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Immobilization on Eupergit C of cyclodextrin glucosyltransferase (CGTase) and properties of the immobilized biocatalyst

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Abstract

The extreme thermophilic cyclodextrin glucanotransferase (CGTase) from *Thermoanaerobacter* sp. was covalently attached to Eupergit C. Different immobilization parameters (incubation time, ionic strength, pH, ratio enzyme/support, etc.) were optimized. The maximum yield of bound protein was around 80% (8.1 mg/g support), although the recovery of β-cyclodextrin cyclization activity was not higher than 11%. The catalytic efficiency was lower than 15%. Results were compared with previous studies on covalent immobilization of CGTase.

The enzymatic properties of immobilized CGTase were investigated and compared with those of the soluble enzyme. Soluble and immobilized CGTases showed similar optimum temperature (80–85 °C) and pH (5.5) values, but the pH profile of the immobilized CGTase was broader at higher pH values. The thermoinactivation of the CGTase coupled to Eupergit C was slower than the observed with the native enzyme. The half-life of the immobilized enzyme at 95 °C was five times higher than that of the soluble enzyme. The immobilized CGTase maintained 40% of its initial activity after 10 cycles of 24 h each. After immobilization, the selectivity of CGTase (determined by the ratio CDs/oligosaccharides) was notably shifted towards oligosaccharide production.

Keywords: Cyclodextrin glucanotransferase; Covalent immobilization; Cyclodextrins; Eupergit C; *Thermoanaerobacter* sp.

1. Introduction

Cyclodextrin glucosyltransferases (CGTases) (EC 2.4.1.19) represent one of the most important groups of microbial amylolytic enzymes [1]. These enzymes catalyze the formation of cyclodextrins (CDs) from starch and related α(1 → 4) linked glucose polymers via a transglycosylation reaction [2]. Three different CDs are known, α^-, β^- and γ-CD, which consist of 6, 7 and 8 α(1 → 4) linked D-glucose units, respectively [3]. These cyclic products are able to form versatile inclusion complexes with many organic and inorganic substances, therefore being used as complexing agents in food, pharmaceutical and cosmetic industries [4].

Apart from making cyclodextrins, CGTases also catalyze other three reactions [5,6]: (a) coupling, where the CD molecule is opened and combined with a linear oligosaccharide to produce a longer linear carbohydrate; (b) disproportionation, which is the transfer of part of a linear oligosaccharide chain to an acceptor; and (c) saccharifying or hydrolysis of starch.

Different kinds of carbohydrates and related compounds such as natural glycosides, sugar alcohols, flavonoids or vitamins can be used as acceptors [7,8]. Transglycosylation often confers new properties (stability, solubility, etc.) to the glucosylated compound

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[9]. The three transglycosylation reactions catalyzed by CGTase (cyclization, coupling and disproportionation) involve the same catalytic residues, and the mechanism is a double-displacement reaction through a covalent enzyme-intermediate complex [5].

The CGTase from the thermophilic anaerobe *Thermoanaerobacter* sp. is able to degrade starch into CDs under jet-cooking industrial conditions, thus avoiding the use of α-amylase pretreatment for the liquefaction of starch [10].

There is increasing interest in developing efficient and scalable synthetic routes to oligosaccharides for addressing applications in the food (sweeteners, stabilizers, bulking agents, functional foods, etc.) and pharmaceutical industries [11]. Possibly, the main bottleneck in the industrial application of enzymes is the price and the stability of the biocatalyst. Enzyme immobilization is one of the most useful approaches to overcome such difficulties. Continuous production of oligosaccharides using immobilized CGTase would offer several advantages allowing reuse of expensive CGTase [12], simplifying product purification process and providing opportunities for scaling up [13]. However, CGTase immobilization is not an easy task. Different approaches have been applied for the immobilization of CGTases, based on adsorption [14], entrapment [15] or covalent binding [13,16,17]. Immobilization by physical adsorption or entrapment were unsuitable because the enzyme readily leaks from the support during the reaction [18]. The recovery of activity in the immobilized biocatalyst was reported to be very low, especially when compared with structurally-related enzymes such as the glycosidases. This has been attributed to the complexity of CGTase 3D-structure (the enzyme presents five domains) and mechanism (four possible reactions).

