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Tight control of growth and cell differentiation in photoautotrophically growing moss (*Physcomitrella patens*) bioreactor cultures

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Abstract The use of the moss *Physcomitrella patens* as a production system for heterologous proteins requires highly standardised culture conditions. For this purpose a semi-continuous photoautotrophic bioreactor culture of *Physcomitrella* was established. This culture grew stably for 7 weeks in a 5-l bioreactor with a dilution rate of 0.22/day. Enrichment of the air for aeration in a batch bioreactor culture with 2% (v/v) CO_2 resulted in an increase in the specific growth rate to 0.57/day. Changes in the pH of the semi-continuous bioreactor culture medium between pH 4.5 and pH 7.0 influenced protonema differentiation; however it did not negatively affect the growth rate compared to uncontrolled pH. The advantages of *Physcomitrella* as a system for the production of heterologous proteins in plants are discussed.

Keywords CO_2 enrichment · pH control · Photoautotrophic culture · Plant cell suspension · Semi-continuous bioreactor culture

Abbreviations *DAPI*: 4,6-Diamidino-2-phenylindole · t_d: Doubling time (in days), *v/v*: Volume/volume (%) · *vvm*: (Aeration volume)/(medium volume) per minute

Introduction

The moss *Physcomitrella patens* (Hedw.) B.S.G. is receiving growing interest as a model system in plant genomics (Reski 1998, 1999; Schaefer 2001). Moreover, *Physcomitrella* can be used as a system for the production of heterologous proteins (Reutter und Reski 1996). The general advantages of plant tissue cultures over microbial cell culture for foreign protein production include eukaryotic post-translational modification and the exclusion of contamination with bacterial toxins. In addition, there is no risk of contamination with mammalian pathogens as could occur for animal cell culture (Doran 2000). Furthermore, in vitro cultures of *Physcomitrella* grow photoautotrophically in a simple inorganic modified Knop medium (Reski and Abel 1985), which is cheap and facilitates the isolation of the desired protein. *Physcomitrella* grows in suspension culture as a fully differentiated plant, mainly as filamentous protonema, which is the juvenile gametophyte. Both of the protonema cell types develop in liquid culture: chloronema cells with a high number of chloroplasts and right-angled cell walls as well as caulonema cells, which are characterised by a reduced number of chloroplasts and oblique cell walls (Reski 1998). Because fully differentiated plants are grown, the risk of somaclonal variation – frequently reported for callus and suspension cultures of higher plants – is reduced (Karp 1991).

In this context bioreactor cultures of *Physcomitrella* are necessary for scale-up and for the exact control of culture conditions in order to study the effect of environmental parameters on growth, differentiation and – in the case of a transgenic genotype – product formation. Although there are a few reports on the growth of *Physcomitrella* in bioreactors (Boyd et al. 1988; Reutter and Reski 1996; Cove et al. 1997), comprehensive and systematic studies on bioreactor culture of *Physcomitrella* are lacking to date. Thus, as a prerequisite for the use of *Physcomitrella* for the production of heterologous proteins, we developed a semi-continuous, long-term bioreactor culture and studied the effect of CO_2 enrichment and pH control on suspension cultures of the *Physcomitrella* wild type.

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Materials and methods

Suspension cultures of *Physcomitrella patens* were grown in modified Knop medium containing 1,000 mg/l $Ca(NO_3)_2 \times 4H_2O$, 250 mg/l KCl, 250 mg/l KH_2PO_4, 250 mg/l $MgSO_4 \times 7H_2O$ and 12.5 mg/l $FeSO_4 \times 7H_2O$, pH 5.8 before autoclaving, as described by Reski and Abel (1985). Where mentioned, this medium was supplemented with 460 mg/l ammonium tartrate.

Bioreactor cultures were carried out in stirred tank glass bioreactors (Applikon, Schiedam, The Netherlands) with working volumes of 5 l or 10 l, respectively, equipped with a marine impeller running at 500 rpm (5-l vessel) or 400 rpm (10-l vessel). The cultures were aerated with either 0.3 vvm air or with air enriched with 2% CO_2 (v/v) and grown at 25°C under a photoperiod regime of 16/8 h (light/dark) with light supplied by Philips TLD 25 lamps at an intensity at the surface of the vessels of either 120 µmol/m^2 per second (5-l vessels) or 190 µmol/m^2 per second (10-l vessels). The pH of the bioreactor cultures was determined off-line and on-line; for the off-line measurements three samples were taken at one time. In the case of pH control, either 0.5 *N* HCl or 0.5 *N* KOH was automatically titrated using an ADI 1030 control device (Applikon, Schiedam, The Netherlands).

