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Lignocellulolytic activities of culturable marine woodborers' gut microbiota

Bosire Carren M*[1], Abubakar Laila U[2]

[1]*Department of Pure and Applied Sciences, Technical University of Mombasa, Mombasa, Kenya*

[2]*Department of Biochemistry, University of Nairobi, Nairobi, Kenya*

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Abstract

In this study, pure bacterial and fungal isolates obtained by culturing inoculum from woodborers' gut were induced to produce lignocellulolytic enzymes in a variety of substrates and their lignocellulolytic activities investigated. The inocula were obtained from woodborers *Dicyathifer mannii* (Wright, 1866), *Sphaeroma terebrans* (Bate, 1866) and *Cirolana* sp. The cultures used enrichment media containing ground *Rhizophora mucronata* wood, filter paper, carboxyl methylcellulose, avicel cellulose, beechwood xylan and cellobiose as sole carbon sources. The microorganisms showed generally low ligninolytic activities but commendable cellulolytic and hemicellulolytic activities. β-glucosidase and xylanase activities were the highest activities exhibited by both bacterial and fungal isolates. The highest was bacterial β-glucosidase activity (94.55 U/ml) shown by *Lysinibacillus boronitolerans* from *S. terebrans* gut cultured in a medium containing avicel cellulose. Xylanase activity was also relatively high (up to 91.7U/ml) cultured in media containing cellobiose and xylan. The fungal isolate with the highest cellulolytic activity was β-glucosidase activity of 38.34 U/ml shown by *Aspergillus niger* obtained from the gut of *S. terebrans* and cultured in a medium containing avicel cellulose as a sole carbon source. Avicel cellulose, cellobiose and xylan beech wood were found to be best inducers of cellulase and hemicellulase production. *Lysinibacilli* and *Aspergilli* in this study present interesting advantages that make them good models for studying physiological approaches to enzyme production and lignocellulose degradation.

* **Corresponding Author:** Bosire Carren M ✉ crrnbosire@gmail.com

Introduction

There are three groups of marine woodborers along the Indian coasts. These are Shipworms, Piddocks and Pill Bugs (Santhakumaran, 1996). Shipworms belonging to Family Teredinidae, are molluscs in class bivalvia (Turner, 1966; Turner, 1971). Piddocks, are also molluscs Family Pholadidae class bivalvia (Turner, 1971). The Pill Bugs on the other hand are arthropods in the class crustacean, order is opoda and family Sphaeromatidae (Pillai, 1961). Three species of woodborer belonging to two of the above-mentioned groups were encountered in this study. They were identified in our earlier work as *Dicyathifer mannii* (Wright, 1866) belong to phylum Molusca, class bivalvia, family Teredinidae; *Sphaeroma terebrans* (Bate, 1866) belong to phylum Arthropoda, class crustacea, family Sphaeromatidae; and *Cirolana sp*. belonging to phylum Arthropoda, class crustacea, family Cirolanidae (Bosire *et al*., 2015). The culturable marine woodborers' gut microbiota in this study were both bacterial and fungal isolates obtained from the above-mentioned marine woodborers' gut inocula. 16S rRNA and ITS gene barcoding identified them respectively, in our earlier work (Bosire *et al*., 2013a). Four strains of bacteria were identified as *Lysinibacillus boronitolerans* (from *D. mannii a*nd *S. terebrans*), *Lysinibacillus fusiformis* (from *S. terebrans* and *Cirolana* sp.), *Lysinibacillus sphaericus* and *Lysinibacillus xylanilyticus* (both from *Cirolana sp.).* Fungal isolates were identified as *Aspergillus niger* (from *D. mannii* and *S. terebrans*), *Neosartorya fischeri*, *A. fumigatus* and *Penicillium* sp. (from *D. mannii*), *Botryotinia fuckeliana* (from *S. terebrans*), *A. costaricaensis* and *A. fumigatus* (from *Cirolanna* sp.).

Lignocellulose is the major structural component of plants composed of a mixture of carbohydrate polymers (cellulose and hemicellulose) tightly bound to lignin, a complex polymer of phenyl propanoid molecules (Lee, 1997). Wood, grass, agricultural, forest and urban solid wastes all contain lignocellulose.

Lignin is degradable by lignin peroxidease (LiP), Mn-dependent peroxidase (MnP), and laccase (Lac) or mono-phenol oxidase (Vaaje-Kolstad *et al*., 2010; Bugg *et al*., 2011; Majumdar *et al*., 2014; Beeson *et al*., 2015 and Pollegioni *et al*.2015). Complete enzymatic hydrolysis of cellulose requires endo-β-1, 4-D- glucanase (EC 3.2.1.4), exo-β-1, 4-D-glucanase or exo-β-1, 4-D-cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (de Vries and Visser, 2001; de Vries, 2003; Watanabe and Tokuda, 2010; van den Brink and de Vries, 2011 and Payne *et al*., 2015). Complete degradation of xylan heteropolymer requires endoxylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), acetylxylan esterase (EC 3.1.1.72), L-arabinose releasing enzymes such as α-L-arabinofuranosidase (EC 3.2.1.55) and arabinoxylan arabinofuranohydrolase, α-glucuronidase (EC 3.2.1.139), feruloyl esterase, and *p*-coumaroyl esterase (Sunna and Antranikian, 1997; Gregory *et al*., 1998). Cellulasesand hemicellulases are produced by a number of microbes, including fungi and bacteria (Beguin, 1990; Pauly *et al*., 1999; de Souza, 2013; Busk and Lange, 2015).

The major constituents in lignocellulose enzyme hydrolysates are glucose and xylose released from cellulose and hemicellulose, respectively (Himmel, 2007; Hahn-Hagerdal *et al*., 2007; Galazka *et al*., 2010, and de Souza, 2013). The fermentation of the sugars from these hydrolysates generates products such as ethanol, acetone, butanol, glycerol, acetic acid, citric acid and fumaric acid (Howard *et al*., 2003; Madson and Tereck, 2004). Ethanol and butanol produced from degraded and fermented lignocellulose can be used as biofuels (Sun and Cheng, 2002). Bioethanol converted from edible sources such as corns and sugarcane is called first-generation bio-ethanol (FGB), whereas that produced from non-food sources is called second-generation bio-ethanol (SGB). The latter offers great promise to replace fossil fuels without causing the feud of food-fuel supply as they are derived from non-edible sources such as lignocellulose biomass (Tengerdy and Szakacs, 2003; Tan *et al*., 2008; Chun *et al*., 2010).

