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Nitrocellulose degradation by a coculture of *Sclerotium rolfsii* and *Fusarium solani*

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Aerobic degradation of nitrocellulose in submerged cultivation (31% in a 3-day period) was accomplished by a combination of *Sclerotium rolfsii* ATCC 24459 and *Fusarium solani* IFO 31093. Evidence for the degradation includes a decrease in the nitrocellulose weight, an increase in the biomass weight, and reduction of the pH to 2.0. The extent of nitrocellulose biodegradation was probably limited by the low pH produced when the buffering capacity of the culture medium was exhausted.

Keywords: nitrate ester; nitrocellulose; propellant; biodegradation; *Sclerotium rolfsii*; *Fusarium solani*

Introduction

The nitrate ester of cellulose, also known as nitrocellulose and cellulose nitrate, is the most commonly used energetic ingredient in gun propellants. Nitrocellulose nitrogen content can vary over a wide range with a maximum nitrogen content of 14.15%. Typically the nitrogen content of the nitrocellulose used in gun propellant compositions is 13.1–13.2%. Nitrocellulose has a limited shelf life because of an autocatalytic decomposition reaction which can occur over long periods of time while in ambient storage. This reaction becomes dangerous when the diphenylamine (stabilizer) present in the propellant is depleted. Each year large quantities of scrap propellant must be disposed of by the current methods of open air burning and incineration. These methods lead to the production of hazardous waste. For this reason it would be appropriate to develop a microbial method that could result in mineralization of this material. Early investigations of the biodegradation of nitrocellulose led to the conclusion that nitrocellulose was not directly attacked by microorganisms, but rather degraded by acidic metabolites produced during their growth [12,14,16].

A number of chemical processes utilizing inorganic sulfides and hydrides have been investigated for the degradation of nitrocellulose. Gold and Brodman [7] reported a method for nitrocellulose degradation which uses organic sulfhydryl compounds to release nitrogen from nitrocellulose in the form of inorganic nitrite ions. Nitrocellulose is decomposed by acid treatment and more readily by alkaline treatment. Wendt and Kaplan [19] reported that the solution resulting from alkaline hydrolysis could be treated efficiently by a combination of anaerobic and aerobic activated sludge processes. Hsieh and Tai [9] showed that 99% of nitrocellulose could be converted to sugars by a single-stage acid hydrolysis at 70° C for 45–60 min. Earlier work carried out on microbial degradation of nitrocellulose has produced conflicting results [2,11–14].

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Aerobic microbial degradation of nitrocellulose was investigated in the present work. A combination of cellulolytic (*Sclerotium rolfsii* ATCC 24459) and denitrifying (*Fusarium solani* IFO 31093) fungi was able to grow and significantly degrade nitrocellulose. To our knowledge, nitrocellulose degradation by the combined cellulolytic and denitrifying fungi under aerobic conditions has never been reported.

Materials and methods

Organisms

Sclerotium rolfsii ATCC 24459, a cellulolytic fungus [1], was purchased from the American Type Culture Collection, Rockville, MD, USA. *Fusarium solani* IFO 31093, a denitrifying fungus [17], was purchased from the Institute for Fermentation, Osaka, Japan.

Chemicals

Nitrocellulose (smokeless grade) was received as a gift from Hercules, Inc, Kenvil, NJ, USA. It contained 13.17% nitrogen and 25.07% moisture. On average, there were 2.3 nitrate ester groups per carbohydrate unit.

Growth medium

The mineral salts medium used for the study contained (g L^{-1}): KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; NaCl, 0.1; $CaCl_2$, 0.1 and trace metal solution, 100 μl. Trace metal salts solution contained (w/v%): 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.

Nitrocellulose sterilization

Steam sterilization (15 psi, 121° C, 30 min) of nitrocellulose released a significant quantity of free nitrite ions indicating decomposition of nitrocellulose. Hence, treatment with ultraviolet light (254 nm) was tested as an alternative for nitrocellulose sterilization. Comparison of the control and the UV-irradiated nitrocellulose revealed that UV treatment did not release free nitrite ions. Nitrocellulose was

sterilized for 45 min using UV light and then added to the cooled steam-sterilized medium.

Inoculum preparation

Mycelial mats were grown in mineral salts medium containing $NaNO_3$ (0.1%) as a nitrogen source and xylan (0.1%) as a carbon source. Cultures contained 50 ml of medium in a 250-ml Erlenmeyer flask. Fungi were grown at 28° C in a gyrotary shaker (~150 rpm) for 4 days and harvested by centrifugation (3014 × *g*, 4° C, 10 min). The fungal mycelia were thoroughly washed under aseptic conditions with cooled basal salts medium lacking any carbon and nitrogen sources. The washed fungal mycelia were then homogenized using a Virtishear explosion proof pilot homogenizer (The Virtis Company, Inc, Gardiner, NY, USA) operated at half of the maximum output for 2 min at 4° C with 15-s interruptions.

