Research Article

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# The thermostable 4,6-α-glucanotransferase of *Bacillus coagulans* DSM 1 synthesizes isomalto-oligosaccharides

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**Abstract:** The 4,6-α-glucanotransferases of the glycoside hydrolase family 70 can convert starch into isomalto-oligosaccharides (IMOs). However, no thermostable 4,6-α-glucanotransferases have been reported to date, limiting their applicability in the starch conversion industry. Here we report the identification and characterization of a thermostable 4,6-α-glucanotransferase from *Bacillus coagulans* DSM 1. The gene was cloned and the recombinant protein, called BcGtfC, was produced in *Escherichia coli*. BcGtfC is stable up to 66 °C in the presence of substrate. It converts debranched starch into an IMO product with a high percentage of α-1,6-glycosidic linkages and a relatively high molecular weight compared to commercially available IMOs. Importantly, the product is only partly and very slowly digested by rat intestine powder, suggesting that the IMO will provide a low glycaemic response *in vivo* when applied as food ingredient. Thus, BcGtfC is a thermostable 4,6-α-glucanotransferase suitable for the industrial production of slowly digestible IMOs from starch.

**Keywords:** food ingredient; family GH70; glucansucrase; slowly digestible; starch.

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## Abbreviations

BcGtfC, *Bacillus coagulans* DSM14,6-α-glucanotransferase; GH, glycoside hydrolase; IMO, isomalto-oligosaccharide; TLC, thin-layer chromatography.

## 1 Introduction

Our diet provides the energy and the nutrients our body requires. A large part of the energy that fuels our body comes from the carbohydrates in our food, mostly starch and sugars. Although it is well known that too much sugar is unhealthy, also starch can be digested rapidly. Starch breakdown starts in the mouth by salivary α-amylase, followed by the action of pancreatic α-amylase and the brush border glucosidases within the small intestine resulting in the same undesirable high blood glucose spikes [1]. A recent study based on food frequency questionnaires following 137,000 adults from 5 continents for nearly 10 years revealed that a diet with a high glycemic index increased the risk of cardiovascular disease and death [2].

Designing starchy foods with slow digestion properties is thus an important target of today's food scientist, as recently discussed in an opinion paper [3]. Potential approaches include optimizing the food matrix in such a way that the starch becomes less accessible to digestive enzymes and the use of resistant starch. The key challenge of applying resistant starch, and other dietary fibers as well, is that they still should provide the desired texturizing functionality and pleasant sensory characteristics in the final food product.

An alternative strategy to slow down the digestion rate of starches is to modify their structure via either chemical or enzymatic modification. About thirty distinct types of starch-acting enzymes are known to date, most of them being hydrolytic enzymes [4,5]. However, transferases acting on starch, such as branching enzyme and cyclodextrin glucanotransferase can be regarded as

more promising for producing slowly digestible starch due to their ability to change the molecular structure without losing yield due to hydrolysis [6-8].

Still another approach to slow down the digestion of starches is to increase the percentage of α-1,6-glycosidic linkages, because they cannot be hydrolyzed by α-amylases. This is either done by increasing the branch density using branching enzymes or trimming the external side chains of amylopectin and as such raising the fraction of α-1,6-linkages [9,10]. Far higher fractions of α-1,6-glycosidic linkages, usually around 50%, are reached by converting starch into isomalto-oligosaccharides (IMOs) [11].

Commercially, IMOs are produced by saccharification of starch to maltose syrup, which is then converted into IMOs by transglucosidase [12,13], and are a collection of short oligosaccharides, mostly di-, tri- and tetrasaccharides [14]. The size and percentage of α-1,6-glycosidic bonds of IMOs can be increased by the action of dextransucrase, which transfers the glucose moiety of sucrose onto acceptor oligosaccharides including IMOs [15]. A further step towards a higher fraction of α-1,6-glycosidic linkages is the preparation of dextran oligosaccharides by the hydrolysis of dextran [16].

