Pure & Appl. Chem., Vol. 67, No. 6, pp. 947–954, 1995.
Printed in Great Britain.
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Aromatic compounds as model substances for environmental pollutions: Energetic and kinetic calorimetric investigations of mineralization by microorganisms

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Abstract: Calorimetric investigations of microbial growth on aromatic compounds are reported with special emphasis on phenol and its derivatives. One bacterial and two yeast strains are used which are known for their phenol degrading capabilities. They show a high tolerance against aromatic poisoning, elevated rates of metabolism and the ability to grow on phenol as sole carbon and energy source in the medium. The calorimetric curves are discussed with respect to the energetic turnover, growth yield and growth kinetics under various experimental conditions. A double kinetics is presented to describe the metabolic events and to model the combination of a flow calorimeter with a fermentor.

INTRODUCTION

Aliphatic and aromatic compounds belong to those contaminations of aquatic and terrestric ecological systems that are most frequently found in industrial countries. They are the heaviest burdens of polluted sites and a real challenge for environmental engineering. Many of these substances are highly toxic for microorganisms as well as for plants and animals. As they are still used in industrial processes or in agricultural crop protection their concentrations continue to increase in many parts of our environment.

Phenol and its catabolic intermediates were chosen as model substances for the present investigations. Phenolic structures are widely distributed in nature and - e.g. - the largest group of substances in wood besides carbohydrates. They are responsible for the colours of many fruits and flowers and for the organoleptic properties of plants. Thus, phenols are naturally occuring due to plant degradation, and not only as discharge of wastes from different branches of industry. Phenol as a troublesome contaminant is listed among the priority pollutants by the US Environmental Protection Agency (1). As phenol is malodorous and generally very toxic its concentration in the environment is strictly limited to very low threshold values by law (2).

Although phenols are toxic to most organisms there are microbial cohorts or pure strains not only tolerating phenolic compounds at higher concentrations but taking them as sole energy and carbon source for growth. Intensiv screening has been performed in the past for microbes with phenol

degrading properties. They resulted in lists of bacterial strains in the genera *Acinetobacter* and *Pseudomonas*, mold strains in the genus *Geotrichum* and yeast strains in various genera, among them *Candida, Rhodotorula* and *Trichosporon* (1,3). Phenol mineralization is often performed anaerobically by microbial consortia (4), e.g. in methanogenic sediments (5), where phenol is degraded to acetate, methan and carbon dioxide via benzoate (4,5). In contrast, yeasts catabolize phenol under strictly aerobic conditions and strong aeration via its oxidation products. These organisms are able to use phenol as their only energy source, phenol being metabolically comparable with glucose or acetate as carbon source. Both, ortho- and meta-pathways are possible, but the chromosomally encoded ortho-cleavage of the benzol ring is preferred against the plasmid-encoded meta-pathway (6). Above a special threshold concentration - specific for each of the applied strains - phenol is just toxic for the organism and no further metabolism takes place. Below this threshold different experimental parameters determine the rate of degradation so that optimal conditions can be found for decontamination processes (3).

In the present investigation one bacterium: *Pseudomonas putida* and two yeasts: *Rhodotorula glutinis* and *Trichosporon beigelii* were chosen. They will be introduced in some more details below.

Phenol mineralization can be followed by means of manometry, polarography, spectrophotometry, fluorimetry, gas chromatography or high-performance liquid chromatography or nowadays with biosensors, e.g. with immobilized tyrosinase (7). Here, we have chosen calorimetry as a non-specific, non-invasive, general and integrative tool to monitor total heat production and heat production rates during microbial metabolism of aromates. Both, flow- and batch-calorimetric set-ups were applied to meet the various experimental demands. Flow calorimetry with its separation of calorimeter and external fermentor facilitates simultaneous determinations of other growth parameters without disturbing the calorimetric signal, but introduces the problem of decreasing oxygen concentrations in the flow line from the fermentor to the calorimeter (see below). Batch calorimeters, on the other hand, offer the chance to investigate the microbial metabolism even in suspensions with gross particles.

