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Encapsulated catalase from *Serratia* genus for H_2O_2 decomposition in food applications

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The recombinant catalase isolated from a psychrotolerant microorganism belonging to *Serratia* genus exhibits a high activity in a wide range of pH. Due to a great catalytic potential in operational conditions, it can be used in various industrial applications whereby it acts as a hydrogen peroxide scavenger. To reduce the cost of biocatalyst the enzyme encapsulation into a hydrogel structure was proposed. The obtained results showed a high activity of encapsulated catalase in acidic conditions (pH in range 4.4 – 6.6) and at low temperatures (6–15°C). Moreover, immobilized catalase exhibited a high stability in natural media, especially in milk. Its activity during peroxide decomposition in milk, the possibility of re-using, as well as the fixed bed reactor performance confirmed wide application possibilities. High values of enzyme and substrate concentrations led to the beads burst due to rapid oxygen diffusion from the capsules, thus they are limited.

Keywords: catalase, hydrogen peroxide, enzyme encapsulation, cold sterilization, *Serratia* genus.

INTRODUCTION

Catalase, the well know hydrogen peroxide (H_2O_2) scavenger is extensively used in food processing due to a widespread use of preservatives and various food additives, among which H_2O_2 plays an essential role[1]. Its sporicidal and bactericidal properties are appreciated in milk and cheese technologies as well as in areas of sterilizing agents in packaging materials[2]. Moreover, H_2O_2 is the acceptable substance by the Joint FAO/WHO Expert Committee on Food Additives as an antioxidant and food preservative[1]. Nowadays, the use of H_2O_2 in raw milk preservation is common in countries with a restricted access to refrigeration facilities, while the EU and US also accept its supplementation during cheese-making and modified whey processing[3]. The protective action of H_2O_2 on raw milk is related to lactoperoxidase system that includes additionally thiocyanate and hydrogen peroxide and exhibits a bactericidal and bacteriostatic effect on gram-negative bacteria with irreversible inhibition of D-lactate dehydrogenase and gram-positive bacteria with reversible inhibition of membrane ATPase, respectively. For activation of lactoperoxidase system, the external supplementation of H_2O_2 is necessary, like in cold pasteurization[4]. A lot of research has shown that H_2O_2 is an economical and effective alternative to other preservative agents (such as $NaHCO_3$, ethanol and boric acids) in the dairy industry since it makes short-term milk preservation possible during transport from the production to the consumption area[5]. Most of the H_2O_2-related industrial applications require rapid and effective H_2O_2 removal to achieve a high quality of final products. The excess of H_2O_2 should be decomposed due to the possible changes in the nutritional value of treated foodstuffs and formation of toxic substances[1]. Additionally, a lot of methods of H_2O_2 detection in food samples including traditional approaches as well as using sensors and strips make it possible to use H_2O_2 in a safe way[3]. The problem of enzyme stability under industrial conditions is the main factor, which limits the popularity of biocatalysis in industrial application. Extreme values of pH and temperature restricted the wide application possibilities of catalase in food and bleaching processes[6, 7]. In most cases, application of fungal and bacterial catalase is restricted to neutral pH and 20–50°C[8]. Extremophilic microbial enzymes may provide a solution to unfavorable operational conditions. The recombinant catalase isolated from a psychrotolerant microorganism belonging to *Serratia* genus shows a high activity in a wide range of pH and temperature[9]. Due to its great catalytic properties, it can be an interesting proposition for applications on the industrial scale. The benefits following by industrial biocatalysis are often covered up by the high processing costs, mainly related to the purchase of enzyme[10]. In spite of that, the methods of enzyme immobilization make it possible to improve the biocatalysis economics.It has been confirmed that porous materials, like organic or inorganic membranes and hydrogels, are recognized as effective supports to commercial catalase immobilization[11–14].

Enzyme encapsulation into a hydrogel structure due to the low cost of hydrogel materials, the simple method of capsules production, as well as the various modifications of capsules (core-shell structure), is increasingly popular in industrial biocatalysis. Furthermore, the rapid enzymes separation from reaction mixture via filtration, the elimination of product inhibition effect, the improving enzymes operational stability and the enzymes re-using for many batches are sought in technological-industrial solutions[10].

The encapsulation method into hydrogel structure of the recombinant catalase was proposed in this study as an effective way to improve its stability and the hydrogen peroxide decomposition efficiency in food applications.

