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Solubilization of low-rank coal by *Trichoderma atroviride*: Evidence for the involvement of hydrolytic and oxidative enzymes by using ^{14}C-labelled lignite

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The deuteromycete *Trichoderma atroviride* is able to solubilize lignite in dependence on a given carbon source for growth. When cultivated on media containing glutamate, this mold excreted a set of different enzymes with hydrolytic activity. Addition of lignite to the growth media induced the synthesis of extracellular lignite-specific esterase activity but no evidence has been provided for its direct involvement in the process of lignite solubilization. Hence, the basic capability of *T. atroviride* enzymes to degrade a variety of ester and ether bonds at the surface or within the bulky lignite structure was tested using coal following its direct labelling with ^{14}C-alkyl iodide. The participation of hydrolytic and oxidative enzymes in lignite degradation was assessed by measuring the release of ^{14}C radioactivity from selectively alkylated carboxylic and phenolic OH groups. *T. atroviride* cleaved both carboxylic esters using esterases and the phenolic ether bonds by using oxidative enzymes, most likely laccases.
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Introduction

The ability of some microorganisms to solubilize and metabolize lignite has been known since the beginning of the 1980s [4,7]. Oxidative ligninolytic enzymes produced by white rot fungi, e.g., manganese peroxidase, lignin peroxidase or laccase, are able to degrade aromatic compounds in the lignite structure and to depolymerize humic acids extracted from coal [9,16,28]. Due to the action of these enzymes, a portion of the attacked humic acids is degraded to oxygen-rich and water-soluble fulvic acids whereas another portion is oxidized to CO_2 [17]. The oxidative enzymes catalyze lignite degradation in an acidic pH range only. In contrast, lignite solubilization occurs predominantly at alkaline pH values, at which the solubility of humic acids is enhanced [19,34]. The participation of extracellular enzymes in the process of lignite solubilization has not yet been proven directly.

A parallel appearance of esterase activity with lignite solubilization was described for some microorganisms such as the white rot fungus *Polyporus versicolor* [4,31] or bacteria, e.g., *Streptomyces viridosporus* [6] and *Pseudomonas* (DLC-62) [30]. The described enzymes operated in a neutral or slightly alkaline milieu. However, a direct correlation between the activity of esterases and the process of lignite solubilization has been rejected for at least two reasons: (i) isolated esterases exhibited no solubilizing effect *in vitro* [5]; (ii) the content of potentially easily hydrolyzable bonds such as esters and glycosidic bonds in lignite was held to be negligible. It has been argued that during the process of lignite genesis these bonds were preferably hydrolyzed by microorganisms [13].

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Contrary to the above arguments, a direct correlation between lignite solubilization and enzymatic hydrolytic activity has recently been demonstrated for the deuteromycete *Trichoderma atroviride* [21]. The esterase activity measured in the culture supernatant increased considerably when the fungus was cultivated on media containing glutamate. Addition of lignite to this culture medium led to a further significant induction of extracellular esterases.

New results on the structure of lignite demonstrated that extracellular esterases have several potential targets. The importance of ester bonds in stabilizing the macromolecular structure of lignite was investigated by Große [11], who found that the fraction of carboxylic ester groups in the total organic matter of lignite (Lithotype A, Bergheim, Germany) amounted to approximately 2.5–5%, corresponding to one to two ester bonds per 100 lignite carbon atoms. Until now, extracellular esterases that would be able to attack nonspecifically heterogeneous complex organic materials such as lignite have not been described in *Trichoderma* sp. Acetyl xylan esterases, on the other hand, were described in detail [12,26] but their substrate specificity was rather high [2]. Therefore, it seemed unlikely that these substrate specific acetyl xylan esterases excreted by *Trichoderma* sp. were able to hydrolyze various esters spread within the bulky lignite structure and thus to play a role in lignite solubilization.

To prove an enzymatic attack on the lignite structure by *T. atroviride*, both carboxylic and phenolic OH groups of lignite were labelled with ^{14}C-alkyl iodides, giving rise to the corresponding ester and ether bonds. Radioactive labeling of coal by the introduction of mixed ^{13}C/^{14}C-alkyls has been used for chemical investigations of coal structure and quantification of ^{13}C NMR spectroscopic results [14], following O alkylation with alkyl iodides as described by Liotta [24]. Specific ^{14}C alkylation of lignite for activity assays of extracellular enzymes has not yet been done. ^{14}C-Ring-labelled aryl substrates were used as models to

investigate degradation of lignin or humic acid-like compounds by manganese peroxidase [15,18].