Cultural conditions

A known volume of homogenized mycelia (~7 mg dry wt) was transferred to the experimental medium (50 ml in a 250-ml Erlenmeyer flask) containing nitrocellulose (0.3%) as a nitrogen source and starch (0.1%) as a co-substrate. The culture was then incubated in a gyrotary shaker (~150 rpm, 28° C) for 0, 3 and 28 days.

Three types of control were run in parallel to the treatment, one lacking nitrocellulose or other nitrogen source but containing the cultures, basal salts medium, and starch; a second control containing NaN_3 (1 mM) and $HgCl_2$ (1 mM) along with the cultures; the third control contained nitrocellulose but lacked cultures and fungicidal agents.

Determination of biomass and unutilized nitrocellulose

Medium containing nitrocellulose and fungi was filtered through a preweighed filter paper (VWR Scientific, Westchester, PA, USA), 5.5 cm, (W1) and the filter cake was washed thoroughly with distilled water, dried at 50° C for 3 h and cooled to room temperature (22° C) in a desiccator containing Drierite (WA Hammond Drierite Company, Xenia, OH, USA). The filter paper with filter cake was dried and weighed until constant weight was attained (W2). The difference (W2 − W1) corresponded to the weight of biomass and residual nitrocellulose in the culture. The dried filter cake was then transferred to a preweighed centrifuge tube (W3). The difference between the weight of the centrifuge plus filter cake (W4) and the weight of the empty tube (W3), was the weight of the filter cake which was subjected to further analysis.

Nitrocellulose in the filter cake was dissolved in acetone. Acetone was added gradually with vigorous stirring until almost all nitrocellulose in the filter cake dissolved. The residual biomass was collected by centrifugation (7716 × *g*, 15° C, 1 h), washed in acetone a second time, centrifuged, and dried to constant weight (W5).

The supernatant acetone was transferred to another preweighed centrifuge tube (W6). Water was added to the supernatant fluid obtained from biomass centrifugation to precipitate nitrocellulose. Residual nitrocellulose in the centrifuge tube was dried at 50° C for 3 h and cooled in a desiccator containing Drierite. Nitrocellulose samples were dried and weighed until a constant weight (W7) was attained.

Residual nitrocellulose and biomass were determined as follows:

$$\text{Weight of Biomass} = \frac{(W2 - W1)\,(W5 - W3)}{W4 - W3}$$

$$\text{Weight of Nitrocellulose} = \frac{(W2 - W1)\,(W7 - W6)}{W4 - W3}$$

Correction factors for biomass and nitrocellulose determinations

In order to establish a correction factor for the fraction of biomass that was extracted by acetone, certain amounts of biomass (20, 50, 100, 200 and 300 mg) were extracted with 10 ml of acetone and the suspension was centrifuged (7716 × *g*, 15° C, 1 h). The supernatant fluid was decanted and saved for determination of a correction factor for nitrocellulose. Residual biomass remaining in the centrifuge tube was again extracted with acetone (5 ml). The suspension was centrifuged as described above and the supernatant fluid was combined with the corresponding first extracts (total volume 15 ml). The collected biomass was dried at 50° C for 3 h and cooled to room temperature (22° C) in a desiccator containing Drierite until a constant weight was attained. A standard curve comparing extracted biomass to original biomass was plotted from which a correction factor was determined.

Some of the extracted biomass reprecipitated with nitrocellulose when water was added to the supernatant phase. To correct for biomass reprecipitated with the nitrocellulose, the combined acetone extracts were mixed with 5.0 ml of a solution of nitrocellulose in acetone (500 mg of nitrocellulose in 35 ml acetone). Water (5 ml) was added to each of these mixtures to precipitate nitrocellulose. The suspension was centrifuged (7716 × *g*, 15° C, 1.5 h) and the supernatant fluid was discarded. Collected nitrocellulose was dried at 50° C for 3 h and cooled to room temperature in a desiccator containing Drierite until a constant weight was attained. The result was used to determine the amount of biomass co-precipitated with nitrocellulose and a correction factor was established.

