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Biological bleaching of water-soluble coal macromolecules by a basidiomycete strain

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Abstract There is need for new effective technologies to convert coal into environmentally acceptable liquid fuels. Thermochemical coal-conversion processes occur under extreme conditions. Thus there is a potential to use the biotransformation of coal as a cheap alternative method. A basidiomycete strain, which decomposes coal macromolecules, was isolated from humic-acid-rich soil of a lignite surface-mining region. The isolate showed the ability to decolorize liquid dark-brown media containing water-soluble coal-derived substances (humic acids). The presence of an easily available substrate is necessary for the biodegradation. The influence of different culture conditions on the bleaching effect was studied. Evidence for decomposition of water-soluble coal substances was provided by measuring the decrease of absorbance and the modification in the distribution of molecular masses. The degradation process resulted in a complete decolorization of the coal-derived humic acids and was also combined with massive alterations in their molecular structure. Solid-state 13C-NMR spectroscopy showed an increase of carboxylic groups as well as hydroxylated and methoxylated aliphatic groups, which indicates an oxidative attack. Enzymatic analysis showed the presence of a Mn peroxidase in the culture supernatant. Extracellular lignin peroxidase and laccase activities were not detectable. The production of the peroxidase was induced by addition of humic acids. But, in vitro, this enzyme did not cause a decolorization or reduction in molecular mass of the coal-derived humic acids.

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Introduction

In the early 1980s the potential of microorganisms was discovered for using coal as substrate and/or transforming coal into liquid products (Fakoussa 1981; Cohen and Gabriele 1982). These effects are very interesting because they could represent an alternative process for coal conversion, as thermochemical processes have a low thermal efficiency and high capital cost.

The complex and very heterogeneous macromolecule lignite (brown coal) consists of the following components: bitumen (waxes and asphaltenes), humic-acid-like material and the macromolecular matrix. German lignites contain between 30% and 80% humic-acid-like material, which is extractable by the use of alkaline substances (e.g. NaOH). These constituents of coal are probably more easily used as substrates by microorganisms than is the coal matrix.

Coal substances extractable by alkali are somehow structurally related to humic acids from other origins, but the real structure is still unknown. Forest soil and water humic acids are usually regarded as very complex and heterogeous macromolecules with aromatic and aliphatic structure elements. These alkali-soluble, but not acid-soluble dark-brown-coloured substances of high molecular mass are very resistant to microbial attack, because of their heterogeneous structure, their physical status/condition as aggregates, their association with metals and the limited accessibility of functional groups (Ziechmann 1980). In spite of these properties, the isolation of fungi capable of decomposing soil humic acids was reported by Burges and Latter (1960). Subsequently forest soil humic acid biodegradation was achieved in vitro by other white-rot fungi (Hurst et al. 1962; Haider and Martin 1988; Blondeau 1989), streptomycetes (Monib et al. 1981; Khandelwal and Gaur 1980; Yanze Kontchou and Blondeau 1990) and different bacterial species (Gordienko and Kunz 1984). The most active humic acid degraders are basidiomycete species (white-rot fungi), which are known as lignin

degraders (Ziechmann 1980). The cometabolic degradation of forest soil humic acids by the lignin degrader *Phanaerochaete chrysosporium* was reported by Haider and Martin (1988). Generally the presence of an easily utilizable carbon source is necessary for the decomposition of soil humic acids. Very few fungi can attack humic acid as the sole source of carbon. The presence of additional carbon and nitrogen sources stimulates the degradation.

The resulting modifications of the structure in humic acid molecules are probably due to the low specificity of the ligninolytic enzyme system, consisting of extracellular lignin peroxidases, Mn peroxidases and laccases.

Materials and methods

Preparation of water-soluble coal macromolecules

Coal-derived humic-acid-like material was extracted under alkaline conditions from German lignite (lithotype A), obtained by the Rheinbraun AG, Cologne. Lignite pieces were ground with a mortar. The lignite powder was subsequently treated with ammonia solution (0.5%, w/v) for 48 h by shaking (80 rpm/min) at room temperature. The extracted humic substances were separated from the matrix by centrifugation at 15 000 *g* for 20 min. The humic acids in the dark-brown-coloured supernatant were recovered by acidification with 1 M HCl to pH 1, followed by centrifugation at 15 000 *g* for 20 min. The humic-acid-containing pellet was resolubilized in distilled water by adding 1 M NaOH to pH 7.0. Subsequently the humic acids were freeze-dried (Christ Alpha I-6) to avoid polymerization and other reactions during storage.

