New urethane prepolymer immobilized fungal bioreactor for decolorization and dechlorination of kraft bleach effluents

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ABSTRACT

A new fluidized bed bioreactor system containing urethane prepolymer immobilized Trametes versicolor *can successfully decolorize and dechlorinate kraft bleach plant effluents. Color reduction ranging from 72–80% and AOX reduction ranging from 52–59% is possible from a continuous bioreactor at a residence time of 24 h. The decolorization process was linearly dependent on the concentration of glucose cosubstrate up to a level of 0.8% by weight. Color reduction followed first-order kinetics in the fluidized bioreactor.*

Application:

The system described offers promise for use as a biotreatment process for decolorization and dechlorination of bleach plant effluents.

AMONG THE VARIOUS IMMOBILIZATION techniques presently available, entrapment of biocatalysts in polymer gels is extremely promising. This technique has wide applicability to the immobilization of enzymes, cellular organelles, microbial cells, plant cells, and animal cells *(1)*. Urethane prepolymer supports offer attractive characteristics for cell entrapment such as mechanical strength and an open-celled structure. The urethane prepolymer supports adapt well to use in bioreactors *(2)*. The porous, open-celled structure of the foam allows a nondiffusion-limited environment for substrate and product.

Although the literature contains a considerable amount of research on the application of this method for immobilization of enzymes *(3, 4)* and microbial cells *(5, 6)*, there has been little attention to the entrapment of fungal mycelium. Fungi are important microorganisms to degrade many environmentally toxic and persistent pollutants. Providing a unique immobilizing support with sufficient mechanical and chemical stability, one can extend the activity and lifetime of the biocatalysts.

White-rot fungi, *Phanerochaete chrysosporium* and *Trametes versicolor*, have a nonspecific ability to degrade many persistently toxic organic chemicals from both bleach plant effluents and other sources *(7, 8)*. For *P. chrysosporium*, two processes are MyCoR and MyCoPor. The MyCoR process uses the biological decolorization of E1-stage bleach plant effluents by fungus immobilized on disks in a rotating biological contactor (RBC) *(9)*. The MyCoPor process is a trickling filter system that immobilizes fungus on poly-urethane foam cubes *(10)*. Both systems employ passive immobilization methods that involve adhesion of cells to a solid support. Active immobilization techniques based on cell entrapment have the advantages of high mechanical resistance and stability against pH and media variations.

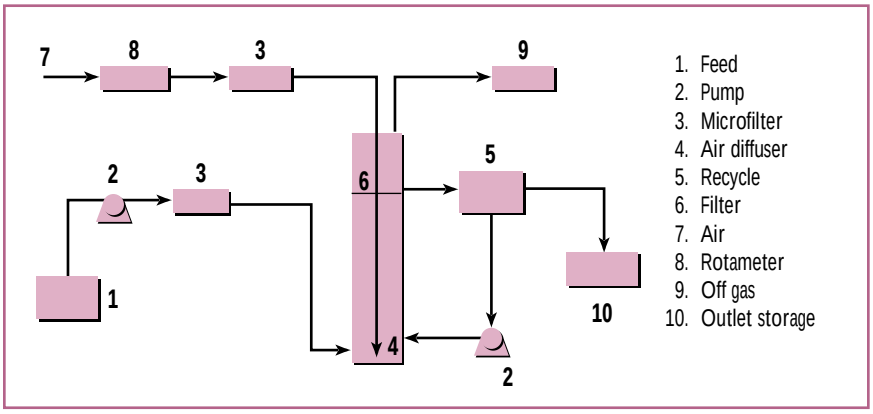
In our laboratory, we previously investigated the use of free-cell and calcium alginate immobilized white rot fungus, *T. versicolor*, for the continuous decolorization and dechlorination of pulp and paper mill wastes *(11)*. Although the alginate immobilized *T. versicolor* system was adequate for laboratory scale studies, it may not be attractive for large-scale applications. Drawbacks of the alginate system include (1) associated diffusional limitations that result in the localization of mycelia to a limited thickness near the outer surface of the alginate bead and (2) the susceptibility of calcium alginate to degradation *(12)*. Many researchers reported similar patterns of growth limitations with calcium alginate immobilized systems *(13, 14)*.

