

## 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.

**Table 1.1** Applications of microbial biotechnology.

| Microbes  | Benefit  | Application in biotechnology  | Cultivation mode                              | Source   |
|---|--|---|---|----------|
| <i>Aspergillus niger</i> ,<br><i>Enterobacteria</i>   | 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 <i>Picrophilus</i> , <i>Thermoplasma</i> , <i>Sulfolobus</i>   | High-temperature stable enzymes  | Food, feed, textile, chemical, pharmaceutical, and other industrial sectors | Continuous cultivation                        | [4, 5]   |
| <i>Thiobacillus/Leptospirillum</i>  | Noble metal recovery   | Bio-oxidation   | Bioleaching                                   | [6]      |
| High diverse group, e.g. <i>R. eutropha</i>   | Conversion of toxic organic compounds, surface binding of heavy metals         | Bioremediation  | Batch and continuous processing               | [7, 8]   |
| <i>E. coli</i> , <i>Bacillus</i> , <i>S. cerevisiae</i> , <i>P. pastoris</i>  | Drug production, antibiotics, etc.   | Biopharmaceutical industry, enzyme industry, agricultural industry          | Fed-batch technology                          | [9, 10]  |
| <i>Lactobacillus</i> and <i>Bifidobacterium</i>   | Functional food  | Probiotics  | Batch cultivation                             | [11]     |
| <i>S. cerevisiae</i> , <i>Zymomonas mobilis</i> , <i>Klebsiella oxytoca</i> , <i>Streptococcus fragilis</i>   | Biomass fuels based on waste streams   | Biofuels  | Batch and continuous cultivations             | [12, 13] |
| Wild type: <i>Ralstonia eutropha</i> , <i>Alcaligenes latus</i> ; Recombinant: <i>Aeromonas hydrophila</i> , <i>E. coli</i><br>Photosynthetic: <i>Synechocystis</i> sp. | Environmentally friendly non-petrochemical-based plastics                      | Bioplastics (polyhydroxyalkanoates)   | Batch and fed-batch cultivation               | [14, 15] |
| <i>Haloferax mediterranei</i> , 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. <i>Proteus vulgaris</i> , <i>Rhodoferrax ferrireducens</i> , <i>Geobacter sulfurreducens</i>   | 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, previously 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].

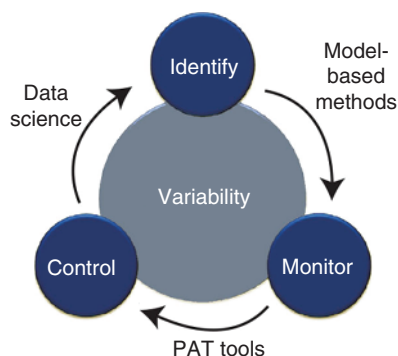
Hence, continuous processing requires a *much earlier definition of the process control strategy directly during the process development and characterization phase*. We need this control strategy earlier as prerequisite for process design. NORs must be defined earlier and turned into a real process control 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].

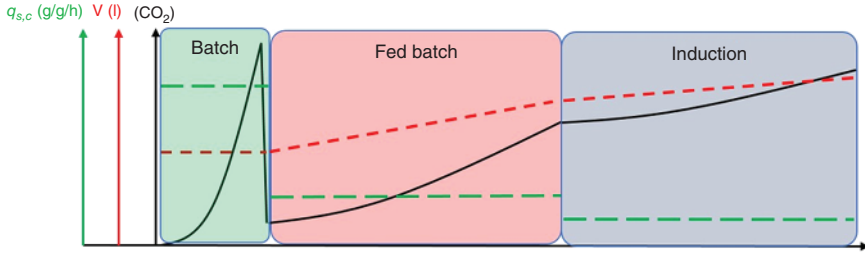


liter per day [26], or for waste streams as the process enabling the highest catalytic capacity to degrade toxic compounds.

### 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)  $K_L a$  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 synthesize 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  $\text{CO}_2$  coding for residual carbon dioxide.

capacities, the Mut<sup>S</sup> 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

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

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),  $\rho_f$  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  $\mu$  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 ( $\mu$ ) can be adjusted easily, with a set  $\mu$  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)

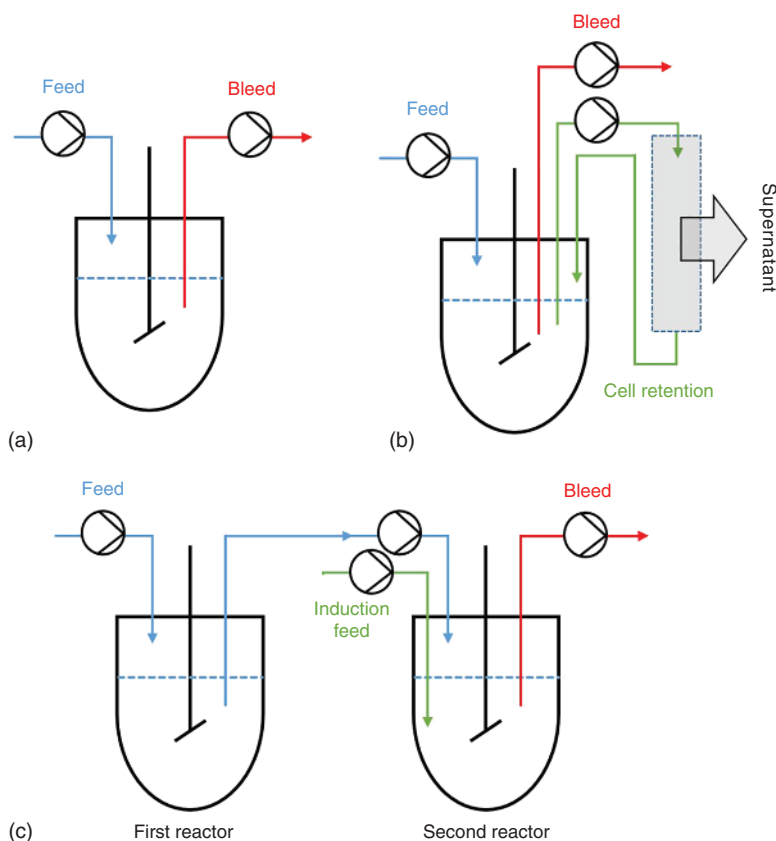
### 1.2.2.1 General Considerations

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  $\mu\text{m}$  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<sub>2</sub> 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):

