*Biodegradation* **12:** 443–453, 2001.



# Biotransformation of linear alkylbenzene sulfonate (LAS) by *Phanerochaete chrysosporium* : oxidation of alkyl side-chain

Jagjit S. Yadav[2], David L. Lawrence[3], Barbara A. Nuck[3], Thomas W. Federle[3] & C. Adinarayana Reddy[1]*

[1]*Department of Microbiology and the NSF Center for Microbial Ecology, Michigan State University, East Lansing, MI 48824-1101, USA;* [2]*Department of Environmental Health, Division of Molecular Toxicology, University of Cincinnati, Cincinnati, OH 45267-0056, USA;* [3]*Environmental Science Department, The Procter & Gamble Company, P.O. Box 538707 , Cincinnati, OH 45253-8707, USA (*author for correspondence)*

Accepted 14 December 2001

*Key words:* biodegradation, biotransformation, linear alkylbenzene sulfonate, *Phanerochaete chrysosporium*, sulfophenyl carboxylate, white rot fungus

**Abstract**

The white rot fungus *Phanerochaete chrysosporium*, which generally mineralizes substituted aromatics to $CO_2$, transformed linear alkylbenzene sulfonate (LAS) surfactants mainly at their alkyl side chain. Degradation of LAS was evidenced by a zone of clearing on LAS-containing agar plates and colorimetric analysis of liquid cultures. Disappearance of LAS was virtually complete within 10 days in low nitrogen (2.4 mM N), high nitrogen (24 mM N) and malt extract (ME) liquid media. After 5 days of incubation in ME medium, transformation of LAS was complete at concentrations $\leq$4 mg $l^{-1}$, but decreased at higher concentrations. The LAS degradation was not dependent on lignin peroxidases (LiPs) and manganese-dependent peroxidases (MnPs). Mineralization of $^{14}$C-ring-LAS to $^{14}CO_2$ by *P. chrysosporium* was $<$1% regardless of the culture conditions used. Thin layer chromatography and mass spectral analyses indicated that *P. chrysosporium* transformed LAS to sulfophenyl carboxylates (SPCs) through oxidative shortening of the alkyl side-chains. While LAS disappearance in the cultures was not dependent on LiPs and MnPs, transformation of the parent LAS moieties to SPCs was more extensive in low N medium that favors expression of these enzymes. The SPCs produced in LN cultures were shorter in chain- length than those produced in ME cultures. Also there was a notable shift in the relative abundance of odd and even chain length metabolites compared to the starting LAS particularly in the low N cultures suggesting the possible involvement of processes other than or in addition to $\beta$-oxidation in the chain-shortening process.

*Abbreviations:* LAS – linear alkylbenzene sulfonate, SPC – sulfophenyl carboxylates, LiP – lignin peroxidase, MnP– manganese peroxidase, ME – malt extract, N – nitrogen, TLC – thin layer chromatography, LC – liquid chromatography, MS – mass spectrometry

## Introduction

*Phanerochaete chrysosporium* is a widely studied white rot fungus which grows rapidly and mineralizes lignin more extensively than most other wood rotting fungi. It has attracted worldwide attention because of its ability to degrade a broad range of aromatic and substituted aromatic pollutants such as dioxins, polychlorinated biphenyls (PCBs), chlorophenols, chlorinated pesticides, polycyclic aromatic hydrocarbons (PAHs), trinitrotoluene (TNT), and azo dyes (Aust 1990; Hammel 1992; Pszczynski & Crawford 1995; Reddy 1995). Moreover, degradation of some of these compounds has been observed to occur when presented either alone or in a mixture with related pollutants. Such versatile degradative ability makes this fungus an attractive candidate for bioremediation of industrial waste disposal sites, which are usually contaminated

with a mixture of chemical pollutants rather than a single compound.

*P. chrysosporium* has been found to mineralize aromatic rings substituted with functional groups such as —OH, —COOH, —Cl, —$NH_2$, —$NO_3$, —$SO_3$ (Hammel 1992). Even compounds with unsubstituted or relatively less substituted aromatic rings such as benzene and toluene are mineralized to $CO_2$ by this organism (Yadav & Reddy 1993a). Degradation of many of the aromatic compounds by *P. chrysosporium* has been ascribed to the activity of its lignin-degrading enzyme system (LDS) including lignin peroxidases (LiPs) and/or manganese-dependent peroxidases (MnPs) (Hammel 1992). However, other aromatic compounds such as DDT, phenanthrene, 2,4,5-T and BTEX hydrocarbons are degraded without the involvement of LiPs and MnPs (Kohler et al. 1988; Sutherland et al. 1991; Yadav & Reddy 1993a, b; 1995).

Linear alkylbenzene sulfonate (LAS) is an anionic surfactant, which is used widely in laundry detergents worldwide. It has been used in the U.S. since 1965 when it served as a replacement for branched alkylbenzene sulfonate in detergent formulations. It accounts for 28% of the total annual production (3–4 million metric tons) of synthetic surfactants in Western Europe, Japan, and the United States (Werdelmann 1984; McEnvoy & Giger 1986). Approximately 270,000 metric tons of LAS have been produced in the U.S. every year over the last decade (Rapaport & Eckhoff 1990). Commercial LAS is a mixture of homologs with alkyl side-chains ranging from 10 to 15 carbons in length. It is degraded by a number of bacteria under aerobic conditions. A community of bacteria rather than a single species is generally involved in its mineralization (Swisher 1987; Sigoillot & Nguyen 1992; Breen et al. 1992; Campos-Garcia et al. 1999; Schleheck et al. 2000).

In this study, we focused on the metabolism of LAS by the fungus *P. chrysosporium*, including the rate and extent of degradation, the possible involvement of its extracellular peroxidases LiPs and MnPs in degradation, and transformation of the parent LAS to metabolites in order to identify the mechanism of fungal degradation. Our results indicate that *P. chrysosporium*, in contrast to its known ability to cleave and mineralise aromatic rings with or without substitution (Hammel 1992; Reddy 1995), shows negligible mineralization of LAS but extensively transforms it into polar metabolites, primarily sulfophenyl carboxylates (SPCs) of varying chain-length. The results further show that transformation of LAS may involve processes other than or in addition to beta oxidative shortening of the side-chain, which has been observed in bacteria (Sigoillot & Nguyen 1992; Schleheck et al. 2000).

