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DEGRADATION OF RECALCITRANT XENOBIOTICS BY *PHANEROCHAETE CHRYSOSPORIUM* IMMOBILIZED BY ADSORPTION ON ACTIVATED CARBON

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EXECUTIVE SUMMARY

The main focus of the research was on the characterization of the microbial (*i.e.* fungal and bacterial) degradation patterns of selected toxic aromatic and polyaromatic compounds. These compounds represent major chemical groups of toxic xenobiotics and include penthaclorophenol (PCP), *p*-nitrophenol (PNP), phenanthrene, aniline and phenolic wastes from the plywood industry. Because of the difficulties presented by the filamentous nature of fungi and change in morphology with cultivation system, the work was extended to bacterial cells as well. The main tasks covered by the research on fungal degradation concerned the characterization of the attachment of *Phaneochaete chrysosporium* to polyurethane foam, identification of the fungal degradation pattern of PCP under diverse conditions, and mineralization of PCP under non-limiting nutrient conditions. Granulated activated carbon, polyurethane foam cubes, rock wool, powdered silica, coke, pumice stone, celite and peat moss were tested as supports for the immobilization of the white rot fungus *P. chrysosporium*. The main tasks covered by the research on bacterial degradation concerned the degradation of aromatic and polyaromatic toxic compounds adsorbed on natural and synthetic adsorbent materials. Powdered activated carbon (PAC) and various synthetic polymeric adsorbents with different chemical polarity and porous structure were used (products of copolymerization of styrene and divinylbenzene and copolymerization of 2-methyl-5-vinylpyridine with divinylbenzene). The combination of physical sorption and biological degradation on an active carrier was compared with the degradation performance of suspended cells and cultures attached to sand.

Another significant issue covered within the framework of the project was the elaboration of a mathematical model of the adsorption-desorption-biodegradation system for the bacterial degradation in batch and continuous regime of aromatic toxic compounds dissolved in water. The initial system of equations contains four parameters which characterize a biological process, namely: u, the specific biomass growth rate; K_s, the saturation constant of the Monod equation; K_e, the specific biomass decay rate; and Y, the biomass yield. A method for the determination of these parameters by the combined utilization of theoretical and experimental data was applied. This methodology is based on minimizing the deviations of experimental kinetic curves from theoretically calculated ones. The mathematical model developed for *p*-aminophenol and aniline degradation can be applied to any complex system consisting of a organic substance-sorbent- microorganisms.

The findings achieved during the course of the project contributed to the understanding of a biological system for the degradation of toxic organic pollutants and established the basis for its implementation, based on the use of powdered adsorbent material as carrier for pollutant adsorption and microbial attachment. The scientific group of the collaborating country developed, within the framework of the project, the skills to deal with degradation of toxic organic compounds in an immobilized microbial system, at the levels of (i) reactor technology and operation; (ii) mathematical modeling and (iii) analytical analyses. Part of the findings of the research have already been published in a peer reviewed journal (J. Biotechnol. 51:265-272, 1996), as well as in several local publications and symposia in Eastern Europe. Another part of research is being prepared for publication in the form of regular papers in scientific journals.

RESEARCH OBJECTIVES

The object of the present research was to study the synergetic effect of physical adsorption and microbial degradation of aromatic and polyaromatic hydrocarbons by microorganisms attached on granular natural and synthetic adsorbents. The degradation of toxic organic compounds, such as aromatic and polyaromatic hydrocarbons, used in modern agriculture and discharged by industrial plants, is a major concern in developed countries. The political, sociological, and technological changes in developed countries has begun to increase awareness of environmental pollution in those countries. Furthermore, the global environmental protection policy undertaken by the industrialized countries obliges all the countries directly involved in commercial linkages with them to adopt an unequivocal attitude towards the control of use and disposal of recalcitrant toxicants.

Traditional large-scale methods for wastewater treatment, such as waste activated sludge, which are very effective for the treatment of domestic and municipal effluents, are inefficient for the treatment of industrial wastewater mainly because of the presence of toxic materials. Because of the variations in concentration and spectrum of the toxicants, it makes difficult to maintain biological reactors under steady conditions for long periods of time. This effect is sometimes amplified when the biological system deals with mixed microbial populations. Suspended growth is ineffective for the treatment of toxic materials because the contact between the microorganisms and the contaminants is frequently limited and hydraulic resident times are too fast for the slower biodegradation rates (and/or growth rates) of the more recalcitrant compounds [Peyton, 1984]. A crucial task in the achievement of the microbial degradation of toxic compounds is the process technology and selection of the right microorganisms able to efficiently degrade these compounds. The microbial purification of industrial wastewater has been reported to be efficient, preferably in immobilized systems, using synthetic and natural carriers.

Several scientific and technological reports, as well as patents, describe the use of immobilized microorganisms for the purification of wastewater. The key to making attached growth systems more efficient is to provide the maximum amount of surface area on which the biomass can grow. Thanks to its great adsorptive surface and structure, activated carbon is suited as supporting material for microorganisms, and also provides high adsorption capacity for organic compounds. It is used as a final polishing step in the purification of groundwater and drinking water as well as directly added to high rate biological reactors for wastewater treatment [Stenzel and Merz, 1989; Voice *et al.*, 1992].

Activated carbon offers a rough and fissured surface on which microorganisms can settle and colonize easily. The outer pores of activated carbon are inaccessible for the organisms, which can therefore attach only to the external surface and enter only some big macropores and fissures [Ehrhardt and Rehm, 1985; Morsen and Rehm, 1987; Voice *et al.*, 1992]. Activated

carbon carrier is thought to act as a modulator, by adsorbing relatively high concentrations of toxic compounds in the aqueous system and regulating the concentration of the free material, while protecting the organisms against the toxicity caused by the lipophilic nature of the pollutants [Morsen and Rehm, 1990]. Degradation of organic compounds by bacterial cultures immobilized on activated carbon occurs as a combination of physical adsorption and biological degradation [Ehrhardt and Rehm, 1985]. The adsorbed organic compounds desorb, diffuse out of the carbon and can then be metabolized. The rate of desorption depends on the culture conditions, fluid dynamics, metabolic activity of the organism, and kind and density of particles [Fan *et al.*, 1990]. Activated carbon was also reported to contribute to the buffering capacity of the microbial system by adsorbing acid and alkaline compounds, as well as enriching dissolved oxygen [Baldauf, 1993].

An adsorbent with good adsorption properties (such as activated carbon), which supports a reasonable rate of desorption and allows degradability, will favor continuous stable operation while avoiding toxicity due to concentration spikes. Obviously, the choice of the right organism and the right bioreactor technology, will ensure the technical and scientific feasibility of the process. The present work focused on the degradation of aromatic and polyaromatic compounds by either bacteria and fungi attached to activated carbon and other porous carriers.

The following specific subjects where addressed within the framework of the project:

- **System components.** The ability of the different carrier materials, according to their characteristics, to adsorb the pollutants under study, and attach the microorganisms were studied. Their ability to immobilize crude and purified enzymes were also studied.

Pollutants: pentachlorophenol, benzo-a-pyrene, aromatic amines and nitro-compounds were studied first as model compounds. Biopurification of waste water and ground water were of next consideration.

Organisms: mixed bacterial populations, aerobic and anaerobic bacteria; white-rot fungi, and specially *P. chrysosporium.*

Enzymes: e.g. extracellular ligninolytic enzymes of *P. chrysosporium.*

- **Carrier materials.** The majority of organic substances found in wastewater in molecular form are expected to adsorb to active carbon at a rate much higher than their rate of bio-degradation. The energy of the adsorption of the adsorbed substances is thought to be so high, that the regeneration of the sorbent by means of microorganisms is expected to be very slow. Hence, chemisorption on polymer sorbents, such as copolymers of styrene ($C_6F_5CH{:}CH_2$) and divinylbenze derivatives [$C_6H_4(CH{:}CH_2)_2$], were tested. However, due to its higher adsorption capacity, lower price and ability to regeneration, activated carbon, either alone or in a mixed-bed with synthetic polymers, remains a preferential support. Two major categories of carriers, were preferentially considered for the study:

Adsorbent materials: activated carbon, coke and synthetic polymeric adsorbents.
Non-adsorbent materials: pumice stone and polyurethane foam.

• **Process.** Two different types of process were studied:

- Batch process, in which the pollutants are first adsorbed and then degraded. This step was considered as a bio-regeneration process of the adsorbent.

- Continuous process, in which the pollutants were degraded concomitantly during their passage through the reactor. Single and multi-step processes have been considered.

• **Mathematical modeling.** The conceptual model of biosorption assumed the existence of biodegradable and bioresistant compounds in the composition of the substrate, and did not take into account either increase or destruction of the biofilm. The initial system of differential equations for the mathematical model included mass balance of toxicant and biomass, kinetics of sorption and biodegradation, sorption isotherms, and rate of biomass growth, as well as algorithms to solve the above equations. The constants of integration have been extracted from experimental results obtained.

The innovative aspects of the project consisted of the employment of a combined system for the physical adsorption/biological mineralization of recalcitrant xenobiotics at high loading rates, based on the use of natural adsorbents and new synthetic carrier materials. The particulate adsorbents acts simultaneously as support for the attachment of the organisms and as an adsorbent for the organic pollutants, and allow close contact between the different phases of the system.

METHODS AND RESULTS

SCIENTIFIC BACKGROUND

1. Fungal degradation of PCP

White-rot fungi are able to biodegrade lignin as well as a broad spectrum of organic chemicals containing carbon skeletons similar to those found within the lignin polymer [Boominathan and Reddy, 1991; Bumpus *et al.*, 1985; Eriksson *et al.*, 1990; Hammel, 1989]. Consequently, they and their extracellular ligninolytic enzymes have been considered for various applications in environmental biotechnology. However, significant improvements in the enzyme productivity and physiological conditions of the white-rot fungi in liquid culture still need to be made. The basidiomycete *Phanerochaete chrysosporium* is one of the best characterized white-rot fungi. It produces no phenol oxidase activity and the major components of its extracellular lignin-degrading enzyme system consist of two families of extracellular glycosylated heme peroxidases designated lignin peroxidase (LIP), and manganese peroxidase (MNP), and glyoxal oxidase (GLOX), which produces extracellular H_2O_2 [Kersten, 1990; Kuwahara *et al.*, 1984; Tien and Kirk, 1983]. LIP and MNP comprise 10-15 different isoenzymes [Gold *et al.*, 1989; Leisola *et al.*, 1987; Pease and Tien, 1991; Tien, 1987]. The expression of ligninolytic enzymes by *P. chrysosporium*, an idiophasic event triggered by nutrient limitation (nitrogen, carbon or sulfur), is particularly active at high O_2 tension, and is highly dependent on culture conditions and medium composition [Buswell and Odier, 1987; Dosoretz and Grethlein, 1991; Faison and Kirk, 1985; Jeffries *et al.*, 1981]. The expression of the multiple genes encoding LIP and MNP isoenzymes has been reported to be differentially regulated at the mRNA level, according to the conditions of nutrient limitation [Boominathan and Reddy, 1991; Holzbaur *et al.*, 1991; Kirk and Farrel, 1987].

Owing to the dependence of the ligninolytic system on nutrient limitation, most studies on the production of lignin-degrading enzymes by *P. chrysosporium* have been performed in unbalanced media, usually with nitrogen limitation, resulting in low productivity of both biomass and biocatalyst. LIP production has never been found at nitrogen sufficiency or in balanced media, whether the fungus was grown as free pellets in shaken cultures or as a filamentous mat in shallow stationary cultures [Dosoretz *et al.*, 1990b; Faison and Kirk, 1985; Jager *et al.*, 1991]. Several strategies have been employed to enhance LIP productivity, such as the use of lignin model-amino acid adducts, veratrylamine, the addition of manganese oxide or phospholipids, and several immobilization systems [Asther *et al.*, 1992; Jager *et al.*, 1991; Janshekar and Fiechter, 1988; Kern, 1989; Liebeskind *et al.*, 1991; Tien *et al.*, 1987]. However these studies were still conducted under limiting nutrient concentrations, or using glycerol, which is a slowly metabolizable substrate [Tonon *et al.*, 1990]. Heterologous expression of *P. chrysosporium* LIP in bacteria, yeast and even fungi has been unobtainable

to date [Holzbaur *et al.*, 1991]. However, the expression of a LIP gene clones, in their active form, in the host insect *Spodoptera frugiperda* (SF-9 cells), upon addition to the growth medium of exogenous heme, was recently reported [Johnson *et al.*, 1992; Pease *et al.*, 1992]. A few N-deregulated mutants have been reported which are able to synthesize part of the ligninolytic system under nonlimiting nutrient conditions in shallow stationary cultures, albeit in relatively low amounts and with multiple expression of isoenzymes [Boominathan *et al.*, 1990; Buswell *et al.*, 1985; Kuwahara and Asada, 1987]. More recently, a lysine auxotrophic mutant capable of producing about 700-900 U/l of LIP activity has been reported [Orth *et al.*, 1991; Tien and Myer, 1990]. Although there was a predominance of H1 and H2 isoenzymes, almost all of the ligninolytic isoenzymes were formed. These results notwithstanding, no LIP production has been obtained with the wild-type strain under nonlimiting nutrient conditions, using glucose as substrate. However, a recent paper has reported the production of LIP activity under nitrogen sufficient conditions (24 mM NH_4) by cultures of the wild-type BKM-F immobilized on polyurethane foam, using 56 mM glucose [Chen *et al.*, 1991].

The role of the high oxygen level required for the synthesis of LIP in liquid culture is thought to overcome problems of oxygen availability arising from culture conditions. An undetectable oxygen level has been reported in the mycelial mat at depths below 2 mm, in non-agitated liquid cultures of *P. chrysosporium*, even in 100% oxygen [Leisola *et al.*, 1983]. A similar result was obtained from a submerged liquid culture [Michel *et al.*, 1992]. It was shown that the addition of Tween 80 or Tween 20 to the growth medium results in higher permeability of oxygen through the cell membranes, and in enhanced production of LIP [Lestan *et al.*, 1994]. Most laboratory studies on the formation of ligninolytic enzymes in *P. chrysosporium* have been performed in submerged or shallow cultures, and have been conducted in pure oxygen or in an oxygen-enriched environment, because these growth conditions are thought to enhance lignin degradation, and are critical for the production of LIP [Rothschild *et al.*, 1995; Faison and Kirk, 1985; Kirk *et al.*, 1978]. On the other hand, there is evidence that the degradation of lignin in solid-state cultures by *P. chrysosporium* and other white-rot fungi proceeds at virtually the same rate in air as in an oxygen-enriched atmosphere [Highley *et al.*, 1982; Kerem *et al.*, 1992]. Recent experiments in our laboratories, in which *P. chrysosporium* was immobilized on polyurethane foam in a non-immersed liquid culture, have demonstrated overproduction of LIP under nonlimiting nitrogen conditions, as a result of increased oxygen availability [Dosoretz *et al.*, 1993]. This cultivation system provides a high surface area, and thereby resembles the growth conditions on woody tissue. It therefore becomes possible to determine whether the differences in oxygen levels required to degrade lignin in nature, *vs.* that required to degrade lignin and produce LIP in the laboratory are due to differences in oxygen availability to the mycelia.

Different reactor configurations have been proposed to improve the production of ligninolytic enzymes from *P. chrysosporium.* Most of them are completely mixed systems, with and without

mechanical agitation, such as the complete stirred tank reactor (CSTR), airlift reactors, and bubble column reactors [Linko, 1988; Janshekar and Fiechter, 1988; Jager et al., 1991; Chen et al. 1991; Bonnarme et al. 1993]. CSTR are easy to control, specially at laboratory scale, and also allow good oxygen transfer rates (OTR), but they generate relatively high shear forces.

Reactors generating low shear forces, including the rotating disk reactor, the silicone membrane reactor and the hollow fiber reactor, have also been considered [Kirkpatrick and Palmer, 1987; Venkatadri and Irvine 1993]. However, low OTR is a major constraint of these reactors, and therefore significant improvements have to be made to obtain adequate performance. Only a few studies have reported continuous production of LIP, in particular, or ligninolytic enzymes in general [Linko and Zhong, 1987; Linko, 1988; Rogalski et al., 1992]. Production of ligninolytic enzymes in continuous regime requires a reactor configuration capable of providing regeneration of mycelium in successive growth/production cycles. Packed-bed reactors are the most widely used low shear reactors, characterized by high mass transfer and reaction rates. Packed-bed reactors have been used to produce lignolytic enzymes from *P. chrysosporium* immobilized in a sintered glass carrier [Rogalski et al. 1992].

A major goal of the research is to combine the immobilization of fungal mycelium and adsorption of toxic xenobiotics to high surface porous supports, in a single or multi-step system, in order to achieve mineralization of recalcitrant xenobiotics under controlled and constant metabolic activity, especially at high organic loadings. The biodegradation of toxic compounds by *P. chrysosporium* occurs via co-metabolism, in the presence of a suitable carbon source, which serves as a growth substrate. Neither lignin nor synthetic polymers are able to serve as growth substrates for *P. chrysosporium* [Bumpus and Aust, 1987]. In systems in which extracellular enzymes are present, a problem of adsorption of the extracellular enzymes themselves, arises, however. According to Shanon and Bartha [1988], the immobilization on soils of the extracellular enzymes of *P. chrysosporium*, obtained from a carbon-limited agitated culture, drastically decreases the enzyme activity against phenolic substances, such as 2,4-dichlorophenol and methyl-phenols. The immobilization of the entire organism also changes its ability to mineralize pentachlorophenol (PCP). In non-immobilized shallow stationary cultures of *P. chrysosporium,* under nitrogen-limited conditions, 23-50% of the PCP was mineralized after 30 days of incubation, whereas in various kinds of soil, only about 2% was mineralized after 2 months, under the same culture conditions [Mileski *et al.*, 1989; Lamar *et al.*, 1990; Lamar and Dietrich, 1990]. Under fungal treatment, on the other hand, a great reduction in PCP in soil took place (measured as PCP disappearance), with an average decrease of 51% and 86%, after 22 and 46 days of incubation, respectively. Under these conditions, pentachloroanisol (PCA) was identified as the major extractable product of transformation of PCP-contaminated soil inoculated with *P. chrysosporium* [Lamar and Dietrich, 1990]. However, the rate of conversion of PCP to PCA was only 9-14%, while most

of the PCP was converted to soil-bound, non extractable products, whose nature is still unknown. It was suggested that the oxidation of PCP in the soil, by ligninolytic enzymes of *P. chrysosporium,* resulted in polymerization reactions that caused irreversible binding to organic matter [Lamar *et al.*, 1990]. This part of the research focus on the attachement of *P. chrysosporium* mycelium on polyurethane foam as a function of reactor configuration and regime as well as on pattern of PCP degradation, as an example of a toxic aromatic compund.