MATERIAL AND METHODS

Microbial Strains

Pseudomonas putida is a gram-negative, polar-flagellated, unicellular bacterium frequently used in degradation experiments of aromatic compounds and in elucidating their possible metabolic pathways (8-10). Strain 548 (ATCC 17514, NCIB 10015) (Deutsche Sammlung der Mikroorganismen DSM/Brunswick) was incubated on a solid complex growth medium at 30 °C. Experiments were run in a synthetic medium with varying amounts of aromates as sole carbon and energy source (11). Initial concentrations ranged from 0 to 333 $mg.l^{-1}$, but some tests were performed with far higher amounts up to 1.5 $g.l^{-1}$ (11).

Rhodotorula glutinis is a wide-spread non-pathogenic yeast isolated from soil, fresh and sea water, plants, food and also from the atmosphere. It forms distinct yellow to red coloured, oval budding cells with no pseudomycel or mycel (advantageous for flow calorimetric experiments!) and is not able to gain energy through fermentation. Strain 70398 (DSM/Brunswick) was cultivated in a universal yeast medium supplemented with various amounts of aromates. In a few experiments a synthetic medium was applied with phenol as sole energy source. Concentrations varied between 0 and 670 $mg.l^{-1}$.

Trichosporon beigelii (also named *T.cutaneum*) is a strictly oxidative yeast with true mycelium, frequently isolated from soil, fresh and sea water, sewage sludge, garden compost and plant detritus. Adapted isolates are able to oxidize phenol and its derivatives and are therefore used in many environmental investigations (12,13) or as immobilized organisms in biosensors (14). The applied *T.beigelii* strain was a friendly gift of Prof.Kraepelin/Technical University Berlin. Since *Trichosporon beigelii* is known to cause a number of severe infections in man and since the used strain was quickly growing around 37 °C, only a few orientating runs were performed with this organism.

Calorimetry

Two different instruments were applied throughout the experiments, both operating at 30 °C. The first was a four-vessel batch calorimeter of the Calvet type (Setaram/Lyon) with a sensitivity of 62.9 $mV.W^{-1}$ and 6 ml stirred suspensions (11). Additionally, a flow calorimeter (type 10700, LKB/Bromma) with a flow-through cell of 0.587 ml, a sensitivity of 54.5 $mV.W^{-1}$ and typical pumping rates around 100 $ml.h^{-1}$ was used for investigations with strong aerations (11). For this end, a small conical 100 ml-fermentor was developed operating in an air-lift mode and guaranteeing a by far better aeration than that possible in batch experiments. The fermentor was connected with the calorimeter via a peristaltic pump and could house different electrodes, mainly for the determination of oxygen tension and pH-value.

Polarography

Since we became aware of the strong decrease of oxygen tension in the flow lines to the calorimeter (15) a second polarographic oxygen electrode (Clark type, Bachofer/Reutlingen or Eschweiler /Kiel) was incorporated in the line directly behind the calorimeter. It showed that at higher cell densities, elevated substrate concentrations and/or reduced pumping rates oxygen became limiting or even zero in the flow line and falsified the calorimetric signals: they were no longer true pictures of the metabolic rates in the fermentor (15). In some experiments a similar batch oxygen electrode was applied simultaneously to determine the microbial respiration rates during growth of the culture. These data were compared with the calorimetrically determined heat production rates.

RESULTS

Growth and Yield

The three applied microbial strains were tested for tolerance against increasing amounts of aromatic compounds and for their ability to grow on these substrates in solid or liquid cultures. Tests were performed by cell counting, photometry and dry weight determinations. These preliminary investigations were followed by the intended calorimetry under various experimental conditions. All three strains showed a sufficient to good growth on phenol in the chosen complex or synthetic media after preculturing and thus adaptation in the presence of phenol. The same holds true for related aromatic compounds like catechol as first metabolite in phenol catabolism, for benzoic acid and for 3-oxoadipic acid. Most experiments were performed at low to moderate aromate concentrations (less than 600 $mg.l^{-1}$) with some orientating runs at initial concentrations up to 1.5 $g.l^{-1}$. A drawback of this approach was that the metabolic activities of the cultures might change significantly from day to day without any plausible reason, a phenomenon already described in the literature (6). This leads to a larger scatter in the results of metabolic rates and thus heat dissipation as function of aromate concentration.

