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Degradation of nitrocellulose by fungi

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Abstract

Three lignocellulolytic fungi, *Trametes versicolor*, *Pleurotus ostreatus*, and *Coprinus cinereus,* and two cellulolytic fungi *Trichoderma reesei* and *Chaetomium elatum* were tested for their ability to degrade nitrocellulose. They were provided with different carbon and nitrogen sources in liquid cultures. Nitrocellulose (N content above 12%) was added as nitrogen source (in solution in acetone) alongside amino acids or as sole N source. Either starch or carboxy-methyl cellulose were provided as carbon sources. After 28 days of growth the highest decrease of nitrocellulose was observed with *Chaetomium elatum* when up to 43% was degraded in a medium containing nitrocellulose as the only nitrogen source. *Coprinus cinereus* caused a 37% decrease of nitrocellulose when provided with amino acids and starch as co-substrate. In cultures of *Trametes versicolor, Pleurotus ostreatus* and *Trichoderma reesei,* only 10%–22% decrease of nitrocellulose was measured in all media. In the presence of nitrocellulose with N content below 12% supplied as 3 mm pellets as the only carbon source, or with nitrocellulose with carboxy-methyl cellulose, the release of nitrite and nitrate from liquid cultures of *Chaetomium elatum* was measured. Between 6 and 9 days of growth in these media, an increase in both nitrite and nitrate was observed with a loss in weight of nitrocellulose up to 6% achieved after 34 days. The physical nature of the NC pellets may have reduced the rate of degradation in comparison with supplying NC in solution in the cultures.

Abbreviations: *C. cinereus* – *Coprinus cinereus*; *Ch. elatum* – *Chaetomium elatum*; DPA – diphenylamine; NC – nitrocellulose; *P. ostreatus* – *Pleurotus ostreatus*; *T. reesei* – *Trichoderma reesei*; *T. versicolor* – *Trametes versicolor*

Introduction

Nitrocellulose (NC), one of the most important and oldest cellulose derivatives with over a century of history, has a wide range of use both in the military and civilian sectors. NC is a major ingredient in gun propellants and has to be stabilised to prevent autocatalytic decomposition reactions (Makashir et al. 1995). Once the stabiliser is depleted the propellant becomes unstable and must be destroyed (Sharma et al. 1995). This scrap propellant coupled with waste from the manufacturing of NC causes widespread contamination especially with the annual world production capacity of NC of over 20,000 metric tons (Chang et al. 2000). Since NC is extremely flammable and highly reactive, the waste containing NC is classified as a non-toxic organic contaminant by the U.S. Environmental Protection Agency (EPA), which does not give any health advisory or water quality standard for NC. Thus its relative recalcitrance in the environment has led to concerns

regarding environmental fate and impact on human and environmental health. Although NC production has a long history, there have been only limited research efforts for NC waste treatment (O'Donnell et al. 2001; Sundaram et al. 1995). Currently, the disposal of gun propellants is carried out by open burning or detonation (OB/OD) (Sharma et al. 1995). This produces air borne particulates and pan residues containing toxic materials. For this reason OB/OD is becoming questionable and more and more countries prohibit its application. An alternative to OB/OD is incineration, which is very costly, dangerous and time consuming (Sundaram et al. 1995). Hence it would be appropriate to develop methods to degrade NC in an environmentally safer and more economical way.

In the middle of the last century it was documented (Siu et al. 1949) that cellulose derivatives, when substituted on even a small percentage of available hydroxyl groups, are not subject to microbial attack. Siu et al. (1949) reported that complete resistance to breakdown by microorganisms usually is obtained with only one substituent on each anhydroglucose unit and that the nature of the substituent itself is of secondary importance, if any. NC itself is a uniformly substituted cellulose compound though with varying degrees of nitration, and is believed to be rather resistant to microbial attack, with hardly any case reported where the biodegradability of NC was established beyond doubt (Pfeil 1999).

The aim of this study was to determine if cellulolytic fungi could degrade NC. During this work, it became necessary to develop a robust, defined analytical method for quantitative estimation of NC.

Materials and methods

Fungal strains

Chaetomium elatum Kunze ex Fries was isolated from straw spiked with NC with a N-content of 13.4% NC. *Coprinus cinereus* (Schaeff ex Fr.) Gray (ATCC 18065), *Trametes versicolor* (Fr.) Pil (FPRL 28A), *Pleurotus ostreatus* (Jacquin ex Fr.) Kummer (CMI 341687) and *Trichoderma reesei* E.G.Simmons (CMI 192656ii) were from the fungal culture collection of the University of Westminster, London, UK. All fungi were grown on potato-dextrose agar (PDA) (BDH) at 26 °C, and stored at 4 °C.

