CHAPTER 5

Exopolysaccharide production by the marine benthic diatom
Cylindrotheca closterium: effects of nutrient conditions

Abstract

During the stationary phase of a batch culture of the marine benthic diatom Cylindrotheca closterium, accumulation of exopolysaccharide and intracellular carbohydrates was observed. When nitrogen was added to the culture in the stationary phase, growth was resumed and accumulation of exopolysaccharides was delayed. This indicated that nitrogen depletion caused cessation of growth, and stimulated exopolysaccharide accumulation. Exopolysaccharide accumulation was also stimulated when cells were either resuspended in medium lacking nitrogen or phosphorous, or when they were inoculated in medium with low concentrations of nitrogen or phosphorous. Growth was not immediately affected by low nitrogen or phosphorous concentrations. Sulphur depletion only resulted in exopolysaccharide accumulation when growth was affected. Silicate or iron depletion did not stimulate exopolysaccharide accumulation, even when growth rates were lowered. Apparently, stimulation of exopolysaccharide accumulation is dependent on the type of nutrient depletion. Intracellular storage carbohydrates did not accumulate when cells were incubated at low nitrogen or phosphorous concentrations. Moreover, cells grown with ammonium as nitrogen source produced more carbohydrates (both extracellular and intracellular) than cells grown with nitrate as nitrogen source, indicating that both exopolysaccharides and intracellular carbohydrates accumulated as a result of overflow metabolism.

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Introduction

Exopolysaccharides produced by diatoms on the surface of tidal mudflats form a matrix covering the sediment (Holland et al., 1974; Dade et al., 1990; Yallop et al., 1994; Paterson, 1997). It seems that the degree of stabilization brought about by the formation of such a biofilm is related to the amount of exopolysaccharide present in the sediment (Sutherland et al., 1998). Hence, it is of interest to understand the factors that control the production of exopolysaccharides. In this chapter the effects of different nutrient conditions on exopolysaccharide accumulation by *C. closterium* are reported.

Much work has been done on the effect of nutrients on extracellular carbohydrate production by pelagic diatom species, indicating that it is influenced by growth rate and type of nutrient depletion (Myklestad & Haug, 1972; Myklestad, 1977; Waite et al., 1995). However, little is known with respect to the effect of nutrient conditions on polysaccharide secretion by benthic diatoms. It is generally assumed that growth of benthic diatoms is not limited by nutrients, because nutrient concentrations in pore water are generally high (Cadée & Hegemann, 1974; Admiraal et al., 1982; Sundby et al., 1992). However, in the thin layer of diatoms on the sediment surface biomass may be highly concentrated, and therefore nutrients may temporarily become depleted (Admiraal, 1977; Flothmann & Werner, 1992). Indeed, evidence is accumulating that at high population densities, growth of benthic diatoms may become nutrient limited, especially by nitrogen (Sullivan & Daiber, 1975; Van Raalte et al., 1976; Hillebrand & Sommer, 1997).

The central question of the experiments was whether exopolysaccharide accumulation was affected by nutrient conditions. When benthic diatoms are grown in batch culture, polysaccharide secretion is enhanced during the transition towards stationary growth (Bhosle et al., 1995; Sutherland et al., 1998; chapter 4 this thesis). It was therefore of interest to know which factor induced stationary growth and exopolysaccharide accumulation in cultures of *C. closterium*. Another question to be solved was, whether the type of nutrient depletion influenced polysaccharide secretion. Finally, we investigated the effect of different inorganic nitrogen sources (ammonium or nitrate) on polysaccharide secretion.
Materials and methods

Organism and culture conditions
Cultivation conditions of C. closterium were as described in chapter 4.

Experimental design
In experiments in which light or nutrient pulses were given to cultures in early stationary phase, samples were taken in triplicate from a single culture. Reproducibility was ensured by repeating each experiment independently.

