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Cell cycle of *Chlorella vulgaris* can deviate from the synchronous binary division model

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Conditioned medium from high density *Chlorella vugaris* cultures was freeze dried extracted into ethanol. The ethanol was volatilized and the resulting powder then introduced with *C. vulgaris* photoautotrophic cultures which underwent asynchronous DNA replication forming cells with two, three, four, and six autospores instead of 2^n characteristic of synchronous DNA replication. These cells were blocked at the division stage of the cell cycle.

Introduction

Chlorella vulgaris, a green eucaryotic unicellular microalga, is believed to replicate its DNA synchronously to produce 2^n daughter cells per division burst (Donnan *et al.*, 1985; John *et al.*, 1973; Morimura, 1959; Tamiya *et al.*, 1961; Tamiya, 1964). A mature cell can produce 2, 4, 8, and, more rarely, 16 autospores (Bold and Wynne, 1978). Studies have shown that the average number of autospores per division burst was dependent on culture conditions (Tamiya *et al.*, 1961; Tamiya, 1964; Morimura, 1959; Donnan *et al.*, 1985). *C. vulgaris* has been cultivated to high cell densities (30 g wet wt/l) in photobioreactors (Javanmardian and Palsson, 1991a; Lee and Palsson, 1994). On-line ultrafiltration was required to achieve these high cell densities. Population distributions of per cell DNA obtained prior to ultrafiltration showed that the cells were blocked at some stage in the cell cycle. Following ultrafiltration, this blockage was relieved and the cells continued through the growth cycle. The presence of autoinhibitors in culture filtrates of *C. vulgaris* was reported over 50 years ago (Pratt, 1942a, 1942b, 1944; Pratt and Fong, 1940; Pratt *et al.*, 1945). In this study the effect of conditioned medium (CM) fractionated by organic dissolution on the growth and DNA replication of *C. vulgaris* is reported.

Materials and methods

The experiments were conducted in 125 ml shake flasks with 40 ml working volume. *C. vulgaris*, UTEX 398, (UTEX Culture Collection of Algae, Texas) was cultivated in N-8 medium (Javanmardian and Palsson, 1991b) at 25ºC under a constant light intensity of 300 $\mu E/m^2$.sec in a incubator shaking at 150 rpm. Samples were collected daily.

The conditioned (spent) medium (CM) was obtained from high density *C. vulgaris* cultures as previously described (Mandalam and Palsson, 1995). The CM was lyophilized to dry powder in a Lyph-Lock 6 liter bench top freeze drying system (Model 77520, Labconco Corporation, Kansas City, MO). Organic dissolution of the freeze-dried powder of the CM were performed to purify the components of the CM. 120 ml of CM was freeze-dried and the powder was dissolved in 100 ml 100% methanol or 100% ethanol. The solution was stirred for twelve hours and the undissolved solids were removed. The methanol and ethanol were evaporated, the residue washed with deionized water and freeze-dried again. The washing step was repeated two more times to remove any residual methanol or ethanol. Only 20% and 5% of the total freeze-dried CM solids dissolved in methanol and ethanol, respectively. This powder was dissolved in water and was used in the experiments. Cells were cultured under four different conditions. 10% fresh medium supplemented with deionized water was used as negative control (flask no. 1). Experiments have shown that *C. vulgaris*, when cultivated in N-8 medium and low CO_2 levels (atmospheric) is not limited by nutrients in 10% fresh medium cultures (Mandalam, 1994). 90% CM (CM freeze-dried and dissolved in the same quantity of deionized water) and 10% fresh medium was used as positive control as the purifications were done from the CM (flask no. 2). 90% methanol dissolving

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fraction of 120 ml of CM (three times concentrated) supplemented with 10% fresh medium was used to study the activity in this fraction (flask no. 3). 90% ethanol dissolving fraction of 120 ml of CM supplemented with 10% fresh medium was used (flask no. 4). It is to be noted that the amount of CM used for this extraction is three times more than that of the untreated CM used in flask no. 2. A similar concentration of conditioned medium could not be used in flask no. 2 as it would result in a detrimental increase of osmolarity. This experiment was repeated twice and the results were found to be consistent within experimental errors. The cell number and average cell volume was measured using Coulter Counter Model ZM with a Channelyzer (Coulter Electronics, Inc., Hialeah, FL). The distribution of per cell DNA content was obtained using flow cytometry as previously described (Mandalam and Palsson, 1995). The channel number for one DNA equivalent was set by the channel number of the peak of a DNA histogram of a stationary phase control culture.

