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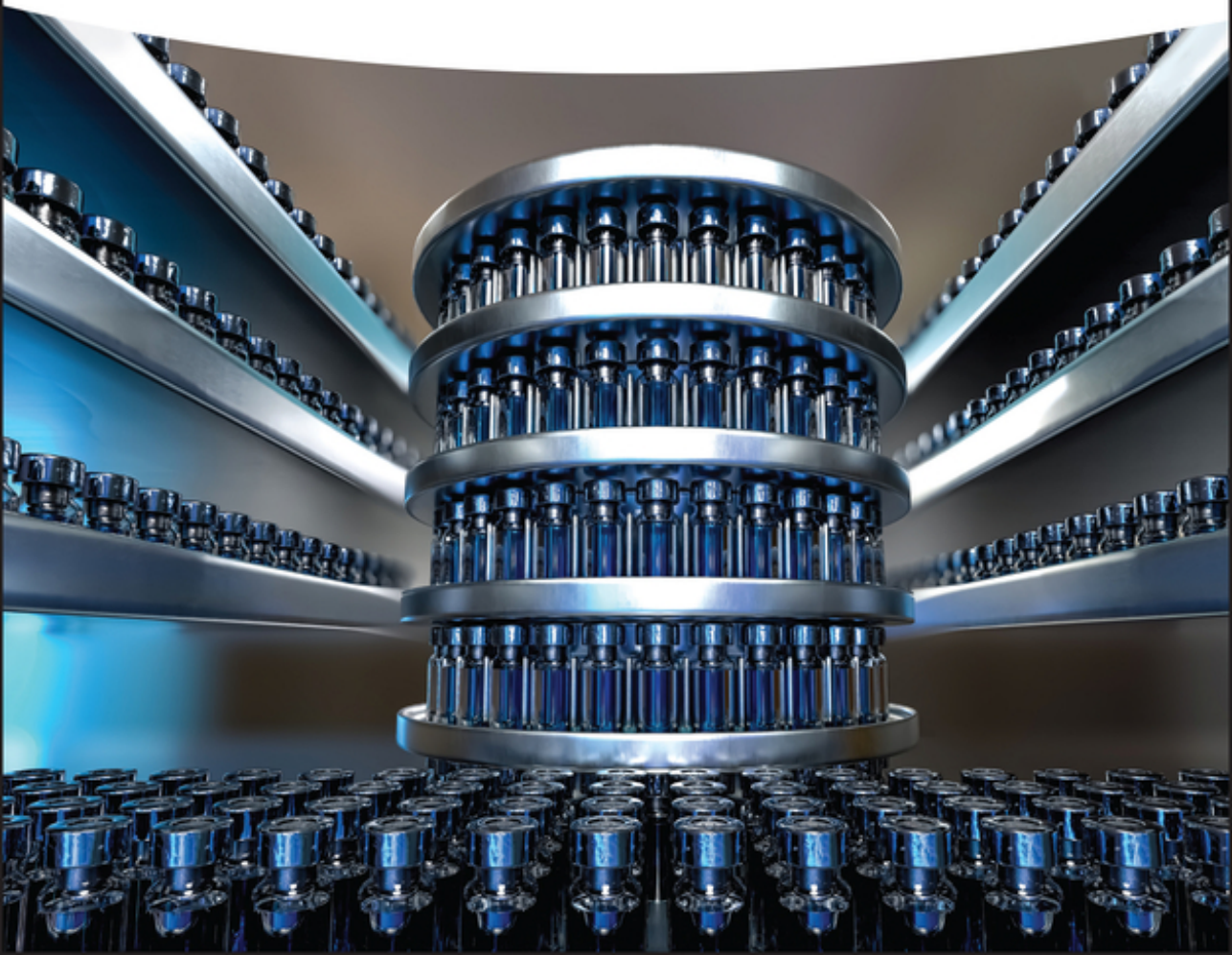

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Preface

During the past two decades, we have seen great trends in the advancements of technologies especially in accelerating the bioprocessing sector for manufacturing. The current Covid-19 pandemic and the currently existing bioprocessing technologies have been accelerated to meet the global needs of vaccines. One of the several issues that the current pandemic has highlighted is the need to be in the forefront to quickly meet the global market demand. The requirements to bring vaccines to the market and scale up the production at a much higher speed have accelerated the research and development. Clinical trials and supply chain strategies were carried out with their established processes. The rapid development of vaccines has set a positive precedent and the ongoing expectation that biological products will continue to reach the market much faster than in the past. The pandemic has certainly disturbed the equilibrium, and hence, Pharma 4.0 is underway.

Over the past ten years, pharmaceutical industries have adopted continuous processing and have invested to manufacture the products economically, to ease the method of operation, and to minimize the operational cost.