Biofuels are emerging as a promising option with which to cope with climate change and a diminishing oil supply (Goldemberg and Coelho, 2004; Jun-Seok *et al*., 2010). Therefore, the focus of the present work was to explore the lignocellulolytic activities of culturable bacteria and fungi from the gut of woodborers *D. mannii*, *S. terebrans* and *Cirolana sp.* The enzymes could find application in lignocellulosic biomass bioconversion industries especially bioethanol production.

Materials and methods

Chemicals (Enzymes, Substrates, Buffers and Reagents)

Laccase (from Trametes versicolor), Mn-dependent peroxidase (MnP), lignin peroxidase (LiP), β-glucosidase from *Aspergillus niger*, p-Nitrophenyl β-D-glucopyranose (pNPG), 4-hydroxy-3, 5-dimethoxybenzaldehyde azine (syringaldazine), D (+)- xylose, microcrystalline cellulose (Avicel PH101), carboxy methylcellulose (CMC) sodium salt, D (+)- cellobiose, 3,4-dimethoxybenzyl alcohol (veratryl alcohol), PMSF (phenyl methyl sulfonyl flouride), sabouraud dextrose agar (SDA), and beech wood xylan were all purchased from Sigma-Aldrich, USA. All other chemicals were of analytical grade.

Enzyme Production Induction of Microorganisms from the Gut of the Woodborers

One *D. mannii*, three *S. terebrans,* and three *Cirolana sp*. were surface sterilized with 70% ethanol, rinsed in distilled water and allowed to air dry for 1 minute. Under aseptic conditions, the entire gut of *D. mannii* and the guts of the 2 isopod species were separately removed with a blade and a pair of tweezers and mixed with 1 ml 0.85% sodium chloride to obtain inoculums.

Bacteria were cultivated in nutrient agar and subsequent culturing and plating carried out to obtain pure colonies. Fungi were cultivated on sabouraud dextrose agar (SDA) medium solidified with 1.6% w/v agar. They were incubated at 30ºC for 2-5 days.

A single agar disc was cut from the actively growing colony margin of a culture to inoculate each assay medium in subsequent culturing and plating to isolate pure colonies.

The bacteria and fungi above were induced to produce lignocellulolytic enzymes by culturing them in enrichment media containing ground *Rhizophora mucronata* wood, filter paper, carboxy methylcellulose, avicel cellulose, beech wood xylan and cellobiose as sole carbon sources. Crude enzyme extracts from these micro organisms were screened for lignocellulolytic activities and the activities compared between different carbon sources.

The bacterial isolates were cultured in liquid culture medium containing 0.1M citrate-phosphate buffer, 5 g/L carbon source (carboxyl methylcellulose sodium salt (CMC)/ filter paper/ beech wood xylan/*R. mucronata* wood dust/cellobiose/avicel cellulose) and 0.2g/L yeast extract. The cultures were incubated at 37ºC for 3 days. Solubilisation of insoluble carbon sources indicated degrading activity. Proteinase inhibitor PMSF (phenyl methyl sulfonylflouride) was added to the culture media. The media were homogenized and centrifuged at 12000 rpm for 30 minutes at 4ºC and the supernatant (crude extract) stored at $^{-}$20ºC.

The fungal isolate were cultured in liquid medium containing 0.1M citrate-phosphate buffer, pH 4, 10g/L yeast nitrogen base and 5 g/L carbon source (carboxyl methylcellulose sodium salt (CMC)/filter paper/beech wood xylan/*R. mucronata* wood dust/cellobiose/avicel cellulose). Solubilisation of insoluble carbon sources indicated degrading activity. Proteinase inhibitor PMSF was added to the culture media. The culture media were homogenized and centrifuged at 12000 rpm for 30 minutes at 4ºC and the supernatant (crude extract) stored at $^{-}$20ºC. The extracts were used in determination of lignocellulolytic activities.

Determination of Lignocellulolytic Activities of Woodborers' Gut Microbiota

Determination of Ligninolytic Activity of the Extract

Ligninolytic activities were determined spectra photo metrically using a Beckman Coulter^{tm} UV/VIS spectrophotometer (DU® 530 Life Science). The least count of absorbance measurement was 0.001 (Risna and Suhurma, 2002; Sahay *et al*., 2008; Yadav *et al*., 2009; Singh *et al*., 2011).

Lignin peroxidase (LiP) was determined by the peroxide-dependent oxidation of 10mm veratryl alcohol to veratraldehyde in 125mM tart rate buffer, pH 3.0 with 2mM H_2O_2 (controls without H_2O_2 were included), according to Orth *et al*. (1993). The molar extinction coefficient value of 9300 M^{-1} Cm^{-1} for veratraldehyde at 310nm was used for calculating the enzyme units. All enzyme assays were carried out in triplicate. The results were also interpreted as percent discolouration compared to the control calculated as (A_{310} for control – A_{310} for test/A_{310} for control) x 100 (Denise *et al.,* 1996).

Manganese-dependent peroxides (MnP) was assayed by oxidation of phenol red, which was measured by monitoring the A_{610} (ε = 22.0 mM^{-1} cm^{-1}) at room temperature (Camarero *et al*., 1999; Salame *et al*., 2012). The reaction mixture contained 250mm lactate, 2mM $MnSO_4$, 0.5% bovine serum albumin, 1mg/ml of phenol red, and 0.5 ml of supernatant in 20mm sodium succinate buffer (pH 4.5) in a total volume of 1ml. The reaction was initiated by the addition of H_2O_2 to final concentration of 2mm and was stopped after 1min with 50µl of 10% NaOH. Omitting MnSO4 from the reaction mixture carried out control assays of phenol red oxidation in the absence of Mn^{2+}. MnP activity was calculated by subtracting the value for phenol red-oxidizing activity in the absence of Mn^{2+} from the value for the activity obtained in the presence of manganese. All enzyme assays were carried out in triplicate.

