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Cellulase Immobilization on Ca-alginate Beads

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Summary

Cellulase (EC 3.2.1.4) was immobilized on inactivated baker's yeast biomass which was trapped in Ca-alginate beads. The specific enzyme activity of free cellulase was 4.72 U/mg protein, while the specific enzyme activity of the immobilized enzyme was 0.56 U/mg protein. The pH optimum of free cellulase was at pH=3.5 and of the immobilized one was at pH=3.0. The immobilized enzyme had a high relative activity over the whole examined pH range which is of great technological importance. The temperature optimum for the free enzyme was between 37 and 60 °C, while the temperature optimum of the immobilized enzyme was found at 60 °C. Free and immobilized enzyme stabilities during 2-hour incubation at 70 °C were very similar. At the end of incubation the initial enzyme activity was drastically reduced. The time profile of enzyme activity showed that immobilized enzyme stored in 0.01 M acetate buffer at 4 °C lost its enzyme activity after 9 days, while in 0.1 M phosphate buffer with DEAE-dextran and lactitol at the same temperature the Ca-alginate beads still had some enzyme activity after 45 days.

Keywords: cellulase immobilization, Ca-alginate, lactitol, DEAE-dextran

Introduction

Cellulose is the world's most abundant biomass (10^{11} metric tons per year) *(1)*, although vegetation utilizes only 0.1% of the energy of the sunlight for it's production. Cellulose has a very wide potential as fuel, feed and food, but for efficient usage it must be hydrolyzed to its monomeric component-glucose *(2)*. Enzyme hydrolysis is the most suitable for this purpose, in spite of some disadvantages including the high price of the enzyme which represents 40% of total enzyme processing costs, and strong inhibition by end products *(3)*.

Second application of cellulases in the processing can be as food processing agent in particular in clarification treatment as grape must, fruit juice *(4)*, oil *(5)*.

Cellulase (EC 3.2.1.4) immobilization on solid carriers gives a promising possibility for recycling or retaining the most expensive component of the process. It enables greater variability in bioreactor design for continuous removal of inhibitory endproducts *(3)*.

Cellulase immobilization is possible in many different modes with different supports, such as dextran *(6)*, agarose gel *(7)*, nylon *(8)*, Fe_3O_4 *(3)*, and modified silica support *(2)*, but in almost all cases immobilization reactions strongly reduced enzyme activity and stability.

Therefore, the goal of this work was to prepare immobilized biocatalysts with cellulase in a two step immobilization procedure. In the first step immobilization of cellulase on inactive yeast biomass was performed, while in the second, the entrapment in Ca-alginate beads was done.

Materials and Methods

Materials

A. niger cellulase EC 3.2.1.4. (Fluka 0.67 U/mg, M_r approximately 31000), glutaraldehyde (Fluka, 25% solution in water, free carboxylic acid<5%), compressed baker's yeast (Union, 25% dry matter) and alginic acid (Industrial grade) were used for the immobilization process. CMC (carboxymethylcellulose) (Tokis) was used as a substrate for the enzyme reaction. BSA (bovine serum

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albumin) (Sigma, M_r=67000, 99% pure) was used as a standard for the protein determination. Lactitol (4-O-β--D-galactopyranosyl-D-glucitol) (Sigma, approx. 99% pure) and DEAE-dextran (Sigma, nitrogen content aprox. 3.2%) were used for prolongation of the enzyme activity. 3,5-dinitrosalicylic acid (Fluka, min. 98% pure) was used as constituent of the reagent for the total reducing sugar determination.

Methods

Total reducing sugars

The concentration of total reducing sugars was determined by the DNS method *(9)*.

Enzyme activity determination

3 g/L CMC solutions were prepared in 0.01 M acetate buffer, pH and temperature were adjusted. After additions of equivalent concentrations of cellulase, the solutions were incubated with mixing (250 rpm) for 15 minutes in a thermostat. The enzyme reaction was stopped by a 5 minutes incubation in boiling water. Specific enzyme activity was expressed in micromols of glucose per minute per mg of enzyme protein. The specific enzyme activity determination of the immobilized enzyme was the same as in the case of the free enzyme.

Protein determination

For protein determination a modified Lowry's method was used with bovine serum albumin as a standard *(10)*.