The direct conversion of starch into isomalto/malto-polysaccharides has also been reported using a 4,6-α-glucanotransferase GtfB from *Lactobacillus reuteri* 121 GtfB [17]. This enzyme, belonging to the glycoside hydrolase family 70 [18], was the first GH70 enzyme that used α-1,4-malto-oligosaccharides, but not sucrose, as glucose donor substrate in disproportionation reactions [19,20]. Subsequent research revealed the existence of more α-1,4-malto-oligosaccharide converting type of GH70 enzymes, not only forming linear IMOs with consecutive α-1,6-glycosidic linkages [21,22], but also branched α-glucans with alternating α-1,4/α-1,6 [23] or alternating α-1,3/α-1,4 linkages [24]. These polymers are synthesized by transferring the non-reducing end glucose moiety of linear α-1,4-glucans onto acceptor α-glucans [18], except for the GtfD type of 4,6-α-glucanotransferases, which transfer maltose units [23]. Most of these enzymes use the non-reducing end of other α-glucan chains as acceptor forming linear α-glucans with α-1,6 linkages, though some are also capable of creating branches. Nevertheless, the "common" GH70 glucansucrases are still forming a wider variety of structurally distinct α-glucans from sucrose, ranging from linear to highly branched polymers, with either α-1,2, α-1,3, α-1,4 and α-1,6 bonds, and combinations thereof [25-29]. The enormous variation in molecular structures results in α-glucan products having distinct functional properties in various applications [30,31].

Nearly all GH70 enzymes originate from lactic acid bacteria and have a limited thermal stability. Here we report the first thermostable 4,6-α-glucanotransferase, *Bacillus coagulans* DSM 1 (BcGtfC), capable of synthesizing IMOs up to 66 °C.

# 2 Materials and methods

## 2.1 Substrates and chemicals

Malto-oligosaccharides and *Chaetomium erraticum* dextranase were obtained from Sigma-Aldrich. Dextran 10 having a molecular weight of 9,000–11,000 Da was obtained from CarboSynth (Compton, UK). The isomalto-oligosaccharide Vitafiber was obtained from the web shop MyProtein (https://nl.myprotein.com/). Maltodextrin Eliane MD2 having a dextrose equivalent of 2.6 g/100 g is produced by Avebe (Veendam). Debranched waxy potato starch was obtained via enzymatic debranching using pullulanase (Promozyme D6; Novozymes). Briefly, 5% waxy potato starch (Avebe, Veendam, The Netherlands) was solubilized in 50 mM sodium acetate pH 4.7 at 121 °C for 30 min, and debranched overnight at 55 °C with 1% (w/w) enzyme. The analytical enzymes, α-amylase from *Aspergillus oryzae* (E-ANAAM), β-amylase from barley (E-BARBL), isoamylase from *Pseudomonas* sp. (E-ISAMY), pullulanase from *Klebiella* (E-PULKP) and mirobial oligo-α-1-6-glucosidase (E-OAGUM), were obtained from Megazyme (Wicklow, Ireland).

## 2.2 Cloning of the *BcGtfC* gene

The gene encoding the putative 4,6-α-glucanotransferase from *Bacillus coagulans* DSM 1 (GenBank accession No. AJH79253.1) was synthesized (GenScript, NJ, USA) and cloned into the *Nde*I-*Bam*HI restriction sites of the *Escherichia coli* expression vector pET28. As such, the recombinant protein (BcGtfC) carries an N-terminal His6-tag to simplify purification. The construct lacks the N-terminal 32 amino acid signal sequence, as predicted using the Signal P4.1 server ([32], http://www.cbs.dtu.dk/services/SignalP/).

## 2.3 BcGtfC protein expression

BcGtfC was expressed in *E. coli* BL21Star(DE3) cultivated in auto-induction medium (VWR) supplemented with

25 mg/L kanamycin at 37 °C. When the $OD_{600}$ reached about 1.2, the cultivation was continued at 15 °C for 16 h. Cells were harvested by centrifugation. The cells were resuspended in 50 mM Tris/HCl, 500 mM NaCl, 10% glycerol and 1 mM $CaCl_2$ of pH 8.0 and lysed by sonication. BcGtfC was purified by $Ni^{2+}$ affinity chromatography, and subsequently dialyzed against the above buffer. Protein concentration was determined by the Bradford protein assay with BSA as a standard, and the purity and molecular weight were confirmed by SDS-PAGE.

## 2.4 Enzyme assays

Reactions were performed in 50 mM sodium acetate buffer pH 4.7 supplemented with 1 mM $CaCl_2$ unless stated otherwise. The optimal pH and temperature were determined over the pH range of 3.5–7 and the temperature range of 37–75 °C, respectively, using 10 mM maltoheptaose as substrate, and by quantifying the release of glucose in time using a glucose detection kit (GOPOP; Megazyme). Reactions were started by adding the enzyme to preheated reaction mixture, and stopped by heating the reaction mixture in boiling water.