In this work, we have investigated the immobilization of a thermophilic CGTase (from *Thermoanaerobacter* sp.) by covalent attachment to Eupergit C. This support has been described as a suitable carrier for covalent immobilization of enzymes for industrial applications. It possesses good properties and is commercially available in different forms [19]. Eupergit C consists of macroporous beads with a diameter of 100–250 μm made by copolymerization of *N*,*N*′-methylen-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide. The optimum conditions for CGTase immobilization, such as the pH of the solution and the ratio enzyme/Eupergit C, were studied. Stability and activity of the immobilized enzyme were also investigated.

2. Materials and methods

2.1. Materials

CGTase from *Thermoanaerobacter* sp. (Toruzyme 3.0L) was kindly provided by Novozymes A/S. Phenolphthalein, maltooligosaccharides (MOS) (G1–G7), α-, β- and γ-CD were purchased from Sigma. Eupergit C and Eupergit C 250L were donated by Röhm Pharma (Weiterstadt, Germany). Potato soluble starch (Paselli SA2) was kindly donated by Avebe (Foxhol, The Netherlands).

2.2. Methods

2.2.1. Assay of β-CD cyclization activity

The production of β-CD was detected spectrophotometrically at 552 nm on the basis of its ability to form a stable, colourless inclusion complex with phenolphthalein [20]. Paselli SA2 (partially hydrolyzed potato starch, with an average degree of polymerization of 50) was used as substrate. The CGTase activity was measured at 85 °C by incubating the enzyme (0.1–0.2 U/ml) in presence of 5% (w/v) starch in 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$. At time intervals, aliquots were removed and added to the phenolphthalein solution. The formation of β-CD was followed for 5 min. One unit of activity was defined as the amount of enzyme able to produce 1 μmol of β-CD per minute under the corresponding conditions. The recovered activity after immobilization refers to the units of activity measured in the immobilized biocatalyst with respect to the total units before immobilization.

2.2.2. Immobilization in Eupergit C

The immobilization was carried out at 25 °C with gentle orbital shaking (150 rpm) in potassium phosphate buffer (final volume 10 ml). After incubation, the beads were collected by filtration in vacuo using a porous glass filter. The beads were rinsed thoroughly on the same filter with approximately 3 × 20 ml of diluted potassium phosphate, and 2 × 20 ml of 10 mM sodium citrate buffer, pH 5.5.

2.2.3. Measurement of protein

The concentration of protein was determined by the Bio-Rad assay (Microassay procedure with a sensitivity of 1–20 μg protein; ≤25 μg/ml) with immunoglobulin G (IgG) as standard. This assay is based on the Bradford method.

The amount of protein bound to the support was calculated by using the following formula: (amount of CGTase before immobilization)−(amount of CGTase recovered in the filtrate)−(amount of CGTase recovered in the washings).

2.2.4. Determination of optimum pH

The effect of pH on native and immobilized enzyme was studied by assaying the preparations at different pH values (4–9) using 10 mM universal buffer (boric acid, citric acid, potassium dihydrogen phosphate and diethylbarbituric acid) [21].

2.2.5. Thermal stability assays

The thermoinactivation assays were performed at 60, 85 and 95 °C in 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$. Periodically aliquots were removed and the activity was measured as described in the standard assay.

2.2.6. pH stability assays

The pH stability of the soluble and immobilized enzyme was examined by incubating CGTase samples at 60 °C for 6 h at different pH values. The residual β-CD-activity was assayed. Universal buffer (10 mM, pH 4.0–9.0) was used.

2.2.7. Operational stability of the immobilized CGTase

The reuse activity was studied in repeated batch experiments at 60 °C using soluble starch (10% w/v) as donor and D-glucose (20% w/v) as acceptor. Reactions were maintained for 24 h with gentle shaking (150 rpm). At the end of each cycle, an aliquot of 250 μl was taken, mixed with 250 μl of 0.4 N NaOH in order to quench the reaction, and analyzed by HPLC. The activity was referred to the disappearance of soluble starch in 24 h. At the end of each cycle, the immobilized enzyme was filtered and washed thoroughly with 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$.

