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IMPROVED IMAGE ANALYSIS BASED MORPHOLOGICAL CONTROL OF RECOMBINANT MOSS IN PHOTO-BIOREACTORS

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Abstract*:* The moss *Physcomitrella patens* has been introduced in the last years as a novel platform for the expression of complex heterologous proteins. For this purpose, strict control of the plant cell culture in bioreactor is required. Recombinant mosses are cultured as filamentous protonemal tissue and therefore, a description of the morphology and development is compulsory. The homogeneity of the biosuspension could be improved in tubular photo bioreactors by means of image analysis supported cell disruption. Algorithms for the quantification of shape parameters and the detection of gametophores have been adapted and evaluated on submerse moss cultures. *Copyright © 2007 IFAC*

Keywords*:* Image analysis, process control, reactor control, biotechnology.

1. INTRODUCTION

The suitability of bryophytes to study cell and developmental processes in plants has been well documented by investigations with diverse mosses (Knight, *et al*. 1995; Rosenbaum-Hofmann, and Theg, 2003; Sarnighausen, *et al*., 2003). Especially the use of *Physcomitrella patens* for comparative plant physiology and functional genomics studies has become standard in the last years. For reviews (Reski, 1999; Schaefer and Zryd, 2001).

Glyco-engineered *Physcomitrella patens* lines that expressed the recombinant human vascular endothelial growth factor $rhVEGF_{121}$ have been developed (Baur, *et al*., 2005; Jost, *et al*., 2005). With this model product, a new platform has been introduced for the expression of complex proteins that before could be obtained only from animal cells. e.g. CHO.

Physcomitrella patens is grown photoautotrophically and requires a simple mineral medium, carbon dioxide and light as energy source. Continuous long-term cultures with moss recirculation in tubular photo-bioreactors may be an alternative to produce VEGF (vascular endothelial growth factor) with transgenic *Physcomitrella patens* under GMP (good manufacturing practice) (Decker, and Reski, 2004; Lucumi, *et al*., 2003). For this reason, a tight control and characterisation of the culture of *Physcomitrella patens* in photo-bioreactors is required. Therefore, the particularities of bryophytes have to be considered for the culture technique and adequate solutions have to be introduced.

1.1 Developmental variations of Physcomitrella patens.

In the nature, the life cycle of *Physcomitrella patens* ranges between weeks and months depending on environmental conditions such as light, minerals and concurrence. From spores, small haploid filamentous cells emerge and build threads, which are composed by a variable proportion of chloronema and caulonema according to the advance of cell differentiation. Both cells conform the protonemal tissue of the moss. Mature chloronema cells have a width and length close to 25 µm and 55 µm, 20-40 chloroplasts per cell and are highly photosynthetic. Chloronema cross-cell walls are perpendicular to the growth direction (Fig. 1A). Further differentiation leads to the formation of caulonema cells, which are longer and thinner than chloronema and own fewer chloroplasts (Fig. 1B). Their cell walls are oriented

obliquely with respect to the apical growth. From some protonemal filaments, small rounded cells called buds come out and configure small leaflets (Fig. 1C and 1D). Young and leafy gametophores grow and develop brown root-like cells, the rhizoids. Sexual organs: antheridium and arquegonium are formed from mature gametophores during the diploid phase of *Physcomitrella patens* (Knoop B, 1984; Reski, 1998).

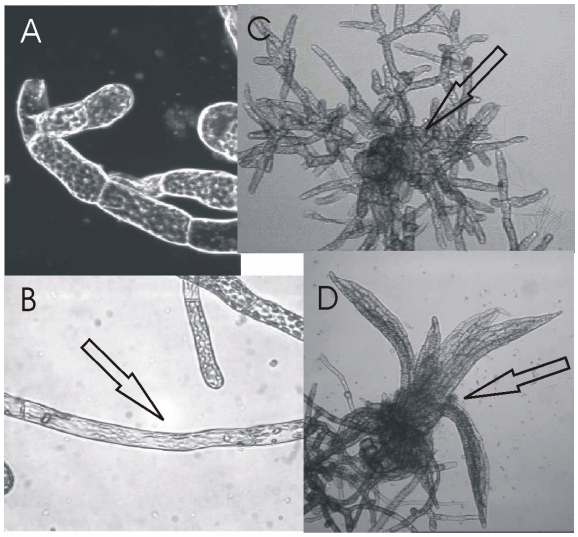

Fig. 1. Typical developmental stages of the moss *Physcomitrella patens* in suspension cultures. (A) Chloronema. (B) Caulonema (arrow). (C) Buds emerging from the core of a moss thread (arrow). (D) Young leafy gametophore (arrow).

