Mass propagation of *Nicotiana tabacum* cv. BY-2 suspension cells in the HyPerforma Rocker Bioreactor

Summary

This application note describes the mass propagation of *Nicotiana tabacum* cv. BY-2 (cultivar tobacco Bright Yellow-2) suspension cells in the Thermo Scientific™ HyPerforma™ Rocker Bioreactor at laboratory scale. Maximum fresh cell weights of 351 ± 9 g/L, corresponding to packed cell volumes of 74% ± 2%, were achieved, which shows the ability of the HyPerforma Rocker Bioreactor to cultivate fast-growing plant suspension cells.

Introduction

The fast-growing tobacco *Nicotiana tabacum* cv. BY-2 suspension cell line has been established as a model cell line in many laboratories and is one of the most widely used plant cell cultures [1]. Successful cell expansion in wave-mixed bioreactors at 1 L scale has previously been demonstrated [2-4], where packed cell volumes (PCV) of up to 70% have been achieved. Using initial cell concentrations of between 10% and 15% PCV, a three-day exponential growth period with growth rates in the range of 0.54 and 0.79 per day has been observed.

In the present study the suitability of the HyPerforma Rocker Bioreactor (Figure 1) for the cultivation of BY-2 suspension cells is demonstrated. The Thermo Scientific™ BioProcess Container (BPC) for the HyPerforma Rocker Bioreactor with a maximum working volume of 5 L is equipped with single-use sensors to measure pH and dissolved oxygen (DO). They are operated via the Thermo Scientific™ HyPerforma™ G3Lab Bioprocess Controller, which incorporates mass flow controllers (MFCs) for up to six gases, a standard set of four peristaltic pumps, a motor driver, and transmitters for single-use or electrochemical sensors. The system is connected to Thermo Scientific™ TruBio™ automation software and a Thermo Scientific™ TruLogic™ Controller, powered by the Emerson™ DeltaV™ Distributed Control Platform for bioreactor control, data acquisition, and process visualization. The cells were cultivated in batch mode using chemically defined MSMOplus (3% sucrose) culture medium.

In order to evaluate the reproducibility, three individual cultivations with each running 10 days were realized. Maximum fresh cell weights of approximately 350 g/L, corresponding to packed cell volumes of 74%, were achieved. Even though the culture becomes increasingly viscous during the cultivation (>100 mPa/sec, based on previous studies), intensifying the rocking motion was sufficient to guarantee well-mixed conditions and high cell growth.

Figure 1. HyPerforma Rocker Bioreactor with PC-based HyPerforma G3Lab Controller.

Materials and methods

Cell line and culture media

For the experiments, the tobacco suspension cell line of *Nicotiana tabacum* cv. BY-2 (established and kindly provided by the group of Dr. Stefan Schillberg, IME Aachen, Germany) was used. The cultivations were realized using MSMOplus medium (Cell Culture Technologies). The composition is summarized in Table 1. The pH value was adjusted to 5.8 using 1 M sodium hydroxide solution.

Table 1. Composition of the MSMOplus culture medium.

Component	Concentration
MSMO macroelements	NA
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Inositol	100 mg/L
Thiamine HCl	1 mg/L
Sucrose	30 mg/L
Monosodium phosphate	230 mg/L
Kanamycin	0.1 mg/L
2,4-dichlorophenoxyacetic acid	0.5 mg/L

Overview of setup procedures

- Day –7: Inoculum production with *Nicotiana tabacum* cv. BY-2 suspension cells in shake flasks.
- Day 0: Medium filling and equilibration, and sensor calibration. Inoculation and startup of the HyPerforma Rocker Bioreactor BPC with a seeding concentration of 10% PCV in 5 L MSMOplus medium, and sampling.
- Day 1–10: Sampling, analytics, and harvesting.

Inoculum preparation

The inoculum for the HyPerforma Rocker Bioreactor was produced in five single-use shake flaks (Corning) at a maximum working volume of 300 mL. The cells were inoculated with cell concentrations of 15% PCV. The flasks were shaken at a rate of 180 rpm and amplitude of 25 mm (Multitron shaker, Infors HT) in darkness. After three days, the cell suspension had a PCV of 50% and cell viability was assessed to be above 95%.

Bioreactor preparation and inoculation

About an hour prior to inoculation, a Sartorius™ Flexboy™ media bag with 3 L MSMOplus culture medium was connected to the HyPerforma Rocker Bioreactor BPC via luer lock connection under laminar flow. The medium was transferred to the HyPerforma Rocker Bioreactor BPC, and then the BPC was reinstalled on the HyPerforma Rocker Bioreactor. About 10 minutes prior to the inoculation, single-use optical pH and DO sensors were both calibrated using a one-point calibration.

