1
Bacterial Suspension Cultures

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Abstract

Bacteria became the primary workhorses of the biotechnology industry due to fast growth on inexpensive media, well understood genetics, and an advanced genetic engineering toolset. This chapter provides a thorough basis for bioprocess and bioreactor design of cultivating bacteria in industrial scale suspension culture. First, an overview of the product spectrum produced by bacteria is presented. Thereafter, industrial expression technologies and strain engineering aspects are discussed with special attention on plasmid stability and predictability of product titers to large scale. Subsequently, bioprocessing strategies for bacteria in suspension culture are discussed, focusing on the production of recombinant products in fed-batch mode. As discussed, bioprocess design has to consider technical constraints as well as physiological constraints of the cell. Based on this, a detailed roadmap for the design of a bacterial bioprocess is provided. Subsequently, insight into bioreactor design, engineering, and instrumentation is given, discussing stirred-tank bioreactors, for multi-use and for single use purposes. The chapter finishes with a discussion of regulatory aspects (quality by design) as well as industrial scale economic aspects. The sections on bioreactor design and "quality by design" should also be regarded as valid for the entire book.

1.1
Introduction

Heterotrophic bacteria are those bacteria that use organic carbon-containing compounds as sources of carbon and energy. Many bacteria in this category are able to produce valuable bioproducts; hence, they play an important role in modern manufacturing. The focus of this chapter is the cultivation of such bacteria in suspension culture. These cultures have been implemented widely in commercial production of many bioproducts. This chapter concentrates on practical and

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industrial aspects of the process and reactor design for the cultivation of heterotrophic bacteria, and the authors expect the reader to have enough scientific background about microbial systems.

In this chapter, first, a thorough review of bacterial products is presented (Section 1.2). Among all products, the one that has the most promising and thriving market is introduced. Recombinant therapeutics, specifically fragment antigen-bindings, were found to be a fast-growing market. Subsequently, industrial aspects of bacterial expression systems, especially those dealing with the production of recombinant therapeutics, are discussed. Section 1.3 explains bioprocess design aspects such as technical and physiological constraints and cultivation strategy. Section 1.4 gives insight into the bioreactor design, engineering, and instrumentation. This section serves as a general basis for the design of bioreactors for cultivation of not only bacteria but also yeasts. Single-use bioreactors are covered in Section 1.5. Various regulatory aspects of microbial systems and key economic parameters are discussed in Sections 1.6 and 1.7, respectively.

1.2 Organisms, Cells, and their Products

1.2.1 Bacteria as Production Platform for Various Products

Bacteria are a very large and diverse group of unicellular organisms. These organisms are a few micrometers long, do not possess a nucleus, and are found in every habitat of the earth. Bacteria as production platforms provide many advantages that make them popular. Their molecular biology is well understood. They grow rapidly on inexpensive media and their characterization is straightforward. Considering recombinant DNA technology, the construction of bacterial vectors is relatively easy and common bacterial expression platforms profit from an established regulatory track record [1].

A wide variety of products are produced by bacteria. They are extensively used in white, red and green biotechnology markets. Many of these products, among them amino acids, solvents, vitamins, and antibiotics, are synthesized naturally. Nevertheless, the invention of recombinant technology facilitated the production of those products that are not synthesized naturally. Recombinant proteins and pDNA are representative for this category. Hence, all bacterial products can be roughly classified into natural and recombinant products (Table 1.1). Of course, natural products are also boosted in productivity via genetically engineered pathways. Depending on the application field and purpose of use, they are produced in different amounts with varying added-value. The production efficiencies of natural products can be greatly enhanced through metabolic pathway engineering. Most of the native products are produced in large quantities while recombinant products are produced in low kilogram ranges. Typically, compounds with applications in the field of red biotechnology are high value, low volume products.

Table 1.1 Some examples of recombinant and native bacterial products. Products are classified in respect to their value, usage, and production tonnage.

	Product	Value			Usage			Production volume	
		Low	Middle	High	White biotech.	Red biotech.	Green biotech.	kg yr^{-1}	tons yr^{-1}
Recombinant products	Proteins	×	×	×	×	×		×	
	Fab fragments			×		×		×	
	Hormones			×		×		×	
	Cytokines			×		×		×	
Natural products	Amino acids		×		×				×
	Antibiotics		×		×	×			×
	Vitamins		×		×				×
	Solvents	×			×				×
	Pesticides		×				×	×	
	Growth factors for animals and plants		×				×	×	

Table 1.2 presents examples of bacterial products with their annual global production volume, bacterium, and the scale of the industrial bioreactor (www.researchandmarkets.com) [2].

Recently, biopharmaceuticals became the fastest growing sector of the pharmaceutical industry, with more than 200 marketed products and hundreds more in development [3]. The growth rate of this market was expected to be at 12% per annum for the next decade [4]. The biopharmaceuticals market segment has expanded rapidly due to the significantly higher clinical success rate compared with new small molecules, their greater potential for curing disease rather than just treating symptoms, and their greater efficacy and reduced side effects [5].

The biopharmaceuticals market consists of mature segments such as the hormone products and traditional vaccines as well as maturing segments like monoclonal antibodies (MAbs) and modern vaccines. MAbs are the dominant product class in the biopharmaceuticals market (see also market information in the introductory chapter) with applications in fields such as cancer treatment; 13% of all deaths in the world are cancer related [5]. MAbs are highly complex biomolecules and their proper synthesis requires post-translational modification machinery. Such machinery is available only in eukaryotic cells. However, antibody fragments like fragment antigen-bindings (Fabs) are suitable for expression in microbial bacterial systems, providing benefits in terms of increased scale and ease of manufacture [6]. For many applications Fabs are perceived to have similar therapeutic effects as full monoclonal antibodies. Hence, Fabs do have the potential to develop into the most important bacterial product in the maturing market.

Table 1.2 Some examples of bulk chemicals, their production volume, and the respective bacterial host.

Bioproduct	Product category	Bacteria	Annual production (tons yr^{-1})	Bioreactor scale (m^3)[a)]
L-Glutamic acid	Native product (primary metabolite)	*Brevibacterium* species	1 600 000	50–500
L-Lysine-HCL	Native product (primary metabolite)	*Brevibacterium lactofermentum*	850 000	50–500
Vitamin C	Native product (primary metabolite)	*Gluconobacter oxydans*	107 000	50–500
Monensin	Native product (secondary metabolite)	*Streptomyces cinnamonensis*	>3000	50–500
α-Amylase	Recombinant product	*Bacillus subtilis*	26 000 (only in China)	50–500
Recombinant proteins for medical purposes	Recombinant product	*Escherichia coli*	10–100 kg	0.05–15

a) The size of the reactors depends on the plant capacity. However, bioreactors as large as some hundreds of cubic meters are common.

The most common bacterial host, which is widely used as production platform for the production of Fabs and other biopharmaceuticals, is *Escherichia coli*. Hence, in the following, key aspects of this bacterial expression system is discussed.

1.2.2 Historical Outline for *Escherichia coli*

The relevance of bacteria for the development of modern life-sciences and the biotech industry is extraordinary. *Escherichia coli*, a rod shaped Gram negative intestinal bacterium, is the most important model organism for microbiology, gene technology, and biotechnology. Major scientific achievements such as bacterial conjugation, topography of gene structure as well as transformation, were obtained from studies on the bacterium *E. coli* [7–9]. These findings paved the way to modern genetic engineering, which enabled efficient production of industrial and pharmaceutical proteins through the use of recombinant DNA technology. The introduction of recombinant DNA technology can be regarded as a quantum leap for the whole biotechnology industry. Its role as an omnipresent companion of biotechnology related research made *E. coli* a well-characterized organism. Access to a wealth of strain-specific information including a fully sequenced genome has obvious advantages. Furthermore, *E. coli* is outstanding regarding growth rate, safety, ease of cultivation due to simple nutritional demand and suitability for high-density cultivation, and the availability of a large tool box of genetic techniques for genome modification [1,10–12]. Nowadays, *E. coli* serves as a host organism for the industrial production of various recombinant proteins,

Table 1.3 Industrial key aspects for the use of bacterial expression systems.

Aspect	Goals
Productivity	$>15\,g\,l^{-1}$ for proteins as inclusion bodies;[a)] $>5\,g\,l^{-1}$ for simple monomeric proteins; $>0.5\,g\,l^{-1}$ for more complex proteins such as heterodimers
Solubility	Inclusion body-free processes for higher yields, reliable, simple DSP, and no refolding tanks
Stability	Genetic stability of host and plasmid (both segregational and structural) to support reliable, predictable, and scalable high cell density cultivation
Scalability	Predictable to large scale
Speed	Short development times to identify the best production clone and suitable process within a few weeks

a) Inclusion bodies: insoluble protein aggregates. To obtain the protein in active form, additional steps in downstream processing are necessary (Section 1.3.3).

including insulin, β-interferon, and numerous technical enzymes. Most microbial recombinant proteins are still produced in *E. coli,* although recombinant expression in other organisms such as other bacterial platforms, yeast, and fungi was proven to be applicable in an industrial context.

1.2.3 Industrial Aspects of Bacterial Expression Systems

For successful process development, the key aspects given in Table 1.3 need to be addressed in the early phases of a strain development program. These key aspects are discussed in more detail in the subsequent sections.

Sufficient Productivity, a Prerequisite for Commercial Success The choice of the production strain background is essential since different hosts and expression systems solve different challenges. For simple monomeric proteins with no solubility issues *E. coli* is good to go. *E. coli* is still the main workhorse, producing nearly 70% of all commercial microbial recombinant proteins.

Nevertheless, at least for the time being, the huge diversity of proteins that nature invented cannot be produced by a single expression technology. No single strain or vector is capable of being the best expression option for all types of biopharmaceuticals. No one system fits all of the target molecules (Table 1.4).

Solubility, Inclusion Body-Free Processes for Higher Yields and Simple DSP In principle, positively and negatively regulated inducible expression systems can be used. The induction kinetics of positively regulated systems (using e.g. promoters of the arabinose, rhamnose, and melibiose catabolic operons) is completely different from negatively regulated expression systems using e.g. the T5, T7, lac or tac promoters which are induced by IPTG or lactose. The maximum product amount using negatively regulated systems is already detectable a few hours after induction start, in contrast to positively regulated systems in which the product peak is detectable after more than 8 h of induction. A slow induction kinetics favors the

Table 1.4 Commercial bacterial expression systems.

Host	Trade name	Company
Escherichia coli	pAVEway	Fujifilm Diosynth Biotechnologies
	T7	Brookhaven National Laboratory
	XS	Lonza
	ESETEC	Wacker Chemie
	BioXcellence	Boehringer Ingelheim
	NAFT system	Sandoz
	PlugBug	DSM
		Genentech
	pBAD	Life Technologies
Pseudomonas fluorescens	Pfenex	Pfenex
Corynebacterium glutamicum	Corynex	Ajinomoto
Bacillus subtilis		MoBiTec
		Takara
	XS	Lonza
		DSM
Bacillus brevis		Itoham Foods
Bacillus megaterium		MoBiTec
Lactococcus lactis	NICE	MoBiTec (NIZO food research)
Brevibacillus choshinensis		Takara

formation of soluble, functional target protein, especially for periplasmic production. IPTG-induced negatively regulated systems may overload the secretion machinery and the periplasmic space with very high target protein amounts leading to aggregation and solubility issues.

In addition, IPTG-induced promoters are not tightly controlled; the non-induced promoter activity is much higher compared to positively regulated systems, leading to product accumulation in the absence of IPTG. Already at very low IPTG concentrations the promoter is fully active and therefore makes promoter tuning very difficult or even impossible. However, process technological approaches aimed at promoter tuning using IPTG induced promoters have been reported [13].

In contrast, the transcriptional activity of the rhamnose promoter is very low in the absence of L-rhamnose, enabling easy plasmid construction and clone selection for proteins that interfere with the cell metabolism and are therefore toxic [14]. Transcription is activated by two regulators, RhaR and RhaS, which interact with rhamnose. Activated rhaR enables its own transcription as well as that of RhaS, which positively regulates the transcription from the rhamnose promoter.

Recently, a process technological method that allows tunable recombinant protein expression using the pBAD promoter via simultaneous feeding of D-glucose and L-arabinose was reported [15]. The method aims to achieve high product titers through moderate long-term expression of recombinant product.

Owing to low titer, solubility, or impurity issues (such as endotoxin), *E. coli* might not always be a suitable host. Then, alternative microbial expression systems need to be evaluated (Table 1.4) such as *Bacillus subtilis* or also yeast such as

Pichia pastoris. It does make sense to cover a broader phylogenetic spectrum of microbial hosts (Gram negative, lower eukaryote, Gram positive) to identify viable microbial production systems for every single protein of interest. Real secretion hosts such as *B. subtilis* are very useful as they accumulate the target protein in the culture supernatant, which therefore sometimes circumvents solubility issues which may appear for periplasmic production in *E. coli*. In addition, cell-free medium with reasonable product titers is the perfect starting point for simple and successful downstream process development.

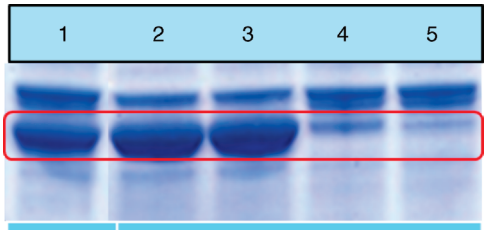
Genetic Stability of Host and Plasmid to Support Reliable, Predictable and Scalable High Cell Density Fermentation A common problem of state-of-the-art bacterial expression technology is host and plasmid instability. For example, the frequently used T7 expression system suffers from three major issues:

1) commonly used production strains contain prophages;
2) related host instability [16];
3) segregational and structural plasmid instability.

BL21 and its recA mutant BLR are very popular *E. coli* host strains, which are available as λDE3 lysogens called BL21(DE3) and BLR(DE3). A lytic cycle of λDE3 can be induced by stress. Though a rare event, spontaneous induction of lambdoid prophages under process conditions is described in literature [17]. For this reason, it is assumed that any culture broth derived from a prophage containing *E. coli* contains a certain number of phages even under standard process conditions. Fortunately, due to the immunity phenomenon, a released free phage does not affect its lysogenic host, but, nevertheless, phage release and contamination is a large-scale issue for multipurpose plants with changing production hosts and needs to be addressed carefully.

Figure 1.1 illustrates host instability in the framework of a use test as part of a cell banking procedure. The cultures were inoculated from the corresponding glycerol stocks from the state-of-the-art host/plasmid combination (BLR(DE3)), a stable production strain and a reference culture from a freshly transformed strain. The cultures were induced and samples collected and analyzed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). For the commercial T7 system a reduced productivity is visible for the master cell cultures and no product at all with working cell cultures. The genetically stable host/plasmid combination always delivers the same product amount, which is the optimal starting point for the development of a robust production process. Sequencing of the genomic DNA of non-producing BLR (DE3) revealed that the T7 polymerase is truncated, thus eliminating or reducing the T7 polymerase activity, which ultimately reduces or eliminates the target protein yield.

