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Carbohydrate Research 339 (2004) 1099–1104
Carbohydrate RESEARCH

Crosslinked enzyme crystals of glucoamylase as a potent catalyst for biotransformations

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Received 24 July 2003; accepted 19 January 2004

Abstract—Glucoamylase (E.C: 3.2.1.3, α-(1→4)-glucan glucohydrolase) mainly hydrolyzes starch and has been extensively used in the starch, glucose (dextrose), and fermentation industries. Immobilized glucoamylase has an inherent disadvantage of lower conversion rates and low thermostability of less than 55 °C when used in continuous operations. We have developed crosslinked enzyme crystals (CLEC) of glucoamylase that overcome the above disadvantages, possess good thermal stability and retain 98.6% of their original activity at 70 °C for 1 h, 77% activity at 80 °C for 1 h, and 51.4% activity at 90 °C for 0.5 h. CLEC glucoamylase has a specific activity of 0.0687 IU/mg and a yield of 50.7% of the original activity of the enzyme under optimum conditions with starch as the substrate. The crystals obtained are rhombohedral in shape having a size ~10–100 μm, a density of 1.8926 g/cm^3 and a surface area of 0.7867 m^2/g. The pH optimum of the glucoamylase crystals was sharp at pH 4.5, unlike the soluble enzyme. The kinetic constants V_{max} and K_m exhibited a 10-fold increase as a consequence of crystallization and crosslinking. The continuous production of glucose from 10% soluble starch and 10% maltodextrin (12.5 DE) by a packed-bed reactor at 60 °C had a productivity of 110.58 g/L/h at a residence time of 7.6 min and 714.1 g/L/h at a residence time of 3.4 min, respectively. The CLEC glucoamylase had a half-life of 10 h with 4% starch substrate at 60 °C.

Keywords: Crosslinked enzyme crystal; CLEC; Glucoamylase; Thermal stability; Starch hydrolysis; Maltodextrin hydrolysis

1. Introduction

Starch is an important industrial raw material produced by the photosynthesis of plants. The most important transformation of starch into glucose is generally carried out by hydrolyzing with mineral acids or with α-amylase to limit dextrins, followed by saccharification using glucoamylase. The glucoamylase enzyme splits off the glucose units from the nonreducing end of the dextrin.[1] Many of the commercially available glucoamylase enzymes are derived from microorganisms of the genera *Rhizopus* and *Aspergillus*. When these enzymes are used to produce glucose (dextrose), they are allowed to react at 55–60 °C for 2–4 days.[2] Hydrolysis time could be effectively reduced if thermally stable forms of the glucoamylase were available. Starch hydrolyzes using soluble and immobilized glucoamylase that have been reported are limited to batchwise and semicontinuous methods, and no continuous process is commercially available.[3]

Industries need enzymes having high productivity and stability for repeated use over an extended period of time. Enzyme immobilization provides many important advantages over the use of soluble enzymes, namely, reusability, continuous operation, controlled product formation, and simplified and efficient processing.[4] Continuous reactors provide high productivities and minimize downtime, enzyme costs, and capital investment.[5] Much work has been carried out to immobilize glucoamylase on various supports,[6] but these preparations had disadvantages that include low-temperature stability and enzyme activities, gradual enzyme

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doi:10.1016/j.carres.2004.01.011

inactivation over the period of storage, and low glucose yields. With immobilized glucoamylase the saccharification can be conducted continuously in a packed-bed reactor, thereby reducing the reaction time and production cost. Glucoamylase has been immobilized by ion-exchange processes, physical adsorption, covalent bonding, gel entrapment, etc.,[7–9] but the drawback is the tendency of glucoamylase to become inactivated above 50 °C. This reduces the commercial feasibility of the process, since at lower temperatures the threat of microbial contamination is quite high. The company, Novozymes, has patented a process for the production of glucoamylase from a new strain of microbe, *Talaromyces dupont*, which is thermostable up to 70 °C.[10]

Crosslinked enzyme crystals (CLECs) have several key characteristics that confer significant advantages over conventional enzyme immobilization methods[11–13] like enhanced temperature stability, absence of an inert support, uniformity across crystal volume, catalysis under harsh conditions, such as elevated temperature, denaturing organic solvents, etc. These combined effects represent a major improvement in the reaction efficiency by maximizing the productivity of a given quantity of enzyme catalyst, which is generally the most expensive component of the reaction process.[14] The insoluble nature of CLECs facilitates easy separation of the enzyme from the reaction medium, which increases the reuse efficiency of the enzyme. Lyophilized CLECs can be stored for commercially attractive periods of time (months to years) in the absence of refrigeration. CLECs of several industrially important enzymes like thermolysin, elastase, asparaginase, lipase, lysozyme, and urease[13] have been prepared and are used in organic synthesis.