Malto-oligosaccharide substrate preference was determined by incubating the enzyme with 34.2 mg/mL malto-oligosaccharides of different lengths. Reaction progress was followed by thin-layer chromatography (TLC) using silica gel 60 plates (Merck) run with butanol/ethanol/water (5:3:3; v/v/v). TLC plates were developed with 10% $H_2SO_4$ in ethanol/water (1:1; v/v), followed by heating at 100 °C.

The thermal inactivation rate of BcGtfC was assessed by incubating the enzyme at a concentration of 0.05 mg/mL in 50 mM sodium acetate buffer pH 5.0 in the presence of 1 mM $CaCl_2$ at temperatures ranging from 20 to 73 °C for 20 min. Samples were then cooled on ice, and the residual activity was quantified by following the release of glucose using maltoheptaose as substrate, as described above. As a measure of thermostability the activity half-life of the enzyme was defined as the temperature, at which half of the initial activity was lost during a 20 min incubation.

The thermostability of BcGtfC in the presence of a high substrate concentration was measured by incubating the enzyme with maltoheptaose substrate at a temperature range of 37 to 72 °C. The maltoheptaose concentration was 115 mg/mL (100 mM), and the enzyme concentration 0.1 mg/mL. Reactions were started by adding the enzyme to a preheated substrate solution. The progress of substrate conversion was followed for 16 h and assessed by TLC.

## 2.5 NMR spectroscopy

The percentage of α-1,6-glycosidic linkages in the product made by BcGtfC was measured by $^1$H NMR spectroscopy. Freeze-dried product was dissolved in $D_2O$ (99.9 atom% D, Sigma-Aldrich), lyophilized, and dissolved again in $D_2O$ at a concentration of 5 mg/mL. The NMR spectra were recorded at 340 K on a Bruker (Billerica, MA, USA) Avance 500 spectrometer at the Wageningen University NMR Centre. The percentage of α-1,6 bonds was calculated by dividing the peak surface area of the α-1,6 signal by the combined areas of the α-1,4 and α-1,6 signals, as described previously [33]. The percentage of reducing ends was calculated by dividing the peak surface area of the reducing end signals by the combined areas of all anomeric signals.

## 2.6 Molecular weight analysis

The molecular weight distribution was measured with a **gel permeation chromatography** system (Thermo Scientific; Waltham, USA), equipped with a refractive index detector (Optilab T-rEx; Wyatt; Santa Barbara, USA) and a light scattering detector (Dawn Heleos II, Wyatt). Samples were dissolved at a concentration of 20 mg/mL in Milli-Q water and filtered through a 0.45 µm cellulose acetate filter. Samples (100 µL) were injected at a flow rate of 0.2 mL/min, and run through a guard-column (OHpak-SB-G, Shodex; New York, USA) and a SEC column (OHpakSB-803 HQ, Shodex) at a flow rate of 0.5 mL/min. The columns and RI detector were held at 25 ºC. Data processing was done with the ASTRA software (Wyatt), using a specific refractive index increment value dn/dc of 0.148 mL/g.

## 2.7 Product analysis with enzymatic hydrolysis

The product made by BcGtfC (20 mg/mL) was incubated with various hydrolytic enzymes in excess. Reactions with isoamylase from *Pseudomonas*, microbial oligo-α-1-6-glucosidase (hydrolyzing the α-1,6-glycosidic linkage of the terminal non-reducing glucose moiety [34]) and dextranase from *Chaetomium erraticum* were performed in 50 mM sodium acetate buffer pH 4.7 at 50 °C for 40 h. Incubations with barley β-amylase were performed at pH 6.5 and 50 °C. Treatment with *Aspergillus oryzea* α-amylase and *Klebiella pullulanase* M1 were done at pH 4.7 and 40 °C. The degree of hydrolysis was assessed by TLC.