Figure 1.2a addresses plasmid stability. Although all strains look good in early strain development, cultivation data show that the strains are not equally usable for upscaling purposes. Fed-batch cultivations (1 L multiple bioreactors) using a state-of-the-art host/plasmid combination were compared with the first- and



Lane	Sample
1	Reference culture
2	Genetically stable MCC
3	Genetically stable WCC
4	MCC BLR (DE3)
5	WCC BLR (DE3)

Figure 1.1 Host instability in the framework of a use test as part of the cell banking procedure. The master and working cell cultures (MCC, WCC) were inoculated from the corresponding glycerol stocks from the state-of-the-art host/plasmid combination (BLR (DE3)), the genetically stable host/plasmid combination (strain W3110 with an expression plasmid using a rhamnose inducible promoter), and a reference culture from a freshly transformed strain. The cultures were induced, samples collected and analyzed by SDS-PAGE.

second-generation and newly developed genetically stable host/plasmid combinations. The first-generation plasmid carries a multimer resolution site (Cer) and the second-generation host/plasmid combination stabilizes the plasmid via an auxotrophic marker. Segregational plasmid stability was tested before and after induction. The commercial expression system showed plasmid instability even before induction and after induction nearly all plasmids were lost (4% plasmid containing cells). Compared to that, the new expression system stably maintains the plasmid throughout the whole cultivation process. A productive and genetically stable production strain is the foundation of a robust and reliable production process.

Figure 1.2b addresses plasmid stability in the case of very difficult to produce products under production conditions with very high selection pressure (11 multiple bioreactor experiments). The producing cells have massive growth limitations (Figure 1.2b) and plasmids are therefore lost very early. In this case 100% plasmid stability is an absolute requirement for production of the desired product. A 100% plasmid retention also offers the possibility of an extended induction time and therefore further potential to improve the titer. On top of that the system also guarantees plasmid retention during master cell banking. Hence, 100% plasmid stability is the foundation of a consistent, reliable, and scalable process.

Scalability, Predictable to Large Scale Scalability means that the results from early strain screening through process development can be translated into later production scale. That means for strain development that the specific

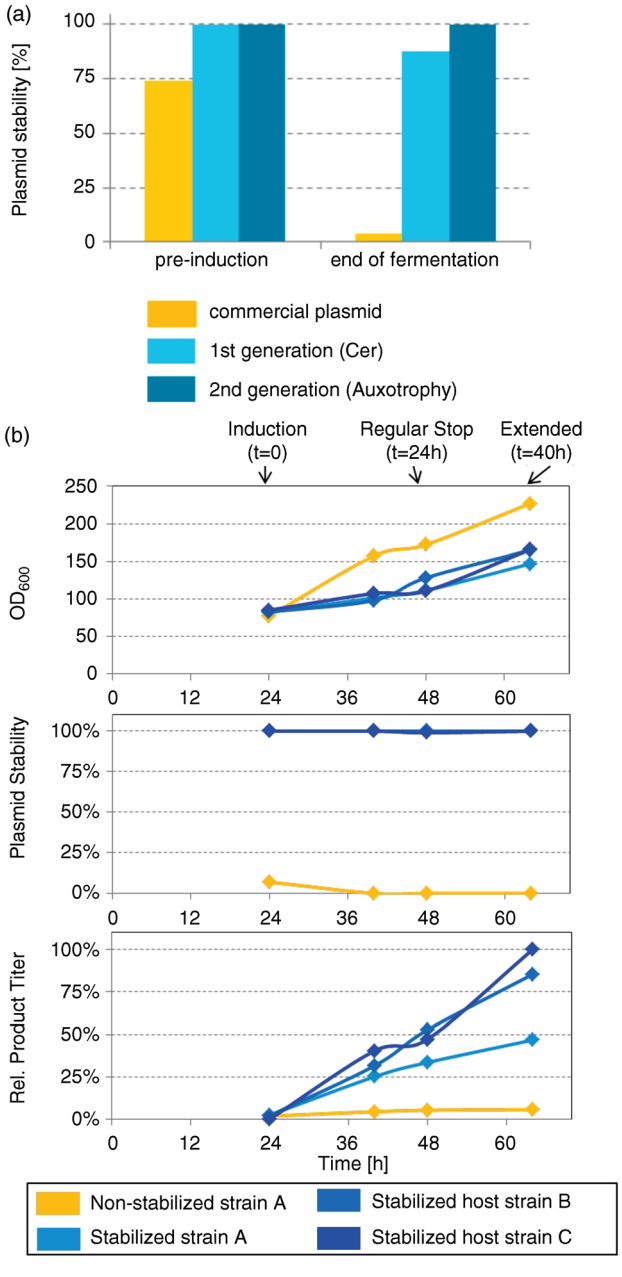


Figure 1.2 (a) Fed-batch fermentation with an open access host/plasmid combination compared to the first and second generation of a newly developed stabilized host/plasmid combination. Segregational plasmid stability was tested before and during induction. (b) Fed-batch fermentation with a non-stabilized and three stabilized host/plasmid combinations for a very difficult to produce product and under production conditions with very high selection pressure (1-l multiple bioreactor experiments). Segregational plasmid stability was tested.

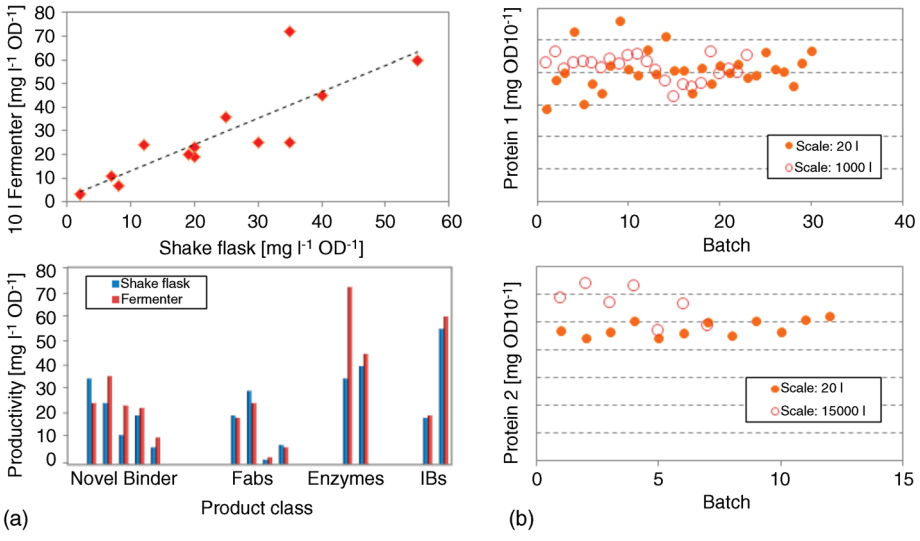


Figure 1.3 Scalability from shake flask to 1000 l and 15000 l. (a) Shake flask to 10 l; (b) 10 l to 1000 l and 15 000 l.

productivities need to be in a similar range and predictable to a certain extent. Figure 1.3a shows a comparison of specific product titer (mg l^{-1} OD^{-1}) of 13 different proteins in a shake flask compared to 10-l fed-batch bioreactor. It is a collection of productivities throughout various protein classes, reflecting a pretty good alignment between shake flask (batch) and 10-l cultivation (fed-batch). Figure 1.3b shows how 10-l fed-batch translates to large scale (1000 or 15 000 l). A comparison of specific titer (mg l^{-1} OD^{-1}) of two different proteins in 10-l high cell density cultivation and large-scale high cell density cultivation (y-axis) is shown. The data show good alignment between 10 l and at-scale process (1000 l, 15 000 l), independent of the protein class for a large number of batches.

Speed, Short Development Times to Identify the Best Production Clone Within a Few Weeks The major challenge in industrial strain development is the limited time. Most strain development programs are part of a process development program that itself is under immense time pressure. A typical industrial strain development feasibility study covers gene optimization and synthesis, primary batch screening in 96/24-well format, including basic analytics (Figure 1.4), secondary fed-batch cultivation runs to verify the early batch screening, and, sometimes, protein supply for first functional assays (Figure 1.4). Gene synthesis might be a time-critical factor since the time for gene synthesis is dependent on gene length, complexity (such as repetitive sequences), and GC content. Sometimes, requested sequences are found to be toxic and/or genetically unstable. Hence, a reasonable development time from gene synthesis to non-GMP product material supply of around 10–12 weeks can be achieved.

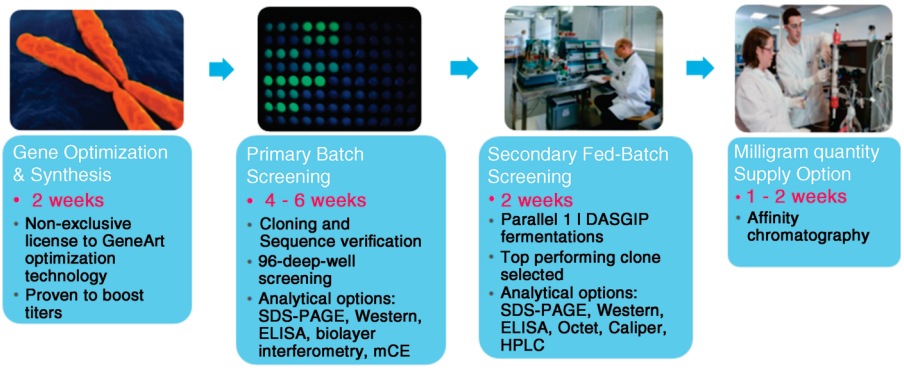


Figure 1.4 Accelerating bioprocess development: workflow from gene to purified product.

1.3 Bioprocess Design Aspects for Recombinant Products

This section discusses bioprocess design considerations for bacterial cultivations. Deviating from the use of the term in other chapters we refer to "cultivation" rather than "fermentation." We refer to "cultivation" rather than "fermentation" because the latter suggests fermentative metabolism, which is to be avoided, while purely aerobic metabolism is targeted. In the same context we replace the term "fermenter" by "bioreactor" in this chapter. Basic aspects of the three most prevailing production modes (batch, fed-batch, and continuous cultures) and basics of the bacterial cell factory are described. Moreover, a detailed roadmap covering technical design aspects of a fed-batch process is provided under consideration of cell-physiological characteristics and product-related aspects.

1.3.1 Bacterial Cultivation Processes

Cultivation processes can be categorized as follows:

1) **Batch culture** – The medium is composed of all necessary nutrients that are used throughout the cultivation. All nutrients are present in excess. The cultivation ends due to depletion of the growth limiting substrate, which typically is the carbon substrate. Hence, the culture grows exponentially at maximum growth rate. This may result in the formation of overflow metabolites such as, for example, formate, acetate, or ethanol, which reduces biomass yield, can inhibit cell growth, or negatively impact production of target molecules [11]. Design aspects of batch processes are covered in Section 1.3.6.1.
2) **Fed-batch culture** – The batch medium usually contains nutrients in excess except the one limiting nutrient that is fed according to a defined feed regime. The culture is typically in a state of nutrient limitation. Therefore, growth

kinetics are controlled by the addition of the growth limiting substrate. Applying an exponential feeding profile allows for control of the specific growth rate at a constant rate. Preferentially, growth rates are chosen such that the formation of overflow metabolites is avoided. Design aspects of fed-batch processes are covered in Section 1.3.6.3.

3) **Continuous culture** – Continuous cultures are characterized by controlled inflow (F_{in}) of nutrients and outflow of culture broth (F_{out}) with F_{in} equal to F_{out}. The dilution rate D ($D = F/V = \mu$) controls growth kinetics. More specifically, it controls the growth rate as D equals the specific growth rate μ. Steady states are typically achieved after five residence times.

1.3.2
Gram Negative Cell Factory: Cellular Compartments and Transport across Membranes

Transcription and translation of the gene product takes place in the bacterial cytoplasm. Several features, such as the presence of protein folding modulators (chaperones) and favorable pH conditions, make the bacterial cytoplasm an ideal compartment for the folding of recombinant proteins. Chaperons are special proteins that assist folding of polypeptides. The pH is maintained actively at 7.2–7.8 through "pH homeostasis" [18]. However, not every desired protein can be functionally produced in the bacterial cytoplasm. Some proteins require post-translational modifications to gain full functionality. *Escherichia coli* does not provide the capability for post-translational modifications. Furthermore, the formation of disulfide bridges is hampered in the reductive environment of the bacterial cytosol.

The space between the inner and outer membrane of Gram negative bacteria is referred to as "periplasmic space" (Figure 1.5). Unlike the cytoplasmic space, pH

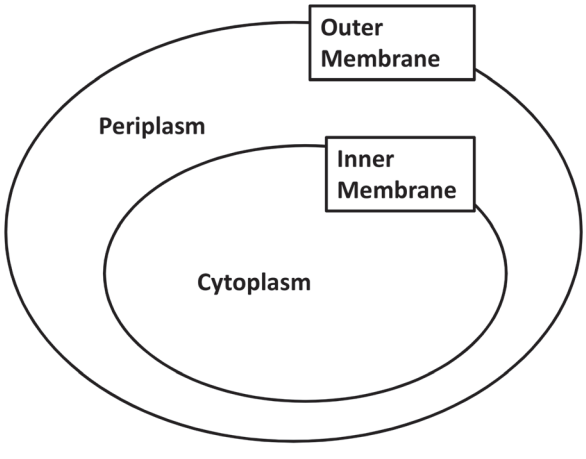


Figure 1.5 Compartments of a Gram negative cell factory.

and osmolarity of this compartment are less well maintained [19]. However, the periplasm features the "disulfide bond formation system" (Dsb system), enzymatic machinery capable of forming and isomerizing disulfide bonds and therefore modulating the correct folding of proteins which contain structural disulfide bonds [20]. This opens up the possibility to produce active and correctly folded recombinant proteins containing disulfide bonds in bacteria. However, as transcription and translation of the recombinant gene product takes place in the cytoplasm, translocation of the polypeptide across the inner membrane to the periplasmic space is necessary. Translocation can be enabled by the general secretory pathway (GSP). Transport across the inner membrane is targeted via a signaling sequence, which is cleaved off during translocation [21]. In the periplasmic space the protein folds into its native conformation. The twin-arginine translocation (TAT) pathway constitutes an alternative translocation pathway [22]. In contrast to the GSP, the protein correctly folds in the cytoplasm and is translocated in its native correctly folded state.

Targeting proteins into the extracellular space is referred to as protein secretion. Following transcription and translation in the cytoplasm, the protein has to be moved across the inner and outer membrane to be secreted into the extracellular space. While targeting of recombinant proteins into the periplasm to achieve active disulfide bridge formation is already established for commercial processes, secretory production is considered more challenging. However, promising commercial technologies are emerging such as the Wacker ESETEC© technology. General strategies for secretory production of recombinant proteins are (i) targeting of the recombinant protein to the periplasmic space and subsequent targeting to the extracellular space (two-step translocation) and (ii) the use of transport proteins mediating the transport across inner and outer membrane simultaneously (one-step translocation) [10,23]. Furthermore, approaches involving the co-expression of bacteriocin release proteins are reported [24,25]. Process technological approaches to increase recombinant protein release have been recently reported [26].

