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Immobilisation of cyclodextrin glucanotransferase from *Bacillus circulans* ATCC 21783 on purified seasand

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Abstract Cyclodextrin glucanotransferase (CGTase) from *Bacillus circulans* (ATCC 21783) was immobilised on a silica-based support: purified seasand. Although adsorption of 98% was achieved, considerable desorption was encountered. This problem was minimised by crosslinking the adsorbed enzyme with glutaraldehyde. The immobilised enzyme after crosslinking could be used repeatedly for cyclodextrin (CD) production in a batch process. The activity retention was 80% at the end of the eighth cycle. The immobilised enzyme showed a shift in the pH optimum towards the alkaline side and also an improvement in the pH stability compared to the free enzyme. It catalysed the formation of β-CD as a major product. A significant amount of α-CD production was also observed on prolonged incubation.

Keywords Cyclodextrin glucanotransferase · Immobilisation · Seasand · Glutaraldehyde · Cyclodextrin

Introduction

Cyclodextrin glucanotransferase (CGTase, E.C. 2.4.1.19) is a unique extracellular transglucosidase, which degrades starch and related compounds to non-reducing, cyclic polymers of D-glucosyl units called cyclodextrins (CDs). The sources of this industrially important enzyme include *Bacillus macerans*, *B. megaterium*, *Klebsiella oxytoca*, *Micrococcus* spp. and *B. circulans* [4]. Economic utilisation of this enzyme for production of CDs can be achieved by immobilisation. Physical adsorption of an enzyme to an insoluble matrix is a simple and inexpensive way of preparing immobilised enzymes. In the present investigation, adsorption and immobilisation of the enzyme on purified seasand, a cheap and easily available inert support, was achieved. The method of covalently coupling enzymes to inorganic carriers such as silica involves treatment of the carrier with chemical reagents that can place an organic functional group on the matrix surface [16]. This method involving prior activation of the support is frequently cited for immobilisation of CGTase [2, 12, 13]. The process of activation was bypassed in the present study. Conditions for immobilisation were optimised and the immobilised enzyme was further characterised.

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Materials and methods

Materials

B. circulans (alkalophilic *Bacillus* sp., ATCC 21783) was procured from the American Type Culture Collection, Rockville, Md. Tapioca starch and purified seasand (synthetically prepared silica) were procured locally from Loba Chemicals, Mumbai, India. Purified seasand was prepared by precipitating sodium silicate and further treating it to remove trace metals. The standard CDs and polyethyleneimine were from Sigma (St. Louis, Mo.). All other chemicals used were of analytical grade. Immobilisation experiments were performed on an orbital incubator shaker (CIS-24; Remi Instruments, Mumbai, India).

Assay methods

Assay of the free enzyme

The assay was carried out using the procedure described by Tomita et al. [14] with a few modifications. Substrate solution (0.24 ml), containing 1.2 mg soluble starch in 0.05 M glycine/NaCl/NaOH buffer, pH 8.5, was incubated with 0.05 ml of suitably diluted enzyme at 40°C for 10 min. The reaction was stopped by addition of 1 ml 0.5 M acetic acid. After addition of 0.5 ml 0.02% iodine/0.2% potassium iodide reagent, the solution was diluted to 10 ml with distilled water. Absorbance was measured at 700 nm. A standard curve for the method was established using concentrations of soluble starch ranging from 0.2 mg to 1.2 mg in 0.29 ml buffer. One unit is defined as the amount of enzyme that can dextrinise 1 mg starch per minute at 40°C.

Assay of immobilised enzyme

Ten millilitres of 1.5% starch solution was added to 1 g immobilised support. After incubation for 10 min at 40°C, an 80 µl aliquot was removed and added to a test tube containing 1 ml 0.5 M acetic acid. Further estimation was carried out as for the free enzyme.

Cyclising activity

The principle for this method [6] is the reduction in the colour of phenolphthalein due to complexation with β-cyclodextrin (CD).

Production of CGTase

The production medium contained (g/l): tapioca starch, 20.0; yeast extract, 15.0; magnesium sulphate, 0.2; dipotassium hydrogen phosphate, 1.0; and sodium carbonate 10.0. Starch was gelatinised by heating. All other components except sodium carbonate were added to starch and autoclaved. Sodium carbonate solution was autoclaved separately. The medium was dispensed in 50 ml aliquots in 500-ml Erlenmeyer flasks. Inoculum (1.0%; OD at 660 nm = 0.1) was added to the flasks, which were incubated on a rotary shaker at 200 rpm, 30°C for 84 h. The broth was centrifuged at 6,000 *g* for 15–20 min and the supernatant was assayed for CGTase activity.