The primary objective of this study was to develop an immobilization technique for the *T. versicolor* that produces a biocatalyst stable to biological activity and mechanical stability. We explored the potential application of fungi immobilized in urethane prepolymers for decolorization and dechlorination of kraft bleach effluents. We also investigated some parameters influencing the color and adsorbable organic halide (AOX) reduction levels. SEM examined the conformational characteristics of the prepolymer entrapped fungal mycelia.

MATERIALS AND METHODS

Chemicals

Hampshire Chemicals Corp., Lexington, MA, supplied the polyurethane prepolymer, HYPOL FHP 2000, and Air Product & Chemicals, Inc., New Milford, CT, provided the surfactant, DABCO DC193. We prepared polyurethane foam by mixing prepolymer with water or blended mycelial solution.



1. Schematic diagram of the experiment

Parameter	Value
Feed color, mean	2476 CU
Product color, mean	772 CU
Product color, SD	147 CU
Reduction percentage, mean	69%
Reduction percentage, mean	5%
Feed AOX, mg/L	23
Product AOX, mg/L, mean	10.6
Product AOX, mg/L, SD	1.7
Reduction percentage, mean	53%
Reduction percentage, SD	5%

SD: Standard deviation

***I. Performance of continuous bioreactor using polyurethane prepolymer immobilized* T. versicolor**

Fungus and immobilization method

The strain of fungus was *Trametes (Coriolus) versicolor*, ATCC 20869. The cultures were grown by inoculating 500 mL shaker flasks containing 150 mL of mycological broth (Difco) at pH 4.8. Four-day-old cultures from different flasks grown under similar conditions were combined and blended in a Waring blender. The blended mycelium was mixed with the urethane prepolymer and vigorously stirred at room temperature for one minute. Surfactant was added at 2% to regulate the cell structure of the foam *(15)*.

After one minute, the foam expanded five-fold in volume. Then the foam was poured onto parafilm, spread into a thin layer, and allowed to stand at room temperature. Polymerization started immediately and produced hardened foam in approximately 30 min. The foam was cut into small cubes of approximately 5 x 5 x 2 millimeters. The HYPOL 2000 contains less than 5% by weight of free toluene diisocyanate (TDI). This is an important feature for immobilized biocatalysts *(4)*. For control studies, mycelia-free foam was made by mixing prepolymer with water. Before use, the foam cubes with entrapped mycelia underwent washing with sterile water.

Bleach plant effluents

The bleach plant effluents (BPE) came from Mill I and Mill II. Using an OD(EO)DED bleaching sequence, Mill I supplied a first D-stage effluent and an EO-stage effluent. Another EO-stage effluent came from Mill II using an OCD(EO)D bleaching sequence. The feed to the bioreactor system contained a mixture of 60% D-stage and 40% EO-stage effluents. Both were from Mill I unless otherwise specified. The feed mixture contained a vitamin and mineral solution and a carbon, nitrogen, and phosphorous source solution containing 0.6% glucose, 0.16% $(NH_4)_2SO_4$, and 0.3% K_2HPO_4 and 0.2% KH_2PO_4 by weight per volume. The composition and final concentration of the vitamin supplement was 0.2 mg/L each of D-pantothenic acid, riboflavin, folic acid, pyridoxal, thiamine-HCL, nicotinic acid, and biotin. The composition of the mineral salt supplement was 0.1 µm $NiCl_2$, 5 µm $CoCl_2$, 20 µm $MnCl_2$, 5 µm $ZnCl_2$, 1 µm $CuSO_4$, 40 µm sodium citrate, 20 µm $FeSO_4$, and 0.5 µm $(NH_4)6Mo_70_{24}$. The final pH of the mixture was adjusted to 5.0 with concentrated HCl.