2.2.8. Production assay

Soluble and immobilized CGTase (0.7 β-CD-units) were incubated at 60 °C with 10% (w/v) Paselli SA2 in 10 ml of 10 mM sodium citrate buffer (pH 5.5) containing 0.15 mM $CaCl_2$. At different times, aliquots of 250 μl were taken and mixed with 250 μl of 0.4 N NaOH in order to quench the reaction. Samples were centrifuged during 15 min at 3000 × g and further analyzed by HPLC.

2.2.9. HPLC analysis

Samples were analyzed by high-performance liquid chromatography (HPLC) using a ternary pump (Varian 9012) and two Aminex HPX-42A columns (300 mm × 7.8 mm, Bio-Rad) put in series. Water was used as the mobile phase (0.7 ml/min). The column temperature was kept constant at 85 °C. Detection was performed using a refraction index detector (Varian 9040). Integration was carried out using Varian Star 4.0 software.

3. Results and discussion

3.1. Optimization of the immobilization procedure

3.1.1. Ratio enzyme: support

Different amounts of support and protein were tested. Table 1 shows that the amount of enzyme immobilized increased when increasing the amount of the polymer. With a 10:1000 ratio (w/w) of enzyme to support, the catalytic efficiency reached the maximum value of 4.4% (70% of the added protein was bound). When the amount of support added was further increased, the recovered activity decreased; nevertheless, the bound protein was slightly higher. This effect is probably caused by structural changes in the enzyme introduced by the insolubilization process and by diffusional restrictions of the substrate to the active site of the protein. A similar effect was reported previously when α- or β-galactosidase were attached onto Eupergit C [22].

3.1.2. Effect of molarity of the immobilization buffer

The results are shown in Table 2. Although the percentage of bound protein was similar (68–74%) in the range 0.2–1.2 M, the maximum recovered activity (2.2%) and catalytic efficiency (3.2%) were

Table 1
Immobilization of CGTase from *Thermoanaerobacter* sp. on Eupergit C at different ratios enzyme/support[a]

Eupergit C (mg)	Enzyme (mg)	Bound protein (%)	Recovered activity (%)[b]	Catalytic efficiency (%)[c]
100	10	26	0.2	0.7
200	10	31	0.4	0.9
400	10	66	0.7	1.1
800	10	71	2.2	3.1
1000	10	70	3.0	4.4
2000	10	75	2.2	2.9

[a] Conditions: 1 M potassium phosphate buffer, pH 7.5; final volume 10 ml, 25 °C; gentle orbital shaking (150 rpm) 24 h.
[b] Measured in the β-cyclodextrin cyclization assay.
[c] The catalytic efficiency was defined as the ratio between the specific activity of the immobilized and native CGTase (the specific activity of the native enzyme was 140 U/mg protein).

Table 2
Immobilization of CGTase at different buffer concentrations[a]

Buffer concentration (M)	Bound protein (%)	Recovered activity (%)[b]	Catalytic efficiency (%)[c]
0.2	68	0.1	0.2
0.6	73	0.2	0.3
0.8	72	1.5	2.0
1.0	74	1.8	2.4
1.2	69	2.2	3.2

[a] Conditions: 10 mg CGTase; 1 g Eupergit C; potassium phosphate buffer, pH 7. 5; final volume 10 ml, 25 °C; gentle orbital shaking (150 rpm) 24 h.
[b] Measured in the β-cyclodextrin cyclization assay.
[c] The catalytic efficiency was defined as the ratio between the specific activity of the immobilized and native CGTase (the specific activity of the native enzyme was 140 U/mg protein).

achieved with 1.2 M potassium phosphate buffer at pH 7.5.

The activity yield of immobilized CGTase was dependent on the molarity of the buffer used for the immobilization. Raising the ionic strength of a solution increases the strength of hydrophobic interactions [23]. This may explain that the best results were obtained with a high buffer concentration.

3.1.3. *Effect of immobilization time*

The results of the influence of the contact time CGTase/support are presented in Table 3. The binding

Table 3
Immobilization of CGTase at different incubation times[a]

Time (hours)	Bound protein (%)	Recovered activity (%)[b]	Catalytic efficiency (%)[c]
24	69	3.1	4.4
48	79	4.7	6.0
72	84	5.0	6.2
96	83	5.2	6.3
120	85	5.4	6.3

[a] Conditions: ratio enzyme:Eupergit C 10:1000; 1.2 M potassium phosphate buffer, pH 7.5; final volume 10 ml, 25 °C; gentle orbital shaking (150 rpm).
[b] Measured in the β-cyclodextrin cyclization assay.
[c] The catalytic efficiency was defined as the ratio between the specific activity of the immobilized and native CGTase (the specific activity of the native enzyme was 140 U/ mg protein).

