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Catalytic membrane with the recombinant catalase from psychrotolerant bacteria *Serratia* sp. in dairy applications

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ABSTRACT

The preparations of catalase immobilized into a membrane are a promising solution for the industrial application, for example, in dairy processes, in which hydrogen peroxide is used as a preservative agent. The activity of the immobilized recombinant catalase from psychrotolerant bacteria *Serratia* sp. confirms its huge application potential, since the preparation is active in cow milk solution (pH 6.6) and at low temperature (8°C–12°C). This work shows the results of above-mentioned catalase, chemically immobilized via divinyl sulfone on the regenerated cellulosic membrane. The immobilized enzyme exhibits kinetics in accordance with Michaelis–Menten equation until 1.8 and 2.5 g/L of H_2O_2, for 12°C and 8°C, respectively. Moreover, the obtained preparation keeps more than 50% of the initial activity for 3.5–6.6 h under process condition (1–2 g/L H_2O_2, pH 6.6, 8°C–12°C).

Keywords: Catalase; Dairy industry; Chemical immobilization; Cellulosic membrane; Catalytic membrane

1. Introduction

Biocatalysis plays an important role in current industrial technologies. Moreover, advanced screening procedures allow for searching of desirable microbial species and their isolation from the extreme environments to create attractive enzymatic preparations [1]. The food industry itself engages a wide range of enzymes, both in native and immobilized form like for example, α-amylase, amyloglucosidase, catalase, cellulase, β-galactosidase, glucose isomerase, oxidase, hemicellulase, xylanase, lipase, pectinase, and protease [2]. Catalases are also applied for hydrogen peroxide (H_2O_2) decomposition.

The problem of food preservatives and additives is widely discussed and regulated by law. According to WHO (World Health Organization) and FAO (Food and Agriculture Organization of the United Nations) regulations, H_2O_2 is recognized as a food preservative, preventing microbial contaminations [3]. H_2O_2 addition is considered as an alternative to the traditional approach, related to other chemical preservatives application, such as $NaHCO_3$, ethanol, or boric acid [4–6]. H_2O_2 is commonly used in cheese-making, dairy, and beverage industry. The process of cold pasteurization related to H_2O_2 addition is well known in countries with a restricted access to refrigeration facilities. H_2O_2 allows for short-term beverages preservation required for the safe transport from production to processing areas [7]. To maintain nutritional values of H_2O_2-treated foodstuffs, all amount of H_2O_2 must be decomposed before consumption. For this purpose, enzymatic strategies involving a catalase addition are appreciated.

Catalase preparations increase the attractiveness of H_2O_2-related technologies, allowing for its rapid decomposition and providing a high quality of H_2O_2-treated final products not only in the food industry, but also in the textile and the paper ones [8]. Moreover, the application of the recombinant catalase isolated from psychrotolerant bacteria belonging to *Serratia* genus increases the catalase application possibilities due to its activity in the wide range of pH and temperature compared with traditional catalase preparations [1].

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Enzyme immobilization increases the profitability of industrial biocatalysis, mainly by the enzyme molecules reuse. Additionally, it may lead to the increase in an enzyme stability under operational conditions. Variety of immobilization methods (chemical and physical), as well as the number of available carriers (hydrogels, biopolymers, and solid supports), confirm the potential of immobilized enzymatic preparations [9,10].

During a chemical immobilization on a membrane surface, enzyme molecules are efficiently bound. It prevents enzyme subunits dissociation and increases the stability of proposed preparations [11]. Furthermore, the enzymatic membrane can be reused many times, or it can be applied in a continuous process [12,13].

Natural polymers, such as cellulose, ensure a favorable price of cellulosic carriers due to the commonness of plant raw materials. Due to the properties of cellulosic membranes such as pH and temperature stability, they can be used in many industrial processes [11]. Particularly, as a natural material, they are recommended to use in food and pharmaceutical applications.

The presented results refer to the practical aspects. Hence, the applied values of temperature and substrate concentrations are like the industrial operational conditions. Obtained preparations of catalase were designed to work in dairy industry or in another drink production. The combination of a cellulosic membrane and a recombinant catalase preparation could allow for a unique preparation creation. The most important was the possibility to use it at low temperatures (≤12°C) and its stability at relatively high substrate concentration.

2. Materials and methods

2.1. Materials

The recombinant catalase preparation isolated from psychrotolerant bacteria *Serratia* sp. (65,000 U/mL, purity > 99%) was donated by Swissaustral (USA). Divinyl sulfone (DVS) was purchased from Sigma-Aldrich (Germany). Hydrogen peroxide and other chemicals were obtained from Avantor Performance Materials Poland S.A. (Poland). The regenerated cellulosic membrane (pore diameters of 0.45 μm) was supplied by Whatman (USA). The natural media solution was cow milk Łaciate UHT 0% fat content (Poland).