Results and discussion

Cell growth was obtained under the four different culture conditions (Figure la). The initial growth rate (0.04 h^{-1}) for the first 46 hours was the same under all four conditions. Growth in the CM cultures ceased around 72 hours, after which there was no significant increase in cell number. The average per cell volume of the cultures over the period of cultivation is shown in Figure 1b. There was an increase and decrease in average cell volume in the fresh medium and CM cultures (flasks no. 1 and 2) in the first 48 hours due to the formation of autospores and subsequent burst of mother cells. The average cell volume increased in flasks no. 3 and 4 in the first twenty four hours indicating the formation of autospores. However, unlike flasks no. 1 and 2, even though there was an increase in cell number, the average volume in flasks no. 3 and 4 did not decrease but continued to increase throughout the period of cultivation. The pH (Figure 1c) of the cultures in flasks no. 2, 3 and 4 increased from their initial values of 6.9, 7.5

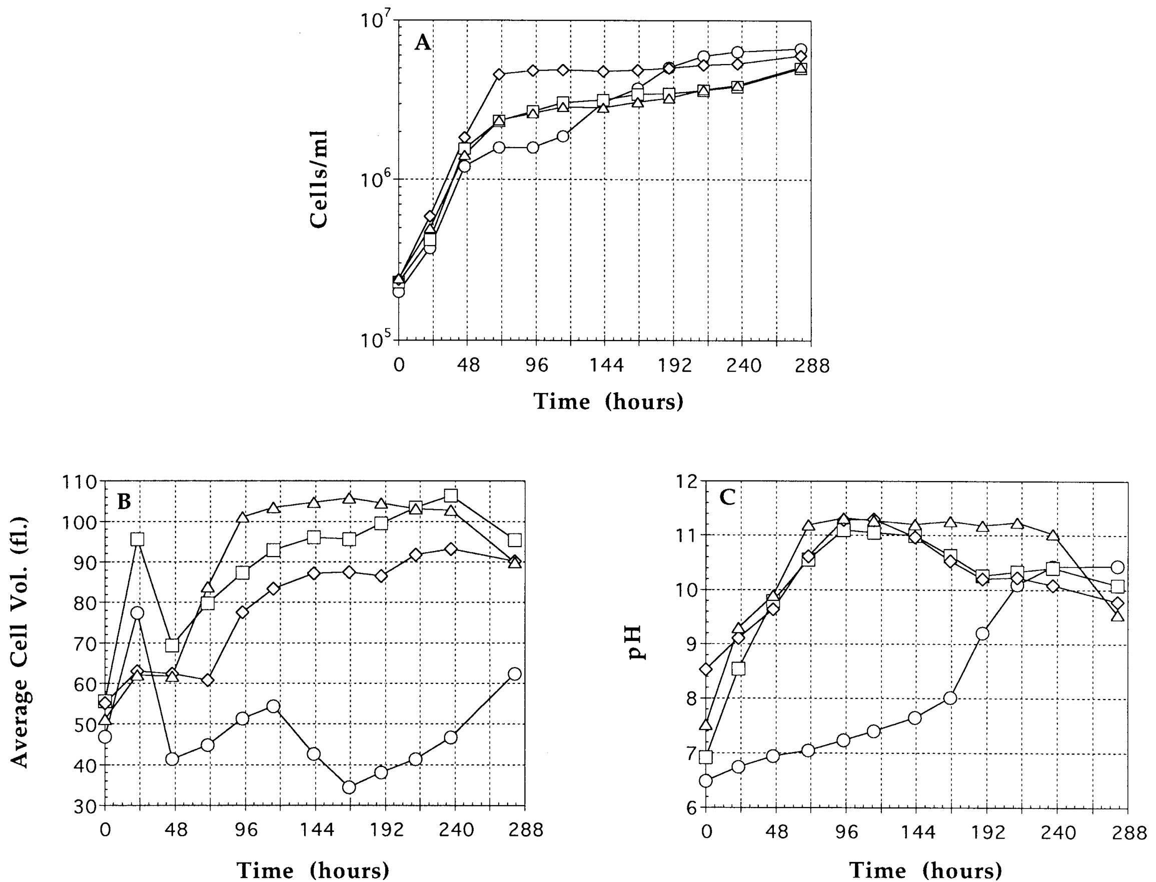


Figure 1 Growth (A), Average cell volume (B), and pH (C) of *C. vulgaris* in 10% fresh medium (circle); 90% CM supplemented with 10% fresh medium (square); 90% CM components dissolving in methanol supplemented with 10% fresh medium (diamond); and 90% CM components dissolving in ethanol supplemented with 10% fresh medium (triangle). The CM was obtamed from a 2 × 10^8 cells/ml culture.

and 8.5 respectively to 11.0 in 96.0 hours at about the same rate. The pH of flask no. 1 (10% fresh medium culture) increased gradually due to the reduced buffering capacity of the 10% fresh medium.

