Folia Microbiol. **50** (4), 301–308 (2005) **http://www.biomed.cas.cz/mbu/folia/**

Continuous Aerobic Phenol Degradation by Defined Mixed Immobilized Culture in Packed Bed Reactors

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Received 20 December 2004
Revised version 13 April 2005

ABSTRACT. A defined mixed culture of *Pseudomonas putida*, *Commamonas testosteroni* and *Candida tropicalis* was immobilized by adsorption on polyurethane foam, cocoa-fibers, expanded slate and sintered glass. Packed bed reactors were used for long-term continuous phenol biodegradations. Loading experiments were done to study the impact of the following parameters: (*1*) hydraulic retention time, (*2*) dissolved oxygen concentration, and (*3*) elimination of the oxygen limitation. After the acclimation period (≈10 d), the loading test with the individual packings showed the following maximum degradation rates: sintered glass 34, polyurethane foam 12, expanded slate 11.5, and cocoa-fibers 7.7 kg m^{-3} d^{-1}. All these values were reached at a removal efficiency >99 % and with oxygen in excess. Under these conditions, the pH of the diluted unbuffered medium in the reactor effluent was 3.2–4.0 and no incompletely oxidized metabolic intermediates were found. The free cell concentration in the effluent increased after the phenol overloading time period.

Abbreviations

c_{in}	inlet phenol concentration, mg/L	PBR	packed bed reactor
c_{out}	outlet phenol concentration, mg/L	pH_{out}	pH of the medium outlet
DOC	dissolved oxygen concentration, %	PUF	polyurethane foam
FBR	fluidized bed reactor	Q	water flow rate, mL/min
HRT	hydraulic retention time, min	q_S	phenol degradation rate, mg L^{-1} h^{-1}
MM	mineral medium	RE	removal efficiency, %
OL	organic loading, mg L^{-1} h^{-1}	V_L	working volume of reactor, mL

Phenol is an aromatic pollutant, which is present in the wastewaters of numerous industries including oil refining, petrochemical, coal-coking and coal-gasification (Singer *et al.* 1977; Young and Cerniglia 1995). Decades of operation involving the leaking of gaskets, bad handling and technical troubles can contaminate both soil and groundwater with phenolic compounds. In companies where phenol is produced, troubles in the cleaning of waste water also occur. During the cleaning and washing periods of the phenol production section, the concentration in waste waters may exceed the limiting value that can be treated by conventional biological methods using activated sludge (Watanabe *et al.* 1996) without harming their regular function. The problem can be solved using a preliminary cleaning step, which can include, *e.g.*, a specially designed packed bed reactor with immobilized cells used in this study. Similar technology can be for a "pump-up and clean" procedure at any location with highly contaminated soils and ground-waters.

The degradation of phenol can be performed under aerobic (Hinterreger *et al.* 1992; Chitra *et al.* 1995; Oh and Han 1997; Páca *et al.* 2002) or anaerobic conditions (Bechard *et al.* 1990) by pure (Heipieper *et al.* 1991; Rigo and Allegre 2004) or mixed cultures (Ehrhardt and Rehm 1985; Mörsen and Rehm 1987, 1990). Using a mixed culture permits faster phenol degradation than a pure culture (Zache and Rehm 1989) and systems with immobilized cells are more stable to shock loadings than submerged cultures with free cells (Holladay *et al.* 1978).

We tested a continuous long-term procedure of phenol degradation by a defined mixed culture, immobilized by adsorption on various packing materials. Loading tests of bench-scale packed bed reactors

with various hydraulic retention times, dissolved oxygen concentrations and the elimination of oxygen limitation are evaluated and compared. The links between the degradation rate and efficiency, the dissolved oxygen concentration, the pH of the medium effluent and the composition and number of cells in biofilm and free cells in effluent are discussed.

MATERIALS AND METHODS

Microorganisms. Pseudomonas putida S7, *Commamonas testosteroni* Pb50 and *Candida tropicalis* Ct2 (all from the collection of the *Laboratory of Bioengineering, Institute of Chemical Technology*, Prague) were used as a mixed culture for phenol degradations. They were chosen as a defined mixed culture because of their ability to use phenol as the only carbon and energy source. The yeast strain was also able to grow on a mixture of phenol and pyrocatechol. Both the bacterial strains are G^- rods and can utilize (as the only C- and energy source) the following compounds: phenol, pyrocatechol, *o*-, *m*- and *p*-cresols, nitrophenols, nitrotoluenes and some aromatic and aliphatic hydrocarbons (Alexieva *et al.* 2002).

