BIOTECHNOLOGY TECHNIQUES
Volume 9 No. 1 (January 1995) p. 49-54
Received 29th November

ON-LINE DETERMINATION OF PIGMENT COMPOSITION AND BIOMASS IN CULTURES OF MICROALGAE

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SUMMARY
Rhodomonas sp. was grown in a photo-bioreactor equipped with a measuring cell in a spectrophotometer as part of an external flow loop. The apparent absorbance from 400 to 800 nm of the cell suspension was recorded at predetermined intervals and stored in a computer. From the spectra, the biomass and the concentrations of the two pigments chlorophyll *a* and phycoerythrin were determined in nitrogen-limited batch cultures.

INTRODUCTION
The biomass concentration in bioreactors is usually estimated from the apparent absorbance of the cell suspension at wavelengths where there is no absorption from intracellular pigments. Such measurements can be made on samples removed from the culture but often an automatic, on-line measuring system will be either advantageous or necessary as in turbidostats (Watson, 1972). The apparent absorbance can be automatically measured either by a probe inserted in the bioreactor (Maxwell *et al.*, 1987) or in a measuring chamber outside the bioreactor (Cook, 1951) where the cell suspension is circulated through the measuring chamber via an external flow loop.

If the apparent absorbance is measured at a wavelength where intracellular pigments absorb light, the measured value is a result of both light scattering (turbidity) and pigment absorption. If this value is corrected by subtracting the apparent absorbance at a wavelength where there is minimal absorption from intracellular pigments, the pigment concentration in the culture can be estimated, as long as light scattering is the same at both wavelengths. We have used this principle for on-line determination of the concentrations of the two photosynthetic pigments chlorophyll *a* and phycoerythrin (PE) in nitrogen-limited batch cultures of the unicellular microalga *Rhodomonas* sp.

MATERIALS AND METHODS
Culture methods: *Rhodomonas* sp. (Cryptophyta) was provided by Grethe M. Christensen,

Marinbiologisk Laboratorium, Helsingør, Denmark. The culture was grown photoautotrophically with a photosynthetic photon flux density of 55 μmol m^{-2} s^{-1} in an Applikon BTS 05 bioreactor with a volume of 2.0 l. The temperature was 22°C and pH was controlled at 8.0 by titration with CO_2, the sole carbon source, added in pulses to the influent air (Sand et al., 1994). The growth medium was seawater (salinity = 17‰) enriched with 2,5 mM $NaNO_3$, 0.3 mM Na_2-EDTA, 1.6 mM H_3BO_3, 0.4 mM Na_2HPO_4, 6.6 μM $MnCl_2$, 14.4 μM $FeCl_3$, 0.05 μM $ZnCl_2$, 0.03 μM $CoCl_2$, 0.002 μM $(NH_4)_6Mo_7$ and 0.02 μM $CuSO_4$ and the vitamins B_1 (6.0 mg l^{-1}), B_{12} (0.3 mg l^{-1}) and biotin (1.5 mg l^{-1}). $NaNO_3$ was the growth limiting nutrient.

On-line determination of apparent absorbance: The bioreactor was equipped with an external flow loop containing the measuring chamber, a flow cell with a light path length of 1.0 cm placed in a spectrophotometer (LKB Ultrospec II, Sweden) connected to a computer via the RS-232C serial port. A program for spectrophotometer control, data acquisition and data reduction was written in Microsoft Quick Basic 4.5. A peristaltic pump, placed between the flow cell in the spectrophotometer and the bioreactor in order to avoid high liquid pressures in the flow cell, continuously pumped the cell suspension from the bioreactor through the loop with a circulation period of 30 seconds. Before an experiment, a baseline signal was measured from 400 nm to 800 nm with distilled water in the flow cell, stored in the computer and automatically subtracted from the recorded spectra of the *Rhodomonas* sp. culture.

At 4-hour intervals the apparent absorbance of the cell suspension from 400 nm to 800 nm was recorded and stored in the computer. The cell density was determined from the apparent absorbance at 606 nm, where pigment absorption is almost absent in *Rhodomonas* sp. Further, we chose the wavelength between the absorption peaks of chlorophyll *a* (680 nm) and PE (560 nm) in order to minimize the difference in apparent absorbance caused by wavelength dependent light scattering. The turbidities at 680 nm and 560 nm were estimated from absorption spectra of cells with low pigment contents to 98% and 101% respectively of the turbidity at 606 nm. The absorbance of chlorophyll *a* is calculated from:

$$A_{680}(Chl.a) = OD_{680} - 0.98 \cdot OD_{606}$$

and the absorbance of PE is calculated from:

$$A_{560}(PE) = OD_{560} - 1.01 \cdot OD_{606}$$

where A is the pigment absorbance and OD is the measured apparent absorbance.