The results presented provide evidence for the ability of *T. atroviride* to synthesize both extracellular esterases, which are able to hydrolyze radioactively labelled alkyl esters, and oxidases (most likely laccases) that are able to degrade the phenolic ether bonds within the lignite structure. The degradation products may be subsequently oxidized to CO_2.

Materials and methods

Fungus

T. atroviride is one of the fungal species isolated from lignite [20]. The fungus was classified by the Centraalbureau voor Schimmelcultures, Baarn, the Netherlands (CBS strain number 349). *T. atroviride* was cultivated under the same conditions as described earlier for another lignite-solubilizing deuteromycete, *Fusarium oxysporum* [19], in a medium containing glutamate as sole carbon source.

Lignite pretreatment

Lignite (lithotype A, Bergheim) was dried and pulverized to increase its reactive surfaces (particle size 0.2 mm). A modified method of Kuczynski and Andrzejak [22] was used to free the polar groups within the lignite. Fifteen grams of the lignite powder was stirred in 75 ml 5 N HCl for 2 h at room temperature. The suspension was filtered and the filtrate was washed with hot demineralized water several times until a neutral pH was reached. Ten grams of demineralized lignite was debituminated by extracting it with 150 ml toluene in a Soxhlet apparatus until the extract was colorless.

Specific labeling of lignite

^{14}C-Esters: Two grams of demineralized and debituminized lignite was stirred for 24 h at room temperature in a suspension containing 5 ml tetrahydrofurane, 2 ml 1 M tetrabutylammoniumhydroxide dissolved in methanol and 1 ml methyl or ethyl iodide, according to Liotta [24]. During this process all acidic OH groups (−COOH as well as phenolic OH) in the lignite were alkylated. To set the COOH groups free again the alkylated lignite was hydrolyzed with 5 N NaOH at 80°C for 2 h. This partially hydrolyzed sample was acidified, filtered, washed several times with demineralized water and dried at 50°C to constant weight. One gram of this demineralized and debituminized lignite, which was protected at the phenolic OH, was alkylated according to the above mentioned method of Liotta for a second time with 0.5 ml ^{14}C[methyl iodide] or ^{14}C[ethyl iodide] (specific radioactivity of 0.3 and 0.11 MBq mmol^{-1}, respectively). The free carboxylic groups in the lignite were thus radioactively labelled. The unbound ^{14}C[methyl iodide] or ^{14}C[ethyl iodide] was removed by washing the suspension several times with hot methanol and water, until no radioactivity was detected in the wash. The labelled lignite was dried to constant weight.

^{14}C-Ethers and ^{14}C-esters: The demineralized and debituminized lignite was alkylated with ^{14}C[methyl iodide] or ^{14}C[ethyl iodide] as described above. ^{14}C labelling of both ester and ether bonds was achieved by omitting the hydrolysis steps.

^{14}C-Ethers: The ^{14}C-ethyl-labelled lignite was hydrolyzed with 5 N NaOH at 80°C for 2 h. This hydrolyzed sample was acidified, filtered, and the filtrate was washed several times with demineralized water and dried at 50°C to constant weight. The resulting lignite was labelled only with ^{14}C[ethyl iodide] connected by ether bonds with the phenolic OH groups within the lignite fraction. The labelled lignite was dried to constant weight.

In vivo assay conditions

T. atroviride was cultivated on solid glutamate medium in Petri dishes [19] at room temperature for four days until the surface was fully covered with fungal mycelium. ^{14}C-Labelled lignite powder (100 mg) was spread over the mycelial surface. Four Petri dishes were sealed in a 25-l desiccator. $^{14}CO_2$ and other labelled volatile substances released by the fungus were flushed out every 24 h so that the total gas volume of the desiccator was exchanged. Volatile compounds were trapped in a flask containing Oxysolve-C-400 scintillation cocktail (Zinsser Analytic, Frankfurt, Germany). The samples were measured in a liquid scintillation analyzer (1600 CA TRI CARB, Packard, Dietrich, Germany).

IR spectroscopic characterization

IR data were obtained with a Nicolet 510 FT-IR spectrometer (Thermo Nicolet, Madison, WI) using the KBr technique.

Chemicals

^{14}C[Methyl iodide] and ^{14}C[ethyl iodide] were obtained from Amersham Life Science, Freiburg, Germany and ICN Pharmaceuticals, Frankfurt, Germany, respectively. All other chemicals were of analytical grade and were purchased from Merck, Darmstadt, Germany.

