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REVIEW ARTICLE

Bioprocessing of mAb (Monoclonal Antibodies) using Chinese Hamster Ovary (CHO) Cells: A Review

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ABSTRACT

Monoclonal antibodies (mAbs) are able to be produced from Chinese Hamster Ovary (CHO) cells. It has become increasingly important throughout the years due to its efficiency and revolutionary treatment for various diseases such as cancer, autoimmune diseases, and so forth. This review examines several upstream and downstream processes involved in the production of mAb including CHO cell maintenance, cellular engineering of CHO, transfection of plasmids into CHO cells, clonal selection and screening, culture of rCHO (recombinant CHO) cells, cell harvesting, purification, and polishing. Several challenges of the bioprocessing process include a lack of large up scaling for industrial purposes, high capital costs, as well as productivity inefficiencies. However, a solution proposed is for biopharmaceutical companies to further research into downstream processing for a continuous, efficient, and productive process.

KEYWORDS

Bioprocessing process; Chinese Hamster Ovary (CHO) cells; Downstream process; Monoclonal antibodies; Upstream process

HIGHLIGHTS

- mAbs can be used to treat cancer and autoimmune diseases.
- This review examines the upstream and downstream processes in the mAb production from CHO cells.
- The upstream processes include gene transfection into CHO cells and their culture.
- Downstream processes include cell harvest, purification, and polishing.
- The future direction is the advancement and increased effectiveness of mAb production.

INTRODUCTION

Therapeutic monoclonal antibodies are becoming increasingly important, as they have been the primary therapy technique for a variety of diseases over the last 25 years (Lu et al., 2020). mAbs could be use as biosimilars, in which they are similar to an already-approved reference biological product (i.e., the "original" or "innovator" drug). Biosimilars are typically manufactured after the expiry of the patent for the reference product. Since they do not have to go through the extensive clinical trials that the original product underwent, they can be produced more efficiently and at a lower cost, making them a more accessible treatment option for patients (Isaacs et al., 2017). Additionally, advances in technology and manufacturing

processes can also contribute to the reduced cost of biosimilars. Biosimilars are used to treat various diseases, including cancer and autoimmune disorders (Rugo et al., 2019). An example of this is rituximab. Rituximab works by reducing the number of B cells in the blood circulation; it does so by binding to the CD20 surface marker on B cells, triggering cell death (Randall, 2016). mAbs have an advantage over polyclonal antibodies because of their great repeatability employing culture methods and specificity (Tyagi et al., 2011). Current technological advancements and high demands have also enabled the discovery and development of mAb therapies to be faster and more efficient (Lu et al., 2020).

Monoclonal antibodies (mAbs) are created by exposing white blood cells to a specific viral protein, which is then cloned to mass generate the antiviral antibodies (Lloyd et al., 2021). The base of creating monoclonal antibodies can be divided into 4 types which are murine monoclonal antibodies (ends with -omab), chimeric monoclonal antibodies (ends with -ximab), humanized monoclonal antibodies (end with -zumab), and human monoclonal antibodies (ends with -umab) (Almagro et al., 2018). Murine monoclonal antibodies are generated from mice that are created in laboratories. They are used to treat a variety of illnesses, particularly, cancer because they are created to replicate the natural antibodies of the human immune system. They act by focusing on particular molecules or cells within the body and preventing them from performing their job, which helps to lessen symptoms and improve health outcomes (Holzlöhner & Hanack, 2017). Chimeric antibodies are structural chimeras created by joining the consistent parts of one species, like a human, with the variable regions of another, like a mouse (Doevendans & Schellekens, 2019). Humanized antibodies are antibodies from non-human species (such as mice or rats), in which their protein sequence have been engineered to resemble human antibodies in terms of their structure and function. The difference between human and humanized monoclonal antibodies is that humanized mAbs are first produced in wild type mice with a native genome harboring the mouse immunoglobulin locus, while fully human mAbs can be made in transgenic mice that have been genetically altered with the human immunoglobulin locus (Mallbris et al., 2016).

The most common cell line for the manufacturing of mAbs is Chinese hamster ovary cells (CHO). CHO cells are known to have a successful record and the ability to undergo post-translational modifications (Orellana et al., 2015). The aim of this review is to assess the production of mAbs using the CHO cells. This review will cover and discuss the upstream and downstream processes of mAbs derived from Chinese hamster ovary cells (CHO) including the type of bioreactors used, the operation mode, the environmental parameters, the monitoring of the process, feeding strategies, harvesting methods, types of chromatography, and filtration methods. Lastly, the advantages and disadvantages of using CHO cells in performing monoclonal antibodies will be explored, as well as the possible methods to overcome these disadvantages. The entire dependence on previously published research, the many search techniques used by the authors, and the accessibility of these studies are the limitations of this literature review.

CHINESE HAMSTER OVARY (CHO) CELLS

The CHO cell line was sub-cultured from the original ovary tissue of *Cricetulus griseus* (or Chinese hamster) in 1957 and found to be immortal. Successive cloning of this cell line resulted in variants of sublines such as CHO-K1, CHO-K1SV, and CHO-S cell lines, which, when mutagenized, will produce further variations of cell lines, such as CHODXB11 (or CHO-DUKX) and CHO-DG44 which lack in dihydrofolate reductase (DHFR) activity (Dhara et al., 2018; Hacker & Wurm, 2017).

Over the decades, CHO cells have been the primary host for commercial recombinant protein manufacturing. According to Zhu et al. (2017), approximately 70% of all recombinant therapeutic proteins are produced in CHO cells. This cell line was used in mAb production to combat the problems arising from using the hybridoma technology (where B cells of mice are fused with myeloma cells), such as the instability of hybridoma cells and the production of human anti-mouse antibodies (HAMA) by patients (Kunert &

Reinhart, 2016). Moreover, CHO cells offer advantageous characteristics which contribute to their popularity. Firstly, their ease of cultivation, capability to grow at high cell densities in single-cell suspension culture, and adaptability to grow in regulatory-friendly serum-free conditions make them a preferred choice in scaling up and bioreactor settings. Secondly, their ease of transfection with plasmid DNA is a benefit of using CHO cells (Hacker & Wurm, 2017). Furthermore, CHO cells do not produce infectious endogenous retroviruses and do not allow the growth of most pathogenic human viruses such as HIV, influenza, and polio in them, decreasing the risk of viral contamination and increasing the safety of mAb production (Hacker & Wurm, 2017; Ho et al., 2013-a). CHO cells can also be genetically modified easily to improve mAb production as their genome consists of large, observable 11 pairs of chromosomes (Wurm & Wurm, 2017). Last but not least, they have a history of producing safe, biocompatible, and bioactive mAb over the past decades, proving them to be safe hosts and enabling their products to gain regulatory approval readily (Ho et al., 2013-a).

