Green Chemistry

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/greenchem

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Using Fluorogenic Probes for the Investigation of Selective Biomass Degradation by Fungi

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

A library of fifteen commercially purchased and synthetic fluorogenic probes were employed for the investigation of biomass degradation using extract of white-rot fungi. These probes were selected or designed to mimic the dominant linkages in celluloses, hemicelluloses and lignin, the three most abundant polymers found in biomass. The results show that white-rot fungi display a high preference for cleaving mannose- and glucose-based probes, which mimic hemicelluloses. Low degree of cleavages were noted for xylose- and cellobiose-based probes. No cleavages were observed for probes that mimic the linkages in lignin. Overall, these discoveries prove that it is possible to employ fungi for selective degradation or release of hemicelluloses from biomass.

Introduction

Celluloses, hemicelluloses (commonly present in forms, such as xylan, arabinoxylan, mannan and glucomannan) and lignin are the three most abundant polymers found in biomass (Figures 1-3). Non-edible biomass is a superior source for alternative energy in the category of 2nd generation biofuels. Unlike solar or wind energy, the supply of feedstock biomass is immense and low cost. Using fungi for biodegradation of biomass and release of carbohydrates is an effective and environmentally friendly way of providing feedstock for biofuel production. However, for many years, the main issue for utilizing non-edible biomass has been the difficulties in developing low-cost and continuous separation methods for extracting fermentable carbohydrates (cellulose or hemicellulose) from the biomass.[1] The common practices for removing hemicellulose from biomass, especially those employed by the paper-making industry during wood pulping, involve the uses of caustic chemicals (acids, alkaline, ammonia, ozone) and/or harsh mechanical conditions (pyrolysis, steam explosion).[2] These practices have the shortcoming of producing chemical wastes that require further treatments or immense energy investments. As a result, there

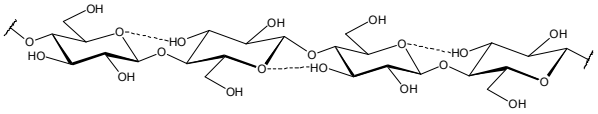


Figure 1. Representative structure of cellulose

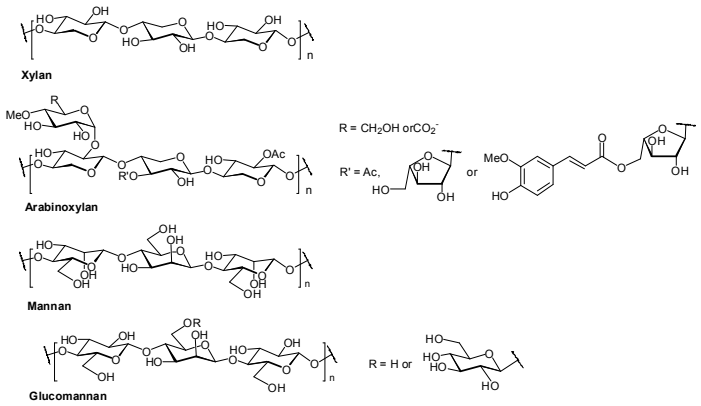


Figure 2. Representative structure of hemicellulose

is an ongoing effort in searching for "green" processes for

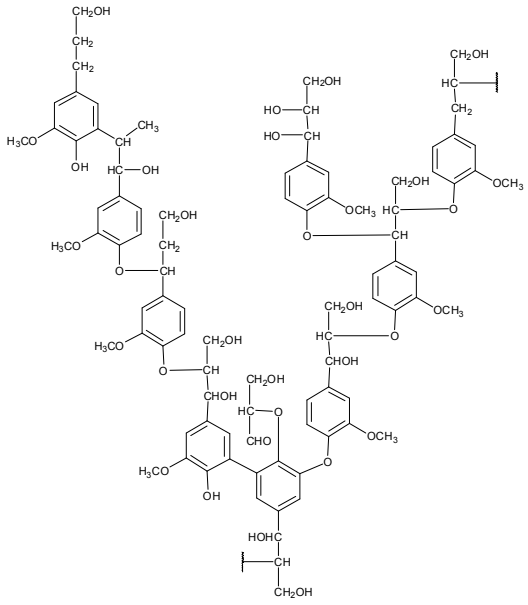


Figure 3. Representative structure of lignin

hemicellulose isolation, and microbes for cost-effective cellulose-based biofuel production.[3]

Due to the capability of fast rotting of wood, fungi have been the focus for biodegradation of biomass. This process can be free of

hazardous chemicals and requires little energy investment.[3a,4] Extensive investigations have been devoted to the search for fungi, studies of fungal enzymes and the genetic modification of microbes.[4c] Nevertheless, little progress has been noted. The major challenges are the complexity of enzymes employed by various fungi: not all the targeted fungi can degrade wood with satisfactory efficiency. Another obstacle is that the degradation of lignin will generate phenols or quinones, which are toxic to the microbes, commonly yeasts, that will be used for the fermentation of the carbohydrates produced from biomass.[5] Over degradation of lignin will also lead to the formation of excess tar during the gasification of biomass.[6] Therefore, chemical treatments are required to remove these phenols or quinones which further complicates the process of ethanol or biofuel production. We believe that an ideal solution is to identify strains of fungi that can selectively degrade or release hemicellulose from biomass without significant degradation of lignin.

Rationale of Designs

Hemicelluloses are thought to link to lignin via covalent bonds. Although the exact linkages have not been fully characterized, they most likely involve anomeric carbon in the form of glycosidic bond or hydroxy groups in the form of ether bond (Figure 4).[7] For achieving the goal of selective degradation or releasing hemicellulose, a library of synthetic and commercially-available fluorogenic probes were screened to show their usefulness in identifying and characterizing such linkages. These probes were designed to contains linkages that mimic those found in biomass, including: a) the first type (class I) with a chromophore attached to the anomeric position of cellobiose to mimic cellulose, b) the second type with chromophores attached to the anomeric position of xylose, mannose and glucose (class IIa) to mimic the anomeric linkages between hemicelluloses or with chromophores attached to the hydroxy group of mannopyranose and xylofuranose to mimic the ether linkages between hemicelluloses and lignin (class IIb), and c) the third type (class III) that are designed to mimic various linkages found in lignin.

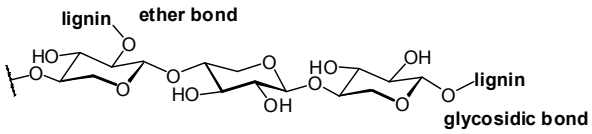


Figure 4. Possible linkages between hemicelluloses and lignin

Two types of chromophores were employed. 4-methylumbelliferone (4MU), **1** will be used in the designs that mimic the phenolic link. 6-methoxynaphthalenecarbonyl (6MN) motif, **2** was used in the designs that mimic linkages between hemicellulose and lignin at benzylic position (Figure 5). These molecules show no fluorescence when linked to carbohydrates or other structural moieties. However, upon degradation or breaking of the designed chemical bonds, fluorescent molecules are released, and are thus termed fluorogenic. In addition, 4MU undergoes hydrolytic cleavage whereas 6MN fluorescence only occurs through oxidative cleavage. Thus, these two probes can also differentiate the possible mechanisms that are involved in the breakage of polymers in biomass. All of the examined probes are shown in Figure 6.

