1 **Multi-functional glycoside hydrolase: Blon_0625 from *Bifidobacterium***
2 ***longum subsp. infantis* ATCC 15697**

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21 **Keywords:** β -D-glucosidase, β -D-xylosidase, multi-functional, cellulase

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25 **Introduction**

26 Lignocellulosic materials are an attractive, environmentally friendly
27 feedstock since sustainable biomass can be converted into bio-based chemicals or
28 liquid fuels such as bioethanol [1, 2]. Lignocellulosic materials, including grass,
29 wood, and their residues, are predominantly composed of cellulose, hemicellulose
30 and lignin. To harness these materials for bioconversion processes using
31 microorganisms, they must be degraded into compounds that can be used as
32 nutrients by microorganisms. Cellulose and hemicellulose consist of
33 polysaccharides, which most microorganisms cannot utilize [3]. Hence, the
34 degradation of lignocellulose to soluble sugars has been a key issue for the
35 production of bio-based chemicals and liquid fuels. Enzymatic hydrolysis
36 methodologies using cellulase are one of the best solutions to this problem as
37 cellulose. There are three major types of cellulases: endoglucanases,
38 exoglucanases, and beta-glucosidases. Endoglucanases (EG; EC 3.2.1.4)
39 randomly cleave the internal bonds of cellulosic materials, exoglucanases such as
40 cellobiohydrolase (CBH; EC 3.2.1.91/EC 3.2.1.176) degrade cellulose to
41 cellobiose units from the reducing/non-reducing end, and β -D-glucosidases (BGL;
42 EC 3.2.1.21) hydrolyze celloorigosaccharides or cellobiose to glucose units.
43 Hemicellulose is broken down into soluble xylose or other types of saccharide by
44 hemicellulases such as xylanase (XYN; EC 3.2.1.8), xylosidase (XYL; EC
45 3.2.1.37) and mannosidase (EC 3.2.1.25) [4].

46 Cellulose and hemicellulose are hydrolyzed to glucose, xylose or other
47 types of monosaccharide units by concerted catalysis by these enzymes. For

instance, *Trichoderma reesei* is a well-known cellulase-overproducing filamentous fungus which secretes at least five types of EG and two types of CBH. Therefore, a *T. reesei*-produced cellulase cocktail has been widely used as a commercial enzyme mixture in order to degrade cellulosic materials [5, 6]. Anaerobic cellulolytic bacteria such as *Clostridium thermocellum* produce a multi-cellulase and hemicellulase complex referred to as a cellulosome. In microbial cellulosomes, dockerin-containing enzymes are immobilized on the scaffoldin subunit to form a docking backbone. This backbone contains a cellulose binding module (CBM) and multiple dockerin-binding cohesion modules [7]. The cellulosome is designed for the efficient degradation of cellulosic materials, but it is not overproduced in bacteria the way cellulases are overproduced in fungi. Therefore, artificial cellulosomes (i.e., designer cellulosomes) have been constructed to provide a better understanding of cellulosomes and also to enhance the utility of cellulosomes [8, 9]. Although the utilization of fungal enzymes or cellulosomes is one solution for the efficient degradation of lignocellulose, a recent paper demonstrated a new approach by reporting the discovery of a new enzyme, a multi-functional glycoside hydrolase. Hess et al. reported metagenomic analyses conducted to characterize biomass degrading genes and genomes from cow rumen microbes [10]. Rumen is a unique environment for the degradation of cellulosic plant materials. Several novel enzymes which display both BGL and XYL activity have been identified from rumen microbes [11, 12]. In addition, a multi-functional BGL has recently been identified from another bacterium [13]. Multi-functional enzymes, which could play a versatile role in the degradation of lignocellulosic

71 materials, have attracted attention because fewer enzymes would be required to
72 degrade lignocellulosic materials, resulting in cost savings for industrial
73 conversion.

