Production and Surfactant Properties of Tert-Butyl α-D-Glucopyranosides Catalyzed by Cyclodextrin Glucanotransferase

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Abstract: While testing the ability of cyclodextrin glucanotransferases (CGTases) to glucosylate a series of flavonoids in the presence of organic cosolvents, we found out that this enzyme was able to glycosylate a tertiary alcohol (tert-butyl alcohol). In particular, CGTases from Thermoanaerobacter sp. and Thermoanaerobacterium thermosulfurigenes EM1 gave rise to the appearance of at least two glycosylation products, which were characterized by mass spectrometry (MS) and nuclear magnetic resonance (NMR) as tert-butyl-α-D-glucoside (major product) and tert-butyl-α-D-maltoside (minor product). Using partially hydrolyzed starch as glucose donor, the yield of transglucosylation was approximately 44% (13 g/L of tert-butyl-α-D-glucoside and 4 g/L of tert-butyl-α-D-maltoside). The synthesized tert-butyl-α-D-glucoside exhibited the typical surfactant behavior (critical micellar concentration, 4.0–4.5 mM) and its properties compared well with those of the related octyl-α-D-glucoside. To the best of our knowledge, this is the first description of an enzymatic α-glucosylation of a tertiary alcohol.

Keywords: biocatalysis; glycosidases; transglycosylation; cyclodextrin glycosyltransferases; alkyl glucosides; biosurfactants

1. Introduction

Cyclodextrin glucanotransferases—also known as cyclodextrin glycosyltransferases (CGTases, EC 2.4.1.19)—are extracellular enzymes included in the so-called α-amylase family GH13 [1] and are able to convert starch and related maltodextrin substrates into nonreducing, cyclic glucooligosaccharides termed cyclodextrins (CDs) [2]. CDs are formed by an intramolecular transglycosylation reaction in which 6, 7, or 8 glucosyl residues are linked by α(1→4) glycosidic bonds, giving rise to the formation of α, β, or γ-CDs, respectively [3]. Besides cyclization, CGTases also catalyze three additional reactions: Coupling, i.e., the aperture of the CD ring and subsequent transfer of glucose residues to acceptors; disproportionation, i.e., the transfer reaction between two linear dextrins to form maltooligosaccharides of different sizes; and hydrolysis, in which the acceptor is a H2O molecule [4]. It is worth noting that CGTases display a weak hydrolyzing activity (1.5–3.0 U/mg protein) [5]. The main reaction catalyzed...
by these enzymes (in terms of specific activity) is the intermolecular transglycosylation (coupling and disproportionation, employing CDs or maltodextrins as glucosyl donors, respectively) [6,7]. In the presence of certain carbohydrates such as sucrose, CGTase also transfers glucosyl moieties yielding the so-called acceptor products [8].

The enzyme CGTase has proved an exceptional capability to glucosylate compounds of different nature employing starch, maltodextrins, or cyclodextrins as glucosyl donors [9,10]. Apart from monosaccharides and disaccharides [11,12], other compounds such as flavonoids [13,14], vitamins [15], sugar alcohols [16], sweet glycosides [17], and polyols [18] have been successfully used as acceptor molecules for the intermolecular transglycosylation. Unfortunately, one of the main drawbacks of CGTases is that their product selectivity is not very high, because the enzyme displays its four activities simultaneously [19]. Furthermore, the formation of a homologous series of polyglucosylated products is normally described with this enzyme [20–22]. As a result, a low yield of the desired glycosylated product is typically obtained [23]. Several strategies have been assessed to improve the transglycosylation activity of CGTases, including protein engineering [24–26], chemical modification [7], immobilization [27,28], and the addition of cosolvents and additives [29–31]. Bacterial CGTases are produced mainly by the genus Bacillus [32], although Micrococcus and Klebsiella species had also been reported as producers.