Culture conditions

The strain RBS 1k (DSM 11132) was isolated in our laboratory. The fungus species could be determined by the Centraalbureau voor Schimmelcultures (Baarn, Niederlande) and by the Mycotheque de l'Université Catholique de Louvain (MUCL), Laboratoire de Mycologie systematique et appliquée (Belgium) only to the order. The fungal strain RBS 1k is a haploid basidiomycete, probably belonging to the *Aphyllophorales* or *Agaricales*. The strain RBS 1k was routinely maintained on malt-extract-agar slants (6 g malt extract, 0.6 g soya peptone, 18 g agar, distilled water to 1000 ml, pH 5.6). A piece of fungal mycelium (0.5 × 0.5 cm) was used as inoculum for submerged cultures. All experiments were conducted in shaken-batch cultures using 250-ml conical flasks, containing 80 ml culture medium. The basal solution (Aust 1990, with modification) contained (per litre of distilled water) 200 mg KH_2PO_4, 50 mg $MgSO_4 \cdot 7H_2O$, 10 mg $CaCl_2$, up to 11 g D (+)-glucose, 220 mg ammonium tartrate, 1 mg thiamine, 1.46 g sodium dimethylsuccinate, 0.5 g Tween 80; the pH of solution was adjusted to 4.3 and the medium was supplemented with 5 ml trace elements (composition 3.0 g: $MgSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 1.0 g NaCl, 100 mg $FeSO_4 \cdot 7H_2O$, 100 mg $CoSO_4$, 82 mg $CaCl_2$, 100 mg $ZnCl_2$, 10 mg $CuSO_4 \cdot 5H_2O$, 10 mg $AlCl_3$, 10 mg H_3BO_3, 10 mg $NaMoO_4$, distilled water to 1000 ml) and 0.5 ml vitamin solution. The vitamin mixture (Kirk et al. 1978; with modification) contained (per litre): 2 mg biotin, 2 mg folic acid, 5 mg thiamine hydrochloride, 5 mg riboflavin, 10 mg pyridoxine hydrochloride, 0.1 mg cyanocobalamine: 5 mg nicotinic acid, 5 mg calcium DL-pantothenate, 5 mg *p*-aminobenzoic acid, distilled water to 1000 ml.

The sterilized humic acids (membrane filtration, pore size: 0.45 μm) were added in different concentrations to the culture flasks. Fungal growth was measured in terms of dry weight after filtration through a paper filter (Schleicher & Schüll, 595 1/2) and by drying at 80°C to constant weight.

The degradation of the coal-derived humic-acid-like material was monitored by the decrease of absorbance at 450 nm (brown colouring). The results were expressed as the rate of decrease of absorbance in relationship to the uninoculated control. All studies were performed in triplicate. Remaining coal substances were recovered by acidification with 1 M HCl to pH 1 and subsequent centrifugation (see above).

Enzyme assays

Peroxidase assays were carried out by using fungal culture supernatant in the presence of 2.5 mM enzyme substrate and 3.0 mM hydrogen peroxide (Kahle *et al*. 1970, with modification). With 2,2 primo-azinobis-3-ethylbenzthiazolinsulphonic acid (ABTS) as substrate the reaction mixture contained citratephosphate buffer (400 mM; pH 3.2) and 150 μm $MnSO_4$ for the Mn peroxidase. The reaction was started by adding hydrogen peroxide. Peroxidase activity was quantified as the initial rate of ABTS oxidation. The increase in absorbance at 420 nm was monitored. One unit was expressed as the amount of enzyme required for oxidation of 1 μmol ABTS in 1 min. The absorption coefficient (420 nm) of the product is 21.6 cm^2 μmol^{-1} (Kahle et al. 1970).

The lignin peroxidase assay was performed by using veratryl alcohol as substrate (Tien and Kirk 1984). In the laccase assay the oxidation of syringaldazine was measured (Harkin and Obst 1973). The presence of hydroxyl radicals in cell-free culture supernatant was determined by the method of Bors et al. (1979) by measuring the bleaching of *p*-nitrosodimethylaniline. Ligninolytic activity was proved by the decolorization of the polymeric dye Poly R-478 (Freitag and Morell 1992).

