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Chlorophyll loss of *Chlorella* after a heat stress. A flow cytometric study

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Abstract

Synchronous culture of *Chlorella pyrenoidosa* was obtained by the light/dark regime. The small vegetative cells were subjected to a mild heat pre-treatment (46.5 °C for 1 h) and cultured again in growth medium. The variation in chlorophyll content with time of the cell population was then monitored using chlorophyll fluorescence by a flow cytometer thereafter. Within about 16 h after stress, cellular chlorophyll vanished in two distinguishable steps. It started with a uniform decrease in chlorophylls of all the cells by about 2-3-fold. In the second stage, the cell population then lost all the remaining pigment by a random process. The culture resumed growth about 3 days after stress. It was also found that chlorophyll bleaching required continuous illumination. The minimal exposure time was 6 h and the minimal intensity was only about 4 μmole $m^{-2}s^{-1}$. Moreover, the pigment loss could be alleviated by dark treatment right after stress. It thus appears that the breakdown of chlorophylls is a programmed process, which requires light as a signal. In addition, the response of large cells, which contained up to 20 autospores, was also studied. It was found that large cells took longer time to lose all the pigments (~24 h), and also took much longer time to restart growth (5 days).

Introduction

Pigment degradation or bleaching is a common process for all the photosynthetic organisms. It occurs during natural senescence or fruit ripening. However, many

environmental stresses also induce pigment breakdown, such as air pollution [1-3], heavy metal contamination [2-4], acid rain [5] or even herbicides [6,7].

Pigment degradation is a complicated process. Its regulation is still not fully understood. Chlorophyll bleaching usually is light dependent [8,9] and is generally believed to involve oxygen radicals [9-11]. In a series of studies on Euglena gracilis, it was found that prolonged incubation of the algae at 33°C induced a time-dependent loss of chlorophyll.

In addition, it was also shown that protein synthesis of bleaching chloroplasts was impaired [12], a polypeptide (24.5 KDa) of light harvesting complex of photosystem II is rapidly degraded [13] and mature transcripts for CP47 and CP43, the chlorophyll binding proteins of photosystem II, declined sharply early during bleaching [14]. Moreover, chloroplast chaperonin was also found to be sensitive to an increase growth temperature [15].

In the present study, green algae Chlorella pyrenoidosa were subjected to a mild heat pre-treatment (46.5°C for 1h). It was found that the treated cells lost all the chlorophylls when they were cultured again in growth medium. The culture regained its green color several days later. To closely examine the processes involved in chlorophyll degradation and the resumption of growth, the flow cytometer was used to continuously monitor the changes in chlorophyll contents of the cell population cultured in liquid medium after stress. The advantages of this technique were the possibility of identification and quantification of subpopulations in heterogeneous cell samples. Our investigation suggests that the process of chlorophyll breakdown is a well-programmed process, which requires light as a signal.

Materials and Methods

Chlorella pyrenoidosa (211-8b, from the Algal Collection Center, University of Göttingen, Germany) were cultured in a medium similar to that of Chen and Lorenzen [16]. Cultures were grown in large glass culture tubes (4 cm diameter, 350 ml volume) at 32°C with slow bubbling at 3.5% CO2 in air [17]. Synchronous cultures of

Chlorella were obtained by the programmed 14-h light/ 10-h dark regime. The illumination was provided by daylight fluorescence tubes at an intensity of 250 μmole $m\text{-}2s^{-1}$. The culture usually started with a cell density of 2×106 cells ml-1 and dilution was made after each 24-h cycle.

The mild heat treatment was applied by incubating 100 ml of culture medium (containing 2×106 cells/ml) at 46.5°C for 1h. The treated cells were then cultured immediately thereafter under the same conditions as before the stress, but were illuminated continuously at 17 μmole $m\text{-}2s^{-1}$ or lower light intensity when specified. To monitor the variation in cellular chlorophyll contents, cell samples (2 ml) were collected at regular time interval.

Flow cytometry analysis was carried out with a FACStarplus flow cytometer (Becton Dickinson) equipped with an argon laser (IN NOVA 90, Coherent). LYSYSII software (Becton Dickinson) was used for instrumental control and sample analysis. FACSFlow sheath fluid (Becton Dickinson) was forced by a pressure of 20 psi though a 70 μm nozzle. For chlorophyll content measurement, the fluorescence of intrinsic chlorophyll a was detected. The excitation at 457 nm was provided by the argon laser at 500 mW output power. The emission was confined by a 685 nm interference filter (10 nm bandpass). 104 particles were analyzed each measurement.