Immobilisation

Wood shavings, glass and ceramic beads, crosslinked sago starch beads, wool threads, chitin and purified seasand (particle size 40–150 mesh) were screened as supports. One gram of the support was weighed and equilibrated with 10 ml 0.1 M phosphate buffer pH 7.0, for 24 h at 10°C. Following equilibration, 16 U crude enzyme in 10 ml buffer was loaded and the flasks were incubated at 10°C and 100 rpm for 3 h. Unadsorbed enzyme activity in the supernatant was assayed. The support was assayed for adsorbed enzyme. Amongst the supports screened, purified seasand gave the best results. Crosslinking of the adsorbed enzyme was carried out at various temperatures (10–30°C) and concentrations (0.5–4.0%) of glutaraldehyde.

Characterisation of the immobilised enzyme

Effect of pH on activity of free and immobilised enzyme

The buffers (0.05 *N*) used were: pH 3.0, glycine/HCl: pH 4.0–5.5, acetate buffer; pH 6.0–8.0, phosphate buffer; pH 8.5–11.0, glycine/NaCl/NaOH buffer. Substrate was prepared separately in each buffer.

pH stability

The free and immobilised enzymes were incubated at 40°C for 30 min in buffer solutions from pH 4.0 to pH 9.5, and residual activity was estimated at pH 8.5.

Effect of temperature on activity of immobilised and free enzyme

The activities of free and immobilised enzyme were measured at temperatures in the range 30–80°C.

Thermal stability

Immobilised enzyme and free enzyme were incubated in glycine/NaCl/NaOH buffer, pH 8.5, at various temperatures for 30 min. Residual enzyme activity was measured.

Reusability of the immobilised enzyme

The immobilised enzyme was incubated with 30 ml 1.5% starch in glycine buffer, pH 8.5 at 40°C. After 30 min, starch utilised was analysed. The supernatant was decanted and immobilised enzyme was washed with phosphate buffer, pH 7.0. The cycle was repeated 15 times.

Percent conversion and yield of β-CD

Immobilised support (2 g) corresponding to 8.0 U immobilised enzyme was incubated at 40°C at 50 rpm on a rotary shaker with 1,000 mg starch substrate (15 ml). The amount of starch converted and yield of β-CDs were estimated and compared with those of free enzyme.

Study of CD profile by thin layer chromatography

Immobilised support (2 g = 8.0 U) was incubated with 1 g starch (15 ml) at 40°C, 50 rpm. Aliquots were collected at time intervals up to 18 h. Using the Linomat applicator, 5–10 µl of each aliquot was applied to Alufolien Kieselgel 60 F_{254} thin layer chromatography (TLC) plates (20 cm/10 cm). Standards of α-CD, β-CD and γ-CD in starch solution were also applied. *n*-Butanol/ethanol/water (4:3:3) was used as the developing solvent. After one run, the plates were dried and rerun in the same system. Spots were developed in an iodine chamber and scanned using a Camag TLC Scanner II (CAMAG, Muttenz, Switzerland).

Results and discussion

Amongst the supports screened, maximum adsorption (45%) was obtained with purified seasand (with 16 U enzyme loaded onto 1 g support). Purified seasand is crude silica prepared by neutralisation of aqueous alkali metal silicate with acid and further calcined to remove trace metals [1]. It has good mechanical stability as it is mainly used as a filler in column packing for chromatography. This material is free from mineral impurities like aluminium and barium, which are found in natural seasand, thus making it suitable for use in pharmaceutical and food applications. The significant advantage is its cost compared to tailor-made matrices like aminopropyl silica.

Immobilisation on purified seasand was carried out for up to 24 h and it was observed that an incubation period of 4 h was sufficient for maximum adsorption. pH plays an important role in immobilisation. In the present study, adsorption improved with increasing pH, was maximum at pH 7.0 and decreased at higher pH. High temperatures can weaken the strength of dipolar interactions such as hydrogen bonds and favour formation of hydrophobic interactions [3]. Decreased temperature can cause the reverse. Silica has silanol groups on its surface, which take part in hydrogen bonding as well as electrostatic interactions during adsorption. Immobilisation was performed at temperatures from 5°C to 20°C. Adsorption increased with decreasing temperature and hence it is possible that hydrogen bonding plays a predominant role in immobilisation. Maximum adsorption occurred at 5°C. Optimisation of enzyme loading was performed in the range of 3–20 U/g

support. Optimum adsorption of 4.88 U (97.6%) was achieved at an enzyme loading of 5 U/g.

After the initial cycle only 50% of the enzyme remained adsorbed. To retain the enzyme and prevent leakage, crosslinking with glutaraldehyde was carried out. Crosslinking occurred only under stationary conditions. The crosslinking effect increased with a rise in temperature, but with concomitant denaturation of the enzyme. Concentrations above 2% glutaraldehyde did not improve the crosslinking and 2 h was sufficient for the reaction. Optimum crosslinking with minimum denaturation could be obtained at 20°C, using 2% glutaraldehyde for 2 h as shown in Table 1.

