ELSEVIER

0032-9592(95)00002-X

Process Biochemistry, Vol. 30, No. 8, pp. 767–772, 1995

0032-9592/95 $9.50 + 0.00

Modelling and Operation of a Turbidity-Meter for On-Line Monitoring of Microbial Growth in Fermenters†

Guillermo R. Castro,[a,b]* Eduardo P. Andribet,[b] Luis M. Ducrey,[a] Oscar A. Garro[a] & Faustino Siñeriz[a]

[a]Cátedra de Microbiología Superior, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, PROIMI MIRCEN Tucumán, Avda. Belgrano y Pje, Caseros, 4000 Tucumán, Argentina
[b]Laboratorio de Microbiología, Departamento Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, 1428 Buenos Aires, Argentina

(Received 27 June 1994; revised manuscript received and accepted 8 January 1995)

The PROIMI-2 prototype turbidity-meter for measuring cell concentrations in stirred tanks, has been designed and constructed using theories based on kinetic laws and the general principles of cell growth. Laboratory assays were carried out with batch cultures of Bacillus amyloliquefaciens, Zymomonas mobilis *and* Saccharomyces cerevisae. *The signals showed a good correlation with optical density and biomass. In the latter case up to 4·0 g per dm³ expressed as dry weight could be monitored successfully.*

INTRODUCTION

Cell growth is one of the most important parameters in fermentation processes.[1] Generally, the methods used most often for quantification of microbial growth involve cell or plate counting, or dry weight.[2] These procedures introduce a high risk of contamination in repeated batch cultures and continuous culture fermenters.

One of the most commonly used methods for monitoring cell growth is based on spectrophotometric techniques, i.e. light scattering or light transmission. For this purpose, equipment design and operation have been developed in several laboratories with rather varied results.[3–8] Most of the equipment installations need several operations in order to be used.[5,7,8] This is inconvenient for automatic on-line monitoring of cell growth. A semi-automatic on-line turbidity-meter has been developed recently, but the cell concentrations measured involve dilution of the sample in special chambers outside the fermenter or alternatively the use of flowcells with different pathlengths in or outside of the fermenter vessel.[6,8,9] Other turbidity-meters developed for the on-line monitoring of cells involve reductions in the fermenter volume and/or vessel modifications. In all cases the equipment complexity and costs had increased considerably.[3,6–10]

The objectives of this work were the construction and operation of a simple turbidity-meter prototype, named PROIMI-2, for on-line monitoring of microbial growth in stirred tank fermenters at laboratory scale. The simple mathematical modelling of the turbidity-meter

*To whom correspondence should be addressed.
†Patent pending.

based on kinetic laws and the general principles of the cell growth is presented.

MATERIALS AND METHODS

Microbial strains

Bacillus amyloliquefaciens MIR-41 and *Zymomonas mobilis* PRO-910, isolated and characterized in our laboratories, and *Saccharomyces cerevisae* K-382 were used for the experiments.[11,12]

Media

The defined medium for *B. amyloliquefaciens* MIR-41 contained in g dm^{-3}: $(NH_4)_2SO_4$, 1·0; KH_2PO_4, 3·0; K_2HPO_4, 6·0; $MgSO_4 \cdot 7H_2O$, 0·01; $CaCl_2 \cdot 2H_2O$, 0·05; $MnSO_4 \cdot 7H_2O$, 0·01; $FeSO_4 \cdot 7H_2O$, 0·001; $ZnSO_4 \cdot 7H_2O$, 0·001; trisodium citrate, 1·0; potato soluble starch (filtered), 10·0, or D-glucose, 5·0; were used as carbon source.[11]

Zymomonas mobilis PRO-910 was grown in media contained in g dm^{-3}: yeast extract, 5·0; $(NH_4)_2SO_4$, 1·0; $MgSO_4 \cdot 7H_2O$, 0·5; K_2HPO_4, 2·0 and D-glucose, 10·0.[12]

The growth medium for *Saccharomyces cerevisae* K-382 contained in g dm^{-3}: yeast extract, 10·0; peptone, 20·0; and D-glucose, 20·0.