MAMMALIAN EXPRESSION VECTORS AND RECOMBINANT DNA TECHNOLOGY

For a CHO cell to produce the antibody of interest, the genes of said antibody must be first expressed in the cell by using recombinant DNA technology to insert the antibody gene of interest into a vector (which is independently replicating DNA molecules used as a vehicle to transfer foreign lengths of DNA). This recombinant vector can then be subsequently transferred into the CHO cell to express the antibody protein. Plasmids (which are circular, extrachromosomal DNA) are commonly used as a mammalian expression vector since recombinant proteins will be expressed in a mammalian CHO cell (Khan, 2013; Zhu & Hatton, 2017).

To insert the antibody gene of interest into the plasmid, restriction enzymes such as BamHI, XbaI, and AgeI are first used to cut the plasmids. Then, the DNA fragments encoding the variable regions of a specific antibody, such as anti-CD20, anti-CD22, and anti-TNFα, are fused with the constant regions of the antibody, producing its respective sequences for HC (heavy chain) and LC (light chain) (Haryadi et al., 2015; Voronina et al., 2016). Controlling the LC:HC peptide ratio is essential to the kinetics of mAb formation. It was reported that excess LC and an LC:HC ratio of more than 1.5 are favored since incompletely formed HCs can cause the mAb clones to aggregate and have inconsistent glycosylation (Ahmadi et al., 2017; Ho et al., 2013-a; Ho et al., 2013-b; Li et al., 2018). The DNA fragments are then joined together to the plasmid by the enzyme ligase, such as T4 DNA ligase, or by overlap extension PCR and cloned (Haryadi et al., 2015; Voronina et al., 2016). This process is compactly demonstrated in Figure 1 below.

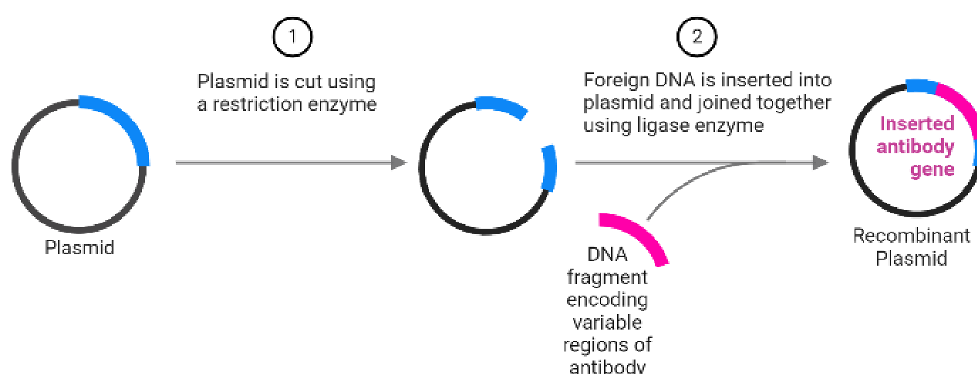

Figure 1. Recombinant DNA technology to induce desired antibody production

BIOPROCESS OF mAb PRODUCTION

Bioprocessing is defined as the synthesis of a product that utilizes living resources (Cossar, 2011). In order to produce mAbs, its bioprocess can be divided into two sections, which is the upstream and downstream processes. The upstream process includes the harvesting and production of the substance from raw materials; in mAb production, this includes CHO cells maintenance, cellular engineering of CHO cells, transfection of mammalian expression vectors into CHO cells, clonal selection and screening of rCHO cells, and culture of rCHO cells. On the other hand, the downstream process involves extracting, purifying, and filtering the final product; in mAb production, it encompasses cell harvest (centrifugation/filtration), purification using Protein A Affinity chromatography, and polishing (Gronemeyer et al., 2014).

