Amperometric Biosensor for Monitoring Respiration Activity of *Saccharomyces cerevisiae* in the Presence of Cobalt and Zinc

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Summary

For efficient control of heavy metal concentrations electrochemical methods, such as polarography and related techniques, are applied. Their advantages are simplicity, short analysis time and small quantities of samples needed. The presence of some heavy metals, such as zinc and cobalt, accelerates the growth of yeast. For the measurements of concentration changes, amperometric biosensor containing yeast *Saccharomyces cerevisiae* was used. The influence of zinc and cobalt on respiratory activity of the yeast *Saccharomyces cerevisiae* was estimated by measuring oxygen in the solution that was earlier enriched with cobalt or zinc. Measurements were performed using modified Clark's oxygen electrode and the investigated concentrations of cobalt and zinc were up to 100 mg/L.

Key words: biosensor, *Saccharomyces cerevisiae*, cobalt, zinc

Introduction

Saccharomyces cerevisiae needs certain mineral compounds that act as functional components of proteins, as activators for enzymes or as stabilisers for proteins. It is known that some mineral compounds may stimulate the growth of yeast and some of them inhibit growth totally or partially (*1,2*).

With respect to this the elements are systematised in groups designated as very poisonous, moderately toxic, slightly toxic and non-toxic. Copper and iron were found to be only slightly poisonous, while zinc and cobalt in small concentrations induce increased activity and growth of *Saccharomyces cerevisiae*.

Interactions of fungi with toxic metals are well described with increasing interest in mechanisms of toxicity, tolerance, uptake, accumulation and cellular fate, as well as biotechnological applications (*3,4*).

Almost every aspect of fungal growth, physiology and metabolism can be affected by potentially toxic concentration of both essential and nonessential metal species, with toxic effects resulting from, *e.g.* blocking of functional groups and sites in enzymes and transport systems, displacement and/or substitution of essential metal ions in molecules and structural components, denaturation and inactivation of enzymes, and perturbation of cell and organelle membranes (*5*). The metal cations K^+, Mg^{2+}, Ca^{2+} and Zn^{2+} are known to directly influence fermentative metabolism of yeast (*6*).

Yeast cell walls are known to bind various metal ions *e.g.* calcium, copper, iron, magnesium, manganese and zinc. Zinc, having a coordination number 4, forms a tetrahedral structure, while cobalt, having a coordination number 6, lies in an octahedral structure.

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Influence of zinc

It is known (*7*) that fermentation troubles in yeast industry arise from the shortage of zinc so the necessary quantity of zinc is in the range from 0.05 to 0.2 mg/kg zinc of initial wort.

The effects of zinc deficiency on protein synthesis in higher plants, such as in *S. cerevisiae* as model organism, were also discussed (*8*). When the zinc concentration in the cells decreased to a level below 100 mg/kg (dry weight), cell growth was depressed.

Zinc ion might also play a role in pathways that are involved in cellular morphogenesis. The micronutrient zinc is nowadays implicated in an ever-growing number of cellular processes. Some proteins with associated zinc include alcohol dehydrogenase, aspartate transcarbamoylase, RNA polymerase, reverse transcriptase and a significant number of DNA binding regulatory proteins containing so-called »zinc fingers«.

Influence of cobalt

The yeast cells display good resistance to cobalt ions, present in culture medium at a concentration of 2–40 mM (*9*). The inhibitory effect of cobalt on the growth of yeast (*9*) was first studied in a natural medium. No visible growth was observed for yeast with applied concentrations of $Co(NO_3)_2 \cdot 6H_2O$ greater than 0.02 % (10^{-4} M). The pH-dependent inhibition of 22 metal salts for the yeast *S. cerevisiae* and the inhibition of growth by copper, cobalt and nickel were also studied (*10*).

The mutagenic action of cobalt salts results in mitochondrial respiratory deficiency in yeast. Low concentration of Co^{2+} in a medium stimulates growth from simple algae to complex higher plants (*11*). It has been found (*12*) that cobalt can selectively inhibit cell division in various yeasts without simultaneously inhibiting growth (defined as an irreversible increase in volume) and other metabolic processes. Thus, they have obtained the growth of yeast as elongated mycelial elements. It has been reported (*13*) that 0.3 mM cobalt is toxic to liquid culture, but not to solid cultures of yeast.

