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# Substrate Inhibition of *Chlamydomonas reinhardtii* by Acetate in Heterotrophic Culture

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*The heterotrophic growth of microalgae has been proposed as a potential mode of culture to achieve high cell densities on a large scale. In this paper, the heterotrophic growth of the green microalga,* Chlamydomonas reinhardtii, *on acetate as sole energy and carbon source in batch and single-stage continuous cultures is reported. No inhibition by acetate was observed at low concentrations (≤ 0·4 g/litre), in which case, cell growth was well described by the Monod model. However, above this acetate concentration, growth was progressively inhibited. Inhibition was observed as a decrease in both the specific growth rate and the observed growth yield. The former was well modelled using the Haldane model. A model based on constant $q_m$ was proposed to describe the latter phenomenon. Whereas it predicted the trend of falling observed cell growth yield with initial acetate concentration in batch cultures, there was significant lack of fit between simulated and experimental data. In continuous cultures, a steady state cell concentration of 0·4 g/litre and maximum cell productivity of 0·02 g/litre/h was obtained for an acetate feed concentration of 0·85 g/litre. This compares well with values achieved by photosynthetic mass culture systems.*

## NOTATION

| | |
|---|---|
| $D$ | Dilution rate ($h^{-1}$) |
| $K_i$ | Substrate inhibition constant (g/litre) |
| $K_s$ | Monod saturation constant (g/litre) |
| $m$ | Maintenance coefficient (g/g/h) |
| $\bar{P}$ | Steady state cell productivity (g/litre/h) |
| $q_m$ | Maximum specific rate of substrate utilisation (g/g/h) |
| $S$ | Substrate (acetate) concentration in the fermentor (g/litre) |
| $\bar{S}$ | Steady state substrate (acetate) concentration (g/litre) |
| $S_f$ | Substrate (acetate) concentration in the feed (g/litre) |
| $S_{mi}$ | Substrate (acetate) concentration at maximum specific growth rate with inhibition (g/litre) |
| $t$ | Time (h) |
| $X$ | Observed cell concentration (g/litre) |
| $\bar{X}$ | Steady state cell concentration (g/litre) |
| $Y$, $Y^{obs}$ | Observed growth yield (g/g) |
| $Y_g$ | True growth yield coefficient (g/g) |
| $Y_i$ | Observed growth yield with inhibition (g/g) |

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$\mu$ Specific growth rate ($h^{-1}$)
$\mu_i$ Specific growth rate with inhibition ($h^{-1}$)
$\mu_m$ Maximum specific growth rate without inhibition ($h^{-1}$)
$\mu_{mi}$ Maximum specific growth rate with inhibition ($h^{-1}$)

## INTRODUCTION

Microalgae constitute a large resource of genetic diversity that has been largely untapped for products of commercial interest. Microalgae have found application in agriculture, waste treatment and aquaculture[1-3] and a number of microalgal products have been commercialised, such as $\beta$-carotene from *Dunaliella salina*[4] and $\gamma$-linolenic acid from *Spirulina*.[5] Many other microalgal products with commercial potential, particularly high value chemical and pharmaceutical compounds have been discovered and are summarised in several recent reviews,[4,6-8] but there has been little progress in bringing them to the market.

Microalgae are predominantly photoautotrophs and most microalgal mass culture is performed using this mode of growth. The low productivity of large scale, photoautotrophic systems with typical cell densities of 0·5–1 g dry weight/litre, however, is a major obstacle to the successful commercialisation of microalgal processes.[9] Typical photoautotrophic productivities are of the order of 15–40 t/ha/yr (about 3 mg/litre/day).

Furthermore, it is now widely accepted that extensive pond systems and open, but more intensive, raceway systems are suitable for the mass culture of only those microalgae which can be grown under conditions hostile to most other photosynthetic competitors, such as *D. salina* (at high salinities) and *Spirulina platensis* (at high pH).

Many microalgae, which synthesise potentially valuable products, require milder photoautotrophic growth conditions. Large-scale, open culture of these is unlikely to be sustainable or economic. Consequently, they must be grown in specialised photobioreactors. These bioreactors can be expensive, and their productivity is limited by mutual shading at high cell densities (i.e. 5 g/litre), and by the difficulty of maintaining optimal environmental conditions and control of microalgal predators.