74 Here we describe a unique beta-glucosidase (BGL; Blon_0625) derived
75 from *Bifidobacterium longum subsp. infantis* ATCC 15697. We previously cloned
76 more than 30 kinds of xylosidases from various microorganisms, and the XYL
77 activities were evaluated by plate assays [14]. Then we found that some of them
78 also have the hydrolyzing activity against *p*NPG. The Blon_0625 gene was
79 expressed by recombinant *E. coli*, and the purified protein was shown to retain
80 hydrolyzing activity against both *p*-nitrophenyl- β -D-glucopyranoside (*p*NPG) and
81 *p*-nitrophenyl- β -D-xylopyranoside (*p*NPX). Furthermore Blon_0625 also retains
82 activity against 4-nitrophenyl- α -L-arabinofuranoside (*p*NPAf) and several kinds of
83 oligosaccharides. To our knowledge, no previously-described BGL retains the
84 same level of *p*NPGase and *p*NPXase activity. In addition, the results of the
85 degradation of cellulosic and hemicellulosic substrates using EG and XYN show
86 that Blon_0625 acts as a BGL and a XYL for hydrolyzing oligosaccharides. These
87 results clearly indicate that Blon_0625 is a multi-functional glycoside hydrolase
88 which retains BGL, XYL and also α -L-arabinofuranosidase activity.

89

90 **Materials and methods**

91 **Chemicals and materials**

92 1,4- β -D-cellobiose, 1,4- β -D-cellopentaose, and 1,4- β -D-xylotriose were purchased
 93 from Megazyme (Bray Co., Ireland). Xylobiose was purchased from WAKO
 94 (Osaka, Japan). 4-Nitrophenyl- β -D-cellobioside (*p*NPC) was purchased from
 95 TOKYO Chemical Industry Co., Ltd. (Tokyo, Japan). *p*NPAf and cellobiose were
 96 purchased from Sigma-Aldrich (St. Louis, MO, USA). KOD FX DNA polymerase
 97 was purchased from TOYOBO (Osaka, Japan), *p*ET22-b(+) and *p*Cold II vector,
 98 TALON metal affinity resin, BCA protein assay kit, and *Escherichia coli*
 99 BL21(DE3) strain were purchased from Takara Bio Inc. (Shiga, Japan), *p*NPG,
 100 *p*NPX and all other chemicals were purchased from Nacalai Tesque (Kyoto,
 101 Japan).

102

103 **Gene expression and protein purification**

104 Three types of BGL (Blon_0625; *Bifidobacterium longum subsp. infantis* ATCC
 105 15697, Tfu_0937; *Thermobifida fusca* YX, Sde_1394; *Saccharophagus*
 106 *degradans* 2-40) were expressed and purified as follows. KOD FX DNA
 107 polymerase was used for polymerase chain reaction (PCR), and PCR-amplified
 108 sequences were verified by DNA sequencing. The gene encoding Blon_0625 was
 109 obtained from the *Bifidobacterium longum subsp. infantis* ATCC 15697 genome
 110 as a template with
 111 5'-GGAGATATACATATGAGCGAGTCCACCTACCCGTCCGTC-3' as the 5'
 112 primer and 5'-GTGCTCGAGTGCGGCCGCC

113 TTCGCGAGCTTGGCGTACTC-3' as the 3' primer. The amplified fragment was
 114 subcloned into the *NdeI/NotI* sites of the pET22-b(+) vector to yield
 115 pET22-Blon_0625, which was introduced into *E. coli* BL21(DE3). In a similar
 116 manner, the BGL genes encoding Sde_1394 was obtained from the
 117 *Saccharophagus degradans* 2-40 (ATCC number: 43961D-5) genome as
 118 templates, respectively, using the following primers:
 119 5'-GAAGGAGATATACATATGAATAGACTTACACTACCGCCTTCTTCTCG-3'
 120 as the 5' primer and 5'-GTGCTCGAGTGCGGCCGCG
 121 CTCCTACTCGAGACAACTC AGCAAATGCG-3' as the 3' primer (Sde_1394).
 122 The amplified fragments were subcloned into the *NdeI/NotI* sites of the
 123 pET22-b(+) vector to yield pET22-Sde_1394, which was introduced into *E. coli*
 124 BL21(DE3). The genes encoding Tfu_0937 was obtained from *Thermobifida*
 125 *fusca* YX (ATCC number BAA629D-5)
 126 5'-CATCATCATCATATGGTGACCTCGCAATCG-3' as the 5' primer and 5'-
 127 ACAAGCTTGAATTCCTATTCCTGTCCGAA-3' as the 3' primer (Tfu_0937).
 128 The amplified fragment was subcloned into the *NdeI/HindIII* sites of the pCold II
 129 vector to yield pCold II-Tfu_0937, which was introduced into *E. coli* BL21(DE3).
 130 In addition, EG and XYN (Tfu_0901; *T. fusca* YX, KSE_59480; *Kitasatospora*
 131 *setae* KM-6054) were expressed and purified as follows. The gene encoding
 132 Tfu_0901 (or KSE_59480) was obtained from the *T. fusca* YX (or *K. setae*
 133 KM-6054) genome (NBRC number 14216G) as a template using the following
 134 primers:
 135 5'-GGAGATATACATATGCTCACCGCCACAGTCACCAAAGAATCCTCG-3 as

