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SHORT CONTRIBUTION

A. Hidalgo · A. Lopategi · M. Prieto · J.L. Serra
M.J. Llama

Formaldehyde removal in synthetic and industrial wastewater by *Rhodococcus erythropolis* UPV-1

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Abstract *Rhodococcus erythropolis* strain UPV-1 is able to grow on phenol as the only carbon and energy source and to remove formaldehyde completely from both synthetic and industrial wastewater. The rate of formaldehyde removal is independent of either initial biomass or formaldehyde concentration. The presence of viable, intact cells is strictly necessary for this removal to take place. Discontinuous and continuous formaldehyde-feed systems were successfully tested with synthetic wastewater in shaken flasks. Once biodegradation was well established in model synthetic wastewater, a real wastewater sample was obtained from a local phenolic and melamine resin-manufacturing company. Incubation of biomass with this wastewater at subtoxic concentrations of formaldehyde resulted in the complete removal of the pollutant. Parameters, such as chemical oxygen demand and toxicity, were assessed as indicators of wastewater cleanup progress.

Introduction

Phenolic resins are polymers originated by the condensation of phenol and formaldehyde. At the end of the process, a formaldehyde-containing wastewater is generated. Such industrial streams cannot be discharged into the environment and are only accepted in wastewater treatment plants below certain chemical oxygen demand (COD) and toxicity limits.

Micro-organisms possess the required genetic diversity that enables them to transform pollutants into less toxic compounds (Dassapa and Loehr 1991). Because of that, inoculating an activated sludge bioreactor with micro-organisms isolated and cultivated for their ability to degrade specific pollutants might prove to be an adequate wastewater treatment strategy (Hallas and Heitkamp 1995).

The high reactivity of formaldehyde with proteins, membranes and DNA must promote in cells the appearance of a detoxifying system which efficiently removes this potentially lethal compound. To date, there are several reports of bacterial degradation of formaldehyde by either aerobic (Hunter et al. 1984; Bonastre et al. 1986; Kato et al. 1986) or anaerobic processes (Qu and Bhattacharya 1997; Lu and Hegemann 1998; González-Gil et al. 1999, 2000; Omil et al. 1999; Garrido et al. 2000, 2001).

In this paper, we focus on formaldehyde removal by *Rhodococcus erythropolis* UPV-1 and its effect on the well established biodegradation of phenol (Hidalgo et al., in prep.). Since phenol and formaldehyde are usually found together in industrial phenolic wastewaters, the removal kinetics of each pollutant were studied separately and in combination, using model synthetic wastewaters. Our preliminary results in discontinuous laboratory-scale systems, reported here, will lead to fluidized-bed bioreactor studies as a necessary step in the design and implementation of a pilot plant.

Materials and methods

Analytical methods

Phenols in culture supernatants (13,000 *g*, 4 °C, 10 min) were determined colorimetrically using the 4-aminoantipyrine method (Greenberg et al. 1992), modified as indicated. The following were added sequentially to a 400-µl sample in mineral medium: 250 µl of 0.87% (w/v) ammonium hydroxide, 170 µl of 1.1 M potassium phosphate buffer (pH 7.0), 40 µl of 0.2% (w/v) 4-aminoantipyrine and 40 µl of 0.8% (w/v) potassium ferricyanide. Formaldehyde was determined using the Hanztsch reaction (Nash 1953). The initial COD of wastewater samples was determined using the open reflux method (Greenberg et al. 1992) and, during wastewater treatment, COD was determined in filtered (0.22-µm filters) and acidified supernatants (13,000 *g*, 4 °C, 10 min), using the appropriate Spectroquant kits and a Spectroquant Nova 60 photometer, all from Merck (Darmstadt, Germany). Toxicity test-

A. Hidalgo · A. Lopategi · M. Prieto · J.L. Serra · M.J. Llama (✉)
Enzyme and Cell Technology Group,
Department of Biochemistry and Molecular Biology,
University of the Basque Country, Faculty of Sciences,
P.O. Box 644, 48080 Bilbao, Spain
e-mail: gbpllfom@lg.ehu.es
Tel.: +34-94-6012622, Fax: +34-94-4648500

ing was performed in supernatants (13,000 *g*, 4 °C, 10 min) supplemented with NaCl (2% w/v), using Toxalert 100 kits from Merck (Darmstadt, Germany). EC_{50} values were calculated from the linearization of inhibition vs wastewater concentration plots as gamma (inhibition/luminescence) vs concentration. All reagents were of analytical grade, unless otherwise stated.

