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The physiology of anthracene biodegradation by the white-rot fungus *Bjerkandera* sp. strain BOS55

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Abstract A recently isolated white-rot strain, *Bjerkandera* sp. strain BOS55, displays high extracellular peroxidase activity, and rapidly degrades polycyclic aromatic hydrocarbons (PAH). In this study, the culture conditions for the biodegradation of the model PAH compound, anthracene, were optimized with respect to O_2, N, and C. An additional objective was to determine if the decolorization of the polymeric ligninolytic indicator dye, Poly R-478, could be correlated to anthracene biodegradation observed under a wide range of culture conditions. The supply of O_2 was found to be the most important parameter in the biodegradation of anthracene. Increasing culture aeration enhanced the biodegradation of anthracene and the accumulation of its peroxidase-mediated oxidation product anthraquinone. Decolorization of Poly R-478 was less affected by inadequate aeration. Provided that ample aeration was supplied, the degradation of anthracene under different culture conditions was strongly correlated with the ligninolytic activity as indicated by the rate of Poly R-478 decolorization. Concentrations up to 22 mM NH_4^+ N did not repress anthracene biodegradation and only caused a 0%–40% repression of the Poly R-478 decolorizing activity in various experiments. A cosubstrate requirement of 100 mg glucose / mg anthracene biodegraded was observed in this study.

Introduction

White-rot fungi are the most extensive degraders of the natural recalcitrant polymer, lignin (Kirk and Farrell 1987). This capacity is due to the extracellular peroxidase enzymes that initiate the attack of lignin. The ligninolytic system consists of lignin peroxidase and manganese-dependent peroxidase, H_2O_2 generating oxidases, substrates for oxidases, and the secondary metabolite, veratryl alcohol (Kirk and Farrell 1987; Buswell 1991; Hammel 1992). Due to the nonspecific character of the radical-mediated reactions of ligninolytic enzymes, a wide variety of xenobiotic compounds, having an aromatic structure like lignin, are also susceptible for attack by these extracellular enzymes (Aust 1990; Bumpus et al. 1989; Hammel 1992; Field et al. 1993). Lignin peroxidase has been shown to oxidize polycyclic aromatic hydrocarbons (PAH) in vitro (Haemmerli et al. 1986; Hammel et al. 1986). Mn-dependent peroxidase has been shown to oxidize Mn(II) to Mn(III) (Glenn et al. 1986), which is a powerful oxidator capable of oxidizing PAH's (Cavalieri and Rogan 1985; Cremonesi et al. 1992).

The ligninolytic peroxidases of the best-studied white-rot fungus, *Phanerochaete chrysosporium* are induced by the onset of secondary metabolism resulting from N, C or S depletion (Kirk and Farrell 1987; Bumpus 1989; Aust 1990). Commonly, N-limited media are used to promote ligninolysis and ligninolytic enzyme production when culturing *Phanerochaete chrysosporium* and other white-rot fungi (Kirk et al. 1978; Buswell 1991). Likewise, aromatic xenobiotic compounds are also mineralized to a much greater extent in N-limited cultures of *Phanerochaete chrysosporium* compared to N-sufficient cultures (Aust 1990; Hammel et al. 1991; Hammel 1992). Among the other culture parameters that are known to influence ligninolysis, aeration is considered to be important. High O_2 levels enhance the production of ligninolytic enzymes as well as ligninolytic activity (Kirk et al. 1978; Buswell 1991; Reid and Seifert 1992). Neither lignin nor xenobiotic aromatics can serve as the sole carbon and energy source; therefore, the degradation of lignin and xenobiotic aromatics depends on readily assimilable cosubstrates such as carbohydrates (Kirk et al. 1976; Fernando et al. 1989; Morgan et al. 1993).

The ability of various white-rot fungal strains to degrade PAH was found to be highly correlated to the

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decolorization of the polyaromatic dye Poly R-478 (Field et al. 1992). From a screening with this dye, a new isolate, *Bjerkandera* sp. strain BOS55, was found to be the best PAH degrader. *Bjerkandera* spp. are known to produce Mn-dependent peroxidase (de Jong et al. 1992a; Kaal et al. 1993) and lignin peroxidase (Kimura et al. 1990; Muheim et al. 1990; Kaal et al. 1993), and to mineralize lignin (Waldner et al. 1988). *Bjerkandera* sp. strain BOS55 has also been shown to produce a novel peroxidase, manganese-independent peroxidase (de Jong et al. 1992b).

The objective of this study was to optimize the culture conditions of *Bjerkandera* sp. strain BOS55 for the degradation of PAH. The three-ring PAH anthracene was chosen as the model compound because its oxidation product, anthraquinone, accumulates in the culture fluid (Field et al. 1992). Anthraquinone is a well-known product of anthracene oxidation by purified lignin peroxidase (Haemmerli 1988; Hammel et al. 1991) and the extracellular fluids of *Bjerkandera* sp. strain BOS55 (Field et al. 1992). Thus anthraquinone accumulation is indicative of the extracellular peroxidative degradation of anthracene.