Heavy metals, mostly toxic for *S. cerevisiae*, probably inhibit their respiration activity.

Biosensor

Biosensors are self-sufficient integrated devices that enable obtaining certain quantitative analytic information through biological element of recognition (biochemical receptors) which are in constant touch with the transmitter. Indicating electrode is either cathode or anode, depending on the fact that the electrons are either brought to or taken away from the examined analyte. In amperometric biosensors, elements for selective biochemical recognition (enzymes, tissues of animal or plant origin, cell organelles, microorganisms *etc.*) are most frequently immobilised on the top of the transducer. Substances that build net structure (*14*), such as agar-agar, gelatine, alginate *etc.* and mechanically prevent scattering of cells into the examined solution are used for immobilisation of microorganisms.

Sensor system often consists of oxygen electrode and immobilised *S. cerevisiae* whose respiration activity is measured by oxygen electrode. Collagen has been widely used for the immobilisation of various cells because the method is mild to the cells, inexpensive and enables easy assemblage in spherical bed. Collagen (w=2 %) membrane and immobilised *S. cerevisiae* are attached to an oxygen electrode by cellophane strip so leakage of *S. cerevisiae* cells is prevented.

This study was undertaken to investigate the influence of heavy metals, *e.g.* cobalt and zinc, on the respiratory mechanism in backer's yeast using chemical biosensors with immobilised *Saccharomices cerevisiae*.

Materials and Methods

Basic studies of the Saccharomyces cerevisiae electrode

The respiration of immobilised microorganisms can be determined by oxygen electrode (*15*). The number of yeast cells in the membrane affects the total respiration activity of *S. cerevisiae* and therefore the current. The current decreases with increasing number of cells.

The respiration activity of *S. cerevisiae* was determined in the growth medium by measuring the current of the oxygen electrode.

The sample solution (growth medium) was saturated with oxygen (100 % of relative saturation). The electrode was inserted in the sample solution (growth medium) and the current of the electrode decreased during 5 minutes and then increased markedly. Consumption of oxygen by *S. cerevisiae* caused a decrease in dissolved oxygen around the membrane until the steady-state was reached. The steady-state indicated that the consumption of oxygen by the *S. cerevisiae* and the diffusion of oxygen from the solution to the membrane were in equilibrium. The steady-state value was attained within 10 min at 28 °C. Oxygen consumption was followed until relative saturation values under 10 % was attained.

Reagents

Yeast was purchased from Pliva, Zagreb, Croatia and polypeptone from Biolife S.r.l., AOCA, Italy. Other reagents were commercially available analytical reagents or laboratory grade materials and were used as received. Deionized oxygen free water was used in all experiments.

The standard cobalt and zinc solutions were made by serial dilution of 10^{-1} M cobalt or zinc perchlorate stock solutions. The standard solution was stored in plastic bottle and renewed weekly.

The supporting electrolyte for voltammetric determination of zinc was NaAc/HAc buffer solution in equimolar concentrations (0.1 M), while for spectrophotometric determinations of cobalt the borate buffer (0.1 M) and 4-pyridyl-2-azoresorcinol solution (PAR) (0.1 M) were used.