Equipment: Stirred Membrane Cell 8010 (Amicon, USA), magnetic stirrer 285-MS11 (Wigo, Poland), ultrathermostat 8012 (PolyScience, USA), spectrophotometer UV-1800 (Shimadzu, USA), thermostated shaking machine KS 4000 ic control (IKA, USA), LDO Dissolved Oxygen Sensor HQ440d multi (Hach, USA).

2.2. The catalytic membrane preparation

The regenerated cellulosic membrane, with pores of 0.45 μm (Whatman) was used in the preparation of a catalytic membrane with the recombined catalase from Swissaustral. The available membrane surface for enzyme binding was 4.9 cm^2. The procedures of a membrane activation and an enzyme immobilization took place in a closed circuit (without a solution circulation) in Amicon Stirred Cell of volume 10 mL.

The activation procedure of the membrane was preceded by its washing with a solution of 1 M Na_2CO_3. Then, 10% DVS (a coupling agent) solution in 1 M Na_2CO_3 of volume 6 mL was added for 2.5 h, 8°C. Next, the membrane was washed with 0.05 M HEPES buffer, pH 6.6 to remove excess of the coupling agent. An immobilization stage was conducted in the presence of a solution of the catalase at concentration 0.34 g/L and 0.5 M $NaHCO_3/Na_2CO_3$, pH 9 at the volume of 4.5 mL, for 24 h at 8°C, 300 rpm. Finally, the unbound enzyme molecules were removed by multiple rinsing with 0.05 M HEPES buffer, pH 6.6. The efficiency of the immobilization process was calculated based on the protein balance in solutions before and after immobilization. The protein concentration was estimated by Lowry's method [14] using the standard curve: A (750 nm) = $C_{protein}$ (g/L)/0.44.

2.3. The recombinant catalase characterization – pH influence

The enzyme activity and stability were tested in pH 4–7 at 25°C, according to the pH values of the most popular beverages. The concentration of H_2O_2 was determined spectrophotometrically at 230 nm using a standard curve A (230 nm) = $1.97C_{H_2O_2}$ (g/L). The enzyme activity was monitored in 7 min cycles. An expected H_2O_2 concentration after each cycle was the concentration below 0.25 g/L. Having assumed H_2O_2 concentration, a new portion of the substrate was added and the further H_2O_2 decomposition in the next cycle was measured. In case of higher H_2O_2 concentration (above 0.25 g/L) after 7 min cycle, the cycle time was extended to 14, 21 min or longer to obtain the expected H_2O_2 concentration. The procedures for longer process durations were the same as in case 7 min cycles.

The reaction with the native catalase was carried out in thermostated at 25°C 25 mL glass tubes. The reaction mixture consisted of catalase preparation 0.4 g/L, 10–20 g/L H_2O_2, and a buffer solution (0.1 M acetic buffer pH 4–6 and 0.1 M Tris–HCl buffer pH 7) in a final volume of 3.6 mL. The measurement of catalase activity in a milk solution (pH 6.6) was performed at 8°C and 12°C and determined by LDO Dissolved Oxygen Sensor. The reaction was carried out in thermostated glass reactors at the volume of 50 mL. The reaction mixture consisted of H_2O_2 1 or 2 g/L in milk solution and catalase 2 μg/L. The oxygen concentration measurement took place in a continuous mode, with 30 s intervals.

2.4. The activity of the immobilized catalase

The activity of the immobilized catalase was tested in Amicon Stirred Cell. The membrane with the immobilized catalase was placed in the cell, into which a cow milk solution with H_2O_2 in range 0.075–2.5 g/L (pH 6.6) in a final volume of 10 mL was then poured. The Amicon Stirred Cell was placed in thermostated shaking machine, maintaining a low-temperature (8°C or 12°C) setpoint. The stirring was kept at 200 rpm. After specific intervals, the oxygen concentration was measured by LDO Dissolved Oxygen Sensor.

2.5. Kinetics with the immobilized catalase

After the immobilization procedure, the obtained enzyme surface concentration was in the range 0.2–0.347 g/m^2. The

kinetic parameters were designated for the presence of H_2O_2 concentration in the range of 0.075–2.6 g/L in the cow milk solution (pH 6.6, at 8°C or 12°C).