2. Bacterial degradation of aromatic and polyaromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) are known as major pollutants, which are discharged as by-products of burned fossil fuels, spent mineral oil and residues of coal processing. Some PAHs are classified as high toxicants, and some are carcinogenic, such as anthracene and benzo[a]pyrene [Cerniglia, 1993; Heitkamp *et al.*, 1988]. The microbial degradation of PAHs has been reported for compounds containing 2 to 4 rings, whereas PAHs with 5 and 6 rings are considered to be more resistant to microbial attack rings [Cerniglia and Heitkamp, 1989; Dreyer *et al.*, 1995; Weibenfels *et al.*, 1991]. Phenolic and nitrophenolic compounds are commonly found as pollutants in aqueous wastes of many different industries, such as dyes, pesticides, plastics, explosives, pharmaceuticals and solvents [Spain and Gibson, 1991; Heitkamp *et al.*, 1990]. Microbial degradation of phenol and *p*-nitrophenol (PNP) has been reported in different natural environments and wastewater treatment systems. The use of activated carbon was shown to be effective for the degradation of high concentrations of phenol (> 9 g/l) employing mixed bacterial cultures [Ehrhardt and Rehm, 1985; 1989]. This part of the research focuses on the bacterial degradation of aromatic and polyaromatic toxic compounds adsorbed on powdered activated carbon (PAC). The combination of physical sorption and biological degradation on an active carrier was compared with the degradation performance of suspended cells and cultures attached to sand, which served as an example of an inert support.

MATERIALS AND METHODS

1. Fungal biodegradation

Organism and culture conditions

P. chrysosporium, wild-type strain BKM-F-1767 (ATCC 27245) was maintained at 37°C on 2% malt extract agar slants. Inoculum was prepared in shallow stationary cultures by inoculating a spore suspension (1.6 absorbance units at 600 nm) in 2.6 l Fernbach flasks containing 75 ml medium and grown at 37°C for 2 days. The mycelium mat was then homogenized and used as inoculum (10% v/v).

a) Serum bottles experiments: The experiments were performed in 120 ml serum bottles under shallow stationary conditions at 37°C. The growth medium used was the N-limited medium, containing 1.2 mM di-ammonium tartrate, as described by Tien and Kirk [1988], but with acetate buffer (20 mM, pH = 4.5).

b) Flasks experiments with polyurethane: The experiments were conducted in the non-immersed liquid culture system reported by Dosoretz *et al.* [1993]. When indicated, control experiments with free, non-immobilized mycelium were performed in submerged cultures in 250 ml shaken flasks according to Dosoretz *et al.* [1990b]. The growth medium was based on that described by Tien and Kirk [1988], but with acetate buffer (20 mM, pH 4.5) instead of dimethyl succinate buffer, as previously reported [Dosoretz *et al.*, 1990b]. Veratryl alcohol was added at inoculation (0.4 mM) and again after 48 h of incubation (2 mM), as previously reported [Dosoretz *et al.*, 1993]. The initial glucose and ammonium concentrations for the three different nutrient conditions were, respectively, 5.6 mM and 4.5 mM for low carbon (C/N = 7.47), 56 mM and 2.2 mM for nitrogen limitation (C/N = 153), and 56 and 45 mM for high nitrogen (C/N ratio = 7.47). When indicated, Mn^{+2}-free medium was used; otherwise media contained 13 mg/l Mn^{+2} (as $MnSO_4$).

Flasks and bottles containing cultures were sealed with rubber stoppers and flushed once a day for 2 min with water-saturated O_2, by means of two hypodermic needles (3 cm, 23 gauge). Cultures grown under free air exchange were stoppered with paper plugs. Oxygen gas was of medical grade.

c) Reactors experiments

c.1) Batch reactors

i) Trickle-bed reactors: The trickle-bed reactors consisted of columns of 60 ml total volume operated in trickle-flow mode. The reactor were packed and directly inoculated with a spore suspension. The medium was recycled at a flow rate of 20 ml/min. Oxygen gas was supplied at the top of the reactor just above the influent liquid inlet, in co-current, at a flow rate of 0.1 ml/min ii) Packed-bed reactors: The packed-bed reactors consisted of jacketed columns of 167 ml working volume operated in up-flow mode in batch regime and equipped with three sampling ports: one at the inlet (bottom, p1), one at the middle (p2) and one at the outlet (top, p3), as described below in Fig. 1. The influent was saturated with pure oxygen gas in a recirculation vessel, at a continuous oxygen flow of about 1 ml/min. The immobilization of the fungi in packed-bed reactors was carried out in two steps. First the fungus was grown in 250 ml Erlenmeyer flasks for 4 days. Thereafter the cubes with pre-grown mycelium attached to them were transferred to the reactor. Eight flasks were used for each reactor. The final volume of the liquid in the reactor system was about 400 ml. Samples (1.5 ml) were taken daily from the three ports with a sterile syringe, and enzymatic activity and glucose concentration were monitored.

c.2) Continuous up-flow Packed bed reactors

i) Medium composition: The growth medium used was the N-limited medium, containing 0.2 g/l di-ammonium tartrate and 10 g/l glucose, as described by Tien and Kirk [1988], but with acetate buffer (20 mM, pH = 4.5). The production medium used for the different production cycles was the same as above, but with 2 g/l glucose and 0.02 g/l di-ammonium tartrate. The events in the operation of the reactors are given in Table 1.

Table 1. Schedule of events in the operation of the packed-bed reactors.

Day	Event
0	Growth medium with 5 g/l glucose
2	Production medium with 1 g/l glucose
11	Production medium with 2 g/l glucose
14	Buffer Solution
21	Growth medium
24	Buffer Solution
32	Production medium with 2 g/l glucose
34	Buffer Solution
37	Growth medium
40	Buffer Solution

ii) Polyurethane foam cubes: Polyurethane foam (15 kg/m^3), purchased from Caesarea Polymers, Mifratz Haifa, was cut into cubes of 1 cm side, washed once in methanol, then three times in double distilled water, autoclaved in double-distilled water and dried until use.

iii) Immobilization: Erlenmeyer flasks of 500 ml capacity, containing 140 ml medium and 0.016 g/ml polyurethane foam, were inoculated (10% v/v) with homogenized mycelium previously grown in Fernbach flasks [Dosoretz et al., 1993]. The cultures were incubated at 37°C and 150 rpm in an orbital shaker (New Brunswick Scientific, NJ), until the glucose concentration decreased to 5 g/l, and then transferred to the reactors (starting time for this experiment). The flasks were flushed every day for 3 min with pure O_2 at 0.5 atm.

iv) Characteristics and operational conditions of the reactors: The reactors consisted of jacketed glass columns of 167 ml working volume (H/D ratio of 2.33) (Fig. 1). The medium was pumped from the bottom by means of a peristaltic pump (Cole & Parmer). The temperature was maintained at 37° C by circulation of temperature-controlled warm water. Each reactor had three

sampling ports, at the entrance (bottom), middle and top. In order to improve fluid distribution and minimize preferential paths glass beads of 1.5 mm diameter were used in the bottom of the reactor. Three packed-bed reactors were operated without (R1) and with recycling flow/feeding flow (R:F) at ratios of 2:1 (R2) and 12:1 (R3), and a hydraulic residence time of 4 h. Sterile medium was saturated with sterilized oxygen to a concentration of 11 mg/l in a previously stirred tank. The reactor were sampled twice a day.

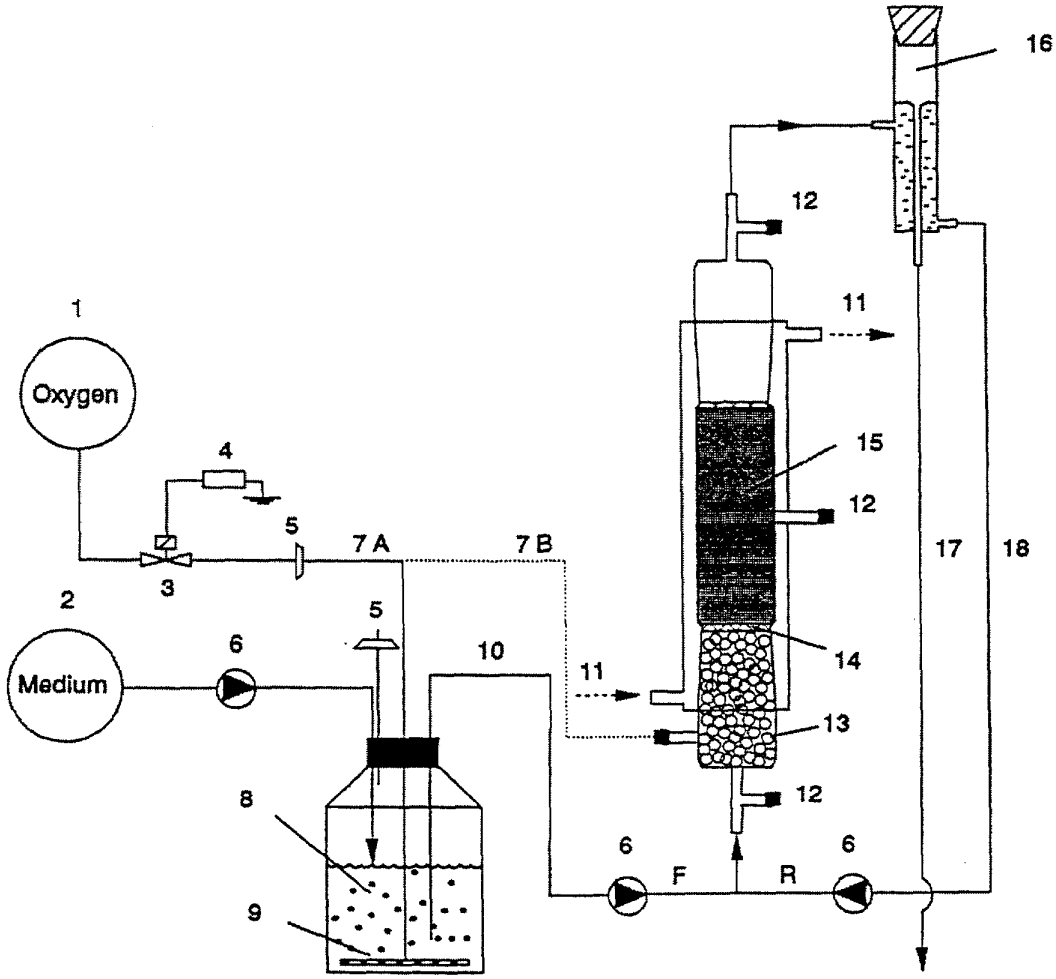

Fig. 1. Scheme of the continuous packed-bed reactor system. 1. Oxygen tank; 2. Medium reservoir; 3. Electric valve; 4. Timer; 5. Sterile filter; 6. Peristaltic pump; 7. Oxygen line, A: To saturation tank, B: Direct oxygenation; 8. Oxygenation stirred tank; 9. Oxygen sparger; 10. Feeding line (F); 11. Tempered-controlled water; 12. Sampling port; 13. Glass beds; 14. Sintered glass; 15. Polyurethane foam cubes; 16. Recycling system; 17. Effluent; 18. Recycling line (R).

v) Residence time distribution (RTD) experiments: To characterize the hydrodynamic behavior of the reactors, a stimulus-response technique was used to determine the RTD curves. A solution of LiCl (77 g/l) was used as a tracer. One ml tracer was injected, resulting in an almost ideal pulse (δ Dirac function). Lithium was determined by atomic absorption

spectrophotometer. The data was fitted into the Tanks-in-Series model to obtain the characteristic parameters.

d) PCP supply: Cold PCP (25 mg/l, unless otherwise stated) was added to the cultures on day 3 or 6 of incubation, as indicated. For labeling experiments PCP (50 mg/l PCP solution containing 0.01 μCi [U-^{14}C]-PCP per bottle) was added to the cultures on day 3 on incubation.

Chemicals

Pentachlorophenol (PCP) and [U-^{14}C]-PCP were obtained from Sigma Chemical Co.; 2,3,4,5,6-pentachloroanisol (PCA) and tetrachloro-1,4-dibenzoquinone (TCHD) were obtained from Aldrich Chemical Co.

Immobilization supports

Granular activated carbon, particle size 2.5 mm and silica powder (Kieselgel 60) were purchased from Merck. Celite was purchased from Fluka: R-630 is the type recommended for whole cell immobilization and R-640 is the type recommended for enzyme immobilization, according to the manufacturer's specifications. Peat moss (sphagnum moss) was obtained from Samen Mauser. These supports were washed clean of dust particles with double distilled water, dried and autoclaved before use. The polyurethane foam (density 15 g/l), rock wool and coke used were commercial products without specification. They were cut into pieces approx. 0.5 x 0.5 cm (polyurethane) and approx. 2 x 2 cm (rock wool and coke), rinsed with distilled water, autoclaved wet, dried and autoclaved again before use.

PCP degradation

At the end of the experiments, the liquid and solid (support) were separated. The liquid fraction was extracted with acidified hexane, as described by Mileski *et al.* [1989]. The carrier with attached mycelium was first extracted with acetone and thereafter with hexane. In the case of polyurethane cubes, the cubes were squeezed out of the water and then extracted as above by squezing and wetting several times with the solvent in a 50 ml glass syringe mounted on a bracket especially designed for this purpose. The concentration of PCP and metabolites was determined by either HPLC or GC as indicated. Each fraction was injected separetly and the results reflect the sum of the concentration of each one corrected by the dilution factor. HPLC was done on a Lichrospher (Merck) RP-18 column with acetonitrile:H_2O_2:glacial acid (75:25:0.3 volumes), at a flow rate of 1 ml/min and monitored at 254 nm. GC was done on a HP-5 (Hewlett Packard) capillary column with He as carrier gas using an electron capture detector (ECD).

Mineralization of PCP

The headspace of the serum bottles was flushed for 20 min with pure O_2 every 3 days. The CO_2 evolved from cultures was trapped in 8 ml of a solution containing ethanolamine:methanol:Safety Solve™ (Packard)-scintillation cocktail (1:4:5), and the $^{14}CO_2$ was determined by liquid scintillation spectrometry.

Enzymatic activities

LIP activity was measured according to Tien and Kirk [1988], with one unit (U) representing 1 μ mol veratryl alcohol oxidized to veratraldehyde per minute. **MNP activity** was measured as described by Kuwahara *et al.* [1984] with phenol red as substrate. One unit of activity was calculated as one mol of phenol red oxidized per min per ml reaction, using the extinction coefficient described by Michel *et al.* [1992].

Heme protein analysis

Equal volumes of defrosted extracellular fluid were concentrated 25-fold by ultrafiltration, using a 10-kDa cutoff PM-10 membrane (Amicon), centrifuged for 10 min at 25,000xg, then dialyzed against 10 mM sodium acetate pH 6.0. Samples were analyzed for heme protein by anion exchange-HPLC, using a MonoQ column (Pharmacia) at a flow rate of 1 ml/min and monitoring at 409 nm [Kirk *et al.*, 1986]. Heme protein nomenclature (H1-H10) was based on elution properties and activity tests, as in previous reports [Boominathan and Reddy, 1991; Dass and Reddy, 1990].

Scanning electron microscopy (SEM)

Polyurethane cubes colonized by mycelia were harvested on day 4, except for the carbon-starved cultures, which were harvested on day 3. The colonized cubes were fixed for 2 h at ambient temperature in 4% (v/v) glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.1, and analyzed by SEM according to Flegler and Baker [1983].

Analytical techniques

Glucose was determined by the dinitrosalicylic acid method, according to Ghose (1987). **Nitrogen-ammonia** was determined by the phenol-hypochlorite method, according to Weatherburn [1967]. **CO_2** was measured by means of a gas chromatograph equipped with a thermal conductivity detector, as previously reported [Dosoretz *et al.*, 1990a]. Samples were taken directly from the head space of the culture flasks by means of a pressure lock syringe. CO_2 values represent μ mol of gas accumulated in the head space between oxygenation periods per ml liquid media. **Fluorescein di-acetate** (FDA) activity was measured according to Stubberfield and Shaw [1990]. **Protein** concentration was measured by the Bio-Rad method according to the manufacturer's instructions. For **biomass** estimation, the contents of

the reactor were subjected to alkaline hydrolysis with 1 N NaOH for 5 min at 70 °C, diluted and finally the soluble protein content was determined.

2. Bacterial biodegradation

2.1. Degradation of phenol, *p*-nitrophenol and phenanthrene

This part of the work was published in "J. Biotechnol. 51:265-272, 1996.

2.2. Degradation of pentachlorophenol

Organisms

Enrichment cultures of microorganisms were produced as follows:

- the samples of soil which was subjected to the action of haloid-organic compounds for a long time;
- the activated sludge processes into which wastewater containing haloid-aromatic substances penetrated by regular manner.

Medium and conditions of growing

In experiments the mineral synthetic medium was used which contained (g/l) :

KH_2PO_4 - 0.87; K_2HPO_4 - 2.96; $MgSO_4$ $7H_2O$ - 0.1; NH_4HCO_3 - 1.0; $NaHCO_3$ - 2.2; $FeSO_4$ $7H_2O$ - trace amounts; yeast extract - 0.05; glucose - 0.5; PCP - 0.01-0.2; distilled water - 1 l; pH 7.2-7.3.

Cometabolic transformation of PCP by the enrichment cultures was studied in anaerobic conditions in hermetically sealed bottles with argon and in microaerophilic conditions (without removal of O_2).

Analytical techniques

Bacterial density was measured as optical density of a suspension at 540 nm. The concentration of dissolved PCP was determined by UV-spectrophotometer. The content of Cl^-ions was measured with selective electrode.

RESULTS AND DISCUSSION

1. IMMOBILIZATION OF FUNGAL MYCELIUM

In order to compare the suitability of the different supports as packing materials for the immobilization of *P. chrysosporium*, a series of experiments was performed.

1a. Characterization of solid carriers

In a first stage, experiments in 120 ml serum bottles containing 10 ml medium, in stationary conditions, were done. Immobilization was performed by inoculating homogenized fungal mycelium, previously grown in shallow stationary conditions. The culture medium, essentially the nitrogen-limited medium described by Tien and Kirk [1988], consisted of 1.2 mM ammonium tartrate, 56 mM glucose, salts and trace elements. The production of CO_2 was measured as an indicator of physiological activity and compared to that of the free, non-immobilized organism. Peat moss, polyurethane foam and rock wool displayed the highest CO_2 evolution rate among the supports studied, even higher than the free organism (Table 2 and Fig. 2).

Table 2. Glucose consumption and CO_2 production by cultures of *P. chrysosporium* immobilized on different supports[a]

Support	Glucose disappearance (mmol)[b]	CO_2 evolved (mmol)[c]	
		Calculated	Experimental
Non-immobilized	0.57	3.4	3.5
Polyurethane foam	0.85	5.1	5.0
Activated carbon	0.01	0.1	1.2
Silica powder	0.08	0.5	0.5
Peat moss	0.57	3.4	5.9
Rock wool	-	-	3.8

[a]All cultures used were nitrogen limited (1.2 mM di-ammonium tartrate). Initial glucose concentration was 56 mM. Cultures were incubated in stationary conditions for 10 days at 37°C in 120 ml serum bottles.

[b]Glucose disappearance was measured as $(C_{initial} - C_{final}) \times V$, where C = concentration of glucose (mmol/ml), at the beginning and end of the incubation period, respectively; and V = volume of culture medium (ml).

[c]CO_2 was calculated as glucose disappearance (mmol) x 6, according to $C_6H_{12}O_6 + 6O_2 \rightarrow 6H_2O + 6CO_2$.

The CO_2 production calculated from the data of glucose consumption fitted almost perfectly the results obtained from direct measurements of CO_2, for all the supports, with the exception of activated carbon. The poor correlation obtained for activated carbon was probably due to

the relatively low efficiency of extraction of the adsorbed glucose due to its strong adsorption under the assay conditions.