EXPERIMENTAL

Material

The catalase preparation was donated by Swissaustral (USA), sodium alginate was obtained from Sigma–Aldrich (USA). The other reagents were purchased from POCh (Poland).

The native catalase stability

The enzyme stability has been described as the number of cycles in which H_2O_2 concentration was decreased below the value of 0.25 g/L. The reaction was carried out in thermo-stated (15°C) glass tubes. The reaction mixture consisted of catalase preparation 0.04 g/L, 10–20 g/L H_2O_2 and a buffer solution (0.1 M acetic buffer pH 4–5 and 0.1 M HEPES buffer at pH 6.6.) in a final volume of 3.6 mL. The H_2O_2 concentration was checked every 7 minutes. If its value was below 0.25 g/L, the new substrate portion was added and the new cycle was begun. The cycle time was extended to the next 7, 14, 21 minutes if the concentration 0.25 g/L was not reached. Hydrogen peroxide concentration was determined spectrophotometrically (Shimadzu UV-1800) at 230 nm, using a standard of curve: $A(230) = 1.97\ C_{H2O2}$ [g/L].

Immobilization of catalase into alginate capsules

The alginate capsules were prepared of 1.8% (w/v) sodium alginate in 0.1 M acetate buffer at pH 3.5–5.0 or 0.1 M HEPES buffer at pH 6.0–6.6. The tested enzyme concentrations, determined by the Lowry method[15], were in the range of 0.2–20 mg/L. The mixture of enzyme and sodium alginate solution was dropped into a crosslinking bath – 15% (w/v) sodium chloride solution in 0.1 M acetate buffer or 0.1 M HEPES buffer by Pasteur pipette. To avoid the enzyme diffusion during capsules creation process, the cross-linking bath included the enzyme at the same concentration as in the dropped solution. The instilled mixture to crosslinked bath solution volume ratio was 1:1.5. The crosslinked bath solution was stirred at 230 rpm. The capsules were crosslinked at 4°C for 1 hour. Then they were washed thrice with the 0.1 M acetate or HEPES buffer solution. The capsules were stored at 6°C in a suitable buffer solution.

The measurement of encapsulated catalase activity

The reactions of hydrogen peroxide decomposition were carried out in thermostated stirred-tank reactors of a total volume of 50 ml at 6–15° C and at stirring velocity of 230 rpm. The volume ratio of capsules (V_c) to hydrogen peroxide solution in buffer (V_b) was 1:10, respectively. 0.1 M acetate buffer at pH 3.5–5.0 or 0.1 M HEPES buffer at pH 6.0–6.6 was used. The substrate concentration was 0.5 g/L, while the catalase concentration inside the capsules was 0.02 g/L. The moment of capsules added to the substrate solution was recognized as the beginning of the hydrogen peroxide decomposition reaction. Substrate concentration during the process was measured spectrophotometrically at 230 nm. The reaction rate was calculated according equation:

$$r\left[\frac{g}{L\cdot h}\right] = \frac{\Delta m_S}{\Delta t\cdot(V_b+V_c)} \quad (1)$$

where:

m_S – mass of substrate, g
r – reaction rate, g/L·h
t – time, h
V_b – buffer volume, L
V_c – capsules volume, L

The operational stability of encapsulated catalase

The encapsulated the catalase stability in natural media was determined in Fortuna vegetable juice (pH 4.4), Pilsner Urquell beer (pH 4.5) and Laciate 0% cow milk (pH 6.6), at 6°C and 0.5 g/L H_2O_2. The ratio of the capsules volume to the volume of media was 1:10, respectively. The time of capsules incubation was 217.25 hours. During that time the concentration of H_2O_2 was monitored and fresh portions of natural media with new portions of substrate at 0.5 g/L were added when this concentration changed by 10%.

Before the enzymatic activity measurement, the capsules were washed for an hour in 0.1 M acetate buffer pH 4.4 and 4.5 for the juice and beer, respectively or in 0.1 M HEPES buffer, pH 6.6 for the milk, at 6°C. The buffer solutions were changed twice to fresh, after 15 and 30 minutes. The time of rinsing was added to the time of capsules incubation in natural media.

During reaction the volume ratio of capsules and buffer was 1:20, respectively. The reaction was carried out in a stirred-tank reactor at 15°C, in the presence of the appropriate buffer solution and H_2O_2 at 0.5 g/L.

