Efficient synthesis of rare disaccharides by engineered β-glucosidase based on hydropathy index

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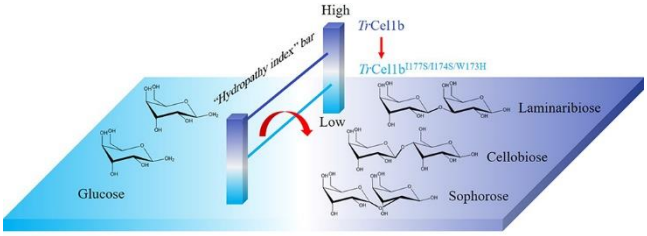

ABSTRACT: Oligosaccharides have important therapeutic applications. A useful route for oligosaccharides synthesis, especially rare disaccharides, is reverse hydrolysis by *β*-glucosidase. However, the low conversion efficiency of disaccharides from monosaccharides limits its large-scale production because the equilibrium is biased in the direction of hydrolysis. Based on the analysis of the docking results, we hypothesized that the hydropathy index of key amino acid residues in the catalytic site is closely related with disaccharide synthesis and more hydrophilic residues located in the catalytic site would enhance reverse hydrolysis activity. In this study, positive variants *Tr*Cel1b^{I177S}, *Tr*Cel1b$^{I177S/I174S}$, and *Tr*Cel1b$^{I177S/I174S/W173H}$, and one negative variant *Tr*Cel1b^{N240I} were designed according to the Hydropathy Index For Enzyme Activity (HIFEA) strategy. The reverse hydrolysis with *Tr*Cel1b$^{I177S/I174S/W173H}$ was accelerated and then the maximum total production (195.8 mg/ml/mg enzyme) of the synthesized disaccharides was increased 3.5-fold compared to that of wildtype. On the contrary, *Tr*Cel1b^{N240I} lost reverse hydrolysis activity. The results demonstrate that the average hydropathy index of the key amino acid residues in the catalytic site of *Tr*Cel1b is an important factor for the synthesis of laminaribiose, sophorose, and cellobiose. The

HIFEA strategy provides a new perspective for the rational design of β-glucosidases used for the synthesis of oligosaccharides.

KEYWORDS: β-glucosidase, hydropathy index, disaccharide synthesis, reverse hydrolysis reaction, site-directed mutagenesis

INTRODUCTION

Oligosaccharides are widely distributed in nature and are used in the food and medical industries.[1-9] Oligosaccharides are mainly prepared by extraction and isolation from a variety of natural plants, by chemical synthesis or biosynthesis.[1,10,11] Extraction from plants is limited by the source plant and its terrestrial distribution. The biosynthesis of oligosaccharides via enzymatic synthesis technique *in vitro* has recently received increasing attention due to attributes including mild reaction temperature and excellent regio- and stereo-selectivity without the need for masking of functional groups.[12] Enzymatic synthesis of oligosaccharides is mainly catalyzed by glycosidases or glycosyltransferases.[10] Synthesis of oligosaccharides by glycosidases has many advantages that include of simplicity, reliability, ease of operation, and inexpensive donor substrates,[13,14] compared with catalysis by glycosyltransferases, which requires activation and expensive donor substrates.[14,15] These facts favor glycosidase as an economically feasible approach in the production of oligosaccharides.[16] Furthermore, some high value-added rare oligosaccharides, such as laminaribiose, gentiobiose, and sophorose, have been produced using glycosidases.[9,17,18] These oligosaccharides reportedly have potential applications in food and enzyme industries.[19-22]

In addition to glycosidic bond cleavage, glycoside hydrolases (GHs) can be used for the synthesis of glycoside bonds *in vitro* via reverse hydrolysis reaction without the need for cofactors, such as uridine diphosphate.[3,10,16,23,24] Recently, several glycosidases, such as endo-α-*N*-acetylgalactosaminidase, *α*-mannosidase, *β*-galactosidase, and *β*-glucosidase, have been used to synthesize glycosides via the reverse hydrolysis

