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PILOT-PLANT CULTIVATION OF BREWERY'S YEAST *SACCHAROMYCES CEREVISIAE* ENRICHED WITH MAGNESIUM

Dorota Nowak[1], Tadeusz Kasiak[1], Piotr P. Lewicki[1], Wanda Duszkiewicz-Reinhard[2]

[1]Department of Food Engineering and Process Management, [2]Division of Biotechnology and Food Microbiology; Warsaw Agricultural University, Warsaw

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Cells of brewer's yeasts *Saccharomyces cerevisiae* No. 1 were used as the experimental material. Cultivation was carried out in a BIOFLOW 3000 bioreactor (New Brunswick Sci.) using the YPD medium. Magnesium ions as $MgCl_2 \cdot 6H_2O$ were added at a dose of 1.25 g/L in two variants. In the first variant (Mg1), magnesium chloride was added to culture medium before the process of propagation had been initiated. In the second variant (Mg2), magnesium was added continuously to the culture medium during the first two hours of propagation.

The supplementation of the culture medium with magnesium ions resulted in higher yields of biomass, compared to the control. Continuous dosing of magnesium was found to be better than a single-dose addition.

Biomass obtained in the presence of magnesium ions contained more colony forming units than the control. Magnesium was absorbed by yeast cells and its level was twice as high as that measured in control experiments.

INTRODUCTION

Due to their great availability, *Saccharomyces cerevisiae* yeasts are the microorganisms especially readily applied in the bioaccumulation of metal ions [Lipke & Ovalle, 1998]. The bioaccumulation is a two-stage process. First, metal ions are adsorbed at the surface of cell walls, then there proceeds their active transport through a cell wall and cytoplasmatic membrane to the cell's interior [Avery & Tobin, 1992]. The cell wall, composed mainly of polysaccharides, proteins and lipids, contains a variety of functional groups (*i.a.* carboxyl, hydroxyl, sulfuric, phosphate and amine groups), which may bind metal ions as well as exchange ions contained therein with the surrounding environment [Sag & Kustal, 1996]. As a result of adsorption of positive charges on the surface of a cell wall, a difference in the concentration of protons appears between the outer and the inner side of the cytoplasmatic membrane. Those electrostatic interactions are the main driving force for ion transport to the cell's interior. The electrochemical potential produced may also be an additional driving force for the movement of ions [Walker & Maynard, 1997].

The phenomenon of bioaccumulation may be used for two purposes: to remove ions of metals, especially the heavy ones, from the environment [Göksungur *et al.*, 2003], and to produce biomass enriched with ions of metals performing important functions in metabolic processes of humans and animals.

One of the most essential elements, in respect of metabolism and physiology, is magnesium. It exercises an important function in the processes of muscle contraction, including that of cardiac muscle, it exerts a beneficial effect on the process of blood coagulation, stimulates defence mechanisms of the organism, affects a proper development of the osseous system, it also demonstrates sedative activity, mitigates effects of stress, pain and headaches. It is indispensable in protein synthesis [Gawlik, 2003].

Magnesium deficiency in the organism may lead to serious somatic and mental disorders [Papierkowski, 2002]. A too low level of that element has bee determined in *ca.* 50% of the population. Its deficiency results from a diet composed of highly-processed products [Wojnowski, 2000], and from civic development enforcing situations that favour magnesium losses (mental and physical strain, stress, coffee or alcohol abuse) as well. Hence, diet supplementation with preparations containing high doses of magnesium, especially in an easily assimilable form, is of great significance these days. An example of such a preparation may be yeasts enriched with magnesium.

In the cell's interior, magnesium ions form complexes, referred to as bioplexes, with proteins, peptides and amino acids. That form is characterised by a much better assimilability and biological activity than that in the form of inorganic salts [Mardarowicz, 1997]. It is linked with the character of the transport of such a complex, which is typical of proteins, and not of a cation. Bioplex may easily penetrate an intestinal wall, and then a cell wall, whereas ionic transport is subject to some limitations resulting from osmotic pressure or ionic equilibrium.