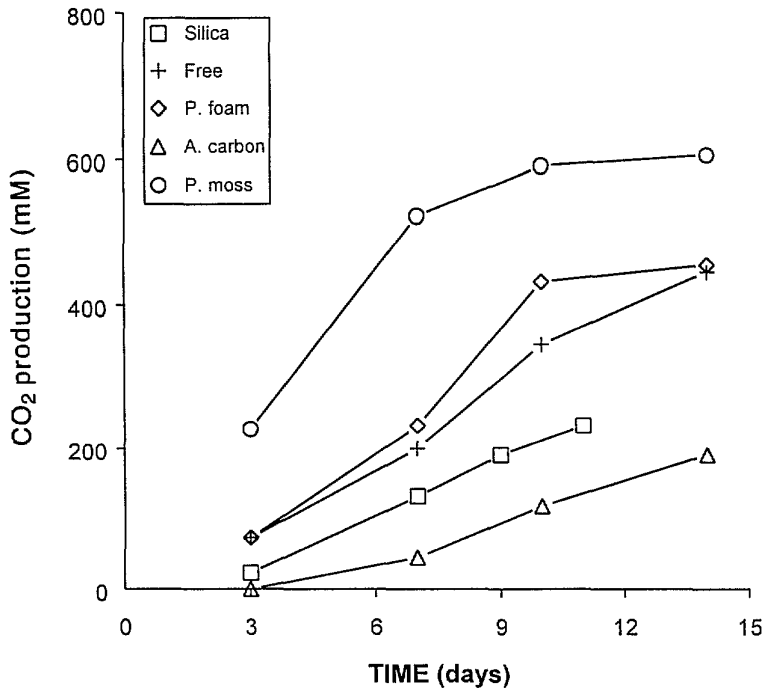

Fig. 2. Effect of support material on CO_2 evolution in cultures of *P. chrysosporium* grown in serum bottles. Conditions as in Table 2.

The effect of the different supports on the growth of *P. chrysosporium* in 60 ml trickle batch reactors was then studied. The reactors were packed alternatively with 12 g celite (R-630), 7 g pumice stone, 25 g activated carbon or 25 g coke. The experiments were conducted with three different volumes of media: 100 ml, 200 ml and 300 ml for each support material. The immobilization was performed by inoculating 1 ml spore suspension (1.6 AU at 600 nm) directly into the reactor.

The efficiency of the different supports to attach the fungal biomass was estimated by two different techniques: i) soluble protein measurement after alkaline hydrolysis of the reactor content (Fig. 3); ii) total enzymatic activity, measured as FDA hydrolysis (Fig. 4). According to the results, activated carbon almost completely inhibited fungal growth while all the other supports displayed an almost similar efficiency to attach fungal biomass. Although polyurethane yielded higher fungal growth than the control without immobilization (data not shown), all the rigid supports yielded, in general, lower biomass than the control. Peat moss and rock wool, like polyurethane, also displayed good growth (data not shown). Nevertheless, due to their low hydromechanical properties these materials collapsed once the mycelium formed a dense mass (around day 4 of incubation), considerably increasing the resistance to flow of the liquid throughout, and even clogging it. In general terms, the trickle reactors

displayed a series of operational difficulties, including channeling (preferential paths), lack of homogeneity and clogging with mycelium.

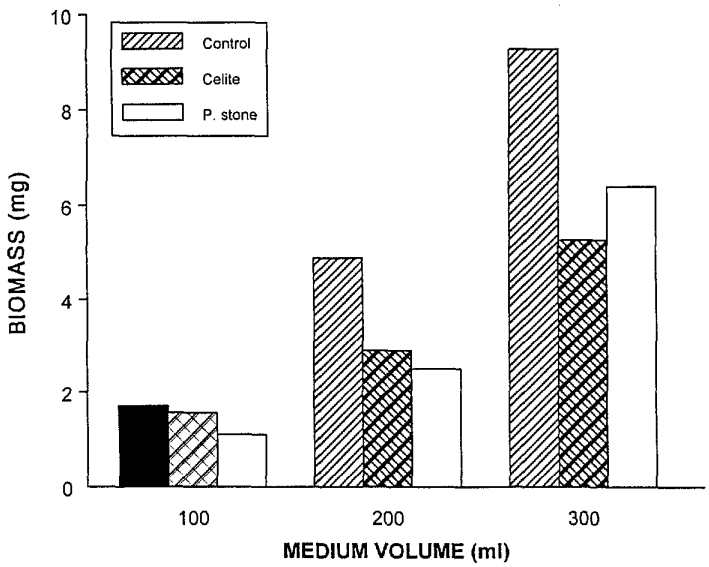

Fig. 3. Biomass production in a trickle reactor in batch culture by *P. chrysosporium* immobilized on celite, pumice stone and non-immobilized (control).

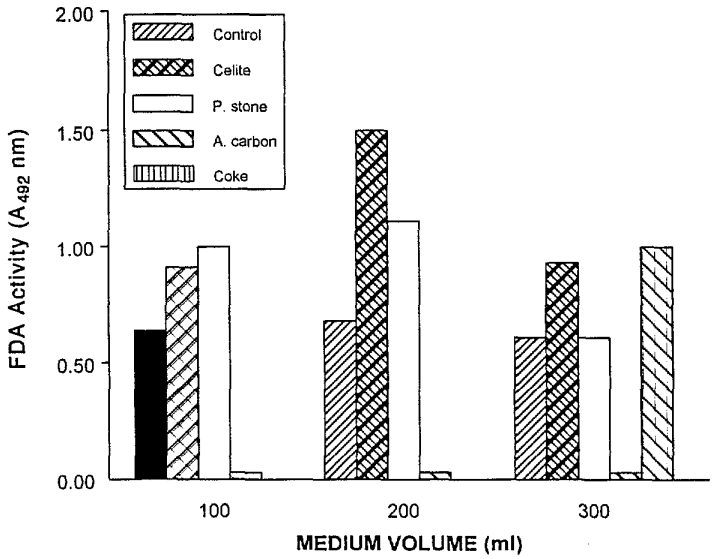

Fig. 4. FDA activity in a trickle reactor in batch culture by *P. chrysosporium* immobilized on coke, celite, pumice stone, activated carbon and non-immobilized (control). No data is presented for coke at 100 and 200 ml medium volume.

An up-flow packed bed batch reactor with polyurethane, with or without an external loop filled with R-640 celite, was tested. The purpose of the external loop was to trap the extracellular enzymes produced. Glucose and LIP activity were monitored at the bottom (inlet), middle and top (outlet) of the reactor. The results are presented in Figs. 5 and 6.

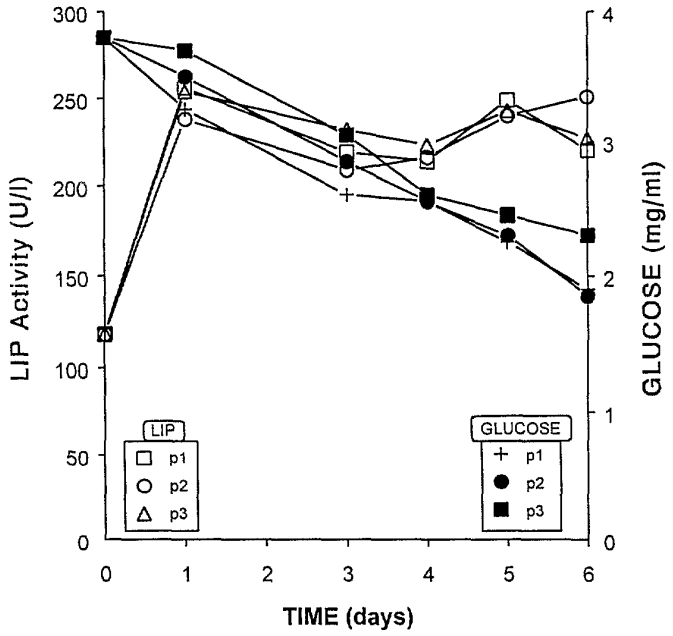

Fig. 5. Glucose consumption and LIP production in a fixed bed, up-flow batch reactor, of *P. chrysosporium* immobilized on polyurethane foam. p1, p2, p3 represent sampling ports at the bottom, middle and top of the reactor.

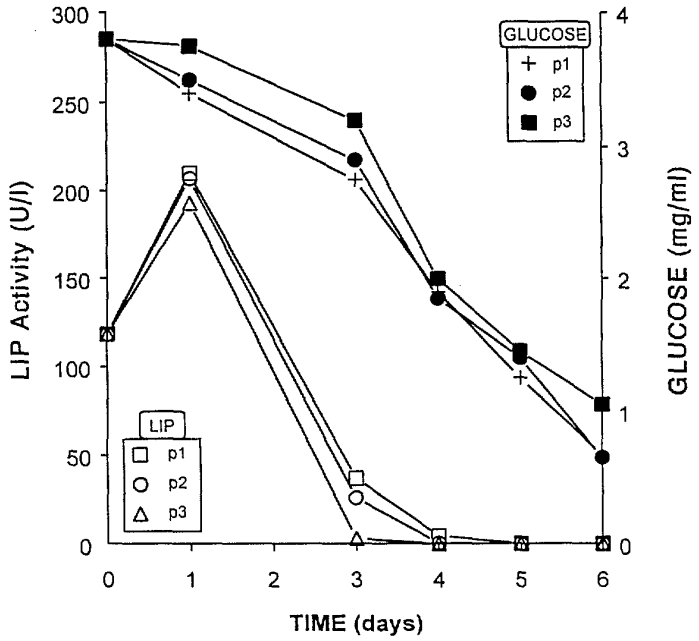

Fig. 6. Glucose consumption and LIP production in a fixed bed, up-flow batch reactor, of *P. chrysosporium* immobilized on polyurethane foam with an external column containing celite R-640. Sampling ports as in Fig. 5.

1b. Immobilization on polyurethane in the non-immersed liquid culture system

Cultures of *P. chrysosporium*, immobilized on polyurethane foam in the non-immersed liquid culture system in 250 ml Erlenmeyer flasks, were grown under two different oxygenation regimes, by exposing them to either a continuos supply of pure oxygen or air. The effect of these treatments on NH_4 and glucose consumption was examined under high nitrogen (Fig. 7). Glucose was completely depleted, within 48 h and 24 h, respectively, regardless of oxygen level (Fig. 7-top). The time-course profile for the consumption of NH_4 was not affected by oxygen level, and nitrogen assimilation ceased after complete depletion of the glucose, followed by a gradual increase in nitrogen level. However, under high nitrogen, 25% of the original NH_4 remained when nitrogen assimilation ceased (Fig. 7-bottom).

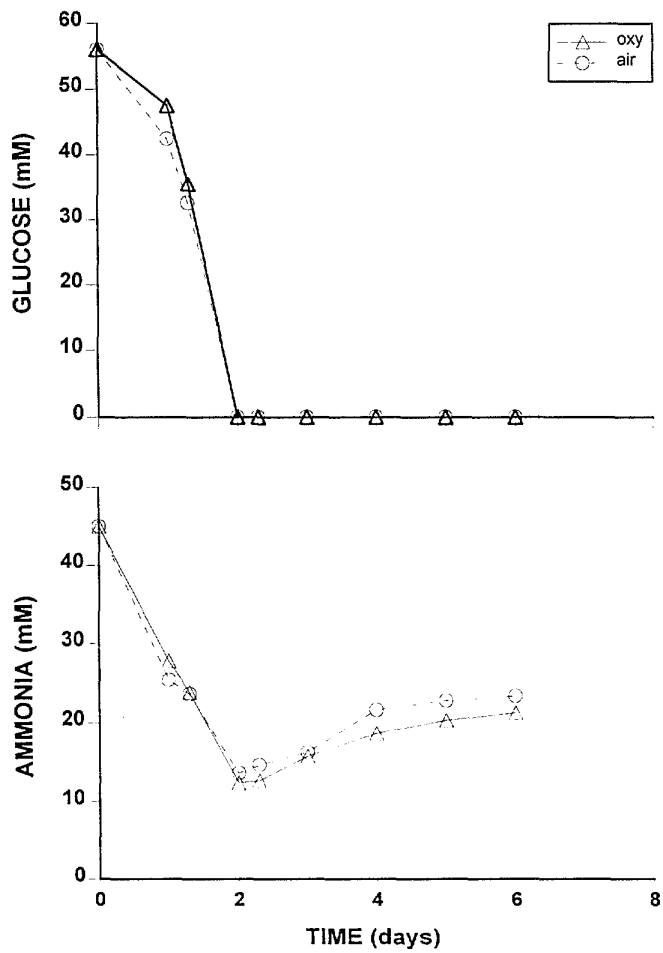

Fig. 7. Time course of nutrient consumption for cultures exposed to air or flushed with pure oxygen. **Top:** glucose concentration; **Bottom:** NH_4 concentration.

The effect of oxygen level on LIP and MNP activity is shown in Fig. 8. High levels of LIP were produced with air and oxygen, reaching a maximum of 1,300 and 1,800 U/l, respectively, on day 4 (Fig. 8-top). The onset of LIP synthesis coincided with the depletion of

glucose from the medium. The level of LIP activity beyond the peak decreased more slowly in air-grown than oxygen-grown cultures. Around 50% higher MNP activity was observed in oxygen than in air (Fig. 8-bottom), though even this peak was only 45% of the peak normally found under nitrogen limitation. In general, it appeared that oxygen level has more influence on MNP activity than on MNP.

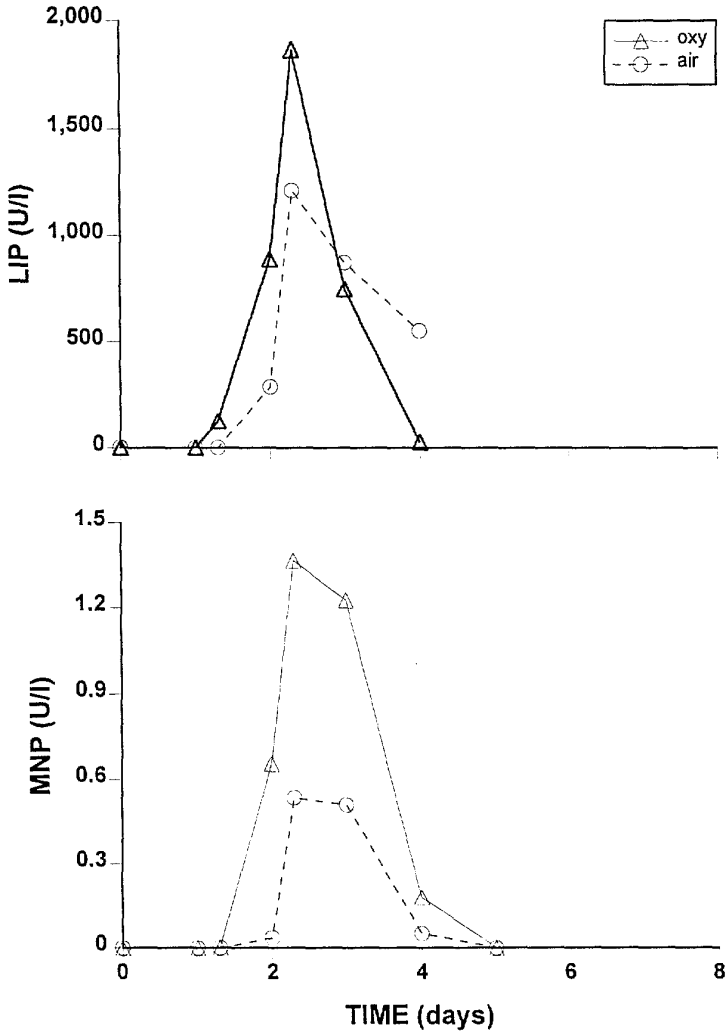

Fig. 8. Effect of O_2 level on lignin peroxidase (LIP) and manganese peroxidase (MNP) activities in *P. chrysosporium* grown under high nitrogen. Cultures were exposed to air or flushed with pure oxygen.

The level of oxygen had little effect on the heme protein profile observed in the extracellular fluid on the day of peak LIP activity. H1 and H2 were the dominant isoenzymes in high nitrogen cultures (Fig. 9). A substantial level of Ha was found under both conditions whereas, under oxygen, H8 appeared at a higher intensity. MNP isoenzymes were almost absent in both cases.

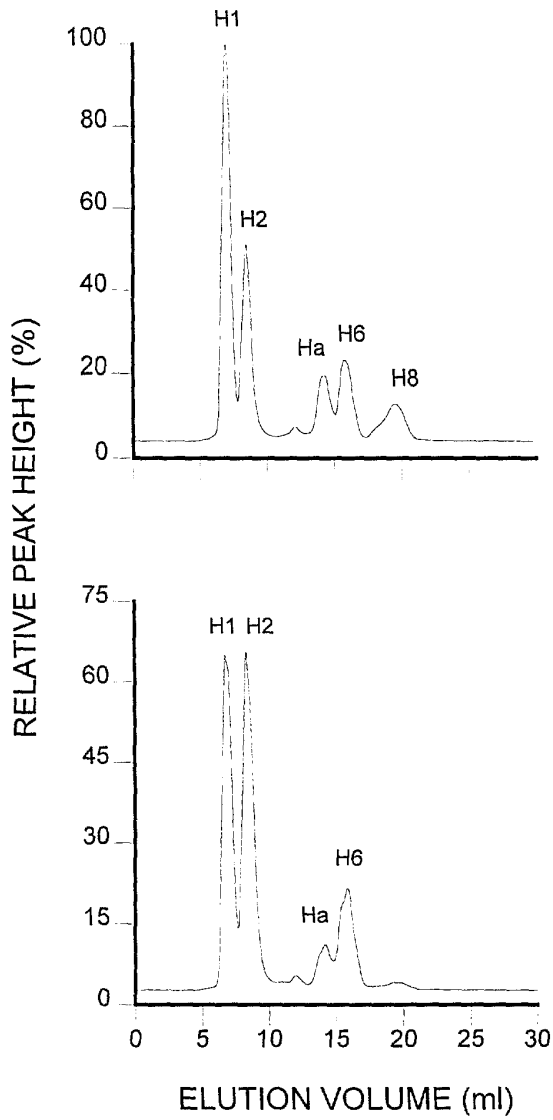

Fig. 9. Typical HPLC profile of heme proteins in the extracellular fluid of *P. chrysosporium* grown under high nitrogen and oxygen (top) and air (bottom). Equal volumes of extracellular fluid from day 4 were analyzed by strong anion-exchange-HPLC at 409 nm.

The profile of the extracellular ligninolytic enzymes obtained under high nitrogen conditions resembled that found by Holzbaur and Tien [1988] under carbon-limiting conditions. However, significant differences can be noted between those carbon-limiting conditions and our system. The carbon-limited systems were attained by decreasing the glucose concentration to one-tenth (5.6 mM) of the initial, whereas our system employed unchanged glucose concentrations (56 mM). As a result, we achieved eight to nine times higher ligninolytic enzyme activity, on par with that observed in the recently reported, improved, overproducing mutants [Orth *et al.*, 1991; Tien and Myer, 1990].

