APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1997, p. 3950–3956 Vol. 63, No. 10
0099-2240/97/$04.00+0

Identification and Retting Efficiencies of Fungi Isolated from Dew-Retted Flax in the United States and Europe

GUNNAR HENRIKSSON,[1,2] DANNY E. AKIN,[2]* RICHARD T. HANLIN,[3] CARMEN RODRIGUEZ,[3] DOUGLAS D. ARCHIBALD,[2] LUANNE L. RIGSBY,[2] AND KARL-ERIK L. ERIKSSON[1]

Department of Biochemistry and Molecular Biology[1] and Department of Plant Pathology,[3] University of Georgia, Athens, Georgia 30602, and Richard B. Russell Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Athens, Georgia 30604[2]

Received 12 February 1997/Accepted 7 August 1997

Seven strains of filamentous fungi and one yeast were isolated from flax that was dew retted in the United States. These filamentous fungi were subcultured to purity and identified, and six appear not to have been reported earlier as isolates from dew-retted flax. Five of the purified U.S. strains, two fungi isolated from flax that was dew retted in Europe, and a laboratory culture of *Aspergillus sojae* were tested for their ability to ret flax stems. The monocultures were evaluated for the degree of retting, fiber strength, dry weight loss, and tactile response (i.e., feel of softness) as reflected in the retted fiber. Structural modifications of representative samples of the retted flax were assessed by scanning electron microscopy. All of the filamentous fungi were able to carry out some retting, whereas the isolated yeast could not. All organisms produced pectinases when they were cultivated in shake flasks on ball-milled flax as the sole carbon source. Some fungi also produced cellulases, mannanases, and xylanases. *Rhizomucor pusillus* and *Fusarium lateritium* were noteworthy as retting organisms by their high level of pectinase activity, ability to attack noncellulosic cell types without attacking cellulose, capacity to penetrate the cuticular surface of the stem, and efficient fiber release from the core. The results indicated that these organisms deserve further study as potential organisms for retting of bast fibers in industrial applications.

Flax is the raw material for such important industrial products as linseed oil, some high-quality papers, and linen fibers. Linen has very high textile quality properties such as strength, water absorption, and comfort and feel, but it occupies only a minor part of the total textile market, i.e., less than 1% (10, 14). The process of retting is required to produce fibers from flax stems. In this process, bast fiber bundles are separated from the core, epidermis, and cuticle and are also separated into smaller bundles and individual fibers. This separation is accomplished by removal of pectin and hemicelluloses from parenchyma cells and the middle lamellae.

That retting is a major limitation to efficient production of linen fibers is obvious from the considerable amount of research invested in improving the process (19). Water retting, which depended on fermentation of matrix polysaccharides by anaerobic bacteria, was the primary method used formerly. Pollution from this fermentation process as well as the high costs of labor and drying caused water retting to be replaced by dew retting as the primary method for isolating fibers in the last several decades (21). In dew retting, harvested flax is spread out in fields where indigenous microorganisms, mainly filamentous fungi, grow on the plant and partly degrade the polysaccharides in cell walls and middle lamellae, thus releasing the fibers for further processing. However, dew retting has several disadvantages: only a few regions worldwide have suitable climates, variable and inferior fiber quality is produced compared with water retting, risks of overretting occur, and land is unavailable for use over long periods of time (21). Dust and fungal contaminants may also cause health problems.

In addition to recently renewed interest in natural fibers in Europe (14, 20), there has also been an interest in reestablishing the flax fiber industry in the United States (2). Flax is grown experimentally and in small commercial fields in Oregon, Connecticut, Maine, and South Carolina; in South Carolina, flax is a winter crop. It is recognized that retting of flax is a major limitation and should be improved to increase the efficiency of flax fiber production. The preliminary success of dew retting in South Carolina suggests that microorganisms different from those in Europe might be important and further suggests that enzymes from new isolates could substantially improve the industrial extraction of fibers. The microbiology of dew retting of flax in the United States has not been studied. Therefore, the present work was undertaken to gain knowledge of the microorganisms responsible for retting in unexplored regions and to search for superior retting organisms and enzyme mixtures.

MATERIALS AND METHODS

Plant materials and substrates. Citrus pectin was from Sunkist, Inc. (Corona, Calif.). Bacto Agar was from Difco Laboratories (Detroit, Mich.). Birch wood xylan and locust bean gum mannan were from Sigma (St. Louis, Mo.). Carboxymethyl cellulose (CMC) type 7H3 SXF PH was from Aqualon Co. (Wilmington, Del.). Flax (cultivar Ariane) was grown in The Netherlands in 1995, and straw that was collected before retting (i.e., unretted) as well as straw that was dew retted by industry standards was supplied by van de Bilt zaden en vlas, b.v. (Sluiskil, The Netherlands). Samples of flax (cultivar Natasja), which were dew retted near Florence, S.C., were supplied by R. B. Dodd (Clemson University). Natasja flax, which was dew retted in Connecticut, was supplied by G. R. Stephens (Connecticut Agricultural Experiment Station). A high-quality sample of dew-retted flax from France was supplied by H. S. S. Sharma (Department of Agriculture, Belfast, Northern Ireland). All other chemicals were of analytical grade. A Cannon-Fenske viscometer (type 13-617E) was from Fisher Scientific, Pittsburgh, Pa.

Isolation and identification of organisms. Pieces of retted flax were placed on agar plates with Vogel medium (22) and citrus pectin as the carbon source. One liter of medium (pH 5.0) contained 2.5 g of sodium citrate monohydrate, 5 g of KH_2PO_4, 2 g of NH_4NO_3, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of $CaCl_2 \cdot 2H_2O$, 10 g of citrus pectin, 5 μl of concentrated HCl, 2.5 mg of $FeSO_4$, 0.98 mg of $MnSO_4 \cdot H_2O$, 0.83 mg of $ZnCl_2$, 1.0 mg of CoCl, and 12 g of Bacto Agar. The organisms from the retted flax growing on the plates were isolated by picking hyphal tips, colonies, or spores for inoculation of new plates and were subcultured in this way

* Corresponding author.

