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GROWTH PARAMETERS IN GREEN PHOTOTROPHIC BACTERIA PURE CULTURES: A STUDY ON «in vivo» ABSORPTION VALUES

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RESUM

L'absorció com a mesura del creixement d'un cultiu és, generalment, un bon paràmetre. En el cas dels bacteris fototròfics no poden utilitzar-se directament els valors d'absorció perquè reflecteixen també les interferències del sofre i la presència de pigments, sobretot quan es coneix que aquests depenen de la intensitat de llum i del grau d'activitat del cultiu. En el cas de les Clorobiàcies, les quals excreten sofre al medi, aquest fet és encara més patent. Un seguit de paràmetres lligats a l'absorció han estat analitzats estadísticament per tal de determinar el més idoni pel seguiment de les fases dels cultius de bacteris fototròfics verds.

RESUMEN

La absorción como medida del crecimiento de un cultivo es, en general, un buen parámetro. En el caso de las bacterias fototróficas no es posible utilizar directamente los valores de absorción porque incorporan también las interferencias del azufre y la presencia de pigmentos, sobre todo cuando se conoce que estos dependen de la intensidad de la luz y del grado de actividad del cultivo. En el caso de las Clorobiaceas, que excretan azufre al medio, este hecho es aún más evidente. Se han analizado estadísticamente una serie de parámetros ligados a la absorción con el fin de determinar el más idóneo para el seguimiento de las fases de los cultivos de bacterias fototróficas verdes.

ABSTRACT

Optical density is, generally, a good parameter to evaluate bascterial growth, but when used with phototrophic bacteria OD is also influenced by the sulfur, either stored inside cells (Chromatiaceae) or accumulated in the medium (Chlorobiaceae), and by the number of cells. In order to ascertain the best parameter to detect the growth phases of pure cultures, several absorption values from *in vivo* spectra were tested as direct indicators of phototrophic bacterial growth in Chlorobiaceae cultures, since they accumulate elemental sulfur out of the cell and increase turbidity to the medium.

Key words: absorption, spectrum, methods, bacterial growth, green phototrophic bacteria, Chlorobiaceae.

INTRODUCTION

Experimental cultures of phototrophic bacteria are usually monitored by optical density measurements (Trentini & Starr, 1967; Schmidt & Kamen, 1970) since this parameter is useful to detect sulfide depletion and sulfur appearance. Elemental sulfur is accumulated inside the cell as globules in Chromatiaceae and released in the medium in Chlorobiaceae (Trüper & Pfennig, 1981). In some cases turbidity can be used as a measure of intracellular sulfur in Chromatiaceas, specially in those steps of growth near to sulfide depletion (Van Gemerden, 1968).

Due to the high turbidity present in culture media with growing green phototrophic bacteria, optical density is unsatisfactory if growth state has to be determined (Van Gemerden, 1968). Another parameter used to estimate cell number is the bacteriochlorophyll content, which can be measured by *in vivo* absorption. In spite of this it is well known that specific pigment concentration could be influenced by the light intensity at light limiting conditions.

In other cases (e.g. *Rhodomicrobium vanielli*, Trentini & Starr, 1967) neither total cell number nor viable cells counts are practical parameters of growt due to a special cell morfologies. Therefore, it seems clear that some methodological problems are present if growth of phototrophic bacteria has to be monitored. Growth parameters in cultures of phototrophic bacteria have to be measured once the growth experiment has finished (total cell number, cell nitrogen, proteins, pigment concentration, etc.). Thus, it is really a difficult task to obtain direct growth measurements with phototrophic bacteria.

Information about the growth process of culture could be necessary in some experiments concerning cell physiology in order to study bacterial behaviour in a determined phase of growth. The work presented here relates some parameters derived from the absorption at different wavelengths with the total cell number in a culture of *Chlorobium phaeobacteroides*, and proposes a new direct absorptional method to follow bacterial growth.

Guillenea *et al.* (1984) studied the logistic growh on *Chlorobium phaeovibrioides* by means of different direct and indirect parameters of cell growth. In the paper presented here a deeper insight on direct growth parameters is carried out.