A comparative SEM study of immobilized mycelia on day 4 of growth (corresponding to peak LIP activity) is presented in Fig. 10. Under nitrogen limitation the formation of polysaccharides was observable also with 100% oxygen (Fig. 10-top), whereas under high nitrogen, no polysaccharides were found attached to the mycelia, even in cultures exposed to air (Fig. 10-bottom). The effect of immobilization on the morphology of the fungus is also visible in Fig. 10. The micrographs show a spaced and linear filamentous mycelium at low density, under all culture conditions, even in the high nitrogen culture which gave rise to a high biomass. This is in contrast to the mycelial structure in pellet (non-immobilized) cultures, especially those grown in high nitrogen, where a very dense and branched mycelium is found.

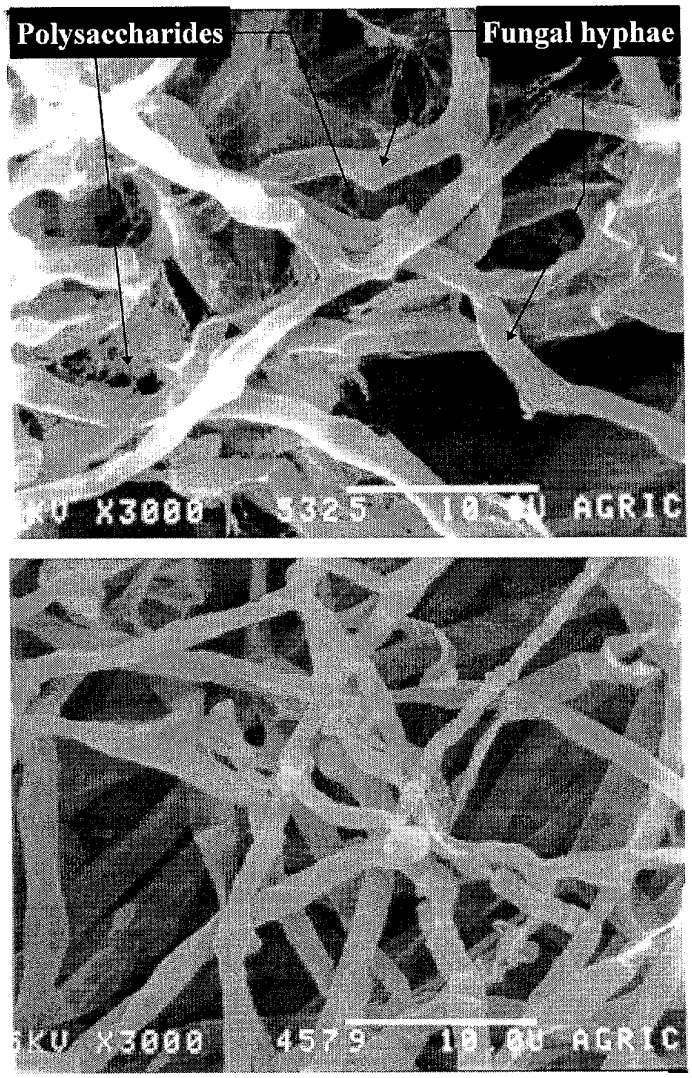

Fig. 10. Typical scanning electron micrographs of *P. chrysosporium* mycelia immobilized on polyurethane in non-immersed liquid culture; sampled on day 4 (Magnification x 3,000). **Top:** nitrogen limitation in oxygen; **bottom:** high nitrogen in air.

The lack of LIP activity in cultures grown in air under nitrogen limitation can also be explained in terms of oxygen availability, as a result of the copious formation of polysaccharides attached to the mycelia. Dosoretz *et al.* [1990a] showed that extracellular polysaccharides are the major secondary metabolite produced by this fungus when grown under nitrogen-limited conditions in air, and suggested that the free and mycelium-attached polysaccharides offer resistance to oxygen and nutrient diffusion. The same authors also reported higher glucose consumption and CO_2 evolution rate in cultures flushed with oxygen instead of air, suggesting that oxygen is a limiting factor. It is therefore likely that the lower rate of glucose consumption obtained under air, as opposed to oxygen, from the fourth day of growth onward, reflects lower availability of oxygen to the mycelium, once significant formation of polysaccharides has occurred.

The advantage of using polyurethane foam (a solid, hydrophobic and very porous material) over other porous supports on the thermodynamic adhesion properties and morphology of *P. chrysosporium*, has been previously reported [Asther *et al.*, 1990; Chen *et al.*, 1991; Kirkpatrick and Palmer, 1987]. The non-immersed liquid culture system used in this work is suggested to increase the availability of oxygen to the mycelia, by preventing the formation of a dense mycelial structure and increasing their surface area, thus enabling LIP to be synthesized under nitrogen-rich conditions [Dosoretz *et al.*, 1993] and even in the presence of air. The results of the present study may explain differences observed with regard to the oxygen level required for the degradation of lignin under natural conditions (woody tissue) *vs.* that in liquid cultures under laboratory conditions. Whereas lignin degradation in solid-state cultures of *P. chrysosporium* and other white-rot fungi is almost identical in air and in an oxygen-enriched atmosphere [Highley *et al.*, 1982; Kerem *et al.*, 1992], the synthesis of LIP and the degradation of lignin in liquid cultures has been reported to be highly dependent on the oxygen level [Bar-Lev and Kirk, 1981; Dosoretz *et al.*, 1990a; Kirk *et al.*, 1978]. Undetectable O_2 levels in the mycelial mat, at depths of lower than 1 mm, have been reported in non-agitated as well as in submerged liquid cultures of *P. chrysosporium* incubated in 100% O_2 [Leisola *et al.*, 1983; Michel *et al.*, 1992].

1c. Continuous up-flow fixed bed reactors with polyurethane

The hydrodynamic performance of the packed-bed reactors was determined by RTD analysis. The RTD curves obtained for each reactor are shown in Fig. 11. The experimental data were adjusted to the Tank-in-Series model. This model allows the characterization of the actual hydraulic behavior of the system by means of an equivalent number (N) of Continuous Stirred Tank reactors (CSTR) coupled in series. Low N values represent well mixed behavior, whereas high N values represent plug-flow behavior. For reactor R1, one single pass without recycling, a Gaussian and symmetrical RTD curve was obtained, with a calculated N value of 20. This value corresponds to clear plug-flow behavior, indicating that negligible axial mixing took place. Reactor R3 with an R:F ratio of 12:1 displayed a RTD curve typical of a

completely mixed system, characterized by a N value of 2.5. Reactor R2 with an R:F ratio of 2:1 fitted an intermediate N value of 12, corresponding to typical plug flow behavior with partial axial mixing, namely a combined plug-flow/CSTR regime.

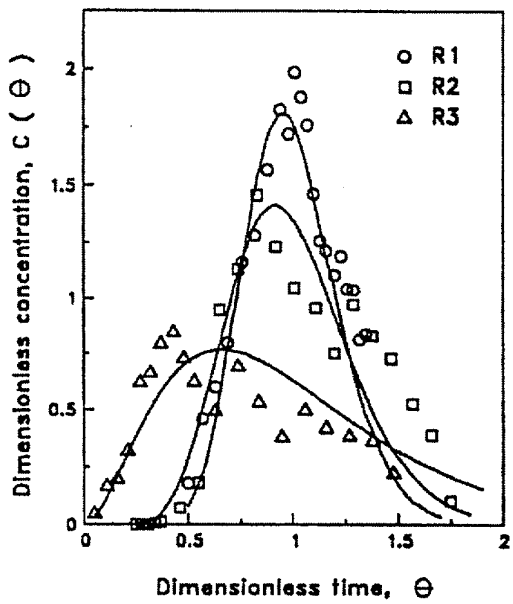

Fig. 11. RTD curves of the packed-bed reactors filled with polyurethane foam cubes. Operating conditions were: hydraulic residence time, 4 h; temperature, 37°C. R1, single pass; R2, R:F = 2:1; R3, R:F = 12:1.

Reactor R2 displayed the highest LIP productivity, measured as either overall activity (U_o) or overall LIP production rate (r_ε), during the entire period of operation (40 days), in which 4 cycles of production were achieved. U_o values of 700, 1462 and 400 U as well as r_ε values of 264, 578 and 151 U/l/day were obtained for reactors R1, R2 and R3, respectively. The results are summarized in Table 3.

Table 3. LIP production by *P. chrysosporium* immobilized on polyurethane foam cubes in continuous up-flow packed-bed reactors.

Reactor	R1	R2	R3
R:F ratio	0	2:1	12:1
N value	20	12	2.5
RTD behavior	plug-flow	plug-flow with axial mixing	completely mixed
U_o (U)	700	1462	578
r_ε (U/l/day)	264	578	151

The overall activity (Uo) was calculated for the entire period of operation as follows:

$$U_o = \sum_{cycles} \int_a^b C.F.dt$$

where:

Uo is the overall activity (U); C is the enzyme concentration (U/l); F is the flow rate (l/day) and t is the cycle production time (day).

The overall LIP production rate (r_ε) was calculated for the entire period of operation as follows:

$$r_\varepsilon = \frac{U_o}{V_R.T}$$

where:

VR is the volume of the reactor (l) and t is the total operation time (days).

These data on LIP production fully agree with the RTD data, indicating that a plug-flow reactor with axial flow is the most appropriate regime for LIP production in up-flow packed-bed reactors. Furthermore, these results indicate that a completely mixed regime, even operated at low shear forces, represses LIP synthesis. This repression in a low shear forces system, as opposed to LIP repression observed in mechanically agitated reactors, suggests a metabolic rather than a mechanical mechanism of repression in completely mixed reactors, in general. The advantages of using of a packed-bed reactor with a moderate recycling ratio, over a single pass plug-flow reactor may be expressed by:

a. sequential renovation of the microbial biofilm, even at low shear forces
b. increased mass transfer rate
c. increased energy transfer rate
d. minimizing preferential paths and dead zones within the reactor.

Further work is required to optimize a continuous fungal reactor to be applied for the degradation of toxic pollutants.

2. PCP DEGRADATION

2a. Effect of solid carriers

Degradation of cold PCP in packed-bed reactors is presented in Table 4. PCP was added to the cultures after 3-4 days of incubation, concurrently with the end of the growth phase and initiation of idiophase. In all the cases a fast and almost complete disappearance of PCP was observed. Analysis of the hexane extract of the packed bed revealed that even though a partial degradation of PCP took place, still a considerable portion of PCP (20-40% of the initial) remained intact. According to the results of our experiments it is not possible to determine decisively the precise contribution of the extracellular ligninolytic enzymes of *P. chrysosporium* into the pattern of PCP degradation. In the absence of ligninolytic activity (reactors with coke), PCP was degraded to a lesser extent (60%) than in the case of polyurethane or celite in which ligninolytic activity was detected (70-90%). However, no correlation was detected between the level of LIP activity and extent of PCP degradation.

Table 4. Disappearance of PCP in immobilized cultures of *P. chrysosporium* in a fixed bed reactor

Support	Polyurethane	Coke	Celite R-630	Polyurethane +Celite R-630
PCP Adsorption	+	-	-	-
Enzyme Adsorption	-	+	+	+
Enzyme Production (U/l)	250	ND	ND	250
Remaining PCP (%)[a]	33	43	20	11

[a]Remaining PCP represents the percentage of the initial PCP concentration found after the incubation period.

The major peaks observed in a typical chromatogram by either HPLC or GC corresponded to the remaining PCP and to PCA, which was identified as the major metabolite of PCP degradation (Fig. 12). TCHD, which was described by Lin *et al.* [1990] as the major extracellular metabolite during PCP degradation was also identified, but as a minor metabolite (Fig. 12 bottom).

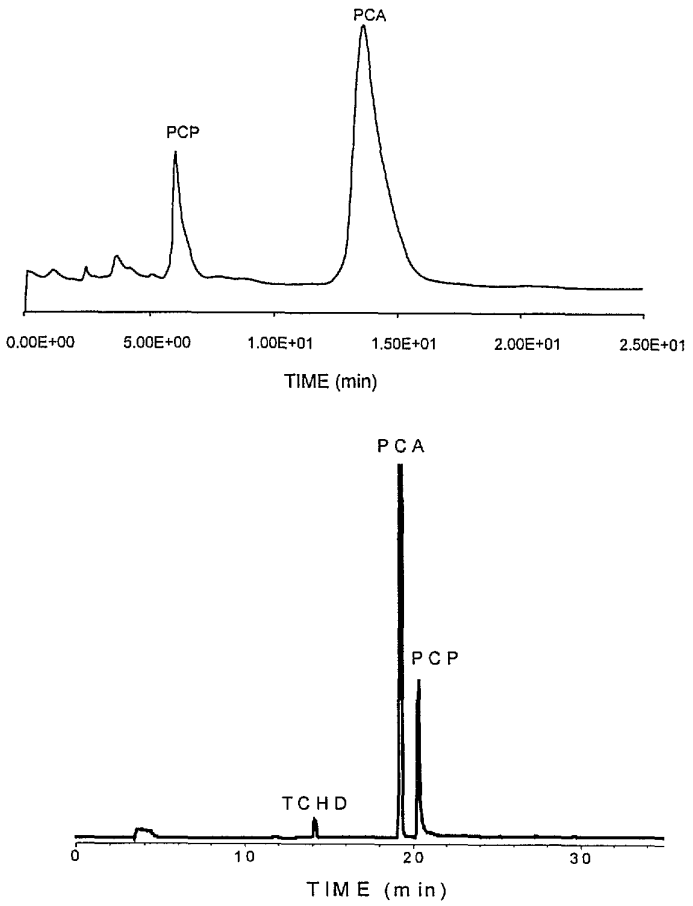

Fig. 12. Typical chromatographic profile of an hexane extract of the polyurethane foam cubes of a *P. chrysosporium* culture four days after PCP addition. Top: HPLC chromatogram; Bottom: GC chromatogram. PCP was addded on day 3 of the incubation. PCP: pentachlorophenol; PCA: pentachloroanisol; TCHD: tetrachloro-1,4-dibenzoquinone.

From the point of view of increasing the rate of PCP destruction, the use of a carrier with the ability to adsorb enzyme is preferable to the use of carriers able to adsorb pollutant. It is noteworthy that the addition of a external column with celite to the reactor with polyurethane cubes improves the degradation of PCP. This may be attributable to the effect of enzymatic pretreatment of pollutant, but further experiments are required before final conclusions can be drawn.

2b. PCP degradation on polyurethane - Effect of time of addition, culture conditions and medium composition

The effect of adding glucose and PCP on LIP activity by *P. chrysosporium* immobilized on polyurethane foam, was studied. The experiments were carried out in 250 ml Erlenmeyer flasks in the non-immersed liquid culture system as described above. The culture medium consisted of N-limited medium with 5 g/l glucose. PCP (5 mg/l) was added on day 2 (Fig. 13) or 4 (Fig. 14) of incubation, as indicated. The results show an increase in LIP activity, when only glucose was added, some retardation of maximum activity when only PCP was added,

and a marked synergetic effect when glucose and PCP were added simultaneously. This effect was more marked when the glucose and PCP were added on day 2 of incubation. These results show that when added at the onset of idiophase or during idophase, PCP did not affect fungal metabolism, at the concentrations studied, and even, appears to improve the conditions for the synthesis of the ligninolytic enzymes.

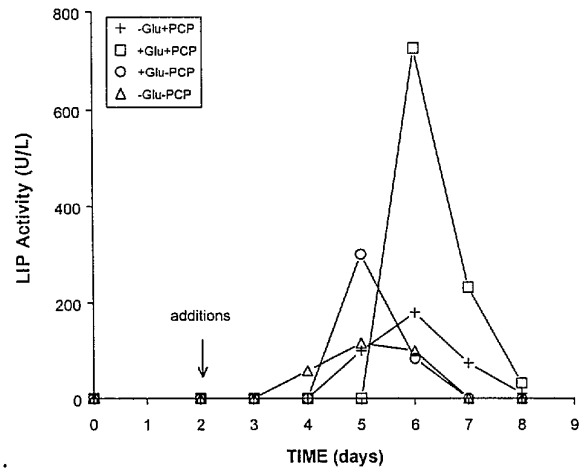

Fig. 13. LIP activity resulting from the addition of PCP and glucose on day 2 on cultures of *P. chrysosporium* immobilized on polyurethane foam in 250 ml Erlenmeyer flasks. Arrow indicates time of addition.

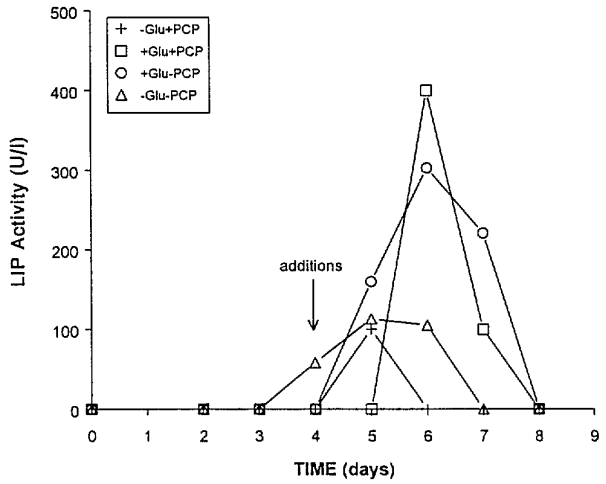

Fig. 14. LIP activity resulting from the addition of PCP and glucose on day 4 on cultures of *P. chrysosporium* immobilized on polyurethane foam in 250 ml Erlenmeyer flasks. Arrow indicates time of addition.

The effect of time of PCP addition and culture conditions on LIP and MNP activities, nutrient consumption and pattern of degradation by *P. chrysosporium* was further studied. Cultures were grown under ligninolytic conditions (*i.e.* pure oxygen gas and limiting nitrogen) in 250 ml shaken flasks in either non-immersed or submerged cultures. PCP was added on day 3 or 6 of the incubation, which is in reach of the expected time for the peak of MNP and LIP

activities, respectively, with the purpose to observe the effect of ligninolytic enzymes on PCP degradation, from one side, and the effect of PCP addition on ligninolytic enzyme synthesis, from the other side. The results for the cultures immobilized on polyurethane foam cubes in the non-immersed liquid culture system are presented in Fig. 15. Besides LIP activity, an almost similar pattern of PCP disappearance, PCA accumulation, glucose consumption and MNP activity were observed for PCP addition in either day 3 (Fig. 15-Top) or day 6 (Fig. 15-Middle). However, PCP addition on day 3 decreased LIP activity, which was at its onset at the time of addition, by almost 50%, as compared to the control (Fig. 15-Bottom). In contrast, PCP addition on day 6 was at the end of the ligninolytic phase and therefore no effect was observable. The kinetic profile of PCP disappearance was almost similar in both cases, suggesting that PCP metabolism is independent of the presence of the ligninolytic enzymes.