TABLE 1. Organisms isolated from retted flax

Organism	Location in which flax was retted	Appearance on pectin agar plate	Appearance on flax (retting expt)
F. equiseti sw (Corda) Sacc.	South Carolina	White mycelium, orange spores	Rich white mycelium, orange spores
SC Orange (yeast)	South Carolina	Orange colonies	No visible growth
R. pusillus sb (Lindt) Schipper	South Carolina	Airy white mycelium, black fruit bodies	Airy white mycelium, black fruit bodies
T. virens sg (Miller, Giddens et Foster) Arx	South Carolina	Green	Green spots, uneven white mycelium
A. alternata sog (Fr.) Keissl.	South Carolina	White mycelium, gray spores	Rich gray mycelium; stains flax dark
F. lateritium sr Nees	South Carolina	White mycelium, red spores	Sparse red slime
F. lateritium cw Nees	Connecticut	White mycelium	Airy white mycelium
C. herbarum cg (Pers.) Link	Connecticut	Green	Grey slime
F. oxysporum fw Schltdl.	France	White mycelium	White mycelium
E. nigrum hp Link	Holland	White mycelia, yellow and black spots; stains the plate peach	Rich peach mycelium, black spots; stains flax peach

three times. Before identification, the microorganisms were inoculated on malt agar plates with penicillin (100 mg/liter) and streptomycin (100 mg/liter). Isolated strains were maintained on flax straw, except for a yeast (labeled SC Orange) that was maintained on pectin agar plates. The isolated fungal strains were identified according to standard taxonomic references (3, 4, 7).

Retting experiment. The organisms were inoculated on Vogel's pectin agar plates, and the plates were incubated at room temperature until the fungi covered the plates. Spores and mycelial fragments (and cells in the case of the yeast) were collected with a sterile spatula and suspended in a sterile 0.9% NaCl solution. Sterilized flax pieces were inoculated with fungal spores of all isolated strains from flax retted in South Carolina, from *Fusarium oxysporum* of flax retted in France, and from *Epicoccum nigrum* of flax retted in The Netherlands. For comparison, *Aspergillus sojae* from the culture collection maintained at the University of Georgia was also inoculated on flax.

For in vitro retting tests, 5-cm pieces were cut from the middle of unretted Ariane flax stems. The flax pieces were freeze-dried, and samples of 1 to 1.5 g were autoclaved (120°C, 103.5 kPa, 15 min) in 6 ml of distilled water. Stem pieces were inoculated by dipping them in a mixture containing 40 ml of 1% autoclaved glucose solution and 3 ml of a spore suspension. The flax was put in a solid-state fermentor (previously autoclaved as described above) on a steel web allowing air to enter from the bottom side. The solid-state fermentors were incubated in a 30°C chamber in which the air humidity varied between 50 and 80%, with the exception of *E. nigrum*, which was incubated at room temperature (about 22°C) at a humidity of between 50 and 80%. The fermentors were incubated for 2, 4, or 6 weeks, and harvested samples were freeze-dried, weighed, and stored at −20°C.

Retting efficiency. The degree of retting was determined by a modification of the Fried test, which indicates separation of fibers from the core and from each other in enzymatically treated flax stems (8, 11, 21). Briefly, in this test 8 ml of boiling water was added to a test tube with three retted flax pieces. The tubes were sealed and immediately agitated for exactly 10 s on a vortex mixer and, thereafter, were manually shaken vigorously four times in a vertical direction. Samples in the vials were numerically scored for fiber release by visual comparison with standard images (21) as follows: 0, flax stem intact; 1, bast fibers starting to be released from the core; 2, bast fibers substantially released from the core and fibers partly separated from each other; and 3, bast fibers totally released from the core and fiber bundles subdivided into smaller units and totally or almost totally separated from each other. To avoid bias in the ranking, each sample was given a random number, and the grading was done by an examiner without knowledge of the treatments. The test was done in triplicate. The validity of this test has been confirmed by increased enzyme concentrations and related enhanced retting efficiencies (10a).

Strength measurement of flax fibers. The tensile strength of retted flax fiber bundles was determined with an Instron Universal Measuring Instrument with 200 kg of maximal force and a breaking velocity of 2.0 mm/min. Samples were equilibrated at 65% relative humidity and room temperature (about 22°C) overnight before testing. Retted fibers from one flax stem were manually removed from the core and cuticle, cut from the middle of the sample to 32 mm in length, and positioned in parallel fashion in a holder. The gap for the breaking strength measurement was 6.0 mm. The broken fibers were collected and weighed on an analytical balance. The maximal load force required to pull fibers apart divided by mass per fiber length was considered the strength value of the fiber. Values obtained for fibers that slipped or did not break simultaneously were excluded; samples in which some of the fibers broke within the holder were accepted.

Tactile response. Fibers removed from the core and cuticle were manually graded for tactile response as stiff (as in unretted material), soft (as in animal fur), or medium (between stiff and soft).

SEM. Samples were prepared for scanning electron microscopy (SEM) as follows. Five intact stem pieces were chosen from vials with selected microbial treatments that represented a range of retting efficiencies and were fixed overnight at 8°C in 3% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The stems were rinsed in 0.1 M cacodylate buffer twice, and 3-mm sections were cut from each end and from the middle of the stem piece. These sections were air dried on filter paper, mounted on SEM stubs with Duco cement, surrounded with silver paste, and stored in the desiccator until coating with Au-Pd alloy and viewing in the JEOL SEM at 3.0 kV.

Enzyme profiles. Eight of the isolated fungal strains were cultivated in shake flasks at 30°C and 140 rpm in 1-liter Erlenmeyer flasks with 300 ml of Vogel medium (22) containing ball-milled flax (sterilized by ethylene oxide gas) as the only carbon source (lactose omitted from original medium). Samples for measurement of pectinase activity were taken periodically during the cultivation, and the culture filtrates were collected when the pectinase activity ceased to increase (about 120 h). Insoluble material was removed from the culture filtrates by centrifugation (Beckman J2-21) at 4,220 × *g*, and supernatants for enzyme analyses were passed through a prefilter and concentrated with an ultrafiltration membrane with a 10-kDa cutoff. NaN_3 was added (0.02% [wt/vol]) to prevent microbial growth.

