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Optimisation of a bioreactor culture of the moss *Physcomitrella patens* for mass production of protoplasts

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Abstract

One prerequisite for plant functional genomic projects is the development of a high-throughput transformation platform. As the moss *Physcomitrella patens* can be transformed via PEG-mediated DNA-uptake into protoplasts, a semi-continuous bioreactor culture of this plant was optimised regarding protoplast isolation efficiencies. Under standard conditions protoplast yields were 2.8×10^4/mg dry weight. This yield was increased sixfold by supplementation of the medium with 460 mg/l ammonium tartrate. The same effect was achieved by controlling the pH-value in the bioreactor culture with a setpoint of 4.5. In contrast, pH control with a setpoint of 7.5 reduced the protoplast yield compared to a culture without pH control to 11%. A semi-continuous culture of Physcomitrella in a 5-l bioreactor grown at pH 4.5 yielded sufficient cell material for more than 100 transformations per day.

Keywords: Ammonium tartrate; pH Control; Protoplast isolation; Protoplast yield; Suspension culture

1. Introduction

The moss *Physcomitrella patens* (Hedw.) B.S.G. is of growing interest as a model system in plant functional genomics as it is the only plant showing high rates of homologous recombination in its nuclear DNA, which allows the analysis of gene functions by targeted knock-outs [1–3]. For transformation of Physcomitrella nearly exclusively PEG-mediated protoplast transformation is applied (for standard protocols see [4,5]). Protoplast isolation and regeneration of Physcomitrella has first been reported by Stumm et al. [6], while Grimsley et al. [7] were the first to use the enzyme mixture 'Driselase' for cell wall digestion of Physcomitrella protonema. The majority of protocols published later on refer to the procedure described by Grimsley et al. [7], working with cultures on solidified medium in petri dishes and using ammonium tartrate as a medium supplement [8]. By addition of ammonium tartrate to the culture medium, moss protonema is growing predominantly in the chloronema stage [8,9]; the cell type developing directly from germinating spores or regenerating moss protoplasts. Lal [10] assumed, that protoplasts can only be obtained from chloronema and young caulonema cells, whereas the cell walls of subsequent developmental stages (old caulonema, gametophores) are not sufficiently susceptible to cell wall degrading enzymes. In contrast, Rother et al. [11] grew Physcomitrella in agitated liquid cultures without supplying ammonium tartrate. By reducing the calcium concentration in the medium these authors increased the protoplast yield and were able to isolate protoplasts from every gametophytic tissue, even from leafy gametophores. However, for producing a huge mutant collection of Physcomitrella [12], a high-throughput transformation platform had to be established, including a novel system for mass production of protoplasts using bioreactors in order to culture large volumes and to reduce manual labour. Moreover, regenerating protoplasts of Physcomitrella are used for developmental studies (e.g. [13]), which demands a highly standardised protoplast quality, which is also ensured by bioreactor culture under tightly controlled environmental conditions. Bioreactor culture of Physcomitrella has already been reported [14–16], however, these reports dealt with either batch cultures or

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short (15 days) continuous cultures, and the cell material was not subsequently used for protoplast isolation.

Here we report on the development of a semi-continuous bioreactor culture of Physcomitrella for protoplast isolation using media supplements and pH control to optimise the protoplast yield.

2. Materials and methods

2.1. Bioreactor culture of Physcomitrella patens

Protonema suspension cultures of *Physcomitrella patens* were grown in modified Knop medium containing 1000 mg/l $Ca(NO_3)_2 \cdot 4H_2O$ (4.24 mM), 250 mg/l KCl (3.36 mM), 250 mg/l KH_2PO_4 (1.84 mM), 250 mg/l $MgSO_4 \cdot 7H_2O$ (1.02 mM) and 12.5 mg/l $FeSO_4 \cdot 7H_2O$ (45 μM), pH 5.8 before autoclaving, as described by Reski and Abel [17]. If mentioned in the results the medium was supplemented with 460 mg/l ammonium tartrate.

