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β-Galactosidase from *Penicillium canescens*. Properties and immobilization

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Abstract: β-galactosidase from *Penicillium canescens* was immobilized on chitosan, sepharose-4B, foamable polyurethane and some other carriers. The highest yield of immobilization (up to 98 %) was obtained by using chitosan as a carrier. The optimum pH and temperature were not significantly altered by immobilization. High stability of immobilized β-galactosidase during storage was demonstrated. Efficient lactose saccharification (over 90 %) in whey was achieved by using immobilized β-galactosidase.

Keywords: β-Galactosidase; immobilization; chitosan; Penicillium canescens

1 Introduction

$\beta-$galactosidases are among the most important industrial enzymes. The natural substrate for these enzymes is lactose, the main sugar of milk and of several dairy products, with poor solubility properties and relatively low degree of sweetness. The hydrolysis of lactose offers some advantages in a dairy product. First, it improves the digestibility of lactose. Moreover, the low solubility of lactose can result in its crystallization in dairy

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food with concomitant appearance of a sandy texture and deposit formation. Only 25 % of lactose needs to be hydrolysed to prevent crystallization during prolonged storage, because small amounts of glucose and galactose inhibit the formation of lactose crystals. Another advantage is the possibility of preparing or improving new food and dairy products with higher solubility characteristics and a higher degree of sweetness.

$\beta-$galactosidases occur rather widely in nature and have been isolated from animals, plants and microorganisms. Yeast and fungal enzymes are used most widely. The fungal β-galactosidases (from *Aspergillus*) with acid pH optima (2.5-4.5) are especially suitable for hydrolysis of lactose in acid whey but they are more sensitive to product inhibition by galactose. The yeast enzymes from *Kluyveromyces* or *Saccharomyces* sp. have neutral pH optima (6-7) making them suitable for the hydrolysis of lactose in milk, and they are less inhibited by galactose. However, they are less thermostable than the fungal enzymes. Many studies have been made with bacterial β-galactosidases obtained from *E. coli* (pH optima 6.5-7.5), although their usage is not viable for products intended for human consumption [1].

A number of publications relating to immobilization of fungal β-galactosidases from *Aspergillus* species [2-5] and yeast β-galactosidases from *Kluyveromyces fragilis* [6-10] have appeared in recent years.

In Russia during the last 20 years fungal β-galactosidase from *Penicillium canescens* has been under investigation and has found some practical applications. The molecular weight of this enzyme is aproximately 120 kDa. It does not dissociate into sub-units during electrophoresis in denaturating conditions, i.e., it behaves as a monomeric protein [11]. But electron microscopy indicates a tetrameric quaternary structure [12]. The isoelectric point of β-galactosidase is 6.7, and the pH optimum for lactose 4–5 [11-13]. After incubation for 8 h at 50 °C the enzyme retains 100 % of initial activity, but after incubation for 15 min. at 60 °C only 30 % of the initial activity is left [13]. The highest reported activity of purified β-galactosidase from *Penicillium canescens* was 427 μmol of hydrolyzed lactose/ min·mg of protein at pH 4.5 and 30 °C [14]. The reported values of K_m for lactose as substrate were 11.2 mM at 55 °C [13] and 16.0 mM at 30 °C [15]. These are lower than K_m for fungal β-galactosidases from *Aspergillus oryzae* and *Aspergillus niger* (K_m = 36-112 mM at 50 °C) [16] and yeast β-galactosidases (K_m = 23-43 mM at 40 °C) [16]. β-galactosidase from *Penicillium canescens* is not sensitive to metal ions. Incubation with 10^{-3} M of Na^+, K^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, or Hg^{2+}did not change the activity. Addition of EDTA or dialysis against EDTA solution also did not affect the activity [13]. Galactose acts as a competitive inhibitor with K_i = 25 mM [15].