Laccase (Lac) was analyzed by monitoring the oxidation of 0.25mM syringaldazine in 100mM citrate–phosphate buffer at pH 5.2 (Fakoussa and Frost, 1999; Lopez *et al*., 2007). Heat inactivated (100ºC for 15min) supernatant and enzyme substrate controls were included. Lac activity was determined by the increase in the absorbance due to the formation of tetramethoxy-azo-bis-methylene quinone, resulting from the oxidation of syringaldazine as described by Leonowicz and Grzywnowickz (1981). The activity was assayed in mixed reactions containing supernatant, citrate-phosphate buffer (pH 5.2, 0.1M) and syringaldazine 0.25mm in methanol. An increase in absorbance at 530 nm (ε = 65000 M^{-1} cm^{-1}) was followed at 25ºC to determine laccase activity in international units (IU) (Salmones and Mata, 2002). All enzyme assays were carried out in triplicate.

All ligninolytic enzyme activity was expressed as IU/ml. An international unit IU (or U) is defined as the amount of enzyme activity, which catalysed the transformation of 1 micromole of substrate per minute under standard conditions. This was calculated using the formula:

Enzyme Activity (U/ml) = (A*V) / (t*ε*v), where A = Absorbance at corresponding wavelength, V = Total volume of reaction mixture (ml), v = enzyme volume (ml), t= Incubation time (min) and ε = Corresponding Extinction Coefficient (M^{-1} cm^{-1}) (Desai *et al*., 2011).

Determination of Cellulolytic Activity of the Extract

The crude extract was assayed for cellulose activity by incubating crude enzyme solution with substrate and measuring amount of reducing sugar released. For general cellulase (FPase) activity, the substrate was What man No. 1 filter paper in 0.05M sodium citrate buffer (pH 4.8).

For CMCase (endoglucanase) and avicelase (exoglucanase) activity determination, the substrates were 1% (w/v) CMC sodium salt and

1 % avicel cellulose respectively, in 50mM sodium acetate buffer (pH 5.0). Boiling at 100ºC for 15 min stopped the reactions. The amount of reducing sugar released was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). Heat inactivated (100ºC for 15min) supernatant and enzyme substrate controls were included. Glucosidase activity was assayed with pNPG as the substrate according to Chen *et al*. (2010). All the enzyme assays were carried out in triplicate.

One unit of cellulase activity is defined as the amount of enzyme that released 1µmol/ml of glucose equivalents per min (Libmond and Savoie, 1993). Glucose was used as standard.

Determination of Hemicellulolytic Activity of the Extract

The crude enzyme extract was assayed for xylanase activity by incubating 0.1 ml of crude enzyme solution with 0.1ml of 0.5% (wt v^{-1}) Beech wood xylan freshly suspended in 0.1m pH 5.5 acetate buffer. The reaction mixture was incubated at 40°C for 30min and then completed to 1.0ml by adding 0.8ml distilled water (Sherief *et al*., 2010). The amount of reducing sugar released was determined by DNS method (Miller, 1959) against boiled enzyme using D-xylose as standard. All enzyme assays were carried out in triplicate. One unit of xylanase is defined as the amount of enzyme that catalyzed the release of one µ mole of D-xylose per ml per min under the assay conditions.

Results

Ligninolytic Activity Assays

There was generally low lignin modifying enzyme activity in both bacterial and fungal isolates (data not shown).

Cellulolytic and Hemicellulolytic Activities of the Crude Extracts

Both bacterial and fungal isolates exhibited endoglucanase, avicelase (exoglucanase), β-glucosidase and filter paper (FPase) activities.

Cellulolytic and Hemicellulolytic Activities of the Crude Bacterial Extract

Bacterial isolates showed significantly high cellulolytic (β-glucosidase) and hemicellulolytic (xylanase) activities. β-glucosidase activity was the highest (94.55 U/ml) shown by *S. terebrans* isolate 1 (*L. boronitolerans*) cultured in a medium containing avicel cellulose substrate as a carbon source.

Xylanase activity was also relatively high (up to 91.7 U/ml) by isolates in media containing cellobiose and xylan beech wood substrates. Highest xylanase activity was shown by all the 3 bacteria species (*L. fusiformis, L. xylanilyticus and L. sphaericus*) isolated from *Cirolana sp*. in media containing cellobiose substrate (Table 1).

β-Glucosidase production was induced in all the substrates used while xylanase was induced moderately in *R. mucronata* wood dust and FP and but highly induced in xylan (2 isolates) and cellobiose substrates.

All the bacteria isolates from the 3 woodborers showed an appreciably high xylanase induction in media containing cellobiose substrate (Fig. 1).

Table 1. Cellulolytic and Hemicellulolytic Activity of Bacterial Isolates.

Culture	Isolate	β-Glucosidase	Xylanase
C_1W	*L. fusiformis*	11.86 ±0.22	11.03 ±0.34
C_2W	*L. xylanilyticus*	28.92 ±0.06	13.43 ±0.3
C_3W	*L. sphaericus*	4.64 ±0.08	11.32 ±0.22
D_1W	*L. boronitolerans*	17.9 ±4.9	13.32 ±0.45
S_2F	*L. fusiformis*	9.53 ±0.35	13.21 ±0.4
S_3F	*L. fusiformis*	11.25 ±0.18	2.21 ±0.25
C_2F	*L. xylanilyticus*	10.51 ±0.46	-
C_3F	*L. sphaericus*	13.9 ±0.75	0.55 ±0.29
S_1CM	*L. boronitolerans*	19 ±0.38	0.59 ±0.34

Culture	Isolate	β-Glucosidase	Xylanase
S_2CM	*L. fusiformis*	31.57 ±0.43	0.51 ±0.25
C_2CM	*L. xylanilyticus*	11.92 ±0.25	0.52 ±0.26
S_1A	*L. boronitolerans*	94.55 ±8.35	0.5 ±0.25
S_3A	*L. fusiformis*	13.64 ±0.76	-
C_1A	*L. fusiformis*	14.62 ±0.37	0.49 ±0.24
D_1A	*L. boronitolerans*	15.84 ±0.23	11.12 ±0.25
S_2X	*L. fusiformis*	10.45 ±0.08	-
C_2X	*L. xylanilyticus*	10.92 ±0.17	-
C_3X	*L. sphaericus*	3.79 ±0.08	79.71 ±0.8
D_1X	*L. boronitolerans*	22.39 ±0.7	89.4 ±1.43
S_1CE	*L. boronitolerans*	27.31 ±0.59	79.78 ±0.13
S_2CE	*L. fusiformis*	29.77 ±3.07	88.66 ±0.46
S_3CE	*L. fusiformis*	6.31 ±0.18	88.6 ±0.39
C_1CE	*L. fusiformis*	1.55 ±0.11	90.14 ±1.18
C_2CE	*L. xylanilyticus*	1.5 ±0.06	91.7 ±1.35
C_3CE	*L. sphaericus*	28.57 ±0.71	90.44 ±1.43
D_1CE	*L. boronitolerans*	8.4 ±0.55	84.37 ±2.22