Results

Characterization of ester groups

Lignite is a heterogeneous complex organic material that is difficult to characterize chemically. The distribution of different ester groups in lignite was investigated by IR spectroscopy applying the method of Supaluknari and coworkers [32]. The IR bands of the carbonyl functional groups appear in the carbonyl-stretching region between

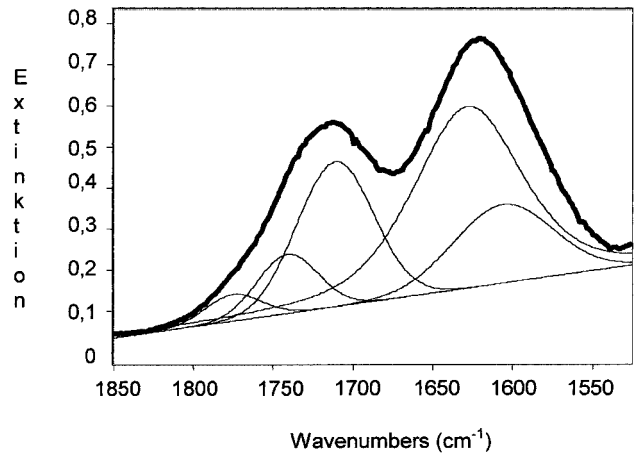

Figure 1 Infrared spectrum of the lignite Bergheim lithotype A. Curve-fitting bands in the carbonyl-stretching region of the spectrum.

Table 1 Radioactive labelling of lignite following alkylation with ^{14}C[methyl iodide] or ^{14}C[ethyl iodide]

	Introduced radioactivity	
	Calculated values[a]	Measured values[b]
Complete methylation	0.161	0.126
COOH methylation	0.078	0.052
Complete ethylation	0.071	0.093
COOH ethylation	0.035	0.037
OH ethylation	0.039	0.054

Comparison of calculated and measured values (in MBq g^{-1} lignite).
[a]Values were calculated from the distribution of carboxylic and phenolic OH groups as described in the text.
[b]Values measured after ^{14}C alkylation.

1780 and 1650 cm^{-1} (Figure 1). Three bands at approximately 1770, 1740 and 1700 cm^{-1} were resolved by a deconvolution. The comparison of the characteristic group frequencies for model substances led to the conclusion that the band at 1770 cm^{-1} represented probably phenolic esters (RCOOAr). The absorption at 1740 cm^{-1} was due to the aliphatic esters (RCOOR′), phenol esters of aromatic acids (ArCOOAr′) and alkyl esters of aromatic acids (ArCOOR). The band at 1700 cm^{-1} was assigned mainly to carboxylic acids of aromatics esters (ArCOOR) and ketonic structures.

^{14}C labelling of lignite

The distribution of acidic OH groups in lignite of lithotype A (Bergheim) was investigated by Weber [33] and Große [11].

The content of alkylation-amenable phenolic OH groups of the lignite was calculated from OCH_3 content before and after methylation according to the equation:

$$\%\ \mathrm{OH} = \frac{0.548\times(\%\ \mathrm{OCH_3^2} - \%\ \mathrm{OCH_3^1})}{1 - 0.00452\times\%\ \mathrm{OCH_3^2}}$$

in which % OCH_3^1 is the content of methoxy groups of nontreated lignite, % OCH_3^2 the content of methoxy groups after total methylation followed by saponification of the resulting esters [33]. The methylated phenolic OH groups amounted to 4% of the total lignite dry weight. The content of COOH groups was estimated by FT-IR spectroscopy and ^{13}C NMR spectroscopy [11,33], at 10% of the lignite dry weight. Thus, the concentrations of phenolic OH and COOH correspond to 2.35 and 2.22 mmol g^{-1} lignite, respectively. Following the method of Liotta [24] these groups react quantitatively with alkyl iodide. The radioactivity in the ^{14}C[methyl iodide] solution used for alkylation was set to 0.6 MBq ml^{-1} corresponding to 16 mmol CH_3I. According to the total concentration of acidic OH which can be alkylated, and in view of the increased weight of the alkylated lignite, 4.57 mmol methyl groups per gram lignite were introduced. The final radioactivity amounted to 0.161 MBq g^{-1} lignite. Taking into account the molar distribution of COOH and phenolic OH in lignite, 0.078 MBq g^{-1} lignite was associated with COOH ester bonds.

