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SCREENING FOR AND ISOLATION OF LIGNOCELLULOLYTIC FUNGI FROM THAILAND FOR ANIMAL FEED PRODUCTION

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Abstract

With the ultimate objective of upgrading ruminant feed by solid fermentation, a collection of approximately 200 filamentous fungi were selected from various sources mostly in Thailand. These have been screened for cellulase activity using acid swollen (Walseth) cellulose and with cellulose azure. They have also been tested for phenol oxidase (PO) activity using guaiacol and for growth on rice straw. Of 200 isolates 160 were able to release dye from cellulose azure and 38 of these released more dye than the reference strain ***Trichoderma reesei*** *QM9414. Ten isolates decolorized the dye and this decolorization was not a pH effect. A total of 122 isolates showed PO activity and 18 of these were representatives of the genus* ***Trichoderma.*** *Approximately half of the isolates (99) gave some substrate weight loss when grown on rice straw screening cultures and 76% of these (75) were positive for PO activity. Ten isolates showing the greatest lignin degredation in these screening cultures were selected for toxicity tests and for retrials on 10g milled rice straw. The resulting fermentation product was analyzed for* ***in vitro*** *digestibility and for various components including lignin and protein. Two of the ten isolates were toxic and none of the isolates gave a product with digestibility higher than untreated rice straw, though protein content was slightly improved.*

Introduction

A recent publication by the National Academy of Sciences, Washington, D.C.[1] has stressed the potential returns developing countries may gain from the use of microbes to upgrade the animal feed value of plant wastes and byproducts or to convert them into fuel alcohol after saccharification. Supportive reviews on microbial conversion of plant biomass[2,3,4] attest to the great interest and extensive research being carried out in this field. One problem in these conversion processes is the lignin content of the substrates since it may inhibit the growth and/or hydrolytic activity of microorganisms and may complex with polysaccharides in such a way as to protect them from enzymatic attack[5]. Among the microorganisms capable of attacking lignin, the filamentous fungi, and

particularly "white rot" fungi, have been considered the most effective[6]. Some species have been employed in attempts to upgrade agricultural wastes as animal feeds[7,8]. The purpose of the work reported here was to establish in Thailand a collection of locally isolated lignolytic and cellulolytic fungi from which ten strains would be selected and further tested for efficacy in upgrading rice straw and other agricultural wastes as animal feeds. The ultimate objective for the work is a simple process requiring limited skills and low capital investment; one which could be utilized with benefit on small rural farms. At the same time, this collection would serve as a source of organisms for Thai scientists interested in other bioconversions.

Materials and Methods

General

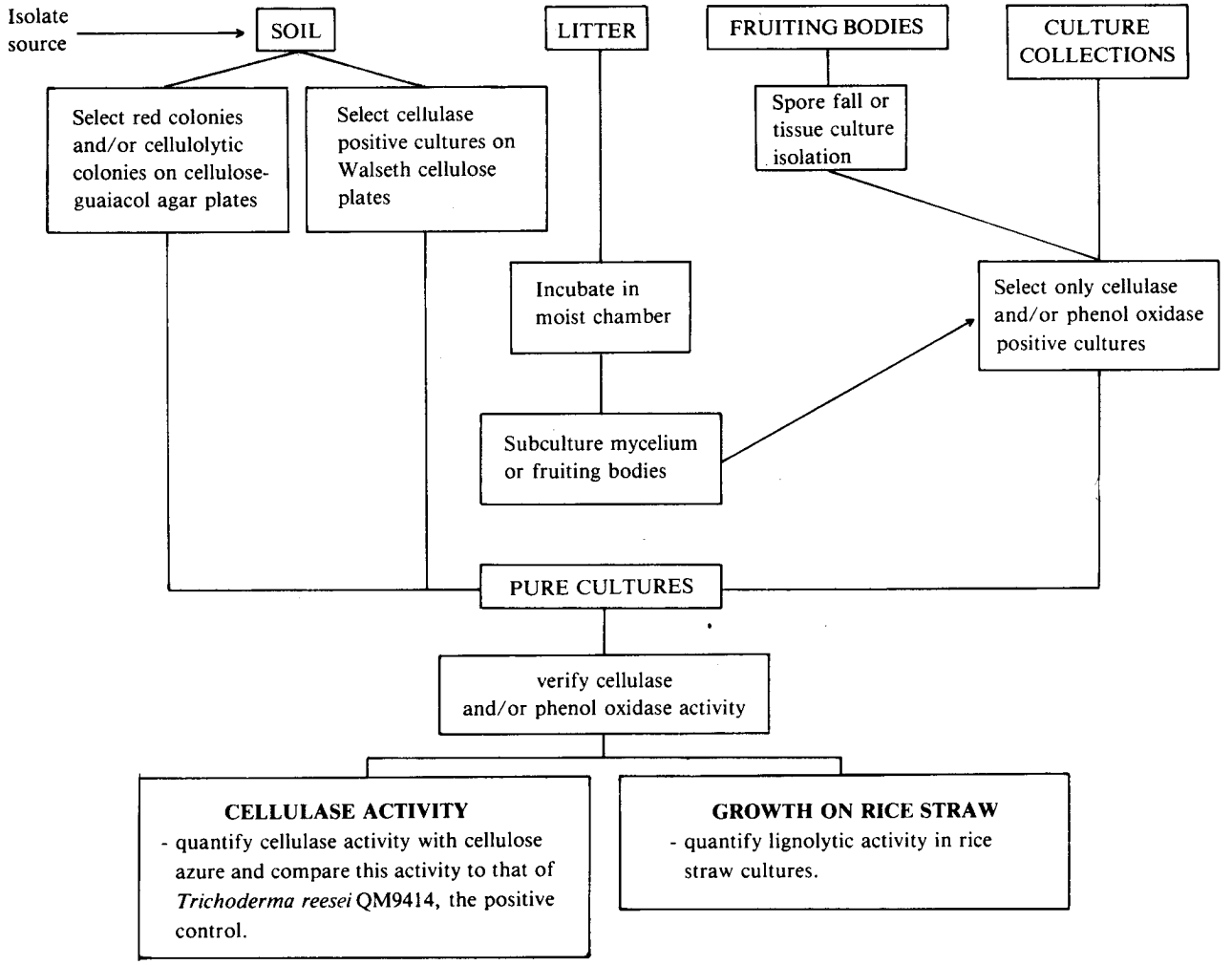
A summary of the screening and isolation procedures followed in this work are given in Fig. 1 and details of individual procedures follow.

Soil samples (approximately lg) were placed in 18 × 150 mm sterile testtubes and shaken vigorously with 10 ml of added sterile distilled water. After a few seconds delay to allow large particles to settle the samples were serially diluted ten times with distilled water for 3 to 6 transfers before 0.1 ml aliquots were removed for plating on screening media. Litter samples were incubated in moist chambers from which mycelial fragments, fruiting body fragments or spores were transferred to screening media. Fruiting bodies collected in the field were either suspended over malt extract agar to obtain pure cultures by the spore fall method, or they were subcultured by direct culture of tissue fragments aseptically removed from the centre of freshly broken sporocarps. Isolates yielding non-sectoring colonies with consistent and uniform morphology upon serial transfer were considered pure cultures. Once in pure culture, isolates were transferred to the screening media described below. Isolates obtained from culture collections were subcultured first on malt extract agar and then transferred to the screening media.

