A glucotolerant β -glucosidase from the fungus *Talaromyces amestolkiae* and its conversion into a glycosynthase for phenolic compounds glycosylation.

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Abstract

Background: The interest for finding novel β -glucosidases that can improve the yields for the production of second-generation (2G) biofuels is still very high. One of the most desired features for these enzymes is the glucose tolerance, which allows them to act optimally under elevated glucose concentrations. Besides, there is an additional focus of attention on finding novel enzymatic alternatives for glycoside synthesis, for which a mutated version of glycosidases, named glycosynthases, have gained much interest in recent years.

Results: In this work, a glucotolerant β -glucosidase (BGL-1) from the ascomycete fungus *Talaromyces amestolkiae* has been heterologously expressed in *Pichia pastoris*, purified, and characterized. The production of the enzyme in the yeast was very high, reaching 75 U/mL, and it allowed purification in just one step with a yield of 80%. Although the enzyme showed good efficiency on p NPG ($K_m = 3.36 \pm 0.7$ mM, $k_{cat} = 898.31 \text{ s}^{-1}$), the activity detected on celooligosaccharides, the natural substrates of the enzyme, was much lower, which could limit its exploitation in lignocellulose degradation processes. Interestingly, when examining the substrate specificity of BGL-1, it showed to be more active on sophorose, the β -1,2 disaccharide of glucose, than on cellobiose. Besides, the transglycosylation profile of BGL-1 was examined, and, for expanding its synthetic capacities, it was converted into a glycosynthase. The mutant enzyme, named BGL-1-E521G, was able to use α -D-glucosyl-fluoride as donor in glycosylation reactions, and synthesized glucosylated derivatives of different p NP-sugars in a regioselective manner, as well as of some phenolic compounds of industrial interest, such as epigallocatechin gallate (EGCG).

Conclusions: In this work, we report the characterization of a novel glucotolerant $1,2\text{-}\beta$ -glucosidase, which also has a considerable activity on $1,4\text{-}\beta$ -glucosyl bonds, that has been cloned in *P. pastoris*, produced, purified and characterized. In addition, the enzyme was converted into an efficient glycosynthase, which can transfer glucose molecules to glucose to a diversity of acceptors for obtaining compounds of interest. The remarkable capacities of BGL-1 and its glycosynthase mutant, both in hydrolysis or in synthesis, suggest that it could be an enzyme with applications for industrial processes.

Background

Lignocellulosic biomass is the most abundant material of biological origin in the world with great opportunities for generating value-added products for the benefit of mankind. It is composed by three distinct polymers: lignin, cellulose, and hemicelluloses. Cellulose is the major polysaccharide in plant cell wall and it is conformed of a linear polymer of glucose, linked by β -1,4 linkages. For a long time it was considered that this polysaccharide was exclusively degraded by the synergistic action of cellulolytic glycosyl hydrolases (GHs): endoglucanases (EGs), cellobiohydrolases (CBHs), and β -glucosidases (BGLs) [1]. However, this classic model underwent a big breakthrough with the discovery of lytic polysaccharide monooxygenases (LPMOs), auxiliary enzymes that break crystalline cellulose by oxidative depolymerization, thus creating new chains, and improving cellulose accessibility by glycosyl hydrolases [2]. The combination of endoglucanases, cellobiohydrolases and LPMOs generates cellobiose and other

small cellobiosaccharides, which are degraded by β -glucosidases, converting them into glucose as the final step of cellulose degradation. BGLs are fundamental to degrade this polysaccharide up to the monosaccharide level since, in their absence or low activity, short chain oligosaccharides will accumulate in the medium, causing product inhibition of the other cellulose degrading enzymes and thus decreasing total yields [3]. However, these enzymes are generally found in low proportion in commercial preparations, which are mostly produced by the fungus *Trichoderma reesei*, which usually represents the major bottleneck in cellulose degradation. Hence, many studies are focused on finding robust and efficient β -glucosidases, since cellulolytic enzyme cocktails must be supplemented with this activity to increase the efficiency of cellulose saccharification. BGLs are widely distributed in GHs families, but, according to the CAZY database, the two most predominant families are GH1 and GH3, being GH1 the family with the largest number of characterized BGLs [4].

One of the most important characteristics of BGLs is that they are frequently inhibited by their own product, glucose. Therefore, glucotolerance is always a sought-out feature in novel BGLs, in order to decrease the enzyme amount needed for a complete lignocellulose hydrolysis, and thus reducing process costs. Nevertheless, despite their high hydrolytic capacity, glucotolerant GH3 BGLs are exceptional [5], while on the contrary this desirable property seems to be commonly observed in BGLs from the GH1 family.

On the other side, the capacity of GHs for catalyzing transglycosylation reactions makes them a great biotechnological tool for the synthesis or modification of molecules of interest, through the addition of one or more sugar units to different compounds. Increasing the solubility of the original compound, making it safer, or improving its stability are among the many beneficial effects reported for glycosides [6, 7]. The application of biocatalysis to synthesize new glycosides has two main advantages compared to classical chemical synthesis: i) the synthetic process is generally much less contaminant and more eco-friendly, and ii) it is usually simpler, since the complex structure of oligosaccharides makes a classical chemical approach difficult and lengthy if the stereospecificity and regiospecificity of the glycosylation are to be controlled [8].

Although GHs can be successfully used to synthesize glycoconjugates, the yields are often poor. The reaction proceeds under kinetic control and the newly-formed products are easily hydrolyzed by the same GH when the initial glycoside donor is exhausted and, thus, the process is not economically viable at a large-scale. However, the hydrolytic capacity of these enzymes can be eliminated using rational design tools, leading to the production of a novel class of enzymes that possess only synthetic activity. In this context, glycosynthases are obtained by directed mutagenesis of the nucleophile amino acid, one of the two catalytic residues of these enzymes, placed in the active center of GHs [9]. The resulting mutant enzymes catalyze the formation of glycosidic bonds using glycosyl fluoride donors as surrogates of the enzyme covalent intermediate. In the absence of the nucleophile residue the enzyme cannot degrade the produced glycosides and the reaction yields are much higher [10]. The use of glycosynthases has been shown as an effective way to generate a wide variety of value-added products, as oligosaccharides of nitrophenol-sugars, methylumbelliferyl-sugars [8], oligosaccharides [11], and human milk oligosaccharides with prebiotic activity [12].