Table 4
Immobilization of CGTase at different values of pH of the binding buffer[a]

pH	Bound protein (%)	Recovered activity (%)[b]	Catalytic efficiency (%)[c]
4.1	76	3.5	4.6
5.0	77	3.3	4.3
6.0	77	4.2	5.5
7.0	71	5.0	7.0
8.0	71	2.8	4.0

[a] Conditions: ratio enzyme:Eupergit C 10:1000; 1.2 M potassium phosphate buffer; final volume 0 ml, 25 °C; gentle orbital shaking (150 rpm) 48 h.
[b] Measured in the β-cyclodextrin cyclization assay.
[c] The catalytic efficiency was defined as the ratio between the specific activity of the immobilized and native CGTase (the specific activity of the native enzyme was 140 U/mg protein).

yield and the recovered activity of the immobilized enzyme are notably dependent on the immobilization time.

CGTase from *Thermoanaerobacter* sp. is a thermostable enzyme. For this reason, the maximum binding yield (>80%) and recovered activity (>5%) were obtained in the range 72–120 h. In some cases, a long incubation time may result in a much higher operational stability of the immobilized enzyme due to increased multipoint attachment [24].

3.1.4. Effect of pH of the immobilization buffer

The influence of pH on the immobilization procedure is demonstrated in Table 4. The oxirane groups of Eupergit C can react with proteins over a wide pH range (from 0 to 12) [19]. Optimum binding was achieved with potassium phosphate 1.2 M at pH 7.0.

3.2. Immobilization of CGTase in Eupergit C and Eupergit C 250L

A ratio of enzyme (mg):Eupergit C (mg) of 1:100, potassium phosphate buffer 1.2 M (pH 7.0) and 72 h were selected as the optimum conditions for immobilization. CGTase was immobilized on Eupergit C and Eupergit C 250L under the above conditions (Table 5).

The main differences between Eupergit C and Eupergit C 250L are: (1) the content of epoxide groups, 600 μmol/g dry for Eupergit C and 200 μmol/g dry for Eupergit C 250L, and (2) the porosity, Eupergit C has smaller pores (<500 Å) than Eupergit C 250L (1000–4000 Å).

With Eupergit C 250L the bound protein (72%) was lower than with Eupergit C (81%). This could be related with the fact that Eupergit C 250L contains less epoxide groups, so it is necessary to use a larger quantity of polymer to achieve the same percentage of binding. Similar results were observed with the β-galactosidase from *Bacillus circulans* [22].

Anyhow, we consider the CGTase immobilized on Eupergit C a good starting point for further applications.

Table 6 summarizes previously reported results on covalent immobilization of CGTases. Other published works could not be included in this table due to the absence of reliable data regarding recovered activity. As shown, only CGTase from *Paenibacillus macerans* shows a comparable specific activity to that of our Eupergit C biocatalyst. The rest of preparations present specific activities lower than 10 U/g.

Although other immobilization methods give rise to higher recoveries of activity than those obtained with

Table 5
Immobilization of CGTase in Eupergit C and Eupergit C 250L[a]

	Protein (before immobilization, mg)	Bound protein		Activity		Recovered activity (%)[b]	Catalytic efficiency (%)[c]
		%	Protein (mg)/support (g)	U/g support	U/mg protein		
Eupergit C	10	81	8.1	147	18	10.2	13
Eupergit C 250L	10	72	7.2	67	9.3	4.8	6.7

[a] Conditions for immobilization procedure: 1 g support; 1.2 M potassium phosphate buffer; pH 7.0, final volume 10 ml, 72 h, 25 °C; gentle orbital shaking (150 rpm).
[b] Measured in the β-cyclodextrin cyclization assay.
[c] Ratio between the specific activity of the immobilized and native enzyme (the specific activity of the native CGTase was 140 U/mg protein).