59 reaction.[3] 4-Butanoic acid-*N*-butyl-amide-1-*O*-*β*-D-glucopyranoside, 3-butanoic acid
60 ethyl ester-1-*O*-*β*-D-glucopyranoside, 2-(trimethylsilyl)-ethyl-1-*O*-*β*-D-
61 glucopyranoside, laminaribiose, sophorose, cellobiose, and gentiobiose have been
62 synthesized by via reverse hydrolysis reaction using *β*-glucosidase derived from
63 almond.[17,25] Furthermore, protein engineering of GHs has been widely used to eliminate
64 hydrolytic activity and improve synthetic activity. An important strategy is to disrupt
65 the binding of catalytic water. Honda et al. reported that hydrogen-bonding interaction
66 with catalytic water that reduced the hydrolytic reactivity of an inverting xylanase was
67 dramatically decreased by eliminating the retention of the nucleophilic water molecule
68 at the key amino acid residue.[26] Other studies have focused on improving the
69 hydrophobicity of the entrance to the active site[27,28] or acceptor subsite.[29]
70 In this study, we report the ability of *β*-glucosidase *Tr*Cel1b from *Trichoderma reesei*
71 to simultaneously catalyze the synthesis of three disaccharides (laminaribiose,
72 sophorose, and cellobiose) from glucose. The three-dimensional structure of *Tr*Cel1b
73 was obtained by SWISS-MODEL and docked with cellobiose as the model of
74 disaccharides. Based on the analysis of the docking results, we hypothesized that the
75 hydropathy index of key amino acid residues in the catalytic site is closely related with
76 disaccharide synthesis and more hydrophilic residues located in the catalytic site would
77 enhance reverse hydrolysis activity.
78 To verify our deduction, the Hydropathy Index For Enzyme Activity (HIFEA)
79 strategy was devised. Three hydrophobic amino acid residues in the catalytic site were
80 mutated into hydrophilic residues, which generated the maximal change in the

hydropathy index. Three variants were obtained: *Tr*Cel1b^{I177S}, *Tr*Cel1b$^{I177S/I174S}$, and *Tr*Cel1b$^{I177S/I174S/W173H}$. Additionally, the variant *Tr*Cel1b^{N240I} was obtained by improving the hydrophobicity in the catalytic site. The production of synthesized disaccharides by the three variants were investigated. Total production (195.8 mg/ml/mg enzyme) of the synthesized disaccharides was increased 3.5 times, compared to that of the wild type. Especially, the production of laminaribiose and sophorose reached 92.3 and 71.1 mg/ml/mg enzyme. The findings indicate the value of the HIFEA strategy in providing a new perspective for the rational design of β-glucosidases used for the synthesis of oligosaccharides.

RESULTS AND DISCUSSION

The rational design of *Tr*Cel1b. *Tr*Cel1b (GenBank no. EGR49111.1) in *T. reesei* belongs to the GH1 family and shares 92, 52, 52, 39, and 38% amino acid sequence identity with GH1 family *β*-glucosidase *Th*Bgl2 (5JBO), *Th*Bgl1 (5JBK), *Hi*BG (AII80277.1), *Nk*Bgl (BAB91145.1), and *Ca*BglA (JX030398.1), respectively. Mature *Tr*Cel1b consists of 484 amino acids with a molecular weight of 55.1 kDa. As shown in Fig. S1, *β*-glucosidases *Th*Bgl1 and *Th*Bgl2, which have a relatively close evolutionary relationship with *Tr*Cel1b, synthesize oligosaccharides.[30] The three-dimensional structure of *Tr*Cel1b was predicted based on the structure of its variant (PDB ID: 6KHT). The structure shared 100% identity with *Tr*Cel1b by SWISS-MODEL. A typical TIM-barrel structure of $(\alpha/\beta)_8$ fold was evident in *Tr*Cel1b (Fig. 2A), as well as the other GH1 family members, such as *β*-glucosidase *Th*Bgl2.