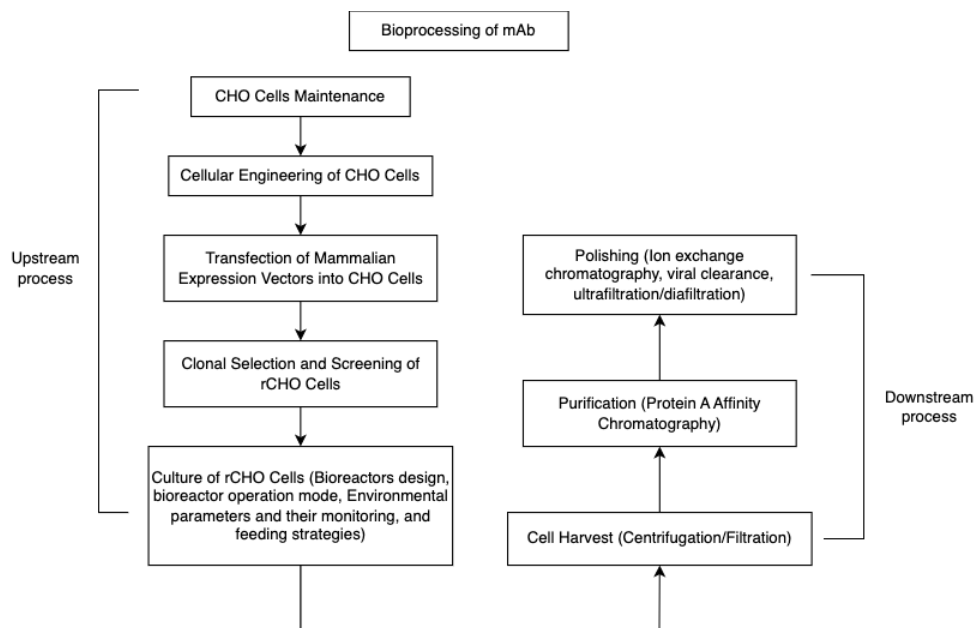

Figure 2. Overall bioprocessing of mAb production

UPSTREAM BIOPROCESSES OF mAb PRODUCTION

CHO cells maintenance

Host CHO cells must be cultured and maintained in a growth medium appropriate for CHO cells prior to being subjected to any processes or treatments. Different CHO cell lines use slightly different modified media (for example, CHO-DG44 requires a growth medium rich in DHFR) with the addition of glutamine, antibiotics to prevent microbial contamination, and anti-clumping agents. The cells are typically incubated in humidified conditions at 37°C and 5% CO_2 and passaged every few days. The common determination of cell viability and density is by using the trypan blue exclusion method, where viable cells will appear colorless and non-viable cells will be stained blue since the dye trypan blue cannot pass through the complete cell membrane of viable cells (Ahmadi et al., 2017; Bayat et al., 2018; Fouladiha et al., 2020; Ho et al., 2013b; Inniss et al., 2017; Noh et al., 2018; Reinhart et al., 2015; Strober, 2015; Voronina et al., 2016; Zboray et al., 2015).

Cellular engineering of CHO cells

The modification of host CHO cells at the cellular level has been proven to improve the yield, quality, and efficiency of mAb production, where its principles lie in the overexpression of exogenous effector genes and the suppression or silencing of endogenous gene expression (Hacker & Wurm, 2017). This is mainly

achieved by slowing down apoptosis, regulating cell cycle progression, improving glycosylation patterns, engineering chaperone proteins, and metabolic engineering (Dangi et al., 2018; Datta et al., 2013).

Transfection of mammalian expression vectors into CHO cells

Transfection refers to the delivery of genes into eukaryotic cells without the use of viruses. Therefore, in the case of CHO cells, plasmids are used to deliver recombinant genes to the cells, so that they are able to express the antibody proteins (Hacker & Wurm, 2017). Transfection comprises both chemical and physical methods. The main chemical methods include lipofection with cationic liposomes, polyfection with the cationic polymers such as polyethylenimine (PEI), and the use of calcium phosphate (CaPi). Since plasmids are too large to pass through the cell membrane, these delivery agents form complexes with the plasmids which help to bind with the membrane, allowing the endocytosis of the plasmids. On the other hand, the main physical methods of transfection include electroporation, microinjection, and ballistics. Even though these methods allow the plasmids immediate access to the nucleus since they directly overcome the physical cellular barrier, they are difficult to scale up. The common transfection methods are the use of CaPi and electroporation (Hacker & Wurm, 2017).

Clonal selection and screening of rCHO cells

Transfected monoclonal antibodies will generate a stable cell pool that will undergo single-cell cloning, followed by the analysis of the mAb activity inside the cells. The commonly applied cloning technique is the limiting dilution technique, where monoclonal cell suspensions are obtained from parent cells that are polyclonal. The procedure typically includes the dilution and cultivation of the transfected cells in 96-well plates that contain a growth medium. Moreover, the ELISA assay is a popular choice in the primary screening of the cell line with low to no productivity in mAb production before sending the cells for scaling up (Ahmadi et al., 2017; Bayat et al., 2018; Inniss et al., 2017; Noh et al., 2018; Voronina et al., 2016; Ye et al., 2021; Zboray et al., 2015). This immunoassay is used to detect the presence and determine the number of substances such as antibodies, proteins, antigens, et cetera by measuring the antibody-antigen complexes produced in the process (Alhajj & Farhana, 2022). However, there is another screening method found which is PCR (polymerase chain reaction) and ddPCR (droplet digital polymerase chain reaction) which in the experiment were utilized, as it can scan the EGFP (enhanced green fluorescent protein) expression in the clone. If the screening process finds a clone that does not contain a landing pad (LP), then they will remove it. In other words, they will maintain the recombinase-mediated cassette exchange (RMCE) landing pad, which allows the reproduction site-specific genomic incorporation, in an excellent manner (Inniss et al., 2017).

Culture of rCHO cells

Bioreactors design. The bioreactor designs that are commonly used in the culturing of rCHO cells are a pitch-blade impeller bioreactor system and a packed-bed bioreactor system — both are stirred-tank bioreactors. The bioreactor helps to provide an ideal environment for the biological reaction to occur and helps to scale up the number of rCHO cells (Bhatia & Bera, 2015).

The packed-bed bioreactor system (Figure 3a) is equipped with a packed-bed impeller that is designed to have punctured metal screens that are horizontally positioned in a basket. In addition to the metal screens, Fibra-Cel disks are placed in between the metal screens to ensnare the suspension cells or create a surface for anchorage-dependent cells to adhere to (Zhang et al., 2015). The Fibra-Cel disk bed creates an environment that permits fresh oxygenated mediums to pass over the cells at a slow pace while also protecting them from external shear stresses, as seen in Figure 3. The motion of the impeller helps to provide a negative pressure that pushes the medium up through the hollow center shaft, where the sparger adds oxygen (Hatton, 2012). Moreover, the packed-bed reactor system is also equipped with cell lift impeller

technology. This acts as an agitator and allows the medium to travel from the bottom to the top, receiving gas, before it is expelled via the three discharge ports situated at the top. The gaseous medium will next circulate through the Fibra-Cel disk bed that holds the cells; hence, the cells are not exposed directly to the gas itself. If the cells were to be directly exposed to the gas, it could damage their growth and overall health (Guertin, 2016; Zhang, 2017).

The pitch-blade impeller bioreactor system consists of a flat blade that is set at a 45° angle, as visualized in Figure 3b. The position of the blade increases the oxygen mass transfer, allows the media to be evenly mixed via a radial and axial flow, and it increases the disruption of the bubbles that are created by the sparger. It is designed to reduce the mixing stress placed upon the shear-sensitive cells (Hatton, 2012). Furthermore, the pitch-blade impeller bioreactor was set with the following parameters: dissolved oxygen levels at about 35%, agitation speed of 120 rpm (+/- 5 rpm), a pH of around 7.1, and a constant temperature of 37° C (Bulnes-Abundis et al., 2012). These parameters are commonly used for mammalian cell culture meaning that these parameters would work for the production of mAbs from Chinese hamster ovary cells. However, several experiments may need to be performed to ensure these parameters are ideal, in this case.