Table 6
Covalent immobilization of CGTases reported in the literature

Support	Microorganism	Recovered activity (%)	Activity (U/g support)	Reference
Silochrome	*Bacillus* sp.	37–49	6	Abelyan and Afrikyan [26]
Trisorperl	*Bacillus macerans*	25	0.7	Steighard and Kleine [27]
Akrilex C	*Bacillus macerans*	3.8	n.r.	Ivony et al. [28]
PVC	*Paenibacillus macerans*	27–45	63–154	Abdel-Naby [13]
Styrene-based resin	*Bacillus stearothermophilus*	≤0.8	≤2	Sakai et al. [29]
Controlled pore silica	*Bacillus* sp.	2.5	8.6	Tardioli et al. [16]
CNBr-Sepharose	*Thermoanaerobacter* sp.	4.4	4 U/ml gel	Martin et al. [17]
EAH-Sepharose	*Thermoanaerobacter* sp.	2.4	2 U/ml gel	Martin et al. [17]

n.r.: not reported.

our acrylic polymers, several advantages arise from the election of Eupergit C as carrier. First, most of the supports in Table 6 (including PVC) need a double activation process (amination + activation with glutaraldehyde). This contrasts with the easy and straightforward coupling method when using epoxy-activated supports. Second, the net price (including support, activators and operation time) of the immobilization process is notably more inexpensive when using Eupergit C.

The immobilized CGTase on Eupergit C showed a specific activity of 18 U/mg protein; therefore, upon immobilization the activity was reduced eight-fold. Reduction in CGTase specific activity after immobilization may be attributed to: (1) steric hindrances within the pores; (2) internal diffusional limitations; (3) modifications of the enzyme conformation and/or the active site; (4) microenvironment of the support matrix, which can differ significantly from the natural environment of the enzyme. In this context, Eupergit C is a very hydrophilic polymer [25], and this can modulate the orientation of the enzyme molecule when it is attached to the support.

3.3. Enzymatic properties of CGTase immobilized on Eupergit C

3.3.1. Effect of temperature on CGTase activity

A common finding of many studies on immobilization is that some properties of the immobilized enzyme, such as its catalytic activity or thermal stability, differ from those of its soluble counterpart [23]. In this way, the enzymatic properties of immobilized CGTase were compared with those of soluble CGTase.

The activity of soluble and immobilized CGTase at pH 5.5 was measured at various temperatures (Fig. 1). The CGTase immobilized on Eupergit C showed an optimum temperature (80 °C) and a temperature profile similar to that of the soluble CGTase (85 °C).

3.3.2. Effect of pH on CGTase activity

The pH/activity curves of soluble and immobilized CGTase at 60 °C are depicted in Fig. 2. Both biocatalysts showed an optimum pH of 5.5, but the immobilized enzyme has a broader pH range of high activity, especially at higher pH values.

3.3.3. Thermal stability

The thermal inactivation of soluble and immobilized CGTase was studied in the temperature range between 60 and 95 °C, at the optimal pH for the catalytic activity (pH 5.5). In general, the immobilization process of the CGTase on Eupergit C protected the enzyme against thermoinactivation (Fig. 3). In particular, a significant improvement of the thermal stability of the immobilized CGTase at 95 °C was observed. This stabilization seems to represent an increase in rigidity; that is, a conformational stabilization of the protein. This improved thermostability might be useful in the application of this system at high temperatures, avoiding the microbial contamination. Furthermore, at 95 °C the solubility of substrate and products is higher, which would allow us to work under industrial conditions (25% starch (w/w)) with better yields.

3.3.4. pH stability

The pH stability of the soluble and immobilized CGTase was examined (Fig. 4). Both biocatalysts were notably stable after 6 h when incubated at different pH

