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Calorimetric investigations of phenol degradation by *Pseudomonas putida* [1]

Ch. Motzkus, G. Welge and I. Lamprecht *

Institut für Biophysik, Freie Universität Berlin, D-14195 Berlin (Germany)

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Abstract

Cyclic and polycyclic aromatic hydrocarbons are among the most widespread contaminants in soils, because they are still used in large amounts. Although poisonous to many organisms, consortia of microorganisms or single species such as *Pseudomonas* have specialized in the catabolism of these compounds and use them as their sole energy and carbon source.

Phenol, together with some of its catabolic intermediates, and the bacterium *Pseudomonas putida* were chosen for microcalorimetric investigations in a batch and a fermentor/flow system. As many of these aromatics are very poisonous, the concentration limits for growth and metabolism were evaluated optically and calorimetrically, rendering significantly prolonged lag-phases at higher concentrations, but no complete inhibition of metabolism. Under such experimental conditions the calorimetric curves for heat production rate versus time showed reactions following a Michaelis–Menten kinetics in agreement with data from the literature. The calorimetric results proved that phenol is mineralized aerobically to water and carbon dioxide and that an energy balance can be established.

INTRODUCTION

Cyclic and polycyclic aromatics were widely used in the past and are used even now in industrial production and in agricultural crop protection. They are hazardous pollutants of air and water as well as of soil and sediments, originating from liquid or gaseous effluents of various industrial activities, including oil refineries and petro-chemical plants, as well as chemical, pharmaceutical, metallurgical, pesticide and textile productions. It is not surprising that they are found in water and in many soils at extremely high concentrations. To ameliorate these polluted sites the soil is either dug up and deposited or decontaminated in situ by microbial treatment. Recent data for Berlin alone suggest around 4500 heavily polluted sites which have to be decontaminated in the next 10–20 years at an expense of 4 to 5 billion

* Corresponding author.

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German marks. Thus, biological in situ mineralization would be the most suitable technique.

It was noticed as early as 1910 [1] that particular microorganisms are able to degrade aromatics, including phenols. These degradations are often performed anaerobically by microbial consortia [2], e.g. in methanogenic sediments [3]. Under such conditions, carboxylation of phenolic compounds to benzoate seems to be a general pathway [2, 3], yielding acetate, methane and carbon dioxide. In addition to this anaerobic degradation some organisms can metabolize phenol or related aromatic compounds aerobically as their only carbon and energy source. The best known representative of this group is the gram-negative, polar-flagellated, unicellular bacterium *Pseudomonas putida* which has been frequently used to investigate possible metabolic pathways of aromatics [4] and which was chosen for the present experiments. Detailed descriptions of the biochemical, genetic and physiological properties of *P. putida* are given in refs. 5 and 6.

Phenol was selected as the most prominent representative of aromatic compounds and because of its frequent use in various applications. It is known as a toxic, malodorous substance whose negative influences on health have been intensely investigated [7].

The microbial degradation of aromatics proceeds aerobically, depending on the primary substrate, to catechol or protocatechuic acid and from there via the ortho- or meta-pathway in several steps to the final metabolites which enter the tricarboxylic acid cycle. The most frequently encountered pathway of chromosomally-encoded ortho-cleavage of the ring between two hydroxyl groups leads to succinate and acetyl-CoA, while the rare plasmid-encoded meta-cleavage proceeds to acetaldehyde and pyruvate [8]. The choice of the two pathways seems to depend only on the catechol precursor [9]. Phenol is metabolized via the meta-pathway, catechol by the ortho-cleavage [9].

Because of the central role of catechol in the aerobic metabolism of aromatic compounds, it was included in our calorimetric investigations. Some orientating experiments were run with benzoic acid as a representative of the ortho-pathway and with 3-oxoadipic acid as a metabolite of this more frequent metabolism. The results with these substrates will be published elsewhere.

MATERIAL AND METHODS

Biological material

All experiments were performed with the bacterium *Pseudomonas putida*, strain 548 (ATCC 17514, NCIB 10015) (Deutsche Sammlung der Mikroorganismen DSM/Brunswick). Stock cultures were kept on agar

slants at 4°C. Two days before an experiment, cells were incubated on a solid complex growth medium at 30°C for 48 h, harvested by washing off with a few milliliters of buffer, and adjusted to the required optical density. For the calorimetric experiments, cells were incubated in a synthetic growth medium at a pre-set optical density of around 0.15. At the end of the experiment, optical density was again determined to calculate the growth yield of biomass.

