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Controlling Soil Pollution Using Biodegradation of PAH Pollutants In Biotechnology

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BY

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Controlling Soil Pollution Using Biodegradation of PAH Pollutants

Introduction:

From a contaminated soil, a strain of Absidia was isolated.

This strain's ability to bio transform two polycyclic aromatic hydrocarbons (PAHs)—fluoranthene and anthracene—was compared to that of a different Absidia sp. isolate taken from uncontaminated soil and used as a control.

The statistical analysis of the results revealed that the isolate from the polluted soil was more effective than the control at removing anthracene from the medium during all stages of the kinetics (90% eliminated versus 45% after 24 hrs.).

The amount of fluoranthene eliminated by both strains was quite high during the first 24 hours, but the control strain was marginally more effective (94% versus 89%), and the outcomes for the two strains were comparable during the rest of the kinetics. This study makes the Absidia sp species potential interest in PAH bioremediation.

Table 1. Multifactorial analysis of variance corresponding to the amount of Anthracene transformed.

Source of variation	dF[b]	SS[c]	MS[d]	F	p
S Factor	1	38.34	38.34	172.03	<0.0001
T Factor	1	1.47	1.47	6.60	0.012
t Facteur	4	48.16	12.04	54.02	<0.0001
S*T[a]	1	2.51	2.51	11.26	0.001
S*t[a]	4	0.82	0.20	0.92	0.46
T*t[a]	4	1.99	0.50	2.23	0.072
S*T*t[a]	4	1.00	0.25	1.13	0.35
Residual	100	22.29	0.22		

[a]Interaction between successively S and T factors, S and t factors, T and t factors, S and T and t factors.

[b]dF: degrees of freedom.

[c]SS: sum of squares.

[d]MS: mean square.

Incomplete combustion of practically any organic material produces fused ring aromatic molecules known as polycyclic aromatic hydrocarbons (PAHs), which are widely dispersed in the environment (Menzie et al. 1992; Cerniglia, 1993; Chaudhry, 1994). Some of them are regarded harmful compounds due to their toxic and mutagenic or carcinogenic potential (Menzie et al. 1992; Nadon et al. 1995), and 16 are on the US Environmental Protection Agency's priority pollutants list (EPA). The environment and human health are seriously put at risk by the presence of PAHs in contaminated soils and sediments. Because they are hydrophobic molecules with a limited water solubility, PAHs are persistent in ecosystems (Chaudhry, 1994). The most prevalent PAH in the environment, fluoranthene, is regarded as a pollution indicator (Chaudhry, 1994). Another representative chemical for the study of PAH degradation is anthracene, which shares structural similarities with carcinogenic PAHs like benzo (a) pyrene and benzo (a) anthracene (Müncnerova and Augustin, 1994).

According to Mahmood and Rama Rao (1993; Kästner and Mahro 1996), microbial biotransformation is a significant environmental activity that affects the destiny of PAHs in both terrestrial and aquatic ecosystems. Numerous bacteria, including Alcaligenes denitrificans, Rhodococcus sp., Pseudomonas sp., and Mycobacterium sp., have been discovered that metabolise PAHs (Cerniglia, 1993; Harayama, 1997; Dean-Ross et al. 2001; Moody et al. 2001). Some PAHs can be entirely broken down by a variety of bacteria into CO2 and metabolic intermediates (Kelley et al. 1993; Müncnerova and Augustin 1994). Less is known about the degradation caused by fungi: By means of a co-metabolic mechanism, PAHs are converted to phenolic metabolites (Cerniglia, 1993; Paszczynski and Crawford, 1995; Cerniglia, 1997; Harayama, 1997). The extracellular enzymes of white rot fungi catalyse a non-specific oxidation reaction that results in the synthesis of a variety of quinones and hydroxylated aromatic molecules (Hammel, 1995; Bogan and Lamar, 1996, Tekere et al. 2005). Cunnighamella elegans was used to carry out a thorough examination into the fungus's metabolism of fluoranthene (Müncnerova and Augustin, 1994). Our team has conducted preliminary research to assess how certain soil fungi degrade or alter anthracene, fluoranthene, and pyrene (Krivobok et al. 1998; Salicis et al. 1999; Ravelet et al. 2000). More recent research has evaluated the possibility of enhancing the biodegradation of anthracene and fluoranthene in fungus isolated from a contaminated military base (Giraud et al. 2001). Additionally, it has been that anthracene may be hazardous to certain soil fungus (Bonnet et al. 2005). Absidia is not well known for being effective at bioremediating environmental pollutants. Except for our most recent research on herbicides (Bordjiba et al.