the 5' primer and 5'- gtgctcgagtgcggccgcGGACTGGAGCTTGCTCCG-3' as the
3' primer (Tfu_0901),
5'-GGAGATATACATATGGCTTCCCCCACCACCCCCACCGCAGAACG-3'
as the 5' primer and
5'-CTCGAGTGCGGCCGCGGCGGTGGTGCAGGAGACGGTCGGCCAGGT-3'
' as the 3' primer (KSE_59480). The amplified fragments were subcloned into the
NdeI/NotI sites of the pET22-b(+) vector to yield pET22-Tfu_0901 and
KSE_59480, which were also introduced into *E. coli* BL21(DE3). Cells were
grown in Luria-Bertani (LB) medium at 37 °C to an optical density (OD; 600 nm)
of 0.5-0.7, then the cells were incubated for an additional 30 min at 25 °C (15 °C
if pCold II vector was used). Protein expression was induced by adding
isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM.
After incubation for an additional 24 h at 25 °C (15 °C if pCold II vector was
used), the cells were harvested by centrifugation. The cell pellets were
resuspended in 20 mM phosphate buffer (pH 8.0) containing 150 mM NaCl, then
lysed using sonication. Each protein was purified from the soluble fraction using
TALON metal affinity resin according to the manufacturer's protocol and then
dialyzed against 50 mM phosphate buffer (pH 8.0) containing 150 mM NaCl. The
concentration of each purified protein was determined using a BCA protein assay
kit.

Enzymatic activity assays

The activity of β -D-glucosidase, β -D-xylosidase and α -L-arabinofuranosidase was

analyzed using *p*NPG, *p*NPC, *p*NPX and *p*NPAf as substrates. The enzyme solutions were incubated in 20 μ l of 10 mM substrate in 100 mM phosphate buffer (pH 6.0) for 10 min at 30 °C, then 500 μ l of 1 M Na₂CO₃ was added to stop the reaction. The amount of obtained *p*-nitrophenol was measured at 400 nm. One unit of specific activity was defined as 1 μ mol nitrophenol generated in one minute. The pH dependence of β -D-glucosidase and β -D-xylosidase activity was also determined at 30 °C for 10 min in various 100 mM buffers: citrate (pH 3.0-6.0), phosphate (pH 6.0-8.0) and carbonic acid (pH 8.0-10.0). The kinetic parameters of BGLs were estimated with *p*NPG and *p*NPX as the substrate. The values of the Michaelis constant, K_m , and the reaction velocity were determined by the activities at several substrate concentrations: 0.1-2.0 mM.

The hydrolysis of oligosaccharides by Blon_0625

To evaluate the hydrolysis ability of Blon_0625, 500 μ l of Blon_0625 solution (50 μ g) was incubated with 500 μ l of 10 mg mL⁻¹ cellobiose, cellotriose, cellopentaose, xylobiose or xylotriose in phosphate buffer (pH 6.0, 50 mM) overnight at 37 °C. Also, mixed sugar (cellobiose and xylobiose) was tested as a substrate to evaluate the simultaneous degradation of oligosaccharides by Blon_0625. The obtained glucose and xylose concentrations were determined using an HPLC equipped with a SUGAR-KS-800 column (SHOWA DENKO, Kanagawa, Japan) at 50 °C using water as the mobile phase at a flow rate of 1.0 ml min⁻¹. Products were detected with a RID-10A refractive index detector (Shimadzu, Kyoto, Japan).

