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STUDIES ON PHOTOSYNTHETIC BACTERIA II. STUDIES ON CHLOROPHYLL AND GROWTH IN *CHLOROBIUM LIMICOLA* AND *CHLOROBIUM THIOSULFATOPHILUM*

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ABSTRACT

Studies were conducted on *Chlorobium limicola* and *Chlorobium thiosulfatophilum* for chlorophyll and growth. Spectrophotometric readings at different wave-lengths and TLC showed two types of chlorophylls (*a* and *b*).

Chlorophyll contents under different growth conditions were also observed. Growth curve was studied by the turbidity assay procedures. The results demonstrated that growth is slow. Maximum growth was observed after 75 hr.

INTRODUCTION

Photosynthetic bacteria are obligately phototrophic and strictly anaerobic. They convert light energy into a chemical energy which occurs only in reaction centres having an enzymatic apparatus (Van Niel, 1941). Light energy absorbed by the pigments is transferred via various pigment molecules to the reaction centres in the form of electron excitation energy after which cyclic or non-cyclic phosphorylation takes place (Duysen and Amesi, 1967).

The group of green photosynthetic bacteria have recently been found to contain different chlorophylls. Metzner (1922) was the first to point that these bacteria have their own type of chlorophyll which he called bacterio-viridin. Later Larsen (1953) studied the two green bacteria *Chlorobium limicola* and *Chlorobium thiosulfatophilum* and proposed that this pigment should be named chlorobium chlorophyll. Some years later, Stanier and Smith (1960) found that of *Chlorobium thiosulfatophilum* contained a similar but yet distinctly different chlorophyll entity, with the main light absorption peak at 650 mμ (measured in ether) compared to 660 mμ for the same absorption peak with the chlorobium chlorophyll of Larsen (1953). They suggested that these chlorophylls should be named chlorobium chlorophyll-660 and chlorobium chlorophyll-650 respectively. Measurements of light absorption spectra (whole cells) of strains of *Chlorobium limicola* made by Pfennig (1964) have indicated that a similar situation exists within this species also.

Chlorophyll pigment content is not stable but differs under different conditions of growth and intensity of light to which the cells are exposed. In recent years extensive work has been carried out by Clayton (1963, 1966), Gest (1951, 1954, 1963, 1972), Holt and

Marr (1965), Olson (1971) and Jensen, Aasmundred and Eimhjellen (1964), on various aspects of pigment production and bacterial photosynthesis.

In the present investigation, *Chlorobium limicola* and *Chlorobium thiosulfatophilum* isolated by Aguiar and D'Souza (1978), from aquatic sediments in Goa, were used to study the associated pigments and growth requirements.

MATERIAL AND METHODS

Cultures of *Chlorobium limicola* and *Chlorobium thiosulfatophilum* were maintained in the agar medium of Larsen (1953) by the roll tube technique described by Hungate (1969). These cultures were transferred into 150 ml of the above liquid medium in MacCartney bottles and incubated near a 40 watt bulb at ambient temperature till a suspension of appoximately 10^8 cells/ml was obtained. This was used for further investigation as described below.

1. *Determination of chlorophyll* : Chlorophyll was determined by two wavelength spectrophotometry of a suspension of intact cells and by direct spectrophotometry after its extraction in methanol (Fraker and Kaplan, 1971).

(a) For estimation of chlorophyll in suspension of intact cells, the OD was measured at about 880 mμ (the absorption maximum for BChl in vivo) and 680 mμ. Since the OD of equivalent concentration of pigmented and non-pigmented cells is equal at 680 mμ the ratio (0.736) of the OD at 880 mμ to that at 680 mμ of suspension of non-pigmented cells can be applied as a correction for the contribution of light scattering to the apparant absorbancy at 880mμ. Thus OD 880 (OD 680) (0.736) is the absorbancy of the chlorophyll in pigmented cells. An absorbancy of 1.0^{-1} cm is equivalent to 9.5 mg of chlorophyll per ml.

(b) For the determination of chlorophyll peaks, the cell suspension centrifuged at 5000 g for 30 minutes washed and recentrifuged. The chlorophyll was extracted with 10 ml methanol for 45 minutes, centrifuged and the absorbancy of the clear solution was observed at different wavelengths within the visible range.