Some algae can utilise organic carbon substances as their sole carbon and energy source.[10] This mode of growth eliminates the requirement for light and therefore offers the possibility of greatly increasing cell concentration and process productivity using high cell density techniques, such as fed-batch cultures and continuous culture with cell recycle. To date, there have been no reports of such processes.

The microalga *Chlamydomonas reinhardtii* can be cultured in darkness on acetate as a source of energy and carbon.[11] It is unable to utilise sugars. It was selected for this study since it is one of the few microalgae for which shuttle vectors have been developed to aid genetic manipulation.[12,13] Furthermore, it has been used extensively in biochemical and physiological studies.[13-15] There are few data published, however, concerning its heterotrophic growth.

Acetate is a poor substrate for heterotrophic growth since it inhibits microbial growth at relatively low concentrations,[16] which makes high cell density culture difficult. The inhibition is highly pH dependent, since the undissociated form is particularly toxic. The present paper reports the heterotrophic growth of *C. reinhardtii* on acetate in batch and single-state chemostat cultures. This knowledge will be useful in the development of high cell density strategies for the organism.

## THEORY

The growth of heterotrophic microorganisms is autocatalytic with cell number increasing exponentially until an essential nutrient becomes limiting or byproducts accumulate to inhibitory levels. The dependence of the specific rate of cell growth on the concentration of a single, limiting nutrient is commonly represented by eqn (1), which is the Monod model,[17] in which the specific growth rate, $\mu$, of a microorganism exhibits a dependence on the limiting nutrient at low nutrient concentrations, but is independent by the same model above a certain threshold concentration.

In practice, most nutrients also inhibit cell growth at high concentrations, often reflected as a reduced specific growth rate. This is especially true of acetate. To describe such substrate inhibition by a single nutrient, the Haldane model[18] is frequently used (eqn (2)). The Haldane model is virtually the Monod model incorporating an inhibition term, which describes competitive inhibition of growth by the substrate itself. Substrate

inhibition mechanisms have been discussed in detail by Edwards.[19]

$$\mu = \frac{\mu_m S}{K_s + S} \tag{1}$$

$$\mu = \frac{\mu_m S}{K_s + S + \frac{S^2}{K_i}} \tag{2}$$

Where a substrate inhibits growth, there will be an optimal substrate concentration, $S_{mi}$, at which the highest specific growth rate, $\mu_{mi}$ is obtained. This specific growth rate, $\mu_{mi}$ and substrate concentration, $S_{mi}$ are given by eqns (3) and (4) respectively.

$$\mu_{mi} = \frac{\mu_m}{1 + 2\sqrt{K_s/K_i}} \tag{3}$$

$$S_{mi} = \sqrt{K_s K_i} \tag{4}$$

This concentration represents the maximum initial substrate concentration that can be effectively used in batch cultures of microorganisms and thus places a limit on the final cell concentration and productivity that can be achieved.

Substrate inhibition can be elegantly overcome by using either chemostat or fed-batch culture methods, in which a concentrated substrate solution is fed to the bioreactor, with or without withdrawal of contents, respectively. In these culture systems, the substrate concentration in the vessel can be maintained below inhibitory levels.

Excessive substrate concentration affects not only the rate of microbial growth, but also the material efficiency of growth. In bioprocesses, the latter is typically expressed as an observed growth yield coefficient, $Y^{obs}$, which is related to cell concentration, $X$, and the limiting substrate concentration, $S$, by eqn (5).

$$Y^{obs} = -\frac{dX}{dS} \tag{5}$$

$Y^{obs}$ is usually considered to be a constant for a given organism and substrate. However, the observed yield exhibits considerable variation in heterotrophic microbial cultures, particularly with respect to specific growth rate. This dependency of observed growth yield on specific growth rate was explained by Pirt[20] as reflecting increased diversion of substrate from cell growth to cell maintenance activities as the specific growth rate decreased, and is expressed by eqn (6).

$$\frac{1}{Y^{obs}} = \frac{1}{Y_g} + \frac{m}{\mu} \tag{6}$$

Where $Y_g$ is the true, or maximum, growth yield coefficient and $m$ is the maintenance coefficient. The former constant expresses the gross cell yield from the substrate, whereas the latter defines the specific rate of substrate consumed for maintenance activity.