There has been no report about glucoamylase CLEC in the literature, and, hence, attempts have been made to provide a process for the preparation of a crosslinked glucoamylase composition with improved stability and activity.

2. Results

2.1. Properties of the crosslinked enzyme crystals (CLEC) of glucoamylase

Glucoamylase catalyzes the hydrolysis of glucosidic linkages with inversion of the anomeric configuration.[15] *Aspergillus niger* glucoamylase occurs in two natural forms. The larger one, GA1, consists of two globular parts with separate functions. Amino acid residues 1–471 form the catalytic globular domain (CD), and AAs 509–616 form the globular starch-binding domain (SBD). The carbohydrates are attached exclusively to the peptide region having AAs 471–508 between the two domains. The smaller, natural proteolytic fragment GA II comprises residues 1–512, only, and thus neither binds to nor hydrolyzes raw starch.[16] We assume that in the *A. niger* glucoamylase only AA residues 1–471, which form the catalytic globular domain (CD), have crystallized. There is a lack of any report that the full length of glucoamylase has ever been crystallized.[15] The crystals formed were separated and crosslinked with glutaraldehyde (2% v/v) solution. Crosslinked crystals of 10–100 μm were obtained (Table 1). The crystals were light brown in color and fluffy. The active site of the glucoamylase enzyme has lysine groups, and hence the active site is blocked by starch before crosslinking with glutaraldehyde to prevent the inactivation of the enzyme. Addition of a small quantity of a protein like bovine serum albumin (BSA) enhances the activity of the crystalline product. After crystallization and crosslinking, 63.15% of the enzyme protein was retained, and the activity yield was found to be 50.66%. The soluble enzyme had a specific activity of 5.56 IU/mg protein where as the CLEC had a specific activity of 11.68 IU/mg crystal protein. Immobilized glucoamylase previously produced in our laboratory[17] has an inherent disadvantage of having a lower conversion rate and a thermal stability of less than 65 °C when used in continuous operations. Crosslinking of glucoamylases via carbohydrate of linker regions is known to decrease thermal stability.[18] Hence crosslinking by glutaraldehyde via lysine residues was tried. The crosslinked glucoamylase crystals that we have developed have circumvented some of the above disadvantages and have exhibited better thermal stability. These CLECS are shown to retain 98.6% activity at 70 °C for 1 h, 77% activity at 80 °C for 1 h and 51.4% of the original activity at 90 °C for 0.5 h in the presence of starch (Table 2). The presence of starch has a protective effect on the enzyme toward denaturation at elevated temperatures. The inactivation coefficient calculated from the Arrhenius equation is $E = -58.67\,\mathrm{kJ\,mol^{-1}}$. Crosslinking of the enzyme by glutaraldehyde seems to prevent the unfolding of glucoamylase induced by heat. In the absence of starch, the thermal stability is moderate, the material having retained 82% of its activity at 70 °C for 1 h and 30.82% of its activity at 80 °C for 0.5 h. Hence the glucoamylase CLEC can be used in biotransformation of other substrates at elevated temperatures, which makes it more versatile.

Table 1. General characteristics of CLEC glucoamylase

pH optimum	4.5
Specific activity	11.68 IU/mg crystal
Crystal shape	Rhombohedral
Crystal size	10–100 μm
Density	1.8926 g/cm^2
Surface area	0.7867 m^2/g
K_m (starch)	5.105 mg/mL

Table 2. Comparison of thermal stability of CLEC glucoamylase in the presence and absence of 4% starch (w/v) at pH 4.5

Temperature (°C)	Duration of incubation (h)	Soluble glucoamylase activity (%)	CLEC glucoamylase activity (%)
70	1 (with starch)	83.46	98.6
80	1 (with starch)	62.62	77.0
90	0.5 (with starch)	44.89	51.4
70	1 (without starch)	Negligible	82
80	0.5 (without starch)	Negligible	30.82
90	0.5 (without starch)	Negligible	Negligible

Figure 1 shows the relationship between the pH and the enzyme activity in the case of the crosslinked enzyme crystals and the soluble enzyme for the hydrolysis of soluble starch (4% w/v). Compared to the soluble enzyme, the activity of the CLEC is slightly lower because of the lower concentration of enzyme in the crystal, as well as due to the hindrance of substrate's access to the active site and the restricted flexibility of the catalytic sites. This CLEC has also a narrow pH optimum at pH 4.5, which in turn warrants strict pH control during hydrolysis.