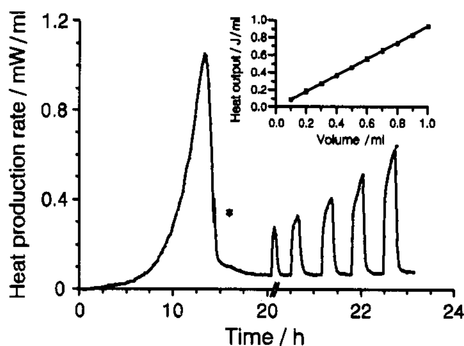

Fig. 1 Flow-calorimetric power-time curve of *Pseudomonas putida* growth on phenol as sole energy source. After termination of growth small amounts of phenol were added in a ballistic manner. The inset exhibits the linear response of total heat production to the added amount of phenol. Note: The weakly indicated second peak (*); the change of time scale after 20 h.

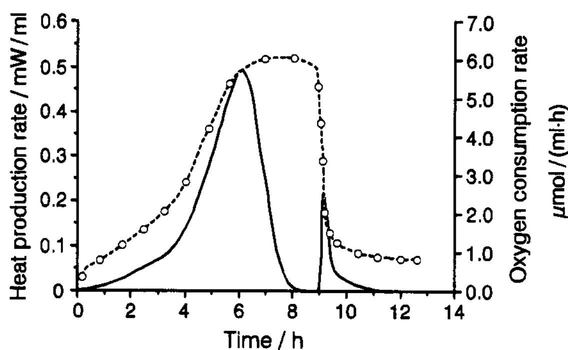

Fig. 2 Simultaneous determinations of heat production rate (full line) and oxygen consumption rate (dashed line) during growth of *Pseudomonas putida* on phenol.

In the case of synthetic media when the aromate serves as sole energy and carbon source linear relationships were established between the initial aromate concentration and the final cell count, the cell yield (g of cells per g of substrate) or the total heat output in calorimetric experiments. A final optical density of 1.90 per g phenol per liter was found in the case of *P.putida* which can be transformed to a metabolic yield of 0.890 g dry weight per g phenol. This figure corresponds well to data seen in the literature (6).

A quick and efficient approach to determine the energetic yield of an aromate is to add increasing amounts of it at the end of a usual calorimetric growth experiment. Figure 1 depicts this situation in the second part of the graph after "20 h". The inset shows the linear response in "J per milliliter medium" to the addition of increasing volumes of phenol (1.0 $g.l^{-1}$). Similar experiments were performed with catechol and benzoic acid. The usual way to determine energetic yields directly from the calorimetric growth curves renders corresponding values of 17.8 $kJ.g^{-1}$ phenol. As there are no earlier energetic investigations of aromates this figure cannot be compared with data from the literature, but it fits well in the energetic balance.

While the polarographic electrodes in the fermentor and in the flow line behind the calorimeter monitor the oxygen concentrations in these two places, a third external sensor was applied to determine the oxygen consumption rate in parallel to the heat flow measurements. To this end 1 ml aliquots were transfered from the fermentor to the electrode container and intensively aerated. Figure 2 exhibits the results in the case of *P.putida*. It shows that oxygen consumption in the fermentor still proceeds with maximum rate while heat production already decreases to zero. At the moment when the second peak appears after a short neutral thermal phase, the respiration rate drops also. Comparing respiratory and heat production rates leads to a figure of 3.4 mol O_2 per mol phenol, lying between a value of 7 for a complete mineralization of phenol to water and carbon dioxide and 3.3 for intensiv growth.