Immobilization method

100 mg of enzyme preparation was added to a baker's yeast suspension (25 g/100 cm^3 compressed baker's yeast) in distilled water. After mixing, 10 cm^3 of 3% solution of glutaraldehyde in 0.1 M phosphate buffer, pH=7.2, was added. The duration of the immobilization process was one hour with gentle mixing at room temperature. Then 200 cm^3 of 3.0% aqueous solution of alginic acid was added to the suspension. The mixture was slowly dropped in a 2% solution of $CaCl_2$ which was gently stirred. After the immobilization, beads were removed from the $CaCl_2$ solution, in which enzyme activity and protein were determined by the methods described above. The yield of immobilization was measured by the Lowry's method for protein determination before and after the immobilization process. The yield of immobilization was also determined by measuring enzyme activity before and after immobilization in 10 cm^3 $CaCl_2$ solution.

Determination of kinetic constants

This was performed by measuring the initial enzyme reaction velocity at different substrate concentrations (CMC) in 0.01 M acetate buffer. The concentrations ranged between 0.5–6 g/L. These experiments were conducted at the temperature and pH optimum of the free (pH=3.5, T=50 °C) and immobilized enzyme (pH=3.0, T= =60 °C). The Lineweaver-Burke modification was used to establish the Michaelis constant and maximal velocity of the enzyme reaction.

Determination of pH and temperature optimum

The experimental procedure was the same as that in the case of determination of enzyme activity. pH was adjusted by the HCl or NaOH addition in the range between 2.5 and 5.5, while the temperature optimum was tested in the temperature range between 10 and 70 °C.

Determination of stability at 4 °C

The stability of immobilized enzymes at 4 °C was determined by measuring the enzyme activity of immobilized enzymes at 24 hour intervals by methods described above. Enzymes were stored in 0.01 M acetate buffer, pH=4.6, and in 0.1 M phosphate buffer, pH=7.0 with and without lactitol and DEAE-dextran addition (weight ratio 5:1) at 4 °C.

Temperature stability

Free and immobilized enzymes were incubated for 2 hours at 70 °C in 0.01 M acetate buffer. Samples were taken every 30 minutes and specific enzyme activities were measured by the methods described above. Changes in the specific enzyme activity as a function of time were a measure of the temperature stability.

Results and Discussion

Direct cellulase immobilization in Ca-alginate gel was unsuccessful, unlike the case of inulase and glucose oxidase *(11)*, and therefore, immobilization on yeast cells was used. Yeast cells also enhance the mechanical stability of attachment to Ca-alginate spheres. Yeast cells were inactivated by glutaraldehyde, which also acted as the linking reagent. The diameter of Ca-alginate beads was 3.5 mm. These immobilized catalysts are very suitable for grape must clarifications (data not shown), because they have some advantages: suitability for stirred tank and packed bed bioreactors, no negative sensoric influences on musts, easy handling, low price and good mechanical and thermal stability.

Many methods for cellulase immobilization are available, but practically all of them reduce enzyme activity, because the steric structure is changed during the enzyme-substrate interaction. The degree of the enzyme activity reduction after immobilization depends on the immobilization method, reaction conditions and specificity *(3)*.

Robillarda spp. cellulase immobilized on aminosilica had specific activity 0.24 U/mg carrier on CMC as a substrate *(2)*. Lower enzyme activity with Avicel as substrate was observed when cellulase was immobilized on Fe_3O_4 (0.090 and 0.039 U/mg protein) *(3)*, while the cellulase immobilized in Sepharose with CMC as substrate retained 17.2% activity of free form *(7)*. This value is similar as in the immobilization of cellulase in Ca-alginate (12%), but the results are only partially comparable, because of very different substrates, enzymes and measuring conditions used.

The immobilized enzyme lost a large part of its activity during the immobilization procedure in comparison with the free enzyme. Similar trends were observed in the case of kinetic constants: the maximal velocity of enzyme reaction of the immobilized enzyme (V_{max}) was

approximately 3.5 times lower, while the Michaelis constant (K_M) was higher (approximately 2.5 times) than in the case of free enzyme (Table 1). These trends were a

Table 1. Specific enzyme activity and kinetic constants of the free and the immobilized enzyme