The effect of starch concentration on the activity of both the soluble enzyme and the CLEC is given in Figure 2. CLEC glucoamylase has a better activity at higher substrate concentrations up to 300 mg/mL of soluble starch. The Michaelis constant, K_m, for the crosslinked glucoamylase crystal is 5.105 mg/mL with a V_{max} of 1782 μg glucose/mg protein/min, which is 10-fold higher than the soluble enzyme where K_m is only 0.470 mg/mL with a V_{max} of 173.4 μg glucose/mg protein/min with starch as substrate at pH 4.5 at 60 °C. The Michaelis constants K_m and V_{max} were obtained from the Lineweaver–Burk plot (Fig. 3). The affinity of the substrate is reduced due to the diffusion limitations of the starch polymer getting into the active site of the enzyme's crystal lattice.

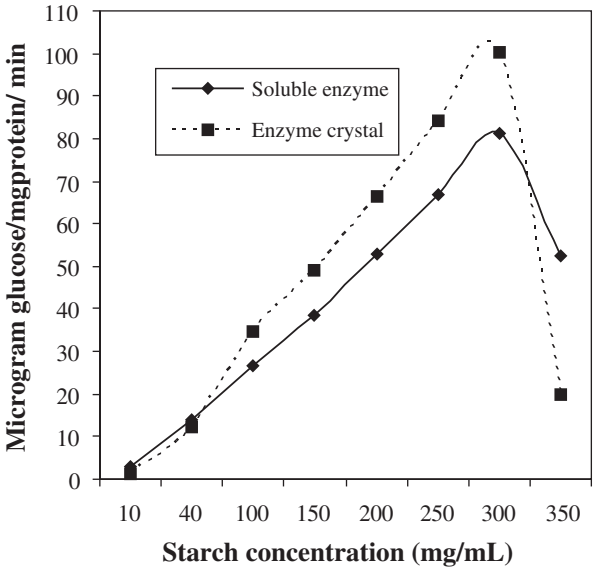

Figure 2. Effect of substrate concentration on CLEC glucoamylase activity.

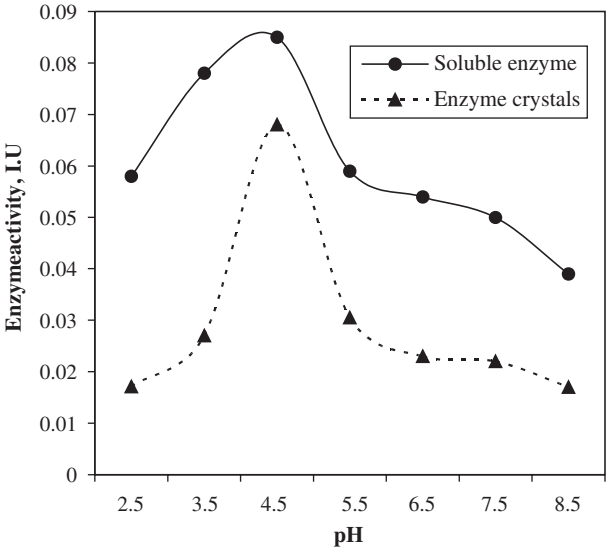

Figure 1. Activity of CLEC glucoamylase crystals and soluble enzyme.