Saccharomyces cerevisiae yeasts are commonly regarded as safe, they are easy in culturing, ensure high biomass yield, and are not demanding as far as the culture medium is concerned. They may be used as ingredients of widely consumed food products and simultaneously as separate nutri-

Author's address for correspondence: Dorota Nowak, Department of Food Engineering and Process Management, Warsaw Agricultural University, ul. Nowoursynowska 159c, 02-776 Warsaw, Poland; tel.: (48 22) 59 375 62; fax: (48 22) 59 375 76; e-mail: Nowak@alpha.sggw.waw.pl

tional preparations, containing – besides magnesium – a number of valuable vitamins, proteins and mineral components. Hence they seem a fine way of diet supplementation with magnesium.

Investigations carried out at a laboratory scale under stationary and batch culture conditions have demonstrated the usefulness of brewer's yeast *Saccharomyces cerevisiae* as a source of protein-magnesium complexes [Błażejak *et al.*, 2002 a, b]. That research indicates also that magnesium content of the biomass produced is determined by the concentration of magnesium ions in the culture medium, the type of chemical compound being a source of magnesium, the growth phase of cells at which medium supplementation with magnesium ions is done, the method of culture propagation or the oxygen saturation of the culture medium. A question was asked then, whether the positive results referring to the bioaccumulation of magnesium ions by brewer's yeast *Saccharomyces cerevisiae* under small laboratory scale are also possible at the application of a large-scale biomass production carried out in bioreactors. Propagation in bioreactors is linked with a 50--fold increased volume of culture medium, compared to the conditions applied by Błażejak *et al.* [2002 a, b]. Thus, it should have been expected that the increased role of the mass transport, micro- and macromixing, distribution of shearing forces in the space, as well as developed surface of contact of liquid-gas phases and foam formation, connected with the enlargement of the scale, might affect the processes of magnesium accumulation and biomass growth. Culture carried out in fermentors enables providing a controlled oxygen supply and better mixing (results in better conditions for convective mass transport of components essential for the physiology and metabolism between the culture medium and a cell). On the other hand, a too high concentration of oxygen or shearing stresses, affecting living organisms and caused by the mixing process, may constitute stress conditions evoking changes in the intensity of propagation or modifying metabolism [Nowak *et al.*, 2003]. Therefore, the objective of this research was to evaluate the course of propagation run in an enlarged scale, especially with reference to biomass yield and the capability of brewer's yeast *Saccharomyces cerevisie* for accumulation of magnesium.

MATERIAL AND METHODS

Preparation and course of culture. *Saccharomyces cerevisiae* No. 1 strain of brewer's yeast originating from the collection of pure cultures of the Institute of Agricultural and Food Biotechnology in Warsaw was used as the material of investigations. The strain was stored at wort slants at a temperature of 4°C and re-inoculated on fresh slants every 4 weeks to refresh the culture.

Inoculum was prepared in 500-mL flat-bottom flasks by inoculating the material from the slants onto 100 mL of liquid YPD medium (2% – peptone, 2% – glucose, 1% – yeast extract) prepared from deionised water. Propagation was carried out at a temperature of 28°C in a reciprocating shaker (Julabo) at 200 rpm for 22–24 h (until a constant value of optical density was obtained).

The YPD medium was the culture medium. Two variants of medium enrichment with magnesium ions were applied: (1) $MgCl_2 \cdot 6H_2O$ supplement added in a single dose of 1.25 g/L in the stage of medium preparation – variant Mg1; and (2) $MgCl_2 \cdot 6H_2O$ supplement at a dose of 1.25 g/L added continuously for the first two hours of incubation starting from inoculum introduction – variant Mg2. The latter variant was applied to attain the effect of gradual adaptation of yeast to the medium with an increased content of magnesium ions.

