

## Regular Paper

# Effect of C-6 Methylol Groups on Substrate Recognition of Glucose/Xylose Mixed Oligosaccharides by Cellobiose Dehydrogenase from the Basidiomycete *Phanerochaete chrysosporium*

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**Abstract:** Cellobiose dehydrogenase (CDH) is a flavocytochrome catalyzing oxidation of the reducing end of cellobiose and cellooligosaccharides, and has a key role in the degradation of cellulosic biomass by filamentous fungi. Here, we use a lineup of glucose/xylose-mixed  $\beta$ -1,4-linked disaccharides and trisaccharides, enzymatically synthesized by means of the reverse reaction of cellobiose phosphorylase and cellodextrin phosphorylase, to investigate the substrate recognition of CDH. We found that CDH utilizes  $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-glucopyranose (Xyl-Glc) as an electron donor with similar  $K_m$  and  $k_{cat}$  values to cellobiose.  $\beta$ -D-Glucopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose (Glc-Xyl) shows a higher  $K_m$  value, while xylobiose does not serve as a substrate. Trisaccharides show similar behavior; i.e., trisaccharides with cellobiose and Xyl-Glc units at the reducing end show similar kinetics, while the enzyme was less active towards those with Glc-Xyl, and inactive towards those with xylobiose. We also use docking simulation to evaluate substrate recognition of the disaccharides, and we discuss possible molecular mechanisms of substrate recognition by CDH.

**Key words:** cellobiose dehydrogenase, *Phanerochaete chrysosporium*, flavin adenine dinucleotide, docking simulation

## INTRODUCTION

Cellulose degradation by filamentous fungi is one of the most important processes in the carbon cycle on Earth, because even though cellulose is an abundant biomass, relatively few organisms can degrade cellulose.<sup>1)</sup> Cellulose-degrading microbes typically utilize hydrolytic enzymes called cellulases and  $\beta$ -glucosidases to obtain glucose, the repeating unit of cellulose, as an energy source.<sup>2)3)4)5)6)7)8)</sup> Although these enzyme reactions require only water molecules, Eriksson and coworkers found that the rate of cellulose decomposition by filamentous fungal cellulases was greater in an oxygen atmosphere than in a nitrogen

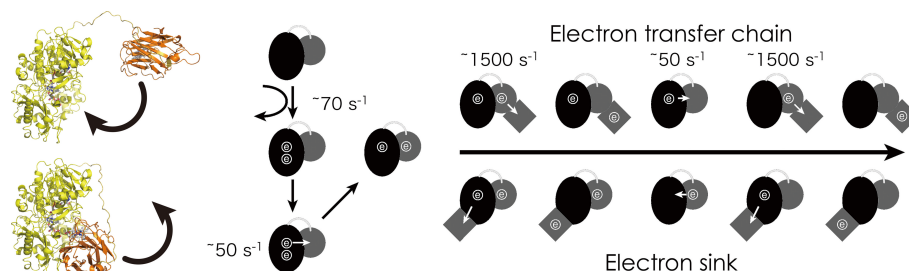
atmosphere, clearly indicating a contribution of oxidation to cellulose degradation by fungi.<sup>9)10)11)12)13)14)15)</sup> Indeed, an oxidative enzyme named cellobiose dehydrogenase (CDH: EC 1.1.99.18), initially known as cellobiose oxidase (CBO: EC 1.1.3.25), was discovered in cellulolytic cultures of the wood-rotting fungus *Sporotrichum pulverulentum*, an anamorph of *Phanerochaete chrysosporium*.<sup>9)11)</sup>

CDH is an extracellular enzyme that oxidizes the reducing end of cellobiose and cellooligosaccharides, hydrolytic products of cellulose, to the corresponding  $\delta$ -lactones, using various quinones and ferric compounds as electron acceptors.<sup>9)</sup> CDH consists of two major domains, i.e., the flavin- and heme-containing domains.<sup>16)</sup> The flavin chromophore in the flavin domain of CDH, which belongs to the glucose-methanol-choline oxidoreductase family,<sup>17)</sup> is flavin-adenine dinucleotide (FAD), and the flavin domain is responsible for the oxidation of cellobiose.<sup>18)19)20)</sup> In the heme domain, one *b*-type heme is located at the surface of the domain;<sup>21)</sup> it receives an electron from flavin when the substrate is oxidized,<sup>22)</sup> and then the electron is transferred

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Abbreviations: CDH, cellobiose dehydrogenase; FAD, flavin adenine dinucleotide.