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

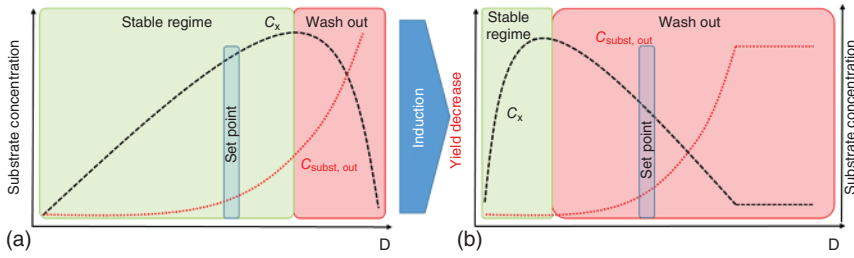
where  $\dot{V}_{\text{in}}$  is the volume flux in the reactor,  $\dot{V}_{\text{out}}$  is the flux of the bleed,  $c_{i,\text{in}}$  is the concentration of component  $i$  in the influx,  $c_{i,\text{out}}$  is the concentration of component in the bleed,  $V_R$  is the reactor volume,  $r_i$  is 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

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

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,

$$r_i = \Delta c_i * D \quad (1.4)$$

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):

$$Y_{\frac{a}{b}} = \frac{r_a}{r_b} \quad (1.5)$$

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- $\beta$ -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:

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

where  $\mu$  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  $\mu_{\max}$ ,  $\mu = D$  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] |
| <i>E. coli</i>     | 0.1–0.49          | up to 300 h                  | 43–212          | [39, 44] |
| <i>P. pastoris</i> | 0.009–0.2         | up to 1000 h                 | 13–290          | [44, 45] |

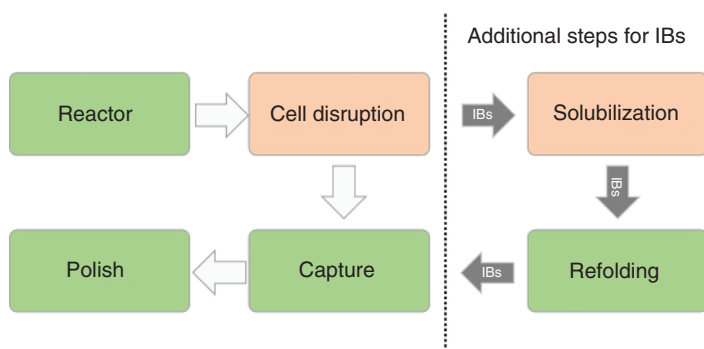
Source: Refs. [23, 27, 28, 33].

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.

Cell disruption is the first bottleneck to be dealt with in integrated CBM as link between up- and downstream for intracellular products. Generally, cell disruption techniques can be separated between mechanical and non-mechanical approaches [54, 55]. The most frequently used techniques for small-scale cell disruption are performed via bead mill or french press technology and suitable for a small volume of broth in a batch approach. Large-scale cell disruption is performed with high-pressure homogenization, as high-pressure homogenization is the only scalable form of cell disruption [56, 57]. These homogenizers can be operated at high velocities, realizing cell disruption within one passage, and the implementation of a continuous cell disruption mode is rather easy [58]. Besides high-pressure homogenization, other methods are currently under investigation for continuous cell disruption. Ultrasonic devices may be used for quick energy-saving cell disruption using flow cells. However, problems with the abrasion of titanium elements responsible for energy transduction must be considered. Strong pulsed electrical fields may also be used for cell lysis, as the cells are effectively opened, but intracellular components are not affected. However, bubble generation and joule heating issue cause problems, and first tests were only performed in microscale [54].

A major bottleneck of misfolded proteins (IBs) is the solubilization and refolding steps. Even though, IBs exhibit some disadvantages, there are many benefits, especially in continuous cultivation, shown in Table 1.3.

Solubilization of IBs is performed using high concentrations of chaotropic detergents, like urea or guanidine hydrochloride [59]. However, mild solubilization has also shown to result in high yields of biological active protein [60]. Continuous approaches for the refolding step revealed positive effects in *in silico* studies (especially buffer consumption) and were found to yield promising results in experimental studies [50, 61]. A fully integrated continuous downstream protocol for the purification of inclusion bodies has been described recently [62]. Even though no integration between upstream and downstream is performed up until now, results for continuous inclusion body treatment in downstream seem promising.

**Table 1.3** Pros and cons of IB expression in *E. coli*.

| Positive aspects   | Negative aspects   |
|--|--|
| Nano-particulate matter, which can be highly concentrated upon cell disruption | Necessary cell disruption for capture of the protein                                   |
| High initial purities before capture step (up to 90%)                          | No posttranslational modifications (also true for soluble proteins)                    |
| Active (so-called nonclassical IBs [ncIBs]), which require no refolding        | Refolding needs to be performed for classical IBs, which reduces the yield drastically |
| High volumetric titers of up to 15 g/l in fed batch                            | Time- and cost-intensive downstream  |
| Expression of inactive toxic proteins  | —  |

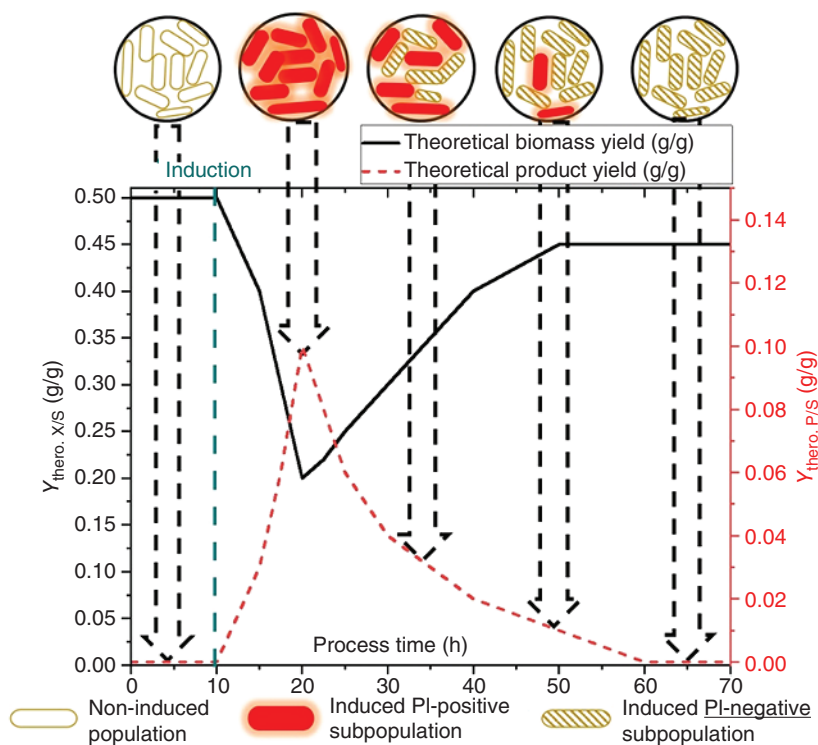
Once continuous cell disruption can be performed and promising capture steps are established, the production of intracellular proteins could be fully integrated in a continuous mode. As continuous chromatography principles are established for the purification of extracellular proteins, these methods are transferable. By adapting separation techniques and resins to the needed downstream step (i.e. capture or polishing [Figure 1.5]), continuous purification might be possible for *E. coli* – derived products. Detailed information for continuous purification of intracellular products has been given in recent reviews [33, 63].