Enzyme purification

The purification of the peroxidase in one step by affinity chromatography on concanavalin-A–Sepharose material was possible because this extracellular enzyme is a glycoprotein. Cell-free culture fluid of the strain RBS 1k was filtered to remove particles and concentrated tenfold by ultrafiltration (Amicon XM 10). Afterwards the pH of the supernatant was adjusted to 6.0. Then 30 ml sample solution was pumped slowly over a column (1.6 cm × 15 cm, filled with 10 ml concanavalin-A–Sepharose (Sigma) with a flow rate of 0.25 ml/min. The affinity material was then washed with four to five column volumes of acetate buffer (10 mM, pH 6.0) for removing the non-bound proteins. The elution of peroxidase was performed with a linear gradient of 0–0.5% methyl α-D-mannopyranoside (Fluka) in acetate buffer (10 mM, pH 4.5) with a flow rate of 0.5 ml/min. The homogeneity of the enzyme was proved by sodium dodecyl sulphate I polyacrylamide gel electrophoresis.

Analytical methods for product characterization

Molecular-mass distribution

Molecular mass changes of humic acids were measured relatively by two methods.

(a) Gel-permeation chromatography was performed on Sepharose CL-6B (Pharmacia) with 50 mM TRIS/HCl buffer, pH 9.0, as eluent. The chromatography was performed with a FPLC system (Pharmacia, Uppsala, Sweden). The flow rate was 0.5 ml/min. The elution of water-soluble brown-coal molecules was monitored at 450 nm (UV/Vis detector Shimadzu SPD-GAV). The column calibration was obtained with the following globular proteins (Pharmacia) because of the lack of humic-acid-like substances with defined molecular mass detectable at 280 nm: egg-albumin (45 kDa), aldolase (160 kDa), catalase (240 kDa), ferritin (450 kDa) and thyroglobin (669 kDa)(Sigma).

(b) Gel-permeation chromatography was carried out on a column (HEMA-Bio 40, 10 μm PSS gel, 8 × 300 mm, PSS Co., Mainz, Germany). The solvent contained 80% purified water and 20%

acetonitrile with addition of 5 g/l $NaNO_3$ and 2 g/l K_2HPO_4 (personal communication: M. Hofrichter and W. Fritsche 1995). The mobile phase was adjusted to pH 9, the flow rate to 1 ml/min. The following equipment was used: an HPLC system (LiChroGraph series, Merck, Darmstadt) with diode-array detector, autosampler, gradient pump and HSM software.

Solid-state ^{13}C-NMR spectroscopy

^{13}C-NMR spectra were run on a Bruker MSL 200 spectrometer (^{13}C: 50.32 MHz) with cross-polarization and magic-angle spinning techniques. The cross-polarization time was 0.6 ms, the pulse-return time 4 s and the rotation frequency 8000 Hz (sample head MAS4.DB.CP.BB.VT). The samples (200 mg) were employed in freeze-dried form. For external calibration the standard adamantane was used.

Elemental analysis

Elemental analysis of coal samples (20 mg), to determine the percentage of carbon, hydrogen, nitrogen and ash, was performed using standard procedures (Laboratory Dornis & Kolbe, Mülheim/Ruhr). The oxygen content was determined by difference formation.

Results

A basidiomycete, called strain RBS 1k, isolated near Cologne, was able to decolorize dark brown, coal-derived water-soluble coal substances.

Figure 1 shows the bleaching of humic acids by this basidiomycete in shaken cultures. The decolorizing activity for humic acids appeared after 3–4 days of incubation and continued over a period of 12–14 days. The adsorption of humic acid molecules onto the fungal cell wall surface may be the first step in their biodegradation by the strain RBS 1k. But the bleaching of the dark culture medium was not the only result of the binding of humic acids on fungal mycelia: after a few days of incubation the dark-coloured mycelia were bright again. The presence of humic acids in the culture medium caused a stimulating effect on growth. Analytical measurements showed chemical changes in the remaining humic acids.