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**Fig. 1.** Domain movement (left) and proposed electron transfer mechanisms (right) of CDH.

Black ellipse, flavin domain; gray circle, cytochrome domain; gray square, cytochrome *c*; CB, cellobiose; CBL, cellobionolactone.

to an electron acceptor such as ferri-cytochrome *c*.<sup>23,24</sup> An electron sink model,<sup>25</sup> in which flavin radical (FADH•) is the electron donor, was proposed for the electron transfer mechanism, as shown at the bottom of Fig. 1. On the other hand, a subsequent pre-steady-state kinetic study using a three-syringe stopped-flow apparatus provided evidence for an electron-transfer chain mechanism (top of Fig. 1) in which one-electron-reduced heme is the electron donor of the ferric compound.<sup>26</sup>

Cellobiose ( $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-glucopyranose) is a good substrate of CDH, whereas xylobiose ( $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose) is not recognized by the enzyme.<sup>27,28</sup> Although the mechanism of inter-domain electron transfer from reduced flavin to heme has been analyzed in detail as noted above, the initial oxidation step of the substrate is still unclear.<sup>29</sup> Considering the chemical structures and contents of polysaccharides in cellulolytic biomass and the products formed from them by hydrolytic enzymes, both cellobiose and xylobiose could be produced during the course of degradation. The crystal structure of the complex of cellobionolactam (5-amino-5-deoxycellobiono-1,5-lactam) with the flavin domain of CDH has been reported.<sup>30</sup> However, the substrate-binding site does not appear to contain any amino acid residue capable of hydrogen-bonding with the C-6 methylol group of reducing- or non-reducing-end glucose units, which makes interpretation of the substrate recognition mechanism difficult.

In the present study, we examined the steady-state kinetics of CDH using gluco/xylo oligosaccharides with  $\beta$ -1,4-glycosidic bonds as substrates in order to clarify the role of the C-6-methylol group in substrate recognition by the enzyme. We also carried out docking simulation of glucose/xylose disaccharides with the flavin domain to find the reason why cellobiose but not xylobiose serves as a substrate.

## MATERIAL AND METHODS

**Materials.** Cellobiose was purchased from ICN Biomedicals, Inc. (Irvine, CA, USA). Xylobiose and bovine heart cytochrome *c* were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Cellotriase and xylotriase were purchased from Seikagaku Co. (Tokyo, Japan). Recombinant wild-type *P. chrysosporium* CDH was heterologously expressed in the methylotrophic yeast *Pichia pastoris* and purified as described previously.<sup>31,32</sup>  $\beta$ -D-Glucopyrano-

syl-(1 $\rightarrow$ 4)-D-xylopyranose (Glc-Xyl) were synthesized using the reverse reaction of cellobiose phosphorylase from *Cellvibrio gilvus*,<sup>33</sup> and  $\beta$ -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-glucopyranose (Xyl-Glc) was chemically synthesized as described previously.<sup>34</sup> Glucose/xylose trisaccharides were synthesized using cellodextrin phosphorylase from *Clostridium thermocellum* as described previously.<sup>35</sup> Chemical structures of all substrates used in this study are summarized in Fig. 2.