Results and Discussion

The synchrony of growth and division of Chlorella pyrenoidosa was accomplished by repeating the light/dark cycle several times. At the beginning of a light period (time zero shown in Fig. 1), Chlorella cells of a synchronous culture were small vegetative cells. As the time went on, the cells gradually matured and contained many autospores. The rupture of parental cell walls and free of the autospores started to took place at about 4 h after the beginning of the dark period (18 h of Fig. 1) and the whole process took about 6 h to complete. As shown in Fig. 1, the cell number increases about 20-fold each cell cycle.

A flow cytometer was also used to the check the cellular chlorophyll contents of the

algal population using intrinsic chlorophyll fluorescence as the gauge. The results are displayed in Fig. 2. Each frequency distribution histogram in Fig. 2 corresponded to 104 particles analyzed. The differences among large cells (Fig. 2a, collected at the time of 18 h shown in Fig. 1), cell population undergoing rupture (Fig. 2b, collected at 20 h) and small cells (Fig. 2c, collected at 0 h) are clearly demonstrated. Note that the fluorescence intensity of large cells is about 20 times of that of the small cells.

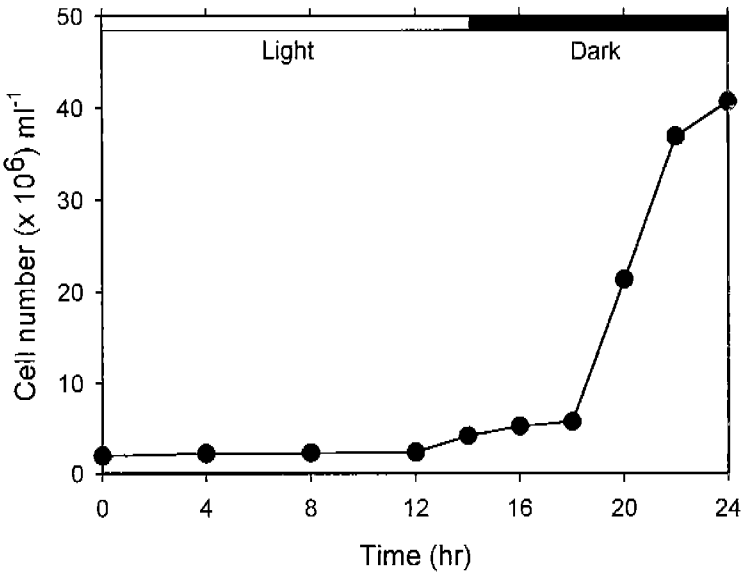

Fig. 1. The cell number of a synchronous culture of *Chlorella pyrenoidosa* as a function of culture time. The synchrony was accomplished by repeating the light/dark cycle several times. Light on at zero time and off at 14 h.

The small vegetative cells were subjected to a mild heat stress (46.5°C for 1h), and cultured immediately thereafter. The variation in chlorophyll contents with time of the whole cell population was again checked by using flow cytometer (Fig. 3). The high fluorescence control was the signal from freshly harvested living cells (Fig. 3b), whereas the low fluorescence control was from the white Chlorella cells which had been baked in an oven at 100°C for 4 h followed by 3-day cultivation to deplete all cellular chlorophyll (white control, Fig. 3a).