1.3.3 Industrial Strategies: Quality, Folding State, and Location of Recombinant Protein Products

Folding state and product location have a big impact on type and number of required downstream processing operations and consequently on overall process economics. Industrial strategies are (i) soluble extracellular production of recombinant products, (ii) soluble intracellular production of recombinant products (with disulfide bonds in periplasm, without disulfide bonds in cytoplasm), and (iii) production of recombinant proteins as insoluble aggregates known as an "inclusion body" (IB), located in the cytoplasm (Figure 1.6).

Ideally, active soluble target protein is secreted selectively by recombinant bacteria to the extracellular space requiring solely separation of soluble product and biomass by means of centrifugation. This way steps such as cell rupture can be

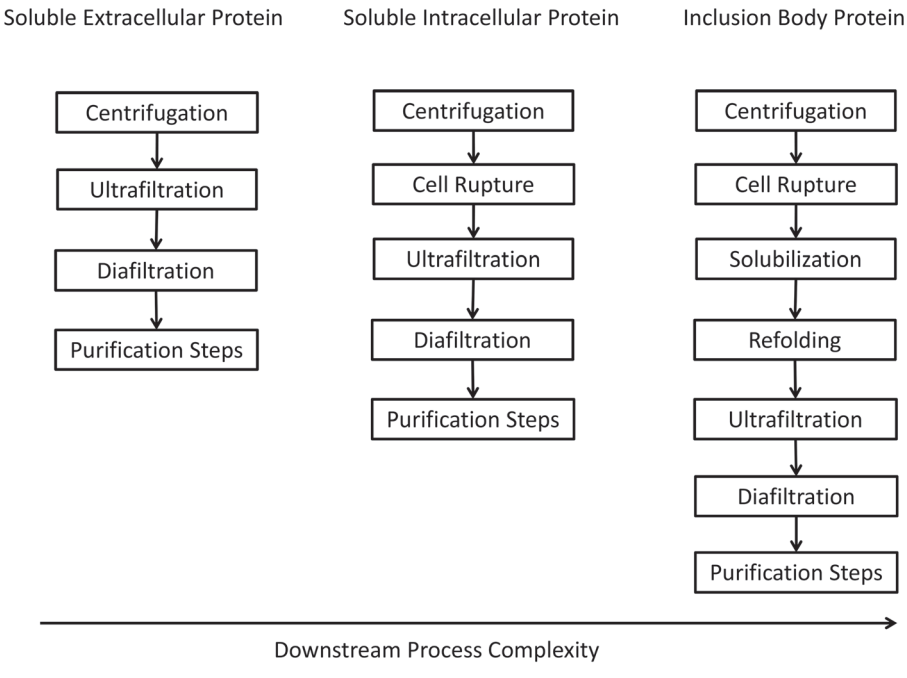


Figure 1.6 Impact of upstream product folding state on downstream process complexity. Protein location (intracellular, periplasm or cell-free medium) and protein solubility determine type and number of unit operations required for obtaining a cell free solution of the functional active protein. Sometimes, conversely to the above scheme, inclusion body processes are designed such that solubilized protein is pre-purified through a chromatography step prior to refolding. Ultrafiltration steps are used for broth concentration. Diafiltration steps are used to change buffer characteristics as required for refolding and chromatographic purification steps.

avoided [24]. However, secretory production is still highly challenging and extracellular protein yields are not yet satisfactory.

Some proteins tend to form insoluble and typically activity restricted aggregates referred to as inclusion bodies. Physical and structural characteristics of inclusion bodies can differ strongly depending on the target protein and cultivation conditions [27,28]. Typical measures aiming to reduce the extent of inclusion body formation and driving a process towards formation of soluble target protein are (i) lowering cultivation temperature and (ii) using weak expression systems, which allow tuning of production kinetics. However, the success of these measures is highly dependent upon the characteristics of the protein product.

Obviously, the same measures are used to promote inclusion body formation as it may be, for example, desirable for the synthesis of proteins that are toxic to the production organism. Recombinant proteins present as inclusion bodies are often highly pure and can be obtained at high titers. However, achieving the native, active protein conformation demands protein refolding, which is typically carried

out via highly diluted batch-, fed-batch, or continuous processes, resulting in large process volumes. For more information on inclusion body processing see References [29,30], for process technology aspects of continuous refolding processes see Reference [31]. Although integration of elaborate refolding steps in the downstream process is required, inclusion body processes may represent the most economical way of processing for a given recombinant protein product. Host-cell proteins encapsulated in recombinant inclusion bodies can negatively impact refolding yields [32]. Therefore, implementing chromatographic steps prior to inclusion body refolding can be beneficial [33].

1.3.4 Approaches towards Bioprocess Design, Optimization, and Manufacturing

Bioprocess development aims to investigate the relationship between product quality and performance attributes, typically via targeted experimentation in laboratory-scale bioreactors. Simplistically speaking, experiments are carried out, varying set-points of process parameters (e.g., temperature, pH, feeding strategy). As a result, product as well as process related variables (concentration of product, formation of carbon dioxide) are measured (Figure 1.7). Using inverse analysis, the relationship between process parameters (e.g., cultivation temperature) and process variables (e.g., product titer) can be explored for the benefit of increased process understanding, process optimization, establishment of process models, and the development of process control strategies. In subsequent manufacturing, the established process knowledge can be exploited for maintaining consistent

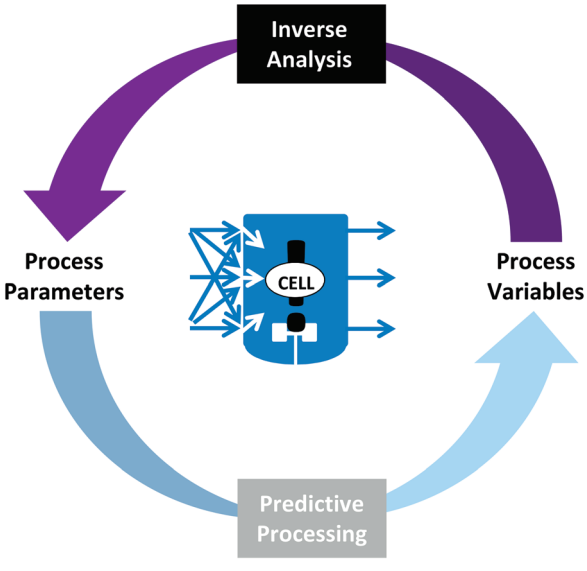


Figure 1.7 Approaches towards bioprocess design/optimization and manufacturing.

product quality and optimal productivity as well as to reduce process failures (predictive bioprocessing).

1.3.5 Bacterial Bioprocess Design

1.3.5.1 Technical and Physiological Constraints for Bacterial Bioprocess Design

The following section describes general bioprocess design aspects for the design of scalable, industrial bioprocesses. Generally speaking, bioprocess design aims at setting of optimal process parameters for optimal growth and product formation conditions, including (i) control of optimal pH value, (ii) control of optimal temperature, (iii) supply of sufficient nutrients, and (iv) supply of sufficient oxygen.

Control of pH and temperature is typically performed using the simple proportional integral (PI) controllers implemented in every commercially available laboratory or industrial scale bioreactor and is not considered further in this chapter. However, sufficient supply of oxygen (oxygen transfer from gaseous phase into the cultivation broth) needs to be considered during basic process design. Processes with expected oxygen uptake rates (OURs) exceeding the maximum oxygen transfer rate (OTR_{max}) capacity need to be avoided. Bacterial bioprocesses produce heat due to the oxidation of substrate needed for growth (460 kJ per mol-O_2). The choice of substrate has a significant effect on the heat produced per biomass formed, as further outlined in Chapter 2 on yeast suspension culture.

Hence, dependent on the biological activity, the bioreactor needs to be cooled to allow the control of cultivation temperature. During bioprocess design, the maximum cooling capacity needs to be considered; the heat production rate (HPR) is not allowed to exceed the maximum heat transfer rate (HTR_{max}). In summary, technical bioprocess design constraints (HTR_{max} and OTR_{max}) need to be considered during bioprocess design. Industrial thresholds for OTR_{max} are typically 300–500 mmol-O_2 l^{-1} h^{-1}, depending on the possibility to gas the reactor with pure oxygen, pressurize the reactor, and the k_La of the bioreactor in use.

Next to those "technical constraints," "physiological bioprocess design constraints" also need to be considered. Bacteria produce unwanted overflow metabolites such as acetate (bacterial Crabtree effect) once a defined specific growth rate, with respect to the specific substrate uptake rate, is exceeded. The accumulation of overflow metabolites reduces biomass yields, may cause inhibition of growth, and may have detrimental effects on recombinant protein production. Therefore, production of overflow metabolites needs to be avoided by controlling the specific growth rate, with respect to the specific substrate uptake rate, below the threshold value for the onset of overflow metabolite production [11,34]. This threshold value can be determined experimentally, for example, through dynamic chemostat experimentation or dynamic decelerostat experimentation [34]. Figure 1.8 gives a typical dependency of the specific acetate production rate as a function of the specific uptake rate q_s. Notably, overflow metabolite formation characteristics can change once the culture is induced and recombinant proteins are produced and may be time dependent [35]. In some processes, it is desired that the cultivation

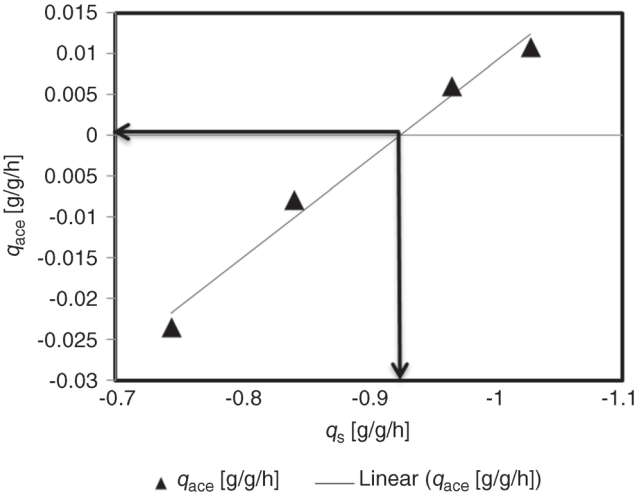


Figure 1.8 Typical dependency of overflow metabolite formation (here: acetate q_{ace}) as a function of the specific substrate uptake rate (q_s). The onset of acetate production is indicated by an arrow. Negative q_{ace} indicate acetate uptake. Adapted from [34] with kind permission from Elsevier.

temperature is reduced, for example to avoid inclusion body formation or to reduce protease activity [28,36]. In this case it is important to consider the maximum specific growth rate or maximum specific substrate uptake rate at the respective temperature, as both rates decrease at lower temperature. When the maximum specific growth rate or maximum specific substrate uptake rate at the respective temperature is exceeded, substrate accumulates in an uncontrolled manner. Notably, the maximum specific growth rate or specific substrate uptake rate can change once the culture is induced and recombinant proteins are produced. Therefore, this physiological capacity should be determined under induced conditions [37]. Table 1.5 summarizes technical and physiological constraints for recombinant bioprocess design.

1.3.5.2 Media Design

Media in use for industrial bacterial bioprocesses can be categorized as (i) fully defined media (fully synthetic), (ii) defined media supplemented with complex components, for example, yeast extract or peptones, and (iii) fully complex media. Processes run with media that are supplemented with complex components or run with fully complex media can show a greater batch to batch variance, which is attributed to lot-to-lot variability of complex media components. Hence, defined media should be used preferentially.

Process economics of biotechnological production of bulk chemicals is strongly driven by raw material costs. Metabolic characteristics of the strain, for example, the ability to metabolize C_5 sugars [38], are to be considered for the design of an optimal and low cost medium. Strategies for media development and optimization are largely empiric or based on stoichiometric analysis of the microorganism [39].

Table 1.5 Technical and physiological constraints for basic recombinant bioprocess design.

Technical constraint	Requirement	Process failure
Maximum heat transfer rate	HPR[a] < HTR_{max}[b]	Temperature cannot be controlled → reactor heats up
Maximum oxygen transfer rate	OUR[c] < OTR_{max}[d]	Dissolved oxygen cannot be controlled → culture runs into oxygen limitation, reductive metabolism occurs
Physiological constraint		
Onset of overflow metabolite production	μ[e] < μ_{acetat_onset}[f]	Overflow metabolites accumulate → reduced biomass and product yields and possible growth inhibition
Maximum growth rate at set-point temperature	$\mu < \mu_{max}$[g]	Substrate accumulates → uncontrolled accumulation and possible growth inhibition

a) HPR: heat production rate = heat produced due to microbial growth.
b) HTR_{max}: maximum heat transfer rate = maximum cooling capacity of reactor.
c) OUR: oxygen uptake rate = oxygen consumed due to microbial growth.
d) OTR_{max}: maximum oxygen transfer rate.
e) μ: specific growth rate.
f) μ_{acetat_onset}: threshold specific growth rate where acetate formation occurs.
g) μ_{max}: maximum specific growth rate.

Preferentially, statistical experimental design (design of experiments, DoEs) methodologies [40] are to be used to minimize the experimental effort towards an optimized medium.

1.3.5.3 Product Titer is Determined by the Biomass Concentration and the Specific Productivity q_p

Industrial processes aim at the production of a maximum amount of product within quality specifications in a minimum amount of time. The product titer (g l^{-1} product) at the end of the process is determined by the integral of the specific productivity q_p multiplied by the biomass concentration x (Equation 1.1):

$$\text{Titer} = \int_{t_{Ind}}^{t_{End}} q_p x \, dt \tag{1.1}$$

Hence, the maximum titer achievable is determined by three distinct process variables:

1) **Specific productivity (q_p):** The biomass specific activity (q_p) describes the amount of product formed per biomass (cells) in a defined time interval. Identifying the interactions between process parameters (temperature, dissolved oxygen, feeding strategy) and the specific productivity (q_p) and its time function

is a major goal of process development. It is important to achieve both a high q_p as well as maintaining q_p high over a maximum amount of time.

2) **Biomass concentration (x):** The volumetric product formation rate (r_p) is proportional to the biomass concentration in the broth. Hence, industrial fed-batch processes aim to maximize biomass concentration, while maintaining a high specific productivity q_p.
3) **Productive time ($t_{End} - t_{Ind}$):** The productive time, that is, the time interval between the start of induction (t_{Ind}) and the end of the process (t_{End}), governs the overall amount of product that can be produced. The end of the process (t_{End}) is initiated once the specific productivity q_p reaches zero or is based on time–space yield considerations (Section 1.3.6.6 and Figure 1.11).