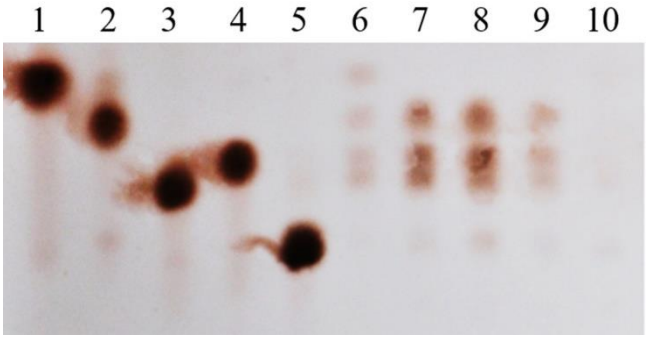

Figure 1. High-performance TLC analysis of products. Lane 1: Glucose; Lane 2: Laminaribiose; Lane 3: Cellobiose; Lane 4: Sophorose; Lane 5: Gentiobiose; Lane 6: Reaction solution synthesized by *Tr*Cel1b; Lane 7: Reaction solution synthesized by *Tr*Cel1b^{I177S}; Lane 8: Reaction solution synthesized by *Tr*Cel1b$^{I177S/I174S}$; Lane 9: Reaction

solution synthesized by *Tr*Cel1b$^{I177S/I174S/W173H}$; Lane 10: Reaction solution synthesized by *Tr*Cel1b^{N240I}.

To assess its function, *Tr*Cel1b was heterologously expressed in *Escherichia coli* was suspended in 4.4 M glucose solution at 30 ºC. Laminaribiose, sophorose, and cellobiose were synthesized by the *Tr*Cel1b suspension based on the result of thin layer chromatography analysis (Fig. 1). The results indicate that the three disaccharides were simultaneously produced though the reverse hydrolysis reaction with inexpensive glucose as the glucosyl donor. Moreover, the production of laminaribiose and sophorose was similar, with the production of cellobiose being markedly lower (Fig. S2 A-L, A-S, A-C).

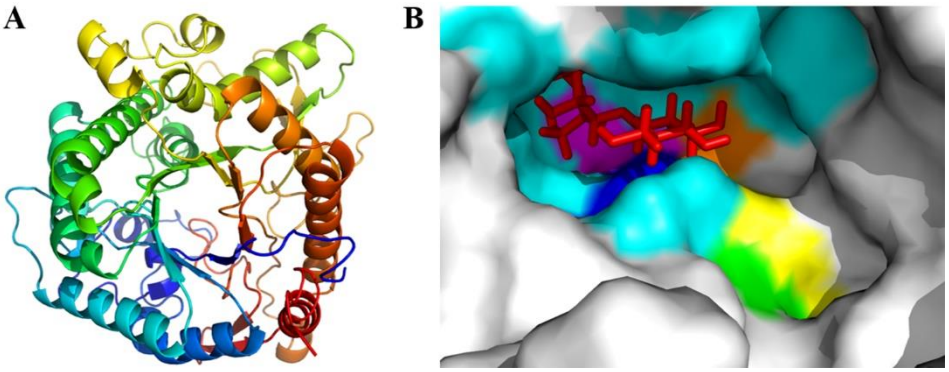

Figure 2. Structural-guided rational design of *β*-glucosidase *Tr*Cel1b. (A) Homology modeling of the 3-D structure of *Tr*Cel1b based on the crystal structure of 6KHT. (B) The flexible docking between *Tr*Cel1b and cellobiose. Cellobiose is depicted in red, the catalytic amino acid residues- E171 and E383 are depicted in magenta, W173, I174, I177 and N240 are depicted in yellow, blue, green, and orange, the hydrophilic amino acid residues around cellobiose are depicted in cyan.

To improve the production of disaccharides, interaction between *Tr*Cel1b and cellobiose as the model of disaccharide was predicted with Autodock 1.5.6. The result is presented in

Fig. 2B. The amino acid residues surrounded by the glucose moiety in aglycone subsite (+1 subsite) reportedly has a significant effect on synthesis capacity compared to the residues surrounded by the glucose moiety of cellobiose at the -1 subsite.[27,29-31] The glucose moiety of cellobiose in the aglycone subsite is surrounded by two hydrophobic residues, W173 and I174, and two hydrophilic residues, Y178 and N240, within a distance of 3.1 Å. The predicted distances between W173, I174, Y178, or N240 and cellobiose were 3.1, 2.8, 2.1, and 2.1 Å, respectively. The extremely hydrophobic residue I177 was also found at the +2 subsite at a distance of 6.4 Å. The hydropathy index of I, W, Y, and N was 4.5, −0.9, −1.3, and −3.5, respectively.