One experiment to test the effect of different nutrient depletions consisted of growing cells in complete medium for 7 days, followed by resuspension in medium devoid of either Fe, Si, P, S or N. Resuspension in complete medium served as a control. Cells that were resuspended in medium without Si were grown in polycarbonate vessels, and no sea sand was used. After 4 days of incubation in depleted medium, part of the culture was harvested and analyzed and the remainder was resuspended in fresh medium (depleted of the same inorganic nutrient). After another 4 days of incubation cultures were analyzed. In addition, cell density was measured 2 days after each resuspension. Samples were taken in triplicate from two independent cultures for each treatment.

In another experiment cells were inoculated in medium with low concentrations of N (95.5 μM NH₄Cl) or P (7.7 μM NaH₂PO₄ * H₂O). In the control the regular medium concentrations were used, which were 50 μM P and 0.5 mM N. Samples were taken in triplicate from a single culture.

The experiment in which cells were grown with either NH₄⁺ (0.5 mM) or NO₃⁻ (5.9 mM) as N source, samples were taken in triplicate from three independent cultures.

Isolation of exopolysaccharides
The isolation of exopolysaccharides is described in chapter 4. For total exopolysaccharide amounts, the sum of the amounts in the medium and water extracts was taken.

Analyses
Analysis of cell density, cell protein, exopolysaccharide and intracellular carbohydrate are described in chapter 4. Soluble reactive phosphorous was analyzed using the method of Murphy & Riley (1962). NH₄⁺ was analyzed by the method of Kempers & Kok (1989).
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Results

In order to assess whether stationary growth of *C. closterium* was induced by shortage of light or by nutrient depletion, a culture entering the stationary phase was divided into three portions. One portion was incubated at a photon irradiance of 100 \( \mu \text{mol phot} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \), another portion received a pulse of nutrients yielding the original medium concentration of day 0, and the third portion served as a control and was left untreated. In the culture to which the concentrated mixture of nutrients was added at the beginning of the stationary phase, growth was resumed and continued for approximately 3 more days (Fig. 5.1a). Protein content of the cells was comparable in all treatments (Fig. 5.1b). Incubation at a higher photon flux density (100 \( \mu \text{mol phot} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \)) had no effect on growth compared to the control (60 \( \mu \text{mol phot} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \)) (Fig. 5.1a). Three days after addition of the nutrient mixture, the culture entered the stationary phase again. There was a lag in exopolysaccharide accumulation in the nutrient treated culture compared to the control (Fig. 5.1c). The nutrient enriched culture started to accumulate exopolysaccharides after day 11, which coincided with the onset of stationary phase. Intracellular carbohydrates also accumulated (Fig. 5.1d), but at a lower rate than exopolysaccharides.

Nitrogen in the medium was supplied in the form of NH\(_4^+\), and therefore it was supposed that an increase in pH might cause a rapid decrease in NH\(_4^+\) concentration caused by dissociation into NH\(_3\) gas. In order to verify whether stationary phase was induced by N depletion, a culture entering the stationary phase was divided into two portions. One portion was given a pulse of NH\(_4\)Cl (final concentration: 0.5 mM), the other portion served as a control and was not treated. The onset of stationary phase in the control culture coincided with depletion of NH\(_4^+\) (Figs. 5.2a and b). The addition of NH\(_4^+\) caused an increase in biomass compared to the control (Fig. 5.2a), whereas cell protein content was similar to the control (Fig. 5.2c).

One set of experiments aimed at obtaining cells starved for a specific nutrient. Cells were grown in complete medium to a high density. Subsequently, they were resuspended in medium devoid of a particular nutrient. Table 5.1 shows the growth rates that were obtained when cells were resuspended in medium without Si, S, P, Fe or N. After the first resuspension, none of the treatments exhibited a lower growth rate than the control (resuspended in complete medium). However, when the resuspension step was repeated, all treatments led to substantial lower growth rates than the control (Table 5.1). Cells transferred to medium without P or N showed much higher rates of exopolysaccharide accumulation than the control.
effects of nutrient conditions

