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## Effects of Partial O<sub>2</sub> Pressure, Partial CO<sub>2</sub> Pressure, and Agitation on Growth Kinetics of *Azospirillum lipoferum* under Fermentor Conditions

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*Azospirillum lipoferum* crt1 was grown in batch cultures under standard conditions at 85% saturation of dissolved oxygen (DO) and 30-g/liter glucose concentrations. Kinetic studies revealed nutritional limitations of growth and the presence of an initial lag phase prior to consumption of glucose. The influences of various gaseous environments and shear stress on growth, i.e., various conditions of agitation-aeration, were characterized. Faster growth in the first stages of the culture and shorter duration of the lag phase were observed at DO concentrations of <30% saturation. The possible influences of dissolved CO<sub>2</sub> concentration or shear stress or both were discounted, and we confirmed the detrimental effect of high DO levels (up to 80% saturation) and the favorable influence of low DO concentrations (lower than 30% saturation) on growth. It was concluded that the gaseous environment, i.e., the DO concentration, needs to be considered as an operating parameter and be accurately controlled to ensure optimal growth of *Azospirillum* cells.

Bacteria of the genus *Azospirillum* are free-living, N<sub>2</sub>-fixing bacteria, which colonize the rhizosphere of nonleguminous plants, mainly, forage and grain grasses. These bacteria are able to produce plant growth hormones and to improve nutrient uptake by roots. These properties may explain beneficial effects of *Azospirillum* inoculation on plant growth and crop yields (18, 21). However, the phenomena implied in crop yield enhancement are complex (22), and several questions arise regarding the variability of responses to the microbial inoculation of plants (21, 26). Properties such as production of cysts under unfavorable conditions (24), accumulation of reserve material such as poly-β-hydroxybutyrate (PHB) (28), utilization of a wide range of carbon sources (8, 12, 17), and ability to move chemotactically (23) provide mechanisms for good colonization and survival of the *Azospirillum* cells in the rhizosphere. Benefits may also depend on the effectiveness of the association between plant and microorganism and therefore on the environmental conditions as well as on plant and bacterial genomes. It must be pointed out that only few results of field experiments take into account bacterial concentration in the inoculum and the viability of cells (28). Studies on *Azospirillum lipoferum* provide information about taxonomy, metabolic aspects of carbon and nitrogen utilization, and PHB formation (8). In addition, biochemical investigations have elucidated the different pathways for utilization of sugars or organic acids. *A. lipoferum* possesses the complete Entner-Doudoroff pathway, glycolytic pathway, and tricarboxylic acid cycle (20). However, only a few reports on growth on organic acids or glucose of this microorganism in submerged cultures are available in the literature (1).

This paper reports kinetic aspects of the growth of *A. lipoferum* cultivated in fermentors with the aim of biomass production for an agricultural inoculant. Varying the dis-

solved oxygen (DO) and carbon dioxide concentrations, we investigated the influence of gaseous environments on biomass formation and glucose consumption as related to ensuring faster growth.

### MATERIALS AND METHODS

**Microorganism and culture conditions.** *A. lipoferum* crt1 was isolated from corn roots by Fages and Mulard (11).

The standard medium used was derived from that proposed by Fages (10). It has the following composition (in grams per liter): MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.2; NaCl, 0.1; CaCl<sub>2</sub>, 0.02; FeSO<sub>4</sub> · 7H<sub>2</sub>O, 0.01; Na<sub>2</sub>MoO<sub>4</sub> · 2H<sub>2</sub>O, 0.002; K<sub>2</sub>HPO<sub>4</sub>, 0.6; KH<sub>2</sub>PO<sub>4</sub>, 0.4; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 3; yeast extract, 3; glucose, 30. The pH was adjusted to 7.5 by NaOH addition. The medium was sterilized at 120°C for 20 min by autoclaving. Working cultures were propagated in 250-ml Erlenmeyer flasks containing 150 ml of medium. The flasks were incubated on a reciprocating shaker (120 strokes per min) at 32°C for 24 h. For batch culture experiments, glucose was sterilized separately and added aseptically at an initial concentration of 30 g/liter.