Materials and methods

Microorganism and inoculum

The organism used, *Bjerkandera* sp. strain BOS55 (CIMW 1.91), was obtained from the Division of Industrial Microbiology culture collection. This strain was maintained on malt extract agar at 4° C; inocula for the experiments were prepared as described by Kaal et al. (1993). However, the inoculum used for the C- and N-limitation experiments was cultivated in a 5-l conical flask with 350 ml N-limited glucose-BIII medium. After 10 days of incubation at 30° C, the mycelium was separated from the medium and washed three times with basal medium without glucose or NH_4^+ N, respectively. The mycelium was milled with a blender (Ystrul, Dottingen, Germany) in 100 ml BIII medium, 200 µl of this mycelial suspension (800 mg dry weight mycelium l^{-1}) was added to each experimental culture (5 ml).

Culture conditions

Two types of media were used: BIII medium (Tien and Kirk 1988), and hemp stem wood (HSW) medium. The standard BIII liquid medium was N-limited, with 2.2 mM NH_4^+ N as diammonium tartrate, and 10 g l^{-1} glucose or cellulose as primary substrate in a 20 mM pH 4.5 2,2-dimethylsuccinate buffer. After autoclaving, a filter-sterilized thiamine solution (200 mg l^{-1}) was added at the rate of 10 ml l^{-1} medium. The standard HSW medium consisted of 10 mM pH 4.5 2,2-dimethylsuccinate buffer, with 2 g l^{-1} milled (particle size 100–1000 µm) HSW added.

Aliquots of 5 ml of these media were incubated statically at 30° C in serum bottles of 30–250 ml as described by Kaal et al. (1993). In one experiment, 30-ml bottles were shaken with 70 strokes of 2.5 cm min^{-1}.

Poly R-478 decolorization

The rate of Poly R-478 decolorization, at an initial concentration 0.8 g l^{-1}, was measured as described by Gold et al. (1988), except that in most experiments Poly R-478 was added prior to inoculation. Samples of 50 µl were drawn aseptically, diluted in 1 ml demineralized water and centrifuged before analysis. Absorbance readings were performed according to Field et al. (1992). The results are shown as the maximum decolorization rate, $[1000 \times \Delta A(520/350)/h]$, generally occurring between days 2 and 6. In one experiment Poly R-478 was added to cultures at distinct culture ages and analyzed after 18–21 hours of incubation.

Carbon dioxide analysis

Carbon dioxide evolution was measured in the headspace by gas chromatography as described previously (Field et al. 1992). The results are shown as the maximum carbon dioxide evolution rate, generally occurring between days 2 and 6.

Enzyme assays

The Mn-dependent and Mn-independent peroxidase activities were measured by the oxidation of 2,6-dimethoxyphenol (Kaal et al. 1993). The molar absorption coefficient used for the dimeric dimethoxyphenol oxidation product was 49600 M^{-1} cm^{-1} as determined by Wariishi et al. (1992). Lignin peroxidase activity was not measured since it was not detected in previous work using N-limited culture conditions (Field et al. 1992; de Jong et al. 1992a; Kaal et al. 1993).

Biodegradation of anthracene

Just before inoculation, anthracene was added to the triplicate cultures in a 50-µl aliquot of acetone, providing a final concentration of 10 ml l^{-1} acetone and either 10 or 50 mg l^{-1} anthracene. After incubation the whole culture was utilized for extraction; teflon liners were added to prevent adsorption of anthracene onto the septa, and 10 ml or 25 ml acetonitrile was added to 30 ml and 250-ml serum bottles, respectively. The bottles were then placed in a Branson 5200 sonicator (Danbury, USA) for 15 min and afterwards shaken by 300 2-cm-long strokes min^{-1} for 1 h on a Janke & Kunkel shaking table (Staufen, Germany) in complete darkness. Analysis of anthracene elimination was carried out according to Field et al. (1992), using HPLC for analysis.

Every experimental point described in this paper was run in parallel with two sets of triplicate abiotic controls in order to confirm that the elimination of anthracene and the formation of anthraquinone were truly biologically mediated. One set of abiotic controls consisted of incubating sterile media with anthracene, the other set consisted of incubating anthracene with killed 12-day-old cultures of *Bjerkandera* sp. strain BOS55. The cultures were killed by addition of 2 g l^{-1} sodium azide or by autoclaving for 20 min at 120° C. After 12 days, losses of anthracene ranged from 0% to 12% in both the sterile media and the autoclaved killed-fungi controls. Formation of anthraquinone was always less than 1% of the added anthracene in these abiotic controls.