## Materials and methods

### *Fungal strains and mutants*

*P. chrysosporium* strain ME-446 (ATCC 34541) was from the American Type Culture Collection (ATCC). Three mutants of this organism were also used. These included: a lignin peroxidase-negative mutant (*lip5b*) which fails to produce LiPs but produces MnPs (Boominathan et al. 1990a), a peroxidase-negative mutant (*per*) which fails to produce both LiPs and MnPs (Kim K-J, Dass S.B., Yadav J.S. & Reddy C.A., unpublished data), and a nitrogen-deregulated mutant (*der8-5*) which, unlike the wild type, produces LiPs and MnPs in high nitrogen medium (Boominathan et al. 1990b).

### *Chemicals and radiochemicals*

An LAS mixture, sodium dodecylbenzene sulfonate (linear side chain with an average length of 12 carbons), was purchased from Sigma. (80% purity). $^{14}$C-(U-ring)-dodecylbenzene sulfonate (DBS) sodium salt with a specific activity of 23.7 mCi $(\text{mMole})^{-1}$ equal to $8.76 \times 10^8$ Bq $(\text{mMole})^{-1}$ and a radiochemical purity >96.3% was synthesized by Dupont NEN products.

### *Media and inocula*

Defined low nitrogen basal III medium (low N medium) with 2.4 mM N, defined high nitrogen medium (high N medium) with 24 mM N, and malt extract medium (ME medium) with 8 mM N and 2% glucose, were described previously (Yadav & Reddy 1993a). Agar plugs (7 mm diameter) for the plate assay were cut from ME agar, which had been inoculated with the fungus and incubated at 37 °C for 7 days. Liquid cultures were inoculated with 10% (v/v) of a blended mycelial inoculum which was prepared as described previously (Yadav & Reddy 1993a).

*Agar plate assays*

Assay plates consisted of agar media amended with a colony inducer (0.02% w/v sodium deoxycholate) and 100 $\mu$g ml$^{-1}$ LAS. The LAS-containing agar plates were inoculated with mycelial plugs (described above) and incubated for 7 days. Degradation of LAS in agar plates was detected as described by Breen et al. (1992). A clear zone surrounding the fungal colony was indicative of LAS degradation. Well-format of the agar plate assay was used to study the role of extracellular peroxidases in LAS clearance. Wells (8 mm) made in the agar matrix were filled with extracellular culture fluid (ECF) from 6 day-old LN cultures showing normal LiP (103 units l$^{-1}$) and MnP activities (3066 units l$^{-1}$) as measured by the procedures of Tien and Kirk (1988) and Paszczynski et al. (1988), respectively. The assay plates were incubated as above for 24 hours and examined for zones of clearance around the wells. The well assay was also performed using simulated LiP and MnP reaction mixtures using conditions as described previously (Yadav and Reddy, 1993a). The MnP reaction also contained $MnSO_4$ (0.4 mm) as source of $Mn^{+2}$.

*Liquid cultures*

LAS liquid cultures (50 ml in 125-ml Erlenmeyer flasks) were inoculated with blended mycelial inoculum (10% v/v) and the culture headspace was flushed with pure oxygen for one min (Yadav & Reddy 1993a). The cultures were incubated with shaking (200 rpm) at 37 °C. Everyday the headspace was reflushed (1 min) with oxygen. Uninoculated medium and cultures without LAS were included as controls. Following incubation, the residual concentration of LAS in experimental and control flasks was analyzed by the colorimetric MBAS method described below.

Sensitivity of *P. chrysosporium* to LAS was determined by growing the fungus in ME medium with increasing concentrations of the compound (2.5 to 400 mg l$^{-1}$). After incubation for 10 days at 200 rpm, mycelial dry weights of the cultures were determined as described previously (Yadav & Reddy 1993a).

*Colorimetric determination of LAS*

The classical methylene blue method (Longwell & Maniece 1955) as modified by Slack (1959) was used to measure LAS in liquid cultures. The procedure was further modified by including an acid hydrolysis prestep for desorption of LAS from the fungal mycelium, as suggested by Allred et al. (1964). The desorption/extraction procedure used for LAS recovery from *P. chrysosporium* cultures was optimized by including heat-killed (autoclaved) controls and chemically-killed (using 1 mM sodium azide) controls. Both these controls were prepared by inactivating a culture pregrown under conditions identical to those used for the experimental cultures, so that the fungal biomass in heat killed- or chemically-killed controls is comparable to that in experimental cultures. The cultures (50 ml) in 125-ml Erlenmeyer flasks were acidified to 1N HCl followed by boiling for 30 min and cooling to room temperature. The acid-hydrolyzed cultures were neutralized with 10% NaOH and the volume was made up to 100 ml with distilled water. The samples were then subjected to a single-step extraction with chloroform as described by Slack (1959). The absorbance (650 nm) of the chloroform extract was measured using a Varian Spectrophotometer (CARY 219, Varian Instruments, Sugar Land, TX). The LAS content was derived from a standard curve prepared by using a set of LAS standards (25 to 150 $\mu$g LAS). The net residual LAS content in a culture was obtained by subtracting the background value obtained for a parallel control culture lacking LAS. Samples containing more than 150 $\mu$g of LAS were diluted to be within the recommended range (25 to 150 $\mu$g) for the assay procedure. The glassware used for the procedure was rinsed with a 10% (v/v) solution of concentrated HCl in methanol as suggested by Webster & Halliday (1959).

*Mineralization studies*

Uniformly ring-labeled $^{14}$C-dodecylbenzene sulfonate sodium salt ($10^5$ cpm) along with its unlabeled form (2 mg l$^{-1}$) were added to 10-ml static cultures (in triplicate) of *P. chrysosporium* immediately after inoculation. At specified intervals during incubation, the $^{14}CO_2$ evolved was trapped by flushing the headspace with $CO_2$-free air for 20 min and quantified as previously described (Yadav & Reddy 1993b). The cultures were reoxygenated for 1 min after each $^{14}CO_2$-trapping.