Cells were collected at regular intervals from the fresh medium and CM cultures and analyzed for the distribution of the DNA content per cell using the flow cytometer. At the start of the experiment the distribution was the same in all the flasks and is shown in the histogram at 0 hours (Figure 2). The cultures containing 10% fresh medium showed a unimodal distribution throughout the period of cultivation (Figure 2). A small tail is observed indicating the formation of autospores in some of the cells in the culture. In the presence of CM, the distribution of the per cell DNA content was not unimodal, but resolved into at least three peaks at one, two and four DNA equivalents (Figure 2). The population of cells at four DNA equivalents (one equivalent is the total amount of DNA in a single cell) and the ratio of the cells with four DNA equivalents (Channel no. 72) to one DNA equivalent (Channel no. 18) increased with time. In flask no. 3 similar activity was observed with peaks emerging at two and four equivalents of DNA (Figure 3). In flask no. 4 the distribution resolved into peaks at two (Channel no. 32), three (Channel no. 48), four (Channel no. 64) and six (Channel no. 90) DNA equivalents (Figure 3). The percentage of cells at one DNA equivalent kept decreasing with a gradual increase in percentage of cells in two, three and four DNA equivalents. At the end of cultivation (around 237 hours) the culture contained cell populations with one, two, three, four and six DNA equivalents.

The pH increase in the flasks containing CM is due to the presence of 10 mM HCO^{-}_{3} in CM (Mandalam and Palsson, 1995). The increase in volume and the accumulation of cells at higher DNA equivalents could be explained by one of the two following explanations: (i) aggregation or clumping of cells, and (ii) inhibition of cell division resulting in accumulation of cells with multiple autospores. The cells with three, four and six DNA equivalents were sorted using the flow cytometer. On examination under an inverted compound microscope (with 40× magnification), they were all found to be single cells containing multiple autospores.