**Growth conditions.** Batch cultures were performed in 2-liter fermentors (S.G.I., Toulouse, France) at 32°C. The agitation system was a Rushton-like turbine, and for standard conditions initial impeller rate and volumetric air flow rate were, respectively, 500 rpm and 0.65 liter/liter/min (unless otherwise specified). The DO concentration was kept constant by changing the impeller speed. The aeration flow rate was measured with a gas mass flow meter which was also used, when necessary, for DO concentration regulation. The pH value was maintained constant at 7.0 by NH<sub>4</sub>OH addition. An increase in pH from 7 to 7.4 was noticed during the first stage of the culture (before initiation of sugar consumption), but no regulation was set up during this growth period. Addition of Silicone J673 antifoam prevented foam formation.

**Analytical methods.** Biomass concentration was determined by cell dry weight measurements, and the number of

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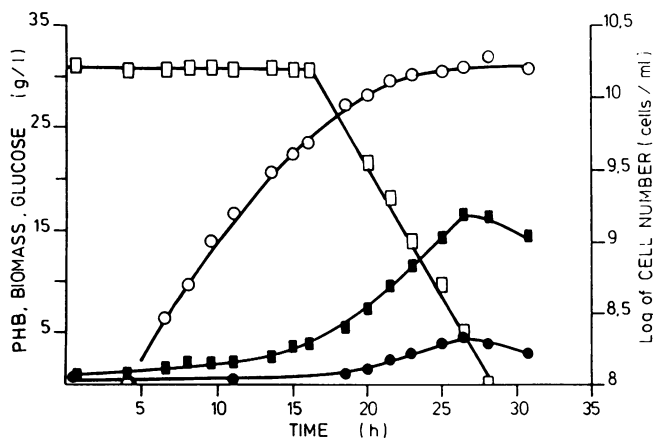


FIG. 1. Time courses of changes in biomass concentration (■), cell number (○), residual sugar (□), and PHB concentration (●) in a reference culture.

cells was determined by enumeration with a hemacytometer slide. Glucose analyses were achieved on supernatants of centrifuged samples with a glucose analyzer (YSI model 27). Intracellular PHB was determined by a method described by Braunegg et al. (3), using a Perkin-Elmer sigma 3B gas chromatograph equipped with a flame ionization detector. The column was made with 10% Carbowax 20M-TPA on Chromosorb W-AW, 80 to 100 mesh (Alltech). Nitrogen was used as a carrier gas at a 30-ml/min flow rate. Injector, detector, and column temperatures were 300, 320, and 130°C, respectively. Analysis of chromatographic data was carried out on an Intersmat ICR1B integrator and was based on the use of benzoic acid as internal standard.

DO concentration in the culture medium was measured with an Ingold amperometric probe. At saturation, the solubility of oxygen in water was 7.2 mg/liter (32°C, 10<sup>2</sup> kPa). The oxygen saturation constant,  $K_{sO_2}$ , was determined by plotting  $1/r_{O_2}$  versus  $1/[O_2]$ , assuming that the oxygen uptake by the cells is described by the usual Monod relation. The carbon dioxide partial pressure was measured with a CO<sub>2</sub> probe and an Ingold CO<sub>2</sub> amplifier and, when necessary, maintained at a constant level by supplying carbon dioxide to the inlet air flow.

## RESULTS AND DISCUSSION

**Growth under reference conditions.** Fermentors were inoculated with flask cultures to give initial cell counts in the range of  $1 \times 10^8$  to  $2 \times 10^8$  cells per ml. The DO concentration was maintained at 85% of saturation level. For one of the standard cultures, the time courses of biomass concentration, cell number, residual sugar, and PHB concentration are given in Fig. 1 and the main growth characteristics are summarized in Table 1.

According to cell number, a short lag phase of <4 h was observed. An exponential growth phase then occurred as shown by the linear increase in the log cell number (Fig. 1). However, no corresponding glucose consumption was observed until about 13.5 h. The early growth is probably supported by yeast extract and intracellular PHB, which can be used as carbon and energy sources (28). During this period, the mean specific growth rate ( $\mu_{C1}$ ), calculated by using cell number increase, was  $0.18 \text{ h}^{-1}$ . At this moment, the cell number reached about  $1.5 \times 10^9$  to  $2.5 \times 10^9$  per ml, equivalent to 1.5 to 2.5 g (dry weight) of biomass per liter.