Samples of enzyme solution were incubated at 50°C in a final volume of 750 μl with 50 mM sodium acetate buffer (pH 5), with 0.3% substrate solution (pectin, xylan, or mannan). Incubation time was 10 min for the pectinase assay, except for samples taken from the nonconcentrated culture filtrate during the cultivation in which the incubation times were 30 and 40 min for the xylanase and mannanase assays, respectively. The reaction was terminated by adding 750 μl of dinitrosalicylic acid reagent (15). Undissolved material was removed by centrifugation (4,220 × *g*), and the samples were boiled for 5 min. The concentrations of released reducing sugars were determined colorimetrically at 640 nm by using a glucose standard. Background compensation was done from control samples without enzyme and substrate. Endoglucanase activity was determined by measuring the decrease in the relative viscosity of CMC. Four milliliters of 0.26% CMC equilibrated at 30°C was mixed with 10 μl of sample, and the mixture was poured into a Cannon-Fenske-type viscometer calibrated at an efflux time of 12.38 s for buffer and at an efflux time of 46.55 s for 0.26% CMC solution at 30°C. The measurements were done in a 30°C water bath. Efflux times were determined at regular intervals. Endoglucanase activity was measured as the decrease in relative viscosity per minute under the conditions described above.

RESULTS

Isolation and identification of organisms. A set of fungi representing prevalent colonizers was isolated from the various flax sources. Six microorganisms were isolated from flax dew retted in South Carolina, two fungi were isolated from Connecticut-retted flax, and one fungus each was isolated from flax retted in The Netherlands and France. Some physical characteristics of these fungi are listed in Table 1. One organism from flax retted in South Carolina, which was labeled SC Orange, was a yeast and was not identified further.

Large differences occurred among the characteristics of the different fungi colonizing the flax (Table 1). The mycelium of the South Carolina strain of *Fusarium lateritium* was almost invisible, whereas *Alternaria alternata*, *Fusarium equiseti*, and *E. nigrum* all formed dense mycelia and *Rhizomucor pusillus* had an airy, fibrous mycelium. *A. alternata* and *E. nigrum* stained the flax black and peach, respectively, whereas none of the other organisms changed the color of the fiber to any large

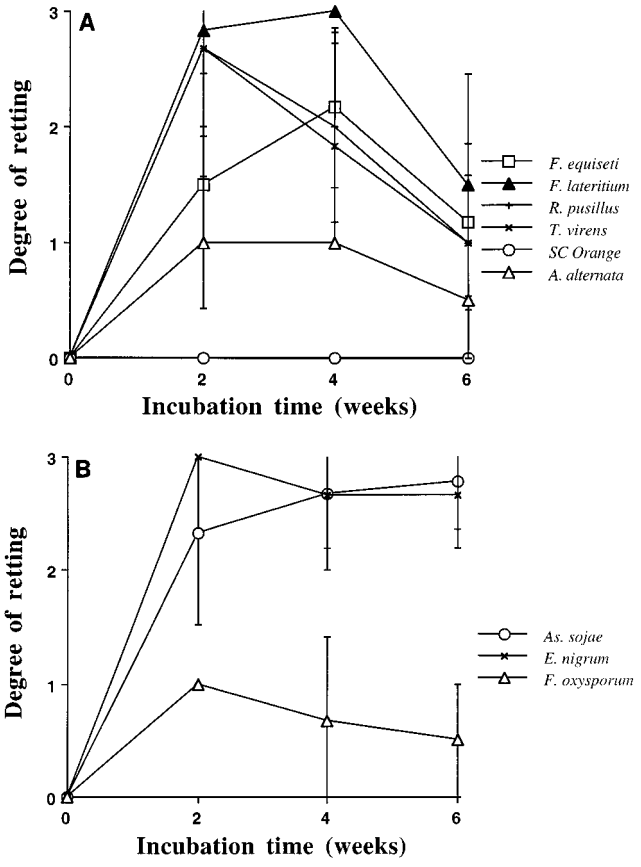

FIG. 1. Degree of retting by the Fried test for flax stems from solid-state incubations with pure cultures of fungi isolated from South Carolina (A) and other fungi (B). Error bars represent the standard deviations from three sets of data.

extent. In the case of *E. nigrum*, the peach color penetrated the fiber as well. Interestingly, the two *F. lateritium* strains looked very different both on pectin agar plates and on flax.

Retting experiment. The Fried test indicated that *F. lateritium*, *R. pusillus*, *Trichoderma virens*, *A. sojae*, and *E. nigrum* were the most efficient retters (Fig. 1). The decline in the Fried score at 4 or 6 weeks for several of the organisms likely occurred because of hyphal growth and subsequent reduction in fiber separation from the stem. Scores for *A. sojae* were variable, with values not different ($P = 0.05$) for 2, 4, and 6 weeks. *A. alternata*, *F. equiseti*, and *F. oxysporum* were less efficient (Fig. 1), and the yeast SC Orange did not ret the flax.

Characterization of retted fiber. *E. nigrum* produced the greatest dry weight losses during the incubation (Table 2). *A. alternata* and *F. oxysporum* also produced considerable weight losses, whereas *R. pusillus*, in spite of its good retting ability, caused small weight losses. *E. nigrum* also produced the largest loss of fiber strength (Table 2). Flax inoculated with *A. alternata*, *R. pusillus*, *F. lateritium*, or *F. oxysporum* also lost some strength, while *T. virens*-treated stems showed high variability in strength loss. Softness, which was subjectively rated, did not totally follow scores in the Fried test (Table 2; Fig. 1). *A. alternata* and *F. oxysporum* produced soft fibers and gave relatively low scores by the Fried test, whereas the *A. sojae*-treated fibers were stiff but ranked high in the degree of retting. Flax retted with *E. nigrum* and *A. alternata* had the softest fibers. Material retted by *T. virens* varied considerably in softness.