Microbial cells

Daily, *Saccharomyces cerevisiae* was cultured aerobically at 28 °C in 50 mL of medium (pH=7) for 6 hours
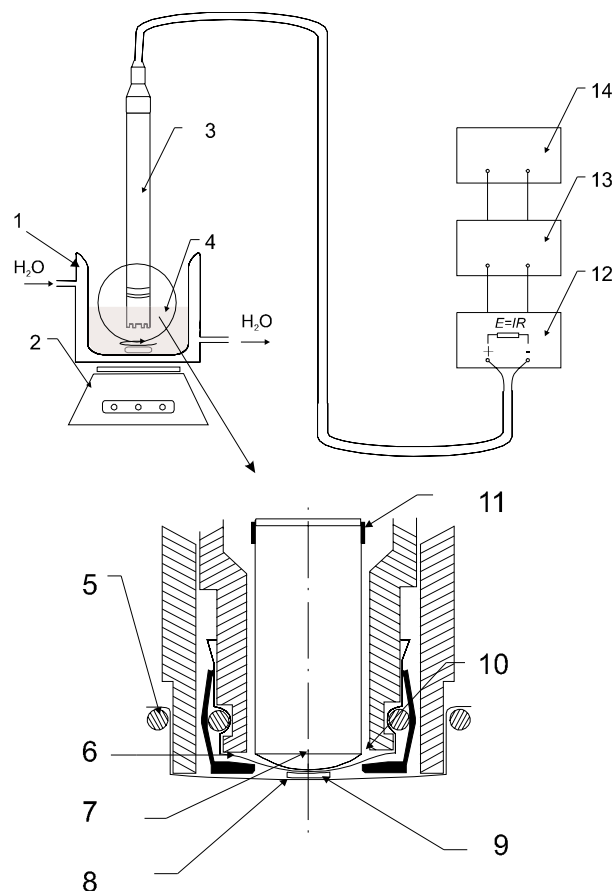
containing 2 mL ethanol (φ=96 %), 0.5 g polypeptone, 0.26 g KH_2PO_4 and 0.1 g of $MgSO_4 \cdot 7H_2O$. The cells were washed twice with physiological saline.

Preparation of the microbial membrane sensor

The yeast (2 mL of physiological saline suspension) was resuspended in collagen (w=2 %) in a suitable test tube at 25 °C and thus immobilised cells were stored in desiccator at 5 °C. Daily, volume of 10 µL suspension of immobilised cells (containing 10^7 c.f.u/mL) were dropped onto filter paper (diameter 3 mm, thickness 100 µm) and attached to oxygen electrode with permeable Teflon membrane just before measurement was performed.

Apparatus

Voltametric measurements were performed, as described in detail elsewhere (*16*) using the polarographic analyser PAR 264A (EG&G PAR, USA) connected to a XY recorder PL 3 (Lloyd, United Kingdom). A three--electrode electrochemical cell was used. The hanging mercury drop electrode (HMDE) was used as working electrode, the saturated calomel electrode (SCE) as reference electrode and Pt wire as an auxiliary electrode. To evaluate the metal ion concentration, standard addition procedure was used. Voltamograms of zinc were observed at approximately –1.0 V *vs*. SCE.

Spectrophotometric determination was performed using double–beam Perkin Elmer Lambda 200 UV/VIS. Radiation sources were deuterium and halogen lamps while absorbance was measured in 1.00-cm quartz cuvette. A sensitive and selective spectrophotometric determination of cobalt is based on the formation of a complex with PAR (*17*). Absorbance measurement was performed at 510 nm. Sensitivity of the mentioned method is between 0.1 and 0.3 µg/mL.

Respiration activities were performed by an oxygen electrode with immobilised *S. cerevisiae* connected to the computer (Fig. 1). Thus relative oxygen saturation and oxygen mass concentration with simultaneous correction for pressure and temperature were measured. All measurements were performed at 28 °C in a thermostated cell.

