Growth Rates of a Pseudomonad on 2, 4-Dichlorophenoxacyclic Acid and 2, 4-Dichlorophenol

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The growth of a pseudomonad on 2, 4-D (2, 4-dichlorophenoxacyclic acid) and 2, 4-DCP (2, 4-dichlorophenol) was studied in batch and continuous culture. The optimum growth rate using 2, 4-D was 0.14/h at 25 C in a pH range from 6.2 to 6.9. Highest specific growth rate using 2, 4-DCP was 0.12/h at 25 C in a pH range from 7.1 to 7.8. Growth was strongly inhibited by 2, 4-DCP above a concentration of 25 mg/liter whereas no appreciable inhibition was observed with 2, 4-D at concentrations up to 2,000 mg per liter. Growth on 2, 4-DCP was described by Monod kinetics at subinhibitory concentrations but the inhibition by 2, 4-DCP exhibited an unusual linear response to substrate concentration, and did not fit a model based on noncompetitive inhibition. The lag phase of batch cultures was found to depend on both 2, 4-DCP concentration and prior adaptation of the inoculum. A study such as this on the kinetics of growth on related substrates may be useful as a method of finding the rate-limiting step in a metabolic sequence.

There are few studies in the literature of the kinetics of growth on unusual substrates such as are often found in industrial waste effluents. As waste treatment becomes increasingly sophisticated it will be necessary to have more detailed kinetic data for design purposes.

Both 2, 4-D (2, 4-dichlorophenoxacyclic acid) and 2, 4-DCP (2, 4-dichlorophenol) are present in the liquid waste from the production of the herbicide, 2, 4-D. Representative concentrations in the waste stream are 300 to 500 mg/liter for each compound. This effluent has been known to contaminate ground water and may persist for years in this environment (8). Although the waste is not extremely toxic, highly objectionable tastes and odors at concentrations as low as 20 μg/liter may result from the 2, 4-DCP (8).

The feasibility of a biological treatment system for wastes of this nature has been demonstrated (4, 15). However there is little information on the kinetics of bacterial growth on 2, 4-D or 2, 4-DCP.

MATERIALS AND METHODS

Organism. The organism selected was Pseudomonas species NCIB9340 isolated by Gaunt and Evans (9) from 2-methyl-4-chlorophenoxacyclic acid enrichments. A basal salts medium of the following compo-

sition was used: K2HPO4, 1.5 g; NH4NO3, 0.5 g; MgSO4·7H2O, 0.2 g; CaSO4·2H2O, 0.05 g; FeSO4·7H2O, 0.0005 g; and distilled water, 1,000 ml. The carbon source, either 2, 4-D or 2, 4-DCP, was added as the sodium salt, and before inoculation the pH was adjusted with H2SO4. All components were reagent grade except 2, 4-D and 2, 4-DCP, which were practical grade from Fisher Scientific Co. Controls were run using highly purified 2, 4-DCP (Fisher) to insure that other chlorinated compounds did not interfere.

Growth rate determination. (i) Chemostat. The organism was propagated in a chemostat with a working volume of 800 ml. Strict sterile precautions were not observed; due to the toxic nature of the substrates contamination was not considered to be a serious problem. Air supplied to the culture varied from 0.225 to 0.350 liter per min depending on the density of the culture. A vibromixer (Chemap Inc.) agitated the culture. Temperature was maintained at 25 ± 0.5 C with an external jacket. The culture was normally held at pH 7.4 ± 0.1 by manual adjustment with 1 N NaOH. Medium containing 2,000 mg of either 2, 4-D or 2, 4-DCP per liter was fed via a peristaltic pump at rates which varied from 30 to 70 ml per h. The fermentor was started up by using 2, 4-D feed which proved to be noninhibitory up to 2,000 mg per liter. After the chemostat had stabilized, the feed was changed to 2, 4-DCP. The continuous culture method was used to measure growth rates at low substrate concentration by determining dilution rate and residual substrate concentration at steady state (14). Inocula for other experiments were taken from the chemostat.