1.3.6 Industrial Production Strategy by Two-Step Cultivation

Typically, industrial fed-batch processes follow a two-step strategy. The first step aims at maximizing the biomass concentration (x) while considering technical and physiological process constraints and involves a non-induced batch and fed-batch process phase. In these phases biomass should be accumulated without the formation of overflow metabolites and while staying within the technical limitations of the bioreactor setup. Subsequently, the culture is induced, for example, by adding a chemical component triggering the formation of recombinant product (indicated by an arrow in Figure 1.9). Feeding is typically continued linearly at maximum feeding rate (Feed Rate A in Figure 1.9) or reduced to a level optimal for recombinant protein production (Feed Rate B in Figure 1.9).

The next subsections describe concepts impacting productivity of recombinant fed-batch processes and basic bioprocess design aspects of the batch, fed-batch, and induction phase of industrial recombinant processes.

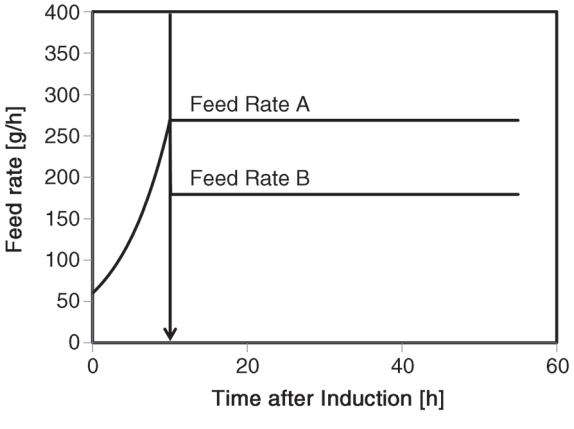


Figure 1.9 Industrial two step strategy.

1.3.6.1 Batch Phase for the Accumulation of Biomass

A sterile medium containing a carbon source (e.g., 20 g l^{-1} glucose) is inoculated with a pre-culture. The initial batch carbon source concentration is limited by technical constraints (e.g., maximum oxygen uptake rates, calculated for unlimited growth at maximum specific growth rate) or by physiological constraints, for example, the formation of unwanted overflow metabolites.

The initial biomass concentration (seed), determined by the inoculum volume and inoculum biomass concentration, is typically of minor physiological importance for most bacterial cultures, whereas when cultivating filamentous organisms it is considered to be very critical. However, the amount of seed drives batch time and is therefore of economic importance. For a typical *E. coli* strain an inoculum volume of 10 ml per liter culture broth, at an OD of 1, is sufficient to allow for lag-phase free unlimited growth.

In the batch phase the culture growth is non-limited at a maximum specific growth rate (μ). Hence the specific substrate uptake rate (q_s) is maximized. Typically, overflow metabolites (in the case of *E. coli* mainly acetate) are formed in this phase. This can be problematic when the accumulated overflow metabolites are not fully consumed in the subsequent fed-batch phase, since the presence of overflow metabolites negatively impacts recombinant protein production. Reduction of cultivation temperature during the batch phase may help to avoid accumulation of overflow metabolites as it results in a reduced specific growth rate. However, this impacts on the duration of the batch phase.

1.3.6.2 Structured Approach Towards Batch Design

The initial substrate concentration determines the biomass concentration at the end of the batch phase, which can be estimated by the biomass yield coefficient in the batch phase. Equation 1.2 shows the calculation of end biomass concentration in the batch phase:

$$x_{\text{Batch,end}} = s_{\text{Batch,0}} Y_{x/s} + x_{\text{Batch,0}} \tag{1.2}$$

where:

- $x_{\text{Batch,end}}$: concentration of biomass after the batch phase (g l^{-1});
- $s_{\text{Batch,0}}$: initial substrate concentration (g l^{-1});
- $Y_{x/s}$: biomass yield coefficient (g g^{-1}), typically ~0.4–0.48 g g^{-1} for *E. coli*;
- $x_{\text{Batch,0}}$: initial biomass concentration (g l^{-1}).

On the basis of the end biomass concentration and the specific growth rate of the respective organism, the volumetric biomass conversion rate at the end of the batch can be calculated (Equation 1.3):

$$r_{\text{xBatch,max}} = \mu x_{\text{Batch,end}} \tag{1.3}$$

where:

- $r_{\text{xBatch,max}}$: maximum volumetric biomass conversion rate in the batch process (g l^{-1} h^{-1});

- μ: specific growth rate of the process (h^{-1});
- $x_{Batch,end}$: biomass concentration at the end of the batch phase ($g\,l^{-1}$).

A design criterion for basic design of batch processes keeps OUR below the maximum oxygen transfer rate of the reactor setup. On the basis of the end biomass conversion rate and the oxygen/biomass yield coefficient, the maximum oxygen uptake rate can be calculated at the end of the batch phase (Equation 1.4):

$$\begin{aligned} &OUR = r_{xBatch,max}\, Y_{O_2/x} \\ &OUR < OTR_{max} \end{aligned} \tag{1.4}$$

where:

- OUR: oxygen uptake rate ($mmol\,l^{-1}\,h^{-1}$);
- OTR_{max}: maximum oxygen transfer rate of reactor setup;
- $r_{xBatch,end}$: metabolic biomass conversion rate at the end of the batch process ($g\,l^{-1}\,h^{-1}$);
- $Y_{O_2/x}$: oxygen/biomass yield coefficient ($mmol\,g^{-1}$).

Table 1.6 gives the process parameters for a batch phase.

1.3.6.3 Fed-Batch Phase Process Design from Scratch

Following the batch phase, a fed-batch process is started. By applying a feed-forward exponential feeding profile, assuming a constant biomass yield coefficient, the fed-batch process mode allows us to control the specific growth rate μ as well as the specific substrate uptake rate q_s. This way formation of overflow metabolites can be avoided. The flow rates F_0 and F are given in $l\,h^{-1}$; in case the flow rates are given in $g\,h^{-1}$ the feed solution density needs to be considered. The initial flow rate can be calculated according to Equation 1.5:

$$F_0 = \frac{x_0 V_0 \mu}{Y_{x/S} c_{in}} \tag{1.5}$$

The feed-forward function can be calculated according to Equation 1.6:

$$F = F_0 e^{\mu t} \tag{1.6}$$

The initial biomass concentration of the fed-batch phase x_0 can be calculated from the initial substrate concentration in the batch phase, as described in the previous section. The biomass yield coefficient of the fed-batch phase must be

Table 1.6 Process parameters batch phase.

Governing process parameters	Design criterion	Ranges
Initial substrate concentration	OTR_{max} [a] / HTR_{max} [b]	10–20 $g\,l^{-1}$
Temperature	OTR_{max} / HTR_{max}	25–37 °C

a) OTR_{max}: Maximum oxygen transfer rate.
b) HTR_{max}: Maximum heat transfer rate.

determined experimentally. Notably, the biomass yield coefficient of the fed-batch phase is typically higher than the biomass yield coefficient of the batch phase, since the formation of overflow metabolites is avoided. The maximum feed concentration (c_{in}) is mainly dependent on the substrate used. Glycerol based substrates can even be pumped in pure form (1261 g l^{-1}, pure glycerol), which is beneficial for not filling up the bioreactor with water. Heating of the transfer pipe and/or direct heating of glucose feeding solution is advisable when the concentration is higher than 500 g l^{-1}.

The specific growth rate of the fed-batch process should be controlled below the specific growth rate threshold for overflow metabolites production [41]. This threshold can be determined by elaborate, time consuming continuous experimentation or dynamic methods [34]. As a rule of thumb no overflow metabolites formation is typically observed at specific growth rates that are below $\frac{1}{4}\mu_{max}$. However, this rule of thumb has to be handled with care since metabolite formation is strongly strain dependent. Table 1.7 gives key design criteria.

The amount of biomass to be formed in the fed-batch phase is strongly dependent on the function of the specific productivity during the induction phase, as described in the next section. Furthermore, similar to the design of batch processes (see previous section), reactor capabilities with respect to maximum oxygen transfer rates are to be considered.

The biomass formation rate at the end of the fed-batch process is driven by the specific growth rate (μ) of the fed-batch process as well as the biomass concentration to be achieved. The maximum biomass formation rate at the end of the fed-batch can be found from Equation 1.7:

$$r_{x\ Fed\_Batch,max} = \mu_{Fed\_Batch} x_{Fed\_Batch,end} \tag{1.7}$$

where:

- $r_{xFed\text{-}Batch,max}$: maximum metabolic biomass conversion rate at the end of the fed-batch process (g l^{-1} h^{-1});
- μ_{Fed_Batch}: specific growth rate of the fed-batch process (h^{-1});
- $x_{Fed\_Batch,end}$: biomass concentration at the end of the fed-batch phase (g l^{-1}).

Table 1.7 Key design criteria in feed-forward exponential fed-batch processes.

Parameters	Criterion/measurement	Ranges
Initial biomass concentration	Process development	5–15 g l^{-1}
Biomass yield coefficient	Process development	0.3–0.5 g g^{-1}
Feed-substrate concentration	HPLC/enzymatic measurements	400–800 g l^{-1}
Specific growth rate	$\mu^{a)} < \mu_{overflow}{}^{b)} < \mu_{max}{}^{c)}$	0.1–0.8 μ_{max}

a) μ: specific growth rate.
b) $\mu_{overflow}$: specific growth rate threshold where formation of overflow metabolites starts.
c) μ_{max}: maximum specific growth rate.

Table 1.8 Key design criteria for exponential fed-batch processes for the accumulation of biomass.

Parameters	Design Criterion/Measurement	Ranges
Biomass concentration	Batch design	20–70 $g\,l^{-1}$
Cultivation temperature	Process development	25–37 °C
Biomass yield coefficient	Process development	0.3–0.5 $g\,g^{-1}$
Feed – substrate concentration	HPLC/enzymatic measurements, gravimetric (in industrial environment)	400–800 $g\,l^{-1}$
Specific growth rate	$\mu < \mu_{overflow} < \mu_{max}$	0.1–0.8 μ_{max}

The maximum oxygen uptake rate at the end of the fed-batch is given in Equation 1.8:

$$\begin{aligned} &OUR = r_{xFed\_Batch,max}\, Y_{O_2/x} \\ &OUR < OTR_{max} \end{aligned} \tag{1.8}$$

Table 1.8 gives key design criteria for the accumulation of biomass.

1.3.6.4 Induction Phase: Product Formation Characteristics

The second step of the industrial two-step strategy is initiated by the induction of the culture, which initiates recombinant protein production. Owing to the redirection of cell resources, better metabolism towards recombinant protein production in highly induced systems (metabolic burden), the cellular population undergoes severe physiological changes.

As a result of the physiological changes, quadratic functions of the specific product production rate (q_p) as well as the volumetric production rate (r_p) are observed (Figure 1.10). The level of q_p as well as how it rises and declines is strongly connected to the applied feeding profile as well as process parameters (T, pH, DO), media components, desired product location as well as folding state, and the host/vector combination used. The final product titer to be achieved depends upon the function of q_p, more specifically on the integral of $r_p dt$ ($=q_p x dt$).

As mentioned above, the final titer achievable in a fed-batch process is influenced by the integral of the specific productivity (q_p) and the biomass concentration (x) in the productive time. Hence, not only the level of q_p drives final product titer, but also how long q_p can be maintained at a high level ($t_{End} - t_{Ind}$). This is illustrated in Figure 1.10, showing real data from a recombinant *E. coli* fed-batch process. Parameter set 1 as well as parameter set 2 resulted in a quadratic r_p and q_p function (Figure 1.10a and c). Parameter set 2 shows a higher maximum q_p and r_p than parameter set 1 (Figure 1.10a and c). However, the high level of q_p and r_p cannot be maintained over a long period in Parameter Set 2, as shown by the strong decrease in q_p and r_p after 5 h of induction. Therefore, despite a lower maximum r_p a higher overall product titer is obtained using parameter set 1 (Figure 1.10d).

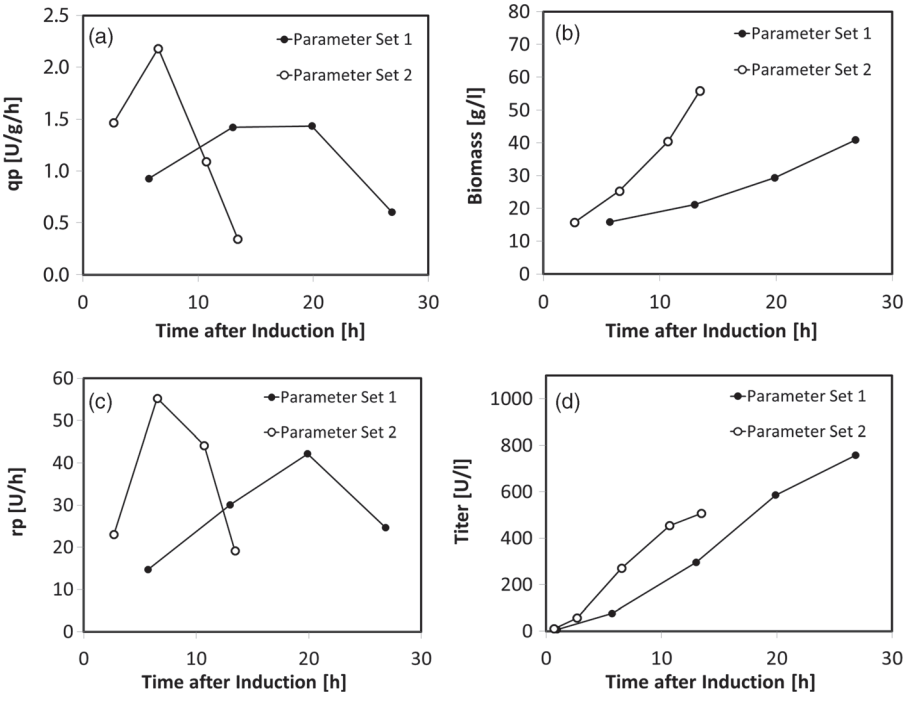


Figure 1.10 Typical product formation characteristics in recombinant bacterial bioprocesses. The specific as well as volumetric product formation rate q_p shows typically a quadratic function ((a) and (c)). The increase in the biomass concentrations is depicted in subplot (b). Final product titer is governed by the integral of r_p (d).

1.3.6.5 Process Parameters Impacting Recombinant Product Formation

As mentioned in the last section, q_p trajectories are strongly dependent upon applied feeding profile as well as process parameters (*T*, pH, DO), media components, desired product location as well as folding state, and the host/vector combination used. Optimization efforts are typically carried out using statistical experimental plans (design of experiments, DoE). For a comprehensive review on the application of DoE methodologies for bioprocesses see Reference [40].

Prior to optimization, process parameters under investigation need to be chosen following clear bioengineering reasoning. Owing to the high number of process parameters that can be optimized, the process parameters investigated should be carefully selected, for example, using risk assessment tools such as Ishikawa diagrams or failure mode and effects analysis (FMEA). For further information on the application of risk assessment tools within bioprocess development see the A-Mab case study in Reference [42]. Table 1.9 provides an overview of factors impacting recombinant protein formation and the rationale for their investigation relating to process productivity and product quality.

Table 1.9 Fed-batch process parameters with impact on induction phase productivities.