The technology of β-galactosidase production has been developed at the Institute of Biotechnology (Moscow) on the basis of the high–productive strain *Penicillium canescens* F-178 [17]. The enzyme was intended for lactose hydrolysis in whey and permeate with the goal of producing glucose-galactose syrup as a sugar substitute. Glucose-galactose syrup possesses palatable sweet taste, golden color, perfect transparency, optimal consistency and good solubility. It could be suitable for applications in the food industry: baking,

confectionery, brewing, sweet drinks as well as weak alcoholic beverages, ice cream, and dietetic products. But for economic reasons industrial production of glucose-galactose syrup in Russia has not yet been realized.

Covalent immobilization of β-galactosidase from *Penicillium canescens* has not been investigated. Only immobilization by adsorption on hydrophobic carrier [18] and on macroporous silica [19-21] has been described. In the latter case adsorption of the enzyme was very much improved in the presence of starch [21].

We describe here immobilization of β-galactosidase from *Penicillium canescens* on various carriers by covalent binding and direct adsorption. Immobilized β-galactosidase has been examined in order to test its utility in the dairy industry for lactose hydrolysis in whey.

2 Materials and methods

2.1 β-Galactosidase

The fungus *Penicillium canescens* strain F-178 was cultured in a medium containing mineral salts and beet pulp, in an industrial scale experiment. Isolation of the enzyme was carried out by ultrafiltration with successive spray–drying of the culture liquid concentrate. Further purification was achieved by precipitation of β-galactosidase from solution in acetate buffer (pH 5.0) with isopropanol (1.0:0.75, v/v). Specific activity was 30-40 U/mg of protein.

2.2 Carriers for enzyme immobilization

Sepharose-4B was obtained from Amersham Pharmacia. Granocell-2000, a macroporous cellulose (exclusion limit $2{\cdot}10^6$, wet bead size 160−315 μm) was a kind gift from dr. J. Liesiene, Kaunas Technological University (Lithuania). Amino groups were introduced into polysaccharide carriers granocell-2000 and sepharose-4B by the action of epichlorhydrin and pentaethylenehexamine, according to Morkeviciene *et al.* [22]. The modified carriers were activated with glutaraldehyde (GA) [23].

Chitosan of various molecular weights (M_η) and degrees of deacetylation (DD) was obtained from Fluka (M_η $7{\cdot}10^4$, DD 77 %; M_η $7.5{\cdot}10^5$, DD 71 %; M_η $2{\cdot}10^6$, DD 68 %). The degree of deacetylation was calculated from overall quantity of nitrogen (estimated by Kyeldal method) and quantity of nitrogen in primary amino groups (estimated by direct titration method with HCl or reversible titration with HBr [24]). The molecular weight of chitosan was determined from viscosity measurements. Solutions of chitosan in 0.5 M acetate buffer were used [25]. Microspheres of chitosan were prepared according to Budriene *et al.* [26]. Powdered chitosan was dissolved in 2 % acetic acid. Into 20 ml of the 1 % chitosan solution at 50 °C under stirring was added gradually 13.2 ml of 0.5 N KOH. 0.5-17.0 weight parts (w.p.) of 25 % GA were added after 10 min (quantity of chitosan in the reaction mixture was 1 w.p.). Stirring was continued for an additional

30 min. The size of wet chitosan microspheres was 15-30 μm (estimated by Leitz optical microscope).

CM-Cellulose was obtained from Sigma. CM-Sephadex C50 was obtained from Amersham Pharmacia. These carriers were swelled in 0.1 M citrate-phosphate buffer (pH 4.5) for several days before the enzyme immobilization by adsorption.

Aminosilochrom C-80 (particle size 160-315 μm, pore diameter 50 nm, surface area 80 m^2/g), a macroporous inorganic carrier modified with γ-aminopropyltriethoxysilane, was obtained from Reachim. Aminosilochrom C-80 was activated with GA [23].

