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White-rot fungal growth on sugarcane lignocellulosic residue

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Summary. Twelve white-rot fungi were grown in solid state culture on sugarcane chips previously fermented by yeast employing the EX-FERM process. The lignocellulosic sugarcane residue had 12.5% permanganate lignin and 81.3% holocellulose. After 5 to 6 weeks at 20° C, all fungi produced a solid residue which had a lower in vitro dry matter enzymatic digestibility than the original bagasse, with the exception of *Coriolus versicolor* which showed a slight increase of 0.6 units. Four fungi produced a residue with higher soluble solids than the original sample. Lignin losses were rather similar for all fungi tested, an average value of 38.64% of the original value was obtained. About the same amount of hemicellulose was degreaded, 32.22%. Most fungi showed a preference for hemicellulose hydrolysis over cellulose degradation. The two fungi that showed greater cellulolytic activity were *Sporotrichum pulverulentum* and *Dichomitus squalens*. No appreciable dry matter losses were detected for *Agrocybe aergerita* and *Flammulina velutipes*.

Introduction

In natural decomposition of lignocellulosic matter, both fungi and aerobic bacteria play an important role in degrading holocellulose and lignin to lower molecular weight compounds, some of which are then further metabolized by facultative and obligate anaerobic soil bacteria and actinomycetes (Bazin et al. 1976). Sugarcane bagasse is no exception (Schmidt and Walter 1978; Sandhu and Sidho 1980). Fungi are usually better lignin degraders, particularly the so-called white rots which completely metabolize the complex polymer, exhibit the highest reported rates and have been the most studied (Kirk and Chang 1981; Crawford and Crawford 1984). Most of them, however, have the enzymatic capacity to use cellulose, hemicellulose and other components of lignocellulosic matter as a source of carbon and energy; hence total biomass breakdown usually occurs and lignin removal is accompanied by removal of polysaccharides (Kirk and Moore 1972). Some of the fungi produce fruiting bodies when growth occurs under solid substrate conditions. These two points make solid state fungal delignification an attractive alternative for converting lignocellulose into human food (fruiting bodies of edible fungi) and animal feed (solid residue with improved digestibility). Most of the work has been done with cereal straws from temperate countries and very few tropical lignocellulosic residues have been studied.

The fibrous residue remaining after juice extraction from the sugarcane stalk is commonly referred as bagasse. In sugar mills most of the bagasse is used as fuel, and although the figure varies within each factory, energy-efficient units can have 2 t (dry wt.) of surplus bagasse for every 100 t of fresh cane processed (Paturau 1982). The lignocellulosic residue left after sucrose conversion into ethanol by the EX-FERM process (Rolz 1981) will be referred here as EX-FERMented sugarcane chips.

In terms of macrocomponents the EX-FERMented sugarcane chips are very similar to sugarcane bagasse from industrial mills; the average values for cellulose, hemicellulose and lignin for EX-FERMented chips from four cane varieties were 44.8%, 37.7% and 13.5% respectively. The corresponding figures for three bagasse samples were 42.1%, 37.0% and 14.6% respectively. The lig-

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nin content of the samples is slightly higher than most cereal straws but digestibility is rather low; an extensive search of the literature on sugarcane bagasse showed that the mean in vitro dry matter enzymatic digestibility (IVDMED) was $17.2 \pm 4.5\%$ and the in vitro dry matter digestibility (IVDMD measured by employing ruminal fluid or the nylon bag technique) was $26.0 \pm 4.4\%$. Hence sugarcane lignocellulose is rather resistant to enzymatic hydrolysis-delignification.

Ibrahim and Pearce (1980) studied the effects of eleven white-rot fungi on the composition and in vitro digestibility of bagasse and found that greater delignification and increased digestibility was obtained with *Peniophora gigantea*. Sengupta et al. (1984) tested *Termitomyces clypeatus* and Madan and Bisaria (1984), *Pleurotus sajor-caju*. Nigam and Prabhu (1985 a, b) isolated three basidiomycetes which showed delignification values up to 59%; unfortunately cellulose and hemicellulose was also degraded extensively.

In this contribution, we report solid state culture of twelve basidiomycetes on EX-FERMented cane chips and their effect on the chemical composition and IVDMED values. None of the basidiomycetes tested were previously studied by Ibrahim and Pearce (1980).