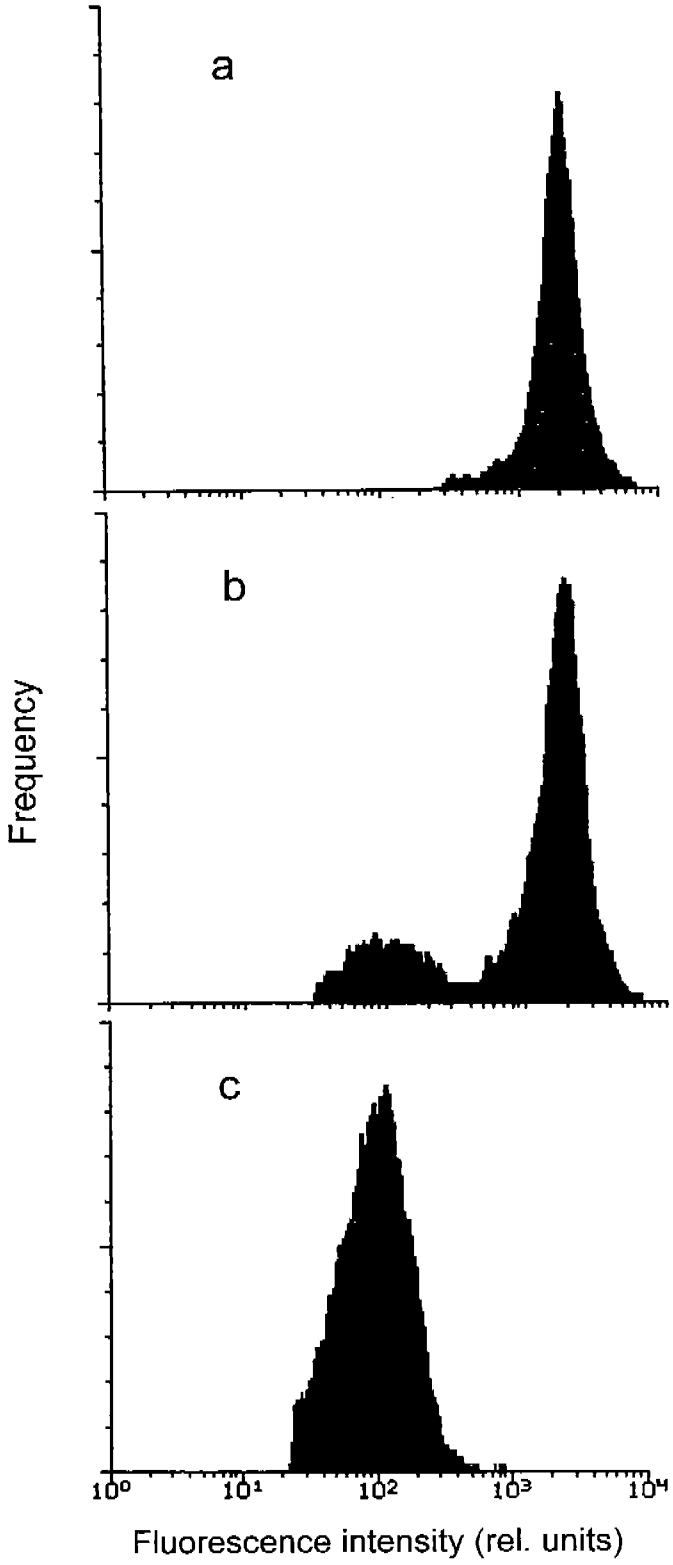

Fig. 2. Frequency distribution histograms of Chlorella cells analyzed for chlorophyll a fluorescence. (a) Large cells (cells collected at the time of 18 h shown in Fig. 1), (b) cell population undergoing rupture (collected at 20 h) and (c) small cells (collected at 0 h).

After stress, there was no change in the chlorophyll contents of *Chlorella* cells, and the cells yielded a frequency distribution pattern (Fig. 3c) similar to that of fresh harvested cells (Fig. 3b). However, Chlorella lost all the chlorophylls within about 16 h after stress. The disappearance of pigments seemed to proceed in two distinguishable steps. It started with a noticeable shift of the frequency distribution peak toward lower fluorescence (Fig. 3d). The fluorescence intensity dropped about 2-3-fold within about 8 h after stress, indicating that the whole population of cells uniformly lost a portion of their pigments. In the second step, a new peak appeared at a low fluorescence position similar to that of the dead white cells (Fig. 3d). The number of these white cells increased rapidly, and was accompanied by a decrease of the high fluorescence peak representing the green cells (Fig. 3e). This conversion completed within 16 h after stress (Fig. 3f). Thus for the second step, in which each cell depleted all of its remaining pigments, the conversion of the cell population was a random process.

At about 48 h after stress, the presence of a small population of green cells was indicated by the appearance of a few highly fluorescent cells (Fig. 3g). The number of these green cells increased rapidly afterward. After 72 h, more than 80% of cells were green (Fig. 3h). The green cells seemed to appear from no where. Since no shift of the frequency distribution peak from low to high fluorescence positions was observed, it was unlikely that the newly appearing green cells derived from the white cells that had undergone pigment resynthesis. The green cells then must originate from a few cells that survived heat stress.

It is worthy to note that many studies have been done to investigate the changes taking place inside the algal cells during stress and it has been generally assumed that the responses of these cells may related to the mechanism of stress tolerance. This study, however, clearly showed that the resumption of growth of *Chlorella* culture started from only a few cells, i.e. most cells lost the ability for reproduction after stress. So one should be careful about the search for the mechanisms of stress tolerance when a study is made on the whole cell population.