### 2.8 *In vitro* digestion analysis

To model digestion in the human small intestine, the product made by BcGtfC was incubated with rat intestinal acetone powder (Sigma-Aldrich). The *in vitro* digestion using rat intestinal powder, instead of the microbial amyloglucosidase, has been shown to much better predict the *in vivo* digestion, because the α-glycosidic linkage specificity of amyloglucosidase is much more restricted than the α-glycosidic linkage specificity of the brush border glucosidases of the small intestine, which are present in rat intestinal powder [35,36]. Briefly, 400 mg rat intestinal acetone powder was incubated with 10 mL ice-cold 0.1 M sodium phosphate buffer of pH 6.9 in a sonification bath with ice water for 15 min. Following centrifugation (13,000 × *g*; 5 min), 500 µL of the supernatant was directly transferred to 500 µL of a 5 mg/mL carbohydrate substrate solution. The digestion was performed at 37 °C. Progress of the digestion was followed in time by taking 100 µL samples, which were placed in a boiling water bath for 10 min to inactivate the digestive enzymes. The amount of glucose formed was subsequently quantified using the glucose detection kit GOPOD (Megazyme).

## 3 Results and discussion

### 3.1 The 4,6-α-glucanotransferase of *Bacillus coagulans* DSM 1

The GH70 family enzymes are well-known for their capacity to synthesize a wide variety of high molecular weight α-glucan polymers from sucrose. In the last decade, it was reported that this family also contains 4,6-α-glucanotransferases, which convert linear α-1,4-glucan chains into linear isomalto/malto-oligosaccharides [19], and branched polymers with alternating α-1,4/α-1,6 [23] or alternating α-1,3/α-1,4 linkages [24]. These enzymes have, however, a limited thermostability, not exceeding 50 °C, which hampers their application in the starch industry. Thus, either the existing enzymes should be engineered towards higher thermal stability, or more thermostable enzymes have to be identified. The majority of the (putative) GH70 family sequences are found in lactic acid bacteria, though some of the sequences are found in other bacteria [23], including in a few *Bacillus coagulans* strains. Because *B. coagulans* strains grow optimally at around 50 °C, it is expected that its enzymes have at least some thermostability.

The putative GH70 sequence of *B. coagulans* DSM 1 (GenBank accession no. AJH79253.1), of which the genome sequence was reported in 2015 [37], encodes for a protein of 965 amino acids, designated BcGtfC. The 32 N-terminal amino acids are predicted to be a signal peptide according to Signal P4.1 [32]. A BLASTp search (https://blast.ncbi.nlm.nih.gov/) with the BcGtfC sequence revealed about 100 putative sequences with a high similarity, with the GtfC homologue of *Exiguobacterium sibiricum* 255-15 being the highest-ranking characterized enzyme at position 29 (BLAST search on March 23, 2021). The *E. sibiricum* GtfC shares 54% sequence identity with a coverage of 83%. The *E. sibiricum* GtfC displays 4,6-α-glucanotransferase activity with malto-oligosaccharide substrates but not with sucrose [22], similar to the *L. reuteri* GtfB. However, the *E. sibiricum* GtfC forms short isomalto/malto-oligosaccharides, whereas the *L. reuteri* GtfB synthesizes longer isomalto/malto-polysaccharides.

### 3.2 Purification and basic properties of BcGtfC

The full-length BcGtfC without its signal sequence was successfully expressed in *E. coli* BL21Star(DE3) as soluble protein. The protein was purified using its N-terminal His-tag, yielding 11 mg of purified protein from 1 L of *E. coli* culture.

BcGtfC showed no activity with sucrose (data not shown). Instead, it displayed clear disproportionation activity with linear malto-oligosaccharides, forming both shorter and longer oligosaccharides (Fig. 1). The appearance of glucose, in addition, showed that the enzyme had a significant hydrolytic side activity. The enzyme had the highest activity at around pH 4.5 (Fig. 2A), basically identical to the pH optimum of *L. reuteri* GtfB, but much lower than the pH 6.0 optimum of *E. sibiricum* GtfC [22]. BcGtfC displayed maximal activity at 60 °C (Fig. 2B), far higher than the 37 °C of *L. reuteri* GtfB [19] and the 45 °C of *E. sibiricum* GtfC [22].

Maltotetraose is the shortest oligosaccharide used as donor substrate by BcGtfC, though the enzyme was clearly more efficient with maltopentaose and longer substrates (Fig. 1). The donor substrate requirements differ somewhat among the 4,6-α-glucanotransferases, with the GtfW of *Lactobacillus reuteri* DSM 20016 already efficiently utilizing maltose as glucose donor [21], while *L. reuteri* GtfB is highly active with maltotetrose and longer oligosaccharides [38]. The TLC reveals that BcGtfC synthesized only small amounts of longer oligosaccharides (Fig. 1), while equivalent reactions of *L. reuteri* GtfB show