The ascomycete *Talaromyces amestolkiae* was studied for its ability to degrade cellulose and hemicellulose. Sequencing and annotation of the genome of this fungus disclosed a high number of genes encoding GHs and specially BGLs, being its number of CAZymes significantly higher than those reported for other organisms used to produce commercial cellulolytic enzyme cocktails [13]. Two of the BGLs produced by *T. amestolkiae*, BGL-2 and BGL-3, belong to the GH3 family and have interesting peculiarities. BGL-2 is the major β -glucosidase secreted by this fungus in the presence of cellulosic inducers. Structurally, this enzyme has a cellulose binding domain, an unusual feature among BGLs [14]. On the other hand, *T. amestolkiae* produces BGL-3 in all carbon sources tested, which is uncommon for β -glucosidases, and it was isolated and characterized from a basal medium with glucose under carbon starvation conditions [15]. Both, BGL-2 and BGL-3, were used as BGL supplements of commercial cocktails for saccharification of lignocellulosic waste, but its product inhibition is handicap for improve more the process. Up to now, the BGLs from the family GH1 produced by this fungus remain completely unexplored. In this work, we report the cloning and expression in *Pichia pastoris* of the β -glucosidase gene *bgl1* of this fungus, the purification and characterization of the recombinant BGL-1, with its classification in GH1, and its conversion into a glycosynthase, aimed to expand the biotechnological applications of this enzyme.

Results And Discussion

Cloning, production, purification and biochemical characterization of BGL-1

T. amestolkiae has been recently postulated as a very interesting option for producing enzymatic cocktails rich in BGLs [13]. All the BGLs from this fungus characterized up to date belong to family GH3 [14, 15], which is usually considered the one encompassing the BGLs with better catalytic efficiency, although these enzymes have some limitations such as their low glucotolerance [16]. In this sense, the GH1 family contains most of the glucose-tolerant BGLs characterized so far [17]. For this reason, investigating the presence of potential glucotolerant GH1 BGLs in *T. amestolkiae* genome and proteome could increase the value of the cellulolytic system of this fungus.

In a previous work [13], the secretome released by *T. amestolkiae* growing in different carbon sources was analyzed and, in every condition tested, one potential BGL from GH1 family (protein g8384, renamed as BGL-1) was detected in very low amounts. Therefore, the *bgl1* gene was cloned and expressed in *P. pastoris* with the goal of increasing BGL-1 production in order to analyze its glucose tolerance, kinetic constants, and physicochemical properties. After identifying the DNA sequence, RNA was extracted from 7-day old cultures of *T. amestolkiae*, obtaining total cDNA by retrotranscription. Amplification of the sequence of the mature *bgl1* gene concluded that the 1906 bp gene contains one intron, and encodes a 619 amino acids protein (Figure S1).

The methylotrophic yeast *P. pastoris* has been widely used as one of the most efficient expression systems for heterologous expression of BGLs. Some of its most notable advantages include its ability to

produce correctly folded protein at high levels, or to perform complex post-translational modifications [18]. A recombinant plasmid with the sequence of *bgl-1* without the intron was constructed using pPIC α as vector. Once transformed *P. pastoris* X-33, the transformants were screened to detect the best β -glucosidase producers. The maximal β -glucosidase activity was 75 U/mL, which is among the highest productions of BGLs reported in the literature. As can be seen in Table 1, this value is only surpassed by those found for the recombinant PtBglu3 from *Paecilomyces thermophila*, and *bgl3A*, from *Talaromyces leycettanus*. It is important to emphasize that the activity determined for this recombinant BGL-1 was 35-fold higher than the total β -glucosidase activity detected in cultures of *T. amestolkiae* [14], which contains a mixture of BGL-1, BGL-2 and BGL-3. This value confirms the very high overexpression of BGL-1 in this system.

Table 1
Comparison of the heterologous production in *P. pastoris* of BGLs from different microorganisms.

Enzyme Name	Microorganism	Production (U/mL)	References
rBgl3	<i>Aspergillus fumigatus</i>	4.9	[46]
rBgl4	<i>Penicillium funiculosum</i>	52.8	[5]
<i>bgl3A</i>	<i>Talaromyces leycettanus</i>	6,000.0	[47]
PtBglu3	<i>Paecilomyces thermophila</i>	274.4	[48]
Nfbgl1	<i>Neosartorya fischeri</i>	33.5	[49]
MtBgl3a	<i>Myceliophthora termophila</i>	41.0	[50]
Bgl3B	<i>T. leycettanus</i>	1.5	[51]
BGL-2	<i>T. amestolkiae</i>	6.0	[14]
BGL-3	<i>T. amestolkiae</i>	8.1	[15]
BGL-1	<i>T. amestolkiae</i>	75.0	This work

BGL-1 was purified in very high yield (around 80%) in just one step, by anion-exchange chromatography using a HiTrap QFF cartridge (Table 2), and 10 mM sodium phosphate buffer, pH 6.

Table 2

Purification of the β -glucosidase BGL-1 secreted from *P. pastoris* cultures.

BGL-1 Purification				
Step	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)
Crude extracts	39.66	4,176.97	105.48	100.0
HiTrap QFF Anion exchange	11.82	3,354.15	282.83	80.1

Table 3

Kinetic constants of BGL-1 hydrolyzing different substrates.

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}.s^{-1}$)
pNPG	3.36 ± 0.7	898.31	267.35
oNPG	2.36 ± 0.6	135.72	57.50
Cellobiose	20.36 ± 3.4	137.77	6.76
Cellotriose	19.39 ± 5.4	196.24	10.12
Cellotetraose	17.62 ± 0.6	276.62	15.69
Cellopentaose	12.41 ± 0.2	260.42	20.98
Cellohexaose	9.18 ± 0.6	217.86	23.73

The isoelectric point of the pure BGL-1 was determined to be 6.7 by isoelectrofocusing, and its molecular mass, measured by MALDI-TOF mass spectrometry, was 88.11 kDa. Considering that the theoretical mass was 23% lower (68.05 kDa), the differences found can be attributed to *P. pastoris* protein hyperglycosylation [18].

Optimal activity of BGL-1 was found at pH 4 and 60 °C (Fig. 1). Although these values are in the ranges reported for other native β -glucosidases [3, 17], BGL-1 has the peculiarity of working unusually well even at more acidic pHs. This behavior was also observed in the two other known BGLs of this fungus [14, 15], which may indicate that these enzymes from *T. amestolkiae* are more tolerant to acidic pHs than most BGLs characterized to date.