Optical density was measured at 532 nm using a spectrophotometer (Shimadzu/Kyoto). The dry weight of the cells was determined in an exsiccator with silica gel at 110°C overnight.

Calorimetry

Most experiments described here were performed in batches with a Calvet microcalorimeter (Setaram/Lyon) with four vessels of 15 ml capacity each. They were filled with 6 ml of suspension and stirred mechanically [10] to avoid sedimentation of the cells and to increase oxygen supply. The sensitivity of the instrument was 62.9 mV W^{-1}, typical recordings were made at 500 μV full scale and 12 mm h^{-1} paper speed. The calorimetric temperature was set to 30°C, the ambient temperature was 22°C.

In addition, a flow calorimeter (type 10700, LKB/Bromma) with a flow-through cell of 0.587 ml capacity and a sensitivity of 54.5 mV W^{-1} was used, connected to a separate fermentor in a water bath controlled at 30°C. In most experiments, a pumping rate of 100 ml h^{-1} was sufficient at the low cell density used.

Because *P. putida* tends to grow on the walls of the vessels, intensive cleaning was necessary after each experiment. The usual rinsing with a solution of 70% ethanol and 2% sulfuric acid was insufficient to lyse the bacterial cells completely and to sterilize the flow lines. Therefore, a solution of 10% hydrogen peroxide was allowed to act for at least 15 min before the routine washing procedure. Without this precaution, normal growth was observed calorimetrically in phenol medium without inoculation.

The areas under the obtained calorimetric curves were determined by means of an electronic planimeter (Digikon, Kontron/Munich), giving the total heat produced during the experiment.

Polarography

In some experiments, a polarographic oxygen electrode of Clark type (Bachofer/Reutlingen) was used to measure the respiration rate of *P. putida* cultures in parallel with the flow calorimetric investigations. This was mainly done to determine if the oxygen tension became a limiting factor

during the flow from the fermentor to the flow-through vessel. Aliquots of 1 ml were transferred at given times of the growth curve from the fermentor to the electrode to determine the oxygen consumption rate and, thus, the interval during which all the oxygen was depleted.

Substances and media

Experiments on the catabolism of aromatics by *P. putida* reported here were run on phenol of analytical grade (Merck/Darmstadt). Stock solutions of 1 g l^{-1} in 0.05 mol l^{-1} KH_2PO_4 buffer (pH 7) were prepared and amounts of between 1 and 6 ml were added to the medium to render a total volume of 18 ml. In this way, the final phenol concentration ranged between 0 and 333 mg l^{-1}. Sometimes, even higher concentrations were applied.

P. putida was precultured on solid meat extract medium (3 g l^{-1} meat extract/Fluka, Buchs; 5 g l^{-1} peptone/Difco, Detroit; 15 g l^{-1} agar/Difco, Detroit; pH 7 in aqua dest.). The synthetic medium for the calorimetric experiments consisted of two solutions which were autoclaved separately and mixed afterwards. Solution 1: 500 mg l^{-1} $MgSO_4 \cdot 7H_2O$ (Merck/Darmstadt) plus 300 mg l^{-1} $CaCl_2 \cdot 2H_2O$ (Merck/Darmstadt). Solution 2: 1.6 g l^{-1} KH_2PO_4 (Roth/Karlsruhe), 3 g l^{-1} $(NH_4)_2SO_4$ (Merck/Darmstadt), 10 ml SL8, pH 7. SL8 contained various trace elements (SL8 after Biebl and Pfennig [11]). These solutions were mixed in a 1:1 ratio and were then made up with the aromatic compound and the cell suspension.

Phenol concentrations were determined colorimetrically following the method of Ochynski [12] which is based on the reaction of phenol with 4-aminoantipyrin (Aldrich/Steinheim) in the presence of the oxidizing agent ammonium persulfate (Merck/Darmstadt) and a buffer solution of sodium tetraborate (Sigma/Deisenhofen). A red complex (540 nm) is formed that is proportional to the amount of phenol in the solution.

