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EFFECT OF CARBON DIOXIDE ON THE GROWTH OF CELL SUSPENSIONS OF **CATHARANTHUS ROSEUS**

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SUMMARY

Growth parameters of **Catharanthus roseus** in suspension cultures were examined under various pCO_2 conditions. In CO_2 -enriched environments (up to 2 %) for Erlenmeyer flask cultures, enhanced maximum growth rates and conversion yields were observed. Fermenter cultures with a constant pCO_2 of 2 %, gave high conversion yields although no growth-promoting activity was observed. High aeration rates led to decreased rates of growth due to CO_2 stripping.

INTRODUCTION

Carbon dioxide is often considered an essential requirement for the culturing of plant cells. **Nesius** and **Fletcher** (1973) have shown that a CO_2 deficient environment inhibited growth of rose cell suspensions. In sycamore cultures initiated at low cell densities growth was induced by an increase of pCO_2 (**Gathercole** et al., 1976). Flushing soyabean cells with CO_2 enhanced growth of the cultures (**Constabel** et al., 1977). The development of plant cell biotechnology has revealed gaseous transfer problems for large-scale cultivation in bioreactors. Some studies have suggested that high aeration rates may be detrimental to cell growth, possibly due to removal of CO_2 from the culture broth (**Pareilleux** and **Chaubet**, 1981 ; **Smart** and **Fowler**, 1981). However few attempts have been made to study kinetic aspects of plant cell cultures with respect to pCO_2. In the present work the effect of various CO_2 conditions on growth parameters were investigated in Erlenmeyer flask and fermenter cultures.

MATERIALS AND METHODS

Plant cells induced from **Catharanthus roseus** (L.) G. Don were used for all experiments. The cell suspensions were subcultured weekly and incubated in rotary shaken Erlenmeyer flasks (100 ml per 250 ml flask) at 120 r.p.m. and 27°C. The nutrient medium was that of **Gamborg** et al. (1968) supplemented

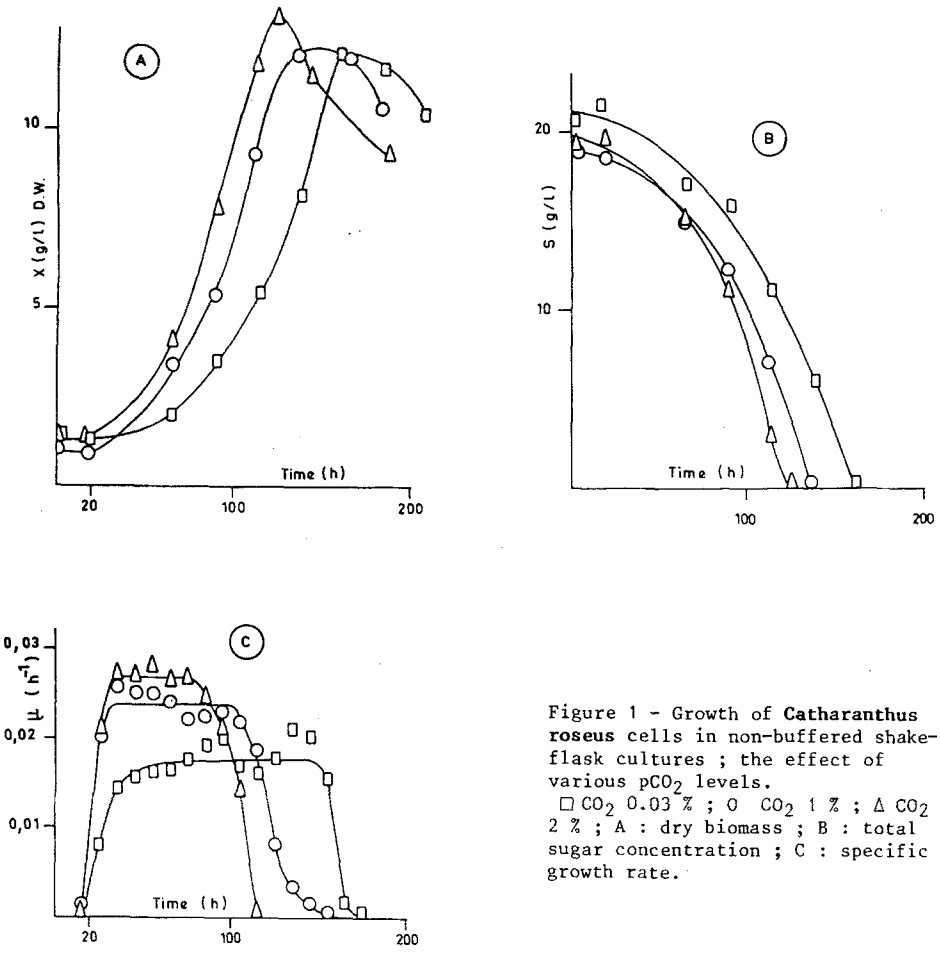

Figure 1 - Growth of **Catharanthus roseus** cells in non-buffered shake-flask cultures ; the effect of various pCO_2 levels.
□ CO_2 0.03 % ; ○ CO_2 1 % ; Δ CO_2 2 % ; A : dry biomass ; B : total sugar concentration ; C : specific growth rate.

