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ORIGINAL PAPER

Obtaining yeast biomass enriched with copper, zinc and manganese

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Abstract

Saccharomyces strains were tested for their ability to resist to high concentrations of Cu^{2+}, Zn^{2+}, and Mn^{2+} as a way to obtain copper, zinc and manganese enriched yeast.

Cell growth was determined by measuring OD at 590 nm, copper, zinc and manganese concentrations were determined by ICP-MS and AAS and culture media was optimized using Hadamard matrix.

Growth kinetics for Saccharomyces cerevisiae ICCF 349 strain in the presence of different concentrations of copper, zinc and manganese was investigated.

A distinct way for addition of minerals in the cultivation media was applied during the fermentation process.

By optimizing media factors we obtained yeast biomass enriched with 1145.8 mg×l^{-1} copper, 1143.4 mg×l^{-1} zinc and 33.9 mg×l^{-1} manganese.

Keywords: yeast enriched with copper, zinc and manganese, ICP-MS, AAS

Introduction

Manganese functions as a component of several enzymes involved in carbohydrate, lipid and protein metabolism. Exposure to high concentrations of manganese leads to manganism, Parkinson's disease [1;2] and Alzheimer’s [3]. Cellular inhibition was observed, indicating that high amounts of Cu^{2+} and Mn^{2+} ions have a toxic effect.

Inorganic copper has a strong pro-oxidant effect and (if not bound to proteins), can stimulate lipid peroxidation in feed or the intestinal tract [4].

To investigate the survival capacity of yeast cells in presence of Cu^{2+} and Mn^{2+} various amounts of Cu^{2+} (0 –10 mM) or Mn^{2+} (0 –12 mM) were added to yeast cell cultures during the exponential growth phase in YPD medium (glucose - 2%, wt/vol, yeast extract - 1%, wt/vol, peptone - 2%, wt/vol, and agar - 2%, wt/vol) and then cell death ratios were analyzed. After 12-h incubation, survival was assayed by CFU counts on YPD plates. The percentage of survival of cells decreased with increasing concentrations of Cu^{2+} in the medium. Significant toxicity was observed starting between 4 and 8 mM, and 10% wild-type (WT) cells survived when they were exposed to 10 – 12 mM Mn^{2+}. These results demonstrated that Cu^{2+} and Mn^{2+} ions induce cell death starting from several millimolar levels [5].

Zinc, copper and manganese play an important role in antioxidant defense as an integral part of SOD. Superoxide dismutase (SOD) constitutes a primary cellular defense against oxidative stress in most organisms. There are two forms of SOD in eukaryotic cells.

In *Saccharomyces cerevisiae,* CuZnSOD and MnSOD are encoded by nuclear genes *SOD1* and *SOD2,* respectively. As in other eukaryotes, yeast MnSOD is located in mitochondrial matrix [6].

Superoxide dismutase, which exists in mitochondrial (Mn-SOD) and cytoplasm forms (Cu/Zn – SOD), catalyses the conversion of two O_2 molecules into H_2O_2 and O_2 [7].

Zinc, copper and manganese ions are very interesting because they have a positive effect on the respiratory activity and the growth rate of *Saccharomyces cerevisiae* [8].

The objective of the researches presented in this paper was to point out the best method for the addition of the copper, zinc and manganese in order to assure their accumulation in the yeast biomass and to evaluate their impact on the yeast cells viability. Optimization of the copper, zinc and manganese concentrations was performed during the fermentation in semi-aerobic conditions, at 28-32^{0}C, pH 4-6.5, aiming to obtain yeast enriched with copper, zinc, and manganese.

Materials and methods

Microorganisms

Saccharomyces cerevisiae ICCF 349 and *Saccharomyces bayanus* ICCF 385 from the Culture Collection of National Institute for Chemical Pharmaceutical R & D were screened; yeast cells resistant to minerals were selected and used in the fermentation process, for obtaining the biomass enriched with copper, zinc and manganese.