*Analysis of residual radioactivity*

After quantifying the total $^{14}CO_2$ released, the triplicate cultures were pooled and acidified to pH 2.0 and centrifuged (10,000 rpm for 10 min) to separate the mycelial pellet from the supernatant. The pellet was

blended (5 min) in an Omni mixer with 5 ml of methanol. This process was repeated twice and the extracts combined. The supernatant was flash frozen, lyophilized and likewise extracted three times with methanol. To quantify $^{14}C$, 1 ml aliquots of the extracts were mixed with scintillation fluid (Safety Solve) and analyzed by a liquid scintillation counter (model 6892 Trackor Analytic).

*Analyses for metabolites*

The extracts were analyzed for metabolites using Radio-TLC and mass spectrometry (MS). Subsamples of the methanol extracts were spotted onto Whatman K6 (250 mm, 60 A) silica gel TLC plates and developed in chloroform/methanol/water/formic acid (80/25/3/1). Radioactivity on the plates was localized and quantified using a Bioscan Imaging Scanner (Bioscan) and $R_f$ values were compared to LAS standard (Dupont NEN Products). The methanol extracts of mycelia and lyophilized supernatants were combined and analyzed for sulfophenyl carboxylate and LAS homologs by flow injection mass spectroscopy. Measurements were carried out with a Quattro tandem mass spectrometer (VG Biotech) using negative ion electrospray ionization. The mass spectrometer measurements were made by scanning over the mass range of 50–500 with a cycle time of 2.1 s. MS/MS measurements were also made by scanning the mass range of 50–450 with a cycle time of 2.1 s. The collision cell was operated at 40 eV with argon as the target gas at a pressure such that the ion beam was attenuated by 75%. Samples were analyzed by flow injection using an integrated LC pump and autosampler (HP1050 series, Hewlett-Packard GmbH). Injections were made into a stream of water/methanol (1:1, v/v) at a flow rate of 0.1 ml $min^{-1}$. To confirm identification and more accurately quantify the various species, the samples also were analyzed by liquid chromatography (LC) combined with negative electrospray mass spectroscopy. The LC separation was achieved using an Alltech $C_3$ column (25 cm $\times$ 4.6 mm) and a linear gradient of water and methanol.

## Results and discussion

*Degradation of LAS*

*P. chrysosporium* showed LAS degradation as evidenced by a distinct zone of clearance underneath and around the colony on ME agar supplemented with LAS. The diameter of the zone of clearance was typically 2.0 to 2.3 cm. Zones of clearance were also seen in low N and high N media supplemented with LAS. The size of the clearance shown by the wild type *P. chrysosporium* was approximately twice as large in high N medium as compared to low N medium (Table 1). Degradation of LAS by *P. chrysosporium* was further confirmed by liquid culture studies (Figure 1) based on the colorimetric determination of residual LAS. Almost 100% of the LAS added (2 mg $l^{-1}$) was degraded in about 6 days by *P. chrysosporium* in ME cultures. Concurrent with the disappearance of LAS, there was a loss of foaming in the cultures. To insure that the disappearance of LAS was not due to sorption to the mycelia, the LAS estimation procedure included a prestep for desorption of LAS from fungal mycelium as described in Methods. The efficiency was verified by recovering the total amount of LAS added to parallel heat killed controls and chemically killed controls. Comparable LAS degradation was observed in low N and high N liquid media (data not shown).

*Table 1.* Degradation of LAS by *P. chrysosporium* and its mutants based on agar plates[a]

| Strain[b] | Diameter of the zone of clearance (cm) | |
|---|---|---|
| | Low N medium | High N medium |
| Wild type | 0.3 | 0.6 |
| *per* mutant | 0.4 | 0.3 |
| *der-8-5* mutant | 0.5 | 0.5 |
| *lip5b* mutant | 0.3 | 0.4 |

[a] The assay was performed by inoculating low N (2.4 mM N) and high N (24 mM N) agar plate media containing 100 $\mu$g $ml^{-1}$ of LAS with each strain followed by incubation at 37 °C for 7 days and staining the plates as described in Materials and methods.
[b] *Per* (peroxidase-negative mutant), *lip5b* (lignin peroxidase-negative mutant), and *der8-5* (nitrogen-deregulated mutant) are mutants of *P. chrysosporium* ME-446.

Virtually 100% of the LAS was transformed when its concentration in the medium did not exceed 4 mg $l^{-1}$, but the rate of transformation was lower at higher concentrations (Table 2). To determine whether the decrease in LAS degradation at higher concentrations was due to inhibition of growth of *P. chrysosporium* by LAS, the influence of increasing LAS concentrations (2.5 to 400 mg $l^{-1}$) on mycelial dry weights was determined (data not shown). LAS did not inhibit the growth at concentrations up to 40 mg $l^{-1}$, but there was an approximate 50% inhibition of growth at 100 mg $l^{-1}$ and growth was negligible at concentrations beyond 200 mg $l^{-1}$. These

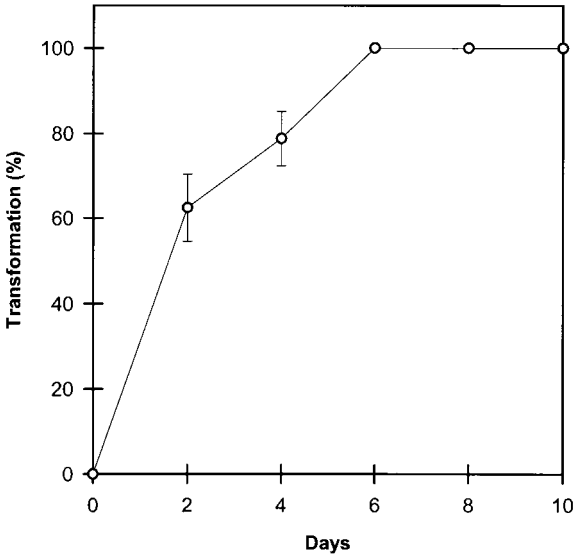


*Figure 1.* Time course of degradation of LAS by *P. chrysosporium*. The fungus was grown as 50 ml shaken cultures in malt extract medium spiked with LAS @ 2 mg $l^{-1}$. Incubation was carried out at 37 °C for varying periods of time. Values represent means ± standard deviations for triplicate cultures.

