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BIOSORPTION OF HEAVY METALS BY YEAST CELLS AND THEIR APPLICATION TO INCREASE THE EFFICIENCY OF WASTEWATER TREATMENT

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1. INTRODUCTION

The aim of mankind was to improve the conditions of living in the course of history. The results of science, especially those of natural sciences, were used, and thus mankind transformed the environment more effectively for its own purposes. In the past one hundred year, in proportion with the growth of industrial production, the use and exploitation of heavy metals have improved, and so has, at the same time, environmental pollution, since environmental protection is in phase delay with industrial development.

With the spread of environmental pollution, more and more attention is paid to the hazards related to heavy metals, because the accumulation of micro-nutrients and toxic heavy metals has crucial human healthcare, ecological and biological significance. The environmental emission of microelements and heavy metals has multiplied with the development of the industrial area, where together with traffic and industrial activity, the modernization of agriculture has also become a potential heavy metal polluter.

Some heavy metals are important, essential for the life processes, (eg, Cu, Ni, Zn, Co, Cr), while we do not know the biologically beneficial effects of others (eg, Cd, Pb, Ag, Hg), however, it is well known that the higher than natural concentration of essential elements induce stress at living cells and is toxic. Soils are able to accumulate heavy metals for many years without their acute toxic effects becoming obvious. Their filtering capacity exhausts over a certain load level, they become permeable and they also appear as a pollution source. Toxic metals appear in the water, they become available for the plants and so they can cause damage if they get into the food chain. In Hungary, more than 90% of residential water usage comes from groundwater (PUZDER et al. 2001), so it is especially important to treat pollution when the aim is to remove the contaminants from ground water or soil.

The purification of industrial wastewater and its return into the environment is a very costly process, it is necessary to develop procedures which are easy, fast and efficient to eliminate the pollution. Several chemical methods have been developed for this purpose. Among the chemical adsorbents only the ion-exchange resins mean an ecologically minimal risk, but they are very expensive. Therefore, there was increased demand for the research of cheap, environmental-friendly alternatives. So the use of biomass as biosorbent appeared as an economical and ecologically friendly possibility. It is important to concentrate primarily on microorganisms that are involved in this process and reproduce well on food or other industrial by-products. One of the benefits of this is that microbes reproduced on by-products with a high content of organic matter and process waters break down a part of the environmentally harmful by-products, such as whey, a dairy by-product, or starch from the

processing of corn or potatoes, respectively; on the other hand the produced biomass can also serve as a filter due to its capabilities in heavy metal accumulation. Immobilized cells as biosorbent may also be used for removing heavy metals from wastewater.

2. OBJECTIVES

During my work I tested different starch and lactose utilizing yeasts to determine if a biomass can be produced which is suitable for the accumulation of heavy metal ions as well as for regenerating and cost-effective biosorbent production.

My aims were the following:

1) A selection of yeast strains that effectively assimilate lactose respectively starch and are suited for the accumulation of heavy metals

2) The examination of the metal binding ability of different strains with the use of live, or dried biomass in the case of heavy metal ions as Cr, Cd, Ni, Cu, Pb and Ag

3) To determine the intracellular localization of accumulated heavy metals, as well as developing a process for bioresorption of heavy metals

4) The optimization of selected yeast strains and culture conditions for biomass production purposes under laboratory conditions, at shaken cultures. Yield and yield constant determination

5) Developing a biosorbent which can be regenerating and can be produced at lower cost

6) Developing the immobilisation methods of the cell of yeast biomass under the laboratory conditions. The immobilisation technology achieve at laboratory level

3. MATERIALS AND METHODS

In the course of my work the strains were tested not only from culture collections but also ones isolated earlier from dairy products.

I defined the cell yield and the yield constant in broth containing starch, lactose and glucose at different strains. I selected the best indicator of biomass yield strains, where I wanted to determine the optimal amount of carbon and nitrogen sources which play an important role in the fermentation medium. Glucose, lactose or starch as carbon source and yeast extract or ammonium sulphate as nitrogen source were used. On the basis of preliminary experimental results 1.5 (w / v)% glucose / lactose / starch and 0.5 (w / v)% yeast extract / ammonium sulphate concentrations were chosen as set point. To complete the optimization task I used Central Composite Design (CCD). The method includes the design of experiments and mathematical-statistical evaluation. To describe the maximum total mass change of biomass the second-order polynomial model was applied according to $Y = b_0 + b_1x_1 + b_2x_2 + b_3x_1^2 + b_4x_1x_2 + b_5x_2^2$ equation. Glucose or lactose optimisation was carried out with Multiskan Ascent (Thermo, Electron Corporation) micro-plate densitometer, which allows the on-line monitoring of growth of the cells. Optimization of the starch source was carried out under slightly shaken conditions, because due to the starch opalescence, the Multiskan instrument is not suitable for measuring the growth of cells inside this broth. The statistical evaluation of results was done by Statistica 9.0 software in each case.