Off-line determination of cell number and pigment concentrations: Samples were taken from the culture and the cell density was measured at 606 nm in a Shimadzu UV-160A spectrophotometer after dilution with growth medium to turbidities between 0.2 and 0.3. We have found a linear relationship (r^2 = 0.98) between OD_{606} and the cell number, x (cells ml^{-1}), determined microscopically on six replicate samples using a 0.064 μl counting chamber:

$$x = 4.89 \cdot 10^6 \cdot OD_{606} - 6.24 \cdot 10^5$$

which is independent of the cellular pigment content.

Chlorophyll *a* was extracted from a 0.5 ml sample of cell suspension in the dark with 5 ml

methanol for 5 min. After filtration (Whatman no. 4) the concentration of chlorophyll *a* was determined from the absorbance at 665 nm using an extinction coefficient of 74.5 l g^{-1} cm^{-1} (MacKinney, 1941).

PE was extracted by two freeze-thaw cycles of the cell suspension. After centrifugation (18,000 g for 3 min.) the concentration in the supernatant was determined from the absorbance at 545 nm using an extinction coefficient of 12.6 l g^{-1} cm^{-1} (MacColl *et al.*, 1976).

RESULTS

Absorption spectra of *Rhodomonas* sp. during a batch culture are shown in Fig. 1. In addition to the absorption peaks due to chlorophyll *a* and PE at 680 nm and 560 nm respectively, there was a broad peak below 500 nm which is a combined absorption peak from chlorophyll *a*, chlorophyll c_2 and carotenoids (Erata and Chihara, 1989). Even at the largest absorption peak (chlorophyll *a*) only about 10% of the apparent absorbance was caused by pigment absorption whereas the rest was caused by light scattering of the cells. In spite of the low absorbance/turbidity ratio, determination of intracellular pigment concentrations was still possible. Fig. 2B and Fig. 2C show pigment absorbance values calculated as the difference between the apparent absorbance and the turbidity. The absorbance values are shown on expanded scales compared to Fig. 1.

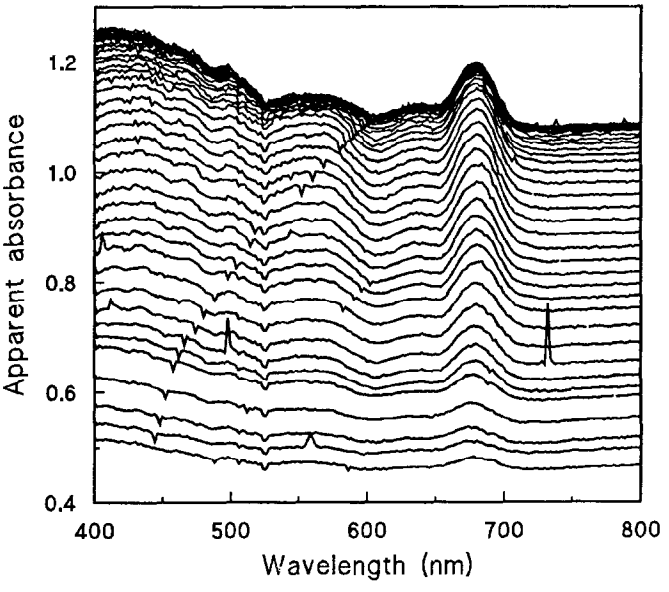

Fig. 1. On-line absorption spectra of a batch culture of *Rhodomonas* sp. The spectra were recorded at 4-hour intervals starting from below (only the first 39 spectra out of a total of 59 are shown). The spikes in the spectra are artifacts caused by air bubbles passing through the flow cell.

Fig. 2A shows on-line (OD_{606}) and off-line (cells ml^{-1}) measurements of the cell density. There was a linear relation (r^2 = 0.99) between the two in the measured range. Growth was light-limited during the first 100 hours of the experiment resulting in linear growth.

