Journal of Environmental Sciences Vol.15, No.5, pp.577—582, 2003

ISSN 1001—0742
CN11—2629/X

Article ID: 1001-0742(2003)05-0577-06 **CLC number**: X7 **Document code**: A

Releasing nitrogen from ammoniated lignin by white rot fungus cometabolizes environmental pollutants

LIN Lu*[1], ZHAO De-qing[1], ZHOU Xian-tao[1], QIU Yu-gui[1], ZHANG Gan[2]

(1. State Key Laboratory of Pulping and Paper Engineering, Research Division of Plant Resource Chemistry, College of Paper and Environment Engineering, South China University of Technology, Guangzhou 510640, China. E-mail: lclulin@scut.edu.cn; 2. State Key Laboratory of Organic Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510641, China)

Abstract: The nitrogen-modified lignocelluloses(NML) produced under oxic ammoniation was metabolized by white rot fungus, NH_4^+-N was released, NO_3^--N concentration was decreased and total nitrogen loss was blocked within incubation period. During releasing nitrogen from the metabolism of NML, white rot fungus cometabolized recalcitrant environmental pollutants and showed higher degradation capability. Results indicated that this NML complex colonized by white rot fungus might be effective with economic feasibility when they are applied into the vast field ecosystem, it might stabilize NH_4^+ nitrogen flux and bioremediate the polluted environmental sites.

Keywords: white rot fungus; ammoniated lignin; cometabolism; pollutants

Introduction

Lignocelluloses is the most abundant biopolymers on the earth, decomposition of lignocelluloses and its derivatives completes the carbon cycling in the global environment(Béguin, 1996; Freeman, 2001). Lignocelluloses is in lack of nitrogen(C/N ratio > 100), however, nitrogen is closely related to its biotic metabolism(Mary, 1996; Fog, 1988), its intensity and duration in an ambient microcosm is limited by its C/N ratio(Berg, 1982). While lignocelluloses is degraded in the soil ecosystem, large amount of nitrogen is immobilized by degraders from soil nitrogen pool, causing pulsed fluctuation of nitrogen flux and retarding their availability to sustain growth of plants(Hauck, 1984; Raghubanshi, 1990), concern about nitrogen flux fluctuation which affects the functioning and stability of the field ecosystem has been actived in recent decades(Post, 1985). Intensive fertilization enhances internal nitrogen store into lignocelluloses, besides a rich harvest of biomass, during vegetation and raises external nitrogen concentration in degradation microcosm during decomposition (Corbeels, 1998; Tam, 1991). Contradictorily, many nitrogen-related problems have hence been generated in the global ecosystem(Convay, 1988), such as water contamination caused by NO_3^- leaching(Burt, 1990), eutrophication in waters(Tyrrell, 1999) and global warming from evolution of greenhouse gases N_2O and even CH_4 and ozone depleting gas NO to atmosphere(Mosier, 1991). Exploration is expected to find new path to increase nitrogen content in lignocelluloses and elucidate ecologic implications of its biotic metabolism process.

1 Materials and method

1.1 Ammoniation reaction and vigor measurement of the products

Ground air-dried bagasse kraft lignin reacted with ammonium under conditions as follows: ratio of lignin to water: 1:7 (w/w); oxygen pressure: 0.8, 1.0, 1.2, 1.4, 1.6 mPa; temperature: 100, 115, 130, 145 and 160℃; reaction time: 120 min. Amount of ammonium added was 2%, 2.5%, 3.0%, 3.5% and 4.0% calculated on the basis of effective N(w/w) separately for 100, 115, 130, 145 and 160℃. 25 batches of treatments were carried out with three replicates for each. The NML products from the above-mentioned conditions was mixed with quartz sand(1/1, w/w) as a media for peanut seeds germination and seedlings growth. Vigor index = weight of seedlings within culture time of 14 days (g) ×

Foundation item: The National Natural Science Foundation of China (No. 30170031); * Corresponding author

germination rate of peanut seeds(%). From the graph it was shown that when nitrogen content was more than 1.21%, NML obviously inhibited germination of peanut seeds and growth of peanut seedlings cultured for 14 days.