Since with the help of micro-densitometer only the growth can be detected, in order to track the changes in oxygen concentration and pH during the fermentation and to make scale-enhancement, shaking flask fermentation experiments were carried out.

After determining the optimal medium for yeast cells, I examined the metal accumulation capacity of both live and heat-inactivated (dried) cells. The cells were exposed to the effect of silver, chromium (VI), lead, copper, nickel and cadmium in the form of the following soluble salts 20 mM $AgNO_3$, 20 mM K_2CrO_4, 20 mM $Pb(NO_3)_2$, 20 mM $CuSO_4$, 20 mM $CdSO_4$ and 20 mM $NiCl_2$. After the cells were removed, the metal concentrations of the supernatants were measured by ICP-AES (ICAP-61, Thermo Ash Jarre, USA). The cellular distribution of heavy metals in the case of living cells from each cell component for extracting metal ions the modified version of the White & Gadd method (1986) was used. Heavy metals adsorbed by the cell wall as cations were obtained by 50 mM EDTA-Na_2 (pH7) as a chelating agent. To extract the intracellularly solved heavy metals 0.7 M sorbitol-DEAE-dextran was used. I checked the complete permeabilization of citosol membrane by painting the

cells with malachite-green paint. The complete membrane disintegration has been achieved by the metil-ethanol treatment of the cells. Soluble fractions of the accumulated metals were determined by washing the cells with 0.7 mol MOPS buffer (pH 7) two times. The fraction which could not be removed by washing was considered as an organically bound fraction in the case of dried cells. Cell wall bound fraction of the living cells was determined by mobilizing the metals using desorption treatments. Heavy metal contents of the remaining cells were considered as intracellular fractions.

I examined the immobilization ability of the cells on the HAP material and parallel with this I monitored the pH of the medium, the cell-surface hydrophobicity, as well as the detergents, proteases and Lysing enzyme (Sigma) treatments of the cell wall with the effects of the immobilization efficiency.

4. RESULTS

Among the yeasts with different morphology and cell-wall structure I was looking for strains which have good starch or lactose utilization ability for economical biomass production. To monitor the growth of microorganisms there are a number of methods available. Among all of these methods the principle of turbidity is one of the most simple procedure with fast response time and enables the on-line measure of growth. The further advantages of it are the automatization and the measuring of a large number of samples. Optimization was carried out by Multiskan Ascent equipment.

There is a small number of yeast species which use starch as carbon source. The *Debaryomyces occidentalis var. occidentalis*, the *Saccharomycopsis fibuligera* and the *Lipomyces kononenkoe* species were examined. According to my results, the *Saccharomycopsis fibuligera* CCY 42-3-1 and *Debaryomyces occidentalis var. occidentalis* Y758 gave the best biomass yield. The effects of nutrient components show that increasing the concentration of the yeast extract and decreasing the concentration of starch at the same time resulted in a higher biomass production in the case of the starch carbon source. Since lactose utilization is under strong genetic regulation, strains forming the microflora of dairy products could improve properties regarding lactose utilization efficiency. Therefore strains not only from culture collections but also ones isolated earlier from dairy products were tested. Yeast strains belonging to three lactose utilizing species (*Kluyveromyces lactis, Kluyveromyces marxianus* and *Dekkera anomala*) were tested during optimization. Yield on lactose sometimes exceeded that of on glucose, which refers to lactofilia. During the experiments, the C- and

N-content, pH and aeration was optimized to achieve a higher yield. The increasing aeration resulted in the increase of the dissolved oxygen concentration in the culture medium but had no significant effect on biomass production. The dairy strains had much better production than the culture collections strains.

I also examined the different yeast species and their accumulation and biosorption ability. A laboratory model of metal solution was prepared which contained the following metals: Ag, Cd, Cr, Cu, Ni. The metal accumulation was studied on living cells, and on gently dried, but dead cells, too. During the preliminary experiments I set the cell and metal concentrations (2mM and 20mM), where the effect of biosorption can be measured on the basis of the decreasing metal concentration of the metal content salt solution by ICP equipment. The accumulated metal ratio was nearly independent of the solution concentration and I found no significant differences between the living and dried cells. Therefore the screening afterwards was made in a 20 mM solution. Experiments were carried out to develop the recovery method of the valuable heavy metal. The application of EDTA (complex-forming organic compound) resulted in almost complete desorption.