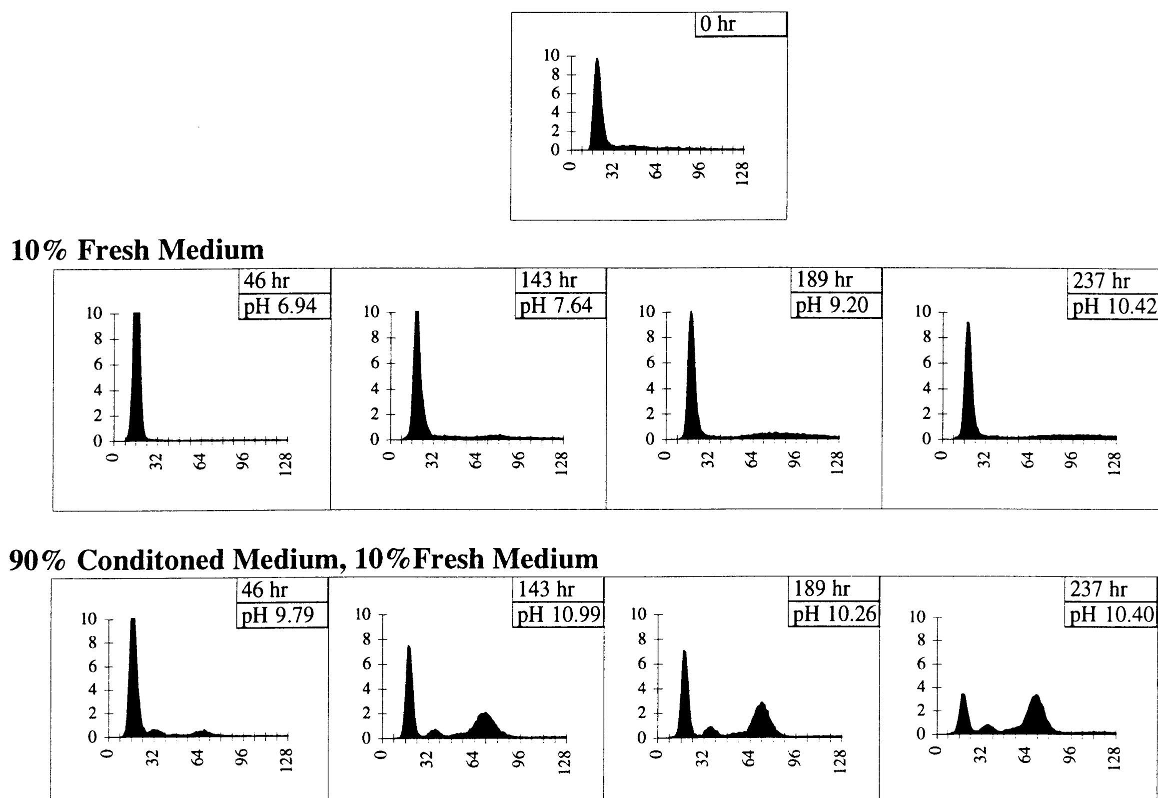
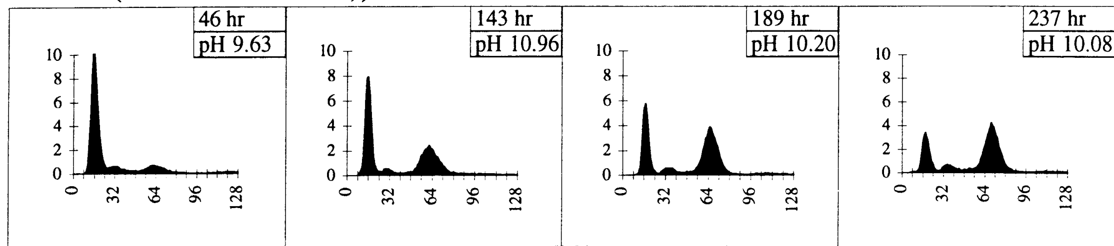


Figure 2 The time sequence of DNA histograms of *C. vulgaris* in 10% fresh medium; and 90% CM supplemented with 10% fresh medium. All histograms are obtained in this figure on analysis of 30,000 cells. The channel no. is on the x-axis and percentage of cells per channel no. in the y-axis.

90% CM (Methanol Dissoln.), 10% Fresh Medium



90% CM (Ethanol Dissoln.), 10% Fresh Medium

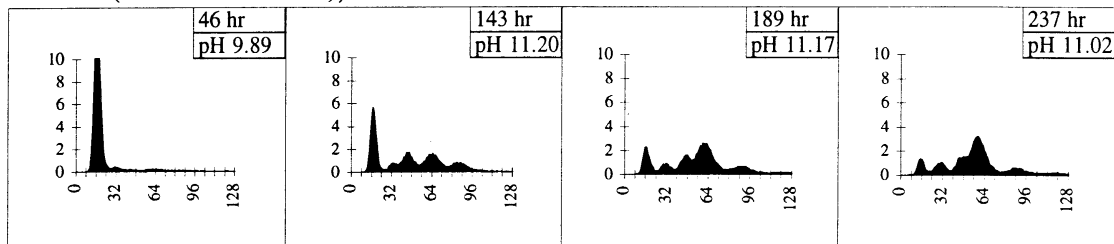


Figure 3 The time sequence of DNA histograms of *C. vulgaris* in 90% CM components dissolving in methanol supplemented with 10% fresh medium; and 90% CM components dissolving in ethanol supplemented with 10% fresh medium. All histograms are obtained in this figure on analysis of 30,000 cells. The channel no. is on the x-axis and percentage of cells per channel no. in the y-axis.

Furthermore, Transmission Electron Microscopy micrographs of cells from CM culture showed spherical cells with a single cell wall containing completely formed autospores in it (Mandalam, 1994). There is also a slow increase in cell number with an increase in cell volume which is contrary to what one would expect in the case of aggregation. Under alkaline conditions, autospore release at the end of the cycle is prevented as a result of change in cell wall structure causing aggregation of cells from several generations (Malis-Arad *et al.*, 1980; Malis-Arad and McGowan, 1982a; Malis-Arad and McGowan, 1982b). Moreover, Guckert and Cooksey (1990) have found that at high pH, the membrane lipids in *Chlorella* are not synthesized, resulting in the inhibition of autospore release after nuclear division. Thus, the results from our investigation and those of others (as cited above) leads us to conclude that the phenomenon observed is due to inhibition of cell division (i.e. prevention of release of autospores).