2. *The effect of Fe and light on chlorophlly production:* The chlorophyll content of the above organisms was measured by growing them under different conditions, *i.e.*, by exposing them to different light intensities (40 & 60 watt bulbs), to filtered light through red cellophane paper, and in the absence of light. They were also grown in the medium lacking iron. The chlorophyll content was then measured as given above.

3. *Thin layer chromatography*: Methanol extracted chlorophylls were used in the TLC experiments carried out on Kieselguhr (Merck) adsorbent using a mixture of petroleum ether and *n*-prophyl alcohol (99:1) as the solvent system, as described by Bolliger and Konig (1969).

4. *Growth curve:* 1 ml of the inoculum was inoculated into 10 ml of the liquid medium of Larsen (1953) with 0.5% Tween 80 in a test tube which was plugged with cotton and sealed with wax to ensure complete anaerobic conditions. The turbidity of this suspension was then determined with the help of a Klett Summerson at zero time. The tube was then incubated near a 40 watt bulb at ambient temperature, and the turbidity determined at different time intervals for 100 hr. The tube was well shaken before

taking a reading. The number of microorganisms at any particular time was determined by dilution pour plate technique or counting the organisms under a haemocytometer.

RESULTS AND DISCUSSION

The studies reveal that the green bacteria have two types of chlorobium chlorophylls giving two peaks. The peaks for *Chlorobium limicola* correspond to the wavelengths 660 mμ and 420–410 mμ which are the absorption maxima for the respective chlorophylls. In the case of *Chlorobium thiosulfatophilum,* the absorption maxima are at 660 mμ and 430 mμ. These results correspond with those reported by Stanier, Doudoroff and Adelberg (1974).

On separation of these chlorophylls on TLC, two green bands were observed at a short distance from each other. The R_f values were found to be 0·93 and 0·85 which correspond to chlorophyll *a* and *b* respectively, as observed by Bolliger and Konig (1969).

Studies conducted on the chlorophyll contents of these cells under different growth conditions gave results which are in accordance with those observed by Holt and Marr (1965). Table I summarises all the results of the chlorophyll content under different growth conditions. These results give some favourable and unfavourable conditions for the production of chlorophyll in the photosynthetic cells.

Table I. Growth and chlorophyll content under different conditions.

	Growth conditions	O.D. 880 mμ	680 mμ	Absorption of chlorophyll	mg. of chlorophyll per ml	No. of cells/ml after 72 hr incubation
Chlorobium thiosulfatophilum						
I	40 watt bulb	0.275	0·310	0·047	0·427	$1·24 \times 10^9$
II	60 watt bulb	0·460	0·585	0 030	0·285	2.34×10^9
III	Red cellophane paper	0.385	0·450	0·054	0·513	1.80×10^9
IV	75 hr incubation	0.380	0·420	0·070	0·665	$1·68 \times 10^9$
V	100 hr incubation	0·280	0·350	0.030	0.285	$1·40 \times 10^9$
VI	Incubation in dark	0.210	0·260	0.020	0.190	$1·04 \times 10^9$
VII	Medium without iron	0·235	0·285	0.026	0 247	$1·14 \times 10^9$
Chlorobium limicola						
I	40 watt bulb	0·280	0 320	0·045	0·427	$1 28 \times 10^9$
II	60 watt bulb	0.390	0·490	0·030	0.285	$1·96 \times 10^9$
III	Red cellophane paper	0 240	0.270	0·038	0 361	$1·08 \times 10^9$
IV	75 hr incubation	0.380	0·410	0 079	0.750	$1·64 \times 10^9$
V	100 hr incubation	0·275	0.340	0.025	0·237	1.46×10^9
VI	Incubation in dark	0.345	0·430	0·029	0·275	1.02×10^9
VII	Medium without iron	0.285	0.360	0.020	0·190	$1 44 \times 10^9$

It was observed that in the presence of bright light (60 watt bulb), the chlorophyll content of the cells went on decreasing and was nearly 50% lesser than the cells grown in the presence of a 40 watt bulb indicating that the chlorophyll production is inversely related to light intensity (Cohen-Bazire, Sistrom and Stanier, 1957; Sistrom, 1962). This