Glucose tolerance, kinetic study and substrate specificity of BGL-1

In general, most of the β -glucosidases used for cellulose degradation belong to the GH3 family, showing high catalytic efficiency values. However, they are often inhibited by glucose, with inhibition constant values lower than 0.1 M. In contrast, some GH1 β -glucosidases are much more glucotolerant than GH3

BGLs, but they usually have lower k_{cat}/K_m values over cellooligosaccharides, which are their natural substrates [3, 16]. The inhibition constants of BGL-1 towards glucose were calculated, displaying two main features that should be highlighted. First, its K_i value was very high (3.8 M), which to the best of our knowledge, corresponds to the second highest reported [19]. Second, the activity of BGL-1 was stimulated by low concentrations of glucose (Fig. 2). This finding has been observed in other β -glucosidases, mostly belonging to the GH1 family [16], but also in few BGLs from the GH3 family [5]. The reason for this glucose-induced stimulation remains unknown, although it could be related either to an allosteric effect triggered by the binding of glucose to some part of the protein, or to an increased hydrolysis rate upon transglycosylation [20]. BGL-1 activity was improved by 1.18-fold in the presence of 0.25 M of glucose. At this point, the activity begins to decrease, although BGL-1 still retained 40% of its initial activity at 3 M glucose (Fig. 2). Both characteristics postulates BGL-1 as a candidate for industrial processes performed at high glucose concentrations.

β -glucosidases can be classified into three groups, according to their substrate preferences: cellobiases, which have high substrate specificity towards cellooligosaccharides, aryl- β -glucosidases, with very high specificity towards synthetic substrates such as p-nitrophenyl- β -D-glucopyranoside (pNPG), and β -glucosidases with broad substrate specificity, that combine both activities [17]. In this sense, the kinetic constants showed that BGL-1 has very high efficiency and good affinity on pNPG and oNPG and, in addition, this enzyme displays a remarkable activity against p-nitrophenyl- β -D-xylopyranoside (specific activity 5.3 U/mg), thus demonstrating to have some versatility. However, its catalytic efficiency against cellooligosaccharides, from cellobiose to cellohexaose is low, as it occurs with other fungal BGLs from the GH1 family (Table 4). Thus, in contrast with the high activity on oligosaccharides shown by BGL-2 and BGL-3, which could be classified as cellobiases from the family GH3, BGL-1 seems to be a member of the group of aryl- β -glucosidases.

Table 4

Comparison of the catalytic efficiency on cellobiose and glucotolerance reported for BGLs from the GH3 and GH1 families.

Enzyme	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ /mM)	K_i (mM) or Glucose tolerance
GH3				
<i>T. leycettanus</i> Bgl3A [47]	10.4	786	75.8	14.0
<i>T. amestolkiae</i> BGL-2 [14]	1.1	630	567	1.0
<i>T. amestolkiae</i> BGL-3 [15]	0.5	1,594	3,308	1.6
<i>P. funiculosus</i> rBgl4 [5]	1.2	4,513	3,610	60% of residual activity at 500 mM
GH1				
Metagenomic library BGL mutant M3 [16]	49.2	48.4	1.1	50% of residual activity at 250 mM
Metagenomic library BGL mutant V174C [16]	45.1	83.1	1.7	50% of residual activity at 3000 mM
Soil metagenomic library BGL [19]	ND*	ND	ND	4,280
<i>Thermoanaerobacterium</i> <i>thermosaccharolyticum</i> BGL [52]	7.9	120	13.3	600
<i>Thermoanaerobacterium</i> aotearoense BGL [53]	25.4	740.5	29.1	800
<i>Actinomadura</i> amylolytica AaBGL1 [54]	95.3	10.7	0.1	40% of residual activity at 2000 mM
<i>Actinomadura</i> amylolytica AaBGL2 [54]	187.7	16.6	0.1	40% of residual activity at 500 mM
<i>Thermotoga</i> thermarum BGL [55]	35.5	19.0	0.5	1,500
BGL-1 (this work)	20.4	137.8	6.8	3,780
*ND = no determined				

However, it is interesting to remark that, in spite of the relatively good k_{cat} values of BGL-1 in the hydrolysis of celooligosaccharides, the K_m values are poor when compared with those observed for other

BGLs from the GH3 family (Table 4). This result confirms that, like other BGLs from the GH1 family, BGL-1 has low affinity for these substrates, which could limit its applicability in hydrolytic processes.

Finally, an interesting discovery was made when examining the regioselectivity in hydrolysis reactions catalyzed by BGL-1. Enzymatic activity was tested against cellobiose, sophorose, laminaribiose and gentiobiose. While the activity over laminaribiose and gentiobiose was low, the activity over cellobiose and sophorose was considerably high, and BGL-1 was 5 fold more active on sophorose (535.82 U/mg) than on cellobiose (110.27 U/mg). These results indicated that BGL-1 could be considered as a versatile β -1,2 BGL, due to its preference for β -1,2 bonds but also being able to hydrolyze β -1,4 bonds. This behavior, although initially unexpected, seems to be more common as has recently been reported by Heins et al. [21], in a recent study in which more than 170 GH1 family enzymes were analyzed, using a high throughput screening approach. In said study, it can be seen how the activities obtained against sophorose were superior to those detected in the hydrolysis of cellobiose in a large number of the enzymes tested, which can establish a pattern within the proteins of this family. But, it is interesting to note that, so far, a very low number of β -1,2-glycosidases have been completely characterized. For example, some glucanases and glucosidases induced by β -1,2-glucan have been discovered in *Acremonium* sp., a filamentous anamorphic fungus [22]. However, their amino acid sequences have not been determined, which precludes their comparison with the BGL-1 from *T. amestolkiae*. Recently, a BGL with activity on β -1,2 bonds was reported in *Listeria innocua*, and related with the β -1,2-glucan metabolism in this bacteria [23], although the physiological role of these BGLs capable of synthesizing the β -1,2 disaccharide, remains poorly understood. However, it is well known that sophorose is the most powerful inducer of cellulases in *T. reesei* [24]. A recent report describes the production of this disaccharide by transglycosylation catalyzed by intracellular BGLs of this fungus, and the regulatory role of another BGL that hydrolyzes this compound, triggering the synthesis of cellulases [25]. Therefore, taking into account that BGL-1 is produced by *T. amestolkiae* in all conditions assayed, and that this enzyme can hydrolyze and synthesize β -1,2 bonds, its physiological role could be related to the regulation of the induction of the cellulolytic system in this organism.

Transglycosylation profile and regioselectivity of BGL-1

In order to test the transglycosylation capacities of BGL-1 a screening with a variety of potential acceptors, including sugars, sterols, phenolic compounds, or amino acids (Table S1) was performed according to a methodology previously developed [26] and also applied for studying the transglycosylation profile of other *T. amestolkiae* BGLs [27]. Unfortunately, in contrast with the good results obtained with the GH3 BGLs of this fungus against a large and diverse panel of glycosylation acceptors, BGL-1 only showed potential for transglycosylating p-nitrophenol sugar derivatives, like pNPG, pNPGal, or pNPX, which ruled out most of the potential acceptors for transglycosylation tested with the wild type enzyme. On the other hand, taking into account the saccharide nature of those acceptors, the regioselectivity of the transglycosylation was assessed analyzing by NMR the products of a model reaction set up with pNPG as donor and ^{13}C -labelled glucose as acceptor. The NMR spectra of the compounds detected in the mentioned reaction were compared with those from sophorose, cellobiose

and laminaribiose, confirming their coincidence with the pattern from sophorose (Fig. 3). This result showed that BGL-1 transglycosylated with high selectivity towards the β -1,2 manner.

