yeast biomass (B, C) it was observed that there was some shift in FT-IR spectra, due to metal binding process with yeast biomass. The shift in band intensity at 1242 cm^{-1} suggested manganese binding onto carboxyl groups of amino acids. Also, disappearance of peak at 1310 cm^{-1} can be observed, suggesting the involvement of N-H groups of secondary amide in manganese binding. Although the region of 900 – 500 cm^{-1} is not specific for special functional groups, some authors reported adsorptions peaks near 900 cm^{-1} as a result of S-O stretching in sulfonate group (Pavasant et al., 2006). The shift in FT-IR spectrum in blank and manganese loaded yeast biomass in region 800-900 cm^{-1} can also be observed. Although the experiments have shown significant impact of manganese salt type on the mass of incorporated manganese ions in yeast biomass, no change has been observed in any of the characteristic absorbance peaks between yeast biomass loaded with sulphate (Fig 5 - B) or chloride (Fig 5 - C) manganese salts, suggesting the same mechanism of manganese binding.

Conclusions

Our study showed that the higher concentration of free Mn^{2+} in a medium causes higher inhibition of fermentation. This inhibition strongly depends on the type of Mn-salt and on media composition. Also, incorporation of Mn^{2+} into yeast cells depends on the aeration conditions (it is higher in static than in aerobic conditions) and does not depend on the *S. cerevisiae* strain. Type of Mn-salt (stability of complex) influences availability of Mn^{2+} in media and consequently strongly influences the accumulation of manganese in *S. cerevisiae*. We have determined marginal concentration of manganese above which growth and ethanol production inhibitions occur. This marginal concentration is optimal for the production of manganese enrichment yeast biomass. On the other hand, although the incorporation of Mn^{2+} into the yeast is higher in static than in shaken or aerated culture conditions, aerobic conditions seem to be a more promising way for Mn removal from solution because of usual higher biomass yield. The *S. cerevisiae* strains were chosen for this study because this yeast possesses several attributes as a biomineral nutrient, including safety (non-pathogenicity), availability (economy), well-developed technology, public acceptability and nutritional value. The change of FT-IR spectrum of manganese loaded yeast biomass in proteins region indicates manganese ions in the form of metalloproteins complex in yeast biomass. This demonstrates that *S. cerevisiae* can be employed for trace enrichment with manganese.

References

Avery S.V., Tobin J.M. (1993). Mechanism of adsorption of hard and soft metal ions to *Saccharomyces cerevisiae* and influence of hard and soft anions. Appl Environ Microbiol 59: 2851-2856

Azenha M., Vasconcelos T., Cabral J.P.S. (1995). Organic ligands reduce copper toxicity in *Pseudomonas syringae*. Environ Toxicol Chem 14(3): 369-373

Bayer K. (1983). Trace element supplementation of cheese whey for the production of feed yeast. J Dairy Sci 66: 214-220

Bergmeyer H.U. (1974). Methods of Enzymatic Analysis. Academic Press, New York.

Blackwell K.J., Singleton I., Tobin J.M. (1995). Metal cation uptake by yeast: a review. Appl Microbiol and Biotechnol 43: 579-584

Blazejak S., Duszkiewicz-Reinhard W., Gniewosz M., Rostkowska Demner E. (2002). The study of *Saccharomyces cerevisiae* brewery yeast strain capacity of binding with magnesium in dynamic conditions. EJPAU 5(2): 1-8

Dobrzanski Z., Jamroz D. (2003). Bioavailability of selenium and zinc supplied to the feed for laying hens in organic and inorganic form. EJPAU 6: 1-8

Freeland-Graves, J., and Llanes, C (1994). Models to study manganese deficiency. In Manganese in Health and Disease (D. J. Klimis-Tavantzis, Ed.), CRC Press, Boca Raton, USA.115–120

Godlewska-Zlkiewicz B. (2006). Microorganisms in inorganic chemical analysis. Anal Bioanal Chem 38(4): 114-123

Husain S. (1997). The effect of chronic exposure of manganese on antioxidant enzymes in different regions of rat brain. Neurosci Res Commun 21: 135-144

Lin Z., Wu J., Xue R., Yang Y. (2005). Spectroscopic characterization of Au^{3+} biosorption by waste biomass of *Saccharomyces cerevisiae*. Spectrochim ActaPart A 61: 761-765

Mrvčić J., Stanzer D., Stehlik-Tomas V., Škevin D., Grba S. (2007). Optimization of Bioprocess for Production of Copper-enriched Biomass of Industrially Important Microorganism *Saccharomyces cerevisiae*. J Biosci Bioeng 103(4): 331-337

Naumann D., Helm D, Labischinski H., Giesbrecht P. (1991). The characterization of microorganism by Fourier-transform infrared spectroscopy. In: Modern Techniques for Rapid Microbiological Analysis (W H Nelson, ed), VCH, New York, 43-96

Pavasant P., Apiratikul R., Sungkhum V., Suthiparinyanont P., Marhaba T.F. (2006). Biosorption of Cu^{2+}, Cd^{2+}, Pb^{2+}, and Zn^{2+} using dried marine green macroalga *C. lentillifera*. Biores Technol 97: 2321-2329

Simić D., Budić I. (2003). Trace elements. Acta Fac Med Naiss 20(4): 189-202

Stehlik-Tomas V., Grba S., Stanzer D., Vahčić N., Gulan-Zetić V. (2003). Uptake of iron by yeast cells and its impact on biomass production. Acta Aliment 32(3): 279-287

Underwood, E.J., in Trace Elements in Human and Animal Nutrition, Academic Press, London, 2nd Ed, 1962, 100-122

Walker M.G. (2004). Metals in yeast fermentation processes. Adv Appl Microbiol 54: 197-229

acs74_57