Data is Mean of triplicates (U/ml); bacterial isolates from the gut of woodborers D, *Dicyathifer manii*; S, *Sphaeroma terebrans* andC, *cirolana sp.*; cultured in media containing substrates W, wood; CM, carboxymethylcellulose; A, avicel cellulose; X, xylan beechwood and CE, cellobiose. (-) No activity.

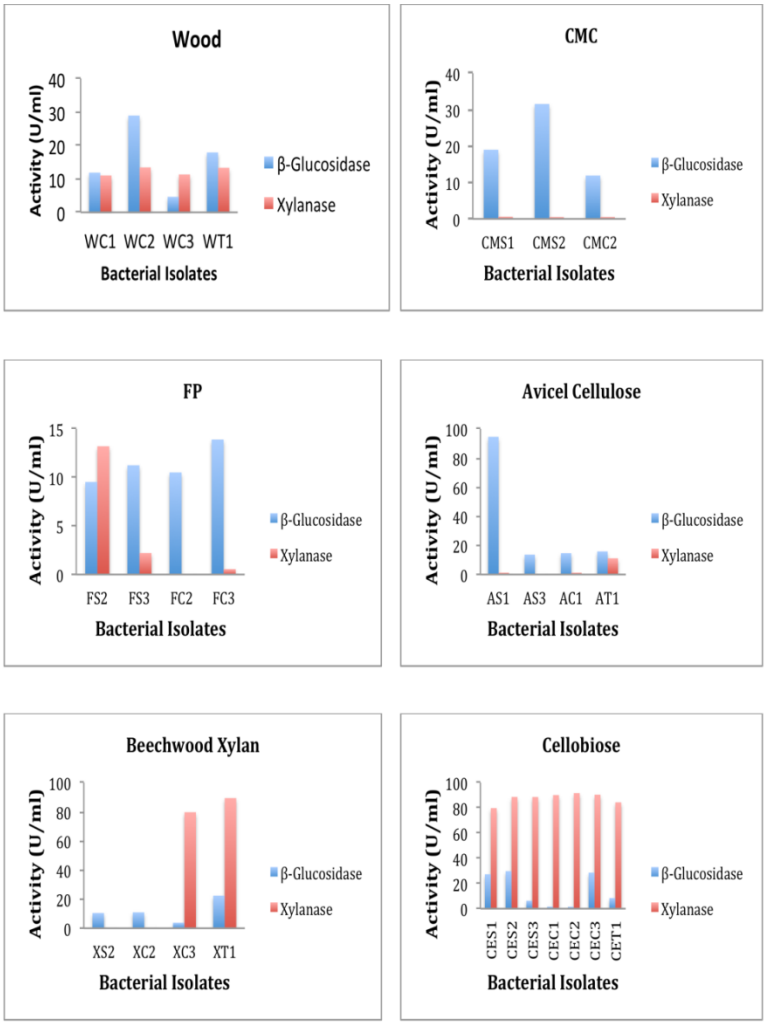

Fig. 1. β-glucosidase and Xylanase Activities of the Crude Supernatants.

Comparison of β-glucosidase and xylanase activities (U/ml) of the crude supernatants of bacterial cultures grown in enrichment media containing 0.5% (w/v) *Rhizophora* wood dust, CMC, FP, Avicel®, xylan beech wood and cellobiose as sole carbon sources. U = µmol reducing sugar produced/min.

Cellulolytic and Hemicellulolytic Activities of the Crude Fungal Extract

Inducement of cellulolytic and hemicellulolytic enzyme production by fungal isolates on a number of substrates was successful. Their activities were however relatively lower compared to bacterial isolates. They showed significantly high cellulolytic (CMCase or endoglucanase and β-glucosidase) activities and hemicellulolytic (xylanase) activity.

The fungal isolate with the highest cellulolytic activity was β-glucosidase activity of 38.34 U/ml shown by *A. niger* obtained from the gut of *S. terebrans* and cultured in a mediumc ontaining avicel as a sole carbon source.

The next highest β-glucosidase activity was by another *A. niger* strain (33.62 U/ml) also from *S. terebrans* cultured in medium containing xylan beech wood substrate. The highest xylanase activity (17.02 U/ml) was exhibited by yet another *A. niger* strain from *D. mannii* cultured in a medium containing cellobiose, whereas the highest CMCase activity (14.14 U/ml) was exhibited by *A. costaricaensis* from *Cirolana* cultured in a medium containing xylan beech wood (Table 2).

FPase and Avicelase activities were very low in all the fungal isolates (data not shown).

Table 2. Cellulolytic and Hemicellulolytic Activity of Fungal Isolates.