Organisms

Soil samples, fruiting bodies and forest litter were obtained from widely separated locations in Central, Northern, Eastern, Western and Southern Thailand and from one location in Burma. In addition, 81 isolates of fungal cultures with xylanase and cellulase activities were kindly provided by Dr. Napha Lothong (59 isolates) and Ms. Sawitri Limtong (22 isolates), respectively, from the Microbiology Department, Faculty of Science, Kasetsart University. Also 101 isolates of Actinomycetes were provided from an existing collection belonging to V. Meevootisom. These preceeding fungi and Actinomycetes were isolated from soils and decaying vegetation in Thailand. Finally, 5 isolates of mushrooms and one isolate of *Trichoderma reesei* were provided by Dr. Wanchern Dangsupa of Bangkok MIRCEN, and one strain of *Sporotrichum pulverulentum* was obtained from Dr. Paul Ander of the Swedish Forest Products Research Laboratory, Stockholm, for use as a reference strain.

Figure 1. Procedure for isolation and selection of lignocellulolytic fungi.



Isolation and maintenance media

The medium used for spore fall isolations, for tissue fragment isolations and for pure stock cultures was malt extract agar (MEA) containing Difco malt extract, 15g; glucose, 10g; Difco peptone, 1g; Difco yeast extract, 1g; Difco agar, 20g; and distilled water 1000 ml. In situations where bacterial contamination could not be eliminated by serial transfer on this medium, tetracycline to make 200mg/1 and chloramphenicol to make 200mg/1 were added after autoclaving dissolved in 70% ethanol.

Screening media

The screening medium used up to collection of isolate 147 was prepared as double layer agar plates using Walseth cellulose[9] (see below) alone as an indicator for cellulase activity. The bottom layer of the plates consisted of 20 ml of mineral salts agar prepared by adding 15g Difco agar Noble to one litre of mineral salts solution. The mineral salts solution, modified from Saunders Sui and Genest[10] by Dr. T.M. Wood of the Rowett Research Institute, Aberdeen, was prepared as described in Table 1. The top layer of the plates consisted of 8 ml of agar medium containing Difco agar Noble 15g; Walseth cellulose (see below), 10g; in one litre of the same mineral salts solution as used for the bottom layer. Selected cellulase positive colonies showed a clearing zone in the cellulose medium.

From the collection of isolate 148 onwards, guaiacol[11] (Nakarai Chemicals Ltd. Japan) at 0.5 ml to 1 ml per litre was added to the top layer medium. On the resulting screening plates, selected colonies produced clearing zones if cellulase active and/or reddish brown zones if phenol oxidase (PO) active. After the formulation of this medium, isolates 1 to 147 were rescreened on it to determine PO activity.

Walseth cellulose

This acid swollen cellulose was, with minor changes, prepared in a cold room following the procedure of Walseth[9]. Whatman CF11 chromatographic cellulose powder was added slowly with rapid stirring to 85% orthophosphoric acid (approximately 13g of cellulose powder per 100 ml of acid) held in a beaker on ice. After 2 hours, the swollen cellulose was washed with 5 volumes of distilled water on a Buchner funnel. Next, 1N Na_2CO_3 solution was added sufficient to suspend the cellulose mass and the suspension was homogenized at full speed in a Waring commercial grade blender for 10 minutes. The homogenate was then left for 24 hours in a cold room (4°C) before rehomogenizing and washing on a Buchner funnel with distilled water until the pH of the filtrate was 6.5. A 10 ml sample of the final homogenate was removed and dried to constant weight to determine its dry weight cellulose content. The homogenate was stored in the refrigerator and added to agar media in appropriate volumes to give 10g dry weight per litre.

TABLE 1. CONTENTS AND PREPARATION OF MODIFIED SAUNDERS, SUI AND GENEST[10] MEDIUM.

Solution A.

KH_2PO_4	0.20g
K_2HPO_4	0.15g
NaH_2PO_4 $2H_2O$	2.30g
Na_2HPO_4	1.50g
NH_4NO_3	0.60g
$MgSO_4$ $7H_2O$	0.30g
$NaNO_3$	3.80g
Distilled water	100 ml

Solution B.

$ZnSO_4$	0.050g
$Fe_2(SO_4)_3$	0.054g
$MnSO_4$ $4H_2O$	0.006g

Using distilled water, dissolve each salt separately in a little water, then mix in a vessel containing 0.8 ml of 12% HCl and make up to 100 ml.

Solution C.

$CuSO_4$ $5H_2O$	0.0025g
H_3BO_3	0.0570g

Using distilled water, dissolve each separately in a little water, then mix and make up to 100 ml.

Note:

To make the final stock solution, add 0.1 ml of each of solutions B and C to 100 ml of solution A. Add 0.02g of yeast extract and then make the mixture up to 1 litre.

Cellulose azure assays

These were carried out using a modification of a dye-release assay of Smith[12] employing cellulose azure (Sigma Chemicals) as the dyed substrate. To each 50 ml Erlenmeyer flask was added 25 mg cellulose azure and 3 ml of the mineral salts medium described in Table 1. The flasks were autoclaved at 121°C for 15 minutes and after cooling were inoculated with a loop of mycelium taken from a colony front on MEA. After two weeks stationary incubation at 28°C and 80% relative humidity in a temperature and humidity controlled incubator (Hotpack, Philadelphia, USA) the contents of each flask were made up to 10 ml with distilled water in a test tube and centrifuged at 3000 rpm for 5 minutes. Finally, the optical density of the supernatant was measured on a Pye Unicam SP 1800 spectrophotometer at 595 nm with an uninoculated culture supernatant as a blank. *Trichoderma reesei* QM9414, a high cellulase mutant originally from Natick Laboratories, was obtained from Bangkok MIRCEN and used as a comparative standard.

Lignin degradation assays

The initial screening procedure was carried out with all PO positive isolates using a liquid medium containing a commercial Kraft lignin with or without added Walseth cellulose[13]. The fungi were cultured for 12 to 15 days after which contents of the flasks were decanted and made up to 20 ml with 1N NaOH. The resulting mixture was filtered and a 10 ml sample of the filtrate was assayed for lignin content by the chlorine consumption method[14]. The replicates in this screening were consistent, but subsequent retrials did not give reproducible results. Consequently, the data was discarded as unreliable. In addition a publication by Setliff and Eudy[15] showed that absence of PO activity did not mean that a fungus was unable to degrade lignin. Consequently, all isolates in the collection were rescreened for growth on rice straw as described below and the lignin content of these cultures was measured after three weeks cultivation. Controls consisted of uninoculated sterilized cultures.

The Klason lignin content of the rice straw cultures was at first measured using the procedure described by Effland[16]. However, this gave values of 12 to 15% lignin for the uninoculated controls; a value which did not agree with the animal fodder literature. The reasons for this discrepancy will be the subject of a future publication. To overcome this problem, lignin was finally determined following Van Soest[17]. An advantage to the Van Soest method is the fact that the values obtained using it have been shown to correlate closely with the ruminant digestibility of analyzed fodders[17, 18].

In addition to this lignin measurement, a supplementary one was attempted by the acetyl bromide technique[19]. However, the values obtained with this method did not correlate with those from the Van Soest determination for 8 samples tested and the method was thus abandoned.

