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Hydrogen Peroxide Removal with Magnetically Responsive *Saccharomyces cerevisiae* Cells

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Hydrogen peroxide (HP) is a promising chemical sanitizer for use in the food industry. Its residues have to be decomposed, usually using an enzyme process employing catalase. In order to offer an inexpensive biocatalyst and to simplify subsequent manipulation, we have prepared magnetically responsive alginate beads containing entrapped *Saccharomyces cerevisiae* cells and magnetite microparticles. Larger beads (2−3 mm in diameter) were prepared by dropping the mixture into calcium chloride solution, while microbeads (the diameter of majority of particles ranged between 50 and 100 μm) were prepared using the water in oil emulsification process. In general, microbeads enabled more efficient HP decomposition. The prepared microparticulate biocatalyst caused efficient decomposition of HP in water solutions (up to 2% concentration), leaving very low residual HP concentration after treatment (below 0.001% under appropriate conditions). The biocatalyst was stable; the same catalytic activity was observed after one month storage at 4 °C, and the microbeads could be used at least five times.

KEYWORDS: Magnetic alginate beads; *Saccharomyces cerevisiae* cells; hydrogen peroxide; catalase; magnetic separation

INTRODUCTION

Several types of chemical sanitizers (e.g., chlorine, chlorine compounds, iodophors, surface active agents, peroxyacetic acid, etc.) are currently used in food technology. Among them, hydrogen peroxide (HP) deserves special attention, being a very effective microbicidal and bleaching agent, whose use is permitted in some countries, including the United States. HP is effective against a wide range of microorganisms, including bacteria, yeast, molds, viruses, and spore-forming organisms (*1*). Its major benefit and the reason for its growing popularity in the food, cosmetic, and medical fields is its low toxicity at applied concentrations, as well as its safe decomposition products (water and oxygen). As stated by 184.1366 of the FDA Code of Federal Regulations, hydrogen peroxide is affirmed as generally recognized as safe. Maximum treatment levels for different types of food range from 0.04% (whey) up to 1.25% (emulsifiers containing fatty acid esters); for specific food, such as dried eggs, instant tea or beef feet, the amount sufficient for the purpose can be used.

Hydrogen peroxide has been used in food industry in several countries, for example, in the production of Swiss cheese (in this case raw milk was treated with 0.2% of a 35% hydrogen peroxide solution at 49 °C for 30 min (*2*)) and for milk cold pasteurization (*3, 4*). Recent research performed in Bangladesh has shown that hydrogen peroxide addition to raw milk (0.05% concentration) enabled milk storage for 20 days compared to that of the control one (5 days only) at refrigerated temperature (8 °C) (*5*). In addition, the FDA has approved the use of HP for sterilization of equipment and food and drinks containers (*1*).

The excess of hydrogen peroxide is usually decomposed by adding catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6). Commercial catalase preparations from molds and bacterial sources are available but tend to be rather expensive. In selected cases, crude microbial extracts from appropriate microbial producers can be used (*6*).

Microbial cells can serve as inexpensive substitutes of purified enzymes. In the case of catalase, readily available *Saccharomyces cerevisiae* cells (baker's yeast) are of special interest because of their broad application in the food industry; they are classified as generally recognized as safe (GRAS). The cells can be used both in the native state (*7*) or in the immobilized form (*8*).

Immobilized form of catalase-producing microbial cells enables relatively simple biocatalyst removal and its repeated application. In order to simplify the manipulation, magnetically responsive cells containing beads are of special interest because they enable work in difficult-to-handle samples, such as suspensions. Magnetic particles of different types can be selectively

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captured using batch or flow-through magnetic separators based on strong permanent magnets. Magnetic properties of the beads are usually caused by the presence of magnetic iron oxides nano- and microparticles (*9, 10*).

In this article, we describe the preparation of two types of magnetically responsive alginate beads containing *Saccharomyces cerevisiae* (baker's yeast) cells and magnetite microparticles. Because of the presence of intracellular catalase, these magnetically responsive biocatalysts have been successfully used to decompose hydrogen peroxide in water solutions.