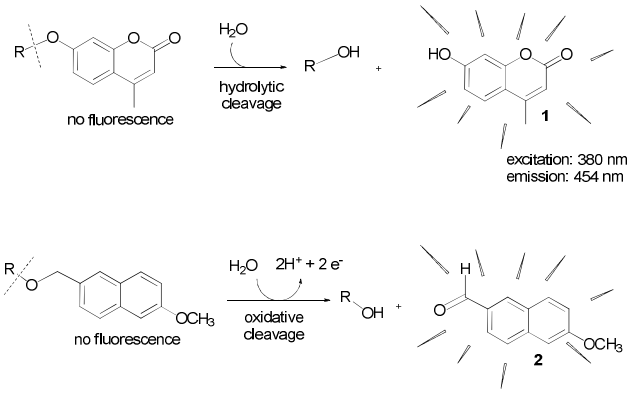


Figure 5. Types of fluorescent chromophores

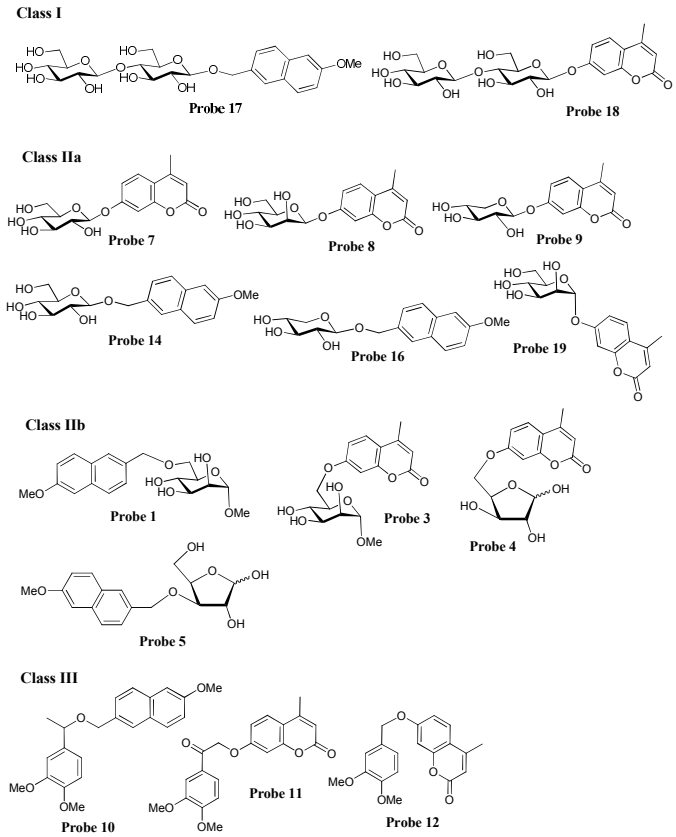
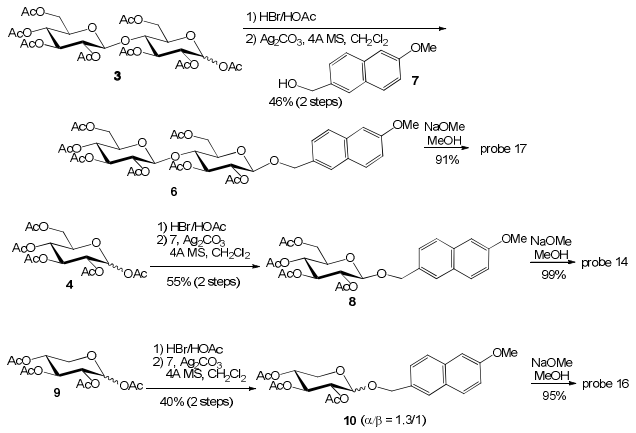


Figure 6. Structures of employed probes

Synthesis of Probes

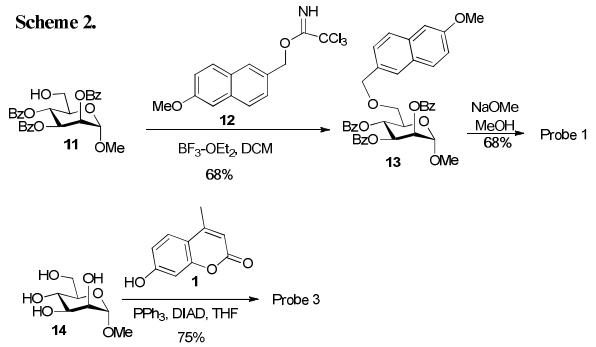
All of the probes used with 4MU at the anomeric position are commercially available. The synthesis of probe 17 began with converting the per-acetylated cellobiose into the glycosyl bromide followed by glycosylation using (6-Methoxy-2-naphthyl)methanol, **7** as the glycosyl acceptor. Deacetylation of **8** afforded the desired probe 17. Similar process was used for the syntheses of probes 14 and 16 (Scheme 1).

Scheme 1.



Probe 1 was synthesized with alkylation of methyl 2,3,4,-tri-*O*-benzoyl-α-D-mannoside, **11** using a 6MN derivative, (6-methoxynaphthalen-2-yl)methyl-2,2,2-trichloroacetimidate, **12** followed by the deprotection of the benzoyl groups (Scheme 2).

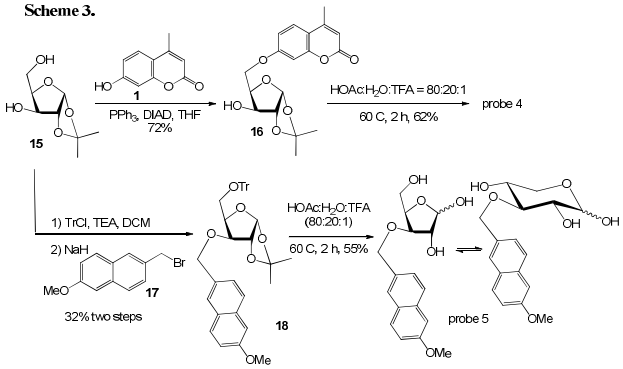
Scheme 2.



Probe 3 was prepared from methyl mannopyranoside, **14** via a Mitsunobu reaction using 4MU as the nucleophile (Scheme 2).

Similar to the synthesis of probe 3, probe 4 was prepared from 1,2-*O*-isopropylidene-a-D-xylofuranose via a Mitsunobu reaction followed by the deprotection of the isopropylidene group (Scheme 3). In a separate route, the 3-OH of compound **15** was alkylated with 6MN. Following the deprotection of both trityl and

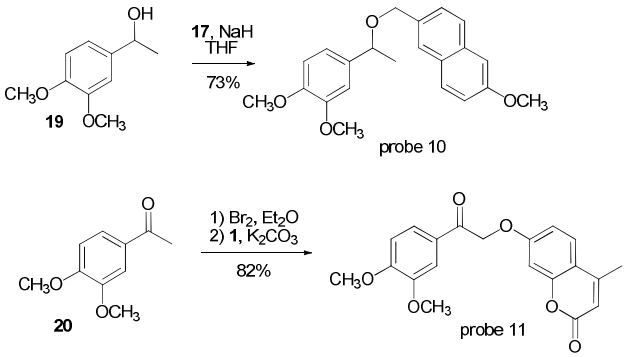
Scheme 3.



isopropylidene groups, probe 5 can be obtained which could exist as an equilibrium of furanose and pyranose forms (Scheme 3).