Culture media:

1. malt extract broth supplemented with copper, zinc and manganese (stock solution), for screening;
2. malt extract - peptone hy soy - agar medium, for strain selection.
3. yeast extract - malt extract - sucrose - peptone hy soy - agar medium (YMSP-A), for strain conservation and for preinoculum (static culture) preparation;
4. yeast extract – malt extract and sucrose (YMS), for inoculum (submerged culture) preparation

Stock solutions

- copper sulfate solution – prepared by dissolving 1 g % in distilled water, heated at 40^{0}C, pH ~5.3.
- zinc sulfate solution - prepared by dissolving 1 g % in distilled water, heated at 40^{0}C, pH ~5.3.
- manganese sulfate solution - prepared by dissolving 1 g % in distilled water, heated at 40^{0}C, pH ~5.3.

All the solutions above mentioned were filtered on G5 filter.

Screening method [9]

The screening method for the yeast strains comprises the selection of the best formed yeast colonies grown in a medium enriched with different concentrations of copper, zinc and manganese, through the following stages:

(1) a 100:1 dilution of each actively growing yeast colony (culture) into malt extract broth was made;

(2) the growth of the cells was monitored by measuring the optical density at 590 nm (OD_{590}) and the cultures with the highest OD values were selected;

(3) the selected yeast cultures were streaked on malt extract agar medium (on plates), and were incubated at 28-30^{0}C for 5-7 days;

(4) the most well – formed yeast colonies were selected.

(5) stock culture (adapted culture) was obtained by growing the selected yeast colonies on YMSP agar, in 3-4 slants at 28-30^{0}C, for 48 h.

Inoculum and fermentation cultures

The inoculum (submerged culture) was prepared from a preinoculum culture (stock culture), in 500 ml Erlenmeyer flasks containing 100 ml YMS liquid medium. Inoculum is a culture developed for 17 hours at 28°C, at 240 rpm, in a shaking incubator, GFL-3033.

The submerged cultures (fermentation stage) were developed in 500 ml Erlenmayer flacks containing 100 ml media, at 28-30^{0}C, with continuous shaking, using an inoculation ratio of 15%.

Cell concentration

The number of cells in the yeast cultures was measured by the viable plate count method. Decimal diluted samples were spread on the MP- agar medium and the number of colonies was counted after incubation at 28-30^{0}C for 48h.

The wet cells weight (g/l) and dry cells weight (g/l) was also measured at the end of the cultivations.

The optimization method

The purpose of optimization is to determine the optimal values for the factors leading to a maximum value of the system response, provided that the parameter which quantifies the answer is technically and economically relevant [10, 11].

The optimization methodology allows the study of a large number of factors in order to determine their interaction and to obtain one or more experimental answers; the answer is the result of an experience (e.g.: concentration of the obtained biomass).

A factorial experimental plan starts from a matrix of experiences that allows to evaluate the influence of the "K" factors with two levels of response, studied in N number of experiments so that N = K +1, with a minimum of attempts (N is a multiple of four). These are square matrix (called Hadamard matrices) that contains elements corresponding to two levels of factors, noted with +1 and -1, against a basic value (control witness).

Matrices are constructed through circular permutation starting from a basic generator, the factors of last experiences being always taken as level -1.

The Box Behnken design experimented by Palukurty [12] allows the estimation and interpretation on the interactions between various variables at a time, during the optimization process.

To optimize the composition of the culture media, we used an experimental plan based on a matrix (Hadamard matrix) with a number of factors K = 7, referring to the concentration of: sucrose, yeast extract, $MnSO_4$, $ZnSO_4$, $CuSO_4$, vitamins, $FeCl_3$ and with two levels of concentration, in a number of eight experiments (N=K+1). According to the method, for a number of factors K = 7, a matrix with eight lines is obtained (Table 1).