Materials and methods

Microorganisms. In Table 1 the twelve basidiomycetes are identified and source documented. They were kept on PDA agar plus 3% yeast extract.

Substrate. Sugarcane was sampled in a sugar mill in the South Coast of Guatemala located around 300 m above sea level, during the month of April which was within the later part of the harvest during the dry season. The sugarcane sample came from the receiving yard and usually one or more cane varieties were being processed on the same day. The cane had been cut by hand and was not burned in the field. It was chipped in a pilot wood chipper (Type 9-72 OY Santsalo). The chips were placed in 7000-l tanks, water was added so as just to cover them and 2% of commerical baker's yeast was added. The EX-FERM cycle to convert all the sucrose into ethanol took 48 h. The fermented chips were pressed in a continuous screwpress (Model VP-GF, Vincent Corp.), washed with water in a mechanically agitated tank and screwpressed again. They were finally sun dried to a final moisture of around 6—7% and packed in plastic ventilated bags. A chip size between 0.5—1.6 cm was separated on circular hole screens (vibrating screen TMI 16-1).

The chemical composition on dry weight basis was: permanganate lignin 12.5%, cellulose 43.3%, hemicellulose 38.0%, ash 2.4% and protein 1.2%. Its neutral detergent fiber value, NDF, or cell wall components, was 92.8%. This value is in agreement with the sum of the individual contents for lignin, hemicellulose and cellulose. Its IVDMED was 19.04% and its saccharification rate at 8 h was 1.51 mg reducing sugars h^{-1}. The soluble solids determined in the IVDMED test were 4.18% of dry weight.

Inoculation and incubation. A recently inoculated culture tube for each fungus was left for 5 to 6 days at ambient temperature (20° C). This was transferred to a petri dish with the same medium. Usually it took 9—10 days at ambient temperature to cover the whole surface. If the dish was free of any contaminant, as seen macroscopically, one sixth of its contents were used to inoculate a 700 ml wide-mouth glass jar with a metal cap, in which 50 g of dried substrate plus 100 ml of water had been placed and sterilized for 30 min at 125° C. Duplicate jars for each fungal culture were left for 5 to 6 weeks at ambient temperature until fungal growth had completely covered the substrate surface. The jars were covered loosely with the metal caps and they were oxygenated every week for 15 min by introducing a flow of air through a tube connection installed within the metal cap. The entire jar contents were dried at 60° C in a forced air laboratory dryer.

Chemical analysis. The dried samples were milled in a laboratory Wiley mill employing a 0.84 mm sieve. The following analyses were made: Kjeldahl nitrogen and ash content (AOAC, 1975); neutral and acid detergent fiber, permanganate lignin and cellulose following the Van Soest techniques (Van Soest and Robertson 1980). In vitro dry matter enzymatic digestibilities (IVDMED) were determined as suggested by Goto and Minson (1977), Adegbola and Paladines (1977), McLeod and Minson (1978, 1980) and Dowman and Collins (1982). Briefly, 200 mg of the material was suspended in 20 ml of a 2 g l^{-1} pepsin solution (No. P-7125 Sigma Chemical Co.) in 1 N HCl and incubated at 40° C for 48 h. Samples were centrifuged, washed once with distilled water, centrifuged and suspended in 20 ml of a 2.5% by weight solution of fungal Onozuka FA cellulase (Maruzen Chemical Co. Ltd.) in 0.2 M acetate buffer pH 4.8 and incubated at 40° C for 48 h. The suspension was filtered in a glass filter crucible, washed with water, dried and weighed. Controls used distilled water instead of enzyme. During incubation the tubes were agitated twice a day. In order to obtain the saccharification rate, 100 mg of cane lignocellulose was suspended in 9 ml of 0.2 M acetate buffer at pH 4.8. One ml of a 1% by weight solution of Onozuka FA cellulase in 0.2 M acetate buffer pH 4.8 was added and the sealed tubes were placed in a rotary shaker (New Brunswick Co.) at 40° C and 300 rpm. At 8 h the solids were discarded and sugars determined in the liquid by the dinitrosalicylic acid method (Miller 1959). Controls used distilled water instead of enzyme. The filter paper activity of the enzyme solution was 0.02 units mg^{-1} protein. Soluble protein was determined by the Lowry method (Lowry et al. 1951). The IVDMED values represent weight loss due to enzymatic action in terms of initial dry matter of the sample but taking into account the weight loss of the control sample. Saccharification rates measured reducing sugars produced in 8 h by enzymatic action again taking into account any reducing sugars present in the control sample. So both parameters indicate net enzymatic action upon the substrate. Of course the figures can be converted from one to the other, however note that IVDMED was measured after 72 h employing 25 times more enzyme per unit weight of substrate.