Growth medium

To examine the effect of different carbon sources on the degradation of NC, starch and carboxymethyl cellulose were used. For the screening experiment the defined media contained (the NC used contained 13.4% nitrogen) (g l^{-1}): *CMC/Starch medium*: CMC or starch 10.0; L-asparagine 2.5; D,L-phenylalanine 0.15; adenine 0.275; thiamine-HCl 5×10^{-5}; KH_2PO_4 1.0; Na_2HPO_4 $2H_2O$ 0.1; $MgSO_4$ 7 H_2O 0.5 and trace metal solution, 100μl. Trace metal salts solution contained (w/v%): $CaCl_2$ 1.0; $FeSO_4$ 1.0; $MnSO_4$ 0.1; $ZnSO_4$ 0.1; $CuSO_4$ 0.2. *NC medium with NC as only C source*: NC 10.0 (replacing CMC/starch), all other constituents as for CMC/starch medium. *NC medium with NC as only N source:* 10.0 NC and all other constituents as for CMC/starch medium except the organic N sources (asparagine and phenylalanine). *NC medium with NC as supplement*: NC 3.0, all other constituents as for CMC/starch medium. For the denitration experiment the defined media contained all constituents as for CMC/starch medium. NC was added with either 3 g/50 ml or 10 g/50 ml medium. The type of NC used for this experiment had a nitrogen content below 12% and was added as solid rectangular chips of approximately 3 mm length.

Potato dextrose agar (PDA)

Potato dextrose agar (BDH) made up according to the manufacturer's directions.

Malt extract agar (MEA)

Malt extract agar (Oxoid) made up according to the manufacturer's directions.

Chemicals

Two batches of nitrocellulose were obtained one with a nitrogen content of 13.4% dissolved in acetone (2%) and one containing 11.9% nitrogen and 30% moisture, supplied as rectangular chips of approximately 3 mm.

Nitrocellulose sterilisation

NC containing 13.4% nitrogen dissolved in acetone was precipitated in water, dried at 50 °C and stored in a desiccator. Both types of NC were sterilised for 45 min using UV light and then added to cooled steam-sterilised medium as powder or chips respectively.

Inoculum preparation and growth conditions

T. versicolor and *P. ostreatus* were grown on MEA agar, *Ch. elatum, C. cinereus* and *T. reesei* on PDA agar at 26 °C and 10 × 5 mm plugs taken from the growing margin of 7-day old cultures were added to 10 ml sterile distilled water containing 5 g glass beads. The bottles were shaken vigorously and 1 and 2 ml of this mycelial solution was used to inoculate 50 and 200 ml of medium (for denitration measurements), respectively. All cultures were incubated at 26 °C on a rotary shaker.

NC recovery from the liquid cultures

Media containing NC (13.4%) and fungi were centrifuged. The pellet (biomass and NC) was dried over night at 50 °C, 20 ml of acetone was then added to each pellet to dissolve the NC and shaken on ice for 1 h. After centrifuging at 10,500 g at 15 °C for 1 h, the supernatant was assayed for quantification of NC (weight 1, W1). Water (20 ml) was added to the supernatant from the first centrifugation step to precipitate residual NC, the same steps were then applied as described above and the NC quantified (weight 2, W2). W1 and W2 were combined to obtain the total amount of residual NC in the liquid cultures. NC containing 11.9% nitrogen, supplied as rectangular chips was recovered from the liquid cultures by filtering through cotton tissue and the residual biomass washed off with distilled water. The NC was then freeze-dried and the weight measured.

NC assay

Aliquots of 20 μl of the supernatants were added to 80 μl of 1% diphenylamine (Sigma Chem. Co) in acetone, followed by 1.4 ml of concentrated sulphuric acid (98% Aristar, BDH).The solutions were incubated statically for 1 h at room temperature (22 °C). Each mixture was thoroughly shaken after incubation until a homogeneous blue solution was observed. One ml of each mixture was transferred into plastic cuvettes and scanned in a Perkin Elmer V Spectrophotometer within the range of wavelength of 550–650 nm. The readings of the highest absorbance were recorded irrespective of the wavelength.

