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EFFECT OF PRE-IMMOBILIZATION CONDITIONS ON PHOSPHATE UPTAKE BY IMMOBILIZED *CHLORELLA*

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SUMMARY

Batch culture studies of phosphate uptake by non-immobilized (free) and immobilized (Ca-alginate-entrapped) *Chlorella emersonii* have shown that exponentially growing free cells remove phosphate from the medium five times more rapidly than cells in late stationary phase. Culture age is also shown to be an important factor in determining the uptake abilities of cells when in their immobilized state. When cells of different ages are immobilized in Ca-alginate and placed in a small-scale packed-bed reactors the effects of culture age are sufficient to produce significant differences in reactor performance lasting in the order of five days.

INTRODUCTION

Immobilized microalgae (including cyanobacteria) have been the subject of considerable research interest, featuring in more than 150 publications to-date. Much of the recent literature involves the application of these cells to the removal of phosphate from waste-waters. Phosphate is of importance since it is known to be an important causal factor in eutrophication, and it is also known that algal cells are highly effective in their uptake and accumulation of this pollutant.

Most studies of phosphate removal by immobilized algae have involved batch culture systems in which uptake is evaluated by measuring the rate of loss from the surrounding medium, though there have been some studies using semi-continuous systems and small-scale continuous-flow photobioreactors (de la Noüe & Proulx, 1988; Robinson *et al.*, 1989; Megharaj *et al.*, 1992; Garbisu *et al.*, 1993; Robinson & Wilkinson, 1994).

Phosphate uptake by microalgae is known to be an active process, which is affected by both external and internal phosphate concentrations (Cembella *et al.*, 1984). Hence it would be expected that the nutritional status of the cells prior to immobilization would have a marked effect on the subsequent ability of these cells to remove phosphate once immobilized. Inspection of the literature, however, reveals that there has been little study of the effects of pre-immobilization conditions on the metabolic activity of immobilized algae, and it is thus the intention of this paper to demonstrate the importance of culture age and the nutritional status of the cells prior to immobilization in affecting the phosphate uptake ability of immobilized cells both in batch and continuous-flow systems.

MATERIALS AND METHODS

Routine Cultivation: *Chlorella emersonii* (LP8A) is an isolate held within the culture collection of the University of Central Lancashire. The strain had originally been obtained from the Culture Collection of Algae and Protozoa, Windermere as CCAP 211/8A, from which it now shows significantly different nutritional preferences. Cells were cultivated in 250 ml glass Erlenmeyer

flasks containing 100 ml Beijerinck's medium (BM) (Nichols, 1973) modified to contain 1 mM bis-Tris buffer (pH 6.5) plus 0.1% (w/v) D-glucose. Cultures were incubated in a Gallenkamp cooled illuminated orbital incubator at 25°C, shaken at 120 rpm and constantly illuminated at 70 $\mu mol/m^2.s$ PAR.

Cell enumeration: Cell number was measured using a Coulter Multisizer (Coulter Electronics, UK).

Immobilization: Ca-alginate beads, 4 mm in diameter, were formed according to the protocol of Kierstan & Bucke (1977).

Phosphate determination: The phosphate concentration of the medium surrounding free and immobilized cells was determined spectrophotometrically using the molybdate-antimony method (Golterman *et al.*, 1978). A 0.3 ml sample was used for each determination. For the assay of phosphate in the medium surrounding free cells, the cells were first removed by centrifugation in a MSE MicroCentaur microfuge at 13,000 rpm (11,600 x g) for 5 minutes.

General experimental conditions: In batch culture *growth* studies *Chlorella*, at an initial stocking density of $5x10^4$ cell/ml, was grown in BM (plus glucose). Cell number and phosphate were measured over the 30 day experimental duration, as described above.