RESULTS

Growth in phenol medium

The growth of *P. putida* in phenol medium was followed photometrically to determine the limits within which catabolism takes place. Up to 500 mg l^{-1} phenol, a linear relationship between the final optical density and the initial phenol concentration was found (Fig. 1). The slope has a value of 1.90 OD per g l^{-1} phenol which transforms to 890 mg dry weight (dw) per g phenol, in good agreement with data from the literature [8, 13]. The straight line indicates that no poisoning effect by phenol occurs at these concentrations. At even higher initial phenol concentrations, the lag phase of the growth curve may be considerably prolonged indicating an altered adaptation period. From the log phase of the calorimetric curves, a generation time of approximately 73 min was calculated for flow experiments at 167 mg l^{-1} phenol concentration and of 120 min in corresponding

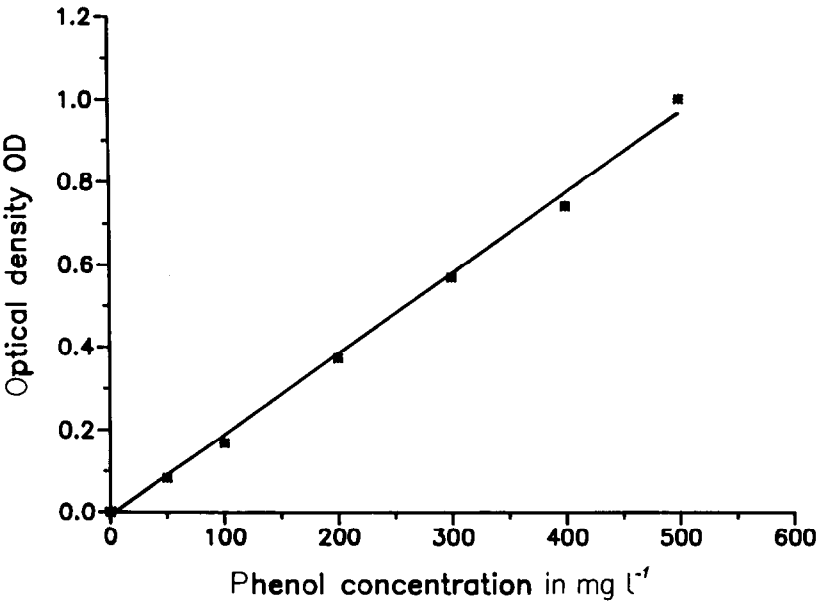

Fig. 1. The final optical density OD at 532 nm for the growth of *P. putida* as a function of the initial phenol concentration *c*.

batch investigations. These values are in the range given in the literature [8, 13]. A substrate inhibition by phenol in higher concentrations would be indicated by a decreasing generation time during the log phase; this was not detectable in our calorimetric curves. In spite of identical preparations of the cells, the metabolic activity of the culture may change significantly from day to day as already stated by Fischer [8] and Fürst [13]. This might be due to a different choice between the ortho- or meta-pathway of catabolism [13]. These changes are manifested in a larger scatter of the maximum heat production rates in calorimetric experiments.

Calorimetric experiments

Figure 2 shows a typical slope of heat production rate versus time for a batch culture of *P. putida* in liquid phenol medium at 167 mg l^{-1} initial concentration. Heat production slowly increases exponentially to a maximum followed by a steep decline to the base line with virtually no further exogenic metabolism. Optical tests for phenol showed no remaining substrate within the normal scatter, proving that phenol was completely catabolized under these experimental conditions. Moreover, the single-peak slope of the heat production rate indicates that no metabolite was enriched in the medium and subsequently degraded in a diauxic fashion. Thus, we may assume that phenol was completely oxidized aerobically to water and carbon dioxide following the stoichiometric equation.