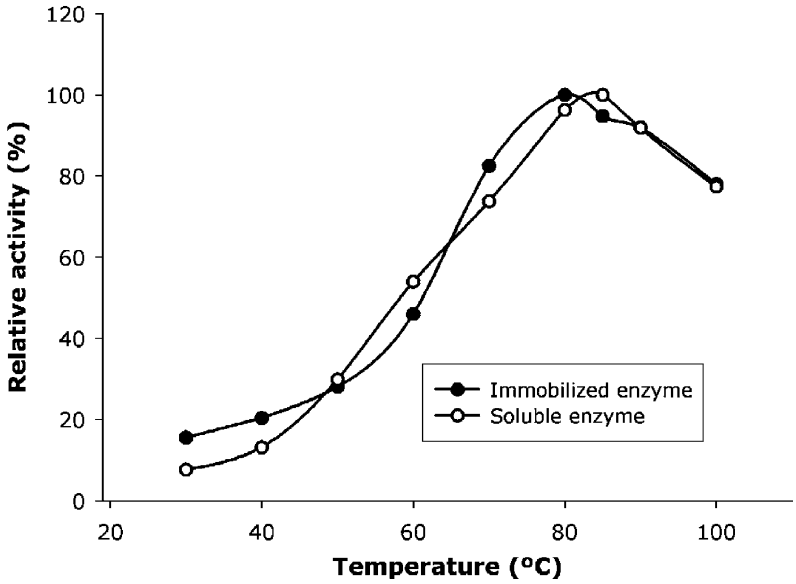

Fig. 1. Effect of temperature on the activity of soluble and immobilized CGTases from *Thermoanaerobacter* sp. (100% of relative activity was 27 U/g biocatalyst and 258 U/mg protein for immobilized and soluble CGTase, respectively).

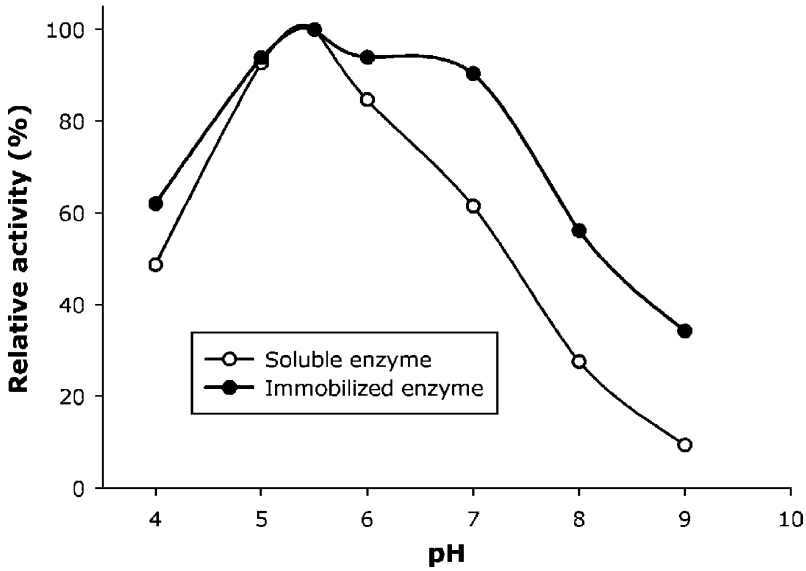

Fig. 2. Effect of pH on the activity of soluble and immobilized CGTase.

values, and no effect of CGTase immobilization on its pH stability was observed. The pH/activity profile of the immobilized CGTase was also shifted to a more alkaline range than that of the soluble enzyme.

3.3.5. Operational stability

For the operational stability study, a disproportionation-acceptor reaction was carried out in order to ascertain the application of this system. The main