Probe 10 can be synthesized by alkylation of the hydroxy group of **19** with **17** (Scheme 4). Probe 11 can be prepared by bromination of the α-carbon of **20** followed by nucleophilic

Scheme 4.



substitution of Br using **1** as the nucleophile. Probe 12 was synthesized as reported in the literature.[9]

Results and Discussion

White rot fungus, *Phanerochaete chrysosporium* (ATCC24725), which uses cellulases and peroxidases to degrade cellulose, lignin and hemicellulose, has been studied for many years.[4d,8] All of the probes were tested using crude extract of *P. chrysosporium*. The fluorescence intensities of the inoculated solutions were observed every ten minutes using excitation at 360 nm and emission at 460 nm. Relative fluorescence intensity (RFI) values were calculated as the difference in fluorescence intensity between the inoculated solutions and controls (no crude extract of *P. chrysosporium* added). The RFI value for each probes was determined 6-9 times and the averaged RFI values for all probes are summarized in Figures 7-10.

Figure 7. RFI values of class I probes

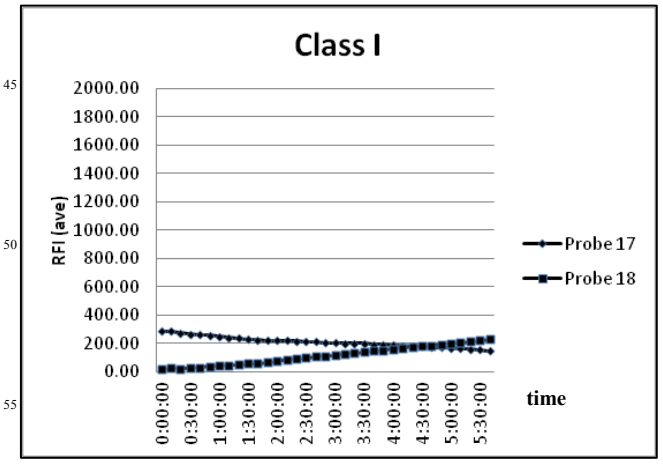


Figure 8. RFI values of class IIa probes

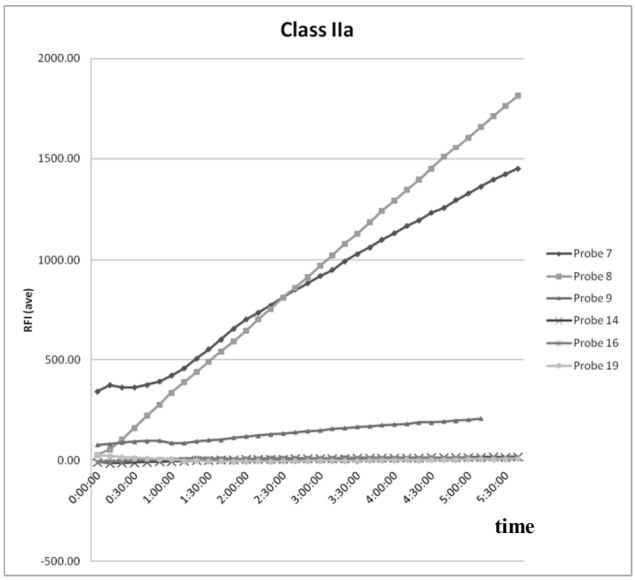


Figure 9. RFI values of class IIb probes

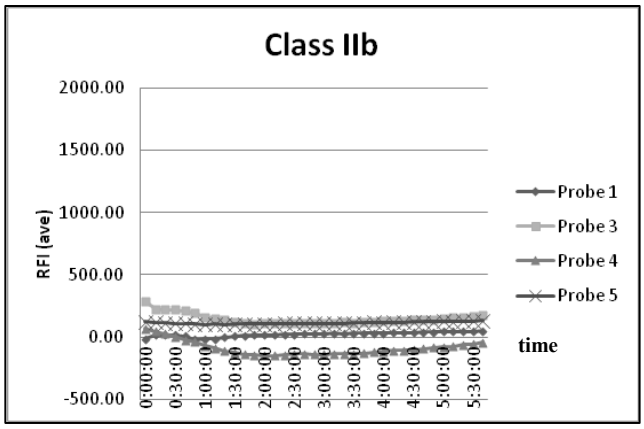
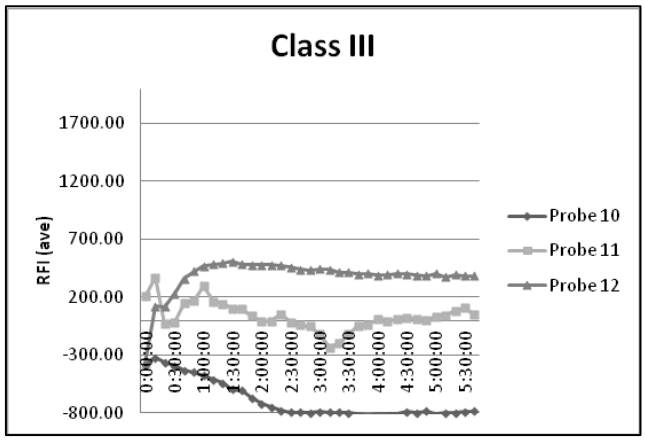


Figure 10. RFI values of class III probes



The data show that only two of the class IIa probes (probes 7 and 8) displayed significant fluorescence increase showing a time-dependent increase in RFI. Probe 9 also manifested a time-dependent but rather small increase in RFI. All three probes have 4MU attached to the anomeric position, whereas similar probes with 6MN attached at the anomeric position (probes 14 and 16) showed no obvious fluorescence increase. Since 6MN can be fluorescent only via oxidative cleavage, this result suggests that the activity of glycosidases, not peroxidases, are responsible for the degradation. However, it is worth mentioning that the lack of fluorescence from probes 14 and 16 cannot be viewed as the lack of fungal enzyme activity. In fact, we believe that fugal glycosidases can degrade these two probes. Nevertheless, the adducts from such degradation were not fluorescent.

The lack of fluorescence increase from class IIb probes attached with either 4MU or 6MN also implies that peroxidases or etherases cannot degrade xylose- or mannose-based hemicelluloses, which are connected to lignin via ether linkages. Therefore, it is possible to release or degrade glucose-, xylose- or mannose-based hemicelluloses when these are connected to lignin via glycosidic bonds.