If the cultures were run semi-continuously, the suspension was harvested and replaced by an equal amount of fresh medium daily. The density of the cultures was controlled by determining the dry weight, and the daily dilution was calculated in order to maintain an average dry weight of 150 mg/l. The dry weight of the cultures was determined by drying the cell material of two 50-ml samples at 105°C for 2 h.

The specific growth rate, µ (per day), was calculated from the dry weight data by fitting an exponential function to the exponential section of the growth curve (Microsoft Excel). From the specific growth rate the doubling time was obtained according to the function $t_d = \ln 2 \, \mu^{-1}$.

Comparisons of different treatments were always carried out in parallel bioreactor runs using an identical inoculum. The reproducibility of all data was confirmed in independent replications of the experiments.

For flow cytometric analysis, the cell material of three 10-ml samples was chopped with a razor blade in 2.5 ml DAPI solution containing 0.01 mg/l DAPI, 1.07 g/l $MgCl_2 \times 6H_2O$, 5 g/l NaCl, 21.11 g/l Tris and 1 ml/l Triton (pH 7.0). After 5 min the material was filtered through a 30-µm sieve in order to remove cell debris. The analysis was performed with a Partec PAS flow cytometer (Münster, Germany) equipped with a high-pressure mercury lamp. In each sample at least 5,000 particles were counted.

Results

A semi-continuous bioreactor culture (5-l working volume) in modified Knop medium supplemented with 460 mg/l ammonium tartrate was run for 49 days (Fig. 1). Starting on day 4, on average 1.1 l of the culture was harvested per day, and except for Mondays (following the weekend) the dry weight of the culture could be maintained between approximately 150 mg/l and 200 mg/l dry weight by diluting the culture on average at a rate of 0.22/day. Due to the addition of ammonium tartrate the culture grew as a pure chloronema culture, and no further differentiation (development of caulonema or gametophores) occurred.

Within the total culture period of 49 days 51 l of suspension was harvested, and the total yield of cell material was 9.1 g (dry weight).

As the medium used did not contain any carbohydrates, the cultures were growing strictly photoautotrophically. Therefore, the effect of supplementing the cultures with additional CO_2 was investigated in the batch cultures of the 10-l bioreactors, which were either aerated with air (control) or with air supplemented with 2% CO_2. In order to avoid differentiation of the plant material, the medium was supplemented with 460 mg/l ammonium tartrate. The bioreactors were run for 10 days (Fig. 2). Within this time period, the control bioreactor reached a maximum culture density of 266 mg/l dry weight, while the culture aerated with air supplemented with 2% CO_2 reached the stationary phase with a maximum dry weight of 685 mg/l on day 9. The specific growth rate determined during the exponential growth phase was 0.25/day (t_d=2.7) for the control bioreactor. Following supplementation of the air with 2% CO_2 the growth rate increased markedly to 0.57/day (t_d=1.2).

The pH of the suspension cultures decreased from 5.8 to 4.1 in the bioreactor aerated with air and from 5.8 to 3.4 in the culture aerated with additional CO_2.

In order to determine the effect of pH on the growth of the *Physcomitrella* bioreactor cultures, we inoculated

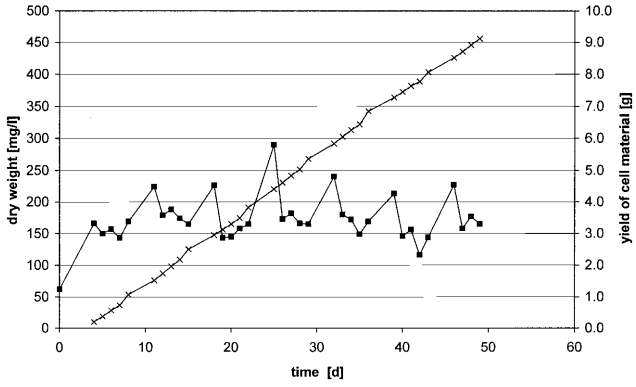

Fig. 1 Semi-continuous culture of *Physcomitrella* in a 5-l bioreactor: dry weight (*black square*) and cumulated cell yield (×) summed up over the total culture period. Average dilution rate during semi-continuous culture mode: 0.22/day