Three stirred tank glass bioreactors (Applikon, Schiedam, The Netherlands) with a working volume of 5 l were operated in parallel. Stirring was performed with a marine impeller running with a speed of 500 rpm, the cultures were aerated with 0.3 vvm [(aeration volume)/(medium volume)/min] air. The culture temperature of 25 °C in the vessels was controlled by a double jacket cooling system. Light intensity was 120 μmol/m^2 s provided by fluorescent tubes (Philips TLD 25) with a light/dark rhythm of 16/8 h. The pH-value in the cultures was measured on-line and controlled by automatic titration of either 0.5 N HCl or 0.5 N KOH. In addition the pH-value was checked off-line by taking three samples of 3 ml per day.

Bioreactor cultures were run in a semi-continuous mode: beginning on day 3–6 after the start of the culture, when the dry weight had reached approximately 150 mg/l, the bioreactors were harvested daily and an equal amount of fresh medium was added. Depending on the experiment, the average dilution rate was 0.19–0.23/d.

Dry weight was determined daily by drying the cell material of two samples of 50 ml at 105 °C for 2 h.

The specific growth rate μ (per day) was calculated by fitting an exponential function to the exponential segment of the growth curve (Microsoft Excel). The doubling time was obtained according to the function $t_d = \ln 2/\mu$.

Bioreactor experiments were always carried out as parallel bioreactor runs using identical inoculum. Results were confirmed in independent replications.

2.2. Protoplast isolation

Protoplasts were isolated according to a modified protocol of Rother et al. [11]. The cell material of 200 ml suspension culture was given into 16 ml of 2% Driselase (Sigma, Taufkirchen, Germany) in 0.5 M (91.1 g/l) mannitol. After agitated incubation for 60 min in the dark the cell material was passed successively through sieves with a mesh size of 100 and 45 μm (Wilson, UK). After 15 min, the resulting protoplast suspension was centrifuged for 10 min at $45 \times g$. The pellet was washed in 0.5 M (91.1 g/l) mannitol and after a second centrifugation the number of protoplasts was determined by counting in a Fuchs-Rosenthal-chamber.

3. Results

3.1. Effect of ammonium tartrate and pH control (pH 4.5) on the protoplast yield

In a parallel experiment, three bioreactors were inoculated with identical cell material, two bioreactors with Knop medium as described above, one bioreactor with Knop medium supplemented with 460 mg/l ammonium tartrate. In one of the bioreactors with Knop medium the pH-value was controlled with a setpoint of 4.5, in the other two bioreactors the pH-value was not controlled resulting in an average pH of 5.8 for Knop medium and 4.4 for Knop medium supplemented with ammonium tartrate (Fig. 1). The bioreactors were run semi-continuously starting from day 4; the average dilution rates were 0.21/d for the culture in Knop medium without pH control, 0.22/d for the culture in Knop medium supplemented with ammonium tartrate and 0.2/d for the culture in Knop medium with pH 4.5. The dry weight of the cultures was kept between approximately 100 and 200 mg/l for the cultures in

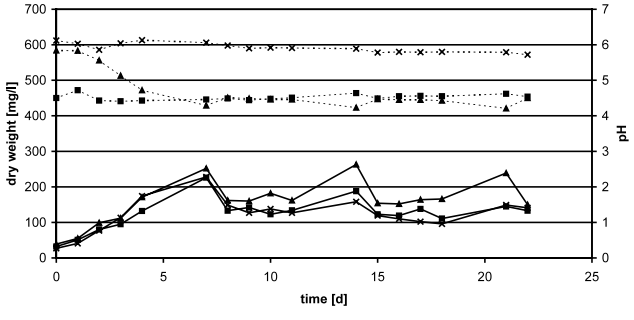

Fig. 1. Dry weight (—) and pH-value (---) of semi-continuous bioreactor cultures of Physcomitrella cultured in Knop medium without pH control (×), in Knop medium supplemented with 460 mg/l ammonium tartrate (▲) or in Knop medium with pH control with a setpoint of 4.5 (■).