Control culture (Control) was run on the YPD medium without magnesium addition.

The selection of a chemical compound being the source of magnesium ions, its dose and manner of its supplementation was made based on an analysis of results obtained by Błażejak *et al.* [2002 a, b] in a study carried out on the same yeast strain under stationary and batch culture conditions.

The proper culture was run in a BIOFLOW 3000 bioreactor (New Brunswick Scientific) with a volume of 5 L. After filling with culture medium and fixing control devices (thermometer, oxygen electrode, and pH-electrode by Mettler-Toledo), bioreactor was sterilized at a temperature of 121°C for 25 min. After cooling, the bioreactor was connected to an operating-controlling unit, the culture medium was adjusted to a desired temperature and 100% oxygen saturation (under the experimental conditions), and then the oxygen electrode was calibrated. After those preliminary procedures have been completed, the culture medium was inoculated. The addition of the inoculum reached 10% of the volume.

The bioreactor's vessel with internal baffles, was also equipped with a double turbine mixer working with the speed of 400 rpm. In addition, sterile air was supplied by a bubbler at a flow rate of 100 L/h. This system enabled obtaining a high oxygen saturation of the culture medium.

The medium saturation with oxygen, pH changes and temperature were monitored continuously over the incubation period.

To avoid the effects of excessive foaming, use was made of a foam level detector. When the level of foam exceeded the specified value, a peristaltic pump was activated that supplied a 10% sterile solution of silicon emulsion at a dose necessary to extinguish foam.

Incubation was carried out for 72 h. Over that period, *ca.* 30-mL samples were collected using the bioreactor's system for sterile sampling.

Optical density (OD) of the samples collected was determined and biomass yield was calculated. In addition, samples collected after 24 h were assayed for the number of living cells and magnesium content of the biomass produced.

Analytical methods. Biomass yield was determined by centrifugation of *ca.* 25 mL of the culture liquid in an MPW 223 centrifuge (MPW MED Instruments) at 3500g x 10 min. Immediately after centrifugation, the fluid left over the sediment was decanted and the content of dry matter was determined using two-stage drying, first at a temperature of 60°C for 2 h, and then finish drying at 105°C till the constant weight was reached.

The optical density (OD) of the yeast cells culture suspended in a culture liquid was determined using a Specol 11 spectrophotometer (Carl Zeiss Jena, Germany) at a wavelength of 600 nm [Pasternakiewicz & Tuszyński, 1997].

The number of live yeast cells was assayed with the plate method by determining the number of colony forming units

(cfu) converted into 1 g d.m. Biological material originating from the three subsequent dilutions was inoculated on YPD medium with 2% agar addition and incubated at 28°C for 72 h [Burbianka & Pliszka, 1983].

After 24 h of propagation, the degree of magnesium ions bioaccumulation by yeast biomass was determined. Magnesium content was assayed in biomass obtained directly from the culture medium by centrifugation and after twofold rinsing with deionised water. In both cases, the centrifuged biomass was dried to a constant weight. The specified amount of dried yeasts was mineralized in a mixture of nitric and perchloric acids in a combustion apparatus (Buchi Digestion Unit K-435, Germany), transferred to a measuring flask and filled with deionised water up to the volume of 50 mL. Magnesium content in prepared samples was measured for with atomic absorption spectrophotometry (AAS). Measurements of absorbance were carried out at the Department of Physicochemical Analyses, Warsaw Agricultural University. The results obtained were expressed in mg Mg^{2+} per g d.m. yeast.

RESULTS AND DISCUSSION

Both the amount of biomass produced as well as magnesium content in that biomass are important in evaluating the content of magnesium accumulated in cells during propagation.