Process intensification is an ongoing trend in the bioprocessing sector that focuses on continuous bioprocessing. Over the years, industries have been regularly adapting process intensification methods.

This book presents the current advances in the intensified bioprocessing process and its application in biomanufacturing. Each chapter brings out detailed information and its values in the bioprocessing sector.

We hope that this volume will stimulate great appreciation of the usefulness, efficiency, and its potential in continuous processing of biological products and propel further progress in advancing continuous processing to meet the ever-increasing challenges and demands in the manufacturing of therapeutic products.

This book has been completed with the help and support of my friends and colleagues. It is a great pleasure for me to acknowledge the authors with deep gratitude for their contribution toward the chapters and for spending their valuable time. During this Pandemic period, one of the contributors and his family has unfortunately been the victim, and I would like to sincerely thank him and his family for still completing the chapter.

Finally, I would like to thank Felix Bloeck, Sakeena Qurashi of Wiley, and their team for their great encouragement and support throughout the preparation of this book.

Maidenhead
19 April 2021

Ganapathy Subramanian

Part I

Continuous Biomanufacturing

1

Strategies for Continuous Processing in Microbial Systems

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1.1 Introduction

1.1.1 Microbial Hosts and Their Applications in Biotechnology

With regard to microbial cultivation technology, first associations might be drawn between classical food technological applications like ethanol fermentation in beer and wine and production of dry yeast for baking dough. Nevertheless, microbial systems play a fundamental role in all parts of biotechnology in a multitude of industrially used processes. Table 1.1 gives a – certainly not complete – list for possible application of microbes in today's industrial biotechnology.

There is a high variety of possible applications for a high number of different microorganisms (MOs) as shown in Table 1.1. There are classical working horses like *Escherichia coli*, *Saccharomyces cerevisiae*, and *Bacillus* spp. that can be cultivated easily to high cell densities and produce high amounts of the desired product. Other applications and microorganism suffer from inhibitory effects (e.g. inhibition from contaminants in waste water) and low biomass and product yields. Continuous cultivations are referred to increase the time–space yield (TSY) of many processes and provide optimal usage of installed assets. Still, most these processes are established for biomass generation or detoxification. Only very few continuously operated processes involve the production of recombinant compounds. The benefits and drawbacks of continuous cultivation will be discussed throughout this book chapter, focusing especially on microbial hosts. Hence, the ideal cultivation mode must be chosen wisely.

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Table 1.1 Applications of microbial biotechnology.

Microbes	Benefit	Application in biotechnology	Cultivation mode	Source
Aspergillus niger, *Enterobacteria*	Overproduction of raw chemical by MOs, e.g. citric acid, lactic acid, vitamins	Bulk chemicals	Batch, fed-batch, and continuous cultivations	[1–3]
Thermophilic microbes – genera *Picrophilus*, *Thermoplasma*, *Sulfolobus*	High-temperature stable enzymes	Food, feed, textile, chemical, pharmaceutical, and other industrial sectors	Continuous cultivation	[4, 5]
Thiobacillus/ *Leptospirillum*	Noble metal recovery	Bio-oxidation	Bioleaching	[6]
High diverse group, e.g. *R. eutropha*	Conversion of toxic organic compounds, surface binding of heavy metals	Bioremediation	Batch and continuous processing	[7, 8]
E. coli, Bacillus, *S. cerevisiae*, *P. pastoris*	Drug production, antibiotics, etc.	Biopharmaceutical industry, enzyme industry, agricultural industry	Fed-batch technology	[9, 10]
Lactobacillus and *Bifidobacterium*	Functional food	Probiotics	Batch cultivation	[11]
S. cerevisiae, *Zymomonas mobilis*, *Klebsiella oxytoca*, *Streptococcus fragilis*	Biomass fuels based on waste streams	Biofuels	Batch and continuous cultivations	[12, 13]
Wild type*: Ralstonia eutropha*, *Alcaligenes latus*; Recombinant*: Aeromonas hydrophila*, *E. coli* Photosynthetic: *Synechocystis* sp.	Environmentally friendly non-petrochemical-based plastics	Bioplastics (polyhydroxyalkanoates)	Batch and fed-batch cultivation	[14, 15]
Haloferax mediterranei, other halophiles	Tolerate high salt concentrations	Detoxification in chemical waste streams	Continuous cultivation	[16, 17]
High diverse groups – depending on application	Waste to value	PHA production; enzymes/organic acids	Batch and fed-batch cultivations	[18, 19]
Mixed cultures, e.g. *Proteus vulgaris*, *Rhodoferax ferrireducens*, *Geobacter sulfurreducens*	Energy generation from waste	Microbial fuel cells	Batch and continuous cultivations	[20]

1.1.2 Regulatory Demands for Their Applied Cultivation Mode

The batch definitions in continuous manufacturing, preciously defined for mammalian cultivations, apply for microbial processes as well: "A Batch means a specific quantity of a drug or other material that is intended to have uniform character and quality, within specified limits, and is produced according to a single manufacturing order during the same cycle of manufacture. In the case of a drug product manufactured by a continuous process, it is a specific identified amount produced in a unit of time or quantity in a manner that assures its having uniform character and quality within specified limits" 21 CFR 210.3 2, or "a batch may correspond to a defined fraction of the production. The batch size can be defined either by a fixed quantity or by the amount produced in a fixed time interval" EU GMP Guide, Part II (ICH Q7).

