Screening of Mycelial Fungi for Nitrocellulose Degradation

S. T. SUNDARAM,[1] Y. Z. ZHANG,[1] A. SHARMA,[1] KOONWING NG,[2] and B. W. BRODMAN[2,*]

[1]Geo-Centers, Inc., Lake Hopatcong, New Jersey 07849; [2]U.S. Army, Armaments Research, Development and Engineering Center Picatinny Arsenal, New Jersey 07806

SYNOPSIS

Several mycelial fungi were screened in liquid medium for their ability to degrade nitrocellulose. Nitrocellulose was tested as the sole source of nitrogen while starch was provided as an additional carbon source. More than 20% nitrocellulose degradation was accomplished with *Acremonium persicinum* ATCC 60921, *Bjerkandera adusta* ESF 620, *Fusarium solani* IFO 31093, and *Sclerotium rolfsii* ATCC 24459 in a 3-day growth period utilizing submerged cultivation. *Cyathus stercoreus* NRRL 6473 degraded only 8% of the nitrocellulose, while *Irpex lacteus* Z 212 degraded 24% of the nitrocellulose in a 7-day growth period. © 1995 John Wiley & Sons, Inc.

INTRODUCTION

Nitrocellulose is the major ingredient in most gun propellant formulations. The propellant is capable of undergoing autocatalytic decomposition, which is prevented by adding a stabilizer, such as diphenylamine, which, in turn, is capable of being nitrated and, thus, terminating the chain reaction. Once the stabilizer is depleted and totally nitrated, the propellant becomes unstable and must be destroyed. This scrap propellant, along with the waste generated during the manufacture, are the major sources of nitrocellulose waste generation. Since nitrocellulose is extremely flammable and highly reactive, the waste-containing nitrocellulose is classified as a hazardous waste. Currently, the disposal of nitrocellulose is carried out by open-air burning or detonation (OB/OD). Recent studies have indicated that OB/OD of the propellants produces air-borne particulates and pan residues containing materials such as 2,4-dinitrotoluene and 2,6-dinitrotoluene that are toxic.[1] Hence, the current focus for nitrocellulose disposal is on biodegradation, which is potentially a more environmentally friendly disposal method than is OB/OD.

Previously, a number of degradation processes based on the chemical and biological treatments of nitrocellulose have been investigated.[2-5] The chemical treatment of nitrocellulose results in toxic residues and subsequent treatments are necessary to convert the toxic residues to environmentally innocuous products.[2] Studies carried out by Kaplan and co-workers indicated that the nitrocellulose is not subject to direct microbial attack and that a chemical pretreatment of nitrocellulose is necessary in order to generate a modified polymer that could be attacked by microorganisms.[2,6,7] Brodman and Devine[8] reported that *Aspergillus fumigatus* can utilize the nitrogen resulting from the hydrolysis of the nitrate ester group of nitrocellulose without attacking the carbon back bone. Recent studies by Gallo et al.[9] indicated that *A. fumigatus*, *Phanerochaete chrysosporium*, and Actinomyces sp. could not degrade nitrocellulose. However, IL'Inskaya and Leshchinskaya reported that *A. fumigatus* F-316 was capable of directly attacking nitrocellulose.[10]

In our earlier work, we demonstrated nitrocellulose degradation in liquid medium by *Penicillium corylophilum* Dierckx[11] and also by a combination of cellulolytic (*Sclerotium rolfsii* ATCC 24459) and denitrifying (*Fusarium solani* IFO 31093) fungi.[12] In the present investigation, we screened several fungi in order to identify a fungal culture that would degrade nitrocellulose much more efficiently.

MATERIALS AND METHODS

Organisms

F. solani IFO 31093 was purchased from the Institute for Fermentation, Osaka, Japan; Acremonium per-

* To whom correspondence should be addressed.

Journal of Applied Polymer Science, Vol. 58, 2287–2291 (1995)
 CCC 0021-8995/95/122287-04

sicinum ATCC 60921, *P. chrysosporium* ATCC 32629, and *S. rolfsii* ATCC 24459 were obtained from the American Type Culture Collection, Rockville, MD; Basidiomycetes sp. NRRL 6464 and *Cyathus stercoreus* NRRL 6473 were obtained from the National Center for Agricultural Utilization Research, Peoria, IL; *Bjerkandera adusta* ESF 620, *Irpex lacteus* Z 212, and *Trametes versicolor* ESF 491 were received from the College of Environmental Science and Forestry, State University of New York, Syracuse, NY; and *Trichoderma pseudokoningii* 228 F was obtained from the Western Forest Products Laboratory, Vancouver, BC.