*Table 2.* Influence of initial concentration of LAS on its degradation by *P. chrysosporium*[a]

| LAS concentration (mg $l^{-1}$) | % Transformation |
|---|---|
| 1.0 | 100.0 ± 0 |
| 2.0 | 94.9 ± 3.9 |
| 4.0 | 96.0 ± 4.4 |
| 10.0 | 65.6 ± 5.2 |
| 20.0 | 47.8 ± 5.4 |

[a] The fungus was grown as 50 mL shaken cultures in malt extract medium at 37 °C for 5 days. Values are based on triplicate cultures.

results show that the lower percent degradation of LAS observed at concentrations >4 mg $l^{-1}$ is not due to growth inhibition. It is also worth noting that the LAS concentrations reported in natural environments are much lower than the concentrations that were found to be inhibitory to growth in this study (Rapaport & Eckhoff 1990).

*Non-involvement of ligninolytic peroxidases*

Agar plate assays indicated that wild type *P. chrysosporium* exhibits higher transformation of LAS on high N medium and ME medium (Table 1) in which the LiPs and MnPs are not produced (Yadav & Reddy 1993a), than in low N medium (Table 1), in which a full complement of LiPs and MnPs are known to be expressed (see Reddy & D'Souza 1994). As mentioned above, similar pattern of degradation was observed in the three media in liquid cultures suggesting that LiPs and MnPs are not essential for LAS transformation by *P. chrysosporium*. To further confirm these results, a *per* mutant which lacks the ability to produce LiPs and MnPs (Kim K-J, Dass SB, Yadav JS & Reddy CA, unpublished data) and a *lip* mutant which lacks the ability to produce LiPs (Boominathan et al. 1990a) were assayed for their ability to transform LAS (Table 1). Both the mutants exhibited LAS transformation analogous to that seen with the wild type (WT) *P. chrysosporium*. Also, comparable transformation was observed in high N medium by the WT and a nitrogen-deregulated mutant (*der-8-5*), which unlike the WT is capable of producing LiPs and MnPs in high N medium (see Reddy & D'Souza 1994). These results further confirm that LiPs and MnPs are not essential for LAS transformation by *P. chrysosporium*. These data on the non-involvement of LiPs and MnPs in LAS degradation are consistent with similar conclusions on the degradation of other organopollutants such as DDT (Kohler et al. 1988), phenanthrene (Sutherland et al. 1991), 2,4,5-trichlorophenoxyacetic acid (Yadav & Reddy 1993b), BTEX compounds (Yadav & Reddy 1993a), chlorobenzenes and PCBs (Yadav et al. 1995) by *P. chrysosporium*. Furthermore, the degradation activity of *P. chrysosporium* in the plate assay was observed only with the whole organism and not with extracellular peroxidases when tested using either the extracellular culture fluid (ECF) containing LiPs (103 units $l^{-1}$) and MnPs (3066 units $l^{-1}$) or using simulated *in vitro* LiP or MnP enzyme reactions as described under Materials and methods.

*Mineralization of LAS*

*P. chrysosporium* has been shown to mineralize the aromatic rings of chlorobenzenes, chlorophenols, chloroanilines, chlorophenoxy herbicides (2,4-D and 2,4,5-T), DDT, PCBs, dioxins, PAHs (phenanthrene, anthracene, benzopyrene etc), TNT, and azo dyes (see Aust 1990; Hammel 1992; Pszczynski & Crawford 1995; Reddy 1995). In experiments with typical incubations of 30 days, the amount of mineralization of these aromatic compounds has been reported to vary from 1% to 75%. In our studies, *P. chrysosporium* was also shown to mineralize unsubstituted and relatively less substituted aromatic rings as in the case of benzene and toluene (Yadav & Reddy 1993a). In another study, sulfonated azo dyes were efficiently mineral-

ized by *P. chrysosporium* and the substitution pattern did not significantly influence the susceptibility of the aromatic ring to degradation (Paszczynski et al. 1992). Our results show that less than 1% of the uniformly ring-labeled $^{14}$C-LAS was mineralized by *P. chrysosporium*. The mineralization values were 0.5%, 0.35%, and 0.63% in low N, high N, and ME cultures after 32 days of incubation. This level of degradation is insignificant in that it may represent impurities present in the radiolabelled preparation. The extent of mineralization remained unchanged irrespective whether LAS was added right after inoculation or to 5 days-pregrown cultures (data not shown). This lack of mineralization of LAS in spite of extensive biotransformation of the parent LAS structure led us to extract and characterize the radioactivity that is present in the culture supernatant and that associated with the mycelia.

*Characterization of degradation products*

The total radioactivity recovered from the cultures and the fractional distribution of the recovered label were found to be different for the low N culture and the ME culture (Table 3). In the ME culture, about 35% of the total extractable radioactivity was associated with the mycelia, while about 65% was in the culture supernatant. In contrast, only about 5% of the extractable radioactivity in low N culture was associated with the mycelia, while the rest (95%) was in the supernatant.

Complete metabolism of LAS in bacterial communities has been shown to involve three key steps: terminal oxygenation and shortening of the alkyl side-chain (via $\omega$-oxygenation and $\beta$-oxidation), desulfonation, and aromatic ring cleavage (Sigoillot & Nguyen 1992; Kertesz et al. 1994; Schleheck et al. 2000). In the light of this, we examined the extracts of LAS-metabolizing fungal cultures for possible metabolic intermediates. Radio-TLC chromatograms of the methanol extracts of the mycelia and culture supernatants from *P. chrysosporium* cultures grown in ME and Low N media are shown in Figure 2. The mycelial extracts from the ME culture contained parent LAS as well as one major peak and two minor peaks, which were more polar than LAS. The mycelial extract from the low N culture contained parent LAS, three minor polar peaks and an indistinct peak, which was slightly less polar than LAS. The latter represented less than 1% of the total radioactivity recovered from the culture. The supernatants from the cultures contained low levels of parent LAS and several polar peaks. The low N culture contained a greater number of peaks, which were more polar, than those present in the ME culture. Based upon the analysis of both the mycelial and supernatant extracts, 14% of the parent LAS remained in the ME culture, while 86% had been converted to more polar metabolites. In low N culture, 9% of the parent LAS remained, while 90% was recovered as polar metabolites and less than 1% as less polar materials. The peaks in low N culture were not only more in number but had higher polarity than those in the ME culture.