2001) and phenolic chemicals, Absidia fusca Linnemann has not been the subject of any reports on the metabolism of xenobiotics (Guiraud et al. 2003).

In this study, the purpose was to investigate the ability of Absidia to degrade these two compounds. Two strains were compared: one from the normal soil, the other isolated from a polluted soil, and the results of the degradation kinetics obtained were statistically analysed.

MATERIALS AND METHODS

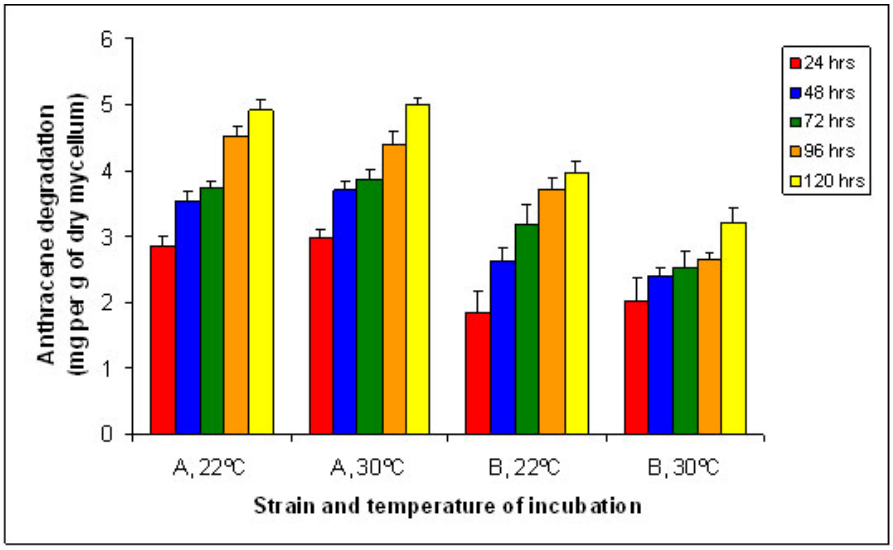

Figure 1. Strain*Temperature*time interaction for anthracene transformation by *A.bsidia* strains A and B (error bars: one standard error).

Chemicals

Anthracene (AC) and fluoranthene (FA)

Agar, Malt Extract, Glucose, Dextrose, Potato Dextrose Agar, Phenol Red, Urea

Media and culture conditions

The solid medium used for maintaining the cultures contained: malt extract 15 g l-1 - agar 15 g l-1 (MEA medium). Galzy and Slonimski (1957) liquid synthetic medium (GS) was slightly changed by adding glucose at a final concentration of 5 g l-1. Prior to usage, the media were autoclaved for 20 minutes at 121 degrees Celsius. Prior to each experiment, the strains were reactivated on MEA medium for 8 days.

Fungal strain isolation

Absidia sp (A) and (B) was isolated from contaminated soil where potato and Tomato were cultivated. Soil samples were collected from 1 to 10 cm depth in sterile tubes. It was stored at 4ºC for further experimental analysis. Absidia was stored at 4ºC in wide neck slant tubes on MEA medium

Biotransformation assays

The strains were cultivated for 1-2 weeks on MEA medium at 22 °C, without any adaptation to the xenobiotics, to obtain enough inoculum for liquid media cultures.

Culture were previously sterilised by autoclaving for 20 minutes at 121°C before being aseptically inoculated as mycelium and spores into sterile GS liquid medium at pH 4.5, containing glucose (5 g l-1). The 35 ml of inoculation medium in the 125 ml Erlenmeyer flasks was incubated at 22 °C and 30 °C for 2 days with agitation (180 rpm, orbital shaker). Glucose assay was done and it was established that there was no longer any glucose in the medium at this point (Roche Diagnostics, Meylan, France). By filtering through 0.2 m Millipore membranes, dimethyl sulfoxide stock solutions of AC and FA were sterilised before being added at a final concentration of 0.01 g l-1.