Results and Discussion

Prior to the determination of the influence of zinc and cobalt on respiration activity of *S. cerevisiae*, it was necessary to examine the possible contents of the mentioned metals in the growth medium. Therefore, two techniques were applied: differential pulse anoding stripping voltammetry (DPASV) for zinc determination and spectrophotometry for cobalt determination. Wet
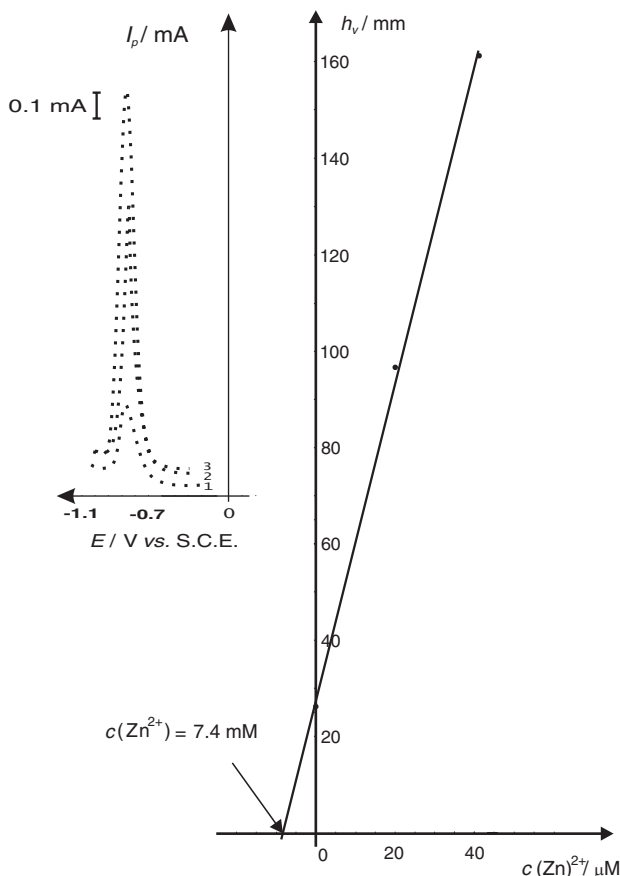

Fig. 1. Shematic diagram of the microbial electrode system
1. thermostatic cell, 2. magnetic stirrer, 3. oxygen electrode, 4. sample solution, 5. rubber ring, 6. membrane, 7. Pt wire, 8. cellophane, 9. immobilized cells of *S. cerevisiae*, 10. electrolyte solution, 11. S.C.E., 12. battery, 13. A/D conversion, 14. computer

Fig. 2. Typical voltamograms (HMDE) and calibration curve for one of the components of the growth medium (KH_2PO_4) at pH= 4.5.
1) sample, 2) and 3) standard addition of 20 µmol/L Zn^{2+}. Three parallel samples were analysed. Confidence level 97 %.
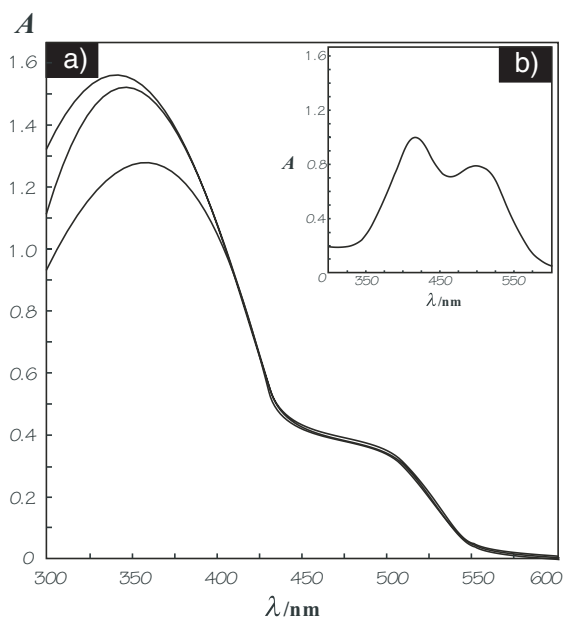
Fig. 3. a) Spectrum of the growth medium (polypeptone + KH_2PO_4 + $MgSO_4$) **with PAR** (measurements were done in triplicate), **b) Spectrum of the standard Co solution + PAR** c(Co) = 8 μM; c(PAR) = 1mM; pH = 8 (borate buffer); blank solution = water; path lenth b = 1 cm; λ(A_{max}) = 511 nm

mineralization of each sample was necessary before applying both techniques. Procedure was described in detail elsewhere (*18*). As shown the Figs. 2 and 3, growth medium does not contain zinc or cobalt in the concentration levels interesting for our investigation.

As aerobic conditions were applied throughout our experiments, ethanol seemed to be the best as substrate for *Saccharomyces cerevisiae.* In the same conditions, other substrates could cause cultivation of some other microorganisms. Likewise, enzymes needed cobalt and zinc, so we decided to investigate the possibilities of ethanol as a substrate.

