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Immobilization of cell wall invertase in a polyacrylamide hydrogel for invert sugar production

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Abstract: Cell wall invertase (CWI) from *Saccharomyces cerevisiae* was immobilized in polyacrylamide hydrogels. The aim of the development of a new biocatalyst was to obtain an improved enzyme for invert sugar production. The monomer concentration and enzyme amount in the immobilizate were optimized, and the obtained biocatalyst had an enzyme activity of 138±6 IU g^{-1}. The pH and temperature optima were 4.0 and 70 °C, respectively. The stability of immobilized enzyme was determined at several temperatures in the absence of substrate and the half-life obtained at 50 °C was 81 days, at 60 °C, 128 min and at 70 ° C, 1.24 min. The biocatalyst was tested at low pH values, 3.0 and 3.5 were tested, and showed great stability. The K_M values were 34.1±1.7 and 126.2±6.3 mM for free and immobilized CWI, respectively. The activation energies were 37.7 and 23.0 kJ mol^{-1} for free and immobilized CWI, respectively. Cell wall invertase immobilized in polyacrylamide hydrogel (CWI––PAA) was tested for the production of high concentrated invert sugar in a batch and a packed bed reactor. After five days of continuous process, the quality and characteristics of the produced invert sugar remained unchanged.

Keywords: immobilization; *Saccharomyces cerevisiae*; enzyme; stability; CWI–PAA.

INTRODUCTION

The main goal of enzyme immobilization is for the industrial use of enzymes, process control and for as many as possible reaction cycles. Simplicity and improvement of enzyme properties are strongly associated with the design of protocols for enzyme immobilization. An immobilized enzyme should be designed to be suitable for large-scale production. There are many protocols for immobilization of enzymes but very few are also very simple and/or very capable

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of improving enzyme properties. In addition, relatively few have been successfully commercialized on a large scale.[1] Immobilization of an enzyme in different types of hydrogels is one of the most frequently used and simplest immobilization techniques. Unsatisfactory mechanical properties and susceptibility to microbial contamination of the enzymes immobilized in hydrogels are the main disadvantages of this kind of biocatalyst. Natural polymers, such as gelatin, carrageenan and alginate, are some of the carriers frequently used for enzyme immobilization with these and some other weaknesses.[2] Synthetic organic polymers have the greatest variability concerning physical and chemical characteristics. In principle, they can be adapted to the requirements of nearly any enzymatic process. Some of polymers can be easily synthesized, and their characteristics controlled by slight changes in the conditions of the polymerization reactions. The main disadvantage of hydrogels as a carrier for enzyme immobilization is unsuitable pore size. It has to be narrow enough to prevent leakage of the enzyme molecules out of the polymer network, but large enough to allow free diffusion of substrates and products. Polyacrylamide (PAA) is one of the immobilization carriers for entrapment that is strong enough to withstand the chemical stress and mechanical forces in a technical process. Moreover, it possesses very suitable properties, such as high elasticity, low abrasion in stirred-tank reactors[3,4] and good stability in almost all relevant reaction conditions.[5] The great advantage of polyacrylamide is the possibility to modify the structure and pore-size of the obtained polymer by changing the ratio of monomer and cross-linking agent in the reaction mixture.

Yeast invertase is a glycoprotein and can be found in the internal (non-glycosylated) or external (glycosylated) form. Invertase is easily extracted enzyme and can be easily obtained in the soluble form[6,7] or in the form of cell wall invertase (CWI).[8] This enzyme has found many applications in the food and beverage industry.[9] Therefore, there are many research papers dealing with different immobilization techniques or immobilization carriers for the immobilization of invertase. The main problem with invertase immobilization by entrapment, *i.e.*, enzyme leakage,[10–12] was resolved by using cell wall invertase instead of a soluble form.[8] In addition, as invertase is one of the inexpensive enzymes, it is used frequently as a model system for the development of new enzyme immobilization techniques.