For many of these acceptor reactions catalyzed by CGTases and other glycosidic enzymes, a cosolvent is needed to increase the solubility of the acceptor in the reaction medium. A requirement of the solvent is that it cannot act as acceptor itself, because this could cause a reduction of the yield of the transglycosylation and lead to the appearance of undesired side products. For this reason, primary and secondary alcohols are scarcely used in these transglycosylations. In fact, the synthesis of alkyl glycosides has been widely reported using the reverse hydrolysis reaction catalyzed by glycosidases employing mainly primary and secondary alcohols as acceptors [33–35]. Typical cosolvents for transglycosylation reactions include acetonitrile, DMSO, ethers, tertiary alcohols, etc. [22,23,36], and more recently, biomass-derived solvents and ionic liquids [37,38].

In this work, while testing the ability of CGTases to glucosylate a series of flavonoids in presence of organic cosolvents, we found out that this enzyme was able to glycosylate a tertiary alcohol (tert-butyl alcohol). We report herein the enzymatic synthesis of two novel alkyl glycoside derivatives, namely tert-butyl-α-D-glucoside and tert-butyl α-D-maltoside. Alkyl glucosides (AGs) are non-ionic surface-active agents with extensive applications in food, cosmetic, and detergent industries [39,40], in part due to their antimicrobial activity, biodegradability, and low toxicity. The surfactant properties of the major synthesized product were compared with those of related alkyl glycosides.

2. Results and Discussion

2.1. Glucosylation of Tert-Butyl Alcohol by CGTases and Characterization of Products

In our laboratory we are studying the application of different CGTases to synthesize glucosylated derivatives of polyphenols with bioactive properties [41]. Many of these flavonoids are scarcely soluble in water, which implies the need of using a miscible cosolvent to increase the solubility of the acceptor. While screening a series of cosolvents (at 30% v/v) for the glycosylation of polyphenols, we observed in the HPLC chromatogram, when using tert-butyl alcohol, the appearance of several peaks with retention times lower than the corresponding to glucose (Figure 1). Such peaks were also present in the control reaction in absence of the acceptor, but not in the control experiments lacking enzyme, sugar donor, or tert-butyl alcohol. Thus, it seemed that CGTase was able to glycosylate the tertiary alcohol tert-butyl alcohol.

We screened four CGTases from different bacterial species (Table 1) for their transglycosylation efficiency towards tert-butyl alcohol at 60 °C. First, the transglycosylation activity of these enzymes was determined with a test based on the use of p-nitrophenyl-α-D-maltoheptaoside-4,6-O-ethylidene (EPS) as glucosyl donor and maltose as acceptor [12]. For commercial CGTases, the preparations
were partially purified by a PD-10 desalting column (GE Healthcare, Chicago, IL, USA) to eliminate low-molecular-weight contaminants that could interfere with our reaction. The transglycosylation experiments towards tert-butyl alcohol were carried out employing the same amount of EPS enzyme units.

![HPLC chromatogram](image)

**Figure 1.** HPLC chromatogram of the reaction mix after 24 h displaying the acceptor products of tert-butyl alcohol synthesized with CGTases from *Thermoanaerobacter* sp. and *Thermoanaerobacterium thermosulfurigenes* EM1. Reaction conditions: Soluble starch (30 g/L), 30% tert-butyl alcohol, 10 mM sodium citrate buffer (pH 5.5), CGTase (0.4 U/mL, EPS method), and 60 °C. (1) Main glucosylation product; (2) minor glucosylation product; and (3) glucose.