The total heat production obtained during an experiment as a function

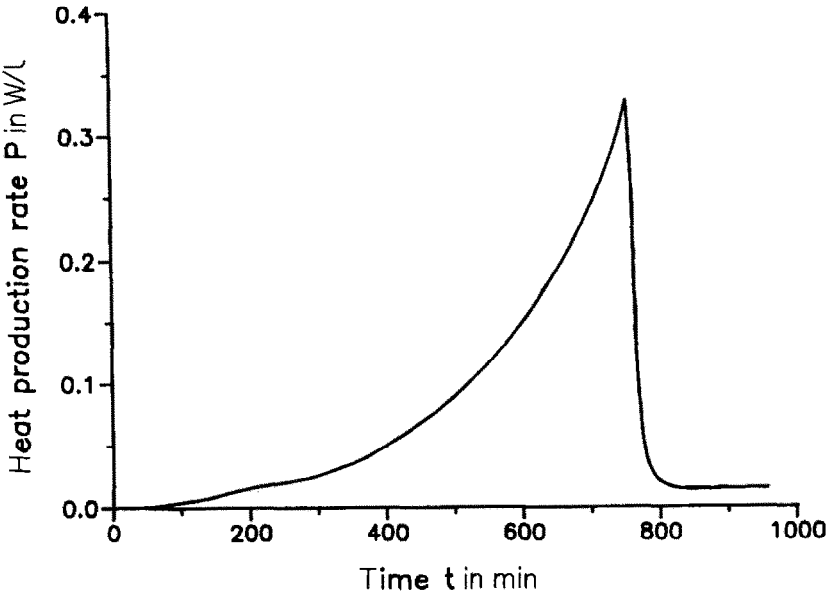

Fig. 2. Heat production rate P versus time t for growth of *P. putida* in a batch experiment with phenol at an initial concentration of 167 mg l^{-1}.

of the initial phenol concentration yields a linear relationship with a slope of 17.8 kJ g^{-1} phenol. As these are, to our knowledge, the first calorimetric experiments with microbial growth on phenol, no comparable data are available in the literature. But as the energy is balanced in the growth experiment, the figure seems reasonable.

The shapes of the calorimetric growth curves are similar at all phenol concentrations. Because their area is proportional to the amount of metabolized phenol, the peak heights have to follow a square-root dependence on the phenol concentration [14]. This is demonstrated in Fig. 3 for initial phenol concentrations of up to 667 mg l^{-1}. But with some of the higher concentrations, the scatter is very large with a strong deviation from the optimal growth slope. Plotting P^2_{max} as a function of c renders the expected linear relationship with a correlation coefficient r of 0.998. The intercept with the abscissa amounts to 37 mg l^{-1} phenol which is not used for cell growth but for maintenance metabolism.

The last part of the growth curve with the steep decline can be described by a Michaelis–Menten kinetics. All necessary information concerning reaction rate, substrate concentration and dry matter of the culture can be taken directly from the calorimetric curve; they are proportional to the momentary heat production rate, the total dissipated heat minus the heat evolved up to that moment, and the heat evolved up to that moment, respectively [15]. At the end of the experiment, the necessary proportionality constants are determined from the total heat and the biomass grown. Figure 4 shows a Lineweaver–Burk plot of phenol degradation by a growing culture of *P. putida*. It renders a Michaelis constant K_m of

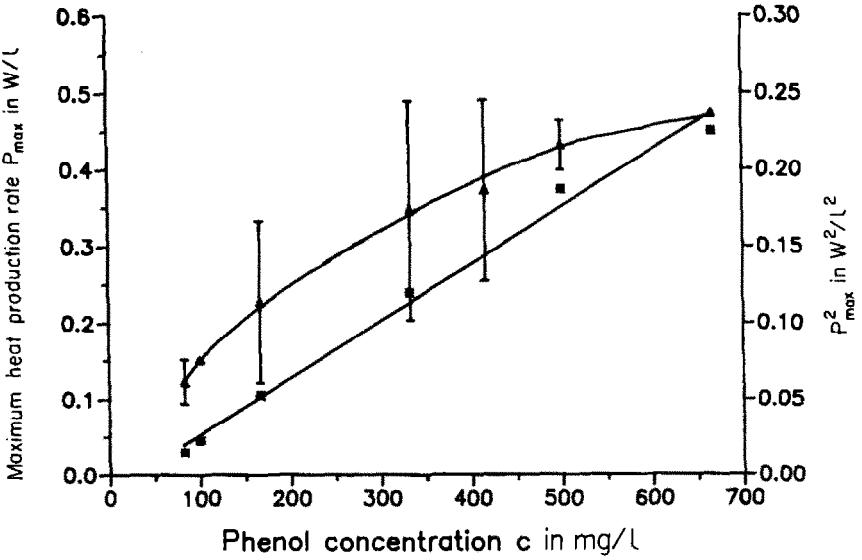

Fig. 3. Maximum heat production rate P_{max} and its square P^2_{max} as functions of the initial phenol concentration c, following a square-root and a linear dependence, respectively.

