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Calorimetric investigations of bacterial growth on phenol–efficiency and velocity of growth as a function of the assimilation pathways[1]

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Abstract

Aromatic compounds (e.g. phenol) are known to be assimilated via two pathways initiated by the cleavage of the ring structure in the intradiol (*ortho*) or extradiol (*meta*) position of catechol intermediate. There is some biochemical and physiological evidence that the *ortho* pathway is energetically more efficient than the *meta* pathway, i.e. the former results in higher growth yield than the latter. *Alcaligenes eutrophus* JMP 134 (now, *Ralstonia eutropha*) possesses both possibilities. As the growth efficiency corresponds to the heat production, calorimetric measurements can help determine (i) which of the two pathways is activated under what conditions and (ii) whether or not the upper limit of carbon conversion into biomass is reached. To answer these questions, a feeding system for a fermentation calorimeter was constructed which allowed to adjust dilution rates, the concentration of substrates and the composition of feed as a function of time. It was shown that during chemistatic growth *A. eutrophus* uses the *ortho* pathway up to a dilution rate of 0.25 h^{-1}. At this point the rate of heat production changed, indicating a shift to the *meta* pathway. Phenol can be used as a sole source of carbon and energy for growth. But the energy, which can be made available, does not reach to assimilate the phenol-carbon consumed. This means that phenol is deficit in energy. Formate can balance the carbon/energy ratio and provides energy to increase phenol-carbon conversion. By adding formate, the yield coefficient grew from 0.56 g dry mass/g phenol to 1.03 g/g, and in the end it was found that the net gain of energy from the formate is not constant. © 1998 Elsevier Science B.V.

Keywords: *Alcaligenes eutrophus*; Aromatic compounds; Assimilation pathways ; Calorimetry; Growth efficiency

1. Introduction

Various metabolic pathways are often established in an organism to utilize a certain organic compound as a source of carbon and energy for growth and multiplication. Phenol, for instance, can be assimilated through the *ortho* or *meta* pathway [1]. *Alcaligenes eutrophus* JMP 134 (now, *Ralstonia eutropha* [2]) is known to possess both metabolic routes. The *ortho* pathway is energetically more efficient than the *meta* cleavage and operates at both low phenol concentrations and low growth rates [3,4]. If *A. eutrophus* is grown chemistatically on phenol and the dilution rate is increased, the *meta* pathway is activated [5]. This shift from the one to the other pathway ought to be possible to monitor via heat production. Phenol as a

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chemo-organo-heterotrophic substrate can be used as a source of carbon and of energy for growth. However, phenol is carbon excess and, thus, phenol must undergo biochemical combustion to generate biologically usable energy. This amount is proportional to the energy liberated as heat. If this amount, expressed as a biological energy equivalent, is provided by an extra energy source (e.g. formate) then there is an increase in the conversion of phenol-carbon into biomass. The difference between the combustion enthalpy of formate and the experimental reaction enthalpy is a measure of the energy utilized biologically.

The gradients for the increasing dilution rates are generated by a peristaltic pump and checked by a personal computer running special software (H.J. Große, unpublished). The increments in the dilution rate can be controlled with a sensitivity of $5\times10^{-4}\,h^{-1}$.

The formate is added by a special system. The mixture of phenol C_P^0 and formate C_F^0 is removed at a constant flow F_2 from a mixing flask. The mixing flask is filled from a reservoir flask at a constant flow F_1. The course of formate concentration over time follows from the mass balance:

$$C_F^0 = C_F^V - \left[(C_F^V - C_F^M)\left(\frac{V_0 + (F_1 - F_2)t}{V_0}\right)^{(F_1/(F_2-F_1))}\right]$$

if	$F_2 = 2F_1$	then	linearly increasing function
if	$F_2 = F_1 = F$	then	$C_F^0 = C_F^V - \left[(C_F^V - C_F^M)e^{-(F/V)t}\right]$
if	$F_2 > 2F_1$	then	potentially increasing function
if	$F_2 < 2F_1$	then	asymptotically increasing function

Calorimetry appeared to be particularly suitable for the purpose of checking the above-mentioned statements and investigating the postulated relations in detail, as it is precise and provides in vivo response immediately. We opted to use the Berghof fermentation calorimeter because it is (i) especially designed for biotechnological purposes, (ii) highly sensitive and stable over a long time, and (iii) commercially available.