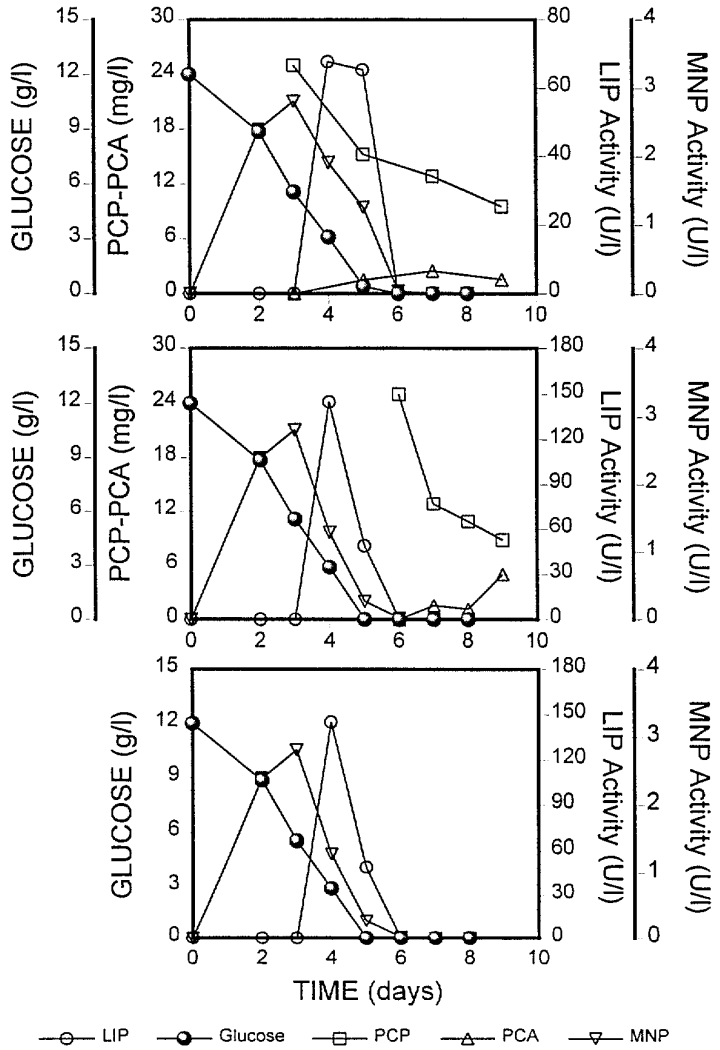

Fig. 15. Pattern of PCP degradation by *P. chrysosporium* grown immobilized on polyurethane foam cubes in the non-immersed liquid culture system under limiting nitrogen and pure oxygen. Top: PCP added on day 3; Middle: PCP added on day 6; Bottom: Control, no PCP addition. Initial PCP concentration was 25 mg/l.

The effect of time of PCP addition on LIP and MNP activities, nutrient consumption and pattern of degradation in free pellets gown in submerged culture are shown in Fig. 16. According to these results, the effect of PCP addition on the course of the culture was substantially different of that observed in the polyurethane cultures. When added on day 3, PCP disappeared almost completely 6 days later (day 9 of the culture), however it dramatically affected the course of the culture (Fig. 16-Top). Indeed, LIP activity was completely absent and MNP remained up to the end of the experiment at the value that it was at the time of addition, roughly close to its maximum, and glucose consumption rate decreased. Apparently, once PCP was added, *de novo* synthesis stopped, as compared to the addition on day 6 (Fig. 16-Middle), or to the control, without PCP addition (Fig. 16-Bottom). Addition of PCP on day 6 affected glucose consumption on a similar manner, however it had no effect on MNP activity, and, on the contrary, slightly increased the level of LIP at peak activity (Fig. 16-Middle). Initial PCP disappearance rate was very similar to that found at addition on day 3, however the remaining concentrations of PCP and PCA were slightly higher. At the time of PCP addition on day 3 the culture was at the beginning of the idophase, as compared to the control (Fig. 16-Bottom), whereas at the time of PCP addition on day 6, cultures were almost at the end of the ligninolytic phase and therefore only a slight effect was observable. As in the case of the polyurethane cultures, the kinetic for PCP disappearance was almost similar in both cases, suggesting that its metabolism is independent of the presence of the ligninolytic enzymes.

The more pronounced effect on synthesis of the ligninolytic enzymes and extent of degradation was found for PCP addition in day 3 for the free pellet cultures. Indeed, while MNP activity decay ceased and LIP activity was completely inhibited, PCP disappeared to almost completion. This behavior may be explained by the slight, but still considerable, adsorption of PCP onto the polyurethane, which buffered the inhibitory effect of PCP towards the synthesis of fungal enzymes. On the other hand, the adsorption of PCP apparently limited its availability and consequently the rate of degradation, as judged by the almost complete disappearance observed in free pellet cultures when PCP was added in day 3. These results strongly suggested that PCP degradation is independent of the presence of the ligninolytic enzymes, at least LIP. The transient accumulation of PCA to only one tenth of the mass of PCP degraded, suggested that PCA is further metabolized or even mineralized by the fungus. Finally, our results indicated that PCA is the primary metabolite transiently accumulated during PCP degradation by *P. chrysosporium*, in contrast to previous works, which reported TCHD as the primary metabolite of PCP degradation by *P. chrysosporium* [Lin *et al.*, 1990].

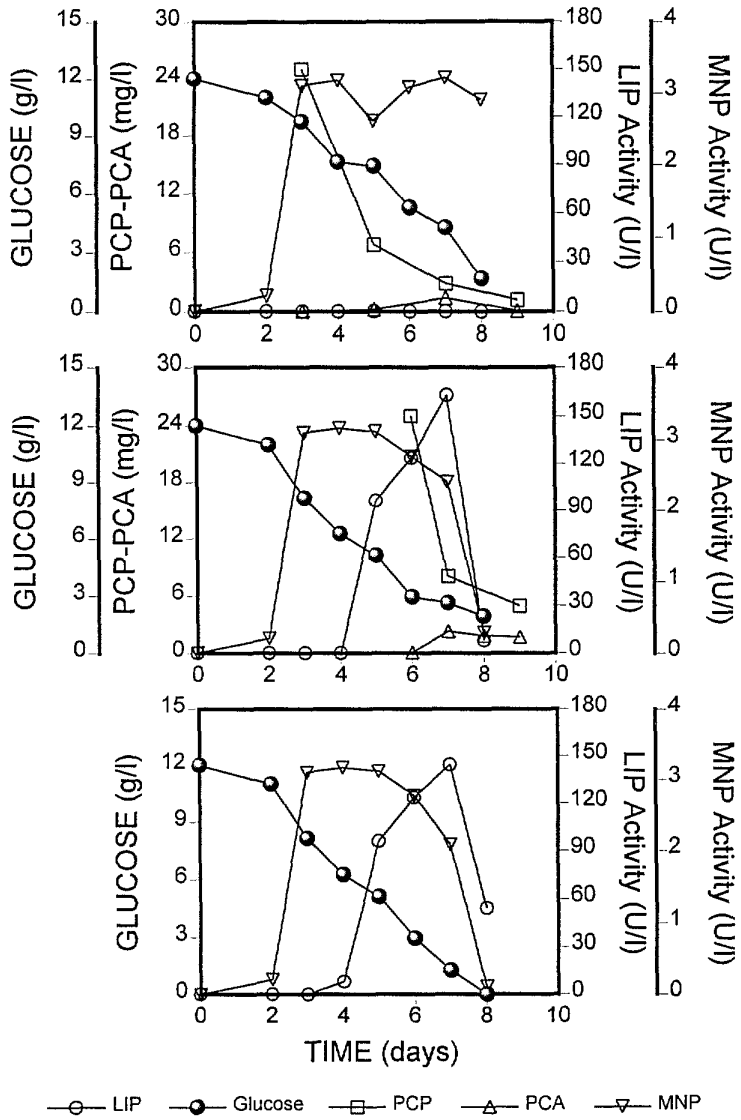

Fig. 16. Pattern of PCP degradation by *P. chrysosporium* grown in pellet form in submerged culture under limiting nitrogen and pure oxygen. Top: PCP added on day 3; Middle: PCP added on day 6; Bottom: Control, no PCP addition. Initial PCP concentration was 25 mg/l.

The contribution of the individual ligninolytic enzymes, LIP and MNP, to PCP degradation by *P. chrysosporium* immobilized on polyurethane foam cubes in the non-immersed liquid culture system was then studied. Experiments were performed under air, closer to the conditions normally found in nature or applied in commercial bioreactors, in limiting and excess nitrogen media, with and without Mn^{+2}, and addition of PCP on day 3 (Fig. 17). A control experiment, without addition of PCP, was also performed (Fig. 18). The following conditions were allowed: LIP alone (Fig. 17,a and b), MNP alone (Fig. 17d) and no ligninolytic enzymes (Fig. 17c), that can be compared with the contribution of both ligninolytic enzymes together showed above (see Fig. 15). Roughly, almost similar patterns of PCP disappearance and PCA accumulation were observed for all the conditions studied, with

or without LIP or MNP, suggesting no contribution of the ligninolytic enzymes to PCP degradation. The remaining final concentration of PCP can be explained by its adsorption onto the polyurethane, as above (see Fig. 15). The transient concentration of PCA reached almost one fifth of the mass of PCP degraded, again indicating that a great part of it is further metabolized or even mineralized. The effect of PCP on the synthesis of the ligninolytic enzymes and glucose consumption was similar for either N-excess or N-limiting conditions, with or without Mn^{+2}. In fact, an almost 50% reduction of LIP (Figs. 17a and b) or MNP (Fig. 17 d) were found upon addition of PCP as compared to the controls without PCP (Figs. 18 a, b and d, respectively). These results are similar to that obtained under oxygen (see Fig. 15).
The slower glucose metabolism and lesser extent of PCP degradation found at N-limiting conditions with Mn^{+2} (Figs. 17d and 18d) can be explained by the copious amount of polysaccharides produced by the fungus (see bottom SEM micrograph in Fig. 10). On the other hand, the higher extent of PCP degradation in cultures without Mn^{+2}, under N-limitation (~80%) compared with N-excess (~50%) (compare Figs. 17 a and b), suggest a much higher specific activity in N-limited cultures.

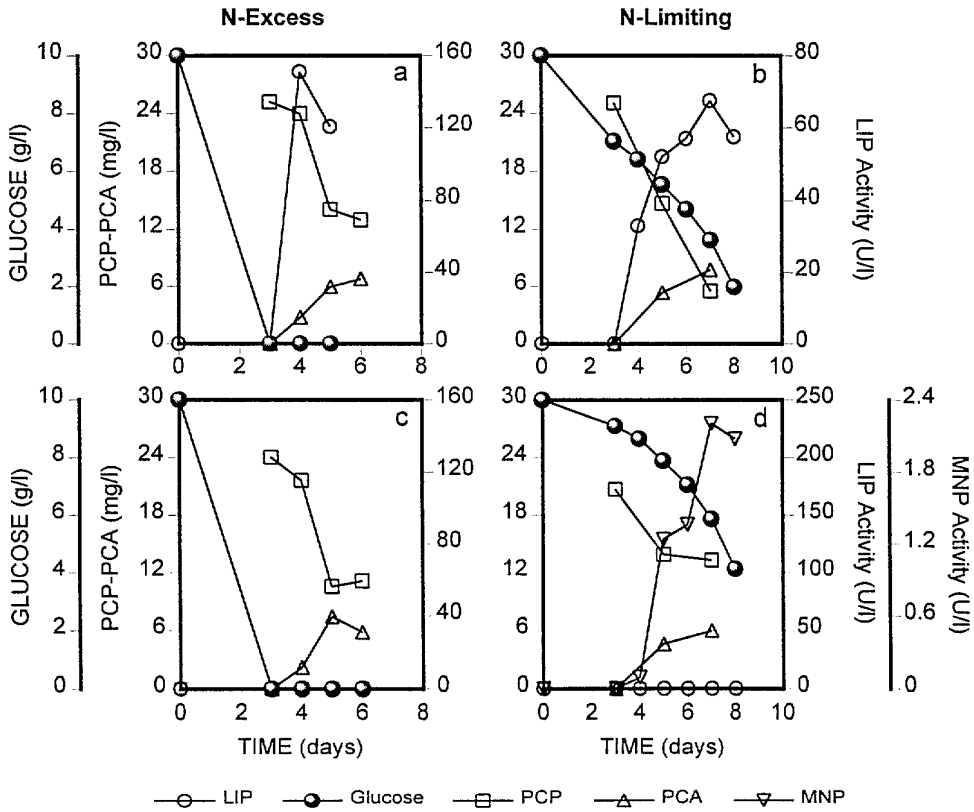

Fig. 17. Effect of medium composition on PCP degradation and ligninolytic enzymes activity by *P. chrysosporium* grown immobilized on polyurethane foam cubes in the non-immersed liquid culture system under air. **a:** N-excess medium without Mn^{+2}; **b:** N-limited medium without Mn^{+2}; **c:** N-excess medium with Mn^{+2}; **d:** N-limited medium with Mn^{+2}. PCP was added on

day 3 at an initial PCP concentration of 25 mg/l. In cultures without Mn^{+2}, top panels, no MNP activity is expected.

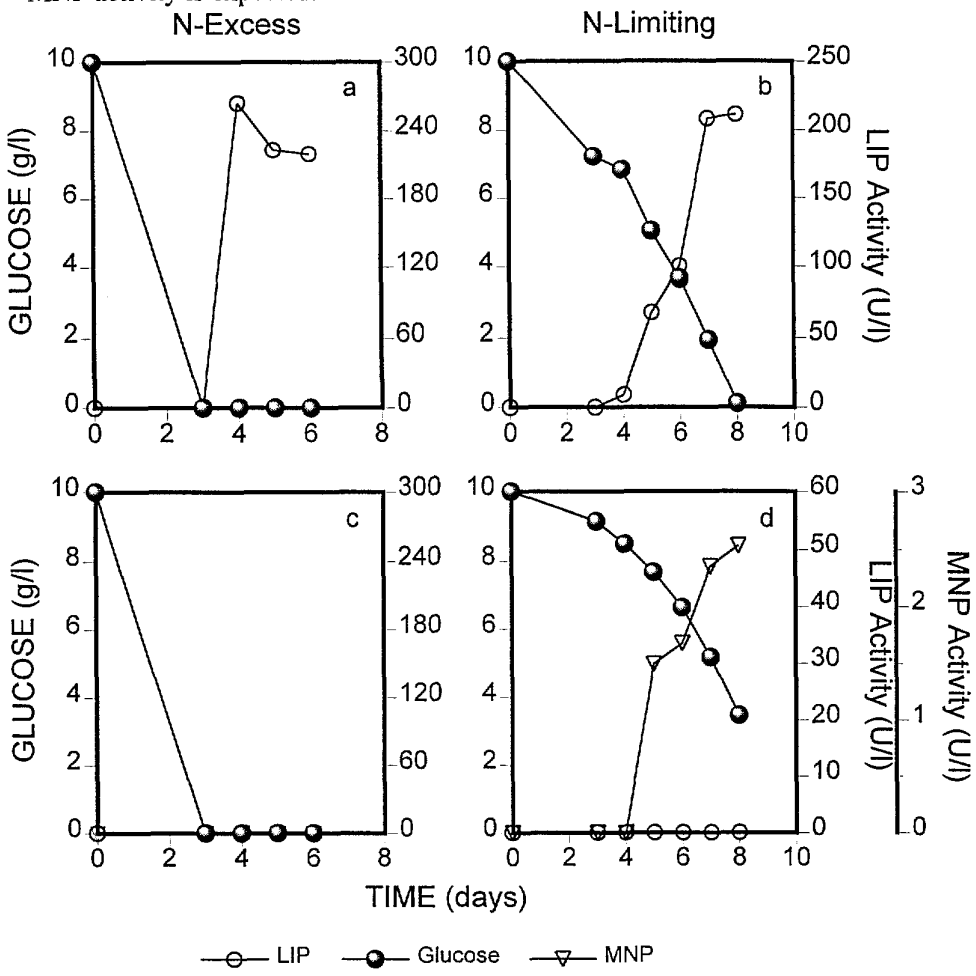

Fig. 18. Effect of medium composition on ligninolytic enzymes activity by *P. chrysosporium* grown immobilized on polyurethane foam cubes under air in the non-immersed liquid culture system in the absence of PCP (control). **a:** N-excess medium without Mn^{+2}; **b:** N-limited medium without Mn^{+2}; **c:** N-excess medium with Mn^{+2}; **d:** N-limited medium with Mn^{+2}. In cultures without Mn^{+2}, top panels, no MNP activity is expected.

2c. PCP mineralization

The results of mass balance experiments carried out in growth flasks in shallow stationary cultures are shown in Table 5. PCP was added to the cultures after 3-4 days of incubation, concurrently with the end of the growth phase and initiation of the idiophase. Total recovered radioactivity (in %) represents the sum of the measurable radioactivity in the gas phase ($^{14}CO_2$) and extracts of the liquid and solid fractions (remaining initial ^{14}C-PCP and ^{14}C-labeled metabolites), relative to the total amount of ^{14}C-PCP initially added. Percent radioactivity represents the measured radioactivity of the indicated fraction (*i.e.* $^{14}CO_2$ in extract of the liquid phase, or extract of the support) relative to the total radioactivity

measured. The total recovered radioactivity varied from 33.8%, in the case of activated carbon, to 96% for polyurethane foam. The variation in total recovery may be correlated to the degree of adsorption, or perhaps the efficiency of extraction (i.e. desorption), from the different supports. Thus, the lowest total recovery was seen in the case of activated carbon, which displayed the highest adsorption.

Table 5. Mass balance analysis of radioactive labeled material during [U-^{14}C]-PCP degradation in cultures of *P. chrysosporium* immobilized on different supports

Support	Percent Radioactivity[c]				Total Recovered Radioactivity (%)
	CO_2	Liquid	Support		
			Hexane	Ethylenamine	
Non-immobilized					
Sample	37.3	56.4	6.3	-	90.6
Control[a]	3.2	96.8	-	-	95.0
Peat moss					
Sample	1.3	4.9	93.8	-	75.6
Control	0.1	3.1	96.8	-	96.8
Activated carbon					
Sample	1.5	5.9	3.9	88.7	33.8
Control	0.1	2.7	97.2	-	94.0
Rock wool					
Sample	33.6	29.5	36.9	-	92.0
Control	0.1	97.0	2.9	-	100.0
Polyurethane foam					
Sample	3.3	0.5	96.2	-	96.0
Sample[b]	1.7	0.7	97.6	-	98.0
Control	0.1	2.3	97.6	-	96.5

[a]Control represent results obtained with non-inoculated bottles containing support, media and PCP solution. For the non-immobilized cultures, the control contained only medium and PCP.

[b]Nitrogen-sufficient culture (12 mM di-ammonium tartrate). All others were nitrogen limited (1,2 mM di-ammonium tartrate).

[c]Percent radioactivity represents the distribution of radioactive material in the gas, liquid and solid phases, relative to the total radioactivity found. Total recovered radioactivity was calculated as the sum of the total radioactivity found at the end of the experiments in the liquid, gas and solid phase, relative to the initial added radioactivity.

In the case of non-immobilized cultures, the major part of non-mineralized PCP (61.7%) was found in the culture liquid, and only 6.3% in the hexane extract of the biomass. For rock wool, the distribution of the measured radioactivity between gas and liquid+solid fractions was close to that found in the non-immobilized cultures. However, due to the fungal growth, the proportion of radioactive material adsorbed to the support, and extracted by hexane, increased significantly (36.9% versus 2.9%) in the non-immobilized cultures. At the same

time, 29.5% of the radioactive material was found in the culture liquid, implying that the biotransformation of PCP is much greater than the 30% mineralization obtained. In analogous experiments with cold PCP, no PCP was found in either the hexane extract or the culture liquid.