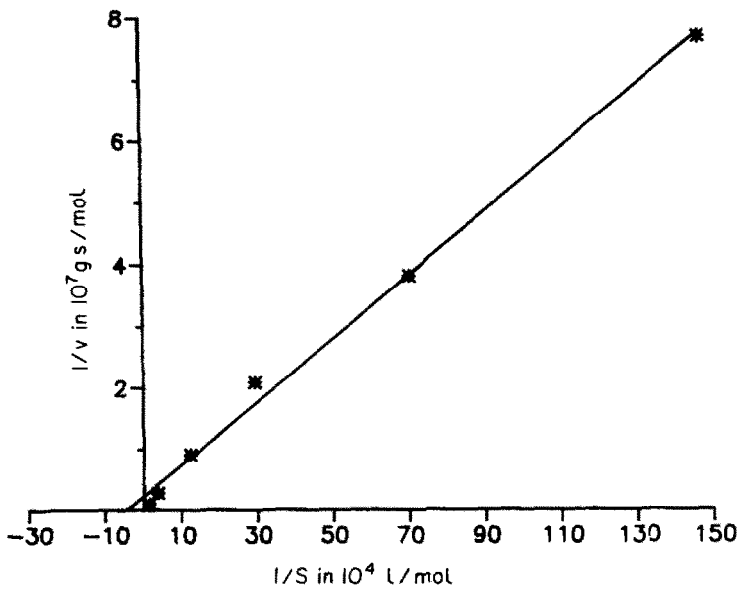

Fig. 4. Lineweaver–Burk plot of growth of *P. putida* on phenol obtained from the last part of a calorimetric curve. Because of the increasing matter during the (growth) experiment, the dimension "g" ("per dry weight") appears along the ordinate in contrast to the usual Michaelis–Menten kinetics.

0.24×10^{-4} mol l^{-1} and a maximum metabolic rate v_{max} of 4.6×10^{-6} mol s^{-1} $(g\,dw)^{-1}$; (dw indicates dry weight). Data for K_m found in the literature show a large scatter between 0.01×10^{-4} and 0.63×10^{-4} mol l^{-1}; v_{max} varies between 0.89×10^{6} and 1.83×10^{-6} mol s^{-1} g^{-1} [13].

Energy balance of phenol mineralization

With a generalized C-molar formula of cell mass for *P. putida* being $CH_2O_{0.52}N_{0.23}$ [16], the growth of the bacterium on phenol may be described as

$$C_6H_6O + 3.26O_2 + 0.8NH_3 \rightarrow 3.5CH_2O_{0.52}N_{0.23} + 0.7H_2O + 2.5CO_2$$

where the stoichiometric coefficients are obtained from an atomic balance of both sides of the equation plus the growth yield of *P. putida* on phenol. Energy contents of the components may be either found in the literature or estimated by different methods. One approach uses the formula of Dulong for the combustion energy of organic substances, as modified by Wang et al. [17]

$$-\Delta H_c = 33.78C + 144.1(H - \tfrac{1}{8}O)\ \text{kJ g}^{-1}$$

where C, H and O are the relative mass portions in the substance. This formula renders a ΔH_c of -31.83 kJ g^{-1} for phenol and a ΔH_c of -23.18 kJ g^{-1} for the cells. Together with a ΔH_c of -20.47 kJ g^{-1} for ammonia in solution [18], the energy balance for one mole of phenol reads

$$-3271\ \text{kJ} \rightarrow -3747\ \text{kJ}$$

which shows a deviation of +15%.

An alternative approach uses the estimation from the reductance degree of an organic substance defined by

$$\gamma^0 = 4 + e_1 - 2e_2$$

for a generalized chemical C-molar formula $CH_{e_1}O_{e_2}N_{e_3}$ [19, 20]. From this value, the C-molar enthalpy of combustion can be estimated as $\Delta H_c \simeq Q_0\gamma^0$ with $Q_0 = -115$ kJ per reductance degree for many organic substances. With $\gamma^0 = 4.66$ for phenol and 4.27 for cells, one obtains $\Delta H_c = -34.2$ kJ g^{-1} for phenol and $= -19.2$ kJ g^{-1} for the cells. In this case the energy balance for one mole of phenol reads

$$-3494\ \text{kJ} \rightarrow -3392\ \text{kJ}$$

with a deviation of -2.9%. This value shows that under the above assumptions the energy is balanced.