2.6. The immobilized catalase stability

The immobilized catalase stability was evaluated in the presence of 1 and 2 g/L H_2O_2 in milk, pH 6.6, at the volume of 2 L. The catalase incubation bounded into membrane surface took place in thermostated shaking machine, 8°C or 12°C, 200 rpm. After appropriate intervals, the membrane with the immobilized catalase was transferred to Amicon Stirred Cell placed in the thermostated shaking machine and the activity of the immobilized catalase at the presence of 1 or 2 g/L H_2O_2 in 0.05 M HEPES buffer, pH 6.6 (the final volume 10 mL) was monitored spectrophotometrically at 230 nm during 22.7 h by taking samples in appropriate intervals. After measurements, the linear change of substrate concentration in time was plotted and thus, an initial reaction rate was estimated.

2.7. The conversion degree of H_2O_2 decomposition by the immobilized catalase

The evaluation of hydrogen peroxide conversion degree by the immobilized catalase was performed in thermostated reactors, at 8°C. The reaction mixture consisted of 0.05 M HEPES buffer solution, pH 6.6, and 2 g/L H_2O_2 at the volume of 20 mL. The membrane of surface 4.9 cm^2 and the enzyme surface concentration of 0.18–0.284 g/m^2 was used.

The reaction was monitored spectrophotometrically at 230 nm by 36 h. Three catalytic membranes with immobilized catalase were used in series. Each membrane was worked for 400 min. After this time, the used membrane was changed to another one. To determine H_2O_2 conversion rate, the samples were taken at appropriate intervals and H_2O_2 concentration was measured spectrophotometrically.

3. Results and discussion

3.1. The recombinant catalase characterization – pH influence

Due to the rapid reaction progress and the fast oxygen generation, the enzyme activity was determined in 7 min cycles. One cycle corresponded to the H_2O_2 decomposition level below 0.25 g/L at the initial concentration 10 and 20 g/L. The obtained results indicated that the recombinant catalase could be used in acidic solutions and had the highest stability at pH 6. The stability depended also on the substrate concentration (Fig. 1).

It is important to note that catalase isolated from psychrotolerant bacteria is an interesting alternative to other commercial catalase preparations, which exhibit the activity in narrower pH range, thus their application may only be related to selected drinks group (water or milk, pH 6.4–7) [15–17]. The presented recombinant catalase preparation may be used additionally to H_2O_2 decomposition from fruit juice, wine, or beer, in which pH is above 4.0. In pH below 4.0 the enzyme is not active.

3.2. The activity of the immobilized catalase

The efficiency of the catalase immobilization was evaluated based on the protein concentration estimation by Lowry

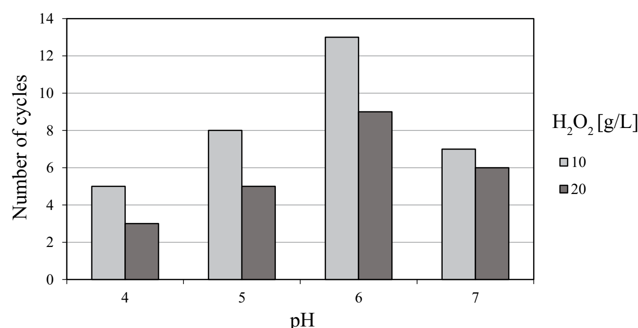

Fig. 1. The stability of the recombinant catalase in native form in different pH at H_2O_2 concentration 10 and 20 g/L, in 0.1 M acetic buffer pH 4–6 and 0.1 M Tris–HCl buffer pH 7, 25°C.

method in solutions before and after immobilization. The enzyme mass bounded on the membrane surface was in the range 0.2–0.347 g/m^2. It corresponded to the immobilization process efficiency 11.56% ± 41.42%. This efficiency could be improved by the enzyme concentration decrease in solution undergone to immobilization.

The operational conditions related to food processes are associated with acidic pH and low temperature. The main application of catalases is H_2O_2 decomposition in milk, therefore the research with the obtained catalytic membrane was carried out in cow milk solutions at cold storage temperature. According to literature reports, the temperature in the truck during transportation depends on the distribution system of a company. Based on Koutsoumanis et al. [18], this temperature was recorded in the range of 3.6°C–10.9°C.

The WHO and FAO suggest the addition of H_2O_2 at concentration 0.5–2.5 g/L to foodstuffs. [3]. Furthermore, many reports indicate that even lower H_2O_2 concentration is sufficient to prevent before food microbial contaminations. It has been presented that H_2O_2 supplementation in the range of 0.03–0.125 g/L effectively reduces the microbial content in milk samples [8,19–21].