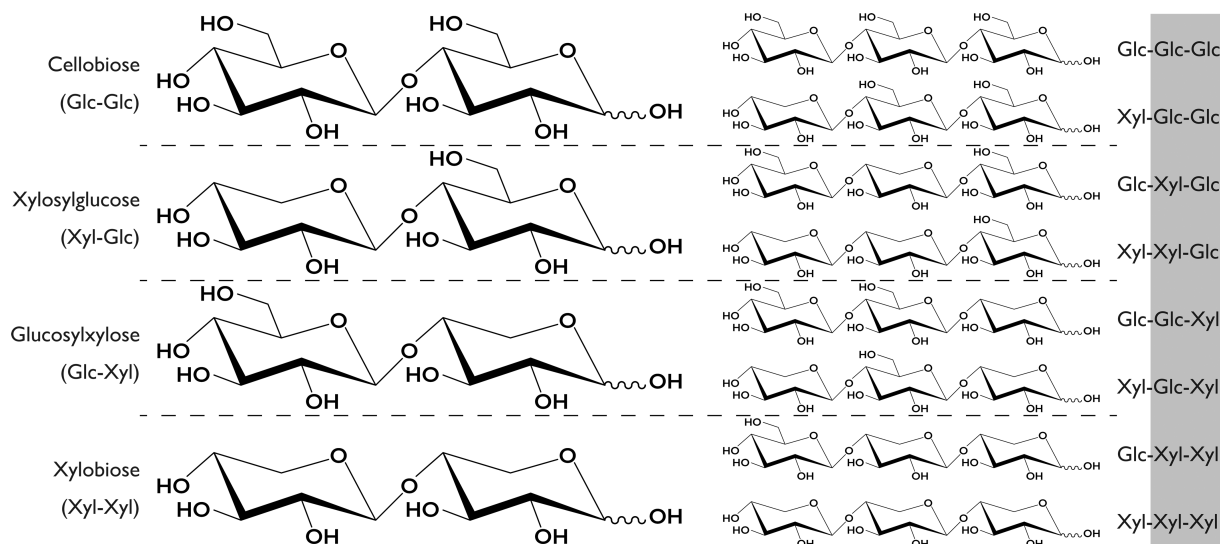
**Steady-state enzyme assays.** To obtain the steady-state kinetic parameters of oligosaccharide oxidation, the reduction rate of 50  $\mu$ M cytochrome *c* was plotted versus substrate concentration (0 to 250  $\mu$ M). The reduction of cytochrome *c* was monitored photometrically at 550 nm with  $\Delta\epsilon_{550} = 17.5 \text{ mM}^{-1} \text{ cm}^{-1}$ .<sup>15,23,26</sup> Each measurement was done in triplicate unless otherwise noted, and steady-state kinetic parameters ( $K_m$  and  $k_{cat}$ ) were estimated by non-linear fitting of the data to the Michaelis-Menten equation using GraFit version 7.0.2 (Erithacus Software Ltd., London, UK).

**Docking simulation of glucose/xylose disaccharides.** The interaction between the flavin domain and cellobiose, Xyl-Glc, Glc-Xyl, or xylobiose was examined using AutoDock Vina<sup>36</sup> running on the UCSF Chimera (Ver. 1.11.2 build 41376). The X-ray structure of the flavin domain of CDH with cellobionolactam as the ligand (PDB ID 1NAA<sup>30</sup>) was used as a template. The cellobionolactam in the PDB file was replaced by cellobiose obtained from the X-ray structure of *P. chrysosporium* endo-glucanase PcCel45A (PDB ID 3X2M<sup>37</sup>) by using MacPyMol (Ver. 1.8.0.6, Schrödinger Inc., NY, USA). After the superposition, the methylol group of the glucose unit at the non-reducing end (Xyl-Glc), reducing end (Glc-Xyl), or both (xylobiose) was eliminated to introduce a xylose unit(s). The PDB files obtained above were input to AutoDock Vina with a 15 Å cubic space as a search area for docking simulation as shown in Fig. 3. The obtained data were evaluated in terms of the position of the ligands relative to FAD (N4), catalytic histidine (His689), and asparagine (Asn732). The most plausible structures of the flavin domain and each glucose/xylose disaccharide were input to LigPlot<sup>+</sup><sup>38</sup> (Ver. 4.5.3) to visualize the interactions with surrounding amino acids.

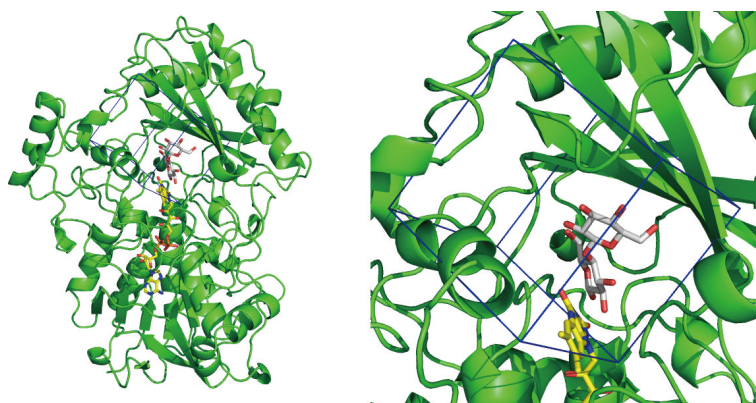
## RESULTS AND DISCUSSION

### Kinetics of glucose/xylose disaccharides.

We first tested the activity of  $\beta$ -1,4-linked mixed disaccharides, Xyl-Glc and Glc-Xyl, in addition to cellobiose



