Carbon Dioxide Inhibition of Photosynthetic Growth of *Chlorella*

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Chlorella cultures were grown in a tubular loop reactor which facilitated both irradiation of the culture and gas mixing compared with a conventional stirred vessel with vortex aeration. Measurements of the inhibition of maximum specific growth rate (proportional to photosynthetic rate) in the tubular reactor showed that CO₂ behaves as a typical inhibitory substrate at partial pressures (P_{CO₂}) up to 0.6 atm. The P_{CO₂} for 50% reduction in maximum specific growth rate was 0.36 atm. At 0.6 atm there was a discontinuity in the inhibitory effect with a sharp increase in the inhibitory effect at higher P_{CO₂} values. Cultures rapidly adjusted to step changes in the P_{CO₂}, up to 0.6 atm. At a P_{CO₂} of 1 atm inhibition was complete but the inhibitory effect was readily reversed.

INTRODUCTION

Photosynthetic production of microbial biomass using solar energy is a potential biotechnological route to renewable resources of carbon compounds (Pirt, 1980). Full control over the photosynthetic process requires knowledge of all the relevant environmental factors. Of these factors the effect of CO₂ partial pressure (P_{CO₂}) is one of the most important. Previous studies on the effect of P_{CO₂} on the kinetics of photosynthesis in growing organisms have been meagre and inconsistent in their findings. Nielsen (1955) found that the rate of algal photosynthesis decreased at a partial pressure above 0.05 atm. Myers (1953) and Amman & Lynch (1967) also reported inhibition of algal photosynthesis at P_{CO₂} values above about 0.05 atm. Most previous studies were made using 'resting cells' derived from cultures grown under different conditions, which, as Hogetsu & Miyachi (1977) reported, influence the photosynthetic CO₂ fixation rate. In contrast, Fowler *et al.* (1972) successfully used a P_{CO₂} of 1 atm to culture *Chlorella* sp. and in a recent study, Watts Pirt & Pirt (1980), using continuous culture, found that, apart from transient effects, the photosynthetic rate of *Chlorella* was not inhibited by CO₂ until the P_{CO₂} exceeded 0.65 atm.

The aim of the present study was to extend the work of Watts Pirt & Pirt (1980) by systematic study of the transient effects caused by step changes in the P_{CO₂}. Whereas they used a conventional stirred culture vessel with vortex aeration we have used the tubular loop reactor (Lee & Pirt, 1981) with gas injection. The tubular loop reactor was advantageous in that it improved the gas-to-liquid mass transfer rate. In a culture of algae growing photosynthetically under substrate- and light-sufficient conditions the maximum specific growth rate is proportional to the maximum photosynthesis rate. By means of the tubular loop reactor, the maximum specific growth rate of *Chlorella* was observed to decrease with increase in P_{CO₂}, above 0.07 atm and there was a sharp increase in the inhibitory effect at P_{CO₂} values above 0.6 atm.

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Table 1. *Symbols and units*

H	Henry's constant (mol l ⁻¹ atm ⁻¹)
I_0	irradiance (W m ⁻²)
K_i	inhibitor constant (mol l ⁻¹)
K_i'	inhibitor constant (atm)
K_s	saturation constant (mol l ⁻¹)
K_s'	saturation constant (atm)
t_c	cycle time in loop reactor (s)
x	biomass concentration (g dry wt l ⁻¹)
V_A	culture volume per m ² (m)
Y_G	growth yield from light (g dry wt J ⁻¹)
P_{CO_2}	partial pressure of CO ₂ (atm)
s	substrate concentration (mol l ⁻¹)
α	illuminated fraction of culture
β	fraction of culture which continues to grow in the dark
ϕ	fraction of irradiance which is available for photosynthesis
μ	specific growth rate (h ⁻¹ or s ⁻¹)
μ_m	maximum specific growth rate (h ⁻¹ or s ⁻¹)
μ_o	observed specific growth rate (h ⁻¹ or s ⁻¹)
$\mu_{m(o)}$	observed maximum specific growth rate (h ⁻¹ or s ⁻¹)

THEORY

Substrate inhibition of growth can be represented by

$$\mu = \mu_m s K_i / (s^2 + s K_i + K_s K_i) \quad (1)$$

(Pirt, 1975) where μ is the specific growth rate; μ_m is the maximum specific growth rate; s is the substrate concentration; K_s is the saturation constant; K_i is the inhibitor constant, that is the substrate concentration which causes 50% inhibition of growth ($\mu = 0.5 \mu_m$) provided $K_s \ll K_i$.

