Chapter 12

Plant Cell-Based Recombinant Antibody Manufacturing with a 200 L Orbitally Shaken Disposable Bioreactor

Nicole Raven, Stefan Schillberg, and Stefan Rasche

Abstract

Tobacco BY-2 cells are an attractive platform for the manufacture of a variety of biopharmaceutical proteins, including antibodies. Here, we describe the scaled-up cultivation of human IgG–secreting BY-2 cells in a 200 L orbitally shaken disposable bioreactor, resulting in cell growth and recombinant protein yields that are proportionately comparable with those obtained from cultivations in 500 mL shake flasks. Furthermore, we present an efficient downstream process for antibody recovery from the viscous spent culture medium using expanded bed adsorption (EBA) chromatography.

Key words Antibody purification, Expanded bed chromatography, *Nicotiana tabacum* Bright Yellow 2 (BY-2), Plant cell suspension culture, Scaled-up manufacture, Single-use technology

1 Introduction

The suitability of different plant-based production hosts for the manufacture of valuable pharmaceutical and industrial proteins such as enzymes, antibodies, vaccine candidates, and other biotherapeutics has been well described [1–3]. Plant-based production systems include intact plants [4, 5], plant tissues and organs [6, 7], simple plants in containment [8, 9], and cell suspension cultures [10, 11]. Tobacco-based cell suspension cultures such as NT-1 and BY-2 [12] are often chosen as production hosts because they can be easily transformed and show favorable growth characteristics and high productivity [13]. Recombinant protein production according to good manufacturing practice (GMP) is made possible by the contained cultivation of plant cell suspension cultures in bioreactor systems with rigid process control [14]. In recent years, disposable bioreactor systems have become well accepted as an alternative to stainless steel bioreactors for mammalian cell cultures by the biomanufacturing industry, mainly due to their cost-effectiveness, flexibility, and safety [15].

Jacqueline MacDonald et al. (eds.), *Recombinant Proteins from Plants: Methods and Protocols*, Methods in Molecular Biology, vol. 1385, DOI 10.1007/978-1-4939-3289-4_12,