Table 1. Experimental matrix for K=7 and N=8

Variants exp (N)	*Medium factors*							*Answer*
	X_1	**X_2**	**X_3**	**X_4**	**X_5**	**X_6**	**X_7**	
1	+	+	-	-	-	+	+	y_1
2	+	+	+	-	-	-	+	y_2
3	+	+	+	+	-	-	-	y_3

	Medium factors							
4	**-**	+	+	+	+	**-**	**-**	y_4
5	**-**	**-**	+	+	+	+	**-**	y_5
6	**-**	**-**	**-**	+	+	+	+	y_6
7	+	**-**	**-**	**-**	+	+	+	y_7
8	**-**	**-**	**-**	**-**	**-**	**-**	**-**	y_8
9	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**Blind sample**

Analytical assay

Total concentrations of copper, zinc and manganese

Atomic spectrometric techniques, AAS, ICP-AES and ICP-MS are widely used for analysis of trace elementals [13, 14]. AAS is probably the most extensively used techniques for determination of metals in different sample matrices [15]. In our study, the zinc and copper concentrations were measured using an atomic absorption spectrophotometer, A Analyst 800 (Perkin Elmer) equipped with air-acetylene flame. An electrode free discharge lamp (EDL) for Zn, whereas hollow cathode lamp (HDL) for Cu were used.

Several ICP-MS procedures have been reported for determination of single and multi-elements in drugs and pharmaceuticals [16]. Determination of manganese concentration in our study was performed with a Perkin Elmer ELAN DRC-e inductively coupled plasma mass spectrometer (ICP-MS), using ^{55}Mn. The ELAN DRC-e uses Dynamic Reaction Cell (DRC) technology to eliminate polyatomic interferences.

Results and discussion

The *Saccharomyces cerevsiae* and *Saccharomyces bayanus* colonies were treated with different concentrations of copper, zinc and manganese as sulfate salts (tables 2 and 3). The yeast colonies that survived formed the static cultures.

We selected the *Saccharomyces cerevisiae* survior colonies with 1000 mg l^{-1} $CuSO_4$, 1000 mg l^{-1} $ZnSO_4$ respectively 1250 mg l^{-1} $MnSO_4$, obtaining 2,14 x 10^4 CFU/ml (table 2). From these, the most developed yeast colonies were selected to form the stock culture.

Table 2. Influence of zinc, copper and manganese on the yeast cells growth and survival

Saccharomyces cerevisiae colony	$ZnSO_4$ mg l^{-1}	$CuSO_4$	$MnSO_4$	OD_{590nm}	UFC/ml
	1250	1000	1000	0,762	0,5 x 10^4
	1250	1000	1250	0,760	1,27 x 10^4
	1250	1250	1250	0,658	0,5 x 10^3
	1000	1250	1250	0,710	0,57 x 10^3
	1000	1250	1000	0,760	0,58 x 10^3
	1000	1000	1250	0,744	2,14 x 10^4

Saccharomyces bayanus colonies were treated with different concentrations of minerals. (table 3).

Table 3. Influence of zinc, copper and manganese on yeast cell growth

Saccharomyces bayanus colony	$ZnSO_4$	$CuSO_4$	$MnSO_4$	DO_{590nm}
	mg l^{-1}			
	500	-	-	1,654
	-	500	-	1,036
	-	-	500	1,768
	500	500	-	0,920
	500		500	1,745
		500	500	0,900
	500	500	500	0,719
	-	-	-	1,942

When, for screening purposes, copper, zinc and manganese were added in the culture media, the optical density was very small, decreasing from 1,942 (without minerals – parental culture) to 0,719 (with minerals' addition). For higher concentrations of microelements the cells viability was small, the cells becoming inactive from the biological point of view.

For *Saccharomyces cerevisiae* yeast strains selected after the screening, we pointed their capacity to grow in submerse conditions, in media containing 100 mg l^{-1} $ZnSO_4$, 150 mg l^{-1} $CuSO_4$ and respectively, 75 mg l^{-1} $MnSO_4$, added at time zero in the fermentation medium (figure 1 A, B).

