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Aeration requirements of *Rhizobium* cultures

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Introduction

The usefulness and shelf-life of an inoculant depend, among other factors, on the initial number of viable micro-organisms (Roughley, 1985). In order to obtain a *Rhizobium* broth culture with a large cell population in a short time, it is necessary to adjust the operating conditions to the medium and the bacterial strain used. High biomass values can be attained if no limiting factors exist for cell growth.

As an inadequate oxygen supply can limit growth, the oxygen supply rate and the dissolved oxygen concentration are two of the most important factors determining cell yield. The oxygen demand of the culture depends on strain, cell concentration and growth rate. If the oxygen demand exceeds the solution rate, oxygen becomes limiting in the culture, but when the medium contains sufficient substrate and oxygen, growth rate attains the maximum value.

Although some research work has been done on the attainment of high cell numbers in *Rhizobium* culture, the adjustment of operating conditions based on oxygen requirements has received little attention. Some authors (Roughley 1970; Date & Roughley 1977; Roughley & Pulsford 1982; Williams 1984) mention air flow rates in a range of 0.5 to 120 v/v/h but the fermenter characteristics, oxygen demand and oxygen absorption rates are not mentioned. The aim of this paper is to provide quantitative data on oxygen absorption rates and on the cell oxygen demand so as to obtain high cell concentrations of *Rhizobium* cultures to be used in inoculant production.

Materials and methods

Micro-organisms

The rhizobia used in this study were obtained from the Unidad de Microbiología del Instituto Nacional de Tecnología Agropecuaria, INTA (Castelar, Argentina) and the Mircen (Porto, Alegre, Brasil).

The strains were: *Rhizobium meliloti* B-36, *R. phaseoli* F-10, *R. trifolii* A-22,

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Rhizobium spp. LL-22 (*Lotus* strain) *R. leguminosarum* D-91 and *R. japonicum* 5019. Pure cultures of rhizobia were maintained by subculturing into tubes containing yeast extract/mannitol/agar medium and storing at 5°C.

Media and inocula

The inocula media were similar to those indicated in Table 1 but contained 5 g of carbon source/l. Furthermore, the inoculum medium for *R. phaseoli* F-10 contained 0.2 mg biotin/l and those for *R. japonicum* 5019 and *Rhizobium* spp. LL-22 contained 0.4 and 0.8 g KNO_3/l, respectively. The volume of inoculum was 5–10% of the volume used in the fermentation experiments. The initial concentration of rhizobia was about 10^8 viable cells/ml in all cases.

Table 1 Composition of media

Component (g/l)	Composition for medium for					
	Rhizobium leguminosarum D-91	*Rhizobium* spp. LL-22	*Rhizobium phaseoli* F-10	*Rhizobium meliloti* B-36	*Rhizobium trifolii* A-22	*Rhizobium japonicum* 5019
KH_2PO_4	–	–	0.6	0.4	–	0.3
K_2HPO_4	0.5	0.5	0.75	0.5	0.5	0.5
$MgSO_4 \cdot 7H_2O$	0.2	0.2	0.2	0.2	0.2	0.2
NaCl	0.1	0.1	0.1	0.1	0.1	0.1
$MnSO_4$	0.01	0.01	0.01	0.01	0.01	0.01
$FeCl_3$	0.01	0.01	0.01	0.01	0.01	0.01
KNO_3	0.8	1.0	1.0	0.8	0.8	0.8
$(NH_4)_2HPO_4$	–	–	–	–	–	0.3
Sucrose	10.0	10.0	10.0	10.0	10.0	–
Glycerol	–	–	–	–	–	10.0
Peptone*	–	–	2.0	–	–	–
Yeast extract	4.0	4.0	2.0	4.0	4.0	4.0
pH	6.8	6.8	6.5	6.8	6.8	6.8

*Peptone from meat trypsin-digested for microbiology (Merck).

Growth

Bacterial growth was determined by optical density (650 nm), dry weight and viable cell numbers.

Oxygen absorption rate

The oxygen absorption rate (OAR) was measured by the sulphite method (Cooper *et al.* 1944).

Oxygen demand

The oxygen demand was measured in a Warburg respirometer at 28°C (Umbreit *et al.* 1944).

Dissolved oxygen

The dissolved oxygen was measured with a Biotech (LKB) galvanic probe.