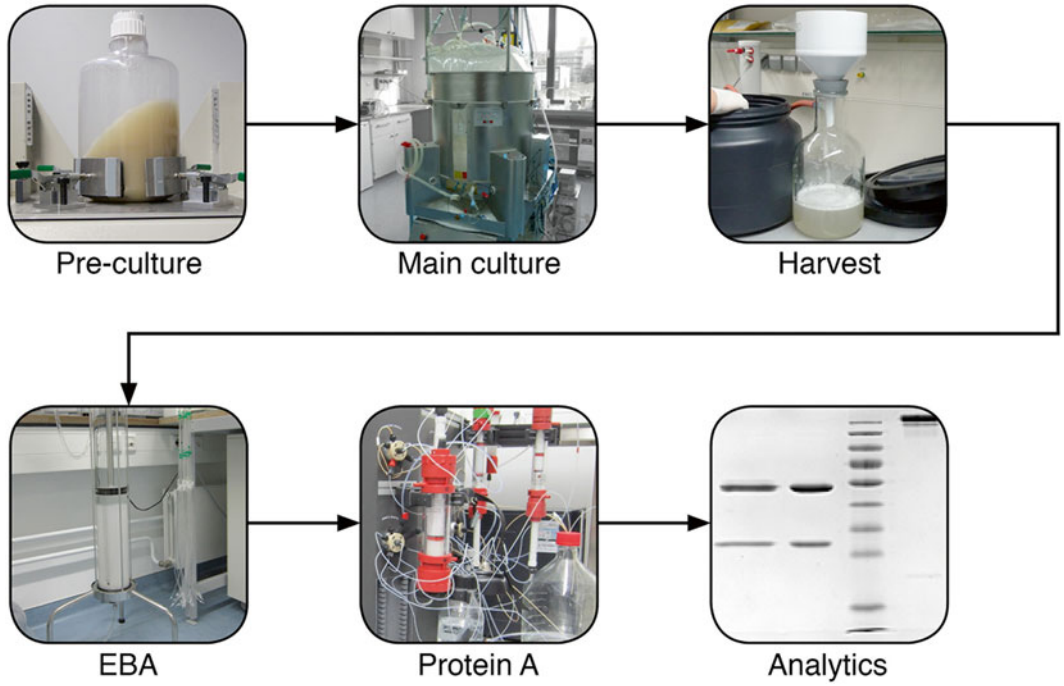

Fig. 1 Upstream and downstream process steps of 100 L scale antibody manufacture from BY-2 suspension culture. EBA is expanded bed adsorption chromatography; Protein A refers to M12 antibody capture using staphylococcal protein A, which binds IgG molecules by their Fc region

Plant cell cultures have been successfully cultivated in various disposable bioreactor systems such as wave-mixed bioreactors or stirred-tank reactors [16–18]. Orbitally shaken bioreactor systems are one of the most recent trends in disposable cultivation technology. The simple bag geometry of orbitally shaken bioreactor systems and their predictable liquid distribution characteristics account for straightforward scalability [19] and facilitate the characterization of bioprocessing capabilities. Here, we describe the cultivation of human M12 antibody-secreting BY-2 suspension cells in a disposable orbital shaker at a working volume of 100 L, a 200-fold greater scale compared to routine shake flask cultivation. After cell removal by vacuum filtration, we applied expanded bed adsorption (EBA) chromatography upstream of the protein A chromatography as a novel strategy for efficient antibody recovery from the viscous spent medium. The overall antibody recovery of this manufacturing process (*see* Fig. 1) ranged between 75 and 85 %, and product purity of >95 % was typically achieved.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when discarding waste materials.

2.1 Plant Cultivation

1. MSN medium: For each liter of medium, weigh 4.3 g Murashige & Skoog (MS) medium, 30 g sucrose, 10 g potassium nitrate, 0.2 g potassium dihydrogen phosphate, and 0.1 g myo-inositol, then transfer the components into an appropriate beaker or vessel. Add ultrapure water to 95 % of the final volume and dissolve the components using a magnetic stirrer. Add 0.2 mg/L 2,4-dichlorophenoxyacetic acid (*see* **Note 1**) and 1 mg/L thiamine–HCl (*see* **Note 2**). Without raising the pH above the desired value, carefully adjust the pH to 5.8 using 1 M potassium hydroxide and fill with ultrapure water to the final volume. Medium sterilization is achieved either by heat sterilization in an autoclave at 121 °C and 100 kPa (15 Psi) for 35 min or by filter sterilization using Sartopore 2 sterile MidiCap filters (Sartorius Stedim AG, Goettingen, Germany) for media volumes of up to 20 L or Sartopore 2 150 sterile capsule filters (Sartorius Stedim AG) for larger media volumes. The smaller filter size is operated at a flow rate of 150 mL/min while the flow rate for the larger filter size is adjusted to 1.5 L/min. The medium is stored at room temperature until usage.
2. Pre-culture cultivation vessels: Tobacco BY-2 suspension cells are routinely cultivated in 250 mL wide neck glass Erlenmeyer shake flasks. A 5 L pre-culture used as inoculum for a 100 L batch cultivation is cultivated in a 20 L polycarbonate carboy vessel.
3. Incubator shaker with a shaking diameter (d_0) of 5 cm (e.g., ISF1-X incubator shaker, Kuhner AG, Birsfelden, Switzerland): This incubator is used for the cultivation of 100–500 mL BY-2 shake flask cultures.
4. Incubator shaker with a shaking diameter (d_0) of 7 cm (e.g., SR200-X shaker, Kuhner AG): This shaker is used for the cultivation of BY-2 cells in a 20-L Nalgene polycarbonate carboy vessel.
5. SB200-X 200 L orbitally shaken bioreactor system: The SB200-X bioreactor system (Kuhner AG) is equipped with a 350-L disposable Cultibag ORB 200 L (Sartorius Stedim AG). The maximal nominal working volume of the bioreactor system is 200 L.

2.2 Culture Broth Clarification

1. Miracloth (Merck-Millipore, Darmstadt, Germany).
2. Nutsche filter with a diameter of at least 17 cm.

3. 15 L gas washing bottle.
4. Membrane vacuum pump with a capacity of 1.7 m/h.