SEM. Unretted, control flax stems (Fig. 2) were used as a basis for characterizing the retting by monocultures of fungi. *R. pusillus*, which showed a high efficiency in retting by the Fried test (Fig. 1A), substantially colonized flax stems at 2 weeks (results not shown). By 6 weeks, the exposed edge of the stems showed heavy fungal colonization and subdivision of fiber bundles (Fig. 3A). Fibers were not degraded to any substantial extent, although the lumen of the fibers indicated some expansion from that in the controls. Stems bisected for evaluation of fungal attack at the center of the sample showed colonization and partial bundle separation (Fig. 3B). The lumen of the fiber cell appeared expanded as at the edge, indicating that the innermost portion of the secondary wall was susceptible to enzymatic attack. Examination of freehand-cut sections by light microscopy also showed expanded cell lumens by *R. pusillus* (results not shown). Examination of attack on the stem surface indicated that *R. pusillus* appeared to partly degrade or remove the cuticular surface (Fig. 3C), thus allowing entry into the stem tissues through the surfaces.

A. alternata, which retted flax poorly according to the Fried test (Fig. 1B), heavily colonized the cut stem surface and often obscured the fibers (Fig. 4). SEM did not indicate that the fibers were separated from other tissues, and examination of bisected stems (not shown) did not indicate a structure different from that of unretted stems.

T. virens at 6 weeks of incubation produced few hyphae as observed by SEM (Fig. 5), despite good retting (Fig. 1A) at 2

TABLE 2. Characteristics of retted flax[a]

Organism	Incubation time (wk)	Dry wt lost (%)	Strength N/(kg/m), 10^{3}[b]	Softness
Uninoculated control	6	3.6 ± 1.4	636 ± 129	Stiff
F. equiseti	2	12.8 ± 2.3	497 ± 192	Medium
	4	11.0[c]	397 ± 103	Medium
	6	13.2 ± 0.3	495 ± 50	Medium
SC Orange (yeast)	2	6.6 ± 0.05	808 ± 182	Medium
	4	4.8[c]	612 ± 114	Stiff
	6	6.1[c]	704 ± 267	Stiff
R. pusillus	2	9.1 ± 0.7	450 ± 86	Soft
	4	8.1[c]	300 ± 72	Soft
	6	11.2 ± 2.1	308 ± 29	Soft
T. virens	2	13.4 ± 1.6	317 ± 211	Uneven
	4	12.6 ± 3.7	320 ± 239	Uneven
	6	10.2 ± 1.6	485 ± 179	Uneven
A. alternata	2	12.2 ± 0.8	339 ± 104	Soft
	4	14.8 ± 1.0	316 ± 98	Soft
	6	15.6 ± 1.0	249 ± 39	Soft
F. lateritium (from South Carolina)	2	10.0 ± 0.5	523 ± 152	Soft
	4	9.6 ± 0.4	380 ± 178	Soft
	6	13.2 ± 0.8	337 ± 113	Soft
F. oxysporum	2	12.1 ± 0.3	335 ± 149	Soft
	4	13.4 ± 1.3	361 ± 169	Soft
	6	14.2 ± 1.2	392 ± 186	Soft
E. nigrum	2	12.2 ± 0.3	210 ± 133	Soft
	4	16.6 ± 0.4	140 ± 120	Soft
	6	21.5 ± 0.9	97.7 ± 27	Soft
A. sojae	2	10.1 ± 1.8[d]	630 ± 193	Stiff
	4	11.1 ± 1.4[d]	781 ± 53	Stiff
	6	10.9 ± 0.5[d]	722 ± 155	Stiff

[a] Blank sample varied between 2 and 5% dry weight loss. Values are averages of duplicates unless otherwise specified. All strength measurements are based on triplicates, except the uninoculated sample, which was based on six samples. Softness was manually estimated as described in Materials and Methods.
[b] Breaking force divided by mass per fiber length. N, newtons.
[c] One sample.
[d] Triplicate.

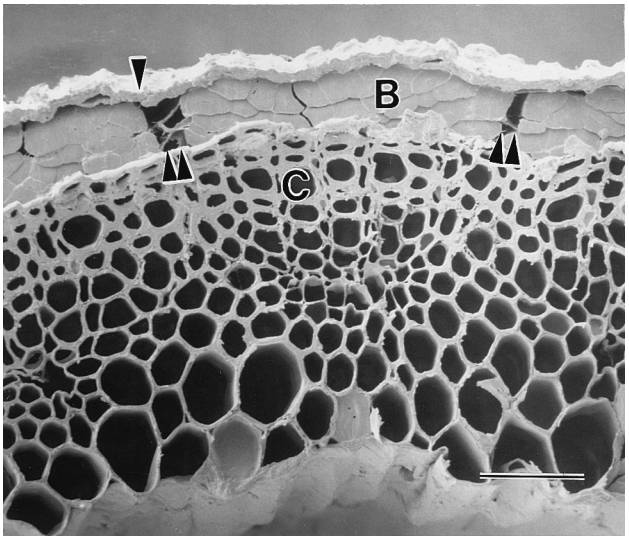

FIG. 2. SEM of unretted, control flax stem showing fibers within bundles (B) between the cutinized epidermis (arrow) and lignified core cell (C). Thin-walled parenchyma cells occur above and below the fiber bundles and between the bundles (double arrows). Bar, 50 μm.

weeks and a relatively high level of dry-matter loss (Table 2). However, the fiber at the exposed end of the section appeared to be partially degraded (Fig. 5). Fibers in the centers of bisected stems were well separated from the cuticle and core but were also partially degraded (not shown).

F. lateritium, which was scored as an excellent retter by the Fried test (Fig. 1A), produced few visible hyphae, appeared to remove cuticle, and degraded fibers at the exposed edge (Fig. 6A). The centers of bisected stems showed exposed fibers and bundles, indicating extensive loss of cuticle (Fig. 6B).

A. sojae and *F. oxysporum* showed variable retting activities (Fig. 1B). SEM indicated that neither fungus appeared to ret flax well, as indicated by separation of fibers from other tissues (not shown). Furthermore, both fungi appeared to degrade the fibers. However, neither of them weakened the fiber strength (Table 2).

E. nigrum, which gave a high score according to the Fried test (Fig. 1B) and degraded large amounts of dry matter while substantially reducing fiber strength (Table 2), separated and degraded the fibers. By 2 weeks, bundles in the center of bisected samples were separated into fibers and the cuticle was extensively removed in some regions (Fig. 7A). By 6 weeks, *E. nigrum* had removed cuticle and separated bundles into fibers, but the fungus also attacked fibers at the exposed edge (Fig. 7B). Few hyphae were observed by SEM.