**Table 1.** Cyclodextrin glucanotransferases (CGTases) screened for the glucosylation of tert-butyl alcohol.

| Source Protein (mg/mL) | Source Protein (mg/mL) | Source Protein (mg/mL) |
|------------------------|------------------------|------------------------|
| *Thermoanaerobacter* sp. 2.88  | *Thermoanaerobacter* sp. 2.88  | *Thermoanaerobacter* sp. 2.88  |
| *Thermoanaerobacterium thermosulfurigenes* EM1 0.77  | *Thermoanaerobacterium thermosulfurigenes* EM1 0.77  | *Thermoanaerobacterium thermosulfurigenes* EM1 0.77  |
| Geobacillus sp. 2.63  | Geobacillus sp. 2.63  | Geobacillus sp. 2.63  |
| Bacillus circulans 251 2.79  | Bacillus circulans 251 2.79  | Bacillus circulans 251 2.79  |
| **Transglycosylation Activity (U/mL)** | **Transglycosylation Activity (U/mL)** | **Transglycosylation Activity (U/mL)** |
| 37.1 | 43.1 | 66.4 |
| 152.7 | 152.7 | 152.7 |

*Measured by the EPS method.*

The TLC screening showed that the only enzymes that led to a noticeable production of glucosylated products, under the tested conditions, were the CGTases from *Thermoanaerobacter* sp. (Toruzyme 3.0L) and *Thermoanaerobacterium thermosulfurigenes* EM1. No appreciable formation of the new acceptor derivatives was observed with the rest of the CGTases. Moreover, in the case of *Bacillus circulans* and *Geobacillus* sp. CGTases, tert-butyl alcohol seemed to produce an inhibitory effect on enzyme activity. Figure 1 shows the HPLC chromatograms obtained with the CGTases from *T. thermosulfurigenes* EM1 and *Thermoanaerobacter* sp. after 24 h of reaction.

Several carbohydrates (maltose, α-cyclodextrin, β-cyclodextrin, and partially hydrolyzed starch) were compared as sugar donors in the transglycosylation reaction towards tert-butyl alcohol with *Thermoanaerobacter* sp. CGTase. All the substrates tested yielded the expected transglycosylation products. However, partially hydrolyzed starch was selected as the best glucosyl donor for further experiments due to its availability and lower cost.

With the aim to clearly identify the nature of the new synthesized products, the two main peaks in the chromatograms of Figure 1 were isolated by semipreparative HPLC. Both products were purified at a high degree (>98%). The major product (1) was a white solid whereas the minor derivative
(2) showed an oily appearance. The molecular weight was established by MS-QTOF using ionization by electrospray (see Supplementary Materials, Figures S1 and S2). For compound 1, the main peak in the MS spectrum (positive mode) was at m/z 259.11 that corresponded to the M+[M+] ion of the tert-butyl glucoside. For compound 2, the major signal of the MS spectrum in positive mode was at m/z 421.17 that fits with the M+[M+] ion of the tert-butyl maltoside. The identity of the products was clearly established by 13C and 1H NMR analysis. NMR data confirmed the α-configuration of the glucosyl moieties. The structure of the synthesized compounds is represented in Figure 2. The major compound was tert-butyl-O-α-D-glucopyranoside and the minor product the corresponding α-D-maltoside. A particular feature of CGTase is the formation of several polyglycosylated products (the so-called homologous series) with different acceptors [8, 42].

tert-butyl-O-α-D-glucopyranoside. 1H-NMR (δ): 5.07 (d, 1H, H-1, J1,2 = 3.9 Hz); 3.78-3.66 (m, 3H, H-5 + H-6a + H-6b); 3.61 (t, 1H, H-3, J2,3 = 9.4, J3,4 = 9.2 Hz); 3.31 (dd, 1H, H-2); 3.28 (t, 1H, H-4, J4,5 = 9.3 Hz); 1.30 (s, 9H, t-butyl). 13C-NMR (δ): 94.6 (C-1); 75.2 (C-3); 73.5 (C-5); 73.1 (C-2); 72.0 (C-4); 62.7 (C-6); 28.9 (tert-butyl).

