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Lignocellulolytic activities of crude gut extracts of marine woodborers *Dicyathifer mannii* and *Sphaeroma terebrans*

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Abstract

Marine woodborers have a close association with tropical mangrove plants whereby they voraciously consume lignocellulose and play a role in nutrient cycling. They represent a rich source of potential lignocellulolytic enzymes that can be harnessed for conversion of biomass into simple sugars and other monomers for a variety of uses. Ligninolytic enzymes find applications in bio bleaching of pulp and decolouration of textile dyes, whereas cellulolytic and hemicellulolytic enzymes find applications in animal feed, manufacture of bread, bioethanol production and xylitol production among other uses. In this study, we obtained crude gut extracts from two marine woodborers, *Dicyathifer mannii* (Wright, 1866) and *Sphaeroma terebrans* (Bate, 1866), from three sampling sites along the Kenyan coast. Lignocellulolytic activities of the gut extracts were investigated in an effort to seek the species with the most lignocellulolytic efficacious extracts. Ligninolytic activities investigated were lignin peroxidase (LiP), manganese-dependent peroxidase (MnP) and laccase (Lac) or monophenol oxidase. Cellulolytic enzymes investigated were glucanases endoglucanase (endo-1-4-β-D-glucanase), exoglucanase (1,4-β-D-glucan-cellobiohydrolase), and β-D-glucosidase or cellobiase (β-D-glucoside glucanohydrolase). Endo-1-4-β-xylanase was investigated in the hydrolysis of xylan, the chief type of hemicellulose. *D. mannii* crude extracts showed an appreciable Lip activity of up to 34.65±0.116 U/L and endoglucanase (CMCase) activity of up to 50.7 U/ml (1 U represents the amount of enzyme which catalyzed the transformation of 1 micromol of substrate min^{-1}). *D. mannii* is implicated as a source of these enzymes for industrial use.

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Introduction

The woodborer *Dicyathifer mannii* (Wright, 1866) belong to phylum Molusca, class bivalvia, family Teredinidae whereas *Sphaeroma terebrans* (Bate, 1866) belong to phylum Arthropoda, class crustacea, family Sphaeromatidae. They consume and digest lignocellulose.

Lignocellulose is a major structural component of plants. It's a complex substrate composed of a mixture of carbohydrate polymers (cellulose and hemicellulose) and lignin. The carbohydrate polymers are tightly bound to lignin mainly by hydrogen bonds but also by some covalent bonds. Lignin is a complex polymer of phenylpropanoid molecules. The biological process degrading lignocellulose requires delignification to liberate cellulose and hemicellulose from their complex with lignin and depolymerisation of the carbohydrate polymers to produce free sugars (Tengerdy and Szakacs, 2003).

Lignin is extremely resistant to chemical and enzymatic degradation. Mainly fungi achieve biological degradation most efficiently by white-rot basidiomycetes, but also by certain actinomycetes. Lignin degradation by these organisms is a secondary metabolic process, occurring under low levels of nutrient nitrogen and requiring the presence of a carbon source such as glucose or cellulose. An extracellular lignin peroxidase (LiP), in the presence of H_2O_2, can degrade lignin by bringing about oxidative cleavage of the C–C backbone, oxidation and hydroxylation of benzylic methylene groups, oxidation of phenols and benzyl alcohols, etc. Reaction of the LiP with H_2O_2 generates a high-redox-potential porphyrin cation radical (oxferryl complex) which can extract a single electron from an aromatic ring in the lignin substrate to generate aromatic cationic radicals; this is followed by a variety of spontaneous degradative reactions via radical and cation intermediates.

Other enzymes implicated in aerobic lignin degradation include Mn-dependent peroxidase (MnP) and laccase (monophenol oxidase). MnP oxidizes Mn^{2+} to Mn^{3+} that acts as a unique redox couple, and in turn oxidizes phenolic substrates to phenoxy radicals that undergo subsequent reactions to yield final products (Lopez *et al*., 2007; Zouari-Mechichi *et al*., 2006). Laccases catalyze the one-electron oxidation of a vast amount of phenolic substrates. Molecular oxygen serves as the terminal electron acceptor and is thus reduced to two molecules of water.