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183 The degradation of xylan or phosphoric acid swollen cellulose (PASC)

184 To degrade xylan or PASC, Blon_0625 (250 nM), KSE_59480 (5 nM) or

185 Tfu_0901 (50 nM) were added to 50 mM phosphate buffer (pH 7.0) containing 5

186 mg mL⁻¹ xylan or PASC. After incubation at 37 °C for 18 h (using xylan as the

187 substrate) or 24 h (using PASC as the substrate), the concentration of reducing

188 sugar was analyzed using the tetrazolium blue (TZ) assay. Briefly, 5 µl of the

189 sample supernatant was mixed with 195 µl of TZ assay buffer [15] at 100 °C for 5

190 min, then the reaction mix was cooled on ice and the absorbance at 660 nm was

191 measured. The concentration of the reducing sugars was calculated from the

192 absorbance at 660 nm against glucose in TZ buffer.

Results

Characterization of purified BGLs

Four BGLs were expressed in recombinant *E. coli* BL21(DE3) strain. Tfu_0937 is a family 1 glycoside hydrolase (GH1) BGL from *Thermobifida fusca* YX. We previously showed that expressed Tfu_0937 efficiently degraded cellooligosaccharide to glucose on the *E. coli* cell surface [16]. We also previously reported that Tfu_0937 was expressed and secreted efficiently by recombinant *Streptomyces lividans* [17]. Sde_1394 is also a GH1 BGL, from *Saccharophagus degradans* 2-40. We previously showed that Sde_1394 expressed on the *Corynebacterium glutamicum* cell surface contributes to cellobiose degradation [18]. The other BGL was GH3 BGL: Blon_0625 is derived from *Bifidobacterium longum subsp. infantis* ATCC 15697. These expressed BGLs were purified from the soluble fraction using His-tag affinity resin and identified by SDS-PAGE analysis as major bands with the following masses: Tfu_0937; 53 kDa, Sde_1394; 50 kDa, Blon_0625; 81 kDa) (Fig. 1).

The kinetic parameters of these BGLs were determined by incubating with *p*NPG and *p*NPX. In Table 1, Blon_0625 exhibited a more distinct response against *p*NPG and *p*NPX compared to the other three BGLs. Although Tfu_0937 and Sde_1394 retained hydrolyzing activity only against *p*NPG (Sde_1394 retains low activity against *p*NPX), purified Blon_0625 retained comparable hydrolyzing activity against both *p*NPG and *p*NPX. To our knowledge, no BGL that retains similar *p*NPGase ($17.3 \pm 0.24 \text{ U mg}^{-1}$) and *p*NPXase ($16.7 \pm 0.32 \text{ U mg}^{-1}$) activity has been previously reported (Table 2). In addition, further investigation showed

216 that Blon_0625 also retains activity against *p*NPAf and *p*NPC, with 32.5%
217 (5.6 ± 0.09 U mg^{-1}) and 38.0% (6.6 ± 0.00 U mg^{-1}) relative activity compared to
218 *p*NPGase, respectively (Table 2). These results clearly indicated that Blon_0625 is
219 a multi-functional glycoside hydrolase which retains the activity of
220 β -D-glucosidase, β -D-xylosidase and α -L-arabinofuranosidase.

221 The pH dependence of *p*NPGase and *p*NPXase activity was also
222 determined. Although Blon_0625 displayed somewhat lower pH stability against
223 *p*NPX compared to *p*NPG, Blon_0625 retained both *p*NPGase and *p*NPXase
224 activity at a broad pH range between 4.0-8.0, with maximum activity at pH
225 5.0-8.0 against *p*NPG and at pH 6.0-7.0 against *p*NPX (Fig. 2). These results
226 showed that Blon_0625 exhibits better pH dependency against *p*NPG than against
227 *p*NPX. These results also indicate that Blon_0625 would have different catalytic
228 mechanisms because they have two catalytic domains in an enzyme.

