Substrate Inhibition of the Growth of Bacterium NCIB 8250 by Phenol

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SUMMARY

Examination of the growth kinetics of Bacterium NCIB 8250 in a two-stage continuous culture showed that growth of this organism on phenol could be accounted for by the Haldane function for substrate inhibition. The magnitude of the constants ($K_s < 1$ mg/l; and K_i , 110 mg/l) was such that inhibition was the dominant factor governing the growth of the organism even at substrate concentrations less than 10 mg/l. The maintenance requirement of the organism was high (0.24 to 0.28 g/g h), probably owing to the energy requirement for rupture of the aromatic nucleus of the substrate.

The influence of such kinetics on the treatment of inhibitory wastes by the activated-sludge process is discussed. Extreme sensitivity of the experimental system to slight changes in operating conditions was explicable by a mathematical model using constants found in the investigations.

INTRODUCTION

As a step towards the elucidation of the biochemical and microbial interactions involved in the treatment of waste waters by the activated-sludge process, a study was made of some of the bacteria concerned in the treatment of carbonization waste liquors. This choice was made since the range of substrates available in this system is limited, and a simpler bacterial ecosystem was expected than in the case of sewage treatment. Of the ten strains isolated from a laboratory-scale plant treating a carbonization waste liquor, only two were capable of growth on phenol and were selected for further study.

One of these strains was identified as a member of the *Acinetobacter-Moraxella* group, very similar in properties to Bacterium NCIB 8250 (Sebald & Véron, 1963; Véron, 1966; Fewson, 1967). When, during preliminary experiments, the original strain was lost, the work was continued with Bacterium NCIB 8250.

Operation of the activated-sludge process to produce a low substrate concentration in the effluent depends on settlement of the organisms leaving the reactor, and subsequent recycle of these organisms (thus reducing the specific growth rate to a value below the dilution rate of the system). The study of pure cultures under conditions obtaining in the activated-sludge process is made difficult by this need to concentrate the culture leaving the reactor to allow recycling. This difficulty was overcome by use of a two-stage continuous-culture apparatus, where culture from the first stage, operated as a normal chemostat, was pumped to the second stage, together with a supply of fresh medium. In this way the second stage was analogous to the reactor of an activated-sludge plant, with the specific growth rate of the organisms less than the dilution rate.

Fig. 1. Flow diagram of two-stage continuous-culture apparatus.

METHODS

Organism. Bacterium NCIB 8250 was subcultured monthly and maintained on nutrient agar slopes stored at 5 °C.

Medium. Constituents added (g/l deionized distilled water) were: KH_2PO_4 , 2.0; $(\text{NH}_4)_2\text{SO}_4$, 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; phenol to required concentration, pH adjusted to 6.8 with m-NaOH . Medium was made in 14 l batches and sterilized by filtration through a Sartorius membrane filter (mean pore size, 0.2 μm ; V. A. Howe & Co Ltd.) and filter-sterilized ferric chloride solution added to give a concentration of 10 mg/l.

Salts solution. In some experiments the ferric chloride was replaced by a salts solution containing (g/l deionized distilled water): $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.1; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.1; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5; di-sodium salt of EDTA, 3.3. Portions (10 ml) were added to each litre of medium.

Apparatus. The two-stage continuous culture apparatus (Fig. 1) consisted of two 'Quick-fit' culture vessels (FV 1 L and FV 500) fitted with multi-socket flat-flanged lids (MAF 2/2 and MAF 1/75). (Jobling Laboratory Division, Stone, Staffordshire). Each fermentor was aerated by a diffuser (1 vol. of sterile air/vol. of culture/min) and stirred by a Teflon-covered magnet. The operating volume of the vessels could be adjusted by changing the level of the overflow. Each overflow was fitted with a sampling device similar to those used by Curds & Cockburn (1971), modified to take 1 oz screw-cap bottles. Connexions between the fermentors and medium reservoirs were made with silicone-rubber tubing and the whole apparatus was sterilized by autoclaving at 105 kN/m² for 1 h.

Sterile medium was pumped continuously from a 20 l storage reservoir to the first-stage fermentor by means of a fixed-speed 'Perpex' peristaltic pump (LKB Instruments Ltd, South Croydon, Surrey). Some of the culture from the first stage was pumped continuously through one or more channels of a 'Quickfit' six-channel variable speed peristaltic pump to the second stage, the remainder of the effluent from the first stage being discharged to waste. In addition to culture from the first stage, a continuous supply of fresh medium was pumped from another

20 l storage reservoir to the second stage through one or more of the remaining channels of the 'Quickfit' pump. By changing the number of channels used for each stream, or by using pump tubes with different internal diameters, the concentrations of micro-organism and substrate entering the second stage could be adjusted. The apparatus was maintained at 20 ± 0.5 °C in a water bath.