The estimation of the reaction progress was based on the measurements of oxygen concentration using LDO (dissolved oxygen sensor), Hach. The hydrogen peroxide concentration was determined based on the calibration equations: $C_{H2O2} = [-0.0017\cdot (C_{O2}-0.0005)/0.001] + 0.1$.

Hydrogen peroxide decomposition in the fixed-bed reactor

The reaction was conducted in a glass chromatography column, at the volume of 120 mL – Fig. 1. Capsules with the encapsulated catalase were packed and flooded by the 0.05 M HEPES buffer, pH 6.6. The capsules at different diameters in the range of 2–3.8 mm were tested. The enzyme concentration inside the capsules in all cases was the same, equal to 0.2 mg/L and the ratio $V_{capsules}/V_{buffer}$ was always 7:1. The concentration of the substrate in the milk dosing to the column was in particular series 0.0075 and 0.15 g/L. Residence times were regulated by the flow of milk solution generated by a rotor pump (Model 75211-15, Cole-Parmer Instrument Company). The flow stream was regulated by two valves and was in the range 0.05–0.31 mL/s while its temperature was kept at 15°C. The oxygen concentration in the entering stream was continuously measured by the LDO sensor, Hach.

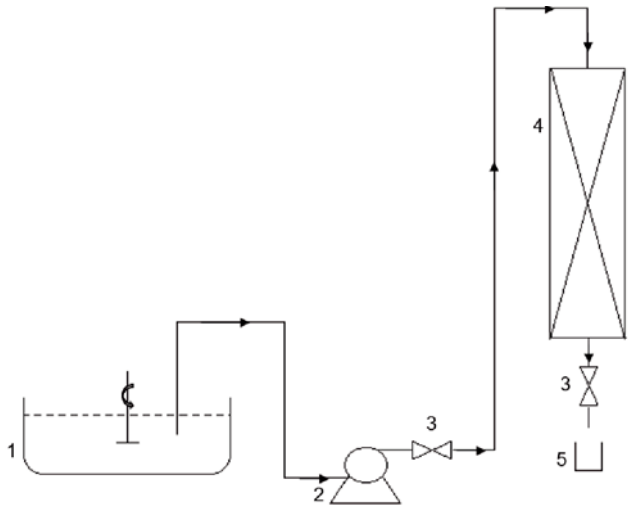

Figure 1. Scheme of the fixed-bed reactor. 1- feed (cow milk with H_2O_2) thermostated tank, 2 – rotor pump, 3 – valve, 4 – packed column, 5 – product sample

RESULTS AND DISCUSSION

The native catalase stability

The activity of native catalase has been examined in acidic conditions, at pH of popular beverages: cow milk, vegetable juice and beer. The preservative function of hydrogen peroxide was noted usually in the range from 0.4 g/L to 12.5 g/L during the preservation of raw milk and cheese making[6, 16, 17].

Catalase in native form (tested at concentrations 0.04 g/L) could be re-used in 13 cycles at pH 6.6 and 10 g/L H_2O_2. The higher (20 g/L) substrate concentration led to the reduction of cycle number to 9. At pH 4 and 5, the native catalase has exhibited the lower stability. In all cases, the activity was lower in the presence of 20 g/L H_2O_2 (Fig. 2) but this concentration is above the concentration applied in the food industry.

Obtained results have shown that catalase from Swissaustral can be considered as a competitive biocatalyst to other commercial preparations, especially in cold milk pasteurization processes, in which pH of treated milk is around 6.6. Anyway, there is a need to improve the enzyme stability at pH 4–5 that corresponds to pH of juices, wines and beers. Therefore, the tests under these pH conditions were performed upon the enzyme encapsulation into the hydrogel.

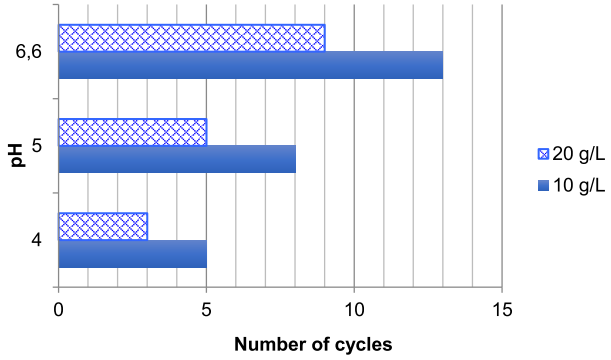

Figure 2. The stability of the native catalase from Swissaustral expressed as the number of cycles, at 10 and 20 g/L H_2O_2, pH = 4–6.6

The measurement of encapsulated catalase activity

The tested pH values were in range 3.5–6.6 corresponding to pH of popular beverages. Considered temperatures were close to the storage temperatures (6–15°C). As it was presented above, the tested enzyme was able to work at very high H_2O_2 concentrations. In the case of the encapsulated enzyme, high substrate concentrations induced the high concentrations of oxygen (the reaction product) which accumulated inside capsules caused their fast bursting. Therefore, the initial concentration of 0.5 g/L H_2O_2 was applied in this case.