Culture conditions

Cultures were run in a 1 dm^3 Gallenkamp fermenter (London, UK) with a working volume of 500 cm^3. For *Bacillus* cultures, aeration was settled at $1·0 \pm 0·2$ v v^{-1} min^{-1} and agitation at 3·0 Hz. The pH was maintained at 7·0 for *Bacillus amyloliquefaciens* batch cultures, and 5·0 for *Zymomonas mobilis* and *Saccharomyces cerevisae* cultures. The pH was adjusted with 1·5 mol dm^{-3} H_3PO_4 or NH_4OH. Samples were withdrawn from the culture, analysed and stored at −20°C.

Analytical procedures

Microbial growth was simultaneously determined with a turbidity-meter, constructed and optimized in our laboratory, and spectrophotometrically by optical density at 560 nm or 660 nm.[11] Samples with an optical density higher than 0·95 were diluted with 145 mmol dm^{-3} NaCl.

Biomass dry weight was determined by centrifuging the cells (10 000 × g during 10 min), washing twice with distilled water and drying at 105°C until the weight was constant.

Description of the turbidity-meter

A diagram of the turbidity-meter PROIMI-2 is shown in Fig. 1. The turbidity-meter consists of an amplifier with an interface output for a computer, a digital multimeter (reading instrument) and a regulated potentiometer to determine light intensity. There are two different probes, one for low density cultures (probe A) and the other for greater densities (probe B). Probe A has two principal components: the cell lamp (W-lamp, 15 V) and on the opposite side of the channel a photoreceptor (BP 104). Both are removable for sterilization. The probe is introduced into the fermenter using the same method for introducing a pH electrode and when the cells pass through the channel of 2 mm diameter they are detected by the photoreceptor (Fig. 2(a)). When the probe is in the upper position, above the liquid–air interface (arrested position), it is surrounded by a plastic rigid hood, connected to the cover of the fermenter, in order to prevent any microbial growth on the walls if the turbidity-meter is used under discontinuous operations.

The probe B, includes a closed loop outside the vessel where the turbidity is read through a glass tube with a variable diameter. In this way it is possible to change the light pathway according to the requirements of higher densities (Fig. 2(b)).

The turbidity-meter signal is sent to an operational amplifier, where it is read by a digital multimeter and sent to a parallel placed analogue-digital converter. The calibration was made setting zero in the digital multimeter when no light was in the probe, and increasing the light intensity while the probe was submerged in a grown culture until the signal saturation was reached.

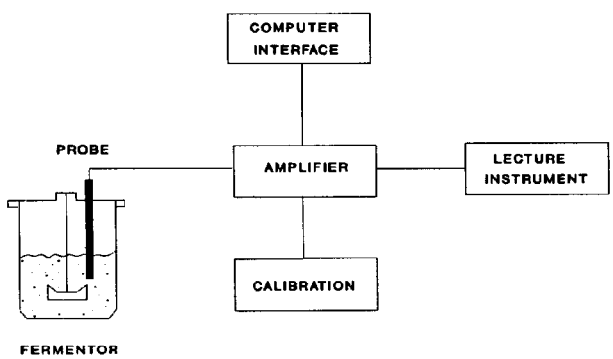


Fig. 1. Diagram of turbidity-meter PROIMI-2.