Statistical procedures. Analysis of variance was performed on the IVDMED values using the F test. Those significant different from the control at the 0.95 level are indicated in the corresponding table.

Table 1. Basidiomycetes used, substrate chemical composition changes, saccharification rate and digestibility results

No.	Fungi[a]	Source	IVDMED (average of two values)	Saccharification rate at 8 h mg reducing sugars h^{-1}	Dry weight loss g	Soluble solids % of dry weight	NDF-SUM[g]	Lignin loss % of control	Cellulose loss % of control	Hemicellulose loss % of control
1.	*Phanerochaete chrysosporium* (F 1107)	USDA-FPL[b]	0.88*	0.58	5.84	6.14	10.3	40.16	13.63	90.16
2.	*Agrocybe aergerita* (F 1090)	CBS-BAARN[c]	0.42*	0.94	0.00	7.79	7.4	31.29	0.00	20.76
3.	*Flammulina velutipes* (F 1100)	CMI-KEW[d]	3.73*	0.69	0.00	3.50	7.3	37.46	4.41	16.13
4.	*Coprinus fimentarius* (F 1094)	NRC-SASK[e]	8.53*	0.82	2.07	3.93	1.6	37.89	2.82	24.83
5.	*Ganoderma applanatum* (F 1102)	CMI-KEW[d]	8.62*	0.58	1.14	1.93	6.7	40.58	1.37	20.02
6.	*Sporotrichum pulverulentum* (F 1113)	CMI-KEW[d]	10.07*	0.36	8.13	6.62	10.4	47.75	24.39	41.83
7.	*Pycnoporus sanguineus* (F 1112)	CMI-KEW[d]	10.78*	0.86	3.52	6.15	8.3	32.33	5.12	31.27
8.	*Dichomitus squalens* (F 1099)	CBS-BAARN[c]	12.44*	1.12	2.90	4.39	4.6	35.94	14.06	24.39
9.	*Ischnoderma resinosum* (F 1105)	USDA-FPL[b]	14.25*	0.79	6.34	3.27	7.6	42.72	10.26	33.13
10.	*Pleurotus flabellatus* (F 1011)	IARI-DELHI[f]	15.54*	0.75	5.35	0.34	8.5	40.70	12.55	27.14
11.	*Bondarzewia berkeleyi* (F 1093)	USDA-FPL[b]	15.80*	0.62	5.41	1.02	5.4	37.93	13.29	26.07
12.	*Coriolus versicolor* (F 1095)	USDA-FPL[b]	19.69	0.55	6.10	2.80	6.3	38.89	0.00	30.91

* Statistically different from control, significant 95%. Control was the analytical data for the EX-FERMented cane chips

[a] ICAITI collection number in parentheses

[b] Forest Products Laboratory, Madison, Ms. Frances F. Lombard, *P. chrysosporium* ME-446; *I. resinosum* (or *Polyporus resinosus* older name) L-13682-Sp; *B. berkeleyi* (or *Polyporus berkeleyi* older name) FP-105839-5; *C. versicolor* (or *Polyporus versicolor* older name) R-105-Sp

[c] Centraalbureau voor Schimmelcultures, Baarn

[d] Commonwealth Mycological Institute, Kew, *F. velutipes* 176670; *G. applanatum* 157818; *S. pulverulentum* 174727; *P. sanguineus* 75002

[e] National Research Council of Canada, Saskatoon, Dr. Ian D. Reid, *C. fimentarius* PRL 3001 (Source ATCC 36567)

[f] Indian Agricultural Research Institute, New Delhi, Dr. L. M. Joshi, *P. flabellatus* 1724

[g] Neutral detergent fiber minus the sum of the lignin, hemicellulose and cellulose contents. It should be close to cero for a balanced composition analytical data (see text)