The tested catalase has shown very good activity at pH 5.0–6.6 – Table 1. The initial reaction rate decreased with the pH decrease. Below pH 4.4 the preparation was practically not active. Moreover, the dependence on temperature has been noticed. Nonetheless it is very important information that the preparation can be applied at very low temperatures, what is a unique property of the analyzed enzyme.

The operational stability of encapsulated catalase

The main benefit of enzyme immobilization is related to an increase of enzyme stability at operational conditions[21, 22]. Therefore, the encapsulated enzyme stability was investigated in natural media as Fortuna vegetable juice (pH 4.4), Pilsner Urquell beer (pH 4.5) and Laciate cow milk (pH 6.6). The initial reaction rates at 0.5 g/L H_2O_2, 6°C and $C_{catalase}$ = 0.02 g/L were 0.0037, 0.0038 and 0.0167 g/L*Min for juice, beer and milk, respectively.

The encapsulated catalase has shown the highest stability in milk. It exhibited 63% remaining activity after 217.5 hours. After incubation for 146 hours in the vegetable juice and beer it maintained 21% and 2% of the activity, respectively (Fig. 3). The pH values of these media were 4.4 and 4.5, respectively, thus very close to the limit value (pH = 4.2) at which the native enzyme was practically inactive. Since the pH of juice and beer were very similar, it means that another compound e.g. ethanol also influences that stability. Obtained results are quite satisfying while the commercial catalase was inactive at pH below 5.5[23].

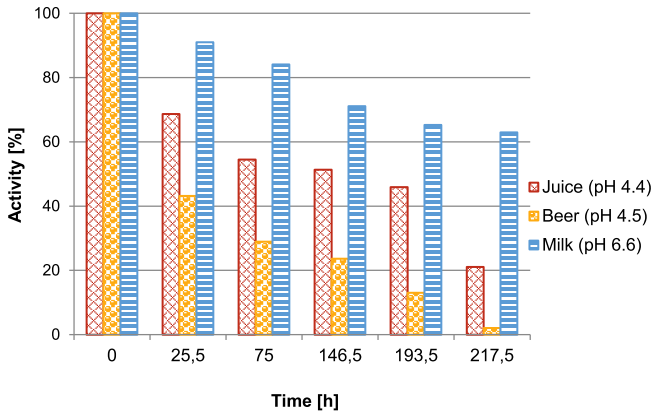

Figure 3. The stability of encapsulated catalase (0.02 g/L) in natural media: Fortuna vegetable juice (pH 4.4), Pilsner Urquell beer (pH 4.5) and Laciate cow milk (pH 6.6), at 6°C, 0.5 g/L H_2O_2

The compounds present in the tested beverages and diffusing into capsules had an influence mainly on the colour of the capsules (Fig. 4). The change of 10–15% in the capsules size was observed also as a result of more water absorbed into the capsules. The presence of calcium ions in milk had a positive impact on the physical stability of alginate capsules. In the other media

Table 1. The activity of encapsulated catalase from Swissaustral at acidic conditions and low temperatures, $C_{catalase}$ = 0.02 g/L, 0.5 g/L H_2O_2. The initial reaction rate was expressed as a percentage of the reaction rate at the best of tested conditions (pH 6.6, 15°C) for which it was estimated on 0.0250 g/L·Min.

pH	6.6	6.0	5.0	4.8	4.6	4.4	4.2	3.5
r(15°C) [%]	100.00	98.64	92.11	72.00	37.99	18.01	0.59	0.0
T [°C]	6	8	10	13	15			
r (pH=6.6) [%]	47.83	67.39	71.74	91.30	100			