In one experiment, anthracene was added at a given culture age and analyzed after 18–21 hours of incubation in order to determine the rate of anthracene biodegradation and anthraquinone formation. The results shown are expressed as mg l^{-1} day^{-1}. In the abiotic controls, which consisted of autoclaved cultures of the same age, the loss of anthracene and accumulation of anthraquinone after incubation were less than 2% and 0.5%, respectively.

Chemicals

Anthracene, 9,10-anthraquinone and 2,6-dimethoxyphenol were obtained from Janssen Chimica (Tilburg, The Netherlands), or

Aldrich (Steinheim, Germany). Poly R-478 was obtained from Sigma (St. Louis, USA). Hemp stem wood (*Cannabis sativa* Fibrimon 56) was kindly supplied by the Agrotechnological Research Institute (Wageningen, The Netherlands).

Results

Four liquid media were tested for their ability to support the biodegradation of 10 mg l^{-1} anthracene by *Bjerkandera* sp. strain BOS55 in 30-ml serum bottles (deep cultures). N-limited BIII-glucose medium, 2.2 mM NH_4^+ N, was compared with N-limited BIII-cellulose and N-sufficient BIII-glucose, containing 22 mM NH_4^+N. These media, which contained 10 g l^{-1} cosubstrate, were compared with hemp stem wood (HSW) medium containing only 2 g l^{-1} natural powdered lignocellulose substrate. No significant differences in the rate of anthracene elimination, anthraquinone formation and Poly R-478 decolorization were found among the media with 10 g l^{-1} defined carbohydrate cosubstrates, including the high-N medium (results not shown). Only the HSW medium provided an enhanced rate of anthracene biodegradation.

Since the best results were obtained in HSW media, we were not sure if this was due to the type or concentration of cosubstrate. The effect of HSW concentration on the biodegradation of 10 mg anthracene l^{-1} was tested in deep cultures (Fig. 1). Surprisingly, considerable degradation of anthracene occurred in cultures receiving no HSW cosubstrate. The cosubstrate present in the inoculum, provided by the malt extract agar plug, was apparently sufficient to support growth and anthracene co-metabolism. The anthracene biodegradation increased up to 2 g HSW l^{-1}. However, higher concentrations of cosubstrate caused a distinct inhibition in the anthracene biodegradation. In contrast, the average Poly R-478 decolorization rate increased up to 10 g HSW l^{-1} and was not inhibited by 30 g HSW l^{-1}. In parallel, the Mn-dependent peroxidase activity, measured on day 5, increased from 5 nmol to 115 nmol dimethoxyphenol oxidized ml^{-1} min^{-1} at HSW concentrations of 2 g l^{-1} and 30 g l^{-1}, respectively (results not shown). These results indicate that the enzyme production and activity towards the dye was not inhibited by high HSW levels, but rather that the inhibition was specific to the anthracene oxidation process.

A possible explanation for the inhibition of anthracene biodegradation at high HSW concentrations could be poor aeration or low redox potentials resulting from oxygen uptake for fungal respiration. To test this hypothesis, biodegradation of 10 mg anthracene l^{-1} in 30 g HSW l^{-1} medium was monitored in deep and shallow cultures of 30-ml and 250-ml serum bottles, respectively. These cultures were incubated under air, and an 80% O_2 atmosphere (Table 1). The anthracene biodegradation as well as the molar yield of anthraquinone per unit of anthracene biodegraded (AQ/A) were remarkably improved in the deep cultures receiving an 80% O_2 atmosphere. The improvement of these parameters was even more pronounced in shallow cultures, irrespective of the oxygen level. In contrast, the production of Mn-dependent and Mn-independent peroxidase, and the decolorization rate of Poly R-478 were not highly affected (results not shown).

The effect of aeration on the biodegradation of anthracene was studied further in the 30 g HSW l^{-1} media in 15-, 30-, 250- and 1000-ml serum bottles. The results, plotted in terms of effective aeration surface area (Fig. 2), show that anthracene biodegradation increased up to 5780 cm^2 l^{-1} (250-ml serum bottle). The AQ/A was again observed to increase with the better aeration.