Calorimetric Growth Experiments

The calorimetric curves reflect the various kinetic aspects during microbial growth in a liquid culture with substrate inhibition in the beginning (at high aromate concentrations) and with reduced rates at the end due to a decreased substrate availability as will be discussed below (see Kinetics). Typical growth curves are given in Figs. 1 to 3. The ideal calorimetric growth curve is presented in Fig. 1 showing the initial lag phase, a long exponential phase, where heat output follows increase in biomass up to a maximum, and a subsequent steep decline (in the beginning stationary phase) due to a Michaelis-Menten kinetics at low substrate concentrations. Figures 3 and 4 are modulations of this shape when decreasing oxygen concentrations in the flow line induce a reduction of metabolism and a more or less pronounced second maximum. The oxygen curve in Fig. 3 indicates the strong decrease in metabolism and the return to saturation after substrate exhaustion.

The area under the curve corresponds to the total dissipated heat and leads to the energetic yield, while the momentary heat production rate equals the metabolic rate and thus the substrate flux. The heat produced up to a certain moment is due to the consumed substrate so that the remaining substrate concentration can be easily calculated for each moment. In this way, a single calorimetric curve renders all necessary data for establishing kinetics of aromate mineralization. Moreover, aromate uptake by the cells can be directly estimated from the heat production rates under the sound assumption that no aromatic compounds are accumulated in the cells. Flow calorimetric ex-

periments render e.g. values of 0.61 mg phenol per hour and mg dry cell weight in good agreement with data from the literature (6,16).

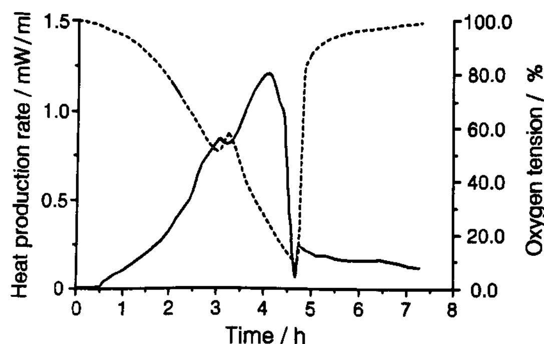

Fig. 3 Flow-calorimetric power-time curve of *Trichosporon beigelii* growth on phenol (full line). Oxygen tension directly behind the flow-through cell (dashed line) indicates the dramatic changes in oxygen concentration in the calorimeter during progressive microbial growth and metabolism.

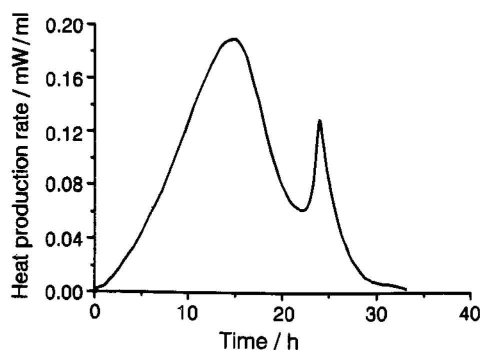

Fig. 4 Flow-calorimetric power-time curve of growth of *Rhodotorula glutinis* on phenol under reduced pumping rate and thus oxygen limitation in the flow line.

Energy Balances

Energy balances for growth on aromatic compounds can easily be established from the calorimetric experiments. They are composed of the combustion heats of substrate, metabolic products and cell mass and the reaction enthalpy. Combustion heats of cells - if not experimentally determined - can be calculated from a generalized C-molar formula of cell mass which reads $CH_2O_{0.52}N_{0.23}$ for *P.putida* (17) and in a mean form $CH_{1.68}O_{0.49}N_{0.17}$ for yeasts (18). One approach is to use the formula of Dulong in the modification by Wang *et al.* (19) to determine the combustion heat of organic substances from their relative composition of C, H and O. A second possibility applies the reductance degree of an organic substance and a mean figure of -115 kJ per degree of reductance. Depending upon the chosen assumptions, growth on aromatic compounds is energetically more or less well balanced. In the case of phenol we could estimate a deviation of only -2.4 % in the energy balance (11).