Enzyme profiles. Enzyme activities produced by several organisms with different retting efficiencies in shake flask cultures with ball-milled flax as the carbon source are shown in Table 3. All organisms produced relatively high levels of pectinases, with *R. pusillus* and *F. lateritium* having the highest specific activities. Cellulases were present in all culture filtrates except *R. pusillus*, *F. lateritium*, and the yeast, with *A. alternata* and *T. virens* being the most prolific producers. Xylanase activity occurred only in culture filtrates of *T. virens*, *F. oxysporum*, and *F. equiseti*, whereas mannanase activity appeared in those of *T. virens*, *A. alternata*, *E. nigrum*, *F. lateritium*, and *F. oxysporum* (Table 3).

DISCUSSION

In a study of fungi isolated from flax which had undergone different retting methods, Sharma (16) reported that *Cladosporium herbarum*, *E. nigrum*, *Fusarium culmorum*, *Mucor* species, and *Rhizopus* species were the most commonly occurring species, with *A. alternata*, *Botrytis cinerea*, *Colletotrichum* species, *Penicillium* species, *Phoma* species, and *Ramularia* species and yeasts being less frequently isolated. In this study, no differences occurred between flax cultivars in the number or types of fungi colonizing the plant, but different retting methods influenced the number of colonizing fungi. Van Sumere (21), in a review of flax retting, reported that the following fungi developed on plants during dew retting: *C. herbarum* (summer retting), *Mucor stolonifer* (autumn retting), *Mucor hiemalis* (retting under snow), *Mucor plumbeus*, *A. niger*, *F. culmorum*, *E. nigrum*, and *Rhizopus* species. Brown (5) reported that *E. nigrum* was the most frequent fungus colonizing retted flax. *E. nigrum* and *Rhizopus* sp. colonized inner tissues of the flax stem, but *Mucor* spp. were not isolated after surface sterilization.

We isolated a total of nine filamentous fungi and one yeast from flax dew retted in the United States, The Netherlands, and France. Of those appearing to be the major colonizers of flax during dew retting, four species of *Fusarium* as well as *T. virens* and *R. pusillus* apparently have not previously been isolated from dew-retted flax or associated with retting fungi. It is noteworthy that *R. pusillus* isolated from South Carolina flax showed a high retting efficiency in our tests, and the South Carolina flax has been reported to have been dew retted very well by technical experts (6a). The climatic conditions in South Carolina might lead to different fungal colonists, as implied by Van Sumere (21).

R. pusillus, *F. lateritium*, *T. virens*, and *E. nigrum* showed efficient retting by our tests. *E. nigrum* caused the highest losses in both the dry weight and strength of flax fibers. *R. pusillus* and *F. lateritium* also weakened the fiber, but to a much lower degree than *E. nigrum*. While the test of tensile strength of fiber bundles is useful for comparing fungi, its relationship to fiber strength in the context of textile properties is unknown and suspect. In a well-retted sample, the strength is expected to decrease somewhat, since the fibers would be more easily separated within the bundles. However, the region of the bundle selected for tensile strength could contain fibers spanning the break zone along with partial fibers that are less rigidly bound within the bundle. In the case of *E. nigrum*, however, the strength loss was so great that cellulosic fibers themselves appeared to be attacked. Indeed, degradation of fibers, i.e., overretting, is indicated by SEM as well as by enzyme profiles. Other work (5, 21) has indicated that *E. nigrum* produced potent cellulases that degraded fiber and reduced quality.

R. pusillus and *F. lateritium* both produced soft, well-separated fibers. Micrographs supported their high score in the Fried test and suggested that the former fungus penetrated the cuticle and thereby gained access to the internal matrix polysaccharides (Fig. 3, 6). Dry weight losses during the solid-state fermentation were rather low (Table 2).

Flax stems inoculated with *T. virens* showed high variability in fiber strength and softness, and the fungus appeared to colonize the flax rather unevenly. When cultivated in shake flasks with ball milled flax as carbon source, *T. virens* had high xylanase and cellulase activities compared with those of pectinase and in this way differed from the other isolates. *Trichoderma* species are well known producers of cellulases (9). Because of these properties, *T. virens* may cause overretting and does not appear to be a good candidate for further testing.