Phenol uptake by P. putida

Assuming that in a steady state no phenol is accumulated in the cells, the heat production rate is proportional to the phenol uptake and turnover. Thus, heat production rates can be converted into phenol uptake rates by means of the total heat dissipated during the experiment.

The dry matter yield is determined only at the end of the experiment, but the formation of new cell material is proportional to the heat evolved up to a given moment, so that at any time on a calorimetric growth curve, the metabolic turnover rate and biomass are known. Taking as an example an experiment with 167 mg l^{-1} phenol, the maximum heat flow amounts to 0.337 mW ml^{-1}, the total heat production to 17.8 kJ (g phenol)$^{-1}$ and the increase in biomass to 0.165 mg dw per ml. These figures render a specific phenol uptake of 0.41 mg (h mg dw)$^{-1}$. Our flow calorimetric investigations show slightly higher uptake rates of 0.61 mg (h mg dw)$^{-1}$. A value of 0.46 mg (h mg dw)$^{-1}$ can be estimated from data of Fischer for similar phenol concentrations [8]. Sokol [21] cites values between 0.53 and 1.87 mg (h mg dw)$^{-1}$.

Oxygen limitation

Figure 5 shows the growth of *P. putida* on phenol in a flow calorimetric experiment. A double kinetics with a two-fold limitation is apparent. The second steep decline indicates a substrate limitation as described by a Michaelis–Menten kinetics mentioned and evaluated above for phenol (see Fig. 4). The first smooth drop of the heat production rate is assumed to depend on an oxygen deficiency during transport from the fermentor to the calorimeter. From the stoichiometry of phenol catabolism, the total heat

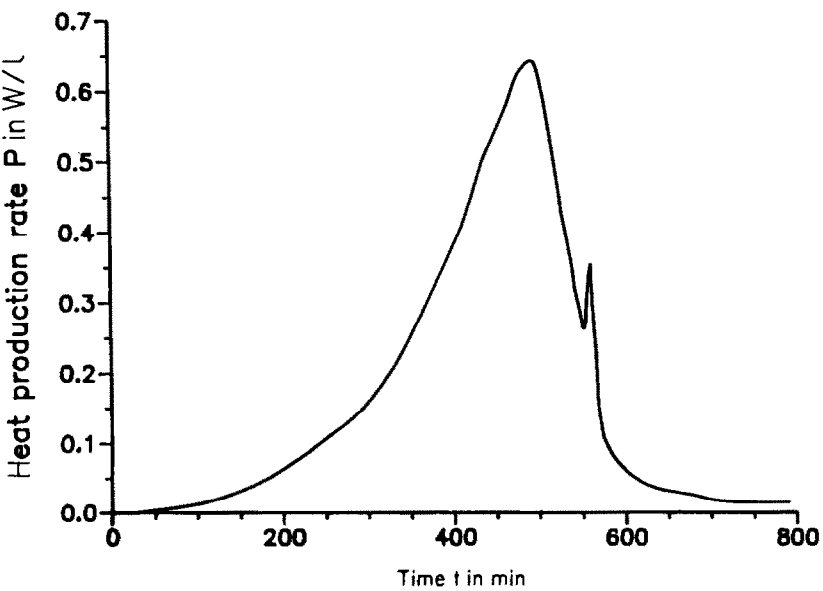

Fig. 5. Double kinetics of heat production rate *P* during the growth of *P. putida* on phenol in a flow experiment with an initial concentration of 333 mg ml^{-1} and a flow rate of 35 ml h^{-1}.

evolved and the heat production rate, the oxygen consumption in the flow line was calculated as follows.