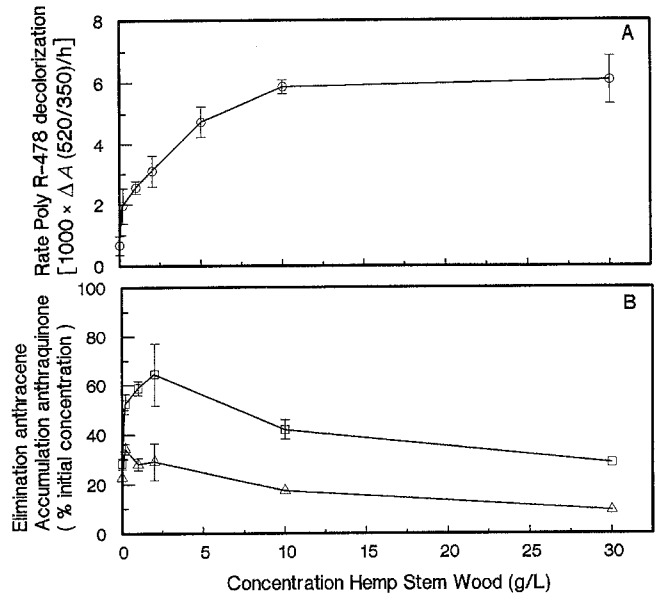

Fig. 1A, B The effect of increasing hemp stem wood (HSW) concentration in deep cultures (1600 cm^2 surface area l^{-1} culture fluid) on (**A**) the rate of Poly R-478 decolorization and (**B**) the elimination of 10 mg anthracene l^{-1} (□) and accumulation of anthraquinone (△) as a molar percentage of the initial anthracene concentration after 5 days

Table 1 Effect of atmospheric oxygen concentration and aeration surface area on the biodegradation of anthracene (10 mg l^{-1}) in static cultures after 5 days with 30 g l^{-1} hemp stem wood cosubstrate. Standard deviations are shown in parentheses ($n=3$). *AQ/A*, the molar yield of anthraquinone relative to anthracene degraded

Culture conditions		Anthracene eliminated	Anthraquinone[a] formed	AQ/A ratio
Surface area (cm^2 l^{-1})	Atmosphere (% O_2)	(%)	(%)	
1600[b]	20	35.4 (3.2)	14.5 (1.2)	0.41
1600	80	63.9 (3.0)	32.8 (4.0)	0.51
5780[c]	20	84.1 (1.8)	48.2 (5.9)	0.57
5780	80	80.5 (5.5)	47.4 (2.1)	0.59

[a] Molar percentage of anthracene added
[b] Deep culture
[c] Shallow culture

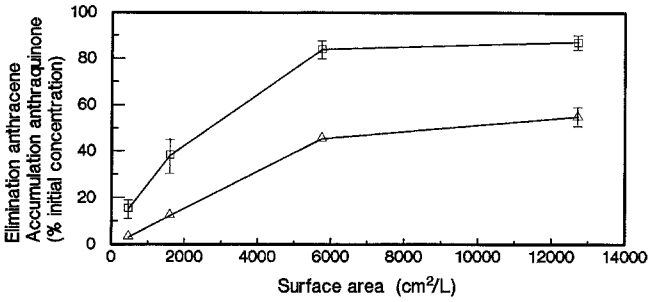

Fig. 2 The effect of aeration surface area in cultures with 30 g HSW l^{-1} cosubstrate on the elimination of 10 mg l^{-1} anthracene (□) and anthraquinone accumulation (△) as a molar percentage of the initial anthracene concentration after 5 days

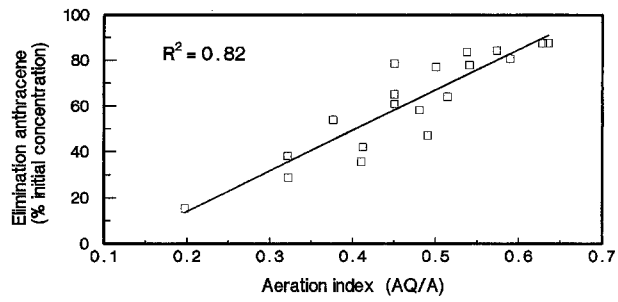

Fig. 3 The correlation between anthracene elimination on day 5 and the ratio anthraquinone formed (mol) of anthracene eliminated (mol) (*AQ/A*) in cultures supplied with a starting anthracene concentration of 10 mg l^{-1}. The coefficient of determination (R^2) was calculated according the product-moment method for the coefficient of linear correlation. The experimental points refer to 1 g l^{-1} cosubstrate or more so that the anthracene elimination was not limited by inadequate levels of biocatalyst (e.g. biomass)

Cultures with 468, 1600, 5780 and 13000 cm^2 l^{-1} aeration surface area had AQ/A ratios after 5 days of 0.20, 0.32, 0.54, and 0.63, respectively. In cultures of 5780 cm^2 l^{-1}, 84% and 100% of the anthracene was eliminated after 5 and 12 days, respectively.

A similar experiment was repeated in defined N-limited BIII medium containing 10 g glucose l^{-1}, and compared with the aeration achieved by shaking deep cultures. The anthracene biodegradation and anthraquinone accumulation obtained in the shaken deep culture were similar to values in the static shallow cultures (results not shown). The CO_2 production and the Poly R-478 decolorization were also found to be influenced by the aeration, albeit to a lesser extent. Dye decolorization in the deepest culture was inhibited by only 50%, whereas anthracene degradation was inhibited by 92% compared to shallow cultures.