2.3 Expanded Bed Adsorption (EBA) Chromatography

1. Streamline BPG 100/950 glass chromatography column (10 cm internal diameter × 95 cm height; GE Healthcare, Munich, Germany) with flow distribution provided by a perforated plate fitted with a 23 μm mesh at the inlet.
2. Masterflex L/S with Easy-LoadII pump head (Cole-Parmer via VWR International GmbH, Darmstadt, Germany).
3. Streamline SP adsorbent (GE Healthcare): 2 L Streamline SP adsorbent is packed into the Streamline BPG 100/950 glass chromatography column, resulting in a packed mode bed height of 31 cm.
4. Analogue pressure gauge with a measuring range of 0–6 bar (Anderson Instruments Company, INC., Fultonville, NY).
5. High salt elution buffer: 50 mM sodium phosphate, pH 8.0, 500 mM NaCl. For each L, weigh 19.0 g tri-sodium phosphate docecahydrate and 29.2 g NaCl. Transfer the components into an appropriate vessel and add ultrapure water to 95 % of the final volume. Dissolve by stirring on a magnetic stirrer. Without raising the pH above the desired value, carefully adjust the pH to 8.0 using 1–10 M NaOH and add the required amount of ultrapure water to reach a final volume of 1 L or equivalent. The buffer is stored at room temperature.

2.4 Preparative Protein A Chromatography

1. XK 50/20 column (GE Healthcare).
2. ÄKTAexplorer 10 (GE Healthcare).
3. Protein A sorbent: 150 mL MabSelect SuRe™ (GE Healthcare).
4. Equilibration and wash buffer: Phosphate buffered saline (PBS), pH 7.4. For each liter, weigh 8.0 g sodium chloride, 0.2 g potassium chloride, 2.9 g sodium dihydrogen phosphate·12 H_2O, and 0.2 g potassium phosphate. Dissolve the components in 900 mL ultrapure water by stirring on a magnetic stirrer and carefully adjust the pH to 7.5 using 1–10 M NaOH. Fill to a final volume of 1 L with ultrapure water, run the buffer through a 0.22 μm filter, and store at room temperature.
5. Elution buffer: 100 mM glycine, pH 2.8. Dissolve 7.5 g glycine in 900 mL ultrapure water by stirring on a magnetic stirrer; carefully adjust the pH to 2.8 using 1–10 M HCl without lowering the pH below the desired value. Fill to a final volume of 1 L with ultrapure water, run the buffer through a 0.22 μm filter, and store at room temperature.

2.5 Desalting

1. Pre-packed HiPrepTM 26/10 desalting column (Sephadex G-25, GE Healthcare).
2. ÄKTAexplorer 100 (GE Healthcare).

3. M12 antibody storage buffer: 10 mM sodium acetate, pH 4.7, 5 mM EDTA. We find the preparation of a 10× stock solution to be the most convenient. For each liter of 10× stock solution, weigh 8.2 g sodium acetate and 37.2 g EDTA·2 H_2O and transfer into a suitable vessel. Add 900 mL of ultrapure water and dissolve by stirring on a magnetic stirrer. Without lowering the pH below the desired value, carefully adjust the pH to 4.7 using acetic acid. Fill to a final volume of 1 L with ultrapure water. The 10× stock solution is stored at room temperature. To prepare the M12 antibody storage buffer, take 100 mL from the 10× stock solution and dilute in 900 mL ultrapure water. Mix by stirring on a magnetic stirrer. Check the pH value and re-adjust to 4.7 if necessary. Run the buffer through a 0.22 µm filter and store at room temperature.

2.6 Storage of Purified M12 Antibody

1. Acrodisc 32 mm sterile syringe filter with 0.2 µm Supor membrane (Pall via VWR International GmbH).
2. 150-mL FlexBoy Bag with luer locks (Sartorius Stedim AG).