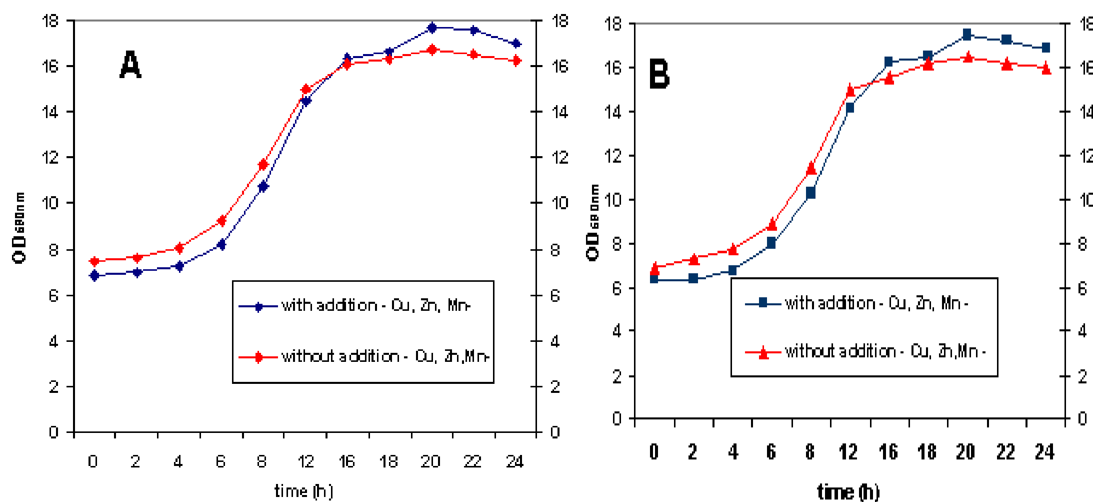

Figure 1. The influence of Cu^{2+}, Zn^{2+} and Mn^{2+} addition on the *S.cerevisiae* cells growth (cultivation A and B)

In accordance with the optimization method, we evaluated the average value of the answers (b_0) = $\sum \frac{y_i}{N}$ and the linear coefficients (b_1,.....,b_7) from the table 4 and 5, corresponding to the optimized medium factors.

Table 4. Corespondence between experimental factors and their levels of concentrations in the culture media

Media factors		*Concentrations levels (g%)*		
		-1	**0**	**+1**
X_1	X_1 succrose (g%)	6,0	7,0	8,0
X_2	X_2 yeast extract (g%)	0,1	0,3	0,5

		Concentrations levels (g%)		
X_3	X_3 manganesse sulfate ml	0,75	1	1,250
X_4	X_4 zinc sulfate ml	0,5	0,75	1
X_5	X_5 copper sulfate ml	0,5	0,75	1
X_6	X_6 vitamins ml	0,5	1,0	1,5
X_7	X_7 feric chlorure ml	0	0,1	0,2

Table 5. Cellular concentrations (yeast biomass) (y_i) obtained in different variants experimented during the optimization of cultivation medium

Variants exp (N)	Medium factors							Answer	
	succrose (X_1) g%	**Yeast extract (X_2) g%**	**$MnSO_4$ (X_3) ml**	**$ZnSO_4$ (X_4) ml**	**$CuSO_4$ (X_5) ml**	**Vitamins (X_6) ml**	**$FeCl_3$ (X_7) ml**	**(y_i)**	**DCW (g/l) (dry cells weight)**
1	8	0,5	0,750	0,5	0,5	1,5	0,2	y_1	5.8
2	8	0,5	1,25	0,5	0,5	0,5	0,2	y_2	5.8
3	8	0,5	1,25	1,0	0,5	0,5	0	y_3	6.7
4	**6**	**0,5**	**1,25**	**1,0**	**1,0**	**0,5**	**0**	y_4	**7.49**
5	6	0,1	1,25	1,0	1,0	1,5	0	y_5	54
6	6	0,1	0,75	1,0	1,0	1,5	0,2	y_6	4.75
7	8	0,1	0,750	0,5	1,0	1,5	0,2	y_7	4.65
8	6	0,1	0,75	0,5	0,5	0,5	0	y_8	4.10
Blind sample	7	0,3	1,0	0,75	0,75	1	0,1	**M**	6.8