Different yeast species having diverse cell morphology (budding, fission, dimorphic, being flocculent or film-forming) were tested regarding their immobilization potential on nanostructured hydroxyapatite (HAP) material. The best results were achieved with the dimorphic yeast species *Saccharomycopsis fibuligera* CCY 42-3-1 and *Kluyveromyces marxianus* NB. The hydrophilic character of the cells was advantageous for immobilization on HAP. Chemical modification of the cell wall by detergents, proteases and cell wall lytic enzyme was tested as a possibility to increase immobilization efficiency. Treatment with cell wall lytic enzyme and certain detergents improved the attachment of cells on the surface. The results show that the enzymatic cell wall treatments and the detergents increased the ability of the cells to the HAP material and helped the formation of biofilm.

5. CONCLUSIONS AND SUGGETIONS

Considering the results obtained during my work I came to the conclusion that the micro-plate densitometer is an efficient tool for the optimization of the medium. The Central Composite Design (CCD) was adopted as an efficient way to find optimal ratio of carbon and nitrogen sources and quantity within a short time. The employment of whey and its optimization could be the continuation of the experiment series. The changes of the nutrient composition could result in changes within the

nutrient- and oxygen uptake, in the pH, which have effect on the reproduction and alcohol production. The aim of my series of experiments would be the examination of the ability of strains producing ethanol from whey (*Kluyveromyces lactis* NU and *Kluyveromyces marxianus* NB) stress-tolerance capability, batch fermentation under oxygen and carbon-limited conditions. The application of the optimal fermentation parameters together with new biotechnological methods can provide the economical ethanol production, rarely achieved so far.

Promising results were obtained through the examination of the heavy metal accumulation capacity of the cells. For a further step, it would be an interesting area of research to explore the heavy metal accumulation capacity of cells to the maximum, and the influence of environmental parameters.

Immobilization to HAP tablet with pseudohyphal form of yeast was much more efficient than with the unicellular organism. Buffering of the solution did not affect the ability of the cells to immobilisation, but cell wall treatments did affect it. Henceforth, I recommend the optimization of immobilized biosorbent on the HAP material for the removal of heavy metals.

6. NEW SCIENTIFIC ACHIEVEMENTS

1. With the analysis of different lactose utilizing strains I showed that the strains from milk products adapted to the environment from the point of view of lactose utilization, because the highest biomass yield performing strains are selected from them, and in some case strains I showed lactofilia. In the case of lactose or starch utilizing yeast strains I determined the optimum C and N sources value which ensure the biomass yield in the case of appropriate strains through the production of biomass: *S. fibuligera* CCY 42-3-1 (starch concentration of 25 g/L and yeast extract 8 g/L or 14 g/L starch and 4 g/L ammonium-sulphate); *L. kononenkoe* CCY 33-4-1 (30 g/L starch and 7 g/L yeast extract); *Deb. occidentalis* Y758 (23 g/L starch and 7 g/L yeast extract or 22 g/L starch and 3.5 g/L ammonium-sulphate). *K. lactis* NU (20 g/L lactose and 5 g/L yeast extract); *K. marxianus* NB and *D. anomala* VT (30 g/L lactose and 8 g/L yeast extract or 23 g/L lactose and 5 g/L ammonium-sulphate). I showed that the growth optimum of these strains is more acidic (4 - 4.5) pH range as compared to *Saccharomyces cerevisiae* strains, where the fermentation is microbiologically safer.

2. Since I wanted to apply yeasts for a heavy metal accumulation I found that both the living and heat inactivated (dried) cells are suitable for biosorption purposes, but between the different strains I found sometimes significant differences, on the one hand in various heavy metals

accumulation (Cr, Cu, Cd, Zn, Ag), and the other hand in the case of living and dried cells. I found that the dried cells are capable of the further effective cleaning of the water from the water purification plants which include metals in low concentration.

3. I detected the amount of accumulated heavy metals in some cell compartments with fractionated digesting cells. Yeast strains belonging to five different species were organically bound the copper (70-90%) by cell wall. All the yeast species, except *Schizosaccharomyces pombe* RIVE 4-2-1, accumulated Cr^{6+} in similar quantity. In the case of lead and nickel the ratio was generally 10 – 20 % higher in the cytosol and in vacuoles.

4. I found that the pseudohyphae forming yeast attach more effectively and form biofilm on the surface of the carrier, than the unicellular yeast species. The best results were achieved with the dimorphic yeast species *Saccharomycopsis fibuligera* CCY 42-3-1 and *Kluyveromyces marxianus* NB. The immobilization in the range between pH 5 and 7 was not affected significantly. In the case of *Saccharomycopsis fibuligera* CCY 42-3-1 both detergents and lysing enzyme (Sigma) treatments increased the adhesion efficiency, while in the case of *Kluyveromyces marxianus* NB only the cell wall lytic enzyme complex treatment was effective. I found that strains of both species fall in the hydrophobic range, but due to effective treatments the cells became hydrophilic.

7. PUBLICATIONS

JOURNALS

In journals with impact factor

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International (abstract)

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