MATERIALS AND METHODS

Materials and Equipment. Sodium alginate was from BDH Laboratory Supplies, United Kingdom. Magnetite (iron (II, III) oxide, declared particle size <5 μm) was from Aldrich, USA. Hydrogen peroxide, sulfuric acid, potassium permanganate, sodium oxalate, calcium chloride, and sodium chloride were obtained from Lachema, Czech Republic. Sunflower oil and compressed baker's yeast (*Saccharomyces cerevisiae*) were purchased in a local market. Yeast cells were suspended in 0.15 M NaCl, and the homogeneous suspension was centrifuged at 2500*g* for 5 min; after pouring off the supernatant, the remaining pellet was used as the starting material (wet yeast cells). The flat magnetic separator was from Qiagen, USA.

Preparation of Magnetically Responsive Alginate Beads with Encapsulated Yeast Cells. The millimeter-sized alginate beads were prepared as follows. Sodium alginate solution (2%; 2 mL) was thoroughly mixed with magnetite (20 mg), then 1 g of wet yeast cells was added. The thoroughly mixed suspension was dropped through a pipet tip in 5% $CaCl_2$ solution. After 10 min, the beads were magnetically separated and transferred to 1% $CaCl_2$ solution for 30 min. The magnetic calcium alginate beads were stored in 0.15 M NaCl containing 0.05 M $CaCl_2$ at 4 °C. Usually, 74 pieces of magnetic alginate beads were obtained.

To prepare magnetic alginate microbeads, 2 mL of 2% sodium alginate, 20 mg of magnetite, and 1 g of wet yeast cells were mixed in a test tube. After adding 8 mL of sunflower oil, the suspension was thoroughly vortex mixed for 5 min in order to create magnetic alginate microbeads. Then 10 mL of 5% $CaCl_2$ was added, and mixing continued for another 2 min. Sunflower oil was washed out with 1% $CaCl_2$, using a flat magnetic separator. The prepared magnetic calcium alginate microbeads were stored in 0.15 M NaCl with 0.05 M $CaCl_2$ at 4 °C. Approximately 2.6 mL of sedimented microbeads was prepared.

Catalase-free magnetic beads were prepared in order to test nonspecific HP decomposition. Catalase-free yeast cells were prepared by steam-boiling the cells suspension (in 0.15 M NaCl) for 10 min. After centrifugation at 2500*g* for 5 min, the pellet was used for magnetic alginate particle preparation. In addition to magnetite-containing beads, nonmagnetic beads were also prepared using analogous procedures; gravity sedimentation was used for nonmagnetic particle separation.

Experimental Setup. All experiments were performed using both magnetic alginate millimeter-sized beads and microbeads. The reaction medium contained 0.15 M NaCl and 0.05 M $CaCl_2$. Different concentrations of HP (range ca. 0.01−2.2%, corresponding to 3−650 mM) and amounts of magnetic beads were tested. Total reaction volume was 50 mL. The reactions were performed at room temperature under shaking at 125 rpm using Rotamax 120 (Heidolph, Germany). After reaction, magnetic alginate particles were separated from reaction medium using a flat magnetic separator. The initial and final concentrations of HP in reaction media were determined by titrimetric method as described below. Each experiment was repeated three times, and the results were statistically evaluated; each point in the graphs represents the arithmetic mean ± SD. The following parameters were tested.

Amount of Yeast Cells. Different amounts of beads (corresponding to 67−670 mg of wet yeast cells) were placed in the reaction mixture containing hydrogen peroxide (initial HP concentration ca. 1%) and incubated for 1 h.

Initial Concentration of Hydrogen Peroxide. Constant amounts of magnetic beads (corresponding to 335 mg of wet yeast cells) were incubated in 50 mL of reaction medium containing HP (concentration range 3 mM−650 mM) for 1 h.