With peat moss, polyurethane foam or activated carbon, mineralization of PCP was negligible. In the case of peat moss, the quantity of radioactive materials extracted from the support was almost the same in samples with and without fungal growth (control), indicating that almost all the PCP was adsorbed to the peat moss during the experiment (13 days). Similar results were obtained with polyurethane foam, using either limiting or sufficient nitrogen medium.

With activated carbon, 98% of the measured radioactive material was recovered by hexane extraction of the support itself, in the control tests, whereas only 4% of the measured radioactive material was recovered from the support after fungal treatment, using hexane; but almost 30% was extracted using ethyleneamine. Similar behavior was observed with peat moss, where the recovery of radioactive material decreased by 20% after fungal treatment. This suggests that products that are insoluble and irreversibly attached to the support may be formed during the process of biodegradation of PCP. These results are consistent with the suggestion that polymerization reactions possibly occur as a result of enzymatic activity, as reported by Lamar *et al.* [1990].

3. BACTERIAL DEGRADATION OF AROMATIC AND POLYAROMATIC COMPOUNDS

The degradation of phenol, *p*-nitrophenol and phenanthrene on powdered activated carbon (PAC) was studied in comparison with free (suspended) and sand-attached cultures, using specialized mixed bacterial cultures adapted to high toxicant concentrations. An advantage of PAC, compared to inert supports and to suspended cultures, is its ability to act as a modulator, by adsorbing high concentrations the toxic compounds immediately, and thus regulate the concentration of the free toxic material, thereby protecting the attached microorganisms against toxicity. Indeed, almost similar profiles of degradation were obtained in the cultures with PAC for the three compounds studied, even at the higher concentrations. On the other hand, degradation was dramatically inhibited, or even ceased completely, in suspended or sand-attached cultures at the same concentrations. A remaining baseline concentration found in the PAC cultures may be explained by the negligible diffusion at low concentrations of adsorbed material, rendering it unavailable to the bacterial film. A similar phenomena was reported by Ehrhardt and Rehm [1985]on granular activated carbon. This part of the work was published in "J. Biotechnol. 51:265-272, 1996.

4. BACTERIAL DEGRADATION OF AROMATIC COMPOUNDS ADSORBED ONTO POROUS POLYMERIC ADSORBENTS

4a. Adsorption equilibrium of pentachlorphenol onto porous polymeric adsorbents

The major task of this part of the work was focused on the studying and characterization of pentachlorphenol adsorption process from water solutions onto porous polymeric adsorbents with different chemical polarity and porous structure. The porous polymeric sorbents used were the following: polysorb 40/100, polysorb 60/100 and porolas VP-3. Polysorb 40/100 and polysorb 60/100 are formed by copolymerization of styrene and divinylbenzene, porolas VP-3 is formed by copolymerization of 2-methyl-5-vinylpyridine with divinylbenzene.

Prior to use, the sorbents were purified to remove unreacted monomers through separation of fine particles by decanting in isopropyl alcohol and extraction in a Soxhlet apparatus for 8 hours [Kun and Kunin, 1968]. Then the copolymer in the column was washed successively with the same solvent and water. The washing was checked spectrophotometrically. The sorption equilibrium on the polymeric sorbents were studied in a stirred reactor. A given amount of swollen sorbent was weighed into sorbate solutions of various concentrations. After standing for 24 hours (enough to obtain equilibrium in the system) the sorbent was filtered off, dried and weighed. The equilibrium sorbate concentration was determined spectrophoto- metrically in the solution.

The first aim was to study the influence of water solution pH-values on the efficiency of PCP adsorption. This is why we determined the values of PCP dissolving index in water solution at the different pH-values. The results obtained are summarized and given below.

pH	2,2	7,2	7,5	8,1
C_s 10^3 ,g/l	10,4	830	1550	5880

Then we studied the adsorption process of PCP onto polysorb 60/100 at different pH values: 2,2 (all substance is in non-ionic form); 5,7 (the pH value when PCP is dissolved in water); 8,3 (all substance is in ionic form). The measured adsorption isotherms are depicted in Fig. 19. It follows that the best adsorption parameters are obtained at pH = 2,2, when all PCP exists in water in non-ionic form.

The adsorption isotherms of PCP onto different polysorbs are given at Fig. 20. One can see that as the divinylbenzene content increased in the copolymer samples polysorb 40/100 and polysorb 60/100, the amount of substance sorbed also increased. Results obtained indicated that polysorb 60/100 is an effective sorbent for PCP extraction from water solution.

We also studied the regeneration process of polysorb 60/100, which had been saturated with PCP. NaOH solution was used as regeneration agent. The sorption isotherms PCP onto polysorb 60/100 after first, second and third circles of adsorption-desorption process are

shown in Fig. 21. These results indicate good regeneration properties of saturated polymeric sorbents in the conditions chosen.

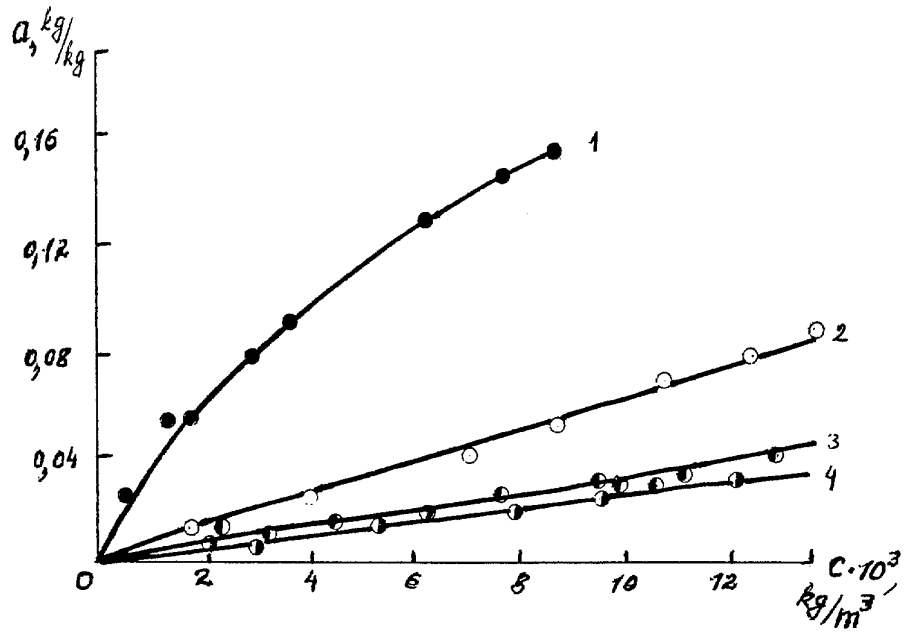

Fig. 19. Adsorption isotherms of PCP onto polysorb 60/100 at the different pH-values. **1**- pH = 2.2; **2**- pH = 5.7; **3**- pH = 8.3; **4**- from NaOH solution with 20 kg/m^3 concentration.

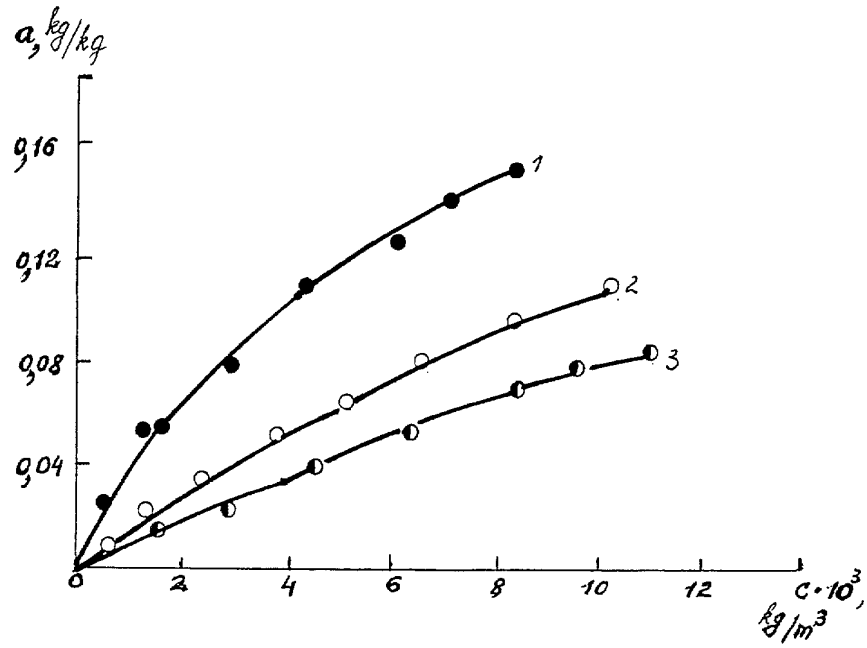

Fig. 20. Adsorption isotherms of PCP onto polysorb 60/100 (1), polysorb 40/100 (2), porolas VP-3 (3).

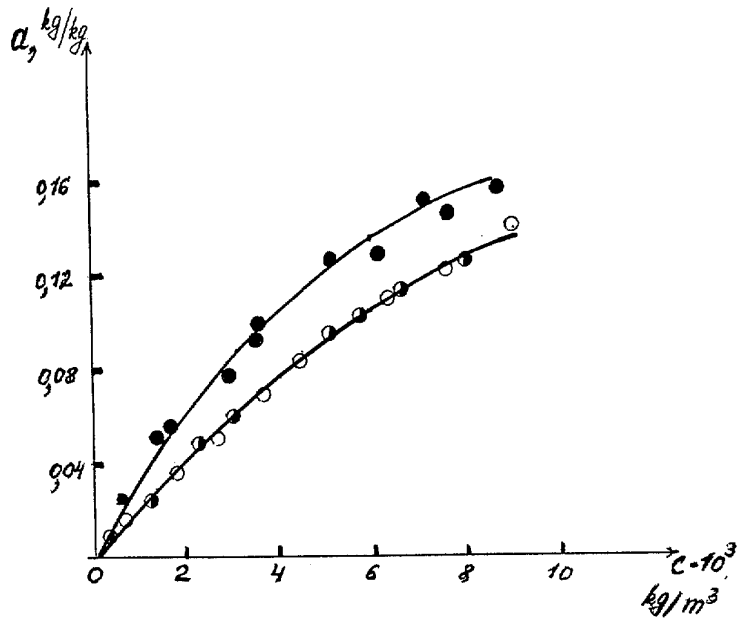

Fig. 21. Adsorption isotherms of PCP onto fresh polysorb 60/100 (•) and polysorb 60/100 after regeneration process (o) and (o).

According to the main goal of the work we studied the batch degradation process of PCP adsorbed by polysorb 60/100, but we did not carry out a degradation process of adsorbed PCP with biological cultures.

4b. Adsorption process on nitro- and amino-containing organic compounds onto porous polymeric sorbents

Different porous polymeric sorbents were characterized for their ability to adsorb nitro- and amino-contained organic compounds from water solutions. The adsorption experiment was carried out as described earlier. The following substances were used as adsorbates: aniline, *m*-aminophenol, *m*-nitrophenol, *o*-aminobenzolic acid and *m*-nitrobenzolic acid. The following synthesized polymer sorbents were employed: porolas T and polysorb R-60. Porolas T-is a copolymer of styrene and divinylbenzene (50 wt-%). Polysorb R-60 is a polar copolymer of ethylene dimethacrylate (40 wt-%) and glycidylmethacrylate.

The calculation of the sorption equilibrium for polymeric sorbents was based on the modified polymolecular adsorption equation, formulated for sorption from aqueous solution. It has the general form:

$$a = \frac{a_m k\left(\frac{C}{C_s}\right)}{1 + k\left(\frac{C}{C_s}\right)\left[1 - k\left(\frac{C}{C_s}\right)\right]^n}$$

where:

a - is the magnitude of the adsorption at concentration C;

a_m - is the magnitude of the adsorption of the monolayer;

k - is a constant in the equation for the polymolecular adsorption;

n - is the value of the exponent,

C - is the concentration of adsorbate in water;

C_s - is the solubility of the sorbed substance in water, found from the literature

The results obtained for the sorption of a number nitro- and amino-contained compounds are given in Table 6.

Table 6. Calculated parameters for the adsorption process of some organic compounds onto porous polymeric sorbents.

Substance	a_m, mmol/g	K	n
Adsorbent - porolas T			
Aniline	2,08	5,80	0,31
o-Aminobenzolic acid	1,37	15,95	0,10
m-Nitrobenzolic acid	2,28	7,63	0,61
m-Nitrophenol	2,02	48,40	0,69
m-Aminophenol	1,50	60,20	0,46
Adsorbent-polysorb R-60			
Aniline	1,60	23,00	0,20
o-Aminobenzolic acid	0,74	9,17	0,25
m-Aminophenol	1,20	10,20	0,30

Analysis of the experimental data leads to the general conclusion that polymeric sorbents are effective materials for the extraction of organic compounds from water solution.

The regeneration properties of polysorb 60/100, which had been saturated with aniline, were studied. The regeneration process was realized in dynamic conditions by filtering regenerating solution through the saturated adsorbent. 2-Propanol was used as regeneration agent. The results obtained are given in Table 7.

Table 7. Results of the polysorb 60/100 regeneration process.

Number of circle	Quantity of adsorbed aniline	Quantity of desorbed aniline	% regeneration
1	14,2	13,0	91
2	14,2	13,0	91
3	14,2	12,5	88

According to the obtained data polysorb was chosen for the next experiments, which deal with studying adsorption-biodegradation process for aniline by *P. putida*.

4c. Transformation of pentachlorophenol by enrichment culture

Halogenated products of metabolism which were found in the cells of animals, microorganisms (such as microscopic fungi and actinomycetes) and seaweed, made it possible to suppose synthesized halogenated-organic compounds can be degraded by microbes. The structure of these compounds differs very much from the compounds found in living organisms. Therefore, the biodegradation of these compounds by microorganisms, especially of halogenated-substituted aromatic, poly- and heterocyclic substances, is difficult.

Pentachlorphenol is one of the most toxic and mutagenic halogenated organic substances which are recalcitrant to the degradation by microorganisms and capable of accumulating in the organisms of men and animals. PCP is a compound widely used as herbicide, algaecide and disinfectant.

The characteristics of pure and mixed microbial populations able to degrade PCP have been extensively reported. These works describe the metabolism of PCP degradation *in vitro* and *in vivo*, as well as the role of plasmids and genetically designed strains. Most investigations on the PCP degradation by microbes are carried out by pure or mixed cultures of microorganisms. Therefore, the course of PCP degradation process in natural systems can be predicted with difficulty. For example, the presence of additional contaminants in wastewater which require the adaptation of microorganisms for their degradation. Since halogenated organic compounds are synthesized for special purposes and introduced into biosphere in a large quantity, such as pesticides, the possibility for designing new technologies for water purification should be considered.

At present, the question about practical utilization of pure cultures, mixed microbial populations or constructed strains for environmental cleaning remains open. Since waste waters contain many components, including PCP, the biotechnology of wastewater purification from halogenated-organic compounds must be worked out and carried out by bacterial associations, that is a microbial consortium having not only a high degradation activity, but also displaying synergistic interactions.

It is known that biodegradation of halogenated-organic compounds by natural microflora occurs seldom and at a very low rate. Therefore the technique of enrichment culture was used to associate the microorganisms which can destroy PCP. The samples of soil (100 g) and activated sludge (50 ml) were incubated in 250 ml bottles containing mineral medium supplemented with 0.01 and 0.2 g/l of PCP (Fig. 22). The total volume of all components was 200 ml. PCP degradation was not marked during the first 16 days of growth in those flasks in which oxygen was removed from the medium and head space. After 18 days of cultivation the content of PCP decreased and after 25 days no remaining PCP was detectable.

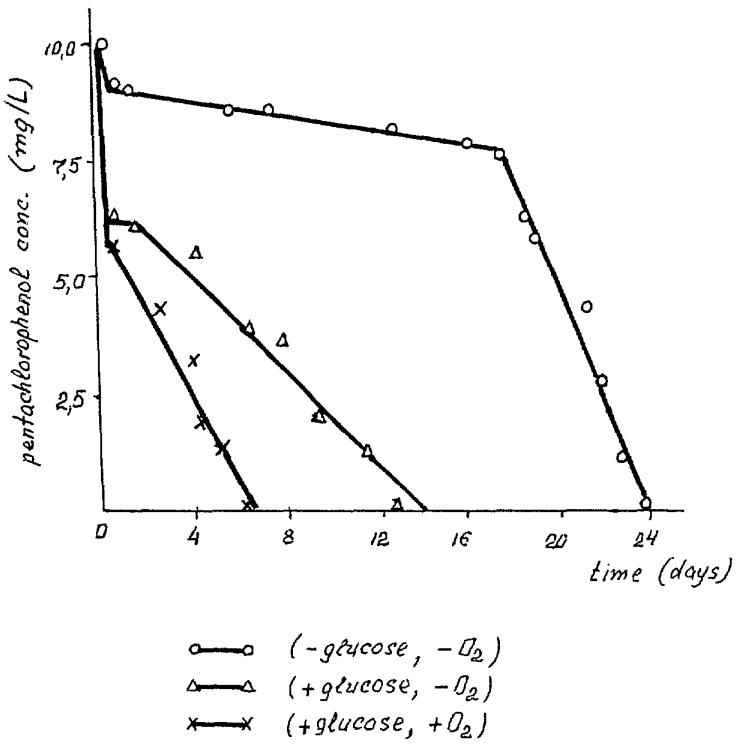

Fig. 22. Biodegradation PCP by the mixed bacterial association.

The transformation of PCP was considerably more intensive in the presence of glucose. Thus, PCP degradation at a initial concentration of 10 mg/l, was completed after 13 days of cultivation. In this case a significant increase of biomass was observed and pH of medium decreased to 6.5. The presence of oxygen in the medium and in head space made PCP degradation more intensive and after six days of cultivation no PCP was detectable. When the bacteria were separated from the medium and washed with a physiological solution, centrifuged and then reused for second experiment, the rate of PCP degradation increased and the lag-period decreased. Thus, in the absence glucose and oxygen, undetectable levels of PCP at a initial concentration of 10 mg/l were achieved after 18-20 days. When glucose was present and oxygen absent, complete PCP transformation (*i.e.* undetectable levels) was achieved after 8-10 days whereas in the presence of both glucose and oxygen complete PCP disappearance was reached after 4-5 days.

Microbiological plate analysis of the bacterial association able to degrade PCP under microaerophilic conditions, showed that about 39% of the colonies counted were *Pseudomonas* species, around 20% *Arthrobacter* species and around 20% *Enterobacteriaceae* species. The remaining 13% contained 11 morphologically different types of colonies. When tested for their contribution to PCP degradation, it was found that the number of colonies of each bacterial group did not corresponded to the extent of PCP transformation found after 48 hours incubation (Table 8). Thus, the number of colonies of *Enterobacteriaceae* species was

practically half of that of *Pseudomonas* species, however the extent of PCP degradation by this two taxonomic groups was practically the same (41% and 59%, respectively). The mixed bacterial culture was also found to have a large number of *Arthrobacter* species, notwithstanding the extent of PCP transformation displayed by them was low.