Media and culture conditions. The microbial biomass for immobilization was prepared in 2-stage shaken flask precultures (pH 7.2) at 30 °C. Cultivation *1* lasted 1 d, cultivation *2* in the mineral medium (MM) lasted 3 d. Phenol was added at the beginning of the cultivation (350 mg/L) and then at 6-h intervals (350 mg/L). The medium contained (g/L): K_2HPO_4 4.3, KH_2PO_4 3.4, $(NH_4)_2SO_4$ 2, $MgCl_2 \cdot 6H_2O$ 0.34, and 1 mL trace element solution (in mg/L: $ZnSO_4 \cdot 7H_2O$ 0.3, $MnSO_4 \cdot 7H_2O$ 0.16, $CaSO_4 \cdot ½H_2O$ 0.15, $FeSO_4 \cdot 7H_2O$ 0.1, $CuSO_4 \cdot 5H_2O$ 0.1, $CoSO_4 \cdot 7H_2O$ 0.1, $NaBO_2$ 0.1, $Na_6Mo_2O_7$ 0.1.

Phenol biodegradations in reactors were done with the following diluted MM (g/L): K_2HPO_4 0.3; KNO_3 0.2; $MgCl_2 \cdot 6H_2O$ 0.2, and 1 mL trace element solution (initial pH 7.2).

Reactor, packing, immobilization procedure and performance. The reactor parameters were: internal diameter 50 mm, total volume 614 mL, working volume 196 mL (Fig. 1). The packing materials were polyurethane foam (PUF) with a particle size of 5–10 mm, sintered glass beads 3–5 mm in size (*Schott Eng.*, Germany), expanded slate with particle size 4–8 mm (*VTS Koop Schiefer*, Germany), and cocoa-fibres (an agricultural waste in Brazil). These packing materials were chosen with respect to their possible industrial

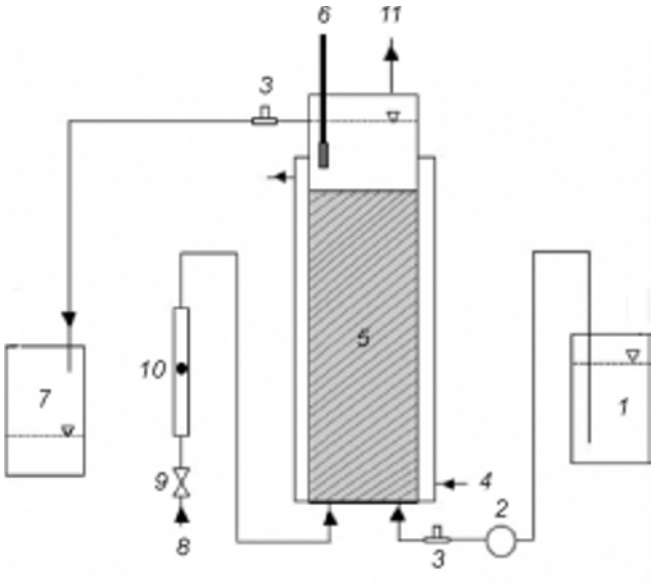

Fig. 1. Experimental setup; *1* – container with phenolic medium, *2* – membrane pump, *3* – sampling ports, *4* – tempering jacket, *5* – packing, *6* – combined electrode for dissolved oxygen concentration and temperature, *7* – container with treated medium, *8* – air inlet, *9* – needle valve, *10* – rotameter, *11* – air outlet.

application (inorganic cheap and expensive materials *vs*. organic ones). The defined mixed culture was immobilized on the packing material by circulating through the reactor the cells suspended in MM without any carbon and energy source for 2 d. The packed bed reactors were operated in a co-current up-flow mode with aqueous phase containing phenol and air. Degradations were done at 30 °C, under nonaseptic conditions to mimic better the actual plant conditions; inlet pH of the medium was 7.2. The following parameters were measured: air flow rate, medium flow rate, inlet and outlet phenol concentrations, pH of the medium outlet (pH_{out}; periodically), dissolved oxygen concentration (DOC) and temperature in the outlet (continuously).

