Cultivation of antibody producing fast-growing suspension tobacco plant cells in ReadyToProcess WAVE 25 bioreactor system

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Cultivation of antibody producing fast-growing suspension tobacco plant cells in ReadyToProcess WAVE™ 25 bioreactor system

This application note demonstrates the performance of ReadyToProcess WAVE 25 bioreactor system in cultivation of plant cells. In developing a culture process for fast-growing tobacco suspension cells, the large increase in culture viscosity plays an important role and represents a great challenge. Results from triplicate batch runs show that cultivation could be successfully achieved, with an average packed cell volume of 70.2%, a doubling time of 1.56 days, and a specific growth rate of 0.45 d^{-1}, using the ReadyToProcess WAVE 25 system.

Introduction

The cultivation of plant cells has become increasingly important during the last years due to their expression of substances that can be used as pharmaceutical and cosmetic ingredients. Recombinant monoclonal antibodies can be produced in genetically modified plant cells (1). Furthermore, non-modified, plant suspension, cell-based secondary metabolites for cosmetics have entered the market (2). The aim of this work was to demonstrate the performance of the ReadyToProcess WAVE 25 for the cultivation of fast growing plant cells. Here, cell growth, along with an increase in culture broth viscosity, resulted in a change in fluid flow behavior from Newtonian fluid flow behavior to non-Newtonian fluid flow behavior.

In this work, a *Nicotiana tabacum* cv. Bright Yellow-2 (BY-2) suspension cell line, secreting IgG, was used. This cell line was established by Fraunhofer IME, Aachen, DE and generated by *Agrobacterium*-mediated stable transformation (1).

The BY-2 suspension cells were cultivated according to standardized protocols developed by the ZHAW work group at the Zurich University, CH in modified Murashige and Skoog medium (MSMO plus) at pH 5.8 and 26°C in dark. In a pilot run, operating parameters for ReadyToProcess WAVE 25 (rocking speed, rocking angle, and acceleration) were set according to previous studies of engineering parameters and determined fluid flow behavior (3). Batch cultivations were performed in triplicate.

Material and methods

Preculture and cell expansion

For maintenance culture, the BY-2 suspension cells were cultivated in shake flasks at 26°C in dark at a shaking speed of 180 rpm on a Multitron™ orbital shaker (Infors HT, CH). For seed inoculum production, a 5 L Optimum Growth™ shake flask system (Thomson Instrument Company, USA) with 2.5 L MSMOplus medium (Cell Culture Technologies, CH) were inoculated with an initial packed cell volume (PCV), that is, the ratio of cells and supernatant after centrifugation, of 10% to 15%. The seed culture was grown until a final PCV of 40% to 50% was achieved. At this stage of the growth, the cells were in the exponential growth phase, which is the favored condition for inoculation of highly vital cells.

Triplicate batch cultivations

Batch cultivations were carried out in a 20 L Cellbag™ disposable culture chamber equipped with optical pH and dissolved oxygen (DO) sensors and screw cap. PCV was determined in the seed inoculum, and the required amount of cells and medium were inoculated with an initial PCV of approximately 15% and a working volume of 10 L. Specific operating parameters are summarized in Table 1. The Cellbag culture chamber was installed on the ReadyToProcess WAVE 25 rocking unit, pH and DO sensor were attached, the filter heater was installed at the gas outlet filter, the gas inlet was connected to the gas inlet filter, and the cultivation was started. Plant cells can tolerate a wide pH range. Hence, pH is generally not controlled in plant cultures (1).

Initially, a rocking speed of 20 rpm and a rocking angle of 6° were used. To avoid strong foaming, maximum rocking speed and angle were set to 30 rpm and 12°, respectively. As the acceleration parameter did not show a significant influence on the mixing time and the oxygen transfer at 10 L working volume in previous experiments (3), it was not investigated here. Parameters for the triplicate batch cultivations are listed in Table 1.

Table 1. Cultivation parameters for triplicate batch cultivations

Inoculum	15% PCV ≥ 90% viability
Working volume	10 L
Cultivation time	10 d
Rocking speed/angle	0-2 d, 20 rpm, 6° 3-5 d, 26 rpm, 8° 6-10 d, 30 rpm, 12°
Acceleration	100% (constant sine oscillation)
Temperature	26°C
Oxygen	Online DO (not regulated)
Aeration rate	0.5 vvm
pH	5.8 (online and offline, not regulated)
Medium	MSMOplus chemically defined pH 5.8 Osmolality: 230 mOsm L^{-1} Conductivity: 5.8 µS cm^{-1}

In process control and analysis

The first sample was taken directly after the inoculation. For the complete cultivation, samples were taken once per day. For sampling, the rocking was stopped, the filter ports were closed and the aeration tube and the filter heater were removed. The Cellbag culture chamber was transferred to a biosafety cabinet and 10–13 mL cell suspension was taken with a wide opening pipet (Sarstedt, DL) via the screw cap port and transferred into a 15 mL Falcon™ tube (VWR International LLC, USA). After reinstalling the bag on the rocking platform, the sample was analyzed for cell viability, PCV, fresh cell weight (FCW), cell dry weight (CDW), conductivity, and pH.