Maintenance of Cultures

The cultures were maintained at 4°C on sabouraud maltose agar plates with monthly transfers.

Chemicals

Nitrocellulose (smokeless grade) was received as a gift from Hercules Inc., Kenvil, NJ, and it contained 13.17% nitrogen (~ 2.33 nitrate ester groups per repeat unit) and 25.07% moisture. Starch and β-1,4-xylan were purchased from Sigma Chemical Co., St. Louis, MO. Unless specified, all other reagents used were of analytical grade.

Determination of Moisture Content in Nitrocellulose

The moisture content of nitrocellulose was estimated using a DuPont thermal analysis system 1090 B equipped with thermogravimetric analyzer 951.

Growth Medium

The mineral salts medium used in this study contained (g/L) KH_2PO_4, 1.0; $MgSO_4$, 0.5; NaCl, 0.1; $CaCl_2$, 0.1, and trace metal solution, 100 μL. Trace metal salts solution contained (w/v%) $C_6H_8O_7$ (citric acid), 5.0; $ZnSO_4$, 5.0; $CuSO_4$, 0.25; $MnSO_4$, 0.25; H_3PO_4, 0.05; $Na_2MoO_4 \cdot 2H_2O$, 0.05, and $CoCl_2$, 2.0. The fungal cultures were grown at 28°C using (0.1%) xylan as a carbon source and ammonium nitrate (0.1%) as a source of nitrogen. The cultures were harvested after 5 days of cultivation and the inoculum for the studies were prepared as described earlier.[11]

Cultural Conditions

A known volume of homogenized mycelia (~ 25–50 mg dry wt) was transferred to 50 mL of the experimental medium (in a 250 mL Erlenmeyer flask) containing nitrocellulose (0.3%) as a sole nitrogen source and starch (0.1%) as a cosubstrate. Inoculated flasks were incubated for various time intervals (0, 3, and 7 days) in a gyrotary shaker (~ 150 rpm, 28°C). Three types of control were run in parallel to the treatment, one of them lacking nitrocellulose but containing the cultures, the other having NaN_3 (1 m*M*), $HgCl_2$ (1 m*M*) along with the cultures, while the third contained only nitrocellulose.

Determination of the Weight of Biomass and Unutilized Nitrocellulose

After 3 and 7 days of fungal growth, the medium containing nitrocellulose and the fungal biomass was filtered through a preweighed filter paper (VWR Scientific, 5.5 cm) (W_1) and the filter cake was washed thoroughly with distilled water, dried at 50°C for 3 h, and then cooled to room temperature (22°C) in a desiccator containing Drierite. The filter paper with a filter cake was dried and weighed until a constant weight was attained (W_2). To a preweighed centrifuge tube (W_3) was added the dried filter cake, and the tube with the filter cake was then weighed (W_4). Acetone was added gradually with vigorous stirring until almost all the nitrocellulose in the filter cake dissolved. This acetone nitrocellulose solution, with a suspended biomass, was centrifuged (8000 rpm, 15°C, 1 h). The supernatant was transferred to another preweighed centrifuge tube (W_5) and the collected biomass remained in the centrifuge tube. Water was added to the supernatant to precipitate the dissolved nitrocellulose and the suspension was centrifuged (8000 rpm, 15°C, 1.5 h). The supernatant was transferred to a flask, and the collected nitrocellulose remained in the centrifuge tube. Acetone was added to the collected biomass with vigorous stirring to let all unutilized nitrocellulose dissolve, and the suspension was centrifuged (8000 rpm, 15°C, 1 h). The supernatant was then transferred to the centrifuge tube containing the collected nitrocellulose and water was added to further precipitate the nitrocellulose. The suspension was centrifuged (8000 rpm, 15°C, 1.5 h). A few drops of water were added to the supernatant to make sure that no more nitrocellulose precipitated, and the supernatant was then combined with the other supernatant solution mentioned above. If there was some precipitate formed, then more water was added and the suspension was centrifuged once again. Finally, the collected nitrocellulose with the centrifuge tube was dried at 50°C for 3 h and cooled to room temperature (22°C) in a desiccator containing Drierite. It was weighed until a constant

weight (W_6) was attained. The collected biomass with the centrifuge tube was dried in the same way and was weighed until a constant weight was attained (W_7). The weight of the collected nitrocellulose and biomass was determined as follows:

$$\text{Weight of biomass} = \frac{(W_2 - W_1)(W_7 - W_3)}{W_4 - W_3}$$

$$\text{Weight of nitrocellulose} = \frac{(W_2 - W_1)(W_6 - W_5)}{W_4 - W_3}$$

Correction Factors for the Determination of the Weight of Biomass and Nitrocellulose

