Research Articles: Biodegradation

Biodegradation of Crosslinked Acrylic Polymers by a White-Rot Fungus

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Abstract

Two synthetic superabsorbent crosslinked acrylic polymers were mineralized by the white-rot fungus *Phanerochaete chrysosporium*. The amount of polymer converted to CO_2 increased as the amount of polymer added to the cultures increased. In the presence of sufficiently large amounts of the superabsorbents, such that all of the culture fluid was absorbed and a gelatinous matrix was formed, the fungus still grew and mineralization was observed. Neither the polymers, nor their degradation products were toxic to the fungus. While the rates of mineralization were low, all of the polymers incubated in the liquid fungal cultures were completely depolymerized to water soluble products within 15–18 days. The depolymerization of the polymers was observed only in nitrogen limited cultures of the fungus which secrete the lignin degradation system, however, the water soluble products of depolymerization were mineralized in both nutrient limited and sufficient cultures of the fungus. The rate of mineralization of the depolymerized metabolites was more than two times greater in nutrient sufficient cultures. Following longer incubation periods, most (> 80 %) of the radioactivity was recovered in the fungal mycelial mat suggesting that carbon of the polymer had been converted to fungal metabolites.

Keywords: *Phanerochaete chrysosporium*; superabsorbents; depolymerization; white-rot fungi; lignin peroxidase; polyacrylate

1 Introduction

Superabsorbents constitute a unique class of synthetic polymers because of their ability to store quantities of aqueous liquid much greater than their own weight. Crosslinked polymers of acrylic acid and comonomers such as acrylamide are effective superabsorbents and have been utilized for a broad range of applications. These superabsorbing polymers have been useful for the development of new hygiene articles with improved liquid holding capacity, for agriculture as soil amendments for improved water management, and for packaging of food products to lengthen shelf life.

Due to the widespread use of these polymers there has been unintentional exposure of these materials to the environment. Therefore, the environmental compatibility of these polymers must be addressed. In-depth ecotoxicological testing has provided no evidence for significant adverse effects of such polymers to marker organisms in water, or plants and birds, due to their chemical inertness. In turn, the crosslinkage of the acrylic polymers and the stability of the polymeric carbon backbone would suggest that these polymers would be very difficult to biodegrade. Indeed, practically no biodegradation was observed in standardized test systems.

Therefore, the ability of white-rot fungi to degrade these superabsorbing polymers was investigated. White-rot fungi are ubiquitous organisms which have been shown to be capable of degrading a wide range of recalcitrant xenobiotics [1, 2]. The proposal for the application of white-rot fungi to degrade man-made polymers was derived from the fact that the ecological niche of these fungi is based on the ability of these fungi to degrade the most recalcitrant biological polymer, lignin [3]. Lignin is a random, stereoirregular, three-dimensional polymer of phenyl propanoid units which provides structure to wood [4]. Due to the size and insolubility of lignin, it can not be internalized by microorganisms. Furthermore, due to the randomness and stereoirregularity of lignin, enzymes designed to cleave specific chemical bonds are unable to depolymerize the polymer. Similarly, the high molecular weight and crosslinking of polyacrylic superabsorbents are properties which render these man-made polymers stable to biological degradation. However, white-rot fungi are equipped with a unique nonspecific, extracellular degradation system. The degradation system is secreted as the fungus enters secondary metabolism in response to nutrient limitation and consists of enzymes and small molecular weight metabolites [2]. The integral enzyme component of the system is a group of peroxidases which oxidize a broad range of chemicals by one electron [2]. This results in the production of free radicals which are highly unstable chemicals. Through the reactions of the peroxidases with the small molecular weight metabolites secreted by the fungi it has been shown that powerful oxidants and reductants can both be produced. The enzymatic mechanisms of the degradation system of white-rot fungi have been reviewed [2]. The production of highly reactive chemical species provides the degradation system of white-rot fungi with an inherent nonspecificity. Indeed it has been shown that white-rot fungi are capable

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of degrading a wide variety of chemicals including DDT, dioxin, trichloroethylene, pentachlorophenol and 2,4,6-trinitrotoluene (TNT) [1,5–7]. Another unique feature is that white-rot fungi degrade many compounds completely to CO_2, a process referred to as mineralization [1]. It has been proposed that, in general, the lignin degrading system is responsible for the initial degradation of compounds and that subsequently the degradation products are internalized and further metabolized to CO_2.