The acid detergent digestion liquids from the Van Soest analyses were further analyzed with crude tests for protein content, total sugar content and lignin content. The 72% sulfuric acid hydrolysis liquid was tested for glucose content. The detergent used in the acid detergent solution interfered with tests for total sugar and for lignin but this interference could be removed by prior treatment of the filtrate with Amberlite IR-120 sodium form (1 ml filtrate, 2 ml distilled water and 2g resin). Total sugar was determined by the phenol-sulfuric acid method[20] and lignin was determined using the tannin-lignin reagent[21]. Protein was determined using ninhydrin[22] and glucose was determined using glucostat reagent (Sigma Chemical Co.).

Rice straw screening cultures

Dried rice straw was collected immediately at harvest from a farm in Samut Prakan Province 30 km from Bangkok on the Bang Na - Trat highway. According to the farmer, no insecticides or fungicides had been used during the cultivation of the crop. The straw was stored in large sealed plastic bags in the laboratory. For growth trials the straw was cut into segments of 1 to 2 cm in length with a hand cutter in sufficient quantity to complete the whole test. The cut straw was mixed thoroughly before dispensing into culure flasks.

Cultures were carried out in duplicate 125 ml Erlenmeyer flasks each containing 2g rice straw. These were autoclaved at 121°C for 15 minutes and then the straw was moistened with 5 ml of mineral salts solution[23]. The contents of this solution are given in Table 2. A moisture content of 70% by weight was chosen on the basis of tests with 31 isolates which showed that growth from 70% to 80% moisture was not significantly different. The lower value was chosen to improve aeration and to gain any advantage fungal growth might have over that of possible bacterial contaminants.

A 10 mm plug of inoculum from the colony front of MEA agar cultures was used as inoculum and the flasks were incubated for 21 days at 30°C and 80% relative humidity in a humidity controlled incubator. At the end of incubation the cultures were dried and weighed to determine dry weight loss. Those without weight loss (no growth) were included with the uninoculated controls to determine lignin content by the Van Soest procedure (see above).

Selection procedure

The object of the selection procedure was to choose 10 fungi from the collection which gave maximum lignin degredation but at the same time minimum loss of the carbohydrate content of the substrate. In addition, the highest possible protein content of the substrate would be an advantage. The following formula was used to calculate an index of effectiveness based on lignin content of the fermentation substrate in comparison to that of the original substrate:

TABLE 2. MINERAL SALTS MEDIUM DESCRIBED BY ANDER AND ERIKSSON[23].

$NH_4H_2PO_4$	0.50g
KH_2PO_4	0.15g
K_2HPO_4	0.10g
$MgSO_4\ 7H_2O$	0.50g
$CaCl_2\ 2H_2O$	74.0 mg
ferric citrate	12.0 mg
$ZnSO_4\ 7H_2O$	6.6 mg
$MnSO_4\ 4H_2O$	5.0 mg
$CoCl_2\ 6H_2O$	1.0 mg
$CuSO_4\ 5H_2O$	1.0 mg
Thiamine	0.1 mg

Contents are listed as amounts per litre of distilled water in the final solution. Each salt should be dissolved separately in a small volume of water before mixing. A stock solution of the first four salts may be prepared at 100X concentration and should be kept separately from a stock of the latter seven constituents which may be prepared at 1000X concentration.

$$\frac{\text{\% lignin in original substrate} - \text{\% lignin in fermented substrate}}{\text{\% lignin in original substrate}} \times 100$$

in situations where carbohydrate was consumed in preference to lignin, the lignin content of the fermented substrate exceeded that of the original and the index gave a minus value. The fungi with the highest positive index were selected for further study.

Toxicity tests

The 10 organisms selected by the above criteria were grown at 30°C on a gyrotory shaker (Psychrotherm, New Brounswick Scientific Co.) in 1 litre of liquid malt extract medium containing 20g glucose, 20g malt extract (Difco), lg yeast extract (Difco) and lg peptone (Difco), for 5 days. The whole cultures were subsequently extracted with one litre of chloroform each for three times. The chloroform extracts for each organism were pooled and concentrated on a rotary evaporaor and toxicity of the concentrates was tested with weanling rats as previously described[24].

In vitro digestibility

The ten fungi selected above were each grown in triplicate for 21 days on 10 g of rice straw milled to pass 20 mesh, in 500 ml Erlenmeyer flasks with the same proportional additions and under the same conditions as described for the smaller rice straw cultures above. Inoculum consisted of 5 ten mm plugs. At the end of cultivation, the cultures were dried and dry weight loss was determined. Samples were removed for lignin determination and for *in vitro* digestibility determination[25]. Rumen fluid for these determinations was obtained from a local slaughterhouse and was inoculated into the digestion flasks within one hour of collection.

Results

Characteristics of fungal strains.

During more than two years of maintainance (subculture of refrigerated stocks on MEA every 6 months) for the isolates collected at the beginning of the project and during the various shorter maintenance times for the others, many isolates died. Those still viable at the time of publication are listed in Table 3 along with data on phenol oxidase (PO) activity and dye release activity with cellulose azure. Strains derived from previously existing collections are so indicated in the table; otherwise, they are original isolates. In Table 4, these same fungi are grouped into taxonomic categories and the collection numbers are included for cross-reference to Table 3. The missing numbers in the sequence

TABLE 3. SEQUENTIAL LIST OF VIABLE ISOLATES REMAINING IN THE COLLECTION AT THE TIME OF PUBLICATION.

Also given in the table are an indication of growth (+) or no growth (−) on rice straw, phenol oxidase (PO) activity (+ or −) and O.D. of the culture broth in the cellulose azure assay for cellulase activity. See the note in Table 4 concerning the names of the organisms. Where an isolate came from an existing collection, the source is given.

Isolate No.	Name	Rice Straw	Cell. Azure	PO
90	Sporotrichum pulverulentum	+	1.6	+
100	Trichoderma reesei QM9414	−	1.4	−
1	Cryptoporus	−	0	−
2	Agonomycete	−	*	−
4	Agonomycete	−	0	−
5	Agonomycete	−	0	−
6	Agonomycete	+	0	−
7	Agonomycete	−	0.2	+
9	Cladosporium	+	0.5	−
11	Agonomycete	−	0.6	−
13	Actinomycete	−	0	−
14	Actinomycete	−	0	−
15	Actinomycete	−	0	−
16	Gymnopilus	−	0.5	+
17	Crinipellis	+	*	+
18	Schizophyllum	−	0	−
20	Schizophyllum	−	0.8	−
22	Paecilomyces	−	0.3	−
23	Trichoderma	+	1.7	−
24	Trichoderma	−	1.8	−
25	Phialophora	−	1.7	−
26	Agonomycete	−	*	−
30	Agonomycete	−	0.5	−
34	Pestalotia	−	0.4	−
37	Agonomycete	−	0.6	−
39	Agonomycete	+	0.5	−
40	Pyrenochaeta	−	0.4	−
41	Aspergillus	−	0.2	−
42	Agonomycete	−	0.3	−