Operating conditions

The flask experiments were carried out in 1-litre Erlenmeyer flasks containing 150 ml

of medium, which were mounted on a rotary shaker operated at 250 rev/min (eccentricity 2.5 cm). The fermenter experiments were performed in an LKB 1601 Ultraferm Fermentation System (LKB-Produkter AB Research Instrument Division, S-16125 Bromma, Sweden). A working volume of 8 l was used (liquid depth/fermenter diameter = 1). The temperature was 28°C, the aeration rate was 0.5 v/v/min, and the agitation rates were between 250 and 450 rev/min.

In all experiments the media and the containers were sterilized at 121°C for 20 min.

Results

Table 2 shows the results obtained in shake flask experiments.

Table 2 Maximum values of dry weight, viable cells and oxygen demand obtained in fermentations in Erlenmeyer flasks with different *Rhizobium* strains

Micro-organism	Dry weight (g/l)	Oxygen demand (ml O_2/l/h)	Viable cells (No. cells/ml)
Rhizobium spp. LL-22	4.6	160	1.9×10^{10}
Rhizobium phaseoli F-10	4.5	195	1.6×10^{10}
Rhizobium leguminosarum D-91	4.2	360	1.2×10^{10}
Rhizobium trifolii A-22	4.6	205	1.6×10^{10}
Rhizobium japonicum 5019	4.8	135	2.4×10^{10}
Rhizobium meliloti B-36	4.5	140	2.3×10^{10}

Operating conditions: 250 rev/min and 2.5 cm eccentricity; volume liquid/volume flask = 0.150; oxygen absorption rate (OAR), 355 ml O_2/l/h; temperature, 28°C.

The maximum dry weight ranged from 4.2 to 4.8 g/l and the maximum number of viable cells was $>10^{10}$ cells/ml in all cases. The cell oxygen demand was between 135 and 205 ml O_2/l/h except in the *R. leguminosarum* culture where the value was higher, 360 ml O_2/l/h.

The process conditions in mechanically stirred fermenters were selected according to the oxygen transfer rates and in relation to the cell oxygen demand of the cultures in shake flasks. The criteria recommended by Pirt (1975) was taken into account, i.e. the oxygen absorption rate, determined by the sulphite technique must be 1.4 to 2.0 times the oxygen demand of the bacterial culture. Table 3 shows the maximum values obtained in dry weight, cell oxygen demand and the number of viable cells for the process conditions that ensure the attainment of a high concentration of biomass. The experiments performed in mechanically stirred fermenters gave similar results to those obtained in shake flasks. The maximum cell concentration was 1.5 to 2.3×10^{10} cells/ml. These values were obtained using agitation rates of 250 to 450 rev/min and an air flow rate of 0.5 v/v/min. These conditions corresponded to oxygen absorption rates of 233 to 661 ml O_2/l/h.

Figure 1, shows typical curves for a fermentation process using a rapid growth strain, *R. meliloti* B-36. The dissolved oxygen concentration decreased by as much as 25% of the saturation level (28 h process); and that coincided with the highest value of cell oxygen demand (140 ml O_2/l/h). At that point, the number of viable cells was $>10^{10}$ cells/ml. Figure 2 shows the results obtained in a fermentation process using a slow growth strain, *R. japonicum* 5019. It could be seen that the maximum growth of 2.3×10^{10} cells/ml was achieved at 70 h although at 50 h the number of viable cells was

Table 3 Typical parameters of *Rhizobium* cultures in mechanically stirred fermenters

Micro-organism	Agitation rate (rev/min)	Oxygen absorption rate (ml O_2/l/h)	Maximum oxygen demand (ml O_2/l/h)	Maximum dry weight (g/l)	Maximum No. viable cells (No. cells/ml)
Rhizobium spp. LL-22	300	315	160	4.6	1.9×10^{10}
Rhizobium phaseoli F-10	350	402	195	4.4	1.5×10^{10}
Rhizobium leguminosarum D-91	450	661	360	4.6	1.5×10^{10}
Rhizobium trifolii A-22	350	402	205	4.6	1.6×10^{10}
Rhizobium japonicum 5019	250	233	135	4.7	2.3×10^{10}
Rhizobium meliloti B-36	250	233	140	4.5	2.2×10^{10}

The air flow and temperature were 0.5 v/v/min and 28°C. The fermenter characteristics were: liquid depth/fermenter diameter = 1; 2 impeller turbines and 4 baffles.