Effect of zinc

It is well known that zinc deficiency remarkably depresses the protein content of the cells. In the evolution of eukaryotes the main role of communicating with DNA has been taken over by cytoplasmic zinc (*19*). It is also known that *S. cerevisiae* lacking copper-zinc superoxide dismutase shows a series of defect including reduced rates of aerobic growth in synthetic glucose medium (*20*).

In order to determine the influence of zinc, a sample solution of growth medium (free of zinc) was placed in thermosthated cell (28 °C). The microbial electrode was inserted in the solution saturated with dissolved oxygen (100 % of relative saturation) which was magnetically stirred. The electrode was connected to the oxygenmetre (Fig.1). Transition time (about 60 minutes, including lag phase) represented stability time (established equilibrium in the membrane) of our sensor, after which the exponential growth started.

Immediately after immersing the sensor in the solution, electrode current rapidly decreased, which could be explained by thin diffusion layer close to electrode

surface where the possibility of oxygen consumption by microbial cells occurred. It was followed by a slight increase of current as a result of increased oxygen concentration from the bulk of the growth medium. Therefore, it takes time to establish a new equilibrium (cca 60 minutes) after which the oxygen consumption began.

Oxygen consumption by *S. cerevisiae* began and its concentration around membrane slowly decreased (less than 10 % of relative saturation was achieved). Fig. 4 shows the response curves in pure growth medium and growth medium with different zinc concentration.

For quantifying our results (after the transition time) the current of 0.55 μA was used as initial measurement value (I_{in}) for the exponential growth of *S. cerevisiae* and the current of 0.2 μA as the end (I_{fin}) of measurement (both lines are discontinuous) (Fig. 4). Therefore, for each curve ΔI was calculated.
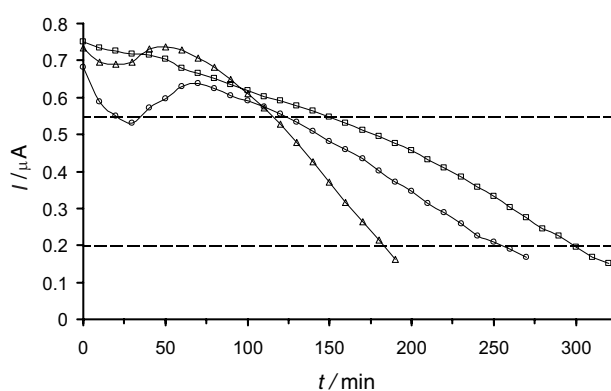
Fig. 4. Influence of different zinc concentrations on the respiration activity of *S. cerevisiae*: o γ(Zn) = 0 mg/L; Δ γ(Zn) = 0.48 mg/L; ◻ γ(Zn) = 100 mg/L Measurements were done in triplicate.

The relationship between those two currents and time (ΔI/Δt) is reverse with oxygen consumption, therefore it is proportional with microbial growth rate, which is also valid for linear and exponential growth model. Concentration of microbial cells in membrane was approximately the same, but for the measurement the same current values were taken, which means that the same measured current corresponds to the same concentration of *S. cerevisiae* at the surface of oxygen electrode. In the mentioned period exponential curve is noticeable. Measurement was interrupted at current value of 0.2 μA when oxygen supply became critical, consequently, further exponential growth of *S. cerevisiae* was limited by the rate of diffusion through the membrane layer. As all measurements were done in triplicate, it is evident (Fig. 5) that shapes of curves are very similar. In spite of possibly different initial volume of cells, the selected current relevant for quantification of results gives for oxygen time consumption an error time of about 10 minutes.