After five days of growing at 22ºC and 30ºC, the compounds' depletion was assessed. With a photoperiod of 12 hours per day, the light level was 1200 lux. Assays were performed in triplicate throughout each series of studies at least three times. Cultures were conducted at 22 °C or 30ºC and stopped 24, 48, 72, 96, and 120 hours after the addition of the PAHs for kinetics investigations. The xenobiotic was given to fungal cultures at the time of harvest and processed right away for the biotic controls. The xenobiotic was contained in cell-free flasks for the abiotic control. Each series' controls worked to offset any losses brought on by mycelium binding or physicochemical deterioration.

Analysis of PAHs

One volume of bidistilled ethyl acetate (25 ml) was used to extract liquid media containing mycelia and either AC or FA by rotary shaking at 250 rpm for five minutes and then 180 rpm for thirty minutes. Mycelia were removed through filtering and washed with ethyl acetate. Two times the extraction was done. The

organic phases were combined, dried over anhydrous Na2SO4, and dried at a reduced pressure of 40 °C. The residue was diluted in acetonitrile (1.5 ml), from which an aliquot of 20 l was taken for HPLC analysis after gently vortexing and filtering through a 0.2 m membrane filter.

HPLC was carried out with a liquid chromatograph outfitted with a LC 6A pump, an SIL-9A automatic injector and a RF-10AXL spectrofluorescence detector. The separation column, Supelcosil TM LC-PAH 5 µm, was 4.6 mm inside diameter x 150 mm. Acetonitrile: water (70:30, v:v) was the mobile phase used . The flow rate was 1 ml min-1 and detection was adjusted at 280 nm (λ excitation) and 450 nm (λ emission) for FA and at 250 nm (λ excitation) and 450 nm (λ emission) for AC. The HPLC detection limit was 0.22 µg.l-1 for FA and 0.49 µg.l-1 for AC. The mean value was computed after each sample was injected three times.

Table 2. Multifactorial analysis of variance corresponding to the amount of transformed fluoranthene.

Source of	dF[b]	SS[c]	MS[d]	F	p
S Factor	1	0.900	0.900	12.16	0.0007
T Factor	1	3.31	3.31	44.75	<0.0001
t Facteur	4	21.44	5.36	72.41	< 0.0001
S*T[a]	1	0.008	0.008	0.10	0.75
S*t[a]	4	1.31	0.327	4.42	0.002
T*t[a]	4	0.215	0.054	0.73	0.58
S*T*t[a]	4	0.157	0.039	0.53	0.71
Residual	140	10.36	0.074		

[a]Interaction between successively S and T factors, S and t factors, T and t factors, S and T and t factors.

[b]dF, degrees of freedom

[c]SS, sum of squares

[d]MS, mean square.

Evaluation of the level of converted xenobiotics

The integrator attached to the HPLC system used a standard to determine the residual concentrations of AC and FA. After accounting for extraction yield (varying from 95 to 98%) and abiotic degradation (not exceeding 5%), the overall biotransformation efficiency (%) was determined. Following that, these data were converted into milligrams of xenobiotic transformed per gram of dry mycelium.

Statistical analysis

The treated data refers to the amount of xenobiotic transformed expressed as mg/g of dry mycelium. Results reported are means (M), standard errors of the means (SEM) and coefficient of variation (CV%).

A multifactorial analysis of variance was performed for each xenobiotic, with the following factors: strain S (two levels: A and B), temperature T (two levels: 22 °C and 30 °C), and time t. (qualitative factor, five levels: 24 hrs, 48 hrs, 72 hrs, 96 hrs, 120 hrs). The Cochran C test was previously used to determine if the 20 variances were homogeneous. Potential interactions between the three parameters taken into consideration were highlighted by the multifactorial analysis. The Fisher PLSD test (Protected Least Significant Difference), which is the most effective in spotting differences, and the Tukey-Kramer test, which allows you to gauge the likelihood of type I error, were the posteriori tests used after the analyses of variance.