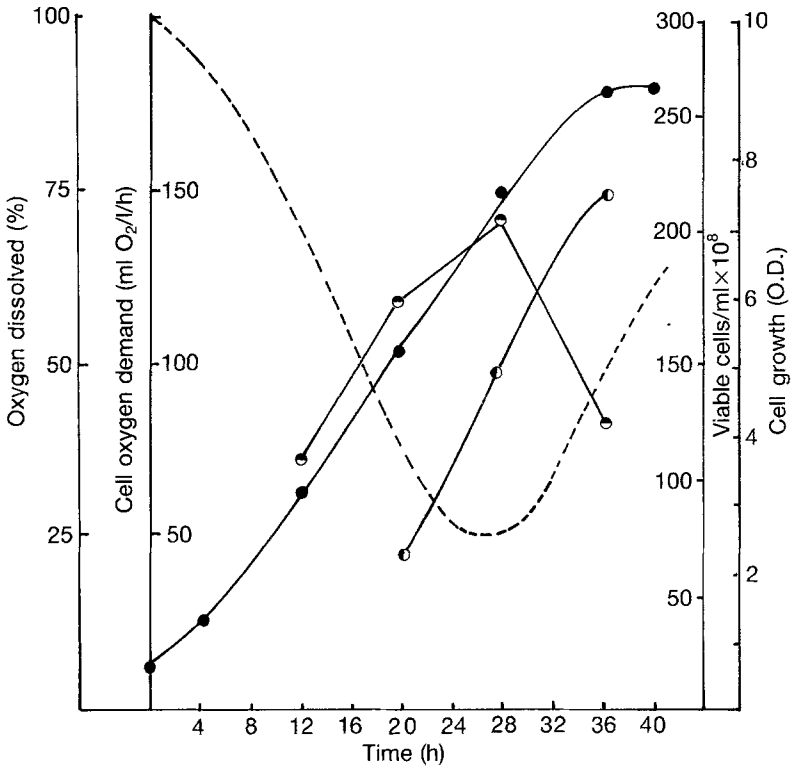

Fig. 1 Parameter changes in a culture of *Rhizobium meliloti* B-36 in a mechanically stirred fermenter. ●, Amount of growth (O.D.); ◐, No. viable cell/ml × 10^8; ◓, cell oxygen demand (ml O_2/l/h);---, % dissolved oxygen.

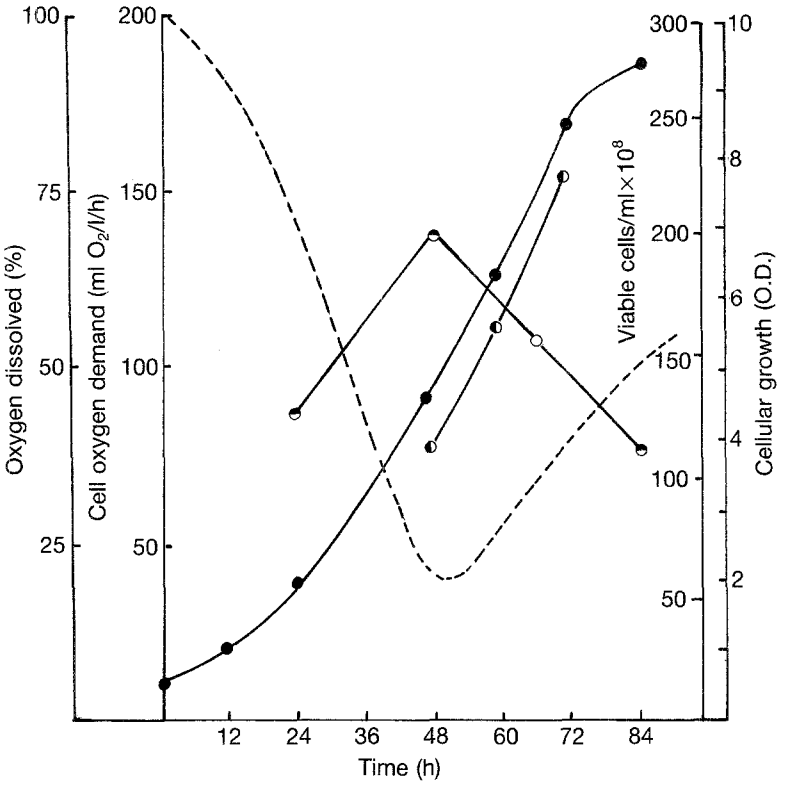

Fig. 2 Parameter changes in a culture of *Rhizobium japonicum* 5019 in a mechanically stirred fermenter. ●, Amount of growth (O.D.); ◐, No. viable cells/ml × 10^8; ◓, cell oxygen demand (ml O_2/l/h); ---, % dissolved oxygen.

already $>10^{10}$ cells/ml. The minimum dissolved oxygen concentration was >20% of the saturation level.