Process parameters	Industrial ranges	Reasoning	Reference
μ	0.01–0.5 h^{-1} (typically declining within induction within two step cultivation strategy)	Impact on cellular energy level; impact on metabolic load; impact on cell stress due to carbon depletion; impact on inclusion body formation	[43–45]
T	10–37 °C	Reduced inclusion body formation at lower temperatures; increased solubility of proteins at lower temperatures; reduced metabolic load at lower temperatures; reduced productivities due to reduced translation at lower temperatures	[28,46]
pH	5–7.2	Interferes with proton motive force	
DO(dissolved oxygen corporation)	>40%, also due to spatial inhomogeneity in large scale bioreactors	Overflow metabolite formation	[47]

1.3.6.6 Concept of Time–Space Yield

The "time–space yield" ($g\,l^{-1}\,h^{-1}$) refers to the product (g) achievable per reactor volume (l) and bioreactor occupancy. Bioreactor occupancy not only includes induction time, but also non-productive process stages such as reactor preparation, cleaning, and sterilization (CIP, cleaning in place, and SIP, steaming in place), batch phase, and fed-batch phase for the accumulation of biomass. Hence, the time–space yield provides a convenient measure of how much product can be produced in the respective bioreactor scale available. Figure 1.11 illustrates the concept of time–space yield. As we recall from the last section, the example "parameter set 1" yields a higher titer due to a higher integral of $r_p dt$ (Figure 1.11a). However, it takes almost 30 h to achieve this titer of 850 $U\,l^{-1}$, compared to the 15 h of time after induction to achieve 600 U using "parameter set 2". Figure 1.11b plots the "time–space yield" and, hence, the product yield per reactor volume and bioreactor occupancy. It can be seen that although higher titers can be obtained using "parameter set 1" the time–space yields of the two parameter sets are almost identical in respect to the overall occupancy of the bioreactor.

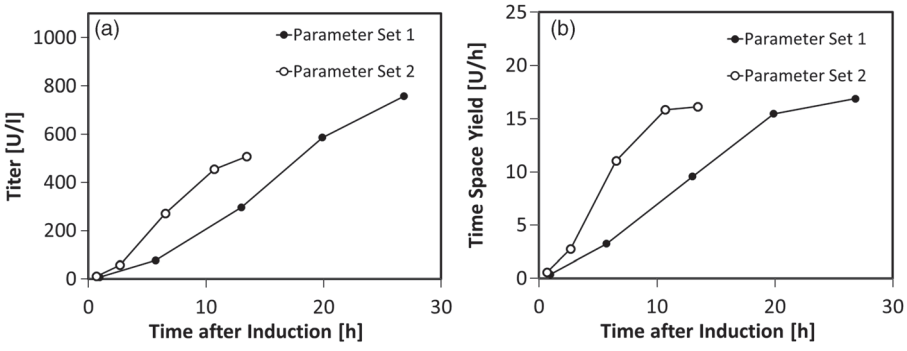


Figure 1.11 Illustration of final product titer and time–space yield of the process.

1.4 Basic Bioreactor Design Aspects

1.4.1 Introduction

In general, a bioreactor is defined as a vessel in which biological reactions take place. Depending on the type of microorganism different types of bioreactors have been designed and introduced so far. Generally speaking, bioreactors are grouped into two main categories based on the presence and absence of oxygen and the requirement of stirring as follows:

- stirred and aerated bioreactors;
- anaerobic bioreactors and non-stirred bioreactors, such as air-lift bioreactors.

Stirred and aerated bioreactors (stirred-tank reactor, STR) are those usually used for production of the products that are the focus of this chapter due to the requirement for a high OTR (oxygen transfer rate). Hence, this chapter describes the design and engineering of STRs. However, disposable bioreactors are briefly introduced and their potential and perspective for bacterial cultivation is discussed.

The main function of a bioreactor is to provide a well-controlled environment for optimal growth of microorganisms and production of products. This encompasses:

- proper control of standard process parameters as pH, temperature, dissolved oxygen concentration, substrate feed rates, gas flow rates, and so on;
- sterility (well-defined, functional sterile boundaries);
- controlled cleaning;
- control of heat and mass transfer;
- control of shear stress.

To design an apparatus that provides such an environment, different aspects concerning design and construction must be considered. In addition, a deep understanding of the mass transfer is indispensable. Here we aim to cover the following factors:

- dimensioning;
- construction principles;
- heat and mass transfer aspects;
- sterilization and cleaning aspects;
- monitoring strategies.

1.4.2 Vessel Design and Construction

For the design and construction of a bioreactor, one must keep in mind some cardinal rules (Table 1.10). These rules help the designer to design a bioreactor that provides for the required well-controlled environment. Generally, the design should follow aseptic design principles as laid down in several design guidelines [48].

1.4.3 Dimensioning

The first step in the design of a bioreactor is the choice of an appropriate reactor volume. This is decided, on the one hand, based on the plant capacity, which is dependent on the economic and market analysis and, on the other hand, by considering the time–space yield. Typically, 75–80% of the reactor net volume represents the working volume and the rest is devoted to the head space, depending of course on foaming of the given process.

Once the decision regarding the total volume of the bioreactor is taken, the dimensions can be easily chosen according to some rules of thumb. Figure 1.12 shows typical dimensions of an industrial stirred tank bioreactor for bacterial and other microbial cultivation:

$$\frac{H}{D_t} = 2.5 - 3; \frac{d_i}{D_t} = 0.3 - 0.5; \frac{H_l}{d_i} = 0.3 - 0.5; \frac{H_i}{d_i} = 1 - 2; 4 \text{ baffles}; \frac{L_b}{D_t} = 0.08 - 0.1; H_b \cong H; \frac{L_c}{D_t} = 0.02$$

1.4.3.1 Materials of Construction

The materials of construction of the bioreactor are of utmost importance. The material of the bioreactor must be chemically inert so that it does not leach elements into the medium. On the other hand, the material should not only fulfill design considerations, it should fulfill economic requirements as well as regulatory requirements, such as from the US FDA (Food and drug administration),

Table 1.10 Cardinal rules for design and construction of a bioreactor.

Process requirements	Cardinal rules	Related bioreactor part
Sterility	Any connection to the reactor should be made accessible for steaming in place (SIP) and cleaning in place (CIP). This also includes prior removal of air, as this can act as insulation	Connections
Sterility	Direct connection between sterile and non-sterile parts of the system should be avoided. Bacteria can grow through closed connections	Connections
Sterility	Flange connections are prone to facilitate contamination due to thermal expansion or equipment vibration. Pipes should therefore be designed and tested for stress free isometry	Connections
Sterility	Welded constructions that are properly polished are preferred	Vessel body
Sterility	The shape of the reactor and its construction should be in such a way that any dead legs and crevices should be prevented. This should also include welding in the product wetting area	Vessel body
Sterility	For cleanability, the reactor must be self-draining	Vessel body
Sterility	Any connection to the reactor and any ports should be steam-sealed	Ports and connections
Sterility	The vessel should always be kept under defined overpressure to avoid any back contamination. As a design suggestion, it also should tolerate positive pressure up to at least three bar gauge to enhance oxygen solubility	Vessel body
Sterility	Valves should be easy to clean, maintain and sterilize	Valves
Sterility	The vessel should be suitable for aseptic long-term operation and meet the requirements of regulatory authorities	Vessel body
Aeration and agitation	Adequate aeration and agitation should be provided to meet the culture requirements	Agitator, engine, baffles, impellers
Agitation	Power consumption should be as low as possible	Motor, agitator, impellers
pH and temperature	pH and temperature control systems should be provided	pH and temperature control systems
Process mode	The vessel should be designed to enable different process modes	Vessel body
Aeration, heat transfer	Evaporation loss from the bioreactor should not be excessive	Sparger, heating and cooling system
Economics	Cheapest materials with satisfactory properties should be used	Materials of construction

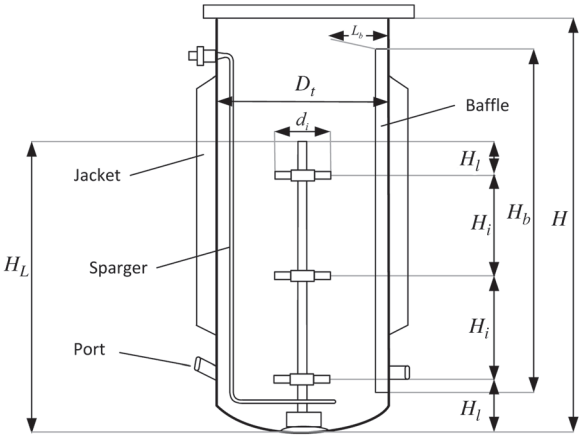


Figure 1.12 Typical dimensions of a stirred tank reactor.

EMA (European Medicine Agency), and MHRA (Medicine and Healthcare Products Regulatory Agency).

The body of bioreactors, which are larger than 10 l, is typically made of stainless steel. Those holding smaller volumes mainly consist of glass. Because of excellent electro-polishing and low leaching characteristics, stainless steel 316 or 316 L (1.4404 and 1.4435) are commonly used. Stainless steel 304 or 304 L are used for parts of the vessels that are not in contact with the product, such as the heating–cooling system or the platform. In addition to the vessel body, the material of the elastomers used for static seals is also important. Silicone, EPDM, and Teflon are commonly used for head plates and elsewhere. Conformity certificates according to FDA CFR Part 170ff should be provided. For further information, the authors recommend References [49,50].

1.4.3.2 Surface Quality and Welding

The smoother the surface the better is the cleaning. For internal surfaces of the vessel and pipes, a roughness of $R_a \leq 0.8\,\mu m$ is suggested. More information about the surface characteristics can be found in Reference [51]. However, surface roughness is not the only factor that affects cleaning. The natural interaction between surface and reactor contents determines the cleaning success. Therefore, for a certain medium and broth, swab tests will be necessary to demonstrate successful cleaning.

Tungsten arc welding is the most common technique used to weld vessels and pipes in biopharmaceutical technology. Automatic welding such as orbital welding is used to connect the pipes wherever the geometry of the pipework allows this technique. In addition to the welding, also welding polishing quality down to above-mentioned roughness specification and its inspection by endoscopy is of utmost importance.

1.4.3.3 Nozzles and Ports

A bioreactor consists of various internal and external parts, each contributing to the proper function of the bioreactor. Baffles, impellers, motor, and sparger play a major role in the mass transfer while ports are indispensable for placing of sensors, sampling, and harvesting. The jacket serves for heating and cooling.

All bioreactors used for cultivation of bacteria have nearly the same configuration and fittings, which facilitates execution of various process modes. Every bioreactor is usually equipped with ports for pH sensors, dissolved oxygen sensors, temperature sensors, antifoam sensor, a port for sampling, and ports for the addition of base, acid, and feeds. In contrast to laboratory scale bioreactors, in pilot and large scale bioreactors the sensors are mounted in the bottom-periphery of the vessel (Figure 1.13).

With reference to the sampling port, various commercial systems are available for aseptic sampling. The sampling system should be designed such that the sterile boundary is maintained during repeated sampling. For a detailed description of sampling systems refer to Reference [52].

1.4.4 Mass Transfer

Providing homogeneous mixing is a prerequisite for successful cultivation. Of course, homogeneous distribution of all medium components is important,

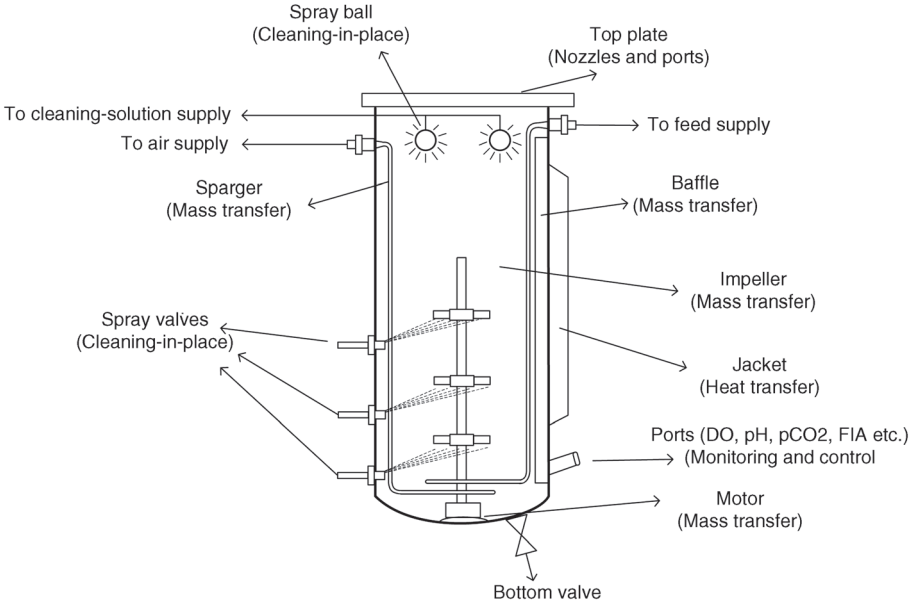


Figure 1.13 Schematic drawing of a standard stirred tank bioreactor. The internal or external components affect the capabilities of the bioreactor to maintain and control the operating conditions.

but the transfer of oxygen from the gas phase to the liquid phase and the transfer of carbon dioxide from liquid phase to gas phase still represent a big challenge in microbial cultivations. This necessitates a proper stirrer design and operation.

The oxygen uptake rate (OUR) is defined as the number of moles of oxygen taken up by the cells per unit volume and time. As mentioned in Section 1.3, this parameter serves as process design criterion. The oxygen demand of the cells correlates with the biomass growth rate (Section 1.3). On the other hand, the oxygen transfer rate (OTR) is defined as the number of moles of oxygen exchanged between the gas phase and the liquid phase per unit volume and time. This is the oxygen that is potentially accessible to the cells. The OTR is expressed as a product of a coefficient (the volumetric gas transfer coefficient (K_{la})) and the driving force (ΔC) as follows (Equation 1.9):

$$\mathrm{OTR} = K_{la}\left(C_{O_2} - C^*_{O_2}\right) \tag{1.9}$$

where C_{O_2} is the dissolved oxygen concentration in the liquid phase and $C^*_{O_2}$ is the equilibrium concentration of oxygen in the liquid at the given temperature and pressure in the bioreactor.

To date, different methods and techniques have been established for the determination of K_{la}. A description and explanation of these techniques is beyond the scope of this book and can be found elsewhere [52]. Here, we focus on the factors influencing OTR and the practical improvement strategy.

The cells take up the oxygen very rapidly. Hence, the rate-limiting step is the availability of oxygen by transfer from the gas phase to the liquid phase. As a design rule, the OTR should be high enough to satisfy OUR requirements. The OTR can be adjusted by means of changing both K_{la} and ΔC. The K_{la} is dependent on the geometry of the reactor, the gas flow rate, and the stirrer speed. Increasing the gas flow rate enhances K_{la} to a certain extent and thereafter has no significant impact [53]. On the other hand, too high a gas flow rate increases foaming tendency. It is reported that a well-designed agitation system should provide for a specific energy input (P/V) of $>5\,\mathrm{kW\,m^{-3}}$ and an impeller tip speed $> 1.5\,\mathrm{m\,s^{-1}}$ [54]. $C^*_{O_2}$ depends on the mole fraction of O_2 in the inlet gas and the total pressure of the bioreactor (Henry's law).

