Combined Carbon Dioxide Inhibition and Oxygen Limitation of the Growth of *Pseudomonas fragi* 72 in Batch and Continuous Culture

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The effect of CO₂ inhibition and O₂ limitation on the growth of *Pseudomonas fragi* strain 72 was studied in carbon and/or energy limited continuous culture using citric acid as the growth substrate. The influence of different CO₂ concentrations on the maximal growth capacity, estimated as the dilution rate at which the culture density begins to fall (D_b) or the dilution rate at which biomass output rate is maximal (D_m), was demonstrated together with the differences in influence of CO₂ inhibition and O₂ limitation, on the steady-state characteristics of the culture. The combined effect of CO₂ inhibition and O₂ limitation was measured. Comparative studies made in batch cultures suggested that continuous culture was more sensitive to change than the batch culture.

The maximum specific growth rate of the organism decreased when the CO₂ concentration was increased. However, the results from the continuous cultures revealed a plateau in the curve of D_b versus CO₂ concentration (between 3.5% and 12% CO₂). This plateau could not be seen from the $\mu_{\max}$ determinations (batch).

Unlike O₂ limitation the CO₂ inhibition did not disturb the regularity of the curves of the steady-state characteristics. Two levels of O₂ limitation were indicated from the curve of biomass versus dilution rate, a minor one, slightly reducing the biomass concentration of the culture and a major one, significantly reducing the biomass. An O₂ concentration of 5% of the incoming gas (dissolved oxygen tension in the culture: 3–5%) had no effect on the steady-state characteristics of the culture, while 0.4% O₂ significantly affected the curve of biomass versus dilution rate (dissolved oxygen tension 0–0.4%).

The combined effect of oxygen limitation and 12% CO₂, further altered the steady-state characteristics and the biomass curve started to decrease at very low dilution rates. Thus, the two inhibitory agents co-operated and their joint growth restricting effect was considerably stronger than the strongest agent alone. The question whether the restricting effect of the two together should be regarded as 'synergistic' or 'additive', is still open to discussion even though some evidence was obtained which suggested the former.

INTRODUCTION

The critical role of O₂ as a regulator molecule for aerobic metabolism is well established. The effect of O₂ limitation of *Pseudomonas* spp. influences the growth rate at O₂ concentrations in the range 0.5 to 4.5% (v/v) of the gas phase (Clark & Burki, 1972; Wells, 1974; Mitchell & Dawes, 1982). The exact concentration is dependent on factors such as O₂ solubility of the substrate, oxygen consumption rate and mixing. A more accurate way to state the actual level of O₂ available to the organism is to give a value of the dissolved oxygen tension (DOT). However, the O₂ limitation of *Pseudomonas* usually starts at such low DOT values that they are difficult to measure.

CO₂ is suspected of being as important a regulator molecule of metabolism as O₂ but understanding of the mechanisms is considerably more vague (Jones & Greenfield, 1982). On the other hand, the growth inhibitory effects of high concentrations of CO₂ have become evident

through reports stretching from Pasteur & Joubert (1877) to Enfors & Molin (1981). Furthermore, the inhibitory effect on *Pseudomonas* spp. in particular, has been frequently studied, e.g. Clark & Lentz (1969), Gill & Tan (1980) and Enfors & Molin (1980). However, many of the results conflict with respect to the pattern of the curve of CO₂ concentration versus growth rate. Explanations could be offered in terms of the effect of growth substrate or the inherent genetic differences between different species or strains. Some of the discrepancies may also be blamed on the application, in some cases, of fairly crude systems to measure the growth rate. All the experiments were carried out in batch cultures.

Knowledge of the combined effects of CO₂ inhibition and O₂ limitation is scarce. The phenomenon has been tentatively studied in batch cultures by Wells (1974) and Enfors & Molin (1980). Wells indicated that the two growth restricting agents were 'antagonistic' (no extra inhibitory effect) but in the latter study it was indicated that they were 'additive' or even 'synergistic'. The effect of high O₂ concentrations together with the effect of CO₂ has been studied in continuous culture on the alga *Chlorella* (Pirt & Pirt, 1980) but no similar reports on O₂ limitation have been found in the literature.