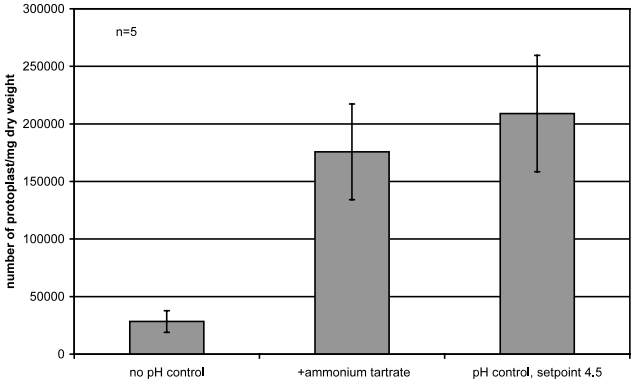

Fig. 2. Average protoplast yield of cell material of Physcomitrella grown in bioreactor cultures in Knop medium without pH control, in Knop medium supplemented with 460 mg/l ammonium tartrate or in Knop medium with pH control with a setpoint of 4.5 (mean ± S.D.)

Knop medium and between 150 and 250 mg/l for the culture in medium supplemented with ammonium tartrate throughout the semi-continuous culture period (Fig. 1). On five different days the protoplast yields from these cultures were determined to be 2.8×10^4/mg dry weight (Knop medium without pH control), 1.8×10^5/mg dry weight (medium supplemented with ammonium tartrate), and 2.1×10^5/mg dry weight (Knop medium at pH 4.5) (Fig. 2).

Protonema growing in the bioreactor with Knop medium without pH control developed chloronema, i.e. cells with a high amount of chloroplasts and right-angled cell walls, as well as caulonema, characterised by a reduced number of chloroplasts and oblique cell walls (Fig. 3A). In contrast, in medium supplemented with ammonium tartrate only chloronema developed (Fig. 3B). In Knop medium at pH 4.5 caulonema development was reduced compared to the culture without pH control, but not completely impeded (Fig. 3C).

3.2. Comparison of the effect of different pH-values

In order to confirm the effect of the pH-value during culture growth on the protoplast yield, three bioreactors with Knop medium without ammonium tartrate were set up in parallel and inoculated with identical material, one bioreactor without pH control, again resulting in an average pH of 5.8, and two bioreactors with pH control, one with a setpoint of 4.5 and one with a setpoint of 7.5 (Fig. 4).

A semi-continuous culture mode was started on day 3, the dilution rates were 0.2/d for the culture without pH control as well as for the culture with pH 4.5 and 0.19/d for the culture with pH 7.5. Again these dilution rates resulted in an average dry weight of the cultures between 100 and 200 mg/l (Fig. 4). Only the culture with pH 7.5 had a dry weight of more than 200 mg/l at the beginning of the semi-continuous culture mode and less than 100 mg/l at the end, indicating that for this culture no steady state conditions were achieved. On four different days the protoplast yields of these cultures were determined to be 2.8×10^4/mg dry weight (culture without pH control), and 2.4×10^5/mg dry weight (culture with pH 4.5), respectively (Fig. 5), these values being nearly identical compared to the first experiment (Fig. 4). For the culture growing at pH 7.5, however, the amount of protoplasts was drastically reduced, yielding only 3×10^3/mg dry weight.

3.3. Effect of the pH of the Driselase digest on the protoplast yield

In order to check whether this pH effect was caused by a pH shift in the digestion solution, which could affect enzyme activity, rather than by the pH-value of the culture itself, the pH-value at the beginning and at the end of the digest was determined (Table 1). There