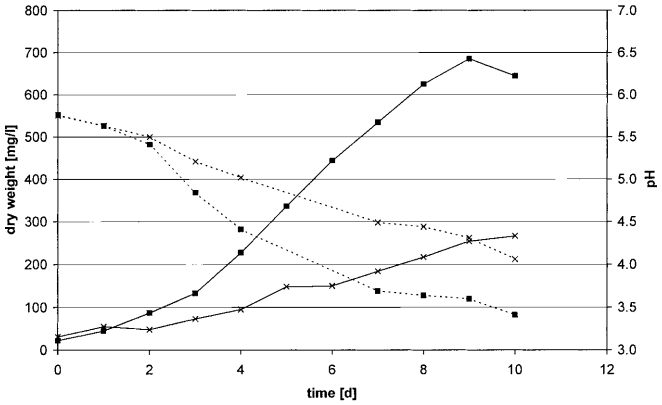

Fig. 2 Batch bioreactor cultures of *Physcomitrella* aerated with air (×) or with air that was enriched with 2% (v/v) CO_2 (*black square*). Growth curves (*solid line*) and pH (*broken line*) were measured from parallel cultures in 10-l bioreactors

three 5-l bioreactors in parallel: one bioreactor without pH control, resulting in a pH of 5.8±0.2 (mean ± standard deviation), and two bioreactors with pH control, with a setpoint of pH 4.5 and pH 7.0, respectively. In this experiment the medium was not supplemented with ammonium tartrate in order to avoid an effect of ammonium nutrition on the pH of the suspension, as shown in Fig. 2. The cultures were run semi-continuously as described above for 21 days (Fig. 3). During the first 3 days – before harvesting began – the suspension with a pH of 7.0 was growing faster (μ=0.41/day, t_d=1.7 day) compared to cultures growing in a medium with a pH of 4.5 (μ=0.25/day, t_d=2.8 day) or no pH control (μ=0.23/day, t_d=3.0 day). As for the semi-continuous bioreactor run in medium supplemented with ammonium tartrate (Fig. 1), in this experiment also the average dry weight of the cultures could be maintained between approximately 100 mg/l and 200 mg/l (Fig. 3). In contrast to the differences in the growth rates at the beginning of the culture, the average dilution rate during the semi-continuous culture was nearly equal for all three bioreactors (0.18–0.2/day), although the dry weight of the culture growing at pH 7.0 was always slightly higher than those growing at a lower pH. Correspondingly, the overall biomass yield (dry weight) was marginally higher for the culture at pH 7.0 (2.7 g) than that at pH 4.5 (2.4 g) and the uncontrolled pH (2.3 g).

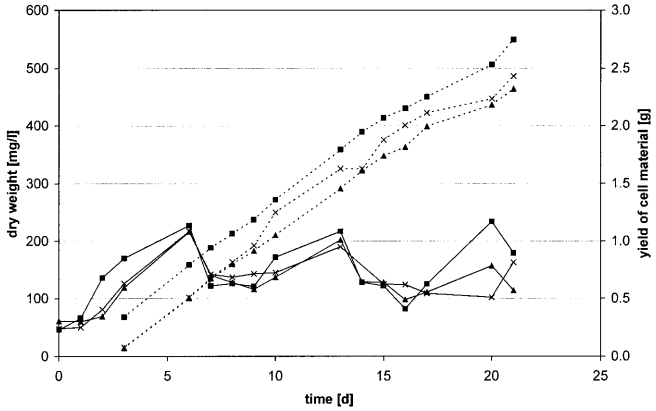

Fig. 3 Semi-continuous bioreactor cultures of *Physcomitrella* with pH control with setpoints of pH 4.5 (×) and pH 7.0 (*black square*), respectively, in comparison to a culture without pH control (*black triangle*), resulting in an average pH of 5.8. Dry weight (*solid line*) and cumulated cell yield (*broken line*) were measured from parallel cultures in 5-l bioreactors