By optimizing the decolorization conditions we obtained the following culture conditions: The bleaching activity was enhanced with an initial pH of 7.0 and a growth temperature of 28°C. High nitrogen concentrations in the culture media tended to reduce the decolorization rate. These findings are in agreement with the results of Haider and Martin (1988), who examined the mineralization of humic acids from soil by *P. chrysosporium.*

Experiments with various carbon sources demonstrated that the best decolorization activities besides those in malt extract medium occurred in mineral salt media supplemented with glucose or glycerol (Fig. 1). A maximum of 0.2 % humic acid was tested and an optimal concentration of 0.05 % humic acids (final concentration) was determined. Lower concentrations did not seem to be sufficient for induction of the degrading enzyme system; higher concentrations are probably inhibitory for the cells under these conditions (Fig. 2). In experiments with 0.05% and 0.075% humic acids the best growth stimulation was also observed.

Molecular mass changes of humic acids during fungal action

A reduction of the predominant average molecular mass of the humic acids was noticed after the fungal treatment. This molecular mass shift towards smaller molecules, measured by two different gel-permeation chromatography methods, indicated the decomposition of the macromolecules by free or cell-wall-bound extracellular enzymes produced by the basidiomycete strain RBS 1k. The molecular masses measured by the two different methods are only relatively comparable. It is difficult to determine the absolute value.

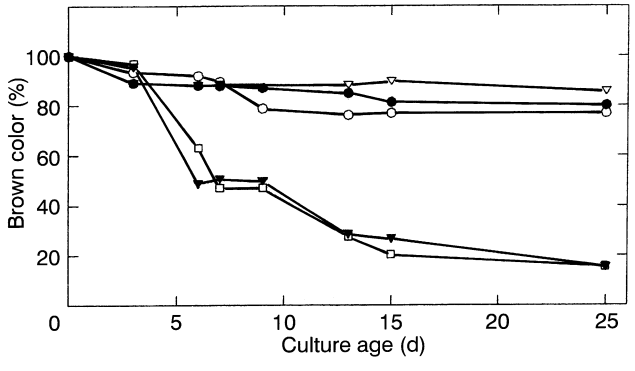

Fig. 1 Decolorization of humic acids from coal (german lignite, lithotype A) in mineral salt medium (see Materials and methods) with different carbon sources (60 mM) 100% brown colouring = absorbance ($A_{420\ nM}$) at the beginning of the culture. ○ Glutamate, ● acetate, ▽ galactose, ▼ glucose, □ glycerine

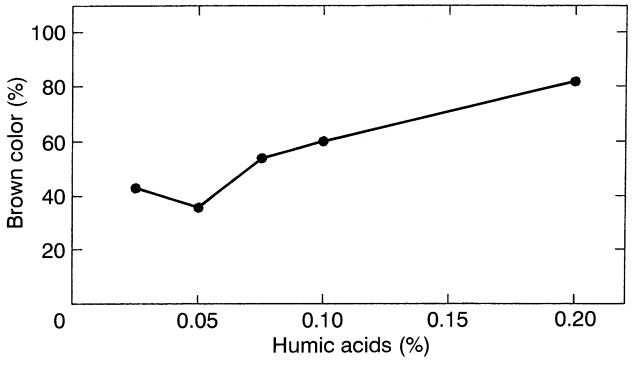

Fig. 2 Bleaching of coal humic acids as a function of their concentration under cometabolic conditions (% w/v) (fungal strain; RBS 1k, culture medium: diluted malt extract); 100% brown colouring = absorbance (A_{420}) at the beginning of the culture

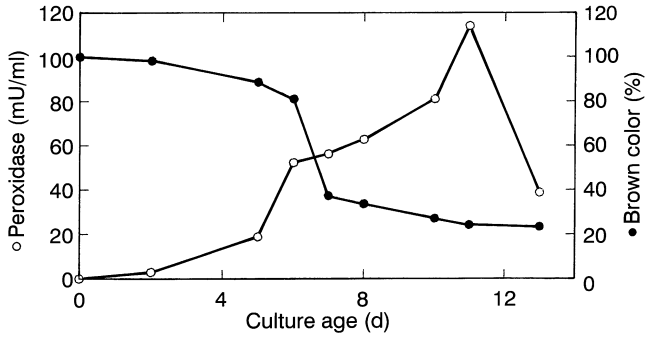

Fig. 3 Relationship between the production of extracellular peroxidase by the fungus RBS 1k and bleaching of humic acids (culture conditions: diluted malt extract medium, 0.05% w/v humic acids from coal; 250–ml conical flask, incubation temperature: 28°C)