The present paper reports the growth inhibitory effect of CO₂ in a steady-state culture and the combined effect of CO₂ inhibition and O₂ limitation in the same test system. Comparisons are made with batch cultures.

METHODS

Organism. *Pseudomonas fragi* 72 was originally isolated from cold stored beef and classified by Molin & Ternström (1982). Stock cultures were stored freeze-dried in skimmed-milk at 4 °C.

Culture equipment. The culture vessel was a fermenter with a working volume of 1.0 l (Chemoferm AB, Hägersten, Sweden). The fermenter was continuously fed with medium by a membrane pump (Precision dosage pump Fe 211, Braun, Melsungen, F.R.G.) and continuous outflow was achieved via an overflow outlet and a tube pump (MHRE Mk 3 flow inducer, Watson-Marlow, Falmouth, U.K.). The stirrer speed was constant at 1200 r.p.m. (impeller: 4 wings, diameter 45 mm). The pH was kept constant (pH = 6.0) by titration with 0.6 M-HCl or 1 M-NaOH (pH M 62 + TTT 60, Radiometer, Copenhagen, Denmark). The temperature was controlled via a heating bath which circulated water around an outer shell of the fermenter.

Gas supply. The chosen gas compositions were supplied to the culture at the following flow rates (l h⁻¹): air, 71; 50% CO₂ + air, 80; 16% CO₂ + air, 81; 12% CO₂ + air, 78; 6% CO₂ + air, 74; 3.5% CO₂ + air, 73; 75% N₂ + air (5% O₂), 95; 98% N₂ + air (0.4% O₂), 72; 11% CO₂ + 0.4% O₂ + N₂, 71. The air was mixed with CO₂ (purity: 99.998%; Alfax AB, Malmö, Sweden) or nitrogen (99.999%; Alfax AB) aided by flow meters (F-150-A, Porter Instrument Co. Ltd, Hadfield, U.K.). The mixture of 11% CO₂ + 0.4% O₂ + N₂ was purchased directly from the manufacturer (Alfax AB).

The CO₂ and O₂ contents of the outgoing gas from the fermenter were monitored continuously by a Binos 1 infrared gas analyser (Leybold-Heraeus, Köln, F.R.G.) and a Servomex oxygen analyser (paramagnetic; OA 540, Taylor Instrument Analytics Ltd, Crowborough, Sussex, U.K.), respectively. The DOT was measured by an oxygen electrode (Radiometer) connected to a TOX 40 oxygen transmitter (Radiometer) and is given as percentage of the value obtained under a theoretical atmosphere of 100% O₂ (the measurement was performed with an electrode calibrated in air).

Medium. The continuous culture medium had the following composition (g l⁻¹): citric acid (1-hydrate; 244, Merck), 1.0; MgSO₄, 0.5; Na₂HPO₄·2H₂O, 0.40; KH₂PO₄, 0.37; (NH₄)₂SO₄, 0.25; CaCl₂, 0.10; MnSO₄·H₂O, 0.05; FeSO₄·7H₂O, 0.01; CuSO₄·5H₂O, 0.005; ZnSO₄·7H₂O, 0.005; 0.10 ml Silicone antifoaming agent (BDH) l⁻¹, pH was adjusted using HCl or NaOH to 5.0 before autoclaving (in 20 l bottles at 121 °C for 60 min) and to 6.0 afterwards. The same medium was used for the batch cultures except that it contained 3.0 g citric acid l⁻¹.

Inoculation. The freeze-dried cultures were recovered in Nutrient broth (Oxoid; shaken bottles) at 25 °C for 8 h and then transferred to a bottle with the medium described above but with 5.0 g citric acid l⁻¹. After 16 h (25 °C; shaken) the fermenter was inoculated. The inoculum was 8% of the fermenter volume.

Growth experiments. The inoculated fermenter, sparged with a chosen gas, was run as a batch culture for 7–9 h after which the continuous culture was started. The continuous culture was run on citric acid as a carbon source and/or energy limited chemostat for a total time of 7–10 d.