## 1.3 Monitoring and Control Strategies to Enable CBM with Microbials

### 1.3.1 Subpopulation Monitoring and Possible PAT Tools Applicable for Microbial CBM

Besides genetic instabilities mentioned earlier (Section 1.2.3.1), microbial cell population can also exhibit phenotypic differentiation. This differentiation arises from biological noise taking place in the intracellular environment, leading cell-to-cell differences in the amount of key intracellular components, such as regulatory proteins or metabolites [64]. Ultimately, this biological noise has been recognized to confer functionality to microbial populations through the appearance of subpopulations with distinct metabolic functions [65]. In natural ecosystems, the occurrence of these subpopulations is generally recognized as a beneficial factor known for increasing the global fitness of the whole population when facing challenging environmental conditions [66]. However, in the context of bioprocesses, such subpopulations are generally unwanted, leading to the simultaneous occurrence of producing and non-producing cells requiring the use of advanced process analytical technology (PAT) tools for the proper characterization of assessing the real productivity of the biological system [67].

Even though some continuous cultivation techniques (i.e. cascaded continuous cultivation) can overcome the issues in microbial continuous bioprocessing, monitoring of occurring dynamics needs to be performed to establish a knowledge platform. PAT applications for *E. coli* continuous manufacturing have been summarized in recent reviews [33, 68]. Classic measurements (such as pH,  $pO_2$ , and oxygen transfer rate [OTR] measurements) are implemented in the process chain, but also new applications have been described. Label-free technologies such as Raman microspectroscopy [69] and nano-SIMS [70] begin to emerge as promising alternatives to fluorescence-dependent approaches. Especially, flow cytometry has been found to be a powerful tool to distinguish between productive and nonproductive subpopulations [71]. As flow cytometry is a single-cell analytic, highly accurate information of the subpopulation state can be given [29]. Online flow cytometry was successfully established to describe the metabolic state of *E. coli* and *P. putida* cultivations [30]. The usage of the fluorescent dye propidium iodide (PI) in flow cytometry analysis can distinguish the so called red-but-not-dead phenotype



**Figure 1.6** Time-dependent behavior of a chemostat cultivation producing a recombinant protein (Figure 1.10a) on a subpopulation level: it can be seen that the un-induced population starts to enhance its PI potential upon induction. However, the formation of PI-negative subpopulation takes over at elongated cultivation times, and no more fluorescence can be monitored.

[65, 66]. These are referred to show enhanced membrane potential, thus allowing PI to penetrate cell wall. Implementation of PI as a marker stain helped to determine and monitor the membrane potential throughout chemostat cultivation with online flow cytometry [30]. The possibility of online flow cytometry in combination with online propidium iodide staining for subpopulation monitoring is given and shown as a hypothetical example for a common chemostat behavior in Figure 1.6.

In large-scale *E. coli* fermentations, a high percentage of the cultivation broth was found to have increased membrane activity, detected via PI staining. Local glucose limitations might have caused enhanced membrane potential, whereas the effects were not monitored in well-mixed scale-down experiments [31]. In this study, green fluorescent protein (GFP), fused to a target protein, was transcribed as a fluorescent marker protein, an interesting approach to gain gene-specific information [29]. Autofluorescent reporter genes and frequent measurement of such with flow cytometry throughout cultivations might help to get more insights in population dynamics. Hence, this may help to distinguish subpopulation noise occurring throughout microbial fermentations [32]. However, reporter genes can



be also be used to derive a real-time metabolic state conditions [72]. Using GFP as a marker protein to distinguish the intracellular burden, in combination with high-frequent flow cytometry measurements, might be a powerful tool to distinguish important gene clusters and test optimal cultivation conditions in real time [33]. Rapid screening and information gained can further be used to (i) accelerate strain optimization and (ii) implement data in model-based approaches.

This example shows the benefit and need of PAT in CBM. Key critical quality attributes that could be measured online and/or at-line are as follows:

- Diversification of cell populations.
- Product concentration.
- Host cell protein (HCP).
- Product aggregates.
- Glycosylation profiles (for eukaryotic hosts).
- Metabolites.

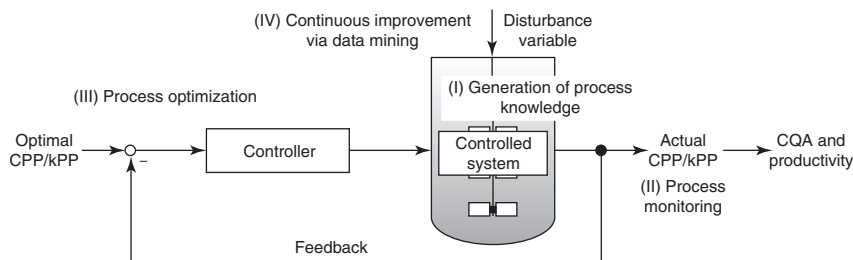
The selection of appropriate PAT tools is a crucial step toward setting efficient monitoring and control strategies in continuous processes. They must possess the following characteristics [24]:

- Easy-to-use instrumentation.
- Measuring frequencies.
- Ability to monitor multiple process parameters.
- Directly measuring CQAs.
- Capturing the real-time process state.
- Eliminating traditional offline techniques and increasing efficiency.