Results

S. rolfsii ATCC 24459 secretes cellulose-degrading enzymes while *F. solani* IFO 31093 is a denitrifying fungus. Results of the time course of nitrocellulose degradation by *S. rolfsii* ATCC 24459 and *F. solani* IFO 31093, measured as the decrease in the weight of nitrocellulose are given in Table 1. More than 30% of the nitrocellulose was utilized by the growing fungi in a 3-day period. An increasing trend of fungal biomass was apparent until day 3 and then declined, possibly due to a drop in pH. The initial pH of the cultures was 6.0. After 7 days of incubation, the pH fell to 2.0 in the culture containing nitrocellulose and starch without biocide. On a dry weight basis, more than a 3-fold increase in mycelial weight was detected in 3 days.

Table 1 Nitrocellulose utilization and biomass production by the combined cultures of *S. rolfsii* ATCC 24459 and *F. solani* IFO 31093 in liquid medium

Cultivation time (days)	Residual nitrocellulose (mg)		Biomass dry weight (mg)	
	Uncorrected	Corrected	Uncorrected	Corrected
0	150.0	150.0	6.9	6.9
3	115.2	103.7	16.9	21.1
28	108.7	97.8	10.6	13.6

Abiotic control on a 28-day incubation under identical conditions resulted in a 2.5% nitrocellulose loss. The biomass dry weight (28 days) in the control lacking nitrocellulose or any other nitrogen source but having basal salts medium and starch was 6.9 mg

The acetone extraction procedure, used for nitrocellulose estimation, resulted in the solubilization of fungal biomass. Our results indicated 25% of the biomass was extracted along with nitrocellulose, and that 10% of the acetone-soluble fungal biomass co-precipitated with water.

Attempts at *in vitro* degradation of nitrocellulose by commercially available cellulase alone or in combinations with homogenate of *F. solani* IFO 31093 were unsuccessful.

Discussion

Much attention has been given to white rot fungi which attack compounds which are difficult to degrade [5,8,15]. Results obtained in this study indicate that partial degradation of nitrocellulose could be accomplished by using the combined white rot fungal cultures of *S. rolfsii* ATCC 24459 and *F. solani* IFO 31093.

Brodman and Devine [3] showed that *Aspergillus fumigatus* could utilize nitrocellulose containing 11.11% nitrogen. They also indicated that the organism did not utilize the nitrogen directly from nitrocellulose but rather relied on a hydrolysis reaction for a source of nitrogen.

Kaplan and co-workers showed that nitrocellulose was not subjected to direct microbial attack. Chemical pre-treatment of nitrocellulose was necessary to generate a modified denitrated polymer that could be attacked by microorganisms [13,16,18–20].

Aspergillus fumigatus F-316 formed reducing sugars from nitrocellulose with a nitrogen content of 11.9% and the fungus also utilized the nitrate ester group when subjected to nitrogen deficiency [11]. It was suggested that deesterification of cellulose nitrate esters might be achieved using immobilized *A. fumigatus* and *Pseudomonas fluorescens*. IL'Inskaya *et al* [10] did not observe any nitrocellulose degradation when six immobilized *Pseudomonas* spp and anaerobic cellulolytic bacteria were tested.

Lack of suitable analytical methods has hampered research on nitrocellulose biodegradation. Gallo *et al* [6] employed a method, based on dry weight, that involved separation of nitrocellulose from the fungal biomass by dissolving the nitrocellulose in acetone. The acetone extract was allowed to evaporate and the residual weight was determined. In the present investigation, we also used an acetone extraction procedure. In our case, 25% of the solids from the biomass were extracted along with the nitrocellulose. Therefore, a correction factor was applied in the biomass and nitrocellulose determinations.

Brodman *et al* [4] used X-ray photon spectroscopy to demonstrate that nitrocellulose interacts with peptides and amino acids. In the present investigation, 10% of the acetone-soluble fungal biomass co-precipitated with nitrocellulose, adding to the nitrocellulose weight, so corrections were applied to the recovered nitrocellulose weight. After applying the correction factor for nitrocellulose estimation to the data obtained by Gallo *et al* [6], we conclude that the authors may have accomplished 2.5–13% nitrocellulose degradation by *Phanerochaete chrysosporium* and *Aspergillus fumigatus*.

In the present investigation, only 35% of the nitrocellulose was degraded by the combined fungal cultures. During the growth of *S. rolfsii* ATCC 24459 and *F. solani* IFO 31093 on nitrocellulose, we noted a drastic drop in pH (6.0 to 2.0) and at such an acidic pH, the cellulose-degrading enzymes are probably inactivated. Studies are under way in our laboratory to enhance nitrocellulose degradation using combined cultures of *S. rolfsii* ATCC 24459 and *F. solani* IFO 31093 by manipulating cultural and media conditions.

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