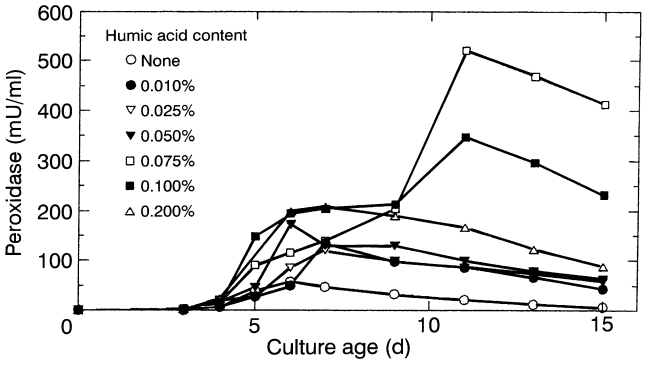

Fig. 4 Induction of extracellular peroxidase activity (strain RBS 1k) by coal humic acids (obtained by alkaline extraction of german lignite (lithotype A), (culture conditons: diluted malt extract medium with various humic acid concentrations w/v)

Interaction with extracellular Mn peroxidase activity

The humic acid degradation is probably due to participation of a nonspecific ligninolytic enzyme system consisting of different extracellular peroxidases and laccases. It would be of interest to know whether the humic-acid-decolorizing fungus RBS 1k produces the extracellular oxidative enzymes known from lignin degraders. Ligninolytic activity of the basidiomycete strain RBS 1k is indicated by the Poly R-478 assay. The results of different enzymatic assays show that the strain RBS 1k excretes a Mn peroxidase, but no lignin peroxidases or laccases.

In our experiments, a relationship between extracellular Mn peroxidase activity and humic acid decolorization has been documented (see Fig. 3). The culture conditions that enhance the production of the peroxidase also stimulate the humic-acid-bleaching activity. The enzyme activity was dependent on the concentration of humic acids. A concentration of 0.075% (w/v) caused the highest enzyme induction, whereas a concentration of 0.1% (w/v) or higher resulted in an inhibition of Mn peroxidase production. These enzyme activities are shown in Fig. 4. All cultures with bleaching activity produced peroxidase. No culture condition with humic-acid-decolorizing activity was found when no extracellular peroxidase was produced.

Cross-polarization/magic-angle-spinning ^{13}C-NMR spectroscopy

The solid-state ^{13}C-NMR spectra of humic acids can be divided into different regions: aliphatic carbon (0–60 ppm), aliphatic hydroxylic carbon (60–90 ppm), aromatic (95–160 ppm) and carboxylic groups (160–185 ppm). The spectrum of humic acids (control) reveals three peaks with maxima at about 175, 130 and 40 ppm, indicating the presence of aliphatic and aromatic constituents in the macromolecular structure of humic acids originating from coal. Comparing the control sample and the fungus-treated one, we observed an increase of carboxylic groups, hydroxylated and methoxylated aliphatic groups and a decrease in aromatics (Table 1 and Fig. 5). The percentage of aliphatic and aromatic carbon was lower than in the control, but there seems to be a shift towards the oxidized aliphatic and aromatic carbon atoms. These results point to an attack by oxidative mechanisms.

Table 1 Relative abundance (%) of different types of carbon atoms, obtained by integration of the # ^{13}C-NMR spectra of coal humic acid samples (control and sample resulting from fungal attack). *NM* not measurable

Constituent	Chemical shift (ppm)	Carbon distribution (% peak area)	
		Control	Fungus RBS 1k
Total aliphatics	0–60	48.5	49.5
Aliphatic Fractions	0–36	NM	30.0
	36–50	NM	13.0
Methoxy	50–60	NM	6.5
—OH, C—O—C	60–90	14.5	12.0
Total aromatics	95–160	30.5	23.0
Aromatic fractions	95–140	NM	16.5
	140–160[a]	NM	6.5
COOH	160–185	6.5	15.5