2. Experimental

2.1. Improvement of the fermentation calorimeter

Each bench-scale calorimeter has problems with baseline stability over a few days or weeks [6]. However, implementing different steady states by using continuous cultivation is a time-consuming procedure. One way out is provided by transient-state-cultivation. A quasi-stationariness can be assumed if the changes of the dilution rate ΔD are small and those of the holding time Δt are large [7].

C_F^V, C_F^M formate concentration in the reservoir flask, in the mixing flask at time zero

V_0 volume of the mixing flask at time zero.

This approach allows high precision and is highly flexible with respect to the choice of the formate gradients.

The heat production was monitored by a fermentation calorimeter (BFK, Berghof, Germany) which is an isothermal heat-flux calorimeter with power compensation and consists of a glass vessel with a working volume of 2 l surrounded by a double-walled adiabatic glass jacket. The calorimeter is described in detail elsewhere [8]. A diagram of the calorimetric system with the additional improvements is given in Fig. 1.

2.2. Organism and culture conditions

All experiments were done with *A. eutrophus* JMP 134. Precultivation was done at 30°C in shaking flasks containing 200 ml minimal medium. Phenol was gradually supplied (30 mg portions) to reach a biomass concentration of ca. 1 g/l. The minimal medium was composed of (mg/l) NH_4Cl 1520, KH_2PO_4 270,