Determination of color and AOX

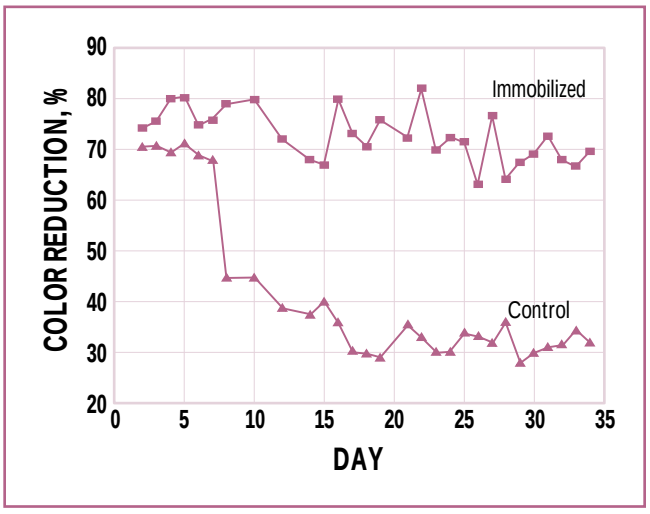
The AOX concentration was determined with a Dohrman DX-20B total organic halide analyzer. Samples were prepared according to EPA method 1650. Color was measured according to NCASI standards *(16)*. The pH of the sample was adjusted to 7.6 with 1*N* NaOH. The absorbance of the sample was measured by a spectrophotometer at 465 nm after filtration through a 0.8-µm membrane filter. The results were reported as platinum-cobalt color units (pcu).

Measurement of glucose

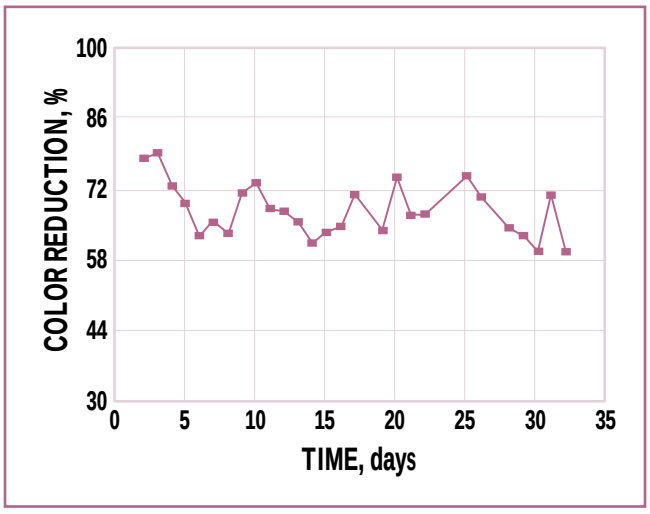
The concentration of glucose in the samples was determined by YSI 2300 STAT PLUS, Yellow Springs, OH.

SEM

To observe mycelial growth in the urethane foam particle, cut sections of the foam were obtained for microscopic observation. The cut sections were prepared by immersing in a 1% glutaraldehyde solution in 0.1 *M* cacodylate buffer at pH 7.0 for 30 min. Then the foam sections were washed in distilled water for 5 min. The fixed particles were dehydrated using a series of ethanol washes with progressively increasing ethanol concentrations of 70%, 85%, 95%, and 100% for 5 min each. Finally, the dehydrated sections were immersed in hexamethyl disilizane (HMDS) for 30 min and air dried at room tem-



2. Color reduction results of sequential batch studies



3. Color reduction performance of continuous bioreactor using polyurethane prepolymer immobilized **T. versicolor**

perature. After 24 h, the specimens were mounted on stainless steel SEM stubs and coated immediately with gold in an ion coater and examined by SEM.

Sequential batch experiments

The batch experiments were conducted by adding approximately 12 cubes of foam entrapped with *T. versicolor* to a 250 mL flask equipped with a foam stopper containing 125 mL of nonsterile effluent. The control experiment was done simultaneously by adding fungus-free foam to another flask prepared identically. Experiments were carried out at 25–30°C while shaken at 180 rpm using a gyratory shaker. Color and AOX were monitored daily beginning on day 2 for 34 days. The incubating liquor was decanted and replaced with a fresh effluent daily.