(ii) Shake flasks. This method was used only with noninhibitory substrates such as glucose, succinate,
or 2, 4-D. One-liter, indented shake flasks (Bellco Glass, Inc.) containing 250 ml of sterile medium were inoculated from the chemostat and shaken at 300 rpm. Cell concentration was measured as optical density at 525 nm using a Spectronic 20 spectrophotometer (Bausch and Lomb, Corp.); the turbidity was previously calibrated to cell dry weight.

(iii) Nephelometry. Monod's method of initial growth rates was used (16). By allowing only a small amount of growth in a batch culture the substrate concentration remains relatively unchanged and can be assumed constant at some mean value. This permits evaluation of the growth rate at the given substrate concentration. The period of time before active growth was considered the lag phase. Small Erlenmeyer flasks containing 25 ml of filtered (0.22 nm, Millipore Corp.) medium were inoculated with approximately 0.25 μg (dry weight) of washed inoculum, and put in a shaking water bath. Cell concentration was measured by the change in light scattering of the bacterial suspension. A fluorometer (Turner Model III) with a 10-mm square cuvette was used. The primary light source had a wavelength of 436 nm. Controls were run using medium made with double-distilled water with no carbon source to insure that the growth observed was not due to organic contamination present in the ordinary distilled water source.

Assays. At concentrations of 5 mg per liter and higher both 2, 4-D and 2, 4-DCP were assayed with a direct ultraviolet absorption spectrophotometric assay used by Ingols et al. (13). For concentrations below 10 mg per liter 2, 4-DCP was estimated by the 4-aminoantipyrine colorimetric technique of Faust and Aly (7).

Cell concentrations were measured as previously mentioned.

RESULTS AND DISCUSSION

Temperature and pH. In batch culture the maximum specific growth of 0.14/h using 2, 4-D as a carbon source was observed at 25 C in a pH range from 6.2 to 6.9. Using 2, 4-DCP the maximum growth rate of 0.12/h was at 25 C and in a pH range of 7.1 to 7.8. Growth rate fell sharply above 25 C for both substrates. Growth was observed to be rapid at higher temperatures using the noninhibitory substrates glucose and succinate. The specific growth rate on glucose was 0.41/h at 25 C and 0.39/h at 30 C, whereas with succinate as the carbon source a growth rate of 0.49/h was obtained at 30 C.

The dependence of pH on growth rate is shown in Fig. 1. There is a pronounced shift in the region of maximum growth rate along the pH axis for 2, 4-D and 2, 4-DCP. This difference between the two curves is probably due to difference between the dissociation constants of the two substrates. The K_s values for 2, 4-D and 2, 4-DCP are 2 \times 10^{-2} and 1.4 \times 10^{-8}, respectively. This difference will affect the amount of substrate present in dissociated and undisassociated form at a given pH, the undissociated form being favored by low pH and low K_s. The undissociated form is the species which is believed to penetrate the cell membrane and to be responsible for both metabolic and inhibitory activity (1, 12). Calculation of the amount of undissociated substrate present at various values of pH did not fully explain the shift of maximum growth rate along the pH axis. There is no simple explanation; however, because although the undissociated form of phenolic compounds is much more active than the ion; the ionic form does have some biological activity (12). The extent to which the ion is active would have to be taken into consideration.

Substrate concentration. Using a combination of batch and continuous culture methods, the relationship of concentration of 2, 4-DCP to growth rate was determined. Batch culture was used in conjunction with continuous methods because of the difficulty of operating at maximum growth rate and of measuring growth rates at inhibitory concentrations. As shown in Fig. 2, the highest growth rate observed (0.12/h) occurred at 25 mg per liter. Above this level growth is strongly inhibited. The solid line drawn through the points at low substrate concentration and extended as a broken line represents the "Monod" equation

$$\mu = \frac{\mu_{\text{max}}}{S}$$

(1)