To establish a correction factor for the determination of the weight of the biomass, specific amounts of each fungal biomass (20, 50, 100, and 200 mg) were extracted with acetone (10 mL) and the suspension was centrifuged (8000 rpm, 15°C, 1 h). The supernatant was decanted and saved for determination of a correction factor for nitrocellulose. The residual biomass remaining in the centrifuge tube was again extracted with acetone (5 mL). The suspension was centrifuged (8000 rpm, 15°C, 1 h) and the supernatant was combined with the corresponding first extract (total 15 mL). The collected biomass was dried at 50°C for 3 h and cooled at room temperature (22°C) in a desiccator containing Drierite until a constant weight was attained. Based on the results, standard curves were plotted from which the biomass correction factors were determined.

Acetone extracts described above (15 mL) were mixed with 7.0 mL of a nitrocellulose acetone solution (1.15 g of nitrocellulose in 50 mL of acetone). Water (5 mL) was added to each of above mixtures to precipitate nitrocellulose. The suspension was centrifuged (8000 rpm, 15°C, 1.5 h) and the supernatant was discarded. Collected nitrocellulose was dried at 50°C for 3 h and cooled at room temperature (22°C) in a desiccator containing Drierite until a constant weight was attained. These results were used to determine the amount of biomass coprecipitated with nitrocellulose and also used to determine the nitrocellulose correction factors.

RESULTS AND DISCUSSION

Ten mycelial fungi were selected based on their known cellulolytic activity and investigated for their ability to degrade nitrocellulose. Nitrocellulose (0.3%) was tested as a sole nitrogen source along with starch (0.1%) as a cosubstrate. The cultures were grown for 3 and 7 days at 28°C and the residual nitrocellulose contents were determined. More than 20% nitrocellulose degradation was observed with *B. adusta*, *A. persicinum*, *S. rolfsii*, and *F. solani* in 3 days (Table I). No further increase in nitrocellulose degradation was measured after the 3-day growth period. At least 7 days of incubation was required with Basidiomycetes sp., *I. lacteus*, *T. pseudokoningii*, and *T. versicolor* to achieve ~ 20% nitrocellulose degradation. *C. stercoreus* degraded only 8% of nitrocellulose in a 7-day growth period. Many of these fungi, particularly, Basidiomycetes sp., *I. lacteus*, *T. pseudokoningii*, *T. versicolor*, *B. adusta*, and *A. persicinum* have not been previously reported to degrade nitrocellulose.

Examination of the biomass weights of *B. adusta*, *C. stercoreus*, *A. persicinum*, *T. versicolor*, *S. rolfsii*, and *F. solani* indicated an increase after 3 days (Table I), suggesting that these cultures utilized nitrocellulose for growth. However, after 7 days of growth, a decrease in the biomass weight was observed with some of the fungal cultures, apparently due to the autolysis of the fungal biomass. In our previous investigation, we measured a drop in pH in the growth medium as well as the lysis of biomass when a combined culture containing *F. solani* and *S. rolfsii* was used.[12] We hypothesized that the acidic pH resulting from the fungal growth might have caused the lysis of the biomass. However, in the present investigation, we observed that except in the medium containing *S. rolfsii* the drop in pH was not very significant. Even though there was a drop in pH in the medium containing *S. rolfsii*, the lysis of the biomass was not apparant (Table I). Hence, it appears that the lysis of the biomass in these cultures occurred due to other factors.

Autolysis of the biomass has been reported to occur in fungi due to the accumulation of toxic products and/or due to the expression of the enzymatic machinery that cause autolysis.[13-16] No direct correlation between the fungal biomass weight and the amount of nitrocellulose degradation was observed with Basidiomycetes sp., *I. lacteus*, *T. pseudokoningii*, and *P. chrysosporium*. It appears that, in these cultures, the autolysis of the fungal biomass started to occur before the 3-day growth period. Lackner et al.[17] suggested a correlation between autolysis and the release of enzymes from the fungal hypha. It is possible that the enzymes, secreted by these fungi at the early stages of growth, might have been released from the fungal hypha. When present in the growth medium, they may have contributed to the degradation of nitrocellulose. Apparently, the degradation products of nitrocellulose inhibited the growth of Basidiomycetes sp. and *I. lacteus*. On the

other hand, it appears that the degradation products marginally supported the growth of *P. chrysosporium* and *T. pseudokoningii* in a 7-day growth period (Table I).