1.2 Requirements for a tight control of the plant tissue culture.

During the submerse culture of filamentous species a reliable method to quantify shape and morphology and detect possible developmental changes in culture is required. Online, not invasive techniques, which allow high grade of automation may constitute in the future a competitive advantage, for example in validation processes. The analysis of microscopic images of recombinant and wild type *Physcomitrella patens* in photo-reactors has been introduced to improve the homogeneity of the suspension. Consequently, the purpose of this work is to describe the progress and challenges of image analysis as a tool to improve the culture of plant cells, with the example of the recombinant moss *Physcomitrella patens.*

2. MATERIALS AND METHODS

2.1 Image analysis strategy.

The steps involved in the analysis of microscopy images have been summarized by Pons, *et al.* (1998) and are illustrated on Fig. 2. The proper image processing usually begins with the conversion of the pictures to grey level and include e.g. binarisation, segmentation, filtering and labelling procedures (Pons and Vivier, 1999; Russ, 1995). However, the steps of the algorithm to calculate selected shape parameters may vary according the final application. *Physcomitrella patens* samples were axenically collected from the reactor and then acquired with a CCD camera that was connected to a light microscope. Lower magnifications give information about the configuration of the moss as organism: filament, thread, agglomerate, aggregate, and pellet (Fig. 3A). From this scale it is also possible to gain information of the branching, projected surface and area of the mosses, and the presence of gametophores in the suspension, as well. Determination of shape distributions of the moss and estimates of cell debris requires intensive sampling to improve the representativity of the source data.

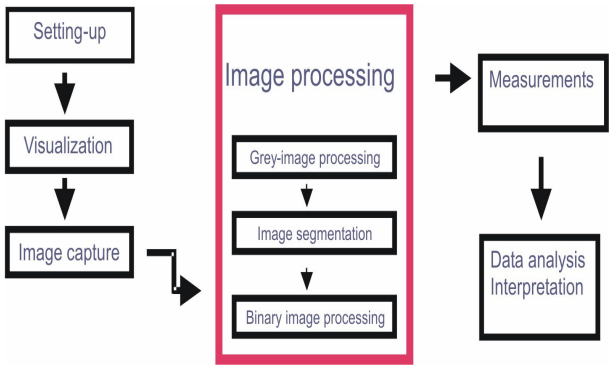

Fig. 2. Overall method for the image analysis of microscopic pictures. The core of the scheme includes the proper image processing. (Pons, *et al.*, 1998).

Several operations as the detection of cell walls and counting of cells pro filament need to be gained from other scales (Fig. 3B). Here, with the help of appropriate algorithms it is possible to recognize individuals, developmental stages (ratio chloronema to caulonema), pigment variations and empty cells.

Higher magnifications may give information about chloroplast shape and number, cell wall integrity and regeneration after stress events (Fig. 3C). However the information at this level is merely descriptive and require high grade of automation to produce statistical data.

2.2 Suspension cultures of moss.

The moss tissue was obtained from suspension cultures. For that reason, batches of *Physcomitrella patens* in a tubular 30-l pilot tubular photoreactor were conduced to describe the morphology, physiology and development of submerse moss at

real culture conditions (Lucumi, *et al*., 2003; Perner, 2003). Carbon dioxide content of the gas supply was hold at 2.16 % (v/v). The average axial velocity of the suspension in the reactor was increased gradually from 0.55 to 0.95 m s^{-1} to test stress responses due to flow turbulence and pumping (Vandanjon, *et al*., 1999). Light was externally irradiated at an average photon flux density (PFD) of 59.3 μE m^{-2} s^{-1}, measured at the external wall of the reactor.

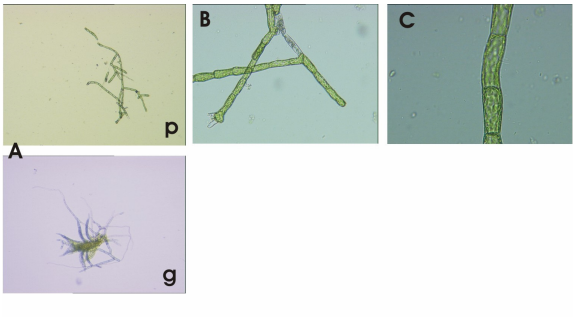

Fig. 3. Types of source data for the definition of selected morphological parameters in filamentous mosses. Recombinant *Physcomitrella patens*. A. Overall morphological characterisation of protonema (p) and gametophores (g). B. Higher magnification for e.g. characterization of pigment distributions and detection of dead cells. C. Cell level. Close up of individual cells to quantify e.g. chloroplast shape and number.