In chemostat culture, the steady state substrate concentration is usually low and can be predicted by the Monod model, in which the dilution rate $D$, is equal to steady state specific growth rate, $\mu$. If no products are formed, then the steady state cell concentration $X$, and cell productivity, $P$, can be estimated by eqns (7) and (8).

$$\bar{X} = \frac{Y_g D\left(S_f - \frac{K_s D}{\mu_m - D}\right)}{D + mY_g} \tag{7}$$

$$\bar{P} = \frac{Y_g D^2\left(S_f - \frac{K_s D}{\mu_m - D}\right)}{D + mY_g} \tag{8}$$

These equations incorporate the effect of maintenance energy requirements using eqn (6) and relate $X$ and $P$ as functions of the feed substrate concentration and the dilution rate.

Growth yield is often decreased at inhibitory substrate concentrations,[21] although this phenomenon has been usually overlooked. The mechanism involved is not clear, since eqn (6) does not account for this reduction in yield. It has been observed that the maintenance coefficient is not constant under these conditions (Chen & Johns, submitted for publication).

To account for the effect of an inhibitor on cell yield, Pirt[22] proposed a linear relationship between cell growth yield and specific growth rate, assuming that there was no change in the maximum specific rate of substrate utilisation, $q_m$, to give eqns (9) and (10).

No inhibition:

$$\mu_m = q_m Y \tag{9}$$

Inhibition:

$$\mu_i = q_m Y_i \tag{10}$$

If the maintenance coefficient is small, then, at the maximum specific growth rate, $\mu_m$, $Y \approx Y_g$. Therefore, by combining eqns (2), (9) and (10), an expression for the cell yield at inhibitory substrate concentrations can be obtained.

$$Y_i = \frac{Y_g}{\frac{K_s}{S} + 1 + \frac{S}{K_i}} \quad (11)$$

At inhibitory substrate concentrations, $S \gg K_s$, and eqn (11) can be reduced to eqn (12).

$$Y_i = \frac{Y_g}{1 + \frac{S}{K_i}} \quad (12)$$

The relationship between growth yield coefficient and specific growth rate for cultures exhibiting substrate inhibition can be obtained from eqns (2) and (12).

## MATERIALS AND METHODS

### Organism and maintenance

*Chlamydomonas reinhardtii* Dangeard (CS-51) was obtained from CSIRO Marine Laboratories, Hobart, Australia and maintained axenically at 4°C on nutrient agar slopes prepared from Bristol's solution[23] with 0·1% bactopeptone and 1·5% agar.

### Medium

Modified Sager & Granick medium,[11] referred to as CR-M1 medium was used in all experiments and consisted of (per litre) 4·15 g $NaH_2PO_4 \cdot 2H_2O$, 1·15 g $K_2HPO_4$, 0·3 g $MgSO_4 \cdot 7H_2O$, 0·053 g $CaCl_2 \cdot 2H_2O$, 0·01 g $FeCl_3 \cdot 6H_2O$, 0·5 g sodium citrate and 1 ml of trace metal solution comprising (per 100 ml) of 125 mg $H_3BO_3$, 125 mg $ZnSO_4 \cdot 7H_2O$, 38 mg $MnSO_4 \cdot H_2O$, 25 mg $CoCl_2 \cdot 6H_2O$, 25 mg $Na_2MoO_4 \cdot 2H_2O$ and 8 mg $CuSO_4 \cdot 5H_2O$. Nitrogen was supplied as urea.

For continuous cultures, an acetate concentration of 0·85 g/litre with 0·2 g/litre urea, was used unless otherwise indicated. For batch cultures, the urea concentration was 0·2 g/litre for actetate concentrations below 1·2 g/litre. For acetate concentrations of 1·2 g/litre or more, 0·5 g/litre urea was used. In all cases, acetate was the growth-limiting substrate.