tert-butyl-O-α-D-maltoside. 1H-NMR (δ): 5.13 (d, 1H, H-1, J1,2 = 3.8 Hz); 3.93-3.65 (m, 6H, H-5 + H-6a + H-6b + H-5'a + H-6'b); 3.82 (dd, 1H, H-3, J2,3 = 9.6, J3,4 = 9.0 Hz); 3.62 (t, 1H, H-3', J2,3' = 9.9, J3,4' = 9.0 Hz); 3.50 (t, 1H, H-4, J4,5 = 9.8 Hz); 3.43 (dd, 1H, H-2': 3.37 (dd, 1H, H-2); 3.26 (t, 1H, H-4', J4,5 = 9.3 Hz); 1.27 (s, 9H, t-butyl). 13C-NMR (δ): 102.9 (C-1'); 94.5 (C-1); 81.9 (C-4); 75.1 + 75.0 (C-3' + C-5'); 74.7 (C-2'); 74.3 (C-3); 73.1 (C-2); 71.8 (C-5); 71.5 (C-4'); 62.8 (C-6); 62.1 (C-6'); 28.9 (tert-butyl).

Figure 2. Chemical structure of the synthesized glycosides: (1) tert-butyl-O-α-D-glucopyranoside and (2) tert-butyl-O-α-D-maltoside.

It is remarkable that CGTases can use a sterically hindered alcohol as acceptor. Alcohols have been frequently used as additives in the reaction media to improve or direct the synthesis of specific cyclodextrins by CGTases. However, to the extent of our knowledge, no other reports describe the synthesis of alkyl glucosides catalyzed by CGTases. Svensson et al. synthesized dodecyl-β-maltodextrin using CGTase from B. macerans by an indirect method based on the lengthening of dodecyl-β-maltoside using α-cyclodextrin as glucosyl donor [43]. In this context, CGTases are able to transglycosylate polyhydroxylated compounds such as trimethylolpropane or glycerol [44].

The synthesis of alkyl glucosides has been manly achieved using the reverse hydrolysis reaction catalyzed by glycosidases [45, 46]. Glycosidases show remarkable chemoselectivity for primary and secondary alcohols as well as phenols, but tertiary alcohols are not easily recognized as substrates. For the glycosylation of phenols, CGTase is one of the best options because its hydrolytic activity is very low. Since phenol is a good leaving group, glycosidases typically fast hydrolyze the synthesized phenyl glycosides, thus lowering the yield.

In general, secondary alcohols are glycosylated more slowly than primary ones by a factor of 3–5 [33]. Tertiary alcohols were considered unreactive in such enzymatic reactions until 1996, when Fischer et al. demonstrated that a tertiary alcohol (2-methyl-2-butanol) could act as nucleophile in a reaction catalyzed by the β-glucosidase from Pyrococcus furiosus, using cellobiose as glucosyl donor [47]. Later, Svasti et al. described the synthesis of tertiary β-glucosides catalyzed by a β-glucosidase from
were produced after 200 h of reaction. In the case of the maltoside derivative, a plateau after 60 h was reached yielding only 4 g/L. Considering that the initial concentration of soluble starch was 30 g/L, and that the sugar donor is the limiting reagent in the reaction, the yield of the tert-butyl glucoside was approximately 44%.

Regarding the formation of alkyl glycosides of tertiary alcohols with α-configuration, Simerska et al. were the first in reporting the enzymatic α-glycosylation of two sterically hindered alcohols, namely tert-butyl and tert-amyl alcohol; the enzyme was a α-galactosidase from Talaromyces flavus, with pNP α-galactoside as donor, yielding the corresponding α-D-galactopyranosides [50]. To our knowledge, our work is the first report of an enzymatic α-glucosylation of a tertiary alcohol. The chemical synthesis of tert-butyl α-glucosides is quite complex and usually gives rise to a mixture of anomers [51].

2.2. Progress of Tert-Butyl Alcohol Glucosylation by Thermoanaerobacter sp. CGTase

In order to dismiss that none of the contaminant enzymes in the Toruzyme preparation was responsible of tert-butyl alcohol glycosylation, the CGTase from Thermoanaerobacter sp. was purified to homogeneity by sequential chromatography steps on DEAE-Sepharose and α-cyclodextrin-activated Superose. We observed that the introduction of an ion-exchange chromatography step before the commonly used affinity step rendered a pure enzyme as confirmed by SDS-PAGE (Figure 3).