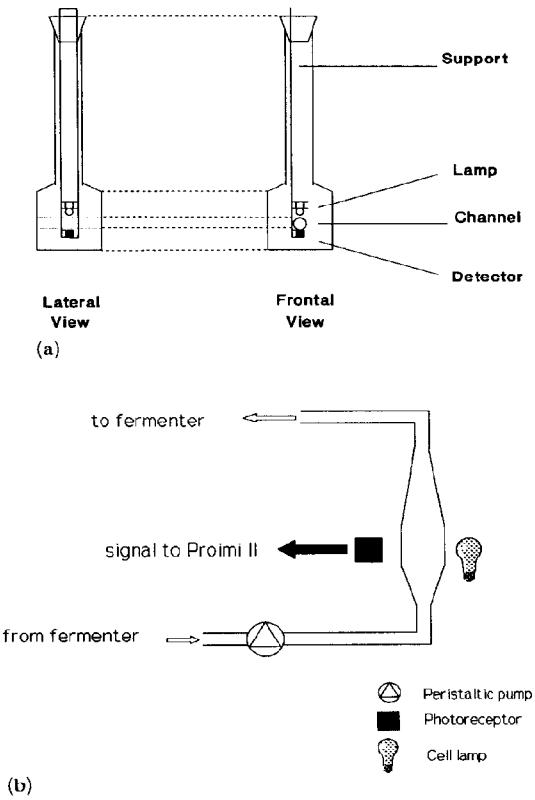


Fig. 2. Scheme of probes A and B of cell counter.

Theoretical aspects

Mathematical modelling of the turbidity-meter
Bacterial growth in the fermenter can be described by the following hypotheses:

(1) The microorganisms are randomly distributed in the fermenter.
(2) The growth rate of the microorganisms is constant under each of the experimental conditions.
(3) The environmental conditions in the vessel are constant (temperature, pH, etc...).
(4) The relative velocities of the microorganisms (particles) are constant during the fermentation process.
(5) No cellular adhesion exists.
(6) The volume of the microorganisms are not significant with respect to the fermenter volume. The microorganisms can be considered as point particles for kinetics considerations.
(7) Cell growth is described by the exponential equation described previously:[1]

$$x(t) = x(t_0).e^{\mu t}, \qquad (1)$$

$x(t_0)$ and $x(t)$ are the cellular concentrations at zero and t times, respectively, and μ is the specific growth rate constant.
(8) The individual velocity of the microorganisms (v_i) is random and has a maximum. The average rate for N microorganisms (α) is defined by:

$$\alpha = \sum_{d\tau} v_i / dN = 0,$$

in which N is the bacterial number in one element of volume $(d\tau)$.[13]
(9) The signal obtained (E) for the interaction of one microorganism-sensor in the solid angle (δw) is considered:[14]

$$\delta w = \int_r \int_\sigma [\cos \sigma / r^2] \, dr \, d\sigma = -\sin \sigma / r + Q,$$

where Q is the integration constant.

The signal E for one microorganism can be considered directly proportional to the conical shadow formed by the interposition of one bacteria to the light beam with respect to the detector. Therefore:

$$E \approx \delta w = E \approx -\sin \sigma / r + Q.$$

For n microorganisms and the distance between light source (lamp) and detector (r), the signal E can be estimated as:

$$E \approx -\int_n \int_r [\sin \sigma / r . \phi(n)] \, dn \, dr$$

$$= E \approx -\sin \sigma . \Omega(n) . \ln(r) + K.$$

E is a function of σ (conical shadow angle) and $\Omega(n)$ a function of the number of cells (n), r is a constant and K is the integration constant.

When the angle σ is very small: $\sigma \approx 0 \Rightarrow \sin \sigma \approx \sigma$,

$$\Rightarrow E \approx \sigma . \Omega(n) . \ln(r).$$

n is related to the cell concentration by a constant (volume of the vessel). In batch operation, the microbial growth follows the exponential equation (eqn (1)). The signal depends on the time and

can be expressed as:

$$E(t) \approx E(t_0) + \phi(\sigma, r).\mu.t,$$

where ϕ is a function of angle (σ) and radius (r), and therefore of the solid angle.

RESULTS AND DISCUSSION

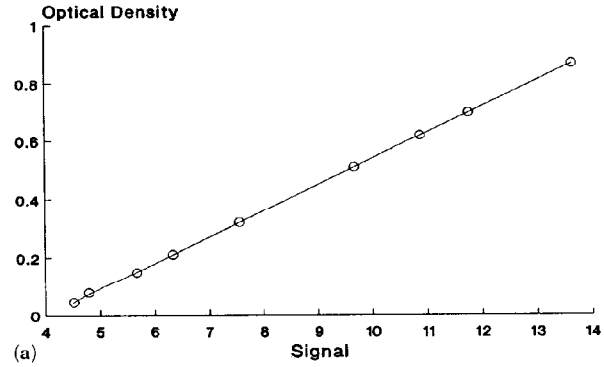
A diagram of the probe is shown in Fig. 2(a). Height and rotation angle of the probe can be varied in order to obtain different readings in the reactor. Changes of the probe position are useful for monitoring the homogeneity of the culture and/or the mixing times in the reactor.