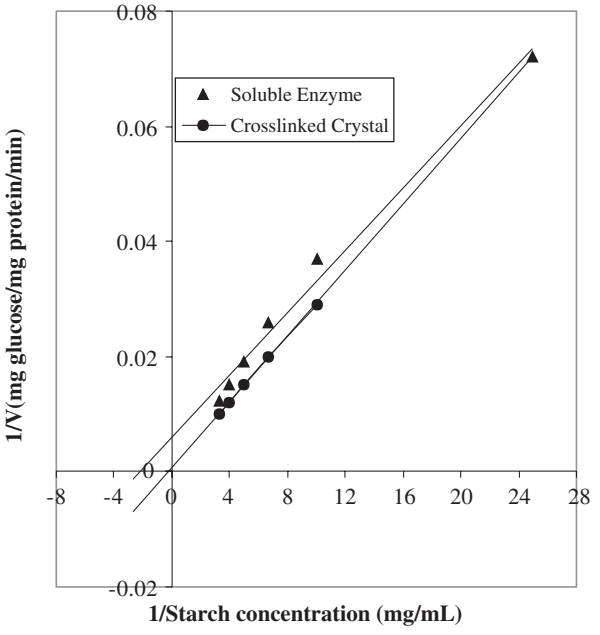

Figure 3. Lineweaver–Burk plot for soluble and CLEC glucoamylase.

2.2. Continuous hydrolysis of starch by CLEC

The hydrolysis of substrates using CLEC glucoamylase packed in a glass column was carried out at 60 ± 2 °C. The productivity of the reactor system at various substrate concentrations and dilutions is given in Table 3. At 4% (w/v) soluble starch feed, a productivity of 55.13 g/L/h was obtained at a residence time of 5.4 min. When the concentration of the soluble starch was increased to 10% (w/v), the productivity was also increased to 110.58 g/L/h, and the residence time was 7.6 min (Fig. 4). When a more suitable substrate for glucoamylase, maltodextrin of DE 12.5, at a concentration of 10% (w/v) was used, the productivity markedly increased, and a value of 463.7 g/L/h was obtained at a residence time of 5.6 min. When the residence time was further reduced to 3.4 min, a higher productivity of 714.1 g/L/h was obtained (Table 3). These results are mainly due to the small size of the substrate, which reduces the diffusion limitations of the hydrolysis.

After 10 h of continuous saccharification of a 4% (w/v) soluble starch solution, the activity of the CLEC declined to 2.3 IU/g from 5.1 IU/g of the crystal. Hence the half-life of the enzyme was about 10 h at 60 ± 2 °C.

2.3. Effect of organic solvents on CLEC activity

When water and organic solvents were taken in ratios of 1:1, the CLEC exhibited high activity in water–hexane, water–*n*-dodecane, and water–chloroform mixtures (27–28 mg of glucose produced per hour). The activity was less in 1:1 mixtures of water–2-propanol, water–1,4-dioxane, and water–Me_2SO (Table 4). Comparatively, CLEC is found to be more active in water-immiscible solvents than in water-miscible solvents. CLEC in hexane and in *n*-dodecane medium hydrolyzed and produced 28.94 mg glucose/h. The catalytic performance of CLEC glucoamylase was lower in 1,4-dioxane and 2-propanol with a glucose production of 11.35 and 13.55 mg glucose/h, respectively, compared to that in aqueous medium, giving 20.39 mg glucose/h. The results show that careful selection of the solvent is necessary for either biotransformations or for the synthesis of oligosaccharides.

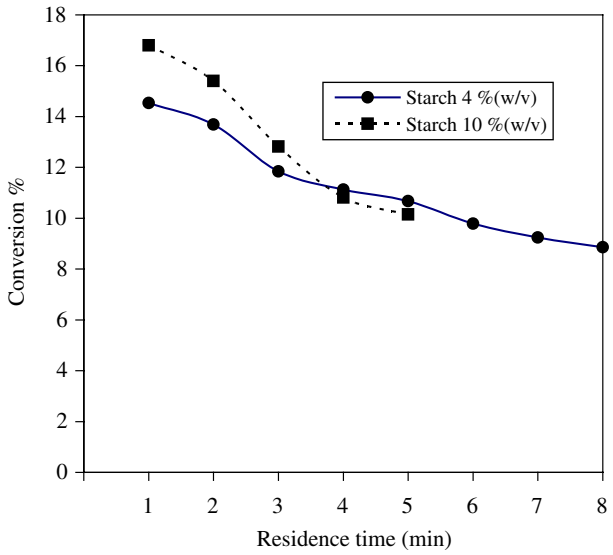

Figure 4. Hydrolysis of starch to glucose by CLEC glucoamylase in a packed-bed reactor (PBR) at 60 °C.