The lack of fluorescence increases from class III probes and very little fluorescence increase from class I probes support the idea of using fungi to selectively degrade or release hemicelluloses without breaking down celluloses or lignin from biomass. To confirm that the aromatic compounds generated from the degradation of class III probes (probes 10, 11 and 12) will not cause fluorescence quenching, we have measured the fluorescence of pre-mixed aromatic compounds and 4MU. The results showed no fluorescence quenching.[10] Nevertheless, there is a slight and time-dependent fluorescence increase for probe 18. It is possible that celluloses may still be degraded but at a much slower rate than that of the hemicelluloses. In contrast to probe 8 (mannose with β-linked 4MU), probe 19 (mannose with α-linked 4MU) did not exhibit an increase in RFI. Plant mannans are mannose-based polysaccharide with mannose linked with β(1-4) linkages while yeasts and mammalian glycoproteins have mannose-based oligosaccharides carrying mannose linked with α(1-6) as the backbone, and α(1-2) and α(1-3) linked at the branches. The lack of RFI increase from probe 19 strongly indicates that the fungal enzymatic degradation is very selective toward the plant mannans.

Finally, based on the time-dependent but small increase in RFI of probe 18, the possibility that the rate of enzymatic degradation/release on hemicelluloses may be slower for polymers consisting of xylose, mannose or glucose cannot be ruled out. It is difficult to design or envision a fluorogenic probe that can mimic the glycosidic bond between two carbohydrate units. However, since the cellobiose-based probe (probe 18) and, likely, probe 17 were degraded by the fungal enzymes, it is possible that the glycosidic bonds between xylose, mannose or glucose-containing hemicelluloses and lignin can also be broken by fungal enzymes albeit, probably, at a slower rate as compared

to the monosaccharide-based probes.

Conclusion

We have screened a library of fifteen commercially purchased and synthetic fluorogenic probes to investigate the feasibility of selective degradation of plant biomass using crude extracts of white-rot fungi. These probes contain linkages that mimic the dominant linkages in celluloses, lignin, and the possible linkages between hemicelluloses and lignin. Our results show that fungal enzymes are very selective in breaking down the β-linked glycosidic bonds of glucose and mannose. Much slower cleavage rate of the β-linked glycosidic of cellobiose and xylose was also noted. No degradation of lignin linkages was observed. Finally, no cleavage of the α-linked glycosidic bond of mannose suggests that fungal enzymes are selectively active toward plant mannans, not mammalian mannans. Fungi are capable of degrading biomass, including celluloses, hemicelluloses and lignin. However, the process often take weeks or months. In our studies, we have shown that the degradation of hemicellulose-based probes occurs much faster (within 6 hrs) than that of the lignin-based probes, which shows no sign of degradation during the incubation period. The observed rate of degradation is following: hemicellulose-based probes >> cellulose-based probes > lignin-based probes. This findings suggests that by controlling the time of incubating fungal enzymes and biomass, it is possible to selectively release and separate hemicelluloses while keeping cellulose and lignin intact. In summary, this study support the use of fungal enzymes as a tool for developing effective and green process for isolating and utilizing hemicelluloses from biomass.

Experimental section

General procedure for the Mitsunobu reaction using primary alcohol and 4-methylumbelliferone (probe 3 and 16). To a solution of starting material (0.5 g) and 4-methylumbelliferone (1 equiv.) in 20 mL of anhydrous THF, triphenylphosphine (1.5 equiv.) and DIAD (1.5 equiv.) was added. The reaction was heated at 50 °C overnight. After completion of the reaction (confirmed by TLC, eluted with EtOAc), the solvent was removed and the product was purified by column chromatography (hexane/EtOAc from 80/20 to 0/100). The obtained product was further purified with recrystallization in a mixture of EtOAc/MeOH.

General procedure for the deprotection of acetyl groups using NaOMe/MeOH. To a solution of peracetylated sugars (0.1 g) in anhydrous MeOH (10 mL), a catalytic amount of NaOMe (ca. 1 M in MeOH) was added. The solution was stirred at room temperature for 6 hours. After completion of the reaction (confirmed by TLC, eluted with EtOAc/MeOH = 9/1), the reaction was quenched by adding Amberlite 120 (H^+) resin. The reaction mixture was filtered through a short column packed with Celite, and the filtrate was collected and concentrated. The product usually would be in good quality but can be purified by a gradient column chromatography (eluted with CH_2Cl_2/MeOH from 100/0 to 4/1). The yields for deacetylation were usually between 90 – 100%.

Methyl-6-*O*-(6-Methoxy-2-naphthalenylmethyl)-mannopyronoside (probe 1)

^{1}H NMR (300 MHz, METHANOL-D3) δ 7.7 (m, 3H), 7.43 (dd, *J* = 9.42, 1.74 Hz,1H), 7.19 (d, *J* = 2.43 Hz, 1H), 7.1 (dd, *J* = 8.91, 2.73 Hz), 4.7 (s, 3H), 4.62 (d, *J* = 1.74, 1H, H1), 3.87 (s, 3H), 3.81 (t, *J* =10.32 Hz, 1H), 3.63-3.76 (m, 5H), 3.36 (s, 3H); ^{13}C NMR (100 MHz, METHANOL-D3) δ 158.3, 134.7, 133.7, 129.3, 129.2, 127.1, 126.7, 126.6, 118.8, 105.7, 101.8, 73.6, 72.4, 71.6, 71.0, 70.1, 67.7, 54.7, 54.3; ESI/APCI calcd for $C19H24O7^+$ ($[M]^+$) *m/z* 364.1522; measured *m/z* 364.1499.

Methyl-6-(4-methyl-umbelliferyl)-*O*-*α*-*D*-mannopyranoside (probe 3).

^{1}H NMR (300 MHz, METHANOL-D3) δ 7.66 (d, *J* = 8.94Hz, 1H), 7.0 (dd, *J* = 8.94, 2.4 Hz, 1H), 6.94 (d, *J* = 2.4 Hz, 1H), 6.14 (d, *J* = 1.02 Hz, 1H), 4.63 (d, *J* = 1.38 Hz, 1 H), 4.37 (dd, *J* = 10.65, 1.71 Hz, 1H), 4.26 (dd, *J* = 10.65, 5.85 Hz, 1H), 3.8 (m, 2H), 3.73 (t, *J* = 8.91 Hz, 1 H), 3.69 (m, 1H), 3.36 (s, 3H), 2.42 (d, *J* = 1.35Hz, 3H). ^{13}C NMR (100 MHz, METHANOL-D3) δ 162.6, 162.3, 155.2, 154.5, 126.1, 113.7, 112.9, 110.9, 101.7, 101.3, 71.4, 71.3, 70.7, 68.3, 62.3, 54.0, 17.4. ESI/APCI calcd for $C_{17}H_{20}O_8^+$ ($[M]^+$) *m/z* 352.1158; measured *m/z* 352.1152.

6-(4-Methyl-umbelliferyl)-*O*-D-xylofuranose (probe 4).

0.2g (0.57 mmol) 1,2-O-isopropylidene-6-(4-methyl-umbelliferyl)-*O*-α-D-xylofuranose was dissolved into a 5mL solution (HOAc:H_2O:TFA = 80:20:1) in a 10mL round bottom flask. The solution was heated at 60 °C under reduced pressure for 2 hours. The completion of the reaction was confirmed by TLC (eluted with EtOAc/hexane = 3/7). Then the solvent was removed under reduced pressure. 5 mL dichloromethane was added and the solvent was removed under reduced pressure, this was repeated 3 times in order to remove AcOH. Then the product was pumped dry by oil pump for 24 hours. Pure product was obtained after chromatography (Hexane/EtOAc = 100/10 to 0/100, yield 110 mg, 0.35 mmol, 62%).