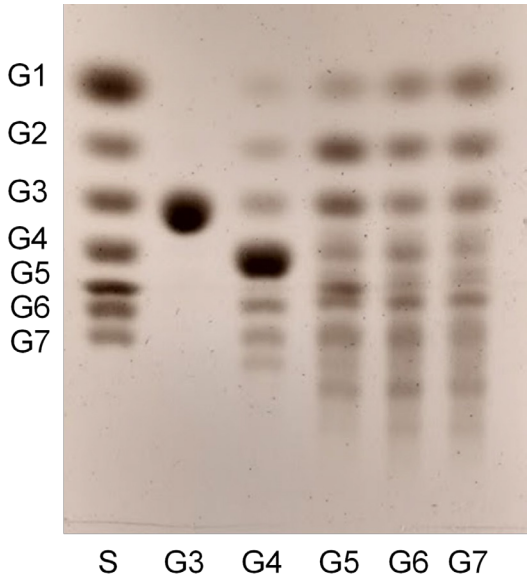


**Figure 1:** TLC analysis of the products synthesized by BcGtfC from linear α-1,4-oligosaccharides (G3-G7). Reactions were performed at 58 °C in 50 mM sodium acetate buffer pH 4.7 and 1 mM calcium chloride. Lane S – standards: G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose.

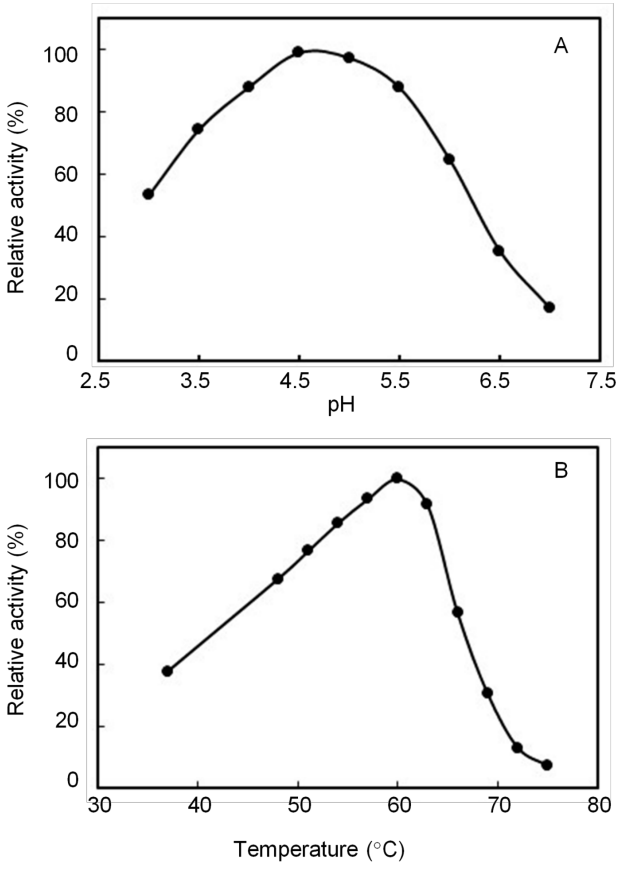


**Figure 2:** The pH (A) and temperature (B) activity profiles of BcGtfC.

intense spots at the origin of TLC plates [19], indicating the synthesis of significant amounts of polymeric material, which was not observed with BcGtfC.

## 3.3 Temperature stability

The thermal stability of BcGtfC was determined by incubating the enzyme at a concentration of 0.05 mg/mL in 50 mM sodium acetate pH 5.0 supplemented with 1 mM $CaCl_2$ at various temperatures for 20 min. The remaining activity was subsequently quantified using maltoheptaose as substrate, revealing an activity half-life of 20 min at 59 °C (Fig. 3A). Thus, BcGtfC had a much higher activity half-life than that of GtfB, which loses half of its activity in only 3.5 min at 45 °C [39].

## 3.4 Thermostability in presence of substrate

From an application viewpoint it is, however, more important how stable the enzyme is under process conditions, *e.g.*, at high substrate concentration and full reaction time. To assess the performance of BcGtfC under "process conditions", 0.1 g/L enzyme was added to a relative high substrate concentration of 115 g/L at various temperatures. The conversion of the substrate maltoheptaose was followed in time, revealing that BcGtfC was most active at 57–66 °C, and fully converted maltoheptaose at temperatures of up to 66 °C (Fig. 3B). Thus, the presence of substrate provided a significant protection against thermal inactivation, because without substrate the enzyme had an activity half-life of only 20 min at 59 °C.