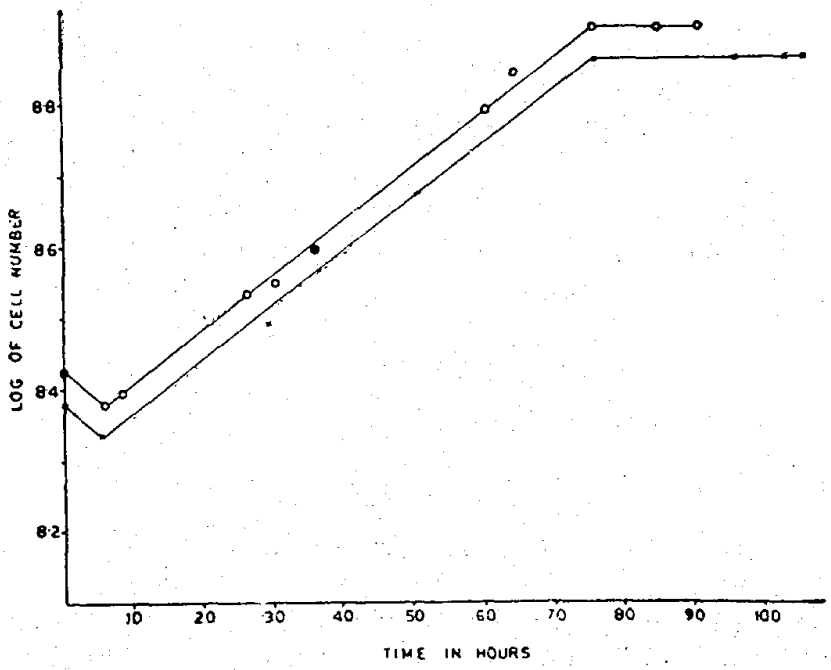
shows that light intensity has an effect on the chlorophyll content of the cells which may be due to the effect on the cell membrane. It is also known that variations in the light intensity may cause alterations in the relative positions of the peaks in the chlorophyll region of the spectrum.

Light on passing through a red cellophane paper did not effect the chlorophyll content much. The absorbance of chlorophyll per number of bacterial cells in this case was slightly less than that obtained in the case of normally grown cells under 40 watt bulb. The reason for this decrease in chlorophyll was probably because of the filtered light, as reported by Frenkel and Nelson (1971).

The chlorophyll in cells tested after 75 hr was much higher than in those cells grown for 100 hr. The chlorophyll content per number of bacterial cells at 75 hr has been found to be twice the amount observed after 100 hr. This may be because of the cells being at the peak of the exponential phase, where the maximum amount of cell growth was observed and hence the invaginations of the cell membrane is increased tremendously thus increasing the amount of chlorophyll.

It is a well known fact that photosynthetic bacteria chiefly depend on solar energy for photosynthesis. Cells incubated in the absence of light possessed low chlorophyll content after 75 hr. The density of the cell suspension also decreased showing that light and chlorophyll are important factors for the proper growth of these bacteria.

Iron is an essential mineral required by photosynthetic organisms. Lack of iron is said to cause cholosis, a disease which results in the discolouring of the organisms. Photosynthetic bacteria require 500μg of iron per litre of the medium. Cells grown in the absence of iron appeared less green. Their chlorophyll content was almost half of



⊙—⊙ = Growth curve of *Chlorobium thiosulfatophilum*
.——. = Growth curve of *Chlorobium limicola*

the normal cells grown under 40 watt bulb. This confirms that iron is essential for chlorophyll and ultimately for photosynthesis.

Unlike other microorganisms, the green sulphur bacteria grow at a very slow rate (Fig. 1). The growth curve by the turbidity method gives us an understanding of their slow growth and the different phases of growth. The lag phase is long and lasts for about 6 hr. Wherein it is observed that the number of cells also decrease giving the curve a slight dip. This may be because of the change to a fresh medium and the exposure to air during the process of transfer which causes death in some cells. After this the exponential phase begins which shows a gradual increase in the number of cells. The exponential phase lasts till 75 hr which is the peak or maximun growth after which the stationary phase begins. The cells during the exponential phase grow faster and the cell mass and cell number increase considerably. Cell density and competition for limited nutrients results in the initiation of the stationary phase after 75 hr. Tween 80 helped in preventing clumping of cells.

Thus it can be concluded that *Chlorobium limicola* and *Chlorobium thiosulfatophilum* used in the investigation are slow growing organisms with two major chlorophylls. These organisms are affected by changes in light intensity and require iron for growth and chlorophyll production.

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