To verify our hypothesis, the HIFEA strategy was applied to improve the reverse hydrolysis activity by reducing the average hydropathy index of key amino acid residues in catalytic site (I_{ah}) of *Tr*Cel1b. I_{ah} was defined as the sum of the hydropathy index of amino acid residues 173, 174, 177, and 240 divided by their number, namely, $I_{ah} = (I_{h,173} + I_{h,174} + I_{h,177} + I_{h,240}) / 4$. Three hydrophobic amino acid residues in the catalytic site were mutated into hydrophilic residues and three variants *Tr*Cel1b^{I177S}, *Tr*Cel1b$^{I177S/I174S}$, and *Tr*Cel1b$^{I177S/I174S/W173H}$ were obtained. Additionally, the variant *Tr*Cel1b^{N240I} was obtained by improving the hydrophobicity in the catalytic site. The I_{ah} of *Tr*Cel1b and its variants was calculated according to the hydropathy index.[32] The I_{ah} of *Tr*Cel1b, *Tr*Cel1b^{I177S}, *Tr*Cel1b$^{I177S/I174S}$, *Tr*Cel1b$^{I177S/I174S/W173H}$, and *Tr*Cel1b^{N240I} was 1.15, −0.175, −1.5, −2.075, and 3.15, respectively. The findings revealed that I_{ah} changed along with the mutation. The hydrophobic interaction between these key amino acid residues and disaccharides was weakened when I_{ah} was reduced by the mutation of the hydrophobic residues located in the catalytic site of W173, I174, and/or I177 to the hydrophilic residues,

149 which facilitated the release of disaccharide. Finally, production of disaccharides
150 synthesized by reverse hydrolysis was improved. On the contrary, reverse hydrolysis was
151 repressed when the hydrophilic residues were mutated into hydrophobic residues.

152 Identification of products synthesized by *Tr*Cel1b wildtype and its variants. Purified
153 *Tr*Cel1b^{I177S}, *Tr*Cel1b$^{I177S/I174S}$, *Tr*Cel1b$^{I177S/I174S/W173H}$, and *Tr*Cel1b^{N240I} displayed a
154 similar molecular weight of 74 kDa, compared to the size of *Tr*Cel1b (Fig. S3), consistent
155 with the molecular weight predicted by the ExPASy website
156 (https://web.expasy.org/compute_pi/). The products synthesized by these variants, except
157 *Tr*Cel1b^{N240I}, were laminaribiose, sophorose, and cellobiose, respectively (Fig. 1, lane 7–
158 9). The hydrolysis activities of the variants decreased as the I_{ah} of variants decreased (Fig.
159 3A) and *β*-glucosidase activity of *Tr*Cel1b$^{I177S/I174S/W173H}$ was almost lost, compared to 0.35
160 U/mg soluble protein of *Tr*Cel1b. However, there was no significant change of *β*-
161 glucosidase activity between *Tr*Cel1b and *Tr*Cel1b^{N240I}. On the contrary, the production of
162 disaccharides was enhanced as the I_{ah} of the variants decreased (Fig. 3B). The disaccharide
163 production of *Tr*Cel1b$^{I177S/I174S/W173H}$ increased 3.5 times, reaching 195.8 mg/ml/mg
164 enzyme, compared with that of *Tr*Cel1b. On the contrary, the variant *Tr*Cel1b^{N240I} with an
165 I_{ah} of 3.15 displayed no disaccharide synthetic activity. As shown in Fig. S2, the
166 disaccharides laminaribiose, sophorose, and cellobiose were synthesized by *Tr*Cel1b^{I177S},
167 *Tr*Cel1b$^{I177S/I174S}$, and *Tr*Cel1b$^{I177S/I174S/W173H}$ the same as that of their wildtype protein.
168 Moreover, the production of laminaribiose, sophorose, and cellobiose synthesized by
169 *Tr*Cel1b^{I177S}, *Tr*Cel1b$^{I177S/I174S}$, and *Tr*Cel1b$^{I177S/I174S/W173H}$ were greater than those of
170 *Tr*Cel1b (Fig. S2). These results indicated that the decreased I_{ah} of *Tr*Cel1b was favorite
171 for the reverse hydrolysis reaction.