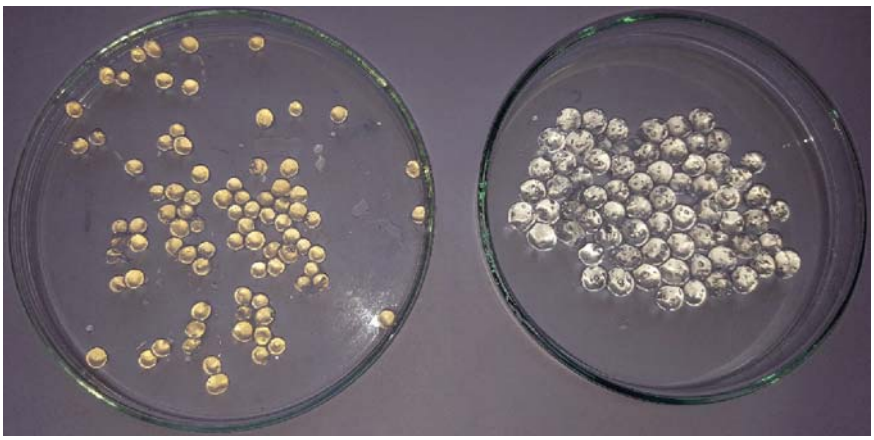

Figure 4. Alginate capsules with the immobilized catalase before (left) and after 217.25 hours (right) of H_2O_2 decomposition in the milk

they lost their physical properties by crosslinking calcium ions stepwise elusion.

Hydrogen peroxide decomposition in the fixed-bed reactor

In a stirred tank reactor, high system turbulence promotes the transpot of oxygen on the outside of capsules. In the case of a fixed-bed reactor, an oxygen accumulation caused capsules floating towards a liquid-air surface and in the next minutes capsules burst. Therefore in the case of the process conducting in the bed reactor the enzyme concentration inside the capsules was decreased to the value 0.2 mg/L and the substrate concentration was decreased to 0.0075 and 0.15 g/L. This H_2O_2 concentration was much lower the previously tested but according to the literature even 0.03 g/L of H_2O_2 significantly reduced the microbial contents in milk samples[24].

The diffusion resistances are recognized as the greatest restrictions reducing the efficiency of industrial technologies based on encapsulated biocatalysts. Among external and internal limitations of the mass transfer, the first group can be overcome by the change of the reactor hydraulic conditions, but limitations related to the second group are much harder to solve due to their connection to the enzyme properties, type of reaction kinetics and capsules mechanical and chemical properties. One of the improved methods for an encapsulated enzyme is the change of capsules performance[25, 26].

In this study, capsules with different diameters in the range 2–3.8 mm were tested. The obtained results showed no significant differences between conversion degrees obtained for different size capsules at the same residence time (Table 2). It means that under these conditions the process is run in the kinetic regime[27].

CONCLUSIONS

Despite some disadvantages of enzyme immobilization into hydrogels, like enzyme leaking during crosslinking or product diffusion limitations, presented results are satisfactory. The measurement of catalase concentration via Lowry method in crosslinking bath no enzyme leakage confirming the high immobilization efficiency of the catalase isolated from psychrotolerant microorganism belonging to *Serratia* genus into sodium alginate beads. Additionally, encapsulated catalase isolated by Swissaustral can be an interesting solution for beverages cold pasteurization, compared to encapsulated catalase from bovine liver, which cannot be used for hydrogen peroxide decomposition in fruit juices, beer and wine, due to the low activity at pH below 5.5.

When the capsules were applied in the stirred tank reactor both - enzyme and substrate concentrations could be higher than in the case of the bed reactor. The operation of the system was stable at the substrate concentration of 0.5 g/l and enzyme concentration inside capsule of 20 mg/l for a stirred reactor and substrate concentration of 0.15 g/l and enzyme concentration in capsule of 0.2 mg/l for a fixed-bed reactor.

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Table 2. Substrate conversion degree in the bed reactor filled with capsules with a diameter of 2 to 3.8 mm including the enzyme at concentration 0.2 mg/L. Cow milk at pH 6.6 at 15°C containing 0.075 and 0.15 g/L H_2O_2 was the dosing stream

Residence time [Min]	C_0 = 0.075 g/L			C_0 = 0.150 g/L		
	Capsules diameter [mm]			Capsules diameter [mm]		
	2.0	3.0	3.8	2.0	3.0	3.8
1.62	0.52	0.47	0.53	0.50	0.43	0.47
4.67	1.50	1.46	1.41	1.51	1.53	1.42
6.44	2.08	2.02	2.06	2.09	2.00	1.97
8.46	2.77	2.59	2.78	2.74	2.85	2.88

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