$b_0 = (5.8 + 5.8 + 6.7 + 7.4 + 5.4 + 4.75 + 4.65 + 4.10)/ 8 = 44.6 /8 = 5.575$
$b_1 = (5.8 + 5.8 + 6.7 - 7.4 - 5.4 - 4.75 + 4.65 - 4.10)/ 8 = 1.3/8 = 0.16$
$b_2 = (5.8 + 5.8 + 6.7 + 7.4 - 5.4 - 4.75 - 4.65 - 4.10)/8 = 6.8/8 = 0.85$
$b_3 = (- 5.8 + 5.8 + 6.7 + 7.4 + 5.4 - 4.75 - 4.65 - 4.10)/8 = 6/8 = 0.75$
$b_4 = (- 5.8 - 5.8 + 6.7 + 7.4 + 5.4 + 4.75 - 4.65 - 4.10)/8 = 3.9/8 = 0.4875$
$b_5 = (- 5.8 - 5.8 - 6.7 + 7.4 + 5.4 + 4.75 + 4.65 - 4.10)/8 = -0.2/8 = - 0.025$
$b_6 = (5.8 - 5.8 - 6.7 - 7.4 + 5.4 + 4.75 + 4.65 - 4.10)/8 = - 3.425/8 = - 0.428125$
$b_7 = (5.8 + 5.8 - 6.7 - 7.4 - 5.4 + 4.75 + 4.65 - 4.10)/8 = - 2.6/8 = - 0.325$

From the obtained results, a classification of the factors with significant influence on the answer was made.

Thus, $b_i > 0$ = positive influence and $b_i < 0$ = negative influence, obtaining the linear objective polynomial function of the form:

$Y = 5,575 + 0,16 (X_1) + 0,85 (x_2) + 0,75 (x_3) + 0,48 (x_4) + (-0,025) (x_5) + (-0,428) (x_6) + (-0,325) (x_7) = 7.037$

Based on the experimentally determined linear coefficients b_1, b_2,............,b_7, we evaluated the variation step of the more important medium factors (table 6).

Table 6. Variation step of the medium factors

Media factors	X1	X2	X3	X4	X5	X6	X7
Base level	7	0,3	1	0,75	0,75	1,5	0,2
Variation unity	1,5	0,2	0,5	0,25	0,25	0,5	0
Linear coefficient c	0.16	0.85	0.75	0.4875	-0.025	-0 .428125	-0.325
U × c	2,4	0,17	0.375	0.12	-0.00625	-0,21	- 0,32

In accordance with the optimization method, the variation step of the most influent factor is chosen within a more reduced space compared to the preceding factorial plan. This allows a better settlement of the optimal regions.

A new experimental plan was established (table 7) in which variable factors are yeast extract and microelements, the rest of the ingredients remaining unchanged.

Table 7: Experimental variants for the optimization of the culture medium

Nutrients	**Experimental variants**		
	(blind samples-V10)	(V11)	(V12)
X_1 succrose (g%)	7,0	7,3	7,6
X_2 yeast extract (g%)	0,3	0,5	0,7
X_3 manganesse sulfate ml	1	1,4	1,8
X_4 zinc sulfate ml	0,75	0,9	1,04
X_5 copper sulfate ml	0,75	0,743	0,736
X_6 vitamins ml	1,0	0,8	0,6
X_7 feric chlorure ml	0,1	0,07	0,04
Monomaniacal phosphate (g%)	0,2	0,2	0,2
Potassium chloride (g%)	0,05	0,05	0,05
Magnesium sulfate (g%)	0,1	0,1	0,1
Distilled water	100 ml	100 ml	100 ml

In experiments with the medium variants from above, a significant growth was obtained concerning the DCW (variant no.12) with respect to the blind sample (table 8). We have thus considered it as the optimum medium for obtaining biomass enriched in copper, zinc and manganese in the laboratory.