![Figure 3. Purification of CGTase from Thermoanaerobacter sp. Lane 1, molecular mass markers; lane 2, crude extract; lane 3, DEAE chromatography step; and lane 4, affinity chromatography step.](image)

We evaluated the production of tert-butyl-glucosides with the purified enzyme in a batch reactor employing 30% tert-butyl alcohol. Although Thermoanaerobacter sp. CGTase has an optimal temperature around 90 °C [52], the reaction was carried out at 60 °C to avoid starch browning and evaporation over time. Figure 4 depicts the progress of the reaction. Approximately 13 g/L of tert-butyl glucoside were produced after 200 h of reaction. In the case of the maltoside derivative, a plateau after 60 h was reached yielding only 4 g/L. Considering that the initial concentration of soluble starch was 30 g/L, and that the sugar donor is the limiting reagent in the reaction, the yield of the tert-butyl glycosides was approximately 44%.
Anomerically pure alkyl glucosides (AGs) are very useful in pharmaceutical and biomedical applications [53] due to their compatibility with biological systems that arise from the presence of the sugar head groups attached to the molecule. AGs do not denature enzymes and proteins, and in consequence are commonly used in protein extraction from cellular membranes [34]. AGs, unlike other sugar-based surfactants, are quite stable under alkaline conditions. In previous work, while studying several resveratrol glucosides, we demonstrated that the α-configuration gave rise to a superior surfactant performance when compared with β-configuration [9].

The surfactant properties of the monoglucosylated derivative were measured and compared with the reference compound octyl-α-D-glucoside (Figure 5). Our results suggest a similar surfactant behavior for both compounds. The synthesized tert-butyl glucoside behaves as a typical surfactant, defined by a linear descent of the surface tension vs. the logarithmic concentration of the surfactant, reaching a well-defined critical micellar concentration (CMC). CMC values of the tert-butyl and octyl α-derivatives were closely similar. It is worth noting that the relatively small tert-butyl group seems to be equivalent to the bulky octyl group for micelle formation.

Figure 4. Progress of tert-butyl alcohol glucosylation by CGTase from *Thermoanaerobacter* sp. Open circles, α-glucoside derivative; filled circles, α-maltoside derivative. Reaction conditions: Soluble starch (30 g/L), 30% tert-butyl alcohol, 10 mM citrate buffer (pH 5.5), 0.4 U/mL CGTase (transglycosylation activity, EPS method), and 60 °C.

Figure 5. Variation of surface tension vs. concentration for tert-butyl and octyl glucosides.
The main surfactant parameters of both compounds are summarized in Table 2. The surface tension at CMC is slightly higher for the tert-butyl glucoside with respect to the octyl derivative. As expected, the average area occupied per each molecule of adsorbed surfactant at the saturated water-air interface \((A)\) was higher for the tert-butyl glucoside compared with the octyl glucoside. The parameter \(pC20\), which is directly related to the efficiency of a surfactant, was only 10% higher for octyl-\(\alpha\)-glucoside compared with the tert-butyl derivative.

Table 2. Surfactant properties of tert-butyl-\(\alpha\)-\(\delta\)-glucoside and octyl-\(\alpha\)-\(\delta\)-glucoside.

| Property                  | Tert-butyl-\(\alpha\)-glucoside | Octyl-\(\alpha\)-glucoside |
|---------------------------|----------------------------------|-----------------------------|
| CMC (mM)                  | 4.0–4.5                          | 4.8–5.0                     |
| Surface tension at CMC (mN/m) | 43.9                            | 37.0                        |
| \(pC20\) \(^a\)            | 2.72                             | 3.00                        |
| \(A\) (\(\text{Å}^2\)) \(^b\) | 66.5                            | 45.1                        |

\(^a\) Minus logarithm of the concentration required to diminish the surface tension of water by 20 mN/m. \(^b\) Area occupied per molecule adsorbed at the saturated interface.