Foamable polyurethane was synthesized from bicomponent composition HYPERLAST, a product of Hyperlast Limited (Derbyshire, UK). HYPERLAST ISOCYANATE 5003 (H1, first component) contains 86 % diphenylmethane diisocyanate. HYPERLAST 7982016 (H2, second component) contains 5-10 % 1,4-butanediol.

Powdered polyurethane was synthesized from 1,4-butanediol (BD) and hexamethylene diisocyanate (HMDI) according to [27] and washed several times with 0.1 M citrate-phosphate buffer (pH 4.5). It was used for immobilization immediately after preparation.

2.3 Enzyme assays

Activity of native β-galactosidase was assayed by incubation of 1 ml of enzyme solution with 2 ml of 7.5 % lactose as substrate in 0.1 M citrate-phosphate buffer (pH 4.5) at 37 °C for 30 min. The glucose produced in this reaction was determined by the glucoseoxidase method ([28]). Activity of the immobilized enzyme was determined in the same way exept that the immobilized enzyme was added by weight (100-300 mg) to the substrate solution and incubation was carried out with intensive stirring. The activity unit (U) is defined as the amount of enzyme that releases 1 μmol of glucose from lactose per min. at 37 °C and pH 4.5.

2.4 Immobilization

The carriers activated with glutaraldehyde and powdered polyurethane were washed with 0.1 M citrate-phosphate buffer (pH 6.5). The solution of β-galactosidase was added, the mixture was stirred at 37 °C for 30 min. and then left at 4 °C overnight. Next day the immobilized enzyme was thoroughly washed with buffer until no enzyme activity was detected in the washes.

To prepare the polyurethane foam-immobilized enzyme, the enzyme solution was mixed with 3-4 w.p. of HYPERLAST ISOCYANATE 5003 and 1 w.p. of HYPERLAST 7982016. The mixture was stirred vigorously for 60 seconds to achieve a homogeneous distribution of enzyme within the composition. After polymerization, foams were cut into small cuboids and washed several times with 0.1 M citrate-phosphate buffer (pH 4.5).

2.5 Effect of pH and temperature on the activity of free and immobilized β-galactosidase

The influence of pH on the activity of β-galactosidase was examined in the range of 2.0-7.5. The effect of temperature on activity was measured at the optimum pH of 4.5 from 15 to 70 °C. The relative activity at each pH and temperature was expressed as a percentage of the maximum activity.

2.6 Treatment of whey in batch operation

The cheese whey (pH 4.7) was heated to a temperature about 80 °C for 10-15 min. Such heating causes pasteurisation of the whey and induces the formation of solid colloidal protein particles in suspension that is eliminated by filtration. The standard hydrolysis was carried out in flasks in the presence of the whey (20 ml) containing 4.8 % of lactose and β-galactosidase immobilized on chitosan (molecular weight $7{\cdot}10^4$) under stirring for 6 h at 55 °C (to 1 ml of whey was added 2 U of immobilized β-galactosidase). Samples were withdrawn periodically at 1 h intervals and analysed for glucose by the glucoseoxidase method [28].

3 Results and discussion

3.1 Activity and stability of immobilized β-galactosidase

We investigated the possibility of using various carriers for immobilization of β-galactosidase. The results of immobilization are presented in Table 1. The best yields of immobilization were obtained using chitosan as a carrier. This method of immobilization, and the properties of immobilized β-galactosidase were investigated in more detail.

Microspheres were prepared from chitosan of various molecular weights. The yield of immobilization and activity of immobilized β-galactosidase were highest in the case of chitosan of molecular weight $7{\cdot}10^4$ (Table 1, No. 4).

The yield of β-galactosidase immobilization on chitosan depended on the quantity of GA used for the microsphere preparation (Fig. 1). The optimal quantity of GA was found to be 4.4 w.p. (yield of immobilization was 98 % in that case). The results of immobilization given in Table 1 were obtained using 2.65 w.p. of GA for microsphere preparation. Therefore optimal yields of immobilization on chitosan are somewhat higher than those presented in the Table 1.