The continuous culture was started at the lowest dilution rate (about 0.1 h⁻¹), the dilution rate then being successively increased until wash-out was almost reached. Each dilution rate was maintained for 24 h before any measurements were made (40 h for *D* = 0.1 and 64 h for *D* < 0.1). All cultures were performed at 25 °C and pH 6.0.

Fig. 1. Maximal growth capacity of *Pseudomonas fragi* 72 at different CO₂ concentrations of the outgoing gas phase: ○, $\mu_{\max}$ estimated from batch cultures; ●, dilution rate of biomass decrease estimated from carbon and energy limited continuous cultures (D_b , the dilution rate where the curve of biomass versus dilution rate had decreased by 0.03 A_{620} units from its maximum value).

Analytical methods. When the culture was in steady-state at a specific dilution rate, the following measurements were performed: absorbance at A_{620} (Turner spectrophotometer, 330; the same 1 cm cuvette was used for all measurements), dry weight, viable count [Tryptone glucose extract agar, (Difco) for 3 d at 25 °C], and citric acid concentration (enzymically; Lebensmittelanalytik, Boehringer-Mannheim).

Both the viable count and the dry weights roughly followed the A_{620} values. However, the latter was generally more reproducible and in the present study the biomass is reported as absorbance.

Determination of maximal growth capacity. The maximum specific growth rate was calculated from the batch culture (Pirt, 1975). In CO₂ the growth curves of the batch cultures (log A_{620} versus time) sometimes showed two exponential phases. The first was short and steep and the second one was considerably longer and somewhat flatter. The latter was used to calculate $\mu_{\max}$.

In contrast, under reduced input of O₂, $\mu_{\max}$ was calculated from the first part of the growth curve. The curve then became non-exponential.

A value of the maximal growth capacity of the continuous culture was obtained by estimating, from the curve of the biomass versus dilution rate, the dilution rate where the biomass had decreased 0.03 A_{620} units from its maximum value (dilution rate of biomass decrease, D_b) and by estimating the dilution rate of the maximum output rate (dilution rate of maximum output rate, D_m).

RESULTS

The effect of different CO₂ concentrations on the maximal growth capacity estimated as $\mu_{\max}$ and D_b is given in Fig. 1. A corresponding curve of D_m versus CO₂ concentration coincides with that of D_b . There was a marked difference in shape between the $\mu_{\max}$ curve obtained from batch cultures and the D_b curve obtained from continuous cultures. The CO₂ concentration of the culture grown under an air atmosphere was 0.2% when the output rate of the culture was at its maximum. The maximum specific growth rate at 50% CO₂ was 0.10 h⁻¹.

The steady-state characteristics of the culture under air and a mixture of 12% CO₂ plus air are shown in Fig. 2(a, b). The biomass at carbon source limited steady-state was slightly lower in 12% CO₂ than in pure air. The yield coefficient was decreased from about 0.44 to 0.39 (A_{620} units per g citric acid). The yield was about 0.44, 0.40 and 0.37 for 3.5%, 6% and 16% CO₂, respectively.

The dry weights at carbon source limited steady-state were 0.2–0.3 g l⁻¹ for all the tested CO₂ concentrations and the viable count was about 3×10^8 c.f.u. ml⁻¹.

The DOT was on no occasion lower than 18% O₂ in any of the cultures of Fig. 1. The maximum specific growth rate as well as D_b was unaffected by a decreased O₂ concentration of

Fig. 2. Steady-state characteristics of *Pseudomonas fragi* 72 in continuous culture. (a) Biomass (●) and substrate concentration (○) in air; biomass (▲) and substrate concentration (△) in 0.4% $O_2 + N_2$. (b) Biomass (■) and substrate concentration (□) in 12% $CO_2 + air$; biomass (▼) and substrate concentration (▽) in 0.4% $O_2 + 12\% CO_2 + N_2$. (c) Biomass output rate/productivity in air (●), 12% $CO_2 + air$ (■), 0.4% $O_2 + N_2$ (▲) and 0.4% $O_2 + 12\% CO_2 + N_2$ (▼).