Only when the results of the second test disagree with those of the Fisher PLSD are mentioned. Utilizing StatView version 5, these studies were conducted (SAS Institute, Cary, NC). When the factors S and T were crossed, four groups were produced: A at 22 °C, A at 30ºC, B at 22 °C, and B at 30 °C. To check the regression's significance and linearity, each of them underwent an analysis of variance of the time-dependent regression. The Student's t test was used to compare the slopes of the linear regression curves. The significance level α for all analyses was 0.05, and p values below or equal to 0.05 were considered significant.

RESULT

Kinetics of anthracene biotransformation by Absidia

For the four groups, the kinetics of AC transformation were run over the course of five days at 22°C, 30°C, 22°C, and 30°C, respectively. Six replicates were performed for each time.

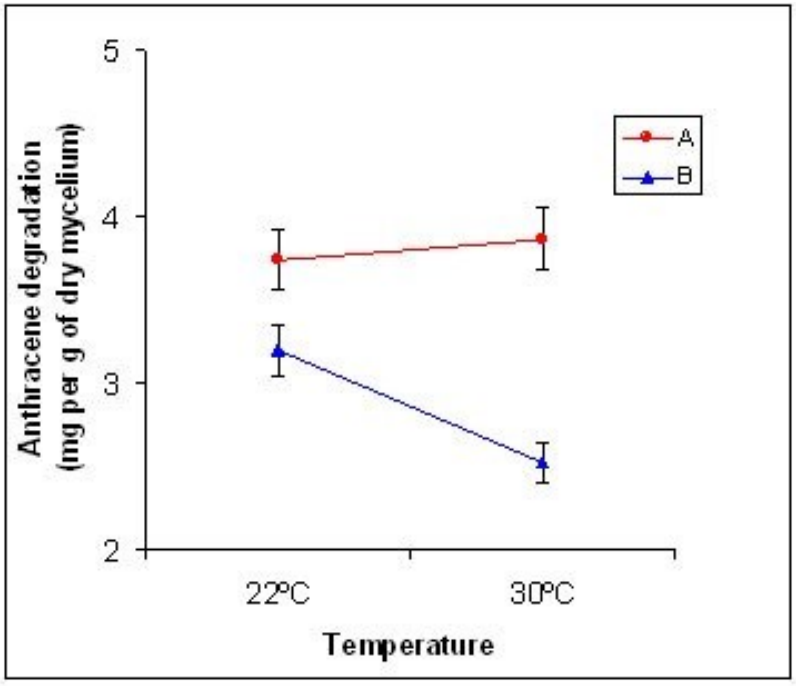

Figure 2. Strain*Temperature interaction for anthracene transformation by *Absidia* strains A and B (error bars: one standard error; time: 72 hrs).

With retention times varying between 0.5 and 2 min, the chromatographic profiles showed three peaks for both strains, while the peak corresponding to AC was discovered at 5 min in our experimental setup. The peak related to strain A was higher in height and area during the first 24 hours, but the peak corresponding to strain AC declined at the same period. In the HPLC profiles of the control extracts corresponding to the medium supplemented by AC without fungus and the medium with fungus but without AC, these peaks were not visible. All of these findings provided strong evidence that the three peaks related to AC-derived metabolites.

Additionally, these peaks fell out within 24 hours, pointing to a biodegradation process. After 24 hours with strain A, the percentages of AC transformation were very high: mean values were 89% (CV = 10.6%) at 22 °C and 88.1% (CV = 6.9%) at 30°C. The figures obtained for strain B were 2 times less favourable: 47% (CV = 17.2%) at 22°C and 41% (CV = 11.9%) at 30°C. Figure 1 displays the comparable values in mg/g of dried mycelium. For all groups, the coefficients of variation were high at 24 hours but fell off after that.