Results and discussion

The IVDMED and saccharification rates, dry weight, lignin, cellulose and hemicellulose losses and production of soluble solids are given in Table 1. Individual component losses are expressed as relative values as % of the original value in the control and were obtained using the analytical data for each component, the solid yield and mass balance. The procedure will be exemplified for the first fungus in Table 1, *P. chrysosporium:* initial lignin content: (50 g) (0.125) = 6.25 g; initial cellulose content: (50 g) (0.433) = 21.65 g; initial hemicellulose content: (50 g) (0.38) = 19.0 g; % lignin on residue after fungal growth according to analysis = 8.4% dry weight; % cellulose on residue after fungal growth according to analysis = 42.0% dry weight; % hemicellulose on residue after fungal growth according to analysis = 4.2% dry weight; final lignin content = (50—5.48) (0.084) = 3.74 g; relative amount of lignin lost = ((6.25—3.74)/6.25)100 = 40.16%; final cellulose content = (50—5.48) (0.42) = 18.7 g; relative amount of cellulose lost = ((21.65—18.7)/21.65)100 = 13.63%; final hemicellulose content = (50—5.48) (0.042) = 1.87 g; relative amount of hemicellulose lost = ((19—1.87)/19)100 = 90.16%; ratio of degraded hemicellulose over degraded lignin = (19—1.87)/(6.25—3.74) = 6.82; ratio of degraded hemicellulose over degraded cellulose = (19—1.87)/(21.65—18.7) = 5.81. A column has been included that gives for each sample of fungus-degraded sugarcane chips, the difference between its NDF value and the sum of the analysed values of lignin, cellulose and hemicellulose. These form the cell wall polymers and should be close then to the NDF value; the difference being possible neutral detergent insoluble ash and other components. The average difference was 7.03 which is an 8% of the average NDF value for all samples. Note the following points: a) all fungi tested produced a solid residue which had a lower IVDMED than the original bagasse, with the exception of *Coriolus versicolor* which showed a slight increase of 0.65 units; b) there was no obvious correlations between IVDMED loss and change in soluble solids or between IVDMED and dry weight, cellulose, hemicellulose and/or lignin losses; c) all fungi tested produced a solid residue which had a lower saccharification rate than the control sample. The residue obtained after growth of *Sporotrichum pulverulentum* showed only (0.36/1.51)100 = 23.8% of the saccharification susceptibility of the original bagasse. This residue had the greatest dry weight and cellulose losses and was the second in hemicellulose loss. The relative dry weight loss was (8.13/50.00)100 = 16.3%; d) the following fungi showed an increase in soluble solids relative to the original value: *A. aegerita, S. pulverulentum, P. sanguineus, P. chrysosporium.* Hence no general patterns emerge. The enzymatic susceptibility of the solid residues cannot be predicted from the chemical composition of the residues only. Undoubtedly the type of bonds hydrolysed, the new compounds synthesized by the fungi and the physical characteristics of the solid residues play a major role in determining the extent and rate of further enzymatic hydrolysis.

The lignin losses were similar for all fungi tested. An average lignin loss of 38.64% was calculated. An average hemicellulose loss of 32.22% was observed and only *P. chysosporium* showed almost total hemicellulose hydrolysis. In absolute terms, however, more hemicellulose was degraded than lignin, as their content ratio in the original material was: 38.0/12.5 = 3.04. Lignin is not only the glueing element of the lignocellulose matrix, but also part of the lignin-carbohydrate complex (LCC) stabilized by phenolic acids such as ferulic and p-coumaric acids and acetyl constituents of the cell walls (Hartley, 1972, 1973, 1981; Chesson et al. 1983; Gordon et al. 1983; Tanner and Morrison 1983). The existance of LCCs in sugarcane bagasse has been reported (Nagaty et al. 1982; Crosthwaite et al. 1984; Atsushi et al. 1984; Molina et al. 1984; du Toit et al. 1984) and at least three different fractions have been identified. Hence it is not unexpected to have concurrent hemicellulose and lignin biodegradation.