Microbial growth conditions

R. erythropolis strain UPV-1 (Spanish Type Culture Collection CECT 3054, http://www.uv.es/cect), isolated in our laboratory (Hidalgo et al., in prep.) from a phenol-polluted site on the Gernika estuary (Bizkaia, Spain), was used. Cells were grown in 250-ml Erlenmeyer flasks containing 100 ml of mineral medium (Tomasi et al. 1995), shaken in an orbital incubator (Gallenkamp INR-401, Leicester, UK) at 160 rpm and 30 °C. Inocula were prepared as follows: colonies were transferred from phenol/agar plates (200 mg phenol l^{-1}) to 100 ml of mineral medium containing 20 mg of phenol. After 96 h, grown biomass was used to inoculate 100 ml of mineral medium containing 40 mg of phenol. After a further 24 h, all the biomass was used to inoculate 400 ml of mineral medium containing 160 mg of phenol. This culture was diluted every 16 h to obtain a balanced growth.

Industrial wastewater samples

A 20-l wastewater sample (termed A5) was obtained from a local phenolic and melamine resin-manufacturing company. Sampling took place at documented points within the plant and the samples sent to our laboratory were kept in polyethylene containers at 4 °C.

Gas chromatographic analysis

Prior to biodegradation, several wastewater A5 samples were analyzed by solid phase extraction (SPE)-gas chromatography (GC) in a HP6890 gas chromatograph equipped with a 5973MSD mass spectrometer (MS; Hewlett Packard, Palo Alto, Calif.). SPE was performed with Sep-Pak Plus tC18 cartridges (Waters Corporation, Milford, Mass.). These cartridges were solvated with 8 ml of methanol (Merck, Darmstadt, Germany) and conditioned with 12 ml of ultrapure water (Milli-Q; Millipore, Milford, Mass.) at pH 2. Samples (4 ml) of undiluted, acidified wastewater were loaded in the conditioned cartridge, which was then vacuum-dried. Extracted analytes were eluted with methanol, and 2 µl were injected directly into the gas chromatograph. The chromatographic conditions were: 5:1 split injection, injector at 250 °C, HP-5 capillary column, 30 m, 0.25 mm i.d., 0.25 µm thickness (Hewlett Packard, Palo Alto, Calif.). The temperature ramp was: 85 °C for 1 min, 84–105 °C at 10 °C min^{-1}, 115–250 at 20°C min^{-1} and 2 min at 270 °C. The interface temperature was 280 °C and the mass detector was set at 70 eV. All organic solvents used were trace analysis-grade from Merck (Darmstadt, Germany).

Biodegradation assays in both synthetic and real wastewater

All experiments were performed at least twice in triplicate cultures of *R. erythropolis* UPV-1 in 250-ml Erlenmeyer flasks containing 100 ml of mineral medium, shaken in an orbital incubator (model INR-401; Gallenkamp, Leicester, UK) at 160 rpm and 30 °C. Phenol (Merck, Darmstadt, Germany) was filter-sterilized (0.22-µm filters), whereas paraformaldehyde (Aldrich, St. Louis, Mo,) was dissolved by autoclaving at 121 °C. Both sterile compounds were added to mineral media just prior to inoculation with cells. Uninoculated controls were incubated in all experiments to demonstrate the lack of phenol and/or formaldehyde removal.

Continuous formaldehyde feed without variation of culture volume was achieved by placing an upstanding polypropylene test tube containing 2 ml of a 20 g paraformaldehyde l^{-1} solution inside the Erlenmeyer flasks, leaning against the wall. When shaken in the orbital incubator, formaldehyde diffused from the tube into the culture headspace and dissolved steadily into the liquid medium.

The initial wastewater A5 concentrations used were determined from studies with synthetic wastewater. First, the amount of phosphate, ammonium and micronutrients required to amend wastewater A5 was determined. For this purpose, formaldehyde removal was assayed using a fixed amount of biomass and different nutrient concentrations, beginning with phosphate, then ammonium and, finally, both wastewater A5 and biomass concentration were increased for the optimum nutrient concentrations. At those selected biomass and wastewater A5 concentrations, COD and toxicity were assessed during the incubation of *R. erythropolis* biomass with such wastewater.