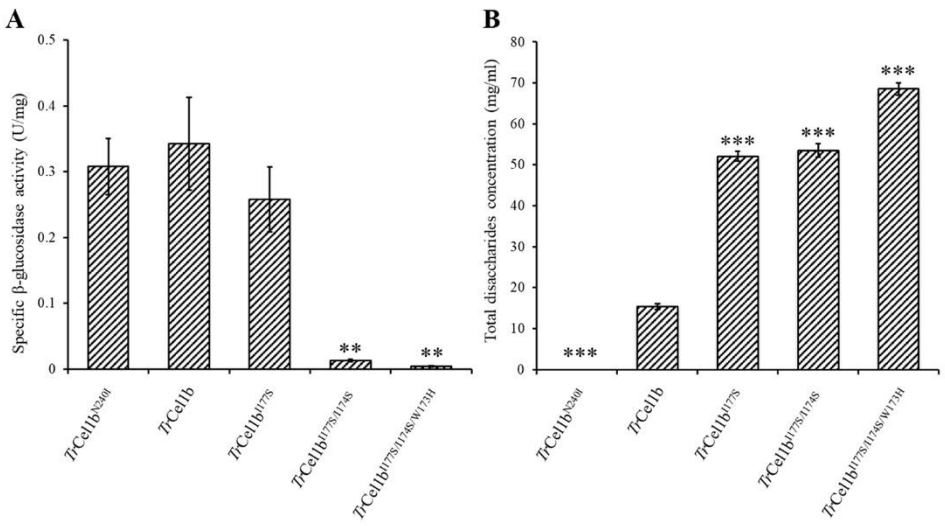

Figure 3. The specific *β*-glucosidase activity (A) of *Tr*Cel1b and its variants and the total disaccharides production (B) synthesized by *Tr*Cel1b and its variants using 80% (w/v) glucose as the substrate for 72 hours. **p*<0.05; ***p*<0.01; ****p*<0.001. The statistically significant difference was performed between *Tr*Cel1b and its variants.

Glucose was used as the substrate (10, 20, 40, 60, and 80%) for reverse hydrolysis and the laminaribiose, sophorose and cellobiose productions were measured by high-performance liquid chromatography (HPLC) (Fig. S2). The productions of laminaribiose, sophorose, and cellobiose productions were increased with increasing glucose concentration (10–80%), and the productions of laminaribiose and sophorose were increased when the I_{ah} value of the variants was decreased (Fig. S3). Laminaribiose production was the highest among the three disaccharides. The maximal laminaribiose production by *Tr*Cel1b^{I177S}, *Tr*Cel1b$^{I177S/I174S}$, and *Tr*Cel1b$^{I177S/I174S/W173H}$ reached 57.1, 78.9, 92.3 mg/ml/mg enzyme, and increased 1.8-, 2.8-, and 3.5 -fold, compared to that of *Tr*Cel1b, respectively. Sophorose production was markedly higher than that of cellobiose, and its maximal productions of *Tr*Cel1b^{I177S}, *Tr*Cel1b$^{I177S/I174S}$, and *Tr*Cel1b$^{I177S/I174S/W173H}$

reached 50.9, 56.9, 71.1 mg/ml/mg enzyme, respectively. These results indicated a direct relationship between the reverse hydrolysis activity and the I_{ah} value of *Tr*Cel1b.

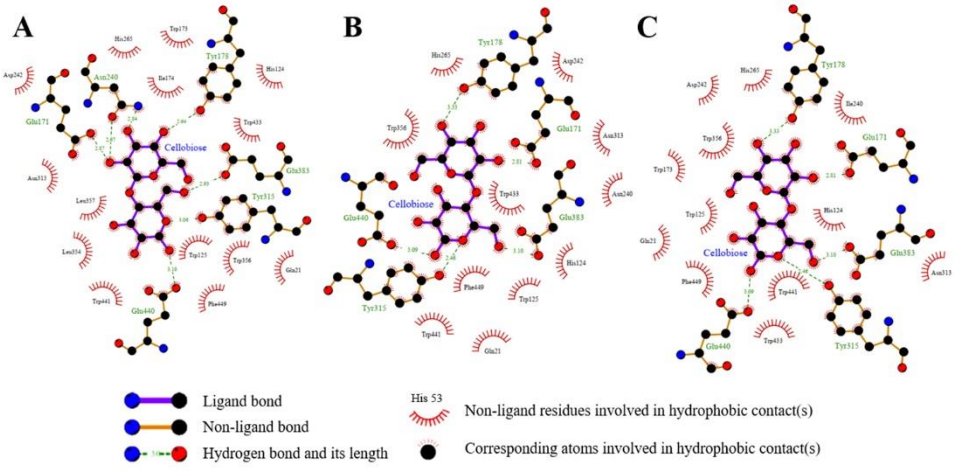

Figure 4. The interaction profiles between *Tr*Cel1b (A), *Tr*Cel1b^{I177SI174SW173H} (B) or *Tr*Cel1b^{N240I} (C) and cellobiose analyzed by LigPlot, respectively.