Polyacrylamide was used the first time for invertase immobilization in the early seventies, but the obtained immobilizate had low activity[13,14] or lower thermal stability than the free enzyme.[15] Usage of PAA as a carrier for invertase immobilization gave immobilized biocatalysts with better performance in combination with gelatin.[12] Invertase from *S. cerevisiae* could also be immobilized on PAA by adsorption,[16] but the obtained immobilized enzyme was used only in a dilute sucrose solutions. Invertase was also immobilized by entrapment in PAA

hydrogel by radiopolymerization.[17] This method gave good immobilization results, but it is not a useful method for the production of an immobilized biocatalyst on the large scale for industrial application. From an industrial viewpoint, usage of low concentrated sucrose solution for hydrolysis followed by concentration step is not economically relevant. There is an example when whole bacterial cell immobilized in a similar polymer was developed for a commercial biotransformation process.[2]

EXPERIMENTAL

Chemicals

All chemicals were of analytical grade (or higher) and were purchased from Sigma–Aldrich and Merck. Baker's yeast was obtained from a local market.

Enzyme isolation

CWI was isolated from the slurry obtained after cell autolysis as described in a previously published paper.[8] The obtained slurry was diluted with an equal volume of 1 % sodium chloride and mixed for 30 min. The yeast cell wall was centrifuged at 4000 g for 15 min at 4 °C, and the pellet was resuspended in 5 volumes of distilled water. The procedure was repeated until proteins could no longer be detected in the supernatant. The resulting solid material was defatted and dried using cold acetone. The pellet was left to dry overnight at room temperature.

Immobilization of CWI

The polymerization mixtures contained different amounts of CWI (invertase activity 9 IU mg^{-1}; in the range of 0.25–3 g), 2.67 mL of 30 % acrylamide solution (with 0.8 % cross-linker monomer *N*,*N*′-methylenebisacrylamide), 4 μL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), 50 μL of 10 % ammonium persulfate (water solution) and water. The total volume was kept to 15 mL by the addition of 1.5 M Tris buffer of pH 8.8. After 1 h polymerization at room temperature, the gels obtained between glass plates were washed with 50 mM acetate buffer pH 4.5 and cut into discs (2 mm in diameter and 0.75 mm thick). The obtained immobilized enzymes were stored at 4 °C in 70 mass % invert sugar solution in 50 mM acetate buffer pH 4.5 prior to use.

Enzyme activity assay

The enzyme activity was assayed in a batch reactor at 25 °C. Fifty μL of free CWI suspension (1 mg mL^{-1}) was mixed with 450 μL of sucrose solution (0.3 M in 50 mM acetate buffer, pH 4.5). After 5 min, the reaction was stopped by the addition of 3,5-dinitrosalicylic acid (DNS) reagent (500 μL) and heated in a boiling water bath for 5 min. For the determination of the activity of immobilized enzymes, 3 discs were taken and mixed with 5 mL of sucrose solution. After 5 min, 500 μL of the reaction mixture was mixed with 500 μL of DNS reagent and heated in a boiling water bath for 5 min. After cooling to room temperature and dilution with 4 mL of water, the amount of reducing sugars was determined spectrophotometrically at 540 nm[18] using a Shimadzu UV-1800 spectrophotometer. One international unit (IU) of enzyme activity is defined as the amount of enzyme that hydrolyses one micromole of sucrose per minute under the assay conditions.

Determination of optimum pH and pH stability

The effect of pH on the activity of free and immobilized CWI was studied at various pH values at 25 °C. The pH of the reaction mixtures were in the range of 3.0−8.0 (0.05 M acetate buffer in the range of 3.0–5.6 and 0.05 M phosphate buffer in the range of 5.7–8.0 were used). The reaction conditions were as described in the previous section.

The effect of different pH values during the 7 days incubation was studied at 25 °C at pH values 2.0, 3.0 and 4.5. In order to determine the pH stability, both free and immobilized CWI were incubated in 50 mM acetate buffer of various pH values. The enzyme activities were measured after various incubation times as described above (at pH 4.5).