The mycelial extracts and supernatants were combined and subjected to negative ion electrospray MS and MS/MS analysis (Figure 3). For illustration, the MS for the starting LAS and the extracts of the ME broth cultures are shown in Figure 4 (top and middle panels). In the control LAS spectrum (top panel), ions representing $C_{10}$, $C_{11}$, $C_{12}$ and $C_{13}$ LAS are prominent. However, in the ME culture sample (middle panel), the levels of these LAS ions were diminished, and replaced with the major ions, *m/z* 313, 327, 341 and 355, which are consistent with the presence of $C_9$, $C_{10}$, $C_{11}$ and $C_{12}$ sulfophenyl carboxylates (SPCs). The identity of these ions was further confirmed by MS/MS, illustrated by the bottom panel for the low N culture. Under MS/MS conditions LAS and SPCs fragment to form a daughter ion of *m/z* 183. By setting the mass spectrometer to scan only the parent ions, which have a daughter ion with *m/z* 183, the analysis became more selective since only LAS and materials derived from LAS are detected. In these analyses, the major ions were consistent with the presence of SPCs. In addition, trace levels of *m/z* 273, 287, 301, 329 343, 357 and 371 were detected, which could represent sulfophenyl dicarboxylates (SPDCs). Further confirmation and quantification of SPCs was obtained by LC/MS/MS using authentic $C_4$, $C_5$ and $C_{11}$ , SPCs as standards.

Figure 4 shows the distribution of chain lengths in the starting LAS as well as those for residual LAS and SPCs recovered following incubation based upon LC/MS/MS. The LAS remaining in the low N culture was very similar to the starting material, while the distribution of chain lengths of LAS remaining in ME grown culture was depleted in $C_{13}$. This observation suggests a preference for oxidizing the longer chain lengths or poorer recovery of these longer chains from the more massive mycelium in the ME cultures. Large differences were observed in SPC distribution in the cultures grown on different media. The SPCs recovered from the ME cultures were primarily $C_9$,

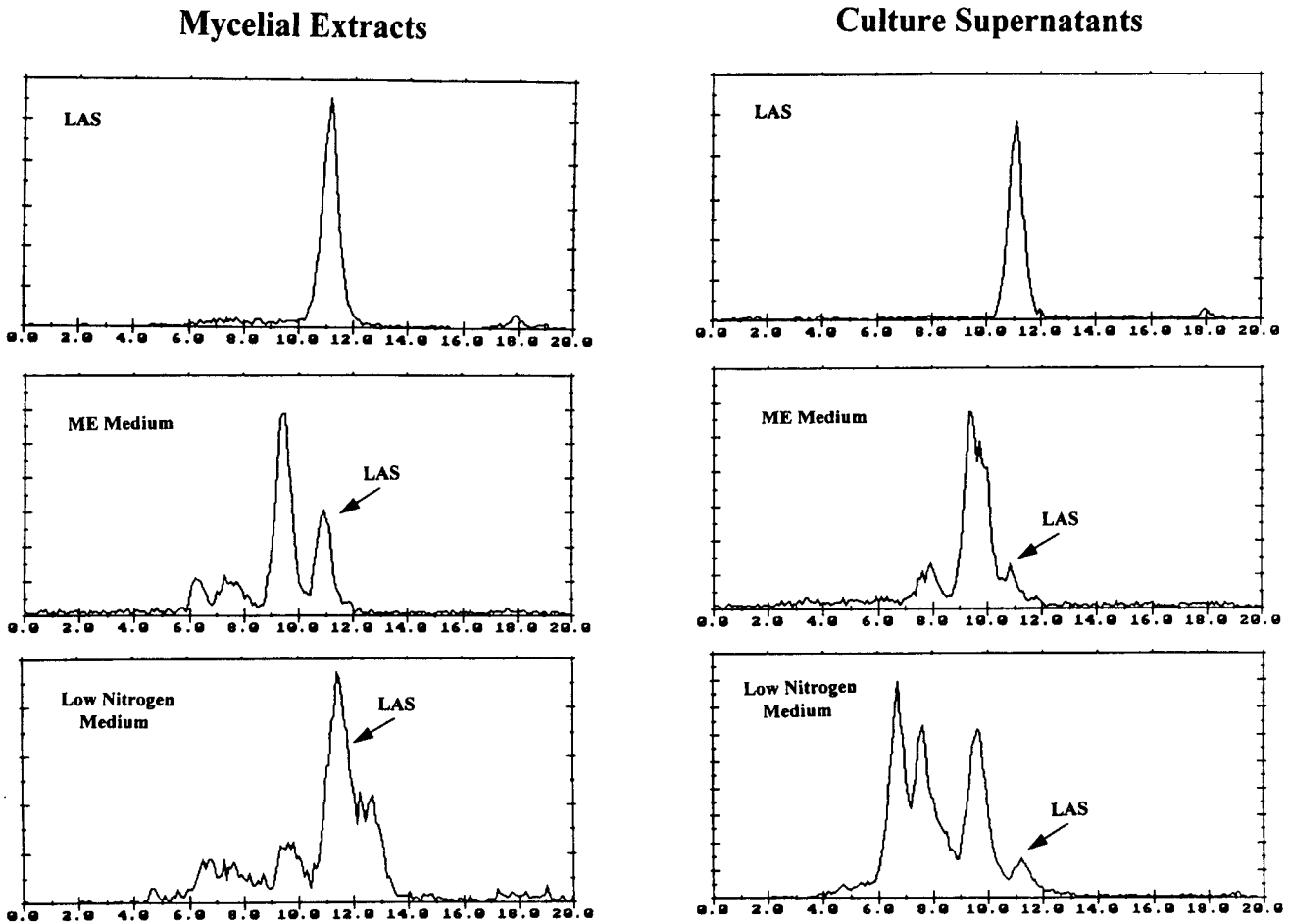


*Figure 2.* Radio-TLC chromatograms of the mycelial extracts (left) and culture supernatants (right) of *P. chrysosporium* cultures grown in ME and low N media as described in Materials and methods. Chromatograms of the LAS standard are given for comparison.