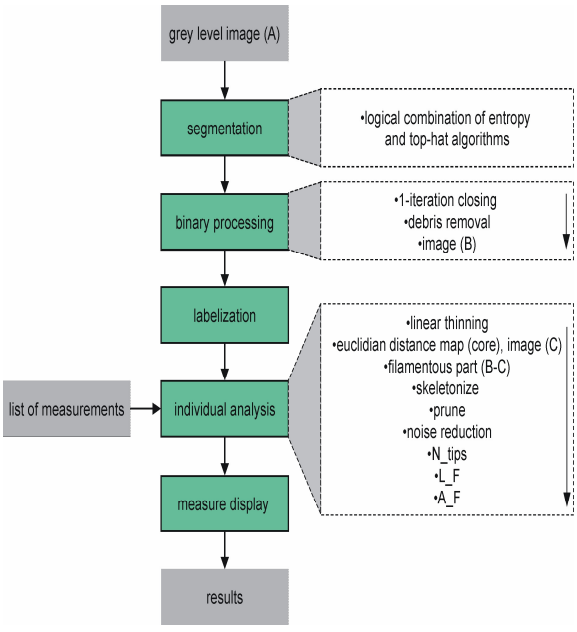

Fig. 4. Main steps of the image analysis algorithm for filamentous species. The determination of shape parameters is obtained from grey level images of *Physcomitrella patens* from suspension cultures.

3. RESULTS AND DISCUSSION

3.1 Image analysis algorithm.

An algorithm for the analysis of filamentous species was adapted for moss with the help of the software for image processing Visilog 6.1. (Noésis, Les Ulis, France) (Lucumi, *et al.*, 2005). The programming of algorithms in this platform was performed according to the guidelines of the Visilog 5 Reference Manual (1999).

Each image was first segmented to detect the biomass. In this step binary images resulting from an entropy-based algorithm (for undamaged filaments and agglomerates) and from a top-hat algorithm (to detect the membrane of damaged cells) were logically combined, see Fig. 4. A 1-iteration closing was then applied to fill small holes in the filaments, without removing empty spaces that are present for example in moss entanglements. Objects smaller than a given area were removed and then labelled as debris. The last produced a binary image B. Each object of this image was individually characterized. The presence of agglomerates was detected by applying a 15-iterations linear thinning, which eliminated the thin part of each object. The remaining part was the core (image C) and its size (core_size) was evaluated from the maximal value taken by its Euclidian distance map. The filamentous part (image F) was retrieved by logical difference between images B and C. To calculate the length of the filaments (L_F), they were skeletonized and pruned to eliminate elements smaller than 10 pixels, which were considered as noise. The number of filament tips (Ntip) was then calculated. The hyphal growth unit based on area was calculated as HGU_A= A_F/Ntip where A_F was the projected area covered by filaments. The hyphal growth based on length was HGU_L=L_F/Ntip. The filamentous fraction was the ratio of A_F to the total projected area of the object under consideration.

In order to refine some steps of the described algorithm, samples from protonemal *Physcomitrella patens* from the pilot 30-l photobioreactor were taken and then evaluated. A further adjustment of the algorithm was obtained after "calibration" with manually determined parameters, microscopic inspection of selected individuals and pigment quantification from biosuspensions. The first part of the basic image treatment of the moss is illustrated in Fig. 5. Here, for example two single individuals were recognized and then were individually analysed.
In order to test the sensibility of the parameter HGU_L to calculate skeleton branching, mosses with similar length were compared. Fig. 6A represented a moss in a lag phase of growth and 6B an individual at the begin of the exponential growth phase. Though the individuals from Fig. 6 belonged to different stages of a suspension culture, they looked similar for not experienced observers. However, it was

possible to detect even light variations of branching by means of the algorithm.

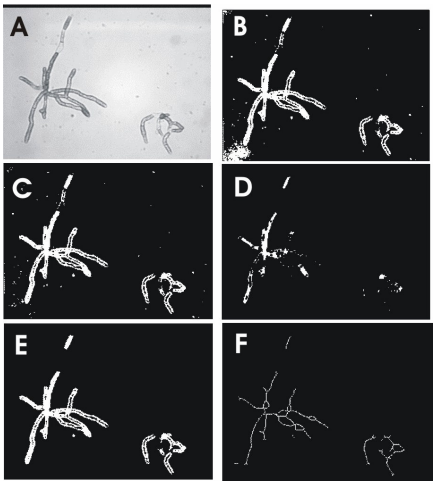

Fig. 5. Basic treatment of *Physcomitrella patens* images. (A) Grey level image. (B) Binarisation. (C) Border kill. (D) Erosion. (E) Reconstruction. (F) Skeleton.