Kinetics

It is known from previous experiments (20) and from the literature (6,21) that aromates inhibit their own enzymatic degradation at higher concentrations and follow a usual Michaelis-Menten kinetics at lower ones. This behaviour can be described by a Haldane kinetics $v = v_{max} \cdot S/(K_m + S + S^2/K_i)$, where v is the reaction rate, v_{max} the maximum rate, S the substrate concentration and K_m and K_i constants equal to those substrate concentrations where $v = v_{max}/2$. The left part of Fig. 1 shows a growth curve that can be described by a Haldane kinetics. The prolonged lag phase is partly due to the metabolic inhibition at high substrate concentrations.

This Haldane kinetics holds true for the situation in the well aerated fermentor with constant oxy-

gen concentration. But with its low solubility of 0.24 mmol.l^{-1} at 30 °C and a stoichiometric ratio of 3.3 mol oxygen per mol phenol oxygen soon becomes limiting in the flow line to the calorimeter. A Michaelis-Menten kinetics can be applied to describe the respiratory rate at constant phenol concentrations: $v = v_{max} \cdot S'/(K_m + S')$ where S' indicates the oxygen concentration, all other symbols as above. The product of these two kinetics - Haldane for phenol and Michaelis-Menten for oxygen - approximated the observed power-time curves well and made unexpected predictions which could be checked experimentally later on. Figure 5 presents such a calorimetric graph calculated with the mathematical model of a double kinetics. It shows the well known growth phases and the main peak with the subsequent decline, but also a late second peak after a neutral thermal period. Such a behaviour was already seen in Fig. 2 with a very distinct second peak. Its appearance can be shifted by choosing the experimental or mathematical parameters, so that positions as in Fig. 1, 3 or 4 can be obtained.

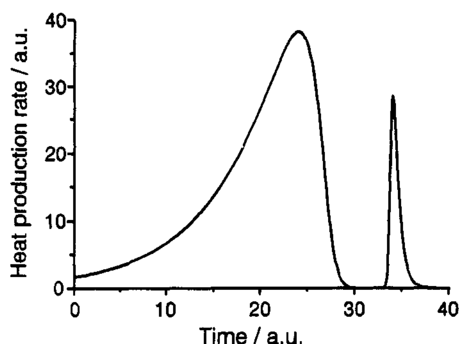

Fig. 5 Mathematical calculation of a calorimetric growth curve after the double kinetics model, in arbitrary units (a.u.). Compare with Fig. 2.

The message from these kinetic analyses is that the calorimetric signal not always renders a true picture of the metabolic situation in the fermentor, that the obtained total heat output might be considerably reduced due to lack of oxygen in the lines, that high flow rates have to be applied in such experiments and that it can be advisable to perform not only a thermal desmearing of the calorimetric curves, but a "metabolic" one, too.

RÉSUMÉ

Aromatic compounds are widely distributed in the environment and often hazardous to living systems even at low concentrations. It is, therefore, necessary to look intensively for natural ways to mineralize these compounds by microorganisms, at best in on-site techniques. Various microbial cohorts or pure strains were isolated in the past from contaminated areas, enriched and studied in the laboratory. It will be a future task to increase their metabolic activity on and their tolerance against the various aromatic substrates found in the environment.

Batch- and flow-calorimetric experiments are appropriate means to proceed in this direction. They offer the chance for bench scale tests and optimization of degradation processes, to find the most suited strains and conditions, to construct effective artificial cohorts of various microbes and to release them to polluted places. The mentioned drawbacks of the flow-calorimetric technique help us

lease them to polluted places. The mentioned drawbacks of the flow-calorimetric technique help us to understand the importance of high oxygen availability and good aeration for those strains which are not able to fermentate aromatic compounds anaerobically as some methanogenic cohorts do. In total, calorimetry should be applied more often in environmental investigations.

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