There is quite some advancement in the availability of at-line tools for CQA measurements using NMR or liquid chromatography coupled to mass spectroscopy [73]. However, for continuous processing, those tools must be deployed as real-time PAT tools. Various solutions may bridge this task in the near future such as (i) data-driven model-based approaches using spectroscopic measurements, (ii) robust online sampling solutions allowing to link gold standard analytics in online mode, and (iii) model-based approaches linking CQAs to easier real-time measurable components, as models, applied with observability analysis, offer a clear advantageous means to measure less. Hence, we need to diversify the PAT program to do the following:

- Minimize incoming material variation.
- Reduce CQA and CPP variations.
- Perform timely in-process measurements.
- Define representative sampling.
- Develop chemometric models and set appropriate acceptance criteria.
- Characterize the propagation of changes and disturbances through the system.

Data gained from adequate PAT measurements can be added to a knowledge platform, establishing model-dependent control, such as digital twins in the future. The usage of model predication can be a powerful tool to facilitate process control strategies, as discussed in Section 1.3.2.



**Figure 1.7** Multivariate control loop for the establishment of a robust process. Source: Kroll et al. [74].

### 1.3.2 Modeling and Control Strategies to Enable CBM with Microbials

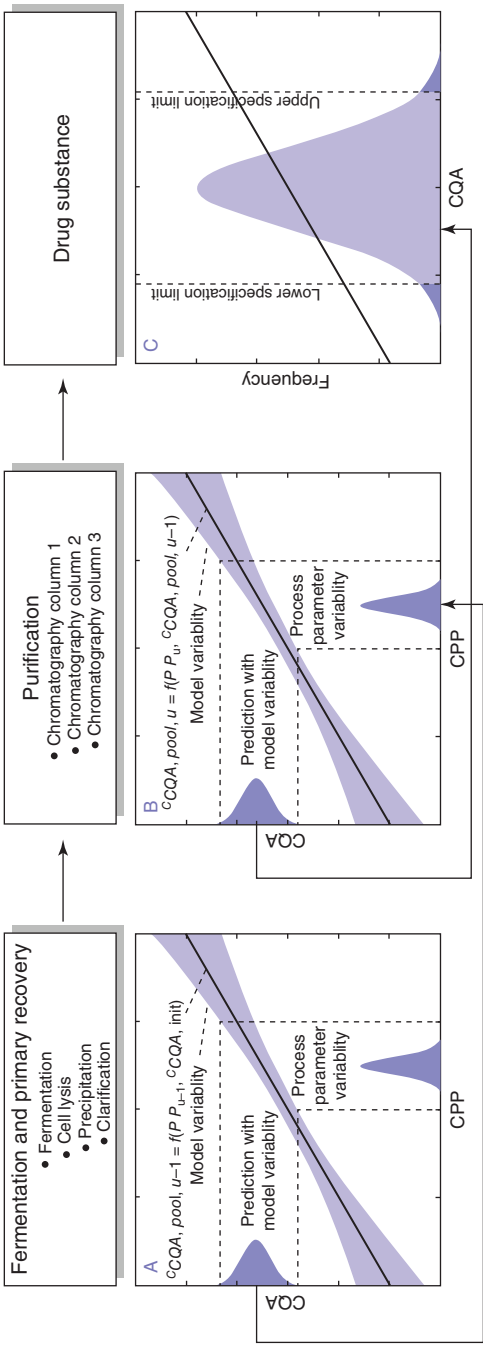
As derived from above sections 1.1.2 and 1.3.1, we need the following prerequisites for a robust process control strategy:

- Analysis of the output process variables of the unit operations (key performance indicators [KPIs] and CQA) using PAT (see Section above).
- A controller varying the CPPs to achieve a robust process to achieve time-invariant process performance and CQAs (Figure 1.7).

The development of the multivariate controller is the more difficult challenge, which allows varying multiple CPPs as function of a multitude of CQAs. Those multiple-input multiple-output (MIMO) controllers are available in other market segments; however, they are hardly applied in R&D bioprocessing labs up until now.

The main trigger for this transition will be the integrated real-time architecture combining data management, Namur Open Architecture (NOA) data architectures, real-time execution of models, and advanced control algorithms and workflows for model generation using good modeling practice [75] and maintenance using SaaS tools [75, 76], as recently reviewed [74]. A possible implementation is given in Figure 1.7:

In addition to the understanding of single unit operations, the interplay between the unit operations needs to be efficiently elaborated for robust continuous processing, because we have to consider the variation of the output of the preceding unit operation (intermediate CQAs or also defined by the FDA as RMA). Integrated models have been used in other market segments successfully (ASPEN, G-Proms) and need to be applied to integrated bioprocesses, irrespective from the mode of cultivation. The integrated process model (IPM) should quantitatively display the process understanding and include the elaborated NORs of individual unit operations [23]. Subsequently, this allows linking the individual unit operations and assessing error propagation within the variation in the NOR using sensitivity studies. Acceptance criteria for any process step can be established and fused together in the total process chain via Monte-Carlo simulations, for example, as shown in Figure 1.8. As a result, integrated process modeling identifies PPs that are holistically critical for the entire process chain and therefore allows identifying necessary control strategies along the entire process to meet acceptance criteria of regulatory authorities [25].



**Figure 1.8** Integrated process modeling, defining the criticality of each unit operation and the effect on the process outcome. Source: Zahel et al. [25].

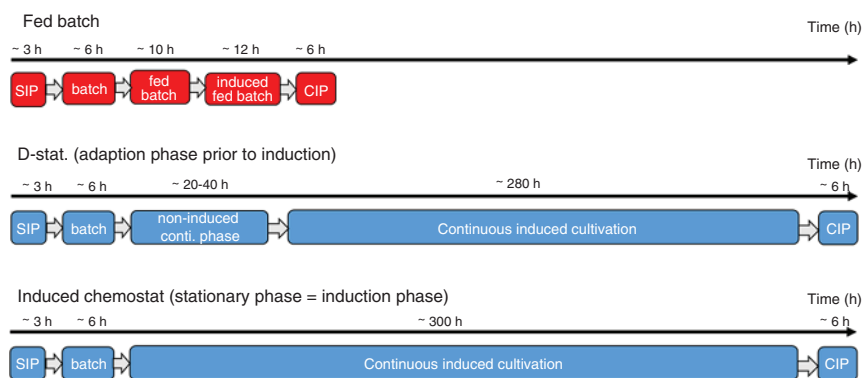
## 1.4 Chances and Drawbacks in Continuous Biomanufacturing with *E. coli*

The choice of a suitable expression host is highly dependent on the produced protein and the final application. For example, the production of monoclonal antibodies (mABs) cannot be performed in prokaryotic hosts due to required PTMs. In contrast, fragment antigen binding (fABs) can be successfully expressed in *E. coli*, due to the lack of PTMs and the oxidizing environment of the periplasm [77]. Expression of recombinant proteins with *E. coli* exhibits certain benefits, such as high cell specific productivity, throughout continuous upstream. TSY might be boosted in microbial continuous cultivation systems, as overall dilution rates and protein expression rates are higher in microbial hosts than in mammalian cells. Hence, we wanted to demonstrate the chances and drawbacks of microbial continuous applications using the host *E. coli* as a model organism.