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.66 (dd, *J* = 8.9, 1.7 Hz), 7.0 - 6.9 (m, 1H), 6.9 – 6.8 (m, 1H), 6.14 (d, *J* =1.1 Hz), 5.39 (d, *J* = 4.1 Hz, 0.4 H), 5.13 (s, 0.4H, this compound is a 1:1 mixture of α and β), 4.5 – 4.4 (m, 1H), 4.4 – 4.3 (m, 1H), 4.3 – 4.2 (m, 2H), 4.2 – 4.1 (m, 1H), 4.1 – 4.0 (m, 1H), 2.42 (d, J =1.1 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) for the sugar ring there are two sets of peaks since it is a 1:1 mixture of α and β. 162.5, 162.3, 155.1, 154.1, 126.1, 113.7, 112.8, 110.9, 103.3, 101.3, 96.8, 81.1, 80.2, 77.0, 76.9, 75.9, 75.8, 68.6, 67.9, 17.4; ESI/APCI calcd for $C_{15}H_{17}O_7^+$ ($[M+H]^+$) *m/z* 309.0947; measured *m/z* 309.0959.

3-(6-Methoxy-2-naphthalenylmethyl)-*O*-D-xylofurnose (probe 5). This compound was prepared using the similar procedure for preparing probe **4** with the isolated yield of 55 %.

^{1}H NMR (300 MHz, MeOH) δ 7.79 (s, 1H), 7.72 (d, *J* =1.7 Hz, 1H), 7.69 (d, *J* = 2.4 Hz), 7.52 (dd, *J* = 8.6, 1.7 Hz, 1H), 7.18 (d,

J = 2.7 Hz, 1H), 7.08 (dd, *J* = 8.9, 1.7 Hz, 1H), 5.01 (d, *J* = 3.4 Hz, 0.6 Hz, α), 4.96 (d, 2H), 4.42 (d, *J* = 7.2 Hz, β), 3.87 (s, 3H), 3.8-3.5 (m, 5H), 3.3 – 3.2 (m, 1H); ^{13}C NMR (100 MHz, MeOH) for the sugar ring there are two sets of peaks since it is a mixture of α and β.157.9, 134.5, 134.4, 129.1, 128.9, 126.8, 126.6, 126.4, 125.3, 118.5, 105.5, 97.8, 93.1, 84.8, 81.9, 74.9, 74.9, 74.8, 72.5, 70.1, 70.0, 65.9, 61.9, 54.5; ESI/APCI calcd for $C_{17}H_{20}O_6^+$ ([M]$^+$) *m/z* 320.1260; measured *m/z* 320.1263.

2-((1-(3,4-dimethoxyphenyl)ethoxy)methyl)-6-methoxynaphthalene (probe 10). 1-(3,4-dimethoxyphenyl)-ethanol was prepared by reducing 3,4-dimethoxyacetophenone using $NaBH_4$ in MeOH. 2-bromomethyl-6-methoxy-naphthalene was prepared by treating 6-methoxynaphthyl-methanol with PBr_3 in Et_2O for 5 hours. The reaction was quenched by adding sat. $NaHCO_3$ to the reaction solution until no bubbling. Then the organic layer was washed by Sat. $NaHCO_3$ for two times and Brine for one time. The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude product was use directly for next step. (0.5 g, 2.7 mmol) 1-(3,4-dimethoxyphenyl)ethanol and 1eq. 2-bromomethyl-6-methoxy-naphthalene was dissolved in THF, then 10 equiv. of NaH was added. After 6 hours, the reaction solution was poured to a flask with ice to quench the reaction. 300 mL of EtOAc was added to extract the product. The organic layer was washed by 100 mL distilled water, 100 mL 1N HCl and Brine, then dried over Na_2SO_4. Filtration and removal of the solvent followed by purification with gradient column chromatography (Hexane/EtOAc = 100:0 to 40/60) afforded desired product (Yield 0.69g, 1.97mmol, 73%).

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.7 – 7.6 (m, 3H), 7.40 (dd, *J* = 8.4, 1.8 Hz, 1H), 7.1 – 7.0 (m, 2H), 6.9 – 6.8 (m, 3H), 4.58 – 4.38 (dd, *J* = 46, 11.67 Hz, 2H), 4.47 (q, *J* = 6.54, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.89 (s, 3H), 1.49 (d, *J* = 6.5 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$,) δ 157.8, 149.4, 148.6, 136.5, 134.3, 133.9, 129.5, 128.9, 127.2, 126.8, 126.7, 119.0, 111.1, 109.3, 105.9, 70.5, 56.1, 56.1, 55.5, 29.9, 24.4; ESI/APCI calcd for $C_{22}H_{24}O_7^+$ ([M]$^+$) *m/z* 352.1673; measured *m/z* 352.1675.

1-(3,4-dimethoxyphenyl)-2-(4-methyl-umbelliferoxy)-ethanone (probe 11). To a cooled (0 Celsius) solution of 3,4-dimethoxyacetophenone (0.5 g, 2.7 mmol) in diethyl ether (50 mL), bromine (0.266 g, 3.32 mmol) dissolved in ether (20 mL) was added slowly. After 10 min, the reaction mixture was worked up, and the organic layer was wash with water and brine. Then organic layer was dried over sodium sulfate. Filtration and evaporation of the solvent gave a crude product which was used directly for the next step. The crude product from last step, potassium carbonate (0.56 g, 4.05 mmol) and 4-methylumbelliferone (0.47 g, 2.7 mmol) was dissolved into 50 mL of acetone. The solution was heated under reflux for overnight. Upon completion of the reaction (check by TLC, eluted with EtOAc/hexane = 1/9), the solvent was removed by compress air. Add 100 ml EtOAc to the crude yellowish solid, there was some insoluble yellowish solid. Filtration to get the yellowish solid. It was confirmed as the right product by H NMR (Yield 0.78 g, 2.2 mmol, 82%).

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.62 (dd, *J* = 8.25, 2.04 Hz, 1H), 7.5 - 7.4 (m, 2H), 7.0 - 6.9 (m, 2H), 6.79 (d, *J* = 2.76 Hz, 1H), 6.14 (d, *J* = 1.38 Hz, 1H), 5.33 (s, 2H), 3.97 (s, 3H), 3.93 (s, 3H), 2.4 (d, 1.38 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.9, 161.3, 161.2, 155.2, 154.5, 152.7, 149.7, 127.5, 125.9, 122.8, 114.5, 112.9, 112.6, 110.4, 110.4, 102.1, 70.7, 56.4, 56.3, 18.7; ESI/APCI calcd for $C_{20}H_{18}O_6^+$ ([M]$^+$) *m/z* 354.1103; measured *m/z* 354.1091.

(6-Methoxy-2-naphthalenylmethyl)-*β*-*D*-glucopyranoside (probe 14).