Analyses. Phenol was determined by the 4-amino phenazone method of Ochynski (1960).

Bacterial concentration. The bacterial dry weight was determined by filtration of 10 ml of sample through a washed (2×10 ml membrane-filtered water) and dried Oxoid membrane filter. The sample was then washed with 2×5 ml membrane-filtered water, and dried at 105 °C for 1 h. The membranes were weighed before and after the two drying periods with a similarly treated membrane as tare, through which 10 ml of membrane-filtered water was passed in place of the sample.

RESULTS

The first-stage fermentor was operated at a constant dilution rate to provide a source of bacteria for the second stage. The second stage was operated over a range of dilution rates and it was assumed that steady states had been reached when there had been a minimum of three vol. changes during which bacterial and substrate concentrations did not change significantly. Concentrations of bacteria and substrate entering the second stage were calculated from the concentrations in the first stage and in the medium reservoir and the flow rates. Thus

$$\text{bacterial concentration entering second stage} = x_{02} = \frac{f_{12} x_1}{f_{02} + f_{12}},$$

where f_{12} is the rate of flow of culture from first to second stage (ml/h), f_{02} is the rate of flow of fresh medium to the second stage (ml/h), and x_1 is the concentration of bacteria in the first stage (mg/l).

$$\text{Substrate concentration entering second stage} = s_{02} = \frac{f_{02} s}{f_{12} + f_{02}},$$

where s is the concentration of substrate in the fresh medium supplied to the second stage (mg/l). (Since the concentration of substrate leaving the first stage was below the detectable limit of 1 mg/l, it was not necessary to make allowance for substrate in this flow when calculating the concentration entering the second stage.)

With a second-stage culture volume of v_2 , the dilution rate of the second stage D_2 (h^{-1}) was given by $(f_{12} + f_{02})/v_2$ and the specific growth rate of the culture in the second stage could then be determined from the relationship

$$\mu_2 = D_2 \frac{x_2 - x_{02}}{x_2}, \quad (1)$$

where μ_2 is the specific growth rate (h^{-1}) and x_2 is the bacterial concentration in the second stage (mg/l).

The concentration of bacteria entering the second stage was maintained in the range 40 to 70 mg/l (nominal value 52 mg/l) and substrate concentration in the range 226 to 236 mg/l (nominal value 229 mg/l). Variations in these values were caused by changes in the pumping rate of the two channels in the Quickfit pump and by some growth which occurred on the walls of the first stage fermentor from time to time. Below a dilution rate of 0.37 h^{-1} in the second stage the culture remained stable, and substrate could not be detected; in the range 0.37 to 0.44 h^{-1} the culture became more susceptible to variations in input bacterial concentration, which could cause the substrate concentration to switch between being

Table 1. Yield constants (Y_2) found for second-stage fermentor operated at various dilution rates (D_2) during substrate-inhibited growth of *Bacterium* NCIB 8250 on phenol ($s_2 > \sqrt{(K_i K_s)}$)

Organisms and substrate are entering at flow rates f_{12} and f_{02} and concentrations x_1 and s_{02} and leaving at concentrations x_2 and s_2 . First-stage dilution rate D_1 , 0.12 h^{-1} ; substrate concentration entering first stage s_{01} , 365 mg/l ; volume of first stage, 700 ml ; volume of second stage, 200 ml .

x_1 (mg/l)	f_{12} (ml/h)	f_{02} (ml/h)	D_2 (h^{-1})	s_{02} (mg/l)	x_2 (mg/l)	s_2 (mg/l)	Y_2
129	19	24	0.22	225	148	< 1	0.41
115	39	51	0.45	228	67	154	0.24
111	39	51	0.45	228	68	153	0.27
126	37	50	0.435	231	144	10	0.39
162	37	50	0.435	231	145	23	0.37
142	37	50	0.435	231	124	37	0.33
130	38	51	0.445	231	76	167	0.33
124	38	51	0.445	231	70	160	0.25
103	30	44	0.37	—	67	95	—
98	29	43	0.36	—	185	< 1	—
91	29	44	0.365	—	72	74	—

undetectable and a high value. With bacterial and substrate concentrations maintained at the nominal values a dilution rate of 0.44 h^{-1} was critical, below this value substrate was not detectable but an increase from 0.44 to 0.45 h^{-1} caused an abrupt increase from 10 to 150 mg/l .