We have investigated the degradation of superabsorbent polymers by *Phanerochaete chrysosporium*, a white-rot fungus which has been studied extensively for its biodegradative capabilities. Two high molecular weight, crosslinked acrylic polymers were utilized. One polymer was crosslinked polyacrylate and the other was a crosslinked copolymer of acrylamide and acrylic acid and is referred to as crosslinked copolymer.

2 Experimental Section

The polymers used in this study were cross-linked, high molecular weight, insoluble polymers of acrylic acid and other comonomers such as acrylamide supplied by Stockhausen GmbH & Co. KG, Krefeld, Germany. They also provided the ^{14}C-radiolabelled form of each polymer in which the ^{14}C was incorporated into the carbon backbone of the polymers. The monomers used for the two radiolabelled polymers included 2,3-(^{14}C)-acrylic acid and 2,3-(^{14}C)-acrylamide. The specific activity of the two polymers was 1.4 mCi/g. In all experiments the cultures contained 200–300 µg of radiolabelled polymer which was weighed accurately using a microbalance. Additional polymer was added as unlabelled material. The polymers were washed repeatedly prior to fungal incubation to remove any water soluble extracts from the polymers. *P. chrysosporium* BKMF-1767 was grown at 37 °C in stationary cultures containing standard liquid media as described previously [6, 8]. Additional glucose supplements (100 mg) were added as 0.25 mL of a 40 % filter-sterilized glucose solution. Analysis of the production of $^{14}CO_2$ in the fungal cultures was performed as previously described [6]. The kinetics of depolymerization were monitored by removing 0.1 mL of the 10.0 mL culture fluid from each culture every three days and analyzing the amount of radioactivity in the 0.1 mL sample by scintillation spectroscopy.

3 Results and Discussion

A small amount of either ^{14}C-radiolabelled crosslinked polyacrylate or copolymer was incubated in liquid cultures of *P. chrysosporium*. The fungus was grown under nutrient nitrogen sufficient conditions (24 mM NH_4) and nutrient limited conditions (2.4 mM NH_4), the latter which induces the production of the lignin degrading system [8]. The mineralization of the polymers was monitored by quantitating the amount of $^{14}CO_2$ produced. As shown in **Figure 1**, both the crosslinked polyacrylate and copolymer were degraded to CO_2 by the fungus. The mineralization of the polymers was dependent on the lignin degrading system since no mineralization occurred in nutrient sufficient cultures of the fungus which do not secrete the degradation system [8]. In addition, the mineralization of the polymers in nutrient limited cultures of the fungus did not occur until after day 3, which corresponds with the time that the fungus secretes the degrading system under these conditions [1, 8].

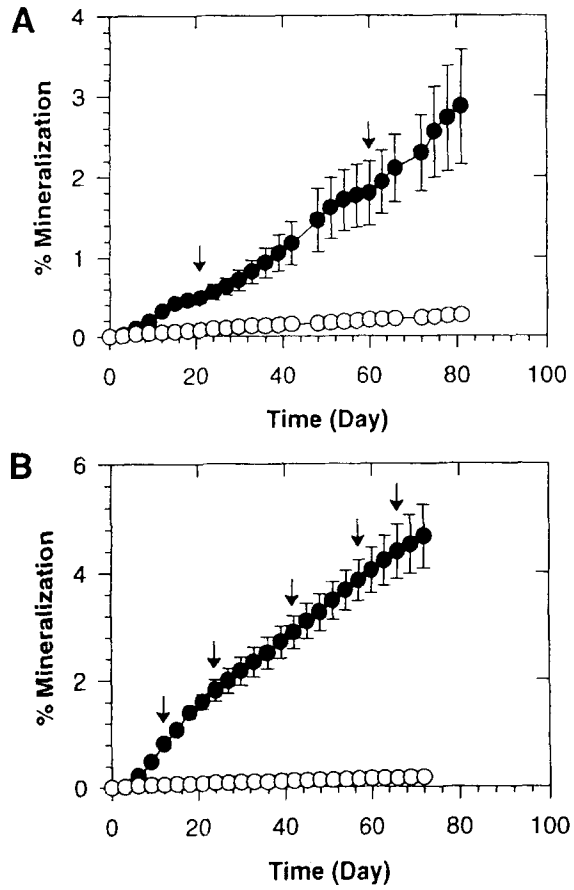

Fig. 1: Mineralization of crosslinked copolymer (A) and crosslinked polyacrylate (B) by nutrient nitrogen limited (●) and nitrogen sufficient (o) liquid cultures of *P. chrysosporium*. Crosslinked copolymer or polyacrylate (200 µg) were added to pentuplicate culture bottles. Additional nutrient (glucose, 100 mg) was added on day 21 and 60 to cultures containing crosslinked copolymer and day 12, 24, 42, 57 and 66 to those containing crosslinked polyacrylate, as indicated by arrows