Results

Formaldehyde removal in synthetic wastewater

The disappearance of the pollutant was studied for different initial concentrations of formaldehyde. Then, each time-course was fit to a linear equation and the velocity (the slope of the linear fit) was plotted vs the initial concentration of formaldehyde in each case. The specific rate of formaldehyde disappearance remained constant throughout the chosen concentration range. Likewise, these specific rates for different initial biomass concentrations remained almost constant, decaying slightly at the higher biomass concentrations tested. The age of cells used as inoculum was irrelevant to the resulting formaldehyde-removal rate, provided such cells were within the exponential phase of growth (data not shown).

Bacterial growth was negligible in all of the cases mentioned above. The linearity of time-courses, together with the absence of detectable cell growth suggested that physical and/or chemical adsorption mechanisms could be involved in the disappearance of formaldehyde. To ascertain the dependence of the process for viable biomass, formaldehyde removal was assayed in the presence of cell-free extracts, obtained as described in Materials and methods, heat-shocked cells (autoclaved for 10 min at 121 °C) and whole, intact cells (positive control). Removal only took place in the presence of whole intact cells; and therefore structurally intact and viable biomass is essential to the process. To obtain inactive but whole cells, a culture was incubated with sodium azide concentrations ranging from 0 mM to 5 mM, leaving cells physically intact. It was observed that concentrations of azide higher than 0.5 mM were required to inhibit formaldehyde removal to some extent, inhibition being almost complete at 5 mM.

When a repeated- and discontinuous-feed approach was used, *R. erythropolis* UPV-1 was able to remove several consecutive doses of 20 mg paraformaldehyde l^{-1} from the culture medium (Fig. 1). The last pulses of formaldehyde took longer to be completely removed than the previous ones, showing some kind of cumulative effect. To overcome this toxicity problem, a continuous formaldehyde delivery system was designed which allowed

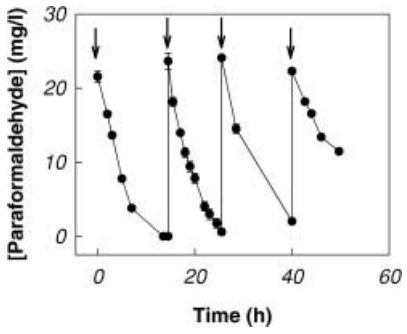

Fig. 1 Removal of sequential pulses of paraformaldehyde by *Rhodococcus erythropolis* UPV-1 cells. A biomass concentration equivalent to 0.2 absorbance units at 600 nm (A_{600} units) was used. Paraformaldehyde was supplied to 20 mg l^{-1} at times indicated by *arrows*. Values represent the mean ±SD of three separate assays

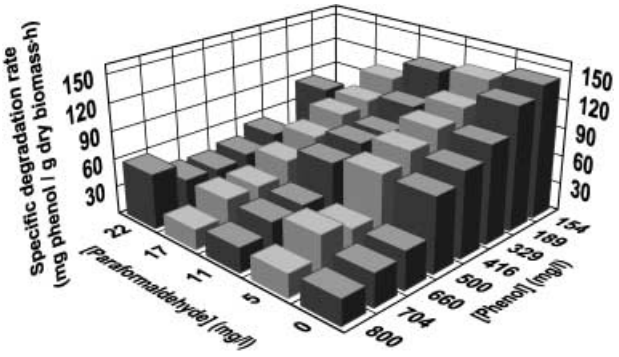

Fig. 2 Specific rate of phenol biodegradation by *R. erythropolis* UPV-1 cells in media containing different concentrations of phenol and paraformaldehyde. Initial biomass concentration was 0.2 A_{600} units in all cases. Experimental details are described in Materials and methods

formaldehyde to diffuse into the medium at a constant rate of 0.41 mg l^{-1} h^{-1}. At this rate, the redissolved formaldehyde in the liquid culture medium was completely removed over long periods of time (more than 40 days).

Effect of formaldehyde on phenol biodegradation

When the calculated specific rates of phenol degradation were plotted vs the corresponding phenol and formaldehyde concentrations (Fig. 2), the specific phenol degradation rate decreased with phenol concentration, because of the reported substrate inhibition effect (Straube et al. 1990), which was also corroborated in *R. erythropolis* UPV-1 cultures (Hidalgo et al., in prep.). An increase in formaldehyde concentration decreased not only the maximum growth rate of cells but also the capacity for phenol biodegradation.