Additional routines to quantify colour profiles in filaments can be incorporated to the algorithm as a way to detect empty cells or measure pigments. The use of grey level images for this purpose may reduce the computational effort in some cases. Main condition for a forecast of pigment content e.g. chlorophyll and carotenoids is an enough acceptable correlation with the grey intensity, see Fig. 7. Here, it was possible to recognize typical pigment patterns of mosses with a high proportion of chloronema cells and empty cells as well. The problem of pigment quantification in image analysis has been intensively reported by several authors (Pons, *et al.*, 1998).

3.2 Image analysis assisted cell disruption in photo reactors

Size control mechanisms for moss entanglements must guarantee low stress and stable cell activity. In the case of randomly distributed stress vectors produced by e.g. reactor impellers, cell debris and free pigments increases, and the carbon dioxide fixation diminishes.

Rotor-stator devices used for dispersion and homogenisation have been also used for cell comminution. Here, the energy input to the cell is efficiently converted in a reduction of pellets content and the average length of the filaments (Fig. 8). Moreover, the energy input required for an analogous size reduction can be around 10 times lower as by means of increased stirring. The mechanical behaviour of the size reduction operation of the moss by means of rotor-stator devices could be reasonably described with the empirical equations developed for dispersions by Wiedmann, (1975), facilitating the fine-tuning of the disruption step in culture.

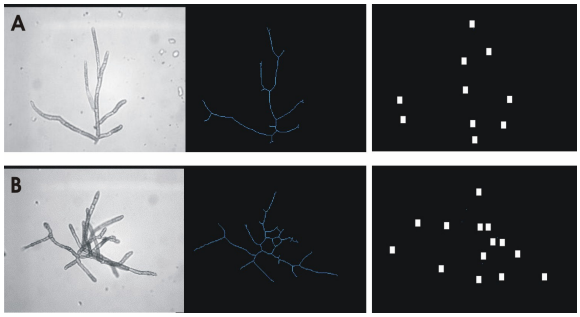

Fig 6. Characterization of side branching in *Physcomitrella patens* by means of the parameters HGU_A and HGU_L. Pictures B shows low HGU_L and high side branching when compared to A. Original grey images, skeletons and end tips are illustrated.

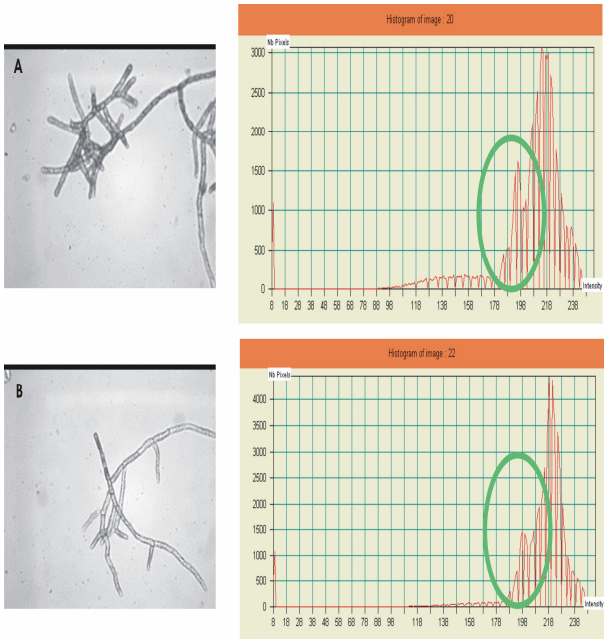

Fig. 7. Grey level images of *Physcomitrella patens* filaments and corresponding histograms. A. Moss with high proportion of pigment-rich chloronema. B. Filament with caulonema.

Although *Physcomitrella patens* shows particular robustness against different kind of stresses (Frank, *et al.*, 2005), metabolic activity can be severely reduced by means of unfocused mechanical stress, erosion and impact. The best balance between tolerable cell damage and size reduction performance, was obtained with quick disruptions at high rotational velocity (Lucumi, *et al.*, 2005). Intense treatments with the rotor-stator disrupter were effective to reduce the number of pellets with a tolerable decrease of carbon fixation rate during the disruption time. Moss disruption by means of high

axial velocity was not effective to reduce the proportion of pellets and the average filament length and caused lessening of the photosynthesis. The residence time and the number of cycles of a single filament in the rotor-stator were used as the main scale up parameters for the operation of cell disruption.