To study the effect of mutation on the interaction between protein and disaccharide, cellobiose was docked with *Tr*Cel1b and its variants using Autodock software and the result of docking was analyzed by LigPlot. The hydrophobic interaction between cellobiose and the amino acid residues of *Tr*Cel1b$^{I177S/I174S/W173H}$ (Fig. 4B) became weak, compared to that of *Tr*Cel1b (Fig. 4A). The number of residues that hydrophobically interacted with cellobiose in *Tr*Cel1b$^{I177S/I174S/W173H}$ and *Tr*Cel1b was 16 and 20, respectively. The hydrophobic interactions between the W173 and I174 residues and cellobiose disappeared since the two residues were mutated into the hydrophilic residues (Fig. 4A and 4B). These results were verified using Discovery studio (Fig. S4).

Compared with *Tr*Cel1b, the I_{ah} of *Tr*Cel1b$^{I177S/I174S/W173H}$ decreased, which was beneficial for the release of the cellobiose product. On the contrary, the I_{ah} of *Tr*Cel1b^{N240I} was enhanced and the synthesis activity of *Tr*Cel1b^{N240I} was completely lost (Fig. 3B)

owing to the mutation of the hydrophilic residue N240 to the hydrophobic residue isoleucine (Fig. 4C). The findings provided an obvious indication of a direct relationship between the I_{ah} value of *Tr*Cel1b and reverse hydrolysis activity. When the I_{ah} value of *Tr*Cel1b became negative, the reverse hydrolysis activity was enhanced. On the contrary, when the I_{ah} value of *Tr*Cel1b increased, the reverse hydrolysis activity was abolished. The findings are consistent with our hypothesis that the hydropathy index of key amino acid residues in the catalytic site is closely related with disaccharide synthesis.

Table 1. Thermodynamic parameters for the reverse hydrolysis from glucose to laminaribiose, cellobiose, and sophorose

Process	K_{eq} ($\times 10^{-3}$)	$\Delta_rG'^0$ (kJ/mol)	Δ_rG' (kJ/mol)
2 Glucose = Laminaribiose + H_2O	3.5	14.0 ± 6.0	-44.7 ± 6.0
2 Glucose = Cellobiose + H_2O	5.4	12.9 ± 3.7	-45.8 ± 3.7
2 Glucose = Sophorose + H_2O	3.4	14.1 ± 5.0	-44.6 ± 5.0

$\Delta_rG'^0$: the change in Gibbs free energy of the chemical reaction in standard 1 M concentrations of substrates and products at pH 7.4 with ionic strength of 0.05 M.

K_{eq}: the equilibrium constant of the chemical reaction in standard 1 M concentrations of substrates and products at pH 7.4 with ionic strength of 0.05 M.

Δ_rG': the change in Gibbs free energy of the chemical reaction in 4.4 M concentrations of substrate at pH 7.4 with ionic strength of 0.05 M.

In this study, an *β*-glucosidase *Tr*Cel1b from *T. ressei* was shown to simultaneously synthesize laminaribiose, sophorose, and cellobiose using a high concentration glucose as substrate. As shown in Table 1, the Δ_rG' of laminaribiose, sophorose, or cellobiose synthesis is <0 indicating it is realizable that laminaribiose, sophorose, and cellobiose were