Isolate No.	Name	Rice Straw	Cell. Azure	PO
47	Aspergillus fumigatus	−	0	−
48	Aspergillus fumigatus	+	0	−
49	Aspergillus flavus	—	0.8	−
51	Aspergillus fumigatus	—	1.3	−
53	Aspergillus aculeatus	+	1.5	−
54	Agonomycete	−	0	−
55	Trichoderma	−	0	−
56	Cladosporium	+	0	−
57	Papulaspora	−	1.8	−
61	Penicillium oxalicum	−	0	−
64	Stachybotrys	−	1.4	−
68	Sclerotium	−	1.3	−
73	Cladosporium	−	0.5	−
74	Diheterospora	−	0.4	−
75	Diheterospora	−	0.6	−
80	Chaetomium	−	0.9	−
82	Diheterospora	−	0	−
83	Cladosporium	+	1.6	−
84	Cladosporium	−	0.6	−
85	Paecilomyces	−	0.4	−
87	Panus	−	0.9	+
91	Chaetomium	−	0.6	−
92	Trichoderma	−	1.2	−
93	Penicillium	−	1.2	−
95	Cephalosporium	−	1.0	+
96	Cephalosporium	−	0.6	+
97	Verticillium	−	0	+
99	Penicillium	−	0	−
105	Geotrichum	−	0	+
107	Agonomycete	−	0	+
109	Auricularia	−	0.5	+
112	Polyporus	−	0.7	+
113	Trichoderma	−	2.0	−
114	Trichoderma	−	1.8	−
115	Trichoderma	−	1.1	−
116	Trichoderma	−	1.2	−
117	Trichoderma	−	1.4	−

Isolate No.	Name	Rice Straw	Cell. Azure	PO
118	Trichoderma	–	1.2	–
119	Agonomycete	–	0	–
120	Agonomycete	+	0	–
122	Aspergillus	–	0.7	–
124	Agonomycete	+	0.5	–
126	Agonomycete	–	0.2	–
127	Agonomycete	–	0	–
128	Agonomycete	–	0.5	+
129	Agonomycete	–	0.1	+
131	Panaeolus	–	0.4	+
133	Pleurotus florida (Mircen)	+	0.2	+
134	Pleurotus sajor-caju (Mircen)	–	0.1	+
135	Pleurotus ostreatus (Mircen)	+	0.3	+
136	Auricularia polytricha (Mircen)	+	0.2	+
138	Aspergillus (Kasetsart)	+	0.8	–
140	Penicillium (Kasetsart)	+	0.7	–
141	Trichoderma (Kasetsart)	+	1.8	–
142	Trichoderma (Kasetsart)	+	1.2	–
143	Aspergillus (Kasetsart)	+	0.5	–
144	Aspergillus (Kasetsart)	+	0.9	–
146	Agonomycete (Kasetsart)	–	0.6	–
147	Fusarium (Kasetsart)	+	0.9	–
148	Auricularia	–	0.3	+
149	Geotrichum	+	1.1	+
150	Trichoderma	+	1.6	+
151	Aspergillus	+	1.1	+
153	Trichoderma	–	0	+
154	Trichoderma	–	1.9	+
155	Trichoderma	+	1.9	+
156	Trichoderma	+	1.9	+
160	Agonomycete	–	1.3	+
161	Trichoderma	+	> 2.0	+
162	Agonomycete	+	0.3	+
163	Trichoderma	+	1.5	+
164	Agonomycete	–	0.7	+
165	Agonomycete	+	*	+
166	Trichoderma	–	>2.0	+

Isolate No.	Name	Rice Straw	Cell. Azure	PO
167	Trichoderma	+	2.0	+
168	Trichoderma	+	2.0	+
169	Trichoderma	+	2.0	+
170	Trichoderma	+	1.8	+
171	Aspergillus	−	1.8	−
173	Agonomycete	+	0.8	+
174	Tubercularia	+	1.6	+
175	Agonomycete	−	0.9	+
177	Agonomycete	+	1.6	+
178	Agonomycete	+	1.8	+
179	Agonomycete	+	1.4	+
180	Marasmius	−	>2.0	+
182	Agonomycete	+	*	+
183	Polyporus	+	0	+
184	Agonomycete	−	0.6	+
185	Agonomycete	−	0	+
186	Agonomycete	+	*	+
187	Polyporus	−	1.0	+
189	Agonomycete	−	1.2	+
190	Agonomycete	+	0.7	+
191	Trichoderma	−	1.9	+
192	Agonomycete	+	0	+
194	Agonomycete	−	0	+
195	Agonomycete	+	1.6	+
197	Gasteromycete	−	0	+
198	Trichoderma	+	1.8	+
199	Agonomycete	−	0.7	+
201	Polyporus	+	0	+
202	Agonomycete	+	0.7	+
203	Trichoderma	+	1.8	+
204	Trichoderma	+	1.4	+
205	Polyporus	+	0.4	+
206	Trichoderma	+	1.7	+
207	Trichoderma	+	>2.0	+
208	Geotrichum	+	1.4	+
209	Cephalosporium	−	0	+
210	Xylaria	+	0.1	+

Isolate No.	Name	Rice Straw	Cell. Azure	PO
219	Trichoderma	−	1.9	−
220	Alternaria	+	1.2	+
222	Trichoderma	+	N.D.	+
223	Coprinus	+	0.6	+
225	Trichoderma	−	> 2.0	+
226	Agonomycete	+	0.1	+
227	Agonomycete	−	0.1	+
232	Cephalosporium	−	1.7	+
233	Geotrichum	+	1.5	+
234	Geotrichum	+	1.3	+
235	Myrothecium	−	0.5	+
251	Agaricales	+	0.8	+
254	Agaricales	+	0.6	+
257	Trichoderma	+	1.4	−
258	Polyporus	+	0.3	+
260	Agaricales	+	0.4	−
261	Polyporus	+	0.4	+
262	Polyporus	+	*	+
263	Polyporus	+	0.3	+
264	Agaricales	+	0.9	+
265	Agaricales	+	0.5	+
269	Polyporales	+	0.3	+
273	Polyporus	−	0.2	−
275	Clitocybe	+	0.2	+
276	Polyporus	+	0.9	+
277	Clitocybe	+	0.1	+
278	Polyporus	+	0.3	+
280	Polyporus	+	0.2	+
283	Polyporus	+	1.5	−
284	Polyporus	+	0.3	+
285	Agonomycete	+	1.4	−
288	Polyporus	+	0.1	+
290	Cyathus	+	0.7	+
293	Polyporus	−	1.2	+
296	Agaricales	+	1.0	+
301	Lenzites	+	*	+
305	Polyporus	−	0.7	+

Isolate No.	Name	Rice Straw	Cell. Azure	PO
306	Polyporus	+	0.8	+
307	Polyporus	+	0.9	+
312	Agaricales	+	0.3	+
314	Agaricales	–	0.8	+
316	Agaricales	+	0.2	+
318	Polyporus	+	0.2	+
319	Marasmius	+	0.1	+
320	Schizophyllum	+	1.0	+
321	Thelephora	–	1.4	–
323	Agaricales	+	0.3	+
325	Agaricales	+	0.3	+
327	Agaricales	–	0.1	+
328	Polyporus	+	0.1	+
329	Agaricales	+	0.2	+
331	Agaricales	+	0.7	+
332	Agaricales	–	*	–
333	Polyporales	+	*	+
334	Agaricales	+	0.9	+
335	Agaricales	+	0.8	+
336	Agaricales	+	0.3	+
341	Crepidotus	+	0.1	+
342	Polyporales	–	0.3	+
343	Agaricales	+	0.2	–
347	Myrothecium	+	N.D.	+

* = decolorized the dye

N.D. = not done

TABLE 4. LIST OF ISOLATES ARRANGED ACCORDING TO TAXONOMIC CATEGORY.

Within each caregory the isolates are listed in alphabetical order. Identification was made as nearly as possible using references 26 and 27 for mushrooms, references 28 and 29 for the Polyporales, references 30 and 31 for Deuteromycetes and reference 33 for species of *Aspergillus*. These identifications should not be considered absolute, especially for the Basidiomycetes since many of the specimens (e.g., those listed as Agaricales or Polyporales) were very small single collections where the fruiting body was destroyed by the isolation procedure.