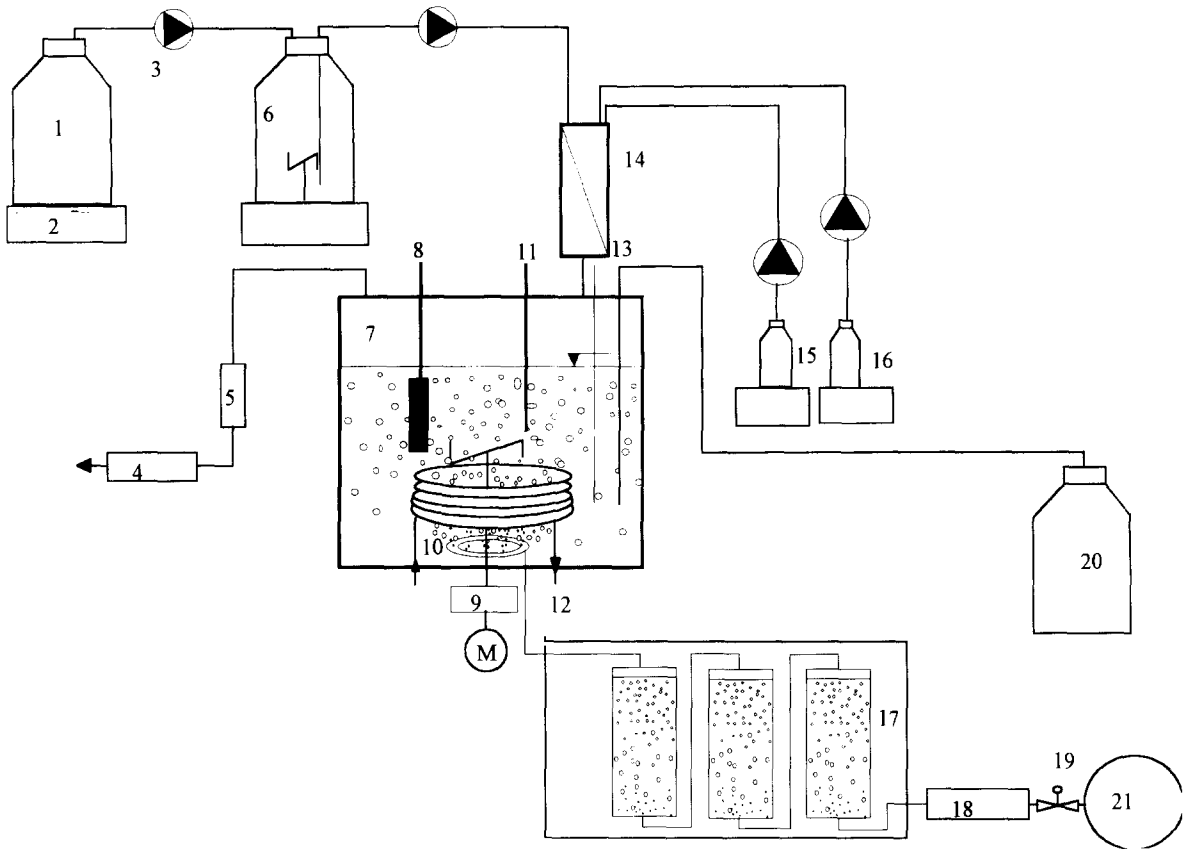

Fig. 1. Experimental setup: 1, reservoir flask; 2, balances; 3, peristaltic pumps controlled by a computer; 4, exhaust gas analysis; 5, gas drier; 6. mixing flask; 7, fermentation calorimeter surrounded by an adiabatic jacket; 8, thermistor; 9, torque measurement; 10, aeration ring; 11, thermopile; 12, heating/cooling unit; 13, sampling probes; 14, heat exchanger; 15, acid; 16, base; 17, humidification and temperature adjustment; 18, flowmeter; 19, electrical valve; 20, fermentor effluent storage; and 21, air.

$MgSO_4 \cdot 7H_2O$ 71.2, $CaCl_2 \cdot 6H_2O$ 5.5, $FeSO_4 \cdot 7H_2O$ 4.98, $CuSO_4 \cdot 5H_2O$ 0.785, $MnSO_4 \cdot 4H_2O$ 0.81, $ZnSO_4 \cdot 7H_2O$ 0.44, and $NaMoO_4 \cdot 4H_2O$ 0.25. The inoculum volume was ca. 200 ml. *A. eutrophus* was chemostatically grown on minimal medium, in one case, with phenol as the sole source of carbon and energy and, in the other, on a mixture of phenol with formate as an auxiliary energy source. The pH of 7.0 was kept constant by titration with either 1 M NaOH or 1 M HCl. The growth temperature was 30°C. The temperature of all feeds was adjusted to reactor temperature before they entered the vessel. The fermenter was aerated with 100 N l/h air, which was saturated with water at reactor temperature to avoid heat loss by evaporation. The agitation rate was constant at 900 rpm to avoid concentrations of oxygen under 30%.

2.3. Analysis

Biomass concentrations were immediately measured after the removal of the samples via the optical density at 700 nm and by determination of the washed pellets. Separation of the biomass from the culture broth within ca. 10 s was achieved by the direct ultrafiltration of the efflux from the reactor. The sampling technique has been described elsewhere [9]. Phenol concentration was detected by HPLC on Nucleosil 100 PR 18 column (250×3 mm inner diameter, Knauer-Säulentechnik, Berlin, Germany) with 70% acetonitrile in water containing 0.1% acetic acid anhydride as the eluant, while the formate was measured ion-chromatographically on an IonPac AS4-SC column (250×4 mm i.d. Dionex, Sunnyvale/Calif. USA). Harvested cells were washed twice with phos-

phate buffer (0.1 M, pH 7.5) and disrupted by three passages through a French pressure cell press (Aminco, Silver Sring, USA) at 124.1 MPa and ca. 4°C. Particle-free supernants were obtained after centrifugation at 15 000×*g* for 20 min at 4°C (centrifuge 5403, Eppendorf, Germany). Catechol 1,2-dioxygenase activity (key enzyme of the *ortho* pathway) was determined by monitoring the formation of *cis,cis*-muconic acid from catechol at 260 nm, and catechol 2,3-dioxygenase activity (key enzyme of the *meta* pathway) was measured via following the formation of 2-hydroxymuconic acid semialdehyde from catechol at 375 nm [10,11]. Protein was assayed by the Bradford reaction [12]. The concentration of oxygen in the exhaust gas was detected electrochemically and the concentration of CO_2 was measured via thermal conductivity (System 27442, Orbisphere Laboratories, Switzerland).