Conversion of BGL-1 into glycosynthases by rational design

Historically, enzyme engineering has been successfully implemented to enhance the transglycosylation activity of glycosidases and, simultaneously, attenuate hydrolysis. With the aim of expanding the transglycosylation capacities of the recombinant BGL-1, it was converted into a glycosynthase. This kind of enzymes were first reported by Withers and coworkers [9], who noticed that a mutated glycosyl hydrolase lacking its catalytic nucleophile can use activated glycosyl fluoride donors with the opposite anomeric configuration for synthesizing glycosides, without hydrolyzing the products. This approach has been successfully applied for instance to convert glycosidases from GH1 family into glycosynthases, from GH36 in galactosynthases, or from GH29 in fucosynthases [10].

In this work, the replacement of the catalytic nucleophile of BGL-1, a glutamic acid at position 521, by a glycine (BGL-1-E521G) or a serine (BGL-1-E521S) produced two novel versions of BGL-1. These mutations have been shown to be much more efficient than the alanine replacement in the synthesis of oligosaccharides and p-nitrophenol derivatives [8]. Both versions of the protein were produced in *P. pastoris* and purified with the same strategy used for BGL-1. The purified glycosynthases showed their ability to use α -GlcF for synthesizing glycosides, and the most efficient mutant was selected from a comparative assay developed using 10 mM α -GlcF as donor and 10 mM pNPG as acceptor. Product formation was analyzed by HPLC, and the outcomes from this experiment showed the glycine mutant as the more efficient with a transglycosylation yield 2-fold higher than for the serine mutant. This is in concordance with data reported in the literature, which are explained considering that the rigid serine side-chain could hamper the departure of the fluoride, which is instead stimulated in the glycine mutants [28–30]. In a similar way, other authors justify this different behavior between the mutants considering the lack of a side-chain in glycine, and thus of reduced steric hindrance compared with the alanine or serine side-chains [31].

Once selected the glycine mutant, the kinetic parameters of this new glycosynthase employing α -GlcF and pNPG, for the formation of pNPG plus glucose, were determined. The results revealed that the affinity of BGL-1-E590G for pNPG (K_m 90.14 mM) was higher than for α -GlcF (K_m 260.86 mM), although the catalytic constants were similar for both substrates (k_{cat} 0.11 s⁻¹ and 0.08 s⁻¹, respectively). The results obtained displayed slightly worst performance in K_m than reported for a xylosynthase [32], but were similar in terms of k_{cat} , confirming that the obtained glycosynthases could be a valuable starting point for optimizing biocatalytic transglycosylation reactions.

Transglycosylation of selected acceptors and analysis of the products

BGL-1-E521G was tested as the catalyst for transglycosylation towards aryl-glycoside acceptors, pNPG, pNPX, pNPGal, and some phenolic compounds, vanillin, hydroxytyrosol, gallic acid, and epigallocatechin gallate (EGCG), using α -GlcF as the donor (Fig. 4). With other glycosynthases pNP-sugars have been frequently used as preferential acceptors of transglycosylation, generating a variety of products, from the expected pNP-disaccharides, to pNP-oligosaccharides of different length and regioselectivity [8]. Besides these acceptors, phenolic compounds are very interesting targets for transglycosylation, because of the possibility of obtaining value-added glycosides from this type of compounds. These molecules have shown a variety of beneficial properties related to human health, and have been reported to confer cardiovascular protection, and to exert a positive effect in neurodegenerative diseases and cancer [33]. One of the main disadvantages of these substances when used in treatments is their low bioavailability, and their glycosylation, which can increase its solubility, has been proposed as a potential solution. In this context, various studies have demonstrated the interesting properties of hydroxytyrosol, vanillin and gallic acid [34–36], and EGCG has recently attracted attention as a potential therapeutic agent [37, 38], even in its glycosylated forms [39]. In a first approximation TLC analysis of the reaction mixtures was used to detect the synthesis of glycosides from the selected acceptors, identifying positive spots for each potential glycoside (figure S4). The presence of the expected compounds was confirmed by obtaining by mass spectrometry (MS) the molecular weight of the newly synthesized molecules. All the molecules were detected in its sodium adduct form in their mass spectra (Table 5). It is interesting to highlight that MS analysis revealed the presence of additional products with higher molecular weights in all reactions. The peaks corresponding to saccharides with two (G2), three (G3), four (G4) and five (G5) glucose units were thus detected, showing that this glycosynthase can also generate oligosaccharides. The presence of non-fluorinated derivatives of these molecules could be due to self-hydrolysis of α -GlcF or the fluorinated derivatives during overnight reactions. Besides, considering the ability of the native BGL-1 hydrolyzing pNPX, and the capacity of BGL-1-E521G to interact with transglycosylation acceptors with xylose, it opens up the possibility of synthesizing oligosaccharides with xylose, or using D-xylosyl fluoride as potential donors of the reactions, which may expand the applications of the enzyme.

Table 5
ESI-MS data glycosides obtained by
transglycosylation catalyzed by BGL-1-E521G. All
the glycosides were detected as Na⁺ adducts.

Glycoside	Intensity	m/z
EGCG-glucose	533,662	643.2
Vanillin-glucose	43,977	337.1
Hydroxytyrosol- glucose	22,386	339.1
Gallic acid-glucose	48,294	355.1
pNPX-glucose	307,637	456.1
pNPGal-glucose	569,165	486.1
pNPG-glucose	430,001	486.2
G2	449,035	365.1
G3	428,105	527.2
G4	287,540	689.3
G5	59,761	851.3

The reactions rendering products with higher intensity in the TLC and MS analysis (those corresponding to pNPG, pNPX, pNPGal, and EGCG as acceptors) were submitted to HPLC analysis to determine the transglycosylation yield and to purify the main glycoside products for further NMR analysis. The conversions were 73.5% of initial pNPG, 89.8% for pNPX, and 36.6% for pNPGal, and, more interestingly, the glucoside of EGCG was obtained with a very significant yield of 48.8%. Besides, a second EGCG derived product was also detected and purified. Besides, a second EGCG product was also detected and purified. The conversion rate of 48.8% in transglycosylation of EGCG is among the best reported in the literature, although yields of 58% [40] and 91% [41], have also been reported for the enzymatic synthesis of the same compound using a cyclodextrin glucanotransferase and a dextranucrase, respectively. However, since the production of this glycoside has not been optimized, adjusting the reaction conditions for BGL-1-E521G transglycosylating EGCG could generate higher yields, and will be explored in future works.