Culture	Isolate	CMCase	β-glucosidase	Xylanase
S_1CM	*A. niger*	3.57 ± 0.67	0.44 ± 0.01	10.58 ± 0.56
S_1A	*A. niger*	2.35 ± 0.14	16.84 ± 2.22	2.37 ± 0.25
S_2A	*A. niger*	2.33 ± 0.15	18.87 ± 0.61	3.09 ± 0.03
S_3A	*A. niger*	0.7 ± 0.36	38.34 ± 0.56	2.44 ± 0.22
S_4A	*B. fuckeliana*	1.46 ± 0.2	11.32 ± 0.3	3.33 ± 0.22
S_5A	*A. niger*	9.33 ± 0.88	14.18 ± 0.28	1.56 ± 0.11
C_1A	*A. costaricaensis*	1.42 ± 0.03	19.6 ± 0.39	1.61 ± 0.53
C_2A	*A. fumigatus*	0.12 ± 0.05	17.76 ± 0.5	0.89 ± 0.44
D_2A	*A. niger*	2.87 ± 0.08	17.6 ± 1.81	2.66 ± 0.22
D_5A	*Penicillium sp.*	2.48 ± 0.28	16.69 ± 0.61	2.37 ± 0.25
S_1X	*A. niger*	7.36 ± 0.61	17.78 ± 0.08	13.84 ± 1.43
S_2X	*A. niger*	10.6 ± 0.53	33.62 ± 1.8	11.77 ± 0.59
S_3X	*A. niger*	10.66 ± 0.51	6.11 ± 0.61	11.92 ± 0.72
S_4X	*B. fuckeliana*	9.7 ± 0.28	16.21 ± 0.24	12.8 ± 0.56
S_5X	*A. niger*	11.55 ± 0.4	8.37 ± 0.56	14.65 ± 0.45
C_1X	*A. costaricaensis*	14.14 ± 0.28	20.72 ± 0.37	13.32 ± 1.11
C_2X	*A. fumigatus*	11.01 ± 0.7	21.22 ± 0.65	10.21 ± 1.18
D_1X	*A. niger*	7.25 ± 0.56	21.09 ± 0.56	14.5 ± 1.12
D_2X	*A. niger*	9.51 ± 0.57	17.19 ± 0.65	11.54 ± 0.59
D_3X	*N. fischeri*	11.32 ± 1.18	14.15 ± 0.25	0.93 ± 0.17
D_4X	*A. niger*	10.4 ± 1.68	14.4 ± 1.51	11.69 ± 0.56
D_5X	*Penicillium sp.*	9.51 ± 0.57	18.67 ± 0.2	4.22 ± 0.22
S_1CE	*A. niger*	7.88 ± 0.59	15.76 ± 0.3	15.77 ± 0.23
S_2CE	*A. niger*	1.41 ± 0.28	18.65 ± 0.19	11.1 ± 1.11
S_3CE	*A. niger*	0.11 ± 0.03	13.45 ± 0.16	16.36 ± 0.9
S_4CE	*B. fuckeliana*	0.43 ± 0.59	12.81 ± 0.09	12.73 ± 0.34
S_5CE	*A. niger*	1.67 ± 0.56	14.66 ± 0.33	10.51 ± 0.56
C_1CE	*A. costaricaensis*	0.22 ± 0.06	15.46 ± 0.12	5.03 ± 0.56
C_2CE	*A. fumigatus*	0.13 ± 0.03	11.72 ± 0.1	7.4 ± 1.7
D_1CE	*A. niger*	0.13 ± 0.04	16.05 ± 0.21	13.99 ± 0.59
D_2CE	*A. niger*	0.14 ± 0.03	18.17 ± 0.13	17.02 ± 0.92
D_3CE	*N. fischeri*	0.88 ± 0.2	18.72 ± 0.29	15.32 ± 0.8
D_4CE	*A. niger*	0.13 ± 0.04	21.93 ± 0.36	14.65 ± 1.18
D_5CE	*Penicillium sp.*	0.13 ± 0.03	15.3 ± 0.26	16.06 ± 0.56

Data is Mean of triplicates (U/ml); fungal isolates from the gut of woodborers D, *Dicyathifer manii*; S, *Sphaeroma terebrans* andC, *cirolana sp.*; cultured in media containing substrates CM, carboxymethylcellulose; A, avicel cellulose; X, xylan (beech wood) and CE, cellobiose.

While avicel cellulose, xylan bech wood and cellobiose substrates showed sufficient inducement of CMCase, β-glucosidase and xylanase production by many fungal isolates, CMC showed. sufficient inducement of only *A. niger* from *S. terebrans* gut. Interestingly, CMCase (endoglucanase) production was significantly induced by xylan beech wood substrate (Fig. 2). Fungal isolates grew slowly in media containing

R. mucronata wood and FP as a sole carbon source. The resulting enzyme activities of these cultures were very low (data not shown).