Nitrite determination

The concentration of NO_2^- was measured by the Griess Reaction (Granger et al. 1995). One ml of the liquid culture was centrifuged at 12,000 g for 30 min. To the supernatant, 1 ml of 0.1% sulfanilamide in 3M HCl and 1 ml of 0.01% (w/v) *N*-(1-naphthyl) ethylenediamine dihydrochloride solution were added, the mixture was incubated at room temperature for 30 min and its absorbance read at 534 nm.

Results and discussion

Quantitative analysis of NC

A method was devised to measure NC using its reaction with DPA. Products with different spectral characteristics formed during the reaction giving a shift in wavelengths with the highest absorbance, as the NC concentration increased (Figure 1). Addition of the reagents in the specified order was important to achieve consistent blue colours of the end product. When the highest absorbance irrespective of wavelength (between 550 and 650 nm) was recorded, the rate of reaction with increasing NC concentration was non-linear, which suggested that the reaction was a second order, not first order reaction. A polynomial regression for calibration gave a correlation coefficient above 0.99 within the range 5–30 μg of NC (Figure 2). This provided a reliable, simple assay for measurement of NC that could be carried out in a reasonable time period.

DPA is commonly used as a stabiliser for NC, reacting with NO_2^- that is liberated during aging of unstable NC propellants (Lindblom et al. 1995). The reactions taking place during degradation of NC are complex and, with DPA, unknown reaction steps occur. Lindblom et al. (1995) suggested a reaction scheme for degradation of DPA where the amine hydrogen is removed stepwise followed by

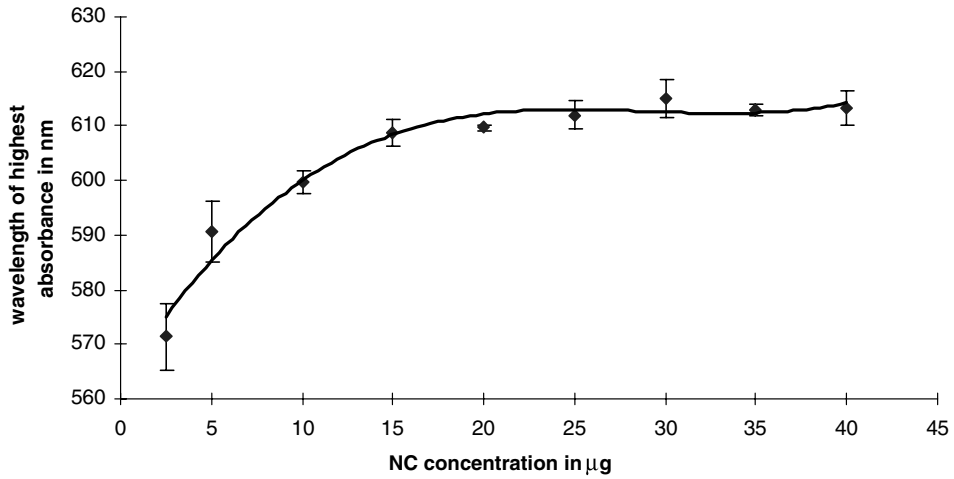

Figure 1. Shift of wavelengths with increasing nitrocellulose concentration. Data points are means with error bars representing standard deviations of the means, n = 4.

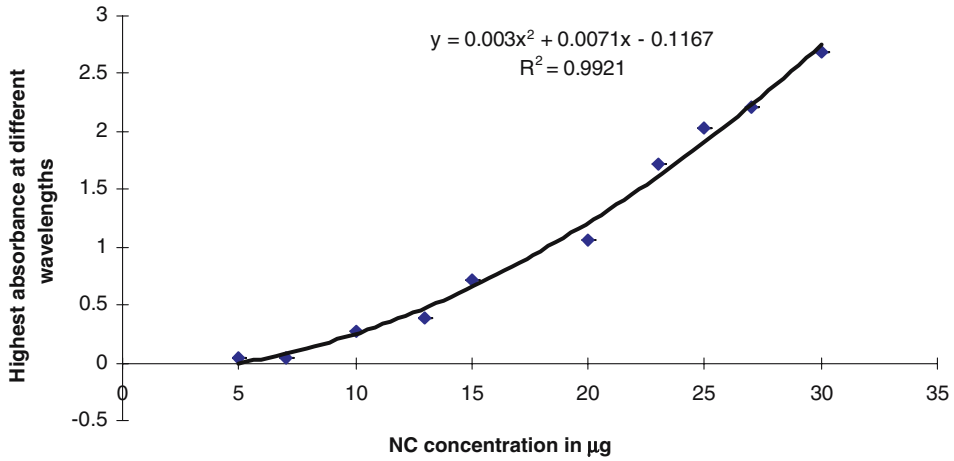

Figure 2. Second order rate reaction of nitrocellulose with diphenylamine (polynomial regression). Reaction conditions were 80 μl of 1% diphenylamine in acetone, added to 20 μl of NC test solution in acetone, then 1.4 ml of concentrated sulphuric acid was added and reaction mixtures incubated for 1 h at 20–22 °C in day-light.