ASCOMYCOTINA

Name	Isolate Numbers
Chaetomium spp.	80, 91.
Xylaria sp.	210.

BASIDIOMYCOTINA

Name	Isolate Numbers
Agaricales	251, 254, 260, 264, 265, 296, 312, 316, 323, 325, 327, 329, 331, 332, 334, 335, 336, 343.
Auricularia polytricha	136.
Auricularia sp.	148.
Clitocybe spp.	275, 277.
Coprinus sp.	223.
Crinipellis sp.	17.
Cyathus sp.	290.
Gasteromycete	197.
Gymnopilus sp.	16
Lenzites sp.	301.
Marasmius spp.	180, 319.
Panaeolus sp.	305.
Panus sp.	87.
Pleurotus florida	133.
Pleurotus ostreatus	318.
Pleurotus sajor-caju	134.
Polyporales	269, 333, 342.
Polyporus spp.	112, 183, 187, 201, 205, 258, 261, 262, 263, 273, 276, 278, 280, 283, 284, 288, 293, 305, 306, 307, 318, 328.

Schizophyllum spp.	18, 20, 320.
Thelephora sp.	321.

DEUTEROMYCOTINA

Name	Isolate Numbers
Alternaria sp.	220.
Aspergillus aculeatus	53.
Aspergillus flavus	49.
Aspergillus fumigatus	47, 48, 51.
Aspergillus spp.	41, 122, 138, 143, 144, 151, 171.
Cephalosporium spp.	95, 96, 209, 232.
Cladosporium spp.	9, 56, 73, 83, 84.
Diheterospora spp.	74, 75, 82.
Fusarium sp.	147.
Geotrichum spp.	105, 149, 208, 233, 234.
Paecilomyces spp.	22, 85.
Papulaspora sp.	57.
Pestalotia sp.	34.
Penicillium oxalicum	61.
Penicillium spp.	93, 99, 140.
Phialophora sp.	25.
Pyrenocheta sp.	40.
Sclerotium sp.	68.
Stachybotrys sp.	64.
Sporotrichum pulverulentum	90.
Trichoderma reesei	100.
Trichoderma spp.	23, 24, 55, 92, 113, 114, 115, 116, 117, 118, 141, 142, 150, 153, 154, 155, 156, 161, 163, 166, 167, 168, 169, 170, 191, 198, 203, 204, 206, 207, 219, 222, 225, 257.
Tubercularia sp.	174.
Verticillium sp.	97.

in Table 3 are for isolates that died during maintenance. These isolates and what data was obtained for them up to the time when they lost viability are not listed since cultures difficult to maintain would not be useful for the ultimate application proposed. Also omitted from this list are the majority of the 101 Actinomycete isolates and the 81 fungal isolates obtained from Kasetsart University. Exceptions were those which showed laccase activity and/or large clearing zones on the Walseth cellulose screening plates. These cultures were absorbed into the collection and assigned numbers in the collection sequence. *Trichoderma reesei* QM9414 and *Sporotrichum pulverulentum* are given as reference strains at the head of Table 3.

With reference to cellulase activity, all of the isolates in Table 3 were able to produce clearing in Walseth cellulose agar plates. However, only 160 of the isolates were able to release dye from cellulose azure. Of those isolates which did release dye from cellulose azure, 38 released more dye than QM9414. One distinctive subgroup of 10 isolates had the capacity to decolorize the cellulose azure substrate almost completely during the incubation period. In this situation cellulase activity could not be evaluated using cellulose azure. In Table 3, these strains are marked with an asterisk in the column showing cellulose azure activity. This effect was not due to a change in pH since additions of acid or base did not restore the colour in the cultures; nor did they decolorize fresh cellulose azure or Coomassie Brilliant Blue R, the dye used in the preparation of cellulose azure. Five of these isolates were derived from spores or tissue cultures of fungi in the Agaricales or in the Aphyllophorales. The remaining five were nonsporulating filamentous fungi isolated from soil and listed as Agonomycetes in Table 3. Two of the latter produced mycelium bearing clamp connections and would thus be considered basidiomycetous. Consequently, 7 out of these 10 decolorizing strains could be confirmed as basidiomycetous fungi.

Of the 200 isolates remaining in the collection, 122 showed PO activity. Originally, we intended to use this feature as our first elimination criterion based upon the assumption (now known to be erroneous[15]) that all lignin degrading fungi would exhibit PO activity. Since the converse of this assumption was not the case, a specific test for ligninolysis was to have served as our second elimination test. Subsequently, upon receiving copies of a recently published Proceedings to a seminar on lignin biodegredation[32], we reevaluated our selection criteria, abandoned our original scheme and tested all the isolates remaining in the collection for ability to grow on rice straw.

Before going on to discuss the rice straw cultures, it is worthy of note that 18 strains of *Trichoderma* in the collection were positive for PO activity, and that this previously unreported phenomenon has been the subject of a separate publication[13]. Further, these PO positive strains included all the *Trichoderma* strains with exceptionally high cellulose azure activity (i.e., greater than 2.0).

Rice straw screening cultures.

The strains of fungi which grew on the 21-day rice straw cultures are shown in Table 5. This table also shows the % loss in substrate dry weight, the final lignin content of the substrate, the effectiveness index (derivation described in the Materials and Methods) and the PO and cellulose azure activities for each of the strains. The isolates are listed in order of effectiveness index from the highest to the lowest.

Of the 200 isolates currently in the collection, 99 yielded some weight loss when grown on rice straw. It is interesting that 75 of these isolates (76%) and nine (90%) of the isolates from the top ten in the Table are positive for PO activity. Also, 75/122 or 61% of the PO positive isolates in the collection grew on rice straw, but only 24/78 or 31% of the PO negative isolates in the collection did so. In addition, out of 34 isolates of *Trichoderma,* 15/20 (75%) of the PO positive ones grew on rice straw but only 4/14 (29%) of the negative ones did.

In Table 6 the 14 fungal isolates giving 10 to 15% substrate weight loss (the highest) are listed in descending order of weight loss. They display cellulose azure activities from zero to 1.7. One isolate, 262, decolorized the dye. The only striking thing in the Table is that all isolates except 138 are positive for PO activity. The exceptional strain was obtained from Kasetsart University as a xylanase positive isolate, but the other xylanase positive isolates from this source (140-147, laccase negative) did not give comparably large losses in dry weight and one (146) did not grow on the substrate.

The lignin determinations by the Van Soest method[16] were quite variable. The mean for 25 samples of nonfermented rice straw was 4.74% with a standard deviation of 0.48% and a range of 4.1% to 5.6%. Consequently, since there were only duplicate tests for isolates 1-235 and only single tests for iolates 251-347, the lignin values were considered useful only as a preliminary screening tool. Confirmatory tests were performed with isolates selected for further tests; the results of which follow below. But first, if the strains in Table 5 are ranked from highest to lowest, not according to effectiveness index but according to the amount of lignin lost (i.e., total weight of lignin in the control substrate minus the total weight remaining in the fermented substrate) a ranking is obtained which gives a measure of lignin degredation not correlated to the loss in dry weight of the substrate. When this was done (list not shown), the top 20 isolates in the list included the same top 20 isolates as in Table 5. However, the order within the 20 isolates in the list was somewhat (not greatly) altered from that in Table 5. This comparison was made to determine whether there might be an exceptional lignin degrader in the collection whose capabilities were missed because of a concomitantly excessive carbohydrate weight loss.