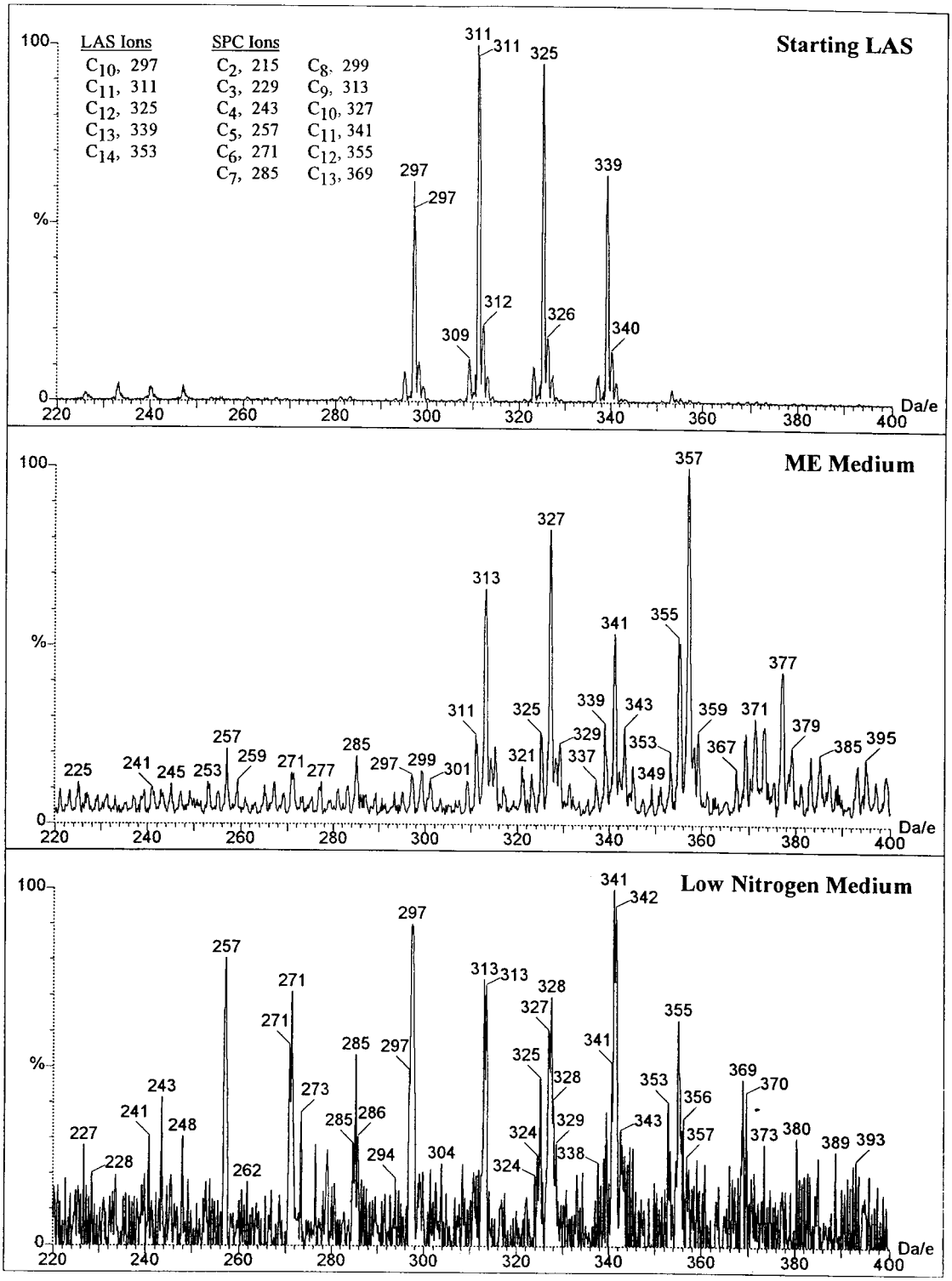


*Figure 3.* Negative ion electrospray mass spectra (MS) of the starting LAS (top) and the combined methanol extracts of mycelia and lyophilized supernatants of a *P. chrysosporium* culture grown in ME medium (middle). Negative ion electrospray MS/MS spectra showing parent ions with a *m/z 183* daughter ion of the combined methanol extracts of mycelia and lyophilized supernatants of a *P. chrysosporium* culture grown in low nitrogen medium (bottom).