### Cultures

A glass fermentor was used in continuous (1 litre) and batch culture (800 ml) experiments, respectively. Cultures were agitated with a magnetic stirrer at 400 rev/min and maintained at 35°C by a waterbath. The cultures were grown in darkness to prevent photosynthetic growth. Sterile air was supplied through a 0·2 $\mu$m filter at a rate of 160 ml/min, which provided a dissolved oxygen concentration greater than 50% saturation. The medium was buffered with phosphate and the pH was usually within the range of $6{\cdot}9 \pm 0{\cdot}5$ during the culture. Media and fermentors were sterilised by autoclaving at 121°C for 20 min. On cooling, the medium was inoculated with 5% (v/v) of an inoculum grown at 25°C on CR-M1 medium containing 0·85 g/litre acetate and 0·2 g/litre urea under a cool white fluorescent tube (1800 lux) on a 16/8 h on/off cycle.

For continuous cultures, cells were grown in batch mode after inoculation. In the late exponential phase, a feed medium containing 0·85 g/litre acetate and 0·2 g/litre urea was added at a constant rate using a peristaltic pump and the culture was discharged via an overflow tube to maintain constant culture volume. Steady state conditions were considered to have been established when cell concentrations from at least three samples collected after a period of three residence times, varied by no more than 10%.

### Acetate concentration

Acetate concentration in culture fluids was determined by HPLC. A sample (5 ml) of the culture was filtered through a 0·2 $\mu$m cellulose acetate membrane and kept for analysis at −20°C. HPLC was performed using a Waters liquid chromatograph equipped with a R401 differential refractometer (Waters Associates, Milford, MA). A portion (20 $\mu$l) of sample was injected onto a 300 mm × 7·8 mm HPX-87H$^+$ column (Bio-Rad Lab, Richmond, CA) with a Micro-Guard ion exclusion cartridge and operated at 65°C. The mobile phase comprised 0·008 N $H_2SO_4$ with a flow rate of 0·6 ml/min. Acetate (retention time generally 14·8 min) was quantified automatically by comparison of peak height with that of a standard (1000 or 1500 mg/litre acetate) injected after every 5 samples.

### Cell density

Cell density was determined by measuring optical density (OD) at 750 nm with a Hitachi U-1100

spectrophotometer, after dilution with deionised water to ensure OD readings were less than 0·8. Cell dry weight was obtained from a calibration curve generated by measuring the cell dry weight and optical density of culture samples as previously described.[24]

## RESULTS AND DISCUSSION

### Batch culture

The growth of *C. reinhardtii* on CR-M1 medium containing 1·2 g/litre acetate in heterotrophic batch culture is shown in Fig. 1. After inoculation, the alga grew slowly for hours and then exponentially until about 60 h by which time the acetate was completely consumed. The maximum cell concentration obtained was 0·485 g/litre, which corresponded to a $Y^{obs}$ of 0·36 g/g. The cells were green, indicating chlorophyll synthesis, despite the dark culture conditions. The specific growth rate of the culture was 0·049 $h^{-1}$, corresponding to a doubling time of 14 h. The culture pH increased slightly as acetate was consumed and the dissolved oxygen concentration was near saturation during the experiment. A fermentor culture performed under identical conditions, except that acetate was omitted from the medium, demonstrated no growth in darkness. This was in agreement with the results of Sager and Granick,[11] who reported that *C. reinhardtii* could not utilise urea carbon for heterotrophic growth.

### Effect of initial acetate concentration on *C. reinhardtii* in batch culture

For high cell densities, it is necessary to use high initial substrate concentrations in batch cultures. Consequently, the effect of initial acetate concentration on the growth of *C. reinhardtii* in batch culture was investigated. Initial acetate concentration had a marked effect on the length of lag phase, specific growth rate and growth yield. The lag phase at 0·85 g/litre initial acetate concentration was about 12 h and increased in length in proportion to the initial acetate concentration. Since the inoculum was grown on 0·85 g/litre, this phenomeon was presumably due to the requirement for adaptation by the cells to the high acetate concentrations or due to a selection for cells able to tolerate the higher acetate concentrations. This would effectively comprise a 'reduced' inoculum concentration.

Acetate concentration also had a profound effect on the specific growth rate of *C. reinhardtii* (Fig. 2). The optimal initial acetate concentration was found to be about 0·4 g/litre, above which concentration, growth inhibition of the microalga was observed. The specific growth rate fell away sharply within about 0·2 g/litre acetate of the optimal value.