More important than batch definition is the application of the quality-by-design (QbD) context to continuous processing. Generally, QbD mainly urges to relate critical quality attributes (CQAs) to critical process parameters (CPPs) and raw material attributes (RMA) to form a design space [21]: "A multidimensional combination and interaction of input variables and process parameters that have been demonstrated to provide assurance of quality of the product" for demonstrating process understanding. As proposed by current validation guidelines [22], stage 1 validation includes the execution of process characterization studies (PCS), which is the "collection and evaluation of data, from the process design stage throughout production. This establishes scientific evidence that a process is capable of consistently delivering quality product." PCS finally leads to the awareness of the mutual interplay of CPPs on CQAs. This demonstrates process robustness within multivariate normal operating ranges (NOR) and therefore finally proposes the control strategy including process and analytical controls. Currently, this is achieved by fusing development and manufacturing data.

Using an enhanced PCS approach, the determination of appropriate material specifications and process parameter ranges could follow a sequence such as the following [23]:

(i) Identify potential sources of process variability.
(ii) Identify the material attributes and process parameters likely to have the greatest impact on drug substance quality.
(iii) Design and conduct studies (e.g. mechanistic and/or kinetic evaluations, multivariate design of experiments, simulations, modeling) to identify and confirm the links and relationships of material attributes and process parameters to drug substance CQAs.
(iv) Analyze and assess the data to establish appropriate ranges, including the establishment of a design space.

Even more, continuous processes require a different level of process understanding: as an example, classical recombinant protein production (RPP) using *E. coli* as a host pools the product solution after four days of processing. The time-variant dependency of CPPs and CQAs is finally integrated in one analytical result, and the process is also registered as such. Hence, batch processes are characterized by operating subsequent steps on the *integral* outcome of the current process step. Implementing

continuous operations, we must *understand the time dependency* between CPPs and CQAs with the goal to have a time-invariant CQA process result. Hence, as microbial processes are more dynamic in terms of kinetics and stoichiometry, proper understanding of a dynamic design space and establishment of a robust control strategy are more relevant from a regulatory point of view ("A planned set of controls derived from product and process understanding that assure process performance and product quality") [21].

For continuous processing, time-variant interrelations between CPPs and CQAs must be transformed into a control concept. This calls for the enhanced use of metabolic and kinetic models integrated in experimental designs for elucidating the design space. Of course, initially, we relate CPPs and CQAs in a classical QbD manner. However, we need to enhance this context: for example, classical design of experiment (DoE) approaches can only capture the response of the system to time-invariant factors of the integral experiment. For continuous operation, and in contrast to conventional development strategies, we aim to operate with time-invariant process variables and CQAs. Therefore, we need to understand the time-variant dependencies on their CPPs for control. Thus, we do not change the CPPs to analyze the integral outcome of the CQAs, but we change the CQAs to analyze the integral outcome of the CPPs, which can compensate for their variability of time. Hence, the development strategy is enhanced. We may need dynamic model-based experimental designs to develop a control strategy able to cope with process variability over time; those experimental setups need to be established in the R&D environments [24].

Hence, continuous processing requires a *much earlier definition of the process control strategy directly during the process development and characterization phase*. We need this control strategy earlier as prerequisite for process design. NORs must be defined earlier and turned into a real process control strategy based on PAT, models, and controls. Hence, the tasks of PCS need to be done already during the development. Those elements may include data mining, risk assessments, characterization of process performance, screening studies, criticality assessment [23], and integrated process modeling [25], as shown in the workflow in Figure 1.1. On the other hand, scale-down model qualification tasks may not be necessary, as the development scale may already be the production scale, since productivity is scaled by processing time or scale-out techniques.

Thus, R&D labs need therefore higher data management and data science orientation, as well as advanced PAT and process control environments as skill set, which will be addressed in Chapter 3.