Determination of the optimum temperature and thermal stability

The effect of temperature on the activity of the enzymes was studied by performing the reaction in the temperature range 20−75 °C (with 5 °C increments). The reaction conditions were as described in the previous section.

Free and immobilized CWI were incubated at selected temperatures (50, 60 and 70 °C) in 50 mM acetate buffer pH 4.5. Enzymes were incubated for various time intervals (for 57 d at 50 °C, for 4 h at 60 °C and for 20 min at 70 °C) during these times, the enzyme activity was monitored as described in the previous section. The inactivation rate constants, k_d were calculated from the equation:

$$\ln(A/A_0) = -k_d t \quad (1)$$

where A_0 is the initial activity and A is the activity after time t. The half-life values of the biocatalysts were calculated for all temperatures used for the thermal stability tests from the equation:

$$t_{1/2} = \ln 2/k_d \quad (2)$$

Determination of kinetic parameters

The activation energy of free CWI and the immobilized CWI was calculated using the Arrhenius equation after measuring enzyme activities at different temperature.[19]

The Michaelis–Menten kinetics was established by studying the effect of substrate concentration (1–200 mM for free CWI and 1–800 mM for immobilized CWI) on the reaction rate at 25 °C. Parameters of Michaelis–Menten kinetics were obtained using non-linear regression (Graph Pad Prism 5.0).

Production of invert sugar in a batch process

Production of invert sugar using CWI immobilized in a polyacrylamide hydrogel was tested in a batch reactor. Five grams of immobilized CWI was incubated in 50 mL of 60 mass % sucrose solution at 50 °C with continuous stirring (150 rpm). The immobilized CWI used in this experiment was pre-equilibrated in 60 % sucrose solution.

The packed bed reactor

Immobilized CWI in a polyacrylamide hydrogel was tested in packed bed reactor. The experiments were realized in a 5 mL water-jacketed glass column (6.5 cm long and 1 cm in diameter) at 50±1 °C. Sucrose solutions (60 mass %) were used as the substrates. Substrate solutions were brought to 50 °C before entering the column and pumped through the bed by means of a peristaltic pump. After steady state was attained, the ratio of conversion was evaluated at the end of the column by determining the reducing sugars, as described in the previous section. The concentration of the sucrose solution was checked using a SinoTech model 2WAJ refractometer.

Statistical analysis

Each data point represents the mean of three independent assays. Statistical significance was determined by standard deviation (*SD*). The data are presented as the mean ± standard error of the mean (*SEM*).

RESULTS AND DISCUSSION

The usage of CWI instead of soluble invertase in a few natural hydrogels showed great enzyme characteristics, good stability and high productivity of invert sugar.[8,20] Polyacrylamide was chosen for the development of a new immobilized biocatalyst because it is hydrophilic and extensive previous experience established it as non-denaturing toward many proteins.[21] The polymerization reaction could cause damage to the structure of the soluble enzyme, but as the stability of CWI in comparison to soluble enzyme is much higher, no damage was expected nor observed.

Immobilization

CWI isolated from yeast cells has an enzyme activity of 9±1 IU mg^{-1}. Optimization of the immobilization of CWI in a polyacrylamide gel included optimization of amount of free CWI in the immobilizate and the percentage of monomer, acrylamide, in the polymerization mixture. Changing of amount of monomer added in the reaction mixtures resulted in significant difference in the mechanical properties of the obtained immobilized biocatalysts. CWI was immobilized by entrapment in PAA containing 8, 10 and 12 % of monomer. The biocatalyst with the best mechanical characteristics was composed of 8 % of monomer (data not shown). Immobilized CWI in PAA with the higher concentration of monomer was fragile, and the same characteristic was described earlier.[22] The amount of CWI was in the range of 17–200 mg mL^{-1} of the immobilization mixture. The optimal concentration of enzyme for immobilization in PAA by entrapment was chosen based on the results of invertase activity obtained for all tested biocatalysts, shown in Fig. 1.