^{1}H NMR (300 MHz, METHANOL-D3) δ 7.8 – 7.7 (m, 3H), 7.48 (dd, *J* = 8.5, 1.7 Hz, 1H), 7.20 (d, *J* = 2.7 Hz, 1H), 7.10 (dd, *J* = 8.9, 2.4 Hz), 5.0 – 4.7 (dd, *J* = 75.9, 11.7 Hz), 4.32 (d, *J* = 7.5 Hz, H-1), 3.9 – 3.8 (m, 1H), 3.88 (s, 3H), 3.7 – 3.6 (m, 1H), 3.4 – 3.2 (m, 3H); ^{13}C NMR (100 MHz, METHANOL-D3) δ 158.1, 134.6, 132.9, 129.2, 128.9, 126.7, 126.6, 118.6, 105.5, 102.0, 76.9, 76.85 (2C), 73.9, 70.7, 70.5, 61.6, 54.5; ESI/APCI calcd for $C_{18}H_{22}O_7Na^+$ ([M+Na]$^+$) *m/z* 373.1263; measured *m/z* 373.1273.

(6-Methoxy-2-naphthalenylmethyl)-*β*-*D*-xylopyranoside (probe 16).

^{1}H NMR (300 MHz, METHANOL-D3) δ 7.8 – 7.7 (m, 3H), 7.46 (dd, *J* = 8.6, 1.7 Hz, 1H), 7.19 (d, *J* = 2.4 Hz, 1H), 7.09 (dd, *J* = 8.9, 2.4 Hz), 4.9 – 4.7 (dd, *J* = 67.3, 11.7 Hz), 4.32 (d, *J* = 7.2 Hz, H-1), 3.9 – 3.8 (m, 1H), 3.88 (s, 3H), 3.6 – 3.4 (m, 2H), 3.3 – 3.1 (m, 2H); ^{13}C NMR (100 MHz, METHANOL-D3) 134.6, 132.9, 129.1, 128.9, 126.8, 126.6, 126.57, 118.6, 1015.5, 102.9, 76.6, 73.8, 70.8, 70.1, 65.8, 54.5; ESI/APCI calcd for $C_{18}H_{22}O_7Na^+$ ([M+Na]$^+$) *m/z* 343.1158; measured *m/z* 343.1167.

(6-Methoxy-2-naphthalenylmethyl)-*β*-*D*-cellubioside (probe 17).

^{1}H NMR (300 MHz, METHANOL-D3) δ 7.8 – 7.7 (m, 3H), 7.48 (dd, *J* = 8.5, 1.7 Hz, 1H), 7.20 (d, *J* = 2.7 Hz, 1H), 7.10 (dd, *J* = 8.9, 2.4 Hz), 5.0 – 4.7 (dd, *J* = 71.8, 11.7 Hz), 4. (s, 2H), 4.40 (dd, *J* = 7.5, 3.1 Hz, 2H), 3.9 – 3.8 (m, 3H), 3.88 (s, 3H), 3.7 – 3.4 (m, 4H), 3.4 – 3.3 (m, 2H), 3.20 (t, *J* = 8.6 Hz,1H); 13C NMR (100 MHz, METHANOL-D3) δ 158.1, 134.6, 132.9, 129.2, 128.9, 126.7, 126.6, 118.6, 105.5, 103.4, 101.9, 79.5, 76.9, 76.6, 75.4, 75.2, 73.7, 70.8, 70.2, 61.2, 60.7, 54.5. ESI/APCI calcd for $C_{24}H_{32}O_{12}Na^+$ ([M+Na]$^+$) *m/z* 535.1791; measured *m/z* 535.1787.

(6-Methoxy-2-naphthalenylmethyl)-2,3,4,6-tetra-*O*-acetyl-*β*-D-glcucopyranoside (8). Bromo-2,3,4,6-acetyl-*O*-α-D-glucopyranoside was prepared by treating 1,2,3,4,6-penta-*O*-acetyl-D-glucose (0.5 g, 1.28 mmol) with HBr (3mL, 33% in AcOH) in a 20 mL flask with 3 mL anhydrous dichloromethane as solvent for 2 hours. The reaction mixture was extracted by 100 mL EtOAc and the organic layer was washed by distilled water, Sat. $NaHCO_3$ for three times and Brine for one time. Then the organic layer was dried over anhydrous Na_2SO4 and filtered. The bromo-2,3,4,6-acetyl-*O*-α-D-glucopyranoside was obtained by removing the solvent under reduced pressure and used directly

for next step. Bromo-2,3,4,6-acetyl-*O*-α-D-glucopyranoside (1.28 mmol) and (6-Methoxy-2-naphthyl)methanol (0.34 g, 1.8 mmol) and 4 Å MS was stirred in 20 mL of anhydrous dichloromethane for 15 minutes before adding in Ag_2CO_3 (0.49 g, 1.8 mmol). The mixture was stirred for overnight before filtered through a syringe packed with Celite and silica gel. Then the solvent was removed and the crude product was loaded to a column and purified with gradient (Hexane/EtOAc = 100/10 to 50/50). Pure product was obtained (0.41g, 0.79 mmol, 65 % yield over two steps).

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.8 – 7.7 (m, 3H), 7.37 (dd, *J* = 8.3, 1.4 Hz), 7.2 – 7.1 (m, 2H), 5.70 (d, *J* = 5.1 Hz, H-1),5.22 (t, *J* = 2.8 Hz, H-2), 4.91 (dd, *J* = 9.3, 2.1 Hz, H-3), 4.67 (s, 2H), 4.35 (dd, *J* = 4.5, 3.1 Hz, H-4), 4.2 – 4.1 (m, 2H), 3.97 (ddd, *J* = 9.6, 5.5, 3.5 Hz, H-5), 3.9 (s, 3H), 2.1 – 1.8 (m, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.9, 169.9, 169.4, 157.9, 134.3, 132.8, 129.6 128.9, 127.3, 126.6, 126.5, 121.7, 119.2, 105.9, 97.22, 73.4, 70.3, 68.4, 67.2, 66.3, 63.3, 55.5, 21.2, 21.0 (3C); ESI/APCI calcd for $C_{26}H_{30}O_{11}Na^+$ ([M+Na]$^+$) *m/z* 541.1686; measured *m/z* 541.1677.

(6-Methoxy-2-naphthalenylmethyl)-2,3,4,-*tri*-*O*-acetyl-D-xylopyranoside (10). This compound was prepared using the similar procedure for preparing compound **8** with the isolated yield of 45 %.