In Table 2 we have calculated the hydrolysis ratios of hemicellulose:lignin and hemicellulose:cellulose components for the twelve fungal strains. The values for the first ratio are close to two with the exception of *P. chysosporium*, which showed a high hemicellulose loss. The values are also within a narrow range 1.30 to 2.91. On the other hand most fungi showed a preference for hemicellulose hydrolysis over cellulose. Very low cellulose degraders were *Agrocybe aergerita* and *C. versicolor*; the best cellulolytic fungi were *S. pulverulentum* and *Dichomitus squalens.* In the table we have included the results of a two-week solid substrate fermentation by two basidiomycetes isolated by Nigam and Prabhu (1985a, b) and designated BH1 and BW1. They showed very similar losses between themselves, so the average has been calculated. A lower hemicellulose to lignin loss ratio was observed. Both were very selective for cellulose hydrolysis, however, showing a

Table 2. Biodegradation ratios of various bagasse components

Fungal strain	Ratio 1[a]	Ratio 2[b]
1	6.82	5.81
2	2.01	very high
3	1.30	3.19
4	1.99	7.74
5	1.50	12.67
6	2.67	1.51
7	2.91	5.30
8	2.06	1.52
9	2.36	2.83
10	2.03	1.90
11	2.09	1.72
12	2.42	very high
BH1 / BH1	0.97	0.43

[a] Ratio of the amounts of hemicellulose degraded to lignin degraded

[b] Ratio of the amounts of hemicellulose degraded to cellulose degraded

rather active cellulase enzyme complex produced in sumerged culture (Nigan and Prabhu 1985a).

Kirk and Moore (1972) first reported that lignin removal from aspen and birch woods by white-rot fungi was always accompanied by removal of polysaccharides, although not necessarily correlated with removal of any particular fraction. In fact lignin degradation by *P. chrysosporium* was stimulated by addition of carbohydrates (Reid 1979). Wood polysaccharides provide the energy required for lignin attack (Hattaka and Uusi-Rauva 1983), so previous to or during lignolysis these fungi show holocellulose degradation. Blanchette (1984), employing scanning and transmission electron microscopy of wood decayed by *G. applanatum* and *Ischnoderma resinosum*, showed that in delignified areas the middle lamella was degraded causing an extensive cell defiberization. Wood sugar analysis by HPLC demonstrated that hemicelluloses were removed in preference to cellulose. This seems to coincide with our observations.

Weight losses for four to six weeks shown in Table 1 are somewhat low. No appreciable weight loss could be detected with *A. aergerita* and *Flammulina velutipes*. Three more fungi showed a loss of less than 10% of initial dry weight and the rest a loss of less than 17%. To our knowledge no similar data have been published on weight losses of bagasse under solid state fermentation by these white rot fungi. Higher weight losses have been reported for cereal straws. In 17 weeks at 30° C *Stropharia rugosoannulata* and *Pleurotus cornucopiae* degraded 60—65% of wheat straw; *Pleurotus florida* 45% and *A. aergerita* only 25% at 22—30° C (Zadrazil 1977). In 7 weeks at 22° C, *S. rugosoannulata* degraded 26%, *P. florida* 26.3%, *Pleurotus eryngii* 10.6%, *L. edodes* 12.7%, *Kuehneromyces mutabilis* 11.8%, *G. applanatum* 28.7%, *F. velutipes* 5.1%, *A. aergerita* 11.4% (Zadrazil and Brunnert 1980). *S. pulverulentum* at 35° C degraded 58 and 72% at 5 and 9 weeks and *D. squalens* 19.1 and 58.9% (Zadrazil and Brunnert 1982). Streeter et al. (1982) found that *P. ostreatus* in mixed culture with *Erwinia carotovora* degraded from 28.8% to 55.9% of wheat straw in 8 weeks. Hattaka (1983) tested 19 strains of white rots for 4 weeks at 28° C; those that degraded more biomass were *Fomes ignarius* 51.8%, *Phlebia radiata* 45.2%, *Phanerochaete sordida* 42.3%, *Polyporus brumalis* 38.5%, *Pycnoporus cinnabarinus* 35.6%, *Lenzites betulina* 35.6% and *C. versicolor* 30.8%.

With oat straw Levonen-Munoz et al. (1983) obtained with 11 white rots a rather wide range of dry matter loss, from 3% to 28%. In cotton straw, flavonoids present stimulated *P. florida* growth and in 3 weeks it degraded 17% of dry matter, compared to only 10.8% for wheat (Platt et al. 1983).