1.2 Overview of Applied Cultivation Methods in Industrial Biotechnology

As an easy rule, the cultivation mode resulting in the highest TSY should be pursued. TSY could be defined in pharmaceutical applications as the highest throughput from pre-culture inoculation until purified drug substance, in gram product per operating

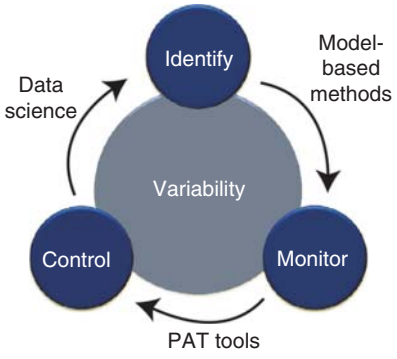

Figure 1.1 Tackling process variability in the process development phase for continuous biomanufacturing through early establishment of identification, monitoring, and control for the identification of a dynamic control strategy. Source: Dream et al. [24].

liter per day [26], or for waste streams as the process enabling the highest catalytic capacity to degrade toxic compounds.

1.2.1 Batch and Fed-Batch Cultivations

1.2.1.1 Conventional Approaches and Their Technical Limitations

The golden standard in RPP is batch and fed-batch cultivations. With regard to batch, all ingredients are added to the reactor, and microorganism react until limiting component inhibits further growth. Common limitation elements in industrial biotechnology are carbon, nitrogen, or phosphor. Problems in batch cultivation are that MOs grow at maximal specific growth rate and causing problems in aeration and heat transfer, discussed in more detail later. This limits the maximal limiting component concentration and results in low overall biomass concentrations. For this purpose, fed-batch technology is currently applied. Additional feeding is conducted (e.g. high concentrated sugar feeds), which results in higher biomass concentrations. Controlled addition of limiting substrate can also overcome several problems like carbon catabolite repression and substrate inhibition [27]. High cell density cultivations are referred to increase the overall titer. However depending on the media and reactor setup employed, biomass concentrations should not exceed physiological levels [28]. This is because in high cell density fermentations, (i) non-controlled nutrient limitation might occur, (ii) K_La levels might not cope for the demands of high cell densities, and (iii) reactor cooling capacity might be exceeded [29]. To cope for demands of limited oxygen transfer, additional oxygen could be supplemented, but at industrial scale, additional oxygen supply might lead to unfeasible cultivation costs. pO_2-limited cultivations tend to increase secondary metabolite production to synthetize their needed reduction equivalents. Furthermore, amino acid mis-incorporation in recombinant produced proteins has been found as a side effect of oxygen-limited cultivations [30, 31]. Moreover, biomass concentrations must be kept within the reactor cooling capacity. High growth rates monitored for many microorganism can cause high heat formation, being especially a problem in yeast fermentation: as methanol is commonly used for the induction in *Pichia pastoris* systems containing alcohol oxidase (AOX) promoters, high heat is generated by methanol on its own [32]. To stay within reactor cooling

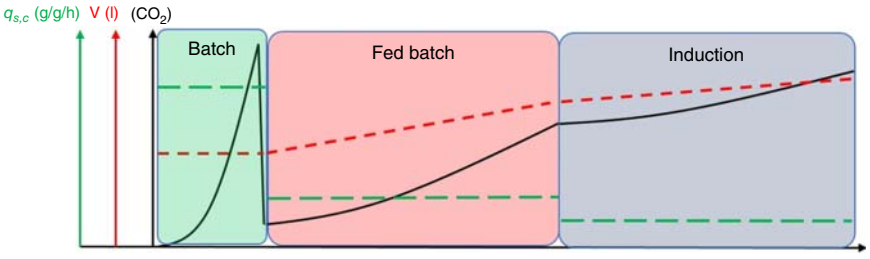

Figure 1.2 General procedure for microbial Fed-batch cultivations: A Batch phase is followed by a non-induced Fed-batch phase and an Induction phase, with $q_{s,C}$ being the specific carbon uptake rate, with V being the reactor volume and CO_2 coding for residual carbon dioxide.

capacities, the Mut^S strain was invented, showing decreased methanol uptake rates in *P. pastoris*. Hence a compromise between maximum biomass concentration and reactor cooling capacity must be made at an industrial level.

1.2.1.2 Feeding and Control Strategies Using *E. coli* as a Model Organism

A sketch for industrial fed batch used for *E. coli* cultivation in red biotechnology is shown in Figure 1.2. Maximum specific feeding rates ($q_{s,\max}$) are generally applied through batch. Fed batches are operated at specific feeding rate values far below the $q_{s,\max}$.