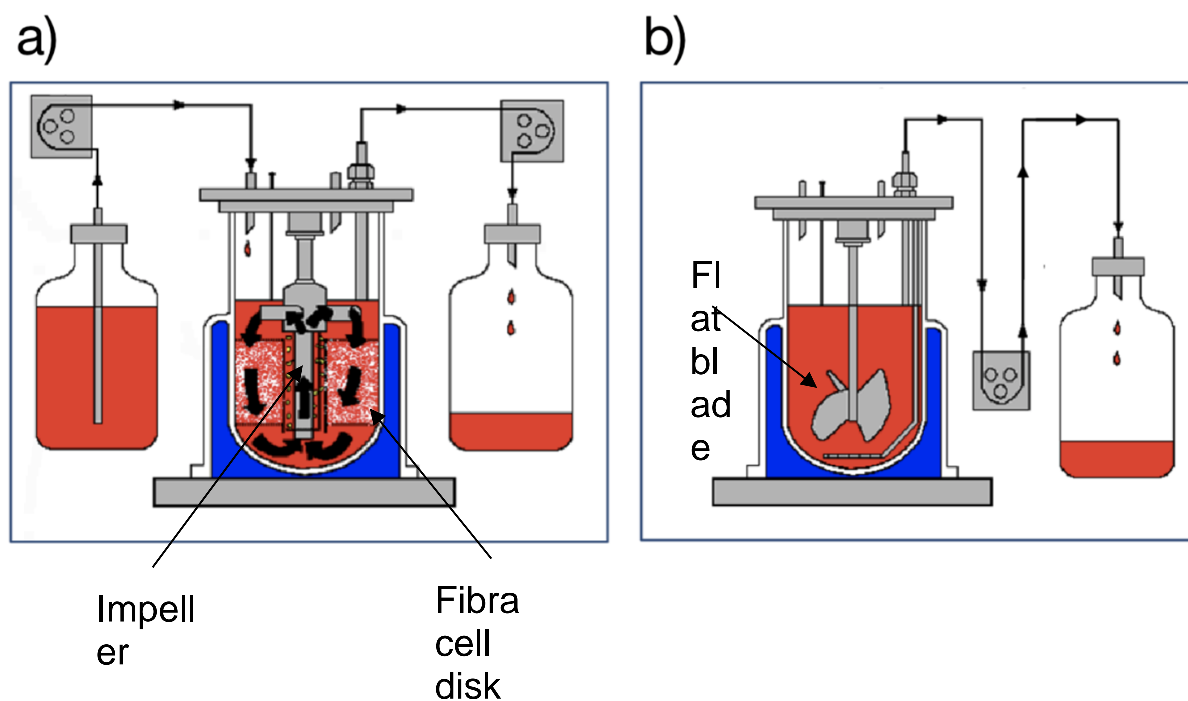

Figure 3. (a) packed-bed bioreactor and (b) pitch-blade impeller system (Adapted from Hatton, 2012)

According to Rashid et al. (2012), in comparison to the rCHO cells grown in a pitched-blade bioreactor, rCHO cells that are produced in a packed-bed bioreactor consumed more glucose and at a faster pace over the course of 5 days, indicating greater cell counts and high metabolic activity. The product yield in a packed-bed bioreactor can result ten-fold compared to other processes due to the large surface area possessed by the bed of the FibraCel disks; this allows the bioreactor to run for a longer period, saving time and money (Zhang, 2017). In addition, since the cells of a packed-bed bioreactor are contained in-between the Fibra-Cel disks, the packed-bed bioreactor allows the media to be changed without affecting the cells (Hatton, 2012). Hence, Rashid et al. (2012), Hatton (2012), and Zhang (2017) concluded that the packed-bed bioreactor is more suitable to be used.

Bioreactor operation mode. Fed-batch and batch are two of the most common types of cultivation. The batch process consists of the cells being grown in a nutrient-rich base medium that has a specific volume,

wherein the entire batch is then harvested once the manufacturing process is complete. The duration of the batch process normally takes a week, and the entire batch would be collected when the viability level reaches below 30% (Templeton et al., 2013). Although it has a simple setup, other studies have shown that the batch process has low levels of productivity, low cell density, rapid depletion of nutrients, and a large accumulation of byproducts (Zhang, 2017).

The fed-batch process involves a nutrient-rich medium being added to the cultivation in order to avoid depletion of nutrients, support large numbers of cell density, and support the production for a longer period of time. However, in fed-batch mode, the byproduct accumulation is unavoidable, and the culture is changing over time, resulting in product quality varying over time (Zhang, 2017).

Other operation modes of bioreactors such as a perfusion culture system incorporate a cell retention function and the bioreactor together, and can also be used in the culturing of rCHO cells as mentioned by Zhang et al. (2015). This bioreactor system functions by housing the cells in a non-woven polyester matrix that is perfused with the culture medium, trapping them, and submerged in a reservoir inside this system as seen in Figure 4 (Zhang et al., 2015). In addition, they are held by attaching them to a support, such as a membrane, matrix, or a cell retention device, such as spin filters, fixed bed, or gravity settlers. Since the cells are connected to a carrier, it allows efficient separation to be possible; several factors that are considered in the retention process include particle size and density (Bielser et al., 2018; Pollock et al., 2012). In perfusion, the secreted products are also automatically broken apart from the cells removing the need for filtration and membrane fouling. Compared to fed-batch processes, the perfusion system is able to reach 4-10 times higher volumetric output rates than fed-batch processes (Zhang, 2017). However, there have been concerns revolving around the cost of perfusion due to the high quantity of media that it consumes (Langer & Rader, 2014).