Analytical methods. The concentration of free cells in the reactor outflow was determined by measuring absorbance A_{500} (Spekol 11; *Carl Zeiss Jena*, Germany). Phenol was determined by HPLC with a UV

detector (Spectro monitor 3200 at 270 nm) and column (Nucleosil 12-5 C18, 250 × 4 mm; *Watrex*, Czechia) under isocratic conditions (eluent mixture methanol–water–H_3PO_4, 50 : 49 : 1). The signal was evaluated by Chromatography Station for Windows version 1.7 (CSW). Carboxylic acids, *e.g.*, succinate (as a possible incompletely oxidized intermediate in the medium), were determined by HPLC with a RI detector K-2300 (*Knauer*, Germany) and Polymer IEX H Form 8 μm, 250 × 8 mm (*Watrex*, Czechia) column at 5 MPa and 50 °C (eluent 9 mmol/L sulfuric acid); the signal was evaluated by CSW v.1.7. The dissolved oxygen concentration was determined using a Pt-Ag–AgCl electrode coupled with Oxytest analyzer (*Developmental Workshops of Academy of Sciences of the Czech Republic*). The porosity of the packing materials was determined according to Hopp and Huegele (1996).

Microbial analyses. In order to distinguish between the total cell number, the number of eukaryotic cells, *Pseudomonas*, and primary phenol degraders of the strains present in the biofilm and free cells in the medium effluent, cultivations were done using the following selective solid agar media: Standard Plate count agar, MM agar, Rose-Bengal chloramphenicol agar base CM 549 with the Selective supplement SR 78 and *Pseudomonas* agar base CM 559 with C-N Supplement SR 102 (all *Oxoid*, UK). Individual cell colonies on selective solid media were determined as colony forming units (CFU). Identification of the individual strains in the biofilm or free cells was performed by biochemical tests, Gram-staining and examining the morphology of colonies and free cells. The cells in the biofilm, immobilized on PUF beads, were resuspended in MM by vigorous mixing before any microbial analyses.

Microscopical observation. Samples of PUF containing the biofilm were taken at a depth of 50 mm from the top of the packing for scanning electron microscopy. The samples were critical-point-dried using the following procedure: fixation in 3 % glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.2) for 4 h at 4 °C, dehydration in a graded ethanol series and transfer to pentyl acetate. Critical point drying was carried out at 6 °C using CO_2. Samples were coated with gold and observed (*Hitachi* S 4700).

Calculated parameters (for abbreviations and units *see* p. 301).

Organic loading	$OL = 0.06\, c_{in} \times Q$
Mean hydraulic retention time	$HRT = V_L/Q$
Removal efficiency	$RE = (1 - c_{out}/c_{in}) \times 100$
Degradation rate	$q_s = 0.06\, (Q/V_L) \times (c_{in} - c_{out})$

DOC is a percentage of its saturation concentration in the diluted mineral medium. To fit the plots in Figs 3, 4, 6 and 7 from repeated measurements during 6 months of the reactor operations, the weighted mean of a discrete set of numbers was used (**`http://mathworld.wolfram.com/WeightedMean.html`**).

RESULTS AND DISCUSSION

Acclimation period of PBR with PUF. The inlet phenol concentration during a continuous performance of the PBR with PUF increased with time (Fig. 2). The phenol removal from the medium proceeded