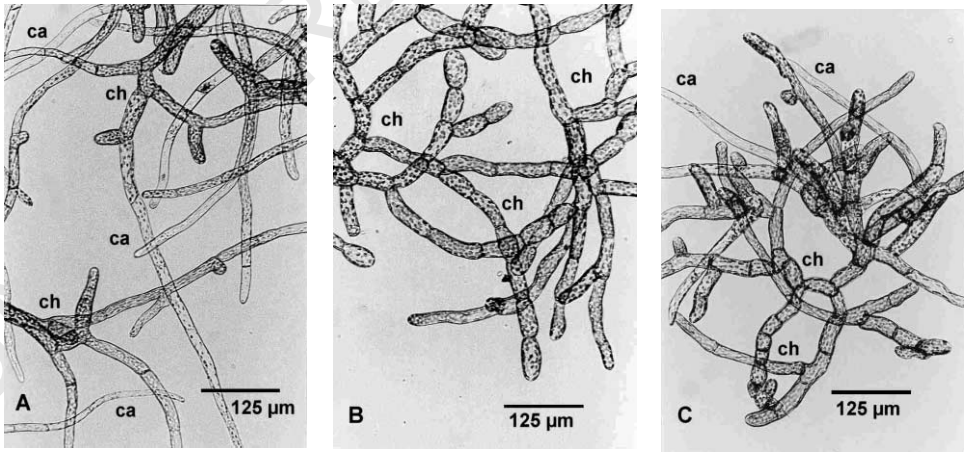

Fig. 3. Microscopic views of cell material in bioreactors in Knop medium without pH control (A), in Knop medium supplemented with ammonium tartrate (B) or in Knop medium with pH control with a setpoint of 4.5 (C). (A) The culture developed chloronema (ch) and caulonema (ca). (B) The culture was growing only in the chloronema (ch) stage. (C) Caulonema (ca) development was slightly reduced in comparison to (A).

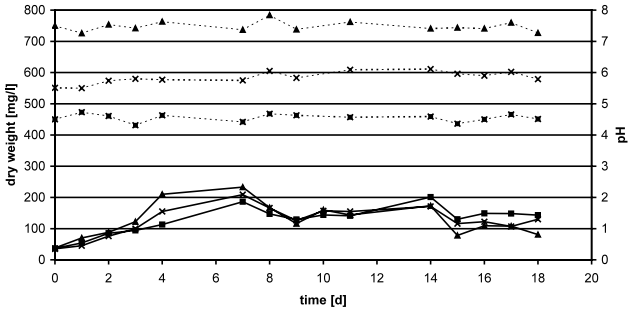

Fig. 4. Dry weight (—) and pH-value (---) of semi-continuous bioreactor cultures of Physcomitrella cultured in Knop medium without pH control (×) and in Knop medium with pH control with setpoints of 4.5 (■) or 7.5 (▲), respectively.

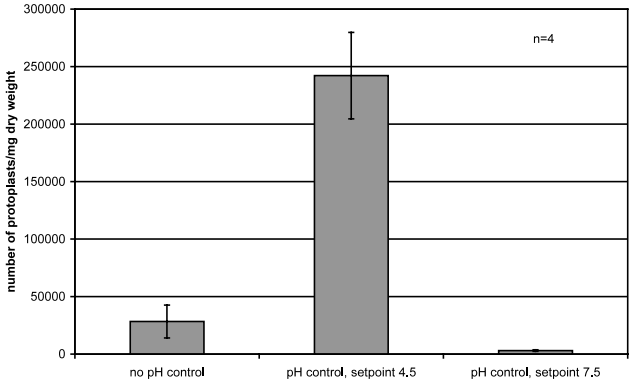

Fig. 5. Average protoplast yield of cell material of Physcomitrella grown in bioreactor cultures in Knop medium without pH control and in Knop medium with pH control with setpoints of 4.5 or 7.5, respectively (mean ± S.D.).