The kinetic parameters for the immobilized enzyme were defined in a wide range of substrate concentrations in 8°C and 12°C. H_2O_2 decomposition occurring in proposed temperature range refers to the milk transportation temperatures [18]. Moreover, it allows for rapid H_2O_2 removal right after transport stage, without the necessity of additional temperature increase. Additionally, the obtained result of thermostability at a lower temperature confirms a great potential of immobilized catalase in dairy processes. The process conditions, which were used to the characterization of immobilized catalase kinetic, were determined on the basis of the real conditions – the pH of cow milk (pH 6.6), without any milk modification. According, to the catalase pH stability in native form, the most favorable pH condition was observed at pH 6. Based on literature data, catalase immobilization may shift the pH optimum [22–24]. Furthermore, the stability of native catalase in various pH ranges is closely related to the substrate concentration, as shown in Fig. 1, while the evaluation of catalase catalytic membrane stability in dairy processes takes place at much smaller H_2O_2 concentrations. The immobilized catalase exhibited a little higher activity at the higher temperature (Fig. 2), which was expected. The fact of being

active at such low temperatures is a great advantage of the used catalase. The maintenance of low temperature during all processing in dairy industry promotes the less microbial contamination risk [25,26].

The problem of substrate inhibition was considered in many reports [27,28]. For the tested preparations the inhibition was observed above 1.8 and 2.5 g/L H_2O_2 for 12°C and 8°C, respectively (Fig. 2). These results were satisfactory, considering concentrations of H_2O_2 in preservative action of foodstuffs. For data obtained in the range of substrate concentration below the inhibition, the kinetic constants of Michaelis–Menten equation were calculated according to Eq. (1) [29] and presented in Table 1.

$$r_S = \frac{dN_S}{v_m \times dt} = \frac{k_3 \times \frac{m_E}{V_m} \times C_S}{K_M + C_S} \quad (1)$$

where C_S – substrate concentration (g/L); K_M – Michaelis–Menten constant (g/L); k_3 – reaction rate constant (min^{-1}); m_E – enzyme mass located on membrane (g); N_S – amount of substrate (g); r_S – rate of substrate decomposition (g/L min); t – time (min); and V_m – volume of substrate solution (substrate solution) (L).

The obtained values of k_3 mean that the reaction was fast and a few minutes were needed for complete substrate decomposition. Moreover, values of K_M inform that in wide range of applied substrate concentration the reaction runs according to first-order reaction. According to the literature, the desired effect of H_2O_2 addition in dairy processes was observed even at very low H_2O_2 concentrations, such as 0.03–0.125 g/L [8,19–21].

Catalases, depending on the enzyme isolation sources, exhibit broad variations related to the kinetic constant values [30], but the tested enzyme presented unique high activity at low temperatures.

3.3. The immobilized catalase stability

The enzyme stability under operational conditions is a key factor deciding about the preparation usefulness.

The stability of the immobilized catalase was studied at 8°C and 12°C for different substrate concentrations, that is, 1 and 2 g/L H_2O_2. Due to the substrate inhibition, the higher substrate concentrations are unlikely to be used and at lower concentrations the catalytic reaction can even be better. Fig. 3 shows exemplary results expressed as a remaining activity (the ratio between the reaction rate at given time to the initial reaction rate).

According to the procedure described in Ref. [13], the half-life of the immobilized enzyme under different process conditions was calculated. The obtained data are presented in Table 2. The stability of the preparation strongly depends on the substrate concentration. It is related to a strong substrate inhibition. The violent oxygen generation associated with rapid H_2O_2 decomposition by immobilized catalase may affect catalytic membrane stability. The obtained results of the immobilized catalase half-life times were satisfactory (Table 2), compared with other immobilized catalase preparations used in milk pasteurization [31,32]. The proposed immobilized catalase preparation, due to its very good activity and relatively good stability, can be used in many batches or in continuous processes at relatively large dosing stream.

3.4. The conversion degree of H_2O_2 decomposition by the immobilized catalase

The aim of this part of the study was to check if it was possible to completely decompose H_2O_2, which was required in food products and drinks. According to kinetics (Section 3.2),

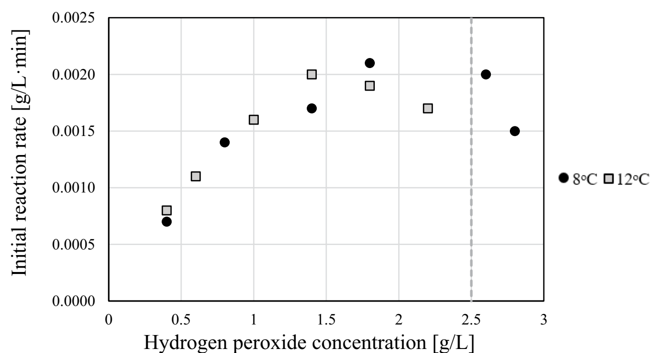

Fig. 2. The kinetics with the immobilized catalase in cow milk solution, pH 6.6, 8 and 12°C.