MATERIAL AND METHODS

Chlorobium phaoebacteroides and *Pelodictyon clathratiforme* strain 7504 were grown on Pfennig's medium with 1 mM of initial sulfide and without added acetate. *Pelodictyon clathratiforme* was isolated by Abella and Garcia-Gil from Corominas lake. Cultures were continuously stirred in 125 mL sealed bottles and samples were taken under N_2 pressure (Turet, 1981). Light was at saturating intensity conditions (over 2000 lux) in order to avoid the self-shading of growing cells and to make sure that no variation in the

specific pigment content took place. As inoculum, 25 mL of exponential growth were used. In the *Chlorobium phalobacteroides* culture a 2 mM refeeding after 24 hours was made to increase cell yield.

Pigments were extracted with 90% acetone. Pigment concentration was calculated using Bchlor e=10-2 (A_{650} -A_{830}) × f, where f=v/V x d and v is the extract volume in milliliters, V is the sample volume in milliliters and d is the pathlength of the used cuvette (Montesinos, 1981). Absorption spectra of each culture were carried out in a spectrophometer SPECTRONIC 2000 with continous X-Y recorder. Elemental sulfur was determined by the cyanolysis method modified by Fisher & Trüper (1977). Statistical treatment was done using and SPSSX pakage.

Total cell number was estimated by epifluorescence microscopy, counting several fields of each sample in Nuclepore filters of 0.1 μm pore diameter using acridine orange (Hobbie *et al.* 1977).

RESULTS AND DISCUSSION

In pure culture of Chromatiaceae (Van Gemerden, 1967) there is a relationship between optical density (OD) and total cell number, since sulfur, (also expresed in this OD) is located inside the cell. This happens if a constant number of S° globules inside the cells is assumed. Further analysis is needed to quantify this relationship in Chromatiaceae at different steps of culture growth.

However, this situation is quite different for Chlorobiaceae cultures, because S° is accumulated outside the cell. Hence, the sulfur desity (OD_{675}: wavelength at minimal cell absorption) differ from those for total cell num-

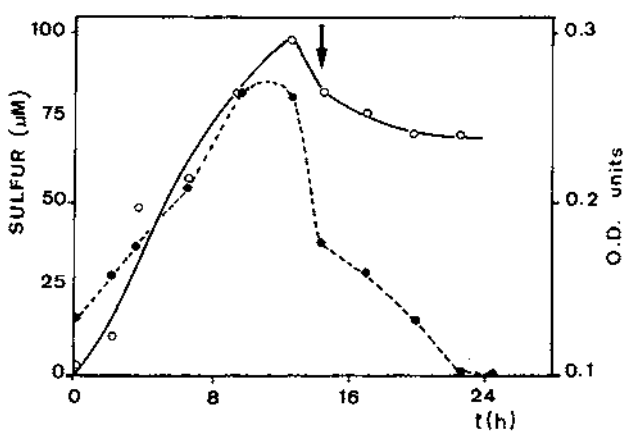

Figure 2. Optical density (o) and sulfur concentration (•), of the culture medium over time course in a growing culture of *Pelodictyon clathratiforme.* Arrow indicates the moment from which OD is not proportional to sulfur concentration. It coincides with the oxidation of sulfur.

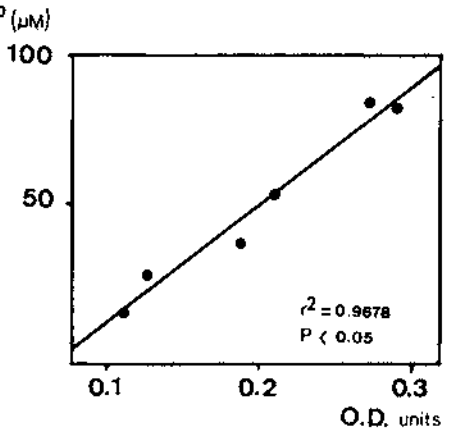

Figure 3. Plot of S° versus OD_{675}, in Those steps of growth concerning sulfur appearance.

ber during growth time course. In the logistic growth curve the stationary phase beggins 23 hours after starting up. In this moment a 2 mM refeeding with H_2S was done and either total number and sulfur concentration increased. Sulfur increasing is a consequence of a high bacterial activity.