	Free enzyme	Immobilized enzyme
Specific enzyme activity	4.72 U/mg protein	0.56 U/mg protein
Kinetic constants	K_M=0.7 g/L	K_M=1.8 g/L
	$V_{max.}$=5.20 μmol/min mg protein	$V_{max.}$=1.32 μmol/min mg protein

consequence of the steric hindrance in the interaction between the enzyme and the substrate, because the protein structure was exposed to different external influences. The transport barrier in Ca-alginate beads was an additional reason for changes in the enzyme activity and kinetic constants. It was not the result of competitive inhibition occurring when the reaction products remain in the microenvironment of active sites as a consequence of slow diffusion. After addition of large amounts of substrate no differences in the enzyme activity were observed. The pH optimum of the free enzyme was at pH=3.5, whereas a small shift was observed in the case of the immobilized enzyme to pH=3.0. In the pH range between 4.0 and 5.5 the pH had only a slight influence on the enzyme activity of the immobilized enzyme. In this pH range the relative activity was very high which is very interesting for an industrial use where pH oscillations are more common (Fig. 1). The pH optimum of

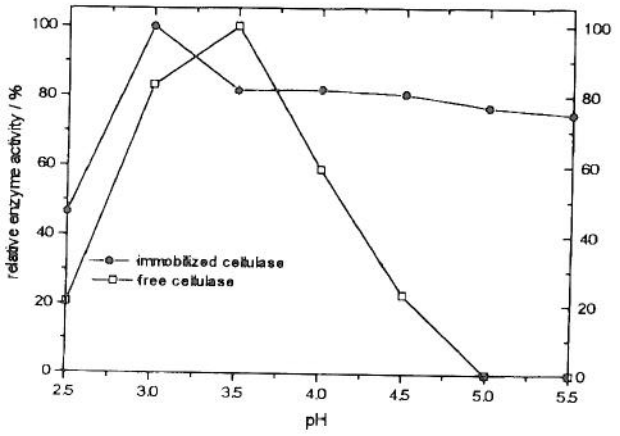

Fig. 1. Relative enzyme activity of the free and immobilized enzyme at 37 °C in acetate buffer as a function of pH

the free enzyme was in the temperature range between 37 and 60 °C. Cellulase enzyme preparations are constituted of at least three enzymes *(12)* which have different biochemical properties. This was probably the reason for the last observation. The immobilized enzyme has a temperature optimum at 60 °C, which indicates that immo-

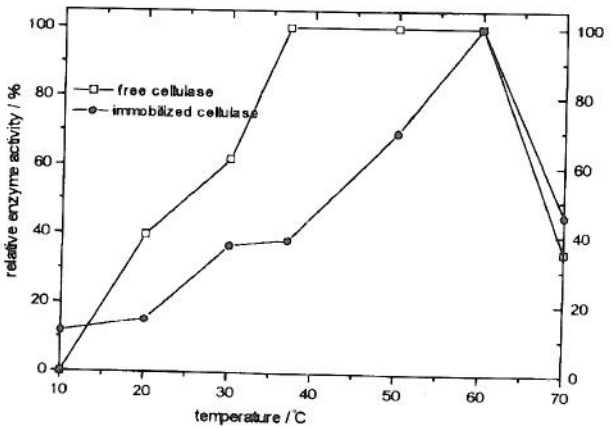

Fig. 2. Relative enzyme activity of the free and immobilized enzyme at pH=3.5 in acetate buffer as a function of temperature

bilization had different effects on different enzyme preparation constituents (Fig. 2). A higher temperature also had an influence on the reduction of viscosity of the macromolecular solution, enabling better substrate diffusion into the beads. Other authors found out that cellulase had enzyme activity in the pH range between 2.0–8.0 and had enzyme activity during a longer incubation at 50 °C *(13)*. Fig. 3 shows that the decrease of the relative enzyme activity (which is a measure for the enzyme stability) as a function of time during 2-hour incubation at 70 °C was similar in free and immobilized forms. This could be explained by canceling out positive and negative influences of immobilization on the enzyme structure. Slightly higher enzyme activity was measured in the case of the free enzyme during a 2-hour incubation at 70 °C. Only a small part of the initial en-

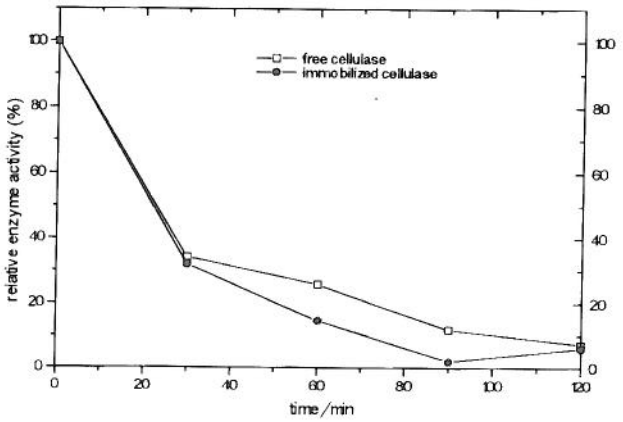

Fig. 3. Relative enzyme activity during two hours of incubation at 70 °C in acetate buffer, pH=4.6 as a function of time

zyme activity remained after this incubation (8% and 7% of initial enzyme activity).