In batch culture *uptake* studies, 100 Ca-alginate beads, each containing 10^7 cell, or an equal number of free cells were added to 100 ml sterile BM (minus glucose) in 250 ml Erlenmeyer flasks. Cultures were incubated in a Gallenkamp cooled illuminated orbital incubator at 25°C, shaken at 120 rpm and constantly illuminated at 70 $\mu mol/m^2.s$ PAR. At time intervals, samples were removed from each flask and analysed for phosphate as described above.

In continuous-flow studies, small-scale packed-bed reactors were constructed from Pharmacia C10/20 chromatography columns, 20 cm in length and 1 cm internal diameter. Pharmacia AC10 column adapters were used to connect inlet and outlet tubing to the columns. Sterile BM (minus glucose) was pumped in to the bottom of each column at a flow rate of 100 ml/day using a Gilson Minipuls III pump (Anachem). Columns were continuously illuminated using six 20W fluorescent tubes, providing an irradiance of 60 $\mu mol/m^2.s$ PAR at the surface of each column. Prior to use, the columns and all associated tubing were sterilised by pumping through 3% (v/v) sodium hypochlorite followed by sterile distilled water. Columns were filled with 100 Ca-alginate beads, with each bead containing 10^7 cell. At intervals, samples of effluent were collected from each reactor and analysed for phosphate, as described above.

RESULTS AND DISCUSSION

Growth of *Chlorella* in batch culture is shown in *figure 1*. Cultures reached stationary phase in about 10 day. Cell growth was accompanied by a depletion of phosphate from the medium (*figure 1*) suggesting that phosphate may be the limiting nutrient in the system. Phosphate was, in fact, totally depleted from the medium within 5 days, at which time cells were still in the exponential growth phase. Thus, exponential growth from day 5 onwards must have resulted from utilisation of intracellular stores. Since there was no further external phosphate available from day 5 onwards it is possible to calculate that the mean cellular phosphate content of cells at day 5 must have been 8 times higher than that of cells at day 10, and 20 times higher than those at day 30.

To test the hypothesis that phosphate depletion resulted in the cells entering stationary phase (once internal phosphate pools were depleted) cells were cultured in media containing elevated levels of phosphate. The resultant growth curves revealed that cultures did attain a significantly higher cell density when provided with elevated levels of phosphate (e.g. using day 15 data in 1-way ANOVA; $F=5.27$, $p<0.05$). This supports the hypothesis that phosphate is the limiting nutrient in the batch culture system. It is therefore reasonable to suggest that, exponentially growing cells (day 0-7) may be considered as phosphate replete, whilst stationary phase cells (day 10 onwards) may be considered as phosphate limited.

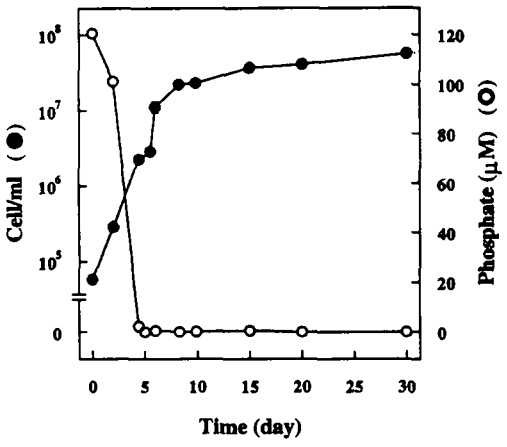

Figure 1 Cell growth (●) and phosphate concentration of medium surrounding *Chlorella* (○) growing in batch culture. (Points are means with n=6.)

Cultures Age (day)	Phosphate removal rate (μmol P/h.10^9cell)
5	15.3
10	10.4
15	8.2
20	5.7
30	2.9

Table 1 Phosphate removal rate of non-immobilized *Chlorella* of different ages. (μmol P/h.10^9cell)

Free cells were cultured for 5-30 day, after which they were harvested, washed and placed in fresh BM (minus glucose) at a stocking density of 10^7 cell/ml. Measurement of the phosphate concentration surrounding the cells revealed a virtually linear rate of removal over the first 30 min of the experiment. These rates are included as *table 1*.