In high cell density cultures, the actual OTR may be lower than the OUR, hence the measured dissolved oxygen concentration (usually annotated as DO or pO_2) may fall below the critical level. Therefore, the OTR needs to be increased. This is achieved by changing parameters as follows:

- head pressure;
- air inflow rate;
- agitator speed;
- oxygen inflow rate.

Usually, a cascaded control strategy is implemented, in which the above-mentioned parameters are changed in accordance with the DO controller output. The

use of oxygen-enriched air should be considered as the last option because addition of the oxygen imposes additional cost to the process.

1.4.5 Cleaning in Place

After harvesting, the bioreactor must be prepared for the next operation. This preparation includes cleaning and sterilization of the bioreactor and all associated transfer lines. Cleaning in place (CIP) is defined as the removal of culture leftovers from process equipment and transfer lines. This typically is accomplished by spraying and rinsing of water and cleaning solutions in recirculation mode without the removal or dismantling of the equipment. The generally applicable cleaning scheme utilizes a pre-rinse to remove gross soils, a hot caustic flush to digest and dissolve remaining soils, a water wash to remove residual caustic, and a potential hot water for injection (WFI) or purified water (PW) wash.

Cleaning methods and cleaning agents used vary, depending on the process (type of microorganism, product produced, and facility, among others). Similar cleanliness can be achieved with different methods. Four important factors affecting CIP are: time, velocity, cleaning agent, and temperature. For a good CIP system design and operation the following recommendations are suggested [48]:

- construction of CIP systems equivalent to the material quality of the main process;
- bioreactors with electro-polished surface of $R_a <= 0.6$ are preferred;
- splashing with CIP spray balls should be between 30 to 40 l per m reactor circumference per min; more spray balls than one may be installed for proper cleaning; the total flow rate can be split to the number of spray balls, which should be run alternately;
- the minimum flow velocity through the CIP and transfer piping is considered to be 1.5 m s^{-1} (6 ft s^{-1}) to avoid biofilm formation;
- in case the minimum flow velocity cannot be kept in large pipes, a minimum Reynolds number of 10 000 is suggested to assure good mass, heat, and momentum transfer of cleaning solution;
- dead legs must be less than three-pipe diameters of the branch to assure adequate cleaning (3D rule);
- 1% (w/v) solution of sodium hydroxide at 75–80 C for 15–20 min is usually used after pre-rinse;
- acid treatment can be used optionally to break pH after caustic treatment;
- final rinse with process water quality is used to verify cleaning was successful. The acceptance criterion is normally a conductivity measurement slightly higher than the feed water quality (1.3 µS cm^{-1}), but still much lower than contaminants.

For pharmaceutical applications, validation of the CIP is necessary to demonstrate a reasonable degree of assurance that cleanliness was achieved as desired

and in a reproducible manner. More information on CIP can be found in Reference [51].

1.4.6 Steaming in Place

Steaming in place (SIP) refers to sanitization or sterilization without dismantling the equipment or lines. This technique is used to sterilize commercial bioreactors. Steam sterilization of the bioreactor is performed either with medium (full sterilization in place) or without medium (empty sterilization in place). Full sterilization is typically applied to *in situ* sterilization of the batched medium. Steam is injected into coils and/or jackets and to some extent into the reactor head space (clean steam). The agitator is turned on for faster heat transfer. In the case of empty sterilization, clean steam is injected into the empty vessel and the system is sterilized similar to the full sterilization. Empty sterilization is preferred when heat sensitive media are used or when it is intended to reduce heating and cooling times. The state-of-art of SIP is a wide area and more insight into this field is available elsewhere [51].

The presence of the air reduces heat transfer during heating phase of sterilization. Therefore, removal of air prior to cleaning and heating up must be ensured before closing all valves. When the system cools down, sterile air or nitrogen must be supplied to avoid the formation of a vacuum. These gases are usually filter sterilized. A sufficient peak capacity of gas to compensate for the collapse of steam to condensate must be available within seconds.

1.4.7 Monitoring and Control of Bioprocesses

1.4.7.1 Standard Instrumentation – Measuring and Control of Process Parameters

Process parameters such as cultivation temperature, pH, and dissolved oxygen are typically controlled at defined set-points that are optimal for bacterial growth and product formation. Hence, a bioreactor must be equipped with measuring sensors to allow for precise and frequent monitoring of these parameters.

Sensor accuracy, long-term stability, measurement range, and sensor robustness must be considered for the choice of an appropriate sensor. Furthermore, in-line bioreactor sensors must be temperature resistant in order to resist multiple sterilization cycles.

Control of standard process parameters is typically performed via simple proportional integral (PI) controllers that are generally implemented in commercially available bioreactor operating software. Control of dissolved oxygen can be approached via a cascade of manipulated variables: First, the controller acts on one input variable, for example, stirrer speed until a maximum is reached. In a second step, the controller acts on another input variable impacting the oxygen transfer rate, for example, reactor pressure, air flow rates, or oxygen flow rates.

Table 1.11 Standard measurement and control in a bioreactor.

Operating condition	Sensor	Range	Accuracy on range	Controller type
Temperature	Pt-100[a)]	0–150 °C	1 °C	PI[b)]
Pressure	Piezo-resistor	0–3 bar gage	20 mbar	PI
Gas flow	Thermal mass flow meter	Air: 0–2 vvm; oxygen: 0–0.4 vvm	2%	PI
pH	pH electrode	2–12	0.05	PI
pO_2	Clark electrode; fluorescence quenching	0–100%	2%	Cascade controller
Agitation speed	Tacho/stroboscope	Along scale up strategy	1%	PI

a) Platinum (Pt) electrical resistance temperature probe with an electrical resistance of 100 Ω at 0 °C.
b) Proportional integral controller.

Temperature, pressure, pH, pO_2, gas flow, and agitator speed are part of standard control equipment for bacterial bioprocesses. Table 1.11 lists typical accuracies and measurement ranges of commercially available sensors.

1.4.7.2 Challenges with Bioreactor Standard Sensors

The pO_2 and pH sensors may show a drift during long-term operation, distorting proper control of process parameters. Since it is very difficult to detect a sensor drift, it is strongly recommended to install redundant pH and pO_2 probes. Moreover, in large bioreactors with a volume larger than 10 m^3, inhomogeneous mixing may occur and the sensor reading may depend on the sensor location. Hence, three sensors of each type may be mounted on the vessel body. Two of them can be mounted near each other at the lower lateral location of the vessel, while the third sensor may be installed between the second and third agitator level.

Furthermore, off-gas analysis has emerged as a standard process analytical tool for monitoring bacterial bioprocesses. Changes in metabolic rates can be very well observed by monitoring carbon dioxide evolution and oxygen uptake.

1.4.7.3 Advanced Bioprocess Analytics: Real-Time Monitoring of Process Variables

Multiple bioprocess variables change during a bacterial bioprocess. Biomass increases (typically exponentially), carbon source and nitrogen sources are consumed, and recombinant product and metabolites are formed. Furthermore, the cells undergo physiological changes, increase or decrease in size, or even segregate in multiple subpopulations. Monitoring of these variables is of great interest for the development and understanding of bioprocesses as well as for bioprocess control during manufacturing.

In recent decades, a plethora of process analytical equipment aimed at measuring these process variables has been developed. Near-infrared, mid-infrared, as well as Raman spectrometers for the use in bioprocesses are commercially available. Placed *in situ* (place in the bioreactor), they allow for the online monitoring of metabolites, substrates, and products. However, typically sophisticated

chemometric methods[1] are necessary for the establishment of calibrations, which need robust reference data. For a recent review of spectroscopic methods for bioprocess monitoring see Reference [55].

HPLC (high-performance liquid chromatography), GC (gas chromatography), enzymatic assays, and flow injection analysis[2] enable accurate quantification of a wide range of chemical and biological components using simple (typically linear) calibrations. However, they are typically used off-line (decoupled in terms of time from the process), demanding sampling from the bioreactor and sample treatment. Furthermore, the obtained offline information can then not be used for bioprocess control purposes. Hence, in recent decades a lot of effort was undertaken to interface these devices directly to the bioreactor, for example, using aseptic filtration probes (e.g. see Reference [56]). This allows monitoring of the components of interest with a high frequency and without manual user interaction. However, challenges such as clogging of interface tube lines and fouling of filtration membranes can hamper the applicability. Furthermore, the interface poses a potential threat to the sterility of the bioreactor.

Biomass acts as catalyst for product and metabolite formation as well as substrate consumption and is therefore of great interest. The biomass concentration in the bioreactor can be measured in real-time using in-line turbidity probes, which are typically based on measuring a back-scattering signal. Furthermore, dielectric spectroscopy in-line probes are commercially available for estimation of the biomass concentration. The latter can also be used to extract morphological information.

Flow cytometers allow for detection of subpopulations of cells and hence grant a segregated view on the biomass. Living and dead cells can be quantified using appropriate dyes. Interfacing a flow cytometer with a bioreactor demands a several-magnitude dilution step. Although challenging, flow cytometers have already been successfully interfaced with bioreactors [57].

Difficult to measure process variables can also be estimated using mathematical models. To do so in real-time, appropriate software processing readily available process data (off-gas analysis, flow rates) into unknown process variables (e.g., the biomass or substrate concentrations) are used. These "sensors" are referred to as "software sensors," or "soft-sensors," and provide the benefit of low cost (no additional analytical equipment required). Furthermore, they do not violate the sterile barrier of the bioprocess. Depending on the type of soft-sensor (data driven[3] or first principle[4]) and the type of process model, a varying amount of training or parameter fitting data is necessary to run a soft-sensor.

1) Science of the extraction of chemical information from large data sets using statistical/mathematical/computational methods.
2) Automated analysis method where a sample is injected in a continuous carrier stream for chemical analysis.
3) Use of large data sets for the calibration of the soft-sensor, typically using chemometric methods, for examples see Reference [58].
4) Use of first-principle relationships (mass balances, kinetic models). For examples see References [34,37].

Along with chemical information, physiological information (e.g., cellular flux distributions) is also of interest to characterize the state of the cellular population in real-time. Specific rates (specific growth rates, specific uptake rates) as well as yield coefficients (distribution of anabolic, catabolic fluxes) grant insight into cellular flux distributions, for example, to assess the change in the physiological state of the cellular population due to the production of recombinant proteins [59]. They can be calculated in real-time by means of soft-sensors from a combination of process signals (off-gas analysis, in-flow of feed solution, and in-flow of air/oxygen) [60,61].

1.5 Single Use Bioreactors for Microbial Cultivation

Single use bioreactors (SUBRs) also referred to as disposable bioreactors, are presterilized plastic containers intended for the cultivation of mammalian, insect, yeast, or bacterial cells. This section discusses the use of SUBRs for the cultivation of microbial cells.[5] Possible benefits and process-technological design constraints for bioprocesses using single use bioreactors are discussed, providing a critical outline of the potential of the use of SUBRs for industrial microbial suspension processes.

1.5.1 Multi-use or Single Use?

Multi-use stainless steel stirred tank reactors that need to be cleaned and sterilized *in situ* (clean in place and steam in place, see Sections 1.4.5 and 1.4.6, respectively) emerged as the primary workhorse for the cultivation of cells in suspension culture. Since stainless steel stirred-tank reactors proved applicable, why should there be a benefit in switching to a plastic disposable alternative? From the manufacturer's perspective, single use bioreactors offer several advantages:

1) no cleaning validation is necessary (reduces paperwork, effort, and cost);
2) no CIP/SIP piping is necessary (lower investment);
3) no CIP/SIP kitchens are necessary (manufacturing sites are smaller);
4) time for reactor preparation is reduced;
5) time for campaign changeover[6] can be significantly reduced;
6) personnel savings;
7) investment cost savings.

In respect to investment, process scheduling, and manufacturing flexibility, single use bioreactors have undisputable advantages. Furthermore, disposable

5) Yeast and bacterial cells (in this context).
6) Change from one product to another in the same manufacturing site.

process monitoring probes are available to monitor basic process variables such as pH and dissolved oxygen. Materials of single use bioreactors that come in contact with the product are considered GMP (good manufacturing practice, the highest standard for the manufacturing of pharmaceutical drugs) compliant. However, several characteristics of single use bioreactors challenge their broad applicability for microbial bioprocesses, which are discussed in the following section.

1.5.2 Challenges for the Use of Single Use Bioreactors in Microbial Bioprocesses

Bacterial and yeast bioprocesses offer the possibility of fast growth and the possibility to achieve high cell densities, resulting in high volumetric productivities. As outlined in Section 1.3, process development takes place in a framework of technical and physiological constraints. Maximum heat transfer rates and maximum oxygen transfer rates of the bioreactor as well as physiological constraints need to be considered for the design of a bioprocess. Stainless steel bioreactors were specifically designed for optimal oxygen transfer through high power inputs and high heat transfer via high performing double jackets. Therefore, they are ideal for high performing bioprocesses (see Section 1.4 and references cited therein).

In contrast, single use bioreactors are characterized by (in comparison with stainless steel reactors) low power input, low oxygen transfer rates, and high mixing times. Plastic has a low heat transfer coefficient; therefore, heat removal from the bioreactor is inferior to steel. Mass transfer rates (oxygen transfer rates in particular) are lower due to low power input and the difficulty in pressurizing disposable single use bioreactors. However, oxygen transfer rates in single use bioreactors can be increased by the addition of pure oxygen. The following discussion mainly focuses on stirred-tank single use bioreactors since they have the most relevance for the cultivation of microbial cells. Wave bag type single use bioreactors as frequently applied for the seed trains in mammalian cell cultivations (Chapter 12) are discussed in other chapters.

1.5.3 Microbial Bioprocess Development Using Single Use Bioreactors

In single use bioreactors, oxygen transfer rates and heat transfer rates are inferior to stainless steel, narrowing the technically feasible process parameter space.

For example, final product titer in recombinant bioprocesses is mainly determined by the (i) biomass concentration and (ii) the specific growth rate (Section 1.3.5.3 and references cited therein). In oxidative processes, possible combinations of final biomass concentrations achievable and specific growth rates are constrained by maximum oxygen transfer rates and maximum heat transfer rates of the bioreactor. Hence, optimal processing parameters in respect to product titer might not be feasible in single use bioreactors.