After reaction, the products NML was ball-grounded, extracted with dioxane/water(9/1, v/v), and further precipitated by ether(1/25, w/v), then freeze-dried and dried in vacuum oven for measurement of total nitrogen content.

1.2 Immobilization of nitrogen from NML and degradation of pollutants

WW-NML(NML not mixed with soil); UW-NiML(NML mixed with soil); T-NML(NML at 1.21% N, mixed with soil), WW-NML and UW-NML was inoculated with white rot fungus *Phanerochaete chrysosporium* ME446, T-NML was inoculated with cellulolytic fungus *Trichoderma viride*. All samples was air-dried before use. Concentration of NH_4^+-N and NO_3^--N of soil from paddy rice field applied in this experiment was 40.8 mg/kg and 38.3 mg/kg soil respectively. The total weight of incubation media was 10g. The ratio of inocula after pre-incubation to WW-NML or UW-NML and soil was 1∶10∶100. The incubation mixture was contained in a 200 ml Erlenmeyer flask and incubated in sterile greenhouse. Incubation time lasted for 60 days at 30℃.

In the experiment for detection of degradation of pollutants by white rot fungus during the process of their immobilization of nitrogen from NML, three treatments have been completed, they were WW-NML (NML after water-washing, with 0.83% total nitrogen and 42.25 C/N ratio), LHE(lignocelluloses without nitrogen-modification, internal nitrogen was 0.15%, 0.68% external nitrogen urea was amended) and LLE(lignocelluloses without nitrogen-modification or amendment of any external nitrogen). All the treatments were inoculated with white rot fungus *Phanerochaete chrysosporium* ME446. Weight of NML for incubation was 10 g. Twenty varieties of pollutants was exerted in the experiment, they were benzene, naphthalene, biphenyl, phenanthrene, anthracene, toluene, ethylbenzene, *o*-xylenes, *m*-xylenes, 1,3,5-trimethylbenzene, chlorobenzene, 1, 2, 3-trichlorobenzene, 1, 2, 3, 4-tetrachlorobenzene, hexachlorobenzene, *o*-chlorophenol, *m*-chlorophenol, 2,4- dichlorophenol, 2,7-dichlorodibenz-*p*-dioxin, pentachlorophenol, nitrobenzene, 3-nitroanilline, *p*-dinitro-benzene, *m*-dinitrobenzene, benzidine. Pollutants were added at incubation day 20. Extraction with chloroform at pH 2.0 and pH 12.0 was carried out at day 14 after addition of pollutants. Detection was conducted on HP 6890 gas chromatograph with FID and ECD detector. The products was identified either by comparison with an authentic sample or by GC-MS (Finnigan MAT (EI. 80 eV, ITD)).

2 Results and discussion

Earlier studies documented that technical lignin, partly degraded lignin fragments extracted from kraft paper mill liquor at economic cost, could react with ammonium, nitrogen was bound to lignin fragments (Capanema, 2001) as potential humic matter (Meire, 1994). In our experiment here, we utilized the kraft lignin of bagasse of sugarcane (*Saccharum officinarum* L.) to react with ammonium and produced a nitrogen-modified lignocelluloses (NML) complex. The increase of nitrogen content and decrease of C/N ratio of NML was consistent with the upgrade of temperature and pressures employed during reaction(Fig. 1a). However,