Near the end of exponential phase (depending on zinc concentration) (I_{fin} at 0.2 μA) a cyanide solution was added (γ(CN⁻)=13 mg/L) in order to inhibit microbial growth. As shown in Fig. 6, maximal current value termed as limited current (I_{lim}) was estimated, and as such it represents the current of oxygen electrode. Exponential part of the curve is expressed by equation:
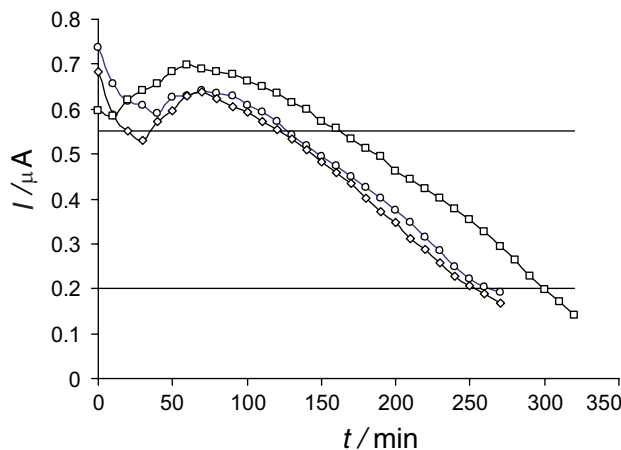
Fig. 5. Reproducibility of measurements in growth medium free of Zn/Co

$I = I_0 \, e^{kt}$ were k is the regression constant and the corresponding time can also be calculated.

It is evident (Fig. 6) that exponential part of the curve varies from the linear model a little bit (k values are relatively small). It is also noticeable (Fig. 6) that curves marked as ❏ represent the current registered on the electrode, which is identical to non consumed oxygen, while Δ represents the difference between limited and residual current (I_{lim}–I_{resid}), were I_{resid} (residual current) represents nonconsumed oxygen.

The graphs for all the investigated concentration levels are shown in Fig. 7.

Effect of cobalt

Yeast cells of *S. cerevisiae* incorporate cobalt in *e.g.* alcohol dehydrogenase (ADH) when grown in increased concentrations of this metal. The substitution zinc-cobalt in enzyme, the ratio of which was determined to be 4:1 (*21*), originates a new ADH form. The modified enzyme extracted from 6 mM of CoCl$_2$ medium still retained its enzymatic activity while in higher concentration a low activity was detected.

Reactivity of cobalt-substituted ADH was tested towards different transition metals (*22*). Cobalt ADH (from 4 mM of CoCl$_2$ yeast culture) displayed higher specific activity (30–50 fold) compared to the native ADH. Introduction of cobalt in the enzymatic molecule alters the affinity of ADH for its ligands, resulting in better catalytic properties (*21*).

In order to determine cobalt influence on the respiration activity of *S. cerevisiae* the microbial electrode was inserted in the pure growth medium (free of cobalt) and after that in the growth medium containing cobalt.

Experiments were performed as those for zinc, so the influence of different cobalt concentrations was investigated using identical microbial sensor under the same conditions. The obtained results are shown in Fig. 8. Results for cobalt were quantified on the same basis as those for zinc. Exponential curve (for oxygen consumed by *S. cerevisiae*) in the current range between 0.55 and 0.2 μA was obtained. The graph for all the investigated metal concentration levels is shown in Fig. 9.

As it could be seen from Figs. 7 and 9 (response curves), our biological sensor with immobilised *S. cerevisiae* shows a maximum stimulation in growth me-
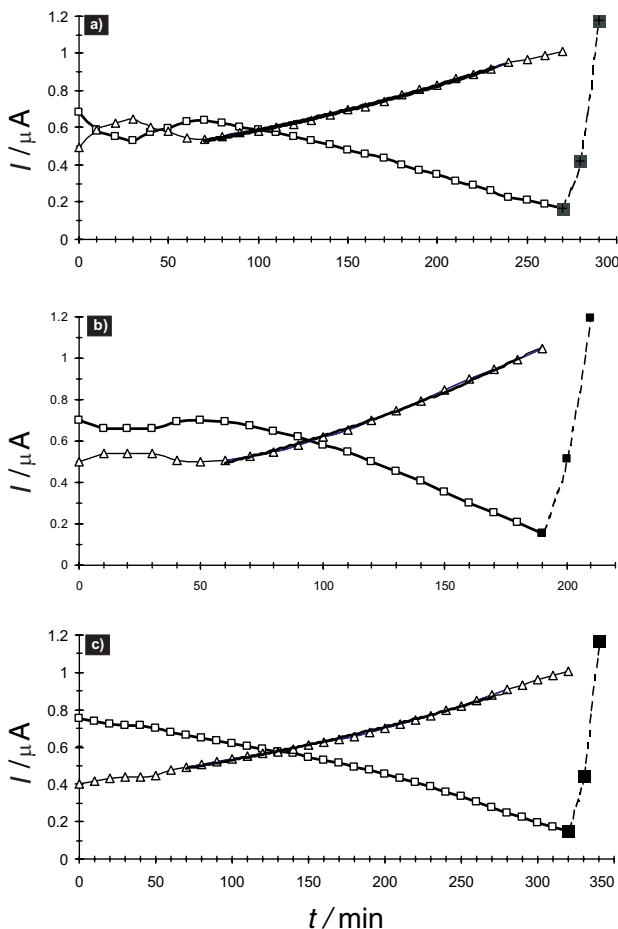