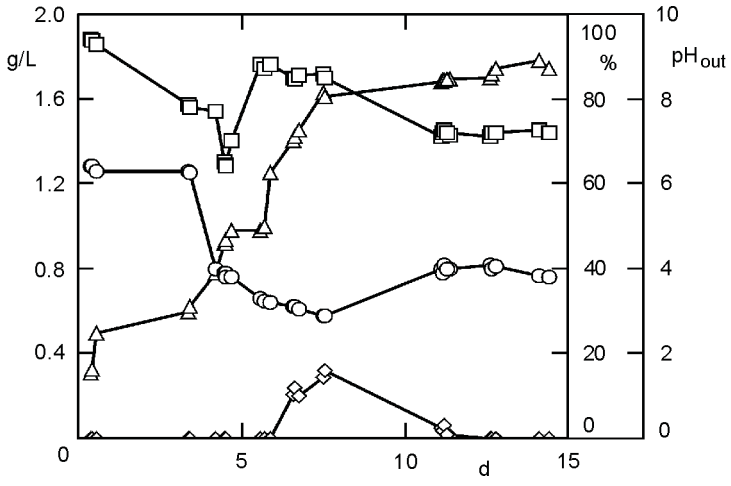

Fig. 2. Acclimation period of the reactor packed with polyurethane foam; HRT = 193 min; *triangles* – c_{in}, *diamonds* – c_{out} (both in g/L); *squares* – DOC (%); *circles* – pH_{out}.

due to two simultaneous phenomena: its adsorption onto the packing and microbial cells, and its biodegradation. While phenol adsorption into PUF particles has not been quantitatively evaluated, its biodegradation

was confirmed by a drop of the dissolved oxygen concentration. After 6 d, when the inlet phenol concentration exceeded 1.2 g/L and increased to 1.65 g/L (on day 7), a transient breakthrough of phenol was observed. However, on day 11 (with c_{in} = 1.7 g/L) steady state conditions were achieved (as confirmed by constant values of DOC, pH_{out} and total phenol removal). Microscopical observation (day 11) showed that the cell colonization of the packing was completed (*not shown here*); with expanded slate and sintered glass beads, the acclimation was reached within 10 d. With a batch circulating culture of *P. putida* 3 weeks were necessary to obtain a thin biofilm on Poraver packing (Sa and Boaventura 2001), our procedure is therefore much more effective. Similarly, Gonzales *et al.* (2001) operated a FBR for 15 d as a batch system before commencing continuous performance.

Effect of HRT on performance characteristics of PBR with PUF. All the experiments in this series were done at a constant oxygen supply rate (at air flow rate of 1.5 L/min). OL increased by increasing the c_{in}. Using HRT 193, 129 and 97 min and to be sure that the system reached the steady state conditions, a time interval of 36 to 48 h was used before starting the measurements under conditions of a next OL (Fig. 3). Regardless of the value of HRT the maximum removal rate was obtained up to an OL of 500 mg L^{-1} h^{-1}. The cluster of both the q_S and RE values at HRT of 129 min (the lower part of them) is a consequence of the channeling effect due to a thick biofilm layer formation after 165 d of continuous operation (visually observed when the reactor was repeatedly operated under the same conditions).

Fig. 3 also shows changes in DOC and pH_{out} during the loading tests. Under conditions of high c_{in} values and a HRT of 193 min, phenol degradation in the reactor at OL > 300 mg L^{-1} h^{-1} proceeded under oxygen limitation; under these conditions pH_{out} dropped to 3.2. Similar pH_{out} values were at HRT of 129 min at OL ≈ 600 mg L^{-1} h^{-1} when the DOC dropped to <20 %, which can also be considered as partial oxygen limitation. Nevertheless, under conditions of both the low c_{in} and HRT, when the DOC values show a large excess of oxygen in the medium outflow, pH_{out} values decreased to the same value of 3.5 as under oxygen limitation conditions. Since the diluted mineral medium or tap water had no buffering capacity, the drop of pH_{out} resulted from a combined effect of cell-membrane transport and ion exhaustion from the mineral medium. HPLC analyses with a refractometry detector did not show any carboxylic acid inherent to the phenol metabolism. A similar decrease in pH_{out} was reported by Mörsen and Rehm (1990). In a continuously operated PBR packed with sintered glass pH_{out} decreased to 4.3 while in batch operation it dropped to 2.5 and DOC almost to zero. In addition, the clusters of both the DOC and pH_{out} values below OL = 600 mg L^{-1} h^{-1} at HRT of 129 min point to a close link between the biodegradation activity and these parameters.