To determine viability, the cells were stained with 0.5% Evans Blue (Sigma-Aldrich Chemie GmbH, CH) and monitored using Leica DFC450 C light microscope (Leica Microsystems AG, CH). Cell viability was determined by counting stained and unstained cells and calculating the ratio. For PCV determination, the sample was centrifuged for 15 min at 3000 rpm (1811 rcf) in an Eppendorf™ 5180 centrifuge (Eppendorf AG, CH). The supernatant was used for determination of pH and conductivity using FiveEasy FE20/EL20 pH meter and FiveEasy FE30/EL30 conductivity meter, respectively (Mettler Toledo GmbH, CH). Supernatant samples were frozen at -20°C for subsequent analysis of the medium components by high pressure liquid chromatography (HPLC). The cell pellet was transferred to a 55 mm Whatman™ 589/2 filter paper, placed in a vacuum filter unit and filtrated for 3 min. The filter cake was transferred into a preweighed petri dish and weighed on an analytical balance for FCW measurement. Afterwards, the petri dishes were sealed with Parafilm™ and stored at -20°C until the end of the cultivation for CDW analysis. Subsequently, all samples were lyophilized and CDW was determined. The concentration of the medium components (sucrose, fructose, glucose, ammonium, and nitrate) was determined by HPLC analysis (BioRad, USA).

Results

The cultivations were started at an initial rocking speed of 20 rpm and rocking angle of 6°. To prevent cell sedimentation, rocking speed and angle were increased to 26 rpm and 8°, respectively, on day 3 and further to 28 rpm and 10°, respectively, on day 6. To prevent foaming, the maximum rocking speed and angle were set to 30 rpm and 12° on day 7 and kept at these values until end of cultivation.

The results of the triplicate batch cultivation are summarized in Table 2. All three cultivation runs showed similar cell growth behavior and viability (Fig 1). The highest PCV was reached on day 6 in run 3 and day 7 in run 1 and 2 at 70.2%, 72.6%, and 70.5%, respectively. The highest FCW was observed on day 7 in all three experiments, with concentrations ranging between 331.3 and 335.7 g L^{-1}, but remained constant until end of cultivation. The viability was constantly higher than 95% in all three cultivations.

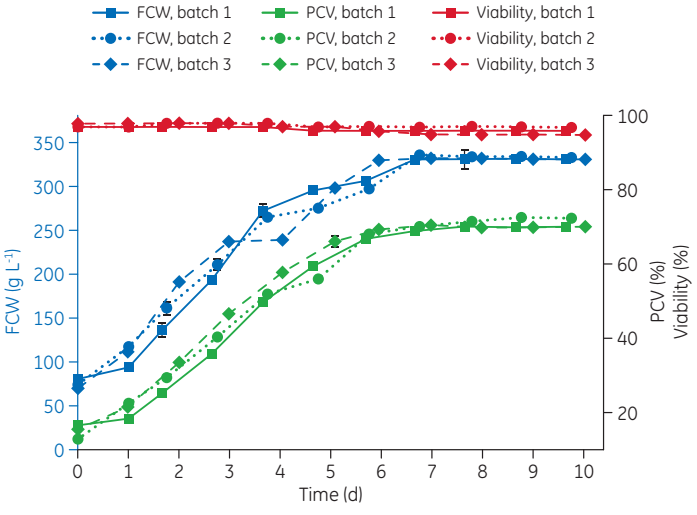

Fig 1. PCV, FCW and cell viability for triplicate cultivations.

Table 2. Results obtained for triplicate cultivations

	Run 1	Run 2	Run 3
PCV inoculation (%)	16.6 ± 0.1	12.9 ± 0.2	15.7 ± 0.1
PCV max. (%)	70.0 ± 0.1	72.4 ± 0.5	70.1 ± 0.3
FCW max. (g L^{-1})	331.3 ± 1.9	335.7 ± 2.5	332.1 ± 1.0
CDW max. (g L^{-1})	11.8 ± 0.1	12.1 ± 0.2	12.1 ± 0.2
Viability (%)	≥ 96	≥ 97	≥ 95
$\mu_{max, FCW}$ (d^{-1})	0.40	0.44	0.50
$t_{d, FCW}$ (d)	1.73	1.56	1.38
$r_{x,FCW}$ (g L^{-1} d^{-1})	132.2	113.2	125.1
$Y_{X/S}$ (g gC^{-1})	24.8	25.2	30.2
r_s (gC L^{-1} d^{-1})	1.05	1.05	0.86
q_s (gC L^{-1} d^{-1})	0.004	0.004	0.003

PCV = packed cell volume, CDW = cell dry weight, FCW = fresh cell weight $\mu_{max, FCW}$ = maximal specific growth rate (for FCW), $t_{d, FCW}$ = doubling time (for FCW), $r_{x,FCW}$ = biomass production rate (for FCW), $Y_{X/S}$ = yield, r_s = substrate consumption rate, q_s = specific substrate consumption rate.