The effects of pH and temperature on the relative activity of native β-galactosidase and of β-galactosidase immobilized on chitosan are shown in Fig. 2, 3. The highest activity of immobilized and of native β-galactosidase is obtained at about pH 4 and 4.5, accordingly. The optimum temperature for native and immobilized β-galactosidase is about 60 °C.

Stability of β-galactosidase immobilized on chitosan during storage in buffer solution

No.	Carrier for immobilization	Method of immobilization	Yield of immobil., %[c]
1.	Sepharose 4B[a]	Covalent binding via GA	34.0
2.	Granocell-2000[a]	Covalent binding via GA	38.5
3.	Aminosilochrom C-80	Covalent binding via GA	73.0
4.	Microspheres of chitosan ($M_\eta 7{\cdot}10^4$)	Covalent binding via GA	89.9
5.	Microspheres of chitosan ($M_\eta 7.5{\cdot}10^5$)	Covalent binding via GA	83.0
6.	Microspheres of chitosan (M_η $2{\cdot}10^6$)	Covalent binding via GA	79.2
7.	Polyurethane foam ([H1] : [H2]= 3.0:1.0, w.p.)[b]	Entrapment and covalent binding via isocyanate group	27.9
8.	Polyurethane foam ([H1] : [H2]= 3.5:1.0, w.p)[b]	Entrapment and covalent binding via isocyanate group	34.7
9.	Polyurethane foam ([H1] : [H2]= 4.0:1.0, w.p)[b]	Entrapment and covalent binding via isocyanate group	43.9
10.	Polyurethane powder ([BD] : [HMDI]=0.050:0.05, mol)[b]	Covalent binding via isocyanate group	6.2
11.	Polyurethane powder [BD] : [HMDI]=0.050:0.06, mol)[b]	Covalent binding via isocyanate group	15.3
12.	Polyurethane powder ([BD] : [HMDI]=0.050:0.07, mol)[b]	Covalent binding via isocyanate group	19.6
13.	CM-Sephadex	Adsorption	40.0
14.	CM-Cellulose	Adsorption	45.4

[a] Modified with epichlorhydrin and pentaethylenehexamine,
[b] Relative quantity of components for polyurethane formation,
[c] Activity of immobilized β-galactosidase in % from the activity of native enzyme used for immobilization. The given values of the yield are means of 2-4 immobilization experiments.

Table 1 Immobilization of β-galactosidase.

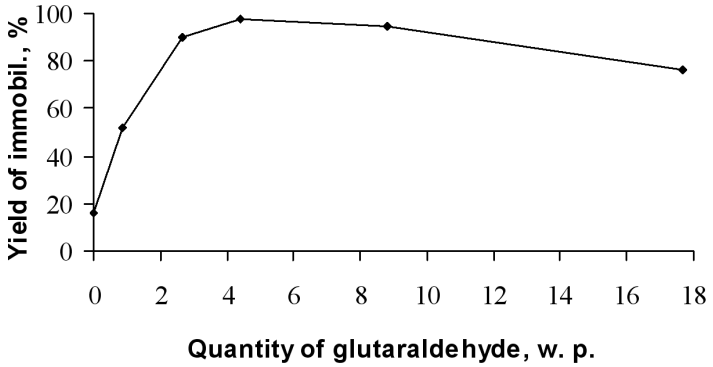

Fig. 1 Yield of β-galactosidase immobilization on chitosan ($M_\eta = 7{\cdot}10^4$) as a function of quantity of glutaraldehyde.

at 4 ^{o}C was acceptably high (Fig. 4). The highest stability was obtained with chitosan of molecular weight $7{\cdot}10^4$. Residual activity of β-galactosidase was 92 % after 90 days of moist storage, and 89 % after 240 days (not shown on the Fig. 4).