229

230 The hydrolysis of oligosaccharides by Blon_0625

231 To harness the multi-functionality of Blon_0625, its hydrolyzing activity against
232 various oligosaccharides was assessed. We first determined the hydrolyzing
233 activity of Blon_0625 against 5 mg mL^{-1} of cellobiose or xylobiose. As shown in
234 Fig 3, Blon_0625 hydrolyzed cellobiose almost completely into glucose ($4.31 \pm$
235 0.00 mg mL^{-1}), and 40% of xylobiose was hydrolyzed into xylose (1.49 ± 0.05 mg
236 mL^{-1}). The other BGLs (Sde_1394, Tfu_0937) could not hydrolyze xylobiose into
237 xylose (Fig 3b). These results clearly showed that Blon_0625 has both
238 β -D-glucosidase (*p*NPGase) and β -D-xylosidase (*p*NPXase) activity, as mentioned

239 above. In addition, Blon_0625 degraded 5 mg mL⁻¹ of various
240 cellooligosaccharides into glucose (46% of cellotriose (2.04 ± 0.25 mg mL⁻¹
241 glucose); 63% of cellopentaose (2.89 ± 0.05 mg mL⁻¹ glucose)). This result
242 indicates that Blon_0625 can hydrolyze several types of cellooligosaccharides.
243 Moreover, we examined the hydrolysis of mixed oligosaccharides (5 mg mL⁻¹
244 cellobiose and 5 mg mL⁻¹ xylobiose) by Blon_0625 and observed that Blon_0625
245 cannot hydrolyze cellobiose and xylobiose simultaneously: although cellobiose
246 was almost totally degraded into glucose (86%, 4.31 ± 0.01 mg mL⁻¹), no
247 xylobiose was degraded into xylose. It is possible that the generated glucose
248 inhibited the hydrolysis of xylobiose. Indeed, Blon_0625 could not hydrolyze
249 xylobiose in the presence of glucose (data not shown). Accordingly, Blon_0625
250 may prefer cellobiose as a substrate to xylobiose, and the generated glucose
251 inhibits the hydrolysis of xylobiose to xylose.

252

253 The degradation of polysaccharides by Blon_0625

254 Blon_0625 was utilized to degrade polysaccharides such as cellulose and
255 hemicellulose. Cellulose and hemicellulose consist of a large number of glucose
256 (xylose) units conjugated with glycosidic bonds. Several types of enzymes are
257 required to degrade polysaccharides. For example, EG (CBH) and BGL are
258 required for the degradation of cellulose, and XYN and XYL are required for the
259 degradation of hemicellulose. As discussed previously, Blon_0625 displays both
260 BGL and XYL activity. Therefore, PASC and xylan were degraded using
261 Tfu_0901 (EG) and Blon_0625 (BGL), and KSE_59480 (XYN) and Blon_0625

(XYL), respectively (Fig 4). Fig. 5 shows that the use of both Tfu_0901 and Blon_0625 to degrade PASC resulted in a higher concentration of produced reducing sugars than when only Tfu_0901 was used. A similar result was obtained for the degradation of xylan: the concentration of produced reducing sugars was higher when both KSE_59480 and Blon_0625 were used than when only KSE_59480 was used (Fig 6). These results indicate that Blon_0625 can degrade cello- and xylooligosaccharide to glucose or xylose.

Discussion

Lignocellulosic materials are an attractive feedstock because they are environmentally friendly, since sustainable biomass can be bioconverted to chemicals and biofuels using microorganisms. However, most microorganisms utilize monosaccharides such as glucose and xylose, and cannot directly process polysaccharides. Hence, a saccharification process is required to first hydrolyze polysaccharides into monosaccharides. Cellulase and hemicellulase play important roles in degrading lignocellulosic biomass. Cellulose and hemicellulose are hydrolyzed to glucose and xylose, respectively, by the concerted catalysis of several cellulases and hemicellulases. Among these, BGL and XYL are key enzymes which catalyze the hydrolysis of oligosaccharides to monosaccharides. BGL (EC 3.2.1.21) is classified as GH1, GH3, GH9, GH30 and GH116 in the CAZy database, while XYL (EC 3.2.1.37) is classified as GH1, GH3, GH30, GH39, GH43, GH51, GH52, GH54, GH116 and GH120. Blon_0625, identified in the present study, is classified as a GH3 carbohydrate-active enzyme, and exhibits