Cellulase enzymes are produced by a number of microbes, including fungi, yeast, and bacteria. The enzymatic hydrolysis of cellulose takes place under the action of a cellulolytic enzyme complex containing at least three types of glucanases. These are endoglucanase (endo-1-4-β-D-glucanase), exoglucanase (1,4-β-D-glucan-cellobiohydrolase), and β-D-glucosidase or cellobiase (β-D-glucoside glucanohydrolase). There is possibility of mutants with both cellulase and cellobiase activity (Romero *et al*., 1999; Brijwan *et al*., 2010).

Endoglucanases cleave intramolecular β-1,4-glucosidic linkages randomly, and their activities are often measured on a soluble high degree of polymerization (DP) cellulose derivative, such as carboxymethylcellulose (CMC). Exoglucanases hydrolyze long chains from the ends in a processive process (Irwin *et al*., 1993; Teeri, 1997; Zhang and Lynd, 2004). Avicel has been used for measuring exoglucanase activity because it has the highest ratio of the fraction reducing ends of all anhydroglucose units of cellulose to fraction of β-glucosidic bond accessible to cellulose (F_{RE}/F_a) among insoluble cellulosic substrates. Endoglucanase and exoglucanase activities can be measured based on a reduction in substrate viscosity and/or an increase in reducing ends determined by a reducing sugar assay. β-D-glucosidases hydrolyze soluble cellobiose and other cellodextrins with a DP up to 6 to produce

glucose in the aqueous phase. (Zhang and Lynd, 2004). β-D-glucosidases are very amenable to a wide range of simple sensitive assay methods, based on colored or fluorescent products released from pnitrophenyl β-D-1, 4-glucopyranoside (Deshpande *et al.*, 1984; Strobel and Russell, 1987), β-naphthyl- β-D-glucopyranoside, 6-bromo-2-naphthyl-β-D-gluco-pyranoside (Polacheck *et al.*, 1987), and 4-methylumbelliferyl-β-D-glucopyranoside (Setlow *et al.*, 2004). In addition, β-D-glucosidase activities can be measured using cellobiose, which is not hydrolyzed by endoglucanases and exoglucanases, and using longer cellodextrins, which are hydrolyzed by endoglucanases and exoglucanases (Ghose, 1987; Gong *et al.*, 1977; McCarthy *et al.*, 2004; Zhang and Lynd, 2004).

The main enzymes involved in xylan degradation are the xylanases. These enzymes are produced mainly by microorganisms that break down plant cell walls, but are also present in marine algae, protozoans, crustaceans, insects, snails and seeds of land plants (Sunna and Antranikian, 1997). Hemicellulolytic enzymes include; endo-β-1, 4-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, acetyl-esterase and feruloyl- or p-coumaroyl esterase (Coughlan and Hazlewood 1993). A full set of enzymes required to release all the constituents of xylan has not yet been isolated from a single bacterial species, but some filamentous fungi secrete an effective xylan-hydrolysing enzyme mixture (Schafer *et al.,* 1996).

Lignin-degrading enzymes are oxidoreductive enzymes that play an important role in degradation and transformation of polymeric substances, which have practical application in bio-bleaching of pulp, decolouration of textile dyes and bioremediation of polluted environment (Castillo *et al.,* 1997) among other uses. Lignin peroxidases are useful in the treatment of coloured industrial effluents and other xenobiotics as it has bioremediation potential to decolourize the effluents (Buzzini *et al.,* 2006). During hydrolysis of lignocellulosic biomass, the major constituents in enzyme hydrolysates are glucose and xylose released from cellulose and xylan, respectively. Cellulases and xylanases find applications in animal feed, manufacture of bread, ethanol and xylitol production among other uses.

D. mannii bore deeply into the wood using two small anteriorly placed roughened shell valves that are small in relation to the rest of the body. The valves are rotated with respect to the foot, carving a circular burrow that is then lined by calcareous secretion of the mantle. They ingest a certain amount of wood and digest it although they posses filter feeding mechanism (George and George, 1979). The stomach is provided with a caecum for sawdust storage, and a section of digestive gland is specialized for handling wood particles. Symbiotic bacteria housed within a special organ that opens into the oesophagus provide cellulose digestion and also, by fixing nitrogen, compensate for the low-protein diet (Barnes, 1987).