For initial acetate concentrations up to the optimum, the Monod model (eqn (1)) fitted the experimental data. Values for the model parameters, $\mu_m$ and $K_s$, were estimated by two methods (Table 1), which yielded identical results. However, as expected, the Monod model failed to account for substrate inhibition of growth by acetate and therefore overpredicted the specific growth rate at initial acetate concentrations above the optimum.

The Haldane model was found to give a superior fit to the experimental data at initial acetate concentrations greater than the optimal concentration (Fig. 2). Parameter values for the Haldane model were estimated by non-linear regression, with and without fixing the value of $K_s$ to that estimated using the Monod model, and are given in

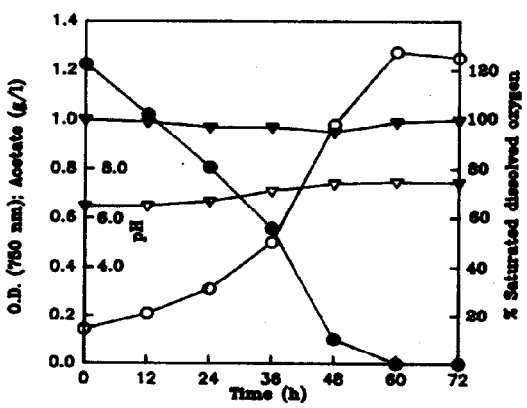


**Fig. 1.** Batch culture of *C. reinhardtii* ((○) optical density; (●) acetate concentration; (▽) pH; (▼) dissolved oxygen).

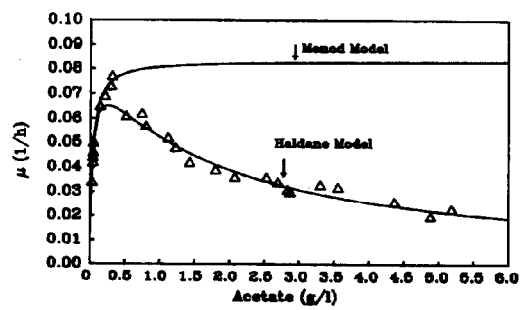


**Fig. 2.** Effect of acetate concentration on the specific growth rate of *C. reinhardtii* ((—) model prediction; (△) experimental data).

**Table 1.** Estimated parameter values for specific growth rate models[a]

| *Model* | *Method* | $\mu_m$ ($h^{-1}$) | $K_s$ (g/litre) | $K_i$ (g/litre) |
|---|---|---|---|---|
| Monod | Non-linear regression | 0·083 ± 0·002 | 0·035 ± 0·005 | |
| | Lineweaver–Burk plot | 0·084 ± 0·003 | 0·036 ± 0·005 | |
| Haldane | Non-linear regression | 0·084 ± 0·003 | 0·028 ± 0·471 | 1·76 ± 0·15 |
| | Non-linear regression[b] | 0·084 ± 0·003 | 0·035 | 1·77 ± 0·15 |

[a]Non-linear regression was performed using computer software NLREG1 based on Marquardt method; values obtained are in 95% confidence intervals.
[b]Adopting $K_s$ values obtained from the non-linear regression of the Monod model.

Table 1. The values obtained of $\mu_m$ and $K_i$ were identical within experimental error. The standard errors for all parameters were small, except for the $K_s$ value estimated using the Haldane model, which had larger error bounds, probably due to the high substrate concentrations used. Therefore the $K_s$ value obtained from the Monod model was preferred. $K_s$ values for *C. reinhardtii* have not been reported, however values for microbial growth on carbon sources generally range from 1 to 50 mg/litre.[22]

Using these parameter values, the maximum specific growth rate with inhibition, $\mu_{mi}$, and the optimal acetate concentration, $S_{mi}$, were calculated using eqns (3) and (4), respectively. Values of $\mu_{mi}$ and $S_{mi}$ of 0·065 $h^{-1}$ and 0·25 g/litre were obtained respectively. Compared to the Monod model, the Haldene model substantially underpredicted the experimentally determined specific growth rates of *C. reinhardtii* near the optimal substrate concentration (approximately 0·4 g/litre).