**Fig. 2.** Chemical structures of oligosaccharides used in this study. In the case of trisaccharides, disaccharides used as primers are highlighted.



**Fig. 3.** Three-dimensional structure of the flavin-containing dehydrogenase domain of CDH (left) and closed view at the active site (right).

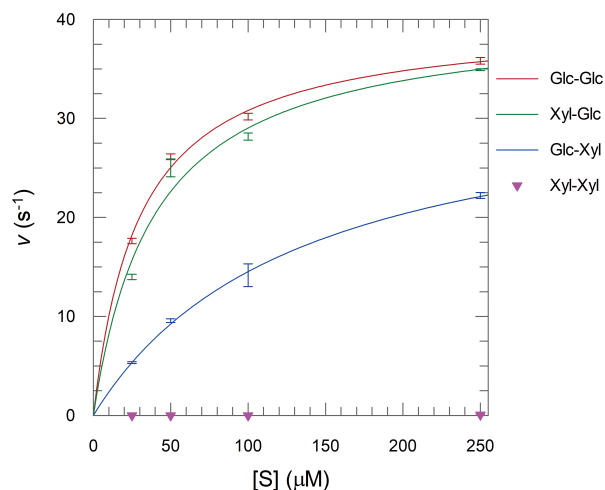
The 15 Å cubic space shown is the search area for docking simulation in AutoDock Vina. The figure was prepared from PDB ID:1NAA with the superposition of cellobiose instead of cellobionolactam.

and xylobiose. As shown in Fig. 4 and Table 1, cytochrome *c*-reducing activity was observed with Xyl-Glc and Glc-Xyl as substrates, as well as cellobiose, whereas xylobiose was not a substrate for the enzyme, as reported previously.<sup>27)28)</sup> The activity of CDH towards mixed disaccharides has been tested previously for  $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 4)-D-mannopyranose (Glc-Man) and  $\beta$ -D-mannopyranosyl-(1 $\rightarrow$ 4)-D-glucopyranose (Man-Glc); Glc-Man was half as good a substrate as cellobiose, while Man-Glc and mannobiose ( $\beta$ -D-mannopyranosyl-(1 $\rightarrow$ 4)-D-mannopyranose) did not serve as substrates.<sup>9)16)18)</sup> These results suggest that the C-2 position of non-reducing-end glucose is important for substrate recognition by CDH, but that of the reducing-end glucose is not. Considering that CDH cannot oxidize xylobiose, one of the C-6 methylol groups should play an important role in the catalytic activity of CDH. A comparison of the  $K_m$  values of the activity for cellobiose and mixed disaccharides indicates that the affinity is decreased in the absence of C-6 methylol groups. The C-6 methylol group at the reducing-glucose unit is especially important, because Glc-Xyl has a 4.4 times higher  $K_m$  value than cellobiose, while the  $K_m$  value is only 1.6 times higher for

Xyl-Glc. The  $k_{cat}$  values of cellobiose, xylosylglucose and glucosylxylose were all like each other, suggesting that the removal of one of the C-6 methylol groups does not affect the oxidative reaction itself.

#### **Kinetics of glucose/xylose trisaccharides.**

CDH catalyzes the oxidation of not only cellobiose, but also cellooligosaccharides with higher DP as substrates.<sup>9)17)29)</sup> Therefore, we next tested the activity of CDH towards glucose/xylose trisaccharides. As shown in Fig. 2, eight different oligosaccharides can be prepared by the permutation of two sugar moieties (glucose and xylose) at three positions (non-reducing, middle, and reducing) in the case of trisaccharides with a single  $\beta$ -1,4-linkage. Their reactivity with CDH are given in Fig. 5 and Table 2. The affinity is decreased when the DP is increased, i.e., the  $K_m$  value for cellotriose is 3.0 times higher than that for cellobiose. This is reasonable because the flavin domain of CDH does not contain any amino acid residue interacting favorably with the third glucose unit from the reducing end, so trisaccharide has a steric disadvantage compared with cellobiose. This is also supported by the higher affinity