Products characterization by NMR

The purified EGCG, pNPG, pNPX and pNPGal derived glucosides synthesized by transglycosylation catalyzed by BGL-1-E521G were analyzed by ¹H and ¹³C-NMR in order to confirm their structure and assign their regiochemistry. The HMBC spectra of the two EGCG-glucose derivatives (figures S5 and 6) showed a correlation between the anomeric position of glucose and the meta-carbon (3"/5") of the gallate

aromatic ring, indicating the position of the linkage between the phenolic and the sugar moieties in these glycoconjugates (Fig. 5). In addition, the second sugar unit in the EGCG-disaccharide is attached to the O-2 of the first one through a β -linkage, as deduced from the value of the coupling constant of the anomeric proton (7 Hz) and in accordance with the spectral assignment (Table 6). The results from the NMR analysis of the three pNP derivatives are shown in Figs. 6, 7 and 8 and their NMR spectra in figures S7, S8 and S9. All of them indicate the regioselectivity of the glycosynthase that specifically forms pNP-disaccharides, incorporating the second sugar unit through a β -1,2 linkage. Finally, the different molecular species produced in a crude transglycosylation reaction mixture of α -GlcF (donor) and pNPG (acceptor) were also analysed by NMR. Interestingly, this sample showed significant heterogeneity as observed in the anomeric region of the ^1H - ^{13}C HSQC spectrum of the mixture (Fig. 9). The presence of some unreacted acceptor pNPG-glucose but not starting donor α -GlcF, was confirmed. Unexpectedly signals tentatively assigned to α -F-sophorose were observed indicating that the α -GlcF itself with the α configuration could fit in the acceptor site. Besides, free glucose was also identified, confirming the auto-hydrolysis of the fluorinated substrate during the reaction. To note, this free glucose also worked as acceptor of α -GlcF, as deduced from the presence of sophorose among the reaction products. Again, the newly-synthesized disaccharides were linked by β -1,2 bonds, confirming the total regioselectivity of the synthase, which follows the same behavior than BGL-1, just being able to transglycosylate in this position.

Table 6
Chemical shifts for EGCG-glucose and EGCG-
sophorose.

EGCG-Glucose			EGCG-Sophorose		
	¹ H	¹³ C		¹ H	¹³ C
2	5.08	77.06	2	5.03	77.05
3	5.60	68.89	3	5.57	68.71
4	2.86	24.91	4	2.84	24.86
	3.01			2.97	
4a	—	99.04	4a	—	99.00
5	—	155.18	5	—	155.34
6	6.08	95.72	6	6.06	96.01
7	—	155.17	7	—	155.34
8	6.08	95.72	8	6.06	96.01
8a	—	155.18	8a	—	155.21
1'	—	106.33	1'	—	106.39
2'	6.48	106.45	2'	6.47	106.40
3'	—	145.42	3'	—	145.51
4'	—	132.10	4'	—	132.62
5'	—	145.44	5'	—	145.65
6'	6.48	106.45	6'	6.47	106.40
1''	—	120.59	1''	—	120.58
2''/6''	7.09	108.61	2''/6''	7.06	108.61
3''	—	144.30	3''	—	144.59
4''	—	139.73	4''	—	139.90
5''	—	144.27	5''	—	144.59
6''/2''	7.06	112.27	6''/2''	7.05	112.18
7''	—	166.50	7''	—	166.43
1 Glc	4.97	100.33	1 Glc	5.09	99.22

EGCG-Glucose			EGCG-Sophorose		
2 Glc	3.53	72.60	2 Glc	3.78	80.98
3 Glc	3.54	75.32	3 Glc	3.69	75.39
4 Glc	3.49	68.65	4 Glc	3.53	68.29
5 Glc	3.22	75.97	5 Glc	3.21	75.63
6 Glc	3.41	59.73	6 Glc	3.41	59.83
	3.66			3.66	
			1' Glc	4.77	102.77
			2' Glc	3.25	73.85
			3' Glc	3.43	75.70
			4' Glc	3.36	69.36
			5' Glc	3.29	76.09
			6' Glc	3.25	60.33
				3.53	

Conclusions

The novel GH1 β -glucosidase BGL-1 from the ascomycete *T. amestolkiae* has been heterologously produced in *P. pastoris* in large amounts. The recombinant enzyme was easily purified in good yield (80%) and then characterized and evaluated in hydrolysis and transglycosylation reactions. The inhibition constant by glucose of BGL-1 was 3.78 M, confirming its outstanding glucotolerance. However, its low catalytic efficiency in the hydrolysis of cellooligosaccharides may limit its applications in saccharification. BGL-1 was also tested as transglycosylation catalyst of a wide array of compounds showing poor efficiency in synthesis. To overcome this problem, the enzyme was converted into a glycosynthase by protein engineering, abolishing its hydrolytic activity. This novel biocatalyst showed to be regioselective too, and catalyzed the transfer of glucose molecules not only to p-nitrophenol sugars, but also to interesting phenolic acceptors such as EGCG, a compound with many potential medical applications. These findings postulate this mutant as a potential candidate to be used in industrial processes devoted to the synthesis of bioactive glycosides.

Materials And Methods

Microorganism and culture conditions

The fungus *T. amestolkiae* A795, deposited in the IJFM collection, at the Centro de Investigaciones Biológicas (Madrid, Spain), was cultured in PDA (potato dextrose agar) plates at 28 °C and maintained at 4 °C. *T. amestolkiae* was also cultured in Mandels medium, as reported before [15], with 1% of Avicel as carbon source, for RNA extraction.

For plasmid propagation, *Escherichia coli* DH5α (Invitrogen) was grown at 37 °C, overnight, in LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L agar) containing 100 mg/L ampicillin for selection of resistant colonies.

The heterologous expression of BGL-1 was performed using *P. pastoris* X33 strain (Invitrogen), previously grown in YPD medium plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 10 g/L of agar). The positive clones were screened in YPD containing 100 mg/L of zeocin as selection marker, and cultured 2–5 days at 28 °C. Recombinant protein was produced in YEPS medium (20 g/L peptone, 10 g/L yeast extract, 10 g/L sorbitol, and 100 mM potassium phosphate buffer, pH 6), with daily addition of 10 mL/L of methanol as inducer. Cultures were incubated for 9 days, at 28 °C and 250 rpm, taking samples daily to measure BGL-1 production. All experiments were performed in triplicate.