As no ammonium tartrate was added to the media in this experiment, differentiation of the plant material was not blocked at the chloronema stage – i.e. filamentous cells with a large number of chloroplasts and right-angled cell walls – and caulonema developed as well, which is characterised by oblique cell walls and a reduced number of chloroplasts. However, caulonema differentiation was more pronounced at pH 7.0 and in cultures with an uncontrolled pH than at pH 4.5 (Fig. 4). Flow cytometric analysis of the cultures confirmed the differences in differentiation (Fig. 5): The culture grown in the bioreactor without pH control and that grown at pH 7.0, respectively, showed a clear 1C peak that corresponded to caulonema cells; these cells are mainly found in the G1 phase of the cell cycle (Reski 1998). In contrast, cultures grown at pH 4.5 only showed one single

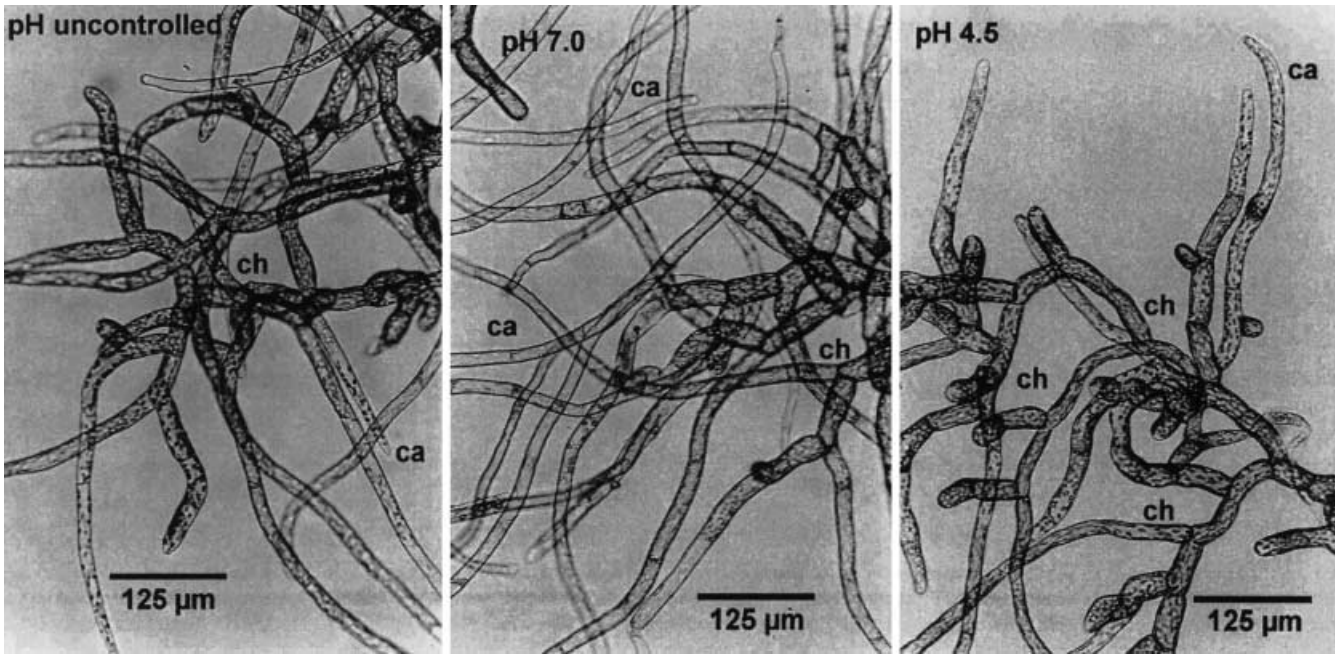

Fig. 4 Microscopic views of cell material from semi-continuous bioreactor cultures of *Physcomitrella* without pH control, resulting in an average pH of 5.8, in comparison to cultures grown at pH 7.0 and pH 4.5, respectively. Without pH control and at pH 7.0 chloronema (*ch*) and caulonema (*ca*) developed; at pH 4.5 caulonema development was impeded