Growth rate

Steady-state specific growth rate and substrate concentration (Table 1) were plotted according to the Haldane (1930) function, which takes the form

$$\mu_s = \frac{\mu_{\max}}{1 + K_s/s + s/K_i} \quad (2)$$

where $\mu_{\max}$ is the maximum growth rate in the absence of inhibition; s is the steady state substrate concentration (mg/l); K_s is the saturation constant, the lowest substrate concentration (mg/l) at which, in the absence of inhibition, the specific growth rate is half the maximum growth rate ($\mu_{\max}$); and K_i is the inhibition constant, which numerically equals the highest substrate concentration (mg/l) at which the specific growth rate is equal to one half the maximum specific growth rate ($\mu_{\max}$).

At high substrate concentrations, if K_s is small, K_s/s is negligible so that in the reciprocal form equation (2) reduces to

$$\frac{1}{\mu} = \frac{1}{\mu_{\max}} + \frac{s}{\mu_{\max} K_i} \quad (3)$$

and a plot of $1/\mu$ against s gives a straight line (Fig. 2) with intercepts of $1/\mu_{\max}$ and K_i . From Fig. 2, values of 0.29 h^{-1} and 110 mg/l were obtained for $\mu_{\max}$ and K_i respectively.

Morphological changes

The microscopic appearance of the organism was observed to change with cultural conditions. When the inhibition constant dominated the growth rate, the cells appeared more rod-like and some filamentous forms were seen. When cultural conditions were returned to those where the saturation constant was dominant the normal cocco-bacillus form reappeared

Fig. 2. Substrate inhibition of *Bacterium* NCIB 8250 growing on phenol. Points on vertical axis were obtained at substrate concentrations below detection limit of phenol.

Saturation constant

A precise evaluation of the saturation constant for this organism growing on phenol was not possible. Since the relationship is still linear at low substrate concentrations (Fig. 2), the saturation constant must also be low. Differentiation of the Haldane equation shows that the maximum attainable growth rate occurs at a substrate concentration given by $\sqrt{(K_i K_s)}$, which is clearly below 10 mg/l, so that the value of K_s must be less than $10^2/K_i$, i.e. approx. 0.9 mg/l.

The detection limit of the 4-amino phenazone test for phenol is about 1 mg/l and, unless the culture was showing inhibition, phenol could not be detected in the effluent even at growth rates in excess of $\mu_{\max}/2$, again showing that K_s was less than 1 mg/l.

Yield and maintenance requirement

In addition to the decrease in growth rate with increasing substrate concentration, the yield of organisms also decreased. To explain such a decrease in yield with reduction of growth rate, Pirt (1965) proposed the relationship

$$\frac{1}{Y} = \frac{1}{Y_g} + \frac{m}{\mu}, \quad (4)$$

where Y is the observed growth yield, Y_g is the 'true' growth yield, i.e. the yield that would be obtained in the absence of a maintenance requirement, and m is the maintenance coefficient (g substrate/g dry wt h).

Plotting observed values of $1/Y$ against $1/\mu$ for specific growth rates in the range 0.11 to 0.28 h⁻¹ gave a straight line ($1/Y = (0.28/\mu) + 1.7$) and values of 0.59 g/g and 0.28 g/g h for Y_g and m respectively (Fig. 3a).

Further experiments were carried out to examine changes in yield with specific growth rate under 'non-inhibitory' conditions, i.e. when specific growth rate theoretically increases with substrate concentration or $s_2 < \sqrt{(K_i K_s)}$. Unfortunately at this time the supply arrangements for the de-ionized distilled water were changed, and using water from the new supply the medium described would not reliably support growth. By replacing the ferric chloride by the 'salts solution' containing trace metals the organism grew readily, and the experiments were continued using this medium.

At high substrate concentrations it was difficult to obtain steady states owing to a ten-

Fig. 3. Change in yield with growth rate of *Bacterium* NCIB 8250 growing on phenol under (a) inhibitory conditions ($s_2 > \sqrt{(K_i K_s)}$) and (b) non-inhibitory conditions ($s_2 < \sqrt{(K_i K_s)}$).