The rate of mineralization of the crosslinked copolymer in the liquid cultures of *P. chrysosporium* (→ *Fig. 1A*) began to decrease after approximately 18 days. Therefore, on day 21 an additional supplement of glucose was added to the cultures and subsequently the rate of mineralization increased. Glucose was also added on day 60 following another cessation in mineralization of the crosslinked copolymer and again the rate increased in response to the addition. Based on these results, during the mineralization of crosslinked polyacrylate (→ *Fig. 1B*) glucose was added more frequently, on days 12, 24, 42, 57 and 66, and no decrease in the rate of mineralization of this polymer was observed.

The effect of adding greater amounts of polymers on the mineralization by the fungus was then investigated. Up to 64 mg of the polymers were added to nutrient limited liquid cultures of *P. chrysosporium*. Since these polymers are superabsorbents, 64 mg of the polymers absorbed virtually all of the culture fluid such that the cultures were gelatinous mixtures. However, the fungus grew on the polymer matrices and mineralization of the polymers was observed. Therefore it was concluded that neither the polymers, nor the polymer degradation products were toxic to the fungus. The amount of polymer mineralized by the fungus continued to increase as the amount of either crosslinked polyacrylate or copolymer added to the cultures increased up to 64 mg (→ *Fig. 2*). This indicated that the degradation system of the fungus was not saturated with polymer up to 64 mg/10 ml.

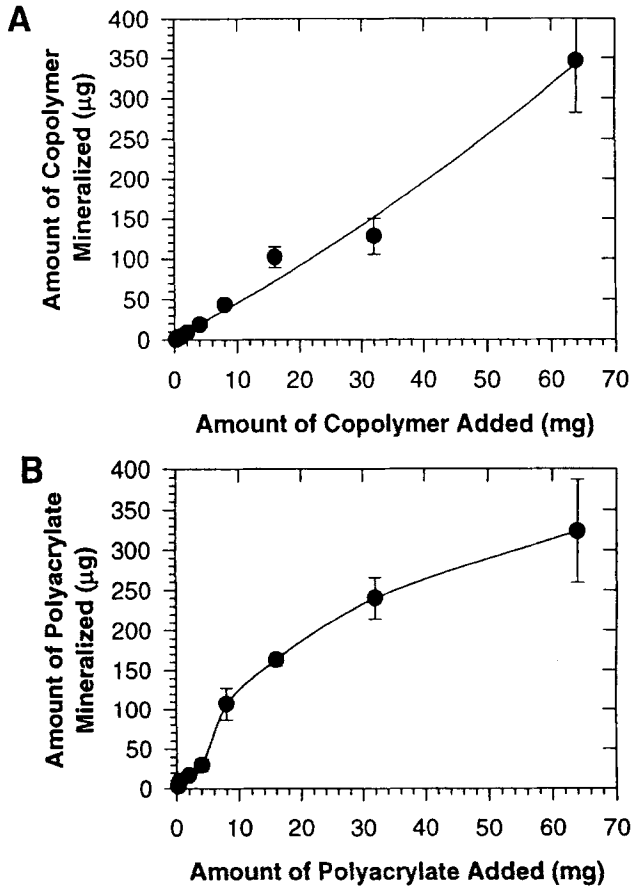

Fig. 2: Effect of the amount of crosslinked copolymer (A) and crosslinked polyacrylate (B) added to liquid fungal cultures on the mineralization of the polymers by nutrient nitrogen limited cultures of *P. chrysosporium*. The cultures were incubated in pentuplicate for 30 days and were supplemented with glucose (100 mg) on day 12 and 21

It was observed in the fungal cultures which contained 64 mg of polymers that the gelatinous polymer matrices liquified during the course of the 30 day experiment. Therefore, while the rate of degradation of the polymer to CO_2 was low, the polymers were rapidly degraded to such an extent that the polymers lost the ability to retain the absorbed liquid. The depolymerization of the polymers in liquid cultures of *P. chrysosporium* was thus investigated. Depolymerization of the superabsorbents from insoluble polymers to water soluble products was monitored by quantitating the formation of water soluble radioactivity in the culture fluid. Liquid cultures of nutrient limited *P. chrysosporium* were incubated with small amounts (250 µg) of ^{14}C-labelled polymer. Following 21 days of incubation the cultures were separated into 3 fractions which contained the fungal mycelia mat, the culture fluid, and any remaining insoluble polymer. In controls, containing no fungus, the insoluble polymers were completely recovered. However, in the nutrient limited fungal cultures, all of the radioactivity of the formerly crosslinked copolymer and polyacrylate (95 % and 107 %, respectively) were recovered in the water soluble fractions.