### 1.4.1 Optimization of Plant Usage Using CBM with *E. coli*

As drug manufacturing is operated in large scales, setup and cleaning are time and energy intensive to meet guidelines of authorities in the industry [78]. Even though energy-effective sterilization and cleaning procedures have been established, a reduction of such “downtimes” would facilitate the overall TSY. Continuous processes meet these demands and in addition can reduce the amounts of chemicals needed for cleaning, thus lowering overall costs.

RPP in *E. coli* is usually employed with inducible promoters [79], where cells are grown to high cell densities prior to induction, therefore resulting in a higher amount of catalyst for ongoing reactions [65–67]. Consequently, time-dependent cultivations (such as batch and fed-batch) profit from non-induced cultivation times as cells grow burden-free [64]. Timeframe for batch cultivation is highly dependent on the achieved cell density throughout pre-culture, the amount of pre-culture added, and the glucose concentration in the batch medium [68]. Generally, sugar concentrations supplied throughout batch phase should be below 30 g/L, as long growth at  $\mu$ -max results in high acetate formation [69, 70], and  $dO_2$  limitations might occur (see Section 1.2.1). Therefore, we assumed an average batch phase to be conducted within six hours. Due to the given reasons, it is beneficial for the overall product yield to conduct a non-induced fed-batch phase. Timeframes for non-induced fed-batch are highly dependent on (i) the set  $\mu$  and (ii) the targeted biomass concentration before induction. Process conditions throughout induction might vary between target products, and thus it is hard to generalize this approach. Theoretical biomass per substrate yields prior to induction should be  $Y_{x/s} = 0.5$  g/g. Therefore, set growth rates in the range of  $0.08$ – $0.15$  h<sup>-1</sup> for *E. coli* cultivations should result in a linear correlation of biomass growth and substrate uptake allowing burden-free doubling of batch biomass within 4.6–8.6 hours [71]. Hence, we assumed fed-batch duration with 8 hours. Induction time is highly dependent on the target product and the achieved biomass prior to induction in combination with the set physiological parameters throughout induction. For the production of fABs in the periplasm



**Figure 1.9** Average time needed for each process step comparing a fed-batch cultivation with a continuous cultivation including an adaption phase and a continuous cultivation mode, where induction is performed directly after batch phase for recombinant protein production in *E. coli*.

and intracellular soluble proteins, induction times in the range of 7–10 hours were applied in literature [80, 81]. Time spans in the range of 10–12 hours showed beneficial results for inclusion body production in previous studies [28, 82, 83]. Hence, induction time was calculated with 10 hours. Setup times and cleaning times in the industry are highly dependent on the operating scale. For the comparison of common process strategies as shown in Figure 1.9, we assumed an USP plant in the range of 5–10 m<sup>3</sup>, with sterilization and cleaning times of three and six hours, respectively.

A time-dependent biomass growth phase in a batch mode should be conducted to reach a targeted biomass before cultivations are switched to a continuous cultivation mode. In case high biomass concentrations are needed prior to continuous mode, a fed-batch phase can be employed to reach targeted biomass, or a continuous adaption phase can result in the same effect. Furthermore, in case induction is employed throughout continuous phase, either (i) a non-induced adaption phase consisting of four residence times has to be performed to establish an equilibrium state prior to induction (D-stat. cultivation), or (ii) an “induced continuous phase” is performed directly after batch phase (Figure 1.9).

For continuous cultivations with *E. coli*, dilution rates in the range of 0.1–0.5 h<sup>-1</sup> are commonly employed for the screening of wild-type strains [84]. However, when producing recombinant proteins, biomass yield might decrease, as lower cell capacities for maintenance are given (Figure 1.4) [72]. Hence lower dilution rates in the range of 0.1–0.2 h<sup>-1</sup> should be employed for induced chemostat cultivation, producing recombinant proteins, to avoid sugar accumulation and minimize the risk of washout.

D-stat cultivation might be an option to adapt cells stepwise to the formation of target molecules. In an induced chemostat, many shifts occur in parallel, as (i) carbon limited growth is started and (ii) a constant washout of cells occurs. As levels of ppGpp and rpoS were shown to alter within minutes when switching to carbon-limited growth, for fed-batch and continuous cultivations [85], “small”

shifts might already cause high deviations in the host cell transcriptome. In induced chemostat cultivation, an inducer is supplemented on top, implementing additional shifts such as (i) the establishment of new transport systems and (ii) recombinant molecule growth. Regarding the overall TSY, it would be beneficial to omit the adaption phase and perform induction phase directly after desired biomass concentration is achieved. Still, its shifts must be investigated for any product and host, whether cells tolerate a harsh shift such as an induced chemostat. Cell stress might cause negative side effects, possibly causing a unstable productivity.

In case a continuous cultivation strategy can be found to maintain stable productivity, the downtime can be reduced significantly. Using continuous systems, the percentage of downtime in comparison with total process time can be reduced from 65.7% to 14.3% or 4.7%, comparing fed-batch cultivations with D-stat and induced chemostat cultivations, respectively (Figure 1.9). Therefore, average downtime in continuous processes with *E. coli* is at maximum 22% (14.3% for D-stat vs. 65.7% for fed-batch downtime) of the downtimes required for conventional fed-batch cultivation. Using the timeframe shown in Figure 1.9, a continuous cultivation producing more than 22% of the total throughput achieved in a fed batch would thus be superior. However, in this calculation, no purification procedure is included, and downstream processing is known to be the bottleneck in microbial production of recombinant proteins [86, 87]. Low target protein concentrations provoke highly difficult and expensive downstream applications [88]. To realize the calculation above, (i) continuous systems would have to achieve the same purity (ratio of target protein to impurities) as achieved at fed-batch harvest and (ii) continuous downstream applications would have to achieve the same purification yields as batchwise downstream. In case the same purification yield can be achieved, a continuous cultivation system reducing overall downtime can have a major increase on total product throughput. Taking timeframes depicted in Figure 1.9, continuous cultivation producing constantly 50% of a fed-batch productivity would thus increase total product throughput by more than double. Within this calculation, the saved costs for chemicals and energy are not even implemented; therefore the high potential of increasing TSY via continuous cultivations is most definitely given.