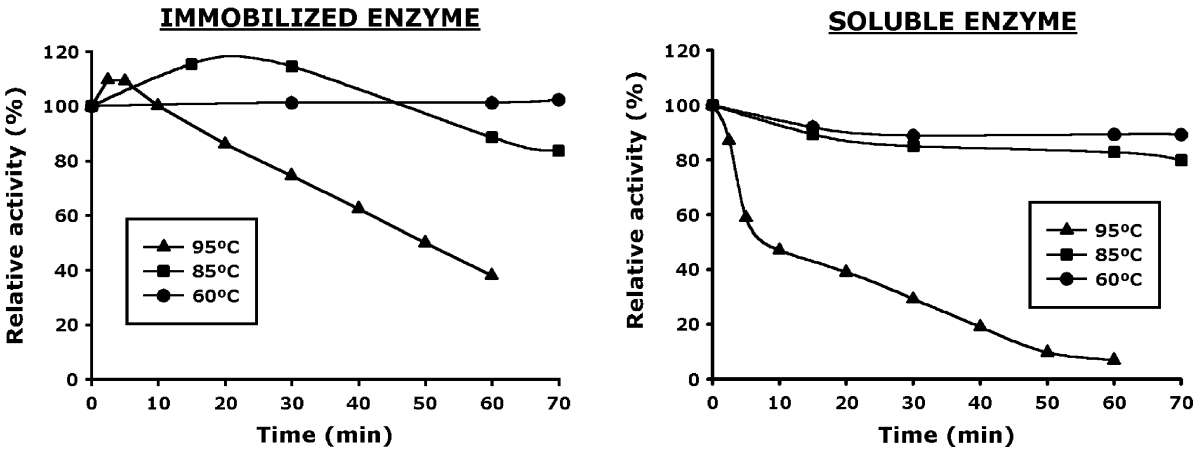

Fig. 3. Thermal stability of soluble and immobilized CGTase at different temperatures.

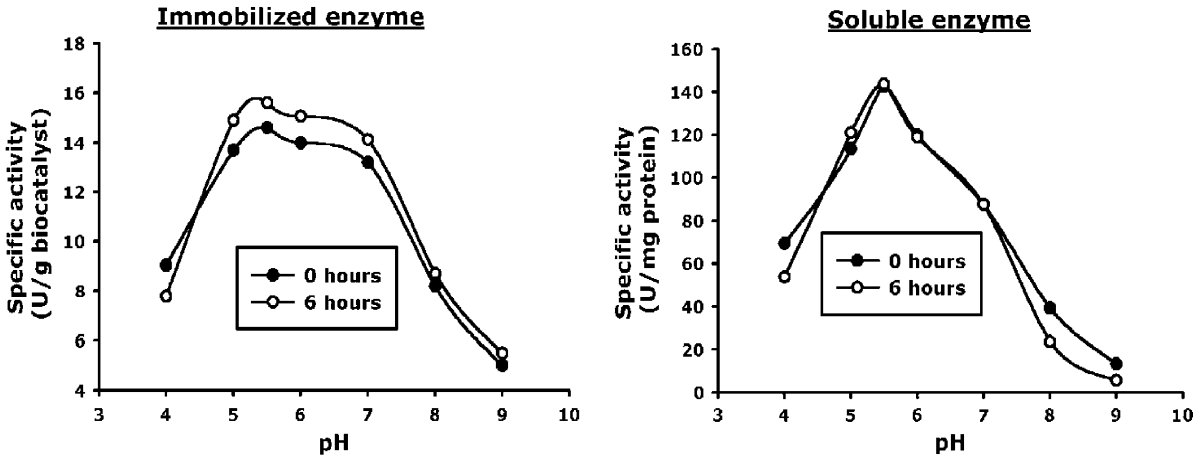

Fig. 4. pH stability of soluble and immobilized CGTase.

products formed were maltooligosaccharides, an important class of sugars with an emergent market in the food industry [30]. The operational stability of the immobilized CGTase was evaluated in a batch process (Fig. 5). It was found that the immobilized enzyme produced a good yield of oligosaccharides in the successive cycles, with 40% of the initial catalytic activity after 10 cycles of 24 h. The significant loss of activity during the 1st and 2nd cycle can be attributed to the fact that CGTase immobilized to Eupergit C may exist in distinct forms or subpopulations.

The operational stability of immobilized CGTase on Eupergit C presented in this work may indicate applicability of immobilized CGTase for continuous production of oligosaccharides.

3.4. Effect of immobilization on activity

The immobilized and soluble enzymes were assayed under the same reactions conditions using 10% (w/v) soluble starch. The time-course of CD and oligosaccharides formation was analyzed at 60 °C (Table 7).