Table 4. Effect of organic solvents on CLEC glucoamylase on glucose-releasing activity

Water–organic solvent mixture (1:1)	Amount of glucose produced (mg/h)
2-Propanol	13.55
n-Dodecane	28.94
1,4-Dioxane	11.35
Me_2SO	14.05
Hexane	28.94
Chloroform	27.02
Water	20.39

3. Discussion

Crystallization followed by crosslinking is an efficient method for the production of a thermostable glucoamylase enzyme that also has good activity in organic media. Crosslinking of the enzyme crystals resulted in a significant change in the kinetic parameters as evident from the increase in K_m and V_{max} of the crosslinked glucoamylase crystal during soluble starch hydrolysis. The CLEC must, however, be used at pH 4.5 to get the optimum activity, and it must also be able to withstand a temperature of 80 °C for 30 min, even without the protection of starch.

Table 3. Productivity of CLEC glucoamylase in a packed-bed reactor (PBR) for the hydrolysis of starch and maltodextrin (12.5 DE) at 60 ± 2 °C

Substrate (w/v)	Residence time (min)	Glucose produced (g/L)	Productivity (g/L/h)
Starch (4%)	5.4	4.972	55.13
Starch (10%)	7.6	14.07	110.58
Maltodextrin (10%)	5.65	43.73	463.69
Maltodextrin (10%)	3.39	40.42	714.10

The CLEC can be used for the continuous production of glucose from soluble starch or maltodextrin at 60 °C in a packed-bed reactor within short reaction times. The crystals are robust and can also be used in solvent media for many biotransformations, including the synthesis of novel oligosaccharides. In addition, our experiments showed that CLECs made by this method when subjected to lyophilization, with or without surfactants, yield a lyophilized CLEC glucoamylase that can be stored at nonrefrigerated temperatures for extended periods of time. Further studies are needed to determine the effects of crosslinking on the CD, SBD, and the linker regions of the glucoamylase enzyme.

4. Experimental

4.1. Materials

Glucoamylase (amyloglucosidase, E.C:3.2.1.3, α-(1 → 4)-glucan glucohydrolase) from *Aspergillus niger* (AMG 3L 3388 IU/mL, protein 609.8 mg/mL) was obtained from Novozymes (Denmark). Glutaraldehyde was from BDH (UK). Soluble starch and ammonium sulfate were purchased from E. Merck (India). Maltodextrin was obtained from Grain Processing Corporation (Muscatine, Iowa, USA). Hexane, dodecane, chloroform, Me_2SO, *n*-octane, 2-propanol, and Tween-80 were obtained from BDH, S.D fine Chemicals and E. Merck (India).

4.2. Purification of glucoamylase

The commercial glucoamylase obtained from Novozymes was further precipitated with ammonium sulfate (40–80% cutoff) and dialyzed against sodium acetate buffer at pH 4.5 or by passing through a column of Sephadex G-25.

4.3. Preparation of crosslinked glucoamylase crystals

The purified glucoamylase enzyme was crystallized by the batch method using ammonium sulfate as precipitant (65% saturation) along with 20% 2-propanol as cosolvent in acetate buffer (0.5 M, pH 4.5). The solution was stirred at 4 °C for 30 min and then kept for 16 h at the same temperature. The crystals formed were separated by centrifugation, and the crystals were suspended in the minimum amount of acetate buffer (0.5 M, pH 4.5) containing starch (1%) and/or bovine serum albumin (BSA, 1 mg/mL). The glucoamylase crystals were chemically crosslinked using glutaraldehyde (50%), and the final concentration was 2% (v/v) in 0.2 M phosphate buffer of pH 7.0. After 1 h, the crosslinked crystals formed were separated by centrifugation at 2000 rpm and washed several times with 0.2 M acetate buffer pH 4.5 until the crystals were free of glutaraldehyde. The preparation was then lyophilized with a nonionic surfactant and stored at room temperature (28 ± 2 °C).

4.4. Determination of physical characteristics

4.4.1. Density. Density of the glucoamylase CLEC was obtained using a helium Auto pycnometer (Micromeritics model 1320).

4.4.2. Surface area. Surface area was measured by the BET (Brunauer, Emmett, and Teller) technique using a Zetasizer (Melvern, UK) in which liquid nitrogen was the adsorbent.

4.4.3. Crystal structure. The crystal structure was observed under a scanning electron microscope (JEOL, Japan) at 10 kV accelerating voltage, after sputtering with gold. A photograph of the material is shown in Figure 5.