Results in Fig. 1 show the enzyme from *B. circulans* to have a pH optimum in both acidic as well as alkaline conditions. It exhibited maximum activity at pH 4.5 in the acidic range and at pH 8.5 in the alkaline range, which was 80% of the activity at pH 4.5. The earlier methods of immobilisation studied for this enzyme report reduced activity in either the acidic or alkaline region [7, 9]. Loss of acid CGTase on immobilisation to a synthetic resin, DIAION HP-20, was reported by Kato and Horikoshi [7]. Loss of alkaline activity was seen on immobilisation of succinylated CGTase from alkalophilic *Bacillus* sp ATCC 21783 to a vinylpyridine copolymer [9]. In the present study, immobilisation yielded an enzyme with reduced activity in the acidic region, but with more activity in the alkaline region compared to the free enzyme. This can be considered a favourable shift as conversion is often carried out under alkaline conditions. The pH optima remained unaltered, i.e. pH 4.5 and pH 8.5. An additional advantage was that, although the free enzyme exhibited sharp peaks of optimum pH, upon immobilisation the peak of optimum pH broadened in the alkaline region with more than 90% activity being retained at pH 9.0.

The free enzyme was unstable in the acidic region, retaining only 49% activity after 30 min at pH 4.5 whereas it was stable in the alkaline region, retaining 96.5% of its activity at pH 8.5. Decreased pH stability of immobilised CGTases in the acid as well as alkaline range has been reported [7, 9]. In the present study, the immobilised enzyme showed better stability than free enzyme in acidic as well as alkaline conditions.

The temperature activity profile of the free enzyme showed increasing dextrinising activity up to 70°C. Activity decreased thereafter (Fig. 2). A shift in temperature optimum from 50°C to 55°C has been reported on immobilisation of CGTase from alkalophilic *Bacillus* sp. no. 38–2 (ATCC 21783) to the synthetic adsorption resin, DIAION HP-20 [7]. Similar results were reported on adsorption of the enzyme to a vinylpyridine copolymer [9]. However, in our study the immobilised enzyme showed a decrease in the temperature optimum to 45°C. (Fig. 2). CGTase immobilised by covalent bonding onto activated Sepharose and alkylamine silica treated with glutaraldehyde also showed a decrease in the temperature optimum [2]. This may be attributed to modification of a site conferring thermostability. A study on cloned CGTase from *Bacillus* spp. revealed a decrease in the

Table 1 Optimisation of crosslinking conditions (in 2% glutaraldehyde). Results are the mean of three determinations

Temperature (°C)	Time (h)	Initial % adsorbed	% retained after three cycles
Control	–	90.0	25.0
10	2	81.5	42.6
10	3	75.4	40.6
15	2	79.0	56.2
15	3	75.0	52.5
20	2	76.5	62.6
25	2	70.4	57.6
30	2	46.2	38.6
30	3	38.4	31.6

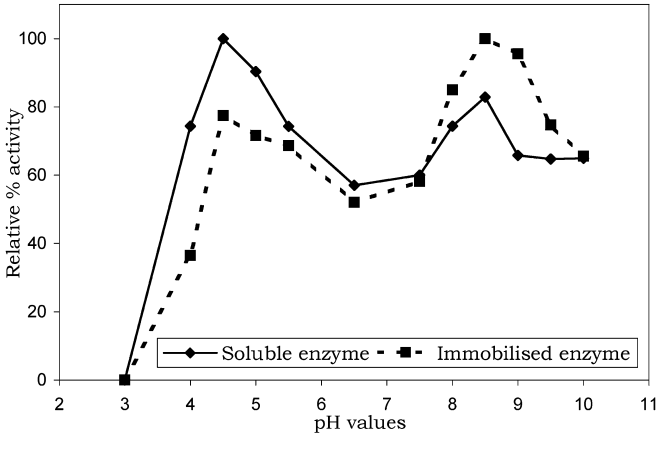

Fig. 1 pH activity profile of soluble and immobilised enzyme. Soluble (0.05 ml; 13.0 U/ml) or immobilised (1 g; 4.5 U/g) enzyme was incubated with 0.24 ml substrate prepared in various buffers and the activity estimated