Checking early steps of growth, just before sulfide depletion, a good correlation between sulfur concentration and OD was observable (Fig. 2). This relationship is plotted in figure 3. After 15 hours of growth sulfur began to decrease due to bacterial activity: optical density also dimisnishes, but with much lower intensity. This is because, when growth finishes, other factors such as cell mass and some pigment absorption play a more important role on OD, than sulfur.

Thus, it could be stated that OD_{675} is not a representative parameter for green phototrophic bacteria growth and only could be useful as indicator of sulfur in medium at the early steps of bacterial culture development using sulfide as electron source (see Fig. 1).

Matheron (1976) demonstrated that bacteriochlorophyll (Bchlor) is quite constant during growth only at saturation light conditions. Therefore, *in vivo* absorption due to Bchlor, could be a good parameter to estimate cell number if sulfur interferences are corrected. The ratio Bchl *e*cell number is shown in table 1 and is practically constant during the experiment time course. From these data it can be stated that, in this case, cell number is proportional to Bchlor *e* concentration, which can be estimated from the values of *in vivo* absorption of Bchlor *e*. This is possible regardles of sulfur absorption at the wavelength of maximal absorption of Bchlor *e*.

In order to evaluate Bchlor *e* concentration, several values from *in vivo* spectra have been taken into account. In Fig. 4 used parameters are shown. A_p is the *in vivo* absorption of bacteriochlorophyll peak at the wavelength corresponding to the peak in a narrow range between 717.5 and 720 nm.

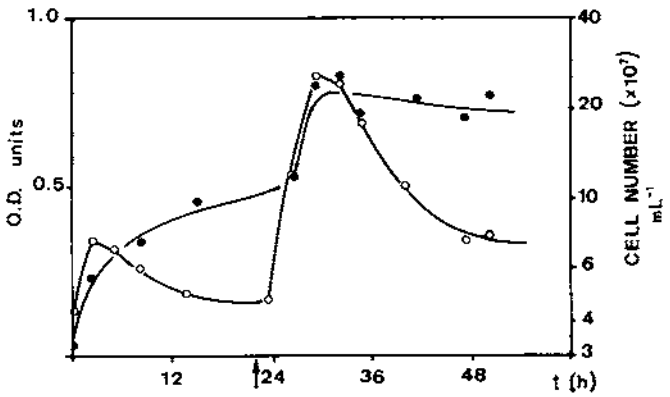

Figure 1. Optical density (o) and cell number (•) over time course in a growing culture of *Chlorobium phaeobacteroides*. Arrow indicates the sulfide refeeding.

Table 1. Bchlor *e*/cell number ratio for experiment time course before sulfide refeeding (First growth)

Sample	Time course (h)	Bchor *e* /cell
1	2	15.2
2	5	22.0
3	8	19.1
4	16	20.3
5	23	22.0

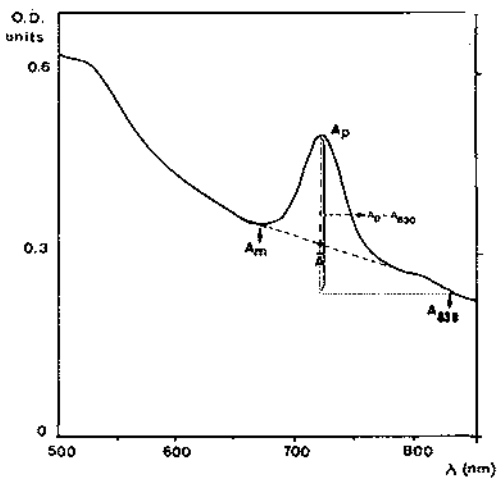

Figure 4. Location of different parameters analized on a *in vivo* spectrum of a growing culture of *Chlorobium phaeobacteroides*. For explanation see text.

A_m is the optical density where minimum pigment absorption and maximal turbidity are both combined. A_p-A_m : this parameter theoreticaly means the *in vivo* absorption due to the pigment, regardless cell mass and sulfur content. A_m-A_{830} implies the absorption due to cell mass exclussively. It would be true assuming small variation between A_m and A_{830} so A_m is the measure of cell plus sulfur content. A_p-A_{830} is Bchlor absorption minus sulfur and cell turbidity absorption in a wavelength where pigment absorption is minimum. A_p-A is a parameter used by some authors to quantify cell mass in cyanobacteria and represents the peak-heigth at selected wavelength (Zevenboom, 1986). In table 2 the values of different parameters during the experiment time course are compiled.