The seeding procedure was realized in a laminar flow cabinet, where 1 L of the BY-2 inoculum suspension and 1 L fresh MSMOplus medium were transferred into the HyPerforma Rocker Bioreactor BPC by using an autoclaved funnel with a male luer lock connector in order to achieve the desired cell concentration of 10% PCV at the desired starting volume of 5 L. Subsequently, the HyPerforma Rocker Bioreactor BPC was reconnected to the control unit; the heating blanket for the exhaust air filter was installed; and all control loops for rocking motion, DO, and temperature were started. The settings were defined as given below. About 60 min after the cell transfer, the first sample was taken using a specially designed sampling device (Figure 2).

Three individual runs with similar culture conditions (maximum rocking rates were lower in runs 2 and 3) were realized in order to evaluate the reproducibility of the results.

Table 2. The cultivation conditions.

Parameters	Settings
Culture volume	5 L
Rocking rate	Run 1: 24–40 rpm Run 2 and 3: 24–36 rpm (increased stepwise daily after the sampling)
Rocking angle	8–10°
pH value	Non-regulated
Temperature	26°C
Dissolved oxygen	50% saturation
Air flow rate	0.25/0.1 slpm*
Maximum O_2 flow rate	0.25/0.1 slpm*
Start cell concentration	10% PCV
Cultivation time	10 days

* In run 1, flow rate was decreased to 0.1 slpm after the clogging of the outlet filter.

The bioreactor was agitated with increasing rocking rates and angles in order to ensure sufficient mixing of the culture broth, which became increasingly viscous (an increase by a factor of 70 or greater [5]) because of biomass production and secretion of polysaccharides.

The DO concentration in the HyPerforma Rocker Bioreactor was controlled solely by the addition of pure oxygen if the controller output of the DO regulation exceeded 70%. No regulation of pH was realized. The temperature was maintained at 26°C via the integrated heater of the rocking platform.

Sampling and analysis

Samples were taken once a day with a specially designed sampling device mainly consisting of a Duran™ bottle (250 mL), which was sealed to the thermoplastic C-Flex™ tube (1/4 inch) of the HyPerforma Rocker Bioreactor BPC by using the Rewelder from ReedElectronics (Figure 2). Samples of about 40–100 mL were taken from the HyPerforma Rocker Bioreactor BPC via a vacuum pump. For an in-process control (IPC), biomass growth was determined directly by analyzing fresh cell weight, dry cell weight, and PCV. Indirect methods to determine the biomass growth included pH and conductivity measurements.

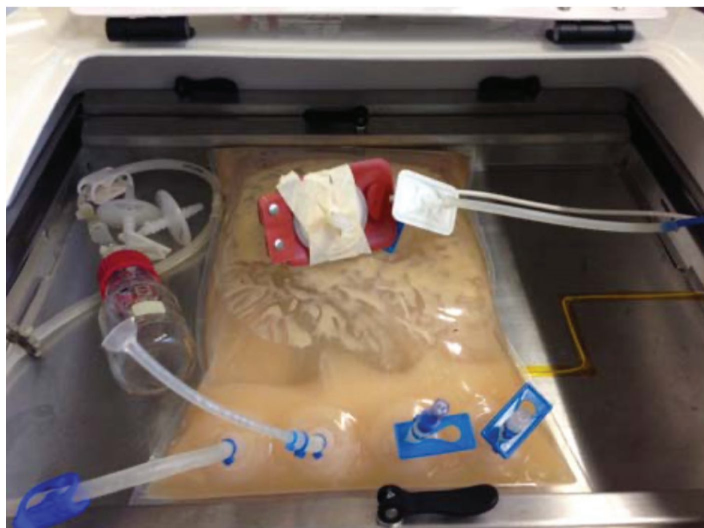

Figure 2. The BY-2 tobacco cell culture in the HyPerforma Rocker Bioreactor BPC after seven days of culture. The specially designed sampling system is shown on the left side.