The batch phase is followed by an exponential fed batch for biomass production according to

$$F(t) = \frac{q_{s,(C)} * X(t) * \rho_f}{c_f} \text{with } X(t) = X(t=0) * e^{\mu * t} \tag{1.1}$$

where F is the feeding rate (g/h), $q_{s,(C)}$ is the specific uptake rate (g/g/h), $X(t)$ is the absolute biomass at the time point t (hours), ρ_F is the feed density (g/l), c_F is the feed concentration (g/l), $X(t=0)$ is the biomass before start of the fed batch in (g), and μ is the specific growth rate (1/h). After the first exponential fed-batch phase, cells are induced for RPP and fed until harvest. Besides the classic exponential fed batch, different feeding profiles can be employed, which is often done throughout induction phase [33, 34]. Cells are mainly grown carbon limited after batch phase, as a desired specific growth rate (μ) can be adjusted easily, with a set μ beneath $\mu_{\max}/2$ to reduce acetate formation and reduce stress onto host cells. Common control strategies for carbon-limited growth are either basic feed-forward protocols (see Eq. (1.1)) or soft-sensor approaches [35]. Throughout feed-forward control strategies, a constant q_s value is set for a fixed timeframe to achieve a targeted biomass within a certain time. The amount of fed carbon is calculated into biomass, assuming a constant biomass yield. As overall biomass is increasing, feed rate is thus increased via higher pump set points, which are adjusted using a PID controller (proportional, integral, and derivative control terms). However, in this strategy, no feedback control is implied. In soft-sensor approaches, a feedback loop to off-gas signals by mass balancing is implemented in the feeding strategy. Hence, feeding rate can be adjusted

to unexpected process deviations. The usage of a noncontrolled feeding strategies might lead to substrate accumulation in carbon-limited feeding approaches. Off-gas signals are used to predict biomass formations due to the stoichiometric balances, hence adjusting pump set points [26, 36].

1.2.2 Introduction into Microbial Continuous Biomanufacturing (CBM)

1.2.2.1 General Considerations

In some branches of biotechnology, continuous processing is already established (i.e. bioleaching and oxidation, using several stirred tank reactors serially connected) [6]. Also in the field of biofuels, the trend leads to a continuous production platform [37, 38]. Moreover, continuous processing is well suited for the degradation of toxic compounds. As cell growth-inhibiting compounds are fed, growth rates can be very low, and thus retentostat setups (Figure 1.3b) can increase the detoxification efficiency as shown for the halophile *Haloferax mediterranei* [16]. Large-scale detoxification can be found in wastewater treatment plants, also using retentostat principles. Retentostat cultivation used a retention device (i.e. 0.2 µm pore size membrane) to maintain a controlled number of cells in the cultivation device. Hence, a feed/bleed system can be maintained at feasible cell densities compared with common chemostat cultivation, especially advantageous for slow-growing organisms. Problems such as changing media composition and changing yields and inhibitory substances often make continuous cultivations challenging in diverse branches. For recombinant protein expression, using microbial hosts, continuous biomanufacturing (CBM) is still far from its industrial application. Despite the several benefits coming with fed-batch cultivation, product quality is highly time dependent. Furthermore, high batch-to-batch variations may result in severe problems in the subsequent downstream process for red biotechnology. The following benefits could be expected from the establishment of CBM for microbial cultivations:

- Small reactor systems reduce investment costs and enable efficient and highly flexible production even for small companies ("**small footprint facilities**").
- Cleaning in place (CIP) and steam in place (SIP) can be reduced to a minimum, as cultivation times are increased from some days to several weeks, making this cultivation mode **sustainable**.
- Quality of the product is not batch performance dependent but can be expressed at **constant quality**.
- Continuous waste streams may be used for certain applications (whey from milk industry, molasses from sugar industry, etc.). This would decrease the costs for the product drastically, leading to **circular economy** approaches.
- Continuous upstream enables continuous downstream, leading to an **integrated process**, and enables robust downstream processing, e.g. usage of "simulated moving bed chromatography."

In this chapter, three different cultivation modes that are often implemented in the upstream processing (USP) of microbial continuous systems will be discussed. Figure 1.3a) shows the classic chemostat process for microbial systems. A feed is