the incoming gas phase down to 5% which corresponded to a DOT of 3–5%. However, at an O_2 concentration of 0.4% of the gas phase (DOT, 0–0.4% O_2), the culture was severely limited (Fig. 2a). The maximum specific growth rate of the batch was $0.24\ h^{-1}$ whereas the biomass curve of the continuous culture dropped in two stages, indicating a step-by-step O_2 limitation at increasing dilution rates, i.e. a primary limitation at $0.19\ h^{-1}$ and a secondary limitation at $0.30\ h^{-1}$. The initial decrease in biomass was also slightly reflected in the curve of the output rate (Fig. 2c). However, the output-rate curve of the O_2 limited culture had a similar appearance to that of the pure air. This is in spite of the significant discrepancies in shape between the curves

Table 1. *Maximal growth capacity of Pseudomonas fragi* 72 in batch and continuous culture

Maximal growth capacity is measured as maximum specific growth rate ($\mu_{\max}$) in batch cultures, and as the dilution rate of biomass decrease (D_b) and the dilution rate of maximum output rate (D_m) in continuous cultures.

Gas atmosphere	Batch culture		Continuous culture*			
	$\mu_{\max}$ (h ⁻¹)	RI† (%)	D_b ‡ (h ⁻¹)	RI† (%)	D_m § (h ⁻¹)	RI† (%)
Air	0.40	0	0.45	0	0.42	0
0.4% O ₂ + N ₂	0.24	40	0.30 (0.19)	33 (58)	0.31	26
12% CO ₂ + air	0.22	45	0.35	22	0.33	21
11% CO ₂ + 0.4% O ₂ + N ₂	0.24	40	0.21 (<0.11)	53 (>76)	0.23	45

* Values within parentheses are based on the initial fall of the A_{620} curve (Fig. 2).

† Relative inhibition, $RI = 100 (r_c - r) r_c^{-1}$ where r_c and r are the values of the control culture (air) and the restricted culture, respectively.

‡ The dilution rate where the curve of biomass versus dilution rate has decreased by 0.03 A_{620} units from its maximum value.

§ The dilution rate where the culture has its maximum output rate.

of biomass versus dilution rate. The yield at both phases (A_{620} constant) was about 0.4 (A_{620} units per g citric acid), which is the same as in air.

The steady-state characteristics of a culture that was simultaneously O₂ limited (0.4% O₂ + N₂) and CO₂ inhibited (12% CO₂ + air), are shown in Fig. 2(b). The A_{620} curve appears somewhat irregular at low dilution rates with a fairly flat slope down to higher dilution rates. The peak of the productivity curve maximum was rounded (Fig. 2c).

The combined effect of CO₂ and low O₂ content is compared in Table 1 with each inhibitory factor alone. According to the batch studies of the $\mu_{\max}$ values, the relative inhibitory effect of O₂ limitation and CO₂ are about equal. Furthermore, the combined effect is about the same as that for each factor on its own. However, the situation drastically altered when the effects were evaluated from the drop in biomass of the steady-state cultures (D_b). The growth preventing effect of O₂ limitation was larger than that of CO₂ inhibition and the combined effect was considerably larger than the O₂ limitation by itself (Table 1).

It can be seen that the sum of the restriction due to CO₂ (20%) and of O₂ limitation (33%) should be 48% if the values of the relative reduction are compared. Addition of reductions is done according to the formula:

$$\text{sum of reductions} = B + C - BC \cdot 100^{-1}$$

where B is the reduction in CO₂ and C the reduction at O₂ limitation. In contrast, the experimentally-found effect of both CO₂ inhibition and O₂ limitation together was 53% (Table 1), i.e. 5% larger than expected from pure addition.

If the calculation is made using the values of primary O₂ limitation (values within parentheses in Table 1) the difference between theoretical and experimental values is >9%. However, when the calculation was made from the D_m values, the difference was only 3%.