Fig. 5.1. Protein concentration (a), protein content of the cells (b), exopolysaccharides (c) and intracellular carbohydrates (d) in batch cultures of *C. closterium*, with addition of concentrated medium at day 7 (▲), incubation at a PFD of 100 μmol photons m$^{-2}$ s$^{-1}$ at day 7 (●) and a control (no additions of nutrients, PFD: 60 μmol photons m$^{-2}$ s$^{-1}$) (●). Mean ± SD of three samples from a culture.
Fig. 5.2. Protein concentration (a), NH$_4^+$ concentration (b) and protein content of the cells (c) in batch cultures of *C. closterium*, with addition of NH$_4^+$ on day 10 (●) and without additions (●●). Mean ± SD of three samples from a culture.
effects of nutrient conditions

Table 5.1. Growth rates ($d^{-1}$) of *C. closterium* when resuspended in medium devoid of a specific nutrient

<table>
<thead>
<tr>
<th>nutrient depletion</th>
<th>4 day starvation</th>
<th>8 day starvation</th>
</tr>
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<tbody>
<tr>
<td>repleted</td>
<td>0.36</td>
<td>0.36</td>
</tr>
<tr>
<td>SiO₃⁻</td>
<td>0.43</td>
<td>0.26</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>0.38</td>
<td>0.17</td>
</tr>
<tr>
<td>PO₄³⁻</td>
<td>0.33</td>
<td>0.24</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>0.40</td>
<td>0.28</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>0.35</td>
<td>0.14</td>
</tr>
</tbody>
</table>

* For experimental design: see text Materials and methods

(Fig. 5.3a). In all treatments rates of intracellular carbohydrate accumulation were slightly negative after the first transfer.

When the transfer to nutrient depleted medium was repeated, exopolysaccharide accumulation rates were higher than after the first transfer in all treatments except in the N depleted medium (Fig. 5.3b). Compared to the control, exopolysaccharide accumulation rates were higher in cells grown without S or P. In contrast, Si, Fe and N depletion did not exhibit elevated exopolysaccharide accumulation rates compared to the control. In all cases rates of intracellular carbohydrate accumulation were higher than after the first transfer.

In another set of experiments cultures were grown at low starting concentrations of N or P. Initially, increases in cell density were comparable in all cultures (Fig. 5.4a). After 5 days, growth in cultures with low concentrations of N and P slowed down compared to the control. This coincided with the depletion of N or P (Figs. 5.4d and e). During the first 5 days, exopolysaccharide content in the cultures grown at low N or P concentrations had increased markedly. This was not the case in the control culture, in which exopolysaccharide content remained constant (Fig. 5.4b). The control culture maintained a high growth rate until day 16, and no increase in exopolysaccharides was observed during that period (Figs. 5.4a and b). In the "low N" culture exopolysaccharide levels remained more or less constant from day 7 onwards. In the "low P" culture, exopolysaccharide content decreased after 9 days. Intracellular carbohydrates showed a different pattern from exopolysaccharides (Fig. 5.4c). In all cultures, intracellular carbohydrate content initially remained constant. In the "low N" and "low P" cultures the content of intracellular carbohydrate started to increase after approximately 7 days, whereas in the control
Fig. 5.3. Accumulation rates of exopolysaccharides (white bars) and intracellular carbohydrates (black bars) of *C. closterium* when grown in medium devoid of Si, S, P, Fe or N. After 7 days of growth in complete medium, cells were transferred to medium lacking one of the nutrients and analyzed after 4 days of incubation (a), followed by a second transfer to fresh nutrient depleted medium and analysis after 4 days (b). Note difference in scale. Mean ± SD of duplicate cultures.

culture it remained low. As was observed for exopolysaccharides, intracellular carbohydrate content in the "low N" culture was higher than in the "low P" culture (Fig. 5.4c).