The kinetics of biomass growth in the cultures run in this experiment is presented in Figure 1. Its course indicates that directly after the inoculation there occurred a short (*ca.* 2-h) adaptation phase, which may result from changes in the concentration of nutrients and in oxygen saturation of the medium as well. This phase was short, which stems from the fact that the YPD medium was used as both the inoculum and the culture medium, and that the inoculated cells were in the growth phase. An intensive growth, irrespective of the medium used, occurred till 22–24 h of propagation, to be then followed by the stationary phase. Up to 72 h of incubation, no symptoms of the lethal phase were observed.

Experimental points illustrating biomass gain in time were described by kinetic equations assuring the best fit.

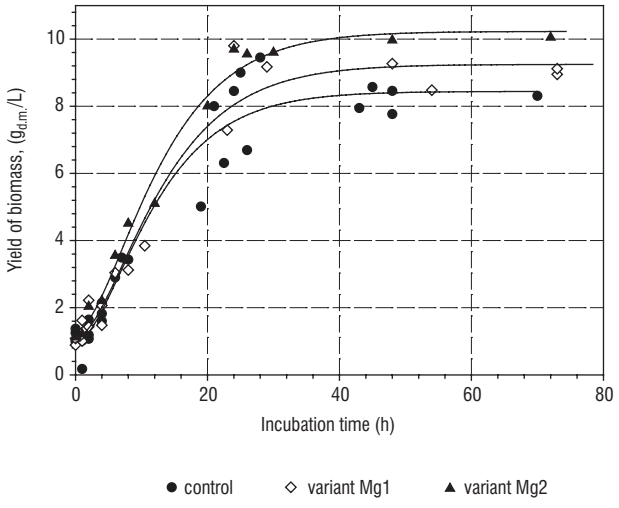

FIGURE 1. Yield of *S. cerevisiae* yeast biomass in control and magnesium-enriched liquid YPD medium.

The coefficients of determination for those equations were high and ranged from 0.96 to 0.98. The amount of biomass in the stationary phase of growth determined based on those equations accounted for 8.4 g d.m./L of control medium, 9.2 g d.m./L of medium in variant Mg1, and 10.2 g d.m./L of medium in variant Mg2. This indicates that medium enrichment with the applied dose of magnesium salt proved to have a positive impact on biomass gain, still gradual supplementation of medium according to variant Mg2 appeared to be more beneficial. A similar tendency was observed by Błażejak *et al.* [2002 b], though biomass yields obtained in their study under stationary culture conditions were 3-fold lower. Under dynamic conditions, after 24 h of propagation, those authors recorded the biomass yield to be lower by 1.6 g d.m./L compared to the culture carried out in a bioreactor.

The curves of yeast growth were used to calculate a rate of biomass gain (Figure 2). Their analysis indicates that, irrespective of the medium applied, the rate of biomass gain increases throughout the first 7 h of calculation. After this period of time, the rate of biomass gain reaches its maximum which accounts for 0.42, 0.42 and 0.47 $g_{d.m.}$/(Lh) for the control culture and variants Mg1 and Mg 2, respectively. After reaching its maximum, the rate of biomass growth rapidly declines. In *ca.* 32 h, the second point of inflexion can be observed on the curve, at which the growth rate attains a very low value ranging from *ca.* 0.04 to 0.05 $g_{d.m.}$/(Lh). From that moment, biomass gain is observed to decrease very slowly and finally terminates completely in *ca.* 52 h of incubation. The diversification of the final biomass yield in individual culture variants results from the higher maximal value in the case of variant Mg2 and from higher rates of yeast growth in the period of the first value decline.