The extent and rate of PCP degradation by the bacterial association depended on the growth phase at which the bacterial population was and on the concentration of bacteria, measured as optical density of the bacterial suspension. The time needed for complete PCP degradation became shorter practically by a factor of two when it was added during the log phase. When PCP was added to a bacterial culture with density of 10^6, 10^5, and 10^4 cells/ml, 90% degradation was achieved in 42, 70 and 180 hours, respectively.

Among bacteria constituting the remaining 13% of the microflora there were microorganisms which were stable in the presence of PCP and preserved viability during several sub-culturing stages. Nevertheless, these culture displayed almost no ability for PCP degradation.

Table 8. Characteristic of the microbial association transforming PCP (10 mg/l) in microaerophilic conditions (after the sixth transfer).

Microorganisms, kind			Utilization of PCP during 48 h (%)
Pseudomonas	Arthrobacter	Enterobacteriaceae	
+	+	+	98
+	-	-	59
-	+	-	13
-	-	+	41

The study showed that the sub-culturing of the association from a medium with an easily metabolized carbon source (*e.g.* glucose) to a medium with PCP as the only source of carbon and energy led to the damage of cells and to the lysis of bacteria. The presence of carbon compounds, which can be easily metabolized and support biomass growth with ability to degrade PCP, may be significant under real conditions of wastewater purification, since it is difficult to imagine wastewater containing only PCP.

Thus, the investigations showed the possibility of co-metabolism of 10 mg/l PCP by bacterial association in microaerophilic conditions in the absence oxygen. The transformation of PCP as the only source of carbon can occur under conditions of co-oxidation of organic compounds released from dead cells. The appearance of Cl^- ions in this medium is an evidence of PCP degradation, but because of the absence of necessary equipment it was impossible to detect the intermediate products of PCP degradation.

5. MATHEMATICAL MODEL OF BIOSORPTION

5a. Theoretical model and experimental data for biosorption in a fixed bed batch reactor

Biosorption tests in non-equilibrium conditions were carried out with bacterial isolates specialized on the degradation *p*-aminophenol (*Pseudomonas sp*) and aniline (*P. putida*). Suspensions of the bacterial isolates were pre-adsorbed on the polymer adsorbent polysorb Y-59. A weighed amount of polysorb Y-59 (0,5 g or 1,0 raw material) was added to 50 ml of cell suspension of known optical density, and mixed for 30 min. Following the period of contact with the carrier, the residual optical density was measured, and the attached bacterial mass was calculated by difference, from a calibration curve (bacterial density *vs.* bacterial mass). Experimental data showed that in this case of *Pseudomonas sp* the attached bacterial mass was 4,97 ± 0,08 mg cells/g carrier and 1,21 ± 0.05 mg cells/g carrier for *P. putida.*

The experimental set-up, consisting of a down flow fixed bed reactor is described in Fig. 23. The feeding tank was saturated with air.

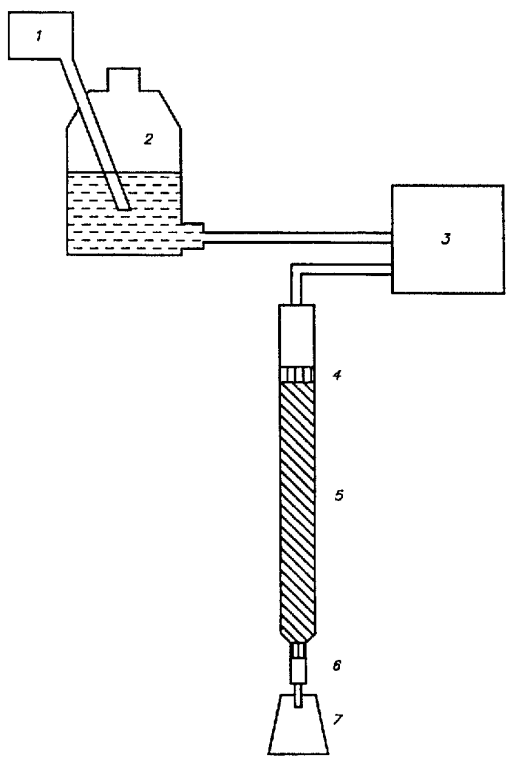

Fig. 23. Experimental set-up: 1. Air pump, 2. Feeding reservoir, 3. Feeding pump, 4. Nozzle to prevent adsorbent loss, 5. Column reactor, 6. Fiberglass outlet, 7. Receiving vessel.

The initial system of equations contains a number of parameters which characterize a biological process [Ying and Weber, 1979; Hutchinson *et al.*, 1990], namely:

- the specific value of biomass growth rate, u;

- the constant of Monod equation, K_s;
- the constant of biomass decay, K_e;
- the substrate yield coefficient, Y.

We worked out the methodology for determination of these values by the combined utilization of theoretical and experimental investigation methods. This methodology is based on minimizing the deviations of experimental kinetic curves from theoretically calculated ones. The curves of biodegradation kinetics were determined in the following manner. A polymer sorbent was saturated by an organic substance up to equilibrium. Then, this sorbent was separated from the solution and subjected to biodegradation during different periods of time. The remained adsorbate was desorbed with an organic solvent miscible with water. The concentration of desorbed adsorbate in the eluent was determined by spectrophotometry and then the time dependence of substance quantity desorbed from the sorbent was plotted. This desorbed quantity was expressed as a percentage of the adsorbed substance. This kinetic process is described by the following equations:

$$\frac{\partial x_s}{\partial t} = \frac{uC_s}{K_s + C_s} x_s - K_e x_s \tag{1}$$

$$\rho_k \frac{da}{dt} + \frac{1}{Y} \frac{uC_s}{K_s + C_s} x_s = 0 \tag{2}$$

$$C_s = \varphi(a) \tag{3}$$

$$x_s(o) = x_{so}, a(o) = a_o \tag{4}$$

In these equations there are also the following parameters:

x_s (t) - the bacterial density on the sorbent surface;

a(t) - the adsorption constant relative to the adsorbent particle;

C_s - the concentration of an organic substance on the surface of sorbent particle;

x_{so} - the initial bacterial density on the surface of sorbent particles;

a_o - the value of preliminary saturation of the sorbent.

In the above system of equations, (1) is the equation of biomass growth rate; (2) is the material balance in a solid phase; (3) is the ratio modeling the adsorption equilibrium.

Thus, the problem is the correlation of a kinetic curve, described by the equations (1) - (4), with an experimental curve. Two parameters, which characterize the biological process, can be determined as a first approximation by means of the growth profiles of the unattached bacteria. These two parameters are the specific rate of biomass growth, u, and the substrate yield coeficient, Y. These values are calculated as the slope of the exponential section of bacterial growth phase, by the set of equations:

$$u = \frac{\lg x_2 - \lg x_1}{\lg e(t_2 - t_1)}$$
$$Y = \frac{x}{C_0 - C_k} \tag{5}$$

were: x_2 and x_1 are the density of cells (i.e. the dry mass of cells) and C_o and C_k at time t_2 and t_1, respectively.

The substrate yield coefficient, Y, shows the dependence of cell harvest with the concentration of substrate consumed. This constant is determined with the help of equilibrium data on the biodegradation of substrate in the solution. When knowing u and Y values, the problem can be solved by calculating K_s and K_e, which depend on the course of the combined process of biodegradation in the system sorbent - adsorbed organic substance - bacterium. This problem can be solved by the minimization method of disclosure functional between the kinetic curves of adsorbed substance biodegraded.

To calculate the kinetic curve of biodegradation, the equations (1) - (4) must be expressed as:

$$\begin{cases} \frac{da}{dt} = -\frac{1}{\rho_k Y} \frac{uC_s}{K_s + C_s} x_s \\ \frac{dx_s}{dt} = \left(\frac{uC_s}{K_s + C_s} - K_e \right) x_s \\ C_s = \varphi(a) \\ x_s(o) = x_{so}, a(o) = a_o \end{cases} \tag{6}$$

The approximation of (6) equations is carried out by the explicit Eiler scheme:

$$\begin{cases} a_{i+1} = a_i - \frac{\tau}{\rho_k Y} \frac{uC_{s,i}}{K_s + C_{s,i}} x_{s,i} \\ x_{s,i+1} = x_{s,i} - \tau \left(\frac{uC_{s,i}}{K_s + C_{s,i}} - K_e \right) x_{s,i} \\ C_{s,i+1} = \varphi(a_{i+1}) \end{cases} \tag{7}$$

were:

τ - is a step according to the time variable;

i - is a number of temporary layer.

To determine the optimal values of K_s an K_e, the minimization of disclosure functional is carried out on the basis of experimental kinetic curves:

$$f(K_s, K_e) = \sum_{i=1}^{N} \left[a(t_i) - a_{\exp}(t_i) \right]^2 \quad (8)$$

where:

$a(t_i)$ - are theoretically calculated values of adsorption in t_i nodes (i=1,2,...);

$a_{exp}(t_i)$ - are the experimental values of adsorption;

N - is the number of experimental points.

The algorithm for determination of K_s and K_e parameters comes from the two-layer minimization of disclosure functional by the method of "golden section". For that, an initial value of K_e parameter is fixed and the one-parametrical minimization in equation (8), according to K_s parameter, was carried out by the method of "golden section". Then, according to the rule of interval formation in this method, a new value of K_e parameter is introduced and the functional minimum is calculated for K_s. The iterative process finishes when the minimum of the function is found with a prescribed accuracy.

Thus, the minimum of disclosure functional is in a two - layer iterative process (i.e. the inner layer for K_s, the outer layer for K_e and on the each layer the algorithm of "golden section" method is used.

This methodology was applied in the biodegradation of aniline by *P. putida* cells with aniline being adsorbed on a polymeric sorbent, polysorb 40/100 and for *p*-aminophenol by *Pseudomonas spp.* on polysorb Y-59. The isotherms of aniline and *p*-aminophenol adsorption on the polymeric sorbents from water solution are shown in Fig. 24 and 25, respectively. The approximation of experimental data by Freundlich equation $a=kC^n$, when [a] = mg/g and [C] = mg/l, are given in the Table 9.

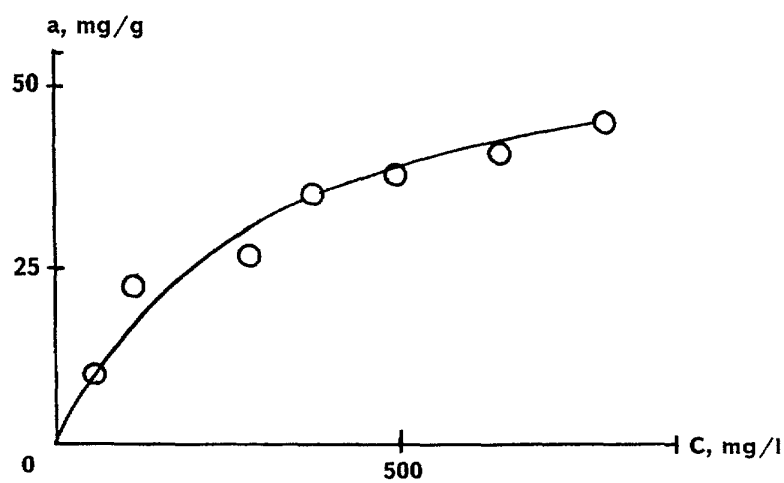

Fig. 24. Isotherm of aniline for polisorb 40/100. Solid line calculated from Freundlich equation. Circles represent empirical data.

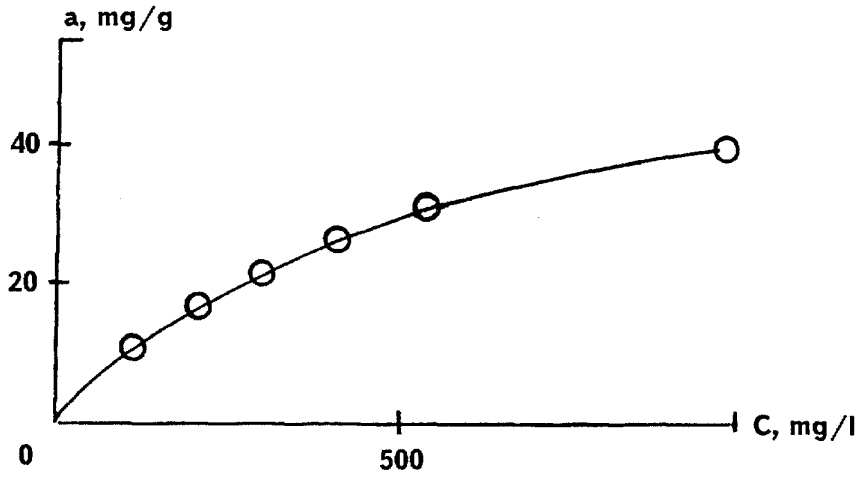

Fig. 25. Isotherm of *p*-aminophenol for polisorb 40/100. Solid line calculated from Freundlich equation. Circles represent empirical data.

Table 9. Parameters of the Freundlich adsorption isotherm, providing [a]=mg/g, [C]= mg/l

System	k	n
Aniline-polysorb 40/100	3,57	0,39
p-aminophenol-Y-59	0,6	0,63

The specific growth rate (u) and substrate yield (Y) for the two cases studied are given in Table 10. These values were calculated from the data presented in Fig. 26 for *P. putida* during aniline degradation, and Fig. 27 for *Pseudomonas sp. d*uring *p*-aminophenol degradation, and applying equations 5. These values were determined on the basis of experimental data in the range of initial concentrations of either pollutants in the range of 300-1000 mg/l.

Table 10. Calculation of specific growth rate (u) and substrate yield (Y).

System	u, 1/h	Y (mg of biomass /mg)
Aniline; *P. putida*	0,25	0,60
p-Aminophenol; *Pseudomonas sp.*	0,30	0,70

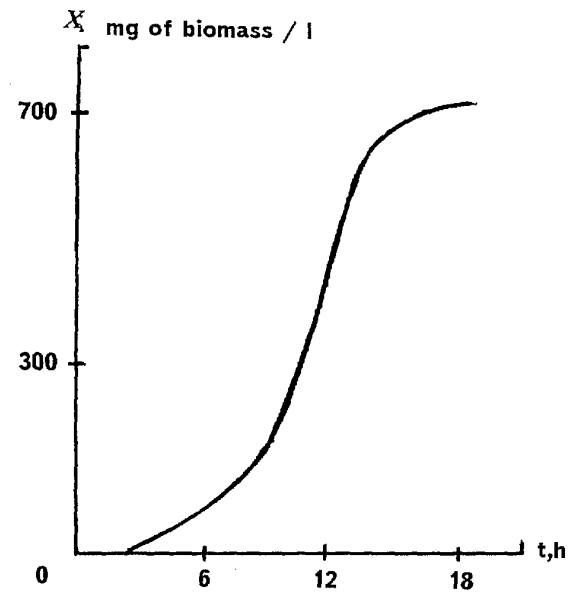

Fig. 26. Growth kinetics for *P. putida* on an aqueous solution of aniline as substrate.

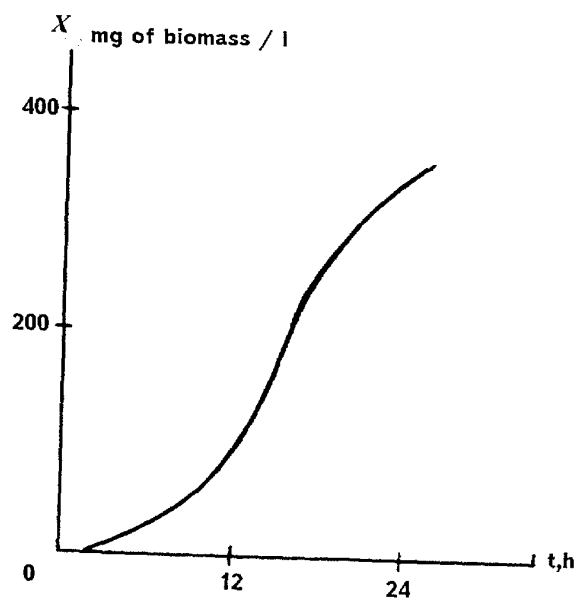

Fig. 27. Growth kinetics of *Pseudomonas sp.* on an aqueous solution of *p*-aminophenol as substrate.

On the basis of the data of the Tables 9&10, the Monod constant, K_s and the biomass decay constant, K_e, were calculated, as given in Table 11. Thus, for example the mean value of biological power constant, Y, in the system aniline solution - *P. putida* is 0.60 mg of biomass/mg. When the values of u, Y, k, n were given and the initial parameters of the

experiment were a_0 = 20 mg/g, ρ_k = 440 g/l, the values of K_s and K_e were determined by the proposed methodology for the system aniline - *P. putida* - polysorb 40/100. The determined values are: K_s = 54.5 mg/l , K_e = 0.00004 1/h.

Table 11. Calculation of specific growth rate (u) and substrate yield (Y).

System	a_o, mg/g	K_s, mg/l	K_e, 1/h
Aniline - *P.putida*-polysorb 40/100	20	54,5	0,00004
P-aminophenol-*Pseudomonas sp.*-Y-59	32,7	292,0	0,00003

The kinetic curves were calculated for the determined values of K_s and K_e so as for other above parameters (Figs. 28 & 29). This Figs. shows a good agreement of calculated and experimental data, what indicates the correctness of the method developed. In conclusion, it must be pointed out that the proposed method can be applied to any complex system consisting of a organic substance - sorbent - microorganism.

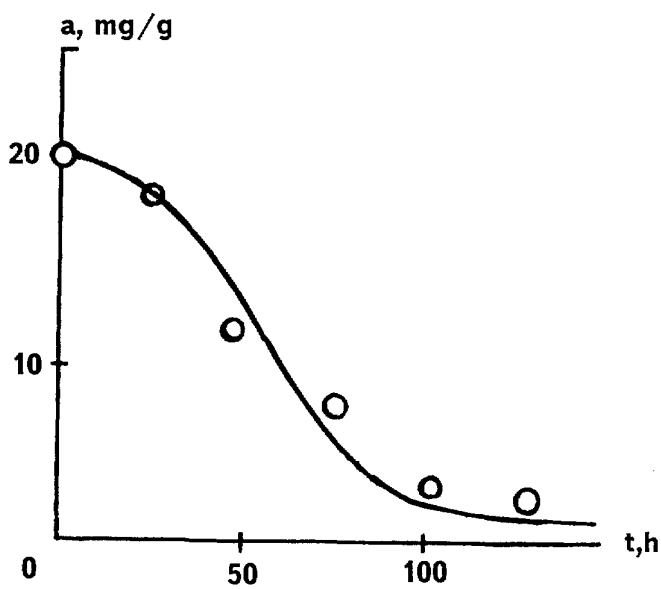

Fig. 28. Experimental and predicted curves of kinetic processes for biodegradation of adsorbed aniline by *P. putida*. Full line is calculated with model, o - experimental data.