## 1.4.2 Reasons Why CBM with *E. coli* Is Not State of the Art (Yet)

### 1.4.2.1 Formation of Subpopulation Following Genotypic Diversification

Biotechnology, unlike many other branches of industry, employs living cells for catalysis. Following Darwin's principles, all living cells always suffer from a certain mutation rate to create a more-fit species [89]. Distinguishing microbial cultivations on a species level would result in a harsh difference; however we can differentiate them into certain subpopulations. Hence, any bacterial cell, bearing a certain mutation, could be the beginning of a new subpopulation formation. Mutation rate probabilities in *E. coli* have been summarized by Rugbjerg and Sommer [90], showing the likelihoods of mutation rates in conventional time-dependent cultivations. Even though mammalian cells bear a higher mutation rate probability than *E. coli*, bacterial cells exhibit much higher growth rates (Table 1.2), and

therefore time-dependent effects might occur faster cultivating bacterial cells than in mammalian cells (Table 1.2) [23, 27, 28, 33].

Alterations in the karyotype (chromosome restructuring) were found to increase majorly in mammalian cell cultivations lasting longer than 50 generation. This results in population heterogeneities upon changing genetic material [91]. Furthermore, cloning in CHO cells is an uncontrolled procedure, resulting in a high number of gene copies in the cell with uncontrolled loci of integration [91]. Epigenetic changes based on DNA methylation (decline of recombinant Mab transcript copy number correlated with increased) and methylation of the Mab human cytomegalovirus (CMV) promoter and gene loss are complex interactions making cell culture cultivation hard to predict [92]. Genetic instability for continuous cultivations with *E. coli* is also based on several different effects [44]. Without additional selection pressure, bacterial cells tend to expel plasmids, leading to the reduced plasmid copy numbers per cell, which can be limited by antibiotic resistances or auxotrophic genes [93, 94]. Besides the general belief that single-nucleotide polymorphism (SNP) is the predominant cause for variations in bacterial cultures, major effects in continuous cultures are based on population inhomogeneity. Metabolic burden, also called product burden, decreases fitness and specific growth rates of the producing cultures, which finally results in an overgrowth of the non-productive subpopulation. For several *E. coli* strains, IS elements were identified to be responsible for the inactivation of genes in the production of mevalonic acid [41].

However, differences on subpopulation level can be difficult to monitor, without genome sequencing or transcriptomic analysis. Distinguishing between productive and nonproductive subpopulation using RPP is rather easy to monitor and will be discussed in the following paragraph.

In red biotechnology, plasmid technology is commonly used to produce recombinant target molecules in *E. coli* [68]. Cultivated cells, which have been transformed with plasmids containing gene sequences for target proteins, generally suffer from decreased growth rates and lower yields than wild-type strains [69]. Furthermore, mutation rate probability is increasing upon addition of inducer for recombinant protein producing strains [95]. The so-called transcription-induced mutation has been described to increase mutation rate probability by a factor of 4 compared to the non-induced state [96, 97]. To form an efficient subpopulation, mutations need to decrease burden onto host cell machinery in such an efficient way that the mutated population yields in higher growth rates [41]. In conventional batch and fed-batch cultivations, induction time is relatively short compared with continuous cultivations (Figure 1.9). Thus, a takeover of a non-efficient subpopulation is unlikely to take place. Taking a set  $\mu$  of  $0.1 \text{ h}^{-1}$  throughout an induction time of 10 hours [28], cells would only double 1.44 times throughout their induced phase, given there is no observed decrease of growth rate [98]. However, a continuous cultivation at a set  $D = \mu = 0.1 \text{ h}^{-1}$  running for an induction time of 280 hours (Figure 1.9) would bear 40.4 generations throughout its induction time. By increasing the generation times, the probability of shifting transposable elements, base-pair substitution, and large gene deletion is increased [29, 67]. Furthermore, in conventional fed-batch cultivation, cells are maintained in the fermenter until the time point of harvest,



keeping all kinds of subpopulations within one reactor, whereas in chemostat a constant washout occurs. Hence, subpopulations growing “more efficiently” than other subpopulations can be detected more quickly in feed/bleed systems, as they will overgrow the initial population, which will be washed out consequently [99].

#### 1.4.2.2 Formation of Subpopulation Following Phenotypic Diversification

Phenotypic diversification of cell population is driven by the stochasticity of the intracellular biochemical reactions. This phenomenon can be also termed biological noise and comprises two components, i.e. extrinsic noise and intrinsic noise [100]. The intrinsic component of noise results from the low abundance of reacting molecules in the cell (i.e. transcription factors and ribosomes), lowering the probability of collision between reacting species. The extrinsic component of noise is driven by cell-to-cell variation in the number of the reacting species due to the external factors. The cultivation environment thus can have a huge impact onto external factors that is majorly influenced by (i) the design of the cultivation device, (ii) the operation mode of the bioreactor (i.e. batch, fed batch, or continuous), and (iii) the used media taking metabolites or substrate accumulation into account also. The understanding of the dynamic adaptation of microbial populations to environmental perturbations is one of the key missing elements, which is required to allow controllability of the biological system under phenotypic diversification. To analyze phenotypic diversification, the development of appropriate biosensors is required. Furthermore, promoter-based biosensors have been used in many studies to track physiological changes at a single-cell resolution [29, 31, 101]. Models that integrate the stochastic components of biochemical reactions can then be incorporated based on the quality of the acquired single-cell data and can be used to reconstruct transcriptional regulatory networks [102]. Such strategies have allowed to precisely control a microbial population with optogenetics approaches, i.e. by controlling gene expression based on light pulses [103, 104]. However, these stochastic modeling frameworks need to be improved to take account of all factors, leading to the biological noise and affecting the internal state of host cells [32]. Hence, factors such as the noise in gene expression and its consequence on metabolic pathways and cell elongation/division need to be investigated more thoroughly [105–107].