DISCUSSION

The maximal growth capacity of the culture was given as $\mu_{\max}$ in the batch cultures. However, it was difficult to calculate any reliable $\mu_{\max}$ values from the continuous cultures.

The method of 'wash out' (Pirt, 1975) was tried and found to be unreliable. Maximum specific growth rates obtained in this way were not constant but highly dependent upon the dilution rate that preceded the wash-out experiment; the dilution rate of the wash-out; the age of the continuous culture. Another way to determine $\mu_{\max}$ had been to use a reciprocal plot of growth rate (dilution rate) versus substrate concentration (Pirt, 1975). However, no reliable values could be calculated from the present data as the plot was not linear.

These difficulties may have been caused by attached growth in the fermenter, a phenomenon which had earlier been demonstrated as being a problem in continuous cultures of *Pseudomonas* (Molin, 1981). Attached growth may also underlie the finding that steady-states in the present study were obtained at dilution rates far above the $\mu_{\max}$ calculated from the batch cultures.

In the absence of reliable $\mu_{\max}$ values the estimated D_b and D_m values make it possible to compare the maximal growth capacity of the continuous culture under different conditions. However, the way D_b or D_m relate to the true $\mu_{\max}$ of a continuous culture and whether the $\mu_{\max}$ value of a batch and its corresponding continuous culture really are equal, cannot be answered by the present study.

The response of the growth rate to different CO_2 concentrations is shown in Fig. 1. The 'batch curve' had a similar shape to that reported earlier for *P. fragi* ATCC 4973 (8–90% CO_2 ; Enfors & Molin, 1980). However, the CO_2 effect on D_b was different in the continuous cultures and a plateau in the decreasing curve was revealed at 3.5–12% CO_2 (Fig. 1). The principal difference between the two test systems may offer an explanation for the apparent discrepancy in CO_2 response. Thus, in the continuous cultures the organism (1) has been accustomed to the CO_2 atmosphere for several days before it had to make use of its maximal growth capacity, and (2) no biological material from the inoculum was left in the fermenter when the maximal growth capacity was determined. Therefore the continuous culture is probably a more accurate testing tool and suggests a truer picture of how growth is influenced by the CO_2 concentration.

The DOT in a continuous culture with a constant input of a limited amount of O_2 will gradually decrease with dilution rate (growth rate) until not enough O_2 is available for full metabolic activity. At this point the biomass will start to decrease and the relationship between growth rate, total biomass and available O_2 will control the shape of the biomass curve. According to Pirt (1975) this fall in the curve is initially steep, subsequently becoming flatter until the point where the culture is washed-out at about the same dilution rate as a carbon source limited culture with full access to O_2 . However, this behaviour was not shown by the O_2 limited continuous culture in the present study (Fig. 2a), where the biomass curve had a plateau indicating a step-by-step O_2 limitation. This is unexpected in view of what has just been said. It was likewise unexpected that the growth curves of the O_2 restricted batch cultures were exponential over a significant length of time from where a $\mu_{\max}$ could be calculated. Both the results found in batch and in continuous culture would suggest that the growth rate of an O_2 limited culture is controlled by the availability of O_2 in a stepwise pattern, i.e. the growth rate is not changing continuously with changes in DOT.

The combined effect of CO_2 inhibition and O_2 limitation gave steady-state characteristics that followed curves of new, and in the case of the biomass, irregular patterns without any clear plateaux (Fig. 2b). However, the curves indicate that some change occurred in the cultures at an approximate dilution rate of 0.1 h^{-1} . This was, unfortunately, also the starting point of the experiment. Nevertheless, it could suggest that there is a primary O_2 limitation around the dilution rate of 0.1 h^{-1} .