3. Results and discussion

3.1. Phenol as the sole energy and carbon source

The heat production curves of the transient-state cultivation of *A. eutrophus* grown on phenol (Fig. 2) changed at the dilution rate of 0.25 h^{-1}. At this point its metabolism shifts from the *ortho* to the *meta* cleavage pathway, as indicated by the expression of the respective catechol dioxygenase (Fig. 3).

Balance equations for the conversion of phenol into biomass suggest that the *ortho* pathway is energetically more efficient than the *meta* pathway:

ortho

$$0.22\text{Phenol} + 0.25NH_3 + 0.44O_2 + 0.06H_2O + 2.54\text{ATP} \rightarrow CH_2O_{0.5}N_{0.25} - 0.24\text{NAD(P)H} + 0.25FADH_2 + 0.25CO_2$$

meta

$$0.33\text{Phenol} + 0.25NH_3 + 0.67O_2 + 0.83H_2O + 3.43\text{ATP} \rightarrow CH_2O_{0.5}N_{0.25} + 0.88\text{NAD(P)H} + 0.25FADH_2 + CO_2$$

If this is true, a higher portion of Gibbs energy is dissipated as heat by using the *meta* pathway than the *ortho* pathway. In fact, at low dilution rates, i.e. at low actual phenol concentration, phenol is assimilated via the *ortho* pathway, whereas at high dilution rates the *meta* pathway is activated. Thus, the whole growth

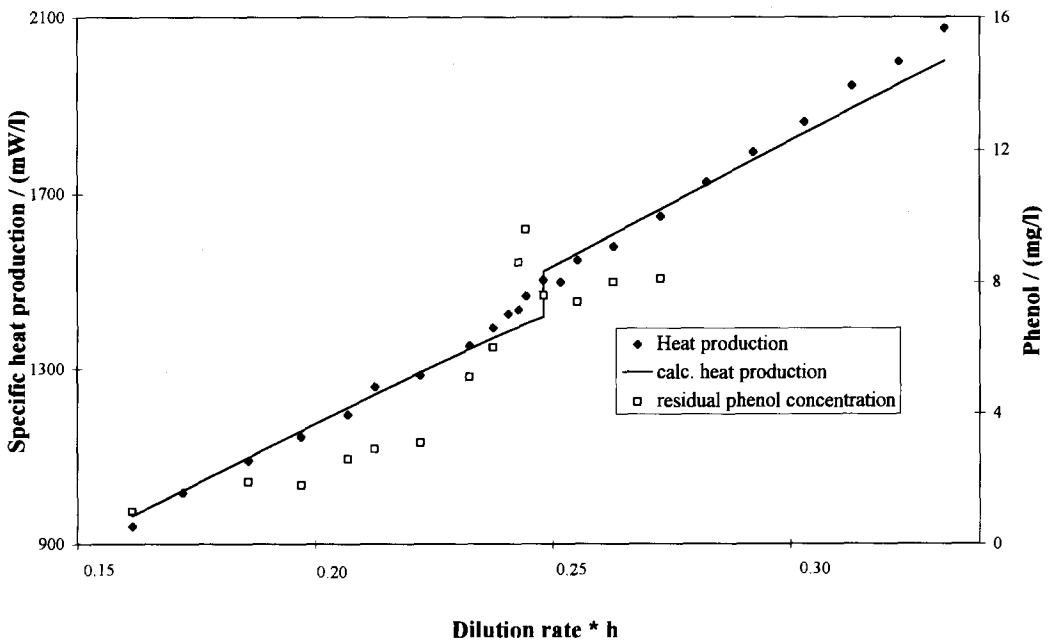

Fig. 2. Heat production curve of *A. eutrophus* during growth on phenol; the heat production was calculated by the Y_{ATP}-concept (*P/O*=2, maintenance coefficient=0.3 μmol mg^{-1} h^{-1}, shift from the *ortho* pathway to the *meta* pathway at the dilution rate *D*=0.25 h^{-1}).