reaction with NO. The intermediate product (N-nitrosodiphenylamine) undergoes a rearrangement and an oxidation to produce 4-nitrodiphenylamine and 2-nitrodiphenylamine, as shown in Figure 3. The suggested end products of reaction of DPA and NC in the presence of H_2SO_4, give a blue colour that can be measured spectrophotometrically. The previous problem of inaccurate and unreliable quantification using this reaction did not account for the multiple reaction products affecting the colouration.

Screening of fungi capable of degrading NC

The screening experiments were undertaken with NC containing 13.4% nitrogen. Residual NC in the fungal cultures was determined by the NC assay. Of the five fungi tested for a capability to degrade nitrocellulose, *Ch. elatum* gave the highest decrease when 43% NC disappeared from the medium where NC was the only nitrogen source along with CMC as carbon source. *C. cinereus* decreased the NC by 37% when NC was the only nitrogen source but

NO_3^- + DPA → $[HNO_3]$ → NO_x

Quinodium salts (blue)

$[HNO_3]$ → $R\text{-}CH_2\text{-}O\text{-}NO_2$ → $R\text{-}CH_2\text{-}O\text{-}$ + NO_2^-

$R\text{-}CH_2\text{-}O\text{-}NO_2$ → HNO_3

autocatalysis

NO_2^- + H_2O → HNO_3

NO_2^- + DPA → N-NO-DPA → 4-NDPA + 2-NDPA

Figure 3. A simplified model for the stabilization of nitrocellulose and the degradation of diphenylamine (DPA).

with starch as carbon source. Under the same conditions but with CMC as co-substrate no growth of *C. cinereus* occurred at all. *P. ostreatus* was the only other fungus tested in the medium where NC was supplied as the only N source, but no growth was observed under these conditions. Comparing the results from the media where NC was present along with either starch or CMC as carbon source and organic nitrogen sources, starch in general proved to be a better co-substrate for all fungi for the decrease of NC except for *Ch. elatum*. Figure 4 shows the reduction of NC in cultures containing CMC or starch as co-substrate.

Denitration of NC with Ch. elatum *and* C. cinereus

As *Ch. elatum* and *C. cinereus* showed the greatest ability to degrade NC of the fungi tested, further investigation of their denitration mechanism was made. The nitrogen content of the NC used in these experiments was below 12%. Two control cultures in which NC (3 g l^{-1} and 10 g l^{-1}) was incubated in water under the same conditions as the fungal cultures were used to assess the release of nitrite due to autocatalytic hydrolysis (dotted lines in Figure 5). For fungal control cultures, both fungi were grown in CMC medium and values obtained for nitrite concentrations in the medium were subtracted from the values measured in the medium containing NC for each time point. This was necessary to account for the amount of nitrite in the medium that was not derived from NC. An increase in nitrite in the medium when NC was supplied as a supplement to CMC and organic nitrogen, was observed in both fungal cultures between day 6 and 7 (relative to the amount of nitrite released from NC in water). No increase in nitrite was measured in the medium with NC as the only nitrogen source with CMC as a carbon source. When *C. cinereus* was supplied with 10 g l^{-1} NC as the only carbon source but with organic nitrogen, nitrite was released between day 10 and 15 of growth. After day 15, nitrite levels fell below those measured in the control (NC in wa-

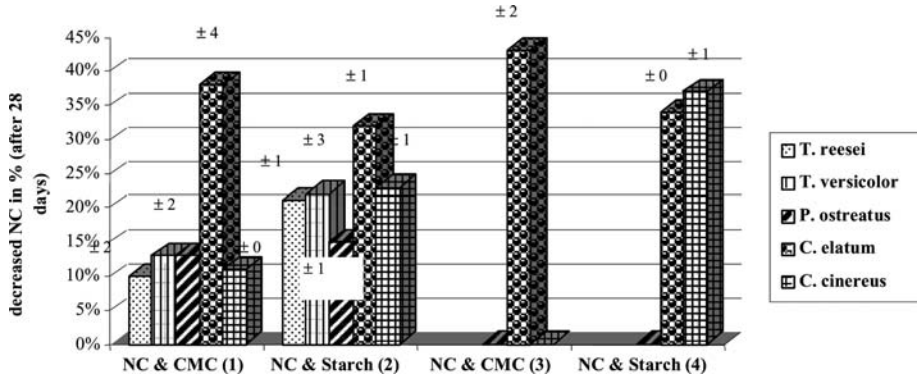

Figure 4. (1) + (2) NC and organic nitrogen sources supplied; (3) + (4) with NC as the only nitrogen source. Data points are means with figures representing standard deviations of the means (n = 3).