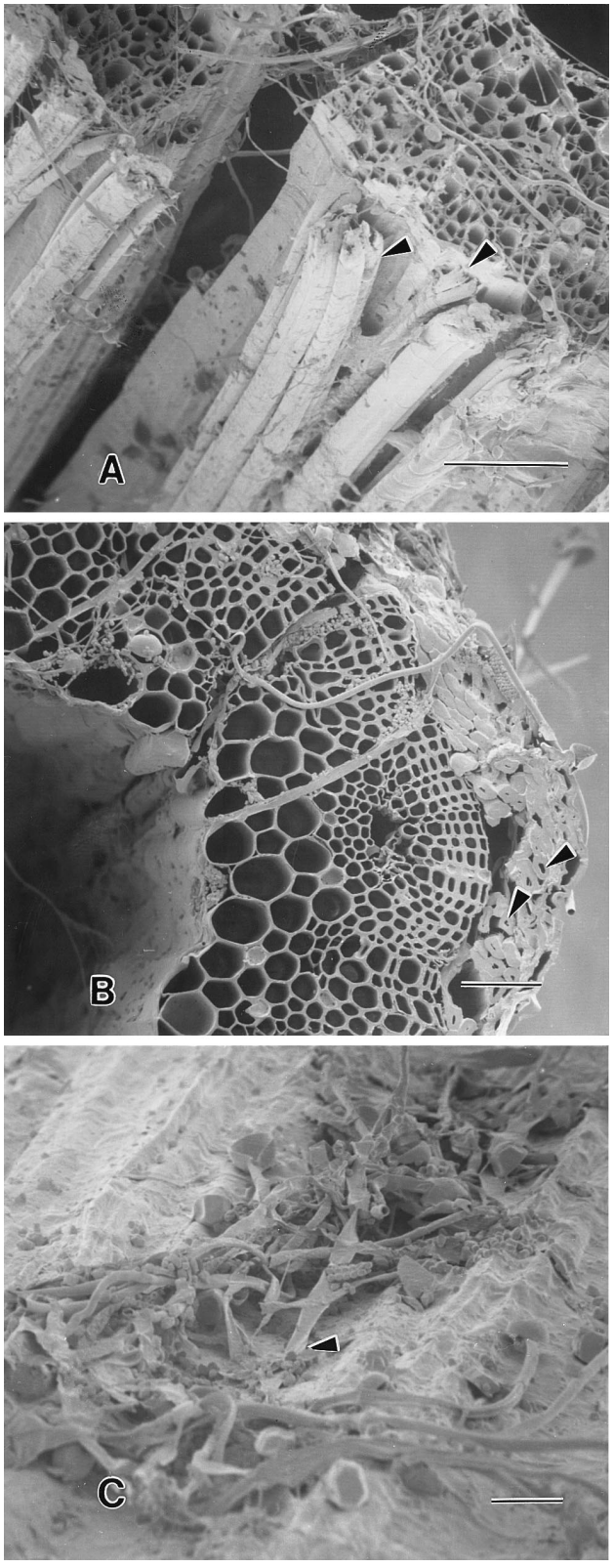

FIG. 3. SEMs of flax stems incubated for 6 weeks with *R. pusillus*. (A) Stem edge showing hyphal colonization, loss of cuticle, and division of bundles into smaller units (arrows). Bar, 100 μm. (B) Bisected stem showing attack on tissues in the sample center and separation of bundles from core into smaller units; expansion of the lumen is apparent in some fibers (arrows). Bar, 50 μm. (C) Surface of stem showing colonization and penetration of the cuticle by hyphae (arrow). Bar, 20 μm.

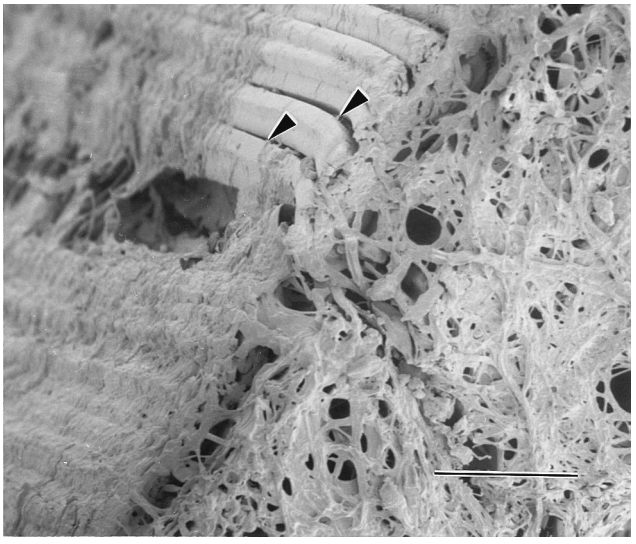

FIG. 4. SEM of flax stem incubated for 6 weeks with *A. alternata* showing heavily colonized stem edge, close association of fibers to core, and loss of epidermis and apparent dissolution of cuticle. Fibers are separated in some regions (arrows). Bar, 50 μm.

A. alternata, *F. oxysporum*, and *F. equiseti* showed less efficient retting than other organisms by the Fried test. However, at least in the case of *A. alternata*, part of the explanation for the relatively low retting rates may be the heavy mycelial growth that could prevent fiber separation from the stem. When flax straw colonized by *A. alternata* or *F. oxysporum* was manually broken, the fibers easily separated and were soft. The Fried test, which was developed to measure enzymatic retting (21), may thus have limitations in evaluating retting by fungi. This binding of fibers by mycelium may also explain the tendency for a decrease in the retting rate in the samples with longer incubation times (Fig. 1).

Sharma and Van Sumere (19) reported that *A. alternata* was mainly established later in the retting process. However, our results indicated that it can also work as a primary colonizer.

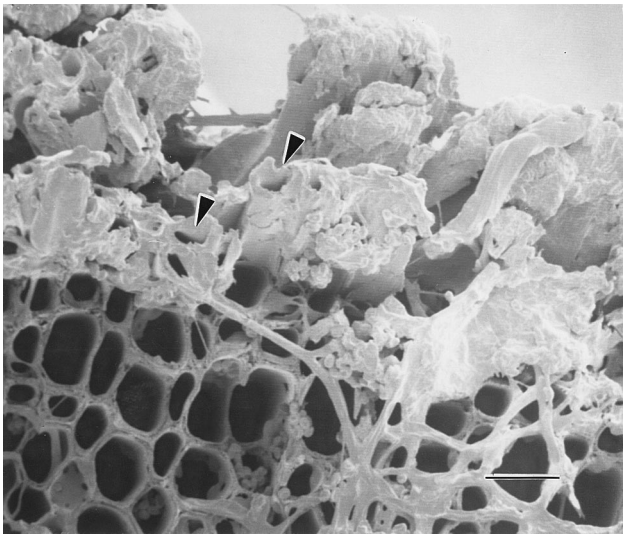

FIG. 5. SEM of flax stem incubated for 6 weeks with *T. virens* showing the cut edge with few hyphae and apparent degradation of fibers (arrows). Bar, 20 μm.