3. Materials and Methods

3.1. Enzymes and Reagents

CGTase from \(Thermoanaerobacter\) sp. (Toruzyme 3.0L) was kindly provided by Novozymes A/S (Bagsværd, Denmark). CGTase from \(Geobacillus\) sp. (CGT-SL) was from Amano Enzyme Inc. (Nagoya, Japan). Both CGTases were partially purified using a PD-10 desalting column (GE Healthcare). CGTases from \(T. \ thermosulfurigenes\) EM1 and \(B. \ circulans\) strain 251 were kindly provided in purified form by L. Dijkhuizen (Groningen University, The Netherlands). The \(\alpha\)-glucosidase (EC 3.2.1.20) from \(Saccharomyces \ cerevisiae\) was from Boehringer Mannheim. \(\beta\)-Cyclodextrin was purchased from Sigma. \(p\)-Nitrophenyl-\(\alpha\)-\(\delta\)-maltotetraoside-4,6-O-ethylidene (EPS) was from Boehringer Mannheim. Starch from potato Paselli SA2 (partially hydrolyzed with a mean degree of polymerization of 50) was kindly provided by Avebe (Foxhol, The Netherlands). Tert-butyl alcohol was from Fluka. All other reagents and solvents were of the highest purity grade available.

3.2. Activity Assay

The transglycosylation (disproportionation) activity of CGTase was measured on the basis of the method described by Nakamura et al. [55], adapted to 96-well plates. EPS (\(p\)-nitrophenyl-\(\alpha\)-\(\delta\)-maltotetraoside-4,6-O-ethylidene) was as the glucosyl donor and maltose the acceptor. In this assay EPS is first cleaved and the maltose molecule is then linked to the free reducing end. Thereafter, the \(p\)-nitrophenol is released from the reaction product by the action of \(\alpha\)-glucosidase. In particular, 20 \(\mu\)L of a maltose solution (50 mM) were mixed with 10 \(\mu\)L of an EPS solution (30 mM) and 65 \(\mu\)L of 0.2 M phosphate buffer (pH 7.0). The reaction, performed by triplicate, started when 5 \(\mu\)L of enzymatic solution was added. The 96-well plate was incubated at 60 °C for 20 min. The transglycosylation was stopped with 10 \(\mu\)L of 1 M HCl and the mixture was incubated at 60 °C for 10 min. Thereafter it was neutralized with 10 \(\mu\)L of 1 M NaOH. The \(\alpha\)-glucosidase (EC 3.2.1.20) from \(S. \ cerevisiae\) was then added (0.005 U) and the mixture was kept at 25 °C for 1 h. Finally, \(\text{Na}_2\text{CO}_3\) (1 M) was added to a final volume of 200 \(\mu\)L and the absorbance was measured at 401 nm. A calibration curve with \(p\)-nitrophenol was made from a stock solution of 0.74 mg/mL. Different volumes between 0–10 \(\mu\)L were taken by triplicate and conveniently diluted with 0.2 M phosphate buffer to a total volume of 100 \(\mu\)L. One unit of activity (U) was established as that corresponding to the release of 1 \(\mu\)mol of \(p\)-nitrophenol per min under these conditions.
3.3. Enzymatic Synthesis of Tert-Butyl Glucosides

Typical reaction mixtures contained 30% v/v tert-butyl alcohol, a 3% w/v sugar donor (Paselli SA2 starch, maltose, or cyclodextrins) and 0.4 U/mL CGTase (transglycosylation activity, chapter 3.2), in 10 mM sodium citrate buffer (pH 5.5). Reaction mixtures were incubated at 250 rpm in an orbital incubator at 60 °C. At regular time intervals, aliquots were removed and analyzed by TLC and HPLC. Controls without enzyme, tert-butyl alcohol, and/or sugar donor were also performed to rule out the presence of non-enzymatic products.