Fig. 6. Relationships between consumed/nonconsumed oxygen with time in the growth medium: ❏ current of oxygen electrode responding to non consumed oxygen; Δ current responding to consumed oxygen; ■ limited current after cyanide addition;

a) γ(Zn) = 0 mg/L; $I = 0.416 \, e^{0.0034t}$; R^2 = 0.0998
b) γ(Zn) = 0.48 mg/L; $I = 0.348 \, e^{0.0058t}$; R^2 = 0.0998
c) γ(Zn) = 100 mg/L; $I = 0.396 \, e^{0.0029t}$; R^2 = 0.0997

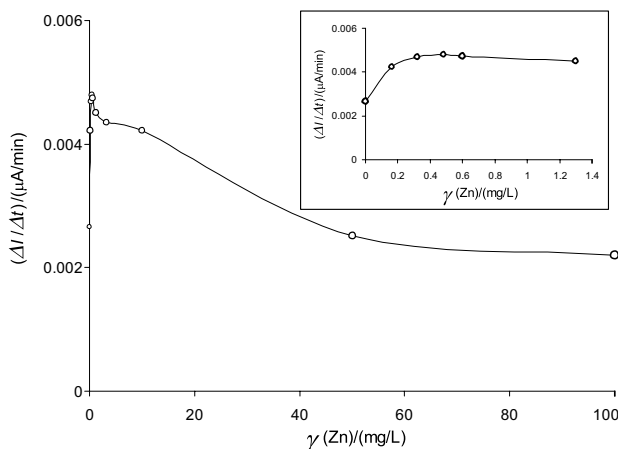**Fig. 7. ΔI/Δt as a function of zinc concentration:** γ(Zn) = 0; 0.16; 0.32; 0.48; 0.60; 1.3; 3.2; 10; 50 and 100 mg/L

dium enriched by zinc/cobalt in the concentration of zinc from 0.16 to 1.3 mg/L (Fig. 7) and that of cobalt from 2 to 10 mg/L (Fig. 9), respectively. Those intensive activities of *S. cerevisiae* were observed on the basis of the relationship of $\Delta I/\Delta t$ for each response curve. It could be seen that respiration activities of *S. cerevisiae* were diminished in the presence of zinc when γ(Zn) >10 mg/L and in the presence of cobalt when γ(Co) >25 mg/L.

It is evident that some zinc and some cobalt concentrations increase oxygen consumption, *e.g.* the current of the electrode decreases with increasing the zinc/cobalt concentration.
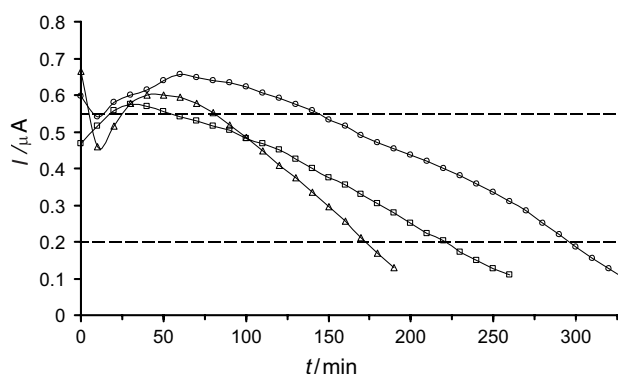

Fig. 8. Influence of different cobalt concentrations on the respiration activity of *S. cerevisiae*: ○ γ(Co) = 0 mg/L; △ γ(Co) = 6 mg/L; ▫ γ(Co) = 100 mg/L. Measurements were done in triplicate