S. terebrans feed on wood, and their hepato-pancreatic secretions include cellulase. They are thought to be attracted to the fungi in the wood (Geyer and Becker, 1980). The fungi add nitrogen to their largely cellulose diet. Cellulose digestion is thought to be accomplished by bacteria, the hindgut playing a major role in the digestive process (Hassall and Jennings, 1975).

For a number of years now, lignocellulolytic activity has been investigated in several organisms. Bacteria and several fungi have been reported to exhibit ligninolytic enzyme (Lac, MnP and LiP) activity (Arora *et al.*, 2002; Zouri-Mechichi *et al.*, 2006; Quaratino *et al.*, 2007; Lopez *et al.*, 2007; Sahay *et al.*, 2008; Fakoussa and Frost, 1999; Desai *et al.*, 2011; Bholay *et al.*, 2012). Cellulolytic and Xylanolytic enzymes have also been demonstrated from bacteria and several fungi (Gong *et al.*, 1977; Romero *et al.*, 1999; Suna and Antranikian, 1997; Karnchanatat *et al.*, 2008). Schafer *et al.* (1996) demonstrated hemicellulose-degrading bacteria and yeasts from termite gut.

Enzymatic properties and primary structures of many cellulases have been investigated and cellulase genes from widely differing origins have been cloned and

sequenced (Hamada *et al.*, 1999, Karnchanatat *et al.*, 2008). Digestive β-glucosidases from wood-feeding termites have been cloned and sequenced (Tokuda *et al.*, 2002; Tokuda *et al.*, 2009). However, little is known about the lignocellulolytic enzymes from marine woodborers.

In the present investigation, we report on lignocellulolytic activities of gut extracts of the two marine woodborers for potential in industrial applications.

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), carboxymethylcellulose (CMC) sodium salt, D (+)-cellobiose, 3,4-dimethoxybenzyl alcohol (veratryl alcohol) and beechwood xylan were all purchased from Sigma-Aldrich, USA. All other chemicals were of analytical grade.

Woodborers

Adult *Dicyathifer mannii* and *Sphaeroma terebrans* were collected from submerged parts of roots (proproots, pneumatophores), stems and branches of mangrove plants *Rhizophora mucronata* and *Avicennia marina* respectively, in the intertidal region of Mida Creek (North coast), Tudor Creek (island) and Gazi Bay (south coast) along the Kenyan coast.

Extraction and Preparation of Extracts

35 *D. mannii* and 200 *S. terebrans* were surface sterilized with 70% ethanol, rinsed in distilled water and allowed to air dry for 1 minute. Under aseptic conditions, the entire guts were separately removed with a blade and a pair of tweezers and immediately washed in sodium phosphate buffer (0.1 M, pH 6.2). The tissues were pooled separately and homogenized (5 min) in 100 ml of chilled sodium phosphate buffer (0.1 M, pH 6.2) using an electric homogenizer. The homogenates were centrifuged at 12000 rpm for 30 minutes at 4°C. The resultant supernatant was stored in aliquots of 5 ml at $^{-}$20°C (Foster *et al.*, 1999) and used for screening for lignocellulolytic enzymes.

Determination of Lignocellulolytic Activities of the Extract

Lignocellulolytic activities were determined spectrophotometrically 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; Yadav *et al.*, 2009; Singh *et al.*, 2011; Sahay *et al.*, 2008).

Ligninolytic Activity

Lignin peroxidase (LiP) was determined by the peroxide-dependent oxidation of 10mM veratryl alcohol to veratraldehyde in 125mM tartrate 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 peroxidase (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 (Salame *et al.*, 2012, Camarero *et al.*, 1999). The reaction mixture contained 250 mM lactate, 2mM $MnSO_4$, 0.5% bovine serum albumin, 1mg/ml of phenol red, and 0.5 ml of supernatant in 20 mM sodium succinate buffer (pH 4.5) in a total volume of 1 ml. The reaction was initiated by the addition of H_2O_2 to final concentration of 2mM and was stopped after 1 min with 50 µl of 10% NaOH. Control assays of phenol red oxidation in the absence of Mn^{2+} were carried out by omitting $MnSO_4$ from the reaction mixture. 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 (100 ^{o}C for 15 min) 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 25 ^{o}C 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).