The maximum in the heat production rate was 0.623 mW ml^{-1}, and the total heat evolved in this experiment was 7.72 J ml^{-1}. With this figure, the maximum transforms to a phenol metabolism of 26.9×10^{-9} g s^{-1} ml^{-1} or 0.286×10^{-9} mol s^{-1} g^{-1}. As 7 moles of oxygen are consumed per mole of phenol in a complete mineralization to water and carbon dioxide, the maximum corresponds to a respiration rate of 2.00×10^{-9} mol s^{-1} ml^{-1}. With an oxygen saturation of 237×10^{-9} mol ml^{-1} in water at 30°C, all the oxygen is consumed within 119 s or 2 min. When cell formation is taken into account in the energy or C-molar balance, only 3.26 moles of oxygen are consumed per mole of phenol. In this case, it takes 254 s or 4.2 min to deplete oxygen. But with the applied flow rate of 35 ml h^{-1}, it takes 5.75 min for a suspension volume to reach the flow-through vessel of the calorimeter, so that the conditions become anaerobic.

The same experiments and calculations were performed with growth on catechol and were checked by parallel polarographic measurements (not shown). At specific times on the calorimetric growth curve, samples were taken, intensively aerated and transferred to the oxygen electrode to determine the maximum respiration rate and the time for oxygen depletion at this particular cell concentration. The results showed that: (i) oxygen consumption rates determined polarographically or calculated from the heat production rate were similar, indicating the complete aerobic mineralization of catechol; (ii) up to the peak in the calorimetric curve, oxygen did not become limiting during the flow time; (iii) oxygen was consumed before the sample entered the calorimetric flow-through cell at higher cell densities.

This example demonstrates that there is a steady competition between the demand for a high flow rate for an optimal oxygen supply of the microbial culture in the flow line, and for a small flow rate because of signal stability of the calorimeter. For the aromatic concentrations and the cell titers used here, a flow rate of 100 ml h^{-1} proved to be sufficient for optimal growth conditions and a stable base line.

DISCUSSION

Aromatics represent a real burden for nature because they are toxic for most organisms even in low concentrations; however, they are widespread in man-made environments due to their manifold use in industrial processes. They are found in the effluent of chemical plants as well as in municipal waste water. Thus, an intensive search for a single organism or microbial consortia which can degrade this class of substance is most important. Some of these organisms can use aromatics as their only energy and carbon source [2, 3, 8, 13, 22], while others destroy them only in the presence of an alternative energy source [4, 23].

In the present investigations, phenol was selected because it is a prominent aromatic compound, well known for its toxic influences and adverse effects on health [7] and because of its application in many technical fields. It is first hydroxylated microbially to catechol by phenol-hydroxylase; this is the rate-limiting step in its mineralization. Its further metabolism by the ortho- or meta-pathway depend upon the applied microorganism.

Pseudomonas putida is the best known representative of the aerobic degraders of aromatics, especially of phenol, and is used in many investigations. But the various *P. putida* strains found in the literature differ considerably. Only a few of them are able to follow the meta-cleavage while most proceed via the ortho-pathway [9]. *P. putida* strain 548, chosen for our experiments, mineralizes phenol via the plasmid-encoded meta-pathway, but its successor catechol as initial substrate mineralizes via the chromosomally-encoded ortho-pathway. The latter is the more stable pathway because the plasmid is lost after some time by the cells. Then the alternative ortho-cleavage is followed, but at a significantly reduced rate. This partly explains why the kinetics of *P. putida* cultures differs from day to day [13] and why the maximum growth rate on phenol and the constant of substrate inhibition vary from author to author over a broad range [4, 8, 13]. Zilli et al. [4] cited values for this that range from 22.9 to 1503 mg l^{-1}. The figures determined by us for maximum growth rate and inhibition are well in the range published by other groups.

Only a few calorimetric experiments with phenol or other aromatic compounds are found in the literature. To our knowledge, none of them has focussed on microbial growth on such substrates, but all were concerned with the toxic effect of these substances. Fortier et al. [24] investigated biodegradation processes in biological wastewater treatment by means of a flow calorimeter and the influence of toxic chemicals in industrial effluents, among them phenol. While some heavy metals led to strong reductions of the heat dissipation in a microbial culture from activated sludge, phenol showed nearly no effect up to 50 ppm [24]. Chemostat-cultures of *Acinetobacter calcoaceticus* obtained from a municipal wastewater treatment plant reacted with a 20% increased heat output rate at medium phenol concentrations and displayed all the signs of poisoning at higher concentrations [25]. The authors calculated a 50% decrease in heat production rate with relatively high phenol concentrations of 830 mg l^{-1}. In similar experiments with *Escherichia coli*, they observed a 50% inhibition at even higher phenol concentrations of 1300 mg l^{-1}, while growth was already inhibited at 450 mg l^{-1} and respiration at 650 mg l^{-1}. Beezer and coworkers [26] determined phenol coefficients by means of flow calorimetry, giving relative efficacies of various disinfectants.