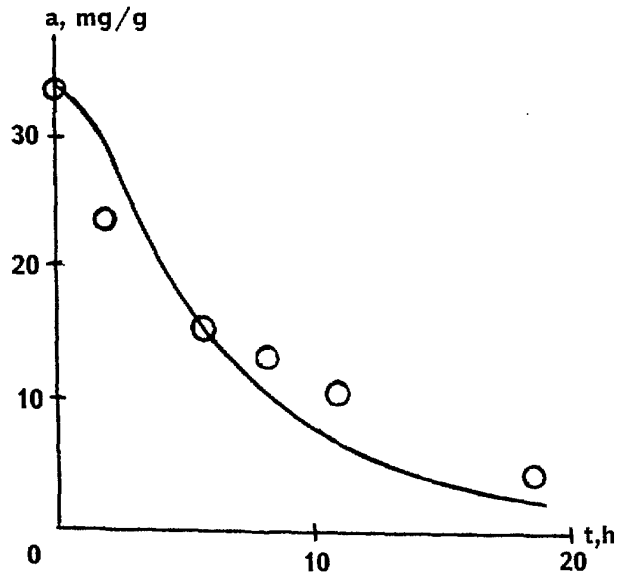

Fig. 29. Experimental and predicted curves of kinetic processes for biodegradation of adsorbed *p*-aminophenol by *Pseudomonas sp.* Full line is calculated with model, o - experimental data.

5b. Theoretical model and experimental data for biosorption in a fixed bed continuous reactor

Our mathematical model of a biosorption process (adsorption & biodegradation) on activated carbon or porous polymer sorbents is a generalization of the adsorption equations in the fixed bed which takes into account a biochemical process. The initial system of the equations involve the equations of mass balance in a liquid phase, the equation of the mass balance in a solid phase, a relationship described in the sorption isotherm equation, the equation of biomass growth rate and the corresponding initial and boundary conditions. We shall discuss these relationships more in detail.

The equations of the mass balance in a liquid phase could be written taking into account no a free longitudinal transfer, which is quite common for mass transfer in the liquid-solid system, and could be written as :

$$V\frac{\partial c}{\partial x} + \beta(C - C_s) = 0 \qquad (8)$$

where:

V- is the average flow rate calculated for the full column section (axial velocity),

C (x,t) - is the substance concentration in the liquid phase;

C_s(x,t)- is the concentration of an organic substance at the liquid-sorbent interface;

β - is the overall mass transfer coefficient.

The equations of the material balance in solid phase is correlated with a local rate of biomass growth on a sorbent particle surface, and could be written in the form:

$$\frac{\partial a}{\partial t} = \frac{eb}{r_H}(C - C_s) - \frac{1}{Y}\tilde{U}\, x_s = \frac{eb}{r_H}(C - C_s) - \frac{1}{Y}\frac{uC_s}{K_s + C_s}x_s \tag{9}$$

where

a(x,t)- the adsorption constant;

ε - the the void fraction:

$_H$ - the sorbent bulk density;

Y - the substrate yield coefficient;

x_s(x,t)- the density of bacteria on the sorbent particle surface;

u and K_s - the constants of the Monod equation.

The adsorption isotherm equation, a = f(C_s), links the equilibrium adsorption values with the local concentration of an adsorbed substance at the interface, and can be expressed by the Freundlich adsorption isotherm:

$$a = k_1\ C_s^{k_2} \tag{10}$$

where k_1 and k_2 are the parameters of the Freundlich isotherm.

The relationship representing the biomass growth kinetics is written in the form:

$$\frac{\partial x_s}{\partial t} = \frac{uC_s}{K_s + C_s}x_s - K_e x_s \tag{11}$$

where K_e is the specific biomass decay rate.

The boundary conditions of the problem illustrate that the substance at an initial concentration C_o, is introduced in the column

$$C(0,t) = C_o \tag{12}$$

at the time zero there is no adsorbed substance in the column with a fixed bed layer

$$a(x,0) = 0 \tag{13}$$

and the initial bacterial concentration on the sorbent surface is equal to

$$x_S(x,0) = x_{SO} \tag{14}$$

The system of equations (8)-(14) is the initial system for the differential equations describing the biosorption process with a definite degree of accuracy.

We shall rewrite equations (8)-(11) :

$$\frac{\partial c}{\partial x} = f_1(C,a), \tag{15}$$

$$\frac{\partial a}{\partial t} = f_2(C,a,x_s) \tag{16}$$

$$\frac{\partial x_s}{\partial t} = f_3(C, x_s) \tag{17}$$

$$C_s = f^{-1}(a) \tag{18}$$

where:

$$f_1(C,a) = \frac{\beta}{v}(C - f^{-1}(a)),$$

$$f_2(C,a,x_s) = \frac{\varepsilon\beta}{\rho_H}(C - f^{-1}(a)) + \frac{1}{y}\frac{uf^{-1}(a)}{K_3 + f^{-1}(a)}x_3,$$

$$f_3(a,x_s) = \left[\frac{uf^{-1}(a)}{K_s + f^{-1}(a)} - K_e\right]x_s.$$

For every fixed β it is possible to obtain the C(t) solution of equations (8-14). On the other hand, the kinetic curve can be obtained experimentally in the form of the concentration sequence measured at different times, $C_{\exp}(t_i)$; i = 1, N where t_i is i-th time; N is the number of experimental points. Changing the β parameter in either way it is possible to bring the calculated curve closer to the experimental one. This could be obtained by the function minimization:

$$f(\beta) = \sum_{i=1}^{N}[C(t_i) - C_{\exp}(t_i)]^2$$

The optimum β value is found by the "gold section" method.

The finite-differential scheme used to calculate a sorbent fixed bed layer is expressed by the following set of equations:

$$C_i^k = C_{i-1}^k - h\frac{\beta}{\vartheta}\left(C_{i-1}^k - C_s\left(a_i^{k-1}\right)\right)$$

$$a_i^k = a_{i-1}^k - \tau\frac{\beta\varepsilon}{\varsigma_H}\left(C_{i-1}^k - C_s\left(a_i^{k-1}\right)\right) - \tau\frac{x\left(a_i^{k-1}\right)x^{k-1}}{\mathrm{Y}}$$

$$x_i^k = x_i^{k-1} + \tau\, x_i^{k-1}\left(x_s\left(a_i^{k-1}\right) - K_e\right)$$

$$C_s(a) = \left(\frac{a}{R_1}\right)^{\frac{1}{R_2}}$$

$$x_s(a) = u\frac{C_s(a)}{K_s + C_s(a)}$$

The set of equations allows us to calculate the functions C(x,t), C_s(x,t), a(x,t), x_s (x,t).

A calculation of biosorption in the fixed bed layer of sorbent was done for two systems: (i)

p-aminophenol/*Pseudomonas sp.*/polysorbY-59; (ii) aniline/*P.Putida*/polysorb 40/100. In some cases the data were compared with experimental ones. The calculations were carried out with parameters given in Table 11.

Table 11. Initial data for biosorption in the fixed-bed layer for the systems: (1) *p*-aminophenol/ *Pseudomonas sp.*/polysorbY-59; (2) aniline/*P. putida*/polysorb 40/100.

Parameters	System 1	System2
k_1	0,6	3,57
k_2	0,63	0,39
x_{50} mg of biomass/g	5	1
C_o, mg/l	625	500
ρ , g/l	325	300
ε	0,4	0,4
ν, m/h	4	4
Y, mg of biomass /mg	0,7	0,6
u, 1/h	0,3	0,25
K_s, mg/l	292	54,5
K_e,1/h	0,00003	0,00004
β, 1/h	37,43	50

Calculations for the given systems were done for a short (small heights) fixed bed, and these data were compared with experimental ones (Figs. 29-32). These data fit the model well confirming that the proposed model for the fixed bed layer is adequate and serves as a tool for the calculation of biological constants. As can be seen from the data presented, for short beds the pollutant concentration in the effluent was in all the cases around 100-200 ppm, *e.g.* breakthrough concentration, perhaps due to mass transfer limitation at one pass. Therefore, the limit effluent concentration of our model for short beds (*i.e.* 0.2 m) is around that value.

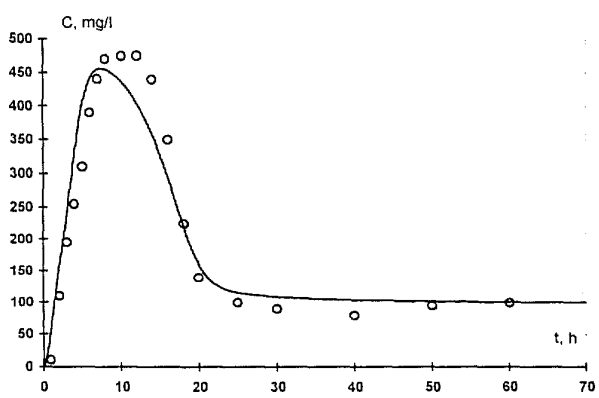

Fig. 29. Breakthrough profile for aniline in a bioactive fixed bed. Full curve was calculated with model, o -experimental data (Lf = 0.22 m; v =5 m/h; Co = 500 mg/l). Lf, bed height; v, linear velocity of the fluid throughout the column; Co, initial pollutant concentration.

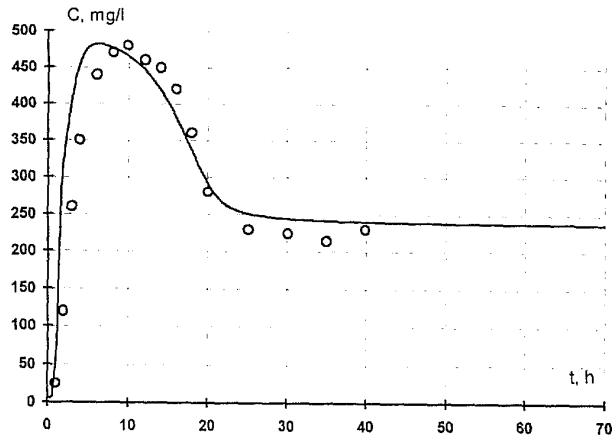

Fig. 30. Breakthrough profile for aniline in a bioactive fixed bed. Full curve was calculated with model, o - experimental data (Lf = 0.10 m; v =5 m/h; Co = 500 mg/l).

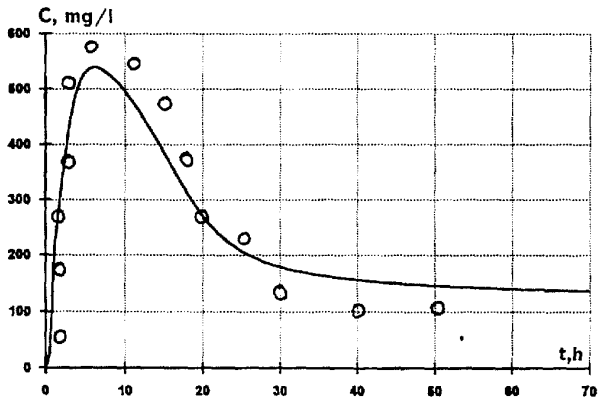

Fig. 31. Breakthrough profile for p-aminophenol in a bioactive fixed bed. Full curve was calculated with model, o - experimental data (Lf = 0.22 m; v =5 m/h; Co = 625 mg/l).

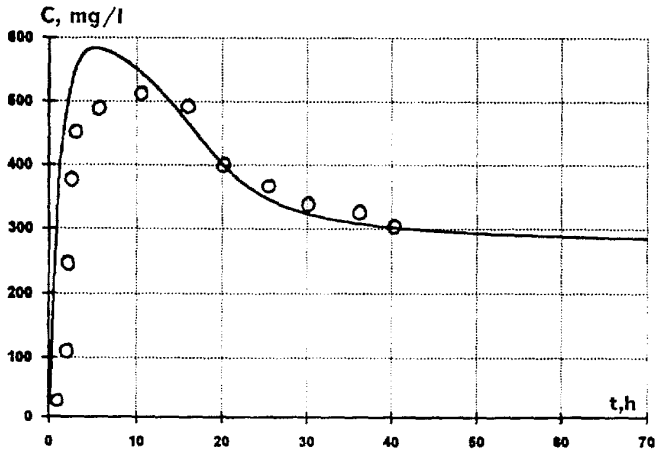

Fig. 32. Breakthrough profile for p-aminophenol in a bioactive fixed bed. Full curve was calculated with model, o - experimental data (Lf = 0.10 m; v =5 m/h; Co = 625 mg/l).

Parameters for the fixed bed layer for tall beds (*i.e.* industrial scale > 1 m) were calculated on the basis of a theoretical model. The data are given in Fig. 33 for system 1. The data show actually a zero concentration of pollutant in the effluent. This behavior confirms the efficacy of biosorption processes and their prospects as an alternative technology for purification of waste water.

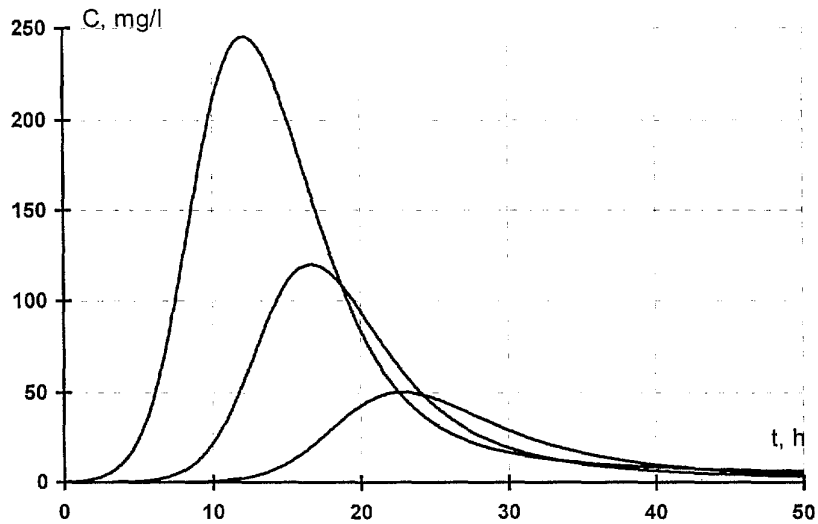

Fig. 33. Breakthrough profile for p-aminophenol in a bioactive fixed bed calculated with model. 1, Lf = 1.0 m; 2, Lf = 1.5 m; 3, Lf = 2.0 m (v =5 m/h; Co = 625 mg/l, x_O = 5 mg/g; ε = 0.4; ρH = 325 g/l, β = 37.4 l/h.

From the point of view of the technology it is necessary to underline the following:

1. Porous polymer materials can be considered as appropriate adsorbents to be used in the biosorption processes, so as the experiments proved that there was no biomass grown in the interstices between the sorbent particles. This was confirmed by no decrease of filtration velocity during long runs (several weeks).
2. The mathematical model developed is a useful theoretical tool to evaluate the parameters that will assure very low concentration of the toxic compounds in the effluent (under a particular system operation).
3. Theoretical calculations and the experimental data show that constant operation conditions can be reached within 50-70 hours of operation.
4. It is crucial under aerobic conditions to maintain an oxygen saturated feeding to ensure enough oxygen all along the height of the fixed bed system.

IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

The results of this project provide fundamental information on the factors affecting the microbial degradation of toxic organic aromatic and polyaromatic pollutants by microbial cells attached to porous supports. A mathematical model has been developed which describes the degradation of aromatic compounds by bacterial microbial biomass attached to carriers with adsorption capacity for the pollutants, which serve as carbon and energy source for the microorganisms. Because of the filamentous form of growth, it was very difficulty to maintain a continuous reactor of fungi, and therefore the modeling studies were performed with bacteria. These results also added new tools to a rational design of processes needed for the disposal of recalcitrant pollutants, and generated new scientific and technological ideas. A specific scientific contribution of the data generated by the project concerns the study of the ability of white-rot fungi (typified by *Phanerochaete chrysosporium*) to attach to natural porous supports, and especially to activated carbon. One clear output of this research is that despite their broad ability to degrade toxic compounds, fungi are less effective than bacteria in degrading pollutants in waste water. However, filamentous fungi are more effective in solid and semi-solid environments.

The scientific collaboration between the teams involved in the program was instrumental in gaining a fundamental understanding on the biosorption of recalcitrant pollutants, *i.e.*, physical adsorption combined with microbial degradation. The joint work improved the skills of the Polish scientist on specific aspects of bioremediation of recalcitrant organic compounds, at the levels of (i) reactor technology and operation; (ii) mathematical modeling and (iii) analytical analyses (Researchers from Institute of Colloids and Water Chemistry of the Ukrainian Academy of Science at Kiev joined in with the efforts of the Polish side). Furthermore, the analytical capabilities of the Polish side, who purchased a GC with funds of the project, were also improved. One major obstacle during the joint work was communication problems, both in personal and electronic contacts, which in part were solved with the aid of translators.

Scientific and technological achievements of the last decade in the field of microbiology and biotechnology allow new biological methods of purification of wastewater contaminated with toxic organic compounds to be applied. A new technology, based on the use of immobilized microorganisms, which gives clean and harmless water for use in closed cycles of water supply or for throw-off into open reservoirs, has economic and ecological advantages. The elaboration and use of this technology, as performed within the framework of the present project, are important, especially for countries of Central and East Europe, because of the need to upgrade the disposal technique of wastewater contaminated with toxic synthetic organic compounds. Even though the research established both the experimental and analytical bases for the application of microbial processes, combined with natural or synthetic

sorbents for the treatment of toxic wastewater, there is still a need for further development before this process can be applied. This includes, inter alia, building a pilot plant under field conditions. The Polish group and its Ukrainian co-partners have now acquired the skills and knowledge needed for the further steps of the project.

PROJECT OUTPUTS

The Israeli group has already managed to produce the following publications/presentations, and at least one more paper is being prepared for publication:

1. Feijoo, G., C.G. Dosoretz, and J.M. Lema. 1995. Production of lignin peroxidase by *Phanerochaete chrysosporium* in a packed bed bioreactor. 6th Intl. Conference on Biotechnology in the Pulp and paper Industry, Vienna, Austria.
2. Abu-Salah, K., G. Shelef, D. Levanon, R. Armon, and C.G. Dosoretz. 1995. Microbial degradation of *p*-nitrophenol and phenanthrene adsorbed on powdered activated carbon. Workshop on Environmental Biotechnology, Technion, Haifa, Israel.
3. Abu-Salah, K., G. Shelef, D. Levanon, R. Armon, and C.G. Dosoretz. 1996. Microbial degradation of aromatic and polyaromatic toxic compounds adsorbed on powdered activated carbon. J. Biotechnol. 51:265-272.

The publications/presentations of the Polish group are in Polish and are therefore unavailable.

PROJECT PRODUCTIVITY

In general terms, all the goals of the proposed research has been accomplished. Nonetheless, because of the filamentous form of growth of white-rot fungi, and the fact that their morphology changes according to growing conditions (*e.g.*, pellet form in submerged agitated growth, linear surface mycelium on solid surfaces) it was very difficulty to maintain a continuous reactor of fungi, and therefore the modeling studies were performed with bacterial and not with fungal reactors.

FUTURE WORK

Two major aspects of the presents project are expected to lead to further scientific work and process implementation. The first is the knowledge acquired on the use of bacterial cells attached to adsorbent carriers, in either fixed-bed or fluidized-bed regime, including the mathematical model developed. The second is the specific ability of white-rot fungi to attach to hydrophobic surfaces in solid or semi-solid environments. This ability, added to the broad ability to degrade toxic compounds displayed by fungi, offers a specific application in which fungi are more effective than bacteria. On the other hand, because of the lower competitiveness of fungi for pollutant degradation in waste water, compared to bacteria, wastewater degradation by continuous systems may in practice be restricted to the use of bacterial cells.

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