## 3.5 IMO synthesis from starch

Subsequently, debranched waxy potato starch, consisting of linear α-1,4-glucan chains with an average degree of polymerization of 26, in the range of 6 to about 60 glucose moieties [40], was explored as substrate. BcGtfC effectively converted debranched waxy potato starch, as revealed by a complete disappearance of iodine staining upon incubation with the enzyme (data not shown). When acting on maltodextrins, partly α-amylase-hydrolysed starches which still contain branches, iodine staining only decreased to about 40% of the initial value (data not shown), indicating that BcGtfC is an exo-acting enzyme that cannot bypass branches.

TLC analysis of the product derived from debranched waxy potato starch showed that the product contained far more shorter oligosaccharides than the starting material (Fig. 4A). The product had a wide molecular weight distribution with 20% of the product below 0.5 kDa and 20% above 2.0 kDa, and an average molecular weight of 1.2 kDa (Table 1), which is clearly higher than the 0.5 to 0.8 kDa of commercially available IMOs [14]. NMR analysis demonstrated that BcGtfC catalyzed the formation of α-1,6-glycosidic linkages, counting for 68% of the glycosidic linkages, the remaining 32% being α-1,4 bonds (Fig. 4B). This percentage is higher than the 44 and 52% reached by the *E. sibiricum* GtfC acting on maltoheptaose or amylose, respectively, but distinctively lower than the 83% of *L. reuteri* GtfB when converting debranched waxy potato starch [17]. Thus, BcGtfC converted debranched starch into relative long chain IMOs.

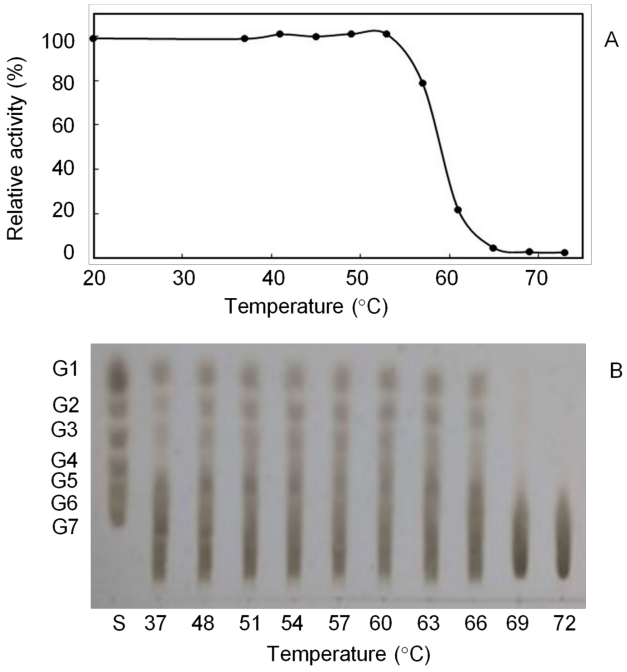


**Figure 3:** Temperature stability of BcGtfC. (A) Activity half-life of BcGtfC challenged at various temperatures for 20 min at a concentration of 50 µg/mL in 50 mM sodium acetate buffer pH 5.0 and 1 mM calcium chloride. (B) Thermostability at a high substrate concentration during the conversion of 115 mg/mL maltoheptaose at various temperatures with 0.1 mg/mL BcGtfC in 50 mM sodium acetate buffer pH 4.7 and 1 mM calcium chloride. Reaction progress was followed by TLC. Lane S – standards: G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose.

**Table 1:** Product properties of the IMO synthesized by BcGtfC from debranched waxy potato starch.

| Parameter | Value |
|---|---|
| α-1,6-Glycosidic linkage | 68% [a] |
| α-1,4-Glycosidic linkage | 32% [a] |
| Reducing ends | 21% [a] |
| Glucose content | 11% [b] |
| Molecular weight | 1.2 kDa [c] |

[a] Determined by $^1$H NMR spectrometry.
[b] Quantified by the glucose detection kit GOPOD.
[c] Determined by **gel permeation chromatography.**