^{1}H NMR (300 MHz, $CDCl_3$) The compound was obtained as a mixture of alpha and beta conformation (α/β = 1.3/1) δ 7.8 – 7.7 (m, 4.6 H), 7.5 – 7.4 (m, 1.6 H), 7.2 – 7.1 (m, 3.1 H), 5.7 (d, *J* = 6.9 Hz,1H, H α-1), 5.58 (d, *J* = 4.5 Hz, 1.3H, H β-1), 5.28 (t, J = 2.8 Hz, 1.3H), 5.20 (t, *J* = 8.3, 1H), 5.1 – 4.8 (m, 4H), 4.68 (s, 2H), 4.3 – 4.2 (m, 1.4H), 4.15 (dd, *J* = 12.0, 5.2 Hz, 1H), 4.0 – 3.9 (m, 1H), 3.90 (s, 5.1H), 3.71 (dd, *J* = 12.4, 6.9 Hz, 1.4H), 3.51 (dd, *J* = 12.0, 8.6 Hz, 1 H), 2.1 – 2.0 (m, 21H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.1, 170.0, 169.5, 169.3, 169.2, 157.9, 134.3, 132.8, 129.6, 128.9, 127.3, 126.7, 126.6, 126.1, 125.7, 122.6, 119.1, 105.9, 96.8, 92.3, 74.5, 71.2, 69.7, 69.6, 68.8, 68.5, 67.6, 65.6, 63.0, 59.9, 55.5, 22.9 (2C), 21.0, 20.9, 20.87, 20.81; ESI/APCI calcd for $C_{23}H_{26}O_9Na^+$ ([M+Na]$^+$) *m/z* 469.1475; measured *m/z* 469.1468.

Methyl-2,3,4-Tri-benzoyl-*O*-6-(6-Methoxy-2-naphthalenylmethyl)-*α*-D-mannopyranoside (13).

6-methoxy-2-naphthalenylmethyl trichloroacetimidate (0.33 g, 1 mmol) and methyl 2,3,4-tri-*O*-benzoyl-α-D-mannopyranoside (0.35 g, 0.7 mmol), 4 Å molecular sieve in anhydrous dichloromethane (20 mL) was stirred for 30 minutes and then cooled to -78 °C. After addition of several drops of $BF_3.Et_2O$, the reaction mixture was stirred overnight. After completion of the reaction (check by TLC, eluted with EtOAc/hexane = 1/9), the reaction mixture was filtered through a short column packed with Celite and silica gel. After removal of most of the solvent, the crude product was purified with a gradient column chromatography (hexance/EtOAc = 100/0 to 75/25) to furnish the desired product (0.48 g, 68%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.1-8.0 (m, 2H), 7.82-7.9 (m, 4H), 7.7-7.2 (m, 10H), 7.0-7.1 (m, 2H), 6.02 (t, *J* = 9.69 Hz, 1H, H4), 5.85 (dd, J = 9.96, 3.09 Hz, 1H, H3), 5.7 (dd, *J* = 3.45, 1.71 Hz, 1H, H2), 5.0 (d, *J* = 1.71 Hz, 1H, H1), 4.71 (dd, *J* = 26.1, 11.67 Hz, 2H), 4.27 (dt, *J* = 9.63, 3.45 Hz, 1H, H5), 3.91 (s, 3H), 3.79 (d, *J* = 3.75 Hz, 2H, H6), 3.54 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 165.8, 165.7, 163.6, 157.8, 134.3, 133.6, 133.4, 133.3, 133.2(2C), 130.1(2C), 130.0(3C), 129.9, 129.6, 129.6, 129.44, 129.4, 128.7(2C), 128.5(2C), 128.5(2C), 127.1, 126.80, 126.7, 118.92, 105.9, 98.8, 74.1, 70.7, 70.5, 70.3, 69.1, 67.5, 55.7, 55.5. ESI/APCI calcd for $C_{40}H_{36}O_{10}^+$ ([M]$^+$) *m/z* 676.2308; measured *m/z* 676.2300.

1,2-*O*-isopropylidene-6-(4-methyl-umbelliferyl)-*O*-α-D-xylofuranose (16).

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.45 (d, *J* = 8.9 Hz, 1H), 6.88 (dd, *J* = 8.9, 2.4 Hz, 1H), 6.84 (d, *J* = 2.4 Hz, 1H), 6.1 (d, 1H), 6.01 (d, *J* = 3.5 Hz, 1H, H-1), 4.59 (d, *J* = 3.8 Hz, 1H, H-2), 4.55 (ddd, *J* = 11.3, 5.5, 2.7 Hz, 1H. H-4), 4.40 (d, *J* = 2.7 Hz, 1H, H-3), 4.32 (ddd, *J* = 16.5, 10.3, 6.2 Hz, 2H, H-6), 2.77 (s, 1H, OH), 2.37 (d, 3H), 1.52 (s, 3H), 1.33 (s, 3H); 13C NMR (100 MHz, $CDCl_3$) 161.7, 155.2, 152.9, 125.8, 114.1, 112.9, 112.3, 105.2, 101.8, 85.5, 78.4, 75.3, 66.4, 29.9, 27.0, 26.4, 22.1, 18.9; ESI/APCI calcd for $C_{18}H_{20}O_7^+$ ([M+H]$^+$) *m/z* 349.1287; measured *m/z* 349.1295.

General procedure for assay of fluorogenic probes.[11] White rot basidiomycete, *Phanerochaete chrysosporium* ATCC24725 was grown on potato dextrose agar (PDA) plates and incubated around 30°C for one week before the fungus was transferred to liquid culture medium. The culture medium contained, per liter: 2 g KH_2PO_4, 0.5 g $MgSO_4$•$7H_2O$, 0.1 g $CaCl_2$•$2H_2O$, 1% glucose, 10 mM pH 4.2 2,2-dimethyl succinate, 1 mg thiamin-HCl stock solution, 0.2 g ammonium tartrate, and 10 mL of trace element solution (see below). The thiamin and KH_2PO_4 stock solutions were filter sterilized; the others are autoclaved as separate solutions. The stock solution of trace elements is made by first dissolving 1.5 g of nitrilotriacetic acid in 800 mL water and the pH of the solution was adjusted to approximately 6.5 with KOH. The following were then added with dissolution: 0.5 g $MnSO_4 \cdot H_2O$, 1.0 g NaCl, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CoSO_4$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 10 mg $CuSO_4 \cdot 5H_2O$, 10 mg $A1K(SO_4)_2 \cdot 12H_2O$, 10 mg H_3BO_3, and 10 mg $Na_2MoO_4 \cdot 2H_2O$. Sterile distilled water was added to make the total volume of one liter. The medium was inoculated with a 100 mL suspension of fungal culture containing spores (absorbance of 0.5 at 650 nm).

For fungal growth, 10 ml portions of liquid culture in the growth medium were incubated in 125-ml Erlenmeyer flasks capped with rubber-stoppers at 30-39°C. Enzyme production was stimulated by including 0.4 mM veratryl alcohol final concentration and trace elements (7 times the above concentration) at the time of inoculation. After one week growth, the 10 mL culture aliquots were centrifuged at 1500 RPM, 5°C for 15 minutes, and the supernatant fluids were collected and directly used for enzymatic assays. In a 96-well microtiter plate, each well contained 100 µL 125 mM sodium tartrate (pH 3), 50 µL 10 mM probes, 50 µL 2 mM H_2O_2, 50 µL culture supernatant. For the control, each well contains 100 µL 125 mM sodium tartrate (pH 3), 50 µL 10 mM probes, 50 µL 2 mM H_2O_2 (freshly prepared), and 50 µL H_2O.

Fluorescence was measured by using excitation 360/40, emission 460/40 at 37°C. Two duplicated experiments were conducted in parallel. Each probes was assayed at least 6-9 times.

Acknowledgment

We acknowledge support from the Sun Grant Western Regional Center/Department of Transportation.