Increasing the aeration of culture fluid stimulated both the degradation of anthracene and the molar yield of anthraquinone (AQ/A). Figure 3 shows a strong relationship between the amount of anthracene degraded after 5 days and the AQ/A ratio. Provided that well aerated culture conditions were supplied, the degradation of anthracene showed a strong correlation with the decolorization rate of Poly R-478 (Fig. 4).

An important parameter for the bioremediation of PAH polluted soils by white-rot fungi is the amount of cosubstrate required per unit of pollutant. To determine this value, the biodegradation of 50 mg anthracene l^{-1} was evaluated at different glucose concentrations in N-limited BIII medium placed in 250-ml serum bottles. As described in Materials and methods, special care was taken to obtain a cosubstrate-free inoculum. After 12 days the cosubstrate was depleted in cultures containing up to 2 g glucose l^{-1}, as indicated by the cessation of CO_2 production. Up to this concentration, there was a linear increase in the amount of anthracene degraded. From this line, a cosubstrate requirement of approximately 100 mg glucose mg anthracene degraded^{-1} was calculated.

Anthracene degradation in cultures without glucose (Fig. 5) was not significantly higher than in abiotic controls, confirming that anthracene cannot be degraded as

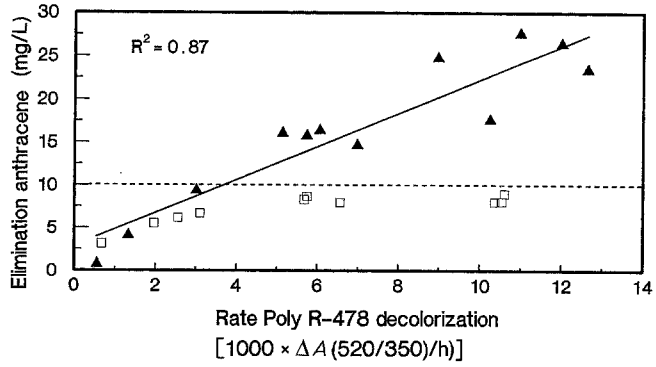

Fig. 4 The correlation between anthracene elimination after 5 days and the rate of Poly R-478 decolorization at two starting concentrations of anthracene; □, 10 mg l^{-1} and ▲, 50 mg l^{-1}. The selected points had an AQ/A ratio higher than 0.45. The coefficient of determination (R^2) was calculated for the points corresponding to a starting anthracene concentration of 50 mg l^{-1}. - - -, The value of 10 mg l^{-1} anthracene elimination

a sole carbon and energy source. The maximum biodegradation of anthracene was obtained with 5 g glucose l^{-1}; after 5 days of incubation, 27 mg l^{-1} anthracene was biodegraded. In parallel, the rate of CO_2 production and Poly R-478 decolorization also reached maximal values at 5 g glucose l^{-1}. Higher glucose concentrations did not inhibit anthracene degradation nor increase the CO_2 production (O_2 uptake) rate. As in the previous experiments, no significant anthracene elimination nor anthraquinone accumulation occurred in the sterile and dead fungus controls, killed by autoclaving. However, dead fungus controls killed by sodium azide, showed a small but significant level of anthracene elimination and anthraquinone accumulation in cultures receiving high levels of cosubstrate. At 30 g glucose l^{-1}, 17 ± 2.0% of the initial concentration of anthracene was degraded, and 7.9 ± 0.6% anthraquinone was accumulated after 12 days, indicating that a heat labile catalyst produced by the fungus was not completely inhibited by sodium azide.