Nucleic acid isolation, enzyme mutagenesis and cloning in *Pichia pastoris*

In order to identify the *bgl-1* DNA sequence, a TBLASTN was performed against the set of predicted proteins of *T. amestolkiae*, which were obtained in a previous work [13]. The gene sequences returned were used to run a local BLASTN against the assembled genome [13]. Once recognized the predicted sequence for *bgl-1*, the presence of a signal peptide in the BGL-1 protein sequence was examined using the SignalP server. RNA was extracted from 7-day old *T. amestolkiae* cultures growing in 1% of Avicel using Trizol reagent [42]. The isolated transcripts were converted to cDNA using the Superscript II Reverse Transcriptase RT-PCR kit, according to the manufacturer's instructions. PCR amplifications were performed in a thermocycler Mastercycler pro S (Eppendorf). The primers were designed based on the nucleotide sequence of the *bgl-1* gene identified in *T. amestolkiae* genome (GenBank accession no. M1KG00000000), excluding the region corresponding to the signal peptide. Restriction sites for XhoI and NotI were included in the forward and reverse primers respectively (BG1FWXH01: 5'-ATCTCGAGAAAAGACAAGAGGTGTACATCAGACT-3', and BG1RVNOTI: 5'-ATGCGGCCGCATATCCCAGCCCATTCTCGC – 3'). The PCR protocol was developed as follows: initial denaturation at 95 °C for 5 min, followed by 36 cycles of amplification: denaturation at 95 °C for 45 s, primer annealing at 55 °C for 45 s, and elongation at 72 °C for 2 min. A final extension step at 72 °C for 10 min was also carried out. The PCR product obtained in the last step was ligated to the yeast expression vector pPICZα (Invitrogen), and it was used to transform *P. pastoris* X-33. For improving transformation efficiency, the vector was linearized with SacI (New England Biolabs). The lithium chloride method was used for transformation according to the manufacturer's instructions. Transformed colonies

were grown on YPD medium plates with zeocin as selection marker. Positive clones were screened with 4-methylumbelliferyl β -D-glucopyranoside as described in Méndez-Líter et al. [14].

Conversion of BGL-1 into the two glycosynthase variants

The plasmid pPICza containing *bgl-1* gene was used to generate two new versions of the protein by directed mutagenesis, replacing the glutamic acid 521 by a glycine or a serine. The identification of the catalytic amino acids of BGL-1, was performed by alignment using clustal omega (see supplementary material, figure S3), with the BGLs sequences of bacterium *Clostridium cellulovorans*, fungus *Trichoderma reesei* and termite *Neotermes koshunensis*, in which the nucleophilic amino acid were previously detected [43]. To do so, the Expand™ Long Template PCR System (Roche) was used as described by the manufacturer. Primers BG1sfwSer (CCCTCGTCCTCAGCTCATTCGGTTTTCCCGTCTAC), BG1sRvSer (GTAGACGGGAAAACCGAATGAGCTGAGGACGAGGG), BG1sfwGly (CCCTCGTCCTCAGCGGATTCGGTTTTCCCGTCTAC) and BG1sRvGly (GTAGACGGGAAAACCGAATCCGCTGAGGACGAGGG) were used for serine and glycine replacements, respectively. After PCR reaction, the product was digested by DpnI (New England Biolabs), in order to hydrolyze the parental methylated DNA that was used as template. Both new vectors were cloned into *P. pastoris* with the same method used before.

Production and purification of BGL-1 and BGL-1 glycosynthase variants

The selected positive *P. pastoris* clones were grown overnight in 250 mL flasks with 50 mL of YPD medium at 28 °C and 250 rpm to obtain the respective preinocula. Then, they were used for recombinant protein production in 2-L flasks with 400 mL of YEPS medium. Cultures were incubated at 28 °C and 250 rpm for 9 days with daily addition of 10 mL/L methanol. For BGL-1 purification, 9 day-old cultures were harvested and centrifuged at 10,000 $\times$ g and 4 °C for 20 min. The supernatant was concentrated and dialyzed against 10 mM phosphate buffer (pH 6.0) using a 30-kDa cutoff membrane (Merck-Millipore). BGL-1 was purified in a single chromatographic step using an FPLC system (Äkta), using a 5 mL QFF HiTrap cartridge (GE Healthcare) equilibrated with phosphate buffer pH 6.0. Elution of the bound proteins was carried out by applying a 25 min-linear gradient from 0 to 0.3 M of NaCl, at 2 mL/min. The column was then washed with 10 mL of 1 M NaCl and re-equilibrated using 10 mL of the starting buffer. Fractions with β -glucosidase activity were dialyzed and concentrated. The glycosynthase variants of BGL-1 were purified using the same protocol.

Protein quantification, enzyme assays and substrate specificity

Total protein was calculated by 280 nm absorbance using a Nanodrop spectrophotometer (Thermo Fisher Scientific), and confirmed by the bicinchoninic acid assay (BCA) method.

The β -glucosidase standard reaction was performed using 3 mM p-nitrophenyl- β -D-glucopyranoside (pNPG, Sigma), at 60 °C, in sodium acetate buffer 50 mM, pH 4.0. The reaction was stopped after 10 min

by adding 2% (w/v) Na_2CO_3 , measuring the release of p-nitrophenol (pNP) in a spectrophotometer at 410 nm. One BGL activity unit was defined as the amount of enzyme capable of releasing 1 μmol of pNP per min (the molar extinction coefficient of pNP is $15,200 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

Glucose tolerance was determined in standard BGL reactions' containing increasing glucose concentrations from 0.1 mM to 3 M. Glucotolerance was calculated comparing the activity measured in reactions without glucose, that was considered as the 100%, with those obtained for reactions in the presence of added glucose. For K_i determinations, the concentrations of glucose used were 1, 1.25, and 1.5 M.

To prevent the activity loss when working with low enzyme concentrations, all enzymatic assays included 0.1% BSA, a protein that does not affect the catalytic activity of BGL-1 [44]. The kinetic constants of the purified BGL-1 were determined against pNPG over a range of concentrations from 10 μM to 5 mM, o-nitrophenyl- β -D-glucopyranoside (oNPG, 40 μM to 20 mM), cellobiose (80 μM to 40 mM), cellotriose (80 μM to 40 mM), cellotetraose (80 μM 40 mM), cellopentaose (40 μM to 20 mM), and cellohexaose (20 μM to 10 mM). The K_m and V_{max} parameters were calculated using SigmaPlot. The reactions containing celooligosaccharides were quantified by measuring the glucose released after hydrolysis, using the Glucose-TR commercial kit (Spinreact) according to the manufacturer's instructions. All reactions were carried out in sodium acetate 100 mM, pH 4.0, in a heating block for 10 min at 1200 rpm. Then, the reactions were stopped by heating at 100 °C for 5 min.