Figure 3 shows *R. meliloti* B-36 and *R. japonicum* 5019 growth curves of processes conducted in shake flasks and in mechanically stirred fermenters. It can be seen that the processes performed in the shake-flask experiments took a shorter time than the others; all the other strains behaved similarly.

Discussion

The values in Table 2 prove that the operating conditions employed in shake-flask experiments were efficient for the media and the strains used, due to the fact that the values of the oxygen absorption rate (355 ml O_2/l/h) were above the values of the cell oxygen demand except for that of *R. leguminosarum* D-91 which was somewhat below. Despite this, growth in the *R. leguminosarum* culture was only slightly affected because the final cell concentration was similar to those obtained with the other strains where oxygen was not limiting.

The high cell concentration obtained in the experiments performed in mechanically stirred fermenters (for each strain and medium used) is due to the selected operating conditions that ensure adequately aerated cultures. In all cases, as shown in Figs 1 and

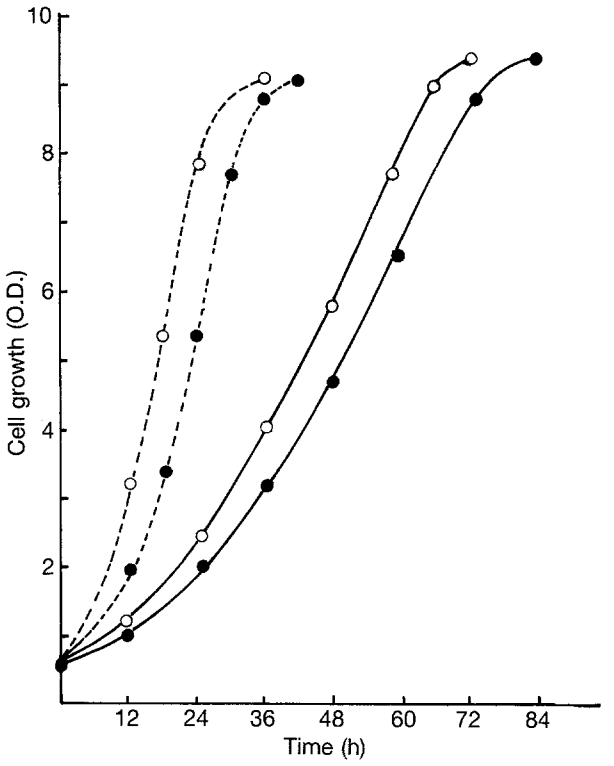

Fig. 3 Comparative growth curves of strains of rhizobia in cultures in shake flasks and in a fermenter. ---, *Rhizobium meliloti* B36; ——, *R. japonicum* 5019; ○, shake flasks; ●, fermenter.

2, the minimum value of dissolved oxygen was above the critical value for bacterial suspensions growing without oxygen limitation (10–15% of the initial saturated dissolved oxygen concentration; Drew 1981).

If we compare the experiments performed in mechanically stirred fermenters and in shake flasks, the similar concentration of viable cells obtained in both systems was due to the selected aerated conditions. The shorter time employed in the processes performed in Erlenmeyer flasks compared to those carried out in the fermenter could be due to the difference in hydrodynamic regime of the liquid media in both systems (Bilinka & Birukov 1972). The conditions established in these studies allow the achievement of a high cell concentration $>10^{10}$ cells/ml, of viable *Rhizobium* strains used for inoculant production, with adequate aeration levels. The quantitative data obtained in these studies could serve as a basis for scaling-up the process.

Acknowledgement

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Summary

To obtain a high concentration of *Rhizobium* in broth cultures, the process conditions should be adjusted to the medium and strain used. Oxygen may be a growth-limiting factor if the micro-organism requirements are not satisfied. In this work the process conditions in shake flasks and stirred fermenters were established, based on the oxygen supply rate, in relation to the cell oxygen demand of the various *Rhizobium* strains used in inoculant preparations. The strains used were *Rhizobium meliloti* B-36, *R. phaseoli* F-10, *R. trifolii* A-22, *Rhizobium* spp. LL-22, *R-leguminosarum* D-91 and *R. japonicum* 5019. They had maximum oxygen demand rates from 135 to 360 ml O_2/l/h in media containing 10 g of carbon source/l, 4 g of yeast extract/l and mineral salts. When the operations were conducted in Erlenmeyer flasks with a volume liquid/volume flask of 0.150 in a rotary shaker at 250 rev/min and 2.5 cm eccentricity, the oxygen absorption rate was 355 ml O_2/l/h. In mechanically stirred fermenters with a liquid depth/fermenter diameter of 1, with an agitation rate of 250 to 450 rev/min and an air flow rate of 0.5 v/v/min, the bacteria had oxygen absorption rates of between 233 and 661 ml O_2/l/h. These conditions allowed the attainment of 1.2 to 2.4 $\times$ 10^{10} viable cells/ml from between 24 and 72 h.