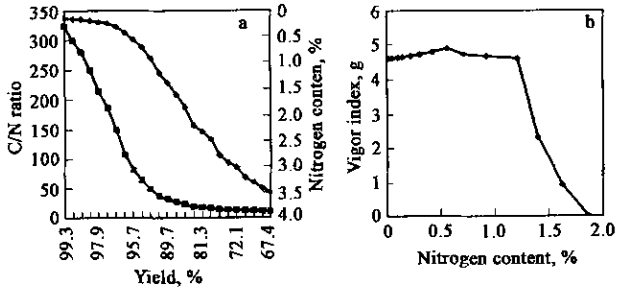

Fig. 1 Relationship between yield and nitrogen content of NML as well as effect of NML on vigor of peanut seeds

a: ◆—nitrogen content of NML; ■—C/N ratio; b: relationship between nitrogen content of NML and vigor index

when nitrogen content was more than 0.92%, the yield of NML dropped down sharply, the resultant products exhibited the effect of inhibition to seeds germination and seedlings growth of peanut (*Arachis hypogaea* L.) indicated by vigor index (Fig. 1b). Analysis of a NML sample at 1.21% N showed that organic and inorganic form was separately 0.83% and 0.38% (Fig. 2a). Inorganic nitrogen was NH_4^+-N, 95.24% of it was water-washable, 4.76% was water-unwashable. Among organic nitrogen, 4.10% was water-soluble, 92.42% was detected to be bound to lignin fraction, 3.48% to celluloses fractions (Fig.2b).

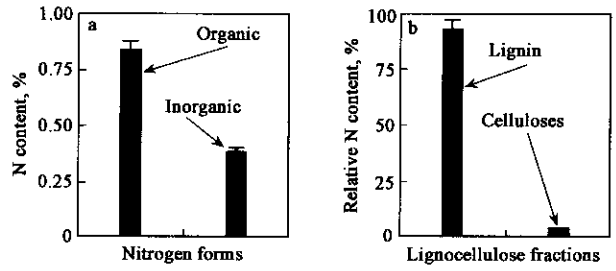

Fig.2 Content of organic and inorganic nitrogen in different fractions of NML

a: organic and inorganic nitrogen in NML. In this sample, ratio of organic form to inorganic form was 2.18. We found that inorganic nitrogen content increased more rapidly than organic nitrogen when temperature and pressure increased, the maximum organic nitrogen content of NML in our experiments was 1.32%, the maximum inorganic nitrogen content of NML was 2.19%; moreover, water-soluble organic nitrogen also increased rapidly at higher grades of oxygen pressure and temperature employed; b: relative nitrogen content in fractions of NML

In the field ecosystem, the dominating colonizers on the bulk of lignocelluloses in the soil or in the mineral litter layer of forest are not the degrader for all the fractions of lignocelluloses (Griffin, 1972). The fraction in lignocelluloses that are most resistant to biodegradation is lignin (Trojanowski, 2001). Compared with other microorganisms, white rot fungi are the only independent but slow degrader for all fractions, especially lignin, of lignocelluloses in the natural environment (Sharma, 2000), however, it is less competitive to colonize lignocelluloses earlier than other microorganisms (Cox, 2000). We examined the capability of white rot fungi to colonize the NML complex. White rot fungus *Phanerochaete chrysosporium* ME446 was inoculated to autoclaved NML and put to pre-incubation for 7 days in aerobic condition (Lopez, 2002). Within 7 days, it colonized NML, the culture became an intra-aggregate bulk complex from loosely granular one before inoculation. The resultant biotic complex was utilized as secondary inocula, blended to the unsterilized mixture of paddy rice soil and NML. White rot fungus colonized the mixture of NML and soil in 3 days, becoming the dominant species throughout the incubation time. But with inocula without pre-incubation, *Trichoderma viride*, a cellulolytic fungus antagonistic to other fungi (Lopez, 2002), naturally colonized the mixture of NML and soil. This result indicated that white rot fungus after pre-incubation is the competitive and stable colonizer of NML, compared to other microorganisms.