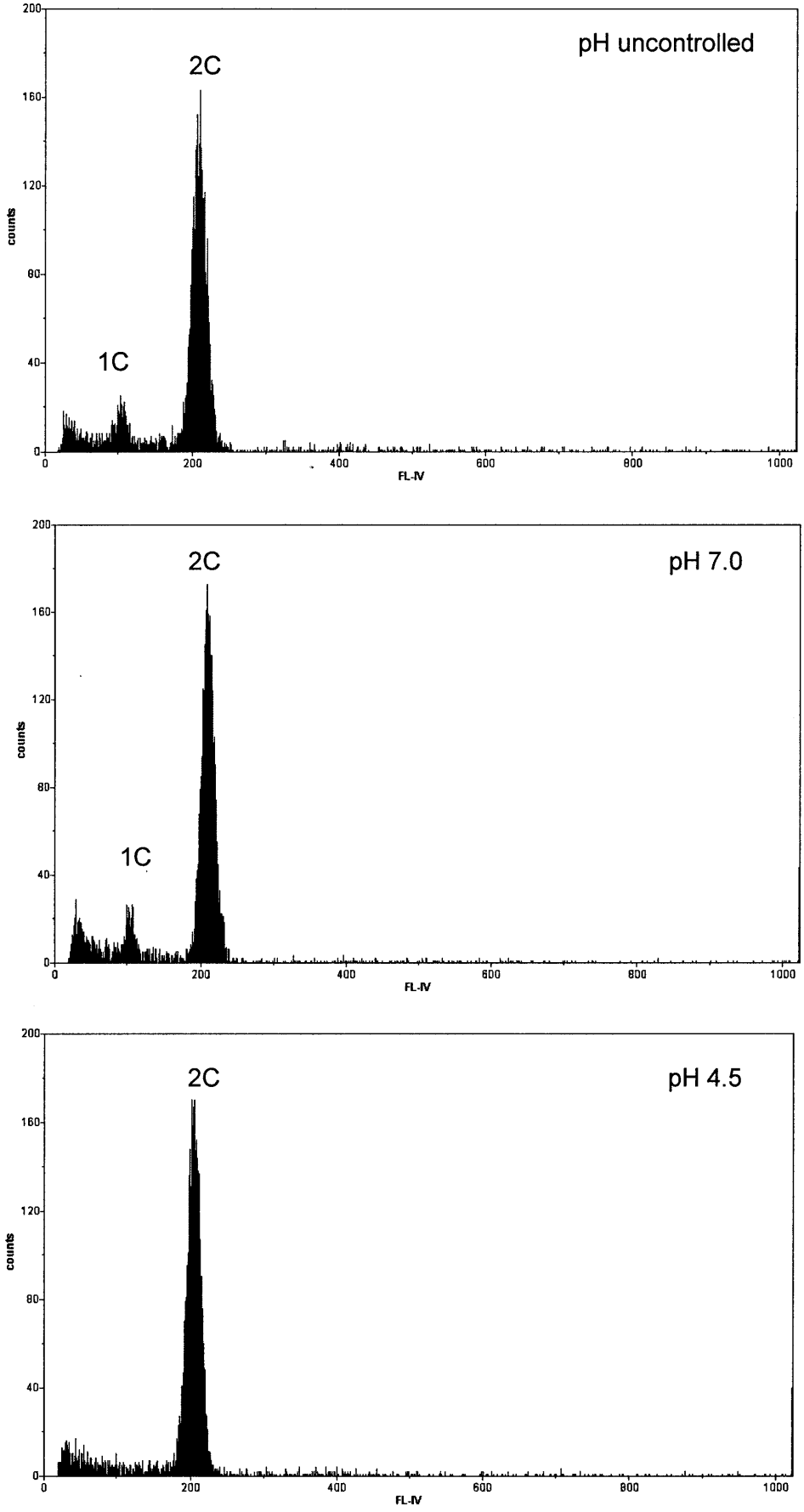

Fig. 5 Flow cytometric analysis of cells from semi-continuous bioreactor cultures of *Physcomitrella* with pH control (pH 4.5 or 7.0) in comparison to a culture without pH control. The histograms from cultures growing without pH control or at pH 7.0 show 1 C and 2 C peaks corresponding to caulonema and chloronema cells, whereas the histogram from the culture growing at pH 4.5 only shows a 2 C peak that corresponds to chloronema cells

2C peak that corresponded to chloronema cells; these cells remain predominantly in G2.

All results were confirmed in at least one independent replication of the experiments.