Résumé

Etudes d'aération en cultures de Rhizobium

Pour obtenir une haute concentration de cellules de *Rhizobium* dans des milieux de fermentation, il faut ajuster les conditions de processus au milieu et aux souches utilisés. L'oxygène peut être un des facteurs limitants de la croissance du microorganisme si ses besoins ne sont pas assouvis. Dans ce travail, pour les milieux employés, on établit des conditions de processus dans des Erlenmeyers et dans des fermenteurs. Ces conditions se basent sur la vitesse d'absorption d'oxygène et sur la demande cellulaire, de différentes souches de *Rhizobium*, utilisées dans la préparation d'inoculants. Les souches employées sont: *Rhizobium meliloti* B-36; *R. phaseoli* F-10; *R. trifolii* A-22; *Rhizobium* spp. LL-22; *R. leguminosarum* D-91 et *R. japonicum* 5019. Elles ont presenté des valeurs du besoin maxima d'oxygène entre 135 et 360 ml O_2/l/h dans des milieux contenant 10 g/l de source de carbone, 4 g/l d'extrait de levure et sels minéraux. Pour les espèces indiquées, en opérant dans un agitateur rotatif à 250 tours/minute et 2,5 cm d'excentricité on a établi qu'il faut utiliser des Erlenmeyers dont le rapport volume de milieu liquide/volume de récipient est 0,150 (pour ces conditions, la vitesse

d'absorption d'oxygène est 355 ml O_2/l/h); tandis que dans des fermenteurs avec agitation mécanique et pour un rapport hauteur de milieu liquide/diamètre de tank égal à 1, les conditions établies se trouvent entre vitesses d'agitation de 250 à 450 tours/minute et un débit d'air de 0,5 litre par volume de milieu et par minute (ces valeurs d'operation correspondent aux vitesses d'absorption d'oxygène de 233 à 661 ml O_2/l/h). Ces conditions permettent d'obtenir des concentrations cellulaires de 1,2 à 2,4 $\times$ 10^{10} cellules viables par millilitre apres 24 à 72 heures de processus.

Resumen

Estudios de aeración en cultivos de Rhizobium

Para obtener alta concentración de células de *Rhizobium* en caldos de fermentación deben ajustarse las condiciones de proceso al medio y cepas utilizadas. El oxígeno puede resultar uno de los factores limitante del crecimiento si no satisfacen las necesidades del microorganismo. En el presente trabajo se establecen condiciones de proceso en Erlenmeyers agitados y en fermentadores en base a la velocidad de absorción de oxígeno y relación a la demanda para los medios y las distintas cepas utilizadas en la preparación de inoculantes. Las cepas empleadas, *Rhizobium meliloti* B-36; *R. phaseoli* F-10; *R. trifolii* A-22; *Rhizobium* spp. LL-22; *R. leguminosarum* D-91; y *R. japonicum* 5019, presentaron máximos valores de demanda de oxígeno en el rango de 135 a 360 ml O_2/l/h en medios que contienen 10 g/l de fuente de carbono, 4 g/l de extracto de levadura y sales minerales. Para las especies indicadas se estableció que operando en agitador rotatorio a 250 r.p.m. y 2,5 cm de excentricidad deben utilizarse Erlenmeyers con una relación de volumen de líquido a volumen de frasco de 0,150 (para estas condiciones la velocidad de absorción de oxígeno es de 355 ml O_2/l/h) mientras que en fermentadores con agitación mecánica y para una relación de altura de líquido a diámetro de tanque igual a uno las condiciones establecidas se encuentran comprendidas en el ámbito de velocidades de agitación de 250 a 450 r.p.m. y caudal de aire de 0,5 v/v/min (estos valores corresponden a velocidades de absorción de oxígeno comprendidas entre 233 a 61 ml O_2/l/h). Las condiciones indicadas permiten obtener concentraciones celulares de 1,2 a 2,4 $\times$ 10^{10} cel/ml en tiempos de 24 a 72 horas de proceso.