When the combined effect of O_2 limitation and a growth inhibiting factor (CO_2) is to be studied, it could be argued that the additional inhibitor slows down growth and O_2 consumption rate leading to a less strict O_2 limitation. In fact, this could be one explanation of the apparently poor inhibition by CO_2 and O_2 limitation when tested in batch cultures (Table 1). On the other hand, this cannot be the explanation for the results obtained with continuous culture where the O_2 restriction started at a considerably lower dilution rate than the CO_2 inhibition (Fig. 2a, b). In the continuous culture O_2 restriction and CO_2 inhibition are not antagonistic but co-operative, i.e. the two factors together gave a greater reduction in growth than each factor alone (Table 1). However, it cannot be determined whether they are 'additive' or 'synergistic'. There is some evidence suggesting the latter, but the comparison of the two growth restricting agents was only made with a single set of conditions. This is particularly important since it has been argued that discussion about additivity/synergism could not be held on the basis of 'an effect' (in the present study the apparent growth rate at a certain condition) but must be based on 'a quantity' (for example, the amount of inhibitory agent necessary to obtain the value $\mu_{\max} 2^{-1}$ where $\mu_{\max}$ is the growth rate at optimum growth conditions; Voorspuij, 1977). Nevertheless, at the

concentrations tested, the combined effect of CO₂ inhibition and O₂ limitation was considerably larger than either agent working alone.

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REFERENCES

- CLARK, D. S. & BURKI, T. (1972). Oxygen requirements of strains of *Pseudomonas* and *Achromobacter*. *Canadian Journal of Microbiology* **18**, 321–326.
- CLARK, D. S. & LENTZ, C. P. (1969). The effect of carbon dioxide on the growth of slime producing bacteria on fresh beef. *Canadian Institute of Food Technology Journal* **2**, 72–75.
- ENFORS, S.-O. & MOLIN, G. (1980). Effect of high concentrations of carbon dioxide on growth rate of *Pseudomonas fragi*, *Bacillus cereus* and *Streptococcus cremoris*. *Journal of Applied Bacteriology* **48**, 409–416.
- ENFORS, S.-O. & MOLIN, G. (1981). The influence of temperature on the growth inhibitory effect of carbon dioxide on *Pseudomonas fragi* and *Bacillus cereus*. *Canadian Journal of Microbiology* **27**, 15–19.
- GILL, C. O. & TAN, K. H. (1980). Effect of carbon dioxide on growth of meat spoilage bacteria. *Applied and Environmental Microbiology* **39**, 317–319.
- JONES, R. P. & GREENFIELD (1982). Effect of carbon dioxide on yeast growth and fermentation. *Enzyme and Microbial Technology* **4**, 210–223.
- MITCHELL, C. G. & DAWES, E. A. (1982). The role of oxygen in the regulation of glucose metabolism, transport and the tricarboxylic acid cycle in *Pseudomonas aeruginosa*. *Journal of General Microbiology* **128**, 49–59.
- MOLIN, G. (1981). The impact of dilution rate and attached growth on steady-state characteristics of *Pseudomonas putida*. *European Journal of Applied Microbiology and Biotechnology* **13**, 102–106.
- MOLIN, G. & TERNSTRÖM, A. (1982). Numerical taxonomy of psychrotrophic pseudomonads. *Journal of General Microbiology* **128**, 1249–1264.
- PASTEUR, L. & JOUBERT, L. (1877). Etude sur la maladie charbonneuse. *Comptes rendus hebdomadaires des séances de l'Académie des sciences* **84**, 900–906.
- PIRT, M. W. & PIRT, S. J. (1980). The influence of carbon dioxide and oxygen partial pressures on chlorella growth in photosynthetic steady-state cultures. *Journal of General Microbiology* **119**, 321–326.
- PIRT, S. J. (1975). *Principles of Microbe and Cell Cultivation*. Oxford: Blackwell Scientific Publications.
- VOORSPUIJ, A. J. Z. (1977). Some fundamental reflections on “antimicrobial potency”. In *Antibiotics and Antibiosis in Agriculture*, pp. 284–295. Edited by M. Woodbine. London: Butterworths.
- WELLS, J. M. (1974). Growth of *Erwinia carotovora*, *E. atroseptica* and *Pseudomonas fluorescens* in low oxygen and high carbon dioxide atmospheres. *Phytopathology* **64**, 1012–1015.