Cellulolytic Activity

The crude extract was assayed for cellulase activity by incubating crude enzyme solution with substrate and measuring amount of reducing sugar released. For general cellulase (FPase) activity, the substrate was Whatman 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 100 ^{o}C for 15 min stopped the reactions. The amount of reducing sugar released was determined by the dinitrosalicylic acid (DNS) method (Miller, 1959). Heat inactivated (100 ^{o}C for 15 min) 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 1 µmol/ml of glucose equivalents per min (Libmond and Savoie, 1993). Glucose was used as standard.

Hemicellulolytic Activity

The crude enzyme extract was assayed for xylanase activity by incubating 0.1 ml of crude enzyme solution with 0.1 ml of 0.5% (wt v^{-1}) Beechwood xylan freshly suspended in 0.1 M pH 5.5 acetate buffer. The reaction mixture was incubated at 40°C for 30 min and then completed to 1.0 ml by adding 0.8 ml 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 catalysed the release of one µ mole of D-xylose per ml per min under the assay conditions.

Data analysis

Rate of enzyme activity can be measured as disappearance of reactant or accumulation of product (Nelson and Cox, 2004). This was monitored spectrophotometrically as change in absorbance. To quantify enzyme activity;

Rate of reaction

= Concentration of substrate disappearing per unit time (mol L^{-1} min^{-1})

= Concentration of product produced per unit time (mol L^{-1} min^{-1})

Enzyme activity

= Moles converted per unit time

= Rate × reaction volume

1 international unit (IU or U) of enzyme activity is defined as enzyme quantity required for consuming 1 μmol of substrate or producing 1 μmol of product per minute (Manole *et al.*, 2008). Data was represented as means ± S.D.

Results

All the gut extracts from the two woodborers showed ligninolytic, cellulolytic and xylolytic activities (Table 1). 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 low. MnP activity ranged from 1.64 ± 0.021 U/L in *S. terebrans* from Gazi Bay to 4.73 ± 0.002 U/L in *S. terebrans* from Tudor Creek. This was the lowest ligninolytic activity. It was followed by Lac activity ranging from 0.36 ± 0.028 U/L in *S. terebrans* from Gazi Bay to 21.16 ± 0.591 U/L in *D. mannii* from the same study site. LiP activity was the highest ranging from 17.65 ± 0.079 U/L in *D. mannii* from Tudor Creek to 34.65 ± 0.116 U/L in *D. mannii* from Gazi Bay. The highest ligninolytic activity (LiP activity of 34.65 U/L) exhibited 68.9% discolouration.

Table 1. Lignin Modifying Enzyme activity of crude gut extracts.

Lignin Modifying Enzyme activity of crude gut extracts (U/L)			
Isolate	Lac (A_{530})	LiP (A_{310})	MnP (A_{610})
DT	8.75±0.229	17.65±0.079	3.76±0.019
DG	21.16±0.591	34.65±0.116	3.15±0.016
DM	10.32±0.276	18.26±0.260	2.41±0.004
ST	4.44±0.316	22.67±0.610	4.73±0.002
SG	0.36±0.028	25.14±0.121	1.64±0.021
SM	3.18±0.217	21.99±0.651	3.71±0.003

Data is Mean of triplicates ±SD; Woodborers D, *Dicyathifer mannii* and S, *Sphaeroma terebrans*; sampling sites T, Tudor Creek, G, Gazi bay and M, Mida Creek; enzymes Lac, Laccase, LiP, Lignin Peroxidase and MnP, Manganese Peroxidase.

There was generally high cellulolytic and hemicellulolytic activities by *D. mannii sp.* from all the three sampling sites (Table 2). Fpase activity ranged from 2.22 ± 0.111 U/ml in *S. terebrans* from Gazi Bay to 34.78 ± 0.714 U/ml in *D.mannii* from Tudor Creek. Avicelase activity ranged from 1.78 ± 0.24 U/ml in *S. terebrans* from Tudor Creek to 17.5 ± 1.399 U/ml in *D.mannii* also from Tudor Creek. β-glucosidase activity ranged from 10.77 ± 0.743 U/ml in *S. terebrans* from Tudor Creek to 22.31 ± 0.462 U/ml in *D. mannii* from Gazi Bay. Xylanase activity ranged from 6.66 in *S. terebrans* from both Mida Creek and Gazi Bay to 35.52 ± 1.539 U/ml in *D. mannii* from Gazi Bay. CMCase activity ranged from 2.59 ± 0.128 U/ml in *S. terebrans* from Gazi Bay to 50.7 ± 1.512 U/ml in *D. mannii* from the same study site. The highest cellulolytic/hemicellulolytic activity was therefore endoglucanase (CMCase) activity of 50.7 U/ml in *D. mannii* sampled from Gazi Bay.