The continuous mode of cultivation (i.e. the chemostat) seems to promote phenotypic diversification since nutrient limitation is a strong driver of such diversification strategies [70, 108]. Switching toward continuous cultivations will have to deal with this kind of phenomena that impair the observability of microbial populations. All the above mentioned methods can be applied to investigate phenotypic diversification of microbial population in CBM systems. However, population control is scarce. A recurrent feature is that population stability cannot be ensured in classical chemostat systems [30, 109]. On the other hand, the very same studies have shown that applying nutrient pulses at given interval during continuous cultivation promoted proteome and subpopulation stabilization. This effect known as periodic forcing has also been shown to be efficient in stabilizing the activity of synthetic gene networks [110]. Whereas the molecular mechanisms behind population stabilization through periodic forcing are still unknown, carbon-limited

growth can be avoided, and phenotypic diversification can be lowered by the application of nutrient pulses. Once the puzzle is solved, substrate and inducer pulses at given frequency and amplitude throughout continuous cultivation might be a new paradigm for ensuring cellular stability during CBM.

#### 1.4.2.3 Is Genomic Integration of the Target Protein an Enabler for CBM with *E. coli*?

Genomic integration is believed to solve the stability issues in continuous cultivations as plasmid loss can be avoided and antibiotic-free medium can be employed, being especially important in large scales [111]. As the expression of recombinant protein bears a high burden onto host cells upon induction [112], plasmid loss can be expected as a consequence. Comparing mutation rate probabilities, the probability of base substitution due to the DNA polymerase errors is in the range of  $10^{-7}$  to  $10^{-10}$  per generation per base pair, and disturbance by transposable elements ranges from  $10^{-5}$  to  $10^{-8}$  per generation per base pair, whereas plasmid loss is documented with a higher probability of  $10^{-2}$  to  $10^{-6}$  per generation [67]. Plasmid loss probability can be decreased by diverse selection pressures [113, 114]. Furthermore, the employment of high copy plasmids (obtaining 500–700 plasmid copies per cell) makes plasmid loss rather unlikely of being responsible for a decrease in productivity during continuous cultivation [115, 116]. Constitutive promoters are rarely employed for *E. coli*, whereas inducible promoters present the dominant fraction [59]. Using inducible promoters, no basal expression should be monitored prior to induction, and thus burden onto cells should not be detectable in non-induced growth phases [117]. Genomic integrated strains were found to show a slightly higher  $\mu_{\max}$  than plasmid-based systems [80].

Even though results are promising for genomic integration, the total replication rates of high copy plasmids tend to exceed the numbers achieved by genomic integrated systems. Moreover, the exact interactions in genome-integrated systems have not been understood up until now [16]. Given that the locus of integration is of high relevance for target molecule expression [118], each target sequence would require an efficient screening for the best locus on the genome. As for these reasons, genomic integration technology still needs to be eased, and a toolbox platform needs to be established, favoring plasmid-based systems in industry up to now [119]. However, the expression system and the applied product locus are highly dependent onto the desired goal. Plasmid technology might be the appropriate choice for inclusion body processes, as high copy rates are needed to meet the high titer demand [120, 121]. For the expression of soluble proteins, genomic integration might be feasible as a soft induction of host cells might lead to properly folded protein and lower copy numbers are needed to not overload chaperones and the folding machinery of *E. coli* [122].

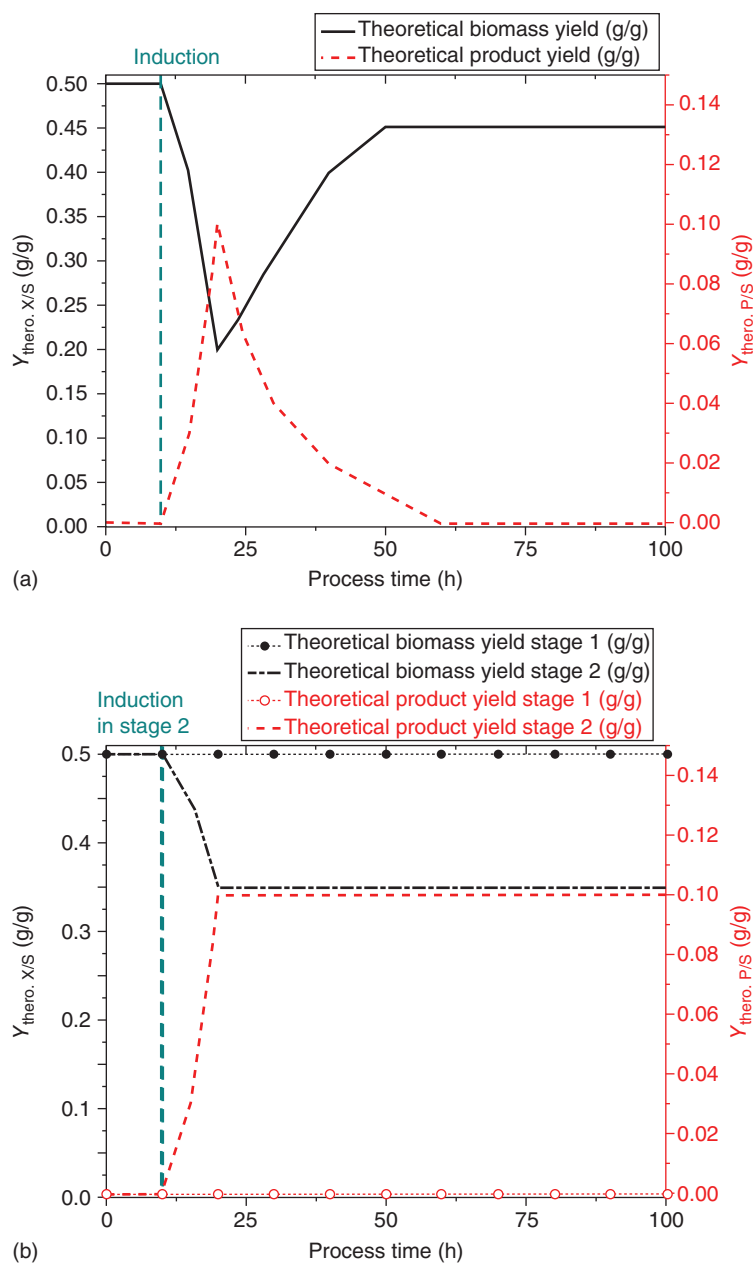
As the plasmid loss seems not to be the reason for observed shifts in productivity, it is most likely that genome-based mutations are responsible to cause a “more-fit” sub-population, having the probability to overtake a cultivation broth. Hence, it would be intriguing to investigate mutation rate likelihood derived (i) by genomic integration vs. plasmid-based systems, (ii) as a function of induction strength, and (iii) as a function of residence time in induced continuous systems.