The PCV, which was defined as the volume ratio of packed biomass to the total suspension volume, was obtained by centrifugation of 2 x 10 mL cell suspension at 201 rcf for 15 min. For the determination of the fresh cell weight, 10 mL of the cell suspension was filtered for 3 min by using a filtration unit equipped with a round filter. The filtrated biomass was transferred to a dried (two days, 80°C) Petri dish and the weight of the filtered biomass was calculated and converted to g/L. The Petri dish with the biomass was sealed by Parafilm™ and stored at –20°C. Subsequently, the Petri dish containing the frozen biomass was placed in the drying oven for two days at 80°C. Conductivity and pH were determined in the supernatant generated during centrifugation for PCV determination using standard probes. For HPLC analysis of sucrose, glucose, fructose, ammonium, and nitrate, 1.2 mL of cell-free supernatant were frozen.

Results

During the experiments good agreement was obtained between the replicates (Figure 3). The highest deviation was found for the fresh dry weight, where all measurement points were within a range of ± 20% around the mean value. However, very similar trends and excellent agreement of the final fresh weights (± 4%) were obtained. Therefore, only mean values of the offline data are given below.

The cells grew exponentially for three days with mean growth rates of 0.62 ± 0.02/day. A one-day lag phase in run 1 was indicated by the fresh cell weight, but this was not confirmed by the packed cell volume (data not shown) and substrate consumption (Figure 3). The determined growth rates are within typical ranges reported for wave-mixed and stirred single-use bioreactors, where growth rates between 0.54/day and 0.79/day have been reached [2]. At day 4, the cells entered the stationary phase, where the biomass increased subsequently only slightly from 301 ± 36 g/L to 331 ± 13 g/L. Even though direct comparison is difficult because of different cultivation systems and operation conditions, the maximum biomass concentration (fresh weight 352 ± 9 g/L) is about 20% lower than values reported for the 3 L Applikon™ stirred bioreactor (fresh weight 412 g/L)[1]. Even higher fresh biomass concentrations of up to 470 g/L and 95% PCV have been achieved in the 3 L Thermo Scientific™ HyPerforma™ Glass Bioreactor in our laboratory [6].

Nevertheless, the cell viability remained high around 95%, except of cultivation run 1, where the viability decreased to ~90% after 6 days of cultivation. This decrease may be explained by the fact that the exhaust filter was clogged. Due to the bag deformation, the cell suspension was less mixed. In subsequent cultivations, the exhaust air filter was changed at day 6, in order to prevent filter clogging.

As shown in Figure 3B, sucrose was rapidly consumed in all cultivations, which led to an increase of glucose and fructose concentrations up to 11 g/L (run 2, day 2) and 12 g/L (run 3, day 1), respectively. Afterwards, the concentrations of both substrates decreased and were below the detection limit of 0.1 g/L after four days of cultivation.

Typical profiles for the nitrate and ammonia ions were also found in all experiments. The nitrate and ammonium concentrations decreased during the exponential growth phase from the initial values of 2.0 ± 0.1 g/L and 275 ± 40 mg/L respectively. The minimum values of 17 ± 23 mg/L (nitrate) and 3.67 ± 3.2 mg/L (ammonium) were detected on day 5, before the concentrations of both ions increased, which may be attributed to cell lysis.

Due to the loss of ions in the culture broth, the conductivity decreased as well. The final value was ~0.5 ± 0.1 mS/cm, whereas the initial conductivity was 5.1 ± 0.1 mS/cm. The offline determined pH values increased over the entire cultivation from pH 5.0 ±0.4 to pH 7.5 ±0.1, since the pH value was non-regulated.

It should be emphasized that, based on our experiences using other single-use bioreactors with optical sensors, it is still challenging to accurately measure the pH of plant cell cultures online. Often the pH value is below the measurement range, in particular at the beginning of the cultivation. Furthermore, some sensors are pre-calibrated by the manufacturer using a temperature of 37°C (instead of the typical 26°C for plant cell cultures). These factors may affect the reliability of the measurement.