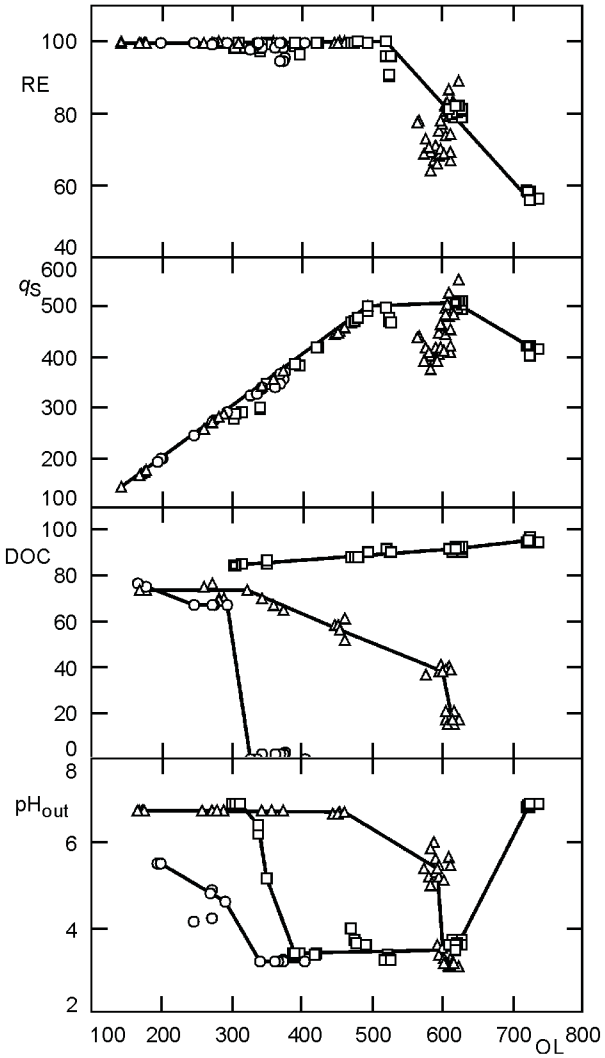

Fig. 3. Effect of hydraulic retention time (HRT; *circles* – 193 min; *triangles* – 129 min; *squares* – 97 min) on RE (%), q_S (mg L^{-1} h^{-1}), DOC (%) and pH_{out} at OL 100–800 mg L^{-1} h^{-1}.

Effect of DOC at different HRT in PBR with PUF. Under steady state conditions the total RE was achieved at both HRT values in excess of oxygen (Fig. 4). The high metabolic activity of immobilized cells under these conditions resulted in a drop of pH_{out} regardless of the HRT value. pH_{out} of 3.2 corresponded with the results obtained at different loading rates (Fig. 3).

A decrease of DOC was set by decreasing the air flow rate to 1 L/min. When the DOC value dropped to <30 %, the degradation rate of phenol also decreased (to 80 %). Further oxygen supply-rate decrease (at air flow rate of 0.5 L/min) was accompanied by the decrease of both the RE and pH. HPLC analyses of the medium samples taken at DOC of zero did not show again any incompletely oxidized intermediate. These findings are important for reactor control and a phenol removal technology.

Cells from PBR with PUF. Two different forms of cells were present in each PBR: immobilized cells on the packing and free cells in the medium effluent that were released from the biofilm; both cell forms participate on the overall degradation capacity. The quantity of free cells (determined every day during the degradation) varied according to the OL rate and HRT (Table I). The total cell number in the biofilm in our experiments was nearly the same as that of Zache and Rehm (1989) who also degraded phenol by an immobilized mixed culture.

A majority of microbial population in the biofilm are the degraders, *Pseudomonas* cells being predominant. This finding coincides with the results of Arquiaga (1995) and Okaygun *et. al.* (1992) who found that the concentration of fungal cells was much lower than that of bacteria.

The concentration of free cells differed when determined under various conditions. The increase in the free cell concentration during the overloading period started after 5 d, culminating on day 7 (Table I). The cells that appeared in the effluent were both individuals and aggregates (making the absorbance measurements difficult). After elimination of phenol overload (by drop in c_{in}) we observed a decrease in free cell concentration in the effluent.