Cultures grown with NH$_4^+$ exhibited a slightly higher growth rate than cultures grown with NO$_3^-$ as N source (Table 5.2). Because growth rates were high and cells were not nutrient depleted, absolute rates of exopolysaccharide and intracellular carbohydrate accumulation were relatively low. Both on a cell and on a protein
Fig. 5.4. Cell density (a), exopolysaccharides (b), intracellular carbohydrates (c), 
NH$_4^+$ concentration (d) and soluble reactive phosphorous concentration (SRP) (e) 
in batch cultures of *C. closterium*, with low starting concentrations of N (●), low 
starting concentrations of P (▲) and regular medium (★). Mean ± SD of three 
samples from a culture.
Table 5.2. Growth rates and rates of accumulation of exopolysaccharide (EPS) and intracellular carbohydrates (ICH) of *C. closterium* calculated over 7 days of exponential growth with NO$_3^-$ or NH$_4^+$. Mean of 3 replicate cultures, standard deviation between brackets. EPS and ICH accumulation rates in µg (10$^6$ cells * day)$^{-1}$ or µg (mg protein * day)$^{-1}$

<table>
<thead>
<tr>
<th></th>
<th>biomass correction: cell number</th>
<th>biomass correction: protein concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO$_3^-$</td>
<td>NH$_4^+$</td>
</tr>
<tr>
<td>growth rate (d$^{-1}$)</td>
<td>0.43 (0.02)</td>
<td>0.46 (0.02)</td>
</tr>
<tr>
<td>EPS accumulation rate</td>
<td>0.14 (0.02)</td>
<td>0.27 (0.03)</td>
</tr>
<tr>
<td>ICH accumulation rate</td>
<td>0.28 (0.02)</td>
<td>0.55 (0.09)</td>
</tr>
</tbody>
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The cultures grown with NH$_4^+$ accumulated more exopolysaccharides and intracellular carbohydrates than the NO$_3^-$ grown cultures. Because protein content after 7 days was higher in NH$_4^+$ grown cells (38.5 ± 4.0 µg (10$^6$ cells)$^{-1}$) than in NO$_3^-$ grown cells (28.0 ± 3.6 µg (10$^6$ cells)$^{-1}$), the differences in carbohydrate content were smaller when expressed on a protein basis than when expressed on a cell basis. The N source did not have any effect on the ratio exopolysaccharide/intracellular carbohydrate, which was 0.5 at day 7 in both NH$_4^+$ and NO$_3^-$ grown cells.

**Discussion**

In chapter 4 it was demonstrated that exopolysaccharides in cultures of *C. closterium* mainly accumulated during the transition from linear to stationary growth. From the results presented in this chapter it is concluded that the cultures entered stationary growth because of nitrogen depletion, and that this stimulated exopolysaccharide accumulation. Also when growth ceased due to sulphur or phosphorous depletion, exopolysaccharide accumulation was enhanced. In contrast, silicate or iron depletion did not stimulate exopolysaccharide accumulation, even though growth rates under phosphorous, silicate and iron depleted conditions were comparable. This indicated that polysaccharide secretion depended on the nature of the nutrient depletion. High rates of exopolysaccharide accumulation under phosphorous limitation were also reported for other diatom...
species, such as *Navicula pelliculosa* (Lewin, 1955), *Chaetoceros affinis* (Myklestad, 1977) and several other planktonic diatom species (Waite *et al.*, 1995).