Three incubation experiments were carried out with NML at 1.21% N, designed to WW-NML (NML after water-washing, organic nitrogen kept, urea of 40.8 mg/kg media was amended), UW-NML (NML without water-washing) and T-NML (NML without water-washing). WW-NML and UW-NML was inoculated with *Phanerochaete chrysosporium* ME446, T-NML was inoculated with cellulolytic fungus *Trichderma viride*. NML in UW-NML and T-NML was mixed with paddy rice soil, WW-NML was not. During incubation, NH_4^+-N concentration rippled with incubation course after a decrease firstly in UW-NML and dropped relaxed firstly followed gradual increase afterwards in WW-NML (Fig. 3a). The phase that shifted from immobilization to mobilization of NH_4^+-N lasted 15 and 24 days in WW-NML and UW-NML respectively. A periodical wave of nitrogen flux was observed in UW-NML. In T-NML, NH_4^+-N concentration decreased throughout the course of incubation, more rapidly in the first 20 days (Fig.3a). This change pattern suggested the nitrogen immobilization or mobilization by white rot fungus was different from that by cellulolytic fungus *Trichoderma viride* during their metabolism on NML. In contrast to NH_4^+-N

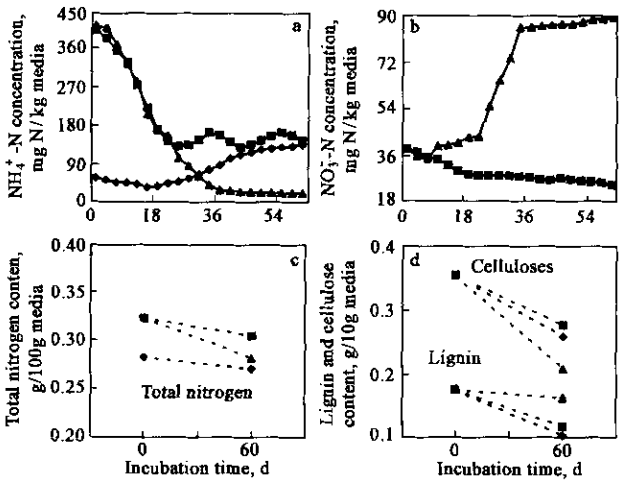

Fig. 3 Changes of NH_4^+-N and NO_3^--N concentration and total nitrogen content during incubation

a: NH_4^+-N; b: NO_3^--N; c: total nitrogen content; d: lignin and celluloses content. ◆—WW-NML; ■—UW-NML; ▲—T-NML.

change, concentration of NO_3^--N dropped down slowly in UW-NML but rose up highly in T-NML (Fig. 3b). NO_3^--N was hardly found in WW-NML. In consideration of 2.78% and 4.26% of the total nitrogen (inorganic + organic form from incubation media) lost respectively in WW-NML and UW-NML, but only 26.82% of total nitrogen disappeared in T-NML (Fig. 3c), these results lead to the suggestion that total nitrogen loss was obviously blocked by white rot fungus during their metabolism on NML. Because no leaching of NO_3^--N and NH_4^+-N would take place during metabolic process when incubation was under way in the flasks, moreover, NO_3^--N is not easily assimilated by microflora (Rice, 1989), total nitrogen loss in T-NML might be attributed to evolution of NO or N_2O derived from nitrification or denitrification (Focht, 1977).

Lignin was degraded 41.01%, 32.19%, 7.90%, celluloses was degraded 27.12%, 22.03%, 41.53% and NH_4^+-N concentration was 147.4 mg/kg, 137.5 mg/kg, 22.7 mg/kg media, respectively, in WW-NML, UW-NML and T-NML until the incubation was stopped (Fig. 3a, d). These results implied that nitrogen was mobilized from the metabolism of lignin fraction of NML by white rot fungus, cellulolytic fungus *Trichoderma viride* mainly immobilized nitrogen from bulk soil pool or inorganic NH_4^+-N of NML not organic one that bound to lignin.