Table I . Growth parameters for various pCO_2 levels (Erlenmeyer flask cultures).

Culture conditions		Exponential growth phase characteristic parameters			Total conversion yield
Medium	CO_2 level (%)	Specific growth rate μ_m (h^{-1})	Sugar consumption before μ_m decline (%)	Conversion yield (g d.w/g sugar)	(g d.w/g sugar)
non-buffered	0.03	0.0175	82	0.49	0.51
	1	0.0235	48.5	0.64	0.58
	2	0.0270	35	0.66	0.61
pH 6-buffered	0.03	0.0160	81	0.55	0.53
	2	0.0250	36.5	0.70	0.59

with 4.5 µM 2-4-dichlorophenoxyacetic acid and 0.28 µM kinetin. Sucrose (20 g/l) was the sole carbon source and the initial pH was adjusted to 5.5 with KOH (1 M). Erlenmeyer flask cultures at the shaking speed and temperature described above were grown in a CO_2 incubator (Forma Scientific model 3157). A fermenter (4 l) containing 3.5 l medium buffered with MES (2-(N-morpholino) ethane sulfonic acid) (0.05 M, pH 6.0) was used for other experiments with an agitation speed of 80 r.p.m. and an incubation temperature of 27°C. The bioreactor was inoculated to give an initial biomass concentration of 1 g d.w/l.

Measurements of dry cell weights were made by a filtration method using Millipore filters (0.45 µm pore size). Residual sugars (sucrose, glucose and fructose) in the culture medium were measured by an HPLC method (NH_2 silica column, acetonitrile-water 80/20).

The dissolved oxygen concentration was measured with an amperometric oxygen analyser and the pCO_2 with an Ingold CO_2 amplifier using a CO_2 probe (Severinghaus type electrode).

RESULTS AND DISCUSSION

The influence of carbon dioxide on cell growth was examined in Erlenmeyer flasks with various CO_2 conditions. Increasing CO_2 levels in the incubator resulted in enhanced biomass formation, faster carbon source utilization and higher specific growth rate (Figure 1). Analysis of cell mass and sugar consumption curves enabled growth parameters to be obtained (Table I). During the exponential growth phase carbon dioxide had a positive effect on both growth rate and conversion yield. Thereafter a reduction in carbon conversion into biomass was observed and consequently the global conversion yields were lower than those obtained during exponential growth. At high pCO_2 the point where growth rates were seen to decline occured with a higher proportion of residual sugars in the medium. Thus it appears that growth was not subject to limitation by substrate availability but rather by an inhibitory effect of carbon dioxide ; indeed, the respiratory metabolism of the cells increased the partial pressure of CO_2 in the culture medium. At the beginning of the culture the pCO_2 in cell suspension was 0.4 % in a normal (non-enriched) atmosphere, and 2.5 % in an enriched environment (2 % v/v CO_2), but during growth its value was seen to increase to 2.4 % and 3.5 % respectively (results not shown). It is therefore conceivable that pCO_2 might reach critical values in CO_2-enriched cultures.

Because the pH changed during cell growth and led to variations in the bicarbonate concentration, the above experiments were repeated in buffered medium (pH 6.0, 0.05 M MES). Growth parameters are given in Table I and as with non-buffered medium, a positive effect of CO_2 on growth rates and conversion yields was observed.

The importance of endogenous CO_2 accumulation in such cultures was investigated by comparing growth data from cultures initiated at different cell

densities under various CO_2 conditions (0.03 % and 2 % v/v CO_2). Biomass concentrations at various stages of the cultures are shown in Table II. As expected, the growth promoting activity of supplied CO_2 was observed whatever the initial cell density, but the ratio between biomass increase observed in the presence of enhanced CO_2 atmospheres and that in control cultures indicated that the efficiency of CO_2 supply was of less importance in cultures initiated at high cell densities. From these results, enhanced pCO_2 levels of up to 3.5 % in the culture medium had a positive effect on both maximum growth rate and conversion yield of **Catharanthus roseus**, but this effect could be attributed to either endogenous or exogenous CO_2.

Further experiments were initiated in a turbine driven fermenter to elucidate the range of aeration rates which would enable the plant cell bioreactors to be operated without growth limitation by gaseous transfer (O_2 limitation and detrimental CO_2 removal). In such experiments the pCO_2 levels varied during the course of the fermentation at the chosen aeration conditions (initial k_{L_a} value or dissolved oxygen level control). Batch cultivations were performed under oxygen non-limiting conditions with or without additional CO_2, the dissolved oxygen level being maintained at 2 mg/l by increasing the aeration rate (from 0.03 to 0.9 v.v.m.) as described previously (**Pareilleux** and **Vinas**, 1983).