TABLE 1. Growth characteristics of *A. lipoferum* for various agitation-aeration levels

Operating conditions			Growth characteristics			
DO (% of saturation)	Initial agitation rate (initial $k_L a$ )		Lag phase prior to glucose consumption (h)	$\mu_{C1}$ ( $\text{h}^{-1}$ ) <sup>a</sup>	$\mu_{C2}$ ( $\text{h}^{-1}$ ) <sup>b</sup>	Specific glucose consumption rate (g/g/h)
	rpm	$\text{h}^{-1}$				
85 <sup>c</sup>	500	90	11	0.18	0.13	0.25
25	200	48	8	0.268	0.185	0.29
25	350	65	8	0.25	0.2	0.25
85	500	90	12	0.17	0.18	0.21
85	800	120	12	0.177	0.146	0.196
25	350		9	0.32	0.217	0.33
100	500		11	0.25	0.19	0.27
10	200		7	0.23	0.17	0.17
25	350		8	0.22	0.16	0.18
25	400		9	0.28	0.2	0.25
85	400		11	0.21	0.18	0.24

<sup>a</sup> Mean specific growth rate during the glucose nonconsumption phase.

<sup>b</sup> Mean specific growth rate during the glucose consumption phase.

<sup>c</sup> Reference culture.

Glucose concentration then began to show a linear decrease and a complete depletion at 26.5 h of culture. The specific consumption rate of glucose ( $q_s$ ) continuously decreased from 0.55 to 0.19 g/g/h. At the end of the incubation, the biomass concentration reached maximal values of 16.5 g (dry weight)/liter and  $1.9 \times 10^{10}$  cells per ml. The mean specific growth rate ( $\mu_{C2}$ ) strongly decreased to  $0.13 \text{ h}^{-1}$ . This change in the  $\mu_C$  value between the two periods of growth may correspond to the depletion of yeast extract, which provides faster growth. PHB production only occurred during the glucose consumption phase with a continuous increasing specific formation rate from 0.035 to 0.06 g/g/h. PHB formation seemed to have been initiated by a metabolism shift due to a nutritional limitation. The scheme of fine control of PHB metabolism proposed by Senior et al. for *Azotobacter beijerinckii* (25) and verified by Tal and Okon for *Azospirillum brasilense* (28) might be applicable to the *A. lipoferum crt1* strain. Indeed, when a nutrient limitation occurs, the concentration of reduced coenzyme NADH increases, excess reducing power being eliminated by PHB synthesis. The assumed nutrient limitation occurring in our culture conditions could explain the decrease of  $q_s$  and the time lag between maximum cell number and maximum biomass dry weight.

As fermentor cultures, carried out after two flask stages with the same medium, did not lead to a reduction of the lag phase, the question of the existence of a delay in glucose consumption arose. In addition, reducing the yeast extract concentration in the medium two- to fourfold did not cause an earlier onset of glucose consumption. Therefore, a diauxic effect could not entirely explain the sugar nonconsumption phase (data not shown). It appears that enhancement of cell productivity could be achieved by reducing this phase.

The differences in growth observed in flask or fermentor cultures, probably due to differences in the gaseous environment, led us to examine the importance of this parameter for cultivation of *A. lipoferum crt1*.

**Effect of gaseous environment on growth.** Batch cultures were performed at two constant DO concentrations, 25 and 85% of saturation. For each DO value, two initial agitation rates were tested, 200 and 350 rpm for the first condition and 500 and 800 rpm for the second condition. The correspond-

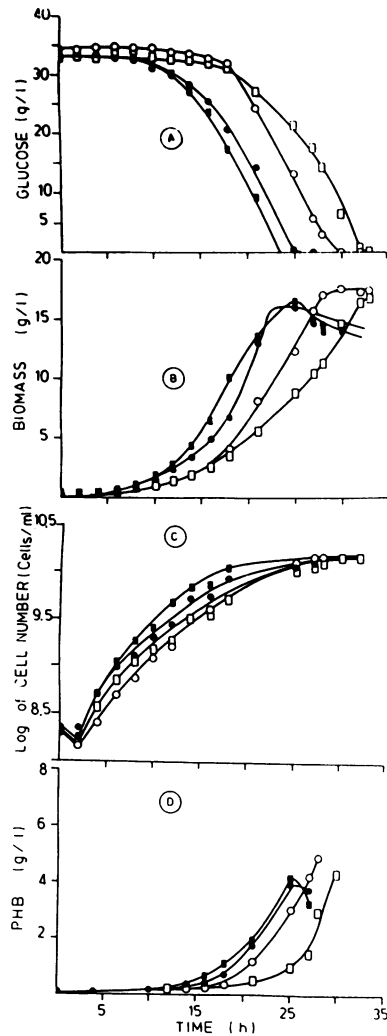


FIG. 2. Time courses of residual sugar (A), biomass (B), cell number (C), and PHB concentrations (D) for various initial agitation-aeration rates: 200 rpm, 0.55 vol/vol/min, 25% DO concentration (■); 350 rpm, 0.55 vol/vol/min, 25% DO concentration (●); 500 rpm, 1 vol/vol/min, 85% DO concentration (○); 800 rpm, 1.3 vol/vol/min, 85% DO concentration (□).