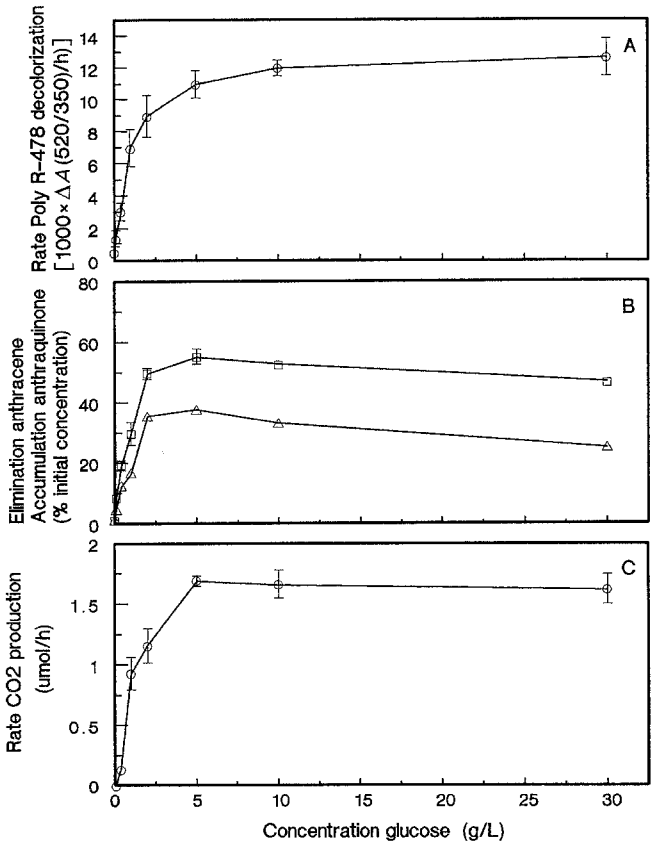

Fig. 5A–C The effect of glucose cosubstrate concentration in shallow cultures (5780 cm^2 surface area l^{-1} culture fluid) on (**A**) the rate of Poly R-478 decolorization, (**B**) the elimination of 50 mg anthracene l^{-1} (□) and anthraquinone accumulation (△) as a molar percentage of the initial anthracene concentration after 5 days, and (**C**) the maximum respiration rate as indicated by the maximum rate of CO_2 production

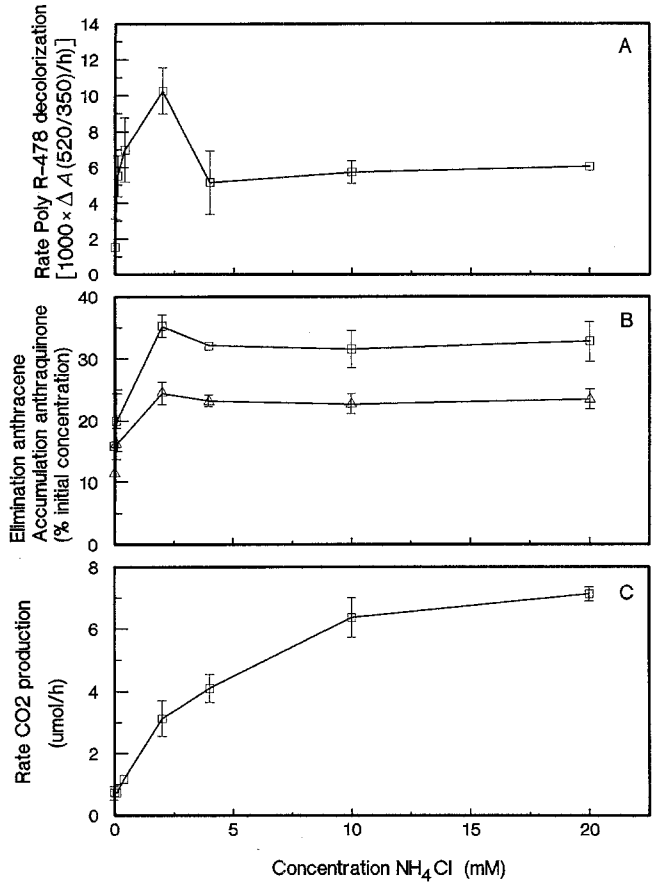

Fig. 6A–C The effect of NH_4^+ N concentration, supplied as NH_4Cl, in shallow cultures (5780 cm^2 surface area l^{-1} culture fluid) on (**A**) the rate of Poly R-478 decolorization, (**B**) elimination of 50 mg anthracene l^{-1} (□) and anthraquinone accumulation (△) as a molar percentage of the initial anthracene concentration after 5 days, and (**C**) the maximum respiration rate as indicated by the maximum rate of CO_2 production.

Since the media experiment showed no repression of ligninolytic activity by 22 mM NH_4^+ N, supplied in the form of diammonium tartrate, the influence of the nitrogen concentration, from 0 to 20 mM NH_4Cl, was monitored in shallow cultures with 5 ml BIII medium containing 10 g glucose l^{-1}, and special NH_4^+ N-free inoculum. Anthracene biodegradation increased until 2 mM NH_4Cl and was not repressed by higher concentrations up to 20 mM NH_4Cl (Fig. 6). The Poly R-478 decolorization rate also increased until 2 mM NH_4Cl; however, partial repression (40%) occurred at higher concentrations although the high-N cultures were still expressing considerable dye decolorizing activity. The rate of CO_2 production increased up to 10 mM NH_4Cl, indicating that below that concentration the medium was truly N-limited. Low but significant levels of biodegradation of anthracene, decolorization of Poly R-478 and CO_2 production occurred in media not supplemented with any NH_4Cl.