In the first 72 hours, they were primarily high for strain B, reaching 40% at 24 hours on AC biotransformation. The findings of the multifactorial variance analysis are presented in Table 1. No matter the time or temperature taken into

account, strain A was more efficient than strain B for AC transformation, as shown by Figure 1's illustration of the S*T*t interaction (not significant, p = 0.35). This was further supported by the evidence of a substantial strain effect (p 0,0001). Regardless of the value of the factor t, the factor T had no impact on strain A, though the temperature effect was substantial (p = 0.012).

The impact of the parameters S and T was then determined by analysing the results. Since the S*T interaction was significant (p = 0.001), the influence of the temperature was dependent on the strain. This interaction, which is depicted in Figure 2, revealed that strain A degraded an average amount of AC at 22 °C and 30 degrees Celsius (3.91 mg/g of dry mycelium versus 3.98 mg/g of dry mycelium, p = 0.57), whereas strain B degraded an average amount that was lower at 30 °C than it was at 22 °C (2.56 mg/g of dry mycelium versus 3.07 mg/g of dry mycelium, p < 0,0001

With the Fisher PLSD test, this difference with strain B was only significant after 72 hours (p = 0.044), 96 hours (p = 0.002), and 120 hours (p = 0.03). With the Tukey-Kramer test, only the difference at 96 hours was significant (p = 0.05). A T*t interaction that was near to the significant value (p = 0.072) and showed that the temperature effect increased during the kinetics supported these findings

Effect of incubation time on the biotransformation of anthracene

This multifactorial analysis's findings demonstrated the impact of the factors S and T on the transformation of AC. The four groups' results were examined independently at 22°C, 30°C, A, and B. When the amount of AC was transformed based on the incubation time, an analysis of the regression variance revealed a positive and significant regression for each group (p 0.0001 for the groups A, 22 °C, B, 22 °C, A 30 °C, and p = 0.0008 for the group B, 30°C). Figure 3 shows a representation of the four kinetics.

For the groups A, 22 °C; B, 22 °C; A, 30 °C; B, 30 °C, the slopes (rate of AC transition) were 0.0213, 0.0221, 0.0198, and 0.0111 mg/g of dry mycelium/h, respectively. These slopes for the first three groups were roughly 0.50 mg/g of dry mycelium/d and did not differ statistically (p > 0.54). The kinetics run at 22 °C and 30 °C for strain A from the polluted sol were similar (Figure 3). Although the amount of xenobiotic converted was less for strain B from the CBS collection, the kinetics run at 22 °C was parallel to that seen with strain A. However, the rate of transformation was two times lower at 30ºC roughly 0.26 mg/g of dry

mycelium/d, and was substantially faster at 22ºC than at 30ºC (difference between the slopes = 0.011 mg/g dry mycelium/h, p = 0.003). (Figure 3).

Overall, these findings supported the multifactorial variance analysis and "a posteriori" test results for strains A and B.

Kinetics of fluoranthene biotransformation by Absidia

The FA transformation's kinetics were run similarly to AC's. For each replicate, eight were performed. In our experimental settings, the peak corresponding to FA was found at 11 min in the chromatographic profiles, which showed four large peaks for both strains with retention durations ranging from 0.9 and 2.1 min and two minor peaks at 3.2 and 4 min.

During the first 24 hours, the peaks' height and surface rose, whereas the peak that corresponds to FA shrank at the same time. In the HPLC profiles of the control extracts corresponding to the medium supplemented by FA without fungus and the medium with fungus but without FA, these peaks were not visible. All of these findings provided compelling evidence that the peaks related to FA-derived metabolites. Additionally, these peaks fell out within 24 hours, pointing to a biodegradation process.

After 24 hrs the percentages of FA transformation were very high: mean values were 89.3% (CV = 4.3%) for the group A at 22ºC, 87.3% (CV = 2.9%) for A, for B at 30ºC, 94.2% (CV = 2.1%), 22ºC and 91.1% (CV = 3.6%) for B, 30ºC. Results shown in mg/g of dry mycelium, are illustrated in Figure 4. All of the coefficients of variation were all below 10% (min = 4.8%, max = 9.2%). They were not significantly different according to the time in each group and also very similar from one group to another (mean values: 6.7% for A, 22ºC, 6.8% for A 30ºC, 6.5% for B 22ºC, 5.9% for B, 30ºC).