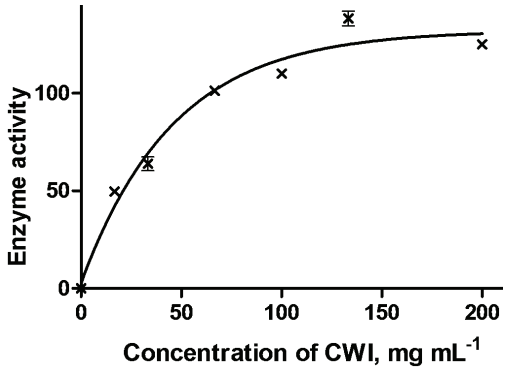

Fig. 1. Optimization of the amount of CWI for immobilization in the polyacrylamide hydrogel. The CWI concentration is indicated in g CWI mL^{-1} of immobilization mixture; enzyme activity is expressed in IU g^{-1}.

The immobilized CWI containing 133 mg mL^{-1} of free enzyme and 8 % monomer, with an activity of 138±6 IU g^{-1}, was selected as optimal. The first immobilized CWI was entrapped in Ca-alginate hydrogel and it had an activity of 71 IU g^{-1};[8] a better activity of 91 IU g^{-1} was registered for CWI modified with glutaraldehyde immobilized in alginate,[23] while the hitherto best published activity of 93 IU g^{-1} was evidenced for CWI immobilized in gelatin.[20] The biocatalyst obtained in this work has 48 % higher activity than the best previously published. The immobilization of CWI within PAA hydrogel was complete, without loss of enzyme in all tested biocatalysts. However, immobilization of soluble invertase in PAA hydrogel without additional treatment resulted in leakage of the enzyme from the immobilizate particles.[24]

pH optimum and stability

The changes in enzyme activity with changing pH values of the reaction mixtures of free and immobilized CWI were studied at 25 °C. Based on the results presented in Fig. 2, it could be concluded that optimal pH values for both free and immobilized CWI were in the range 3.0–5.0. However, maximum activity of free CWI was shown in the pH range 4.5–5.0 while immobilized CWI had a maximum activity at pH 4.0.

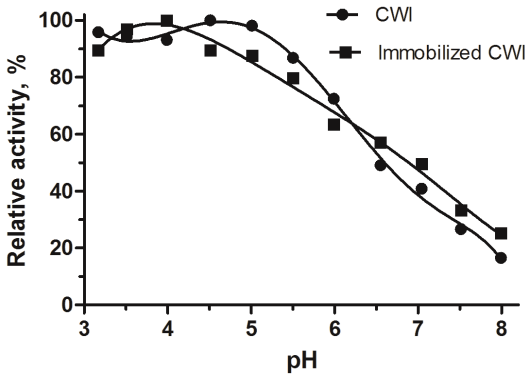

Fig. 2. Effect of pH on the activity of free and immobilized CWI.

Similar pH optimums of free and immobilized soluble invertase from *Saccharomyces cerevisiae* in a PAA hydrogel[14] and other carriers[10,12,25] and also of free and immobilized CWI[8,20] were published previously. High activity of immobilized CWI was observed also at lower pH value, 97 and 90 % of the highest activity at 3.5 and 3.0, respectively. The ability to hydrolyze sucrose at low pH could be a significant advantage of this biocatalyst, because microbial contamination is prevented under such conditions.[26] Invertase would not be used at pH values lower than 3.0, since under these conditions, a rapid acid hydrolysis of sucrose occurs.[27] The slightly shifted pH optimum of immobilized CWI into the acid region could be the effect of secondary interactions, such as ionic interact-

ions between the enzyme and the polymeric matrix or due to decreased hydrogen ion concentration in the microenvironment of the enzyme, which would require a more acid assay medium for the maximum activity of the enzyme.[12]

Since both free and immobilized CWI showed high activity in the pH range of 3.0 to optimal, the pH stability was examined in terms of potential industrial application of this immobilized biocatalyst for invert sugar production at low pH values. The pH stabilities of both forms of the enzyme were tested at two pH values. The biocatalysts were incubated at pH 2.0, 3.0 and 4.5 at 25 °C for one week. No changes were observed in the enzyme activity (both free and immobilized enzymes) after 7 days incubation at pH 3 and 4.5, while at pH 2.0, both biocatalysts showed low stability. After 20 h at pH 2.0, only 5 % residual activity of the free CWI was detected and 11 % residual activity of the immobilized enzyme.