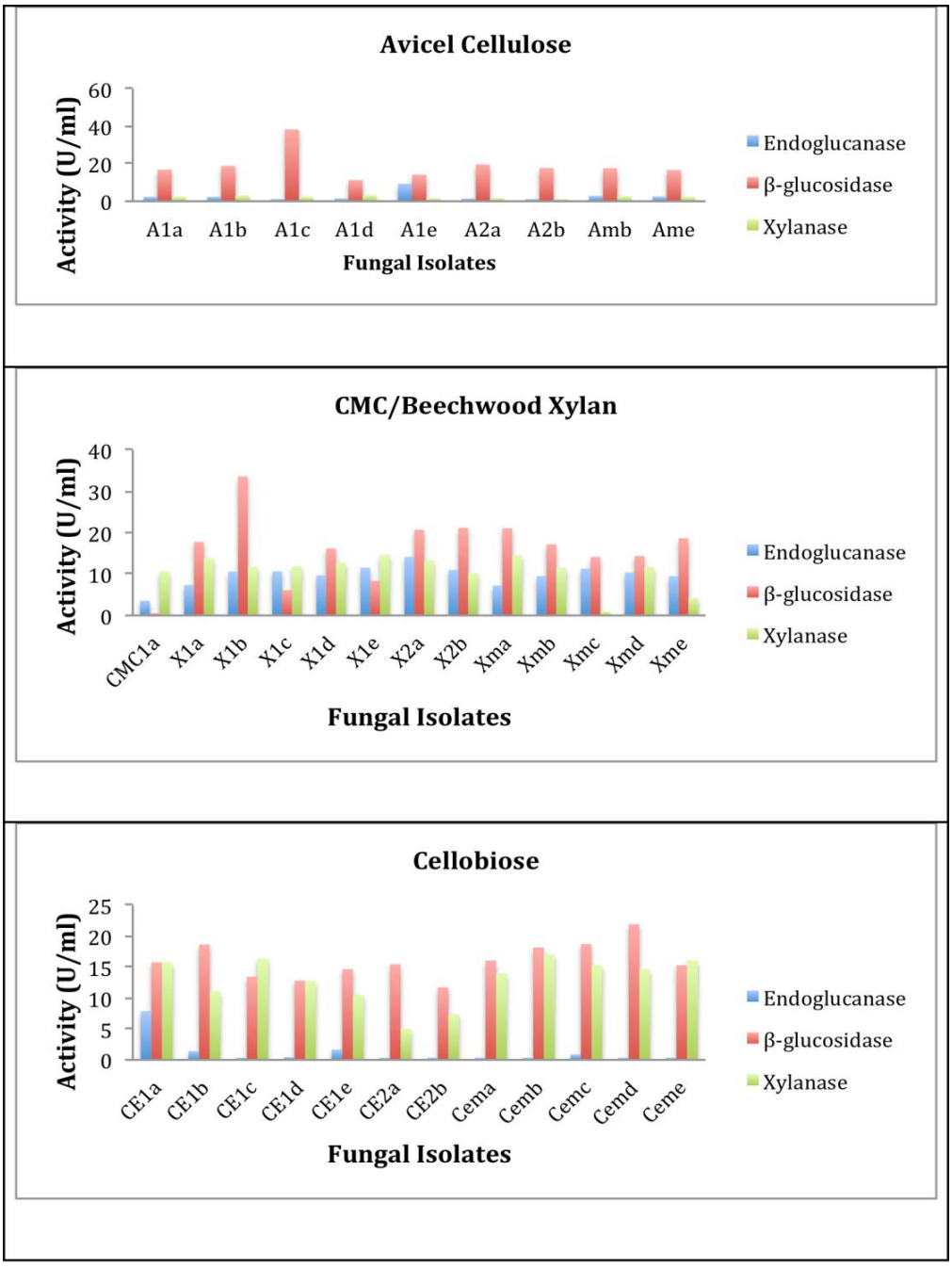

Fig. 2. Activities of the Crude Fungal Supernatants.

Comparison of CMCase (endoglucanase), β-glucosidase and xylanase activities (U/ml) of the crude supernatants of fungal cultures grown in enrichment media containing 0.5% (w/v) Avicel®, CMC or xylan beech wood and cellobiose as sole carbon sources. U = µmol reducing sugar produced/min.

Discussion

In this study, pure bacterial and fungal isolates obtained by culturing inoculum from woodborers gut were induced to produce lignocellulolytic enzymes in a variety of substrates and their lignocellulolytic activities were investigated. The cultures used enrichment media containing ground *R. mucronata* wood dust, filter paper, CMC, avicel cellulose, beech wood xylan and cellobiose as sole carbon sources.

The micro organisms showed generally low ligninolytic activities but commendable cellulolytic and hemicellulolytic activities. This is in agreement with a similar study using soil fungal strain on wheat straw as a model agro-waste substrate by solid-state fermentation (Acharya *et al*., 2010). The study showed a low MnP activity of between 0.108 ± 0.031U/g and 2.849 ± 0.143 U/g but high cellulolytic and hemicellulolytic activities of between 0.424U/g and 84.87U/g. β-glucosidase and xylanase activities were the highest activities exhibited by both bacterial and fungal isolates in our study (Table 3). The highest was bacterial β-glucosidase activity (94.55U/ml) shown by *L. boronitolerans* from *S. terebrans* gutcultured in a medium containing avicel cellulose. Xylanase activity was also relatively high (up to 91.7U/ml) cultured in media containing cellobiose and xylan.

The fungal isolate with the highest cellulolytic activity was β-glucosidase activity of 38.34 U/ml shown by *A. niger* obtained from the gut of *S. terebrans* and cultured in a medium containing avicel as a sole carbon source.

Table 3. Activity maxima of woodborers' gut microbiota.

Culture	Substrate	Isolate	Activity	Units (U/ml)	±	SD
S_1A	Avicel	*L. boronitolerans*	β -glucosidase	94.55	±	8.35
C_2CE	Cellobiose	*L. xylanilyticus*	Xylanase	91.70	±	1.35
C_3CE	Cellobiose	*L. sphaericus*	Xylanase	90.44	±	1.43
C_1CE	Cellobiose	*L. fusiformis*	Xylanase	90.14	±	1.18
D_1X	Xylan	*L. boronitolerans*	Xylanase	89.40	±	1.43
S_2CE	Cellobiose	*L. fusiformis*	Xylanase	88.66	±	0.46
S_3CE	Cellobiose	*L. fusiformis*	Xylanase	88.60	±	0.39
D_1CE	Cellobiose	*L. boronitolerans*	Xylanase	84.37	±	2.22
S_1CE	Cellobiose	*L. boronitolerans*	Xylanase	79.78	±	0.13
C_3X	Xylan	*L. sphaericus*	Xylanase	79.71	±	0.80
S_3A	Avicel	*A. niger*	β -glucosidase	38.34	±	0.56
S_2X	Xylan	*A. niger*	β -glucosidase	33.62	±	1.80
S_2CM	CMC	*L. fusiformis*	β -glucosidase	31.57	±	0.43
S_2CE	Cellobiose	*L. fusiformis*	β -glucosidase	29.77	±	3.07
C_2W	Wood	*L. xylanilyticus*	β -glucosidase	28.92	±	0.06
C_3CE	Cellobiose	*L. sphaericus*	β -glucosidase	28.57	±	0.71
D_2CE	Cellobiose	*A. niger*	Xylanase	17.02	±	0.92
S_3CE	Cellobiose	*A. niger*	Xylanase	16.36	±	0.9
D_5CE	Cellobiose	*Penicillium sp.*	Xylanase	16.06	±	0.56
S_1CE	Cellobiose	*A. niger*	Xylanase	15.77	±	0.23
D_3CE	Cellobiose	*N. fischeri*	Xylanase	15.32	±	0.80

Data is Mean of triplicates (U/ml); bacterial and fungal isolates from the gut of woodborers D, *Dicyathifer manii*; S, *Sphaeroma terebrans* andC, *cirolana sp.*; cultured in media containing substrates CM, carboxy methylcellulose; A, avicel cellulose; X, xylan (beech wood); CE, cellobiose; and W, *Rhizophora mucronata* wood dust.

Our study showed *A. niger* β-glucosidase activity of between 0.44U/ml to 38.34U/ml, endoglucanase 0.11U/ml to 11.15U/ml, and xylanase 0.89U/ml to 17.02U/ml. FPase and exoglucanase activities were the lowest. In another study (Acharya *et al*., 2010), lignocellulolytic soil fungal strains activity profile using wheat straw as a model agro-waste by solid-state fermentation showed *A. niger* activities of β-glucosidase 6.171U/g to 9.259U/g, endoglucanase 15.43U/g to 64.811U/g, xylanase 11.102U/g to 84.87U/g and FPase 3.858U/g to 9.259U/g.