2.7 Antibody Analytics

1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE): XCell SureLock Mini-Cell protein gel electrophoresis system using Novex NuPAGE 4–12 % Bis-Tris protein gels and NuPAGE MES SDS running buffer (all Life Technologies, now Fisher Scientific).
2. Coomassie Brilliant Blue staining of SDS-polyacrylamide gels according to Fairbanks et al. [20]: Fairbanks A solution: 0.05 % (w/v) Coomassie Brilliant Blue R-250, 25 % (v/v) 2-propanol, 10 % (v/v) acetic acid; Fairbanks B solution: 0.05 % (w/v) Coomassie Brilliant Blue R-250, 10 % (v/v) acetic acid; Fairbanks C solution: 0.002 % (w/v) Coomassie Brilliant Blue R-250, 10 % (v/v) acetic acid; Fairbanks D solution: 10 % (v/v) acetic acid; Whatman paper.
3. Analytical protein A chromatography: YMC EcoPlus 5/125 glass chromatography column (YMC Europe GmbH, Dinslaken, Germany) packed with 0.3 mL MabSelect SuRe™ and Äktaexplorer 10 (both GE Healthcare). Running buffer: PBS, pH 7.4 (*see* **item 4** in Subheading 2.4).

3 Methods

3.1 Tobacco BY-2 Pre-culture Cultivation

1. Routinely cultivate 100-mL aliquots of transgenic BY-2 suspension cells in 250 mL glass Erlenmeyer shake flasks in the dark on an incubator shaker ($d_0 = 5$ cm) at 180 rpm and 26 °C (*see* **Note 3**).

2. Inoculate 5 L MSN medium inside a 20 L Nalgene polycarbonate carboy vessel with 250 mL of 7-day-old M12 antibody-producing BY-2 suspension cells (from shake flask cultivation) using sterile techniques.
3. Insert and fix the 20 L Nalgene vessel to an incubator shaker ($d_0 = 7$ cm) and incubate the culture at 26 °C shaking at 180 rpm for 7 days.

3.2 100 L Scaled-Up Tobacco BY-2 Cultivation in an SB200-X Orbital Shaker

1. Insert the 350-L disposable bag into the SB200-X (*see* Fig. 2) and connect the supply and exhaust air filters to the bag (*see* **Note 4**).
2. Fill the bag with air by gassing and adjust the position of the bag in the SB200-X bioreactor if necessary (*see* **Note 4**).
3. Connect the tubing of one bag with a Sartopore 2 150 sterile capsule using MPC connectors under sterile conditions. Then, sterilize 100 L of freshly prepared MSN medium by pumping it through the filter at a flow rate of 1.5 L/min directly into the 350 L bag (*see* **Note 5**).
4. The medium is incubated at 26 °C and 80 rpm for 24 h to confirm the sterility of the bioreactor system (*see* **Note 6**).