Optimum temperature

The effects of temperature on enzyme activity of immobilized in PAA and free CWI were obtained in the temperature range 20–75 °C and the results are shown in Fig. 3. The results are expressed as percentage of the maximum activity. Optimal temperatures for free and immobilized CWI were found to be 60 and 70 °C, respectively. The activation energies were calculated by the Arrhenius method and found to be 37.7 and 23.0 kJ mol^{-1} for free and immobilized CWI, respectively. A slightly better stability of the immobilized compared to that of the free enzyme could be used as an explanation of the obtained results. Similar results, *i.e.*, higher temperature optimum of immobilized than free enzyme were previously reported[8,17,28,29] and the same result, shifted temperature optimum of invertase immobilized in the PAA hydrogel to about 10 °C higher and a change in the temperature dependence curves was observed.[14] Activation energy is an important parameter as it may indicate diffusion limitation.[30,31]

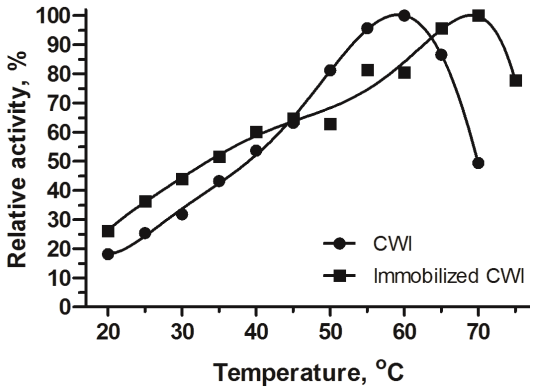

Fig. 3. Effect of temperature on the activity of free and immobilized CWI.

Thermal stability is one of the most important parameters of the improved biocatalysts, especially for their industrial application or application at higher temperatures. A study of thermal stability of CWI free and immobilized in PAA was performed at 50, 60 and 70 °C. Both biocatalysts were incubated at these temperatures and their activities were measured in determined intervals, during 57 d at 50 °C, 4 h at 60 °C or 20 min at 70 °C. The kinetic parameters of the thermal denaturation data of free and immobilized CWI were calculated and are given in Table I. Significant decreases in the stability of the free and immobilized CWI were observed, which is similar with the results observed for soluble invertase above 55 °C.[14,32] The thermal stability of immobilized CWI was 65 % higher at 50 °C, 47 % at 60 °C and 42 % at 70 °C. The improvement of the thermal stabilities of immobilized soluble as well as CWI invertase are well known characteristics.[8,9,20,23]

TABLE I. Half-life of CWI and soluble invertase at different temperatures

CWI	$t_{1/2}$ at 50 °C, days	$t_{1/2}$ at 60 °C, min	$t_{1/2}$ at 70 °C, min
Free	49	87	0.87
Immobilized	81	128	1.24

Determination of kinetic parameters

The kinetic parameters Michaelis constant (K_M) and maximum reaction rate (V_{max}) for free and immobilized CWI were determined by varying the concentration of sucrose in the reaction mixture. As expected, increasing values of K_M for the immobilized enzyme were observed. The obtained values for K_M were 34.1±1.7 and 126.2±6.3 mM for free and immobilized CWI, respectively, the values for V_{max} were 11.6±1.1 and 96.4±8.7 mM min^{-1}, respectively. The increase in the K_M values for immobilized CWI could be the result of the limited accessibility of sucrose molecules to the active sites of the immobilized enzyme. Similar results were published previously in many papers.[8–10,12,20,33]

Production of invert sugar in a batch process and a packed bed reactor

The obtained immobilized CWI was used in a batch reactor for hydrolysis of a high concentrated sucrose solution. The percent of invert sucrose was measured using DNS reagent at various time intervals and the obtained results are presented in Fig. 4. Almost complete sucrose conversion (96.5 %) was achieved after 29 h. In a previous paper, CWI immobilized in alginate hydrogel was used in a similar experiment, but complete conversion was only achieved in almost double the time.[8]