It has been accepted that white rot fungi are capable of degrading a broad specificity of ubiquitous anthropogenic contaminants (Bumpus, 1985, Simonich, 1995). The degradation of pollutants by white rot fungi usually takes place in nitrogen-limiting (C/N ratio is 25 - 50) microcosms (Bumpus, 1985). The nitrogen-limiting microcosm is regulated by amendment of external easily-available nitrogen or carbon (Bumpus, 1985), this is the regulation mode of external nitrogen amendment (ENA). However, under ENA regulation mode, the nitrogen flux in a microcosm of the vast field ecosystem is usually in widely pulsed fluctuation because of runoff, leaching, nitrification, denitrification, uptake or assimilation by microbiota or plants (Post, 1985; Focht, 1977), thus ENA is shifty, unstable and not adjusted by white rot fungi. However, from the above-discussed results (Fig. 3a, b, c, d) it was shown that NML complex colonized by white rot fungus stands for a novel nitrogen flux regulation mechanism, nitrogen is released from the metabolism of NML by white rot fungus, this is the regulation mode of internal nitrogen metabolism (INM), INM is delicate, stable and adjusted by white rot fungus.

Here, we examined the effect of NML on potentiality of white rot fungi to metabolize environmental pollutants. Three treatments with concentration of 10 or 500 mg/kg incubation media (designed to concentration Ⅰ and Ⅱ separately) were carried out, namely WW-NML (NML after water-washing, with 0.83% total nitrogen and 42.25 C/N ratio), LHE (lignocelluloses without nitrogen-modification, internal nitrogen was 0.15%, 0.68% external nitrogen urea was amended) and LLE (lignocelluloses without nitrogen-modification or amendment of any external nitrogen), all treatments were inoculated with white rot fungus and not mixed with soil. In WW-NML, high degradation rate of 24 varieties of pollutants tested

whether at concentration Ⅰ and Ⅱ was found, degradation rate of organopollutants, especially phenanthrene, *o*-chlorophenol, pentachlorophenol and *p*-dinitrobenzene, was higher(Fig. 4a). In LHE and LLE compared with WW-NML, degradation rate at concentration Ⅱ was drastically lower than that at concentration Ⅰ (Fig. 4b, c). The average degradation rate of 24 varieties of pollutants tested in WW-NML, LHE and LLE was 91.33%, 48.07%, 78.13% and 97.17%, 5.97% and 7.95% respectively at concentration Ⅰ and Ⅱ. These results indicated that white rot fungus metabolize environmental pollutants depending on releasing nitrogen integrated in lignin fraction, resistant to degradation by most microbes, from NML under INM regulation mode (Fig. 3a, c, d), this is a new cometabolism phenomenon, different from that easily-available substrates are metabolized to cometabolize pollutants in bacteria (Chang, 1995). Bioremediation of polluted sites in the field ecosystem with NML colonized by white rot fungus might be perspective. Through oxic ammoniation, lignocelluloses could be changed from nitrogen-scarce (C/N > 100) to nitrogen-limiting state (C/N 25 - 50 in NML at 0.92% - 1.62% N), constituting the basis of regulation mode of INM. Within NML, lignin-rich niches of the inner cell wall layers that integrated nitrogen are fabricated, these integrated nitrogen provide a driving force for white rot fungus to metabolize NML by way of tunneling and caving(Daniel, 1994). As a result, hyphae of white rot fungus build a favorable garden in NML, the internal C/N ratio (25 - 50) falls in their palatable range, these metabolic substrates are hardly utilized by other microbiota after white rot fungus colonize them stably with the method of pre-incubation as indicated in our experiment. Under regulation mode of INM, mycelia of white rot fungus is in the proper metabolic phase(Barr, 1994). When NML is contacted with environmental pollutants even at high concentration, cells of white rot fungus colonizing NML might be protected and still grow well in those inner nitrogen-integrated niches, demolishing pollutants efficiently outside their cell with their lignin-modifying enzyme system(Tien, 1983) that are regulated under INM mode. However, white rot fungi failed to do so in LHE and LLE because of lack of nitrogen-integrated niches to invite them, under this circumstance(internal C/N > 100), they only utilized easily-available external nitrogen and bloomed on the surface of bulk lignocelluloses, endurance capability to toxicity of environmental pollutants is less than that in NML, especially in cause of higher concentration, the capability of degradation for pollutants are thus attenuated and lost. Moreover, NML might also significantly increase organic matters as the long-term growth media for white rot fungi after it is amended to the soil profile, it is sorptive to nonionic organic pollutants and improve the retardation capabilities of soils of low organic matter, and the mobility of pollutants are attenuated (Boyd, 1988), more of them adhere to the surface of the bulk lignocelluloses that exists extracellular lignin-modifying