Asynchronous initiation of DNA synthesis in *C. vulgaris* is contrary to the accepted cell cycle model of multiple synchronous divisions (Donnan *et al.*, 1985; John *et al.*, 1973; Morimura, 1959). Evidence has been published showing asynchronous initiation of DNA in procaryotic cyanobacterium (Binder and Chisholm, 1990) and some mutant *E. coli* (Skarstad *et al.*, 1986). The presence of three DNA equivalents would result from the replication of only one of the two nuclei formed by the initial nuclear replication. It appears that subsequently, each of the nuclei undergo another round of DNA replication resulting in a mother cell with six DNA equivalents. The mechanism of formation of cells with three and six DNA equivalents is not clear at this point and needs to be elucidated. Our observations suggest that either one of the factors present in the CM dissolving in ethanol or specific nutrient imbalance initiates this asynchronous replication under the culture conditions studied. Similar asynchronous activity was not observed in untreated CM or in components of CM dissolving in methanol.

In summary, the regulation of the cell cycle of *C. vulgaris* is altered when the cells are cultivated in the presence of components of CM obtained from high density cultures that dissolve in ethanol. Our observations show the formation of cells with three and six autospores in addition to the expected two and four, suggesting asynchronous initiation of DNA synthesis and subsequent nuclear division. This finding shows that *C. vulgaris* can deviate from the binary division cell cycle model.

Acknowledgements

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References

Binder, B. and Chisholm, S. (1990). *J. Bacteriol.* **172**, 2313–2319.

Bold, H.C. and Wynne, M.J. (1978). *Introduction to the Algae, Structure and Reproduction*, Englewood Cliffs, N.J.: Prentice-Hall.

Donnan, L., Carvill, E.P., and John, P.C.L. (1985). *New Phytology* **99**, 1–40.

Guckert, J. and Cooksey, K. (1990). *Journal of Phycology* **26**, 72–79.

Javanmardian, M. and Palsson, B.O. (1991a). *Biotechnol. Bioeng.* **38**, 1182–1189.

Javanmardian, M. and Palsson, B.O. (1991b). *Biotechnol. Bioeng.* **39**, 487–497.

John, P.C.L., McCullogh, W., Atkinson, W.J., Forbe, B.J., and Cunning, B.E.S. (1973). The cell cycle in *Chlorella*. In: *The Cell Cycle Development and Differentiation*, M. Balls and F. Billet, eds. pp. 61–76, Cambridge: Cambridge University Press.

Lee, C.-G. and Palsson, B.O. (1994). *Biotechnol. Bioeng.* **44**, 1161–1167.

Malis-Arad, S., Friedlander, R.B.-A.M., and Richmond, A. (1980). *Plant & Cell Physiol.* **21**, 27–35.

Malis-Arad, S. and McGowan, R. (1982a). *Plant & Cell Physiol.* **23**, 11–17.

Malis-Arad, S. and McGowan, R. (1982b). *Plant & Cell Physiol.* **23**, 397–401.

Mandalam, R. and Palsson, B.O. (1995). *Am. J. Botany*, **82**, 955–963.

Mandalam, R. (1994). Ph.D. Thesis, University of Michigan, Ann Arbor, MI.

Morimura, Y. (1959). *Plant & Cell Physiol.* **1**, 49–62.

Pratt, R. (1942a). *Am. J. Botany* **29**, 142–148.

Pratt, R. (1942b). *Am. J. Botany* **30**, 32–33.

Pratt, R. (1944). *Am. J. Botany* **31**, 418–421.

Pratt, R. and Fong, J. (1940). *Am. J. Botany* **27**, 431–436.

Pratt, R., Oneto, J.F., and Pratt, J. (1945). *Am. J. Botany* **32**, 405–408.

Skarstad, K., Steen, H., and Boye, E. (1986). *EMBO J.* **163**, 661–668.

Tamiya, H. (1964). Growth and cell division of *Chlorella*. In: *Synchrony in Cell Division and Growth*, E. Zeuthen, ed., pp. 247–305, New York: Interscience Publishers.

Tamiya, H., Morimura, Y., Yokota, M., and Kunieda, R. (1961). *Plant & Cell Physiol.* **2**, 383–403.

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