Kinetic parameters were calculated for two transglycosylation experiments catalyzed by the BGL-1-E521G variant with D-glucosyl fluoride (α -GlcF) and pNPG as substrates. Each substrate was used in one experiment at a fixed concentration of 10 mM, and with varying concentrations in the other. When pNPG was examined, its concentrations ranged between 500 mM and 12.5 mM. When calculating kinetic constants α -GlcF, it was used in a range from 25 mM to 1 M.

BGL-1 activity towards cellobiose (β -D-glucopyranosyl-1,4- β -D-glucopyranose), sophorose (β -D-glucopyranosyl-1,2- β -D-glucopyranose), laminaribiose (β -D-glucopyranosyl-1,3- β -D-glucopyranose) and gentiobiose (β -D-glucopyranosyl-1,6- β -D-glucopyranose), was determined using 10 mM of the disaccharides in sodium acetate buffer 100 mM, pH 4.0, with the appropriate amount of enzyme. Reactions were performed for 10 min at 60 °C and 1200 rpm, and quantified by measuring the glucose released using the Glucose-TR commercial kit (Spinreact).

Physicochemical properties

The molecular mass of the native BGL-1 was determined using MALDI-TOF according to the protocol explained in [15]. Isoelectric point (pI) was determined by isoelectrofocusing (IEF), and the gel was revealed with 4-methylumbelliferyl- β -D-glucopyranoside (Sigma Aldrich), following the same procedure explained in [14]. Optimal pH was assayed for 10 min using pNPG as substrate and Britton-Robinson buffer (100 mM) in a range from 2 to 10. Optimal temperature was assayed using standard conditions but varying the temperature from 30–70 °C.

Screening for transglycosylation acceptors of BGL-1

In the search of potential transglycosylation acceptors for the wild type BGL-1, a library of 70 compounds (Supplementary material, Figure S2), was used to conduct a preliminary screening. The compounds were used to perform recovery inhibition assays as described in a previous work [27], and those that produced higher reaction rates than the controls without acceptor were considered potential hits for transglycosylation.

Transglycosylation reactions catalyzed by the glycosynthases. Analysis of the products

In these reactions, α -GlcF was prepared as described previously [45], and used as donor in every reaction. The efficiency of both glycosynthase variants (BGL-1-E521G and BGL-1-E521S) was first compared in reactions containing 10 mM pNPG, 10 mM α -GlcF 10 mM, 0.4 mg/mL of the corresponding mutant enzyme, and 50 mM acetate buffer pH 4. The reaction mixture was incubated at room temperature for 16 h at 500 rpm, and then analyzed by High Performance Liquid chromatography (HPLC) as explained below.

Other potential acceptors tested were: pNPG, p-nitrophenyl- β -D-xylopyranoside (pNPX), p-nitrophenyl- β -D-galactopyranoside (pNPGal), vanillin, hydroxytyrosol, gallic acid, and epigallocatechin gallate (EGCG). The standard transglycosylation reaction contained 20 mg/mL of α -GlcF, 5 mg/mL of each acceptor and 1 mg/ mL of BGL-1-E521G in acetate buffer 50 mM, pH 4, with 0.1% of BSA, and it was performed at room temperature for 16 h at 500 rpm. The synthesis of glycosides was first checked by thin layer chromatography (TLC) in silica gel G/UV254 polyester sheets, (0.2 mm thickness and 40 × 80 mm plate size) provided by Macherey–Nagel, using ethyl acetate/methanol/water 10:2:1 (v/v) as running solution. Substrates and glucosides were detected under 254 nm UV light, since all the potential acceptors possess an aromatic ring detectable at this wavelength.

The reaction mixtures were also analyzed by mass spectrometry in a HCT Ultra ion trap, in order to identify the expected products. The samples were analyzed by electrospray ionization-mass spectrometry (ESI-MS) with methanol as ionizing phase in the positive reflector mode, and data were processed with the Masshunter Data Acquisition B.05.01 and Masshunter Qualitative Analysis B.07.00 software (Agilent Technologies).

Finally, the most interesting glycosides were purified by HPLC, in an Agilent 1200 series LC instrument equipped with a ZORBAX Eclipse plus C18 column (Agilent). The column was first equilibrated in a mix of acetonitrile and H₂O with 0.1% acetic acid, with a flow of 2 mL/min, and the reaction products were separated isocratically in 8 min. For the glycosides of pNPG and pNPGal, they were purified with a proportion of 14:86 (v/v) acetonitrile:H₂O, while for the pNPX glycoside this proportion changed to 20:80 (v/v) and for the EGCG products it was 13:87 (v/v). After isocratic elution, the column was washed for 3 min with 95:5 acetonitrile:H₂O, and the system was finally re-equilibrated to the initial conditions for

4 min. Every product peak was detected by monitoring the absorbance at 270 nm, and quantification was done using a calibration curve of each non glycosylated parental. The fractions containing the glycosides were collected to be further analyzed by nuclear magnetic resonance (NMR) to determine their structure. After collection, the products were lyophilized and stored at -20 °C. The reactions conducted to determine the kinetic parameters of BGL-1-E521G were analyzed isocratically in the equilibration buffer, as described above for the glycosides of pNPG and pNPGal.

Nuclear Magnetic Resonance

The structure and regiochemistry of the purified glucosides of EGCG, pNPG, pNPX and pNPGal synthesized by BGL-1-E521G was elucidated by NMR. The samples for the NMR analysis were prepared by dissolving the purified compounds in 500 µL of deuterated water (D₂O). NMR spectra were acquired at 298 K, using a Bruker AVANCE 600 MHz spectrometer equipped with a cryogenic probe. 1D ¹H NMR spectra, ¹H-¹³C HSQC and HMBC experiments were acquired to assign all NMR signals. For 1D ¹H, ¹H-¹³C HSQC, and HMBC experiments, the zg, zgpr, hsqcedetgp, and hmbcgpndqf sequences were employed. Crude reaction mixtures without further purification were also submitted to NMR analysis. First, a transglycosylation reaction mixture using BGL-1-E521G, with 10 mM pNPG, 10 mM α-GlcF 10 mM, 0.4 mg/mL of the enzyme, in 50 mM acetate buffer pH 4. The reaction mixture was incubated at room temperature for 16 h at 500 rpm. Second, a reaction using the native BGL-1, with 3 mM of pNPG as donor and ¹³C-labelled glucose as acceptor, in 50 mM acetate buffer pH 4. The reaction was set directly in the NMR at room temperature. Both were analyzed for determining the regioselectivity of this β-glucosidase and its mutant in transglycosylation.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors read and approved the final manuscript.