It was observed that the biomass produced during the fungal growth was trapped in the nitrocellulose fibers. Hence, it was necessary to separate the biomass from nitrocellulose to determine the individual amounts. In our laboratory, we established an analytical method to determine both components.[11] The method involves acetone extraction of the nitrocellulose–biomass mixture. As it is known that some of the components of the biomass such as proteins, carbohydrates, and lipids are also soluble in acetone, it was necessary to establish a correction factor in order to determine the correct weight of biomass. Since the amount of carbohydrates, proteins, lipids, and the other acetone soluble materials in the biomass varies from species to species,[14] it was necessary to establish correction factors for all the tested fungal cultures. In the present study, we observed that the correction factors for the acetone extractions varied from 0.4 to 5.1%.

Brodman and co-workers observed that amino acids and peptides can interact with nitrocellulose.[18] Since the proteins and peptides can remain bound to the nitrocellulose,[19–21] it was necessary to establish a correction factor for the nitrocellulose estimation. A known amount of nitrocellulose in acetone was added to the biomass acetone extracts and subjected to the same procedure used in the analytical determination. The increase in the nitrocellulose weight was determined, from which a correction factor was established. As the amount of acetone-soluble materials decreased (less than 2.5%) in *A. persicinum*, Basidiomycetes sp., *C. stercoreus*, *I. lacteus*, *P. chrysosporium*, and *S. rolfsii*, the correction factor for nitrocellulose estimation was not determined. The correction factor for the quantity of biomass extract bound to nitrocellulose in the other cases varied from 1.5 to 3.1% and, thus, appropriate corrections were applied to the nitrocellulose weight data. In our previous investigation, we observed a 10% nitrocellulose correction factor when the combined *F. solani* and *S. rolfsii* cultures were used.[12] However, in the present investigation, when *F. solani* and *S. rolfsii* were cultivated individually, the nitrocellulose correction factors were found to be 0 and 1.1%. The difference in growth metabolism may have contributed to this variation. Since there is a large variation in the acetone extraction for different cultures, it becomes necessary to establish nitrocellulose correction factors whenever the acetone extraction determination is used.

Table I Screening of Mycelial Fungi for Nitrocellulose Utilization in Liquid Medium Containing a Cosubstrate[a]

Fungus	Biomass (mg Dry Weight)			Residual Nitrocellulose (mg)		Nitrocellulose Removed (%)	
	0 Days	3 Days	7 Days	3 Days	7 Days	3 Days	7 Days
Control[b]	0	0	0	144.6	143.4	3.6	4.4
Acremonium persicinum ATCC 60921	26.0	58.4	57.4	118.4	134.8	21.1	10.1
Basidiomycetes sp. NRRL 6464	49.8	40.1	33.9	140.5	125.9	6.3	16.1
Bjerkandera adusta ESF 620	27.3	64.9	53.2	112.8	125.3	24.8	17.7
Cyathus stercoreus NRRL 6473	40.6	57.8	58.5	142.5	138.2	5.0	7.9
Irpex lacteus Z 212	23.1	22.5	14.3	136.4	113.6	9.1	24.3
Phanerochaete chrysosporium ATCC 32629	48.7	43.0	48.2	132.1	126.8	11.9	15.5
Trametes versicolor ESF 491	41.7	46.0	47.9	128.2	118.2	14.5	21.2
Trichoderma pseudokoningii FTK 228	33.6	33.2	38.0	124.3	120.8	17.1	19.5
Sclerotium rolfsii ATCC 24459	30.8	83.5	88.4	118.0	120.8	21.3	19.4
Fusarium solani IFO 31093	26.4	77.5	55.0	114.6	114.6	23.6	23.6

Starting concentration of nitrocellulose, 150 mg. Starting pH of the growth medium 6.0; final pH 5.5 except for the growth medium containing *S. rolfsii* (pH 2.0).

[a] Starch (0.1%) was used as a cosubstrate.

[b] Values given are average of four replicates.

Recently, Gallo et al.[9] reported that *P. chrysosporium* does not have the potential to degrade nitrocellulose. In the present investigation, we observed about 20% nitrocellulose degradation by *P. chrysosporium* ATCC 32629. The *P. chrysosporium* strain used in the present study may have been different from the strain used by Gallo et al.[9] Although 10 fungal cultures were screened in the present study, only 8–24% nitrocellulose degradation was achieved. Factors responsible for the limited nitrocellulose utilization may include (i) the composition of the medium, (ii) cultural conditions, (iii) accumulation of products inhibiting the key enzymes involved in the degradation, and (iv) the presence of the functional groups in an inaccessible steric environment.