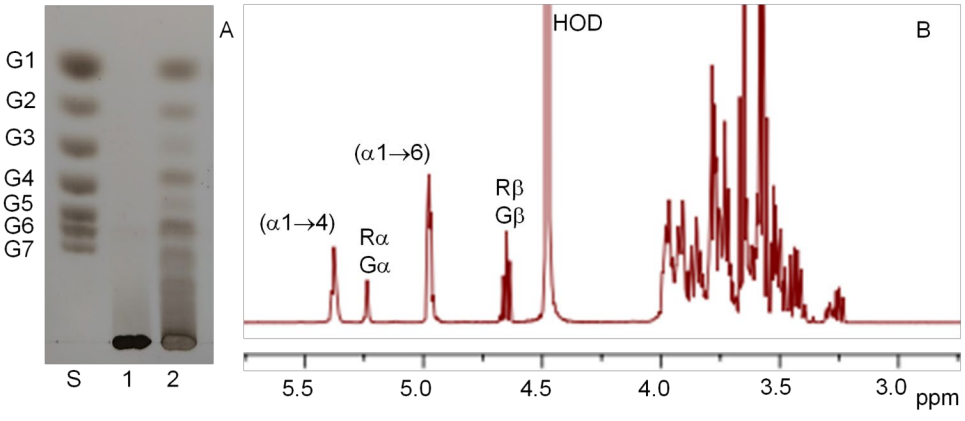


**Figure 4:** The product derived from debranched waxy potato starch by BcGtfC. (A) TLC analysis and (B) the 1H-NMR spectrum recorded at 340 K in $D_2O$. The chemical shift for the α-1,4-glycosidic linkage at ~5.4 ppm and the α-1,6-glycosidic linkage at ~5.0 ppm relative to the internal standard acetone was set to 2.225 ppm. The anomeric signals of free glucose and the reducing ends of glucan chains are indicated with Gα/β and Rα/β, respectively. Lane S – standards: G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose; 1, substrate; 2, product formed by BcGtfC.

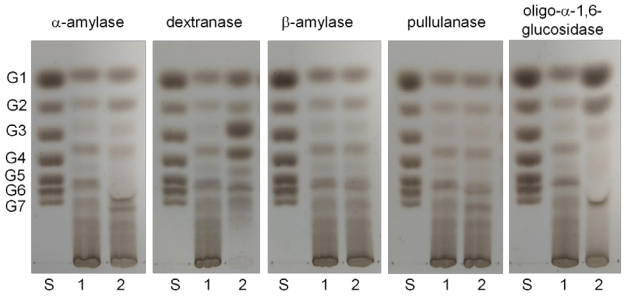


**Figure 5:** Enzymatic hydrolysis of the IMO derived from debranched waxy potato starch by the action of BcGtfC. Degradation was assessed by TLC analysis. Lane S – standards: G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose; 1, product formed by BcGtfC; 2, product after incubation with the indicated hydrolytic enzyme.

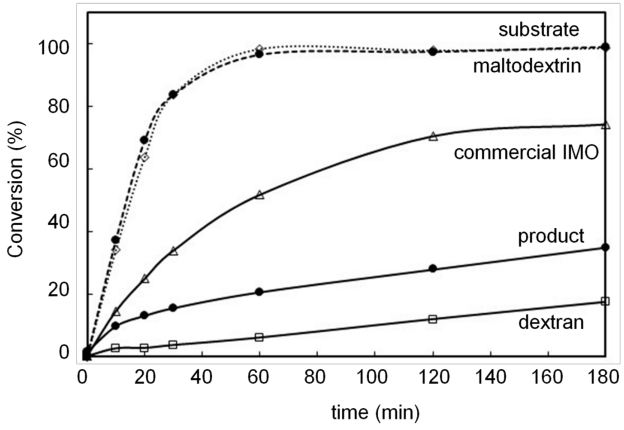


**Figure 6:** Digestion of the BcGtfC product with rat intestinal powder. The digestion was followed in time by quantifying the amount of glucose formed, showing the average of duplicate experiments. The controls included are the debranched waxy potato starch used as substrate, potato maltodextrin (Eliane MD2, Avebe), the commercially available IMO VitaFiber and dextran.