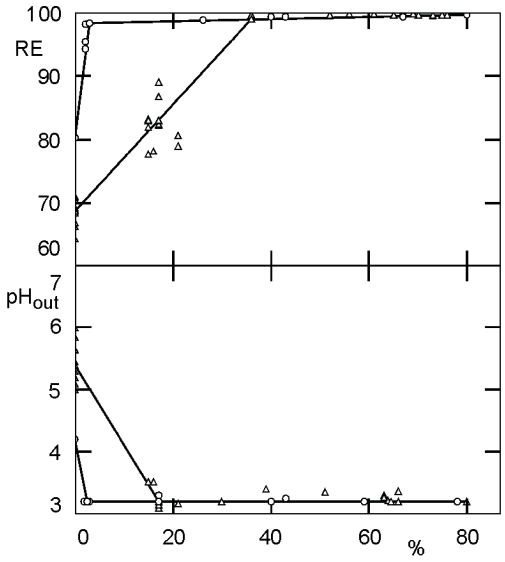

Fig. 4. Effect of dissolved oxygen concentration (%) on RE (%) and pH_{out}; HRT: *circles* – 193 min at OL = 350 mg L^{-1} h^{-1}; *triangles* – 129 min at OL = 450 mg L^{-1} h^{-1}.

The following procedure was suggested as a solution to the operational problem linked with increasing biofilm thickness: (*1*) eliminating nutrition from the influent (if possible) when the biofilm completely covers the packing, then the channeling effect accompanied by the formation of oxygen-limited zones in PBR can occur later; (*2*) controlling the nutrient supply; the biofilm is able to maintain its biodegradation activity by switching to cryptic growth and nitrate respiration of bacteria in anoxic zones and the PBR can operate well for 6 months (in contrast to PBR clogging after a mere 2 weeks; Prieto *et al.* 2002); (*3*) a washing procedure followed by an addition of NO_3^- ions into tap water is recommended to decrease the quantity of biomass in the reactor bed.

Table I. Microbial populations after 7 d of phenol overloading (day 67) and after 14 d of total phenol removal (day 143)[a]

Cell group	Biofilm[b] × 10^{-3}, day 143	Free cells in effluent[c]	
		day 67	day 143
Total cell number	240 ± 13.0	5000 ± 200	80.0 ± 3.6
Pseudomonas	98.0 ± 5.0	4600 ± 350	58.0 ± 2.2
Degraders	96.0 ± 6.1	4200 ± 86	45.0 ± 3.2
Eukaryots	0.19 ± 0.05	14.0 ± 4.8	2.4 ± 0.2

[a]HRT = 193 min, oxygen in excess in both cases; means of data from repeated 6-month reactor operations ± SD.
[b]CFU per mg dry packing. [c]CFU/mL.

In a pure culture or an artificially constructed mixed microbial culture (our case) in long-term continuous phenol degradations under nonaseptic conditions, the proportion of strains in the biofilm changed. All our strains were primary phenol degraders; nevertheless, during a long-term continuous nonaseptic degradation a large quantity of air-borne microorganisms enters the reactor in air used for oxygen supply. The result-

Table II. Identification and growth ability of microorganisms in biofilm after 4 months

Microorganism	Growth ability[a]
Gram-negative bacteria	
Commamonas testosteroni	++
Pseudomonas putida	+++
Sphingomonas sp.	+++
Fungi	
Aspergillus oryzae	+
Penicillium sp.	+

[a](+++) – high, (++) – medium, (+) – weak.

ing microbial composition of both the biofilm and free cells then depends on an ability to survive under the following conditions: (*1*) high and changing phenol loading, (*2*) long-term exposure to phenol loading, (*3*) nutrient starvation (diluted MM), (*4*) various HRT used, (*5*) competition among the microorganisms present.

Identification of the individual genera and strains of a mixed culture from the PBR was performed after 4 months (Table II). Both the inoculated bacterial strains *P. putida* S7 and *C. testosteroni* Pb50 survived varied loading conditions. Both fungal strains were able to use phenol as the sole C- and energy source but their growth was much slower.

Microscopical observation of biofilm in PBR with PUF (Fig. 5). Cell colonization of the packing particle was not yet complete after 6 d of reactor operation. The biofilm surface, on the other hand, was almost homogeneous and the bacterial cells appeared to be trapped in some extracellular polymeric material. The production of extracellular polymer by bacterial cells degrading pollutants has been reported by several autors (Eighmy *et al*. 1983; Acuna *et al*. 1999; Annadurai *et al*. 2002).