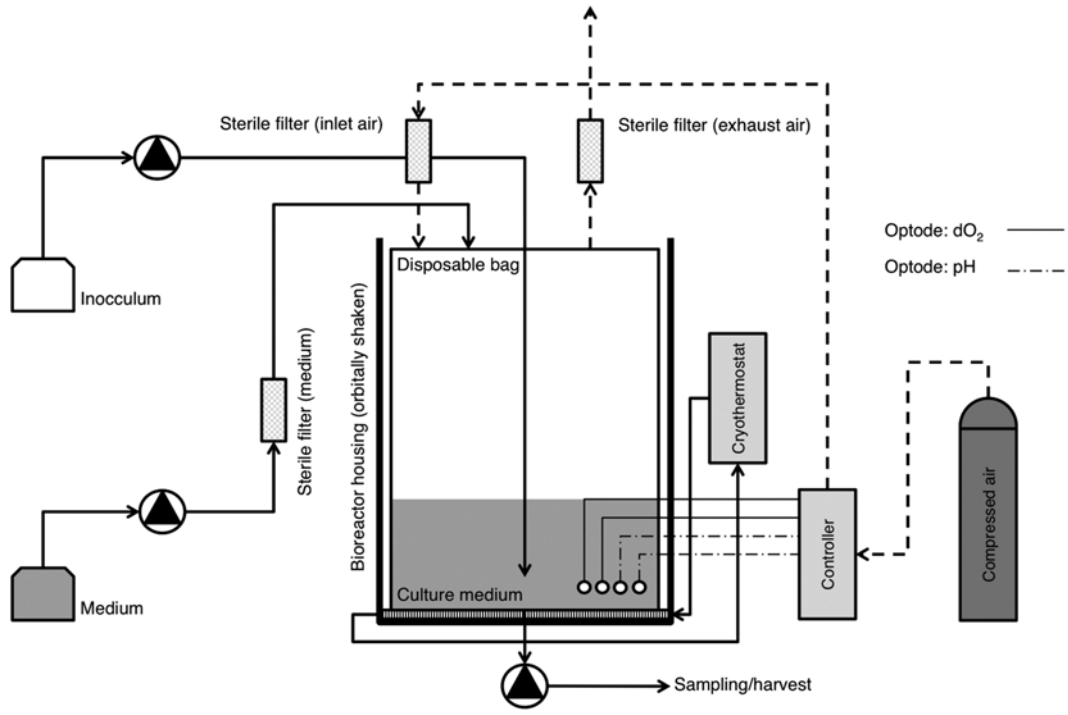

Fig. 2 Process diagram of 100 L scale plant cell-based recombinant antibody manufacture

5. Place the 5 L pre-culture from the Nalgene vessel into the SB200-X bioreactor system using MPC connections that are attached to both cultivation vessels.
6. Cultivate for 5–7 days at 26 °C and 80 rpm (*see* **Note 7**).

3.3 Harvest and BY-2 Cell Removal

1. Harvest the culture broth by opening the harvest tubing of the 350-L disposable bag (*see* Fig. 2) and transferring into an appropriate container (*see* **Note 8**).
2. Prepare the vacuum filtration unit to separate the BY-2 cells from the spent medium by placing two layers of miracloth onto the sieve bottom of the Nutsche filter, then mount the Nutsche filter to the gas washing bottle and connect the gas washing bottle to a vacuum pump. Switch on the pump and pour the harvested culture broth into the Nutsche filter. The BY-2 cells are retained in the Nutsche filter while the spent medium containing the M12 antibody is collected in the gas washing bottle (*see* **Note 9**).
3. Take 20 L from the clarified spent medium and dilute 1:3 with ultrapure water to attain a conductivity of less than 12 mS.

3.4 Expanded Bed Adsorption (EBA) for M12 Antibody Capture

1. Fill the streamline BPG 100/950 glass chromatography column to approximately 1/3 full with deionized water via the bottom valve. Then, reverse the flow direction of the pump to suck out any air that might be trapped underneath the endpiece net. Leave approximately 5 cm of water in the column.
2. Suspend 2 L of Streamline SP adsorbent in PBS to a slurry of approximately 50 %, then quickly pour the slurry into the column and let the resin settle. The resulting bed height should be approximately 31 cm.
3. Fill the column to the rim with deionized water and insert the top adapter without trapping air under the adapter net. Lower the adapter carefully until the gasket is submerged in water. Fill the remaining space with deionized water, then close the column lid, and connect the column to a second masterflex pump that serves as a hydraulic drive to lower the adapter (Fig. 3a).
4. Set all valves to up-flow conditions according to Fig. 3a.
5. Equilibrate the resin with ultrapure water at a flow rate of 550 cm/h, resulting in a stable height of $H/H_0 = 2.0$ (*see* **Note 10**).
6. Pump 60 L of the diluted spent medium through the bed at 183–260 cm/h, resulting in a bed height that does not exceed 80 cm. Avoid further expansion of the bed height by reducing the flow rate if necessary.