285 activity against several types of substrates. Although similar multi-functional
286 enzymes have been recently reported, to our knowledge no multi-functional
287 enzyme that retains the same level of both *p*NPGase and *p*NPXase activity has
288 been previously reported. For example, bi-functional BGL/XYL RuBG3A and
289 RuBG3B, cloned from yak rumen microbes, display higher *p*NPGase activity than
290 *p*NPXase activity [11]. BglX has been cloned from *Lactococcus sp. FSJ4*, and
291 displays higher *p*NPXase activity than *p*NPGase activity [13]. Gruninger et al.
292 identified Bgxa1 from cow rumen and showed it to be highly active against *p*NPG,
293 *p*NPX and *p*NPAf. However, its catalytic efficiency towards XYL, and its
294 α -L-arabinofuranosidase activity, is 100 times lower than its BGL activity [12]. In
295 contrast, Blon_0625 retains hydrolyzing activity towards *p*NPAf, *p*NPC,
296 cellobiose, celloligosaccharide and xylobiose. A synergetic effect was observed in
297 the breakdown of cellulosic and hemicellulosic substrate using Tfu_0901 and
298 KSE_59480, respectively, and Blon_0625. It is therefore clear that Blon_0625 is a
299 bi-functional BGL/XYL enzyme.

300 The results of the degradation of mixed oligosaccharides showed that
301 Blon_0625 cannot hydrolyze cellobiose and xylobiose simultaneously. The
302 competitive inhibition of BGL by glucose is a common problem [19], so the
303 generated glucose likely inhibited the hydrolysis of xylobiose. We separately
304 demonstrated that Blon_0625 cannot hydrolyze xylobiose in the presence of
305 glucose. Although Blon_0625 is not a glucose-tolerant XYL, several BGLs have
306 been identified which are highly tolerant to glucose [20, 21]. Therefore, further
307 structural investigation may lead to improved glucose-tolerance of Blon_0625.

308 This is important since the practical enzymatic hydrolysis of lignocellulose
309 produces monosaccharides and several kinds of residual oligosaccharides, so the
310 co-assimilation of mixed oligosaccharides is important for microbial
311 bioconversion [22, 23]. Hence, a system in which the produced glucose is
312 immediately consumed, such as simultaneous saccharification and fermentation
313 (SSF), would facilitate the use of multi-functional Blon_0625 [24].

314 In conclusion, a multi-functional glycoside hydrolase, Blon_0625, was
315 identified from *Bifidobacterium longum subsp. infantis* ATCC 15697. The purified
316 Blon_0625 retains comparable hydrolyzing activity against both *p*NPG and *p*NPX.
317 Also, Blon_0625 retains hydrolyzing activity against *p*NPAf, cellobiose,
318 xylobiose and several kinds of cellooligosaccharide. The utilization of
319 multi-functional Blon_0625 may help facilitate both the efficient degradation of
320 lignocellulosic materials and the realization of commercial bioconversion
321 processes.

322 **Acknowledgements**

323 This work was supported by Special Coordination Funds for Promoting Science
324 and Technology from the Creation of Innovation Centers for Advanced
325 Interdisciplinary Research Areas (Innovation Bioproduction Kobe), MEXT, Japan,
326 and by the Advanced Low Carbon Technology Research and Development
327 Program (ALCA) of the Japan Science and Technology Agency (JST).