When $s \gg K_s$ it follows from equation (1) that

$$1/\mu = 1/\mu_m + s/\mu_m K_i \quad (2)$$

Then a plot of $1/\mu$ against s should be a straight line with intercept, $1/\mu_m$ and slope, $1/\mu_m K_i$, from which the values of μ_m and K_i can be estimated.

In a tubular loop reactor in which a fraction (α) of the culture is illuminated and light sufficient and the remainder ($1 - \alpha$) is in the dark, Lee & Pirt (1981) showed that the overall or observed maximum specific growth rate $\mu_{m(o)}$ is related to the actual maximum value (μ_m) by the expression

$$\mu_{m(o)} = (\alpha + \beta) \mu_m \quad (3)$$

where β is the fraction of the culture which continues to grow in the dark using light energy stored during the illuminated phase. Since Lee & Pirt (1981) found that light-sufficient cells of *Chlorella* 211/8k could continue growing at the maximum rate for 9 s in the dark it follows that the value of $\beta = 9/t_c$, where t_c (s) is the cycle time of the culture in the loop reactor.

For a gaseous substrate such as CO₂, $s = HP_{CO_2}$, $K_s = HK_s'$ and $K_i = HK_i'$ where H is Henry's constant, P_{CO_2} is the partial pressure of CO₂, and K_s' and K_i' are given in atm. Making these substitutions, from equations (1) and (3) we obtain

$$\mu_o = \mu_{m(o)} K_i' P_{CO_2} / (P_{CO_2}^2 + K_i' P_{CO_2} + K_s' K_i') \quad (4)$$

Also equation (2) becomes

$$1/\mu_o = 1/\mu_{m(o)} + P_{CO_2}/\mu_{m(o)} K_i' \quad (5)$$

Equations (4) and (5) are used to analyse quantitatively the CO₂ inhibition of photosynthetic growth.

METHODS

Organism. An axenic culture of *Chlorella vulgaris* 211/8k (Sorokin strain) was used; it was obtained from the Culture Centre of Algae and Protozoa, Cambridge, UK. Stock cultures were maintained on slopes of urea/mineral salts medium plus agar (2%, w/v) under 5% CO₂ in air at 37 °C.

Culture medium. Chemically defined medium had the following composition: (amounts in g l⁻¹): urea, 2.15; KH₂PO₄, 1.31; MgSO₄·7H₂O, 0.75; CaCl₂·2H₂O, 0.08; FeCl₃·6H₂O, 0.048; MnSO₄·4H₂O, 0.008; ZnSO₄·7H₂O, 0.009; CuSO₄·5H₂O, 0.004; Na₂B₄O₇·10H₂O, 0.00175; NaVO₃, 0.00025; NiSO₄·7H₂O, 0.0004; Na₂EDTA·2H₂O, 0.168. The pH was 6.7. The medium was made up in the following five solutions, which were autoclaved separately at 15 lbf in⁻² (103.5 kPa) for 15 min and then mixed after cooling: (a) urea, (b) KH₂PO₄ adjusted to pH 6.7 with NaOH, (c) CaCl₂ and MgSO₄, (d) FeCl₃ + EDTA (2 mol per mol Fe), (e) other salts + EDTA (1 mol per mol metal) adjusted to pH 6.7 with NaOH.

Loop reactor. The culture was grown in a tubular loop reactor similar to that used by Lee & Pirt (1981). It consisted of 28 Pyrex glass tubes, each about 31 cm long with 0.4 cm bore, connected by Pyrex and silicone rubber U-bends. The degasser had a bore of 4 cm. The culture volume, excluding the gas hold up, was 150 ml. The culture was cycled by means of a peristaltic pump (MHRE 200, Watson Marlow, Falmouth, UK). The recycle rate was 256 ml min⁻¹ which gave a Reynolds number (Pirt *et al.*, 1983) of 1000. The cycle time (*t_c*) was 35 s. The temperature of the culture was maintained at 37 °C by means of a fan heater operated by a temperature controller (Pirt *et al.*, 1979). The pH was controlled at 6.7 ± 0.1 by automatic addition of 2 M-NaOH. The pH was measured by a PYE Ingold 425-60 steam-sterilizable glass electrode connected to an EIL pH meter/controller 9160, which operated a Watson Marlow delta pump to feed the alkali. Illumination was provided by four horizontal warm-white fluorescent tubes (20 W, Philips) placed 5 cm below the reactor tubes and parallel to them. The average light intensity at the surface of the reactor, measured by a pyranometer (J. Crump, Rayleigh, Essex, UK), was 40 W m⁻². Practically all the light was photosynthetically available (wavelength 400–700 nm), that is $\phi = 1$. The fraction of the culture volume exposed to the light (α) was 0.33.