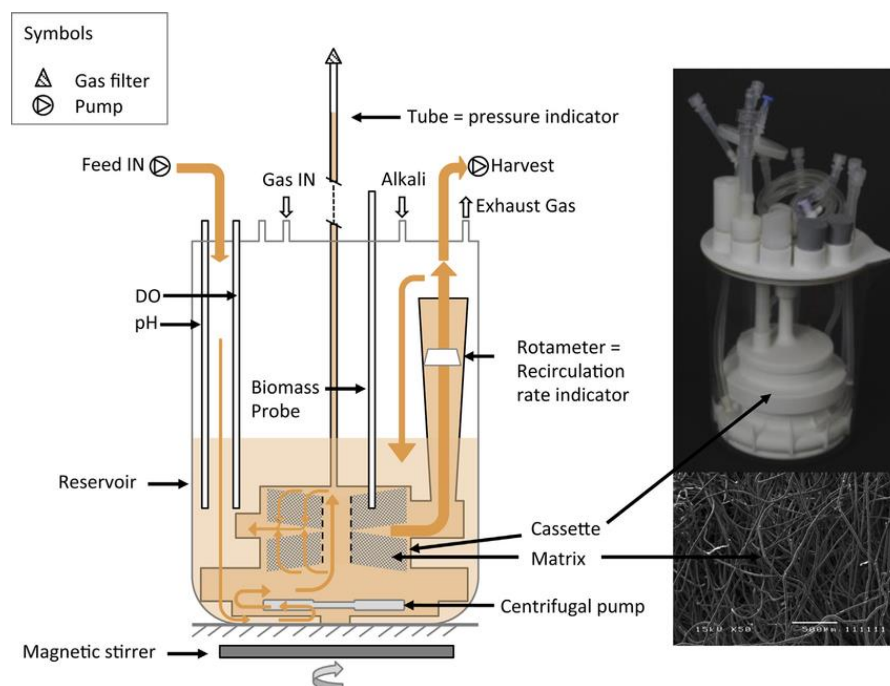

Figure 4. Perfusion culture system (Zhang et al., 2015)

Environmental parameters and their monitoring. The most important step to gain high-quality product yields is by optimizing the cell culture parameters, which can be divided into physical parameters (consisting of temperature, impeller speed, and gas flow), chemical parameters (such as dissolved oxygen and carbon dioxide concentration, redox potential, pH, and osmolality), and biological parameters (such as cell cycle, viability, mitochondrial activity, NADH and LDH levels) (Kunert & Reinhart, 2016). However, the

main parameters that are usually monitored in rCHO cell culture of mAb production involve pH, dissolved oxygen (DO), temperature, and osmolarity, and they are volume-independent parameters (Batra & Rathore, 2016).

pH of the cell culture is generally maintained at 6.8-7.8, where optimum pH is at the neutral level of 7.0-7.1. Slightly basic pH has also been reported to be preferable during the initial phase of cell growth (Batra & Rathore, 2016; Karst et al., 2017). Accumulation of toxic by-products during cell growth, such as lactic acid and ammonia, is able to deviate the pH of the culture, where even the slightest changes in the pH can be detrimental to the culture growth and metabolism (Kunert & Reinhart, 2016). pH is therefore monitored by electrochemical pH sensors in large scale-ups while smaller bioreactors usually use online fluorescence-based optical sensors and fiber optic sensors (Biechele et al., 2015; O'Mara et al., 2018; Zhao et al., 2015). If any changes in the pH are detected, the processing system will either sparge carbon dioxide to make the medium more acidic or add a base, usually the buffer sodium bicarbonate, to make it more alkaline (Li et al., 2010).

DO level is maintained to be 20-50%, as a value lower than this range could lead to anaerobic respiration of the cells, and a value higher could lead to oxygen toxicity (Karst et al., 2017; Li et al., 2010). Low DO has also been reported to alter the final mAb product quality by reducing its glycosylation (Batra & Rathore, 2016). Clark-type electrochemical sensors, which are membrane-covered amperometric electrodes that allow oxygen to pass through the membrane and get reduced at the platinum cathode to generate a voltage proportional to DO concentration, are generally used to monitor DO levels. Meanwhile, optical sensors can also be used to detect DO levels in smaller scales (Biechele et al., 2015; O'Mara et al., 2018; Stine et al., 2020; Zhao et al., 2015). DO level adjustments are achieved via alterations to the agitation, gas flow rate, and the opening diameter of the oxygen sparger which control how much oxygen is released into or how much carbon dioxide is removed from the medium. The adjustment of agitation is based on specific energy dissipation rate by varying the type, speed, and positioning of the impeller in the bioreactor to promote adequate mixing of the gases and oxygen mass transfer. Meanwhile, gas flow rate is adjusted by altering oxygen concentration and the volumetric flow of oxygen. Oxygen volumetric flow can also be adjusted by either increasing the diameter of the oxygen sparger for larger delivery volumes or decreasing the diameter for smaller volumes (Li et al., 2010).

Temperature is maintained at 37℃ (Karst et al., 2017; Liu et al., 2014) but can be lowered to a range of 30-35℃ in the later stages of the growth cycle (Clavaud et al., 2013). It is measured by platinum resistance thermometers (PRT) and thermocouples, where PRT is used if a high degree of accuracy and faster response time is required (O'Mara et al., 2018).

Lastly, osmolality refers to the concentration of dissolved particles in the medium and it plays a significant impact on mAb productivity and cell viability. High osmolality may result in the growth inhibition of the CHO cells and it is a direct result of all the dissolved nutrients in the medium (e.g., gases, glucose, amino acids) and the accumulation of growth by-products. The osmolality of mAb production is maintained at the range of 270-330 mOsm/kg but it has been reported that an osmolality of between 450-470 mOsm/kg shows increased mAb production. It is measured by vapor pressure osmometers or freezing point depression osmometers (Alhuthali et al., 2021; Li et al., 2010; Nasseri et al., 2014).

Feeding strategies. Culturing CHO cells are commonly done with 1% up to 20% fetal bovine serum growth media as the base. However, there is a great risk when using FBS serum growth media which is prone to contamination by animal-derived materials. In order to increase process performance and robustness, the implementation of fully chemical-defined media is important, one of them is by developing serum-free media. Instead of serum, soy, yeast, and wheat can be processed for the animal-component free protein hydrolases. Serum production should be minimized as it is non-standardized and may lower the outcome of bioprocess (Kunert & Reinhart, 2016). This results in the desire of replacing the serum with serum-free media. For the serum-free media, it can be replaced with a chemically defined medium such as DMEM/F-12 which is common in serum-free cultures. Other examples of the effort of replacing the serum media are using

transmissible spongiform encephalopathy or animal-derived hydrolysate-free media. Though it is important to pick the most suitable basal media to optimize the mAb production, it is also common to add supplements for media enhancement (Kishishita et al., 2015).