The depolymerization of larger amounts of polymers to water soluble products was then monitored over time by removing small samples of the culture fluid during the course of the experiment and quantitating the amount of radioactivity. As shown in **Figure 3**, even in cultures which contained up to 4.0 mg of either crosslinked copolymer or polyacrylate, essentially all of the polymer was depolymerized following 18 days of incubation with the fungus. In some cultures containing less than 1.0 mg of polymer, the polymer was completely depolymerized to water soluble products following only 12 days of incubation.

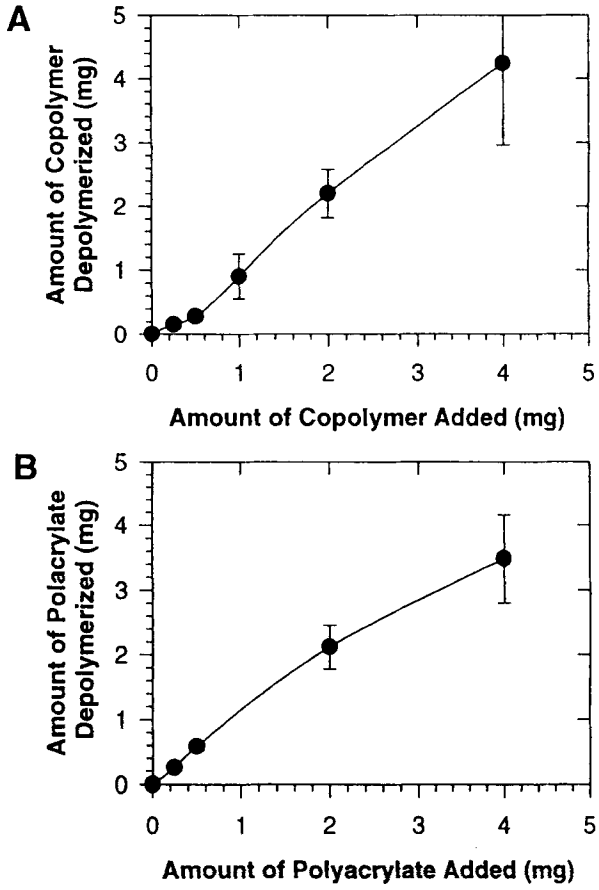

Fig. 3: Effect of the amount of crosslinked copolymer (A) and crosslinked polyacrylate (B) added to liquid fungal cultures on the depolymerization of the polymers by *P. chrysosporium*. The cultures were incubated in pentuplicate (under nutrient nitrogen limited conditions) for 18 days with supplemental glucose (100 mg) added on day 12 (A) and day 9 (B)

The kinetics of the depolymerization of 4.0 mg of crosslinked copolymer and polyacrylate over time are shown in **Figure 4**. The role of the lignin degrading system in the depolymerization process was confirmed by the fact that no depolymerization was observed in nutrient sufficient cultures and that in nutrient limited cultures, depolymerization did not occur until after day 3 [1, 8]. Therefore, while a small amount of the polymers were mineralized, all of the polymer added to the cultures was rapidly depolymerized to water soluble products. The water soluble products of depolymerization have not been characterized yet, but it is known that in order to convert these polymers to water soluble products, a significant amount of depolymerization must occur.