Continuous bioreactor

These studies used a fluidized bed bioreactor with a 600 cc working volume. The bioreactor was aerated with air using a glass dispersion tube. With continuous supply of a new feed and withdrawal of treated product, the bioreactor operated continuously. The inlet air and inlet feed to the reactor passed through an in-line air purifying membrane filter and a microfilter (pore size 0.2 microns from Gelman Sciences), respectively. Figure 1 shows the schematic diagram of the fluidized bioreactor system. Fungus-entrapped foam cubes occupying a volume of about 200 mL containing approximately 450 mg of mycelia by dry weight were placed in the reactor for the continuous study. Before transferring to the reactor, the foam cubes were incubated in a shaking flask containing mycological broth (10% MB) at 180 rpm to obtain an optimum level of immobilized fungus growth. Daily monitoring of AOX and color started on day 2.

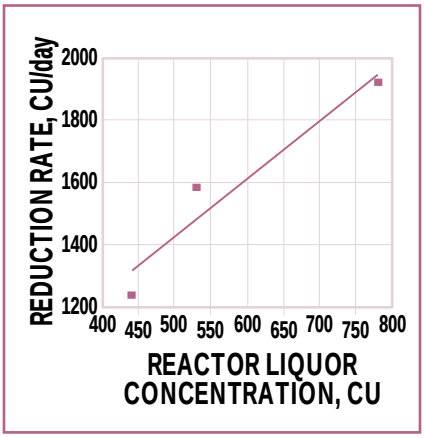
RESULTS

Batch studies

We investigated the color and AOX reduction ability of urethane prepolymer immobilized *T. versicolor* using sequential batch studies by replacing the treated effluent in the flask daily with fresh effluent. During the first seven days of batch operation, the flask containing the polymer immobilized fungus showed an average color reduction of 77% per day. The flask containing cell-free urethane foam showed an average color reduction of 70% per day. Starting on day 8, however, the decolorization efficiency of the cell-free foam declined rapidly as Fig. 2 shows. Foam containing fungus showed a consistent color reduction of 72% per day and AOX reduction of 52% per day during the batch operations. These results indicate that in the initial stage of the batch operation adsorption of color bodies and organic halogenated compounds by the polyurethane immobilizing matrix played the most important role in reducing total color and AOX of the effluents. Following several days of batch operation, the immobilized fungus had grown to an optimum level and adapted to the bleach effluent environment. Because of this adaptation and growth, the fungus-entrapped foam exhibited rapid biodegradation while the cell-free foam approached saturation.

Continuous studies

We used a fluidized bed bioreactor system for the continuous reactor studies. A flask containing fungus-immobilized foam cubes in 10% MB was shaken for one day at 180 rpm before transferring to the bioreactor. This allowed mycelia growth within the cubes. Table I and Fig. 3 show the continuous color reduction per-



4. Decolorization rate (CU/day) as a function of reactor outlet color concentration (CU), $R^2 = 0.93$

Concentration	Cosubstrate (glucose)	Mineral media (N, P and trace metal)
Base	73 ± 2% a	73 ±2% a
50% of base	67 ± 2% b	68 ±4% a
25% of base	60 ± 6% c	-
Trace	-	64 ±3% d

Base glucose concentration is 8 g/L

Results compared using ANOVA and least significant difference (lsd). Percentages of color removal followed by the same letter are not significantly different (a = 0.05).

II. Influence of cosubstrate and mineral media concentration on decolorization from continuous bioreactor

formance of polyurethane immobilized *T. versicolor* using the fluidized bed bioreactor. An average decolorization of 69% and an average AOX reduction of 53% were observed with a residence time of 24 h over the length of the continuous operation. This bioreactor operated for 34 days to determine the active life of the biocatalyst. Operation was terminated without any observable decline in removal rates. The physical form of the mycelia-immobilized foam cubes remained constant for the duration of the operation.

INFLUENCE OF OPERATING PARAMETERS

We studied the influence and optimization of some operating parameters such as glucose cosubstrate concentration, amount of added chemicals (N, P, etc.), and liquor concentration on the decolorization process. This used a series of continuous experiments with EO-stage effluent received from Mill II.