ing initial values of the global oxygen transfer rate,  $k_L a$ , were 48, 65, 90, and 120 h<sup>-1</sup>. The DO concentration was kept constant by sparging nitrogen at the beginning of the cultures or by increasing the agitation rate afterwards. The time courses of glucose consumption, cell number, dry matter, and PHB concentration are given in Fig. 2A, B, C, and D, respectively. The main kinetic parameters are summarized in Table 1.

Compared with the 85% saturation DO level condition, maintaining the DO concentration at 25% of saturation resulted in a shorter lag phase before the onset of glucose consumption and a higher  $q_s$  value. In addition, whereas no difference was observed in glucose consumption rate for the two lowest  $k_L a$  values, higher and different consumption rates were noticed for the 90- and 120-h<sup>-1</sup>  $k_L a$  values (Fig. 2A).

Maximal biomass concentrations (dry weight and cell number) were almost the same for the four culture conditions tested and compared favorably with those obtained in

the reference culture. According to the values of the mean specific growth rate,  $\mu_{C1}$ , calculated during the first growth period, a faster growth rate was noticed when DO concentration was regulated at 25%. However, the values of the growth rate,  $\mu_{C2}$ , calculated during the glucose consumption phase were quite similar regardless of the DO level (Table 1). For the two culture conditions, PHB production presented the same profile, but the longer the lag phase prior to glucose consumption, the later the initiation of PHB formation (Fig. 2D).

No significant changes in kinetic parameters were observed for the same DO concentration when the agitation profile was changed. In contrast, increasing the DO concentration resulted in an increase of the lag phase and a decrease of  $\mu_{C1}$ . The DO concentration appears to be a key parameter for control growth kinetics.

For further analysis of the effect of oxygen concentration on growth, cultures were grown at a DO concentration either higher than 85%, using pure oxygen, or at 10% of saturation. According to the value of the saturation constant relative to oxygen,  $K_{sO_2}$ , found for the strain (0.25 mg/liter), the latter DO value was chosen to ensure the oxygen demand of the culture without O<sub>2</sub> limitation. The initial agitation rates were, respectively, 500 and 200 rpm. Comparing the growth kinetic parameters calculated in these two culture conditions with those of their corresponding standard cultures, the negative effect of a high DO concentration and the favorable influence of a low DO concentration on growth were emphasized (Table 1). Nevertheless, as the various DO levels were obtained by varying the agitation and aeration rates, possible negative influences of shear stress or CO<sub>2</sub> stripping or both were investigated.

Two cultures were grown at 25 and 85% DO concentration with a 400-rpm initial agitation rate and the same agitation profile. Despite the same agitation conditions, the time lag in glucose consumption and the differences in  $\mu_{C1}$  values during the first stage of growth were found once again. Thus, any possible influence of shear stress on growth was discounted (Table 1). As measurement of the carbon dioxide partial pressure ( $p_{CO_2}$ ) in a reference culture revealed a CO<sub>2</sub> concentration of 30 ± 10 mbar (3.06%) at the onset of the glucose consumption phase, a culture was performed at a constant  $p_{CO_2}$  value of 30 mbar during the early stage of growth and compared with a reference culture carried out without  $p_{CO_2}$  regulation. The desired CO<sub>2</sub> concentration was

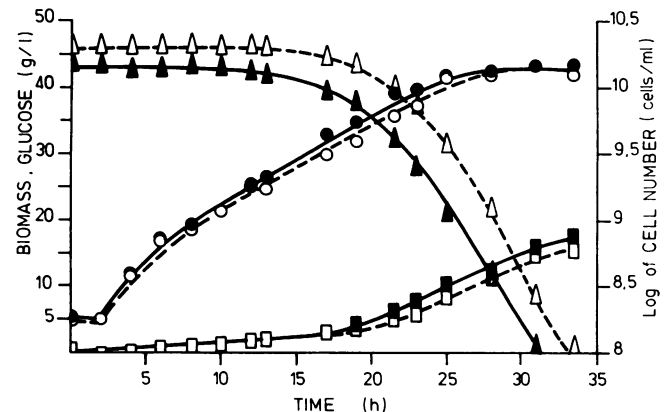


FIG. 3. Time courses of biomass concentration (□, ■), cell number (○, ●), and residual sugar (Δ, ▲) for constant (---, open symbols) or nonregulated (—, closed symbols) CO<sub>2</sub> partial pressure.