328

329 **Conflict of interest**

330 The authors declare that they have no conflict of interest.

331

References

- [1] Elkins JG, Raman B, Keller M. Engineered microbial systems for enhanced conversion of lignocellulosic biomass. *Curr Opin Biotechnol* 2010;21:657-62
- [2] Peralta-Yahya PP, Zhang F, del Cardayre SB, Keasling JD. Microbial engineering for the production of advanced biofuels. *Nature* 2012;488:320-328
- [3] Huffer S, Roche CM, Blanch HW, Clark DS. *Escherichia coli* for biofuel production: bridging the gap from promise to practice. *Trends Biotechnol* 2012;30:538-545
- [4] Percival Zhang YH, Himmel ME, Mielenz JR. Outlook for cellulase improvement: screening and selection strategies. *Biotechnol Adv* 2006;24:452-481
- [5] Montenecourt BS and Eveleigh DE. Preparation of mutants of *Trichoderma reesei* with enhanced cellulase production. *Appl Environ Microbiol* 1977;34:777-782
- [6] Henrissat B, Driguez H, Viet C, Schülein M. Synergism of Cellulases from *Trichoderma reesei* in the Degradation of Cellulose. *Nat Biotech* 1985;3:722-726

- 355 [7] Doi RH and Tamaru Y. The *Clostridium cellulovorans* cellulosome: an enzyme
356 complex with plant cell wall degrading activity. Chem Rec 2001;1:24-32
357
- 358 [8] Caspi J, Barak Y, Haimovitz R, Gilary H, Irwin DC, Lamed R, Wilson DB,
359 Bayer EA. *Thermobifida fusca* exoglucanase Cel6B is incompatible with the
360 cellulosomal mode in contrast to endoglucanase Cel6A. Syst Synth Biol
361 2010;4:193-201
362
- 363 [9] Vazana Y, Morais S, Barak Y, Lamed R, Bayer EA. Designer cellulosomes for
364 enhanced hydrolysis of cellulosic substrates. Methods Enzymol 2012;510:429-452
365
- 366 [10] Hess M, Sczyrba A, Egan R, Kim TW, Chokhawala H, Schroth G, Luo S,
367 Clark DS, Chen F, Zhang T, Mackie RI, Pennacchio LA, Tringe SG, Visel A,
368 Woyke T, Wang Z, Rubin EM. Metagenomic discovery of biomass-degrading
369 genes and genomes from cow rumen. Science 2011;331:463-467
370
- 371 [11] Bao L, Huang Q, Chang L, Sun Q, Zhou J, Lu H. Cloning and
372 characterization of two β -glucosidase/xylosidase enzymes from yak rumen
373 metagenome. Appl Biochem Biotechnol 2012;166:72-86
374
- 375 [12] Gruninger RJ, Gong X, Forster RJ, McAllister TA. Biochemical and kinetic
376 characterization of the multifunctional β -glucosidase/ β -xylosidase/ α -arabinosidase,

- 377 Bgxa1. Appl Microbiol Biotechnol 2014;98:3003-12
378
- 379 [13] Fang S, Chang J, Lee YS, Guo W, Choi YL, Zhou Y. Cloning and
380 characterization of a new broadspecific β -glucosidase from *Lactococcus sp.* FSJ4.
381 World J Microbiol Biotechnol 2014;30:213-223
382
- 383 [14] Tanaka T, Hirata Y, Nakano M, Kawabata H, Kondo A. Creation of
384 Cellobiose and Xylooligosaccharides-Coutilizing *Escherichia coli* Displaying both
385 β -Glucosidase and β -Xylosidase on Its Cell Surface. ACS Synth. Biol.,
386 2014;3:446-453.
387
- 388 [15] Jue CK and Lipke PN. Determination of reducing sugars in the nanomole
389 range with tetrazolium blue. J Biochem Biophys Methods 1985;11:109-115
390
- 391 [16] Tanaka T, Kawabata H, Ogino C, Kondo A. Creation of a
392 celooligosaccharide-assimilating *Escherichia coli* strain by displaying active
393 beta-glucosidase on the cell surface via a novel anchor protein. Appl Environ
394 Microbiol 2011;77:6265-6270
395
- 396 [17] Noda S, Ito Y, Shimizu N, Tanaka T, Ogino C, Kondo A. Over-production of
397 various secretory-form proteins in *Streptomyces lividans*. Protein Expr Purif
398 2010;73:198-202
399