Table 1
pH-value in the Driselase digest for protoplast isolation of cell material of Physcomitrella grown in bioreactor cultures at different pH-values ($n = 2$)

pH-value of the bioreactor culture	pH-value at the beginning of the digest	pH-value at the end of the digest
Not controlled, resulting in 5.8	5.4 ± 0.02	5.0 ± 0.05
4.5 (pH control)	5.3 ± 0.02	5.1 ± 0.19
7.5 (pH control)	6.4 ± 0.04	6.5 ± 0.08

was nearly no differences in pH-values of the digests of cell material cultured in a bioreactor without pH control and pH 4.5, respectively. For these cultures the pH at the beginning of the digest was 5.4 and 5.3, respectively, at the end of the digest, the pH had decreased to pH 5.0 and 5.1, respectively. In contrast, the pH in the digest of cell material, that has been cultured at pH 7.5, was 6.4 at the beginning and 6.5 at the end of the digest.

As a control, the effect of different pH-values (4.5–6.5) in the digest of identical cell material (bioreactor, Knop medium, pH 4.5) on the protoplast yield was

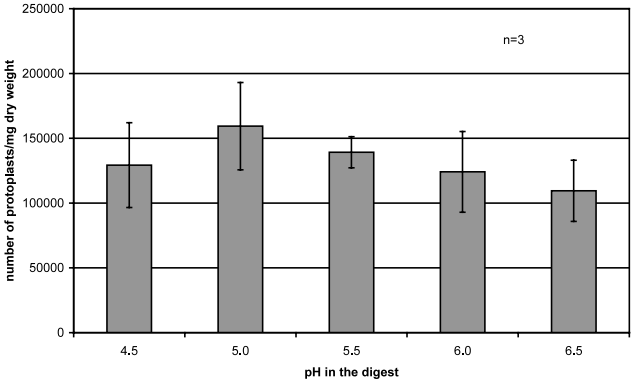

Fig. 6. Protoplast yield of cell material of Physcomitrella digested with 2% Driselase at different pH-values.

subsequently analysed. A pH of 5.0 in the digest resulted in 1.6×10^5 protoplast/mg dry weight, decreasing continuously to 1.1×10^5 with increasing pH (Fig. 6).

3.4. Time course of the pH effect

In order to generate a time course of the pH effect in the bioreactor culture, cells were cultivated in Knop medium without ammonium tartrate and without pH control until day 6. At this day, a semi-continuous culture mode was started as described above (dilution rate: 0.23/d). Simultaneously, pH control with a setpoint of 4.5 was applied and the protoplast yield was determined regularly (Fig. 7). Starting from 1×10^4/mg dry weight on day 6, this yield was continuously increasing for the first 4 days of the semi-continuous culture mode, resulting in 2.5×10^5/mg dry weight on day 10. After that, the protoplast yield remained between 2.5 and 3×10^5/mg dry weight until the end of the experiment on day 16.

4. Discussion

Prerequisite for the establishment of the moss *Physcomitrella* as a model plant in functional genomics approaches is the establishment of a high-throughput transformation platform. To date, most moss transformation protocols use the PEG-mediated DNA-uptake into protoplasts.

Such protoplasts are usually obtained by enzymatic digestion of the moss protonema, which mainly consists of two different cell types, namely chloronema and caulonema. As ammonium tartrate in the culture medium promotes the development of chloronema [8,9], which is thought to be the best cell type to isolate protoplasts from [10], most protoplast isolation protocols rely on this medium additive (e.g. [7,9,18–21]). However, to our knowledge there was no systematic study, that was quantifying this effect.

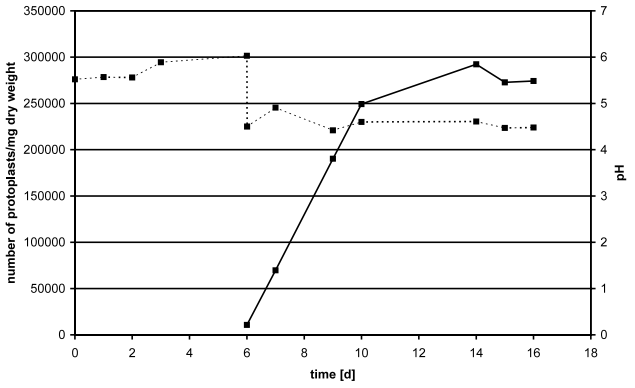

Fig. 7. Protoplast yield (–■–) and pH (--■--) of a semi-continuous bioreactor culture of Physcomitrella: day 0–6: no pH control, day 6–16: pH control with a setpoint of 4.5.