Analysis of the acid detergent hydrolysis liquid and the 72% sulfuric acid digestion liquid for five control samples from the Van Soest lignin analysis procedure gave values for lignin, protein and sugars with different degrees of variation. This affected the

TABLE 5. LIST OF ISOLATES WHICH GREW ON RICE STRAW RANKED ACCORDING TO THE INDEX OF EFFECTIVENESS IN REMOVING LIGNIN FROM THE SUBSTRATE.

In addition, the table gives the % loss in dry weight of the substrate and the % lignin in the substrate at the end of the fermentation. Also given are the PO activity (+ or −) and the cellulose azure activity. For details, see Materials and Methods.†

Isolate No.	Name	%D.W. Loss	% Lignin	Cell. Azure	PO	Index
233	Geotrichum	14	2.47	1.5	+	48
136	Auricularia polytricha	6	2.87	0.2	+	39
226	Agonomycete	6	3.00	0.1	+	37
133	Pleurotus florida	4	3.06	0.2	+	35
190	Agonomycete	8	3.16	0.7	+	33
208	Geotrichum	12	3.45	1.4	+	27
56	Cladosporium	6	3.47	0	−	27
135	Pleurotus ostreatus	7	3.76	0.3	+	21
202	Agonomycete	13	3.81	0.7	+	20
192	Agonomycete	12	3.88	0	+	18
347	Myrothecium	5	3.89	N.D.	+	18
178	Agonomycete	8	3.91	1.8	+	18
6	Agonomycete	7	3.93	0	−	17
163	Trichoderma	10	3.94	1.5	+	17
198	Trichoderma	6	3.97	1.8	+	16
206	Trichoderma	10	4.06	1.7	+	14
186	Agonomycete	6	4.09	*	+	14
83	Cladosporium	5	4.12	1.6	−	13
147	Fusarium	9	4.12	0.9	−	13
142	Trichoderma	7	4.21	1.2	−	11
167	Trichoderma	4	4.25	2.0	+	10
17	Crinipellis	7	4.26	*	+	10
144	Aspergillus	7	4.26	0.9	−	10
201	Polyporus	2	4.27	0	+	10
203	Trichoderma	7	4.27	1.8	+	10
155	Trichoderma	4	4.29	1.9	+	9
207	Trichoderma	8	4.31	2.0	+	9

Isolate No.	Name	%D.W. Loss	% Lignin	Cell. Azure	PO	Index
210	Xylaria	4	4.32	0.1	+	9
280	Polyporales	5	4.34	0.2	+	8
168	Trichoderma	3	4.38	2.0	+	8
9	Cladosporium	3	4.39	0.5	–	7
140	Penicillium	5	4.41	0.7	–	7
312	Agaricales	3	4.41	0.3	+	7
120	Agonomycete	3	4.42	0	–	7
182	Agonomycete	3	4.42	*	+	7
124	Agonomycete	5	4.43	0.5	–	6
177	Agonomycete	7	4.43	1.6	+	6
143	Aspergillus	4	4.44	0.5	–	6
90	Sporotrichum pulverulentum	7	4.46	1.6	+	6
205	Polyporus	3	4.46	0.4	+	6
23	Trichoderma	3	4.48	1.7	–	5
48	Aspergillus fumigatus	3	4.48	0	–	5
169	Trichoderma	4	4.48	>2.0	+	5
275	Agaricales	4	4.48	0.2	+	5
331	Agaricales	12	4.49	0.7	+	5
156	Trichoderma	4	4.53	1.9	+	4
284	Polyporales	3	4.55	0.3	+	4
334	Agaricales	11	4.55	0.9	+	4
141	Trichoderma	3	4.57	1.8	–	4
318	Polyporales	5	4.60	0.2	+	3
335	Agaricales	8	4.60	0.8	+	3
257	Trichoderma	4	4.62	1.4	–	3
333	Polyporales	5	4.63	*	+	2
165	Agonomycete	8	4.64	*	+	2
174	Tubercularia	6	4.65	1.6	+	2
161	Trichoderma	3	4.66	>2.0	+	2
39	Agonomycete	4	4.67	0.5	–	1
285	Agonomycete	4	4.69	1.4	–	1
234	Geotrichum	13	4.72	1.3	+	0
262	Polyporales	15	4.73	*	+	0
343	Agaricales	4	4.74	0.2	–	0
138	Aspergillus fumigatus	11	4.76	0.8	–	0

Isolate No.	Name	%D.W. Loss	% Lignin	Cell. Azure	PO	Index
251	Agaricales	7	4.77	0.8	+	−1
261	Polyporales	3	4.79	0.4	+	−1
162	Agonomycete	4	4.91	0.3	+	−4
288	Polyporales	4	4.91	0.1	+	−4
290	Cyathus	3	4.91	0.7	+	−4
323	Agaricales	1	4.91	0.3	+	−4
149	Geotrichum	13	4.95	1.1	+	−4
170	Trichoderma	9	4.96	1.8	+	−5
265	Agaricales	3	5.00	0.5	+	−5
296	Agaricales	7	5.00	1.0	+	−5
301	Polyporales	3	5.00	*	+	−5
320	Schizophyllum	6	5.00	1.4	−	−5
220	Alternaria	9	5.01	1.2	+	−6
150	Trichoderma	5	5.02	1.6	+	−6
53	Aspergillus aculeatus	2	5.03	1.5	−	−6
329	Agaricales	4	5.04	0.2	+	−6
151	Aspergillus	5	5.07	1.1	+	−7
341	Agaricales	10	5.09	0.3	+	−7
283	Polyporales	4	5.12	1.5	−	−8
319	Agaricales	5	5.16	0.1	+	−9
195	Agonomycete	5	5.18	1.6	+	−9
328	Polyporales	4	5.19	0.1	+	−9
316	Agaricales	3	5.30	0.2	+	−12
336	Agaricales	4	5.30	0.3	+	−12
276	Polyporales	5	5.32	0.9	+	−12
260	Polyporales	4	5.35	0.4	−	−13
269	Polyporales	5	5.37	0.3	−	−13
307	Polyporales	4	5.51	0.9	+	−16
254	Agaricales	5	5.57	0.6	+	−18
223	Coprinus	7	5.67	0.6	+	−20
173	Agonomycete	7	5.88	0.8	+	−24
204	Trichoderma	3	6.20	1.4	+	−31
179	Agonomycete	5	6.41	1.4	+	−35
264	Agaricales	4	6.48	0.9	+	−37
277	Agaricales	13	6.48	0.1	+	−37

Isolate No.	Name	%D.W. Loss	% Lignin	Cell. Azure	PO	Index
263	Polyporales	4	7.84	0.3	+	−65
278	Polyporales	3	8.78	0.3	+	−85

† For 1-235 the values given represent the average of two tests but for 236-347 there was no duplicate.

* = decolorized the dye

N.D. = not done

TABLE 6. ISOLATES GIVING THE HIGHEST DRY WEIGHT LOSS OF SUBSTRATE ARRANGED IN DESCENDING ORDER OF WEIGHT LOSS.
Also given for each isolate are PO activity (+or -) and cellulose azure activity.