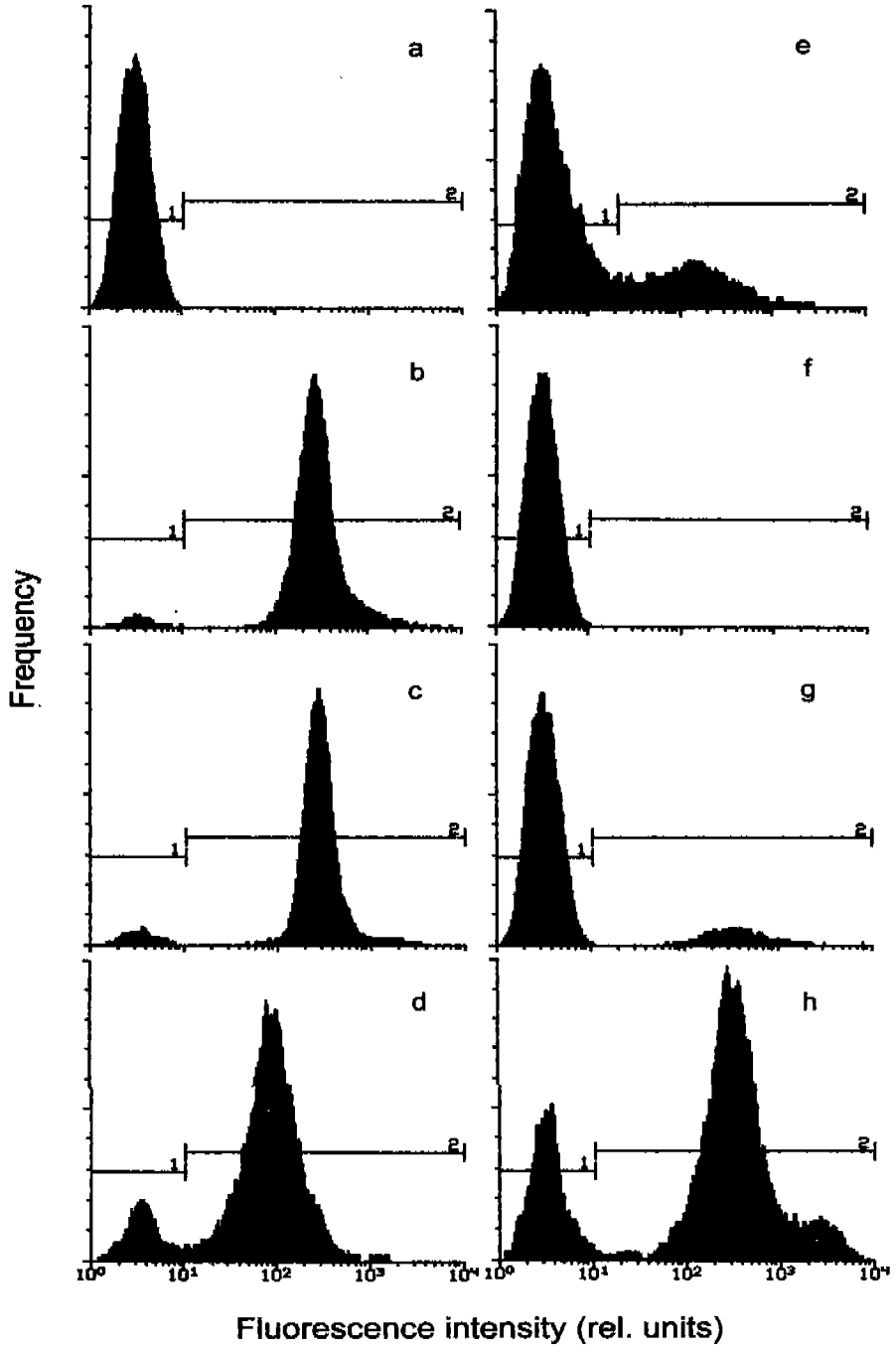

Fig. 3. Frequency distribution histograms of *Chlorella* cells analyzed for chlorophyll a fluorescence. (a) Low fluorescent (white) cell control - 4 h at 100°C, then 3-day cultivation, (b) high fluorescent (freshly harvested) cell control and cells subjected to a mild heat pre-treatment followed by cultivation in growth medium for (c) 0, (d) 8, (e) 12, (f) 16, (g) 48 and (h) 72 h.