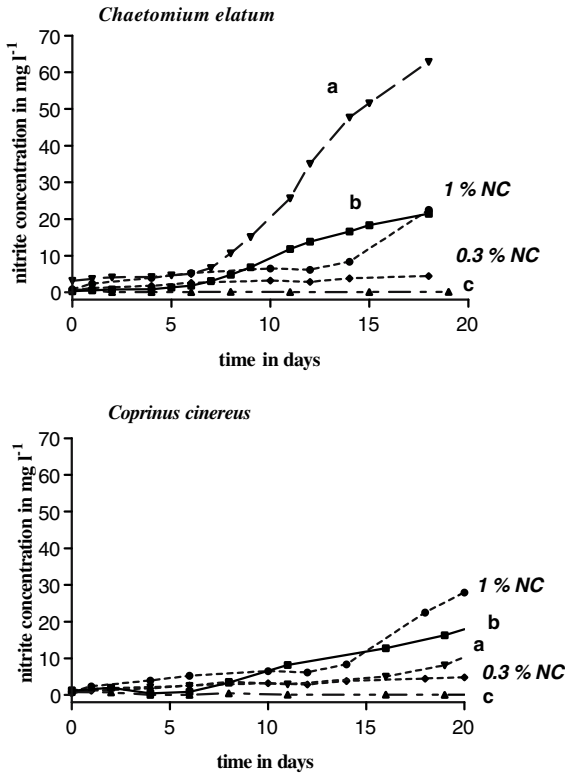

Figure 5. Denitration of NC with *Ch. elatum* and *C. cinereus* in liquid culture. NC with a nitrogen content of 11.9% was used in all cases: (a) 1% NC (2000 mg) and organic nitrogen, (b) 0.3% (600 mg) NC, CMC and organic nitrogen, (c) 0.3% (600 mg) NC as only nitrogen source and CMC.

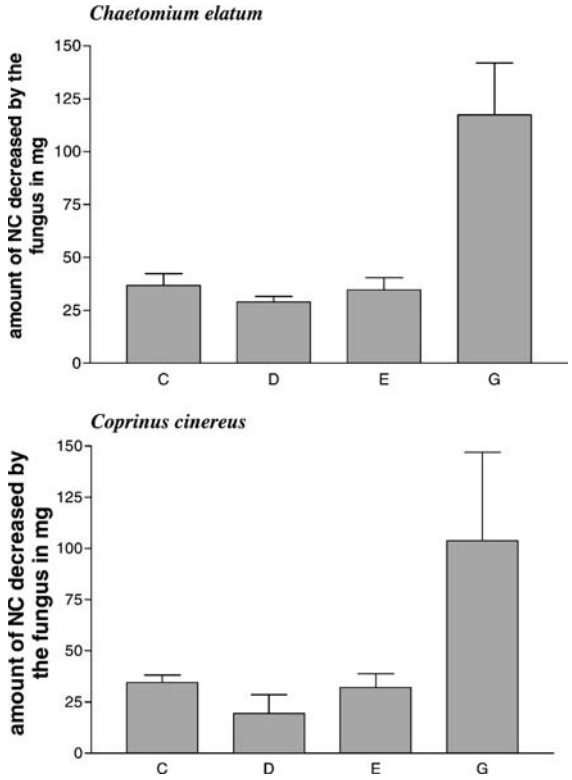

Figure 6. The amount of NC (N content below 12%) decreased by the fungi *Ch. elatum* and *C. cinereus* after a growth period of 34 days. The culture media were supplemented with two different amounts of NC; culture C, D and E contained a total of 600 mg, culture G contained 2000 mg initially (Error bars show the standard deviation (n = 6).

ter). The total amounts of NC decreased within 34 days of growth depended on the initial amount of NC added to the medium. An addition of 600 mg (3 g l^{-1}) NC was decreased by up to 35 mg (5.8% removal) whereas 2000 mg (10 g l^{-1}) was decreased by up to 103 mg (5.1%) (Figure 6).