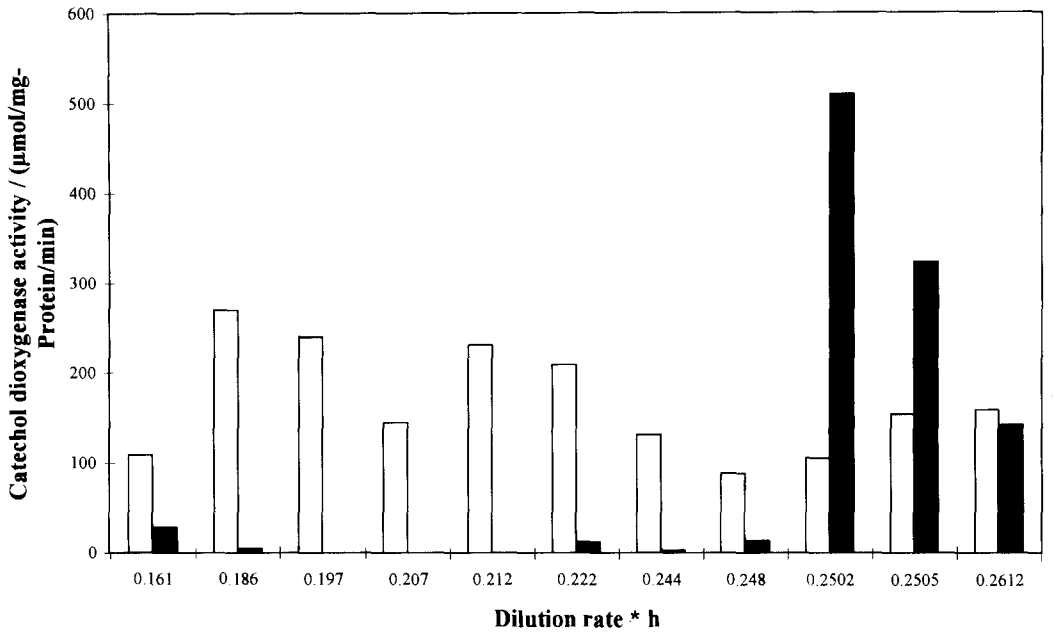

Fig. 3. Catechol dioxygenase activity: open columns catechol 1,2 dioxygenase; filled columns catechol 2,3 dioxygenase (the key enzyme of the *meta* cleavage).

curve is characterized by two sets of kinetic parameters. After the exhaustion of the capacity of the *ortho* assimilation pathway at 0.25 h^{-1}, the *meta* pathway was introduced. The wash out occurs at 0.39 h^{-1} (not shown). The existence of two different metabolic pathways is supported by the K_S-values with phenol: a value of 7 µM was obtained for the *ortho* pathway in contrast to a value of ca. 60 µM after introduction of the *meta* pathway (data not shown).

On the basis of the above-mentioned reaction equations and the Y_{ATP}-concept [13,14] (P/O=2), a power time curve can be calculated which closely resembles the experimentally obtained heat production curve (Fig. 2).

The yield coefficient was Y_{ortho}=0.7 g/g at a dilution rate of 0.22 h^{-1}. This value corresponds to the theoretically possible yield coefficient of Y_{ortho}=0.76 (P/O=2).