It has been shown that the process of chlorophyll degradation requires light [8,9]. This was in line with our observation that if *Chlorella* culture was kept in the dark after stress, the chlorophyll content of cells remained virtually unchanged for up to 5 days tested (data not shown). The next question we asked was what happened if illumination was stopped in the middle of the conversion process from the green to the white cells. For cells which were illuminated for only 6 h after stress (in the middle of the first step of the conversion), there was a shift of the distribution peak corresponding to about 40% decrease in fluorescence intensity (data not shown), but without formation of any white cells (see Fig. 4). If illumination lasted for 12 or 16 h (in the middle of the second step of the conversion), the decrease in fluorescence intensity of the first step was about 3-fold, but the second step resulted in only about 30% or 85% of the cell conversion, respectively (Fig.4). The result shows that the conversion process stops and stays where it is whenever illumination is terminated i.e. light must be present during the conversion of each individual cell. Here the proportions of the green and the white cells were determined from the frequency distribution histograms. As presented in Fig. 3d, cells covered by the horizontal bar no. 1 were considered to be white cells, and those under horizontal bar no. 2 were green cells.

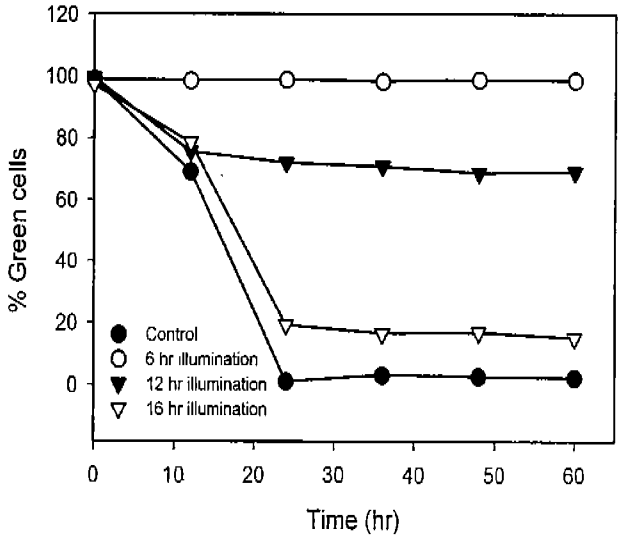

Fig. 4. The percentage of the green cells as a function of culture time. The small vegetative *Chlorella* cells subjected to heat pre-treatment were cultured immediately thereafter and illuminated for 6, 12, 16 h or continuously (control). The heat pre-treatment ends at zero time.

We then proceeded to find out the requirement for the conversion in term of light intensity. As presented in Fig. 5, there was no significant change in the rate of the cell conversion when illumination intensity was decreased down to 4 μmole $m^{-2}s^{-1}$. However, a dramatic slowing down in the process of chlorophyll degradation was observed at a light intensity of 2 μmole $m^{-2}s^{-1}$. The data indicate that a very weak light is enough to keep the cell conversion going. It also suggests that light only plays a role as a signal for triggering the process, instead of a direct driving force causing the bleach.

Since a continuous dark treatment after stress can completely block the chlorophyll degradation process of *Chlorella*, it would be interesting to find out what happen if dark treatment is terminated after a certain period of time. As displayed in Fig. 6, chlorophyll bleaching still occurred when light was turned on after 12-h dark treatment, but the conversion to the white cells was not complete. It also resulted in an earlier recovery of the green cell population. The degree of chlorophyll loss was decreased further when dark incubation after stress was prolonged to 24 h or 48 h. It clearly indicates that the pigment degradation can be alleviated by dark treatment right after stress.

From the above, it appears that the conversion from the green to the white cells is a well-programmed process. It is initiated by heat stress, and then proceeds in two distinguishable steps. Light must be present during the conversion. A very weak light is enough to trigger it, but a certain period of dark treatment can stop it.

In addition, we have also studied the response of large cells, which contained up to 20 autospores, to the mild heat stress. The result is shown in Fig. 7. It was found that large cells took longer time to lose all the pigments (~24 h), and also took much longer time to restart growth (5 days), probably hindered by the rupture of cell walls. It suggests that large cells are more vulnerable at an elevated temperature.