Sucrose was completely hydrolyzed within the first day of the cultivations. Accordingly, glucose increased from 2 to 8 g L^{-1} and fructose from 2 to 12 g L^{-1}. Both glucose and fructose were completely incorporated into the vacuole after a total cultivation time of four days (Fig 2).

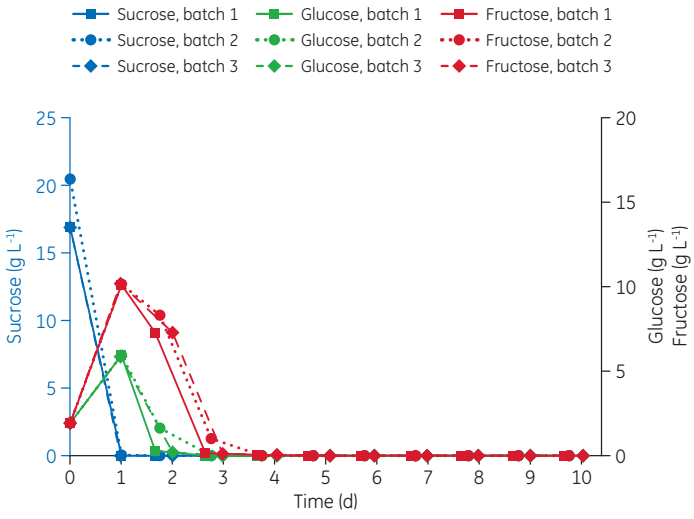

Fig 2. Sucrose, glucose, and fructose concentration for triplicate cultivations.

The graphs for conductivity and pH of the triplicate batch cultivation are shown in Figure 3. The conductivity decreased from 4.5 mS cm^{-1} at inoculation to 0.3-0.4 mS cm^{-1} on day 5 and remained constant with a slight increase at the end of the cultivation, which indirectly correlates to cell growth. The pH value of 5.2–5.5 at the beginning of the cultivation slightly decreased within the first day to 5.1 and subsequently increased to pH 7.2–7.3 by end of cultivation.

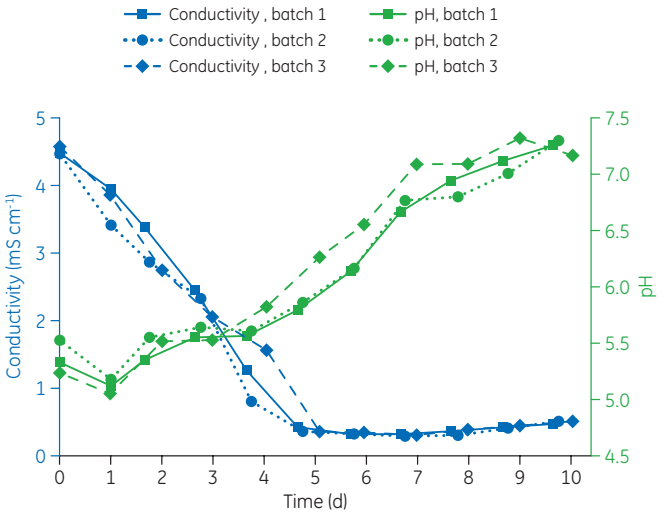

Fig 3. Conductivity and pH for triplicate cultivations.

The results listed in Table 2 are comparable to literature data (1). For example, PCVs between 60% and 80% were reached with the same cell line under comparable conditions; a PCV of 74% was reached in batch cultivation of another tobacco cell line in a 10 L wave-mixed bag bioreactor system with 5 L working volume (4); and a PCV of 63% was achieved in a 2 L bag with 1 L working volume (5). Additionally, comparable FCW's were reached with 332 and 358 g L^{-1} in other types of rocking bioreactor systems in 1 and 5 L scales. Even orbital shaken shake flasks or orbital shaken bioreactors led to comparable results (6, 7).

Conclusion

Triplicate cultivations of *N. tabacum* BY-2 suspension cells were successfully conducted using the ReadyToProcess WAVE 25 bioreactor system equipped with a 20 L Cellbag disposable bioreactor. The starting rocking speed and angle were set to 20 rpm and 6°, respectively. To prevent cell sedimentation, these parameters were stepwise increased to 28 rpm and 9° over day 3 to 6. To prevent too heavy agitation of the culture broth, with the risk of cells accumulating in the outlet air filter causing filter blockage, final rocking speed and angle were set to maximum 30 rpm and rocking angle of 12° from day 7 and maintained at this level until end of cultivation. In the triplicate batch cultivations, an average PCV of 70.2%, a doubling time of 1.56 days, and a specific growth rate (μ_{max}) of 0.45 d^{-1} could be achieved.

In summary, these results show that the ReadyToProcess WAVE 25 bioreactor system is a suitable device for successful cultivation of fast growing plant cell lines.

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Ordering information

Product	Description	Product code
ReadyToProcess WAVE 25	Rocker unit	28988000
ReadyToProcess™ CBCU	Measurement and controller unit	29044081
ReadyToProcess Pump 25	Pump unit	29032003
Cellbag 20 L	Culture chamber with DO and pH optical sensors with screw cap	CB0020L10-33

GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden

gelifesciences.com/wave

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