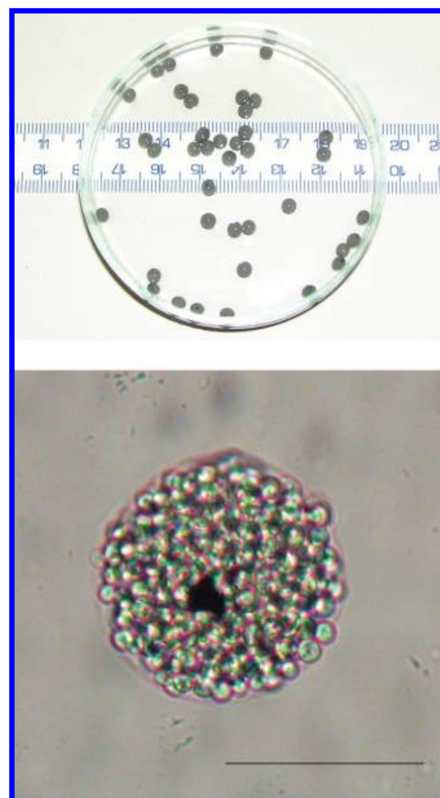

Figure 1. Magnetically responsive alginate beads containing entrapped *Saccharomyces cerevisiae* cells and magnetite microparticles. Millimeter-sized beads (top) and microbeads (bottom). The scale bar corresponds to 50 μm.

Repeated Application of Magnetic Beads. Constant amounts of magnetic beads (corresponding to 335 mg of wet yeast cells) were used five times for the decomposition of different HP concentrations (3 mM−630 mM) for 1 h.

Time Dependence of Hydrogen Peroxide Decomposition. Constant amounts of magnetic beads (corresponding to 335 mg of wet yeast cells) were incubated in 50 mL of reaction medium containing 320 or 650 mM of HP for 1 to 5 h.

Biocatalyst Stability. Magnetic beads were stored in 0.15 M NaCl with 0.05 M $CaCl_2$ at 4 °C, and their ability to decompose hydrogen peroxide was monitored during one month. The reaction conditions were similar as described in the Initial Concentration of Hydrogen Peroxide section; the initial HP concentration was ca. 330 mM.

Determination of Hydrogen Peroxide. Hydrogen peroxide concentrations in beads-free solutions were determined by a titrimetric procedure. The assay was performed as described recently (*11*). Briefly, the sample (volume 1 to 50 mL; mixed with appropriate amount of water to make a total volume of 60 mL) was mixed with 15 mL of sulfuric acid (490 g/L) in a 500-mL conical flask. Potassium permanganate (0.25 mol/L) was added to the mixture drop by drop under continuous shaking until a slight pink coloration was obtained, which persisted for 30 s. The HP concentration was expressed in percentage and recalculated into millimolar concentration.

RESULTS AND DISCUSSION

Magnetically responsive yeast-cells containing alginate beads had spherical shape. Their diameters ranged between 2 and 3 mm in the case of larger beads (**Figure 1**, top), and between 20 and 150 μm for microbeads; however, a majority of microbeads had diameters from 50 to 100 μm (**Figure 1**, bottom). Prepared beads were stable during one month of storage at 4 °C.

Both amount of yeast cells and the type (size) of the particles dramatically influenced the velocity of HP decomposition

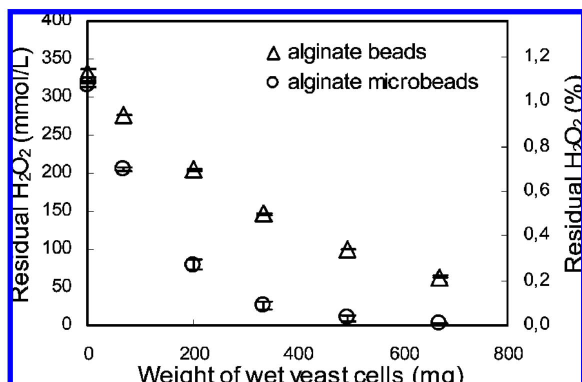

Figure 2. Dependence of hydrogen peroxide decomposition (initial concentration 320 mM) on the wet yeast cells weight in magnetic alginate particles.

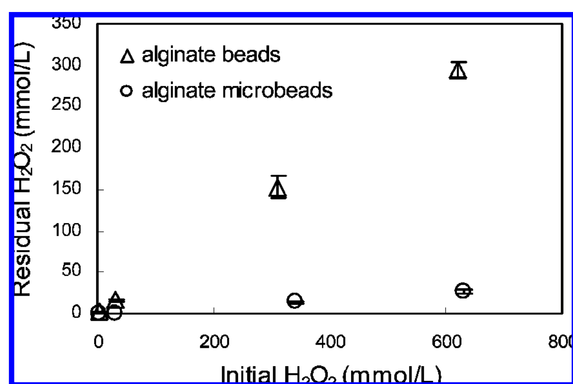

Figure 3. Dependence of hydrogen peroxide decomposition by immobilized yeast cells on the initial concentration of hydrogen peroxide (wet yeast cell weight, 335 mg).