The channel of probe A and the tube of probe B possibly function as a Venturi tube, because no cell growth on the channel walls was observed in submerged position in continuous culture operation for several days with high cell density cultures of the assayed microbes. On the contrary, cell growth was observed in other parts of the fermenter (vessel walls, electrodes, etc.).

In order to correlate the turbidity-meter signal with any other parameter, i.e. dry weight, optical density, etc., it was necessary to withdraw samples from a culture while the turbidity-meter was monitoring growth. The plot of the optical density (560 nm) of a batch culture of *Bacillus amyloliquefaciens* MIR-41 versus the signal of the turbidity-meter using the probe A showed a linear response (Fig. 3(a)), as was the case of *Zymomonas mobilis* PRO-910 batch cultures (Fig. 3(b)). The correlation between biomass and optical density showed some deviation from the linear relationship at high dry weights, as previously reported.[1] However, the correlation between biomass and turbidity-meter signal was linear in the range of cell density obtained (0·1–1·0 g dm^{-3}) (Fig. 3(b)).

For monitoring high cell density cultures a strain of *Saccharomyces cerevisae* was used in batch cultures. The turbidity-meter was equipped with probe B, and simultaneously the dry weight of the culture and optical density (660 nm) with appropriate dilutions were measured (Fig. 4). Linear correlation between dry weight up 4·0 g dm^{-3} and turbidity-meter-probe B signal was observed.

In order to evaluate the behaviour of the turbidity-meter *in vivo*, batch cultures of *Bacillus amyloliquefaciens* MIR-41 with starch (40 g dm^{-3}) as the carbon source were used. During the



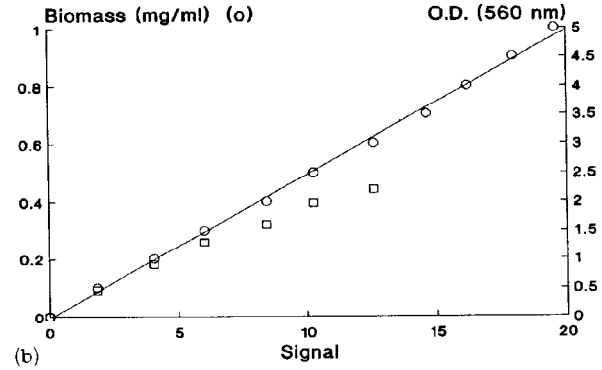


Fig. 3. (a) Correlation of the optical density (560 nm) with turbidity-meter probe A signal of *Bacillus amyloliquefaciens* MIR-41. (b) Correlation between the turbidity-meter probe A signal (○) and optical density (560 nm) (□) with biomass as dry weight (g dm^{-3}) of *Zymomonas mobilis* PRO-910 batch culture.

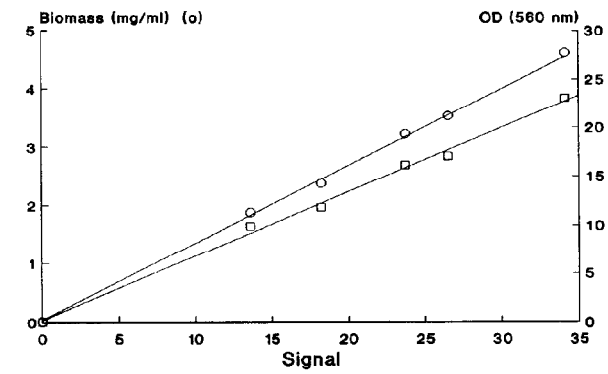


Fig. 4. Correlation between the turbidity-meter probe B signal with biomass as dry weight (g dm^{-3}) of *Saccharomyces cerevisae* batch culture (○) and optical density at 560 nm (□).

experiments the values of the turbidity-meter and the optical density were both measured and were followed in time (Fig. 5). Starch medium has a high turbidity and presents some problems for cell