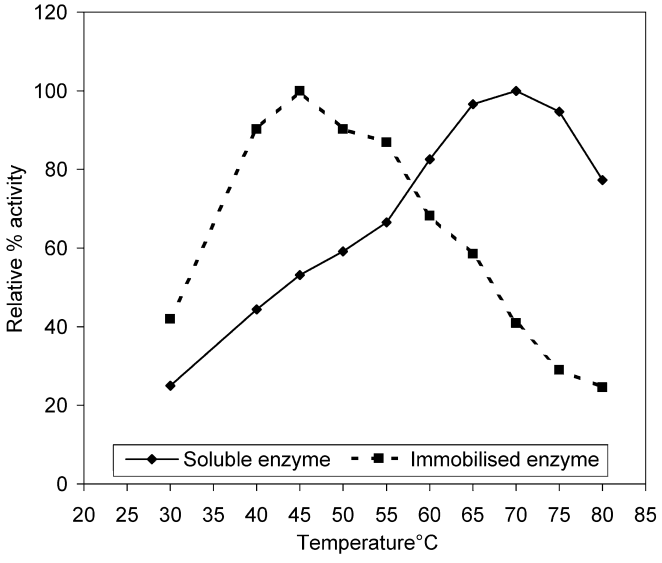

Fig. 2 Temperature activity profile of soluble and immobilised enzyme

Table 2 Yield of cyclodextrin (CD). Results are the mean ± SD of three determinations

Time (h)	Immobilised enzyme		Soluble enzyme	
	Starch converted (mg)	CD (mg)	Starch converted (mg)	CD (mg)
2	725 ± 9.5	22.3 ± 1.7	799 ± 10.8	54.3 ± 2.8
4	901 ± 25.2	53.2 ± 2.0	929 ± 29.2	65.6 ± 2.3
8	958 ± 24.6	78.6 ± 3.3	966 ± 30.2	86.6 ± 6.1
10	981 ± 29.5	111 ± 6.9	981 ± 35.1	120 ± 6.3
12	993 ± 54.5	127 ± 9.3	994 ± 42.7	137 ± 10.6
16	996 ± 41.4	145 ± 12.5	997 ± 25.7	153 ± 9.7
18	998 ± 29.7	152 ± 11.0	998 ± 32.5	159 ± 11.0

Table 3 Profile of CD production. Results are the mean of three determinations. *N* Negligible

Time of incubation (h)	Free enzyme α-CD:β-CD:γ-CD	Immobilised enzyme α-CD:β-CD:γ-CD
1	0:100:0	8.1:100:0
2	10:100:0	8.8:100:0T
4	13.0:100:0	12.5:100:0
6	15.4:100:0	15.8:100:0
8	15.2:100:0	14.7:100:0
12	17.5:100:N	17.5:100:0
16	32.8:100:19.0	32.3:100:0
18	40.0:100:29.8	35.7:100:0

temperature optimum without a decrease in activity [5]. The authors attributed this to selective modification of certain amino acids, leading to decreased thermostability. In the present study, a similar mechanism may be responsible, with glutaraldehyde crosslinking selectively with those amino acids, conferring thermostability. The free enzyme was stable at temperatures up to 50°C and was inactivated at higher temperatures. The immobilised enzyme was stable up to 40°C. Denaturation thereafter was gradual up to 55°C.

The batch reusability of the immobilised enzyme was studied to determine the number of cycles for which it could be used without significant loss of activity. The enzyme retained 82% of its activity after the initial cycle, and there was no further activity loss up to the ninth cycle. In the ensuing cycles a gradual decrease was encountered, with a residual activity of 55% after 14 cycles. The dextrinising activity of free and immobilised enzymes were compared. The rate of conversion of starch to CDs remained relatively the same for both free and immobilised enzyme. About 99.8% of soluble starch was converted to linear dextrins. The yield of β-CD from 1,000 mg starch with the immobilised enzyme was 152.1 mg after 18 h whereas with free enzyme it was 159.5 mg (Table 2). The difference in yield was marginal, but the suitability of the immobilised enzyme for reuse is considered a major advantage.

Although *B. circulans* is a β-CD producer it also produces small amounts of α-CD and γ-CD [10]. The present study investigated the formation of these CDs by TLC of the reaction products. β-CD was the major product of CGTase action throughout the reaction period. As shown in Table 3, the ratio of α-CD to β-CD increased as the reaction progressed. Gradual formation of γ-CD on prolonged incubation was observed by the action of the free enzyme. γ-CD was, however, not detected in the reaction mixtures of the immobilised enzyme. The active site of CGTase has certain amino acid residues, such as tyrosine, asparagine and phenylalanine, that determine the type of CD produced. Studies to investigate and modify product specificity have been reported [8, 11, 15]. It would be interesting to study the production of specific CDs by manipulating various reaction conditions.

Immobilisation of *B. circulans* CGTase onto purified seasand gave promising results and CD production could be conducted efficiently using this immobilised preparation. As purified seasand is used as a column packing material, scaling up to continuous bioconversion in column reactors is feasible.

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