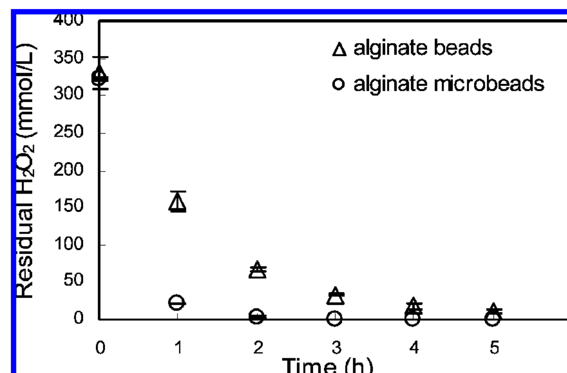

Figure 4. Dependence of hydrogen peroxide decomposition by immobilized yeast cells on the reaction time. Initial concentration of hydrogen peroxide: 320 mM.

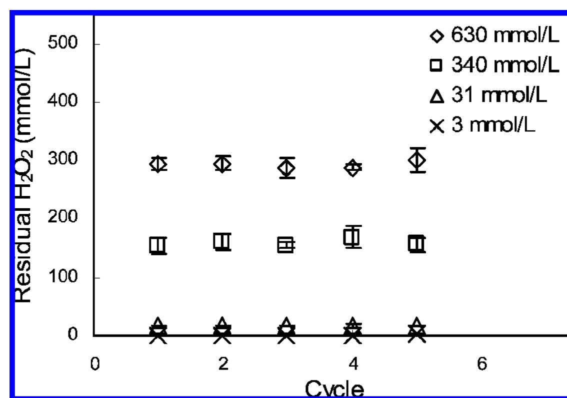

Figure 5. Dependence of hydrogen peroxide decomposition by magnetic alginate beads on the number of cycles.

(**Figure 2**). As expected, the same amount of yeast cells encapsulated in alginate microbeads was substantially more efficient in HP decomposition in comparison with millimeter beads. This fact can be explained by better access of HP to yeast cells entrapped in microparticles. This is a well-known phenomenon, which for example explains phenol degradation by mixed biomass immobilized in alginate beads: the larger the bead size, the slower the phenol degradation rate (*12*). The present data on HP decomposition can be interpreted by using a three step model: (i) mass transfer of HP from bulk liquid to the surface of alginate beads; (ii) intra particle HP diffusion; and (iii) the enzyme reaction by catalase present within *Saccharomyces cerevisiae* cells; because of catalytic properties of catalase, it can be assumed that the enzyme reaction is rapid with respect to the first two steps (*13*).

On the basis of these results, the amount of wet yeast cells encapsulated in magnetic beads was chosen to be 335 mg for the next experiments; this mass of wet yeast cells decomposed about 50% of HP using magnetic alginate beads and about 92% of HP using magnetic alginate microbeads during 1 h of incubation (ca. 320 mM HP initial concentration).

Initial concentration of HP in the reaction mixture can influence the ability of encapsulated yeast cells to decompose HP in samples. To study this effect, the initial HP concentration in the reaction mixture ranged from 3 mM to 630 mM (ca. 0.01−2.15%; see **Figure 3**). Approximately 50% HP decomposition was observed using alginate beads for all initial HP concentration during 1 h of incubation. In the case of alginate microbeads, HP decomposition was substantially better; 96 to 98% HP decomposition was observed for the above-mentioned initial HP concentrations. For HP concentration of 0.05%, allowed by the FDA for milk treatment, the residual HP concentration would be approximately 0.001%.

Time dependence of HP decomposition is shown in **Figure 4**. Using magnetic alginate beads and 320 mM initial HP concentration, approximately 50% of HP was decomposed during the first hour and 97% in five hours. In the case of magnetic alginate microbeads, 95% of HP was decomposed during the first hour and 99.9% in five hours; similar results were obtained for both 320 and 630 mM HP initial concentrations (data not shown).