The growth data from a culture aerated with air without additional CO_2 are presented in Figure 2. An exponential growth phase accounted for the majority of the culture time with a maximum specific growth rate (μm) of 0.0185 h^{-1}. The experimental conversion yield remained constant, averaging 0.56 g d.w/g sugar. The pCO_2 in the cell suspension was highest during early stages of growth when aeration was minimal, decreasing rapidly at higher aeration rates to establish a constant level at the end of the growth. At a constant level of pCO_2 (2 %) in the medium, cell mass and sugar concentration data were determined (Figure 3). The observed μm of 0.0178 h^{-1} was of the same magnitude as previously reported but a reduced rate of growth was observed after just 40 % of the sugar had been consumed, although this slower growth rate cannot be explained by increased CO_2 concentrations since it also occurred in shake-flask cultures. The global conversion yield in this experiment was shown to increase (up to 0.675 g d.w/g sugar), and during exponential growth, observed conversion yield reached 0.80 g d.w/g sugar.

It would therefore appear that the conversion rate was dependent on the average pCO_2 value, although no growth promoting activity was observed. In order to demonstrate that carbon dioxide tension operated upon the growth

Table II. Growth promoting efficiency of CO_2 supply during cultures initiated at different densities (Erlenmeyer flask cultures).

Age of culture (h)	Cell mass concentration (g d.w/l)		Ratio between the biomass increases, $\Delta X_B/\Delta X_A$, in time intervals 0-70 h and 70-126 h
	pCO_2 : 0.03 % (Exp. A)	pCO_2 : 2 % (Exp. B)	
0	0.285	0.285	
70	0.743	1.174	1.94
126	1.88	3.45	2.00
0	0.681	0.681	
70	1.705	2.51	1.786
126	5.463	7.49	1.325
0	1.092	1.092	
70	3.31	4.36	1.47
126	10.51	12.53	1.13

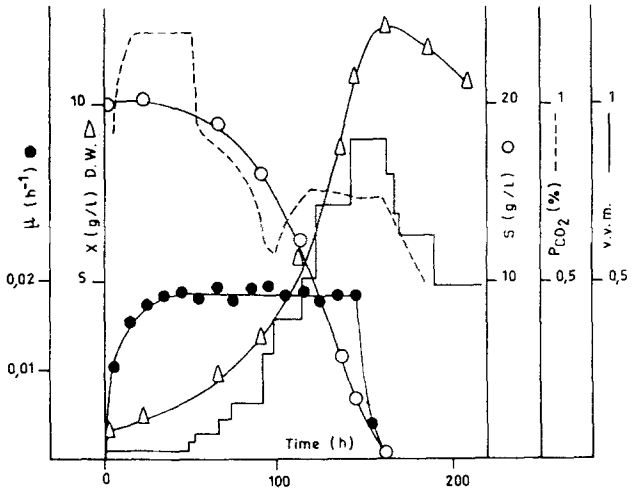

Figure 2 - Growth of **Catharanthus roseus** cells in an oxygen non-limited batch culture (Fermenter culture).
Δ dry biomass ; O total sugar concentration ; ● specific growth rate ; --- average pCO_2 values in the culture medium ; —— aeration rate.

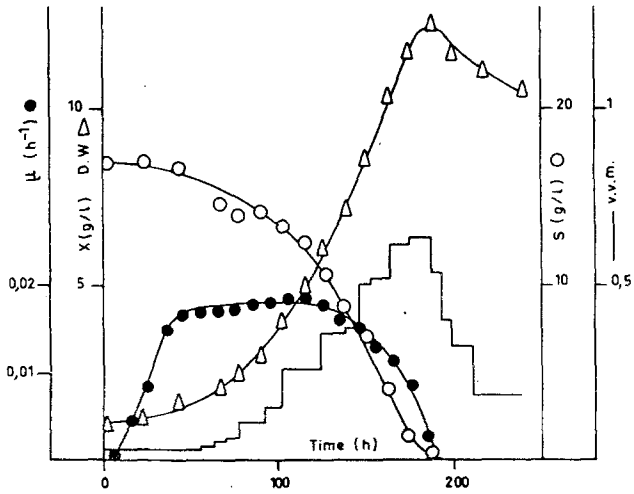

Figure 3 - Growth of **Catharanthus roseus** cells in a batch culture with constant pCO_2, 2 % (Fermenter culture).
Δ dry biomass ; O total sugar concentration ; ● specific growth rate ; —— aeration rate.

rate, a culture was carried out using high aeration rates (0.2 to 1.0 v.v.m), restricting the pCO_2 in the medium to less than 0.25 %. In such conditions, the biomass concentration reached only 5 g g d.w/l after 160 h, insted of 12 g d.w/l as in the fermenter culture performed with a lower rate of aeration (Figure 2), and the growth proceeded with a low specific growth rate ie. 0.01 h^{-1}.

CONCLUSIONS

The results presented in this report emphasize the importance of the gaseous environment in heterotrophic plant cell cultures. A distinct positive effect on growth parameters in shake-flask culture was shown with increased pCO_2 levels. In aerated fermenter-grown cultures CO_2 stripping correlated with oxygen supply was found to be an important constraint. Further detailed study of the optimal growth conditions is necessary to enable successful mass cultivation of plant cells. Such studies should include a thorough analysis of the CO_2 supply.

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