Discussion

For the production of any compound in cell culture it is desirable to establish a long-term growth system – preferably under steady-state conditions – which ensures uniform growth conditions and avoids labour-intensive frequent bioreactor set-up. Earlier bioreactor cultures of *Physcomitrella* were either batch cultures (Reutter and Reski 1996) or only short continuous cultures (15 days, Boyd et al. 1988). We have succeeded in establishing and maintaining a stably growing semi-continuous bioreactor culture of *Physcomitrella* for 7 weeks (Fig. 1). As the average dry weight of the culture did not change markedly throughout the culture period, steady-state conditions could be assumed. Thus, the dilution rate (0.22/day) equalled the specific growth rate. This growth rate was nearly identical to the one determined in the exponential growth phase of a batch culture (0.25/day, Fig. 2), indicating that in the semi-continuous culture the maximum growth rate of the system was realised.

Ashton and Cove (1977) and Jenkins and Cove (1983) described the arrest of *Physcomitrella* in the chloronema stage following the addition of ammonium tartrate. In our experiments also, the use of ammonium tartrate as a medium supplement in bioreactor culture resulted in a homogenous chloronema suspension. Thus, in contrast to the cultures described by Boyd et al. (1988) and Reutter and Reski (1996), no further differentiation of the plant material occurred, which can limit the stability and duration of a long-term culture.

When the aeration gas was supplemented with 2% CO_2 (v/v), the growth rate of the cultures doubled (Fig. 2). The observed decrease in the pH of these cultures was probably due to ammonium uptake, as it was never found in culture media that had not been supplemented with ammonium tartrate (data not shown). Thus, the more pronounced decrease in the pH of the CO_2-enriched culture probably reflects a higher ammonium uptake due to the higher growth rate. Although Boyd et al. (1988) also enriched the air of their bioreactor culture with additional CO_2, they did not carry out parallel bioreactor runs and growth kinetics. The addition of 1–5% CO_2 (v/v) is also used for the growth enhancement of photoautotrophic suspension cultures of higher plants (e.g. Hüsemann 1985; Widholm 1992; Fischer and Alfermann 1995). However, the growth rate of the CO_2-enriched culture shown in Fig. 2 (0.57/day, corresponding to a doubling time of 1.2 days) is very high in comparison with the doubling times of 2–3 days of most suspension cultures of higher plants (Scragg 1995).

pH control or buffered media are not commonly used in plant cell cultures: routinely, the pH of the medium is adjusted to 5.2–5.8 before autoclaving, and characteristic pH shifts are allowed to occur throughout the course of the culture (Minocha 1987). In controlling the pH in the semi-continuous bioreactor cultures of *Physcomitrella* we were able to slightly influence differentiation of the cultures – if differentiation was not blocked by the addition of ammonium tartrate. Whereas at pH 4.5 the culture was mainly growing as chloronema, at pH 5.8 (not controlled) and pH 7.0 caulonema also developed (Figs. 4, 5). As the transition from chloronema to caulonema is triggered by auxin and given that more than 90% of the total auxin of a *Physcomitrella* suspension culture accumulates in the medium (Reutter et al. 1998; Reski 1998), the pH of the culture medium might well interfere with this differentiation process by affecting the excretion, stability in the medium or uptake of auxin. Effects of the pH of the medium – especially on auxin uptake – are well known for higher plants (Minocha 1987).

These deviations in culture differentiation did not negatively affect the growth rate of the cultures compared to a culture without pH control (Fig. 3). Thus, *Physcomitrella* cultures may be grown in a very broad range of pH – from 4.5 to 7.0. This insensitivity offers the possibility of adjusting the pH of a culture of transgenic *Physcomitrella* producing a heterologous protein (as described by Reutter and Reski 1996) according to the requirements of the production of this specific compound, which might be especially important if it is excreted into the medium.

Moreover, in contrast to most other plant cell cultures, *Physcomitrella* suspension cultures must be extremely robust against hydrodynamic forces, as the cultures were stirred at a speed of 400–500 rpm. Susceptibility to shear stress is a major constraint in bioreactor cultures of higher plants (e g. Doran 1999; Sajc et al. 2000). The tolerance of *Physcomitrella* cultures allows the use of standard stirred tank bioreactors; there is no need for special bioreactor or stirrer design in order to reduce shear stress.

In summary, compared to most higher plant cell cultures, *Physcomitrella* is a very robust, fast and stably growing bioreactor culture. Thus, *Physcomitrella* bioreactor cultures are very well suited as a system for the production of heterologous proteins in plant tissue culture.

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