Covalent attachment to polyurethane foam has been used to immobilize several en-

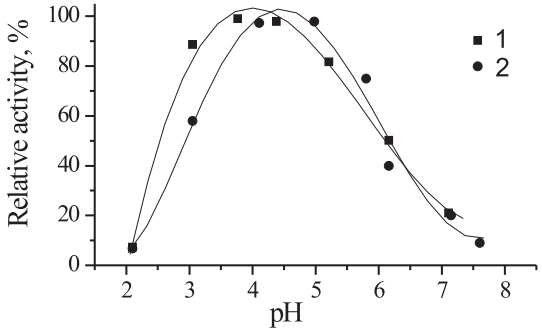

Fig. 2 Effect of pH on the activity of β-galactosidase immobilized on chitosan (1) and native β-galactosidase (2).

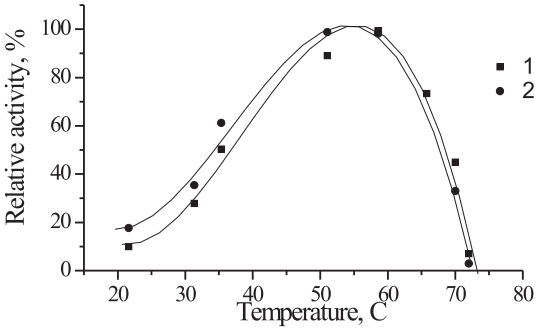

Fig. 3 Effect of temperature on the activity of β-galactosidase immobilized on chitosan (1) and native β-galactosidase (2); pH 4.5, incubation time 30 min.

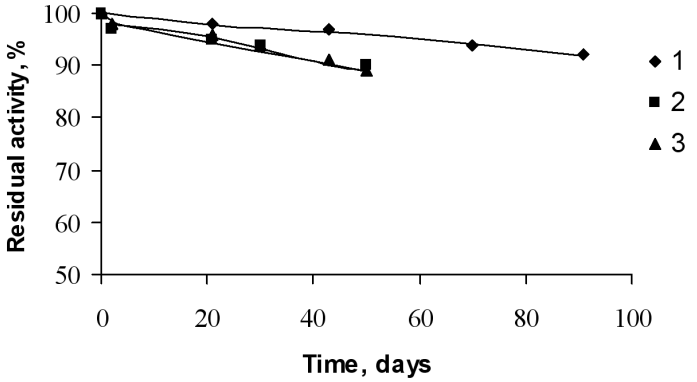

Fig. 4 Stability of β-galactosidase immobilized on chitosan during storage in 0.1 M citrate-phosphate buffer, pH 4.5, at 4 °C. Molecular weight of chitosan: 1 - $7{\cdot}10^4$, 2 - $7.5{\cdot}10^5$, 3 - $2{\cdot}10^6$.

zymes [3, 29]. We used foamable and powdery polyurethane for immobilization of β-galactosidase. The activity of immobilized β-galactosidase and the yields of immobilization were higher in the case of foamable polyurethane (Table 1). In the case of powdered polyurethane, only covalent binding between amino groups of the β-galactosidase

and isocyanate groups of polyurethane may cause immobilization. With polyurethane foam, entrapment in the micropores assists the immobilization of β-galactosidase [29]. In both cases the efficiency of immobilization increased in parallel with the ratio of diisocyanate/diol components (Table 1, No. 7-12) because of the increasing content of active isocyanate groups on the carriers.

Immobilization on foamable polyurethane was very simple and rapid. But the yield of immobilization was below 50 %. Maximum activity of immobilized β-galactosidase was only about 3 U/g of wet carrier. In the case of immobilization on microspheres of chitosan the activity was 10-15 U/g of wet carrier.