Exoglucanase showed the lowest activity of 0.424U/g to 0.831. Both studies implicate *A. niger* as a potent lignocellulolytic fungi.

The filamentous fungus *A. niger* is known to produce a wide range of hemicellulose-degrading enzymes and it has been used for many industrial applications. Our study confirms its consideration as one of the most important microorganism for cellulase/hemicellulase production.

It constitutes the source of these enzymes for industrial applications, including the production of biofuels from plant biomass (de Souza, 2013). This study discovered that fungal isolates grew slowly in media containing *R. macronata* wood and FP as a sole carbon source. The resulting enzyme activities of these cultures were very low, indicating that wood and FP were not good carbon sources for the inducement of production of cellulolytic and hemicellulolytic enzymes from the isolates.

Cellulolytic bacteria have been reported in different genus such as *Clostridium, Ruminococcus, Caldicellulosiruptor, Butyrivibrio, Acetivibrio, Cellulomonas, Erwinia, Thermobifida, Fibrobacter, Cytophaga, and Sporocytophaga*. Bacterial degradation of cellulolytic material is more restricted to biomass containing low amounts of lignin, since bacteria are poor producers of ligninases (Lynd *et al*., 2002). This explains the low inducement by *R. mucronata* wood and FP substrates in this study, which have high lignin content.

The low ligninolytic activities in our study could be due to use of single cultures. Co-culturing could improve inducement of production of ligninases, cellulases and hemicelluloses. Noratiqah *et al*., 2016 reported four micro organisms from *Bulbitermes* sp. termite gut (A1, B1, B2, and Br3) with high lignocellulolytic enzyme activity. They were identified as *Aspergillus* sp., *Bacillus* sp., *Bacillus* sp. and *Brevibacillus* sp., respectively. *Aspergillus* sp. A1 showed highest activities of lignin peroxidase (729.12 U/g) and β-glucosidase (22.97 U/g). The highest endoglucanase (138.77 U/g) and manganese peroxidase (47.73 U/g) activity were observed in *Bacillus* sp. B1. The *Bacillus* sp. B2 produced highest activities of exoglucanase (32.16U/g) and laccase (71.18U/g). The highest xylanase (104.96U/g) activity was observed in *Brevibacillus* sp. Br3. The production of enzymes particularly endoglucanase, β-glucosidase, xylanase, lignin peroxidase and laccase were approximate 17-93% higher in co-culture compared to single culture. Their findings suggested that saw dust can be used as a cheap renewable raw material for the induction of production of lignocellulolytic enzymes. The study also indicated that the fungal-bacterial co-culture could be a good alternative for the enzymes production.

Bacterial isolates in this study showed significantly high cellulolytic (β-glucosidase) and hemicellulolytic (xylanase) activities. β-glucosidase activity was the highest (94.55U/ml) shown by *L. boronitolerans* cultured in a medium containing avicel cellulose substrate as a carbon source. Xylanase activity was also relatively high (up to 91.7U/ml) by isolates in media containing cellobiose and xylan beech wood substrates. Highest xylanase activity was shown by all the 3 bacteria species (*L. fusiformis, L. xylanilyticus and L. sphaericus*) in media containing cellobiose substrate.

Inducement of cellulolytic and hemicellulolytic enzyme production by fungal isolates on a number of substrates was relatively lower compared to bacterial isolates. They showed significantly high cellulolytic (CMCase or endoglucanase and β-glucosidase) activities and hemicellulolytic (xylanase) activity. The fungal isolate with the highest cellulolytic activity was β-glucosidase activity of 38.34U/ml shown by *A. niger* cultured in a medium containing avicel as a sole carbon source. The next highest β-glucosidase activity was by another *A. niger* strain (33.62U/ml) cultured in medium containing xylan beech wood substrate.

The highest xylanase activity (17.02U/ml) was exhibited by yet another *A. niger* strain cultured in a medium containing cellobiose, whereas the highest CMCase activity (14.14U/ml) was exhibited by *A. costaricaensis* from *Cirolana* sp. cultured in a medium containing xylan beech wood.

This showed that both bacterial and fungal isolates were induced to produce enzymes by the various substrates used and the highest induction was by avicel cellulose, cellobiose and xylan beech wood.

The biochemistry of the process behind lignocellulosic degradation has been studied in detail (Gregory *et al*., 1998; de Vries and Visser, 2001; Pollet *et al*., 2010; Vaaje-Kolstad *et al*., 2010; Bugg *et al*., 2011; Majumdar *et al*., 2014; Beeson *et al*., 2015 and Pollegioni *et al*., 2015). However, the mechanism by which the microorganisms detect the substrate and initiate the overall process of enzyme production is still unsolved.

Some researchers have proposed that a low constitutive level of cellulase expression is responsible for the formation of an inducer from cellulose, amplifying the signal (Amore *et al*., 2013). Others have suggested that the microorganisms initiate a starvation process, which could in turn activate cellulase expression (de Souza, 2013). It is also possible that an inducing sugar derived from carbohydrates may be released from the organism's cell wall, which acts as a de-repressing molecule for hydrolase induction. Despite the fact that the true mechanism behind natural cellulase production induction is still lacking, some individual molecules have been reported to induce production of hydrolases.

The fungus *T. reesei* is an impressive producer of cellulases and most studies concerning the regulation of cellulase genes have been performed in this species.

The most powerful inducer of cellulases in *T. reesei* is sophorose, a disaccharide composed of β-1, 2-linked glucose units. Sophorose appears to be formed from cellobiose through trans glycosylation activity by β-glucosidase (Gritzali, 1979; Vaheri *et al*., 1979; Fowler and Brown, 1992). In addition to *T. reesei*, sophorose is also known to induce cellulase expression in *A. terreus* and *Penicillium purpurogenum* (Bisaria and Mishra, 1989; Hrmova *et al*., 1991).

Another important inducer of cellulase is cellobiose (two β-1, 4-linked glucose units). It is formed as the end product of cellobiohydrolases activity. It has been reported to induce cellulase expression in many species of fungi, which include *T. reesei, Volvariella volvacea, P. janthinellum* and *A. nidulans* (Hrmova *et al*., 1991; Mernitz *et al*., 1996; Ilmen *et al*., 1997; Chikamatsu *et al*., 1999; Ding and Buswell, 2001).