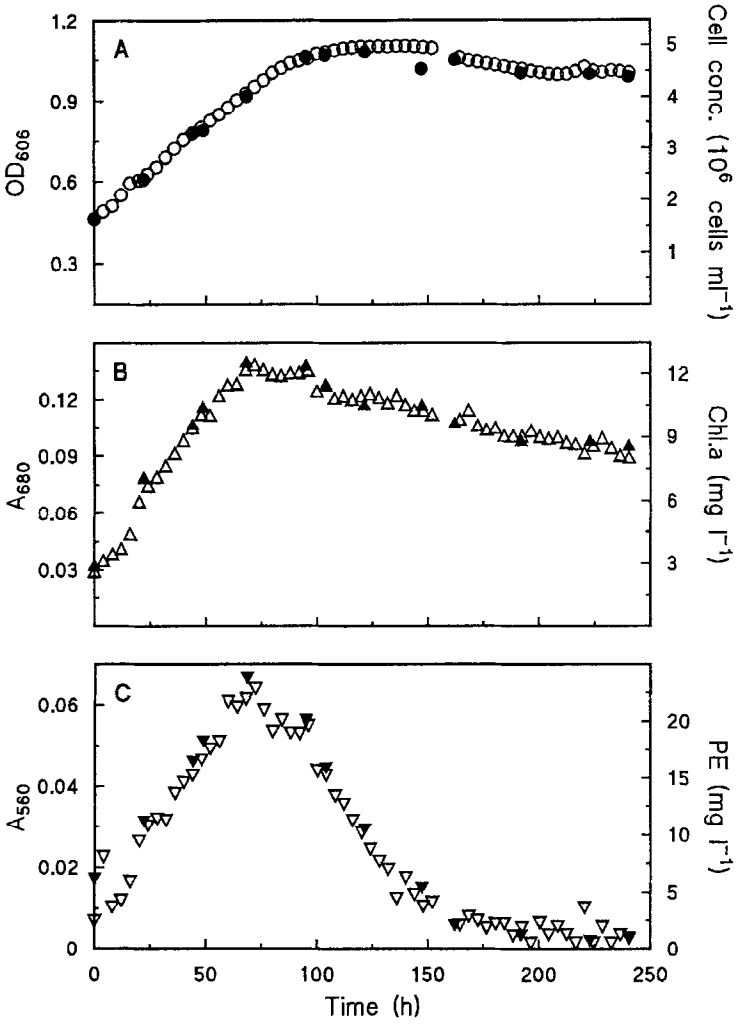

Fig. 2. Comparisons of *in situ* optical density and pigment absorbance values measured in the external flow cell and cell number and pigment concentrations measured on samples removed from a batch culture of *Rhodomonas* sp. All on-line data are calculated from the absorption spectra in Fig. 1. **A.** Cell number: (○) on-line, (●) off-line. **B.** Chlorophyll *a* concentration: (△) on-line, (▲) off-line. **C.** PE concentration: (▽) on-line, (▼) off-line.

Fig. 2B compares the on-line measurements of absorbance at 680 nm with the chlorophyll *a* concentration measured in samples from the bioreactor after extraction in methanol. In Fig. 2C the on-line measured absorbance at 560 nm is compared with the PE concentration measured in samples

from the bioreactor after two freeze-thaw cycles. In both cases there is an excellent correlation between concentration and absorbance with correlation coefficients of $r^2 = 0.96$ (chlorophyll *a* and A_{680}) and $r^2 = 0.98$ (PE and A_{560}). The *in situ* extinction coefficient of chlorophyll *a* at 680 nm is 11.5 l g^{-1} cm^{-1} and the *in situ* extinction coefficient of PE at 560 nm is 2.6 l g^{-1} cm^{-1}

We tested the long-term stability of the system at 100 hours after inoculation by repeating the measurement of the apparent absorbance at 800 nm with distilled water in the flow cell (the base line signal) and compared it with the value before the start of the experiment. The increase of apparent absorbance was only 0.01 or about 1% of the range of measured values of Fig. 1. This control was repeated after 250 hours with the same result. Therefore, the apparatus was stable and cells attached to the walls of the flow cell did not significantly influence the accuracy of the measurements.

DISCUSSION

The majority of methods concerning on-line surveillance of cell cultures by spectrophotometry seems to focus on biomass determination and not on determination of intracellular or extracellular molecules. Photosynthetic pigments of microalgae and photosynthetic bacteria are among the cell components which can be most easily detected by spectrophotometry since they have high extinction coefficients and usually are present in sufficient concentrations.