We here report, that supplementation of the medium with ammonium tatrate increased the protoplast yield from semi-continuous Physcomitrella bioreactor cultures sixfold (Fig. 2). Concomitantly, this medium additive suppressed the differentiation of caulonema cells (Fig. 3), confirming several earlier reports.

In contrast to what was assumed to date, these two effects were not connected in our experiments: when media without ammonium tartrate where titrated to a constant pH of 4.5, Physcomitrella protonema did develop caulonema cells but these cultures yielded an even higher number of protoplasts compared to cultures in ammonium tartrate supplemented medium, that were growing only in the chloronema stage.

Therefore, the ammonium tartrate-effect on the protoplast yield cannot be a secondary effect of an altered proportion of chloronema and caulonema in the culture, but is probably caused by a modified cell wall composition. It is known that protoplast isolation from cultures older than 3–4 weeks, i.e. from mature caulonema or gametophore tissue, is markedly impeded. Lal [10] assumed that the chemical composition of the cell walls is significantly altered in these tissues. Thus, in our experiments, this variation of cell wall composition is probably affected by the pH in the culture medium.

This effect of the pH-value of the culture medium was confirmed in a subsequent experiment comparing pH 4.5 and 7.5 to a culture without pH control (resulting in pH 5.8), and resulting in an extremely reduced number of protoplasts from cultures with pH 7.5 (Fig. 5). In this experiment the pH-value in the digest itself was nearly identical (pH 5.0–5.4) for the cell material coming from the culture without pH control and the culture with pH 4.5. In contrast, it was much higher (pH 6.5) for the cell material, that was cultured at pH 7.5 (Table 1). However, it was shown that an increase of the pH-value to 6.5 affected Driselase activity only slightly, reducing the protoplast yield to 69% compared to pH 5.0 and to 79% compared to pH 5.5 (Fig. 6). In contrast, culturing the cell material at pH 7.5 reduced the protoplast yield to 11% compared to a culture without pH control and to 1.2% compared to a culture at pH 4.5. Thus, a reduced enzyme activity due to an altered pH-value in the digest was clearly not the reason for the great differences in protoplast yield from cell material grown in media with different pH-values. In fact, this effect could definitely be attributed to a difference of the cell material itself caused by the pH-values during culture growth as discussed above. When the pH-value of a bioreactor culture was changed from 5.8 (not controlled) to 4.5 (controlled) at the beginning of the semi-continuous culture mode, the linear increase of the protoplast yield for 4 days (Fig. 7) indicates, that probably predominantly the cells that were newly formed at pH 4.5 were the ones, whose cell walls were easily digested.

From these results, we deduced a semi-continuous culture at pH 4.5 in medium without ammonium tartrate as a standard culture protocol for large scale suspension cultures of Physcomitrella for protoplast isolation and transformation. This protocol has the advantage of a large protoplast yield while the use of ammonium tartrate with its severe effect on moss development [8,9], which might also affect transformation and protoplast regeneration, is avoided. From an average daily harvest of 930 ml from a 5-l bioreactor (corresponding to a dilution rate of 0.2/d) protoplasts sufficient for 114 transformations using the protocol described by Reutter et al. [5] or 105 transformations using the protocol described by Schaefer et al. [4] can be obtained. Protoplasts obtained from these high-yield treatments differed neither in viability nor in regeneration ability from protoplasts obtained from published standard methods (data not shown).

Thus we succeeded in the establishment of a long-term bioreactor culture for mass protoplast production for high-throughput transformation or huge developmental screenings on protoplast level. Moreover, although suspensions cultures are frequently used for protoplast isolation of higher plants, especially important monocotyledonous species (e.g. wheat [22] and rice [23]), to our knowledge this is the first report on the distinct effect of a pH control in a plant cell suspension culture on the protoplast yield.

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