The present investigations have confirmed that batch- and flow-calorimetry are suitable techniques to investigate the metabolism and

kinetics of microbial cultures growing on aromatics as their sole energy source. Moreover, calorimetric experiments with resting cells and various aromatics may supplement such investigations, yielding rapid information on the toxic effects and metabolic kinetics of different substrates. Finally, the calorimetric comparison of diverse aromatic compounds and their mineralization by the meta- or the ortho-pathway may help to understand this complex metabolism and the energetics behind it. Such investigations are being carried out.

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REFERENCES

1 G.J. Fowler, E. Arden and W.T. Lockett, Proc. R. Soc. London Ser. B, 83 (1911) 149.
2 C. Gallert and J. Winter, Appl. Microbiol. Biotechnol., 37 (1992) 119.
3 X. Zhang and J. Wiegel, Appl. Environ. Microbiol., 58 (1992) 3580.
4 M. Zilli, A. Converti, A. Lodi, M. Del Borghi and G. Ferraiolo, Biotechnol. Bioeng., 41 (1993) 693.
5 R.Y. Stanier, N.J. Palleroni and M. Douderoff, J. Gen. Microbiol., 43 (1966) 195.
6 P.H. Clarke and L.N. Ornston, in P.H. Clarke and M.H. Richmond (Eds.), Genetics and Biochemistry of Pseudomonas, John Wiley, London, 1975, p. 191.
7 N.I. Sax, Phenol. Dang. Prop. Ind. Mat. Rep., 3 (1983) 77.
8 P. Fischer, Thesis, Technical University of Berlin, 1989.
9 C.F. Feist and G.D. Hegeman, J. Bacteriol., 100 (1969) 869.
10 I. Lamprecht and C. Meggers, Z. Naturforsch. Teil B, 24 (1969) 1205.
11 H. Biebl and N. Pfennig, Arch. Microbiol., 117 (1978) 9.
12 F.W. Ochynski, Analyst, 85 (1960) 278.
13 M. Fürst, Diploma Thesis, Free University of Berlin, Department of Biology, 1992.
14 I. Lamprecht and C. Meggers, Biophysik, 8 (1972) 316.
15 J.P. Belaich, J.C. Senez and M. Murgier, J. Bacteriol., 95 (1968) 1750.
16 J.A. Roels, Energetics and Kinetics in Biotechnology, Elsevier Biomedical Press, Amsterdam, New York, Oxford, 1983.
17 H.Y. Wang, D.G. Mou and J.R. Swartz, Biotechnol. Bioeng., 18 (1976) 1811.
18 C.E. Vanderzee, M. Mansson, I. Wadsö and S. Sunner, J. Chem. Thermodyn., 4 (1972) 541.
19 L.E. Erickson, in A.M. James (Ed.), Thermal and Energetic Studies of Cellular Biological Systems, Wright, Bristol, 1987.
20 U. von Stockar and I.W. Marison, Thermochim. Acta, 193 (1991) 215.
21 W. Sokol, Biotechnol. Bioeng., 32 (1988) 1097.
22 G. Knoll and J. Winter, Appl. Microbiol. Biotechnol., 30 (1989) 318.
23 H. Yanase, K. Zuzan, K. Kita, S. Sogabe and N. Kato, J. Ferment. Bioeng., 74 (1992) 297.
24 J.L. Fortier, B. Reboul, P. Philip, M.A. Simard, P. Picker and C. Jolicoeur, J. Water Pollut. Control Fed., 52(1) (1980) 89.
25 P. Weppen and D. Schuller, Thermochim. Acta, 72 (1984) 95.
26 A.E. Beezer, W.H. Hunter and D.E. Storey, J. Pharm. Pharmacol., 33 (1981) 65.