A packed bed reactor is usually very useful for industrial application of an immobilized biocatalyst, especially if product inhibition occurs. Immobilized CWI–PAA was tested for invert sugar production in a packed bed reactor. For

this purpose, 3 g (3 mL) of immobilized CWI were used. During 5 days, the percent of sucrose inversion at a constant flow rate of 2 bed volume per hour (bvh) was measured. The invert sugar production was constant during these days and the percentage of sugar inversion was about 30 %.

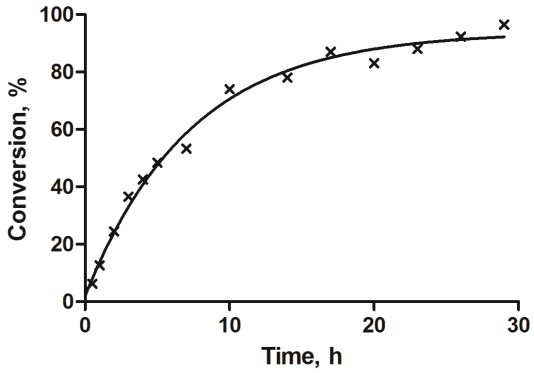

Fig. 4. Sucrose hydrolysis with immobilized CWI in a stirred batch reactor.

CONCLUSIONS

In this study, CWI was successfully immobilized in a polyacrylamide hydrogel cross-linked with *N*,*N*′-methylenebisacrylamide. The obtained biocatalyst had a high invertase activity, 48 % higher than the previously published immobilized CWI in a gelatin hydrogel.[20] Since the aim of this study was to obtain immobilized enzyme with high microbial resistance, the high stability at low pH values is proof that the goal is attained, because the possibility of microbe contamination of sugar at low pH values and high sugar molarity is considerably less than at the pH value usually used for enzymatic invert sugar production. The significantly better thermal stability of immobilized than free enzyme shown for CWI–PAA qualifies it for potential industrial use. This presumption was proved by testing the obtained biocatalyst in a packed bed reactor when it showed good characteristics under conditions similar to industrial ones.

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И З В О Д

ПРОИЗВОДЊА ИНВЕРТНОГ ШЕЋЕРА ЗИДНОМ ИНВЕРТАЗОМ ИМОБИЛИЗОВАНОМ У ПОЛИАКРИЛАМИДНОМ ХИДРОГЕЛУ

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Зидна инвертаза изолована из ћелија квасца *Saccharomyces cerevisiae* имобилизована је у хидрогелу полиакриламида. Циљ добијања овог новог имобилизата је развитак и побољшање биокатализатора који се користи за производњу инвертог шећера. Оптимизо-

вана је концентрација мономера и ензима у имобилизату, а као оптималан изабран је имобилизат са ензимском активношћу од 138±6 IU g^{-1}. Одређени су оптимални услови за рад имобилизата, pH 4,0 и температура 70 °C. Испитивана је стабилност имобилизата на неколико различитих температура без присуства супстрата, добијени резултати изражени су као полуживот имобилизованог ензима и износе 81 дан на 50 °C, 128 min на 60 °C и 1,24 min на 70 °C. Тестирана је и стабилност имобилизата и слободног ензима на ниским pH вредностима (3,0 и 3,5), а добијени резултати су показали бољу стабилност имобилизата. K_M вредност за слободну и имобилизовану зидну инвертазу износе 34,1±1,7 односно 126,2±6,3 mM, док енергија активације износи 37,7 односно 23,0 kJ mol^{-1}. Продуктивност имобилизоване зидне инвертазе у полиакриламиду у производњи инвертног шећера тестирана је у високо концентрованим растворима шећера у шаржном и континуалном реактору. Након пет дана континуалне производње инвертног шећера квалитет и карактеристике добијеног производа нису биле промењене.

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