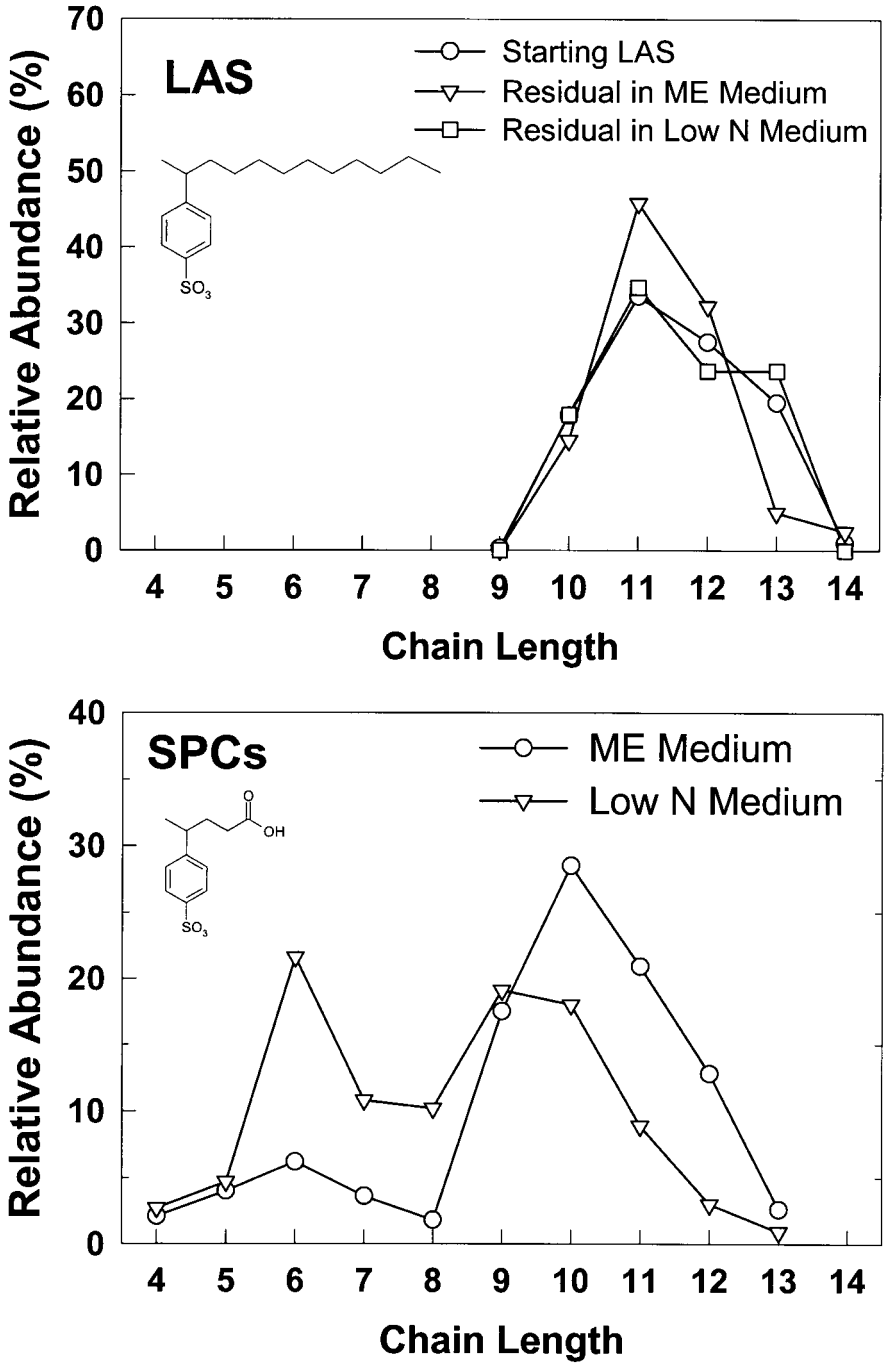


*Figure 4.* Distribution (relative abundance) of chain lengths of the starting LAS and residual LAS in *P. chrysosporium* cultures grown in ME and low nitrogen media (top). Distribution (relative abundance) of chain lengths of the sulfophenyl carboxylates (SPCs) formed by *P. chrysosporium* when grown in ME and low N media (bottom).

*Table 3.* Mass balance analysis of $^{14}$C-ring-LAS degradation by *P. chrysosporium*[a]

| Culture | Percent distribution of recovered $^{14}$C in different fractions | | | | Total $^{14}$C recovery (%) |
|---|---|---|---|---|---|
| | | | Mycelial fraction | | |
| | $^{14}CO_2$ | Culture supernatant | Methanolic extract | Unextractable | |
| Low N culture | 0.4 | 89.2 | 4.7 | 0.1 | 94.4 |
| ME culture | 0.3 | 52.5 | 21.3 | 6.9 | 80.9 |

[a] Cultures were grown in low N medium and malt extract medium at 37 °C for 10 days as described in Materials and methods.

$C_{10}$, $C_{11}$ and $C_{12}$ chain lengths, which accounted for 80% of the total. SPCs shorter than $C_9$ accounted for only 18% of the total. Notably, no $C_{14}$ SPCs and a very low level of $C_{13}$ SPCs were observed, indicating that the $C_{14}$ and $C_{13}$ LAS side-chains were being shortened in addition to being oxidized. Furthermore, the distribution of $C_9$ to $C_{13}$ SPCs relative to that of the $C_{10}$ to $C_{14}$ LAS homologs was suggestive of a single carbon reduction in chain length.

The SPCs recovered from the low N culture were shorter on average than those from the ME culture. $C_9$, $C_{10}$, $C_{11}$ and $C_{12}$ chain lengths accounted for 49% of the total., while SPCs shorter than $C_9$ accounted for 50%. A significant level of *m/z* 273 also was observed indicating the potential presence of $C_4$ SPDC. These observations are consistent with the more polar radio-TLC peaks and the higher level of radioactivity in the supernatant compared to the mycelial extract in this culture. Interestingly, the distribution of $C_9$ to $C_{13}$ SPCs relative to that of the $C_{10}$ to $C_{14}$ LAS homologs was suggestive of two or more (rather than a single) carbon reduction in chain length. One approach for examining the mechanism of side-chain shortening is to compare the ratio of odd to even chain lengths for the SPCs and LAS. Normal $\beta$-oxidation would preserve this ratio, while $\alpha$ or a combination of $\alpha$- and $\beta$-oxidation would shift this ratio. The starting LAS had an odd-even ratio of 1.15 : 1. In contrast, the odd-even ratio for SPCs recovered from the ME and low N incubations were 0.94 : 1 and 0.80 : 1, respectively, further suggesting that side chain shortening does not occur solely by $\beta$-oxidation. This is interesting in view of the fact that bacterial transformation of LAS predominantly involves $\beta$-oxidation of the side chain (Sigoillot & Nguyen 1992; Schleheck et al. 2000).

Our results provide the first evidence for the ability of a white rot fungus to transform a long, saturated n-alkane-type side-chain of an aromatic structure, as in LAS. However, metabolism of a substituted short aliphatic side-chain, as in 2,4,5-trichlorophenoxyacetatate (2,4,5-T), by *P. chrysosporium* has been observed in previous work (Yadav & Reddy 1993a; Reddy et al. 1997). In a study using model sulfonated aromatic compounds with substituted nucleus (Muralikrishna & Renganathan 1993), LiP isolated from *P. chrysosporium* has been shown to catalyze desulfonation of 3,5-dimethyl-4-hydroxy and 3,5-dimethyl-4-aminobenzenesulfonic acids. In low N cultures which express a full complement of LiPs and MnPs, desulfonated products were theoretically expected from a sulfonated aromatic such as LAS. However, no desulfonated intermediates of LAS were detectable either in low N or in ME cultures indicating the role of alkyl chain length and presence of other substituents on the aromatic ring in desulfonation.

In conclusion, *P. chrysosporium* oxidizes and shortens the side-chain of LAS resulting in the formation of sulfophenyl carboxylates. On the average, oxidation and chain shortening is more extensive in low N cultures than in ME cultures. Chain shortening appears to involve mechanisms other than or in addition to $\beta$-oxidation, which is divergent from that documented in bacterial systems.

## Acknowledgments

This work was supported in part by grant DE-FG02-85 ER 13369 from the U.S. Department of Energy and grant BIR 912-006 from the NSF Center for Microbial Ecology. We thank Alec Breen of the University of Tennessee for providing the commercial LAS mixture for initial exploratory experiments. Technical assistance by R. J. Wiederholt and C. S. Merritt is acknowledged.