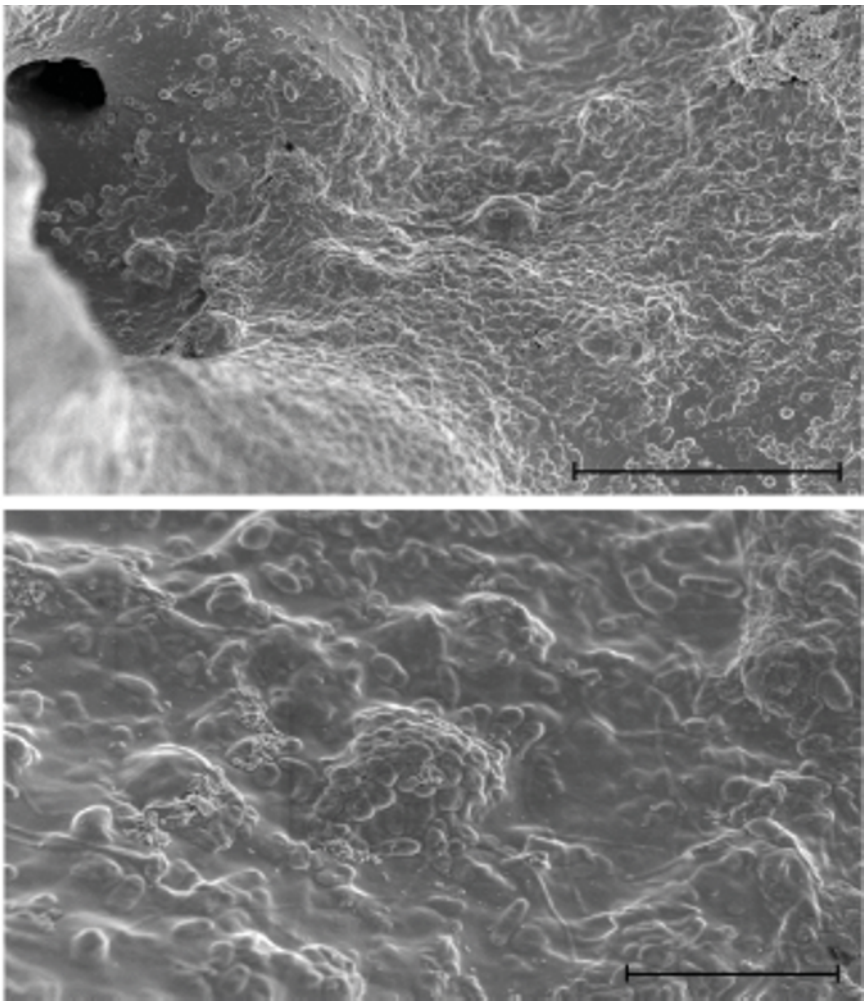

Fig. 5. Scanning electron microscopy of (*above*) cell colonization 6 d after the start of phenol loading (*bar* = 20 μm) and (*below*) the biofilm surface after 4 months of operation; *bar* = 5 μm.

Comparison of performance characteristics of PBRs with various packing materials. The loading tests of PBR were done with various packing materials but with the same mixed microbial culture (Figs 6 and 7). Cocoa-fibers gave total phenol removal up to OL = 250 mg L^{-1} h^{-1}. The highest degradation rate of q_S = 500 mg L^{-1} h^{-1} (12 g L^{-1} d^{-1}) was reached but with a reduced RE (70 %). However, the maximum degradation rate of 500 mg L^{-1} h^{-1} was also reached with both PUF and expanded slate with RE > 99 %. The highest performance was reached using sintered glass beads with maximum degradation rate of 1.45 g L^{-1} h^{-1} (34.8 g L^{-1} d^{-1}) with RE > 99 %.

According to a preliminary calculation of investment costs and running costs for our packing materials economically optimal, despite the lower degradation rate, was polyurethane foam (or expanded slate).