One of the concerns during cell development is maintaining cell viability in the system, therefore, a sophisticated approach to feeding should also be implemented (Spearman et al., 2014). Hydrolysate is a protein that is a product of hydrolysis which is commonly used to enrich the basal media. Hydrolysate itself can come from either animal or plant, however, for cell culture, the plant-derived or chemically defined (CD) hydrolysate is preferred to keep the product animal component-free. Hydrolysate feed can come from yeast, wheat, or soybean and it is an easy method since it uses easily-sourced materials when upscaling, until large-scale manufacturing processes (Ho et al., 2016). A recent study found that choline limitation caused lower cell viability, therefore fortifying the medium with choline chloride can further enhance the mAb titer, especially in fed-batch culture (Kuwae et al., 2018).

Lactate, which is a major waste product in mammalian cell culture, can potentially affect the culture performance. By-product accumulation of lactate can cause the system to experience the Warburg effect, leading to an increase in anaerobic glycolysis even in normal oxygen environments (Buchsteiner et al., 2018). Earlier studies showed that CHO cells have the tendency to produce lactate. This was because of the impairment of mitochondrial oxidative metabolism; however, this is also found to be affected by media composition (Zagari et al., 2013). A common method to control the byproduct accumulation is by minimizing the glucose content which directly reduces the lactic acid levels. However, a decrease in glucose causes undesirable uptake of glutamine, which subsequently increases ammonia as another byproduct. Therefore, the accumulation of lactic acid and ammonia can be avoided by controlling both glucose and glutamine simultaneously (Freund & Croughan, 2018). Alternatively, to reduce lactate accumulation, inducing lactate consumption is also feasible. According to Yuk et al. (2014), a higher copper concentration can increase the net lactate consumption of the culture cell, while still maintaining both the cells viability. Furthermore, CHO cells are known to have a high toxic tolerance to copper, therefore it will increase the mAbs yield if copper is added to the CHO cell culture to decrease lactate accumulation.

DOWNSTREAM BIOPROCESSES OF mAb PRODUCTION

Downstream processing is the recovery and purification of a biological product from cell culture broth to a final purified product (Jungbauer, 2013). This portion of bioprocessing first involves the harvesting of the CHO cells and the recovery of the extracellularly secreted mAb in the cell culture broth, followed by a series of intermediate purification and polishing procedures for the product. Factors such as different cell culture processes, expression systems, and types of the mAb product can customize the methods of the downstream approach; however, they all should satisfy the criteria that the system should be robust and applicable to extensive IgG (Immunoglobulin G) molecules without significant changes and that the downstream processing should be time-minimizing (Paul et al., 2014; Shukla et al., 2017).

Cell harvest by centrifugation/filtration

Cell harvest is the removal of suspended matter such as cells, cell debris, or impurities to isolate the product for the subsequent chromatography processes (Marichal-Gallardo & Álvarez, 2012). mAbs are usually soluble in the cultures and the amount inside the cells is negligible. Consequently, the harvesting system typically starts by suspending the broth, and then the suspended matter is removed, and one of the most common methods used is centrifugation with a vertically mounted disk stack design. This harvesting technique makes use of the insoluble solids and liquid medium density difference to create solid suspension components through the centrifugal forces, it separates the product with the mixed liquids from cells, other

particulates, and cellular debris (Marichal-Gallardo & Álvarez, 2012). When using this type of harvesting method, a few variables such as g-force, residence time, and discharge frequency are often subjected to evaluations, and cell culture feedstock quality such as cell density and viability at harvest have an impact on the clarification efficiency of the centrifuge (Mehta & Vedantham, 2018).

G-force is the centrifugal driving force of the conical disk stack that separates solid particles; nevertheless, working at high g-force can produce severe shear stress, causing cell damage and the release of soluble impurities such as DNA and HCP. Another component, residence time, can also have an effect on volumetric throughout, with longer residence times increasing clarifying efficiency but decreasing volumetric throughout with the risk of product deterioration. The discharge frequency parameter relates to the activity of opening a release valve to release solid waste that may build in the stream. Because each discharge wastes a portion of the product supernatant, thus lowering the yield, it is advised that the discharge duration be calculated using the following formula (Pieracci et al., 2018).

$$T_{discharged} = \frac{(V\ x\ F)}{(Q\ x\ S)}$$

Figure 3. Discharge Time Equation where; Q is feed flow rate, S is % solids mass, V is bowl volume, and F is safety factor (Pieracci et al., 2018).

The clarification of mAb products can also be done through filtration. One of the methods is depth filtration — it removes impurities by physically capturing them in their narrow pore spaces and also through electrostatic and hydrophobic adsorptive interactions. Depth filtration is often done to remove the cells and cell debris from the product. This process should be done before the chromatography as it can cause the column to become fouled. Depth filtration is done in between two processes, after harvest centrifugation, specifically to promote DNA clearance from the culture broth and also debris, whereas depth filtration before the affinity chromatography, is conducted to reduce the host cell protein (Yamada et al., 2017). However, the clarification method that is applicable is not only depth filtration but there is also flocculation, acoustic cell retention devices, or dynamic body feed filtration (Kruse et al., 2019). Moreover, filtrations are done in between two processes respectively, after harvest centrifugation, and before purification or chromatography, in order to produce a more clarified mAb product.

Purification by Protein A Affinity Chromatography

Protein A affinity chromatography is the commonly used initial capture method in the purification of almost all commercialized mAb (Natarajan & Zydney, 2013). It is involved in the separation of the IgG/mAb molecules from host cell proteins (HCP) and other impurities under the basis of a reversible binding between the immobilized ligands in the chromatography matrix and a part of the mAb (Bolton & Mehta, 2016).