Cosubstrate and mineral media

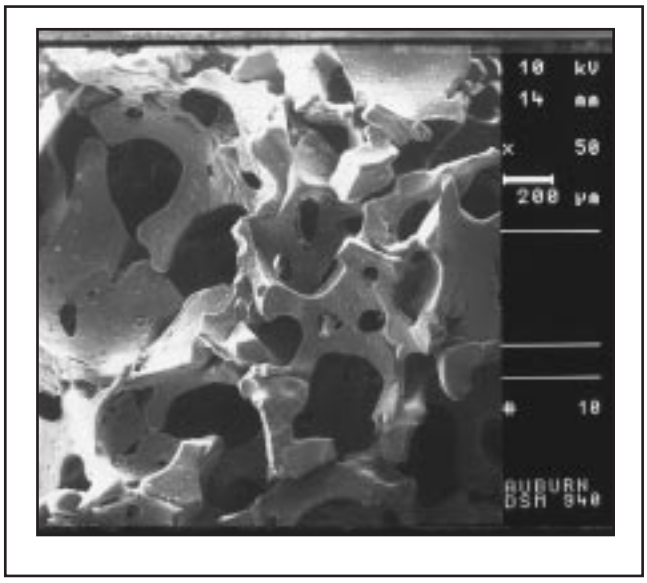
In this study, glucose concentrations ranged from 2–8 g/L. The 8 g/L was the base level. The bioreactor operated at each concentration level until biological stability was achieved as denoted by a constant level of color reduction. Table II illustrates decolorization levels at different supplied concentrations of glucose and nutrients. The highest decolorization of 73 ± 2% occurred with the glucose and nutrient sources supplied at the base level given in the feed composition. As the concentration of glucose at the bioreactor inlet decreased from 8 g/L to 2 g/L, the color reduction declined from 73 ± 2% to 60 ± 6%. There was no improvement in the decolorization performance of the bioreactor with an increase of glucose concentration beyond the base level (data not shown). Concentration levels of trace minerals did not affect the decolorization process in the bioreactor. Table II shows that the color reduction difference produced by halving the supplied concentration of the trace mineral media was statistically insignificant. The bioreactor yielded significant color reduction of 64 ± 3% even without adding trace mineral media.

Continuous experiment with different liquor concentrations

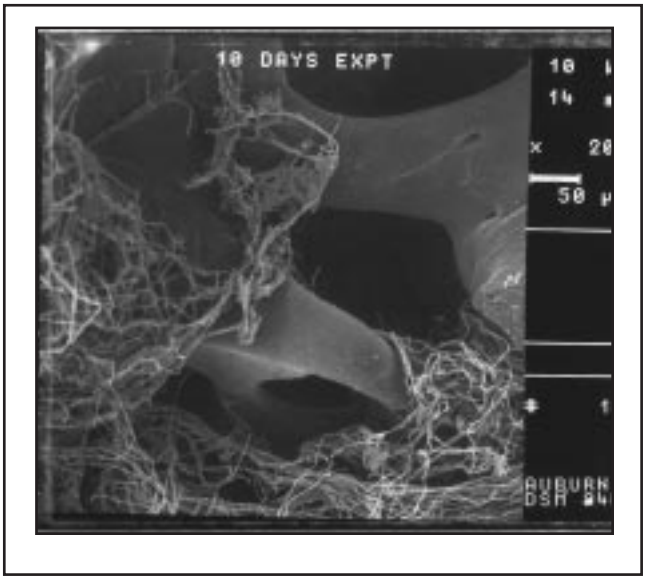
We evaluated the efficacy of a continuous bioreactor containing urethane prepolymer entrapped *T. versicolor* at different inlet liquor concentrations. The inlet liquor color ranged from 1600–2700 color units (CU). The continuous bioreactor started with a liquor concentration of 1600 CU color and operated at the same inlet concentration for 4 days. Once the bioreactor attained constant operation for color reduction, the inlet concentration was changed to a liquor of 2100 CU color. After steady state was achieved at 2100 CU color, the inlet liquor concentration was increased to 2700 CU color. Figure 4 shows the influence of inlet liquor concentration on the performance of the continuous bioreactor. The color removal rate is proportional to the reactor effluent color concentration for the studied range. The highest decolorization rate of 1900 CU/day was obtained at a liquor concentration of 2700 CU. This corresponded to a reactor outlet concentration of 780 CU.