Notes and references

1 (a) Keller, R. "Biofuel from Algae Has Problems" *Ag Professional*, June 11, **2011**. (b) Corma, A.; Iborra, S.; Velty, A. "Chemical Routes for the Transformation of Biomass into Chemicals." *Chem. Rev*. **2007**, *107*, 2411-2502. (c) Wright, J. D. "Ethanol from biomass by enzymatic hydrolysis." *Chem. Eng. Prog*. **1998**, *84*, 62–74. (d) Cadoche, L.; Lopez, G. D. "Assessment of size reduction as a preliminary step in the production of ethanol from lignocellulosic wastes." *Biol. Wastes* **1989**, *30*, 153–157.

2 (a) Mäki-Arvela, P.; Salmi, T.; Holmbom, B.; Willför, S.; Murzin, D. U. "Synthesis of Sugars by Hydrolysis of Hemicelluloses- A Review." *Chem. Rev*. **2011**, *111*, 5638-5666. (b)Aguilar, R; Ramirez, J. A.; Garrote, G.; Vazquez, M. "Kinetic study of the acid hydrolysis of sugar cane bagasse." J. *Food Eng*. **2002**, *55*, 309-318. (c) "Biorefineries-Industrial Processes and Products: Status Quo and Future Directions." Kamm, B., Gruber, P. R., Kamm, M., Eds.; Wiley-VCH: Weinheim, **2006**; Vol. 1. (d) Bjerre, A. B.; Olesen, A. B.; Fernqvist, T. "Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose." *Biotechnol. Bioeng*. **1996**, *49*, 568–577. (e) Azzam, A.M. " Pretreatment of cane bagasse with alkaline hydrogen peroxide for enzymatic hydrolysis of cellulose and ethanol fermentation." *J. Environ. Sci. Health. B*. **1989**, *24*, 421–433. (f) Dale, B.E., Henk, L.L., Shiang, M., "Fermentation of lignocellulosic materials treated by ammonia freeze-explosion." *Dev. Ind. Microbiol*. **1984**, *26*, 223–233.

3 (a) Sanchez, O. J.; Cardona, C. A. "Trends in Biotechnological Production of Fuel Ethanol from Different Feedstocks." *Bioresource Technology*, **2008**, *99*, 5270-5295. (b) Wang, M., Saricks, C., Santini, D., 1999. "Effects of Fuel Ethanol use on Fuel-Cycle Energy and Greenhouse Gas Emissions." Argonne National Laboratory, Argonne, IL. (c) Ritter, S. K. "Microbiome Mining" *C&EN News*, **2012**, December 3, 13-16. (d) Reshamwala, S.; Shawky, B. T.; Dale, B. E. "Ethanol production from enzymatic hydrolysates of AFEX-treated coastal Bermuda grass and switchgrass." *Appl. Biochem. Biotechnol.* **1995**, *51/52*, 43–55. (e) Duff, S. J. B.; Murray, W. D. "Bioconversion of forest products industry waste cellulosics to fuel ethanol: a review." *Bioresour. Technol*. **1996**, *55*, 1–33.

4 (a) Boominathan, K;, Reddy, C. A. " cAMP-mediated differential regulation of lignin peroxidase and manganese-dependent peroxidase production in the white-rot basidiomycete Phanerochaete chrysosporium." *Proc. Natl. Acad. Sci*. **1992**, *89*, 5586–5590. (b) Hatakka, A. I. "Pretreatment of wheat straw by white-rot fungi for enzymatic saccharification of cellulose." *Appl. Microbiol. Biotechnol*. **1983**, *18*, 350–357. (c) Have, R; Teunissen, P. J. "Oxidative Mechanisms Involved in Lignin Degradation by White-Rot Fungi." *Chem. Rev.* **2001**, *101*, 3397-3413. (d) Carlile, M. J.; Watkinson, S. C. *The Fungi* **1994**, Academic Press.

5 (a) Lebo, S. E. Jr.; Gargulak, J. D.; McNally, T. J. "Lignin". Kirk-Othmer Encyclopedia of Chemical Technology. **2001**, John Wiley & Sons, Inc. (b) Zakzeski, J.; Bruijnincx, P. C. A.; Jongerius, A. L.; Weckhuysen, B. M. "The Catalytic Valorization of Lignin for the Production of Renewable Chemicals." *Chem. Rev*. **2010**, *110*, 3552-3599.

6 (a) de Lasa,H.; Salaices, E.; Mazumder, J.; Lucky, R. "Catalytic Steam Gasification of Biomass: Catalysts, Thermodynamics and Kinetics." *Chem. Rev*. **2011**, *111*, 5404-5433. (b) Milne, A.; Evans, J.; Abatzoglou, N. NREL/TP-570-25357; National Renewable Energy Laboratories: Golden, CO, **1998**. (c) Blasi, C. D.; Branca. C. "Kinetics of Primary Product Formation from Wood Pyrolysis". *Ind. Eng. Chem. Res*. **2001**, *40*, 5547-5556.

7 Laine, C. "Structures of Hemicelluloses and Pectins in Wood and Pulp." **2005**, Doctoral Dissertation, Helsinki University of Technology, Department of Chemical Technology.

8 (a) Cameron, M. D.; Post, Z. D.; Stahl, J. D.; Haselbach, J.; Aust, S. D. "Cellobiose dehydrogenase-dependent biodegradation of polyacrylate polyers by *Phanerochaete chrysosporium." Enviorn. Sci. & Pollut. Res*. **2000**, *7*, 130-134. (b) Cameron, M. D.; Aust, S. D. "Degradation of Chemicals by Reactive Radicals Produced by Cellobiose Dehydrogenase from *Phanerochaete chrysosporium*." *Biochemistry and Biophysics* **1999**, *367*, 115-121.

9 Kajikawa, H., Kudo, H., Kondo, T., Jodai, K., Honda, Y., Kuwahara, M., & Watanabe, T. "Degradation of benzyl ether bonds of lignin by ruminal microbes." *FEMS microbiology letters*, **2000**, *187*, 15-20.

10. We selected three aromatic adducts from the possible degradation of class III probes for this investigation: 3,4-dimethoxyacetophenone, 3,4-dimethoxybenzyl alcohol and 1-(3,4-dimethoxylphenyl)ethanol. There was only a slight decrease in fluorescence when 4MU was mixed with 3,4-dimethoxyacetopheone and no changes on the other two combinations. Therefore, we were certain that there was no degradation of class III probes by fungal enzymes. Please refer to Supporting Information for more details.

11. This procedure was modified from the reported procedure in the literature: Kirk, T. K.; Croan, S.; Tien, M.; Murtagh, K. E.; Farrell, R.L. "Lignin Peroxidase from Fungi: *Phanerochaete chrysosporium*." *Enzyme Microb. Technol*. **1986**. *8*, 27-32.

A library of fifteen fluorogenic probes was designed to mimic the dominant linkages in celluloses, hemicelluloses and lignin, and screened against crude extracts from white-rot fungi. The results show that fungal enzymes display a high preference for cleaving mannose- and glucose-based probes, which mimic hemicelluloses. This finding may enable the uses of fungal enzymes as a tool for developing an effective process for isolating and utilizing hemicelluloses from biomass.