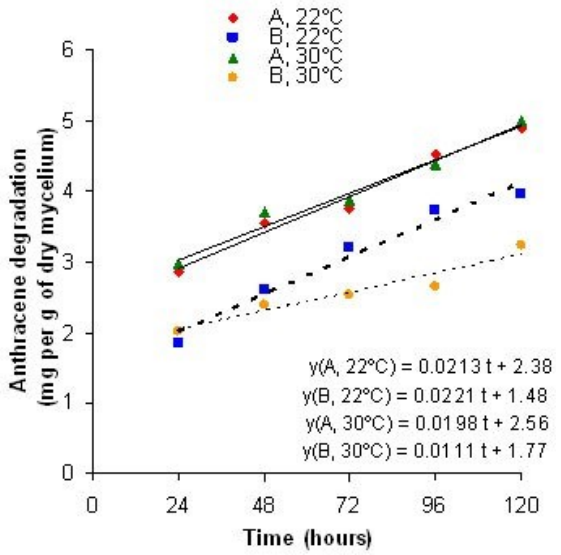

Figure 3. Kinetics of anthracene transformation by *Absidia* strains A and B as a function of the temperature (mean values of the amount

degraded).

Effect of the strain and the temperature on fluoranthene biotransformation

Table 2 presents the findings of the multifactorial analysis. The amount of FA transformed was always (regardless of the strain and time considered) lower at 22 °C than at 30ºC (temperature effect significant: p 0.0001), according to the analysis of the interaction S*T*t (not significant, p = 0.71). Between 24 and 72 hours, the disparities in the amount converted were more pronounced (Figure 4 and Table 2).

The strain effect was prominent (p = 0.0007), but has to be related to the significant S*t interaction (p = 0.002) shown in Figure 5. The graphic shows that the mean amount of FA transformed for strain A was lower during the first 72 hrs (3.29 mg/g of dry mycelium versus 3.72 at 24 hrs, 3.83 versus 4.06 at 48 hrs, 4.01 versus 4.19 at 72 hrs). During this time, there was a decrease in the differences between the two strains, and an inversion was then seen (4.46 versus 4.40 at 96 hrs, 4.55 versus 4.52 at 120 hrs). Using the Fisher PLSD test, the difference between the two strains at 22ºC was only significant at 24 hours (difference A - B = 0.44 mg/g of dry mycelium, p = 0.002), and at 24 and 48 hours for 30ºC (difference A - B = -0.41 and -0.33 mg/g of dry mycelium, p = 0.003 and 0.02 respectively; the Tukey-Kramer test only found the difference The strain effect was only noticeable for the first 48 hours of the kinetics, in other words.

Influence of the time of incubation on fluoranthene biotransformation

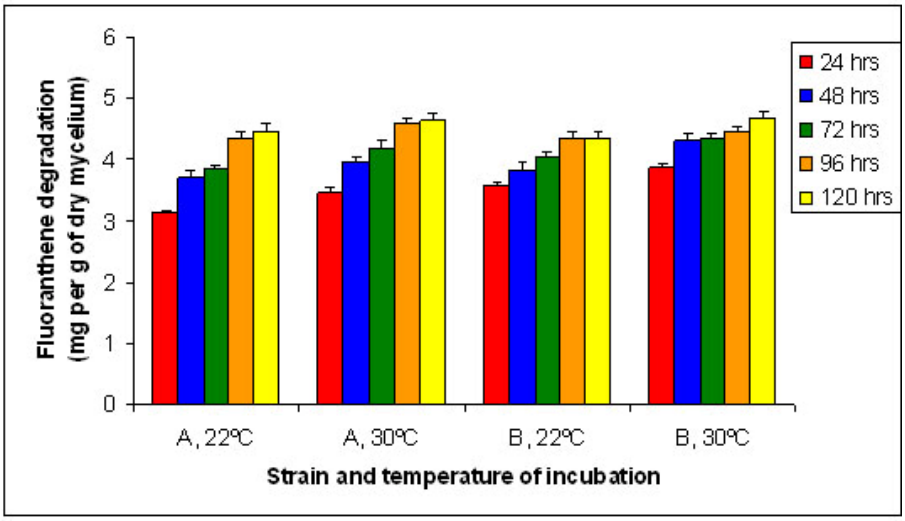

Figure 4. Strain*Temperature*time interaction for fluoranthene transformation by *Absidia* strains A and B (error bars: one standard error).