3.2. Formate as auxiliary energy source

During growth on phenol, part of the consumed phenol must be dissimilated to generate energy for the incorporation of phenol into biomass. This part of phenol can be substituted, for instance, by formate which is oxidised by a formate dehydrogenase.

$$HCOOH + NAD^+ \rightarrow CO_2 + NADH + H^+$$

The energy of the liberated reducing equivalents is transduced via an electron transport chain into ATP. The amount of formate which is necessary to reach the upper limit of carbon conversion efficiency or the maximum growth yield (and the mixing ratio of formate to phenol) is determined by the efficiency of the oxidative phosphorylation, i.e. the P/O quotient. The biomass increase ought to be linearly proportional to the formate added. This is actually true. However, three different phases of the heat production were observed (Figs. 4 and 5):

1. The first phase (at time zero up to the molar mixture of sodium formate to phenol of 4.3 : 1) is characterized by an energetically inefficient usage of formate. The heat production increased from 560 up to 800 mW/l. The gradient of the yield coefficient of 0.006 g/g h corresponds to these values.
2. In the second phase, the gradient of the yield coefficient increased to ≈0.011 g/g h. The maximum yield coefficient of 1.03 g dry mass/g phenol

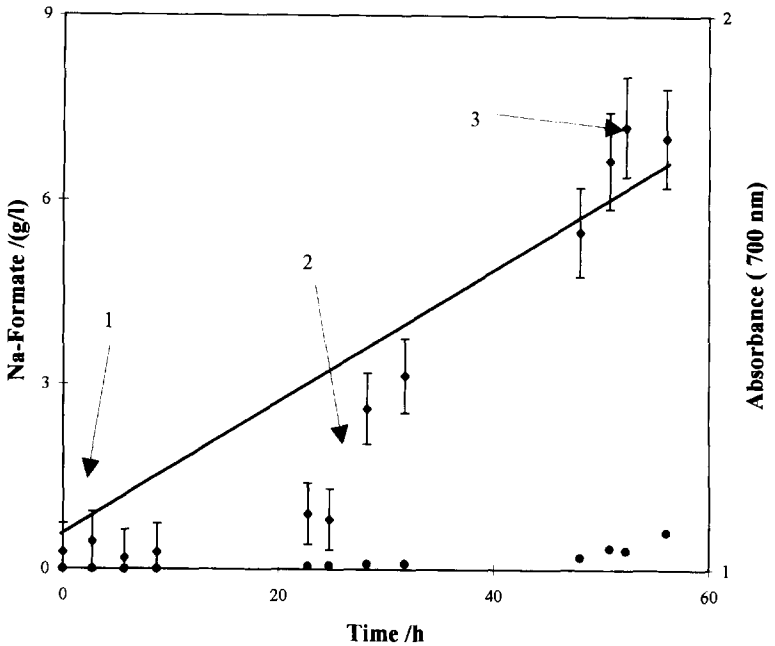

Fig. 4. Growth yield pattern of *A. eutrophus* during continuous cultivation on phenol and simultaneous supply of a linear gradient of formate. The culture was chemostatically precultivated on 1 g/l phenol and 0.2 g/l sodium formate. At $t=0$ the gradient was generated. The concentration of the format input is indicated by the solid line. The black points stand for the residual formate concentration in the fermentor and the points with an error bar stand for absorbance at 700 nm.