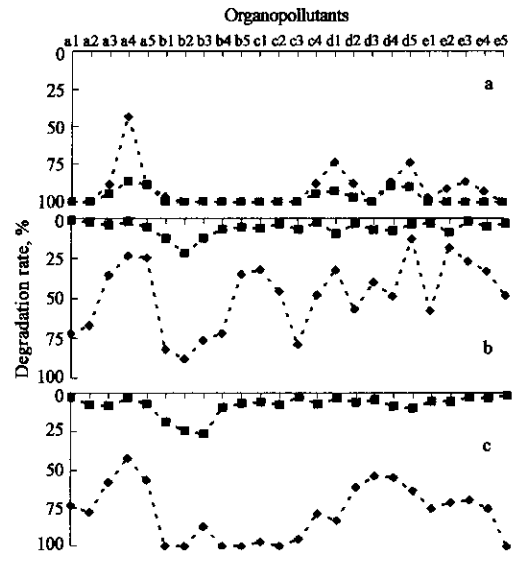

Fig. 4 Degradation of organopollutants by wite rot fungi *Phanerochaete chrysosporium* ME446 on NMLl. Three treatments the three replicate were carried out is as follows: a: WW-NML; b: LHE; c: LLE. Two concerntions of 24 varieties of organopollutants were chosen: ◆-concentration Ⅰ; ■-concentration Ⅱ. Organopollutants exerted were: a1: benzene; a2: naphthalene; a3: biphenyl; a4: phenanthrene; a5: anthracene; b1: toluene; b2: ethylbenzene; b3: *o*-xylenes; b4: *m*-xylenes; b5: 1, 3, 5-trimethylbenzene; c1: chlorobenzene; c2: 1,2,3-trichlorobenzene; c3: 1,2,3,4-tetrachlorobenzene; c4: hexachlorobenzene; d1: *o*-chlorophenol; d2: *m*-chlorophenol; d3: 2,4-dichlorophenol; d4: 2,7-dichlorodibenz-*p*-dioxin; d5: pentachlorophenol; e1: nitrobenzene; e2: 3-nitroanilline; e3: *p*-dinitrobenzene; e4: *m*-dinitrobenzene; e5: benzidine

enzymes of white rot fungus and tend to be degraded.

We expect that a quantity of NML colonized by white rot fungus, while amended to soil pedon, might not only sustain a stable N nutrient flux as nitrogenous fertilization buffers to support biomass augmentation of plants, but also persistently cometabolize persistent environmental pollutants as a novel biological detergent in the vast field ecosystem.

References:

Barr D P, Aust S D, 1994. Mechanisms white rot fungi used to degrade pollutants[J]. Environ Sci Technol, 28:78A—87A.

Béguin P, Aubert J P, 1996. The biological degradation of cellulose[J]. FEMS Microbiol Rev, 13: 25—58.

Berg B, Wessen B, Ekbohm G, 1982. Nitrogen level and decomposition in Scots pine needle litter[J]. Oikos, 38: 291—296.

Boyd S A, Lee J F, Mortband M M, 1988. Attenuating organic contaminant mobility by soil modification[J]. Nature, 333: 345—347.

Bumpus J A, Tien M, Wright D, Aust S D, 1985. Oxidation of persistent environmental pollutants by a white rot fungus[J]. Science, 228: 1434—1436.

Burt N H T, 1990. Handling excess nitrates[J]. Nature, 348: 291.

Capanema E A, Balakshin M Y, Chen C L *et al*., 2001. Oxidative ammonolysis of technical lignins—Part 1. Kinetics of the reaction under isothermal condition at 130 degrees C[J]. Holzforschung, 55:397—404.