produced from glucose by *Tr*Cel1b. Ravet et al. reported that the disaccharides were produced by *β*-glucosidase derived from almonds.[17] However, most of these disaccharides were gentiobiose, rather than laminaribiose and sophorose. This was the reason why the equilibrium constant (K_{eq}) of the reaction to synthesize laminaribiose, sophorose, and cellobiose (Table 1) was markedly lower than that of gentiobiose (53.8×10^{-3}). There are few reports on laminaribiose and sophorose synthesis, reflecting their low production. To improve the production of disaccharides synthesized by *Tr*Cel1b, protein engineering was performed using the HIFEA strategy. The production of laminaribiose, sophorose, and cellobiose synthesized by *Tr*Cel1b$^{I177S/I174S/W173H}$ was increased 3.5-, 2.6-, and 3.9-fold, respectively, compared to that of *Tr*Cel1b (Fig. S2). Compared with reported *β*-glucosidases from different species (Table S1), the maximal productions of laminaribiose and sophorose by *Tr*Cel1b$^{I177S/I174S/W173H}$ reached 92.3 and 71.1 mg/ml/mg enzyme, respectively, and were higher than the results produced by *β*-glucosidases from *Aspergillus niger, Corynascus* sp.*, Penicillium verruculosum*, *T. reesei*,[18] and almond.[17] To our knowledge, this is the highest production of laminaribiose and sophorose simultaneously synthesized by *β*-glucosidase.

CONCLUSIONS

In summary, *β*-glucosidase *Tr*Cel1b from *T. reesei* simultaneously synthesized laminaribiose, sophorose, and cellobiose. Three variants (*Tr*Cel1b^{I177S}, *Tr*Cel1b$^{I177S/I174S}$, and *Tr*Cel1b$^{I177S/I174S/W173H}$) with improved disaccharide production were obtained using the HIFEA strategy. The I_{ah} of *β*-glucosidase *Tr*Cel1b is an important factor for the production of laminaribiose, sophorose, and cellobiose. The decreased I_{ah} value of *Tr*Cel1b

248 improved the synthetic activity and reduced the hydrolytic activity. The HIFEA strategy is
249 implicated as a new avenue for the production of high value-added rare disaccharides.
250
251 EXPERIMENTAL SECTION
252 Chemicals, plasmids, and culture media. Laminaribiose, sophorose, *p*-nitrophenol (*p*NP),
253 and *p*-nitrophenol-*β*-D-glucoside(*p*NPG) were purchased from Sigma-Aldrich Corporation
254 (St. Louis, MO, USA). Kanamycin and isopropyl-1-thio-*β*-D-galactopyranoside (IPTG)
255 were purchased from Gen-view Scientific Inc. (El Monte, CA, USA). The KOD-Plus-
256 Mutagenesis Kit was purchased from Toyobo Co., Ltd. (Osaka, Japan). All other chemicals
257 were from Sangon Biotech Co., Ltd. (Shanghai, China). Plasmid pET-32a was purchased
258 from Invitrogen (Carlsbad, CA, USA). Restriction enzymes and T4 DNA ligase were
259 purchased from Thermo Fisher Scientific (Shanghai, China). Primers were synthesized by
260 Sangon Biotech Co., Ltd. *Escherichia coli* DH5α and *E. coli* BL21 (DE3) was purchased
261 from TransGen Biotech (Beijing, China).
262 Site-directed mutagenesis. *Tr*Cel1b (GenBank accession number: AAP57758.1) was
263 amplified from the cDNA of *T. reesei* QM6a (ATCC 13631) with primers harboring *EcoR*I
264 and *Hind*III sites, and ligated into pET-32a after it was digested with the same enzymes to
265 obtain the recombinant vector pET-32a-WT. The recombinant vectors pET-32a-I177S,
266 pET-32a-I177S/I174S, pET-32a-I177S/I174S/W173H, and pET-32a-N240I were
267 constructed using the aforementioned KOD-Plus-Mutagenesis Kit. Oligonucleotides used
268 in this study for plasmid constructions are listed in Supplementary Table S2.
269 Expression and purification. The constructed vectors were introduced into *E. coli*
270 BL21(DE3) for protein expression and transformants were selected on LB plates