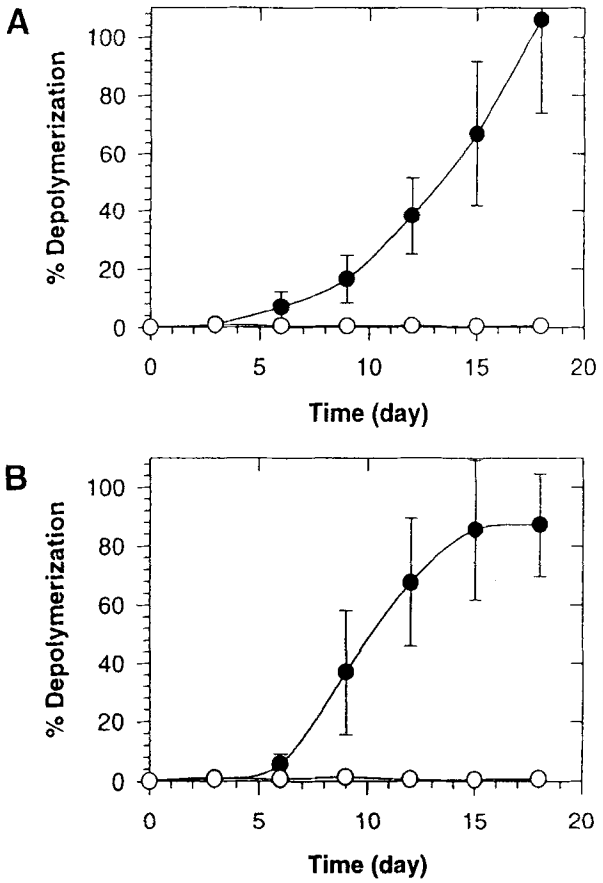

Fig. 4: Depolymerization of 4.0 mg of crosslinked copolymer (A) and crosslinked polyacrylate (B) by nutrient nitrogen limited (●) and nitrogen sufficient (o) liquid cultures of *P. chrysosporium*. The cultures were inoculated in pentuplicate and were supplemented with glucose on day 12 (A) and day 9 (B)

Based on these results, it was clear that nutrient limiting conditions, and thus the lignin degrading system, were required for the depolymerization of the polymers. However, since this process was completed in less than 20 days, it was proposed that the subsequent internalization and mineralization may not require the lignin degrading system. If this were the case, then mineralization of the products of depolymerization may occur at a greater rate in nutrient sufficient cultures of *P. chrysosporium* since the fungus grows better under such conditions. Therefore, fungal cultures containing a small amount of crosslinked polyacrylate were incubated for 33 days under nutrient limiting conditions. Complete depolymerization of the crosslinked polyacrylate to water soluble metabolites was verified. Half of the fungal cultures were then converted to nutrient sufficient conditions and the other half were not manipulated. The subsequent rates of mineralization following day 33 were more than two times greater in the cultures which had been converted to nutrient sufficient fungal cultures (→ *Fig. 5*). No mineralization was observed in cultures which were nutrient sufficient since the time of inoculation. Again, the rates of mineralization were dependent on the addition of glucose. Since nutrient sufficient cultures of the fungus mineralized the water soluble products of depolymerization at a much greater rate, it was proposed that an ideal fungal bioreactor for the degradation of these polymers would consist of two stages. The first stage would expose the insoluble polymers to nutrient limited *P. chrysosporium* to perform the depolymerization of the polymers. The water soluble products of depolymerization would then be transferred to the second stage reactor which would contain fungus grown under nutrient nitrogen sufficient conditions to maximize the subsequent mineralization.

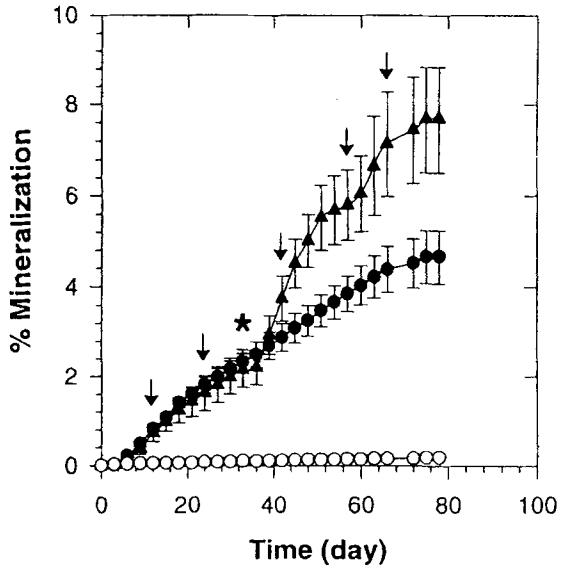

Fig. 5: Mineralization of crosslinked polyacrylate by liquid cultures of *P. chrysosporium*. Two sets of cultures were inoculated under nitrogen limited conditions initially (▲, ●). On day 33 (✳), one was made nitrogen sufficient by reinoculation under high nitrogen conditions (▲). The other was not manipulated (●). The third set was inoculated under nutrient sufficient conditions throughout the entire experiment (o). The cultures were supplemented with additional glucose on days 12, 24, 42, 57 and 66, as indicated by arrows