(μ, specific growth rate per hour; \(\mu_{\text{max}}\), maximum specific growth rate per hour; K_s, Monod constant substrate limited, milligrams per liter;
S, substrate concentration, milligrams per liter) with constants of $\mu_{\max} = 0.14/\text{h}$, $K_i = 5.1 \text{ mg per liter}$. This curve shows good fit to the data up to 25 mg per liter of 2,4-DCP. The above values for the constants were obtained from the intercepts of a double reciprocal plot using a least squares fit to the data. A correlation coefficient of 0.98 was obtained. It is interesting that the extrapolated value of $\mu_{\max}$ (0.14/h) for 2,4-DCP is identical to that observed for the relatively nontoxic 2,4-D. With the latter substrate no inhibition was observed at concentrations up to 2,000 mg per liter. Since dichlorophenol is believed to be the first degradation product of 2,4-D according to one of the major dissimilatory pathways (6), we can conclude from the kinetics that this first step is not a rate-limiting one in the overall metabolism assuming that the two substrates permeate into the cell at the same rate. To verify this conclusion cell-free systems should also be examined. Growth is probably limited by some later step since much higher values for $\mu_{\max}$ can be obtained on substrates such as glucose or succinate. From similar kinetic analysis, excluding inhibition and pH effects, it should be possible to pinpoint the rate-limiting enzymatic reactions in complex pathways.

The straight line in Fig. 2 drawn in the region of substrate inhibition is best described by the empirical equation:

$$\mu = 0.156 - 0.00155 S$$  \hspace{1cm} (2)

An attempt was made, however, to fit the data for 2,4-DCP inhibition to a Haldane function based on the analogy to non-competitive enzyme inhibition, namely

$$\mu = \frac{\mu_{\max}S}{(K_a + S)(1 + S/K_i)}$$  \hspace{1cm} (3)

where $K_i$ is a substrate inhibition constant, milligrams per liter. Such an equation was successfully used by Boon and Laudelout (2) to describe nitrite inhibition for the growth of *Nitrobacter winogradskyi*. Likewise, Jones et al. (11) accounted for phenol inhibition of a phenol-utilizing bacterium by such an expression. As shown in Fig. 2 though, equation 3 does not fit our data very well. The calculated parameters from a least squares computer curve fitting devised by Edwards (5) were:

$$\mu_{\max} = 0.228/\text{h}$$

$$K_a = 11.7 \text{ mg per liter}$$

and

$$K_i = 35.7 \text{ mg per liter}$$

Other inhibition models discussed by Edwards (5) would not afford any better fit. Since the inhibition action of 2,4-DCP probably stems from its tendency to complex with cellular and respiratory proteins and thus uncouple oxidative phosphorylation (12, 17), the use of more complex models was not justified. The observa-
tion of a threshold concentration at 25 mg per liter and subsequent linear decline in growth rate with increasing concentration is perhaps more consistent with such general toxicity than with any single-enzyme model. Hinshelwood (10) observed linear inhibition curves for ethanol as a product inhibitor on *Klebsiella aerogenes*.

**Lags.** Batch cultures of the pseudomonad on 2,4-DCP showed interesting lags, the duration of which depended on substrate concentration and on adaptation of the inoculum. The lag times of batch cultures were measured nephelometrically at different substrate concentrations for inocula taken from the chemostat at steady state levels of 2.8 mg per liter and 10.5 mg per liter of 2,4-DCP. The two curves pictured in Fig. 3 resemble those describing the response of yeast to 2,4-dinitrophenol as an inhibitor rather than as a substrate (18). The finite lag predicted by extrapolation of the curves to zero concentration could be due to osmotic shock since the inoculum was washed with distilled water to prevent carryover of substrate with the inoculum. However, Dean and Hinshelwood (3) in their review of adaptation to drugs also consider some lag curves with non-zero intercepts. The cells adapted to the higher substrate level (10.5 mg per liter) responded faster particularly at inhibitory concentrations to increases in substrate concentration in batch subculture than the cells adapted to 2.8 mg per liter of 2,4-DCP. The difference between the two curves is due to the ability of bacteria grown at a higher growth rate and higher substrate concentration to adapt more rapidly to changes in environment.

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