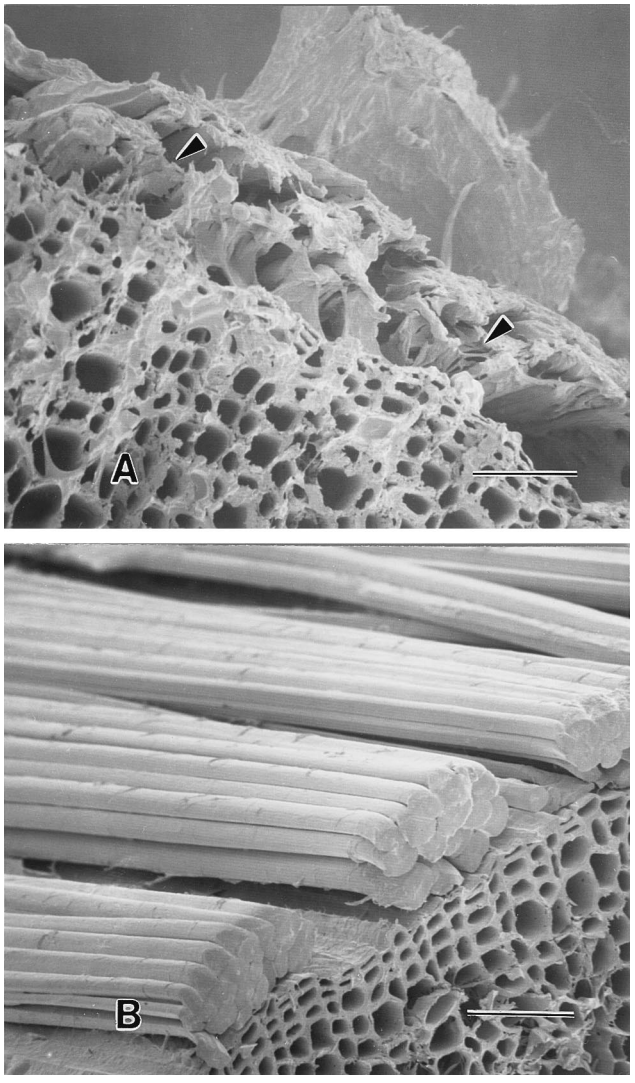

FIG. 6. SEMs of flax stems incubated for 6 weeks with *F. lateritium*. Bar, 50 μm. (A) Edge of cut sample showing degraded fibers (arrows); (B) center of bisected stem sample showing loss of epidermis and cuticle and exposure of bundles.

The decrease in fiber strength and loss of dry matter caused by this organism were the greatest following those of *E. nigrum*. These results, together with the high level of specific cellulase activity (Table 3), indicated that this fungus may to some extent cause overretting. Strains of *F. oxysporum*, as well as other *Alternaria* species and *Cladosporium* species and other *Fusarium* species, can be phytopathogenic (1). *F. equiseti* was a frequent colonizer of flax during dew retting in South Carolina; however, neither the Fried test nor the softness result suggested that it was an efficient retter.

The yeast SC Orange did not ret stems when it was inoculated on flax. Yeasts have earlier been isolated from dew-retted flax (6), but at least this yeast appears not to have any retting ability and is thus probably a secondary colonizer. However, SC Orange was pectinolytic (Table 3), and although the losses in the dry weight of flax inoculated with this organism were low, they were higher than those of the control (Table 1). Thus, we cannot rule out that some pectin was degraded, and the yeast may act synergistically with filamentous fungi during the retting process.

In recent years, particularly in the 1980s, considerable research has been undertaken to improve retting through microbial and enzymatic methods (18, 21). A consortium of enzymes, namely, pectinases, xylanases, and cellulases, are produced by retting fungi (5, 12, 18, 21). Enzymes from predominant retting fungi such as *C. herbarum* (12) and *E. nigrum* (5) were tested for flax-retting abilities. Furthermore, Sharma (17) used the enzymes from *Ceraceomyces sublaevis*, which contained the phenol oxidase laccase along with polysaccharidases, and reported effective depolymerization of noncellulosic materials in dew-retted fibers. The most promising enzyme mixture for retting of flax was found in a product from Novo Nordisk called Flaxzyme, which was produced by *A. japonicus* or *A. aculeatus* (21).

All of the fungi that we have described herein produced pectinases when they were cultivated in shake flasks with ball-milled flax as the sole carbon source, supporting their impor-

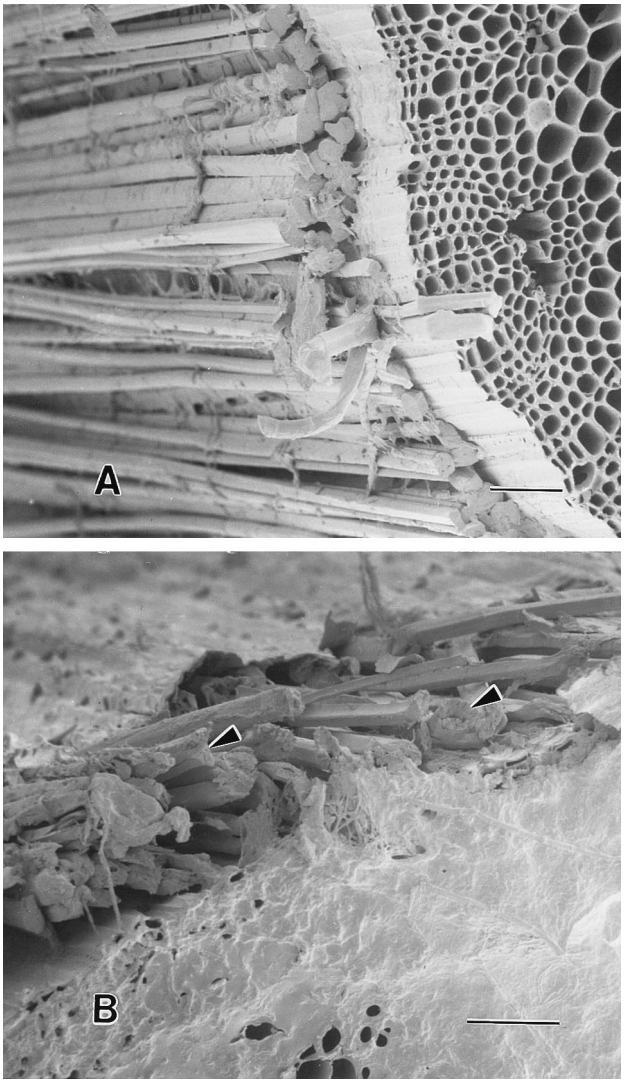

FIG. 7. SEMs of flax stems incubated with *E. nigrum*. Bar, 50 μm. (A) Center of bisected stem after incubation for 2 weeks showing loss of cuticle and separation of bundles into fibers in several areas. (B) Edge of sample incubated for 6 weeks showing fibers undergoing degradation (arrows).