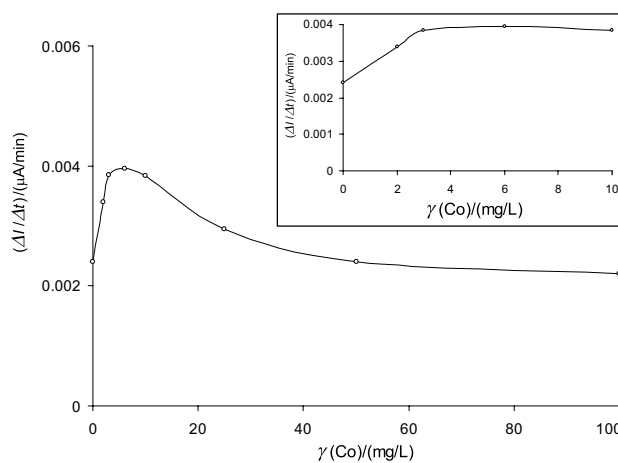**Fig. 9. $\Delta I/\Delta t$ as a function of cobalt concentration:** γ(Co) = 0; 2; 3; 6; 10; 25; 50 and 100 mg/L

Conclusion

Our experiments evidently show that cobalt and zinc influence the microbial growth. When *Saccharomyces cerevisiae* was introduced in fresh growth medium no immediate increase in cell number or mass occurred in the so-called lag phase. The main reason for prolonged lag phase is stabilisation within membrane containing collagen and microbial cells of *S. cerevisiae*. Transition time (including lag phase) was decreased depending on

optimal metal concentrations. Initial volume of cells probably has an influence on prolongation of the whole experiment, in other words, on time of oxygen consumption but as the initial concentration of microbial cells is practically the same, the time at which the current values are between 0.55 and 0.20 µA causes a possible error of about 10 minutes or the error is 10 %.

After 60 minutes, the growth phase starts. In relatively short time (about two hours) of measurements, very delicate exponential decrease is recognised but linear model can also be suitable. In the case of the addition of zink the first part of microbial growth curve was identical to the one when zinc is not added. The growth phase is more rapid, which can be seen from the curve. This positive zinc influence indicates greater oxygen consumption of *S. cerevisiae* but also exponential decrease of current on the electrode. When zinc concentration increases above 10 mg/L, negative influence on metabolic processes is evident (population growth is slower) and respiration activity slows down. Zinc concentration above 25 mg/L indicates the inhibition of growth and dividing of *S. cerevisiae*. Similar behaviour of *S. cerevisiae* was noticed in the growth medium enriched with cobalt. Positive influence of cobalt is noticed in the concentration range between 2 to 25 mg/L but greater concentrations of cobalt limit the growth of *S. cerevisiae*, or even inhibit it.

The developed biosensor is applicable for determination of oxygen consumption of *Saccharomyces cerevisiae*, the increase of the microorganisms population, in detection of respiration activity and the influence of activators and inhibitors, which is important in optimization of cultivation and production of yeast.

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Amperometrijski biosenzor za praćenje respiracijske aktivnosti kvasca *Saccharomyces cerevisiae* u prisutnosti kobalta i cinka

Sažetak

Učinkovita kontrola koncentracije teških metala provedena je primjenom polarografije i spektrofotometrije. Prednost je primijenjenih metoda i u njihovoj jednostavnosti i u kratkoći vremena potrebnog za analizu s relativno malim količinama uzoraka. Prisutnost nekih teških metala kao što su cink i kobalt pospješuje brzinu rasta kvasaca. Amperometrijski senzor koji sadržava kvasac *Saccharomyces cerevisiae* primijenjen je za uspješnu kontrolu promjena koncentracije metala. Utjecaj cinka i kobalta na respiracijsku aktivnost kvasca *Saccharomyces cerevisiae* procijenjen je mjerenjem udjela kisika u otopinama obogaćenim tim metalima. Mjerenja su provedena pomoću modificirane Clarkove kisikove elektrode u otopinama što sadržavaju kobalt i cink u koncentracijskom rasponu do 100 mg/L.