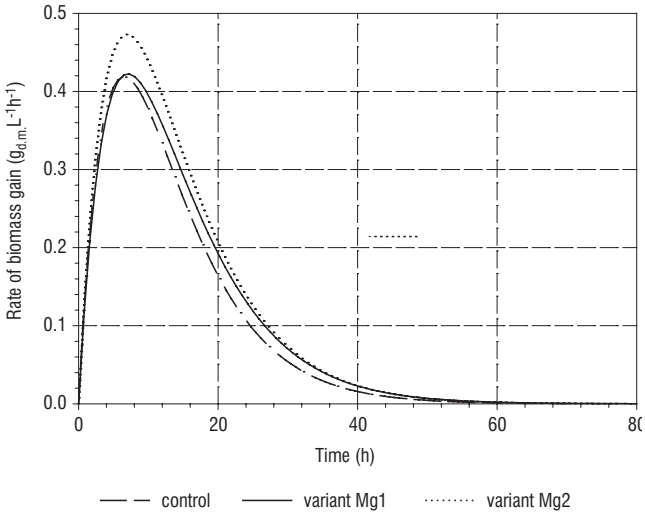

FIGURE 2. Changes in the rate of *S. cerevisiae* biomass gain in control and magnesium-enriched liquid YPD medium.

One of the indicators of the number of cells present in culture medium may be optical density (OD) expressing the number of cells suspended in liquid as objects capable of both reflecting and absorbing light. From the point of running a culture in a bioreactor, this parameter may be readily applied for the on-line determination of biomass gain. In this research, the estimation of that parameter in parallel with biomass yield aimed at verifying whether the presence

of magnesium ions affects absorbance measurements and to what extent the optical density correlates with biomass yield. The results obtained are presented in Figure 3. Their analysis indicates that linear correlation between the optical density and biomass yield (w/w) occurs up to biomass content in the medium reaching *ca.* 5 g d.m. of yeast/L of medium. Such a yield is obtained within 12 h of incubation. The correlation coefficient for that correlation was high and amounted to 0.96. In the range at which the linear correlation was observed to occur, there were no statistically significant differences between the values obtained in the control and magnesium-enriched media. In addition, within the first 12 h of incubation, the differences in biomass yield recorded for different media were not statistically different (Figure 3). Hence it proves that the presence of magnesium ions does not affect the results of optical density measurements.

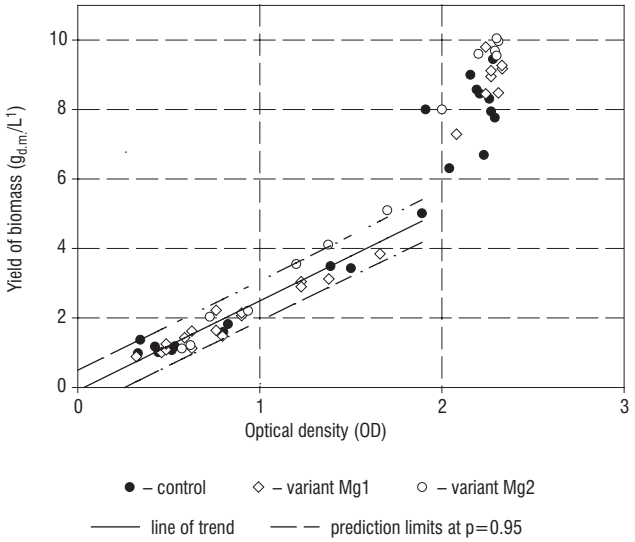

FIGURE 3. Correlation between the values of optical density and biomass yield during propagation of yeast.

At biomass content in medium exceeding 5 g d.m. yeast/L (which corresponds to the absorbance of *ca.* 1.8), the increase in biomass yield results in relatively small changes in OD, which points to the necessity of diluting a medium sample before measurement in order to obtain reliable results. This, in turn, renders the application of that parameter for on-line control of biomass gain impossible.

Changes in the life activity of cells linked to their propagation may also be monitored based on oxygen saturation of the culture medium (Figure 4).

The level of oxygen saturation results from the rate of oxygen solubility in the medium and the rate of its consumption by microorganisms. Under specified conditions (mixing speed, temperature, chemical composition *etc.*), the solubility rate is a constant value. Hence, a decreasing level of oxygen saturation indicates that more oxygen is being consumed than supplied. It indicates increased yeast cells demand for oxygen. A high consumption of oxygen is observed up to *ca.* 20 h of propagation. In the 20th h, oxygen saturation reaches its minimal level, *i.e. ca.* 20%. This demonstrates that the oxygen supply applied under the experimental culture conditions was properly adjusted – there was no incidence of the lack of oxygen availability.