Table 2. Yield constants (Y_2) found for second-stage fermentor operated at various dilution rates (D_2) during 'non-inhibited' growth of *Bacterium* NCIB 8250 on phenol ($s_2 < \sqrt{(K_i K_s)}$)

Organisms and substrate are entering at flow rates f_{12} and f_{02} and concentrations x_1 and s_{02} and leaving at concentrations x_2 and s_2 . First-stage dilution rate D_1 , substrate concentration entering first stage s_{01} , and cell concentration in first stage x_1 shown for each group of experiments. Volume of first stage, 760 ml; volume of second stage, 250 ml.

	f_{12} (ml/h)	f_{02} (ml/h)	D_2 (h ⁻¹)	s_{02} (mg/l)	x_2 (mg/l)	s_2 (mg/l)	Y_2
(a) $D_1 = 0.12 \text{ h}^{-1}$	10.8	28.5	0.16	254	112	< 1	0.32
$s_{01} = 362 \text{ mg/l}$	13.4	34.2	0.19	252	122	< 1	0.36
$x_1 = 112 \text{ mg/l}$	16.2	43.1	0.24	255	144	< 1	0.44
	19.4	50	0.28	252	152	< 1	0.48
	22	55	0.32	251	145	< 1	0.45
(b) $D_1 = 0.12 \text{ h}^{-1}$	25.3	57	0.33	243	172	< 1	0.5
$s_{01} = 384 \text{ mg/l}$	32.4	76.7	0.44	246	166	< 1	0.47
$x_1 = 165 \text{ mg/l}$	30.3	74.1	0.42	249	188	< 1	0.6
	37.9	89.6	0.51	246	221	< 1	0.7
(c) $D_1 = 0.12 \text{ h}^{-1}$	38.6	97.9	0.54	362	247	< 1	0.57
$s_{01} = 350 \text{ mg/l}$							
$x_1 = 136 \text{ mg/l}$							
(d) $D_1 = 0.14 \text{ h}^{-1}$	32.5	41.5	0.3	322	209	< 1	0.4
$s_{01} = 388 \text{ mg/l}$	35.3	42.6	0.31	314	239	< 1	0.5
$x_1 = 179$							

dency for the culture to grow on the wall of the reactor and in the overflow. However, results were qualitatively the same, with a decrease in specific growth rate at high substrate concentrations. Under non-inhibitory conditions no difficulties were experienced with wall growth, and specific growth rates and yields were determined over a range of dilution rates from 0.16 to 0.54 h⁻¹ (Table 2); their reciprocals are plotted in Fig. 3(b), which gave values for the 'true' growth yield and maintenance coefficient of 0.9 g/g and 0.24 g/g h respectively.

DISCUSSION

The occurrence of substrate inhibition in the growth of *Bacterium* NCIB 8250 has been described previously by Kennedy & Fewson (1968), who showed that during growth in batch culture on certain aromatic substrates the specific growth rate decreased with increasing substrate concentration. Our results show that growth of this organism on phenol was substrate inhibited and could be described by the Haldane function which has been used extensively in enzymology. While there may be no theoretical justification for using this function to describe bacterial growth kinetics as well as enzyme kinetics, Andrews (1968) pointed out that the widely used Monod relationship is similar in form to the Michaelis-Menten expression on which the function is based. Additionally, Boon & Laudelout (1962) showed that the function could be used to fit the behaviour of *Nitrobacter winogradskyi* using nitrite as substrate.

Stability of the system

Spicer (1955) in a theoretical study of continuous-culture systems showed that a stable equilibrium could only occur when $d\mu/ds > 0$. In the case of substrate inhibition such a criterion cannot be met when $s > \sqrt{(K_1 K_s)}$, so that instability will occur. Both Andrews (1968) and Yano & Koga (1969) examined this instability with mathematical models based on the Haldane equation for substrate inhibition. Yano & Koga (1969) described the existence of three non-negative steady states, of which two were stable and the third unstable. The two stable steady states were the normal condition of high cell concentration and low substrate concentration and that which they called the wash-out steady state. In a two-stage continuous-culture system, because of the continuous supply of organisms to the second stage, wash out cannot occur, and the wash-out steady state of Yano & Koga (1969) will correspond to a condition of high substrate and low cell concentration. The steady states obtained at high and low dilution rates correspond to the stable steady states, and the unstable steady state ($d\mu/ds_{02} < 0$) lies in the region where the culture was susceptible to fluctuations in the input bacterial concentrations.