These results clearly show that exponentially growing free cells (day 5) are capable of removing phosphate from the surrounding medium approximately five times faster than cells in the late stationary phase of growth (day 30). This might seem surprising since the results shown above would suggest that exponentially growing cells already had a much higher phosphate content than cells in stationary phase. However, the result likely reflects the high demand for phosphate for polynucleotide and phospholipid biosynthesis by rapidly growing (exponential phase) cells.

When cells were grown for 5 day (exponential phase) and 15 day (stationary phase) prior to immobilization in Ca-alginate, the subsequent effect on phosphate uptake ability was noticeable (*figure 2*). The mean uptake rate over the first two hours of the experiment was 3.1 μmol/h.10^9 cell for 5-day cells and 1.4 μmol/h.10^9 cell for 15-day cells. These uptake rates were noticeably slower than those observed for free cells. However, as with free cells, removal of phosphate from the medium (over the early part of the experiment) was about twice as rapid when cells were in the exponential phase of growth (5 day) prior to immobilization as when in early stationary phase (15 day). This result shows the importance of preculture condition on the ability of cells to remove phosphate once immobilized.

To show how this phenomenon would influence the performance of cells in a continuous-flow system, packed-bed reactors were stocked with cells which had been grown for 5 day (exponential phase) and 15 day (stationary phase). The performances of these reactors (*figure 3*) shows that the culture age greatly influences the operational performance of the reactors for approximately 5 days of operation. Indeed over the first few hours of operation the system packed with exponential phase cells had a 4-fold higher removal efficiency. However, after this initial period the performances of the two systems were remarkably similar over the 17 day experimental duration

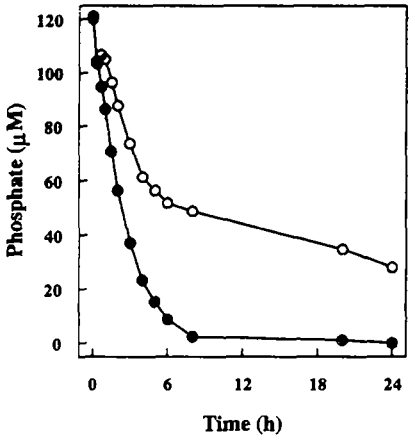

Figure 2 Removal of phosphate by 5-day old (●) and 15-day old (○) cells immobilized in Ca-alginate. (Points are means with n=3.)

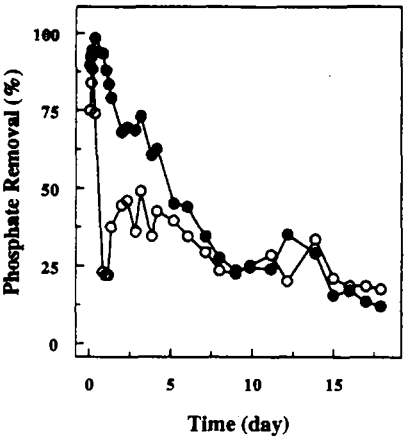

Figure 3 Phosphate removal by packed-bed reactors containing 5-day old (●) and 15-day old (○) *Chlorella*. (Points are means with n=2.)

CONCLUSIONS

Results show that even in continuous-flow studies, the pre-immobilization environment (in this case culture age and P-nutritional status) has a marked and prolonged effect on the activity of immobilized cells. Thus results from studies which have employed continuous-flow reactors for periods of only a few days (Megharaj *et al.*, 1992; Garbisu *et al.*, 1993) or weeks (Robinson *et al.*, 1989) will undoubtedly be strongly influenced by the pre-immobilization history of the cells as well as the actual operating conditions within the reactor during the experimentation.

Results also show the greatly superior phosphate uptake performance of exponentially growing algal cells. It is clear, therefore, that in immobilized algal cell systems designed to remove phosphate from waste-waters, there is a need to encourage rapid cell growth whilst fully maintaining cells in the immobilized state. This situation is likely to favour the development and application of membrane bioreactors to such processes.

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