Indeed, no signal of the pH sensor was obtained during

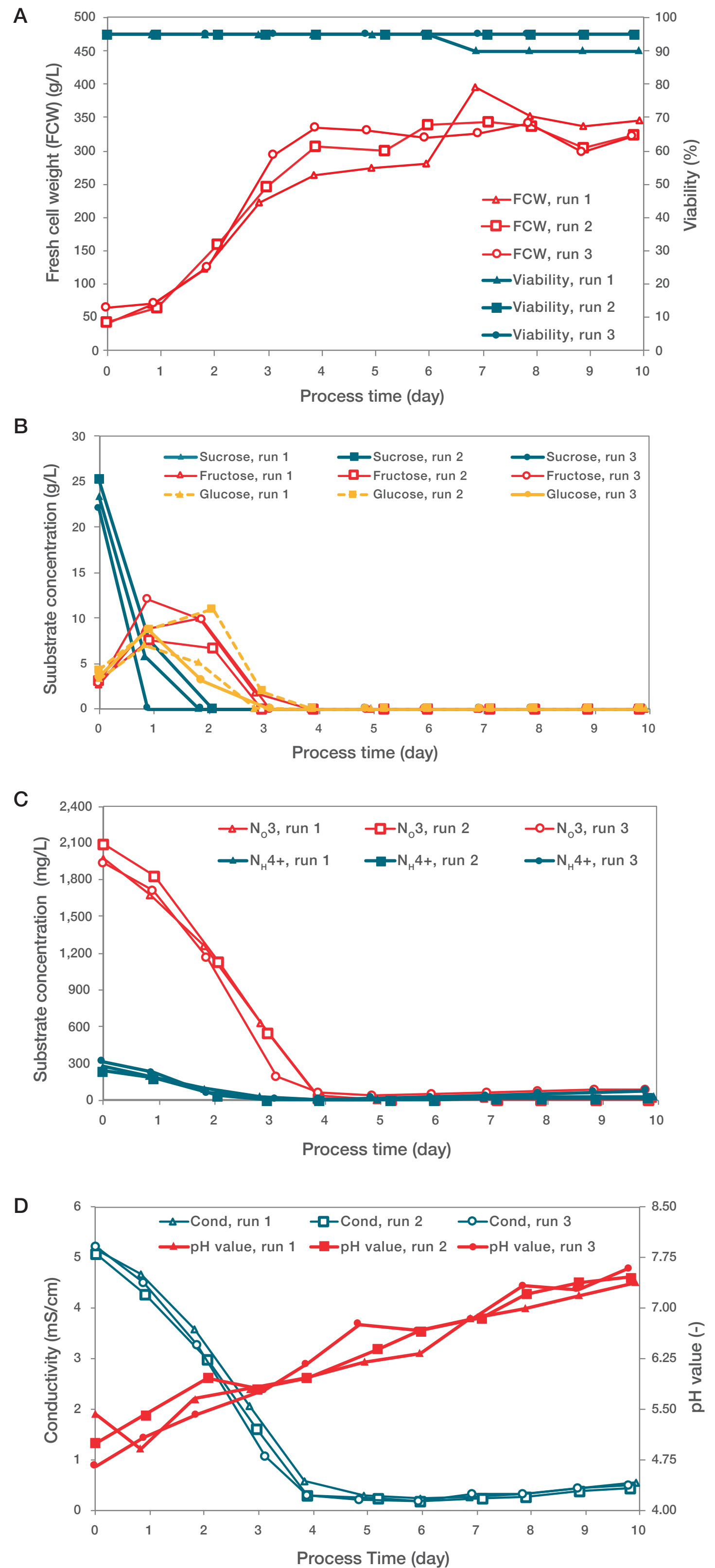

Figure 3. Profiles of offline data determined in a batch experiment with *Nicotiana tabacum* cv. BY-2 suspension cells. (A) Fresh cell weight and viability. (B) Concentrations of sugars sucrose, glucose and fructose. (C) Concentrations of nitrate and ammonium ions. (D) Conductivity and pH value.

the first four days of the first experiment (run 1), which may be caused by the low pH of 5.4 (as determined offline) at the beginning of the cultivation, being at the lower limit of the sensor's measurement range. But, the sensors worked appropriately over the complete duration of the other two runs, even though recalibration was required almost daily (Figure 4A). The maximum deviation from offline to online pH value was 0.6 units. Recalibration was realized if the offline measured pH values of the samples differed more than 0.1 pH unit from the online data.

In contrast, the DO measurement worked well over almost the complete process. Some obstacles appeared for about 12 hours between the second and third day of cultivation, when the cells started to enter the stationary phase. By the addition of pure oxygen in the gas supply and increase of rocking rate and angle, as shown in Figure 4C, the DO remained among the setpoint and increased continuously after five days, so that an oxygen limitation can be excluded. Because of the surface aeration in the wave-mixed, no flotation was observed and no foam was produced (see Figure 2), as often observed in sparged cultures [2]. Hence, no addition of antifoam agent was required.

Nevertheless, rather high DO fluctuations between 10% saturation and 70% saturation were observed, which can be explained by the low oxygen mass transfer rates and probe response in the increasingly viscous culture broth. Perhaps the DO controller settings should be adapted for further investigations.