The biodegradation of anthracene in the previous experiments was measured after 5 and 12 days. In order to determine the anthracene-degrading activity and the Poly R-478 decolorization rate as a function of culture age, anthracene and Poly R-478 were added to cultures of different ages and incubated for 19–21 h. The respiration rate and the production of Mn-dependent and Mn-dependent peroxidases were also monitored at each culture age. The rate of anthracene biodegradation increased rapidly to 10.3 mg l^{-1} day^{-1} (97% of initial concentration) at day 5 (Fig. 7). The rate remained high during the rest of the experiment; on day 67, half of the maximal rate was still evident. No significant anthracene degradation occurred in the autoclaved fungi controls. The anthracene-degrading activity parallelled the amount of peroxidase enzymes present in the culture fluid, and, initially, the Poly R-478 decolorization activity. Adsorption of the dye onto the increasing amount of fungal biomass disturbed the Poly R-478 decolorization measurements. The adsorbed dye was decolorized before the soluble dye became decolorized. Thus the apparent activity in solution measured in the first day after addition, when predominately the adsorbed dye was being decolorized, is lower than the actual activity. The CO_2 production confirmed growth

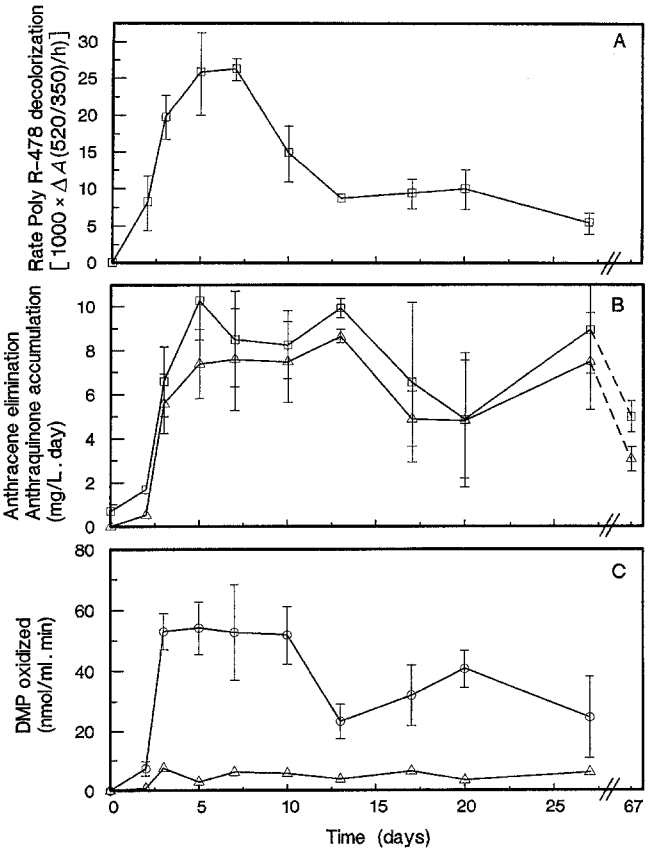

Fig. 7 **A** The Poly R-478 decolorization rate, **B** anthracene biodegradation rate (□) and anthraquinone accumulation rate (△), both expressed as mg l^{-1} day^{-1}, **C** activity of Mn-dependent (○) and Mn-independent peroxidases (△) in 30 g l^{-1} hemp stem wood medium in shallow cultures (5780 cm^2 surface area l^{-1} culture fluid). Poly R-478 (0.8 g l^{-1}) and anthracene (10 mg l^{-1}) were added to separate triplicate cultures at the indicated times. DMP 2,6-dimethoxyphenol

during the experiment, and only a slight decrease in growth rate could be detected towards the end of the experiment (results not shown). In a similar experiment, a single dose of anthracene (10 mg l^{-1}) was added on day zero. By day 5, anthracene was completely eliminated and up to 6 mg l^{-1} anthraquinone accumulated. In the following 30 days, only 25% of the anthraquinone was metabolized (results not shown).

Discussion

Under the wide variety of culture conditions tested in this study, anthracene was consistently oxidized to anthraquinone in high molar yields (20%–80%). Anthraquinone has been found earlier as an oxidation product in N-limited whole cultures of *Phanerochaete chrysosporium* and *Bjerkandera* spp (Hammel et al. 1991; Field et al. 1992). The same oxidation product is found in in vitro experiments with purified lignin peroxidase (Hammel et al. 1986, 1991; Haemmerli 1988). Quinones are also formed from the chemical reaction with the one-electron oxidant, Mn(III), with various PAH (Cavalieri and Rogan 1985; Cremonesi et al. 1992). These observations suggest that peroxidases, including the Mn-dependent enzyme are also involved in vivo. Anthraquinone was shown to be degraded by *Phanerochaete chrysosporium* to phthalic acid and CO_2 (Hammel et al. 1991). However, as was reported previously (Field et al. 1992), we have again observed that anthraquinone tends to behave as a dead-end metabolite in *Bjerkandera* sp. strain BOS55. Thus anthraquinone levels are a useful indicator for the peroxidative biodegradation of PAH by this fungus.

The most important factor that influenced the biodegradation of anthracene was oxygen. Increasing the effective aeration surface areas of static cultures or increasing the O_2 concentration in the headspace above the culture fluids resulted in higher rates of anthracene degradation. When the degradation of anthracene was optimized by increasing the effective aeration surface area in shallow cultures, no further increase of anthracene degradation could be achieved by supplying higher O_2 tensions in the headspace. So the O_2 transfer into the culture rather than the O_2 partial pressure was the limiting factor. In previous experiments, where the O_2 profiles in stationary cultures of *Phanerochaete chrysosporium* were measured, the importance of O_2 limitation was clearly indicated (Leisola et al. 1983). Even 1 mm into the mycelium mat, O_2 was completely depleted. The O_2 limitation could be overcome by decreasing the culture depth or increasing the O_2 concentration.