**Fig. 4.** Substrate concentration vs catalytic velocity (S-V) plot for Glc/Xyl disaccharides.

**Table 1.** Kinetic parameters of CDH for glucose/xylose mixed disaccharides.

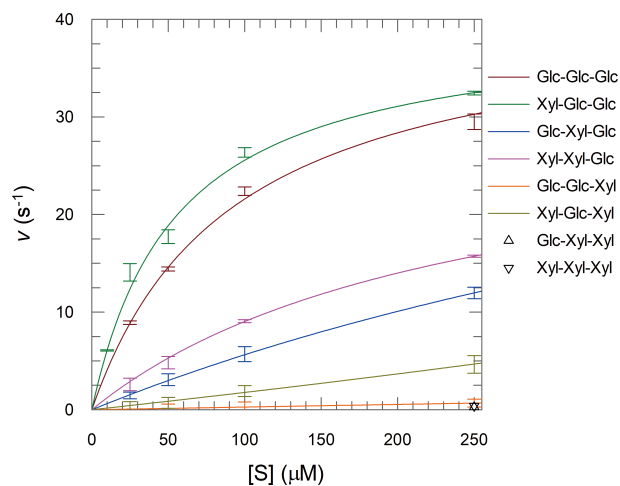
Substrate	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$k_{\text{cat}}/K_m$ ( $\text{s}^{-1} \cdot \text{mM}^{-1}$ )
Cellobiose	$30.1 \pm 3.7$	$40.1 \pm 1.5$	$1332 \pm 123$
Xyl-Glc	$47.6 \pm 4.9$	$41.6 \pm 0.8$	$873 \pm 74$
Glc-Xyl	$132 \pm 6$	$34.0 \pm 1.0$	$258 \pm 6$
Xylobiose	n.d.	n.d.	$< 0.5$

n.d., not determined due to high  $K_m$  values.

(lower  $K_m$  value) of Xyl-Glc-Glc than cellobiose, because xylose is smaller than glucose and so its steric effect should be less than that of glucose. The activity of CDH towards glucose/xylose trisaccharides was dependent on the nature of the reducing-end two-sugar unit. The activity was high for trisaccharide with a cellobiose unit at the reducing end, and the trend is similar to that for disaccharides, i.e., catalytic efficiency ( $k_{\text{cat}}/K_m$ ) is in the order cellobiose > Xyl-Glc > Glc-Xyl, and a non-reducing xylose unit is favored over glucose as described above. These results are not unexpected, considering that CDH oxidizes the reducing end of oligosaccharides, and the active site in the flavin domain has a pocket-like architecture.

#### Docking simulation of glucose/xylose disaccharides.

To understand the effect of C-6 methylol groups at the reducing and non-reducing glucose unit, interactions between CDH and glucose/xylose disaccharides were evaluated by means of molecular docking simulations. Since the X-ray structure of the flavin domain of CDH complexed with a substrate analogue (cellobionolactam) is available,<sup>30</sup> the docking simulation was performed using the protein structure as a starting template, and the substrate analogue was replaced with cellobiose, Xyl-Glc, Glc-Xyl, or xylobiose. The results of the simulations are summarized in Table 3. For each docking simulation, nine probable docking states were selected based on the strength of binding affinity, which was similar for all 4 disaccharides ( $-6.6$  to  $-7.4$  kcal/mol). However, when the conformations of the bound ligands are evaluated in terms of the distance from