Radioactivity in the ^{14}C[ethyl iodide] solution used for alkylation was set to 0.22 MBq ml^{-1} corresponding to 12 mmol CH_3CH_2I. According to the above calculations the radioactivity of the completely labelled lignite as well as that associated with COOH esters and with phenolic ethers amounted to 0.07, 0.035 and 0.039 MBq g^{-1} lignite, respectively.

These calculated values based on the available chemical data for the distribution of acidic OH groups are in accordance with the measured values in the labelled lignite, which were used as reference values in the following microbial degradation experiments (Table 1).

Ability of T. atroviride to cleave the introduced bonds

The methyl esters were hydrolyzed by *T. atroviride* 5 days after the labelled lignite was placed on the surface of pregrown mycelium. A continuous release of labelled volatile substances was measured for 21 further days. Surprisingly the ^{14}C radioactivity was released faster from the completely methylated coal than from that labelled only in COOH groups. Radioactivity in the Oxysolve-C-400 scintillation cocktail increased with completely methylated coal at a

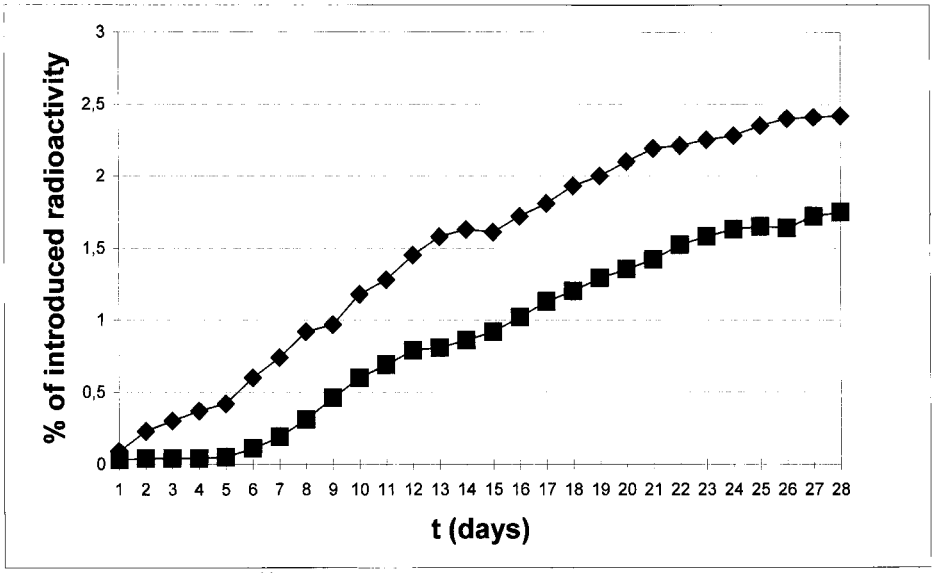

Figure 2 Cumulative release of radioactivity from ^{14}C-methylated lignite by *T. atroviride* (in percent of the measured total radioactivity before fungal treatment). (◆) Completely methylated lignite; (■) carboxylic OH methylated lignite.