The effect of initial acetate concentration on observed cell growth yield in batch cultures is presented in Table 2. At the optimum acetate concentration for specific growth rate, $Y^{obs}$ was approximately 0·5 g/g. However, with increasing initial acetate concentration, $Y^{obs}$ fell rapidly and asymptotically approached 0·15 g/g at acetate concentrations greater than 2 g/litre. This value is typical of cell yields obtained from growth in which energy is derived solely from glycolytic metabolism. The fit of eqn (12) to the experimental yield data is presented in Fig. 3. The simulated yield coefficient data have considerable error due to the uncertainty in the value of $K_i$. Although eqn (12) conveys the general trend of the experimental data, it deviates significantly, especially at low values of acetate concentration, probably due to the fact that the $K_i$ value estimated is lower than the reality (i.e. $K_i$ value is usually high at low substrate concentrations) in this region.

**Table 2.** Effect of initial acetate concentration on growth of *C. reinhardtii* in batch culture[a]

| *Acetate concentration (g/litre)* | $X_{max}$ (g/litre) | *Final pH* | $Y^{obs}$ (g/g) |
|---|---|---|---|
| 0·29 | 0·15 | 6·67 | 0·51 |
| 0·31 | 0·16 | 6·69 | 0·51 |
| 0·52 | 0·28 | 6·75 | 0·50 |
| 0·68 | 0·38 | 7·17 | 0·49 |
| 0·74 | 0·39 | 6·92 | 0·49 |
| 0·80 | 0·40 | 7·01 | 0·40 |
| 1·23 | 0·49 | 7·40 | 0·36 |
| 1·43 | 0·44 | 7·66 | 0·26 |
| 1·80 | 0·44 | 7·31 | 0·20 |
| 2·07 | 0·45 | 7·43 | 0·16 |
| 2·53 | 0·50 | 7·76 | 0·18 |
| 3·30 | 0·49 | 8·01 | 0·13 |

[a]Only trace residual acetate concentrations were detected.

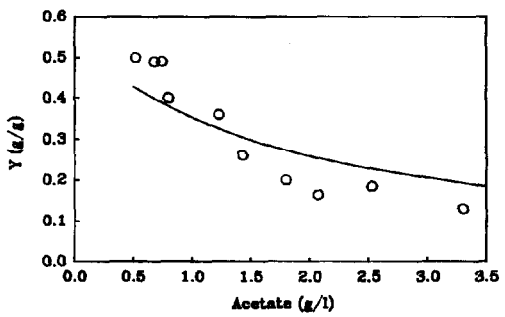


**Fig. 3.** Relationship between acetate concentration and observed cell growth yield of *C. reinhardtii* in batch culture. ((—) model prediction; (○) experimental data; $Y_g = 0·55$ g/g; $K_i = 1·77$ g/litre).

Acetate is known to be soluble in the lipids of cell membranes.[25] Samson[26] indicated that acetate inhibition of cell growth occurs by chemical interference with the membrane transport of phosphate, which might result in more expenditure of ATP. Disruption of cell membranes was also

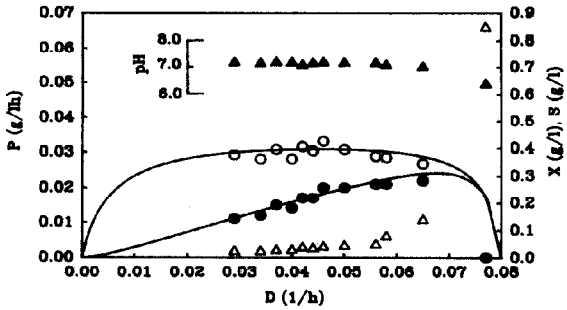


Fig. 4. Effect of dilution rate on steady state cell and acetate concentrations, pH and cell productivity of *C. reinhardtii*. ((○) cell concentration; (●) cell productivity; (△) acetate concentration; (▲) pH; (—) model prediction; $\mu_m = 0{\cdot}084$ $h^{-1}$; $K_s = 0{\cdot}035$ g/litre; $Y_g = 0{\cdot}55$ g/g; $m = 0{\cdot}011$ g/g/h).