Table 8. Results of the experimental variants for the optimization of culture medium

Exp.variants	**OD_{590nm}**							**pH**					**Reducing sugar (g%)**		**DCW (g/l)**
	0h	**16h**	**18h**	**20h**	**21.30h**	**22h**	**22.30h**	**0h**	**16h**	**18h**	**20h**	**22.30h**	**0h**	**16h**	22.30h
V10 (blind sample)	8.84	19.08	19.09	19.43	19.35	19.60	19.21	6.5	3.8	4.1	4.0	3.9	7	0.2	6.5
V11	8.98	19.37	19.48	19.88	19.84	20.08	19.43	↓	3.7	4.4	4.1	4.0	7.3	0.12	5.9
V12	7.98	19.47	19.45	19.50	20.00	23.1	20.25		3.8	4.7	4.3	4.3	7.6	0.12	7.87

Various approaches were experimented for the addition of cooper, zinc and manganese (one part at 0 h and another during the exponential phase of the growth).

It was observed that the biomass increase was influenced by the minerals concentration and the way they were added. Two experimental variants were selected as the best (table 9) for the next study, aiming the isolation of yeast enriched with Cu, Zn and Mn.

Table 9. Composition of fermentation medium used for obtaining yeast enriched with copper, zinc and manganese

Nutrients		V13b/1	V13b/2
Sucrose g%		9	9
Yeast extract g%		0,7	0,7
$MnSO_4$	**0h**	180	100
mg l^{-1}	**15h**	0	80
$CuSO_4$	**0h**	73,6	53,6
mg l^{-1}	**15h**	0	20
$ZnSO_4$	**0h**	100	50
mg l^{-1}	**15h**	0	50
Vitamins ml		0,5	0,5
$NH_4H_2PO_4$ g%		0.2	0.2
KCl g%		0.05	0.05
$MgSO_4$ g%		0,5	0,5
Distilled water		100 ml	100 ml

Downstream processing

The processing of the resulting fermentation medium was performed in several steps:

- separation of the yeast biomass
- purification of the yeast biomass
- drying of the yeast biomass, to 5% humidity, leading to the organometalic preparate.

The separation of the yeast biomass – a mixture of yeast cells enriched with copper, zinc and manganese – from the fermented culture media (obtained by cultivation in agitated flasks) was made by centrifugation at 2900 rpm for 20 minutes.

After the enriched yeast biomass had been separated by centrifugation, it was washed several times in order to remove the unprocessed extra cellular metallic residues.

The purification was achieved by repeated washings with an 0.1M EDTA-Na_2 solution, 0.01M Na_2HPO_4 buffer solution and distilled water.

After each washing, samples were taken from the effluent wash waters in order to determine the extra cellular inorganic copper, zinc and manganese.

By increasing the number of washes we noticed a decrease in the concentrations of copper, zinc and manganese in the effluent, but also a change in the biomass aspect.

After purification, the yeast cream was pasteurized at 75-80°C, for 45-50 minutes, in order to inactivate the microorganisms.

Table 10. Concentrations of copper, zinc and manganese from the yeast biomasses

Samples with yeast enriched with copper, zinc and manganese	Microelement contents (mg l^{-1})		
	copper	zinc	manganese
($P_{13b/1}$)	1016	1123.8	33.1
($P_{13b/2}$)	1145.8	1143.4	33.9

The Cu, Zn, Mn enriched yeast biomass (more than 10 g/l) was obtained after pasteurization and drying at 5% humidity, with a content in microelements accumulated in the dry biomass as follows: 1145.8 mg l^{-1} copper, 1143.4 mg l^{-1} zinc and 33.9 mg l^{-1} manganese (table 10).

Conclusions

Saccharomyces cerevisiae ICCF 349 and *Saccharomyces bayanus* ICCF 348 were screened for their ability to resist to Cu^{2+}, Zn^{2+} and Mn^{2+} and colonies resistant to concentrations of: 1000 mg l^{-1} $CuSO_4$, 1000 mg l^{-1} $ZnSO_4$ and 1250 mg l^{-1} $MnSO_4$ were selected.