Single use bioreactors pose an additional challenge to bioprocess design in respect to scale ability of bioprocesses. Maximum volumetric power input,

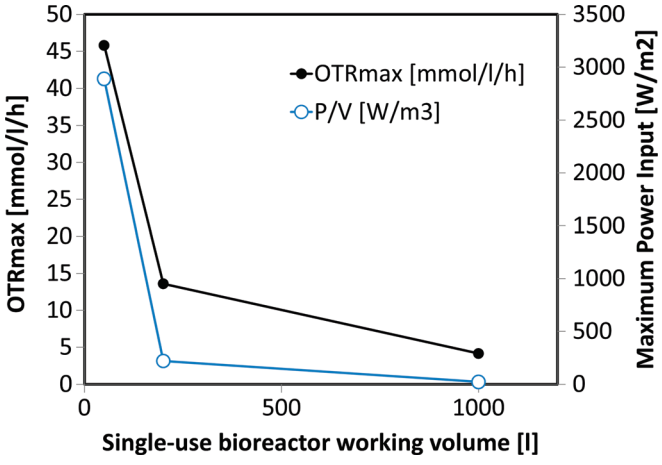


Figure 1.14 Maximum oxygen transfer rates (OTR_{max}s) and volumetric power input (*P/V*) for commercially available single use stirred bioreactors of different sizes. OTR_{max} was calculated based on K_{la} values measured in coalescing media assuming 25% oxygen saturation.

maximum heat transfer rates, and maximum oxygen transfer rates of commercially available single use bioreactors decrease as a function of scale (Figure 1.14). This is due to a decrease in the area/volume ratio (decrease in maximum heat transfer rates) and mechanical limitations of disposable stirrers.

Figure 1.15 depicts the maximum biomass concentration that can be achieved at a given specific growth rate without running into oxygen limitation. Single use bioreactors of different sizes and a typical stainless steel stirred tank reactor are compared. The iso-lines indicate the maximum biomass concentration achievable

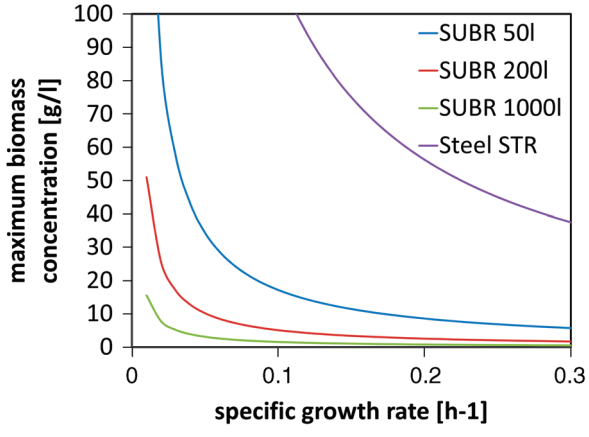


Figure 1.15 Maximum biomass concentrations feasible with different types of single use bioreactors (SUBRs) as a function of the specific growth rate (controlled in fed-batch mode or maximum specific growth rate in batch processes). Maximum oxygen transfer rates as the basis for the calculation are depicted in Figure 1.14.

at a given specific growth rate without running into oxygen limitation. It can be seen, that with steel stirred tank reactors high biomass concentrations at high specific growth rates are feasible. For example, at a specific growth rate of $0.2\,h^{-1}$ biomass can be grown exponentially up to a biomass concentration of $55\,g\,l^{-1}$ without running into oxygen limitation, as indicated by an arrow. With SUBRs, high densities can only be achieved at very low specific growth rates without running into oxygen limitation. Since the productivity is growth rate dependent (Section 1.3.6.5 and references cited therein), processes might be forced to be run at suboptimal conditions. This affects the potential bioprocess design space.

1.5.4
Applications for Single Use Bioreactors in Microbial Suspension Cultures

In the previous sections, the advantages and disadvantages of single use bioreactors were discussed. In summary, SUBRs show distinct advantages in respect to process scheduling (reduced downtime necessary between batches, no cleaning validation) and saving of initial investment costs. However, this *manufacturing flexibility* comes at cost of *process design flexibility*. Designing high performing high cell density processes in single use bioreactors is much more challenging using single use bioreactors compared to stainless steel bioreactors. However, single use bioreactors can be the first choice for processes with low specific growth rates or for the cultivation of anaerobic microorganisms or production of secondary metabolites, where mass and heat transfer are less challenging. Hence, single use bioreactors have the potential to be a rewarding alternative to stainless steel bioreactors.

Possible applications for single use bioreactors encompass:

1) products required in low quantities;
2) multi-product facilities with a high number of product changeovers;
3) personalized medicine pharmaceuticals;
4) cultivation of anaerobic organisms;
5) seed trains.

For further information on design, constraints, and applications of single use bioreactors the reader is referred to the literature [62,63].

1.6
Quality by Design: Vision or Threat for Twenty-First Century Pharmaceutical Manufacturing

Biopharmaceutical drug products produced in living organisms are under tight control of regulatory authorities to ensure product quality, safety, and efficacy. To date, this goal is mainly achieved through final product testing and fixed manufacturing ranges. Manufacturing ranges are defined based on mainly empiric process

development efforts. These take place prior to the submission stage of a new pharmaceutical drug. Thereafter, during the entire product lifecycle, the manufacturer has limited means to change and improve the manufacturing process. This regulatory inflexibility made the implementation of novel technologies in existing processes practically impossible, slowing down innovation in the pharmaceutical industry.

In the early 2000s, the regulatory authorities realized that there is a necessity to change the regulatory submission process of new pharmaceutical and biopharmaceutical drugs. In an attempt to grant the manufacturers more flexibility and allow the efficient implementation of new technologies, novel concepts summarized under the term "quality by design" (QbD) were promoted by the regulatory authorities. In contrast to "testing quality into the product," "quality by design" aims to ensure product quality through a thorough investigated manufacturing process. In terms of the regulatory authorities "quality should be built-in or be by design . . . " and not solely " . . . tested into products" [64]. Following QbD principles, manufacturers are encouraged to thoroughly investigate their process following a science and risk based approach to achieve process understanding. This should allow them to be capable of actively handling wanted or unwanted process variability. In return for the demonstrated process understanding, regulatory trust is earned by the manufacturers. This should lead to increased manufacturing flexibility.

The first part of this section summarizes the basic concepts of QbD. The regulatory guidance in respect to the task of the practical implementation of QbD is reviewed and a discussion of benefits and challenges of QbD from the manufacturer's perspective is conducted. Thereafter, we discuss the regulatory demand of "process understanding" in respect to biopharmaceutical QbD manufacturing processes (where highly complex molecules are produced in cells as manufacturing units). In the last part of the section we discuss who will shape the future of QbD in pharmaceutical manufacturing: Will the manufacturers' readily accept and develop the concepts of QbD or will QbD evolve to a unilateral regulatory imperative?

1.6.1 Regulatory Drive towards the Implementation of QbD

From 2003 to 2012 the pharmaceutical QbD framework was developed in a series of documents developed by International Conference of Harmonization (ICH), United States food and drug administration (FDA) as well as its European and Japanese equivalents (EMA and MLHW (Japanese Ministry of Health and Welfare)). Primary documents concerning the implementation of QbD, including the principal aim of the document, are listed in Table 1.12.

QbD is referred to as a "holistic" approach towards pharmaceutical manufacturing. QbD covers drug and product development, manufacturing process development, and also stretches to post-drug and process approval manufacturing. Hence, QbD is not limited to process development but covers the whole lifecycle of a

Table 1.12 Leading quality by design related regulatory documents.

Document	Principal aim	Reference
Pharmaceutical cGMPs for the twenty first century – A Risk-Based Approach	Communicate the regulatory goal to push a risk based approach in the pharmaceutical industry	[65]
ICH Q8R2	Guidance for the implementation of QbD concepts in process development	[64]
ICH Q9	Guidance for implementation of quality risk management tools in the drug product lifecycle	[66]
ICH Q10	Guidance for the implementation of a pharmaceutical quality system	[67]
Guidance for Industry PAT-A framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance	Provide a framework for the development and implementation of process analytical technology in the pharmaceutical industry	[68]

pharmaceutical drug. The current understanding of QbD for pharmaceuticals was mainly shaped by a series of documents of the "International Conference on the Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use" ("ICH" for short). These documents will now be reviewed briefly.

"Risk" and "risk management" are key elements of QbD. Risk is defined as the product of "severity" and "probability" and "detectability." The main goal of risk management is to reduce the risk to drug product quality throughout the product lifecycle. ICH Q9 aims to provide guidance on how to implement modern risk management tools in the pharmaceutical industry. ICH Q8 specifies how risk- and science-based process development within a QbD framework can be structured and communicated within the regulatory submission. More precisely, ICH Q8 specifies what the QbD contents within the Pharmaceutical Development section of a regulatory submission in the ICH M4 common technical document (CTD) can look like. Overarching and complementing documents ICH Q8 and ICH Q9, document ICH Q10 gives guidance about how to implement a quality management system, including guidance for management responsibilities as well the current understanding about what a pharmaceutical quality system can look like. The most recent document, ICH Q11 (2011), tries to provide guidance on what information should be provided in the CTD section of a regulatory submission. Furthermore, ICH Q11 discusses the possibility of submission of "enhanced" and "classical" approaches and also discusses the possibility of submitting a combination of both. However, ICH Q11 still remains vague concerning its guidance about how QbD elements can now be effectively put into practice.

Quality risk management tools as presented in ICH Q9 are ineffective without a product-lifecycle spanning quality management concept as presented in ICH Q10. Furthermore, quality management is difficult without a solid risk- and science-based knowledge basis derived from process development as specified in ICH Q8. Hence, in our opinion full quality by design can only be achieved using the

Figure 1.16 The QbD puzzle is composed of risk management (ICH Q9), risk-based manufacturing process development (ICH Q8), and a quality management system (ICH Q10).

combinations of tools and concepts presented in the ICH Q8, ICH Q9, and ICH Q10 documents (Figure 1.16).

1.6.2 Process Development along QbD Principles

QbD starts with predefined objectives. As outlined in ICH Q8 a "quality target product profile (QTPP)" needs to be established, summarizing all quality, safety, and efficacy characteristics of the drug product [64]. Based on the QTPP, chemical, biological, and physical characteristics and their respective ranges are identified, which form the set of critical quality attributes (CQAs) for the drug product. Subsequently, a manufacturing process is to be designed to ensure the entire properties of the drug quality attributes as defined in the CQAs stay within the defined ranges. Using risk assessment tools and prior knowledge on the manufacturing process, process parameters with possible impact on drug product quality are identified, called critical process parameters (CPPs). From an early stage in process development onwards, the impact of CPPs on CQAs is thoroughly investigated. The toolset for this investigation is statistical experimental design (design of experiments, DoEs), process analytical technology, as well as mechanistic modeling. Quantifying and describing (on a statistical or mechanistic level) the interactions of CPPs and CQAs can be considered the key step to the demonstration of process understanding and also of a quality by design submission. The mathematical description of CPP and CQA interactions is referred to as "knowledge space" (Figure 1.17). This multidimensional space of process parameters carries the information on how CQAs change as a function of CPPs. The subset of CPP ranges where the quality attributes of the drug product as defined by the CQAs are met is referred to as "design space." Within the design space all combinations

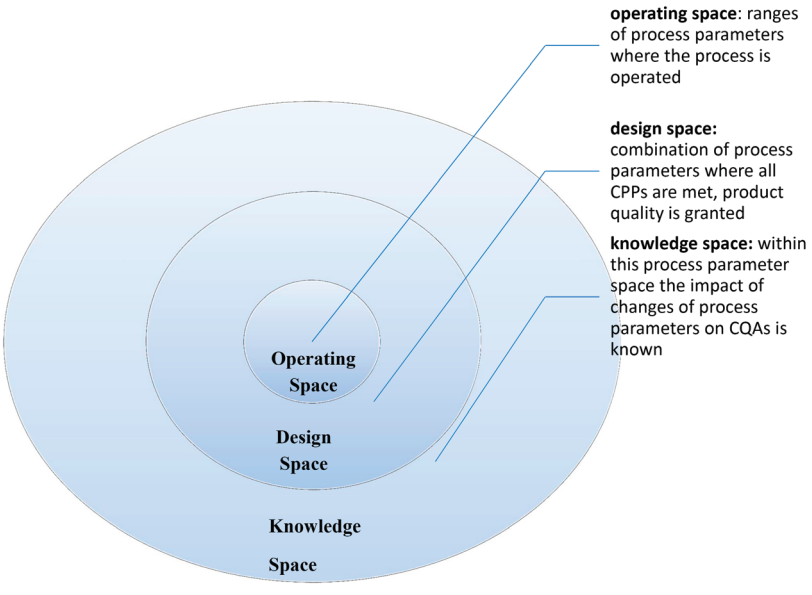


Figure 1.17 Knowledge space, design space, and operating space.

of CPPs lead to the desired CQAs of the drug and therefore also meet all requirements as defined in the QTPP. Within the design space, intended and not intended changes of critical process parameters do not compromise drug product quality. This allows the regulatory authorities to grant the freedom to the manufacturer to operate freely within the boundaries of the design space. This regulatory flexibility is highly desired by the manufacturing industry, for example, to respond to unintended changes such as variations in material quality attributes or other process variations. Basic elements of QbD including risk assessment, the definition of CPPs and CQAs, as well as the development of the design and knowledge space are exemplified in the A-mAb case study [42]. The basic QbD concept for process development is outlined, reviewed, and commented on in several recent contributions and textbooks [69–74].

Process analytical technology (PAT) emerged as a key element of process development and manufacturing along QbD principles. PAT is defined as a "system to design, analyze and control manufacturing through timely measurements." Ideally, critical process parameters (CPPs) or even critical quality attributes (CQAs) can be measured in real-time (i.e., at the time of processing). In combination with the process understanding developed during process development, PAT aims to assess (analyze) and even control drug quality. Depending on the objective, a plethora of analytical devices based on various measurement principles capable of being implemented as PAT are commercially available, as discussed for biopharmaceutical applications elsewhere [75]. However, it should be stressed at this point that timely measurements alone or just linking an online monitoring device to the process are not PAT approaches. PAT evolves as a combination of timely

measurements and its purposeful use is for the development of process understanding (during process development). PAT can also be a tool to make use of process understanding within manufacturing (control on the basis of process understanding). Typically, a PAT system is composed of the process analytical measurement device embedded in a control or process analysis strategy.

Understanding of the CPPs impact on the process as well as timely information on the process state through PAT also acts as the basis for the development of a control strategy. A control strategy aims at ensuring that the CQAs stay within the desired range. Although not considered the final goal of QbD and PAT, QbD can lead to potential real-time release. Hence it can grant the possibility to release products without final product testing.

1.6.3 Entry Points to QbD for Manufacturers

ICH Q11 distinguishes between "classical" and "enhanced" submission processes for pharmaceutical drugs, whereby the latter refers to the implementation of QbD elements [76]. Furthermore, future submission processes can also be a combination of both [76]. Hence, the industry is not forced to enforce full QbD in a few years but can implement step by step QbD elements. As an example, the industry can choose to introduce a PAT application for a single unit operation. However, since QbD is conceptually holistic, this gradual implementation is in the authors' opinion not believed to show the full industrial benefit. In the authors' opinion QbD will unfold the most benefit if (i) implemented as early as possible (immediately after drug discovery) and (ii) applied in a holistic fashion spanning the whole product lifecycle using the combination of tools outlined in ICH Q8, Q9, and Q10. Furthermore, existing processes can possibly be upgraded to QbD processes using historical data to allow process optimization, which would show an immediate benefit.