Wastewater characterization

As indicated, the pH of the original wastewater A5 was neutralized with 0.66 M potassium phosphate buffer, pH 6.8. The following parameters were determined in supernatants (13,000 *g*, 4 °C, 10 min): phenol concentration (1,780±15 mg l^{-1}), formaldehyde concentration (12,900±320 mg l^{-1}), COD (19,750±80 mg O_2 l^{-1}), EC_{50} (0.07%, v/v). COD values are in good correlation with those calculated theoretically from the phenol and formaldehyde content, assuming a contribution of 2.4 mg O_2 mg^{-1} phenol and 1.1 mg O_2 mg^{-1} paraformaldehyde, as determined in standard solutions. Therefore, there is probably no other organic matter present in the wastewater A5 sample, just phenol and formaldehyde. GC analysis revealed, too, that phenol was the only phenolic compound present in the wastewater A5 sample.

Formaldehyde removal in real wastewater

First, the amount of phosphate, ammonium and micronutrient added to amend the real wastewater A5 was determined. Formaldehyde removal proceeded at the same rate when this wastewater was supplemented, to a final concentration of 33 mM potassium phosphate, 66 mM phosphate, or complete mineral medium (i.e., 66 mM potassium phosphate plus micronutrients). Similarly, when optimizing the minimum ammonium concentration needed, the presence of concentrations higher than 0.6 mg l^{-1} of this nitrogen source did not increase the rate of formaldehyde removal (data not shown). Finally, at the nutrient concentrations specified above, the maximum dilution for wastewater A5 was established as 0.6% (v/v) for discontinuous experiments, using a biomass concentration equivalent to 2.0 absorbence units at 600 nm. At this dilution, the formaldehyde concentration is about 90 mg l^{-1} and phenols were not detected.

COD and toxicity reduction in real wastewater

The COD of wastewater A5 was monitored during incubation with biomass; and a 56% COD reduction was observed. The profile for the time-course of formaldehyde removal in real wastewater yielded a linear response, just as in synthetic wastewater. Accordingly, the profile of the COD removal was linear, too (data not shown).

Toxicity was also assessed during the treatment of wastewater A5; and a 32% reduction was calculated. However, when toxicity was assayed in synthetic wastewater, a 90% reduction was routinely obtained.

Discussion

Despite the fact that formaldehyde-disappearance rates are independent of both initial biomass and formaldehyde concentration (for the range of formaldehyde concentrations used), the lack of removal in the presence of 5 mM azide, or autoclaved cells or cell-free extracts suggest that metabolically active cells are required for the process. In fact, most formaldehyde removal mecha-

nisms reported are related to bacterial metabolism, rather than originated by physicochemical processes. Such mechanisms include oxidation to formate, catalyzed by a NAD^+-dependent formaldehyde dehydrogenase (Barber et al. 1996; Barber and Donohue 1998), or a dismutation reaction catalyzed by NAD^+-dependent formaldehyde dismutase (Kato et al. 1984, 1986). At this point, we are still investigating the pathway used by *R. erythropolis* UPV-1 to deal with formaldehyde, but the fact that azide blocks formaldehyde removal strongly suggests either a metabolic use of formaldehyde or its participation in an electron-detoxifying system. Furthermore, a NAD^+-dependent formaldehyde dehydrogenase activity has already been detected in *R. erythropolis* UPV-1 cell-free extracts and further work is oriented to continue elucidating the pathway.

Regarding the interaction of two pollutants, such as phenol and formaldehyde in synthetic wastewater, similar interactions between two inhibitory substrates have been described before. One such case is that of phenol in the presence of heavy metal ions (Nakamura and Sawada 2000), although the most frequently reported case is the coexistence of an inhibitory substrate with a second Monod-type substrate, generally a sugar (Brinkmann and Babel 1996; Wang et al. 1996). This interaction has been successfully described, using a classic enzyme-uncompetitive inhibition pattern, although the equation governing this model did not fit our experimental data satisfactorily.

When real phenolic wastewaters were used as a substrate, neither COD nor toxicity removal was complete, although the calculated reduction percentages were higher than those achieved for other phenolic wastewaters (Hidalgo et al., in prep.). Assuming safely from the GC-MS analysis that no other phenols are present and taking into consideration that COD is completely removed in synthetic wastewater a remaining 46% of unidentified organic matter is still present. The existence of unidentified chemical compounds other than phenol and formaldehyde, which probably are responsible for a fraction of COD and toxicity in treated formaldehyde-containing wastewaters, has been suggested before (Garrido et al. 2000).

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