4.5. Determination of enzyme activity

Enzyme solution (0.2 mL) is added to 1 mL of 4% starch and 4% maltodextrin solution in 0.2 M acetate buffer (pH 4.5), and the mixture is incubated at 60 °C for exactly 1 h. The enzyme reaction was then terminated by adding 0.8 mL of 4 N NaOH. The dextrose formed was determined using the Lane–Eynon method.[16] Activity of glucoamylase CLEC was determined by the same procedure using 454 mg of CLEC. The amount of enzyme that is capable of producing 1 μmol of dextrose per minute under experimental conditions is defined as 1 enzyme unit (1 IU).

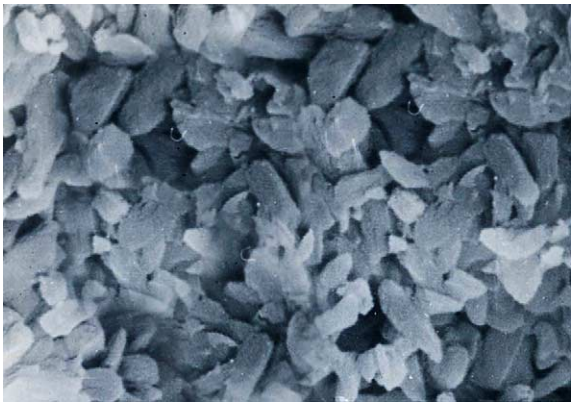

Figure 5. Scanning electron micrograph of CLEC glucoamylase (×1000).

4.6. Determination of protein

The determination of protein was carried out by Lowry's method.[19] BSA was used as the standard, and the absorbance was read at 660 nm. The crosslinked enzyme crystals were washed thoroughly to remove excess reagents before estimation.

4.7. Determination of reducing sugar and dextrose equivalent (DE)

The dextrose equivalent was determined by the Lane–Eynon titrimetric method,[20] and reducing sugar was determined by the DNS method.[21] Percentage conversion was calculated as glucose obtained × 0.9. DE [dextrose (glucose) equivalent] refers to the reducing sugar content of a material, calculated as glucose (dextrose) and expressed as percent of total solids.

4.8. Thermostability of glucoamylase CLEC

CLEC (454 mg, specific activity 0.068 IU/mg protein) was incubated for 0.5–1 h at temperatures ranging from 60 to 90 °C in the presence of 4% (w/v) starch and in the absence of any starch. After incubation, 1 mL of 4% starch solution at pH 4.5 was added and incubated at 60 °C for 1 h, and the glucose produced was monitored.

4.9. Effect of pH and substrate concentration

In order to study the effect of pH and substrate concentration on the activity of the crosslinked enzyme, 454 mg CLECs (specific activity 0.068 IU/mg protein) was incubated with 1 mL of 4% starch solution of pH varying from 2.5 to 8.5, and soluble starch concentration ranges from 10 to 350 mg/mL at 60 °C for 1 h. The glucose produced by hydrolysis was estimated.[20] The same procedure was repeated using 0.2 mL of the soluble enzyme (specific activity 0.085 IU/mg protein), and the activities were compared.

4.10. Effect of organic solvents on the activity of crosslinked enzyme crystals (CLEC) of glucoamylase

To 10 mL of 2% (w/v) maltodextrin (DE 12.5) in different water–organic solvent mixtures (1:1) at pH 4.5 was added 20 mg of CLEC glucoamylase, and the mixtures were incubated at 60 °C for 1 h. The resultant reducing sugar was estimated by the DNS method.

4.11. Continuous hydrolysis of starch in a packed-bed reactor (PBR) using CLEC glucoamylase

Continuous hydrolysis of soluble starch to glucose was carried out in a packed-bed reactor. A jacketed glass column (114 × 8 mm and $V_0 = 3.88$ mL) with 3.5 g of CLEC glucoamylase was used, and the hydrolysis was carried out at 60 °C. Continuous saccharification was carried out by passing 4% and 10% (w/v) solution of starch (pH 4.5) and 10% maltodextrin (DE 12.5, pH 4.5) through the column in different residence times using a pump at a dilution rate of 7–17 bed volumes per hour.

Acknowledgements

We are grateful to the Director, RRL, Trivandrum, for providing the necessary facilities. The second author acknowledges CSIR, India, for awarding him a Senior Research Fellowship. Our thanks are also to Dr. Lalithambika of RRL, for the surface area measurements and Dr. Prabhakar Rao, Scientist, RRL for the SEM of the crystal.

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