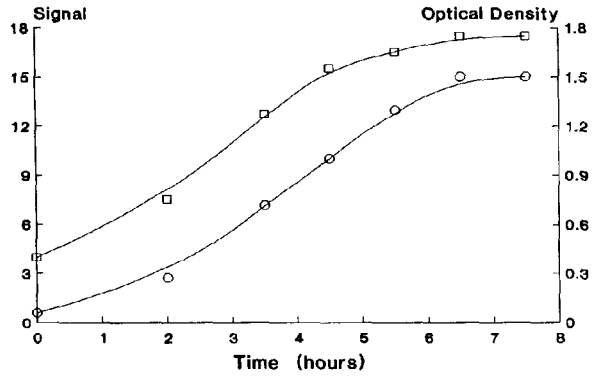


Fig. 5. Batch culture of *Bacillus amyloliquefaciens* MIR-41 in starch medium. Cellular growth was determined with the PROIMI-2 turbidity-meter probe A (□) and optical density at 560 nm (○).

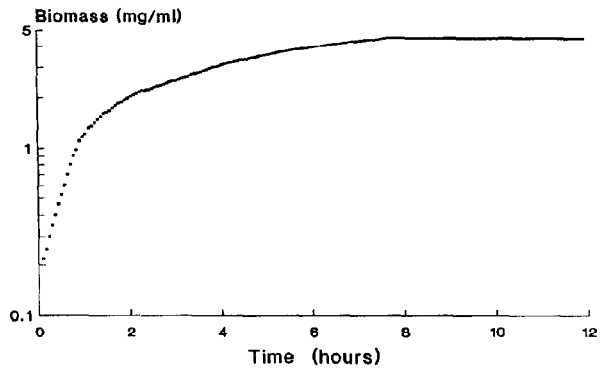


Fig. 6. Batch culture of *Saccharomyces cerevisae* K-382. Cellular growth was determined with PROIMI-2 turbidity-meter probe B.

quantification. It is not possible to use the dry weight method, because of the content of high molecular weight compounds, which cannot be filtered out. Optical densities are usually measured in such microbial cultures. However, spectrophotometric determinations have an increased error because of the dilution of the samples in order to decrease the starch concentration and adjust the readings below one unit of absorbance. The same error occurs, when the microbial culture reaches high cellular concentrations and has to be diluted. In previous reports the determination of low and high biomass concentrations involves either the use of different flowcells or different lengths of the light paths.[8,9] Alternatively, a system for high cell density measurements involves a circulating system and samples have to be taken outside the vessel.[10] However, *Saccharomyces cerevisae* was cultured and biomass was determined automatically with the turbidity-meter PROIMI-2 probe B according to a previous calibration, without dilution of samples and flow-cell changes (Fig. 6). With the turbidity-meter presented here, at high cell concentrations, one flow-cell was used and the upper limit of detection was approximately twice the cell concentration reported previously.[9]

In other reports, it has been suggested that gas bubbles present in the medium may interfere with the readings in turbidity-meters.[3] However, the results obtained with the PROIMI-2 turbidity-meter used in aerated cultures of *Bacillus amyloliquefaciens* MIR-41 (0–1 v v^{-1} min^{-1}) did not show interference (Fig. 5). This may be due to the constant conditions under which the experiments were carried out. Moreover, gas production by the microorganisms themselves might be negligible compared with the air influx. Similarly, no effects of aeration rates were reported in a plant cell bioreactor with a laser probe of 780 nm.[15] However, the problem associated with the laser probes is the monochromatic light, which can be interfered with due to the coloured compounds or pigmented cell products.

The development of a simple mathematical model would facilitate the automation of the fermentation process. It could also be used in culture techniques where the addition of nutrients is necessary once the cellular concentration is established, such as in fed-batch cultures.[16]

CONCLUSIONS

The turbidity-meter PROIMI-2 designed and manufactured in our laboratory has the following characteristics and advantages: linear correlation with the readings obtained spectrophotometrically at 560 and 660 nm; similar response in low and high absorbance media; fast and reliable installation without modifications of the fermenter; no microbial growth on the walls of the detector; possibility of changing the position of the probe in the fermenter during the experiment, and low costs of construction and operation.