The inoculum for the loop reactor was obtained either from a shake-flask culture or by dilution of the previous culture in the loop reactor. For shake-flask culture 10 ml medium were placed in a 250 ml conical flask with a rubber stopper, inoculated, gassed with 5% CO₂ in air and incubated at 37 °C on an illuminated rotary shaker.

Gas supply. Chosen mixtures of CO₂/air were supplied to the reactor at a flow rate of 100 ml min⁻¹ through a hypodermic needle installed downstream from the pump. The required mixtures in terms of CO₂/air were obtained by mixing pure CO₂ and air. Flow controllers (G. A. Platon) and flowmeters (Rotameter Co.) were used to regulate the supply of each gas into the reactor. The gas supply was humidified by passage through a sintered filter in a bottle of water at 37 °C.

Biomass determination. The culture optical density was measured in a Unicam SP500 spectrophotometer at a wavelength of 680 nm with a 1 cm light path. Under the conditions used the optical density was proportional to the biomass dry weight concentration.

RESULTS

Cultures were operated batchwise with light-sufficient conditions in the illuminated fraction of the loop reactor. The fact that the cultures were light-sufficient and not light-limited follows from any one of three observations. First, constant exponential growth of the culture occurred whereas if the culture were light-limited the biomass would increase at a linear rate as shown by Lee & Pirt (1981). Secondly, the energy balance shows that the light input exceeded the light requirement. As shown by Pirt *et al.* (1980) the light requirement is given by

$$\phi I_0 = \mu \times V_A / Y_G \quad (6)$$

where ϕ = fraction of the incident light available for photosynthesis; I_0 = light intensity at the culture surface (W m⁻²); μ = specific growth rate (s⁻¹); x = biomass concentration (g dry wt l⁻¹); V_A = culture volume per m² (m); Y_G = growth yield (g biomass dry wt J⁻¹). Relation (6) has been shown to hold up to a ϕI_0 value of 38 W m⁻² (Pirt *et al.*, 1983). Taking the value of Y_G as 0.02×10^{-3} (Pirt *et al.*, 1980), the maximum value of $V_A = \pi r^2$ where r = the tube radius (Pirt *et al.*, 1983), the available light intensity $\phi I_0 = 40$ W m⁻², then it follows from equation (6) that with μ at its maximum value (0.17 h⁻¹ = 0.472×10^{-4} s⁻¹) there would be excess light available up to a biomass concentration of 2.5 g l⁻¹; in fact the maximum biomass concentration was maintained well below that value. Thirdly, on dilution of the culture the specific growth rate was maintained unchanged, whereas if the culture were light-limited an increase in specific growth rate would be expected.

Fig. 1 shows the maximum overall specific growth rate ($\mu_{m(o)}$) at different P_{CO₂} values. Each specific growth rate was maintained for seven or more generations. The specific growth rates were found to be reproducible within 5% of the mean. The plot of $1/\mu_{m(o)}$ against P_{CO₂}, to test equation (5), is shown in Fig. 2. The relation is linear from 0.1 to 0.6 atm but there was a marked

Fig. 1

Fig. 2

Fig. 1. Effect of CO_2 partial pressure (P_{CO_2}) on the observed maximum specific growth rate ($\mu_{m(0)}$) of *Chlorella* 211/8k in a tubular loop reactor.

Fig. 2. Plot of reciprocal growth rate ($1/\mu_{m(0)}$) versus CO_2 partial pressure (P_{CO_2}). The correlation coefficient of the linear regression line between P_{CO_2} 0.07 atm and 0.60 atm was 98.7%.

Table 2. Effect of stepwise changes in the CO_2 partial pressure (P_{CO_2}) on the observed maximum specific growth rate ($\mu_{m(0)}$) of *Chlorella* 211/8k in the tubular loop reactor

Inoculum conditions*	P_{CO_2} (atm)	$\mu_{m(0)}$ (h^{-1})
Shake flask culture, P_{CO_2} 0.05 atm	0.1	0.100
Shake flask culture, P_{CO_2} 0.05 atm	0.6	0.057
Shake flask culture, P_{CO_2} 0.05 atm	1	No growth
Diluted loop culture, P_{CO_2} 0.6 atm	0.1	0.066
From inhibited loop culture, P_{CO_2} 1 atm	0.1	0.100

* The inoculum concentration was $0.092 \text{ g dry biomass l}^{-1}$ (OD_{680} 0.9).

increase in the slope of the graph at P_{CO_2} value > 0.6 atm. These results indicate that CO_2 behaves as a simple inhibitory substrate at P_{CO_2} values up to 0.6 atm with $K_i' = 0.36$ atm, which is equivalent to a dissolved CO_2 concentration of 10 mM at 37°C . The intercept on the ordinate in Fig. 2 gives 0.133 h^{-1} for the value of $\mu_{m(0)}$, hence the uninhibited real maximum growth rate $\mu_m = \mu_{m(0)}/(\alpha + \beta) = 0.23 \text{ h}^{-1}$.