Different outcomes were observed with *Ch. elatum* in the same medium. An increase in nitrite concentration was observed after 6 days of growth. After 20 days of growth, at the termination of the experiment, nitrite in the fungal culture was nearly 2.5 times the amount measured in the NC control culture (Figure 5). However the degradation rate of NC was similar to that by *C. cinereus*. After 34 days of growth *Ch. elatum* decreased an initial amount of NC of 600 mg in the medium by up to 36 mg (6%), when 2000 mg NC were supplied 117 mg was metabolised by this fungus (5.9%) (Figure 6).

Conclusion

Screening of fungi capable of transforming NC

The ability of the fungi to decrease NC in growth media over 28 days was dependant on the carbon and the nitrogen sources supplied. NC was removed from the culture medium by all tested fungi with starch as co-carbon substrate, and organic nitrogen added. Degradation of NC depending on a certain co-substrate has been reported by others (Duran et al. 1994; Sharma et al. 1995). The highest reduction in NC was found in cultures of *Chaetomium elatum* when CMC was supplied as carbon source, with and without organic nitrogen. *Coprinus cinereus* degraded NC when starch was the carbon source and no organic N supplied.

Denitration of NC with Ch. elatum and C. cinereus

NC undergoes autocatalytic hydrolysis when maintained in liquid medium. To account for the amount of nitrite in the control medium, lacking the culture, those data were subtracted from that obtained from the media containing NC. The white-rot fungus *C. cinereus* was able to hydrolyse more nitro groups from the NC polymer than were hydrolysed by autocatalytic hydrolysis when other organic nitrogen was present in the culture. This might indicate that 'active agents' required for hydrolysis, were produced by the fungus only when organic nitrogen additional to NC was supplied. Such agents might be enzymes, reactive oxygen species or other radicals. Nitrite released could be absorbed by the fungus and used for metabolism, including that arising from autocatalytic hydrolysis or by active hydrolysis by the fungus. This hypothesis was supported by measurements of nitrite in the NC control medium being higher than those in the fungal cultures. Comparing the amounts of residual NC measured in the liquid cultures showed that when the fungus was supplied with 3 g l^{-1} NC the decrease in NC was similar to the cultures containing 10 g l^{-1} (5.8% decrease and 5.1%). Similar observations were made with the soft-rot fungus *Ch. elatum*, when NC provided with CMC and organic nitrogen resulted in nitrite release. All nitrite released from the polymer in autocatalytic hydrolysis was utilised by the fungus when no other organic N was supplied, whether or not *Ch. elatum* actively hydrolysed the nitro groups under these conditions. Nevertheless when NC was the only carbon source a 2.5 fold increase in nitrite concentration was measured. The soft-rot fungus would have to hydrolyse the nitro groups to be able to attack the cellulose backbone for use as a carbon source. Amounts of NC decreased were similar to those measured in the cultures of *C. cinereus* when the initial 3 g l^{-1} and 10 g l^{-1} (in 200 ml) of NC were decreased by approximately 6%.

These experiments show that of the fungi studied, the most effective degrader of NC with a total nitrogen content of above 12%, when supplied with organic nitrogen and CMC in the culture medium was *Ch. elatum*. This fungus also had a high capability of denitration of NC (with a total nitrogen content below 12%). Such a mechanism may be a necessary initial step in NC degradation since it may render the substituted polymer more susceptible to microbial attack. The fact, that the fungus degraded more NC of the explosive grade (N content above 12%) in a shorter time period than non-explosive grade NC (N content below 12%) may be due to the physical state of the NC. The explosive-grade NC was added to the medium dissolved in acetone, thus displaying a much higher surface available for the fungal attack. The non-explosive grade NC, however, was added as rectangular chips of about 3 mm length, which means a much lower surface per weight ratio may have lead to the lower degradation rate. Also by adding solid material to a liquid culture, this results in a semi-solid culture and may have an influence on the fungal metabolism, with different enzymes or enzyme-patterns being excreted. The 'active agent' responsible for NC degradation could be an enzyme(s), reactive oxygen species or other radicals.

Ch. elatum is a natural coloniser of straw and if straw were mixed with NC this organism would be capable of continued growth and denitrify NC. Provision of organic nitrogen may be needed to provide sufficient readily available amino nitrogen for protein synthesis. *Ch. elatum* was the most effective of the fungi tested in denitrifying and removing NC from liquid cultures.

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