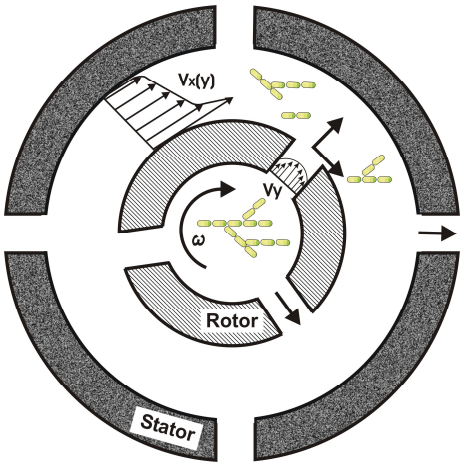

Fig. 8. Schematic representation of a rotor-stator device for inline disruption of moss treads and pellets during a suspension culture in a pilot photobioreactor. A focused cut of the moss is produced by the incidence of two velocity gradients mainly at the exit of the rotor channels.

3.3 Shape control in plant cell culture

The expression of recombinant proteins with transgenic *Physcomitrella patens,* implies that cell stages with high product expression and secretion have to be maintained in long-term cultures. Moss pellets and big agglomerates induce transport problems in the reactor and therefore are to be avoided.

The use of few disruption cycles with the rotor-stator at 22000 rpm during 1minute in batch-cultures helped to delay up to 4 days the formation of buds and gametophores. Gametophores detection was performed offline by an algorithm subroutine based on the parameter core size (Pons, *et al.*, 1998). The algorithm was able to discriminate gametophores and filaments from suspension cultures in the 30-l tubular photobioreactor. Young gametophores showed core size almost three times higher than protonema threads.

Even late size reduction treatments showed to be advantageous in terms of keeping high the carbon dioxide uptake rate RCO_2 between 30-50 mg g^{-1} h^{-1}. In the last case, though the number of mature gametophores present in the suspension could not be decreased without producing severe damage to undifferentiated aggregates, the percentage of gametophores in the total moss mass kept ca. constant until the end of the culture at less than 1% (w/w). Additionally, the proportion of leafy gametophores in disrupted suspension was definitively lower than in cultures without size reduction. Shape variations of *Physcomitrella patens* in suspension cultures could be expressed according the relation between the ratio filament to pellet, and the average filament size. Both parameters could be effectively steered in moss suspensions in the pilot photobioreactor by means of inline mechanical size reduction. Absent or inappropriate shape control during the batches favoured the formation of gametophores from pellet cores and enhanced the morphological and developmental heterogeneity of the mosses, as well. Nevertheless, it was necessary to analyze more than 1300 individuals to obtain a confident determination of shape distributions for the moss in culture.

The combination of size control and perfusion in continuous cultures has been shown to be effective to stabilize the production rate of VEGF, where the inclusion of foreign phytohormone inhibitors was undesirable (Lucumi, *et al*., 2005). In addition, the culture strategy must consider the risks involved by intensive cell stress on the stability of target proteins caused e.g. by free proteases as well as the complications on down stream processing. Links between morphology, protein expression and stability in cultured recombinant moss are still to be investigated.

Population models to predict growth of filamentous organisms by means of morphological parameters such as middle length, hyphal growth unit and shape factors (Bergter, 1978; Metz and Kossen, 1977) could not be properly utilized for *Physcomitrella* patens. Several models do not consider cell differentiation and are inadequate for photoautotrophs. Well established correlations between hyphae size and growth in fungi, could not be validated in mosses.

4. CONCLUSIONS

Although the morphological parameters obtained by means of image processing cannot substitute the information from classical metabolic analysis, and are subject to proper limitations: intensive sampling, high cost for online measurements (Pons and Vivier, 1999), a better understanding of the behaviour of the moss *Physcomitrella patens* in suspension has been achieved. A description of morphological distributions of *Physcomitrella patens* in suspension was settled and the feasibility of plant cell cultures with strict environmental and morphologic control was introduced.
In this work, the suitability of novel reactors for the culture of recombinant *Physcomitrella patens* supported by image analysis was discussed. Long-term bryophyte cultures in fully scalable reactors to produce complex heterologous proteins are only

possible under high controlled conditions. In this way, the preliminary characterization of a new production platform for future transgenic moss lines was introduced. Online sampling and image analysis in plant cell suspensions for the production of high value products represents the next challenge, as it has been developed for other organisms (Frerichs, 2002; Treskatis, *et al*., 1997).

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