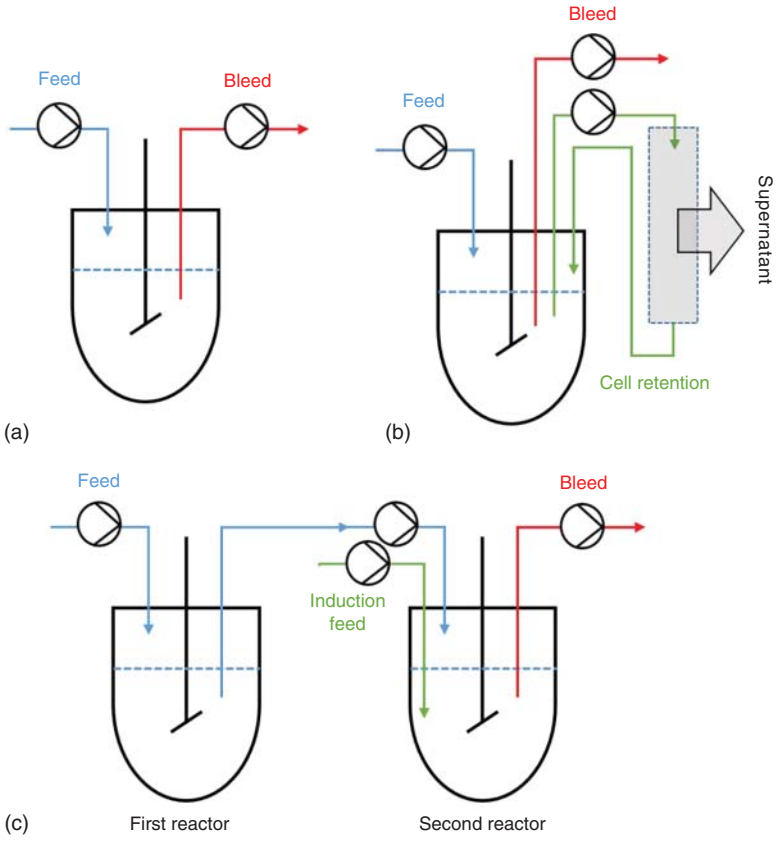

Figure 1.3 (a) Classical microbial chemostat for fast-growing organism. Feed is pumped at a fixed dilution rate and bled out at a certain volume including the product. (b) Cell retention system for slow-growing microorganism. Cell concentrations are increased until the theoretical biomass to substrate yield is reached. Product is usually concentrated after (c) Cascade systems for sequential/serial addition of bioreactors. First reactor is used for biomass production only, biomass is transferred from reactor 1 to reactor 2 indicated by the blue line. Only reactor 2 is fed with an induction feed and bleed out of reactor 2 is containing target product. This cultivation system could be used for different MOs digesting the same feedstock or for recombinant protein production. Source: Refs. [39–41].

added at a constant rate to the reactor. The bleed is removed using pneumatic valves, connected to peristaltic pumps, enabling constant volume throughout cultivation. This system is preferably used for fast-growing MOs. The main benefit is easy process control as generally only monitoring is necessary and no control circuits need to be used, like PI or PID controllers.

Retentostats, also called perfusion systems, shown in Figure 1.3b), contain a common chemostat setup with an additional hollow fiber membrane to retain cells in the reactor. Pumps (feed, bleed, cell retention) must be adjusted accordingly to guarantee a stable process performance. Retentostats are common for slow-growing cultures and are therefore often used in cell culture. The second advantage is that extracellular product can easily be harvested using cell retention modules

and waste-to-value approaches can clear contaminants effectively through higher biomass concentrations inside the reactor. The third cultivation system is shown in Figure 1.3c, which is regarded as a serial combination of chemostats or retentostat systems. Cascaded cultivation systems can be successfully applied for red biotechnology approaches in decoupling biomass production from induction of the cells in a spatially resolved manner [39, 40] (see Section 1.4.3). Hereby cells in reactor one is grown "burden-free," whereas the second reactor is operated in an induced stage. Continuous application is given as two feed/bleed systems are serially connected with each other: feed, free of inducer, is supplemented to the burden-free stage (first reactor), and non-induced biomass is transferred to the induced reactor (second reactor). Further ongoing, the second reactor is supplied with an inducer-containing feed to initiate RPP [40]. Using this system, the benefits of time-dependent cultivations can be included in a continuous system as (i) burden-free cell growth, equal to non-induced biomass growth, can be maintained in the first stage and (ii) adequate induction times can be set via the residence time in the induced stage. Cascaded or serial combinations can also be used in waste-to-value approaches and circular economy thoughts combining aerobic cultures producing CO_2 that may be recycled in the second reactor using autotroph/chemolithotroph MOs [39], implementing a neutral carbon footprint.

The cultivation method of choice has of course always to be adapted to the current aim. A rough overview about the desired aim can be gained via proper mass balances.

1.2.2.2 Mass Balancing and the Macroscopic Effects in Chemostat Cultures

Mass balancing can be perfectly used to highlight benefits of a continuous system in favor of the classical fed-batch approach. The general macroscopic mass balance for an ideal stirred tank reactor is given in Eq. (1.2):

$$\dot{V}_{\text{in}} * c_{i,\text{in}} + \dot{V}_{\text{out}} * c_{i,\text{out}} + V_R * r_i = V_R * \frac{\partial c_i}{\partial t} + c_i * \frac{\partial V_R}{\partial t} \tag{1.2}$$

where $\dot{V}_{\text{in}}$ is the volume flux in the reactor, $\dot{V}_{\text{out}}$ is the flux of the bleed, $c_{i,\text{in}}$ is the concentration of component i in the influx, $c_{i,\text{out}}$ is the concentration of component in the bleed, V_R is the reactor volume, r_i is the reaction rate for component i, and t is the time. As one of the strong benefits of continuous reactor systems is the time independence of the reactor upon tuning, the balance reduces to

$$\dot{V}_{\text{in}} * c_{i,\text{in}} + \dot{V}_{\text{out}} * c_{i,\text{out}} = -V_R * r_i \tag{1.3}$$