An interesting phenomenon was observed showing a rapid decrease in oxygen demand, which was reflected by

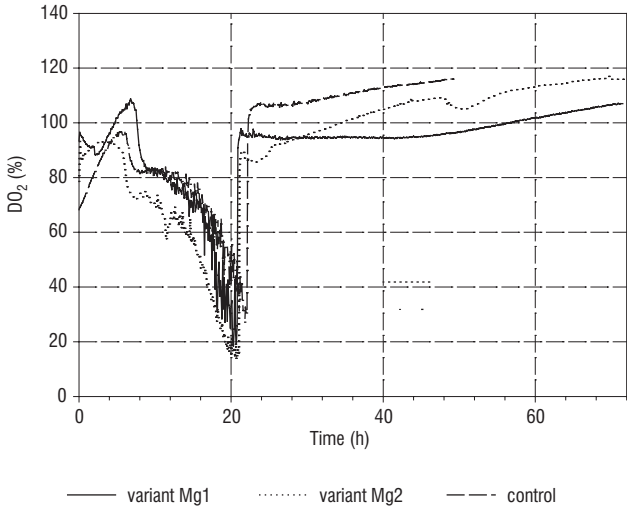

FIGURE 4. Changes in the intensity of oxygen consumption during propagation of brewery's yeasts *Saccharomyces cerevisiae* in a bioreactor.

an immediate increase in the concentration of oxygen practically to the maximal value under those conditions. This points to the fact that there is a stage in the development cycle of yeasts when suddenly large amounts of oxygen are no longer necessary, which is likely to result from such a concentration of yeast cells that evokes the inhibition of the multiplication process.

After 24 h of incubation, 1 mL of culture medium contained $(2.14\pm0.2)x10^8$ cfu (control); $(2.96\pm0.24)x10^8$ cfu (Mg1); and $(3.26\pm0.28)x10^8$ cfu (Mg2) of yeast cells, which after taking into account the differentiated biomass yield accounted for $2.54x10^{10}$, $3.20x10^{10}$, and $3.19x10^{10}$ cfu when related to 1 g d.m. of biomass, respectively. In considering standard deviations from mean values, it should be stated that the method of medium enrichment with magnesium ions applied in this experiment had no effect on the number of living cells, whereas medium supplementation with magnesium ions evoked *ca.* 20% increase in the number of colony forming units. This may indicate that the biomass obtained from magnesium-enriched medium contained more living cells (part of biomass is formed by dead cells) or/and that the grown cells had lower individual weights. Such conclusions may be supported by the fact that although the doses of magnesium ions applied in the study did not appeared to be toxic and provided a high biomass yield, yet they were higher than the optimal doses for those yeasts [Mayhard, 1993].

After 24 h of propagation, the yeast biomass of the control culture contained 1.45 ± 0.2 mg Mg^{2+}/g d.m., whereas yeast biomass obtained from variants Mg1 and Mg2 contained 2.86 ± 0.15 and 3.03 ± 0.38 mg Mg^{2+}/g d.m., respectively. There were no statistically significant differences between magnesium contents of the two variants enriched with magnesium, whereas medium supplementation with magnesium ions caused *ca.* twofold increase in magnesium content of yeasts. The values obtained in this experiment were lower than those obtained under stationary and dynamic conditions [Błażejak *et al.*, 2002 a, b]. This is likely to result from better conditions of mass exchange in a bioreactor between the cell and the surrounding medium, which in turn results from intensive agitation.

While expressing the amount of accumulated magnesium ions in respect to the amount of biomass produced from 1 L of culture medium, the following values are obtained: 12.2, 26.3, and 30.9 mg Mg^{2+}/L. For all culture variants, those values were comparable with the results obtained under dynamic conditions by Błażejak *et al.* [2002 b]. Lower values expressed per 1 g d.m. were compensated for due to a higher content of biomass obtained from incubations carried out in a bioreactor.