Protein A, the ligand used in this chromatography, can either be obtained naturally from the cell walls of *Staphylococcus aureus* or from *Escherichia coli* as a recombinant protein ligand (where the cell wall domain is deleted) (Marichal-Gallardo & Álvarez, 2012). It is immobilized in a natural base matrix, such as agarose and cellulose, or a synthetic base matrix, such as polyvinyl ethyl (Ramos-de-la-Peña et al., 2019). The clarified cell culture supernatant obtained from the previous step is loaded into the chromatography column and as it passes through the Protein A resin at pH 6-8, the Fc region of the IgG, specifically between CH_2 and CH_3, binds to Protein A due to hydrophobic interactions, allowing the impurities to flow out of the column (Marichal-Gallardo & Álvarez, 2012; Ramos-de-la-Peña et al., 2019). The column is then washed to further remove impurities such as product isoforms, nucleic acids, HCP, product aggregates, and leached Protein A. The products bound to the resin are eluted at pH 3-4 by a low pH buffer, usually glycine since it promotes the detachment of the IgG-Protein A complex by mutually repellant charges. Finally, the resin is regenerated by

sanitization using acetic acid and ethanol to remove any residual bound to it (Bolton & Mehta, 2016; Marichal-Gallardo & Álvarez, 2012; Ramos-de-la-Peña et al., 2019).

Protein A affinity chromatography offers various advantages, such as the exclusion of any pretreatment of the clarified cell culture broth before loading to directly capture the mAb. Its high selectivity and flow rate also make it quicker and easier to bind to the mAb and eliminate most HCP, providing a supply of product with high purity and a yield of more than 95% in a single batch (Natarajan & Zydney, 2013; Saraswat et al., 2013). Moreover, it has been reported that this chromatography is able to indirectly remove viruses such as SV40 (Saraswat et al., 2013). These benefits are able to outweigh some of the drawbacks of Protein A affinity chromatography, which are its expensive cost, Protein A leaching which decreases resin selectivity, caustic instability caused by the acidic environment during elution which shortens resin life, and resin fouling by contaminants overtime which decreases the binding affinity and capacity of the resin (Ramos-de-la-Peña et al., 2019; Saraswat et al., 2013).

Furthermore, interest in integrating both upstream and downstream processes of mAb production into a continuous process has been gaining due to considerations of reduced capital cost and improved productivity, flexibility, and product quality (Zydney, 2015). The use of multi-column chromatography (MCC), either parallel or concurrent, has been suggested for the purification of mAbs to allow the continuous processing of the clarified supernatant. The number of columns is at least equivalent to the number of steps in the purification process, where one column is responsible for loading, while another is for washing, another one is for elution, and so on. This greatly reduces the residence time for purification of mAb in the chromatography apparatus, from 3 minutes in batch mode to 0.25-1 minute in continuous, without compromising the loading capacity (Jungbauer, 2013; Steinebach et al., 2017; Xenopoulos, 2015).

Polishing

Ion exchange chromatography. This step ensures the safety of the product by reducing the contaminants including host cell proteins, high and low molecular ionic solutes, and also leached protein A that remains after the purification process (Moustafa & Morsi, 2013). The removal of the aggregate can increase the risk of producing an immunogenic response in patients (Moussa et al., 2016).

There are two steps that are in this process which is the cation-exchange chromatography, also known as CEX, and anion-exchange chromatography (AEX). However, anion-exchange chromatography is not used often for mAb due to the mAb has the tendency to be basic, hence, not suitable for use (Liu et al., 2022). CEX is one of the most widely used chromatography processes as the aggregates bind more strongly to the CEX media resulting in a more efficient polishing process (Madadkar et al., 2017). In this process, strong acidic ligands such as sulphopropyl, sulfoethyl, or sulfoisobutyl groups, as well as weak acidic ligands like carboxyl groups, are immobilized in the resin. During the loading process, the antibody is attached to the resin and eluted with increasing conductivity or pH in the elution buffer. The load and wash fractions eliminate impurities (e.g., host cell proteins, DNA, and leached protein A) associated with the most negatively charged process (Erkal et al., 2021).

Several components that are needed to take into account when using CEX are the resin and separation condition. Stone et al. (2019) mentioned that the development of CEX using a continuous loading instead of the bind/elute loading poses a greater advantage as it permits the resin to be supplied with the mAb feed until the aggregates entirely occupy it, which is substantially greater than bind/elute method; the bind/elute method requires the resin to bind both the monomer and the aggregate. In addition to this, by utilizing a continuous loading process with a higher CEX resin, they only need much fewer quantities of both resin and buffer, reducing the downstream purification footprint of the mAb (Stone et al., 2019).

An alternative to CEX is by using frontal chromatography. Frontal chromatography occurs when the column is continuously loaded in which all of the components would then bind with the resin. It allows the product to have a high purity by removing large molecular weight aggregates from the intended product (Shi

et al., 2021). Although it is not often employed for preparative scale purification of proteins, Hill et al. (1990) as cited in Stone et al. (2019) showed that it is possible to use frontal chromatography to purify the weakest interacting component in a combination preparative. Since the monomer of mAbs would interact weakly with the CEX resin compared to the aggregates, they would be eluted first from the column. The process would continue until the column is fully packed with the aggregates. However, there are times when there is a small percentage of monomers that are likely to be retained in the column. Nevertheless, it still has a higher percentage of monomers recovered (Stone et al., 2019).