β−galactosidase from *Penicillium canescens* has pI 6.7. Therefore at pH 4.5 it is positively charged and ought to be adsorbed on negatively charged carriers. Indeed, the yields of immobilization of β-galactosidase by direct adsorption on CM-Sephadex and CM-Cellulose (Table 1, No.13, 14) were 40.0 % and 45.4 %, accordingly, but leaking of enzyme from these carriers was already apparent after a few days of storage.

3.2 Batch and column hydrolysis of whey by immobilized β-galactosidase

β−galactosidase immobilized on chitosan showed high stability, and was tested in the processing of whey. The saccharification of whey by immobilized *Penicillium canescens* β-galactosidase was studied as a function of reaction time (Fig. 5). The obtained results showed that for whey containing 4.8 % of lactose and 2 U of immobilized or native β-galactosidase /ml whey, hydrolysis time 4 h and temperature 55 °C may be considered as the optimum conditions, resulting in over 90 % of lactose conversion. Repeated batch whey hydrolysis by immobilized β-galactosidase allowed five 4 h cycles without any notable decrease in lactose saccharification (Fig. 6).

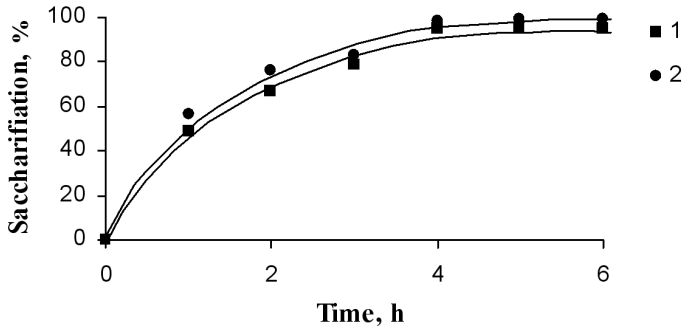

Fig. 5 Saccharification as a function of reaction time (temperature 55 °C, pH 4.7): 1 – immobilized β−galactosidase, 2 – native β−galactosidase.

β−galactosidase immobilized on chitosan was also tested for saccharification of lactose in column operation. The column became clogged after a few hours. Presumably, some components of whey were adsorbed on chitosan particles. So, this type of carrier is not suitable for continuous processing of whey.

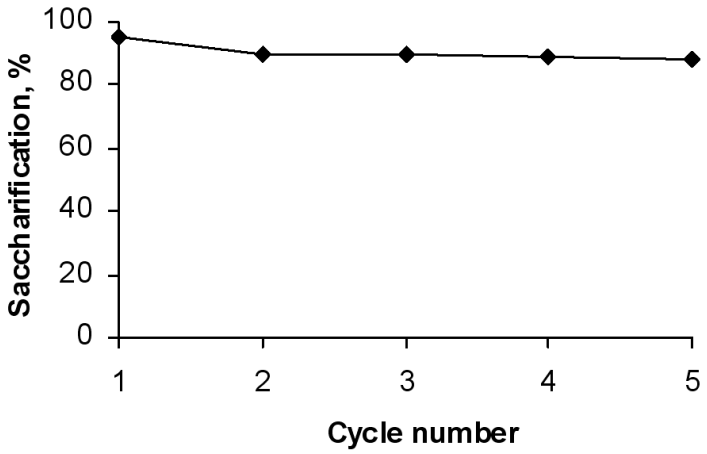

Fig. 6 Repeated batch hydrolysis of lactose in whey using β-galactosidase immobilized on chitosan (temperature 55 °C, pH 4.7).

4 Conclusions

A number of carriers were used for immobilization of β-galactosidase from *Penicillium canescens*. The highest yield of immobilization (98 %) was obtained on chitosan (molecular weight $7{\cdot}10^4$) as a carrier. Immobilized enzyme retained 89 % of its original activity after 240 days at 4 °C. It hydrolyzed over 90 % of lactose in whey within 4 h at 55 °C and retained full activity after 5 cycles of whey hydrolysis.

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