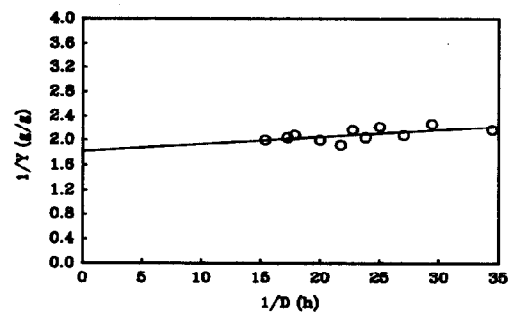


Fig. 5. Determination of maintenance coefficient $m$ and true growth yield coefficient $Y_g$ of *C. reinhardtii* for growth on acetate in chemostat culture.

reported leading to changes in cell morphology with the cells becoming irregular, and elongated.[16] These alterations of cell morphology were observed in this study.

The toxic effect of acetate on microbial cultures has often been attributed to the concentration of the undissociated form, which is highly pH dependent.[27,28] At the pH used in this work (above 6·4), the acetate ion concentration was at least 44 times that of the undissociated acetic acid. Nevertheless, the resulting inhibition and its effect on the growth rate and cell growth yield of *C. reinhardtii* were quite profound.

### Continuous culture of *C. reinhardtii*

In view of the inhibitory effects observed in batch cultures at even low initial acetate concentrations, continuous culture studies were performed in the chemostat mode to characterise the growth of *C. reinhardtii* at different dilution rates and at low acetate concentrations. The results are presented in Fig. 4.

At all dilution rates investigated, steady state cell concentration was approximately 0·4 g/litre and the observed growth yield was largely constant at $0{\cdot}48 \pm 0{\cdot}01$ g/g. Steady state acetate concentrations were less than 0·2 g/litre at all dilution rates. The pH of the cultures was relatively constant. At dilution rates greater than 0·065 $h^{-1}$, the steady state acetate concentration began to rise, corresponding to lower cell concentrations, and stable steady state conditions were difficult to achieve. The washout dilution rate was found to be approximately 0·077 $h^{-1}$, which was close to the maximum specific growth rate obtained in batch cultures.

The Monod model was used to simulate the experimental data using the parameter values presented in Table 1 and the comparison is given in Fig. 4. The growth of *C. reinhardtii* on acetate in a chemostat is well described by Monod kinetics, since the residual acetate concentrations in the bioreactor are non-inhibitory. A maximum cell productivity of 0·022 g/litre/h was obtained at a dilution rate of 0·065 $h^{-1}$. At this dilution rate, the volumetric and specific rates of substrate consumption were 0·045 g/litre/h and 0·130 g/g/h, respectively.

The true growth yield coefficient, $Y_g$, and the maintenance coefficient, $m$, were determined according to eqn (6) and were found to be $0{\cdot}55 \pm 0{\cdot}02$ g/g and $0{\cdot}011 \pm 0{\cdot}002$ g/g/h respectively, by linear regression (Fig. 5). The contribution of maintenance to acetate consumption was, therefore, small.

The biomass levels achieved in this work are similar to those that are obtained in photosynthetic culture in open ponds.[9] Higher biomass concentrations using a heterotrophic culture of *C. reinhardtii* on acetate might be obtained by either increased acetate concentrations in the medium, or by cell recycle techniques. There are no reports of the use of the former for the heterotrophic growth of *C. reinhardtii* on acetate and the use of the latter has not been reported for the heterotrophic culture of any microalgae. The results of this work have demonstrated the limitations of the use of higher acetate concentrations in batch culture, namely that of substrate inhibition with resultant decrease in both the rate and the efficiency of growth. Neither is the problem entirely solved by the use of continuous culture, since for cases of substrate inhibition, there are two steady states at a given value of dilution rate, one with a

steady state substrate concentration which does not inhibit cell growth and the other which does cause growth inhibition. This makes operation of the fermentor vulnerable to perturbations in substrate feed concentration, since at the latter steady state, any increase in substrate concentration will ultimately result in the washout of cells from the fermentor.[29]

Further research is in progress to evaluate the potential of fed-batch and membrane cell recycle techniques for increasing concentrations of *C. reinhardtii* grown in heterotrophic cultures on acetate.

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