The fact that nutrient sufficient cultures of *P. chrysosporium* were capable of mineralizing the water soluble products of depolymerization indicated that the process of mineralization must involve primary metabolism, which must be an internal process. Therefore, the internalization of the depolymerized metabolites into the fungus was monitored. Following the incubation of nutrient limited liquid cultures of *P. chrysosporium* with either crosslinked copolymer or polyacrylate for 96 and 144 days, respectively, the distrib-

ution of the remaining radioactivity was analyzed. The cultures were separated into fractions containing the liquid culture fluid, remaining insoluble polymer, and the fungal mycelial mat. In the control bottles containing no fungus, essentially all of the radioactivity was recovered as remaining insoluble polymer (→ *Table 1*). However, following incubation of the polymer with the fungus, only trace amounts of the radioactivity were recovered as insoluble polymer. This is in agreement with the previous results in which the polymers were depolymerized to water soluble products within 20 days. However, following further incubation only approximately 10 % of the original radioactivity remained as water soluble products in the culture fluid (→ *Table 1*). Almost all of the radioactivity, 73 and 85 % for crosslinked polyacrylate and copolymer, respectively, was recovered in the washed mycelial mat. Only a small fraction (2–3%) was mineralized, however, these cultures were not continually supplemented with glucose.

Table 1: Distribution of radioactivity of crosslinked ^{14}C-polyacrylate and copolymer following incubation with nutrient limited liquid cultures of *P. chrysosporium*

Fraction	% Radioactivity			
	Crosslinked Copolymer		Crosslinked Polyacrylate	
	Fungus	No Fungus	Fungus	No Fungus
mycelial mat	85 ± 15	–	73 ± 10	–
culture fluid	9 ± 4	1	10 ± 4	2
insoluble polymer	1 ± 0	90	3 ± 2	102
CO_2	3 ± 1	–	2 ± 1	–
total recovery	98		88	

Therefore, while the rate of mineralization was low, all of the polymer was depolymerized in 20 days and then virtually all of the depolymerized products were internalized by the fungus. Although the actual chemical form of the radioactivity in the fungal mycelia has not been identified, it is believed that most of the carbon from the polymer was converted into fungal metabolites. Since the mycelial mats were washed extensively and the incorporation of the radioactivity of the water soluble metabolites of depolymerization into the fungus was a time dependent process (virtually no radioactivity was found in mycelia between day 20 and day 30), the possibility that the depolymerized products were merely bound to the exterior of the fungus was rejected. The nature of this material is currently being investigated.

4 Conclusion and Future Outlook

The results of this investigation have demonstrated that the white-rot fungus, *P. chrysosporium*, was capable of degrading man-made crosslinked acrylic superabsorbent polymers. The degradation process consisted of three stages. The insoluble polymers were first rapidly depolymerized to water soluble products by the extracellular nonspecific degrading system. Over longer time periods the water soluble metabolites were incorporated in the fungal mycelial mat. Mineralization of the water soluble metabolites by the fungus occurred throughout the time course and although the overall amount of polymer mineralized was low, the results suggested that almost all of the polymer was degraded to fungal metabolites. Therefore, there seems to be sufficient and increasing evidence that the tested crosslinked acrylic polymers do not constitute persistent, nondegradable man-made polymers. Our future research will involve scaling up the fungal-based degradation system and maximizing the rate and extent of degradation of these crosslinked polymers. We will also investigate the ability of other white-rot fungi to degrade these polymers.

Acknowledgements

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5 References

[1] J.A. BUMPUS; M. TIEN; D. WRIGHT; S.D. AUST (1985): Science 228, 1434–1436
[2] D.P. BARR; S.D. AUST (1994): Environ. Sci. Technol. 28, 78A-87A
[3] T.K. KIRK (1987): Ann. Rev. Microbiol. **41**, 465–505
[4] K.V. SARKANEN; C.H. LUDWIG (1971) in: Lignins – Occurrences, Formation and Structure. New York, Wiley-Intersciences, pp. 1–8
[5] A. KHINDARIA; T.A. GROVER; S.D. AUST (1994): Environ. Sci. Technol. 29, 719–725
[6] G.J. MILESKI; J.A. BUMPUS; M.A. JUREK; S.D. AUST (1988): Appl. Environ. Microbiol. **54**, 2885–2889
[7] T. FERNANDO; J.A. BUMPUS; S.D. AUST (1990): Appl. Environ. Microbiol. **56**, 1666–1671
[8] T.K. KIRK; E. SCHULTZ; W.J. CONNORS; L.F. LORENZ; J.G. ZEIKUS (1978): Arch. Microbiol. 117, 277–285

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