Isolate No.	% D.W. Loss	PO	Cellulose Azure
262	15	+	*
233	14	+	1.5
149	13	+	1.1
202	13	+	0.7
234	13	+	1.3
277	13	+	0.1
192	12	+	0
208	12	+	1.4
331	12	+	0.7
138	11	-	0.8
334	11	+	0.9
163	10	+	1.5
206	10	+	1.7
341	10	+	0.1

* = decolorized the dye

confidence that could be placed in the differences measured. For the tannin-lignin reagent the variation for the control tests was small (± 1% for 1 S.D. and a range of 11 to 14%). Great variation between tests would not be surprising since the reagent will react with reducing substances in general, and the various fungi used in the tests could have widely different capabilities to produce these. Originally, it was hoped that these values would give some indication of fungi which solubilized lignin rather than degraded it to carbon dioxide. However, none of the test solutions gave values different from the control (appoximately 12% lignin equivalent).

The variation with the sugar analysis was also small (for the controls, acid-detergent soluble sugar was 22 ± 2% and cellulose was 38 ± 3%) and allowed a tentative measure of relative preference for acid-detergent soluble sugar (ADS sugar) or cellulose by the fermenting fungi. This was done by comparison of the cellulose to ADS sugar ratio in the control and test substrates. The rationale behind this comparison was based on the fact that the acid detergent solutions contained the hydrolyzed hemicellulose and other soluble sugars from the test samples and that the sulfuric acid hydrolysate contained the cellulose in the form of glucose[18]. The ratio of cellulose to ADS sugar for the control was 1.75 ± 0.2. Approximately half of the test fungi gave ratios of less than this (ranging from 1.0 to 1.7) indicating that degradation of hemicellulose was slower than degradation of cellulose. The other half gave ratios greater than 1.75 indicating a preference for ADS sugar. There was a tendency (not absolute) for isolates with a high effectiveness index to have lower ratios than the control, thus showing a relative preference for cellulose over ADS sugars.

The ninhydrin analysis was intended to give some indication of the protein content of the final fermentation substrate, and it was hoped that this measure would give some assistance in the selection of candidate fungi for further tests in the upgrading of rice straw as a ruminant feed. The variation in the control samples (1.25 ± 0.28%, 1 S.D.) was quite high, reducing the confidence that could be placed in any measured differences. Consequently, protein analysis from these hydrolysates was not considered useful. More controlled tests were performed with the top ten lignin degraders and these are described below.

Rice straw retrial cultures.

The top ten fungi in Table 5 were regrown in triplicate on 10g of milled rice straw as described in the Materials and Methods, in order to reproduce the results obtained in Table 5, to carry out more controlled tests for protein content of the final substrate and to carry out *in vitro* digestibility tests. The results are shown in Table 7. With the exception of isolate 136, all the fungi gave considerably higher weight loss (i.e., better growth) in the enlarged trials.

TABLE 7. Results from 10g fermentations of the ten fungi with the highest effectiveness indices.

Isolate No.	% D.W. Loss	% Digest.	% Lignin	% Protein	Cell. to ADS Sugar
56	12	10	5.2	2.1	1.38
133	14	25	7.2	2.2	1.38
135	15	34	6.3	1.6	0.88
136	3	24	7.0	1.2	1.13
190	22	5	5.6	2.1	1.13
192	23	17	6.7	2.9	1.13
202	23	30	4.8	2.4	1.00
208	15	13	6.4	1.1	1.25
226	21	20	5.8	2.2	1.38
233	25	26	5.5	2.1	1.13
CONTROL	0	40	4.7	1.9	1.75

TABLE 8. Results of acute toxicity tests for extracts[a] of the top ten isolates..

Isolate No.	PEI Weight (mg)	Mortality (Dead/Tested)	
		PEI	PES
56	62	0/5	0/5
133	13	0/5	0/5
135	22	0/5	0/5
136	17	0/5	0/5
190	44	0/5	0/5
192	41	5/5[b]	0/5
202	42	3/5[b]	0/5
208	27	0/5	0/5
226	18	0/5	0/5
233	34	0/5	0/5
CONTROL	15	0/5	0/5

[a]**PEI** = petroleum ether insoluble; PES = petroleum ether soluble.
[b]PEI contained the toxic substance (s).

The lignin content of the final substrate for each of these isolates is also given in Table 7. The comparative results show that the lignin contents of all of the final fermentation substrates were greater than the control value and this is in sharp contrast to the results given in Table 5.

Lignin assay of the acid detergent solution for the top ten isolates gave values which were the same for all tests and for the control samples (approximately 13%). This indicates that reducing substances which react with the lignin-tannin reagent are decreased in proportion to dry weight loss, leaving the final amount relative to other substrate components constant. This was in agreement with results from the earlier screening trials.

The ADS sugar assay of the 10g trials gave results higher than the control for most isolates. When compared to the results for cellulose content, the trend was the same as that noted in the preliminary screening trials. The ratio of cellulose to ADS sugars was higher than in the control, indicating a preference for cellulose over other carbohydrates by the test fungi.

Ninhydrin assays for total protein content of the whole fermentation substrates for the 10g trials of the top ten isolates showed that protein contents of the trials were higher than the controls for 7 of the ten isolates.

The *in vitro* digestibility assays for the final fermentation product from the 10g rice straw trials are also shown in Table 7. These results are not what would be expected by examination of the lignin content data. Those isolates giving the highest lignin content for the substrate did not give the lowest digestibility. However, all of the trial fermentations gave much lower digestibility than the control straw and considering the overall objective of the work, these results were discouraging.

The toxicity tests showed that two isolates, 192 and 202, were toxic to weanling rats. The toxic extracts were fatal only for the petroleum ether insoluble fraction from the original chloroform extract. All isolates, including the toxic ones gave no detectable mycotoxins for those tested by thin layer chromatography.

Discussion

Characteristics of fungal strains.

With respect to cellulase activity, the 38 strains with dye releasing activity higher than *T. reesei* QM9414 would be good starting material for Thai investigators interested in examining cellulase production or bioconversion of cellulosic substrates, and these strains are available to them upon request. It is curious that all the *Trichoderma* strains with exceptional cellulose azure activity (i.e., greater than 2.0) were also phenol oxidase positive strains. Tests are currently underway to determine whether this activity correlates with better degradation of the native cellulose in rice straw and sugar cane bagasse. For animal feed production, strains with low cellulose azure activity would be advantageous if they still retained good lignolytic activity. A limited ability to attack crystalline cellulose

would leave more nutrient in the fermentation product. The ten strains which decolorized the dye (marked with an asterisk for cellulose azure activity in Table 3) may be worth testing by those interested in microbial decolorization of industrial wastewater, to see whether decolorization of cellulose azure is an indication of a general ability to decolorize substrates. Since most of these fungi (7) also showed positive PO activity, they might be of particular interest in tests to decolorize paper mill wastewater.

Rice straw screening cultures.

The most striking feature of the data from tests on these cultures was the correlation between PO activity and ability to grow on rice straw, i.e., 76% of the strains that grew on rice straw were PO positive. This was not a result of bias in the screening process, since the correlation held even for the PO positive strains alone (61% grew on the substrate) and the PO negative strains alone (only 31% grew on the substrate). In the genus *Trichoderma,* 75% (15/20) of the PO positive ones grew on rice straw while only 29% (4/14) of the negative ones did. In addition, 13 of the 14 fungi giving the highest rice straw weight loss were PO positive. Cellulose azure dye release ability, on the other hand, did not correlate with weight loss. These facts argue that PO activity provides some advantage for growth on rice straw and it might be suggested that such activity indicates an ability to degrade lignin in the substrate[34] This may be true for some of the isolates. However, several of the *Trichoderma* strains have been tested with ^{14}C labeled artificial lignin (DHP) and they do not release $^{14}CO_2$ (Ian D. Reid, personal communication), indicating that they are not capable of catabolizing it. This does not, of course, mean that they cannot *modify* lignin in some manner similar to brown rot fungi[35] or produce soluble materials[36] which are not further metabolized to CO_2

Lignin analysis in these rice straw screening cultures indicated that some of the strains were capable of good preferential degradation. However, from isolate 307 onwards in Table 5, the lignin content greatly exceeded the control value even if weight loss was accounted for. There are several possible explanations for this phenomenon, but no further tests have been done to examine it. Tannin-lignin reagent analysis of the acid detergent hydrolysates from these screening cultures gave values of approximately 12% lignin equivalent in spite of substrate weight losses of up to 15%. This indicated that the reagent reactive material was used up in direct proportion to general weight loss. We are currently doing tests to determine whether this material could be, in good part, acid-detergent soluble lignin.