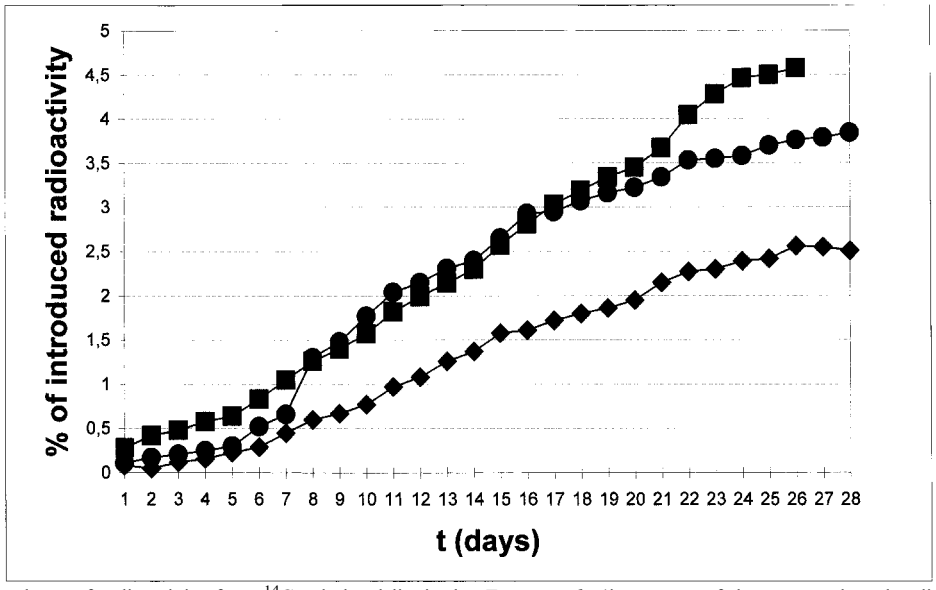

Figure 3 Cumulative release of radioactivity from ^{14}C-ethylated lignite by *T. atroviride* (in percent of the measured total radioactivity before fungal treatment). (◆) Completely ethylated lignite; (■) carboxylic OH ethylated lignite; (●) phenolic OH ethylated lignite.

constant velocity from the first day after application of the labelled lignite on the mycelium for the next 21 days, indicating that not only esters were hydrolyzed but also the phenolic ether fraction was degraded. A plateau of released radioactivity was reached with both ^{14}C-labelled lignite samples after 28 days. A total amount of 913 Bq g^{-1} lignite (representing 1.75% of the introduced radioactivity) was released from the carboxylic labelled lignite, 3045 Bq g^{-1} lignite (representing 2.42% of the introduced radioactivity) from the completely labelled lignite (Figure 2).

To prove the contribution of enzymes able to cleave ether bonds we investigated the release of radioactivity from lignite specifically labelled with ^{14}C[ethyl iodide] at the phenolic OH groups. The volatile compounds in experiments with ^{14}C[ethyl iodide]-labelled lignite were trapped by bubbling the perfusing gas at defined intervals through two flasks in series, the first containing saturated NaCl solution and the second Oxysolve-C-400 scintillation cocktail. No trapped radioactivity was detected in the NaCl solution, indicating that the radioactivity from ^{14}C[ethyl iodide] groups was released as $^{14}CO_2$.

The introduced ethyl ether and ester bonds were cleaved more efficiently than the corresponding methyl bonds. The radioactivity released by *T. atroviride* increased 3 to 5 days after addition of labelled lignite on the surface of pregrown fungal mycelium regardless of the introduced label (carboxylic, phenolic or completely labelled coal). After 25 days the velocity of radioactivity release decreased and reached a plateau in all three cases. Total amounts of 1770 (representing 4.78% of the introduced radioactivity), 2335 (representing 2.51% of the introduced radioactivity) and 2037 Bq g^{-1} lignite (representing 3.77% of the introduced radioactivity) were released from the carboxylic, completely and phenolic OH-labelled lignites, respectively (Figure 3).

Comparing the release of radioactivity from ^{14}C-methylated and ^{14}C-ethylated lignites led to the following conclusions: Contrary to the completely ^{14}C-labelled lignite from which radioactivity was released at comparable rates regardless of methyl or ethyl alkylation, ^{14}C-ethyl esters were hydrolyzed at a higher rate than the corresponding ^{14}C-methyl esters. Only coal labelled at phenolic OH showed small droplets of solubilized lignite on the mycelial surface, indicating that free carboxylic groups are necessary for coal solubilization.

To exclude nonenzymatic ester hydrolysis due to the pH of the culture medium, which increases during the growth of *T. atroviride* on media containing glutamate up to pH 9, control experiments were done with completely labelled coal. This coal was placed on solid agar in Petri dishes, which were not inoculated with the fungus, but contained a buffered medium at pH 9.2. Under these conditions small amounts of radioactivity (not more than 0.3% of the introduced radioactivity after 28 days) were released with a constant slope from the first day on. These values have been subtracted from those given in Figures 2 and 3.

Discussion

The participation of oxidizing and radical-forming enzymes in lignite depolymerization by the unspecific cleavage of C–C bonds has been investigated for 20 years. In contrast, the participation of hydrolytic enzymes in this process was a matter of dispute. Fakoussa and Hofrichter [8] postulated that an enzyme must possess at least two properties in order to be able to depolymerize lignite: (i) a low substrate specificity and (ii) an efficient participation of a mediator substance, which can penetrate into the complex lignite structure. Neither of the two properties seemed to be featured by hydrolytic enzymes. However, this report presents evidence that esterases secreted by *T. atroviride* contribute to lignite depolymerization. A recent analytical search for possible targets for extracellular hydrolytic enzymes in lignite structure revealed that 1–2% of the total carbon in lignite can be attributed to carboxylic esters [11]. Lissner and Thau [25] showed that ester groups are mainly in the aliphatic portion of lignite (bitumen) that can be extracted with toluene. This portion of ester groups can be characterized by determining the "saponification number" which can be assessed by saponification of the bitumen and titration of the carboxyl acids with chlorine acids. The presence of ester groups was confirmed by IR spectra of Australian lignite by