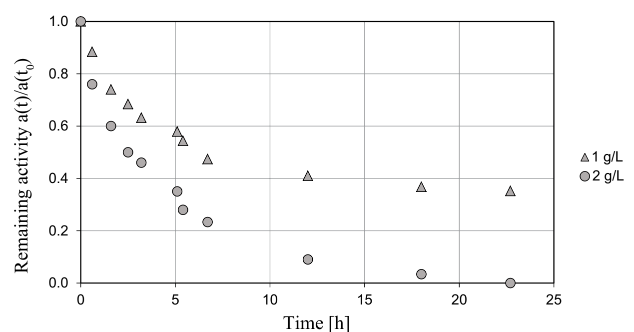

Fig. 3. The immobilized catalase stability in the presence of H_2O_2, 12°C.

Table 1
The values of the constants k_3 and K_M of Michaelis–Menten equation for the immobilized catalase

T (°C)	k_3 (min^{-1})	K_M (g/L)
12	1.034	1.55
8	1.014	1.74

Table 2
The values of half-life for the immobilized catalase at the substrate presence

T (°C)	0 g/L	1 g/L	2 g/L
8	>120 h	6.6 h	4.8 h
12	>120 h	5.9 h	3.5 h

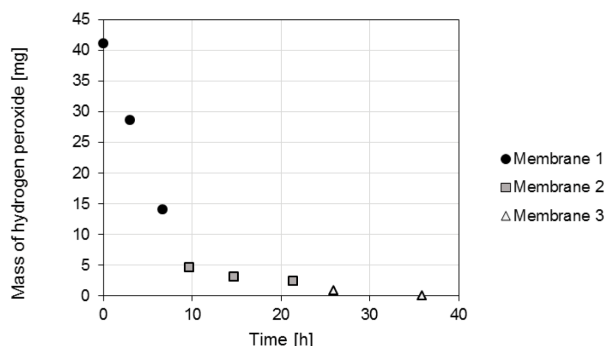

Fig. 4. The conversion rate of 2 g/L H_2O_2 by the immobilized catalase, 0.05 M HEPES, pH 6.6, 8°C. Three membranes in series were used.

the reaction was very slow at very low substrate concentrations, for example, at concentration 0.05 g/L H_2O_2 the rate was equal 0.028 g_S/g_E min. As a consequence, it was necessary to extend the reaction time (or the residence time in case of a continuous process) or to use a large area of the catalytic membrane (that is, a high amount of the immobilized enzyme).

The proposed process was a simulation of a cascade membrane modules. First catalytic membrane worked at high substrate concentration, thus the reaction rate should have been high, but the enzyme stability in this case was low and the reaction rate decreased in time of using the preparation. The last membrane in series was in contact with a solution of a very low substrate concentration, which in turn meant it did not lose its activity quickly.

The obtained results showed that the complete decomposition of H_2O_2 using the prepared catalytic membrane at 8°C was possible (Fig. 4). The carried out tests corresponded to a low concentration of the enzyme on the membrane (0.18–0.284 g/m^2) and a small area of the membrane in relation to the volume of the system (0.245 cm^2/mL). In consequence, the reaction time had to be extended to 36 h. The obtained result can be improved by the increase of the catalytic membrane surface and thus, the increase of the enzyme mass.

4. Conclusions

Catalases play an important role in food applications related to H_2O_2 preservative function. The possibility of H_2O_2 addition to foodstuffs and its rapid and efficient decomposition by catalase treatment is an attractive method.

Enzyme chemical immobilization on a membrane gives the possibility to obtain mechanically, chemically, and biologically stable preparation. Oxygen created during reaction does not cause bonded enzyme to be easily detached.

Moreover, a catalytic membrane creates an opportunity for a continuous process performance with the fast product removal. It is particularly important at a product inhibitory effect. The proposed simulation of a cascade of membrane modules allows for complete H_2O_2 decomposition.

Good stability under process conditions as well as the activity at storage temperatures makes the obtained catalytic membrane preparations of psychrotolerant bacteria *Serratia* sp. useful in continuous processes of dairy industry.

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