The effects of step changes in the P_{CO_2} on the maximum specific growth rate were investigated as detailed in Table 2. When the P_{CO_2} was increased from 0.05 to 0.6 atm the maximum specific growth rate fell by 43%. The adjustment took place without significant lag and the specific growth rate remained constant thereafter for at least seven generations; thus there was no sign of adaptation and development of resistance to the CO_2 inhibition. Complete inhibition of growth occurred when the P_{CO_2} was increased stepwise from 0.05 to 1 atm. When the P_{CO_2} was stepped down from 0.6 atm or more to 0.1 atm either partial or complete reversal of the inhibition could occur. As Table 2 shows, in one experiment on changing the P_{CO_2} from 0.6 atm to 0.1 atm the μ_0 increased, from 0.057 to 0.066; in another experiment, on changing from 1 atm to 0.1 atm the

reversal of inhibition was complete. The partial reversal of inhibition in the one case and the complete reversal in the other suggests that the CO_2 inhibition of photosynthesis is reversible, but there may be a variable lag before the reversal is complete.

DISCUSSION

The application of the tubular loop reactor to study the kinetics of CO_2 inhibition of photosynthesis in growing *Chlorella* cells has revealed aspects which were masked in the study made previously using a conventional stirred vessel with vortex aeration (Watts Pirt & Pirt, 1980). Whereas Watts Pirt & Pirt (1980) identified a strong CO_2 inhibition at CO_2 partial pressures > 0.6 atm the present study shows that at $P_{\text{CO}_2} < 0.6$ atm, CO_2 behaves as a typical inhibitory substrate. At P_{CO_2} values > 0.6 atm a large increase in the sensitivity to CO_2 inhibition was observed. The main cause of the masking of the CO_2 inhibition at the lower partial pressures of CO_2 in the previous studies using a stirred culture vessel could have been lack of equilibrium between the gas phase and the liquid phase. Lack of CO_2 equilibrium between the gas and the liquid phases was much less likely in the loop reactor where the gas-liquid contacting was intense. In contrast in the stirred vessel the gas-liquid contacting was relatively slight.

Watts Pirt & Pirt (1980) found that in a urea-limited chemostat culture, transient reversible inhibition by step changes in $P_{\text{CO}_2} < 0.6$ atm occurred, although the effect was not quantified. This effect could not be produced in the loop reactor but the conditions in the two systems differed in that the loop reactor cultures were substrate-sufficient batch cultures.

The effect of P_{CO_2} on the bicarbonate concentration in the culture needs to be considered in relation to the effects observed. There was no pH change with variation of the P_{CO_2} , due to the automatic pH control; under such a condition at equilibrium the bicarbonate concentration must increase with the P_{CO_2} . Given the CO_2 solubility (24 mM with $P_{\text{CO}_2} = 1$ atm at 37°C) and the pH value (6.7) the concentration of bicarbonate can be calculated (Pirt, 1975, p. 76) and it is found that with P_{CO_2} at 1 atm the bicarbonate concentration would be 60.3 mM. At other P_{CO_2} values the bicarbonate concentration would be $60.3 P_{\text{CO}_2}$ (mM), where P_{CO_2} is given in atm. The molar concentration of $\text{CO}_2 + \text{H}_2\text{CO}_3$ would be $0.4 \times$ bicarbonate molarity. It is possible that the inhibition with increase in P_{CO_2} is partly due to the osmotic effect of increase in NaHCO_3 concentration; however, in our view, the change in osmolality is unlikely to account for the inhibition. The extent to which the HCO_3^- ion, or free CO_2 or H_2CO_3 are specifically responsible for the inhibitory effects cannot be decided by these experiments.

By means of the loop reactor the K_i for CO_2 inhibition of photosynthesis in a growing culture of *Chlorella* has been determined for the first time. It would be of interest to correlate the inhibition with the enzyme activities, in particular with that of ribulosebisphosphate carboxylase, in the growing cells. Finally the quantification of the CO_2 inhibition in the tubular reactor is of particular importance to the development and scale-up of photobioreactor technology for biomass production based on solar energy.

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