Using an experimental factorial plan (Hadmard matrix), the influence of seven media factors (sucrose, yeast extract, manganese sulfate, zinc sulfate, copper sulfate, vitamins, ferric chloride) was evaluated, which were found to have significant effect on dry cells weight.

A distinct way for addition of minerals in the cultivation media was established during the optimization experiments. The biomass increase was influenced by the trace minerals' concentrations and the way they were added.

The optimum result of the experiments described in this study was: 14g/l yeast biomass with an enriched content in microelements accumulated, as follows: 1145.8 mg l^{-1} copper, 1143.4 mg l^{-1} zinc and 33.9 mg l^{-1} manganese.

References

1. PAL, P.K., A. SAMII, AND D.B. CALNE, "Manganese neurotoxicity: A review of clinical features, imaging and pathology", *Neurotoxicology* **20**:227-238. 1999;
2. KAISER.J., "Manganese: a high-octane dispute". *Science 300*, 926–928, 2003;
3. WAGGONER, D.J., BARTNIKAS, T. B., AND GITLIN, J. D, "The role of copper in neurodegenerative disease", *Neurobiol. Dis*. **6**, 221–230, 1999;
4. SURAI KP, SURAI PF, SPEAKE BK, SPARKS NHC "Antioxidant – prooxidant balance in the intestine", *Nutritional Genomics and Functional Foods,* **1**, 51-70, 2003;
5. Q. LIANG and B. ZHOU, "Copper and Manganese Induce Yeast Apoptosis via Different Pathways", *Molecular Biology of the Cell*, **18**, 4741–4749, December 2007;
6. K.M.O'BRIEN, R. DIRMEIER, "Mitochondrial Protein Oxidation in Yeast Mutants Lacking Manganese- (MnSOD) or Copper- and Zinc-containing Superoxide Dismutase (CuZnSOD)", *The Journal of Biological Chemistry* **279**, No. 50, Issue of December 10, pp. 51817–51827, 2004;
7. D. AL. NITA, V. NITA, "Oxidative damage following cerebral ischemia depends on reperfusion - a biochemical study in rat", *J.Cell.Mol.Med,* **5,** 163-170, 2001;
8. JONES, R.P; GADD, G.M; "*Enzyme Microbiol. Technol*" **12**, 402-418, 1990;
9. IONIȚĂ A., BĂRBULESCU I.D.,, Screening of some yeast strains for chromium yeast production with terapeutic and bioproductive effect" *Lucrări ştiinţifice Zootehnie şi Biotehnologii*, vol. XXVIII, Timişoara, 774-779, 2005;
10. SMIGELSCHI,O; WOINAROSCHY,A; "Optimizarea proceselor in industria chimica" Ed.Tehnica, Bucuresti, 179-182, 1978;
11. CHERUY,A; DUMENIL,G; SANGLIER.J, "Optimizare si modelare", 290-308, 1985;
12. M.A. PALUKURTY, "Screening and optimization of metal ions to enhance ethanol production using statistical experimental design", *African Journal of Microbiology Research,* **2**, 087-094, 2008;
13. N.H. BINGS, A. BOGAERTS, J.A.C. BROEKAERT, *Anal. Chem.* 78 3917-3946, 2006;
14. D. BEUCHEMIN, "Inductively Coupled Plasma Mass Spectrometry", *Anal. Chem.* **78,** 4111-4136, 2006;
15. J.W. JACKSON, L. SHIJUN, "Atomic Absorption, Atomic Emission, and Flame Emission Spectrometry", *Anal. Chem.* **70,** 363 – 384, 1998;
16. R. NAGESWARA RAO, M.V.N. K. TALLURI, "Review, An overview of recent application of inductively coupled plasma-mass spectrometry (ICP-MS) in determination of inorganic impurities in drugs and pharmaceuticals", *Journal of Pharmaceutical and Biomedical Analysis* 43 1-13, 2007;