The turbidity-meter PROIMI-2 can be used to predict the development of the fermentation process, the on-line estimation of kinetic constants of the microorganisms, and also the automatic control of the cellular growth.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support for this work from CONICET (Argentina), Third World Academy of Sciences (Italy) and Sarec (Sweden). We express our appreciation to Drs D. M. and J. T. F. Spencer for language advice. We thank Mrs H. A. L. Abregú and Mr S. Borchia for material preparation.

REFERENCES

1. Pirt, S. J., In *Principle of Microbe and Cell Cultivation*, ed. S. J. Pirt. Blackwell Scientific Publications, London, 1975, pp. 15–21.
2. Koch, A. L., Growth measurement. In *Manual Methods for General Bacteriology*, ed. P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. Briggs Phillips. American Society for Microbiology, Washington DC, 1981, pp. 179–217.
3. Hancher, C. W., Thacker, L. H. & Phares, E. F., A fiber-optic retroflective turbidity-meter for continuously monitoring cell concentration during fermentation. *Biotechnol. Bioeng.*, **15** (1974) 475–84.
4. Lima Filho, J. L. & Ledingham, W. M., Continuous measurement of biomass concentration in laboratory scale fermenters using a led-electrode system. *Biotechnol. Tech.*, **1** (1987) 145–50.
5. Nielsen, J., Nikolajsen, K. & Villadsen, J., FIA for on-line monitoring of important lactic acid fermentation variables. *Biotechnol. Bioeng.*, **33** (1989) 1127–34.
6. Ohashi, M., Watabe, T. & Watanabe, Y., Sensors and instrumentation: steam-sterilizable dissolved oxygen sensor and cell mass sensor for on-line fermentation system control. *Biotechnol. Bioeng. Symp.*, **9** (1979) 103–16.
7. Ortmanis, A., Patterson, W. I. & Neufeld, R. J., Evaluation of a new turbidimeter design incorporating a microprocessor-controlled variable pathlength cuvette. *Enzyme Microb. Technol.*, **13** (1991) 450–5.
8. Thatipamala, R., Rohani, S. & Hill, G. A., Spectrophotometric method for high biomass concentration measurements. *Biotechnol. Bioeng.*, **38** (1991) 1007–11.
9. Konstantin, K. B., Pambayun, R., Matanguihan, R., Yoshida, T., Peruish, C. M. & Hu, W. S., On-line monitoring of hybridoma cell growth using a laser turbidity sensor. *Biotechnol. Bioeng.*, **40** (1992) 1337–42.
10. Yano, T., Madsuki, A. & Nishizawa, Y., Photometric measurement of high cell density by continuous dilution of broth with circulating system. *J. Ferment. Bioeng.*, **74** (1992) 100–3.
11. Castro, G. R., Méndez, B. S. & Siñeriz, F., Amylolytic enzymes produced by *Bacillus amyloliquefaciens* MIR-41 in batch and continuous culture. *J. Chem. Tech. Biotechnol.*, **53** (1993) 289–94.
12. Rodríguez, E. & Callieri, D. A. S., High yield conversion of sucrose into ethanol by a flocculent *Zymomonas* sp. isolated from sugarcane juice. *Biotechnol. Lett.*, **8** (1986) 745–8.
13. Glasstone, S., The Maxwell–Boltzman distribution law. In *Textbook of Physical Chemistry*. D. Van Nostrand Co. Inc., New York, 1946, pp. 270–5.
14. Granville, W. A., In *Elements of the Differential and Integral Calculus*. Ginn & Co., Boston, 1952, pp. 641–9.
15. Zhong, J. J., Fujiyama, K., Seki, T. & Yoshida, T., On-line monitoring of cell concentration of *Perilla frutescens* in a bioreactor. *Biotechnol. Bioeng.*, **42** (1993) 542–6.
16. Yamane, T., Fed batch culture automated by use of continuously measured cell concentrations and cell volume. *Biotechnol. Bioeng.*, **39** (1992) 550–5.