Mycelial growth within urethane foam particles

We observed the growth of the immobilized fungus using SEM. This gives an idea about the proliferation and development of mycelia within the foam cubes. The cubes were collected at different stages of a continuous operation and divided into several slices. The middle slice from the cube was used for SEM studies. Fig-



5. Photomicrograph of a foam particle without fungus



6. Photomicrograph of a sliced section from the bioreactor on day 10 of a continuous experiment

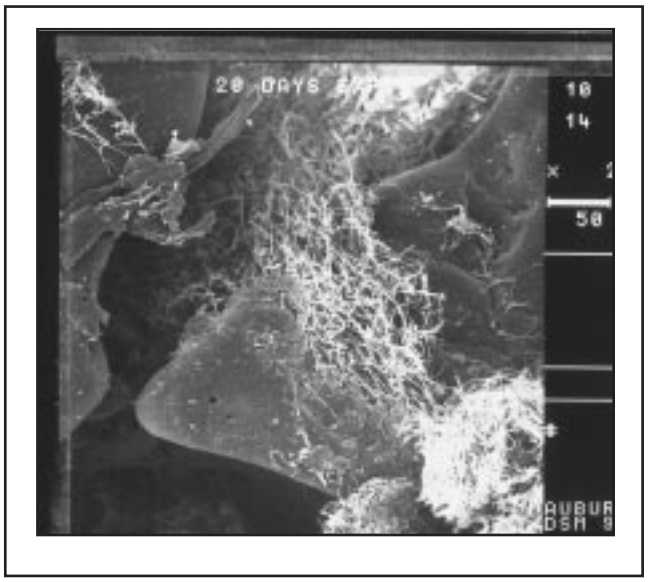
ure 5 is the control showing the micrograph of a foam particle without fungus. The figure shows that developed foam consists of interconnected series of pockets. These pockets were formed by the production of CO_2 during the polymerization process *(5)*. Figures 6–8 show the photomicrographs of sliced sections collected from the bioreactor on days 10, 20, and 28 of a continuous experiment, respectively. Extensive mycelia growth was evident within the pockets. The mycelia remained stable and active even on day 28.

DISCUSSION

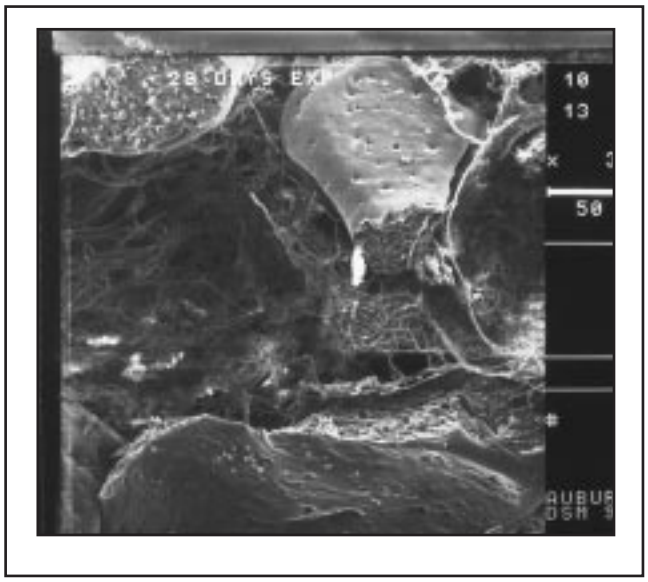
Observation from batch studies showed that the immobilized mycelia took a long time to grow in the presence of kraft bleach effluents before acclimating to the bleach plant effluent environment. This suggested that the immobilized fungi should have incubation in growth media before transfer into the continuous bioreactor. This would reduce the lag time in the degradation process. Incubation of the polyurethane immobilized cells in nutrient media allows the immobilized cells to grow to an optimum level before using them for degradation *(17)*. The role of adsorption in the total reduction of color and chlorinated organics in studies using *T. versicolor* entrapped foam cubes initially incubated in growth media was insignificant. Adsorption was prevalent in the entrapped fungus not previously incubated in growth media.