Supaluknari and coworkers [32]. They found a relationship between the amounts of ester groups and the H/C ratio in lignite and concluded that lignite with a higher H/C ratio contained a higher concentration of ester groups than lignites with a lower H/C ratio. The presence of ester groups in humic substances of soil, which can be seen as a precursor of lignite, was documented by Grasset and Ambles [10]. The authors showed that the main portion of humic substances was soluble in an acid milieu after saponification. Identification of the saponification products by gas chromatography revealed that ester groups were localized mainly in aliphatic fractions of the soil. Recently, hydrolysis experiments with several Rhenish lignites showed that the macromolecular portion of lignite, the insoluble matrix, can be partially hydrolyzed by strong bases [11].

The fraction of free carboxylic and phenolic OH groups, which can be alkylated, was estimated at 10% and 4% of the ash-free lignite dry weight, respectively [11,33]. The amounts of radioactivity introduced by ^{14}C ethylation of specific groups for the purpose of investigating the microbial attack were in keeping with these results.

Alkylated lignite was a less suitable substrate for fungal attack due to the introduction of alkyl groups, which considerably altered the chemical nature of lignite. Hydrophilic functional groups such as the carboxylic and phenolic OH were thus masked, increasing the hydrophobicity of lignite. Consequently, biosolubilization of lignite was decreased. The fact that only lignite, which was ethylated at phenolic OH groups, was partially solubilized indicates that lignite biosolubilization requires free carboxylic groups. Lignite solubilization by both ascomycetes and deuteromycetes should not be mixed up with the process of lignite mineralization by basidiomycetes, especially by the white rot fungi. Ralph and Catcheside [29] reported that the white rot fungus *Phanerochaete chrysosporium* depolymerized "cold" methylated lignite more effectively than untreated lignite. The authors postulated that nonphenol methylation hindered a possible repolymerization of the phenoxy radicals formed by oxidation of nonphenolic aromatic rings through the involved lignin peroxidase.

Although water was supposed to be a limiting factor for the hydrolytic cleavage of bonds in the hydrophobic alkylated coal, *T. atroviride* was able to cleave ester bonds as well as phenolic ether structures, ethyl ester being hydrolyzed best. Independent chemical analysis of the biodegradation products showed that up to 50% of ester bonds in lignite were not detectable following fungal attack by *T. atroviride* (unpublished data). Since also ^{14}C-phenolic ether structures were degraded, an oxidative attack on them can be concluded, probably due to the action of laccases. Laccase, although only with low degradation potential, was described in *Trichoderma* sp. by Assavaning and coworkers [1]. The ability of laccase to demethylate lignin, a polymer comparable to lignite, in dependence on various mediator substances has so far been reported only for white rot fungi [3].

The completely ethyl-labelled lignite was degraded with the lowest rate referred to the percentage of released radioactivity. This can be due to a different treatment of lignite samples for specific ^{14}C labelling. Specific introduction of ester and ether bonds requires an additional saponification step, which might decrease the mean diameter of the lignite particles and thus increase the surface to volume ratio. Due to the increased hydrophobicity of the alkylated lignite, it is probable that only ligands at the surface of the particles could be attacked. Consequently, the radioactivity released from the completely ethylated lignite (with lower surface to volume ratio) was lower as compared with specifically labelled samples (after saponification). In contrast, the release of radioactivity from the completely ^{14}C-methylated lignite was higher than from the esterified samples. This does not indicate that *T. atroviride* is able to oxidize methanol to CO_2. It would be more reasonable to postulate that the suggested laccase of the fungus also caused lignite fragmentation. The resulting fragments would contain the labelled carbon atom, which could be further oxidized intracellularly without requiring a specific C_1 metabolism, which is known in eucaryotes only in some methanol-utilizing yeasts (e.g., *Candida* or *Pichia* spp.) [23,27]. However, the metabolic fate of the introduced label in *T. atroviride* is only of minor interest for this communication. The objective of this work was to provide evidence for the ability of the fungus to use extracellular enzymes to hydrolyze esters and also to attack phenolic ethers in the bulky lignite structure.

Acknowledgements

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