After the first transfer to depleted medium, exopolysaccharide accumulation rates were high when cells were resuspended in medium without nitrogen or phosphorous, but growth was not affected. This suggested that cells reacted to low ambient concentrations of nitrogen or phosphorous by secreting polysaccharides. This was further confirmed by growing cultures in media with low initial concentrations of nitrogen or phosphorous. Exopolysaccharide content had already markedly increased during the first 5 days of incubation in the "low nitrogen" and "low phosphorous" cultures, but not in the control. During this period growth was not affected by the low concentrations of nitrogen or phosphorous. In the control culture, cell density continued to increase until day 16, while concentrations of nitrogen and phosphorous were already very low after 10 and 7 days, respectively. The increase in cell density may have been caused by a decrease in cell size, to which *C. closterium* was subject during long term incubations (chapter 4). This would also explain why no stimulation of exopolysaccharide accumulation was observed in the control treatment. Of the nutrients studied, only low nitrogen and phosphorous concentrations caused stimulation of exopolysaccharide accumulation without affecting growth, since sulphur-, silicate- and iron-depleted cultures initially exhibited high growth rates but produced little exopolysaccharide. Similar results have been obtained with the green alga *Micrasterias radiata*, which produced more extracellular fibrils when phosphorous concentration in the medium was low, while growth was not limited (Strycek *et al.*, 1992). Similarly, *Eremosphera sp.* was able to grow faster at low than at high concentrations of phosphorous in the medium, while producing more extracellular fibrils (Strycek *et al.*, 1992).

The accumulation of exopolysaccharides as observed upon transfer to nitrogen- or phosphorous-depleted medium was not accompanied by accumulation of intracellular carbohydrate. This was confirmed by the experiment where cells were inoculated with low nitrogen or phosphorous concentrations, in which intracellular carbohydrate content remained constant during the first 5 days of incubation, when exopolysaccharide accumulation rates were high. Apparently, the induction of polysaccharide secretion by low ambient nitrogen or phosphorous concentrations did not result in intracellular carbohydrate accumulation, and intracellular storage and secretion were subject to different controlling mechanisms.

It remained unclear why polysaccharide secretion was stimulated at low nitrogen or phosphorous concentrations but not at low iron or silicate concentrations. The ecological significance of the stimulation of polysaccharide secretion to low ambient nitrogen or phosphorous concentrations may be that exopolysaccharides
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could function as polyelectrolytes that bind organic and inorganic nutrients (Admiraal & Werner, 1983; Armstrong & Bärlocher, 1989; Decho, 1990). Also, exopolysaccharides may function as sites where phosphatases could be active (Burkholder et al., 1990). Furthermore, it has been suggested that the oxygenation of the sediment that accompanies exopolysaccharide production is important (Sutherland et al., 1998). The depth to which the sediment is oxygenated influences both nitrification/denitrification processes (Wiltshire, 1992) and sorption/desorption of phosphorous to sediment particles (Sundby et al., 1992), thereby influencing availability of both nitrogen and phosphorus.

Exopolysaccharide secretion may also be the result of overflow metabolism. This became apparent when cultures were compared that were grown with either ammonium or nitrate as nitrogen source. With ammonium as nitrogen source, more electrons are available for reduction of carbon dioxide than with nitrate as nitrogen source, which may result in a higher carbohydrate production rate (Turpin, 1991; Smith et al., 1992). However, growth on ammonium resulted in a higher protein content of the cells compared with nitrate grown cells. Therefore, only when expressed on a cell basis differences in carbohydrate accumulation rates reflected differences in electron flow. The two-fold difference in carbohydrate accumulation rates between ammonium and nitrate grown cells indicated that supplying ammonium as nitrogen source had a considerable impact on carbohydrate accumulation rate. The fact that the ratio exopolysaccharide/intracellular carbohydrate remained unaltered with either ammonium or nitrate as nitrogen source suggested that both exopolysaccharides and intracellular carbohydrates were produced as a result of overflow metabolism. In natural populations overflow metabolism (induced by nitrogen limitation) may be a steering factor for exopolysaccharide production, as was shown by Ruddy et al. (1998a).

In summary, in batch cultures of *C. closterium* exopolysaccharide accumulation was enhanced during stationary phase induced by nitrogen depletion. However, depletion of other nutrients such as silicate or iron did not lead to enhanced accumulation of exopolysaccharides. In addition, exopolysaccharide accumulation was enhanced at low, but not growth limiting, concentrations of nitrogen or phosphorus, respectively. Furthermore, accumulation of both exopolysaccharides and intracellular carbohydrates were subject to overflow metabolism.