In a small PBR with a mixed culture immobilized on ceramic foam packing the maximum q_S = 14.2 g L^{-1} d^{-1} but with a RE of only 80 % was described by Branyik *et al.* (1998). Other authors found maximum phenol degradation rates to be 18 g L^{-1} d^{-1} (every 2 weeks clogged by biomass; Prieto *et al.* 2002), 9.2 g L^{-1} d^{-1} (Mörsen and Rehm 1990), 7.5 g L^{-1} d^{-1} (Chen *et al.* 2002; Bettmann and Rehm 1984), 4.0 g L^{-1} d^{-1} (Gonzales *et al.* 2001; Lakhwala *et al.* 1992), and 0.25 g L^{-1} d^{-1} (Sa and Boaventura 2001; Holladay *et al.* 1978). All the authors used PBRs or FBRs, a membrane reactor, rotating contactors with a better and/or worse RE for relatively short time-periods (1–3 months). In contrast, Gallego *et al.* (2003) used with similar results FBR for a 6-month degradation of a mixture of phenols.

Using PUF or expanded slate as a packing in a PBR together with the mixed culture of phenol degraders and adequate process control we showed that PBR can be considered to be a very efficient preliminary phenol-degradation step preceding biological wastewater treatment in coal processing and petrochemical refineries. We simulated real industrial conditions (wastewater) by tap water or distilled water with low nutrients containing phenol; the working pH_{out} of the reactor was then 4.0–3.2.

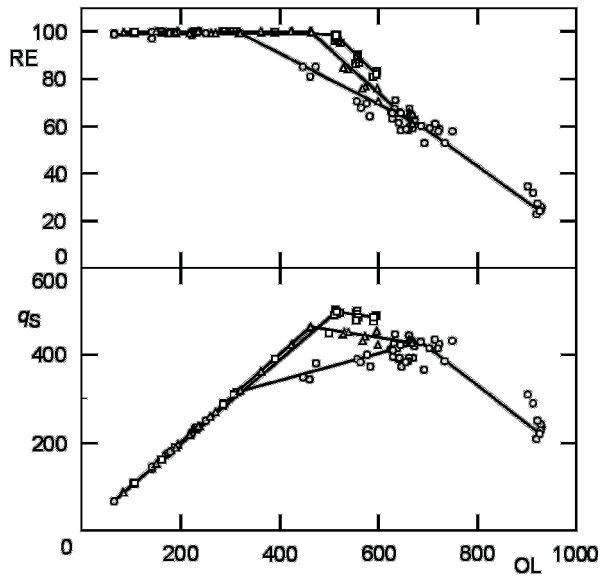

Fig. 6. RE and q_S reached in loading tests (OL in mg L^{-1} h^{-1}) with various packing materials (HRT = 193 min); packing: *circles* – cocoa-fibers; *squares* – polyurethane foam; *triangles* – expanded slate.

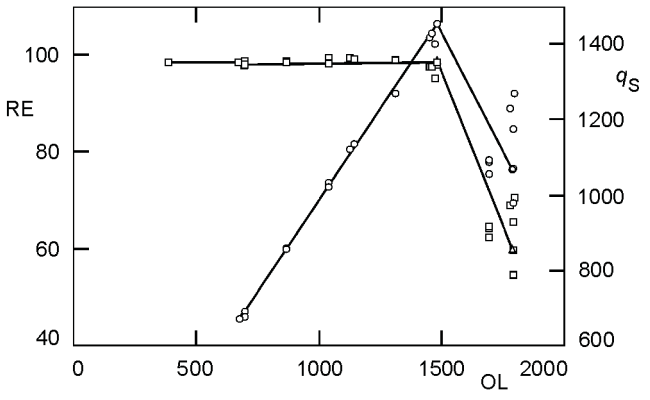

Fig. 7. RE (*squares*)and q_S (*circles*)of the reactor packed with sintered glass beads (HRT = 193 min; OL in mg L^{-1} h^{-1}).

The system with the immobilized mixed microbial culture can reach the maximum phenol-degradation rate regardless of the HRT value used, under conditions of the same biomass quantity, metabolic activity of the culture and with an excess of oxygen. Using nutrient limitation, PBR can be maintained in operation with low but sufficient biofilm thickness, reaching the degradation rate near maximum with RE > 99 % for a time period of half a year. Active biofilms formed under nonaseptic conditions contain predominantly *P. putida*. Some other fungi are also present, eliminating possible incompletely oxidized phenol intermediates as no metabolic intermediates with selected mixed cultures were determined, both with oxygen in excess and under oxygen-limitation conditions.

The work was supported by the *Czech Science Foundation* (project 104/03/0407).

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