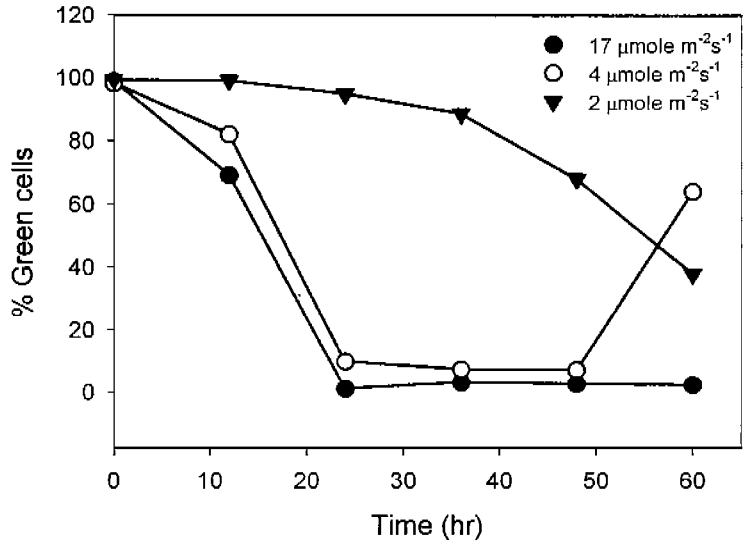

Fig. 5. The percentage of the green cells as a function of culture time. The small vegetative *Chlorella* cells subjected to heat pre-treatment were cultured immediately thereafter and illuminated continuously at the intensity of 2, 4 or 17 μmole m-2s-1. The heat pre-treatment ends at zero time.

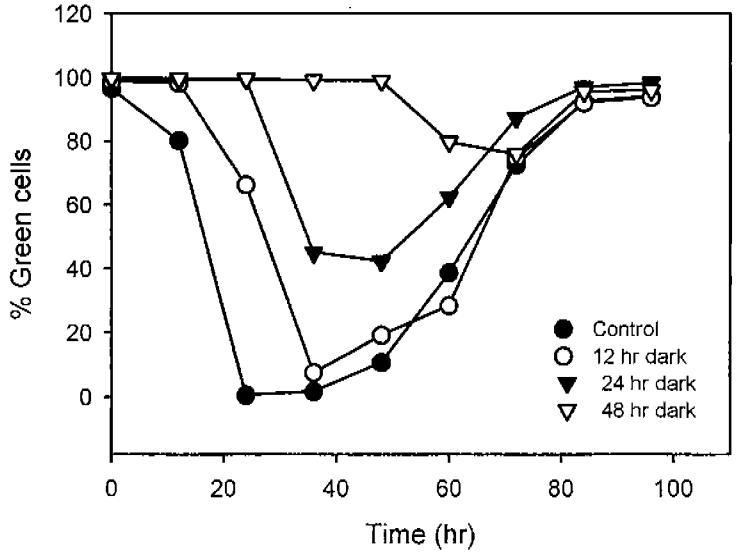

Fig. 6 . The percentage of the green cells as a function of culture time. The small vegetative *Chlorella* cells subjected to heat pre-treatment were cultured immediately thereafter in the dark for 12, 24, 48 h before light on or illuminated continuously (control). The heat pre-treatment ends at zero time.

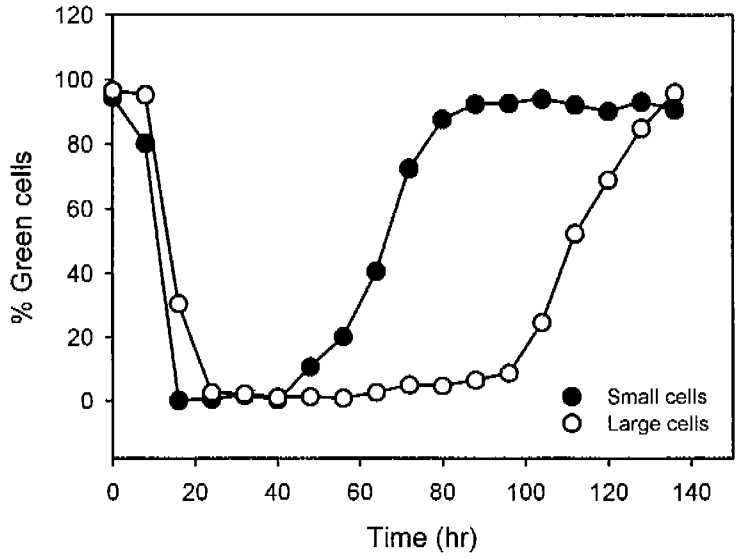

Fig. 7. The percentage of the green cells as a function of culture time. The small vegetative *Chlorella* cells and the large cells (containing up to 20 autospores) were subjected to heat pre-treatment. They were then cultured immediately thereafter under continuous illumination. The heat pre-treatment ends at zero time.

Acknowledgements

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