**Fig. 5.** Substrate concentration vs catalytic velocity (S-V) plot for Glc/Xyl trisaccharides.

**Table 2.** Kinetic parameters of CDH for glucose/xylose mixed trisaccharides.

Substrate	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$k_{\text{cat}}/K_m$ ( $\text{s}^{-1} \cdot \text{mM}^{-1}$ )
Cellobiose	$91.5 \pm 8.8$	$41.4 \pm 2.4$	$452 \pm 19$
Xyl-Glc-Glc	$55.0 \pm 1.7$	$39.6 \pm 0.5$	$721 \pm 16$
Glc-Xyl-Glc	$752 \pm 102$	$48.0 \pm 5.1$	$63.8 \pm 2.0$
Xyl-Xyl-Glc	$243 \pm 13$	$31.0 \pm 0.9$	$128 \pm 3$
Glc-Glc-Xyl	n.d.	n.d.	$2.7 \pm 0.1$
Xyl-Glc-Xyl	n.d.	n.d.	$17.1 \pm 2.2$
Glc-Xyl-Xyl	n.d.	n.d.	$< 2$
Xylobiose	n.d.	n.d.	$< 2$

n.d., not determined due to high  $K_m$  values.

the cofactor (FAD) and catalytically important amino acids (His689 and Asn732), 3 binding states are within a catalytically feasible distance for cellobiose, whereas the only single binding state was reasonable for Xyl-Glc or Glc-Xyl.

The interactions of amino acids at the active site with the disaccharides (cellobiose, Xyl-Glc, and Glc-Xyl) at their most probable positions, i.e., where the binding energy is high enough to keep the substrate at an appropriate location, at suitable distances from the cofactor and the catalytic residues, are summarized in Fig. 6. As predicted from the previous liganded structure of the flavin domain, there is little interaction around the C-6 methylol group at the reducing-end glucose unit, and the role, if any, of this group in fixing the substrate is uncertain. However, the total number of hydrogen bonds for cellobiose (=8) is higher than that for Xyl-Glc (=6) or Glc-Xyl (=6), in good accord with the lower  $K_m$  value of cellobiose, compared with the other disaccharides. It is interesting that in 4 of 9 possible binding states of xylobiose, the non-reducing glucose unit points towards the cofactor and catalytic amino acids; in other words, the substrate binds in a “reversed” direction. There are 9 hydrogen bonds fixing the reversed binding of xylobiose, and this binding mode is the strongest, with the lowest binding energy, among the substrates examined in the docking simulations. Since this raises the possibility that xylobiose acts as an inhibitor of the enzymes, we ex-



**Table 3.** Results of AutoDock simulation for disaccharides.

Ligand	Rank	Score (kcal/mol)	RMSD l.b. (Å)	RMSD u.b. (Å)	Binding state <sup>a</sup>	Distance (Å)		
						FAD(N4)-C1	His689-O1	Asn732-O5
Cellobiose	1	-7.1	0	0	High	2.4	2.9	3.3
	2	-7.0	2.188	6.755				
	3	-6.9	1.733	5.846				
	4	-6.9	1.402	1.821	High	2.3	3.2	3.1
	5	-6.9	1.492	2.865				
	6	-6.8	2.137	3.584				
	7	-6.6	1.685	6.270				
	8	-6.6	1.655	1.713	High	1.9	3.0	3.7
	9	-6.6	2.073	6.439				
Xyl-Glc	1	-7.2	0	0				
	2	-7.2	2.572	6.224				
	3	-7.0	2.786	5.262	Low	3.6	2.9	4.3
	4	-7.0	2.611	4.211	High	2.8	3.0	3.6
	5	-6.9	2.964	5.679				
	6	-6.9	2.929	5.123				
	7	-6.8	2.427	5.169				
	8	-6.8	2.494	5.949				
	9	-6.8	2.480	5.343				
Glc-Xyl	1	-7.2	0	0				
	2	-7.1	1.847	3.287	High	2.2	2.9	3.1
	3	-7.0	1.368	6.683				
	4	-6.9	1.956	6.279				
	5	-6.9	2.043	5.843				
	6	-6.9	1.976	6.598				
	7	-6.8	2.581	6.587				
	8	-6.6	1.770	5.797				
	9	-6.6	1.549	3.332				
Xylobiose	1	-7.4	0	0	Rev			
	2	-7.3	2.794	6.799				
	3	-7.2	1.267	6.499	Rot			
	4	-7.1	2.103	6.684	High	2.0	2.9	3.0
	5	-7.0	2.215	3.219	Rev			
	6	-7.0	2.514	3.701	Rev			
	7	-7.0	1.490	2.405	Rev+Rot			
	8	-6.9	2.040	2.514				
	9	-6.9	2.095	6.234				

<sup>a</sup>The state of ligand binding is evaluated in terms of the relative position with respect to FAD. High, likely to react; Low, unlikely to react; Rev, non-reducing end of the ligand points towards FAD; Rot, upside down between  $\alpha$ - and  $\beta$ -face. No indication means that the closest atom is too close (less than 1.5 Å) or too far (more than 4.0 Å) from FAD.