After 5 days of cultivation, the dissolved oxygen started to increase because of the decreasing oxygen demands of the culture, which can be explained by the progressive cell death. At the end of the process, the culture broth was nearly oxygen saturated.

In summary, it can be stated that *Nicotiana tabacum* cv. BY-2 suspension cells were successfully expanded using the novel wave-mixed HyPerforma Rocker Bioreactor at 5 L scale. Experiments with other plant cell types in our laboratory are currently under preparation.

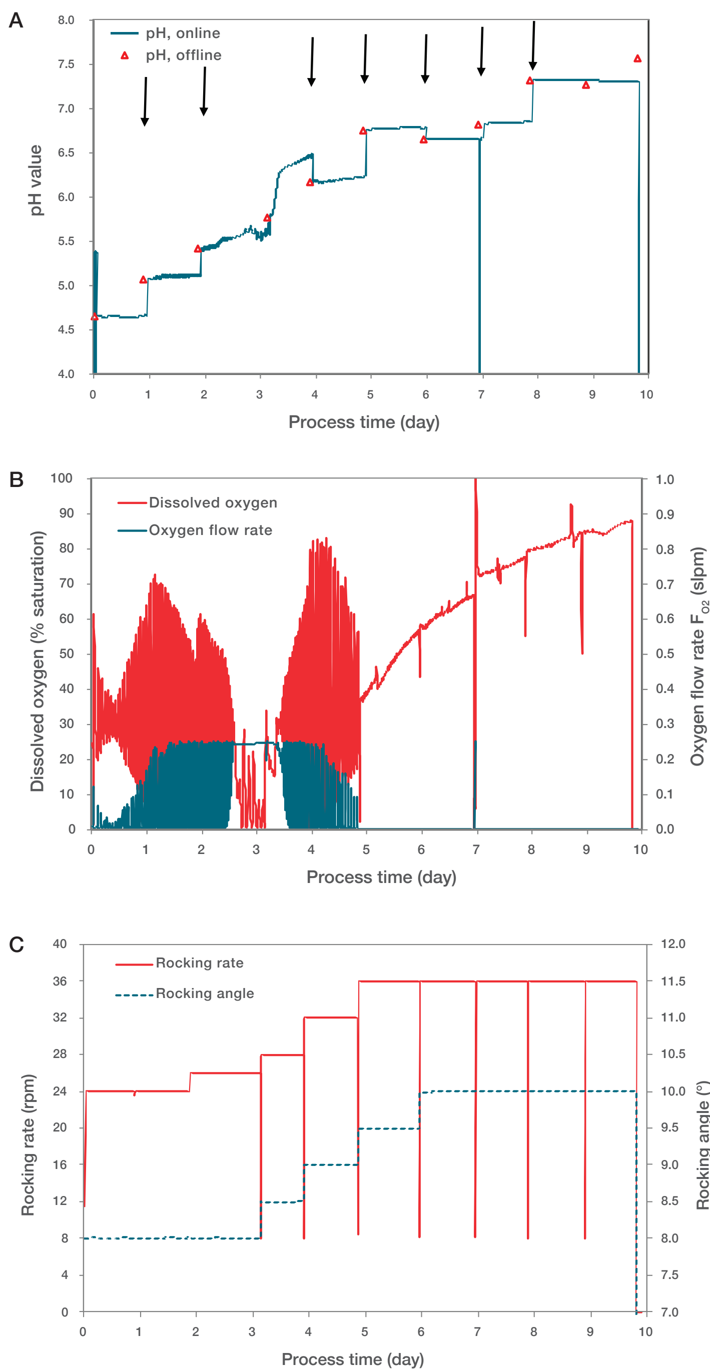

Figure 4. Profiles of online data determined in the batch experiment run#3 with *Nicotiana tabacum* cv. BY-2 suspension cells. (A) pH value measured online and offline—arrows indicate recalibration. (B) Dissolved oxygen and oxygen flow rate. (C) Rocking rate and angle.

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Authors

Nadezda Perepelitsa, GMP Process Engineer, Johnson & Johnson, Basel, Switzerland

Stephan C. Kaiser, Staff Scientist, Research and Development, Thermo Fisher Scientific

Dieter Eibl, Professor, ZHAW School of Life Sciences and Facility Management, Centre for Biochemical Engineering and Cell Cultivation Technique, Wädenswil, Switzerland

Nicole Imseng, Process Expert USP/DSP, CSL Behring, Lengnau, Switzerland

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