Table III shows a comparative illustration of color and AOX reduction results from a continuous bioreactor using calcium alginate immobilized *(11)* and polyurethane prepolymer immobilized *T. versicolor*. The table shows that the activity of the polyurethane immobilized fungus is lower than the calcium alginate immobilized fungus when comparing color and AOX reduction. Lower activity may be from the cross linking TDI contained in the prepolymer. TDI may exhibit a toxic effect on the mycelium during immobilization in the conformational structure of the polyurethane immobilized fungus compared with the calcium alginate fungus. Previous workers reported the toxicity effects of TDI on activity yield *(6)*. The polyurethane biocatalyst was more robust in its retention of activity, stability, and mechanical strength even over an extended operation. The biocatalyst remained intact and stable after an extended 32-day operation. Alginate immobilized fungus remained viable for only 22 days.

The decolorization process was linearly dependent on glucose concentration up to the base level of 8 g/L. The literature reports that white-rot fungi require an additional carbon source for efficient decolorization *(9)* and degradation of lignin *(18)*. Other investigators *(19, 20)* examined the dependence of fungal decolorization on cosubstrate concentration from a variety of carbon sources. Archibald *et al. (20)* observed a continuous increase in color reduction with an increase in glucose concentration from 0.35–3.5 g/L. Studies *(21, 22)* in RBC using fungus *P. chrysosporium* showed that decolorization was influenced by the amount of glucose provided a critical amount of glucose was present. During a 4-day batch study using mycelial pellets, mycelial growth was minimal. When



7. Photomicrograph of a sliced section from the bioreactor on day 20 of a continuous experiment



8. Photomicrograph of a sliced section from the bioreactor on day 28 of a continuous experiment

KEYWORDS
Adsorbable organic halogen, bibliographies, biological treatment, bleaching effluents, chlorine compounds, decoloration, effluent treatment, fluidized beds, fungi, polyurethanes, reactors.

Parameter	Calcium immobilized	Urethane prepolymer immobilized
Color reduction	75 ± 4%	69% ± 5%
AOX reduction	59 ± 6%	53% ± 5%
Longest period of continuous operation	22 days	32 days

III. Comparison of mean color and AOX reduction levels by* T. versicolor *from calcium alginate (12) and urethane prepolymers immobilized bioreactor system

the batch study went past 6 days, mycelial mass remained constant, although the nutrients and mineral media were present in the incubation liquor. Using batch results, we believe that the immobilized fungus in the continuous operation initially grew until attaining a steady-state level. Then little growth occurred. Supplied nutrients and mineral media had slight effect on decolorization.

The linear relationship between the color reduction rate and reactor outlet color concentration shows a first-order reaction for decolorization produced by the immobilized fungus in the fluidized bioreactor. A similar observation was made by Royer *et al. (23)* by using mycelial pellets of *T. versicolor* and by Prouty *(24)* with *P. chrysosporium*. Initial color concentration of the effluent is a factor influencing the color removal rate for both batch and plug flow reactors. Royer *et al. (23)* reported a maximum mean decolorization rate of 904 CU/g mycelia per day using *T. versicolor* mycelial pellets at an initial color concentration of 3268 CU/L. Campbell *(25)* obtained the fastest color removal rate of 2000 CU/L per day using an oxygen enriched atmosphere at an elevated temperature of 40°C using the MyCoR process employing *P. chrysosporium*. He also observed a color removal rate of 600 CU/L per day using an air atmosphere. Prouty *(24)* reported an average color removal rate of 1090 CU/L per day using *P. chrysosporium*. During this study, we observed the highest color removal rate of 1920 CU per day (9600 CU/L_{biocat} per day) at an initial color concentration of 2700 CU.

Electron microscopy studies showed that the interconnected pocket structure of the developed foam favored the filamentous growth of the immobilized fungus and allowed retention of more mycelia. Observations at different locations of the same section revealed that the mycelia developed well within pockets throughout the foam. Some pockets extended to the surface of the particle by a series of interconnections. A micrograph of the outer surface displayed the

mycelial growth extending from the particle through surface pockets. Apparently the porous structure of the foam facilitated the development of straight filamentous hyphae and prevented the formation of a mycelial mat at the outer surface. The mat prevention is essential, since development of biomass covering the outer surface will promote diffusional limiting.