271 containing 10 μg/ml ampicillin as previously described.[33] These proteins were purified
272 from the supernatant with His SpinTrap columns (GE Healthcare, Uppsala, Sweden) as
273 previously described.[34] The purified protein of *Tr*Cel1b and its variants was analyzed by
274 sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described.[35]
275 Measurement of *β*-glucosidase activity and enzymatic synthesis of disaccharides. *β*-
276 glucosidase activity was measured as we previously described, except that the temperature
277 was 30 ºC.[36] The unit of *β*-glucosidase activity were defined as the amount of enzyme
278 required to release total reducing sugar equivalent to 1 μmol *p*NP per min. Ten milliliter
279 reaction mixtures, containing 100, 200, 400, 600, or 800 g glucose/l, 500 μl glycerol, and
280 10 mg sodium nitride in 50 mM phosphate buffer at pH 7.4, were loaded with 3.5 mg of
281 *Tr*Cel1b and its variants. The reactions were carried out in 50 ml centrifuge tube at 30 °C
282 for 72 h. Three independent replicates were carried out and reaction samples were taken at
283 0, 2, 4, 6, 8, 10, 19, 24, 34, 48, and 72 h.
284 The products were analyzed by HPLC as we previously described.[37] An Inertsil NH_2
285 column (250 mm × 7.8 mm; Shimadzu, Kyoto, Japan) and 80% acetonitrile as the mobile
286 phase (1.0 ml/min, 45 °C) were used. Synthesized disaccharides products were detected by
287 TLC following the removal of glucose from the solution. TLC was performed on
288 aluminum-backed sheets of silica gel $60F_{254}$ (E. Merck) that were 0.2 mm thick. Elution
289 was carried out with n-butanol: ethanol: water (5:3:2). The plates were visualized by
290 exposure to staining solution containing 3 g phenol, 5 ml concentrated sulfuric acid and 95
291 ml alcohol followed by charring. Thermodynamic parameters for the reverse hydrolysis
292 reaction from glucose to laminaribiose, sophorose, and cellobiose at pH 7.4 with an ionic
293 strength of 0.05 M was calculated using the eQuilibrator.[38]

The phylogenetic and structural analysis. The phylogenetic tree of *Tr*Cel1b (GenBank accession no. EGR49111.1) from *T. reesei* QM6a, *Th*Bgl2 (5JBO) from *T. harzianum*, *Th*Bgl1 (5JBK) from *T. harzianum*, *Hi*BG (AII80277.1) from *Humicola insolens*, *Nk*Bgl (BAB91145.1) from *Neotermes koshunensis* and *Ca*BglA (JX030398.1) from *Caldicellulosiruptor* sp. F32 was generated using MEGA. The three-dimensional structures of *Tr*Cel1b, *Tr*Cel1b^{I177S}, *Tr*Cel1b$^{I177S/I174S}$, *Tr*Cel1b$^{I177S/I174S/W173H}$, and *Tr*Cel1b^{N240I} were predicted using SWISS-MODEL[39] with the crystal structure of *Tr*Cel1b-H13 (PDB ID: 6KHT) as the template. The structures were illustrated using PyMOL software (Delano Scientific, Palo Alto, CA). The interaction between protein and cellobiose was analyzed by LigPlot[40] and Discovery Studio Software (Accelrys, San Diego, CA, USA).

Statistics. The Student's t-test was performed for significant differences between two groups of data. $P<0.05$ was considered statistically significant and standard deviations (SD) were calculated at least in triplicate.

ASSOCIATED CONTENT

Supporting Information

Figure S1. The phylogenetic tree of *Tr*Cel1b and other GH1 family *β*-glucosidases.

Figure S2. The disaccharides synthesis of *β*-glucosidase and its variants under different concentrations of glucose.

Figure S3. SDS-PAGE analysis of purification of *Tr*Cel1b and its variants.

Figure S4. The interaction profiles between *Tr*Cel1b (A), *Tr*Cel1b^{I177SI174SW173H} (B) or *Tr*Cel1b^{N240I} (C) and cellobiose analyzed by Discovery Studio 4.5, respectively.

Table S1. The comparation of the production of laminaribiose and sophorose synthesized by *β*-glucosidase from different species.

Table S2. Primers used in this study.

AUTHOR INFORMATION

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Author Contributions

K.N., Z.L., Y.F. and T.G. carried out protein mutagenesis and purification. K.N., Z.W., P.Z., D.G. carried out biochemical assays. K.N., Z.L. and X.F. carried out structural modeling and docking. Z.W. and X.F. conceived of the study. Z.D. and X.F. oversaw experimental and computational work. K.N., Z.D. and X.F. designed experiments and wrote the manuscript. X.F. coordinated the project.

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Notes

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