The immobilized enzyme was stored in 0.01 M acetate buffer, pH=4.6 at temperature 4 °C. It was active for 9 days, while the same catalyst when stored at the same temperature, but in 0.1 M phosphate buffer, pH=7.0,

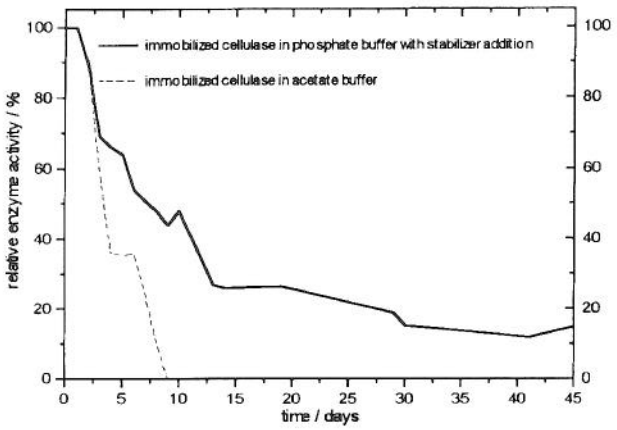

Fig. 4. Relative enzyme activity of the immobilized enzyme in 0.01 M acetate, pH=4.6 and 0.1 M phosphate buffer, pH=7.0 with the addition lactitol and DEAE-dextran at 4 °C, as a function of time

with the addition of lactitol and DEAE-dextran in the weight ratio 5:1, was active for more than 45 days. The relative enzyme activity of the immobilized enzyme in phosphate buffer dropped relatively fast in the first days, but later the activity was very stable during a longer period (Fig. 4). The theoretical model which was developed by Gibson *et al. (14)*, predicted that polyelectrolytes (DEAE-dextran) associate with molecules of the opposite charge in aqueous solutions and in some cases the precipitation occurs. These aggregates dissolve when the ionic strength is raised, but some enzyme-polyelectrolyte interaction still occurs even at a high ionic strength. These effects stabilize the enzyme structure. Ca-alginate beads stored only in 0.1 M phosphate buffer, pH=7.0 without the stabilizer addition (data not shown) and in 0.01 M acetate buffer had practically the same stability at 4 °C (Fig. 4).

Conclusion

The procedure with lactitol and DEAE-dextran addition to phosphate buffer presented in this article is promising in terms of prolongation of shelf life of immobilized biocatalysts containing cellulase activity, for different industrial processing applications. However, in terms of the theoretical explanation of the stabilizing activity of substances (such as lactitol and DEAE-dextran) much remains to be elucidated.

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Imobilizacija celulaza na Ca-alginatne nosače

Sažetak

Celulaze (3.2.1.4) imobilizirane su na inaktiviranoj biomasi kvasca koja je poslije ugrađena u Ca-alginatne nosače. Specifična je enzimska aktivnost slobodnih celulaza iznosila 4,72 U/mg proteina, a kod imobiliziranih 0,56 U/mg proteina. Optimalna je vrijednost pH za slobodne celulaze bila 3,5, a za imobilizirane 3,0. Imobilizirane su celulaze imale vrlo veliku relativnu enzimsku aktivnost u cijelom istraživanom pH-području između 3,0–5,5. Temperaturni optimum za slobodne celulaze bio je između 37 i 60 °C, za razliku od imobiliziranih celulaza koje su ga imale pri 60 °C. Enzimska aktivnost tijekom dvosatnog inkubiranja pri 70 °C bila je vrlo slična za oba enzima, pri čemu je smanjena na manje od 10% početne aktivnosti. Celulaze imobilizirane na kvaščevu biomasu u Ca-alginatnom gelu pokazale su enzimsku aktivnost 9 dana u 0,01 M acetatnom puferu, dok su u 0,1 M fosfatnom puferu, uz dodatak laktitola i DEAE-dekstrana, bile aktivne više od 45 dana.