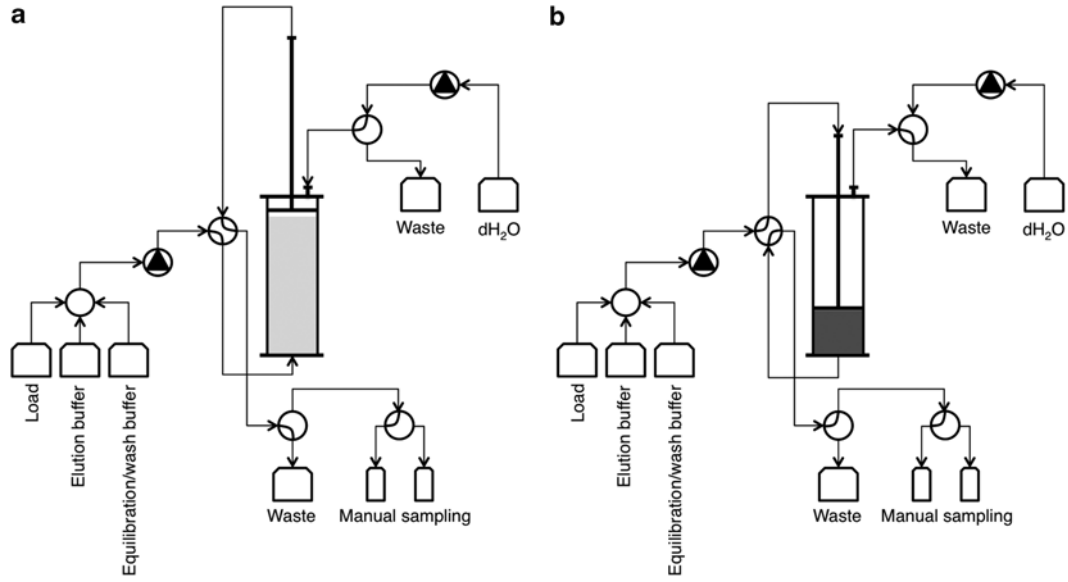

Fig. 3 Process diagram of expanded bed adsorption (EBA) chromatography: (**a**) shows the expanded bed mode used during equilibration, sample loading, and washing, (**b**) shows the packed bed mode used for elution of the antibody

7. Take samples every 10–20 L and determine the M12 concentration in the flow through (e.g., by analytical protein A chromatography) to monitor the capacity of the resin.
8. Wash with 20 L (equivalent to 10 column volumes, CVs) of ultrapure water at 183 cm/h.
9. Enable packed bed mode by setting all valves to down-flow conditions according to Fig. 3b and pump deionized water at 183 cm/h until a stable packed bed is reached. Lower the top adapter by pumping deionized water in the housing between adaptor and lid. Leave approximately 0.5–1 cm space between the packed bed and the adapter.
10. Bound M12 antibody is eluted using high salt elution buffer that is applied in packed bed mode at 183 cm/h. Collect elution fractions of 1-L portions.
11. The antibody content in the elution fractions is analyzed by analytical protein A chromatography (*see* **Note 11**). The pooled antibody-containing elution fractions comprise a volume of approximately 12 L. Insoluble contaminants are removed by pumping the pooled fractions through a filtration unit consisting of a SUPERCAP 100 PDH4 depth filtration module (Pall, Crailsheim, Germany) connected to a Sartopore 2 150 sterile capsule filter (Sartorius Stedim AG). The clarified solution is further processed in a subsequent round of preparative protein A chromatography.

3.5 Preparative Protein A Affinity Chromatography

1. Pour 150 mL of MabSelect SuRe™ into an XK 50/20 column and pack the column according to the manufacturer's instructions.
2. Connect the packed column to the ÄKTAexplorer 100.
3. Equilibrate the column with 3 CVs of glycine, pH 2.8, followed by 10 CVs of PBS, pH 7.4, at 60 cm/h.
4. Load the pooled EBA eluate onto the column at a flow rate of 60 cm/h.
5. Wash the column with 5 CVs of PBS at a flow rate of 60 cm/h.
6. Elute the bound proteins using 5 CVs of 100 mM glycine, pH 2.8, in 20-mL fractions (*see* **Note 12**).
7. Determine the M12 antibody content in the elution fractions by analytical protein A chromatography.
8. Pool the M12-containing elution fractions and use for desalting.