In our previous investigation, by using a combined culture containing a denitrifying and a cellulolytic fungus, we obtained 28% degradation with a drop in pH of the growth medium.[12] We hypothesized that the acidic pH resulting from the fungal growth caused the inhibition of certain key enzymes involved in the degradation and thus inhibited the degradation beyond 28%. One can also presume that the acidic pH resulting from the fungal growth might have caused the hydrolysis of nitrocellulose and the resulting end products were utilized by the growing fungal cultures. However, we were able to improve the degradation to 38% by buffering the medium at pH 6.0.[22] Also, in the present investigation, we have not observed a drastic drop in pH of the growth media except that containing *S. rolfsii*. These observations indicate that the fungal cultures directly degraded nitrocellulose and that other factors apart from pH may have been responsible for the limited nitrocellulose utilization.

The authors are thankful to Dr. Peterson, National Center for Agricultural Utilization Research, Peoria, IL; Dr. Worrall, SUNY - College of Environmental Science and Forestry, Syracuse, NY, and Dr. Johnson, Western Forest Products Laboratory, Vancouver, Canada, for providing some of the fungal cultures used in the present study.

REFERENCES

1. M. Johnson, *Technical Report DAAAD09-87-D-0008,* U.S. Army Armament, Munitions and Chemical Command, Bangbox Test Series, Rock Island, IL, 1992, Vol. 2.
2. J. Quinchon and J. Tranchant, in *Propellants, Explosives and Other Industries,* Wiley, West Sussex, U.K., 1989.
3. T. M. Wendt and A. M. Kaplan, *J. Water Poll Control Fed.,* **48,** 660–668 (1976).
4. K. Gold and B. W. Brodman, U.S. Pat. 4,814,439 (1989).
5. H. N. Hsieh and F. J. Tai, in *Proceedings on Nitrocellulose Fines, Separation and Treatment,* J. E. Alleman, Ed., U.S. Army–Purdue University, West Lafayette, IN, 1993, pp. 110–121.
6. D. L. Kaplan, P. A. Railey, J. Pierce, and A. M. Kaplan, *Int. Biodeterior.,* **23,** 233–248 (1987).
7. P. A. Railey, D. L. Kaplan, and A. M. Kaplan, *Technical Report 85-004,* U.S. Army Natick Research, Development and Engineering Center, Natick, MA, 1984.
8. B. W. Brodman and M. P. Devine, *J. Appl. Polym. Sci.,* **26,** 997–1000 (1981).
9. B. Gallo, A. Allen, R. L. Bagalawis, C. Woodbury, A. Yang, P. Yang, and D. Kaplan, in *Proceedings on Nitrocellulose Fines, Separation and Treatment,* J. E. Alleman, Ed., U.S. Army–Purdue University, West Lafayette, IN, 1993, pp. 78–91.
10. O. N. IL'Inskaya and I. B. Leshchinskaya, *Biotekhnologiya,* **4,** 495–500 (1988).
11. A. Sharma, S. T. Sundaram, Y. Z. Zhang, and B. W. Brodman, *J. Appl. Polym. Sci.,* **55,** 1847–1854 (1995).
12. A. Sharma, S. T. Sundram, Y. Z. Zhang, and B. W. Brodman, *J. Indust. Microbiol.,* **15,** 1 (1995).
13. R. Watanabe and N. Ogasawara, *Agric. Biol. Chem.* **54,** 2–6 (1990).
14. D. H. Griffin, *Fungal Physiology,* Wiley, New York, 1981.
15. F. Reyes, P. Villanueva, and C. Alfonso, *FEMS Microbiol. Lett.,* **69,** 67–71 (1990).
16. F. Reyes, P. Villanueva, and C. Alfonso, *Lett. Appl. Microbiol.,* **10,** 175–180 (1990).
17. R. Lackner, E. Srebotnik, and K. Meessner, *Can. J. Micobiol.,* **37,** 665–668 (1991).
18. B. W. Brodman, S. Schwartz, and M. P. Devine, *J. Macromol. Sci. Chem. A,* **14,** 1139–1143 (1980).
19. H. Towbin, T. Staehelin, and J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.,* **76,** 4350–4355 (1979).
20. Millipore publication number TB001, Millipore Corp., Bedford, MA, 1983.
21. L. F. Luescher, *Electrophoresis,* **8,** 508–514 (1987).
22. A. Sharma, S. T. Sundaram, Y. Z. Zhang, and B. W. Brodman, *Proceedings of the 3rd International Symposium on In Situ and On-Site Bioreclamation,* San Diego, CA, to appear.

Received March 17, 1995
Accepted May 26, 1995