[a] ArOH, ArOCH3, ArOAr

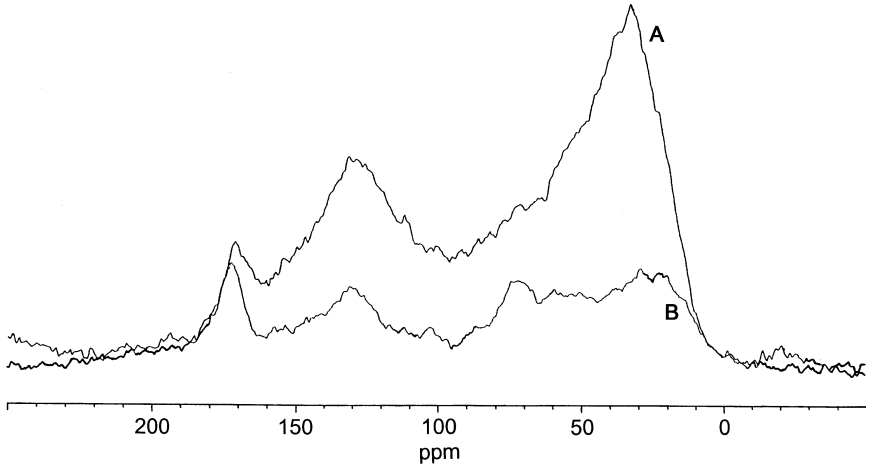

Fig. 5 Cross-polarization/magic-angle-spinning ^{13}C NMR spectra of (A) control and (B) humic acids from coal, modified by the fungus RBS 1k

In Fourier-transform/infrared spectroscopy only small differences could be observed between the control sample and the microbially bleached humic acids.

Elemental analysis

Table 2 shows an analysis of the elemental composition of coal-derived humic acids and of the product of microbial bleaching activity. These results give new information about the degradation mechanisms. The increased content of oxygen indicates an oxidative attack. The hydrogen content decreased, whereas the nitrogen content rose. The ash content declined.

Investigations into the mechanism of humic acid degradation

The purified extracellular peroxidase of RBS 1k (500 mU/ml, pH 4.9, 0.05 % w/v humic acids, 3 mM hydrogen peroxide, 150 μM $MnSO_4$) did not cause bleaching or molecular mass reduction of the humic acids in vitro.

With the cell-free supernatant of the basidiomycete RBS 1k, cultured under optimal conditions for bleaching, no decolorizing activity was measured after the addition of different humic acid concentrations (0.01 %, 0.025 % 0.05 % w/v). Neither a variation in the time for taking samples (24, 48, 72, ... 336 h incubation), nor the addition of hydrogen peroxide, nor variation of the pH (3.0–8.0) nor induction with humic acids (concentration: 0.05 %) led to a change in brightness of the humic-acid-enriched culture fluids. Also a tenfold concentration of cell-free culture supernatant by ultrafiltration (Amicon, cut off: 10.000 da) did not show a positive result.

Discussion

We were able to demonstrate the enzymatic bleaching of dark-brown-colored humic acids, prepared by alkaline extraction from German lignite, by a fungus isolated from an open-cast mining region (Bergheim, near Cologne). Our studies also revealed the reduction of molecular mass of the biodegraded humic acids (Willmann 1994). Little is known about the mechanism of the degradation of humic acids originating from soil. The high molecular mass of humic acids demands an extracellular enzymatic attack. We examined the changes in chemical structure of these macromolecules by cross-polarization/magic-angle-spinning ^{13}C-NMR spectroscopy and elemental analysis. All modification of the structure confirm a strong oxidative degradation process of humic acids from coal by the basidiomycete strain RBS 1k. The aromatic and aliphatic structures within the humic acid seemed to be modified. Dehorter et al. (1992) analysed microbially bleached soil humic acids by ^{13}C-NMR spectroscopy but, in contrast to our results, they did not record important changes in their main constituents and found no increase of carboxyl content. Their results are contrary to the experiments of Yanze Konzchou and Blondeau (1990), who described the degradation of humic

Table 2 Elemental composition of coal-derived humic acids (control) and of humic acids, microbially bleached by the basidiomycete strain RBS 1k