No statistically significant differences were observed between magnesium content of biomass separated from the culture liquid and that additionally rinsed with deionised water. This effect could have been due to the scale of the experiment. As a consequence of applying a relatively large sample in the process of centrifugation, the yeast sediment, usually highly compressible, was devoid of the culture liquid, which present in the intercellular spaces could have resulted in overestimating magnesium content in yeast cells.

CONCLUSIONS

The incubation of brewer's yeast *Saccharomyces cerevisiae* in a bioreactor at an increased production scale produced satisfactory results. Conditions created during the propagation process enabled increasing the efficiency of biomass yield, compared to stationary and batch cultures run with the same yeast strain and on the same media.

Medium enrichment with magnesium ions, using a 1.25 g/L dose of magnesium chloride, resulted in higher biomass yields obtained, compared to the control culture. Yet, the continuous supplementation of medium within the first 2 h of propagation yielded better results than a single addition of a full magnesium salt dose to the medium.

Biomass produced from the magnesium-enriched medium contained more colony forming units, compared to the biomass of the control culture. This is of significance to the application of technologies based on fermentative capabilities of yeast for determining the properties of a product.

Yeast biomass produced in a bioreactor on magnesium--enriched medium was characterised by over twice as high content of magnesium permanently bound with a cell, compared to the biomass of the control culture. Culturing run at an enlarged scale resulted in lower concentrations of magnesium ions when expressed per 1 g d.m. of yeast, when compared to the results reported by Błażejak *et al.* [2002 a, b]. It is worth emphasising that the accumulated magnesium is so firmly bound with cells that it is not washed out during biomass rinsing with deionised water. The amount of magnesium ions bound with the yeast biomass in respect to the unit volume of the culture medium was comparable with the results obtained at a small laboratory scale.

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HODOWLA DROŻDŻY PIWOWARSKICH *SACCHAROMYCES CEREVISIA*E WZBOGACONYCH W MAGNEZ W SKALI PÓŁTECHNICZNEJ

Dorota Nowak[1], Tadeusz Kasiak[1], Piotr P. Lewicki[1], Wanda Duszkiewicz-Reinhard[2]

[1]Katedra Inżynierii Żywności i Organizacji Produkcji, [2]Zakład Biotechnologii i Mikrobiologii Żywności Katedry Biotechnologii, Mikrobiologii i Oceny Żywności, Wydział Technologii Żywności Szkoły Głównej Gospodarstwa Wiejskiego, Warszawa

Materiałem badawczym były drożdże browarnicze *Saccharomyces cerevisiae* No. 1 Hodowlę prowadzono w bioreaktorze BIOFLOW 3000 (New Brunswick Sci.) na podłożu płynnym YPD. Podłoże wzbogacano w jony magnezowe, których źródłem był $MgCl_2 \cdot 6H_2O$ dodawany w ilości 1.25 g/L na dwa sposoby. W pierwszym wariancie (Mg1), chlorek magnezowy podawany był w jednej dawce do podłoża przed zaszczepieniem. W drugim (Mg2), taka sama dawka soli magnezowej podawana była w sposób ciągły przez pierwsze dwie godziny hodowli.

Wzbogacenie podłoża hodowlanego w jony magnezu spowodowało wzrost wydajności plonu biomasy w porównaniu z podłożem kontrolnym, przy czym wariant Mg2 był korzystniejszy w tym aspekcie niż wariant Mg1.

Biomasa wyhodowana na podłożu wzbogaconym w magnez zawierała więcej jednostek tworzących kolonie w porównaniu z hodowlą kontrolną. Ilość jonów magnezu związanych przez komórki drożdży była około dwukrotnie większa w porównaniu z biomasą wyhodowaną na podłożu kontrolnym.