Increase in bacterial concentrations in the second stage is given by

$$x_2 - x_{02} = Y(s_{02} - s_2). \quad (5)$$

By applying equations (1), (2), (4) and (5) to the second-stage fermentor, and eliminating x_2 and Y , it can be shown that

$$D_2 = \frac{\mu_{\max}}{1 + K_s/s_2 + s_2/K_i} \left[\frac{x_{02}}{Y_G(s_{02} - s_2)} + 1 \right] + \frac{x_{02}m}{s_{02} - s_2}. \quad (6)$$

Solution of this equation for D over a range of values for s_2 at constant x_{02} and s_{02} produces a hysteresis curve as shown in Fig. 4. The upper (C–D) and lower (A–B) parts of the hysteresis curve correspond to two stable steady states, while the intermediate values of substrate concentration (B–C) correspond to an unstable steady state (Edelstein, 1970).

Using the constants obtained from the data (assuming $K_s = 0.1$ mg/l) and values for x_{02} and s_{02} of 52 and 229 mg/l respectively, the curve is consistent with the observed behaviour of the culture in the second stage. Thus when the dilution rate was increased from 0.44 to 0.45 h⁻¹, the abrupt increase in substrate concentration represented a change between two stable steady states. The overlap of the two stable states could explain the tendency for the substrate concentration to wander with variations of incoming cell and substrate concentration, as was found in the dilution rate range 0.37–0.44 h⁻¹. Outside this range of dilution rates the system would be stable since at each extreme there exists only one positive steady-state solution.

Fig. 4. Relationship between substrate concentration and dilution rate in second stage of a two-stage continuous culture growing on an inhibitory substrate. $K_s = 0.1$ mg/l, $K_i = 110$ mg/l, $s_{02} = 229$ mg/l, $x_{02} = 52$ mg/l (for explanation of points A, B, C, and D see text).

Fig. 5. Comparison of theoretical curves for normal (solid line) and substrate inhibited (broken line) growth with values of $K_s = 2$ mg/l, $\mu_m = 0.4$ h⁻¹ and $K_i = 100$ mg/l.

Maintenance coefficients

The values obtained for the maintenance coefficient of *Bacterium* NCIB 8250 growing aerobically on phenol (0.24 and 0.28 g/g h) were considerably higher than those quoted by Pirt (1965) for aerobic growth of *Aerobacter aerogenes* on glycerol ($m = 0.076$ g/g h) and *A. cloacae* on glucose ($m = 0.094$ g/g h). Such a difference might be expected since growth of an organism on phenol requires the cleavage of the aromatic nucleus needing a considerable expenditure of energy.

The maintenance coefficients obtained for 'inhibited' and 'non-inhibited' growth were not strictly comparable since different media had to be used in the two series of experiments. However, the two values obtained were of the same order and it is possible that the maintenance requirements for the two states are not very different, particularly when compared with the energy required for the disruption of the aromatic nucleus.

Yield coefficients

Yields of organisms growing on an aromatic substrate are high when expressed as g bacteria/g substrate, which may be accounted for by the high carbon content of the substrate. The highest observed yield was 0.72, or 67.7 g/mol of substrate, which is within the range (50 to 72) of values given by Kennedy & Fewson (1968) for growth of this organism on other aromatic substrates.

Practical implications

Treatment of wastes containing inhibitory substrates is often necessary, and the question of stability is of considerable importance if the substrate concentration in the effluent is to be as low as possible. The main property of substrate inhibition kinetics is that for each specific growth rate below the maximum attainable growth rate there are two possible steady-state substrate concentrations (Fig. 5). Under normal conditions a completely mixed treatment plant should operate in the region below inhibition at values of s below $\sqrt{(K_i K_s)}$, but under certain conditions, e.g. start-up, organism recycle failure, or an increase in flow rate, the system could be driven into a situation where K_i becomes dominant, s is greater than $\sqrt{(K_i K_s)}$, and wash out ensues.

In such a system the limiting specific growth rate will be that obtained when the substrate concentration in the reactor is equal to that in the influent, i.e. $\mu = \mu_{\max}/(1 + K_s/s_{02} + s_{02}/K_i)$. For an organism to be maintained within an activated-sludge system, the growth rate must be able to exceed that given by the relationship

$$\mu = D[1 - a(b - 1)], \quad (7)$$

where a is the recycle ratio (flow of recycle/flow of influent) and b is the concentration ratio (concentration of cells in recycle/concentration of cells in reactor). Thus, in the case of an organism exhibiting substrate inhibition, provided that $\mu_{\max}/(1 + K_s/s_{02} + s_{02}/K_i) > D[1 - a(b - 1)]$ the system will remain stable and wash out cannot occur.

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