Table 2. Cellulolytic and Hemicellulolytic activity spectrum of gut extracts.

Cellulolytic /Hemicellulolytic activity spectrum of wood-borers' crude gut extracts (U/ml)					
Wood-borer	Fpase	CMCase	Avicelase	β-glucosidase	Xylanase
DT	34.78±0.714	37.37±1.829	17.5±1.399	17.39±0.213	26.64±1.154
DG	34.41±1.017	50.7±1.512	15.02±0.583	22.31±0.462	35.52±1.539
DM	31.82±0.128	27.75±1.635	15.8±0.423	13.91±0.607	28.86±1.539
ST	6.29±0.501	8.51±0.650	1.78±0.240	11.77±0.916	8.88±0.385
SG	2.22±0.111	2.59±0.128	2.00±0.327	11.47±0.678	6.66±0.000
SM	2.96±0.170	12.95±1.025	3.63±0.367	10.77±0.743	6.66±0.000

Data is Mean of triplicates ± SD; woodborers D, *Dicyathifer mannii* and S, *Sphaeroma terebrans*; sampling sites T, Tudor Creek, G, Gazi bay and M, Mida Creek.

Discussion

In this study, we have demonstrated that the gut extracts of marine woodborers *D. mannii* and *S. terebrans* exhibit lignocellulolytic activities. The highest activities (LiP and endoglucanase) were shown by *D. mannii*, a wood boring teredinid.

In a recent study, Kalmis *et al.* (2008) investigated ligninolytic enzyme activities of different fungal species (six commercial and 13 wild) in solid and liquid culture media. Highest Lac activity of 941.66 ± 1.67 U/L was obtained from wild *Pleurotus ostreatus*-4 (PO-4) after 12 days cultivation, the highest MnP activity of 267.63 ± 0.55 U/L was from wild *P. eryngii* (PE-1) after 10 days and the highest LiP activity of 17.84 ± 0.11 U/L was from a commercial strain of *P. sajor-caju* (PS) after 14 days cultivation.

While Lac and MnP activity in our study is not comparable with the activity maxima obtained in their study, the highest LiP activity of 34.65±0.116 U/L obtained in our study is much higher (two times) than that from their LiP activity maxima. Also, in another study to investigate bacterial LiP activity on industrial effluents (Bholay *et al.,* 2012), the activity ranged between 30% to 76% discolouration while in our study the highest LiP activity exhibited 68.9%. In addition, the highest endoglucanase activity of 50.7 U/ml observed in this study is appreciable as it is comparable to a purified endoglucanase activity (50.2 U/mg) from *Penicillium notatum NCIM NO-923* produced under mixed solid-state fermentation of waste cabbage and Bagasse (Das *et al.,* 2012). Therefore *D. mannii* is implicated as a source of these enzymes for industrial use.

The lignocellulolytic activities we observed are as a result of lignocellulolytic enzymes, but it is not clear whether the enzymes are produced by gut microbiota symbionts or they are endogeneous, produced by glands in the digestive tract. Ability of Teredinidae to feed on wood is thought to depend on intracellular bacterial endosymbionts contained within specialized cells (bacteriocytes) of their gills. These bacterial endosymbionts are thought to produce cellulolytic enzymes that aid the host in digestion of wood (Distel, 2003), and they are known to fix nitrogen (Lechene *et al.,* 2007; Waterbury *et al.,* 1983) that may supplement the host's nitrogen deficient diet. Lignin degrading enzymes are essentially extracellular in nature due to the large and complex structure of lignin that cannot enter the cell for intracellular action. This implies that enzymes that digest lignin are either produced by microorganisms in the gut or are secreted by glands in the digestive tract and released into the gut for action. Therefore, there is need to culture microorganisms from the gut of these woodborers and investigate their lignocellulolytic activities. These microorganisms could be potential sources of commercial enzymes for industrial use in bio-bleaching of pulp, decolouration of textile dyes and bioremediation of polluted environment (Castillo *et al.,* 1997) for lignin modifying enzymes; and in animal feed, manufacture of bread, ethanol and xylitol production for cellulolytic enzymes, among other uses.

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