When the amount of FA was changed based on the incubation time, a regression analysis of the variance revealed a positive and significant relationship (p 0.0001 for the four groups). Figure 6 shows a representation of the four kinetics. The slopes were 0.0138, 0.0123, 0.0086, and 0.0074 mg/g of dry mycelium/h for groups A at 22ºC, A at 30ºC, B at 22ºC, and A, B at 30ºC, respectively. The rise in the mean amount of FA converted for strain A was 0.31 mg/g of dry mycelium/d, and the kinetics were parallel at 22ºC and 30ºC (difference between the slopes = 0.0014 mg/g dry mycelium/h not significant, p = 0.40).

The same findings were made for strain B, with an increase in the mean amount transformed of 0.19 mg/g of dry mycelium/d (difference between slopes = 0.0012 mg/g of dry mycelium/h not significant, p = 0.50). However, the rate of FA transformation was increased by 1.6 times for strain A compared to strain B, with a difference in slopes of 0.005 mg/g of dry mycelium/h (p = 0.01). These results lined up with those from the "a posteriori" tests and the multifactorial variance analysis.

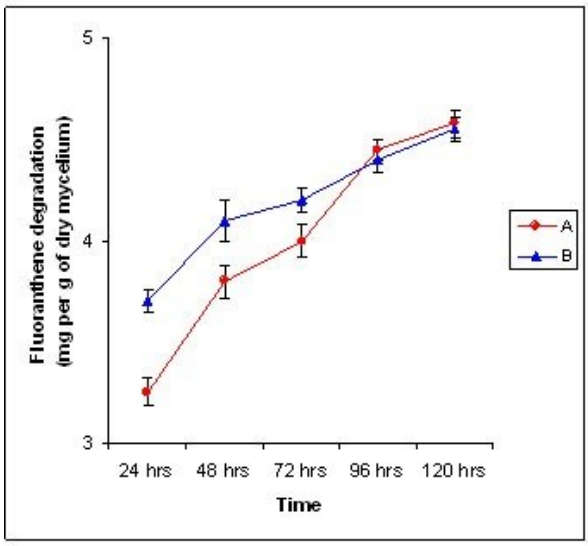

Figure 5. Strain*time interaction for fluoranthene transformation by *Absidia* strains A and B (error bars: one standard error; temperature: 30ºC).

Comparison of the kinetics of anthracene and fluoranthene biotransformation

For strain A, the kinetics were faster for AC than for FA at both 22ºC and 30ºC, with the difference between the slopes at 22ºC being 0.0075 mg/g of dry mycelium/h significant (p 0.001) and 0.0074 mg/g of dry mycelium/h significant (p 0.003). At 30ºC, the difference was 0.0074 mg/g of dry mycelium/h significant (p 0.003). This was also seen for strain B: the kinetics were 1.5 times quicker at 30ºC but the difference was not significant in this case. The kinetics were 2.6 times faster at 22ºC (difference between the slopes = 0.0135 mg/g of dry mycelium/h significant p = 0.0004).

DISCUSSION

The ability of the species Absidia for the transformation or degradation of two PAHs, AC and FA, as well as the alteration of its effectiveness depending on the habitat it was isolated from, is revealed by the current investigation. Absidia was used in both AC and FA bioremediation assays, and the biodegradation activity of Absidia was demonstrated by the differences in efficiency seen between the strains, the appearance of new peaks on the HPLC profiles after 24 hours of incubation, the progressive decrease of these peaks between 24 hrs and 120 hrs, as well as the decrease of the peak corresponding to the xenobiotic.