TABLE 2. Growth characteristics of *A. lipoferum* at constant or nonregulated CO<sub>2</sub> partial pressure

Operating condition			Growth characteristics			
DO (% of saturation)	CO <sub>2</sub> concentration (%)	Initial agitation rate (rpm)	Lag phase prior to glucose consumption (h)	$\mu_{C1}$ (h <sup>-1</sup> ) <sup>a</sup>	$\mu_{C2}$ (h <sup>-1</sup> ) <sup>b</sup>	Specific glucose consumption rate (g/g/h)
85	0.05	600	12	0.19	0.16	0.23
85	3.06	600	13	0.19	0.154	0.24

<sup>a</sup> Mean specific growth rate during the glucose nonconsumption phase.

<sup>b</sup> Mean specific growth rate during the glucose consumption phase.

maintained with CO<sub>2</sub>-enriched air. In the two cultures, the DO concentration was kept constant at 85% of saturation and the agitation rate was fixed at 600 rpm to increase CO<sub>2</sub> stripping. The time courses of biomass concentration, cell number, and residual sugar are given for both cultures in Fig. 3, and the main kinetic parameters are summarized in Table 2. Since similar curves were obtained, it could be concluded that in the range tested, a constant p<sub>CO<sub>2</sub></sub> did not improve growth in contrast to the results obtained with other microorganisms, for example, streptococci (13).

It is therefore obvious that the DO concentration is the main parameter of the gaseous environment which affects growth and glucose consumption, mainly in the first stages of culture. A faster growth was achieved with low DO concentration (<30% of saturation), while the mean growth rate,  $\mu_{C1}$ , decreased at a high DO concentration (>80% of saturation). However, the glucose nonconsumption phase could not be entirely removed regardless of the DO concentration.

Our results demonstrating the unexpected influence of oxygen on growth could be related to those obtained in cultures of *Azotobacter* species, another diazotroph. A decrease of growth yield and a rapid increase of respiration rate were reported for increased DO concentrations (19, 25). In the same way, increasing oxygen supply provided a decrease in activities of the enzymes of the Entner-Doudoroff pathway but an increase of activity of the tricarboxylic acid cycle (5, 14, 15). However, it was reported that the pathway of glucose metabolism was unaffected by oxygen concentration (5, 27). These changes in metabolic activities linked to variations of DO levels are representative of N<sub>2</sub>-fixing organisms. They were assumed to correspond to a manifestation of a respiratory protection in various *Azotobacter* species (6, 9).

Although *A. lipoferum* is described as a strict aerobe displaying an oxidative metabolism in the presence of combined nitrogen (16), our results demonstrate a sensitivity to oxygen, this specific behavior being characteristic of N<sub>2</sub>-fixing organisms. This assumption is supported by the high values of oxygen uptake found in *Azospirillum* (7) or *Arthrobacter* (4) species at low DO concentration either in N<sub>2</sub>-fixing conditions or in the presence of ammonium. Information is lacking to understand whether it is molecular oxygen itself or intermediate molecules which control the specific growth rate in the first stages of the culture. A relation between the sensitivity towards oxygen and PHB utilization during the first period of growth should be considered. Further investigations are needed regarding the possible regulation by oxygen of the activities of both the Entner-Doudoroff pathway and the tricarboxylic acid cycle in *A. lipoferum*.

In addition, studies concerning nutritional requirements and physiological behavior of *A. lipoferum* under various

culture conditions are necessary to reduce the lag phase, to provide increased biomass productivity, and to control both the maximal cell number and the PHB content. The latter improves the resistance of the cells to dehydration stress (28). An inoculant technology as proposed by Bashan (2) and Fages (10) should then be applied with success since the physiological state of the bacteria before harvesting has been considered.

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