Reuse of immobilized biocatalysts is one of the necessary assumptions for their potential applications in food and biotechnology industries. HP decomposition efficiency was tested with both types of alginate particles during five cycles, using four different initial HP concentrations (630 mM, 340 mM, 31 mM, and 3 mM). Different results were obtained for larger magnetic alginate beads (**Figure 5**) and for microbeads (**Figure 6**).

Larger magnetic yeast alginate beads exhibited the same HP decomposition efficiency in all five cycles in the whole range of tested HP concentrations. As already shown in previous experiments, relatively high residual HP concentration has been observed using this type of biocatalyst. On the contrary, magnetic alginate microbeads enabled almost complete HP decompostion in all five cycles when using HP solutions up to concentrations of 340 mM (i.e., 1.2%). Higher HP concentration (630 mM; 2.1%) caused gradual inactivation of the catalase activity present in the yeast cells entrapped within the microbeads, which resulted in high residual HP concentration during the fifth cycle (approximately 60% of the initial HP concentration).

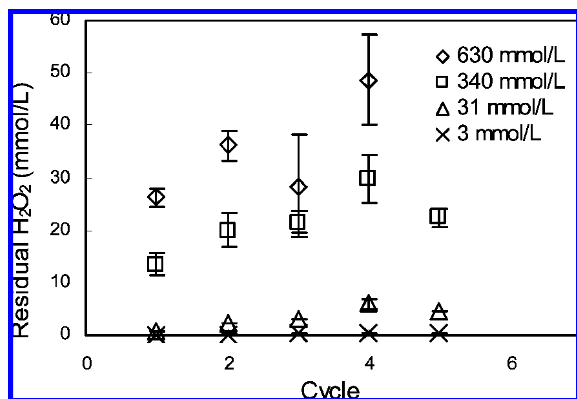

Figure 6. Dependence of hydrogen peroxide decomposition by magnetic alginate microbeads on the number of cycles.

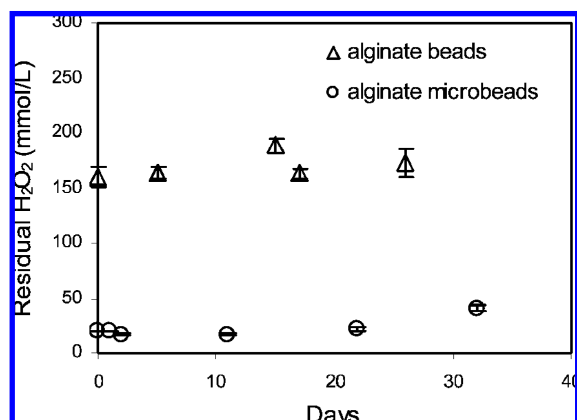

Figure 7. Stability of magnetic alginate beads and microbeads during storage at 4 °C. The initial hydrogen peroxide concentration was 330 mM, and the residual HP concentration is presented.

Magnetically responsive yeast-cells containing alginate beads and microbeads were stable during one month of storage at 4 °C in the presence of calcium ions. As shown in **Figure 7**, there was very low change in HP decomposition efficiency during this period. This finding corresponds to previously published data showing that alginate-immobilized *Saccharomyces cerevisiae* cells can be stored for a long time without loosing enzyme activity and viability (*14*).

In order to check the possibility of nonenzymatic HP decomposition by the biocatalyst, catalase free alginate beads were prepared. It was shown that dead yeast cells entrapped in alginate beads (both with and without magnetite particles) had almost no effect on HP decomposition; at maximum, 0.6% of HP was decomposed using alginate beads without magnetic particles, and at maximum, 2.7% of HP was decomposed using alginate beads with both dead yeast cells and magnetic particles after 1 h of incubation in 330 mM HP initial concentration. Similar results were obtained using catalase-free alginate microbeads.

Presented results clearly demonstrate that immobilized *Saccharomyces cerevisiae* cells (in fact, one of the cheapest and most safe biological materials) can easily decompose hydrogen peroxide. Their immobilization, together with magnetite microparticles, into alginate microparticles led to the formation of a magnetically responsive biocatalyst with high activity and high stability, which can be used repeatedly. Such a biocatalyst could be efficiently used to remove hydrogen peroxide residues in appropriate food technology processes. Becaue of the variety of enzymes present in the entrapped yeast cells, this inexpensive biocatalyst can also be used for other biotechnology processes.

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