The 9 white rots tested by Ibrahim and Pearce (1980) attacked very little the lignin from sugarcane bagasse, however the authors did not record dry matter losses. None of these white rots were tested by us. The two basidiomycetes isolated by Nigam and Prabhu (1985b) degraded up to 60% of cane bagasse lignin with high dry weight losses of up to 58% (Nigam and Prabhu 1985a). In submerged culture Ibrahim and Pearce (1985a) reported a 18.3% lignin degradation by *P. ostreatus* and 20.9% by *C. versicolor*. These figures were increased to 26% and 29.8% when peptone was added to the medium. As shown in Table 1 our result for *C. versicolor* is higher, namely 38.89% lignin loss. The difference is difficult to explain but different culture conditions, cane varieties and fungal strains might be reasons. *C. versicolor* effectively degrades lignin from grasses and hard and softwoods; for an incubation of 10 weeks at 28° C Antai and Crawford (1982) obtained values of 61.6%, 58.8% and 63.3% respectively. These values are higher than ours in about twice the incubation time.

After 3 weeks four *Pleurotus* strains degraded from 10% up to 56% of lignin in cotton straw (Platt et al. 1984). With 8 white rot strains, Zadrazil and Brunnert (1980) reported from 0% *(F. velutipes)* to 54.7% *(P. florida)* lignin degradation in wheat straw. With sunflower hulls and rice husks,

S. rugosoannulata showed 46.8% and 42.1% degradation; *P. florida* 60.3% and 21.6%; *P. cornucopiae* 68.1% and 59.6% and *A. aergerita* 13.4% and 12.6% (Zadrazil, 1980). In 9 weeks with wheat straw, Zadrazil and Brunnert (1982) reported very high lignin degradation values 60%—70% with *S. pulverulentum* and up to 80% with *D. squalens*. About the same values were obtained by Streeter et al. (1982), 69% in eight weeks employing *P. ostreatus* with *E. carotovora*.

The response of various fungi in different lignocellulosic substrates is somewhat contradictory towards modifying susceptibility to enzymatic hydrolysis or in vitro dry matter enzymatic digestibility. Zadrazil (1977) was successful in increasing by 75% the IVDMED of wheat straw after 17 weeks either at 25 or 30° C with *S. rugosoannulata*, but only 50% at 22° C with *P. cornucopiae*. In only 8 weeks Streeter et al. (1982) did not improve the IVDMED of wheat straw employing *P. ostreatus*; however it was increased by 45.9% when a mixed culture of *P. ostreatus* and *E. carotovora* were employed.

Hattaka (1983) was very successful in increasing the amount of carbohydrates produced in 72 h with an enzyme from *Trichoderma reesei* from samples of wheat straw that had been incubated with *P. ostreatus* (an increase of 2.89 times the control), *Pleurotus* sp. (an increase of 2.67 times), *P. cinnabarinus* (an increase of 2.67 times) and *Ischnoderma benzoinum* (an increase of 2.94 times).

Zadrazil (1980) reported unsuccessful attempts of increasing the in vitro dry matter digestibility of rice husks; a 36%, 64% and 53% decrease of the control with *S. rugosoannulata, P. florida* and *A. aergerita*, respectively.

These brief comments on results obtained with other fungi and substrates show in essence what we said before, that the major parameters influencing the extent and rate of further enzymatic hydrolysis of solid residues obtained after the solid state substrate growth of basidiomycetes, are as yet not completely determined. The production of more water soluble compounds or the extent of lignin removal do have an influence but are not the only causes. In fact contradictory results have been published in the past and our data with cane bagasse prove this point.

Some fungi prefer certain substrates. For example, Sengupta et al. (1984) showed that bagasse enriched with minerals was digested with *Termitomyces clypeatus* for one week at 55° C and pH 5.0. This fungus saccharified 71% of the carbohydrate contents. However, only 10% was saccharified for wheat bran and 3.3% for green coconut coir. No clues were given by the authors to explain these differences.

In the last few years both basic and applied research on microbial conversion and modification of lignocellulosic materials have vastly expanded as reviewed by Eriksson (1984), but certainly some new experimental approaches need to be developed in order to better understand how white-rot enzymes interact with surface components and how this activity is regulated.

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