## 3.6 Structural analysis of the BcGtfC product with hydrolytic enzymes

The structural properties of the IMO product synthesized by BcGtfC, with 68 and 32% of α-1,6- and α-1,4-glycosidic bonds, respectively, were further analysed using hydrolytic enzymes with distinct reaction specificities. The product was largely resistant to the endo α-1,4 hydrolyzing activity of α-amylase demonstrating that the product contained only a limited amount of linear α-1,4 chain segments (Fig. 5). In contrast, the product was extensively digested by the endo α-1,6 hydrolyzing activity of dextranase (Fig. 5), proving the presence of consecutive α-1,6-glycosidic linkages within the product. Moreover, the extensive digestion by dextranase implies that BcGtfC synthesizes linear IMOs, because branches would hamper extensive hydrolysis by dextranase. The product did not contain α-1,4 segments at its non-reducing ends because the exo-acting α-1,4 hydrolyzing β-amylase was not acting on the IMO made by BcGtfC (Fig. 5). The product was also virtually resistant to pullulanase M1 (Fig. 5), which hydrolyses the α-1,6 linkages in pullulan, reuteran and α-1,4,6 branches [23], indicating that the product did not contain alternating α-1,4/α-1,6 linkages nor α-1,4,6 branches. The IMO generated by BcGtfC was also significantly digested by the exo α-1,6 hydrolyzing activity of oligo-α-1,6-glucosidase (Fig. 5), revealing the presence of α-1,6-glycosidic linkages at the non-reducing end. The digestion was, however, not as extensive as with the dextranase, and some larger material remained after the oligo-α-1,6-glucosidase treatment. Because such larger material was not seen following the α-amylase treatment, this cannot be long α-1,4 glucan chains, *e.g.*, remaining substrate with or without α-1,6 linked glucose moieties at its non-reducing end. It therefore indicates that segments of consecutive α-1,6 bonds were occasionally interspersed with an α-1,4-glycosidic linkage. Indeed, it has been reported that the products generated by the homologue *L. reuteri* GtfB possess an occasional α-1,4 bond within segments of consecutive α-1,6-glycosidic linkage [38].

Collectively the analysis with the hydrolytic enzymes demonstrated that the main activity of BcGtfC is the transfer of glucose moieties from linear α-1,4-glucans onto the non-reducing end of another glucan chain via an α-1,6-glycosidic linkage creating linear IMOs. In addition, the enzyme has a low hydrolytic activity and most likely a low α-1,4 transferase activity. The fact that the IMO products contain a high percentage of 68% α-1,6-glycosidic linkages indicates that the enzyme preferentially elongates chains already possessing an α-1,6 linked terminal glucose residue. This acceptor substrate preference has also been reported GtfB of *L. reuteri* [41].

## 3.7 *In vitro* digestibility

The product generated from debranched waxy potato starch by BcGtfC was incubated with rat intestinal powder to simulate the digestive system of the human small intestine, and to assess if the product is a non-digestible or slowly digestible carbohydrate. We choose rat intestinal powder instead of the combination of pancreatic α-amylase and microbial amyloglucosidase, because the digestive power of rat intestinal powder better resembles the *in vivo* digestion [35,42,43]. Indeed, some α-glycosidic

carbohydrates are not digested by pancreatic α-amylase and microbial amyloglucosidase, while they are known to provide a glycemic response in humans [1,36]. This issue is resolved by using rat intestinal powder instead of amyloglucosidase, because of the wider substrate specificity of the brush border glucosidases [44,45].

The digestion data show that the IMO made by BcGtfC was digested slowly, with only 35% digestion within 3 h, while the substrate (debranched waxy potato starch) and a maltodextrin control were fully digested within 1 h (Fig. 6). Applying the definition of rapidly digestible starch (within 20 min), slowly digestible starch (between 20-120 min) and resistant starch (undigested in 120 min) [46], the product consisted of 13% rapidly, 17% slowly and 70% resistant starch. The digestion rate of dextran with a molecular weight of ~10 kDa was about half, reaching 18% in 3 h. Importantly, the BcGtfC product was digested much slower than the commercial IMO VitaFiber (Fig. 6), suggesting that the IMO product made by BcGtfC will provide a lower glycemic response *in vivo*.

## 4 Conclusions

BcGtfC synthesizes IMOs with a high content of α-1,6-glycosidic linkages from linear α-1,4-glucan chains at high temperatures of up to 66 °C. Conversion of debranched starch yielded an IMO product with a relative high molecular weight of about 1.2 kDa compared to commercially available IMOs. Moreover, the product is only very slowly digested by rat intestine powder, suggesting that the product will have low glycemic response properties *in vivo*. Together these data indicate that the thermostable BcGtfC 4,6-α-glucanotransferase is a suitable candidate for the industrial production of slowly digestible IMOs from starch.

**Conflict of interest:** The authors declare no conflict of interest.

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