	Composition (%)					
Humic acid samples	C	H	N	O	ash	Sum formula
Control	40.3	4.0	5.1	24.9	25.6	$C_{100}H_{119}N_{11}O_{46}$
After microbial attack	47.2	4.3	6.4	30.3	11.7	$C_{100}H_{109}N_{12}O_{48}$

acids, extracted from forest soil, as sole carbon source, by bacterial cultures, and measured an increase in carboxylic group content. Ziechmann (1980) supposed that hydroxyl groups and aliphatic side-chains would be attacked first. Hurst et al. (1962) postulated the inclusion of a reductive step during the attack on soil humic acids, like reduction of carboxylic groups. We have no indications for such step in our experiments. Blondeau (1989) demonstrated the biodegradation of natural and synthetic humic acids by the white-rot fungus *P. chrysosporium.* His results suggest that the degrading activity for humic acids is extracellular. Most likely the lignin-degrading non-specific enzyme system of this fungus plays a role in the decay of humic acids. In the case of *P. chrysosporium* the degrading enzyme appears to be extracellular; probably the attack is catalysed by the so-called lignin peroxidase (Blondeau 1989; Haider and Martin 1988). The pure decomposition of water-soluble coal macromolecules by *P. chrysosporium* is due to the action of lignin peroxidase. This conclusion was based on the results of gel-permeation chromatography, in which the average molecular masses of soluble coal macromolecules treated by the enzyme are reduced in comparison to the control (Wondrack et al. 1989). But no bleaching activity was observed. In contrast, our results show that the bleaching activity of the basidiomycete RBS 1k seems to be extracellular, bound to the cell wall or acting only near the cell wall. The adsorption of humic acid molecules onto the fungal hyphae may be required for their biodegradation. The degrading system seems to be primarily localized on the surface of the fungal hyphae or the conditions of incubation of the enzyme assay are not appropiate, e.g. certain cofactors are missing. Also, the fungal strain RBS 1k does not produce extracellular ligninase or laccase activity, only a Mn peroxidase. This exoenzyme can act as an electron carrier by generating Mn^{3+} and Mn^{2+}, which is freely diffusible and able to oxidize complex macromolecules such as lignin (Jong et al. 1994; Messner and Srebotnik 1994).

The results demonstrate a correlation between this extracellular peroxidase activity and humic acid decolorization and oxidation. Moreover, the enzyme production is inducible by adding humic acid to the culture medium. These results indicate a participation of the peroxidase in humic acid degradation and bleaching. Dehorter and Blondeau (1992) also described a relationship between the biodegradation of soil humic acids and extracellular lignin peroxidase and Mn dependent peroxidase activity by *P. chrysosporium* and *Trametes versicolor.* They suggested that surfactant properties of humic acids are responsible for the increase of enzymatic activities. Mangler and Tate (1982) also supposed a correlation between peroxidase production and oxidation of humic acid derived from soil by an unidentified fungus. The participation of fungal laccase and peroxidase activities in bleaching of kraft pulp (paper industry) are described by Addleman and Archibald (1993) and Reid and Paice (1994).

These relationships between peroxidase activity and humic acid bleaching support the hypothesis that this enzyme takes part in the degradation of water-soluble coal material. On the other hand, our results show that the purified enzyme in vitro does not cause a decolorization or molecular mass reduction of humic acids under the conditions described above.

Whereas the bleaching of humic acid by *Arthrobacter* sp. is due to the generation of analogous hydroxyl radicals (Blondeau 1987), in cell-free supernatants of RBS 1k cultures no reactive hydroxyl radicals were found. Possibly such radicals may be produced in the immediate neighbourhood of the cell wall.

Alkali-soluble American low-rank coals can be used as growth substrates by different fungi (Do Nascimento et al. 1987; Polman et al. 1994) and some bacterial species (Crawford and Gupta 1981; Gupta et al. 1990). In most of these studies degradation of the water-soluble coal molecules was also described, but in none of them was a decolorization of the water-soluble coal substances observed.

In vitro coal degradation was also observed in free culture fluids of *Penicillium citrinum* (Polman et al. 1994). But, *in vivo*, more variety in molecular masses was detectable, so it was concluded that not all enzyme activities involved in the degradation process were present in the culture supernatant. Our results show that either the enzyme system of strain RBS 1k, which degrades the alkali-soluble coal substances, is not freely extracellular but bound to the cell wall or an unknown cofactor is necessary for the decomposition. Perhaps defined conditions, e.g. redox potential, are important for the bleaching process.

For the first time it has been demonstrated that up to 50% of any lignite (German lithotype A), namely the alkali-extractable humic acid fraction, is microbially degradable in the presence of a second easily available substrate.

Further investigations should concentrate on the questions why higher concentrations of humic acids are inhibitory and why the enzyme does not react in a cell-free system.

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