The results of this study demonstrate that the immobilization of *T. versicolor* in urethane prepolymers leads to significant reductions in color and chlorinated organic levels in the treatment of kraft bleach effluents under optimized conditions. By immobilizing the fungus in urethane prepolymer, the lifetime of the bioreactor was significantly longer compared with alginate immobilized fungus. TJ

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LITERATURE CITED

1. Fukui, S. and Tanaka, A., *Adv. Biochem. Biotechnol.* 29: 1(1984).
2. Klein, J. and Kluge, M., *Biotechnol. Lett.* 3(2): 65(1981).
3. Storey, K. B., Duncan, J. A., and Chakrabarti, A. C., *Appl. Biochem. Biotechnol.* 23(3): 221(1990).
4. Hu, Z. C., Korus, R. A. and Stormo, K. E., *Appl. Microbiol. Biotechnol.* 39(3): 289(1993).
5. O'Reilly, K. T. and Crawford, R. L., *Appl. Environ. Microbiol.* 55(9): 2113(1989).
6. Sumino, T., Nakamura, H., Mori, N., *et al.*, *Appl. Microbiol. Biotechnol.* 36(4): 556(1992).
7. Livernoche, D., Jurasek, L., Desrochers, M., *et al.*, *Biotechnol. Bioeng.* 25(8): 2055(1983).
8. Bergbauer, M., Eggert, C. and Kraepelin, G., *Appl. Microbiol. Biotechnol.* 35(1): 105(1991).
9. Eaton, D. C., Change, H.-M., and Kirk, T. K., *Tappi* 63(10): 103(1980).
10. Messner, K., Ertler, G., Jaklin-farcher, S., *et al.* in Biotechnology in Pulp and Paper Manufacture: Applications and Fundamental Investigations (T. K. Kirk and H. M. Chang, Eds.), Butterworth-Heinemann, Boston, 1990, pp. 245-251.
11. Pallerla, S. and Chambers, R. P., *J. Environ. Sci. Health* A30(2): 423(1995).
12. Pallerla, S., *Investigation of fungal bioreactors for decolorization and adsorbable organic halides reduction of bleach plant effluent*, M.S. thesis, Auburn University, Auburn, AL, 1994.
13. Kwak, M. Y. and Rhee, J. S., *Appl. Microbiol. Biotechnol.* 36(5): 578(1992).
14. Gosmann, B. and Rehm, H. J., *Appl. Microbiol. Biotechnol.* 29(6): 554(1988).
15. Anon., Introductory guide, Hampshire Chemical Corporation, Lexington, 1993, pp. 1–5.
16. Anon., 1971 Technical Bulletin No. 253, NCASI, New York.
17. Hu, Z. C., Korus, R. A., Levinson, W. E., *et al.*, *Environ. Sci. Technol.* 28(3): 491(1994).
18. Kirk, T. K., Connors, W. J. and Zeikus, J. G., *Appl. Environ. Microbiol.* 32(1): 192(1976).
19. Belsare, D. K. and Prasad, D. Y., *Appl. Microbiol. Biotechnol.* 28(3): 301(1988).
20. Archibald, F., Paice, M. G., and Jurasek, L., *Enzyme Microb. Technol.* 12(11): 846(1990).
21. Pellinen, J., Yin, C. F., Joyce, T. W., *et al.*, *J. Biotechnol.* 8(1): 67(1988).
22. Yin, C. F., Joyce, T. W., and Chang, H.-M., *J. Biotechnol.* 10(1): 67(1989).
23. Royer, G., Yerushalmi, L., Rouleau, D., *et al.*, *J. Ind. Microbiol.* 7(4): 269(1991).
24. Prouty, A. L., *Appl. Microbiol. Biotechnol.* 32(4): 490(1991).
25. Campbell, A. G., Gerrard, E. D., Joyce, T. W., *et al.*, *1982 TAPPI Research & Development Conference Proceedings*, TAPPI PRESS, Atlanta, p. 209.