## References


Allred RC, Setzkorn EA & Huddleston RL (1964) A study of detergent biodegradability as shown by various analytical techniques. J. Amer. Oil. Chem. Soc. 41: 13–17

Aust SD (1990) Degradation of environmental pollutants by *Phanerochaete chrysosporium*. Microbial Ecol. 20: 197–209

Boominathan K, Dass SB, Randall TA, Kelley RL & Reddy C A (1990a) Lignin peroxidase-negative mutant of the white-rot basidiomycete *Phanerochaete chrysosporium*. J. Bacteriol. 172: 260–265

Boominathan K, Dass SB, Randall TA & Reddy CA (1990b) Nitrogen-deregulated mutants of *Phanerochaete chrysosporium* – a lignin-degrading basidiomycete. Arch. Microbiol. 153: 521–527

Breen A, Jimenez L, Sayler GS & Federle TW (1992) Plasmid incidence and linear alkylbenzene sulfonate biodegradation in wastewater and pristine pond ecosystems. J. Industr. Microbiol. 9: 37–44

Campos-Garcia J, Esteve A, Vazquez-Duhalt R, Ramos JL & Soberon-Chavez G (1999) The branched-chain dodecylbenzene sulfonate degradation pathway of *Pseudomonas aeruginosa* W51D involves a novel route for degradation of the surfactant lateral alkyl chain. Appl. Environ. Microbiol. 65: 3730–3734

Hammel KE (1992) Oxidation of aromatic pollutants by lignin-degrading fungi and their extracellular peroxidases. In: Sigel H & Sigel A (Eds) Metal Ions in Biological Systems, Degradation of Environmental Pollutants by Microorganisms and their Metalloenzymes, Vol. 28 (pp 41–60). Marcel Dekker Inc., New York

Kohler A, Jager A, Willershausen H & Graf H (1988) Extracellular ligninase of *Phanerochaete chrysosporium* Burdsall has no role in the degradation of DDT. Appl. Microbiol. Biotechnol. 29: 618–620

Kertesz MA, Kolbener P, Stockinger H, Beil S & Cook AM (1994) Desulfonation of linear alkylbenzene sulfonate surfactants and related compounds by bacteria. Appl. Environ. Microbiol. 60: 2296–2303

Longwell J & Maniece WD (1955) Determination of anionic detergents in sewage, sewage effluents and river waters. Analyst 80: 167–171

McEnvoy J & Giger W (1986) Determination of linear alkylbenzene sulfonates in sewage sludge by high-resolution gas chromatography/mass spectrometry. Environ. Sci. Technol. 20: 376–383

Muralikrishna, C. & Renganathan, V. (1993) Peroxidase-mediated desulfonation of 3,5-dimethyl-4-hydroxy and 3,5-dimethyl-4-aminobenzenesulfonic acids. Biochem. Biophys. Res. Commun. 197: 798–804.

Pszczynski A & Crawford RL (1995) Potential for bioremediation of xenobiotic compounds by the white rot fungus *Phanerochaete chrysosporium*. Biotechnol Prog 11: 368–379

Pszczynski A, Crawford RL & Huynh VB (1988) Manganese peroxidase of *Phanerochaete chrysosporium*. Methods Enzymol. 161: 264-271

Paszczynski A, Pasti-Gigsby M B, Goszczynski S, Crawford R L & Crawford DL (1992) Mineralization of sulfonated azo dyes and sulfanilic acid by *Phanerochaete chrysosporium* and *Streptomyces chromofuscus*. Appl. Environ. Microbiol. 58: 3598–3604

Rapaport RA & Eckhoff WS (1990) Monitoring linear alkylbenzene sulfonate in the environment: 1973–1986. Environ. Toxicol. Chem. 9: 1245–1257

Reddy CA (1995) The potential of white rot fungi for the treatment of pollutants. Curr. Opinion Biotechnol. 6: 320–328

Reddy CA & D'Souza TM (1994) Physiology and molecular biology of the lignin peroxidases of *Phanerochaete chrysosporium*. FEMS Microbiol. Rev. 13: 137–152

Reddy GVB, Joshi DK & Gold MH (1997) Degradation of chlorophenoxyacetic acids by the lignin-degrading fungus Dichomitus squalens. Microbiol 143: 2353–2360

Schleheck D, Dong W, Denger K, Heinzle E & Cook AM (2000) A-proteobacterium converts linear alkylbenzene sulfonate surfactants into sulfophenyl carboxylates and linear alkyldiphenylether sulfonate surfactants into sulfodiphenylethercarboxylates. Appl. Environ. Microbiol. 66: 1911–1916

Sigoillot JC & Nguyen MH (1992) Complete oxidation of linear alkylbenzene sulfonate by bacterial communities selected from coastal seawater. Appl. Environ. Microbiol. 58: 1308–1312

Slack JG (1959) The determination of anionic detergents in river water and sewage effluents. Analyst 84: 193

Sutherland JB, Selby AL, Freeman JP, Evans FE & Cerniglia CE (1991) Metabolism of phenanthrene by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 57: 3310–3316

Swisher RD (1987) Surfactant Biodegradation, 2nd ed. Marcel Dekker Inc., New York

Tien M & Kirk TK (1988) Lignin peroxidase of *Phanerochaete chrysosporium*. Methods Enzymol. 161: 238–249

Webster HL & Halliday H (1959) Determination of alkylbenzene sulfonate in river waters and sewage. Analyst 84: 555–559

Werdelmann BW (1984) Tenside in unsere Welte-heute und morgen. In Proceedings of the Second World Surfactant Congress, Vol. 1, pp 3–21. Syndicat National des Fabricants d'Agents de Surface et de Produits Auxiliaires Industriels, Paris.

Yadav JS & Reddy CA (1993a) Degradation of benzene, toluene, ethylbenzene, and xylenes (BTEX) by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 59: 756–762

Yadav JS & Reddy CA (1993b) Mineralization of 2,4-dichlorophenoxyacetic acid (2,4-D) and mixtures of 2,4-D and 2,4,5-trichlorophenoxyacetic acid by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 59: 2904–2908

Yadav JS, Quensen III JF, Tiedje JM & Reddy CA (1995) Degradation of polychlorinated biphenyl mixtures (Aroclors 1242, 1254, and 1260) by the white rot fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 61: 2560–2565