3.4. TLC and HPLC Analyses

Reaction samples were filtered on UltraFree centrifugal filters (0.45 µm, Millipore, Burlington, MA, USA) and analyzed in silica gel plates (10 × 5 cm, Merck) developed with ethyl acetate/methanol/water (77/15/8, v/v/v). Spots containing the residual carbohydrates and products were visualized after staining with a p-methoxybenzaldehyde solution (ethanol/sulphuric acid/p-methoxybenzaldehyde, 19/1/1, v/v/v) and heated at 90 °C for 15 min. The reaction progress was followed by normal-phase high-performance liquid chromatography (HPLC) employing a quaternary pump (Delta 600, Waters, Milford, MA, USA) and a Luna NH2 column (250 mm × 4.6 mm, 5 µm, Phenomenex). The starting mobile phase was acetonitrile:water 78:22 (v/v) and the gradient is outlined in Table 3. The solvents were conditioned with helium and the flow rate was 1.0 mL/min. The temperature of the column was kept constant at 30 °C. An evaporative light-scattering detector (model PL-ELS 1000, Polymer Laboratories, Salop, UK) was used and fixed to a nebulization and evaporation temperatures of 80 °C and 90 °C, respectively. The data obtained were analyzed using the Millennium Software, employing purified compounds as external standards for calibration.

| Time    | Acetonitrile | Water |
|---------|--------------|-------|
| 0–12 min| 78%          | 22%   |
| 12–15 min| 78% → 50%  | 22% → 50% |
| 15–20 min| 50%         | 50%   |
| 20–21 min| 50% → 78%  | 50% → 22% |
| 21–30 min| 78%         | 22%   |

3.5. Purification of Tert-Butyl Glucosides

The reaction mixture (100 mL) contained 30% v/v tert-butyl alcohol, 3% w/v Paselli SA2 (sugar donor), and 0.4 U/mL CGTase (transglycosylation activity) in 10 mM sodium citrate buffer (pH 5.5). The mixture was incubated at 250 rpm in an orbital shaker and 60 °C. After 200 h the reaction was stopped, and products were purified as follows. Reaction medium was filtered (0.45 µm, Millipore) and water and residual tert-butyl alcohol were evaporated by rotary evaporation at 60 °C. The dried products were then dissolved in a mixture of acetonitrile:water (88:12 v/v) and purified in a semipreparative HPLC pump (Delta 600, Waters) coupled to a Kromasil-NH2 column (250 mm × 10 mm, Analisis Vinicos, Spain) and to a refraction-index detector (model 9040, Varian, Palo Alto, CA, USA). Compounds were eluted in an isotropic regime using acetonitrile:water (88:12 v/v) as mobile phase at a flow rate of 8.0 mL/min. Fractions containing the desired products were collected and pooled. The mobile phase was evaporated by rotary evaporation at 60 °C and the dried products were stored at −20 °C. The purity of the isolated compounds was assessed by analytical HPLC. The equivalent isolated yields were approximately 1.1 g of tert-butyl-glucoside and 0.2 g of tert-butyl-maltoside.

3.6. Mass Spectrometry (MS)

The MS analysis of purified glucosides was carried out with a mass spectrometer equipped with hybrid QTOF analyzer (QSTAR, Pulsar i, AB Sciex, Madrid, Spain). Samples were analyzed by direct
infusion using electrospray ionization in positive reflector mode. Methanol containing 1% of NH$_4$OH was employed as ionizing phase.

3.7. Nuclear Magnetic Resonance (NMR)

NMR spectra were determined on a Varian Unit (1H-NMR, 500 MHz; 13C-NMR, 125 MHz) spectrometer, equipped with a gradient unit and a reverse probe. Samples (6–10 mg) were dissolved in 0.6 mL of semiheavy water (HDO and the spectra were obtained at 40 °C. Proton chemical shifts were referred to residual HDO (4.61 ppm). Carbon chemical shifts were referred to external acetone (31.07 ppm). 2D-homo- (DQCOSY, TOCSY (HOHAHA), NOESY) and hetero- (HMQC and HMBC) NMR experiments were carried out by using the standard software from Varian.