3.6 Desalting

1. Connect a pre-packed HiPrepTM 26/10 desalting column to the ÄKTAexplorer 100.
2. Equilibrate the column with 5 CVs of M12 storage buffer.
3. Pass 15-mL aliquots of the elution fractions containing M12 over the column (*see* **Note 13**).
4. Filter the purified antibody through an Acrodisc 32 mm sterile syringe filter with 0.2 μm Supor membrane that is connected to a 150 mL FlexBoy Bag with a luer lock. The bag is stored at 4 °C. Save a small aliquot to determine the antibody concentration and the final recovery.

3.7 Antibody Analytics: SDS PAGE and Coomassie Brilliant Blue Staining

1. Perform an SDS PAGE with aliquots of the purified antibody according to the manufacturer's instructions.
2. Place the SDS-polyacrylamide gel into a box and cover it with Fairbanks A solution.
3. Close the box with a lid and heat the solution in a microwave until it begins to boil.
4. Incubate the box at room temperature for 5 min with gentle agitation.
5. Discard the Fairbanks A solution and wash the SDS-polyacrylamide gel shortly with deionized H_2O.
6. Cover the SDS-polyacrylamide gel with Fairbanks B solution and repeat **steps 3** and **4**.
7. Cover the SDS-polyacrylamide gel with Fairbanks C solution and repeat **steps 3** and **4**.
8. Cover the SDS-polyacrylamide gel with Fairbanks D solution, add a piece of Whatman paper to remove the remaining dye and repeat **steps 3** and **4**.

9. Discard the Fairbanks D solution and remove the Whatman paper. Add fresh Fairbanks D solution and a new piece of Whatman paper and repeat **steps 3** and **4**.

3.8 Antibody Analytics: Analytical Protein A Chromatography

1. Pour 0.3 mL MabSelect SuRe™ into a YMC EcoPlus 5/125 glass chromatography column and pack the column according to the manufacturer's instructions.
2. Connect the packed column to the ÄKTAexplorer 10.
3. Equilibrate the column with 10 CVs of PBS, pH 7.4, at 611 cm/h.
4. Load the sample onto the column at a flow rate of 92 cm/h.
5. Wash the column with five CVs of PBS at a flow rate of 611 cm/h.
6. Elute the bound proteins using 20 CV of 100 mM glycine, pH 2.8.
7. Determine the antibody content in the elution fractions by comparing the peak area of the M12 elution peak with an M12 standard calibration curve ranging from 4 to 500 µg/mL.

4 Notes

1. Prepare a 2 mg/mL stock solution of 2,4-dichlorophenoxyacetic acid by adding 100 mg 2,4-dichlorophenoxyacetic acid to 50 mL dimethyl sulfoxide (DMSO). Dissolve by stirring on a magnetic stirrer. Aliquot the solution in 1 mL portions and store at 4 °C. Add 100 µL stock solution to 1 L MS medium.
2. Prepare a 4 mg/mL stock solution of thiamine-HCl by adding 200 mg thiamine-HCl to 50 mL ultrapure water. Dissolve by stirring on a magnetic stirrer. Aliquot the solution in 1 mL portions and store at 4 °C. Add 250 µL stock solution to 1 L MS medium.
3. Weekly passaging of the cultures is performed, and fresh cultures are inoculated using 3–5 % (v/v) of a 7-day-old pre-culture.
4. The manual B071_030 "How to fit the disposable bag into SB200-X" provides an excellent illustrated guidance through all of the steps. Please contact Kuhner AG for a copy (http://kuhner.com).
5. Ensure that the exhaust filter is open at all times.
6. Confirm the absence of microbial contamination by microscopic evaluation of a medium sample from the 350 L disposable bag. We use 32-fold magnification with a Leitz Diavert phase contrast inverted microscope (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany).

7. The duration of the cultivation is dependent on the time point at which the cells become oxygen-limited. The dissolved oxygen tension (DOT) is continuously recorded during the cultivation process. Once the DOT drops to 0 %, harvesting should be initiated within the next 24 h.
8. We use 25-L rain barrels.
9. The separated BY-2 cell material is transferred into an autoclaving bag and inactivated by heat sterilization in an autoclave at 121 °C and 100 kPa (15 Psi) for 35 min.
10. The column inlet pressure is constantly measured using an analogue pressure gauge and kept below 100 kPa.
11. Alternatively, M12 antibody quantitation can be determined by sandwich ELISA as described by Kirchhoff et al. [21].
12. Protein elution is monitored by measuring absorbance at 280 nm.
13. The volume will slightly increase (~20 %) during desalting to avoid any loss of protein.