### 1.4.3 Solutions to Overcome the Formation of Subpopulations and How to Realize CBM with *E. coli* in the Future

Engineered *E. coli* cells face a certain metabolic burden visible throughout induction time. In batch and fed-batch cultivations, the metabolic burden can be eased during biomass growth due to time dependency, i.e. growing biomass throughout non-induced phases and applying a relatively short induction time. However, recombinant protein formation is a time-dependent process; hence a trade-off between optimal product yield and too high burden has to be done [28, 82]. In continuous cultures, a time-dependent separation is impossible, as cell growth and recombinant protein formation are occurring in parallel [84]. It was clearly shown that biomass and product yields are counteracting throughout a chemostat cultivation [39]. Therefore, separation of growth and recombinant protein formation should be performed in a continuous mode to enhance the product yield, which can be realized in cascaded cultivation mode (Figure 1.3a,c) [41]. In Figure 1.10a), the biomass and product yield trends for chemostat and cascaded continuous cultivation are depicted. The chemostat is producing only for a short duration before a nonproductive subpopulation takes place as shown in the literature [30, 32, 33, 99, 123, 124]. Throughout induction phase, the biomass yield drops as a major part of the energy derived by the substrate is needed to cope for the needs of target molecule production (Figure 1.4). Depending on the set dilution rate and chosen product, the yield decrease might vary; however decreasing biomass yields upon induction cannot be avoided. Throughout the time span of a chemostat cultivation, biomass yield recovery can be observed. However, the yield recovery unfortunately happens at the expense of product formation. As we suggest that a part of the population still tries to produce the target protein, the biomass yield in chemostat cultivation is believed to be lower than the initial biomass yield observed throughout non-induced growth phases.

The two-stage continuous fermentation first described back in 1991 (Figure 1.3c) [125] should enable stable production of recombinant target protein via spatial separation of biomass and product formation. Plasmid concentration was found to stay stable throughout target molecule production using this cultivation mode [125]. For this process mode, the biomass yield in the non-induced reactor should stay stable at the theoretical level of 0.5 g/g, as host cells are not confronted with metabolic burden (Figure 1.10b). Throughout the second stage, there is a short time-dependent behavior, as cells have to adjust their metabolism to induction, and therefore a certain adaption phase can be observed [33]. In case process conditions challenge host cells with a tolerable metabolic burden, a stable production and biomass yield can be achieved, which should stay constant over time. Hence, it has to be kept in mind that the mutation rate probability in the seed reactor increases also with ongoing cultivation time, probably causing time- or generation-based effects in either one of the reactors [41].

Promising results were found using a BL21(DE3) strain transformed with conventional pET-plasmid expressing target proteins under control of the lac promoter. Independently from the target molecule employed, all studies favored higher dilution rates throughout the second stage [39, 40, 125]. We hypothesize that lower



**Figure 1.10** Showing theoretical biomass and product yields for (a) chemostat cultivation and (b) cascaded continuous cultivation, showing trends of the seed reactor (stage 1) and the induced producing reactor (stage 2).

residence times are beneficial, as cells do not bear the load of induction for a long duration and are washed out more quickly than in systems where low dilution rates are applied. In fed-batch cultivation, the specific feeding rate was found to have a high dependency on the production of the recombinant protein, thus making the time point of harvest crucial [81]. High feeding rates led to a peaking productivity in the range of 6–8 hours for an inclusion body process [28, 83]. Hence, residence times applied (i) should be set close to the peaking productivity monitored throughout fed-batch cultivation, (ii) should not exceed the peaking productivity to maintain cells in non-stressed metabolic state, and (iii) should not be set too low, as a certain time of induction is necessary to yield in sufficient sugar uptake and RPP. Using high dilution rates, cells might have little time to adapt to cultivation conditions; hence, the evolution of a nonproductive population can be reduced as only little time is given in the cultivation device. However, it must be considered that residence times only calculate the average proportion of cells, which are maintained in the reactor. Therefore, cell populations might also show evolutionary tribes at low residence times. Nevertheless, it has to be kept in mind that very low residence times also implement little time for sugar and inducer uptake. Therefore, the dilution rate has to be adapted to avoid washout upon yield decrease (Figure 1.4), and a trade-off between washout and emerging probability of nonproductive subpopulation must be found. Still, from an industrial point of view, higher productivities at higher dilution rates would be desired as the overall mass flow of product out of the reactor is increased, implementing an increased TSY.

## 1.5 Conclusion and Outlook

Continuous systems have a huge opportunity to exceed the TSY of time-dependent cultivations (i.e. fed-batch systems). However, due to the high amount in generations achieved by microbial continuous cultivations, the formation of different subpopulations can be observed. Hence, population heterogeneity needs to be dealt with. Flow cytometry measurements of populations via specific staining (red-but-not-dead phenotype) or the use of reporter genes coupled to fluorescent markers might allow to adjust feeding and dilution rates to keep populations at a constant level. Control strategies known as “segrestat” have been recently developed where subpopulations are monitored via online flow cytometry and steered at a certain level via glucose pulses. Furthermore, cascaded continuous cultivation, separating biomass formation and RPP, has been referred to allow feasible productivity throughout continuous processes. Nevertheless, process steps must be investigated thoroughly for their criticality. A yield comparison between time-dependent and time-independent process modes should therefore be carried out for any production process. A continuous process is only sufficient if all unit operations can be combined properly, enabling a fully functioning process chain. Technologies for continuous upstream have been developed, and the continuous purification of inclusion bodies has also been demonstrated. Fusing current state-of-the-art techniques might thus allow to implement a fully integrated microbial continuous process. Once control strategies

for microbial continuous systems are sophisticated enough for regulatory authorities, they should be implemented in the corresponding unit operations, and hence continuous processing with microbials should be authorized from a regulatory point of view. So, if companies are questioning whether they should also pursue continuous systems or not, just give it a try - It might increase process efficiency.

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