Rice straw retrial cultures.

In the 10g rice straw cultures with the top ten lignin degraders from Table 5, nine isolates grew much better than in the initial screening trials using 2g substrate. This was perhaps because the straw in the 10g trials had been milled to 20 mesh in order to reduce the vessel volume required to contain the substrate. The improved growth over the 2g

trials must have resulted from the smaller substrate particle size, the greater vessel volume or the greater substrate volume used in the 10g trials since all other conditions were unchanged. These physical parameters can effect growth singly or in combination by altering aeration and temperature and these two factors can in turn effect the substrate moisture content during the course of growth. It was not immediately clear which of these factors or combination of these factors was responsible for the improved growth of most of the isolates and for the reduced growth of isolate 136. The matter is currently under examination as it will be critical to proposed scaled-up trials.

The final lignin contents of the substrates in the retrials (Table 7) were much higher than they were for the same fungi in the screening trials. This again may relate to the milling of the straw. It is possible that the destruction of the integrated straw structure made the polysaccharide components more accessible to degradation without prior removal of protecting lignin. There is some support for this proposal from the analytical data both in the screening tests and in the 10g trials. In both cases, the selected fungi showed a preference for cellulose over ADS sugars (represented in good portion by hemicellulose). In unmilled straw, the intact cellular structure would probably necessitate prior lignin attack before the cellulose were available for use, whereas in milled straw the cellulose would be physically exposed and enzymatic attack of the lignin would not be required so early in the course of fermentation. The situation may have been recouped by longer incubation beyond three weeks, but the resulting increase in weight loss would have been detrimental. This result was unexpected and it demonstrates the importance of the fermentation conditions in determining the content of the final fermentation product. Further trials are underway using 10g of fermentation substrate but with a larger particle size (around 2 cm lengths of straw as originally used).

Analysis of the acid detergent hydrolysis solutions for these retrials gave results similar to those for the screening trials, indicating again that tannin-lignin reagent reactive material was reduced in proportion to dry weight loss in the substrate. Also in agreement with the screening trials, these 10 fungi showed a preference for cellulose over hemicellulose.

Total protein in the final fermentation product for these tests was measured with ninhydrin, using glycine as the standard. Consequently, they are approximately two times lower for the control than published figures obtained by total Kjeldahl nitrogen multiplied by 6.25. The contents obtained were considerably less than those required in a good quality ruminant feed, but they were obtained without substantial nitrogen addition to the substrate. Further tests with nitrogen supplements are underway.

By considering all preceding analytical results, both for the initial screening tests and the later scaled-up trials, several significant trends were apparent which may be worked into a general hypothesis that will serve as a testable guide for our further work. Briefly, this hypothesis proposes that the fungi which grew on rice straw to degrade

significant Klason lignin did so because their preferred carbohydrate substrate was cellulose and they had to remove closely bound (covalently bound?) lignin in order to reach this cellulose. By finely milling the straw, its cellular structure was destroyed sufficiently to leave its cellulose components open to more ready attack without prior lignin removal. In this way, the lignin component was either not degraded or was degraded together with the cellulose component in proportion to growth with no net loss in lignin resulting from fermentation. This means that fermentation conditions and especially substrate conformation are critical to the final composition of the fermentaion product.

The poor digestibility of the fermentation products was a negative outcome of this work. However, further tests with altered fermentation conditions are necessary before a final judgement on their usefulness to improve digestibility can be made. The extremely low digestibility of isolate 190 is surprising and it may be that this isolate produces an antimicrobial agent which inhibits rumen bacterial metabolism or growth.

The toxicity of isolates 192 and 202 means that they cannot be studied further for animal feed production. Since these two isolates were nonsporulating fungi, it is difficult to select a limited test group of reference fungal toxins from all of those currently known to be produced by fungi[37]. In addition, the toxins produced could be as yet undescribed. Consequently, further work with these toxic fungi would require cultivation of large batches for extraction and concentration of material for chemical analysis. Since this is beyond the scope of the current project, it will not be persued.

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บทคัดย่อ

คณะผู้วิจัยได้แยกราชนิดเส้นใยจากจังหวัดต่าง ๆ ของประเทศไทยได้จำนวน 200 สายพันธุ์ โดยมีจุดประสงค์ที่จะนำมาใช้เจริญบนฟางข้าว เพิ่มคุณค่าอาหารของฟางเพื่อใช้เป็นอาหารสัตว์ต่อไป ราที่แยกได้ได้ถูกนำมาทดสอบหาความสามารถในการย่อยสลายเซลลูโลส แอสเซอร์ (Cellulose azure) นอกจากนี้ยังทดสอบความสามารถในการสร้างฟีนอลอ๊อกซิเดส (phenol oxidase) และความสามารถที่จะเจริญบนฟางข้าวได้จากราจำนวน 200 สายพันธุ์ พบว่า 160 สายพันธุ์ สามารถย่อยสีออกมาจากเซลลูโลส แอสเซอร์ได้ และในจำนวน 160 สายพันธุ์นี้มีถึง 38 สายพันธุ์ ที่ย่อยสีจากเซลลูโลส แอสเซอร์ ได้ดีกว่า *Trichoderma reesei* QM 9414 มี 10 สายพันธุ์ที่สามารถ decolorize สีน้ำเงินของเซลลูโลส แอสเซอร์ โดยความสามารถอันนี้ไม่ขึ้นกับ พี เอช (pH) มี 122 สายพันธุ์สร้างฟีนอลอ๊อกซิเดส และในจำนวนนี้ 18 สายพันธุ์เป็น *Trichoderma spp.* จาก 200 สายพันธุ์ที่แยกได้ 99 สายพันธุ์ สามารถเจริญบนฟางข้าวได้ และส่วนใหญ่ (76%) ของสายพันธุ์ที่เจริญบนฟางข้าวได้สามารถสร้างฟีน่อลอ๊อกซิเดส คณะผู้วิจัยได้เลือกราที่สามารถย่อยสลายลิกนินได้ดีที่สุดมา 10 สายพันธุ์ เพื่อนำมาเลี้ยงบนฟางข้าว และได้ทดสอบว่า ฟางข้าวหมักนี้เป็นอันตรายต่อสัตว์ทดลองหรือไม่ พบว่ามี 2 สายพันธุ์ที่สร้างสารพิษทำให้หนู (weanling rat) ตาย นอกจากนี้ฟางข้าวหมักที่เลี้ยงด้วย 10 สายพันธุ์นี้ ย่อยยากกว่าฟางข้าวก่อนหมัก และปริมาณโปรตีนในฟางข้าวหมักไม่แตกต่างจากฟางก่อนหมักด้วย