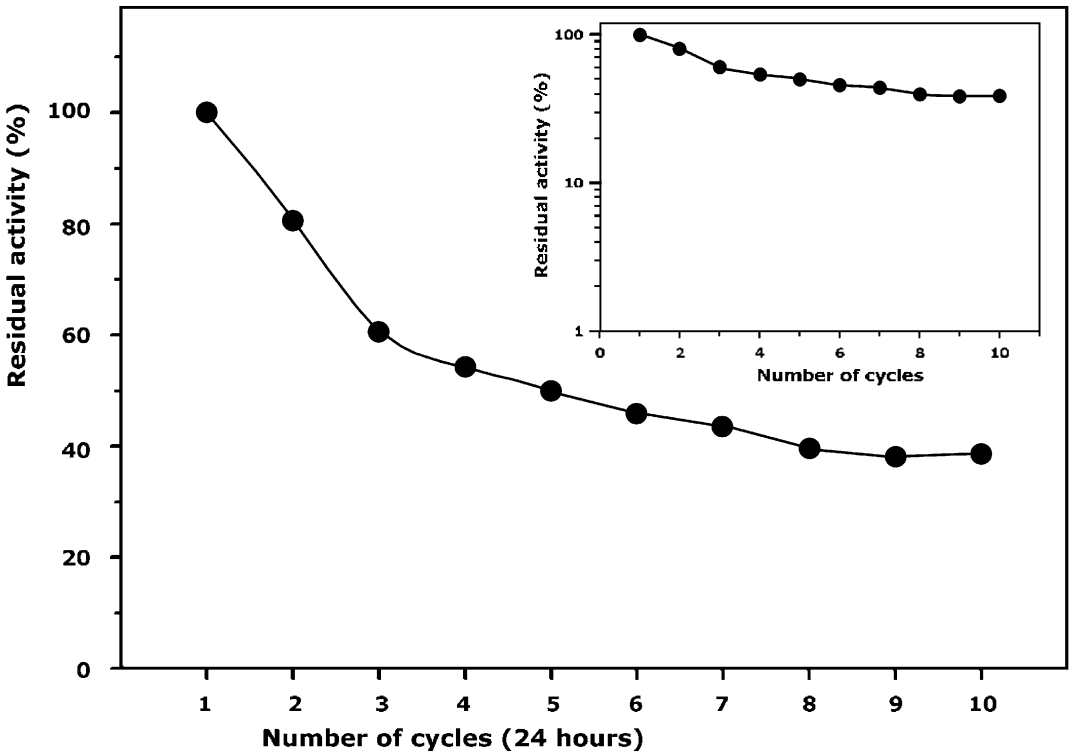

Fig. 5. Operational stability of immobilized CGTase in the acceptor reaction with glucose. (100% of relative activity was 81% of conversion of starch.) The inset shows the semilog plot.

Table 7
Products of the reaction catalyzed by the soluble and immobilized CGTase from *Thermoanaerobacter* sp. using 10% of soluble starch (w/v)

	Products formed (g/l)													Conversion of starch (%)[a]	
	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	α-CD	β-CD	γ-CD	G1–G10	CDs
Immobilized enzyme															
6 h	–	–	1.3	1.6	–	–	–	–	–	–	14	17	5	2.9	36
48 h	4.1	3.9	4	4.5	4.2	4	3.5	4.5	4.6	–	8	8.8	–	37	17
Soluble enzyme															
6 h	–	–	–	–	–	–	–	–	–	–	13	15	5	–	33
48 h	–	1.4	1.4	1.4	–	–	–	–	–	–	16	23	5	4.6	44

Reaction conditions are described in Section 2.

[a] Percentage of initial soluble starch transformed into maltooligosaccharides (MOS) and cyclodextrins.

At the first stages of the reaction (6 h) the appearance of α-, β- and γ-CD is clearly the main process with respect to the formation of short oligosaccharides. But this ratio is inverted at the end of reaction (48 h) in the case of immobilized CGTase. Both immobilized and soluble CGTases reached the maximum production of oligosaccharides around 24–48 h.

Using immobilized CGTase, at the point of maximal productivity (48 h) the weight ratio CDs:oligosaccharides was 31:69 (with a conversion of 54% starch), whereas the ratio for the soluble enzyme was 91:9 (with a conversion of 49% starch). As shown, the immobilization process substantially affects the enzyme selectivity.

4. Conclusions

Our experiments have shown that the *Thermoanaerobacter* sp. CGTase immobilized on Eupergit C exhibits an improved resistance against thermal and pH denaturation, and has a promising operational stability for the production of maltooligosaccharides.

The Eupergit C/CGTase system could be useful not only for *Thermoanaerobacter* CGTase but also for other CGTases.

This method may be effective for the scale-up of oligosaccharides production employing other acceptors than glucose opening an array of applications for the food industry.

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