TABLE 3. Enzyme profiles of culture filtrates cultivated on flax

Organism	Amt of reducing sugar released per protein amt and time[a]			Decrease in relative viscosity for cellulase[b]
	Pectinase	Xylanase	Mannanase	
F. equiseti	17.0	2.2	NS[c]	4.5
SC Orange (yeast)	15.4	NS	NS	NS
R. pusillus	47.2	NS	NS	NS
T. virens	25.2	21.8	0.78	20
A. alternata	9.0	NS	11.3	34
F. lateritium	33.8	NS	1.6	NS
F. oxysporum	16.0	4.9	7.1	16
E. nigrum	1.9	NS	7.6	6.5

[a] Values are micromoles per milligram of protein per minute.
[b] Values are per milligram of protein per minute.
[c] NS, no significant activity detected.

tance in this process (21). The necessary involvement of cellulases and hemicellulases for efficient retting is not clear from our study of these organisms by shake culture methods, and it is possible that enzymes from solid-state fermentation may differ from those of shake flasks. Treatment of flax with pure xylanase alone did not ret flax stems (1a), suggesting that other enzymes are required in this process. Further, culture flask filtrates from *R. pusillus* appear to efficiently ret flax stems (10a). Research to identify enzymes with optimal specificity appears to be a fruitful approach to enhance enzymatic retting.

Based on our results, *R. pusillus* and *F. lateritium* appeared to be the best fungi for further evaluation to improve retting. *A. alternata* and *F. oxysporum* produced soft fiber, but the Fried test and SEM suggested that the fibers were not as well released from flax stems as they were by the first two organisms. *E. nigrum* might be employed, but the process must then be terminated before the fibers are weakened by the aggressive cellulose degradation of this organism.

ACKNOWLEDGMENTS

This work was supported by National Research Initiation (USDA) grant no. 95 37500-1909.

We thank Nirav Patil and Katrina Redmond for skillful technical assistance. We gratefully acknowledge the following for providing flax samples: R. B. Dodd, Clemson University; G. R. Stephens, Connecticut Agricultural Experiment Station; and H. S. S. Sharma, Department of Agriculture, Belfast, Northern Ireland.

REFERENCES

1. **Agrios, G. N.** 1988. Plant pathology, 3rd ed. Academic Press Inc., San Diego, Calif.
1a. **Akin, D. E.** Unpublished data.
2. **Anderson, J. F., and M. A. Schiavoni (ed.).** 1994. Proceedings of the World Flax Symposium. The Connecticut Agricultural Experiment Station, New Haven, Conn.
3. **Bissett, J.** 1991. A revision of the genus *Trichoderma*. III. Section Pachybasium. Can. J. Bot. **69:**2373–2417.
4. **Booth, C.** 1971. The genus *Fusarium*. Commonwealth Mycological Institute, London, United Kingdom.
5. **Brown, A. E.** 1984. *Epicoccum nigrum*, a primary saprophyte involved in the retting of flax. Trans. Br. Mycol. Soc. **83:**29–35.
6. **Brown, A. E., and H. S. S. Sharma.** 1984. Production of polysaccharide-degrading enzymes by saprophytic fungi from glyphosate-treated flax and their involvement in retting. Ann. Appl. Biol. **105:**65–74.
6a. **Dodd, R. B.** Personal communication.
7. **Domsch, K. H., W. Gams, and T. H. Anderson.** 1980. Compendium of soil fungi, vol. I and II. Academic Press, London, United Kingdom.
8. **Dujardin, A.** 1942. Vlas-roten. De Westvlaamsche Boekhandel s.v. Groeninghe drukkerij, Kortrijk, Belgium.
9. **Eriksson, K.-E. L., R. A. Blanchette, and P. Ander.** 1990. Microbial and enzymatic degradation of wood and wood components. Springer-Verlag, New York, N.Y.
10. **Hamilton, I. T.** 1986. Linen. Textiles **15:**30–34.
10a. **Henriksson, G.** Unpublished data.
11. **Henriksson, G., K.-E. L. Eriksson, and D. E. Akin.** The influence of chelating agents and mechanical pretreatment on enzymatic retting of flax. Text. Res. J., in press.
12. **Hunter, S. A., and A. E. Brown.** 1984. Effect of the application of *Cladosporium herbarum* inoculum to flax plants on retting. Rec. Agric. Res. (Belfast) **32:**77–86.
13. **Jauneau, A., A. Driouich, and C. Morvan.** 1996. A clear-cut zonation of pectic polysaccharides in the cortex of flax plantlets, p. 495–498. *In* Proceedings of the Workshop of the FAO Network on Flax. Institut Technique du Lin, Paris, France.
14. **Kozlowski, R.** 1996. Look at flax in 21st century, p. 7–19. *In* Proceedings of the Workshop of the FAO Network on Flax. Institut Technique du Lin, Paris, France.
15. **Miller, G. L.** 1959. Use of dinitrosalicylic acid reagents for determination of reducing sugars. Anal. Chem. **31:**426–428.
16. **Sharma, H. S. S.** 1986. An alternative method of flax retting during dry weather. Ann. Appl. Biol. **109:**605–611.
17. **Sharma, H. S. S.** 1987. Enzymatic degradation of residual non-cellulosic polysaccharides present on dew-retted flax fibres. Appl. Microbiol. Biotechnol. **26:**358–362.
18. **Sharma, H. S. S., J. Lefevre, and J. Boucaud.** 1992. Role of microbial enzymes during retting and their effect on fibre characteristics, p. 199–212. *In* H. S. S. Sharma and S. F. Van Sumere (ed.), The biology and processing of flax. M Publications, Belfast, Northern Ireland.
19. **Sharma, H. S. S., and C. F. Van Sumere.** 1992. Enzyme treatment of flax. Genet. Eng. Biotechnol. **12:**19–23.
20. **van Dam, J. E. G., G. E. T. van Vilsteren, F. H. A. Zomers, W. B. Shannon, and I. T. Hamilton.** 1994. Industrial fiber crops. Increased application of domestically produced plant fibres in textiles, pulp and paper production, and composite materials. ATO-DLO, Wageningen, The Netherlands.
21. **Van Sumere, C. F.** 1992. Retting of flax with special reference to enzyme-retting, p. 157–198. *In* H. S. S. Sharma and S. F. Van Sumere (ed.), The biology and processing of flax. M Publications, Belfast, Northern Ireland.
22. **Vogel, H. J.** 1964. Distribution of lysine pathways among fungi: evolutionary implications. Am. Nat. **98:**435–446.