Chang H L, Alvarez-Cohen L, 1995. Model for the cometabolic biodegradation of chlorinated organics[J]. Environ Sci Technol, 29: 2357—2367.

Convay G R, Pretty J N, 1988. Fertilizer risks in the developing countries[J]. Nature, 334:207.

Corbeels H, Hofman G, Van Cleemput O, 1998. Residual effect of nitrogen fertilization in a wheat-sunflower cropping sequence on a Varisol under semi-arid Mediterranean conditions[J]. Eur J Agron, 9: 109—116.

Cox P, Wilkinson S P, Anderson J M, 2000. Effects of fungal inocula on the decomposition of lignin and structural polysaccharides in *Pinus sylvestris* litter[J]. Bio Fert Soils, 33:246—251.

Daniel G, 1994. Use of ecectron microscopy for aiding our understanding of wood biodegradation[J]. FEMS Microbiol Rev, 13: 199—233.

Focht D D, Verstraete W, 1977. Biochemical ecology of nitrification and denitrification[J]. Advances in Microbial Ecology, 1:135—214.

Fog K, 1988. The effect of added nitrogen on the rate of decomposition of organic matter[J]. Biological Review, 63: 433—462.

Freeman C, Evans C D, Monteith D T *et al*., 2001. Export of organic carbon from peat soils[J]. Nature, 412:785.

Griffin D M, 1972. Ecology of soil fungi[M]. London: Chapman and Hall.

Hauck R D, 1984. Technological approaches to improving the efficiency of nitrogen fertilizer use by crop plants[M]. In: Nitrogen in crop production(Hauck, R. D. ed.). Madison, Wis.: Am Soc Agron. 551—560.

Lopez M J, Elorrieta M A, Vargas-Garcia M C *et al*., 2002. The effect of aeration on the biotransformation of lignocellulosic wastes by white-rot fungi[J]. Bioresour Technol, 81:123—129.

Mary B, Recous S, Darwis D, 1996. Interactions between decomposition of plant residues and nitrogen cycling in soil[J]. Plant Soil, 181: 71—82.

Meire D, Zuniga-Partida V, Ramirez-Cano F *et al*., 1994. Conversion of technical lignin into slow release nitrogenous fertilizers by ammoxidation in liquid phase[J]. Bioresour Technol, 49: 21—128.

Mosier A D, Valentine S D, Bronson K *et al*., 1991. Methane and nitrous oxide fluxes in attrite, fertilized and cultivated grassland[J]. Nature, 350: 330—332.

Post W M, Pastor J, Zinke P Z, 1985. Global pattern of soil nitrogen storage[J]. Nature, 317: 613—616.

Raghubanshi A S, Svivastava S C, Singh R S, 1990. Nutrient release in leaf litter[J]. Nature, 346: 226.

Rice C W, Tiedje J M, 1989. Regulation of nitrate assimilation by ammonium in soils and in isolated soil microorganisms[J]. Soil Biol Biochem, 21:597—602.

Sharma J R, 2000. Wood-decomposition and succession in wood-rotting fungi[J]. Indian J Fores, 23:123—128.

Simonich S C, Hites R A, 1995. Global distribution of persistent organochlorine compounds[J]. Science, 269: 1851—1854.

Tamm C O, 1991. Nitrogen in the terrestrial environment. Questions of productivity, vegetational changes and ecosystem stability[M]. In: Ecological Studies. Berlin: Springer Verlag. 81—116.

Tien M, Kirk T K, 1983. Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds[J]. Science, 221: 661—663.

Trojanowski J, 2001. Biological degradation of lignin[J]. International Biodeterior Biodegrad, 48 :213—218.

Tyrrell T, 1999. The relative influence of nitrogen and phosphorus on oceanic primary production[J]. Nature, 400:525—531.

(Received for review June 12, 2002. Accepted September 27, 2002)