The measurement of the light absorbed by pigments is independent of the construction of the spectrophotometer whereas turbidity measurements are strongly influenced by the distance between the flow cell and the photomultiplier. A spectrophotometer where the flow cell is placed close to the photomultiplier, compared to the spectrophotometer used in this study, would result in a more favourable ratio between cellular turbidity and molecular absorbance in the measurements. This would increase the sensitivity of the pigment determinations, and pigments with lower extinction coefficients could also be detected by this method.

Non-linearity over wide ranges of biomass concentrations, interference from bubbles and growth on optical surfaces are problems which limit the application of optical on-line measurements of cell cultures (Clarke *et al.*, 1984; Clarke *et al.* 1986). The problem of non-linearity can be minimized by decreasing the length of the light path (Lee and Lim, 1980), bubbles can be trapped before they reach the flow cell and growth on the optical surfaces can be prevented by cooling the flow cell or by automatic cleaning of the optical surfaces (Blachère and Jamart, 1969). In this study wall growth did not occur and only momentary interference from air bubbles was seen as spikes in the spectra (Fig. 1).

The residence time in the external flow loop was short (30 seconds) compared to the doubling time, t_d, of the cells ($t_{d,max} = 0.8$ days). Consequently, no effects from the external circulation of the culture was seen on cell morphology, pigment contents or growth curves.

As can be seen from the data presented in this paper, the specific pigment composition of

the alga changes throughout growth in batch cultures. During the first phase of growth, chlorophyll *a* and PE accumulated. After 70 hours, the nitrogen source of the growth medium was exhausted (results not shown) and the chlorophyll *a* concentration decreased to 70% of its maximum value. This has been reported by others and explained by either reduced resynthesis of damaged chlorophyll (Coleman *et al.*, 1988) or active breakdown of the chlorophyll (Perry *et al.*, 1981). In contrast, PE was almost depleted at the end of the experiment (Fig. 2C). PE is therefore believed to play a dual role, both as a light harvesting pigment and as an intracellular nitrogen store from which nitrogen is released when extracellular nitrogen is absent, thereby supporting further growth (Lewitus and Caron, 1990). Therefore, the on-line system for pigment determination described here provides important information about the nutritional status of algal cultures. Also it makes a control of the pigment composition of growing cultures possible.

Our results demonstrate that growth of photosynthetic microorganisms should be followed by turbidity measurements rather than measurements of apparent absorbance at pigment absorption maxima. The latter method is often preferred in order to increase the sensitivity of optical measurements, but can only be used in a meaningful way in cultures where the specific pigment concentrations are constant. Otherwise the increase in sensitivity is at the expense of accuracy.

We thank Raymond P. Cox for a critical reading of the manuscript and Helle Kvist for excellent technical assistance.

REFERENCES

Blachère, H. and Jamart, G. (1969). *Biotehcnol. Bioeng.* 11, 1005-1010.

Clarke, D.J., Blake-Coleman, B.C., Calder, M.R., Carr, R.J.G., and Moody, S.C. (1984). *J. Biotechnol.* 1, 135-158.

Clarke, D.J., Blake-Coleman, B.C., Carr, R.J.G., Calder, M.R. and Atkinson, T. (1986). *Trends Biotechnol.* 4, 173-178.

Cook, P.M. (1951). *Ind. Engng. Chem.* 43, 2385-2389.

Coleman, L.W., Rosen, B.H. and Schwartzbach, S.D. (1988). *Plant Cell Physiol.* 29, 423-432.

Erata, M. and Chihara, M. (1989). *Bot. Mag.* 102, 429-443

Lee, C. and Lim, H. (1980). *Biotechnol. Bioeng.* 22, 639-642.

Lewitus, A.J. and Caron, D.A. (1990). *Mar. Ecol. Prog. Ser.* 61, 171-181.

MacColl, R., Berns, D.S. and Gibbons, O. (1976). *Arch. Biochem. Biophys.* 177, 265-275.

MacKinney, G. (1941). *J. Biol. Chem.* 140, 315-322.

Maxwell, G.M., Allen, E.R. and Freese, E. (1987). *Appl. Environ. Microbiol.* 53, 618-619.

Perry, M.J., Talbot, M.C. and Alberte, R.S. (1981). *Mar. Biol.* 62, 91-101.

Sand, O., Petersen, I.M. and Iversen, J.J.L. (1994). *Antonie van Leeuwenhoek* 65, 133-142.

Watson, T. G. (1972). *J. Appl. Chem. Biotechnol.* 22, 229-243.