In whole cultures of *Bjerkandera* sp. strain BOS55, the yield of anthraquinone per unit of anthracene degraded (AQ/A) increased with better aeration. The AQ/A ratio is also reported to increase with increasing O_2 concentrations during the in vitro oxidation of anthracene by lignin peroxidase (Haemmerli 1988). Therefore the AQ/A ratio can be viewed as an index for the level of aeration. Moreover, since anthraquinone is only slowly metabolized in our experiments, the ratio would only be susceptible to negligible changes from further metabolism by whole cells. The fact that a strong correlation exists between the AQ/A ratio and anthracene degradation indicates that aeration was an important factor determining the rate of anthracene biodegradation. Hammel et al. (1986) demonstrated that the oxygen incorporated into the PAH quinone by lignin-peroxidase-mediated oxidation of PAH is provided by H_2O, and not by molecular oxygen. Thus high levels of aeration might be required for maintaining a high redox potential in the culture fluid rather than the supply of molecular oxygen itself.

Our results show that the Poly R-478 decolorization is less repressed by non-optimal aeration than the anthracene degradation. The utilization of Poly R-478 decolorization as an indicator of ligninolytic activity towards compounds such as anthracene (Field et al. 1992) is therefore doubtful under low-oxygen. However, provided that the aeration of the culture was adequate, a

high correlation between the Poly R-478 decolorization and the degradation of anthracene was found, which is supported by the fact that both PAH and the dye are oxidized by the same ligninolytic enzymes. As discussed previously, anthracene is initially attacked by peroxidases. Ligninolytic peroxidases as well as Mn(III) have also been shown to be responsible for the decolorization of Poly R-478 (de Jong et al. 1992b; Glenn and Gold 1985; Paszczynski and Crawford 1991; Kuwahara et al. 1984; Glenn et al. 1986). In our experiments, Mn-dependent and Mn-independent peroxidases were produced in parallel with the onset of Poly R-478 decolorization and anthracene biodegradation (Fig. 7).

Fungal growth and H_2O_2 production, which are required for the production and activity of peroxidases, respectively, are dependent on the availability of readily biodegradable cosubstrates (Kirk et al. 1976; Fernando et al. 1989; Morgan et al. 1993). In this study, we have shown that *Bjerkandera* sp. strain BOS55 was not able to degrade anthracene nor to decolorize Poly R-478 without glucose supplementation. Approximately 100 mg glucose was needed / mg anthracene degraded. High HSW cosubstrate concentrations in deep cultures inhibited anthracene biodegradation. The AQ/A ratios in these cultures were low and the inhibition could be overcome by providing better aeration. The inhibition could be due to the poor aeration resulting from the competition between O_2 needed to maintain a high redox potential for anthracene oxidation and O_2 consumed for fungal respiration. Although the maximum O_2 uptake in N-limited cultures had already been reached at 5 g l^{-1} of glucose, N of ill-defined bioavailability provided by high HSW concentrations (60 mM HSW N at 30 g l^{-1} HSW), could have enhanced the respiration rate beyond that of the N-limited cultures. It is also possible that reducing equivalents, accumulated by the fungus under O_2 limitation, interfere with the anthracene oxidation. The fact that Poly R-478 decolorization was not inhibited at high HSW concentrations indicates that O_2 needed for H_2O_2 producing oxidases was not rate limiting.

Generally, N or C limitation has been considered essential for triggering the onset of secondary metabolism, when the production of ligninolytic enzymes occurs (Kirk and Farrell 1987; Aust 1990; Buswell 1991). However, ligninolytic enzyme activity; degradation of anthracene and decolorization of Poly R-478, were not severely repressed by high N in *Bjerkandera* sp. strain BOS55. CO_2 production curves indicate that the metabolic activity was N-limited below 10 mM NH_4^+ N. Nonetheless, the cultures were expressing full anthracene-degrading activity at the highest NH_4^+ N concentrations tested (20–22 mM). Recently it has been shown that organic N-nutrients up to 34 mM N stimulate Manganese-dependent peroxidase and lignin peroxidase production; moreover, organic N induced lignin peroxidase, which is usually not detectable in N-limited media nor in the high NH_4^+ N media of *Bjerkandera* spp. (Kimura et al. 1990; Kaal et al. 1993). Since N-limitation is not required for ligninolytic activity in *Bjerkandera* sp. strain BOS55, future studies should attempt to increase the PAH degradation rate by organic N-supplements, which are known to stimulate peroxidase production.

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