As flux in and flux out are constant in a classic chemostat and solving for the reaction rate and substituting $\frac{\dot{V}}{V_R} = D$, with D being the respective dilution rate in 1/h,

$$r_i = \Delta c_i * D \tag{1.4}$$

It is clearly visible that every volumetric rate r_i is dependent upon the applied dilution rate of the bioreactor and on the concentration of components in the media. TSY, being the volumetric productivity, is directly dependent on these two

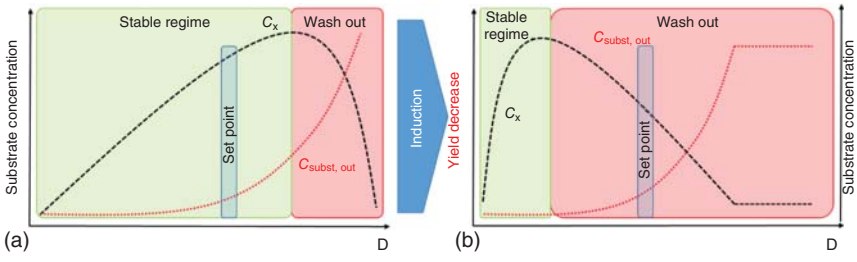

Figure 1.4 (a) A set point for stable biomass production was chosen, all fed substrate is consumed, and biomass is formed based on the yield coefficient. Upon induction, the $Y_{X/S}$ changes, and less substrate can be metabolized. (b) shows that the stable set point before might now suffer from a decrease in yield. Hence sugar biomass formation might be reduced at the given set point, and substrate is accumulating.

factors. Consequently, high dilution rates and high concentrations of the limiting component in the feed should positively influence the TSY. Connections between the different rates can be easily drawn in using yield coefficients in Eq. (1.5):

$$Y_{\frac{a}{b}} = \frac{r_a}{r_b} \tag{1.5}$$

Postulating constant yield coefficients, the continuous reactor can be set up and operated at optimal conditions. In general, this hypothesis holds true for chemostats with defined media of constant quality and sole biomass production. However, in RPP using, for example pET plasmids, induction with isopropyl-β-D-thiogalactopyranosid (IPTG), lactose or related inducer is necessary. The effects of induction onto the cell itself will be discussed in a later chapter. We regard the reactor as a black box for now and just look at the effects of a changing substrate to biomass yield ($Y_{X/S}$). A visualization is given in Figure 1.4.

Starting with induction feeds at identical carbon concentrations, the yield coefficient changes within some hours as several stress responses affect cell growth. With decreasing yield, the same set point of dilution rate might possibly lead to sugar accumulation. As the stable set point moves toward the washout regime and consequently biomass concentrations are reduced, substrate is washed out of the reactor. This is no stable process and brings again a time dependence of the yield coefficient into consideration. These effects act also upon productivity and make single-vessel chemostat cultures very unstable at fast-growing MOs.

Similar problems are observed upon changes in the feed substrate quality. These changes may be based on fluctuations in substrate concentration but could also be fluctuating in inhibitory substances. Simple Michaelis–Menten kinetic considerations show effects upon the process in Eq. (1.6). We assume competitive inhibition as cells are directly affected by the inhibitory substance in the reactor:

$$\mu = \mu_{\max} * \frac{[S]}{[S] * K_S * \left(1 + K_I * [I]\right)} \tag{1.6}$$

where μ is the specific growth rate, which is identical to the dilution rate, [S] is the substrate concentration, $\mu_{\max}$ is the maximal possible growth rate, K_S is the reaction

constant for substrate uptake, $[I]$ is the inhibitor concentration, and K_I is the reaction constant for the inhibitory reaction. Therefore, changing inhibitory concentrations $[I]$, as well as substrate concentrations $[S]$, has effects on the specific growth rate and may shift the critical specific growth rate. Close to μ_{max}, $\mu = D$ is not valid anymore, as washout starts and hence results in an unstable process.

So even simple macroscopic mass balance and kinetic considerations, considering the biomass in the reactor, show the complexity of the system. Further cell physiological effects might occur in RPP. However, the high expression of recombinant protein and the extremely high doubling rates may make microbial continuous cultivation a promising alternative to state-of-the-art fed-batch approaches.