Acknowledgement

This work was supported by the European Union Seventh Framework Program under grant agreement no. 227420 (CoMoFarm).

References

1. Christou P (2013) From medicinal plants to medicines in plants: plant factories for the production of valuable pharmaceuticals. Curr Pharm Des 19:5469–5470
2. Doran PM (2013) Therapeutically important proteins from in vitro plant tissue culture systems. Curr Med Chem 20:1047–1055
3. Fischer R, Schillberg S, Buyel JF et al (2013) Commercial aspects of pharmaceutical protein production in plants. Curr Pharm Des 19:5471–5477
4. Twyman RM, Stoger E, Schillberg S et al (2003) Molecular farming in plants: host systems and expression technology. Trends Biotechnol 21:570–578
5. Basaran P, Rodríguez-Cerezo E (2008) Plant molecular farming: opportunities and challenges. Crit Rev Biotechnol 28:153–172
6. Hofbauer A, Stoger E (2013) Subcellular accumulation and modification of pharmaceutical proteins in different plant tissues. Curr Pharm Des 19:5495–5502
7. Häkkinen ST, Raven N, Henquet M et al (2014) Molecular farming in tobacco hairy roots by triggering the secretion of a pharmaceutical antibody. Biotechnol Bioeng 11:336–346
8. Rival S, Wisniewski J-P, Langlais A et al (2008) *Spirodela* (duckweed) as an alternative production system for pharmaceuticals: a case study, aprotinin. Transgenic Res 17:503–513
9. Woodard SL, Wilken LR, Barros GOF et al (2009) Evaluation of monoclonal antibody and phenolic extraction from transgenic Lemna for purification process development. Biotechnol Bioeng 104:562–571
10. Hellwig S, Drossard J, Twyman RM et al (2004) Plant cell cultures for the production of recombinant proteins. Nat Biotechnol 22:1415–1422
11. Walmsley AM, Doran PM (2012) Foreign protein production using plant cells: opportunities and challenges. Biotechnol Adv 30:385–386
12. Nagata T, Nemoto Y (1992) Tobacco BY-2 cell line as the "HeLa" cell in the cell biology of higher plants. Int Rev Cytol 132:1–30
13. Schillberg S, Raven N, Fischer R et al (2013) Molecular farming of pharmaceutical proteins

using plant suspension cell and tissue cultures. Curr Pharm Des 19:5531–5542

14. Fischer R, Schillberg S, Hellwig S et al (2012) GMP issues for recombinant plant-derived pharmaceutical proteins. Biotechnol Adv 30: 434–439
15. Eibl R, Kaiser S, Lombriser R et al (2010) Disposable bioreactors: the current state-of-the-art and recommended applications in biotechnology. Appl Microbiol Biotechnol 86:41–49
16. Eibl R, Eibl D (2006) Design and use of the wave bioreactor for plant cell culture. In: Gupta SD, Ibaraki Y (eds) Plant tissue culture engineering. Springer, Dordrecht, Netherlands, pp 203–227
17. Eibl R, Eibl D (2009) Design of bioreactors suitable for plant cell and tissue cultures. Phytochem Rev 7:593–598
18. Georgiev MI, Eibl R, Zhong J-J (2013) Hosting the plant cells in vitro: recent trends in bioreactors. Appl Microbiol Biotechnol 97: 3787–3800
19. Zhang X, Stettler M, De Sanctis D et al (2010) Use of orbital shaken disposable bioreactors for mammalian cell cultures from the milliliter-scale to the 1,000-liter scale. Adv Biochem Eng Biotechnol 115:33–53
20. Fairbanks G, Steck TL, Wallach DF (1971) Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry 10:2606–2617
21. Kirchhoff J, Raven N, Boes A et al (2012) Monoclonal tobacco cell lines with enhanced recombinant protein yields can be generated from heterogeneous cell suspension cultures by flow sorting. Plant Biotechnol J 10:936–944