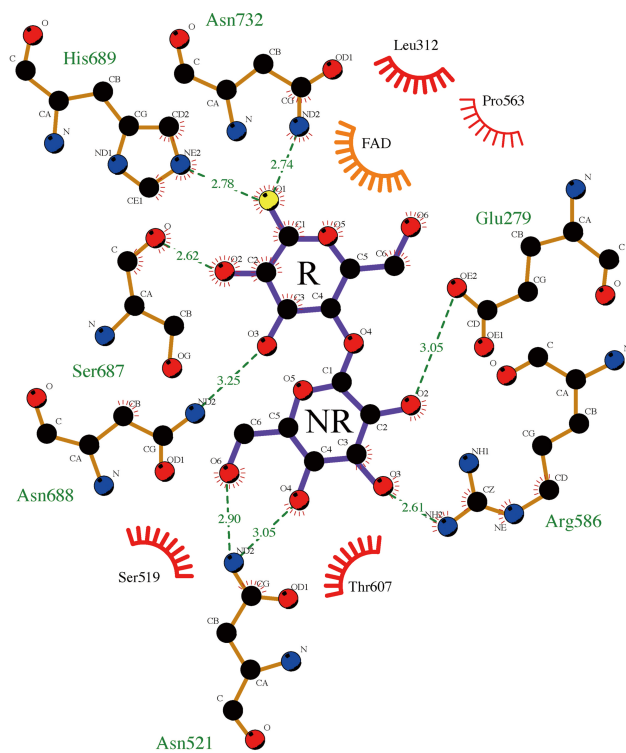
aminated its effect on cellobiose oxidation by CDH. However, 10 mM xylobiose did not inhibit oxidation of cellobiose at the tested concentrations (10, 100, and 1,000  $\mu$ M), suggesting that substrate recognition by CDH is highly specific for substrates containing at least one of the C-6 methylol groups of cellobiose.

Physiologically, CDH is proposed to be a source of reducing activity for Fenton-type reactive oxygen species formation<sup>39)</sup> and/or lytic polysaccharide monooxygenases.<sup>40)</sup> However, these proposals do not consider the possible role of the oxidative half reaction, i.e. conversion of neutral oligosaccharides such as cellobiose or cellooligosaccharides to the corresponding lactones. This reaction could have a role in substrate recognition, transportation of the reaction products, *etc.* The present analysis using Glc/Xyl mixed

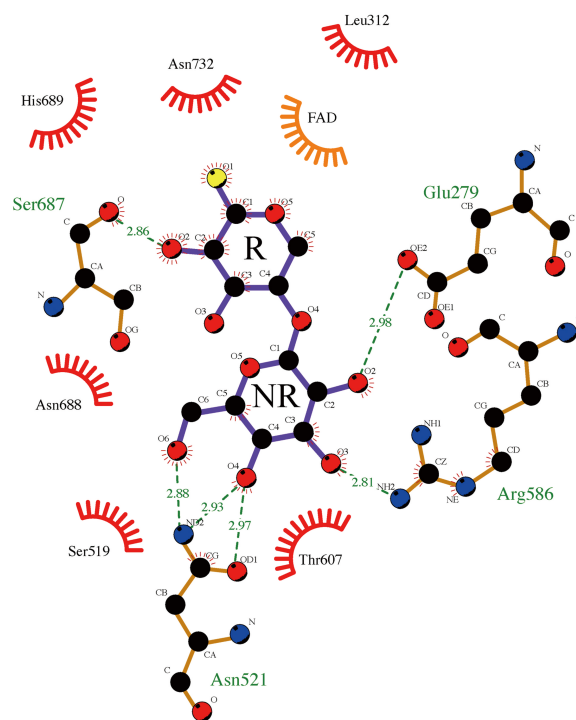
oligosaccharides clearly indicates that fungi can distinguish cellobiose and xylobiose extracellularly by utilizing the reaction of CDH, which could be advantageous in terms of induction of other degrading enzymes.