- 400 [18] Adachi N, Takahashi C, Ono-Murota N, Yamaguchi R, Tanaka T, Kondo A.
401 Direct L-lysine production from cellobiose by *Corynebacterium glutamicum*
402 displaying beta-glucosidase on its cell surface. Appl Microbiol Biotechnol
403 2013;97:7165-7172
404
- 405 [19] Andrić P, Meyer AS, Jensen PA, Dam-Johansen K. Reactor design for
406 minimizing product inhibition during enzymatic lignocellulose hydrolysis: I.
407 Significance and mechanism of cellobiose and glucose inhibition on cellulolytic
408 enzymes. Biotechnol Adv 2010;28:308-324
409
- 410 [20] Jabbour D, Klippel B, Antranikian G. A novel thermostable and
411 glucose-tolerant β -glucosidase from *Fervidobacterium islandicum*. Appl
412 Microbiol Biotechnol 2012;93:1947-1956
413
- 414 [21] Rajasree KP, Mathew GM, Pandey A, Sukumaran RK. Highly glucose
415 tolerant β -glucosidase from *Aspergillus unguis*: NII 08123 for enhanced
416 hydrolysis of biomass. J Ind Microbiol Biotechnol 2013;40:967-975
417
- 418 [22] Jojima T, Omumasaba CA, Inui M, Yukawa H. Sugar transporters in efficient
419 utilization of mixed sugar substrates: current knowledge and outlook. Appl
420 Microbiol Biotechnol 2010;85:471-480
421
- 422 [23] Kim SR, Ha SJ, Wei N, Oh EJ, Jin YS. Simultaneous co-fermentation of

423 mixed sugars: a promising strategy for producing cellulosic ethanol. Trends
424 Biotechnol 2012;30:274-282.

425

426 [24] Bokinsky G, Peralta-Yahya PP, George A, Holmes BM, Steen EJ, Dietrich J,
427 Lee TS, Tullman-Ercek D, Voigt CA, Simmons BA, Keasling JD. Synthesis of
428 three advanced biofuels from ionic liquid-pretreated switchgrass using engineered
429 *Escherichia coli*. Proc Natl Acad Sci U S A. 2011;108:19949-19954

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433 **Tables**434 **Table 1** Kinetic parameters for the hydrolysis of *p*NPG and *p*NPX using BGLs

	<i>p</i> NPG			<i>p</i> NPX		
	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$
Tfu_0937	26.6±0.4	0.39±0.03	68.9±3.3	ND	ND	ND
Sde_1394	45.5±0.9	0.74±0.03	61.5±2.8	ND	ND	ND
Blon_0625	23.7±1.0	0.27±0.02	89.3±8.7	20.2±1.2	0.28±0.03	73.3±10.6

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436 ND: not detected

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438

439 **Table 2** Substrate specificity of Blon_0625

Substrates	Specific activity (U mg ⁻¹)	Relative activity (%)
<i>p</i> NPG	17.3±0.2	100
<i>p</i> NPX	16.6±0.3	96.4
<i>p</i> NPC	6.6±0.00	38.0
<i>p</i> NPAf	5.6±0.09	32.5

Figure Legends

Fig. 1 SDS-PAGE analysis of the purified BGLs. Lane M: Dual color standard marker. Lane 1: Tfu_0937 (53 kDa). Lane 2: Sde_1394 (50 kDa). Lane 3: Blon_0625 (81 kDa)

Fig. 2 The influence of pH on the activity of Blon_0625 against *p*NPG (closed circle) and against *p*NPX (open circle).

Fig. 3 The degradation of cellobiose (a) or xylobiose (b) using Blon_0625 (column 1), Sde_1394 (column 2) and Tfu_0937 (column 3). Error bars represent the standard error from triplicate measurements.

Fig. 4 The schematic illustration of polysaccharides degradation using Blon_0625.

Fig. 5 The degradation of phosphoric acid swollen cellulose (PASC) using only Blon_0625 (open circle), using only Tfu_0901 (closed square), or using Tfu_0901 and Blon_0625 (open square). Error bars represent the standard error from triplicate measurements.

Fig. 6 The degradation of xylan using only Blon_0625 (open circle), using only KSE_59480 (closed square), or using KSE_59480 and Blon_0625 (open square). Error bars represent the standard error from triplicate measurements.

Figure. 1

Figure. 2

Figure. 3

Figure. 4

Figure. 5

Figure. 6