The work did not attempt to identify the generated metabolites. Since the mean amount of AC transformed was two times higher, strain A of Absidia from the contaminated soil was significantly more effective than strain B in converting AC. While strain A's ability to convert AC was unaffected by incubation at 30°C, strain B was significantly inhibited between 72 and 120 hours. A, 22ºC, A, 30°C, and B22ºC all had similar transformation rates; B, 30°C had a lower transformation rate. For strain A over the first 48 hours, the mean amount of FA converted was marginally but significantly lower.

In comparison to strain B, strain A showed a faster rate of transformation. For strains A and B, there were no variations in the transformation rates as a function of temperature. Overall, the transformation rate with both strains was higher for AC than for FA (1.5 to 2.6 times). Since it has been documented in the literature for several fungi and xenobiotics, the high levels of transformation that were seen after 24 hours were not a unique characteristic. FA was more readily broken down by fungi than AC, according to earlier research by Giraud et al. (2001); in this study, strain B from a collection was an example of this. This was reported for strain A isolated from a contaminated soil, and other investigations using the two molecules (Krivobok et al. 1998; Salicis et al. 1999) demonstrated comparable outcomes. The majority of reports noted Absidia cylindrospora as well as Rhizopus arrhizus and the Cunninghamella species as having high PAHs degradation efficiency (Cerniglia, 1993; Krivobok et al. 1998; Salicis et al. 1999). (Giraud et al. 2001). In this study, we demonstrated that Absidia has a potential to alter or degrade PAHs.

In the Basidiomycete group, and especially the white-rot fungi, environmental xenobiotics, particularly PAHs, have been extensively researched through fungal biotransformation and/or degradation (Pointing, 2001; Tekere et al. 2005). In two earlier studies, we demonstrated how effectively Absida can break down a variety of xenobiotics, including various kinds of herbicides (mostly metribuzin and metobromuron), phenolic chemicals (primarily ferulic acid), and pentachlorophenol (Bordjiba et al. 2001; Guiraud et al. 2003).

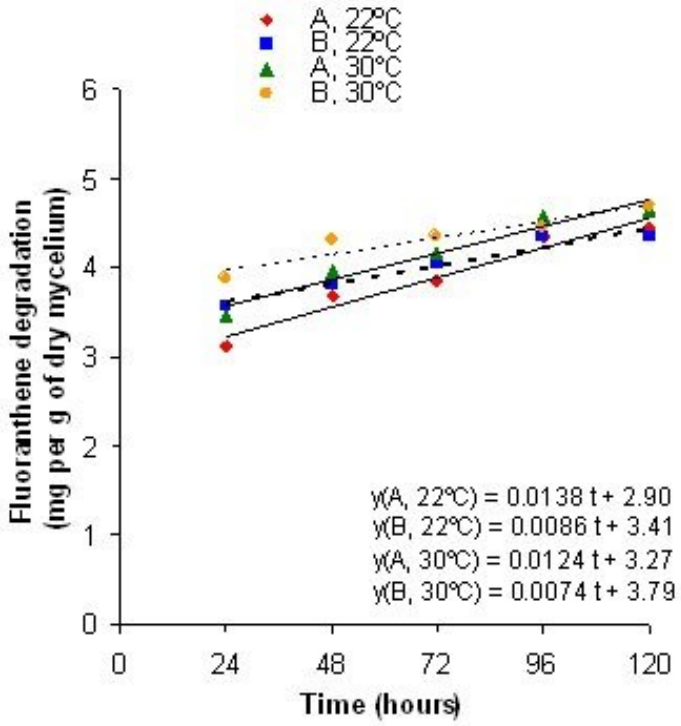

Figure 6. Kinetics of fluoranthene transformation by *A. fusca* strains A and B as a function of the temperature (mean values of the amount degraded).

In both the studies, it was shown that the strain isolated from a polluted environment had significantly greater biodegradation capabilities (strain A). Here, we saw that Absidia is also effective at breaking down PAHs like AC and FA, with strain A having an improved capacity, at least for AC degradation. Furthermore, strain A responded to temperature fluctuations less sensitively. The selection of a strain expressing low specific but effective enzyme systems capable of breaking down a wide range of compounds was likely influenced by the contaminated environment. The importance of researching microbial communities that can adapt in degraded ecosystems for bioremediation purposes is highlighted by this study.

Acknowledgment

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