## CONCLUSION

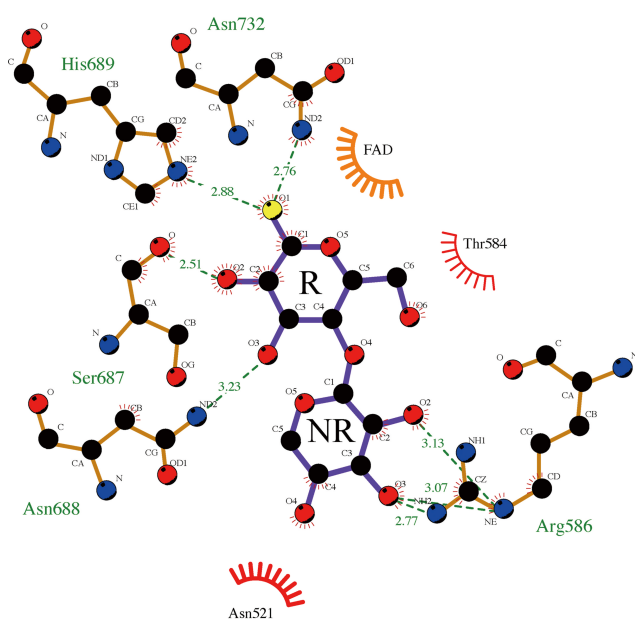
The substrate specificity of CDH was evaluated by using a series of glucose/xylose mixed disaccharides and trisaccharides as substrates, in order to throw light on the function of the C-6-methylol group. The kinetic parameters of trisaccharides were dependent on the nature of the disaccharide unit at the reducing end, which is consistent with recognition of the reducing end of oligosaccharides by CDH. The results of biochemical study and docking



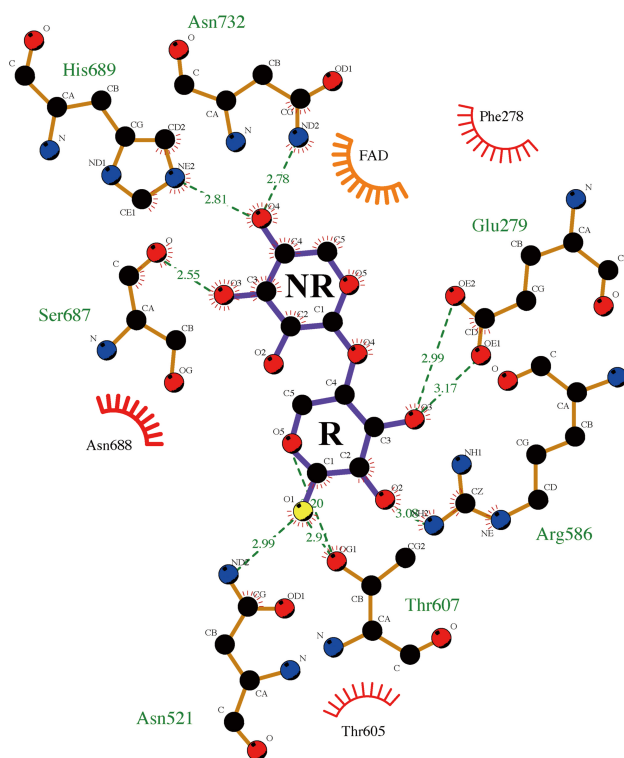
Glc-Glc



Glc-Xyl



Xyl-Glc



Xyl-Xyl

Fig. 6. Schematic diagrams of protein-ligand interactions after docking with AutoDock, visualized by LigPlot<sup>†</sup>.

simulation indicate that the C-6-methylol group at the reducing glucose unit stabilizes the productive position of the reducing end, while groups of both non-reducing and reducing units influence the direction of substrate loading into the active site. Thus, CDH substrate recognition may enable CDH-producing microorganisms to differentiate the products of cellulose or xylan degradation and induce the appropriate hydrolytic enzymes, cellulases or xylanases. Further connection of the biochemical characteristics of each enzyme with fungal physiology will be necessary to understand the overall system used by fungi to degrade plant cell walls.

### CONFLICTS OF INTEREST

The authors declare no conflict of interests.

### ACKNOWLEDGMENTS

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