Some studies concerning the inducing effect of cellobiose on cellulase expression are however controversial (Aro *et al*., 2005). For instance, cellobiose can be trans glycosylated by β-glucosidases, producing sophorose, which could be the true inducer of cellulases. In addition, β-glucosidases are able to cleave the cellobiose into glucose, which may cause repression by carbon catabolite repression (CCR). Therefore, the outcome in cellobiose cultures appears to be dependent on the balance between hydrolysis and trans glycosylation, as well as the subsequent uptake of the generated sugars and the intracellular signals they initiate.

Interestingly the disaccharide lactose (1,4-O- β-D-galactopyranosyl-D-glucose), which is not a component of plant cell wall polymers, has been shown to be economically viable in inducing cellulase expression in *T. reesei*. Lactose induction of cellulase genes requires the β-anomer of D-galactose (Fekete *et al*., 2008).

Hemicellulase expression has been studied mostly in *Aspergilli* and *T. reesei* (Pel *et al*., 2007; Martinez, *et al*., 2008). It is reported that the presence of the hemicelluloses xylan, xyloglucan, arabinan and mannan usually induces a high production of hemicellulases. However, just as for cellulases, the mechanism is unknown.

In most cases, small hemicellulose-derived molecules are able to induce the expression of a wide range of hemicellulases.

The monosaccharide D-xylose is a well-known inducer of xylanolytic enzymes as well as other hydrolase genes such as α-glucuronidase (agu A), acetylxylan esterase (axe A) and feruloyl esterase (faeA) in *Aspergillus* species (de Vries and Visser, 1999; de Vries *et al*., 2002). Some studies have demonstrated that xylose can act as a repressor carbon source of hemicelluloses induction at high concentrations (de Vries *et al*., 1999).

On the other hand, other studies have demonstrated that utilization of a high D-xylose concentration was beneficial for the induction of hemicelluloses-encoding genes (Mach-Aigner *et al*., 2012). In addition to xylose, xylobiose and D-glucose-β-1, 2-D-xylose have been demonstrated to induce expression of xylanolytic genes in *A. terreus* (Hrmova *et al*., 1991).

In *T. reesei*, the induction of hemicelluloses was observed during growth in the presence of cellulose, xylan, sophorose, xylobiose and L-arabitol (Aro *et al*., 2005). For instance, a xylanase gene (xyn2) was induced by sophorose and xylobiose (Mach *et al*., 1996; Zeilinger *et al*., 1996). Xylobiose has also been reported to be able to induce genes involved in xylan degradation in *T. reesei*, such as the xylanase genes xyn1 and xyn2 and the β-xylosidase gene bxl1. In addition, sophorose induced some genes encoding enzymes that cleave the side chains of xylan such as agl1 and agl2 (α-galactosidase genes), and glr1, encoding a α-glucuronidase gene (Margolles-Clark *et al*., 1997).

Complex mixtures of polysaccharides have also been shown to induce a wide range of cellulase and hemicelluloses genes in *A. niger*. A good example is sugarcane biogases, a by-product of sugarcane that has been shown to induce lignocellulolytic enzyme production (de Souza *et al*., 2011).

Induction of ligninase production using carboxyl methylcellulose (CMC), filter paper (FP), beech wood xylan, *R. mucronata* wood dust, cellobiose and avicel cellulose was very poor in this study. There was generally low lignin modifying enzyme activity in both bacterial and fungal isolates. In our earlier work (Bosire *et al*., 2013b), all the crude gut extracts from two of the woodborers in this study showed ligninolytic, cellulolytic and xylolytic (hemicellulolytic) activities. Generally, among the lignin modifying enzymes, there was a high lignin peroxidase activity in the crude gut extracts obtained from all the woodborers screened whereas laccase and manganese peroxidase activities were relatively lower. MnP activity ranged from 1.64 ± 0.021U/L to 4.73 ± 0.002U/L. This was the lowest ligninolytic activity. It was followed by Lac activity ranging from 0.36 ± 0.028U/L to 21.16 ± 0.591U/L. LiP activity was the highest ranging from 17.65 ± 0.079U/L to 34.65 ± 0.116U/L. The highest ligninolytic activity (LiP activity of 34.65U/L) exhibited 68.9% discolouration. It was not clear whether the enzymes were produced by gut micro biota symbionts or they are endogeneous, produced by glands in the digestive tract. The fact that bacterial and fungal isolates in this study showed very low ligninolytic activities, implies that ligninolytic enzymes are endogenously produced in the digestive glands in the gut of the woodborer hosts while cellulases and hemicelluloses produced by the endosymbionts complete the digestive process into oligomers and monomers.

Conclusion

The study showed that bacterial and fungal strains of *Lysinibacilli* and *Aspergilli* used are highly potential and useful for β-glucosidase and xylanase production.

It is therefore concluded that *Lysinibacilli* and *Aspergilli* in this study present interesting advantages that make them good models for studying physiological approaches to enzyme production and lignocellulose degradation, including inhibition studies, with the aim of developing the key enzymes. Their genomic features and systematic studies on their enzyme systems need to be investigated to facilitate directed strain engineering for improved performance in degradation of plant biomass.

The natural ability of these bacteria and fungi to degrade cellulosic biomass, due their highly efficient enzymatic systems, is very attractive for the development of new strategies concerning industrial processes. It is evident that complete digestion of wood in the woodborers guts require both ligninolytic endogenous enzymes produced by host glands and cellulolytic as well as hemicellulolytic enzymes produced by the endosymbionts. Therefore, there is need for the elucidation of woodborers digestomics, which will define the collective pool of host and symbiont genes that collaborate to achieve a high-efficiency lignocellulose digestion. This study has shown that avicel cellulose, cellobiose and xylan beech wood were the highest inducers of cellulases and hemicelluloses. These substrates are therefore recommended for induction of production of these enzymes.

Lastly, the high efficiency of marine woodborers gut bioreactors makes them promising models for the industrial conversion of lignocellulose into microbial products, enzymes and enzyme combinations of potential value to biomass-based industries, such as cellulosic biofuel production.

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