One method to evaluate which of those parameters is the best to estimate cell number is to study statistically cell numbers and absorption values for each one of the parameters described above. Linear regression was calculated by conventional statistical methods, using $P<0.05$ as significance level. In table 3 correlation level (r^2) foe each parameter is shown.

Our results indicate that A_p-A_{830} is the optimal parameter among those studied, according with those found by Guillenea *et al.* (1984) and it can be very helpful to follow cell growth in green phototrophic bacteria. The usefulness using this parameter is explained in part by suppression of sulfur

Table 2. Numerical values of different absorption parameters defined in the text during phototrophic green bacteria experiment timecourse.

Sample	A_p	A_m	A_{830}	A_p-A_m	A_m-A_{830}	A_p-A_{830}	A_p-A
0	0.222	0.138	0.085	0.084	0.053	0.237	0.100
1	0.408	0.334	0.225	0.074	0.109	0.183	0.105
2	0.401	0.320	0.228	0.081	0.092	0.173	0.115
3	0.341	0.256	0.170	0.085	0.086	0.171	0.110
4	0.268	0.168	0.110	0.100	0.058	0.158	0.122
5	0.602	0.540	0.350	0.062	0.190	0.252	0.120
6	0.882	0.828	0.577	0.054	0.251	0.305	0.115
7	0.875	0.808	0.578	0.067	0.230	0.297	0.130
8	0.755	0.688	0.451	0.067	0.237	0.304	0.140
9	0.492	0.347	0.222	0.145	0.125	0.270	0.185
10	0.511	0.352	0.226	0.159	0.126	0.285	0.200

Table 3. Statistical correlation (r^2) between cell number and studied parameters.

Parameter:	A_p	A_m	A_p-A_m	A_m-A_{830}	A_p-A_{830}	A_p-A
Correlation: (r^2)	0.83	0.76	0.15	0.76	0.93	0.59

interferences measured with A_{830}. The proximity between both wavelengths makes unnecessary any scattering correction.

From data shown above, we can conclude that while Bchlor *e* concentration is proportional to cell number, Bchlor *e in vivo* absorption is not a good measure of cell number, because using Chlorobiaceae, sulfur is not proportional to cell number. As a consequence of this, the absorption due to turbidity of the medium has to be substracted. The main difference between A_{830} and A_m in order to evaluate the turbidity of the culture is that, besides sulfur, A_m includes cell mass and also some pigment absorption. Guillenea *et al.* (1984) concluded that A_p-A_{830} is not valid for other growth conditions whereas we demonstrate by sulfide refeeding that relationship between A_p-A_{830} and cell number keeps significatively (Table 3).

An interesting point of discussion appears when direct growth parameters between heterotrophic and autotrophic bacteria are compared. In heterotrophic microorganism OD at wavelength at minimal cell absorption reflects cell turbidity. Energy, carbon and reducing power sources are the same for this organisms and hence are expressed in cell turbidity measurements. In phototrophic bacteria turbidity also contains pigment and sulfur absorption. For this reason A_p-A_{830} is used as a measurement of pigment concentration which is directly related to the cell number provided that constant saturating light conditions. These parameter allow to compare growth at different sulfide and carbon sources concentrations only with the same light intensity.

On this basis, and for green sulfur bacteria, the parameter A_p-A_{830} can be used as a good direct method to estimate bacterial growth and it can be helpful when information about the growth phase of the culture is needed. This parameter was found linearly related to total cell number according to the equation $Y = 115.769X\text{-}12.14$ where Y is cell number x 10^7 mL^{-1}, and X is A_p-A_{830}. In the other hand, A_m provides a good estimation of accumulation of S^o, coming from sulfide utilization, and can be useful in kinetic experiments in which cellular growth has not to be measured.

Further studies should be developed in order to find similar parameters, not only for other species of Chlorobiaceae but also for other groups of phototrophic bacteria such as Chromatiaceae species which change turbidity of medium during growth.

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