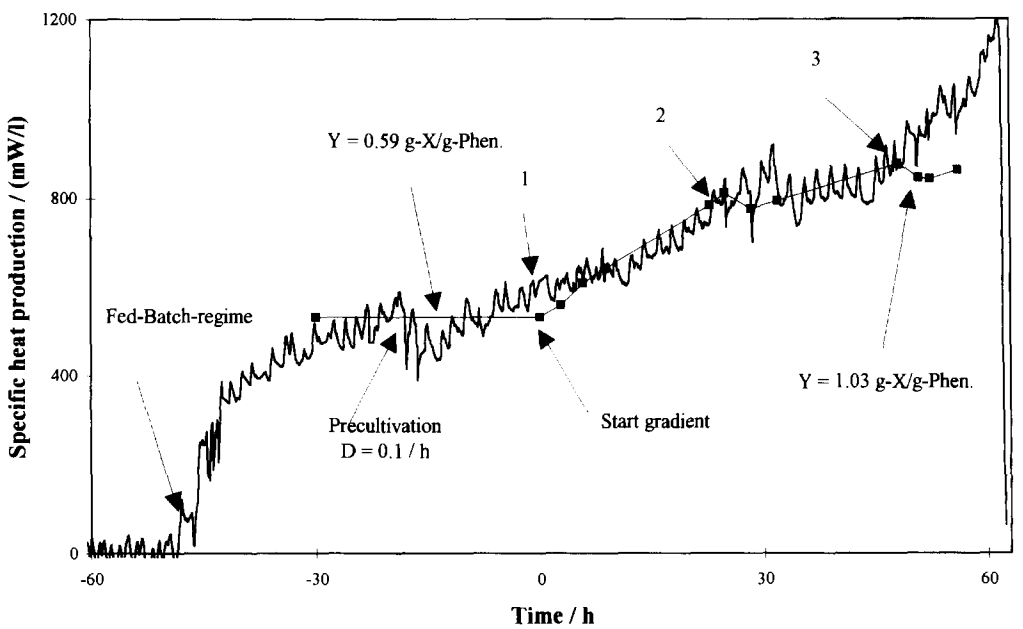

Fig. 5. Formate concentration linked heat production of *A. eutrophus* during growth on phenol. The solid line indicates the experimental heat production and the straight line stands for the calculated heat production from the consumption of phenol and formate and the production of biomass.

was reached at a molar mixture of sodium formate to phenol of 8 : 1. The theoretically possible yield coefficient Y_{ortho}=1.22 g/g ought to be attained at a molar mixture of 6 : 1 and a yield increasing to 0.013 g/g h, calculated on the basis of the Y_{ATP}-concept with a *P/O* quotient of 2. This discrepancy can be explained if a *P/O* quotient of 1.5 is assumed.

3. In the third phase, the heat production grew dramatically. This may indicate uncoupling effects. As long as the capacity for the oxidation of formate is exhausted, formate accumulates in the medium. If the concentration exceeds a certain threshold, formate can operate as an uncoupler. The energy contained is wasted in the form of heat. If a cessation or a decrease of growth actually occurs, the heat production will immediately increase and the biomass concentration decrease slowly. Consequently, the calculated heat production curves on the basis of the input–output streams should be lower than the experimental curves.

4. Conclusions

A. eutrophus JMP 134 (now *R. eutropha*) is known to assimilate phenol via different pathways. The *ortho pathway* is used at low phenol concentrations and the *meta* pathway at high concentrations. As both routes differ bioenergetically, it ought to be possible to monitor the shift from one pathway to the other via the liberation of heat. Calorimetric devices are basically suitable although commercially available calorimeters are not. Therefore, we constructed a feeding system which allowed increasing dilution rates and gradients of substrate concentration to be adjusted. By means of the "improved" calorimeter, we were able to study the relation between the efficiency and growth rate on the one hand, and the energetic utilization of an additional substrate, on the other. We monitored the kinetics of the heat production and found that the slope changed. This shift corresponds to the expression of both pathways. While the key enzyme of the *ortho* path (1,2 dioxygenase) could be measured over the whole range considered, the key enzyme of the *meta* route (2,3 dioxygenase) was introduced at a dilution rate of 0.25 h^{-1}. Evidently, the capacity of the *ortho* pathway is exhausted. *A. eutrophus* is able to overcome the limit by activating the faster but less efficient *meta* route. Thus, the growth rate behaviour is described by two sets of kinetic parameters (*ortho*: μ_{max}=0.26 h^{-1}, K_S=7 μM; *meta*: μ_{max}=0.39 h^{-1}, K_S=60 μM).

With growth on an energy deficient substrate, the part of substrate consumed for energy generation can be replaced by an extra energy source, such as formate. In the case of *A. eutrophus*, it cannot serve as a carbon source for growth. The amount of formate added is proportional to the yield increase. Surprisingly, the energy which can be made available does not linearly depend on the phenol/formate ratio.

As we demonstrated by using a highly sensitive calorimeter, it is possible to examine biochemically postulated pathways. Moreover, variations in the assimilation of substrates can be elucidated and the efficiency of energy conservation can be determined.

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