1.6.4 Challenges for Putting QbD Into Practice

The concepts of QbD have been developed and discussed in the pharmaceutical industry for more than a decade. However, there is, to our knowledge, still no single full QbD process including design space approved by the regulatory authorities. The main challenges for putting QbD into practice are listed here:

- **Industrial doubt and vague regulatory guidance:** From a manufacturers' perspective submitting a QbD dossier is accompanied with high risk. A significant higher amount of investment must be made in the initial phases of product development. In these early stages, the additional regulatory flexibility granted by the regulatory authorities cannot be predicted. Even more difficult is the prediction of the economic benefit of a possible future higher degree of regulatory flexibility (which is not specified in detail). Summarizing, it is difficult to assess

whether submitting a QbD process is an economically reasonable decision. Furthermore, the regulatory guidance is still vague, complicating the assessment of risk and benefit of a QbD submission.

- **Industrial hesitation to share information with the regulatory authorities:** For decades it has been pharmaceutical practice to share as little processing information as possible with the regulatory authorities. Sharing information on failed batches is still unthinkable for manufacturers. However, the development of mutual trust between industry and regulatory authorities demands open communication. To put QbD into practice, the regulatory authorities and the industry have to communicate openly and at eye level.
- **Validity of risk assessment:** Risk assessments for ranking of the criticality of process parameters are carried out using a team of experts. Parameters selected as critical within the risk assessment are then selected for the design and knowledge space development. Risk assessments are per se highly subjective and can rarely be reproduced once the team of experts is changed. However, the decision of criticality propagates to design space development. In cases where the regulatory authorities disagree with the criticality assessment (e.g., one parameter is assessed as non-critical although the regulatory authorities considered this parameter critical within the review process), the design space cannot be approved.
- **Validation of the design space:** Following risk assessment, multivariate studies take place leading to the development of a design space. These studies are typically carried out in laboratory-scale bioreactors. Here the question of *scalability* of the design space arises. Are the effects at 10 000-liter scale the same as at 10-liter scale? Do scale dependent effects interfere with CPP/CQA interactions? Is the down-scale model correct and how can it be validated? In cases where the scalability of the design space is not proven, it is highly questionable whether it can be approved by the regulatory authorities. However, it is generally understood that the design space cannot be validated entirely at the target scale. As a resolution to this dilemma, the current solution for the validation of the design space is the demonstration that the process delivers the expected product quality at some operating points only.

1.6.5 Process Understanding for Biopharmaceutical Processes

In biopharmaceutical upstream processes, highly complex molecules are assembled by living organisms. The cell can be considered as the primary production unit (Figure 1.18). Hence, highly complex cellular processes are responsible for quality, safety, and efficacy of the drug product. In a QbD context, this poses immense challenges to the demonstration of process understanding. Changes in process parameters do not directly impact critical quality attributes, but initiate a change in the physiological status and biochemistry of the cell [59]. The question arises, whether the sound description of the cellular changes (changes in the

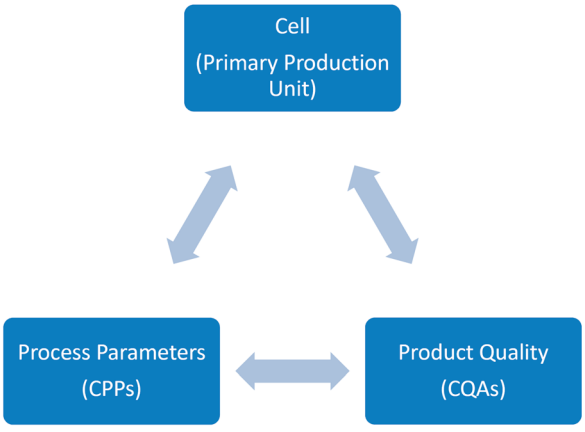


Figure 1.18 The cell as primary production unit determines product quality. In extracellular production, process parameters can also influence product quality attributes directly. Figure is adapted from Reference [59].

assembling unit) is obligatory for the demonstration of "process understanding." In this context, the authors' aim at differentiating three different levels of process understanding:

1) Statistical process understanding: using statistical tools to prove and quantify the impact of CPPs on CQAs. This is typically done using multi-linear regression models.
2) Statistical process understanding plus mechanistic hypothesis: the identified statistical correlations can be interpreted on the basis of scientific hypotheses (e.g., multi-linear regression coefficients can be clearly interpreted).
3) Mechanistic process understanding: the impact of CPPs on the production unit as well as its impact on the CQAs can be described using a mechanistic model.

While the A-mAb case study [42] only focuses on statistical process understanding, the level and quality of process understanding demanded by the regulatory authorities is still a topic of speculation. However, it is safe to assume that the level and quality of process understanding demonstrated within the regulatory submission will directly impact the degree of regulatory flexibility and trust granted by the regulatory authorities.

1.6.5.1 Quality by Design – Opportunity or Threat for the Pharmaceutical Industry?

QbD was conceived by the regulatory authorities to promote science- and risk-based approaches in pharmaceutical manufacturing and to facilitate innovation. Although a full QbD process is not yet approved by the regulatory authorities, QbD has already initiated change in both the pharmaceutical industry and the regulatory authority organizations:

Table 1.13 Benefits of the implementation of QbD (quality by design).

	Benefit
1	Higher degree of process understanding
2	Less failed batches through better investigated manufacturing processes
3	More efficient and effective control of change
4	Increased regulatory flexibility
5	Return on investment
6	Continuous improvement of processes

1) QbD fueled investment in research and development in the pharmaceutical process sciences;
2) QbD initiated the increased use of risk assessment and risk management tools;
3) QbD fueled the hire of statisticians both on side of the industry as well as on side of the regulatory authorities.

QbD may result in increasing costs during process development (design space, PAT strategy, control strategy establishment). On the other hand, this initial investment can result in cost savings at later stages of the product lifecycle. Table 1.13 summarizes further benefits of implementing QbD.

The realization of QbD in the pharmaceutical world is still in its infancy and many open questions need to be resolved. However, despite the slow process of adapting QbD principles in pharmaceutical manufacturing, it is a common understanding that QbD "won't go away." Hence, in our opinion the industry must eagerly respond to these new paradigms of manufacturing and actively shape the future of QbD by using mechanistic approaches, as encouraged by ICH Q11. If not, QbD is likely to become a regulatory imperative rather than a mutual effort to increase drug product quality and therefore patient safety.

1.7 Process Economics

This section briefly discusses general design aspects with impact on process economics.

1.7.1 Optimization of Overall Productivity and Capital Expenses of the Production Facility

Operating a production facility aims at optimizing the product output, while making optimum use of the installed assets. Hence there is a trade-off to be found between capital and operating expenses.

Capital expenses consist of the installation of equipment of an integrated biopharmaceutical process. The first principle objective for an appropriate facility

design is balancing the durations of USP (upstream processing) and DSP (downstream processing) unit operations. USP usually consists of seed stage, production, harvesting, and isolation. DSP consists of purification steps, usually a sequence of chromatography and ultra- and diafiltration steps. The rate-limiting step of batch scheduling is determined on the one hand by the longest unit operation and on the other hand by the longest occupation of a production suite (in which only one batch at a time can be processed and which consists of multiple unit operations). This can be analyzed using time and motion analysis tools (e.g., Batch Plus of Aspen Tech, Schedule Pro of Intelligen).

The unit operation of the longest duration in mammalian cultures is the production step itself. Typically, multiple bioreactors are run for cascaded harvesting. In microbial processes, however, the identification of the unit operation of the longest duration is not that obvious. For bacterial processes, the production can be finished in three days including cleaning and preparation. In contrast to all other biopharmaceutical processes, the intracellular product location in *E. coli* may shift the rate-limiting unit operation to the refolding step. Irrespective of whether continuous or fed-batch processing is selected, slow refolding kinetics cannot be overruled in cases were reasonable refolding recovery yields are targeted. When the rate limiting unit operation is not the production bioreactor, the seeding tasks can be shifted into the production bioreactor and seed steps can be omitted. This is possible as bacteria have the ability to grow also at very small inoculum fractions.

1.7.2
Further Economic Effects by Intracellular Product Location

In cases of intracellular product location, additional unit operations have to be installed (Figure 1.6). Such unit operations require not only additional equipment in the process chain itself (homogenizer, high speed centrifuge, solubilization vessel, diafiltration for solvent removal, refolding tank) but also additional buffer tanks for the operation.

As a consequence, those buffer tanks may need their own additional floor for preparation and holding of buffers. Additionally, corrosion resistant material of construction (1.4539, 904L or Hastelloy C22) is needed for the harsh process conditions (using 3 M urea or 6 M guanidine·HCl) in the solubilization steps of inclusion bodies. The reader should also keep in mind that any solvent or chemical added to the process must also leave the process. Hence, the solubilization buffers may also require a separate waste collection system and, subsequently, solutions for the disposal of the waste.

1.7.3
Comparison of Product Yields, Intracellular Versus Extracellular

We finish this chapter with a comparison of the final product yield, which can be obtained by extracellular or intracellular product location.

Table 1.14 Comparison of titers and step yields for extracellular, periplasmic, and intracellular inclusion body (IB) processes.

Unit operation	Step yield ($g\ g^{-1}$)			Concentration ($g\ l^{-1}$ bioreactor volume)		
	Extracellular	Intracellular PPS	Intracellular IBs	Extracellular	Intracellular PPS	Intracellular IBs
Production	—		—	3.00	3.00	20.00
Centrifugation/microfiltration	0.90	0.90	0.90	2.70	2.70	18.00
Cell disruption	—	0.90	0.85	—	2.43	15.30
Centrifugation of cell debris	—	0.90	0.90	—	2.19	13.77
Solubilization of inclusion bodies	—	—	0.85	—	—	11.70
Diafiltration for solvent removal	—	—	0.95	—	—	11.12
Refolding	—	—	0.50	—	—	5.56
Chromatography 1	0.90	0.90	0.90	2.43	1.97	5.00
Ultrafiltration 1	0.90	0.90	0.90	2.19	1.77	4.50
Chromatography 2	0.90	0.90	0.90	1.97	1.59	4.05
Ultrafiltration 2	0.90	0.90	0.90	1.77	1.43	3.65
Chromatography 3	0.90	0.90	0.90	1.59	1.29	3.28
Ultrafiltration 3	0.90	0.90	0.90	1.43	1.16	2.95
Concentration = final bulk	0.95	0.95	0.95	1.36	1.10	2.81
Overall process yield	**0.45**	**0.37**	**0.14**	**1.36**	**1.10**	**2.81**

In the near future product titers of 3–5 $g\,l^{-1}$ in the extracellular medium and in the periplasmic space exiting the bioreactor can be expected. However, when expressed as inclusion bodies, a product titer of up to 20 $g\,l^{-1}$ at the exit of the bioreactor is an expected value. Hence, which process would yield the higher productivity? Table 1.14 uses typically achieved step recoveries to compare the performance of the overall process of the three product scenarios. The total yield of the inclusion body process decreases drastically due to the multiple additional unit operations and the still lousy refolding recovery. The overall titer remains favorable, though, compared to soluble products. However, factors like batch cycle time, lower investment costs, and lower processing risk will clearly drive development to extracellular and soluble products in the future!

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Summary Box: Characteristics of Production System

Production systems, cells, and products	Expression systems type of cells	Yeast, *Saccharomyces cerevisiae* and *Pichia pastoris* as typical representatives
	Typical products preferred for the production of	Primary and secondary metabolites, heterologous proteins and biomass
	Special products	Biomass, ethanol, riboflavin, biopharmaceutical proteins (e.g., hepatitis B vaccine, insulin, etc.)
	System maturity Products on the market	ISO and GMP yeast products on the market; potential not yet exhausted; system development continues
Characteristics affecting product quality	Secretion, product location, proteolysis	Intracellular production and secretion
	Post-translational modifications as disulfide bonds, glycosylation, protein multimers, product size	All post-translational modifications possible. Glycosylation in *S. cerevisiae:* high-mannose type glycosylation, in *P. pastoris*: low-mannose type (human like gylco-engineered *P. pastoris* strains are available)
	System robustness, solubility issues, endotoxins	Good robustness, no solubility problems and endotoxins
Systems characteristics concerning growth and productivity	Maximum specific growth rate, μ_{max} (h^{-1})	*S. cerevisiae* approx. 0.4 *P. pastoris* approx. 0.25
	Maximum dry biomass concentration, x_{max} ($g\,l^{-1}$)	*S. cerevisiae* 60–100 *P. pastoris* 80–150
	Maximum specific product production rate, q_P ($mg\,g^{-1}\cdot h^{-1}$)	*S. cerevisiae* 0.21 (insulin) *P. pastoris* 0.375 (insulin)
	Maximum volumetric product production rate, r_P ($mg\,l^{-1}\cdot h^{-1}$)	*S. cerevisiae* 25 (HSA extra cellular protein) *P. pastoris* 42 (extracellular protein) *P. pastoris* 160 (intracellular protein)
	Maximum product titres, $c_{p,max}$ ($g\,l^{-1}$)	Ethanol (15%), protein intracellular 10–20 Protein secreted <5
	Specific maintenance, m_s ($g\,g^{-1}\cdot h^{-1}$)	$0.016\,g\cdot g^{-1}\cdot h^{-1}$ (*P. pastoris*)
	Yield coefficients $Y_{X/S}$ and $Y_{P/S}$ ($g\,g^{-1}$)	*P. pastoris* $Y_{X/Glucose} = 0.55$ $Y_{X/Methanol} = 0.42$
	Energy and carbon source Special nutritional requirements	Carbon 46–52% (w/w) Nitrogen 6–9% (w/w) Phosphor 0.8–2.6% (w/w) Sulfur 0.01–0.25% (w/w)
	Maximum oxygen uptake rate, OUR_{max} ($mmol\,l^{-1}\cdot h^{-1}$) Specific oxygen consumption ($mmol\,g^{-1}\,h^{-1}$)	$480\,mmol\cdot l^{-1}\cdot h^{-1}$ for a *P. pastoris*/methanol culture with a biomass concentration of $100\,g\,l^{-1}$. $4.8 \pm 0.5\,mmol\,g^{-1}\,h^{-1}$
	Heat production rate ($W\,m^{-3}$) Specific heat production	67 000 (*P. pastoris*/methanol, $100\,g\,l^{-1}$) $0.67 \pm 0.007\,W\,g^{-1}$
	Typical duration from inoculation of a production culture to harvest (days)	3–5
	Systems shear sensitivity	no special shear sensitivity
	Preferred bioreactor design Equipment standard and typical production scale	Continuous stirred-tank reactor ($10\,m^3$), disk stack centrifuge
	Most important production cost drivers of production culture	Man power, maintenance cost of bioreactor system, DSP
	Process development cost aspects Duration of process development	Development of strain, analytical methods, fermentation and purification; basic development 6 ± 2 months
	Key strengths of system	High biomass yields
	Key weaknesses of system	Low specific production rates
	Issues to be addressed in the future	Increase of specific productivity, uncoupling of growth from product formation