1.2.3 Microbial CBM vs. Mammalian CBM

1.2.3.1 Differences in Upstream of Microbial CBM Compared with Cell Culture

The first remarkable difference between microbial and cell culture-based expression systems are the differences in cell doubling times. While cell culture-based cultivations take up several hours for a cell division, the maximal doubling time in *E. coli* can be 20 minutes. Table 1.2 compares the three most important organisms regarding their growth rates upon the production of recombinant proteins. Absolute values may differ from strain to strain and expressed recombinant protein but give a certain lead to compare different continuous approaches.

Cell cultures (Chinese hamster ovary [CHO] cells) exhibit exceptionally low doubling rates (13.8–85 hours per cell doubling). This also results in long preparation times for pre-cultures (seed flasks) up to four weeks and the starting batch phase before enabling continuous feeding. For *E. coli* preparation, pre-culture and batch phase take approximately 30 hours, depending on applied sugar concentrations [35, 46]. It was already stated that microbial systems show very high dynamics in metabolism and recombinant protein expression, based on the high number of cell divisions during a continuous process [44]. Taking mean dilution rates in Table 1.1 and comparing generation times to one week of cultivation, which corresponds to 168 hours, CHO cells doubled in mean 4.3 times, *E. coli* cells doubled 50 times, and *P. pastoris*, as frequently used expression host for yeast-based expression, doubled 18.5 times.

While CHO and yeast cells have a eukaryotic translation and posttranslational modification (PTM) mechanism (through golgi apparatus), prokaryotic

Table 1.2 Growth rates and approximated generations for cultivation times found in the literature.

Organism	Growth rate (1/h)	General process duration (h)	Generations (–)	Source
CHO cells	0.0008–0.05	650–2160	2–47	[42, 43]
E. coli	0.1–0.49	up to 300 h	43–212	[39, 44]
P. pastoris	0.009–0.2	up to 1000 h	13–290	[44, 45]

Source: Refs. [23, 27, 28, 33].

microorganism lack these systems [47, 48]. As the product is generally secreted into cultivation supernatant, cell culture processes rely mainly on retentostat/perfusion technology, where product can be harvested in the broth, without dealing with the intact host cell. Yeasts also have the possibility to translocate the product to the broth while having a sufficient high growth rate. Recombinant proteins produced in yeast, however, are highly mannose glycosylated, and no human like N-glycosylation can be performed. Hence, products need cost- and time-intensive treatment prior to clinical application [49]. Recombinant proteins in *E. coli* are located primary inside the cell. Most of these products are expressed in the cytoplasm and kept in this place, where no signal sequences for transport into the periplasm are attached to the protein. The reducing milieu in the cytoplasm does not allow disulfide bond creation and makes correct folding of complex proteins difficult. The result is often the expression of inclusion bodies (IBs), misfolded proteins with hydrophobic character. Hence, continuous purification in microbial systems might be leading to challenging technical applications, owing to different product loci.

1.2.3.2 Downstream in Microbial CBM

An integration of the process from up- to downstream would be the desired future perspective in a modular design. This would ease the way for "small-footprint facilities" as high modular elements can be easily exchanged and stuck together for a new product. Furthermore, costs can be strongly reduced especially in the downstream, heading toward smaller columns [50–52]. Continuous purification methods for extracellular proteins have been established [53]. Filtration steps, followed by continuous chromatography systems (making use of simulated moving bed principles), have been established for the purification of products derived from mammalian cells [51].

However, other downstream unit operations, especially such operations for intracellular proteins, are still considered problematic. Figure 1.5 shows the schematic downstream chain for intracellular proteins and highlights the additional steps needed for misfolded protein aggregates derived by *E. coli*, which are known as IBs.

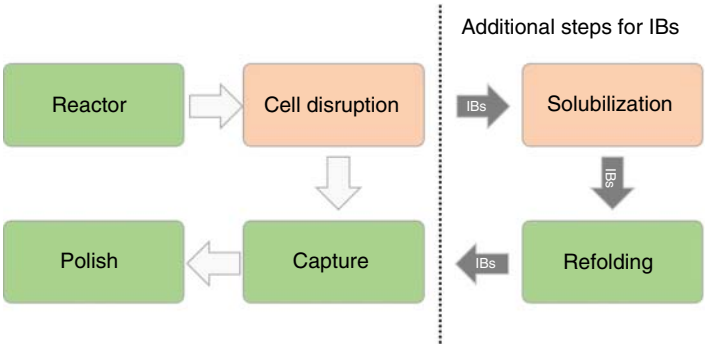

Figure 1.5 Simplified process chain for production of a recombinant product in *E. coli*. Green unit operations can be accessed in a continuous mode; red operations are hard to realize. IBs need at least two additional steps during downstream.