3.8. Enzyme Purification

*Thermoanaerobacter* sp. CGTase was purified from the commercial extract (Toruzyme 3.0L, Novozymes A/S, Bagsværd, Denmark) through successive steps of ion exchange and affinity chromatography on a FPLC LCC-500 equipment (Pharmacia, Sweden). The commercial extract was dialyzed against 20 mM Tris-HCl buffer (pH 7.5) and loaded onto a DEAE-Sepharose column equilibrated with the same buffer. Proteins were eluted using a linear salt gradient from 0 to 1 M of NaCl in the Tris-HCl buffer. The fractions containing CGTase were pooled, dialyzed against 10 mM acetate buffer (pH 5.5), and concentrated in an ultrafiltration cell (Amicon, Merck KGaA, Darmstadt, Germany) fitted with a 10 kDa membrane. The concentrate was then loaded onto a column of Sepharose activated with α-cyclodextrin and equilibrated with the acetate buffer. After washing, the proteins were eluted with the above buffer containing α-cyclodextrin (10 mg/mL). CGTase-enriched fractions were merged, concentrated as before and stored at −20 °C until its use. Purity was assessed by Coomasie-Blue stained SDS-PAGE electrophoresis.

3.9. Critical Micellar Concentration

The measurement of the surface tension in H$_2$O was carried out at 20 °C using different aqueous solutions of alkyl glycosides at various concentrations, according to the Wilhelmy plate method [56] with a tensiometer (Processor tensiometer K-12, Krüss, Hamburg, Germany), which measures the real tension values at the equilibrium. Critical micelle concentrations (CMCs) were calculated graphically from the sharp change in the slope of the surface tension values vs. the logarithm of surfactant concentration, expressed in mM.

The average area ($A$) occupied per molecule of surfactant adsorbed in the saturated water-air interface was estimated from the equation

$$A = \frac{10^{16}}{N_A} \times \Gamma_m$$

in which $N_A$ is the Avogadro’s number, $\Gamma_m$ is the maximum concentration of surfactant molecules adsorbed in the saturated interface (moles/cm$^2$), and the resulting $A$ is expressed in squared Angstroms. The value of $\Gamma_m$ can be calculated by applying the Gibbs equation:

$$\Gamma_m = -\frac{(d\gamma/d\log c)}{2.3030 \cdot n \cdot R \cdot T}$$

where $(d\gamma/d \log c)$ is the maximum slope of the linear plot representing surface tension vs. logarithm of surfactant concentration that appears immediately below the CMC, $R = 8.31$ J·mol$^{-1}$·K$^{-1}$, and $T$ is the temperature in K. The value of $n$ (the number of species into which the surfactant dissociates) is taken as one for nonionic surfactants.
4. Conclusions

The present work describes, for the first time, the enzymatic synthesis of α-glucosides of a tertiary alcohol (tert-butyl alcohol). The process is catalyzed by the enzyme CGTase, in particular from the strains *Thermoanaerobacter* sp. and *T. thermosulfurigenes*. The biotransformation is carried out under mild conditions, using starch as glucose donor, and gives rise to a significant yield (44%) of α-glucoside (major product) and α-maltoside (minor product). The synthesized tert-butyl-O-α-D-glucopyranoside exhibited the typical surfactant behavior and its properties were comparable to those of the related α-octyl derivative.

**Supplementary Materials:** The following are available online at [http://www.mdpi.com/2073-4344/9/7/575/s1](http://www.mdpi.com/2073-4344/9/7/575/s1), Figure S1: ESI-TOF spectrum (positive mode) of tert-butyl-O-α-D-glucopyranoside, Figure S2: ESI-TOF spectrum (positive mode) of tert-butyl-O-α-D-maltoside.

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