Availability of data and material

T. amestolkiae whole genome shotgun project is deposited at DDBJ/ENA/GenBank under the accession number MIKG000000000. BGL-1 sequence is deposited in GenBank under the accession number KM393204.1.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

JAML drafted the manuscript and performed the experiments excluding NMR assays. BFT and FJC designed and performed the NMR assays and analyzed the data. AGS and JLA, synthesized the α -D-glucosyl-fluoride used as donor in the glycosynthase reactions, and the 2,4-dinitrophenyl-2-deoxy-2-fluoro- β -D-glucopyranoside used for the screening of potential transglycosylation acceptors. MND, AP, LIE and MJM designed, coordinated the study, helped to draft, and critically reviewed the manuscript. All authors read and approved the final manuscript.

Additional Information

Supplementary information is accompanied

Figure S1. DNA and protein sequences of BGL-1. **Figure S2.** List of compounds tested in transglycosylation screening assay. **Figure S3.** Thin layer chromatography of the different compounds in transglycosylation reactions using BGL-1-E521G. **Figure S4.** Alignment of BGL-1 amino acid sequence. **Figure S5.** NMR Spectra of the EGCG glycoside. **Figure S6.** NMR Spectra of the EGCG plus sophorose. **Figure S7.** NMR Spectra of the Glucose-Glucose-*p*NP derivative. **Figure S8.** NMR Spectra of the Glucose-Galactose-*p*NP derivative. **Figure S9.** NMR Spectra of the Glucose-Xylose-*p*NP derivative.

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Figures

Figure 1

Effect on BGL-1 activity of: A) pH and B) temperature.

Figure 2

Inhibitory effect of glucose on the activity of BGL-1 in hydrolysis of pNPG.

Figure 3

^1H - ^{13}C HSQC NMR spectra of the reaction mixture catalyzed by native BGL-1 on pNPG as substrate (3 mM) in presence of ^{13}C -labelled glucose. A) Detail of the region of the spectra over ^{13}C 75 ppm. The anomeric region is presented for only the signals of labelled glucose, either unreacted or as part of the transglycosidation product. The regiochemistry corresponding to substitution on position 2 on the labeled glucose is deduced from the superimposition of the spectra of the reaction mixture (in blue) with the spectra of: B) sophorose (in red). The signals from H1' of α - and β -sophorose are not observed in the spectrum of the reaction mixture (blue) since the non-reducing residue comes from the unlabeled donor. C) cellobiose (in red). D) laminaribiose (in red).

Hydroxytyrosol

Gallic acid

*p*NPG

Vanillin

*p*NPGal

EGC'G

*p*NPX

Figure 4

Molecular structure of the acceptors selected for transglycosylation catalysed by the glucosynthase BGL-1-E521G.

Figure 5

Structures, deduced from the NMR analysis, of the two glucosides produced by transglycosylation of EGCG with the synthase BGL-1-E521G. Every C atom in the molecules is numbered to clarify the identification of the signals.

	¹ H	¹³ C		¹ H	¹³ C		¹ H	¹³ C
1' Glu	4.77	102.83	1 Glu	5.41	97.90	1 pNP	---	161.19
2' Glu	3.22	73.88	2 Glu	3.82	81.29	2 pNP	8.20	126.15
3' Glu	3.44	75.46	3 Glu	3.74	75.28	3 pNP	7.18	116.18
4' Glu	3.32	69.23	4 Glu	3.49	68.98	4 pNP	---	142.52
5' Glu	3.27	76.03	5 Glu	3.63	76.13	5 pNP	7.18	116.18
6' Glu	3.26	60.20	6 Glu	3.68	60.71	6 pNP	8.20	126.15
	3.48			3.86				

Figure 6

Structure, deduced from NMR analysis, of the pNPG-glucoside produced by transglycosylation of pNPG with the synthase BGL-1-E521G. Every C atom in the molecules is numbered to clarify the identification of the signals. Chemical shifts (ppm) are indicated in the table.

	¹ H	¹³ C		¹ H	¹³ C		¹ H	¹³ C
1' Glu	4.74	103.00	1 Gal	5.35	98.28	1 pNP	---	161.43
2' Glu	3.20	73.68	2 Gal	3.98	79.60	2 pNP	8.20	126.05
3' Glu	3.45	75.42	3 Gal	3.90	72.22	3 pNP	7.19	116.18
4' Glu	3.30	69.00	4 Gal	3.97	68.02	4 pNP	---	142.52
5' Glu	3.25	75.94	5 Gal	3.87	75.32	5 pNP	7.19	116.18
6' Glu	3.18	59.83	6 Gal	3.70	60.73	6 pNP	8.20	126.05
	3.46			3.70				

Figure 7

Structure, deduced from NMR analysis, of the pNPGal-glucose product obtained by transglycosylation of pNPGal with the synthase BGL-1-E521G. Every C atom in the molecule is numbered to clarify the identification of the signals. Chemical shifts (ppm) are indicated in the table.

	¹ H	¹³ C		¹ H	¹³ C		¹ H	¹³ C
1' Glu	4.74	102.91	1 Xyl	5.40	98.39	1 pNP	---	161.22
2' Glu	3.22	73.65	2 Xyl	3.82	80.95	2 pNP	8.20	126.06
3' Glu	3.44	75.36	3 Xyl	3.72	74.44	3 pNP	7.17	116.13
4' Glu	3.32	69.15	4 Xyl	3.71	68.71	4 pNP	---	142.56
5' Glu	3.28	75.89	5 Xyl	3.49	64.94	5 pNP	7.17	116.13
6' Glu	3.25	60.01		3.98		6 pNP	8.20	126.06
	3.48							

Figure 8

Structure, deduced from NMR analysis, of the pNPX-glucose product obtained by transglycosylation of pNPX with the synthase BGL-1-E521G. Every C atom in the molecule is numbered to clarify the identification of the signals. Chemical shifts (ppm) are indicated in the table.

Figure 9

1) Structures of pNPG (a), Glc (b), sophorose (c), sophorose-F (d) and sophorose-pNP (e). 2) Anomeric region of the ^1H - ^{13}C HSQC spectrum of a transglycosylation reaction mixture which pNPG as acceptor and α -Glc-F as donor catalyzed by BGL-1-E521G. The peaks are labeled with their corresponding assignment. Red circles indicate where the signals corresponding Glc-F, which has been fully consumed, should appear.

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