Viral clearance (viral inactivation, viral filtration). Viral clearance is a key component process for producing biological products, including mAbs. The difference between viral safety measures in upstream and downstream processes is the aim of the process. In the upstream processes, it focuses on reducing the risk of contaminants entering the production process, whereas the downstream is designed to clear a specific virus target (Bohonak et al., 2020). Viral filtration also shows a robust and effective clearance and proves significant reduction through the size exclusion method. The size of a monomeric mAb is approximately 10 nm and 20 nm in diameter for viruses; therefore, a common median pore size for virus filter is 19 nm which allows mAb to flow through while entrapping viruses and also potentially aggregating high molecular weight species also caught in the pore. However, there are quite a lot of factors that can affect viral filtration flux, such as pH, type of buffer salt, conductivity, and impurities (Rayfield et al., 2015)

The development of viral filtration mainly focuses on improving the filter capacity and filtration times, especially for batch processes, time is a crucial parameter. Depending on the bioreactor processes, batch culture mainly depends on a short but fast filtration, while a continuous system requires a slow but long operating time. However, in the market setting, filtration with larger areas and high-pressure resistance are the dominating compared to continuous viral filtration which mainly focuses on a slow and long-time operation. In the batch system, they usually operate for hours at a maximum of 8 hours, compared to the continuous system, they are desired to run for several days, which results in the necessity to alter the filtration used. General filtration such as minidisc is common for a batch experiment, however, this filter does not meet the criterion of continuous filtration because the necessary flow between 1.5 and 4.8 µL/min does not meet. In exchange, filter capsules needed to be utilized as it is suitable because they can lead to a larger amount of virus spike, moreover increase the hold times of the filtration, which is necessary for a continuous system (David et al., 2019).

Furthermore, to perform viral filtration there are several options of filters that can be utilized, such as Pall SV4 and Sartorius Virosart CPV for the first-generation type of filters, and Pall Pegasus Prime and Sartorius Virosart HF for the second-generation filters. However, a sufficient viral clearance specifically for continuous viral filtration, was only found on second generation filters, as for the first generation it did not show a sufficient viral clearance. The key difference first- and second-generation filter is their membrane modification, which leads into an increased in force of the retention due to surface effects, additionally the pore size distribution (PSD) of each filter is different (David et al., 2019).

As mentioned before, PSD of each filter types are different that result in the efficiency of viral filtration. To obtain a suitable PSD, there are several techniques that can be used, technique like gas-liquid displacement porometry (GLDP) and mercury porosimetry, and liquid-liquid displacement porometry (LLDP) can be done to determine the required PSD, however, GLDP and mercury porosimetry both required high pressure which exceeds the limit for polymeric viral filtrate. Meanwhile, for LLDP, the pressure required is significantly lower thus it had been applied to virus filters successfully, determining the PSD of the separation active layer (Kosiol et al., 2017).

Ultrafiltration/diafiltration (UF/DF). The ultrafiltration/diafiltration step is referring to the process in downstream purification in which the buffer exchanges the product into the formulation buffer (Pinto et al., 2015). The UF/DF is used in the last part, specifically for product concentration in biotherapeutic, for instance in monoclonal antibody production (Baek et al., 2018). According to Erkal et al. (2021), the principle

of UF/DF step (in particular, ultrafiltration) is a separation process that works by retaining large molecules and only letting go of the small molecules, using the help of transmembrane pressure. In this UF process, the retentate will be recycled back to the feed reservoir, then the permeate will be eliminated (Baek et al., 2018). Besides that, DF is just UF but it maintains a constant retentate volume by the addition of DF buffer, leading to buffer exchange. Moreover, both UF and DF are conducted in the same pressure (Baek et al., 2018). The steps for both UF and DF are commonly operated by the tangential flow filtration (TFF) systems, where fluid is passed along the filter surface rather than across it. The benefit of using TFF is where flow of fluid parallel to the filter will prevent membrane fouling by sweeping the filter surface off of contaminants, thus decreasing the material precipitation and increase the efficiency of filtration (Erkal et al., 2021).

UF has a membrane with various structure of polymer, including regenerated cellulosic membrane which have an obvious low protein binding, ease of clean up, and having a high mechanical strength or durability as well (Baek et al., 2018; Erkal et al., 2021; Fernandez-Cerezo et al., 2019; Jabra et al., 2021). The utilization of the TFF method is performed with the help of TFF cassettes, along with the membrane filtration. Moreover, hollow fiber UF membrane arrangement allows more constant flow of liquid which can minimize the shear-induced protein aggregation, especially in high concentrations (Hung et al., 2016). Membrane cleaning involves NaOH; however, increase in membrane pore size is closely correlated to increasing NaOH concentrations which will result in an increase of membrane permeability. Instead using NaOH, an alternative cleaning with deionized water should be considered, where the water enters reversely (from the retentate exit and through the feed port) which increases the cleaning productivity (Baek et al., 2018).

FUTURE DIRECTION AND GAP ANALYSIS

There are multiple challenges that can still be further addressed to optimize the production of monoclonal antibodies using Chinese Hamster Ovary (CHO) cells. Higher titer (> 10g/L) produced via the upstream process may pose a challenge for the downstream process as limitations can occur when selecting the capacity of the purification process, efficiency, as well as cost of the purification technology. In addition, due to the size of tanks, cleaning system, changeovers, validation of the system, steaming process, large-scale production may prove to be less adaptable (Ding et al., 2019).

The appeal of a continuous mAb processing system has been receiving abundant attention due to the increase in mAb market size and the further need to improve the productivity of mAb production. Although continuous upstream processing has been established by the use of perfusion bioreactors for CHO cell culture, a lack of research in continuous downstream processing methods has made it unable to complement the progress in upstream productivity, leaving the entire mAb production to be semi-continuous at best. Several methods have been suggested, such as the use of multi-column chromatography during purification and membrane technology; however, most of the studies revolving around these are conducted either on a laboratory scale or *in silico.* Therefore, further research and studies, especially in the continuous downstream processing of mAb, are required to establish an ideally continuous mAb processing system with desired, improved productivity.

CONCLUSION

Nowadays, monoclonal antibodies (mAbs) are sought after in treatments for various diseases and are produced using Chinese Hamster Ovary (CHO) cells and recombinant DNA technology through a series of fixed upstream and downstream processes. The review properly evaluates each of the significant elements in the processes for optimal production as well as analyzing the challenges that can possibly occur in each process. The upstream processing consists of the transfection of genes encoding selected mAb into CHO cells and their culture in pitched-blade or packed-bed bioreactors, followed by downstream processing which

includes centrifugation/depth filtration, Protein A affinity chromatography, ion-exchange chromatography, viral inactivation and filtration, and UF/DF. The increasing demand for mAb products requires cell culture and purification productivity to advance. From this, biopharmaceutical companies need to design and constantly improve the bioprocessing system of producing mAb from CHO cells to turn a continuous laboratory procedure into a large-scale production process.

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